Action of Multiple Cell Wall–Degrading Enzymes Is Required for Elicitation of Innate Immune Responses During *Xanthomonas oryzae* pv. *oryzae* Infection in Rice

Lavanya Tayi,¹ Roshan Maku,¹ Hitendra Kumar Patel,¹ and Ramesh V. Sonti¹

¹CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad, Telangana State, India

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Xanthomonas oryzae pv. *oryzae* secretes a number of plant cell wall–degrading enzymes (CWDEs) whose purified preparations induce defense responses in rice. These defense responses are suppressed by *X. oryzae* pv. *oryzae* using type 3 secretion system (T3SS) effectors and a type 3 secretion system mutant (T3SS[−]) of *X. oryzae* pv. *oryzae* is an inducer of rice defense responses. We assessed the role of individual CWDEs in induction of rice defense responses during infection, by mutating them in the genetic background of a T3SS[−]. We mutated the genes for five different plant CWDEs secreted by *X. oryzae* pv. *oryzae*, including two cellulases (*clsA* and *chsA*), one xylanase (*xyn*), one pectinase (*pglA*), and an esterase (*lipA*), singly in a T3SS[−] background. We have demonstrated that, as compared with a T3SS[−] of *X. oryzae* pv. *oryzae*, a *chsA*[−]T3SS[−], a *clsA*[−]T3SS[−], and a *xyn*[−]T3SS[−] are deficient in induction of rice immune responses such as callose deposits and programmed cell death. In comparison, a *lipA*[−]T3SS[−] and a *pglA*[−]T3SS[−] is as efficient in induction of host defense responses as a T3SS[−]. Overall, these results indicate that the collective action of *X. oryzae* pv. *oryzae*–secreted ClsA, ChsA, and Xyn proteins is required for induction of rice defense responses during infection.

Plants are constantly exposed to a wide range of potential pathogens. Due to the lack of mobile defender cells and an adaptive immune system, plants depend upon the powerful inducible innate immunity of each cell to fight against pathogens that show diverse strategies to survive within the host (Jones and Dangl 2006). The first step in inducible defenses is perception of a pathogen in the vicinity and elicitation of pathogen triggered immunity (PTI), wherein conserved microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) and damage-associated molecular patterns (DAMPs) released upon damage caused by the pathogen to the host are perceived by transmembrane pattern recognition receptors on the host cell membrane to initiate defense responses (Chisholm et al. 2006). PTI is suppressed by successful pathogens, using effectors that are directly secreted into the host cells via the type 3 secretion system (T3SS) (Büttner and He 2009). Bacterial mutants lacking functional T3SS are virulence-deficient, suggesting a key role of T3SS secreted effectors in pathogenicity (Alfano and Collmer 2004).

The T3SS mutant (T3SS[−]) strains of pathogens like *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *phaseolicola*, and *Pseudomonas syringae* pv. *tomato* DC3000 have been shown to induce cell wall–associated basal defenses, like deposition of callose and lignin (Bartetzko et al. 2009; Bestwick et al. 1995; Hauck et al. 2003). Transient as well as transgenic expression of T3SS effectors have been shown to suppress defense responses induced by PAMPs and DAMPs as well as defenses induced by T3SS[−] strains that, presumably, produce PAMPs and DAMPs (Hauck et al. 2003; Keshavarzi et al. 2004; Li et al. 2005; Sinha et al. 2013).

As part of their virulence repertoire, gram-negative plant-pathogenic bacteria secrete a wide range of plant cell wall–degrading enzymes (CWDEs), such as cellulases, pectinases, xylanases, and proteases, using a type 2 secretion system (T2SS) (Barras et al. 1994; Jha et al. 2005; Kang et al. 1994). Mutational studies clearly indicated that the CWDEs and the T2SS are important virulence determinants of the pathogen (Gough et al. 1988; Kang et al. 1994; Ray et al. 2000). Action of these CWDEs on the wall results in release of endogenous oligosaccharide molecules that are sensed by the host and lead to induction of immune responses. Elicitor activity to the oligosaccharides was reported almost four decades ago by Albersheim and his colleagues (1984), who have demonstrated that hepta β-glucosyl fragments released from the pathogenic fungal cell walls by the action of plant hydrolytic enzymes are elicitors of phytoalexins (antimicrobial compounds) in the soybean cultures (Darvill and Albersheim 1984). Later on, oligogalacturonide (OG) fragments of the plant cell wall released by the action of pectinases and fragments of fungal cell-wall polysaccharide chitin were also shown to be potent inducers of plant defense responses (Ryan and Farmer 1991). A number of studies have shown that purified preparations of the CWDEs secreted by pathogens induce defense responses in plants. To mention a few, *Erwinia carotovora* pv. *carotovora* secreted pectic enzymes, including an endopolygalacturonase and two pectate lyases, work collectively with a cellulase to induce expression of a pathogenesis-related gene β,1-3 glucanase in tobacco (Vidal et al. 1998). Cellulase treatment in sweet pepper increased endogenous levels of salicylic acid (a defense hormone) and conferred resistance to mild mottle virus attack (Sato et al. 2011). OGs, particularly of degree of polymerization 10–15, released from the homogalacturonan part of pectin, induce various defense responses and protect *Arabidopsis* against infection by *Botrytis cinerea*, a necrotrophic fungus (Galletti et al. 2009). Are these defense responses that are induced by purified CWDEs relevant to in vivo conditions? This question arises because, in vivo, the CWDEs may not be produced at the same concentrations as they have been used in the

Corresponding author: R. V. Sonti; E-mail: sonti@ccmb.res.in

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purified preparations. Therefore, despite the well-established action of purified preparations of CWDEs in induction of immune responses, we do not know whether these enzymes do, indeed, induce defense responses during infection. We addressed this issue in the interaction between *Xanthomonas oryzae* pv. *oryzae* and its host, the rice plant.

