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Agrobacterium tumefaciens-mediated Transformation in *Colletotrichum falcatum* and *C. acutatum*

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***Agrobacterium tumefaciens*-mediated transformation (ATMT) is becoming an effective system as an insertional mutagenesis tool in filamentous fungi. We developed and optimized ATMT for two *Colletotrichum* species, *C. falcatum* and *C. acutatum*, which are the causal agents of sugarcane red rot and pepper anthracnose, respectively. *A. tumefaciens* strain SK1044, carrying a hygromycin phosphotransferase gene (*hph*) and a green fluorescent protein (GFP) gene, was used to transform the conidia of these two *Colletotrichum* species. Transformation efficiency was correlated with co-cultivation time and bacterial cell concentration and was higher in *C. falcatum* than in *C. acutatum*. Southern blot analysis indicated that about 65% of the transformants had a single copy of the T-DNA in both *C. falcatum* and *C. acutatum* and that T-DNA integrated randomly in both fungal genomes. T-DNA insertions were identified in transformants through thermal asymmetrical interlaced PCR (TAIL-PCR) followed by sequencing. Our results suggested that ATMT can be used as a molecular tool to identify and characterize pathogenicity-related genes in these two economically important *Colletotrichum* species.**

Keywords: *C. falcatum*, *C. acutatum*, insertional mutagenesis, pepper anthracnose, sugarcane red rot, transformation

Colletotrichum falcatum Went (teleomorph: *Glomerella tucumenensis*) and *C. acutatum* Simmonds (teleomorph: *G. cingulata*) are the causal agents of sugarcane red rot and pepper anthracnose diseases, respectively. Red rot is one of the most serious diseases of sugarcane and continues to be a threat in some subtropical countries, especially in Indian sugarcane cultivation [2]. *C. acutatum* causes anthracnose diseases in a wide range of hosts and is one of the major anthracnose pathogens of hot pepper in Korea [8]. To infect host plants, *Colletotrichum* species require diverse morphological changes such as conidial germination, appressorium formation and maturation, penetration from appressoria, and invasive growth in host plants [28]. Although much study has focused on the developmental, biochemical, and molecular analyses of the plant-pathogen interactions, the complete genetic basis for disease development has yet to be defined [11, 33]. A better understanding of the pathogenicity mechanisms of these fungi is needed to develop novel strategies for the effective management of the diseases.

Fungal transformation technology has been considered essential in the study of fungal pathogenicity genes at the molecular level, either for creating targeted or random gene disruptions. Several different methodologies using protoplasts with polyethylene glycol (PEG), electroporation, or both have been developed and used widely in many fungi [22, 31]. For large-scale mutagenesis, restriction enzyme-mediated integration (REMI) has also been used in various plant pathogenic fungi including *Alternaria alternata* [35], *Magnaporthe grisea* [3], and *Colletotrichum* spp. [29, 36]. However, disadvantages of REMI such as large deletions of nucleotides, multiple copies of plasmid, and untagged mutagenesis including chromosomal rearrangements require the development of alternative transformation systems [20, 33, 35].

Agrobacterium tumefaciens-mediated transformation (ATMT) has long been used for gene transfer and gene tagging in plants [14]. ATMT has also been used to transfer genes from yeast to filamentous fungi [4, 23] as well as human cells [19]. The decisive advantage of ATMT compared with other transformation techniques in filamentous fungi is the flexibility in choosing the starting material for transformation; *i.e.*, protoplasts, hyphae, spores, or blocks of the mycelial tissue could serve as starting materials [7, 10, 38]. Significantly, T-DNA-mediated insertional

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mutagenesis is effective compared with other mutagenesis in fungi, in that T-DNA-tagged mutated genes or flanking sequences can be identified easily [18, 24]. Furthermore, T-DNA of *A. tumefaciens* can also integrate through homologous recombination in fungi, mainly for targeted gene disruption [5, 23]. ATMT has also been successfully employed in many plant-pathogenic fungi [23], including several species of *Colletotrichum* [10, 12, 26, 34, 37]. However, there have been no previous attempts of ATMT in *C. falcatum* and *C. acutatum*.

