Establishment of Agrobacterium tumefaciens-Mediated Transformation System for Rice Sheath Blight Pathogen Rhizoctonia solani AG-1 IA

YANG Ying-qing¹, ², #, YANG Mei¹, #, LI Ming-hai¹, LI Yong¹, HE Xiao-xia¹, ZHOU Er-xun¹

(¹Department of Plant Pathology, South China Agricultural University, Guangzhou 510642, China; ²Institute of Plant Protection, Jiangxi Academy of Agricultural Sciences, Nanchang 330200, China; # These authors contributed equally to this paper)

Abstract: To construct the T-DNA insertional mutagenesis transformation system for rice sheath blight pathogen Rhizoctonia solani AG-1 IA, the virulent isolate GD118 of this pathogen was selected as an initial isolate for transformation. The conditions for transformation of isolate GD118 were optimized in five aspects, i.e. pre-induction time, co-culture time, acetosyringone (AS) concentration at the co-culture phase, co-culture temperature and pH value of induction solid medium (ISM) at the co-culture phase. Finally, a system of Agrobacterium tumefaciens-mediated transformation (ATMT) for R. solani AG-1 IA was established successfully. The optimal conditions for this ATMT system were as follows: the concentration of hygromycin B at 30 μg/mL for transformant screening, 8 h of pre-induction, 20 h of co-culture, 200 µmol/L of AS in ISM, co-culture at 25 °C and pH 5.6 to 5.8 of ISM at the co-culture phase. The transformants still displayed high resistance to hygromycin B after subculture for five generations. A total of 10 randomly selected transformants were used for PCR verification using the specific primers designed for the hph gene, and the results revealed that an expected band of 500 bp was amplified from all of the 10 transformants. Moreover, PCR amplification for these 10 transformants was carried out using specific primers designed for the Vir gene of A. tumefaciens, with four strains of A. tumefaciens as positive controls for eliminating the false-positive caused by the contamination of A. tumefaciens. An expected band of 730 bp was amplified from the four strains of A. tumefaciens, whereas no corresponding DNA band could be amplified from the 10 transformants. The results of the two PCR amplifications clearly showed that T-DNA was indeed inserted into the genome of target isolate GD118.

Key words: rice sheath blight; Rhizoctonia solani; Agrobacterium tumefaciens-mediated transformation; T-DNA insertional mutagenesis; methodology

Rice sheath blight is one of the three most serious rice diseases worldwide (Lee and Rush, 1983; Peng et al, 1986). With the extension of high-yield and semi-dwarf rice varieties, the damage of sheath blight to rice becomes greater and leads to severe economic loss of rice production, especially in the southern China (Zhou et al, 2002a, b; Huang et al, 2009). The rice sheath blight disease is caused by a soil-borne fungus Rhizoctonia solani Kühn, with a teleomorph of Thanatephorus cucumeris (Frank) Donk, which can infect more than 260 plant species including rice, maize, soybean, potato, tomato, cucumber, cabbage and so on (Peng et al, 1986; Huang et al, 2008; Xiao et al, 2008). R. solani was considered as a complex species because of its complicated intraspecific members (Cubeta and Vilgalys, 1997). As a result, R. solani was ordinarily classified into different anastomosis groups (AGs) (Ogosh, 1987). In recent years, with the development of molecular biology and its application in plant pathology, fungal transformation has gained more attention due to the relative simplicity to obtain target genes. Currently, more than 100 filamentous fungi have been transformed successfully (Wang and Li, 2001). There are some methods for fungal transformation, and Agrobacterium tumefaciens-mediated transformation (ATMT) has many advantages such as simple manipulation, high transformation efficiency and high single-copy rate. Therefore, it has become the most powerful tool for fungal transformation and gene cloning (Mullins and Kang, 2001; Combier et al, 2003; Michielse et al, 2005; Li et al, 2009; Wu and O’Brien, 2009). In regarding to the transformation in R. solani, the PEG-mediated DNA integrated technique was used to transform R. solani and several transformants were obtained (Robinson and Deacon, 2001). However, these transformants could not grow normally and transient transformation existed in R. solani, which was similar...
The transformations of *R. solani* AG-3, AG-4 and AG-6 were carried out, and only AG-6 was transformed successfully (Wu and O’Brien, 2009). Rice sheath blight is one of the most important diseases, however, the transformation system of rice sheath blight pathogen *R. solani* AG-1 IA has not yet been reported. In the present study, we intend to carry out genetic transformation and construct the transformation system for *R. solani* AG-1 IA, the causal agent of rice sheath blight. This study aims at providing a foundation for understanding the pathogenic mechanism and cloning of pathogenicity-related genes of *R. solani*, which would be of great theoretical and practical significance.

