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Full Length Research Paper

Host induced changes in plasmid profile of *Xanthomonas axonopodis* pv. *malvacearum* races

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We investigated host-induced changes of plasmid profile in two laboratory subcultured races of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*). Laboratory subcultured isolates contained fewer plasmids (i.e. two plasmids of size 60 and 40 kb) presumably due to loss or undetectable low copy number during subculturing. When these isolates were grown in the presence of leaf extract and intercellular fluid obtained from cotton differentials, the number of plasmids increased and the plasmid profile resembled those of the natural isolates. Based on known facts about genome rearrangement, we speculate this as an adaptation strategy for *Xam* to increase copy number of genes involved in pathogen aggressiveness which are otherwise present as single copy in bacterial chromosome and this possibly occurred by induction from host elicitors present in leaf extracts.

Key words: *Xanthomonas axonopodis* pv. *malvacearum*, plasmid profile, leaf extract, intercellular fluid.

INTRODUCTION

Bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* (E.F. Smith) Dye (*Xam*) is known to be one of the most devastating disease in cotton (Delannoy et al., 2005). The disease is most prevalent in regions with high relative humidity which provides congenial conditions for proliferation and spread of the causative agent (*Xam*) (Voloudakis et al., 2006). Several isogenic cotton lines carrying bacterial blight resistance genes (designated as *B*-genes) that govern resistance to *Xam* races have been developed (Brinkerhoff, 1963; Essenberg et al., 2002). However, highly virulent races attacking major blight resistant genes (*B*-genes namely B_7, B_2, B_{In} and B_N) are reported to be widely distributed in India (Verma, 1986).

Most plant pathogenic bacteria are known to harbor plasmids carrying genes responsible for virulence, antibiotic resistance and exopolysaccharide production (Sundin, 2007). Sathyanarayana and Verma (1993) found that highly virulent races of *Xam* also harbor at least five plasmids. Furthermore, avirulent and plasmid cured strains of *Xam* races were defective in exopolysaccharide production and virulence indicating that the plasmids harbored by *Xam* races might be the primary determinants of virulence (Chakrabarty et al., 1995). Das (1997) worked on several races of *Xam* and concluded that the large sized plasmids (60 and 40 kb) (responsible for maintaining pathogenicity) were common to most races while there was great variability in the number of smaller plasmids which are required for extra aggressiveness. In our previous study we not only found variations in number of miniplasmids harbored by different *Xam* races, but also noticed the instability of these miniplasmids during continuous storage and subculturing conditions resulting in reduced virulence. However, complete restoration of plasmid profile was observed when laboratory isolates were inoculated and re-isolated from the host (Narra et al., 2004).

Verma and Singh (1974) observed similar loss of virulence in *Xam* isolates stored on Yeast-Glucose-Chalk-Agar media. Based on infectivity studies on highly susceptible barbadense cultivars, they concluded that the extra aggressiveness (virulence for *B*-genes) was lost

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Abbreviations: HR, Hypersensitive response; **ICF,** intercellular fluid; **IS,** insertion sequence.

and the race maintained/retained only sufficient virulence required for attacking barbadense cultivars. The regaining of extra aggressiveness by the pathogen through reinoculation/ passing through the host genotypes (Narra et al., 2004) coupled with the fact that *Xam* proliferates in the protected sites of the substomatal chambers (Wilson et al., 1999), show that elicitor molecules present in host tissue are playing a crucial role in the regeneration of complete virulence in the otherwise less aggressive strains. Moreover, several organic compounds present in leaf extract and intercellular fluid are known to act as signal molecules/activators for virulence gene expression during the infection process (Li et al., 1998). Considering the role of plasmids in virulence and its rejuvenation upon passage through the host, the influence of host leaf extracts and intercellular fluid on the plasmid number and profile still remain elusive. Therefore, the present study is aimed at understanding the effects of host extracts on plasmid profile of two highly virulent *Xam* races.

MATERIALS AND METHODS

Maintenance and preservation of bacterial cultures

Two races of *Xam*: race 27 (overcomes three *B*-genes i.e. B_7, B_2 and B_{In}) which can produce a susceptible reaction (watersoaking symptoms) on all cotton differentials except for 20-3 (B_N+B_{Sm}) and 101-102.B (B_2B_3+B_{Sm}) and race 32 (overcomes five *B* genes *i.e.* B_7, B_2, B_4, B_{In} and B_N) which can produce a hypersensitive response (HR) only on cotton differential 101-102.B (B_2B_3+B_{Sm}) (attacks all other differentials) were subcultured 35 times at two week intervals. This repeated subculturing resulted in isolates which retained only 60 and 40 kb plasmids and were termed as “laboratory subcultured races (LS-races)” and were used in this study. The LS-races were stored in 50% glycerol at -70°C.

