Research Report

TEOSINTE BRANCHED1/CYCLOIDEA/ PROLIFERATING CELL FACTOR4 Interacts with WRINKLED1 to Mediate Seed Oil Biosynthesis[1][OPEN]

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Cross-family transcription factor (TF) interactions play critical roles in the regulation of plant developmental and metabolic pathways. WRINKLED1 (WRI1) is a key TF governing oil biosynthesis in plants. However, little is known about WRI1-interacting factors and their roles in oil biosynthesis. We screened a TF library using Arabidopsis (*Arabidopsis thaliana*) WRI1 (AtWRI1) as bait in yeast two-hybrid assays and identified three TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) family TFs, namely TCP4, TCP10, and TCP24, as AtWRI1-interacting partners. The physical interaction between AtWRI1 and TCPs was further validated using bimolecular fluorescence complementation assays. TCPs play important roles in various plant developmental processes; however, their involvement in fatty acid biosynthesis was not previously known. Coexpression of TCP4, but not TCP10 or TCP24, with AtWRI1 reduced AtWRI1-mediated oil biosynthesis in *Nicotiana benthamiana* leaves. Transcriptomic analysis in transgenic Arabidopsis plants with enhanced TCP4 activity engineered by expressing *rTCP4* (i.e. miR319-resistant *TCP4*) revealed that AtWRI1 target genes were significantly repressed. TCP4 expression is strongly correlated with AtWRI1 during embryo development. A *tcp4* loss-of-function mutant, the *jaw-D* mutant with a strong reduction of *TCP4* expression, and a *tcp2 tcp4 tcp10* triple mutant accumulated more seed oil than wild-type Arabidopsis. In addition, TCP4 repressed the AtWRI1-mediated transactivation of the promoters of fatty acid biosynthetic genes. Collectively, our findings suggest that TCP4 represses fatty acid biosynthetic gene expression through interaction with AtWRI1, leading to a reduction of AtWRI1-mediated seed oil accumulation.

Plant oils are important for both human diet and industry as renewable feedstocks. Many plants synthesize triacylglycerol (TAG) in seeds as a crucial mechanism for storing and providing energy for seedling development (Chapman and Ohlrogge, 2012). The APETALA2 (AP2) transcription factor (TF), WRINKLED1 (WRI1), is a key regulator of plant oil biosynthesis (Cernac and Benning, 2004; Masaki et al., 2005; Kong and Ma, 2018). The Arabidopsis (*Arabidopsis thaliana*) loss-of-function mutant *wri1-1* has ~80% reduced seed oil content compared with the wild-type plant (Focks and Benning, 1998). Microarray analysis revealed that the repressed genes in the *wri1-1* mutant mainly encode fatty acid biosynthetic and late glycolytic enzymes (Ruuska et al., 2002). Subsequent studies validated that the genes encoding proteins of late glycolysis and fatty acid biosynthesis are targets of AtWRI1 (Baud et al., 2007; Maeo et al., 2009). *WRI1*

[1]This work was supported by a Department of Energy-Great Lakes Bioenergy Research Center Cooperative Agreement (grant no. DE–FC02–07ER64494 to C.B.), the Harold R. Burton Endowed Professorship (to L.Y.), a Nanyang Technological University Startup Grant (to W.M.), and the Ministry of Education of Singapore Tier 1 (grant no. 2018–T1–002–019 to W.M.).

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Q.K., C.B., and W.M. conceived and designed the experiments; Q.K., S.K.S., J.J.M., S.P., and W.M. performed the experiments; Q.K., S.K.S., S.P., L.G., L.Y., C.B., and W.M. analyzed the data; Q.K., S.K.S., S.P., L.G., L.Y., C.B., and W.M. wrote the article.

[OPEN]Articles can be viewed without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.20.00547

orthologs have been identified from many plant species, such as *Brassica napus* (Liu et al., 2010), *Zea mays* (Shen et al., 2010), *Elaeis guineensis* (Ma et al., 2013), *Brachypodium distachyon* (Yang et al., 2015), *Glycine max* (Chen et al., 2020), and rice (*Oryza sativa*; Mano et al., 2019). In addition, transgenic plants overexpressing *AtWRI1* or other *WRI1* orthologs produce higher oil in seeds and leaves (Cernac and Benning, 2004; Shen et al., 2010; Yang et al., 2015). AtWRI1 is regulated at the posttranslational level and subject to 26S proteasome-mediated degradation (Chen et al., 2013). A PEST motif in the C-terminal intrinsically disordered region of AtWRI1 mediates the protein stability, which, in turn, affects oil biosynthesis (Ma et al., 2015). The 14-3-3 proteins interact with AtWRI1 in the mediation of AtWRI1 stability, transcriptional activity, and, consequently, oil production (Ma et al., 2016). In addition, phosphorylation of AtWRI1 by the Suc nonfermentation-related kinase KIN10 was recently reported to be crucial for AtWRI1 degradation (Zhai et al., 2017). A subsequent study showed that trehalose 6-phosphate stabilizes AtWRI1, resulting in increased fatty acid production through KIN10 activity suppression (Zhai et al., 2018).

