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Studies on biotic and abiotic elicitors inducing defense responses in tomato

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Per Elisabetta…
…immaginandoti ridere

Man muss noch Chaos in sich haben,
um einen tanzenden Stern gebären zu können.

Abstract

Tomato (*Lycopersicon esculentum* Mill., *Solanum lycopersicon* L.) is one of the most popular vegetable throughout the world, and the importance of its cultivation is threatened by a wide array of pathogens. In the last twenty years this plant has been successfully used as a model plant to investigate the induction of defense pathways after exposure to fungal, bacterial and abiotic molecules, showing triggering of different mechanisms of resistance. Understanding these mechanisms in order to improve crop protection is a main goal for Plant Pathology.

The aim of this study was to search for general or race-specific molecules able to determine in *Solanum lycopersicon* immune responses attributable to the main systems of plant defense: non-host, host-specific and induced resistance.

Exopolysaccharides extracted by three fungal species (*Aureobasidium pullulans, Cryphonectria parasitica* and *Epicoccum purpurascens*), were able to induce transcription of pathogenesis-related (PR) proteins and accumulation of enzymes related to defense in tomato plants cv Money Maker, using the chemical inducer Bion® as a positive control.

During the thesis, several *Pseudomonas* spp. strains were also isolated and tested for their antimicrobial activity and ability to produce antibiotics. Using as a positive control jasmonic acid, one of the selected strain was shown to induce a form of systemic resistance in tomato. Transcription of PRs and reduction of disease severity against the leaf pathogen *Pseudomonas syringae* pv. *tomato* was determined in tomato plants cv Money Maker and cv Perfect Peel, ensuring no direct contact between the selected rhizobacteria and the aerial part of the plant.

To conclude this work, race-specific resistance of tomato against the leaf mold *Cladosporium fulvum* is also deepened, describing the project followed at the Phytopathology Laboratory of Wageningen (NL) in 2007, dealing with localization of a specific R-Avr interaction in transfected tomato protoplast cultures through fluorescence microscopy.

Keywords:

Tomato, biotic elicitor, glucans, *Pseudomonas* spp., *Cladosporium fulvum*, induced resistance, abiotic inducer.

Summary

1 Introduction

1.1 The plant immune system: overview

Knowledge about the damage caused by plant diseases to mankind has been mentioned in some of the oldest books available (Old Testament, 750 B.C.; Homer, 1000 B.C.). In agricultural practice worldwide, plant diseases regularly cause severe crop losses that may devastate the staple of millions of people, thus causing famines, and collectively result in economic damage of billions of euros (Van Esse *et al.*, 2008). Famous examples from the past are the Irish potato famine (1845-1847), caused by the oomycete pathogen *Phytophthora infestans,* and the great Bengal famine (1942-1943) when the rice pathogen *Helmintosporium oryzae* caused a food shortage that resulted in the death of two million of people (Padmanab, 1973). Among crops, the total global potential loss due to pests varies from about 50% in wheat to more than 80% in cotton production. The responses are estimated as losses of 26-29 % for soybean, wheat and cotton, and 31, 37 and 40 % for maize, rice and potatoes, respectively (Oerke, 2006). Since 70% of total calories consumed by human population come from only four of the six cultures previously mentioned (Raven *et al.*, 1999), it's easy to understand the relevance of crop protection to pests.

Phytopathology is a branch of Plant Science that studies plant diseases and their management; its central role as a medical discipline for plant defense is widely recognized. The progresses made in molecular biology in the last thirty years have allowed scientists to achieve amazing results and insights in this field, explaining the intimate relations between hosts and pathogens.

Understanding the dynamics of plant-microbes interactions has enormously and positively affected the management of plant diseases worldwide. Widening the knowledge about microorganisms and hosts creating a "pathosystem", Phytopathology has explained most of the successful/unsuccessful mechanisms of attack of pathogens and unraveled many of the pathways leading to plant resistance/susceptibility. Nowadays we're able to take advantage of this knowledge. In the last twenty years the plant immune system has become a primary topic for Plant Science: inducing forms of resistance in plants through processes of immunization, or genetically engineering a cv in order to express resistance factors to a particular pathogen, are not challenges anymore, but real scenarios for plant defense (Stuiver and Custers, 2001). In this first chapter I'm going to describe the general mechanisms of pathogen detection in plants and the subsequent activation of the main forms of resistance, focusing on the molecules involved in the early stages of perception and signal transduction and reporting the main classes of molecules implied in plant defense.

The capacity of plants to resist infection, recover from diseases, and then avoid future infections has been reported in botanical studies since the beginning of the twentieth century (Chester, 1933). Pathogen activities focus on colonization of the host and utilization of its resources, while plants are adapted to detect the presence of pathogens and to respond with antimicrobial defenses and other stress responses. The ability of a pathogen to produce a disease in a host plant is usually the exception, not the rule. This is because plants have an innate ability to recognize potential invading pathogens and to set up successful defenses. On the other hand, successful pathogens produce diseases because they are able to evade detection or suppress host defense mechanisms, or both (Borrás-Hidalgo, 2004). Plant-pathogen interactions result either in a compatible reaction, causing disease symptoms in a *host* plant, or in an incompatible reaction, preventing multiplication and spread of the *non-host* pathogen. In the latter case one speaks of resistance, in the former of susceptibility of the plant.

Plants rely on an innate immune system to defend themselves. Unlike mammals, plants lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Ausubel, 2005). The key for an effective activation of a defense response in the plant is a rapid detection of an external molecule as "extraneous", a concept known in Immunology as "non-self recognition". Non-self determinants are often referred as "elicitors" and are constituted by a bewildering array of compounds including different oligosaccharides, lipids, peptides and proteins (Montesano *et al.*, 2003). The broader definition of elicitor includes both substances of pathogen origin (exogenous elicitors) and compounds released from plants by the action of the pathogen (endogenous elicitors) (Boller, 1995; Ebel and Cosio, 1994). Their recognition occurs directly, via receptor ligand interaction, and indirectly, via host-encoded intermediates (Da Cunha *et al.*, 2006).

Although they do not always gain the attention of animal immunologists, plants also have quite complex and efficient immune systems (Woods, 2000). Two main strategies of defense mechanisms have evolved in plants that are similar to innate and adaptive immunity seen in animals: preformed defenses, and induced-resistance mechanisms (Menezesa and Jared, 2002). Innate (or non-adaptive) immunity can be defined as the battery of first-line host defense or resistance mechanisms employed to control infections immediately after host exposure to microorganisms, and it includes morphological and chemical structures of the plant. Pathogens able to penetrate beyond this barrier of non-host resistance may seek a subtle and persuasive relationship with the plant. For some, this may be limited to molecular signals released outside the plant cell wall, but for others it includes penetration of the cell wall and the delivery of signal molecules to the plant cytosol. Direct or indirect recognition of these signals triggers a host-specific resistance, similar to adaptive immunity in mammals. (Jones and Takemoto, 2004).

According to recent studies (Jones and Dangl, 2006; Chisholm *et al.*, 2006), plants respond to infection using a two-branched innate immune system. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly

evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs). The second acts largely inside the cell and responds to pathogen virulence factors (also called "effectors") using receptor proteins (RPs) encoded by most plant resistance genes. Both primary and secondary immune responses in plants depend on germ line-encoded PRRs and RPs (de Wit, 2007*)* and are also referred as PTI (PAMP-triggered immunity) and ETI (effector-triggered immunity), respectively.

Recent studies have revealed intriguing similarities in elicitor recognition and defense signaling processes in plant and animal hosts suggesting a common evolutionary origin of eukaryotic defense mechanisms (Montesano *et al*., 2003; Zipfel & Felix, 2005; Nürnberger *et al.,* 2004). Some general elicitors (PAMPs) are also recognized as antigenic by animals and appear to trigger innate immunity both in animals and plants (Nürnberger and Brunner, 2002). This is for example the case of flagellin, the protein forming the bacterial flagellum. Similarities also exist between molecules involved in signal transduction processes and defense genes expression: numerous DNA-binding proteins that interact with plant promoters have been identified and the corresponding cDNAs have been cloned. Some of these proteins are structurally similar to well-characterized transcription factors in animal or yeast cells, while others seem to be unique to plants (Yanagisawa, 1998).

Recognition of a potential pathogen results in several defense responses of the plant, like activation of enzymes and generation of reactive oxygen species (ROS) (Chai and Doke, 1987; Legendre *et al.,* 1993). These early events are followed by other defense responses including production of antimicrobial compounds, such as defense proteins and phytoalexins, and induction of a hypersensitive response (HR), a localized cell death around the site of infection able to stop the spread of the pathogen. Before introducing the different forms of resistance in plants, it's necessary to deepen the process at the basis of plant-pathogen recognition: the first and essential encounter between host and microbe molecules.

1.2 Plant-pathogen interactions: elicitors and receptors

How does a plant recognize a microorganism as harmful? Which are the main players involved in recognition? When a plant and a pathogen come into contact, close communications occur between the two organisms (Hammond-Kosack and Jones 2000). Plant-pathogen communications rely on the interaction among a wide and heterogeneous world of molecules, distinguished between those produced by the pathogen, often referred as "elicitors", and those produced by the plant and responsible for the detection of the elicitors, called "receptors". At first this distinction may look simple but the nature of these molecules gives an idea of the complexity of this communication. Oligo and polysaccharides, enzymes and

toxins, proteins and small peptides, fatty acids and gases: specific and non-specific interactions between these players determine and influence the outcome of the challenge among plants and pathogens.

Originally the term elicitor was used to describe molecules able to induce the production of phytoalexins (a class of defense molecules in plants) but it is now commonly used for compounds stimulating any type of plant defense (Ebel and Cosio, 1994). They were first described in the early 1970 (Keen, 1975). Elicitors may be classified into two groups, "general elicitors" or "PAMPs", and "race specific elicitors" or "effectors", depending on the specificity of the defense response induced in the plant. While general elicitors are able to trigger defense both in host and non-host plants (Nürnberger, 1999), race specific elicitors induce defense responses leading to disease resistance only in specific host cultivars (Angelova *et al.*, 2006). A list of several general and race-specific elicitors is presented in Table 1. Another classification is based on the source of these molecules, distinguishing between biotic and abiotic elicitors. While the first class encloses all molecules derived from living microorganisms, the second class include environmental stress factors, like UV lights and heavy metals ions, and chemical compounds acting as hormones or signaling molecules in the plant.

Regardless of whether the elicitor is race-specific or a general elicitor, the downstream events that the elicitor-receptor binding triggers are often similar. As a general trend, PAMPs induce basal defenses and effectors induce an HR, but both types of elicitors can induce both types of responses. For instance, the general elicitor flagellin induces basal defense in *Arabidopsis*, but its over-expression can induce a strong non-host HR response in tomato plants (Shimizu *et al*., 2003). In fact, neither the types of pathogenic molecules that elicit resistance nor the molecules used by the host to recognize pathogens are strictly correlated with the class of resistance or the type of defensive response of the plant (Da Cunha *et al*., 2006). The final defense response depends on which/how many defense pathways are triggered and how strongly those pathways are activated. Plant defense responses due to elicitor-receptor binding will be discussed in the next paragraphs.

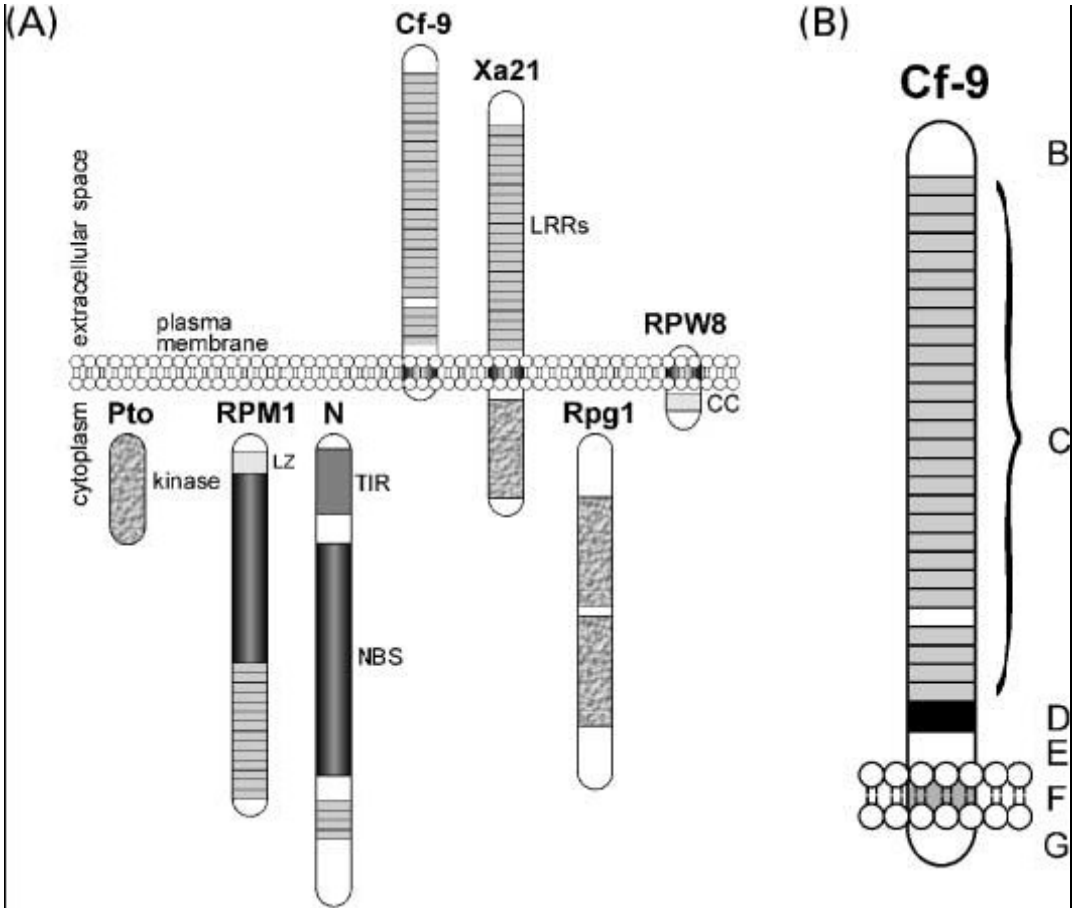
	Elicitor	Source	Function in producing organism	T	Effects in plants
I	Branched (1,3-1,6)-β-glucans	Oomycetes	Component of the fungal cell wall	G	Phytoalexin in soybean, rice
	Chitin oligomers	Higher fungi	Chitin of the fungal cell wall	G	Phytoalexin in rice; lignification wheat
I+II	Pectolytic enzymes degrading plant cell walls (»endogenous elicitors)	Various fungi and bacteria	Enzymes provide nutrients for the pathogen	G	Protein inhibitors and defense genes in *Arabidopsis*
II	Endoxylanase	*Trichoderma viridae*	Enzyme of fungal metabolism	G	HR+defense gene in tobacco
	Avr gene products	*Cladosporium fulvum*	Role in virulence	Rs	HR in tomato (Cf genes)
	Viral coat protein	TMV	Structural component	Rs	HR in tomato, tobacco
	Harpins	Some Gram- bacteria	Involved in type III secretion system	G	Callose and defense genes in tobacco
	Flagellin	Gram- bacteria	Part of bacterial flagellum	G	Callose deposition, defense genes, ROS
	Victorin (toxin)	*Helminthosporium victoriae* (rust)	Toxin for host plants	Rs	Programmed cell death (PCD) in oat
III	Glycoproteins	*Phytophtora sojae*	?	G	Phytoalexin defense genes in parsley
	Glycopeptide fragments invertase	Yeast	Enzyme in yeast metabolism	G	Defense genes and ethylene in tomato
IV	Syringolids (acyl glycosides)	*Pseudomonas syringae* pv.	Signal compound for the bacterium?	Rs	HR in soybean (Rpg4 gene)
	Nod factors (lipochitooligo-saccharides	*Rhizobium* and other rhizobia	Signal in symbiosis communication	G	Nod formation in legumes
V	FACs (fatty acid amino acid conjugates)	Various *Lepidoptera*	Emulsification of lipids during digestion?	G	Monoterpenes in tobacco-"indirect defence"
	Mycotoxins (eg. fumonisin B1)	*Fusarium moniliforme*	Toxin in necrotrophic interaction; disturb metabolism	G	PCD and defence genes in tomato, *Arabidopsi*

Montesano *et al.*, 2003

Table 1: Elicitors of defense and defense-like responses in plants. **I=** oligosaccharides; **II=** peptides and proteins; **III=** lycopeptides and proteins; **IV=** glycolipids; **V=** lipophilic elicitors; **T**=type; **G**=general elicitor; **Rs**=race-specific elicitor.

As previously mentioned, general elicitors are often referred as “PAMPs". Most of them share conserved domains which have been preserved through evolution and constitute the basis of antigens and virulence factors: a viral coat protein or a peptide from a bacterial flagellum are typical examples of molecules appeared million of years ago on Earth. During evolution plants have adapted themselves to the external environment, evolving receptors able to detect the presence of these world-spread molecules. Their recognition is therefore indiscriminate and induces a kind of non-specific resistance in a broad range of host species. However, some general elicitors are still recognized by a restricted number of plants (Shibuya and Minami, 2001). Examples of general elicitors include cell-wall glucans, chitin oligomers and glycoproteins from fungi, lypopolisaccharides (LPS) and harpin proteins from several Gram-negative bacteria, and even volatile compounds as FACs (fatty-acid amino-acid conjugates) produced by variousc *Lepidoptera.* It's interesting to see that some general elicitors are constitutively present in the pathogen as structural components, while others are expressly encoded to act as virulence factors in the host.

The latter function is a distinguishing feature of race-specific elicitors, able to induce a host-specific response in some cultivars of a certain plant species. This mechanism relies on the so called "gene for gene" theory (Flor, 1942; 1971): a specific elicitor encoded by an avirulence gene (Avr) present in a specific race of a pathogen, will elicit resistance only in a host plant cultivar carrying the corresponding resistance gene (R). The absence of either gene product will often result in disease (Cohn *et al.,* 2001; Hammond-Kosack and Jones, 1997; Luderer and Joosten, 2001; Nimchuk *et al.,* 2001; Nürnberger and Scheel, 2001; Tyler, 2002).Many plant resistance genes (R-genes) have been cloned and characterized (Dangl and Jones, 2001). Most of them encode proteins with a predicted trimodular structure (Inohara and Nunez, 2003; Martin *et al.,* 2003). These molecules act as receptors, mediating Avr protein recognition, and are categorized in seven distinct classes (Figure 1). At their carboxy terminus, they carry a Leucine-rich repeat (LRR) domain believed to be the initial recognition domain: LRRs are found throughout the tree of life and mediate protein-protein interactions (Kobe and Kajava, 2001). Various studies indicate that the pathogen specificity resides in this domain (Thomas et al., 1997; Jia *et al.*, 2000; Seear and Dixon, 2003).

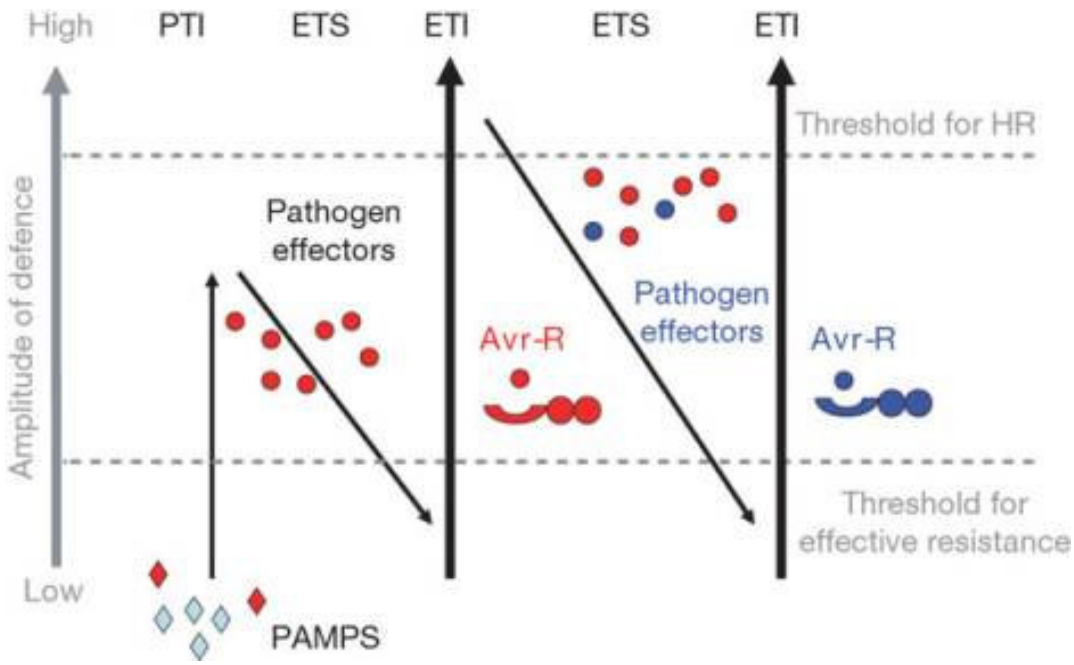


Kruijt *et al.*, 2005

Figure 1: Schematic representation of the seven major structural classes of plant R proteins. **(A)** Overall structure of one representative R protein of each of the seven classes. From left to right: **Pto**, resistance protein against *Pseudomonas syringae* pv. *tomato* of *Lycopersicon pimpinellifolium* (tomato) (Martin *et al.*, 1993); **RPM1**, resistance protein against *P. syringae* pv. *maculicola* of *Arabidopsis thaliana* (Grant *et al.*, 1995); **N**, resistance protein against Tobacco Mosaic Virus of *Nicotiana tabacum* (tobacco) (Whitham *et al.*, 1994); **Cf-9**, resistance protein against *Cladosporium fulvum* of *L. pimpinellifolium* (Jones *et al.*, 1994); **Xa21**, resistance protein against *Xanthomonas oryzae* pv. *oryzae* of *Oryza sativa* (rice) (Song *et al.*, 1995); **Rpg1**, resistance protein against *Puccinia graminis* f. sp. *tritici* of *Hordeum vulgare* (barley) (Brueggeman *et al.*, 2002); **RPW8**, broad spectrum powdery mildew resistance protein of *A. thaliana* (Xiao *et al.*, 2001).

LRRs, leucine-rich repeats; TIR, Toll/Interleukin-like receptor domain; LZ, leucine-zipper; NBS, nucleotide-binding site; CC, coiled coil. **(B)** Detailed structure of the mature *C. fulvum* resistance protein Cf-9 from tomato. Cf-9 comprises several functional domains (Jones and Jones, 1997; Jones *et al.*, 1994), indicated as domains B–H. The signal peptide for extracellular targeting (domain A) is not present in the mature protein. Domains B–E are located in the extracellular space; domain F is located in the plasma membrane; domain G is cytoplasmic. Domain B (white) is cysteine-rich; domain C comprises 27 LRRs (grey) and a loop-out (white) between LRRs 23 and 24. Domain D (black) has no distinct features; domain E (grey) is acidic; domain F (grey) is a transmembrane domain; domain G (white) is a basic cytoplasmic tail.

Race-specific elicitors (or effectors) are thought to be virulence factors evolved to target specific regulatory components of the basal defense system stimulated by PAMPs (Dangl and Jones, 2001; Hauck *et al.*, 2003; Jones and Takemoto, 2004; Kim *et al.*, 2005; Li *et al.*, 2005; de Torres *et al.*, 2006; He *et al.*, 2006; Ingle *et al.*, 2006; Van Esse *et al.*, 2008). Natural selection has driven pathogens to avoid recognition of race-specific elicitors by the plant either by diversifying them, or by acquiring additional effectors that suppress ETI. At the same time, evolution favours new plant resistance genes able to encode for proteins that can recognize the newly acquired effectors. It's a slow, everlasting ping-pong game where the player tries to adapt to the opponent's attack strategies, which continuously change. This process can be illustrated with a zig-zag model (Figure 2) which shows that the susceptibility to new effectors in a plant is continuously balanced by the appearance of new receptors able to recognize the pathogen molecules as harmful. This is the main difference between PAMPs and effectors: while some Avr proteins can evolve substantially or may be entirely absent from certain strains of a pathogen, PAMPs are defense elicitors that are evolutionarily stable, forming a core component of the microorganism that cannot be sacrificed or even altered much without seriously impairing viability (Bent and Mackey, 2007).



Jones and Dangl, 2006

Figure 2: A zigzag model illustrating the quantitative output of the plant immune system (**PTI**: PAMPs triggered immunity, **ETS**: effector-triggered susceptibility, **ETI**: effector-triggered immunity).

Since recognition of pathogen-derived molecules is the crucial point for a successful response of the plant, one the main research field in Phytopathology is to identify elicitors (and respective receptors) able to trigger a complex set of defenses of the plant and to provide enhanced resistance to subsequent infections by the same or even unrelated pathogens (Montesano *et al.*, 2003).

1.3 Mechanisms of resistance in plants

Mechanisms of plant resistance to pathogens have developed through time following the evolution of microorganisms and the appearance of new diseases on Earth. As introduced in the first paragraph of this chapter, the specificity of plant responses to pathogens can be classified into two broad categories.

Since most pathogens exhibit narrow host specificity, and will not infect "non-host" species, the resistance of plants to the vast majority of potential pathogens is termed "non-host resistance" (Dangl *et al.,* 1996; Heath, 2000; Kamoun, 2001; Thordal-Christensen, 2003; Mysore and Ryu, 2004). Non-host resistance (non-specific or basal resistance) is a response to all races of a particular pathogen, and occurs in all cultivars of a plant species. It relies on successful passive defenses, such as a preformed barrier or toxic chemical, but can also result from active defenses induced upon pathogen recognition, like synthesis and accumulation of antimicrobial reactive oxygen species, phytoalexins, and translation products from pathogenesis-related genes (Nürnberger and Brunner, 2002; Thordal-Christensen, 2003). Recognition of the pathogen by non-host plants is assumed to be brought about by general elicitors (PAMPs) (Gomez-Gomez and Boller, 2002; Montesano *et al.*, 2003; Nürnberger *et al.*, 2004). Non-host resistance is the most common and durable form of plant resistance to disease-causing organisms but it's still poorly understood due to its multigenic trait (Heat, 1996; Kang *et al*., 2003).

In contrast, host-specific resistance (race-cultivar specific resistance) is dependent upon the presence of a particular pathogen race, a particular host plant cultivar, or both. It is often governed by single resistance (R) genes, the products of which directly or indirectly interact with the specific elicitors produced by the avirulence (avr) genes of pathogens (Mysore and Ryu, 2004). Perception of the specific elicitors activates plant defense, including the HR. Host-specific resistance has been studied intensively in several model systems to elucidate the gene-for-gene theory: *Arabidopsis-Pseudomonas syringae*, tobacco-TMV, tomato-*P.syringae* and tomato-*Cladosporium fulvum* (Tao *et al.*, 2003; Peart *et al.*, 2005; Tang *et al.*, 1996; Joosten and de Wit, 1999).

Host and non-host resistance share many common defense pathways (Navarro *et al.*, 2004; Tao *et al.*, 2003). Collectively, PAMP-induced non-host resistance, as well as Avr-induced cultivar-specific resistance, should be considered two complementary elements of plant innate immunity that have been shaped in an arms race with coevolving microbial pathogens (Espinosa and Alfano, 2004;

Nürnberger *et al.*, 2004). A publication by Peart *et al*. (2002) demonstrated that the gene SGT1, which encodes a ubiquitin ligase-associated protein, is required for both non-host and R gene-mediated resistance against certain pathogens in *Nicotiana benthamiana*, supporting the idea that similar mechanisms are required for both types of resistance. However the timing, intensity and sequence of these responses are not the same in each instance.

If defense mechanisms are triggered by a stimulus prior to infection by a plant pathogen, disease can be reduced. This is the basic theory of induced resistance, one of the most intriguing forms of resistance, in which a variety of biotic and abiotic treatments prior to infection can turn a susceptible plant into a resistant one (Heat, 1996). Induced resistance is not the creation of resistance where there is none, but the activation of latent resistance mechanisms that are expressed upon subsequent, so-called "challenge" inoculation with a pathogen (Van Loon, 1997). Induced resistance can be triggered by certain chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens, or by virulent pathogens under circumstances where infection is stalled due to environmental conditions (Tuzun *et al*., 1992; Tuzun and Kúc, 1991; Benhamou *et al.*, 1998; Fought and Kúc, 1996). Plant resistance and induced forms of resistance are generally associated with a rapid response, and the defense compounds are often the same.

Generally, induced resistance is systemic, because the defensive capacity is increased not only in the primary infected plant parts, but also in non-infected, spatially separated tissues. Induced systemic resistance is commonly distinguished between systemic acquired resistance (SAR: Ross, 1961a; Ryals *et al.*, 1996; Sticher *et al.*, 1997) and induced systemic resistance (ISR: Van Loon *et al.*, 1998; Knoester *et al.*, 1999; Pieterse *et al.*, 1996;), which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved, as demonstrated in model plant systems (Ward *et al.*, 1991; Uknes *et al.*, 1992; Pieterse *et al.*, 1998; Schenk *et al.*, 2000; van Wees *et al.*, 2000; Maleck *et al.*, 2000 Yan *et al.*, 2002).

The SAR defense signalling networks appear to share significant overlap with those induced by basal defenses against pathogen-associated molecular patterns (PAMPs) (Ton *et al.*, 2002; Mishina and Zeier, 2007). Basic resistance involves the recognition of PAMPs by pattern recognition receptors (PRRs), whereas SAR-responding leaves must decode one or more unknown mobile signals (Grant and Lamb, 2006). The nature of the molecule that travels through the phloem from the site of infection to establish systemic immunity has been sought after for decades. Accumulation of salicylic acid (SA) is required for SAR, but only in the signal-perceiving systemic tissue and not in the signal generating tissue (Vernooij *et al.*, 1994). Reactive oxygen species (ROS), nitric oxide (NO), jasmonic acid (JA), ethylene and lipid-derived molecules are all implicated in systemic signalling (Maldonado *et al.*, 2002; Buhot *et al.,* 2004; Truman *et al.,* 2007). A major future

challenge will be to determine how the different factors interact to facilitate their integration into a signaling network (Vlot *et al.*, 2008).

