Opinion

Current knowledge on the *Ralstonia solanacearum* type III secretion system

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*Ralstonia solanacearum* was ranked in a recent survey the second most important bacterial plant pathogen, following the widely used research model *Pseudomonas syringae* (Mansfield et al., 2012). The main reason is that bacterial wilt caused by *R. solanacearum* is the world’s most devastating bacterial plant disease (http://faostat.fao.org), threatening food safety in tropical and subtropical agriculture, especially in China, Bangladesh, Bolivia and Uganda (Martin and French, 1985). This is due to the unusually wide host range of the bacterium, its high persistence and because resistant crop varieties are unavailable. In addition, *R. solanacearum* has been established as a model bacterium for plant pathology thanks to pioneering molecular and genomic studies (Boucher et al., 1985; Salanoubat et al., 2002; Cunnac et al., 2004b; Occhialini et al., 2005; Mukaihara et al., 2010). As for many bacterial pathogens, the main virulence determinant in *R. solanacearum* is the type III secretion system (T3SS) (Boucher et al., 1985; 1994), which injects a number of effector proteins into plant cells causing disease in hosts or a hypersensitive response in resistant plants. In this article we discuss the current state in the study of the *R. solanacearum* T3SS, stressing the latest findings and future perspectives.

A regulatory cascade controls T3SS expression

Synthesis of the T3SS machinery—encoded by some 20 *hrp/hrc* genes—is tightly controlled in all species studied, probably due to its high metabolic cost. *Ralstonia solanacearum* is the only bacterial species for which a regulatory cascade linking T3SS gene expression to plant host contact has been described (Brito et al., 2002). In *R. solanacearum* the *hrp/hrc* gene induction is triggered upon recognition of an unidentified non-diffusible cell wall component by the outer membrane receptor PrhA (Aldon et al., 2000), which transfers the activation signal through a cascade of transcriptional regulators (Brito et al., 2002). HrpG is a central regulator in this cascade (Brito et al., 1999; Valls et al., 2006), whose downstream activator HrpB directly controls transcription of the T3SS genes and its associated effectors (Genin et al., 1992; Occhialini et al., 2005). Interestingly, these two regulators have homologues in various *Xanthomonas* ssp. and *Burkholderia* ssp. strains, including the human pathogen *B. pseudomallei* (Wengelnik and Bonas, 1996; Zou et al., 2006; Li et al., 2011; Lipscomb and Schell, 2011), whereas the PrhA receptor and the upper regulators in the cascade are not conserved in other species.

A regulatory network with connections to many cellular processes

In addition to the activation by the presence of plant cell wall components, expression of the T3SS genes is also induced by metabolic and environmental inputs. It has been known for a long time that *hrpB* expression is repressed when the bacterium grows in complete medium, as compared with a minimal medium that is thought to mimic plant apoplastic fluids (Arlat et al., 1992; Genin et al., 2005). More recently, it was found that other regulatory circuits impact *hrp* gene expression. The global regulator PhcA, which activates expression of many virulence activities including motility, plant cell wall degradation, and exopolysaccharide synthesis (Genin and Denny, 2012) has been reported to repress *hrpB* expression by two orders of magnitude during growth in complete medium (Genin et al., 2005). PhcA can also bind directly to the promoter of upstream regulators in the Hrp cascade but it only downregulates their transcription to one half of...
the normal levels (Yoshimochi et al., 2008). Recent findings showed that PrhG – a HrpG parologue – also influences expression of the HrpB regulon (Plener et al., 2010) and that this pathway is modulated by an unrelated virulence operon (Zhang et al., 2011). Thus, the actual view is that of a complex network of regulators controlling hpr gene expression in connection with a number of environmental and physiological cues.

The hpr regulatory system thus integrates different inputs but it also brings about various output responses by co-regulating transcription of the T3SS and effector genes to that of genes likely associated to metabolic adaptation to parasitic life in the plant (Occhialini et al., 2005; Valls et al., 2006). Indeed, transcriptomic studies have revealed that HrpG controls expression of some 400 genes, half of them independently of the downstream regulator HrpB. Some of these additional genes encode lectins and enzymes that degrade plant polysaccharides or drive the synthesis of polyamines or phytohormones (Valls et al., 2006). Further analyses may detect additional targets of the T3SS regulatory system that have escaped our notice due to experimental or technical limitations. In this sense, it is expected that RNA sequencing experiments can identify small RNAs involved in virulence controlled by the hpr regulators, as has been found in Xanthomonas campestris, which bears a closely-related regulatory system (Chen et al., 2011; Schmidtk et al., 2012).

