

ORIGINAL ARTICLE

Role of the FnIII domain associated with a cell wall-degrading enzyme cellobiosidase of *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Cellobiosidase (CbsA) is an important secreted virulence factor of *Xanthomonas oryzae* pv. *oryzae* (Xoo), which causes bacterial blight of rice. CbsA is one of several cell wall-degrading enzymes secreted by Xoo via the type II secretion system (T2SS). CbsA is considered a fundamental virulence factor for vascular pathogenesis. CbsA has an N-terminal glycosyl hydrolase domain and a C-terminal fibronectin type III (FnIII) domain. Interestingly, the secreted form of CbsA lacks the FnIII domain during in planta growth. Here we show that the presence of the FnIII domain inhibits the enzyme activity of CbsA on polysaccharide substrates like carboxymethylcellulose. The FnIII domain is required for the interaction of CbsA with SecB chaperone, and this interaction is crucial for the stability and efficient transport of CbsA across the inner membrane. Deletion of the FnIII domain reduced virulence similar to $\Delta cbsA$ Xoo, which corroborates the importance of the FnIII domain in CbsA. Our work elucidates a hitherto unknown function of the FnIII domain in enabling the virulence-promoting activity of CbsA.

KEYWORDSexoglucanase, FnIII domain, rice, secretion, type II secretion system, virulence, *Xanthomonas*

1 | INTRODUCTION

When a phytopathogen encounters a potential host, the plant cell wall serves as a physical barrier to limit access of the pathogen to cellular contents (Kubicek et al., 2014). The plant cell wall is a recalcitrant exoskeleton that surrounds the cell protoplast and is composed of a complex network of polysaccharides including cellulose, hemicellulose, and pectin (Cosgrove, 2005). Successful plant pathogens possess essential virulence attributes such as cell wall-degrading enzymes (CWDEs) that degrade different components of the plant cell wall (Kubicek et al., 2014).

The gram-negative bacterial genus *Xanthomonas* includes species that cause diseases in almost 400 plants, including agronomically important crops (Jacques et al., 2016; Ryan et al., 2011). One

member of this genus, *Xanthomonas oryzae* pv. *oryzae* (Xoo), causes bacterial blight disease of rice. Xoo secretes a battery of CWDEs including cellulase (ClsA), cellobiosidase (CbsA), xylanase (XynB), lipase (LipA), and pectinase (PglA) through the type II secretion system (T2SS) (Jha et al., 2007; Rajeshwari et al., 2005; Tayi et al., 2016). Disruption of genes encoding these enzymes in Xoo leads to either partial virulence deficiency, as in the case of $\Delta clsA$ and $\Delta lipA$, or a more severe virulence deficiency, as seen in the case of $\Delta cbsA$ (Jha et al., 2007). The CWDEs are double-edged swords as the damage that they cause serves as a mark of infection and results in induction of plant immune responses such as callose deposition and programmed cell death responses (Jha et al., 2007).

CbsA is an extracellular exoglucanase and its biochemical activity is critical for virulence (Tayi et al., 2018). Recently, the presence

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and absence of the *cbsA* gene in *Xanthomonas* was shown to be central to the switch between vascular and nonvascular pathogenesis, respectively (Gluck-Thaler et al., 2020). Genomic comparison between pathogenic and nonpathogenic *Xanthomonas* sp., which are inhabitants of the same host, shows an association of the *cbsA* gene with pathogenicity (Bansal et al., 2019). Interestingly, CbsA is among the highest expressed proteins during the growth of Xoo in the vascular tissue (González et al., 2012; Jacobs et al., 2012). In addition, the LuxR (quorum sensing-related transcription regulator) subfamily member of Xoo, OryR, was shown to induce expression of CbsA on treatment with macerated rice tissue (Ferluga et al., 2007; Gonzalez & Venturi, 2013). Collectively, the above reports suggest that CbsA is expressed in planta and is an important virulence factor of Xoo.

The 566-amino acid CbsA protein has an N-terminal glycosyl hydrolase domain and a C-terminal fibronectin type III (FnIII) domain (Figure 1a). The FnIII domain is one of the three types of internal repeats (FnI-FnII-FnIII) originally identified in its eponym fibronectin, a eukaryotic plasma protein (Main et al., 1992; Ruoslahti, 1988). The FnIII domain is present in almost 2% of animal proteins with a few instances in yeast and plants as well (Bateman & Chothia, 1996; Bork & Doolittle, 1992; Tsyguelnaia & Doolittle, 1998). In the case of animals, the FnIII domain is mostly present in extracellular matrix proteins, where it is involved in protein-protein interaction. The FnIII domain also acts as a spacer between domains to facilitate unhindered biological action of the respective domains (Bork et al., 1996; Campbell & Spitzfaden, 1994).

In prokaryotes, the FnIII domain was first identified in a chitinase enzyme of *Bacillus circulans* (Watanabe et al., 1990). Most of the initially identified FnIII domains were found to be associated with carbohydrate-hydrolysing enzymes, but later they were also identified

in the fibronectin-binding proteins and other prokaryotic protein types (Henderson et al., 2011; Konkel et al., 2010). FnIII domains associated with bacterial hydrolases have been shown to have different roles, which include increasing thermostability of the associated protein (Brunecky et al., 2012) and acting as a spacer between the hydrolysing domain and the substrate-binding domain for enhanced catalysis (Kataeva et al., 2002). In the case of *Dickeya dadantii* (a plant pathogen), genetic and biochemical studies showed that the FnIII domain associated with a pectin-hydrolysing enzyme pectate lyase harbours a loop that acts as a type II secretion signal for the transport of the protein across the outer membrane (Pineau et al., 2014).

