AWARDS AND HONOURS

J.P. Verma Memorial Award Lecture

Dialogue between Xop-T3SS effectors of *Xanthomonas oryzae* pv. *oryzae* and rice during bacterial blight pathogenesis

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ABSTRACT: Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is a major biotic threat to rice production globally. Xoo initiates dialogue with rice plant through its effectors that are secreted via type 3 secretion system (referred to as T3SS effectors). The result of this dialogue leads to the phenotype of either susceptible or resistance reaction. Xoo secretes two type of T3SS effectors, Xop (Xanthomonas outer protein) and TAL (Transcription activator like) effectors. Screened the total composition of T3SS effectors in one of the most virulent Xoo race 4 from India. Xoo race4 contains 21 Xop and 18 TAL effectors. This paper discusses recent insight into the dialogue between Xop T3SS and rice. Studied inducible, *hrp*-dependent expression pattern of the Xop T3SS effectors. We created null mutants for each Xop T3SS effectors to understand their function in blight induction. Our subsequent investigation revealed that major Xop T3SS effectors like XopF, XopR plays crucial role during blight pathogenesis. The major effectors regulate the *in planta* Xoo growth and suppress PAMP-triggered immune (PTI) response in rice. Though there was no aberration in phenotypic as well as *in vitro* growth characters between wild and mutants, but mutants showed significant reduction during *in planta* colonization relative to the wild strain. The RT-qPCR analysis indicated significant fold increase in expression of the PTI marker genes in rice upon challenge-inoculation with mutants suggesting the role of effectors in suppressing rice PTI. It is evident that mutants induced more callose deposition in infected rice leaves. When transiently expressed in *Nicotiana benthamiana*, fusion effector gene products get localized to the plasma membrane. Collectively, our insight into the Xop T3SS effectors leads to the understanding that the major T3SS effectors like XopF, XopR suppress rice immune responses, and thereby favours Xoo for its *in planta* growth and pathogenicity during bacterial blight development.

Keywords: Bacterial blight, rice, Type III effectors, *Xanthomonas*, Xop effectors

Through this article, I pay my tribute to the Renowned Plant Bacteriologist Late Professor Jeevan Prakash Verma, who mentored my PhD programme and still remain in my heart as great source of inspiration to carry out my research in the bacterial world.

Bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is an important biotic constraint to the rice productivity globally. Xoo suppress innate immune responses of rice through secreting effectors directly into rice cells via a secretory channel referred to as type III secretion system (T3SS) (Jones and Dangl, 2006). The T3SS are regulated by *hrp* (hypersensitive response and pathogenicity) genes and in absence of a functional T3SS, bacteria cannot grow into the host plants (Mudgett, 2005). The effectors of *Xanthomonas* outer

protein (Xop) family (non-TAL effectors) function as major virulence determinants (Kim *et al.*, 2008; Kumar and Mondal, 2013; White *et al.*, 2009) in Xoo. The role of many Xop effectors, including XopD, XopJ, XopN, XopR, XopZ in governing virulence to *Xanthomonas* were demonstrated and the functional motifs of these Xop effectors were identified based on similarities with the known motifs or sequences in other *Xanthomonads* (Akimoto-Tomiyama *et al.*, 2012; Bartetzko *et al.*, 2009; Kim *et al.*, 2009; Kumar and Mondal, 2013; Song and Yang, 2010). XopN of *X. campestris* pv. *vesicatoria* was predicted to interact with tomato a typical receptor-like kinase 1 (TARK1) and four tomato 14-3-3 isoforms (Kim *et al.*, 2009) during the suppression of PTI in tomato. XopD of the same pathogen was reported to desumolyze plant proteins (Kim *et al.*, 2008) while XopJ was shown to be involved in the ubiquitin/proteasome system and help in removing SUMO proteins, resulting in the inactivation of SUMOylated proteins (Bartetzko *et al.*, 2009). XopN of *Xanthomonas axonopodis* pv. *punicae*, causing bacterial blight of pomegranate was known to

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suppress cell wall associated immune response in pomegranate (Kumar and Mondal, 2013, Kumar *et al.*, 2016). XopZ of Xoo Philippine strain PXO99A was reported to be crucial for the suppression of PTI in rice and two identical copies of XopZ are essential for full virulence (Song and Yang, 2010). XopR of Xoo Japanese strain, MAFF311018 was found to act as a repressor of MAMP-induced plant innate immune responses (Akimoto-Tomiya *et al.*, 2012). Xop effectors, like XopF and XopR of Indian Xoo strain were recently investigated for their possible role in virulence and in the suppression of PTI responses in rice. This chapter will discuss few highlights of the recent progress in this direction (Mondal *et al.*, 2016).

