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DOTTORATO DI RICERCA IN PATOLOGIA VEGETALE

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**Tolleranza a *Monilinia Laxa* nel corso
dell'accrescimento delle pesche
e variazione dell'espressione genica**

(Variation in peach fruit susceptibility
to *Monilinia laxa* and gene expression)

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Chapter 1

Introduction

Brown rot caused by *Monilinia laxa* and *Monilinia fructigena* is considered one of the most important diseases affecting *Prunus* species. It can attack either the peach blossom or fruits during growth. Normally, the first symptoms appear on blossom; from here they can invade the whole flower and stem. In humid weather, the fungus brown conidia become evident on infected organs which then start to dry. Sunken cankers at the base of the infected flowers may also become apparent (fig.1.1).

Symptoms on fruits consist of small, circular brown rot, which can enlarge and involve the entire fruit (fig. 1.2). This can become completely rotten and dry up into a mummy (fig.1.3).

Although some losses can result from the rotten fruits in the orchard, major economics losses occur during the post-harvest phase. In the most severe cases, up to 50% -75% of the fruit may rot in the orchard, whereas the rest may be lost after harvest, before reaching the market.



Figure 1.1: *Monilinia* brown rot on a peach blossom.



Figure 1.2: *Monilinia* brown rot on a peach fruit.



Figure 1.3: Mummies of peach fruits.

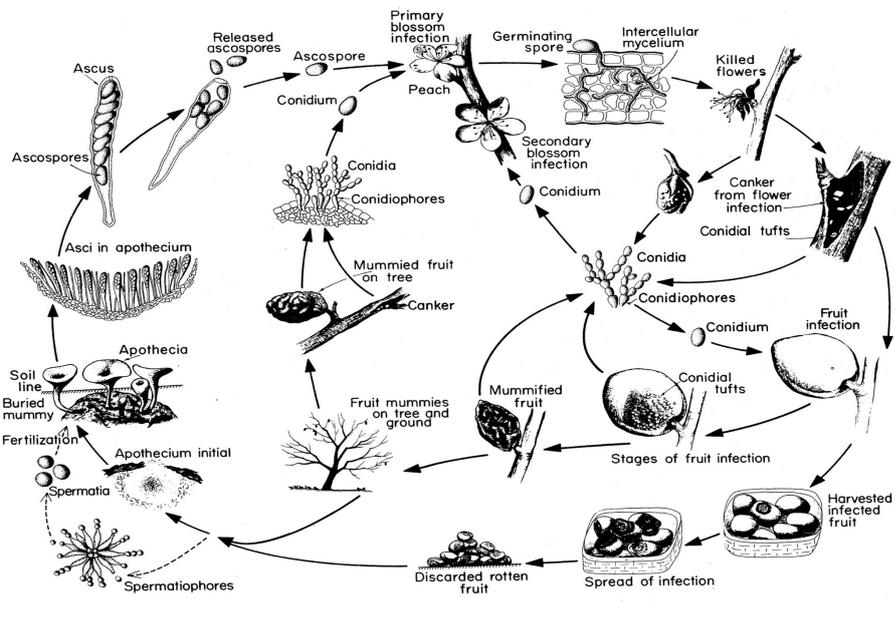


Figure 1.4: The disease cycle of brown rot of stone fruits (From George N. Agrios: Plant Pathology, Fourth Edition.)

Fungicide applications are mostly based on the use of strobilurins during the fruit growth phase. However, due to the recent concern about the environment and human health, a considerable interest in development of disease resistant cultivars and in the identification of appropriate markers associated with resistance, has recently emerged among researchers, farmers and consumers [1].

Several studies reported that brown rot incidence during fruit development highly varies; green fruits appear more resistant to the pathogen than later ripening stages [2]. The molecular basis of this phenomenon is still not well understood. Different hypothesis has been proposed to explain the association between the decrease in *Monilinia* susceptibility and the green fruit stage: i) the higher mechanical resistance, ii) to the inhibitory substances abundant in the tissue of this type of fruits, iii) to a different biochemical response between unripe and ripe fruits. More recently, susceptibility of peach fruits to *M. laxa* has been deeper characterized by monitoring it during the entire period of fruit growth. It was found that, at a period corresponding to the the pit hardening stage, fruit susceptibility drastically decreases [3, 4, 5], to be quickly restored afterwards. Furthermore, no difference in the rot incidence was found between wound and un-wound fruits, suggesting that resistance associated more to a specific biochemical response of the fruit, rather than to a higher mechanical resistance [3].

The South American peach cultivar Bolinha is known to have a high degree of resistance to brown rot [1]; among the Bolinha fruit typical features, such as an high compactness of epidermal and subepidermal cells, few trichomes and an high cuticle, they differ from more susceptible ones also for a higher concentration of phenolic compounds. Similarly, an inverse relation between the severity of brown rot and the polyphenolic content was already

found in apples [6]. Some of these compounds, such as chlorogenic acid, caffeic acid, the catechin and epicatechin, were used in *in vitro* assays to evaluate their antifungal potential as inhibitors of spore germination and of fungal growth. However, despite they showed inhibition of the activity of fungal lytic enzymes, which are fundamental for pathogen infection, such as cutinase, no direct inhibition on *M. fructicola* growth was detected [1].

The antimicrobial activity of several phenolic compounds, such as anthocyanins and chlorogenic acid (3-caffeyl-quinic) is well established [7]. Consistently, an increase in the total phenolic compounds in peach skin during the pit hardening stage was already shown; in particular, catechin and chlorogenic acid were found as the major phenolic compounds [1].

With respect to fruit size, peach fruits, during growth, display a typical double sigmoid pattern, with four standard growth stages (S1–S4):

- **S1 phase** in which the pericarp enlarges
- **S2 phase** in which pit hardening occurs and fruit stop to growth
- **S3 phase**, the pre-climateric phase, during which the mesocarp increases
- **S4 phase** the climateric phase, in which fruit ripening occurs

Consistently, in the peach cv.K2, the level of chlorogenic acid was found to strongly increase in the fruit peel, from the late S1 phase for the entire S2 phase; on the other hand, the level of cathechin increases later on the S2 phase, when the pit hardening is already completed (Mari, data not shown).

So far, the interaction *Monilinia*-peach was analyzed through chemical approaches. In this study, a bio-molecular approach was undertaken, in order to reveal alteration in gene expression associated to the variation of susceptibility.

Regulation of the gene expression is one of the mechanism used by cells to respond to external stimuli. Although the enzyme activity determines the real level of a specific function, measurement of the transcripts level has proven to be a valuable tool for highlighting the genes encoding for the important activities in each specific cellular process [8].

In this thesis three different methods of gene expression analysis were used to analyze the alterations in gene expression occurring in peach fruits during the pit hardening stage, in a period encompassing the temporary change in *Monilinia* susceptibility. Real time PCR analysis was performed to study the variation in expression of those genes encoding for enzymes of the phenylpropanoid pathway and of the jasmonate pathway, both involved in the production of important defence compounds.

The phenylpropanoid biosynthetic pathway is a plant specialized metabolism which provides important secondary compounds. Among these, there are anthocyanins for pigmentation, flavonoids, such as flavons, for protection against UV photo-damage, polymeric lignin, for structural support, and a series of antimicrobial compounds classified as inducible phytoalexins and preformed phytoanticipins. Moreover, phenylpropanoid products may play an important role as signal molecules, both in plant development and plant defence [9]. These natural products are mainly derived from the amino acid L-phenylalanine, through a three steps commitment of the carbon flow, from primary metabolism to the general phenylpropanoid pathway. The first step is catalyzed by the phenylalanine ammonia-lyase (PAL) enzyme, and consists of the deamination of the phenylalanine to produce cinnamic acid, the phenylpropanoid skeleton (fig.1.5). The second step is catalyzed by the cinnamic acid 4-hydroxylase (C4H) enzyme, which catalyzes the introduction of a hydroxyl group in the cinnamic acid, producing the 4-coumarate compound.

The third reaction is catalyzed by p-coumaroyl:CoA ligase (4CL), which converts the 4-coumarate to its corresponding CoA thiol ester. From this step on, the phenylpropanoid metabolism branches, to produce thousands of compounds, many of which are plant species specific. An important branch leads to the production of flavonoids, including flavonols, anthocyanins and tannins. The first committed step in this pathway is catalyzed by chalcone synthase (CHS). The phenylalanine ammonia-lyase and the chalcone synthase are the key enzymes in phenylpropanoid and flavonoid assembly, respectively; for this reason they are the most studied and the most regulated ones [10].

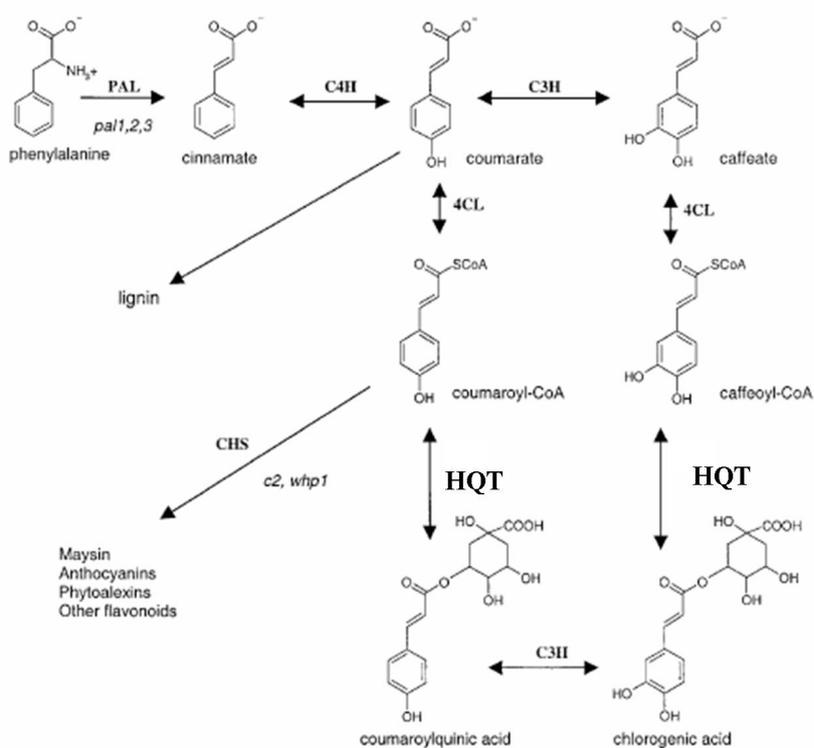


Figure 1.5: **The phenylpropanoid pathway.**

Jasmonic acid (JA) and the methyl ester MeJA, generate the best characterized fatty acid derived signals in plants. The cellular membranes have

an evident important function in separating the cell from the environment; they are the site of signal perception and transmission and therefore are an important source of signalling molecules, many of which derived from the fatty acids, the components of the membrane itself. JA is known to have important role not only in defence response to biotic or abiotic stress, such as microbe interactions and wounding, but also in plant development [11]. The first step to generate JA and MeJA is committed in the chloroplast by the lipoxigenase (LOX) enzyme, which catalyzes the production of fatty acid hydroperoxides, substrates for the subsequent enzymes in the pathway (fig. 1.6). In general, the correlation between LOX activity and defence was well characterized in transgenic plants, in which *LOX* expression was silenced. Fatty acids hydroperoxides can be in turn metabolized to produce not only jasmonate, but also antifungal compounds and plant-specific volatile compounds.

In this study, real time PCR analysis was used to analyze the variation in gene expression of *PAL* and *CHS*, as the key enzymes of the phenylpropanoid pathway. Moreover, the expression pattern of the second gene of the pathway, *C4H*, of the gene involved in the last step of the catechin synthesis, *leucoanthocyanidin reductases (LAR)*, and of the key gene involved in the synthesis of the chlorogenic acid, *hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT)*, were analyzed. The variation of *LOX* gene, which commits the first biosynthetic step in the jasmonate synthesis, was analyzed.

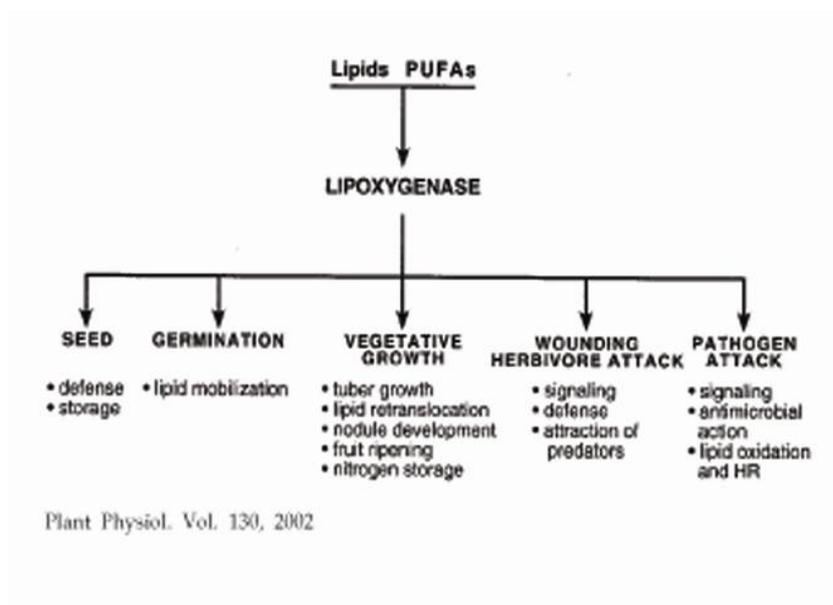


Figure 1.6: The LOX pathway and its biological function. PUFA: Polyunsaturated fatty acids.

As further methods of gene expression analysis, the microarray and the cDNA AFLP (amplified fragment-length polymorphism) techniques were used to study the whole 'transcriptome' and to investigate on gene expression differences between *Monilinia* susceptible and resistant peach fruits.

Microarray technology is an efficient and accurate method for large-scale analysis of gene expression variations; it allows the acquisition of a large amount of biological information from multiple samples. It is considered a hybridization-based method and its major advantages are miniaturization, automation and the simultaneous relative expression analysis of large numbers of genes on two samples, through the use of two different fluorescent labeling dyes. On the other hand, one considerable limit can be the availability of a large amount of sequences, either derived from the genome analysis or from expressed sequence tag (EST) library. For plant tree species this holds particularly true, since, so far, the genome is totally or partially known only for few species.

Microarray is based on the specific hybridization of two labeled samples to the probe immobilized on the chip. This consists of collections of DNA sequences. Data have to be normalized and for each gene an expression ratio is obtained; finally the biological meaning are speculated from data analysis between one or more experiments [8], (1.7).

Two different types of microarray are available: cDNA microarray and oligonucleotide microarray. The first is prepared directly from existing cDNA libraries with the appropriated instrumentation. Its production depends only on sequences availability and they can be created also as smaller array with a sub-set of genes, for a specific research.

Oligo microarray are more expensive and their production is more time consuming with respect to the cDNA microarray. The advantages are the

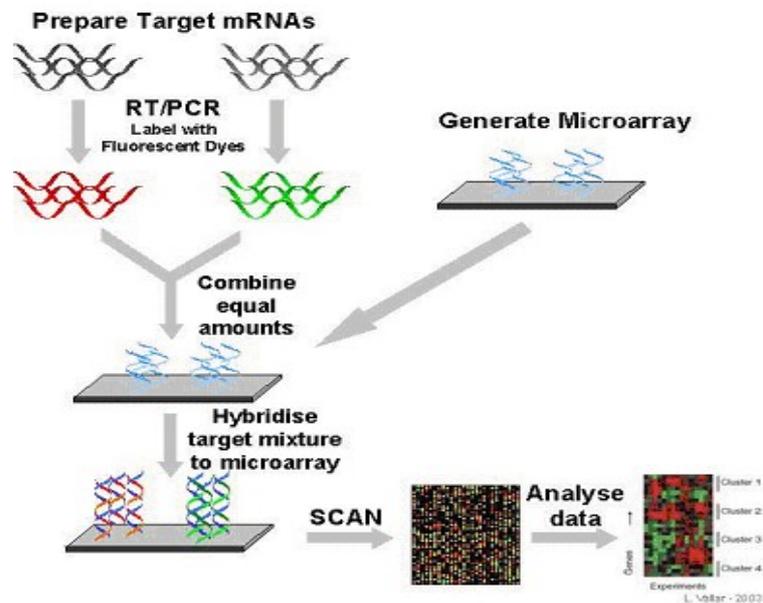


Figure 1.7: Schematic diagram of the microarray technique.

uniqueness of the probe sequence, the uniformity of probe length and melting temperatures, which can significantly reduce experimental variation and increase precision [12].

Many microarray chips for different plant species are now available; for *Arabidopsis* and the other plant sequenced genome, chips with sequences representing almost the complete 'transcriptome' are available. In *Arabidopsis*, the genes upregulated during stress defence and upon wounding were revealed using cDNA microarrays [13, 14, 15]. For the fruit gene expression analysis, some smaller microarrays are also available, such as the one for tomato [12] with 8500 tomato genes, the one for strawberry [16], for pear [17], citrus [18], grape [19] and peach[20].

Peach is a well studied species, and lately has been considered a good model for both ripening and genome analysis of fruit tree species. The first microarray on peach fruit was named μ PEACH1.0. It contains 4800 oligonu-

cleotides probes, derived either from a cDNA library of ripening fruits or from the sequences available in the Genome Database for Rosaceae at Clemson University Genomics Institute (<http://www.genome.clemson.edu/gdr/projects/prunus/unigene/>). μ PEACH1.0 was developed with the specific purpose of studying the changes occurring from the pre-climacteric to climacteric phase. However, in this study, it was shown to be a powerful tool also to analyze variation in gene expression during the physiological change in *Monilinia* susceptibility, which occurs earlier in the fruit growth (S2 phase).

Gene expression analysis in susceptible and resistant peach fruits was also performed using the cDNA AFLP method. This can be used also when genome or EST sequences are not available. It consists of a PCR-based method. cDNAs are prepared and restricted to generate cDNA specific tags; these are then amplified by PCR and separated on polyacrylamide high resolution gels. The differences in intensity of the bands reflect differences in gene expression (1.8).

cDNA AFLP analysis was already used to study many plant-microbe interactions. For instance, it was used to study the interactions between tomato and the fungal pathogen *Cladosporium fulvum* [21], the interactions between the aphid *Dysaphis plantaginea* and susceptible and resistant cultivars of apple [22], to investigate the expression variation in apricot fruits *P.armeniaca* upon inoculation with *Plum pox virus* [23], or also to simultaneously study the signal transduction pathways activated after different stress treatments [24]. cDNA AFLP has been proven to be a useful technique also to study change in expression during plant development, such as seed germination in *Arabidopsis thaliana* [25] and seed dormancy in *Nicotiana plumbaginifolia* [26].

In our work, cDNA AFLP was carried out following the method of Bryene

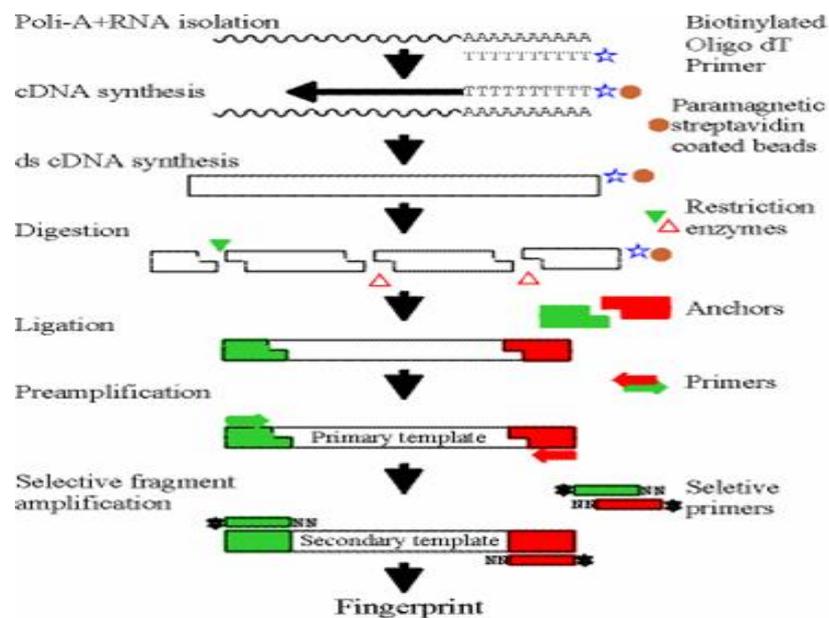


Figure 1.8: **The cDNA-AFLP technique.** Double stranded cDNA is cleaved with BstYI and MseI restriction enzymes. After adapter ligation, the AFLP templates are pre-amplified with BstYI-T or BstYI-C and Mse-0 primers and then selectively amplified with primers carrying either one or two additional selective nucleotides. The amplification products are then separated by polyacrylamide gel electrophoresis and visualized by autoradiography [27].

and Zabeau [27], developed on the basis of the original one published by Bachem et al. [28]. Bryene and Zabeau demonstrated also that the method could be used to perform genome-wide expression studies and it could be considered a valid alternative to the microarray [27].

The aim of this thesis was to investigate on the molecular basis of the physiological change in *Monilinia* susceptibility of peach fruits during growth. Three gene expression analysis methods were used: real time PCR, microarray and cDNA AFLP. Among the most interesting genes found differentially expressed between susceptible and resistant fruits, genes encoding for pathogenesis related (PR) proteins were found. To investigate on the association of *Monilinia* resistance and PR biological function, some PR proteins were expressed in heterologous system and *in vitro* assayed for their anti-microbial activity.

