Plant Signaling & Behavior 8:10, e25678; September 2013; © 2013 Landes Bioscience

Hrp mutant bacteria as biocontrol agents

Toward a sustainable approach in the fight against plant pathogenic bacteria

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Keywords: *hrp* mutant, phytopathogen, biological control, ABA, basal resistance, bacteria

Abbreviations: ABA, Abscisic acid; *A. thaliana*, *Arabidopsis thaliana*; BCA, Biological Control Agent; *E. amylovora*, *Erwinia amylovora*; *Hrp*, Hypersensitive Response and Pathogenicity; *P. syringae*, *Pseudomonas syringae;* PAMP, Pathogen-Associated Molecular Pattern; PTI, Pamp Triggered Immunity; *R. solanacearum, Ralstonia solanacearum;* T3SS, Type Three Secretion System; *X. campestris*, *Xanthomonas campestris*

Sustainable agriculture necessitates development of environmentally safe methods to protect plants against pathogens. Among these methods, application of biocontrol agents has been efficiently used to minimize disease development. Here we review current understanding of mechanisms involved in biocontrol of the main Gram- phytopathogenic bacteria-induced diseases by plant inoculation with strains mutated in *hrp* (*hypersensitive response and pathogenicity*) genes. These mutants are able to penetrate plant tissues and to stimulate basal resistance of plants. Novel protection mechanisms involving the phytohormone abscisic acid appear to play key roles in the biocontrol of wilt disease induced by *Ralstonia solanacearum* in *Arabidopsis thaliana*. Fully understanding these mechanisms and extending the studies to other pathosystems are still required to evaluate their importance in disease protection.

Introduction

Diseases have a major impact on plant yield, quality, and safety. Disease control constitutes therefore a major challenge for agriculture. One option for controlling plant disease consists in developing synthetic chemicals respecting public health and environment. Alternatively, using living organisms called biocontrol agents (BCA) constitutes a way to biologically control pests or pathogens and is a potentially important component of sustainable agriculture.

Prior exposure to eliciting organisms renders frequently plants more tolerant to subsequent infection. Non-pathogenic rhizobacteria termed plant-growth-promoting rhizobacteria (PGPR) induce the well documented induced systemic response (ISR).[1] Systemic acquired resistance (SAR) is another well-known form of resistance induced via local inoculation of a pathogen and provides long-term resistance to subsequent attack.[2]

In contrast, resistance induced by plant inoculation with bacteria mutated in *hrp* genes (*hypersensitive response and pathogenicity*), namely *hrp* mutants, remains poorly documented.

To successfully infect a plant, bacterial pathogens have to counteract plant defense mechanisms and redirect host metabolism for nutrition and growth. Type III Secretion System (T3SS) is a major determinant of pathogenicity of many gram-negative bacteria. It allows delivery within plant cells, of a battery of proteins so-called type III effector proteins known to collectively suppress plant defense and to favor bacterial multiplication and nutrition.[3,4] *Hrp* genes, required to set up a functional T3SS, are necessary for disease development in susceptible plants and elicitation of the hypersensitive response in resistant plants. They are highly conserved across the main gram-negative phytopathogenic lineages and exhibit extensive homologies with their animal counterparts, thus establishing a link between plant and animal pathology.[5] These genes have been grouped in 3 classes. The first class includes genes highly conserved among diverse animal and plant pathogenic bacteria and are named *hrc* (*hrp*-conserved). The second class contains transcriptional regulators of T3SS regulon genes, whereas the third one includes structural proteins and some secreted proteins like chaperone or other post-transcriptional regulatory proteins. *Hrp* genes clustered in pathogenicity islands have been

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Submitted: 05/28/13; Revised: 07/08/13; Accepted: 07/09/13
Citation: Hanemian M, Zhou B, Deslandes L, Marco Y, Trémousaygue D. *Hrp* mutant bacteria as biocontrol agents: Toward a sustainable approach in the fight against plant pathogenic bacteria. Plant Signal Behav 2013; 8: e25678; http://dx.doi.org/10.4161/psb.25678

subjected to intensive mutagenesis leading, in most of the cases, to loss of pathogenicity.[6]

The great majority of studies on *hrp* mutants aimed at a better understanding of the role of *hrp* genes. This review will focus on the plant responses to *hrp* mutants in order to gain some insights on their protective effect against virulent bacteria.

Hrp mutants were indeed used to reduce or completely abolish disease symptoms caused by virulent bacteria in several pathosystems involving the main gram-negative phytopathogenic bacteria (*Pseudomonas syringae, Ralstonia solanacearum, Erwinia amylovora, Xanthomonas campestris species*). Natural occurrence of *hrp* mutants in the environment was recently demonstrated, making them potential interesting BCA.[7]

In this review, we first describe how *hrp* mutants colonize plants and induce host responses, focusing on the bacterial species mentioned above. The molecular mechanisms underlying biocontrol exerted by the *R. solanacearum hrp* mutants in the model plant *Arabidopsis thaliana* are then presented in more details.

