Analysis of Plant Gene Expression Responses to the Pathogen and Natural Genetic Engineer *Agrobacterium tumefaciens*

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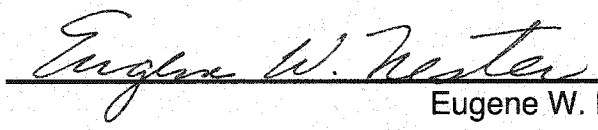

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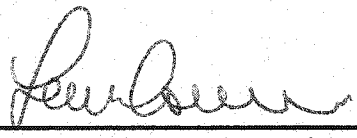

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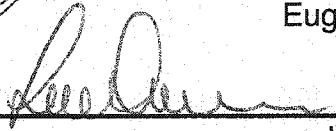

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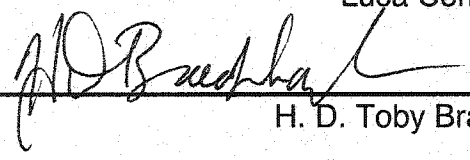

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Abstract

Analysis of Plant Gene Expression Responses to the Pathogen and Natural Genetic Engineer *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens genetically transforms its natural plant host and other eukaryotic cells, representing both an intriguing pathogen and a vehicle for genetic engineering. Host responses to this bacterium have not been studied extensively in the past, although this understanding is critical for both practical applications in biotechnology and insights into host-microbe interaction. Here we present a study of plant gene expression responses to *Agrobacterium* infection. Two different plant species and two different large-scale approaches have been used to identify genes with altered expression during infection. By cDNA-AFLP we identified several transcripts from *Ageratum conyzoides* cell cultures that were regulated at 24 and 48 hours after infection with *Agrobacterium.* A number of these transcripts code for plant defense-related proteins which are also regulated by a non-pathogenic bacterium. However, a nodulin-like gene was regulated uniquely by *Agrobacterium*, suggesting that the closely-related symbiotic Rhizobium and

Agrobacterium can provoke similar responses in plants. We observed that an attachment-deficient *Agrobacterium* mutant hyper-induces a set of plant defense genes and we propose that *Agrobacterium* can dampen plant defense responses by an attachment-dependent mechanism. The plant defense system is likely important in regulating infection since *Ageratum* cells with heightened defenses hinder transformation. By using microarrays, we also investigated the transcriptome of *Arabidopsis thaliana* cell cultures during a time-course of infection by *Agrobacterium.* Statistically significant alterations in gene expression are observed at 48 hours after infection, but not at earlier time points. The identity of the differentially expressed genes suggests similarities with classes of genes characterized in our previous studies and with other defense-related genes. Future microarray studies comparing infection with other pathogens, symbionts and various *Agrobacterium* mutants will further advance our understanding of this fascinating plant-bacterium interaction.

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Chapter I

INTRODUCTION

All living organisms must respond to the environment that surrounds them. Abiotic and biotic stresses are perceived, signals are transmitted and an appropriate response is formulated. Plants cannot move to escape the environmental challenges they face and have therefore evolved sophisticated and flexible mechanisms to respond to the environment.

Wounding, drought, poor nutrient availability and extreme temperatures are just some of the abiotic stresses to which plants are constantly exposed. Plants also have to respond to a vast number of different life forms closely associated with them, ranging from viruses, bacteria, fungi, nematodes, insects and even to other plants. Those organisms can be harmful or beneficial to the plant, so an appropriate response by the plant is crucial to its survival.

Agrobacterium tumefaciens is one of the organisms that live in close association with the plant. Its unique and sophisticated mechanism of infecting and colonizing the plant prompted us to ask how the plant perceives and responds to this bacterial pathogen. In this chapter I present an overview of the current knowledge on molecular plant responses to both pathogenic and beneficial organisms, comparing and contrasting them with animal responses and pointing out overlaps with abiotic stress responses. I review the virulence system of *Agrobacterium*, the recent findings on plant responses to this pathogen and how they relate to responses to other pathogenic and beneficial organisms.

Innate Immunity in Plants and Animals

Differentiating between self and non-self is an essential ability in all living organisms. In animal cells, an evolutionarily ancient process of non-self recognition known as innate immunity forms the first line of defense against

attempted microbial infection (3, 172). In this system, molecules derived from the surface of potential pathogens, the so-called pathogen-associated molecular patterns or PAMPs, bind to pattern recognition receptors present in the host cell surface. Binding triggers the expression of immune response genes and production of antimicrobial compounds. Innate immunity is also important for initiating the longer-lasting, specific responses of the more evolutionary recent adaptive immunity system, present only in vertebrates (3, 178).

A remarkable feature of pattern recognition receptors in innate immunity is the ability to recognize a vast range of PAMPs from microbes. The only properties shared by most (if not all) PAMPs are their absence from host cells, their importance in the microbial metabolism or virulence, and therefore, their high conservation. PAMPs that can trigger an immune response in vertebrates and invertebrates include the lipopolysaccharide (LPS) fraction of Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, bacterial flagellin, unmethylated bacterial DNA fragments, and glucans, mannans, chitins and proteins that are derived from fungal cell walls (3, 172, 248). Recently, single-stranded RNA, which is associated with viral infection, has been identified as another ligand recognized by innate immunity receptors in mammals (59, 102).

In a seminal discovery eight years ago, the membrane-localized *Drosophila* Toll gene, initially described as having a developmental role, was found to be crucial for the immune response in flies, acting as a pattern recognition receptor (140). Since then, a number of Toll and Toll-like receptors (TLR) have been identified in insects and mammals and their signal transduction pathway in innate immunity has been elucidated. These receptors recognize PAMPs through an extracellular leucine-rich repeat (LRR) domain and transduce the signal through a cytoplasmic TIR (*Drosophila* Toll and human Interleukin-1 Receptor) domain (3, 248).

Plants differ from animals in many obvious aspects. For example, plants lack a blood circulatory system capable of transporting cells specialized in immunity; therefore, each plant cell must be capable of responding independently to an invading pathogen. Plants also lack an adaptive or acquired immune system, but possess a form of innate immunity that shares striking similarities to innate immunity in animals. Recent comparative studies support the idea of a common ancient origin for non-self recognition systems in eukaryotes, before the separation of animal and plant lineages (158, 172, 173, 178).

It has been known for some time that defense responses in plants can be triggered by a number of the same PAMPs (also called general elicitors in the plant literature) that activate defenses in animal cells. Fungal peptides (18, 97, 174), compounds derived from fungal cell wall and membrane, such as chitin and ergosterol (21, 92), as well as LPS from Gram-negative bacteria (46) and bacterial peptides such as harpins (139, 267), are strong elicitors of defense reactions in plants. Elicitor activity can also be attributed to self-determinants such as plant cell wall fragments that are released by the action of hydrolytic enzymes secreted by fungal pathogens (125). High affinity binding sites in plant cells have been identified in some of these studies but the isolation and characterization of the corresponding receptors for general elicitors has proven difficult until recently.

In what perhaps is the biggest contribution to our understanding of innate immunity and responses to general elicitors in plants, the Boller lab discovered that flagellin, the main protein component of the bacterial flagellum that induces innate immunity in animals, is also a potent elicitor of plant defense responses (73). By screening different *Arabidopsis* ecotypes and mutants for insensitivity to flagellin, they have identified the locus FLS2 involved in flagellin recognition. FLS2 encodes a protein with an extracellular leucine-rich repeat (LRR), a transmembrane domain and a cytoplasmic serine/threonine kinase domain,

sharing structural similarities with both Toll-like receptors from animal immunity and other plant proteins involved in pathogen recognition (86, 87). In addition, an entire MAP kinase signalling cascade downstream of FLS2 was identified in *Arabidopsis*, indicating another level of overlap with animal innate immunity in which MAP kinase signalling is utilized for signal transmission following microbe recognition (6). Even though general elicitors such as flagellin can induce defense responses, their role in contributing to natural resistance is more difficult to determine. However, Boller's group has recently demonstrated that perception of flagellin indeed contributes to pathogen resistance in plants (295).

The strongest elicitor activity of flagellin was localized to a N-terminal peptide fragment that shows high conservation in a wide variety of bacteria pathogenic to plants and animals, such as *Pseudomonas syringae* (different pathovars), other *Pseudomonas* species, *Erwinia*, *E. coli* and *Bordetella* (73). Most interestingly, flagellin preparations from *Agrobacterium tumefaciens*, *Rhizobium meliloti* and *Xanthomonas* species were completely inactive in their assays, even at high concentrations. Flagellin sequences from *Agrobacterium tumefaciens* and *Rhizobium meliloti* were extremely divergent from the N-terminal domain conserved in other species and the authors speculate that microbes that live in close association with the plant may be under selective pressure to modify or lose these molecules that could potentially alarm the plant surveillance of their presence and trigger a defense response (73). This concept may be easier to understand in the context of a microbe like *Rhizobium meliloti*, so well adapted to live associated with the plant that it actually forms a symbiotic interaction, while this phenomenon may be more intriguing for *Agrobacterium*, traditionally considered a plant pathogen.

Types of Resistance in Plants

Non-Host Resistance

The observation that "most plants are resistant to most plant pathogens" (51) is a testimony to the effectiveness of innate immunity in plants. Immunity of an entire plant species to a pathogen, known as non-host resistance, is the most common and durable form of disease resistance (99, 101). Its durability results in part from the fact that non-host resistance is composed of several layers of defense mechanisms, probably under complex genetic control, which are not easily overcome by the pathogen. Although inducible defenses are more commonly studied, preformed defenses such as constitutive toxins against pathogens and herbivores (274) and physical barriers such as the plant waxy cuticule and cell wall reinforcements contribute significantly to non-host resistance (239). Because of its durability and broad spectrum, non-host resistance has attracted a renewed interest recently as an alternative in plant breeding for resistance (99, 101). Genetic screens in *Arabidopsis* for mutants that allow the growth of bacterial (148) and fungal pathogens (44) that normally cannot grow on this plant have led to the identification of genes involved in non-host resistance. Mutations in the *PEN1* gene allowed plant cell penetration by the fungus and resulted in alterations to the plant cell wall and membrane, consistent with a function for these initial physical barriers in non-host resistance (44).

Race-Specific Resistance

It is assumed that during evolution, certain strains or races of a pathogen species acquired virulence determinants that allowed them to overcome plant species (non-host) resistance by evading or suppressing defenses, effectively transforming these plants into susceptible hosts and causing disease. Even in these susceptible hosts, weak levels of resistance responses are still present, characterizing what is called basal resistance (99). As a consequence of co-evolution however, certain cultivars of an otherwise susceptible host, in turn,

evolved resistance genes that specifically recognize pathogen virulence factors, becoming resistant (host or race-specific resistance) to these pathogen strains or races (173). Race-specific resistance conforms to the classical gene-for-gene hypothesis put forward by Flor when studying flax-rust fungus interactions (74). According to this hypothesis, resistance results from the presence of a matching pair of genetic determinants: a plant-encoded resistance gene (*R*) and a pathogen-encoded avirulence (*avr*) gene. The absence of either one in a host-pathogen interaction results in disease. Although it seems counterintuitive that a pathogen would have an avirulence gene that would indicate its presence to the plant and limit its growth, the name was adequately used in the past to indicate the pathogen phenotype for lack of virulence. However, after several years of molecular studies, it is known today that avirulence genes are in fact determinants of pathogen virulence in hosts that lack a resistance gene (36, 124) and therefore the more inclusive name effector protein has been used recently to indicate the product of an *avr* gene (124, 224). Effector proteins can be defined as race-specific elicitors, based on their ability to trigger defense responses in a host cultivar-pathogen race specific manner. They are extremely variable and usually non-essential to the pathogen (20), contrasting with the essential character of general elicitors or PAMPs described before. Interestingly, an equivalent level of recognition for pathogen race-specific elicitors is not known in innate immunity in animals (173). Bacterial effector proteins are delivered to host cells via a complex secretory system termed type III secretion, which is stimulated upon contact with host signals and is present in both animal and plant pathogenic bacteria (77, 224). Recent evidence indicates that fungal pathogens may use their nutrient-acquisition structure called haustorium to deliver effector proteins into plant cells (61), while in other cases fungal effectors could be recognized extracellularly.

Resistance (*R*) Genes

Since the early days after the gene-for-gene model was described, it has been expected that *R* gene products serve directly or indirectly as receptors for *avr* gene products. To test these ideas and because of the obvious interest in plant breeding, a significant effort from several laboratories resulted in the identification and isolation of dozens of *R* genes. Their function and structure are presented in several excellent reviews (16, 51, 67, 99, 157, 171, 232, 286). Based upon their predicted protein structure, *R* genes can be divided into just five groups, even though they are able to provide plants with resistance to a wide variety of pathogens with very different lifestyles such as viruses, bacteria, fungi, nematodes and insects. By far the largest group of *R* genes is comprised of the cytoplasmic nucleotide-binding/leucine-rich repeat proteins (NB-LRR), which can be further subdivided into two classes based upon their N-terminal structure: many have domains with homology to animal Toll and Interleukin receptors (TIR), while others have coiled-coil (CC) domains. The importance of this group of proteins is highlighted by the fact that *Arabidopsis* is estimated to have about 200 sequences with homology to NB-LRR proteins (67). Representatives of these classes include: the *Arabidopsis* proteins RPS2 (130) and RPM1, which contains functional homologues in pea, bean and soybean (93), conferring resistance to *Pseudomonas syringae* strains; the tobacco protein N, conferring resistance to tobacco mosaic virus (269); and the flax protein L6, conferring resistance to the rust fungus *Melampsora lini* (137). More recently, the *Arabidopsis* NB-LRR protein RRS1 conferring resistance to the bacterium *Ralstonia solanacearum* was identified. It contains a C-terminal WRKY domain, a class of plant-specific zinc-finger transcription factor commonly induced during defense responses and known to bind to pathogen-induced promoters, providing a link between resistance genes and their phenotype (58). The other three groups of *R* genes include: proteins with an extracellular LRR domain and a transmembrane

domain, represented by the tomato Cf proteins that confer resistance to *Cladosporium fulvum* (238); proteins with an LRR and transmembrane domains in addition to an intracellular kinase domain, represented by the rice Xa21 protein that confers resistance to *Xanthomonas campestris* pv *oryzae* (218); and finally the group represented by the tomato gene *Pto*, encoding a Ser/Thr kinase which requires a NB-LRR protein (Prf) to confer resistance to *Pseudomonas syringae* strains carrying *avrPto* (194). Intriguingly, the perception of a general elicitor such as flagellin and of a race-specific elicitor such as the *X. campestris* pv *oryzae* elicitor is coordinated by two close homologues, FLS2 and Xa21, respectively. More than 200 kinases related to FLS2 and Xa21 (LRR receptor-like kinases, LRR-RLK) are present in the *Arabidopsis* genome (173), and in addition to microbe perception, LRR-RLKs also have a role in development (205, 214), plant steroid hormone perception (264) and perception of the peptide hormone systemin, involved in response to wounding (200). Moreover, besides Toll receptors, which contain extracellular LRRs, intracellular NB-LRR proteins are also employed in animal innate immunity (51). These evidences indicate an overlap of race-specific resistance in plants with more general mechanisms, such as plant development, wounding response, recognition of PAMPs and animal innate immunity.

The "Guard Hypothesis" for *R* Gene Function

A natural interpretation of the gene-for-gene model is that R proteins and effector proteins interact directly. *R* genes code for protein-protein interaction domains such as LRR, supporting this idea. But despite several attempts to demonstrate this interaction biochemically for a number of R/Avr pairs, in only one case has it been demonstrated *in vitro* (113). Besides, the RPM1 protein recognizes two different *Pseudomonas* effectors, AvrRpm1 and AvrB and the tomato NB-LRR Mi protein is able to confer resistance against such distantly related pests as a nematode and an aphid (190). It is harder to interpret a direct

interaction between an R protein and such diverse effectors. The Pto kinase from tomato does interact with its corresponding AvrPto protein but it requires a NB-LRR protein, Prf, for activation of downstream responses (233). In an attempt to reconcile these data, an alternative interpretation was proposed: the Pto kinase could be a general component of host defenses, for example, an activator of defenses in response to a non-specific elicitor. Bacterial AvrPto could have evolved to target and inactivate Pto, suppressing defenses, while Prf could have evolved to detect a Pto/AvrPto complex and activate defenses (252). This model has been called the "guard hypothesis" (51, 106) and it predicts that R proteins "guard" particular components of the plant cell (the "guardees"), which are targets for effector proteins, by sensing the interference of the guardee by the effector protein and activating defenses. It also predicts that in the absence of an R gene, the host target is not guarded, resulting in disease. Increasing evidence supports the guard hypothesis: the tomato Cf-2 protein requires the cysteine protease Rcr3 for recognition of its corresponding avirulent protein (127). The related NB-LRR *Arabidopsis* proteins RPM1 and RPS2 interact with and guard the cellular target RIN4, perceiving its modification or elimination, respectively, by three *Pseudomonas syringae* effector proteins, AvrRpm1, AvrB and AvrRpt2, and subsequently activating defenses (8, 150, 151). Another R protein from the NB-LRR class, RPS5, detects the effector AvrPphB from *P. syringae* indirectly via AvrPphB proteolytic activity that cleaves the "guardee" protein kinase PBS1 (211). If the multitude of pathogens encountered by plants only target a limited set of plant proteins that, for example, regulate defenses and/or nutrient acquisition, an important consequence of the guard hypothesis is that plants could potentially detect a large number of pathogen effectors with only a limited number of surveillance R proteins (150, 211).

In the context of these different mechanisms of resistance evolved in plants, it is interesting to reflect upon the *Agrobacterium tumefaciens*-plant

interaction. Even though there is host range variation between strains, *A. tumefaciens* has one of the broadest host ranges known among plant pathogens, being capable of infecting virtually all dicotyledoneous plants. It has been reported that, for a single strain, at least 643 plant species from 331 genera were susceptible (168). So, even though "most plants are resistant to most pathogens", most plants are not resistant to *Agrobacterium*. How was this bacterium capable of efficiently overcoming non-host resistance in so many different plant species? Why did plants not evolve resistance genes to defeat this pathogen? We do not know the answers to these questions but perhaps by revisiting plant responses to pathogens and symbionts and recent studies of plant responses to *Agrobacterium* in the next sections, we can begin to gain more insights into plant-*Agrobacterium* interactions.

Plant Responses to Pathogens

Several studies using both general and race-specific elicitors in plant cell cultures as well as pathogen inoculation on whole plants have revealed a number of plant responses to pathogens involving both physiological changes and transcriptional reprogramming (98, 191, 199, 201). Interestingly, responses to avirulent pathogens (incompatible interaction, plant is resistant, pathogen growth is limited) and to virulent pathogens (compatible interaction, plant is susceptible, pathogen grows and colonizes plant) can overlap significantly, varying mostly by timing and magnitude (84, 170). Faster and more pronounced activation of defense responses during *R* gene-mediated responses (incompatible) compared to a compatible interaction has been observed in small-scale studies (15, 39, 257), but a recent large-scale study confirmed these observations and proposed a model to clarify how these quantitative differences came about (235). It appears that basal defenses operating in susceptible plants are not mechanistically different from the very efficient *R* gene-mediated defenses. *R*

gene function may be highly correlated with a rapid perception and/or transmission of the pathogen signal. Overlap in defense signaling pathways is not limited to compatible and incompatible responses. A recent review describes the significant overlap between responses to pathogens and herbivores (236). Moreover, some of the responses to pathogens and insect pests also overlap with responses to several abiotic stresses, such as wounding, drought and temperature stress (122, 149, 216, 240). For example, similarities were observed on the gene expression profile of an incompatible plant-fungal interaction with that of wounding stress (66).

Some of the earliest events during plant defense responses are changes in cell ion fluxes, such as calcium influx, which can occur minutes after pathogen inoculation or addition of elicitors (19). Calmodulin (123), calcium-dependent protein kinases (149, 186), MAP kinases (6, 122, 143, 187) and G proteins (199, 229) are also components of signal transduction pathways activated very early during plant defense responses. Ion fluxes across the plasma membrane mediate another important and fast plant response, the oxidative burst and the production of reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (111). ROS can have a direct antimicrobial activity, are involved in the oxidative cross-linking of hydroxylproline-rich glycoproteins to reinforce cell walls, and can also function as downstream signals in the activation of transcriptional responses (21, 134). Similar to what occurs in animal cells during defense, ROS are likely produced by a plant homologue of a mammalian NADPH oxidase (243). Ion fluxes and ROS were shown to be essential for triggering the production of antimicrobial phytoalexins from phenylpropanoid metabolism (111). Phenylpropanoids form a large class of plant compounds with diverse functions such as structural components (lignin), pigments, protectors from UV-light and other abiotic stresses, and signalling molecules in symbiotic and pathogenic interactions (177, 268). Key enzymes of this pathway are

phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), which are known to be induced during several plant-microbe interactions and abiotic stresses (15, 126, 273). The phenolic hormone-like compound salicylic acid (SA) has long been recognized to have a role in plant defense and it is synthesized via PAL, althought recent reports argue that this is not the only pathway for its synthesis (210). In any case, salicylic acid levels increase after pathogen attack and exogenous application can induce defense responses and enhance resistance to a broad range of pathogens (64, 193). Several studies have shown that SA is required for both local defenses and systemic acquired resistance (SAR) (131). SAR is a state of heightened defense to secondary infection that is activated systemically upon a primary infection by pathogens that form necrotic lesions (193). SAR is effective against a broad range of pathogens and is induced by SA and its analogs. A similar form of heightened resistance is also induced independently of necrotizing pathogens and SA by non-pathogenic *Pseudomonas* rhizobacteria. This induced systemic resistance (ISR) requires jasmonic acid (JA) and ethylene (ET), hormone signalling molecules that, like AS, are closely involved with regulation of plant defense responses (131, 253). JA is also involved in the systemic response to wounding upon insect attack which leads to the production of proteinase inhibitors (228). Extensive cross-talk between the signalling pathways involving SA, JA and ET are observed and are thought to have evolved in order to finely adjust appropriate responses to different pathogens (85, 131). Recently, a putative SA receptor, SABP2, was identified that binds to SA and has lipase activity stimulated by the SA binding (128). The authors speculate that this activity may generate a lipid-derived signal that transmits the information systemically during SAR. Interestingly, the evidence that lipid signaling is important in defense responses is not new. JA is a lipid-derived molecule and the *Arabidopsis* proteins EDS1 and PAD4, required by a group of *R* genes in the defense signaling pathway, have lipase signature

sequences (71, 114). More recently, mutations in the *Arabidopsis* apoplastic lipid transfer protein DIR1 were shown to be compromised in systemic but not local resistance, and the authors suggest that DIR1 promotes the long distance transmission of a lipid-derived molecule during SAR (153). Proteins induced by SA or pathogen such as PR-1, PR-2 and PR-5, are typical markers of SAR and they belong to a larger group of proteins collectively called pathogenesis-related proteins or PRs, which are commonly induced during plant defense and stress responses (193, 225). Some PR proteins such as chitinases and glucanases have antimicrobial activity via degradation of pathogen cell walls, while the role of others remains unclear (225). Induction of PR-1 as well as PAL was observed in plants after addition of nitric oxide (NO) donors (65) and endogenous NO acted synergistically with ROS in the activation of defense responses (55). NO has been known to have signaling roles in several physiological responses in animals, including immune and inflammatory responses. In mammalian macrophages, ROS and NO function together to kill bacterial pathogens (165), and it has becoming increasingly evident that similar processes operate in plant cells (166). ROS have been shown to play a key role as local triggers of the plant hypersensitive response (HR) (141) and NO can potentiate that function (55). HR is an extensively studied plant cell defense response that is correlated with rapid and localized cell death at the site of infection (50). The development of HR results in the formation of visible, defined, necrotic lesions, which are thought to contribute to pathogen limitation (50, 133). Although HR cell death is frequently associated with *R*-gene mediated resistance responses, not all incompatible *R-avr* interactions result in an obvious HR and some compatible interactions can also express cell death, probably as a disease symptom (98). HR constitutes a genetically programmed form of cell death in the sense that plant mutants can mimic the lesions in the absence of pathogen (50, 147). HR in plants has been frequently compared with the apoptotic cell death in animals, and although some

of the basic underlying mechanisms are conserved, plants lack many of the animal homologues of apoptosis regulators (7, 133). The protease caspase is a potent executor of animal apoptosis and an encouraging recent finding is a caspase-like activity in tobacco plants during HR (37). The HR expressed during an incompatible plant-bacterium interaction is dependent upon the delivery of bacterial effector proteins to the plant cell via the type III secretion system (77, 224). The bacterial *Hrp* locus is a cluster of genes encoding the type III machinery components, which are induced upon sensing the host. The locus name is derived from the effects of mutations in *Hrp* genes which abolish hypersensitive response in resistant hosts and bacterial pathogenicity in susceptible hosts (77, 224). One of the most intriguing aspects of plant-*Agrobacterium* interactions is that this bacterium can deliver several proteins to the plant cell via its type IV secretion system (41), and yet it does not trigger a typical HR (185, 276). One interesting exception is the species *Agrobacterium vitis* which has a much more limited host range than *A. tumefaciens* and can cause necrosis on grape and an HR on tobacco (289), even though *A. vitis* lacks an *Hrp* locus and the HR response involves another, unknown, mechanism. In addition, it has been reported that one super-virulent strain of *A. tumefaciens* can cause necrosis on grape (57) and an apoptosis-like cell death was observed in maize (100). Apart from these isolated examples, no HR has been observed on either *Agrobacterium*-resistant plants or the several susceptible ones.

