Class-I TCP Transcription Factors Activate the *SAUR63* Gene Subfamily in Gibberellin-Dependent Stamen Filament Elongation[1][OPEN]

Victoria Gastaldi, Leandro E. Lucero, Lucía V. Ferrero, Federico D. Ariel, and Daniel H. Gonzalez[2,3]

Instituto de Agrobiotecnología del Litoral, Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, 3000 Santa Fe, Argentina

ORCID IDs: 0000-0003-4453-8964 (L.E.L.); 0000-0002-1558-6476 (L.V.F.); 0000-0001-8478-8808 (F.D.A.); 0000-0002-3137-8095 (D.H.G.).

In autogamous plants like Arabidopsis (*Arabidopsis thaliana*), stamen filament elongation must be finely regulated to ensure that anthers reach the pistil at the correct developmental stage. In this work, we studied the roles of Arabidopsis TEOSINTE BRANCHED1, CYCLOIDEA, PCF15 (TCP15), and related class-I TCP transcription factors in stamen filament elongation. Plants with decreased expression of class-I TCPs and plants that express a fusion of TCP15 to a repressor domain (*pTCP15::TCP15-EAR*) had shorter stamens, indicating that class-I TCPs stimulate filament growth. These plants also showed reduced expression of several *SMALL AUXIN UP RNA* (*SAUR*)*63* subfamily genes, which contain TCP target motifs in their promoters. Mutational analysis indicated that the TCP target motif in the *SAUR63* promoter is required for expression of *SAUR63* in stamen filaments. Moreover, TCP15 directly binds to the *SAUR63* promoter region that contains the TCP target motif in vivo, highlighting the role of the TCPs in this process. Class-I TCPs are also required for the induction of *SAUR63* subfamily genes by gibberellins (GAs). In addition, overexpression of *SAUR63* restores filament growth in *pTCP15::TCP15-EAR* plants, whereas overexpression of *TCP15* rescues the short stamen phenotype of GA-deficient plants. The results indicate that TCP15 and related class-I TCPs modulate GA-dependent stamen filament elongation by direct activation of *SAUR63* subfamily genes through conserved target sites in their promoters. This work provides insight into GA-dependent stamen filament elongation.

Stamen filament elongation is particularly important in self-pollinating, autogamous species, like Arabidopsis (*Arabidopsis thaliana*). In these plants, the pollen fertilizes ovules from the same flower, usually before the flower opens. Stamens must elongate for anthers to reach the top of the gynoecium at the correct developmental stage (i.e. when the stigma becomes receptive and anthers dehisce). Incorrect stamen filament elongation may severely affect successful reproduction. Not surprisingly, the elongation of stamen filaments is a strictly controlled process and occurs during specific stages of flower development (Tashiro et al., 2009;

Cardarelli and Cecchetti, 2014). Stamen filaments are first recognizable from the anther at flower stage 7. At stage 9, the filaments represent only 20% of the length of stamens. A phase of filament elongation, known as preanthesis growth, takes place between stages 10 and 13 and is mainly due to cell expansion. During this phase, the stamens reach the length of the pistil at stage 13, when the flower opens. Postanthesis growth occurs until stage 14, when the stigma becomes receptive for pollination.

Several hormones, including auxin, gibberellins (GAs), and jasmonic acid (JA), are involved in stamen filament elongation (Song et al., 2013). Plants defective in auxin biosynthesis, transport, or perception and double mutants in the *AUXIN RESPONSE FACTOR* genes *ARF6* and *ARF8* show defects in stamen filament elongation and anther maturation (Nagpal et al., 2005; Cecchetti et al., 2008; Tashiro et al., 2009; Tabata et al., 2010; Reeves et al., 2012). Particularly, a splice variant of *ARF8* (*ARF8.4*) is required for stamen filament elongation and the correct expression of the auxin inducible gene *AUX/IAA19* (Ghelli et al., 2018). Auxin transport from the tapetum through the middle layer and toward the filament is required to coordinate anther maturation with filament growth (Cecchetti et al., 2017). Mutations in JA biosynthesis genes, or in certain components of the JA signaling pathway, also affect filament elongation (Xie et al., 1998; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002). The response to JA in

stamens is mediated by two JA-inducible MYB transcription factors, MYB21 and MYB24 (Mandaokar et al., 2006), which are targeted by Jasmonate-ZIM domain proteins (Song et al., 2011). It has been reported that ARF6 and ARF8 induce the expression of JA biosynthesis genes during late stages of stamen development, indicating that auxin acts upstream of JA (Nagpal et al., 2005; Tabata et al., 2010; Reeves et al., 2012). However, the fact that stamen filament elongation is not rescued by JA treatment of *arf6 arf8* mutants (Nagpal et al., 2005) suggests that additional pathways are involved. Among the genes downregulated in *arf6 arf8* mutant flowers, there are several *SMALL AUXIN UP RNA* (*SAUR*) genes from the *SAUR63* subfamily (Nagpal et al., 2005). SAUR proteins promote cell expansion by activating plasma membrane H$^+$-ATPases (Spartz et al., 2014) and the overexpression of *SAUR63* subfamily members stimulates stamen filament elongation (Chae et al., 2012). Thus, induction of *SAUR* genes by ARF6 and ARF8 may be required, in addition to JA biosynthesis, to stimulate filament elongation.

Plants defective in GA biosynthesis or perception also show defects in stamen filament elongation (Cheng et al., 2004; Tyler et al., 2004; Rieu et al., 2008). GAs induce the synthesis of JA and the expression of MYB transcription factors to modulate stamen development; however, the short stamen phenotype of GA-deficient plants cannot be rescued by exogenous JA, suggesting that other GA-dependent, JA-independent pathways are required for correct stamen filament elongation (Cheng et al., 2009). Notably, analysis of available microarray data indicates that several *SAUR63* subfamily genes are also induced by GAs (Bai et al., 2012; Ren and Gray, 2015), suggesting that GA-dependent stamen filament elongation may involve the induction of *SAUR* genes. However, the mechanism involved in this process is largely unknown.

Teosinte branched1, cycloidea, PCF (TCP) transcription factors regulate several aspects of plant development, including plant architecture, leaf morphogenesis and maturation, inflorescence stem growth, and floral organ development (Martín-Trillo and Cubas, 2010; Manassero et al., 2013). Twenty-four TCP proteins (TCPs), assigned to either class I (13 proteins) or class II (11 proteins), are encoded in the Arabidopsis genome. Class-I proteins show a high degree of functional redundancy, and thus developmental phenotypes are usually observed only in higher-order mutants or plants that express fusions of the TCPs to the EAR domain (Kieffer et al., 2011; Uberti-Manassero et al., 2012; Aguilar-Martínez and Sinha, 2013). Fusions to the EAR domain convert transcription factors into strong dominant repressor forms (Hiratsu et al., 2003). This strategy is useful in cases of genetic redundancy and has been widely used to study the role of transcription factors, including those of the TCP family (Koyama et al., 2007, 2010; Kieffer et al., 2011; Li et al., 2012; Uberti-Manassero et al., 2012; Aguilar-Martínez and Sinha, 2013). This type of analysis revealed that class-I

TCPs either positively or negatively modulate cell proliferation and expansion depending on the organ/tissue involved (Kieffer et al., 2011). Interplay of TCPs with hormone action was also described (Nicolas and Cubas, 2016). As an example, TCP14 and TCP15 negatively modulate both auxin biosynthesis genes during gynoecium development and the expression of the auxin reporter *DR5:GUS* in vegetative and reproductive tissues (Lucero et al., 2015). TCP14 and TCP15 also participate in GA-dependent germination, flowering, and inflorescence stem elongation (Davière et al., 2014; Resentini et al., 2015; Lucero et al., 2017). TCP20 and TCP9, more distantly related class-I TCPs, inhibit JA biosynthesis through the repression of the JA biosynthesis gene *LIPOXYGENASE2* (Danisman et al., 2012).

In this work, we investigated the role of TCP15 and related class-I TCPs in stamen filament elongation. We report that class-I TCPs participate in GA-dependent stamen filament elongation by directly inducing the expression of *SAUR63* subfamily genes. These results contribute to a more comprehensive understanding of the molecular pathway between GA action and stamen filament elongation.

