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Full Length Research Paper

Development of a transformation method for the nematophagous fungus *Dactylellina cionopaga*

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Dactylellina cionopaga **is a trapping fungus that produces adhesive columns and a two-dimensional network. The factors that influence protoplast preparation and regeneration of** ***D. cionopaga*** **were analyzed, and poly ethylene glycol (PEG)-$CaCl_2$- or** ***Agrobacterium tumefaciens*****-mediated transformation was conducted to develop a transformation system for the fungus and provide a tool for studying the function of nematode infection-related genes. The results indicate that between $4.175\pm1.025\times10^6$ and $3.08\pm1.4\times10^7$, protoplasts/ml were obtained under optimized conditions and that the protoplasts could be regenerated on potato dextrose agar (PDA), RA and IM regeneration media.** ***D. cionopaga*** **transformation using PEG-$CaCl_2$ or** ***A. tumefaciens*** **displayed 4.2 to 11 resistant colonies/µg DNA using 10^6 protoplasts and 180-270 resistant colonies using 10^6 conidia. Molecular analysis and microscopy of randomly selected transformants showed that the target genes were integrated into the genome of** ***D. cionopaga*** **and that green fluorescence could be detected in transformants containing pK2-BarGFP, which carried a glufosinate ammonium resistance gene and the enhanced green fluorescence protein gene. The methods used in this study for protoplast preparation and convenient** ***Agrobacterium*****-mediated transformation of** ***D. cionopaga*** **represent useful tools for genetic research on this nematophagous fungus. This is the first report on protoplast generation and transformation of** ***D. cionopaga*****.**

Key words: Nematophagous fungi, *Dactylellina cionopaga*, *Agrobacterium tumefaciens*-mediated transformation, PEG-$CaCl_2$-mediated transformation, protoplast preparation and regeneration.

INTRODUCTION

Nematophagous fungi have attracted attention because of their potential roles as biocontrol agents for phytonematodes. The development of effective biocontrol agents depends on a thorough understanding of the mechanisms of nematode infection by nematophagous fungi. Serine protease genes have been frequently identified as having roles in the parasitism of nematophagous fungi. Currently, at least 16 serine proteases from trapping fungi, endoparasites or opportunisticspecieshave been purified and characterized (Yang et al., 2005; Wang et al., 2006, 2007, 2009). The introduction of multiple copies of genes encoding serine proteases led to increased nematicidal activity by *Arthrobotrys oligospora* and *Paecilomyces lilacinus* (Åhman et al., 2002; Yang, 2011). In infections by fungi showing egg parasitism, chitinase, which degrades chitin, was involved in egg parasitism; it was present on the surface of nematode eggs and inhibited their growth (Gan et al., 2007 Tikhonov et al., 2002). In addition, Ahrén et al. (2005) found a number of genes that were

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differrentially expressed in adhesive knobs versus mycelium and might play a role in the parasitism of *Monacrosporium haptotylum*. These genes included morphogenesis and cell polarity genes such as *rho*1, *rac*1 and *ras*1, a *rho* GDP dissociation inhibitor (*rdi*1) and genes involved in stress responses, protein synthesis and degradation, transcription and carbon metabolism.

However, the genes implicated in the *in vivo* pathogenicity of nematophagous fungi await functional identification. The development of a transformation system for nematophagous fungi is the first step toward functional identification of infection-related genes *in vivo* and transformation of candidate genes into fungi to improve the efficiency of nematode biocontrol. Thus far, available transformation systems in adhesive network-producing fungi have been limited to *A. oligospora*, *Monacrosporium sphaeroides* and opportunistic *P. lilacinus* (Åhman et al., 2002; Xu et al., 2005; Yang et al., 2011). Transformation methods for fungi include PEG-$CaCl_2$-mediated transformation, restriction enzyme-mediated introduction (REMI), lithium acetate treatment, electroporation, *Agrobacterium tumefaciens*-mediated transformation (ATMT) and biolistic transformation. PEG-$CaCl_2$-mediated transformation was traditionally applied to fungi, while ATMT was recently found to be efficient for various fungi (Gao and Yang, 2004). Different fungus transformation methods often use the same recipients and have similar initial steps; thus, a fungus without a known procedure of introducing foreign genes could be assayed simultaneously (or in a reasonable time) using various transformation approaches (Sánchez-Torres et al., 1994).

Dactylellina cionopaga is an adhesive column-developing fungus that is a parasite of *Meloidogyne javanica* and *Heterodera schachtii* (Khan et al., 2006; Jaffee and Muldoon, 1995). It could easily be developed for commercial purposes due to its rapid growth. *Caenorhabditis elegans* can be trapped by *D. cionopaga*, and *D. cionopaga-C. elegans* may provide a model system for studying the interaction between trapping fungi and plant parasitic nematodes. A cDNA library of *D. cionopaga* was constructed, and the secretory protein-encoding genes were trapped and sequenced (Duan, 2007). In the present study, the effects of different factors on protoplast preparation and regeneration of *D. cionopaga* were analyzed. PEG-$CaCl_2$- or *A. tumefaciens*-mediated transformation was then performed to develop an effective and convenient transformation system for this fungus. To our knowledge, this is the first reported transformation of *D. cionopaga*.

