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SHORT COMMUNICATION

# Simple transformation of the rice false smut fungus *Villosiclava virens* by electroporation of intact conidia

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**Abstract** A simple procedure is reported for transformation of the rice false smut fungus *Villosiclava virens* (anamorph: *Ustilaginoidea virens*) using electroporation of intact conidial cells. The transformation vector pCB1004eGFP was constructed with a green fluorescent protein (eGFP) gene under a constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene of *Cochliobolus heterostrophus*. When a linearized vector was applied, eGFP-expressing transformants were successfully acquired. An inoculation test in rice plants showed that the eGFP-expressing transformants were able to form rice false smut balls.

**Keywords** Hygromycin B · Infection · Rice disease · Secondary conidia · *Ustilaginoidea virens*

False smut is a globally and economically important disease in rice. The causal fungus is *Villosiclava virens* (Nakata) E. Tanaka & C. Tanaka (Ascomycota, Clavicipitaceae), and its anamorph is *Ustilaginoidea virens* (Cooke) Takahashi (Tanaka et al. 2008). The teleomorph has been assigned the names *Claviceps virens* Sakurai ex Nakata and *Claviceps oryzae-sativae* Hashioka because the teleomorphic characteristics of *U. virens* are similar to those of *Claviceps* (Hashioka 1971). However, several molecular phylogenetic studies have revealed that *Ustilaginoidea* is not congeneric with *Claviceps* (Bischoff et al. 2004; Tanaka and Tanaka 2008).

The false smut balls are formed in the place of a rice grain and consist of a powdery mass of verrucose and thick-walled dark green conidia. These thick-walled conidia are borne pleurogenously on hyaline spore-bearing hyphae with short sterigmata (Takahashi 1896), and they germinate and form secondary conidia in liquid media and on plate media. The secondary conidia are hyaline and globose to subglobose.

Despite a number of studies, the infection process of this fungus is still poorly understood. Recent molecularly based studies have detected this fungus in the field and the host (Zhou et al. 2003; Ashizawa et al. 2010). In attempts to reveal the relationship between this fungus and its rice host, reporter genes are of obvious use. Previous studies have reported transformation of this fungus with the green fluorescent protein gene (*gfp*) using an *Agrobacterium*-mediated method (Zhang et al. 2006). However, the transformation procedure is problematic and time consuming. An alternative transformation system is needed that will facilitate further investigations of this fungus.

In this study, we employed electroporation of intact secondary conidial cells to easily transform the rice false smut fungus. Electroporation has been used to transfer DNA into many organisms. Usually, electroporation procedures for fungi require protoplasts or germinating conidia (Richey et al. 1989; Ozeki et al. 1994; Robinson and Sharon 1999; Dobrowolska and Staczek 2009). However, a simple electroporation procedure has been developed using intact yeast cells or conidia of *Neurospora crassa* Shear & B.O. Dodge (Gray and Brendel 1992; Vann 1995). Because the secondary conidia of *V. virens* look like yeast and are readily available, we focused on those present in our fungus.

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The cultures of false smut fungus were isolated from rice false smut balls collected at rice paddies in Kanazawa, Ishikawa, Japan, on September 5, 2007 (38°36′N, 41°136′E). The cultures were deposited under the identifier MAFF240420 in the Ministry of Agriculture, Forestry, and Fisheries Genebank at the National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan. To simplify the experimental procedures used in this study, we used a conventional medium, YT medium, consisting of 0.1% (w/v) yeast extract, 0.1% (w/v) tryptone, and 1% (w/v) glucose.

The transformation vector pCB1004eGFP (Fig. 1) was constructed by inserting a green fluorescent protein gene (*egfp*) into a pCB1004 plasmid under a constitutive promoter of a gene that encodes glyceraldehyde-3-phosphate dehydrogenase (*gpd*) amplified from *Cochliobolus heterostrophus* Drechsler HITO7711 (Saitoh et al. 2010). The pCB1004 plasmid contained an *Escherichia coli* Castellani & Chalmers hygromycin B phosphotransferase gene (*hph*) controlled by the *Aspergillus nidulans* G. Winter tryptophan synthase (*trpC*) promoter as a fungal selection marker (Carroll et al. 1994). Hygromycin B is a widely used selection marker in the transformation of fungi (Nakazawa et al. 2009; Yi et al. 2009; Kano et al. 2010). No terminator is present in the downstream region of *egfp* and *hph* genes because it is unnecessary for function. The plasmid also contained a chloramphenicol resistance gene for bacterial selection.