**MATERIALS AND METHODS**

**Fungal isolate and plasmid**

The fungal isolate used was the virulent isolate GD118 of the rice sheath blight pathogen *R. solani* AG-1 IA, which was conserved by our laboratory. The plasmid pTHPR1 and the strain AGL-1 of *A. tumefaciens* were kindly provided by Professor ZHANG Lianhui at the Institute of Molecular and Cell Biology (IMCB), Singapore. Other *A. tumefaciens* strains of EHA105, MP90 and LBA4404 were conserved by our laboratory.

**Biochemical reagents**

Hygromycin B was purchased from Roche, Germany. Universal genomic DNA extraction kits and PCR reagents were purchased from TaKaRa, Japan. Acetosyringone (AS) and Morpholineethanesulfonic acid (MES) were purchased from Sigma, USA. Bacterial genomic DNA extraction kits were purchased from TIANGEN, China. Other common reagents were purchased from companies in China.

**Sensitivity detection of *R. solani* AG-1 IA to hygromycin B**

A suitable amount of hygromycin B was added to PDA (potato dextrose agar) medium (potato 200 g, dextrose 20 g, agar 18 g. Adjust the total volume to 1000 mL with ddH₂O) and the final concentrations of hygromycin B were adjusted to 0, 5, 10, 15, 20 and 25 μg/mL, respectively. Three replications of each concentration were set. The wild type isolate GD118 of *R. solani* AG-1 IA was incubated on PDA plates for 36 h and mycelial plugs were cut from the colony edge with a 5-mm-diameter borer, and then transferred onto the centre of PDA plates with different concentrations, and incubated at 26 °C for 8 d. For further determining the screening concentration of hygromycin B, the wild type isolate GD118 of *R. solani* AG-1 IA was incubated in Erlenmeyer flask with PDB (potato dextrose broth) solution (potato 200 g, dextrose 20 g. Adjust the total volume to 1000 mL with ddH₂O) and then the 36-h-old mycelia were ground into small pieces. Add water to suspend the small pieces of mycelia and spread evenly onto PDA plates with hygromycin B at different concentrations of 0, 5, 10, 15, 20, 25, 30 and 35 μg/mL, respectively. Three replications of each concentration were set and incubated at 26 °C for 8 d.