MATERIALS AND METHODS

Strains and plasmids

D. cionopaga AS 3.6776 (SQ27-3) isolated from Panax root-zone soil in the Yunnan province of China, was stored on potato dextrose agar (PDA) slants at 4°C for the experiments (Drechsler, 1950; Yang, 2006). *A. tumefaciens* AGL-1 and *Escherichia coli* containing pAN7-1 or pK2-BarGFP were stored in 15% glycerol at -80°C. Plasmid pK2-BarGFP was a construct containing the herbicide resistance gene *bar* and the enhanced green fluorescent protein gene *egfp*; it was generated by inserting a *bar*::*gfp* fusion gene into the *Eco*RI and *Hind*III sites of the binary vector pPK2, from which an *hph* cassette was deleted (Zhang et al., 2010; Jin et al., 2008; McCluskey, 2003).

Protoplast preparation and regeneration

D. cionopaga was activated by culture on PDA plates at 25°C for 5 days, ground by mortar and pestle and inoculated into a synthetic broth (glucose 10 g/L, peptone 5 g/L, yeast extract 2 g/L, KH_2PO_4 1.52 g/L, NaCl 5 g/L, $MgSO_4 \cdot 7H_2O$ 0.52 g/L, pH 5.5 to 6.5). The fresh filaments that grew for 20, 24, 36, 39, 45 or 48 h were collected by filtering through a piece of cheesecloth and cleaned with sterile water. Wet mycelia were first treated with β-mercaptoethanol at 30°C for 20 min and then cleaned sequentially with sterile water and lysis buffer before lysis. The protoplasts of *D. cionopaga* were released with enzyme system I, consisting of 10 mg/ml lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich (Shanghai) Trading Co., Ltd.), 1.25 µL/ml β-glucuronidase (Sigma-Aldrich (Shanghai) Trading Co., Ltd.) and 1 µL/ml lyticase (Tiangen Biotech (Beijing) Co., Ltd.), or enzyme system II, consisting of 10 mg/ml lysing enzyme and 5 mg/ml cellulase R-10 (Yakult, Japan).

To assay the influence of pH on protoplast formation by *D. cionopaga*, enzyme system I was used in 50 mmol/L potassium acid phthalate (pH 4.0), 18 mmol/L MES (pH 5.3), 10 mmol/L sodium phosphate (pH 6.4), 10 mmol/L Tris-HCl (lysing buffer I; pH 7.5) or 10 mmol/L Tris-HCl (pH 8.3), with 1.2 mol/L sorbitol as the osmotic pressure stabilizer. To test different digestion temperatures, enzyme system I in lysing buffer I was used at 28, 31, 34 and 37°C. Both pH and temperature were tested using 39 h-old hyphae. After 2 h, the digestion was filtered through 2 layers of lens paper to remove residual filaments, and the protoplasts were precipitated by centrifuging at 700 *g* for 10 min, washed twice with STC (1.2 mol/L sorbitol, 10 mmol/L Tris-Cl, 20 mmol/L $CaCl_2$, pH 7.5) and resuspended in 100 µL STC. Regeneration was performed by mixing the protoplasts with PDA containing 1 mol/L sucrose, with regeneration agar (RA) medium (when used as a selective medium containing hygromycin: potato dextrose broth 10 g/L, yeast extract 2 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, $NaNO_3$ 3 g/L, sorbitol 218.6 g/L, agar 3.5 g/L, pH 5.5 to 6.5; when used as a selective medium containing glufosinate ammonium: glucose 10 g/L, $NaNO_3$ 6 g/L, KCl 0.52 g/L, $MgSO_4 \cdot 7H_2O$
0.52 g/L, KH_2PO_4 0.25 g/L, sorbitol 218.6 g/L, agar 3.5 g/L, pH 5.5 to 6.5) or with induction medium (IM) with 1.2 mol/L sorbitol (Bundock et al., 1995). To calculate the regeneration rate, the protoplasts were diluted and incubated with water for 30 min. The frequencies of regeneration were computed following the equation $Rpf = (Cr - Ch) / Np$, where *Rpf* is the regeneration rate of the protoplasts, *Cr* is the number of colonies on the regenerative medium, *Ch* is the number of colonies on the regenerative medium on which the protoplasts treated by sterilized water were spread, and *Np* is the number of protoplasts (Li et al., 2008).