X. oryzae pv. *oryzae* causes bacterial leaf blight, a serious rice disease. This bacterium secretes a cocktail of CWDEs, including cellulases, xylanases, lipase/esterase, and more, using a T2SS (Jha et al. 2007; Rajeshwari et al. 2005). Purified prep-

arations of *X. oryzae* pv. *oryzae*-secreted cellulase (ClsA), cellobiosidase (CbsA), and an esterase (LipA) have been shown to induce defense responses in rice (Jha et al. 2007). These defense responses were suppressed by some of the T3SS effectors of *Xanthomonas oryzae* pv. *oryzae* (Sinha et al. 2013). A T3SS⁻ of *X. oryzae* pv. *oryzae* has been shown to induce defense responses like callose deposition in rice leaves and programmed cell death (PCD) in rice roots. A T2SS/T3SS double mutant is deficient in induction of these defense responses, suggesting a role for T2SS proteins in induction of defense responses during infection (Jha et al. 2007).

In this study, we assessed the role of five major plant CWDEs secreted by *Xanthomonas oryzae* pv. *oryzae*—two cellulases, including an endoglucanase (ClsA) and an exoglucanase (CbsA), one xylanase (Xyn), one pectinase (PglA), and an esterase (LipA)—in induction of host innate immunity during infection. We generated mutations in the genes for each of these five enzymes singly in the genetic background of a T3SS⁻ of *X. oryzae* pv. *oryzae*. We observed that the ClsA, CbsA, and Xyn proteins, indeed, have a role in inducing defense responses during infection, as mutations in genes for any of these three enzymes drastically affected the ability of a T3SS⁻ to induce defense responses. This is the first report showing the in vivo role of any CWDE in induction of defense responses during infection.

RESULTS

The *clsA*⁻ T3SS⁻, *xyn*⁻ T3SS⁻, *pglA*⁻ T3SS⁻, and *lipA*⁻ T3SS⁻ strains are deficient in the enzyme activities.

The culture supernatants of *clsA*⁻ T3SS⁻ and *lipA*⁻ T3SS⁻ double mutant strains are deficient in acting on their respective substrates carboxy methyl cellulose and tributyrin as compared with a T3SS⁻ mutant, in the plate assays performed. The culture supernatant of T3SS⁻ produced a zone of clearance, around the well in agarose plates containing the substrate, in which mutants were inefficient in substrate clearance, indicating loss of enzyme activity. The supernatants of the complementing clones *clsA*⁻ T3SS⁻/pHM1+*clsA* and *lipA*⁻ T3SS⁻/pHM1+*lipA* showed a halo (substrate clearance) as T3SS⁻ (Fig. 1A and B).

Similarly, the *xyn*⁻ T3SS⁻ and *pglA*⁻ T3SS⁻ double mutant strains are deficient in acting on their respective substrates, Remazol brilliant blue (RBB)-xylan and Na-polygalacturonic acid (PGA), as compared with a T3SS⁻ strain. The T3SS⁻ produced a zone of clearance around the colony on peptone sucrose agar (PSA) plates containing the substrate, whereas mutants were inefficient in substrate clearance, indicating loss of enzyme activity. The complementing clones *xyn*⁻ T3SS⁻/pHM1+*xyn* and *pglA*⁻ T3SS⁻/pHM1+*pglA* produced a similar halo (substrate clearance) as the T3SS⁻, while the strains with empty vector, *xyn*⁻ T3SS⁻/pHM1 and *pglA*⁻ T3SS⁻/pHM1, did not show any activity (Fig. 1C and D).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the culture supernatant of the T3SS⁻ mutant shows CbsA protein along with other proteins like LipA and ClsA (Supplementary Fig. S1-d). The culture supernatant of the *cbsA*⁻ T3SS⁻ strain lacks the CbsA protein. The SDS-PAGE profile of the strain with the complementing clone (*cbsA*⁻ T3SS⁻/pHM1+*cbsA*) indicates restoration of CbsA protein production. The *cbsA*⁻ T3SS⁻/pHM1 exhibits a protein profile that is similar to the profile of the *cbsA*⁻ T3SS⁻ mutant.

X. oryzae pv. *oryzae* secreted cellulases CbsA and ClsA are inducers of defense responses during infection.

Callose deposition is considered as an innate immune response that strengthens the cell wall at the site of pathogen entry. Callose deposition was assessed after infiltration of rice leaves with the following *X. oryzae* pv. *oryzae* strains: T3SS⁻,

Fig. 1. Phenotypes of mutations in genes for specific cell wall-degrading enzymes. **A**, Endoglucanase assay on carboxymethyl cellulose (CMC) substrate. Culture supernatants of *Xanthomonas oryzae* pv. *oryzae* strains T3SS⁻ (type 3 secretion system mutant), *clsA*⁻ T3SS⁻, *clsA*⁻ T3SS⁻/pHM1, and *clsA*⁻ T3SS⁻/pHM1+*clsA* were loaded in the wells present in agar containing CMC. A halo surrounding the well indicates the presence of active protein in the sample. **B**, Esterase activity on tributyrin substrate. Culture supernatants of *X. oryzae* pv. *oryzae* strains T3SS⁻, *lipA*⁻ T3SS⁻, *lipA*⁻ T3SS⁻/pHM1, and *lipA*⁻ T3SS⁻/pHM1+*lipA* were loaded in the wells present in agar containing tributyrin. A halo around the well indicates substrate clearance by active protein in the sample. **C**, Xylanase activity on RBB-xylan substrate. *X. oryzae* pv. *oryzae* strains T3SS⁻, *xyn*⁻ T3SS⁻, *xyn*⁻ T3SS⁻/pHM1, and *xyn*⁻ T3SS⁻/pHM1+*xyn* were patched onto a peptone sucrose agar plate containing RBB-xylan. Presence of a halo around the colony is indicative of xylanase proficiency. **D**, Pectinase activity on Na-polygalacturonic acid (PGA) substrate. *X. oryzae* pv. *oryzae* strains T3SS⁻, *pglA*⁻ T3SS⁻, *pglA*⁻ T3SS⁻/pHM1, and *pglA*⁻ T3SS⁻/pHM1+*pglA* were patched on PSA plate containing PGA. The presence of a halo around the colony indicates pectinase proficiency.

