

TCP1 Modulates Brassinosteroid Biosynthesis by Regulating the Expression of the Key Biosynthetic Gene *DWARF4* in *Arabidopsis thaliana* [©]^W

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Brassinosteroids (BRs) are essential phytohormones regulating normal plant growth and development. *TCP1*, a gene thought to be involved in floral organ symmetric control, was identified as a genetic suppressor of a weak BR receptor mutant, *bri1-5*, in an activation-tagging genetic screen. *TCP1* encodes a putative transcription factor possessing a basic helix-loop-helix domain. The dominant allele of *TCP1*, *tcp1-1D*, suppresses the defective phenotypes of *bri1-5*. Overexpression of a dominant-negative form of *TCP1*, *TCP1-SRDX*, with a 12-amino acid repressor sequence fused to *TCP1* at its C terminus, results in dwarfed plants resembling BR-deficient or insensitive mutants. The defective phenotypes can be rescued by exogenously applied brassinolide but cannot be recovered by auxins, gibberellins, or cytokinins. BR profile assay (quantitative analysis of BR biosynthetic intermediates) strongly suggests that *TCP1* expression level positively coordinates with the function of *DWARF4* (*DWF4*), a key enzyme in BR biosynthesis. Real-time RT-PCR analysis further demonstrated that *TCP1* regulates the transcription levels of *DWF4*, and chromatin immunoprecipitation experiments showed that *TCP1* indeed interacts with the *DWF4* promoter. Confocal microscopy indicated that *TCP1* is mainly confined to the nucleus. The expression of *TCP1* appears to be regulated by BR levels. These studies demonstrate another level of regulation through which BRs mediate plant growth and development.

INTRODUCTION

Brassinosteroids (BRs) are a class of polyhydroxyl steroidal hormones naturally found in almost all plant species examined (Clouse and Sasse, 1998). BRs play critical roles in multiple physiological processes during normal plant growth and development, from seed germination to leaf senescence. Mutant plants unable to biosynthesize or perceive BRs exhibit typical phenotypes, including extremely dwarfed statures, shortened leaf petioles, rounded and curled leaves, prolonged life spans, reduced male fertility, and deetiolated hypocotyls when grown in darkness. Within the last two decades, detailed information regarding BR signal transduction and BR biosynthesis has been uncovered (Kim and Wang, 2010). A number of BR signaling regulators, such as the cell surface BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) (Li and Chory, 1997) and its coreceptor BRI1-ASSOCIATED PROTEIN KINASE1 (BAK1), were identified via genetic and biochemical methods (Li et al.,

2002; Nam and Li, 2002). Other important regulatory proteins, such as a secreted Ser carboxypeptidase designated as *bri1* SUPPRESSOR1 (Li et al., 2001a), a BRI1 inhibitory protein BKI1 (Wang and Chory, 2006), several putative BRI1 substrates, including an *Arabidopsis thaliana* paralog of TGF-BETA RECEPTOR-INTERACTING PROTEIN-1 (Jiang and Clouse, 2001; Ehsan et al., 2005), a TRANSTHYRETIN-LIKE protein (Nam and Li, 2004), and three homologous BR SIGNALING KINASES (Tang et al., 2008), a negative regulator called BRASSINOSTEROID INSENSITIVE2 (Li et al., 2001b; Li and Nam, 2002), a protein phosphatase (BSU1) (Mora-Garcia et al., 2004), 14-3-3 proteins (Bai et al., 2007; Gampala et al., 2007; Ryu et al., 2007), and two novel transcription factors, BZR1 (Wang et al., 2002) and BES1 (Yin et al., 2002), have also been identified using various approaches. Although a more detailed mechanistic understanding as to how the aforementioned proteins coordinate various steps in BR signaling is needed, evidence to date strongly indicates that they are key signaling components in BR signal transduction that relay information from the cell surface to nuclear transcription factors. A proposed pathway for BR signal transduction starts with the ligand (BR) binding to the extracellular domain of BRI1, which triggers a sequential phosphorylation between BRI1 and BAK1 (Wang et al., 2008). Activated receptor/coreceptor complex initiates a phosphorylation/dephosphorylation cascade that can transduce the BR signal from the cell surface to the cytoplasm and eventually to the nucleus where gene expression patterns are altered through the action of two transcription

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www.plantcell.org/cgi/doi/10.1105/tpc.109.069203

factors BZR1 and BES1 (Kim et al., 2009). As a consequence of these events, the plant is able to fine-tune its growth and development.

The entire BR biosynthetic pathway was initially elucidated using cultured *Catharanthus roseus* cells (Fujioka and Yokota, 2003). Several genes encoding key BR biosynthetic enzymes have also been cloned using BR-deficient mutants identified from a number of plant species, such as *Arabidopsis*, pea (*Pisum sativum*), tomato (*Solanum lycopersicum*), and rice (*Oryza sativa*; Fujioka and Yokota, 2003). For example, *de-etiolated2* (*det2*) was identified as a deetiolated mutant from *Arabidopsis* (Chory et al., 1991). *DET2* encodes a protein sharing sequence similarity with the mammalian steroid 5 α -reductase (Li et al., 1997). Feeding experiments revealed that *DET2* is involved in a 5 α -reduction step of multiple related sterols during BR biosynthesis (Fujioka et al., 1997). An ortholog of *Arabidopsis det2*, named *Ik*, was also identified from pea as an extremely dwarfed mutant (Nomura et al., 2004). *dwarf4* (*dwf4*) is another BR-deficient mutant isolated in *Arabidopsis* (Choe et al., 1998). The dwarfed stature of *dwf4* can be rescued by brassinolide (BL), the final product of the BR biosynthetic pathway and the most active form of BRs. *DWF4* encodes a 22-hydroxylase and is responsible for multiple 22-hydroxylation steps during BR biosynthesis. It was proposed that *DWF4* catalyzes a rate-limiting step during BR biosynthesis (Kim et al., 2006). *constitutive photomorphogenesis and dwarfism* (*cpd*) is another dwarf mutant isolated by T-DNA insertion analysis. It was shown that *CPD* encodes a 23 α -hydroxylase and participates in a critical 23 α -hydroxylation step in BR biosynthesis (Szekeres et al., 1996). However, recent feeding and biochemical analyses indicated that two P450 proteins, CYP90C1 and CYP90D1, act as true 23 α -hydroxylases (Ohnishi et al., 2006). The severe phenotype of the *cpd* mutant indicated that it should be involved in a step earlier than the 23 α -hydroxylation reaction. Another gene involved in BR biosynthesis is *BR6ox*, which was first identified in tomato by transposon tagging (Bishop et al., 1996). *BR6ox* catalyzed the C-6 oxidation of a number of different 6-deoxoBRs (Bishop et al., 1999). *BR6ox* orthologs from *Arabidopsis* and rice have conserved functions, which are responsible for linking the early and late C-6 oxidation pathways (Bishop et al., 2006).

Unlike other phytohormones, BRs are unable to be transported through a long distance mechanism in a plant (Symons and Reid, 2004). This suggests that homeostasis of bioactive BRs must be precisely controlled at tissue or even at cellular levels to ensure normal growth and development. There are a number of mechanisms for a plant to maintain adequate levels of bioactive BRs. For instance, excessive amounts of BRs can be inactivated by modifications of BRs (Fujioka and Yokota, 2003). Mechanisms for the inactivation of BRs include sulfonation at a 22-OH group by a steroid sulfotransferase named BNST3, identified in *Brassica napus* (Rouleau et al., 1999); 26-hydroxylation by BAS1, found in *Arabidopsis* (Neff et al., 1999); conjugation by a UDP-glycosyltransferase named UGT73C5 (Poppenberger et al., 2005); and a putative reduction step catalyzed by BEN1 (Yuan et al., 2007). Plants also use a feedback mechanism to monitor the BR biosynthetic rate, which is tightly linked with the BR signaling pathway (Mathur et al., 1998; He et al., 2005; Kim et al., 2006). If BRs are available, they can trigger a series of

cellular processes, resulting in the accumulation of unphosphorylated BZR1 and BES1 in nuclei. Unphosphorylated BZR1 and BES1 have dual roles: repressing biosynthetic gene expression to slow down the biosynthetic rate and activating BR response genes to promote growth. How BR biosynthesis is positively regulated, however, is still poorly understood. In this article, we describe the identification of a transcription factor, which plays a positive role in regulating BR biosynthesis.