In this study, we developed and optimized ATMT for these two species. Transformation efficiency was optimized based on co-cultivation period and *Agrobacterium* cell volume. A high frequency (65%) of single-copy T-DNA insertions into fungal genomes suggested that ATMT is a useful insertional mutagenesis tool in these two species of *Colletotrichum*.

MATERIALS AND METHODS

Bacterial and Fungal Strains

Conidia of *C. falcatum* Cf-1148 and *C. acutatum* JC-24 strains were obtained from 7-day-old cultures grown on oatmeal agar medium (OMA: 50 g of oatmeal for 1 l) at 25°C under continuous fluorescent light to promote conidiation. Both *Colletotrichum* species were initially tested for hygromycin B sensitivity using different concentrations (up to 500 μg/ml). *A. tumefaciens* strain SK1044 that harbors the EGFP construct under the control of the *Aspergillus nidulans trpC* promoter in T-DNA binary vector pBHt2 [24] was used for transformation of these two *Colletotrichum* species. The vector pBHt2 contains a T-DNA harboring hygromycin B resistance (hygromycin phosphotransferase, *hph*) in the backbone of pCAMBIA1300 [24].

Development of Transformation System

A. tumefaciens strain SK1044 was grown at 28°C for 48 h in minimal medium [15] (MM: supplemented with kanamycin, 75 μg/ml). Two ml of bacterial cell aliquot was harvested and washed with an induction medium (IM); the cells were then resuspended in 5 ml of IM [4] with (IM+AS) or without (IM-AS) acetosyringone (AS). Subsequently, the bacterial cells were grown for an additional 6 h before mixing them with an equal volume of conidial suspensions of *C. falcatum* and *C. acutatum*. This mixture (150 μl) was placed on nitrocellulose filters (0.45-μm pore and 45 mm diameter; Whatman, Chicago, IL, U.S.A.) on a co-cultivation medium (CM; same as IM except that it contained 5 mM of glucose instead of 10 mM) in the presence or absence of acetosyringone (200 μM).

Following co-cultivation for 24, 36, and 48 h at 28°C, the fungal and bacterial cells on the nitrocellulose filter were harvested in selection medium and plated on medium containing hygromycin B as a selection agent for fungal transformants of *C. falcatum* (50 μg/ml), *C. acutatum* (300 μg/ml), and Cefotoxime (200 μg/ml) to eliminate the *A. tumefaciens* cells. Individual transformants were transferred into 24-well plates (Costar, Cambridge, MA, U.S.A.) containing 1.5 ml of OMA with an appropriate concentration of hygromycin B and incubated at 25°C until conidiation of *C. falcatum* and *C. acutatum*. Conidia of the individual transformants were harvested, resuspended in sterile distilled water, and plated on water agar to obtain monoconidial cultures. A single conidium from each of the transformants was picked and transferred to an OMA plate.

The effect of the length of cocultivation period using IM+AS and IM-AS was evaluated at 12-h intervals. In addition, three concentrations (50, 100, and 200 μl) of *A. tumefaciens* cell volume were assessed to determine the cell volume for optimum transformation efficiency.

DNA Extraction and Southern Blot Analysis

Fungal genomic DNA was extracted from the mycelia following the method described previously [17]. To verify the integration of T-DNA in the fungal transformants, randomly selected transformants of *C. falcatum* and *C. acutatum* were analyzed by PCR using the *hph*-specific primers Hy_F (5'-TCAGCTTCGATGTAGGAGGG-3') and Hy_R (5'-TTCTACACAGCCATCGGTCC-3'). For Southern blot analysis, fungal genomic DNA isolated from putative transformants of *C. falcatum* and *C. acutatum* was digested with EcoRI, separated on a 0.7% agarose gel, and transferred onto a Hybond N^+ membrane (Amersham Biosciences, Buckinghamshire, U.K.). The blots were hybridized with the (α-^{32}P) -dCTP- labeled 1.4-kb HpaI fragment from pCB1004 as a probe [6]. The hybridization was carried out using standard protocols [32].

