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XopN-T3SS effector of *Xanthomonas axonopodis* pv. *punicae* localizes to the plasma membrane and modulates ROS accumulation events during blight pathogenesis in pomegranate

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ABSTRACT

Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Xap) is a major disease of pomegranate. Xap secretes effector proteins *via* type III secretion system (T3SS) to suppress pathogen-associated molecular pattern (PAMP)-triggered plant immunity (PTI). Previously we reported that XopN, a conserved effector of Xap, modulate *in planta* bacterial growth, and blight disease. In continuation to that here we report the deletion of XopN from Xap caused higher accumulation of reactive oxygen species (ROS) including H_2O_2 and O_2^-. We quantitatively assessed the higher accumulation of H_2O_2 in pomegranate leaves infiltrated with Xap *ΔxopN* compared to Xap wild-type. We analysed that 1.5 to 3.3 fold increase in transcript expression of ROS and flg22-inducible genes, namely *FRK1, GST1, WRKY29, PR1, PR2* and *PR5* in *Arabidopsis* when challenged with Xap *ΔxopN;* contrary, the up-regulation of all the genes were compromised when challenged with either Xap wild-type or Xap *ΔxopN+xopN*. Further, we demonstrated the plasma-membrane based localization of XopN protein both in its natural and experimental hosts. All together, the present study suggested that XopN-T3SS effector of Xap gets localized in the plasma membrane and suppresses ROS-mediated early defense responses during blight pathogenesis in pomegranate.

1. Introduction

Pomegranate (*Punica granatum*) is one of the exportable fruit crops of India. Bacterial blight incited by *Xanthomonas axonopodis* pv. *punicae* (Xap) causes substantial losses to pomegranate cultivation in India (Hingorani and Mehta, 1952; Mondal and Mani, 2009; Mondal et al., 2012a). The disease manifests itself on all plant parts, including stem, leaf, and fruit (Mondal and Mani, 2012). The lesions on fruits appear as watersoaked, which later become dark necrotic with prominent yellowish halo. The most economic losses occur due to cracking of the infected fruits resulting in reduced market value and export quality. Considering the devastating nature the disease is being studied extensively addressing both basic as well as applied aspects including phenomics, genomics and disease management. Our earlier studies on phylogenetic analysis based on house-keeping genes, 16S rRNA and *gyrB*, indicated the close relation of Xap with *Xanthomonas citri* subsp. *citri* as well as subsp. *malvacearum* (Mondal et al., 2012b). This was corroborated with the sequenced draft genome of Xap (Sharma et al., 2012). Presently the disease management through application of antibiotics and chemicals has got limited success due to the resistance development in the pathogen population. Other options including use of antagonists and copper based nano-formulations were shown to be potential but their effectiveness essentially relies on the purity of the strains or nano-formulation (Mondal and Mani, 2012).

In search of novel strategies for disease management, our recent efforts are focused on the host-pathogen interaction. Xanthomonads secret effector proteins directly into the eukaryotic cytosol through type three secretion system (T3SS) (Chisholm et al., 2006; Buttner and Bonas, 2006; Buttner and He, 2009). There are two types of T3SS-effectors namely, TAL (transcription activator like domain) effector or *avrBs3/pthA* family and Xop (Xanthomonas outer protein) effector family (Bonas et al., 1989; Casper-Lindley et al., 2002). The Xop effectors have been documented to be major virulent factor in various *Xanthomonas*:eukaryotic host systems (Mudgett and Staskawicz, 1999; Metz et al., 2005; Mudgett, 2005; Kim et al., 2008). We previously identified six effectors of the Xop family (Xanthomonas outer protein) namely, XopC2, XopE1, XopF2, XopL, XopN, XopQ and XopZ in Xap and demonstrated the