MATERIALS AND METHODS

Bacterial strains and media used

Xoo strain was grown at 28°C in PSA medium (peptone 10g, Sucrose 10g, Na-glutamate 1g, Agar 20g). Xoo $\Delta hrpX$, a T3SS-defective mutant and Xoo $\Delta xopF$, $xopF$ gene specific mutant, Xoo $\Delta xopR$, $xopR$ gene specific mutant were employed for this study. Xoo $\Delta hrpX$ was used as a negative control set.

Hrp-dependent expression of *xop* genes

Bacteria were cultured at 28°C with shaking at 200 rpm in NB (Difco Laboratories) or XOM2 medium (Tsuge *et al.*, 2002). Total bacterial RNA was extracted by using kit (Fermentus, USA) according to the instructions of the manufacturer. cDNA synthesis was done using cDNA synthesis kit (Thermo Scientific Inc., USA) following the user's manual. cDNA derived from 50 ng of RNA was used for each reaction of semiquantitative PCR (semi-qPCR). Semi-qPCR for *Xop* genes expression was performed by using gene-specific primers. Ribosomal 16S RNA expression was used as an internal control.

Studying the role of Xop effectors in bacterial growth and virulence

Fully expanded leaves of 30 days old rice seedlings (cv. pusa basmati 1) was inoculated with bacterial suspension (1×10^9 CFU ml⁻¹) by syringe infiltration techniques and kept in glass house with 100% relative humidity. The Xoo wild as well as mutants and complemented strains were used. The bacterial count was taken at 24h intervals, up to 5 days. For virulence assays leaf-clipping method (Kauffman *et al.*, 1973) was used and after 21 days. The lesion area (in cm²) was measured using Image J software. The data were statistically validated using one way ANOVA analysis.

Assays for callose deposition in rice leaves

Callose deposition assay was performed using a modified protocol of Goel *et al.* (2008). Briefly, 30 days old plants were inoculated with Xoo wild and mutant strains using syringe infiltration technique. After 12 h, the infiltrated regions were washed and stained with 0.01% aniline blue

in 70 mM K₂HPO₄, pH 9.5 and incubated at 65°C for 30 min until they were completely cleared of chlorophyll. The autofluorescent callose spots were examined using confocal microscope (Leica Microsystems, Solms, Germany).

Expression analysis of PTI-marker genes

The relative expression of PTI-marker genes, *PR10*, *OsWRKY13*, *OsRLCK16* and *OsFLS2* was analysed in rice challenged with Xoo strains both in presence or absence of XopF. Total RNA from inoculated rice leaves was extracted using an RNA easy mini kit (Thermo Scientific Inc., USA) at 2 and 4 h of post inoculation. cDNA synthesis for RT-qPCR was done. Real-time PCR was performed using SYBR Green Kit (Qiagen). The expression was normalized using rice *Actin1*.

Localization assay of transiently expressed Xop fusion protein

The subcellular localization of XopF::EYFP fusion protein was studied in two host systems, *Nicotiana benthamiana*, and onion. In both cases, the *Agrobacterium tumefaciens* EHA105 transformed with XopF::EYFP fusion plasmid construct, were infiltrated. Two days after infiltration, the expression and accumulation of XopF::EYFP protein in cells was observed using confocal microscope (Leica Microsystems). An empty construct (without XopF gene) was used as mock. The experiment was repeated twice with three replications each time.

RESULTS

Hrp-dependent expression of *xop* genes

Both the *xop* genes, *xopF* and *xopR* were shown to be *hrp*-dependent for their expression. The expression of the *xop* genes was observed only in XOM2 medium while no amplification was seen in un-induced conditions as in the case of wild Xoo and Xoo $\Delta hrpX$ grown in NA. However, in all cases, amplification of 16S rRNA (used as control) was observed irrespective of the media used (Table 1).

Xop effectors are dispensable for *in vitro* Xoo growth

We investigated the involvement of Xop effectors during bacterial growth *in vitro*. We did not find any difference in bacterial growth in Xoo wild and mutants, which clearly indicated that Xoo does not require Xop effectors for its growth *in vitro* (Fig. 1). The phenotypic characteristics of the mutant wild colonies does not show any aberration.

