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# Generation of an Arginine Auxotrophic Mutant of *Colletotrichum acutatum* as a Recipient Host for Insertional Mutagenesis

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***Colletotrichum acutatum*** **was the main cause of the recent outbreaks of anthracnose on pepper fruit in Korea. To facilitate molecular analysis of *C. acutatum*, we generated an arginine auxotrophic mutant of the *C. acutatum* strain JC24 using a targeted gene replacement strategy. A 3.3-kb genomic region carrying an ortholog (designated *CaARG2*) of the fungal gene encoding N-acetylglutamate synthase, the first enzyme of arginine biosynthesis in fungi, was deleted from the fungal genome. The mutant exhibited normal growth only when arginine was exogenously supplied into the culture medium. Transformation of the arginine auxotrophic mutant with a plasmid DNA carrying an intact copy of *CaARG2*, which was smaller than the deleted region in the mutant, not only caused random vector insertions in the fungal genome, but also recovered both hyphal growth and pathogenicity of the mutant to the wild-type level. Using this new selection system, we have successfully developed a restriction enzyme-mediated integration procedure, which would provide an economically efficient random mutagenesis method in *C. acutatum*.**

***Keywords*** : *Colletotrichum acutatum*, acetylglutamate synthase gene, arginine auxotrophy, insertional mutagenesis

*Colletotrichum acutatum* (teleomorph: *Glomerella acutata*) is a filamentous ascomycete with a ubiquitous geographic distribution. This fungus is an important plant pathogen because it causes anthracnose diseases in a wide range of host plants, including hot pepper (Karunakaran et al., 2008; Sreenivasaprasad and Talhinhas, 2005; Talhinhas et al., 2008). In Korea, the annual yield loss caused by the pepper anthracnose has been estimated to be more than US $100 million, and *C. acutatum* is known to be the main cause of the recent 2002 outbreak (Kim et al., 2008) rather than another species, *C. gloeosporioides*, which was reported to be the dominant pathogen for this disease in the late 1980s (Park and Kim, 1992). These two *Colletotrichum* species differ from each other in their responses to benzimidazole fungicides as well as in conidia morphology and growth rate (Kim et al., 2008). *C. acutatum* is less sensitive to these fungicides, which have been frequently used to control pepper anthracnose, than *C. gloeosporioides* (Chung et al., 2006; Kim et al., 2008; Nakaune and Nakano, 2007). This inherent resistance of *C. acutatum* to antifungal agents is extended to compounds such as hygromycin B, geneticin (G418), and bialaphos, which have been widely used as selection agents for the transformation of several *Colletotrichum* species (Brooker et al., 1996; Dickman, 1988; Epstein et al., 1998; Venard and Vaillancourt, 2007).

Despite the economic importance of plant diseases caused by *C. acutatum*, molecular analyses of the fungal pathogenicity mechanism on diverse host plants are still in the early stages (Chen et al., 2005; Sreenivasaprasad and Talhinhas, 2005; You et al., 2007). Pathogenicity-related genes have recently been identified by insertional random mutagenesis, such as restriction enzyme-mediated integration (REMI) or *Agrobacterium tumefaciens*-mediated transformation (ATMT), in *C. acutatum* (Chen et al., 2005; You et al., 2007), and all of these methods are based on a genetic transformation system using hygromycin B as a selection agent (Horowitz et al., 2002; Karunakaran et al., 2008; Talhinhas et al., 2008). However, high-level resistance of *C. acutatum* to hygromycin B makes the experimental procedures costly, and easily leads to the selection of false-positive transformants that normally abort growth upon re-transfer (Kim et al., unpublished data). To circumvent these problems, we focused on the development of a new genetic complementation system for *C. acutatum* using its auxotroph as a recipient strain. Here, we report the generation of an arginine auxotrophic mutant of *C. acutatum* and its use, in combination with an intact copy of the homologous gene responsible for the mutation, for an economically efficient transformation and REMI procedure, a useful alternative to methods using dominant drug resistance markers.

