Direct Regulation of the *EFR*-Dependent Immune Response by *Arabidopsis* TCP Transcription Factors

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Accepted 11 November 2018.

One layer of the innate immune system allows plants to recognize pathogen-associated molecular patterns (PAMPs), activating a defense response known as PAMP-triggered immunity (PTI). Maintaining an active immune response, however, comes at the cost of plant growth and development; accordingly, optimization of the balance between defense and development is critical to plant fitness. The *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR* (TCP) transcription factor family consists of well-characterized transcriptional regulators of plant development and morphogenesis. The three closely related class I TCP transcription factors TCP8, TCP14, and TCP15 have also been implicated in the regulation of effector-triggered immunity, but there has been no previous characterization of PTI-related phenotypes. To identify TCP targets involved in PTI, we screened a PAMP-induced gene promoter library in a yeast one-hybrid assay and identified interactions of these three TCPs with the *EF-Tu RECEPTOR* (*EFR*) promoter. The direct interactions between TCP8 and *EFR* were confirmed to require an intact TCP binding site in planta. A *tcp8 tcp14 tcp15* triple mutant was impaired in *EFR*-dependent PTI and exhibited reduced levels of PATHOGENESIS-RELATED PROTEIN 2 and induction of *EFR* expression after elicitation with elf18 but also increased production of reactive oxygen species relative to Col-0. Our data support an increasingly complex role for TCPs at the nexus of plant development and defense.

Keywords: *Arabidopsis thaliana*, *EFR*, plant immunity, *Pseudomonas syringae*, PTI, TCP, transcription factors

Eukaryotic organisms are exposed to a variety of potentially pathogenic microbes in their native environments. Plants are genetically equipped with a sophisticated, multilayered innate immune system that provides basal resistance to most

pathogens (Cook et al. 2015). In an initial layer of defense, plants utilize specific pattern recognition receptors (PRRs) to detect the extracellular presence of conserved pathogen-associated molecular patterns (PAMPs), including structural elements like bacterial flagellin or fungal chitin. PRR activation triggers a series of intertwined signal transduction events that coalesce in the nucleus to produce changes in defense gene expression and, ultimately, host physiology to inhibit pathogen virulence, a response known as PAMP-triggered immunity (PTI) (Boller and Felix 2009; Macho and Zipfel 2014). A counterattack in the ‘evolutionary arms race’, pathogens have developed mechanisms for suppression of host PTI responses to re-establish virulence. This strategy is facilitated by the secretion of potent effector molecules into the host cell, which often target and influence critical host signaling hubs to disrupt immune signaling and other host processes (Ahmed et al. 2018; Gassmann and Bhattacharjee 2012; Mukhtar et al. 2011). To protect themselves, plants deploy a second layer of defense, using intracellular immune receptors called resistance (R) proteins that activate on detection of pathogen effector activity. This triggers a stronger and more-sustained version of the PTI response, called effector-triggered immunity (ETI), that is often manifested by a hypersensitive response and localized cell death, an effective strategy against biotrophic pathogens (Cui et al. 2015; Peng et al. 2018).

Though effective, the plant immune response is metabolically expensive and comes at the cost of growth, development, and yield in model and crop species (Brown 2002; Ning et al. 2017). The tradeoff is exemplified classically in both *Arabidopsis thaliana* seedlings continually exposed to PTI elicitors and in mutants exhibiting constitutively active immune signaling (Gómez-Gómez et al. 1999; van Wersch et al. 2016). Accordingly, plants exert tight control over the expression of defense genes to maintain an environmentally contextual balance between growth and immunity (Couto and Zipfel 2016; Garner et al. 2016). A goal, then, for pathogens is to disrupt that balance, using effectors to influence host prioritization toward growth and increased susceptibility. Our understanding of the mechanisms by which plants mediate this tradeoff and the many proteins involved is still very limited, but as the knowledge base of effector targets expands, so does the pool of interesting candidates for further investigation.

The plant-specific *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR* (TCP) gene family is a newer addition to the catalog of eukaryotic transcriptional regulators that encodes a group of transcription factors (TFs) featuring a variant basic helix-loop-helix ‘TCP’ domain associated with protein-DNA and protein-protein interactions. (Nicolas and

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Funding: This work was supported by a Missouri University Division of Plant Sciences Daniel F. Millikan Fellowship to B. J. Spears, a scholarship from the Alabama State Graduate Research Scholars Program to T. C. Howton, a grant by the University of Missouri Research Board (RB 11-29) to W. Gassmann, and National Science Foundation Division of Integrative Organismal Systems (IOS) grants 1121114 and 1456181 to W. Gassmann and 1557796 to M. S. Mukhtar.

*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures and two supplementary tables are published online.