T3SS regulation in planta

An experimental limitation of the above described regulatory circuits is that they were all defined based on experiments carried out in vitro using synthetic media. Recent research has focused on determining their relevance and expression timing in planta during infection. The creation of a gene delivery system to integrate gene constructs in a permissive site of the R. solanacearum chromosome (Monteiro et al., 2012b) has been key to monitor transcription in these conditions. This tool enables the analysis of promoter output from single-copy fusions to fluorescent or luminescent reporters during plant infection, as the constructs remain stably integrated in the modified strains. Surprisingly, the master T3SS regulator hrbB was found to be transcribed in bacteria growing inside wilting plants, causing expression of hrb genes under these conditions (Monteiro et al., 2009; 2012a). These findings have been recently validated by an independent transcriptome analysis approach, which has confirmed that half of the HrpB regulon is induced in bacteria recovered from wilting plants (Jacobs et al., 2012). These results are in contradiction with the widespread view that the T3SS is only required during the first stages of host colonization. This notion was based on the observations that the T3SS genes are induced immediately after plant contact (Kamoun and Kado, 1990; Thwaites et al., 2004; Ortiz-Martin et al., 2010) and that this system is involved in suppression of host defence responses to promote bacterial multiplication early after infection (Deslandes and Rivas, 2012). Thus, it will be interesting to ascertain whether the T3SS remains active in late stages of disease development in other plant pathogens or if this is a particularity of R. solanacearum, and to elucidate what is the functional importance of the T3SS – if any – during the R. solanacearum saprophytic life cycle.

A large effector repertoire

One of the key questions in bacterial pathogenicity is defining the whole inventory of the type III effectors (T3E) present in a given strain or species. The pioneering genome sequencing and annotation of R. solanacearum strain GMI1000 identified a first set of effector candidate genes based on homology to known effectors from other species or presence of domains typically eukaryotic (Salanoubat et al., 2002). The existence of well-defined T3SS transcriptional regulators greatly contributed to complete the list. Two approaches were followed to identify candidate effectors co-regulated with the T3SS: (i) the search for promoters with a HrpB binding sequence, similar to the Pip box described in X. campestris (Cunnac et al., 2004a; Koeblnik et al., 2006) and (ii) transcriptomic studies using HrpB-deficient and overexpressing strains (Occhialini et al., 2005). Translocation analyses with the cyaA reporter or T3SS-dependent secretion to the medium have been used to validate most effector candidates (Cunnac et al., 2004b; Tamura et al., 2005; Mukaihara et al., 2010; Solé et al., 2012), so that the reference strain GMI1000 is thought to bear 72 type III effectors (Poueymiro and Genin, 2009; Mukaihara et al., 2010). Compared with animal pathogens, bacterial plant pathogens contain larger numbers (~30–40) of effectors, but the R. solanacearum effector repertoire is exceptionally large, probably due to its wide host range.

A pan-genomic analysis of R. solanacearum will determine the super-effector repertoire and help define core and variable effectors in this species, providing evolutionary cues on host range determination. A recent study comprising 19 P. syringae strains yielded a super-repertoire of 57 effector genes (Baltrus et al., 2011). Considering that the average effector number per strain analysed is considerably lower in P. syringae compared with R. solanacearum (15–30 in P. syringae versus 72 in R. solanacearum GMI1000), it is reasonable to expect that the super-effector repertoire of R. solanacearum will be correspondingly larger. Up to date, the genomes of 11 R. solanacearum strains have been sequenced (GMI1000, RS1000, UW551, Po82, CFBP2957, PSI07,
CMR15, Molk2, IPO1609, K60 and Y45) and many others are on their way. These genomes, representative of the whole range of strains composing the *R. solanacearum* species complex, will facilitate pan-genomic analyses in the near future and shed light on effector conservation and function in this species. It will be interesting to ascertain whether in *R. solanacearum* divergent repertoires can be found in strains that are pathogenic on the same host, as it is the case for *P. syringae* (Baltrus et al., 2011; Lindeberg et al., 2012).