RESULTS

TCP15 and Related Class-I TCPs Affect Stamen Filament Elongation

The analysis of plants bearing the *TCP15* promoter region controlling the expression of the *GUS* reporter gene revealed that the *TCP15* promoter is active in stamen filaments (Fig. 1A). Expression of the reporter gene was mainly observed in the vasculature and was extended to peripheral tissues. To evaluate a possible role of TCP15 in this organ, we analyzed stamen length in plants expressing a fusion of TCP15 to the EAR repressor domain under the control of the *TCP15* promoter (*pTCP15::TCP15-EAR* plants). In this case, the assumption is that expressing TCP15-EAR within the expression domain of the *TCP15* promoter will cause the downregulation of TCP15 target genes. Through this strategy, we expected to uncover a possible role of TCP15 in stamen filaments even in the presence of genetic redundancy with other class-I TCPs. We observed that the stamen length of *pTCP15::TCP15-EAR* plants was reduced by ~30% to 40% relative to wild type (Fig. 1, B and C). As a complementary approach, we used plants that express TCP15 from the *35S Cauliflower mosaic virus* (*CaMV*) promoter, either in its native form or fused to the red fluorescent protein (*35S::TCP15* and *35S::TCP15-RFP* plants, respectively), to analyze the effect of increased TCP15 expression. Plants of both lines exhibited longer filaments than wild-type plants (Fig. 1, B and C). This result suggests that TCP15 stimulates stamen filament elongation. Because the expression of a repressor form (TCP15-EAR) causes the opposite effect of overexpressing the native protein,

Figure 1. TCP15 and related class-I TCPs participate in stamen filament elongation. A, GUS expression pattern in flowers of plants that contain a fusion of the *TCP15* promoter region to the *gus* reporter gene. Scale bars = 1 mm. B, SEM images of stage 13–15 flowers from wild-type (Col-0) plants and plants with altered TCP function, as indicated. Organs from the outer two whorls were removed to allow visualization of the stamens. Scale bars = 1 mm. C, Stamen filament length, relative to wild type, in flowers at stage 13 of different plant lines with altered TCP function. Bars indicate the mean ± SE (n = 19–43 stamens, depending on the line). D, Stamen filament length of wild-type, *pTCP15::TCP15-EAR*, and *amiR-27* plants at stages 10–13 of flower development. Bars indicate the mean ± SD (n = 10–54 stamens, depending on the stage). Asterisks in (C) and (D) indicate significant differences with wild-type plants ($P < 0.05$; Student's t test). E, GFP (left; scale bars = 0.2 mm) and GUS expression (right; scale bars = 1 mm) in stamen filaments and flowers of plants that contain a fusion of the *TCP15* promoter region to the *TCP15*, *GFP*, and *GUS* coding regions (*pTCP15::TCP15-GFP-GUS*). Stamens from flowers at different stages are shown.
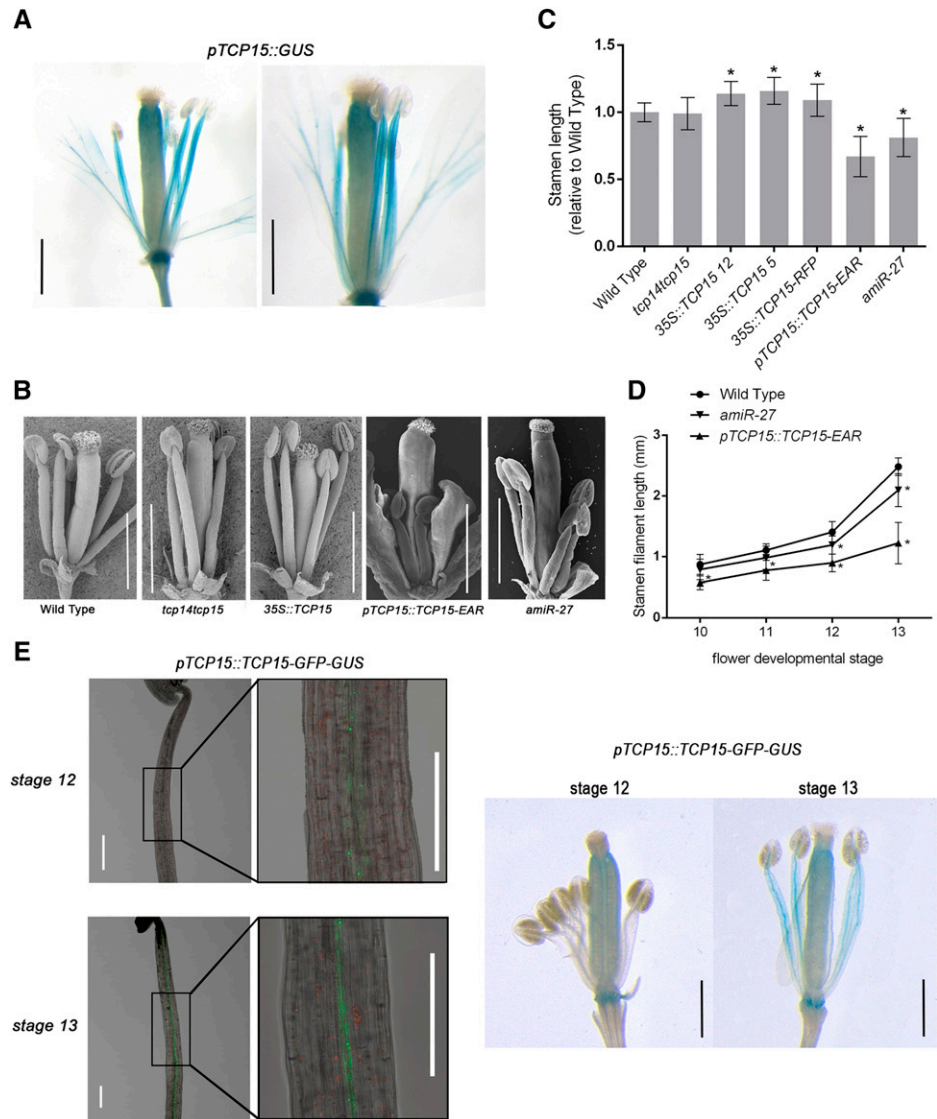
it can be concluded that TCP15 most likely acts as an activator during this process. If TCP15 stimulates filament elongation, it is expected that loss-of-function of *TCP15* will affect stamen length. We then analyzed filament length in a line carrying mutations in *TCP15* and the closely related gene *TCP14*, but found that this double mutant showed no significant difference with wild type (Fig. 1, B and C). We speculate that this is likely due to functional redundancy with other class-I proteins, as observed in other processes (Kieffer et al., 2011; Uberti-Manassero et al., 2012; Aguilar-Martínez and Sinha, 2013). Analysis of expression levels of class-I TCPs in stamen filaments using data available in the Transcriptome Variation Analysis database (http://travadb.org/; Klepikova et al., 2016) indicated that several *TCP* genes are expressed in this organ, including *TCP7*, *TCP8*, *TCP14*, *TCP15*, *TCP21*, and *TCP22* (Supplemental Table S1). Among these, *TCP7* is the most highly expressed, accounting for 29% of all class-I *TCP* transcripts, similar to the sum of those of *TCP14*

and *TCP15* (27%). Whereas available *tcp7* insertional mutants do not show reduced transcript levels (Aguilar-Martínez and Sinha, 2013), plants that express an artificial micro RNA targeting the class-I *TCP* genes *TCP8* and *TCP22* in a *tcp14 tcp15* mutant background were described before (Davière et al., 2014). We measured *TCP8* and *TCP22* transcript levels in stamen filaments of these plants (*amiR-27* plants) and found that they were reduced by ~35% and 70%, respectively, whereas the expression levels of *TCP7* and *TCP21* were not significantly affected (Supplemental Fig. S1). Analysis of *amiR-27* plants indicated that they showed shorter stamen filaments than wild type (Fig. 1, B and C), leading to the conclusion that downregulated expression of *TCP8* and *TCP22*, in addition to *TCP14* and *TCP15*, affects filament elongation. Thus, our results indicate that several TCP15-related class-I TCPs redundantly participate in stamen filament elongation. In the case of *pTCP15::TCP15-EAR*, no changes in expression were observed for class-I *TCP* genes other than

Table 1. *Expression levels of genes related to stamen filament elongation in* pTCP15::TCP15-EAR *plants*

Expression data were obtained from GEO datasets GSE57742, GSE57743 and GSE57744 (Lucero et al., 2015). AGI, Arabidopsis Genome Initiative Locus Code.

Probe ID	AGI	Name	Fold-Change[a]	Adjusted P Value
JA synthesis genes				
A_84_P18289	AT2G44810	DAD1	0.34	0.3335
A_84_P15574	AT3G45140	LOX2	0.28	0.6584
A_84_P13078	AT5G42650	AOS	−0.38	0.4058
A_84_P853664	AT2G06050	OPR3	−0.26	0.8151
JA-regulated MYB genes				
A_84_P19845	AT3G27810	MYB21	0.09	0.6923
A_84_P825353	AT5G40350	MYB24	−0.13	0.8349
A_84_P767898	AT5G06100	MYB33	0.03	0.7715
A_84_P15465	AT3G01530	MYB57	0.20	0.4211
A_84_P16408	AT3G11440	MYB65	0.00	0.9881
Auxin response factor genes				
A_84_P21873	AT1G30330	ARF6	−0.70	0.0442
A_84_P18731	AT5G37020	ARF8	−0.20	0.4085
SAUR63 subfamily genes				
A_84_P537376	AT1G29420	SAUR61	−1.19	0.0395
A_84_P10257	AT1G29430	SAUR62	−1.73	0.0136
A_84_P279980	AT1G29440	SAUR63	−1.97	0.0076
A_84_P19713	AT1G29450	SAUR64	−2.29	0.0077
A_84_P15930	AT1G29460	SAUR65	−1.91	0.0085
A_84_P11207	AT1G29500	SAUR66	−2.06	0.0085
A_84_P22555	AT1G29510	SAUR67	−2.08	0.0076
A_84_P563710	AT5G27780	SAUR75	−1.40	0.0488

[a]Log_2 (pTCP15::TCP15-EAR/wild-type).

for *TCP15* (Supplemental Fig. S1). *TCP15* transcript levels were ~2.5-fold higher in these plants that in wild-type plants (Supplemental Fig. S1). Because this represents the sum of transcripts arising from the endogenous gene and the *TCP15-EAR* transgene, it can be concluded that the transgene was expressed at roughly similar levels as the endogenous *TCP15* gene in these plants. Altogether, our results suggest that the short stamen phenotype is due to a direct effect of the repressor form of TCP15 and not caused by changes in the expression of the other class-I TCPs.