Plant Responses to Symbionts

Agrobacterium tumefaciens is most similar to the nitrogen-fixing, nodule-forming plant symbiont *Sinorhizobium meliloti*, according to phylogenetic analyses based on genome sequence (275) (Figure I-1).

Indeed, *Agrobacterium* and *Rhizobium* species, all from the family Rhizobiaceae, form a monophyletic group based on 16S ribosomal DNA analysis

(284), and it has been proposed, not without controversy, that the two genera be combined in one genus, Rhizobium (285). Interestingly, *A. tumefaciens* harboring *Rhizobium* symbiotic plasmids can form nodules in host plants (1) and even fix nitrogen, although with lower efficiency (155), supporting their common ancestry.

Much is known on the symbiotic relationship between rhizobia and their legume hosts and so the question of similarity is also pertinent to the interaction with plants. Did a common ancestor evolve pathways critical to a non-pathogenic outcome? This question is complicated by the difference in host range between the two taxa: rhizobia interact exclusively with legumes, which presumably have developed unique mechanisms to foster the successful symbiosis. The host range is further limited within legumes, with each particular rhizobial species infecting only one or a few genera of legumes. Agrobacteria, on the other hand, have a broad host range (see above), although legumes are generally not efficiently colonized by these bacteria. Nevertheless, the biology of rhizobia-plant interaction is relevant to the present discussion. This interaction initiates when soil inhabiting rhizobia perceive specific flavonoids produced by plant hosts and trigger the synthesis of bacterial lipochito-oligosaccharides, or Nod factors (146). Nod factors are determinants of host specificity and can induce a series of remarkable plant responses: root hair curling, which encapsulates the bacteria, formation of an infection thread through which the bacteria penetrate, and finally, plant cell division that results in nodule formation. Nodules can be viewed as new plant organs, specialized in nitrogen fixation via the bacterial nitrogenase enzyme. In the nodule, the bacterium radically modifies its physiology and metabolism, becoming an intracellular bacteroid, providing the plant with ammonium and receiving carbon assimilates in return (226). Recently it has been shown that, in addition to carbon compounds, bacteroids also receive amino acids from the plant, enabling the bacteria to shut down their ammonium assimilation (145). This example illustrates the sophistication of the interaction:

bacteroids must secrete ammonium to the plant in order to obtain their amino acids, revealing the partners interdependence and providing a selective force for evolution of symbiosis.

Besides the morphological modifications associated with nodule formation, a number of plant proteins, called nodulins, are found at elevated levels in nodules. While the function of some nodulins, such as leghemoglobin, which delivers oxygen to the bacteroid and protects the oxygen-sensitive nitrogenase, are obvious, the role of most nodulins is unknown (146, 226). Some presumably have a role in cell division and nodule formation, but nodulins can even be related to plant defense genes (78, 231). Some nodulins are proline-rich proteins, which form the major component of the infection thread (96). It becomes apparent that the interaction depends on a complex exchange of signals between plant and bacteria and, as expected, a high level of regulation is observed. When the plant is well fed with nitrogen and the symbiont is perceived as no longer useful, elevated levels of nitrate indicate the need to suppress new interactions, by a genetic process of auto-regulation of nodulation controlled by the plant (208, 226). This regulation likely involves the plant defense system. A hypersensitive response (HR) has been observed in abortive alfalfa infections resulting from the process of auto-regulation of nodule number (254). Additionally, the symbiont could initially be perceived as enemy, triggering defense responses, while later, some of these responses could be suppressed by unknown mechanisms. General defense responses are induced by exopolysaccharide (EPS)-deficient rhizobium mutants and it has been suggested that EPS acts as a suppressor of the plant defense system (88, 169, 179) . An oxidative burst in alfalfa plants in response to infection by *Sinorhizobium meliloti* was observed, and it is consistent with the bacteria being initially perceived as a pathogen invader by the plant (198). There is extensive evidence for induction and suppression of defense responses by rhizobia infection: a chalcone synthase (CHS) gene was induced

by both wild-type and Nod factor mutants (126); different members of the pathogenesis-related protein PR10 family were either induced or suppressed by nodulation (78, 215) ; a receptor-like kinase induced during pathogen attack is suppressed by *Rhizobium* infection (136). During the symbiotic interaction between plant roots and mychorrizal fungi a similar restriction of the microbial symbiont colonization is observed: phosphate is provided to the plant during the interaction and high levels of phosphate can inhibit the establishment of the symbiosis, probably through induction of plant defense responses (135). Transient and/or localized induction followed by suppression of defense genes is also observed during this plant-fungus interaction (52, 135). Therefore, a balance between induction and repression of defenses could limit the colonization without completely rejecting the symbiont. Surprisingly, in a study comparing the expression of eight chitinase genes during mycorrhiza formation, nodulation, and pathogen infection, the pattern of expression during nodulation was more related to fungal pathogen infection than to mycorrhiza formation (195). However, because rhizobia lack chitin and because this chitinase activity in the nodule is unlikely to interact directly with the bacterium, the response could be seen as a general defense mechanism triggered by the plant. The authors nevertheless speculate that these chitinases could cleave, and therefore regulate, Nod factors and moreover, the chitinase activity could provide a barrier to fungal infection in the nodule. Early and transient induction of several defense-related genes, including a basic chitinase, was observed in pea roots inoculated with *Rhizobium* and the mycorrhizal fungus *Glomus mosseae*, and interestingly, the accumulation of these transcripts was higher in mycorrhiza-resistant/non-nodulating mutant plants than in wild type (192). Unlike rhizobia, mycorrhizal fungi have a broad host range and can form symbiotic associations with the majority of land plants. Despite these differences, the two associations share a common symbiotic pathway, as illustrated by the identification of legume mutants

that are arrested early in both rhizobial and mycorrhizal colonization (62, 192, 266). It is thought that the more evolutionary recent legume-rhizobial interaction has recruited components from a pre-existing plant-fungal pathway (182). A breakthrough was achieved independently by two groups with the identification and cloning of a gene belonging to this common symbiotic pathway employed during rhizobial and micorrhizal interactions. The *Lotus SYMRK* (Symbiosis Receptor-like Kinase) and the alfalfa and pea *NORK* (Nodulation Receptor Kinase) gene encode a leucine-rich repeat, receptor-like kinase (LRR-RLK) that perceives both the mycorrhizal and rhizobial signal (68, 227) . More recently, mutations in a LysM-type serine/threonine receptor kinase were identified in *Lotus* (152), alfalfa and pea (144), that have a wild-type mycorrhization phenotype, but specifically lack the early root hair curling response to rhizobia. These proteins are putative Nod-factor receptors that act upstream of the SYMRK receptor and the common pathway. Because these receptors are not required for mycorrhization and act upstream of SYMRK, they probably serve to link rhizobial interaction to the common symbiotic pathway. Transfer of these receptor genes to non-legume important crop plants, to obtain nitrogen-fixing nodules and reduce the use of polluting nitrogen fertilizers, is an exciting future possibility (182).

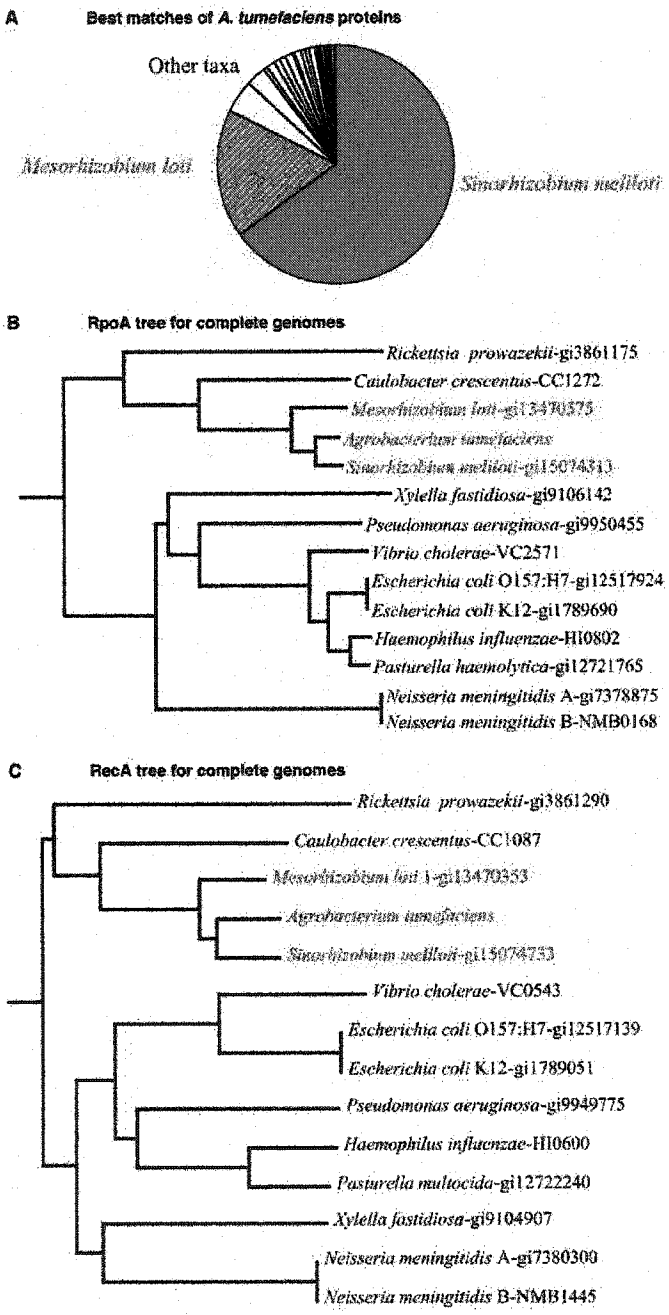

Figure I-1. Phylogenetic comparisons of *A. tumefaciens* with fully sequenced genomes. (**A**) Distribution of best hits based on a comparison of predicted proteins of *A. tumefaciens* with proteins from all published genomes. (**B** and **C**) Phylogenetic trees generated using two broadly conserved proteins (From Wood et al, 2001, reference 275).

Agrobacterium tumefaciens Virulence System

Agrobacterium tumefaciens is a ubiquitous soil-borne α-proteobacterium and a member of the family *Rhizobiaceae* (275). It is the causal agent of the plant disease crown gall and although not usually lethal, the disease can result in reduced yield and/or vigor and significant agronomical losses, mostly in some perennial crops, despite the bacterium's broad host range (69). This Gram-negative rod has numerous features that have contributed to intensive research on both the organism and its unique ability to genetically transform plants. Many of the principles that have been revealed through these studies serve as paradigms not only for other bacterial-plant interactions but also many pathogen-animal systems. The genome organization of *Agrobacterium* is unique, consisting of a circular chromosome, a linear chromosome, a large 200 kb plasmid (Ti) required for tumor formation on plants, and a 540 kb cryptic plasmid that appears not to be necessary for tumor formation (90). The sequencing and annotation of the *A. tumefaciens* strain C58 genome has been completed and comparisons with the genome of *Sinorhizobium meliloti* reveals their close evolutionary relationship (89, 275).

A. tumefaciens induces crown gall tumors on wounded plants by transferring a piece of its own DNA, the T-DNA, from its tumor-inducing (Ti) plasmid into the plant nucleus where the T-DNA is integrated into the plant genome and expressed. The expression of the oncogenes on the T-DNA leads to unbalanced and unregulated production of plant growth hormones triggering tumorigenesis. The expression of a second set of genes on the T-DNA directs the production of opines, compounds that the bacteria can use as nutrients, creating a unique ecological niche in the plant tumor (for reviews see (167, 290)). The T-DNA is transferred via a type IV secretion system that has evolved from bacterial conjugation (41).

The studies on this process of natural genetic engineering and the interaction between *Agrobacterium* and plant cells have fostered the development of plant molecular biology and genetics, contributed to the fundamental discovery of two-way signaling between hosts and pathogens and have been instrumental in the development of plant biotechnology. Recent studies have shown that under laboratory conditions *Agrobacterium* transformation can be extended to recalcitrant monocotyledoneous plants (105, 109, 110) *Saccharomyces* (24, 180), other yeast species (26), commercial mushrooms (35), the human pathogen *Coccidioides* (2), many filamentous fungi (54, 91), the rice blast fungus *Magnaporta grisea* (183), and the oomycete that causes late-blight of potato, *Phytopthora infestans* (258). The T-DNA has even been shown to be transferred and integrated into chromosomes in mammalian cell lines (129). It seems likely that the many experimental and biotechnological applications that *Agrobacterium* serves in its natural plant hosts, such as the high-throughput generation of insertional mutants for genetic studies (9, 209), will be transferable to a wide variety of other eukaryotes. As the need for a more efficient, environmentally safe agriculture increases and the controversy about genetically modified organisms (GMO) grows, it becomes more important to have a greater understanding of the basic processes of genetic transformation by *Agrobacterium* (249). The global area cultivated with GM crops reached 67.7 million hectares in 2003, an increase of 15% since 2002 and a more than 40-fold increase since the initial commercialization of GM crops in 1996 (http://www.afaa.com.au/biotechpdf/05_2004_World_GM_Crop_Statistics.pdf). These numbers will likely increase even more in the future as the technology improves and becomes more accepted and cost-effective. Although most commercially available GM foods were produce in the past by biolistic bombardment, *Agrobacterium*-mediated transformation will probably become increasingly common (249). Biolistic methods are expensive and frequently result

in undesirable multi-copy and rearranged insertions while *Agrobacterium*-based methods have lower costs and usually result in single-copy, intact insertions (109, 237). In addition to its biotechnological applications, continued study of the *Agrobacterium*-crown gall system should lead to new insights into the biology of bacterial conjugation, cell-cell communication, nucleoprotein transport, and the pathogenesis of certain animal and human diseases.

More than a decade ago, Stachel and his colleagues made the seminal discovery that the ability of *Agrobacterium* to transform plants required the activation of a set of genes, the virulence (*vir*) regulon on the Ti plasmid by the signal molecules acetosyringone, monosaccharides and acids secreted by wounded plants (27, 213, 220). This was the first recognition that genes required for the virulence of an organism and the interaction with its host are often only expressed in the environment of the host. These studies also revealed that *Agrobacterium* exploits the wounded plant as a necessary step in the complex process of tumorigenesis. The *vir* genes are organized as a large virulence regulon (221) and include both loci required for tumor formation as well as some which apparently are not required (at least under laboratory conditions). The *vir* genes in the first category are clustered into at least six operons in octopine strains of *A. tumefaciens*: *virA, B, C, D, E and G* (for review see (115)). The genes under control of plant signal molecules which are not required for virulence include *virF, H, K, L* and *M* (117). Plant signals are recognized and transcription of the *vir* genes is activated by the sensor protein VirA and the response regulator VirG, members of a two-component regulatory system (223, 271, 272). The proteins encoded by the *virD* and *virE* operons are involved in the production of a nucleoprotein T-complex, which is transferred from the bacterium to plant cells. VirD1/VirD2 possess endonuclease activity and nick the T-DNA border sequences thereby generating a single-stranded T-DNA intermediate (T-strand), which has been shown both genetically and biochemically to be the transferred

intermediate (82, 222, 241, 280, 287). The VirD2 protein remains covalently bound to the 5' end of the T-strand at the nick site (103, 265, 283). VirD2, as well as VirE2, contain nuclear localization signals which direct the T-strand to the plant cell nucleus (43, 108, 189, 293). The VirE2 protein has also been suggested to form a channel in the plant membrane that allows the passage of the T-strand (63). The T-DNA border sequences are 25 base pair imperfect direct repeats, which delimit the length of the T-DNA (263). However, what lies between the border sequences is not important for T-DNA transfer, which makes *Agrobacterium* an ideal tool for gene transfer. Other proteins, including the single stranded DNA binding protein VirE2, as well as VirF and VirD2 molecules, can be transferred independently of the T-DNA (176) most likely by a two step process (34). Several laboratories have demonstrated that the 11 member *virB* operon and the *virD4* gene encode proteins required for the transfer of the T-complex from the bacterium to plant cells, suggesting that these proteins form the T-complex transport apparatus in the bacterial membrane (for reviews see (40, 296)). This view is supported by the fact that VirB and VirD4 are related to transport proteins involved in export of pertussis toxin by the human pathogen *Bordetella pertussis* as well as other proteins by a number of animal and human pathogens and plasmid transfer by Gram-negative bacteria (45). The similarity in nucleotide sequence of genes coding for the transport apparatus defines a new secretion pathway termed Type IV, of which the T-DNA transporter of *Agrobacterium* serves as the paradigm (41). The *VirB* operon encodes the proteins required for the formation of a Type IV pilus (76) termed the T-pilus (132). How the T-pilus functions in the transfer of T-DNA and other proteins into eukaryotic cells is currently under intense investigation in a number of laboratories. Recently, a breaktrough was achieved with the definition of an orderly pathway of T-DNA contact with specific members of the *VirB* operon (31). In addition to the *vir* genes encoded in the Ti plasmid, chromosomal genes also

play a role in the virulence of *Agrobacterium*. These include *chvA* and *chvB*, which are concerned with the transport and synthesis of β-1,2 glucan to the periplasm. Mutations of either gene result in avirulent strains which do not attach to plant cells (30). Mutations in homologous genes result in the production of defective nodules in *Sinorhizobium* (22, 81) and avirulence in the animal pathogen *Brucella abortus* (188). Other non-attaching *Agrobacterium* mutants include those blocked in phosphoglucomutase (*exoC*) which do not synthesize either β-1,2 glucan or cellulose fibrils (29). Other pleiotrophic *chv* mutants include a poorly characterized two component regulatory system (*ChvG* and *ChvI*), also important for nodulation in *Sinorhizobium* and virulence in *Brucella* (217).

Plant responses to *Agrobacterium tumefaciens*

Although the role of bacterial genes in the interaction between *Agrobacterium* and its host have been intensively studied, much less is known about the host components involved in recognition, transfer and integration of the T-DNA. Recently, a number of studies have begun to unravel some of the host factors that may play a role in these processes (80, 245). Several *Agrobacterium* virulence proteins are transferred to the host cell (206, 256) and these proteins were used in interaction screens to identify candidate host partners. VirD2 was found to interact with an *Arabidopsis* importin-α (karyopherin) protein, which mediates nuclear import of proteins containing nuclear localization signals (NLS) (12), suggesting that this protein may guide VirD2 and the associated T-DNA to the plant nucleus. A plant cyclophilin also interacts with VirD2 and it has been proposed that this protein may serve as a chaperone that maintains the stability of the T-complex inside the plant cell (56). Another VirD2-interacting protein is a type 2C serine-threonine protein phosphatase which may be a negative regulator of T-complex nuclear import (234). The involvement of phosphorylation/ dephosphorylation during T-complex nuclear import is supported by evidence

that a cyclin-dependent kinase-activating kinase interacts with and phosphorylates VirD2 (11). Additionally, these authors report that VirD2 tightly associates with a TATA box-binding protein, providing a possible explanation for the frequent observation of T-DNA integration into transcriptionally active regions of the genome. VirE2 interacts with the *Arabidopsis* VIP1 protein, which contains basic leucine-zipper (bZIP) domains and it is also suggested that this protein facilitates nuclear import and T-DNA integration (246). Accordingly, overexpression of VIP1 can increase transformation in plants (247). VirF is also transferred to plant cells, although the role of this protein during transformation is unclear. VirF was found to have an F box domain and to interact with a Skp1p plant homologue, both of which are part of an E3 ubiquitin ligase complex involved in specific proteolysis by the proteasome (207). Thus, VirF may be involved in targeted degradation of specific host proteins to facilitate transformation.

Unlike transposable elements, the T-DNA does not encode enzymes that mediate its integration and it has been thought that the Vir proteins that accompany the T-DNA, such as VirD2 and VirE2, have a role in this process. While VirD2 has a site-specific ligase activity and it is important for accurate integration of the T-DNA, it is not involved in the efficiency of integration (241). And indeed, an *in vitro* study showed that a plant enzyme, but not VirD2, was required for T-DNA integration, indicating a role for host factors in the process (294). T-DNA integration in plants occurs by a process of illegitimate (non-homologous) recombination (83). Integration in yeast occurs by a similar process, unless the T-DNA carries homology with the yeast genome, in which case integration occurs via homologous recombination (25). This contrasts with the situation in plants, where even in the presence of extensive homologies, the T-DNA still integrates mostly via non-homologous recombination (175), suggesting that the process of T-DNA integration is predominantly determined by

the host. In yeast, it has been demonstrated that several non-homologous end-joining proteins, including ligase IV, are required for T-DNA integration (250). However, ligase IV *Arabidopsis* mutants were not impaired in *Agrobacterium* transformation, although they were hypersensitive to DNA-damaging agents, indicating that this enzyme is necessary for repair of DNA damage but not required for T-DNA integration (251). The authors indicate that other ligases could takeover the role of ligase IV in T-DNA integration and/or other non-homologous end-joining proteins could be important in the process. Because T-DNA integration occurs via non-homologous recombination, the plant DNA repair machinery has always been thought to play a role in integration, even though evidence for this hypothesis is scarce and contradictory. *Arabidopsis* mutants hypersensitive to irradiation were described as being deficient in T-DNA integration (219), although it was later demonstrated that these mutants were not impaired in this process (162, 181). By looking at natural variation in *Arabidopsis*, an ecotype was identified that was more sensitive to γ radiation and had a reduced level of stable transformation and therefore, T-DNA integration (161). More recently, a number of *Arabidopsis* rat (resistant to *Agrobacterium* transformation) mutants were identified (163) and although several have been characterized, none so far has obvious roles in plant DNA repair (292). One of the most well characterized rat mutants contains an insertion into a histone H2A gene and is deficient in T-DNA integration (159). The expression of this gene is correlated with plant tissues that are more susceptible to transformation and overexpression of this gene increases transformation (281). The authors suggest that plant chromatin structure is important for T-DNA integration. Other rat mutants include an insertion into an arabinogalactan protein which renders *Arabidopsis* cells unable to bind *Agrobacterium* (79, 292) and an insertion into a cellulose synthase-like gene, which results in reduced number and growth of lateral roots and also reduced ability to bind *Agrobacterium* (291). Earlier work

attempted to identify plant cell surface molecules involved in *Agrobacterium* binding has identified a vitronectin-like protein (261) and a rhicadhesin-binding protein (230) as possible candidates, although genetic evidence confirming their role in *Agrobacterium* infection is lacking. In addition to plant rat mutants, a genetic screen performed in yeast has identified mutants with altered transformation sensitivities. Yeast with disrupted purine synthesis resulted in hypersensitivity to transformation and application of purine synthesis inhibitors to plant cells greatly improved transformation, although it is not yet clear how purine synthesis specifically affects transformation (184).

Different plant species, cultivars, and even particular plant tissues vary greatly in their competence for transformation and unraveling the molecular basis for these differences will contribute to expanding the usefulness of Agrobacterium as a genetic engineering tool. A recent study quantified the significance of plant cell competence: approximately half of the *Arabidopsis* cells that were selected for a first transformation event (therefore, were competent) were also transformed with a second T-DNA, a ten-fold higher frequency than that observed for a single transformation event, indicating that plant cell competence is crucial for successful transformation (53). Cell competence for transformation is stimulated by phytohormone treatment (4, 33, 196), which suggests that cell division and/ or a specific stage of the cell cycle is associated with higher transformation frequency. In *Petunia*, only DNA-duplicating (S-phase) cells were able to take up and/or integrate the T-DNA, while further cell division was required for stable transformation (259). Alternatively, an S-phase may only be required in order to replicate the T-DNA, converting it into a double-stranded molecule that can be expressed. In addition, T-DNA replication has recently being shown to stimulate T-DNA recombination (288). DNA during replication and/or transcription would not only be in a more accessible state that likely facilitates integration, but also during these events, errors can occur and the DNA

repair machinery is activated, correlating cell-cycle phase and DNA repair with the process of T-DNA integration via illegitimate recombination (83).