Using a gain-of-function genetic approach, we identified a number of suppressors for a weak *BRI1* mutant allele, *bri1-5* (Noguchi et al., 1999; Li et al., 2001a, 2002; Zhou et al., 2004; Yuan et al., 2007). One of these suppressors is *tcp1-1D*. *TCP1* encodes a TCP transcription factor, which contains a basic helix-loop-helix (bHLH) domain. Previous studies suggested that *TCP1* may play a role in regulating flower organ symmetry (Cubas et al., 2001; Busch and Zachgo, 2007). The activation-tagged locus, *tcp1-1D*, can suppress the defective phenotypes of *bri1-5*. Overexpression of a dominant-negative mutant *TCP1-SRDX* in wild-type plants, conversely, resulted in dwarfed transgenic plants similar to typical BR deficient mutants such as *det2* (Li et al., 1996) or signaling defective mutants such as *bri1-5* (Noguchi et al., 1999). Our detailed genetic, biochemical, and molecular analyses demonstrated that *TCP1* positively regulates the expression of the key BR biosynthetic gene, *DWF4*, via a direct or indirect interaction with the promoter region of *DWF4*. Thus, our findings provide a molecular pathway in the regulation of BR biosynthesis under certain endogenous and external stimuli. The discoveries will significantly advance our knowledge about the functions of BR in regulating normal plant growth, development, and adaptation to various environments.

RESULTS

tcp1-1D Was Identified as a Gain-of-Function Genetic Suppressor of *bri1-5*

Activation tagging is a gain-of-function genetic approach that activates gene expression via inserting strong enhancers in the genome. The enhancers are commonly engineered in the T-DNA region of the transformation construct and inserted arbitrarily in the genome by *Agrobacterium tumefaciens*-mediated gene transformation (Clough and Bent, 1998). Usually only the genes in the vicinity of the enhancers can be transcriptionally activated (Weigel et al., 2000). This investigation takes advantage of *bri1-5*, a weak BR receptor mutant, in which a single Cys has been substituted by a Tyr at the N terminus (C69Y) (Noguchi et al., 1999). The *bri1-5* protein is largely retained in endoplasmic reticulum and degraded through a proteasome-mediated degradation pathway (Hong et al., 2008). *bri1-5* plants show a semidwarfed but fertile phenotype. The activation tagging-based *bri1-5* genetic modifier screen has become an effective approach to identify components regulating BR signaling, catabolism, and biosynthetic pathways (Li et al., 2001a, 2002; Zhou et al., 2004; Yuan et al., 2007). One of the *bri1-5* suppressors we identified by this method is called *tcp1-1D*.

The *bri1-5 tcp1-1D* double mutant shows partially suppressed phenotypes compared with the *bri1-5* single mutant (Figure 1A).

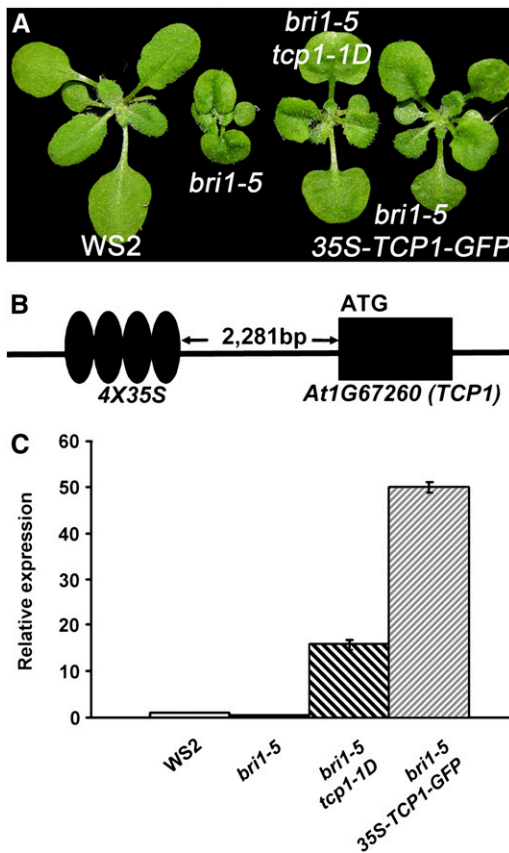


Figure 1. *tcp1-1D* Was Identified as a Dominant Genetic Suppressor of *bri1-5* by an Activation-Tagging Screen.

(A) Phenotypes of 16-d-old WS2, *bri1-5*, *bri1-5 tcp1-1D*, and *bri1-5 35S-TCP1-GFP* plants.

(B) In *bri1-5 tcp1-1D*, four copies of 35S enhancer were inserted at 2281 bp upstream of the start codon of *At1G67260* (*TCP1*).

(C) The expression of *TCP1* was elevated in *bri1-5 tcp1-1D* as well as in *bri1-5 35S-TCP1-GFP* plants as shown by real-time RT-PCR. The data were the average of three replicates. The error bars represent the SD. *TCP1* expression level in the wild type was set to 1. *TCP1* expression levels in other genotypes were determined relative to that of the wild type.

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A *bri1-5* mutant exhibits characteristic BR mutant phenotypes, such as rounded leaves, shortened petioles, and delayed flowering time. Although the leaf shapes of the double mutant plants remain unaltered, the petioles of *bri1-5 tcp1-1D* are significantly elongated. The inflorescences of the double mutant plants are twice as tall as those of *bri1-5* plants at maturity. In addition, the delayed flowering time of *bri1-5* was also significantly suppressed. Genetic analysis indicated that the double mutant phenotype was caused by a single dominant locus because the *bri1-5* suppression phenotype is closely linked with the *basta* resistance gene from the T-DNA of the activation-tagging construct, *pBASTA-AT2* (Yuan et al., 2007).

We cloned the flanking sequences of the T-DNA insertion by thermal asymmetric interlaced PCR (Liu and Whittier, 1995) and

found that the T-DNA is inserted at 2281 bp upstream of the initiation codon of *TCP1* (At1G67260) (Figure 1B). To determine whether *TCP1* is the gene responsible for the suppression phenotype in the double mutant, we cloned the full-length cDNA of *TCP1* and overexpressed it in *bri1-5* driven by a constitutive cauliflower mosaic virus 35S promoter. Over 50% of the transgenic plants showed elongated petiole phenotypes similar to the originally identified activation tagging line (Figure 1A). Real-time RT-PCR analysis confirmed that the *TCP1* expression levels in both *bri1-5 tcp1-1D* and *bri1-5 35S-TCP1-GFP* (for green fluorescent protein) were elevated by at least 10-fold compared with those in WS2 and *bri1-5* (Figure 1C). These results demonstrated that increased expression of *TCP1* is the cause of the suppression phenotype seen in the double mutant.

A Functional BRI1 Is Required for *tcp1-1D* to Regulate Plant Growth and Development

To determine whether *tcp1-1D* affects general growth or has a specific role in BR-related pathways, we conducted a series of genetic crosses. We first backcrossed the *bri1-5 tcp1-1D* with its background ecotype WS2 and segregated out *bri1-5* after self-pollination. The *tcp1-1D* single mutant showed an elongated leaf phenotype reminiscent of *BRI1*-overexpressed or *DWF4*-overexpressed transgenic plants (Choe et al., 2001; Wang et al., 2001) (Figure 2A). A similar suppression outcome was observed when *tcp1-1D* was crossed with *det2* (Figure 2B). *det2* was first identified as a deetiolated mutant when grown in the dark (Chory et al., 1991). *DET2* encodes a steroid reductase responsible for a reduction step from campesterol to campestanol during BR biosynthesis (Li et al., 1996, 1997; Fujioka et al., 1997). It was found that *det2* mutants were still able to synthesize ~5 to 10% wild-type levels of BRs. Therefore, the *det2* null mutant was considered as an intermediate biosynthetic mutant (Fujioka et al., 1997; Fujioka and Yokota, 2003). To further test whether BR signaling is necessary for the function of *tcp1-1D* in regulating plant growth, we crossed *tcp1-1D* with *bri1-4*. *bri1-4* has a 10-amino acid deletion at the N terminus of *BRI1* resulting from an T-DNA insertion, which causes a premature stop codon after amino acid 153 (Noguchi et al., 1999). Therefore, this mutant is regarded as a null mutant of *BRI1*. The *bri1-4 tcp1-1D* double mutant did not show any leaf suppression phenotypes (Figure 2C). Thus, our genetic analyses clearly indicated that the role of *tcp1-1D* in regulating leaf growth is dependent on the presence of BRs and the BR signaling pathway.