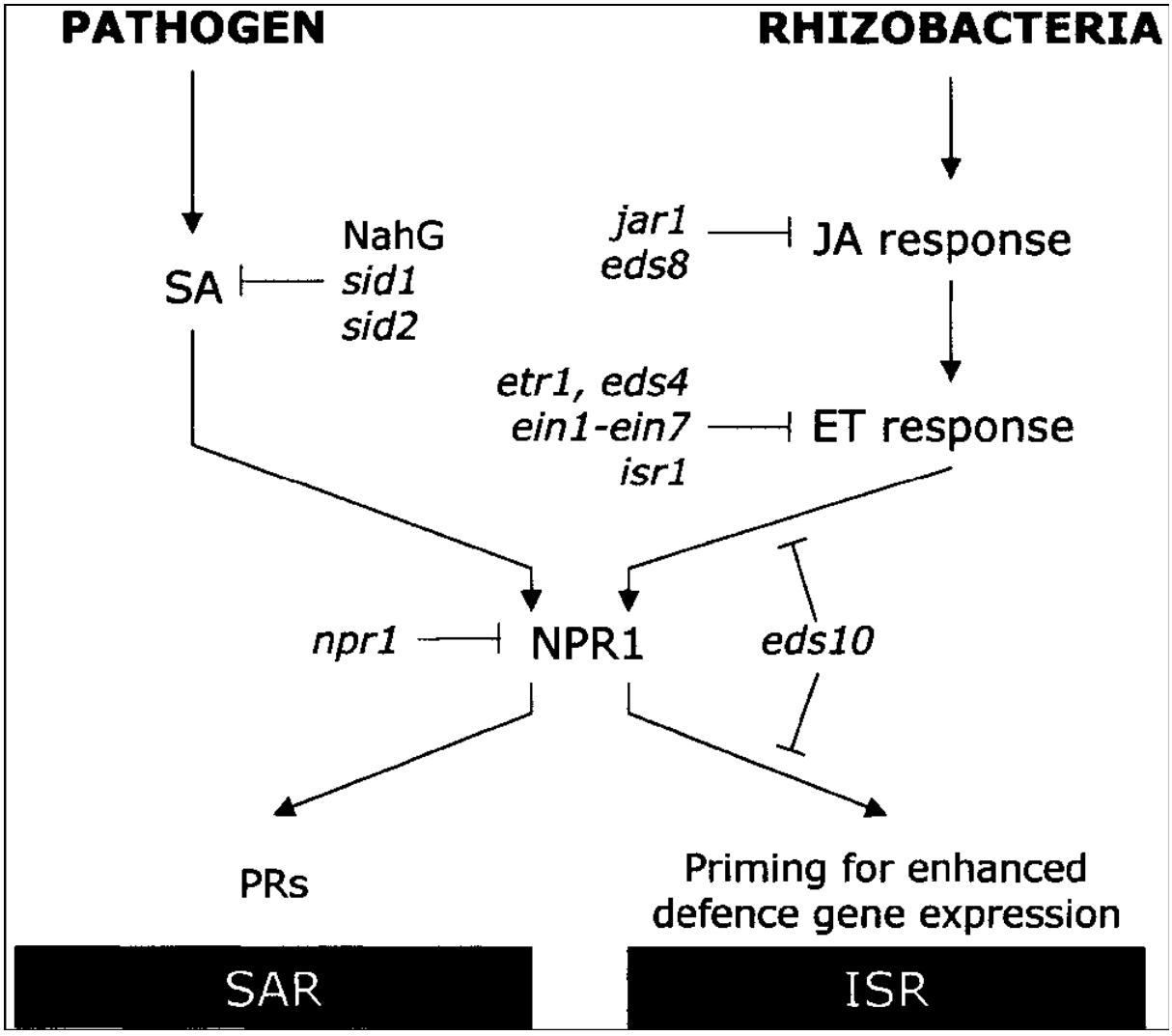
SAR is usually induced by infection of leaves with necrotizing pathogens that induce hypersensitive cell death (HR), although an HR is not obligatorily required to generate the long-distance signal (Cameron *et al.*, 1994;). It can also be triggered by exposing the plant to avirulent and non pathogenic microbes, or artificially with chemicals such as salicylic acid, 2,6-dichloro-isonicotinic acid (INA) or acibenzolar-S-methyl (BTH) (Lawton *et al.*, 1996). This form of systemic resistance is normally associated with the accumulation of pathogenesis-related (PR) proteins (Kessmann *et al.*, 1994; Ryals *et al.*, 1996; Sticher *et al.*, 1997) and is effective against a broad spectrum of plant pathogens. In tobacco, SAR activation results in a significant reduction of disease symptoms caused by the fungi *Phytophthora parasifica, Peronospora tabacina* and *Cercospora nicotianae,* the viruses tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV), and the bacteria *Pseudomonas syringae* pv *tabaci* and *Erwinia carotovora* (Vernooij *et al.*, 1995).

The plant gene NPR1 (non-expresser of PR gene 1) is the most known regulatory factor of the SAR pathway (Cao *et al.*, 1994; Delaney *et al.*, 1994; 1995). Mutant *npr1* plants accumulate normal levels of SA after pathogen infection but are impaired in the ability to express PR genes and to activate a SAR response, indicating that NPR1 functions downstream of SA. Accumulation of SA induces a change in cellular redox potential triggering the reduction of NPR1 from cytosolic, disulphide-bound oligomers to active monomers that translocate to the nucleus and interact with TGA transcription factors, activating PR genes expression (Ryals *et al.*, 1997; Zhang *et al.*, 1999; Kinkema *et al.*, 2000; Mou *et al.*, 2003).

In the last fifteen years, another form of induced resistance, effective against a broad range of diseases and associated with the colonization of plant roots by certain beneficial soil-borne microbes, has been widely documented (Wei *et al.*, 1991, 1996; Pieterse *et al.*, 1996; van Loon *et al.*, 1998). Induced systemic resistance (ISR) is an aspecific defense response of the plant triggered by the presence in the soil of plant growth-promoting rhizobacteria (PGPR), especially fluorescent pseudomonads. The bacterial determinants responsible for the induction of resistance in the aerial parts of the plant are not yet characterized but seem to depend on multiple traits (Van Loon *et al.,* 1998; Pieterse and van Loon, 1999; Pieterse *et al.* 2001a). Besides inducing resistance in the plant, antagonistic PGPR strains control plant diseases by suppressing soil-borne pathogens through the synthesis of various antimicrobial compounds and the competition for colonization sites at the root surface (Baker *et al.* 1985; Schippers *et al.*, 1987; Raaijmakers *et al.*, 1997).

Unlike SAR, ISR is independent by the accumulation of salicylic acid and doesn't seem to involve the synthesis of pathogenesis-related protein (Pieterse *et al.*, 2000), but instead, relies on pathways regulated by jasmonic acid and ethylene (Pieterse *et al.*, 1998; Knoester *et al.*, 1999; Yan *et al.*, 2002).

Curiously, also the rhizobacteria-dependent ISR relies on the regulatory gene NPR1, which functions downstream of JA and ET (Pieterse *et al.*, 1998). This suggests that NPR1 differentially regulates defense responses, depending on the signals that are elicited during induction of resistance. The main differences between the SAR and ISR pathways are showed in Figure 3.



Pieterse *et al.*, 2002

Figure 3: Schematic model describing the pathogen-induced SAR and the rhizobacteria-mediated ISR signal transduction pathways in *Arabidopsis*. Minuscule letters indicate the *Arabidopsis thaliana* mutants affected in establishing induced resistance (*sid*: SA-induction deficient; *eds*: enhanced disease susceptibility; *ein*: ET insensitive; *jar*: affected in JA response).

It is important to realize that SAR and ISR are probably only two outcomes out of an array of possibilities. It is likely that other forms of induced resistance exist that vary in their reliance on salicylic acid, ethylene, and jasmonate and other as yet discovered plant regulators (Vallad and Goodman, 2004). Generally it can be stated that pathogens with a biotrophic lifestyle are more sensitive to SA-mediated induced defenses, whereas necrotrophic pathogens and herbivorous insects are resisted more through JA/ET-mediated defenses (Thomma *et al.*, 2001; Kessler and Baldwin, 2002; Glazebrook, 2005). Cross talks between the two distinct pathways help the plant to minimize energy costs and create a flexible signaling network that allows the plant to finely tune its defense response to the invaders encountered (Reymond and Farmer, 1998; Pieterse *et al.*, 2001b; Bostock, 2005).

Induced resistance is not always expressed systemically: localized acquired resistance (LAR) occurs when only those tissues exposed to the primary invader become more resistant (Ross, 1961b). Localized resistance is generally triggered by necrotizing pathogens able to induce an HR and involves the accumulation of ROS and SA only in a limited number of cells surrounding the site of the lesion (Dorey *et al.*, 1997; Chamnongpol *et al.*, 1998; Costet *et al.*, 1999). However, strengthening of the cell wall, oxidative burst and local expression of PR proteins may also occur after localized treatment of the plant with biotic elicitors (e.g. chitosan), non-host pathogens and even chemicals as benzothiadiazole (Faoro *et al.*, 2008).

1.4 Plant inducible defenses

Plant defense mechanisms against pathogens are classified in two main categories, distinguished by preformed and inducible defenses. In the first case plants prevent the spread of the pathogen through preformed (constitutive) structural and chemical factors. In the latter case plants synthesize *ex-novo* antimicrobial compounds able to defeat the pathogen. These mechanisms are also referred as passive and active defense respectively.

Preformed physical and biochemical barriers constitute a plant's first line of defense against pathogens. These passive defenses include the presence of preformed surface wax and cell walls, antimicrobial enzymes, and secondary metabolites. The plant cell wall is the first and the principal physical barrier (Cassab and Varner, 1988). This cellulose-rich structure consists of a highly organised network of polysaccharides, proteins, and phenylpropanoid polymers that forms a resistant layer surrounding the cell plasma membrane (Menezesa and Jared, 2002). Cutin, suberine, and waxes also provide protection through the reinforcement of the epidermal layer of the leaves. Also lignin acts as a barrier and is characteristically found in plants that have recently endured pathogen attack. The size of the stomatal pores can affect the success with which a pathogen

invades a host and many plant species have leaves that respond to insect damage by increasing their density and/or number of trichomes (Traw and Bergelson, 2003). Preformed chemical barriers have a wide chemical spectrum. All low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents, are often referred as "phytoanticipins" (name coined by J.W. Mansfield) and can be composed of compounds acting as antimicrobial agents or repellents, such as terpenoids, hydroxamic acids, cyanogenic glucosides, phenolic and sulphuric compounds, saponins and peptides (Schonbeck and Schlosser, 1976; Bennett and Wallsgrove, 1994; Van Etten *et al.*, 1994; Osbourn, 1996). Inhibiting compounds may be excreted into the external environment, accumulated in dead cells or be sequestered into vacuoles in an inactive form.

An important group of preformed defensive compounds are plant defensins, small basic peptides which interfere with pathogen nutrition and retard their development. In *Arabidopsis thaliana*, at least 13 putative plant defensin genes (PDF) are present, encoding 11 different plant defensins (Thomma *et al.*, 2002). Lectins are carbohydrate-binding proteins which accumulates in the storage organs of many plants species. These proteins are capable of recognizing and binding glycoconjugates present on the surface of microorganisms (e.g. bacteria and fungi) and some of them are potent inhibitors *in vitro* of animal and human viruses, which have glycoproteins in their virions (Broekaert *et al.*, 1989; Balzarini *et al.*, 1992; Ayouba *et al.*, 1994). Saponins are a class of phytoanticipins that destroy membrane integrity in saponin-sensitive parasites, and which are stored in an inactive form in the vacuoles of the plant cell, becoming active when hydrolase enzymes are released following wounding or infection. Many enzymes contribute to the overall health status of the plant, in terms of metabolism and protection against external agents. Proteases are one of the main classes involved in plant defense mechanisms: some of them are apparently implicated in the degradation of extracellular pathogenesis-related proteins, others have been found to be involved in pathogenesis in virus-infected plants (Tornero *et al.*, 1997; Beers *et al.*, 2000; van der Hoorn, 2008).

Although these barriers can prevent invasion, pathogens have evolved strategies to overcome them. In addition to these pre-existing defense mechanisms, plants are also able to induce biochemical defenses in response to pathogens or potential pathogenic organisms that succeed in crossing the first pre-formed barriers. Biosynthesis of induced defense compounds is often controlled by complex feedback mechanisms which make a hard task to list all the plant molecules involved in resistance responses upon pathogen recognition. In these paragraphs we will only recall the major players of the different forms of resistance described so far, including signaling molecules and focusing on the main and extreme mechanism of defense of the plant: the hypersensitive response.

The host membrane appears to be involved in the earliest stages of pathogen recognition and signal transduction. A change in membrane permeability after exposure to pathogen elicitors causes fluxes in ions, such as K^+, H^+ and Ca^{2+}

(Vera-Estrella *et al.*, 1994; Gelli *et al.*, 1997). Many cellular processes, including plant defense responses, are regulated by changes in cytosolic Ca^{2+} levels, where Ca^{2+} ions can serve to transduce a particular stimulus or stress to target proteins that guide the cellular response (Bush, 1993). Subsequently, cytosolic Ca^{2+} would contribute to the rephosphorylation of the plasma membrane H^{+}-ATPase by Ca^{2+}-dependent protein kinases, resulting in the restoration of normal cellular functions (Xing *et al.*, 1996). Another change at the membrane level is the oxidative burst: a rapid, transient response which involves the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical ($^{\bullet}O_2$-) and hydroxyl radical ($^{\bullet}OH$). These molecules are toxic and produced at the site of infection in quantities capable of killing microorganisms, but they mainly function as defense compounds indirectly, acting as signaling molecules activating gene expression and influencing important biochemical pathways of the plant. H_2O_2 has been shown to induce the expression of defense related genes (Desikan *et al.*, 1998; Grant *et al.*, 2000; Levine *et al.*, 1994) and it is now widely accepted that SA signaling is mediated with ROS production and an increase in $[Ca^{2+}]$ (Larkindale and Knight, 2002; Yoshioka *et al.*, 2001). Major ROS sources are NADPH-oxidase located in the plasma-membrane and cell wall peroxidases (POX), which also participate in various physiological processes, such as lignification, suberization, auxin catabolism and wound healing (Hiraga *et al*., 2001).

The hypersensitive response (HR) is a complex, early defense response of the plant that causes a rapid death of cells in the local region surrounding an infection, in order to stop the spread of a potential pathogen. This phenomenon is a typical response of the cv-specific resistance mediated by the recognition of pathogen effectors through R-proteins of the host. The localized programmed cell death of the HR is closely correlated with the oxidative burst and it was initially attributed to the toxicity of ROS (Levine *et al.* 1994, Wojtaszek 1997, Desikan *et al.,* 1998). However, evidence is accumulating that the connection between ROS and cell death is less direct and more complex than initially conceived (Hoeberichts and Woltering, 2003). Even if lipid peroxidation and membrane damage caused by ROS may be partially responsible for the establishment of the HR, studies have suggested that the actual mode and sequence of disrupting the plant cellular components depends on each individual plant-pathogen interaction, but all HR seem to require the involvement of caspases, a family of cysteine proteases that serve as a critical switch for apoptosis in animal cells (Del Pozo and Lam, 1998; Green, 2000; Uren *et al.,* 2000; Chichkova *et al.,* 2004). The variable role of ROS in triggering hypersensitive cell death is also demonstrated by the fact that ROS-scavenging enzymes (e.g. superoxide dismutase, SOD) can inhibit elicitor-induced cell death in some situations (Lamb and Dixon, 1997), but not in others (Yano *et al*., 1999). The induction of cell death and the clearance of pathogens also require the presence of salicylic acid, which appears to play a central role in the HR, possibly related to its inhibition of mitochondrial function (Xie and Chen, 1999). In fact, recent studies in animal systems have pointed to the importance of

compartmentalization in general, and the mitochondrion in particular, in the regulation of apoptosis (Lam *et al.,* 2001; Ferri and Kroemer, 2001). Moreover, an increase in cytosolic calcium precedes, and seems necessary for, hypersensitive cell death triggered by rust fungi (Xu and Heath, 1998) and the calcium channel blocker La^{3+} prevents bacterial-induced HR in soybean leaves (Levine *et al*., 1996). As a conclusion, cell death associated with the HR may be only one of a larger set of cellular responses that are coordinately activated by different stress signals. Understanding the functional role of each player involved in plant hypersensitive response will require further work.

In some cases, the cells surrounding the HR lesion synthesize antimicrobial compounds, including phenolics and pathogenesis-related (PR) proteins. These compounds may act by puncturing bacterial/fungal cell walls or by delaying maturation or disrupting the metabolism of the pathogen in question.

Many plant phenolic compounds are known to be antimicrobial, function as precursors to structural polymers such as lignin, or serve as signal molecules (Nicholson and Hammershmidt, 1992; Dakora, 1996). The term "phytoalexins" was coined by K.O. Müller for those plant antibiotics that are synthesized *de novo* after the plant tissue is exposed to microbial infection (Müller and Börger, 1941). Phytoalexins are low molecular, lipophilic, antimicrobial substances produced as secondary metabolites by many plant species. These compounds accumulate rapidly in incompatible pathogen infections and also in response to an extensive array of biotic and abiotic elicitors (Smith, 1996). They tend to fall into several chemical classes, including flavonoids, isoflavonoids and sequiterpenes, and their biosynthesis occurs mainly through the mevalonate or schikimic acid pathways. The mode of action of phytoalexins is highly diversified: many of them (e.g. the well-known pisatin from *Pisum sativum* and camalexin from *Arabidopsis thaliana*) disrupt the integrity of bacterial/fungal membranes (Shiraishi *et al.,* 1975; Rogers *et al.,* 1996), others, like phaseolin and rishitin, have been reported to inhibit respiration of whole tissue (Skipp *et al.,* 1977; Lyon, 1980), while kaempferol, a flavonoid found in several higher plants, inhibits mitochondrial electron flow and phosphorylation of plant cell cultures (Koeppe and Miller, 1974; Ravanel *et al.,* 1982).

The defense strategy of plants against pathogens and other environmental factors involves various types of stress proteins with putative protective functions. The term "pathogenesis-related protein" (PR protein) was introduced in the 1970s in reference to proteins that are newly synthesized or present at substantially increased levels after a plant has been infected (Gianinazzi *et al.,* 1970; van Loon and van Kammen 1970). These host-specific proteins are induced both by biotic and abiotic agents, comprising necrotizing and non-necrotizing viruses, viroids, fungi, bacteria, specific physiological conditions and a variety of chemicals. Pathogenesis related proteins are able to resist to acidic pH and proteolytic cleavage and thus survive in the harsh environments where they occur: the vacuolar compartment, the cell wall or the apoplast (Niderman *et al.,* 1995; Van

Loon, 1999). At present, a large number of PR-proteins have been characterized and grouped into 17 families based on their biochemical properties (Table 2): some show β-1,3-glucanase activity, others chitinase and proteolitic activity (Sticher *et al.*, 1997; Van Loon, 2006). Pathogenesis related proteins belonging to the PR-1 family are considered markers for SAR, since their accumulation is induced by salicylic acid (Gu *et al.,* 2002).

Family	Type member	Properties
PR-1	Tobacco PR-1a	antifungal
PR-2	Tobacco PR-2	β -1,3-glucanase
PR-3	Tobacco P, Q	chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco "R"	chitinase type I, II
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P6g	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco "lignin forming peroxidase"	peroxidase
PR-10	Parsley "PR1"	"ribonuclease like"
PR-11	Tobacco class V chitinase	chitinase type I
PR-12	Radish Rs AFP3	defensin
PR-13	*Arabidopsis* THI2.1 thionin	thionin
PR-14	Barley LTP4	Lipid transfer protein
PR-15	Barley OxOa (germin)	oxalate oxidase
PR-16	Barley OxOLP	'oxalate oxidase-like'
PR-17	Tobacco PRp27	unknown

http://www.bio.uu.nl/~fytopath/PR-families.htm

Table 2: Recognized families of pathogenesis-related proteins.

1.5 Molecules involved in plant defense signaling

Another field of great interest in plant-microbe interactions is the complex system of signaling activated after recognition of the pathogen, also referred as "plant signal transduction". Plant enzymes, proteins, lipids, ions and gases are the main characters involved in signaling system. Cell surface or intracellular receptors react to external stimuli by binding directly external agents or by recognizing them indirectly through modifications of guard molecules. This receptor/ligand binding initiates the transmission of a signal across the plasma membrane by inducing a change in the shape or conformation of the intracellular part of the receptor, leading to activation of enzymatic processes. Such processes are usually rapid, lasting on the order of milliseconds in the case of ion fluxes, or minutes for the activation of protein- and lipid-mediated enzymatic cascades. Intracellular signal

transduction is then carried out by secondary messengers of the cell, including most of the ions and gases mentioned so far. Last events of the signal transduction pathway lead to the activation of plant transcription factors, DNA-binding proteins which initiate a program of increased defense-related genes transcription. Cross talks and connections between signaling pathways responding to diseases or environmental stresses make this system even more complex.

In eukaryotic cells, most intracellular proteins activated by a ligand/receptor interaction possess an enzymatic activity. These enzymes include tyrosine kinase, GTPases, various serine/threonine protein kinases, phosphatases, lipid kinases, and hydrolases. Proteins phospho and dephosphorylation play a key role in diverse biological signal transduction systems (Peck, 2003; Thurston *et al.*, 2005; de la Fuente van Bentem and Hirt, 2007), and phosphorylation events are essential for the ethylene-mediated pathogenesis response in tobacco plants (Raz and Fluhr, 1993).

Mithogen-activated protein kinases (MAPK) transfer information from sensors to cellular responses through protein phosphorylation in all eukaryotes. It is therefore not surprising that several MAP kinases have been implicated in plant defense signaling (Menke *et al.,* 2005; Nakagami *et al.,* 2004). Extracellular stimuli lead to activation of a MAP kinase via a signaling cascade ("MAPK cascade"), which phosphorylates a variety of substrates including transcription factors, other protein kinases, and cytoskeleton-associated proteins. MAPKs are stimulated not only during plant-microbe interactions but also in response to many stresses such as wounding, salt, temperature, and oxidative stresses (Jonak et al. 2002).

Plants also use signal transduction pathways based on heterotrimeric guanine nucleotide–binding proteins (G proteins) to regulate many aspects of development and cell signaling. G-proteins are bound to the membrane receptor in their inactive state. Once the ligand is recognized, the receptor shifts conformation and thus mechanically activates the G protein, which detaches from the receptor. Cell division, ion channel regulation, and disease response are processes regulated by G proteins in both plants and animals (Assmann, 2005).

Secondary messengers can be divided in three main classes:

- ► Hydrophobic molecules like diacylglycerol (DAG), and phosphatidylinositols, which are membrane-associated and diffuse from the plasma membrane into the intermembrane space where they can reach and regulate membrane-associated effector proteins.
- ► Hydrophilic molecules: water-soluble molecules, like cAMP, cGMP, IP_3, and Ca^{2+}, that are located within the cytosol.
- ► Gases: nitric oxide (NO) and carbon monoxide (CO), which can diffuse both through cytosol and across cellular membranes.

As previously introduced, changes in ions concentration at the apoplastic and simplastic level, are one the first signals triggering cascade of responses. Reactive oxygen species (ROS) function as intracellular signaling molecules in a diverse

range of biological processes. In signal transduction pathways induced by pathogens or elicitors, ROS participate in MAPK activation (Lebrun-Garcia *et al.,* 1998; Kovtun *et al.,* 2000). Activation of K^+ and Ca^{2+} ions channels play a critical role in the mediation of early events of signal transduction. Calcium is one of the most important second messengers in plants. Ca^{2+}-binding proteins can regulate the activity or function of a large number of target proteins (approximately 200 putative targets in *Arabidopsis*) or directly regulate gene expression (Asai *et al.,* 2002; Reddy and Reddy 2004; Bouché et al. 2005). Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein which regulates many Ca^{2+}-dependent cellular processes in both plant and animal cells (Lu and Means, 1993; Zielinsky, 1998). More than 50 enzymes and ion channels are regulated by CaM, and the number of CaM-modulated proteins is ever increasing (Lee *et al.,* 2000).

The enzyme phospholipase C (PLC) cleaves the membrane phospholipid PIP2 (phosphatidylinositol-4,5-bisphosphate) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG remains bound to the membrane due to its hydrophobic properties, and IP_3 is released as a soluble structure into the cytosol. IP_3 then activates particular calcium channels in the endoplasmic reticulum; this causes the cytosolic concentration of Ca^{2+} to increase, causing a cascade of intracellular responses. PIP2 can serve as a substrate not only for phospholipases, but also for phosphoinositide kinases, thereby generating additional lipid second messengers implicated in signal transduction (Toker, 1998). The other product of phospholipase C, diacylglycerol, activates protein kinase C (PKC), which in turn activates other cytosolic proteins by phosphorylating them. The activity of another cytosolic enzyme, protein kinase A (PKA), is controlled by cyclic adenosine monophosphate (cAMP), a molecule derived from adenosine triphosphate (ATP), functioning as a second messenger for intracellular signal transduction in many different organisms. Cyclic guanosine monophosphate (cGMP) is another important molecule for signaling (Bowler *et al.,* 1994). It acts much like cAMP, by activating intracellular protein kinases like PKG.

Components of the early signal transduction pathway include nitric oxide (NO) that activates G proteins and opens Ca^{2+} channels. In tobacco plants it has been demonstrated that nitric oxide is able to induce the expression of the defense-related genes PR1 and PAL (phenylalanine ammonia lyase), and that exogenous NO treatment resulted in a transient increase in cGMP levels (Durner *et al.,* 1998). Among these signalling molecules, three are considered the major regulators of plant defense responses: salicylic acid, jasmonic acid, and ethylene, which all fall into the broad class of plant hormones.

Salycilic acid (SA) is a phenol which acts through binding to a high-affinity SA-binding protein (SAPB2) with subsequent activation of a SA-inducible protein kinase (SIPK), which is a MAP kinase family member. Another component acting downstream of SIPK is the cytosolic protein NPR1, which contain a BTB/POZ and an ankyrin repeat-domain. Both domains are known to mediate protein–protein interactions and are present in proteins with diverse functions (Bork, 1993; Aravind and Koonin, 1999), including the transcriptional regulator I_kB, which

mediates animal innate immune responses (Baldwin, 1996). Accumulation of SA induces a change in cellular redox potential triggering the reduction of NPR1 from cytosolic, disulphide-bound oligomers to active monomers that translocate to the nucleus and interact with TGA/OBF family of basic leucine zipper (bZIP) transcription factors (Zhou *et al.,* 2000). These bZIP factors physically interact with NPR1 and bind the SA-responsive element in promoters of several defense genes, such as the SAR marker pathogenesis-related genes PR-1, PR-2 and PR5.

SA-dependent, NPR1- independent defense responses also exist, and may involve the transcription factor *Why1* whose DNA-binding activity is induced by SA independently of NPR1 (Desveaux *et al.,* 2004). Two important genes related to plant defense, PAD4 (Phytoalexin-Deficient 4) and EDS1 (Enhanced Disease Susceptibility 1), encode lipase-like proteins and are required for activating SA accumulation in response to some, but not all, SA-inducing stimuli (Zhou *et al.,* 1998; Falk *et al.,* 1999). Their expression levels are enhanced by the application of SA, suggesting that these genes are regulated by SA-dependent positive feedback (Jirage *et al.,* 1999; Feys *et al.,* 2001).

Jasmonic acid (JA) is a plant hormone biosynthesized from linolenic acid by the octadecanoid pathway. The function of JAs in defense was proposed by Farmer and Ryan (Farmer and Ryan, 1992), who provided evidence for a causal link between wounding (as caused by insect herbivores), the formation of JAs, and the induction of genes for proteinase inhibitors that deter insect feeding. JA signaling can also be induced by a range of abiotic stresses, including osmotic stress (Kramell *et al.,* 1995), wounding, drought, and exposure to biotic elicitors, which include chitins, oligosaccharides, oligogalaturonides (Doares *et al.,* 1995), and extracts from yeast (Parchmann *et al.,* 1997; Leon *et al.,* 2001).

JA induces systemic expression of the genes VSP, JR1 (encoding lectins), and Thi2.1 (encoding a thionin) in response to wounding, but it is negatively regulated by the local synthesis of ET (Rojo *et al.,* 1999). However, JA and ET play an essential role in the induction of ISR and can also cooperate synergistically to activate basic pathogenesis-related (PR) proteins such as chitinase (β-CHI), PR-3, PR-4 and the plant defensin PDF1.2 (Xu *et al.,* 1994; Penninckx *et al.,* 1996, 1998; Thomma *et al.,* 1999; Dombrecht *et al.,* 2007). Studies on *Arabidopsis thaliana* showed that the JA signaling pathway requires the activation of several proteins: lypoxygenases (LOX) are enzymes involved in JA biosynthesis while WIPK (Wound Induced Protein Kinase) and COI1 (Coronatine Insensitive 1) are positive regulators acting upstream and downstream of JA, respectively. In particular, the gene *COI1* encode for a leucine-rich repeats (LRRs) and F-box motif protein which is required to degrade a repressor of the jasmonate signaling pathway (Liechti *et al.*, 2006). The MAPK cascade MKK3-MPK6 plays an important role in the JA signal transduction (Takahashi *et al.,* 2007), and a MAP kinase (MPK4) has been identified as a negative regulator of SA signaling and a positive downstream regulator of JA/ET-dependent response (Petersen *et al.,* 2000). Jasmonate-Resistant 1 (JAR1) is an important enzyme for signaling belonging to the luciferase family (Staswick *et al.,* 2002), acting downstream of JA and

upstream of the transcription factor JIN1/MYC2 (Jasmonate-Insensitive 1), which acts as both activator and repressor of distinct JA-responsive gene expression in *Arabidopsis* (Lorenzo *et al.,* 2004).

The plant hormone ethylene regulates a variety of stress responses and developmental adaptations in plants. This gaseous molecule is well known for its participation in physiological processes as diverse as fruit ripening, senescence, abscission, germination, cell elongation, sex determination, pathogen defense response, wounding and nodulation (Bleecker and Kende, 2000; Diaz *et al.,* 2002; Valverde and Wall, 2005; Ortega-Martinez *et al.,* 2007). ET signaling involves a family of membrane-anchored receptors (ETR1, ETR2, EIN4, ERS1, and ERS2), the ETR1-associated protein kinase CTR1, that negatively regulates ET signaling, and members of the family of EIN3-like (Ethylene Insensitive 3) transcription factors, which have been shown to physically interact with two F-box proteins of the ubiquitine ligase family, EBF1 and EBF2 (EIN3-Binding F BOX) (Potuschak *et al.,* 2003).

In the synergic JA/ET pathway, the protein NPR1 and the transcription factor ERF1 (Ethylene-Response Factor 1) play a key role in the integration of JA and ET signals, explaining at the molecular level the cooperation between both hormones in the activation of plant defenses (Berrocal-Lobo *et al.,* 2002; Lorenzo *et al.,* 2003; Pieterse and Van Loon, 2004). WRKY transcription factors are other DNA-binding proteins involved in SA- and JA-dependent defense responses, which further downstream regulate the SA and JA/ET signaling and modulate cross talks between the two pathways (Li *et al.,* 2004; Wang *et al.,* 2006). SA and JA signaling interact on many levels, and in most cases, this relationship seems to be mutually antagonistic (Kunkel and Brooks, 2002). It can be concluded that defense pathways influence each other through a network of regulatory interactions (Figure 4), and thus, plant responses to various biotic and abiotic stimuli are a result of this complex interplay.

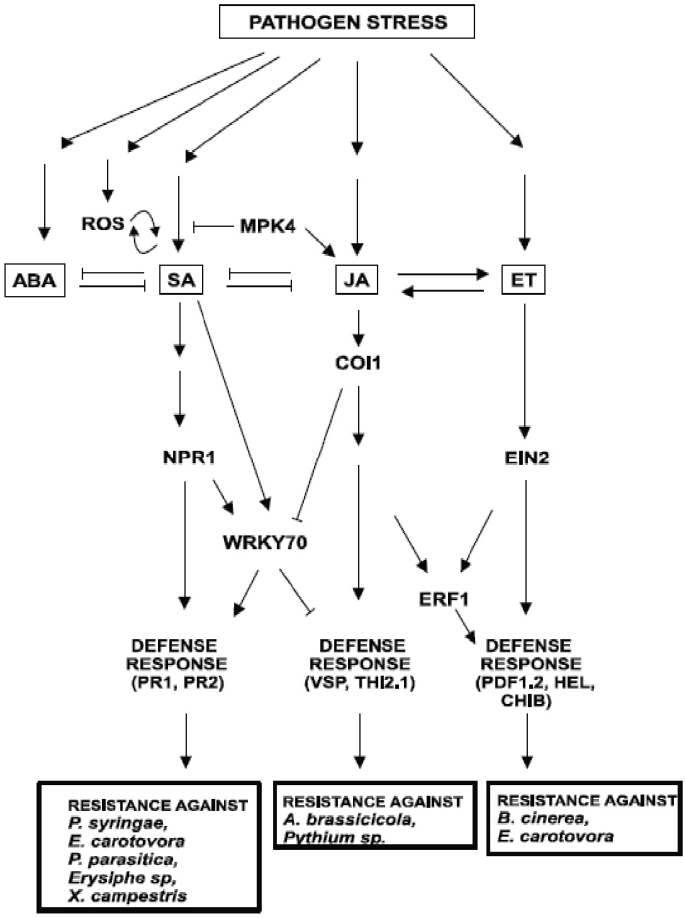


Figure 4: Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) defense signaling pathways and signal cross talks in *Arabidopsis* (modified from Kunkel and Brooks, 2002; Durrant and Dong, 2004).