The minimal inhibitory concentration of hygromycin B for rice false smut fungus was determined by inoculating the secondary conidia in 1.5% agar containing YT medium supplemented with 0, 25, 50, or 100 μg/ml hygromycin B. Growth was totally inhibited at 50 μg/ml. On the basis of these tests, 100 μg/ml was chosen for the selection of resistant colonies.

To produce secondary conidia in the culture, the fungus was incubated in 50 ml YT medium at 25°C on a rotary shaker (180 rpm) in a 300-ml conical flask for several days until the beginning of sporulation. Mycelia with germinating secondary conidia were transferred into a 50-ml conical tube and homogenized with a Polytron mixer (Kinematica, Switzerland). The homogenized fungal culture was filtered through a Miracloth (Calbiochem, San Diego, CA, USA) under vacuum to remove the mycelia. The filtrate was washed twice with distilled water and once with cold 1 M sorbitol. Secondary conidia cells were collected by centrifugation. By counting the cell population with a Thoma hematometer, the conidial concentration was estimated and adjusted to $1.0 \times 10^9$ cells/ml in cold 1 M sorbitol.

We performed electroporation under 15 sets of conditions with circular and linear plasmids. The transformation protocol using electroporation was based on the methods described by Robinson and Sharon (1999) with several

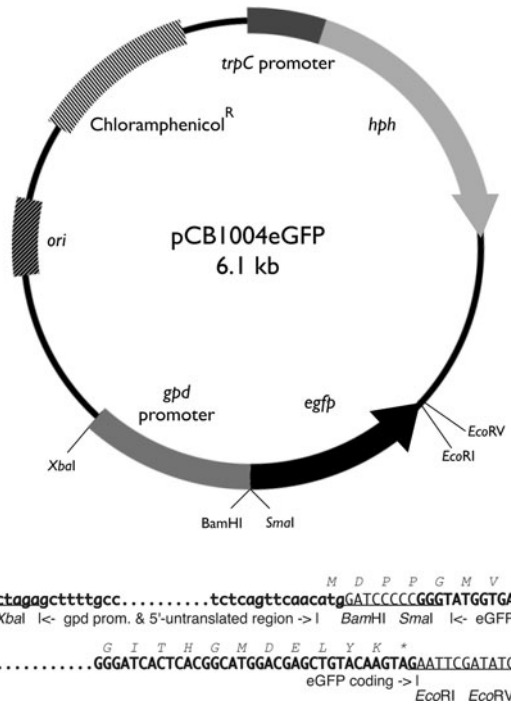


**Fig. 1** Plasmid pCB1004eGFP map. A red-shifted mutant green fluorescent protein (GFP) cDNA (*egfp*) from pBAGFP (Kimura et al. 2001) was fused to the promoter and 5′-untranslated region of *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*; GenBank Acc. No. X63516). The nucleotide sequence of polymerase chain reaction (PCR) products and junctions were experimentally confirmed by dideoxy sequencing. *Underlined sequences*, restriction enzyme recognition sites in pCB1004; *oblique letters*, predicted amino acid residues of eGFP in this construct; *bold lowercase letters*, PCR product amplified from *C. heterostrophus gpd* gene; *bold capitals*, PCR product from *egfp* in pBAGFP; *hph*, *Escherichia coli* hygromycin B phosphotransferase gene; *trpC*, *Aspergillus nidulans* tryptophan synthase gene