Xop effectors is crucial to support *in planta* bacterial growth and virulence

The CFU counts indicated that significant reduction in bacterial growth in rice plants inoculated with the mutants. However, normal growth was found in rice inoculated with complemented strain, which clearly showed that the

Table 1. Induced expression of Xop effectors in wild Xoo grown in XOM2 medium, while no expression in NA medium. 16S rRNA gene was used as control

Xoo strains	Amplification status of Xop genes on <i>hrp</i> inducing medium					
	<i>xopF</i>		<i>xopR</i>		16S rRNA (control)	
	NA	XOM2	NA	XOM2	NA	XOM2
Wild Xoo	-	+	-	+	+	+
Xoo $\Delta hrpX$	-	-	-	-	+	+

- Amlicon not seen, + amplicon seen

Fig. 1. Mutant lacking *xopF* gene showing no-significant *in vitro* growth aberration when compared with Xoo wild. $P < 0.001$ vs Xoo wild by student's t test

suppression was regulated by Xop effectors in Xoo (Fig. 2). We also evident a significant fold reduction in the lesion area on rice leaf inoculated with mutants compared to Xoo wild. Contrary, the lesions produced by the complemented strains was similar to that of Xoo wild. This suggests that major Xop effectors including XopF and XopR plays crucial role in governing virulence to the Xoo.

Xop effectors regulate callose deposition in rice leaves

We noticed that callose deposition on rice leaf inoculated with mutants was significantly higher compared to that of with Xoo wild (Fig. 3). The reduced callose deposition on rice leaf inoculated with complimented strains further confirmed that the increased deposition was due to the deletion of *xop* gene. As callose is an indicator for PTI responses, therefore, suppression of callose deposition as mediated by Xop effectors of Xoo may be correlated with the suppression of PTI.

Xop effectors suppress the expression of PTI-marker genes

Activation of early defense related genes (or PTI-genes) is one of the important events during plant-pathogen interaction at molecular level. We analysed the expression of PTI genes, namely *PR10*, *OsWRKY13*, *OsRLCK16* and *OsFLS2*. The expression of all the genes of rice plants were found to be increased upon inoculation with mutants. The expression of all the PTI-genes were compromised upon challenge inoculation with complemented strains which directed us that the induced expression was due to the absence of Xop effector(s) in Xoo only. The suppression of the induced expression of all the PTI-genes was not achieved using a T3SS-defective mutant, Xoo $\Delta hrpX$, which further validated that

Fig. 2. *In planta* growth assay. Bacterial population in rice leaves was determined at 5 days of post inoculation (A) Plates showing reduced multiplication of mutants over Xoo wild (B) The CFU counts as observed in mutants over Xoo wild. * $P < 0.001$ vs wild type by 2way ANOVA test

Fig. 3. Callose deposition assay. The rice leaves were processed for callose deposits 12 h post-inoculation and were observed by fluorescence microscope. Mean callose deposits per 36 mm² field of view. Data are mean of three independent observations. * $P < 0.001$ vs Xoo wild control by paired t test. (Source: Mondal *et al.*, 2015)

the functional T3SS is required to suppress the PTI genes by Xoo invading rice plants.

Xop effectors localize to the cytoplasmic membrane of rice

Xop effectors, including XopF, XopR were found to localize on plasma membrane of *N. benthamiana* (Fig. 4) as well as in onion cells. The EYFP based fluorescence

signals were detected on the cytoplasmic membrane of the cells, while no fluorescence signals were observed in nuclei of the cells. The result clearly demonstrated the membrane based localization of XopF and XopR.

DISCUSSION

Xanthomonas oryzae pv. *oryzae* (Xoo) causing bacterial blight of rice is major biotic constraint to rice production worldwide. The role of T3SS effectors in bacterial pathogenesis has been demonstrated in several host-pathogen systems (Alfano and Collmer, 2004; Mondal *et al.*, 2016; Mudgett, 2005; White *et al.*, 2009; Yang *et al.*, 2005). Though a large number of *xop* effector genes from xanthomonad strains including *Xanthomonas oryzae* pv. *oryzae* (Mondal *et al.*, 2014; Song and Yang, 2010), *X. campestris* pv. *vesicatoria*, *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris*, *Xanthomonas perforans* have been documented (Kim *et al.*, 2009; Lee *et al.*, 2005; Ochiai *et al.*, 2005; Salzberg *et al.*, 2008), only a few of them have been studied extensively for their functional role in disease induction (Akimoto-Tomiya *et al.*, 2012; Bartetzko *et al.*, 2009; Kim *et al.*, 2009; Kumar and Mondal, 2013; Mondal *et al.*, 2016; Song and Yang, 2010). The Indian Xoo strain has been studied recently in details for their composition of T3SS effectors. It was found that one of the virulent Indian Xoo strain (pathotyped as race 4) possesses 21 Xop effectors (Mondal *et al.*, 2014). The core members of these, effectors XopF and XopR, were investigated in details