1.6 Model plants for plant-pathogen interactions: *Solanum lycopersicum* (*Lycopersicon esculentum*)

A model organism is a species that is extensively studied to understand particular biological phenomena, with the expectation that discoveries made in the organism model will provide insight into the workings of other organisms (Fields and Johnston, 2005). The *Arabidopsis thaliana* plant model system has contributed much in the remarkable progresses made in plant molecular biology during the last twenty years, unraveling many signaling pathways involved in plant-pathogen interactions. The main reasons for the *Arabidopsis* success are its small size, short lifecycle, relatively small genome (the first plant complete genome to be sequenced), and easy transformability (Bechtold *et al.,* 1993). However, the number of *Arabidopsis* pathogens is relatively small, and additional models are

desirable for comprehensive evaluation of plant-pathogen interactions (Arie *et al.,* 2007).

Solanaceous plants have provided excellent model systems to study plant-pathogen interactions (Meissner *et al.,* 1997; Meissner *et al.,* 2000; Emmanuel & Levy, 2002) and represent economically important crop plants, such as tomato and potato. *Nicotiana tabacum* and *Capsicum annum* are well-known examples of model plants studied to elucidate gene regulation; tobacco is commonly used to test the induction of HR by presumed pathogenic bacteria. *Nicotiana benthamiana* is the most widely used experimental host for plant virology and plant hormones signaling research. Moreover, because it can be genetically transformed and regenerated with good efficiency and is amenable to facile methods for virus-induced gene silencing (VIGS) or transient protein expression, *N. benthamiana* is rapidly gaining popularity in plant biology, particularly in studies requiring protein localization, protein interaction or plant-based systems for protein expression and purification (Goodin *et al.,* 2008).

Tomato (*Lycopersicon esculentum* Mill.*, Solanum lycopersicon* L.) is one of the most popular vegetables worldwide. Its cultivation, however, has been limited by an abundance of diseases caused by fungi, bacteria, viruses, and nematodes. This diversity of pathogens emphasizes the importance of the tomato pathosystem as a favorable model for studying plant-pathogen interactions. Moreover, *Lycopersicon esculentum* carries several specific resistance (R) genes against a variety of pathogens (Table 3), which make this plant suitable for genetic studies of plant host-specific resistance based on the gene for gene theory. Famous models are the interactions with the fungal mold *Cladosporium fulvum* (Joosten and de Wit, 1999), the bacterial speck *Pseduomonas syringae* pv. *tomato* (Ronald *et al.,* 1992), and the fungal wilt *Verticillium dahliae* (Fradin and Thomma, 2006). Most of these resistance genes have been found in south american wild tomato species (eg. *L. hirsutum* or *L. pimpinellifolium*), which seem to be more resistant against diseases and have been used as a source of resistance genes in modern tomato breeding (Arie *et al.*, 2007). Tomato expresses a large number of defense compounds and is also used as a model plant to test whether an elicitor or a particular pathogen are able to induce basal resistance or to activate forms of induced resistance through SA or JA/ET signaling pathways.

The appearance of new pathogen races and diseases often invalidates the efforts made in tomato breeding; however, this may further stimulate the study of plant responses to pathogens and lead to the discovery of new defense genes and signaling pathways.

Disease	Pathogen	R-gene	Origin
Fungal diseases			
Alternaria cancer	*Alternaria alternata* tomato pathotype	*Asc*	*Lycopersicon esculentum*
Corky root	*Pyrenochaeta lycopersici*	*Py*	*L. hirsutum*
Crown/root rot	*Fusarium oxysporum* f. sp. *radicis*	*Frl*	*L. peruvianum*
Early blight	*Alternaria solani*	*	*L. hirsutum*
Late blight	*Phytophthora infestans*	*Ph*	?
Leaf mold	*Cladosporium fulvum*	*Cf*	*L. peruvianum*
Leaf spot	*Stemphylium lycopersici*	*Sm*	*L. pimpinellifolium*
Powdery mildew	*Leveillula taurica*	*Lv*	
	Oidium neolycopersici	*Ol*	*L. hirsutum*
Wilt	*Fusarium oxysporum* f. sp. *lycopersici* race 1	*I*	*L. pimpinellifolium*
	Fusarium oxysporum f. sp. *lycopersici* race 2	*I2*	*L. pimpinellifolium*
	Fusarium oxysporum f. sp. *lycopersici* race 3	*I3*	*L. pennellii*
Verticillium wilt	*Verticilliium dahliae*	*Ve*	*L. esculentum*
Bacterial disease			
Bacterial speck	*Pseudomonas syringae* pv. tomato	*Pto*	*L. pimpinellifolium*
Viral diseases			
Mosaic	*Tomato mosaic virus* (ToMV)	*Tm-1*, *Tm-2*, *Tm2a*	*L. hirsutum*, *L. peruvianum*
Spotted wilt	*Tomato spotted wilt virus* (TSWV)	*Sw*	*L. peruvianum*
Yellow leaf curl	*Tomato yellow leaf curl virus* (TYLCV)	*Ty-1*, *Ty-2*	*L. peruvianum*, *L. hirsutum*
Nematode disease			
Root knot	*Meloidogyne arenaria*	*Ma*	*L. peruvianum*
	Meloidogyne incognita	*Mi*	*L. peruvianum*
	Meloidogyne javanica	*Mj*	*L. peruvianum*

Arie *et al.,* 2007

Table 3: Diseases and resistance genes in present tomato cultivars.

2 Aim of the thesis

Tomato (*Solanum lycopersicon*) is one of the most popular vegetable throughout the world, and the importance of its cultivation is threatened by a wide array of pathogens. In the last twenty years this plant has been successfully used as a model plant to investigate the molecular events triggering the induction of defense pathways in the plant cell, after exposure to fungal, bacterial and abiotic molecules, thus leading to the expression of different resistance mechanisms. Understanding these molecular events will improve the development and implementation of newer and more advanced strategies in crop protection.

The aim of this study was to search for general or race-specific molecules able to determine in *Solanum lycopersicon* immune responses attributable to the main systems of plant defense: non-host, host-specific and induced resistance.

In the third chapter, exopolysaccharides extracted by three different fungal species are investigated for their ability to act as general elicitors (PAMPs) and induce a non-host resistance characterized by transcription of plant defense genes. In particular, the transcription of the pathogenesis-related proteins PR-1, PR-5 (marker genes for SAR) and PR-4 (induced by JA/ET pathway), and the expression of plant chitinases and peroxidases have been evaluated.

The fourth chapter describes the research project developed at the Laboratory of Phytopathology of Wageningen (NL) during the second year of the PhD, concerning a specific protein-protein interaction between tomato and the fungus *Cladosporium fulvum*. This recognition between R and Avr gene products is the basis of race-specific resistance of the host against the leaf mold, and culminates in an hypersensitive response (HR). The aim of this study was to tag these proteins with fluorescent tags and to localize their interaction in cultures of tomato protoplasts. Unluckily the project was only partially accomplished due to the lack of time.

In the fifth chapter, the triggering of induced systemic resistance (ISR) by a rhizobacteria selected during the last year of the PhD is investigated in tomato plants, evaluating transcription of pathogenesis-related proteins and the ability of the strain to suppress disease caused by *Pseudomonas syringae* pv. *tomato*.

Apart from the period spent abroad, all the research was conducted at the Laboratory of Phytobacteriology, Di.S.T.A., Bologna.

3 Non-host resistance and general elicitors

3.1 Introduction

Non-host resistance refers to resistance shown by an entire plant species to a specific parasite (Heat, 1987). This resistance is expressed by every plant toward the majority of potential phytopathogens and can be induced by general elicitors (PAMPs) of pathogen or plant origin, including oligosaccharides, lipids, polypeptides, and glycoproteins (Nürnberger *et al.,* 2004). General elicitors are recognized by plant receptors based on their common molecular pattern, and are able to trigger an unspecific, basal resistance response through the activation of several defense pathways, leading to a multitude of events like increase of cytosolic [Ca^{2+}], generation of ROS, increased enzyme activity, cell wall modifications, synthesis of new resistance factors, accumulation of secondary metabolites with antimicrobial properties and even a localized cell death (HR). Well-known examples of general elicitors include lipopolysaccharide (LPS) fraction of Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, the bacterial protein flagellin, methylated bacterial DNA fragments, fungal cell-wall derived glucans, chitins, mannans and proteins, and oligogalacturonides degrading plant cell walls and releasing endogenous elicitors (Aderem and Ulevitch, 2000; Girardin *et al.,* 2002; Medzhitov and Janeway, 2002; Nürnberger and Lipka, 2005).

Non-host resistance has often been considered less effective than cv-specific resistance, maybe because the term is deeply connected to “basal resistance” and may recall a primary but insufficient level of protection. However, non-host resistance is durable, involves multiple gene/protein interactions and relies on a wide and layered pool of defense molecules; its genetic basis, however, are still poorly understood. M.C. Heat (2000) raised the question if non-host resistance could be the result of specific recognition events. Indeed, non-host resistance shares some common features with the cv-specific resistance based on R-Avr recognition, like the regulatory gene SGT1 in *Nicotiana benthamiana* (Peart *et al.,* 2002). Moreover, non-host resistance against bacteria, fungi and oomycetes can induce the HR, a typical response of the cv-specific resistance. Mysore and Ryu (2004) classified non-host resistance in two types: type I non-host resistance does not produce any visible symptoms of necrosis, and the type II non-host resistance is always associated with a rapid hypersensitive response. During type II non-host resistance the non-host pathogen is able to overcome preformed and general elicitor-induced plant defense responses, probably by producing detoxifying enzymes and specific elicitors which target regulatory components of the PAMPs signaling pathway. Race-specific elicitors are then recognized by the plant surveillance system and this triggers plant defense leading to a hypersensitive

response (HR). PR gene expression and SAR signaling can be induced during both types of non-host resistance.

The type of non-host resistance triggered in a non-host plant is dependent on both the plant species and the pathogen species. For example, *P. syringae* pv. *phaseolicola* triggers type I non-host resistance in *Arabidopsis* and type II non-host resistance in tobacco (Lu *et al.,* 2001; Lindgren *et al.,* 1986), while *Nicotiana benthamiana* exhibits type I against *Xanthomonas campestris* pv. *campestris* and type II against *P. syringae* pv. *tomato* (Peart *et al.,* 2002). Because type I non-host resistance does not involve a HR, it is ideal to exploit this type of resistance for durable disease control in cultivated plants (Mysore and Ryu, 2004): testing the effect of general elicitors and investigating induced signaling pathways in reliable model plants is a valuable system to select biotic and abiotic resistance inducers for crop protection.

3.1.1 Polysaccharides produced by fungi and bacteria

Different types of constitutive fungal and bacterial molecules, including cell wall oligo- and polysaccharides, have been found to serve as elicitors of basal defense responses in plants. Plants detect the presence of several non-pathogenic fungi and bacteria by recognizing an essential structural component of the cell walls of the microorganism. Thus, many bacteria and fungi cannot easily evade detection by altering the structure of this component, which is often an highly conserved oligo- or polysaccharide.

Lipopolysaccharides (LPS), also known as lipoglycans, are large molecules found in the outer membrane of Gram-negative bacteria, consisting of a lipid moiety and a polysaccharide chain joined by a covalent bond. LPS have been shown to induce the synthesis of antimicrobial compounds in several animal system (eg. *Drosophila*), as well as the production of immunoregulatory and cytotoxic molecules in humans (Lemaitre, 1996; Alexander and Rietschel, 2001; Medzhitov and Janeway, 2002). In plants, LPS act as general elicitors by inducing NO synthase *AtNOS1* as well as activate several defense genes (Zeidler et al. 2004; Keshavarzi *et al.,* 2004).

Oligosaccharides (OLS) act as general elicitors and signal molecules in plants (Farmer *et al.,* 1991; Fry *et al.,* 1993). Hormonal concentrations of biologically active oligosaccharides, called oligosaccharins, regulate growth and development as well as defense reactions by regulating gene expression (Usov, 1993). The first oligosaccharide shown to possess biological activity was a hepta-β-glucoside isolated from the mycelial wall of a fungal pathogen of soybeans (Ayers *et al.,* 1976). However, oligogalacturonide fragments of cell wall homogalacturonans isolated from a plant cell wall polysaccharide can also act as oligosaccharins.

Linear α-1,4-D-oligogalacturonides containing 12 to 14 galactosyluronic acid residues have the same biological effect as the active hepta-b-glucoside: they elicit soybean seedlings to produce phytoalexins. Leaves of wounded tomato plants release oligogalacturonides which induce the synthesis and accumulation of two serine proteinase inhibitors, basic components of basal defense (Bishop *et al.,* 1984).

Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer of N-acetylglucosamine, a derivative of glucose, and is found in many places throughout the natural world. It's a main component of the fungal cell wall and the core structure of the arthropods exoskeleton. Chitin perception by plants in response to microbial invasion plays an integral role in cell signaling during pathogenesis (Wagner, 1994; Stacey and Shibuya, 1997) and putative chitin receptors required for fungal recognition have been found in soybean (Day *et al.,* 2001) and *Arabidopsis* (Wan et al. 2008). Interestingly, modified chitin oligosaccharides play a central role in the establishment of a host-specific symbiosis between legumes and their rhizobial symbionts (Cohn *et al.,* 1998). Nonetheless, chitinases are a major class of defense-related plant enzymes.

Glucans are polysaccharides of D-glucose monomers linked by glycosidic bond and are present in many eukaryotes. Plant cellulose is a β-1,4-glucan, while the storage polymer starch is a α-1,4- and α-1,6-glucan. The α- and β- letters and the numbers clarify the type of glycosidic bond. Many fungi produce extracellular glucan homopolymers. Some of them have commercially important functional properties (eg. biofilm production), others have been shown to induce an immune response in plants and animals (Ikewaki *et al.,* 2007).

Beta-(1,3)-glucan is a major structural component of the cell wall of yeasts and fungi (Kobayashi *et al*., 1974). These polysaccharides have been extensively studied for their immunological and pharmacological effects and today more than 900 papers describing the biological activities of β-(1,3)-glucans exist (Jamois *et al.,* 2005). Some of them exhibit antitumor activity (Morikawa *et al.,* 1985; Hong *et al.,* 2003), others are able to decrease post-surgical human infections through processes of immunization like leukocyte activation (Babineau *et al.,* 1994; Adachi *et al.,* 1997). This activity is believed to be related to the organization of the (1.3)-β-linked backbone into a triple helix (Falch *et al.,* 2009) and to the complexity of their side-branching (Bohn and BeMiller, 1995).

Lentinan is a form of β-glucan (β-1,6; β-1,3-glucan) derived from the fungus *Lentinula edodes*. In human pathology is one of the host-mediated anti-cancer drugs which has been shown to affect host defense immune system (Nakano *et al.,* 1999). Most *Epicoccum nigrum (*syn. *Epicoccum purpurascens)* strains synthesize an extracellular, ethanol-insoluble mucilage containing a β-linked glucan named epiglucan (Michel *et al.,* 1981), which also seems to induce immunological effects.

Also α-glucans are critical to the normal function of yeast cell walls and play an important role in the virulence of multiple fungal pathogens, including

Aspergillus, Histoplasma, and *Cryptococcus* (Beauvais *et al.,* 2005; Rappleye *et al.,* 2004; Reese *et al.,* 2007). Pullulan, is an α-glucan (α-1,4; α-1,6-glucan) produced by the fungus *Aureobasidium pullulans*. This exopolysaccharide has been extensively studied for its ability to form oxygen-impermeable biofilms, thickening or extending agents, or adhesives (McNeil and Kristiansen, 1990), but it also exhibits various biological activities. Pullulan has been shown to induce antitumor and antimetastatic activity (Kimura *et al.,* 2006), antiosteoporotic effects (Shin *et al.,* 2007), and to prevent food allergies (Kimura *et al.,* 2007).

It's evident that many bacteria- and fungi-derived polysaccharides are able to induce a variety of defense responses in animal and plant systems. Previous reports indicate that the physicochemical properties of glucans may be important determinants for recognition and interaction with pattern recognition receptors (PRPs) in the innate immune system (Mueller, *et al.,* 1996; Mueller, *et al.,* 2000) and β-glucans have been recently identified as fungal PAMPs (Williams, *et al.,* 2004; Brown and Gordon, 2003). Therefore, searching for natural biopolymers able to induce a kind of basal and durable resistance in plant is an intriguing research. Indeed, a series of commercially available polysaccharides, oligosaccharides, and simple sugars have also been tested for elicitor activity, but with poor results.

3.1.2 Pullulan: chemical and biological properties

Pullulan is a neutral, water-soluble, homopolysaccharide consisting of maltotriose and maltotetraose units (Figure 5) with both α-(1,4) and α-(1,6) linkages (Bouveng *et al.,* 1963; Cateley and Whelan, 1971; Taguchi *et al.,* 1973; Catley *et al.,* 1986). The regular alternation of α-(1,4) and α-(1,6) bonds results in two distinctive properties: structural flexibility and enhanced solubility (Leathers, 1993). These properties suggest that pullulan may be used for both medical and industrial purposes (LeDuy *et al.,* 1988).

Pullulan is a fungal exopolysaccharide produced from starch by *Aureobasidium pullulans*. The early observation on this exopolymer was made by Bauer in 1938 and this exopolysaccharide was named as "pullulan" by Bender *et al*. in 1959. Pullulan is naturally occurring, since *Aureobasidium pullulans* is ubiquitous. It is found in soil, lake water, on the surface of latex paint films, synthetic plastic materials, shared-used cosmetic and foods such as cereals, fruits, cheese and tomato (Vadkertiová, 1994; Zabel and Terracina, 1980; Webb *et al.*, 1999; Mislivec *et al.*, 1993). Because it forms a black pigment (melanin), this organism is also known as "black yeast" (Cooke, 1961; Durrell, 1967; Domsch *et al*., 1993; Gibbs and Seviour, 1996).

Pullulan is produced on an industrial scale by fermentation of liquefied starch under controlled conditions using a specific, not genetically modified, non-

pathogenic and non-toxigenic strain of *Aureobasidium pullulans*. The film-forming properties of pullulan are the basis for its proposed use as a substitute for gelatin in the production of capsule shells (for dietary supplements), as an ingredient of coated tablets (dietary supplements), and as an ingredient of edible flavored films (breath fresheners). It has been used as an additive and as a food ingredient in Japan since 1976.

Some important parameters that control the production of pullulan are temperature (McNeil and Kristiansen, 1990), the initial pH of the medium (Lacroix *et al.,* 1985), the oxygen supply (Rho *et al.,* 1988; Wecker and Onken, 1991), the nitrogen concentration (Auer and Seviour, 1990), and the carbon source (Badr-Eldin *et al.,* 1994). The molecular weight of pullulan varies depending on the culture conditions and strain.

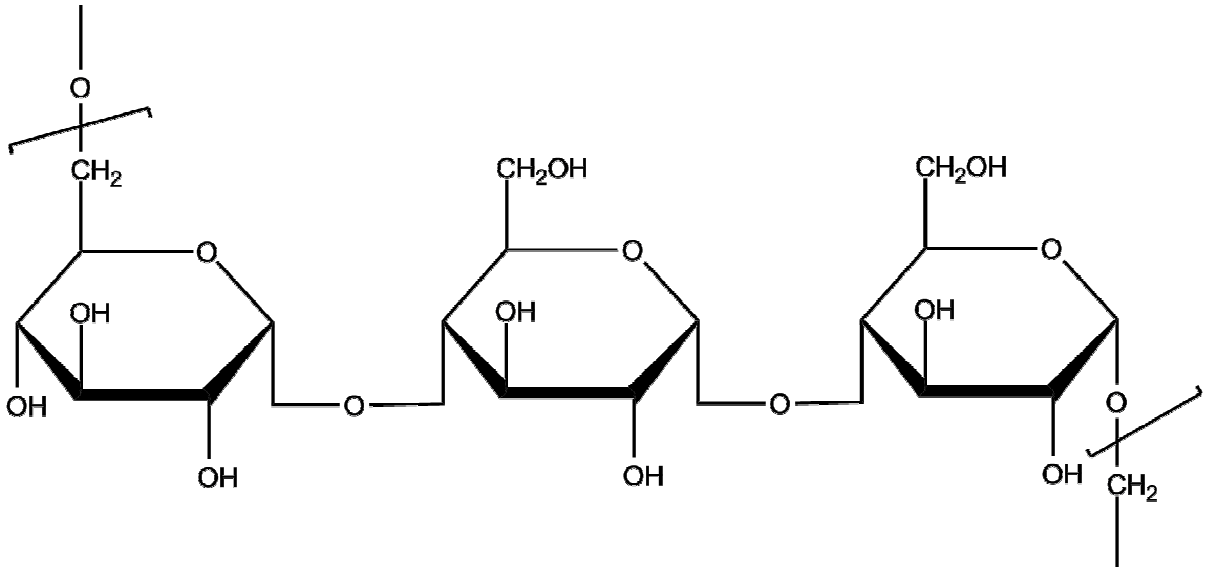


Figure 5: A pullulan maltotriose unit consists of three glucose units connected by α-(1,4) glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α-(1,6) glycosidic bond.

As recently reported by Leathers (2002), pullulans are also produced by other fungi than *A. pullulans*. In particular, scientific papers from Italian authors (Evidente *et al.,* 1997; Corsaro et al, 1998; Forabosco *et al.,* 2006) reported that pullulans are also produced by *Cryphonectria parasitica*, the fungal agent of chestnut blight, and some of them play a role in the virulence of the microorganism (Molinaro *et al.,* 2002).

High-affinity binding sites for β-glucans exist in the membrane of plants (Schmidt and Ebel, 1987; Yoshikawa and Sugimoto, 1993), but little is known about α-glucans specific receptors. Adams *et al*. (2008) have demonstrated that the

mammalian pattern recognition receptor Dectin-1 is highly specific for glucans that have a (1-3)-β-D-glucopyranosyl backbone. Dectin-1 doesn't recognize non-β-linked carbohydrate polymers (e.g. mannan or pullulan) and doesn't interact with plant derived glucans (e.g. barley glucan) that have a mixed linkage polymer backbone characterized by alternating regions of (1-3)-β and (1-4)-β linkages (Aman and Graham, 1987). However, pullulan immunological effects in animal systems suggest that this polysaccharide may be detected by plants as a general elicitor (PAMP).

To test the effect of pullulans as biotic elicitors in the induction of plant defense, exopolysaccharides produced by three fungal species (*Aureobasidium pullulans, Cryphonectria parasitica* and *Epicoccum purpurascens*), were extracted and evaluated for their biological activity *in vitro* and *in planta*. In particular, the ability of these molecules to induce the accumulation of pathogenesis-related proteins and enzymes related to defense was tested in tomato plants cv Money Maker.

3.2 Materials and methods

3.2.1 Microorganisms

Aureobasidium pullulans strains n° 3998, 4958, 4960, 4766 and 4768 were obtained from the collection of Carlo Bazzi (DiSTA) and screened for selection. All strains were collected from the skin of pomaceous and drupaceous fruits. Additional strains were isolated from leaves of *Quercus* sp. and plum skin in the southern area of Bologna. *Cryphonectria parasitica* strain EP67 (accession number ATCC 38751) and *Epicoccum nigrum* were obtained from Antonio Prodi (DiSTA). Additional *E. nigrum* strains were obtained as contaminants in media plates. All strains were maintained by monthly transfers to potato dextrose agar (PDA) at 27 °C and observed through a Leitz-Laborlux microscope.

3.2.2 Culture conditions and exopolymers extraction

SDW-mycelia preparations were transferred to 250 ml conical flasks containing 100 ml of culture medium (pH range 5-6) of the following composition (g/L): glucose 20.0, $(NH_4)_2SO_4$ 0.6, yeast extract 2.5, K_2HPO_4 5.0, $MgSO_4 \cdot 7H_2O$ 0.2 and NaCl 1.0 (Ueda *et al.,* 1963). The flasks were incubated at 28 °C for 72 h in a rotary shaker incubator at 150 rpm. After three days, liquids were transferred into plastic cylindrical containers and cells were collected through centrifugation in a Sorvall RC2B ultracentrifuge (15000g for 20 min at 4°C). Supernatants were separated through a Miracloth sheet (Calbiochem Biochemical, La Jolla, CA, USA), mixed with 2 volumes of cold absolute ethanol (Carlo Erba, Italia), that produced phase separation, and left at -20°C for 18 h. During storage, the phase containing the extracellular polysaccharides separated into floating and settling materials. Crude products were collected through centrifugation (15000g for 30min at 4°C) and supernatants were discharged. Raw polysaccharides were then washed two times with a 1:1 (v/v) solution of acetone and diethylether (Merck, Germany). Washing solution was discharged and raw products were dissolved in 2 mL filter-sterilized SDW. The viscous materials were then frozen at -80°C, lyophilized (BVL2, Brizio Basi) and grinded in mortars to obtain from bright/white to white/creamy powders. Stock solutions (1% w/v) of the raw polysaccharides were promptly prepared.

3.2.3 Polysaccharides *in vitro* and *in vivo* assays

Direct antimicrobial activity of the extracted polysaccharides against two pathogenic bacteria (*Erwinia amylovora* and *Xanthomonas arboricola* pv. *pruni*) was tested *in vitro* at two different concentration (0.1 % and 0.01% w/v). A 10 µl drop of the pullulan solution was loaded on an antibiogram disc placed in a King's B-agar Petri dish. After 15 min, 2 mL of bacterial suspensions (10^6-10^7 cfu/ml)

were poured into the plate. After drying, plates were incubated overnight at 27°C. Water and streptomycin were used as a negative and positive control, respectively.

Phytotoxicity of the different extracts was tested on tobacco and tomato plants in climate chamber. Pullulans solutions (0.1% w/v) were sprayed on the upper leaves of tobacco cv White Burley and tomato plants cv Money Maker. Plants were kept at 24°C under 18 h of light and monitored for the next two weeks.

The different extracts were also tested for their ability to prevent the hypersensitive response induced by the non-host pathogenic strain 6285 of *Pseudomonas syringae* pv. *syringae* in leaves of tobacco plants (cv White Burley). Raw extracts solutions (0.1% and 0.01% w/v) were infiltrated with a 1 mL-syringae into the inferior surface of tobacco panels. Water and strain 6285 were used as negative and positive control respectively, while strain m5 of *Pseudomonas putida* and a 0.1% sucrose solution were used as additional controls. After 24 h, the possible induction of necrosis was monitored and a bacterial suspension of *P. syringae* (10^7 cfu/mL) was infiltrated in the same and in the lower and upper panels. After 24 h, the absence of necrosis in the newly infiltrated panels indicated the prevention of HR respect to the negative control. In order to test how much the concentration of these solutions influences the process, three dilutions (0.1%, 0.01%, 0.001% w/v) were tested in adjacent panels.

3.2.4 Plant materials, protein extraction and IEF of peroxidases and chitinases

Two weeks old tomato plants (cv Money Maker) grown in climate chamber at 22°C with 16 h of light, were sprayed with bacterial OLS (0.2%), chitosan (0.2%, J. Haidebei LTD) and pullulan solutions (0.02% w/v) derived from *Aureobasidium pullulans* strain 4958 and 3998. Plant chitinase and peroxidase activity was monitored at different times (0 h, 24 h, 96 h, 7 days) through isoelectrofocusing (IEF) technique. Water and the SAR inducer Bion® (acibenzolar-S-methyl 50%, Syngenta) were used as negative and positive control, respectively.

Samples (500 mg) of frozen leaves were grinded with liquid nitrogen in a ice-cold mortar to obtain a fine powder, mixed with 1 mL (2 µl/µg) of extraction buffer (TRIS base 20 mM, pH 6.8, 1% PVPP from Sigma), transferred in 2 mL eppendorf and gently shaked for 2 h at 4°C. Samples were then filtered through a Miracloth sheet (Calbiochem Biochemical, La Jolla, CA, USA) and centrifuged at 14000 rpm for 30 min at 4°C. Aliquots (50 µl) of the supernatant were kept at -20°C for maximum 2 weeks (Buzi, 2001).

Qualitative analysis of the isoenzymatic forms of peroxidase and chitinase present in tomato leaf extracts was performed through isoelectrofocusing on polyacrylammide gel (PAGE IEF), using a Multiphor® II 2117 horizontal electrophoresis unit.

A 5% polyacrylammide gel (Pharmacia Biotech, Uppsala, Sweden: 0.8 mm×12 cm×24.5 cm) containing 0.15% bisacrylammyde (N,N'-methylenebisacrylamide, LKB, Produkter AB, Bromma, Sweden), 15% glycerol, 0.04% ammonium persulphate, 0.1% TEMED (N,N,N',N'-tetrametylendiamine, ultra PURE™, BRL

Bethesda, Research Laboratories, Geithersburg, USA) and 5% Ampholine™ (Amersham Pharmacia Biotech AB, Uppsala, Sweden), was rehydrated for 2 h before electrophoresis with water, glycerol and Ampholine (pH 3-10). Each well was loaded with an amount corresponding to 100 µg of total protein. Electrophoresis was run at 4°C following protocol's running conditions.

Peroxidase activity was determined following the protocol of Caruso *et al.* (1999). After the run, the gel was first washed in a 0.1 M sodium phosphate buffer solution (pH 5.4) for 10 min and then in a 4.4% (v/v) guaiacol solution (Farmitalia Carlo Erba S.p.A., Milano, Italia) in 0.1 M sodium phosphate buffer (pH 5.4), for 10 min at room temperature. After immersion in distilled water, the gel was stained in 18% (v/v) hydrogen peroxide solution (Panreac Montplet & Esteban SA, Barcelona, Espana) in 0.1 M sodium phosphate buffer (pH 5.4), till the appearance of the typical dark-red bands. Chitinase activity was determined following the procedure of Trudel and Asselin (1989). After electrophoresis, the gel was washed in a 0.1 M sodium acetate buffer solution (pH 5.2), gently shaked for 10 minutes. Subsequently, a 2.9 % polyacrylammide overlay gel containing 0.12% glycol chitin (w/v), 3.5% glycerol (w/v), 0.012% ammonium persulphate (w/v), 0.14% TEMED (v/v), 14 mM sodium acetate buffer solution (pH 5.0) was incubated for 2 h at 37°C in contact with the separation gel. Glycol chitin was prepared following the procedure from Molano *et al.,* (1977). Lytic (dark) zones were revealed by UV illumination with a transilluminator, after staining the overlay gel for 10 min at dark with 0.01% (w/v) Fluorescent Brightener 28 (Calcofluor White M2R, Sigma Chemical Co.) in 0,5 M Tris buffer solution (pH 8.9).

3.2.5 Plant materials, total RNA extraction and multiplex RT-PCR assay

Three weeks old tomato plants (cv Money Maker), grown in climate chamber at 22°C with 16 h of light, were sprayed with solutions (0.02 % w/v) of the three kinds of extracted fungal exopolysaccharides, and the transcript level of pathogenesis-related genes (PR-1, PR-4, PR-5) and the regulatory component NPR1 was monitored at different times (0 h, 24 h, 72 h, 7 days) through multiplex RT-PCR technique (Reverse Transcriptase PCR). Water and Bion® were used as a negative and positive control, respectively.

Starting from 100 mg/sample of leaf material, total plant RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) applying two main modifications to the protocol: extraction buffer (4 M guanidine isothiocyanate, 0.2 M $CH_3COO^-Na^+$ pH 5.0, NaEDTA 25 mM and 2,5 % PVP) was freshly prepared, and the sample (leaf tissue + extraction buffer) was mixed with 3% sarcosine (Sigma) and incubated for 10 minutes at 70°C before starting the standard procedure. All RNA samples were treated with RQ1 RNase-free DNase (M6101, Promega).

For each single sample, Reverse Transcriptase mix to obtain the cDNAs was as followed: 1X MLV-Buffer, 1 mM dNTPs (U1330, Promega), 50 µM random primers (C1181, Promega), 50U of M-MLV (Moloney Murine Leukemia Virus

Reverse Transcriptase, M1705, Promega) and 1/10 (v/v) of total RNA. Reverse transcriptase reaction was run in a AB2720 thermocycler (Applied Biosystems) at 37°C for 1 h, with additional 5 min at 94°C to deactivate M-MLV.