Protein interactions between TFs from the same family or different families play crucial roles in regulating metabolic or developmental pathways. In Arabidopsis, about half of more than 2,000 TF–TF interactions are cross-family interactions (Bemer et al., 2017). Transcriptional networks are frequently formed by cross-family TF interaction, which enables the integration of various signals that dictate the outcome of a plant metabolic or developmental pathway. However, the potential interactions of AtWRI1 with other TFs in oil biosynthesis are not known. TFs frequently involved in cross-family interaction form transcriptional hubs (Bemer et al., 2017). One such TF family is TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP). TCPs are plant-specific TFs that play important roles in diverse biological processes, such as shoot apical meristem and leaf development, phytohormone biosynthesis, regulation of circadian clock rhythm, and immunity (Palatnik et al., 2003; Schommer et al., 2008; Giraud et al., 2010; Li et al., 2012; Kim et al., 2014a). In Arabidopsis, 24 TCP TFs are divided into class I and class II subfamilies (Cubas et al., 1999; Palatnik et al., 2003). Of note for our study, the Arabidopsis *jaw-D* mutant overproducing the microRNA319 (miRNA319) leads to the repression of several class II *TCPs*, including *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* (Palatnik et al., 2003). The miRNA319-regulated TCPs interact with the TF ASYMMETRIC LEAVES2, thus repressing class I *KNOX* genes (Li et al., 2012). Arabidopsis TCPs show unique and redundant functions (Danisman et al., 2013). TCP3 interacts with MYB12 or MYB111, thus regulating the expression of flavonoid biosynthetic genes (Li and Zachgo, 2013). Six TCPs have been identified as interacting partners of SUPPRESSOR OF rps4-RLD1 (SRFR1), and the interaction between SRFR1 and TCPs is a key mechanism to balance plant development and immune responses (Kim et al., 2014a). TCPs also interact with key circadian regulators (e.g. CCA1 and LHY; Giraud et al., 2010). Therefore, TCPs are known to control plant development, defense, and redox regulation. However, no report thus far indicates the involvement of TCPs in regulating oil biosynthesis.

In this work, we found that AtWRI1 interacted with TCPs in the regulation of fatty acid biosynthesis. We discovered that expression of *TCP4* displayed strong correlation with *AtWRI1* during embryo development and that TCP4 negatively affected AtWRI1-stimulated oil biosynthesis. Arabidopsis loss-of-function mutants, including *tcp4*, *tcp2 tcp4 tcp10*, and *jaw-D* (overproducing miRNA319, resulting in reduced expression of *TCPs*), were found to have higher seed oil content compared with the wild type. Our findings suggest that TCP4 acts as a transcriptional repressor of AtWRI1 through protein-protein interactions. This interaction possibly plays a role in the fine-tuning of plant oil biosynthesis.

RESULTS

TCPs Physically Interact with AtWRI1 in Yeast and Plant Cells

TFs function in the nucleus. We demonstrated that a nuclear localization signal at the N terminus (AtWRI1$^{33\text{-}41}$) enables AtWRI1 to localize to the nucleus, where it likely interacts with other factors in the regulation of target gene expression (Supplemental Fig. S1). In order to identify AtWRI1-interacting factors, we employed the yeast two-hybrid (Y2H) assay to screen an Arabidopsis TF library (Kim et al., 2014b). We used two truncated AtWRI1 variants (AtWRI1$^{1\text{-}306}$ and AtWRI1$^{58\text{-}240}$) as baits for the screening, as the AtWRI1 C-terminal part of the protein shows high autoactivation in yeast cells (Ma et al., 2015). Our initial assay identified three TCP family TFs, namely TCP4, TCP10, and TCP24, as interacting partners of AtWRI1. We next performed independent Y2H assays to confirm the interaction between the TCPs and AtWRI1. Our results showed that TCP4, TCP10, and TCP24 interacted with AtWRI1 in yeast cells (Fig. 1A; Supplemental Fig. S2A). To further validate the physical AtWRI1-TCP interaction in plant cells, we performed bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana* leaves. For this purpose, the coding sequences for the TCPs (TCP4, TCP10, and TCP24) were individually fused at their N-terminal ends to the C-terminal half of YFP (cYFP), and the N-terminal half of YFP (nYFP) was fused to the N terminus of AtWRI1. Reconstituted YFP fluorescence was detected in the nucleus when *nYFP-AtWRI1* and *cYFP-TCPs* were transiently coexpressed in *N. benthamiana* leaves (Fig. 1B; Supplemental Fig. S2B). YFP signal was not detected when only one of the fusion proteins, meaning nYFP-AtWRI1 or one of the cYFP-TCP fusions, was present (Fig. 1B; Supplemental Fig. S2B). Collectively, our results suggest that AtWRI1 physically interacts with TCPs.