Chapter 2

Materials and methods

2.1 DNA isolation and cloning

All primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/primer3_code.html) or on the basis of multiple protein alignment. Oligonucleotides were synthesized by PRIMM s.r.l. (Milan, Italy) with PCR grade of purification. All PCR reactions were performed with the GoTaq DNA polymerase (Promega, Madison, WI, USA) and provided buffers, on a T3 thermocycler (Biometra, Göttingen, Germany) and the amplifications were analyzed on ethidium bromide agarose gel. PCR products of the correct size were gel-purified with Nucleospin Extracts II kit (Macherey-Nagel, Düren, Germany) and cloned in the pGEM-T Easy Vector (Promega, USA), following the manufacturer's instructions. The PCR products used for *E. coli* expression (PruP1 and PruP2) were cloned in the pHAT vector [29], following the standard procedure [31].

The *E. coli* DH5 α strain was used to amplify the plasmids and *E. coli* BL21 (DE3) strain for protein expression experiments. Plasmids were introduced in *E. coli* cells with electroporation technique, according to the standard proto-

cols [30]; competent cells were previously prepared with the standard method [34]. Transformant cells were grown over night at 37 °C in Luria-Bertani solid media. Liquid cultures were prepared from a single colony and the cells were grown in Luria-Bertani media with agitation. For plasmid selection, ampicillin was added to all media to a final concentration of 100 µg/ml. Plasmids were purified from an overnight liquid culture of *E.coli* DH5α cells, using the Nucleospin Plasmid kit (Macherey-Nagel, Germany) and following the manufacturer instructions, the presence of the insert was verified by restriction [31].

DNA fragments were sequenced by BMR-genomics (Padova, Italy; www.bmr-genomics.it); DNA fragments cloned into the plasmids were sequenced using the universal T7 forward primer, whereas the other PCR fragments were sequenced using the specific forward primer. The chromatograms were analyzed with Chromas lite software, freeware version, available on the BMR-genomics site and the obtained sequences were analyzed using BLAST sequence alignment programs [31].

2.2 Fruits and fungal materials

In 2005 peach fruits (*cv.*K2) were weekly harvested during a 17-week long-period, starting from the fourth week after full bloom (4th May), until full maturity (17th August). The fruits were harvested in a local fungicide-free orchard, located in Crespellano, Bologna.

M.laxa cultures were grown on 25% V8 juice in solid agar plates (4% bacto-agar) at room temperature; conidial suspensions were obtained by scraping off one week-old fungal plate culture. The conidia were eluted in 0.05% Tween 80, quantified through microscope analysis (Carl Zeiss Mi-

croscope) of a Thoma cells counting chamber previously filled with conidia suspension, and properly diluted to a concentration of about 10^5 conidia/ml for the fruit infection trials.

At each sampling time, three replicates of five fruits each, were dipped for 1 min in the *M.laxa* conidial suspension or in distilled water, as negative control. The fruits were maintained at room temperature for 3 hours; afterwards, they were peeled with a scalpel; the peel was immediately frozen in liquid nitrogen and transferred to $-80\text{ }^{\circ}\text{C}$ until use.

Similarly, for the analysis of susceptibility, three replicates of 20 fruits each, were inoculated with conidial suspension or with distilled water as negative control, and stored at $20\text{ }^{\circ}\text{C}$ and 95% of humidity for one week. After that time, the degree of susceptibility of peach fruit to the pathogen was determined as percentage of infected fruits.

The fruit growth was evaluated by weekly measuring the diameter of 30 fruits.

2.3 Analysis of conidial germination

In vivo conidial germination was assessed at time zero and after 3, 6, 12 and 24 hours of inoculation on 3 replicates of five fruits each. The fruits were inoculated rolling them for 1 min in a *M.laxa* conidial suspension (10^7 conidia/ml). After incubation at room temperature, the conidia were scraped off with adhesive tape in three different positions of each fruit surface, and the tape was placed on a glass microscope slide surface and fixed with a drop of nail varnish. The conidia were stained with 1-2 drops of lactophenol blue solution, covered, incubated for 5 min and finally fixed under the flame. The conidia were visualized on a microscope (Carl Zeiss, Microscope).

2.4 Expression of genes of the phenylpropanoid and jasmonate pathway

The fruit samples harvested from 6th week after full bloom (end of S1 phase) to 12th week after full bloom (end of S2 phase), were chosen for this analysis. From the peel of each fruit sample, either inoculated with the pathogen or mock inoculated, three replicates of RNA extraction and c-DNA first strand synthesis were made.

2.4.1 Small scale RNA extraction

Total RNA was extracted from 0.1 g of the peach skin using the Concert Plant RNA reagent buffer (Invitrogen, Carlsbad, CA, USA) with some modifications. Briefly, two extractions were done for each replicate; of these, each was precipitated overnight in 3 M LiCl at 4 °C, to increase the RNA yield. RNA was dissolved in 35 μ l of 0.1% DEPC (diethyl pyrocarbonate) water solution, incubated at 60 °C for three min and then centrifuged at maximum speed for two min. The supernatant was transferred in a fresh tube, frozen and stored at -80 °C. Before use, the RNA was heated at 60 °C and again centrifuged to eliminate the precipitated fraction.

The RNA integrity and purity was evaluated on agarose gel and by measuring the absorbance ratios $A_{260}:A_{230}$ and $A_{260}:A_{280}$. Sample concentration was calculated from the absorbance at 260 nm.

Seven μ g of RNA were treated with the TURBO DNA-freeTM kit (Ambion, Applied Biosystem, Foster City, CA, USA) to remove any trace of contaminating DNA, following the manufacturer's instructions. RNA losses were evaluated by a second absorbance reading at 260 nm.

2.4.2 c-DNA first strand synthesis

To generate first strand c-DNA, 360 ng of total RNA was used with the ImProm-II Reverse TranscriptaseTM (Promega, Madison, WI, USA) and oligo(dT) as primers.

2.4.3 Target gene isolation

Primer design

Multiple alignments of CHS, C4H, LOX, LAR and HQT proteins were generated using CLUSTALX software (<http://bips.u-strasbg.fr/fr/Documentation/ClustalX/>). Degenerated primers coding for protein conserved regions were designed (table 2.1) and synthesized by PRIMM srl (Milano, Italy). Specific primers were designed for the peach *PPrich protein*, *Actin*, *PAL*, *β -glucanase* gene sequences available on GenBank database (www.ncbi.nlm.nih.gov).

PCR, gene cloning and sequencing

Using the degenerated primers and the c-DNA first strand as template, amplicon of the target peach genes were generated by PCR reactions; reaction conditions (concentration of primers, template and MgCl₂, and annealing temperatures) were optimized for each amplification as shown in the table 2.2.

Amplicon of the correct size were purified cloned and sequenced using the procedures described in the DNA isolation and cloning.

Gene	Degenerate primer sequences 5'-3'		Specific primer sequences 5'-3'
<i>PAL</i>	F		TGCCATAATGGAGCACATTT
	R		CAGCCATTGGGGAGAAGTT
<i>CHS</i>	F	ATHAARGARTGGGGNCARCC	AGATCACCGCAGTCACCTTC
	R	TANCGNCCNGGNGGCCYAC	CTTCTCGATCTCGGAATTG
<i>C₄H</i>	F	TTYGAYATHTTYACNGGNAARGG	AGAATTCGGCTGTTCAAGGA
	R	ACRTTDATRTTYTCNACDATRTA	CGTTGATCTCTCCCTTCTGC
<i>LAR</i>	F	ATHTGYTGAAAYWSNATHGC	TTCACAATGAAAACGGTGGA
	R	TGRCANCCNTTADATRAADATRTCRTG	GCCAATTTTCTTCTCCCACA
<i>HQT</i>	F	AAYTGAAAYGGNGARGGNGT	AGCTCGTTCCTGCTGTTGAT
	R	GGRTGRTAYTCNACRTGYTCRAA	TGGAGACCAGAAAAGCCATC
<i>LOX</i>	F	ACNGAYGARGARTTYGCNMG	TTTTCCAGCCATCTTGGAAC
	R	TGNCCRAARTTNACNGCNGC	AGGTTCTGTGACGCAGTGTG
<i>ACT</i>	F		ATCATGTTTGAGACCTTCAATG
	R		AGAGTCCAGCACAAATACCAGTT
<i>PP-rich protein</i>	F		ACCTTCCACACCTTCCACAG
	R		TTGGATGAGACTGCAGCAAG

Table 2.1: Primer sequences

Gene	Melting T	[MgCl ₂]	[Primers]	Amplicon length
<i>CHS</i>	50 °C	2 mM	50 pmol	600 bp
<i>C₄H</i>	45 °C	3 mM	50 pmol	600 bp
<i>LOX</i>	44 °C	3 mM	50 pmol	600 bp
<i>LAR</i>	48 °C	2 mM	50 pmol	400 bp
<i>HQT</i>	46 °C	1.8 mM	50 pmol	380 bp

Table 2.2: PCR conditions

Gene	Annealing T
<i>PAL</i>	57 °C
<i>CHS</i>	60 °C
<i>C4H</i>	60 °C
<i>LAR</i>	58 °C
<i>HQT</i>	60 °C
<i>LOX</i>	60 °C
<i>ACT</i>	57 °C
<i>PP-rich</i>	60 °C

Table 2.3: Real Time PCR annealing Temperatures

2.4.4 Real Time PCR analysis

Suitable primers for real time PCR were designed on the basis of the specific sequences, using Primer3 software (http://frodo.wi.mit.edu/primer3/-primer3_code.html), (fig. 2.1).

Real Time PCR was performed on an MX3000 machine (Stratagene, La Jolla, CA, USA) using the Brilliant SYBR Green QPCR Master mix (Stratagene, USA). Each reaction mixture contained 1:50 of cDNA first strand as template, 120 nM of each specific primer, 15 nM ROX as reference dye and 1x Brilliant SYBR Green QPCR Master mix (Stratagene, USA). Two replicates of each reaction were always run in the same experiment. All thermal cycles started with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of a denaturation step at 95 °C for 30 sec, an annealing step at a specific temperature (tab. 2.3) and an extension step at 72 °C for 1 min.

Fluorescence was monitored at the end of each annealing step. To assess the amplification specificity, melting curve analysis was always performed at the end of each experiment by monitoring the fluorescence from 55 °C to 95

°C every 0.1 °C.

Amplified products were checked by agarose gel electrophoresis and sequencing (BMR-genomics, Italy). Quantification was carried out using the standard curve generated by serial dilutions (1:1.5, 1:2, 1:5, 1:10, 1:30) of a cDNA first strand randomly chosen. Data were analyzed using MXPro QPCR Software, Version 3.0 (Stratagene, USA).

Quantifications were normalized through Real Time amplification of the *actin* housekeeping gene, whereas amplification of the *PP-rich* protein gene (whose transcript abundance in S1 and S2 was known) was used to check the data reliability (Data not shown).

Data were analyzed by ANOVA and means were separated by the least significance difference test at the 0.05 probability level. STATISTICA software was used (Statsoft INC., Tulsa, OK, USA). Unless noted otherwise, only results significant at $P \leq 0.05$ are discussed.

2.5 Microarray analysis

The samples harvested on the 8th week after full bloom (AFB) (named **S**, for susceptible ones) and on the 10th week after full bloom (named **R**, for resistant ones) were compared on microarray analysis; both type of fruits, inoculated with the pathogen (**SI** and **RI**) or mock inoculated (**SH** and **RH**), were analyzed. All the microarray experiments were carried out at the University of Padua (Dept. of Environmental Agronomy and Crop Science). The μ PEACH1.0 microarray together with the suited protocols were used [20, 32]. Each microarray experiment was run in three replicates, using three preparations of labeled cDNA first strand, generated from as many RNA extractions, for each sample.

Tris-HCl pH8	100 mM
NaCl	100 mM
EDTA pH8	5 mM
Ascorbic acid	5 mM
PVP 40	1%
PVPP	1%
Proteinase K	10 μ g /ml
β -mercaptoethanol	2.5%
SDS	1%

Table 2.4: **RNA extraction buffer**

2.5.1 Large scale RNA extraction

The RNA was extracted following the protocol published by Bonghi *et al.* [32], with some modifications. 2.5 g of frozen peach skin were ground to a fine powder in liquid nitrogen with mortar and pestle, and the ground tissue was suspended in 10 ml of 65 °C preheated extraction buffer (tab. 2.4) and leaved at 65 °C for 10 min.

One volume of 65 °C preheated phenol was added to the mixture and samples were then centrifuged at 14000 x g for 6 min. The aqueous phase was extracted with an equal volume of phenol:chlorophorm:isoamylalcohol (25:24:1), re-centrifuged as above, and re-extracted with one volume of chloroform:isoamylalcohol (24:1). After centrifugation at 14000 x g for 6 min, the RNA was precipitated in one volume of isopropyl-alcohol and 0.3 M sodium acetate (pH 4.8), washed with 1 ml of 70% ethanol and resuspended in 6 ml of TBE 1x. The RNA was then precipitated in 3M LiCl overnight at 4 °C, centrifuged at 30000 x g for 30 min at 4 °C and washed with 1 ml of 70 % ethanol. The pellet was resuspended in 1 ml of 0.1% DEPC water, transferred in a 2 ml eppendorf tube and precipitated with one volume of

isopropyl-alcohol and 0.3 M of Sodium Acetate pH 4.8 at -80°C for 60 min. After a centrifugation at $14000 \times g$ for 5 min and one washing step in 70 % ethanol, the pellet was air dried and resuspended in $50 \mu\text{l}$ of 0.1% DEPC water. DNA was removed from the samples by Turbo DNase treatment (Ambion, USA) following the manufacturer's instructions. Two additional purification steps with one volume of phenol:chloroform 3:1 and with one volume of chloroform: isoamylalcohol 24:1 were always performed. RNA purity was analyzed by measuring the $A_{260} : A_{230}$ and $A_{260} : A_{280}$ ratios; RNA quantity was calculated from the absorbance at 260 nm; to analyze the integrity of the samples and to confirm the quantification, $0.5 \mu\text{g}$ of RNA was run on agarose gel (fig. 2.1).

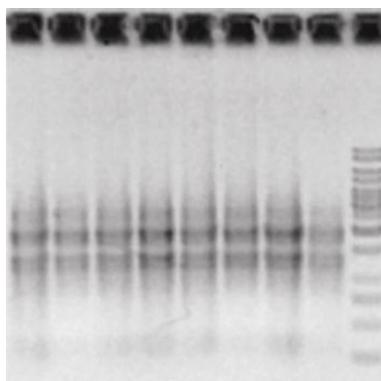


Figure 2.1: **Agarose gel of total RNA.** The integrity of RNA was indicated by the presence of the two ribosomal RNA bands. In the last lane the 1kb Gene Ruler (Fermentas) was loaded. RNA yield ranged from 40 to $120 \mu\text{g}$, from 2.5-3 g of tissue. Only high quality RNA with $A_{260} : A_{230}$ and $A_{260} : A_{280}$ ratios ≥ 2 was used to generate the labelled cDNA.

2.5.2 cDNA synthesis, labeling and quantification

20 μg of high-quality RNA (OD ratios higher than 1.8) were used to prepare cDNA first strand with the SuperscriptTM Indirect Labeling System (Invitrogen, USA) and 5 μg of Oligo(dT)₂₀ associated with the system as primers; the manufacturer's procedure was followed. The term "indirect system" refers to the fact that, instead of fluorescent nucleotides, the cDNA first strand contains aminoallyl- and aminohexyl-modified nucleotides together with other dNTPs; compared to direct labeling systems, this system ensures a greater incorporation of fluorescent dye and an higher signal intensity.

A purification step was performed with the S.N.A.P.TM columns (Invitrogen, USA), associated with the system, to remove unincorporated nucleotides. Subsequently, the cDNA first strands were coupled to monoreactive *N*-hydroxysuccimide (NHS)-ester fluorescent dyes (specifically, the green-fluorescent cyanine3 (Cy3) or the red-fluorescent cyanine5 (Cy5)(GE Healthcare, Milano, Italy), at room temperature for 1 hour, as suggested by the instructions. A second purification step was performed with the S.N.A.P.TM columns (Invitrogen, USA) to eliminates unincorporated dyes.

Labeled c-DNA quantity and purity were analyzed at the spectrophotometer (Ultrospec 2100 pro, GE Healthcare,Italy) using the procedure and formulas specified in the SuperscriptTM Indirect Labeling System Manual (fig. 2.2).

2.5.3 Microarray hybridization

The microarray was pre-hybridized for two hours with the pre-hybridization solution (5X SSC, 0.1% SDS, 1xX Denhardt's and 100 ng/l carrier DNA), washed with 0.2X SSC and isopropanol and dried. 150 pmol of Cy3 and Cy5 labeled samples were mixed, ethanol precipitated, suspended in 37 μ l of hybridization solution (5X SSC, 0.1% SDS, 25% formamide and 40 ng/l carrier DNA) and boiled. The hybridization was performed in Corning chambers for 48 hours and the unbound material was washed away from the slide once with 1x SSC 0.2% SDS, once with 0.1 SSC 0.2% SDS and twice with 0.2 SSC [20].

The following samples were directly compared: **RH** vs. **SH**; **RI** vs. **SI**; **RH** vs. **RI**. To increase the accuracy of the experiment, each couple was analyzed three times, of which, one with the cDNA labeled with inverted fluorescent dyes in order to avoid artefact due to the dye incorporation.

2.5.4 Microarray data analysis

The samples were scanned with a two channel confocal microarray scanner (ScanArray Lite, Perkin-Elmer, Waltham, Massachusetts, USA) using the associated software (ScanArray Express 3.0.0., Perkin-Elmer). The laser power was set between 78 and 81%, the photomultiplier tube (PMT) was set between 73 and 81 %. The dyes excitation-emission wavelengths were set on 543-570 nm for Cy3 and on 633-670 nm for Cy5. The scan was performed at a resolution of 5 μ m.

The fluorescence emission of each dye was converted into a digital output and was stored as a TIFF image. The images were then processed with the TIGR Spotfinder 2.2.3 software, available online at www.tigr.org.

A grid was superimposed on the image; this was built on the basis of parameters specific for the μ PEACH1.0, such as the spot spacing, the printing pins, the distances between them and the number of columns and rows of each grid. For each experiment, the left top grid was manually modified and aligned, then the alignment was automatically propagated to the rest of the grid. Each spot was carefully analyzed in order to eliminate artifacts. A tab delimited text file with all slide data was generated by the software.

These data were manually processed: for each dye the mean intensity of the randomly negative controls was calculated, representing the background fluorescence caused by non-specific binding; the 60% of the mean was arbitrarily attributed to those spots having, only in one channel, a fluorescence value less than the mean of the negative control. This avoided over-evaluation of the two channel ratio.

The so processed data were used as the input file for Genespring Software (Agilent Tech., USA): the data were normalized and the differentially expressed genes identified. The genes with an expression ratio >1.7 (up-regulated) or <0.5 (down-regulated), as processed using a Student's test, were considered differentially expressed genes.

2.5.5 Real Time analysis

The results obtained with microarray analysis were validated with Real Time PCR. 1 μ g of the same RNA samples used in microarray analysis was retro-transcribed using ImProm-II Reverse TranscriptaseTM (Promega, USA) and oligo(dT) as primers. Three replicates of c-DNA first strand were synthesized for each sample. Specific primers were designed with Primer3 software (http://frodo.wi.mit.edu/primer3/primer3_code.html), on the basis of gene sequences deposited in the ESTree database (www.itb.cnr.it/estree),

Gene		Primer sequence
Major allergen Pru av 1 (Pru a 1)[<i>Prunus avium</i>]	F	CCCCGATGCCTACAACATAAA
	R	GCAACAAGAAAAGCCACACA
ATP sulfurylase [<i>Brassica juncea</i>]	F	CGAACACTTGCAAGGAACAA
	R	CTGCTTCTGGGACTTTACCG
S-adenosylmethionine decarboxylase.[<i>Malus domestica</i>]	F	GGGATACTGTTGCGGAGAAA
	R	CAACTTCCTCGTCCTTCTCG
harpin inducing protein [<i>Nicotiana tabacum</i>]	F	TGACAGCACCCCTCCACTACA
	R	GTCGAGGTGTACGTGCTGAA
heat shock protein 70, cytosolic [imported] - spinach	F	GGTGAAGAGAGGCAGTTTGC
	R	AGCCTGACGCTGAGAATCAT
Defensin protein 1[<i>Prunus persica</i>]	F	CGCTCCATGCGTTTATTTTC
	R	TCACAGGTCCTAGCCTCAGC
Ferritin 1, chloroplast precursor.[<i>Brassica napus</i>]	F	TCTCTTGCTCGTCAGCGTTA
	R	TATGCAAACAGGGCATGGTA
Histidine-containing phosphotransfer protein[<i>Catharanthus roseus</i>]	F	CCAGCATGTGAAGCATGAGT
	R	CCCCTGGGTTTCATCATT
Cinnamyl alcohol dehydrogenase (Fragment).[<i>Zinnia elegans</i>]	F	GGCTGAAATGGTGCTTTTCAT
	R	CACAGTCATCATCCCACACC
Calmodulin	F	AGCCTTCAGCCTATTTCGACA
	R	TGGTTCATTACCATCAGCA
Zinc finger	F	TGGGGTCAATTCCCTGGTTA
	R	GTTGCTTATGCCCTCTGCTC

Table 2.5: Specific primers used in real time PCR.

(tab. 2.5).

The same procedure mentioned above (sec. 2.4.4) was followed. All genes were amplified using an annealing temperature of 60 °C, except for the *actin* gene, for which an annealing temperature of 57 °C was used.

