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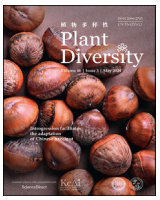

Short communication

BrrTCP4b interacts with BrrTTG1 to suppress the development of trichomes in *Brassica rapa* var. *rapa*

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ABSTRACT

The number of trichomes significantly increased in *CRISPR/Cas9*-edited *BrrTCP4b* turnip (*Brassica rapa* var. *rapa*) plants. However, the underlying molecular mechanism remains to be uncovered. In this study, we performed the Y2H screen using BrrTCP4b as the bait, which unveiled an interaction between BrrTCP4b and BrrTTG1, a pivotal WD40-repeat protein transcription factor in the MYB-bHLH-WD40 (MBW) complex. This physical interaction was further validated through bimolecular luciferase complementation and co-immunoprecipitation. Furthermore, it was found that the interaction between BrrTCP4b and BrrTTG1 could inhibit the activity of MBW complex, resulting in decreased expression of *BrrGL2*, a positive regulator of trichomes development. In contrast, AtTCP4 is known to regulate trichomes development by interacting with AtGL3 in *Arabidopsis thaliana*. Overall, this study revealed that BrrTCP4b is involved in trichome development by interacting with BrrTTG1 in turnip, indicating a divergence from the mechanisms observed in model plant *A. thaliana*. The findings contribute to our understanding of the regulatory mechanisms governing trichome development in the non-model plants turnip.

1. Introduction

Trichomes, epidermal protuberances situated on above-ground plant tissues, function as a physical barrier to safeguard plants against biotic threats such as fungal infections and insect herbivory. They also provide protection against abiotic stressors such as water loss, freezing, and UV radiation (Hauser, 2014). The morphology of trichomes exhibits a diverse range, with glandular trichomes capable of synthesizing metabolites for the medicinal purposes. Given their simple structure, the development of trichomes serves as an ideal model for studying the cell differentiation in plants (Ishida et al., 2008).

Trichome development has been extensively studied in model plants over the past decades, and numerous transcription factors that regulate trichome development have been identified in *Arabidopsis*. Among them, the R2R3 MYB-related transcription factor GLABRA1 (GL1), the Basic Helix–Loop–Helix transcription factor GL3, or its homolog ENHANCER OF GL3 (EGL3), and the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) collectively form a trimeric activator complex known as MYB-bHLH-WD40 (MBW) (Oppenheimer et al., 1991; Payne et al., 2000; Walker et al., 1999). Lose-of-function in *GL1*, *GL3* or *EGL3* leads to an obvious lack of trichomes on the leaves. Complete function mutations in TTG1 result in a loss of trichome initiation, indicating that TTG1 is crucial in regulating trichome development (Wang et al., 2021). The MBW complex can promote the trichome formation by directly activating the expression of *GLABRA2* (*GL2*), which encodes a conserved homeodomain-leucine zipper (HZ-Zip) regulator (Rerie et al., 1994). Lan et al. (2021) found that TCP4 directly interacts with GL3 to interfere with the transactivation activity of MBW complex, thereby inhibits the formation of trichomes. Our

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recently published study also showed that the trichome number was significantly increased in *CRISPR/Cas9*-edited *BrrTCP4b* turnip (*Brassica rapa* var. *rapa*) plants (Liu et al., 2022). This observation led us to speculate about a potential interaction between BrrTCP4b and BrrGL3 in turnip. The yeast two-hybrid (Y2H) and bimolecular luciferase complementation (BiLC) results showed that there was no interaction between BrrTCP4b and BrrGL3 (Fig. S1a and b). These data prompted us to investigate the molecular mechanism of BrrTCP4b in regulating trichome formation.

2. Materials and methods

2.1. Yeast two-hybrid analysis

The coding sequences of BrrTCP4b was fused in-frame with the GAL4 DNA-binding domain of the bait vector pGBKT7 (Takara Bio, Beijing, China) and transformed into yeast strain Y2HGold yeast strain. The library of turnip seedling cDNA fused with the GAL4 activation domain of the prey vector pGADT7-Rec was in the Y187 yeast strain (Taraka Bio). Y2HGold yeast strains were mated with Y187 yeast strains, suspended in synthetic medium dropout of SD-Leu-Trp-His-ADE (SD/-LTHA) and incubated at 30 °C. The positive interactions were further tested through cloning the full-length CDS of *BrrGL3* and *BrrTTG1* were cloned into pGADT7 vectors, respectively. The respective combinations of vectors were co-transformed into the yeast strain Y2HGold. pGADT7 and pGBKT7 vectors were cotransformed as negative controls. And selection medium supplemented with SD-LTH containing 5 mM 3-AT was used. The interactions were observed after 3 days of incubation at 30 °C. Primers used are listed in Table S1.