modifications. The pCB1004eGFP plasmid was linearized with *Eco*RI. First, 1 μg of plasmid was added to the intact secondary conidial cell suspension in 40 μl cold 1 M sorbitol. The resulting suspension was then transferred to a cold 0.2-cm electroporation cuvette, and each cuvette was placed on ice. The conidia were subjected to a high-voltage electric pulse using a Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA). The voltage ranged from 1.0 to 2.0 kV in 0.25-V increments, and the parallel resistance was set at 200, 400, and 800 Ω. Capacitance was held constant at 25 μF, and the time constant varied between 5 and 20 ms. Following the electroporation pulses, 1 ml cold 1 M sorbitol was immediately added to the cuvette. The suspended conidia were placed into Petri dishes. Then, 10 ml molten (about 50°C) YT agar medium containing 100 μg/ml chloramphenicol was poured and mixed thoroughly with the conidial suspension. The plates were incubated at 25°C overnight and overlaid with 10 ml molten YT agar medium containing 200 μg/ml hygromycin

B and 100 μg/ml chloramphenicol. The combined medium eventually contains about 100 μg/ml hygromycin B. After 1 week, colonies were selected using a sterile needle and transferred to fresh YT plates containing 100 μg/ml hygromycin B. The colonies were screened and selected by eGFP fluorescence using epi-fluorescence microscopy. Colonies displaying eGFP expression and resistance to hygromycin B were counted as successful transformants.

The rice false smut fungus was successfully transformed with electroporation of intact secondary conidial cells. When a circular plasmid was applied, no transformant was obtained. When a linearized plasmid was applied, transformants were obtained under the following conditions: 1.50 kV, 800 Ω and 1.75 kV, 800 Ω. In both conditions, the average numbers of transformants obtained from each cuvette were 3.8 ± 2.3/μg plasmid DNA. No significant number of transformants was obtained under the other conditions. The experiments were repeated five times. Successful transformation showed that both the *A. nidulans trpC* promoter and *C. heterostrophus gpd* promoter work in this fungus.

The level of eGFP fluorescence varied among transformants. We obtained 25 transformants that have relatively strong and even eGFP green fluorescence when excited by blue light (Fig. 2). Although this fungus exhibits yellow autofluorescence when excited with blue light (Fig. 2d), the green fluorescence of eGFP and autofluorescence are obviously discriminated.