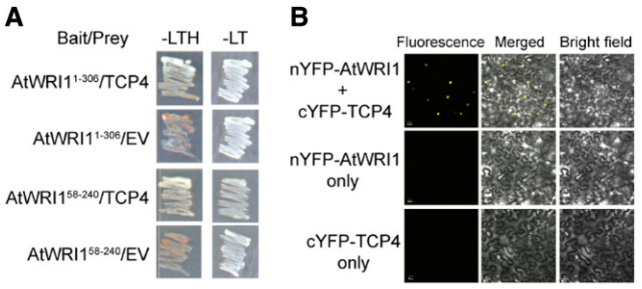

Figure 1. The TCP4 TF physically interacts with AtWRI1. A, TCP4 shows physical interaction with AtWRI1 variants in the Y2H assay. Yeast growth on either stringent selective (-Leu/-Trp/-His [-LTH]) or permissive (-Leu/-Trp [-LT]) medium is shown. Empty vector (EV) was used as a negative control. B, AtWRI1 interacts with the TCP4 TF in planta. A BiFC assay was performed to validate the interaction between AtWRI1 and TCP4. *N. benthamiana* epidermal cells were transiently cotransformed with plasmids encoding nYFP-AtWRI1 and cYFP-TCP4 as indicated. Bars = 20 μm.

Expression of *TCP4* Strongly Correlates with *AtWRI1* during Seed Development

The Arabidopsis genome harbors 24 TCPs, which are broadly divided into two major groups, namely class I and class II (Supplemental Fig. S3). The class II TCPs are further divided into two subgroups: The CINCINNATA clade-containing genes are involved in lateral organ development and the CYC/TB1/ECE clade-containing genes are involved in axillary meristem development. TCP4, TCP10 and TCP24 belong to the CINCINNATA subclade. To determine the temporal expression correlation of *TCPs* with *AtWRI1*, we conducted coexpression analysis of *AtWRI1* and *TCPs* at various stages of Arabidopsis embryo development (Le et al., 2010). Expression data of Arabidopsis embryos at five developmental stages (24 h, globular, cotyledon, mature-green, and post-mature-green stages) were used to generate a heat map. Expression of 16 of 24 *TCPs* was detected during embryo development. Hierarchical clustering analysis showed that *TCPs* form two major clusters, as indicated in Figure 2A by red and green bars, and *AtWRI1* is grouped together with *TCP4* and *TCP24*, whereas *TCP10* is found in the other cluster. *TCP4* displayed strong correlation with the expression of *AtWRI1* during embryo development (Fig. 2A). In addition, using the eFP browser (Winter et al., 2007), we also found that *TCP4* expression during embryo development mirrors that of *AtWRI1* (Fig. 2B). Although *TCP10* and *TCP24* are expressed during embryo development, their expression patterns are different from that of *WRI1* (Supplemental Fig. S4). Taken together, the spatial and temporal expression relationship of their respective genes suggests that AtWRI1 and TCP4 meet the criteria for possible interaction in the regulation of seed oil biosynthesis.

TCP4 Represses AtWRI1-Regulated Oil Biosynthesis

Recent studies have validated that the transient expression of coding sequences for plant oil regulators in *N. benthamiana* leaves followed by TAG analysis is a robust and fast approach to study the regulation of plant oil biosynthesis (Vanhercke et al., 2013; Ma et al., 2015). We therefore evaluated the effects of the TCPs on AtWRI1-regulated oil biosynthesis using an established *N. benthamiana* transient expression system (Ma et al., 2015). As demonstrated previously, expression of *AtWRI1* induced oil accumulation in *N. benthamiana* leaves; however, coexpression of *TCP4* with *AtWRI1* significantly reduced oil production compared with expression of *AtWRI1* alone (Fig. 3A). By contrast, coexpression of *TCP10* or *TCP24* with *AtWRI1* had no effect on AtWRI1-mediated oil biosynthesis. Expression of *TCP4*, *TCP10*, or *TCP24* alone did not lead to oil production (Fig. 3, A and B).

To gain further insight into the function of TCP4 in AtWRI1-regulated oil biosynthesis, we searched publicly available transcriptome data of *TCP4* transgenic plants. We examined the previously published microarray data of *rTCP4* transgenic Arabidopsis, in which the miR319-resistant *TCP4* is more abundantly expressed (Schommer et al., 2008). We discovered that the expression of *AtWRI1* target genes in the oil biosynthetic pathway (e.g. *BCCP2*, *ACP1*, and *PKP-β1*) was down-regulated in *rTCP4* transgenic plants compared with that in lines producing the native form of *TCP4* (Supplemental Fig. S5). We hence hypothesized that TCP4 plays a role in repressing oil biosynthesis through interaction with AtWRI1.