PEG-$CaCl_2$- or *A. tumefaciens*-mediated transformation

The resistance levels of *D. cionopaga* to hygromycin and glufosinate ammonium were assayed on PDA or on RA and IM, respectively. For PEG-CaCl2-mediated transformation, 5 to 10 µg linearized pAN7-1 (GenBank ID: Z32698) and 2.5 µL carrier DNA (25 µg salmon sperm DNA) were added to 1.0 to 2.5×10^6 protoplasts in 100 µL STC and incubated on ice for 30 min. Then 25 µL 60% PEG-4000 (50 mmol/L $CaCl_2$, 10 mmol/L Tris-Cl pH 7.5)

was then added, and the mixture was incubated on ice for 10 min. An additional 500 µL of 60% PEG-4000 (50 mmol/L $CaCl_2$, 10 mmol/L Tris-Cl pH 7.5) was slowly added, and the mixture was incubated at room temperature for 10 min. The solution was diluted with 2 ml of RM (RA without agar) and mixed with 50 ml of molten PDA or RA (42°C). Aliquots (10 ml) were inoculated on plates and incubated at 25°C for 16 to 24 h, and then 10 ml of PDA or RA containing 2.5 µg/ml hygromycin B, 100 µg/ml ampicillin, and 0.5% agar was overlaid on the medium. After 3 to 10 days, the putative resistant colonies were picked up onto new selective media.

The fresh hyphae of *D. cionopaga* grown on PDA for 5 days were also transferred using cellophane to water agar and cultured continually for 5 days to generate conidia. The conidia of *D. cionopaga* were separated by vortexing the culture with glass beads in sterile water for 20 min, centrifuged at 3811 *g* for 20 min and suspended in 100 µL of IM to a concentration of about 1×10^6 conidia/ml. For ATMT transformation of pK2-BarGFP, the plasmid was first transformed into *A. tumefaciens* AGL-1 by the freezing-melting method. A transformant of *A. tumefaciens* AGL-1 was stored at -80°C and used to mediate the transformation of *D. cionopaga*. ATMT was performed using the modified method of Gao and Yang (2004). Briefly, *A. tumefaciens* AGL-1 carrying pK2-BarGFP was grown in yeast extract broth (YEB; sucrose 5 g/L, yeast 1 g/L, peptone 10 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, pH 7.0) at 28°C until the culture reached an OD_{660} of about 0.6 and resuspended into IM with 10 mmol/L glucose and 200 µmol/L acetosyringone (AS) to an OD_{660} of 0.15. The virulence of the AGL-1 cells was induced by 4 h of incubation at 180 rpm at 28°C. Equal volumes of the AGL-1 cells and previously prepared hyphae or conidia of *D. cionopaga* were mixed and spread on cellophane on IM plates with 5 mmol/L glucose and 200 µmol/L AS. After co-cultivation at 28°C for 48 h, the mixture was scraped off, suspended in sterile water and transferred to new IM plates with 50 µg/ml glufosinate ammonium and 500 µg/ml cephalosporin, but without 5 mmol/L glucose, to kill *A. tumefaciens* and screen the putative resistant colonies. When protoplasts of *D. cionopaga* were utilized as the starting material, the plates used for co-cultivation were not covered by cellophane and were instead directly overlaid with selective RA or IM containing glufosinate ammonium and cephalosporin.

Molecular analysis of transformants

Genomic DNA of *D. cionopaga* transformants was extracted with cetrimonium bromide (CTAB) following the method of Cubero et al. (1999). PCR was performed to identify transformants using primers specific to the terminator of *trpC* (forward: 5'-gcccggatccacttaa-cgttactgaaat-3'; reverse: 5'-ggccggatccaagaaggattacctctaa-3') and to the hygromycin B phosphotransferase gene (*hph*). Reported primers specific to *hph* (Zhong et al., 2007; Aimi et al., 2005; Weld et al., 2006) were used in addition to primers designed in this study (forward: 5'-gcataacagcggtcattg-3'; reverse: 5'-cggcatctactctattcctt-3'). For spot blot analysis, an approximately 1000-bp DNA fragment, obtained by digestion of pK2-BarGFP with *Not*I/*Bam*HI and agarose gel purification, was used as a template for the probe labeling of the phosphinothricin N-acetyltransferase gene (*bar*) and the enhanced green fluorescence protein gene (*egfp*). The procedures for probe labeling and spot blot analyses were conducted according to the manufacturer's instructions for the digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science).
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Microscopy

The filaments of *D. cionopaga* transformed with pK2-BarGFP were observed with a confocal laser-scanning microscope. Fluorescence emission was examined with a krypton/argon laser. The excitation and emission wavelengths were set at 488 and 525 nm, respectively.

RESULTS

Factors affecting protoplast preparation and regeneration

Growth time, enzyme system, digestion temperature and pH were all assayed for their influence on protoplast formation by *D. cionopaga*. The number of protoplasts initially increased with mycelial age and then decreased when the filaments were cultured for longer than 39 h. The filaments cultured for 39 h at 25°C at 170 rpm in the synthetic broth medium produced $4.65\pm0.22\times10^6$/ml protoplasts with enzyme system I in lysing buffer I at 37°C; filaments cultured for 20, 24, 36, 45 and 48 h and treated identically produced $1.75\pm0.35\times10^6$, $2.5\pm0.35\times10^6$, $4.02\pm0.21\times10^6$, $1.1\pm0.05\times10^6$ and $1.68\pm0.43\times10^6$/ml protoplasts, respectively (Figure 1A). Two enzyme systems, both consisting mainly of lysing enzyme, were checked for protoplast release by *D. cionopaga*. Both enzyme systems I and II generated the same order of magnitude of protoplasts ($25.4\pm3.3\times10^6$ and $30.8\pm1.4\times10^6$/ml, respectively), but the number of protoplasts produced by enzyme system I was slightly greater (Figure 1B).