**Transformation method**

The transformation method described by Li et al (2009) was adopted with minor modifications. The *A. tumefaciens* strain AGL-1 with the plasmid pTHPR1 was incubated in 3 mL minimal medium (MM) solution [KH₂PO₄ 2.05 g; KH₂PO₄ 1.45 g; NH₄NO₃ 0.5 g; CaCl₂ 0.01 g; Glucose 2 g; (NH₄)₂SO₄ 0.3 g; FeSO₄ 0.01 g; 5 mL Z-buffer (ZnSO₄·7H₂O, CuSO₄·H₂O and MnSO₄·H₂O at the concentration of 0.01% respectively); Adjust the total volume to 1000 mL with ddH₂O and final pH value to 6.7 with 4 mol/L NaOH and 4 mol/L HCl], amended with spectinomycin at 50 μg/mL and rifampicin at 25 μg/mL at 28 °C for 48 h, when the OD₆₀₀ value was above 0.8, the bacterium suspension was diluted to the OD₆₀₀ value of 0.15 with induction medium (IM) solution (KH₂PO₄ 2.05 g; KH₂PO₄ 1.45 g; NH₄NO₃ 0.5 g; CaCl₂ 0.01 g; MgSO₄·7H₂O 0.6 g; NaCl 0.3 g; (NH₄)₂SO₄ 0.5 g; Glucose 1 g; MES 7.808 g; Adjust the total volume to 1000 mL with ddH₂O and final pH value to 5.6 with 4 mol/L NaOH and 4 mol/L HCl), followed by pre-induction at 28 °C for 8 h. When the OD₆₀₀ value was about 0.3, it was suitable for co-culture. The isolate GD118 of *R. solani* AG-1 IA was incubated in PDB solution amended with ampicilin at 50 μg/mL at
26 °C for 36 h. The mycelia were harvested and ground into small pieces, followed by addition of ddH₂O for dilution until the amount of mycelial piece was up to 1×10⁷ fragments in 1 mL, then 100 μL of mycelial piece solution and 100 μL of pre-induced A. tumefaciens solution were mixed and spread onto the nitrocellulose membrane placed on the surface of an solid induction medium (SIM) (K₂HPO₄ 2.05 g; KH₂PO₄ 1.45 g; NH₄NO₃ 0.5 g; CaCl₂ 0.01 g; MgSO₄·7H₂O 0.6 g; NaCl 0.3 g; (NH₄)₂SO₄ 0.5 g; Glucose 1 g; MES 7.808 g; agar 18 g; Adjust the total volume to 1000 mL with ddH₂O and final pH value to 5.6 with 4 mol/L NaOH and 4 mol/L HCl) plate amended with AS at 200 μmol/L. After co-culture at 26 °C for 20 h, the nitrocellulose membrane was transferred to a PDA plate amended with hygromycin B at 30 μg/mL and cephamycin at 300 μg/mL, and incubated at 26 °C for 8 d, followed by re-screening on PDA plates as above. The screened transformants were conserved in a low-temperature refrigerator and used for further experiments.

Effects of different factors on transformation efficiency

Effects of different factors on the transformation efficiency were investigated. These factors included pre-induction time (4–12 h), co-culture time (12–36 h), concentrations of AS during co-culture (0–400 μg/mL), temperatures during co-culture (22–34 °C), and pH values during co-culture (5.2–6.4). The transformation efficiency was expressed as the numbers of transformants per 10⁶ mycelial pieces in each Petri dish (Transformation efficiency = No. of transformants / 10⁶ mycelial pieces).

PCR verification of transformants

A total of 10 transformants were randomly selected, and their genomic DNAs were extracted with the Universal DNA Extraction Kit from TaKaRa (Japan). The genomic DNA of A. tumefaciens was extracted with Bacterium Genomic DNA Extraction Kit from TIANGEN (Beijing, China).

Specific primers of hph-F (5’-GCAAGACCTGCCTGAACCCG-3’) and hph-R (5’-GGTCAGACCAATGCCGGAGC-3’) were designed according to the hph gene sequence. PCR was carried out in a volume of 20 μL containing 10 mmol/L Tris-HCl (pH 8.4), 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 0.25 mmol/L each dNTP, 0.2 mmol/L each primer, 20 ng of template DNA and 1 U of Taq DNA polymerase using the following program: 95 °C for 2 min, then 30 cycles of 94 °C for 40 s, 56 °C for 40 s and 72 °C for 1 min, with a final extension of 72 °C for 10 min.

