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Short Communication

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A Simple Method of Mass Inoculation of Rice Effective for Both Pathovars of *Xanthomonas oryzae*, and the Construction of Comparable Sets of Host cDNA Libraries Spanning Early Stages of Bacterial Leaf Blight and Bacterial Leaf Streak

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Abstract

Xanthomonas oryzae pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) are important bacterial pathogens of rice (*Oryza sativa*). Despite being very closely related, they infect in different ways and cause distinct diseases. Xoo invades the xylem to cause bacterial blight, whereas Xoc colonizes the leaf mesophyll to cause bacterial leaf streak. As the modes of infection of these two pathogens are different, traditional methods of inoculation effective for Xoo are not effective for Xoc and vice versa. We discovered that dipping plants in bacterial suspension is an effective inoculation method both for Xoo and Xoc and can be used to achieve uniform and synchronized infection of multiple plants. Using this method, comparable sets of rice Gateway® cDNA libraries spanning five time points in the early stages of bacterial blight and bacterial leaf streak were constructed. Prepared under identical conditions, the libraries are ideal for probing differential rice transcription during development of the diseases. Furthermore, by capturing transcripts expressed in response to these unique sources of biotic stress, the libraries are likely to contain cDNA clones not available elsewhere.

Introduction

Bacterial blight and bacterial leaf streak are widely distributed diseases of rice caused by *Xanthomonas oryzae* pathovar (pv.) *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc) respectively. Both pathogens cause significant losses particularly in humid tropical and sub-tropical areas of Asia and Africa where, in the last 30 years, susceptible elite varieties of rice have been cultivated on a large scale (Mew, 1993; Tang et al., 2000). In countries where the diseases are not endemic, such as the US, strict quarantine measures are in place. While the genomes of Xoo and Xoc are very similar (Vauterin et al., 1995; Ochiai and Kaku, 1999) the two cause disease via distinct modes. Xoo spreads systemically through the xylem, typically having entered through hydathodes or wounds. As the disease develops, the pathogen also invades the surrounding mesophyll. In contrast, Xoc does not spread through the xylem, but colonizes the mesophyll parenchyma following entry typically through the stomata (Ou, 1985). Differential inoculation methods for Xoo and Xoc have been reported that support these observations (Xie et al., 1991). Several resistance genes behaving in a Mendelian fashion have been identified in rice against Xoo (Song et al., 1995; Yoshimura et al., 1998; Sanchez et al., 1999; Wang et al., 2001; Blair et al., 2003; Porter et al., 2003); however, no resistance gene has been identified against Xoc. Nevertheless, quantitative (polygenic) resistance against Xoc has been identified in some varieties and is being used in breeding programmes (Tang et al., 2000). As the diseases are economically significant, the pathogens closely related, and the host a staple crop and a model for cereal biology, bacterial blight and bacterial leaf streak constitute an important and powerful comparative system for understanding tissue-specific susceptibility in plant–bacterial interactions.

An important resource for the study of the interactions between rice and *X. oryzae* pathovars is high-quality cDNA from transcripts expressed during development of the diseases. A large collection of full-length cDNA clones generated from different rice tissues under different environmental conditions is available (Kikuchi et al., 2003); however, these conditions do not include biotic stress. Among other published libraries, a handful are derived from pathogen-challenged rice (Chittoor et al., 1997; Chern et al., 2001; Chu et al.,

2004; Lu et al., 2004); however, most of these are from plants undergoing resistance responses. A cDNA library from susceptible plants infected with Xoo has been described (Cho et al., 2004), but to our knowledge none has been published for rice infected with Xoc, and none suitable for direct comparison of Xoo- and Xoc-infected plants is available.

Isolation and characterization of host genes that condition susceptibility in bacterial blight and bacterial leaf streak of rice is an important goal. Towards this goal, we set out to generate comparable cDNA libraries for Xoo- and Xoc-infected rice. For this purpose, we sought a method that would allow uniform inoculation of multiple plants simultaneously, and be effective for both *X. oryzae* pathovars. We report here the development of this method and the construction of high-quality cDNA libraries it enabled.

Materials and Methods

Plant material

Oryza sativa ssp. *japonica* cv. Nipponbare plants were grown four to six per pot of 5 cm^2 containing LC-1 soil mixture (Sungro, Bellevue, WA, USA), and arranged in flats of 20 pots, in growth chambers under a 12-h, 28°C light and a 12-h, 25°C dark cycle. To allow inversion of the flats for inoculation, seeds were sown through 1-cm perforations in a fibreglass tray resting on top of the pots. Fertilizer (Peters Professional, St Louis, MO, USA) and iron chelate micronutrient (Becker Underwood, Ames, IA, USA) were applied with daily watering at rates of 0.25 and 4.5 g/l, respectively, until the day before inoculation.

