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Mini review

In planta regulation of phytopathogenic bacteria virulence genes: relevance of plant-derived signals

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Abbreviations: EPS – exopolysaccharide; HR – hypersensitive response; HSL – homoserine lactone; PL – pectate lyase.

Interactions between plants and pathogens are complex dynamic and multistep processes involving invasion of the host, avoidance or overcome of plant defence responses and multiplication of the pathogen, eventually culminating with expression of disease symptoms. In all these steps communication at different levels is important either between the pathogen and its host or between the members of the pathogen community. It is therefore not surprising that the genes involved in plant-pathogen interactions are under the control of complex regulatory circuits responding to numerous stimuli. The discovery by Stachel et al. (1985) that the *Agrobacterium vir* genes, normally expressed at a very low level, are specifically induced by cocultivation of the bacterium with plant cell suspensions and the further identification of plant-derived inducing signals, was the first demonstration of the importance of the direct signalling role of the plant in the initiation of the infection. Subsequently, the role of signals coming from the host in the regulation of genes involved in plant-bacteria interactions was investigated. In gram-negative plant pathogenic bacteria, several factors playing a role in their interactions with plants have been identified. These include the production of toxins or hormones interfering with the normal plant metabolism or development, the secretion of enzymes or signal proteins interacting with plants, polysaccharide production, and the expression of efficient iron acquisition systems. In all bacterial pathosystems studied, the production of these virulence factors is very finely tuned by complex regulatory circuits and several recent overviews on the role of these factors and their regulation in plant-pathogenic bacteria interactions are available (Daniels et al., 1988; Gross, 1991; Zambryski, 1992; Winans, 1992; Leigh and Coplin, 1992; Dow and Daniels, 1994; Barras et al., 1994; Fuqua et al., 1994; Expert et al., 1996; Schell, 1996). The goal of this review is to analyse the data relevant to the possible importance of plant signals in the regulation of the production of the different virulence factors so far characterised. The approaches which were investigated to directly identify genes which would be specifically induced in planta in a search for additional important genes in plant-bacteria interactions will then be discussed.

Plant signalling: the *Agrobacterium* paradigm

Throughout its infection cycle *Agrobacterium*, the causal agent of plant crown gall tumour disease, is in communication with its host via a two-way complex signalling network. All virulent *Agrobacterium* strains contain a self-transmissible virulence plasmid called Ti (or Ri for *A. rhizogenes*). These plasmids have been classified into different types according to the opine genes they carry (see below). *Agrobacterium* infects its host specifically at wounded sites and a chemotactic attraction by compounds produced in wounded tissues has been reported (see Binns and Howitz, 1994 for references). When in contact with wounded tissue, a cluster of about 25 genes carried by the Ti plasmid, the

