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Research Article

Screening and Functional Verification of Selectable Marker Genes for *Cordyceps militaris*

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The selectable marker genes are necessary resistance genes for gene knockout, gene complementation, and gene overexpression in filamentous fungi. Moreover, the more sensitive the filamentous fungi are to antibiotics, the more helpful it is to screen the target transformants. In order to obtain the antibiotic (or herbicide) which can effectively inhibit the growth of *Cordyceps militaris* and verify the function of the corresponding resistance gene in *C. militaris*, the sensitivity of *C. militaris* to hygromycin and glufosinate ammonium was compared to determine the resistance gene that was more suitable for the screening of *C. militaris* transformants. The binary vector of the selectable marker gene was constructed by combining the double-joint PCR (DJ-PCR) method and the homologous recombination method, and the function of the selectable marker gene in *C. militaris* was verified by the *Agrobacterium tumefaciens*-mediated transformation method. The results showed that *C. militaris* was more sensitive to glufosinate ammonium than hygromycin. The growth of *C. militaris* could be completely inhibited by 250 μg/mL glufosinate ammonium. The expression cassette of the glufosinate ammonium resistance gene (*bar* gene) was successfully constructed by DJ-PCR. The binary vector pCAMBIA0390-Bar was successfully constructed by homologous recombination. The *bar* gene of the vector pCAMBIA0390-Bar was successfully integrated into the *C. militaris* genome and could be highly expressed in the transformants of *C. militaris*. This study will promote the identification of *C. militaris* gene function and reveal the biosynthetic pathways of bioactive components in *C. militaris*.

1. Introduction

Cordyceps militaris is a well-known edible and medicinal fungus that is widely consumed in Southeast Asian countries. As a model species of *Cordyceps*, *C. militaris* contains a variety of high-value bioactive ingredients, such as cordycepin [1], pentostatin [2], novel water-soluble pigments [3], ergosterol [4, 5], immunomodulatory proteins [6], and polysaccharides [7]. These ingredients endow *C. militaris* with anticancer [8], antitumor [9], antioxidation [10], and immunity enhancement [11] properties. Based on the above advantages, the demand for *C. militaris* has increased year by year. However, due to the low content of bioactive ingredients in *C. militaris*, it cannot meet the market demand, which leads to a high price of bioactive ingredients in *C. militaris*. Therefore, scientists have been studying the biosynthetic pathways of *C. militaris* bioactive ingredients [2, 12], identifying the functions of biosynthetic genes of bioactive ingredients [13, 14] and aiming to improve the content of *C. militaris* bioactive ingredients by means of genetic engineering.

Gene knockout [15, 16], gene complementation [17], and gene overexpression [16, 18] are often used to identify the gene function of filamentous fungi, but selectable marker genes are required in the above experiments. If strains are sensitive to antibiotics or herbicides, which is helpful for screening target mutants. The hygromycin resistance gene (*hph* gene) and the glufosinate ammonium resistance gene (*bar* gene) have been used as selectable marker genes for *C. militaris* [19, 20]. At the same time, hygromycin and glufosinate ammonium have also been used to screen the transformants of *C. militaris*, but which antibiotic or

herbicide is *C. militaris* more sensitive to? Up to now, the comparison of hygromycin and glufosinate ammonium on the growth inhibition of *C. militaris* has not been reported. Therefore, the purpose of this study is to determine an antibiotic (or a herbicide) that is more effective in inhibiting the growth of *C. militaris* by comparing the inhibition of hygromycin and glufosinate ammonium on *C. militaris*. Then, the double-joint PCR (DJ-PCR) method was used to construct an expression cassette of the selectable marker gene, and its binary vector was constructed by homologous recombination. Finally, *Agrobacterium tumefaciens*-mediated transformation (ATMT) method was used to verify the effectiveness of the binary vector constructed in this study and the function of the related resistance gene in *C. militaris*. This study will contribute to the identification of *C. militaris* gene function and the elucidation of the biosynthetic pathways of bioactive components in *C. militaris*.