The effect of pH on protoplast preparation of *D. cionopaga* was also analyzed by dissolving enzyme system I in lysis buffers with different pH values and with 1.2 mol/L sorbitol as the osmotic pressure stabilizer. The results of this experiment showed the generation of $0.725\pm0.275\times10^6$/ml protoplasts in potassium acid phthalate buffer (pH 4.0), $1.025\pm0.075\times10^6$/ml in MES buffer (pH 5.3), $2.30\pm0.6\times10^6$/ml in sodium phosphate buffer (pH 6.4), $4.175\pm1.025\times10^6$/ml in Tris-HCl buffer (pH 7.5) and $1.75\pm0.55\times10^6$/ml in Tris-HCl buffer (pH 8.3) (Figure 1C). The effect of enzymolysis temperature on protoplast generation is presented in Figure 1D; elevated temperature enhanced the number of protoplasts to some extent, but reduced the yield. At the optimal 31°C treatment temperature, $4.9\pm0.2\times10^6$/ml protoplasts could be harvested, whereas $1.6\pm0.05\times10^6$/ml protoplasts were obtained with 37°C treatment. Thus, the optimized conditions for protoplast release of *D. cionopaga* were a 39 h culture followed by digestion with lysing enzyme, β-glucuronidase and lyticase at pH 7.5 and 31°C. The protoplasts harvested after digestion were regenerated on PDA, RA or IM regeneration media, and the regeneration rates were calculated. The regeneration frequency of *D. cionopaga* was 3.05±0.2% on PDA regeneration medium, whereas 0.11±0.02 and 0.07±0.01% were achieved on RA and IM regeneration media, respectively. No differences in morphology were found between the regenerated and wild-type colonies.

