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Review

Key steps in type III secretion system (T3SS) towards translocon assembly with potential sensor at plant plasma membrane

HONGTAO JI AND HANSONG DONG*

Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, Jiangsu Province, China

SUMMARY

Many plant- and animal-pathogenic Gram-negative bacteria employ the type III secretion system (T3SS) to translocate effector proteins from bacterial cells into the cytosol of eukaryotic host cells. The effector translocation occurs through an integral component of T3SS, the channel-like translocon, assembled by hydrophilic and hydrophobic proteinaceous translocators in a two-step process. In the first, hydrophilic translocators localize to the tip of a proteinaceous needle in animal pathogens, or a proteinaceous pilus in plant pathogens, and associate with hydrophobic translocators, which insert into host plasma membranes in the second step. However, the pilus needs to penetrate plant cell walls in advance. All hydrophilic translocators so far identified in plant pathogens are characteristic of harpins: T3SS accessory proteins containing a unitary hydrophilic domain or an additional enzymatic domain. Two-domain harpins carrying a pectate lyase domain potentially target plant cell walls and facilitate the penetration of the pectin-rich middle lamella by the bacterial pilus. One-domain harpins target plant plasma membranes and may play a crucial role in translocon assembly, which may also involve contrapuntal associations of hydrophobic translocators. In all cases, sensory components in the target plasma membrane are indispensable for the membrane recognition of translocators and the functionality of the translocon. The conjectural sensors point to membrane lipids and proteins, and a phosphatidic acid and an aquaporin are able to interact with selected harpin-type translocators. Interactions between translocators and their sensors at the target plasma membrane are assumed to be critical for translocon assembly.

Keywords: harpins, plasma membrane, sensor, translocators, type III translocon.

**Correspondence*: Email: hsdong@njau.edu.cn

INTRODUCTION

A variety of Gram-negative bacteria which are pathogens of plants (Alfano and Collmer, 2004; White *et al*., 2009) or animals (Chatterjee *et al*., 2013; Mueller *et al*., 2008) utilize the type III secretion system (T3SS) to transport proteins into the surrounding milieu or directly into the cytosol of eukaryotic host cells. The T3SS deploys a proteinaceous needle complex in animal pathogens (Kubori *et al*., 1998; Deane *et al*., 2006) and a proteinaceous pilus complex in plant pathogens (Jin and He, 2001) as an essential pathway for protein secretion and translocation. In the needle or pilus complex, an integral apparatus that governs protein translocation is called the type III translocon, which consists of T3SS accessory (structural) proteins, namely type III translocators, in association with sensory components potentially present in the target plasma membrane (PM) of host cells (Büttner, 2012; Matteï *et al*., 2011; Mueller *et al*., 2008). Translocated bacterial proteins serve as either virulence effectors to cause diseases or defence activators to confer disease resistance in eukaryotic hosts (Galán and Collmer, 1999). In particular, virulence effectors employ different mechanisms, avoiding or defeating the innate immunity and manipulating the transcriptomes of plants (Alfano and Collmer, 2004; Bogdanove and Voytas, 2011; Lindeberg *et al*., 2012; Scholze and Boch, 2010; White *et al*., 2009; White and Yang, 2009) and animals (Bliska *et al*., 2013; Plano and Schesser, 2013), respectively, to secure the bacterial ability to multiply and cause diseases in hosts (Galán and Collmer, 1999).

In the last 10 years, significant progress has been acquired in demonstrating T3SS components and architectures in animal (Büttner, 2012; Chatterjee *et al*., 2013; Matteï *et al*., 2011; Mueller *et al*., 2008) and plant (Alfano and Collmer, 2004; Choi *et al*., 2013; Kay and Bonas, 2009) pathogens. The essential compositions of the type III translocon and the characteristics of the translocators have been elucidated extensively for animal pathogens and have also attracted increasing attention from the plant bacteriology community. The accumulated knowledge has led to new research goals directed towards the determination of the accurate assembly of the translocon and the characterization of recognition sensors of the translocators putatively present in the target PM. This review briefly discusses T3SS organization and then focuses on the biochemical and pathological characteristics

of the translocators from the pathogens of plants compared with animals, models of translocator associations to form the translocon, potential sensors of the translocators in the target PM, especially from plants, and possible interactions between the translocators and their sensors to facilitate effector translocation.

GENERAL ARCHITECTURE OF T3SS

In all bacteria studied to date, the T3SS is exquisitely yet similarly assembled as a nanomachine by refined associations of more than 20 structural proteins that are either diverse or conserved among different bacterial species (Abrusci *et al*., 2014; Bogdanove *et al*., 1996; Chatterjee *et al*., 2013; Matteï *et al*., 2011; Plano and Schesser, 2013). In every bacterial species, different structural proteins are produced hierarchically to build up the T3SS machinery as two associated parts: a membrane-traversing basal body plus an extracellular outgrowth: a needle complex in animal pathogens and a pilus complex in plant pathogens (Fig. 1). The basal body spans bacterial inner (IMs) and outer (OMs) membranes, comprises ring structures that are presumably connected by a periplasmic rod (P-rod) across the peptidoglycan mesh and associates with the needle or pilus complex. The needle or pilus elongates outwards from the bacterial surface and reaches the surface of host cells when the bacterium contacts with host cells (Abrusci *et al*., 2014; Matteï *et al*., 2011; Mueller *et al*., 2008).

In essence, the extracellular needle or pilus appendage comprises an approximately 1.2–3.5-nm inner channel (Fig. 1), which hypothetically functions as a pathway for the secretion of unfolded/semi-unfolded proteins through a trafficking route all the way from the bacterial cytoplasm to the bacteria–host interface (Jin and He, 2001; Büttner and Bonas, 2002; Kubori *et al*., 1998; Schraidt *et al*., 2010). The subsequent translocation of type III effectors from the tip of the needle or pilus appendage into the cytosol of host cells is facilitated by the channel-like type III translocon situated at the distal end of the needle or pilus complex. The translocon extends to penetrate the target PM (Daniell *et al*., 2001; Faudry *et al*., 2006; Neyt and Cornelis, 1999; Ryndak *et al*., 2005; Schoehn *et al*., 2003) and has an opening inwards to the cytosol of host cells (Ide *et al*., 2001), allowing for direct injection of type III effectors from bacteria into host cells (Alfano and Collmer, 2004; Chatterjee *et al*., 2013; Kay and Bonas, 2009; Matteï *et al*., 2011).