Identification of T-DNA Flanking Sequences

Amplification of the region flanking the T-DNA insert in the transformants was carried out by thermal asymmetrical interlaced PCR (TAIL-PCR) as previously described [24]. We used specific primers (SP), RB1 (GGCACTGGCCGTCGTTTTACAAC), RB2 (AACGTCGTGACTGGGAAAACCCT), and RB3 (CCCTTCCCA-ACAGTTGCGCA), for the right border (RB), and LB1 (GGGTTC-CTATAGGGTTTCGCTCATG), LB2 (CATGTGTTGAGCATATAA-GAAACCCT), and LB3 (GAATTAATTCGGCGTTAATTCAGT) for the left border (LB), in combination with three short arbitrary degenerate (AD) primers, AD1 (AGWGNAGWANCAWAGG), AD2 (WAGTGNAGWANCANAGA), and AD3 (WAGTGNAGWANCA-NGTT), so that the relative amplification efficiencies of specific and nonspecific products could be thermally controlled [21]. The number of PCR products was reduced following the secondary and tertiary PCRs, and only single bands in the range from 0.4 to 2.0 kb were obtained in the tertiary PCR. The surplus dNTPs and primers within PCR products were discarded by ExoSAP-IT (USB, Cleveland, OH, U.S.A.), per the manufacturer's descriptions. The tertiary TAIL-PCR products presumed to correspond to the inserted T-DNA junctions were sequenced on an ABI 3770 DNA sequencer (Applied Biosystem). Sequences were analyzed using the BLAST algorithms available on the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov).

RESULTS

Sensitivity of *C. falcatum* and *C. acutatum* to Hygromycin B

Wild-type strains of *C. falcatum* Cf-1148 and *C. acutatum* JC-24 were determined for their sensitivity to hygromycin B. These strains were chosen for their high level of

virulence toward the appropriate host, sugarcane and hot pepper, respectively. Freshly grown mycelial edges were transferred to complete medium for *C. falcatum* or potato dextrose agar for *C. acutatum*, amended with various concentrations of hygromycin B (up to 500 μg/ml). Growth of *C. falcatum* was completely inhibited at 50 μg/ml, whereas in *C. acutatum*, a concentration of 300 μg/ml was most effective in inhibiting mycelial growth (Table 1). This fact may be attributed to the species-specific hygromycin B sensitivity of these fungi. The addition of AS to induction media prior to cocultivation has variable effects on the transformation efficiency in fungi [23, 30], and in particular, it decreases the number of transformants with integration at a single site [24].

Development of ATMT for *C. falcatum* and *C. acutatum*

We optimized the experimental conditions for efficient ATMT in *C. falcatum* and *C. acutatum*. There was no transformant when co-cultivation medium without AS was used. The transformation efficiency or the copy number of T-DNA in individual transformants or both were examined in relation to the following three factors: (i) culturing the bacterial cells in the presence or absence of AS prior to cocultivation, (ii) length of the co-cultivation period, and (iii) the number of *A. tumefaciens* cells initially used for co-cultivation. Addition of AS prior to co-cultivation increased the number of transformants, although the effects were variable between the experimental settings or species (Fig. 1). The influence of AS prior to co-cultivation in increasing the transformation efficiency was higher in *C. falcatum* than in *C. acutatum*.

Duration of the co-cultivation period was also examined in relation to the transformation efficiency in both *Colletotrichum* species. *A. tumefaciens* cells grown in IM+AS prior to co-cultivation generated more transformants than in IM−AS in the 24 h co-cultivation period. However, AS caused little effect in the 36 h co-cultivation in relation to the number of transformants (Figs. 1A and 1C). A prolonged co-cultivation period (>48 h), which promoted excessive mycelial growth on co-cultivation media, made it difficult to subsequently identify the individual transformants.