As can be concluded from the above studies, there is abundant circumstantial evidence for the involvement of plant factors in *Agrobacterium* transformation but, with very few exceptions, mostly provided by the isolation of plant mutants affected in transformation and by the identification of Vir protein-interacting factors, we do not know which factors from the plant are exploited by *Agrobacterium* in order to achieve transformation. We also do not know how the transfer of Vir proteins and T-DNA alters the expression of plant proteins and how the changes in expression compare to those changes effected by other pathogens, symbionts and general stresses. As emphasized earlier, plant responses to pathogens and symbionts can involve both induction and suppression of defenses. Differences in timing of responses and the combination of plant/microbe species and environmental conditions will determine the final outcome of the interaction. Also, host components can be subverted for the benefit of the microbe. For example, plant viruses can interfere with cell-to-cell trafficking (47, 138) and regulation of the plant cell cycle (95). Viruses are perceived by the plant RNA silencing machinery, but can encode suppressors of gene silencing that evade plant responses (260). Like plant-virus and plant-*Rhizobium* interactions, plant-*Agrobacterium* interactions exhibit a great deal of evolutionary sophistication, with bacterial genes that bear plant transcriptional and translational elements and catalyze the formation of plant hormones, to bacterial proteins that contain plant nuclear localization signals. Therefore, a high degree of regulation is expected in both partners during the interaction and our laboratory has undertaken a broad approach to initiate the understanding of plant responses to *Agrobacterium*. We identified several differentially regulated genes upon plant cell culture infection with *Agrobacterium* that appear to be related to signal perception, transduction and plant defense ((60) and Chapter II). The

induction of defense responses is somewhat surprising due to the non-inducing nature of the general flagellin elicitor from *Agrobacterium* (73). Interestingly, some of the defense responses were commonly induced by a non-pathogenic bacterium and they probably represent a general plant response to stress, stimulated by an unknown general elicitor. However, a few genes were induced specifically by *Agrobacterium*, such as the one encoding a nodulin-like protein, revealing that, not only *Agrobacterium* and rhizobial species are closely related, but also trigger similar responses from plants. Additionally, two plant enzymes involved in the phenylpropanoid metabolism have contrasting expression patterns: one is induced and the other (a cytochrome P450) is suppressed by *Agrobacterium*, which suggests that a balance between defense induction and repression, similarly to what occurs during *Rhizobium*-plant interactions, can also operate during plant-*Agrobacterium* interactions. Repression of a cytochrome P450 by wild-type Agrobacterium was also recently observed during later stages of the interaction in potato tumors (119). Differential expression of several genes upon inoculation with different strains of *Agrobacterium* was also observed in tobacco cells (255). A subset of defense-related genes was studied further and while most of them do not appear to be regulated differently by different strains, three genes appear to be more induced by a transfer-deficient strain than by a transfer-competent strain of *Agrobacterium*. These experiments were not replicated and therefore the results should be viewed with caution. Nevertheless, the authors suggest that defense responses are suppressed during a successful transformation. Because the expression of genes encoding histones and ribosomal proteins was induced during later stages of the infection by a transfer-competent strain, the authors speculate that these cell division-related genes are induced to mediate the transformation process. Preliminary observations from our laboratory have indicated the intriguing possibility of an attachment-dependent dampening of plant defense responses by *Agrobacterium*. The

attachment-deficient, avirulent *Agrobacterium* mutant (*chvB*) appears to hyper-induce a small set of defense genes when compared to virulent (transfer-competent) strains or to other avirulent strains that are able to attach to plant cells (Ditt et al., unpublished observations and Chapter III). Further studies of plant responses to different *Agrobacterium* strains and mutants as well as to other pathogens and symbionts will broaden our insights into this unusual plant-microbe interaction.

Conclusions

The study of the interaction of *Agrobacterium* with its host plants is especially interesting because of the unique nature of the interaction. *Agrobacterium* does not induce the hypersensitive response, its peptide flagellin is unable to induce defense-related responses in plants and *Agrobacterium* is closely related to symbiotic bacteria from the genus *Rhizobium*. Can *Agrobacterium* be considered a near-symbiont? Most plants are infected by *Agrobacterium* without extremely detrimental effects and presumably this bacterium has evolved ways to overcome and/or suppress defenses. For example, in our studies we observed the induction of genes involved in the production of reactive oxygen species (ROS) (60), but this plant response is probably overcome by an *Agrobacterium* enzyme that can convert certain ROS to nontoxic products (276). Additionally, evidence is emerging for a possible specific suppression or dampening of defenses by *Agrobacterium* ((255) and our unpublished observations). Why plants did not evolve resistance genes and/or defense responses that effectively hinder this bacterium? Interestingly, the vast majority of *Agrobacterium* isolates from soils and roots are nonpathogenic (17). It is not known whether these strains can become pathogenic upon acquiring a Ti plasmid from a pathogenic strain or they simply have a more restricted host range. Additionally, nonpathogenic mutants were isolated from apple tumors with

high frequency (14). These mutants were altered in the Ti plasmid and presumably retained normal plant-attaching functions, which are encoded chromosomally. If our hypothesis for an attachment-dependent dampening of defenses is correct, these nonpathogenic strains and mutants would still be able to dampen defenses, maybe even more so than wild-type. Because they do not pose harm to the plant, it can be speculated that the high frequency of nonpathogenic strains and mutants in nature could maintain a low selective pressure that would not promote the development of effective plant defense mechanisms. Intriguingly, the apple host appeared to have played a direct role on the loss of pathogenicity by these mutants: the clonal nature of mutants isolated from the same tumor indicated that the plant influenced agrobacterial populations by favoring growth of nonpathogenic mutants over wild type (14).

The model for evolution of eukaryotic mitochondria from an α-proteobacterium endosymbiont is now generally accepted (94). *Agrobacterium tumefaciens* 16S ribosomal RNA sequences identified this bacterium, as well as rhizobia and rickettsias, as the closest living relatives of the ancestral endosymbiont that gave rise to mitochondria (278). Could this indicate that plant associations with Agrobacterium-like organisms are ancient and therefore highly evolved? Rhizobia and rickettsia have contrasting interactions with their hosts, rhizobium being a symbiont and rickettsia a pathogen, although both live intracellularly. *Agrobacterium* is thought to associate extracellularly with its host, althought evidence is lacking for either an extracellular or an intracellular mode of invasion. At any rate, it has been shown that transfer and integration of T-DNA is possible once bacterial cells are injected inside plant cells (70). Intracellularity is a reality in this group of related bacteria, but in which direction will evolution go: to become intracellular or extracellular? Does it matter? Will Agrobacterium evolve to become more antagonistic or beneficial to the host? Future research into Agrobacterium-plant interactions promises exciting discoveries.

Chapter II

cDNA-AFLP Analysis of Plant Responses to *Agrobacterium*

SUMMARY

To elucidate the nature of the plant response to infection and transformation by *Agrobacterium tumefaciens,* we compared the cDNA-AFLP pattern of *Agrobacterium*-inoculated and mock-inoculated *Ageratum conyzoides* plant cell cultures. From 16,000 cDNA fragments analyzed, 251 (1.6%) were differentially regulated (0.5% down-regulated) 48 hours after co-cultivation with *Agrobacterium*. From 75 strongly regulated fragments, 56 were already regulated 24 hours after co-cultivation. Sequence similarities were obtained for 20 of these fragments and RT-PCR analysis was carried out with 7 to confirm their cDNA-AFLP differential pattern. Their sequence similarities suggest a role for these genes in signal perception, transduction and plant defense. RT-PCR analysis indicated that four genes involved in defense response are regulated in a similar manner by non-pathogenic bacteria while one gene putatively involved in signal transduction appeared to respond more strongly to *Agrobacterium*. A nodulin-like gene was regulated only by *Agrobacterium*. These results demonstrate a rapid plant cell response to *Agrobacterium* infection, which overlaps a general response to bacteria, but also has *Agrobacterium*-specific features.

INTRODUCTION

Agrobacterium tumefaciens infects and transfers a piece of its tumor inducing (Ti) plasmid, the T-DNA, to most dicotyledonous plants, thereby modifying their genome and inducing a hyperplastic response that results in a crown gall. This is the only verified example of natural interkingdom DNA transfer and, as a consequence, *Agrobacterium* is widely used to genetically engineer plants and to generate insertional disruptions in genes, facilitating functional genomics of

plants (9). Its uses may broaden as *Agrobacterium* is capable of transferring its T-DNA to fungi (24, 180) and even human cells (129). Despite these extensive applications and biological significance, very little is known about the events that take place in the host cell during genetic transformation by *Agrobacterium*.

In contrast to our lack of knowledge regarding the host partner, the molecular events that occur within the bacterial partner during the interaction have been intensively studied. The mechanism of T-DNA transfer is adapted from bacterial conjugation (42) and involves a number of virulence genes encoded mostly in the Ti plasmid but also in the bacterial chromosome (116). Their expression is induced by signal molecules secreted from wounded plants (270) and results in the formation and export of the T-DNA (212). What host components are involved in recognition, transfer and integration of the T-DNA into the host genome remain largely unknown. Recently, a number of studies have begun to unravel some of the host factors that may play a role in these processes (79). A plant cyclophilin (56) and a plant karyopherin α protein (12) were identified by an interaction screen, and a plant histone H2A was identified by mutational analysis (159). Additionally, a plant DNA-ligase was reported to determine T-DNA integration efficiency by an *in vitro* assay (294).

So far, no attempt has been made to systematically explore the host gene expression response to *Agrobacterium*. In addition to identifying factors that might be relevant for transformation, a study of changes in gene expression should help elucidate the general response of the plant to *Agrobacterium* infection. This information could be compared to the responses of plants to other pathogens and symbionts. The various studies of interactions with pathogens (66, 195, 204) and symbionts (135, 192, 215) such as *Rhizobium* (5, 48, 126), have demonstrated that host defense responses are both induced and repressed and host components are subverted for the benefit of the microbe.

The relationship of *Agrobacterium* to host plants is unique among plant pathogens. *Agrobacterium* does not induce the hypersensitive response (185), even though the bacterium introduces several proteins into the host cell. *Agrobacterium* is closely related to symbiotic bacteria from the genus *Rhizobium* (270). Interestingly, the peptide flagellin from *Agrobacterium tumefaciens* and from *Rhizobium meliloti* is unable to induce defense-related responses in plants, while the corresponding peptide from several other bacteria, including *Pseudomonas* species and *E. coli*, acts as a potent elicitor (73). Taken together, these lines of evidence raise the question of whether *Agrobacterium* is capable of altering plant gene expression and, more specifically, if it can alter the expression of plant defense-related genes. Here we report the use of a differential screen, the cDNA-AFLP (10), to examine the initial response of gene expression in plant cells exposed to *Agrobacterium*. We show that a number of plant transcripts have their expression altered at 24 and 48 hours after interaction with *Agrobacterium* and that the proteins encoded by these genes have a putative role in plant signal transduction and in defense response.

RESULTS

A plant cell culture highly competent for *Agrobacterium* transformation. In order to facilitate the identification of genes differentially expressed in response to *Agrobacterium* transformation, a system where a high percentage of host cells are competent for transformation is essential to prevent signal dilution. To identify changes in gene expression during the early events of transformation, possibly before or during the first integration events, selection for transformed cells was not applied in this study. The model plant *Arabidopsis thaliana* would be a natural choice for the present work, but without selection, the efficiency of T-DNA transfer and expression in *Arabidopsis* root cells is less than 5%, while stable transformation is less than 0.5% (53). This response is likely to be not unique to

Arabidopsis plants and represents a major limitation to our study. In addition, it is critical that the system be highly reproducible, which argues against using intact plants, where the location of transiently transformed cells is not predictable (53). Therefore, we explored the use of plant cell suspension cultures, which should overcome these limitations.

We compared two available suspension cultures that are rapidly growing and consist of very small clumps of cells: BY-2, from *Nicotiana tabacum* (160) and a suspension culture from *Ageratum conyzoides* (118). Kanzaki et al reported that transient transformation and expression of the GUS reporter gene in *Ageratum* cells was nearly 100 times higher than in BY-2 cells. We confirmed these results. We transformed *Ageratum* and tobacco BY-2 cell suspension cultures and *Arabidopsis* plants with *Agrobacterium tumefaciens* strain EHA105 (pBISN1) containing a GUS-intron construct that allows expression in plants but not in the bacteria (164). The data in Figure 1 confirm the high transformation competence of these cells compared to BY-2 cells and to *Arabidopsis* root and leaf tissue. GUS expression was analyzed by a colorimetric assay (staining) and by RT-PCR detection of the GUS transcript at 24, 48 and 72 hours after transformation. GUS enzymatic activity measured by a fluorometric assay (data not shown) also agrees with these data and with data obtained by Kanzaki et al. Therefore, the plant cell culture from *Ageratum* represents the best system for the present study and any other type of study where reproducible high levels of transformation are required.

Analysis of alterations in *Ageratum* plant cell gene expression 48 hours after *Agrobacterium* inoculation. The data in Figure 1 illustrate that high levels of GUS expression in *Ageratum* cells are observed at 48 hours after inoculation. Therefore, we chose this time point to first analyze changes in gene expression by AFLP. *Ageratum* cells were co-cultivated with *Agrobacterium* strain EHA105

(pBISN1) and after 48 hours these cells as well as cells from mock-inoculated controls were harvested for RNA extraction and compared by cDNA-AFLP analysis. The cDNAs from control and treated samples of two independent assays were amplified using all possible 256 primer combinations. A section of a typical AFLP gel obtained is shown in Figure 2. A total of 16,000 cDNA fragments were displayed and 251 were differentially regulated by the treatment (Table 1). Even though most of the bands displayed were reproducible between the two independent experiments, approximately 1% of all bands were specific to either experiment (Fig. 2, Box C). We believe that these signals represent intrinsically variable genes, which can be identified by experimental replication. Only bands reproducibly altered by the treatment in two experiments were studied further.

Analysis of fragments altered at both 24 and 48 hours after *Agrobacterium* inoculation. To identify factors regulated earlier than 48 hours after contact with *Agrobacterium*, the expression of 74 selected differential fragments was analyzed by AFLP 24 hours after inoculation. GUS expression was not evident at this time (Figure 1). These 74 fragments all showed a strong differential expression pattern at 48 hours. Among them, 56 showed the same differential expression pattern at 24 hours after inoculation (Table 1). Sections from AFLP gels resulting from this analysis are shown in Figure 3. Bands were observed that were altered at 24 but not at 48 hours. These fragments would potentially represent interesting candidates to be analyzed as well. However, this class of fragments was not followed further in this study. All 56 fragments altered at both 24 and 48 hours after inoculation were isolated from the AFLP gels, re-amplified by PCR and sequenced.

Compilation of sequences from differentially expressed cDNA fragments. DNA sequence data were obtained for 50 of the 56 fragments isolated.

Sequence similarity to known genes was found for 20 fragments by BLAST searches (E value cut-off = 5e-4). Their length, pattern of expression and homology to known proteins are shown in Table 2. Four fragments are likely involved in signal perception/transduction, including homologues to a zinc finger protein (AC18), a putative nodulin (AC168), a putative receptor kinase (AC181) and a lectin-like protein kinase (AC321). These four fragments were up regulated, except AC181, which was down regulated. Eight fragments are likely involved in disease and stress response, including a homologue to a PR (pathogenesis-related) protein (AC365), factors involved in biosynthesis of phenylpropanoids (AC121 and AC290), a peroxidase precursor (AC275), and factors involved in response to wounding (AC174 and AC315), starvation (AC178) and drought (AC274). These eight fragments were up regulated, except AC290, which was down regulated.

The role for the remaining eight fragments in *Agrobacterium*-plant interaction is unclear: their sequence homology suggests that they are related to metabolism (glyoxalase - AC95, glucosaminyl N-acetyl transferase - AC167, hydroxymethyl glutaryl-CoA lyase - AC282), membrane transport/synthesis/ degradation (anion exchange protein - AC19, ferric reductase-like transmembrane component - AC35, and lysophospholipase - AC50), degradation of proteins through the ubiquitination pathway (F-box protein - AC79) and possibly regulation of the cell cycle (phosphate-induced protein - AC325) (197). These eight fragments were up regulated, except AC50 and AC79, which were down regulated.

RT-PCR confirms the differential expression pattern for seven selected fragments. To verify the expression pattern, primers specific for seven of the sequenced fragments were designed and used for RT-PCR analysis. The proteins encoded by these fragments include the homologues to a nodulin-like

protein (AC168), a starvation-induced ribonuclease (AC178), a putative receptor kinase (AC181), a bacterial-induced peroxidase precursor (AC275), a lectin-like protein kinase (AC321), a phosphate-induced protein (AC325) and a PR protein NtPRp27 (AC365). These fragments were up regulated in the AFLP analysis, except AC181, which was down regulated. Control primers for constitutively expressed genes were also used in this analysis to normalize for amounts of starting cDNA. Degenerate primers for plant actins were used to clone an *Ageratum*-specific actin for which primers were designed. In addition, we isolated, sequenced and designed primers for a putative glycerol 3-phosphate dehydrogenase that experimentally showed constitutive expression pattern in the AFLP analysis. cDNAs from control cells and from cells treated with *Agrobacterium* for 48 hours were amplified by one of the seven gene-specific primers in a multiplex PCR reaction containing also primers for the constitutive genes, actin and putative glycerol 3-phosphate dehydrogenase (G3PD). Fragment AC321 represented a gene expressed at low levels and therefore only the control primers for G3PD were used, as G3PD was expressed at a lower level than actin (actin was saturated at the cDNA concentrations used in these reactions). The opposite was true for fragment AC325, which was expressed at high levels. Therefore, only actin was used as a control (G3PD expression was undetectable at the cDNA concentrations used in these reactions). We found that 30 cycles of PCR provided the sensitivity needed to detect the differences in expression for most fragments. However, 25 cycles were used for fragment AC325. The products were analyzed by agarose gel electrophoresis and the results are shown in Figure 4A. The analysis was repeated in four independent experiments (two of those are shown in Fig.4A), confirming the differential expression pattern for all seven fragments.

The specificity of the responses to *Agrobacterium*. The seven selected fragments were then further analyzed by RT-PCR to determine if their pattern of expression represented a response specific to *Agrobacterium* infection or if some of these transcripts, particularly the ones involved in defense response, could also be regulated by another type of stress, for example, exposure to non-pathogenic bacteria. We performed RT-PCR analysis with cDNA from plant cells exposed to *E. coli* cells for 48 hours. The results show that genes induced by *Agrobacterium* which are involved in defense or stress response, such as the starvation-induced ribonuclease (AC178), the bacterial-induced peroxidase precursor (AC275) and the PR protein NtPRp27 (AC365), are also induced by *E. coli* (Figure 4B). The gene encoding a receptor kinase homologue (AC181) is repressed by both bacteria. The lectin-like kinase gene (AC321) was induced reproducibly by *Agrobacterium* but in only one experiment by *E. coli* (Fig. 4B). This gene is expressed at a very low level and the non-reproducible behavior may reflect quantitative differences in mRNA accumulation between treatments. However, the gene encoding a nodulin-like protein (AC168) is induced by *Agrobacterium* but not by *E. coli.* Additionally, using AFLP-cDNA from plant cells exposed to *E. coli* for 48 hours, we analyzed expression of the 20 genes represented by the fragments listed in Table 2, confirming the RT-PCR results for the seven fragments listed above (at least one *Agrobacterium*-specific gene and five genes commonly regulated by *E. coli*). Among the remaining 13 fragments, at least three more (AC35, AC121 and AC274) appeared to be regulated by *Agrobacterium* but not by *E. coli* (data not shown).

DISCUSSION

To understand how plants respond to *Agrobacterium tumefaciens* infection, we have taken a broad approach and screened changes in gene expression in response to *Agrobacterium* as compared to mock-inoculated controls. Such

screen should identify possible responses, from pre-attachment phase, to cell attachment, to T-DNA transfer. To avoid plant responses induced by hormonal perturbations we employed an *Agrobacterium* strain whose modified T-DNA contained a reporter gene but lacked all wild-type T-DNA genes. Using the cDNA-AFLP technique we identified several cDNAs whose expression was altered. This study represents the first demonstration that plants can modulate their gene expression in response to *Agrobacterium* exposure and that *Agrobacterium* can trigger the plant defense machinery. Additionally, we showed that many of these cDNAs are also induced by a non-pathogen such as *E. coli*. However, some genes, such as one encoding a nodulin-like protein, can be uniquely regulated by *A. tumefaciens*. The identification of this gene as a nodulin suggests a possible commonality with the interaction between *Rhizobium* and legumes. The induction of common defense responses by *Agrobacterium,* which was somewhat unexpected given the non-inducing properties of its flagellin (73), suggests that *Agrobacterium* may have non-flagellin elicitors. Because defense responses were also induced by *E. coli*, which does not bind to the plant cell, we can conclude that the responses observed do not require binding and should involve a diffusible elicitor.

In the detection of changes in gene expression, the cDNA-AFLP technique has significant advantages over differential display (DD, (142)) and microarrays (202). Because longer primers allow the use of more stringent annealing conditions, cDNA-AFLP is more reproducible than DD. In contrast to microarray analysis, cDNA-AFLP can be performed in the absence of DNA sequence data. This allowed us to exploit the high transformation rate of *Ageratum*, which was not available in the plant model system *Arabidopsis*. Additionally, the cDNA-AFLP analysis requires small amounts of RNA when compared to the amounts needed for microarrays, and it also provides the opportunity to be automated with the use of fluorescent dyes in a manner similar to the microarray technology (38).

A high percentage of the differentially regulated cDNAs identified in this study has homology to genes commonly regulated during plant defense/stress responses, such as the gene encoding the pathogenesis-related (PR) protein from tobacco NtPRp27. PR proteins are markers of induced defense responses and some have a defined role in pathogenesis, such as degradation of the cell wall of microbes (225). Included in the class of defense response are proteins involved in biosynthesis of phenylpropanoids, such as a glucosyltransferase from *Arabidopsis* and a cytochrome P450 monooxygenase from *Pisum sativum*. Phenylpropanoids are secondary plant metabolites that can have antimicrobial activity and also serve in signaling and chemotaxis to both pathogenic and symbiotic microorganisms (177). Plant defense responses also include the synthesis of reactive oxygen species and the crosslinking of the cell wall, reactions that involve peroxidases (134). One of the fragments isolated is homologous to a bacterial-induced gene encoding a peroxidase precursor from *G. hirsutum*. Interestingly, *Agrobacterium* appears to have evolved ways to counteract the production of reactive oxygen species by the plant. Inactivation of an *Agrobacterium* catalase, which converts certain reactive oxygen species to non-toxic products, attenuates the ability of this mutant to cause tumors on plants (276). It is very likely that *Agrobacterium* evolved ways to cope with other plant defense responses, since it can infect and live in close association with a wide variety of plants.

Another fragment isolated in this study encodes a product similar to the receptor kinase Xa21, a disease resistance protein from rice. Interestingly, we found that this putative receptor kinase is repressed by *Agrobacterium* as well as by *E.coli* infection. Generally, disease resistance genes of the receptor kinase type are either constitutively expressed (32) or induced by the infection of the recognized pathogen (282), during an incompatible interaction leading to resistance. This difference observed during the interaction with *Agrobacterium* or

with *E. coli*, raises the possibility that surveillance receptors for specific pathogens may be suppressed during compatible or non-specific interactions.

We have identified one cDNA that is induced only by *Agrobacterium* and not by *E. coli* and another that may respond quantitatively more to *Agrobacterium*. They encode respectively products homologous to a nodulin-like protein and to a lectin-like protein kinase. Nodulins form a group of proteins induced in the root nodule of leguminous plants in response to *Rhizobium* infection or to oligosaccharides (nod factors) produced by *Rhizobium*. Even though the function of most nodulins is still unclear, some may be involved in cell division/differentiation and are thought to regulate the organogenesis of the nodule (146, 226). Lectins are carbohydrate-binding proteins whose function in plants is also unclear, although they have been implicated in cell-cell recognition processes. Kinases containing a lectin domain form a new class of kinases in plants, largely undescribed, but with potential important roles in cellular communication and in the transduction of oligosaccharides signals (104), perhaps including nod factors from rhizobia. The finding of these two cDNAs reflects the relatedness of *Agrobacterium* and *Rhizobium*, and suggests that these two bacteria may produce compounds that are recognized by plants in a similar manner, although the final outcome of the two interactions is strikingly different.

In conclusion, we have demonstrated altered gene regulation in *Ageratum conyzoides* plant cells in response to *Agrobacterium* infection. We have shown that *Agrobacterium* induced responses which are commonly triggered by abiotic stress and by non-pathogenic bacteria, as well as responses that appear unique to *Agrobacterium*, and perhaps shared with its close relative, *Rhizobium*. Differential screens that compare challenge by a non-pathogen to challenge by *Agrobacterium* should facilitate the identification of genes responding to *Agrobacterium*-specific stimuli, such as binding to plant cell walls and T-DNA

entry in the plant cell. *Agrobacterium*-specific genes can be further analyzed by comparing plant responses to wild-type *Agrobacterium* with the ones generated by exposure to avirulent mutants, defective at different steps of the interaction and transformation process. Such studies should further dissect the interaction of plant cells with this unusual plant pathogen.

MATERIALS AND METHODS

Plant and cell cultures conditions and treatments. *Ageratum conyzoides* cell cultures (118) were maintained in 100mL of an organic MS medium (Gibco BRL) containing 1μM NAA. The cultures were shaken at 140rpm at room temperature and 10mL were sub-cultured every 2 weeks. Tobacco BY-2 cell cultures (160) were maintained under the same conditions, except that 2,4-D (0.25μg/mL) was used as the growth regulator and 5mL of cells were sub-cultured every 2 weeks. The plant cells were inoculated with *Agrobacterium tumefaciens* 2 to 3 days after sub-culture. An overnight culture of *Agrobacterium* was transferred to induction broth (28) containing 100μM acetosyringone (AS), and the bacteria were induced for 16 hours, at 30°C. Just prior to inoculation, the cells were centrifuged and resuspended in MS medium to OD_{600} = 1.0. Each 100mL of plant cells received AS (100μM) and 1mL of the bacterial suspension. The mock-inoculated controls received AS and 1mL of MS medium. *Arabidopsis thaliana* (Columbia ecotype) leaf tissue was inoculated by vacuum infiltration as described (13), and root tissue was co-cultivated with *Agrobacterium* as described (4). The *Agrobacterium tumefaciens* strain EHA105 (pBISN1) used in this study is a non-oncogenic, hypervirulent strain (107), harboring a binary plasmid containing a GUS-intron construct that allows expression in plants but not in bacteria. The GUS gene is under the control of a "super-promoter" (164). Inoculation of *Ageratum* cells with *E. coli* DH5α was performed in the same manner as described for

Agrobacterium, except that the *E. coli* cells were grown in LB medium at 37°C without AS.

GUS colorimetric (histochemical) and fluorometric analysis. Plant cells or tissue were incubated at 37°C overnight in GUS-staining solution (0.5mg/mL of X-gluc, 10mM EDTA, 0.5mM ferricyanide, 0.5mM ferrocyanide, in 0.1M phosphate buffer, pH 7.0). GUS activity was detected by a fluorogenic assay, as described (112).