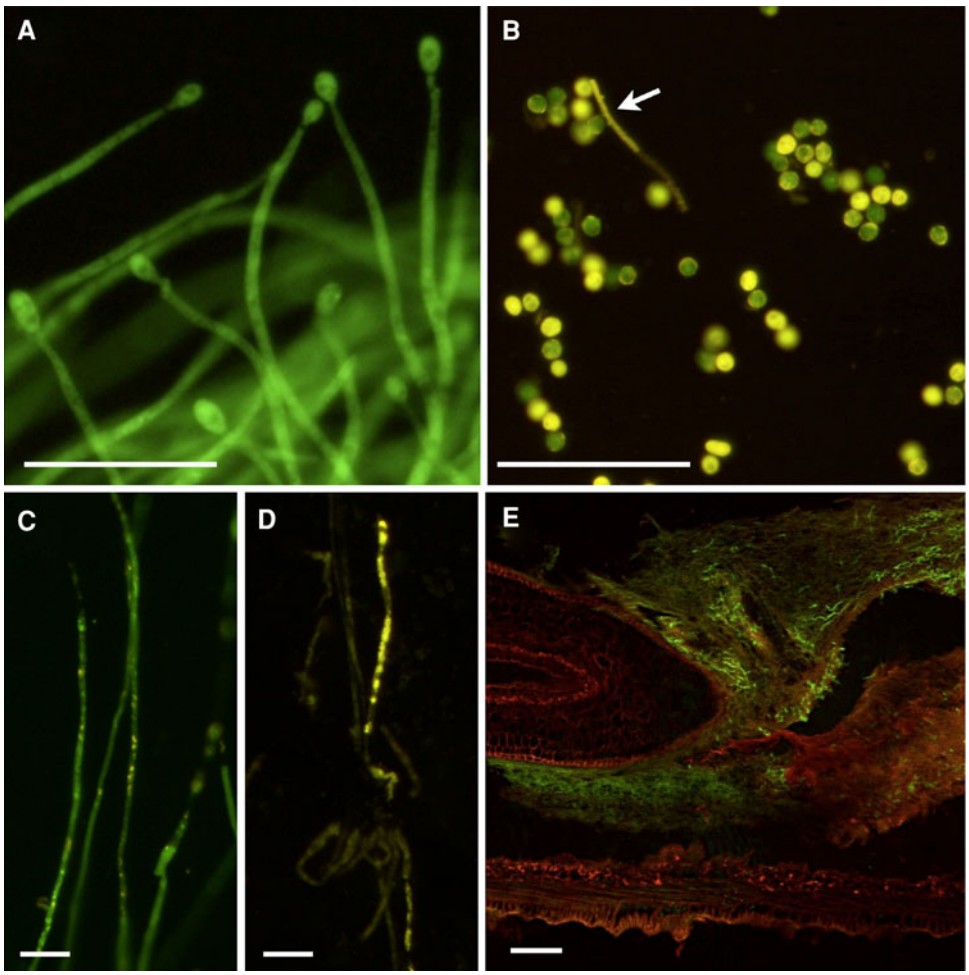


**Fig. 2** Fluorescence images of the rice false smut fungus. *Green* indicates eGFP expression in the transformant. **a**–**d** Epifluorescence images when excited by blue light. The images were acquired using an Eclipse E-800 microscope (Nikon, Tokyo, Japan) equipped with a 40× lens (Plan Fluor; numerical aperture, 0.75). **a** Secondary conidia of transformant. The conidia are holoblastically and sympodially produced at the apex of each conidiophore cells. **b** Thick-walled conidia of transformant. Thick-walled conidia are pleurogenous and born on a conidiophore (*arrow*). *Yellow* shows autofluorescence of the fungus. **c** Hyphae of transformant. **d** Autofluorescence of wild-type hyphae. **e** Confocal fluorescence image of section of rice false smut ball that was formed on rice inoculated with the transformant. Image was acquired using a confocal laser-scanning microscope (LSM510 META version 3.2; Zeiss, Fotografieren, Germany) attached to a microscope (Axioplan2; Zeiss) equipped with a 40× lens (Apochromat; numerical aperture, 1.2). Multitrack laser scanning (*line mode*) was used to avoid fluorescence overlapping. *Red* shows autofluorescence of rice tissue. *Bars* **a**, **b** 50 μm; **c**, **d** 10 μm; **e** 100 μm

To confirm the presence of the *hph* gene and to determine the copy number, Southern blot analysis was performed for six selected transformants expressing eGFP (Fig. 3). For Southern blot analysis, genomic DNA was extracted from the cultured mycelia of the transformants. Southern blot analysis was performed using a partial *hph* gene as a probe. Samples of total genomic DNA (~20 μg) were double-digested with *Eco*RI and *Eco*RV because these restriction enzymes cut once at an adjacent site in pCB1004eGFP but do not cut the *hph* gene (Fig. 1). The number of hybridizing bands indicated the copy number integrated into the genomic DNA. Multicopy integration was observed in lanes 3 and 6, and single-copy integration was observed in lanes 1, 2, 4, and 5, resulting in bands of different sizes. These banding patterns indicated that the *hph* gene was randomly integrated in the genomic DNA.