Bacterial strains and preparation of inoculum

Xoo strain KXO85 and Xoc strain BLS256 (provided by Jan Leach, Colorado State University, USA) were used. For each, a single colony from a glucose yeast extract (GYE; 20 g glucose, 10 g yeast extract per litre) agar plate was transferred to 5 ml of GYE liquid medium and incubated for 24 h at 28°C with constant shaking at 250 r.p.m. Subsequently, 1 ml of this culture was transferred to 260 ml of fresh GYE liquid medium and incubated as above for an additional 18 h. Cells were pelleted by centrifugation at $3200 \times \boldsymbol{g}$ for 10 min, washed twice and resuspended in sterile 10 mM $MgCl_2$ to an OD_{600} of 0.08, yielding approximately 7 l of inoculum. Tween-20 was added to a final concentration of 0.5%.

Inoculation and tissue harvest

Twenty-one days after sowing, and 1 h before the light period, seedlings were inverted into inoculum for 2 min, one flat each for Xoo, Xoc and mock inoculum consisting of sterile 10 mM $MgCl_2$ with 0.5% Tween-20. Following inoculation, plants were returned to the growth chamber. Three replicate inoculations were carried out at 1-week intervals. During growth, plants were cycled weekly in the same order through three growth chambers. In this way all plants could be incubated in the same final chamber following inoculation. For each replicate, position of the flats in the growth chamber (used also for the order of inoculation and harvest) was determined by a Latin square design. Within flats, assignment of pots for the harvest of inoculated tissue at 2, 4, 8, 24 and 96 h was made using a separate randomized complete block design. Tissue was harvested from plants in four pots (approximately 2 g fresh weight) for each time point per inoculum per replication. Harvested tissue was immediately frozen in liquid nitrogen and stored at −80°C until processing.

Bacterial population growth assays

In each replicate, population growth of KXO85 and BLS256 in inoculated plants was monitored by dilution plating of leaf homogenates every 2 days beginning on day 1 and ending on day 7 after inoculation. For each sampling, a single plant was selected at random from each inoculated flat and its leaves ground in 35 ml of sterile water using a Polytron (Brinkmann Instruments, Westbury, NY, USA). Dilutions of the homogenates were spotted on selective agar plates (GYE containing 50 μg/ml of cycloheximide and 20 μg/ml of cephalexin). Numbers of CFUs per square centimetre of leaf were determined following incubation of the plates at 28°C for 3 days.

cDNA library construction and characterization

Total RNA was isolated from frozen tissue following the hot Trizol protocol described by Caldo et al. (2004). PolyA+ RNA was purified from 2.5 mg of total RNA using Oligotex mRNA Spin-Columns (Qiagen, Valencia, CA, USA), and samples from each replicate were pooled in equal amounts. Five micrograms of each pool were used for the construction of each library. Ten Gateway® cDNA libraries, one for each of the five time points following inoculation with KXO85 or BLS256, were constructed using the CloneMiner Library Construction Kit (Invitrogen, Carlsbad, CA, USA). Titre for each library was determined by dilution plating according to the CloneMiner protocol. Percentage of recombinants and average insert length were estimated by analysis of restriction fragments resulting from digestion of 30 plasmids from each library with *Bsr*GI (New England BioLabs, Beverly, MA, USA).

Library expansion

Libraries were expanded by amplifying the primary transformants in semi-solid 2xLB medium with 50 μg/ml kanamycin at room temperature without disturbance for 45 h, according to a protocol supplied by the CloneMiner manufacturer (Invitrogen). For each library, cells were finally resuspended in 100 ml LB supplemented with 15% glycerol. Plasmid DNA was extracted from a 1/10 volume aliquot of each library using the HiSpeed Plasmid Midi Kit (Qiagen), dissolved in TE buffer at 1 μg/μl, aliquoted at 5 μl, vacuum-dried, and stored at −80°C for future use. The

remaining suspension was aliquoted in 10 ml, 1 ml and 100 μl and stored at −80°C for future use.