PEG-$CaCl_2$-mediated transformation of protoplasts

The hygromycin resistance of hyphae, conidia and

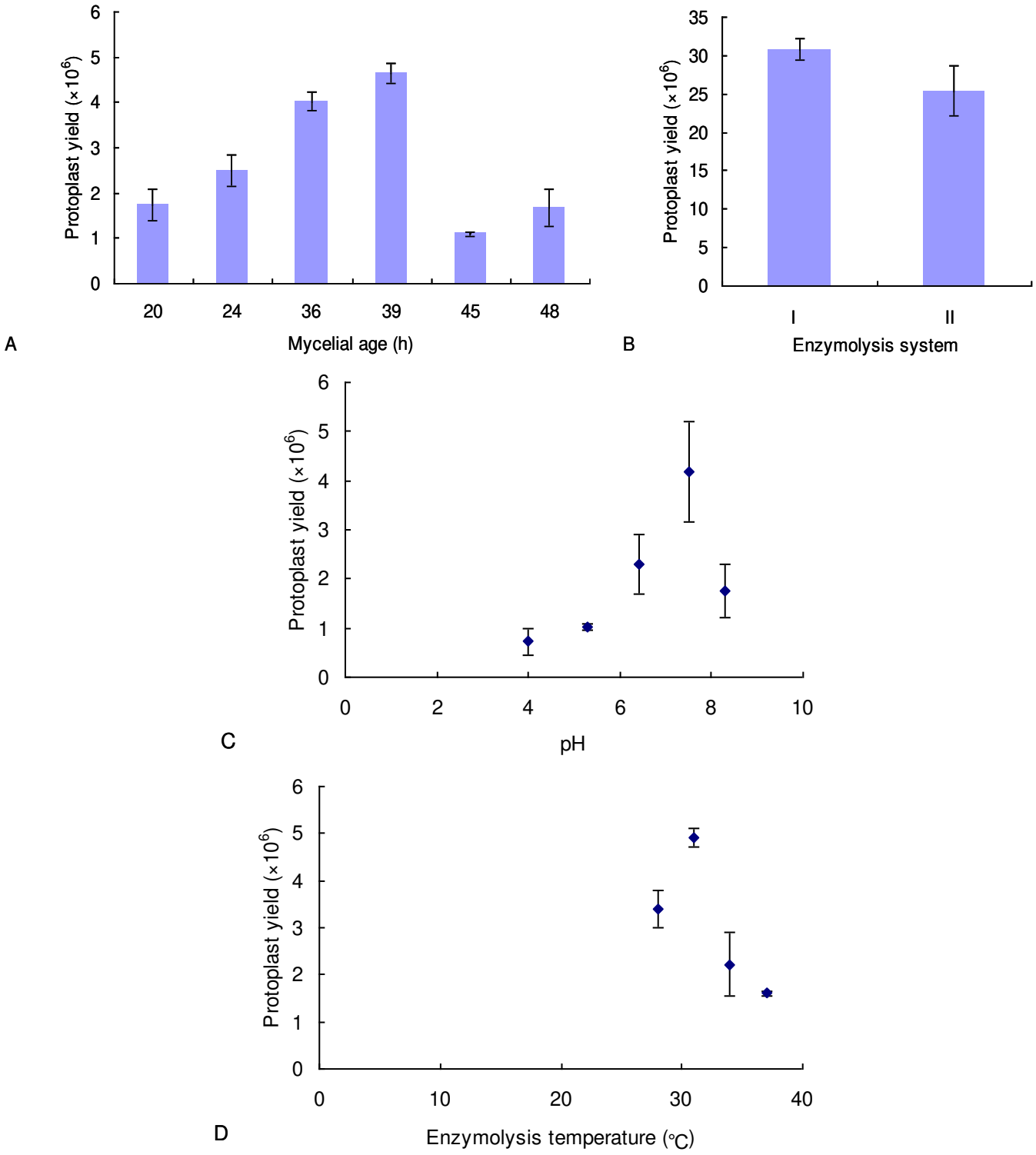


Figure 1. Effects of factors on protoplast yields. Error bars indicate standard error. **A**, Mycelial age, the protoplasts were generated at 37°C. **B**, Enzymolysis system, the protoplasts were formed from 39 h-old filaments at pH7.5 and at 37°C. **C**, pH, the protoplasts of 39 h-old filaments were prepared at 37°C. **D**, Enzymolysis temperature, the protoplasts of 39h-old filaments were released with lysing enzyme, β-glucuronidase and lyticase at pH7.5.

protoplasts of *D. cionopaga* was assayed on PDA. The growth of hyphae, conidia and protoplasts was completely inhibited by 2.0 µg/ml hygromycin B, and 2.5 µg/ml hygromycin B was thus used for transformant

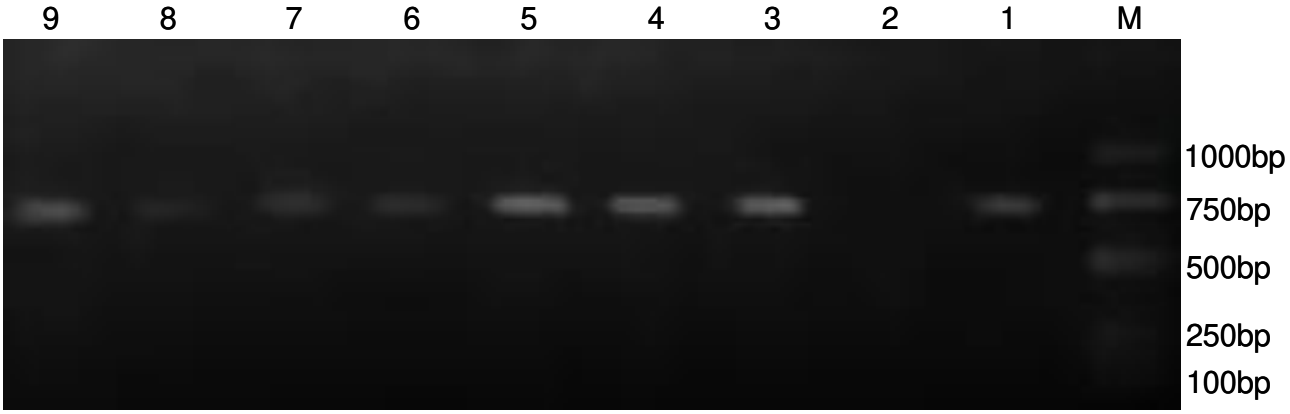


Figure 2. PCR analysis of the putative transformants achieved by PEG-$CaCl_2$ mediated transformation. Lane M, The molecular markers; lane 1, pAN7-1; lane 2, untransformed wild-type strain; lanes 3 to 9, the putative transformants. The vector pAN7-1 contains the hygromycin phosphotransferase (*hph*) construct under the control of the *Aspergillus nidulans gpdA* promoter and the *A. nidulans trpC* terminator.

screening to avoid false-positive colonies. The optimal protoplast preparation conditions, in which hyphae cultured for 39 h and pretreated with β-mercaptoethanol at 30°C for 20 min were digested with enzyme system I in lysing buffer I at 37°C for 1.5 to 2 h, were utilized for protoplast formation and subsequent transformation. The putative resistant colonies appeared on the regeneration media after 3 to 10 days and were then screened by several transfers onto selective media.

PCR using *hph*-specific primer sets designed in this study or previously reported was performed to identify the putative transformants. A weak band appeared in the negative control lane with wild-type *D. cionopaga* DNA, while obvious bands appeared in the lanes with putative transformant DNA. Primers (Trp-L and Trp-R2) specific to the terminator of *trpC* on pAN7-1 were additionally employed to identify the transformants (Figure 2). The transformation rate was calculated from the number of colonies on the selective plates, the quantity of plasmid DNA and the number of protoplasts used, and was obtained as 4.2 to 11 resistant colonies per µg DNA per 10^6 protoplasts.