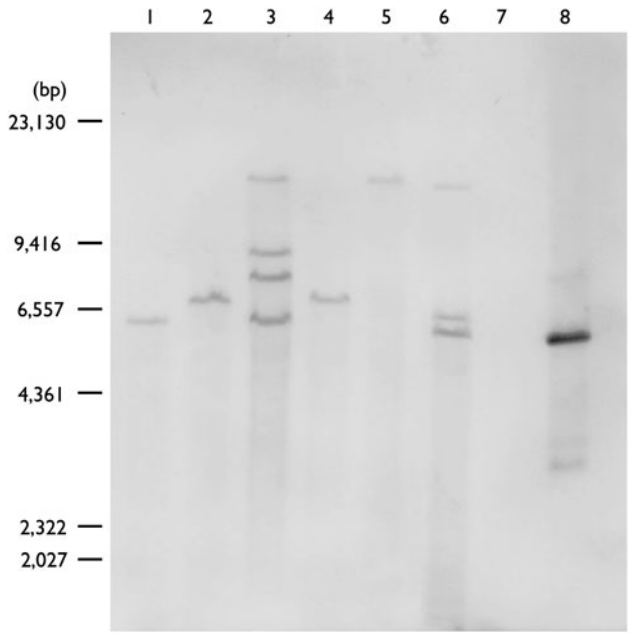


**Fig. 3** Southern blots of wild type and transformants of rice false smut fungus. Genomic DNAs were double-digested with *Eco*RI and *Eco*RV. Both restriction enzymes cut once at an adjacent site of the *hph* gene in pCB1004eGFP but do not cut the *hph* gene (Fig. 1). The membrane was probed with a partial *hph* fragment. The 722-bp fragment of the *hph* gene was amplified with primers hph1 (5′-CAG CGA GAG CCT GAC CTA TTG C-3′) and hph2 (5′-TCT ACA CAG CCA TCG GTC CAG AC-3′). Probe labeling and hybridization utilized the Alkphos Direct Labeling Module (GE Healthcare, UK). Samples were electrophoresed on 0.7% (w/v) agarose gels and transferred onto nylon membranes (Hybond-N+; GE Healthcare). Filters were reacted with a stabilized substrate for alkaline phosphatase (Western Blue; Promega, WI, USA). *Lanes 1–6*, independent transformants; *lane 7*, wild type (negative control); *lane 8*, pCB1004eGFP digested with *Eco*RI (positive control). Hybridization signals showed multiple integration of the *hph* gene in *lanes 3* and *6* and suggested single-copy integration of the *hph* gene in *lanes 1*, *2*, *4*, and *5*. No hybridization signal was observed in the negative control (*lane 7*). *Numbers on the left* indicate molecular sizes estimated by a *Hin*dIII-digested λDNA marker. *bp*, base pair

Inoculation tests were performed to confirm that the transformants maintained their pathogenicity. Random integration of foreign DNA in the genome raises the possibility that the integrated DNA could have disrupted pathogenicity-related genes. Two transformants that showed integration of one copy of the *hph* gene in their genomic DNA were selected for the inoculation tests. Booting stage rice (Nipponbare) plants were inoculated with the transformants, using the method described by Fujita et al. (1989) and Ashizawa et al. (2011) with several modifications. Briefly, a secondary conidial suspension was injected into the rice panicles at the booting stage. The inoculated rice plants were incubated at 15°C for 2 days in the dark in a humidified environment, and then at 26°C for 5 days in the dark in a humidified environment. The plants were then exposed to a normal light (~50,000 lux) and dark cycle. The experimental plants were conditioned in a growth chamber (LH-220S-HH; NK System, Osaka, Japan) with an ultrasonic humidifier. Inoculation tests showed that the eGFP-expressing transformants parasitized the rice and formed rice false smut balls (see Fig. 2e). These results indicate that eGFP-expressing transformants can be used to elucidate interactions between this fungus and host rice plants. Further studies are needed to reveal the infection process of this fungus.

The inoculated transformants formed false smut balls on the rice plants, and eGFP expression was detected from the fungal hyphae in the plants (see Fig. 2). These results show that the eGFP gene was stably integrated into the fungal genome. The preparation of intact secondary conidia for transformation is a simple procedure compared with preparation of a protoplast or of germinated conidia for electroporation. Furthermore, electroporation of intact conidia is an easy and rapid procedure to perform, compared with procedures that require the use of *Agrobacterium* or polyethylene glycol. Our results suggest that electroporation of secondary conidia is a viable and easy alternative procedure for transformation of rice false smut fungus.