Cubas 2016). The 24 family members identified in *Arabidopsis thaliana* are divided by TCP domain structure and preferred recognition sequence into two classes (Cubas et al. 1999). Phenotypic analysis in the family is often difficult due to high levels of redundancy and both intra- and interclass dimerization that facilitates TCP activity (Danisman et al. 2013). Both classes of TCPs have been described as critical regulators of plant development in a wide range of processes including cell proliferation, seed germination, stature, flowering, and leaf morphology, likely integrating the signals of multiple potentially antagonistic phytohormones (Aguilar-Martínez and Sinha 2013; Danisman et al. 2012; Li et al. 2012; Lucero et al. 2015, 2017; Resentini et al. 2015; Steiner et al. 2012; Uberti-Manassero et al. 2012). Plant immunity has recently been added to the growing list of TCP-regulated biological processes. Several class I TCPs have been characterized as positive regulators of ETI activated by the hemibiotrophic bacterial pathogen *Pseudomonas syringae* and have been shown to interact with the negative immune regulator SUPPRESSOR OF rps4-RLD1 (SRFR1), possibly to modulate their activity (Kim et al. 2014). Other TCPs, including TCP8, have also been shown to directly promote the expression of *ICS1* (Wang et al. 2015), a biosynthesis gene for the phytohormone salicylic acid (SA) and critical regulator of immunity to biotrophic pathogens (Pieterse et al. 2012). The targeting of TCPs by effectors from multiple pathogen species strongly supports a critical role for this TF family in the regulation of plant immunity, with current evidence pointing toward a manipulation of phytohormone balances to promote pathogen virulence (Lopez et al. 2015; Mukhtar et al. 2011; Sugio et al. 2011; Weßling et al. 2014; Yang et al. 2017). As a protein family situated at the interface of plant development and defense with expanding roles on both sides of the tradeoff, TCPs are prime candidates for further dissection of the signaling networks governing plant-pathogen interactions.

With such a wide array of developmental roles characterized for class I TCPs, it is likely that TCP-mediated regulation of plant immunity similarly occurs through multiple signaling networks by unexplored mechanisms. In this study, we have characterized a novel role for several class I TCP TFs as enhancers of the plant PTI response. Direct interaction was observed between several TCPs and the promoter of the PRR gene *EF-Tu RECEPTOR (EFR)* in *Arabidopsis thaliana* in a TCP binding site-dependent manner. In accordance with these results, a higher-order *tcp* mutant exhibits several *efr* mutant phenotypes, including reduced *EFR*-dependent elicitation of PTI and resistance to *P. syringae*.

RESULTS

Class I TCP TFs interact with the *EFR* promoter in yeast.

To evaluate a potential role for TCP activity in PTI, we aimed to test the direct binding of TCP proteins to the regulatory regions of a set of 28 genes known to be involved in the PAMP or SA response that also contain predicted binding elements for class I or class II TCPs. Toward this, we cloned the 800-bp fragments immediately upstream of the transcription start site of these genes to be used as the bait in a pairwise yeast one-hybrid (Y1H) assay. In parallel, we also cloned coding sequences of eight members of the TCP family, namely, TCP2, TCP3, TCP4, TCP8, TCP10, TCP14, TCP15, and TCP21, belonging to both class I and class II families, into a prey vector. The selection of these particular family members was based on their individual roles in the regulation of either jasmonic acid biosynthesis and signaling controlled by miR319a for the class II TCPs (Schommer et al. 2008) or potentially antagonistic SA biosynthesis and signaling for the class I TCPs (Lopez et al. 2015). Of

the 28 promoters, 23 could be integrated successfully into the yeast genome and could be tested against the selected TCP TFs; those data are presented in Supplementary Table S1. In our pairwise Y1H test, we found that TCP8 and TCP14 strongly interact with the *EFR* promoter, while TCP15 and the class II TCP2 exhibit relatively weak interactions. All other class II TCPs tested and the class I TCP21 failed to interact at even the lowest stringency (Fig. 1; Supplementary Fig. S1). The specificity of the TCP8 interaction with the *EFR* promoter is supported by failed interactions with other tested promoters (Supplementary Fig. S2). These interactions suggest that specific members of the TCP family play a vital role in driving the expression of *EFR* and provide a link between the TCP proteins and PTI, at least via *EFR*. The general activity of TCP8 has been relatively unexplored as compared with either TCP14 and TCP15, which, combined with its very recently identified role in plant immunity, made it an attractive candidate to pursue further.

TCP8 directly binds and activates the *EFR* promoter in planta via class I TCP binding site.

To validate the interactions observed in our Y1H experiments, we generated native promoter-driven *pTCP8:TCP8-HA* transgenic *Arabidopsis thaliana* lines for use in chromatin immunoprecipitation (ChIP) assays. Functionality of the tagged TCP8-HA was evaluated through complementation of effector-triggered susceptibility to infection by *P. syringae* DC3000 expressing *avrRps4* in the *tcp8 tcp14 tcp15* triple mutant background (Supplementary Fig. S3), as few other clear *tcp8* phenotypes have been characterized to date (Kim et al. 2014; Wang et al. 2015). The *EFR* promoter contains a putative class I TCP binding site 99 bp upstream of the transcription start site (TSS) (−99), around which we designed primers for quantitative ChIP polymerase chain reaction (PCR) analysis (Fig. 2A). After immunoprecipitation of crosslinked TCP8-DNA complexes by anti-hemagglutinin (anti-HA) antibody and subsequent purification, we observed significant enrichment of this region of the *EFR* promoter relative to both nontarget (*pAt18s*) and no-antibody controls (Fig. 2B), supporting the direct interaction of TCP8 with the *EFR* promoter in planta.