A. tumefaciens-mediated transformation of protoplasts, filaments and conidia

A. tumefaciens-mediated transformation of protoplasts, filaments and conidia was conducted to develop a convenient transformation method for *D. cionopaga*. Plasmid pK2-BarGFP, containing *bar* (encoding the glufosinate ammonium resistance protein) and *egfp* (encoding green fluorescent protein), was used for ATMT of *D. cionopaga*. Filaments and conidia of *D. cionopaga* were resistant to glufosinate ammonium at 50 µg/ml on IM and the resistance of protoplasts was the same on both RA and IM regeneration media. ATMT produced no resistant colonies when protoplasts of *D. cionopaga* were used as the recipient. The putative transformants achieved by mycelium-based transformation could only form tiny colonies on the selective transferring medium whenever 39- or 60-h-old filaments were used, which suggested that those transformants were abortive. However, conidia of *D. cionopaga* were shown to be the most favorable starting material. Ten plates (Φ = 90 mm) of culture produced about 1×10^6 conidia in 100 to 200 µL IM, which was well suited for ATMT. In three independent experimental replicates, the transformation rate of ATMT, with a co-cultivation time of 48 h at 28°C, was 180-270 resistant colonies/10^6 conidia (Figure 3). The filament tips and conidia of the putative transformants were transferred to new selective medium and further screened.

To detect the mitotic stability of the selected transformants, they were also transferred four times on IM without glufosinate ammonium. After this treatment, the transformants still grew normally on the selective medium with 50 µg/ml glufosinate ammonium, indicating that those strains were stable through the mitotic cycle. In addition, the transformants were analyzed by spot blotting with a probe specific to *bar* and *egfp* and by microscopic observation. Spot blotting showed that *bar::egfp* was inserted into the genome of *D. cionopaga*, and exogenous *egfp* was microscopically visible in the transformants (Figure 4).

DISCUSSION

Among nematophagous fungi, transformation systems for *A. oligospora*, *M. sphaeroides* and *P. lilacinus* have been developed using successful protoplast preparation as their basis (Åhman et al., 2002; Xu et al., 2005; Yang et al., 2011). Protoplasts are used not only for transformation, but also for cell fusion, mutagenesis,

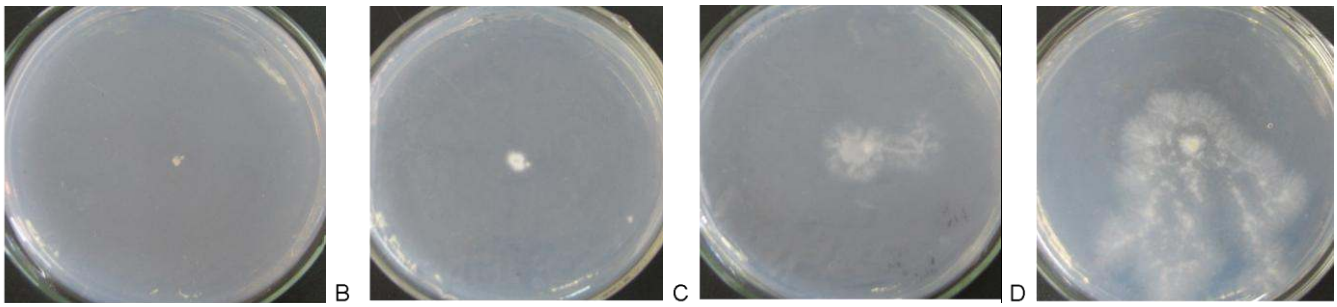
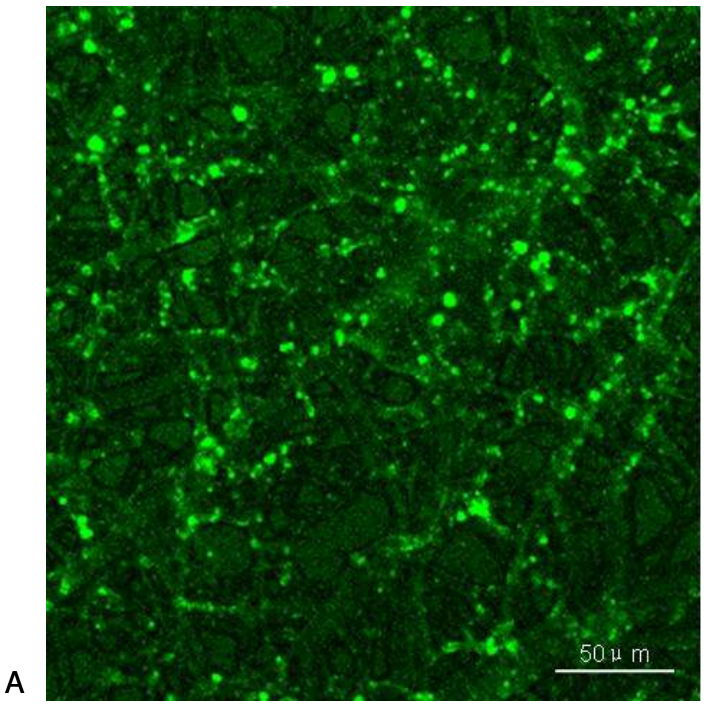