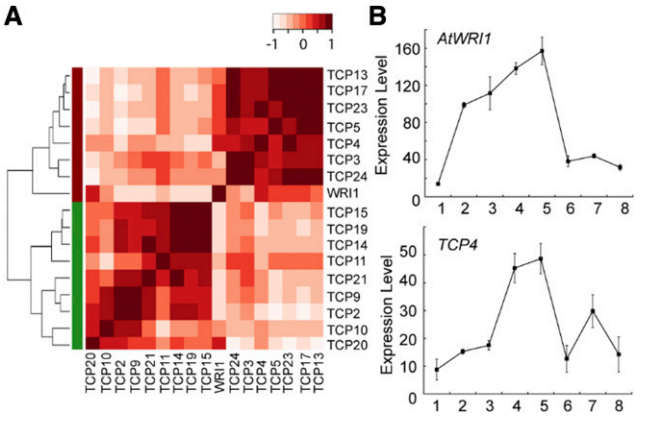

Figure 2. In silico analysis of *AtWRI1* and *TCP* expression. A, Coexpression of *AtWRI1* with *TCPs* in Arabidopsis embryo tissues. Hierarchical clustering and heat map shows that *AtWRI1* strongly correlates with and clusters with *TCP4*. B, Expression profiles of *AtWRI1* and *TCP4* during various stages of embryo development. Expression data are taken from the Arabidopsis eFP browser. Points on the graphs are as follows: (1) seed stage 3 with siliques; (2) seed stage 4 with siliques; (3) seed stage 5 with siliques; (4) seed stage 6 without siliques; (5) seed stage 7 without siliques; (6) seed stage 8 without siliques; (7) seed stage 9 without siliques; and (8) seed stage 10 without siliques. Results are shown as means ± SD (n = 3). Experimental details were described for the eFP browser (Winter et al., 2007).

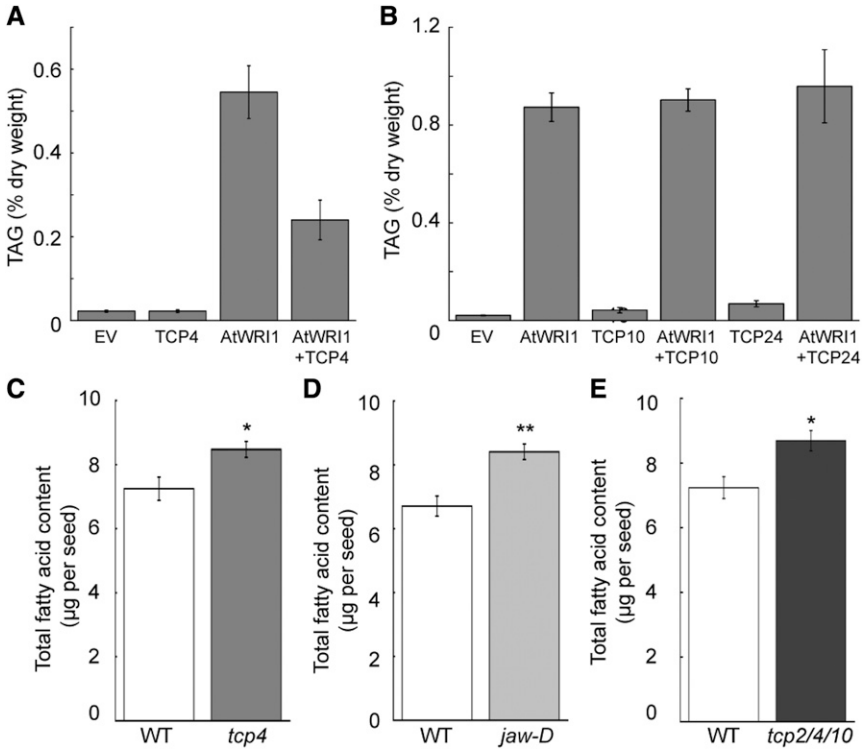

Figure 3. TCP effects on AtWRI1-mediated plant oil biosynthesis. A, TAG content in *N. benthamiana* leaves transiently coproducing AtWRI1 and TCP4. Results are shown as means ± SE ($n = 3$). B, TAG content in *N. benthamiana* leaves transiently coproducing AtWRI1 and TCP10 or TCP24. Results are shown as means ± SE ($n = 3$–4). Empty vector (EV) was used as a control. C, Fatty acid content of seeds of the wild type (WT) and *tcp4*. Results are shown as means ± SE ($n = 3$). D, Fatty acid content of seeds of the wild type and *jaw-D*. Results are shown as means ± SE ($n = 3$–4). E, Fatty acid content of seeds of the wild type and *tcp2 tcp4 tcp10*. Results are shown as means ± SE ($n = 3$). Asterisks indicate significant differences between the mutant and the wild type by one-way ANOVA (*$P < 0.05$ and **$P < 0.01$).