vir genes, are induced by specific phenolic compounds such as acetosyringone. This induction is potentiated by certain sugars, mainly monosaccharides, especially at low concentrations of the phenolic inducers. The *vir* gene products promote the transfer to the plant nucleus of a DNA fragment also carried by the Ti plasmid, the T-DNA. The T-DNA contains two types of genes, those that are involved in the biosynthesis of plant hormones and those that encode enzymes for the synthesis of amino acid:sugar acid conjugates called opines. The production of hormones then directs the unlimited proliferation of tumour cells while the synthesised opines are exuded. These opines are then taken up by the bacteria where they induce the expression of a set of Ti-localised genes involved in opine catabolism thus providing the bacterium with a carbon and nitrogen source. Although other soil microorganisms can utilise opines as nutrients, the fact that a given Ti plasmid carries both the biosynthetic genes for a specific opine (on its T-DNA) and the corresponding catabolic genes, provides to the bacterium strain carrying this specific Ti plasmid a favourable ecological niche in the vicinity of the infected plant (Winans, 1992; Zambryski, 1992; Winans et al., 1994 and references therein). Clearly, the induction of *Agrobacterium* genes by plant-derived signals is a crucial part of the interaction. However, the discovery that the *vir* genes are induced by plant-derived phenolics was immediately accompanied by the observation that environmental conditions such as acidic pH or low temperature are also very important for efficient induction. Thus the expression of *vir* genes is under the control of at least four stimuli; phenolics, sugars, pH and temperature. At least three bacterial proteins are important for this regulation: VirA, VirG and ChvE. VirA is the inner membrane spanning 'sensor' and VirG the cytoplasmic transcriptional 'response regulator' of a two-component system activated by phosphorylation. ChvE is a periplasmic protein homologous to periplasmically located sugar-binding proteins involved in chemotaxis toward sugars. Structure-function analysis of the VirA protein indicates that the ChvE-sugar complex may interact with the periplasmic domain of VirA, although pH and phenolic signals should interact, maybe via intermediary proteins (see Winans et al., 1994; Binns and Howitz, 1994 for discussion), with the cytoplasmic 'linker' region of VirA located between the periplasmic domain and the domain thought to interact with VirG (for more details, see Winans et al., 1994). The *virA* and *virG* genes are also submitted to regulation, they are part of the *vir* regulon and are thus both induced by plant-released phenolics. Moreover the *virG* gene possesses two promoters which are induced by different stimuli such as phosphate starvation, acidic pH, heavy metals or DNA-damaging agents (Winans, 1990; Mantis and Winans, 1992). A chromosomally encoded two-component regulatory system ChvI/G, though very pleiotropic, also seems to be involved in the pH inducible expression of *virG* (Charles and Nester, 1993; Mantis and Winans, 1993).

The VirA/VirG system also regulates genes which are not directly involved in the transfer of the T-DNA. In some *Agrobacterium* strains for example, the cytokinin biosynthetic gene *tzs* (for trans-zeatin synthase) which is carried by the Ti plasmid but is not transferred to plant cells, is induced by plant phenolics through the VirA/VirG regulatory cascade (John and Amasino, 1988).

Another class of plant-released signals regulates *Agrobacterium* genes: the opines produced after transfer of the T-DNA into plant cells. Some of these, the so-called conjugal opines, not only 'classically' activate the Ti carried genes needed for their catabolism but are also transcriptional inducers of Ti plasmid conjugal transfer (*tra*) genes. The regulation of *tra* genes differs in nopaline and octopine Ti plasmids. In octopine-type plasmids, they are positively regulated by the OccR protein in the presence of octopine while in nopaline-type plasmids they are repressed by the AccR protein in the absence of agrocinopines. In both cases the presence of opines allow the transcription of the *traR* gene encoding the TraR activator which allows expression of other *tra* operons. The TraR protein is homologous to quorum-sensing proteins such as LuxR and requires an homoserine lactone called AAI (for *Agrobacterium* autoinducer) for activity. The activity of the TraR-AAI complex is modulated by the product of the *traM* gene, a gene which is also induced by the presence of opines, presumably via TraR activation (Hwang et al., 1995; Fuqua et al., 1995). Furthermore, these two regulatory networks might be even more intertwined as *vir* genes may influence conjugal transfer of the Ti plasmid (Gelvin and Habeck, 1990) and opines may stimulate induction of the *vir* genes (Veluthambi et al., 1989).

What might be the biological significance of these complex networks of regulation? The conditions for induction of the *vir* genes fit perfectly with the conditions encountered by *Agrobacterium* near wounded plant tissue. A healing plant wound produces phenolic inducers (precursors of lignin) and sugar oligomers that are needed for making and strengthening cell walls. In

this respect, it is worth noting that among the 11 most potent inducing sugars, 7 are constituents of major plant cell wall polysaccharides including galacturonic acid and cellobiose, the basic units of pectin and cellulose (Ankenbauer and Nester, 1990; Cangelosi et al., 1990; Shimoda et al., 1990). Acidic conditions are also very likely to occur in wounded tissues due to lysis of vacuoles and the plant tissue healing process might also produces DNA-damaging agents. On the other hand, the induction of Ti plasmid conjugal transfer when bacteria are in close contact to plants and at a high density assures the wide spread of this 'selfish' DNA at the right time.