We further confirmed the in-planta capabilities of TCP8 to directly bind and activate the *EFR* promoter using a β -glucuronidase (GUS)-based transactivation assay in *Nicotiana benthamiana* (Jefferson et al. 1987). Briefly, we generated *pEFR:GUS* reporter constructs and transformed them into *Agrobacterium tumefaciens* GV3101. After coinfiltration of strains expressing either the reporter constructs or HA-TCP8 in *N. benthamiana* leaves, we measured GUS expression qualitatively by staining and quantitatively by fluorescence assays

Fig. 1. TCP8, TCP14, and TCP15 interactions with the *EFR* promoter in yeast. A yeast one-hybrid assay was performed using an 800-bp fragment of the regulatory region of *EFR* as bait and TCP8, TCP14, and TCP15 as preys. The diploid yeast was spotted on synthetic dropout lacking histidine and tryptophan media containing 20 mM and 50 mM 3-amino-1,2,4-triazole (3-AT).

with methylumbelliferyl- β -glucuronide (4-MUG) as a substrate to measure promoter binding and transactivation. We observed strong transactivation of *pEFR:GUS* by HA-TCP8 relative to the reporter-only control, further demonstrating that TCP8 directly regulates the *EFR* promoter in planta (Fig. 3). TCP15 was also capable of activating the reporter construct but to much lower levels, despite levels of protein expression similar to TCP8 (Supplementary Fig. S4). These data could suggest that TCP8 may be the primary binding protein or, possibly, that the interaction of TCP14 or TCP15 with the promoter sequence may require TCP8 or the cooperative efforts of multiple TCP proteins. Interestingly, mutating the putative TCP binding site at -99 bp (TGCCCCAC to AAAATTTT) heavily attenuated the activation by HA-TCP8, with no impact on endogenous background activation in the absence of TCP8 protein. Combined with our ChIP-PCR data, this suggests that activation of the *EFR* promoter is dependent mainly on TCP8 and its direct interaction with the class I TCP binding site.

PAMP-induced transcriptional output is inhibited in the *tcp* triple mutant.

With evidence pointing to the direct regulation of *EFR* expression by TCP8 and other class I TCPs, we reasoned that *tcp8* and other related mutants would exhibit phenotypes associated with the *efr* mutant, though possibly dampened due to functional redundancy within the TCP family. Previously established changes in ETI phenotypes were masked in single and double mutants, requiring a higher-order *tcp* triple mutant to become evident (Kim et al. 2014). Accordingly, we decided to interrogate this line for *EFR*-dependent phenotypes.

Given that previous studies have demonstrated induction of *EFR* by PAMP treatment (Tintor et al. 2013), we first determined the levels of *EFR* mRNA in the *tcp* triple mutant relative to Col-0 with and without elicitation. Interestingly, no differences in transcript level were observed in the mock treatment, while a

two- to threefold induction of *EFR* transcript in Col-0 was observed that is compromised in the *tcp* triple mutant (Fig. 4). This suggests that TCP-mediated activation of *EFR* gene expression is dependent on elicitation or larger activation of plant immune signaling and could also indicate a more significant function of TCP regulatory activity in later PTI responses.

The *tcp* triple mutant exhibits enhanced *elf18*-induced reactive oxygen species (ROS) production.

Some of the earliest observable outputs of the plant response to PTI elicitation include the rapid production of ROS in an oxidative burst and an increase in cytosolic calcium levels and resulting Ca^{2+} -dependent gene induction (Nicaise et al. 2009). Null and low-expression mutants of the receptors *EFR* and *FLS2* have been shown to exhibit heavily impaired ROS production in response to elicitation by *elf18* and *flg22*, respectively (Gómez-Gómez and Boller 2000; Zipfel et al. 2006).

With the intention of better characterizing the role of class I TCPs in the early stages of *EFR*-dependent immune signaling, we quantified ROS production in leaf discs from 4-week-old *Arabidopsis thaliana* plants in a time course following elicitation with *elf18* peptide. To our surprise, we could repeatedly observe that the *tcp* triple mutant exhibits slightly higher peak ROS production and total ROS after 30 min than did Col-0 (Fig. 5A and B). To determine if this unexpected ROS production phenotype was indicative of a larger differential role for TCP TFs between early and late PTI signaling, we also examined mRNA levels of the Ca^{2+} -dependent marker gene *PHI1*, transcription of which is rapidly induced after elicitation with both *elf18* and *flg22* (Boudsocq et al. 2010). The *tcp* triple mutant similarly exhibited slightly higher *PHI1* mRNA levels than Col-0 30 min after elicitation with *elf18* peptide, representing increased activation of Ca^{2+} -dependent immune signaling (Fig. 5C).

The *tcp* triple mutant is susceptible to transient transformation by *A. tumefaciens*.

In *Arabidopsis thaliana*, the native DC3000 EF-Tu elicits a relatively weak immune response that is difficult to measure

Fig. 2. Direct interaction between TCP8 and the *EFR* promoter in *Arabidopsis*. **A**, A gene model of the 1-kb upstream region of *EFR* with the putative class I TCP binding site at -99 bp. **B**, Chromatin immunoprecipitation polymerase chain reaction was performed with primers directed at the class I TCP binding site after immunoprecipitation with anti-HA (hemagglutinin) antibody-conjugated beads in *pTCP8:TCP8-HA*. Significant enrichment was observed relative to both no-antibody (HA-) and nontarget (*At18s*) controls, reported here as a percentage of total input. Similar results were observed in at least three independent experiments. Bars are 1 standard deviation, $n = 2$ or 3, significance determined by analysis of variance at $P < 0.05$ with Tukey multiple comparison test.