The *tcp4* Mutant Accumulates Higher Seed Oil Compared with the Wild Type

We subsequently measured the seed oil content in the *tcp4* loss-of-function mutant and found a significant increase (17%) compared with the wild type (Fig. 3C). The activation-tagging mutant *jaw-D* exhibited increased levels of miRNA319, which consequently led to reduced expression of several miRNA319-targeted *TCPs*, including *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* (Palatnik et al., 2003). Among the several miRNA319a-targeted *TCPs*, expression of *TCP4* displayed the strongest reduction in the *jaw-D* mutant (Palatnik et al., 2003). When we measured the seed oil content of the *jaw-D* mutant, it was significantly increased (23%) compared with the wild type (Fig. 3D). We also measured the seed oil content of the *tcp2 tcp4 tcp10* triple mutant. Notably, the *tcp4* allele in this triple mutant is another allele (SAIL_1174_02), different from the *tcp4* single mutant in Figure 3C. The seed oil content of the triple mutant was 20% higher compared with the wild type (Fig. 3E).

TCP4 Represses AtWRI1 Transactivation Activity

We next examined whether the interaction of TCP4 with AtWRI1 affects AtWRI1 transactivation activity in plant cells. We used a transient expression system (Pattanaik et al., 2010) in which the promoters of two AtWRI1 target genes ($_{pro}$*BCCP2* and $_{pro}$*PKP-β1*) were fused to the firefly *Luciferase* (*LUC*) reporter gene. Vectors expressing *AtWRI1* and *TCP4*, driven by the cauliflower mosaic virus (CaMV) 35S promoter, served as effectors. The reporters were introduced into *Nicotiana tabacum* protoplasts, alone or in combination with the effectors (Fig. 4A). Cointroduction of *AtWRI1* with $_{pro}$*BCCP2-LUC* or $_{pro}$*PKP-β1-LUC* led to a significant increase in LUC activity, indicating that AtWRI1 activated the promoters of *BCCP2* and *PKP-β1*. However, coexpression of *TCP4* with *AtWRI1* significantly reduced the activation by AtWRI1 (Fig. 4B). TCP4 by itself did not affect the promoter-driven LUC activity (Fig. 4B). These results indicate that the interaction between TCP4 and AtWRI1 represses the transactivation activity of AtWRI1.

DISCUSSION

TFs often work together in a complex. Interactions between TFs of the same or different families, evident in numerous developmental or metabolic pathways, dictate the ultimate outcome of a biological process in plants. Accumulating evidence suggests that TCPs are transcriptional hubs, as they interact with various regulatory proteins to control many biological processes (Bemer et al., 2017). However, TCPs have not been implicated in seed oil biosynthesis until now. Here, we provide evidence that TCP4 is involved in AtWRI1-mediated oil biosynthesis in Arabidopsis.

We identified TCP4, TCP10, and TCP24 in an Arabidopsis TF library as interacting partners of AtWRI1 using a Y2H assay and further validated the interactions by BiFC (Fig. 1). AtWRI1 is a major regulator of seed-specific acyl lipid biosynthesis in Arabidopsis.

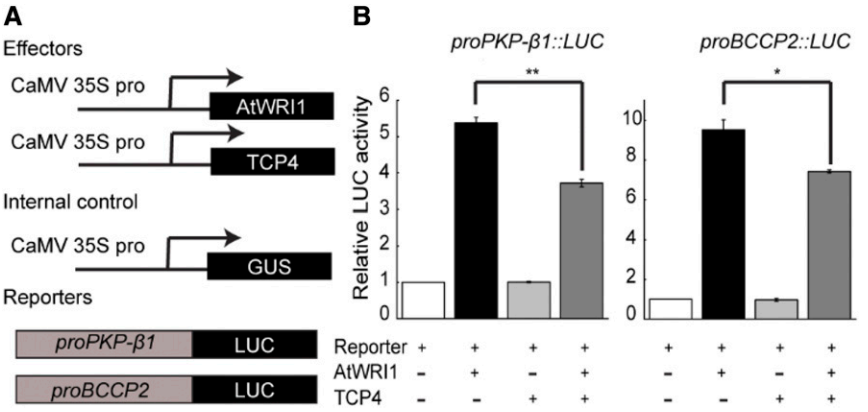

Figure 4. TCP4 repressed the transactivation activity of AtWRI1. A, Schematic representation of constructs used in a transient expression assay in protoplasts. B, Coexpression of *TCP4* with *AtWRI1* repressed the transactivation activity of AtWRI1 on the promoters of *PKP-β1* and *BCCP2* in protoplasts. Results are shown as means ± SE ($n = 3$). Asterisks indicate significant differences by one-way ANOVA ($^*P < 0.05$ and $^{**}P < 0.01$) between AtWRI1 alone and coproduction of TCP4 as indicated.

