Ribozyme-mediated CRISPR/Cas9 gene editing in pyrethrum (*Tanacetum cinerariifolium*) hairy roots using a RNA polymerase II-dependent promoter

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**Abstract**

**Background:** Traditional CRISPR/Cas9 systems that rely on U6 or U3 snRNA promoters (RNA polymerase III-dependent promoters) can only achieve constitutive gene editing in plants, hampering the functional analysis of specifically expressed genes. Ribozyme-mediated CRISPR/Cas9 systems increase the types of promoters which can be used to transcribe sgRNA. Therefore, such systems allow specific gene editing; for example, transcription of the artificial gene Ribozyme-sgRNA-Ribozyme (RGR) is initiated by an RNA polymerase II-dependent promoter. Genetic transformation is indispensable for editing plant genes. In certain plant species, including pyrethrum, genetic transformation remains challenging to do, limiting the functional verification of novel CRISPR/Cas9 systems. Thus, this study’s aim was to develop a simple *Agrobacterium rhizogenes*-mediated hairy root transformation system to analyze the function of a ribozyme-mediated CRISPR/Cas9 system in pyrethrum.

**Results:** A hairy root transformation system for pyrethrum is described, with a mean transformation frequency of 7%. Transgenic hairy roots transformed with the pBI121 vector exhibited significantly increased beta-glucuronidase staining as a visual marker of transgene expression. Further, a ribozyme-based CRISPR/Cas9 vector was constructed to edit the *TcEbFS* gene, which catalyzes synthesis of the defense-related compound (E)-β-farnesene in pyrethrum. The vector was transferred into the hairy roots of pyrethrum and two stably transformed hairy root transgenic lines obtained. Editing of the *TcEbFS* gene in the hairy roots was evaluated by gene sequencing, demonstrating that both hairy root transgenic lines had DNA base loss at the editing target site. Gas chromatography–mass spectrometry showed that the (E)-β-farnesene content was significantly decreased in both hairy root transgenic lines compared with the empty vector control group. Altogether, these results show that RGR can be driven by the CaMV35S promoter to realize *TcEbFS* gene editing in pyrethrum hairy roots.

**Conclusion:** An *A. rhizogenes*-mediated hairy root transformation and ribozyme-mediated CRISPR/Cas9 gene editing system in pyrethrum was established, thereby facilitating gene editing in specific organs or at a particular developmental stage in future pyrethrum research.

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Background

CRISPR systems can modify target genes in plants by substituting, removing, inserting, or knocking out DNA [1]. In these systems, a single guide RNA (sgRNA) forms a complex with the Cas9 protein and guides the Cas9 protein to bind to a specific target DNA for gene editing [2]. Since the first successful application of a CRISPR system [3], this technology has been continuously refined and optimized to improve editing efficiency and accuracy [4, 5]. In recent years, CRISPR systems have been successfully applied to food crops, such as maize (Zea mays L.) [6]; cash crops, such as cotton (Gossypium hirsutum) [7]; fruits, including strawberry (Fragaria ananassa) [8]; and medicinal crops, including Chinese wolfberry (Lycium ruthenicum) [9], and is considered a new and effective tool for use in plant gene function research [10].

In the CRISPR/Cas9 system, the transcription of sgRNA is key. Transcription in eukaryotes is conducted by three RNA polymerase enzymes [11]: RNA polymerase I transcribes large rRNAs; RNA polymerase II synthesizes mRNAs; and RNA polymerase III transcribes small noncoding RNAs, such as the U6 snRNA [12]. Accordingly, their corresponding promoters were also divided into three types, namely RNA polymerase I-dependent promoter (pol I promoter), RNA polymerase II-dependent promoter (pol II promoter) and RNA polymerase III-dependent promoter (pol III promoter) [13–15]. Specific gene silencing requires specific promoters [16]; however, specific promoters are usually driven by RNA polymerase II and cannot be directly used for sgRNA transcription because the RNA undergoes extensive processing and modification at both ends to become a mRNA [17]. Previous researchers have used a root-cap-specific promoter or stomatal lineage-specific promoter (both are pol II promoters) to initiate Cas9 mRNA transcription, with the AtU6-26 promoter (a pol III promoter belonging to non-tissue specific constitutive promoters [17]) used to initiate sgRNA transcription, and thereby achieved tissue-specific gene editing in Arabidopsis [18]. Yet the mRNA can be transported long distances to other tissues [19], and constitutive expression of sgRNA, driven by the U6 or U3 snRNA promoters, may bind to the Cas9 protein outside of the specific tissue of interest, resulting in gene editing occurring in unintended locations.