Measurement of stamen filament length at different stages of flower development indicated that *amiR* plants show shorter filaments than wild type at stages 12 and 13 (before and at anthesis, respectively; Fig. 1D), suggesting that the TCPs are particularly important during the late stages of pre-anthesis growth, when faster elongation occurs (Cecchetti et al., 2008). By contrast, *pTCP15::TCP15-EAR* flowers showed shorter filaments already by stage 10 (Fig. 1D). This implies that TCP15 and other TCPs may also have a role during early stages of filament growth. Nevertheless, the growth rate was more markedly decreased between stages 12 and 13, again pointing to a particular role of the TCPs during these stages. Accordingly, analysis of GFP and GUS reporters indicated that the *TCP15* promoter is active in stamen filaments by stage 12 and its activity increases at stage 13, mainly in vascular tissues (Fig. 1E). Expression of the reporters was not detected at earlier stages.

Class-I TCPs Modulate the Expression of *SAUR63* Subfamily Genes

To gain insight into the action mechanism of TCP15, we analyzed an available microarray experiment of gene expression changes in *pTCP15::TCP15-EAR* plants (Lucero et al., 2015). Among the genes that were previously related to stamen filament elongation, consistent expression changes were not evident for those related to JA metabolism or for JA-regulated *MYB* genes (Table 1). *ARF8* expression was also not significantly affected, whereas *ARF6* showed only an ~40% decrease in expression relative to wild type (Table 1). Because the microarray experiment of *pTCP15::TCP15-EAR* plants was performed with RNA prepared from rosettes, we confirmed this result by measuring *ARF6* and *ARF8* transcript levels in *pTCP15::TCP15-EAR* flowers by reverse-transcription quantitative PCR (RT-qPCR; Supplemental Fig. S2). We also found that most members of the *SAUR63* gene subfamily (composed of *SAUR61-68* and *SAUR75*; Ren and Gray, 2015) were repressed in *pTCP15::TCP15-EAR* plants (Table 1). The only exception was *SAUR68*, whose expression was below background levels in the microarray. Because decreased expression of *SAUR63* subfamily genes causes reduced stamen filament elongation (Chae et al., 2012), this may explain the shorter filament phenotype of *pTCP15::TCP15-EAR* plants.

To confirm the repression of *SAUR63* subfamily genes in floral organs of *pTCP15::TCP15-EAR* plants,

Figure 2. Transcript levels of *SAUR63* subfamily genes in plants with altered TCP function. A and B, Transcript levels, determined by RT-qPCR, in flowers and stamens, respectively, of *pTCP15::TCP15-EAR* plants. C, Transcript levels, determined by RT-qPCR, in flowers of *amiR-27* plants. D, *SAUR63* transcript levels, determined by RT-qPCR, in different organs of flowers from wild-type, *pTCP15::TCP15-EAR*, and *amiR-27* plants. Flowers at stage 13 were used. The bars indicate the mean ± SE of three biological replicates. Asterisks indicate significant differences with wild-type plants ($P < 0.05$; Student's *t* test).
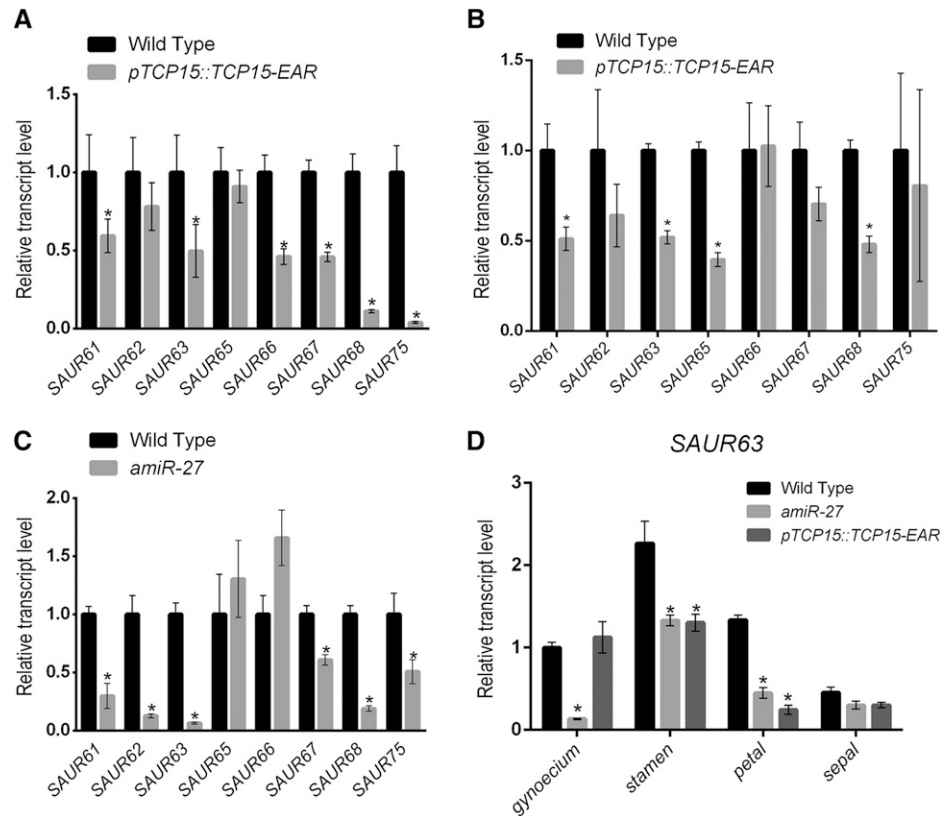
we performed RT-qPCR analyses using RNA prepared from flowers. Decreased expression of most *SAUR63* subfamily genes, with the exception of *SAUR62* and *SAUR65*, was observed in these samples (Fig. 2A). Repression of several *SAUR63* subfamily genes in *pTCP15::TCP15-EAR* plants was also evident when RNA isolated from stamens was tested (Fig. 2B). Most *SAUR63* subfamily genes, except *SAUR65* and *SAUR66*, also showed reduced expression in flowers of *amiR-27* plants (Fig. 2C), indicating that class-I TCPs are required for the correct expression of these genes. The differences in expression observed in *pTCP15::TCP15-EAR* and *amiR-27* plants probably reflect changes in the preference of class-I TCPs for distinct *SAUR* genes. Notably, *SAUR63* was repressed in all the samples analyzed (Fig. 2, A–C).

We then took *SAUR63* as a representative gene of this group and measured its expression in different flower organs. The highest expression of *SAUR63* was observed in stamens of wild-type plants (Fig. 2D). A significant decrease in *SAUR63* transcript levels was evident in stamens and petals of both *pTCP15::TCP15-EAR* and *amiR-27* plants (Fig. 2D). For *amiR-27* plants, but not for *pTCP15::TCP15-EAR* plants, significantly decreased *SAUR63* expression was also observed in the gynoecium (Fig. 2D), indicating that class-I TCPs other than TCP15 likely regulate *SAUR63* expression in this organ. These results show that the regulation of *SAUR63* by class-I TCPs probably extends to other flower organs, in addition to stamens.

Overexpression of *SAUR63* Restores Stamen Filament Elongation in *pTCP15::TCP15-EAR* Plants