Fig. 4. Localization assay of XopF. *Agrobacterium* mediated transient expression of XopF::EYFP fusion protein in *Nicotiana benthamiana* (l). Empty constructs used as mock showing both nuclear (marked with white arrow) and membrane localization (A); XopF::EYFP fusion protein showing localization only on cytoplasmic membrane (B); DAPI-stained nuclei (C), superimposed B and C images (D). Scale bar 25 μ m. (Source: Mondal *et al.*, 2015)

for its role in Xoo virulence as well as in blight development (Mondal *et al.*, 2016). This paper highlighted the recent progress in understanding the role of Xop effectors including XopF and XopR in Xoo virulence.

The inducible and *hrp*-dependent expression of the *xop* genes was confirmed. This clearly showed type III secretion-dependent translocation of XopF. We further, demonstrated that the Xop effectors is dispensable for Xoo *in vitro* growth and there was no visible phenotypic aberration in mutants lacking *xop* genes (either *xopF* or *xopR*) over the wild. However, the mutants showed significant reductions *in planta* growth relative to wild strain. There are several reports wherein deletion of Xop effectors were shown to reduce *in planta* growth of the bacteria, including XopN of *X. axonopodis* pv. *punicae* in pomegranate (Kumar and Mondal, 2013; Kumar *et al.*, 2016), of *X. campestris* pv. *campestris* in radish (Jiang *et al.*, 2008) and of *Xanthomonas campestris* pv. *vesicatoria* in tomato (Kim *et al.*, 2009). The reduced blight intensity on rice leaf produced by Xoo $\Delta xopF$ established the role of the *xopF* effector in contributing to virulence. The reduced lesion due to loss of T3SS effectors was documented in many host-bacterial systems, including bacterial blight lesion induced by *X. axonopodis* pv. *punicae* in pomegranate, bacterial speck lesion induced by *Xanthomonas campestris* pv. *vesicatoria* in tomato, bacterial blight lesions induced by *X. oryzae* pv. *oryzae* in rice (Kim *et al.*, 2009; Kumar and Mondal, 2013; Song and Yang, 2010). The rice leaf inoculated with Xoo $\Delta xopF$ had more callose deposition which clearly indicated the XopF's role in cell-wall associated immune response. Callose deposition in plant cell was demonstrated to be regulated by many T3SS effectors of plant pathogenic Xanthomonads (Jiang *et al.*, 2008; Kim *et al.*, 2009; Kumar and Mondal, 2013; Song and Yang, 2010).

Xop effectors is localized in the plasma membrane when transiently expressed in the tobacco as well as onion epidermis cell. T3SS effectors from phytopathogenic Xanthomonads as well as *Pseudomonas* spp. were shown to be localized differently within the plant cells depending on their function. HopA11 of *Pseudomonas syringae* (chemically an enzyme, phosphothreonine lyase) is reported to be localized in the cytosol from where it dephosphorylates mitogen activated protein kinases (MAKs), MPK3 and MPK6 to suppress MAMP signalling (Zhang *et al.*, 2007). Likewise our studies showed that Xop effectors is required to be localized on the plasma membrane to exert its possible function in the suppression of cell wall associated defense response including callose deposition.

The RT-qPCR analysis indicated significant fold increase in expression of the PTI marker/ROS-associated genes in rice upon challenge-inoculation with mutants suggesting the role of effectors in suppressing rice PTI. It is evident that mutants induced more callose deposition as well as ROS accumulation in infected rice leaves. When transiently expressed in *Nicotiana benthamiana*, fusion effector gene products get localized to the plasma membrane. Collectively, our insight into the Xop T3SS

effectors leads to the understanding that the major T3SS effectors like XopF, XopR suppress rice immune responses, and thereby favours Xoo for its *in planta* growth and pathogenicity during bacterial blight development. This understanding would help in elucidating the disease susceptibility pathways and thereby would provide insight into strategies to exploit for the broad and durable resistance to blight.

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