The development of the ribozyme-mediated CRISPR/Cas9 system represents a remarkable improvement, because it can theoretically enhance gene editing’s specificity by using the pol II promoter. The common feature of this system is that ribozyme sequences, such as the hammerhead-type ribozyme (HH) [20] or hepatitis delta virus ribozyme (HDV) [21], are added at the ends of an sgRNA sequence, and when transcribed into RNA, the covalent bond of RNA is broken via the self-cleavage property of such ribozymes, with additional structures at both ends of RNA separated with ribozyme. In the first report [17], an ADH1 promoter (a pol II promoter of yeast) was used to drive the expression of a ribozyme-sgRNA-ribozyme (RGR) artificial gene, and an sgRNA was generated by ribozyme self-cleavage, facilitating site-specific gene editing.

Pyrethrum (Tanacetum cinerariifolium) is a perennial herbaceous plant belonging to the Asteraceae family that has been cultivated for centuries to extract botanical insecticides from its dried flowers [22]. (E)-β-farnesene is a volatile organic compound expressed in different tissues of pyrethrum that is considered to be related to insect resistance [23–25]. Although the (E)-β-farnesene synthase gene (TcEbFS) has been identified, and (E)-β-farnesene synthase expressed in vitro can catalyze (E)-β-farnesene synthesis [24], it has been postulated that TcEbFS has diverse functions in different pyrethrum tissues [23–25]. Therefore, further characterization of TcEbFS function and tissue-specific gene editing in pyrethrum is desirable.

To conduct gene editing research, an efficient transformation system is essential; however, no transformation system suitable for pyrethrum has yet been developed. Agrobacterium rhizogenes-mediated hairy root transformation is considered a rapid, simple, and highly efficient method that enables rapid characterization of improved CRISPR/Cas9 systems in hairy roots [26–29]. This report describes an A. rhizogenes-mediated hairy root transformation system that can quickly verify the effect of gene editing in pyrethrum, and tests the gene editing effect of a ribozyme-mediated CRISPR/Cas9 system using a pol II promoter in this context. Compared with traditional gene editing methods that rely on U6 or U3 snRNA promoters, this versatile method has unique advantages for use in pyrethrum breeding and gene function research that could be applied in future studies.

Results

A strategy for A. rhizogenes-mediated hairy root transformation system in pyrethrum

Leaves from pyrethrum tissue culture plantlets were used as explants, infected with A. rhizogenes, and roots appeared on explants after 20 days (Fig. 1a). Root tips were excised and placed on culture medium with the
root tip upward (Fig. 1b). When the root tips were placed upside down for 7 days, two kinds of roots were identified: one type exhibiting geotropism and another without geotropism (Fig. 1c). Specific primers for the rol B gene on the Ri plasmid were used for PCR amplification of DNA extracted from the two types of root as template, and roots without geotropism were identified as containing the rol B gene (Fig. 1). Therefore, in the present study, geotropism was used to select hairy roots carrying the Ri plasmid.

