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Note

Construction of an efficient RNAi system in the cellulolytic fungus *Trichoderma reesei*

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ABSTRACT

An improved RNA interference method was developed in *Trichoderma reesei*, using convergent dual promoters for efficient and high-throughput RNA silencing. This new vector allowed for the silencing of the eGFP gene and target genes to occur simultaneously, significantly facilitating the rapid screening of the transformants using eGFP as a reporter.

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Trichoderma reesei (teleomorph *Hypocrea jecorina*) is a famous filamentous fungus that is widely used for the production of hydrolytic enzymes, and is recognized as a potential candidate for the conversion of renewable lignocellulosic biomass to biofuels (Wilson, 2009). As one of the most important cellulase producing strains, the yield and the performance of *T. reesei* have been remarkably improved by random mutagenesis which has contributed a lot to the modification of industrial strains. However, the classical strain improvement method has many limitations, such as labor- and time-consuming, lack of inherent stability and so on. The publication of the genome sequence of *T. reesei* has made it possible to introduce state-of-the-art gene manipulation methods into the strain improvement programs (Martinez et al., 2008). Therefore, developing efficient molecular tools is a feasible way to further study the mechanisms of cellulase or hemicellulase gene regulation and improve protein secretion in *T. reesei*.

RNA silencing is an efficient tool for knocking down the expression of a target gene in cells. In *T. reesei*, the highly expressed genes *cel6a* and *cbh1* were reported to be suppressed by means of RNAi based on a hairpin design (Brody and Maiyuran, 2009; Qin et al., 2012). Generally, RNA silencing is performed using silencing vectors which produce hairpin RNA in filamentous fungi (Nakayashiki et al., 2005). When constructing an hpRNA silencing vector, the target sequence needs to be cloned twice in opposite directions to form the hpRNA needed to produce double-stranded RNA and trigger the RNA silencing machinery (Nakayashiki, 2005). Therefore, application of the hpRNA expression vector is relatively limited.

In this study, an improved RNA silencing system with dual promoters was constructed in *T. reesei*. A *trpC* promoter from *Aspergillus nidulans* (Sakai et al., 2008), and an *rp2* promoter from *T. reesei* (He et al., 2013) were subsequently cloned into the SacI/KpnI and SmaI/BamHI sites of pCAMBIA1300-1 to generate pCAMBIA1300-1-dual (Fig. 1a). For eGFP gene silencing, a 500 bp eGFP fragment was inserted into the SmaI site in pCAMBIA1300-1-dual to generate pCAMBIA1300-1Sgfp (Fig. 1b). For co-silencing of the target genes and the eGFP gene, pCAMBIA1300-1-dual-based vectors were constructed by inserting of a coding fragment of the target gene (Fig. 1c).

A transformant named GFP-11 with strong green fluorescence was acquired in a previous study (He et al., 2013). To evaluate the applicability of pCAMBIA1300-1dual vector for inducing RNA silencing, the enhanced green fluorescence protein gene (eGFP) was used as the reporter. The obtained transformants were screened under fluorescence microscopy. Green fluorescence appeared to be reduced in 11 of the total 20 transformants, compared with GFP-11 (data not shown). Transformants T13, T14, and T2, which appeared to have different intensities of green fluorescence, were selected and cultivated on PDA for 3 days. In comparison with GFP-11, green fluorescence was scarcely observed in T13 and T14, whereas T2 showed strong green fluorescence in both the hyphae and conidia (Fig. 2a). The expression level of *gfp* mRNA was examined by quantitative RT-PCR of T13, T14, T2, and GFP-11. As expected, the expression level of *gfp* was significantly reduced in T13 and T14 while no significant reduction was detected in the non-silenced transformant T2 (Fig. 2b). The results demonstrated

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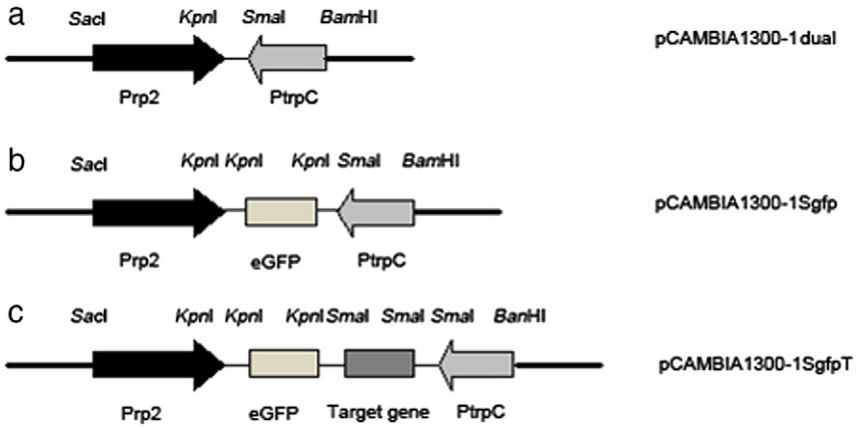

Fig. 1. Schematic of the RNA silencing vectors pCAMBIA1300-1dual (a), pCAMBIA1300-1Sgfp (b), and pCAMBIA1300-1SgfpT (c) with two dual promoters; PtrpC, *trpC* promoter from *Aspergillus nidulans* and Prp2, *rp2* promoter from *Trichoderma reesei*.

that the eGFP gene was successfully silenced using the pCAMBIA1300-1-dual vector.

