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Transformation of *Corynespora cassiicola* by *Agrobacterium tumefaciens*

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ABSTRACT

The fungus causing target spot disease, *Corynespora cassiicola* (Berk. & M. A. Curtis) C. T. Wei, poses an increasing threat to watermelon (*Citrullus lanatus*), muskmelon (*Cucumis melo*), and cucumber (*Cucumis sativus*); the most economically important cucurbit crops grown in China. An understanding of the molecular mechanisms underlying the pathogenicity of *C. cassiicola* is essential for the development of new strategies to control this disease-causing fungus. *Agrobacterium tumefaciens*-mediated transformation (ATMT) might be useful to obtain transformants of *C. cassiicola*, for the ultimate identification of genes involved in pathogenicity. In the present work, we established and optimized an ATMT protocol using *A. tumefaciens* strain AGL-1 carrying the vector pATMT1 for *C. cassiicola*. Efficiency of ATMT was 102–148 transformants per 10^6 conidia and successive subculturing of transformants on non-selective and selective media demonstrated that the integrated transfer (T)-DNA was stably inherited in *C. cassiicola* transformants. The integration of the hygromycin B phosphotransferase (*hph*) gene into *C. cassiicola* was validated by PCR and Southern blot analyses, which revealed that nearly 90 % of the transformants contained single-copy T-DNA. The transformants with altered phenotypes were characterized. Three of these transformants completely lost pathogenicity and other three showed strongly impaired pathogenicity relative to the *Cc*-GX strain on muskmelon leaves. These results strongly suggest that ATMT may be used as a molecular tool for identifying genes relevant to pathogenicity in the fungus *C. cassiicola*, an emerging threat to several agronomically important plants in China.
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1. Introduction

Corynespora cassiicola (Berk. & M. A. Curtis) C. T. Wei (1950) causes a disease commonly known as target spot disease, which results in lesions on flowers, stems, fruits, and roots (Silva et al., 1995, 2000), and has been reported as a fungal pathogen in 390 tropical and subtropical host plants (Farr and Rossman, 2016). *C. cassiicola* also causes serious economic losses in cucurbit crops, including watermelon, muskmelon (Wang et al., 2013; Wei, 1950), and particularly cucumber (Blazquez, 1967; Li et al., 2008), which are three of the most economically important cucurbit crops grown in China. *C. cassiicola* was first described in Europe more than 100 y ago, on cucumber, and reportedly caused angular, necrotic, brown leaf spots, and was thus designated as “leaf fire” disease (Güssow, 1906; Quanjer, 1908). Once considered a weak pathogen that affects cucumber production in China, *C. cassiicola* was first reported in Liaoning province in 1993 (Fang and Fu, 1994). Since then, target spot incidence on cucumber increased until it occurred frequently in Chinese cucumber growing regions due to the over utilization of high-yield susceptible cultivars (Li et al., 2008). *C. cassiicola* was also considered a weak pathogen of muskmelon in China, however, a report published in 2013 highlighted its increased prevalence (Wang et al., 2013) and ability to cause severe target spot disease in this plant, as well as the possibility of this pathogen emerging as a potential risk for cucurbit crops.

Given the economic importance of this disease on cucurbit crops, in-depth studies on the detection and identification of the pathogen and on epidemic characteristics and infection control strategies have been conducted (Dixon et al., 2009; Qi et al., 2011; Shimomoto et al., 2011). However, useful information on the biochemical and molecular analyses of plant–pathogen interactions is very limited, and the genetic mechanisms underlying the progression of plant diseases are still not clear. Specifically, the pathogenicity mechanisms of *C. cassiicola* remain poorly understood and need to be further clarified. Given the economic

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importance and potential impact of this fungal pathogen, the development of novel strategies and optimal methodologies for the effective management of *C. cassiicola* are urgently needed.

The *Agrobacterium tumefaciens*-mediated transformation (ATMT) technique has been widely used in randomized mutagenesis experiments to determine which genes are responsible for the pathogenic interaction between fungi and insects, plants, mammals, and even other fungi (Leclerque et al., 2004; Mullins and Kang, 2001; Sugui et al., 2005; Talhinhas et al., 2008). Compared to other transformation methods, ATMT offers are several benefits largely due to its higher transformation efficiency and percentage of single-copy patterns of T-DNA added into the fungal recipient, which enables the tagged genes to be distinguished more easily than in multi-copy integration (Islam et al., 2012; Jiang et al., 2013; Michielse et al., 2008). Moreover, because ATMT can transform conidia and other fungal material directly, ATMT does not required protoplast preparation, which is complicated and laborious. Therefore, in recent years, ATMT has been feasibly applied to develop random mutants during the transformation of filamentous fungi that cause plant diseases, as this approach is easy and renewable (Michielse et al., 2005; Sugui et al., 2005). However, only one study reported on the transformation of *C. cassiicola*, which was isolated from rubber trees (*Hevea brasiliensis*) by PEG-mediated protoplasts transformation (Liu et al., 2014). Until now, no studies had reported on the successful application of ATMT to *C. cassiicola*. Thus, we aimed to establish an optimized ATMT method to generate random insertion transformants in *C. cassiicola* in the present study.