cbsA⁻*T3SS*⁻ double mutant, *cbsA*⁻*T3SS*⁻/pHM1 (empty vector control), *cbsA*⁻*T3SS*⁻/pHM1+*cbsA* (complementing clone), *clsA*⁻*T3SS*⁻ double mutant, *clsA*⁻*T3SS*⁻/pHM1 (empty vector control), *clsA*⁻*T3SS*⁻/pHM1+*clsA* (complementing clone), and BXO43 (wild type [wt]). A *T3SS*⁻ deficient strain of *X. oryzae* pv. *oryzae* has been shown to be an inducer of innate immune responses in rice (Jha et al. 2007). This has been attributed to its reduced ability to suppress host immune responses. Rice leaves infiltrated with a *T3SS*⁻ strain (Fig. 2A and B) exhibited a higher number of callose spots as compared with the BXO43 (wt) strain (Fig. 2A and B), whereas the rice leaves infiltrated with the *cbsA*⁻*T3SS*⁻ and *clsA*⁻*T3SS*⁻ strains exhibited significantly lesser number of callose deposits (Fig. 2A and B). This clearly suggested that deleting either a *cbsA* or *clsA* gene in a *T3SS*⁻ strain has drastically affected the ability of the *T3SS*⁻ to induce callose deposition in rice leaves. The strains with the complementing clones (*cbsA*⁻*T3SS*⁻/pHM1+*cbsA* and *clsA*⁻*T3SS*⁻/pHM1+*clsA*) expressing *cbsA* and *clsA* genes, respectively, from plasmid pHM1 exhibited a similar number of callose deposits as a *T3SS*⁻ mutant (Fig. 2A and B). Infiltration of control strains carrying only the vector (*cbsA*⁻*T3SS*⁻/pHM1 and

clsA⁻*T3SS*⁻/pHM1) resulted in callose deposition that was at the same level as that observed with *cbsA*⁻*T3SS*⁻ and *clsA*⁻*T3SS*⁻ (Fig. 2A and B).

Jha et al. (2007) have also shown that the *X. oryzae* pv. *oryzae* *T3SS*⁻ induces PCD in rice roots. We assessed the role of *ClsA* and *CbsA* proteins in the induction of PCD by the *T3SS*⁻. The assay for PCD was the internalization of propidium iodide (PI). In living plant cells, PI stains the cell wall while it is internalized and stains nucleic acids in cells undergoing PCD. Rice roots were treated with cultures of the following *X. oryzae* pv. *oryzae* strains: *T3SS*⁻, BXO43 (wt), *cbsA*⁻*T3SS*⁻ double mutant, *cbsA*⁻*T3SS*⁻/pHM1 (empty vector control), *cbsA*⁻*T3SS*⁻/pHM1+*cbsA* (complementing strain), *clsA*⁻*T3SS*⁻ double mutant, *clsA*⁻*T3SS*⁻/pHM1 (empty vector control), and *clsA*⁻*T3SS*⁻/pHM1+*clsA* (complementing strain). Cells of rice roots treated with *T3SS*⁻ showed extensive internalization of PI (Fig. 3A). The PI staining material was dispersed within the cells, suggesting nuclear fragmentation as a consequence of PCD. In comparison, the BXO43 (wt)-treated roots showed only cell wall-bound PI staining (Fig. 3B). The *cbsA*⁻*T3SS*⁻ double mutant and the *clsA*⁻*T3SS*⁻ double mutant treated roots did not exhibit PI internalization, indicating that

Fig. 2. The *cbsA*⁻ and *clsA*⁻ type 3 secretion system–negative mutants (*T3SS*⁻) of *Xanthomonas oryzae* pv. *oryzae* are deficient in induction of callose deposition in rice leaves. **A**, Ten- to 15-day-old rice leaves were infiltrated with one of the following strains of *X. oryzae* pv. *oryzae*: *T3SS*⁻, *cbsA*⁻*T3SS*⁻ double mutant, *cbsA*⁻*T3SS*⁻/pHM1 (empty vector control), *cbsA*⁻*T3SS*⁻/pHM1+*cbsA* (complementing clone), or BXO43 (wild type [wt]). **B**, Similarly, rice leaves were also infiltrated with one of the following strains of *X. oryzae* pv. *oryzae*: *T3SS*⁻, *clsA*⁻*T3SS*⁻ double mutant, *clsA*⁻*T3SS*⁻/pHM1 (empty vector control), *clsA*⁻*T3SS*⁻/pHM1+*clsA* (complementing clone), or BXO43 (Wt). The leaves were subsequently stained with aniline blue and were visualized under an epifluorescence microscope. Bright spots in the images are the callose deposits. The graphs illustrate the average number of callose spots from at least four leaves and three to four different viewing areas plotted in each experiment. Error bars represent standard deviation. A student's two-tailed *t* test for independent means was performed in pairwise combinations for all the values. Values with the same letter (either a or b) are not significantly different at the *P* < 0.05 level. Similar results were obtained in three independent experiments.

these strains do not induce cell death (Fig. 3C and D). Roots treated with the complementing strains *cbsA*⁻ T3SS⁻/pHM1+*cbsA* and *clsA*⁻ T3SS⁻/pHM1+*clsA* exhibited extensive PI internalization, indicating that they are restored in the ability to

Fig. 3. The *cbsA*⁻ and *clsA*⁻ type 3 secretion system–negative mutants (T3SS⁻) of *Xanthomonas oryzae* pv. *oryzae* are deficient in induction of programmed cell death (PCD) in rice roots. Rice roots were treated with one of the following strains of *X. oryzae* pv. *oryzae*: **A**, T3SS⁻, **B**, BXO43 (Wt), **C**, *cbsA*⁻ T3SS⁻ double mutant, **D**, *clsA*⁻ T3SS⁻ double mutant, **E**, *cbsA*⁻ T3SS⁻/pHM1 (empty vector control), **F**, *cbsA*⁻ T3SS⁻/pHM1+*cbsA* (complementing clone), **G**, *clsA*⁻ T3SS⁻/pHM1 (empty vector control), or **H**, *clsA*⁻ T3SS⁻/pHM1+*clsA* (complementing clone). Following treatment, the roots were stained with propidium iodide (PI) and were visualized under a confocal microscope. Internalization and dispersion of PI inside the cells is indicative of PCD. Scale bar measures 20 µm.

induce PCD (Fig. 3F and H). The rice roots treated with the control strains *cbsA*⁻ T3SS⁻/pHM1 and *clsA*⁻ T3SS⁻/pHM1 do not exhibit internalization of PI and exhibit PI staining only in the wall (Fig. 3E and G).

