NOTAS CIENTÍFICAS

Agrotransformation of *Phytophthora nicotianae*: a simplified and optimized method

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Data de chegada:11/02/2016. Aceito para publicação em: 27/04/2016.

10.1590/0100-5405/2159

ABSTRACT

Dalio, R.J.D.; Santos, P.J.C.; Máximo, H.J.; Kawakami, P.A.; Goulin, E.; Machado, M.A. Agrotransformation of *Phytophthora nicotianae*: a simplified and optimized method. ***Summa Phytopathologica,*** v.42, n.3, p.254-256, 2016.

Phytophthora nicotianae is a plant pathogen responsible for damaging crops and natural ecosystems worldwide. *P. nicotianae* is correlated with the diseases: citrus gummosis and citrus root rot, and the management of these diseases relies mainly on the certification of seedlings and eradication of infected trees. However, little is known about the infection strategies of *P. nicotianae* interacting with citrus plants, which rises up the need for examining its virulence at molecular levels. Here we show an optimized method to genetically manipulate *P. nicotianae* mycelium. We have transformed *P. nicotianae* with the expression cassette of fluorescence protein DsRed. The optimized AMT method generated relatively high transformation efficiency. It also shows advantages over the other methods since it is the simplest one, it does not require protoplasts or spores as targets, it is less expensive and it does not require specific equipment. Transformation with DsRed did not impair the physiology, reproduction or virulence of the pathogen. The optimized AMT method presented here is useful for rapid, cost-effective and reliable transformation of *P. nicotianae* with any gene of interest.

Keywords: genetic transformation, DsRed, *Agrobacterium tumefaciens.*

RESUMO

Dalio, R.J.D.; Santos, P.J.C.; Máximo, H.J.; Kawakami, P.A.; Goulin, E.; Machado, M.A. Agrotransformação de *Phytophthora nicotianae*: um método simplificado e otimizado. ***Summa Phytopathologica,*** v.42, n.3, p.254-256, 2016.

Phytophthora nicotianae é um fitopatógeno responsável por danos em culturas e ecossistemas naturais em todo o mundo. *P. nicotianae* está relacionada às doencas: gomose e podridao de raízes e o manejo destas doencas depende principalmente de certificação de mudas e erradicação de plantas infectadas. No entanto, pouco se sabe sobre a estratégia de infecção do patógenos em plantas de citrus, o que eleva a necessidade de examinar a sua virulência em níveis moleculares. Neste trabalho nós apresentamos um método otimizado para manipular geneticamente o micélio de *P. nicotianae*. *P. nicotianae* foi transformada com um vetor de expressão da proteína fluorescente DsRed. O método AMT otimizado gerou relativa alta eficiência de transformação. Também apresenta vantagens sobre os outros métodos porque é o de mais simples execução, não requer protoplastos ou esporos como alvos, é menos dispendioso e não requer equipamento específico. A transformação com DsRed não prejudicou a fisiologia, reprodução ou virulência do patógeno. O método AMT otimizado aqui apresentado é útil para rápida, eficaz e confiável transformação de *P. nicotianae* com qualquer gene de interesse.

Palavras-chave: transformação genética, DsRed, *Agrobacterium tumefaciens*.

Phytophthora nicotianae Breda van Haan (syn. *Phytophthora parasitica* Dastur) is an aggressive plant pathogen (10). It is distributed worldwide and has a very broad host range, capable of infecting more than 250 plant families, including model plants such as *Arabidopsis thaliana* and *Nicotiana benthamiana* (6). The main *P. nicotianae* diseases are the black shank disease of tobacco and citrus gummosis. The latter has attracted more attention in the last years due to damaged trees in citrus nurseries and orchards. In Brazil, *P. nicotianae* is correlated with citrus gummosis and citrus root rot and the management of these diseases relies mainly on the certification of nursery trees before their planting on orchards and eradication of infected trees (3). Understanding the molecular mechanisms of oomycete virulence is essential for the development of efficient control strategies; in this sense, genetic manipulation is a fundamental tool (7).