2. Materials and methods

2.1. Microbial strains and growth conditions

C. cassiicola was isolated as a plant pathogen from muskmelon in Wuming, Guangxi Province, China. The *C. cassiicola* strain (*Cc*-GX) used as the recipient in this study was maintained on potato dextrose agar (PDA) at 25 °C. The AGL-1 strain of *A. tumefaciens* carrying the binary vector pATMT1 was the T-DNA donor for fungal transformation. The pATMT1 carried a T-DNA harbouring the resistance gene: hygromycin B (*hph*), and was remodelled based on the backbone of pCAMBIA1300 (CAMBIA) (Zheng et al., 2011). The bacterial AGL-1 strain was maintained on Luria Bertani (LB) media supplemented with 50 μg mL^{-1} kanamycin (Sigma, China) and 25 μg mL^{-1} rifampicin (Sigma, China) at 28 °C.

Induction medium (IM) [K_2HPO_4 2.05 g, KH_2PO_4 1.45 g, NaCl 0.15 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 6H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.0025 g, $(NH_4)_2SO_4$ 0.5 g, glucose 2 g, glycerol 5 mL, MES 7.8 g, and 1000 mL distilled water, adjusted to pH 5.6 by using 1 M HCl or 1 M NaOH] was prepared for *A. tumefaciens* precultivation. Co-cultivation medium (CM, similar to IM but containing 1 g instead of 2 g glucose, and with 15 g agar added for solid medium) was prepared for co-cultivation. Selective PDA medium [containing 100 μg mL^{-1} hygromycin B (Roche, Germany), 400 μg mL^{-1} cefotaxime (Sigma, China), and 200 μg mL^{-1} timentin (Solarbio, China)] was applied to eliminate *A. tumefaciens* and to selected putative transformants.

The phenotypic characteristics of transformants cultured on PDA, including colony morphology and growth rates were observed and recorded during the growing period. The transformants with phenotypes contrasting those of the parental strain were selected for the pathogenicity assay.

2.2. Hygromycin B sensitivity of C. cassiicola

The *Cc*-GX strain was grown on PDA containing various concentrations of hygromycin B (0, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120 μg mL^{-1}), and incubated at 25 °C, in darkness, for 7 d to monitor its sensitivity level.

2.3. Transformation of C. cassiicola by A. tumefaciens

To explore the optimal conditions for the transformation of C. cassiicola by *A. tumefaciens*, the *A. tumefaciens* strain AGL-1 (containing the binary vector pATMT1) was applied to *C. cassiicola* using the ATMT protocol as described by Michielse et al. (2008), with slight modifications. The AGL-1 strain containing the binary vector pATMT1 was grow on LB agar plates supplemented with 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} rifampicin at 28 °C for 48 h. A single colony was selected from the LB plates and then cultured for 12 h in 5 mL LB medium containing 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} rifampicin at 28 °C and shaken at 11 g. Samples of the culture (2 mL) was centrifuged at 20 °C and 7378 g for 1 min, and the obtained pellet was washed twice with 1 mL IM. The cell pellet was resuspended in IM medium with or without 200 μM acetosyringone (AS) (Sigma, USA). The bacterial solution was then cultured in the dark for 5–6 h at 28 °C and under continuous shaking at 11 g.

Cc-GX strain conidia were harvested from colonies grown on PDA plates for 20 d at 25 °C with a photoperiod of 16:8 h (light:dark) and surfaced sterilised with 0.01 % sterile Tween-20 solution. The *C. cassiicola* conidia suspension was percolated through three layers of lens paper to remove the mycelia, and then centrifuged for 10 min at 10 625 g. The resulting pellet was cleaned twice with sterile water, and resuspended in IM to achive a concentration of 1×10^6 conidia mL^{-1}. Haemocytometers were used to calculate the number of conidia.

