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Note

Development of a dual promoter-mediated gene silencing system in *Flammulina velutipes*

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ABSTRACT

This study aimed to establish an RNAi system based on a dual promoter construct to interfere with the expression of target genes in *Flammulina velutipes*. In this study, the endogenous laccase gene, which was cloned as a silencing reporter, was introduced into *F. velutipes* through electroporation-mediated transformation. Our data unequivocally indicate that the dual promoter silencing system significantly reduced the expression and activity of *lac6*. Additionally, reductions in *lac6* mRNA levels and enzyme activity were correlated (minimum of a 50% reduction). The molecular tools developed in this study should facilitate the functional characterization of genes in this important species.

Flammulina velutipes (Curt. ex Fr.) Sing. is a well-known edible mushroom cultivated all over the world. It is one of the six most actively cultivated mushrooms in the world; over 300,000 tons of this mushroom are produced per year (Kim et al. 2014). For the past several years, many studies have described the important biological activity of *F. velutipes*, such as its antioxidant and immunomodulatory activities (Zhang et al. 2013; Wu et al. 2014; Ma et al. 2015). The number of studies investigating the molecular genetics of *F. velutipes* has increased, with some studies including genome sequence analysis (Kim et al. 2014; Park et al. 2014; Kurata et al. 2016) and gene function analysis (Nakai et al. 2000; Kuo et al. 2004; Yamada et al. 2005, 2008). The research on the development and metabolism of *F. velutipes* is still at an early stage, although some gene transfer procedures have been established (Cho et al. 2006; Kim et al. 2010; Sekiya et al. 2013). The roles of the genes involved in the developmental and metabolic process of *F. velutipes* are still unclear, and investigations have been limited by a lack of effective reverse genetic procedures. Improving the genetic tools for this organism will provide significant insight into the mechanisms regulating the development of fruiting bodies and the production of secondary metabolites and open new possibilities for enhancing the production of biologically active compounds.

Because of its simplicity and effectiveness in down-regulating target genes, RNA interference (RNAi) has been widely used in several types of organisms (Bi et al. 2015; Pfender et al. 2015; Weinheimer et al. 2015) RNAi was first reported in *Neurospora crassa* among filamentous fungi

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(Cogoni and Macino 1997) and has been used for gene function analysis in many ascomycetes and several basidiomycetes (de Jong et al. 2006; Costa et al. 2009; Mu et al. 2012). Several gene-silencing systems have been reported in filamentous fungi; of these systems, the dual promoter silencing system was reported to have high silencing efficiency and simplicity in execution. However, there have been no successful reports on the use of the dual promoter silencing system in *F. velutipes*.

In this study, the laccase gene (*lac*) was cloned as a reporter gene for monitoring RNAi in *F. velutipes*, and the simple and effective dual promoter silencing system was established. This method will facilitate future molecular genetic studies of this fungus.

Construction of RNAi vectors for lac6

The *F. velutipes* strain used in this study, SH, was obtained from the Shanghai Academy of Agricultural Sciences, Shanghai, China. The strain was bred by the Institute of Edible Fungi, and as a heterokaryon, its mycelia grow rapidly and exhibit vigorous growth. In addition, the fruit body of SH appears white in color and measures 12–14 cm in length, including the cap and stipe. The strain was grown at 25 °C on CYM (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 2 mM $MgSO_4 \cdot 7H_2O$, and 33 mM KH_2PO_4) and used as the recipient host strain for transformation. The *Escherichia coli* DH5α strain was used for plasmid amplification and grown on Luria–Bertani (LB) medium (Sambrook and Russell, 2001) containing 100 μg/mL ampicillin or 50 μg/mL kanamycin as required.

Previous studies have reported 11 *lac* genes in *F. velutipes* by genome analysis (Wang et al. 2015). The GenBank accession numbers of eight genes with high homology in *F. velutipes* are as follows: *lac3*: KF557726, *lac5*: KF557728, *lac6*: KF557729, *lac7*: KF557730, *lac8*: KF557731, *lac9*: KF557732, *lac10*, KF557733; and *lac11*: KF557734. Among these laccase genes, *lac6* was selected as the target for gene silencing due to its high expression in mycelia and its significant differences in expression between mycelia and primordium as well as its extracellular secretion properties (Wang et al. 2015).