Genetic transformation of *Phytophthora* species remains difficult nowadays, being reported for only some species and showing low efficiency compared to other plant pathogens (8). There are four main methods: gene bombardment, electroporation, polyethylene glycol (PEG)-mediated transformation, and *Agrobacterium tumefaciens*-mediated transformation (AMT). Our aim was to take the easiest and fastest method of all four (AMT), break its complexity down and optimize it for *P. nicotianae* to the point that it would become a routine method in the lab. Here we show an efficient, rapid, cost-effective and optimized method to genetically transform *P. nicotianae* via *A. tumefaciens*. We decided to use the fluorescence protein DsRed from reef coral (*Discosoma* sp.), placed in the vector pCAMDsRed, for transformation because the successfulness of the method could be easily assessed by using an epifluorescence microscope.

The optimized AMT method goes as follows: *Agrobacterium tumefaciens* strain EHA105 (4) harboring the binary vector pCAMDsRed with the *DsRed*-Express gene is placed under the control of the constitutive glyceraldehyde 3-phosphate promoter *(PgpdA)*

and followed by the sequence of the *trpC gene (TtrpC)* (Fig.1 A). The plasmid also contains the hygromycin gene conferring resistance to hygromycin *(hph)* and the neomycin phosphotransferase II gene conferring resistance to kanamycin for selection in bacteria (2). *A. tumefaciens* was grown at 28 °C in yeast extract peptone (YEP) supplemented with 100 mg/mL kanamycin. Thereafter, the bacteria were incubated for 48 hours in Luria-Bertani (LB) medium also with kanamycin (100 $\mu g.mL^{-1}$) in a shaker (180 RPM); the grown culture was transferred to Induction Medium (IM) (9) with 200 µM of acetosyringone (AS), at an OD_{660} nm of 0.2, shaking under the same conditions until an OD_{660} nm of 0.4 was obtained. Sterile filter paper discs were placed on Petri dishes with *IM agar medium supplemented with AS* (200 µM). Mycelia from newly cultured *P. nicotianae* (IAC 01-95, isolated from three-year-old infected *Citrus sunki* trees and cultivated in carrot-agar medium, at 25°C, in the dark) were transferred to *the plates and A. tumefaciens culture (100 µl,* OD_{660} nm of 0.4, one day old) was inoculated on the filters and co-cultivated at 25° C for 2 days. This method was done to ensure contact between *P. nicotianae* and *A. tumefaciens,* enabling the transfer of DNA. The filter papers were then relocated onto carrot dextrose agar supplemented with hygromycin B (100 $\mu g.ml^{-1}$), to select transformants, and 200 µM cefatoxime sodium salt, which is toxic to *Agrobacterium*. The first transformants appeared after six days. Mycelia from the edge of the cultures were picked with a toothpick and grown on fresh carrot dextrose agar plates for additional 5 days. After repeating this procedure for five times, the mycelia from each transformant were transferred to carrot dextrose agar plates containing hygromycin B (100 µg/ml) to ensure mitotic stability. At this time, a portion of the mycelia was transferred, using a needle, to a microscopic glass for analysis. Under the epifluorescence microscope, mycelia from wild type *P. nicotianae*