Expression of a *TCP1-SRD* Chimeric Repressor Gene in Wild-Type Plants Results in a Typical BR Mutant Phenotype

Our genetic data suggested that *TCP1* is involved in BR-related pathways. To further understand this role of *TCP1*, we searched for T-DNA null mutants of *TCP1* from various resources. Unfortunately, no T-DNA alleles were identified from the available databases. We then tried to generate *TCP1* knockdown mutants using an artificial microRNA strategy (Schwab et al., 2006). All the resulting plants did not show any obvious phenotypes, possibly due to gene redundancy. Therefore, we employed a gene silencing system, named chimeric repressor gene-silencing

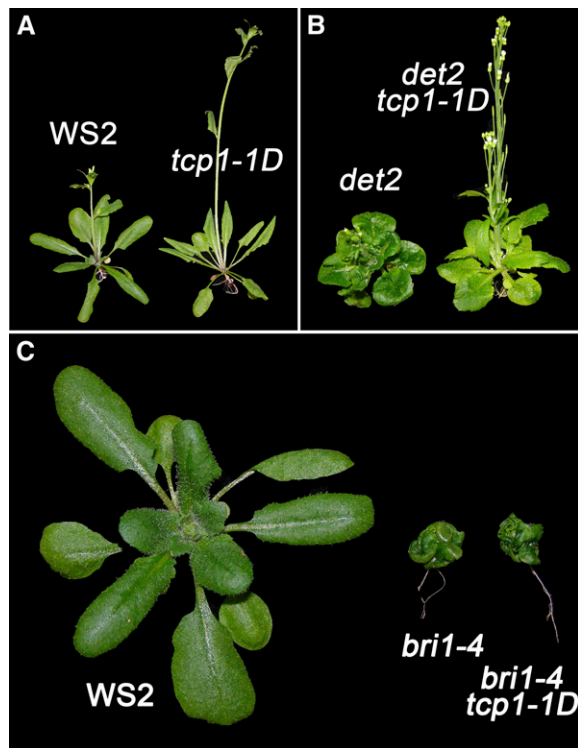


Figure 2. *tcp1-1D* Can Suppress Weak BR Signaling and Biosynthetic Mutants but Cannot Suppress a Null Allele of *BRI1*.

(A) WS2 *tcp1-1D* showed a phenotype reminiscent of the phenotypes of *BRI1*- or *DWF4*-overexpressed plants. The plants were photographed 4 weeks after germination.

(B) *tcp1-1D* can partially suppress the defective phenotypes of a BR biosynthetic mutant *det2*. The plants were photographed 5 weeks after germination.

(C) *tcp1-1D* cannot suppress a *BRI1* null allele mutant *bri1-4*. The plants were photographed 3 weeks after germination.

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technology, in which TCP1 was fused with a 12–amino acid EAR motif repressor domain (SRDX) (Figure 3A) (Hiratsu et al., 2003). Previous experiments indicated that the chimeric version can effectively repress the expression of the target genes of a number of transcription factors, including TCP genes (Koyama et al., 2007). Expression of *TCP1-SRDX* driven by the constitutive 35S promoter resulted in dwarfed transgenic plants similar to BR-deficient or signaling mutants, such as *det2*, *dwf4*, and *bri1-5* (Figure 3B). The dominant-negative plants showed phenotypes opposite to those of *tcp1-1D* plants. Whereas *tcp1-1D* plants showed elongated leaves and petioles, *TCP1-SRDX* plants displayed rounded and epinastic leaves, shortened petioles, and reduced statures (Figures 3B and 3C). When grown under darkness, *TCP1-SRDX* plants exhibited a typical deetiolated phenotype with opened cotyledons resembling that of *det2* and *bri1-4* mutants (see Supplemental Figure 1 online). The dominant-negative phenotypes were likely caused by the competitive binding of TCP1-SRDX with native TCP1 and its paralogs to the target gene(s) via the bHLH domain. Overexpression of

TCP1^{ΔbHLH} (in which *bHLH* domain sequence was deleted) or *TCP1-SRDX^{ΔbHLH}* failed to alter the growth of the transgenic plants (see Supplemental Figure 2 online), suggesting that the bHLH domain of TCP1 is prerequisite to the functions of TCP1 and TCP1-SRDX in transgenic plants. To ensure that the phenotypes of *TCP1-SRDX* transgenic plants were caused by the overexpression of *TCP1-SRDX*, we crossed the transgenic plants with *tcp1-1D* and isolated homozygous plants for both *TCP1-SRDX* and *tcp1-1D* loci. We found that *tcp1-1D* can partially complement the dominant-negative effect of *TCP1-SRDX*; this suggests that the phenotypes were indeed caused by *TCP1-SRDX* overexpression (see Supplemental Figure 3 online). These results suggested that the target gene(s) of the TCP1 transcription factor plays a significant role in regulating BR biosynthesis or signal transduction.

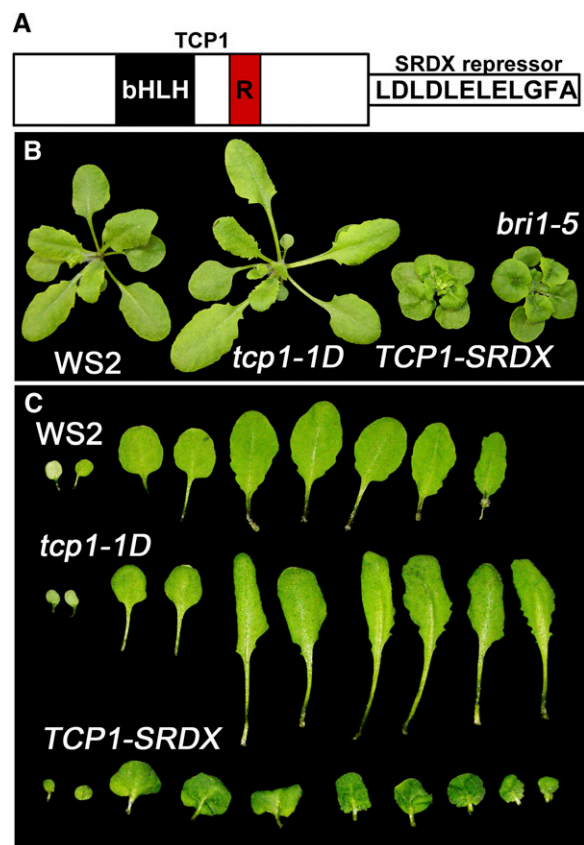


Figure 3. Transgenic Plants Overexpressing a *TCP1* Dominant-Negative Construct (*TCP1-SRDX*) Show a Typical BR Mutant Phenotype.

(A) Protein structures of the 359–amino acid TCP1. TCP1 contains a typical bHLH domain and an R domain. For the dominant-negative version, a 12–amino acid SRDX repressor sequence was fused at the C terminus of TCP1.

(B) Phenotypes of WS2, *tcp1-1D*, *TCP1-SRDX*, and *bri1-5*. The plants were photographed 3 weeks after germination.

(C) Leaf phenotypes of 3-week-old WS2, *tcp1-1D*, and *TCP1-SRDX* plants.