Figure 3. Colony morphology of *D. cionopaga* and the screened transformants on selective medium containing 50 µg/ml glufosinate ammonium. **A**, *D. cionopaga*. **B**, The putative transformants using hyphae as the recipient. **C and D**, The putative transformants using conidia as the recipient. Transformation was achieved by ATMT with pK2-BarGFP, which contains the fusion gene *bar::egfp* construct under the control of the *A. nidulans gpd* promoter (*PgpdA*) and the *A. nidulans trpC* terminator. The appearance of the cultures after 7 days is shown.



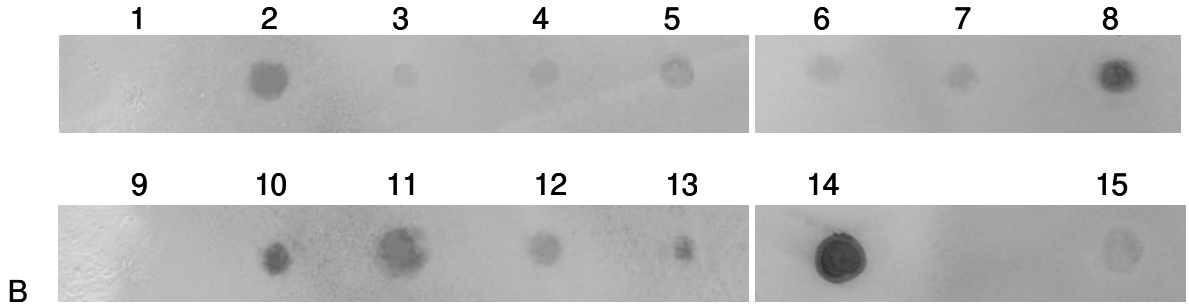


Figure 4. Analysis of *Agrobacterium tumefaciens*-mediated transformants. **A**, Filaments under fluorescence microscope. **B**, Spot blot. 1, untransformed *D. cionopaga*; 2 to 13, putative transformants; 14, pK2-BarGFP; 15, plasmids extracted from *A. tumefaciens* AGL-1 carrying the vector pK2-BarGFP by the method of alkaline lysis. Hybridization was performed with a probe homologous to *bar::egfp* from pK2-BarGFP and labeled by DIG with 1 µL DNA solution applied on a charged nylon membrane.

cytoplasmic organelle isolation, investigation of cell wall synthesis, synchronized cell growth, karyotype analysis and genome shuffling (Barrett et al., 1989). No universal protocol existed for protoplast preparation and regeneration of fungi. In this study, the conditions for protoplast preparation of *D. cionopaga* were optimized, and the regeneration frequencies on different media were computed. Under optimal conditions, between $4.175\pm1.025\times10^6$ and $3.08\pm1.4\times10^7$/ml protoplasts of *D. cionopaga* could be consistently achieved. The yield was similar to the presented protoplast yields of most fungi, such as *Rhizoctonia solani* (Liu et al., 2010), *Aspergillus niger* (de Bekker et al., 2009) and *Pleurotus ostreatus* (Peng et al., 1993), but was less than the 10^8/ml achieved using fungi such as *Metarhizium anisopliae* (Dhar and Kaur, 2009). The protoplasts obtained from *D. cionopaga* were differently sized, similar to those of *R. solani* (Liu et al., 2010), and could regenerate on all tested media, although with lower rates than most fungi, including *M. sphaeroides* (Xu et al., 2005), *Curvularia lunata* (Osiewaez and Weber, 1989) and *A. niger* (Kück et al., 1989). RA was employed for PEG-$CaCl_2$-mediated transformation to produce more consistent results than with the semi-synthetic PDA regeneration medium. The regeneration rate on PDA regeneration media was similar to those of a mycorrhizal fungus (Barrett et al., 1990) and *P. ostreatus* (Lau et al., 1985).

ATMT is a method that introduces exogenous DNA into the recipient with the aid of T-DNA in the plasmid. This approach requires co-cultivation of the recipient and *A. tumefaciens* containing a binary vector, and the transfer process requires induction of *vir* by AS, the efficiency of which is influenced by co-cultivation time and temperature, the particular *Agrobacterium* strain and the concentration and ratio of recipient and *Agrobacterium* cells (Combier et al., 2003; Kano et al., 2010). Successful ATMT of *Aspergillus* spp., *Fusarium venenatum*, *Colletotrichum gloeosporioides*, *Neurospora crassa*, and others has been reported (Sugui et al., 2005; de Groot et al., 1998). ATMT is more convenient compared to the laborious protoplast production, the purified and concentrated DNA preparation and the low transformation rate of PEG-CaCl2-mediated transformation. It was found that the gene replacement frequencies obtained by *Agrobacterium*-mediated transformation were 3- to 6-fold higher than the frequencies obtained with PEG-CaCl2-mediated protoplast transformation (Michielse et al., 2005). Different materials can be subjected to ATMT, although conidia are frequently favored. Research on *T. reesei* confirmed that protoplasts produced higher transformation frequency for ATMT (Yao et al., 2006). It was also shown that using filaments of *Agaricus bisporus* as the recipient could result in single-copy insertion in the genomic DNA (Mikosch et al., 2001).