To exclude the probability of false positive caused by the contamination of A. tumefaciens, specific primers of VCF (5’-ATCATTTTGTAGCGACT-3’) and VCR (5’-AGCTCAACCTGCCTTC-3’) were designed based on the Vir gene published by Sawada et al (1995) and were used for PCR amplification with four strains of A. tumefaciens, i.e. AGL-1, EHA105, MP90 and LBA4404, as positive controls. The amplification program was: 95 °C for 2.5 min, then 40 cycles of 95 °C for 40 s, 55 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 7 min.

Stability test of transformants

Ten transformants were selected randomly and incubated on PDA plates at 26 °C for 36 h, then stored at 4 °C for 10 d. Four cycles of such test were repeated. The 5th generation transformants were transferred to PDA plates with 30 μg/mL hygromycin B and incubated at 26 °C for 36 h.

RESULTS

Sensibility of R. solani AG-1 IA to hygromycin B

After incubation for 8 d, the mycelial mass could not grow on the treatment plates containing 25 μg/mL hygromycin B, but could grow on the plates with hygromycin B lower than 25 μg/mL at different levels. Similarly, after incubation for 8 d, the hyphal fragment could not grow on the treatment plates containing 20 μg/mL hygromycin B, but could grow on the plates with hygromycin B lower than 20 μg/mL at different levels. As a result, 30 μg/mL hygromycin B was selected as the concentration for later screening of transformants.

Effects of pre-induction time on transformation efficiency

The pre-induction times were set as 4, 6, 8, 10 and 12 h in the present study. The OD₆₀₀ values of IM
solutions were determined and used for transformation followed the method mentioned above. The results revealed that the transformation efficiency reached a high value when *A. tumefaciens* was pre-induced for 8 h with the OD$_{600}$ value of 0.27 (Fig. 1-A). When *A. tumefaciens* was pre-induced for 12 h, the OD$_{600}$ value was 0.32 (Fig. 1-A). Thus, IM solutions with the OD$_{600}$ value of about 0.3 were suitable for *R. solani* transformation. Though the transformation efficiency at 10 or 12 h as the pre-induction time was a little higher than that of 8 h, the difference among them was not significant (Fig. 1-A). Thus, 8 h was chosen as the optimal pre-induction time.

**Effects of co-culture time on transformation efficiency**

The co-culture times were set as 12, 16, 20, 24 and 36 h in the present study. The results indicated that the difference of transformation efficiency was not significant when the co-culture time was over 20 h. Though co-culture for 24 and 36 h could produce a little more transformants, 20 h was chosen as the best co-culture time considering time-saving and convenience to pick transformants (Fig. 1-B).

**Effects of AS concentration on transformation efficiency during co-culture**

AS concentrations at 0, 100, 200 and 400 µmol/L were set in the present study. The results showed that the transformation efficiency reached a relatively high level with the AS concentration of 200 µmol/L. Though the transformants at 400 µmol/L of AS were a little more than those at 200 µmol/L, the difference between them was not significant (Fig. 1-C). Thus, 200 µmol/L of AS was selected as the optimal concentration.

**Effects of temperature on transformation efficiency during co-culture**

Temperatures of 22, 25, 28, 31 and 34 °C were set during co-culture in the present study. As the transformation efficiency was the highest at the temperature of 25 °C during co-culture, therefore, 25 °C was chosen as the optimal temperature during co-culture (Fig. 1-D).

**Effects of pH value on transformation efficiency during co-culture**

The SIM media with pH values of 5.2, 5.4, 5.6, 5.8, and 6.0 were set in the present study. The results showed that the transformation efficiency was highest at the pH value of 5.8. Though the transformants at pH 5.6 were a little more than those at pH 5.8, the difference between them was not significant (Fig. 1-E). Thus, 5.8 was chosen as the optimal pH value.
5.8, 6.0, 6.2 and 6.4 were prepared and used for co-culture. The results showed that the transformation efficiency maximized when the pH value was between 5.6 and 5.8 (Fig. 1-E).