To evaluate if reduced expression of *SAUR63* subfamily genes is the cause of the short stamen phenotype observed in *pTCP15::TCP15-EAR* plants, we generated and analyzed *pTCP15::TCP15-EAR* plants expressing SAUR63 under the control of the *35SCaMV* promoter. Because *pTCP15::TCP15-EAR* plants are largely infertile, we first obtained plants transformed with the *35S::SAUR63-GFP* construct that contain flowers with elongated stamens (Supplemental Fig. S3). It is worth noting that the fusion to protein tags is necessary to stably express SAUR proteins, which are otherwise rapidly degraded (Chae et al., 2012; Spartz et al., 2012). We then transformed wild-type and *35S::SAUR63-GFP* plants in parallel with the *pTCP15::TCP15-EAR* construct and analyzed the phenotype of at least 10 independent plants from the T1 population in each background. In the wild-type background, the expression of TCP15-EAR caused defects in rosette and inflorescence development, similar to those previously reported by Kieffer et al. (2011) and Uberti-Manassero et al. (2012). Particularly, the inflorescence showed a decrease in stem and pedicel lengths, flowers showed shorter petals and stamens, sepals were curved inwards, and the protrusion of stigmatic tissue from the replum was observed in the gynoecium (Fig. 3A, left). Similar phenotypes were observed in the *35S::SAUR63-GFP* background, except for stamens, which were
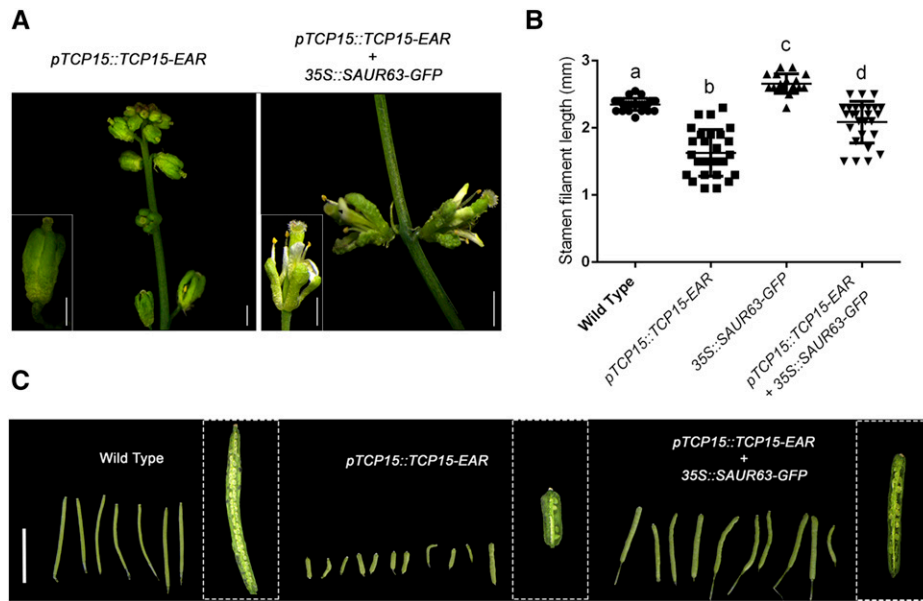
Figure 3. Overexpression of *SAUR63* rescues the short stamen and infertility phenotypes of *pTCP15::TCP15-EAR* plants. A, Phenotype of *pTCP15::TCP15-EAR* plants in wild-type (Col-0) and *35S::SAUR63-GFP* backgrounds. A representative image of 10 independent transformants analyzed for each background is shown. Scale bars = 1 mm. B, Stamen filament length in flowers at stage 13 of wild-type plants, *35S::SAUR63-GFP* plants, and plants that express *TCP15-EAR* under the control of the *TCP15* promoter in either a wild-type or a *35S::SAUR63-GFP* background. The bars indicate the mean ± SE (n = 18–27 stamens, depending on the line). Different letters indicate significant differences ($P < 0.05$; one-way ANOVA for multiple comparisons). C, Images of siliques obtained from wild-type plants, *pTCP15::TCP15-EAR* plants, and *pTCP15::TCP15-EAR* plants that express *SAUR63-GFP* from the *35SCaMV* promoter. Scale bar = 1 cm.

considerably longer, protruding from sepals and almost reaching the top of the gynoecium (Fig. 3A, right). Petals were also elongated in comparison with transformants in the wild-type background (Fig. 3A, right). Increased elongation of stamens and petals in *pTCP15::TCP15-EAR* plants that express SAUR63-GFP agrees with the observation that *SAUR63* transcript levels are reduced in both organs in *pTCP15::TCP15-EAR* plants (Fig. 2D).

Measurement of stamen filament length indicated that the effect of expressing TCP15-EAR was more pronounced in a wild-type than in a *35S::SAUR63-GFP* background (Fig. 3B). Indeed, the median of stamen filament length for the population of plants analyzed was decreased by only 15% in the *35S::SAUR63-GFP* background and by 36% in the wild-type background after TCP15-EAR expression. *SAUR63-GFP* expression also partially restored fertility, as judged from silique growth and seed production, which were considerably affected in *pTCP15::TCP15-EAR* plants (Fig. 3C). Thus, our results suggest that reduced stamen filament elongation in *pTCP15::TCP15-EAR* plants is largely due to repression of *SAUR63* and related genes by TCP15-EAR.

SAUR63 is a direct TCP15 target

To analyze in more detail the regulation of *SAUR63* subfamily genes by TCP15, we focused on *SAUR63*

itself as a representative member of the subfamily. We obtained plants that express a translational fusion of *SAUR63* with the *GUS* reporter gene under the control of the *SAUR63* promoter. The analysis of independent lines revealed strong GUS activity in stamen filaments, as well as in petals (Fig. 4A, left), as previously reported by Chae et al. (2012). We then transformed one of these lines with the *pTCP15::TCP15-EAR* construct. A strong decrease in GUS expression was evident in the stamen filaments of the transformed lines (Fig. 4A), indicating that TCP15-EAR represses the activity of the *SAUR63* promoter in filaments.

Remarkably, the promoter regions of all *SAUR63* subfamily genes, except *SAUR68*, contain sequences that closely match the consensus target site bound by TCP transcription factors (GGGNCC; Fig. 4B; Kosugi and Ohashi, 2002; Viola et al., 2012). In fact, motif enrichment analysis in *SAUR63* subfamily gene promoters using the Regulatory Sequence Analysis Tool platform (http://rsat.eead.csic.es/plants/) yielded the sequence TGGGACC as an enriched motif in these promoters (Fig. 4B; Supplemental Table S2), which is recognized by TCP15 and other class-I TCPs in vitro (Kosugi and Ohashi, 2002; Viola et al., 2011) and in vivo (Lucero et al., 2017). This suggests that *SAUR63* and related genes may be direct targets of the TCPs. To evaluate the role of the putative TCP target sequence in the *SAUR63* promoter, we mutated the TGGGACC sequence to TG<u>AAA</u>A<u>C</u> and obtained plants that

A

B

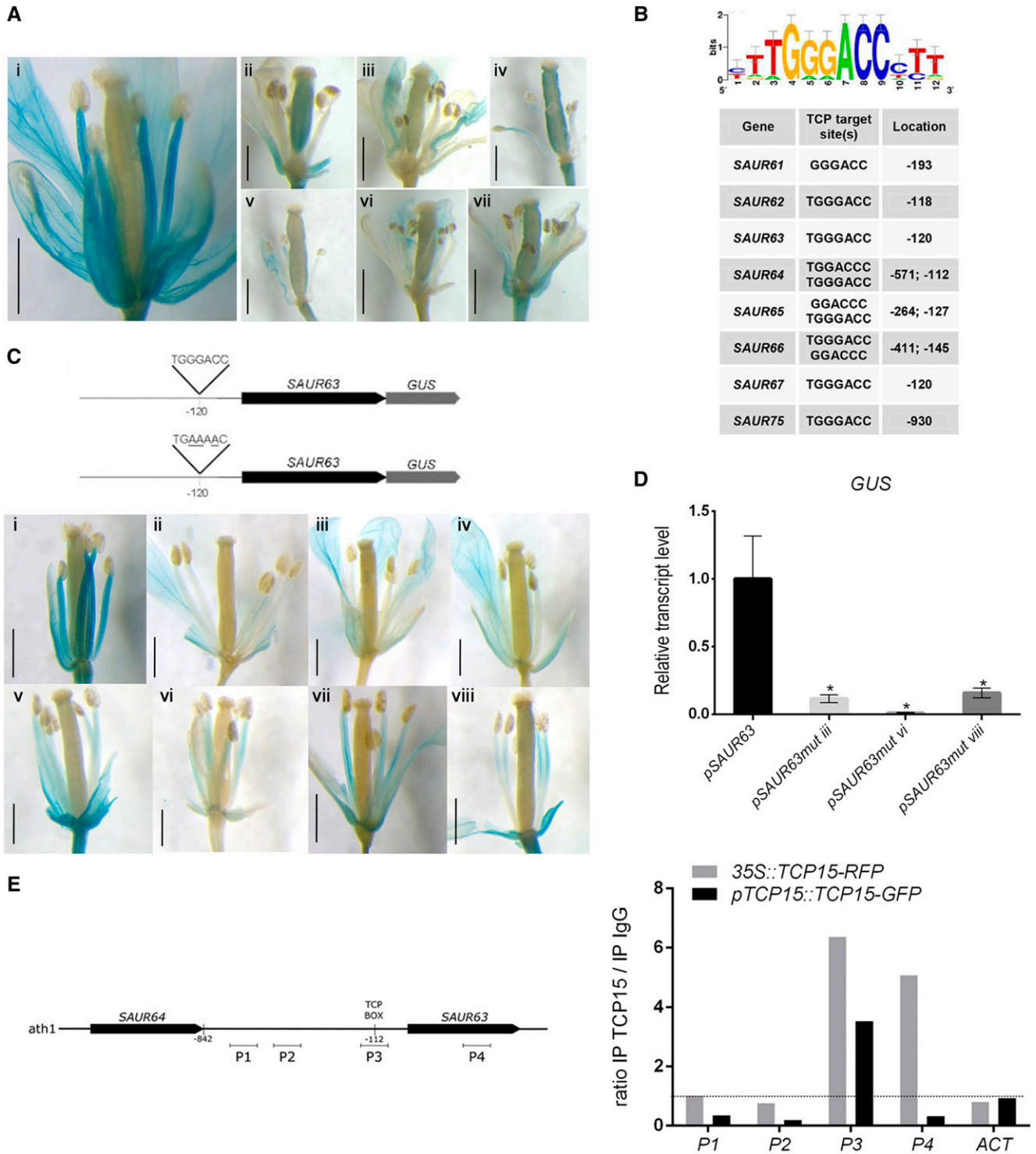| Gene	TCP target site(s)	Location
SAUR61	GGGACC	-193
SAUR62	TGGGACC	-118
SAUR63	TGGGACC	-120
SAUR64	TGGACCC TGGGACC	-571; -112
SAUR65	GGACCC TGGGACC	-264; -127
SAUR66	TGGGACC GGACCC	-411; -145
SAUR67	TGGGACC	-120
SAUR75	TGGGACC	-930