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## Materials and Methods

**Strains and media.** *Colletotrichum acutatum* strain JC24, obtained from infected pepper fruits and highly resistant to hygromycin B as well as benzimidazoles (Kim et al., 2008), was used as the wild-type strain. Arginine auxotrophic mutants of *C. acutatum* carrying a deletion of *CaARG2* and their complementation strains were generated from JC24. *Gibberella zeae* strain Z03643, obtained from Dr. Robert L. Bowden (USDA, Manhattan, KS, USA) was used as a positive control for PCR amplification. All strains were stored in 25% glycerol at −80°C and maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). For the arginine auxotrophy test, fungal strains were inoculated into minimal medium (MM: 0.6% NaCl, 0.05% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% KCl) or MM supplemented with 0.25 mM arginine and grown at 25°C for 7 days. For genomic DNA extraction, fungal strains were grown in 50 ml of PD broth at 25°C for 3 days with shaking (150 rpm). For conidia production, mycelial plugs of each strain were grown on PDA for 7 days. Recombinant *Escherichia coli* HB101 strain (Takara Korea Biomedical Inc., Seoul, Korea) was grown on Luria-Bertani agar or in liquid medium supplemented with 75 μg/ml ampicillin for the propagation of plasmids used in fungal transformation.

**DNA manipulation, PCR conditions, and sequencing.** Fungal genomic DNA was extracted as described previously (Han et al., 2004). Plasmid DNA was isolated from *E. coli* cultures using a plasmid DNA purification kit (NucleoGen Biotech, Siheung, Korea). Standard procedures were used for restriction endonuclease digestion, ligation, agarose gel electrophoresis, gel blotting, $^{32}P$ labeling of probes, and hybridization (Sambrook and Russell, 2001). Primers used in this study (Table 1) were synthesized by the Bioneer oligonucleotide synthesis facility (Bioneer Corporation, Chungwon, Korea), dissolved in 100 μM sterilized water, and stored at −20°C. For inverse PCR, about 2 μg of fungal genomic DNA was digested with an appropriate restriction enzyme, purified by phenol extraction and ethanol precipitation, and set up for self-ligation in 400-μl reaction mixture (including 1× ligation buffer and 40 units of T4 ligase) at 16°C. After overnight ligation, the reaction solution was purified again and resuspended in 10 μl of sterilized water. Aliquots of 1 μl of the self-ligated DNA were used as the template for PCR with appropriate primer pairs. PCR was performed as described previously (45°C as an annealing temperature when using degenerate primers; Lee et al., 2001). For sequencing of PCR products cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA), the primers T7 and SP6 were used, and further sequencing was performed using the primers (Table 1) derived from the previously determined sequences.

**Vector construction.** A fungal transforming construct for targeted gene deletion was made using a modified DJ-PCR

**Table 1.** Primers used in this study

| Number[a] | Primer name | Primer sequences[b] (5'-3') | bp position[c] |
|---|---|---|---|
| 1 | ARG2-5 | GGNGANTAYGARGGNGGNGCNAT | 3786-3809 |
| 2 | ARG2-3i | AARTACCAYTTRTTNACNGGRT | 3999-4020 |
| 3 | ARG2-3ii | GGNGTNGTCCARAACATNGYCCA | 4062-4048 |
| 4 | ICaARG-F1 | AAGTGGTGGAGTTGCAGATGTGGTTTTC | 3914-3941 |
| 5 | ICaARG-R1 | ACCGGTCTTATACGCAGTCTCTTCATCCA | 3835-3863 |
| 6 | ICaARG-R2 | GCCGTGCTTCTGATCCTGTAGGGTGCGTGAG | 3249-3279 |
| 7 | ICaARG-F3 | CTGGGTATCTCTGAGTAAGGTAAG | 4532-4556 |
| 8 | CaARG2-5for | CTGGGAGCGATGGTTGAGCAC | 135-155 |
| 9 | CaARG2-5revtail | <u>ACCTCCACTAGCTCCAGCCAAG</u>CAAAAAGATCCTGAATATGTTGTAA | 1060-1083 |
| 10 | CaARG2-3fortail | <u>TATGAAAATTCCGTCACCAG</u>ACTGGCTTACTTGTGGGACTTA | 4373-4394 |
| 11 | CaARG2-3rev | AAAGGCGGCGGCGATCAAGAAAATAGG | 6258-6284 |
| 12 | hyg-5for | CTTGGCTGGAGCTAGTGGAGGT | |
| 13 | hyg-3rev | GGCTGGTGACGGAATTTTCATA | |
| 14 | CaARG-5nest | AAGTTTCTGCTGGGATGCGGTTGC | 176-199 |
| 15 | CaARG-3nest | GATCTCATAGGCCTTCTTGTGGT | 6068-6090 |
| 16 | CaARG-7for | GGCGGGAGCGTATGCGGAGTTGA | 1108-1120 |
| 17 | CaARG-7rev | TCTCGATTTTTGATTTCTTTTGTT | 4275-4298 |