Wash-binding buffer	Elution buffer	Low salt buffer
0.5 M NaCl	10 mM Tris-HCl (pH 7.5)	0.15 M NaCl
20 mM Tris-HCl (pH 7.5)	1 mM EDTA	20 mM Tris-HCl (pH 7.5)
1 mM EDTA		1 mM EDTA

Table 2.6: **Poly(A)⁺ RNA isolation buffers.**

2.6 cDNA AFLP analysis

Total RNA was isolated from the same fruit samples used for microarray analysis: these were the fruits harvested on 8th week AFB (**S** fruits), and on 10th week AFB (**R** fruits), both inoculated with the pathogen (**SI and RI**) or mock inoculated (**SH and RH**). The large scale extraction method, as previously described in the microarray analysis, was used except for the DNase treatment which was not performed.

2.6.1 Poly(A)⁺ RNA isolation

poly(A)⁺ RNA was isolated from 100 μ g of total RNA. The streptavidin magnetic beads (New England Biolabs, Ipswich, MA, USA) and biotinylated oligo d(T)(PRIMM,Italy) were used following the manufacturer's instructions. In the table 2.6 the composition of the used buffers are reported. The mRNA was resuspended in 50 μ l of Elution Buffer (tab. 2.6).

2.6.2 Double stranded cDNA synthesis

30 μ l of poly(A)⁺ RNA (corresponding approximately to 0.6 μ g of mRNA) were mixed with 56 pmol biotinylated oligo d(T) (PRIMM, Italy) and heated at 72 °C for 5 min for denaturation. First-strand was synthesized using M-MuLV Reverse Transcriptase (NEB, USA); the mixture (tab. 2.7) was

First strand synthesis mixture	Second strand synthesis mixture
20 μ l poly(A) ⁺ RNA	50 μ l first strand mixture
56 pmol oligo d(T)	1x <i>E. coli</i> ligase buffer (NEB)
1x first strand buffer	12 mM dTT
8 mM dTT	0.6 μ M dNTP
0.4 μ M dNTP	30 U <i>E. coli</i> DNA ligase (NEB)
200 U M-MuLV reverse transcriptase	50 U <i>E. coli</i> DNA polymerase I (NEB)
water up to 50 μ l	1.6 U RNase H (NEB)
	water up to 160 μ l

Table 2.7: **Double strands cDNA synthesis reactions**

incubated at 42 °C for 2 hours. Second strand synthesis was carried out with RNase H (NEB, USA), *E. coli* DNA polymerase I (NEB, USA), which possess exonuclease activity, and *E. coli* DNA ligase (NEB, USA); the mixture was incubated for one hour at 12 °C and one hour at 22 °C.

Double strand cDNA was purified with an equal volume of phenol: chloroform: isoamylalcohol 25:24:1, precipitated with 2 volumes of ethanol and resuspended in 50 μ l of Elution buffer (tab. 2.6). The purified cDNA was quantified by measuring the OD at 260 nm (Nanodrop spectrophotometer) and analyzed on 1% agarose gel.

2.6.3 Template preparation

cDNA was double digested with a rare-cutting enzyme (BstYI) and a frequent-cutting enzyme (MseI). 500 ng of double strand cDNA were digested in a final volume of 40 μ l with 10 U of BstYI endonuclease (NEB, USA) at 60 °C for two hours. After digestion, an equal volume of streptavidin magnetic beads (NEB, USA) were added to immobilize the biotinylated 3'-terminal cDNA fragments, the reaction mixture was incubated at room temperature

Primer name	Primer sequence (5'- 3')
BstYI-top-adapter	CTCGTAGACTGCGTAGT
BstYI-bottom-adapter	GATCACTACGCAGTCTAC
MseI-top-adapter	GACGATGAGTCCTGAG
MseI-bottom-adapter	TACTCAGGACTCAT
BstT0	GACTGCGTAGTGATCT
BstC0	GACTGCGTAGTGATCC
Mse0	GATGAGTCCTGAGTAA
Mse-X	GATGAGTCCTGAGTAAX
BstT-X	GACTGCGTAGTGATCTX
BstC-X	GACTGCGTAGTGATCCX

Table 2.8: **cDNA-AFLP oligonucleotides**

for 30 min in gentle agitation. The beads together with the anchored biotinylated fragments, were collected by magnet and washed four times with the Wash buffer (tab. 2.6). The bead-coupled c-DNA fragments were re-suspended in 30 μ l of Elution buffer (tab. 2.6) and digested with 10 U of MseI restriction enzyme; the reaction mixture (40 μ l final volume) was incubated at 37 °C for two hours in gentle agitation. After that time, the beads were collected by magnet and the supernatant, containing the digested template fragments, was transferred in a new clean tube for the adapter ligation reaction.

Before ligation, BstYI and MseI adapter were prepared by mixing the nucleotides named as top and bottom adapter (tab. 2.8) at room temperature for 30 min. The adapters were ligated to cDNA fragments using T4-DNA ligase (NEB, USA) in the following reaction mixture:

- 5 pmol **BstY1 adapter**
- 50 pmol **Mse1 adapter**

- 10 mM **ATP**
- 1x buffer 2 NEB
- 1 U **T4 DNA Ligase** (NEB)
- 10 U **BstY1** (NEB)
- 40 μ l digested DNA fragments

The mixture (final volume 50 μ l) was incubated at 37 °C for 3 hours. The product of the ligation reaction is termed 'primary template'.

2.6.4 Pre-amplification

Two pre-amplification reactions were performed on each ligation; in these, BstT0- or BstC0-primers (tab. 2.8) were combined with Mse0-oligonucleotide (tab. 2.8). The reaction mixture (50 μ l) contains:

- 5 μ l of 10- fold diluted **ligation**
- 75 ng BstT0 or BstC0 **primer**
- 75 ng **Mse0 primer**
- 0.2 μ M **dNTP**
- 1x Taq polymerase specific **buffer**
- 1.5 mM **MgCl₂**
- 1 U **Go-Taq polymerase** (Promega)

The pre-amplification reactions consisted of an initial denaturation step at 94 °C for 3 min, 25 cycles (40 sec at 94 °C, 1 min at 56 °C and 1 min at 72 °C) and a final elongation step at 72 °C for 2 min. 1/10 of the pre-amplification reaction (termed 'secondary templates') were checked on 1% agarose gel.

2.6.5 Selective amplification reaction with labeled primer

Secondary templates were diluted 50-fold and used in a second round of PCR performed with selective primers, which contained a specific nucleotide at the 3' end. In this amplification, the primer complementary to the BstYI site was labeled with γ -³³P-ATP using the T4 polynucleotide kinase (NEB, USA). The selective amplifications were carried out with an initial denaturation step at 94 °C for 10 min, followed by 13 cycles of 30 sec at 94 °C, 30 sec from 65 °C to 56 °C (−0.7 °C / cycle) and 1 min at 72 °C; subsequently, other 23 cycles were performed with the conditions of the last cycle, followed by

a final extension step for 10 min at 72 °C. For each condition (**SI**, **SH**, **RI**, **RH**) 32 different primer combinations were analyzed, each reaction mixture contained:

- 5 μ l of 50-fold dilution of the **pre-amplification mix**
- 5 ng ^{33}P -labeled **BstYI selective primer**
- 30 ng **MseI selective primer**
- 0.08 mM **dNTPs**
- 1x Taq-polymerase **buffer** (Promega)
- 1.5 mM **MgCl₂**
- 0.6 U **Go-Taq polymerase** (Promega)
- water up to 50 μ l

2.6.6 Gel analysis

Gel analysis was done as in conventional AFLP protocol [33]. The PCR products were mixed with an equal volume of loading dye (98 % formamide, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, 10 mM EDTA pH 8.0) and put at 4 °C overnight. 6 μ l of each PCR sample were analyzed on 6% denaturing Sequagel polyacrylamide gel (Polymed s.r.l., Firenze, Italy), using the Sequigel system (Biorad, Hercules, CA, USA). The gel matrix was previously prepared following the manufacturer's instructions. Electrophoresis was performed at a constant power of 90 W for about 2 hours, in 0.5% TBE buffer. After running, the gel was transferred on Whatmann paper 3MM (Whatmann, Maidstone ,UK) and vacuum dried. Then it was exposed overnight, at -80 °C on a Biomax MR autoradiogram (Kodak).

2.6.7 Isolation, re-amplification and sequencing of AFLP fragments

Fragments that were differentially expressed in the selected conditions, were excised from polyacrilamide gels with a scalpel, eluted in 100 μ l of distilled water and put for at least one night at -20 °C; 5 μ l of the solution were amplified using 75 ng of the specific AFLP primers, with an annealing temperature of 56 °C. The PCR products were loaded on 1.5% agarose gel, purified with Nucleospin Extracts II kit (Macherey-Nagel, Germany) and sequenced (BMR-genomics, Italy).

2.6.8 Sequences analysis and bioinformatics

Each sequence was analyzed with the BLAST programs [31] against all sequences in the EStree databases (www.itb.cnr.it/estree) and the NCBI (www.ncbi.nlm.nih.gov/blast/Blast.cgi) databases. Only homologies with an e value $\leq 10^{-4}$ were accepted. The resulting sequences were annotated in according to the Gene Ontology system (http://mips.gsf.de/proj/funcat_DB/search_main_frame.html).

2.7 Pru P1 and Pru P2 expression and activity test

2.7.1 c-DNA cloning

The full length c-DNA of Pru P1 (GenBank::AM493970) and of the Ctg1 [20] (termed Pru P2) were amplified from a first strand cDNA synthesis product used for the microarray analysis. Specific oligonucleotides were designed

Primer name	Primer sequence (5'- 3')
Pru P1 forward	TAT ACCATGGG TGTCTTCACATATGAGAGCGAGTT
Pru P1 reverse	TATA AGCTT AGTTGTAGGCATCGGGGTGGCCCTTA
Pru P2 forward	TAT ACCATGGG TGTCTTCACATACTCAGACGAGTC
Pru P2 reverse	TATA ATGCAT TAGTTGYAGGCATCWGGGTTGGC

Table 2.9: **Pru P1 and Pru P2 primer sequences.** In bold the restriction site sequences

using the Primer3 software and the following restriction enzyme recognition sites were introduced for cloning reasons: NcoI in both the forward primers; HindIII and AvaIII, in the Pru P1 and Pru P2 reverse primers, respectively (tab.2.9).

1/10 of the first strand cDNA synthesis product was amplified in PCR reactions with 25 pmol of each primer, 2 mM dNTP mix, 1,5 mM MgCl₂, 1x buffer without MgCl₂ and 1 U of GoTaq polymerase (Promega, USA). PCR started with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles (95 °C for 30 sec, 58 °C for 1 min and 72 °C for 1 min) and a final extension at 72 °C for 10 min. PCR amplification was analyzed and purified as previously described in the section DNA isolation and cloning and the total amount of purified PCR product was digested with 25 U of each restriction enzyme (Fermentas, Burlington, Ontario, Canada), at 37 °C overnight. 50 μg of pHAT plasmid [29] were digested with 25 U of the same restriction enzymes. The digested products were gel-purified (Nucleospin Extracts II kit, Macherey-Nagel, Germany) properly eluted in sterile water and quantified as band intensity on agarose gel. 60 ng of purified PCR product were ligated with 18 ng of pHAT plasmid with 2 U of T4 ligase (NEB, USA). The reaction was incubated at 16 °C overnight. Cloning and

plasmid purification were performed as described in the section DNA isolation and cloning.

2.7.2 Protein expression and purification

Over-expression of the His-tagged PruP1 and PruP2 were obtained using *E.coli* BL21(DE3) cells. The bacteria were transformed with 1 μ l of each plasmid preparation and 10 μ l of the transformed bacteria were plated on LB- ampicillin (section DNA isolation and cloning). The proteins were expressed from an overnight culture of *E.coli* BL21 (DE3), diluted 100x in 1 L LB liquid medium. The culture was grown to an OD₆₀₀ of 0.45, afterwards the expression was induced with 0.4 mM IPTG for 3 h. Bacteria were pelleted at 5000xg for 15 min at 4 °C and the pellet was suspended in 25 ml buffer A (20 mM phosphate buffer pH 7.5 and 300 mM NaCl). Cells were disrupted with French press (SLM AMINCO I) at 1200 psi; DNaseI (10 μ g/ml) and MgCl₂ (10 mM) were added to the solution prior to the lysis. The lysate was centrifuged at 75600xg (Avant J-25, Beckman Coulter, Germany).

The supernatant was subjected to Ni-affinity purification using the 1 ml HiTrap affinity columns (GE Healthcare, USA). The columns were previously activated with 0.5 volume of NiSO₄ 0.1 M and equilibrated with 5 volumes of buffer A, according to the manufacturer's instructions. The supernatant fraction of *E. coli* lysate was loaded onto the column, washed twice with 5 volumes of wash buffer I (buffer A with 20 mM imidazole) and with 5 volumes of wash buffer II (buffer A with 50 mM imidazole), and eluted in 3 fractions of 2 ml each with elution buffer (buffer A with 200 mM imidazole); a last elution step with buffer A with 500 mM imidazole was performed. All fractions, included the total lysate, the supernatant and the pellet were analyzed on tricine SDS page, following the standard protocols [34].

The proteins were dialysed overnight against a buffer containing phosphate buffer 50 mM pH 7.5 and 150 mM NaCl and quantified as OD 280 nm.

2.7.3 *In vitro* antimicrobial activity of recombinant proteins

Inhibition of *M. laxa*, *M. fructigena*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Rhizopus* and *Penicillium expansum* hyphal growth by Pru proteins were assayed. The fungi were grown on potato dextrose agar plates at room temperature, until the colony reached a diameter of 3.5 cm. Sterile filter paper discs of 6 mm diameter were put at 0.5 cm from the growing front and 5 or 10 μg of the recombinant protein, or the lysate supernatant of a BL21(DE3)- pHAT vector, or protein buffer, as a control, were applied onto the discs. After 12 hours of incubations the growth of the fungi were analyzed.

2.7.4 Ribonucleolytic activity assay

Total RNA from peach fruit was extracted and purified as previously described (section Large scale RNA extraction). Both proteins were analyzed for RNase activity by incubation with RNA for 2-4 hours at 37 °C. The reaction mixtures contained 15 μg of peach total RNA and 10 μg of the recombinant protein in a final volume of 50 μl of phosphate buffer; three negative controls were performed in which the purified protein was replaced by the boiled purified protein, the protein buffer and the lysate supernatant of a BL21(DE3)- pHAT vector. Three replicates of each experiment were performed. The proteins were removed from the reactions mixture by ex-

traction with phenol-chloroform and the RNA was analyzed on 1% agarose gel and ethidium bromide stained.

Chapter 3

Results

3.1 Fruit growth and susceptibility

The fruits caliber of peach fruits was weekly measured and the growth curve showed the typical double sigmoid pattern with the four standard growth stages (S1–S4) (fig. 3.1) [20]:

- **S1 phase** in which the pericarp enlarges (from 4th to 7th week after full bloom)
- **S2 phase** in which pit hardening occurs and fruit stop to growth (from 8th to 12th week after full bloom)
- **S3 phase**, the pre-climateric phase, during which the mesocarp increases (from 13th to 18th week after full bloom)
- **S4 phase** the climateric phase, in which fruit ripening occurs

Peach fruit susceptibility to *M.laxa* was weekly evaluated as percentage of infected fruit upon artificial infections. During the S1 phase, fruits were

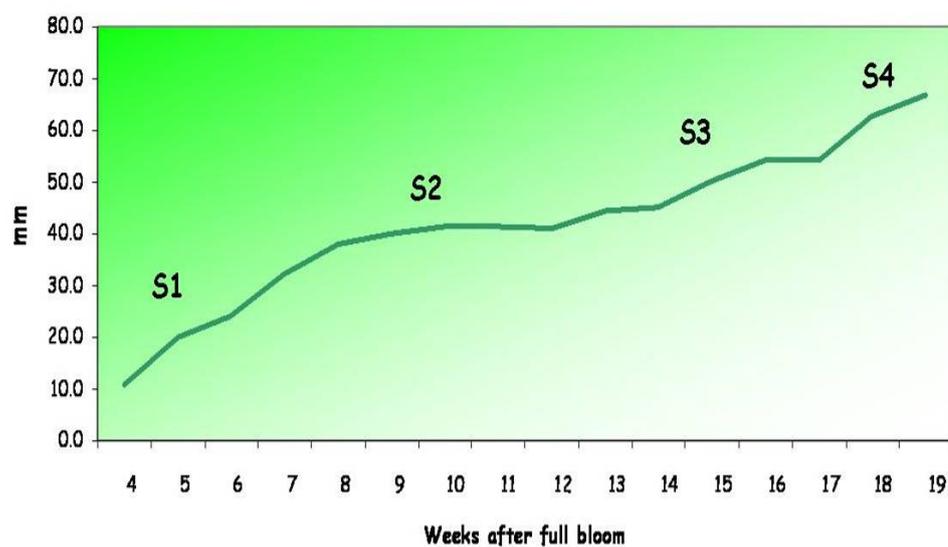


Figure 3.1: **Peach fruit growth curve.** Variation of fruit caliber (mm) during the weekly sampling times from the 4th may (4th weeks after full bloom, to 17th august (19th week after full bloom). Data are the average of 30 samples.

highly susceptible to *Monilinia* (50 to 90% fruits showed infection symptoms); after this period, susceptibility strongly decreased from 58% of infected fruits (at the 9th weeks after full bloom) to 10% at the 10th. At 12th week all fruits showed resistance to *Monilinia* infections. Susceptibility increased again, thereafter (fig. 3.2). The variation in susceptibility showed by peach fruits from the 6th to the 12th weeks AFB, is very reproducible among different years [3], whereas the one before and after this period varies each year.

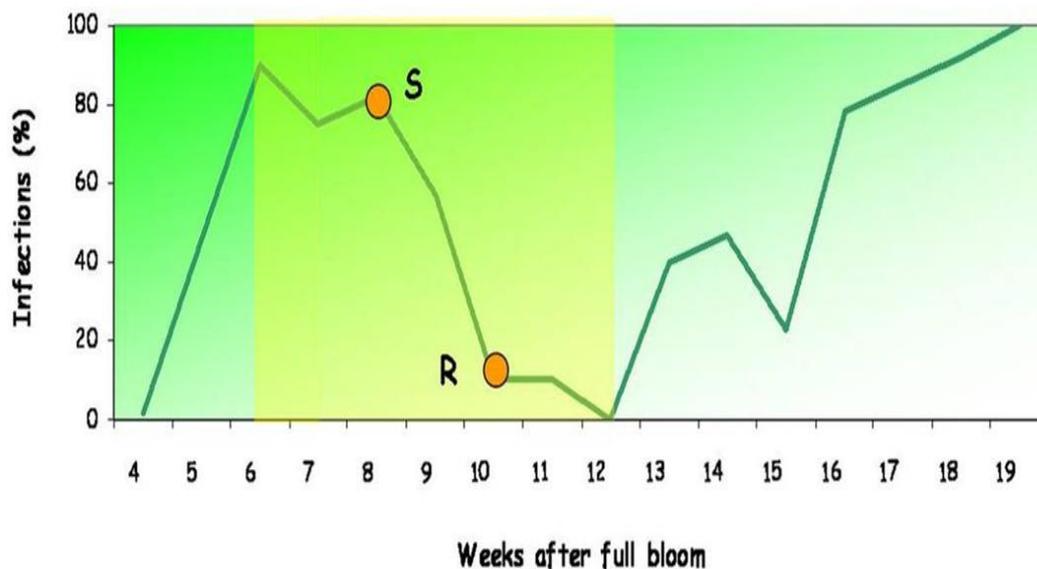


Figure 3.2: Variation of peach fruit (cv.K2) susceptibility to infection of *Monilinia* rot during fruit growth. Fruits were weekly harvested. Three replicates of 20 fruits were inoculated by dipping in a conidia suspension (10^5 /ml) for 1 min or mock inoculated with water. Infections were analysed after one week incubation at 20°C and the percentage of infected fruit was calculated. The yellow box indicates the period in which susceptibility follows a reproducible pattern through years and samples selected for real time analysis. **R** and **S** indicate the sample selected for microarray and cDNA AFLP analysis.

3.2 Analysis of conidia germination

The timing of *Monilinia laxa* conidia germination on the peel of peach fruit was analyzed. After three hours of inoculation about the 50% of the *M.laxa* conidia were germinated and the germinative tube reached a length of 13-16 μm ; whereas after 6 hours after inoculation, 70% of conidia appeared germinated with a germinative tube length ranging from 25 to 54 μm . After 12 and 24 hours after the inoculation 90% of the conidia appeared germinated (figs. 3.3, 3.4).



Figure 3.3: **Un-germinated *Monilinia* conidia on the cv. K2 peach fruit peel at time 0.** Conidia, stained with lactophenol blue, appear among the peel trichomes.



Figure 3.4: **Germinated *Monilinia* conidia on the cv. K2 peach fruit peel after 3 hour of inoculation.** Conidia are considered germinated when the germination tube length is equal or greater than the conidial body larger diameter.

3.3 Expression of genes of the phenylpropanoid and jasmonate pathway

In order to investigate on the possible causes of the temporary loss of susceptibility during S2 phase, a specific gene expression analysis was undertaken on the peach fruits harvested from the 6th to the 12th week after full bloom.

Alteration in expression of the genes encoding for enzymes of the phenylpropanoid metabolism, phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), cinnamate 4-hydroxylase (C4H), leucoanthocyanidine reductase (LAR), hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT), and for enzymes of the jasmonate pathway, involved in plant defense response, such as lipoxygenase (LOX) was monitored by Real Time PCR analysis on fruit samples of a period encompassing the pit hardening stage and the corresponding temporary resistance to *M.laxa* infections. This consisted of fruits harvested weekly from the 6th to the 12th week after full bloom. Expression was analyzed on fruits both inoculated with *M. laxa* or mock-inoculated (fig. 3.2).

No difference in any gene expression was detected between mock inoculated and inoculated fruits.