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TCP1 Regulates BR Biosynthesis

The phenotypes of BR-deficient and signaling mutants are morphologically similar. To determine whether the dwarfed phenotype of *TCP1-SRDX* plants was caused by the failure of the transgenic plants to respond to endogenous BRs or by disruptions in specific steps of the BR biosynthetic pathway, we thus tested their response to exogenous BRs in the form of BL. If BR signaling is altered, the mutant should show reduced response to exogenous BL. Conversely, if BR biosynthesis is impeded, the dwarf mutant should be rescued by exogenous applied BL. Our results show that the hypocotyl growth of the *TCP1-SRDX* plants can be greatly recovered by the addition of BL. In half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose and 1 μ M BL, the hypocotyl length of the *TCP1-SRDX* seedlings was increased by fourfold, whereas those of wild-type *WS2* and *tcp1-1D* seedlings were increased only by 20 to 40% (Figures 4A and 4B). Comparison analysis indicated that the growth of the *TCP1-SRDX* transgenic plants respond to exogenously applied BL in a similar manner as other BR-deficient mutants, such as *det2* (see Supplemental Figure 4 online). Application of 1 μ M BRZ, a specific BR biosynthetic inhibitor (Asami and Yoshida, 1999; Asami et al., 2000), did not further

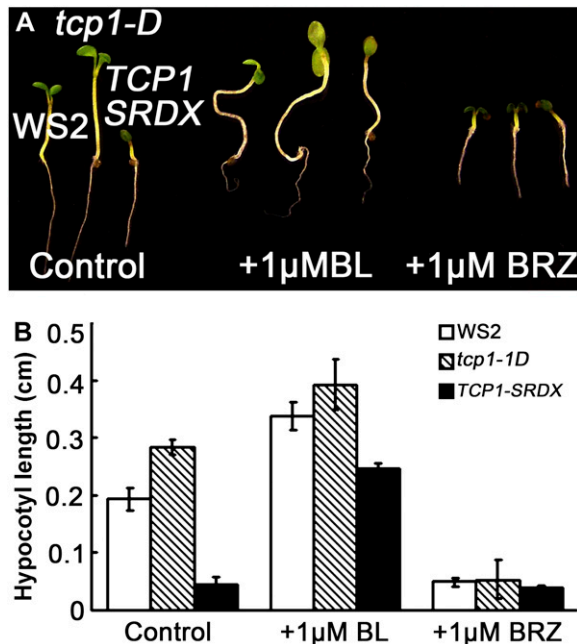


Figure 4. BL Treatment Rescues the Shortened Hypocotyl Phenotype of *TCP1-SRDX*.

(A) Phenotypes of representative *WS2*, *tcp1-1D*, and *TCP1-SRDX* seedlings grown on half-strength MS medium under light condition with no BL treatment, 1 μ M BL treatment, or 1 μ M BRZ treatment, respectively. The seedlings were photographed 5 d after germination.

(B) The measurements corresponding to the treatments shown in (A). Twenty seedlings per genotype were used for the measurements. The error bars represent the SD.

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reduce the growth of *TCP1-SRDX* plants. Under the same treatment, the lengths of *WS2* and *tcp1-1D* seedlings were reduced by four- to eightfold, respectively (Figures 4A and 4B). Furthermore, exogenous application of other known growth hormones, such as gibberellic acid 3, kinetin, and indole-3-acetic acid, showed no rescuing effects (see Supplemental Figure 5 online). These results suggest that expression of *TCP1-SRDX* specifically blocked the BR biosynthetic pathway.

TCP1 Expression Levels Are Positively Correlated with the Catalytic Ability of *DWF4*, a Key Enzyme in the BR Biosynthesis Pathway

To investigate how TCP1 is involved in regulating BR biosynthesis, we performed BR profile analyses (quantitative analyses of BR biosynthetic intermediates) using 4-week-old *WS2*, *tcp1-1D*, and *TCP1-SRDX* plants. It was apparent that *tcp1-1D* enhanced the catalytic capability of *DWF4*, whereas *TCP1-SRDX* greatly reduced the catalytic ability of *DWF4*. For example, one of the substrates of *DWF4*, campestanol, is quantitatively alike in three different genotypes. Although the product of *DWF4*, 6-deoxocathasterone, is increased by approximately twofold in *tcp1-1D* seedlings, it decreased \sim 10-fold in *TCP1-SRDX* seedlings in comparison with that of wild-type seedlings (Figure 5). Similar results were observed for the reaction from campesterol to (22S)-22-hydroxycampesterol, which is also catalyzed by *DWF4* (Figure 5). None of the other reactions were affected in such a dramatic way. Because TCP proteins are transcription factors, TCP1 likely regulates *DWF4*-catalyzed reactions via the control of *DWF4* expression.

The Expression Level of *DWF4* Is Positively Regulated by TCP1

To test our hypothesis that TCP1 transcriptionally regulates *DWF4* expression, we first generated *TCP1p* (*TCP1* promoter)- β -glucuronidase (*GUS*) and *DWF4p*-*GUS* transgenic plants and examined the expression patterns of *TCP1* and *DWF4*. If TCP1 directly regulates the expression of *DWF4*, we would expect to see overlapping expression patterns of the two genes. We analyzed six independent transgenic lines for each construct. Each transgenic event showed consistent *GUS* staining results, which clearly indicated that both genes have overlapped expression patterns (see Supplemental Figure 6 online). Both genes are expressed relatively strongly in the roots and at the junctions of roots and hypocotyls. In the leaves, both genes are mainly expressed in vascular tissues. The expression level of *DWF4* in the leaves, however, is apparently much less than that of *TCP1*. The net *DWF4* expression level is determined by both the positive effect of TCP1 and negative action of BZR1/BES1. To examine whether changing expression levels of *TCP1* can affect the transcription of *DWF4*, we isolated total RNA from *WS2*, *tcp1-1D*, and *TCP1-SRDX* entire seedlings and performed real-time RT-PCR analyses. Our results clearly indicated that the expression level of *DWF4* is elevated in the *tcp1-1D* gain-of-function mutant seedlings but reduced in the *TCP1-SRDX* dominant-negative mutant seedlings (Figure 6A). The expression levels of all other known *Arabidopsis* BR biosynthetic genes were