[a] corresponding to the primers shown in Figs. 2 and 3.
[b] Abbreviations: R=A or G; Y=C or T; N=A, T, C, or G.
The underlined sequences in CaARG2-5revtail and CaARG2-3fortail are complementary to the sequences of hyg-5for and hyg-3rev, respectively.
[c] Refer to the 6.3 kb-*CaARG2* region deposited in GenBank under Accession Number GQ429002.

method (Kim et al., 2007). DNA fragments corresponding to 5' (948 bp) and 3' (1911 bp) regions of the *CaARG2* gene were amplified from genomic DNA of JC24 using the primer pairs CaARG2-5for/CaARG2-5revtail and CaARG2-3fortail/CaARG2-3rev, respectively (Table 1). A 2.1-kb fragment containing the hygromycin B resistance gene (*hygB*) cassette was amplified from the plasmid DNA pBCATPH (Kim et al., 2007) using the primers hyg-5for and hyg-3rev (Table 1). Three amplicons were mixed in a 1:2:1 molar ratio and used as the template for a second round of PCR without primers. A 4.38-kb final fusion PCR product was amplified from the second PCR product using the nested primer pair CaARG-5nest and CaARG-3nest (Table 1). For genetic complementation, a 3.2-kb genomic region including the *CaARG2* open reading frame (ORF; 2.1 kb), its putative promoter (949 bp), and 3'-flanking sequence (120 bp) was amplified from genomic DNA of the JC24 strain using the primer pair CaARG-7for and CaARG-7rev (Table 1) and cloned into the pGEM-T Easy vector (Promega), yielding the plasmid pCaARG2.

**Fungal transformation, REMI procedure, and virulence test.** Fungal conidia harvested from PDA culture were spread on cellophane sheets placed on the surface of the PDA (or PDA supplemented with 2.5 mM arginine for a *CaARG2*-deleted strain) and grown for 20 h to achieve conidial germination. Young mycelia from germinating conidia were harvested from the cellophane sheets and incubated in 80 ml of 1 M $NH_4Cl$ containing lysing enzyme (20 mg/ml, catalog number L1412; Sigma, St. Louis, MO, USA). Protoplasts generated from the young mycelia of the *C. acutatum* wild-type JC24 or the *CaARG2*-deleted (Δ*CaARG2*) strain, TDCaARG2-3, were transformed by a polyethylene glycol (PEG)-mediated method as described previously (Chung et al., 2002). For the REMI procedure, pCaARG2 was linearized with *Apa*I or *Eco*RI, and added to the fungal protoplasts along with an excess volume (100 or 200 U) of each enzyme. Fungal transformants carrying *hygB* were selected on regeneration medium (1 g of yeast extract, 1 g of casein hydrolysate, 342 g of sucrose, and 16 g of agar per liter) supplemented with 300 μg/ml hygromycin B. For the new transformation system involving the arginine auxotrophic strain as a recipient, prototrophic transformants were selected on MM plates containing 1 M sucrose as the sole carbon source. For virulence tests, conidia harvested from PDA cultures grown for 7 days were suspended in sterile water at $10^6$ spore/ml and dropped onto detached pepper fruits (cv. Nokkwang) as described previously (Kim et al., 2008).

**Nucleotide sequence accession number.** The sequence of a 6.3 kb-*CaARG2* region obtained from *C. acutatum* JC24 has been deposited in GenBank under Accession Number GQ429002.