As a prerequisite for translocon assembly at the bacteria–host interface, the distal end of the bacterial needle and pilus must reach to the outer plane of host cell PMs (Fig. 1). This requires a common mechanism by which the P-rod of the T3SS basal body traverses the bacterial periplasm and, by this step, the basal body is able to associate with the OM-located ring structure, which is connected outwards to the needle or pilus complex. Owing to the structural difference between plant and animal cells, the bacterial

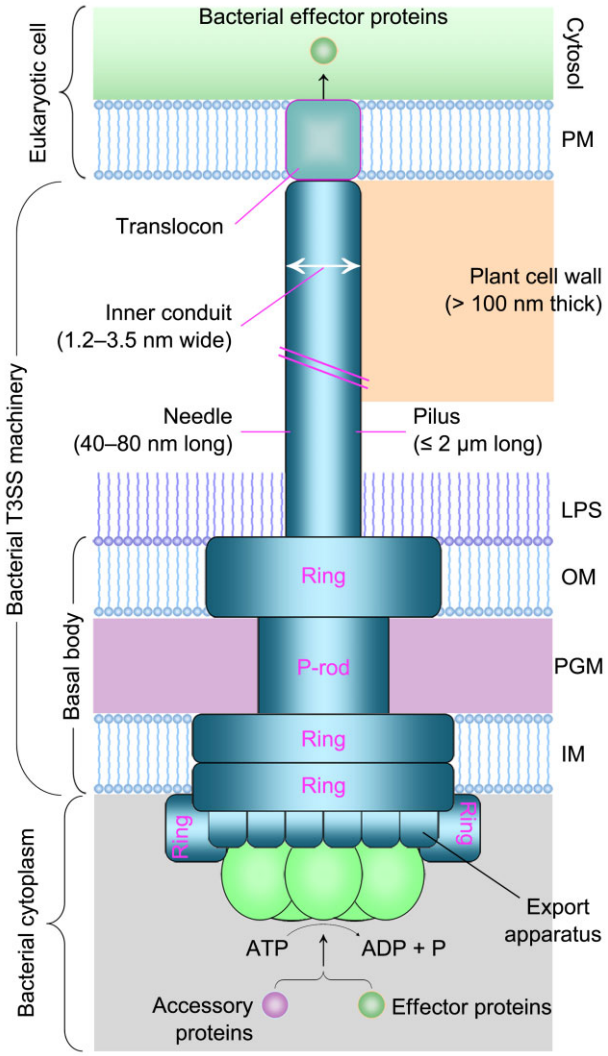

Fig. 1 Schematic diagram illustrating type III secretion system (T3SS) architectures of animal- and plant-pathogenic Gram-negative bacteria. The basal body of the T3SS spans the bacterial inner membrane (IM) and outer membrane (OM), and comprises ring structures that are presumably connected by a periplasmic rod (P-rod) across the peptidoglycan mesh (PGM) (Büttner 2012). The basal body associates with an extracellular needle (animal pathogens) or pilus (plant pathogens) appendage, which elongates across an OM peripheral layer containing lipopolysaccharide (LPS) and forms a channel-like translocon that inserts into the host plasma membrane (PM). The energy for the docking and unfolding of T3SS substrates, including effector proteins and T3SS-accessory extracellular proteins, is probably provided by a cytoplasmic ATPase associated with the T3SS (Büttner 2012). The width and length of the bacterial needle or pilus, as well as the thickness of the plant cell wall, are indicated in the diagram, and in the text these parameters are correlated with key steps of the needle or pilus growth towards the translocon assembly into the target PM of animal or plant cells.

pilus needs to overcome an additional physical barrier, the plant cell wall (CW), which is at least 100 nm thick under most circumstances (Rose, 2003). Perhaps as a result of this particularity, the pilus elongating up to 2 μm is maximally 25-fold longer than the needle extending, at most, to 80 nm in length (Blocker *et al*., 2001; Jin and He, 2001; Journet *et al*., 2003). Penetrations of bacterial periplasmic layers and plant CWs accordingly represent the characteristic similarity and difference between plant- and animal-pathogenic bacteria in their T3SS organization towards the translocon assembly.

PENETRATION OF BACTERIAL PERIPLASM BY T3SS BASAL BODY

The periplasm of Gram-negative bacteria comprises a peptidoglycan mesh (Fig. 1) with natural pores of approximately 2 nm in radius (Demchick and Koch, 1996). As argued circumstantially by Matteï *et al*. (2011), such natural pores are too narrow to accommodate the P-rod, which is much wider than 3.5 nm (Fig. 1). Thus, bacteria need to deploy a mechanism for their T3SS machinery to traverse the periplasmic layer. This mechanism has been characterized as the production of lytic transglycosylases (LTs), which are assumed to locally degrade the peptidoglycan substrate (Mushegian *et al*., 1996; Ward *et al*., 2002) and to enlarge pores in the periplasmic mesh to accommodate the T3SS machinery (Koraimann, 2003; Oh *et al*., 2007; Wang *et al*., 2008; Zahrl *et al*., 2005).

LTs typically recognize a single motif with an invariant glutamate in the substrate and catalyse the cleavage of the β-1,4-glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid in peptidoglycan, producing the terminal 1,6-anhydromuramyl cleavage product (Boch *et al*., 2002; Mushegian *et al*., 1996). This catalytic activity can broaden natural pores in the peptidoglycan mesh, allowing the T3SS machinery to traverse the periplasmic barrier in animal-pathogenic bacteria (Demchick and Koch, 1996; Zahrl *et al*., 2005) and possibly also in plant pathogens (Boch *et al*., 2002; Oh *et al*., 2007; Wang *et al*., 2008; Ward *et al*., 2002). In agreement with this hypothesis, the hypersensitive response and pathogenicity (Hrp) in non-host and host plants, respectively, are regarded as the translocation-associated phenotype of bacteria, and Hrp-associated (Hpa) protein Hpa2 from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*), the pathogens that cause bacterial blight and bacterial streak of rice (*Oryza sativa*), respectively, has been characterized to be an LT enzyme with the ability to degrade the bacterial envelope (Zhang *et al*., 2008). The LT orthologue HpaH from *X. campestris* pv. *vesicatoria* (*Xcv*), the bacterial spot pathogen of pepper (Noël *et al*., 2002), may also be enzymatically active, because HpaH is almost identical in amino acid sequence to Hpa2, contributes to virulence and T3SS assembly, and promotes the secretion and translocation of effector proteins (Büttner *et al*., 2007). In *Xcv*, moreover, the function of HpaH is regulated by HrpB1 and HrpB2 in the Hrp peninsula (Büttner *et al*., 2007; Rossier *et al*., 2000). HrpB1 localizes to the bacterial periplasm and physically interacts not only with peptidoglycan but also with HrpB2 and several other T3SS components (Hausner *et al*., 2013), suggesting that these proteins are part of a periplasmic substructure of the T3SS machinery related to the potential role of HpaH in degrading the peptidoglycan substrate.

Predicted LTs from plant-pathogenic bacteria (Boch *et al*., 2002; Oh *et al*., 2007; Wang *et al*., 2008; Ward *et al*., 2002) perform in a similar manner to their orthologues in animal pathogens, such as IpgB and IpgF from the animal diarrhoea pathogen *Shigella* spp. (Allaoui *et al*., 1993; Zahrl *et al*., 2005), with regard to the positive effect on T3SS and/or virulence. However, single LTs do not contribute significantly to T3SS and virulence, both of which may require cooperative functions of the LTs. For example, *Pseudomonas syringae* pv. *tomato* (*Pst*) produces different effectors, namely Hrp proteins or Hrp-out proteins (Hops), to cause bacterial speck in host plants, such as tomato (*Solanum lycopersicum*). The pathogen deploys three LTs (HrpH, HopP1 and HopAJ1) under co-regulation with the T3SS regulon HrpJ, which controls effector translocation by regulating the secretion of the corresponding translocators (Crabill *et al*., 2012; Fu *et al*., 2006). These LTs function collaboratively and contribute to the translocation of effector HopA1 and its virulence to the plant (Oh *et al*., 2007). HrpH and HopP1, but not HopAJ1, are T3SS substrates, and are themselves secreted (Oh *et al*., 2007), possibly to prevent further peptidoglycan degradation after the secretion machinery has traversed the periplasmic barrier (Büttner, 2012).