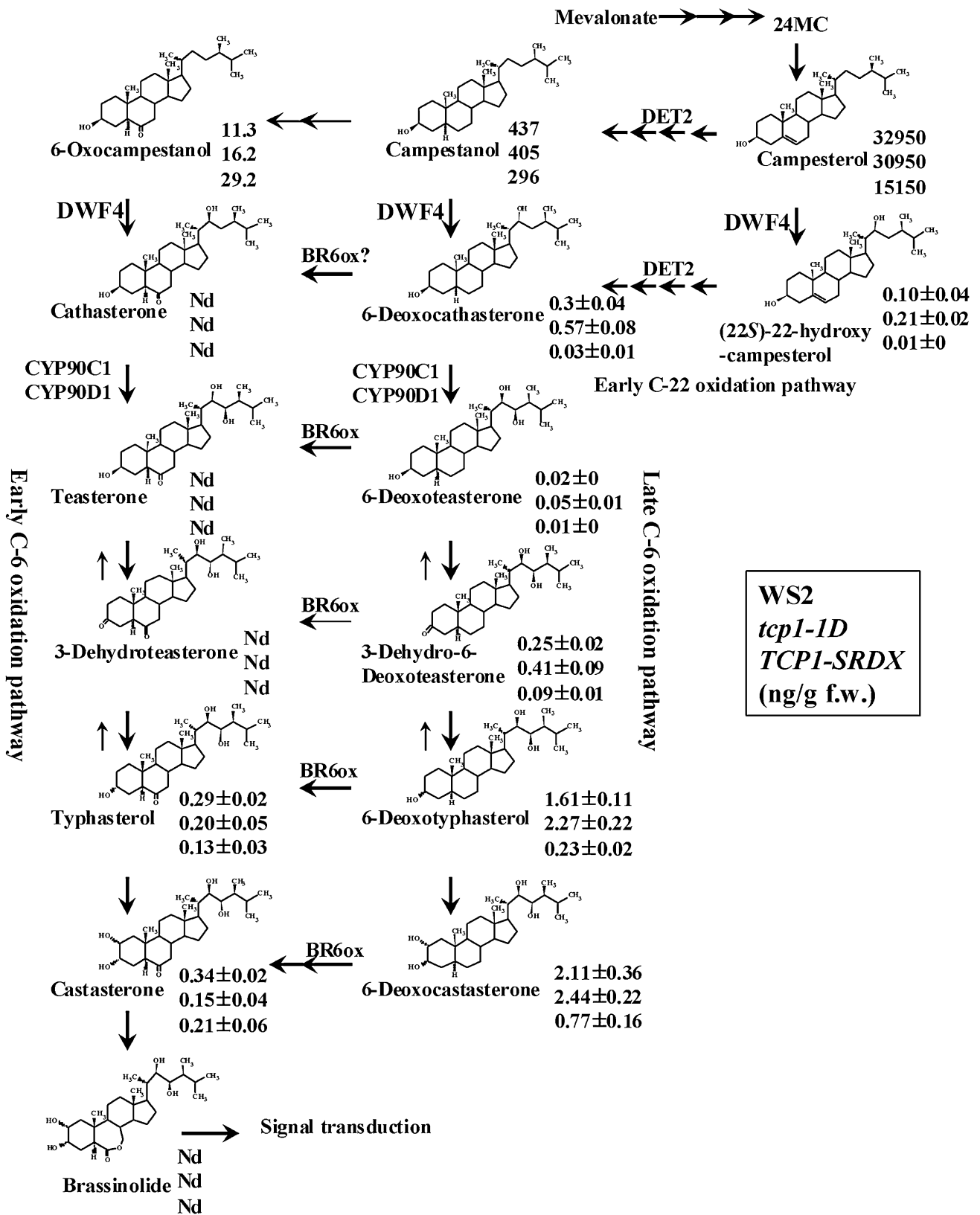


Figure 5. BR Profile Analyses of Four-Week-Old Soil-Grown WS2, *tcp1-1D*, and *TCP1-SRDX* Plants.

Levels (ng/g fresh weight) for each of the indicated molecules in the BR synthesis pathway were determined in WS, *tcp1-1D*, and *TCP1-SRDX* plants (top, middle, and bottom numbers, respectively). The numbers shown are the averages from three independent replicas. The ± represents SD. nd, not detected (below detection limit).

not significantly altered in *tcp1-1D* and *TCP1-SRDX* seedlings (data not shown). We also crossed the homozygous lines of *tcp1-1D* and *TCP1-SRDX* with homozygous lines of *DWF4p-GUS* plants, respectively, and obtained F1 plants. These F1 plants, only containing one copy of each of the transgene, were used to test whether overexpression of *TCP1* (*tcp1-1D*) or *TCP1-SRDX* can influence the expression levels of GUS by an immunoblotting analysis using anti-GUS antibody. If *TCP1* controls *DWF4* transcription via regulating the function of *DWF4* promoter, we expect to see that overexpression of *TCP1* upregulates the level of GUS and overexpression of *TCP1-SRDX* downregulates the level of GUS. Our immunoblotting results indeed confirmed these predictions (Figure 6B). These results confirmed that *TCP1* functions as a positive regulator in controlling *DWF4* transcrip-

tion. In addition, when *tcp1-1D* was crossed into a null *DWF4* mutant background, *dwf4-1*, the resulting *dwf4-1 tcp1-1D* double mutant plants showed the same phenotype as *dwf4-1* single mutant plants. (Figure 6C). *dwf4-1* is a T-DNA insertion mutant originally used to clone *DWF4* (Choe et al., 1998). The null *dwf4-1* mutant is more severe than a null *det2* mutant but much milder than a *cpd* null mutant (Szekeress et al., 1996; Fujioka et al., 1997; Choe et al., 1998). BR profile analysis suggested that *dwf4-1* still makes trace amount of BRs (Choe et al., 2001). The inability of *tcp1-1D* to suppress *dwf4-1* is consistent with our notion about *TCP1*'s role in regulating *DWF4* expression. Without a functional target gene, overexpression of *TCP1* becomes inconsequential as it would not be able to regulate the expression of the target gene and the subsequent downstream events leading to modified plant growth.

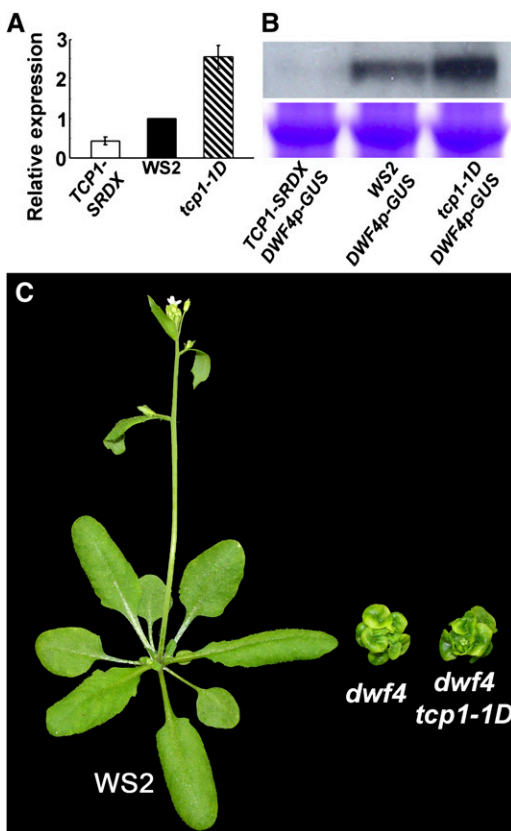


Figure 6. *TCP1* Positively Regulate the Expression of *DWF4*.

(A) Real-time RT-PCR results indicate that the expression of *DWF4* is elevated in *tcp1-1D* but reduced in *TCP1-SRDX* plants. *DWF4* expression level in WS2 was set to 1. *DWF4* expression levels in other genotypes were relative to that in WS2. The data were the average of three replicates. The error bars represent the SD.

(B) The top panel is the immunoblotting data indicating that *tcp1-1D* positively, whereas *TCP1-SRDX* negatively regulates the expression of *DWF4p-GUS*. The bottom panel is a duplicate polyacrylamide gel stained with Coomassie blue to show equal loading.

(C) *tcp1-1D* does not rescue the phenotypes of a null *dwf4* mutant. The plants were photographed 4 weeks after germination.

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

TCP1 Associates with the Promoter Region of *DWF4*

Our genetic and biochemical analysis data suggested that *TCP1* is a transcription factor positively regulating *DWF4* expression. To further examine *TCP1* function, we conducted a *TCP1* sub-cellular localization analysis to examine whether *TCP1* is localized in the nucleus and a chromatin immunoprecipitation (ChIP) assay to determine whether *TCP1* associates with the promoter of *DWF4*. We overexpressed a *TCP1-GFP* fusion gene in WS2 background, selected multiple transgenic lines, and identified homozygous plants. Confocal microscopy of primary roots from 4-d-old *TCP1-GFP* seedlings indicated that fluorescent signals originate exclusively from the nuclei. Representative results are shown in Figures 7A to 7D. These *TCP1-GFP* homozygous transgenic lines were then used to do ChIP experiments. According to predicted DNA binding sites of TCP family proteins from previous reports in rice, consensus TCP binding sites were found in the promoter regions of both *BES1* and *BRZ1* but not in the promoter region of *DWF4*. So we first checked whether the *TCP1* protein binds to the promoter regions of *BZR1* or *BES1* through ChIP analysis. As shown in Figure 7E, *TCP1* does not seem to associate with either the *BES1* or *BZR1* promoter. ChIP analysis, however, did demonstrate that the *TCP1* protein is associated with the *DWF4* promoter sequences, either via a novel binding sequence on the *DWF4* promoter (Figure 7E) or via its interaction with another transcription factor that directly binds to the promoter sequence of *DWF4*. Our ChIP analysis results were consistent with our real-time RT-PCR results because we did not find any altered expression of *BES1* and *BZR1* in *tcp1-1D* or *TCP1-SRDX* plants compared with that in wild-type plants (data not shown). We did find, however, that *DWF4* was upregulated in *tcp1-1D* and downregulated in *TCP1-SRDX* transgenic plants (Figures 6A and 6B).