## Results

**Amplification of the *CaARG2* region from *C. acutatum*.** To amplify part of the gene (designated *CaARG2*) encoding a putative *N*-acetylglutamate synthase required for arginine biosynthesis by *C. acutatum*, two pairs of degenerate primers (ARG2-5/ARG2-3i, and ARG2-5/ARG2-3ii; Table 1) were made corresponding to highly conserved regions of known fungal enzymes (Fig. 1A). The latter amplified the same expected size product (~300 bp) from both *C. acutatum* and *G. zeae* (Fig. 1B). Sequencing of the PCR product indicated that it was similar to fungal genes encoding *N*-acetylglutamate synthase. The remaining ORF of *CaARG2* and both the 5'- and 3'-flanking regions were amplified by several rounds of inverse PCR (Fig. 2). A BLAST search of the amplified 6.3-kb genomic region of *C. acutatum* indicated that it carries the entire *CaARG1* ORF and two other ORFs (*CaORF1* and *CaORF2*), all of which are similar to the corresponding region of the *G. zeae* (*Fusarium graminearum*) genome (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) in both genomic location and direction of transcription (Fig. 2). *CaARG2* encodes an ORF of 665 amino acids, interrupted by two putative introns, and shows a high degree of sequence similarity to *Neurospora crassa* ARG2 (Accession No. AAC37502), with 47% amino acid similarity. The deduced products of *CaORF1* and *CaORF2* are a putative 6-phosphogluconate dehydrogenase and a conserved hypothetical protein, respectively.

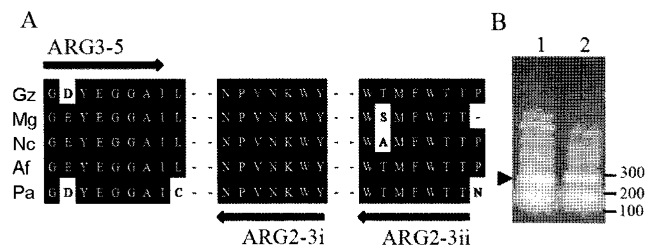


**Fig. 1.** PCR amplification of *CaARG2* from genomic DNA of *Colletotrichum acutatum* using degenerate primers. (A) Amino acid sequences of fungal N-acetylglutamate synthases used for design of degenerate primers (see Table 1). Conserved residues are indicated by a black background. Gz, *Gibberella zeae* (accession no: XP_382115); Mg, *Magnaporthe grisea* (XP_363581); Nc, *Neurospora crassa* (AAC37502); Af, *Aspergillus fumigatus* (XP_363581), Pa, *Podospora anserina* (XP_001910126). Dashes indicate discontinuity. Arrows indicate 5' to 3' direction of each primer. (B) Amplification of *CaARG2* using the primers ARG2-5 and ARG2-3ii. Lane 1, *G. zeae* strain Z03643; lane 2, *C. acutatum* JC24. The expected size bands are indicated by an arrowhead. DNA size markers (in bp) are indicated on the right of the gel.