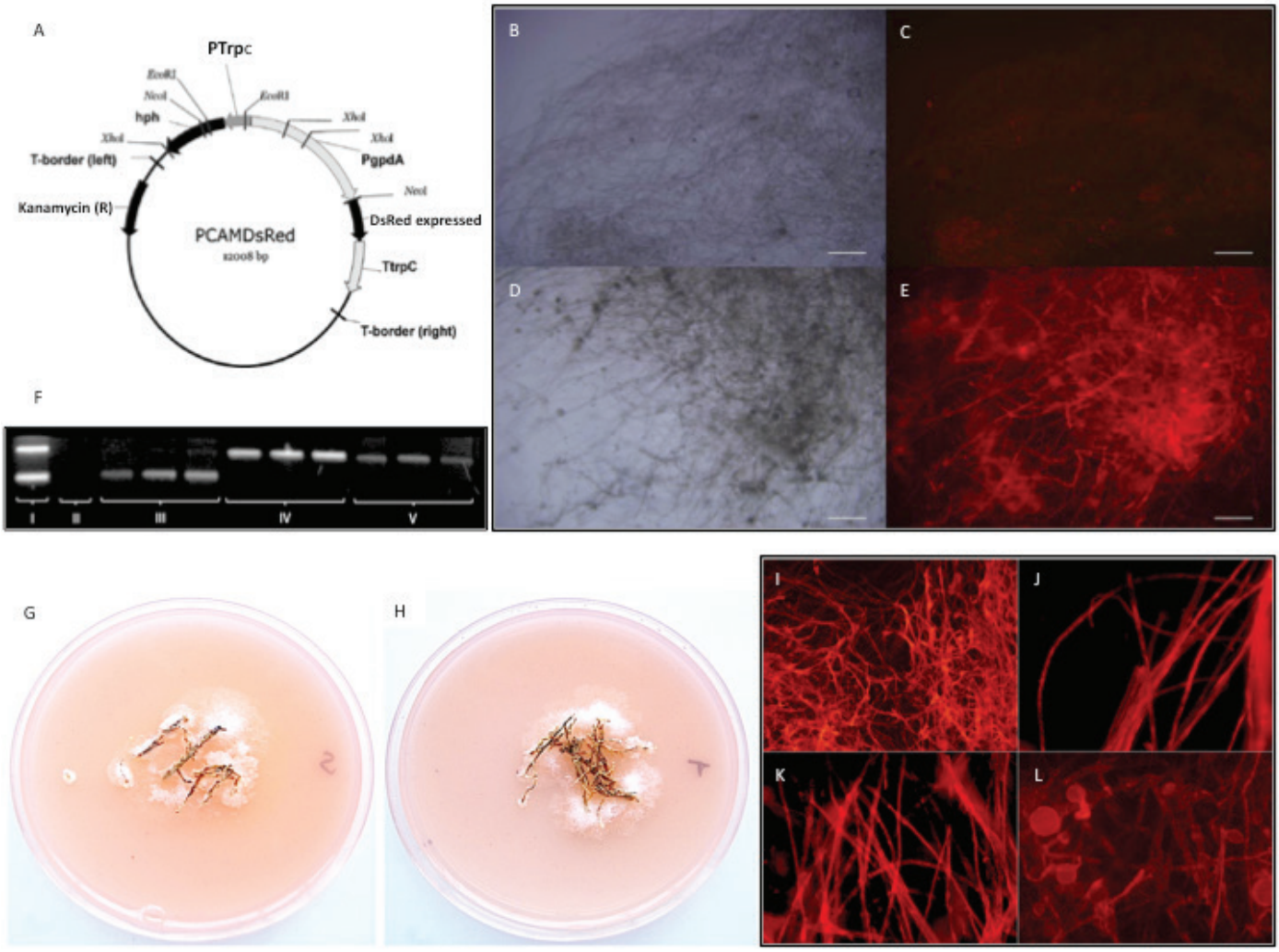

Figure 1. A: Schematic diagram of the PCAMDsRed plasmidial vector used in the transformation of *P. nicotianae* through *A. tumefaciens* (adapted from Eckert et. al.; 2005). B and C: light and epifluorescence microscopy, respectively, of the same area of mycelia from wild type *P. nicotianae*. D and E: light and epifluorescence microscopy, respectively, of the same area of mycelia from the DsRed-*P. nicotianae*. It is possible to notice the stronger fluorescence from the transformed *P. nicotianae* (400x magnification, bars: 20 nm). F: PCR gel electrophoresis confirming the transformation of *P. nicotianae*. I: ladder; II: water (negative control); III: Bands from wild type P. nicotianae DNA (specific primers for ITS region of *P. nicotianae* – PN5 – PN6b); IV: Bands from plasmid DNA (positive control – primers for Hygromycin gene); V: Bands from DsRed-*P. nicotianae* (primers for Hygromycin gene). G and H: DsRed-*P. nicotianae* re-isolated from roots of *C. sunki* and *P. trifoliate,* respectively, in PARPH selective media. I, J, K, and L: Epifluorescence microscopy of the F1 DsRed-*P. nicotianae* re-isolated from *C. sunki* (I and J) and *P. trifoliata* (K and L) roots confirming the stability of the transformation.