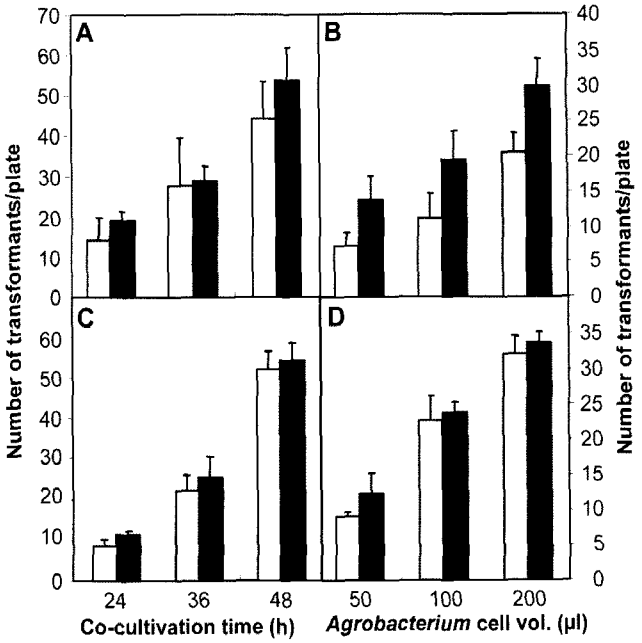

Fig. 1. Effect of co-cultivation period and *A. tumefaciens* cell volume on transformation efficiency of *C. falcatum* (**A** and **B**) and *C. acutatum* (**C** and **D**).
A. tumefaciens cells were grown for 6 h in induction medium (IM) in the presence (solid bars) and absence (open bars) of acetosyringone (AS) prior to co-cultivation. Data presented are the average of five plates per treatment. Error bars indicate standard errors.

Table 1. Comparative analysis of different *Colletotrichum* species transformations using *Agrobacterium tumefaciens*.

Species (Sources)	*Agrobacterium* strains	Vectors	Hygromycin B concentration (μg/ml)	Best co-cultivation time (h)	Percent single T-DNA	No. of transformants	Spore no.
C. gloeosporioides (de Groot *et al.*, 1998)	pUR5750	pBIN 19	100	48	-	130	1×10^6
C. lagenarium (Tsuji *et al.*, 2003)	C58C1	pBIG2RHPH2	100	48	85.71	150–300	1×10^6
C. trifolii (Takahara *et al.*, 2004)	C58C1	pBIG2RHPH2	100	48	75.00	20–30	1×10^6
C. higginsianum (O'Connell *et al.*, 2004)	AGL-1	pBin-GFP-hph	-	-	40.00	250	1×10^6
C. graminicola (Flowers and Vaillancourt, 2005)	AGL-1	pBin-GFP-hph	250	72	16.00	300–1,706	1×10^5
C. falcatum (This study)	SK1044	pBHt2	50	24	62.00	300–500	1×10^6
C. acutatum (This study)	SK1044	pBHt2	300	36	65.00	200–250	5×10^5

-, Not given exact data

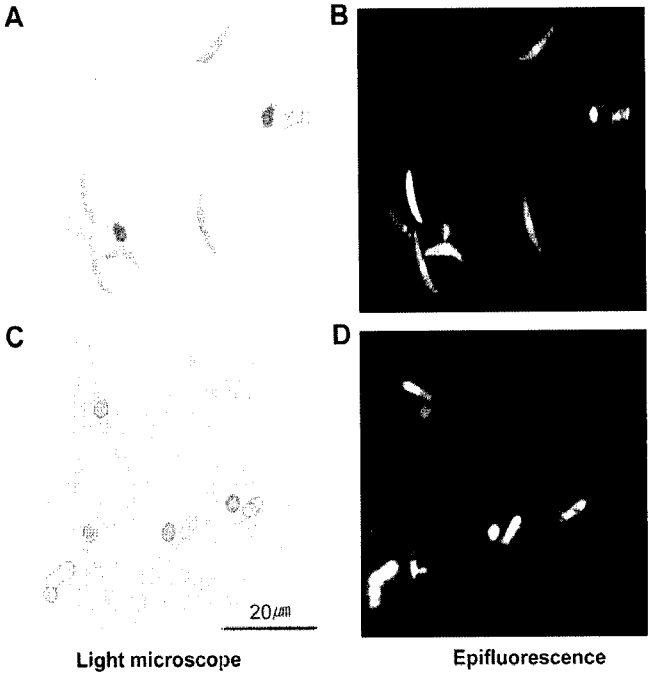

Fig. 2. Expression of green fluorescent protein in *C. falcatum* and *C. acutatum* transformants.
Light (**A** and **C**) and fluorescence (**B** and **D**) microscopies of conidia (magnification, 200×). **A** and **B**, *C. falcatum*; **C** and **D**, *C. acutatum*.