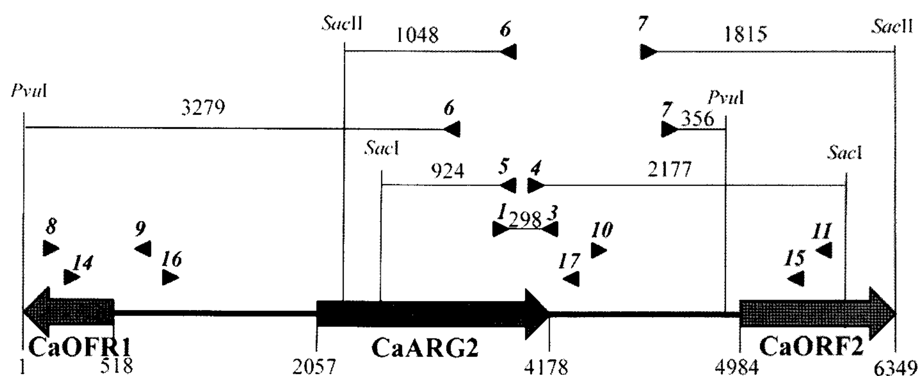


**Fig. 2.** PCR strategies for amplification of the 6.3 kb-*CaARG2* region from *C. acutatum* JC24. The solid arrows indicate the locations of ORFs and their transcription directions. The connecting lines represent noncoding DNA regions. The black arrowheads indicate PCR primer location and orientations. Bold italic numbers above the black arrowheads indicate the primer used (see Table 1 for the corresponding primer name and sequences). The numbers on the lines connected by the black arrowheads indicate the sizes (in bp) of amplified PCR products. The bp positions of each ORF are indicated by underlined numbers.

**Construction of the *CaARG2* auxotroph.** To confirm that *CaARG2* is essential for arginine biosynthesis in *C. acutatum,* we deleted a 3290-bp genomic region [including *CaARG2* and its 5' (974 bp) and 3' (195 bp) flanking sequences] from the JC24 strain using a targeted gene-replacement strategy (Fig. 3). Integration of the DJ-PCR product carrying the *hygB* gene cassette fused to the 5'- and 3'-regions flanking *CaARG2* via a double crossover resulted in the deletion of *CaARG2* (Fig. 3A). In total, only 15 out of 70 fungal colonies appeared on the regeneration medium supplemented with 300 μg/ml hygromycin B turned out to be stable hygromycin B-resistant transformants after two successive transfers on a selection medium, and 9 of the 15 transformants (60%) showed arginine auxotrophy on MM plates. The gene deletions in fungal transformants were verified by PCR amplification with two different primer sets (CaARG2-5for/CaARG2-3rev, and ICaARG-F1/CaARG-7rev; Table 1); the latter was specific to the *CaARG2* ORF and unable to amplify the corresponding *CaARG2* regions from the genomic DNAs of the Δ*CaARG2* strains, whereas it successfully amplified *CaARG2* from JC24 and the Δ*CaARG2* strain complemented with an intact copy of *CaARG2* (Fig. 3B).

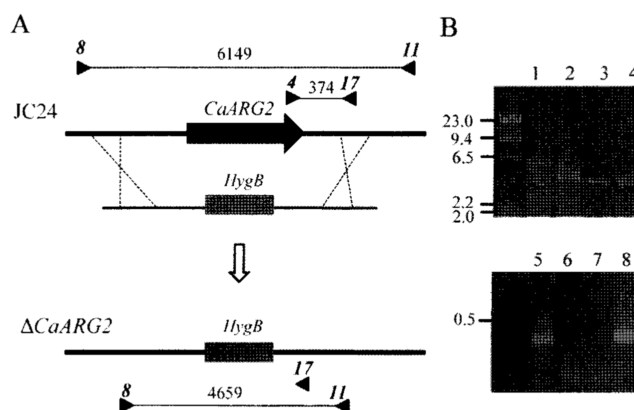


**Fig. 3.** Deletion of *CaARG2* from the genome of *C. acutatum* wild-type strain JC24. (A) Deletion strategy. JC24, genomic DNA of the wild-type strain JC24; Δ*CaARG2*, genomic DNA of the strain with *CaARG2* deleted. The positions and names of the primers used (Table 1) in PCR analysis (in panel B) are indicated by black arrowheads and bold italic numbers, respectively. Numbers on the connecting lines indicate the sizes (in bp) of amplified PCR products. (B) PCR analysis. PCR products amplified using the primer pairs 8/11 and 4/17 (Table 1) are shown in lanes 1-4 and lanes 5-8, respectively. Lane 1, JC24; lanes 2-4, Δ*CaARG2* strains, TDCaARG2-1, TDCaARG2-3, TDCaARG2-4; lane 5, JC24; lanes 6-7, Δ*CaARG2* strains, TDCaARG2-1, TDCaARG2-3; lane 8, a complemented Δ*CaARG2*::*CaARG2* strain, ComARG2-2. The sizes of lambda DNA digested with *Hin*dIII (in kilobase) are indicated on the left of the gels.