C

D

E

Figure 4. *SAUR63* is a direct TCP15 target. A, GUS expression in a representative plant line that contains a fusion of *SAUR63* to *gus* under the control of the *SAUR63* promoter, either in a wild-type background (i) or after transformation of this line with the *pTCP15::TCP15-EAR* construct (ii–vii are different independent transformants). Scale bars = 1 mm. B, Enrichment of sequences bound by TCPs in the promoter regions of *SAUR63* subfamily genes. A sequence logo of a sequence overrepresented in the promoters of *SAUR63* subfamily genes, obtained using the Regulatory Sequence Analysis Tool platform (http://rsat.eead.csic.es/plants/), together with a list of the sequences and their locations in the respective promoters, is shown. Sequences are from the coding strand and numbers are relative to the putative transcription start site, except for *SAUR75*, which corresponds to the complementary strand and is relative to the translation start site. C, Expression of *SAUR63-GUS* under the control of the native

express SAUR63-GUS under the control of the mutated promoter. Several lines of these plants (eight out of 10 independent lines analyzed) showed a strong decrease in GUS expression in stamen filaments when compared with lines that express SAUR63-GUS under the control of the native promoter (Fig. 4C). Notably, expression in petals was less significantly affected, whereas strong expression in rosettes was observed in all the lines (Supplemental Fig. S4). Reduced GUS expression in plants expressing SAUR63-GUS from the mutated promoter was also observed by RT-qPCR (Fig. 4D). The results indicate that TGGGACC is an active cis-acting element in the promoter region of *SAUR63*, driving expression in stamen filaments. Furthermore, this result suggests that *SAUR63* may be a direct target of TCP15 and other class-I TCPs.

Direct binding of TCP15 to the *SAUR63* promoter in vivo was analyzed by chromatin immunoprecipitation (ChIP) in *35S::TCP15-RFP* and *pTCP15::TCP15-GFP* plants. ChIP-qPCR resulted in a relative enrichment of fragment P3 of the *SAUR63* promoter, including the TGGGACC site, in both lines analyzed (Fig. 4E). By comparison, two fragments located further upstream on the *SAUR63* promoter and *ACTIN* genes showed no enrichment, whereas a fragment located downstream, within the *SAUR63* coding region, was enriched only in samples from *35S::TCP15-RFP* plants (Fig. 4E). Therefore, *SAUR63* can be considered a direct in vivo target of TCP15.

Genes Induced By GAs and ARF6/8, But Not JA-Induced Genes, Are Repressed in *pTCP15::TCP15-EAR* Plants

It was previously shown that mutants with deficiencies in GA synthesis or signaling show defects in stamen filament elongation (Cheng et al., 2004; Tyler et al., 2004; Rieu et al., 2008). We thus analyzed available microarray data for GA-regulated genes in flowers (Cao et al., 2006) and compared these with those genes exhibiting modified expression in the microarray of *pTCP15::TCP15-EAR* plants. As shown in Supplemental Figure S5, a significant overlap was observed between genes downregulated in *pTCP15::TCP15-EAR* plants and genes induced by GA or repressed by DELLA proteins, which are negative regulators of GA responses. This, together with previous reports on the inhibition of class-I TCPs by interaction with DELLAs

(Davière et al., 2014; Resentini et al., 2015), suggests that class-I TCPs may be involved in mediating GA responses in flowers. It was also shown that GAs promote JA synthesis during stamen filament elongation, but the short stamen phenotype of GA-deficient plants cannot be rescued by JA treatment (Cheng et al., 2009), leading to the suggestion that GA-dependent pathways that are independent of JA exist. In agreement with the induction of JA synthesis by GAs, there is a significant overlap between genes induced by GAs, or repressed by DELLAs, and JA-upregulated genes (Nemhauser et al., 2006; Supplemental Fig. S5). However, there is no significant overlap between genes repressed in *pTCP15::TCP15-EAR* plants and genes induced by JA (Supplemental Fig. S5). In fact, several genes repressed by JA are also repressed in *pTCP15::TCP15-EAR* plants and those induced by JA are induced in these plants (Supplemental Fig. S5), suggesting that TCP15 affects stamen filament elongation independently from the JA-related pathway.

JA-dependent filament elongation was shown to be mediated by MYB21, MYB24, and MYB57 (Cheng et al., 2009; Reeves et al., 2012). As for JA-responsive genes, most (331 out of 345; listed in Supplemental Table S3) genes upregulated by MYB21 and MYB24 (e.g. genes with reduced expression in *myb21 myb24* mutant flowers; Reeves et al., 2012) were not repressed in *pTCP15::TCP15-EAR* plants (Supplemental Fig. S5), which was further confirmed by RT-qPCR analysis of a subset of five genes with reduced expression in *myb21 myb24* mutants using RNA prepared specifically from flowers of *pTCP15::TCP15-EAR* plants. None of these genes showed reduced expression in *pTCP15::TCP15-EAR* flowers and even some of them were upregulated (Supplemental Fig. S6). Thus, the function of TCP15 and related class-I TCPs is probably not related to JA- and/or MYB21/24-dependent stamen filament elongation.

We also found a significant overlap between genes repressed in *pTCP15::TCP15-EAR* plants and those induced by ARF6 and ARF8 (i.e. genes with reduced expression in *arf6 arf8* flowers; Reeves et al., 2012; Supplemental Fig. S5; genes listed in Supplemental Table S4). We confirmed this result by measuring the expression of four genes selected from this group in *pTCP15::TCP15-EAR* flowers (Supplemental Fig. S6). Three of these genes (*IAA19*, *IAA3*, and *EXP8*) are preferentially expressed in stamen filaments

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Figure 4. (*Continued.*)
SAUR63 promoter (i; a representative line is shown), or under the control of a mutated version of the promoter in which the sequence TGGGACC located at −120 was mutagenized (ii–viii are different independent lines). Scale bars = 1 mm. Schematics of the constructs used for transformation are shown above. D, Quantification by RT-qPCR of *GUS* transcript levels in flowers of representative lines from those shown in (C). The bars indicate the mean ± SE of three biological replicates. Asterisks indicate significant differences (*P* < 0.05; Student's *t* test). E, ChIP analysis of the binding of TCP15-GFP to the *SAUR63* promoter region. The results of two independent experiments, one with *35S::TCP15-RFP* plants and the other with plants that express TCP15-GFP under the control of the native *TCP15* promoter, are shown. Primer pairs for the amplification of different regions of the *SAUR63* gene (P1–P4) and of *ACT2* and *ACT8* genes (ACT; control) were used. A schematic of the genomic region analyzed, indicating the location of the different fragments, is shown to the left. The results are expressed as the ratio of the signal obtained after immunoprecipitation with specific antibodies and with anti-IgG (control).

(Supplemental Fig. S7), suggesting that the observed expression changes in flowers are related to changes in this organ. In addition, *IAA19* expression levels are related to stamen filament elongation (Tashiro et al., 2009; Cecchetti et al., 2017; Ghelli et al., 2018). Most of the aforementioned *SAUR63* subfamily genes also belong to this group of genes with reduced expression in *pTCP15::TCP15-EAR* and *arf6 arf8* flowers accompanied by preferential expression in stamen filaments (Supplemental Fig. S7). These results raise the possibility that class-I TCPs also participate in auxin-dependent responses. On the other hand, none of the class-I TCP genes show significant expression changes in *arf6 arf8* flowers (Reeves et al., 2012). Many of the genes induced by ARF6 and ARF8 are also induced by GAs or repressed by DELLAs (Supplemental Fig. S5). Based on this analysis, it can be speculated that TCP15 and related class-I TCPs participate in GA-dependent and/or auxin-dependent stamen filament elongation pathways. The action of class-I TCPs may be at least partially related to the induction of *SAUR63* subfamily genes, which are direct targets of TCP15 and probably other TCPs. However, it is conceivable that other genes either directly or indirectly regulated by the TCPs are also involved.

Overexpression of TCP15 Restores Stamen Filament Elongation in a GA-Deficient Background

To analyze a possible role of class-I TCPs in GA-dependent expression of *SAUR63* subfamily genes, we analyzed transcript levels of these genes in flowers of *ga1*-mutant plants, deficient in an early step of GA biosynthesis. Most *SAUR63* subfamily genes, except *SAUR61* and *SAUR75*, showed reduced expression in GA-deficient plants (Fig. 5A), indicating that they are under GA regulation. If reduced expression of *SAUR63* subfamily genes in GA-deficient plants is related to decreased activity of the TCPs, overexpression of TCP15 would restore the expression levels of these genes. As shown in Figure 5A, expression of TCP15 from the *35SCaMV* promoter restored the expression of most *SAUR63* subfamily genes that were repressed in the GA-deficient background.