2. Materials and Methods

2.1. Strains and Vectors. *Escherichia coli* DH5*α* was used as a host for the construction of the vector pCAMBIA0390-Bar. The *C. militaris* strain CM10 (GIM5.271) was used to verify the function of the *bar* gene. The vector pCAMBIA3301 contains the *bar* gene. The vector pAg1-H3 contains the *trpC* promoter (P*trpC*) and the *trpC* terminator (T*trpC*) derived from *Aspergillus nidulans*. The binary vector pCAMBIA0390-Bar was reconstructed based on the binary vector pCAMBIA0390 (GenBank: AF234291.1).

2.2. Concentration Gradient Test of Hygromycin and Glufosinate Ammonium. According to previous studies, the concentrations of hygromycin used to screen the transformants of *C. militaris* are 400 μg/mL [21], 500 μg/mL [22, 23], and 800 μg/mL [24]. Therefore, different concentrations of hygromycin (0 μg/mL, 200 μg/mL, 400 μg/mL, 600 μg/mL, 800 μg/mL, 1200 μg/mL, 1600 μg/mL, and 2000 μg/mL) were used to inhibit the growth of *C. militaris* to obtain the minimum concentration of hygromycin that can completely inhibit the growth of *C. militaris*. The concentrations of glufosinate ammonium used to screen the transformants of *C. militaris* were 150 μg/mL [25] and 400 μg/mL [23, 26]. Hence, different concentrations of glufosinate ammonium (0 μg/mL, 100 μg/mL, 150 μg/mL, 200 μg/mL, 225 μg/mL, 250 μg/mL, 300 μg/mL, 350 μg/mL, 400 μg/mL, and 500 μg/mL) were used to inhibit the growth of *C. militaris* to obtain the minimum concentration of glufosinate ammonium that can completely inhibit the growth of *C. militaris*.

The *C. militaris* strain CM10 was inoculated into the PDA medium (200.0 g potato, 20.0 g agar, 10.0 g dextrose, 3.0 g KH_2PO_4, 1.0 g $MgSO_4$, and constant volume to 1000 mL with distilled water) and cultured at 25°C for 20 days under dark conditions. After activation, the activated *C. militaris* was cut into pieces of 0.5 cm × 0.5 cm size. Then, the activated pieces of *C. militaris* were inoculated on the PDA medium containing different concentrations of hygromycin (or glufosinate ammonium) and incubated at 25°C for 20 days under dark conditions. Finally, the inhibitory effect of hygromycin (or glufosinate ammonium) on the growth of *C. militaris* was observed.

2.3. Construction of the Bar Gene Expression Cassette. According to the results of the concentration gradient test, glufosinate ammonium could completely inhibit the growth of *C. militaris*. Because the *bar* gene is the glufosinate ammonium resistance gene, the *bar* gene expression cassette needs to be constructed in this study. The primer pairs F1/R1 and F3/R3 were used to amplify the promoter P*trpC* and the terminator T*trpC* from the vector pAg1-H3, respectively. The primer pair F2/R2 was used to amplify the *bar* gene from the vector pCAMBIA3301. Primers F1 and R3 were used to fuse the promoter P*trpC*, the *bar* gene, and the terminator T*trpC* by the DJ-PCR method to obtain the fusion fragment PtrpC-bar-TtrpC (Supplementary Figure S1 and Supplementary Table S1).