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## References

Ashizawa T, Takahashi M, Moriwaki J, Hirayae K (2010) Quantification of the rice false smut pathogen *Ustilaginoidea virens* from soil in Japan using real-time PCR. Eur J Plant Pathol 128:221–232

Ashizawa T, Takahashi M, Moriwaki J, Hirayae K (2011) A refined inoculation method to evaluate false smut resistance in rice. J Gen Plant Pathol 77:10–16

Bischoff J, Sullivan R, Kjer K, White J Jr (2004) Phylogenetic placement of the anamorphic tribe Ustilaginoideae (Hypocreales, Ascomycota). Mycologia 96:1088–1094

Carroll A, Sweigard J, Valent B (1994) Improved vectors for selecting resistance to hygromycin. Fungal Genet Newsl 41:22

Dobrowolska A, Staczek P (2009) Development of transformation system for *Trichophyton rubrum* by electroporation of germinated conidia. Curr Genet 55:537–542

Fujita Y, Sonoda R, Yaegashi H (1989) Inoculation with conidiospores of false smut fungus to rice panicles at the booting stage. Ann Phytopathol Soc Jpn 55:629–634

Gray M, Brendel M (1992) A ten-minute protocol for transforming *Saccharomyces cerevisiae* by electroporation. Curr Genet 22:335–336

Hashioka Y (1971) Rice diseases in the world. VIII. Diseases due to Hypocreales, Ascomycetes. (Fungal diseases, no. 5). Riso 20:235–258

Kano S, Kurita T, Kanematsu S, Morinaga T (2010) *Agrobacterium tumefaciens*-mediated transformation of the violet root-rot fungus, *Helicobasidium mompa*, and the effect of activated carbon. Mycoscience 52:24–30

Kimura A, Takano Y, Furusawa I, Okuno T (2001) Peroxisomal metabolic function is required for appressorium-mediated plant infection by *Colletotrichum lagenarium*. Plant Cell 13:1945–1957

Nakazawa T, Kaneko S, Murata H, Kamada T, Shishido K (2009) The homologue of *Lentinula edodes ctg1*, a target for CDC5 and its interacting partner CIPB, from *Coprinopsis cinerea* is involved in fruiting-body morphogenesis of *C. cinerea*. Mycoscience 50:331–342

Ozeki K, Kyoya Fujii F, Hizume K, Kanda A, Hamachi M, Nunokawa Y (1994) Transformation of intact *Aspergillus niger* by electroporation. Biosci Biotechnol Biochem 58:2224–2227

Richey M, Marek E, Schardl C, Smith D (1989) Transformation of filamentous fungi with plasmid DNA by electroporation. Phytopathology 79:844–847

Robinson M, Sharon A (1999) Transformation of the bioherbicide *Colletotrichum gloeosporioides* f. sp. *aeschynomene* by electroporation of germinated conidia. Curr Genet 36:98–104

Saitoh Y, Izumitsu k, Morita A, Shimizu K, Tanaka C (2010) ChMCO1 of *Cochliobolus heterostrophus* is a new class of metallo-oxidase, playing an important role in DHN-melanization. Mycoscience 51:326–336

Takahashi Y (1896) On *Ustilago virens* Cooke and a new species of *Tilletia* parasitic on rice plant. Bot Mag Tokyo 10:16–20

Tanaka E, Tanaka C (2008) Phylogenetic study of clavicipitaceous fungi using acetaldehyde dehydrogenase gene sequences. Mycoscience 49:115–125

Tanaka T, Ashizawa T, Sonoda R, Tanaka C (2008) *Villosiclava virens* gen. nov., com. nov., teleomorph of *Ustilaginoidea virens*, the causal agent of rice false smut. Mycotaxon 106:491–501

Vann D (1995) Electroporation-based transformation of freshly harvested conidia of *Neurospora crassa*. Fungal Genet Newsl 42A:53

Yi R, Tachikawa T, Mukaiyama H, Mochida Y, Ishikawa M, Aimi T (2009) DNA-mediated transformation system in a bipolar basidiomycete, *Pholiota microspora* (*P. nameko*). Mycoscience 50:123–129

Zhang Z, Du X, Chai R, Mao X, Qiu H, Wang Y, Wang J, Sun G (2006) *Agrobacterium tumefaciens*-mediated transformation of the pathogen of *Ustilaginoidea virens*. Chin J Rice Sci 20:440–442 (in Chinese)

Zhou Y, Izumitsu K, Sonoda R, Nakazaki T, Tanaka E, Tsuda M, Tanaka C (2003) PCR-based specific detection of *Ustilaginoidea virens* and *Ephelis japonica*. J Phytopathol 151:513–518