PCR verification of transformants

The specific PCR amplification of hph gene revealed that an expected band of about 500 bp could be amplified from all genomic DNAs extracted from 10 randomly selected transformants of R. solani and pTHPR1 plasmid, whereas nothing from that of the wild type isolate GD118 (Fig. 2-A). To eliminate the possibility that the genomic DNAs from 10 transformants were contaminated by that of A. tumefaciens (containing plasmid pTHPR1 and Vir gene), the wild type isolate GD118 and 10 transformants were tested for the presence of Vir gene with four strains of A. tumefaciens i.e. AGL-1, EHA105, MP90 and LBA4404 as positive controls. The specific PCR amplification of Vir gene showed that an expected band of 830 bp could be amplified from four strains of A. tumefaciens, whereas no corresponding DNA band was amplified from wild type isolate GD118 and 10 transformants (Fig. 2-B), indicating that the genomic DNAs from the wild type isolate GD118 and 10 transformants were not contaminated by that of A. tumefaciens. From the results of the two PCR amplifications mentioned above, we concluded that T-DNA insertion indeed existed in the genomes of 10 transformants, which proved that the constructed transformation system was efficient in carrying out transformation of R. solani AG-1 IA.

Stability of transformants

Ten randomly selected transformants were incubated on PDA plates without hygromycin B for five generations, and then transferred onto PDA plates with hygromycin B. It is observed that the 10 transformants after five generations could grow normally on PDA plates containing hygromycin B with the colony diameter of 7 to 8 cm after incubation for 36 h, which indicated clearly that the T-DNA indeed inserted into the genome of R. solani AG-1 IA and could inherit stably.

DISCUSSION

To date, there has been no report on genetic transformation in R. solani AG-1 IA, the causal agent of rice sheath blight. The transformation system for R. solani AG-1 IA was constructed successfully in our study, which would undoubtedly promote the research progress on pathogenic mechanism and pathogenisity-related genes of R. solani AG-1 IA.

In respect to pre-induction time, 6 h was taken as the pre-induction time in some previous reports (Combier et al, 2003; Li et al, 2009), whereas 8 h was confirmed as the best pre-induction time through gradient experiments of pre-induction time in the present study, and the pre-induction time more than 8 h could not improve transformation efficiency observably.

In regarding to co-culture time, we observed that with the increase of co-culture time, the transformation efficiency increased, which supported the same conclusions made by Combier et al (2003), Li et al (2009) and Tsuji et al (2003). The results on Aspergillus giganteus showed that there was no transformant produced when the co-culture time was less than 24 h or more than 72 h (Meyer et al, 2003).

![Fig. 2. PCR amplifications of genomic DNAs from transformants.](image-url)

A, PCR for hph gene. Lane 1, Wild type isolate GD118 of R. solani; Lanes 2 to 11, Ten randomly selected transformants; Lane 12, Plasmid pTHPR1 (positive control for hph gene); M, DL2000 marker.

B, PCR for Vir gene. M, DL1000 marker; Lanes 1 to 4, A. tumefaciens strains of AGL-1, EHA105, MP90 and LBA4404 (positive controls for Vir gene), respectively; Lanes 5 to 14, Ten randomly selected transformants; Lane 15, Double distilled water (negative control).
Takahara et al (2004) and Li et al (2009) thought that it was difficult to screen single colony of transformant due to the excessive growth of mycelia when the co-culture time exceeded 48 h. Furthermore, with the extension of co-culture time, it was prone to more multi-copy and false positive transformants. Therefore, the optimal co-culture time was determined as 48 h. Taken together, our study suggested that co-culture time of 12 h was enough for efficient transformation of \textit{R. solani} AG-1 IA, and when the co-culture time reached 20 h, the transformation efficiency reached a higher level. Owing to the rapid growth, \textit{R. solani} AG-1 IA would grow excessively when the co-culture time exceeded 36 h, leading to much growth of mycelia before being transferred onto nitrocellulose membranes. Thus, for the transformation of \textit{R. solani} AG-1 IA, the co-culture time could not exceed 36 h, which was different from that for most fungi (Meyer et al, 2003; Takahara et al, 2004; Li et al, 2009).