The transformation efficiency was also correlated with the bacterial cell volume at the start of co-cultivation. In *C. falcatum*, however, increased cell volume in IM+AS proportionately increased the number of transformants compared with IM-AS. In *C. acutatum*, the addition of AS prior to co-cultivation did not greatly influence the transformation efficiency. Correspondingly, an increasing bacterial cell volume enhanced the number of transformants in both *C. falcatum* and *C. acutatum* (Figs. 1B and 1D).

We could usually obtain 300–500 transformants per 1×10^6 conidia of *C. falcatum* when *A. tumefaciens* was grown in the presence of AS prior to co-cultivation. For *C. acutatum*, 5×10^5 conidia were used to obtain 200 to 250 transformants, because 1×10^6 conidia produced a fungal lawn making it impossible to separate individual colonies from the plate.

Five randomly selected transformants of each of the two *Colletotrichum* species were subcultured for five successive generations in the absence of hygromycin B, and were subsequently screened for hygromycin B resistance in the sixth generation. All fungal transformants were resistant to hygromycin B, confirming stable maintenance of the marker gene across successive generations (data not shown). Strong expression of GFP was also detected in conidia, germtubes, and appressoria in the transformants of both species (Fig. 2).

Integration of T-DNA into Fungal Chromosomes

To determine the copy number of T-DNA in the transformants, Southern blot analysis for the randomly selected transformants was conducted. About 65% of *C. acutatum* (13 out of 20) and 62% of *C. falcatum* transformants (13/21) contained a single copy of T-DNA in their genomes (Fig. 3A). We further analyzed whether T-DNA copy number varies according to increasing co-cultivation period. Thirteen to 28 transformants at each time were randomly chosen for Southern blot analyses (Fig. 3B). The increased co-cultivation period resulted in an increase in the copy number of the T-DNA integration in both species. About 73% of transformants (20/28) obtained