Regulation of toxin production in *Pseudomonas syringae*

Another set of genes for which activation by plant-derived molecules is well documented are the genes involved in the biosynthesis of certain toxins in *P. syringae*. The *syrB* gene, involved in syringomycin production in *P. syringae* pv. *syringae*, is induced *in vitro* by phenolic β-glucosides. A few mono- or disaccharides, such as D-fructose or sucrose, also weakly activate the *syrB* gene even in the absence of phenolics. Furthermore, as in the case of *Agrobacterium vir* gene induction, these sugars, and some others having no inducing ability 'per se', enhanced about 10-fold the signal activities of the phenolic inducing compounds (Mo and Gross, 1991). The biological significance of this induction process was proved by determining the nature of the activating molecules present in leaves of sweet cherry, a plant known to be susceptible to *P.s.* pv. *syringae*. This analysis demonstrated that phenolic β-glucosides are indeed present in plant leaves in significant quantities, compatible with the concentration range needed for *syrB* activation *in vitro*. *P. syringae* senses plant metabolites of different categories, including flavonoids, coumarins and phenols, all of these having in common the presence of an intact glucoside linkage (Mo et al., 1995). Like several other toxin biosynthetic genes, *syrB* is regulated by the LemA protein, a member of the transmembrane histidine protein kinase sensor-regulator family, and presumably by the corresponding GacA response regulator. It seems however, that regulation by β-glucosides is mediated by a second not yet characterised regulatory circuit (Rich et al., 1994). Although the activation of *syrB* is highly reminiscent of what occurs in *Agrobacterium*, neither the inducing phenolics nor the potentiating sugars are similar in the two systems. Acetosyringone is not able to induce *syrB* and the phenolic β-glucosides inducing *syrB* have no effect on *vir* gene induction. The potentiating sugars are also specific for each system. The nature of the inducing compounds is related to the site of infection or multiplication – wounded tissue or the apoplast of leaves – of the respective pathogen.

Activation in planta of the production of another toxin, coronatin, by *P. syringae* pv. *tomato* was also reported, but the inducing signal was not identified (Ma et al., 1991).

Regulation of cell wall degrading enzymes production

Most phytopathogenic bacteria produce a battery of depolymerizing enzymes that degrade plant cell walls. In all these organisms, it is striking that multiple isoenzymes are produced and that the corresponding genes are regulated by highly complex global regulatory networks (reviewed in Daniels et al., 1988; Barras et al., 1994; Dow and Daniels, 1994; Schell, 1996).

The pectinases produced by soft rot Erwiniae were the most extensively studied. Some aspects of their regulation including analysis of their expression in planta is presented below as an example of the complexity of stimuli which may influence the expression of such genes.

E. chrysanthemi produces 4 to 5 major pectate lyases (PLs) encoded by the *pelA* to *pelE* genes. These genes are organised in two clusters, *pelADE* and *pelBC*, within which the genes are closely related suggesting that gene duplications have occurred. Each *pel* gene however, is under the control of its own promoter and, in spite of the high degree of similarity between some of the PLs, the importance of the different isoenzymes in virulence depends on the species of plant infected (Beaulieu et al., 1993). A search for mutants overproducing pectinases *in vitro* allowed the identification of three regulatory loci *kdgR*, *pecS-pecM* and *pecT* (see references in Barras et al., 1994; Reverchon et al., 1994; Surgey et al., 1996). KdgR is a transcriptional repressor controlling all of the genes of the pectin degradative pathway as well as *out* genes involved in the secretion of pectinases and cellulases; its repressor activity is inactivated by intermediates of the pectin degradative pathway such as KDG (ketodeoxy-gluconate). It was shown however, that in a *kdgR* mutant, the *pel* genes are still subject to induction

