Establishment of *Agrobacterium*-mediated genetic transformation and application of CRISPR/Cas9 genome-editing system to *Brassica rapa* var. *rapa*

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Abstract

**Background:** Genome editing is essential for crop molecular breeding. However, gene editing in turnip (*Brassica rapa* var. *rapa*) have not been reported owing to the very low transformation efficiency.

**Results:** In this study, we established a transformation procedure involving chemical-inducible activation of the *BrrWUSa* gene, which resulted in high transformation frequencies of turnip. Estradiol-inducible *BrrWUSa* transgenic plants were fertile and showed no obvious developmental defects. Furthermore, we used CRISPR/Cas9 gene-editing technology to edit *BrrTCP4b* and generated 20 *BrrTCP4b*-edited seedlings with an increase in leaf trichome number.

**Conclusion:** The results demonstrate that *BrrWUSa* improves the regeneration efficiency in turnip. The transformation procedure represents a promising strategy to improve genetic transformation and for functional characterization of genes in turnip.

**Keywords:** Turnip, *Brassica rapa* var. *rapa*, *BrrWUSa*, Genetic transformation, Genome editing

Background

The development and optimization of genome-editing tools has enabled precise genome modifications and research on gene function has been boosted. Owing to their accuracy, efficiency, and cost-effectiveness, CRISPR/Cas systems have been widely applied to diverse organisms [1]. *Agrobacterium*-mediated genetic transformation is an important means to achieve efficient gene delivery and editing for CRISPR/Cas systems. However, low efficiencies in plant regeneration and dependence on a limited number of transformable genotypes hinder gene-editing tool implementation for crop improvement [2, 3]. Thus, improvement of regeneration efficiency remains an important research focus.

Research on development regulators is crucial to improve plant regeneration, given the key functions of development regulators in cell differentiation and morphogenesis. A suite of development regulators that promote plant regeneration in tissue culture has been reported, such as WUSCHEL (WUS) [4], LEAFY COTYLEDON 1 (LEC1) [5], BABY BOOM (BBM) [6], and AGAMOUSLIKE 15 (AGL15) [7]. Overexpression of such regulators can promote regeneration of various transformation-recalcitrant genotypes and species. WUS, which encode a homeodomain transcription factor, has been exploited to promote cell differentiation and...
plant regeneration, and plays a critical role in stem cell fate determination and maintenance in the shoot apical meristem of higher plants [8]. For example, overexpression of *A. thaliana* WUS induces initiation of stem cells in vegetative tissues, which can differentiate into somatic embryos in the absence of exogenous plant hormones [4]. Recent study shows that overexpression of the wheat gene *TaWOX5* significantly increases wheat transformation efficiency [9]. Expression of the maize development regulators BBM and WUS2 results in high transformation frequencies of maize and other transformation-recalcitrant monocotyledonous species [6].

Turnip (*Brassica rapa* var. *rapa*) is used mainly as a vegetable and for fodder. The antihypoxic activity of turnip provides the potential to prevent altitude stress [10, 11]. Therefore, an improved understanding of the bioactivities is of medicinal importance. However, further research on gene function of turnip has been greatly hampered by the low efficiency of transformation and shoot regeneration. Establishment of a high-efficiency *Agrobacterium*-mediated genetic transformation method for turnip requires optimization of callus proliferation and shoot regeneration during tissue culture.

In this study, we show that inducible expression *BrrWUSa* promotes callus formation and shoot regeneration, and establish a high-efficiency *Agrobacterium*-mediated genetic transformation system for turnip. Furthermore, we successfully generated *BrrTCP4b*-targeted editing in turnip, providing a technological foundation to gain insight into gene function in turnip in future studies.