In Xoo, the secreted CbsA lacks the FnIII domain (Figure 1a) (Kumar et al., 2012), and this secreted form is biochemically active on soluble oligosaccharides and the polysaccharide substrate carboxymethylcellulose (CMC) (Tayi et al., 2018). In addition, it can also induce immune responses in rice tissues (Jha et al., 2007). However, the role of the FnIII domain in CbsA is not known. In the current study, we have used genetic and biochemical approaches to understand the role of the FnIII domain in CbsA function.

2 | RESULTS

2.1 | Secreted CbsA lacks the FnIII domain during in planta growth of Xoo in rice

To understand the possible reason for the removal of the FnIII domain, wild-type Xoo strain BXO43 was grown and the extracellular fraction was analysed using anti-CbsA antibody at different time points. Up to

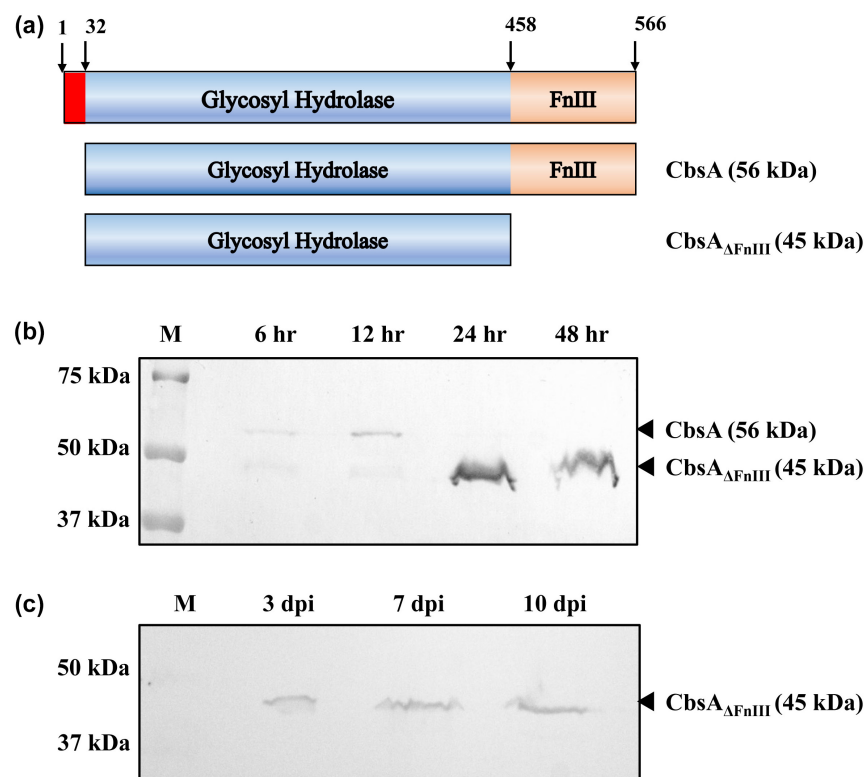


FIGURE 1 Secreted CbsA lacks the FnIII domain. (a) Schematic representation of domain organization of *Xanthomonas oryzae* pv. *oryzae* (Xoo) CbsA. CbsA has an N-terminal signal sequence followed by the catalytic domain (glycosyl hydrolase-6 family) and a C-terminal fibronectin type III (FnIII) domain. (b) Western blot analysis of secreted Xoo CbsA at different time points, using anti-CbsA antibody, showing absence of FnIII domain in the secreted CbsA at later stages of growth. (c) Western blot analysis of Xoo-infected rice leaf exudate at 3, 7, and 10 days postinoculation, showing absence of the FnIII domain in the secreted CbsA during in planta growth