**Phenotypes of the Δ*CaARG2* strains.** All of the Δ*CaARG2* mutants examined differed from the wild-type progenitor strain JC24 in several traits, such as mycelial growth, conidiation, pigmentation, and virulence on host plant. The mutants showed a similar level of radial growth, with fewer aerial mycelia and less pigmentation compared to JC24 on nutrient-rich media, such as PDA or complete medium; they did not grow on MM (Fig. 4A). Conidial production was also severely affected even on PDA. The wild-type JC24 strain typically produced conidia 7 days after inoculation onto PDA and more than 90% of conidia germinated within 20 h after conidiation, whereas the conidiation rates of the Δ*CaARG2* mutants examined were reduced to ~50% of the wild-type level ($P$<0.05); conidial germination in the Δ*CaARG2* mutants was not significantly different from that in JC24. All of the growth defects in the Δ*CaARG2* mutants were recovered to the wild-type level when exogenous arginine was supplied, confirming their arginine auxotrophy (Fig. 4A). In addition, the mutants

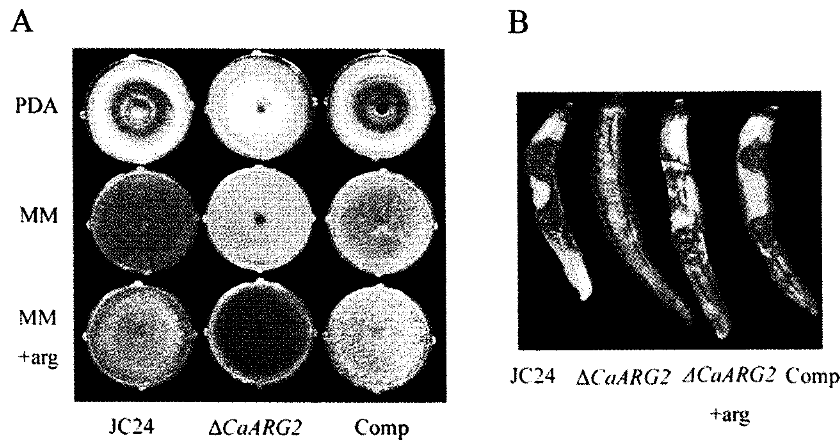


**Fig. 4.** Phenotypes of the Δ*CaARG2* strains. (A) Fungal mycelial growth on PDA, MM, and MM supplemented with 0.25 mM arginine (MM + arg). JC24, *C. acutatum* JC24 strain; Δ*CaARG2*, a Δ*CaARG2* strain, TDCaARG2-3; Comp, a complemented strain, ComARG2-2. (B) Fungal pathogenicity on pepper fruits. Δ*CaARG2* + arg, inoculated by the conidia of TDCaARG2-3 strain dissolved in 0.25 mM arginine solution.

showed almost no disease symptoms in pepper fruits by wound inoculation, whereas the JC24 strain and the Δ*CaARG2* strain, when supplied by arginine, caused typical anthracnose lesions 7 days after inoculation (Fig. 4B).

**Genetic complementation of Δ*CaARG2*.** The circular plasmid DNA pCaARG2 carrying the entire *CaARG2* ORF and its flanking sequences was introduced into the genome of the Δ*CaARG2* mutant (TDCaARG2-3). The flanking regions of *CaARG2* on pCaARG2 were 100 bp shorter than the deleted *CaARG2* region in TDCaARG2-3 (25 bp from the 5'-end and 75 bp from the 3'-end) to prevent homologous recombination between pCaARG2 and the TDCaARG2-3 genome. Putative transformants were selected for normal growth on MM plates supplemented with 1 M sucrose. As a negative control, no transforming vector was added to the same number of protoplasts. An average of two or three colonies appeared on each regeneration plate, while no colonies grew on negative control plates; the average transformation frequency (from three different experiments) was 15.6/μg DNA. PCR amplification indicated that the circular, nonhomologous plasmid was integrated into the genomic DNA of the auxotrophic recipient strain (data not shown). All of the prototrophic transformants examined were mitotically stable during five successive transfers on both nonselective (PDA) and selective medium (MM), and showed no differences in asexual growth (e.g., mycelial growth, conidiation, conidial germination) and pathogenicity compared to the wild-type JC24 strain (Fig. 4).