Results and Discussion

Xie et al. (1991) reported differential methods of inoculation for Xoo and Xoc. They observed that inoculation into guttation fluid in early morning favoured Xoo infection, and conversely brushing bacterial suspension on abaxial leaf surfaces was effective only for Xoc. In order to construct comparable sets of cDNA libraries for rice plants developing bacterial blight and bacterial leaf streak, we sought an inoculation method that would be effective for both Xoo and Xoc. First we assayed a leaf-clip technique commonly used in screening for resistance to Xoo in breeding programmes (Kauffman et al., 1973), and a syringe infiltration method typically used in screening for the resistance-associated hypersensitive response (Reimers and Leach, 1992). The leaf-clipping method was ineffective for Xoc, and the syringe-infiltration method, although resulting in rapidly spreading lesions for Xoc, produced only localized lesions for Xoo (Fig. 1a). We then assayed briefly inverting plants in bacterial suspension supplemented with a surfactant, followed by incubation in a growth chamber. This method resulted in symptom development and growth of the pathogen population throughout the plant for both Xoo and Xoc. Greyish white, opaque lesions typical of bacterial blight developed on Xoo-inoculated leaves by day 6. On leaves of Xoc-inoculated plants, interveinal water soaking was apparent by day 4, which developed into yellow, translucent lesions with droplets of bacterial exudates typical of bacterial leaf streak by day 6 (Fig. 1a). Mock-inoculated plants showed no symptoms or signs of infection (not shown). Over a 7-day period, the population of Xoo increased an average of 100-fold. Xoc numbers increased almost 1000-fold (Fig. 1b). This difference in growth may be due to inherent differences in the development of the two diseases as well as in the relative virulence of the two strains used in this study. In addition, the conditions under which the assays were carried out (e.g. temperature, plant age, etc.) may have been more favourable to Xoc. Nevertheless, the dip method is simple and was effective for both diseases, and, in contrast to the clip and syringe-infiltration methods, permitted uniform and synchronous inoculation of many plants. In addition to Nipponbare, the dip method was also effective for the Indica variety IR24 (data not shown). This method therefore is well suited to many different applications, including comparative studies of molecular pathology or disease physiology, and experiments requiring large amounts of inoculated tissue or moderate amounts harvested over a time course. Depending on the objective, the method could be modified for even greater throughput. For disease resistance breeding or mutant screening, for example, planting through wire or nylon mesh instead of perforated trays would allow growth and inoculation of 300 or more seedlings per flat. In a greenhouse, tens of thousands of plants could be readily screened.

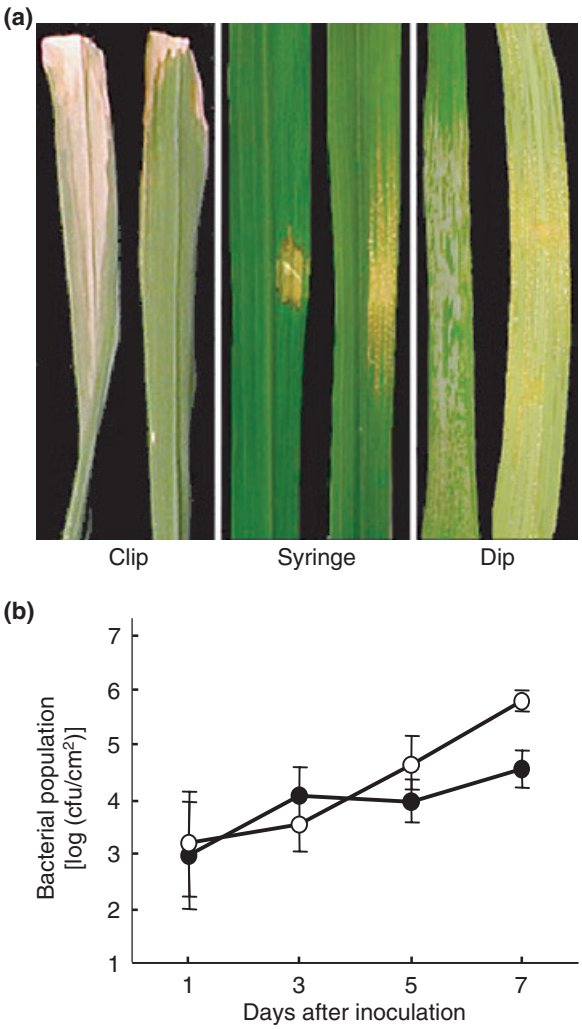

Fig. 1 Dip inoculation, in contrast to clip inoculation or syringe infiltration, is effective for both *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc). (a) Symptoms on rice leaves 7 days following inoculation with either Xoo (left) or Xoc (right) using the indicated methods. (b) Population growth of Xoo strain KXO85 (filled circles) and Xoc strain BLS256 (open circles) in rice plants following dip inoculation. The mean values of three replicate experiments are shown. Capped vertical bars denote standard error