After screening for roots without geotropism, root tips were cut off and placed in kanamycin screening medium to select hairy roots carrying Ti plasmids (Fig. 2a). The resulting proportion of explants inducing roots was 51% ± 4% (100 explants, four replicate experiments), that of roots without geotropism was approximately 26% (10 roots, five replicate experiments), and the mean proportion of resistant roots was 7% (50 roots, four replicate experiments). Next, resistant roots were cut into 1-cm-long segments and inoculated in the same medium. These resistant roots continued to grow and could grow lateral roots (Fig. 2b). To further determine whether T-DNA was transferred into the hairy roots, resistant roots were stained with X-gluc reagent and Lines 1–5 were dyed blue (Fig. 2c). Next, DNA samples were extracted from these roots for PCR analysis. All roots were found to contain the NPTII gene fragment from the pBI121 vector (Fig. 2d). These results indicated that hairy roots stably transformed with pBI121 could be obtained via geotropism and kanamycin screening, and that the CaMV35S promoter was suitable for high level foreign gene expression in pyrethrum hairy roots.

**Analysis of gene editing in transgenic hairy roots**

The control group (CK) hairy root line (Fig. 3a), in which the CaMV35S promoter drives GUS; and the resistant hairy root lines, line A (Fig. 3b) and line B (Fig. 3c), in which the CaMV35S promoter drives RGR and Cas9, were obtained. DNA was extracted from resistant roots, and PCR analysis showed that an RGR fragment of approximately 229 bp was amplified from the positive control, as well as lines A and B, whereas the amplicon was not detected in CK hairy root samples. These results confirm that lines A and B were transgenic roots carrying the CRISPR/Cas9 vector (Fig. 3d). Furthermore, PCR amplification followed by restriction enzyme digestion was used to detect editing of target sites in lines A and B. In the control group,
two fragments of 162 and 272 bp were produced after the complete digestion by BstbI, while the TcEbFS gene fragments amplified from lines A and B were not completely cleaved by BstbI (Fig. 3e). These findings indicated that no TcEbFS gene fragments in the CK group were edited and contained the BstbI restriction site (TT/CGAA), while the TcEbFS gene fragments from lines A and B contained edited TcEbFS gene fragments, in which the restriction site was destroyed. The TcEbFS gene fragment amplified from the CK group, and the BstbI-resistant TcEbFS gene fragments from lines A and B were then sequenced. The results showed that the TcEbFS sequence of the CK group was identical to the published TcEbFS sequence (MF682058), whereas the BstbI-resistant TcEbFS gene fragments from lines A and B carried mutations, including nucleotide insertions and deletions causing amino acid deletions and frame shifts (Fig. 3f).
The (E)-β-farnesene content of CK, line A, and line B was determined by GC–MS. That of the CK group was 87.19 ± 2.89 µg/g (fresh weight (FW)) (Fig. 4b), that of line A was 49.77 ± 1.05 µg/g (FW) (Fig. 4c), and that of the line B was 57.13 ± 0.52 µg/g (FW) (Fig. 4d). These results showed that pyrethrum hairy roots’ (E)-β-farnesene content was very high, being significantly lower in lines A and B than the CK group (Fig. 4e).

Fig. 3 Analysis of gene editing of transgenic hairy roots 1 month after subculture. a Control group (CK). b Line A. c Line B. d Detection of the RGR sequence by PCR. M, marker; +, ribozyme-based CRISPR/Cas9 vector; −, CK; 1, line A; 2, line B. e PCR amplification followed by restriction enzyme digestion detection of target sites: M, marker; 1−, CK TcEbFS amplicon; 1+, CK TcEbFS fragments following digestion with by BstB1; 2−, Line A TcEbFS amplicon; 2+, Line A TcEbFS fragments following digestion with BstB1; 3−, Line B TcEbFS amplicon; 3+, Line B TcEbFS fragments following digestion with BstB1. f DNA sequencing confirming TcEbFS gene mutations in pyrethrum hairy roots. The net length of insertions and/or deletions are presented in the column to the left. Numbers in parentheses indicate the number of clones. g Diagram showing the TcEbFS genomic locus with the sgRNA targeting site and primer locations (arrows). Scale bars in a–c, 1.0 cm.
Discussion