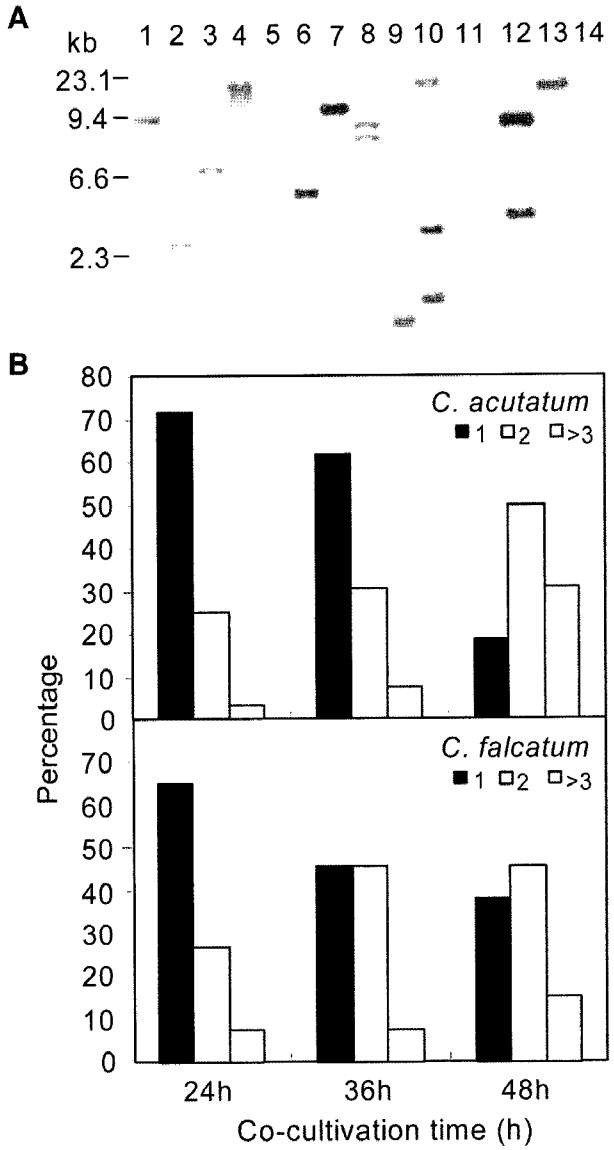

Fig. 3. Copy number of T-DNA in *C. falcatum* and *C. acutatum* transformants.
A. Southern hybridization of transformants. Genomic DNA was digested with EcoRI, separated on 0.7% agarose gel, transferred to a Hybond N^+ membrane, and hybridized with a radiolabeled fragment of 1.4 kb of *hph*. Lanes 1–13, transformants; 14, wild-type strain. **B**. Copy number of T-DNA at each time of co-cultivation was analyzed. Proportions of transformants with each copy number out of 28, 26, and 16 for *C. acutatum*, and 26, 13, and 13 for *C. falcatum* at 24 h, 36 h, and 48 h co-cultivation periods, respectively, were plotted.

Table 2. Sequence analysis of flanking regions from *C. falcatum* and *C. acutatum* transformants using BLAST algorithms.

ID	Border	T-DNA Copy No.	BLAST Best Hits	Origins	E-value
C. falcatum					
Cft-212	LB	2	Calcineurin temperature suppressor Cts1, AAN85205	*Cryptococcus neoformans*	4e-04
Cft-250	LB	1	FGSC A4 hypothetical protein, XM_655792	*Aspergillus nidulans*	1e-08
Cft-267	LB	1	Hypothetical protein, XP_965361	*Neurospora crassa*	6e-24
Cft-305	LB	1	Unnamed protein, BAE66072	*Aspergillus oryzae*	5e-15
Cft-309	LB	2	Hypothetical protein, XP_965376	*Neurospora crassa*	5e-04
C. acutatum					
CAT-1118	RB	1	18S rRNA gene, AJ301918	*Colletotrichum lupini*	0.0
CAT-05	LB	1	Zn_2Cys_6 transcription factor, XP_748391	*Aspergillus fumigatus*	2e-05
CAT-06	LB	2	DNA-directed RNA polymerase II larger subunit, XP_570943	*Cryptococcus neoformans*	4e-07
CAT-16	RB	1	Hypothetical protein, CAD70321	*Neurospora crassa*	5e-04
CAT-07	RB	1	Hypothetical protein, EAQ87798	*Chaetomium globosum*	6e-10
CAT-08	RB	1	Hypothetical protein, XP_962136	*Neurospora crassa*	8e-08

BLAST searches were performed against an available fungal genome database (http://www.ncbi.nlm.nih.gov) using default parameters. The top hit with an e-value lower than $1e^{-2}$ is indicated.

by 24 h co-cultivation contained the single copy of T-DNA in both species. Co-cultivation for 36 h resulted in somewhat different results between the two species. About 61% of transformants (16/28) contained single T-DNA molecules in the *C. acutatum* genome, but only 46% (6/13) had this for *C. falcatum*. Proportions of single-copy integration were further decreased for transformants from 48 h co-cultivation in both species (Fig. 3B). The effect of AS prior to co-cultivation did not seem so conspicuous, but it slightly decreased the single-copy integration (data not shown).

Identification of Flanking Sequences of T-DNA

T-DNA flanking sequences were rescued by Thermal Asymmetrical Interlaced-PCR (TAIL-PCR) [21]. Sixty and 23 sequences from transformants of *C. falcatum* and *C. acutatum*, respectively, were obtained and analyzed. Five *C. falcatum* flanks showed weak or moderate homology to NCBI database entries (Table 2). Left border (LB) flanking sequences of Cft-212 showed homology to the calcineurin temperature suppressor *Cts1* [13] of the mammalian pathogenic fungus *Cryptococcus neoformans*, whereas the other four were matched to hypothetical protein of other fungi. Six out of 23 flanking sequences of *C. acutatum* matched to database entries, one of which (CAT-1118) was the 18S rRNA sequence of *C. lupini*. The LB flank of CAT-05 showed homology to $Zn(II)_2Cys_6$ transcription factors of *Aspergillus fumigatus*, which is known to be present only in fungal species regulating diverse cellular processes including pathogenicity [25]. Because very low percentages of flanking sequences of both species matched to database entries, we questioned whether the sequences really originated from *Colletotrichum* genomic DNA. To address this question, 20 primers from flanks of *C. acutatum* were designed, sixteen of which successfully amplified flanks, suggesting these sequences are really of *C. acutatum* genome (data not shown).