Xylanase is also required for induction of immune responses by a T3SS⁻ strain of *X. oryzae* pv. *oryzae*.

Having observed that cellulases are essential for induction of defense responses under in vivo conditions, we proceeded to assess the role of the major xylanase in induction of innate immunity during infection. For this purpose, we generated in frame deletion in the xylanase encoding gene (*xyn*) of *X. oryzae* pv. *oryzae* in a T3SS⁻ mutant background and assessed the ability of the *xyn*⁻ T3SS⁻ double mutant strain to induce PCD and callose deposition.

The PCD assay was performed in rice roots, as mentioned below, with the following strains of *X. oryzae* pv. *oryzae*: T3SS⁻, *xyn*⁻ T3SS⁻ double mutant, *xyn*⁻ T3SS⁻/pHM1 (empty vector control), *xyn*⁻ T3SS⁻/pHM1+*xyn* (complementing strain), and BXO43 (wt). As expected, the T3SS⁻ induced PCD in rice roots (Fig. 4B), whereas mutation of the *xyn* gene (which is responsible for the major xylanase activity under laboratory conditions) affected the ability of T3SS⁻ to induce PCD in rice roots. Rice roots treated with the *xyn*⁻ T3SS⁻ double mutant showed no PI internalization and nuclear fragmentation (Fig. 4B). Expression of this gene product in the mutant, i.e., the complementing strain *xyn*⁻ T3SS⁻/pHM1+*xyn* has restored the PCD-inducing capacity (Fig. 4B). The *xyn*⁻ T3SS⁻ double mutant with the empty pHM1 vector does not induce any PCD, as only cell wall-bound PI staining is seen (Fig. 4B).

The effect of mutation in the *xyn* gene on the ability of the T3SS⁻ to induce callose deposition was also assessed. The *xyn*⁻ T3SS⁻ double mutant was deficient in inducing callose deposition (Fig. 4A and C). Introduction of the *xyn* gene using pHM1 plasmid (Fig. 4A and C) but not the control empty pHM1 vector (Fig. 4A and C) restored the ability of the *xyn*⁻ T3SS⁻ double mutant to induce callose deposition.

LipA, an esterase secreted by *X. oryzae* pv. *oryzae*, is not essential for induction of immune responses during infection.

The purified LipA protein has been shown to induce immune responses in rice. To assess its role in eliciting immune responses during infection, a *lipA*⁻ T3SS⁻ double mutant was generated. The *lipA*⁻ T3SS⁻ strain is as efficient at inducing callose deposition in leaves as the T3SS⁻ (Fig. 5B and C). Also, *lipA*⁻ T3SS⁻ is as proficient in inducing PCD in roots as the T3SS⁻ (Fig. 5A). This suggests that, although purified LipA can induce rice defense responses, it is not essential for elicitation of immune responses during an actual infection condition.

The polygalacturonase PglA has no role in induction of immune responses during infection.

Pectinases are an important class of CWDEs, whose role as key virulence factors of pathogens and as inducers of host defense responses is very well established. PglA is the major pectin-degrading enzyme secreted by *X. oryzae* pv. *oryzae* (L. Tayi and R. V. Sonti *unpublished results*). To study its ability to induce defense responses under infection conditions, a *pglA*⁻ T3SS⁻ strain was generated and immune response assays were performed. The *pglA*⁻ T3SS⁻ is as efficient in inducing callose deposition in leaves and PCD in roots as the T3SS⁻ (Fig. 6A, B, and C). This clearly indicates that there is no major role for PglA in eliciting defense responses in rice during infection.

DISCUSSION

In a previous study, Jha and coworkers (2007) have shown that defense responses are induced in rice tissues upon treatment with

purified preparations of *X. oryzae* pv. *oryzae* T2SS secreted proteins ClsA, CbsA, and LipA. They have also demonstrated that a T3SS[−] of *X. oryzae* pv. *oryzae* functions as an inducer of rice innate immunity and a T2SS[−] T3SS[−] double mutant is deficient in inducing these defense responses, indicating that T2SS secreted proteins play a key role in the ability of the T3SS[−] to induce defense responses in rice (Jha et al. 2007). In the genetic background of T3SS[−], in this study, we made mutations in the genes for each of five T2SS secreted proteins, to study the contribution of these different CWDEs in induction of host defense responses during infection. Our results indicate that two cellulases (ClsA and CbsA) and a xylanase (Xyn) are essential for the ability of a T3SS[−] of *X. oryzae* pv. *oryzae* to induce immune responses during infection. The loss of any one of these functions

affects the ability of a T3SS[−] to induce immune responses. In contrast, a secreted lipase/esterase (LipA) and a polygalacturonase (PglA) are not essential for elicitation of immune responses during infection. A double mutant of *cbsA* and *clsA* in T3SS[−] background is as defective in induction of defense responses as either of the single mutants *cbsA* or *clsA* in T3SS[−] background (L. Tayi and R. V. Sonti *unpublished data*), indicating that there is no additive effect of deleting both cellulases ClsA and CbsA in cell wall damage-induced defense responses during infection.