by the addition of pectin, indicating that at least another regulatory pathway responsive to pectin or its degradation products exists. The stimuli interacting with PecS, PecM or PecT are not known. PecS is a member of a new class of small (about 20 kDa) regulatory proteins and was shown to bind to the promoter regions of the genes it regulates (Praillet et al., 1996). PecM sequence indicates it is a membrane-anchored protein whose mutation leads to the same phenotype as a *pecS* mutation. For this reason, PecM was proposed to be the sensor controlling PecS activity thus forming with PecS a new type of two-component regulatory system (Reverchon et al., 1994; Praillet et al., 1996). PecS and PecM are global regulators which also regulate other genes such as the *celZ* gene encoding the endoglucanase EGZ, *out* genes and genes involved in the production of a blue pigment (Reverchon et al., 1994; Condemine and Robert-Baudouy, 1995). PecT belongs to the LysR family of regulators, it represses the expression of some *pel* genes but activates *pelB* (Surgey et al., 1996). In planta analysis of the expression of these regulatory genes was reported only for *kdgR*. The *kdgR* gene is expressed in planta quite late after bacterial infection and clearly after induction of the major *pel* genes. For the *pelD* gene for example, expression in a *kdgR* mutant as compared to the wild type background became different only 15 h post inoculation i.e. when pectin degradation products, the ligands inactivating the KdgR protein, are already produced. This indicates that the role of the KdgR regulator might deal more with the modulation of pectinolysis in later stages of the bacterium-plant interaction (Masclaux et al., 1996).

The expression of the *pel* genes is also modulated by changes in environmental conditions such as temperature, anaerobiosis, osmolarity, growth phase, nitrogen or iron concentrations as well as by catabolic repression (Hugouvieux-Cotte-Pattat et al., 1992; Expert et al., 1996).

When grown in presence of plant extracts, these major *pel* genes are not induced. Although the *pelA* gene is only weakly induced by pectin alone, Bourson et al. reported that it is activated by carrot extracts in the presence of pectin. The inducing factor present in the plant extract is a low molecular weight, heat stable product which has not been characterised further (Bourson et al., 1993).

Although some PLs have the same activity in degrading pectin (at least *in vitro*) (Preston et al., 1992), the importance of one given *pel* gene for virulence depends to the plant infected. Furthermore, there is no correlation between the abundance of one PL found at a late stage of the infection in a given plant and the importance of this isoenzyme for symptom development in this same plant (Beaulieu et al., 1993). Analysis of *pel::lac* fusions and comparison of the isoenzyme profiles found in rotted plants clearly indicate that there is differential induction of the various *pel* genes from one plant to another and even from one organ to another of the same plant (Beaulieu et al., 1993; Lojkowska et al., 1993). Kinetic studies of the activation of the different *pel* genes in planta also reveal that this induction is sequential and a role for iron in this process has been proposed (Masclaux et al., 1996).

From all what is known on the expression of *pel* genes in planta, it emerges that it is a very finely tuned sequential process which is still poorly understood. Many signals, either environmental or produced by the plant, and other bacterial regulatory genes remain to be discovered to tackle this complex regulatory circuit allowing a given PL protein to be produced in the adequate amount and at the right time during infection.

In addition to those 'major' pectate lyases, *E. chrysanthemi* produces another set of PLs, called plant-inducible because they were only detected after growth of the bacteria in planta or in the presence of plant extracts. The profiles of production of this new set of PLs also vary according to the species of plant infected (Beaulieu et al., 1993; Kelemu et al., 1993; Lojkowska et al., 1995). One of these secondary PLs, has been recently characterised. As shown for the other *pel* genes, the transcription of the *pelL* gene is dependent on various environmental conditions. Its expression is similarly weakly induced either by pectin or by chrysanthemum extracts and is under the control of PecS and PecT but not KdgR (Lojkowska et al., 1995).

In *E. carotovora*, the production of extracellular enzymes is also regulated in various ways. At least three kinds of signals are implicated: plant extracts, bacterial cell density and DNA-damaging agents (Barras et al., 1994 for a review). A regulatory cascade comprising the *aepA* and *aepB* genes (for activator of extracellular protein production) is involved in the activation of exoenzymes by plant extracts and interestingly, the expression of the *aepA* activator gene is also inducible by plant extracts (Liu et al., 1993). Mutations in a third locus, *aepH* (or *rex*) led to overproduction of exoenzymes (Murata et al., 1994; Salmond et al., 1994). This locus may encode for a 47 amino acids peptide which may be involved in activation (Murata et al., 1994) but the region upstream of this putative ORF is needed for activation and Salmond et al. proposed that the activation of this locus may function