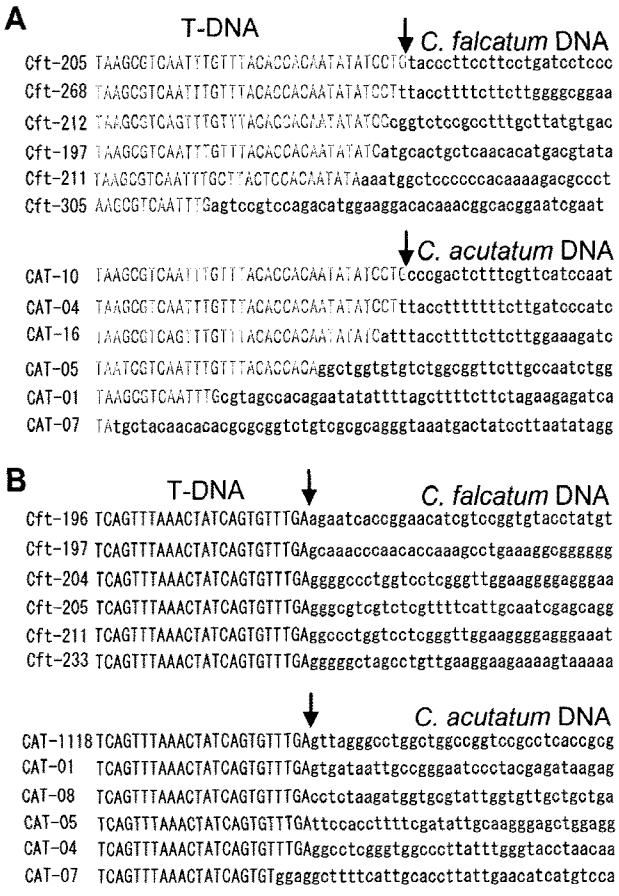

Fig. 4. Alignment of the sequences at the junction of T-DNA integration of *C. falcatum* and *C. acutatum*, rescued using TAIL-PCR.
A. Left border; **B**. Right border.

T-DNA border and flanking sequences were scrutinized to examine how T-DNA molecules integrate into the *Colletotrichum* genome. Bases next to the T-DNA border sequences were regarded as flanks, because there are no genome sequences of *Colletotrichum* species in public. As observed in other fungal species, more truncations of the T-DNA border were found in the LB (up to 40 bp) than in the RB (<10 bp) region in both *Colletotrichum* species.

DISCUSSION

A. tumefaciens has been successfully used to transform a variety of filamentous fungi [23] including *Colletotrichum* species [12, 34, 37]. Because of the ease of selecting starting materials and the higher transformation efficiency of ATMT, many fungi have recently been exploited for random insertional mutagenesis. However, transformation efficiencies vary among fungal species, indicating their biological differences [10]. In this study, we optimized the ATMT protocols for *C. falcatum* and *C. acutatum*. Factors affecting the transformation efficiency, such as influence of acetosyringone on activation of T-DNA transfer, duration of co-cultivation period, and the amount of bacterial cells, were analyzed. Acetosyringone during co-cultivation of conidia with *A. tumefaciens* cells seemed essential, as reported in other fungal systems [10, 23, 38]. The addition of AS to induction media prior to co-cultivation increased the transformation efficiency in both of these *Colletotrichum* species. The effect of AS during preculture of *A. tumefaciens* seemed variable according to fungal species [23, 30] even in the different species within the same genus. In fungal species such as *Beauveria bassiana*, *Fusarium oxysporum*, and *M. grisea*, AS increased the transformation efficiency, whereas in *Hebeloma cylindrosporum* and *C. trifolii* did not [23].

The transformation efficiency in both of these *Colletotrichum* species increased as the bacterial cell volume increased. These results are also in agreement with those of previous reports on other fungal species, such as *M. grisea*, *Coccidioides immitis*, and *F. oxysporum* [1, 24, 30]. Increasing the bacterial cell volume positively affected the number of transformants in *C. lagenarium* [37] and *C. graminicola* [12], but not in *C. trifolii* where excess bacterial cells resulted in a large number of false-positive colonies [34].

