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ORIGINAL ARTICLE

pFungiway: a series of plasmid vectors used for gene manipulation in fungi

Ryohei Nishikawa[1] · Masumi Yoshida[1] · Tomotsugu Noda[1] · Toru Okuhara[1] · Goro Taguchi[1] · Satoshi Inatomi[2] · Makoto Shimosaka[1]

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Abstract A series of plasmid vectors, named pFungiway, was constructed for the purpose of simple and versatile gene manipulation in a wide range of fungi. The backbone of these vectors consists of the binary plasmid pCAMBIA2200, which can be introduced into fungal cells by *Agrobacterium tumefaciens*-mediated transformation. Objective genes can be easily inserted into the vector by the use of Gateway technology, without relying on any restriction cleavage sites. The pFungiway vectors are composed of two types: expression vectors, in which the expression of the objective genes is driven by a constitutive promoter; and repression vectors, in which the expression of the endogenous target gene is repressed by RNA interference. Either of two genes conferring resistance to hygromycin B or G418 can be used as a selective marker. The availability of pFungiway vectors was confirmed by the use of fluorescent reporter genes in the basidiomycete *Flammulina velutipes* and in the plant pathogenic fungus *Fusarium oxysporum*.

Keywords *Agrobacterium tumefaciens*-mediated transformation · Fungi · *Flammulina velutipes* · *Fusarium oxysporum* · Gateway technology · RNA interference

✉ Makoto Shimosaka
mashimo@shinshu-u.ac.jp

[1] Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan

[2] Mushroom Research Laboratory, Hokuto Corporation, 800-8 Shimokomazawa, Nagano 381-0008, Japan

Introduction

A number of species of filamentous fungi have been utilized as producers of useful metabolites and enzymes at the industrial level (Bennett 1998; Kirk et al. 2002), and many efforts have been made to improve their productivity. For instance, a selection of mutants having favorable phenotypes leading to high productivity was achieved through random mutagenesis, by treating fungal cells with a chemical or physical mutagen. In the meantime, recombinant DNA technology is a powerful tool, not only for the analysis of gene function, but also for the molecular breeding of fungal strains. It is also possible to introduce foreign genes into fungal cells and to obtain a large amount of recombinant proteins (Daly and Hearn 2005; Tsuboi et al. 2005).

Various types of plasmid vectors have been constructed in certain species of bacteria that were used for fundamental research or industrial application. Most of these vectors are able to replicate autonomously within host cells, since their origin of replication is derived from extranuclear replicons, such as plasmids and bacteriophages. Contrary to this, the availability of plasmid vectors is very restricted in fungi, because they lack plasmid-like replicons in their cytoplasm. In limited cases, replicating-type vectors have been developed by the incorporation of autonomously replicating sequences (*ars*), which supposedly play a role in the process of chromosomal DNA replication (Gems et al. 1991). However, these vectors are generally unstable and easily lost from the host cells in the absence of selective pressure. Therefore, integrating-type vectors have been preferentially used in fungi, in which the vector DNA is integrated into the genome of the host cell via homologous or non-homologous recombination. The integrated vector DNA is usually stable, and, in some cases, multiple copies of vector DNA are tandemly integrated. This incidence would guarantee the production of a

larger amount of recombinant proteins encoded by the introduced genes.

Another problem making gene manipulation in fungal cells difficult is the absence of an easy and reliable genetic transformation method. A protoplast–polyethylene glycol (PEG) method is widely used, in which foreign DNA molecules are introduced into protoplasts, under the condition that protoplasts are fused to each other in the presence of PEG (Ruiz-Díez 2002). However, this method is time-consuming, because many tedious experimental steps are required for the preparation and regeneration of fungal protoplasts. A substitute for this method is *Agrobacterium tumefaciens*-mediated transformation (ATMT), which has been widely used in the genetic transformation of plants (Michielse et al. 2005; Frandsen 2011). In ATMT, foreign DNA molecules are introduced and integrated into the genome of fungal host cells with the aid of T-DNA transfer, carried out by the plant pathogenic bacterium *A. tumefaciens*. ATMT is an easy and efficient method for genetic transformation, and it has been used for the genetic manipulation of certain species of fungi. In this paper, we report the construction of versatile plasmid vectors that can be integrated into the genome of fungal cells through ATMT. This series of vectors, named pFungiway, has adopted the Gateway technology so that any type of DNA fragment may be easily introduced into the designed region of vectors, with a defined orientation, without relying on the presence of restriction endonuclease cleavage sites. The pFungiway vectors may be used for the expression of both endogenous and foreign genes at higher levels under the control of a constitutive promoter. They may also be used for the repression of endogenous target genes through RNA interference (RNAi). These vectors are promising tools for the analysis of gene function and for the molecular breeding of useful strains in a wide range of fungi.