by 'pumping-out' a negative regulatory factor. As in many other bacteria, cell density (quorum)-sensing is achieved by proteins of the LuxR-LuxI family through the production of a homoserine lactone (HSL) autoinducer (Jones et al., 1993; Pirhonen et al., 1993). HSL production is in turn negatively regulated by the product of the *rsmA* gene (for repressor of secondary metabolites) and *rsmA* mutations lead to the overproduction of extracellular enzymes in the absence of HSL (Chatterjee et al., 1995; Cui et al., 1995). Overproduction of RmsA in different Erwinia strains suppresses other traits important in bacterial virulence such as extracellular polysaccharide production, motility, or elicitation of HR, indicating that it might be a very general global regulator (Mukherjee et al., submitted).

One *E. carotovora* pectinase gene *pnlA*, is specifically induced by DNA-damaging agents through a regulatory cascade including the *recA*, *rdgA* and *rdgB* gene products (Barras et al., 1994; Liu et al., 1994). Expression of *pnl* genes occurs in planta in response to plant metabolites produced either constitutively or in response to different stresses.

Such complex regulation of extracellular enzyme production have also been reported for other plant pathogenic bacteria (Dow et al., 1994; Schell, 1996). In *Ralstonia* (formerly *Pseudomonas*) *solanacearum* for example, virulence factor production is controlled by a complex interconnecting and cascading regulatory network. The central regulatory protein PhcA, a member of the LysR family, controls the expression of various cell wall degrading enzyme genes (*egl, pme, pglA*) but also the production of EPS and motility. While PhcA seems to directly control some of these genes, it acts in conjunction with at least 5 additional regulatory proteins – the two VsrA/D and VsrB/C two-component systems and XpsR – to control EPS biosynthetic genes. Bacterial cell density controls levels of active PhcA protein through a new type of endogenous extracellular signal, 3OH-PAME (palmitic acid methyl ester). Production of PAME requires PhcB, response to it requires another putative two-component regulatory system encoded by *phcS/R*. Expression of the *pglA* gene encoding polygalacturonase, is negatively controlled by PhcA through the PehS/R two-component regulators but also presumably by VsrC/B (Schell, 1996 for a review). The observation that the level of polygalacturonase is 10-fold increased when bacteria are grown in planta led to postulate the existence of an inducing plant factor sensed by the PehS/R system (Allen et al., 1991; Schell, 1996). This substance however remains to be identified.

hrp and *avr* genes

Another class of genes important for plant-bacteria interactions which were claimed to be plant-inducible are the *hrp* and *avr* genes (Kamoun and Kado, 1990; Rahme et al., 1992; Schulte and Bonas, 1992; Huinh et al., 1989).

When the *hrp* genes were first identified by Lindgren et al. (1986) after transposon mutagenesis of *P. syringae*, they were shown to be essential for the bacterium-plant interaction. Indeed *hrp* mutants were both unable to produce disease on susceptible plants and unable to elicit the HR on resistant or non-host plants. *hrp* genes are present in all gram-negative phytopathogenic bacteria except maybe *Agrobacterium*. As already shown by hybridisation studies, sequence analysis revealed the existence of two classes of *hrp* gene clusters, one found in *Erwinia* and *Pseudomonas syringae* genera and the other one present in *R. solanacearum* and *Xanthomonas* strains (Willis et al., 1991; Van Gijsegem et al., 1995). The genetic organisation and the proteins involved in *hrp* regulation differ between these two classes. In all systems however, nine Hrp proteins are conserved (for references see Bogdanove et al., 1996). Homologues of these proteins are also present in several animal pathogenic enterobacteria such as *Yersinia, Salmonella* or *Shigella* and they were shown to be components of a new so called type III secretion machinery which delivers to host cells, proteins that are able to interact with the host (Bonas, 1994; Van Gijsegem et al., 1993, 1995; Huang et al., 1995).