Plant/*Hrp*-Mutant Bacteria Interactions

Infectiveness and invasiveness of *hrp* mutants

Hrp mutants are prototrophic and generally not impaired in their ability to grow in culture.[5] Most of them are able to colonize and invade, to some extent, plant tissues.[8] *Hrp* mutants are generally able to enter into the apoplastic compartment, and to invade plant tissues although their multiplication in a susceptible host is affected.

A well-documented example concerns the vascular pathogen *R. solanacearum,* the causative agent of bacterial wilt disease, that infects plants through root tips and lateral root cracks and reaches xylem vessels leading to their spread throughout the host.[9] Most *R. solanacearum* mutants altered in different *hrp* genes could be detected, after tomato root inoculation, within similar tissues than wild type strains, i.e., root tips, lateral root emergence sites, and root xylem vessels. However, they propagated only in the lower part of the stem and did not reach the fruits.[10] Bacteria numeration in root collar and stem, as well as microscopic observations, showed that some of the *hrp* mutants were significantly impaired in their ability to multiply and colonize tomato plants.[11] On petunia, *R. solanacearum hrp* mutants failed to induce the formation of root lateral structures that constitute efficient colonization sites allowing extensive bacterial multiplication.[12]

Hrp mutants from other gram-negative phytopathogenic bacteria that are able to invade their hosts have been also described. In the case of *E. amylovora*, the agent of fire blight, bacteria penetrate the plant apoplast primarily via natural openings in flowers or through wounds on young aerial vegetative parts.[13] *E. amylovora hrp* mutants could be detected in xylem vessels but formation of lysigenous cavities (structures appearing in the later stages of infection and filled with bacteria) were never observed.[14] *P. syringae* bacteria, that elicit leaf spots and other foliar necroses in host plants, enter via stomata or wounding sites. Then bacterial colonization becomes systemic via the host vascular system.[15] In *A. thaliana* leaves, efficient multiplication of *P. syringae hrp* mutants was impaired in comparison to wild type strain multiplication.[16] In cantaloupe, *P. syringae hrp* mutants inoculated in seedlings were detected in plant tissues, but population stabilized around the initial size after inoculation.[7] *X. campestris* virulent bacteria, infect plants through hydathodes at the leaf margins or through stomata and colonize the vascular system,[17] causing tissue necrosis and severe leaf wilting symptoms.[18,19] Similarly, *X. campestris hrp* mutants failed to grow to the extent of wild type in plant tissues as attested by population counts or microscopy observations.[20,21]

Plant responses to *hrp* mutants

Although *hrp* mutants do not trigger any disease or HR symptoms, inoculated plants often display important developmental, molecular and biochemical alterations, thereby suggesting the elaboration of plant defense responses.

Following pathogen attack, the first line of active plant defense, called basal defense or PTI (Pathogen-associated molecular patterns—PAMP-triggered immunity), involves plant pathogen recognition receptors, the pattern-recognition receptors (PRRs) that recognize PAMPs. This perception triggers many signaling events through cGMP, mitogen-activated protein kinases (MAPKs), Ca^{2+} and H^{+} influxes, early accumulation of reactive oxygen species, cell-wall thickening leading in some cases to papillae formation, and altered expression of many genes.[22] Proteins involved in primary metabolism, redox modulation, molecular chaperoning and cytoskeleton rearrangement are some of the key components of the PTI.[23] In addition, PAMPs modify mitochondrial and chloroplast proteomes and reconfigure proteins into membrane rafts enabling efficient host signal transduction and downstream responses after the initial recognition.[24,25]

Cellular, molecular and metabolic changes observed upon inoculation by *hrp* mutant strains, clearly indicate that basal defense mechanisms are generally highly induced.