A rapid and highly efficient A. rhizogenes-mediated hairy root transformation system for pyrethrum

To date, there has been only one report of pyrethrum transformation using an A. tumefaciens-mediated system based on leaf explants; however, such methods are unsuitable for functional verification of novel CRISPR/Cas9 systems, because they are time-consuming and extremely inefficient [30]. Therefore, in the present study a new pyrethrum transformation method for the rapid verification of novel CRISPR/Cas9 systems was established. It achieved a high hairy root transformation frequency (7%) and the process took only 49 days (2 days of co-culture, 20 days of root formation, 7 days of screening for roots without geotropism, and 20 days of screening for kanamycin-resistant roots).

Transgenic hairy roots are transformed by pRi and pTi [31]; therefore, both plasmids’ characteristics were used to screen for transgenic hairy roots. In a study of Brassica napus, pRi-transformed roots were less sensitive to gravity than normal roots [32]. This phenomenon of reduced geotropism was also reported in sweet potato hairy roots [33] and is caused by aux genes carried on pRi that interfere with hairy root gravitropism [34]. Therefore, this characteristic loss of gravitropism was employed here to screen for hairy roots transformed by pRi. Kanamycin
resistance was used to screen for transgenic hairy roots transformed by pTi. Due to the co-transformation of pRi and pTi, transgenic hairy roots are chimeras (Additional file 1: Fig. S1). This phenomenon has also been reported in *Arachis hypogaea*, *Taraxacum hybernum*, and *Althaea officinalis* [35–37]. Nevertheless, this method is suitable only for qualitative, but not for quantitative, research; hence, gene editing efficiency could not be determined in the later experiments.

**Advantages of the ribozyme-mediated CRISPR/Cas9 system for pyrethrum research**

High expression of sgRNA in cells is critical to the success of gene editing [38]. In general, sgRNA is expressed from the *U6* or *U3* promoter in the CRISPR/Cas9 system; however, as in most plants, no *U6* or *U3* promoters have been identified in pyrethrum, making it difficult to select a functional *U6* or *U3* promoter for use in CRISPR/Cas9 systems [17]. Therefore, gene editing methods using a pol II promoter were sought. Post-transcriptional modification of RNA at both ends influences the ability of an sgRNA to guide Cas9 to cut a target site [39, 40]. Hence, separation of the modified structures at both ends of an sgRNA and conversion of the mRNA into sgRNA is desirable. Using the characteristic of ribozyme self-cleavage, an sgRNA with editing ability can be separated from the cap and tail structures of mRNA. This method is referred to as the *RGR* strategy and was first successfully applied to yeast [17], and later to zebrafish (*Danio Rerio*) [41], human cells [42], mice [43], *Plasmidum yoelli* [44], *Arabidopsis thaliana* [45], and *Oryza sativa* [46]. Moreover, some pol II promoters are more efficient than the *U6* or *U3* promoter [47]. The results reported here are similar to those of other studies, suggesting the *RGR* strategy is generally applicable to eukaryotes. Applying the *RGR* strategy reduces the difficulty of promoter selection for use in pyrethrum gene editing.

In addition to the easy availability of the promoter, ribozyme-based CRISPR/Cas9 technology has other advantages. *U6* and *U3* promoters mediate constitutive expression in plants, and the CRISPR/Cas9 technology that relies on *U6* or *U3* to initiate sgRNA transcription cannot be used for specific editing [46]. The *RGR* strategy increases the types of promoters, allowing CRISPR/Cas9 system to use a wide variety of pol II promoters besides *U6* and *U3* promoters [17]. Therefore, this technology could greatly promote pyrethrum’s gene function research. For example, pyrethrum has three types of (E)-β-farnesene with reported differences in release mode, as follows: large release after leaf damage, large release during flower development, and sustained and stable low-level release [23, 24]. The (E)-β-farnesene released in these three modes can interfere with one another, rendering it challenging to study the biological significance of (E)-β-farnesene in each mode. The best way to investigate the particular biological functions of these types of (E)-β-farnesene is to silence each separately, and applying the *RGR* strategy could theoretically realize such tissue-specific gene editing [17].