2.2. Co-immunoprecipitation assay

For Co-immunoprecipitation (Co-IP) assay, the coding sequences of *BrrTCP4b* and *BrrTTG1* were cloned into the pRI101-GFP and pRI101-6flag, respectively. The resulting constructs were transformed into *Agrobacterium* strain GV3101 and subsequently co-infiltrated into *Nicotiana benthamiana* leaves. The leaves were harvested after 3 days infiltration and ground to powder in liquid nitrogen. Total protein was extracted with lysis buffer [25 mM Tris−HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% (v/v) NP-40, 1 mM PMSF] and incubated with GFP-Trap (ChromoTek, Germany) at 4 °C for 3 h. The magnetic beads were washed three times with washing buffer, and then eluted into a volume of 30 μL using loading buffer before immunoblot analysis.

2.3. Bimolecular fluorescence complementation assay and bimolecular luciferase complementation assay

For Bimolecular fluorescence complementation (BiFC) assay, BrrTCP4b or BrrGL3 were fused to the N or C terminus of GFP respectively, and BrrTTG1 was fused with mCherry. The constructs were separately transformed into *Agrobacterium* strain GV3101. *Nicotiana benthamiana* leaves were observed at 3 d after co-infiltration. Confocal laser scanning microscopy was performed using epi-fluorescence microscopy (Olympus 80i, Olympus Corporation, Japan).

For Bimolecular luciferase complementation (BiLC) assay, BrrTCP4b was fused to the N-terminus of LUC, BrrGL3 and BrrTTG1 were fused to the C-terminus of LUC respectively. The resulting constructs were transformed into *Agrobacterium* strain GV3101, respectively. Luciferase activity was measured using Tanon 5200S Luminescent Imaging Workstation at 3 d after co-infiltration in *Nicotianabenthamiana* leaves. Three biological replicates were analyzed. Primers used are listed in Table S1.

2.4. Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed according to Meng et al. (2018). Two-week-old BrrTCP4b-GFP transgenic plants were used. Immunoprecipitation was carried out using an Anti-GFP antibody (ab290, Abcam, Shanghai, China). The enriched DNA fragments were analyzed through qRT-PCR. The primers used were: P1−F:5′ TAGCTGACGATGAGGATCGGA 3′, P1-R:5′ TGATCCTCCTCACCCTCCAAA 3′, P2−F:5′ ACATACATATACGCTAATGAG 3′ and P2-R: 5′ GTGTAAGCATGTGCAAACAT 3′.

2.5. Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR), total RNA was extracted using the Eastep® Super Total RNA Extraction Kit (Promega). 2 μg total RNA was used to reverse transcription using GoScript™ Reverse Transcriptase (Promega) according to the manufacturer's instructions. qRT-PCR was performed using Fast-Start Universal SYBR Green Master Mix (ROX). The data analysis was performed using Step One Plus Real-Time PCR System (Applied Biosystems). Three biological replicates were carried out for each sample. Primers used are listed in Table S1.

2.6. Transient transcriptional activation assay

For generating the reporter constructs, 2 Kb of *BrrGL2* promoter was amplified from the genomic DNA of turnip and cloned into the *pRI101-LUC*. ProGL2:LUC was transformed into *Agrobacterium* strain GV3101, and co-injected with 35S:BrrTCP4b, 35S:BrrTTG1 and 35S:BrrGL3 were used as effectors. The infiltrated leaves of *Nicotiana benthamiana* plants were sprayed with luciferin solution at 3 d after infiltration, and imaged using a Tanon 5200S Luminescent Imaging Workstation. Three biological replicates were analyzed. Primers used are listed in Table S1.

3. Results and discussion

In order to investigate the molecular mechanism by which BrrTCP4b regulates trichome development in turnip, we performed a Y2H screen using BrrTCP4b as the bait. Our finding revealed that BrrTTG1, a key WD40-repeat protein transcription factor in the MBW complex, interacts with BrrTCP4b in yeast (Fig. 1a).