2.4. Construction of the Binary Vector pCAMBIA0390-Bar. In order to construct the binary vector pCAMBIA0390-Bar, the restriction endonuclease *Hin*d III and *Eco*R I was used to digest the vector pCAMBIA0390, and the linear vector pCAMBIA0390 was obtained. The 5′ and 3′ ends of fusion fragment (PtrpC-bar-TtrpC) are homologous to the two ends of the linear vector pCAMBIA0390, respectively. The One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China) containing homologous recombinase was used to homologous recombine the fusion fragment (PtrpC-bar-TtrpC) and the linear vector pCAMBIA0390 to obtain the recombinant vector pCAMBIA0390-Bar (Supplementary Figure S2). The reaction system and reaction conditions of homologous recombination were carried out according to the manufacturer's protocol. The fusion fragment (PtrpC-bar-TtrpC) was inserted into the transfer DNA (T-DNA) region of the vector pCAMBIA0390.

2.5. Functional Verification of the Bar Gene in C. militaris. To verify the effectiveness of the vector (pCAMBIA0390-Bar) constructed in this study and the function of the *bar* gene in *C. militaris*, the vector pCAMBIA0390-Bar was firstly transformed into the competent cells of the *Agrobacterium tumefaciens* AGL1, and the *Agrobacterium tumefaciens* strain AGL1-pCAMBIA0390-Bar was obtained. Then, the conidia of the wild-type *C. militaris* strain CM10 were collected according to the method described by Lou et al. [27]. According to the ATMT method reported by Zheng et al. [21], the conidia of the wild-type *C. militaris* strain CM10 and the *Agrobacterium tumefaciens* strain AGL1-pCAMBIA0390-Bar were cocultured in order to integrate the *bar* gene expression cassette into the genome of the wild-type *C. militaris* strain CM10. The selective medium contains 300 μg/mL glufosinate ammonium and 300 μg/mL cefotaxime. The transformants of *C. militaris* on the selective medium were selected and inoculated to a new PDA medium containing 300 μg/mL of glufosinate ammonium for resistance screening. If transformants of *C. militaris* can

grow on the PDA medium containing 300 μg/mL of glufosinate ammonium, they are considered to be resistant to glufosinate ammonium. The genome of *C. militaris* transformants with resistance to glufosinate ammonium was extracted. Primers F4 and R4 were used to amplify an 836 bp fragment of the *bar* gene expression cassette from the genome of *C. militaris* transformants. The PCR products were sequenced to determine whether the *bar* gene expression cassette was integrated into the genome of the wild-type *C. militaris*.

The function of the *bar* gene in *C. militaris* was further verified by quantitative real-time PCR (qRT-PCR) using the *tef1* gene (GenBank: DQ070019) as the internal control gene [28]. The PCR-positive mutant of *C. militaris* and the wild-type *C. militaris* were inoculated on the PDA medium and cultured at 25°C. The mycelia of the PCR-positive mutant and the wild-type *C. militaris* were obtained by culturing for 20 days under dark conditions and then culturing for 7 days under light conditions. The Fungal RNA Kit (Omega, Stamford, CT, USA) was used to extract the total RNA of *C. militaris* mycelia, and the TransScript All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) was used for reverse transcription. The primers *tef1*-F/*tef1*-R and JCBar-F/JCBar-R were used to detect the expression levels of the *tef1* gene and the *bar* gene, respectively (Supplementary Table S1). The qRT-PCR experiment was performed using the TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) with a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, MA, USA) according to the method described by Lou et al. [13]. The relative expression level of the *bar* gene relative to the *tef1* gene was calculated using the $2^{-\Delta\Delta CT}$ method [29]. The qRT-PCR experiment was repeated three times.

3. Results

3.1. Inhibition of Hygromycin and Glufosinate Ammonium on the Growth of C. militaris. *C. militaris* cultured on the PDA medium without hygromycin and glufosinate ammonium grew well, and the colony diameter of *C. militaris* was larger (Figures 1 and 2). The colony diameter of *C. militaris* cultured on the PDA medium containing hygromycin was smaller than that of *C. militaris* cultured on the PDA medium without hygromycin. With the increase of hygromycin concentration, the colony diameter of *C. militaris* decreased gradually (Figure 1), which indicated that hygromycin could inhibit the growth of *C. militaris*. Previous studies showed that 400–800 μg/mL hygromycin could completely inhibit the growth of *C. militaris* [21–23], but 2000 μg/mL hygromycin still could not completely inhibit the growth of the *C. militaris* strain CM10 in this study, which might be caused by the difference of *C. militaris* strains. The *C. militaris* strain CM10 used in this study was less sensitive to hygromycin. Therefore, hygromycin was not suitable as an antibiotic to screen the transformants of the *C. militaris* CM10.