Materials and methods

Strains and media

The basidiomycete, *Flammulina velutipes* dikaryotic strain MH092086, and the plant pathogenic fungus, *Fusarium oxysporum* SUF1018, were cultivated and maintained on potato-dextrose agar (PDA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The *A. tumefaciens* strain, LBA4404 (Clontech Laboratories, Inc., Palo Alto, CA, USA), was used as a host to harbor binary plasmids. The minimal medium and induction medium used for ATMT were as described previously (Okamoto et al. 2010). General techniques for gene cloning, plasmid construction, and polymerase chain reaction (PCR) were performed according to Sambrook and Russell (2001). *Escherichia coli* DH5α was primarily used as a host cell to propagate constructed plasmids, and it was grown in LB medium supplemented with ampicillin (50 μg/mL) or chloramphenicol (50 μg/mL).

Construction of pFungiway vectors

Detailed steps for the construction of the binary vectors, pFungiway1–8, are described in Online Resource 1. *E. coli* One Shot *ccdB* Survival T1 Phage-Resistant Cells (Life Technologies, Carlsbad, CA, USA) were used for the propagation of pFungiway vectors. A complete nucleotide sequence of the T-DNA region of each pFungiway vector was determined in order to confirm its expected structure, and was deposited in the DDBJ/EMBL/GenBank database under the accession nos. LC029918–LC029925.

Construction of pFungiway plasmids expressing fluorescent reporter genes

Recombinant pFungiway2 and pFungiway4 plasmids expressing any of the three fluorescent reporter genes were constructed. The three reporter genes used were as follows: enhanced green fluorescent protein (*egfp*), originated from *Aequorea victoria* (Gerdes and Kaether 1996); red fluorescent protein (*DsRed*) from *Discosoma* sp. (Baird et al. 2000); and urogen III methyltransferase (*umt*) from an uncharacterized bacterium (Lan et al. 2008). An in vitro recombination reaction, using BP or LR Clonase Enzyme Mix and a successive selection of *E. coli* clones possessing the desired recombinant plasmids, was performed according to the supplier's manual for the Gateway cloning system (Life Technologies). The oligonucleotides used for PCR are listed in Table 1. In the case of EGFP, the entire length of *egfp* cDNA was PCR-amplified using pEGFP (Clontech Laboratories, Inc.) as a template. A set of primers, EGFPfw and EGFPrv, was designed in which

Table 1 Oligonucleotides used for polymerase chain reaction (PCR) amplification

Oligonucleotide	Sequence (5′-3′)
EGFPfw	caccATGGTGAGCAAGGGCGAGGA
EGFPrv	TTACTTGTACAGCTCGTCCATGCC
DsRedfw	caccATGGCCTCCTCCGA
DsRedrv	CCGCTACAGGAACAGGTG
UMTfw	caccATGCATGACATCGACTATCG
UMTrv	GAAATACCTGCCGAGGCG
UMT-Intfw	caccATGACATCGACTAT
UMT-Intrv	TTGTCACTGCCGAACCGT
Tubfw	AAATCGGTGCTGCTTTCTGG
Tubrv	ACGACGGTGTCAGAGACCTT