The mRNA level of *PAL* gene, the key enzyme of phenylpropanoid pathway, strongly decreased during the S2 phase; it significantly decreased by 50% from the 6th to the 7th week after full bloom (AFB) and by 62.5% from the 7th to the 8th week AFB. Then its expression level did not significantly change until the 12th week AFB. On the other hand, the expression level of the *C4H* gene, gradually decreased during the seven weeks analyzed by 87.5%; significant changes occurred from the 6th to the 7th week AFB, when

C4H expression decreased by 37.5%, and from 10th to the 11th week AFB, when its transcript level decreased by 50%. Conversely, the mRNA levels of *CHS* showed an increment of 5- fold from the 7th to the 8th week AFB; during the following weeks its expression gradually decreased by 75% at the 12th week AFB. *LAR* expression pattern appears similar to the *CHS* pattern: it doubled its value from the 7th to the 8th week AFB. Afterwards, its expression level remained stable until the 11th week AFB, when it showed a decrease of 50%. The expression level of *HQT* did not show significant change until the 8th week AFB. Afterward it significantly decreased by 67% during the 9th week AFB; after which, its expression level remained constant. *LOX*, a gene involved in jasmonate synthesis, increased to a value 2.7- fold higher from the 6th to the 8th week AFB; after this, it decreased significantly by 76.3% the week after (9th week AFB) and it remains constant thereafter.

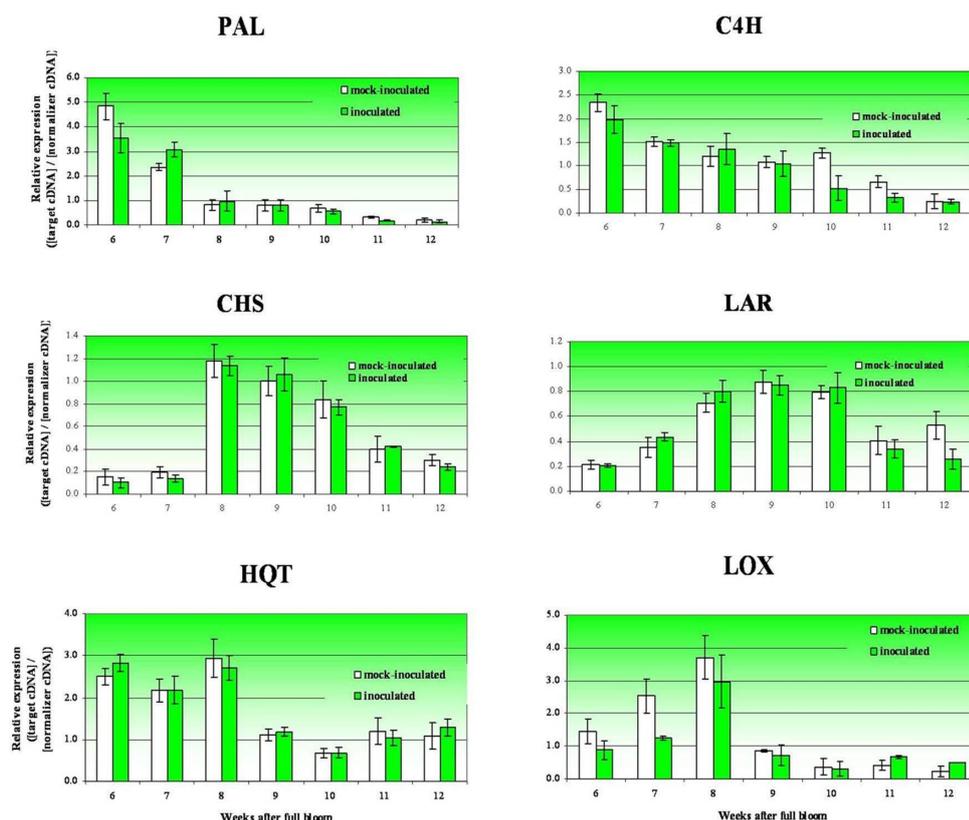


Figure 3.5: Expression levels of genes of the phenylpropanoid pathway (*PAL*, *C₄H*, *CHS*, *HQT*, *LAR*) and of the jasmonate pathway (*LOX*), during the S2 growth phase. Three replicates of cDNA were synthesized from three separated RNA preparations. Concentration of each target cDNA was normalized to the amount of actin cDNA. Two replicates for each reaction and two experiments for each gene were performed.

3.4 Microarray

To investigate on the transcriptome variation underneath the temporary loss of susceptibility of peach fruits to *Monilinia* rot during the S2 phase, the μ Peach1.0 microarray was used [20].

Total RNA was extracted from the peel of the susceptible fruits (**S**) (harvested on the 8th week after full bloom) , and of the resistant ones (**R** harvested on the 10th week after full bloom); both samples were either inoculated with *Monilinia* (**SI** and **RI**) or mock inoculated with water (**SH** and **RH**). Three independent experiments in which three pairs of samples were compared, were performed: 1) **RH** vs. **SH**, 2) **RI** vs. **SI**, 3) **RH** vs. **RI**.

Upon the array image confocal scanning and the TIGR Spotfinder analysis (figs.3.6, 3.7) a .TAV file containing values for fluorescence intensity of each spot of both channel and for the background was generated.

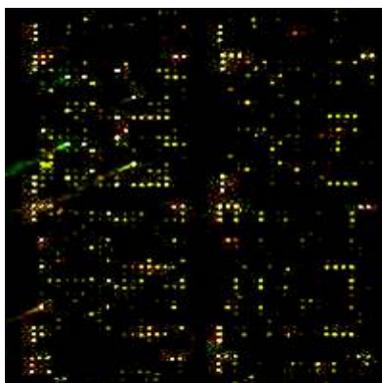


Figure 3.6: **Fluorescent image**. The samples were scanned with the confocal microarray scanner and a hybridization image was obtained from the overlapping of the single channel images. Intensity and colour of each spot are associated to a different expression in the samples.

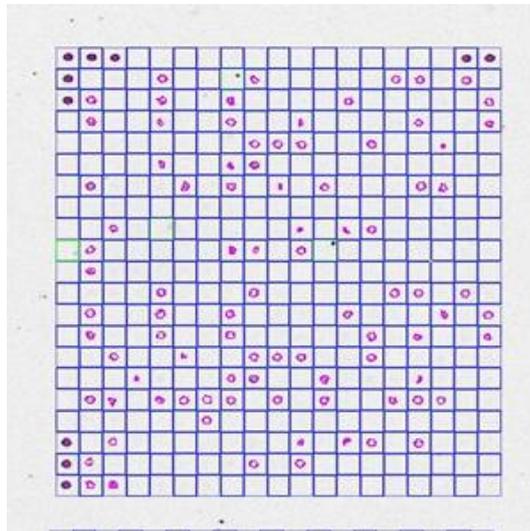


Figure 3.7: **Grid with the spots visualized on the TIGR Spotfinder software.** Hybridization images were processed with TIGR Spotfinder software. Each spot was manually analyzed in order to eliminate unspecific fluorescence.

The Genespring analysis of the TAV file showed that 30 genes (corresponding to the 0.6% of the total sequences (4806) contained in the μ Peach1.0 microarray) were found up-regulated and 31 (0.6%) down regulated in **RH** vs. **SH** fruits. On the other hand, 20 genes (0.4%) were shown to be up-regulated and 13 (0.3%) down-regulated in the **RI** vs. **SI** fruit (fig. 3.8). No genes were found differentially expressed in the mock-inoculated resistant fruits (**RH**) vs. the inoculated resistant fruits (**RI**). 15 genes were found differentially regulated in all the **R** vs. all the **S** fruits (namely, genes found similarly regulated both in **RH** vs. **SH** and in **RI** vs **SI** experiments). Of these five genes were up-regulated (*ATP sulfurylase*, *heat shock protein 70*, *major allergen Pru P1*, *harpin inducing protein* and *S-adenosylmethionine decarboxylase*) and ten genes down-regulated (*Cinnamyl alcohol dehydrogenase*, *photosystem I reaction center subunit PSI-N*, *histidine- containing phosphotransfer protein*, *ferritin 1*, *PGPD14* and other unknown proteins) (fig.3.8).

All sequences were grouped following the *Arabidopsis thaliana* Munich Information Center for Protein Sequences, MIPS (www.mips.biochem.mpg.de) functional classification (fig.3.9).

Furthermore, the susceptible mock inoculated fruits (**SH**) vs. the susceptible inoculated ones (**SI**) were not directly compared, because the differentially expressed genes could be deduced from the other experiments.

The fact that no gene was found differentially expressed between **RH** and **RI** sample, whereas the genes found differentially expressed between **RH** and **SH** experiment partially diverge in quality and quantity from those found in the **RI** vs. **SI** experiment, indicate differences in the expression between **SH** vs. **SI**. This allows drawing indirect conclusion also for **SH** vs. **SI** comparison for which there is no experimental evidence [35] (fig.3.10).

Contig name	Mean	Standard deviation	Condition	Contig size	Best match in EMBL database	E value	cellular component	molecular function	biological process
Metabolism									
2770	0,46	0,002	down RHvsSH	417	OSJNB008SF13.5 [Oryza sativa japonica cultivar-]	1,00E-15		arginase activity	polyamine metabolism
494	0,38	0,075	down RHvsSH	3153	nitrate reductase [Prunus persica]	0,00E+00		reductase activity	nitrate assimilation-response to stimuli
31	0,56	0,172	down RHvsSH	960	glyoxalase family protein (lactoylglutathione lyase family protein)[Arabidopsis thaliana]	4,00E-40		lyase activity	carbohydrate metabolism
1206	0,49	0,046	down RHvsSH	339	Cinnamyl alcohol dehydrogenase (Fragment) [Zirnia elegans]	2,80E-02		dehydrogenase activity	lignin biosynthesis
1398	1,75	0,038	up RHvsSH	719	lvs201 protein, hypersensitivity-related - common tobacco	1,00E-53		transferase activity	
1153	1,74	0,341	up RHvsSH	1386	NAD-dependent epinase/dehydratase family [Arabidopsis thaliana]	1,00E-175	plasma membrane		carbohydrate metabolism
5088	2,47	0,14	up RHvsSH	639	ATP sulfurylase [Brassica juncea]	6,00E-42	chloroplast	transferase activity	sulfate assimilation - metabolism
4668	1,66	0,097	up RHvsSH	401	diphenol oxidase	2,00E-34		oxidase activity	lignin biosynthesis
4543	1,67	0,32	up RHvsSH	595	mevalonate diphosphate decarboxylase [Hevea brasiliensis]	7,00E-93		decarboxylase-kinase activity	isoprenoid biosynthetic process
4148	1,68	0,666	up RHvsSH	655	hexokinase [Citrus sinensis]	7,00E-33		kinase activity	carbohydrate metabolism and transport
1206	0,41	0,114	down RIvsSI	339	Cinnamyl alcohol dehydrogenase (Fragment) [Zirnia elegans]	2,80E-02		dehydrogenase activity	lignin biosynthesis
5088	3,67	1,51	up RIvsSI	639	ATP sulfurylase [Brassica juncea]	6,00E-42	chloroplast	transferase activity	sulfate assimilation - metabolism
1003	1,96	0,22	up RHvsSH	1944	S-adenosylmethionine decarboxylase [Mahua domestica]	0,00E+00	unknown	decarboxylase activity	polyamine biosynthetic metabolism
1003	2,24	0,73	up RIvsSI	1944	S-adenosylmethionine decarboxylase [Mahua domestica]	0,00E+00	unknown	decarboxylase activity	polyamine biosynthetic metabolism
64	2,26	0,368	up RIvsSI	1428	1-aminocyclopropane-1-carboxylate oxidase (1-aminocyclopropane 1-carboxylic acid oxidase) [Prunus persica]	1,00E-168		oxidase activity	ethylene biosynthesis
Energy									
3203	0,43	0,02	down RHvsSH	530	Photosystem II 10 kDa polypeptide, chloroplast precursor (Lightinducible tissue-specific ST-LS1 protein [Solanum tuberosum])	3,00E-43	chloroplast	e- binding	energy pathway- stress response
945	late	0,05	down RHvsSH	921	Light harvesting chlorophyll A/B binding protein [Prunus persica]	1,00E-123	chloroplast	chlorophyll binding	energy pathway
5456	0,48	0,17	down RHvsSH	688	photosystem I reaction center subunit PSI-N precursor or (PSI-N)[Arabidopsis thaliana]	8,00E-44	chloroplast	e- binding	energy pathway
5456	0,38	0,12	down RIvsSI	688	photosystem I reaction center subunit PSI-N precursor or (PSI-N)[Arabidopsis thaliana]	8,00E-44	chloroplast	e- binding	energy pathway
5028	2,99	0,57	up RIvsSI	472	ATP citrate-lyase-related [Arabidopsis thaliana]	4,00E-68	mitochondrion	lyase activity	energy pathway
2496	1,74	0,04	up RIvsSI	1101	putative UDP-glucose dehydrogenase [Oryza sativa]	0,00E+00	endomembrane system	dehydrogenase activity	e- transport energy pathway
137	1,61	0,54	up RHvsSH	638	glutathione reductase [Betula pendula]	1,00E-104		reductase activity	cell redox homeostasis
59	2,05	0,88	up RIvsSI	834	SAH7 protein (Hypothetical protein) (Allergen-like protein BR5n20) [Arabidopsis thaliana]	1,00E-52	endomembrane system	unknown	glucosogenesis
Protein synthesis									
1524	1,79	0,04	up RHvsSH	1085	ribosomal-protein S6 kinase (ATPK19)-related [Arabidopsis thaliana]	1,00E-122	nucleus	kinase activity	response to heat
4939	1,75	0,30	up RHvsSH	679	ribosomal protein L28-like [Oryza sativa japonica cultivar-group]	4,00E-59	ribosome	structural constituent ribosome	translation
Protein fate									
3462	1,94	0,29	up RHvsSH	1071	heat shock protein 70, cytosolic [imported] - spinach	1,00E-149	cytosol	ATP binding	protein metabolism
5271	1,74	0,23	up RIvsSI	653	Endoplasmic reticulum protein precursor or (GRP94 homolog) heat shock protein 90	6,00E-99	endomembrane system	protein binding-ATPase activity	protein metabolism in the cell wall
1128	1,69	0,08	up RHvsSH	644	muclain protein, putative [Arabidopsis thaliana]	4,00E-65	endomembrane system	peptidase activity	proteolysis - response to stress?
4105	1,57	0,59	up RHvsSH	362	cathepsin B-like cysteine protease, putative [Arabidopsis thaliana]	1,00E-13	endomembrane system	peptidase activity	proteolysis
3462	2,31	0,61	up RIvsSI	1071	heat shock protein 70, cytosolic [imported] - spinach	1,00E-149	cytosol	ATP binding	protein metabolism
Transcription									
971	0,40	0,11	down RHvsSH	1192	Dehydration-responsive protein RD22 (Fragment) [Prunus persica]	3,00E-85	endomembrane system	nutrient reservoir activity	response to stress
2757	0,45	0,04	down RHvsSH	549	ethylene-responsive element binding protein [Nicotiana tabacum]	2,00E-06	nucleus	DNA binding	regulation of transcription-ethylene mediated signaling pathway
2499	2,14	0,62	up RHvsSH	705	ethylene responsive element binding protein, putative [Arabidopsis thaliana]	1,00E-41	nucleus	DNA binding	regulation of transcription-ethylene mediated signaling pathway
4674	1,69	0,45	up RHvsSH	495	AP2 domain transcription factor, putative [Arabidopsis thaliana]	4,00E-06	nucleus	DNA binding	stress (dehydration-wounding)
1779	1,68	0,65	up RHvsSH	630	transcriptional regulator-related [Arabidopsis thaliana]	8,00E-90	nucleus	DNA binding	DNA metabolism
4012	1,94	0,10	up RIvsSI	638	CCCH-type zinc finger protein-related [Arabidopsis thaliana]	6,00E-32	nucleus	nucleic acid binding	regulation of transcription
Cellular transport									
1470	1,94	0,38	up RIvsSI	1194	Putative peptide transport protein [Arabidopsis thaliana]	1,00E-104	plasma membrane	protein binding-transport	transport
38	1,69	0,33	up RIvsSI	3424	PFA2/Plasma membrane H+ ATPase (EC 3.6.1.3) [Prunus persica]	0,00E+00	plasma membrane	ion binding	transport
4903	2,01	0,77	up RIvsSI	436	ABC transporter family protein [Arabidopsis thaliana]	5,00E-13	membrane	ATPase activity	transmembrane movement of substances
Cellular communication-signal transduction									
2370	0,30	0,09	down RHvsSH	577	Histidine-containing phosphotransfer protein [Catharanthus roseus]	2,00E-51		kinase activity	signal transduction
2370	0,42	0,14	down RIvsSI	577	Histidine-containing phosphotransfer protein [Catharanthus roseus]	2,00E-51		kinase activity	signal transduction

1391	3,20	0,60	up RHvsSH	675	ESI-1 protein precursor (TR132) [<i>Lycopersicon esculentum</i>]	1,00E-40	endomembrane system		response to gibberellin stimulus
3123	1,60	0,50	up RHvsSH	628	putative mitogen-activated protein kinase 1 [<i>Medicago sativa</i>]	3,00E-68		kinase activity	response to oxidative stress
834	0,39	0,05	down RHvsSI	765	leucine-rich repeat transmembrane protein kinase, putative [<i>Arabidopsis thaliana</i>]	3,00E-12	plasma membrane	kinase activity, binding activity	signal transduction
986	2,09	0,42	up RHvsSI	628	Calmodulin 1 [<i>Medicago truncatula</i>]	6,00E-80		calcium ion binding	response to stress
Cell rescue and defense									
1162	0,35	0,09	down RHvsSH	625	Defensin protein 1 [<i>Prunus persica</i>]	9,00E-17	cell wall		defense response
412	0,29	0,04	down RHvsSH	1090	Ferritin 1, chloroplast precursor [<i>Brassica napus</i>]	6,00E-60	plastid	ion binding	response to stimuli
1701	2,25	0,38	up RHvsSH	819	Major allergen Pru av 1 (Pru a 1) [<i>Prunus avium</i>]	3,00E-86		unknown	response to stress
2225	4,21	0,54	up RHvsSH	774	harpin inducing protein [<i>Nicotiana tabacum</i>]	3,00E-54	unknown	unknown	response to stress
412	0,31	0,10	down RHvsSI	1090	Ferritin 1, chloroplast precursor [<i>Brassica napus</i>]	6,00E-60	plastid	ion binding	response to stimuli
2225	5,92	1,88	up RHvsSI	774	harpin inducing protein [<i>Nicotiana tabacum</i>]	3,00E-54	unknown	unknown	response to stress
1702	4,07	2,42	up RHvsSI	750	Major allergen Pru ar 1 [<i>Prunus americana</i>]	7,00E-66	unknown	unknown	response to stress
1701	2,33	0,58	up RHvsSI	819	Major allergen Pru av 1 (Pru a 1) [<i>Prunus avium</i>]	3,00E-86	unknown	unknown	response to stress
3171	2,37	0,38	up RHvsSI	597	glycosyl hydrolase family 19 (class IV chitinase) [<i>Arabidopsis thaliana</i>]	2,00E-73	endomembrane system	chitinase activity	carbohydrate metabolism, response to stress
Unknown									
3366	0,36	0,08	down RHvsSH	561	PGPD14 [<i>Petunia x hybrida</i>]	1,00E-37		protein- ion binding	
1501	0,46	0,06	down RHvsSH	1089	unknown protein [<i>Arabidopsis thaliana</i>]	5,00E-96	unknown	unknown	unknown
3237	0,29	0,01	down RHvsSH	625	no hits found		unknown	unknown	unknown
5197	0,48	0,01	down RHvsSH	461	no hits found		unknown	unknown	unknown
2103	0,39	0,24	down RHvsSH	489	unknown protein [<i>Arabidopsis thaliana</i>]	3,00E-46	unknown	unknown	unknown
1556	0,48	0,07	down RHvsSH	937	AKIN gamma [<i>Medicago truncatula</i>]	6,00E-95	unknown	unknown	unknown
3420	0,54	0,19	down RHvsSH	622	unknown protein [<i>Arabidopsis thaliana</i>]	4,00E-15	unknown	unknown	unknown
4450	0,57	0,17	down RHvsSH	621	unknown protein [<i>Arabidopsis thaliana</i>]	3,00E-49	unknown	unknown	unknown
5306	0,37	0,04	down RHvsSH	562	base scores	4,00E-34	unknown	unknown	unknown
1793	0,67	0,48	down RHvsSH	628	no hits found		unknown	unknown	unknown
1711	0,48	0,03	down RHvsSH	658	B1147A04.10 [<i>Oryza sativa</i> (japonica cultivar-group)]	2,00E-06	unknown	unknown	unknown
2317	0,55	0,16	down RHvsSH	613	C2 domain-containing protein [<i>Arabidopsis thaliana</i>]	9,00E-43	unknown	unknown	unknown
5401	0,39	0,03	down RHvsSH	444	hypothetical protein [<i>Plasmodium yoehi yoehi</i>]	8,90E-01	unknown	unknown	unknown
3224	0,41	0,01	down RHvsSH	598	unknown protein [<i>Arabidopsis thaliana</i>]	7,00E-42	unknown	unknown	unknown
4808	0,58	0,27	down RHvsSH	580	unknown protein [<i>Arabidopsis thaliana</i>]	3,00E-25	unknown	unknown	unknown
196	0,46	0,23	down RHvsSH	475	PO638D12.11 [<i>Oryza sativa</i> (japonica cultivar-group)]	7,00E-12	unknown	unknown	unknown
3547	0,49	0,03	down RHvsSH	628	no hits found		unknown	unknown	unknown
5400	0,46	0,02	down RHvsSH	612	hypothetical protein [imported] - <i>Arabidopsis thaliana</i>	2,00E-62	unknown	unknown	unknown
1764	2,34	0,89	up RHvsSH	622	Chain A, Agglutinin In Complex With T-Disaccharide	4,00E-11	unknown	unknown	unknown
4548	1,60	0,72	up RHvsSH	625	unknown protein [<i>Arabidopsis thaliana</i>]	1,00E-76	unknown	unknown	unknown
209	1,76	0,78	up RHvsSH	695	hypothetical protein [<i>Plasmodium falciparum</i>]	2,60E-02	unknown	unknown	unknown
4226	1,88	0,92	up RHvsSH	577	At1g68060/T23K23_9 [<i>Arabidopsis thaliana</i>]	1,80E-02	unknown	unknown	unknown
2899	1,84	0,17	up RHvsSH	631	unknown [<i>Arabidopsis thaliana</i>]	4,00E-50	unknown	unknown	unknown
1046	1,77	0,34	up RHvsSH	897	unknown protein [<i>Arabidopsis thaliana</i>]	1,00E-51	unknown	unknown	unknown
4162	1,57	0,43	up RHvsSH	517	hypothetical protein T16K5.70 - <i>Arabidopsis thaliana</i>	2,00E-22	chloroplast	unknown	unknown
2000	1,67	0,60	up RHvsSH	348	hypothetical protein [<i>Plasmodium falciparum</i> 2D7]	3,00E+00			unknown
2964	2,44	0,17	up RHvsSH	840	F14FR.3 protein [<i>Caenorhabditis elegans</i>]	5,80E+00			unknown
196	0,44	0,03	down RHvsSI	475	PO638D12.11 [<i>Oryza sativa</i> (japonica cultivar-group)]	7,00E-12	unknown	unknown	unknown
2103	0,42	0,05	down RHvsSI	489	unknown protein [<i>Arabidopsis thaliana</i>]	3,00E-46	unknown	unknown	unknown
3224	0,44	0,17	down RHvsSI	598	unknown protein [<i>Arabidopsis thaliana</i>]	7,00E-42	unknown	unknown	unknown
3366	0,46	0,05	down RHvsSI	561	PGPD14 [<i>Petunia x hybrida</i>]	1,00E-37		protein- ion binding	unknown
5401	0,57	0,16	down RHvsSI	444	hypothetical protein [<i>Plasmodium yoehi yoehi</i>]	8,90E-01	unknown	unknown	unknown
1272	0,46	0,06	down RHvsSI	1069	Hypothetical 16.1 kDa protein (AT5g02020/T7H20_70) [<i>Arabidopsis thaliana</i>]	6,00E-22	chloroplast	unknown	unknown
3237	0,42	0,16	down RHvsSI	625	no hits found		unknown	unknown	unknown
5207	2,21	0,56	up RHvsSI	631	no hits found		unknown	unknown	unknown
217	1,56	0,46	up RHvsSI	1452	RNA-binding protein, putative [<i>Arabidopsis thaliana</i>]	1,00E-129		RNA binding	unknown
96	2,08	0,10	up RHvsSI	666	Putative embryo-abundant protein (At2g1380) [<i>Arabidopsis thaliana</i>]	2,00E-73		transferase activity	unknown
4458	2,25	0,04	up RHvsSH	421	merozoite surface antigen 2 [<i>Plasmodium falciparum</i>]	1,40E-01			unknown
215	0,46	0,04	down RHvsSI	810	unknown protein [<i>Arabidopsis thaliana</i>]	3,00E-30			unknown