In different host plants, localized strengthening of cell walls due to the accumulation of hydroxyproline-rich glycoproteins, phenolics and callose is often detected in cells adjacent to the inoculation sites of *X. campestris* and *P. syringae hrp* mutants.[16,21] In lettuce, in response to *P. syringae hrp* mutants, cell wall alterations were associated with H_2O_2 accumulation and increases in peroxydase activity, which probably strengthens plant cell wall structures.[26] In *A. thaliana* tissues responding to *P. syringae hrp* mutants, a rapid flux of indole carboxylic acid compounds to the cell wall correlates with a limitation of bacterial multiplication.[27] In response to inoculation by *R. solanacearum hrp* mutants, vascular coating, a non specific plant defense reaction, was observed on tomato roots.[11]

Changes in chloroplastic and mitochondrial leaf nuclear proteomes were also described in *A. thaliana* after *P. syringae hrp* mutant inoculation, which reveals a regulation of primary metabolism through redox-mediated signaling components and the existence of a rapid communication system between organelles.[25]

Plant gene expression was monitored following *hrp* mutant inoculation in several pathosystems. Pioneering work by Jakobek and Lindgren identified defense-associated transcripts, such as phenylalanine-ammonia-lyase (PAL), chalcone synthase, chalcone isomerase, and phytoalexins, accumulating in bean following challenge by a *hrp* mutant of *P. syringae*.[28] More recently,

several studies established that inoculation with *hrp* mutants leads to an extensive reprogramming of gene expression, a requirement for elaboration of immune responses during plant–pathogen interactions.[29-31] In the study of Truman et al.,[30] a set of genes induced by *hrp* mutants whose expression is also modulated in response to many PAMPs and to virulent *P. syringae* strains, was proposed to represent the primary host response to bacterial infections. Transcriptional reprogramming was also investigated in *A. thaliana* following root inoculation with a *R. solanacearum hrp* mutant strain.[31] Despite the absence of apparent symptoms, in response to *hrp* mutants, many plant genes were regulated in a similar way than after inoculation of a susceptible plant with a *R. solanacearum* virulent strain.[32] 27% of the up-regulated genes are related to abscisic acid (ABA) biosynthesis and signaling according to Li et al.[33] Additionally, several *Arabidopsis* mutants altered in the biosynthesis (*aba1-6*) or signaling (*abi1-1, abi2-1*) associated to this hormone exhibit an altered response to *R. solanacearum*.[34] Interestingly, among these ABA-related genes, several genes are also responsive to *P. syringae hrp* mutants in the early stages of infection,[29] suggesting that ABA signaling is also associated to plant response to *P. syringae hrp* mutants (our unpublished observations). It is noteworthy that according to genetic approaches, the limited multiplication of *P. syringae hrp* mutants monitored in *A. thaliana* leaves was not related to SA- or ethylene-mediated mechanisms.[16] Actually, the effect of ABA in this process remains to be evaluated. The importance of ABA in plant responses to *hrp* mutant is also strengthened by the fact that it does positively regulate callose deposition, a plant basal defense response-related which is stimulated following *hrp* mutant inoculation.[36,37]

Altogether, these data support well the enhancement of plant basal defenses in response to *hrp* mutant inoculation. Molecular mechanisms underlying this response remain to be fully elucidated and one can question their importance in protecting plants against virulent bacteria. Actually, *hrp* mutants have been successfully used in bioprotection experiments. For instance, when *X. campestris* pv. *vesiculata hrp* mutants were inoculated on tomato leaves prior to inoculation with wild type virulent strains, disease severity was reduced, both under controlled and field conditions.[35] *Hrp* mutants of *P. syringae* pv. *tomato* strain DC3000 were also able to provide significant reductions in bacterial speck severity on tomato caused by a subsequent inoculation with wild type bacteria, under greenhouse conditions.[36] *E. amylovora hrp* mutants were effective in controlling fire blight disease when inoculated on apple seedlings or apple flowers.[14] *Hrp* mutants of *R. solanacearum* were able to protect susceptible tomatoes from virulent strains under growth chamber conditions or green-house conditions.[10,37,38] Molecular mechanisms occurring after inoculation of protected plants with virulent *R. solanacearum* bacteria have been investigated in *A. thaliana*.[31] The following chapter will focus on biocontrol resulting from *R. solanacearum hrp* mutant inoculation, which it is to date the best documented interaction.