Lowercase letters denote an additional sequence required for a directional cloning toward the pENTR/D-TOPO vector

the forward primer had an additional CACC sequence at its 5′ termini, so that the amplified fragment was cloned into the pENTR/D-TOPO vector (Life Technologies) with a defined direction. Then, the constructed plasmid (entry clone) and pFungiway4 vector (destination clone) were mixed and subjected to an in vitro recombination reaction using the LR Clonase Enzyme Mix (Life Technologies). During this step, the reading frame cassette B, located in pFungiway4, was replaced with *egfp* cDNA. The resulting reaction mixture was transformed into *E. coli* DH5α, and the transformant colonies possessing the objective recombinant plasmid were selected on an LB plate containing chloramphenicol (50 μg/mL). The resulting recombinant plasmid was named pFungiway4-*egfp*. Similarly, pFunigiway4-*DsRed* was constructed using *DsRed* cDNA that was PCR-amplified using a set of oligonucleotides (DsRedfw and DsRedrv) and a template of pDsRed-Express (Clontech Laboratories, Inc.). To construct pFungiway2-*cob*, the *cob* gene was PCR-amplified using a set of primers, UMTfw plus UMTrv, and using the plasmid pRF1.5 DNA as a template (Lan et al. 2008). The constructed plasmids were used for the genetic transformation, by ATMT, of *Flammulina velutipes* dikaryotic mycelia and *Fusarium oxysporum* conidia.

Construction of pFungiway plasmid repressing the expression of *cob*

An internal DNA fragment of *cob* was PCR-amplified using a set of primers, UMT-IntFw and UMT-IntRv, using pRF1.5 as a template. The amplified fragment (ca. 400 bp) was cloned into the pENTR/D-TOPO vector to obtain an entry clone. Two cassettes of reading frame B, located in pFungiway6, were replaced with the *cob* internal fragment by LR recombinase reaction. The resulting recombinant plasmid, pFungiway6-*cob*, was obtained from *E. coli* DH5α and was used for the genetic transformation of *Fusarium oxysporum* that was previously transformed with pFungiway2-*cob*.

Genetic transformation of *Flammulina velutipes* and *Fusarium oxysporum*

First, each of the pFungiway recombinant plasmids were introduced into *A. tumefaciens* LBA4404, and chloramphenicol-resistant transformant colonies were selected. The ATMT of *Flammulina velutipes* dikaryotic mycelia was performed, as described previously (Okamoto et al. 2010). Transformant colonies were selected on PDA plates containing cefotaxime (200 μg/mL), to eliminate co-existing *A. tumefaciens* cells, and hygromycin B (2 μg/mL) or G-418 (25 μg/mL), to select for *Flammulina velutipes* transformant colonies. The ATMT of *Fusarium oxysporum* conidia was performed according to the method described by Mullins et al. (2001), and transformant colonies were selected on PDA plates containing cefotaxime (200 μg/mL), and hygromycin B (30 μg/mL) or G418 (50 μg/mL). Fluorescence emitted by transformant cells of *Flammulina velutipes* and *Fusarium oxysporum* was visualized by a fluorescence microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan). Filters, B-2A, G2-A, and UV-1A, were used for the detection of fluorescence derived from EGFP, DsRed, and UMT, respectively.

Semi-quantitative reverse transcriptase PCR

The amount of *cob* transcript in the mycelia of *Fusarium oxysporum* was estimated by semi-quantitative reverse transcriptase PCR (RT-PCR). Total RNAs were prepared from mycelial cells using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the supplier's manual. The first strand of cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) with a primer of Oligotex-dT30 (Takara Bio, Otsu, Japan). The amplification of the *cob* cDNA fragment (830 bp) was carried out with a primer set of UMTfw and UMTrv, and Ex Taq (Takara Bio), under the following conditions: 30 cycles each, at 98 °C for 10 s, 64 °C for 30 s, and 72 °C for 30 s. The cDNA fragment (540 bp) of the β-tubulin gene was amplified using a primer set of Tubfw and Tubrv, and was used as an internal control for quantitative standardization.

Results and discussion

Construction of pFungiway vectors

We constructed a series of vectors, named pFungiway, which may be used for gene manipulation in fungi (Fig. 1, Table 2). These included two types of vectors: expression vectors (pFungiway1, 2, 3, 4, and 7), in which endogenous or foreign genes that are inserted into the vector are strongly expressed; and repression vectors (pFungiway5, 6, and 8), in which the expression of target genes may be repressed through RNAi, caused by hairpin RNA molecules that are homologous to the target transcript. Various groups have previously reported the construction of vectors for gene manipulation in fungi, in which the Gateway technology was adopted (Toews et al. 2004; Oliveira et al. 2008; Saitoh et al. 2008; Shafran et al. 2008; Zhu et al. 2009). To our knowledge, only a series of the pFungiway vectors mentioned in this article have a backbone of binary vectors, featuring both expression and repression (RNAi) vectors. pFungiway vectors possess several characteristics, as listed below:

i. Binary vector
   pFungiway vectors have a backbone comprised of the binary vector, pCAMBIA2200 (provided by Cambia,