Preparation of leaf extract

Leaf extracts were prepared from young leaves collected from *Gossypium hirsutum* differentials- Stoneville-2B.S9, Stoneville-20, Mebane-B.1, 1-10.B, 20-3 and 101-102.B carrying *B*-genes namely B_{Sm}, B_7+B_{Sm}, B_2+B_{Sm}, B_{In}+B_{Sm}, B_N+B_{Sm} and B_2B_3+B_{Sm}, respectively. Two grams of leaf tissue was rinsed in sterile water and ground in 5 ml of extraction buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 2 mM NaCl) and centrifuged at 6000 rpm for 10 min in a Sorvall RC-5C centrifuge. The supernatant was then sterilized by filtration through 0.2 µm filters (Gelman Sciences Inc., USA) and stored at -70°C for further use.

Preparation of intercellular fluid

Extraction of intercellular fluid (ICF) was performed as described by Hogue and Asselin (1987). Young leaves were collected from *G. hirsutum* differential 101-102.B (B_2B_3+B_{Sm}), rinsed and washed in sterile water for 15 min. The leaves were vacuum infiltrated with sterile water for 10-15 min at room temperature, blot dried on sterile filter paper and rolled into a plastic syringe. The syringe was placed hub down in an appropriate sized centrifuge tube and centrifuged at 6000 rpm for 10 min. The ICF collected at the bottom of the tube was sterilized by filtration through 0.2 µm filters (Gelman Sciences Inc., USA), and stored at -70°C until use.

Nutrient amendment assay

Nutrient amendment was tested by adding 100 µl of the leaf extract or ICF to 50 ml of nutrient broth which was then inoculated with a single bacterial colony and incubated at 28°C overnight at 110 rpm before plasmid isolation. Addition of more than 100 µl of leaf extract or ICF to the media had an inhibitory effect on growth of *Xam* (Data not shown).Three independent replicates were carried out for each experiment. Plasmid isolation and gel electrophoresis were performed as described earlier (Narra et al., 2004). A 20 kb band was consistently detected in all plasmid isolations that is, LS-races before treatment with host leaf extract or ICF (control) and after treatment. However, other plasmids, except for 60 and 40 kb, were detected only under certain nutrient amended conditions indicating that the 20 kb band corresponded to the genomic DNA of *Xam*, and was excluded from the analysis. Similar observations were reported by Sathyanarayana and Verma (1993) and Das (1997).

RESULTS AND DISCUSSION

When ICF from cotton differential 101-102.B (carrying *B*-genes B_2B_3+B_{Sm}) was added to the medium in which the LS-races of *Xam* were grown, race 32 showed the presence of plasmids ca 60, 40, 23, 8.2, 3.7 and 1.6 kb and the later four plasmids were induced by ICF that were earlier not detected in the LS-races (control). In race 27, three plasmids, earlier undetected in LS-race were found and corresponded to size ca 23, 8.2 and 3.7 kb (Table 1). However, the concentration of the 3.7 kb plasmid was extremely low indicating a low copy number plasmid.

When exposed to host leaf extract from *G. hirsutum* differentials, LS-race 27 showed the presence of a 23 kb plasmid in all treatments irrespective of the leaf extract tested (Table 1). Leaf extract from Mebane B-1 and 20-3 induced the appearance of 8.2 kb plasmid while leaf extract from 101-102.B induced the detection of a 3.7 kb plasmid in addition to the 8.2 kb plasmid (Table 1).

Growth of LS-race 32 in media supplemented with host leaf extract from Mebane B.1, 20-3 and 101-102.B induced the appearance of two plasmids ca 23 and 8.2 kb plasmid in addition to the 60 and 40 kb plasmids which were already present in this strain. An additional 3.7 kb plasmid was detected after treatment with leaf extracts from 20-3 and 101-102.B, and a 1.6 kb plasmid only after treatment with a leaf extract from 101-102.B (Table 1). Thus, leaf extract from Mebane B.1 induced detection of two additional plasmids (23 and 8.2 kb), differentials 20-3 and 101-102.B induced the detection of three (23, 8.2 and 3.7 kb) and four additional plasmids (23, 8.2, 3.7 and 1.6 kb), respectively.

Sathyanarayana and Verma (1993) using the method of Kado and Liu (1981) isolated five plasmids from a highly virulent race 32 and three plasmids from moderately virulent race 26. Later on, more than three plasmids were consistently reported from virulent races of *Xam* (Chakrabarty et al., 1995). In this study, using commercial Qiagen® medi kit, we were able to detect six

Table 1. Influence of host intercellular fluid (ICF) and leaf extract from *G. hirsutum* differentials (with *B*-genes) on the plasmid profile of *Xanthomonas axonopodis* pv. *malvacearum* races 27 and 32.