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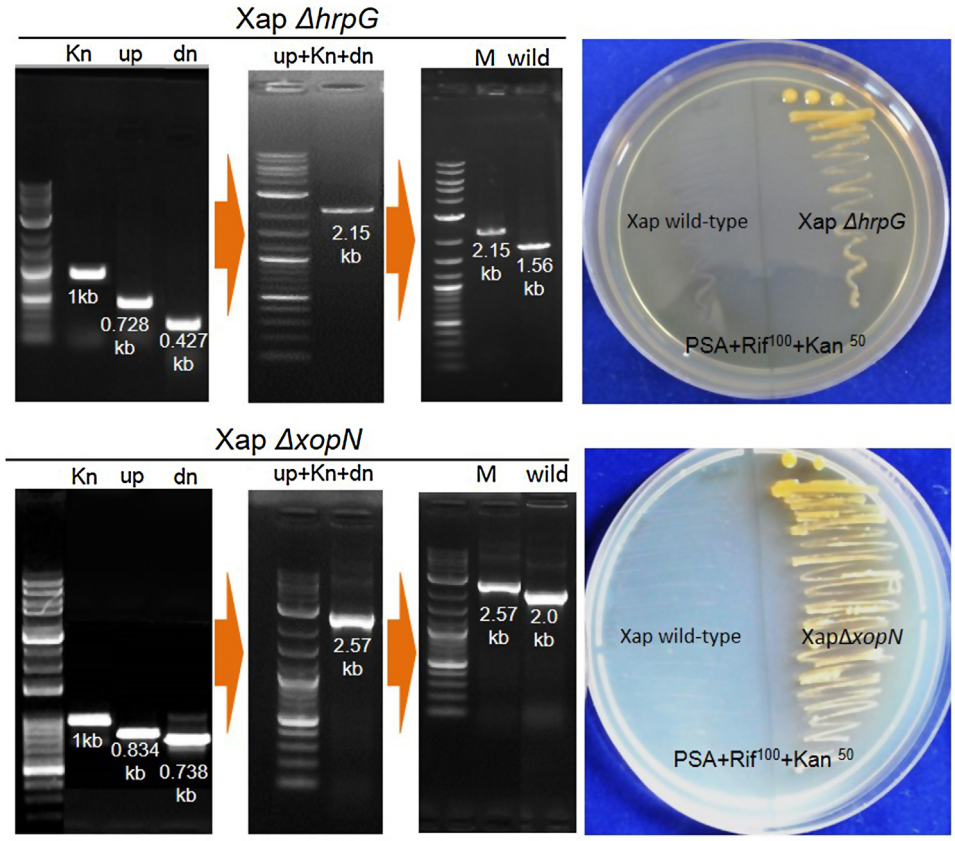

Fig. 1. Confirmation of mutants of the Xap. Amplicons produced during mutant construction for T3SS component gene, Xap *ΔhrpG* (upper panel) and effector gene, Xap *ΔxopN* (lower panel). Subsequent confirmation of the respective mutants showing selective growth of mutants on PSA supplemented with Rif^{100} +Kan^{50} but not of Xap wild-type. Kn = kanamycin gene; up = upstream fragment of the target gene; dn = downstream fragment of the target gene, up + kn + dn = total cassette for homologous recombination containing upstream fragment, kanamycin and downstream fragment; M = mutant. Size in kb are provided below the respective amplicons.

T3SS-dependent manner of secretion of these effectors (Kumar and Mondal, 2013). Further our functional analysis with XopN effector led to the fact that XopN is involved in the suppression of PAMP (pathogen associated molecular patterns)-triggered immunity (PTI) and thus helps the bacteria to cause disease in pomegranate (Kumar and Mondal, 2013).

Plants responds to the pathogen attack through activating a series of defense reactions including hypersensitive response (HR), oxidative burst, cell wall strengthening, and synthesis of defense-associated proteins (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997; Jones and Dangl, 2006). The very early response of plants to pathogen attack is the oxidative burst that leads to the accumulation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), and superoxide (O_2^-) (Mehdy, 1994; Lamb and Dixon, 1997; Kawano, 2003; Laloi et al., 2004; Choi et al., 2007). ROS production during oxidative burst not only limits the growth of the invading pathogens through HR responses but also triggers the defense process in plants (Tenhaken et al., 1995; Jabs et al., 1997). The ROS triggered defense response in plant is attained either by strengthening cell walls through cross-linking of glycoproteins (such as the Pro-rich protein) (Bradley et al., 1992), or by directly killing pathogens (Levine et al., 2016). ROS was also been documented to induce signal network underlying systemic acquired resistance (Alvarez et al., 1998; Lee and Hwang, 2005). Inoculation of avirulent strain of *Xanthomonas campestris* pv *vesicatoria* (Xcv) into the lower leaves of pepper (*Capsicum annuum*) induced SAR in the uninoculated upper leaves (Lee and Hwang, 2005). The SAR response of pepper plants is linked with a rapid oxidative burst that produces H_2O_2 and subsequent activation of defense-associated genes in uninoculated leaves (Lee and Hwang, 2005). Thus, ROS initiates the activation of a set of defense-associated genes, referred to as SAR genes. There are evidences that H_2O_2 being an intercellular or intracellular elicitor induces various defense-associated genes in plants (Orozco-Cárdenas et al., 2001). The oxidative burst triggered upon perception of pathogen avirulence signals is finally lead to the defense response in plants (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997).

In this study, we estimated the accumulation of reactive oxygen species (ROS) like H_2O_2, O_2^-, both qualitatively and quantitatively, during pomegranate:Xap interaction in presence or absence of XopN. We studied ROS-associated gene expression to validate the role of XopN in ROS accumulation events in *Arabidopsis* system. We also demonstrated the subcellular localization of XopN to predict the exact site of its function both in natural as well as experimental hosts. Altogether the present study intended to provide experimental evidences supporting the possible involvement of XopN in the suppression of early defense responses to induce blight lesions on pomegranate.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A rifampicin-resistant wild type Xap (Xap wild-type) strain ITCCBD 0003 and a *xopN*-specific mutant of the same strain (Xap *ΔxopN*) were used in the present study. The mutant was developed using a PCR-based double crossing over mediated homologous recombination strategy, wherein kanamycin resistance gene was