Fig. 3. Transactivation of *pEFR:GUS* by TCP8 through the class I TCP binding site in *Nicotiana benthamiana*. *p35S:HA-TCP8* and *pEFR:GUS* were transformed into *Agrobacterium tumefaciens* and were transiently expressed in *N. benthamiana* leaves for 72 h. TCP8 strongly activates the full-length construct (*EFR*) but fails to activate the truncated promoter construct (*efr*). Activation of the full-length construct with a mutated class I TCP binding site (*tcpEFR*) is heavily attenuated. Total protein was extracted from leaf tissue and was evaluated by methylumbelliferyl- β -glucuronide (4-MUG) fluorescence assays as a measure of β -glucuronidase (GUS) production. Control samples expressed *pEFR:GUS* alone as background. Similar results were observed in at least three independent experiments. Bars are 1 standard deviation, $n = 4$, significance determined by analysis of variance at $P < 0.05$ with Tukey multiple comparison test.

through bacterial growth assays, while the *A. tumefaciens* EF-Tu is a potent elicitor (Kunze et al. 2004). Although transient transformation in the *N. benthamiana* system is unaffected by PTI induction due to EF-Tu insensitivity, a strong inhibitory effect is observed in *Arabidopsis thaliana* (Wroblewski et al. 2005). In line with these observations, the *efr* mutant exhibits increased susceptibility to agrobacterium-mediated transformation (Zipfel et al. 2006). To see if the *tcp* triple mutant exhibited similar susceptibility to transformation, we infiltrated leaves with *A. tumefaciens* C58C1 expressing a 35S:HA-GUS construct and then evaluated GUS expression levels by 4-MUG assays. Across multiple experiments, we observed similar, mild increases in GUS expression relative to Col-0 in both the *tcp* triple mutant and *efr-2*, while the addition of TCP8 was sufficient to complement the mutant phenotype in the *pTCP8:TCP8-HA* line (Fig. 6). These data suggest that TCP8 is a required component of *EFR*-dependent resistance to transient transformation by *A. tumefaciens*, likely through its activity at the *EFR* promoter.

The *tcp* triple mutant is compromised in the *EFR*-dependent immune response to *P. syringae*.

Likely due to the weak elicitation properties of the *P. syringae* EF-Tu, compromised resistance to *P. syringae* DC3000 is not observed in *efr-2* at low inoculum concentrations. However, activating host PTI responses by elicitation with elf18 or elf26 peptide prior to bacterial infiltration significantly enhances resistance in wild-type (WT) plants but has no effect on the insensitive receptor mutant (Tintor et al. 2013; Zipfel et al. 2006). We previously observed that the *tcp* triple mutant does not exhibit higher levels of *P. syringae* DC3000 colonization relative to Col-0, although growth of *P. syringae* DC3000 (*avrRps4*) was slightly enhanced (Kim et al. 2014). Pre-treatment by infiltration with elf26 peptide prior to bacterial inoculation revealed a partial inhibition of the host immune response, resulting in intermediate bacterial growth levels in the *tcp* triple mutant between the peptide-sensitive Col-0 and fully insensitive *efr-2* (Fig. 7a). TCP8-HA was able to fully complement this bacterial growth phenotype, pointing toward

Fig. 4. Pathogen-associated molecular pattern-induced *EFR* expression is reduced in the *tcp* triple mutant. Induction of *EFR* expression is inhibited in the *tcp* triple mutant relative to wild type, while no differences are observed with mock treatment. Total RNA was collected from 4-week-old plants 24 h after syringe infiltration with 1 μ M elf18. *EFR* transcript levels were determined relative to the housekeeping gene *SAND*. No differences were observed in the mock treatment. Bars are 1 standard deviation, $n = 3$ or 4, significance determined by analysis of variance at $P < 0.01$ with Tukey multiple comparison test. Similar results were observed in at least three independent experiments.

Fig. 5. The *tcp* triple mutant is enhanced in several early *EFR*-dependent pathogen-associated molecular pattern (PAMP)-triggered immunity responses. **A**, A 30-min time course of reactive oxygen species (ROS) production in *Arabidopsis thaliana* leaf discs after elicitation with 100 nM elf18. Solid shapes indicate PAMP treatment, open shapes represent mock treatment of the same genotype. **B**, Total ROS production in each genotype after 30 min. Increased ROS production was observed in the *tcp* triple mutant relative to wild type. Bars are 1 standard deviation, $n = 6$ for mock, 10 to 14 for PAMP treatment, significance determined by analysis of variance (ANOVA) at $P < 0.001$ with Tukey multiple comparison test. **C**, *EFR*-dependent *PHI1* induction determined in 10-day-old plants grown in liquid one-half Murashige Skoog. The *tcp* triple mutant exhibits increased *PHI1* transcript levels relative to Col-0 30 min after elicitation with 40 nM elf18. Bars are 1 standard deviation, $n = 3$, significance determined by ANOVA at $P < 0.001$ with Tukey multiple comparison test. For A, B, and C, similar results were observed in at least two independent experiments.

redundancy between some class I TCPs in the regulation of *EFR*-dependent activation of PTI.

These data agree with the pattern of *EFR* expression that we previously observed but are in contrast with the heightened ROS induction in the *tcp* triple mutant. These seemingly contradictory roles of class I TCPs between early and late immune signaling may not be so unusual, as it is becoming increasingly apparent that disconnections can be made between unique signaling pathways contributing to the PTI response (Smith et al. 2014; Tsuda et al. 2009; Xu et al. 2014). Combined, these results suggest that class I TCPs may be acting as negative regulators of early branches of PTI immune signaling while promoting later elements of immune signaling and, ultimately, resistance to pathogen infection.

Enhanced SA-mediated immune signaling largely inhibits the growth of hemibiotrophic pathogens like *P. syringae* (Caarls et al. 2015; Pieterse et al. 2012). Peaking at hours to days after peptide elicitation or bacterial infiltration, SA-dependent enhancement of defense gene expression is a relatively slow component of the plant response during PTI. In mutants exhibiting an impaired *EFR*-dependent immune response, dampened induction of marker genes, such as the β -1,3-glucanase *PATHOGENESIS-RELATED GENE2* (*PR2*), have been observed (Cordelier et al. 2003; Dong et al. 1991; Lu et al. 2009; Tintor et al. 2013). Using a native PR2 antibody, we determined that, similar to *efr-2*, the *tcp* triple mutant exhibited reduced PR2 protein levels relative to Col-0 24 h after elicitation with elf18 peptide (Fig. 7b). As with enhanced susceptibility to *P. syringae* infection, this phenotype is complemented by *pTCP8:TCP8-HA*, restoring PR2 induction to levels observed in Col-0. More work is needed to dissect the individual roles of each participating TCP in the *EFR*-mediated immune response.