The *hrp* genes are induced in plants. The involvement of plant signals for their expression is quite controversial. None of the *hrp* genes are expressed in complex medium. A defined minimal synthetic medium supporting *hrp* genes expression has been defined in all of the systems studied so far (Rahme et al., 1992; Arlat et al., 1992; Wei et al., 1992; Xiao et al., 1992; Wengelnik et al., 1996). In several cases, a level of induction comparable to that found in plants was observed in the synthetic medium. For *P. s.* pv. *phaseolicola* however, *in vitro* expression of the *hrp* genes never reached the level of expression observed in planta which led to postulate the existence of an inducing plant signal (Rahme et al., 1992). In *X. campestris* pv. *vesicatoria*, the existence of such a plant inducing factor was also proposed (Schulte and Bonas, 1992). However the discovery of a novel synthetic medium efficiently inducing all the *hrp* transcription units showed that plant-derived molecules are

not needed for *hrp* genes induction (Wengelnik et al., 1996). These results clearly pose the general problem, also encountered with *Erwinia pel* genes, of what is a gene specifically induced in plants. The fact that no synthetic medium supporting expression is available clearly does not prove that a plant-derived factor is needed for expression. Confirmation of this requirement is only possible by the purification of such a factor.

As for other virulence factors already described, *hrp* gene expression is modulated by environmental conditions such as pH, osmotic strength, the nature of the carbon source or temperature. High concentrations of nitrogen sources or amino acids repressed *hrp* gene expression (Rahme et al., 1992; Arlat et al., 1992; Wei et al., 1992; Xiao et al., 1992). These environmental conditions mimic to some extent the conditions which might be encountered by the bacteria in the intercellular fluids.

The two classes of *hrp* gene clusters are regulated by different cascades. In *R. solanacearum* and *Xanthomonas, hrp* genes are regulated by the HrpB and HrpX proteins which are related to each other and both share similarity with transcriptional activators of the AraC family at their carboxy terminal ends (Genin et al., 1992; Oku et al., 1995; Wengelnik and Bonas, 1996). The expression of these two genes is also regulated: similarly to the other *R. solanacearum hrp* genes, the *hrpB* gene is only expressed in minimal medium. The expression of the *Xanthomonas hrpX* gene was reported to be inducible only in plants (Kamoun and Kado, 1990) but this assumption should be re-evaluated now that an inducing synthetic medium has been devised for expression of the *Xanthomonas hrp* genes (Wengelnik and Bonas, 1996).

In *P. syringae, hrp* gene expression is controlled by a multicomponent regulatory cascade. Three regulatory genes, HrpL, HrpR and HrpS, are involved in this process (Xiao et al., 1994; Grimm et al., 1995). The HrpL protein shows similarities to class III sigma factors, the σ30 family exemplified by the *Bacillus subtilis* SpoOH protein and needs a functional σ54 for its expression. A consensus recognition sequence for HrpL was deduced from the comparison of sequences upstream of *hrp* genes as well as *avr* genes (see below) and a 34-bp fragment carrying this motif was shown to confer HrpL-dependent promoter activity (Xiao and Hutcheson, 1994). The HrpR and HrpS proteins exhibit 55–60% identity and show strong similarities with the central parts of proteins in the σ54-dependent regulator family, these regulators being triggered by environmental or metabolic signals (Shingler, 1996). The domain of this protein family which is conserved in HrpR and HrpS is thought to be involved in binding and hydrolysis of ATP and in interaction with the σ54 RNA polymerase holoenzyme. A model where HrpR and HrpS in conjunction with σ54 promotes the production of HrpL which in turn activates the expression of the other *hrp* genes has been proposed (Xiao et al., 1994; Grimm et al., 1995). The intriguing next question is of course to know at which level(s) of this regulatory cascade the different signals implicated in the regulation of *hrp* genes play a role.