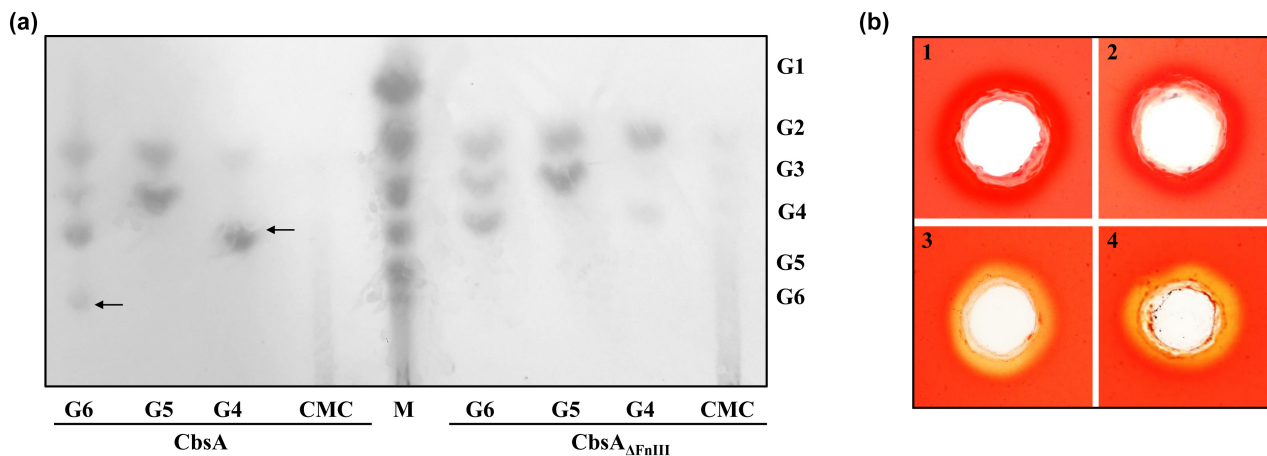


FIGURE 2 FnIII domain has an inhibitory effect on the biochemical activity of CbsA. (a) The activity of the catalytic domain of CbsA in the presence and absence of the FnIII domain was assessed on oligosaccharides and carboxymethylcellulose (CMC). CbsA and CbsA $_{\Delta}$ FnIII were incubated overnight with oligosaccharide substrates G6-cellohexaose, G5-cellopentaose, G4-cellobiose, and polysaccharide substrate CMC. The hydrolysed products were separated and detected using thin-layer chromatography as described in the experimental procedures. A mix of oligosaccharides (G1–G6) is shown in the lane labelled M. The unhydrolysed substrate is marked by a black arrow. (b) CMC-agarose plate assay showing hydrolysis by (1) buffer, (2) CbsA, (3) CbsA $_{\Delta}$ FnIII, and (4) CbsA after cleavage of FnIII domain. The presence of a halo around the well after Congo red staining indicates the activity of the enzyme on CMC

12 h of growth, the full-length form of the protein was present in the extracellular fraction (Figure 1b). From 24 h onwards, the major form present in the extracellular fraction was the cleaved form of CbsA lacking the FnIII domain (CbsA $_{\Delta}$ FnIII). This observation led us to check the form of CbsA that is present during Xoo infection in rice. For this purpose, wild-type Xoo was inoculated on the susceptible rice variety Taichung Native-1 (TN-1), and leaf exudate was collected 3, 7, and 10 days post-inoculation (dpi). Western blot analysis of the leaf exudate showed the presence of a single band corresponding to the cleaved form of CbsA (45 kDa) (Figure 1c). These observations indicate that during infection in rice, the secreted CbsA protein lacks the FnIII domain.

2.2 | Presence of the FnIII domain modulates the biochemical activity of CbsA

CbsA $_{\Delta}$ FnIII is known to hydrolyse soluble oligosaccharides and polysaccharide substrate CMC in *in vitro* assays (Tayi et al., 2018). To assess the effect of the FnIII domain on the biochemical activity of the protein, CbsA and CbsA $_{\Delta}$ FnIII were purified from *Escherichia coli* (Figure S1a–c). The biochemical activity was assessed on cellobiose, cellopentaose, cellohexaose, and CMC. CbsA showed reduced activity on cellobiose and cellohexaose compared to CbsA $_{\Delta}$ FnIII as shown by the presence of unhydrolysed substrates (Figure 2a). CbsA did not hydrolyse CMC, but CbsA $_{\Delta}$ FnIII showed the release of cellobiose, cellobiose, and cellobiose. This difference in activity was also observed in a CMC-agarose plate assay where detection of enzyme activity is achieved by staining with Congo red solution and the presence of a halo indicates hydrolysis. The CbsA protein showed absence of a halo in the assay while CbsA $_{\Delta}$ FnIII showed presence of a halo (Figure 2b). Intriguingly, purified CbsA (c.3-day-old protein) from *E. coli* is spontaneously cleaved into a smaller protein that corresponds to CbsA $_{\Delta}$ FnIII in

size (Figure S1d). The cleaved form showed a gain of activity on CMC (Figure 2b), indicating that it is indeed the presence of the FnIII domain that inhibits the activity on the polysaccharide substrate. In conclusion, the enzymatic activity of CbsA protein is negatively modulated by the presence of the FnIII domain.

2.3 | Deletion of the FnIII domain affects the levels of CbsA protein in the extracellular space and within the cell

To investigate the requirement for the FnIII domain in CbsA, an in-frame deletion of the FnIII domain-coding region was made in the *cbSA* gene of Xoo, and the CbsA protein levels were analysed in the extracellular fraction and the whole-cell lysate using anti-CbsA antibody. The CbsA $_{\Delta}$ FnIII mutant showed more than 95% reduction in the secreted CbsA level as compared to the wild type (Figure 3a,b). Additionally, in the whole-cell lysate, the protein level showed more than 60% reduction in the mutant as compared to the wild type (Figure 3c). Although the mRNA level of *cbSA* was same as the wild type (Figure S2b), the total amount of CbsA protein was drastically reduced on deletion of the FnIII domain (Figure 3). These results illustrate that in absence of the FnIII domain, secretion of CbsA is heavily compromised and the level of CbsA protein within the cell is reduced.

2.4 | CbsA interacts with SecB chaperone through the FnIII domain

Type II secretion of proteins takes place in two steps. In the first step, the protein is transported across the cytoplasmic membrane