Purified preparations of LipA protein have been earlier shown by Jha et al. (2007) to induce defense responses when infiltrated into rice tissues. Mutations in LipA protein affect virulence of *X. oryzae* pv. *oryzae* (Jha et al. 2007) and, so, it appears that this protein is produced during growth of the

Fig. 4. A *xyn*[−] type 3 secretion system-negative mutant (T3SS[−]) is deficient in induction of programmed cell death (PCD) in rice roots and callose deposition in rice leaves. **A**, Rice leaves were treated with one of the following strains of *X. oryzae* pv. *oryzae*: T3SS[−], *xyn*[−] T3SS[−] double mutant, *xyn*[−] T3SS[−]/pHM1 (empty vector control), *xyn*[−] T3SS[−]/pHM1+*xyn* (complementing clone), or BXO43 (wt). The leaves were subsequently stained with aniline blue and were visualized under an epifluorescence microscope. Bright spots in the images are the callose deposits. **B**, Rice roots were treated with one of the following strains of *X. oryzae* pv. *oryzae*: T3SS[−], *xyn*[−] T3SS[−] double mutant, *xyn*[−] T3SS[−]/pHM1 (empty vector control), *xyn*[−] T3SS[−]/pHM1+*xyn* (complementing clone), or BXO43 (Wt). Following treatment, the roots were stained with propidium iodide (PI) and were visualized under a confocal microscope. Internalization and dispersion of PI inside the cells is indicative of PCD. Scale bar measures 20 μ m. **C**, Mean of the callose counts collected from at least four leaves and three to four different viewing areas from the infiltrated region of each leaf were plotted. Error bars represent standard deviations. Values with the same letter (either a or b) are not significantly different at the $P < 0.05$ level in a student's two-tailed t test performed for independent means. Similar results were obtained in three independent experiments.

pathogen in planta. Therefore, the question arises as to why the LipA protein is not required for elicitation of innate immune responses by the T3SS[−]. In this connection, it is important to note that our results merely show that LipA and PglA are not essential for elicitation of innate immunity during infection, as it is possible that their role in elicitation of innate immunity is redundant and that some other functions may substitute for the same.

Purified preparations of ClsA and CbsA can elicit rice immune responses such as callose deposition and PCD (Jha et al. 2007). Purified preparations of Xyn protein elicit PCD in rice (R. Ramanan and R. V. Sonti *unpublished results*). Paradoxically, it appears that the lack of even one of these enzymes affects the ability to elicit immune responses during infection. We propose that, during infection, the rice plant is monitoring damage caused by multiple CWDEs before a decision is made to mount a defense response. As many as six cellulases, three β -glucosidases, one cellobiosidase, four xylanases, six xylosidases, one polygalacturonase, three pectin-degrading enzymes

(one pectin methylesterase, two pectate lyases), six proteases, are encoded in the *X. oryzae* pv. *oryzae* genome (Lee et al. 2005). We have tested the roles of only five such enzymes in elicitation of innate immunity during infection. It is possible that *X. oryzae* pv. *oryzae* secreted CWDEs other than ClsA, CbsA, and Xyn are involved in elicitation of rice innate immunity during infection.

Cellulases, xylanases, and pectinases have all been shown to elicit defense responses in various plants. In the case of ethylene-inducing xylanase of *Trichoderma reesei*, enzyme activity is not required and the protein is recognized as a PAMP by the host (Enkerli et al. 1999; Ron and Avni 2004). Recently, a cellulase from *Rhizoctonia solani* has been shown to be an inducer without the requirement of its enzymatic activity (Ma et al. 2015). However, in a majority of the cases, activity of the CWDE is recognized via the cell wall fragments—the oligosaccharide molecules released by the action of the CWDE on the host cell

Fig. 5. A *lipA*[−] type 3 secretion system–negative mutant (T3SS[−]) of *Xanthomonas oryzae* pv. *oryzae* is proficient in induction of programmed cell death (PCD) in rice roots and callose deposition in rice leaves. **A**, Rice roots were treated with one of the following strains of *X. oryzae* pv. *oryzae*: T3SS[−], *lipA*[−] T3SS[−] double mutant, or BXO43 (Wt). Following treatment, the roots were stained with propidium iodide (PI) and were visualized under a confocal microscope. Internalization and dispersion of PI inside the cells is indicative of PCD. Scale bar measures 20 μ m. **B**, Rice leaves were treated with one of the following strains of *X. oryzae* pv. *oryzae*: T3SS[−], *lipA*[−] T3SS[−] double mutant, or BXO43 (Wt). The leaves were subsequently stained with aniline blue and were visualized under an epifluorescence microscope. Bright spots in the images are the callose deposits. **C**, Mean and standard deviation were calculated for number of callose deposits from a field of view. Data were collected from at least four leaves in each experiment and three to four different viewing areas from the infiltrated region of each leaf. A student's two-tailed *t* test for independent means was performed in pairwise combinations for all the values. Values with the same letter (either a or b) are not significantly different at the *P* < 0.05 level. Similar results were obtained in three independent experiments.

Fig. 6. A *pglA*[−] type 3 secretion system–negative mutant (T3SS[−]) of *Xanthomonas oryzae* pv. *oryzae* is proficient in induction of programmed cell death (PCD) in rice roots and callose deposition in rice leaves. **A**, Rice roots were treated with one of the following strains of *X. oryzae* pv. *oryzae*: T3SS[−], *pglA*[−] T3SS[−] double mutant, or BXO43 (Wt). Following treatment, the roots were stained with propidium iodide (PI) and were visualized under a confocal microscope. Internalization and dispersion of PI inside the cells is indicative of PCD. Scale bar measures 20 μ m. **B**, Rice leaves were treated with one of the following strains of *X. oryzae* pv. *oryzae*: T3SS[−], *pglA*[−] T3SS[−] double mutant, or BXO43 (Wt). The leaves were subsequently stained with aniline blue and were visualized under an epifluorescence microscope. Bright spots in the images are the callose deposits. **C**, Mean and standard deviation were calculated for number of callose deposits from a field of view. Data were collected from at least four leaves in each experiment and three to four different viewing areas from the infiltrated region of each leaf. A student's two-tailed *t* test for independent means was performed in pairwise combinations for all the values. Values with the same letter (either a or b) are not significantly different at the *P* < 0.05 level. Similar results were obtained in three independent experiments.

wall. At least for ClsA and CbsA, Jha et al. (2007) have presented evidence indicating that soluble elicitors that are released by the action of these proteins on crude preparations of rice cell walls are involved in elicitation of innate immunity.