Figure 3.8: Table of the differentially expressed genes. The table indicates the contig name [20], the fluorescence ratio mean value with standard deviation, the relative sample condition, the best Genbank matching sequences with its E value and the GO classification [20]. In orange are indicated the genes differentially regulated in all the **R** vs. all the **S** fruits.

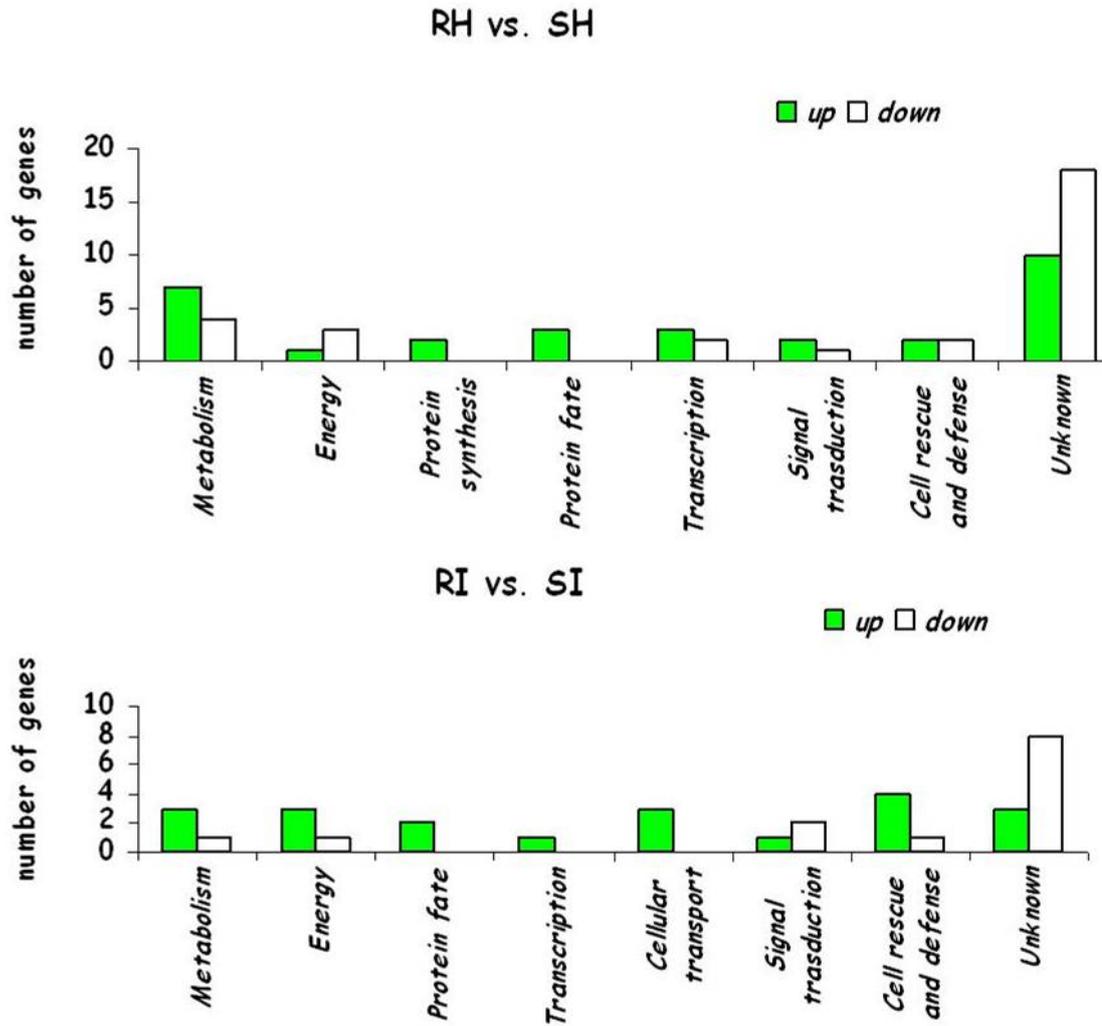


Figure 3.9: **Functional group classification.** Distribution of up- and down-regulated genes among MIPS groups.

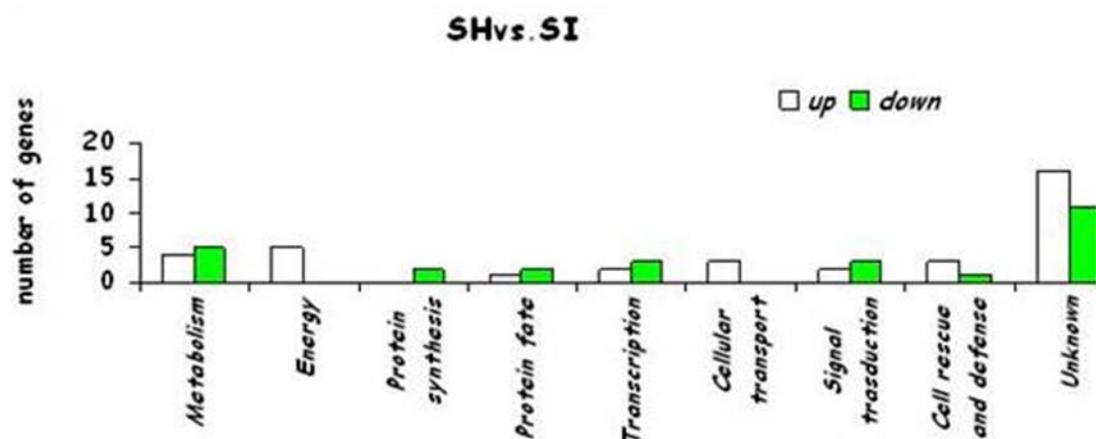


Figure 3.10: **Functional group classification.** Distribution of up- and down-regulated genes among MIPS groups.

3.4.1 Real time PCR analysis of microarray data

The microarray experimental results and the data indirectly derived, were tested by Real Time PCR analysis. Among the 91 total genes found differentially expressed in the microarray analysis, 11 genes (seven up regulated and four down regulated in **R** samples) were assayed. These were chosen on the basis of their functional role on plant defence against fungal pathogens.

Real time PCR analysis showed a variation in the expression of *ATP sulfurylase*, *Heat shock protein 70 (hsp70)*, *Major allergen Pru P1*, *SAMDC* and *harpin induced protein* qualitatively and quantitatively similar to the one shown by microarray (fig.3.11 and tab.3.1); genes appeared up-regulated in the **R** fruits with respect to the **S** fruits with a ratio of about two. Variation of the *harpin induced protein* gene also appeared up regulated in the **R** fruits as in the microarray; for this gene the real time PCR showed a much higher expression ratio (15 by real time PCR vs. 5.9 by microarray).

In the microarray analysis, *calmodulin* was found down-regulated in **RH** vs. **SH**, but not in **RI** vs. **SI**, indirectly indicating a down regulation also in **SH** vs. **SI**. However, the *calmodulin* up regulation in **SI** was not significantly supported by the real time PCR data (fig. 3.11), suggesting that, for some gene, the value of 1.7 chosen as minimal fluorescence intensity ratio for significance, may be misleading. Similarly, real time PCR data on gene expression of the *CCCH-type Zinc finger protein* did not support the microarray findings; this gene was found up regulated in the microarray analysis in the **RI** sample with a ratio **RI/SI** of 2.25, but not in **RH** vs. **SH**; it was then considered down regulated in **SI** fruits. However, real time PCR showed up regulation of the *CCCH-type Zinc finger protein* both in **RH** and **RI** samples.

On the other hand, among the genes down regulated in **R** samples, the

Gene	RH/SH		RI/SI		RH/RI		SH/SI	
	<i>Micro</i>	<i>Real Time</i>	<i>Micro</i>	<i>Real Time</i>	<i>Micro</i>	<i>Real Time</i>	<i>Micro</i>	<i>Real Time</i>
ATP sulfurylase	2.47	4.12	3.67	3.24	-	1.18	-	0.93
Heat shock protein 70	1.94	2.41	2.31	2.59	-	0.78	-	0.84
Pru P1	2.25	2.39	2.33	2.14	-	1.28	-	1.15
SAMDC	1.96	2.43	2.24	2.19	-	1.3	-	1.17
Harpin inducing protein	4.21	15.3	5.92	14.73	-	0.92	≥ 1.7	0.88
Calmodulin	2.09	2.01	$1.7 \leq x \leq 0.5$	1.83	-	1.06	≤ 0.5	1.17
CCCH-zinc finger protein	$1.7 \leq x \leq 0.5$	1.55	2.25	1.94	-	0.84		1.23
CAD	0.49	0.54	0.41	0.28	-	1.78	-	1.78
Ferritin 1	0.29	0.24	0.31	0.14	-	1.42	-	0.83
Histidine protein	0.3	0.41	0.42	0.27	-	1.54	-	1.02
Defensin 1	0.35	0.42	$1.7 \leq x \leq 0.5$	0.31	-	1.45	≥ 1.7	1.09

Table 3.1: Comparison of the expression ratios of the selected genes in microarray and in real time analysis

real time PCR showed a variation in expression for *CAD*, *ferritin*, and *histidine containing phosphotransfer protein* qualitatively and quantitatively similar to the one shown by microarray analysis. For *defensin* gene, which was indirectly classified as differentially expressed in **SH** vs. **SI** comparison, real time PCR analysis showed that this gene was up-regulated in both **SH** and **SI**.

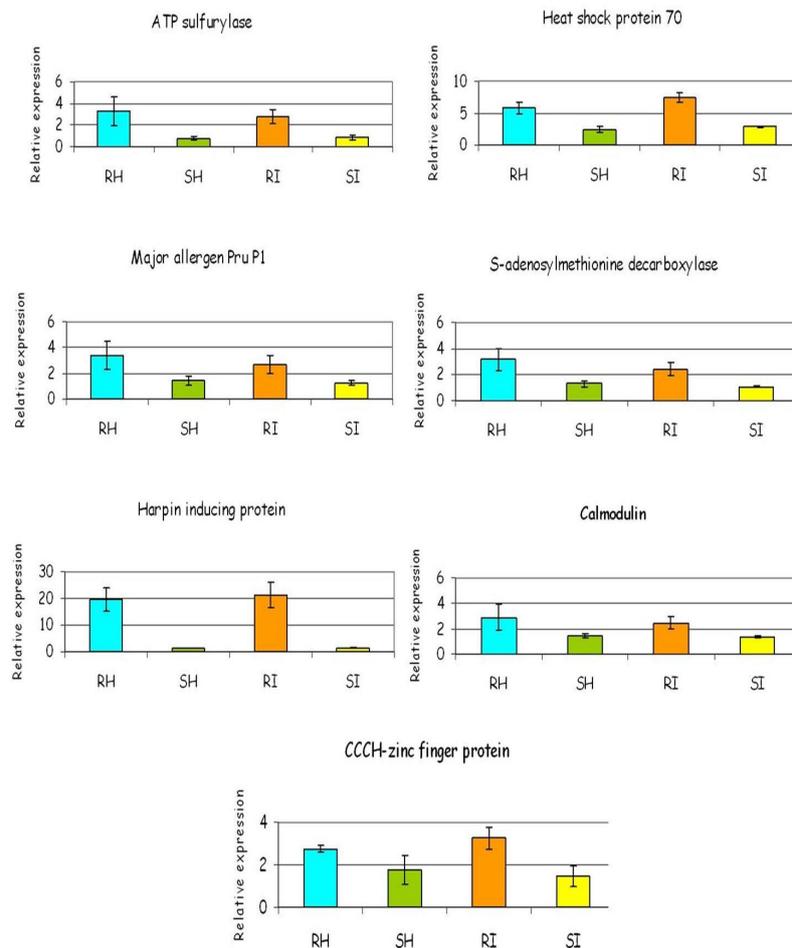


Figure 3.11: Real time analysis of the genes up-regulated in the R fruits vs. S fruits. Three replicates of cDNA were synthesized from three separated RNA preparation. Concentration of each target cDNA was normalized to the amount of actin cDNA. The mean value and the standard deviation were calculated for each replicate.

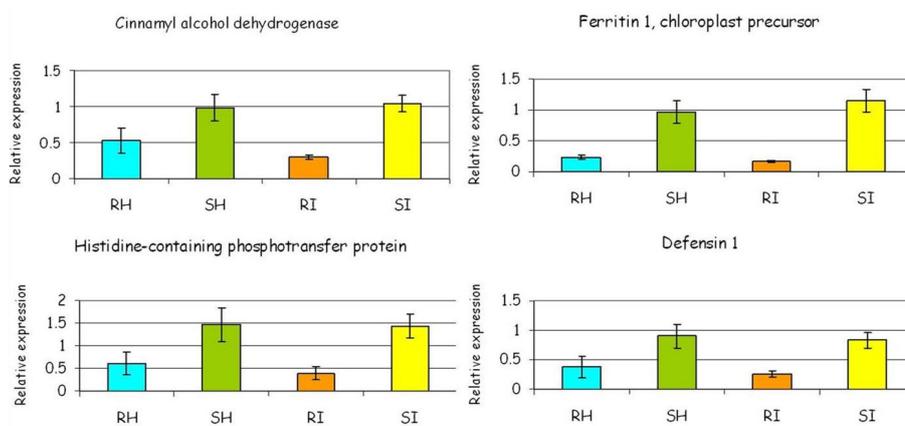


Figure 3.12: **Real time analysis of the genes down-regulated in the R fruits vs. S fruits.** Three replicates of cDNA were synthesized from three separated RNA preparation. Concentration of each target cDNA was normalized to the amount of actin cDNA. The mean value and the standard deviation were calculated for each replicate.

3.5 cDNA AFLP

The same fruits samples analyzed on the μ Peach1.0 were also used in a cDNA AFLP analysis. cDNA was generated using high purity RNA. The cDNA fragments were amplified using all possible 32 primers combinations.

For each primer combination 50- 70 AFLP bands were observed. The 20-35% of these transcript derived fragments (TDF) appeared differentially expressed; they ranged in size from 100 to 1100 bp (fig.3.13).

TDF were considered significant for *Monilinia* resistance and selected for excision using the following criteria:

- 1) Fragments up- or down-regulated only in the susceptible fruits inoculated with *Monilinia*. **SI** vs. **SH, RH, RI**
- 2) Fragments up- or down-regulated only in the resistant fruits inoculated with *Monilinia*. **RI** vs. **RH, SH, SI**
- 3) Fragments expressed in both resistant and susceptible fruits inoculated with *Monilinia* (**I**) vs. mock inoculated (**H**). **I** vs. **H**
- 4) Fragments expressed only in the resistant fruits (both inoculated or mock) (**R**) or only in the susceptible fruits (both inoculated or mock) (**S**) **R** vs. **S**

A total of 339 TDF were excised from the gel, re-amplified with specific primers and sequenced. Sequences were both analyzed using BLAST software [31] against the ESTree database (<http://www.itb.cnr.it/estree/>), specific for *Prunus persica* EST sequences and against the NCBI databases (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) (fig. 3.14).

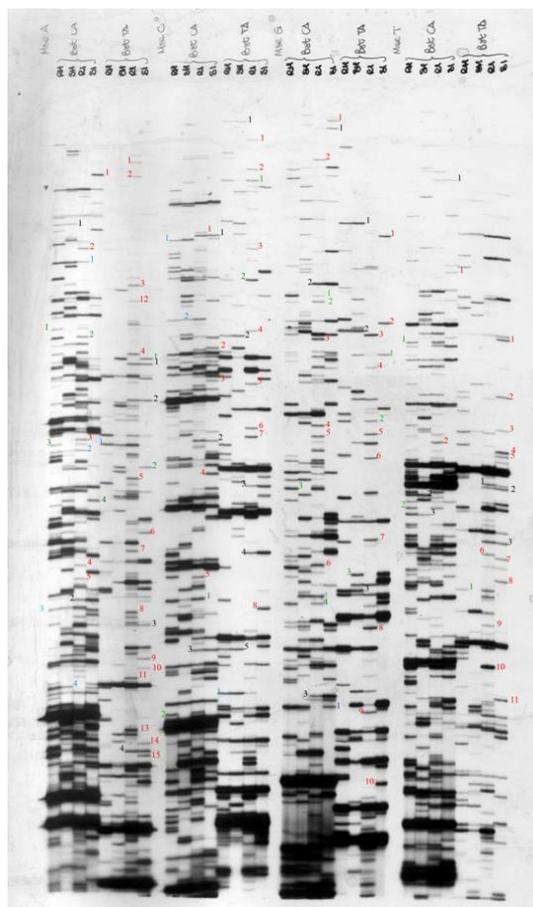


Figure 3.13: **cDNA AFLP analysis**. The autoradiography shows the expression pattern of the four fruit samples (RH-SH-RI-SI). On the top, the primer combination (BstY-CA/TA with each MseX) and the sample (**RH**, **SH**, **RI**, **SI**) are indicated. The numbers indicates the excised TDF.