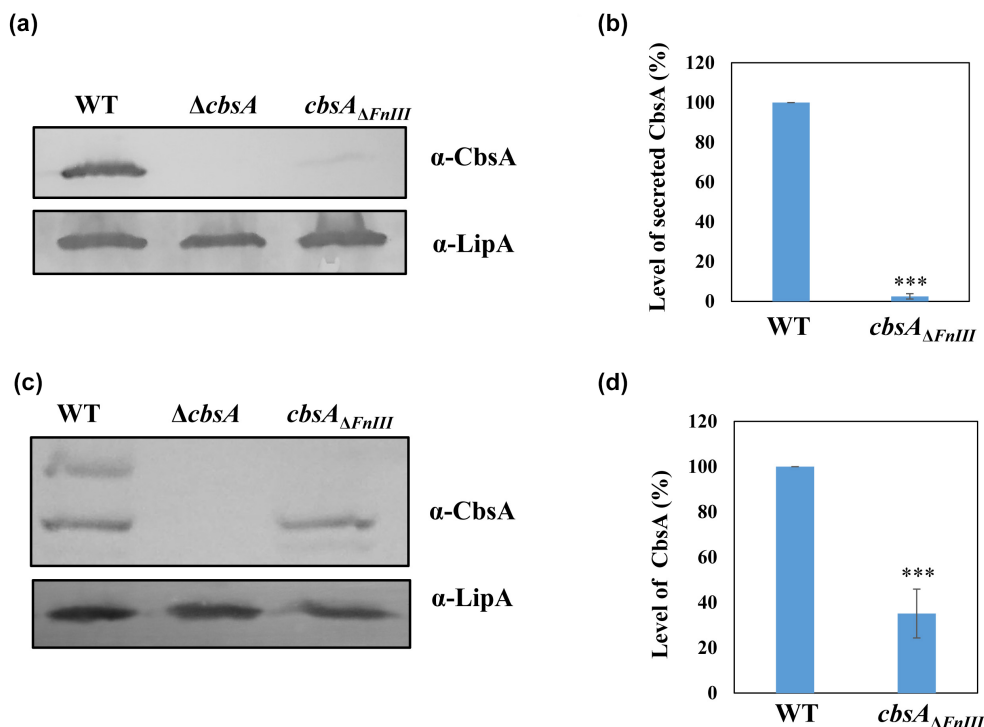


FIGURE 3 Deletion of the FnIII domain leads to a reduction in the level of CbsA protein in the extracellular space as well as within the cell. (a) Western blot analysis of the secreted CbsA protein in the culture supernatant of wild type (WT), $\Delta cbsA$, and $cbsA_{\Delta FnIII}$ *Xanthomonas oryzae* pv. *oryzae* (Xoo). The data indicates that the deletion of the FnIII domain results in more than 95% reduction in the level of the secreted CbsA protein. LipA protein was used as a loading control and $\Delta cbsA$ Xoo was used as a negative control. (b, d) Densitometry analysis of western blots of three independent experiments using ImageJ software. Student's two-tailed t test was performed and compared groups were significantly different at $p < 0.001$ (marked with ***). (c) Western blot analysis of CbsA protein in the cell lysate of WT, $\Delta cbsA$, and $cbsA_{\Delta FnIII}$ Xoo indicates that deletion of the FnIII domain results in more than 60% reduction in the level of the CbsA protein

via either the Sec system or the TAT system, depending on the signal sequence present in the secreted protein (Korotkov et al., 2012). From the periplasm, the protein is secreted into the extracellular milieu by the T2SS. Analysis of the CbsA protein sequence, using SignalP v. 5.0 (Armenteros et al., 2019), indicated the presence of a Sec-dependent signal sequence at its N-terminal region (Figure 4a). Proteins that are transported across the cytoplasmic membrane via the Sec system are known to remain loosely folded in the cytoplasm after their translation and are protected from aggregation and cellular protein degradation machinery by binding to the SecB chaperone (Randall & Hardy, 1995; Sakr et al., 2010). Classically, SecB recognizes its substrates via a signal sequence; however, in addition to the signal sequence, other regions of the protein may also participate in this recognition (Huang et al., 2016; Mallik et al., 2002). SecB recognition sites are known to be enriched in hydrophobic residues and the presence of acidic residues is disfavoured (Bechtluft et al., 2010; Huang et al., 2016; Knoblauch et al., 1999). Analysis of the CbsA protein sequence for hydrophobicity shows a patch of hydrophobic residues in the FnIII domain sequence (Figure 4b). Based on these observations and earlier results that deletion of the FnIII domain affects the level of CbsA protein and its secretion, we hypothesized that the FnIII domain could be the region through which CbsA binds to the SecB chaperone.

To investigate this, an in vitro affinity pull-down experiment was done. For this purpose, SecB-His, FnIII-HA, CbsA, and $CbsA_{\Delta FnIII}$ proteins were expressed in *E. coli* using the isopropyl- β -D-1-thiogalactopyranoside (IPTG)-inducible pET28b vector. Pull-down followed by western blotting showed that SecB interacted with CbsA but not with $CbsA_{\Delta FnIII}$. The FnIII domain alone showed binding with SecB (Figure 4c). These results suggest that CbsA interacts with SecB via the FnIII domain, which in turn facilitates stability and efficient transport of the protein across the inner membrane.

2.5 | The FnIII domain of CbsA is essential for virulence of Xoo on rice

Our results indicate that the FnIII domain influences catalytic activity as well as secretion of the CbsA protein. We further investigated the virulence of $cbsA_{\Delta FnIII}$ Xoo in comparison to wild-type Xoo. For this purpose, the Xoo wild-type strain BXO43, $\Delta cbsA$, and $cbsA_{\Delta FnIII}$ mutants were inoculated onto rice leaves and lesion lengths were measured 15 dpi. The $cbsA_{\Delta FnIII}$ Xoo strain exhibited reduced virulence and the level of reduction was comparable to the $\Delta cbsA$ Xoo strain (Figure 5a,b). This observation confirms that the presence of