This technology is also promising for breeding of pyrethrum disease resistance. The most damaging foliar disease of pyrethrum is ray blight caused by *Stagonosporopsis tanaceti* [48]. The emergence of CRISPR/Cas9 technology provides a new approach to address this problem, and this technology has worked to achieve resistance to pathogens in various crops [49–51]; however, to reduce the negative effects of a transgene on the whole plant, the most efficient way to confer resistance is to restrict its expression only to infected cells. Pathogen-induced promoters are unique and valuable tools for engineering resistance to plant disease [52, 53]. Therefore, integration of the CRISPR/Cas9 system and pathogen-induced promoters (pol II promoter) to develop a pathogen-inducible gene editing system is of great significance for devising pyrethrum resistance to *S. tanaceti*, as this method can restrict gene editing only to infected cells and reduce metabolic disruption caused by gene editing, thus minimizing the impact on plant growth and development.

**Conclusions**

This work established a hairy root transformation system mediated by *A. rhizogenes* in pyrethrum. Using this system, a self-cleaving ribozyme-mediated genome editing method using the *CaMV35S* promoter was evaluated. This system increased the types of promoters capable of transcribing sgRNA and can potentially be expanded to other RNA polymerase II-dependent promoters with strong capacity for expression in specific tissues or under certain conditions, to achieve site-specific gene editing in pyrethrum.

**Methods**

**Plant material**

Pyrethrum clone W99 was obtained from the Key Laboratory for Biology of Horticultural Plants, Ministry of Education, College of Horticulture & Forestry Sciences, Huazhong Agricultural University. Clones were inoculated on Murashige and Skoog (MS) solid medium for 30 days after subculture, and their leaves used for transformation. Cultures were maintained at 25 ± 2 °C, with a photoperiod of 16/8 h at light intensity 40 μmol m⁻² s⁻¹.

**Construction of ribozyme-based CRISPR/Cas9 vectors**

According to Liang et al.’s methods [54], a target site with a PAM sequence in exon 2 of the *TcEbFS* gene was
screened (Fig. 3g). The target site contained a BstB1 (TT/CGA) restriction site, which can be used for an editing analysis by the restriction enzyme site-loss method [55]. The plasmids, pRGEB32-GhU6.7-NPT2 and pGTR4, were gifted from the National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University [56]. Ribozyme-based CRISPR/Cas9 vectors were constructed using the TcEbFS gene target sequence, based on pRGEB32-GhU6.7-NPT2. The process of vector construction is illustrated in Fig. 5. The primers used in the experiment are listed in Additional file 2: Table S1. A plasmid with the correct sequence was transferred into A. rhizogenes MSU440.

Establishment of a stable hairy root transformation system

Leaves were excised from pyrethrum clone W99 and infected with A. rhizogenes MSU440 containing the pBIN121 vector. The process of Agrobacterium infection is now well described in the literature [30]. First, hairy roots stably transformed with Ri plasmids were screened. Then, infected leaves were placed on solid 1/2 MS medium containing 400 mg/L cefotaxime. After 20 days of cultivation in the dark, roots appeared in the pyrethrum leaves and root tips were excised, placed horizontally on culture medium, and culture dishes oriented vertically, to ensure that the root tip was pointing upward. After 1 week of cultivation in the dark, two different hairy roots appeared: those exhibiting geotropism and those showing no geotropism. DNA was extracted from these two different types of root, and three agrobacterium-free samples extracted from each type of root. PCR-based amplification of vir genes was used to test for agrobacterium contamination [57, 58]; primers specific for the TcEbFS gene fragment (approximately 456 bp) was expected from positive hairy roots, and positive samples according to the PCR analysis were used for subsequent experiments.