Another class of genes important in bacteria-plant interactions are the *avr* genes. These genes, to date found mainly in *P. syringae* and in *Xanthomonas* pathovars, are responsible for the race-cultivar specific resistance (see Dangl, 1994 for a review). Many if not all *avr* genes of *P. syringae* are dependent on the *hrpRSL* regulatory cascade for their expression. Similarly to the *hrp* genes, they are expressed in minimal medium but not in complex medium and their expression is modulated by the carbon source used. In contrast, the *avrBs3* gene from *Xanthomona campestis* pv. *vesicatoria* is constutively expressed in an *hrp*-independent manner (see references in Dangl, 1994).

Search for genes specifically induced in planta

After the discovery of *Agrobacterium vir* gene induction by plant-derived phenolics, several approaches aimed at the identification of genes specifically induced either directly in planta or by plant extracts have been undertaken. Osbourn et al. (1987) devised an elegant technique allowing the detection of bacterial promoters induced in planta using an antibiotic resistance gene as an reporter. The subsequent analysis of the genes under the control of the promoters thus identified was however disappointing. Analysis of two such genes were reported. Mutation in one of them appeared to affect bacterial growth as well in culture as in the plant. Mutants in the other are not affected in virulence (Osbourn et al., 1990). A similar approach was however successfully used later in animal pathogenic bacteria using the *purA* biosynthetic gene as reporter (Mahan et al., 1993). Five genes that were expressed at a higher level in the host than *in vitro* (called i*vi* for *in vivo* induced) were further analysed: two of them showed no sequence homology in data bases, the 3 other fusions were located in genes involved in the general metabolism. Strains carrying mutations in

genes belonging to these two classes were isolated and tested for virulence in mice: all three mutants tested were shown to be impaired in virulence. This technique allowed thus not only the characterisation of unknown genes but also revealed the importance of genes involved in the general metabolism for survival in the host. Whereas in this case the reporter gene used led to repair of an auxotrophy, the less successful approach of Osbourn et al. used an antibiotic resistance gene necessitating the external addition of antibiotic to plants for the selection of bacteria carrying plant-induced promoters. As *purA* mutants are impaired for growth in hosts for several genera of bacteria including plant pathogens, the potential of adopting such an approach might be interesting to reconsider.

Other approaches for the identification of plant-induced genes were based on the use of transposable elements, mainly phage Mu derivatives, carrying a promoterless reporter gene suitable for the production of gene fusions. This method permits mutant strains to be tested directly for their phenotypes in interactions with plants. One disadvantage, however, is that any mutations in genes essential for survival in plants cannot be recovered. To circumvent this problem, plant extracts were used to look for induction of the reporter gene. In *E. chrysanthemi*, such an approach using a mini-Mu carrying a promoterless kanamycin resistance gene led to the identification of several so called *pin* genes (for plant inducible). Out of ten of these *pin* mutants further characterised, 8 were affected in virulence and one was shown to be hypervirulent in certain plants but not on others (Beaulieu and Van Gijsegem, 1990; 1992). The functions of these genes as well as the putative plant-derived inducing compounds have yet to be identified.

Mu derivatives were also used in *Agrobacterium* for isolating chromosomal genes inducible by plant extracts. One of these genes, *picA*, has been characterised. The *picA* locus seems to influence the surface properties of the bacterium and affects virulence in certain conditions. Expression of the *picA* gene is activated by pectin oligomers of 6-16 units and interestingly, the presence of a DNA fragment carried on a high-copy number vector and encoding a protein having high similarities with polygalacturonases has a negative regulatory effect on *picA* expression (Rong et al., 1990, 1991). Partial characterisation of the *picA* gene inducing compound in carrot roots revealed it is a complex pectic polysaccharide in which both unesterified uronic acids and arabinosyl units are important for inducing activity. This compound is 100-fold more potent than oligogalacturonides in inducing the *picA* gene, it also induces the *pgl* locus adjacent to *picA*. As *Agrobacterium* cannot utilise pectin as carbon source and the inducer compound is active at very low concentrations (maybe lesser than 1 μm), it was assumed that this molecule is a signal rather than a metabolite (Rong et al., 1994).

By comparing the profiles of proteins synthesised by an *Agrobacterium* devoid of the Ti plasmid in minimal medium supplemented or not by carrot extracts, Rong et al. (1990) noticed that the synthesis of several proteins was increased in the presence of plant extracts. With the development of protein microsequencing techniques, this kind of approach might also lead to the identification genes specifically induced in planta.