To investigate the effect of co-silencing using the pCAMBIA1300-1-dual vector, three genes (*RhoA*, *Cla4*, and *Ras2*) were targeted for co-silencing. To facilitate the screening of transformants, the eGFP gene was used as a reporter for co-silencing in the pCAMBIA1300-1-dual vector. The target genes were fused with the eGFP gene in pCAMBIA1300-1SgfpT and then transformed into *T. reesei* GFP-11 through AMT method. The green fluorescence intensity of the obtained transformants decreased by varying levels compared with the parent strain GFP-11 (data not shown). For each gene, three transformants with different levels of reduction in their green fluorescence intensity were selected for further quantitative PCR analysis. As shown in Fig. 3a–c, the relative expression levels of *RhoA* in RhoT11/17/19, *Ras2* in RasT6/9/13 and *Cla4* in ClaT3/8/21 were significantly decreased, suggesting that the pCAMBIA1300-1-dual based vector can induce gene silencing of *RhoA*, *Ras2* and *Cla4* to different degrees. In a previously study, these three genes were reported to be involved in fungal morphology, especially in hyphae branching. The gene

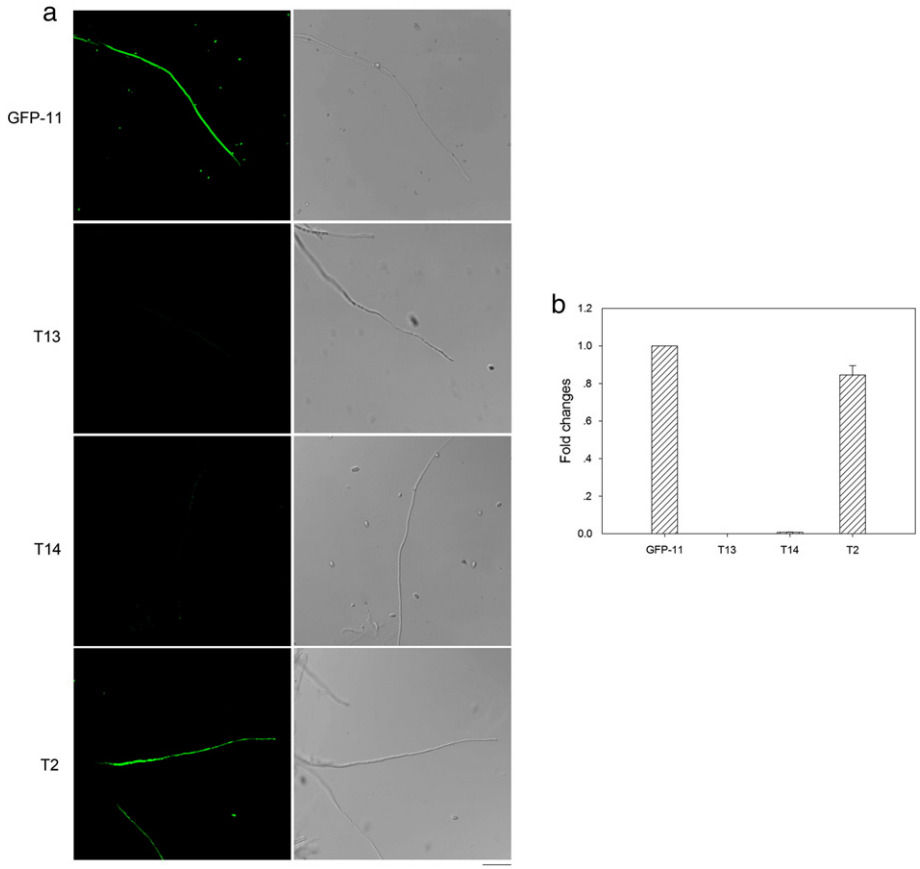

Fig. 2. Green florescence and mRNA expression level of the eGFP gene in the silenced strains and GFP-11. (a). GFP-11, the parent strain; T13 and T14, strongly silenced transformants; T2, non-silenced transformants. Left column, images of green fluorescence; right column, images of bright fields. Bars, 10 μm. (b). Expression levels of eGFP gene in the silenced strains and GFP-11. The expression level of the eGFP gene was analyzed by qRT-PCR and normalized with an endogenous control *gpd1*. The respective ratio in GFP-11 was set to 1. The experiment was repeated three times. The error bars represent the deviation from three independent experiments.

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