The expression of the pathogenesis-related genes PR-1, PR-4, PR-5 and the regulatory component NPR1 was compared with the expression of the gene encoding for the elongation factor 1 α (EF1) of *Lycopersicon esculentum* (Accession Number: X14449), which was used as internal control in the multiplex RT-PCRs.

Two sets of primer pairs (LycNPR1 and TomNPR1, Table 4) were designed on the partial coding sequence of the *Lycopersicon esculentum* NPR1-interactor protein (Accession Number: AF143442), obtained from the National Centre for Biotechnology Information (NCBI, http://www.ncbinim.nih.gov/). TomNPR1 sequences amplified from cDNAs of tomato plants cv Money Maker and cv Perfect Peel were cloned in pGEMT Easy Vector (Promega, Madison, WT), sequenced by BMR genomics (Padova, Italia), and aligned with the reference sequence using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers for PR-1, PR-4, PR-5 and EF1 amplification (Table 4) were obtained from Enrico Biondi, DiSTA. Standard multiplex PCR reaction was as followed: 1X Buffer (M890A, Promega), 2 mM MgCl2, 0,4 mM dNTPs, 500 nM internal control primers, 500 nM target gene primers, 1 U Taq DNA polymerase (GoTaq Flexi DNA Polymerase, M830A, Promega) and 5 µl of cDNA to obtain a final volume of 25 µl. Sequences were amplified setting the following standard thermal profile on a AB2720 thermocycler: pre-denaturing step at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and elongation 72°C for 1 min, and a final elongation step at 72°C for 10 min. Amplicons were run at 70 V on a 1 % agarose gel, stained with ethidium bromide (46027, Fluka) and visualized on a UV transilluminator.

Target gene	Accession number		Primers (5'→3')	Length
EF1	X14449	**F** **r**	AGC TGG TAT CTC CAA AGA TGG TCA GAC TCA TCT TAA CCA TAC CAG CAT CAC CGT	805 bp
NPR1	AF143442	**F** **r**	CAT GCA AGT GAC CCT GAA CTG CGA CTG TTG ACG CAG GTT GTC CGC CTG	456 bp (TomNPR1)
NPR1	AF143442	**F** **r**	GCA GAT CAA TCA AAT GGA GCG GGC TTG CTC TCG TGG TCT GGC AAG CCA	699 bp (LycNPR1)
PR-1	M69247	**F** **r**	CAC TCT TGT GAG GCC CAA AAT TCA CC TAC TTT AAT AAG GAC GTT CTC CAA CC	427 bp
PR-4	X58548	**F** **r**	TGT CAT CAA CAT GAT GAT GGC GGT GGC ATA GCC CAA TCC ATT AGT GTC CAA TCG	349 bp
PR-5	X70787	**F** **r**	GAC TTA CAC TTA TGC TGC CAC TTT CGA G GGT AGC TAT ACG CAT CAG GAC ATC TTT G	560 bp

Table 4: Accession numbers, primers sequences and length of the amplicons of the internal control and the different target genes in the multiplex RT-PCR assay.

3.3 Results

3.3.1 Polysaccharides *in vitro* and *in vivo* assays

All the strains grown on PDA medium showed the typical morphological structures of the referred species (Figure 6). None of the extracted polysaccharides showed a direct antimicrobial activity towards *Erwinia amylovora* or *Xanthomonas arboricola* pv. *pruni* at any concentration (0.1% and 0.01%), while only the pullulan solution (0.1% w/v) from *Cryphonectria parasitica* induced a weak spot-like necrosis in leaves of tomato plants cv Money Maker one week after the treatment.

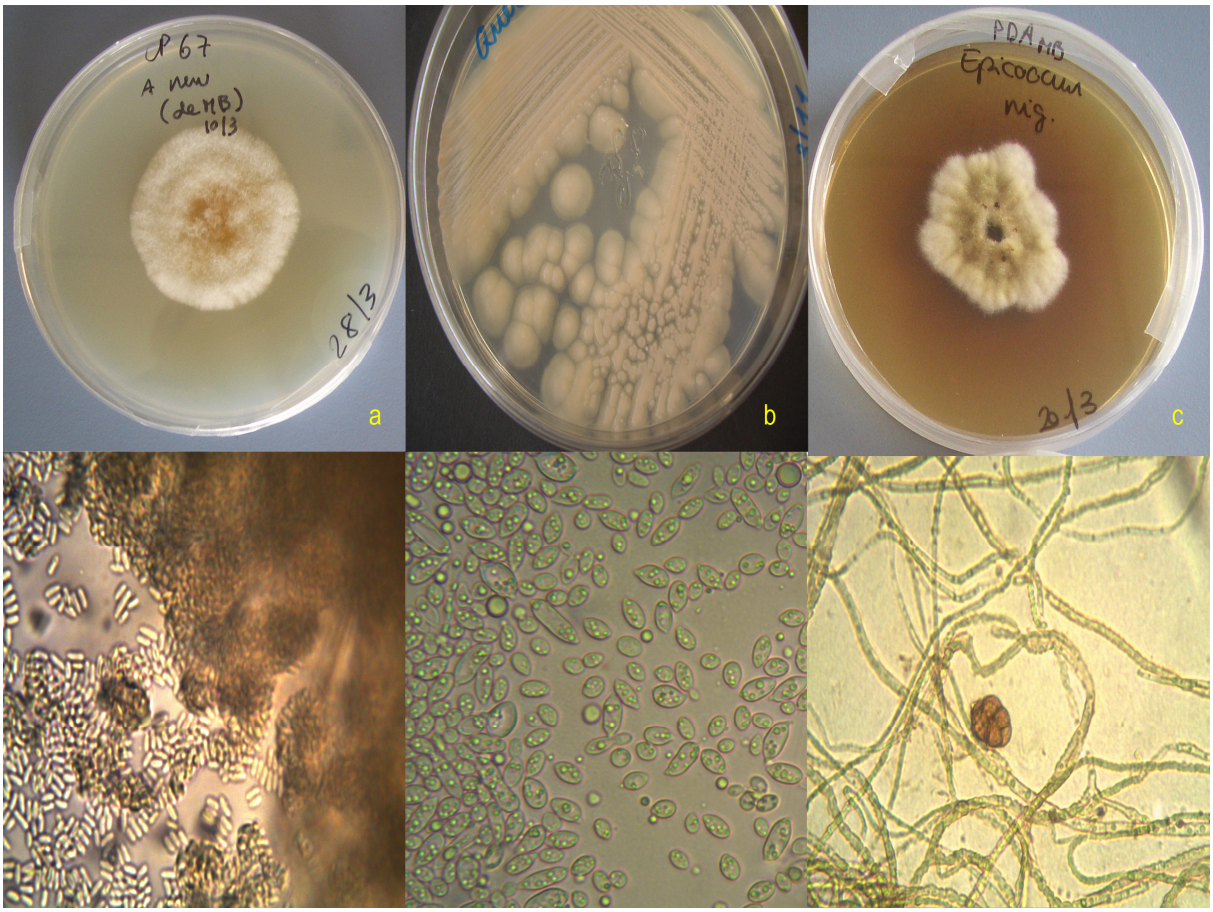


Figure 6: From left to right, *Cryphonectria parasitica*, *Aureobasidium pullulans* and *Epicoccum nigrum* on PDA medium (top), and relative conidia (bottom).

Both the concentrations (0.1% and 0.01% w/v) of polysaccharide solutions from *Aureobasidium pullulans* strain 3998 and 4958, *Cryphonectria parasitica* EP67 (named CP67 in the assays) and *Epicoccum purpurascens,* were able to prevent the

induction of HR normally triggered by *Pseudomonas syringae* pv. *syringae* on leaves of tobacco plants cv White Burley. Interestingly, prevention of necrosis in the infiltrated panel seems to be directly correlated with the concentration of the polysaccharide solution (Figure 7), which reaches its maximum level of protection at 0.1% (w/v). However, inoculation of a 0.1 % solution (w/v) of saccarose didn't prevent the hypersensitive response. Differently, inoculation of the non-pathogenic strain m5 of *Pseudomonas putida* 24 hours before inoculation of *P. syringae*, prevented the process of necrosis and triggered a response of the plant, expressed through a weak chlorosis restricted to the inoculated area (picture not shown). The experiment was repeated several times, and showed that pullulans derived from *Cryphonectria parasitica* EP67 were the most effective in term of HR prevention.

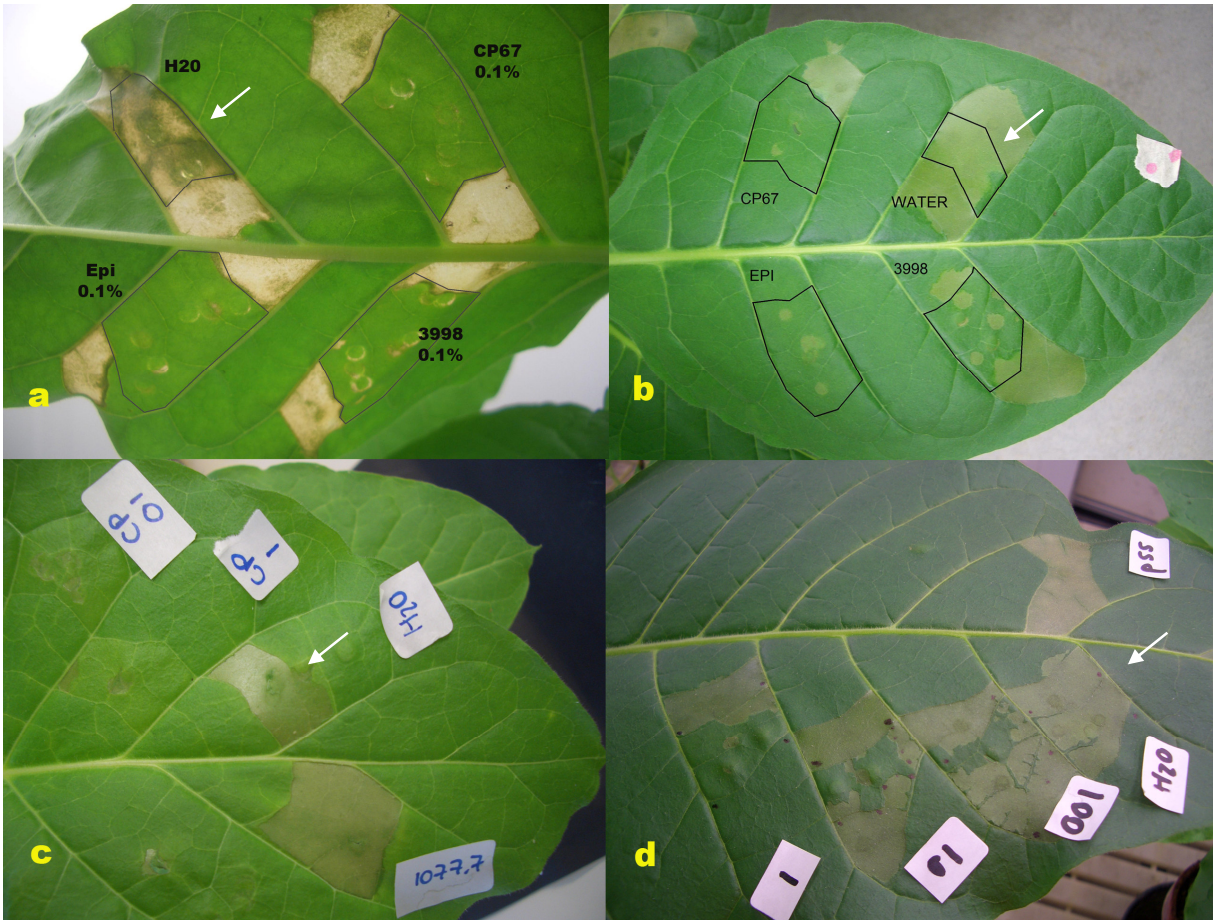


Figure 7: Pre-infiltrating tobacco panels with polysaccharide solutions (0.1%) from *A. pullulans* (3998), *C. parasitica* (CP67) and *E. purpurascens* (Epi), caused prevention of HR induced by strain 6285 of *Pseudomonas syringae* pv. *syringae* with respect to the water control (a and b, white arrow). Local HR prevention obtained by pre-infiltrating panels with a 0.1% and 0.01% polysaccharide solution from *C. parasitica* (c) and effect of the decreasing concentration of pullulan solutions derived from *A. pullulans* 3998 on the extent of necrosis prevention (d). The number "1" stays for 1 mg/mL (0.1% w/v).

3.3.2 Isoelectrofocusing (IEF): chitinase and peroxidase activity

Activity of both basic and acidic isoforms of plant chitinases and peroxidases was induced by all treatments respect to the negative control (Figure 8). In particular, activity of three main isoforms of acidic chitinases ($3.5 \leq pH \leq 4.3$) was induced in plants treated with Bion® and pullulan solutions from *A. pullulans* strain 4958 and 3998. Intense basic peroxidase activity (isoforms with $pH \geq 9$) was observed in tomato plants treated with chitosan and bacterial OLS (0.2% w/v), while plants treated with Bion® and pullulan solutions from *A. pullulans* strain 4958 and 3998 showed weaker bands, maybe due to staining problem.

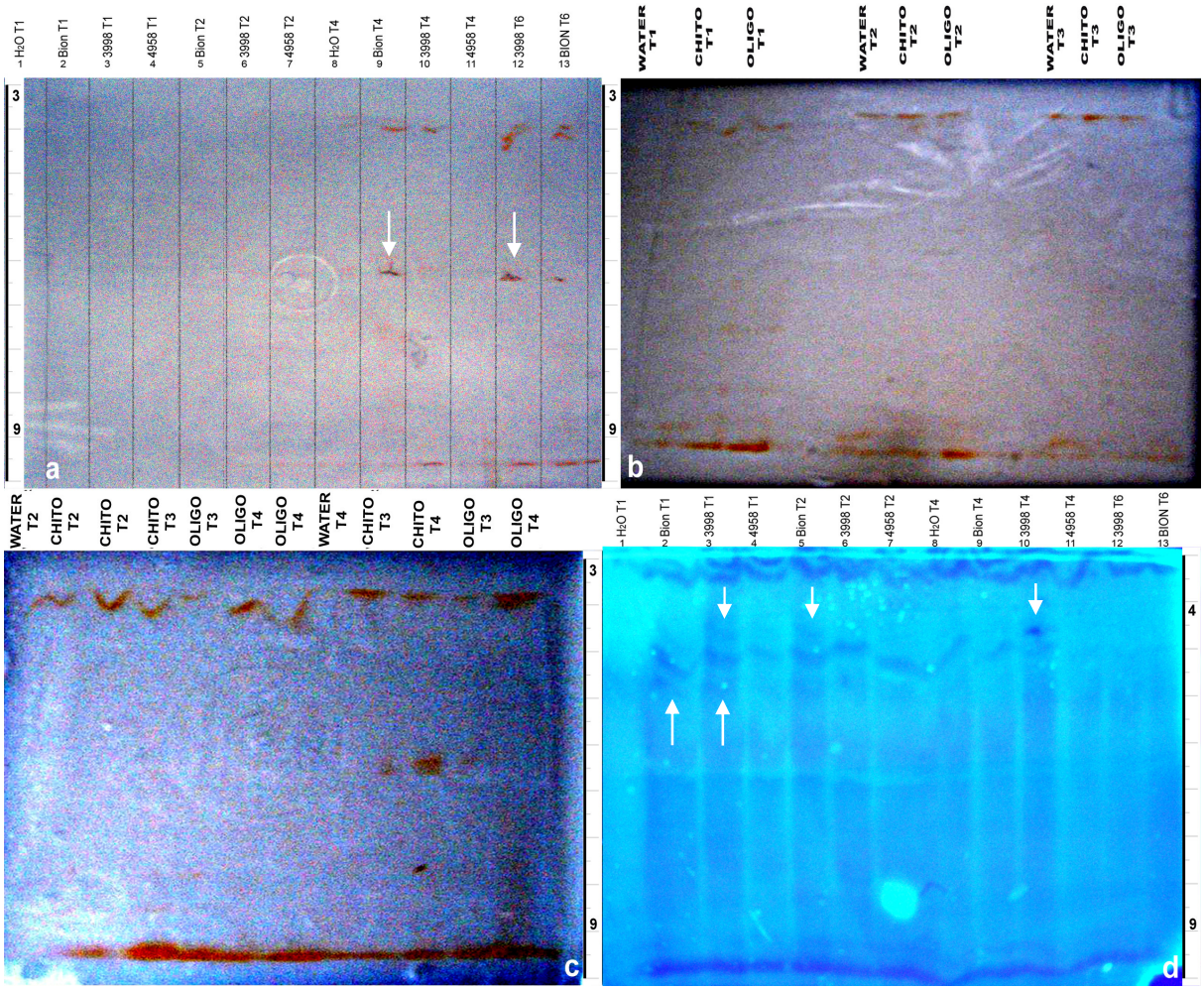


Figure 8: Basic and acidic isoforms of tomato peroxidase (a, b and c) and chitinase (d) induced by pullulans solutions, bacterial OLS, chitosan and the abiotic resistance inducer Bion®. Arrows show particular isoforms expressed in plants treated with Bion® and pullulan from *A. pullulans* strain 3998 (T1=0h; T2=24h; T3=72h; T4=96h; T6=7 days). A pH scale is indicated beside every single picture.

3.3.3 NPR1 cloning

The 456 bp NPR1 sequences amplified from cDNA of tomato (*Lycopersicon esculentum*) cv Money Maker (MM) and Perfect Peel (PP) by primers TomNPR1 and cloned in pGEMT Easy Vector, showed high identity with the NPR1 interactor protein used as reference (Accession Number AF143442, Zhang *et al.,* 1999). Sequences of clones TomNPR1-MM and TomNPR1-PP were blasted in Genbank database showing respectively 93% and 97% identity with the reference sequence (Figure 9). Both the inserts also share homology with the *Nicotiana tabacum* leucine zipper transcription factor TGA2.1 (87% identity in the case of TomNPR1-MM and 91% identity for TomNPR1-PP). Sequences have been submitted to Genbank.

```
MM GENE ID: 543600 NIF1 | NPR1-interactor protein 1 [Solanum lycopersicum]
(10 or fewer PubMed links)
Score = 612 bits (331), Expect = 5e-172
Identities = 388/415 (93%), Gaps = 5/415 (1%)
Strand=Plus/Minus

Query  4    TCCCTAAATTTTCCCCATAGCCATTGCCATCTGA-CCATATAATTAGCTACTTCTCCTGA  62
            ||||| || ||||||||||||||||||||||||| ||||||||||||||| ||||||||
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Query  63   TGACCCTTCAGAAGCAGGAGATCCGTTAACTGATGTCTCTGCCAAGGATTGCTGCCCCTC  122
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Sbjct  184  CGTAATGTGCAGTGACATTATTCACAATACTTCGCAGTTCAGGGTCACTTGCATG  130


PP GENE ID: 543600 NIF1 | NPR1-interactor protein 1 [Solanum lycopersicum]
(10 or fewer PubMed links)
Score =  715 bits (387),  Expect = 0.0
Identities = 411/422 (97%), Gaps = 4/422 (0%)
Strand=Plus/Plus

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Query  374  GGAAAAAAATTAGGGACTCTTGAAGGTTTTCTCCGTCAGGCGGACAACCTGC--CAACA-ACA 432
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Sbjct  528  GGGGAAA-TTAGGGACTCTTGAAGGTTTTCTCCGTCAGGCGGACAACCTGCGTCAACAGACA 588
```

Figure 9: BLAST alignments of TomNPR1-MM and Tom NPR1-PP sequences with the reference NPR1 interactor protein sequence (Genbank: AF143442).

3.3.4 Multiplex RT-PCR assay

A map of the treatments for the multiplex RT-PCR assay is shown in Table 5. First multiplex reverse transcriptase PCRs immediately showed that NPR1 (456 bp) is constitutively expressed in *Lycopersicon esculentum* and its transcript levels are constant and comparable with those of the internal control EF1 (805 bp).

As shown in Figure 10, no difference can be noticed in transcription of the two genes between the negative control and plants treated with Bion® and glucans from *A. pullulans* 3998, *C. parasitica* EP67 and *E. purpurascens.* This confirms that NPR1 is constitutively expressed in an inactive oligomeric state and its transcripts increase can only be observed at the protein level (Cao *et al.,* 1998; Dong, 2004).

1	W α1		***24 h***		***72 h***		***7 days***
2	W α1	**17**	W α2	**32**	W α3	**47**	W α4
3	W β1	**18**	W β2	**33**	W β3	**48**	W β4
4	W γ1	**19**	W γ2	**34**	W γ3	**49**	W γ4
5	3998 α1	**20**	3998 α2	**35**	3998 α3	**50**	3998 α4
6	3998 β1	**21**	3998 β2	**36**	3998 β3	**51**	3998 β4
7	3998 γ1	**22**	3998 γ2	**37**	3998 γ3	**52**	3998 γ4
8	Epi α1	**23**	Epi α2	**38**	Epi α3	**53**	Epi α4
9	Epi β1	**24**	Epi β2	**39**	Epi β3	**54**	Epi β4
10	Epi γ1	**25**	Epi γ2	**40**	Epi γ3	**55**	Epi γ4
11	CP67 α1	**26**	CP67 α2	**41**	CP67 α3	**56**	CP67 α4
12	CP67 β1	**27**	CP67 β2	**42**	CP67 β3	**57**	CP67 β4
13	CP67 γ1	**28**	CP67 γ2	**43**	CP67 γ3	**58**	CP67 γ4
14	BION α1	**29**	BION α2	**44**	BION α3	**59**	BION α4
15	BION β1	**30**	BION β2	**45**	BION β3	**60**	BION β4
16	BION γ1	**31**	BION γ2	**46**	BION γ3	**61**	BION γ4

Table 5: Map of the treatments and repetitions for the multiplex RT-PCR assay testing the effects of exopolysaccharides solutions on tomato plants cv Money Maker. W=water control. Glucans extracted from: *A. pullulans* (3998), *C. parasitica* (CP67) and *E. purpurascens* (Epi).

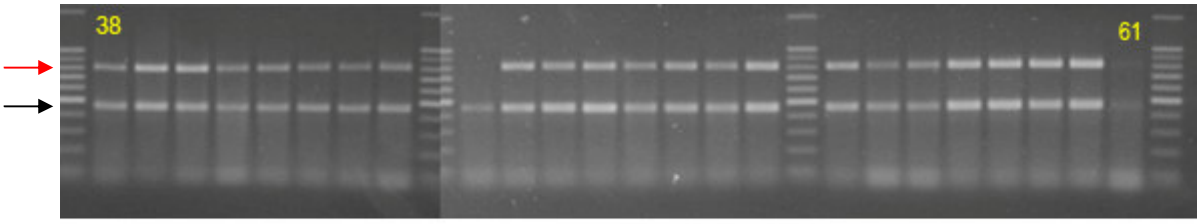


Figure 10: Multiplex RT-PCR showing the constant and comparable transcription of the target gene NPR1 (→456 bp) with the internal control EF1 (→805 bp) between the series of treatments from n° 38 to n° 61.

Regarding transcription of the pathogenesis-related protein PR-1 in tomato, it can be confirmed the efficacy of the abiotic elicitor Bion® in inducing plant resistance: increase in PR-1 transcription was noticed in all the three replica (n° 30, 31, 44, 45, 60, 61), starting 24 hours after the treatment. Between the glucan solutions, CP67 (n° 11, 26, 28, 41, 56, 57) and Epi (n° 23, 25, 39, 53, 55) were the most effective in inducing expression of the pathogenesis related protein, while the pullulan solution derived from *Aureobasidium pullulans* 3998 induced transcriptional increase of PR-1 only in two of the plants tested (n° 5 and 7) at time 0 (Figure 11 and Table 5).

Bion® strongly induced transcription of the pathogenesis-related protein PR-4 in all the plants monitored, starting from 24 hours after the treatment (n° 29, 30, 31, 44, 45, 46, 59, 60, 61). Glucans from *A. pullulans* 3998 were shown to increase the transcript level of PR-4 already at time 0 and were the most effective between the tested solutions (n° 5, 7, 20, 21, 22, 35, 36, 37, 50, 51, 52). Exopolymers from *Epicoccum purpurascens* (n° 23, 25, 38, 39, 53, 54, 55) and *C. parasitica* CP67 (n° 12, 26, 28, 41, 42, 57) were also able to induce expression of PR-4 (Figure 12). Curiously, PR-4 transcription was also induced in the same control plants which previously showed an increase in the transcript level of PR-1 (n° 17, 33, 48, 49).

Again, tomato plants treated with Bion® showed a high transcript level of the pathogenesis-related protein PR-5 (n° 29, 31, 44, 45, 46, 59, 60, 61). Also glucans extracted from *Epicoccum purpurascens* strongly induced transcription of PR-5 (n° 23, 25, 38, 39, 40, 53, 54, 55) compared to tomato plants treated with polysaccharides extracted from *A. pullulans* (n° 21, 22, 35, 36) and *C. parasitica* (n° 26, 41, 43, 56, 57, 58). Also in this multiplex RT-PCR assay, the water control plants n° 17, 33, 48 and 49 showed transcription of PR-5 (Figure 13), suggesting that stressing conditions may have occurred conditioning the result.

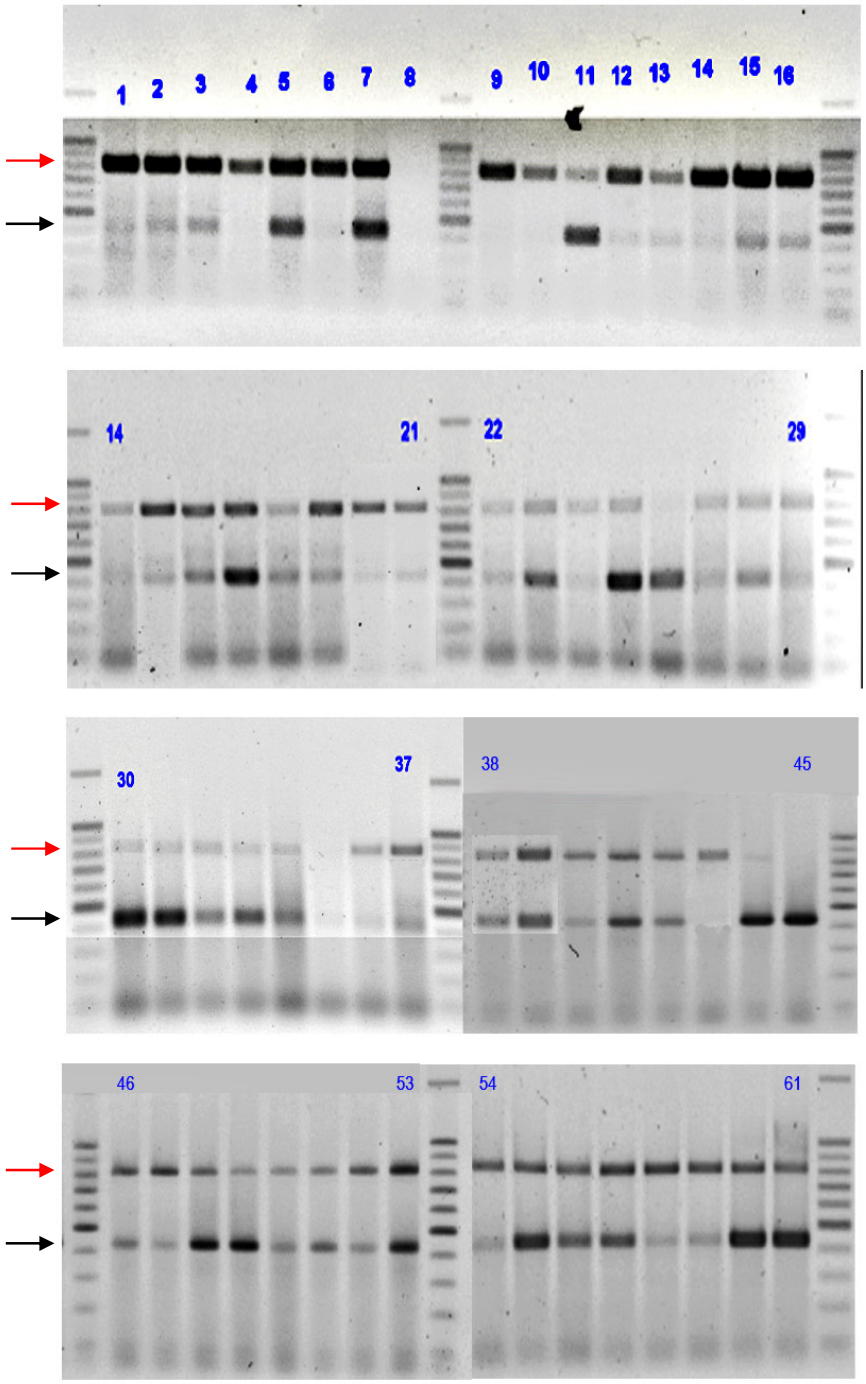


Figure 11: Transcription of PR-1 (427 bp, black arrow) monitored through multiplex RT-PCR in *Lycopersicon esculentum* cv Money Maker following treatments with glucan solutions and the abiotic inducer Bion®. The internal control EF1 (805 bp) is indicated with a red arrow.

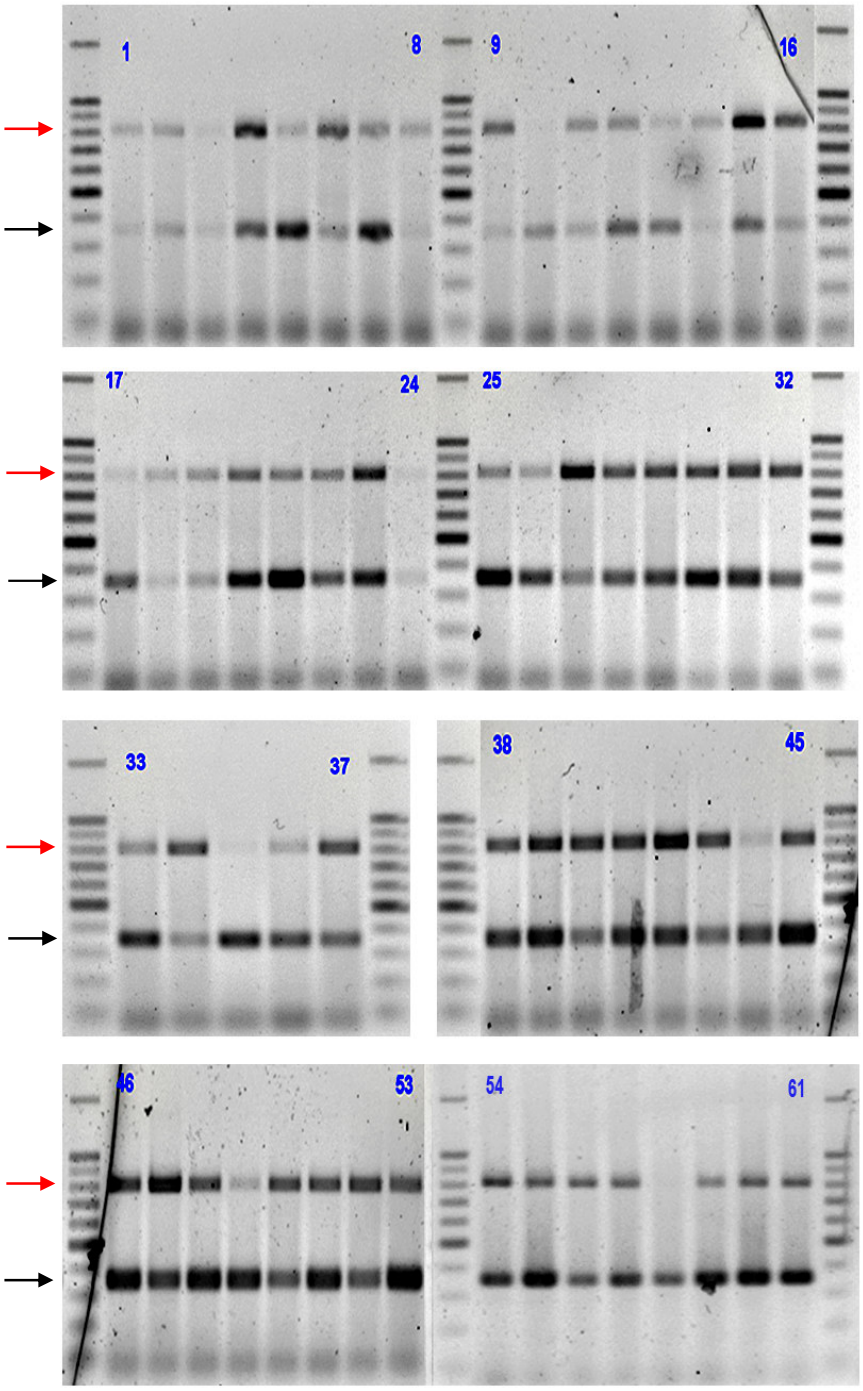


Figure 12: Transcription of PR-4 (349 bp, black arrow) monitored through multiplex RT-PCR in *Lycopersicon esculentum* cv Money Maker following treatments with glucan solutions and the abiotic inducer Bion®. The internal control EF1 (805 bp) is indicated with a red arrow.