With the increase of glufosinate ammonium concentration in the PDA medium, the colony diameter of *C. militaris* decreased significantly (Figure 2). When the concentration of glufosinate ammonium in the PDA medium reached 250 μg/mL, the growth of *C. militaris* was completely inhibited, which indicated that *C. militaris* was sensitive to glufosinate ammonium. Therefore, glufosinate ammonium was suitable as a herbicide for screening *C. militaris* transformants.

3.2. Construction of the Bar Gene Expression Cassette. The extracted vectors pAg1-H3, pCAMBIA3301, and pCAMBIA0390 are shown in Figure 3. The promoter P*trpC* (399 bp), the *bar* gene (582 bp), and the terminator T*trpC* (773 bp) were obtained by PCR amplification. The promoter P*trpC*, the *bar* gene, and the terminator T*trpC* were successfully fused by the DJ-PCR method, and the fused *bar* gene expression cassette (1694 bp) was obtained (Figure 3).

3.3. Construction of the Binary Vector pCAMBIA0390-Bar. The restriction endonuclease *Hin*d III and *Eco*R I were used to digest the vector pCAMBIA0390, and the linear vector pCAMBIA0390 was obtained (Figure 3). The *bar* gene expression cassette and the linear vector pCAMBIA0390 were successfully homologous recombined by the homologous recombinase, and the binary vector pCAMBIA0390-Bar was obtained (Figure 3).

3.4. Functional Verification of the Bar Gene in C. militaris. The conidia of *C. militaris* were round or oval (Figure 4(a)), which was consistent with previous studies [27, 30]. Many white colonies of *C. militaris* appeared on the selective medium containing 300 μg/mL glufosinate ammonium and 300 μg/mL cefotaxime (Figure 4(b)). The colony of the *C. militaris* transformant picked from the selective medium grew well on the PDA medium containing 300 μg/mL of glufosinate ammonium, while the growth of the wild-type *C. militaris* cultured on the PDA medium containing 300 μg/mL of glufosinate ammonium was completely inhibited, which indicated that the transformant of *C. militaris* picked from the selective medium had glufosinate ammonium resistance (Figure 4(c)). The results of amplification of the *bar* gene from *C. militaris* transformants with resistance to glufosinate ammonium are shown in Figure 4(d). The sequencing results of PCR products confirmed that the *bar* gene was successfully integrated into the genome of *C. militaris*.

The wild-type *C. militaris* and its transformants grew well on the PDA medium without glufosinate ammonium (Figures 5(a) and 5(b)). However, the colony diameter of the *C. militaris* transformant was smaller than that of the wild-type *C. militaris*, which indicated that the insertion of the *bar* gene reduced the growth rate of the *C. militaris* transformant. In addition, the color difference between the wild-type *C. militaris* and its transformant might be due to the mutation of genes related to pigment synthesis caused by the insertion of the *bar* gene. The results of qRT-PCR suggested that the expression of the *bar* gene were not detected in wild-type *C. militaris*, which indicated that there was no *bar* gene in the wild-type *C. militaris*. However, the relative