Plasmid size (kb)	Plasmid profile of LS races (control)		Plasmid profile of LS races after treatment with ICF from 101-102.B (B_2B_3+B_{sm})		Plasmid profile of LS races after treatment with host leaf extract from cotton differentials											
					Stoneville 2B.S9 (Bsm)		Stoneville 20 (B_7+B_{sm})		Mebane B.1(B_2+B_{sm})		1-10B (B_{in}+ B_{sm})		20-3 (B_N + B_{sm})		101-102.B (B_2B_3 +B_{sm})	
	27	32	27	32	27	32	27	32	27	32	27	32	27	32	27	32
60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.2	-	-	+	+	-	-	-	-	+	+	-	-	+	+	+	+
3.7	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
1.6	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+

LS-races: Laboratory subcultured races; +, plasmid detected; -, plasmid not detected.

plasmids (ca 60, 40, 23, 8.2, 3.7 and 1.6 kb) in the most virulent race 32 (which can overcome five *B*-genes) and five plasmids (ca 60, 40, 23, 8.2 and 3.7 kb) in race 27 (which can overcome three *B*-genes). The variation in the number of plasmids detected is attributable to the differences in the protocols followed by plasmid isolation as low copy plasmids can potentially be lost during the purification procedure.

Leaf extract and ICF contain sugars, carbohydrates, proteins which support bacterial growth (Theobald et al., 2005). Furthermore, citric, malic, shikimic and quinic acids and β-glucanases and chitinases present in leaf extract and ICF may also act as signal molecules involved in virulence gene activation in the pathogen leading to infection (Cavalcanti et al., 2006; Li et al., 1998). Rahme et al. (1992) observed an elevated *hrp* gene expression in *Pseudomonas syringae* pv. *syringae* grown in minimal media supplemented with high concentrations of citric acid (present in leaf extract). Similar increase in gene expression was observed when *P. syringae* pv. *tomato* DC3000 was grown in media supplemented with crude tomato leaf extract which contained citric and malic acids (Li et al., 1998). In this study, gradual restoration of plasmid profile was observed when LS-races were exposed to leaf extract from cotton differentials and the increase in plasmid number correlated with the degree of resistance confirmed by cotton differentials (Verma, 1986). A 23 kb plasmid was detected in both races when leaf extract from susceptible differentials (produce watersoaking symptom during infection) was added to the growth media. However, smaller plasmids (3.7 and 1.6 kb) were detected only when leaf extract and ICF from resistant differentials (produce HR) was added to the growth media. Occurrence of pathogenicity genes on large sized plasmid was reported in *P. syringae* pv. *tomato* (Hanekamp et al., 1997). Yang et al. (1996) was able to identify six plasmid-borne effector genes in *Xam* which were responsible for the production of water soaking (susceptible reaction) symptom during infection. Based on these reports and results obtained in this study, it may be concluded that genes required for generation of watersoaking symptoms are harbored mostly on a 23 kb plasmid whereas smaller plasmids especially the 3.7 and 1.6 kb harbor genes involved in HR.

Most bacterial plasmids are dynamic in nature. Gain and loss of genes on plasmids was shown to be high when compared to the chromosome (Sundin, 2007). Formation of high frequency recombinants by incorporation of plasmids into the chromosome is well documented in bacteria (Hofemeister et al., 1983). Plasmids are also known to carry integrases and transposases which facilitate rapid DNA rearrangements and exchange (Ruan and Xu, 2007). The integration and excision mechanisms of plasmids are known to occur by general recombination or site-specific recombination depending on the size of the recombining DNA regions (Rohmer et al., 2003). Zhao and co-workers (2005) hypothesized that the integration of plasmids into the chromosome might be the first step for potentially stabilizing the virulence determinants in the genome. Earlier studies have confirmed that mitochondrial and chloroplast DNA can incorporate into the nuclear DNA of the host (Huang et al., 2003; Ricchetti et

al., 1999) and the inclusion of plasmid-borne genes on the chromosome by recombination events or gene duplications has been reported (Pérez-Martínez et al., 2008).

However, site of integration, stabilization and precise excision of the plasmids from host chromosome play a predominant role in maintaining their pathogenicity against the host. Szabo and Mills (1984a, b) found that recombination events between the repeats in the chromosome showing sequence similarity with insertion sequence (IS) elements led to imprecise excision of *P. s.* pv. *phaseolicola* plasmid pMMC7501 into eight smaller plasmids. Jackson et al. (2000) reported that a 42 kb plasmid carrying *avrPphB* in *P. s.* pv. *phaseolicola* could successfully be excised from the host chromosome but could not be maintained due to deleterious genomic rearrangements. Considering these reports, it may be concluded that detection of plasmids is largely due to the excision of chromosome fragments induced by host elicitors. More importantly, the present study provides a viable *in vitro* assay mimicking pathogen growth in a host plant.

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