Plant protection against *R. solanacearum* triggered by *hrp* mutants

Wilt disease caused by the soil-borne bacteria *R. solanacearum* is of substantial economic importance due to its broad host range, aggressiveness and long persistence in soils. Means to control this disease are limited. Thus, alternative ways to control disease such as biological control have been investigated with an increasing interest. In this context, mutant strains able to colonize tomato plants without causing disease symptoms have been tested for their protective effect.[39] The authors showed that root pre-inoculation with a *hrp* mutant leads to high protection rates against a subsequent inoculation with virulent strains.[37] Furthermore, this strategy provided a durable protection by persisting several months within the plant without affecting fruit number and weight.[10] Protection was also achieved in the model plant *Arabidopsis thaliana* using a similar approach.[31] *Arabidopsis* plants were inoculated with a *hrpB* regulatory mutant and simultaneously or subsequently challenged with the wild type virulent *R. solanacearum* strain. HrpB regulatory activity is well characterized and its contribution to *R. solanacearum* virulence resides essentially in the control of T3SS function.[40] Simultaneous root inoculation by both the wild type and *hrp* mutant strains did not induce protection, although the mutant strain was favored by a high mutant to wild type strain inoculum ratio. These results suggested that protection may not be caused by a spatial competition between the 2 strains as previously proposed.[38] Indeed, when both *hrp* and virulent *R. solanacearum* strains were co-inoculated in tomato, they colonized separate xylem vessels.[38] (Similar observations had been made in apple seedlings inoculated simultaneously with a *hrp* mutant and a wild type strain of *E. amylovora*).[14] On the other hand, a subsequent inoculation with the virulent strain allowed a high protection rate associated with a decrease in the multiplication of the virulent strain. The delay required between *hrp* mutant and wild-type strain inoculations suggested that some plant signaling pathways had to be established before inoculation of virulent bacteria. Heat-killed *hrp* mutant bacteria were also able to induce resistance but to a lower extent than live ones, which suggested that an active metabolism for both partners was required for full protection. Genetic analyses established that, despite the fact that this mode of protection by root inoculation resembles ISR, neither jasmonic acid, nor ethylene participated in the establishment of this resistance which rather relies on ABA signaling.[31] As previously mentioned, *hrp* mutant inoculation in *A. thaliana* led to extensive genome re-programming.[31] Subsequent inoculation of protected plants with the virulent strain indeed reversed the expression of 70% of the genes whose expression was altered by the *hrp* mutant pre-inoculation. This reprogramming affected many ABA-related genes, associated with disease development. Thus, upon inoculation of protected plants by a virulent *R. solanacearum*, the pattern of modulation of gene expression is opposite to the pattern of expression observed after infection of unprotected plants. Regulation of disease-associated genes in *hrp* mutant protected plants may have generated a hostile environment for the invading pathogen and a priming of resistance through stimulation of yet unknown pathways by *hrp* mutants cannot be excluded.

Opening questions

Mechanisms underlying the biological control using *hrp* strains remain poorly understood. By using *R. solanacearum*, a soil-borne vascular pathogen, it was shown that the molecular

basis for *hrp*-induced protection differs from the well-studied mechanisms underlying SAR and ISR and has yet to be fully explored.

The prominent role of ABA in this process requires additional studies. This phytohormone has emerged as a crucial actor in plant stress monitoring.[41] A model has been proposed involving ABA as a multifaceted actor, depending on the phase of the infection and the nature of a given microorganism.[42] Its intricated role in the plant response to pathogens, driving increased resistance or increased susceptibility depending on the case, is documented in a recent publication.[43] Typically, it is plausible that this phytohormone, whose role in water stress responses is well known, plays an important function in plants exposed to water deprivation due to the vessel obstruction following *R. solanacearum* invasion and facing simultaneously abiotic and biotic stresses. In this context, it should be of interest to test if ABA signaling is more generally associated to vascular pathogens. A specific role for ABA in the plant response to soil-borne pathogens such as *R. solanacearum* can be also questioned. ABA mutants impaired in biosynthesis or signaling in the model plant *A. thaliana*, could help to address these points.

Several studies illustrate indeed the role of ABA in response to various root-applied stresses. Its synthesis, and transport through xylem vessels up to the aerial parts of the plant, is induced by several abiotic stresses applied on roots (e.g., salt stress, ammonium nutrition, phosphate, and potassium deficiencies).[44] Soil attackers also influence ABA signaling in plants. For instance, ABA acts as an important signal to prime above ground defenses during below ground aggressions by herbivorous.[45] Soil application of the chemical B-aminobutyric acid (BABA) induced resistance through ABA-dependent signaling.[46,47] It is noteworthy that plants treated with *R. solanacearum hrp* mutant exhibit an increased resistance to *P. syringae,* a foliar pathogen whose entry through stomata is prevented by ABA-mediated basal defenses.[31,48] This observation suggests that, following *R. solanacearum hrp* mutant inoculation, a signal migrates from roots to leaves leading to protection against *P. syringae.*

Another interesting point concerns the possible inheritance of the protective effect. Priming against environmental challenges may be inherited in the progeny of the primed plants.[49] Epigenetic components acting on gene expression regulation and more largely on chromatin structure and organization contribute to plant stress responses.[50] ABA signaling pathways appears to be connected to chromatin remodelling complexes.[51] It might therefore be interesting to check whether *hrp*-induced protection is inherited in the progeny of protected plants.

Despite an obvious lack of knowledge on the molecular mechanisms supporting the ABA-dependent biocontrol observed with *hrp* mutant bacteria, this strategy of natural vaccination of plants that requires further investigations from scientists working in this field, could provide a sustainable approach in the battle against plant pathogens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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