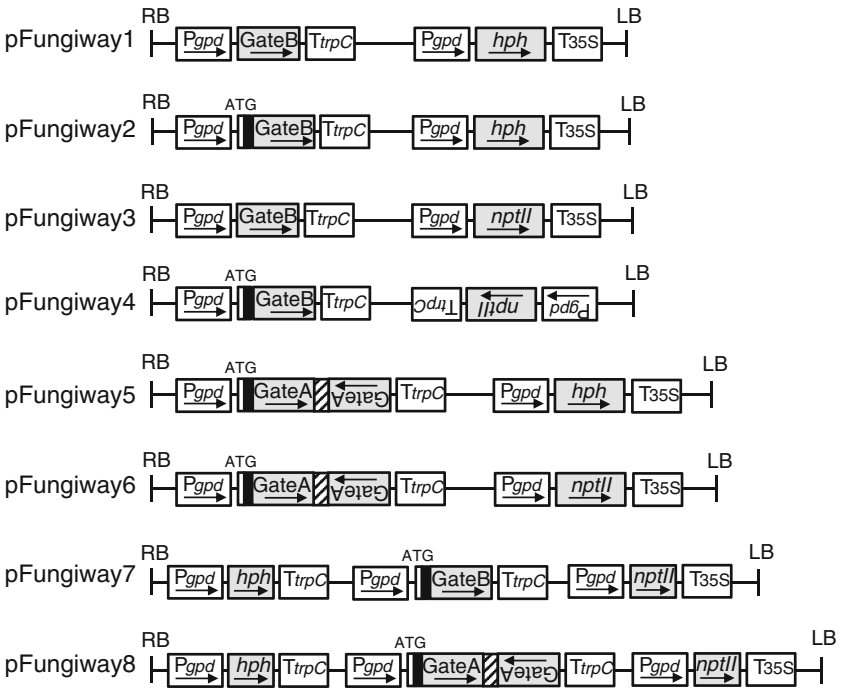

Fig. 1 Structure of pFungiway vectors. The structure of the DNA construct inside the T-DNA region of each pFungiway vector is indicated. pFungiway1, 2, 3, 4, and 7 are expression vectors, while pFungiway5, 6, and 8 are repression vectors. The *black boxes* indicate the first intron of the glyceraldehyde 3-phosphate dehydrogenase gene (*gpd*) derived from *Flammulina velutipes*. The *boxes with diagonal lines* indicate the truncated *egfp*. The *arrows* indicate the direction of transcription and the letters *ATG* denote the transcription initiation codon. The apparent size of each box is not proportional to the actual length of each component. Abbreviations: *LB* left border of T-DNA, *RB* right border of T-DNA, *Pgpd* promoter of the *gpd* gene of *Flammulina velutipes*, *GateA* and *GateB* reading frame cassette A and B provided by the Gateway technology, *TtrpC* terminator of the *trpC* gene of *Aspergillus nidulans*, *T35S* terminator of the cauliflower mosaic virus 35G gene, *hph* hygromycin B phosphotransferase, *nptII* neomycin phosphotransferase

Canberra, Australia), which can be propagated in the plant pathogenic bacterium, *A. tumefaciens*. The T-DNA region of the binary vector is known to be transferred and integrated into the genome of host plant cells during infection by *A. tumefaciens* cells. The pCAMBIA2200 binary vector is an improved vector that is characterized by a high copy number in *E. coli*, high stability in *A. tumefaciens* due to the presence of the pVS1 replicon, and the possession of chloramphenicol-resistant genes, and is available as a selectable marker in both *E. coli* and *A. tumefaciens*. Similarly, T-DNA is known to be transferred and integrated into the genome of certain fungi, when the fungal cells and *A. tumefaciens* cells are co-cultivated. Thus, ATMT is an easy and convenient method of fungal transformation, compared to a traditional method using protoplasts and PEG. However, the number of fungal species able to be transformed by ATMT is limited, and the cause of this remains unknown (Frandsen 2011).