To determine whether BrrTCP4b can interact with TTG1 in planta, BiLC was performed. The luminescence was obviously detected in the co-injection of *BrrTCP4b* and *BrrTTG1* in *Nicotiana benthamiana*, but not in the control (Fig. 1b), suggesting that BrrTCP4b interacted with BrrTTG1 *in vivo*. To further confirm the interaction, the co-immunoprecipitation (Co-IP) assay was performed by co-expressing the GFP-tagged BrrTCP4b and Flag-tagged BrrTTG1 protein using the transient expression system in *N. benthamiana*. The results showed that BrrTTG1 was pulled down by BrrTCP4b (Fig. 1c). These results indicated that BrrTCP4b physically interact with BrrTTG1. And BrrTTG1 could interact with BrrGL3, it suggested that BrrTTG1 and BrrGL3 might form the MBW complex in turnip (Fig S1c). To test whether BrrTCP4b affected the transactivation activity of MBW complex, we firstly detected the interaction between BrrTCP4b and MBW complex. We found that there was no fluorescence signal when the constructs of nGFP-BrrTCP4b and cGFP-BrrGL3, driven by the CaMV 35S promoter, were co-injected in leaves of *N. benthamiana* (Fig. 1d). However, co-injection of *35S:nGFP-BrrTCP4b*, *35S:cGFP-BrrGL3* and *35S:mCherry-BrrTTG1* into the leaves of *N. benthamiana* resulted in the detection of a fluorescence signal. The signal was also observed to be co-localized within the nucleus (Fig. 1e). These results implied that BrrTCP4b could interact with MBW complex. In the MBW complex,