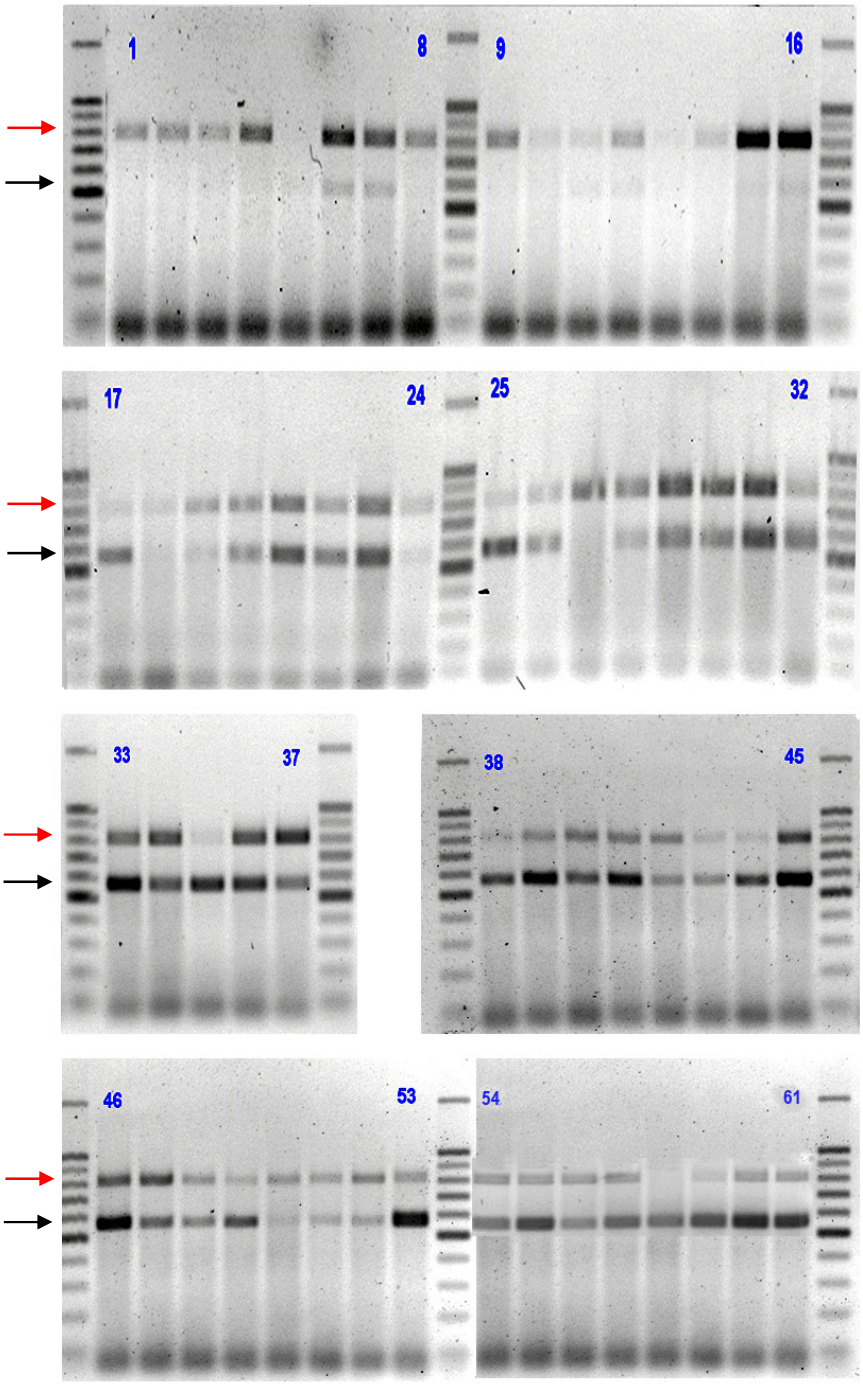


Figure 13: Transcription of PR-5 (560 bp, black arrow) monitored through multiplex RT-PCR in *Lycopersicon esculentum* cv Money Maker following treatments with glucan solutions and the abiotic inducer Bion®. The internal control EF1 (805 bp) is indicated with a red arrow.

3.4 Discussion

Antigenic activity of extracellular polysaccharides produced by many opportunistic fungi is widely investigated in animal systems. The first year of the PhD was also spent studying literature about glucans able to induce an immune response both in animal and plants. By the next year, activity of polysaccharides extracted by the three selected strains was determined in tomato plants cv Money Maker. None of them showed direct antibacteric activity *in vitro*.

The infiltration of pullulan and fungal polysaccharide solutions into panels of tobacco leaves prevented the induction of HR by *P. syringae* pv. *syringae*. This result is not surprising: highly conserved components of the cell wall of microorganisms have often a role in virulence and they may induce a plant defense response similar to the one induced by the microorganism itself. This is for example the case of bacterial flagellin. Also lipopolysaccharides from Gram negative bacteria are important molecules with antigenic activity, and some of them have been shown to prevent the induction of HR caused by *Erwinia amylovora* in leaves of tobacco plants (Bazzi *et al.*, 2003b). It's clear, however, that injecting a polysaccharide solution in the intercellular environment creates mechanical and osmotic stresses, and this may invalidate a hypothesis of direct induction of local resistance by a biotic elicitor. For this reason, it was decided to test whether a 0.1% sucrose solution and a suspension of a non-pathogenic bacterial strain could also trigger prevention of HR. Confirming the first hypothesis, the bacterial suspension did prevent the HR (in spite of chlorosis), while the sucrose solution did not. This experiment has demonstrated that molecules derived from the microorganism are the real determinants of HR prevention. All the tested polysaccharides were able to influence the plant immune response in leaves of tobacco, confirming their role as potential elicitors referable to PAMPs (Bent and Mackey, 2007).

The effect of the fungal polyasaccharides in triggering tomato defense responses was evaluated monitoring accumulation of plant peroxidases and chitinases and transcription of the pathogenesis-related proteins PR-1, PR-4 and PR-5.

Pullulans produced by the fungal species *Aureobasidium pullulans* 3998, have been shown to trigger the accumulation of plant chitinases and to strongly induce transcription of the pathogenesis-related protein PR-4 already at time 0, while low transcription profiles have been determined for PR-1 and PR-5. Since expression of the latter genes is strongly induced by accumulation of salicylic acid in several plant species, while expression of PR-4 has been correlated with the accumulation of jasmonic acid in vine and *Arabidopsis* (Hamiduzzaman *et al.*, 2005; Van Loon *et al.*, 2006), I hypothesize that α-glucans could activate this last signaling pathway in tomato. The fact that pullulan from *Cryphonectria parasitica* could also induce

transcription of PR-1, is possibly related to its role as a virulence factor in plant pathology. In fact, *C. parasitica,* causal agent of chestnut blight, is the only phytopathogenic microorganism between the ones selected for this thesis. Exopolymers produced by some strains can even elicit phytotoxicity in tomato (Corsaro *et al.*, 1998), and pullulans extracted from strain EP67 of *C. parasitica* were the most effective in terms of HR prevention in tobacco. Therefore, it's not surprising that perception of this molecule has induced additional defense responses of the plant.

It would have been very useful to compare these results with transcriptional profiles of PR-4 in plants treated with jasmonic acid, but in this experiment only Bion® was used as positive control.

However, also Bion® strongly induced an increase of transcription for PR-4, as well as for PR-1 and PR-5. Since this compound is able to trigger plant resistance against a large number of fungal and bacterial pathogens, it probably activates more than one single pool of defense genes depending on the pathogen and the host species, or it may induce the synthesis of new plant metabolites.

In the isoelectrofocusing assay, problems occurred in the staining of one gel for the assessment of peroxidase synthesis in plants treated with *A. pullulans* 3998. However, comparing the weak results with the ones obtained in the chitinase assay, it can be deduced that also pullulans may induce accumulation of these defense enzymes.

Exopolysaccharides extracted from the fungus *Epicoccum purpurascens* have been shown to induce high transcription levels of the pathogenesis-related gene PR-5. The PR-5 proteins include a large family of proteins that play a role in membrane permeability and osmotic stress, but most importantly they show ability to bind polymeric β-1,3-glucans, or exhibit endo-β-1,3-glucanase activities (Trudel *et al., 1*998; Menu-Bouaouiche *et al.,* 2003; van Loon *et al.,* 2006). Considering that glucans from *E. purpurascens* also induced a weak transcription of PR-1 and PR-4, it seems promising to use exopolysaccharides derived from this ubiquitous species not only to stimulate the human immune system (as already proved), but also to induce accumulation of defense compounds in plants.

As a conclusion, the multiplex RT-PCR assay has demonstrated that all the polysaccharides object of this study were able to differently induce transcription of pathogenesis-related (PR) proteins with respect to the negative control (water). Only plants treated with the abiotic inducer Bion® (positive control) showed transcription of all the three PRs at time 0.

Perception of these molecules probably involves epidermal receptors or mechanosensors bound to the cell wall and, possibly, to stomata. Indeed, stomata function as innate immunity gates that perceive bacterial virulence factors (PAMPs) and actively prevent their entry in the plant cell (Melotto *et al.*, 2006).

Even if it was planned to compare the chemical structure of the polysaccharides extracted using as a reference molecule pullulan from *Aureobasidium pullulan*s (82550, Fluka), a molecular characterization of the exopolymers couldn't be performed on time.

The regulatory component of the SAR and ISR pathway, NPR1, was cloned and chosen as a target gene because the initial aim of the thesis implied a combination of the multiplex RT-PCR assay with the Real Time PCR technique to quantify amplified DNA.

NPR1 is constitutively expressed in *Arabidopsis*, and can be further induced by pathogen infection or by salicylic acid (SA) or 2,6-dichloro-isonicotinic acid (INA) treatment (Cao *et al*., 1997; Ryals *et al*., 1997). When I realized from literature that levels of its transcripts are increased only two-fold following SA treatment (Cao *et al.*, 1998), the experiment was already started. Real Time PCR will be performed in order to confirm a NPR1 transcript increase (even if small) in tomato plants treated with fungal glucans and Bion®. Comparing transcription of NPR1 with the internal control EF1 has demonstrated that this gene is constitutively expressed also in tomato. Moreover, submission to Genbank of the partial sequences of NPR1 cloned from two cultivars of *Lycopersicon esculentum* (Money Maker and Perfect Peel) has provided additional information about this gene.

However, problems related to transcription of the gene EF1 have also occurred in some cases. Regardless of the experimental technique employed, appropriate normalization is essential for obtaining an accurate and reliable quantification of gene transcript levels. The success of this normalization strategy is highly dependent on the choice of the appropriate control gene: expression levels of the internal control should be relatively constant across the tissues, and shouldn't be altered by the applied experimental procedures (Huggett *et al.*, 2006). A widely used housekeeping gene, β-actin, has been reported to be an unsuitable internal control for RT-PCR since it's highly regulated by matrigel (Selvey *et al.*, 2001) and studies aimed to selection of housekeeping genes for the oomycete *Phytopthora parasitica* have shown that not only β-actin, but also elongation factor 1 α (EF1), are not suitable internal controls for real-time quantitative RT-PCR due to their variable expression levels (Yan and Liou, 2006).

Only at the end of the last year, a work assessing the expression stability of 11 housekeeping genes in tomato was published: showing the widest range of expression level, EF1 was ranked only tenth in the list of the candidate control genes (Expósito-Rodríguez *et al.*, 2008). Comparison with transcription of other housekeeping genes and optimization of PCR settings would have been useful in order to critically evaluate the choice of EF1 as an internal control in this assay.

4 Race specific resistance: the pathosystem *Cladosporium fulvum*-tomato

4.1 Introduction

Race-specific resistance is the most specific response of a plant to a harmful microorganism, since it results only from the interaction of a particular pathogen race with a particular cultivar of the host plant. It is the proof of adaption and evolution of plant resistance mechanisms towards the appearance of new pathogen races able to defeat non-host basal defense responses. This type of resistance is usually referred to as gene-for-gene resistance (Flor, 1942; 1971), because in most cases it requires the presence of both a race-specific avirulence (avr) gene in the pathogen and one or more complementary cultivar-specific resistance (R) genes in the host plant. Surprisingly, R genes that confer resistance to different types of pathogens encode very similar proteins, indicating that in plants, flexible recognition systems are used to monitor attacks by a diverse array of pathogens (van der Hoorn *et al.,* 2001).

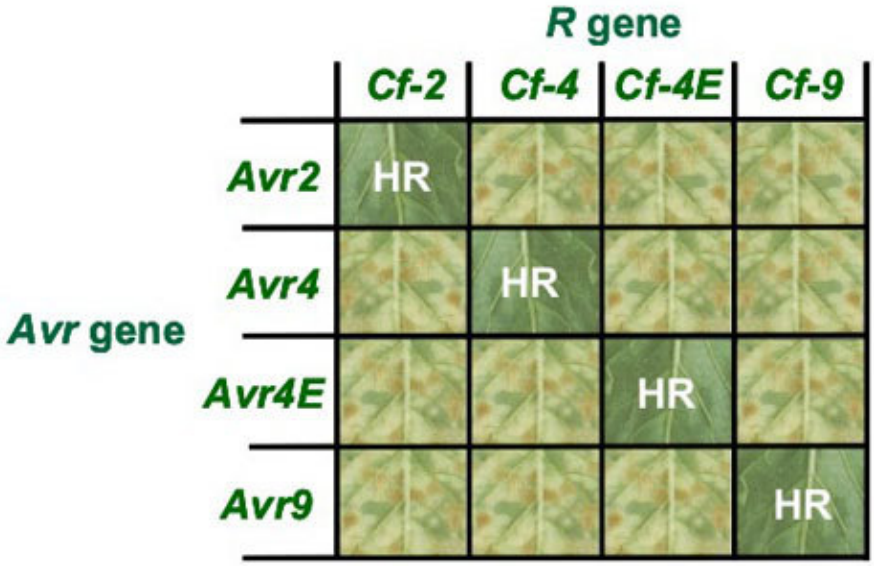
Race-specific resistance is normally shown towards biotrophic pathogens, which are efficiently defeated through the hypersensitive response (HR), typically triggered in plants by specific R-Avr recognition. Well known host-pathogen interactions obeying to the gene-for-gene theory are presented in Table 6.

Host	Disease	Pathogen	Reference
Flax (*Linum ultissimum)*	Rust	*Melampsora lini*	lslam and Shepherd (1991)
Wheat (*Triticum aestivum*)	Stem and leaf rusts	*Puccinia* spp	Roelfs (1988)
Barley (*Hordeum vulgare*)	Powdery mildew	*Erysiphe graminis* f. sp. *hordei*	Jørgensen (1994)
Lettuce (*Lactuca sativa*)	Downy mildew	*Bremia lactucae*	Crute (1991)
Maize (*Zea mays*)	Rust	*Puccinia sorghi*	Hulbert and Bennetzen (1991)
Tomato (*Lycopersicon esculentum*)	Leaf mold	*Cladosporium fulvum*	de Wit (1992); Jones *et al.* (1993)
Tomato (*L. esculentum*)	Bacterial speck	*Pseudomonas syringae* pv. *tomato*	Ronald *et al.* (1992)
Common bean (*Phaseolus vulgaris*)	Halo blight	*Pseudomonas syringae* pv. *phaseolicola*	Jenner *et al.* (1991)
Common bean (*P. vulgaris*)	Bean common mosaic virus	BCMV	Spence and Walkey (1995)

Table 6: Well known race-specific resistance models.

The interaction between the biotrophic fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva*) and its only host, tomato, is one of the best-studied pathosystems that follows the gene-for-gene relationship (de Wit, 1995; Joosten and de Wit, 1999; Thomma *et al.,* 2005) and it's a useful model system to study the molecular basis of signaling events between a pathogen and its host.

Race-specific resistance against *C. fulvum* in tomato is governed in a gene-for-gene manner by dominant *C. fulvum* (Cf) resistance genes that mediate recognition of corresponding race-specific avirulence proteins (Avr) of the fungus and activate a defense cascade, culminating in a hypersensitive response (HR) and host immunity (Joosten and de Wit, 1999; Rivas and Thomas, 2005). Lack of recognition leads to a compatible interaction. During pathogenesis, hyphae remain strictly intercellular without penetrating the mesophyll cells; close contact between the fungal hyphae and plant cells suggests that the pathogen actively withdraws nutrients from the host (Lazarovitis and Higgins, 1976). Typical symptoms of the disease are chlorotic spots that gradually become necrotic and are visible on both sides of the infected leaves. Since *C. fulvum* strictly grows in the apoplastic space of its host, various race-specific avirulence proteins produced by the fungus have been isolated from apoplastic fluid. Upon purification of a specific Avr protein, its presence is monitored by injection of partly purified fractions into leaves of a tomato plant expressing the R gene corresponding to the Avr protein of interest. When the Avr protein is present, Cf-mediated necrosis will appear in the injected areas one or two days after injection (Figure 14). This has resulted in the identification and characterization of different Avrs and their corresponding resistance genes. Notably, Cf-9 (Jones *et al.,* 1994), Cf-2 (Dixon *et al.,* 1996), Cf-4 (Thomas *et al.,* 1997), Cf-4E (Takken *et al.,* 1998) and Cf-5 (Dixon *et al.,* 1998) have been cloned from tomato.



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http://www.php.wur.nl/UK/Research/Cladosporium/

Figure 14: The gene-for-gene theory in the model *Cladosporium fulvum*-tomato: specific resistance mediated by matching R-Avr gene interaction culminates in HR.

The fact that most Avr genes are maintained within *C. fulvum* races suggests that their products, in addition to their role as avirulence factors, have a function that is beneficial for the pathogen (White *et al.,* 2000). Plant pathogens secrete molecules called effectors that contribute to the establishment of disease to their hosts. Besides the avirulence gene products, *Cladosporium fulvum* secretes a number of extracellular proteins (ECPs) into the apoplast which act as virulence factors in compatible interactions (Wubben *et al.,* 1994; Laugé *et al.,* 1997; 2000). Like Avrs, Ecps induce a resistance response in tomato accessions carrying not yet identified Cf-Ecp resistance genes. Remarkably, although all *C. fulvum* effector proteins share some common feature as their small size and even number of cysteine residues, they display no significant sequence similarity to each other or to protein sequences deposited in public databases (Van Esse *et al.,* 2008).

4.1.1 The role of the protease Rcr3 in the Cf2-Avr2 interaction

The basic assumption in the gene-for-gene interaction is that R proteins behave like receptors for the effector ligands (Gabriel and Rolfe, 1990; Keen, 1990). Structural features of the R proteins support this model, as a majority of the R proteins have well-conserved leucine rich repeat (LRR) domains which mediate protein-protein interaction (Dangl and Jones, 2001).

Even if direct interaction between R and Avr gene products has been demonstrated in a few cases (Jia *et al.,* 2000; Leister and Katagiri, 2000; Deslandes *et al.,* 2003; Ron and Avni, 2004), a direct physical interaction between Cf proteins and Avr proteins has not been detected (Luderer *et al.,* 2001). In light of such observations, the original receptor-ligand model was amended to add a new dimension to the R-Avr interaction. The R protein has been assigned the role of a sentinel of cellular machinery, guarding key virulence targets inside the cell (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). This “guard hypothesis” proposes that Avr proteins are virulence factors that interact with host targets to facilitate pathogen growth in the host. The Cf protein perceives the altered status of the virulence target and induces a rapid defense response.

Avr2-producing *C. fulvum* strains trigger an HR in tomato plants harboring the Cf-2 resistance gene. For this response, an additional plant factor is required, a cysteine protease named Rcr3 (required for *C. fulvum* resistance) which is monitored by Cf2 (Krüger et al 2002). By using the irreversible protease inhibitor DCG-04, Rooney et al. (2005) showed that Avr2 binds and inhibits Rcr3, and that the Avr2-Rcr3 complex enables the Cf-2 protein to activate a HR. Recently, it has been demonstrated that Avr2 inhibits several other cysteine proteases that are required for basal defense in tomato and *Arabidopsis* (Van Esse *et al.,* 2008), thus confirming the role of the race-specific elicitor in targeting PAMPs-induced defense mechanisms.

Similarly, Avr4 was found to be a lectin with chitin-binding activity that can protect fungal cell walls against the deleterious effects of plant chitinases (van den Burg *et al.,* 2006), thus promoting the virulence of several fungal pathogens in *Arabidopsis* and tomato (Van Esse *et al.,* 2007). It has been proposed that Avr4 may be recognized directly by the tomato Cf-4 resistance protein (Westerink et al. 2002). This is not the case for the Cf2-Avr2 interaction: further studies are necessary to unravel whether and where Cf-2 identifies the Avr2-Rcr3 complex, and possibly, if other players are involved in the recognition process. The interaction must take place in the apoplast, where Rcr3 performs its defense role and Avr2 is secreted. However, the complex is thought to interact with the extracellular LRR domain of Cf-2 in the proximity of the cell membrane (Figure 15).

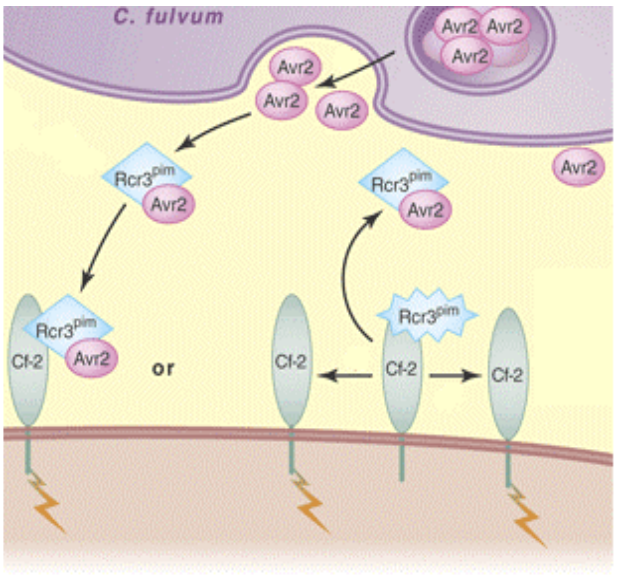

Figure 15: Possible modes of interaction between the Avr2-Rcr3 complex and Cf-2. On the left, the complex is directly recognized by the extracellular LRR domain of the receptor protein. On the right, conformational changes in Rcr3 due to Avr2 activity are perceived by Cf-2.

4.1.2 Use of fluorescent tags for protein-protein interaction study

Identifying the destination or localization of a protein is key both to understanding its function and to facilitating its purification (Lu *et al.,* 2005). Optical microscopy has been very useful to obtain information about the sub-cellular location of proteins. However, classical light microscopy, for example, cannot reveal whether proteins interact with one other (Hink *et al.,* 2002).

The discovery and cloning of the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* have revolutionized studies in cell

biology by enabling the dynamic monitoring of protein localization in the living cell using fluorescent microscopy (Prasher *et al.,* 1992; Sheen *et al.,* 1995). Because of their intrinsic fluorescence ability and minimal toxicity, fluorescent proteins have been widely used as non-invasive markers in many living organisms. Fusing an open reading frame to a fluorescent protein, such as green, yellow, red or cyan fluorescent proteins (GFP, YFP, RFP or CFP, respectively), can be useful for determining the subcellular localization of a protein and for testing interactions with other fluorescently tagged proteins (Hanson and Kohler, 2001; Earley *et al.,* 2006).

A further development in the use of fluorescent proteins was the demonstration that protein-protein interaction in the living cell could be detected by Forster resonance energy transfer (FRET) in fluorescence lifetime imaging microscopy (FLIM), by which the fluorescence lifetime of a fluorescent dye can be determined as a function of intracellular space (Hink *et al.,* 2002). FRET-FLIM is a technique used for identifying and quantifying the distance between two molecules conjugated to different fluorophores (fluorescent tags). FRET is also known as fluorescence resonance energy transfer because it implies the non-radiative transfer of energy between a fluorophore in the excited state (donor) to another other one in the ground state (acceptor). When the distance between the two molecules is small (≤10 nm) the emission spectrum of the excited donor overlaps with the absorption spectrum of the acceptor, which emits energy in the form of fluorescence (Figure 16). In conjunction with the recent development of a variety of mutant fluorescent proteins, FRET microscopy provides the potential to measure the interaction of molecules in intact living cells where the donor and acceptor fluorophores are actually part of the molecules themselves. By combining FLIM with FRET it is possible to obtain quantitative temporal and spatial information about the binding and interaction of proteins *in vivo*. Cultures of mesophyll protoplasts have been utilized in several plant species to investigate protein-protein interactions, since they can be transiently transformed with plant vectors expressing the fusion proteins of interest.

The first successful experiment for the introduction of nucleic acid into protoplasts was accomplished by Aoki and Takebe using tobacco mesophyll protoplasts and tobacco mosaic virus RNA (Aoki and Takebe, 1969). Compared with cell culture lines, the use of fresh tissues as protoplast sources offers unique advantages. For example, protoplasts isolated from plant tissues retain their cell identity and differentiated state; they show high transformation efficiency with low maintenance. These freshly isolated protoplasts have proven to be physiological and versatile cell systems for studying a broad spectrum of plant signaling mechanisms (Sheen, 2001). For example, freshly isolated mesophyll protoplasts perform active photosynthesis and respiration (Kanai and Edwards, 1973). Protoplasts also retain cell membrane potentials similar to intact cells and have served as a model system to study membrane transporters (Bauer *et al.,* 2000; Hamilton *et al.,* 2000). In the last ten years, tobacco, maize, potato (*Solanum*

tuberosum), and *Arabidopsis* protoplast transient expression assays have also been used to study protein stability control (Worley *et al.,* 2000), protein targeting and trafficking (Kleiner *et al.,* 1999; Jin *et al.,* 2001; Ueda *et al.*, 2001; Aker *et al.*, 2007) and protein-protein interactions (Subramaniam *et al.*, 2001).

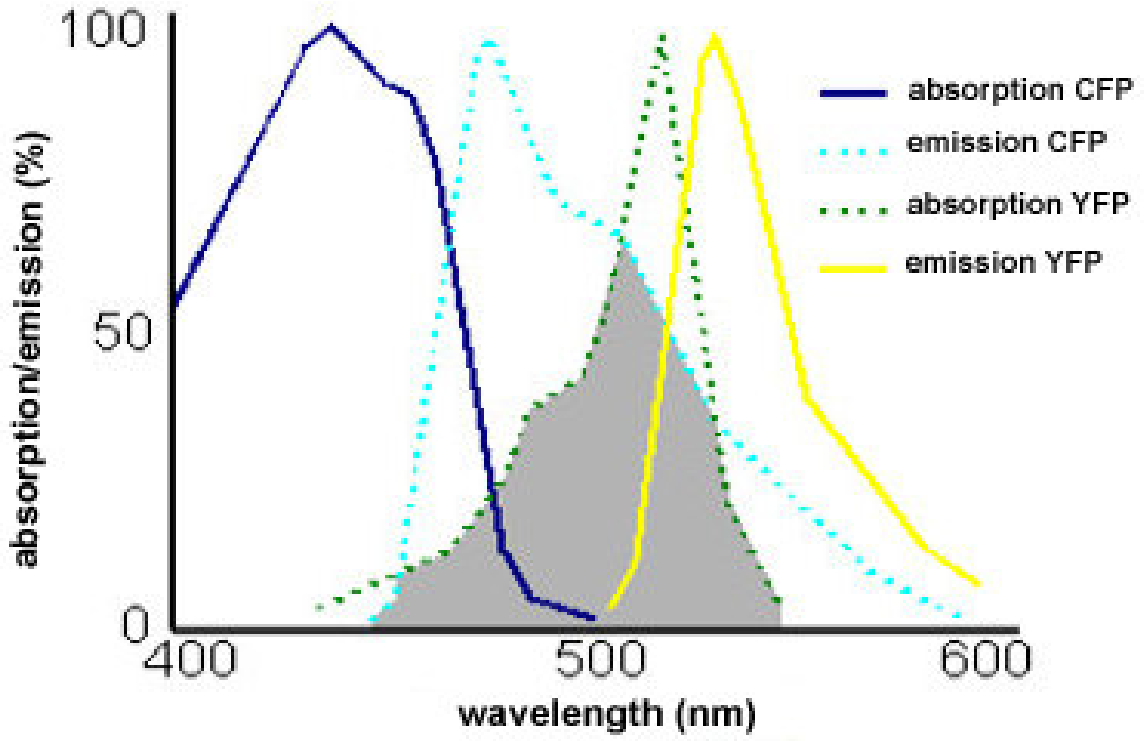

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Figure 16: Absorption and emission spectra of Cyan Fluorescent Protein (CFP, donor) and Yellow Fluorescent Protein (YFP, acceptor) in FRET-FLIM.

In order to localize the interaction of the Rcr3-Avr2 complex with the Cf2 resistance protein of *Cladosporium fulvum*, protoplast cultures of tomato leaves can be transiently transformed with plant vectors containing the sequences of the cysteine protease Rcr3 and the resistance protein Cf2 respectively fused to the fluorescent tags mCherry and EGFP. Adding a signal peptide for extracellular targeting and a 35S promoter for proper expression *in planta*, the interaction of the tagged proteins can be revealed by FRET-FLIM microscopy. The sequences encoding for these "fusion proteins" can also be cloned in *Pichia pastoris*, a methylotrophic yeast frequently used as a recombinant protein expression system (Sreekrishna *et al.* 1997), to perform biochemical studies on the interaction of the tagged molecules.

4.2. Materials and methods

4.2.1 Protoplasts isolation protocol (adapted from Sheen, J. 2002)

Leaves of *Lycopersicum esculentum* cv 'Moneymaker' were collected from the isogenic line carrying the resistance protein Cf2, the Cf0 line that contains no genes for resistance to *Cladosporium fulvum*, and the Cf2 rcr3-3 mutant, lacking the plant cysteine protease needed to establish an interaction with the *C. fulvum* avirulence protein Avr2. In order to digest the middle lamella and the cell walls, leaves were first infiltrated through a vacuum pump with a 0.7 M mannitol solution containing 1% cellulase and 0.5% pectinase (maceroenzyme). Mannitol is a disaccharide which helps to maintain an osmolarity similar to that of the protoplasts. Additional isolation procedures were carried out with a 0.4 M mannitol solution and 0,25 % or 0,05 % pectinase. The pH of the enzyme solution was always adjusted to 5.5 to mimic Rcr3 apoplastic environment. Greater numbers of protoplasts can be obtained by adding 0.1 N NaOH to the mannitol solution to obtain pH 6-7. Leaves were then placed in a Petri dish, cut with a sharp razor blade in small pieces (2×2 mm) and incubated in 15 mL of enzyme solution for 2 to 3 hours, shaking gently at room temperature. Leaves can also be cut before vacuum infiltration of the enzyme solution. Protoplasts were released swirling gently the dish by hand, and the solution was filtered through a 50 µM nylon mesh into a round-bottomed tube. Protoplasts were spinned down at 50g for 5 minutes/RT in an eppendorf centrifuge and washed in 5 mL of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7). Further washing steps were carried out with a 0.4 M mannitol solution.