RNA extraction. Total RNA was extracted from *Ageratum* and BY-2 cells and from *Arabidopsis* tissues at different times after inoculation with *Agrobacterium* using the Trizol reagent (Gibco Life Technologies). The procedure was done according to instructions from the manufacturer with the following modifications for the plant cell suspensions: the RNA was precipitated in 0.2 volume of 1M acetic acid and 0.7 volume of 100% ethanol, at –20°C, overnight. The pellet was washed twice in 3M sodium acetate pH 5.5 and once in 70% ethanol before being resuspended in DEPC (diethyl pyrocarbonate)-treated water.

cDNA-AFLP procedure. mRNA was isolated from *Ageratum* total RNA using biotinylated oligo dT and streptavidin magnetic beads (PolyAtract IV, Promega). Sequence of adapters, and primers, cDNA synthesis, template preparation and analysis of the products by polyacrylamide gel electrophoresis were done as described (10), except that the pre-amplification cycle consisted of 1min at 94°C, 1min at 56°C and 2min at 72°C, and the *Taq*I primer instead of the *Ase*I primer was end-labeled with radioactivity. cDNA fragments were visualized by autoradiography after positionally marking gel and film.

Isolation and sequencing of fragments. The film and gel were aligned and the bands of interest were cut out from the gel with a razor blade. The gel slices were

then hydrated in 100μL of water and incubated at 95°C for 15min. The eluted cDNA was amplified with the same primers and under the same conditions as for the cDNA-AFLP analysis, except that the PCR cycle consisted of: 3min at 96°C; 30 cycles of: 1min at 94°C, 1min at 60°C, 1min 30sec at 72°C; and one final extension step of 10min at 72°C. The fragments were sequenced using the Big Dye Terminator technology (PE Applied Biosystems) and an automated sequencer, and their homology determined by comparison with the database using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and at TAIR (http://www.arabidopsis.org/ Blast/).

RT-PCR analyses. Equal amounts of RNA were treated with DNAse and cDNA was synthesized using reverse transcriptase and random hexamers. The cDNA was amplified in a multiplex PCR reaction containing primers specific for the gene of interest and primers for two constitutive control genes, actin (left primer: CAGCAACTGGGATGATATGG; right primer: ATTTCGCTTTCAGCAGTGGT) and putative glycerol 3-phosphate dehydrogenase (left primer: TCACCC ATATCAAGGCTCAG; right primer: GGGTACCTAATCGGGCAACT). For the GUS expression analysis, the control primers consisted of either cyclophilin (right primer: CACGACCTGCCCAAACAC; left primer: AAAACCCCTTCACTTCAA) or actin and the GUS primers consisted of the following: left primer: TATCAGCGCGAAGTCTTTATACC and right primer: CAGTTGCAACCACCTGTT GAT. The PCR cycle was described previously (except that for fragment number 325, the number of PCR cycles was 25 instead of 30) and the products were analyzed by agarose gel electrophoresis.

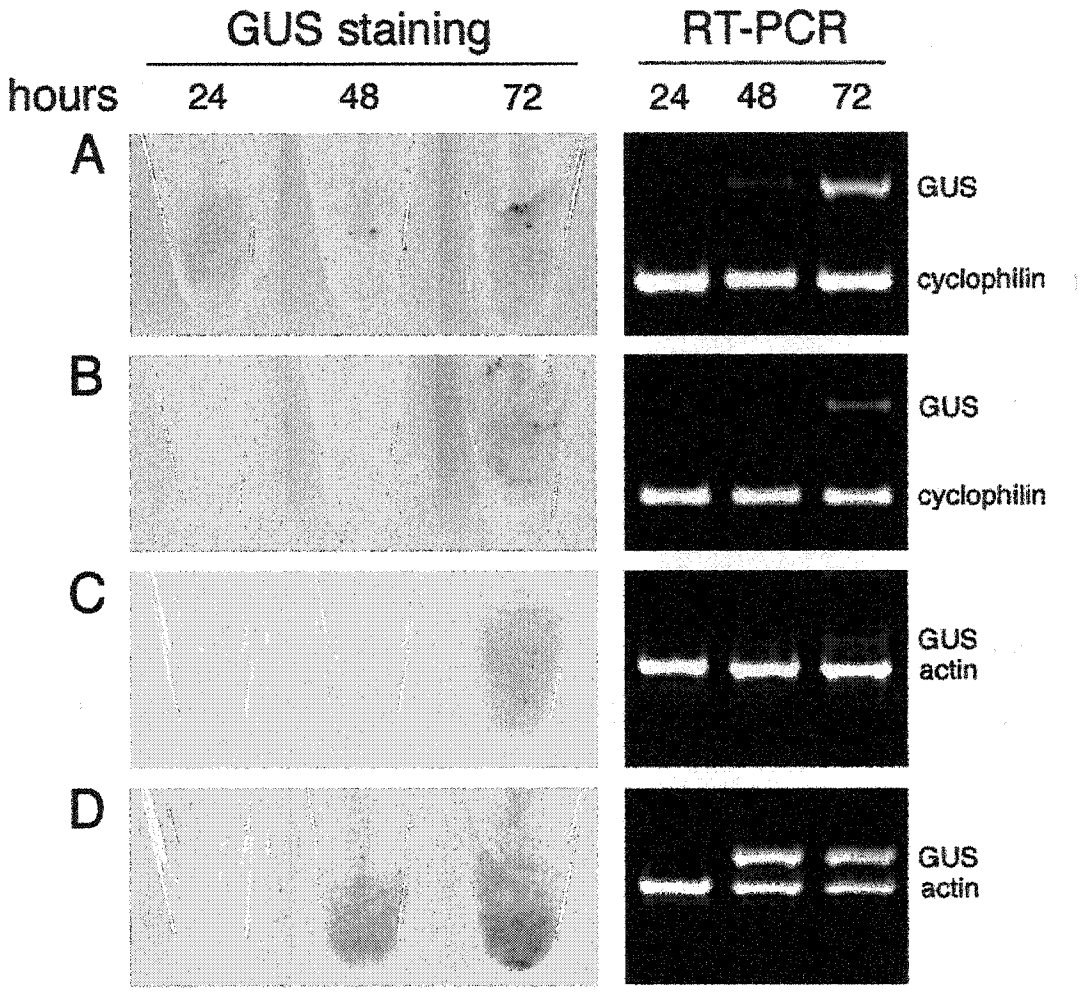

Figure II-1. GUS expression analysis. *Arabidopsis thaliana* root (A) and leaf tissue (B), tobacco BY-2 cells (C) and *Ageratum conyzoides* cells (D) at 24, 48 and 72 hours after inoculation with *Agrobacterium* containing a GUS-intron construct. Primers for constitutive control genes were used in the RT-PCR analysis: cyclophilin primers for *Arabidopsis* and actin primers for BY-2 and *Ageratum*.

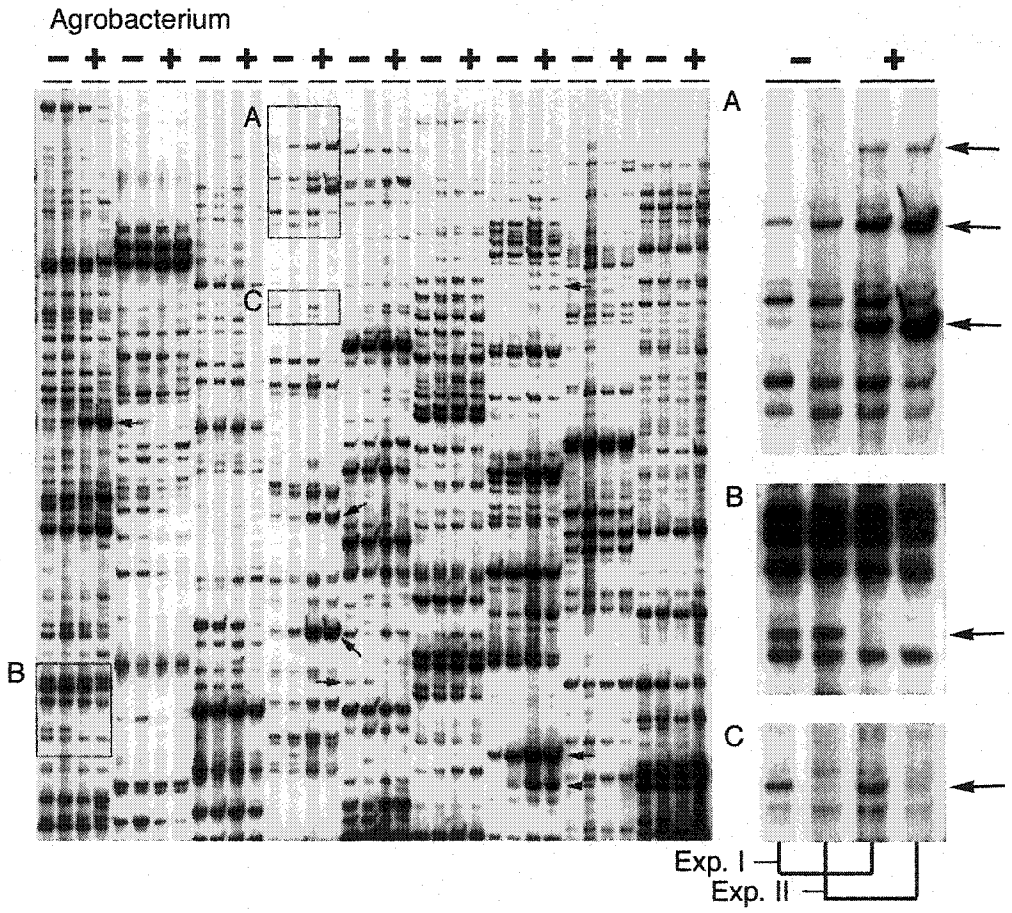

Figure II-2. *Ageratum conyzoides* cDNA-AFLP display after inoculation with *Agrobacterium* for 48 hours. *A. conyzoides* cDNAs from mock-inoculated (-) and *Agrobacterium*-inoculated (+) cells from two independent experiments (Exp. I and Exp. II) were amplified with different primer combinations. Each set of four lanes represents the amplification by one particular primer combination. Arrows indicate differential bands. (A) Enlarged view of box A with arrows indicating bands induced by the treatment. (B) Enlarged view of box B with arrow indicating a band repressed by the treatment. (C) Enlarged view of box C with arrow indicating a non-reproducible (experiment-specific) band.

Table II-1. Overall results of cDNA-AFLP analysis

I. Expression profile at 48 hours	**Number**	**%**
a. Bands or cDNA fragments displayed	16,000	100
b. Differential fragments	251	1.6
b.1. Up-regulated	179	1.1
b.2. Down-regulated	72	0.5
c. Non-reproducible ("noisy") fragments	165	1
II. Expression profile at 24 hours (75 selected fragments differentially regulated at 48 hours)		
a. Fragments tested	75	
b. Fragments regulated at 24 hours	56	
b.1. Up-regulated	39	
b.2. Down-regulated	17	
III. Sequence analysis of 56 fragments regulated at 24 and 48 hours		
a. DNA sequence data obtained	50	
b. Similarity with the database (Table 2)	20	

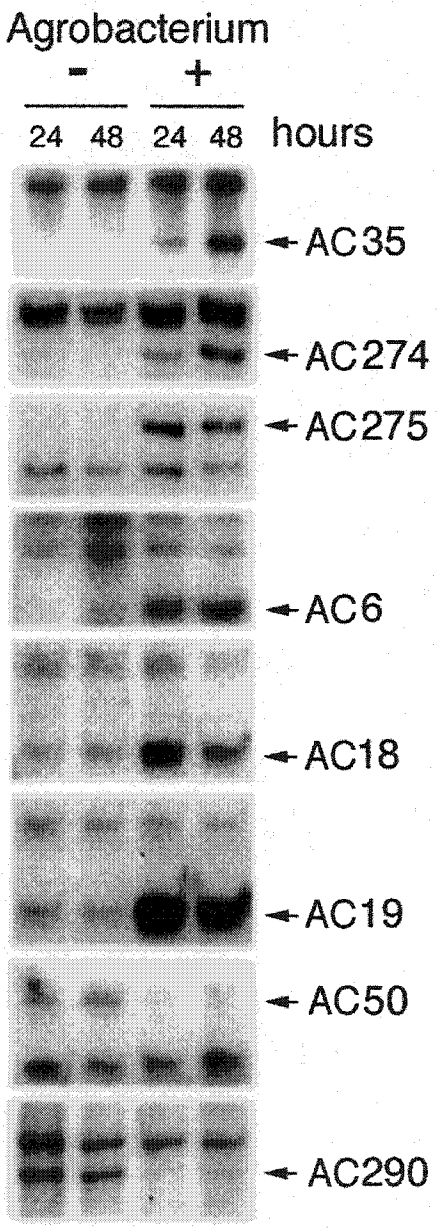

Figure II-3. cDNA-AFLP fragments regulated at 24 and 48 hours after inoculation with *Agrobacterium*. *A. conyzoides* cDNAs from mock-inoculated (-) and *Agrobacterium*-inoculated (+) cells were amplified with primer combinations known to result in differential fragments at the 48-hour time point. Differential fragments are indicated by their numbers, as listed on Table 2. Fragments that are induced (AC6, 18, 19, 35, 274 and 275) and repressed (AC50 and 290) at both time points are shown.

Table II-2. Homologies of AFLP fragments to sequences in the databases

AFLP Fragment	GeneBank Accession #	Length (bp)	Change	Homology[a]	BLASTX Score
AC18	BI397491	210	up	ZPT2-14 (zinc-finger protein) from Petunia x hybrida (AB006601)	2 e-11
AC19	BI397492	180	up	Putative anion exchange protein from *Arabidopsis* (AC007236)	1 e-19
AC35	BI397493	230	up	Hypothetical protein - ferric reductase-like transmembrane component (At1g01590)	4 e-9
AC50	BI397494	210	down	Lysophospholipase isolog from *Arabidopsis* (U95973)	8e-12
AC79	BI397495	260	down	Putative protein, similar to F-box protein Fb12 - *Homo sapiens* (AT5g01720). F-box proteins are involved in protein degradation (ubiquitination)	2e-17
AC95	BI397496	420	up	Member of the PFI0093 glyoxalase family from *Arabidopsis* (AC007591)	2e-15
AC121	BI397497	500	up	Putative glucosyltransferase from *Arabidopsis* (AC006248). Involved in phenylpropanoid metabolism, salicylic acid induced.	8e-34
AC167	BI397498	420	up	Unknown protein similar to glucosaminyl (N-acetyl) transferase - *Homo sapiens* (At1g71070)	2e-12
AC168	BI397499	180	up	Putative nodulin from *Oryza sativa* (AP002747)	1e-5
AC174	BI397500	230	up	Strong similarity to extracellular dermal glycoprotein (EDGP) precursor from *D. carota* (AC005278). Involved in response to wounding	5e-9
AC178	BI397501	400	up	Intracellular ribonuclease LX precursor - RNAseLX (P80196). Starvation induced	9e-37
AC181	BI397502	300	down	Hypothetical protein F23E13.70 - Putative receptor protein kinase from *Arabidopsis* (AL022141). Similar (4e-8) to Xa21, a receptor kinase disease resistance protein from rice.	2e-13
AC274	BI397503	300	up	Probable short chain alcohol dehydrogenase CPRD12, drought-inducible, from cowpea (D88121). S-locus specific stigma protein (SSP) from sporophytic self-incomp. *I. trifida*	2e-9
AC275	BI397504	270	up	Bacterial-induced peroxidase precursor from *Gossypium hirsutum* (AF155124)	2e-18
AC282	BI397505	300	up	Putative hydroxymethylglutaryl-CoA lyase from *Arabidopsis* (AC005168)	2e-21
AC290	BI397506	340	down	Cytochrome P450 monooxygenase, putative similar to cytochrome P450 from *Pisum sativum* (AT3g25180). Methyl jasmonate-inducible, involved in biosynthesis of phenylpropanoids	5e-4
AC315	BI397507	650	up	Elicitor inducible protein from *N. tabacum* (AB040410). Similar (4e-43) to potato wound-induced protein	8e-44
AC321	BI397508	260	up	Lectin-like protein kinase from *P. nigra* (AB030083)	2e-33
AC325	BI397509	290	up	Phi (phosphate induced)-1-like protein from *Arabidopsis* (AB008268)	7e-26
AC365	BI397510	200	up	NtPRp27 from *N. tabacum* (AB024600). Induced by virus, mechanical wounding and drought treatment	2e-24

a Database is non-redudant (all organisms), except fragments 35, 79, 167 and 290, for which the database is *Arabidopsis*.

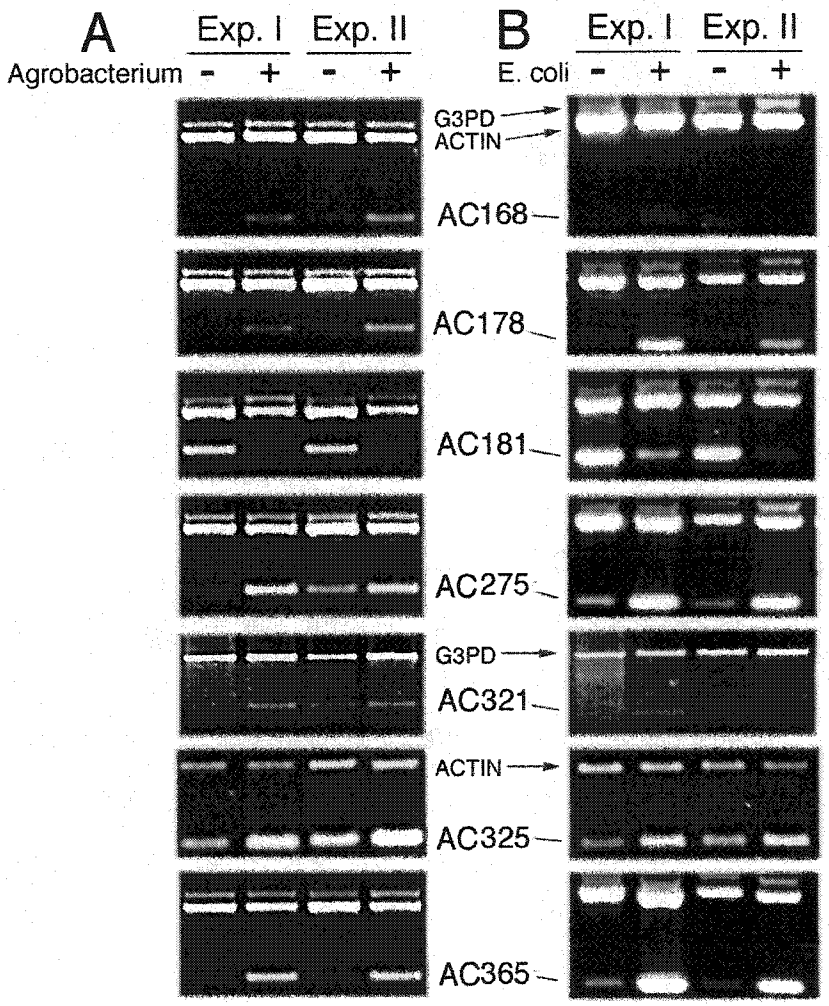

Figure II-4. RT-PCR analysis of seven cDNA-AFLP fragments. *A. conyzoides* cDNAs from mock-inoculated (-) and bacterial-inoculated (+, panel A and B) cells were amplified with primers specific for each fragment and for two control genes, actin (lower band) and putative glycerol 3-phosphate dehydrogenase (G3PD). Fragments AC321 and AC325 were amplified with G3PD alone and actin alone, respectively. **A.** One cDNA is repressed (AC181) and six cDNAs are induced (AC168, 178, 275, 321, 325 and 365) by *Agrobacterium*. The analysis was repeated in four independent experiments, of which two are shown. **B.** One cDNA is repressed (AC181), four cDNAs are induced (AC178, 275, 325 and 365), and two cDNAs are not altered by *E. coli*. The analysis was repeated in two independent experiments.

Chapter III

Further Analysis of Plant Responses to *Agrobacterium* Including the *Arabidopsis* Transcriptome

SUMMARY

We have previously identified changes in gene expression in *Ageratum conyzoides* plant cell cultures inoculated with transfer-competent *Agrobacterium tumefaciens* by using the cDNA-AFLP technique ((60) and Chapter II). Here we show that a subset of defense-related genes is differentially regulated by an *Agrobacterium* attachment-deficient mutant. We also observed that the final outcome of *Ageratum* transformation by transfer-competent *Agrobacterium* correlates with the expression status of these defense-related genes. In order to expand our analysis on *Ageratum* and identify global patterns of gene expression we started a comprehensive study using a cell culture from the model plant *Arabidopsis thaliana* and microarray technology. Because little is known about the timing of responses to *Agrobacterium*, we initiated our analysis by comparing mock-inoculated and *Agrobacterium*-inoculated *Arabidopsis* cells harvested at four time points after inoculation. From a pilot study with a single array, we confirmed the differential expression of four genes by RT-PCR. Their identity suggests similarities with genes characterized in our previous studies and with other defense-related genes. Later, a comprehensive microarray analysis with four biological replicates showed that statistically significant responses are observed at 48 hours after inoculation, but intriguingly, not at earlier time points.

INTRODUCTION

Plants apply both specific and general mechanisms to respond to microorganisms that live in close association with them. One of these microorganisms is *Agrobacterium tumefaciens*, a soil bacterium that is able to

infect a broad range of plants, introducing a piece of its Ti plasmid, the T-DNA, into the plant genome (167, 290). The expression of genes in the T-DNA leads to the synthesis of plant growth regulators, resulting in uncontrolled plant cell division and the crown gall disease (69). Studies on this process of genetic transformation contributed to fundamental discoveries of signaling exchange between hosts and pathogens, promoted research on plant molecular biology and genetics and have found practical applications in plant biotechnology. Under laboratory conditions *Agrobacterium* transformation can be extended to recalcitrant plants (105, 109, 110), a number of fungi and oomycetes (2, 24, 26, 35, 54, 91, 180, 183, 258) and even human cells (129). Therefore, the many experimental and biotechnological applications in plants will likely be transferable to a wide variety of other eukaryotes.

Despite the importance of understanding host responses to this natural genetic engineer, studies have traditionally concentrated on the bacterium. The processing and transfer of the T-DNA have been intensively studied and are mediated by a number of Ti plasmid-encoded virulence (Vir) proteins, some of which are transferred to the plant, in addition to the T-DNA (206, 256). A number of recent studies have revealed potential host factors explored by *Agrobacterium* in order to achieve transformation (79, 245). These host factors have been uncovered by identifying Vir protein-interactors (11, 12, 56, 207, 234, 246) or by genetic screens (163, 184, 292). However, we do not know how the transfer of Vir proteins and T-DNA specifically alters the expression of plant proteins. We also do not know the timing of plant responses to *Agrobacterium* and how this compares with responses to other pathogens, symbionts and general stresses.

In our previous studies, we have used the cDNA-AFLP technique to identify changes in gene expression in cell cultures of the plant *Ageratum conyzoides* upon infection with a transfer-competent strain of *Agrobacterium tumefaciens* (60). Several of the genes identified are related to plant defense

responses and we have showed that many of these genes are also regulated in response to a general stress caused by inoculation with a non-pathogen. However, a nodulin-like gene was regulated only by *Agrobacterium*, in line with the close evolutionary relationship between *Agrobacterium* and rhizobial symbionts (275). In that study we only compared a mock-inoculated control with cells inoculated with transfer-competent *Agrobacterium*. By using different strains and mutants affected in specific steps of the transformation process, one could determine the bacterial components which are responsible for changes in plant gene expression. Indeed, recently it was shown, by using different *Agrobacterium* strains, that the transfer of T-DNA and Vir proteins can modulate the expression of plant genes (255). Specifically, these authors propose that transfer of T-DNA or Vir proteins can suppress plant defense gene expression. Here we show that an attachment-deficient mutant hyper-induces a set of defense genes and we propose that *Agrobacterium* can suppress plant defenses in an attachment-dependent manner. The same set of plant defense genes was used to compare experiments that resulted in different transformation efficiencies. Plant cells that had higher expression levels of these genes were not transformed by *Agrobacterium*. We have only characterized genes from *Ageratum* which were regulated by a non-oncogenic strain at both 24 and 48 hours after inoculation. Here we use an *Arabidopsis* cell culture to take advantage of available microarray technology and expand this analysis to include additional time-points. We also use an oncogenic strain of *Agrobacterium* to gain insights into the responses of plants to a wild-type strain. A preliminary analysis of a single microarray experiment yielded four genes whose differential expression was confirmed by RT-PCR in independent replicated samples. Their sequence similarities support our previous findings. A more thorough microarray analysis using four independent replicates surprisingly revealed that statistically significant

responses are observed at 48 hours after inoculation, but not at earlier time points.