A 100 μL conidial suspension mixed with a 100 μL bacterial culture was spread on the 0.45 μm pore nitrocellulose filter laid on the CM with or without 200 μM AS. The co-cultivation was conducted in the dark. The nitrocellulose filters were then moved to PDA plates in the presence of hygromycin B (100 μg mL^1) as a screening agent for putative transformants, and 200 μg mL^{-1} timentin and 400 μg mL^{-1} cefotaxime were applied to eliminate *A. tumefaciens*. The plates were cultured for 5–7 d at 25 °C and the mycelia of putative transformants were removed with inoculating needles and transferred to PDA containing 100 μg mL^{-1} hygromycin B in a new round of validation tests. The transformants were subsequently purified with single-spore isolation or hyphal tip isolation to ensure genetic uniformity.

The effects of pH (4.4, 4.7, 5.0, 5.3, 5.6, 5.9, 6.2 and 6.5) on the transformation efficiency were determined by inoculating 100 μL of *C. cassiicola* conidia (1×10^6 conidia mL^{-1}) and 100 μL of *A. tumefaciens* (OD_{600} = 0.5) on CM media at 23 °C for 65 h with 200 μM AS. To confirm the effects of temperature (15, 17, 19, 21, 23, 25 and 27 °C) on the transformation efficiency, 100 μL samples of *C. cassiicola* conidia (1×10^6 conidia mL^{-1}) were cocultured with 100 μL of *A. tumefaciens* (OD_{600} = 0.5) on CM media (pH = 5.6) for 65 h with 200 μM AS. The effects of co-cultivation time (51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, and 70 h) on the transformation efficiency were confirmed by inoculating 100 μL of *C. cassiicola* conidia (1×10^6 conidia mL^{-1}) and 100 μL of *A. tumefaciens* (OD_{600} = 0.5) on CM media (pH = 5.6) at 23 °C with 200 μM AS.

To identify the effects of *A. tumefaciens* cell concentration on the transformation efficiency, 100 μL of *C. cassiicola* conidia (1×10^6 conidia mL^{-1}) were cocultured with 100 μL of *A. tumefaciens* (OD_{600} = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8) on CM media (pH = 5.6) at 23 °C for 65 h with 200 μM AS. Experiments were performed in triplicate.

2.4. Confirmation of hph in the transformants

Ten transformants were randomly selected and individually cultured on PDA to identify the mitotic stability of transformants.

Mycelia were acquired from the margin of the colony and then cultured on fresh PDA plates at 25 °C for 10 d in darkness without hygromycin B. This procedure was repeated five times. Finally, the resistance of the transformants to hygromycin B was evaluated by incubating the colonies on PDA medium supplemented with hygromycin B (100 μg mL^{-1}).

For the molecular analysis of transformants, resistant colonies were successively moved to new PDA plates for three generations to eliminate plasmid DNA contamination in the presence of hygromycin B. Mycelia were collected, frozen, and ground. Genomic DNA was extracted from grounded samples using the HP Fungal DNA kit following the manufacturer's protocol (Genview, China) to verify that fungal transformants were T-DNA integrated by PCR. The extracted DNA was then eluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and DNA concentrations were measured in a Beckman DU series spectrophotometer. The 1355 bp *hph* gene fragment was amplified using primers HPH-F (5′-TAGTGGAGGTCAACAATGAATG-3′) and HPH-R (5′-CATCTACTCTATTCCTTTGCCC-3′) (Gu et al., 2016) in a 25 μL PCR mixture containing 2 μL fungal DNA (20 ng), 1 μL (10 μM) of each primer, 0.3 μL Taq DNA polymerase (Takara, USA) (5 U μL^{-1}), 2 μL dNTP mixture (2 mM each), 2.5 μL 10× PCR buffer, and 16.2 μL sterile distilled water. The PCR protocol was conducted as follows: first denaturation at 94 °C for 2 min; 35 cycles of denaturation at 98 °C for 10 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. Amplicons were visualized on 0.8 % agarose gel dyed with ethidium bromide and photographed.

2.5. Southern blot analysis of the T-DNA integration

Southern blot analyses of whole genome transformations were performed to define the copy numbers of the T-DNA integration. Genomic DNA (0.6 μg) from 20 transformants or *Cc*-GX isolates were fragmented by *Eco*RI (no restriction site in T-DNA) and *Hin*dIII (single restriction site in the T-DNA but outside the probe region), according to the manufacturer's recommendations. Resulting fragments were visualized on 0.75 % agarose gel in 1× TAE buffer and then transferred to positively charged nylon membranes. The probe was obtained from the template vector pATMT1 and primers HPH-F and HPH-R were used to amplify the coding region of *hph*. Preparation of digoxigenin (DIG)-labelled probes, hybridization, and chemiluminescence detection were conducted using the DIG High Prime DNA Labelling and Detection Starter Kit (Roche, Germany) following the manufacturer's protocol.