The role of oligosaccharides derived from the cell walls of fungi and plants as potent elicitors of defense signaling cascades is very well established in several plant systems. Hepta β -glucosides, laminarioligosaccharides, and chitoooligosaccharides are well known oligosaccharides of fungal cell-wall origin, whereas OGs are the molecules derived from pectic polysaccharides of the plant cell wall whose role as elicitors of defense responses is best characterized. Oligogalacturonides of degree of polymerization 10-20 appear to be the most active elicitors (Shibuya and Minami 2001). Recently, it has been shown that OGs, indeed, act as DAMPs in planta and induce defense responses. Evidence is provided for in vivo production of OGs in transgenic *Arabidopsis* expressing a fungal polygalacturonase fused to a plant polygalacturonase-inhibiting protein expressing under pathogen-inducible promoter. This chimeric construct-expressing plant is shown to be resistant to *Botrytis cinerea*, *Pectobacterium carotovorum*, and *Pseudomonas syringae* (Benedetti et al. 2015).

Based on our results, we hypothesize that, during infection, the damage caused to the cell wall by the activity of a single enzyme may not be sufficient to mount defense responses and that cooperativity among different classes of CWDEs is required for induction of defense responses. The two cellulases, CbsA and ClsA, and a xylanase, Xyn, must be acting together on their respective components of the cell wall, the cellulose and xylan, to efficiently degrade the highly complex structure to a level that is sensed as an alarming signal by the host, to initiate the activation of defense responses. In this connection, it is pertinent to note that ClsA is an endoglucanase and that CbsA is an exoglucanase. The cooperative action of endo- and exo-acting glycosyl hydrolases in efficient degradation of cellulose is very well demonstrated (Nidetzky et al. 1995; Woodward 1991; Zhou and Ingram 2000). Synergism and cooperativity among different groups of CWDEs is also documented. Palva and co-workers (Vidal et al. 1998) have shown that treatment of tobacco plants with combinations of pectic and cellulase enzymes of *Erwinia carotovora* subsp. *carotovora* induced pathogenesis-related β -1,3 glucanase gene expression to a higher extent than

treatments with individual enzymes. Interestingly, combinations of the same class of enzymes, such as pectinases (PehA + PelA/D) and cellulases (CelV1 + CelS), did not result in such an additive effect. This suggested that pectic enzymes and cellulases co-operate in eliciting the induction of defense genes in response to *Erwinia carotovora* subsp. *carotovora* (Vidal et al. 1998). Similarly cellulosomal xylanase XynA and cellulase from *Clostridium cellulovorans* were found to degrade corn cell walls synergistically (Murashima et al. 2003).

The model we propose is that action of multiple CWDEs is required to release the elicitors from the complex structural barrier of the plant cell. Collective action of key categories of CWDEs, like cellulases and xylanases, which act on the main components of the rice cell wall, may be absolutely required to bring down the complexity to a level that is sensed by the plant as a danger signal. In the absence of even a single major CWDE, proper degradation of the cell wall will not occur and, thus, DAMP-triggered immunity is not mounted. What could be the identity of the DAMP released from rice cell walls? Oligomers of glucose and xylose, the constituents of the cellulose and xylan, respectively, could be elicitor molecules that will be perceived by rice as DAMPs. Further experiments are required to determine the exact nature of one or more DAMPs that are released by action of ClsA, CbsA, and Xyn on rice tissues.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers and culture media used.

Bacterial strains and plasmids used in the study are listed in Tables 1 and 2, respectively. Primers used in the study are listed in Supplementary Table S1. *X. oryzae* pv. *oryzae* strains were grown at 28°C in peptone sucrose (PS) media. *Escherichia coli* strains were grown in Luria Bertani media at 37°C, as previously described (Ray et al. 2000). *X. oryzae* pv. *oryzae* strains carrying the plasmid pK18mobsacB integrated in their genomes (the single crossovers/integrants) were grown in 0.5% peptone and 0.3% beef extract-containing medium (nutrient agar/broth medium) instead of PS medium, as these strains are sensitive to sucrose. The concentrations of antibiotics used were: rifampin, 50 μ g/ml; ampicillin, 50 μ g/ml; spectinomycin, 50 μ g/ml; kanamycin, 25 μ g/ml for *E. coli* and 15 μ g/ml for *X. oryzae* pv. *oryzae*.

Table 1. List of bacterial strains used in this study

Strain	Relevant characteristics	Reference/source
<i>Escherichia coli</i> strains		
DH5 α	λ^- f80d lacZDM15 D(lacZYA-argF) U169 recA1 endA hsdR17 (r $_{K^-}$ m $_{K^-}$) supE44 thi-1 gyrA relA1	Invitrogen
S17-1	RP4-2 Tc::Mu-Km::Tn7 pro hsdR recA Tra+ used as mobilizing strain	Simon et al. 1983
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> strains		
BXO1	Wild type; Indian isolate	Laboratory collection
BXO43	rif-2; derivative of BXO1	Laboratory collection
T3SS $^-$	hrpB6::bla rif-2; HR $^-$, Ap r derivative of BXO43	Jha et al. 2007
cbsA $^-$ T3SS $^-$	Δ cbsA:: rif-2; Ap r derivative of T3SS $^-$	This study
cbsA $^-$ T3SS $^-$ /pHM1	cbsA $^-$ T3SS $^-$ /pHM1; rif-2; Ap r , Sp r ; derivative of cbsA $^-$ T3SS $^-$	This study
cbsA $^-$ T3SS $^-$ /pHM1::cbsA	cbsA $^-$ T3SS $^-$ /pHM1::cbsA; rif-2; Ap r , Sp r ; derivative of cbsA $^-$ T3SS $^-$	This study
clsA $^-$ T3SS $^-$	Δ clsA:: rif-2; Ap r derivative of T3SS $^-$	This study
clsA $^-$ T3SS $^-$ /pHM1	clsA $^-$ T3SS $^-$ /pHM1; rif-2; Ap r , Sp r ; derivative of clsA $^-$ T3SS $^-$	This study
clsA $^-$ T3SS $^-$ /pHM1::clsA	clsA $^-$ T3SS $^-$ /pHM1::clsA; rif-2; Ap r , Sp r ; derivative of clsA $^-$ T3SS $^-$	This study
xyn $^-$ T3SS $^-$	Δ xyn:: rif-2; Ap r derivative of T3SS $^-$	This study
xyn $^-$ T3SS $^-$ /pHM1	xyn $^-$ T3SS $^-$ /pHM1; rif-2; Ap r , Sp r ; derivative of xyn $^-$ T3SS $^-$	This study
xyn $^-$ T3SS $^-$ /pHM1::xyn	xyn $^-$ T3SS $^-$ /pHM1::xyn; rif-2; Ap r , Sp r ; derivative of xyn $^-$ T3SS $^-$	This study
pglA $^-$ T3SS $^-$	pglA $^-$ T3SS $^-$:: pK18mob rif-2; Km r ; pglA $^-$; Km r , Ap r derivative of T3SS $^-$	This study
pglA $^-$ T3SS $^-$ /pHM1	pglA $^-$ T3SS $^-$ /pHM1; rif-2; Km r , Ap r , Sp r ; derivative of pglA $^-$ T3SS $^-$	This study
pglA $^-$ T3SS $^-$ /pHM1::pglA	pglA $^-$ T3SS $^-$ /pHM1::pglA; rif-2; Km r , Ap r , Sp r ; derivative of pglA $^-$ T3SS $^-$	This study
lipA $^-$ T3SS $^-$	Δ lipA:: rif-2; Ap r derivative of T3SS $^-$	This study
lipA $^-$ T3SS $^-$ /pHM1	lipA $^-$ T3SS $^-$ /pHM1; rif-2; Ap r , Sp r ; derivative of lipA $^-$ T3SS $^-$	This study
lipA $^-$ T3SS $^-$ /pHM1::lipA	lipA $^-$ T3SS $^-$ /pHM1::lipA; rif-2; Ap r , Sp r ; derivative of lipA $^-$ T3SS $^-$	This study