Fragment name	Condition	Size (bp)	Database	Similar sequences from databases	E value	TAIR locus	Cellular component	Molecular function	Biochemical process
Metabolism									
C70	2+	220	estdb	PF_LE00160138 - (T10101 Acetate hydratase catabol) (w:acetylhydrolase)	7.00E-71		cytosol	acetate hydratase activity	Carbohydrate metabolism; glycolysis and gluconeogenesis; metabolism
C76	11+	330	blastx	g MEN0698 Transferrin (Medicago truncatula. Analog a ester di AEA (w:acetylhydrolase))	7.00E-15		unknown	transferase activity	cell org subunit and biogenesis
C184	3R+	104	estdb	BC_PFL12_406 (C04667) 51NVA1LING1 - Gh. lumen synthase (Gh.lumen-synthase, lumen synthase)	2.00E-33	AT3G03300	cytosol	ligase activity	nitrogen metabolism; process
C205	3R+	176	estdb	PF0_A1873919 (A0114E38) 1 - adenosine kinase isoform (Zm11c010m1b00m)	4.00E-84			adenosine kinase activity	adenosine salvage
C226	2R+	418	estdb	PF02_plate1_D17C(AA7338) 1 - 2-oxoacyl-CoA synthetase (Arabidopsis thaliana. 2-oxoacyl-CoA synthetase)	0	AT1G22410	chloroplast	3-oxoacyl-CoA synthetase activity	aromatic aa family biosynthetic process; alkaloid pathway
T187	4R+	376	estdb	PF_LE00202247 NP_001038311.0 (O5964) 75480 [Oryza sativa]	0			ubiquitin-protein ligase activity	aa transport and metabolism
T196	4S+	166	estdb	PF04_plate12_C03 (AA164356) 1 - putative mitochondrial 5-oxopropionyl-CoA synthase (Arabidopsis thaliana)	6.00E-80			ubiquitin-protein ligase activity	tryptophan metabolic process
C231	7S+	252	estdb	PF_331al_A_4_C10(AA73607) 1 - arginase (Panicum amaranum)	1.00E-134	AT4G08900.1		arginase activity	arginine catabolic process; polyamine metabolic process
C236	3S+	120	estdb	PF0_A1873979 (ABE92367) 1 - Putative methyltransferase (Medicago truncatula)	1.00E-64		cellular component	methyltransferase activity	acetate biosynthetic process; fructan carbon monoxide synthase catabolism
C106	7R+	414	estdb	PFU1_plate7_HI6 (AA37338) UDP-phenylethanol decarboxylase (Panicum amaranum)	0	AT3G59290	cytosol	methyltransferase activity	D-Xylose metabolic process
T106	6R+	256	tblastx	g CN493192 ACYL CARBOXYL PROTEIN THIOESTERASE (Panicum amaranum)	2.00E-08	AT1G08510	plastid	decarboxylase activity	fatty acid metabolism
T133	7R+	268	tblastx	g JEA49668 Alcohol acyl-transferase (Panicum amaranum)	2.00E-10	AT5G048930		transferase activity	fatty acid metabolism
T149	1R+	415	tblastx	ent CAN60576 hydrophobic protein (Vitis vinifera) - Elongator protein 3 (Musa sapientum)	5e-25	AT2G043800	unknown	biotin binding	biotin biosynthesis
C57	6S+	480	estdb	PFU2_plate22_O01(AA94477) 1 - biotin esterase carrier protein subunit (Elymus repens)	0	AT3G15690.2	chloroplast	transferase activity	fatty acid metabolism
C68	3S+	138	estdb	PFU2_plate40_E21(AA118927) 1 - neovalonate diaphorase decarboxylase (Panicum amaranum)	2.00E-33	AT3G54230.1	unknown	decarboxylase activity	isoprenoid metabolism
C71	2S+	443	estdb	PFU2_plate37_C14 (CAC44502) beta-galactosidase (Panicum amaranum)	0		endoplasmic reticulum	beta-galactosidase activity	carbohydrate metabolism
T17	1S+	257	estdb	PF04_plate7_I11 - (ABP22890) 3,4,5-tri-O-methyl-L-ascorbic acid oxidase (Panicum amaranum)	1.00E-139		unknown	hydroxylase activity	carbohydrate metabolism
T70	4S+	514	estdb	S_kan0612_(NP_830172) 1 - acetolactate synthase-aminoo acid binding (Arabidopsis thaliana)	1.00E-46	AT2G21810	chloroplast	acetolactate synthase activity; aa binding	branched chain family aa biosynthetic process
Energy									
C96	3+	166	estdb	PFU2_plate3_O04 (CAA33078) 1,3-bisphosphoglycerate kinase B (Arabidopsis thaliana)	4.00E-90	AT1G04710	cytosol; mitochondrion; peroxisome	acetyl-CoA C-acyltransferase activity	calicoid catabolic process; ortho-cleavage; protocatechuate catabolic process; ortho-cleavage; fatty acid oxidation
C182	1R+	192	estdb	PFU2_plate22_B05 (AA112084) 1 - putative acetylase (Panicum amaranum)	1.00E-37		mitochondrion or chloroplast	hydro-lyase activity	metabolic process-response to oxidative stress
C186	1R+	263	estdb	PFU1_plate31_F144_NP_268778.1 oxidoreductase, acting on NADH or NADPH (Arabidopsis thaliana)	2.00E-45		mitochondrion	oxidoreductase activity	e-transport or energy pathways
C188	3R+	265	estdb	BC_PFL21-E10 (E29790 - A) PFL2_TOEAC - ATP synthase gamma chain, chloroplast precursor	6.00E-57		chloroplast	enzyme regulator activity	ATP synthesis- energy pathway
C210	3R+	181	estdb	PFU1_plate9_N07 (NP_564091) 1-carboxylate diaphorase (GAMMA.CA1)	3.00E-20		mitochondrial membrane; aspartic complex I	carboxylate diaphorase activity	photosynthesis
T174	1R+	495	estdb	PF_LE0007E167(P26685 - ATP synthase subunit beta, mitochondrial precursor (Panicum amaranum))	1.00E-121			synthase activity; hydrogen ion transporting ATP synthase activity	response to oxidative stress
T188	2+	238	estdb	BC_HFL10-F05 - thylakoid lumenal 29 kDa protein - 5 A induced	7.00E-52		mitochondrion	calcium ion binding	photosynthesis-e-transport or energy pathways
C150	8R+	240	ESTREE	PFU4_plate11_D24(AA06304) 1 - Complex I protein (LYR family-component of the higher eukaryote NADH complex)	1.00E-131		chloroplast	transferase activity	e-transport or energy pathway
C152	10R+	137	tblastx	g DVG47267 PP_LE0019E00F Pechth. shoot Panicum persea cDNA clone	7.00E-22	ATCG00540.1	chloroplast thylakoid membrane	photosynthetic e-transport	e-transport or energy pathway

C169	381+	399	estee	PP_LE_0003814f (AF020690) ubiquitin-specific protease 6 (Aa.)	4,00E-06	ATI651710	cytosol	calmodulin binding, ubiquitin specific protease activity	protein deubiquitination
T139	881+	206	estee	>PU2_plate16_C015 - Clapenram Cpr60707Cp1-1-Phosphatidylinositol-4-phosphate 5-kinase; Zinc finger, FYVE/PHD-type. M. trancubula (ABE86459.1)	5,00E-25	AT5G14270	mitochondria	1-phosphatidylinositol-4-phosphate 5-kinase activity, zinc ion binding	cellular protein metabolic process
C	851+	167	estee	PU4_plate26_P11 (U-box domain-containing protein (Arabidopsis thaliana) NP_568315)	1,00E-90	AT5G15400	ubiquitin ligase complex	ubiquitin-protein ligase activity	protein ubiquitination
C7	251+	839	Blactx	Os10g054500 [Oryza sativa (Oryza sativa) callose group-A; arginine methyltransferase-interacting protein; contains RING Zn finger]	6E-100 (bit 367)			protein binding	posttranslational modification protein turnover, chaperones, intracellular trafficking and secretion
C29	281-	108	nchi-blactx	NM_102340.3 Arabidopsis thaliana nucleotide binding protein	3,00E-07	AT1G27730	unknown	nucleotide binding	unknown
C85	551+	220	blactx	C1638887.1-malvaceae-nucleic-acid-hydrolytic-protein-NP_192673 DNA heat shock protein	1,00E-14	AT4G09930	unknown	heat shock protein binding	protein folding
C86	651+	199	blactx	NM_001066575.1 oryza sativa rps19a proteinase inhibitor	1,00E-08	AT1G08900	unknown	metallopeptidase activity	proteolysis
T14	651+	238	estee	PP_LE_0007013c - (ADE53112.1) Pepsin-like serine, catalytic; DPA-like conserved domain (DPA)	1,00E-145	AT5G13235	unknown	aspartic type endopeptidase activity	ubiquitin cycle
T34	751+	192	estee	PP_LE_002619f - Oligo-acetyl transferase S173 subunit (Osteocalcin complex) (Bat1432.1) hypothetical protein (Arabidopsis thaliana)	3,00E-33	AT1G34130	endoplasmic reticulum	transferase activity	protein glycosylation, protein metabolism
Cellular transport									
T78	2H+	528	estee	PP_LE_00030070f (AA044082.1) putative sorbitol transporter protein (cassia)	0	AT2G16120	membrane	carbohydrate transportase	carbohydrate transport, transport
C11	4+	210	estee	PU3_plate8_N09 - (F38546) Rami-Solammi hexosepinin - RAN1_LYCES GTP-binding nuclear protein (Ran1)	1,00E-109		nucleus and cytosol	transporter activity, sugar, nucleoside, nucleotide activity	transport
C12	3+	372	estee	PU2_plate25_J10 (BA021121) psammis atrium Rab protein - GTP binding protein	2,00E-66		plasma membrane and cytosol	GTP binding	protein transport, small GTPase mediated signal transduction
C98	2+	240	estee	PP_LE_0013E23f (BA0404838.1) ORF (hmm longiniform as perase)	1,00E-130		endoplasmic system	asparaginase activity, transporter activity	as transport -TRANSPORT
T16	1I+	390	blactx	ef15139488 (b) (BAD38112.1) magnesium transporter, Cora-like (Oryza sativa)	3,00E-43	AT1G18010	membrane	metal ion transportase	metal ion TRANSPORT
C191	2R+	242	estee	FP0_A1875694 (NP_187788.1) SYP121, t-SNARE (Arabidopsis thaliana)	6,00E-32	AT3G11820	plasma membrane	t-SNARE activity	response to stress (GMP)- transport
C193	2R+	134	estee	PP_LE_0022422f (AA040645.1) chitin coat assembly protein (Gossypium babadense J)	8,00E-25		cellular vacuole coat	unknown	intracellular protein transport
C203	3R+	297	estee-blactx	PU2_plate26_M17 (NP_569465.1) transport (Aa) - EA2A3615.1, hypothetical protein (Oryza sativa)	1E-79 (bit 23)	AT5G10800	integral to membrane	transporter activity	transport
T212	6+	151	estee	PU4_plate54_C20 (NP_001007068.1) karyophilin importin beta	3,00E-38		cytoplasm, nuclear pore, nucleus	RNA binding?	RNA export from nucleus
C115	3R+	297	estee	Shan17001-(AA068523.1) glucose-6-phosphate/inositol-transporter precursor (di Peam sativum)	1E-131		plasma membrane	transporter activity	transport
C118	4R+	148	blactx	g1C0052598.1 ABC-TYPE TRANSPORTER-LIKE PROTEIN malvaceae-nucleica	3,00E-20		plasma membrane	ATPase activity, transporter activity	transport
C132	6R+	265	blactx	g1(AA065366.1) cyclic nucleotide-gated channel C Phaeocystis vulgaris	2,00E-09		plasma membrane	calmodulin binding, cyclic nucleotide binding, ion channel activity	ion transport, response to pathogen
C136	6R+	246	estee	F30_A1873308-(ATNAP4) ARABIDOPSIS THALIANA NON-INTENSIC ABC PROTEIN 4)	1,00E-128	AT1G03900	plasma membrane	ATPase activity, transporter activity, coupled to basemembrane movement of substance	extracellular transport
C146	4R+	354	estee	PU3_plate28_D11 (AB051934.1) sucrose transporter2A (di leuca, brasiliensis)	1,00E-147		plasma membrane endoplasmic system	transporter activity	carbohydrate transport
C161	1R+	530	blactx	NP_195221.1 - nuclein family protein arabidopsis	4,00E-70	AT2G16600	plasma membrane endoplasmic system	transporter activity	transport
T107	1R+	230	estee	PU2_plate44_P15 homologous to sec61 - protein transporter (ABE0240.1 medicago truncatula)	1,00E-49	AT5G60540		transporter activity	protein metabolism
T144	10R+		estee	PU1_plate39_C17 - AFP-GAP (CA086037.1) VARI3 Arabidopsis thaliana	1,00E-28	AT5G13300	transGolgi network	phosphonucleotide binding, ATP/GTPase activator activity	response to auxin stimulus, leaf morphogenesis, pollen and pollen formation
C55	351+	354	Blactx	g1NEA25206.1 hypothetical protein Os1_021689 (Oryza sativa)	9,00E-09		transport vesicle	nucleic acid binding	intracellular protein transport

C89	2S1-	495	E:his	Slm78501 (NP_974985.1: vascular protein sorting 55 family protein / VPS55 TRANSPORTER)	0,00E+00	ATI6324101	endomembrane system	transport activity	transport
C95 <td>4H+</td> <td>230</td> <td>b:ketx</td> <td>CH907672.1.MAL15 x domestica NP_820805.1: molecular protein-aa</td> <td>3E-36 ZE-18</td> <td>AT5311010</td> <td>chloroplast</td> <td></td> <td></td>	4H+	230	b:ketx	CH907672.1.MAL15 x domestica NP_820805.1: molecular protein-aa	3E-36 ZE-18	AT5311010	chloroplast		
C179	2I+	287	etsee	PI4_plat11_006 (NP_172944.ZIGM4 (ARF GAB1-LIKE ZINC FINGER-CONTAINING PROTEIN ZIG44): DNA binding (Arabidopsis thaliana))	e-139	AT1008880	nucleus	DNA binding	regulation of GTPase activity
C200	7R+	393	etsee	PP_LE4007721(AAM20722.1) putative G protein-coupled receptor (Arabidopsis thaliana)	5,00E-60		membrane	G-protein coupled receptor activity	mitotic cell cycle flower development response to cytokinin stimulus
C110	1SE+	94	b:ketx	CY090049.EST_14366 Eup. Apricot Fruit-unknown function and BRASINAZOLE-RESISTANT 2 protein	0,14		membrane	protein binding	brassinosteroid mediated signaling
C123	3RE+	384	b:ketx	g1ABE93062.2] Response regulator, RegA/Fra1/AtR1 type [Medicago truncatula]	1,00E-30	AT4605420	nucleus	transcription factor activity, two component response regulator activity	signal transduction in cyanobacterial diatom
C149	7RE+	249	b:ketx	ent1(AAM707029.1) Vigna radiata p1c3 gene for phospholipase C	3,00E-12	AT5638700	plasma membrane	hydrolyase activity	signal transduction, lipid metabolic process
C157	5RE+	307	etsee	PP_33101.A2_F01-(CALMODULIN-BINDING PROTEIN)	1,00E-139		cytosol	calmodulin binding	signal transduction
T132	6RE+	280	etsee	Skin67007 (NP_001053900.1: hypothetical protein Oryza sativa, NP176960) golgi transport complex protein related Aa)	1,00E-139	AT1667930	chloroplast, nucleus	steroid hormone receptor activity, transcription factor activity	regulation of transcription, DNA-dependent
C92	4S1+	337	etsee	PU3_plat36_F02 (NP_001091633: transcription factor Arabidopsis thaliana)	e-136	AT4617310	chloroplast	transcription factor activity	regulation of transcription, DNA-dependent
T30	2S1+	303	etsee	F03_plat41_006 -putative transcription factor	7,00E-94	AT1653500	nucleus	transcription factor activity	signal transduction
C227	4S+	239	b:ketx	AB482788.1 putative receptor kinase [Malus x domestica]	1,00E-40		endomembrane system	kinase activity, ATP binding activity	protein as phosphorylation, transmembrane receptor
C125	5RE+	295	etsee	FU1_plat22_A23 - AT260812_1 putative Hsp70-1-like receptor [Elymus repens]	1E-167		unknown	unknown	metastable resistance
C221	3I+	378	etsee-rbb1	Pa_EST1974 (AAN77145.1: fiber protein P52 [Coccyzus americanus])	4,00E-86	AT16027301	unknown	unknown	response to water deprivation
C181	4S+	146	b:ketx	PP_LE500301.3C (NP_001063066.1: samein ORYZA SATIVA)-EAZ44634.1 (bip. protein, Oryza sativa)	0,2e-66	AT1635720	cytosol-membrane	calcium ion binding, calcium-dependent phospholipid binding	response to osmotic stress, oxidative stress and abiotic acid stimulus
C164	5RE+	247	etsee	AAM66024.1 beta-1,3-galactase-like protein [Arabidopsis thaliana]	2,00E-18	AT1613830	endomembrane system	unknown	defense
C177	2RE+	508	T:b:ketx	F80_A1876305 (ABE84254.1: Universal stress protein (Usp) [Medicago truncatula])	3,00E-59		unknown	unknown	response to stress
T162	4RE+	466		DY256448.1 Apple_EST_MdIs Malus x domestica cDNA 3' similar to ratNP1_76350.1 seven transmembrane MLO protein family	9,00E-71	AT1611000	plasma membrane	calmodulin binding	cell death, defense response
T2	2S1-	288	etsee	Slm77D02 -(AAV36361.1 RD22-like protein [Vitis vulpina])BURP domain	1,00E-103		endomembrane system	unknown	response to abiotic stress
T11	1S1+	845	b:ketx	ent1(CAN12302.1) hypothetical protein [Vitis vulpina]	2,00E-97		unknown	unknown	cellular copper ion homeostasis
T29	1S1+	375	rbb1:b:ketx	AAM12890.1 IAF494597_cds11: chitinase [Malus x domestica] E: vva chitinase II	8,00E-41	AT46017001	endomembrane system	e-transport (other binding), chitinase activity	response to pathogen
T36	11S1+	143	etsee	F80_A1872444 -(Q08935: chlorophenolpropan A, chloroplast precursor - CF29A, Nicotiana glauca)	1,00E-22	AT2637220	plastid	F66-binding	response to cold
T62	8S1+	241	etsee	F80_A1876325 -CADM209.1-catalase minus peroxis-CAT2	1,00E-134	AT4635090	peroxisome	catalase activity	hydrogen peroxide catabolic process- response to stress

T96	151-	137	estee	F30_A1872730 - FD-22 o BUPEP domain protein clone T162	1.00E-11	AT1049320	unknown	response to abiotic stress
Development								
T4	11+	707	estee	PP_LEA0015K007F-C04062: PME, PRU1E Pectinesterase PF25B precursor (Pectin methyltransferase) (PME) (Ponsus pestsis)	0	AT35443701	Pectinesterase activity-lysozyme	cell wall modification DEVELOPMENTAL PROCESSES
T164	7R+	364	estee	PP_LE0002K167 (NP_671778.1) ATP binding / RNA ligase/threonine-RNA ligase (Arabidopsis thaliana)	1.00E-119	AT2304842	threonine-RNA ligase activity	embryonic development ending in seed dormancy, chloroplast
T7	4S1+	242	estee	Pa_EST1040: (Pse-nRNA processing ribonucleoprotein, binding region domain containing protein)	5.00E-129	AT105601701	unknown	embryonic development ending in seed dormancy
Bio genesis of cellular component								
C109	11R1+	209	estee	PP_LE0021M174(NP_001078531) Arabidopsis thaliana-nucleolar matrix protein-related)	8.00E-39	AT2627730	unknown	unknown
Unknown								
C49	2H+	255	estee	gIDY646938 F03_plate34_118 F03 hypothetical domain containing calcium binding EF-hand	2.00E-78		calcium binding activity	unknown
C77	2H+	231	blact	B129692 Arabidopsis thaliana cDNA	2.00E-40		unknown	unknown
C23	2H+	330	rbct-blact	gIDY75243 2865 Arabidopsis thaliana CYP7C_G4 cytochrome P450	7.00E-58		unknown	unknown
C84	6+	95	estee	F30_A1872645-WASHING SEQUENCE CONTAINING TWO TAILS	7.00E-58		unknown	unknown
C99	3+	143	Estee	F30_A1873592 (AAO19365 unknown protein (Oryza sativa J))	3.00E-66		unknown	unknown
T59	3+	214	estee	F30_A1873522-unknown	1.00E-41		unknown	unknown
T40	6+	330	rbct-blact	ent(CAN61653 hypothetical protein (Vitis vinifera))	6.00E-21		unknown	unknown
T41	7+	190	rbct-blact	F30_A1873248 unknown function- gIDY256453 MdR3L3302.e1 Apple EST	8e-19 -2e-24		unknown	unknown
T75	1+	271	estee	F01_plate4_M06 (CAB39632) putative protein Arabidopsis thaliana weak similarity to some nucleoside-sulfhydrylase family protein	6.00E-61		unknown	unknown
T79	3+	263	flact-est	NP_177792.1 unknown protein Aa_EE710147.1 homologous to induced protein in Mabs x domestica after V. anguillar accumulation	5e-07 8E-41	AT1076660	unknown	unknown
C178	1R+	300	estee	F01_plate26_H09 -(AB191657.1: Protein of unknown function, DUF384 Medicago truncatula)	1.00E-109		unknown	unknown
C183	2R+	133	estee	BG_HPL-04-C12 - no hit found	2.00E-26		unknown	unknown
C189	4R+	190	estee	Pe-S4_EST2689 - no hit found	8.00E-30		unknown	unknown
C198	5R+	220	blact	xe(NP_564753.1) radical SAM domain-containing protein (e-s-chlorate reductase)	2.00E-15		unknown	unknown
C204	4+	205	estee-blact	F01_plate16_A22 (NP_567786) unknown protein (AFA) - CAH58645.1 putative transporter Plantago major	1.00E-113	AT25493101	unknown	unknown
C207	3R+	268	estee	S346311 - no hit found	7.00E-96		unknown	unknown
C224	4R+	153	blact	ent(CAN181894.1 - hypothetical protein vitis vinifera-similar to nucleolar protein ga2-related)	1.00E-16		unknown	unknown
C227	3R+	339	estee	S317710 - no hit found	1.00E-108		unknown	unknown
C239	6R+	108	estee	F01_plate45_M19 (no hit found - or unknown protein)	2.00E-16		unknown	unknown
T175	2R+	311	estee	S38K11 - unknown protein	2.00E-43		unknown	unknown
T179	1R+	389	blact	NP_565101.1 JAC10 domain protein required for chloroplast accumulation responses (Ara thaliana)	3.00E-39	AT1075100	unknown	chloroplast accumulation movement, chloroplast avoidance movement
T181	3R+	130	estee	PP_LEA016A10F - unknown protein	6.00E-24		unknown	unknown
T183	1R+	207	estee	F04_plate55_L13 (AB193614 hypothetical protein 2340026 (Brassica oleracea)) SNAPE associated G2e1 protein	1.00E-35		unknown	unknown
T184	1R+	515	estee	CAN67793.1 hypothetical protein (Vitis vinifera)-Cys450 - F01_plate9_ED2	1e-59 8E-46	AT253601301	oxygen binding	electron transport
T192	2R+	130	estee	S316514 - no hit found	9.00E-69		unknown	unknown
T203	5R+	151	blact	gIDYAD39083 putative auxin-independent growth promoter (Oryza sativa (spikes cluster group))	6.00E-13		unknown	unknown
T205	2R+	125	blact-EST	gIDYCB822107 EST 2938 Halcyon Apricot Fruit	4.00E-37		unknown	unknown

48.7% of the analyzed sequences showed high similarity to plant genes, whereas 38.9 % belonged to bacteria, particularly to *Pseudomonas spp.*.