In general, AS and its concentrations during co-culture contributed to transformation efficiency greatly, namely, if no AS was added to medium, no transformant was obtained, and the transformation efficiency improved with the increase of AS concentrations (Combier et al, 2003; Rogers et al, 2004; Takahara et al, 2004; Chi et al, 2005; Michielse et al, 2005; Samils et al, 2006; Li et al, 2009). The results of our research suggested that high transformation efficiency of \textit{R. solani} AG-1 IA could also be achieved without AS, which was different from that for most fungi. However, AS could improve the transformation efficiency of \textit{R. solani} AG-1 IA and the transformation efficiency peaked at the AS concentration of 200 μmol/L; but if AS concentration was higher than 200 μmol/L, it could not benefit transformation obviously. In other words, 200 μmol/L was the best AS concentration during co-culture, which was identical with the views of most researchers (Combier et al, 2003; Rogers et al, 2004; Takahara et al, 2004; Chi et al, 2005; Michielse et al, 2005; Samils et al, 2006; Li et al, 2009).

In respect to pH values during co-culture, Li et al (2009) thought pH 5.5 was the best co-culture pH value in transformation of \textit{Fusarium oxysporum}. In this study, it was concluded that pH 5.6–5.8 during co-culture was the optimal pH value for transformation of \textit{R. solani} AG-1 IA. Turk et al (1991) thought that pH values during co-culture could affect the expression of VirA protein, and subsequently affect the transfer of T-DNA. It was once reported that the pH value for the highest activity of VirA protein expression was between 5.3 and 5.8 in different strains of \textit{Agrobacterium}, and the T-DNA transferring activity decreased obviously when the pH value was over 5.8 or below 5.3 (Stachel and Zambryskil, 1986; Rogowsky et al, 1987). Therefore, the most suitable pH values of 5.6 to 5.8 obtained in the present study were within the ranges (pH 5.3–5.8) of VirA protein expression activity based on the conclusions made by Stachel et al (1986) and Rogowsky et al (1987).

In regarding to the transformation of other AGs of \textit{R. solani}, Robinson et al (2001) carried out the transformation of \textit{R. solani} AG-3 mediated by plasmids pES200 from \textit{Aspergillus nidulans} and pAXHY2 from \textit{Cryphonectria parasitica}, and obtained some transformants. However, the transformants grew slowly (25–35 mm diameter after 14 d) and then ceased growth. They never showed rapid growth phase, indicating the presence of nonintegrated plasmid DNA confirmed by high stringency hybridization of DNA extracted from the transformants, using pES200 and pAXHY2 as radiolabelled probes. Therefore, the concept of transient transformation was included to explain that phenomenon in their transformation, which was similar to other basidiomycetes. Wu et al (2009) attempted to conduct the transformation of \textit{R. solani} AG-3, AG-4 and AG-6, but only five transformants from \textit{R. solani} AG-6 grew normally, whereas the transformations of \textit{R. solani} AG-3 and AG-4 were not successful. To date, there is no report on the transformation of \textit{R. solani} AG-1 IA. The transformation system constructed in the present study filled the gap of the transformation in \textit{R. solani} AG-1 IA, and established foundation for study of pathogenic mechanism and cloning of pathogenisity-related genes. It would benefit the further research on the pathogenisity-related genes of rice sheath blight pathogen \textit{R. solani} AG-1 IA.

ACKNOWLEDGEMENTS

The authors thank Professor ZHANG Lianhui at
the Institute of Molecular and Cell Biology (IMCB), Singapore for providing *A. tumefaciens* isolate AGL-1 and plasmid pTHPR1 with *hph* resistance gene. This research was supported by a ‘Special Fund for Agro-scientific Research in the Public Interest’ from the Ministry of Agriculture of China (Grant No. nyhyzx3-16).

**REFERENCES**