ii. Gateway technology

A reading frame cassette of the Gateway vector conversion system (Life Technologies), which contains a chloramphenicol-resistant gene and a *ccdB* gene flanked

Table 2 A series of Fungiway vectors

Vector name	Type	Selective marker for antibiotic resistance	Accession number[a]
pFungiway1	Expression	Hygromycin B	LC029918
pFungiway2	Expression	Hygromycin B	LC029919
pFungiway3	Expression	G-418	LC029920
pFungiway4	Expression	G-418	LC029921
pFungiway5	Repression	Hygromycin B	LC029922
pFungiway6	Repression	G-418	LC029923
pFungiway7	Expression	Hygromycin B, G-418	LC029924
pFungiway8	Repression	Hygromycin B, G-418	LC029925

[a] Complete nucleotide sequence of the T-DNA region of each vector has been deposited

by attR1 and attR2 sites, is inserted into the T-DNA region of the pFungiway vectors. The *ccdB* gene codes for the CcdB toxin that inhibits the growth of most *E. coli* strains by targeting DNA gyrase. Therefore, pFungiway vectors must be propagated in *E. coli* CcdB-resistant strains, such as One Shot *ccdB* Survival T1 Phage-Resistant Cells (Life Technologies). The cassette can be precisely replaced with an objective DNA fragment that is flanked by attL1 and attL2 sites by in vitro LR recombination reaction. The presence of *ccdB* in the cassette guarantees a recovery of only the desired clones when the LR reaction mixture is transformed into the *E. coli* standard host cells, such as DH5α. Thus, the Gateway technology makes it possible to insert any DNA fragment into the pFungiway vectors, in a defined orientation and in a designed reading frame.

iii. Selection marker

Either of the genes conferring resistance to hygromycin B (*hph*) or G418 (*nptII*) may be used as a selectable marker in fungal transformation.

iv. Promoter and terminator

A constitutive promoter originated from the glyceraldehyde 3-phosphate dehydrogenase gene (*gpd*) of *Flammulina velutipes* was used to drive the transcription of cloned genes and DNA constructs for RNAi (Hannon 2002). In most cases (with the exception of pFungiway1 and 3), the *gpd* promoter (P*gpd*) region was followed by the first exon (6 bp) and first intron (43 bp), since it was expected that the presence of the intron in the synthesized transcripts and the successive splicing led to a stabilization of the transcripts (Lugones et al. 1999; Burns et al. 2005). On the other hand, the *Pgpd* region alone (excluding the exon and intron) was used to drive selectable marker genes. To terminate the transcription of the cloned genes and DNA constructs, the *trpC* terminator (T*trpC*), derived from *Aspergillus nidulans*, was used (Cullen et al. 1987).

v. DNA construct for RNAi

The DNA construct for RNAi contains two reading frame A cassettes, which were inversely repeated by the placement of the cDNA encoding enhanced green fluorescent protein (*egfp*) between them. When the whole DNA construct was transcribed, the resulting RNA molecules spontaneously formed hairpin structures, with a loop composed of *egfp*. The double-stranded RNA within the hairpin is known to induce the degradation of homologous messenger RNA molecules through RNAi, resulting in