To check the viability of protoplasts 500 µL of the cultures were stained at dark for 15 minutes with 50 µL fluorescein diacetate (FDA, 100 µg/mL dissolved in acetone) and 50 µL propidium iodide (PI, 20 µg/mL dissolved in SDW) and analyzed by fluorescence microscopy. The principle of staining with FDA relies on the non polar FDA molecule crossing the plasma membrane and its ester bonds being hydrolyzed in the cytoplasm to release fluorescein. The polar fluorescein molecule remains in the cytoplasm because it cannot pass through either the plasma membrane or the tonoplast of living cells (Huang, 1986). Propidium Iodide can only penetrate dead protoplasts and it's used to asses plasma membrane integrity. It binds to DNA of damaged cells which subsequently show a red fluorescent colour that provides an excellent contrast to FDA.

4.2.2 Cloning: Rcr3-mCherry and Cf2(Cf4)-EGFP

First cloning attempts were carried out in order to obtain the C-terminal fusion protein Rcr3-mCherry (Figure 17). The tag is an enhancement of the monomer RFP, derived from the *Dicosoma* sp. fluorecent protein "DsRed" (Shaner *et al.*, 2004). Sequence of the protease Rcr3 was PCR amplified from plasmid DNA of

the vectors pPIC9-His-HA-Rcr3p (sequence derived from *Lycopersicon pimpinellifolium*) and pPIC9-His-HA-Rcr3e (derived from *L. esculentum*) with forward primer Rcr3-prepro and reverse Rcr3-Xho (Table 7), and from colonies of *Agrobacterium tumefaciens* GV3101 carrying the vector pBIN19-Rcr3p and pBIN19-Rcr3e. mCherry fragment, carried on pGEM®-T Easy vector in *Escherichia coli* strain DH5α, was obtained from Wladimir Tameling (Phytopathology, WUR). Vector pPIC9-6His, containing a multiple cloning site (MCS) with a polyhistidine-tag, was double digested with Promega restriction enzymes SmaI/NotI (keeping the His-tag) or SnaBI/NotI (cleaving the His-tag off). Rcr3 PCR product was digested with XhoI and purified on GFX® column (GE Healthcare, UK). mCherry fragment was gel extracted from SalI/NotI digested plasmid DNA and purified by GFX® or QIAGEN® columns. The linear vector and the two fragments were fused through a three point ligation at 14°C overnight, using T4-DNA ligase (M1804, Promega) and adding 1 µL of ATP to the mix. Product of ligation was cloned in *E. coli* DH5α competent cells (Invitrogen) following manufacturer's protocol. Colonies were screened through PCR using vector primers 5AOX and 3AOX (Table 7). Size of the plasmid (9.8 Kb) was checked through double digestion with NotI/EcoRV. Plasmid DNA of positive *E. coli* DH5α transformants was always purified using the QIAprep Spin Miniprep Kit (27106, Qiagen) following manufacturer's protocol. Plasmid DNA of *Agrobacterium tumefaciens* was purified following a standard protocol (http://www.arabidopsis.org/comguide/table_of_contents.html) from Paul Ebert (Institute of Biological Chemistry, Washington State University, U.S.A.).

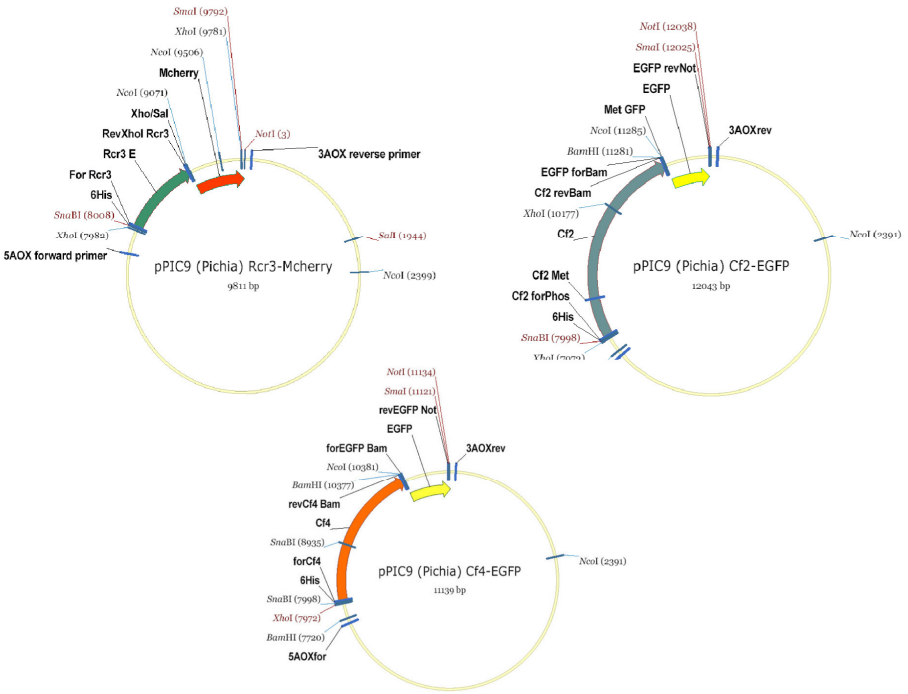

Figure 17: The three fusion constructs inserted in vector pPIC9-6His and cloned in *E. coli* DH5α.

The second step of cloning within the project was obtaining the C-terminal constructs Cf2-EGFP and Cf4-EGFP (Figure 17). The fluorescent tag is an enhanced version of the original wild-type green fluorescent gene found in the jellyfish *Aequorea victoria*. Cf2 sequence, carried on vector pSLJ7291 in *E. coli* DH5α, was PCR amplified with primers Cf2-forphos and Cf2-revBam (Table 7). Cf4 sequence, carried by vector Mog101 in *A. tumefaciens* GV3101, was PCR amplified with primers Cf4-forphos and Cf4-revBam (Tab. 7). The fluorescent tag EGFP, carried on vector pRAPN, was furnished by Jan Roosien (Nematology, WUR). The sequence (around 770 bp) was amplified from plasmid DNA by PCR with primers EGFP-forBam and EGFP-revNot (Tab. 7).

Target sequence	Length	Primers
MCS of pPIC9-6His	0.5 Kb	5AOX for 5' gactggttccaattgacaagc 3' 3AOX rev 5' gcaaatggcattctgacatcc 3'
Rcr3	1 Kb	Rcr3 preprofor 5' cgcagccagccaaaactgtccgtg 3' Rcr3 xhorev 5' ggcctcgagtgctatgtttggataagaaga 3'
mCherry	0.7 Kb	mCherryR5'ctgtacaagctcgagtaacccgggaaatcactagtgaattcgcggccgc 3'
Cf2	3.2 Kb	Cf2 forPhos 5' tcgactgaggaggcaactgccc 3' Cf2 revBam 5' cgcggatccgaagtgattatttcttcttctg 3'
Cf4	2.3 Kb	Cf4 forPhos 5' tcatccttacctcatttgtgccccg 3' Cf4 revBam 5' ggatcctatcttttcttgtgctttttcattttcg 3'
EGFP	0.7 Kb	EGFP forBam 5' aataatcacttcggatccatggtgagcaagggcgag 3' EGFP revNot 5' ttaattcgcggccgcccagatctcccgggtacc 3'
Cf2_EGFP	3.9 Kb	Cf2OE-EGFPf 5'agaagaagaaataatcacttcatggtgagcaagggcgaggag 3' Cf2OE-EGFPrev 5' ctcctcgcccttgctcaccatgaagtgattatttcttcttct 3'
Cf4_EGFP	3.0 Kb	Cf4OE-EGFPf 5'atgaaaaagcacaagaaaagaatggtgagcaagggcgaggag 3' Cf4OE-EGFPrev 5' ctcctcgcccttgctcaccattcttttcttgtgctttttcat 3'

Table 7: Target sequences and primers used in this work to obtain the vectors for the expression of the fusion proteins Rcr3-mCherry, Cf2-EGFP and Cf4-EGFP.

Cf2 and Cf4 PCR products were purified by GFX® column and digested with BamHI. EGFP PCR fragment was purified by GFX® column and double digested with BamHI/NotI. The two fragments were ligated into vector pPIC9-6His-MCS digested with SmaI/NotI (keeping the His-tag), and transformed in *E. coli* DH5α. In order to overcome problems encountered in cloning, an OE-PCR (overlap PCR) approach was chosen. Thus, four overlapping primers (Table 7) were designed and used to amplify Cf2/Cf4 and EGFP in two separate PCR reactions. The fragments obtained were then joined and amplified in a subsequent PCR reaction using the external primers of the two regions of interest, giving as a final product the fusion sequences Cf2-EGFP and Cf4-EGFP (Figure 18). PCR products of the third reaction were gel extracted, purified through GFX® column and digested with NotI.

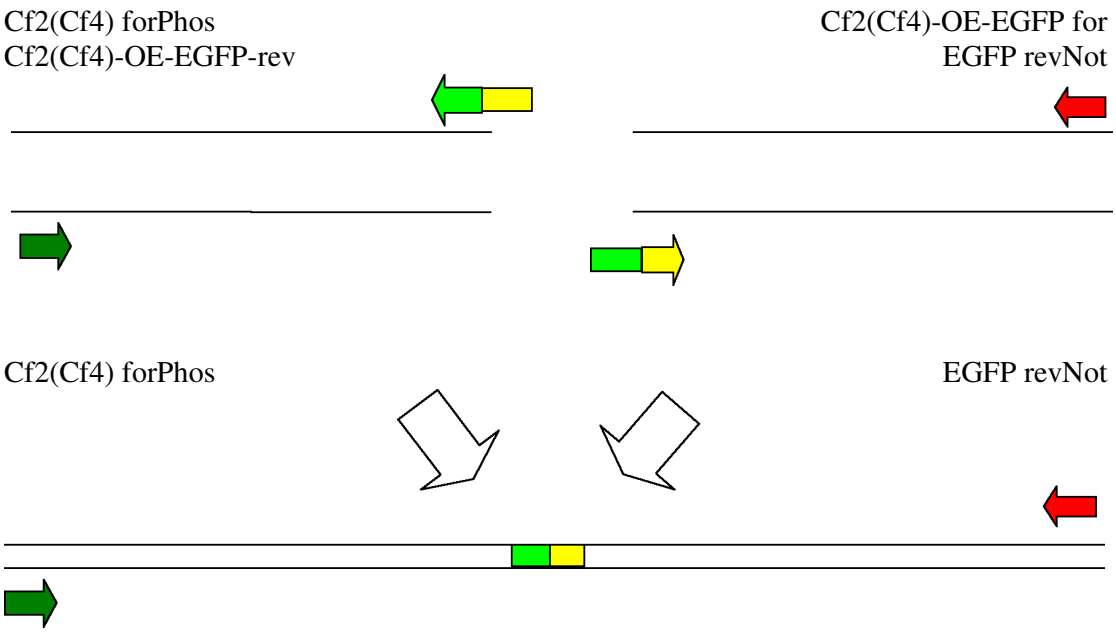

Figure 18: Overlap-PCR technique. Two short overlapping sequences (bicoloured arrows) are used as forward and reverse primers in two separate PCR reaction. Amplicons obtained are joined together and used as a template to run a third PCR with the external primers of the two sequences of interest (red and green arrow).

A new strategy was finally adopted to obtain a better fusion product through a normal PCR reaction. BamHI digested Cf2/Cf4 fragments and BamHI digested EGFP fragment were ligated at 14°C overnight, using T4-DNA ligase (M1804, Promega). The next day, fused fragments were amplified by PCR with the external primers (Cf2/Cf4 forward and EGFP reverse). Final product was digested with NotI and purified through GFX® column, ligated overnight with the SnaBI/NotI or SmaI/NotI digested vector pPIC9-6His-MCS and finally transformed into *E. coli* DH5α. Colonies were screened through PCR using forBam EGFP and 3AOX as primers. Size of the two plasmids (12 and 11 Kb) was double-checked by restriction analysis using NcoI/XhoI and BamHI (Promega).

4.2.3 Transformation of *Pichia pastoris*

Plasmid DNA of pPIC9-6His-derived vectors described above was purified through MIDI-prep (QIAGEN®) and linearized with SalI (Promega) to be cloned into *Pichia pastoris* strain GS115 (Invitrogen) for expression of the fusion proteins.

The yeast strain was grown in 50 mL of YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose) at 30°C overnight, shaking at 140 rpm in a New Brunswick/Innova 4230 incubator. The next day, 100 mL of fresh medium were inoculated with 2 mL of GS115 culture. After aproximatively 5 hours (OD_{600}=1.4),

cells were centrifuged at 1500g for 5 min at 4°C. The washing step was repeated two times and the pellet was finally resuspended in 2-3 ml of ice-cold 1 M sorbitol. Aliquots (80 µL) of the cells from the previous step were mixed gently with 15 µg of SalI-linearized plasmid DNA, transferred to an ice-cold 0.2 cm electroporation cuvette and incubated on ice for 5 min. Cells were pulsed according to the parameters for yeast (*Saccharomyces cerevisiae*) suggested by the manufacturer of the specific electroporation device being used (Biorad Gene Pulser). After electroporation, 1 mL of ice-cold 1 M sorbitol was immediately added to the cuvette and gently mixed. The cuvette contents were spread (200 µL/aliquot) on minimal dextrose (MD) plates and incubated at 30°C until the appearance of colonies. Transformants were grown in BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 1% methanol, 100mM K H2PO4, pH 6.0) daily supplemented with methanol to a final concentration of 1% (v/v). After removing cells by centrifugation, proteins in the culture supernatant (CS) were separated on Tricine SDS-PAGE and analyzed on Western blots, using specific antibodies against the two tagged proteins.

4.2.4 Western blotting and protease activity profile (DCG-04 assay)

Production of Rcr3-mCherry by *P. pastoris* was checked by SDS-PAGE, western blotting with Rcr3-specific antibody (α-Rcr3, Eurogentec) and protease activity profiling DCG-04.

To monitor Rcr3 activity, protease activity profiling was performed at pH = 5 by using DCG-04, a biotinylated derivative of the irreversible cysteine protease inhibitor E-64 that has been used to profile cysteine protease activities from mammals, insects and plants (Greenbaum *et al.*, 2000; Kocks *et al.*, 2003; van der Hoorn *et al.*, 2004). DCG-04 treatment leads to irreversible labeling of cysteine proteases with biotin. Since avidins bind preferentially to biotin, biotin-tagged molecules can be extracted from a sample by mixing them with beads with covalently-attached avidin. Protease activity profiling with DCG-04 was performed as described by Greenbaum *et al.* (2000). Biotinylated proteins were captured on streptavidin beads (Promega), run on a SDS gel, and probed with streptavidin–horseradish peroxidase (SA-HRP, Sigma).

Briefly, CS of *P. pastoris* was diluted 10 fold in DCG-04 assay buffer (50 mM NaAc, 10 mM L-cysteine, pH 5.0) to a final volume of 500 µl, DCG-04 (220 nM final concentration) was added and the reaction mixture was incubated at room temperature for 5 hours. Proteins were precipitated by adding 1 ml of ice-cold acetone, washed with 70% (v/v) acetone and subsequently dissolved in 500 µl TBS buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.5). Biotinylated Rcr3-mCherry protein was bound to magnetic streptavidin beads (Promega) by incubating for 16 hours at 4°C. Proteins were eluted from the beads by boiling in SDS sample buffer and analysed by SDS-PAGE and western blotting with streptavidin-HRP. Sample protocol for western blot was derived from Rooney *et al.* (2005).

4.2.5 Gateway® Cloning Technology

Gateway® cloning technology exploits the bacteriophage lambda recombination system, thereby bypassing the need for traditional ligase-mediated cloning. Once captured in a Gateway®-compatible plasmid 'entry vector' (pENTR™/D or pENTR™/TEV/D), an open reading frame or gene flanked by recombination sites can be recombined into a variety of "destination vectors" that possess compatible recombination sites. These plant destination vectors have been designed for a variety of specific purposes including protein localization, promoter functional analysis, gene overexpression, gene knockdown by RNA interference, production of epitope-tagged proteins for affinity purification, or analysis of protein/protein interactions (Earley *et al.*, 2006). For the Protoplast Transient Expression Assay, Cf2(Cf4)-EGFP and Rcr3-Mcherry needed a PR-1a sequence for extracellular targeting and a constitutive promoter, like the CaMV 35S, for proper expression *in planta*. For this last purpose, Gateway® plant destination vectors were chosen. PR-1a sequence was added to Cf2(Cf4)-EGFP and Rcr3-Mcherry through PCR on plasmid DNA, using as reverse primers EGFP-revNot and mCherry-rev respectively, and adding to the 5' end of the forward primers a 96 bp sequence encoding the signal peptide. Products of the first PCR reaction were gel extracted on GFX® column, and amplified through a second PCR, using the same reverse primers and a common forward primer (VAP_PR1_GateF_b), made up of the first 21 bp of PR-1a but with a CACC site at the 5' end (Table 8).

Primer	Sequence (5'→3')
PR-1a	**atgggatttgttctcttttca**caattgccttcatttcttcttgtctctacacttctcttattcctagtaatatcccactcttgccgtgcccaaaat
VAP_PR1_GateF_b	cacc**atgggatttgttctcttttca**

Table 8: PR-1a and VAP_PR1 forward primers used for extracellular targeting and incorporation of the sequence in the pENTR™/D-TOPO® entry vector

This sequence facilitates directional incorporation into Invitrogen's pENTR™/D-TOPO® entry vector. Two different series of plant destination vectors for protein overexpression and epitope tagging and affinity purification were chosen: series pGWB from Tsuyoshi Nakagawa (Research Institute of Molecular Genetics, Shimane University, Matsue, Japan) and series pMDC from Mark Curtis (Institute of Plant Biology, University of Zurich, Switzerland). The first includes vectors pGWB n° 16, 17 and 18, carrying a C or N-terminal 4×Myc-tag, with or without 35S promoter upstream of the cloning site. Among the second series we have opted for pMDC32, a constitutive expression vector harboring a dual 35S promoter. The three "entry" vectors have also been recombined in pK2GW7, another plant destination vector expressing a constitutive 35S promoter and commonly used at Nematology, WUR. Recombination between the entry and the destination vector (LR reaction) is carried out in 1 hour at RT; cloning protocol was downloaded from www.untergasser.de/lab. The resulting recombinant plasmids were transformed in *E. coli* DH5α.

4.3 Results

4.3.1 Protoplasts isolation

Protoplasts isolation protocol from tomato leaves, adapted from Sheen J. (2002), gave high yield of viable mesophyll and epidermal protoplasts, whose survival lasted for almost 24 hours, thus making them suitable for transient expression assay. Viability of stained protoplasts was observed through a Zeiss confocal microscope. Red fluorescent cells indicate the break of the plasma membrane, thus a dead protoplast. Green fluorescent cells indicate the release of fluorescein in the cytoplasm, thus a living protoplast (Figure 19).

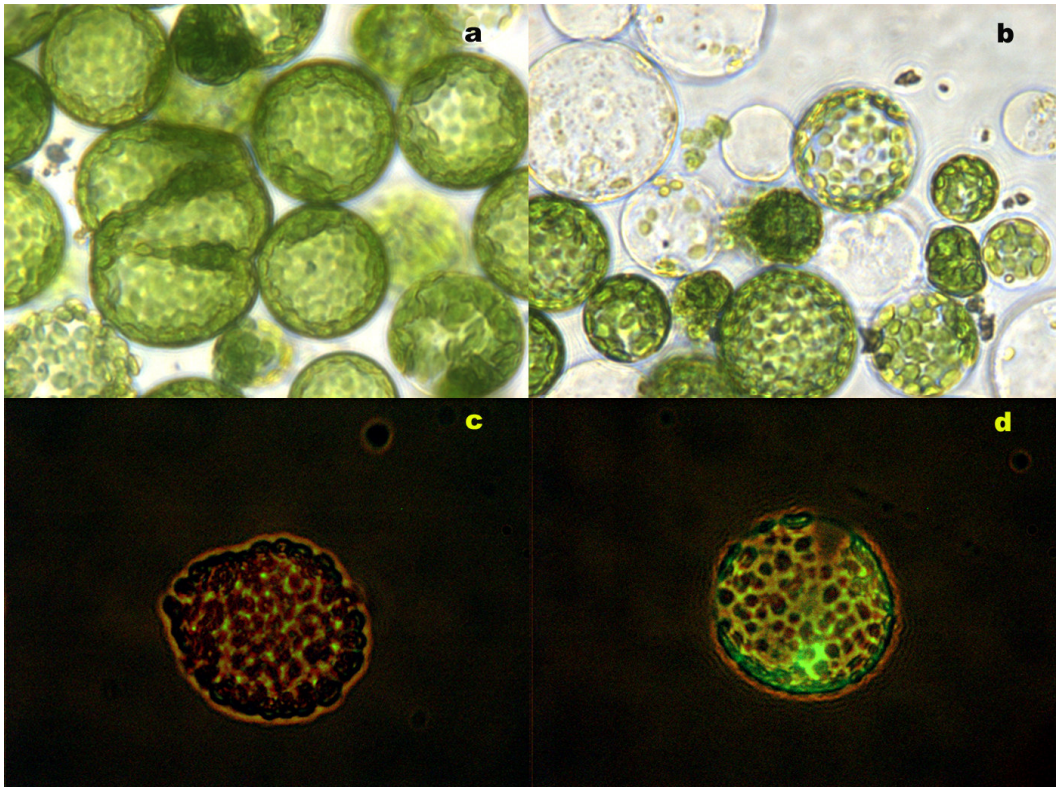

Figure 19: Mesophyll and epidermal protoplasts isolated from tomato leaves (a+b) and confocal microscope images of a dead (c) and a viable (d) protoplast stained with FDA and PI.

4.3.2 Cloning: Rcr3-mCherry and Cf2(Cf4)-EGFP

Three *E. coli* DH5α transformants were found to carry the pPIC9 vector harboring the Rcr3-mCherry sequence: M3CG n° 19 (SmaI clone), N3AC n° 1 and N3AC n°13 (SnaBI clone). Six positive clones were obtained in the cloning of Cf2(Cf4)-EGFP: A1 (Cf2-EGFP without His-tag) and B3(Cf2-EGFP with His-tag), E12, F10 (Cf4-EGFP with HIS-tag) and H2, H3 (Cf4-EGFP without His-tag).

Colonies of *E. coli* DH5α containing the pPIC9-(Rcr3-mCherry) vector showed a weak pink-red color as a probable consequence of the fluorescent protein's expression.

4.3.3 *Pichia pastoris* expression system: western blotting and DCG-04 assay

Both fusion proteins were successfully expressed in *Pichia pastoris* expression system. However, protease activity profiling with DCG-04 showed that the fluorescent tag mCherry is cleaved off when the cysteine protease Rcr3 is activated. In fact, even if the expected size of the Rcr3-mCherry fusion protein (63.4 kD) was confirmed by immunoblotting with α-Rcr3 antibodies, the protease activity profiling with DCG-04 detected only the non-tagged Rcr3 protease (23.3 kD, Figure 20). Conversion of the inactive proprotein to the mature and active form of Rcr3 is dependent on a short sequence present at the 5'end and encoding for one or more polypeptides. The removal of this sequence in the DCG-04 assay buffer probably caused Rcr3 to cleave off itself, thus removing the fused fluorescent protein mCherry. Confocal microscope analysis of *Pichia pastoris* cells carrying the construct Cf2-EGFP showed fluorescence. However, also control transformants expressing Avr4 (with no fluorescent tag) showed clear fluorescent cells. Apparently, *Pichia* cells emitted light via self-induced fluorescence through absorption of another invisible wavelength.

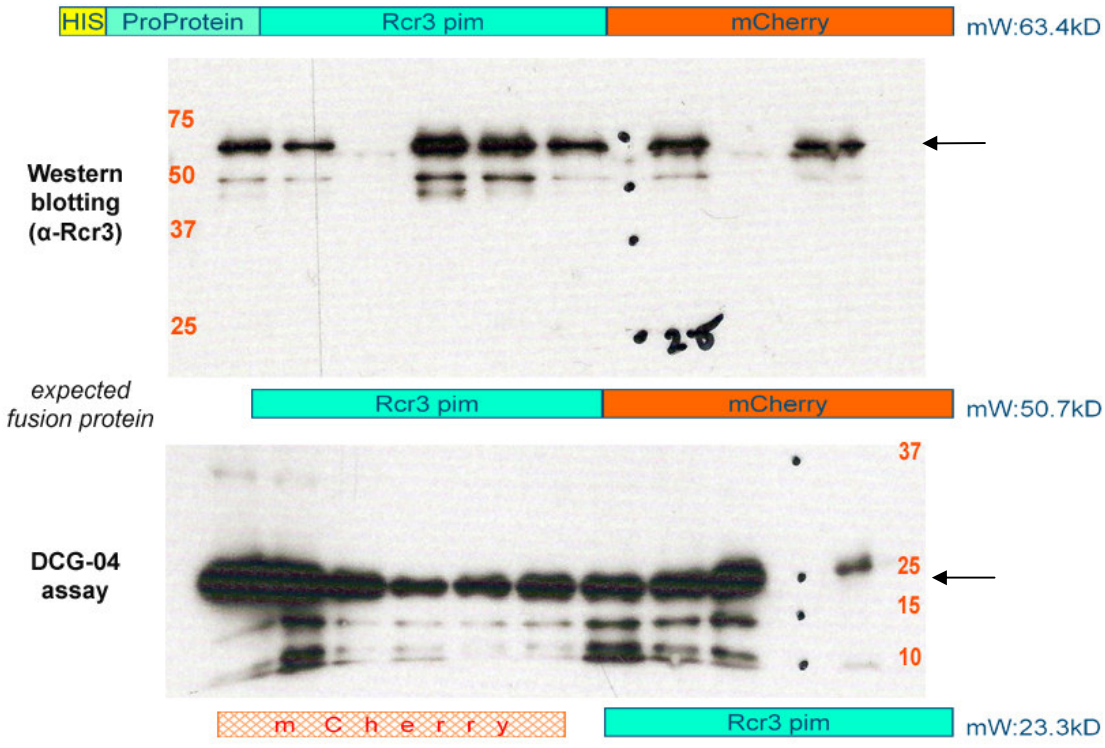

Figure 20: Protease activity profiling (DCG04-assay) detected only Rcr3 (23.3 kD), suggesting that the fluorescent tag mCherry is cleaved off by the activity of the cysteine protease.

4.3.4 Gateway® vectors

All the three constructs (Cf2-EGFP, Cf4-EGFP and Rcr3-mCherry), containing the PR-1a sequence for extracellular targeting of the fusion protein, have been cloned in the entry vector pENTR™/D and transformed in *E. coli* DH5α.

Destination vectors pMDC32, containing Cf2-EGFP and Cf4-EGFP with a double 35S promoter, and vectors pK2GWB, carrying the sequence of all the three constructs with a single 35S promoter, were successfully cloned in *E. coli* DH5α. Vector pGWB, carrying a 4×Myc-tag, wasn't received before the end of the project and is currently under construction (Figure 21). Several positive transformants derived from the two reactions were chosen (Table 9).

ENTRY VECTORS	pENTR™/D(Cf2-EGFP) 11 pENTR™/D(Cf4-EGFP) 4_1, 4_3, 4_4 pENTR™/D(Rcr3-Mcherry) R_1, R_2, R_3
DESTINATION VECTORS	pMDC32(Cf2-EGFP) 2_1, 2_2, 2_3, 2_4, 2_5, 2_6 pMDC32(Cf4-EGFP) 4_1, 4_2, 4_3, 4_4, 4_5 pK2GW7(Rcr3-Mcherry) R_1, R_2, R_3, R_4, R_5 pK2GW7(Cf2-EGFP) 2_2, 2_5, 2_6, 2_7, 2_8, 2_9, 2_{10} pK2GW7(Cf4-EGFP) 4_1, 4_2, 4_3, 4_4, 4_5, 4_6

Table 9: *E. coli* DH5α positive transformants obtained through Gateway® cloning technique

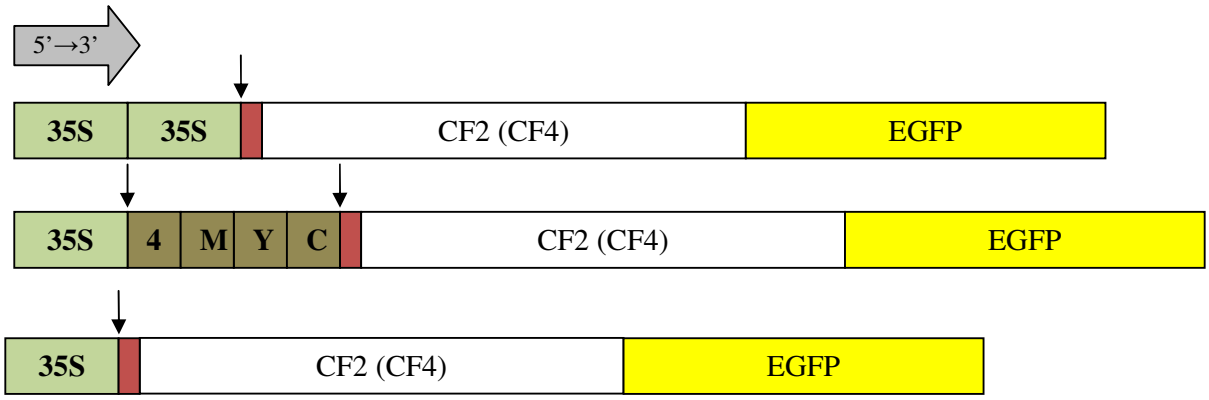

Figure 21: Plant destination vectors for Gateway® cloning technique. From the top to the bottom: pMDC32, pGWB and pK2GW7. Arrows indicate recombination sites with the "entry" vector.

4.4 Discussion

The project dealt with during the research period spent abroad couldn't be completed in the time agreed with the Department. Since the beginning, it was clear that the aim of this project was highly above my knowledge and skills, and I was immediately warned about difficulties and problems I could have encountered. Considering my poor experience with molecular biology, cloning sequences encoding for fusion proteins of 100 kDa required a long time. When the materials and methods for the protoplast transfection and FRET-FLIM microscopy were ready, it was time for me to go back to Italy. However, the support, the confidence and the unique work environment I found during my stay, helped me to face problems and disappointments, and improved my experience and my will to accomplish the project.

Race specific resistance elucidated by the gene-for-gene theory and triggered by the specific interaction between the tomato resistance protein Cf2 and the avirulence protein Avr2 of *Cladosporium fulvum*, is mediated by the plant protease Rcr3. The guard hypothesis has always fascinated me: investigating through fluorescence microscopy where this self-modified recognition occurs in the cell, and unraveling if others players are involved in this interaction was an extremely interesting objective. Although the use of fluorescent tags to track individual proteins in cells has a long history, the availability of new confocal microscopy and cloning techniques has furnished tools of great diversity and utility. Protoplast-based transient assay systems have provided advantages for many types of assays in plants.They have proven very useful for dissecting a broad range of plant signal transduction pathways, transcriptional regulatory networks, and evaluation of reporter gene expression (Mazarei *et al.*, 2008).

Unluckily, protease activity of Rcr3 cleaved off the mCherry tag and microscope analysis of protoplast cultures transfected with plant destination vectors couldn't be performed. Obtaining a fusion protein with a N-terminal fluorescent tag instead of the C-terminal mCherry tag could be a possible solution to overcome this problem, but it could also expose the tag to a higher risk of cleavage by the activity of exopeptidases. Another option resides in the use of Gateway® technology, since some destination vectors are compatible with protein secretion from *Pichia pastoris* (Esposito *et al.*, 2005). A Chinese post-doctoral researcher is currently following the project.