**Results**

*BrrWUSa* promotes callus formation and shoot regeneration in turnip

Although tissue culture of turnip readily produces callus, the shoot regeneration has proved to be a bottleneck. To enhance shoot formation, we aimed to reprogram plant meristems by expressing a development regulator. Three *WUS* homolog genes were identified in the turnip genome, and based on its higher expression levels in shoot apical meristem (SAM), *BrrWUSa* was selected for assessment of regeneration frequencies (Additional file 1: Fig. S1). The hypocotyls of 4-day-old turnip seedlings were cut into 3–5 mm segments as explants. The explants were inoculated with *Agrobacterium* harboring 3SS:GFP (as a control) or 3SS:*BrrWUSa*-GFP for 15 min, and then transferred to callus-induction medium (Fig. 1a, b). The explants were cultured for 2 days in darkness condition to increase the infection efficiency, then were transferred to long-day condition (16-h light/8-h darkness, 22 °C). After 2 weeks, explants of control hypocotyl segments and 3SS:*BrrWUSa*-GFP hypocotyl segments gave rise to callus (Fig. 1c, d). After 4 weeks, no shoots regenerated from control hypocotyl segments (Fig. 1e; Table 1), whereas shoots regenerated from hypocotyl segments inoculated with 3SS:*BrrWUSa*-GFP (Fig. 1f). Hypocotyls transformed with 3SS:*BrrWUSa* showed 13% regeneration efficiency, whereas no shoots regenerated in the control (Table 1). The regenerated shoots were transferred to rooting medium, and whole plantlets formed after 30 days (Fig. 1g). The transformants were verified by PCR and confocal laser scanning microscopy. Using gene-specific primers containing the upstream sequence of *GFP* gene (F) and downstream of *BrrWUSa* (R), the amplified band was detected in all regeneration lines, but not in wild type (Fig. 1h). Confocal laser scanning microscopy observed fluorescence in the nucleus of root cells in 3SS:*BrrWUSa*-GFP transgenic plants, which indicate the successful expression of *BrrWUSa*-GFP fusion protein at the root apical meristem and verifies the transformants (Fig. 1i). These results suggested that *BrrWUSa* could promote shoot regeneration in turnip.

**Inducible expression of BrrWUSa improves shoot regeneration and generates fertile plants**

Overexpression of *BrrWUSa* in turnip resulted in sterile plants with abnormal leaf phenotype (Additional file 1: Fig. S2). To avoid the harmful effects of *BrrWUSa*, *BrrWUSa* was subcloned into the pER8 [12]. Turnip hypocotyl explants transformed with *Agrobacterium* carrying pER8-*BrrWUSa* were cultured on callus-induction medium supplemented with or without estradiol (Fig. 2a, d). After 4 weeks co-culture, explants on both medium gave rise to callus (Fig. 2b, e). The result show that explants untreated with estradiol were unable to generate shoots (Fig. 2c), the callus produced shoots after incubation for 4 weeks on medium supplemented with estradiol (Fig. 2e, f). The regenerated shoots were transferred to soil after incubation in root-induction medium (Murashige and Skoog medium) (Fig. 3). The regenerated shoots developed into complete plants with estradiol treatment, and transgenic plants were fertile without obvious developmental defects (Fig. 3e). These results indicated that inducible expression of *BrrWUSa* in turnip increased the efficiency of plant regeneration.

**Characterization of BrrTCP4b-targeted editing in turnip transformants**

To demonstrate the utility of plant transformation to generate genome-edited plants, turnip *TEOSINTE BRANCHED1/CYCLOIDEA/PCF 4b* (*BrrTCP4b*) was selected for genome editing. The constructs expressing gRNA targeting the *BrrTCP4b* gene were transformed...
into pER8-BrrWUSa transformant hypocotyls and incubated in the dark for 72 h. After incubation for 2 weeks on callus-induction medium supplemented with 2 μM estradiol, all explants developed embryogenic calli and were then transferred to shoot-induction medium supplemented with 2 μM estradiol and 20 mg/L kanamycin. Then we transferred the regenerated shoots to root-induction medium supplemented...
with 20 mg/L kanamycin. All shoots formed complete plants and were fertile (Fig. 4a). We extracted genomic DNA and examined mutations or deletions within the targeted region (Fig. 4b, c). Sequencing of the cloned PCR products verified successful deletions at the expected positions. Twenty gene-edited T₀ plants were transferred to soil. Phenotype analysis revealed that the number of trichomes in CRISPR-Cas9-BrrTCP4b plants was approximately 150% greater than the pER8-WUSa mock plant (Fig. 4d, e). TCP4 directly activates the expression of GLABROUS INFLORESCENCE STEMS (GIS) and lipoxygenase2 (LOX2) to regulate the formation of trichome [13, 14]. Therefore, quantitative RT-PCR assays were conducted to detect the expression levels of targeted genes. The expression levels of BrrGIS and BrrLOX2 in CRISPR-Cas9-BrrTCP4b plants were significantly decreased compared with those of the mock plant (Fig. 4f). These results demonstrated that BrrWUSa may significantly improve the regeneration efficiency and CRISPR/Cas9-mediated genome editing in turnip.