TCP1 Expression Can Be Regulated by BR

To examine whether BR levels affect the expression of *TCP1*, seeds from a representative homozygous *TCP1p-GUS* line were germinated in half-strength MS medium supplemented with 1% sucrose and 0 μ M BL or BRZ, 1 μ M BL, and 1 μ M BRZ. The plants were grown under either complete darkness or continuous light conditions for 5 d. The seedlings were then stained for GUS

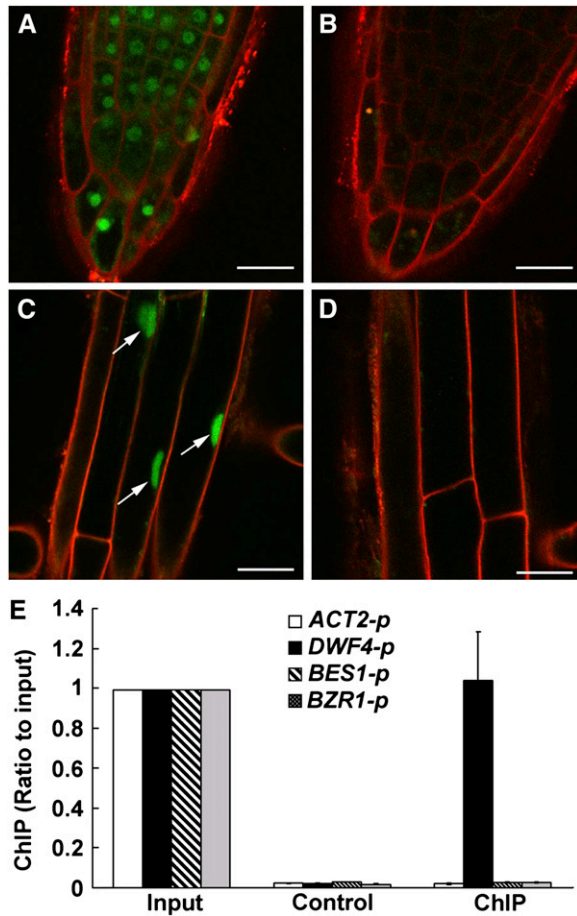


Figure 7. TCP1 Is Localized in the Nucleus, and ChIP Results Suggest That TCP1 Associates with *DWF4* Promoter.

(A) and (C) Confocal microscopy shows that the TCP1-GFP fusion is localized in the nucleus. Homozygous transgenic plants harboring 35S-*TCP1-GFP* were used for the analysis.

(B) and (D) Wild-type plants were used as controls. No fluorescent signals were detected in the analysis. Roots were counterstained with propidium iodide to visualize the outline of the cells. Bars = 20 μ m.

(E) CHIP results show that anti-GFP antibody can coimmunoprecipitate the *DWF4* promoter region but not the promoter regions of *ACT2*, *BES1*, and *BZR1*. Input: using the samples collected before immunoprecipitation as real-time PCR templates. ChIP: using diluted immunoprecipitated (with anti-GFP antibody) samples as real-time PCR templates. Control: using diluted immunoprecipitated (without adding any antibody) samples as real-time PCR templates. Each input was set to 1. Error bars represent the SD of three replicates.

activity and were also subjected to immunoblotting analyses using an anti-GUS antibody (Figures 8A to 8C). Our results indicated that application of 1 μ M BL can significantly stimulate *TCP1* expression as revealed by both GUS staining and immunoblotting results, whereas depletion of endogenous BRs by BRZ treatment can greatly reduce *TCP1* expression (Figures 8A to 8C). We also grew *WS2*, *det2-28* (in *WS2* ecotype), and *bri1-4* (in *WS2* background) for 5 d in half-strength MS medium

supplemented with 1% sucrose and 1 μ M BL. Real-time RT-PCR analysis indicated that *TCP1* expression levels were upregulated in *WS2* and *det2-28* but not in *bri1-4* in the presence of 1 μ M BL (see Supplemental Figure 7 online). The increasing level was quite dramatic in *det2-28*. These data suggest that the expression of *TCP1* can be regulated by exogenous and endogenous levels of BL, and the regulation may rely on a functional BRI1 receptor (Figure 8D).

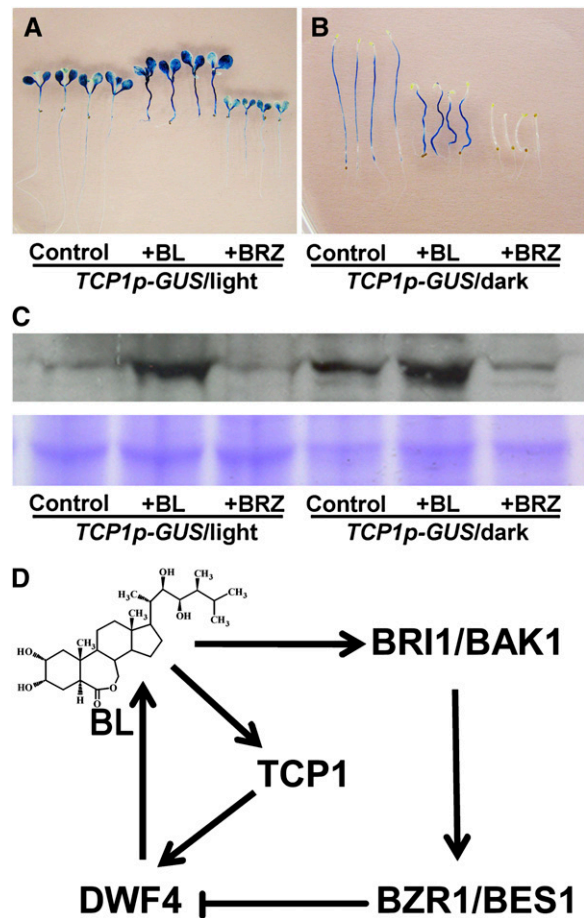


Figure 8. The Expression of *TCP1* Is Upregulated by BL.

(A) GUS-stained light-grown transgenic seedlings harboring *TCP1* promoter-GUS shows that the expression levels of *TCP1* can be positively regulated by the BL treatment and negatively regulated by the BRZ treatment.

(B) Dark-grown seedlings were used for the same experiments shown in (A).

(C) Immunoblotting analysis to confirm that GUS expression is positively regulated by BL. The top panel shows the immunoblotting results. The orders are the same as in (A) and (B). The bottom panel shows a duplicate polyacrylamide gel stained with Coomassie blue, indicating the equal amounts of total proteins were used in each lane.

(D) A model suggesting that the expression of *DWF4* can be negatively regulated by BZR1/BES1 via a feedback loop and also can be positively regulated by TCP1.

DISCUSSION

BR homeostasis is critical for normal plant growth and development. BR-deficient mutants show a severely retarded growth phenotype mainly due to lack of cell elongation. Excessive amounts of BR, on the other hand, greatly inhibit root growth and trigger leaf senescence (Clouse, 1996; Clouse and Sasse, 1998). Bioactive levels of BR are mainly balanced by the rates of biosynthesis and inactivation. Within the last a few years, a feedback inhibitory regulation has been elucidated, which involves two novel transcription factors, BZR1 and BES1 (Mathur et al., 1998; He et al., 2005). Previous studies indicated that the expression levels of five BR-specific biosynthetic genes, including *DET2*, *DWF4*, *CPD*, *BR6ox1*, and *ROT3*, are upregulated in plants treated with the inhibitor BRZ (Bancos et al., 2002; Tanaka et al., 2005). The expression levels of four of the five genes (*DWF4*, *CPD*, *BR6ox1*, and *ROT3*) are significantly downregulated in response to exogenous BL treatment. Previous studies also revealed a number of biochemical reactions that plants use to inactivate BRs if an excessive amount of BR is present. How BR biosynthesis is positively regulated, however, is poorly understood. When additional BR is needed at certain developmental stages or under various biotic/abiotic stresses, how plants perceive internal or external signals to trigger the production of more BR is poorly understood. Generally, it is thought that the plants can accumulate additional BR via two different mechanisms, including acceleration of the biosynthetic rate and deceleration of inactivation speed or slowing down the feedback regulation. The most effective way is to speed up BR biosynthesis by elevating the expression of key BR biosynthetic genes.