Indeed, protocols for protoplast isolation, cloning of the fusion sequences and exploitation of Gateway® cloning technology were improved. The high number of positive clones obtained and the expression of the fusion proteins Rcr3-mCherry and Cf2-EGFP in *Pichia pastoris* were remarkable achievements.

5 Induced systemic resistance (ISR) and PGPR

5.1 Introduction

Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as plant growth promoting rhizobacteria (PGPR) (Nelson, 2004). Plant growth-promoting rhizobacteria were first defined by Kloepper and Schroth (1978) to describe soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth. The widely recognized mechanism of biocontrol mediated by PGPR against pathogens is competition for an ecological niche/substrate and production of inhibitory allelochemicals. Selected PGPR, mainly fluorescent *Pseudomonas* spp, have been demonstrated to control plant diseases effectively by suppressing pathogens and deleterious microorganisms through siderophore-mediated competition for iron, or antibiosis (Thomashow and Weller, 1995).

Under conditions of low iron availability, most aerobic and facultative anaerobic microorganisms, including fluorescent *Pseudomonas* spp., produce low-molecular weight Fe^{3+}-specific chelators, so-called siderophores, which sequester ferric ions in the environment thus making them not available for the growth of other microorganisms (Höfte, 1993). Also the synthesis of antimicrobial compounds by PGPR plays a major role in the suppression of soilborne plant pathogens. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), 2,4-diacetylphloroglucinol (DAPG) and phenazine-1-carboxylic acid (PCA), are a major focus of research in biological control (Hammer *et al.*, 1995; Kraus *et al.*, 1995; Bangera and Thomashow, 1996; Raaijmarkers *et al.*, 1997). Several reports have also pointed to the synergistic interactions between PGPR and arbuscular mycorrhizal (AM) fungi in stimulating plant growth and resistance to parasites (Sanchez *et al.*, 2004; Artursson *et al.*, 2006).

At the beginning of the nineties, research on mechanisms of biological control by PGPR revealed that some PGPR strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves (van Peer *et al.*, 1991; Wei *et al.*, 1991). The protection is typically manifested as both a reduction in disease symptoms and inhibition of pathogen growth and appears to be phenotypically similar to pathogen-induced SAR. This effect of PGPR is referred to as induced systemic resistance (ISR) and has been demonstrated in different plant species, including bean, carnation, cucumber, radish, tobacco, tomato, and the model plant *Arabidopsis thaliana* (Alström, 1991; Kloepper *et al.*, 1992; Maurhofer *et al.*, 1994; Leeman *et al.*, 1995; Van Loon *et al.* 1998).

ISR generally results in a non-specific resistance against different pathogens characterized by the accumulation of basal defense compounds, and the level of

protection has been observed to vary depending on the PGPR strain, the colonized plant and the challenging pathogen (Ryu *et al.*, 2003; Spencer *et al.*, 2003; Meziane *et al.*, 2005). Peroxidase, chitinase, polyphenol oxidase and phenylalanine ammonium lyase (PAL) are common defense enzymes induced by PGPR. A well-studied example of this phenomena is the interaction between *Pseudomonas fluorescens* and *A. thaliana*: *P. fluorescens* strain WCS417r applied on roots protects leaves from *Pseudomonas syringae* pv. *tomato* DC3000 and *Fusarium oxysporum* f. sp. *raphani*. Resistance is induced independently of SA and PR accumulation, but requires an intact response to the plant hormones jasmonic acid (JA) and ethylene (ET) (Hoffland *et al.*, 1995; Pieterse *et al.* 1996). The JA/ET signaling pathway is often designated as induced systemic resistance (ISR) but this term is also used to refer to quite different processes than those initiated by rhizobacteria. So far, most of the forms of resistance triggered by PGPR have been shown to be systemically induced in the plant by at least one of the two plant hormones. It was already mentioned in the first chapter that plant defense responses may be tailored to the attacking pathogen, with SA-dependent defenses acting against biotrophs, and JA- and ET-dependent responses acting against necrotrophic pathogens (McDowell and Dangl, 2000; Thomma *et al.*, 2001). This indicate that pathogens with a hemi-biotrophic lifestyle may trigger both pathways (van Wees *et al.*, 2000) but doesn't explain how saprophytic rhizobacteria can induce a JA-ET-mediated defense response.

There are at least two theories that elucidate the triggering of ISR by PGPR: rhizobacteria may produce molecules acting as general elicitors and recognized as PAMPs by the plant, or, in a fascinating but unlikely way, specific molecules of the bacteria may be perceived as distinguishing, specific features of a host-microbe symbiotic relation. In general, the mechanisms involved in rhizobacteria-mediated ISR appear to vary among bacterial strains or pathosystems and much remains to be discovered about the nature and variety of bacterial determinants responsible for the elicitation of defense mechanisms (Ongena *et al.*, 2005).

5.1.1 *Pseudomonas* spp. and bacterial determinants of ISR

Both antagonism and ISR are very important mechanisms in biological control of plant pathogens by PGPR. The antagonists could directly suppress pathogens with metabolites or antibiotics in the rhizosphere. In addition, induced systemic resistance may also establish a further strengthening of defense responses against pathogens living outside of the soil. Fluorescent pseudomonads such as *P. putida, P. fluorescens* and *P. aeruginosa,* are among the most effective rhizosphere bacteria in reducing soil-borne diseases (Weller, 1988). Biological control of these bacterial species is mainly due to their ability to produce antibiotics such as PCA and 2,4-DAPG (Keel *et al.*, 1996; Raaijmakers *et al.*, 1997; 1998; 2002). Even if some *Bacillus* spp. have been found to trigger systemic resistance (Yan *et al.*,

2002; Kloepper *et al.*, 2004), most ISR-inducing PGPR strains belong to the genus *Pseudomonas* (sensu stricto, Group I). Bacterial determinants of these species have been widely investigated for their capacity to trigger a systemic resistance in the host plant (Table 10). Till the end of the nineties, determinants of *Pseudomonas* spp. responsible for ISR elicitation could be divided into two classes: cell surface components, such as membrane lipopolysaccharides (LPS) or flagella, and iron-regulated metabolites with siderophore activity (Van Loon *et al.* 1998).

Determinant	PGPR strain	Host	Reference
Cell surface components			
Flagella	*P. putida* WCS358	*Arabidopsis*	Meziane *et al.*, 2005
Lipopolysaccharides	*P. fluorescens* WCS374	Radish	Leeman *et al.*, 1995
	P. fluorescens WCS417	*Arabidopsis* Carnation Radish	Van Wees *et al.*, 1997 Van Peer and Schippers, 1992 Leeman *et al.*, 1995
	P. putida WCS358	*Arabidopsis* Bean Tomato	Meziane *et al.*, 2005
Fe-regulated metabolites			
N-alkylated benzylamine derivative	*P. putida* BTP1	Bean	Ongena *et al.*, 2005
Pseudobactin siderophore	*P. fluorescens* CHA0 *P. fluorescens* WCS374 *P. putida* WCS358 *P. putida* WCS358 *P. putida* WCS358 *P. putida* WCS358	Tobacco Radish *Arabidopsis* Bean Eucalyptus Tomato	Maurhofer, *et al.*, 1994 Leeman *et al.*, 1996 Meziane *et al.*, 2005 Meziane *et al.*, 2005 Ran *et al.*, 2005 Meziane *et al.*, 2005
Antibiotics			
2,4-Diacetylphloroglucinol (2,4-DAPG)	*P. fluorescens* Q2-87 *P. fluorescens* CHA0	*Arabidopsis* *Arabidopsis* Tomato	Weller *et al.*, 2004 Iavicoli *et al.*, 2003 Siddiqui *et al.*, 2003
Massetolide A	*P. fluorescens SS101*	*Tomato*	Tran *et al.*, 2007
Pigments/others			
Salicylic acid	*P. aeruginosa* 7NSK2 *P. aeruginosa* 7NSK2 *P. fluorescens* P3 *pchBA*	Bean Tobacco Tobacco	De Meyer *et al.*, 1997; 1999a De Meyer et al., 1999b Maurhofer *et al.*, 1998
Pyocyanin and pyochelin (and/or salicylic acid)	*P. aeruginosa* 7NSK2 *P. aeruginosa* 7NSK2	Tomato Rice	Audenaert *et al.*, 2002 De Vleesschauwer *et al*, 2006
N-acylhomoserine lactones (N-AHLs)	*P. putida* IsoF	Tomato	Schuhegger *et al.*, 2006
Unknown	*P. fluorescens* WCS374 *P. fluorescens* WCS417	Radish Radish	Leeman *et al.*, 1996

Adapted from Bakker *et al.*, 2007.

Table 10: Determinants of *Pseudomonas* spp. involved in ISR triggering.

However, in the last ten years evidence has raised about the involvement of other bacterial specific molecules in the establishment of ISR (Figure 22). The pigment pyocyanin from *P. aeruginosa*, an N-alkylated benzylamine derivative from *P. putida*, bacterial signaling molecules as *N*-acylhomoserine lactones (N-AHLs) and the volatiles 2,3-butanediol and acetoin, have all been shown to induce systemic resistance (see Table 10 for references). Interestingly, it was recently demonstrated that also antibiotics produced by different *Pseudomonas* spp., like the cyclic lipopetide Massetolide A and 2,4-DAPG, are able to trigger ISR. The importance of DAPG production in ISR was further supported by observations that mutants that do not produce DAPG do not induce resistance, and ISR triggering is restored in complemented mutants (Iavicoli *et al.*, 2003; Weller *et al.*, 2004). A role of bacterial antibiotics in the activation of host defense was also demonstrated for surfactin, a lipoprotein produced by *Bacillus subtilis* (Ongena *et al.*, 2007) and it's possibly related to the mode of action of iturin, a broad-spectrum antibiotic produced by strain GB03 of *B. subtilis*, commercially available as Kodiak® (Gustafson Inc., TX, USA).

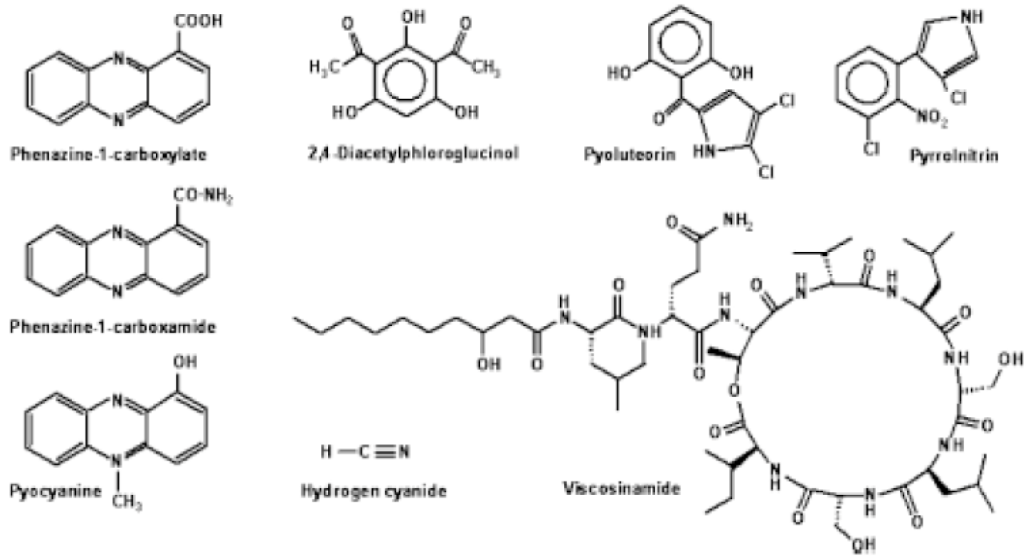

Figure 22: Molecules synthesized by *Pseudomonas* spp. shown to induce resistance in plants.

Unluckily, apart from the genes involved in JA/ET biosynthetic pathways, it's difficult to assess induced systemic resistance by rhizobacteria through common, interspecific traits. Plant responses to bacterial determinants may vary, and a molecule produced by different strains of the same species may induce different effects in the same host. Development of indicator plants that contain a reporter gene that is expressed when ISR occurs would be instrumental in identifying additional bacterial triggers of ISR (Bakker *et al.*, 2007).

5.1.2 Abiotic resistance inducers

Synthetic compounds called chemical inducers can effectively trigger induced resistance responses (Sticher *et al.*, 1997). Some of the best characterized examples are 2,6-dichloro-isonicotinic acid (INA) and acibenzolar-S-methyl (BTH). These compounds induce the same spectrum of resistance as pathogen-induced SAR with concomitant activation of SA-dependent PR genes (Vernooij *et al.*, 1995; Friedrich *et al.*, 1996; Lawton *et al.*, 1996). Since BTH has been shown to be so effective in crop protection against bacterial and fungal diseases, it was commercially released under the name of Bion® (Europe) and Actigard® (USA).

The non-protein amino acid β-aminobutyric acid (BABA) has been shown to protect *Arabidopsis* against different virulent pathogens by potentiating plant defense mechanisms, such as callose deposition, HR, and the formation of trailing necroses (Zimmerli *et al.*, 2000; Jakab *et al.*, 2001). In the case of necrotrophic pathogens, BABA protected mutants insensitive to JA and ethylene (Zimmerli *et al.*, 2001). Moreover, application of 10 mM BABA on tobacco led to the formation of ROS, lipid peroxidation and an increase in SA content of leaves (Siegrist *et al.*, 2000).

Recently, the plant growth retardant prohexadione-Ca (ProCa, commercial name Regalis®, BASF, Germany) was found to block the synthesis of growth-active gibberellins (Rademacher, 2000; Rademacher & Kober, 2003), leading to the formation of the novel antimicrobial compound luteoforol (Halbwirth *et al.*, 2003; Spinelli *et al.*, 2005). This molecule seems to induce resistance in many economically important species, including pear and apple (Bazzi *et al.*, 2003a,b).

The jasmonate pathway is phylogenetically conserved and found in many plants, and results in the production of many secondary metabolites and the expression of a wide set of defense genes (Creelman and Mullet, 1997; Constabel and Ryan, 1998). Exogenous jasmonate application have been shown to induce the production of a diverse array of putatively defensive compounds in both monocots and dicots, but commercial products based on jasmonic acid formulations are still under development (Crane *et al.*, 2003; Pena and Vargas, 2007).

Several *Pseudomonas* spp. strains were isolated and tested for their antimicrobial activity and the ability to produce the antibiotics 2,4-DAPG and PCA. The induction of a form of systemic resistance in tomato plants treated with a DAPG-positive strain was investigated, using as a positive control jasmonic acid. Reduction of disease severity by the same strain against the bacterial speck caused by *Pseduomonas syringae* pv. *tomato* was also assessed in tomato plants cv Perfect Peel ensuring no direct contact between the selected bacterium and the pathogen.

5.2 Materials and methods

5.2.1 Microorganisms

Fluorescent strains of *Pseudomonas* spp. were isolated from several vegetal samples, including *Carpobrotus edulis, Datura stramonium, Verbena* spp., *Apium graveolens* and *Euphorbia pulcherrima*. All strains were mantained at 27°C by weekly transfer on King's B (KB) agar (King *et al.*, 1954).

5.2.2 Strains identification: LOPAT and biochemical tests

The LOPAT determinative tests (L, levan production; O, oxidase production; P, pectinolytic activity; A, arginine dehydrolase synthesis; and T, tobacco hypersensibility) are widely applied to differentiate *Pseudomonas* spp. isolates. (Lelliott *et al.*, 1966).

Hypersucrose medium (5%, NSA) was used to assess the production of the exopolymer levan by the bacterial strain of interest. For the oxidase test, a single, purified colony was spread over a filter disc containing a 10 µl drop of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride, an aromatic amine able to detect the presence or absence of cytochrome c oxidase in the microorganism. In the reduced state (negative), the reagent is colorless, while in the oxidized state (positive) the reagent is deep blue/purple. *Pseudomonas fluorescens* and *P. syringae* were used as positive and negative control, respectively. Production of pectolytic enzymes was assessed putting a 10 µl drop of a 10^6 cfu/ml bacterial suspension over a potato cylindrical slice, using *Erwinia carotovora* and water as positive and negative control, respectively. Degradation of arginine was assessed in tubes containing 5 mL of Thornley's 2A medium (g/L: peptone 1.0, NaCl 5.0, K_2HPO_4 0.3, phenol red 0.01, arginine HCl 10, agar 3.0, pH 7.2). After sterilization, 2 mL of liquid paraffin were added to create anaerobiosis and the tube is inoculated with a colony of the bacteria. After 4 days of incubation at 27°C, presence of arginine dehydrolase was determined through change of the medium color from weak orange to intense pink. *Pseudomonas fluorescens* and *P. syringae* were used as positive and negative control, respectively. Suspension (10^6 cfu/ml) of the bacterial strain of interest are injected in panels of tobacco leaves to determine the induction of hypersensitive response using water and *P. syringae* pv. *syringae* strain 6285 as negative and positive control.

Reduction of nitrate (NO_3) to nitrite (NO_2) by nitrate-reductase was determined by the method of Follet and Ratcliff (1963) using *P. fluorescens* and *P. syringae* pv. *syringae* strain 6285 as positive and negative control, respectively. Production of acidic compounds from utilization of sucrose was determined inoculating the bacterial strains in tubes containing 6 mL of Ayers medium (0.1% w/v $NH_4H_2PO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.015% bromothymol blue, 1.2% agar, 2%

sucrose, pH 7.0) (Ayers *et al.*, 1919). After three days of incubation at 27°C, positive strains turn the color of the medium from blue to yellow. *P. syringae* pv. *syringae* was used as negative control. To determine the ability to produce the sugar 2-ketogluconate the strains were inoculated in tubes containing 10 mL of medium (pH 7.0) of the following composition (g/L): tryptone 1.5, yeast extract 1.0, K_2HPO_4 1.0, sodium gluconate 40. Tubes were incubated in a rotary shaker at 27°C for 7 days. After 3 days, 3 mL of the cultures were transferred in a sterile tube adding 0.6 mL of Benedict's reagent. Tubes were then placed in boiling water for 10 minutes, and the precipitation of copper oxidule (dark orange-brown color) was considered positive for the production of 2-ketogluconate. *Pseudomonas fluorescens* and *P. syringae* were used as positive and negative control, respectively. The ability of the selected strains to liquefy gelatin, to grow at 4°C and 42°C, and the utilization of meso-inositol, inositol and trehalose as carbon sources, were also tested on minimal media.

5.2.3 Strains identification: PCR assays

Genomic DNA of the bacterial strains was extracted using the QIAamp DNA Mini Kit (51304, Qiagen), following manufacturer's protocol. Three different primer pairs were used to amplify inter- and intraspecific DNA regions of the bacteria of interest (Table 11). For amplification of *Pseudomonas* 16S rRNA genes, the highly selective PCR primer pair Ps-for and Ps-rev (Widmer *et al.*, 1998) were used. PCR product was gel purified using the Wizard® Gel and PCR Clean-Up System kit (A9281, Promega) and sent for sequencing to BMR Genomics (Padova, Italia). Sequences were blasted in Genbank database. DNA 16S specific region for *Pseudomonas fluorescens* amplification was performed using the primer set 16SPSEfluF and 16SPSER (Scarpellini *et al.*, 2004). Primer forward is species specific, while the reverse is family specific. *Pseudomonas putida* was detected using the specific primers Xylr-For and Xylr-rev. This primer pair was designed to amplify a 259-bp fragment of the *xylR* gene on the pWW0 plasmid of the bacterium (Kuske *et al.*, 1998). Amplifications were performed in a AB2720 thermocycler, setting the thermal profiles reported by the authors. Amplicons were run at 70 V on a 1 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

Target	Primer pair	Sequence (5'→3')	Length
Pseduomonas	Ps-for	GGTCTGAGAGGATGATCAGT	1007 bp
spp.	Ps-rev	TTAGCTCCACCTCGCGGC	
P. fluorescens	16SPSEfluF	TGCATTCAAAACTGACTG	850 bp
	16SPSEr	AATCACACCGTGGTAACCG	
P. putida	XylR-F1	TCGCTAAACCAACTGTCA	259 bp
	XylR-R1	GCACCATAAGGAATACGG	

Table 11: Primer pairs, sequences and length of the amplicons for the identification of *Pseudomonas* spp. tested in this experiment.

5.2.4 Antagonism and mechanism of action

Antimicrobial activity and mechanism of action of the selected strains against the phytopathogenic bacteria *Erwinia amylovora* and *Xanthomonas arboricola* pv. *pruni* was determined *in vitro*. A spot of the colony was spread in the center of a KB or GA medium Petri dish and incubated for 48 hours at 27°C. The spot was then removed with a glass stick and the plate was exposed to chloroform vapors to kill residual cells. After 30 min, a bacterial suspension (10^6 cfu/ml) of the pathogens *X. a.* pv *pruni* and *E. amylovora* was poured into the same Petri dish, and incubated at 27°C. After 24 h, production of antimicrobial compounds by the antagonistic strain is associated with an "inhibition ring" around the spot.

Mechanism of action was determined by the method of Galasso *et al.* (2002). Also in this case, a spot of the antagonist was spread in the center of a KB or GA-medium plate and, after 48 hours of incubation at 27°C, the cells were removed and exposed to chloroform. Small holes (2-3 mm deep) were made around the perimeter of the removed bacterial spot with a pipette tip and filled with 10 µL of the following solutions: pronase (20mg/mL), proteinase-K (10 mg/mL), $FeCl_3$ (50 mM $FeCl_3$ in 10 mM HCl) and sterile water. Drops were let drying, and after 30 minutes 5 mL of a semi-solid (7%) agar medium containing a suspension (10^6 cfu/mL) of *E. amylovora* or *X. a*. pv. *pruni* was poured into the plate. Petri dishes were incubated for 24 hours at 27°C. The absence of inhibition near one of the holes indicates the inactivation of siderophoric or peptidic antimicrobial molecules produced by the antagonist.

5.2.5 Production of 2,4-DAPG and PCA

Production of the antibiotics 2,4-diacetylphloroglucinol (DAPG) and phenazine-1-carboxylic acid (PCA) by the selected strains was determined through PCR technique using primer pairs Phl and PCA (Table 12) developed by Raaijmakers *et al.* (1997). The PCR thermal profile consisted of an initial denaturation step at 94°C for 2 min followed by 30 cycles of 94°C for 60 s, 67°C for 45 s, and 72°C for 60 s, and a final elongation step at 72°C for 10 min. Amplicons were run at 70 V on a 1 % agarose gel, stained with EtBr and visualized with a UV transilluminator. Positive strains were furnished by Jos Raaijmakers (WUR, NL) and primers by Enrico Biondi, DiSTA.

Target	Primer pair	Sequence (5'→3')	Length
2,4 DAPG	Phl2a (F)	GAGGACGTCGAAGACCACCA	745 bp
	Phl2b (r)	ACCGCAGCATCGTGTATGAG	
PCA	PCA2a (F)	TTGCCAAGCCTCGCTCCAAC	1150 bp
	PCA3b (r)	CCGCGTTGTTCCTCGTTCAT	

Table 12: Primer pairs, sequences and length of the amplicons for the detection of the *Pseudomonas* spp. antibiotics 2,4-DAPG and PCA.

5.2.6 Induced resistance assay: plant material and treatment

Three week old tomato plants (cv Perfect Peel) were grown in climate chamber under a 16 h light/8 h dark cycle at 25°C and 80% humidity. A 10 mM MgSO4 suspension of the DAPG-producing strain m5 of *Pseudomonas putida* (10^7 cfu/mL) was used for root irrigations of 20 tomato plants, 1 week, 3 days and 24 hours before the foliar treatment with a 10^6 cfu/mL suspension of the leaf pathogen *Pseudomonas syringae* pv. *tomato* (strain DC3000). Bacterial inoculum was prepared by harvesting cells from KB-agar plates incubated at 27°C for 24 h. Immediately after inoculation of strain DC 3000, the plants were kept in 100% humidity covering the shelves containing the plants with a wet, plastic film (Figure 23). Irrigation with water was used as negative control and plants were randomly disposed in the climate chamber. Incidence of the disease was assessed two weeks after inoculation, adopting as parameter the number of bacterial specks for plant.

Figure 23: A successful inoculation of *P. syringae* pv. *tomato* requires high humidity conditions.

5.2.7 Plant materials, RNA extraction and multiplex RT-PCR

Two weeks old tomato plants cv Money Maker, grown in climate chamber at 22°C with 16 h of light, were treated with root applications (10^7 cfu/mL) of strain m5 of *P. putida*, five, three and one day before the foliar treatment with a 250 µM solution of jasmonic acid (J2500, Sigma) and water, respectively used as positive and negative control. Four non-treated plants were used as additional controls. Transcription of the pathogenesis-related genes PR-1, PR-4, PR-5 was monitored at different times (0 h, 6 h, 24 h, 72 h) through multiplex RT-PCR technique. Since transcription of EF1 gave alternate results in the experiment with pullulans, the regulatory component TomNPR1 (456 bp) was also chosen as internal control. Starting from 100 mg/sample of leaf material, total plant RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) applying two main modifications to the protocol as previously described. Primers, thermal profiles and multiplex RT-PCR reaction mix were the same described in chapter 3 (Table 4).

5.3 Results

5.3.1 Strains identification

Several strains producing fluorescent pigment on King's B (KB) agar and showing *in vitro* antimicrobial activity towards *Erwinia amylovora* and *Xanthomonas arboricola* pv. *pruni* were isolated: strain "IS" from *Apium graveolens,* "verbena+" and "m5" from *Verbena* spp., "FluoSdN" from *Euphorbia pulcherrima* and "KB+succula" from *Carpobrotus edulis.* Profiles derived from LOPAT and biochemical tests (Figure 25) are showed in Table 13. Three strains were finally selected for further studies: m5, IS and KB+succula (Figure 24).

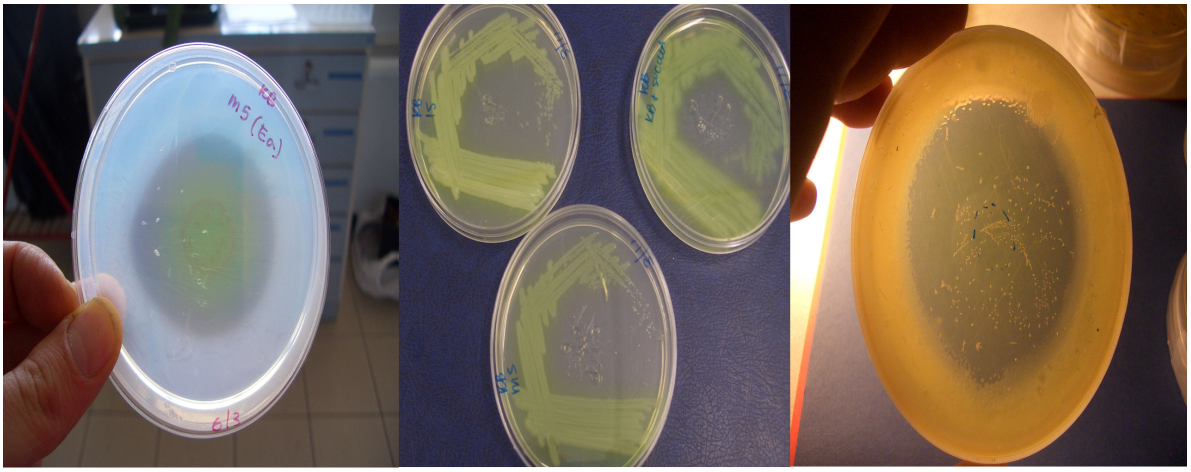

Figure 24: From left to right: inhibition ring of m5 against *E. amylovora*; the three strains object of this study on KB-medium; inhibition ring of KB+succula against *X. a.* pv *pruni.*

	L	O	P	A	T	ketoG	NO_3Red	AcidSac	4°C	42°C	Gel	M	I	T
m5	-	+	-	+	-	+	+	-	+	-	-/+	+	+	+
IS	+	+	-	+	-	+	-	+	+	-	+	+/-	+	+
KB+succula	-	+	v	+	-	+	+	-	-	+	+	+	-	-
Verbena+	-	+	-	+	-	+	-	-						
FluoSdN	-	+	-	+	-	+	-							
P. aeruginosa	-	+	v	+	-	+	+		-	+	+	+	-	-
P. fluorescens														
Biovar I	+	+	-	+	-	+	-		+	-	+	+	+	+
Biovar II	+	+	-	+	-	+	+		+	-	+	+	+	+
Biovar III	-	+	-	+	-	+	+		+	-	+	+	+	+
Biovar IV	+	+	-	+	-	+	+		+	-	+	+	+	+
Biovar V	-	+	-	+	-	+	-		+	-	-	+	+	+
P. putida	-	+	-	+	-	+	-		+	-	-	-/+	-	-

Table 13: LOPAT and additional biochemical profiles are useful to differentiate *Pseudomonas* spp. isolates (v=alternate response; M=meso-inositol; I=inositol; T= trehalose).

From biochemical tests, IS appeared to belong to Biovar I of *P. fluorescens* and m5 to Biovar III. KB+succula was most likely a *P. aeruginosa* able to grow at 42°C and occasionally isolated from plants originating from the south of Italy. FluoSdN and verbena+ could be identified both as *P. putida* or *P. fluorescens* Biovar V.

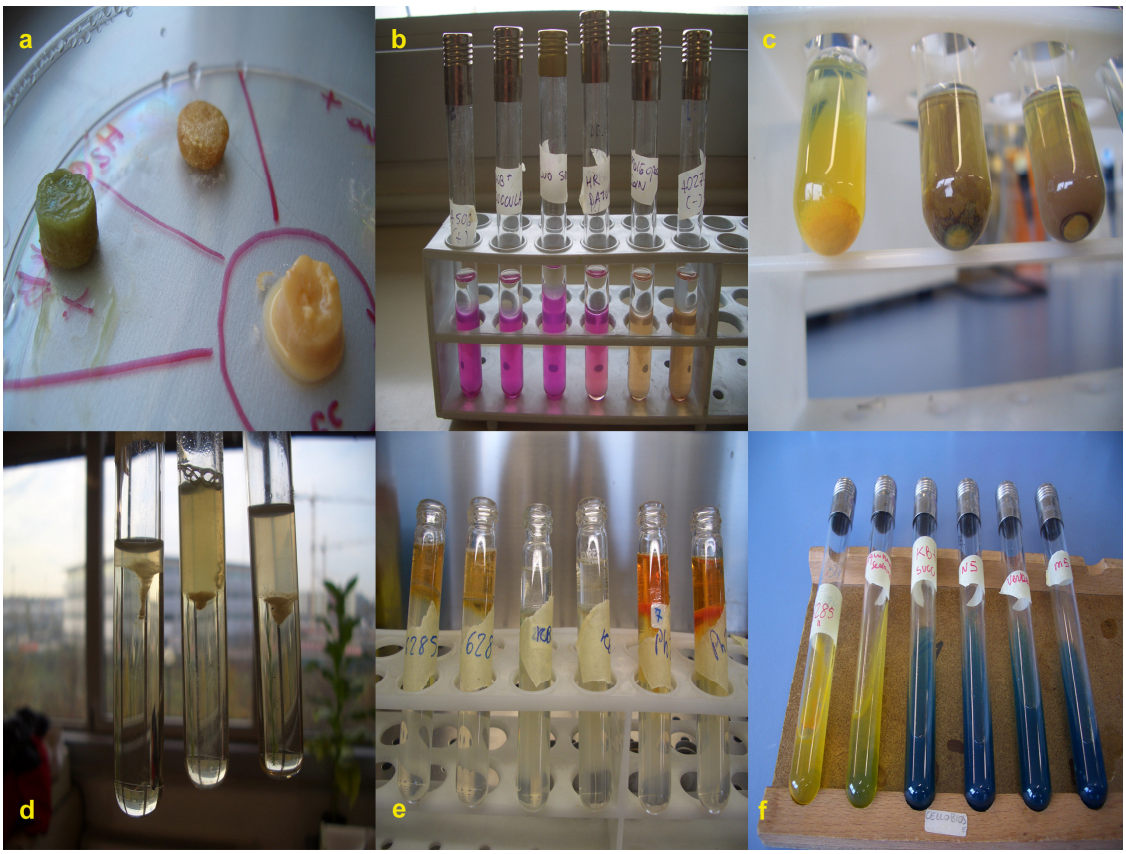

Figure 25: LOPAT and biochemical tests: a) Potato rot; b) Arginine dehydrolase; c) Production of 2-ketogluconate; d) Liquefaction of gelatin; e) Nitrate reduction test; f) Acidity from sucrose.