Coexpression analysis of *AtWRI1* and *TCP* genes during embryo development showed a strong correlation between *AtWRI1* and *TCP4* expression (Fig. 2), suggesting a potential role of TCP4 in seed oil biosynthesis. Although *TCP10* and *TCP24* are expressed during embryo development, the expression patterns are different from that of *AtWRI1* (Supplemental Fig. S4), suggesting that TCP10 and TCP24 are possibly involved in other biological processes during embryogenesis. Using an *N. benthamiana* leaf infiltration assay, we demonstrated that TCP4, but not TCP10 and TCP24, repressed AtWRI1-induced oil biosynthesis (Fig. 3, A and B). Furthermore, oil accumulation is moderately but significantly higher in the *tcp4* and *tcp2 tcp4 tcp10* loss-of-function mutants (Fig. 3, C and E). The class II TCPs, including TCP4, are posttranscriptionally regulated by miRNA319 (Palatnik et al., 2003). We showed that seed oil accumulation was significantly higher in the *jaw-D* mutant, in which the expression of up to five class II TCP genes (*TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*) was altered due to the elevated expression of *miRNA319* (Fig. 3D). We reasoned that the increased seed oil content in *jaw-D* is due to altered expression of *TCP4* because (1) *TCP4* expression showed the strongest reduction compared with the other four *TCPs*, (2) TCP10 and TCP24 had no effect on AtWRI1-mediated oil biosynthesis (Fig. 3B), (3) TCP2 and TCP3 are not known to regulate fatty acid biosynthesis thus far, and (4) the seed oil content of the *jaw-D* mutant is comparable to that of the *tcp4* single mutant and the *tcp2 tcp4 tcp10* triple mutant. Collectively, these findings indicate that TCP4 is a negative regulator of seed oil biosynthesis in Arabidopsis.

Synergistic or antagonistic interactions between TFs are known to regulate metabolic or developmental pathways. Synergistic interaction between R2R3 MYB, bHLH, and WD40 proteins form the MYB-bHLH-WD40 complex that regulates anthocyanin biosynthesis in a wide range of plant species (Patra et al., 2013). Antagonistic interactions between HLH and bHLH TFs regulate brassinosteroid-mediated cell elongation in rice and Arabidopsis (Zhang et al., 2009). Similarly, interactions between the bHLH TF MYC2 and the basic Leu zipper proteins GBFs regulate hypocotyl elongation in Arabidopsis (Maurya et al., 2015) and terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Sui et al., 2018). Interaction between the TCP TF OsTB1 and the MADS domain protein OsMADS57 regulates tillering in rice (Guo et al., 2013). Here, we demonstrated that the interaction between TCP4 and AtWRI1 repressed the transcriptional activity of AtWRI1 acting on key fatty acid pathway promoters (Fig. 4). We envision three possible mechanisms by which TCP4 decreases AtWRI1 activity toward its target promoters (Fig. 5): (1) the TCP4-AtWRI1 complex occupies a target gene promoter through binding to the TCP- and WRI1-binding cis-elements (Fig. 5B); (2) the complex binds to the promoter only through the WRI1-binding cis-element (Fig. 5C); and (3) TCP4 interaction with AtWRI1 reduces AtWRI1 binding to its binding cis-element (Fig. 5D). TCP proteins bind to specific consensus DNA motifs (Kosugi and Ohashi, 2002). We hence searched the promoter regions of the known AtWRI1 target genes, including *BCCP2* and *PKP-β1*, for potential TCP-binding sites. We found TCP consensus-binding motifs in the promoter of *PKP-β1* but not in the *BCCP2* promoter (Supplemental Fig. S6). TCP2, TCP3, and TCP10 have been found to bind to the promoters of *BP* and *KNAT2*, although the classical TCP binding cis-elements are not present in these promoters (Li et al., 2012). Therefore, it is possible that TCP4 binds to the *BCCP2* promoter at an unidentified cis-element or is recruited to the promoter by AtWRI1. TCP4 alone has no activity on the *BCCP2* and *PKP-β1* promoters (Fig. 4B), suggesting that TCP4 acts as a repressor of AtWRI1 activity. As interaction between TFs affects the promoter-binding specificity, it is possible that the interaction between AtWRI1 and TCP4 affects the AtWRI1-binding affinity to the promoters of its target genes.

Fatty acids are essential components of plant cells. A question thus arose about the biological significance of a negative regulator of the fatty acid biosynthetic pathway. AtWRI1 and its orthologs are key regulators of seed-specific acyl lipid biosynthesis in Arabidopsis and other plant species. Seed oil accumulation during embryo development changes slightly during the initial and final stages but changes dramatically during the

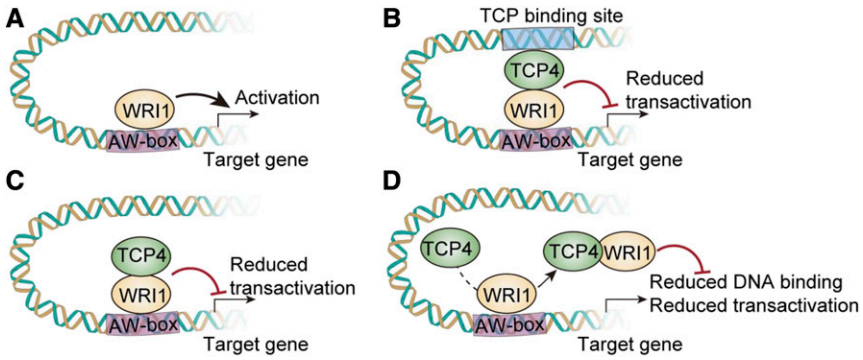

Figure 5. Model of the proposed TCP4-mediated repression of AtWRI1 transactivation. A, AtWRI1 activates the expression of target genes for oil biosynthesis by its binding to the AW-box. B to D, The interaction of TCP4 and AtWRI1 results in acting as a repressor of AtWRI1 (binding to promoters of AtWRI1 target genes; B), acting as a repressor of AtWRI1 (not binding to promoters of AtWRI1 target genes; C), or interference of AtWRI1 binding to the promoters of AtWRI1 target genes (D). In any case, the AtWRI1-TCP4 protein complex displays decreased AtWRI1 target transactivation activity as a result of the AtWRI1-TCP4 interaction.