DISCUSSION

Class I TCP TFs have been characterized as positive regulators of ETI, SA-mediated basal immunity, and systemic acquired resistance, but direct involvement in the regulation of PTI has not previously been demonstrated. We have expanded

Fig. 6. The *tcp* triple mutant exhibits enhanced susceptibility to transformation by *Agrobacterium tumefaciens*. Transformation of the *tcp* triple mutant with a *p35S:HA-GUS* transgene was similar to the susceptible *efr-2* mutant, while the *pTCP8:TCP8-HA* line (Comp.) exhibited full complementation of wild-type (WT) levels of resistance to transformation. *Arabidopsis* leaves were syringe-infiltrated with *A. tumefaciens* expressing the construct at 2×10^8 CFU per milliliter. Total protein was extracted after 72 h and β -glucuronidase production was evaluated by methylumbelliferyl- β -glucuronide fluorescence assays. Expression is presented relative to WT (Col-0). Bars are 1 standard deviation, $n = 6$ to 9, significance determined by analysis of variance with Tukey multiple comparison test. Results were normalized and combined from three independent experiments.

the known role of TCPs in plant immunity through characterization of the partial inhibition of *EFR*-dependent immune responses in the *Arabidopsis thaliana* class I *tcp* triple mutant. The identification of direct interaction with the *EFR* promoter by TCP8 in *Arabidopsis thaliana* and activation of the promoter in *N. benthamiana*, support a mechanism for transcriptional control over *EFR* by TCP8 during PTI. In the yeast system, the interactions between TCP proteins and the *EFR* promoter seem to favor members of the class I TCP family, consistent both with characterized interactions with SRFR1 and with those members' involvement in SA-mediated signaling processes. With dual roles in promotion of both development and defense, TCPs and their interactors may represent an important node in the plant transcriptional regulatory network governing the dynamics of these processes.

Class I TCPs redundantly contribute to activation of *EFR*-dependent PTI to *P. syringae*.

Our data suggest that TCP8 is not acting alone to promote PTI but, rather, as part of a larger network of TCPs interdependently

Fig. 7. The *tcp* triple mutant is impaired in *EFR*-dependent pathogen-associated molecular pattern-triggered immunity. **A**, Bacterial growth levels in the *tcp* triple mutant are intermediate between fully resistant Col-0 and fully susceptible *efr-2* after 4 days of growth with a 24-h pre-elicitation with 1 μ M elf26. No difference in bacterial growth was observed with mock treatment. *Pseudomonas syringae* pv. *tomato* DC3000 was infiltrated at 5×10^8 CFU per milliliter. **B**, PR2 accumulates to a lower level in the *tcp* triple mutant than in wild type 24 h after infiltration with 1 μ M elf18. The *pTCP8:TCP8-HA* line (Comp.) is fully complemented in PR2 induction. Total protein levels determined in 4-week-old plants by Western blot with PR2-directed antibody. Bars are 1 standard deviation, $n = 3$ or 4, significance determined by student's *t* test, an asterisk (*) indicates $P < 0.05$. For A and B, similar results were observed in at least three independent experiments.

governing multiple facets of the plant immune response. This is not an uncommon feature of redundant TF families and has been carefully described in the PHYTOCHROME INTERACTING FACTOR (PIF) bHLH TF family, some of which are thought to interact genetically with TCPs in the coordination of multiple developmental phenotypes (Perrella et al. 2018; Zhou et al. 2018). Although a *pif* quadruple mutant generally exhibits reduced expression of genes directly activated by individual PIFs in the quartet and increased expression of those repressed, further analysis in the triple mutant backgrounds suggests a complicated system of intrafamily regulation in which individual members contribute to varying degrees, depending on the target (Pfeiffer et al. 2014; Zhang et al. 2013). Further clarifying the redundant activities of these TFs in detail is a daunting task; dissection of the individual roles of TCPs in the regulation of *EFR* poses a comparable challenge. The capability of transactivation of the *EFR* promoter in *N. benthamiana* by multiple TCPs to differential levels in addition to positive interaction data in yeast points toward a similarly complicated system of TCP-mediated transcriptional regulation of *EFR*. The fact that the direct interaction of TCP8 and the *EFR* promoter could be observed by ChIP even in the absence of TCP14 and TCP15 suggests that TCP8 may function as a primary binding partner, with dimerization between family members adding additional layers of control over transcriptional activity, as has previously been described in the TCP family (Danisman 2016). The intermediate levels of *EFR*-dependent resistance to virulent *P. syringae* observed in the *tcp* triple mutant and complementation of this mutant by *TCP8-HA* alone both suggest that additional TCPs could redundantly contribute to the PTI response, knockouts of which may be necessary to completely abolish sensitivity. However, we cannot rule out the possibility that the *TCP8-HA* complementation line is masking the role of either TCP14 or TCP15 by overcompensating with higher levels of *TCP8* transcript, despite being driven by the native promoter; in fact, qPCR analysis identifies nearly twice as much *TCP8* transcript in the complemented line relative to WT Col-0 (Supplementary Fig. S5). ChIP experiments with native promoter-driven TCP8, TCP14, and TCP15 lines with confirmed native levels of expression and bacterial growth curves in higher-order *tcp* mutants would be useful in clarifying the dynamics of *EFR* transcriptional regulation in this system.