Among the BR biosynthetic genes isolated, *DWF4* was believed to be one of the key genes in the BR-specific biosynthetic pathway from campesterol to BL (Kim et al., 2006). *DWF4* encodes a P450 protein that catalyzes multiple 22 α -hydroxylation steps in BR biosynthesis (Choe et al., 1998). Relative to other BR biosynthetic genes, such as *CPD* and *DET2*, *DWF4* is expressed at an extremely low level (Kim et al., 2006). Although BR profile analysis indicated that *DWF4* substrates are always plentiful in plants, the products of the *DWF4*-catalyzed reactions are considerably low or even undetectable. Previous studies also indicated that *DWF4* is mainly expressed in actively growing tissues, such as root and shoot apices, where BRs are significantly enriched (Kim et al., 2006). In addition, overexpression of *DWF4* in wild-type *Arabidopsis* or tobacco (*Nicotiana tabacum*) plants can significantly increase biomass and seed production (Choe et al., 2001). These results suggest that *DWF4* catalyzes a flux-determining step in the BR biosynthesis pathway (Kim et al., 2006). Elucidating how *DWF4* expression is regulated will greatly advance our knowledge about the mechanisms controlling BR homeostasis in a plant.

Our detailed analyses clearly demonstrated that TCP1 is an important transcription factor positively regulating the BR biosynthesis by regulating the expression of *DWF4*. This notion is supported by several key observations. First, wild-type plants harboring *tcp1-1D* allele showed a phenotype similar to that of the *DWF4*-overexpressing plants (Choe et al., 2001). The transgenic plants overexpressing a dominant-negative chimeric gene, *TCP1-SRDX*, on the other hand, exhibited a typical BR deficiency

or signaling mutant phenotype. The dwarfed phenotypes of the transgenic seedlings can be rescued by supplementing the culture medium with BL but not with any other growth-promoting hormones, such as auxins, gibberellins, or cytokinins, suggesting that *TCP1-SRDX* transgenic plants are BR-specific deficient mutants, rather than BR signaling mutants. Secondly, our BR profile analysis results clearly indicated that the expression levels of TCP1 affect the function of *DWF4* but not other known BR biosynthetic enzymes. Our real-time RT-PCR results showed that overexpression of *TCP1* upregulates *DWF4* expression, whereas overexpression of *TCP1-SRDX* downregulates the expression of *DWF4*. Finally, our ChIP analysis using transgenic plants overexpressing *TCP1-GFP* demonstrated that TCP1 can interact with the promoter of *DWF4* directly or indirectly, but not with the promoters of *BZR1*, *BES1*, and *ACTIN2*.

It is worth noting, however, that although *tcp1-1D* showed an organ elongation phenotype resembling plants overexpressing *DWF4*, BR profile analysis did not reveal any accumulation of castasterone (CS)/BL in *tcp1-1D* plants. As a matter of fact, several intermediates in the later BR biosynthetic pathway, such as CS, were reduced in both *tcp1-1D* and *TCP1-SRDX* plants. However, the phenotypes were opposite. BR profile analysis was also conducted in plants overexpressing *DWF4* by Choe et al. (2001), and their results also showed there was no CS or BL accumulation in *DWF4*-overexpressing plants. Choe et al. (2001) proposed that BL, CS, or other bioactive BR in *DWF4*-overexpressing plants could be turned over rapidly after triggering the BR signal transduction chain. This idea is supported by the fact that in mutants impaired in BR signaling, such as in *bri1*, BL levels became elevated possibly because the signaling pathway has been disrupted in *bri1* mutants (Noguchi et al., 1999). The similarity of the BR profiles in *tcp1-1D* and *DWF4*-overexpressing plants in comparison with wild-type plants is another piece of evidence linking TCP1 function to the regulation of *DWF4* expression.

Although significant progress has been made in elucidating both the BR biosynthesis and signal transduction pathways, very little is known about how the expression of BR biosynthetic and signaling genes is positively regulated. Our identification of TCP1 as a positive regulator for *DWF4* expression reveals a mechanism through which plants are able to modulate BR biosynthesis during normal plant growth and development. TCP proteins are plant-specific transcription factors regulating a number of processes during plant growth and development, such as floral symmetry (Luo et al., 1999; Broholm et al., 2008), embryonic growth (Tatematsu et al., 2008), morphology of shoot lateral organs (Koyama et al., 2007), and jasmonate biosynthesis and senescence (Schommer et al., 2008). The name TCP was used after TEOSINTE BRANCHED1 from maize (*Zea mays*), CYCLOIDEA from *Anthirrinum majus*, and PCF from rice (Cubas et al., 1999). These proteins contain a bHLH domain, which is thought to be involved in DNA binding. There is also an R-domain whose function is not yet known. In *Arabidopsis*, there are 24 TCP transcription factors. Based on similarity in the amino acid sequence of the TCP domain, 13 were grouped into class I, and 11 were grouped into class II. It was found that the two classes have distinct but overlapping binding sequences. For example, rice class I TCP proteins prefer to bind GGNCCCAC, whereas

class II TCP proteins favor binding to GTGGNCCC (Kosugi and Ohashi, 2002). They share the core sequence GGNCCC. It is not known, however, whether *Arabidopsis* TCP transcription factors bind to the same DNA sequences. Since TCP1 belongs to class I of TCP transcription factors, we searched the promoter region sequence of *DWF4* to see whether it contains a GGNCCCAC motif. We failed to identify the consensus sequence for either class I or class II TCPs in the *DWF4* promoter region. This suggested that the possible binding sequence for TCP1 in *DWF4* promoter may not be strictly identical to what was reported in rice. Alternatively, even though we have determined that TCP1 and *DWF4* promoters are in the same complex as assayed by ChIP experiments, we cannot rule out the possibility that TCP1 is indirectly involved in activating *DWF4* transcription by its association with another true *DWF4* promoter binding transcriptional factor. Interestingly, in the promoters of both BZR1 and BES1, there are typical TCP binding sequences revealed from rice TCP orthologs. For instance, ~400 bp upstream of the translation initiation codon of *BES1*, there are two putative TCP binding motifs, GGACCCAC and GGCCCCAC. Similar sequences have also been detected in the promoter region of *BZR1*. However, it appears that the expression of *BES1* and *BZR1* is not directly regulated by TCP1, as the expression levels of these two transcription factors are not altered in *tcp1-1D* or *TCP1-SRDX* plants (data not shown). It is possible that these transcription factors are regulated by other members of the TCP family. Previous studies suggested that TCP1 is involved in floral symmetric regulation (Cubas et al., 2001; Busch and Zachgo, 2007). The detailed molecular mechanism controlling organ mono- or polysymmetry is poorly understood. Our identification of TCP1 in regulating BR biosynthesis suggests that unequal expression of *TCP1* may result in uneven distribution of BRs in floral meristems, which may contribute to the asymmetric regulation of floral organs. Future studies will focus on understanding how the expression of TCP1 is regulated by internal and external factors. The information about how TCP1 expression is regulated will facilitate our understanding of how BR biosynthesis is controlled.

METHODS

Plant Materials and Activation Tagging for *bri1-5* Genetic Modifiers

All plants used in these studies, including *bri1-5*, *dwf4-1*, and *bri1-4*, were in *Arabidopsis thaliana* ecotype WS2 background. Unless specified, plants were grown under 16 h light (150 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 8-h dark condition. The *bri1-5 tcp1-1D* double mutant was obtained by a large-scale activation-tagging screen for *bri1-5* suppressors as described previously (Li et al., 2001a, 2002; Zhou et al., 2004; Yuan et al., 2007). Briefly, a homemade activation-tagging construct, *pBASTA-AT2* (Yuan et al., 2007), was transformed into *bri1-5* to generate transgenic plants with resistance to Basta herbicide. *bri1-5 tcp1-1D* was identified as one of the *bri1-5* suppressors.