Using this method, we constructed separate, comparable sets of cDNA libraries from leaf tissue of susceptible rice, harvested over a time course following inoculation with Xoo and Xoc. Cultivar Nipponbare was used because its genome has been completely sequenced by the International Rice Genome Sequencing Project (http://rgp.dna.affrc.go.jp/IRGSP/). For inoculation, we chose Xoo strain KXO85 and Xoc strain BLS256, which are both virulent to Nipponbare. Furthermore, the entire KXO85 genome sequence has been published recently (Lee et al., 2005), and the BLS256 genome is currently being sequenced by our laboratory in collaboration with others. The cDNA libraries were constructed for entry into the Gateway® system (Invitrogen). This system, based on *in vitro* recombination technology, allows rapid transfer of inserts, individually or *en masse* without loss of orientation, frame or

representation, into destination vectors for a variety of applications, including gene expression, epitope-tagging, and yeast-two-hybrid analysis. Thus, these library sets are ideally suited not only for isolation of cDNAs corresponding to genes differentially expressed in response to Xoo and Xoc, but also for straightforward manipulation of the cDNAs.

Our purpose for the libraries is to provide cDNA for genes of interest identified through microarray analyses ongoing in our laboratory. By capturing transcripts expressed in response to these unique sources of biotic stress, the libraries are likely to contain cDNA clones not available elsewhere. They are of value as a potential source for these and other cDNAs, and as a resource for other approaches to differential gene expression analysis as well (e.g. subtractive hybridization and cDNA-amplified fragment length polymorphism analysis). For the latter, we anticipate that they will be useful by themselves or in combination with other libraries, such as those generated from rice plants undergoing a resistance response to Xoo, displaying quantitative resistance to Xoc, or undergoing other kinds of biotic or abiotic stress. The libraries may be particularly useful to researchers in countries where Xoo and Xoc are under quarantine, who are interested in comparing rice responses with different types of stress, but do not have the necessary permits and containment facilities for these pathogens.

Across all libraries, the estimated average number of clones was 9.3×10^6, the average percentage recombinants was 96% and the average insert length was 997 bp (Table 1). Distribution of observed insert lengths ranged from 0.2 to 3.4 kb, with 43% of the clones containing inserts between 0.6 and 1.2 kb (Fig. 2). Procedures for obtaining the libraries can be found at http://www.xoos.org.

Table 1
Estimated titre in CFUs per millilitre, average insert length, and percentage of recombinants for 10 cDNA libraries generated from rice leaves undergoing infection by *Xanthomonas oryzae* pv. *oryzae* (Xoo) or *X. oryzae* pv. *oryzicola* (Xoc) following dip inoculation

		Insert length (kb)			
Library	CFUs	Minimum	Maximum	Average	% recombinants
XOC2	1.0×10^7	0.2	2.5	0.9	97
XOC4	7.1×10^6	0.2	1.9	0.9	93
XOC8	5.7×10^6	0.2	3.1	1.0	93
XOC24	6.9×10^6	0.2	1.8	0.8	93
XOC96	5.1×10^6	0.3	2.3	1.1	97
XOO2	1.4×10^7	0.4	3.0	1.0	97
XOO4	1.8×10^7	0.4	3.0	1.3	100
XOO8	5.1×10^6	0.2	2.9	1.1	93
XOO24	5.1×10^6	0.2	2.3	0.9	97
XOO96	1.6×10^7	0.4	1.8	1.0	100

Each library ID displays the pathovar of *X. oryzae* used for inoculation and the hours after inoculation at which plant tissue was collected.

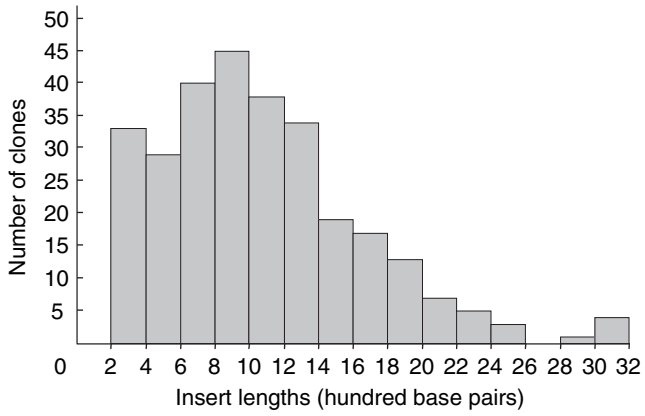

Fig. 2 Distribution of insert lengths for 300 clones from 10 cDNA libraries (30 from each library) generated from rice cv. Nipponbare leaves undergoing infection by *Xanthomonas oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola* following dip inoculation

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