In addition, HopP1 is similar to a distinct group of T3SS-secreted, effector-helper, non-effector proteins, collectively designated as harpins, with respect to the properties of lacking cysteine (except for Hpa1 from bacteria in the *Xanthomonas* genus; Zhu *et al*., 2000) and eliciting the hypersensitive response (HR) in leaves of tobacco (*Nicotiana tabacum*) (Kvitko *et al*., 2007), a non-host plant of *Pst* and many other bacteria. Harpins are basically distinct from other T3SS-secreted proteins, especially virulence effectors (He, 1998; Kim and Beer, 2000). A fundamental distinction is that harpins function outside plant cells; by contrast, to perform their pathological roles, effectors must translocate into the cytosol of plant cells (Alfano and Collmer, 2004; Galán and Collmer, 1999; He, 1998). Multiple effects of harpins elucidated in the last 23 years (since the first report by Wei *et al*., 1992) and the newly appreciated function of some harpins in effector translocation have been summarized in a recent review (Choi *et al*., 2013). This review fully discusses the structural, biochemical and functional features of harpins from different bacterial species. Below, we focus on the structure–function relationship of tested harpins that affects bacterial pilus growth towards PMs of plant cells.

POTENTIAL ROLES OF TWO-DOMAIN HARPINS IN BACTERIAL PILUS PENETRATION OF THE PLANT CW BARRIER

To contact with the outer plane of plant PMs, the bacterial pilus must traverse at least 100-nm-wide plant CWs (Fig. 1). This process perhaps requires the aid of particular T3SS accessory proteins that have activities of CW-degrading enzymes. Candidates have been found in so-called two-domain harpins, which have been defined, in contrast with one-domain harpins, to emphasize the obvious structural difference related to pilus growth towards the target PM, but not to preclude other functional domains (Kvitko *et al*., 2007), such as predicted α-helical and glycine-rich motifs (Ji *et al*., 2011; Kvitko *et al*., 2007; Liu *et al*., 2006; Meyer *et al*., 2006; Wang *et al*., 2007, 2008). One-domain harpins refer to early defined conventional type III accessory proteins that are characteristically acidic, hydrophilic and glycine-rich, with the dual role of inducing HR in non-host plants and/or contributing to virulence on hosts of the bacteria (He *et al*., 1993; Wei *et al*., 1992). Two-domain harpins are hybrids of the corresponding canonical proteins, and each hybrid contains a residue-indexed shortened version of a particular harpin at the nitroxyl (N) terminus and a carboxyl-terminal (C-terminal) enzymatic domain characteristic of either LT enzymes, which have been implicated in the degradation of the bacterial periplasmic layer (Kvitko *et al*., 2007; Zhang *et al*., 2008), or pectate lyase (PL) enzymes, which may catalyse the decomposition of plant CWs (Charkowski *et al*., 1998; Kim and Beer, 1998). So far, nine one-domain harpins (HrpN, HrpZ1, HrpZ, $HrpZ^{j}$, Hpa1, HpaG, XopA, HreX and Hrp_{Xm}) and six two-domain harpins (HrpW, HopAK1, HrpW1, HopP1, PopW and $HrpW^{k}$) have been identified in plant-pathogenic bacteria and a rhizobium (Choi *et al*., 2013). The two classes of harpin are basically different with regard to potential targets in the bacterial envelope or plant CWs.

Although LT-type two-domain harpins have been proposed to act on the peptidoglycan mesh to facilitate the penetration of the bacterial periplasm by the T3SS basal body, as discussed above, PL-containing two-domain harpins have been found to target the compositions of plant CWs and are thought to be essential for the bacterial pilus to traverse the plant CW barrier in the pectin-rich middle lamella. Early characterized two-domain harpins were HrpW1 from *Pst* (Charkowski *et al*., 1998) and HrpW from *Erwinia amylovora* (*Ea*), the pathogen which causes fire blight of rosaceous plants (Kim and Beer, 1998). *Ea* HrpW contains 447 amino acids and comprises two domains connected by an intermediate sequence with abundant proline and serine residues. The N-terminal domain is similar to the partial sequence of HrpN, especially with regard to glycine-rich repeats, and the C-terminal domain is characteristic of polysaccharide lyase family 3 (PL3) PLs (Kim and Beer, 1998). Similar dual domains have been found in *Pst* HrpW1 (Charkowski *et al*., 1998; Kvitko *et al*., 2007). At 424 amino acids in size, HrpW1 appears to be a canonical protein hybrid, in which the N-terminal 1–188 region is equivalent to a residue-indexed HrpZ1 and the C-terminal fragment sequence is similar to PL3 PLs. The PL3 fragment truncated from HrpW1 is able to bind with calcium pectate, a major constituent of plant CWs, suggesting that the target of HrpW1 is in the plant CW (Charkowski *et al*., 1998).

However, neither the truncated PL3 fragment nor the full-length HrpW protein is able to degrade pectate substrates (Charkowski *et al*., 1998; Kim and Beer, 1998; Li *et al*., 2010). By contrast, the HrpW orthologue of *Rhizobium etli*, called $HrpW^{k}$ (Choi *et al*., 2013), exhibits pectinolytic activity, which, however, is eliminated by replacing tryptophan-192 with alanine (Fauvart *et al*., 2009). The alanine residue in HrpW1 is equivalent to tryptophan-192 in $HrpW^{k}$, whereas the tryptophan residue is conserved in all active PL enzymes, but not in HrpW and HrpW1 (Fauvart *et al*., 2009), suggesting that HrpW and HrpW1 are destined to be inactive. Presumably, the loss of enzymatic activity is a congenital strategy for the bacterial pilus machinery to translocate virulence effectors in a manner of friendship with plants, or without breakage of plant cell structures. If this hypothesis is true, HrpW or HrpW1 binding to, instead of degrading, the middle lamella is sufficient for this thin, soft, cementing layer to be penetrated by the pilus. Alternatively, pectate degradation may be provided by a different two-domain harpin from the same bacteria, such as HopAK1, under the particular circumstance (Kvitko *et al*., 2007) in which HrpW1 binding to the middle lamella serves as a platform for HopAK1 to locally degrade the substrate. Such an 'arena-player' mode is commonly used by hydrophilic translocators (Fig. 2A-a), namely needle tip proteins (Chatterjee *et al*., 2011; Johnson *et al*., 2007; Mueller *et al*., 2005), of animal-pathogenic bacteria to assist their hydrophobic counterparts to burrow into the target PM during type III translocon assembly (Büttner, 2012; Mueller *et al*., 2008).