In addition, TCP15 overexpression increased stamen elongation of *ga1*-mutant plants (Fig. 5, B and C), indicating that decreased activity of the TCPs is a likely cause of decreased filament elongation in these plants. Notably, flowers from *ga1* plants that were over-expressing TCP15 remained smaller than wild-type flowers, but their stamen filaments reached the top of the gynoecium, unlike those of *ga1* plants (Fig. 5C). This suggests that other GA-dependent pathways, not related to TCP15, are also important to determine the size of flower organs. Despite that stamens reached the top of the gynoecium, we did not observe restoration of fertility in TCP15-overexpressing *ga1* plants. This may be due to the fact that *ga1* plants show male and female infertility (Plackett et al., 2012). In addition to stamens,

the length of petals was also increased upon TCP15 overexpression (Fig. 5C), suggesting that TCP15 may also be involved in promoting the elongation of these organs.

Measurement of *TCP15* transcript levels indicated that *TCP15* and *TCP22* are also repressed in the *ga1* mutant (Fig. 5D). For *TCP15*, this agrees with results of a microarray experiment with *ga1* flowers (Cao et al., 2006). A slight repression was also observed for *TCP14*, whereas *TCP8* was induced in the mutant (Fig. 5D). Thus, decreased expression of class-I TCPs in the GA-deficient background may partly explain the repression of *SAUR63* subfamily genes observed in these plants. The reported inhibition of class-I TCPs by DELLA proteins (Davière et al., 2014), which would be more pronounced in the GA-deficient background, is likely another factor playing a key role in this regulation.

We also analyzed the response of *SAUR63* to GA in wild-type plants. For this purpose, we treated inflorescences with the GA-synthesis inhibitor paclobutrazol (PAC). The expression of *SAUR63* was significantly reduced in wild-type plants 4 h after a single PAC treatment (Fig. 5E), most likely due to a decrease in endogenous GA levels. In agreement, treatment with GA, in addition to PAC, restored normal *SAUR63* transcript levels (Fig. 5E). To analyze if class-I TCPs are required for the correct expression of *SAUR63* in response to GA, we also treated *amiR-27* plants with PAC and PAC + GA. Contrary to the wild type, *SAUR63* expression was not significantly modified by the treatments in *amiR-27* plants (Fig. 5E), indicating that TCP15 and related class-I TCPs participate in GA-dependent modulation of *SAUR63* gene expression. We also measured the expression of other *SAUR63* subfamily genes with reduced expression in *amiR-27* plants. For *SAUR61* and *SAUR75*, we observed a similar behavior to that of *SAUR63* (Supplemental Fig. S8). Notably, these two genes did not show reduced expression in *ga1* plants (Fig. 5A). For *SAUR62* and *SAUR68*, we did not observe a significant effect of the PAC treatment on expression, although *SAUR68* was induced by GA in wild-type plants and not in *amiR-27* plants (Supplemental Fig. S8). The differences observed between *ga1* and wild-type plants treated with PAC may reflect differences in the response of *SAUR* genes to constitutive versus transient GA deprivation. Nevertheless, the results indicate that most *SAUR63* subfamily genes undergo regulation by GA levels in flowers, and that TCP15 and related class-I TCPs participate in this regulation.

Induction of *SAUR63* Subfamily Genes By Auxin Is Not Compromised in Class-I TCP-Deficient Plants

SAUR genes were discovered based on their rapid response to auxin (McClure and Guilfoyle, 1987). Despite later studies showing that not all *SAUR* genes are induced by auxin, those belonging to the *SAUR63*
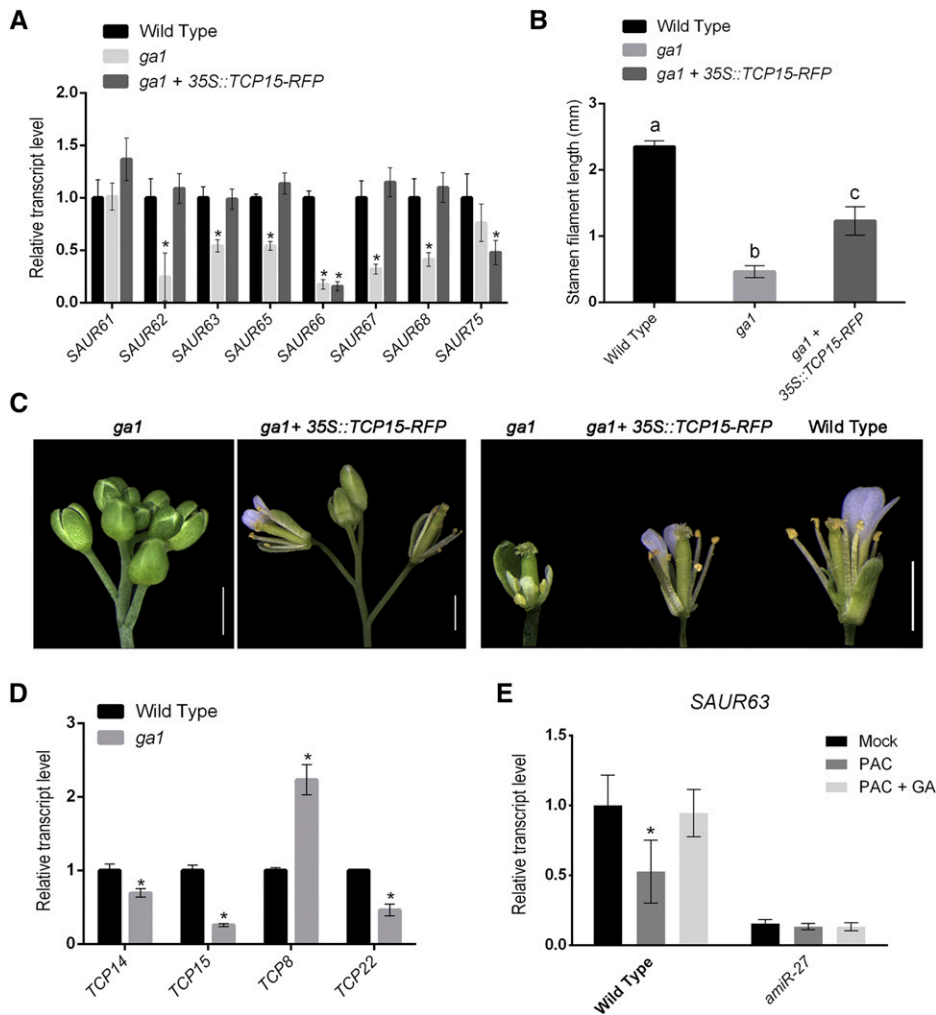
Figure 5. Class-I TCPs participate in GA-dependent stamen filament elongation and *SAUR63* subfamily gene expression. A, Transcript levels of *SAUR63* subfamily genes in flowers from wild-type, *ga1* plants, and *ga1* plants transformed with the *35S::TCP15-RFP* construct. The results are expressed relative to wild type. Values represent the mean ± SE of three biological replicates. Asterisks indicate significant differences with wild-type plants ($P < 0.05$; Student's *t* test). B, Stamen filament length in flowers at stage 13 of wild-type plants, *ga1* plants, and *ga1* plants that express *TCP15-RFP* under the control of the *35SCaMV* promoter. The bars indicate the mean ± SD (n = 11–23 stamens, depending on the line). Different letters indicate significant differences ($P < 0.05$; one-way ANOVA for multiple comparisons). C, Phenotype of *ga1* plants before and after transformation with a *35S::TCP15-RFP* construct. A representative image of five independent lines is shown. A flower of a wild-type plant is included for comparison. Scale bars = 1 mm. D, Transcript levels of class-I TCP genes in flowers from wild-type and *ga1* plants. The results are expressed relative to wild type. Values represent the mean ± SE of three biological replicates. Asterisks indicate significant differences with wild-type plants ($P < 0.05$; Student's *t* test). E, *SAUR63* transcript levels in wild-type and *amiR-27* plants 4 h after a single treatment with 10 μM of PAC (PAC) or 10 μM of PAC plus 100 μM of GA$_3$ (PAC + GA). Control plants (Mock) were mock-treated with solvent solution. Values are expressed as relative to wild type under control conditions and represent the mean ± SE of three biological replicates. Asterisks indicate significant differences between control and treated plants of the same genetic background ($P < 0.05$; Student's *t* test).