However, no ATMT transformants of *D. cionopaga* with protoplasts or mycelia as the starting material have been screened. RA and IM regeneration media were used for ATMT of *D. cionopaga* protoplasts, the colony growth of which on rich medium could not be controlled by glufosinate ammonium. The regeneration rates of *D. cionopaga* on RA and IM were similar to those of the ectomycorrhizal fungi *Hebeloma cylindrosporum* in PPD liquid and *Laccaria laccata* S444 in PRM liquid (Barrett et al., 1989). The lower regeneration rate of protoplasts of *D. cionopaga* might be caused by the nutrient-poor IM and RA regeneration media, which may explain the failure of ATMT with protoplasts as the recipient. *D. cionopaga* produced relatively few conidia, but higher transformation efficiency offset this disadvantage. The concentration of conidia used in this study was lower than those of *Aspergillus fumigatus* and *Leptosphaeria* spp. (Sugui et al., 2005; Eckert et al., 2005) and comparable to *Beauveria bassiana* (Fang et al., 2004), but higher than that of *Helminthosporium turcicum* (Degefu and Hanif, 2003). *A. tumefaciens* AGL-1 was used in this study, which has often been more effective than other *Agrobacterium* strains (Khang et al., 2005). The concentration of AS that was necessary for the induction of the virulence genes in this study resembled that used for most fungi (Wang and Li, 2008; Sugui et al., 2005). The efficiency of ATMT for *D. cionopaga* was significantly higher than that of PEG-$CaCl_2$-mediated transformation. It was also higher than the transformation rate of *Penicillium digitatum* mediated by *Agrobacterium* (Wang and Li, 2008) and similar to that of *Trichoderma reesei* (Zhong et al., 2011), but lower than that of *H. turcicum* (Degefu and Hanif, 2003). T-DNA randomly integrates into the recipient genome.

Moreover, among the obtained ATMT transformants of *D. cionopaga*, it is interesting to note that some small, dry colonies with fewer aerial hyphae were found in addition to the loose diffusing colonies (Figure 4) that were almost identical to the wild-type strain. The mechanism underlying this phenomenon is not clear. In contrast, various transformant morphologies appeared because of random integration of foreign DNA into the genome of *V. dahliae* (Maruthachalam et al., 2011). No obvious side effects on growth appeared after ATMT of *T. harzianum* (Gao and Yang, 2004). Hygromycin B and glufosinate ammonium were used as selective markers for the transformation of *D. cionopaga*. The use of drug resistance markers does not require the genotype of the recipient to be known. However, one disadvantage of drug markers is that the resistance allele must be isolated so that the wild-type strain can be transformed to resistance, and the resistance allele may not show significant dominance over the wild-type allele, resulting in selection difficulties (Ruiz-Díez, 2002). Higher levels of resistance may depend on multicopy integration in the genome that commonly occurs in homologous recombination (Yao et al., 2006). The low hygromycin resistance of *D. cionopaga* was advantageous compared to the relative resistance to glufosinate ammonium; it offset the expense disadvantage of a drug resistance marker,

but required careful preparation of the selective medium to ensure a reasonable compound concentration for transformant selection. In addition, the *egfp* gene in the vector pK2-BarGFP provided a convenient way to screen transformants.

A series of transformation approaches have been developed for fungi. However, not all fungi can be easily transformed (Skory, 2002; Zhang et al., 2011). The use of multiple different transformation methods in parallel is a helpful way to improve the chances of successfully transforming fungi for which no established protocol exists; as the starting material might be the same across various transformation procedures, there is additional efficiency in a parallel approach. In this study, we presented the development of a transformation procedure for *D. cionopaga*, a species that produces relatively few conidia based on PEG-$CaCl_2$-mediated and *Agrobacterium*-mediated transformation methods that do not require special instruments. The loose mycelia used for protoplast preparation were obtained by inoculating pulverized filaments of *D. cionopaga* into a synthetic broth. This new transformation procedure might provide useful insights for the development of transformation protocols for other nematophagous or related fungi, thereby advancing genetic research in other fungi.

The development of *D. cionopaga* protoplast generation and ATMT methods provides a basis for useful tools in studying the parasitism of this fungus through mutant libraries, identification of gene function and improved pathogenicity. Moreover, the strain produced in this work carrying exogenous *egfp* could be used to observe *D. cionopaga* during the infection of nematodes and to analyze its ecological behavior in the environment. The infection-related genes cloned from a cDNA library of *D. cionopaga* now await identification with the new transformation system presented in this work.

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