In addition to direct regulation, there is very likely a contribution of indirect effects on *EFR* expression as well. TCP8 has been identified as one of several class I TCPs directly regulating *ICS1*. It is, therefore, possible that our observations are one consequence of reduced activation of *ICS1* expression and lower total SA levels (Wang et al. 2015). However, unlike the SA-deficient *sid2* mutant, the *tcp* triple mutant exhibits no increased susceptibility to virulent DC3000 relative to WT in the absence of PAMP elicitation and induction of both *PR2* and *PR5* has been demonstrated to be unaffected in *sid2* (Nawrath and Métraux 1999). These data point to an *ICS1*-independent mechanism for regulation of *EFR*-dependent PTI by TCP8 and other related TFs.

A nuanced role in management of the growth-development tradeoff.

While most of our data support positive regulation of *EFR*-dependent PTI by class I TCPs, phenotypes associated with early branches of PTI signaling point toward regulation in the opposite direction or no regulatory role at all. These seemingly contradictory roles of class I TCPs between early and late immune signaling may not be so unexpected, as it is becoming increasingly apparent that disconnections can be made between unique signaling pathways independently contributing to the PTI response. Other mutants that have been characterized as

positive regulators of PTI by bacterial growth assays similarly exhibit no phenotype or the opposite phenotype when examined for early signaling events like apoplastic ROS production, Ca^{2+} -dependent marker gene induction, or mitogen-activated protein kinase activation (Smith et al. 2014; Tsuda et al. 2009).

The mechanisms by which class I TCP TFs promote either development or defense are not well understood, but it is hardly surprising that such multifaceted functionality would beget complicated immune phenotypes. The influence of growth-promoting signaling factors on immunity-specific PRR activities has become an interesting focal point in addressing the tradeoff between signaling pathways. Several models have been proposed of partial antagonism between brassinosteroid (BR) signaling through the BR receptor BRI1 and EFR/FLS2-dependent PTI responses (Albrecht et al. 2012; Belkhadir et al. 2012). Interestingly, one TCP TF has already been directly implicated as a regulator of BR signaling through activation of the biosynthesis gene *DWARF4* (Guo et al. 2010). Increasing the scope of our knowledge of direct regulatory targets of TCP TFs, particularly those, such as TCP14, that have been identified as the targets of pathogen effectors should bring some clarity to the role of TCPs in managing the tradeoff between defense and development. Combined, our current results suggest that class I TCPs may be acting as negative regulators of early branches of PTI immune signaling while promoting later elements of immune signaling and, ultimately, resistance to colonization by *P. syringae*. As such, more work is needed to carefully dissect these individual roles for each TCP of interest and identify potential mechanisms for the contrasting phenotypes described in this study.

TCPs as targets of effector activity.

A key component of pathogen virulence is the successful manipulation of host gene expression to suppress the degree of an immune reaction or to avoid detection altogether. TCP family TFs in diverse plant species have been identified as the targets of multiple pathogen effectors, producing alterations in stability, potential interacting partners, and gene expression (Janik et al. 2017; Sugio et al. 2011; Yang et al. 2017). Suppressive effector activity has been observed on very upstream components of signaling networks behind PTI, as in the case of direct interaction of multiple *P. syringae* effectors with BAK1, a coreceptor for multiple PRRs including EFR, FLS2, and the brassinosteroid receptor BRI1 (Shan et al. 2008; Zhou et al. 2014). Like the TCPs, BAK1 seems to represent an attractive pathogen target in its broad functions as a coregulator of both growth and immune signaling through perception of BR and PAMPs, respectively (Yasuda et al. 2017). In addition to the protein level, *P. syringae* effectors have been demonstrated to regulate the activity of FLS2 and EFR at the translational level through blocking of the interaction between host translational machinery and the transcripts of those receptors (Nicaise et al. 2013). While transcriptional regulation of FLS2 has been well-characterized (Li et al. 2016), relatively little is known about the same regulation of EFR and even less about the influence of effectors on the TFs regulating either. It will be interesting to see if future work will show effector activity directly manipulating the expression of EFR or other PRRs as another mechanism for modulation of promoting virulence.

If class I TCPs are regulating critical PTI components as part of the plant immune response, the question of who regulates the regulators remains. This is particularly interesting, considering that expression of *TCP8*, *TCP14*, and *TCP15* does not appear to be induced by elf18 treatment and is only mildly influenced by infiltration with *P. syringae* (Tintor et al. 2013; Wang et al. 2015). Posttranslational modification of TFs involved in plant immunity is an attractive model by which TF activity can be

very rapidly altered to respond to environmental changes without the need for increasing protein levels. Protein levels of TCP-HA were also unaffected by elicitation with elf18 peptide in our hands (Supplementary Fig. S6). Although we did not observe protein band shifts indicative of possible posttranslational modifications, we cannot rule it out as a possible regulatory mechanism for TCP8 activity at this time.