The specific primers 16SPSEfluF and 16SPSEr confirmed IS to be *P. fluorescens* Biovar I (Figure 26), amplifying the 850 bp sequence also in the positive control (strain IPV-BO G19), while no amplification was noticed for strain m5.

Partial sequencing of the 16S rRNA gene, common to all *Pseudomonas* spp., confirmed KB+succula to be *P. aeruginosa* (96% identity with the 16S rRNA gene of *P. aeruginosa* strain PB11, Accession number EU360107), but BLAST results also indicated a strong identity (99%) of strain m5 with the 16S rRNA gene of *Pseudomonas putida* strain JM9 (Accession Number: FJ472861). However, specific primers for *P. putida* didn't confirm this result, amplifying a 259 bp fragment only for FluoSdN (Figure 26). Moreover, m5 was found to be positive for the nitrate test and able to use trehalose and inositol as carbon sources.

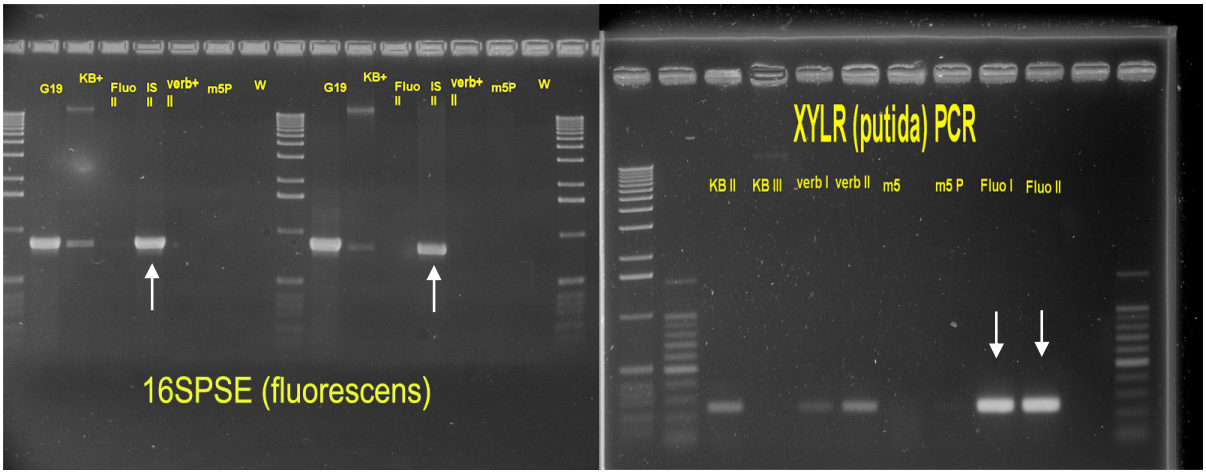

Figure 26: Identification of *P. fluorescens* (strain IS, left) and *P. putida* (strain FluoSdN, right) through PCR using the specific primer pairs 16SPSEfluF/16SPSEr and XylR-F1/XylR-R1 amplifying a 850 bp and a 259 bp amplicon, respectively.

5.3.2 Mechanism of action and production of antibiotics

All the three antagonistic strains investigated were shown to produce Fe-dependent metabolites, possibly siderophores, but no production of peptidic molecules was observed (Figure 27).

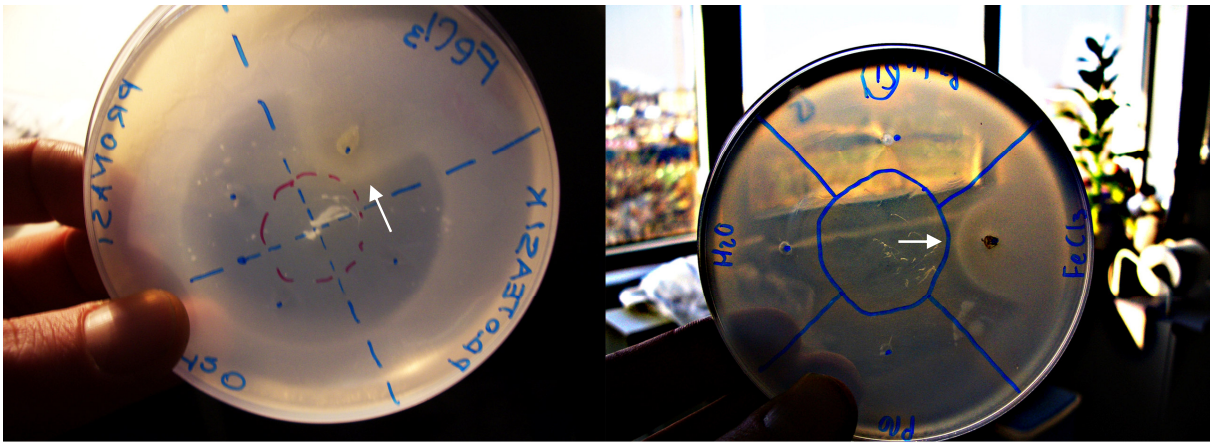

Figure 27: Growth inhibition of the pathogen *Erwinia amylovora* by strain IS of *P. fluorescens* (right) and strain KB+succula of *P. aeruginosa* (left) is suppressed by Fe^{3+} ions (white arrow), demonstrating a siderophore-like activity by the tested microorganisms.

Only strain m5 was shown to posses the *Phl* loci for the synthesis of the antibiotic 2,4-diacetylphloroglucinol (DAPG), while none of the strains harbored the PCA gene encoding for phenazine-1-carboxylic acid (Figure 28).

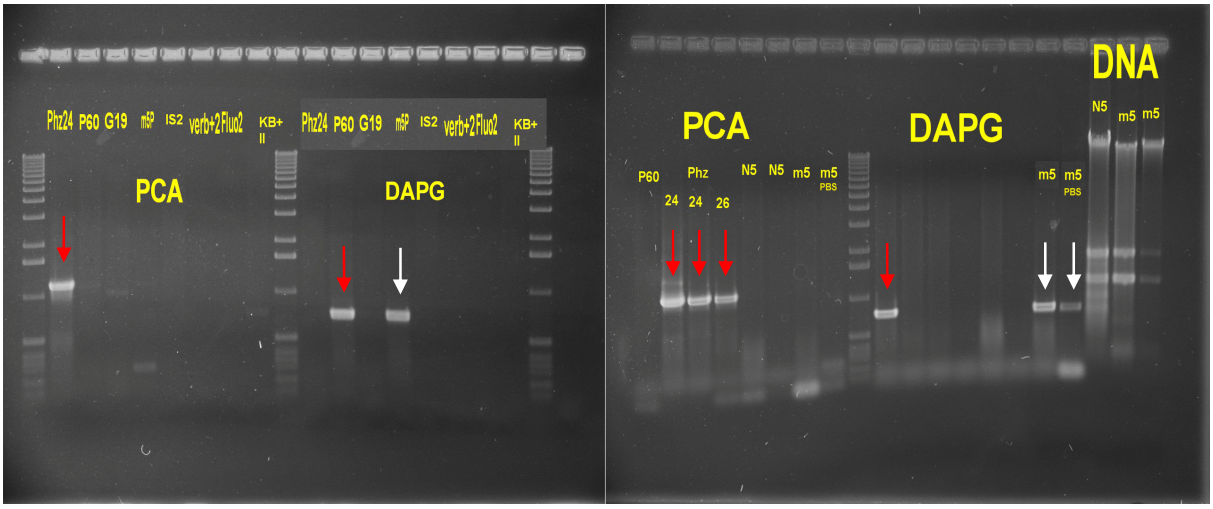

Figure 28: Amplification of the *Phl* loci required for the synthesis of diacetylphloroglucinol in strain m5 (white arrows). Positive controls for 2,4-DAPG and PCA production are indicated with red arrows.

5.3.3. Induced resistance against *Pseudomonas syringae* pv. *tomato*

First symptoms of bacterial speck were already noticed in the inferior surface of the leaves 6 days after inoculation of strain DC3000 of *P. syringae* pv. *tomato*, and kept increasing in their extent. Three days after inoculation of the pathogen, it was immediately noticed that some of the plants were showing confluent necrosis in the apex of their top, younger leaves (second/third branch), where the bacteria normally initiate to penetrate the host through stomata (Figure 29).

Induced resistance-mediated disease suppression by strain m5 of *P. putida* was determined as a drastic and significative decrease of bacterial specks with respect to the non-bacterized control treatment. Statistical analysis (StatGraph) confirmed a statistically significant difference (ANOVA test: p=0.0047) between the two treatments (Table 14 and Figure 30).

ANOVA table					
Bacterial specks by treatment	**Sum of squares**	**Df**	**Mean square**	**F-ratio**	**P-value**
Between groups	12638.0	1	12638.0	9.02	0.0047
Within goups	53246.3	38	1401.22		
Total (corr.)	65884.4	39			
Necrotic lesions by treatment	**Sum of squares**	**Df**	**Mean square**	**F-ratio**	**P-value**
Between groups	81.225	1	81.225	8.40	0.0062
Within goups	367.55	38	9.67237		
Total (corr.)	448.775	39			

Table 14: ANOVA table showing differences between the treatments for both the parameters.

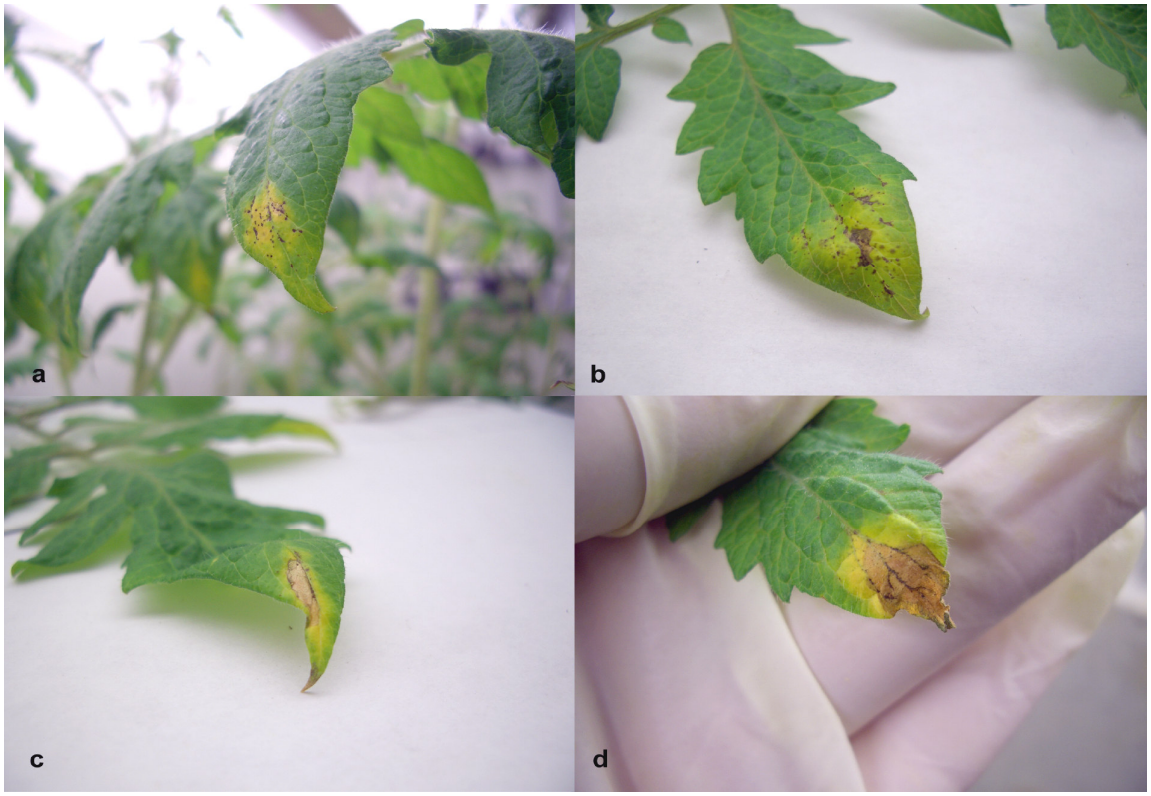

Figure 29: Classic symptoms of *Pseudomonas syringae* pv. *tomato* on leaves of cv Perfect Peel (a+b), and confluent necrosis of the apex in plants treated with the rhizobacteria (c+d).

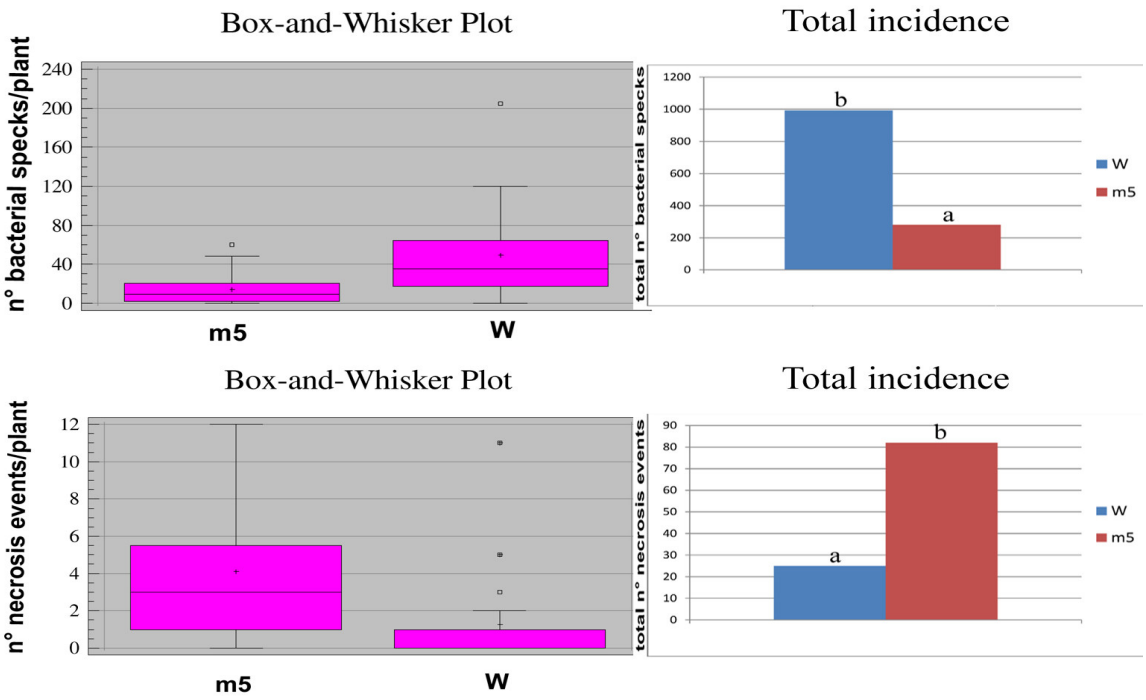

Figure 30: Statystical analysis for the induced resistance assay: StatGraph plots and total incidence of disease (top) and apex necrosis (bottom) between the two treatments.

Normalized to the water control (100% disease), incidence of the disease in plants treated with the rhizobacteria didn't exceed the 28%, clearly demonstrating an induction of resistance by strain m5.

A statistically significant difference (ANOVA test: p=0.0062) between the two treatments was also determined in the distribution of confluent necrosis. Plants treated with root applications of the rhizobacteria showed events of necrosis 70% more with respect to the water control (Table 14 and Figure 30).

5.3.4 Multiplex RT-PCR assay

A map of the treatments for the multiplex RT-PCR assay is shown in Table 15. Curiously, transcription of the pathogenesis-related protein PR-1 was assessed also in control (n° 1, 2, 12, 23) and non-treated plants (n° 10, 20). It has to be mentioned that all plants (apart from the nT controls), were treated outside of the climate chamber to avoid side effects. Compared to the first multiplex RT-PCR assay, testing the effect of fungal exopolysaccharides on tomato plants of the same cv, this experiment have demonstrated that transcription of defense genes may also occur in non treated plants kept at standard conditions (Figure 31). The possible reasons for this induction will be discussed in chapter 5.4.

	0 h		***6 h***		***24 h***		***72 h***
1	W α1	**11**	W β1	**21**	W γ1	**31**	W δ1
2	W α2	**12**	W β2	**22**	W γ2	**32**	W δ2
3	W α3	**13**	W β3	**23**	W γ3	**33**	W δ3
4	m5 α1	**14**	m5 β1	**24**	m5 γ1	**34**	m5 δ1
5	m5 α2	**15**	m5 β2	**25**	m5 γ2	**35**	m5 δ2
6	m5 α2	**16**	m5 β3	**26**	m5 γ3	**36**	m5 δ3
7	JA α1	**17**	JA β1	**27**	JA γ1	**37**	JA δ1
8	JA α2	**18**	JA β2	**28**	JA γ2	**38**	JA δ2
9	JA α2	**19**	JA β3	**29**	JA γ3	**39**	JA δ3
10	nT α1	**20**	nT β1	**30**	nT γ1	**40**	nT δ1

Table 15: Map of the treatments and repetitions for the multiplex RT-PCR assay testing the responses of tomato plants cv Money Maker to treatments with strain m5 and jasmonic acid.

While weak PR-1 transcription was detected in plants treated with strain m5 at time 0, transcripts increase could be noticed in all the other surveys (n° 15, 16, 24, 25, 26, 34, 35, 36), showing a clear induction of this defense molecule by the rhizobacteria when compared to transcription of the internal control used in this assay (EF1). Also the positive control jasmonic acid (250 μM) induced transcription of PR-1 (n° 7, 17, 18, 19, 27, 37, 38, 39), even if this pathogenesis-related protein has always been considered a marker for SAR and its expression is normally related to accumulation of salicylic acid.

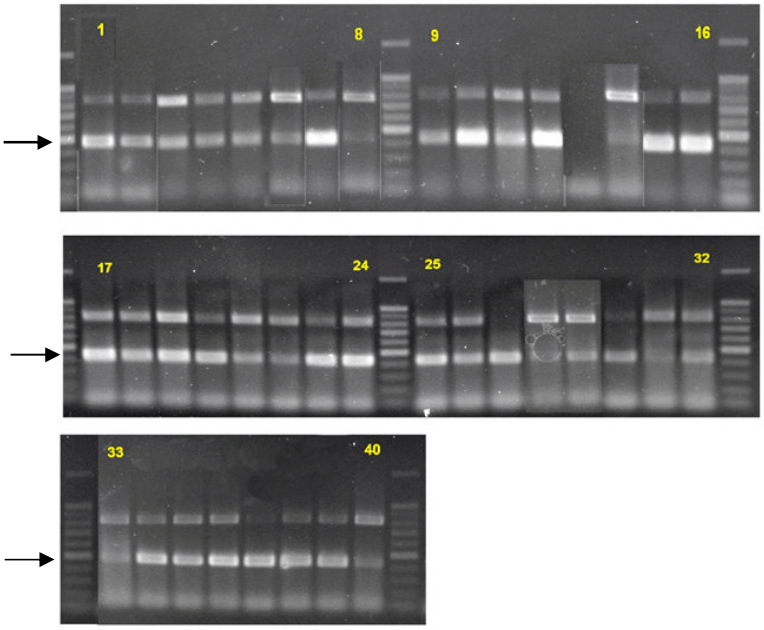

Figure 31: Transcription of PR1 (427 bp, black arrow) monitored through multiplex RT-PCR in *Lycopersicon esculentum* cv Money Maker following root and foliar applications with strain m5 and jasmonic acid, respectively.

Again, transcription of the pathogenesis-related protein PR-4 was shown also in control (n° 1, 3, 12, 23, 31) and non treated plants (n° 10, 20, 30, 40). Transcripts increase of PR-4 (Figure 32) was strongly induced by foliar applications of jasmonic acid (n° 7, 17, 18, 19, 27, 37, 38, 39) and only partially triggered by root applications of strain m5 (n° 14, 15, 16, 24, 25, 36).

Between the three pathogenesis-related proteins investigated, PR-5 has shown the most atypical transcriptional profile. After a first multiplex RT-PCR using EF1 as internal control highlighted problems in its transcription, the choice fell on TomNPR1(Figure 33). However, transcripts level of the two genes were shown to be induced in all the thesis to some extent. Especially in water controls and non treated plants, transcription of PR-5 (560 bp) was comparable to the one of TomNPR1 (456 bp). An analogue transcription profile was also noticed in some of the plants treated with JA (n° 9, 37, 38, 39).

A clear increase in the transcription of PR-5 was shown in plants treated with strain m5 (n° 5, 6, 14, 15, 16, 24, 25, 26, 34, 35), jasmonic acid (n° 7, 8, 17, 18, 19, 27) and also in the negative control (n° 21, 23).

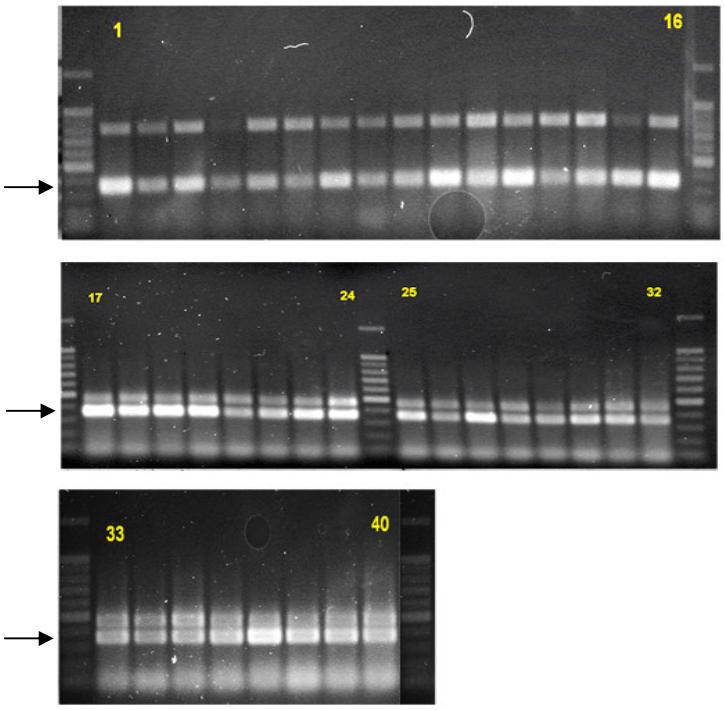

Figure 32: Transcription of PR-4 (349 bp, black arrow) monitored through multiplex RT-PCR in *Lycopersicon esculentum* cv Money Maker following root and foliar applications with strain m5 and jasmonic acid, respectively.

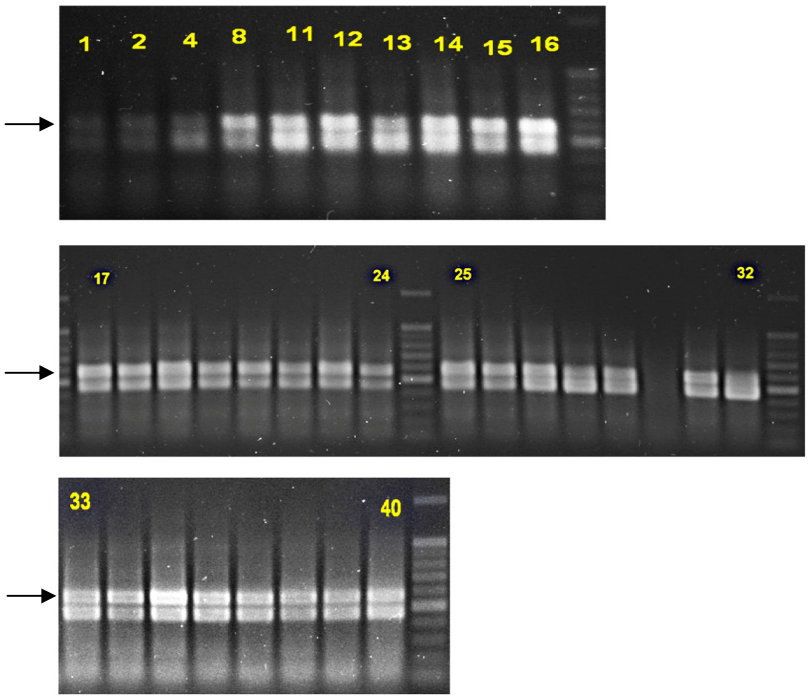

Figure 33: Transcription of PR-5 (560 bp, black arrow) monitored through multiplex RT-PCR in *Lycopersicon esculentum* cv Money Maker following root and foliar applications with strain m5 and jasmonic acid, respectively.

5.4 Discussion

Since my master thesis dealt with biological control of fireblight on pear caused by the bacterium *Erwinia amylovora*, I've been able to select several antagonistic strains belonging to the genus *Pseudomonas* in a short time. Moreover, my colleague Dr Enrico Biondi and Dr Jos Raaijmakers from the Laboratory of Phytopathology of Wageningen were both studying antibiotics produced by *Pseudomonas* spp. at that time.

Inhibition of pathogen growth and production of molecules with antimicrobial activity, were the two main parameters observed before selecting the strains m5, IS and KB+succula. All the three strains were able to inhibit the growth of *Erwinia amylovora* and *Xanthomonas arboricola* pv. *pruni in vitro*, interfering with Fe^{3+} assimilation through the production of siderophores.

Biochemical profiles and specific amplification of *Pseudomonas* spp. 16S region, identified strain KB+succula and IS as *P. aeruginosa* and *P. fluorescens* Biovar I, respectively. Sequencing of the 16S region indicated strain m5 to be *Pseudomonas putida* (99% identity), showing a contrasting result with respect to the biochemical profile. Further analysis are needed in order to identify this species.

Indeed, strain m5 is able to synthesize diacetylphloroglucinol (2,4-DAPG), an antibiotic produced by several *Pseudomonas* spp. who has been reported to induce systemic resistance in *Arabidopsis* against the leaf pathogen *Pseudomonas syringae* pv. *tomato* (Iavicoli *et al.*, 2003; Weller *et al.*, 2004). The ability to induce systemic resistance in tomato plants after root applications with the antagonistic strain was evaluated with two different approaches: monitoring transcription of the pathogenesis-related proteins PR-1, PR-4 and PR-5 in cv Money Maker and determining the level of protection against *P. s.* pv. *tomato* in Perfect Peel (susceptible cv).

Since treatments with the antagonist were exclusively addressed to the roots prior to spraying a suspension of the pathogen *Pseudomonas syringae* pv. *tomato* on the leaves, direct antagonistic effect or competition between the two strains can be excluded. The success in decreasing the incidence of the disease couldn't be better represented than through a significative, drastic reduction of the number of bacterial specks with respect to the negative control. Confluent necrosis of the apex in leaves of plants treated with the rhizobacteria can be possibly related to a fast hypersensitive-like response of tomato. This is also suggested by the fact that small bacterial specks could still be noticed on the necrotized tissues.

In the multiplex RT-PCR assay, strain m5 clearly induced transcription of the pathogenesis-related protein PR-1 and PR-5 after 24 hours, while only partially triggered transcription of PR-4 when compared to the water control and non-treated plants. Since PGPR have been shown to induce resistance in plants through the JA/ET signaling pathway (Pieterse *et al.*, 1998), it would have been expected to observe a high transcriptional level of PR-4 in plants treated with the rhizobacteria.

However, also plants treated with jasmonic acid showed transcription of all the three pathogenesis-related proteins investigated in this thesis, particularly PR-1 and PR-4, indicating that also this chemical can induce transcription of different sets of defense genes.

Unexpectedly, this multiplex RT-PCR assay has also shown transcription of pathogenesis-related proteins in negative control and non-treated plants. I exclude that reason of this triggering could be the transfer of the plants from the outside, where plants were treated, into the climate chamber. In fact, non treated plants were grown and kept inside the climate chamber for all the duration of the experiment, and showed transcription of PRs as well. Since the experiment was run in a small space where inoculation of *P. syringae* pv. *tomato* occurred one month earlier, an incomplete sterilization and removal of bacterial cells or the presence of volatile elicitors may have contributed to the triggering of defense responses.

As previously discussed in chapter 3, the control gene EF1 did not show a constant transcription profile: paradoxically, the use of TomNPR1 as an internal control to compare transcription of the pathogenesis-related proteins PR-4 and PR-5, has provided a more reliable and constant result.

6 Conclusion

This study has confirmed that general or race-specific molecules derived from microorganisms can elicit defense responses in tomato (*Lycopersicon esculentum* Mill., *Solanum lycopersicon* L.).

Since the beginning of the PhD, general elicitors as cell wall polysaccharides and race-specific elicitors obeying to the gene for gene theory were one of the main interests of my research, and source of hundreds of questions and doubts.

The fact that highly conserved molecules as fungal or bacterial exopolysaccharides can trigger forms of resistance in plants, focused my attention on the use of beneficial or non-pathogenic microorganisms for the induction of plant defense responses. In this thesis, the activity of fungal glucans and an ISR-inducing bacterial strain was investigated using tomato as a model plant and abiotic inducers like Bion® and jasmonic acid (JA) as positive controls.

Between the two years of research spent in Italy I had the great opportunity to take part as a "guest-PhD" in the project *"Perception of avirulence proteins by resistance tomato plants"*, supervised by Professor Pierre de Wit and John van't Klooster, from the influential *Cladosporium fulvum* research group of the Laboratory of Phytopathology, part of the Department of Plant Science of Wageningen University (NL). This project, even if incomplete, gave me the chance to deepen my knowledge of race-specific resistance and improve my experience in molecular biology, and it has provided a complete connection between the different types of resistance occurring in tomato and investigated in this thesis.

A molecule can be classified as an elicitor only when it's able to trigger a defense response in the plant at very low concentrations (even at nanomolar levels) and when it doesn't compromise the integrity of the cellular compartmentalization. Fungal exopolymers are frequently reported to fall into this category, but some of them should be carefully tested prior to use since they often play a role in virulence and may cause phytotoxicity in plants. The hypersensitive response is an extreme defense mechanism of the plant leading to cellular apoptosis: preventing this mechanism means less damage to tissues and probably less energy costs for the plant through activation of other defense pathways. Prevention of HR in tobacco plants could be reasonably seen as a positive feature for the selection of molecules with an elicitor-like activity. It would be interesting to test the effect of fungal glucans on tomato plants challenged with different pathogens, in order to value their spectrum and consider the opportunity to use them as defense-triggering molecules for crop protection.

The selection of rhizobacterial strains with antagonistic activity drove my interest in phenomena of induced resistance by PGPR. The induction of trasncription of pathogenesis-related proteins and a significantly effective control

of the leaf pathogen *Pseudomonas syringae* pv *tomato,* have confirmed strain m5 of *Pseudomonas putida* to be able to elicit a form of induced systemic resistance (ISR) in tomato. The increasing number of studies on mechanisms of ISR and the practical use of PGPR-based products in the last twenty years, suggest to broaden studies on this strain and possibly to evaluate its level and repeatability of protection in different plant-pathogen interactions.

As a conclusion, it can be confirmed that tomato is a useful model plant to study plant resistance mechanisms induced by biotic and abiotic elicitors. Using tomato mutants impaired in JA or SA synthesis would be useful to identify signaling pathways triggered by a selected microorganism and/or molecules derived from it. However, it's important to realize that plant responses to external agents may vary in terms of transcription and expression of defense molecules depending on a multitude of factors. Besides the fact that many chemicals and biotic elicitors have been tested only on a small number of model plants and new molecules continue to appear in the plant-pathogen interaction scenario, much remains to be discovered about the plant immune system.

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