Identification of the *tcp1-1D* Locus

Thermal asymmetric interlaced PCR was used to amplify the flanking genomic sequence of the T-DNA of *pBASTA-AT2* as described previously (Liu and Whittier, 1995; Terauchi and Kahl, 2000). The T-DNA insertion site was determined by sequencing the flanking genomic DNA. The T-DNA

was inserted in the promoter of *TCP1* (At1g67260), 2281 bp upstream from its start codon.

Construction of Expression Vectors

Full-length *TCP1* cDNA was amplified with primers TCP1-attb1 and TCP1-attb2, and *TCP1-SRDX* was amplified with primers TCP1-attb1 and TCP1-SRDX-attb2 (see Supplemental Table 1 online). Both sequences were cloned into gateway vector *pB35GWG* as described previously (Gou et al., 2010) and overexpressed in *bri1-5* and WS2 plants. Transgenic lines were analyzed for phenotypes.

Real-Time RT-PCR Analyses

Total RNA was extracted using RNeasy plant mini kits with on-column DNase-treatment (Qiagen). Total RNA (2 μg) was reversely transcribed to the first strand of the cDNA in a 20- μL volume using the SuperScript III first-strand synthesis system (Invitrogen). For non-real-time PCR, 1 to 2 μL RT product was used as PCR template in a 20- μL volume reaction. Different PCR cycles were conducted to get better results. PCR products were separated by 1% agarose gel electrophoresis and visualized under a UV scanner. For quantitative RT-PCR experiments, 10 μg total RNA was reverse transcribed into first-strand cDNAs using the SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. The resulting cDNAs corresponding to 100 ng total RNAs (4 μL) were then used as templates for real-time PCR analyses of transcripts of interest using a MyIQ system (Bio-Rad) with gene-specific primers (see Supplemental Table 1 online). The relative expression level was calculated from three replicates using the comparative Ct method after normalized to an *ACT2* control.

Hypocotyl Measurements

All of the seeds used for hypocotyl analysis were surface-sterilized as described previously (Yuan et al., 2007) and planted on half-strength MS medium supplemented with 1% sucrose, 0.8% agar, and 24-epiBL or BRZ. The plates were kept at 4°C for 2 d and then were grown vertically in white light at room temperature. Hypocotyl length of seedlings was measured after 5 d. All measurements were obtained from three independent experiments, and at least 20 seedlings were measured each time.

Immunoblotting Analysis

Immunoblotting analysis was performed as previously described (Li et al., 2002; Wang et al., 2005). Both anti-GFP (A11120) and anti-GUS (A-5790) antibodies were from Invitrogen.

BR Profile Analysis

Aerial parts of 4-week-old WS2, *tcp1-1D*, and *TCP1-SRDX* seedlings were harvested and lyophilized. The tissues (15 g of lyophilized tissues per assay) were extracted twice with 250 mL of methanol. Deuterium-labeled internal standards were added to the extracts. Purification and quantification of BRs and sterols were performed according to the method described previously (Fujioka et al., 2002).

Confocal Microscopy Analysis

Seeds of transgenic plants overexpressing *TCP1-GFP* were surface sterilized in 95% ethanol and 20% bleach and washed with sterile double deionized water prior to planting. Sterilized seeds were germinated on 62 \times 48-mm glass cover slips coated with 0.5% agar supplemented with

half-strength MS salts, 0.5 mg/mL pyridoxine-HCL, 0.5 mg/mL nicotinic acid, 1 mg/mL thiamine, 0.10 g/L myo-inositol, 0.5 g/L MES, and 1% sucrose. The pH of the agar nutrient medium was adjusted to 5.7 with 10 M KOH. After 3 to 4 d, the primary roots were imaged with a Leica TCS SP2 AOBs laser scanning confocal microscope equipped with a $\times 63$ water immersion objective (Leica Microsystems). Seedlings were counterstained with 5 μ M propidium iodide for simultaneous visualization of GFP and the cell wall. Propidium iodide and GFP were excited with the 488-nm line of the argon laser, and emission was detected at 510 and 620 nm, respectively.

CHIP Analysis

The EpiQuik Plant ChIP kit (Epigentek) was used to conduct ChIP analysis. The experiments were performed based on the specifications from the manufacturer. Briefly, seedlings (1 to 2 g) were used to get chromatin pellets. The chromatin pellet was resuspended in 500 μ L CP3F containing protease inhibitor cocktail (Roche). DNA was sheared to \sim 500-bp fragments by sonicating and then centrifuged to get supernatants. The supernatant was diluted, transferred to an antibody (anti-GFP)-bound strip well, and incubated at room temperature for 60 to 90 min with gentle shaking. TCP1-GFP specifically associated DNA fragments were purified and eluted from the column. The obtained DNA fragments were tested with real-time PCR. Primers for amplifying *DWF4* were *DWF4pF* and *DWF4pR*; for amplifying *BES1*, they were *BES1pF* and *BES1pR*; for amplifying *BZR1*, they were *BZR1pF* and *BZR1pR*; and for amplifying *ACT2*, they were *ACT2pF* and *ACT2pR* (see Supplemental Table 1 online).

Generation of Promoter-GUS Transgenic Plants

A 1.5-kb promoter fragment of *TCP1* was amplified from the *Arabidopsis* genomic DNA by PCR with primers *TCP1p1* and *TCP1p2* (see Supplemental Table 1 online). A 1.1-kb promoter fragment of *DWF4* was amplified from genomic DNA by PCR with primers *DWF4p1* and *DWF4p2* (see Supplemental Table 1 online). Then, they were cloned into gateway vector *pBIB-BASTA-GUS-GWR* (Gou et al., 2010) and were transformed into *Arabidopsis* ecotype WS2. Homozygote transgenic plants were planted on soil or half-strength MS medium with 1% sucrose for GUS activity assay. *TCP1p-GUS* plants were planted on half-strength MS medium containing 1% sucrose with or without 1 μ M epi-BL (Sigma-Aldrich) or 1 μ M BRZ (Asami et al., 2000) for 5 d under light or darkness before determining for GUS activity. Seedlings or tissues were vacuum infiltrated in X-Gluc solution, followed by overnight incubation at room temperature, then destained with 70% ethanol and visualized for blue color.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *TCP1* (At1G67260), *ACT2* (AT3G18780), *DWF4* (AT3G50660), *BZR1* (AT1G75080), and *BES1* (AT1G19350).

Supplemental Data

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

Supplemental Figure 1. *TCP1-SRDX* Transgenic Plants Show a Typical Deetiolated Phenotype When Grown in Darkness.

Supplemental Figure 2. bHLH Domain in TCP1 Is Critical for the Functions of TCP1 and TCP1-SRDX in Regulating Plant Growth.

Supplemental Figure 3. The Dwarfed Phenotype of *TCP1-SRDX* Plants Can Be Partially Rescued by an Additional *tcp1-1D* Allele.

Supplemental Figure 4. Growth of *TCP1-SRDX* Plants in Response to BL Treatment Is Similar to a BR Deficiency Mutant *det2*.

Supplemental Figure 5. The Dwarfed Phenotype of *TCP1-SRDX* Plants Can Be Rescued by the Treatment of BL but Not by Other Major Plant Hormones.

Supplemental Figure 6. Representative Expression Patterns of Transgenes, *DWF4p-GUS* and *TCP1p-GUS*, by GUS Staining.

Supplemental Figure 7. *TCP1* Transcription Level Regulated by BL in Different Mutant Backgrounds.

Supplemental Table 1. Primers Used in This Study.

ACKNOWLEDGMENTS

We thank Frans Tax for providing the *dwf4-1* and *det2* mutants. This work is partially supported by grants from the National Science Foundation (IBN-0317729 to J.L.) and a National Natural Science Foundation of China grant (90917019 to J.L.), as well as a National Science Foundation Arabidopsis 2010 grant (MCB-0419819 to S.D. Clouse, J.L., S. Huber, and M. Goshe). This work was also supported in part by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to S.F. (Grant 19380069). We also thank Suguru Takatsuto (Joetsu University of Education) for supplying deuterium-labeled internal standards.

Received June 8, 2009; revised March 27, 2010; accepted April 7, 2010; published April 30, 2010.

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