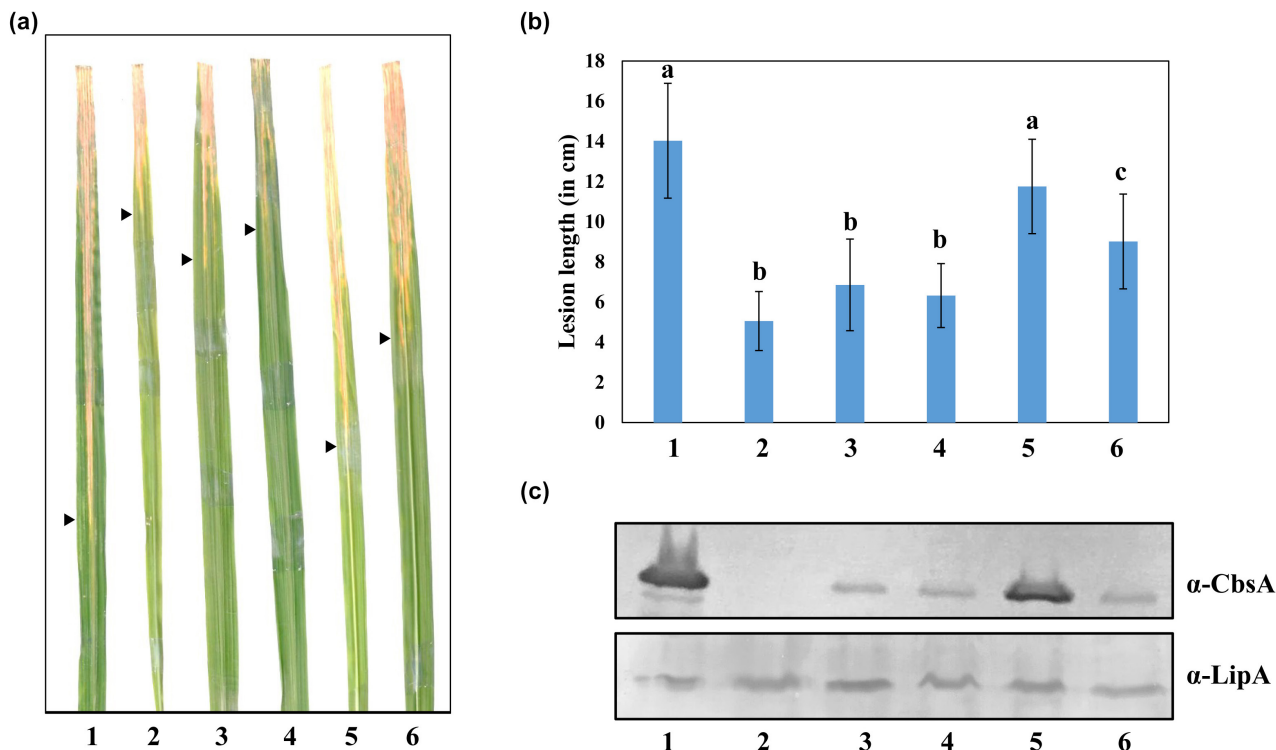


FIGURE 5 The FnIII domain of CbsA is critical for virulence of *Xanthomonas oryzae* pv. *oryzae* (Xoo) on rice. (a) Rice leaves showing lesions caused by (1) wild type, (2) $\Delta cbsA$, (3) $cbsA_{\Delta FnIII}$, (4) $cbsA_{\Delta FnIII}$ + pHM1 (empty vector), (5) $cbsA_{\Delta FnIII}$ + pHM1-*cbsA*, and (6) $cbsA_{\Delta FnIII}$ + pHM1-*cbsA_{\Delta FnIII}* Xoo strains 15 days postinoculation. Black arrowheads mark the leading edge of the lesion. (b) Columns and vertical bars indicate the mean lesion length caused by the above strains and standard deviation of at least 20 rice leaves, respectively. Student's two-tailed t test was performed, groups with different letters were significantly different at $p < 0.001$. Similar results were obtained in three independent experiments. (c) Western blot analysis showing the level of secreted CbsA in the above-mentioned strains. LipA protein was used as a loading control

Xoo CbsA contains a Sec-dependent secretion signal at its N-terminus. Sec-secreted proteins are known to remain loosely folded in the cytoplasm, which is their transport-competent form (Cranford-Smith & Huber, 2018; Hardy & Randall, 1991). SecB is a cytoplasmic chaperone that has an antifolding activity (Huang et al., 2016). The binding of a secretory protein with SecB serves three purposes: first, it keeps the secretory protein in the partly unfolded form; second, it protects this partly unfolded secretory protein from aggregation and cellular degradation machinery; and third, SecB hands over the bound secretory protein to SecA, which is part of the Sec translocon (Cranford-Smith & Huber, 2018; Hardy & Randall, 1991; Sakr et al., 2010). In the case of Xoo, deletion of the FnIII domain of CbsA led to impaired secretion and a reduction in the total amount of CbsA protein, which is indicative of insufficient SecB binding. Our *in vitro* pull-down experiment showed that SecB bound to CbsA but not to $CbsA_{\Delta FnIII}$. This finding elucidates a unique function of the FnIII domain, wherein it mediates interaction with the SecB chaperone to achieve efficient transport across the cytoplasmic membrane as well as stability of the CbsA protein. It is evident that an optimal level of CbsA expression is critical for pathogenesis, which warrants an efficient transport mechanism, and it appears that it may be achieved in this case by appending an FnIII domain to this virulence factor.