2.6. Pathogenicity assay of transformants

Transformants were first cultured on PDA for 10 d at 25 °C and colony morphology and growth rates were compared with those of the *Cc*-GX strain. The transformants with phenotypes that contrasted those of the parental strain were selected for the pathogenicity assay. The pathogenicity of the *Cc*-GX strain and transformants was evaluated using two inoculation methods, i.e. mycelial discs (5 mm in diameter) and spore suspension, on 14-day-old detached muskmelon (Haoyun 8) leaves that had previously been surface-sterilized with a 0.5 % NaOCl solution for 2 min and then rinsed in sterile water three times. In the mycelial discs method, the transformants and *Cc*-GX strain were cultured on PDA media. Mycelial discs were collected from the margin of transformant or *Cc*-GX colonies that contained hyphae and were applied to the muskmelon leaves. Control leaves were treated with 1.2 % water agar solution. In the spore suspension method, conidia of the transformant or *Cc*-GX strains were harvested from PDA plates and suspended in sterile water to 1×10^6 conidia mL^{-1}. Leaves of muskmelon were inoculated with 10 μL of the conidia suspension or sterile water (control leaves). All inoculated and control leaves were incubated in sealed moist petri dishes at 25 °C, in darkness, for 3 d. Leaf symptoms were assessed after this period. The experiments were performed in triplicate.

3. Results

3.1. Hygromycin B sensitivity of C. cassiicola

Hygromycin B sensitivity of *Cc*-GX *C. cassiicola* was determined by incubating the isolate on PDA that contained hygromycin B at various concentrations (up to 120 μg mL^{-1}) at 25 °C, in darkness, for 7 d. Growth was completely inhibited at 100 μg mL^{-1} hygromycin B (Fig. S1), therefore, this concentration was used to select for the resistant transformants in the ATMT experiments.

3.2. ATMT of C. cassiicola

We optimized the conditions for ATMT to yield efficient transformation. Firstly, we assessed the effects of pH and temperature on the transformation efficiency. The optimal pH range was 5.3–5.6 (Fig. 1A) and the optimal temperature range was 21–23 °C (Fig. 1B). Subsequently, the co-cultivation period and the concentration of bacterial cells were assessed to optimize the efficiency of the transformation. No transformation occurred before 54 h of co-incubation and the optimal co-incubation period ranged between 63 and 66 h (Fig. 1C). The number of transformants increased with increasing bacterial cells until the absorbance at OD_{600} reached 0.5 (Fig. 1D). In addition, no transformants were obtained in the absence of AS (Fig. 2), which confirmed its necessity for *C. cassiicola* transformation. Nitrocellulose filters were then moved from the co-cultivation medium to the antibiotic selection PDA plates containing hygromycin B (100 μg mL^{-1}) as a screening agent for the transformants and cultured for 5–7 d at 25 °C in darkness. Resistant colonies were difficult to isolate if the cultured period was prolonged for more than 7 d because they began overlapping each other. After co-cultivation for 65 h in the presence of 200 μM AS under pH 5.6, OD_{600} 0.5, and 23 °C conditions, 102–148 transformants were able to be harvested from each of the 1×10^6 conidia mL^{-1} *C. cassiicola* samples.

3.3. Confirmation of hph presence in the transformants

Mitotically stable integrated T-DNA was assessed by analysing 10 randomly selected transformants. After cultivating *Cc*-GX and transformant strains on PDA plates without 100 μg mL^{-1} hygromycin B for five generations, they were moved to the screening medium that contained the antibiotic. All randomly selected transformants grew normally while the *Cc*-GX strain did not (Fig. 3). The acquired resistance was therefore shown to be stably inherited in the transformants.

For the molecular analysis of transformants, resistant colonies were successively moved to new PDA plates for three generations to eliminate plasmid DNA contamination in the presence of hygromycin B. The third-generation of 10 randomly selected individual transformants that could grow at 100 μg mL^{-1} hygromycin B were maintained on PDA at 25 °C and subjected to molecular analysis. To verify that the single colonies of fungal transformants were T-DNA integrated, a 1355 bp *hph* gene fragment was amplified by PCR using primers HPH-F and HPH-R. The expected 1355 bp amplicon was amplified from all transformants but not from the *Cc*-GX strain, which indicated that the vector pATMT1 successfully conveyed the *hph* gene into the *C. cassiicola* genome (Fig. S2).

