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Efficient method for isolation of pure genomic DNA and hybridization for TALe gene typing of *Xanthomonas oryzae* pv *oryzae*

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ABSTRACT: *Xanthomonas oryzae pv. oryzae (Xoo) is a a gram-negative ?-proteobacterium pathogenic bacteria causing bacterial leaf blight of rice. The major players in the pathogenicity of Xanthomonas pathovar is a type III secretion system (TTSS) and its associated effectors. Transcription Activator Like effectors (TALe) secreted by Xoo utilizing Type III secretion system (TIIISS) inside the host nucleus form the major class of effectors involved in this disease. Hence, TALe based pathotyping of diverse Xoo strains is opted as a faster way for pathotyping. The TALe gene content in each strain can be profiled by Southern hybridisation using a TALe gene specific probe. However, considering Xoo an excessive polysaccharide producer, the method for pure genomic DNA isolation, probe preparation and Southern hybridization method needed modifications for successful interpretation, which is presented in this study.*

Key words: *Xanthomonas oryzae pv. oryzae, Transcription Activator Like effectors, genomic DNA, southern hybridization, rice*

INTRODUCTION

Bacterial leaf blight (BLB) of rice (*Oryza sativa* L.) is one of the most significant agronomic problems, widely distributed in all the major irrigated rice growing regions of Asia (Gnanamanickam *et al*., 1999). The causal pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), The BLB pathogen, is a a gram-negative ?-proteobacterium which invades the host through natural openings in leaves, including hydathodes or wounds, and colonizes the xylem vessels (Parkinson *et al*., 2007; Salzberg *et al*., 2008). The major players in the pathogenicity of *Xanthomonas* pathovar is a type III secretion system (TTSS) and its associated effectors. The largest T3SS effector family found exclusively in *Xanthomonas* spp.is the AvrBs3/PthA or TALe (**T**ranscription **A**ctivator **L**ike **e**ffector) family (Nino-Liu *et al*., 2006). All TAL effectors have a conserved architecture with a N-terminus required for type III secretion, a central region consisting of a varying number of near-perfect 34-amino-acid repeats, and a C-terminus containing nuclear localization signals (NLSs) and an acidic transcription activation domain (AD) (Gu *et al* 2009, Yang *et al.* 2006). The number and order of the 34-amino-acid repeats determine the specificity between a TAL effector and its target DNA sequence (Boch and Bonas, 2010).With the discovery of the code used by TALes to recognize the binding site in the host, there has been an accelerated need to identify more members from this family (Moscou and Bogdanove, 2009; Boch *et al*., 2009).

Since the host and pathogen evolution is a continuous process, each new pathogenic strain is studied for its structure before deploying a resistant source. Usual method followed for pathotyping is disease assays on different near isogenic lines developed in rice *indica* variety IR24 with various resistance genes (Lore *et al.* 2011). This process being lengthy and labor intensive, molecular typing methods utilized are variable number tandem repeats (VNTR), multi-locus sequence typing (MLST) using IS1112 which is a repeat element native to *Xoo* (Zhao *et al*., 2014; Mishra *et al*., 2013), which provide an easier alternative. In this context, a developing strategy to type the TALe gene content is coming up as an efficient method (Yu *et al.* 2011). This method however is limited by necessary requirements of pure genomic DNA free of polysaccharide contamination as well as clean Southern autoradiograms. The present paper describes the protocol optimised for TALome profiling of Indian *Xoo* isolates.

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MATERIAL AND METHODS

Isolation of genomic DNA

The previous published protocol was further modified as follows. *Xoo* isolate was inoculated in 50 ml GYE media (10 g/l of yeast extract, 20 g/l of glucose) and cells were pelleted at 10,000 xg for 5 min at 4°C. The cell pellet was washed twice with NE (0.2M NaCl, 50mM EDTA). Following washes, pellet was resuspended in 1X TE buffer (10mM Tris-HCl, and 1 mM EDTA (pH-8.0)) after which 400 µl of lysozyme (conc. 100mg/ml) and 100ul of Rnase (10mg/ml) was added and mixed well. Proteins were lysed by proteinase K treatment with incubation for 3 hours at 37°C. Further, 3 ml of 5 M NaCl and 3 ml of CTAB/NaCl (100 mM Tris-HCl (pH8.0), 1.2 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% â- mercaptoethanol, pre-heated to 65°C was added, mixed well and incubated at 65°C for 10 min. To this mixture, 15 ml chloroform: isoamyl alcohol (24:1) was added, briefly vortexed and spun at max speed for 10 min at room temperature. The upper aqueous phase was then carefully transferred to a clean microcentrifuge tube. This C: I purification step was repeated twice followed by precipitation of DNA with 0.6 volumes of isopropanol at -20°C overnight. Next day the sample was spun at max speed for 15 min at 4°C.The supernatant was discarded and pellet was washed with 70% ethanol, air dried at room temp and resuspended in 100 µl of DNase-free water. Concentration and purity of isolated DNA was determined spectrophotometrically. DNA separation was carried out by loading 2 µl of isolated DNA on a 0.8% agarose gel and photographed by an Alpha imager gel documentation system.

Complete digestion of genomic DNA

Two microgram (2 ìg) of genomic DNA samples isolated by the modified method were used for restriction digestion utilizing *Sph*I enzyme which flanks the repeat region of TAL effectors giving digested fragment range of 1.5-4kb. Reaction tubes were incubated at 37°C incubator for overnight and analysed by agarose gel electrophoresis on 1.5% agarose gel along with 1kb+ ladder, run at low voltage for 24 hrs.