ROLES OF ONE-DOMAIN HARPINS IN PILUS TARGETING TO PLANT PM

Owing to their hydrophilicity, one-domain harpins of plant-pathogenic bacteria (Fig. 2B-a) represent analogues of needle tip proteins from animal pathogens (Fig. 2A-a) and are likely to locate at the tip of the pilus with direct access to the outer plane of plant PMs. Similar to the spatially contrapuntal situation of needle tip proteins of animal pathogens at the interface between the distal end of the needle and the targeted PM of host animal cells, one-domain harpins of plant pathogens can localize to plant PMs or membranes of other eukaryotic cells, such as oocytes of *Xenopus laevis*, an African clawed frog frequently used in protein localization experiments (Maurel, 2007; Racape *et al*., 2005). Direct evidence has been obtained for *Ralstonia solanacearum*

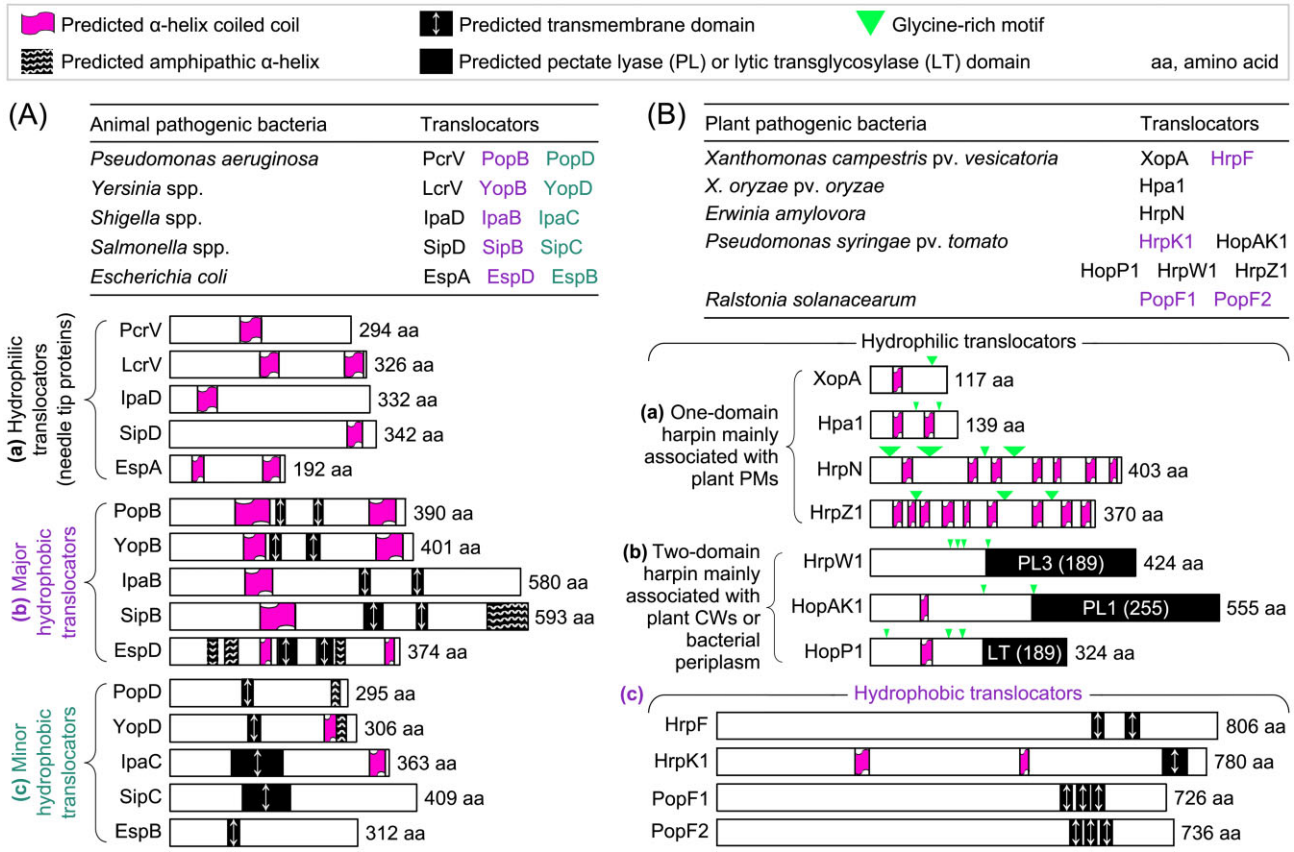

Fig. 2 Schematic analysis of type III translocators from animal- and plant-pathogenic Gram-negative bacteria. The predicted characteristic domains are indicated by the symbols described in the figure and, in (A) and (B), they are located pro rata in the graphic protein sequences. Predicted α-helical regions form a coiled coil, called an α-helix coiled coil, or contain hydrophobic residues, designated as an amphipathic α-helix. Additional information is to be stated below in (A) and (B). In both panels, translocators that belong to the same category are shown together in subpanels indicated by lowercase letters in parentheses. (A) Overview of translocators from animal pathogens. The depicted features of the proteins are constant in the literature, except for EspB, EspD and PcrV. For EspB, only the predicted transmembrane (TM) domain is shown, and the predicted 11 α-helical regions, which vary in length and are located throughout the protein sequence (Hamada *et al*., 2005), are neglected. For PcrV and EspD, the number of amino acid residues shown here is the same as in the original research papers (Delahay and Frankel, 2002; Goure *et al*., 2004), but different from an in-depth review article (Matteï *et al*., 2011). (B) Overview of translocators from plant pathogens. Hpa1 is a candidate for the translocator of the TAL effector PthXo1 based on studies shown in Fig. 4, whereas the other translocators have been studied previously (Choi *et al*., 2013). Predicted TM domains present in the hydrophobic translocators (Büttner *et al*., 2002), the enzymatic domains in two-domain harpins (Kvitko *et al*., 2007) and predicted α-helices present in HrpN, HrpZ1 and Hpa1 (Choi *et al*., 2013) are situated according to the literature. The predicted α-helices present in other harpins were not stated in the literature and were determined by analysis with the online SIB (Swiss Institute of Bioinformatics) ExPASy program (http://www.ch.embnet.org/software/COILS_form.html).

PopA1 binding to *X. laevis* oocytes (Racape *et al*., 2005), and for *Ea* HrpN (Oh and Beer, 2007) and *Xoo* Hpa1 (Sang *et al*., 2012) expressed in *Arabidopsis thaliana*. Direct evidence has also been found for HrpZ from *P. syringae* pv. *phaseolicola*, and this harpin binds to the PM of tobacco cells (Lee *et al*., 2001a). In addition, isolated or synthetic liposomes and/or synthetic planar lipid bilayers bind HreX from *X. campestris* pv. *pelargonii* (Engelhardt *et al*., 2009) and HrpZ orthologues of *Pseudomonas* spp. (Engelhardt *et al*., 2009; Lee *et al*., 2001b). Targeting to the plant PM may allow HrpN (Bocsanczy *et al*., 2008), HrpZ1 (Kvitko *et al*., 2007; Petnicki-Ocwieja *et al*., 2005), Hpa1 (Sang *et al*., 2012) and XopA (Büttner *et al*., 2006; Noël *et al*. 2002) to integrate into the type II translocon assembly and facilitate pore formation in the target PM.