Pathogens are under continuous selection pressure, imposed by their host environment, which leads to the evolution of virulence factors that facilitate colonization within their hosts (Goldstein, 2008; Thornton et al., 1999). Despite low sequence identity among different FnIII domains, the structural fold remains common, which is a Greek key β -sandwich fold made up of seven or eight strands (Banner et al., 1996; de Pereda et al., 1999; Sharma et al., 1999; Zhao et al., 2017). This feature of the FnIII domain allows it to be highly mutated and repurposed to confer novel functions. In particular, one study has shown that the FnIII domain can accommodate multiple mutations within the same fold to become a novel binding protein (Koide et al., 1998). Possibly, the FnIII domain of CbsA has also evolved in a similar way to bind to SecB for proficient secretion.

Intriguingly, the FnIII domain of Xoo CbsA is cleaved from the protein in the later stages of laboratory-grown cultures and during *in planta* infection. Similar observations have been made in *Trichoderma reesei*, where cellobiohydrolase I and cellobiohydrolase II undergo postsecretion proteolysis at the later growth stages to yield multiple forms of cellulases (Hagspiel et al., 1989). However, in both cases, it is not clear whether this cleavage takes place by the action of a specific protease or by spontaneous cleavage. The whole-cell lysates of Xoo (Figure 3c) and *E. coli* expressing CbsA (Figure 4c) show the presence of both full-length and cleaved forms of CbsA,

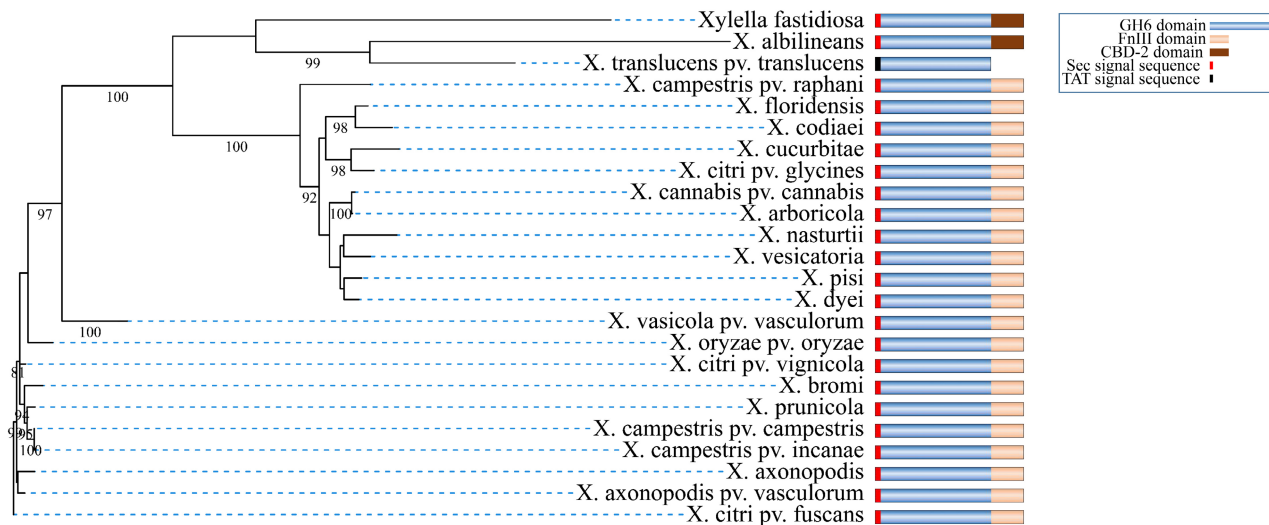


FIGURE 6 Association of Sec-dependent secretion of CbsA and the FnIII domain in *Xanthomonas*. Maximum-likelihood phylogenetic tree of CbsA protein from different *Xanthomonas* species and their close relative *Xylella fastidiosa*. Bootstrap values (>80) are shown at the branches. Domain organization and type of signal sequence present in CbsA protein sequence are shown schematically for the respective organism. Analysis of CbsA protein sequence from different *Xanthomonas* species showed association of the FnIII domain with the presence of a Sec-dependent signal sequence. *Xanthomonas translucens* pv. *translucens* CbsA does not have an FnIII domain and shows a higher probability for the presence of a TAT-dependent signal sequence over a Sec-dependent signal sequence

which would argue for a spontaneous cleavage. Presumably, the FnIII domain has to be removed because its presence inhibits the activity of CbsA on polymeric substrates, making it less efficient for cell wall degradation. CMC is cellulose with carboxymethyl modifications. The action of exoglucanases on such substrates is facilitated by the flexibility of the substrate-binding tunnel-enclosing loops (Varrot et al., 1999; Zou et al., 1999). It is possible that in the presence of the FnIII domain, the movement of these loops is restricted and thus the enzyme is inactive on CMC. Furthermore, it is tempting to propose that the inhibition of the enzyme activity of the CbsA catalytic domain by the FnIII domain serves the purpose of reducing detection by the host immune system during the early phase of infection.