Molecular biology and microbiology techniques.

Genomic DNA isolation was performed as described by Leach et al. (1990). Plasmid DNA was isolated by the alkaline lysis method (Sambrook et al. 1989) or by using a Qiagen plasmid mini/midi kit. Phusion polymerase (Finnzymes) was used for polymerase chain reaction (PCR) amplification in applications that required high-fidelity DNA synthesis, while *Taq* polymerase was used for all other applications. Restriction digestions were done with enzymes from New England Biolabs. PCR products and restriction enzyme-digested DNA fragments were purified using a QIAquick PCR purification kit and a QIAquick nucleotide removal kit (Qiagen), respectively. Other techniques, such as ligation with T4 DNA ligase, agarose gel electrophoresis, and transformation of *E. coli*, were performed as described previously (Sambrook et al. 1989). Plasmids were transferred from *E. coli* to *X. oryzae* pv. *oryzae*, using either broad host-range mobilizing strain S17-1 (Simon et al. 1983) via biparental matings or electroporation (Ray et al. 2000; Subramoni and Sonti 2005).

Sequencing and analysis.

DNA sequencing was performed using an ABI Prism 3700 automated DNA sequencer (Perkin-Elmer). The sequences were analyzed using the BLAST algorithm in the National Center for Biotechnology Information database (Altschul et al. 1990).

Generation of in frame deletion mutants of *chsA*, *lipA*, *clsA*, and *xyn* in a genetic background of T3SS[−] of *X. oryzae* pv. *oryzae*.

Using genomic DNA of *X. oryzae* pv. *oryzae* wt strain BXO43 as template and two primer pairs for each gene, F1/R1 and F2/R2, two PCR fragments were amplified (the R1 and F2 primers designed for all the genes contained engineered *Xba*I restriction enzyme site at its 5' end, whereas F1 and R2 primers for all the genes contained engineered *Hind*III restriction enzyme site at its 5' end). The PCR-amplified fragments were gel-extracted, digested with *Xba*I, and ligated with each other, using T4 DNA ligase. To amplify the ligated fragment, the ligation mixture was used as template and primers F1 and R2 were used, so that the ligated fragment contained *Hind*III restriction enzyme sites at its 5' and 3' ends. The ligated fragment after digestion with the *Hind*III enzyme was cloned into the *Hind*III site of the vector pK18mobsacB (Schäfer et al. 1994). The recombinant plasmids pK18mobsacB with the ligated fragment were then transformed into S17-1 (Simon et al. 1983). Biparental matings were then set up between the S17-1 *E. coli* cells containing the construct and the T3SS[−] of *X. oryzae* pv. *oryzae*. T3SS[−] strains that received and integrated the plasmid into the genome by homologous recombination were selected by kanamycin resistance. Integration of plasmids at the correct

locus in *X. oryzae* pv. *oryzae* genome was confirmed by PCR, using a pair of primers, ICF and ICR. Sucrose sensitivity of these single recombinants was also tested. Single recombinants in T3SS[−] background that were ampicillin- and kanamycin-resistant and sucrose-sensitive were grown in liquid media containing 0.3% beef extract and 0.5% peptone without kanamycin. Subculturing was done twice and, finally, cultures were plated on 1% PSA and ampicillin-containing plates. Putative double recombinants, which include mutants and wt cells that are sucrose- and ampicillin-resistant but kanamycin-sensitive, were isolated. They were confirmed by PCR, digestion of amplified PCR product with *Xba*I and sequencing of the PCR product, and by checking the protein profile of the extracellular fractions for the loss of the corresponding protein, by SDS PAGE.

Generation of the *pglA*[−] T3SS[−] strain of *X. oryzae* pv. *oryzae*.

A *pglA*[−] T3SS[−] strain of *X. oryzae* pv. *oryzae* was generated by gene disruption, using homologous plasmid integration of a suicide vector pK18mob. A 759-bp internal fragment of the *pglA* gene was amplified by PCR, using genomic DNA of a *X. oryzae* pv. *oryzae* wt strain and the gene-specific PglAFP/PglARP oligonucleotide primer pair. This fragment was cloned into the *Xba*I- and *Hind*III-digested pK18mob cloning vector to obtain recombinant plasmid. The plasmid was then electroporated into T3SS[−] and clones were selected for kanamycin resistance to obtain a *pglA*[−] mutant by plasmid integration. Gene disruption was confirmed by PCR, using gene-specific flanking primers PglA ICF and PglA ICR in combination with vector-specific primers M13F and M13R, and the sequencing of PCR products. The *pglA* gene fragment was cloned in the same transcriptional orientation as the lacZ promoter of the pK18mob vector, so that mutation caused by vector integration did not have a polar effect on downstream genes due to activity of the outwardly directed lacZ promoter.