HYDROPHILIC TRANSLOCATORS OF PLANT-PATHOGENIC BACTERIA

With respect to the role in effector translocation, needle tip proteins are also called hydrophilic translocators (Fig. 2A-a), which depend on a unitary motif, the predicted α-helical coiled coil (Espina *et al*., 2006a,b; Markham *et al*., 2008), to oligomerize into the tip of the needle complex (Yip *et al*., 2005). To date, three one-domain harpins (HrpN, HrpZ1 and XopA) have been implicated

in effector translocation from plant-pathogenic bacteria (Fig. 2B-a). These harpins resemble the orthologues of animal pathogens in their structural characteristics (Fig. 2B-a compared with Fig. 2A-a), especially with regard to the presence of predicted α-helical coiled-coil motifs (Ji *et al*., 2011; Kvitko *et al*., 2007; Liu *et al*., 2006; Wang *et al*., 2007, 2008). The three harpins have been demonstrated to play substantial roles in supporting the virulence and/or translocation of different effectors. XopA of *Xcv* is implicated in the translocation of type III effectors, as the *Xcv xopa* mutant incurs partial impairment in the ability to induce plant responses, but does not affect effector secretion (Büttner *et al*., 2006; Noël *et al*. 2002). Hence, XopA is regarded as a hydrophilic translocator candidate, but its direct role in effector translocation has not been determined. By contrast, HrpN of *Ea* has been convincingly elucidated to be an essential translocator for effector DspA/E of the bacterium (Bocsanczy *et al*., 2008).

Two-domain harpins have also been implicated in effector translocation. It is quite interesting that *Pst* produces four harpins, one-domain HrpZ1 and two-domain HrpW1, HopP1 and HopAk1 (Fig. 2B-b), which all affect effector translocation and virulence or HR induction (Kvitko *et al*., 2007). These harpins synergize hydrophobic translocator HrpK1 (Petnicki-Ocwieja *et al*., 2005) on the basis of the following findings: (i) the quadruple harpin gene polymutant carrying the *hrpK1* gene displays moderate reductions in AvrPto1 translocation and the ability to elicit HR or cause disease; (ii) these events are strongly reduced in the quadruple polymutant if *hrpK1* is knocked out; and (iii) the quinary polymutant, in which *hrpK1* and the four harpin genes are concurrently knocked out, can be complemented by any of *hrpZ1*, *hrpW1* and *hopAK1*, but not *hopP1* (Kvitko *et al*., 2007). Thus, HrpZ1, HrpW1 and HopAK1 are functionally redundant with HrpK1, but HopP1 is not, to mediate AvrPto1 translocation. However, HopP1 plays a role in the translocation of a different effector called HopA1 (Oh *et al*., 2007). This difference is attributable to the T3SS regulon HrpJ, which controls effector translocation by regulating the secretion of the corresponding translocators (Crabill *et al*., 2012; Fu *et al*., 2006). Indeed, HrpJ regulates the secretion of HrpZ1, HrpW1 and HopAK1, but not HopP1 (Crabill *et al*., 2012). These analyses indicate that secretory regulation received by translocators is important for their roles in effector translocation. These analyses also suggest that a particular translocator may be responsible for the translocation of some, but not all, of the effectors from a bacterium.

HYDROPHOBIC TRANSLOCATORS OF PLANT-PATHOGENIC BACTERIA

So far, four hydrophobic translocators have been identified in plant-pathogenic bacteria (Fig. 2B-c), representing a very small number of potential translocators compared with the repertoire of animal pathogens (Fig. 2A-bc). Moreover, each species of animal-pathogenic bacterium possesses a major hydrophobic translocator that carries two predicted transmembrane (TM) domains (Fig. 2A-a) and a minor one that contains a single predicted TM domain (Fig. 2A-b). Predicted TM domains guide the hydrophobic translocators into the target PM to create a translocon pore (Faudry *et al*., 2006; Hakansson *et al*., 1996; Ryndak *et al*., 2005). In addition to TM, predicted α-helix coiled-coil and/or amphilipathic α-helix motifs (Fig. 2A) are also present in most hydrophobic translocators (Derewenda *et al*., 2004; Espina *et al*., 2007; Johnson *et al*., 2007). Amphilipathic α-helix and α-helix coiled-coil motifs allow for the dimerization and oligomerization of proteins, respectively (Twyman, 1998), and sometimes dimerization induces oligomerization by the same protein (Delahay and Frankel, 2002). In contrast with these extensive studies on translocators of animal pathogens, the current knowledge is far from sufficient to allow for a consideration of whether or not the attributive 'major' and 'minor' are pertinent to define the hydrophobic translocators of plant pathogens.

The first type III hydrophobic translocator discovered in plant-pathogenic bacteria was HrpF of *Xcv* (Büttner *et al*., 2002). This protein locates within the HrpF pathogenicity peninsula conserved in different species of the *Xanthomonas* genus (Sugio *et al*., 2005) and contains two predicted TM domains (Büttner *et al*., 2002; Huguet and Bonas, 1997; Fig. 2B-c). The role of *Xcv* HrpF as a translocator has been well demonstrated by Büttner *et al*. (2002). They elucidated that the C-terminal region of the HrpF sequence plays an essential role in supporting the translocation of effector AvrBs3, and that the N-terminal region contains a signal for secretion, but not for translocation. This suggests that secretion and translocation are sequential, but independent, processes under the control of different functional domains in the translocator. The same workers further showed that HrpF was able to bind with artificial lipids formed in silicon beads to induce altered electrical current fluxes, indicative of pore formation, in a synthetic planar lipid bilayer system. Quite interestingly, the C-terminus required for lipid binding and effector translocation is dispensable for pore formation in the bionic membrane. This discrepancy may be caused by the higher sensitivity of the planar bilayer assay relative to the lipid-binding measurement for the detection of the activity provided by the region towards the N-terminus.

With a similarity (E value = 6e – 14) to *Xcv* HrpF, HrpK secreted by *Pst* contains two predicted α-helical coiled-coil motifs in the middle and a single predicted TM domain at the C-terminal region of the protein sequence (Fig. 2B-c). The predicted TM domain is required for the translocation of HrpK itself into plant cells and this translocation is subject to T3SS. Furthermore, HrpK is a putative type III translocator, as it is required for type III effector HopA1 to induce HR in the non-host plant tobacco and to cause leaf speck in the host plants tomato and *Arabidopsis* (Petnicki-Ocwieja *et al*., 2005).