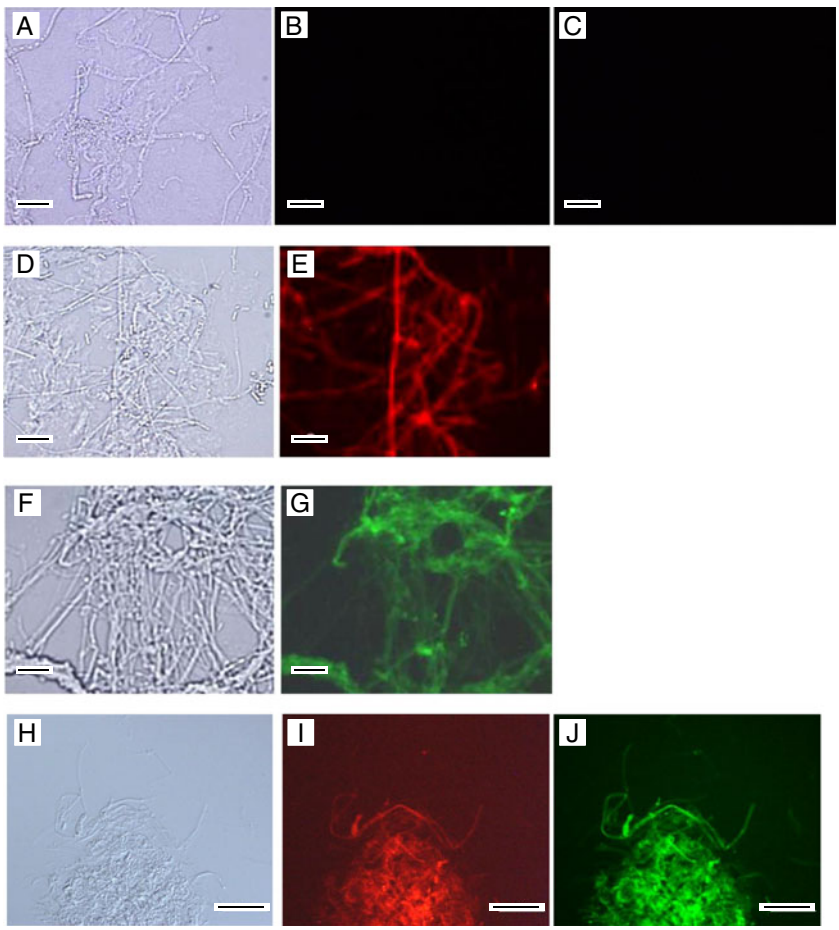

Fig. 2 Fluorescence emitted from *Flammulina velutipes* transformant cells. Mycelial cells of the parental strain (**a**, **b**, **c**), transformant cells with pFungiway4-*DsRed* (**d**, **e**), transformant cells with pFungiway4-*egfp* (**f**, **g**), and transformant cells with both pFungiway4-*DsRed* and pFungiway2-*egfp* (**h**, **i**, **j**). Differential interference contrast (**a**, **d**, **f**, **h**), red fluorescence (**b**, **e**, **i**), and green fluorescence (**c**, **g**, **j**) micrographs were taken of mycelial cells suspended in water. Size bar, 10 μm