The minimal functional set of core effectors has not been yet determined in *R. solanacearum*. In *P. syringae* DC3000 it has been recently shown to comprise AvrPtoB, HopE1, HopG1, HopAM1, AvrE, HopM1, HopAA1 and HopN1 (Cunnac et al., 2011). These effectors function together in host immune suppression, chlorosis and lesion formation, in addition to bacterial growth. Among these, HopG1 is the most widespread in *R. solanacearum* sequenced strains, being only absent in PSI07, K60 and Y45 HopAA1 is the second most represented, as it can be found in GMI1000, Po82, Molk2, IPO1602, CFBP2957 and CMR15. AvrE homologues are identified in Po82, Molk2, IPO1602 and CFBP2957, although the picture is more complex, as distantly-related orthologues may be present in other strains. Finally, an AvrPtoB homologue is only present in Molk2 and the remaining four *P. syringae* predicted core effectors (HopM1, HopN1, HopE1, HopAM1) are absent in *R. solanacearum*. The fact that only half of the *P. syringae* core effectors have members in *R. solanacearum* may indicate that the core effector in this species is constituted by either functional analogues with no sequence similarity to their *P. syringae* counterparts or by a total different set of activities. Functional genetics studies will clarify in the future which of these hypotheses is true.

**Type III effector function**

Deciphering effector function is essential to understand the molecular interactions between pathogens and their hosts in terms of host specificity and pathogenicity. In *P. syringae*, it has been suggested that a small subset of core effectors target antimicrobial vesicle trafficking in plants, whereas a larger and more variable set would interfere with plant kinase-based pathogen recognition pathways (Lindeberg et al., 2012). Whether these two strategies to defeat plant immune processes are conserved in *R. solanacearum* remains an open question.

Up to date 23 *R. solanacearum* T3E have been assigned a function *in planta* using biochemical and/or pathology assays (Table 1). To study the contribution of each individual effector to bacterial fitness *in planta*, three methods have been used: (i) to measure growth of *R. solanacearum* mutant strains inside of natural hosts (tomato, eggplant); (ii) to measure growth of *P. syringae* heterologously expressing *R. solanacearum* T3E in Arabidopsis (Solé et al., 2012); (iii) competitive index assays between co-inoculated wt and mutant strains, which have proved to be a highly sensitive method to detect minor contributions to pathogenicity (Macho et al., 2010). These methods have revealed that several effectors promote growth in *R. solanacearum* natural hosts (Table 1): AvrPphF, AWR1, AWR2, PopP2 and Rip34 (HopD1-like) in tomato; AvrPphF, AWR1, AWR2, Rsp0842 (PopC-like), PopP2, SKWP4, Rip19 (AvrBs3-like), Rip39, Rip64, Rip3, Rip55 and Rip23 in eggplant; and AvrPphF, PopP2, Rsp0842 (PopC-like) and Rip34 (HopD1-like) in bean. Interestingly, two members of the AWR family show contrasting phenotypes, restricting growth in Arabidopsis and tomato (AWR4) or eggplant and Arabidopsis (AWR5), which may indicate a certain degree of recognition of these T3S in certain host cellular contexts. Other *R. solanacearum* T3E have been ascribed an avirulence function: AvrA is considered the major determinant leading to resistance of tobacco to some strains (Carney and Denny, 1990; Robertson et al., 2004; Poueymiro et al., 2009). Other avirulence reactions are triggered by PopP1 in resistant tobacco plants and in petunia (Lavie et al., 2002), PopP2 in Arabidopsis (Deslandes et al., 2002; 2003; Bernoux et al., 2008) and AWR2 and AWR5 in various contexts (Solé et al., 2012). Together, these results evidence that the interaction of *R. solanacearum* with its different plant hosts partly results from a combination of synergistic and antagonistic interactions between specific effectors within a single strain.