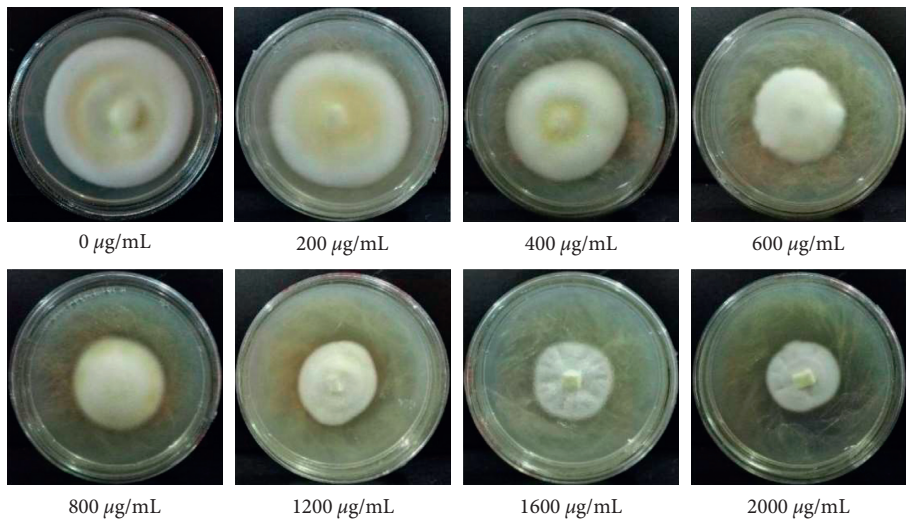

Figure 1: Inhibition of different concentrations of hygromycin on the growth of *C. militaris*. (a) 0 μg/mL. (b) 200 μg/mL. (c) 400 μg/mL. (d) 600 μg/mL. (e) 800 μg/mL. (f) 1200 μg/mL. (g) 1600 μg/mL. (h) 2000 μg/mL.

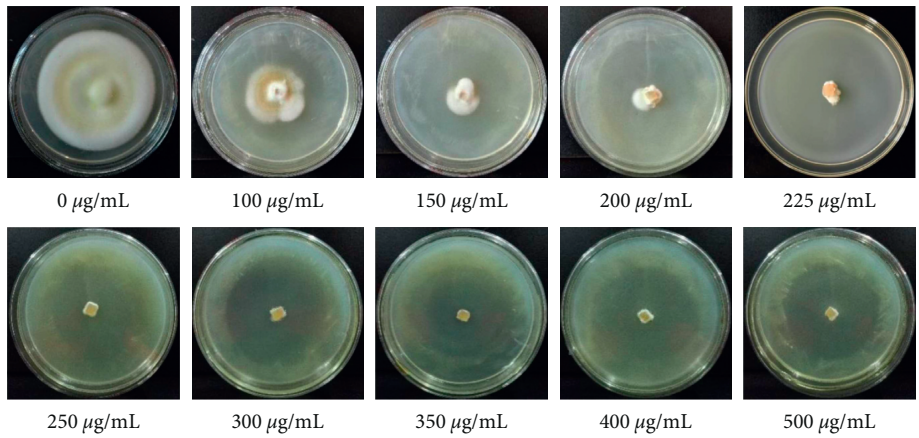

Figure 2: Inhibition of different concentrations of glufosinate ammonium on the growth of *C. militaris*. (a) 0 μg/mL. (b) 100 μg/mL. (c) 150 μg/mL. (d) 200 μg/mL. (e) 225 μg/mL. (f) 250 μg/mL. (g) 300 μg/mL. (h) 350 μg/mL. (i) 400 μg/mL. (j) 500 μg/mL.