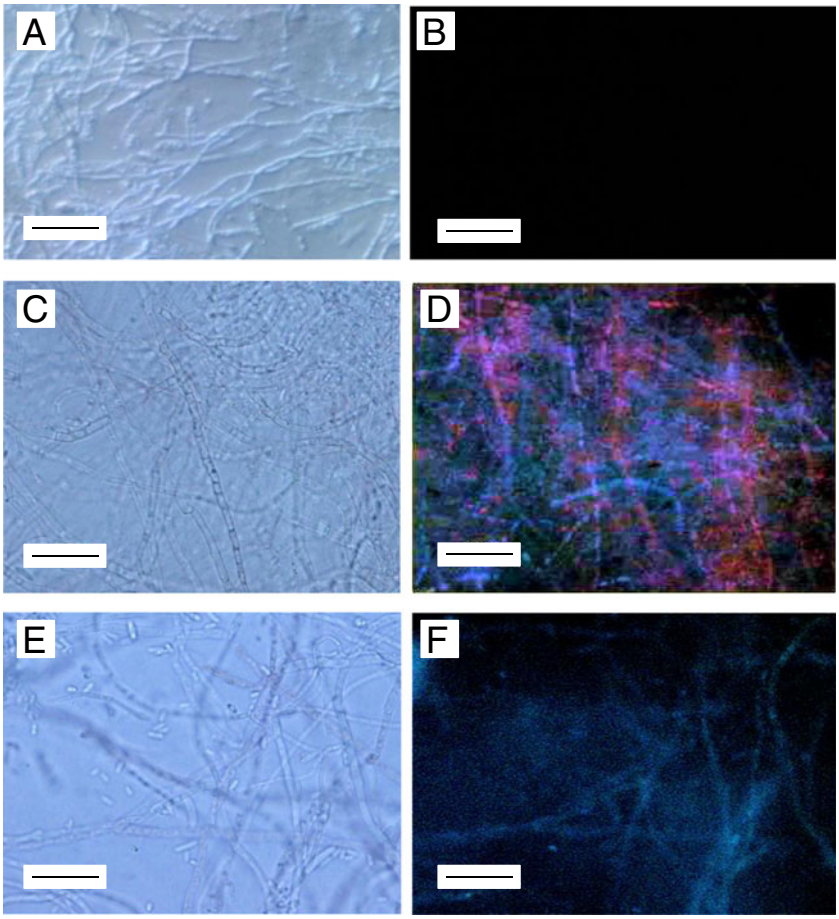

Fig. 3 Repression of red fluorescence emitted from *Fusarium oxysporum* transformant cells by RNA interference (RNAi). Mycelial cells of the parental strain (**a**, **b**), transformant cells with pFungiway2-*cob* alone (**c**, **d**), and cells of transformant strain no. 3 (see lane number in Fig. 4) in which the transformant strain with pFungiway2-*cob* (above) was further transformed with pFungiway6-*cob* (**e**, **f**). Differential interference contrast (**a**, **c**, **e**) and fluorescence (**b**, **d**, **f**) micrographs were taken of mycelial cells suspended in water. Size bar, 10 μm

the repression of the function of the target gene at the transcription stage.

Expression of fluorescent reporter genes in the basidiomycete *Flammulina velutipes*

To confirm the availability of pFungiway vectors, two genes coding for fluorescent reporters, EGFP and DsRed, were used, and two recombinant plasmids, pFungiway4-*DsRed* and pFungiway4-*egfp*, were constructed. Finally, the T-DNA of each recombinant plasmid was introduced into the dikaryotic mycelia of *Flammulina velutipes* by ATMT, and G-418-resistant colonies were obtained on PDA plates containing both G-418 and cefotaxime (to eliminate co-existing *A. tumefaciens* cells). When the mycelial cells of stable transformants with pFungiway4-*DsRed* were observed under the fluorescence microscope, they generated a bright red fluorescence (Fig. 2e), while no fluorescence was detected in the control cells (Fig. 2b). Similarly, the transformants with pFungiway4-*efgp* emitted a green fluorescence (Fig. 2g). *Flammulina velutipes* dikaryotic cells are sensitive to hygromycin B, as well as G-418. Therefore, we constructed a recombinant plasmid of pFungiway2-*DsRed* and introduced its T-DNA into the mycelial cells of G418-resistant transformants with pFungiway4-*egfp*. As a result, the cells of transformants resistant to both G-418 and hygromycin B emitted both red and green fluorescence (Fig. 2i, j).

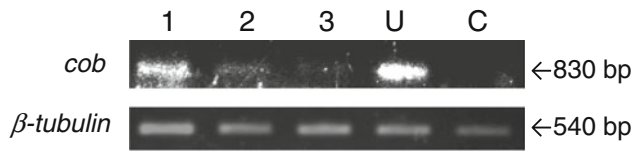

Fig. 4 Effect of RNAi on the amount of *cob* transcript in cells of *Fusarium oxysporum* transformants. The amount of transcript was evaluated by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), using the β-tubulin gene as an internal control for quantitative standardization. Lanes: *1–3* independent transformant strains possessing both full-length *cob* (by pFungiway2-*cob*) and the RNAi construct of partial *cob* (by pFungiway6-*cob*); *U* transformant strain with pFungiway2-*cob* alone; *C* parental strain (control)

In pFungiway1 and 3, an initiation codon, ATG, must be located in the inserted fragment in order for translation to begin. On the other hand, pFungiway2, 4, and 7 contain the *gpd* promoter, plus the first exon and intron, upstream of the reading cassette in expectation of an accumulation of a higher amount of the transcript caused by splice-mediated

stabilization. However, it should be noted that an adjustment of the reading frame is required for the design of the entry clone, and that the recombinant protein possesses 43 additional residues (MAAMGGSSRVDLQACK LDIEFLQPGGSSTSLYKKAGSAAAPFT), derived from the first exon and multicloning site, at the N-termini. These additional residues may be detrimental to the proper function of the protein, although this was not the case for the fluorescent reporters EGFP, DsRed, and UMT used in this experiment.