Induction of at least one SUMO paralog has been observed after induction of PTI by flg22 and SA (van den Burg et al. 2010). Furthermore, sumoylation of multiple TCP TFs, including TCP8, TCP14, and TCP15, has been demonstrated in an *Escherichia coli* expression system in addition to their interactions with key components of the sumoylation machinery (Mazur et al. 2017). Modification by the SUMO protein family is rapid, reversible, and a well-established regulatory mechanism for plant TFs, controlling both plant immunity and growth by affecting stability, localization, and protein-protein interactions (Augustine and Vierstra 2018; Liu et al. 2016). In this regard the SUMO protease activity exhibited in planta by the *Xanthomonas euvesicatoria* effector XopD is a striking adaptation considering that sumoylation is a eukaryotic-specific process (Hotson et al. 2003). More recently, XopD was shown to actively desumoylate a TF that promotes ethylene biosynthesis in tomato, altering the host's hormone balances to promote virulence (Kim et al. 2013). An exciting possibility is that sumoylation status of TCP proteins could be key to the prioritization of their diverse regulatory functions toward growth or immunity through changes in either promoter occupancy or interactions with various coregulators. If so, they may be targeted by pathogen effectors like XopD to alter sumoylation, directing transcriptional activation away from plant immunity under the guise of growth promotion to enhance virulence.

MATERIALS AND METHODS

Molecular cloning.

To generate *pTCP8:TCP8-HA* transgenic lines, genomic *TCP8* with 1 kb upstream and 300 bp downstream sequence was cloned into pGEM-T (Promega) at *Xba*I and *Pme*I restriction sites. A single HA tag was added to the C-terminus in the subclone using overlap-PCR and was inserted via *Kpn*I and *Apa*I restriction sites. The entire genomic sequence was inserted into the plant binary vector pBIB-hyg (Becker 1990) at an *Xba*I restriction site.

To generate constructs for promoter transactivation experiments, the 2 kb upstream of genomic *EFR* was cloned into the Gateway-compatible donor vector pDONR201. For the truncated *efr* construct, only 90 bp upstream was cloned in pDONR201. For the *tcpEFR* construct, PCR-based site-directed mutagenesis with digestion by *Dpn*I was performed with the full-length *EFR* donor clone as template. All donor clones were moved into the Gateway-compatible vector pYXT1 (Xiao et al. 2005), producing complete promoter-reporter constructs. Primer sequences for molecular cloning can be found in Supplementary Table S2.

Plant materials and growth conditions.

Arabidopsis plants used in ChIP experiments and *PHI* transcript level analysis were grown in liquid half-strength Murashige Skoog (MS) media on a light rack under 24 h light at room temperature. All other *Arabidopsis* plants were grown in an E-7/2 reach-in growth chamber (Controlled Environments Ltd.) under an 8-h light and 16-h dark cycle at 24°C, 70 to 80% relative humidity, and a light intensity of 140 to 180 μ mol photons per square meter per second. *N. benthamiana* plants used in transactivation assays were grown under a 8-h light and 16-h dark

cycle at 22°C and 55% relative humidity. To generate the *pTCP8:TCP8-HA* transgenic line in the *tcp8 tcp14 tcp15* mutant background, plasmids were moved into *A. tumefaciens* C58C1 and were transformed into *Arabidopsis* by floral dipping (Clough and Bent 1998). Selection of transformants was performed on one-half MS containing 15 mg of hygromycin per milliliter. The *tcp* triple mutant (Kim et al. 2014) and *efr-2* mutant (Zipfel et al. 2006) lines have been previously described.

Y1H analysis.

Y1H was performed according to established protocols (Zwack et al. 2016). Briefly, an 800-bp fragment of the regulatory region directly upstream of the TSS of our genes of interest were cloned into the bait vector pMW#2 containing the *HIS3* reporter gene. Eight TCP genes (*TCP2*, *TCP3*, *TCP4*, *TCP8*, *TCP10*, *TCP14*, *TCP15*, and *TCP21*) were cloned into a pDEST-AD vector to serve as prey. The bait and prey clones were transformed into YM4271 and Yal867 yeast strains, respectively. The bait and prey transformants were selected on minimal synthetic dropout media lacking histidine (SD-H) and tryptophan (SD-T), respectively. The empty pDEST-AD vector was also transformed into Yal867 as a negative control. The haploid yeast strains were mated in yeast extract peptone dextrose medium for 24 h and were subsequently diploid selected in SD-HT media for 48 h. The diploid yeast strains were spotted onto SD-HT solid media containing 20 and 50 mM 3-amino-1,2,4-triazole (3-AT). Positive interactions were recorded based on growth and no growth of yeast on SD-HT containing various concentrations of 3-AT.

Real-time qPCR.

Adult leaf or whole-seedling tissue was ground to a powder in liquid nitrogen and 0.1 g was mixed with 1 ml of TRIzol reagent (Invitrogen). Total RNA was extracted by chloroform, then isopropanol at room temperature before overnight precipitation in 100% EtOH at -20°C. The RNA pellet was resuspended in diethyl pyrocarbonate H₂O and a total of 10 μ g was treated with TURBO DNase (Invitrogen). For reverse transcription PCR experiments, cDNA was synthesized from 2 μ g of total purified RNA using an oligo(dT)15 primer and Moloney murine leukemia virus reverse transcription (Promega), following the manufacturer's protocol. qPCR was performed with 5 μ l of 20 $\times$ diluted cDNA and a primer concentration of 1 μ M in a 20- μ l reaction with Brilliant III Ultra-Fast SYBR GREEN master mix (Agilent) with a BioRad CFX Connect thermocycler (Bio-Rad). Unless otherwise stated, transcript levels of target were normalized to the housekeeping gene *SAND*.

ChIP.