RESULTS

An attachment-deficient *Agrobacterium* mutant hyper-induces defense-related genes in *Ageratum conyzoides*. Three defense-related genes identified in our previous cDNA-AFLP analysis (60) were used in an RT-PCR analysis comparing *Ageratum* cells inoculated with different *Agrobacterium* strains. These genes were identified as differentially expressed in *Ageratum* cells inoculated with an *Agrobacterium* virulent strain, EHA105(pBISN1), and they encode a starvation-induced ribonuclease (GeneBank accession BI397501), a peroxidase (BI397504) and a pathogenesis-related (PR) protein (BI397510). We compared expression of these genes in plant cells inoculated with EHA105(pBISN1) and three avirulent *Agrobacterium* strains: *chvB* (an attachment-deficient mutant), A136 (a Ti plasmid cured strain) and LBA4404 (a T-DNA deleted strain). The results were repeated in two independent experiments. Figure III-1 shows that the genes were induced in a similar manner by EHA105(pBISN1), Ti plasmid cured, and T-DNA deleted strains. However, the attachment-deficient strain causes a hyper-induction of this set of defense-related genes. The avirulent strains A136 and LBA4404 are affected only in the Ti plasmid and retain chromosomally-encoded attachment functions. Therefore, the hyper-induction is uniquely observed for an avirulent mutant that is unable to attach to plant cells.

Defense gene expression effects on plant cell competence for transformation. We used the same set of defense-related genes described above to compare *Ageratum* cells that have been successfully transformed by EHA105(pBISN1) with cells that were not successfully transformed by the same strain. Transformation efficiency was assessed visually by detecting the

expression of the reporter GUS gene. Strong staining for GUS expression was observed at 48 hours after inoculation with *Agrobacterium* in successful experiments (60). Unsuccessful experiments did not show any sign of GUS expression at 48 hours, and even at later time-points in some cases (data not shown). The occurrence of these GUS-negative experiments was infrequent and we do not fully understand the reasons for these observations. Based on our results however, we propose that one of the causes may be a heightened state of resistance in plant cells. The results of an RT-PCR analysis in Figure III-2 show that the mock-inoculated control from a GUS-negative experiment expressed the defense-related genes at a higher level than the mock-inoculated control from a GUS-positive experiment. In fact, the mock-inoculated control from the GUS-negative experiment expressed these genes at the same levels observed for plant cells inoculated with *Agrobacterium*. The reasons for this heightened state of defense is unknown, but may have been caused by subtle changes in temperature or other cell culture conditions that we could not control. Whatever the reasons, it appears that this state resulted in resistance to *Agrobacterium* transformation.

An *Arabidopsis thaliana* cell culture is transformed by *Agrobacterium* with varying levels of efficiency. Because we wanted to expand our expression studies by using microarrays, we established an *Agrobacterium* transient transformation protocol in the model plant *Arabidopsis*. From our previous studies (60), we noted that transient transformation of *Arabidopsis* tissues was inefficient and inconsistent. When a cell culture of the *Arabidopsis* Ler ecotype (156) became available to us, we tested its ability to be transformed by *Agrobacterium* using the GUS gene as a reporter. As can be seen in Figure III-3, this cell culture can be transformed, although with different degrees of efficiency. In most cases, the *Arabidopsis* cell culture did not attain the same levels of

transformation observed for *Ageratum*, where high levels of GUS expression are normally observed at 2 days post inoculation (60). Only 13% of the transformation experiments conducted with *Arabidopsis* resulted in comparable GUS expression at 2 days after inoculation (Figure III-3 A). The majority of experiments resulted in strong (34%) and intermediate (21%) GUS expression only at 3 days after inoculation (Figure III-3 B and C). A considerable percentage (21%) of the experiments resulted in very low GUS expression, only weakly visible at 4 or more days after inoculation (Figure III-3 D). There was also a small percentage (11%) of experiments that did not result in any GUS expression, even at later times after inoculation (Figure III-3 E). It is important to note that the lower overall levels of *Arabidopsis* transformation, when compared to *Ageratum*, may be in part a consequence of a different *Agrobacterium* strain used. For our studies with *Ageratum*, we used a super-virulent strain (EHA105) while in *Arabidopsis* the wild-type strain A348 was used. However, even when using EHA105 to transform *Arabidopsis*, we could not obtain the same levels of transformation achieved in *Ageratum* (data not shown). The reason for utilizing strain A348 in our *Arabidopsis* studies is twofold: first, we wanted to identify plant responses to a "natural" *Agrobacterium* strain, as opposed to a heavily manipulated laboratory strain such as EHA105. Second, most mutants available in our laboratory collection were developed in the A348 background and because we are interested in later comparing plant responses to the wild-type with responses to different mutants, strain A348 was the best choice.

Microarray experimental design. A crucial aspect when planning a microarray experiment is the selection of an adequate design. Considering that microarray experiments are costly, time-consuming and generate large amounts of data, a careful design becomes essential to extract the most out of the data, with high reliability. Several experimental and practical aspects should be taken into

consideration when choosing the best type of design, such as number of experimental treatments, which samples will be compared, budget, etc. Due to the importance of the topic, a number of recent articles consider different options for design in detail (120, 121, 279). For our studies, we considered two options that seemed appropriate: a "loop" design (Figure III-4 A) and a "reference" design (Figure III-4 B). In the commonly used reference type of design, every RNA sample of interest is compared to a reference RNA. Therefore, a great advantage of this kind of design is that every sample of interest can be compared to each other indirectly because they are all compared to the reference directly (120). One disadvantage is that large amounts of data are collected on the reference sample, for which there is usually no scientific interest. The loop design overcomes this drawback by only comparing samples of interest, in a sequential manner. The loop is more efficient than the reference design because twice as many data are obtained for each sample, for the same number of arrays used. However, analysis of data from a loop design can be complicated. Moreover, the loop design is not robust or extensible, which means that loss or addition of samples can be problematic (120). On the other hand, the reference design allows both the loss of samples due to technical problems and addition of further treatments that may be of interest. Because of this higher flexibility, we opted for the reference type of design.

A preliminary microarray analysis reveals candidate differentially expressed genes. To initiate a microarray analysis of plant responses to *Agrobacterium*, we carried out a time-course experiment. *Arabidopsis* cell cultures were mock-inoculated (control) or inoculated with strain A348 and samples were collected at 4, 12, 24 and 48 hours after inoculation. These experiments were repeated four times independently and RNA was extracted from the cells. Strain A348(pBISN1) was used to monitor transformation

efficiency in each experiment. Samples were collected at 2, 3, 4 and 7 days after inoculation and stained for GUS expression. Only experiments that resulted in transformation levels equivalent to the ones presented in Figure III-3 A, B and C were used for further analysis. A reference RNA was obtained by pooling a large number of RNA extractions from *Arabidopsis* cells treated like 24h mock-inoculated controls. In order to validate our microarrays before proceeding into more extensive analysis, we initially only hybridized a mock-inoculated and an A348-inoculated sample collected at 24 hours after inoculation. Each sample was co-hybridized with the reference RNA and following washing and scanning, image analysis was performed to obtain log ratios of dye intensities. Genes with a log ratio higher than 1.5 (3-fold induction in mock or A348) were listed for both mock/reference and A348/reference comparisons. Features in common between the two lists represent genes induced in both mock- and A348-treated samples in relation to the reference and they were discarded. Table III-1 shows the list of genes possibly induced only in the A348-inoculated sample.

Four genes identified in the microarray have their differential expression confirmed. We designed primers for nine genes selected from Table III-1 and performed RT-PCR analysis in four independent experiments to confirm their differential expression. Control primers were designed for an *Arabidopsis* tubulin-2 gene whose expression did not change in these arrays. Out of the nine genes tested, four had their differential expression confirmed and these results are shown in Figure III-5. The other five genes were either false-positives or their differential expression could not be detected under our PCR conditions. A nodulin-like protein was clearly induced at both 24 and 48h after inoculation in experiment IV, but only at 48h in experiments I, II and III (Figure III-5). A lectin-like protein was expressed at very low levels and its differential expression was not discernable in experiments I, II and III, but it was clear in experiment IV. A

proline-rich extensin-like protein and a glycosyl hydrolase protein were clearly induced in all four experiments at 48h and in some cases also at 24h after inoculation. Interestingly, even though these genes were identified from a microarray experiment performed with 24h samples, their differential expression at this time point was inconsistent in the RT-PCR reactions. It is possible that the samples used for the microarray experiment had a faster response to *Agrobacterium* (similar to experiment IV in Figure III-5), in comparison with experiments I, II and III. Alternatively, the microarrays may have been more sensitive in detecting differential expression at 24h than the RT-PCR.

A thorough microarray analysis reveals differential expression at 48h after infection. In order to identify statistically significant and highly reproducible changes in gene expression over a time-course of *Agrobacterium* infection, we hybridized in microarrays four independently obtained RNAs from mock-inoculated and *Agrobacterium*-inoculated samples collected at 4, 12, 24 and 48h after inoculation. Surprisingly, using the SAM methodology (see Materials and Methods), the first three time-points yielded no significant changes when one considered the large number of genes being screened. For the last time-point (48h), strong evidence of differential expression was seen. With the SAM methodology, the analyst has the option to choose any significance threshold with which to make a list of promising genes. SAM further estimates the "False Discovery Rate" (FDR) associated with that list. The approach makes explicit the fact that there is no pre-determined cut-off on which to base a gene list. One can choose to make a shorter list of only the most significant genes, and have higher confidence that they are truly differentially expressed, or one can opt for a longer list of genes but with less confidence that they are all truly differentially expressed. Table III-2 shows the results from the 48h time-point analysis. If we choose a list with the 64 most significant genes, the estimated false discovery

rate is only 1.3%, i.e. less than 1 gene is expected to be a false positive. Table III-3 shows the fold-change and the identity for these 64 genes significantly regulated in *Agrobacerium*-inoculated plant cells. These genes were classified into functional categories using the gene ontology annotation search tool available at the *Arabidopsis* web page (http://www.arabidopsis.org/). Figure III-6 shows the classification according to Biological Process, Figure III-7 according to Molecular Function, and Figure III-8 according to Cellular Component.

DISCUSSION

To continue our studies on plant responses to *Agrobacterium tumefaciens*, we first used a set of defense genes identified in our cDNA-AFLP analysis to further dissect *Ageratum-Agrobacterium* interactions. Subsequently, we initiated a time-course analysis of *Arabidopsis* infection by *Agrobacterium* using microarrays. From our studies with *Ageratum* inoculated with different *Agrobacterium* mutants we observed that an attachment-defective mutant hyper-induces defense-related genes. This raises the intriguing possibility that *Agrobacterium* can dampen plant defenses in an attachment-dependent manner. Other transfer-deficient strains lacking T-DNA or Vir proteins, but attaching normally to plant cells, do not cause this hyper-induction. These findings contrast with recent results that indicate the transfer of T-DNA and Vir proteins can suppress plant defense-related proteins (255). It is possible that these discrepancies are due to the use of different plant species and the analysis of different plant transcripts. We used an attachment-deficient mutant, *chvB*, that is defective in the production of the exopolysaccharide (EPS) β-1,2 glucan (30). Interestingly, in *Rhizobium*, it has long been recognized that EPS-deficient mutants trigger plant defense responses that hinder the establishment of the symbiosis (75, 169, 179), and it has been proposed that EPS or a related compound can suppress plant defenses, allowing colonization. A similar mechanism could operate in the closely related

Agrobacterium, where attachment itself, an EPS compound, or both could suppress plant defense responses, allowing *Agrobacterium* colonization of a broad range of plants. However, this suppression is not complete, since we still observe induction of defenses by wild-type *Agrobacterium* and probably a balance between suppression and induction of defenses operates in nature. The plant defense system does appear to have a role in controlling infection by *Agrobacterium*, since we observed that *Ageratum* cells that express defense-related genes at higher levels cannot be efficiently transformed by *Agrobacerium*. Accordingly, *Arabidopsis* plants that constitutively express PR proteins have reduced susceptibility to *Agrobacterium* (Veena and S. B. Gelvin, unpublished). It would be interesting to compare at large-scale by microarrays, the pattern of expression of plant cells that were efficiently transformed with that of cells that were not transformed, to confirm our observations that an increased state of defense limits *Agrobacterium* infection. Additionally, other factors thought to be involved in plant cell competence for transformation, such as cell cycle-related components, could be identified by this approach.

Microarrays have become an increasingly powerful tool, allowing not only large-scale expression studies but also other applications such as profiling of DNA methylation (242), mapping of protein-DNA interactions (23) and genome annotation (277). In plant-pathogen interactions, a number of microarray expression studies have already identified similarities and distinctions between different defense pathways (203, 204, 235, 262) and have also revealed regulatory elements in co-regulated genes (154). A comprehensive analysis using microarrays, in particular an analysis of the timing of plant responses, has not been done for plant-*Agrobacterium* interactions. The results of such studies can be compared to other plant-pathogen interactions and stress-inducing conditions. Here we used an oligonucleotide array representing the complete transcriptome of *Arabidopsis* to identify genes regulated by infection with wild-

type *Agrobacterium*. Samples of *Agrobacterium*-inoculated and mock-inoculated cells were collected at 4, 12, 24 and 48 hours after inoculation and four completely independent experiments were done at separate times. Genes with statistically significant changes between mock and *Agrobacterium* treatments were identified at 48 hours after inoculation, but not at earlier time-points. It is likely that genes were regulated at earlier time-points but because of our stringent criteria for statistical significance, the use of four biological replicates, and taking into consideration the size of the array, these genes did not achieve high levels of significance and were below the level of detection for our analysis. It is also possible that *Arabidopsis* cells responded more slowly to *Agrobacterium* when compared to *Ageratum*, which can be implied by the faster transformation rate of *Ageratum* in relation to *Arabidopsis* cells. The results of our RT-PCR analysis with four genes identified from a pilot microarray experiment indeed confirm that the changes in *Arabidopsis* gene expression at 24h after inoculation are less pronounced and less reproducible when compared to the changes observed at 48h. A gene coding for a beta-fructosidase/ cell wall invertase significantly altered at 48h is also present in our list of candidate genes altered at 24h from the pilot study, confirming its significance. Other genes identified in our pilot study, such as the one encoding a nodulin-like protein, showed a clear trend of differential expression in the statistical analysis but due to the unreproducibility in one of the four replicates (data not shown), they fall below our level of significance and therefore are not present in the list of statistically significant alterations at 48h. Even though we did not attempt to confirm its differential expression, it is interesting that an expressed protein similar to an arabinogalactan protein was detected in our pilot study. An *Arabidopsis* mutant that is resistant to *Agrobacterium* transformation (*rat1*) has an insertion in the promoter region of a gene encoding an arabinogalactan protein (292). These authors report that *Agrobacterium* does not attach to roots of this *Arabidopsis*

mutant, indicating the importance of proteins involved in cell wall structure during plant-*Agrobacterium* interactions.

In general, genes belonging to the same classes, such as cell wall-related, defense-related, secondary metabolism, carbohydrate metabolism and signal transduction are observed in our studies with both *Ageratum* and *Arabidopsis*. Also, several of the regulated genes have an unknown function. For example, by classifying the 64 genes significantly altered at 48h into functional categories, we observed that a high percentage of these genes have unknown molecular functions and are involved in unknown biological processes. But also, as expected, a high percentage of these genes were classified as being involved in responses to stress and to biotic and abiotic stimulus.

The use of a cell culture of *Arabidopsis thaliana* facilitated the present study. The validity of this system can be exemplified by the identification of a gene (coding for a lysine and histidine transporter) induced by *Agrobacterium* at 48h after inoculation which was also identified in our previous cDNA-AFLP studies as induced by different *Agrobacterium* strains in *Arabidopsis* root tissue (our unpublished observations).

It will be interesting in the future to compare microarray expression profiles of plants inoculated with different pathogens and non-pathogens, to identify genes commonly and uniquely regulated by *Agrobacterium*. In addition, inoculation with different *Agrobacterium* mutants can reveal the bacterial components responsible for changes in plant gene expression and help elucidate the mechanisms of this intriguing plant-microbe interaction.

MATERIALS AND METHODS

Cell cultures conditions and treatments. *Ageratum conyzoides* cell cultures (118) were maintained as described (60). An *Arabidopsis thaliana* cell culture (156) was a kind gift of Cayle Lisenbee (Mayo Clinic, Scottsdale, AZ). These

cells were maintained in 100mL of MS complete medium (M9500 US Biological) containing 4.2mg/L of NAA and 0.02mg/L of kinetin. The cultures were shaken at 140rpm at 23°C and 10mL were sub-cultured every week. Plant cells were inoculated with bacteria 3 days after sub-culture. An overnight culture of bacteria grown at 29°C was centrifuged and resuspended in MS medium to OD_{600} = 1.0. Each 100mL culture of plant cells received acetosyringone (AS, 200μM) and 1mL of the bacterial suspension. The mock-inoculated controls received AS and 1mL of MS medium. The *Agrobacterium tumefaciens* strain A348 is a wild-type oncogenic strain. The non-oncogenic strain EHA105 has the binary plasmid pBISN1 containing the GUS gene (164). *Agrobacterium* mutants used in this study belong to the Nester lab collection. Four independent inoculation experiments were performed at separate times and used as biological replicates for the microarray analysis.

GUS colorimetric (histochemical) analysis. Plant cells were incubated at 37°C for at least 24 hours in GUS-staining solution (0.5mg/mL of X-gluc, 10mM EDTA, 0.5mM ferricyanide, 0.5mM ferrocyanide, in 0.1M phosphate buffer, pH 7.0).

RNA extraction. Total RNA was extracted from *Ageratum* cells as described before (60). Total RNA was extracted from *Arabidopsis* cells using the Trizol reagent (Gibco Life Technologies), according to instructions from the manufacturer for plant material containing high levels of polysaccharides.

Preparation of samples for microarray hybridization. Labeled cDNA samples were prepared using 50μg of *Arabidopsis* total RNA in reverse transcriptase reactions and subsequently coupled to either Cyanine-3 (Cy3) or Cyanine-5 (Cy5) fluorophores using an established amino-allyl coupling procedure (72).

Cy3- and Cy5-labeled cDNA samples were combined and co-hybridized to the array.

Microarray slide preparation and hybridization conditions. Microarrays were constructed employing a set of 26,090 70-mer oligo probes commercially available from Qiagen (Alameda, CA). Each oligo was designed to probe for a unique coding region in the *Arabidopsis thaliana* genome. Oligo probes were individually spotted onto poly-lysine coated microscope slides using an OmniGrid 300 high-precision robotic gridder (GeneMachines, San Carlo, CA). Each printed array underwent a series of post-processing steps prior to use. Briefly, they were humidified and snap-dried on a 100°C heat block, UV cross-linked at 450 mJ, and blocked with succinic anhydride and sodium borate in 1-methyl-2-pyrrolidinone. Arrays were subsequently washed twice in deionized water, rinsed with 100% ethanol and immediately spun-dried in a centrifuge at 500 rpm. Arrays were stored under vacuum and screened for imaging artifacts prior to use. Arrays were pre-hybridized in a 5x SSC solution of 3% powdered-milk at 42°C for one hour, then washed in H_2O, propanol, and centrifuged dry. Arrays were hybridized on a GeneTAC Hyb Station for 16 hours at 42°C, followed by washings at 42°C for five cycles in 2X SSC and three cycles in 0.1X SSC, and then centrifuged dry. Arrays were scanned using a GenePix 4000 scanner (Axon Instruments, Union City, CA). Image analysis was performed using GenePix Pro 3.0.

Microarray data analysis. The time-course data from four replicates were normalized simultaneously for systematic spatial and intensity-dependent variation (49). Each array was normalized separately. The SAM methodology (244), as implemented in the Bioconductor package "siggenes" (www.bioconductor.org), was used to identify genes with strong evidence for differential expression between *Agrobacterium* and mock treatment at each

timepoint. The SAM procedure relies on a permutation test and accounts for the large number of genes being studied in assessing statistical significance.

RT-PCR analyses. Equal amounts of RNA were treated with DNAse and cDNA was synthesized using reverse transcriptase and random hexamers. The cDNA was amplified in a multiplex PCR reaction containing primers specific for the gene of interest and primers for constitutive control genes: glycerol 3-phosphate dehydrogenase for *Ageratum* (left primer: TCACCC ATATCAAGGCTCAG; right primer: GGGTACCTAATCGGGCAACT) and Tubulin-2 for *Arabidopsis* (left primer: CAACTCTGACCTCCGAAAGC; right primer: TGTGAATTCCATCTCGTCCA). The PCR cycle consisted of: 3min at 96°C; 30 cycles of: 1min at 94°C, 1min at 60°C, 1min 30sec at 72°C; and one final extension step of 10min at 72°C.

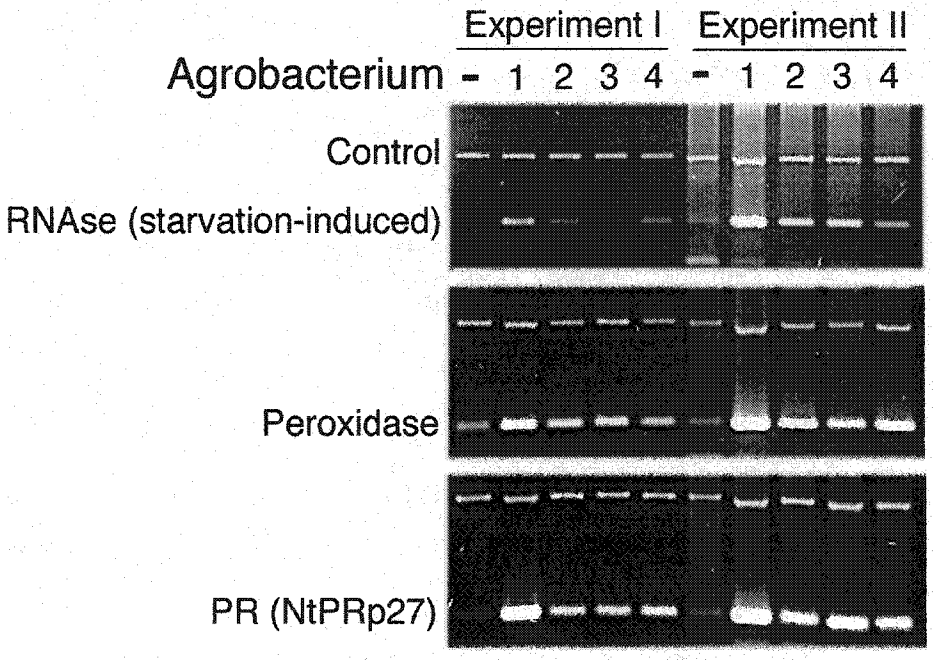

Figure III-1. RT-PCR analysis of *Ageratum* defense gene expression upon treatment with different *Agrobacterium* strains for 48 hours. 1. Attachment-deficient *chvB* mutant; 2. Ti plasmid cured A136; 3. T-DNA deletion mutant LBA4404; and 4. Virulent EHA105 (pBISN1).

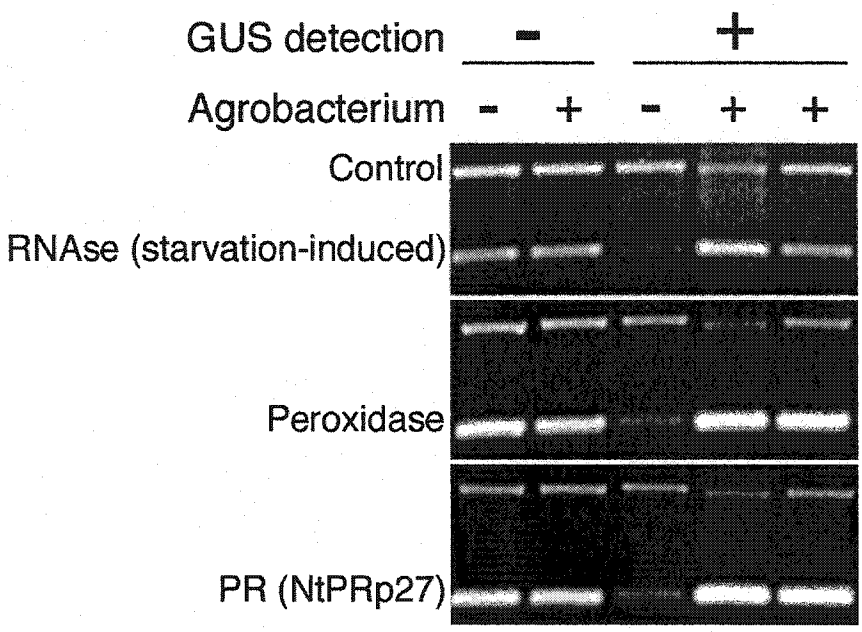

Figure III-2. RT-PCR analysis of *Ageratum* defense gene expression in GUS-negative and GUS-positive experiments. RNA extraction was performed in samples collected at 48 hours after infection.