Conclusions and perspectives

The most striking overall observation concerning the control of virulence gene expression is the complexity of interactions in networks of regulatory genes that integrate a wide variety of signals including environmental and most probably metabolic signals, bacterial cell-to-cell communications as well as plant-derived signals. In *R. solanacearum* for example, as many as 8 regulatory proteins are involved in the regulation of EPS production (Schell, 1996).

What is the importance of specific molecules produced by plants in these processes? Three kinds of plant-derived compounds were found to be implicated in several systems: phenolics, sugar derivatives and, perhaps less generally, DNA-damaging agents. Sugars may have a dual role; in certain cases, they are metabolised by the bacteria and thus might be involved in regulation via the general metabolism while in other systems, their role is really signalling because they are not metabolised. Although the same classes of signal molecules are recognised by different bacteria, there are clearly adaptations to the mode of infection in each system according to which molecules are present in the particular conditions of infection. A further level of sophistication is encountered with the signals derived from plant cell walls such as the pectin oligomers. On one hand the amount of these signals might be modulated by the bacterium itself via the production of degrading enzymes while on the other hand these pectin oligomers are also signals recognised by the plants in different processes including the induction of defence response against pathogens (discussed in

Barras et al., 1994). We are again faced with the idea of dynamic very finely tuned interactions with constant crosstalks between the bacterium and the plant.

The crucial importance of plant-derived signals seems to depend on the system considered. For Rhizobiaceae, *Agrobacterium* and also the symbiotic interaction between legumes and *Rhizobium* (Göttfert, 1993 for a review), gene induction by plant-derived signals is one of the first essential steps in the interaction. The importance of such signalling in other interactions is less obvious. Compared to that of other pathogens, the interaction between *Agrobacterium* and its host is very short, just the time of T-DNA delivering after which *Agrobacterium* may grow more like a saprophyte at the expenses of the tumour. Could this difference in timing be a clue for explaining the differential importance of plant-derived signals in both type of interactions? Or is this discrepancy only apparent, simply reflecting our ignorance in the complex subtle more long-term interactions? Indeed, a phenomenon which is not at all understood for the moment is for example the basis for the differences of expression of the different Erwinia *pel* genes in various plant species. Are specific inducing molecules involved or might the environmental conditions encountered by the bacterium be sufficiently different from one plant to another?

Another important debate concerns the role of plant-derived signals in the specificity of plant-bacteria interactions. In Rhizobiaceae, two opposing situations are encountered. The *Agrobacterium vir* genes are induced by a large number of phenolic compounds consistent with its wide host range. In contrast, *Rhizobium* strains have a very limited host range, each strain nodulating only one or a few plant species; accordingly, expression of the nodulation genes is activated by highly specific flavonoids depending on which *nod* regulatory genes are present (reviewed in Göttfert, 1993).

The role of induction level in specificity was also analysed for *hrp* and *avr* genes. In *R. solanacearum* or *P.s.* pv. *glycinea* the same level of *hrp* gene expression was found in host and in non-host plants (Arlat et al., 1992; Xiao et al., 1992). In *E. amylovora* however, *hrp* genes were shown to be rapidly induced in the non-host plant tobacco and more slowly in pear, a host plant (Wei et al., 1992). For *avr* genes, no differences in the level of expression were noticed in susceptible versus resistant plants, indicating that host-specific induction may not determine host species specificity (discussed in Lorang and Keen, 1995).

In conclusion, although the regulatory network controlling virulence in *Agrobacterium* is quite well understood, the regulatory studies in the other systems are still in their infancy and little is known about the complex crosstalks which must exist between these different regulatory networks each controlling the production of one or a few virulence factors. Master regulators integrating all the different stimuli in a concerted way remain to be discovered. The deciphering of these connections will require direct in planta investigations. It is also important to develop systems which disturb these networks as little as possible. In this respect ways to study gene expression in non mutant backgrounds must certainly be devised.

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