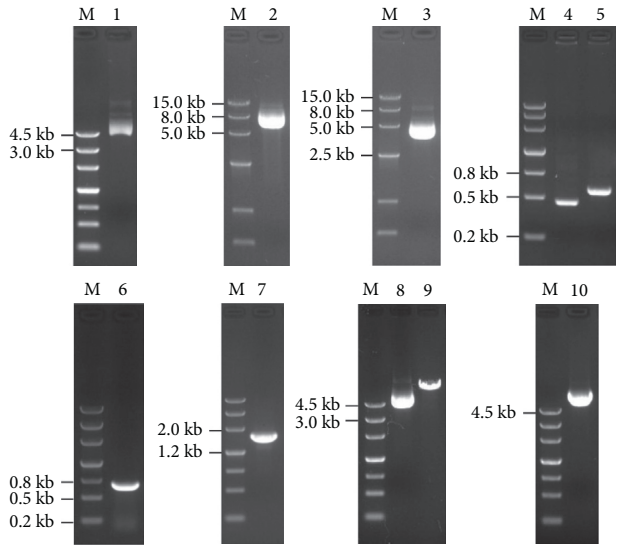

Figure 3: Construction of the binary vector pCAMBIA0390-Bar. M:DNA marker; lane 1: vector pAg1-H3; lane 2: vector pCAMBIA3301; lane 3: vector pCAMBIA0390; lane 4: promoter P*trpC*; lane 5: *bar* gene; lane 6: terminator T*trpC*; lane 7: *bar* gene expression cassette; lane 8: vector pCAMBIA0390; lane 9: linear vector pCAMBIA0390; lane 10: binary vector pCAMBIA0390-Bar.

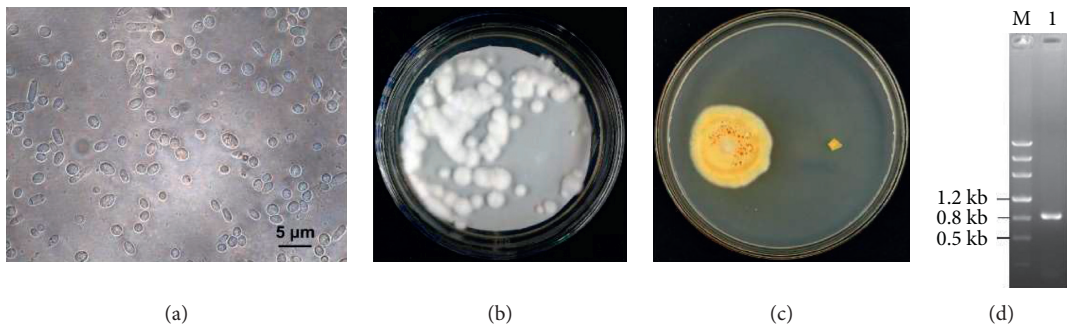

Figure 4: Functional verification of the *bar* gene in *C. militaris*. (a) Conidia of *C. militaris*. (b) Colonies of *C. militaris* on the selective medium containing 300 μg/mL glufosinate ammonium and 300 μg/mL cefotaxime. (c) A *C. militaris* transformant (left) and a wild-type *C. militaris* (right) were inoculated on the PDA medium containing glufosinate ammonium (300 μg/mL) and cultured at 25°C for 18 days (including 12 days under dark conditions and 6 days under light conditions). (d) PCR amplification of the *bar* gene from a randomly selected *C. militaris* transformant. M:DNA marker; lane 1: 836 bp fragment of the *bar* gene expression cassette.