1.5% of the sequences analyzed belonged to fungi and 10.9% of the sequences did not match any significant homology.

51 genes were found to meet the 1°) criterion of TDF selection (genes differentially expressed in the susceptible fruits upon *Monilinia* infection (**SI**): of these, 39 genes (76%) were induced and 12 (23%) were repressed.

Similarly, 46 genes were found for the 2°) class of genes (genes differentially expressed in resistant fruit upon *Monilinia* infection **RI**) of these, 39 genes (85%) were found up-regulated and 7 (15 %) down-regulated.

31 TDF belonging to the 3°) class of genes (genes differentially expressed in **Hvs.I**) were selected; of these, 9 genes were induced in **H** (repressed in **I**), and 22 repressed in **H** (induced in **I**).

Finally, 46 genes were selected for the 4°) class of genes (genes differentially expressed in **S** vs. **R**). Of these, 13 genes were down-regulated in **S** (up- in **R**) and 33 genes up-regulated (down- in **R**).

All sequences were functionally classified following the same functional classification used by *Arabidopsis thaliana* MIPS. Genes potentially involved in cell rescue and defence were well represented (8%); several genes (12.1%) involved in the protein folding, post-transductional modification and genes (9.2%) involved in cellular transport were also found. A further 10.3% of genes were classified as involved in the metabolism of amino-acid, carbohydrate and fatty acid. On the other hand, genes involved in the protein synthesis (5.7%) and in signal transduction and communication (5.7%) were a smaller group (tab.3.2 and fig.3.14).

Functional group classification	Number of sequences analysed	%
Cell cycle and DNA processing	1	0.6
Metabolism	18	10.3
Energy	11	6.3
Transcription	5	2.9
Protein synthesis	10	5.7
Protein fate	21	12.1
Cellular transport	16	9.2
Cellular communication-signal transduction	10	5.7
Cell rescue, defence and detoxification	14	8
Development	9	5.2
Biogenesis of cellular component	1	0.6
unknown	30	17.2
unclassified	28	16.1

Table 3.2: **Functional group classification.**

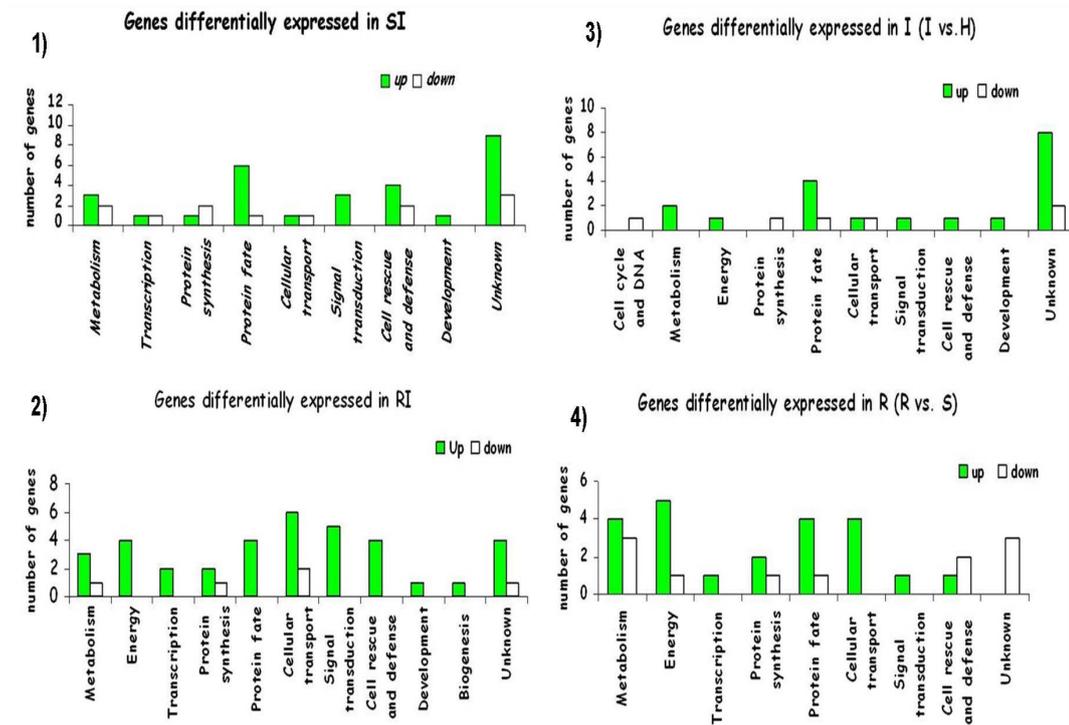


Figure 3.15: Functional group classification of peach genes and their distribution in the four selected class.

3.6 Pru P1 and Pru P2

Microarray analysis showed that two genes encoding PR (pathogenesis related) 10 proteins were up-regulated in the **R** fruits. These consisted of the major allergen Pru P1 (GenBank accession AM493970) and its isoform Ctg1 [20] (here named Pru P2). To investigate on their antimicrobial properties, Pru P1 and Pru P2 were cloned, expressed in *E. coli* system, purified and used in functional assay.

Full length c-DNAs were isolated from the **R** fruit c-DNA first strand, and expressed as His tag fusion proteins.

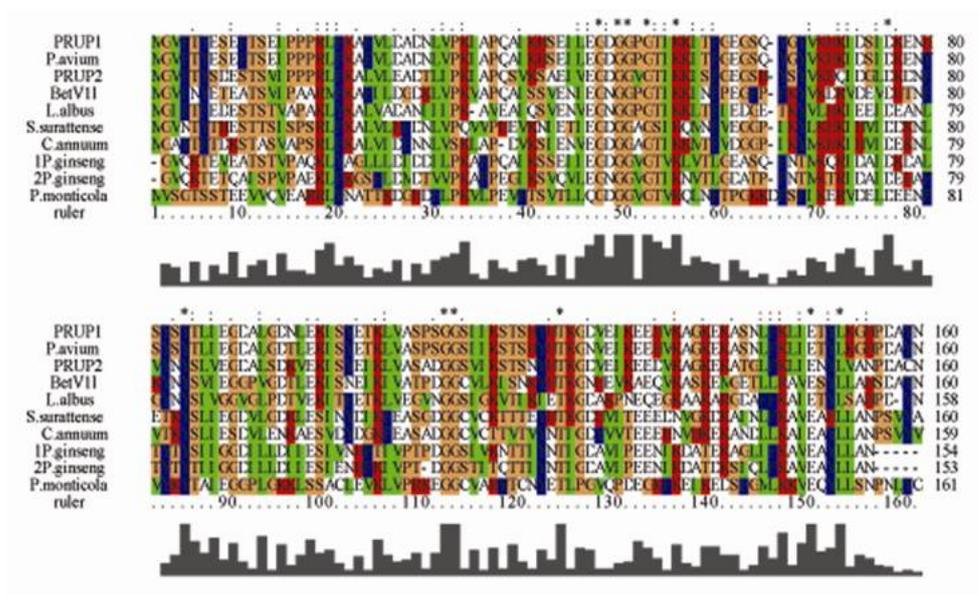


Figure 3.16: Multiple sequences alignment of PR10 protein from different plant species.

Pru P1 and Pru P2 were both expressed as partially soluble proteins, with a protein yield of 7.3 mg/l and 5.4 mg/l, for Pru P1 and for Pru P2, respectively. Despite the molecular mass was 18.5 and 18.1 kDa, respectively

(figs. 3.17, 3.18), both proteins migrated on SDS PAGE as 22 kDa proteins.

Proteins were purified by nickel affinity chromatography both eluting with 200 mM imidazole (fig. 3.17 and fig.3.18).

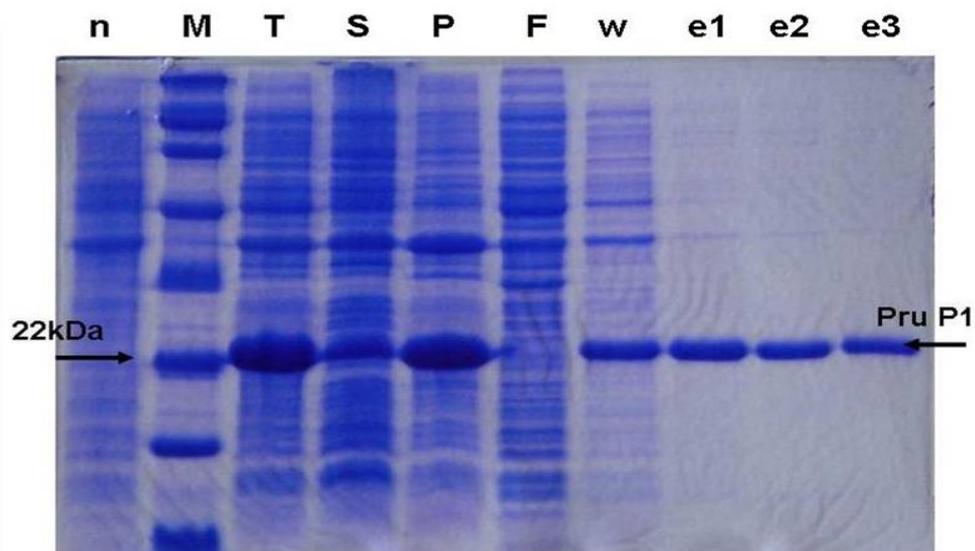


Figure 3.17: **SDS PAGE of *E. coli* BL21 (DE3) expressing PRU P1.** n) total lysate of *E. coli* BL21 transformed with the pHAT vector alone. M) protein marker. T) total extract from *E. coli* cells transformed with pHAT-PRU P1. s) Supernatant fraction of the cellular lysate. P) insoluble fraction of the cellular lysate. F) nickel column through. W) column wash with 20 mM of imidazole. e1, e2, e3) PRU P1 fractions eluted with 200 mM of imidazole.

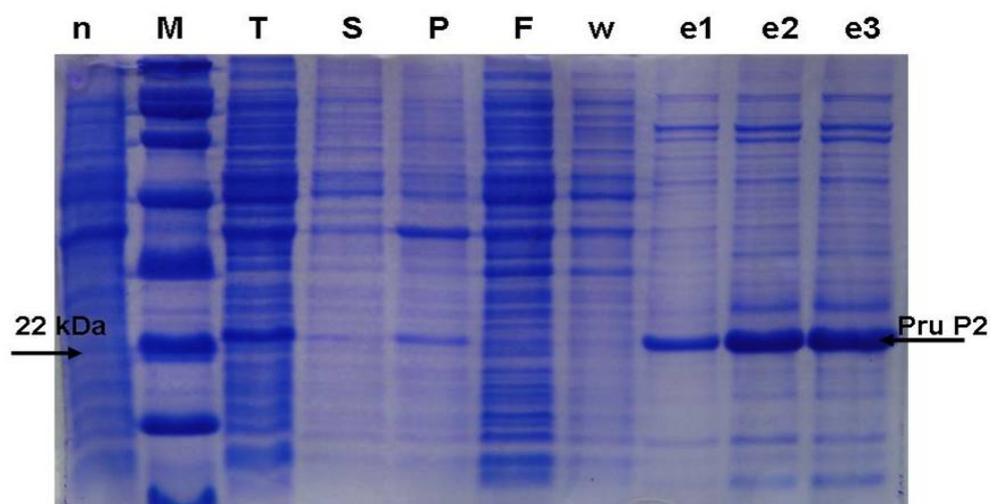


Figure 3.18: **SDS PAGE of *E.coli* BL21 (DE3) expressing PRU P2.** n)total lysate of *E. coli* BL21 transformed with the pHAT vector only. M)protein marker. T) total extract from *E.coli* cells transformed with pHAT-PRU P2. s) Supernatant fraction of the cellular lysate. P)Insoluble fraction of the cellular lysate. F) nickel column flow through . W) column wash with 20 mM of imidazole. e1, e2, e3) PRU P2 fractions eluted with 200 mM of imidazole.

3.6.1 Ribonuclease activity and *In vitro* antimicrobial activity

The recombinant proteins showed ribonucleolytic activity after 2 and 4 hours at 37 °C. RNA was totally and specifically degraded by PRU P1 and PRU P2; no degradation was detected when RNA was incubated with the protein buffer, nor with the lysate of *E.coli* transformed with the vector alone, nor when incubated with the boiled PRU P1 and PRU P2. Moreover, Pru P2 showed a more efficient degradation activity than PRU P1; after 2 hours, the RNA was completely degraded by PRU P2, and only partially degraded by PRU P1 (fig.3.19, 3.20).

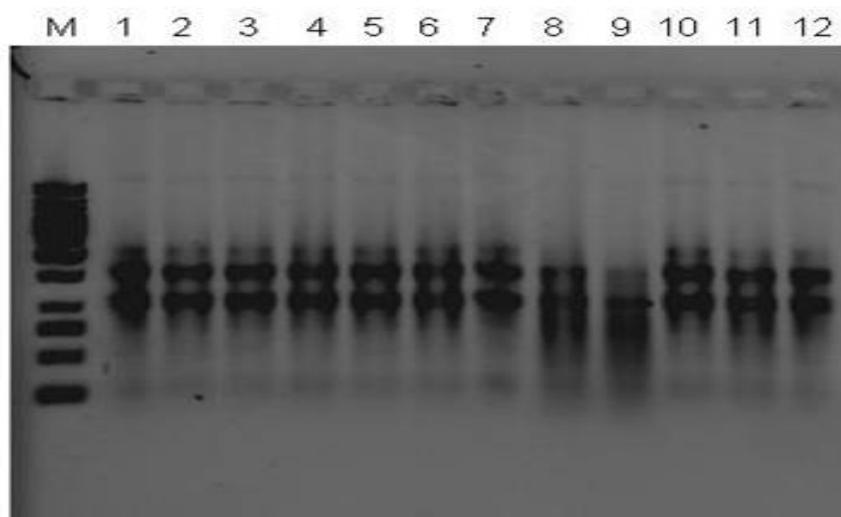


Figure 3.19: **RNase activity of recombinant Pru P1 protein against peach RNA at time 0, and after 2 h and 4 h of incubation at 37°C.** M) DNA marker; 1-3) RNA and phosphate buffer; 4-6) RNA and purified lysate of *E. coli* transformed with the pHAT vector alone; 7-9) RNA and Pru P1 recombinant protein (10 μg); 10-12) RNA and boiled recombinant Pru P1 protein (10 μg)

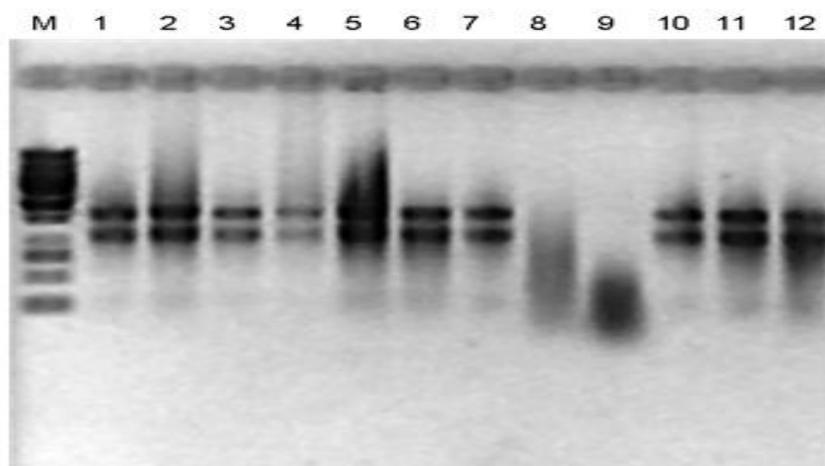


Figure 3.20: **RNase activity of recombinant Pru P2 protein against peach RNA at time 0, and after 2 h and 4 h of incubation.** M) DNA marker; 1-3) RNA and phosphate buffer; 4-6) RNA and purified lysate of *E. coli* transformed with the pHAT vector only; lanes 7-9, RNA and Pru P2 recombinant protein (10 μg); lanes 10-12, RNA and boiled recombinant Pru P2 protein (10 μg)

Because many PR 10 are known to have antimicrobial properties, Pru P1 and Pru P2 recombinant proteins were also checked for direct antimicrobial activity against *M. laxa*, *M. fructigena*, *Botrytis cinerea*, *Collethotrichum acutatum*, *Rhizopus* and *Penicillium expansum*. The effect of the protein on hyphal growth was analyzed by placing them on filter paper discs in proximity of the hyphal growth. After 12-24 hours of incubation, depending of the fungal growth speed, no inhibitory effect of PRU P1 or PRU P2 was detected (fig.3.21).

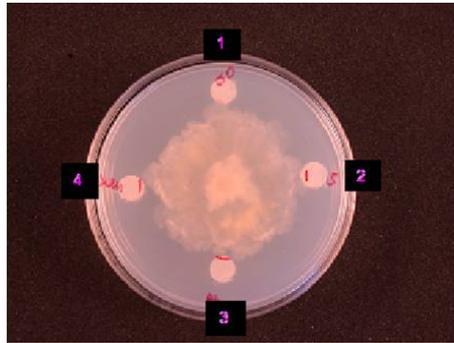


Figure 3.21: **Antimicrobial activity of recombinant Pru P1 and Pru P2 proteins.** Filter paper discs were placed 0.5 cm from the growing front of the pathogen; the plates were photographed after 19 hours of incubation. 30 and 15 μg of Pru P1 or Pru P2 were placed on disc 1 and 2, respectively; 30 μg of the boiled protein were placed on disc 3 and 30 μg of the lysate of *E.coli* cells transformed with the pHAT vector alone, was placed on disc 4.

Chapter 4

Discussion

The variation of susceptibility of peach fruits to *Monilinia laxa* during fruit development has been studied for several years and in different conditions. First investigations revealed that green fruits are more resistant than the ripe ones [2]; more recent studies revealed that peach susceptibility strongly decreased during the pit hardening stage and increased thereafter [3, 4]. The reason of this dramatic decrease of susceptibility is not yet clear, many hypothesis have been proposed; these take into consideration the mechanical resistance of green fruits, the presence of inhibitory substances during this period, or some host physiological mechanisms that can prevent fungal development. This last hypothesis was made on the basis of a similar behaviour of wounded and un-wounded fruits [3].

In this study a molecular approach was undertaken to investigate on the genetic bases of this temporary loss of susceptibility, by evaluating either the constitutive or the pathogen induced differences, between susceptible and resistant fruits .

4.1 Expression of genes of the phenylpropanoid and jasmonate pathway

To associate the presence of inhibitory compounds in the peel of peach fruits during pit hardening with variation in gene expression of key genes of the phenylpropanoid pathway, transcript levels of these genes were determined in susceptible and resistant fruits. Several phenylpropanoid compounds are involved in plant defence, either as preformed compound or as inducible products after pathogen attack, both in local and systemic resistance [36]. An increase in chlorogenic acid and epicatechin was shown by HPLC analysis (data not shown) during the peach pit hardening in correspondence to the apparent fruit temporary resistance to *M. laxa*. Gene expression was analyzed on fruits harvested from the 6th to the 12th week after full bloom in a period encompassing the pit hardening stage; fruits were either inoculated with *M.laxa* or mock inoculated.

These data suggest a critical change in the expression level of the phenylpropanoid pathway from the 7th to the 8th week AFB; such change could be directly physiologically associated to the peach growth and could indirectly determine the decrease of susceptibility of peach fruit to *Monilia* rot during the subsequent weeks.

It is known that the activity of the phenylpropanoid enzymes is regulated at the transcriptional level [10]. The promoter sequences of the key genes of this pathway have been extensively studied, revealing regions regulated during development and after both biotic and abiotic environmental stimuli [9]. Therefore, difference in gene expression of these genes during the S2 phase could be correlated to a difference in the enzyme activity and, as a consequence, to a change in the concentration of the metabolites produced.

Consistent to this, the changes in expression of the genes involved in phenylpropanoid pathway reflect the standard physiological requirements of each fruit growth phase. The process of pit hardening requires the production of several antioxidant compounds necessary for the lignification process. According to this, transcription of the key genes of the phenylpropanoid pathway *PAL* and *C₄H*, and of the *HQT* gene, which regulates the metabolic branches leading to production of chlorogenic acid and the lignin, are down-regulated from 6th and the 8th week AFB, respectively, when seed lignification has completed.