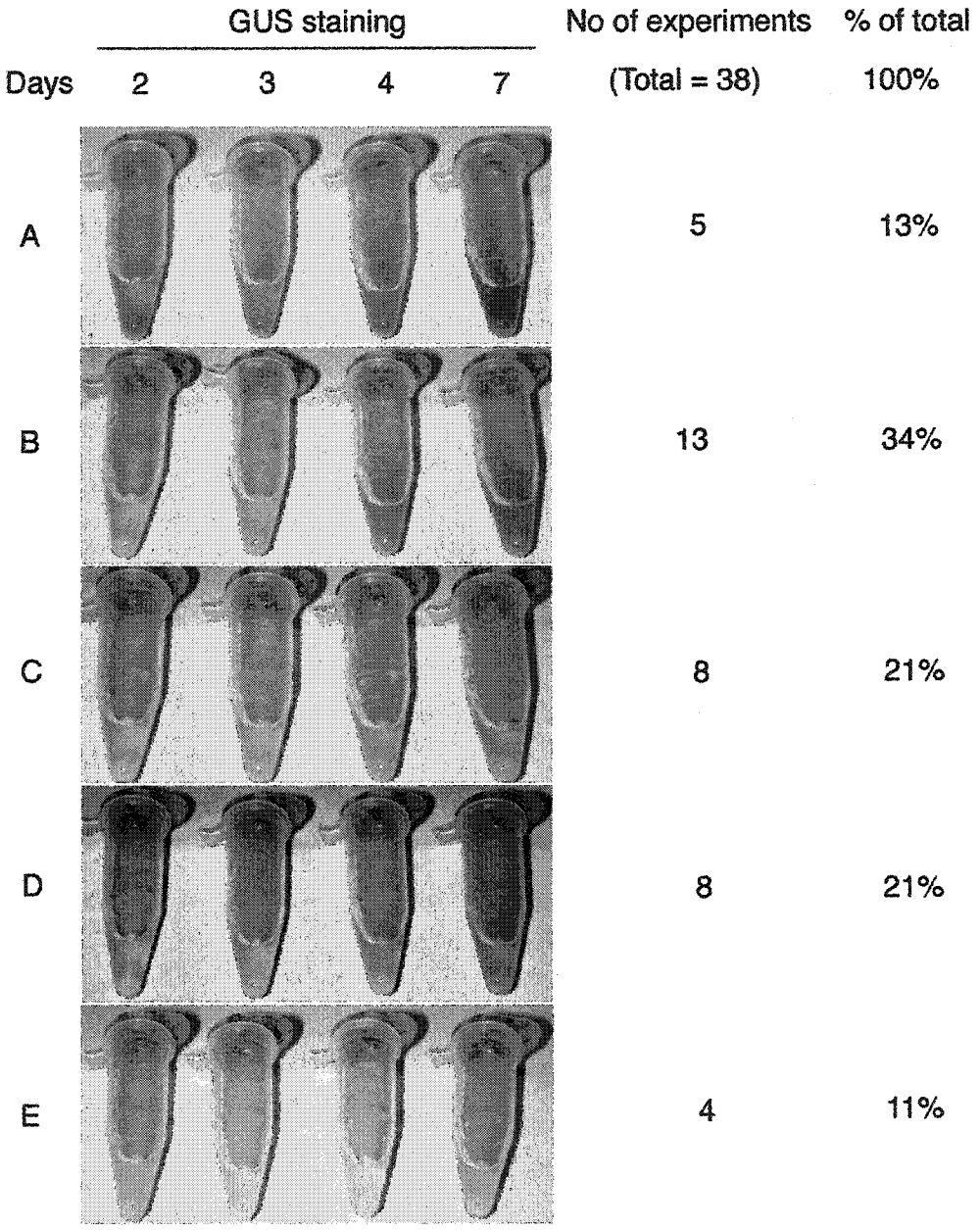

Figure III-3. GUS expression analysis in *Arabidopsis thaliana* cell cultures. Samples were collected at 2, 3, 4 and 7 days after inoculation with *Agrobacterium tumefaciens* strain A348(pBISN1) and stained for GUS expression. A total of 38 inoculation experiments were conducted and the number (and %) of experiments in each category of transformation efficiency (A through E, more to less efficient) are shown.

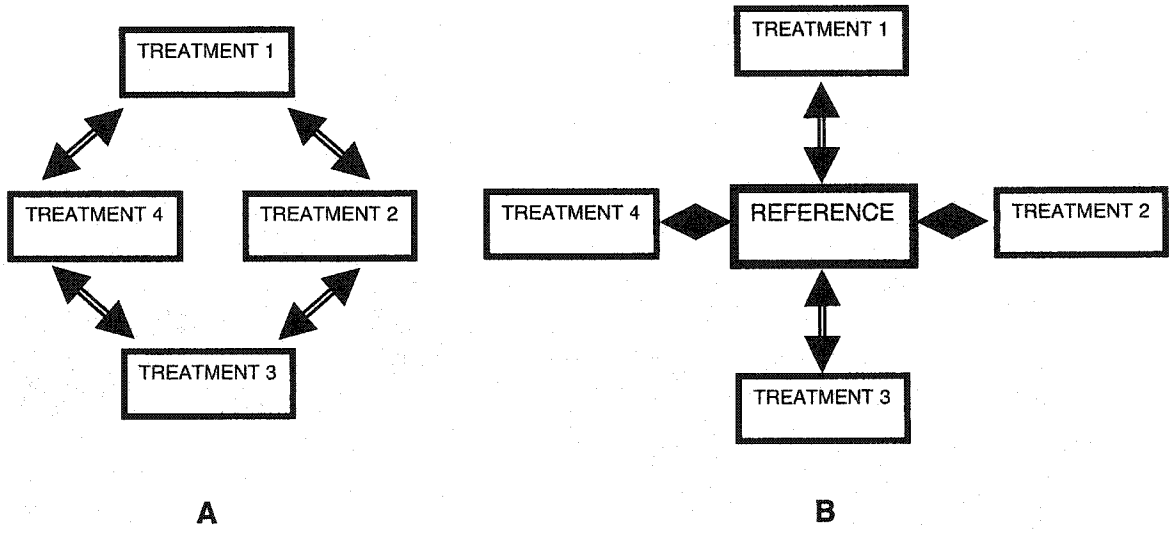

Figure III-4. Microarray experimental design. A. The loop design compares treatments sequentially. B. In the reference design, treatments are each compared to a reference RNA.

Table III-1. Genes putatively induced in *Arabidopsis* cells inoculated with A348 for 24h

Locus	Log Ratio	Description	Confirmed by RT-PCR
At2g25240	2.804	Probable serpin (serine protease inhibitor)	-
At5g45400	2.486	Putative replication protein	n/a
At3g15356	2.479	Lectin-like protein	+
At1g65840	1.903	Putative protein kinase	n/a
At1g76930	1.773	Proline-rich extensin-like family protein	+
At3g13790	1.749	Beta-fructosidase (BFRUCT1) / cell wall invertase	n/a
At3g57810	1.718	Hypothetical protein, similar to OTU-like cysteine protease	n/a
At3g10690	1.704	DNA gyrase subunit A family protein	-
At3g29220	1.701	Unnamed protein product, contains similarity to copia polyprotein	n/a
At1g71380	1.699	Putative beta-glucanase; glycosyl hydrolase family 9 protein	-
At5g26220	1.672	Hypothetical protein; contains similarity to E. coli cation transport protein ChaC	n/a
At1g03820	1.665	Expressed protein, similar to arabinogalactan-protein	n/a
At2g25790	1.649	Putative leucine-rich repeat transmembrane protein kinase	-
At2g10090	1.623	Hypothetical protein, probable retroelement pol polyprotein	n/a
At2g44460	1.605	Probable beta-glucosidase; glycosyl hydrolase family 1 protein	+
At5g04870	1.566	Calcium-dependent protein kinase isoform AK1	-
At4g08850	1.559	Receptor protein kinase-like protein	n/a
At1g04680	1.556	Pectate lyase family protein	n/a
At5g22930	1.537	Hypothetical protein	n/a
At2g39210	1.522	Nodulin family protein, similar to nodule-specific protein Nlj70 (Lotus japonicus)	+
At3g28390	1.504	Putative multidrug resistance p-glycoprotein; ABC transporter-like protein	n/a

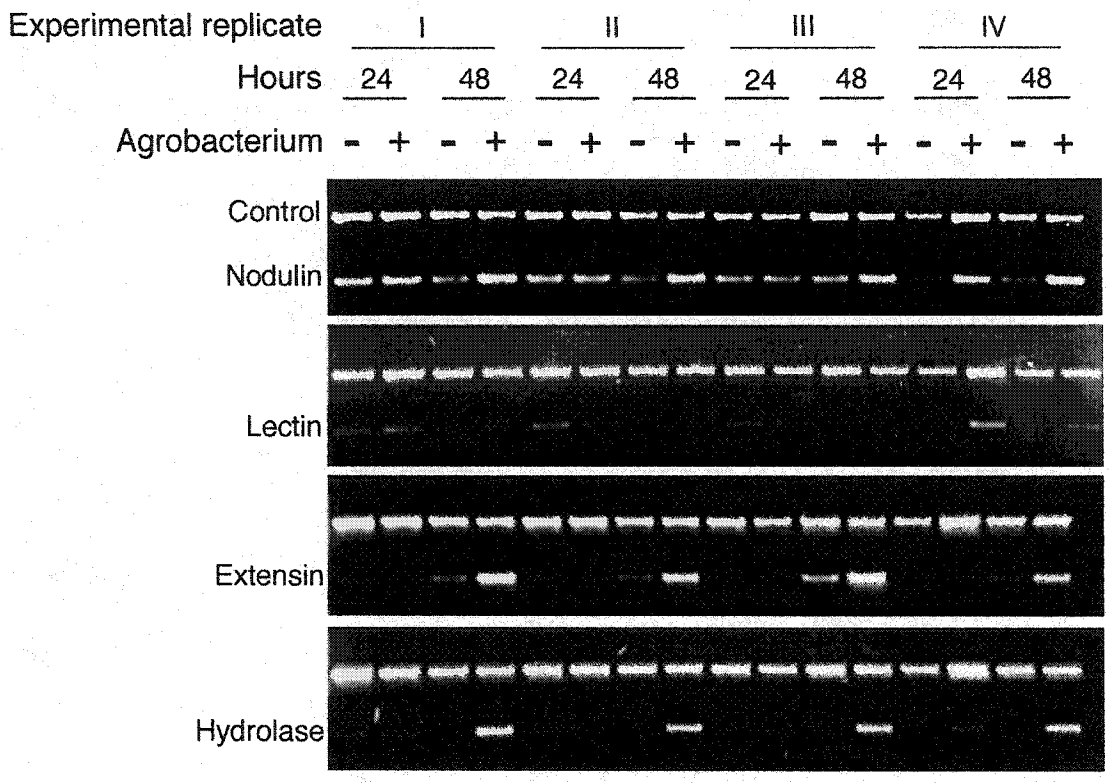

Figure III-5. RT-PCR analysis of four *Arabidopsis* genes identified by microarray. Primers were designed for a control gene encoding tubulin-2 and for genes encoding a nodulin-like, a lectin-like, a proline-rich extensin and a glycosyl hydrolase protein, identified by microarray as putatively induced by *Agrobacterium* at 24h. The analysis was carried with mock-inoculated and *Agrobacterium*-inoculated samples collected at 24 an 48h after inoculation in four independent experiments.

Table III-2. Results of SAM analysis for genes differentially expressed by *Agrobacterium* at 48h after inoculation		
Length of Gene List	Estimated Number of False Calls Under Global Null	Estimated FDR
1061	268	21.2%
418	39	7.8%
180	6	2.8%
105	3	2.4%
64	1	1.3%
58	0	0.0%

Table III-3. Genes significantly regulated in *Arabidopsis* cells at 48h after inoculation with *Agrobacterium* A348

Locus	Fold change	Description
At2g06850	0.2413	Xyloglucan endo-1,4-beta-D-glucanase
At4g08685	0.5644	SAH7 protein, pollen allergen-like protein
At2g46890	0.6001	Hypothetical protein
At2g31870	1.281	Probable poly(ADP-ribose) glycohydrolase
At1g62800	1.3302	Aspartate transaminase
At2g43620	1.339	Probable endochitinase
At2g17950	1.3395	Probable homeodomain transcription factor (WUS)
At5g40590	1.3431	DC1 domain-containing protein
At2g01905	1.3686	Cyclin J18
At1g67920	1.4512	Unknown protein
At2g31570	1.4587	Probable glutathione peroxidase
At1g17190	1.4618	Probable glutathione transferase
At4g23260	1.4643	Receptor-like protein kinase
At5g62070	1.4826	Calmodulin-binding family protein
At2g17220	1.5026	Putative protein kinase
At1g34510	1.5187	Putative peroxidase
At3g01570	1.5272	Putative oleosin, glycine-rich protein
At5g38900	1.546	FrnE protein-like
At1g79180	1.581	Myb-related protein
At1g30730	1.5876	Probable berberine bridge enzyme
At2g26560	1.5924	Patatin-like
At1g65295	1.6042	Unnamed protein product
At3g19930	1.6095	Monosaccharide transport protein STP4
At2g16720	1.6141	Probable MYB family transcription factor
At4g38540	1.6213	Monooxygenase
At5g01210	1.6341	Anthranilate N-benzoyltransferase-like protein
At1g69850	1.637	Nitrate transporter (NTL1)
At5g43520	1.6551	DC1 domain-containing protein
At1g11820	1.674	Glycosyl hydrolase family 17 protein, similar to elicitor inducible chitinase Nt-SubE76
At2g28270	1.6802	DC1 domain-containing protein
At1g48640	1.6874	Putative lysine and histidine specific transporter
At3g46250	1.7018	Hypothetical protein, similar to receptor protein kinase
At1g51810	1.7741	Probable protein kinase
At1g78340	1.8074	Similar to glutathione transferase
At3g43190	1.8318	Sucrose synthase-like protein
At5g39580	1.8961	Myb-related transcription factor
At2g22880	1.9059	VQ motif-containing protein
At5g19440	1.9128	Putative cinnamyl-alcohol dehydrogenase
At3g44780	1.9775	Hypothetical protein
At1g52200	2.0177	Unknown protein
At3g03960	2.0451	Chaperonin, putative T-complex protein 1, theta subunit; TCP-1-Theta
At3g58890	2.0491	Syntaxin-related
At2g44370	2.1997	DC1 domain-containing protein
At3g21240	2.2637	4-coumarate:CoA ligase 2
At4g02380	2.3452	LEA3 family protein. Hypothetical protein, similar to small proteins induced by heat, auxin, ethylene and wounding

Table III-3 continued

At1g32100	2.3459	Pinoresinol-lariciresinol reductase, isoflavone reductase domain
At3g23250	2.5091	Glutathione S-transferase
At1g78380	2.5482	Glutathione transferase
At3g01290	2.6442	Unknown protein, similar to hypersensitive-induced response protein
At3g13790	2.7691	Beta-fructosidase (BFRUCT1) / cell wall invertase
At4g26220	2.8024	Putative caffeoyl-CoA O-methyltransferase
At4g15610	2.8772	Unknown protein
At1g48850	3.1803	Putative chorismate synthase
At5g40780	3.238	Lysine and histidine specific transporter
At5g42830	3.5958	Proanthranilate N-benzoyltransferase-like protein
At1g14870	3.6201	Unknown protein
At5g36925	3.6963	Unnamed protein
At2g43120	3.8309	Probable pirin
At5g24760	3.9657	Alcohol dehydrogenase
At5g39580	4.0156	Peroxidase ATP24a
At5g64120	4.157	Peroxidase
At5g36920	5.0291	Unnamed protein product
At2g30490	6.6359	Cinnamate-4-hydroxylase, cytochrome P450 73 (CYP73)
At4g34050	6.8724	Probable caffeoyl-CoA O-methyltransferase

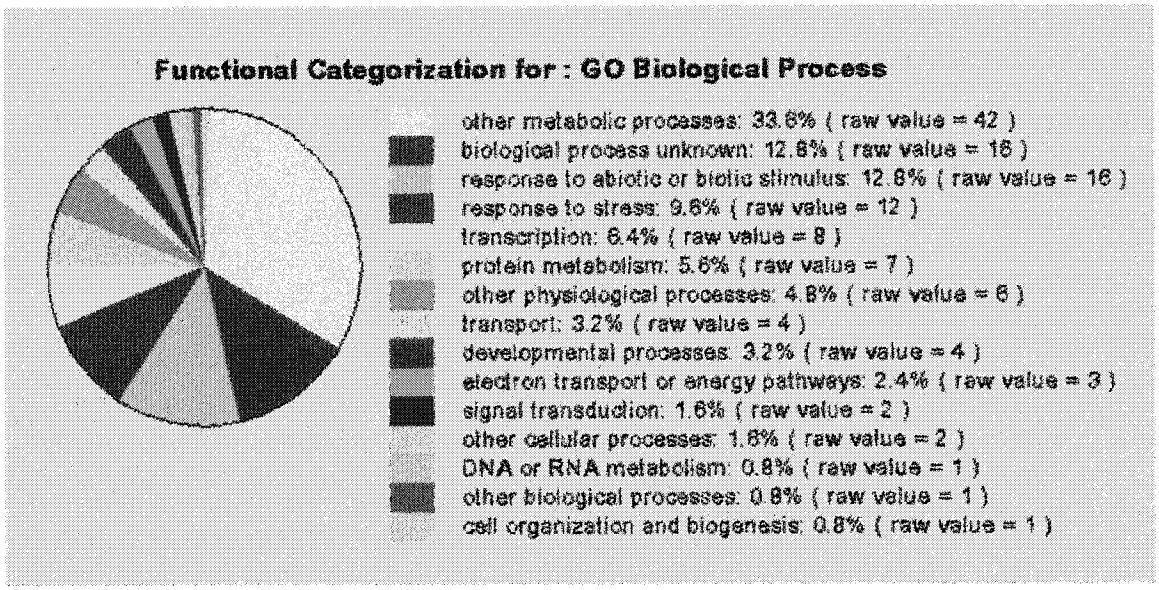

Figure III-6. Functional categorization of genes listed on Table III-3 according to gene ontology (GO) for biological process.

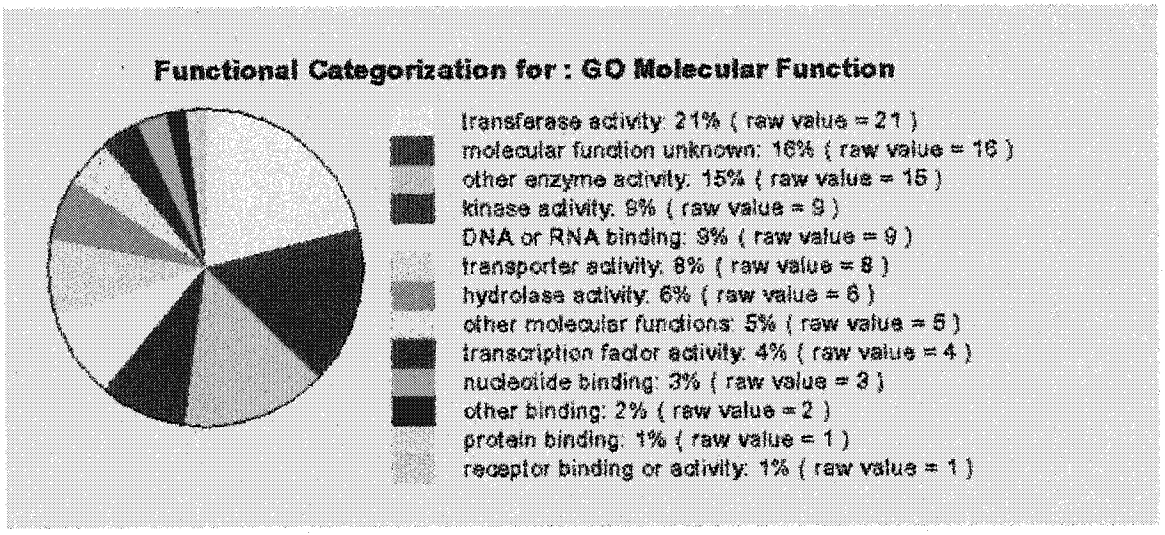

Figure III-7. Functional categorization of genes listed on Table III-3 according to gene ontology (GO) for molecular function.

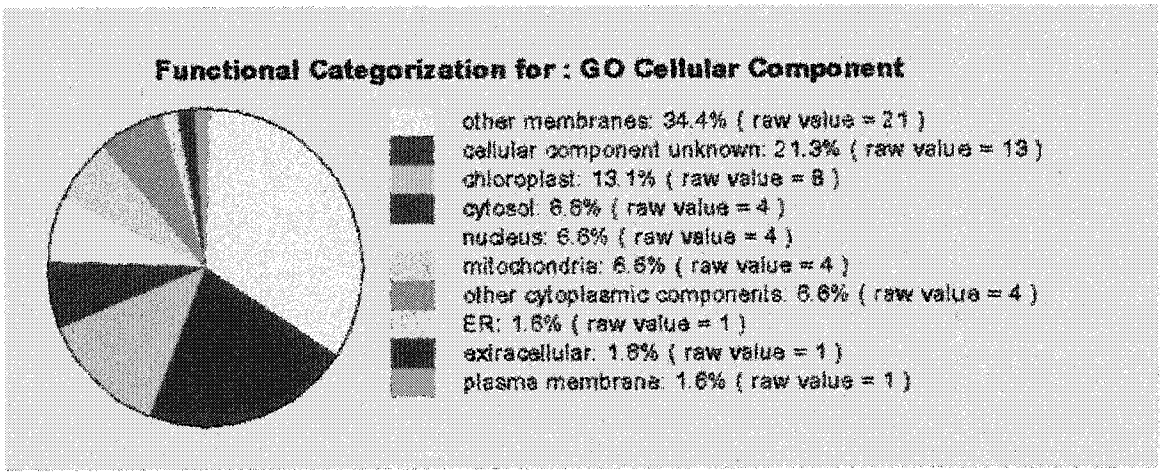

Figure III-8. Functional categorization of genes listed on Table III-3 according to gene ontology (GO) for cellular component.

Chapter IV
Conclusions and Future Directions

In conclusion, we have shown differential expression in response to *Agrobacterium* inoculation in two different plant species, *Arabidopsis thaliana* and *Ageratum conyzoides*, and by using two different technologies, the cDNA-AFLP and microarrays.

In Chapter I we reviewed innate immunity in plants and animals, described plant responses to beneficial and detrimental microbes and reviewed the virulence system of *Agrobacterium tumefaciens*. We can conclude that *Agrobacterium* has a unique way of infecting plants and that it can be considered a near-symbiont: it is a close relative of symbiotic bacteria; it infects a broad range of plant species without causing extremely detrimental effects; it does not trigger a hypersensitive defense response in plants, even though it introduces proteins and DNA into the plant cell; and it has evolved ways to avoid detection by modifying its general elicitor flagellin. Despite these intriguing aspects and the importance of this bacterium in genetic modification of organisms, only recently studies have been aimed at understanding host responses to *Agrobacterium*.

In Chapter II we have shown that the plant defense system is indeed triggered by inoculation with *Agrobacterium* and that some of the same responses are also triggered by the general stress of exposing plant cells to a non-pathogen, indicating that *Agrobacterium* possess general elicitors other than flagellin. However, it is likely that *Agrobacterium* can overcome defense responses: one of the induced defense responses we identified was related to the production of reactive oxygen species (ROS) but *Agrobacterium* possess an enzyme important for its virulence that can convert ROS into nontoxic products (276). We also identified at least one gene, encoding for a nodulin-like protein, which is induced by *Agrobacterium* but not by non-pathogenic *E. coli*. The

identity of the gene suggests that the close relatives *Agrobacterium* and *Rhizobium* may produce compounds that are recognized by plants in a similar manner.

In Chapter III we have shown that an *Agrobacterium* attachment-defective mutant can hyper-induce defense responses and therefore we propose that *Agrobacterium* can suppress plant defenses by an attachment-dependent mechanism. Because this mutant is defective in the production of certain exopolysaccharides (EPS) and *Rhizobium* EPS-deficient mutants also hyper-induce plant defense responses and are unable to establish the symbiosis (75, 169, 179), we believe that a similar mechanism may operate in *Agrobacterium*, where EPS, attachment itself, or both could suppress plant defenses. Even though *Agrobacterium* appears to be able to avoid, overcome or suppress defenses, it is likely that the plant defense system is still important in controlling the infection since we observed that plant cells expressing higher levels of defense genes are resistant to *Agrobacterium* infection. Using microarrays, we confirmed our observations that a general defense response is triggered by *Agrobacterium*. For example, several peroxidases and other defense-related genes were significantly induced by *Agrobacterium* at 48h after inoculation. We also conclude that several aspects of the *Agrobacterium*-plant interaction are not yet understood, as indicated by the high percentage of genes identified by microarray that have an unknown role. These unknown genes represent an opportunity to learn more about plant gene function and the responses to *Agrobacterium*. For example, databases of gene expression could be surveyed in order to identify what other conditions can result in regulation of these genes.

The microarray system that we developed can be used as a powerful tool to dissect even further the intriguing interaction between plants and *Agrobacterium*. By comparing the gene expression pattern of plants inoculated with *Agrobacterium* and plants inoculated with other bacteria, such as non-

pathogenic *E. coli* and pathogenic *Pseudomonas syringae*, we can define which genes are commonly and uniquely induced by *Agrobacterium*. We can also define which components from *Agrobacterium* are responsible for inducing changes in gene expression in plants by comparing the gene expression pattern of plants inoculated with wild-type and with several mutants, defective at different steps of the transformation process. The bacterial treatments and corresponding types of genes possibly identified are presented in Table IV-1.

The microarray data obtained can also be used in comparative studies, using microarray data publicly available that tested the responses to other pathogens, stress conditions and symbionts.

Additionally, microarray data can be used to extend beyond a simple analysis of gene expression. By clustering co-regulated genes, putative roles for unknown genes can be assigned, based on the function of known genes belonging to the same cluster. Also, the promoter region of co-regulated genes can be defined and new insights can be gained on the mechanism of regulation of these genes, as it is already being done for other plant-pathogen interactions (154). To expand our understanding of gene regulation, microarray data can also be compared to proteomic data. These comparisons can give us insights into the mechanisms of transcriptional and post-transcriptional regulation.

Finally, plant mutants can be obtained that are affected in some of the genes regulated by *Agrobacterium*, so that the requirement for these genes during the interaction can be tested in a functional analysis.

Table IV-1. Future bacterial treatments of plant cells and category of genes likely to be identified.