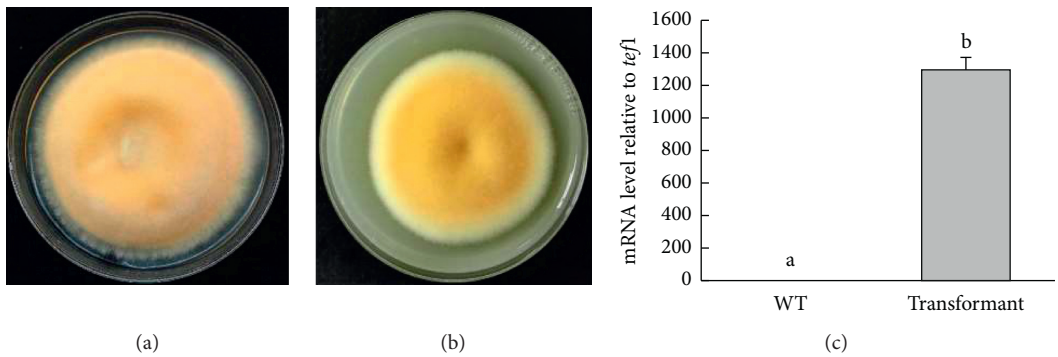

Figure 5: qRT-PCR analysis of the relative expression level of the *bar* gene in *C. militaris* transformants using the wild-type *C. militaris* as a control. (a) Wild-type *C. militaris* cultured on the PDA medium. (b) A *C. militaris* transformant cultured on the PDA medium. (c) Relative expression levels of the *bar* gene in *C. militaris*. Different letters (a, b) indicated that the relative expression levels of the *bar* gene were significantly different.

expression level of the *bar* gene in the *C. militaris* transformant was relatively high, which demonstrated that the *bar* gene expression cassette and the binary vector pCAMBIA0390-Bar constructed in this study could be effectively applied to *C. militaris* (Figure 5(c)). Furthermore, the *bar* gene can be used as a selectable marker gene for *C. militaris*.

4. Discussion

Selectable marker genes are indispensable for gene knockout, gene complementation, and gene overexpression. It is well known that the workload of screening target mutants is very large. If the strain is sensitive to antibiotics, the corresponding resistance gene can effectively reduce the workload of screening target mutants. If the strain is less sensitive to antibiotics, a lot of work is needed to screen target mutants. The *hph* gene and the *bar* gene have been used as selectable marker genes for *C. militaris*, but the comparison of the sensitivity of *C. militaris* to hygromycin and glufosinate ammonium has not been reported. In this study, the inhibitory effects of hygromycin and glufosinate ammonium on the growth of *C. militaris* were compared for the first time. The *bar* gene expression cassette was constructed by the DJ-PCR method, and then the binary vector pCAMBIA0390-Bar was constructed by homologous recombination. Furthermore, the *bar* gene was successfully integrated into *C. militaris* genome by the ATMT method and the transformant of *C. militaris* with resistance to glufosinate ammonium was obtained. The *bar* gene expression cassette constructed in this study could be efficiently expressed in *C. militaris* transformants.

Hygromycin is an aminoglycosidic antibiotic that inhibits protein synthesis by disrupting translocation and promoting mistranslation at the 80S ribosome [31]. The hygromycin phosphotransferase encoded by the *hph* gene can convert hygromycin into phosphorylated products that are not biologically toxic [31]. Based on this, hygromycin has been used as an antibiotic to screen the transformants of *C. militaris*, and the *hph* gene has also been used as a selectable marker gene for *C. militaris* [21, 23, 24]. However, in this study, 2000 μg/mL hygromycin still failed to completely inhibit the growth of the *C. militaris* strain CM10, which indicated that the *C. militaris* strain CM10 was less sensitive to hygromycin. Glufosinate ammonium strongly inhibits the activity of glutamine synthetase (GS) that can convert NH_4^+ to glutamine. The presence of glufosinate ammonium causes cells accumulate NH_4^+, which leads to cell death or growth