A RNA interference procedure with hairpin structures and *Agrobacterium tumefaciens*-mediated transformation (AMT) was previously developed for *F. velutipes* (Sekiya et al. 2013). However, this method can result in a high number of false-positive transformants. To the best of our knowledge, four different RNAi methods have been developed, utilizing sense, antisense, dual promoter, and hairpin constructs in filamentous fungi. Mu et al. (2012) systematically examined the

Table 1 – Oligonucleotide primers used in this study.

Name	Sequence
Fv-GPD-F1	5′-GATCccaacatggtggCGCATTACTTGAATACTTCG-3′
Fv-GPD-R1	5′-GATCccaacatggtggGATTGTAGATGAGGAGATGG-3′
Fv-35S-F	5′-TCATCTAGAAGAGATAGATTTGTAGAGAG-3′
Fv-35S-R	5′-GATAAGCTTCGTAATCATGGTCATAGCTG-3′
HPH-DET-F	5′-GTCGTGGCGATCCTGCAAGC-3′
HPH-DET-R	5′-CCTGCGGGTAAATAGCTGCGC-3′
Fv-LAC6sf-F	5′-GTACggatccATGTCGCGGTCTCTTACCGC-3′
Fv-LAC6sf-R	5′-GTACtctagaTCAAAGGTCATCTTCAGAC-3′
Fv-LAC6rt-F	5′-ACTCTGCCATTCTTCGTTATT-3′
Fv-LAC6rt-R	5′-TCGTCTGCTCCGACTTCC-3′
Fv-LAC3rt-F	5′-ATAGAGGTGCAAAGTCGC-3′
Fv-LAC3rt-R	5′-CTTGTGCATACGATACGG-3′
Fv-LAC5rt-F	5′-TGGACTGTCAACGGACTC-3′
Fv-LAC5rt-R	5′-CGTGAAGATGGAATGGGT-3′
Fv-LAC7rt-F	5′-CTGGCGTTCCCGTGAGTA-3′
Fv-LAC7rt-R	5′-CCCACCTAATGCCCGAAT-3′
Fv-LAC8rt-F	5′-AATTCCCGATCTCAAAGC-3′
Fv-LAC8rt-R	5′-TTCGAGCAAACTTGTAGG-3′
Fv-LAC9rt-F	5′-GGCGAGAATGGGAAGAAT-3′
Fv-LAC9rt-R	5′-AGCGTGGAGTTGGCGATG-3′
Fv-LAC10rt-F	5′-TATCGAGGTTGATGGTGTT-3′
Fv-LAC10rt-R	5′-GTTCATAATGGAGGACAGC-3′
Fv-LAC11rt-F	5′-GTCCACGTCTCCAATTCTC-3′
Fv-LAC11rt-R	5′-TGGTATGGCATAGTCAAGC-3′
Fv-ACTrt-F	5′-CCATAGGTTTCTCTCTTCCTCAC-3′
Fv-ACTrt-R	5′-CCACGTTCCATCAGGTTCTT-3′
Oligo(dT) 17	5′-TTTTTTTTTTTTTTTT-3′

Fv-GPD-F1 and R1 were used for amplification of the promoter region of *gpd* gene. Fv-35S-F and R were used for amplification of the promoter region of 35S gene. HPH-DET-F and R were used for detection the transformants' selectable marker *hph* (hygromycin phosphotransferase) gene. Fv-LAC6sf-F and R were used for amplification of the silenced region of *lac6* gene. Fv-LAC6rt-F and R, Fv-LAC3rt-F and R, Fv-LAC5rt-F and R, Fv-LAC7rt-F and R, Fv-LAC8rt-F and R, Fv-LAC9rt-F and R, Fv-LAC10rt-F and R, Fv-LAC11rt-F and R were used for transcriptional detection of *lac6*, *lac3*, *lac5*, *lac7*, *lac8*, *lac9*, *lac10*, and *lac11* respectively. Fv-ACTrt-F and R were used for transcriptional detection of *actin* gene. oligo(dT) 17 were used to reverse-transcribed to cDNA.

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