Treatment	**Possible types of genes identified in comparison with *A. tumefaciens* wild-type treatment**
Mock	*A. tumefaciens*-induced and other bacterial-induced
A. tumefaciens Ti plasmid deletion (A136)	Virulence-specific
A. tumefaciens T-DNA deletion	T-DNA-specific (resulting from T-DNA invasion and/or expression of T-DNA genes)
A. tumefaciens virB1 mutant	Type IV secretion-specific
A. tumefaciens virD2 mutant	VirD2 and/or T-DNA-specific
A. tumefaciens virE2 mutant	VirE2-specific
A. tumefaciens attachment deficient (*exoC*)	Attachment-specific
E. coli	*A. tumefaciens*-specific and plant pathogen-specific (When each is compared to mock, genes in common will indicate general responses to bacteria)
P. syringae DC3000 *avrRpm1*	*A. tumefaciens*-specific (When each is compared to mock, genes in common will indicate defense response)
P. syringae DC3000	*A. tumefaciens*-specific (When each is compared to mock, genes in common will indicate disease or basal defense response)

List of References

1. Abe M, Kawamura R, Higashi S, Mori S, Shibata M, Uchiumi T. 1998. Transfer of the symbiotic plasmid from Rhizobium leguminosarum biovar trifolii to Agrobacterium tumefaciens. *J Gen Appl Microbiol* 44: 65-74

2. Abuodeh RO, Orbach MJ, Mandel MA, Das A, Galgiani JN. 2000. Genetic transformation of Coccidioides immitis facilitated by Agrobacterium tumefaciens. *J Infect Dis* 181: 2106-10.

3. Aderem A, Ulevitch RJ. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406: 782-7

4. Akama K, Shiraishi H, Ohta S, Nakamura K, Okada K, Shimura Y. 1992. Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. *Plant Cell Reports* 12: 7-11

5. Arsenijevic-Maksimovic I, Broughton WJ, Krause A. 1997. Rhizobia modulate root-hair-specific expression of extensin genes. *Mol Plant Microbe Interact* 10: 95-101.

6. Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, et al. 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* 415: 977-83

7. Assaad FF. 2001. Of weeds and men: what genomes teach us about plant cell biology. *Curr Opin Plant Biol* 4: 478-87

8. Axtell MJ, Staskawicz BJ. 2003. Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* 112: 369-77

9. Azpiroz-Leehan R, Feldmann KA. 1997. T-DNA insertion mutagenesis in Arabidopsis: going back and forth. *Trends Genet* 13: 152-6

10. Bachem CW, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J* 9: 745-53.

11. Bako L, Umeda M, Tiburcio AF, Schell J, Koncz C. 2003. The VirD2 pilot protein of Agrobacterium-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. *PNAS* 100: 10108-13

12. Ballas N, Citovsky V. 1997. Nuclear localization signal binding protein from Arabidopsis mediates nuclear import of Agrobacterium VirD2 protein. *Proc Natl Acad Sci U S A* 94: 10723-8.

13. Bechtold N, Ellis JG, Pelletier G. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. Paris, Sci. la Vie/ Life Sci.* 316: 1194-9

14. Belanger C, Canfield ML, Moore LW, Dion P. 1995. Genetic analysis of nonpathogenic Agrobacterium tumefaciens mutants arising in crown gall tumors. *J Bacteriol* 177: 3752-7

15. Bell JN, Ryder TB, Wingate VP, Bailey JA, Lamb CJ. 1986. Differential accumulation of plant defense gene transcripts in a compatible and an incompatible plant-pathogen interaction. *Mol Cell Biol* 6: 1615-23.

16. Bent AF. 1996. Plant Disease Resistance Genes: Function Meets Structure. *Plant Cell* 8: 1757-71

17. Binns AN, Thomashow MF. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. *Annual Review of Microbiology* 42: 575-606

18. Blein J-P, Coutos-Thevenot P, Marion D, Ponchet M. 2002. From elicitins to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. *Trends in Plant Science* 7: 293-6

19. Blume B, Nurnberger T, Nass N, Scheel D. 2000. Receptor-Mediated Increase in Cytoplasmic Free Calcium Required for Activation of Pathogen Defense in Parsley. *Plant Cell* 12: 1425-40

20. Bonas U, Lahaye T. 2002. Plant disease resistance triggered by pathogen-derived molecules: refined models of specific recognition. *Current Opinion in Microbiology* 5: 44-50

21. Bradley DJ, Kjellbom P, Lamb CJ. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70: 21-30

22. Breedveld MW, Miller KJ. 1994. Cyclic beta-glucans of members of the family Rhizobiaceae. *Microbiol Rev* 58: 145-61

23. Buck MJ, Lieb JD. 2004. ChIP-chip: considerations for the design, analysis, and application of genome-wide chromatin immunoprecipitation experiments. *Genomics* 83: 349-60

24. Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJ. 1995. Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. *Embo J* 14: 3206-14.

25. Bundock P, Hooykaas PJ. 1996. Integration of Agrobacterium tumefaciens T-DNA in the Saccharomyces cerevisiae genome by illegitimate recombination. *Proc Natl Acad Sci U S A* 93: 15272-5.

26. Bundock P, Mroczek K, Winkler AA, Steensma HY, Hooykaas PJ. 1999. T-DNA from Agrobacterium tumefaciens as an efficient tool for gene targeting in Kluyveromyces lactis. *Mol Gen Genet* 261: 115-21.

27. Cangelosi GA, Ankenbauer RG, Nester EW. 1990. Sugars induce the Agrobacterium virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc Natl Acad Sci U S A* 87: 6708-12.

28. Cangelosi GA, Best EA, Martinetti G, Nester EW. 1991. Genetic analysis of *Agrobacterium. Methods in Enzymology* 204: 384-97

29. Cangelosi GA, Hung L, Puvanesarajah V, Stacey G, Ozga DA, et al. 1987. Common loci for Agrobacterium tumefaciens and Rhizobium meliloti exopolysaccharide synthesis and their roles in plant interactions. *J Bacteriol* 169: 2086-91.

30. Cangelosi GA, Martinetti G, Leigh JA, Lee CC, Theines C, Nester EW. 1989. Role for [corrected] Agrobacterium tumefaciens ChvA protein in export of beta-1,2-glucan. *J Bacteriol* 171: 1609-15

31. Cascales E, Christie PJ. 2004. Definition of a Bacterial Type IV Secretion Pathway for a DNA Substrate. *Science* 304: 1170-3

32. Century KS, Lagman RA, Adkisson M, Morlan J, Tobias R, et al. 1999. Short communication: developmental control of Xa21-mediated disease resistance in rice. *Plant J* 20: 231-6

33. Chateau S, Sangwan RS, Sangwan-Norreel BS. 2000. Competence of Arabidopsis thaliana genotypes and mutants for Agrobacterium tumefaciens-mediated gene transfer: role of phytohormones. *Journal of Experimental Botany* 51: 1961-8

34. Chen L, Li CM, Nester EW. 2000. Transferred DNA (T-DNA)-associated proteins of Agrobacterium tumefaciens are exported independently of virB. *Proc Natl Acad Sci U S A* 97: 7545-50.

35. Chen X, Stone M, Schlagnhaufer C, Romaine CP. 2000. A fruiting body tissue method for efficient Agrobacterium-mediated transformation of Agaricus bisporus. *Appl Environ Microbiol* 66: 4510-3.

36. Chen Z, Kloek AP, Boch J, Katagiri F, Kunkel BN. 2000. The Pseudomonas syringae avrRpt2 gene product promotes pathogen virulence from inside plant cells. *Molecular Plant Microbe Interactions* 13: 1312-21

37. Chichkova NV, Kim SH, Titova ES, Kalkum M, Morozov VS, et al. 2004. A plant caspase-like protease activated during the hypersensitive response. *Plant Cell* 16: 157-71

38. Cho YJ, Meade JD, Walden JC, Chen X, Guo Z, Liang P. 2001. Multicolor fluorescent differential display. *Biotechniques* 30: 562-8, 70, 72

39. Choi D, Ward BL, Bostock RM. 1992. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to Phytophthora infestans and to its elicitor arachidonic acid. *Plant Cell* 4: 1333-44

40. Christie PJ. 1997. Agrobacterium tumefaciens T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J Bacteriol* 179: 3085-94.

41. Christie PJ. 2001. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol Microbiol* 40: 294-305.

42. Christie PJ, Vogel JP. 2000. Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* 8: 354-60.

43. Citovsky V, Zupan J, Warnick D, Zambryski P. 1992. Nuclear localization of Agrobacterium VirE2 protein in plant cells. *Science* 256: 1802-5

44. Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, et al. 2003. SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425: 973-7

45. Covacci A, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. 1999. Helicobacter pylori virulence and genetic geography. *Science* 284: 1328-33.

46. Coventry HS, Dubery IA. 2001. Lipopolysaccharides from Burkholderia cepacia contribute to an enhanced defensive capacity and the induction of pathogenesis-related proteins in Nicotianae tabacum. *Physiological and Molecular Plant Pathology* 58: 149-58

47. Crawford KM, Zambryski PC. 1999. Plasmodesmata signaling: many roles, sophisticated statutes. *Curr Opin Plant Biol* 2: 382-7

48. Crockard MA, Bjourson AJ, Cooper JE. 1999. A new peroxidase cDNA from white clover: its characterization and expression in root tissue challenged with homologous rhizobia, heterologous rhizobia, or Pseudomonas syringae. *Mol Plant Microbe Interact* 12: 825-8.

49. Cui X, Kerr MK, Churchill GA. 2003. Transformations for cDNA Microarray Data. *Statistical Applications in Genetics and Molecular Biology* 2 http://www.bepress.com/sagmb/vol2/iss1/art4

50. Dangl JL, Dietrich RA, Richberg MH. 1996. Death Don't Have No Mercy: Cell Death Programs in Plant-Microbe Interactions. *Plant Cell* 8: 1793-807

51. Dangl JL, Jones JD. 2001. Plant pathogens and integrated defence responses to infection. *Nature* 411: 826-33

52. David R, Itzhaki H, Ginzberg I, Gafni Y, Galili G, Kapulnik Y. 1998. Supression of tobacco basic chitinase gene expression in response to colonization by the arbuscular mycorrhizal fungus *Glomus intraradices. Molecular Plant-Microbe Interactions* 11: 489-97

53. De Buck S, De Wilde C, Van Montagu M, Depicker A. 2000. Determination of the T-DNA transfer and the T-DNA integration frequencies upon cocultivation of Arabidopsis thaliana root explants. *Mol Plant Microbe Interact* 13: 658-65.

54. de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG. 1998. Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nat Biotechnol* 16: 839-42.

55. Delledonne M, Xia Y, Dixon RA, Lamb C. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* 394: 585-8

56. Deng W, Chen L, Wood DW, Metcalfe T, Liang X, et al. 1998. Agrobacterium VirD2 protein interacts with plant host cyclophilins. *Proc Natl Acad Sci U S A* 95: 7040-5.

57. Deng W, Pu XA, Goodman RN, Gordon MP, Nester EW. 1995. T-DNA genes responsible for inducing a necrotic response on grape vines. *Mol Plant Microbe Interact* 8: 538-48.

58. Deslandes L, Olivier J, Theulieres F, Hirsch J, Feng DX, et al. 2002. Resistance to Ralstonia solanacearum in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc Natl Acad Sci U S A* 99: 2404-9

59. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. 2004. Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA. *Science* 303: 1529-31

60. Ditt RF, Nester EW, Comai L. 2001. Plant gene expression response to Agrobacterium tumefaciens. *Proc Natl Acad Sci U S A* 98: 10954-9

61. Dodds PN, Lawrence GJ, Catanzariti A-M, Ayliffe MA, Ellis JG. 2004. The Melampsora lini AvrL567 Avirulence Genes Are Expressed in Haustoria and Their Products Are Recognized inside Plant Cells. *Plant Cell* 16: 755-68

62. Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S. 1989. First report of non-mycorrhizal plant mutants (Myc-) obtained in pea (Pisum sativum L.) and fababean (Vicia faba L.). *Plant Science* 60: 215-22

63. Duckely M, Hohn B. 2003. The VirE2 protein of Agrobacterium tumefaciens: the Yin and Yang of T-DNA transfer. *FEMS Microbiol Lett* 223: 1-6

64. Durner J, Shah J, Klessig DF. 1997. Salicylic acid and disease resistance in plants. *Trends in Plant Science* 2: 266-74

65. Durner J, Wendehenne D, Klessig DF. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci U S A* 95: 10328-33

66. Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE, Jones JD. 2000. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* 12: 963-77.

67. Ellis J, Dodds P, Pryor T. 2000. Structure, function and evolution of plant disease resistance genes. *Curr Opin Plant Biol* 3: 278-84

68. Endre G, Kereszt A, Kevei Z, Mihacea S, Kalo P, Kiss GB. 2002. A receptor kinase gene regulating symbiotic nodule development. *Nature* 417: 962-6

69. Escobar MA, Dandekar AM. 2003. Agrobacterium tumefaciens as an agent of disease. *Trends Plant Sci* 8: 380-6

70. Escudero J, Neuhaus G, Hohn B. 1995. Intracellular Agrobacterium can transfer DNA to the cell nucleus of the host plant. *Proc Natl Acad Sci U S A* 92: 230-4

71. Falk A, Feys BJ, Frost LN, Jones JD, Daniels MJ, Parker JE. 1999. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proc Natl Acad Sci U S A* 96: 3292-7

72. Fazzio TG, Kooperberg C, Goldmark JP, Neal C, Basom R, et al. 2001. Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol* 21: 6450-60

73. Felix G, Duran JD, Volko S, Boller T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant Journal* 18: 265-76

74. Flor HH. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9: 275-96

75. Fraysse N, Couderc F, Poinsot V. 2003. Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. *Eur J Biochem* 270: 1365-80

76. Fullner KJ, Lara JC, Nester EW. 1996. Pilus assembly by Agrobacterium T-DNA transfer genes. *Science* 273: 1107-9.

77. Galan JE, Collmer A. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284: 1322-8

78. Gamas P, de Billy F, Truchet G. 1998. Symbiosis-specific expression of two Medicago truncatula nodulin genes, MtN1 and MtN13, encoding products homologous to plant defense proteins. *Mol Plant Microbe Interact* 11: 393-403

79. Gelvin SB. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51: 223-56

80. Gelvin SB. 2003. Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiol. Mol. Biol. Rev.* 67: 16-37

81. Geremia RA, Cavaignac S, Zorreguieta A, Toro N, Olivares J, Ugalde RA. 1987. A Rhizobium meliloti mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form beta-(1----2) glucan. *J Bacteriol* 169: 880-4

82. Ghai J, Das A. 1989. The virD operon of Agrobacterium tumefaciens Ti plasmid encodes a DNA-relaxing enzyme. *Proc Natl Acad Sci U S A* 86: 3109-13.

83. Gheysen G, Villarroel R, Van Montagu M. 1991. Illegitimate recombination in plants: a model for T-DNA integration. *Genes Dev* 5: 287-97.

84. Glazebrook J. 2001. Genes controlling expression of defense responses in Arabidopsis -- 2001 status. *Current Opinion in Plant Biology* 4: 301-8

85. Glazebrook J, Chen W, Estes B, Chang HS, Nawrath C, et al. 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J* 34: 217-28

86. Gomez-Gomez L, Boller T. 2000. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol Cell* 5: 1003-11

87. Gomez-Gomez L, Felix G, Boller T. 1999. A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *Plant J* 18: 277-84

88. Gonzalez JE, Reuhs BL, Walker GC. 1996. Low molecular weight EPS II of Rhizobium meliloti allows nodule invasion in Medicago sativa. *Proc Natl Acad Sci U S A* 93: 8636-41

89. Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, et al. 2001. Genome sequence of the plant pathogen and biotechnology agent Agrobacterium tumefaciens C58. *Science* 294: 2323-8

90. Goodner BW, Markelz BP, Flanagan MC, Crowell CB, Jr., Racette JL, et al. 1999. Combined genetic and physical map of the complex genome of Agrobacterium tumefaciens. *J Bacteriol* 181: 5160-6.

91. Gouka RJ, Gerk C, Hooykaas PJ, Bundock P, Musters W, et al. 1999. Transformation of Aspergillus awamori by Agrobacterium tumefaciens- mediated homologous recombination. *Nat Biotechnol* 17: 598-601.

92. Granado J, Felix G, Boller T. 1995. Perception of Fungal Sterols in Plants (Subnanomolar Concentrations of Ergosterol Elicit Extracellular Alkalinization in Tomato Cells). *Plant Physiol* 107: 485-90

93. Grant MR, Godiard L, Straube E, Ashfield T, Lewald J, et al. 1995. Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. *Science* 269: 843-6

94. Gray MW, Burger G, Lang BF. 1999. Mitochondrial Evolution. *Science* 283: 1476-81

95. Gutierrez C. 2000. DNA replication and cell cycle in plants: learning from geminiviruses. *Embo J* 19: 792-9

96. Gyorgyey J, Vaubert D, Jimenez-Zurdo JI, Charon C, Troussard L, et al. 2000. Analysis of Medicago truncatula nodule expressed sequence tags. *Mol Plant Microbe Interact* 13: 62-71

97. Hahlbrock K, Scheel D, Logemann E, Nurnberger T, Parniske M, et al. 1995. Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells. *Proc Natl Acad Sci U S A* 92: 4150-7

98. Hammond-Kosack KE, Jones JD. 1996. Resistance gene-dependent plant defense responses. *Plant Cell* 8: 1773-91

99. Hammond-Kosack KE, Parker JE. 2003. Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Curr Opin Biotechnol* 14: 177-93

100. Hansen G. 2000. Evidence for Agrobacterium-induced apoptosis in maize cells. *Mol Plant Microbe Interact* 13: 649-57

101. Heath MC. 2000. Nonhost resistance and nonspecific plant defenses. *Curr Opin Plant Biol* 3: 315-9

102. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, et al. 2004. Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science* 303: 1526-9

103. Herrera-Estrella A, Chen ZM, Van Montagu M, Wang K. 1988. VirD proteins of Agrobacterium tumefaciens are required for the formation of a covalent DNA--protein complex at the 5' terminus of T-strand molecules. *Embo J* 7: 4055-62.

104. Herve C, Dabos P, Galaud JP, Rouge P, Lescure B. 1996. Characterization of an Arabidopsis thaliana gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain. *J Mol Biol* 258: 778-88

105. Hiei Y, Komari T, Kubo T. 1997. Transformation of rice mediated by Agrobacterium tumefaciens. *Plant Mol Biol* 35: 205-18.

106. Holt I, Ben F, Hubert DA, Dangl JL. 2003. Resistance gene signaling in plants -- complex similarities to animal innate immunity. *Current Opinion in Immunology* 15: 20-5

107. Hood EE, Gelvin SB, Melchers LS, Hoekema A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* 2: 208-18

108. Howard EA, Zupan JR, Citovsky V, Zambryski PC. 1992. The VirD2 protein of A. tumefaciens contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. *Cell* 68: 109-18.

109. Hu T, Metz S, Chay C, Zhou HP, Biest N, et al. 2003. Agrobacterium-mediated large-scale transformation of wheat (Triticum aestivum L.) using glyphosate selection. *Plant Cell Rep* 21: 1010-19

110. Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T. 1996. High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. *Nat Biotechnol* 14: 745-50.

111. Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D. 1997. Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *PNAS* 94: 4800-5

112. Jefferson RA. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular biology Reporter* 5: 387-405

113. Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* 19: 4004-14

114. Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, et al. 1999. Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc Natl Acad Sci U S A* 96: 13583-8

115. Johnson TM, Das A. 1998. Organization and regulation of expression of the *Agrobacterium* virulence genes. In *The Rhizobiaceae*, ed. HP Spanik, A Kondorosi, PJJ Hooykaas. Dordrecht, The Netherlands: Kulwer

116. Kado CI. 1998. *Agrobacterium*-mediated horizontal gene transfer. *Genetic Engineering* 20: 1-24

117. Kalogeraki VS, Winans SC. 1998. Wound-released chemical signals may elicit multiple responses from an Agrobacterium tumefaciens strain containing an octopine-type Ti plasmid. *J Bacteriol* 180: 5660-7.

118. Kanzaki H, Kagemori T, Asano S, Kawazu K. 1998. Improved bioassay method for plant transformation inhibitors. *Biosci. Biotechnol. Biochem.* 62: 2328-33

119. Kato H, Yamada T. 2003. Characterization of a wound-inducible cytochrome P450 gene (CYP72A29) that is down-regulated during crown gall tumorigenesis in potato tuber. *Molecular Genetics and Genomics* 270: 139-46

120. Kerr MK. 2003. Design considerations for efficient and effective microarray studies. *Biometrics* 59: 822-8

121. Kerr MK, Churchill GA. 2001. Experimental design for gene expression microarrays. *Biostatistics* 2: 183-201

122. Kim CY, Liu Y, Thorne ET, Yang H, Fukushige H, et al. 2003. Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. *Plant Cell* 15: 2707-18

123. Kim MC, Panstruga R, Elliott C, Muller J, Devoto A, et al. 2002. Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature* 416: 447-51

124. Kjemtrup S, Nimchuk Z, Dangl JL. 2000. Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Current Opinion in Microbiology* 3: 73-8

125. Knogge W. 1996. Fungal Infection of Plants. *Plant Cell* 8: 1711-22

126. Krause A, T. LV, Broughton WJ. 1997. Induction of chalcone synthase expression by rhizobia and nod factors in root hairs and roots. *Mol Plant Microbe Interact* 10: 388-93

127. Kruger J, Thomas CM, Golstein C, Dixon MS, Smoker M, et al. 2002. A Tomato Cysteine Protease Required for Cf-2-Dependent Disease Resistance and Suppression of Autonecrosis. *Science* 296: 744-7

128. Kumar D, Klessig DF. 2003. High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl Acad Sci U S A* 100: 16101-6

129. Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V. 2001. Genetic transformation of HeLa cells by Agrobacterium. *Proc Natl Acad Sci U S A* 98: 1871-6.

130. Kunkel BN, Bent AF, Dahlbeck D, Innes RW, Staskawicz BJ. 1993. RPS2, an Arabidopsis disease resistance locus specifying recognition of Pseudomonas syringae strains expressing the avirulence gene avrRpt2. *Plant Cell* 5: 865-75

131. Kunkel BN, Brooks DM. 2002. Cross talk between signaling pathways in pathogen defense. *Curr Opin Plant Biol* 5: 325-31

132. Lai EM, Kado CI. 2000. The T-pilus of Agrobacterium tumefaciens. *Trends Microbiol* 8: 361-9.

133. Lam E, Kato N, Lawton M. 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* 411: 848-53

134. Lamb C, Dixon RA. 1997. The Oxidative Burst in Plant Disease Resistance. *Annu Rev Plant Physiol Plant Mol Biol* 48: 251-75

135. Lambais MR, Mehdy MC. 1993. Suppression of endochitinase, β-1-3-endoglucanase, and chalcone isomerase expression in bean vesicular-arbuscular mycorrhizal roots under different soil phosphate conditions. *Molecular Plant-Microbe Interactions* 6: 75-83

136. Lange J, Xie Z-P, Broughton WJ, Vogeli-Lange R, Boller T. 1999. A gene encoding a receptor-like protein kinase in the roots of common bean is differentially regulated in response to pathogens, symbionts and nodulation factors. *Plant Science* 142: 133-45

137. Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG. 1995. The L6 gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene RPS2 and the tobacco viral resistance gene N. *Plant Cell* 7: 1195-206

138. Lazarowitz SG, Beachy RN. 1999. Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell* 11: 535-48

139. Lee J, Klessig DF, Nurnberger T. 2001. A Harpin Binding Site in Tobacco Plasma Membranes Mediates Activation of the Pathogenesis-Related Gene HIN1 Independent of Extracellular Calcium but Dependent on Mitogen-Activated Protein Kinase Activity. *Plant Cell* 13: 1079-93

140. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86: 973-83

141. Levine A, Tenhaken R, Dixon R, Lamb C. 1994. H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583-93

142. Liang P, Pardee AB. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257: 967-71.

143. Ligterink W, Kroj T, Nieden Uz, Hirt H, Scheel D. 1997. Receptor-Mediated Activation of a MAP Kinase in Pathogen Defense of Plants. *Science* 276: 2054-7

144. Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R. 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302: 630-3

145. Lodwig EM, Hosie AH, Bourdes A, Findlay K, Allaway D, et al. 2003. Amino-acid cycling drives nitrogen fixation in the legume-Rhizobium symbiosis. *Nature* 422: 722-6

146. Long SR. 1996. Rhizobium symbiosis: nod factors in perspective. *Plant Cell* 8: 1885-98

147. Lorrain S, Vailleau F, Balague C, Roby D. 2003. Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends Plant Sci* 8: 263-71

148. Lu M, Tang X, Zhou JM. 2001. Arabidopsis NHO1 Is Required for General Resistance against Pseudomonas Bacteria. *Plant Cell* 13: 437-47

149. Ludwig AA, Romeis T, Jones JDG. 2004. CDPK-mediated signalling pathways: specificity and cross-talk. *J. Exp. Bot.* 55: 181-8

150. Mackey D, Belkhadir Y, Alonso JM, Ecker JR, Dangl JL. 2003. Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112: 379-89

151. Mackey D, Holt BF, Wiig A, Dangl JL. 2002. RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell* 108: 743-54

152. Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, et al. 2003. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425: 637-40

153. Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK. 2002. A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature* 419: 399-403

154. Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, et al. 2000. The transcriptome of Arabidopsis thaliana during systemic acquired resistance. *Nat Genet* 26: 403-10

155. Martinez E, Palacios R, Sanchez F. 1987. Nitrogen-fixing nodules induced by Agrobacterium tumefaciens harboring Rhizobium phaseoli plasmids. *J Bacteriol* 169: 2828-34

156. May MJ, Leaver CJ. 1993. Oxidative Stimulation of Glutathione Synthesis in Arabidopsis thaliana Suspension Cultures. *Plant Physiol* 103: 621-7

157. McDowell JM, Woffenden BJ. 2003. Plant disease resistance genes: recent insights and potential applications. *Trends in Biotechnology* 21: 178-83

158. Meyerowitz EM. 2002. Plants compared to animals: the broadest comparative study of development. *Science* 295: 1482-5

159. Mysore KS, Nam J, Gelvin SB. 2000. An Arabidopsis histone H2A mutant is deficient in Agrobacterium T-DNA integration. *Proc Natl Acad Sci U S A* 97: 948-53.