maturation phase. In addition, partitioning of fatty acids, proteins, and carbohydrates during embryo development is well balanced. Oxidation of fatty acids releases more energy than oxidation of carbohydrates or proteins, and acyl lipid biosynthesis is a high-energy-demanding process. Therefore, fatty acid biosynthesis must be tightly regulated. One mechanism to efficiently fine-tune fatty acid biosynthesis is activity modulation of the key transcriptional regulator. Posttranslational regulation of AtWRI1 by phosphorylation and proteasomal degradation has been previously reported (Chen et al., 2013; Ma et al., 2015; Zhai et al., 2017; Kong et al., 2020). Here, we propose an additional regulatory mechanism by which TCP4 and possibly other regulators act in concert to modulate acyl lipid accumulation in seeds. Whereas multiple TFs are likely involved in regulating the basal fatty acid biosynthetic gene expression in embryonic development, AtWRI1 is a major activator that enables high-level oil accumulation. Nevertheless, AtWRI1 activity must be spatiotemporally regulated in order to respond to developmental cues and to prevent hyperactivation that leads to metabolic imbalance. Such higher level regulation can be effectively achieved by combinatorial action of both transcriptional activators and repressors. Simultaneous expression or coordinated induction of activators and repressors in response to stimuli is evident in many metabolic pathways, likely serving to modulate the amplitudes of target gene expression (Memelink and Gantet, 2007; Sui et al., 2018). Our study suggests that the AtWRI1-TCP4 interaction is one of the mechanisms that allow fine-tuning of the oil biosynthetic pathway. A recent study showed that TCP4, similar to AtWRI1, is a target of posttranslational modification. Mass spectrometry analysis identified nine phosphorylated residues in TCP4 (Kubota et al., 2017). Our in silico analysis using different phosphorylation analysis tools (P^{3}DB, Musite, PhosPhAt 4.0, and DISPHOS 1.3) identified more than 30 potential phosphorylation residues in TCP4. Among the nine phosphorylated residues in TCP4 identified by mass spectrometry (Kubota et al., 2017), eight residues are found in our in silico analyses (Supplemental Fig. S7; Supplemental Table S1). It is well known that phosphorylation affects the stability, transcriptional activity, protein-protein interaction, and subcellular localization of proteins (Ptacek and Snyder, 2006). As TCP4 interacts with and represses WRI1 activity, we hypothesize that reversible phosphorylation in response to stimuli affects the interaction with WRI1 and, ultimately, the expression of fatty acid biosynthetic genes. Identification of candidate kinases and elucidation of the molecular mechanism governing phosphorylation of the AtWRI1-TCP4 module to fine-tune oil production remain to be explored. In summary, our findings uncover a previously uncharacterized role for TCP TFs and open new doors to investigate the complicated regulatory network centered on AtWRI1-regulated gene expression.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) and *Nicotiana benthamiana* plants were grown in a growth chamber at 22°C with a photoperiod of 16 h of light (100–150 μmol m^{-2} s^{-1} illumination)/8 h of dark (Ma et al., 2013, 2015). Arabidopsis wild-type ecotype Columbia was used in this study. The *jaw-D* mutant (Palatnik et al., 2003) and the *tcp2 tcp4 tcp10* triple mutant were described previously (Schommer et al., 2008). Seeds of the *tcp4* mutant (SALK_125700) were obtained from the Arabidopsis Biological Resource Center. Seed sterilization and germination were performed as previously described (Ma et al., 2013).

Bioinformatics Analysis

The nuclear localization signal was predicted using the PSORT II program (Nakai and Horton, 1999). Publicly available transcriptome data for Arabidopsis seeds at different stages of development were used to extrapolate the gene expression correlation among *TCPs* and *WRI1* (Gene Expression Omnibus accession no. GSE680; Le et al., 2010). The correlation between every sample pair was calculated as the Euclidean distance and presented as a heat map using the heatmap.2 function with the gplots R package (http://CRAN.R-project.

org/package=gplots). The full-length protein sequences of Arabidopsis TCPs were aligned using ClustalW (Larkin et al., 2007) with default settings. MEGA software (version X; Kumar et al., 2018) was used to construct the phylogenetic tree using the neighbor-joining method with bootstrap values set at 1,000 replicates. The expression of *TCP4* and other oil biosynthesis-related genes was analyzed in the microRNA-resistant (rTCP4-GFP) and -sensitive (TCP4-GFP) transgenic Arabidopsis lines as previously reported (ArrayExpression accession no. E-MEXP-469; Schommer et al., 2008). TCP binding site analysis was performed using AthaMap (Steffens et al., 2005). Phosphorylation site analysis was performed using P^3DB (Gao et al., 2009), Musite (Gao et al., 2010), PhosPhAt 4.0 (Zulawski et al., 2013), and DISPHOS 1.3 (Iakoucheva et al., 2004).