The characterization of the molecular/biochemical function of the increasingly large number of *R. solanacearum* effectors remains a major challenge. So far only a limited number of its T3E have been biochemically characterized (Table 1). Several members of the GALA family (Gala1, Gala3, Gala5, Gala6 and Gala7) have been shown to interact with SKP1-like proteins, and are thought to mimic plant E3 ubiquitin ligases (Angot et al., 2006). PopP2 has been shown to trigger re-localization of the cysteine protease RD19 to the nucleus, where it is thought to form a protein complex with the atypical WRKY-containing NB-LRR protein RRS1-R leading to disease resistance (Deslandes et al., 2002; 2003; Bernoux et al., 2008). However, direct interaction has only been shown for PopP2/RRS1-R and RRS1-R/RD19, but not for PopP2 and RD19. Recent work suggests that RRS1-R activation of the plant immune responses upon PopP2 recognition involves perception of PopP2 auto-acetylation (Tasset et al., 2010). Finally, the harpin-like T3E PopA has been shown to localize to the membrane of tobacco cells, where it forms ion-conducting pores, likely facilitating translocation of bacterial proteins into the cytoplasm of plant cells (Racapé et al., 2005).
| Gene name | Alternative name | Protein name | Family | Predicted domains | Role in planta | Hosts tested | Mode of action | References |
|-----------|-----------------|-------------|--------|------------------|----------------|-------------|----------------|------------|
| RSc0608   | rs1000          | avrA        | AvrA   | –                | Avirulence/Promotes growth | Nicotiana spp./ Tomato | –            | Carney and Denny (1990); Robertson et al. (2004); Turner et al. (2009); Macho et al. (2010) |
| RSp0822   | rip40           | AvrPphF     | HopF2/AvrPphF | – | Promotes growth | Tomato, Eggplant, Bean | –            | –          |
| Rsc2139   | –               | Awr1        | AWR    | –                | Promotes growth | Tomato, Eggplant | –            | Solé et al. (2012) |
| RSp0099   | rip29           | hpx31/ripA  | Awr2   | –                | Avirulence/Promotes growth | Nicotiana spp. | –            | –          |
| RSp0847   | rip45           | hpx4        | Awr4   | –                | Restricts growth | Arabidopsis | –            | –          |
| RSp1024   | rip56           | hpx10       | Awr5   | –                | Avirulence/Restricts growth | Nicotiana spp./ Tomato, Eggplant, Arabidopsis | –            | –          |
| RSp0914   | rip53           | gala1       | Gala1  | LRR repeats – F-box | – | – | Interaction with SKP1-like proteins | Angot et al. (2006) |
| RSp0028   | rip28           | gala3       | Gala3  | –                | – | – | – | – |
| RSc1801   | rip18           | hpx16       | Gala5  | –                | – | – | – | – |
| RSc1356   | rip13           | hpx13       | Gala6  | –                | – | – | – | – |
| RSc1357   | rip14           | hpx14       | Gala7  | –                | Host specificity factor | Medicago truncatula | – | – |
| RSp0877   | rip49           | popA        | PopA   | –                | Harpin | Nicotiana | Formation of plasma membrane ion-conducting pores | Racapé et al. (2005) |
| Rsp0842   | –               | –           | –      | –                | – | – | Interaction with SKP1-like proteins | Angot et al. (2006) |
| RSc0826   | rip7            | popP1       | PopP1  | LRR Yop/J AvrRxv Ser/Thr acetyltransferase, functional NLS | Promotes growth | Eggplant, Bean Petunia | – | – |
| RSp0888   | rip8            | popP2       | PopP2  | LRR       | Avirulence | Arabidopsis/Tomato, Eggplant, Bean | Nuclear relocalization of RRS1-R and RD19, binds RRS1-R | Deslandes et al. (2002); Bernoux et al. (2008); Macho et al. (2010) |
| RSc1839   | rip20           | hpx30       | Skwp4  | SKWP Heat/bradillo-related repeats | Promotes growth | Eggplant | – | – |
| RSc1815   | rip19           | hpx17       | Rip19  | AvrBs3 central repeat | Promotes growth | Eggplant | – | – |
| RSp0304   | rip34           | hpx25       | Rip34  | HopD1/AvrPphD | Promotes growth | Tomato, Eggplant, Bean | – | – |
| RSp0732   | rip39           | hpx27       | Rip39  | HopAV1 Coiled-coil | Promotes growth | Eggplant | – | – |
| RSp1281   | rip64           | hpx24       | Rip64  | HopR1 | Promotes growth | Eggplant | – | – |
| RSp0257   | rip3            | –           | Rip3   | – | – | Promotes growth | Eggplant | – | – |
| RSp1022   | rip55           | hpx21       | Rip55  | – | – | Promotes growth | Eggplant | – | – |
| RSc2359   | rip23           | hpx28       | Rip23  | – | – | Promotes growth | Eggplant | – | – |
Despite all our current knowledge on *R. solanacearum* T3E derived from the combination of genomic, biochemical and pathology data obtained in the last two decades, there is still a considerable number of effectors with no assigned function. These are usually effectors with no similarity to known proteins or domains or no apparent role in virulence or avirulence. The lack of assigned function in planta for many effectors is likely due to redundancy and specialized functionality restricted to certain host plant contexts. To dissect such complex interface between a pathogen and its host a novel genetic screening (insertional mutagenesis and depletion, iMAD) has been successfully used (O‘Connor et al., 2012). This method systematically combines bacterial and plant mutations, and would be extremely helpful to characterize the interaction of *R. solanacearum* with its multiple hosts. Still, 30% of *R. solanacearum* T3Es have no counterpart in other bacteria (Mukaihara et al., 2010), making this species a good model to explore novel effector functions.

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**Conflict of interest**

None declared.

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