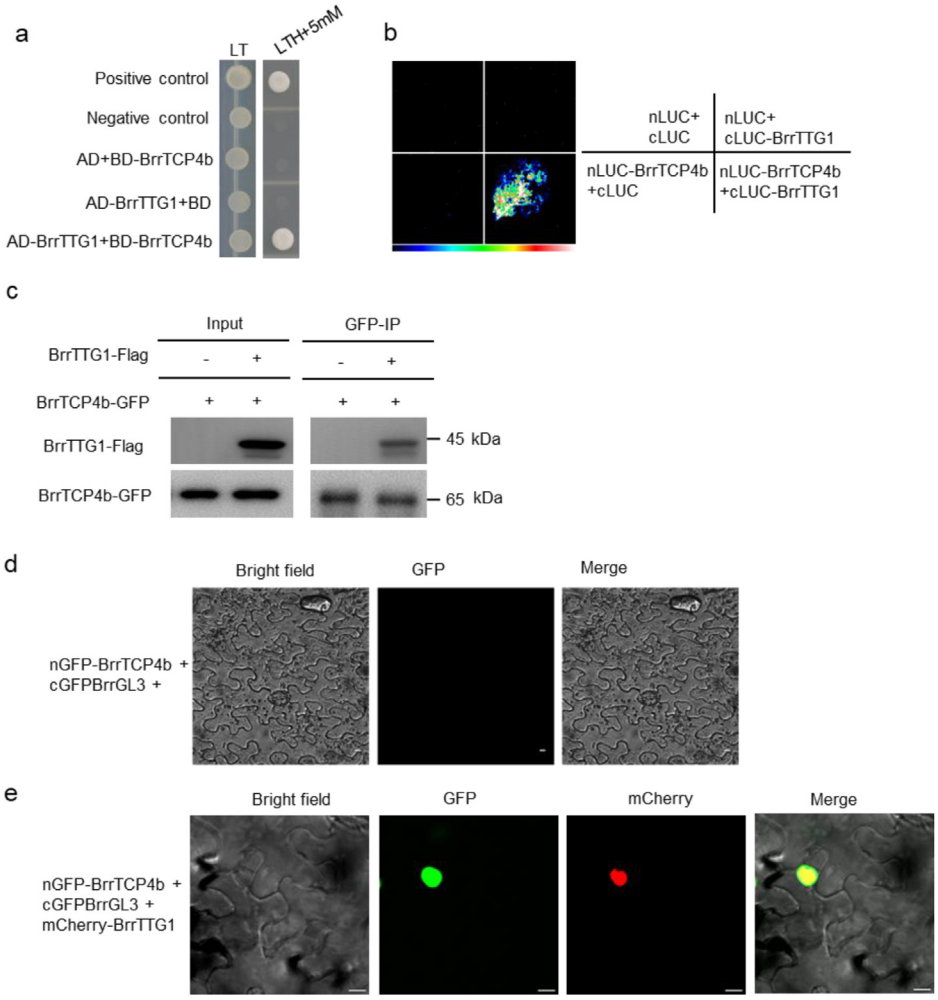

Fig. 1. BrrTCP4b physically interacts with BrrTTG1. a, Y2H assays of BrrTCP4b and BrrTTG1 proteins. The transformed yeasts were spotted on selection medium (SD-Leu-Trp-His) containing 5mM 3-AT (3-amino-1, 2, 4-triazole). 3-AT was used to inhibit the self-activation for the co-transformed yeast. AD, activation domain. BD, DNA-binding protein. b, Firefly luciferase complementation assay to test the interaction between BrrTCB4b and BrrTTG1 in *Nicotiana benthamiana*. BrrTTG1 was fused with cLUC at its N-terminus. c, CoIP assay to confirm the interaction between BrrTCP4b and BrrTTG1 in vivo. d, Bimolecular fluorescence complementation assay to test the interaction between BrrTCP4b and BrrGL3 in *Nicotiana benthamiana*. BrrTCP4b was fused with nGFP at its C-terminus and BrrGL3 was fused with cGFP at its N-terminus. e, BrrTCP4b interacts with MBW complex in *Nicotiana benthamiana*. BrrTTG1 was fused with mCherry. BrrTCP4b, BrrGL3 and BrrTTG1 co-localized in nucleus and formed a complex, Scale Bar = 10 μm. All experiments repeated three times, yielding consistent results.

TTG1 functions as a scaffold, on which the DNA-binding MYB and bHLH proteins interact to generate the transcriptional complex (Airoldi et al., 2019). In this study, BrrTCP4b cannot interact with BrrGL3. When BrrTTG1 is present, BrrTCP4b can interact with BrrGL3. These results implied that BrrTCP4b might regulate trichome development through BrrTTG1 in turnip.

GL2 is a direct target of MBW in regulation of trichome formation (Zhao et al., 2008). The expression level of *BrrGL2* was detected in the *CRISPR/Cas9*-edited *BrrTCP4b* turnip, the result showed that the expression level of *BrrGL2 CRISPR//Cas9*-edited *BrrTCP4b* turnip was obviously increased (Fig. 2a). ChIP-qPCR analysis revealed that BrrTCP4b directly binds to the promoter of *BrrGL2* (Fig. 2b). This finding suggests that BrrTCP4b may directly bind to the *GL2* promoter, thereby inhibiting its expression. Subsequently, a transient expression assay was performed to further validate this interference. The data demonstrated that co-injection of *35S:BrrTTG1* and *35S:BrrGL3* with *ProBrrGL2:LUC* could significantly enhanced the LUC activity. Whereas the LUC activity of the co-injection of *35S:BrrTCP4b*, *35S:BrrTTG1*, and *35S:BrrGL3* with *ProBrrGL2:LUC* was significantly reduced (Fig. 2c and d). BrrTTG1 and BrrGL3 could active the expression of *BrrGL2* in turnip (Fig. S2). These results suggested that BrrTCP4b inhibited the activity of MBW complex by directly interacting with the WD-40 repeat protein BrrTTG1 and was a negative regulator in the development of trichomes in turnip.

In summary, our findings revealed that BrrTCP4b physically interacts with BrrTTG1 to suppress trichome formation in turnip, which is different from the model plant *Arabidopsis*. The *Brassica* lineage underwent an additional whole genome triplication (WGT) event after its divergence from the *Arabidopsis* lineage (Cheng et al., 2014). Multiple copies of genes can arise from WGT and these copies must provide a function to the organism to be preserved in the genome (Birchler and Yang, 2022). This led us to hypothesize that BrrTCP4b might acquire a new function following the genome triplication in turnip. These findings contribute to our understanding of the regulatory mechanism of trichome development in the non-model plants turnip.