Generally, more transformants are obtained by a longer co-cultivation period in most fungi [23, 24, 30]. In our study on *Colletotrichum*, the transformation efficiency was also improved by increasing the co-cultivation period up to 48 h. However, a thick layer of mycelia developed on nitrocellulose filters after 4 days of co-cultivation, which made the observation of individual transformants more difficult. On the other hand, increasing the length of the co-cultivation period has often been associated with multiple T-DNA integration [30]. Single-copy T-DNA integration is generally preferred, as it enables association of an observed phenotype with a single locus alteration in the genome [23]. Southern analysis showed that almost 65% of the transformants had a single copy of the T-DNA in these two species following 24 h co-cultivation. These single-copy percentages are higher than previously reported in other *Colletotrichum* species, except for *C. lagenarium* [37]. Compared with ATMT of other *Colletotrichum* species reported earlier [10, 12, 26, 34, 37], our study suggests that the transformation efficiency is higher in *C. falcatum* and *C. acutatum*. Moreover, the transformation efficiency of *C. acutatum* achieved in our study was higher than those of previously reported transformation systems in this species [9, 16]. Using these optimized ATMT protocols, we could generate 300 to 500 transformants from 1×10^6 conidia of *C. falcatum* and 200 to 250 transformants from 5×10^5 conidia of *C. acutatum*. This indicates that within a short time, thousands of *C. falcatum* and *C. acutatum* transformants can be produced with this protocol in a few experiments.

We could reasonably amplify the flanking sequences from transformants using a TAIL-PCR procedure. Flanking regions thus sequenced contained both T-DNA border as well as fungal flanking sequences. In *C. graminicola*, a falcate spore species, rescue of the flanking sequence itself was difficult with PCR methods, because nearly 80% of the transformants showed tandem integration of the whole plasmid [12]. In contrast, in our study, *C. falcatum*, which is also a falcate spore species, showed significant recovery of flanking DNA from both RB and LB regions.

The flanking sequence from one of the *C. acutatum* transformants, CAT-1118, matched the *Colletotrichum* 18S rRNA sequence available in GenBank with high homology. However, among the other recovered TAIL-PCR sequences, only <10% weakly matched the sequences available in GenBank; two sequences from *C. falcatum* and one from *C. acutatum* had significant homology with other hypothetical and predicted fungal protein sequences. Similar observation was also reported in *C. trifolii*, where the tagged sequences did not match with available genome data [34]. This may be a result of the limited genome information available for *Colletotrichum* in the public domain. The percentage of unmatched sequences of the two *Colletotrichum* species in this study is comparable to the expressed sequence tag (EST) dataset of *C. acutatum*, wherein <8.5% of the sequences out of 455 putative open reading frames (ORFs) matched either the hypothetical or predicted protein sequences or some of the known genes available in GenBank. The remaining 90% of the sequences did not match any known sequences of other fungi (unpublished data). This result strongly suggests that the *Colletotrichum* genome could be distinct from other fungi; however, further experiments are needed to verify its uniqueness. Even though more than 50 fungal genome sequences have

been completed to date [27], the genome of even one *Colletotrichum* species is not sequenced yet. Further analysis of some of the TAIL-PCR sequences recovered from our study should be carried out either by comparing with the upgraded GenBank sequences in the near future or by using other alternative strategies like systematic analysis of every predicted gene and enabling its gene function and expression. Our finding that truncations were more frequent at the LB, as revealed by the TAIL-PCR sequence analysis, is consistent with results reported in several fungi [24, 37].

In summary, we developed an efficient ATMT protocol as a molecular tool for *C. falcatum* and *C. acutatum*. No significant difference was observed in the transformation efficiency of these two closely related species, suggesting that *A. tumefaciens* operates in a similar way to transform these fungi. The developed protocol is expected to be useful for generating a large number of mutants carrying single-copy T-DNA insertions at random, which in turn will facilitate genome-wide analysis of novel genes including pathogenicity factors of these two important pathogenic fungi.

Acknowledgments

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