160. Nagata T, Nemoto Y, Hasezawa S. 1992. Tobacco BY-2 Cell Line as the "HeLa" Cell in the Cell Biology of Higher Plants. *International Review of Citology* 132: 1-30

161. Nam J, Matthysse AG, Gelvin SB. 1997. Differences in susceptibility of Arabidopsis ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell* 9: 317-33.

162. Nam J, Mysore KS, Gelvin SB. 1998. Agrobacterium tumefaciens transformation of the radiation hypersensitive Arabidopsis thaliana mutants uvh1 and rad5. *Mol Plant Microbe Interact* 11: 1136-41.

163. Nam J, Mysore KS, Zheng C, Knue MK, Matthysse AG, Gelvin SB. 1999. Identification of T-DNA tagged Arabidopsis mutants that are resistant to transformation by Agrobacterium. *Mol Gen Genet* 261: 429-38.

164. Narasimhulu SB, Deng XB, Sarria R, Gelvin SB. 1996. Early transcription of Agrobacterium T-DNA genes in tobacco and maize. *Plant Cell* 8: 873-86.

165. Nathan C. 1995. Natural resistance and nitric oxide. *Cell* 82: 873-6

166. Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J Exp Bot* 53: 1237-47

167. Nester EW. 2000. DNA and protein transfer from bacteria to eukaryotes-the *Agrobacterium* story. *Molecular Plant Pathology* 1: 87-90

168. Nester EW, Gordon MP, Amasino RM, Yanofsky MF. 1984. Crown Gall: A Molecular and Physiological Analysis. *Annual Review of Plant Physiology* 35: 387-413

169. Niehaus K, Kapp D, Puhler A. 1993. Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS-I)-deficient *Rhizobium meliloti* mutant. *Planta* 190: 415-25

170. Nimchuk Z, Eulgem T, Holt BF, 3rd, Dangl JL. 2003. Recognition and response in the plant immune system. *Annu Rev Genet* 37: 579-609

171. Nimchuk Z, Rohmer L, Chang JH, Dangl JL. 2001. Knowing the dancer from the dance: R-gene products and their interactions with other proteins from host and pathogen. *Curr Opin Plant Biol* 4: 288-94

172. Nurnberger T, Brunner F. 2002. Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Current Opinion in Plant Biology* 5: 318-24

173. Nurnberger T, Brunner F, Kemmerling B, Piater L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* 198: 249-66

174. Nurnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D. 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* 78: 449-60

175. Offringa R, de Groot MJ, Haagsman HJ, Does MP, van den Elzen PJ, Hooykaas PJ. 1990. Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium mediated transformation. *Embo J* 9: 3077-84.

176. Otten L, DeGreve H, Leemens J, Hain R, Hooykaas P, Schell J. 1984. Restoration of virulence of vir region mutants of *Agrobacterium tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacteium* strans. *Molecular and General Genetics* 195: 159-63

177. Paiva NL. 2000. An Introduction to the Biosynthesis of Chemicals Used in Plant-Microbe Communication. *Journal of Plant Growth Regulation* 19: 131-43

178. Parker JE. 2003. Plant recognition of microbial patterns. *Trends Plant Sci* 8: 245-7

179. Parniske M, Schmidt PE, Kosch K, Muller P. 1994. Plant defense responses of host plants with determinate nodules induced by EPS-defective exoB mutants of Bradyrhizobium japonicum. *Molecular Plant Microbe Interactions* 7: 631-8

180. Piers KL, Heath JD, Liang X, Stephens KM, Nester EW. 1996. Agrobacterium tumefaciens-mediated transformation of yeast. *Proc Natl Acad Sci U S A* 93: 1613-8.

181. Preuss SB, Jiang CZ, Baik HK, Kado CI, Britt AB. 1999. Radiation-sensitive Arabidopsis mutants are proficient for T-DNA transformation. *Mol Gen Genet* 261: 623-6.

182. Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, et al. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425: 585-92

183. Rho HS, Kang S, Lee YH. 2001. Agrobacterium tumefaciens-mediated transformation of the plant pathogenic fungus, Magnaporthe grisea. *Mol Cells* 12: 407-11

184. Roberts RL, Metz M, Monks DE, Mullaney ML, Hall T, Nester EW. 2003. Purine synthesis and increased Agrobacterium tumefaciens transformation of yeast and plants. *Proc Natl Acad Sci U S A* 100: 6634-9

185. Robinette D, Matthysse AG. 1990. Inhibition by Agrobacterium tumefaciens and Pseudomonas savastanoi of development of the hypersensitive response elicited by Pseudomonas syringae pv. phaseolicola. *J Bacteriol* 172: 5742-9.

186. Romeis T, Ludwig AA, Martin R, Jones JD. 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. *Embo J* 20: 5556-67

187. Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones JDG. 1999. Rapid Avr 9- and Cf-9 –Dependent Activation of MAP Kinases in Tobacco Cell Cultures and Leaves: Convergence of Resistance Gene, Elicitor, Wound, and Salicylate Responses. *Plant Cell* 11: 273-88

188. Roset MS, Ciocchini AE, Ugalde RA, Inon de Iannino N. 2004. Molecular cloning and characterization of cgt, the Brucella abortus cyclic beta-1,2-glucan transporter gene, and its role in virulence. *Infect Immun* 72: 2263-71

189. Rossi L, Hohn B, Tinland B. 1993. The VirD2 protein of Agrobacterium tumefaciens carries nuclear localization signals important for transfer of T-DNA to plant. *Molecular & General Genetics 239(3):345-53, 1993 Jun* 239: 345-53

190. Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE, Williamson VM. 1998. The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *PNAS* 95: 9750-4

191. Rowland O, Jones J. 2001. Unraveling regulatory networks in plant defense using microarrays. *Genome Biology* 2: reviews1001.1 - reviews.3

192. Ruiz-Lozano JM, Roussel H, Gianinazzi S, Gianinazzi-Pearson V. 1999. Defense genes are differrentially induced by a mycorrhizal fungus and *Rhizobium* sp. in Wild-Type and symbiosis-defective pea genotypes. *Molecular Plant Microbe Interactions* 12: 976-84

193. Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD. 1996. Systemic Acquired Resistance. *Plant Cell* 8: 1809-19

194. Salmeron JM, Oldroyd GE, Rommens CM, Scofield SR, Kim HS, et al. 1996. Tomato Prf is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the Pto kinase gene cluster. *Cell* 86: 123-33

195. Salzer P, Bonanomi A, Beyer K, Vogeli-Lange R, Aeschbacher RA, et al. 2000. Differential expression of eight chitinase genes in Medicago truncatula roots during mycorrhiza formation, nodulation, and pathogen infection. *Mol Plant Microbe Interact* 13: 763-77.

196. Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B. 1992. Characterization of competent cells and early events of Agrobacterium-mediated genetic transformation in Arabidopsis thaliana. *Planta* 188: 439-56

197. Sano T, Kuraya Y, Amino S, Nagata T. 1999. Phosphate as a limiting factor for the cell division of tobacco BY-2 cells. *Plant Cell Physiol* 40: 1-8

198. Santos R, Herouart D, Sigaud S, Touati D, Puppo A. 2001. Oxidative burst in alfalfa-Sinorhizobium meliloti symbiotic interaction. *Mol Plant Microbe Interact* 14: 86-9

199. Scheel D. 1998. Resistance response physiology and signal transduction. *Curr Opin Plant Biol* 1: 305-10

200. Scheer JM, Ryan CA, Jr. 2002. From the Cover: The systemin receptor SR160 from Lycopersicon peruvianum is a member of the LRR receptor kinase family. *PNAS* 99: 9585-90

201. Scheideler M, Schlaich NL, Fellenberg K, Beissbarth T, Hauser NC, et al. 2002. Monitoring the Switch from Housekeeping to Pathogen Defense Metabolism in Arabidopsis thaliana Using cDNA Arrays. *J. Biol. Chem.* 277: 10555-61

202. Schena M, Shalon D, Davis RW, Brown PO. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467-70.

203. Schenk PM, Kazan K, Manners JM, Anderson JP, Simpson RS, et al. 2003. Systemic Gene Expression in Arabidopsis during an Incompatible Interaction with Alternaria brassicicola. *Plant Physiol.* 132: 999-1010

204. Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, et al. 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proc Natl Acad Sci U S A* 97: 11655-60.

205. Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T. 2000. The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 100: 635-44

206. Schrammeijer B, den Dulk-Ras A, Vergunst AC, Jurado Jacome E, Hooykaas PJ. 2003. Analysis of Vir protein translocation from Agrobacterium tumefaciens using Saccharomyces cerevisiae as a model: evidence for transport of a novel effector protein VirE3. *Nucleic Acids Res* 31: 860-8

207. Schrammeijer B, Risseeuw E, Pansegrau W, Regensburg-Tuink TJ, Crosby WL, Hooykaas PJ. 2001. Interaction of the virulence protein VirF of Agrobacterium tumefaciens with plant homologs of the yeast Skp1 protein. *Curr Biol* 11: 258-62.

208. Schultze M, Kondorosi A. 1998. Regulation of symbiotic root nodule development. *Annu Rev Genet* 32: 33-57

209. Sha Y, Li S, Pei Z, Luo L, Tian Y, He C. 2004. Generation and flanking sequence analysis of a rice T-DNA tagged population. *Theor Appl Genet* 108: 306-14

210. Shadle GL, Wesley SV, Korth KL, Chen F, Lamb C, Dixon RA. 2003. Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of -phenylalanine ammonia-lyase. *Phytochemistry* 64: 153-61

211. Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE, Innes RW. 2003. Cleavage of Arabidopsis PBS1 by a bacterial type III effector. *Science* 301: 1230-3

212. Sheng J, Citovsky V. 1996. Agrobacterium-plant cell DNA transport: have virulence proteins, will travel. *Plant Cell* 8: 1699-710.

213. Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama K, et al. 1990. Control of Expression of Agrobacterium vir Genes by Synergistic Actions of Phenolic Signal Molecules and Monosaccharides. *PNAS* 87

214. Shpak ED, Berthiaume CT, Hill EJ, Torii KU. 2004. Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by promoting cell proliferation. *Development* 131: 1491-501

215. Sikorski MM, Biesiadka J, Kasperska AE, Kopcinska J, Lotocka B, et al. 1999. Expression of genes encoding PR10 class pathohenesis-related proteins is inhibited in yellow lupine root nodules. *Plant Science* 149: 125-37

216. Singh KB, Foley RC, Onate-Sanchez L. 2002. Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology* 5: 430-6

217. Sola-Landa A, Pizarro-Cerda J, Grillo MJ, Moreno E, Moriyon I, et al. 1998. A two-component regulatory system playing a critical role in plant pathogens and

endosymbionts is present in Brucella abortus and controls cell invasion and virulence. *Mol Microbiol* 29: 125-38.

218. Song WY, Wang GL, Chen LL, Kim HS, Pi LY, et al. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270: 1804-6

219. Sonti RV, Chiurazzi M, Wong D, Davies CS, Harlow GR, et al. 1995. Arabidopsis mutants deficient in T-DNA integration. *Proc Natl Acad Sci U S A* 92: 11786-90.

220. Stachel SE, Messens E, Montagu MV, Zambryski P. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens. Nature* 318: 624-9

221. Stachel SE, Nester EW. 1986. The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of Agrobacterium tumefaciens. *Embo J* 5: 1445-54.

222. Stachel SE, Timmerman B, Zambryski P. 1987. Activation of Agrobacterium tumefaciens vir gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5' virD gene products. *Embo J* 6: 857-63.

223. Stachel SE, Zambryski PC. 1986. virA and virG control the plant-induced activation of the T-DNA transfer process of A. tumefaciens. *Cell* 46: 325-33.

224. Staskawicz BJ, Mudgett MB, Dangl JL, Galan JE. 2001. Common and contrasting themes of plant and animal diseases. *Science* 292: 2285-9.

225. Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, et al. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* 75: 687-706

226. Stougaard J. 2000. Regulators and regulation of legume root nodule development. *Plant Physiol* 124: 531-40

227. Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, et al. 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417: 959-62

228. Stratmann JW. 2003. Long distance run in the wound response--jasmonic acid is pulling ahead. *Trends Plant Sci* 8: 247-50

229. Suharsono U, Fujisawa Y, Kawasaki T, Iwasaki Y, Satoh H, Shimamoto K. 2002. The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. *PNAS* 99: 13307-12

230. Swart S, Logman TJ, Smit G, Lugtenberg BJ, Kijne JW. 1994. Purification and partial characterization of a glycoprotein from pea (Pisum sativum) with receptor activity for rhicadhesin, an attachment protein of Rhizobiaceae. *Plant Mol Biol* 24: 171-83

231. Taipalensuu J, Falk A, Rask L. 1996. A wound- and methyl jasmonate-inducible transcript coding for a myrosinase-associated protein with similarities to an early nodulin. *Plant Physiol* 110: 483-91

232. Takken FLW, Joosten MHAJ. 2000. Plant resistance genes: Their structure, function and evolution. *European Journal of Plant Pathology* 106: 699-713

233. Tang X, Frederick RD, Zhou J, Halterman DA, Jia Y, Martin GB. 1996. Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase. *Science* 274: 2060-3

234. Tao Y, Rao PK, Bhattacharjee S, Gelvin SB. 2004. Expression of plant protein phosphatase 2C interferes with nuclear import of the Agrobacterium T-complex protein VirD2. *Proc Natl Acad Sci U S A* 101: 5164-9

235. Tao Y, Xie Z, Chen W, Glazebrook J, Chang H-S, et al. 2003. Quantitative Nature of Arabidopsis Responses during Compatible and Incompatible Interactions with the Bacterial Pathogen Pseudomonas syringae. *Plant Cell* 15: 317-30

236. Taylor JE, Hatcher PE, Paul ND. 2004. Crosstalk between plant responses to pathogens and herbivores: a view from the outside in. *J. Exp. Bot.* 55: 159-68

237. Taylor NJ, Fauquet CM. 2002. Microparticle bombardment as a tool in plant science and agricultural biotechnology. *DNA Cell Biol* 21: 963-77

238. Thomas CM, Jones DA, Parniske M, Harrison K, Balint-Kurti PJ, et al. 1997. Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. *Plant Cell* 9: 2209-24

239. Thordal-Christensen H. 2003. Fresh insights into processes of nonhost resistance. *Current Opinion in Plant Biology* 6: 351-7

240. Timmusk S, Wagner EG. 1999. The plant-growth-promoting rhizobacterium Paenibacillus polymyxa induces changes in Arabidopsis thaliana gene expression: a possible connection between biotic and abiotic stress responses. *Mol Plant Microbe Interact* 12: 951-9.

241. Tinland B, Schoumacher F, Gloeckler V, Bravo-Angel AM, Hohn B. 1995. The Agrobacterium tumefaciens virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. *Embo J* 14: 3585-95.

242. Tompa R, McCallum CM, Delrow J, Henikoff JG, van Steensel B, Henikoff S. 2002. Genome-wide profiling of DNA methylation reveals transposon targets of CHROMOMETHYLASE3. *Curr Biol* 12: 65-8

243. Torres MA, Dangl JL, Jones JDG. 2002. Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *PNAS* 99: 517-22

244. Tusher VG, Tibshirani R, Chu G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 98: 5116-21

245. Tzfira T, Citovsky V. 2002. Partners-in-infection: host proteins involved in the transformation of plant cells by Agrobacterium. *Trends Cell Biol* 12: 121-9

246. Tzfira T, Vaidya M, Citovsky V. 2001. VIP1, an Arabidopsis protein that interacts with Agrobacterium VirE2, is involved in VirE2 nuclear import and Agrobacterium infectivity. *Embo J* 20: 3596-607.

247. Tzfira T, Vaidya M, Citovsky V. 2002. Increasing plant susceptibility to Agrobacterium infection by overexpression of the Arabidopsis nuclear protein VIP1. *Proc Natl Acad Sci U S A* 99: 10435-40

248. Underhill DM, Ozinsky A. 2002. Toll-like receptors: key mediators of microbe detection. *Curr Opin Immunol* 14: 103-10

249. Valentine L. 2003. Agrobacterium tumefaciens and the plant: the David and Goliath of modern genetics. *Plant Physiol* 133: 948-55

250. van Attikum H, Bundock P, Hooykaas PJ. 2001. Non-homologous end-joining proteins are required for Agrobacterium T-DNA integration. *Embo J* 20: 6550-8

251. van Attikum H, Bundock P, Overmeer RM, Lee LY, Gelvin SB, Hooykaas PJ. 2003. The Arabidopsis AtLIG4 gene is required for the repair of DNA damage,

but not for the integration of Agrobacterium T-DNA. *Nucleic Acids Res* 31: 4247-55

252. Van Der Biezen EA, Jones JDG. 1998. Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* 23: 454-6

253. van Wees SCM, de Swart EAM, van Pelt JA, van Loon LC, Pieterse CMJ. 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsisthaliana. *PNAS* 97: 8711-6

254. Vasse J, Billy F, Truchet G. 1993. Abortion of infection during the *Rhizobium meliloti* alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. *Plant J* 4: 555-66

255. Veena, Jiang H, Doerge RW, Gelvin SB. 2003. Transfer of T-DNA and Vir proteins to plant cells by Agrobacterium tumefaciens induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. *Plant J* 35: 219-36

256. Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CM, Regensburg-Tuink TJ, Hooykaas PJ. 2000. VirB/D4-dependent protein translocation from Agrobacterium into plant cells. *Science* 290: 979-82.

257. Veronesi C, Rickauer M, Fournier J, Pouenat ML, Esquerre-Tugaye MT. 1996. Lipoxygenase gene expression in the tobacco-Phytophthora parasitica nicotianae interaction. *Plant Physiol* 112: 997-1004

258. Vijn I, Govers F. 2001. *Conference Literature: Gene silencing and Agrobacterium tumefaciens mediated transformation of the oomycete Phytopthora infestans.* Presented at 10th International Congress on Molecular PLant Microbe Interactions, Madison, WI, USA

259. Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Sangwan-Norreel BS. 1997. Role of the host cell cycle in the Agrobacterium-mediated genetic transformation of Petunia: evidence of an S-phase control mechanism for T-DNA transfer. *Planta* 201: 160-72

260. Voinnet O. 2001. RNA silencing as a plant immune system against viruses. *Trends Genet* 17: 449-59

261. Wagner VT, Matthysse AG. 1992. Involvement of a vitronectin-like protein in attachment of Agrobacterium tumefaciens to carrot suspension culture cells. *J Bacteriol* 174: 5999-6003.

262. Wan J, Dunning FM, Bent AF. 2002. Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. *Funct Integr Genomics* 2: 259-73

263. Wang K, Herrera-Estrella L, Van Montagu M, Zambryski P. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from agrobacterium to the plant genome. *Cell* 38: 455-62.

264. Wang ZY, Seto H, Fujioka S, Yoshida S, Chory J. 2001. BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature* 410: 380-3

265. Ward ER, Barnes WM. 1988. VirD2 Protein of Agrobacterium tumefaciens Very Tightly Linked to the 5 ' End of T-Strand DNA. *Science* 242: 927-30

266. Wegel E, Schauser L, Sandal N, Stougaard J, Parniske M. 1998. Mycorrhiza mutants of *Lotus japonicus* define genetically independent steps during symbiotic infection. *Molecular Plant Microbe Interactions* 11: 933-6

267. Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, et al. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen Erwinia amylovora. *Science* 257: 85-8

268. Weisshaar B, Jenkins GI. 1998. Phenylpropanoid biosynthesis and its regulation. *Curr Opin Plant Biol* 1: 251-7

269. Whitham S, McCormick S, Baker B. 1996. The N gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. *Proc Natl Acad Sci U S A* 93: 8776-81

270. Winans SC. 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiological Reviews* 56: 12-31

271. Winans SC, Ebert PR, Stachel SE, Gordon MP, Nester EW. 1986. A gene essential for Agrobacterium virulence is homologous to a family of positive regulatory loci. *Proc Natl Acad Sci U S A* 83: 8278-82.

272. Winans SC, Kerstetter RA, Nester EW. 1988. Transcriptional regulation of the virA and virG genes of Agrobacterium tumefaciens. *J Bacteriol* 170: 4047-54.

273. Wingender R, Rohrig H, Horicke C, Wing D, Schell J. 1989. Differential regulation of soybean chalcone synthase genes in plant defence, symbiosis and upon environmental stimuli. *Mol Gen Genet* 218: 315-22.

274. Wittstock U, Gershenzon J. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr Opin Plant Biol* 5: 300-7

275. Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, et al. 2001. The genome of the natural genetic engineer Agrobacterium tumefaciens C58. *Science* 294: 2317-23

276. Xu XQ, Pan SQ. 2000. An Agrobacterium catalase is a virulence factor involved in tumorigenesis. *Mol Microbiol* 35: 407-14.

277. Yamada K, Lim J, Dale JM, Chen H, Shinn P, et al. 2003. Empirical analysis of transcriptional activity in the Arabidopsis genome. *Science* 302: 842-6

278. Yang D, Oyaizu Y, Oyaizu H, Olsen GJ, Woese CR. 1985. Mitochondrial origins. *Proc Natl Acad Sci U S A* 82: 4443-7

279. Yang YH, Speed T. 2002. Design issues for cDNA microarray experiments. *Nat Rev Genet* 3: 579-88

280. Yanofsky MF, Porter SG, Young C, Albright LM, Gordon MP, Nester EW. 1986. The virD operon of Agrobacterium tumefaciens encodes a site-specific endonuclease. *Cell* 47: 471-7.

281. Yi H, Mysore KS, Gelvin SB. 2002. Expression of the Arabidopsis histone H2A-1 gene correlates with susceptibility to Agrobacterium transformation. *Plant J* 32: 285-98

282. Yoshimura S, Yamanouchi U, Katayose Y, Toki S, Wang ZX, et al. 1998. Expression of Xa1, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. *Proc Natl Acad Sci U S A* 95: 1663-8

283. Young C, Nester EW. 1988. Association of the virD2 protein with the 5' end of T strands in Agrobacterium tumefaciens. *J Bacteriol* 170: 3367-74.

284. Young J, Kuykendall L, Martinez-Romero E, Kerr A, Sawada H. 2001. A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of Agrobacterium Conn 1942 and Allorhizobium undicola de Lajudie et al. 1998 as new combinations: Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola and R. vitis. *Int J Syst Evol Microbiol* 51: 89-103

285. Young JM, Kuykendall LD, Martinez-Romero E, Kerr A, Sawada H. 2003. Classification and nomenclature of Agrobacterium and Rhizobium - a reply to Farrand et al. (2003). *Int J Syst Evol Microbiol* 53: 1689-95

286. Young ND. 2000. The genetic architecture of resistance. *Curr Opin Plant Biol* 3: 285-90

287. Yusibov VM, Steck TR, Gupta V, Gelvin SB. 1994. Association of single-stranded transferred DNA from Agrobacterium tumefaciens with tobacco cells. *Proc Natl Acad Sci U S A* 91: 2994-8.

288. Zhao X, Coats I, Fu P, Gordon-Kamm B, Lyznik LA. 2003. T-DNA recombination and replication in maize cells. *Plant J* 33: 149-59

289. Zheng D, Zhang H, Carle S, Hao G, Holden MR, Burr TJ. 2003. A luxR homolog, aviR, in Agrobacterium vitis is associated with induction of necrosis on grape and a hypersensitive response on tobacco. *Mol Plant Microbe Interact* 16: 650-8

290. Zhu J, Oger PM, Schrammeijer B, Hooykaas PJ, Farrand SK, Winans SC. 2000. The bases of crown gall tumorigenesis. *J Bacteriol* 182: 3885-95

291. Zhu Y, Nam J, Carpita NC, Matthysse AG, Gelvin SB. 2003. Agrobacterium-mediated root transformation is inhibited by mutation of an Arabidopsis cellulose synthase-like gene. *Plant Physiol* 133: 1000-10

292. Zhu Y, Nam J, Humara JM, Mysore KS, Lee LY, et al. 2003. Identification of Arabidopsis rat mutants. *Plant Physiol* 132: 494-505

293. Ziemienowicz A, Merkle T, Schoumacher F, Hohn B, Rossi L. 2001. Import of Agrobacterium T-DNA into Plant Nuclei. Two distinct functions of vird2 and vire2 proteins. *Plant Cell* 13: 369-84.

294. Ziemienowicz A, Tinland B, Bryant J, Gloeckler V, Hohn B. 2000. Plant enzymes but not Agrobacterium VirD2 mediate T-DNA ligation in vitro. *Mol Cell Biol* 20: 6317-22.

295. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, et al. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428: 764-7

296. Zupan J, Muth TR, Draper O, Zambryski P. 2000. The transfer of DNA from agrobacterium tumefaciens into plants: a feast of fundamental insights. *Plant J* 23: 11-28.

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