In addition to HrpF and HrpK, PopF1 and PopF2 secreted by *Ralstonia* (previous *Pseudomonas*) *solanacearum*, a soil-borne pathogen that causes bacterial wilt disease in more than 200 plant species (Arlat *et al*., 1994; Mansfield *et al*., 2012; Meyer *et al*., 2006), are also characteristic of hydrophobic translocators (Fig. 2B-c). According to Meyer *et al*. (2006), PopF1 and PopF2 share significant homology with one another, with 81% identity and 86% similarity, and exhibit some degree of similarity to HrpF from *Xanthomonas* spp. and YopB from *Yersinia* spp. at the C-terminal 200-amino-acid region, which contains the predicted TM domains (Fig. 2B-c). Although PopF1 contributes more than PopF2 to virulence on tomato and HR induction in tobacco, both PopFs act similarly to mediate the translocation of effector AvrA. Both PopFs are partially exchangeable with *Xcv* HrpF, as HrpF can compensate for part of the compromised AvrA translocation in the *popF1 popF2* double mutant. This finding and the similarity of PopFs and HrpFs suggest that translocators are functionally conserved in bacteria of the genera *Ralstonia* and *Xanthomonas* (Büttner, 2012; Meyer *et al*., 2006).

MODELS OF TYPE III TRANSLOCON ASSEMBLY

As shown in Fig. 2A, every species of animal-pathogenic bacterium possesses one hydrophilic and two hydrophobic translocators, each forming homogeneous oligomers before participating in translocon assembly (Chatterjee *et al*., 2013). Matteï *et al*. (2011) proposed two distinct models of type III translocon assembly by animal pathogens. One involves a hetero-oligomer of two hydrophobic translocators, spontaneously contacting the hydrophilic counterpart (Fig. 3A). The other is called the 'three-tiered ring' model: two hydrophobic translocators exist in oligomeric form, with the major partner inserting stably into the target PM, whereas the minor translocator, situated at the needle tip, serves as the link with the hydrophilic counterpart (Fig. 3B). Although most biochemical results point to the second model (Büttner, 2012; Faudry *et al*., 2006; Goure *et al*., 2004), the possibility of translocon assembly as a hetero-oligomer cannot be ruled out at present, as argued by Matteï *et al*. (2011).

Known translocators of plant-pathogenic bacteria diversify largely in terms of biochemistry (Fig. 2B), indicating the complexity of the process of translocon assembly, which may involve two steps (Fig. 3C). First, the pilus penetrates the plant CW with the aid of PL-type two-domain harpins. Additional CW-degrading enzymes (Yang *et al*., 2008) or physical force only is required for pilus penetration of the primary wall, a thin, flexible and extensible layer formed by growing cells in young tissues that favours bacterial infection. Second, one-domain harpins serving as

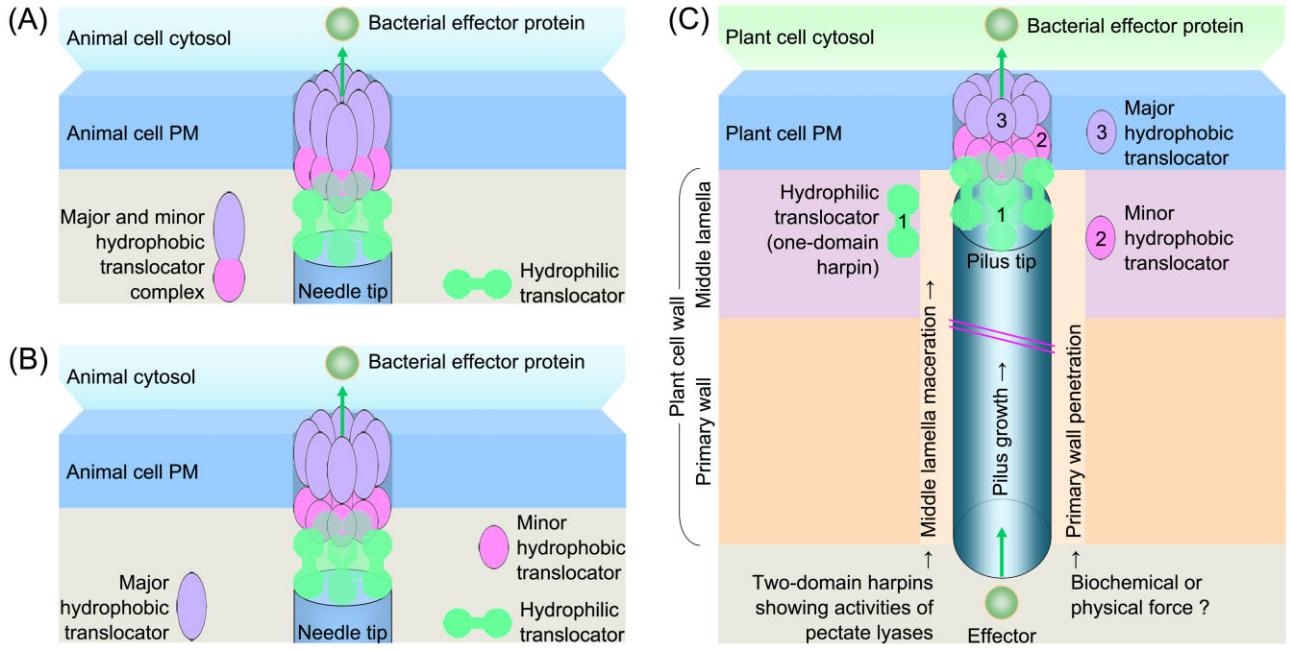

Fig. 3 Models of the translocon assembly by bacteria that are pathogens of animals (A, B) and plants (C). (A, B) Both diagrams were created according to previous definitions (Matteï *et al*., 2011). (C) The diagram was created according to previous studies, which have been partially discussed in a recent review on harpins (Choi *et al*., 2013). The numbers indicate the potential location of translocator complexes. In (A)–(C), the number of a protein complex, five and eight in hydrophilic and hydrophobic translocators, respectively, is shown based on previous studies partially summarized in several review articles (Büttner, 2012; Chatterjee *et al*., 2013; Matteï *et al*., 2011; Mueller *et al*., 2008).

hydrophilic translocators may assist the hydrophobic counterparts to burrow into the target PM. A similar 'two-step model' has been proposed by Kvitko *et al*. (2007). They also presented a 'consortium model': the pilus machinery has an intrinsic ability to breach the plant CW, whereas harpins and HrpF homologues function as a consortium for effector translocation. The intrinsic CW-breaching mechanism may be provided in the manner stated above, i.e. direct pilus penetration of the CW after binding of an inactive two-domain harpin, or by cooperation of two-domain harpin partners in the 'arena-player' mode. The 'consortium' is, indeed, a translocon inserted into the target PM (Büttner, 2012; Mueller *et al*., 2008). In addition to the translocators, LTs may also participate in translocon assembly. For example, *X. oryzae* Hpa2 has been shown to degrade bacterial cell envelopes (Zhang *et al*., 2008), to target plant PMs and to interact with the hydrophobic translocator HrpF (Li *et al*., 2011).