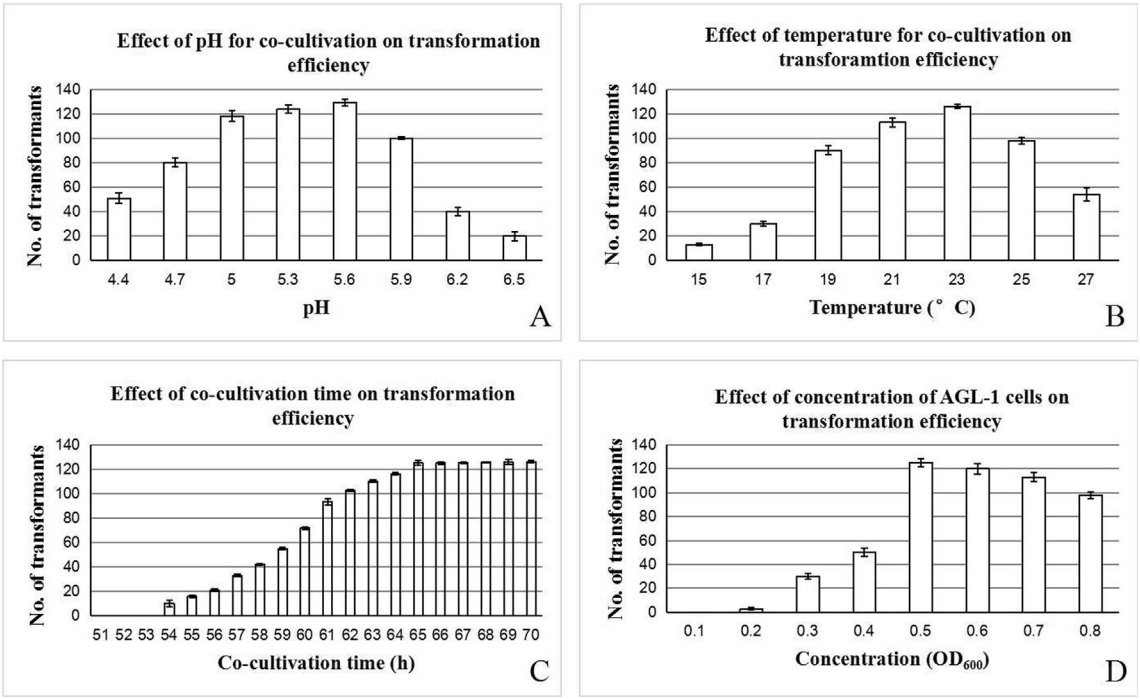

Fig. 1. Factors affecting the transformantion efficiency of *C. cassiicola* ATMT. Effect of pH value for co-cultivation on transformation efficiency (A); Effect of temperature for co-cultivation on transformation efficiency (B); Effect of co-cultivation time on transformation efficiency (C); Effect of concentration of *A. tumefaciens* cells on transformation efficiency (D); Data were the average numbers of transformants per 10^6 conidia. Error bars indicate standard errors.

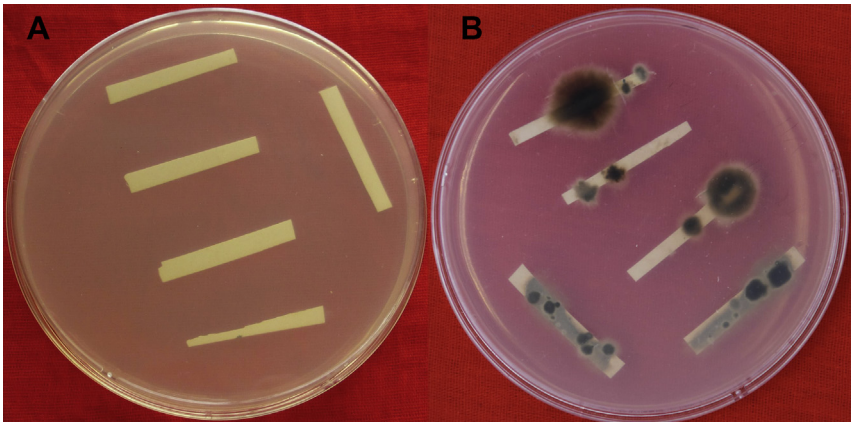

Fig. 2. Transformants grown on potato dextrose agar plates with or without acetosyringone. No transformants were obtained in the absence of acetosyringone (A). Individual colonies of putative transformants appeared on nylon membranes after 65 h of co-cultivation and selection on a medium containing hygromycin B, after 5–7 d of incubation at 25 °C (B).