subfamily are indeed reported as auxin-inducible (Nagpal et al., 2005; Chapman et al., 2012) and targets of ARF6 (Oh et al., 2014). In addition, most members of the subfamily show reduced expression in flowers of *arf6 arf8* mutants (Nagpal et al., 2005; Reeves et al., 2012). We thus analyzed the effect of auxin treatment on *SAUR63* subfamily gene expression in wild-type and *amiR-27* flowers, the latter deficient in several class-I TCPs. A significant induction by auxin was

observed for all tested *SAUR63* subfamily genes in wild-type plants and *amiR-27* plants (Fig. 6A). Particularly for *SAUR61-63* and *SAUR68*, transcript levels after auxin treatment were lower in *amiR-27* plants than in wild-type plants (Fig. 6A). However, the relative induction of *SAUR63* subfamily genes by auxin was similar (or even higher in the case of *SAUR68* and *SAUR75*) in *amiR-27* and wild-type plants when compared with mock-treated plants (Fig. 6B).
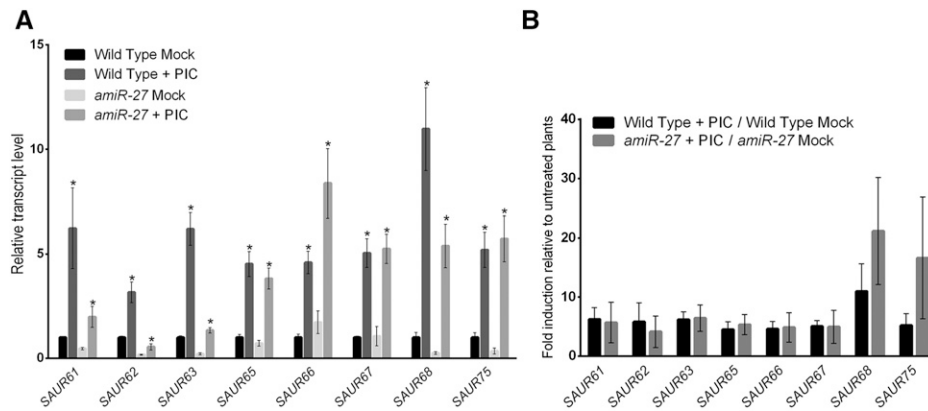
Figure 6. Induction of *SAUR63* subfamily genes by auxin is not compromised in class-I TCP-deficient plants. A, Transcript levels of *SAUR63* subfamily genes in wild-type and *amiR-27* plants 4 h after a single treatment with 100 μM of the synthetic auxin picloram (+ PIC). Control plants (Mock) were mock-treated with solvent solution. Values are expressed as relative to wild type under control conditions and represent the mean ± SE of three biological replicates. Asterisks indicate significant differences between control and treated plants of the same genetic background ($P < 0.05$; Student's *t* test). B, Relative induction of *SAUR63* subfamily genes by auxin in wild-type and *amiR-27* plants. The fold induction of each gene, relative to mock-treated plants, is indicated.

These results indicate that the response of *SAUR63* subfamily genes to auxin is not significantly impaired by a deficiency in the class-I TCPs studied here, suggesting that these TCPs do not participate in the induction of these genes by auxin. The results also suggest that, at least for *SAUR61-63* and *SAUR68*, auxin cannot fully replace the lack of class-I TCPs, indicating that the TCPs are required, in addition to auxin, for maximal expression of these genes.

DISCUSSION

In this work, we report that plants affected in the function of class-I TCPs show defects in stamen filament elongation. This was observed for plants that express a fusion of TCP15 to the EAR repression domain under the control of the *TCP15* promoter (*pTCP15::TCP15-EAR* plants), but not in loss-of-function mutants in *TCP15* and the related class-I gene *TCP14*. Because TCP15-EAR most likely represses the expression of genes that are under direct control of TCP15, the results suggest that TCP15 is involved in the regulation of genes that affect stamen filament elongation. This is also supported by the fact that overexpression of TCP15 yielded plants with longer filaments. The opposite effects on filament elongation observed after expressing the native and repressor forms of TCP15 indicates that TCP15 acts as an activator in this process. The lack of appreciable changes in filament length after loss-of-function of *TCP15* is probably due to redundancy of TCP15 with other class-I TCPs, which was previously observed among members of this class in different developmental contexts (Kieffer et al., 2011; Uberti-Manassero et al., 2012; Aguilar-Martínez and Sinha, 2013). In agreement, plants affected in *TCP8* and *TCP22*, in

addition to *TCP14* and *TCP15* (*amiR-27* plants), show shorter filaments.

Members of the *SAUR63* subfamily of *SAUR* genes are required for correct stamen filament elongation (Chae et al., 2012), a process mainly governed by cell expansion (Tashiro et al., 2009). The fact that several members of the *SAUR63* subfamily show reduced expression in *pTCP15::TCP15-EAR* and *amiR-27* plants and that overexpression of *SAUR63* rescues the short stamen and infertility phenotypes of *pTCP15::TCP15-EAR* plants suggest that reduced expression of *SAUR63* subfamily genes is responsible for defective stamen elongation in these plants. Interestingly, overexpression of *SAUR63* also promotes the elongation of petals, which are shorter than wild type in *pTCP15::TCP15-EAR* plants. This is consistent with the fact that *SAUR63* subfamily genes are also required for petal growth (Chae et al., 2012) and suggests that the regulation of *SAUR63* subfamily genes by class-I TCPs described here may also be important for this process. Indeed, reduced expression of *SAUR63* was observed in both petals and stamens of *pTCP15::TCP15-EAR* and *amiR-27* plants. Notably, reporter gene assays indicated that TCP15 is mainly expressed in the vasculature of stamen filaments, whereas organ elongation is thought to be limited mainly by epidermal cell expansion (Savaldi-Goldstein and Chory, 2008). Chae et al. (2012) observed that SAUR63 is also more prominently expressed in filament vascular tissues and hypothesized that it may have a role in redirecting auxin flux to peripheral tissues. The similar expression characteristics of *TCP15* and *SAUR63* within stamen filaments supports a role for TCP15 in regulating *SAUR63* gene expression.

Analysis of the proximal promoter regions of different members of the *SAUR63* subfamily revealed the existence of putative TCP binding motifs. At least for

SAUR63, mutation of this motif leads to considerably reduced expression in flowers, and particularly in stamen filaments, as deduced from GUS histochemical assays. This, together with the observed binding of TCP15 to the promoter region containing this motif, strongly indicates that *SAUR63* subfamily genes are direct targets of TCP15 and related class-I TCPs. It is noteworthy that several members of the *SAUR63* subfamily were also uncovered as target genes of other transcription factors, namely BZR1 (Sun et al., 2010), PIF4 (Oh et al., 2012), and ARF6 (Oh et al., 2014). Thus, class-I TCPs can be added to this set of transcription factors that directly impinge on *SAUR63* subfamily gene expression to regulate cell growth. We show here that the response of members of the *SAUR63* subfamily to auxin treatment of flowers is not significantly compromised in plants defective in TCP15 and related class-I TCPs, suggesting that the TCPs are not essential for the response to auxin, which is most likely mediated by the ARFs. Previous reports indicated that *35S::TCP15* and *pTCP15::TCP15-EAR* plants show lower and higher expression, respectively, of the auxin reporter *DR5::GUS* (Uberti-Manassero et al., 2012; Lucero et al., 2015; Viola et al., 2016), indicating that TCP15 has a negative impact on expression from auxin response elements, to which ARF transcription factors bind. In fact, a higher response to auxin could be observed for some members of the *SAUR63* subfamily, considering that basal expression levels were lower in TCP-deficient plants. Thus, auxin-dependent expression of *SAUR63* subfamily genes would not be mediated by class-I TCPs. However, the TCPs, as well as ARFs, seem to be required for correct expression of *SAUR63* subfamily genes during stamen filament elongation, most likely directly acting through their respective target sites in the promoters of these genes.

Previous reports showed that GAs promote the synthesis of JA during late stages of stamen development (Cheng et al., 2009). JA, in turn, induces the expression of three *MYB* genes required for filament growth and anther maturation (Mandaokar et al., 2006). Further analysis indicated that induction of these *MYB* genes after JA treatment did not restore normal stamen growth in a GA-deficient background (Cheng et al., 2009), leading to the conclusion that alternative GA-dependent pathways exist. In agreement with this, the short stamen phenotype of plants deficient in the GA transporter GTR1 can be rescued by GA treatment, but not by JA, whereas the anther maturation defects of JA-deficient plants can be rescued by JA, but not by GA (Saito et al., 2015). Based on this, Saito et al. (2015) proposed that GA and JA pathways act in parallel and coordinate each other to modulate stamen development, and that anther maturation is primarily controlled by JA, whereas filament elongation is under GA control. The fact that overexpression of TCP15 rescues the short stamen phenotype of GA-deficient plants suggests that TCP15 and related class-I proteins are major players in a GA-dependent pathway controlling
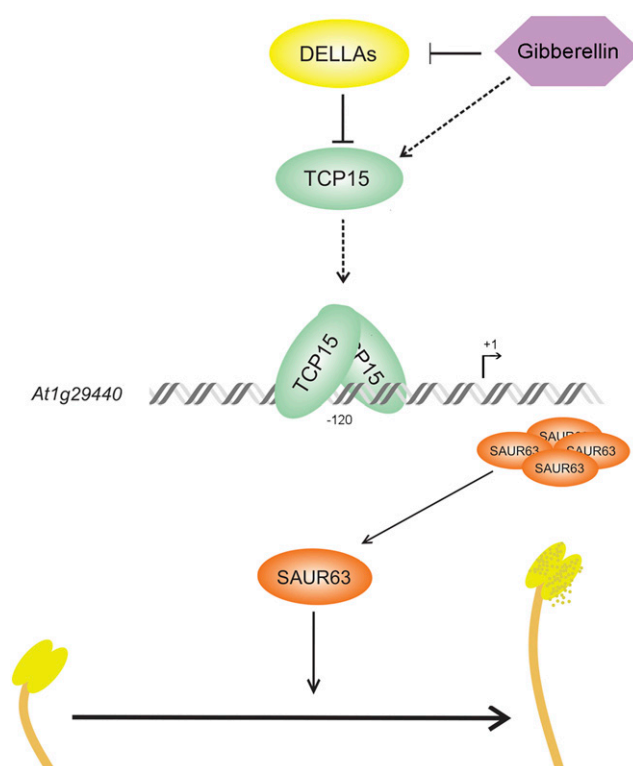