**Discussion**

Genome editing shows strong potential for crop improvement, but its application is restricted by low plant regeneration frequencies and genotype dependence [2, 15]. In recent years, efforts to improve plant regeneration frequencies have continued. Overexpression of plant development regulators greatly increases regeneration and transformation frequencies. Research on genetic transformation of turnip, a valuable cruciferous crop, is hardly a blank.

Given that shoot regeneration efficiency is essentially genotype-dependent, we estimated the genotype effect of more than one hundred turnip landraces. However, plant regeneration was unsuccessful for all landraces. Studies have reported improvements in the efficiency of plant regeneration from tissue culture by overexpression of plant development regulators, such as WUS and BBM [4, 6, 16] and GRF-GIF chimeras [2, 15, 17]. Recent studies indicate overexpression of TaWOX5 is an efficient way to help wheat overcomes genotype dependency and promote genetic transformation [9]. To increase the regeneration efficiency of turnip calli, we cloned BrrWUSa, which showed highest expression levels among WUS homologs in turnip. Our study indicate that BrrWUSa...
promotes callus formation and shoot regeneration in turnip. However, the breakthrough and improvement of both regeneration frequency and genome-editing efficiency is confined to overexpression of the specific plant development regulators which can reprogram somatic cells to embryogenic cell [4, 6, 16]. Based on the high regeneration efficiency of BrrWUSa overexpression, we transfer the target gene into the transgenic plants of BrrWUSa and to assess the genome-editing efficiency. Besides, to avoid sterility and obvious developmental defects in transformants caused by BrrWUSa overexpression, we generated the estrogen-inducible construct pER8-BrrWUSa. Hypocotyl segments transformed with pER8-BrrWUSa successfully generated shoots under estrogen treatment. The regenerated shoots developed into complete plants and were fertile without obvious developmental defects observed. Taking advantage of this transformation procedure, CRISPR/Cas-mediated genome editing was conducted. Twenty T0 plants all showed clear DNA deletion and mutation. TCP4 plays a negative effect on trichome differentiation and suppress trichome branching in Arabidopsis leaves and inflorescence stem [13]. Our research showed the number of trichomes in CRISPR-Cas9-gRNA-BrrTCP4b plants increased significantly in gene-edited compared with mock plant, and the relative expression levels of downstream target genes of BrrTCP4 significantly decrease. These results demonstrated that BrrWUSa may enhance CRISPR/Cas9-mediated genome editing in turnip.

In summary, we successfully established a stable genetic transformation and gene-editing procedure for turnip. Inducible expression of BrrWUSa significantly increased the regeneration efficiency and generated a large number of fertile gene-edited plants. The method may play an important role in functional characterization of genes for turnip and provides a powerful tool for future improvement of turnip by CRISPR/Cas-mediated gene editing.

Materials and methods
Brassica rapa var. rapa Landrace KTRG-B-103 (Xichang, Sichuan, China) was selected owing to its high differentiation rate determined in a preliminary experiment. KTRG-B-103 homozygous F1 seeds were harvested after self-pollination. The seeds were sterilized in petri dishes containing 1% sodium hypochlorite solution for 10 min and rinsed five times with sterilized deionized water. The sterilized seeds were sown on half-strength MS medium (pH 5.8) supplemented with 3% (w/v) sucrose.
and 0.5% (w/v) Phytagel. The seeds germinated for 2 days in the darkness, and then transferred to long-day condition (16-h light/8-h darkness, 22 °C) and gernimated for 2 days. Subsequently, hypocotyl explants (about 3–5 mm in length) were prepared with a scalpel and infected by Agrobacterium.

**Plasmid constructions**

The full-length coding DNA sequence of *BrrWUSa* was cloned in accordance with the method of Li et al. [18] and subcloned into the pRI101-GFP vector using the ClonExpress II One Step Cloning Kit (Vazyme Biotech) (Additional file 1: Fig. S3a). For cloning, the primers F (5'-GCA GCGGCGTCCGACATGGAGCAACCGCAACATCA-3') and R (5'-GTTAGATTCAGGATTC TTAATCCGGTTGACACCGCTG-3') were used.

The *pER8-BrrWUSa* plasmid was reconstructed in accordance with the method of Guo et al., [19]. *BrrWUSa* was subcloned into pENTR™/D-TOPO (Invitrogen) using the primers F (5'-CACCAtggagcaaccgcaacatca-3') and R (5'-atccggtgagacgcctg-3') for the entry clone. The pENTR-BrrWUSa plasmid was chosen to proceed to the LR reaction with pER8-GATEWAY-3Flag and generated the pER8-BrrWUSa plasmid (Additional file 1: Fig. S3b). Then BrrTCP4b target sequence was fused into CRISPR/Cas9 vector to generate the genome-editing construct (Additional file 1: Fig. S3c).