inhibition. Phosphinothricin acetyltransferase encoded by the *bar* gene can acetylate the free amino groups of glufosinate ammonium, which makes glufosinate ammonium unable to inhibit the activity of GS [32]. Therefore, glufosinate ammonium has been used as herbicide for screening the transformants of *C. militaris*, and the *bar* gene has also been used as a selectable marker gene for *C. militaris* [26]. Furthermore, 250 μg/mL glufosinate ammonium could completely inhibit the growth of the *C. militaris* strain CM10 in this study. In addition, we also conducted a concentration gradient test on *C. militaris* strains CM11 and CM12 and found that 2000 μg/mL hygromycin could not completely inhibit the growth of *C. militaris* strains CM11 and CM12, while 250 μg/mL glufosinate ammonium could completely inhibit the growth of *C. militaris* strains CM11 and CM12. Therefore, we believe that *C. militaris* is more sensitive to glufosinate ammonium than hygromycin. Xiong et al. believed that the concentration of glufosinate ammonium to completely inhibit the growth of *C. militaris* was 150 μg/mL [25], while Wang et al. suggested that the concentration of glufosinate ammonium to completely inhibit the growth of *C. militaris* was 400 μg/mL [23], but they were inconsistent with the results of this study, which might be due to the different sensitivity of different *C. militaris* strains to glufosinate ammonium. Based on this, we believe that it is necessary to conduct a concentration gradient test of glufosinate ammonium to inhibit the growth of *C. militaris* before the transformation experiment, so as to determine the minimum concentration of glufosinate ammonium that completely inhibits the growth of *C. militaris*. Furthermore, we believe that an appropriate increase in the concentration of glufosinate ammonium can reduce the appearance of false-positive transformants and the workload of screening target transformants. Therefore, 300 μg/mL of glufosinate ammonium was used to screen the transformants of *C. militaris* in this study.

Three DNA fragments (such as the promoter P*trpC*, the *bar* gene, and the terminator T*trpC*) could be fused in two steps by DJ-PCR [33]. However, it was required that the 5′ end sequences of primers R1 and R2 were homologous with the 5′ end sequences of the *bar* gene and the terminator T*trpC*, respectively, and the 5′ end sequences of primers F2 and F3 were homologous with the 3′ end sequences of the promoter P*trpC* and the *bar* gene, respectively. The length of homologous sequence was about 15 bp. It was worth noting that PCR products needed to be purified after amplifying the three elements (the promoter P*trpC*, the *bar* gene, and the terminator T*trpC*). In this way, the influence of primers and DNA templates of the first round of PCR on DJ-PCR could be avoided. In order to avoid base mutation, it was necessary to use high fidelity DNA polymerase and reduce the cycle times of PCR amplification [34].

When restriction endonucleases were used to prepare linear vectors, it was necessary to purify the digestion products to reduce the occurrence of false positive vectors. In the construction of knockout vectors, two homologous sequences are often inserted on both sides of the resistance gene expression cassette. In the construction of a complementary vector or an overexpression vector, one side of the resistance gene expression cassette needs to be inserted with a complementary gene or an overexpression gene. We believed that it was necessary to retain the restriction sites on both sides of the resistance gene expression cassette during the construction of a vector. Therefore, the *Hin*d III and *Eco*R I sites were retained on both sides of the *bar* gene expression cassette when the vector pCAMBIA0390-Bar was constructed. Because the constructed vector pCAMBIA0390-Bar could be effectively applied to *C. militaris*, so it can be used to construct target gene knockout vectors, complementary vectors, and overexpression vectors.

5. Conclusions

The sensitivity of *C. militaris* to hygromycin and glufosinate ammonium was compared for the first time. It was found that *C. militaris* was more sensitive to glufosinate ammonium, and glufosinate ammonium was more suitable as herbicide for screening the transformants of *C. militaris*. 250 μg/mL glufosinate ammonium could completely inhibit the growth of *C. militaris*. The *bar* gene expression cassette and the binary vector pCAMBIA0390-Bar were efficiently constructed by the DJ-PCR method and the homologous recombination method, respectively. The *bar* gene of the vector pCAMBIA0390-Bar was successfully integrated into the genome of *C. militaris* by the ATMT method, and *C. militaris* transformants with resistance to glufosinate ammonium were obtained. It was confirmed that the *bar* gene was more suitable as a selectable marker gene for *C. militaris*. This study will promote the identification of the functions of *C. militaris* genes and reveal the biosynthetic pathways of bioactive components in *C. militaris*.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table S1. Primers used to construct the *bar* gene expression cassette and analysis of mutants. *Supplementary Figure S1.* Construction of the *bar* gene expression cassette by the DJ-PCR method. *Supplementary Figure S2.* Construction of the binary vector pCAMBIA0390-Bar. (*Supplementary Materials*)

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