Translocon assembly by a plant-pathogenic bacterium probably resembles the 'three-tiered ring' architecture with modifications (Fig. 3C): (i) the two assumed hydrophobic translocators simultaneously burrow into the target PM; and (ii) the harpin-type hydrophilic translocator commits a dual association with the target PM at one side and with the minor hydrophobic counterpart at the other. This hypothesis is in agreement with certain discoveries: (i) HrpN and Hpa1 can bind to the outer surfaces of plant PMs (Oh and Beer, 2007; Sang *et al*., 2012); (ii) Hpa2 and HrpZ1 can bind to plant PMs or with the planar bilayer to create ion-conducting pores (Lee *et al*., 2001a; Li *et al*., 2011); (iii) Hpa2 can interact with HrpF inside plant cells (Li *et al*., 2011); and (iv) Hpa1 and HrpZ1 oligomerize via the α-helical coiled-coil motif (Haapalainen *et al*., 2011; Ji *et al*., 2011). In addition, four translocators lack an α-helix (Fig. 2B) and may oligomerize through hydrogen bonds (Twyman, 1998).

The possibility of dual association relies on multiple motifs in harpin sequences (Fig. 2B). The C-terminal region containing the first three of nine predicted α-helices determines HrpZ1 oligomerization and its ability to create pores in the bionic membrane (Haapalainen *et al*., 2011). Although the N-terminus containing the first of two α-helices is essential for Hpa1 to oligomerize and to induce plant defences and growth enhancement (Ji *et al*., 2011; Li *et al*., 2014b; Wang *et al*., 2007), the last two effects are inhibited by the glycine-rich motif (Liu *et al*., 2006). Thus, the glycine-rich motif is adverse to plants, but may be propitious to bacteria. Both motifs locate with a distance in the Hpa1 sequence and may function independently, enabling the translocator to associate with the pilus tip and the target PM or the assumed hydrophobic counterpart, such as HrpF (Li *et al*., 2011). Further studies are needed to determine the number and characters of translocators and their oligomerization into the translocon, as proposed in Fig. 3C, which differs from the models shown in Fig. 3A, B with regard to the particular translocators that associate with the postulated sensors in the target PM.

POTENTIAL PM SENSORS FOR TYPE III TRANSLOCATORS

As discussed in detail in several recent reviews (Büttner, 2012; Cortes *et al*., 2014; Matteï *et al*., 2011), the recognition of type III translocators by postulated sensors in the target PM is critical for the creation and functionality of the translocon pore. Membrane lipids are curently known common sensors of translocators from animal and plant pathogens. Phospholipids are major components of cell membranes and are essential for the formation of the translocon pore in animal cells (Matteï *et al*., 2011). Cholesterol is a high-density lipoprotein involved in cellular signalling and is implicated in IpB binding to the target PM and in the bilayer binding of PopB and PopD (Cortes *et al*., 2014). In plants, phosphatidic acid is implicated in the sensing of HrpZ1. HrpZ1 interacts with this compound and requires it to create pores in artificial lipid vesicles and in vesicles prepared from PMs of *Arabidopsis* cells (Haapalainen *et al*., 2011). It is possible that the plant PM-integral phosphatidic acid may serve as a receptor of the HrpZ1 translocator.

In addition to membrane lipids, PM-integral proteins are also implicated in plant recognition of harpin-type translocators. Oh and Beer (2007) identified a HrpN-interacting protein from apple (*Malus sylvestris*), HIPM (HrpN-interacting protein from *Malus*), and found an orthologue in *Arabidopsis*, AtHIPM. Both HIPMs contain an N-terminal signal peptide and a central TM domain, and associate with PMs of plant cells. Thus, HIPM and AtHIPM are membrane-integral with a stretch to the cellular periphery in contact with the HrpN translocator.

One further potential PM sensor is an isoform of PM intrinsic proteins (PIPs), which constitute a unique aquaporin (AQP) family in plants (Abascal *et al*., 2014; Bansal and Sankararamakrishnan, 2007). Rice OsPIP1;3 interacts with the canonical form of Hpa1, but not the mutant version (Fig. 4A) generated by removal of the N-terminal region, which contains the first of two α-helical coiled coils (Fig. 2B) and is essential for the oligomerization of Hpa1 and its bioactivity in plants (Ji *et al*., 2011; Li *et al*. 2014a, b; Wang *et al*., 2008). Hpa1 contributes to the virulence of *Xoo* strain $PXO99^{A}$ on the rice variety Nipponbare, as the severity of bacterial blight can be markedly reduced when Hpa1 is deleted (Fig. 4B). The virulence is provided mainly by the effector PthXo1 (Yang *et al*., 2006; Yang and White, 2004), the deletion of which causes a substantial alleviation of blight symptoms (Fig. 4B). Single Hpa1 or PthXo1 protein deletion and double deletion result in similarly alleviated blight symptoms (Fig. 4B). Because virulence is executed only after injection of the effector into rice cells (Gürlebeck *et al*., 2006; White *et al*., 2009), these analyses provide a basis for further studies to determine the possible effects of OsPIP1;3 and Hpa1 on the virulence and translocation of PthXo1.

AQP interacting with non-AQP proteins is a recently appreciated mechanism that governs the physiological roles of distinct