Detection of TcEbFS editing

A TcEbFS gene fragment (approximately 456 bp) was amplified using specific primers (F-up and F-low) across the editing site. As the fragment contained the TT/CGAA restriction site, the TcEbFS gene amplification product from the control group could be recognized and completely digested into 162-bp and 272-bp fragments by BstB1; however, amplified TcEbFS gene products from edited transgenic hairy roots contained fragments could not be recognized and cleaved by BstB1. Undigested bands were ligated into the pCloneEZ vector, and the vector was transferred into Escherichia coli DH5a. Clones were randomly selected and sequenced to detect gene mutations, and eight clones were selected from each line. Hairy roots transformed with the pBIN121 vector alone served as the control.
Detection of (E)-β-farnesene in gene edited hairy roots

Gas chromatography-mass spectrometry (GC–MS) was used to analyze secondary metabolites of hairy root lines containing the ribozyme-based CRISPR/Cas9 vector grown on 1/2 MS medium for 60 days. After freezing in liquid nitrogen and grinding using a mortar and pestle, hairy roots (500 mg) were transferred into a 5-mL tube, and 1 mL of methyl-tert-butyl ether (MTBE) containing...
Fig. 6 Pyrethrum hairy root transformation workflow using leaf explants. At 0 days: Agrobacterium infection. Agrobacterium cell density with an OD$_{600}$ = 0.5. Co-cultivation began. At 2 days: Co-cultivation was over. Roots’ induction began. At 22 days: Roots’ induction was over. Screening of roots transformed with Ri began. At 29 days: Screening of roots transformed with Ri was over. Screening of roots transformed with Ti began. At 49 days: Screening of roots transformed with Ti was over. Sub-cultivation began.
tetradecane (0.01 ng/mL) added as an internal standard. The tube was vortexed for 3 min at maximum speed, incubated at 24 °C with a rotation speed of 50 rpm, then dried using Na₂SO₄. For GC–MS analysis, 1-µL sample aliquots were injected into a GC/MS-QP 2010 Ultra instrument (Shimadzu Corporation, Japan) with an HP-5 MS column. The relative concentration of (E)-β-farnesene was calculated by the area ratio of the (E)-β-farnesene peak to that of the internal standard.

Hairy roots transformed with the pBI121 vector were used as a control. Helium (1.4 mL/min) was used as a carrier gas. The injection temperature was set at 240 °C. The oven temperature program went as follows: initial temperature 50 °C, followed by a ramp from 50 to 150 °C at a rate of 20 °C/min, held for 1 min, and then from 150 to 180 °C at 20 °C/min, held for 1 min, and finally increased to 300 °C at 30 °C/min, held for 5 min. Identification of volatiles was conducted by comparing their retention times and mass fragmentation values with those reported in the literature, and the NIST (2017) and PESTE1_3.lib databases. Spectral data were also compared to (E)-β-farnesene standards (Sigma, Germany), these diluted to 1 ng/µL with MTBE.

Statistical analysis
Data obtained from all experiments are presented as means ± standard error, and separations performed using the least significant difference (LSD) test. Percentage data were subjected to angular transformation and evaluated by ANOVA using SPSS software v17.0. Values with different letters show significant differences at P ≤ 0.05 (LSD).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13007-022-00863-5.

Additional file 1: Fig. S1. Transgenic roots (pBI121) stained with x-gluc reagent showing transgenic hairy root chimeras.

Additional file 2: Table S1. Primers and construction process of ribozyme-based CRISPR/Cas9 vectors used in the experiment.

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Authors’ contributions
JWL, JL, and CYW conceived the research. JWL and TZ wrote the manuscript. QF prepared the materials. JWL, ZZX, and LZ conducted the experiments. TZ analyzed the data. JWL, ZZX, JLL, HH, RRZ, JL, and CYW revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analyzed in 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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