Southern hybridisation

The transfer of digested DNA to nylon membrane was done following standard method (Ausubel *et al.*, 1988). Probe was prepared by restricting the pZW*avrXa7* clone (3ug) (Yang and White, 2004) with *Sph* I generating three bands, out of which the 3.1kb band which was gel eluted using Qiagen kit for PCR purification. This DNA was labelled non radioactively with digoxygenin using kit (Roche Applied Science). Instructions as given in the kit manual was further followed with slight on table modifications like replacing glass trays for incubations with glass tubes. The temperature for washing was also standardized at 68°C with 1XSSC (Saline Sodium citrate) (Southern 1975). The probe concentration for maximum yield of radiolabelling was standardized using dilution series. The X ray films were developed after exposure of 5 minutes with Kodak developer and fixer.

RESULTS AND DISCUSSION

The modified procedure for isolation of genomic DNA yielded high quality DNA as documented by spectrophotometer readings taken on nanodrop. The A260/280 ratio was found to be ≥1.8 and A260/230 substantially increased from 1.8 to ≥ 2.0. Therefore, the purity of isolated DNA with much reduced polysaccharide contamination was successfully achieved as any contamination in DNA gives high background in Southern hybridisation. Moreover, our objective being DNA profiling of TAL effectors, it is necessary to have pure DNA to give discreet hybridised bands. The yield of DNA isolated using this method was found to be around 500ng/ul (Fig. 1).

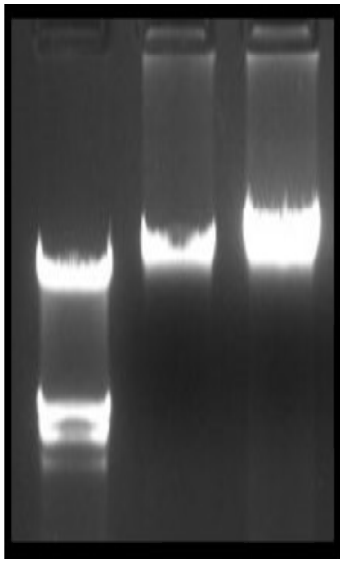

Figure 1: Agarose gel electrophoresis of genomic DNA isolated from *Xoo* with present method.

There was a decrease in comparison to previous protocol which yielded >1ug/ul as shown by

nanodrop. However, this is compensated by the purity which is more relevant in present context. The stickiness experienced earlier with *Xoo* genomic DNA while pipetting it out for complete digestion (Fig. 2) was drastically reduced by this procedure.

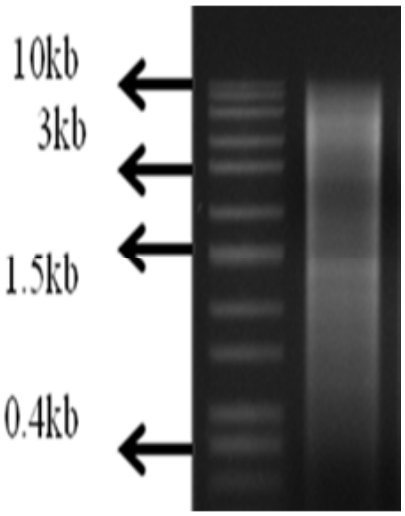

Figure 2: Complete digestion of genomic DNA.

Use of nylon positively charged membrane was preferred. The probe was prepared as described in materials and method (Fig. 1c) critical probe concentration limit was standardized using unloaded membrane and 0.3pg/ul was found to be optimal for our analysis. The minor changes done in the protocol of labelling and hybridization gave a clean profile with no spots and background as was being seen earlier (Fig. 1d) and the DNA bands transformed from thick blobs to discreet sharp bands (Fig 1e).

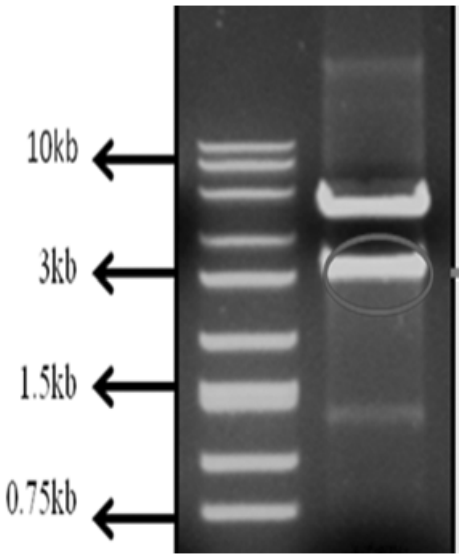

Figure 3: Agarose gel showing the 3.1kb band used as probe

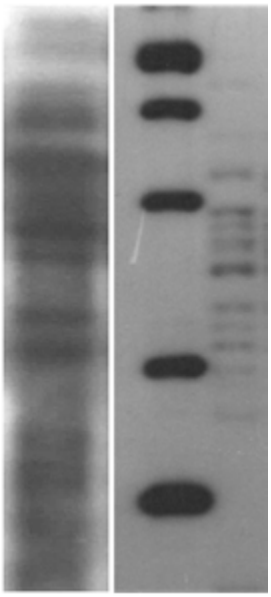

Figure 4: Autoradiogram representing TALe gene profile with polysaccharide contaminated DNA. (Left panel). Autoradiogram representing TALe gene profile developed using protocol described here. (Right panel)

The long running of gel for 24 hrs separated the closely spaced bands between 2-3.5kb which are very crucial to be identified for a TALome profile. Thus, the protocol described here can be followed for Indian *Xoo* isolates TALe profiling successfully.

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