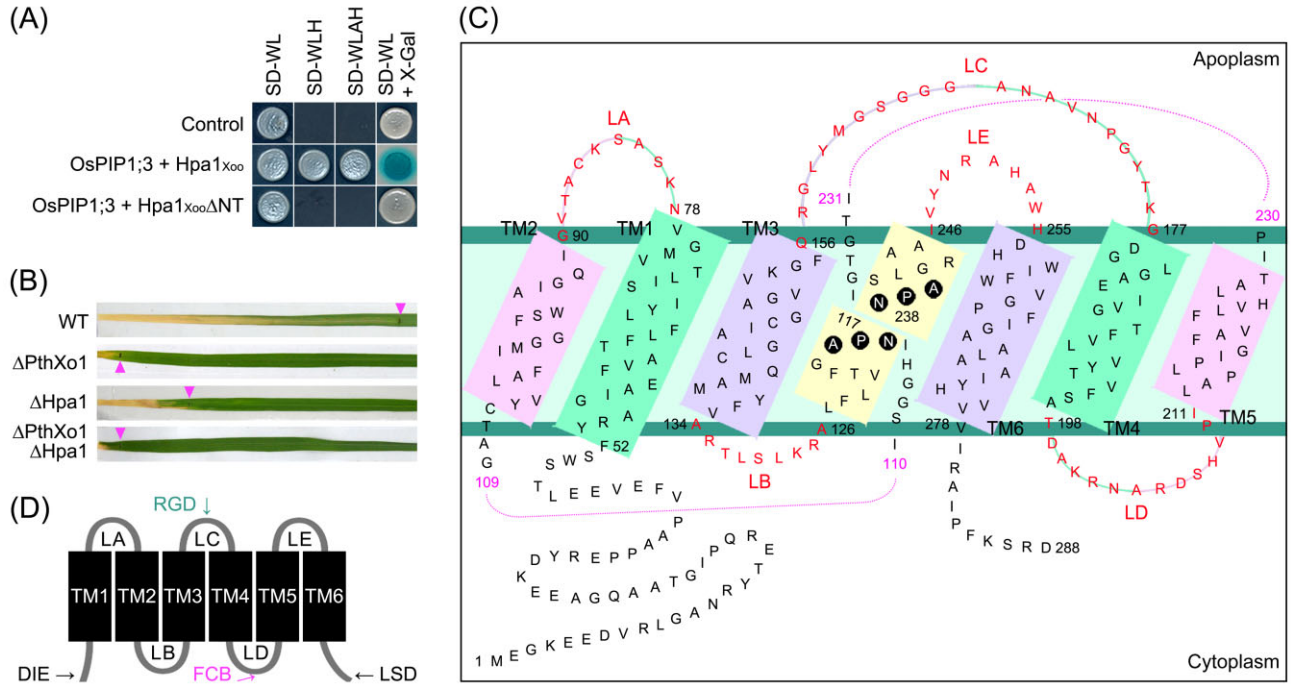

Fig. 4 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Hpa1 interacts with rice aquaporin OsPIP1;3 (A) and affects the virulence of *Xoo* effector PthXo1 on rice variety Nipponbare (B). The aquaporin structural complex is sketched according to the current model (C) which allows for the characterization of protein-interacting motifs (D). (A) The OsPIP1;3–Hpa1 interaction analysed with a split ubiquitin-based yeast two-hybrid system (Dualsystems Biotech., Zurich, Switzerland). This assay was performed on the control protein combination (control) provided by the system manufacturer and the combination of OsPIP1;3 with $Hpa1_{Xoo}$ or $Hpa1_{Xoo}\Delta NT$, a $Hpa1_{Xoo}$ mutant version generated by deleting the N-terminal region, which contains 53 amino acids and the first of two predicted α-helices (Li *et al*., 2014b). Three types of synthetic dropout (SD) amino acid nutrient medium were used in the screening of yeast hybrids. SD-WL medium allows the growth of yeast cells irrespective of protein interactions. Yeast cells are able to grow on both SD-WLH and SD-WLAH media only when an interaction of the tested proteins occurs. The interaction can also be detected by the X-Gal assay of colonies grown on SD-WL (Möckli and Auerbach, 2004). (B) Bacterial blight symptoms in Nipponbare leaves photographed at 14 days after inoculation with the bacterial strain $POX99^{A}$ and its mutants. The symbol Δ prefixed to a protein name indicates that the protein has been deleted from $POX99^{A}$ by the method described previously (Song and Yang, 2010). Purple triangles indicate the end of bacterial blight lesions. WT, wild-type. (C) Topological structure of OsPIP1;3 (accession number Q9SXF8.2) analysed according to a previously established model (Chaumont *et al*., 2001). Six transmembrane (TM) domains, TM1–TM6, are located in the plasma membrane (PM) in an alternate manner and are linked by five connecting loops, LA–LE. NPA is highlighted with black circles as it forms the sandglass-shaped filter that decides substrate selectivity. Numbers indicate the positions of NPA turning points or actual adjacent residues, but plotted separately to yield space. (D) Protein-interacting motifs or regions found in different aquaporins. DIE, maize (*Zea mays*) ZmPIP2 diacidic DIE motif (4–6 Asp-Ile-Glu); FCB, filensin- and calmodulin-interacting region in lens AQP0; LSD, human AQP1 and AQP4 regions binding to the light-sensing protein Killer Red; RGD, integrin β1-binding RGD motif (Arg-Gly-Asp) in renal AQP2 (You *et al*., 2013).

AQPs (Qin and Boron, 2013; Sjöhamn and Hedfalk, 2014) on the basis of the topological structures conserved in all AQPs, including OsPIP1;3. In the current topological model (Gomes *et al*., 2009; Maurel, 2007), OsPIP1;3 is characterized by six α-helical TM domains (TM1–TM6) that are tilted along the plane of PM and linked one to the other by five connecting loops (LA–LE) (Fig. 4C). LB, LD and both termini are located in the cell cytosol and potentially bind to cytosolic substrates, similarly to lens AQP0 binding with filensin through its C-terminus (Hu *et al*., 2012; Lindsey Rose *et al*., 2006; Sjöhamn and Hedfalk, 2014; Fig. 4D). By contrast, LA, LC and LE face the apoplasm and have the opportunity to contact with apoplastic substrates, similarly to renal AQP2 LE binding to integrin β1 (Jin *et al*., 2012). Presumably, PIPs depend on the extracellular LA, LC and LE to sense environmental cues (Chen *et al*., 2013; Maurel *et al*., 2008) and biotic signals (Ruiz-Lozano *et al*., 2009; You *et al*., 2013), and thus extend their physiological roles beyond substrate transport. The experimental performances of wild-type and mutant versions of OsPIP1;3, Hpa1 and PthXo1 (Fig. 4A, B) suggest that OsPIP1;3 potentially interacts with Hpa1 to facilitate PthXo1 translocation from *Xoo* cytoplasm into the cytosol of rice cells.

CONCLUSIONS

As an integral element of T3SS (Fig. 1), the type III proteinaceous translocon is believed to be assembled by refined associations of one hydrophilic and two hydrophobic translocators identified in animal-pathogenic bacteria, or similar proteins from plant pathogens (Fig. 2). In a particular bacterium, the translocator counterparts may rely on the predicted α-helix motifs to

oligomerize into the translocon in a hierarchical and contrapuntal manner (Fig. 2). The translocon organization presumably follows distinct models (Fig. 3), which similarly comprise the essential role of translocator sensors potentially present in the target PM of animal or plant cells (Fig. 4). The aims of future studies will be to determine the accurate contrapuntal location of each translocator at the bacterium–host interface and to generate a complete atomic model of translocon assembly (Taufik *et al*., 2013; Wager *et al*., 2013).

This general perspective remains a great challenge for studies on plant-pathogenic bacteria because their type III translocon and translocators are not as well understood as those of animal pathogens. Numerous studies are needed to address many basic questions regarding: (i) the accurate mechanisms by which the pilus machinery traverses plant CWs; (ii) the composition of the translocon and the number of translocators, especially hydrophilic counterparts other than harpins; (iii) the hydrophobic counterparts of the hydrophilic translocators; (iv) the oligomerization of each translocator; (v) the location of each translocator at the bacterium–plant interface; and (vi) the physical interaction of translocator counterparts into the translocon assembly.

Future studies to demonstrate how the translocators and putative PM sensors interact to facilitate effector translocation will also be a promising field of research, especially for the emerging rice–*Xoo* interaction system that possibly involves functional relationships among OsPIP1;3, Hpa1 and PthXo1 (Fig. 4). The OsPIP1;3–Hpa1 interaction assumed to occur at the target PM probably leads to a channel-like pore which resembles the so-called translocon pore. Critical studies are required to elucidate: (i) the structural basis for OsPIP1;3–Hpa1 interaction; (ii) the topological structure of the channel-like pore at the target PM following molecular interaction; and (iii) the rice cell cytosol-directional opening of the translocon in relation to the dynamic translocation of *Xoo* effectors, PthXo1 and possibly other proteins into the cytosol of rice cells. Similar studies are also needed to elucidate the translocon assembly in other bacterial pathosystems in addition to the rice–*Xoo* interaction.

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