3.4. Southern blot analysis of transformants

For further characterisation of T-DNA insertion patterns in the mutant library, 20 transformants were arbitrarily selected and then subjected to Southern blot hybridization of whole genome by applying the *hph* probe. The different transformants displayed distinct DNA bands when hybridized with the DIG-labelled probe harbouring *hph*. Transformants with a single-copy T-DNA insertion at one site would displayed a unique band after the application of restriction enzymes, and multiple-copy insertions displayed more than one DNA band. Eighteen transformants that were co-cultivated for 65 h displayed one copy insertion at several sites. One of the transformants harboured two T-DNA insertion sites, and the other harboured three sites (Fig. 4). These results indicated that ~90 % of the transformants displayed one-copy integration.

3.5. Identification of pathogenic C. cassiicola transformants

In a preliminary screening, we obtained insertion mutants with phenotypic characteristics that were clearly different from that of the *Cc*-GX strain (Fig. 5). Eleven of the representative phenotypically altered transformants, which differed from *Cc*-GX strain in terms of colony shape and colour, were used in the pathogenicity assay. Three of these transformants (CT0001, CT0022, and CT0013) produced no visible disease symptoms in detached muskmelon leaves and, interestingly, CT0001 and CT0022 did not produce conidia on the PDA plates, while CT0013 displayed severely impaired conidial production (Fig. S3). Transformants CT0005, CT0075, and CT0087 exhibited strongly impaired virulence on detached leaf assays, but CT0009, CT0067, and CT0088 displayed only slightly higher than the *Cc*-GX strain. The other two candidates

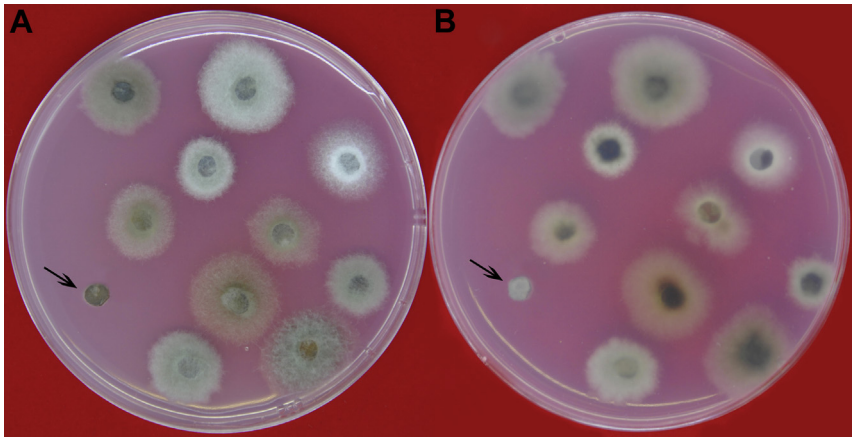

Fig. 3. Mitotic stability of *Corynespora cassiicola* transformants. After cultivating *Cc*-GX and transformant strains on potato dextrose agar plates in the absence of antibiotic for five generations, randomly selected colonies were transferred to screening plates containing 100 μg mL^{-1} hygromycin B. All the randomly selected transformants grew normally whereas the *Cc*-GX strain (arrow) did not. Photos were taken from the top (A) and bottom (B) of plates.