ChIP was performed in a standard manner (Yamaguchi et al. 2014) with several modifications. All solutions involved in crosslinking were chilled and preparations were performed at 4°C. After initial isolation of nuclei from 1.5 g of crosslinked 10-day-old liquid one-half MS-grown seedling tissue in extraction buffer, the nuclear pellets were washed twice with an additional series of extraction buffers (Fiil et al. 2008). Sheared (approximately 300 to 1,000 bp) chromatin was precleared with protein A agarose beads (P6486; Sigma) and was precipitated with either anti-HA-conjugated agarose beads (E6779; Sigma) or a protein A bead control. Five microliters of the resulting purified DNA was analyzed by qPCR as described above.

Transactivation in *N. benthamiana* leaves.

A. tumefaciens C58C1 was electroporated with various *pEFR:GUS* constructs and strain GV3101 was electroporated with 35S promoter-driven *HA-TCP8*, *HA-TCP14*, or *HA-TCP15*

constructs (Kim et al. 2014). Overnight cultures were generated for each transformant at 30°C, then, were pelleted and resuspended in 5 ml of 10 mM MgCl₂ buffered with 1 mM morpholineethane sulfonic acid (MES) (pH 5.6). Acetosyringone was added to a final concentration of 100 nM and the bacterial suspension was incubated for 4 to 5 h. Bacterial inocula were mixed at an optical density at 600 nm of approximately 0.2 for each strain in a solution of 10 mM MgCl₂ and 1 mM MES (pH 5.6) and were syringe-infiltrated into mature *N. benthamiana* leaves. Tissue was collected 3 days after inoculation with a 1 cm in diameter hole punch, four leaf discs per sample. Protein was isolated in extraction buffer and GUS was quantified according to established protocol (Jefferson et al. 1987). Protein concentration was determined by Bradford assay and GUS levels were reported as picomole 4-MUG per minute per microgram of protein.

Immunoblot analysis of HA-TCP construct expression was performed with the same *N. benthamiana* tissue. One gram of total protein was extracted in 1 ml of 2× sodium dodecyl sulfate (SDS) buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 250 mM dithiothreitol [DTT]), was cleared by centrifugation at 13,000 rpm, and was loaded onto an 8% bis-acrylamide SDS-polyacrylamide gel electrophoresis (PAGE) gel. Protein was detected with 1:2,000 horseradish peroxidase (HRP)-conjugated anti-HA antibody (Roche).

Quantitative transformation of *Arabidopsis*.

A. tumefaciens C58C1 was transformed with a 35S promoter-driven HA-GUS construct and was then syringe-infiltrated into 3-week-old *Arabidopsis* leaves at 2×10^8 CFU per milliliter. Inoculum preparation and leaf disc sampling (4 days post-inoculation) was performed in the same manner as in the *N. benthamiana* transactivation assay and GUS was quantified as described earlier. Results presented are combined from three independent experiments in which normalization to Col-0 was performed by determining mean levels of GUS expression in Col-0 samples and, then, calculating the ratios between each sample measurement and that mean.

Bacterial growth curve assay.

P. syringae pv. *tomato* DC3000 was grown on *Pseudomonas* Agar F (Becton Dickinson). Bacterial suspensions were syringe infiltrated into 4-week-old *Arabidopsis* leaves at 5×10^4 CFU per milliliter 24 h after pretreatment, with either 1 µM elf26 (Genscript) or mock solution, by infiltration. Four leaf discs with a total area of 0.5 cm² were ground in 10 mM MgCl₂ and were serially diluted before plating on plates with 50 µg of rifampicin per milliliter and 25 µg of kanamycin per milliliter, in triplicate. Colonies were counted after two days of growth.

Measurements of PAMP-induced responses.

For analysis of *EFR* transcript levels, 4-week-old *Arabidopsis* leaves were infiltrated with 1 µM elf18 or mock solutions and tissue was collected after 24 h. Total mRNA was collected as described above, and *EFR* transcript was quantified with normalization to housekeeping gene *SAND*.

Immunoblot analysis of PR2 induction was performed with leaf tissue infiltrated with 1 µM elf18 or mock solutions 24 h prior to collection. Total protein was extracted in a 2× SDS buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 250 mM DTT), was cleared by centrifugation at 13,000 rpm, and was loaded onto a 12% bis-acrylamide SDS-PAGE gel. Protein was detected with 1:10,000 anti-PR2 (Agrisera) primary and 1:15,000 anti-rabbit-HRP (Genscript) secondary antibodies.

ROS production was measured by an H₂O₂-dependent luminescence assay as previously described (Heese et al. 2007). Briefly, 1 cm in diameter *Arabidopsis* leaf discs were halved and were then floated in 100 µl of water in a 96-well plate,

under full light, overnight. Water was carefully removed and replaced with elicitation solution containing 100 nM elf18 or dimethyl sulfoxide mock. Luminescence was quantified in 2-min increments over the course of 30 min immediately after elicitation.

For analysis of *PHI1* transcript levels, seedlings were grown in liquid one-half MS media on a light rack under 24 h light at room temperature. At 10 days, seedlings were treated with 40 nM elf18 peptide (Genscript) and tissue was collected after 30 min. Total mRNA was collected as described above, and *PHI1*-specific primers were used in quantification (Smith et al. 2014).

The Arabidopsis Information Resource (TAIR) accession numbers.

The TAIR accession numbers for referenced genes are as follows: At5g20480 (*EFR*), At4g37460 (*SRFR1*), At1g74710 (*ICS1*), At1g58100 (*TCP8*), At3g47620 (*TCP14*), At1g69690 (*TCP15*), At5g46330 (*FLS2*), At2g28390 (*SAND*) At4g39400 (*BR1*), and At3g50660 (*DWF4*).

ACKNOWLEDGMENTS

We greatly appreciate J. Chul Nam (University of Missouri) and P. Blair (University of Alabama Birmingham) for technical assistance.

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