(WT-Ppar) and the DsRed-transformed *P. nicotianae* (DsRedPpar) were compared at the same wavelengths, light intensity and gain to check for differential fluorescence. The DsRedPpar showed the same *in vitro* hyphal morphology, growth rate and sporulation rate, but brighter fluorescence emission was visible only for the transformed one, compared with WT-Ppar, which indicated that the DsRed protein production has no impairment in the oomycete physiology (Fig. 1 B, C, D and E). Sporulation induction was performed by daily flooding the Petri dishes with distilled water until sporangium development. The sporangia and the release of zoospores were observed via light microscopy. Furthermore, the T-DNA insertion did not disrupt essential genomic genes or phytopathogenic-related genomic regions. To confirm the successful incorporation of the DsRed gene in the genome of *P. nicotianae*, we extracted the genomic DNA of the WT-Ppar and DsRedPpar mycelia (from 100 mg of ground mycelia, using the Wizard Genomic DNA purification kit, following the manufacture instructions) to perform a PCR with specific vector primers. The DsRedPpar transformants were confirmed by the PCR analysis (Fig. 1 F) and were maintained in our collection for further use.

In order to check if the DsRedPpar still had the ability to reproduce via zoospores and infect citrus plants, we induced sporulation and inoculated 1×10^5 zoospores (per mL per plant) in roots of the citrus rootstocks: *Citrus sunki* and *Poncirus trifoliata*. DsRedPpar was indeed able to infect the plants and was further re-isolated in selective media PARPH (Fig. 1 G and H). The mycelia re-isolated from both citrus rootstocks were again checked for DsRed production by epifluorescence microscopy. Figure 1 I, J, K and L shows the mycelia of DsRedPpar transformants producing DsRed protein after inoculation and re-isolation, which confirms the genetic stability of the transformation.

The first description of a reliable genetic manipulation of oomycetes was done by Judelson et al. (5) in *P. infestan* via PEG-mediated transformation. This method was later successfully expanded to other oomycetes, such as *P. sojae, Saprolegnia monoica, P. palmivora, P. brassica, Pythium aphanidermatum,* and also *P. nicotianae* (7). The PEG-mediated transformation of *P. nicotianae* was done with the insertion of GFP gene (1). This method generated stable transformants and the GFP-transformed *P. nicotianae* was later used for cytological analysis and gene silencing studies (7). However, application of PEG-mediated transformation requires the acquisition of protoplasts of the target organism prior to the transformation, which is the bottleneck of this method, since it is not easy to obtain stable and viable protoplasts of *P. nicotianae* for instance. Other methods such as electroporation and gene bombardment were also used to transform *P. capsici* and *P. infestans*, respectively (8); nonetheless, these alternative methods are clearly more costly and laborious than the AMT method applied in this study.

The highlights of the optimized method presented in this study are: a) It is faster, more cost-effective and less laborious than PEG-mediated transformation, electroporation and gene bombardment. b) It can be done directly on mycelia; there is no need to obtain protoplasts or spores. c) The acetosyringone, which is a relatively expensive compound required for high expression of the virulence genes in transferring the T-DNA, was used at a minimum concentration without compromising the transformation rate. d) Few transformants were lost after sub-culturing for five generations in the absence of hygromycin B (mitotic stability test), and the mitotic stable ones that maintained the DsRed production did not impair their physiology, reproduction or virulence.

The method described here embodies the best alternative to transform *P. nicotianae* and can be used not only with DsRed but also with any other genes of interest, widening the possibilities to study the molecular basis of pathogenicity and the development of control strategies against this plant-pathogen.

ACKNOWLEDGEMENTS

We thank CNPq and FAPESP for financial support, grant numbers: 400675/2013-7, 2015/14498-6 respectively.

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