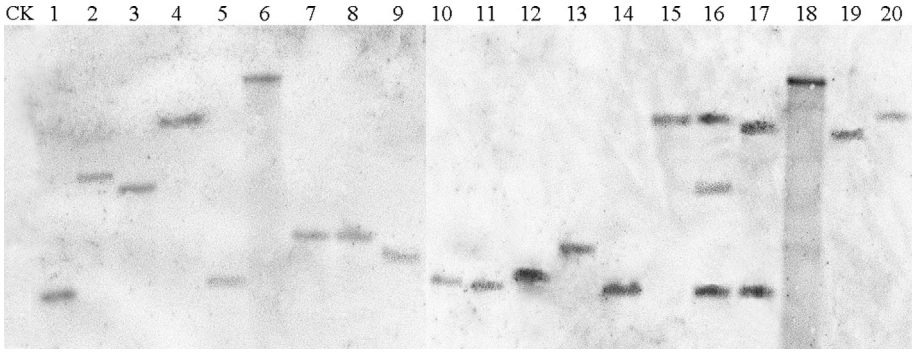

Fig. 4. Transfer (T)-DNA copy number analysis in *Corynespora cassiicola* transformants. Genomic DNA was isolated from 20 randomly selected transformants and digested with *Eco*RI (no restriction site in the T-DNA) and *Hin*dIII (one restriction site in the T-DNA but outside the probe region), separated on 0.75 % agarose gel, transferred to a nylon membrane, and hybridized with a digoxigenin-labelled 1355 bp fragment of gene *hph*. CK, negative control, *Cc*-GX strain; Lanes 1–20, transformants of *C. cassiicola*, CT0001, CT0005, CT0013, CT0022, CT0040, CT0058, CT0067, CT0079, CT0092, CT0161, CT0178, CT0188, CT0203, CT0234, CT0260, CT0290, CT0304, CT0311, CT0332 and CT0350.

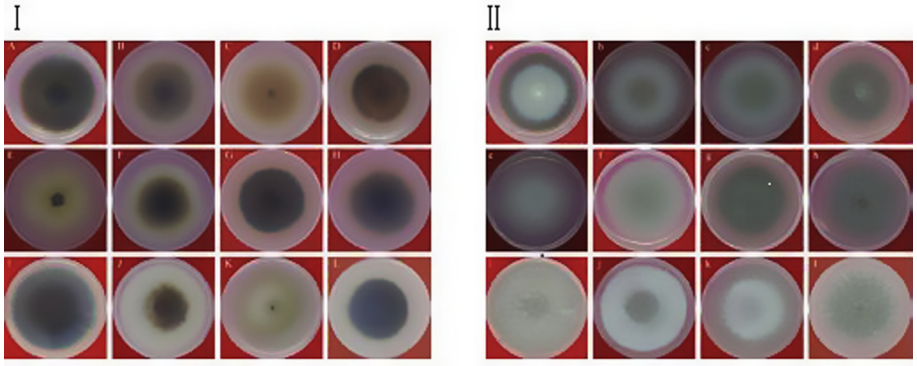

Fig. 5. Colony morphology of transfer (T)-DNA insertion transformants (a-k/A-K) and *Cc*-GX strain (l/L) grown on potato dextrose agar medium at 25 °C for 10 d. Photos were taken from the bottom (I) and top (II) of plates. Transformants with distinctive phenotypes were selected for further analysis: CT0001 (a/A), CT0005 (b/B), CT0009 (c/C), CT0013 (d/D), CT0022 (e/E), CT0028 (f/F), CT0067 (g/G), CT0075 (h/H), CT0087 (i/I), CT0088 (j/J), CT0166 (k/K) and Cc-GX (l/L).

(CT0028 and CT0166) consistently presented similar pathogenicity to the *Cc*-GX strain (Fig. 6).

4. Discussion

Fungi transformation technologies create gene disruptions in a random or targeted mode (Talhinhas et al., 2008). There are several examples of successful ATMT applications in different fungi and that this method is useful for deleting or disrupting targeted genes (Alexander et al., 2017). Until now, ATMT had not been applied in *C. cassiicola*. The present research optimized the ATMT protocol for *C. cassiicola* which was useful for successfully creating T-DNA transformants carrying different morphological features and various pathogenicity levels.