**REMI using the Δ*CaARG2* strain.** Protoplasts of the TDCaARG23 strain were transformed with linearized pCaARG2 in the presence of a restriction enzyme (*Apa*I or *Eco*RI) and selected for prototrophy as described above. All

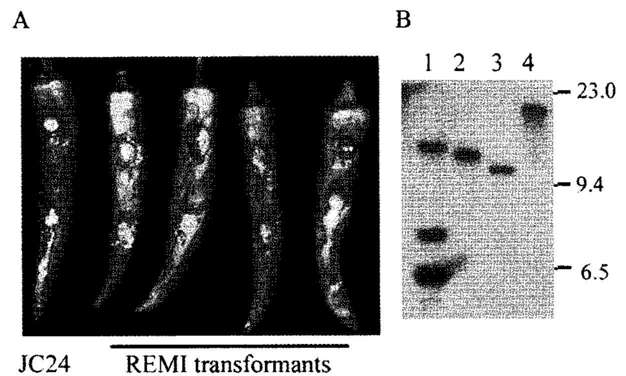


**Fig. 5.** (A) Pathogenicity of the randomly selected REMI transformants generated from *C. acutatum* JC24 on pepper. (B) Gel blot of the fungal genomic DNAs (lanes 1-4) digested with *Xba*I whose recognition site is absent in the vector DNA, probed with pCaARG2. The sizes of marker DNA (in kilobase) are indicated on the right of the blot.

transformants grew normally on MM; the 15 selected clones were as virulent as JC24 on pepper fruits (Fig. 5A). The REMI transformation frequency was varied among different kinds and concentrations of restriction enzymes added, but was not significantly higher than with the same circular plasmid without adding the restriction enzyme. DNA blotting analysis indicated that the transforming plasmid DNA integrated randomly into the genome as single or multiple copies (Fig. 5B).

## Discussion

The significance of the results presented in this study can be assessed both practically and fundamentally. First, the availability of the arginine auxotrophic mutant of *C. acutatum* will enable the development of both new trans-

formation and insertional mutagenesis systems in this fungus. Genetic transformation of *C. acutatum* had been accomplished using several conventional methods, such as electroporation, PEG-mediated protoplast transformation, and ATMT-mediated transformation (Chung et al., 2002; Horowitz et al., 2002; Karunakarn et al., 2008; Talhinhas et al., 2008), all of which are based on selection for resistance to antifungal compounds, mainly hygromycin B. However, the insensitivity of *C. acutatum* field isolates to this antibiotic requires unexpectedly high concentrations (up to 800 μg/ml, the concentration almost 10 times higher than that usually used in the selection in other fungi) to repress the growth of non-transformed cells (Nakaune and Nakano, 2007), which makes the entire transformation procedure expensive, and inefficient, sometimes leading to selection of abortive transformants at a high frequency (~80% in this study; Kim et al., unpublished data). Therefore, this new transformation system can be used as an alternative to the conventional transformation procedures for *C. acutatum*. In addition, it can also be applied to the ATMT procedure, which has been shown to work efficiently in *C. acutatum* (Karunakaran et al., 2008; Talhinhas et al., 2008; Xue et al., 2004).