Plasmid Construction

The coding sequences of *AtWRI1* (including truncated *AtWRI1* variants) and *TCPs* were amplified by PCR and introduced into the pENTR4 vector (Life Technologies). Entry constructs were combined with destination vectors (Y2H vectors, BiFC vectors, and pEarlygate binary vectors) through Gateway LR reactions (Life Technologies) as previously described (Ma et al., 2015). Entry constructs of *TCP4* and *TCP24* were obtained from the Arabidopsis Biological Resource Center. The construct for TCP4-GFP was previously described (Schommer et al., 2008). A list of the primers used for plasmid construction in this study is provided in Supplemental Table S2.

Y2H Assays

The Arabidopsis TF library was a gift from Dr. Michael Thomashow. For library screening, TFs were cloned into the pDEST22 vector (prey) and introduced into yeast strain Y187 (Clontech). *AtWRI1* variants were cloned into pDEST32 vector (bait) and transformed into yeast strain AH109. The prey and bait were mated and spotted on -Trp-Leu medium. After 3 d of growth, the colonies were streaked onto -Trp-Leu-His medium for screening of positive interactions. *TCPs* were cloned into pDEST22 as the prey and *AtWRI1* variants were cloned into pDEST32 as the bait. The Y2H assay was done as previously described (Ma et al., 2015).

Transient Expression in *N. benthamiana*, BiFC, and Confocal Microscopy

Agrobacterium tumefaciens-mediated transient expression in *N. benthamiana* leaves, BiFC, and confocal microscopy were performed as previously described (Ma et al., 2015, 2016). The plasmid pEAQ HT expressing the P19 protein was coinfiltrated with other constructs to ensure high-level expression in *N. benthamiana* (Sainsbury et al., 2009).

Transient Expression Assay in Protoplasts

The reporter plasmids used in the protoplast assay contained the firefly *LUC* coding sequence driven by *BCCP2* or *PKP-β1* promoters and the *rbcS* terminator. The effector plasmids were generated by cloning the full-length coding sequences of *AtWRI1* and *TCP4* into a pBluescript vector containing the CaMV 35S promoter and *rbcS* terminator. The primers for these constructs are listed in Supplemental Table S2. Isolation of protoplasts from tobacco cell suspension cultures and electroporation of tobacco protoplasts with plasmid DNA were performed as previously described (Pattanaik et al., 2010). A plasmid containing the *GUS* gene under the control of the CaMV 35S promoter and *rbcS* terminator was coelectroporated with each promoter construct as an internal control. After 20 to 22 h, protoplasts were harvested for GUS and LUC activity assays. All constructs were tested in at least three independent experiments. The GUS activity was normalized against LUC activity.

Fatty Acid Analysis

Lipid analysis in seeds and leaves was done as previously described (Ma et al., 2013, 2015).

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource database (www.arabidopsis.org) under accession numbers: WRI1 (AT3G54320), TCP4 (AT3G15030), TCP10 (AT2G31070), TCP24 (AT1G30210), BCCP2 (AT5G15530), PKP-β1 (AT5G52920), and ACP1 (AT3G05020).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Identification of the nuclear localization signal in AtWRI1.

Supplemental Figure S2. Assays of physical interaction between AtWRI1 and TCPs.

Supplemental Figure S3. Phylogenetic analysis of the Arabidopsis TCP gene family.

Supplemental Figure S4. Expression profiles of *TCP10* and *TCP24* during various stages of embryo development.

Supplemental Figure S5. Expression analysis of *TCP4*, *WRI1*, and *WRI1* target genes in *TCP4* transgenic plants.

Supplemental Figure S6. In silico analysis of TCP-binding sites in the promoter region of AtWRI1 target genes.

Supplemental Figure S7. Phosphorylation site analysis of TCP4.

Supplemental Table S1. TCP4 phosphorylation sites identified in this study and a previous study.

Supplemental Table S2. Primers used for plasmid construction in this study.

ACKNOWLEDGMENTS

We thank Dr. Detlef Weigel (Max Planck Institute for Developmental Biology) for the seeds of the *jaw-D* and *tcp2 tcp4 tcp10* mutants and TCP4 constructs, Dr. Michael Thomashow (Michigan State University) for the Arabidopsis TF library, Dr. Melinda Frame (Michigan State University Center for Advanced Microscopy) for confocal microscopy experiments, and Dr. Eva Farre (Michigan State University) for helpful discussion.

Received May 5, 2020; accepted June 27, 2020; published July 6, 2020.

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