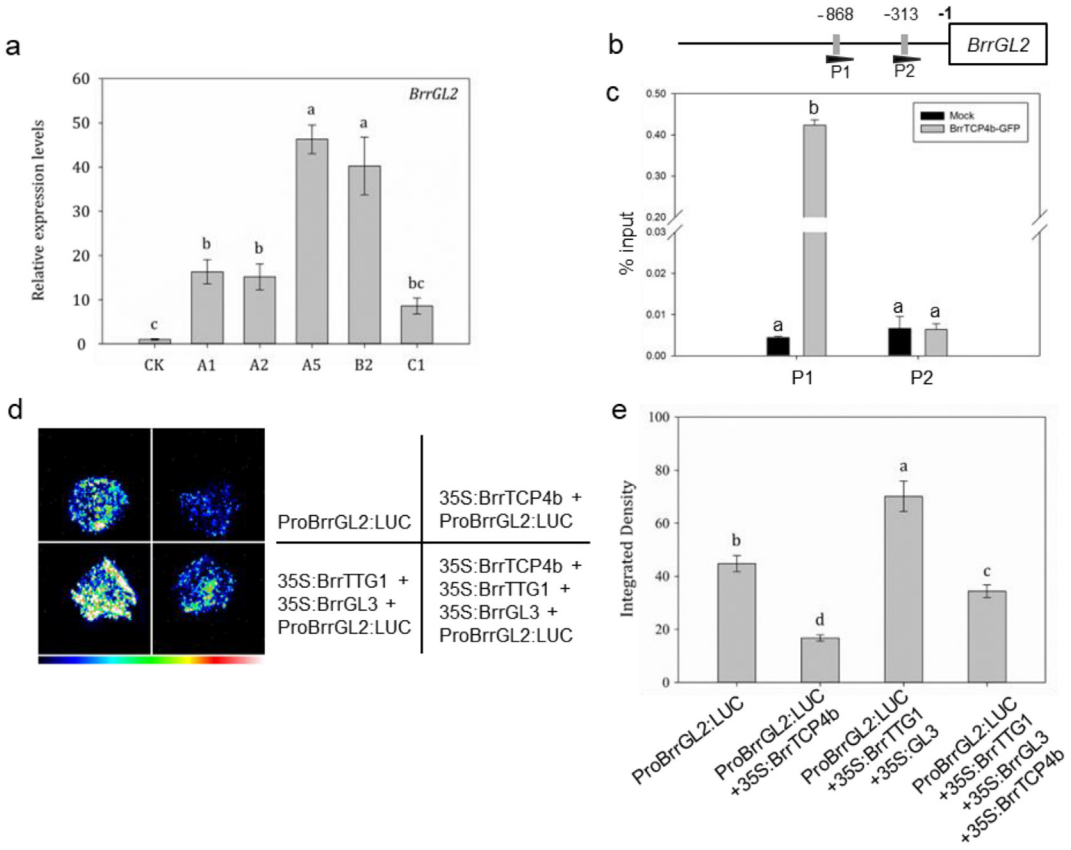

Fig. 2. BrrTCP4b disrupts the transactivation activity of MBW complex. a, Relative expression level of *BrrGL2* in *CRISPR-Cas9-BrrTCP4b* turnip. A1, A2, A5, B2 and C1 represent five *BrrTCP4b* gene-edited lines. The expression level of *BrrGL2* in wild-type turnip was set as one. *BrrTUB* was used as the reference gene. The data are means (±SE) of three biological replicates. b, Schematic of the location of BrrTCP4b-binding motif in the promoter of *BrrGL2* gene. P1 represents BrrTCP4b-binding site. P2 represents the non-binding site of BrrTCP4b, as a negative control. c, Chromatin immunoprecipitation assay of BrrTPC4b binding to the promoter region of *BrrGL2*. Relative enrichment was calculated as the value of the amplified signal normalized against that of the input DNA. Data presented are the means ± SD of three biological replicates. d, Transient expression assay showing that the activation of *GL2* expression by BrrTTG1/BrrGL3 was repressed by BrrTCP4b. The ProBrrGL2:LUC reporter was co-injected with corresponding constructs. e, Quantification of relative luminescence. The data are the means (±SE) of four biological replicates. Letters indicate statistically significant difference in the means ($P < 0.05$). Statistical analyses were performed by one-way ANOVA analysis.

CRediT authorship contribution statement

Cheng Li: Writing – original draft, Data curation. **Li Zhang:** Formal analysis, Data curation. **Hefan Li:** Resources. **Yuanwen Duan:** Resources, Data curation. **Xuemei Wen:** Writing – review & editing. **Yongping Yang:** Writing – review & editing, Funding acquisition. **Xudong Sun:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2024.03.003.

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