Auxotrophic markers are widely used as alternatives to dominant selectable markers (e.g., drug resistance genes) for genetic transformation in filamentous fungi (Fincham, 1989). For example, the *argB* gene encoding ornithine carbamoyltransferase from *Aspergillus nidulans* (John and Peberdy, 1984; Johnstone et al., 1985) has frequently been used to transform arginine auxotrophic strains of *Aspergillus* species and many other filamentous fungi (Antal et al., 1997; Hahm and Batt, 1988; Parsons et al., 1987; Ventura et al., 1992; Xue et al., 2004). Although the auxotrophic markers have disadvantages compared to drug resistance markers in transformation systems (e.g., the need for mutant recipient strain constructions), several other auxotrophic markers, such as adenine, methionine, and uracil are still utilized for stable transformation of fungi (Fu et al., 2006; Han et al., 2004; Kim et al., 2007; Manczingerx et al., 1995; Sunagawa and Magae, 2002). In particular, auxotrophic markers are mainly used to transform *A. nidulans* because this model fungus displays a high level of resistance to hygromycin B, as does *C. acutatum*. However, the auxotroph-based transformation system for *C. acutatum* developed here is different from the previous methods in terms of the recipient strain and complementing gene. The auxotrophic recipient strains used in most studies described above were generated by UV mutagenesis, so their genomes still carry an endogenous allele of the gene responsible for the auxotrophic mutation, whereas that of *C. acutatum* is an isogenic strain lacking the *CaARG2* gene, generated by targeted gene deletion. In addition, the resulting prototrophic transformants in most cases were made by heterologous complementation (i.e., complementing genes were from heterologous origins), while prototrophy was obtained in this study by transformation with a homologous gene (*CaARG2*). However, this homologous selectable marker does not result in homologous integration of the vector into the fungal genome because the *CaARG2* region on the vector is shorter than the deleted *CaARG2* region in the auxotrophic recipient strain. These two points not only increase the frequency of stable transformants, but also prevent homologous integration of the transforming vector pCaARG2 into the recipient genome, which is very important for both ectopic insertion of the heterologous gene, when inserted into pCaARG2, and transformation-mediated random insertional mutagenesis. Moreover, the relatively high generation rate (~60%) of Δ*CaARG2* by a targeted gene replacement strategy compared to the previous study (You et al., 2007) suggests that gene deletion studies can be performed efficiently in *C. acutatum* using this system and that the same procedure can also be employed to generate new arginine auxotrophs in other *C. acutatum* genetic backgrounds.

The altered phenotypes of the Δ*CaARG2* strains demonstrated that arginine biosynthesis is involved in several important traits of *C. acutatum*, such as mycelial growth, conidiation, and virulence on host plants, similar to the arginine auxotrophs of other fungi (Kim et al., 2007; Namiki et al., 2001; Xue et al., 2004). Despite the similarity of the altered phenotypes between these auxotrophic mutants, note that an exogenous supply of arginine was not able to recover conidiation of the Δ*CaARG2* strains to the wild-type level. Therefore, conidia of a Δ*CaARG2* strain should be grown on cellophane sheets on PDA supplemented with arginine to obtain a sufficient volume of young mycelia from germinating conidia. This suggests that downstream arginine biosynthetic steps after that catalyzed by the CaARG2 product (*N*-acetylglutamate synthase) cannot be restored by direct arginine supply, and are involved in normal conidiation. In addition, it also suggests that the formation of melanized appressoria of the Δ*CaARG2* strain on the plant surface, which is essential for the infection process, may also be affected (Sreenivasaprasad and Talhinhas, 2005). If this is the case, it could be one of the direct explanations for the non-pathogenicity of the Δ*CaARG2* strains on pepper fruit. However, further investigations are required to confirm this possibility. The wild-type level of pathogenicity in the complemented Δ*CaARG2* strains (REMI transformants) indicated that random ectopic insertions of the intact copy of *CaARG2* are sufficient to recover the ability of the nonpathogenic Δ*CaARG2* strain to cause disease on pepper fruits, which is desirable in using the Δ*CaARG2* strain as a recipient for random insertional

mutagenesis to investigate the pathogenicity of *C. acutatum*. However, note that positional effects of *CaARG2* gene insertion may occur on the virulence of complemented transformants, as seen in the case of *Candida albicans*, in which the activity of the auxotrophic marker (*URA3*) is loci-dependent to some extent and has an influence on fungal virulence (Staab and Sundstrom, 2003).

In summary, this is the first report on the generation of an arginine auxotroph in *C. acutatum* as a recipient host for genetic transformation and REMI, which are efficient and economic, and will be useful in large-scale genomic studies (e.g., gene disruptions and random insertional mutagenesis) of *C. acutatum*, especially when its complete genome sequence becomes available. All of the arginine auxotrophic strains of *C. acutatum* and the plasmid DNA pCaARG described in this paper will be made available upon request.

## Acknowledgments

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