What is the fate of the FnIII domain after cleavage? Does it have any other function in the extracellular milieu when it is attached to CbsA in the initial growth phase? Some of these questions need further investigation. In the case of *Vibrio cholerae*, RbmA is one of the proteins required for biofilm formation. Structural analysis of this protein showed that it has two tandem FnIII domains that participate in biofilm formation (Giglio et al., 2013). For *Xanthomonas* spp. that dwell in the xylem, biofilm formation during the initial stages and dispersal in the later stages is critical for pathogenesis (Crossman & Dow, 2004; Dow et al., 2003). In addition, factors involved in biofilm formation and dispersion are often regulated by quorum sensing. Interestingly, one such factor regulates CbsA expression in the presence of macerated rice tissues (Degrassi et al., 2007; Ferluga et al., 2007). Given this information and the fact that CbsA is critical for vascular pathogenesis, it would be worthwhile to assess the role of CbsA in the context of biofilm formation during the growth of Xoo in rice.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and growth conditions

The primers, bacterial strains, and plasmids used in this study are listed in Tables S2 and S3. Xoo strains were grown at 28°C in peptone sucrose (PS) medium and *E. coli* strains were grown in Luria Bertani (LB) medium at 37°C unless mentioned otherwise. The concentrations of antibiotics used were rifampicin (Rf) 50 µg/ml, spectinomycin (Sp) 50 µg/ml, ampicillin (Ap) 50 µg/ml, and kanamycin (Km) 50 µg/ml for *E. coli* and 15 µg/ml for Xoo.

4.2 | Construction of *cbsA*_{ΔFnIII} mutant in Xoo

Based on the structure of Xoo-secreted CbsA (Kumar et al., 2012; Tayi et al., 2018), the C-terminal region that is missing in the secreted CbsA was deleted in the Xoo genome. Genomic DNA of Xoo wild-type strain BXO43 was used as a template and two primer pairs, fragment A-FP/ fragment A-RP and fragment C-FP/ fragment C-RP (Table S2), were used to amplify fragments A and C (fragments A and C include the flanking regions of the FnIII domain). The A + C fragment was cloned in the *Hind*III restriction site of pK18*mobsacB* plasmid (Schäfer et al., 1994). The recombinant plasmid containing the fragment A + C was transformed into *E. coli* S17-1 (Simon et al., 1983). This recombinant plasmid was transferred from *E. coli* S17-1 to the wild-type Xoo BXO43 by biparental mating. Xoo cells were selected for kanamycin resistance and sucrose sensitivity. Integration of the plasmid at the expected locus was confirmed by PCR. The PCR-confirmed single recombinants were subcultured twice in liquid

medium (0.3% beef extract, 0.5% peptone) without kanamycin and plated on PS agar plates. Putative double recombinants, which include both wild-type and mutant cells (kanamycin sensitive and sucrose resistant), were isolated. The double recombinants were screened for the desired deletion by PCR and sequencing.

4.3 | Antiserum preparation

The catalytic domain of CbsA was purified from Xoo culture supernatant as described earlier (Kumar et al., 2012; Tayi et al., 2018). The purified protein was used to generate anti-CbsA antibodies in rabbit following the method described (Aparna et al., 2009). CbsA-specific antibodies were purified from the serum using affinity purification as reported earlier (Burbelo et al., 2002).

4.4 | Protein sample preparation for western blotting

Leaf tips of 40-day-old Taichung Native-1 (TN-1) rice plants were clip inoculated with Xoo strains. At different time points, infected leaves were cut at a point 2 cm below the visible lesion, dipped into water, and incubated at 4°C overnight to collect the leaf exudate. The exudate was centrifuged at 6700 × g for 10 min to remove cells. The supernatant was concentrated and subjected to western blotting analysis.

Secreted proteins and whole-cell lysates from Xoo cells were prepared as reported earlier (Gautam & Sharma, 2002) with some modifications. Xoo strains were grown to saturation, centrifuged, and the supernatant was taken as the extracellular fraction (EF). The EF was concentrated and an equal amount of the protein was loaded on a gel for western blotting analysis. The cell pellet from the above-mentioned sample was washed and resuspended in sterile Milli-Q water. Cell number was normalized based on OD₆₀₀ for different strains to obtain similar protein concentrations. Cells were lysed by heating at 95°C for 10 min with 2× protein loading buffer. After the heating step, samples were chilled on ice for 5 min and centrifuged at 9700 × g for 10 min. An equal amount of supernatant was separated by SDS-PAGE for western blotting analysis.

4.5 | Western blotting

Samples were run on 12% SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membrane was subjected to 2 h of blocking with 5% Blotto powder (Bio-Rad). After this, the membrane was washed with phosphate-buffered saline containing 0.05% Tween (PBST) and incubated with primary antibody (α-CbsA antibody, 1:1000; α-LipA antibody, 1:2000; α-HA antibody, 1:4000; abcam) or α-polyHis antibody (1:2000; Sigma Aldrich) at room temperature for 2 h. After thorough washing with PBST, the membrane was incubated with alkaline phosphatase (ALP)-conjugated

secondary antibody (1:30,000 dilution; Sigma Aldrich) for 1 h at room temperature. This was followed by washing and incubation with ALP substrate (33 μg/ml nitroblue tetrazolium, 17 μg/ml 5-bromo-4-chloro-3-indolyl phosphate; Roche) in ALP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris pH 9.5). Once the colour developed, the reaction was stopped by adding 0.5 M EDTA solution.