**Agrobacterium-mediated transformation**

The reconstructed plasmids pRI101-BrrWUSa-GFP and pER8-BrrWUSa were transferred into *A. tumefaciens* strain EHA105 by electroporation, respectively. Positive transformants were identified by PCR, then incubated for two days in 50 mL liquid YEB medium at the
ratio of 1:50. The culture was centrifuged at 5000 × g for 10 min and adjusted to OD₆₀₀ = 0.3 for the inoculation buffer (half-strength MS supplemented with 100 mM acetosyringone). The explants were infected for 15 min and then transferred to the MS medium. After co-cultivation for 3 days in the dark, the explants were rinsed five times with sterile water supplemented with 500 mg/L cefalexin and transferred to MS medium supplemented with 6-benzylaminopurine (4.0 mg/L), naphthaleneacetic acid (1.5 mg/L), and Timentin (100 mg/L). The medium was renewed every 2 weeks. The regenerated shoots were excised and transferred to root induction medium (MS medium).

**Gene expression analysis**
Total RNA was extracted using the Eastep® Super Total RNA Extraction Kit (Promega, Beijing, China) according to the manufacturer’s instructions. One microgram total RNA was used for the first-strand cDNA synthesis in 20μL reaction volumes containing GoScript™ Reverse Transcriptase (Promega). 2 μL cDNA, 6 μL distilled water, 10 μL FastStart Universal SYBR Green Master Mix (ROX), and gene-specific primers was mixed to generate 20 μL qPCR reaction mixtures. The Step One Plus Real-Time PCR System (Applied Biosystems) was used for the PCR program, which comprised one cycle (50 °C, 2 min), one cycle (95 °C, 10 min), 40 cycles (95 °C for 5 s, 60 °C for 15 s, 72 °C for 5 s), and one cycle (72 °C, 10 min). Three independent biological replicates were performed. *Tubulin* was amplified as a constitutive control. Relative expression levels were calculated using 2⁻ΔΔCt method. The primers used for quantitative RT-PCR are listed in Additional file 1: Table S1.

**Fluorescence microscopy**
Root tips from transgenic plants were excised and GFP fluorescence was observed with an OLYMPUS confocal microscope equipped with a 480/530 nm excitation filter.

**Cryo-scanning electron microscopy**
Cryo-scanning electron microscopy was used to observe the trichomes on the leaf. A section of leaf (approximately 3- × 3-mm) along the length of the lamina and midway between the margin and the mid-vein was fixed in a custom sample holder and submerged in liquid nitrogen for 2 min. A cryo-transfer shuttle Quorum pp3010t was used to transfer the sample to a precooled chamber under vacuum for coating. The samples were then observed at an accelerating voltage of 7.0 kV with a scanning electron microscopy (ZEISS Sigma 300, Zeiss, Oberkochen, Germany) with a cryogenic stage maintained at – 140 °C.

**Molecular analysis of CRISPR/Cas9 target sites**
Genomic DNA was extracted from seedlings according to CTAB method. The primers BrrTCP4bF and BrrTCP4bR were used to amplify BrrTCP4b. The PCR products were sequenced and aligned with the MEGA11 [20].

**Statistical analysis**
Statistical analyses of the experimental data were performed using single-factor analysis of variance or Student’s t-test implemented in SPSS (Version 17.0).

**Accession numbers**
Sequence data for most genes studied in this article can be found in the National Center for Biotechnology Information under the following accession numbers: *BrrTCP4b* (KY608005); *BrrWUSa* (MN481054); *BrrGIS* (ON887158) and *BrrLOX2* (ON887159).

**Supplementary Information**
The online version contains supplementary material available at https://doi.org/10.1186/s13007-022-00931-w.

Additional file 1: Fig. S1. Relative expression levels of *BrrWUS*, *BrrWUSa*, and *BrrWUSb* in turnip. Fig. S2. Phenotype of 35S:BrrWUSa transgenic plants. Fig. S3. Schematic structure of three vectors. Fig. S4. Raw result for Fig. 1h. Fig. S5. Raw result of Fig. 4b. Table S1, Primers used in this study.

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**Author contributions**
XS designed the experiments, analyzed the results. XS and YL wrote the manuscript. YL and LZ participated in most of the experiments. Other authors helped with the field work. All authors discussed the results and provided feedback on the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**
**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.
Competing interests
The authors declare that they have no competing interests.

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