Figure 7. Proposed model for the involvement of TCP15 and other class-I TCPs in the stimulation of stamen filament elongation by GAs. GAs induce expression of *TCP15* and activate class-I TCP proteins through GA-dependent DELLA degradation. Class-I TCP proteins induce *SAUR63* and related genes by direct interaction with TCP target sites present in their promoters. SAUR63 proteins stimulate stamen filament elongation.

stamen filament elongation. Furthermore, we showed that the expression of *TCP15* is reduced in GA-deficient plants. However, the observed repression of *TCP15* would not be enough to explain the short stamen phenotype of GA-deficient plants, because the single *tcp15* mutant shows normal stamens. The reported interaction of class-I TCPs with DELLA proteins (Davière et al., 2014; Resentini et al., 2015) provides an additional mechanism for the regulation of TCP action by GAs. In addition, many of the genes induced by GAs in flowers show reduced expression in *pTCP15::TCP15-EAR* plants, suggesting that they may be under the control of TCP15 and related class-I TCPs. Specifically, our experiments showing that the changes in expression of *SAUR63* and related genes observed after PAC or GA treatment are attenuated in TCP-deficient plants indicate that the TCPs are required for the induction of these genes by GA. Moreover, the restoration of filament growth in GA-deficient plants brought about by TCP15 overexpression was accompanied by an increase in the expression of most *SAUR63* subfamily genes. Thus, one of the mechanisms involved in GA-dependent stamen filament elongation most likely involves the activation of class-I TCPs to induce

growth through changes in the expression of *SAUR63* subfamily genes.

In conclusion, our results show that TCP15 and related class-I TCPs link GA action to stamen filament elongation by direct transcriptional activation of *SAUR63* subfamily genes through target sites present in their promoters (Fig. 7). The results presented here contribute to a more comprehensive understanding of the pathway behind GA-dependent stamen filament elongation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) lines used in this study were in the Columbia (Col-0) ecotype. Lines that express TCP15 and TCP15-RFP from the *35SCaMV* promoter (*35S::TCP15* and *35S::TCP15-RFP*) were described in Lucero et al. (2015) and Viola et al. (2016), as well as lines that express GUS or TCP15-EAR under the control of the *TCP15* promoter (Uberti-Manassero et al., 2012). The *tcp14-4 tcp15-3* mutant was described by Kieffer et al. (2011) and provided by Dr. Simona Masiero (Universitá degli Studi di Milano), whereas *amiR-27* plants, which express an artificial micro RNA directed against *TCP8* and *TCP22* in the *tcp14-4 tcp15-3* background (Davière et al., 2014), were kindly sent by Drs. Jean-Michel Davière and Patrick Achard (Institut de Biologie Moléculaire des Plantes). A homozygous *ga1* (SALK_109115) T-DNA insertion mutant was provided by Dr. Stephen Thomas (Rothamsted Research). Plants were grown in pots filled with soil under long-day conditions (16-h light/8-h dark) at an intensity of 100 μmol m^{-2} s^{-1} and a temperature of 22°C to 24°C. For auxin, GA, and PAC treatments, the inflorescences of plants were sprayed with 100 μM of picloram, 100 μM of GA$_3$, and 10 μM of PAC (dissolved in ethanol) and samples were harvested 4 h later for expression analysis. Controls were mock-treated with the respective solutions without the reagent.

DNA Constructs and Plant Transformation

For expression of TCP15 or SAUR63 fused to GFP, the respective coding regions, without the stop codons, were amplified with specific primers (Supplemental Table S5) and cloned into entry vector pENTR3C (Thermo Fisher Scientific). For expression driven by the *35SCaMV* promoter, the inserts were then recombined into the pGreen-based binary vector pFK248 (Hellens et al., 2000) using the Gateway cloning system. For expression of TCP15-GFP from the *TCP15* promoter, an ~1,500-bp sequence located upstream of the translation start codon used previously to analyze *TCP15* expression (Uberti-Manassero et al., 2012) was amplified (Supplemental Table S5) and cloned into the *Sal*I and *Kpn*I sites of the pENTR3C construct that contained the *TCP15* coding region between *Kpn*I and *Xho*I. The fragment containing the *TCP15* promoter and coding region was then recombined into vector pFAST-R07 (Shimada et al., 2010). The *pTCP15::TCP15-GFP-GUS* reporter construct was generated by recombining the fragment into vector pKGWFS7. The constructs expressing SAUR63-GUS under the control of the native or mutated *SAUR63* promoters were obtained by amplification of a 1,256-bp fragment comprising sequences located upstream of the *SAUR63* translation stop codon (Supplemental Table S5), followed by cloning into the *Sal*I and *Bam*HI sites of pBluescript SK$^-$. After mutagenesis of the *SAUR63* promoter through the QuikChange method (Xia et al., 2015) using Phusion DNA polymerase (Thermo Fisher Scientific), the fragments were cloned into pBI101.3. All constructs were verified by DNA sequencing. The *pTCP15::TCP15-EAR* construct, used to express a fusion of TCP15 to the EAR repressor domain, was previously described by Uberti-Manassero et al. (2012). Plants were transformed using floral dip (Clough and Bent, 1998).

Phenotypic Analysis

Stamen lengths shown in Figure 1C were measured with a caliper under a stereoscopic microscope. Alternatively, flowers at different stages were photographed using a model no. MZ10F stereomicroscope (Leica) equipped with a digital camera and filament length was determined using the software ImageJ

(Schindelin et al., 2012). Because wild-type Arabidopsis flowers have four long and two short stamens, only the long stamens in each flower were considered. For SEM analysis, flowers were fixed in formalin/acetyl alcohol/acetic anhydride/water (10:50:5:35 v/v) during 24 h, transferred to 70% (v/v) ethanol, and dissected under an SMZ-10 stereomicroscope (Nikon). After dehydration with successive rounds of ethanol solutions (80%, 96%, and 100% [v/v]), samples were transferred to acetone for desiccation with CO$_2$ in an Emitech K850 critical point drier (UK-RAS Network). Finally, they were coated with gold-palladium and photographed with either an FEI Quanta 200 (Thermo Fisher Scientific) or a model no. XL30 TMP New Look SEM (Philips) at Centro Científico Tecnológico Consejo Nacional de Investigaciones Científicas y Técnicas, Rosario (Argentina) or Museo Bernardino Rivadavia, respectively.

Analysis of Gene Expression

Transcript levels were measured through RT-qPCR. RNA was prepared from inflorescences (complete bolts) or isolated organs from flowers at stage 13 using TRIzol reagent followed by LiCl precipitation. Reverse transcription was performed with an oligo(dT)$_{18}$ primer and MMLV reverse transcriptase (Promega) on 1.5–2.0 μg of RNA. An aliquot of the complementary DNA was used for qPCR with specific primers for the genes of interest (Supplemental Table S5). Amplified products were monitored by SYBR Green detection in an Applied Biosystems StepOnePlus apparatus (Thermo-Fisher Scientific). A comparative C_t method, with *ACT2* and *ACT8* actin genes for normalization (Charrier et al., 2002), was used to calculate relative transcript levels. For histochemical analysis of GUS expression, flowers were incubated in 1 mM of 5-bromo-4-chloro-3-indolyl-beta-D-GlcA, 50 mM of sodium phosphate (pH 7.0), and 0.1% (v/v) Triton X-100. Vacuum was applied for 5 min and reactions were incubated at 37°C until satisfactory staining was observed. When comparing different lines, incubation times were the same for all lines analyzed. GFP fluorescence in stamens at different stages was detected using a model no. TCS SP8 confocal microscope (Leica) with excitation at 488 nm and detection at 498–531 nm.

ChIP

ChIP was performed on inflorescences of plants that express TCP15-RFP or TCP15-GFP under the control of the *35SCaMV* promoter or the *TCP15* promoter, respectively, with anti-RFP (632496; Clontech), anti-GFP (ab290; Abcam), and anti-IgG (ab6702; Abcam) antibodies, mainly as described by Ariel et al. (2014). After cross linking and extraction, chromatin was sonicated in a Bioruptor Pico water bath (Diagenode; 10 cycles of 30 s on-/30 s off-pulses at high intensity, using Bioruptor microtubes). For immunoprecipitation, samples were incubated for 12 h at 4°C with Protein A Dynabeads (Invitrogen) precoated with the corresponding antibodies. Immunoprecipitated DNA was recovered using phenol/chloroform/isoamyl alcohol mix (25:24:1) and ethanol precipitation. The amount of specific genomic regions in the immunoprecipitated DNA was analyzed by qPCR using primers listed in Supplemental Table S5. Untreated sonicated chromatin was processed in parallel and considered the input sample.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers: *TCP8* (At1g58100), *TCP22* (At1g72010), *TCP14* (At3g47620), and *TCP15* (At1g69690). Accession numbers for other genes are listed in Table 1 and Supplemental Tables S3 and S4.

Supplemental Data

The following supplemental information is available.

Supplemental Figure S1. Transcript levels of class-I TCPs in stamen filaments of *amiR-27* and *pTCP15::TCP15-EAR* plants.

Supplemental Figure S2. *ARF6* and *ARF8* transcript levels in flowers of *pTCP15::TCP15-EAR* plants.

Supplemental Figure S3. Stamen length of *35S::SAUR63-GFP* plants.

Supplemental Figure S4. Expression in rosettes of *SAUR63-GUS* under the control of the native or mutated versions of the *SAUR63* promoter.

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