4.6 | Purification of recombinant CbsA and CbsA_{ΔFnIII} protein and enzyme activity assay

Sequences encoding CbsA and CbsA_{ΔFnIII} protein without the signal sequence were amplified from Xoo genomic DNA using specific primer pairs (Table S2). The amplified sequence was cloned into a pETM40 expression vector (Invitrogen) between NcoI and XhoI restriction sites to add an N-terminal maltose-binding protein tag (MBP, to improve solubility of the recombinant protein) and a C-terminal 6× histidine tag. A tobacco etch virus (TEV) protease cleavage site was present between the MBP and the cloned protein sequence. These constructs were overexpressed in *E. coli* BL-21 codon plus cells under IPTG induction. The proteins were purified using Ni-NTA (nitrilotriacetic acid) affinity chromatography followed by TEV protease cleavage to remove MBP. For this purpose, TEV protease and the MBP-tagged protein were mixed in a 1:100 ratio (wt/wt) and incubated at 4°C overnight. The protein of interest was further purified from this reaction mixture by Ni-NTA affinity chromatography followed by gel filtration. The purity of the protein was checked using SDS-PAGE. Enzyme activity assays were performed on soluble oligosaccharides and CMC as mentioned by Tayi et al. (2018).

4.7 | Reverse transcription quantitative real-time PCR

Total RNA was isolated from Xoo wild-type and *cbsA*_{ΔFnIII} cells using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. The RNA quality was assessed by agarose gel electrophoresis followed by DNase I (NEB) treatment and quantification. Primer pairs were checked for amplification efficiency using 10-fold dilutions of genomic DNA and the pair that was closest to 100% efficiency (slope of 3.3 for C_t versus log DNA copy number) was picked for further experiments. cDNA was synthesized from 5 μg of total RNA with random hexamers primers using RNA to cDNA EcoDry Premix kit (Clontech). The cDNA was diluted 10 times, and 1 μl of it was subjected to quantitative PCR (qPCR) using a DyNAmo Color Flash SYBR green qPCR kit (Thermo Scientific). The experiment was performed on a ViiA 7 real-time PCR system (Applied Biosystems). ΔC_t was calculated for Xoo wild type and *cbsA*_{ΔFnIII} by subtracting the C_{t mean} of respective samples from the C_{t mean} of 16S rRNA, which was used as an endogenous control. The experiment was done for three independent biological replicates. Mean ΔC_t and standard error of a representative experiment were plotted.

4.8 | Pull-down assay

CbsA, CbsA_{ΔFnIII}, HA-tagged FnIII domain, and polyhistidine-tagged SecB proteins were overexpressed in *E. coli* using pET28b (Novagen) expression vector. The strains were grown at 37°C until OD₆₀₀ 0.6 and induced with 0.1 mM IPTG. After induction, cells were grown at 18°C overnight. Cells were harvested by centrifugation and resuspended in a lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl). CbsA/CbsA_{ΔFnIII}/FnIII-expressing cell lysates were incubated overnight with TALON beads with and without SecB-expressing cell lysate. After binding to the cell lysate, beads were washed successively with lysis buffer and lysis buffer containing 20 mM imidazole. Finally, the bound protein fraction was eluted using elution buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 250 mM imidazole). The eluted proteins were probed by western blotting.

4.9 | Virulence assay

The following Xoo strains were used for virulence analysis: BXO43, $\Delta cbsA$, $cbsA_{\Delta FnIII}$, $cbsA_{\Delta FnIII}$ complemented with wild-type *cbsA*, and *cbsA* without FnIII domain-encoding region on pHM1 plasmid (Innes et al., 1988). Strains were grown to saturation followed by centrifugation at room temperature. The cell pellet was washed and resuspended in Milli-Q water to achieve OD₆₀₀ = 1.0. The leaf tips of 40-day-old TN-1 rice plants were cut with surgical scissors dipped in the bacterial culture. Lesion lengths were measured 15 dpi.

4.10 | Bioinformatics analysis

CbsA sequences from different *Xanthomonas* species were retrieved from the NCBI database. A multiple sequence alignment was generated using Clustal Omega on the EMBL-EBI server (Sievers et al., 2011). The alignment was submitted to the IQ-TREE web server (Trifinopoulos et al., 2016) to generate a maximum-likelihood phylogenetic tree. Domain identification was done in different CbsA sequences using the InterPro online tool (Blum et al., 2021). The SignalP v. 5.0 web tool (Armenteros et al., 2019) was used to predict the type of signal sequence in CbsA proteins from different *Xanthomonas* species/pathovars. The hydrophobicity score of the Xoo CbsA protein sequence was calculated using the ProtScale web tool (Kyte & Doolittle, 1982).

4.11 | Codon usage pattern

The codon usage pattern was analysed as described earlier (Patil & Sonti, 2004) using “The Sequence Manipulation Suite” web tool (Stothard, 2000). The first group was chosen containing the following housekeeping genes encoding tonB-dependent siderophore receptor (BXO1_013815): *Xanthomonas* adhesin-like protein (BXO1_013910), *rpfF* (BXO1_008735), phytase (BXO1_006505),

shikimate dehydrogenase (BXO1_016165), and secreted xylanase (BXO1_019245). The second group contained genes from the lipopolysaccharide cluster, which was previously shown to be horizontally transferred into Xoo (Patil & Sonti, 2004). This group included *smtA* (BXO1_014260), *wxoA* (BXO1_014255), *wxoB* (BXO1_014250), *wxoC* (BXO1_014240), and *wxoD* (BXO1_014235). The average GC content for the Xoo genome was taken based on the genome sequence of BXO1 (Kaur et al., 2019).

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

AUTHOR CONTRIBUTIONS


R.N., R.V.S., and R.S. conceived the project and designed the experiments. R.N. and R.V.M. performed the experiments. R.N., H.K.P., R.S., and R.V.S. analysed the data, and finalized the manuscript, which was approved by all the authors. H.K.P., R.S., and R.V.S. contributed reagents/materials.

DATA AVAILABILITY STATEMENT

All data has been included in the manuscript.

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