Generation of complementing strains.

For *chsA*[−] T3SS[−] complementation, a 1,782-bp DNA fragment containing the full length of the *chsA* gene was amplified by PCR, using primers CBCF and CBCR and genomic DNA of BXO43 as template. The PCR-amplified DNA fragment was cloned as a *Hind*III-*Eco*RI fragment into the broad host-range vector pHM1 (Innes et al. 1988), to create the recombinant plasmid pTC1. The pTC1 plasmid and pHM1 vector were individually introduced into the *chsA*[−] T3SS[−] strain by electroporation, and the recombinants were isolated by selection for spectinomycin resistance. The presence of pTC1 was confirmed by PCR, using *chsA* gene-specific primers CBCF and CBCR and universal primers M13F and M13R and the sequencing of PCR

Table 2. List of plasmids used in this study

Plasmids	Relevant characteristics	Reference/source
pK18mobsacB	Allelic exchange suicide vector SacB derivative of pK18mob; <i>sacB</i> Tra [−] Mob ⁺ , km ^r does not replicate in <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	Schäfer et al. 1994
pK18mob	pUC18 derivative; Mob ⁺ Tra [−] Km ^r ; does not replicate in <i>X. oryzae</i> pv. <i>oryzae</i>	Schäfer et al. 1994
pHM1	Broad-host-range cosmid vector (13.3kb); Sp ^r	Innes et al. 1988
pTL1	pK18mobsacB+1,373-bp of A+C fragment of <i>chsA</i> gene	This study
pTL2	pK18mobsacB+1,357-bp of A+C fragment of <i>clsA</i> gene	This study
pTL3	pK18mobsacB+1,719-bp of A+C fragment of <i>xyn</i> gene	This study
pTL4	pK18mob+759-bp of internal fragment of <i>pglA</i> gene	This study
pTL5	pK18mobsacB+1,481 bp of A+C fragment of <i>lipA</i> gene	This study
pTLC1	pHM1+1,782-bp gene fragment containing 1,701-bp <i>chsA</i> gene	This study
pTLC2	pHM1+1,685-bp gene fragment containing 1,449-bp <i>clsA</i> gene	This study
pTLC3	pHM1+1,017-bp gene fragment containing 978-bp <i>xyn</i> gene	This study
pTLC4	pHM1+1,853-bp gene fragment containing 1,671-bp <i>pglA</i> gene	This study
pGJ5	pHM1+1,511-bp gene fragment containing 1,281-bp <i>lipA</i> gene	Aparna et al. 2007

products. Similarly for *clsA*⁻T3SS⁻ and *xyn*⁻T3SS⁻ strain complementation, a 1,685-bp DNA fragment containing the *clsA* gene and a 1,017-bp DNA fragment containing *xyn* gene were PCR-amplified and cloned into *HindIII*-*SacI* sites of pHM1 vector to generate pTLC2 and pTLC3 plasmids, respectively. For *pglA*⁻T3SS⁻ strain complementation, a 1,853-bp fragment containing the *pglA* gene was PCR-amplified and cloned into *HindIII*-*EcoRI* sites of pHM1 vector to generate pTLC4. For *lipA*⁻T3SS⁻ strain complementation, pGJ5 (pHM1+1,511 bp of DNA fragment containing the *lipA* gene) was used. These recombinant plasmids were moved into their respective mutants. Empty pHM1 vector was also introduced into mutant strains. Spectinomycin-resistant putative complementation clones were confirmed by PCR and sequencing and were shown to restore either enzymatic activity or protein production.

Protein purification and activity assays.

Saturated cultures of *X. oryzae* pv. *oryzae* strains were pelleted by centrifugation at 4°C. Proteins in the culture supernatant were precipitated by ammonium sulfate (55%) and suspended in 0.01 M potassium phosphate buffer (pH 6.0). Excess salt is removed by dialysis. Total protein (30 µg) of each strain was loaded and separated by SDS-PAGE. Cellulase activity in the extracellular fractions of *X. oryzae* pv. *oryzae* strains was checked on 1.2% agarose suspension containing 0.2% carboxymethyl cellulose solidified in petri plates, as described earlier (Jha et al. 2007). Pectinase activity was performed by growing the *X. oryzae* pv. *oryzae* strains on PSA plates containing 0.1% Na-PGA as substrate and staining with 0.05% ruthenium red dye solution in water. Xylanase and lipase activities were performed as described earlier (Rajeshwari et al. 2005).

Innate immune responses in rice: PCD assay in rice roots.

Rice (TN-1) seeds were surface-sterilized with sodium hypochlorite and were germinated on 0.5% sterile agar in petri dishes for 3 to 4 days at 28°C. Root tips, 1 to 2 cm long, were treated with cultures of *X. oryzae* pv. *oryzae* strains of an optical density (OD) of 1.5 in 1× phosphate buffered saline. After incubation for 16 h, roots were washed with sterile MQ water and were stained with 1 mg of PI solution per milliliter for 1 h. The roots were mounted on a microscopic slide in 50% glycerol. Longitudinal optical sections (1 µm thick) were acquired on a Zeiss LSM-510 Meta confocal microscope using 63× oil immersion (NA 1.4). A HeNe laser at 543 nm excitation and emission above 560 nm (LP) was used to detect PI internalization. All images were analyzed using LSM software and were further edited using Photoshop (Adobe).

Innate immune responses in rice: callose deposition assay in rice leaves.

TN-1 rice seedlings about 10 to 15 days old were infiltrated with cultures of *X. oryzae* pv. *oryzae* strains of OD 1.0, using a needleless syringe. After 16 h, infiltrated zones (approximately 1 cm long) were cut from the leaf and were destained by heating at 60°C with alcohol and, then, were stained with 0.05% aniline blue solution in water overnight and were analyzed under an Axioplan2 epifluorescence microscope, using a blue filter and ×10 objective.

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