Repression of target genes by RNAi in the fungus *Fusarium oxysporum*

The availability of repression vectors was confirmed using a gene (*cob*) coding for uroporphyrinogen III methyltransferase (UMT). The *cob* gene was isolated from a metagenomic library constructed, by our group, from soil bacteria (Lan et al. 2008). UMT catalyzes the methylation of uroporphyrinogen III in the biosynthetic pathway of porphyrinogen. When the *cob* gene was excessively expressed in *E. coli*, an overmethylated product, trimethylpyrrocorphin, accumulated in the cytoplasm. This product has no obvious physiological role, but it emits a strong red fluorescence under UV exposure. Therefore, it is expected that the *cob* gene can be used as a transcription reporter in versatile cells, as porphyrinogen is an essential compound for living cells. We observed that a red fluorescence was emitted from the dikaryotic mycelia of *Flammulina velutipes* (Okamoto et al. 2010) and in the mycelia of the plant pathogenic fungus, *Fusarium oxysporum* (Lan et al. 2008), when the *cob* gene was introduced by ATMT using a prototype of pFungiway vectors.

First, pFungiway2-*cob* was constructed and used for the transformation of the conidia of *Fusarium oxysporum* SUF1018. Hygromycin B-resistant mycelial colonies were purified by single-spore isolation. Mycelial cells of the transformant were confirmed to generate red fluorescence (Fig. 3d). Then, a repression-type recombinant plasmid, pFungiway6-*cob* was constructed, which possesses two copies of the internal region of the *cob* gene (400 bp) in the DNA construct for RNAi. Transfer of the T-DNA of pFungiway6-*cob* was conducted by ATMT of the conidia of *Fusarium oxysporum*, which had been previously transformed by pFungiway2-*cob*. Three independent transformants (resistant to both hygromycin B and G-418) were purified by single-spore isolation. Analysis of semi-quantitative RT-PCR indicated that an amount of the *cob* transcript was significantly reduced in two of the three transformants (transformant numbers 2 and 3), compared to that of the parental transformant with pFungiway2-*cob* alone (Fig. 4). This result implies that the expression of *cob* was successfully suppressed through RNAi, mediated by hairpin RNA containing the partial *cob* sequence. In combination with a decrease of *cob* transcripts, the intensity of the red fluorescence of one transformant (number 3) was significantly reduced, compared to that of the parental transformant (Fig. 3f).

Gene disruption has been widely used to elucidate the function of unknown genes; however, it is ineffective in the case of the dikaryotic mycelia of the basidiomycete, because it is difficult to knock out two homologous genes in individual nuclei at one time. We have previously reported a successful repression of the gene coding for the adenosine deaminase-related growth factor in *Flammulina velutipes*, using the prototype of a vector mainly identical to pFungiway5 (Sekiya et al. 2013).

Improved vectors having two selection markers

During ATMT experiments with the expression vector possessing fluorescent reporter genes, some of the antibiotic-resistant transformant cells did not produce any fluorescence. Judging from the PCR analysis, they appeared to have lost a portion of the reporter gene (data not shown). During the process of the infection of plants by *A. tumefaciens* cells, an entire length of T-DNA, flanked by the left border (LB) and right border (RB), was replicated and excised from the Ti plasmid, and then the resulting T-DNA was transferred into the plant cells in the direction starting from the RB side (Gelvin 2003). It is known that an occasional malfunction of the transfer machinery will give incomplete transformants, in which the T-DNA integrated into the genome has lost a part of its DNA located on the LB side. In consideration of this problem, a selectable marker gene was placed on the LB side of the T-DNA of pFungiway vectors; however, the RB-sided fluorescent marker gene appeared to be lost for unknown reasons. To circumvent this problem, we constructed two additional vectors, pFungiway7 (expression) and pFungiway8 (repression). These vectors possessed two selectable marker genes, *hph* and *nptII*, on the RB and LB sides of the T-DNA, respectively, with the Gateway cassette located between them. It is expected that a selection of transformants resistant to both hygromycin B and G-418 would retain the objective insert inside the T-DNA integrated into the fungal genome. The effectiveness of this selection was confirmed using pFungiway7 possessing the *egfp* gene in *Flammulina velutipes* (data not shown).

In conclusion, a series of pFungiway vectors may be used as a practical tool for gene manipulation in fungi, for which ATMT is applicable.

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