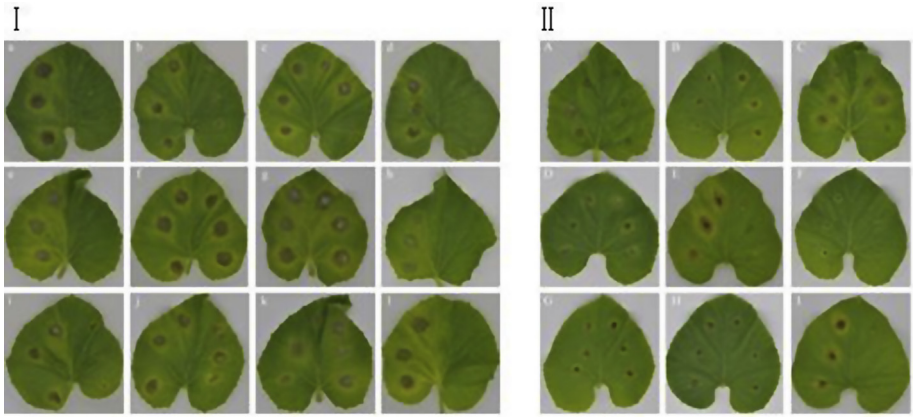

Fig. 6. Pathogenicity of *Corynespora cassiicola* transformants. Pathogenicity of *Cc*-GX strain and some phenotypically distinct transformants was evaluated using mycelial discs (I) or spore suspension (II) methods on 14-day-old detached muskmelon leaves. The mycelial discs (5 mm in diameter) or 10-μL spore suspension liquid (1×10^6 conidia mL^{-1}) of transformants were cultured on the right side of the leaves while *Cc*-GX strain were cultured on the left. Control leaves were treated with 1.2 % water agar plus or sterile water. Inoculated and control leaflets were placed in darkness at 25 °C in sealed moist Petri dishes. Three days after incubation, leaf symptoms were assessed for CT0001 (a), CT0005 (b, A), CT0009 (c, B), CT0013 (d), CT0022 (e), CT0028 (f, C), CT0067 (g, D), CT0075 (h, E), CT0087 (i, F), CT0088 (j, G), CT0166 (k, H), and control (l, I) treatments.

To perform this transformation, we first defined the inhibitory concentration of hygromycin B for selecting *C. cassiicola* transformants. Because the *Cc*-GX isolate was unable to grow at 100 μg mL^{-1} hygromycin B, this was determined as the selection concentration for resistant transformants in the ATMT experiments. The *vir* genes are usually induced by AS (Bundock et al., 1995; Michielse et al., 2005) and the inducer was shown to be necessary for *C. cassiicola* transformation because no transformants were obtained in the absence of AS. The co-culture period was another important factor that influenced the transformation of *C. cassiicola*. The process of transformation requires a certain period of time; thus, an insufficient co-culturing period would greatly affect the transformation outcome (Meyer et al., 2003; Nyilasi et al., 2005; Tanaka et al., 2007). The transformation efficiency of *C. cassiicola* was shown to be affected by the co-cultivation period in the present study, which was consistent with the findings for other fungal transformations (Betts et al., 2007; Knight et al., 2009; Michielse et al., 2005). Generally, the number of obtained transformants is proportional to the time of co-cultivation. In the present study, no transformants were obtained before 54 h of co-cultivation, and the number of transformants increased by extending the time of co-cultivation. The optimal co-cultivation time for *C. cassiicola* transformation was shown to be 65 h. Additionally, molecular analyses revealed that the T-DNA region was integrated in the genome of randomly selected *C. cassiicola* transformants. Single or multiple copies of T-DNA can account for insertion events. Thus, the mode and frequency of T-DNA insertion into the recipients are two key factors for defining the disrupted genes. Southern blot analyses revealed that 90 % of the integration events in *C. cassiicola* by ATMT were single-copy T-DNA insertions, indicating the high potential of this methodology for isolating the genes responsible for the phenotypes of transformed *C. cassiicola*.

The collection of transformants by ATMT and the isolation of target genes is widely used to determine virulence factors in various fungi (Jeon et al., 2007; Talhinhas et al., 2008). In our study, the *C. cassiicola* transformants displayed different morphological and culture characteristics on PDA plates. The *C. cassiicola* transformants with different phenotypes were compared with the *Cc*-GX strain and selected for a pathogenicity assay. Three of the eleven selected transformants showed no pathogenicity and another three transformants showed significantly impaired pathogenicity on detached muskmelon leaves compared to the *Cc*-GX strain. These results strongly supported the hypothesis that the ATMT technology can be used to isolate functional genes and identify pathogenicity-related genes in *C. cassiicola*.

In conclusion, *C. cassiicola* was successfully transformed by *A. tumefaciens* as 102–148 transformants were obtained per 10^6 *C. cassiicola* conidia. This is the first report of successful ATMT in *C. cassiicola*. The suitable ATMT protocol for the insertional transformant of *C. cassiicola* presented here is an important step in the identification of the genes associated with *C. cassiicola* pathogenicity. Although the optimized protocol presented here was helpful in rapidly producing a group of random transformants, mainly single-copy T-DNA transformants, the transformation efficiency obtained was relatively low compared to that reported for other filamentous fungi. This finding may be related to the biological diversity of fungi. Thus, continuous optimization of the ATMT protocol might improve the transformation efficiency of *C. cassiicola*. However, it is more important to systematically improve the confirmation process and effectively acquire the targeted genes. A newly-developed gene library for *C. cassiicola* is currently being extended and molecular analyses of the pathogenicity of transformants are being conducted.

Conflicts of interest

The authors declare there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.05.011.

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