Conversely, *CHS* gene undergoes a dramatic increase in expression from the 7th to the 8th week AFB. Similarly *LAR* expression shows a significant up-regulation from the 7th to the 10th week AFB. *CHS* is the first enzyme committing the phenylpropanoid pathway into the branch that leads to the production of flavons and antocyanins, whereas, *LAR* is a downstream enzyme, specifically involved in the synthesis of conjugated tannins. The physiological reason underneath the production of such compounds is still unknown, however, it is known that flavonoids and tannins have an established role in defence against different type of plant parasite [9]. In order to determine whether they play a role also in conferring fruit resistance to *M.laxa*, by direct inhibition, or by inhibition of crucial pathogenesis components, further investigation are needed. Furthermore, also the up-regulation of the *LOX* gene during the 8th week AFB could be important for conferring temporary resistance to *Monilinia*. *LOX* is a key enzyme in the production of jasmonate precursor. Jasmonate is one of the several lipid-derived plant growth regulators. The action of jasmonate are wide-ranging. In the last few years, it has become clear that is a key component of the wound-signaling pathway that allows plants to protect themselves from insect attack. Jas-

monate induces expression of genes that lead to the production of proteinase inhibitors and other defence compounds. Furthermore, the use of *Arabidopsis* mutants, has demonstrated that jasmonate, along with ethylene, is an important chemical signal in non-host resistance against fungal pathogens [37].

4.2 Microarray and cDNA AFLP analysis

Transcriptome analysis can identify even complex genetic pathways that underlie important physiological processes and intricate pathogen-host interactions. Among these, microarray technique has a lot of advantages because it can measure expression in a semi-quantitative manner, it is sensitive to low abundance of transcript and it is a rapid method, exploiting existing EST sequences. On the other hand, since microarray analysis is restricted to the number of EST sequences available, the data obtained with this method can be partial.

In the case of peach fruit, the μ Peach1.0 microarray is the only available microarray. It contains 4806 unigenes, estimated as the 20% of the entire transcriptome. Moreover, the chip was built to study changes in gene expression during the S3 - S4 ripening phase transition [20]. All together these features, could make the μ Peach 1.0 not totally suited for studying changes in the S2 ripening phase. Consistently, only few genes were found differentially expressed in **RH** vs. **SH** fruits and **RI** vs. **SI**, whereas no differences were found between **RH** and **RI** fruits. For 11 genes, the expression pattern revealed by microarray was also analyzed by real time PCR; the differences between the **RH** vs. **SH** and **RI** vs. **SI** fruits were always qualitatively and quantitatively confirmed, except for the harpin protein, for which real time

PCR showed a much higher expression ratio (15 by real time PCR vs 5.9 by microarray). On the contrary, all the differences resulting from the indirect comparison between **SH** and **SI**, once tested in real time PCR, did not meet the expected results. This could possibly be due to the value of fluorescence ratio used as lower limit of data significance. This was 1.7, slightly lower than the one usually used for microarray analysis, which is 2. In our analysis, the value was lowered on purpose, to highlight even mild differences, which could anyway be relevant when comparing fruits differing in growth for only two weeks time. This, however, may have led to the collection of some noisy, as shown by the real time analysis.

In order to overcome the above mentioned limits of μ Peach 1.0 and to complement the microarray transcriptome analysis, a cDNA AFLP analysis was also performed on **SH**, **RH**, **SI** and **RI** samples. This technique, although less sensitive, allows investigation of poorly characterized genome, as in the case of peach; it does not require preliminary sequence information, and hence enables identification of novel genes. Furthermore cDNA AFLP allows the analysis of a large number of samples at the same time.

Four groups were selected and sequenced:

- TDFs differentially expressed during fruit development (**S** vs. **R** fruits) were selected to investigate on their possible role in physiological decrease of the susceptibility to *Monilinia* rot.
- TDFs differentially expressed in the **SI** fruits (up- or down-regulated) were selected to identify the genes that are possibly induced or inhibited during the early phase of the interaction and to investigate on the possible relationship with the susceptible phenotype.
- TDFs differentially expressed in the **RI** fruits (up- or down-regulated)

were selected to identify the genes that are possibly induced or inhibited during the early phase of the interaction and to investigate on the possible relationship with the resistant phenotype.

- TDFs differentially expressed in **H** vs. **I** were selected to identify the genes up- or down-regulated upon infection both in the susceptible and resistant fruits. Moreover TDFs of the **I** fruits could be derived from the pathogen.

TDFs were functionally classified following the same functional classification used by *Arabidopsis thaliana* MIPS.

4.2.1 Fragments differentially expressed in **R** fruits or in **S** ones

Metabolism

Polyamine metabolism

Two genes implicated in the polyamine metabolism were found differentially expressed in the **R** and **S** fruits: these encode for an arginase and S-adenosylmethionine decarboxylase. The *arginase* was found down-regulated in the **R** fruits, both in the microarray and in cDNA-AFLP analysis. This enzyme commits the biosynthesis of ornithine from arginine pathway which leads to the synthesis of polyamine putrescine. The S-adenosylmethionine decarboxylase, enzyme located downstream in the pathway, was found up-regulated in the **R** fruits in the microarray analysis. The real time analysis showed an increase of 2.34 times in **RH**vs.**SH** and of 2.19 times in **RI**vs.**SI**.

Polyamines are polycationic compounds of low molecular weight, ubiquitous in plant cells. Their function is associated with the tissue development

and their metabolism is also altered after the interactions of plants with fungal pathogens [38]. Also it has been found that polyamines conjugated to the phenolic compounds hydroxycinnamic acid amides (HCAAs) accumulated during incompatible interactions [39]. The expression levels of the genes and the enzyme activity of the polyamine pathway was already studied in peach, however Ziosi *et al.* founded that *SAMDC* expression level in mesocarp remained quite stable during fruit development [40].

A physiological increase of polyamine synthesis during the S2 phase could be associated with a decrease in *M. laxa* susceptibility. Also the role played by the hydroxycinnamic acid amides, known as the main phenolic constituents of seed, could of relevance during plant defence; further investigations on this compound during the pit hardening stage of peach could be useful to find relationship with *M.laxa* resistance.

ATP sulfurylase and glutathione metabolism *ATP sulfurylase* gene was found up-regulated in the **RI** and **RH** fruits of 3.24 and 4.12-fold vs **SH** and **SI** respectively, in microarray analysis. This enzyme catalyzes the first step in sulfate assimilation and sulfate is a crucial element in glutathione (GSH) biosynthesis, a well-known antioxidant compounds. Consistently, in the microarray analysis, a glutathione reductase gene was found 1.6-times up-regulated in the **RH** fruits with respect to **SH** .

It is known that not only GSH is essential for protection against chilling injury and other stress, but also a large portion of sulfated metabolites play various roles in plant defence against biotic and abiotic stress [41]. It could be speculated that a general increase in the sulfate metabolism in the **R** fruits could contribute to protect fruits against the pathogen attack.

Shikimate, aromatic aminoacids and lignin biosynthesis

Cynnamoyl alcohol dehydrogenase gene (*CAD*) is down regulated of 0.45-

fold in **R** fruits, whereas *diphenol oxidase* appeared up regulated in the **RH** fruits, (but of only 1.7 times) in the microarray analysis. CAD is the first enzyme of the lignin biosynthesis, whereas the laccase diphenol oxidase acts downstream in the lignin pathway. The increase in expression of laccase diphenol oxidase gene could be associated with the seed lignification occurring in this period. The presence of the diphenol oxidase in the peach mesocarp has been shown [42] and the association between lignification, as a preformed or induced barrier, and defence has already been found [43].

Energy

Four genes encoding for mitochondria and for the chloroplast proteins involved in the energy metabolism, an aconitase (TDF C182), a NADH oxidoreductase (TDF C186), a gamma chain of ATP-synthase (C188) and a carbonate dehydratase (C210), were found in cDNA AFLP analysis as up-regulated genes in **R** fruits.

It is known that altered mitochondrial function is a requirement for the programmed cell death (PCD), mechanism involved in every plant development process as well as induced after pathogen attack and after the exposure to abiotic stresses [44]. In fact the hypersensitive response occurring when an avirulent pathogen attacks a plant cell, is considered a form of PCD. The PCD process in plants involves enhanced respiration with the occurrence of an oxidative burst [45].

Transcription

Among the genes involved in transcription, the microarray analysis showed that an ethylene responsive element binding protein (*ERF-binding protein*) and an *AP2 transcription factor* were up-regulated in **R** fruits. ERF and AP2 are both involved in the regulation of disease resistance pathway. In fact, it has been shown that, in several cases, over-expression of the AP2

TFs results in resistance to multiple pathogens. The *A.thaliana* gene *ERF1* influences the resistance to *Sclerotinia sclerotium* and to *Botrytis cinerea*, which are fungi phylogenetically close to *M.laxa* [46].

Protein fate

Protein fate includes all post-transduction modifications that can occur to protein. Plants possess an innate immune system that recognize the pathogens and rapidly activate the defence system. The perception of the stress and the downstream signal transduction are controlled by post-translational modification of protein, which is rapid, reversible, highly specific, and provide a tool to regulate protein stability, activity and localization. With respect to this group, the microarray analysis revealed 4 genes up-regulated in the **R** fruits : the heat shock protein 70 and two proteins involved in the proteolysis (a nucellin and a cysteine protease) were up-regulated in the **RH** fruits, whereas an heat shock protein 90 is found up-regulated in the **RI** fruits. cDNA AFLP found only a gene up-regulated in the susceptible fruits (C235, a translocase involved in protein secretion), whereas there are three fragments up-regulated in resistant ones: a chaperone of the endomembrane system (T200) and two component of the proteasome-signalosome (C228, T131). Ubiquitin- and proteasome- mediated protein degradation is a well regulated process that play a crucial role in plant, in fact as sessile organism, they must change continuously their proteome to be ready to bear the different circumstances. It has been shown that several ubiquitin components displayed an altered expression level in response to biotic stress, the reasons and causal relationship of which are still ignored. Fungal elicitors from *Phytophthora megasperma* causes up-regulation of ubiquitin transcripts in soybean cells, similarly, in rice cells treated with fungal elicitors, an increment of ubiquitin transcripts is evident [47, 48, 49].

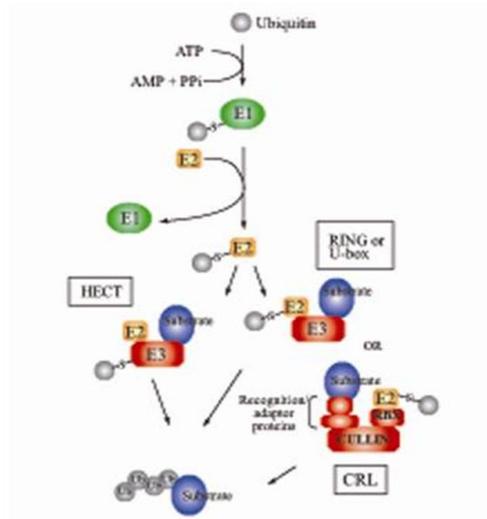


Figure 4.1: Ubiquitin pathway

Cop9-signalosome is a protein complex that controls the ubiquitin-proteasome-mediated protein degradation. It was found involved in a large number of developmental processes. The chaperonin heat shock protein 90 (HSP90), found up-regulated in the **R** fruits, is also involved in several defence pathways and it could also contribute to resistance protein accumulation. In this context, up-regulation of the ubiquitin system in the R fruits could correlate with the increase of pathogen resistance.

Cellular transport

Genes involved in the cellular transport were found up-regulated in the resistant fruits, both in microarray and cDNA AFLP analysis. Among these genes there is the PPA2 ATPase (*Prunus persica atpase 2*). In microarray analysis, this was found up-regulated of 1.7 times in **SI** vs **RI**. On the other hand, cDNA AFLP revealed up-regulation in **S** of SYP121 (syntaxin protein 121), forming the t-SNARE complex (soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor complex), and of a protein of the clathrin coat. It was demonstrated that cellular trafficking, in particular vesicle trafficking (including endocytic and exocytic processes), is an important component of plant innate immune response [50]. Moreover cytoskeleton rearrangement was observed in response to fungal pathogens [51].

Plant immune system functions at two levels; the first occurs at the cellular surface, where the perception of the pathogen-associated molecular pattern (PAMPs) is mediated by the cognate recognition receptor (PRRs). This first level concerns the aim of this study, that was to analyze the early interactions between peach fruits and *Monilinia* rot. It was demonstrated that several PRRs are internalized in the endomembrane system after the interactions with their specific ligand [50]. This molecular mechanism was originally shown in the *Arabidopsis*-bacteria flagellin interactions [50], however, recently, it was also appreciated for fungal-plant interactions[52]. Moreover, endocytosis also seems to play a role in effector-triggered immunity [53], suggesting that the receptor endocytosis may be a general phenomenon in plant defence signaling and regulation. At the first level of interaction, the appressorium, formed by the germinated spores, can exert high pressure on the plant cell walls, to allow the fungal hyphae to enter. This creates an invagination of the cell membranes, and a remodeling of the cell endomembrane

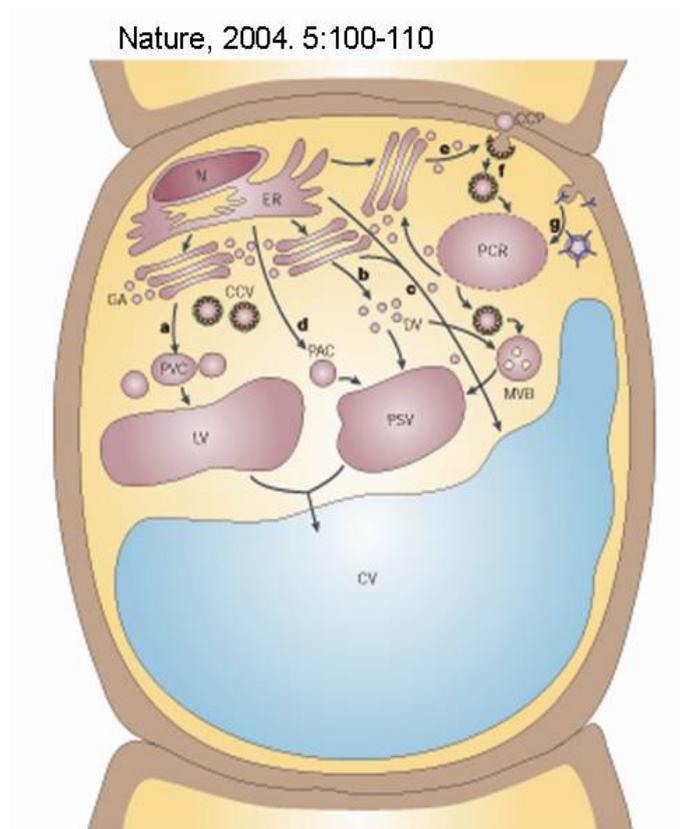


Figure 4.2: Plant cell endomembrane system

system; these changes could represent a signal to block the pathogens (fig. 4.2).

t-SNARE SYP-121, found up-regulated in the skin of the **R** peach fruit encodes a plasma membrane resident syntaxin. This protein was first characterized in *A. thaliana*; PEN1(*penetration mutant 1*)(SYP-121) *Arabidopsis* mutants are susceptible to penetration of powdery mildew, but not to the further growth, suggesting that PEN1 contributes to the pre-invasive immunity of the fungus [54]. Subsequently, two other members of the SNARE were found and the following model was proposed to explain the function of these proteins: in response to fungal attack, a local accumulation of the syntaxin PEN1/AtSYP121 and of the SNAP-25-like protein (AtSNAP3) on the membrane, at the site of fungal penetration occurs [54]. These proteins form a binary t-SNARE complex marking the target membrane. Cellular vesicles labelled with cognate v-SNAREs are trafficked to the target membrane where they form a ternary complex. Here the vesicle fusion occurs and compounds with antimicrobial activities are thus delivered and secreted to contrast fungal ingress. The importance of membrane trafficking in plant-pathogen interaction was further highlighted by the finding that Brefeldin A (BFA), a compound from the fungus *Alternaria carthami*, promotes plant disease through interference in the formation of Golgi-derived vesicles [55], indicating that intracellular trafficking of vesicles can be a target for pathogen effector proteins and compounds. Therefore, the presence of the SYP-121 protein in the peach **R** fruit could have a role in blocking the *M. laxa* penetration 4.3.

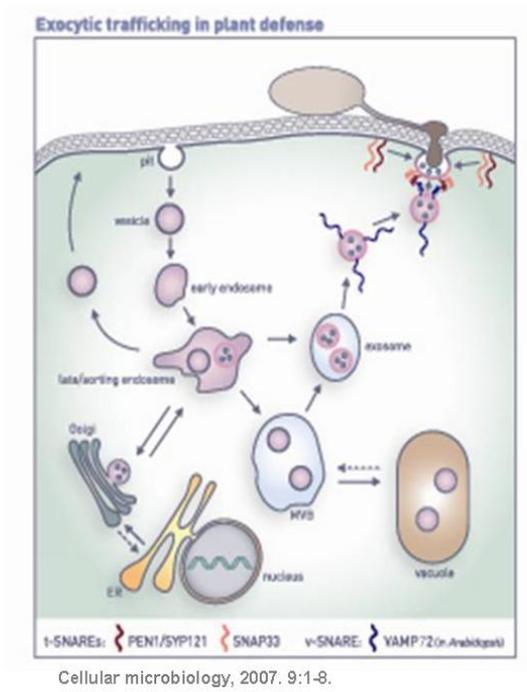


Figure 4.3: t-SNARE pathway

Cellular communication and signal trasduction

Microarray analysis revealed that an *histidine containing phosphotransfer protein* and *leucin rich repeat transmembrane kinase* were down regulated in **R** fruits, whereas a protein responsive to gibberellin stimuli, a *MAP kinase* and the *calmodulin 1* were up-regulated.

Histidine phosphotranfer protein is an intermediate between the sensor His kinase and the response regulator in the bacterial two-components system. This system has a modular design and occur in a variety of organisms, including plants, in order to allow cell signaling circuits to sense and respond to changes in environmental conditions [56] (fig.4.4) Leucine-rich repeats (LRRs) motif is present in different proteins with different functions; however all the proteins containing the motif are involved in the formation of protein-protein interactions [57]. LRR motifs are principally known as related to defence, in fact the most common plant resistance proteins, that recognize specific pathogen virulence factors, possess an extracellular LRR motif [58], however the Tomato *curl3* gene, an LRR receptor kinase, is involved in the brassinosteroid hormones signaling [59]. Moreover the CLAVATA1 gene of *A. thaliana* is a LRR receptor kinase with a role in the development pathway [60], and the ERECTA-family receptor like kinases of *A. thaliana* regulated the inflorescence mechanism [61]. The up-regulation of these genes in the **S** fruits could be correlated better to a developmental function more than a defence function.

Map-kinase is implicated in the signal transduction via phosphorylation; MAP kinases take part to the kinase cascade that transduce diverse extracellular stimuli into intracellular responses. These proteins are ubiquitous in most eucaryotic system, from yeast to mammals and plants. The rice MAPK1 was found involved in multiple stress responses, including pathogen

infection, as it was demonstrated in rice [62], through a tissue-specific regulation [63].

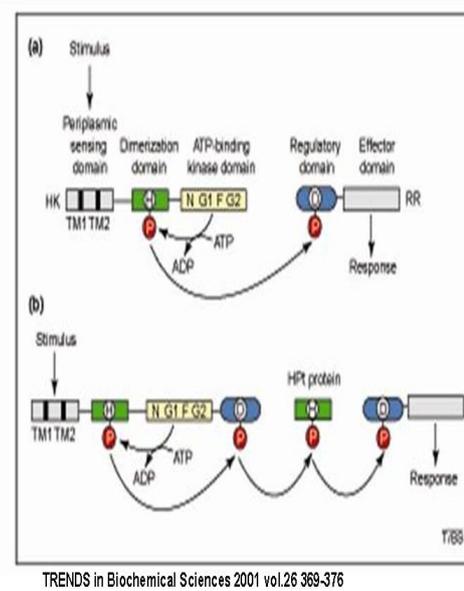


Figure 4.4: **Two component phosphotrasfer scheme.**

PR proteins

Plants have evolved a variety of potent defense mechanisms, including the synthesis of low-molecular-weight compounds, proteins, and peptides which show antimicrobial activity and appear to be involved in either constitutive or induced resistance to fungal attack. These antifungal proteins are known as pathogenesis related proteins, they were classified in 13 classes on the basis of sequence homologies and functions [64]. In our study several PR proteins were found to be altered in expression when comparing **R** and **S** or **H** and **I** fruits. A peach defensin was found up-regulated in **S** fruits by microarray and real time PCR analysis. Defensins are low molecular, cysteine-rich proteins, belonging to the innate immunity system. It has been shown that plant defensins cause fungal inhibition by the modification of ion efflux [65]. Peach defensin (*PpDfn1*) has been firstly isolated from the bark tissue during winter time. Its transcript was found associated with early fruit development, and absent in the mature fruits. Consistently our data show that defensin gene was found up-regulated in **S** fruits vs **R** ones. Expression of defensin in the early stages of fruit development was associated with the evolutionary role of fruit, which is to disperse the seed, maintaining the embryo alive. Defensin induction would then contribute in protecting the embryo during the very early stage of fruit development, when the pit has not hardened yet. Upon pit lignification, defensin gene is then down-regulated [66].

c-DNA AFLP analysis revealed another PR protein up-regulated in the **S** fruits. This is a beta-1-3 glucanase like protein (C181, fig. 3.14). Beta glucanases form the class 2 of PR proteins. They are vacuolar or extracellular proteins and their antifungal activity appear to be displayed through the hydrolysis of glucan compounds in fungal cell wall [64].

So far, three peach beta-1-3 glucanase have been characterized for ex-

pression in the reproductive organ of peach and it was found a spatial and temporal regulation in flower and in young fruit [67, 68, 69].

Microarray analysis revealed other 3 PR proteins up-regulated in the resistant fruits. These are the major allergen of peach (Pru P1) and its isoform, named in this work Pru P2, and a class IV chitinase. Pru P1 and Pru P2 proteins belong to the class 10 of the PR proteins. They were expressed as recombinant proteins and tested for their antimicrobial activity. Data on this part of work will be discussed further on. The class IV chitinases (PR-3 proteins), similarly to the class I, contained an N-terminal cysteine-rich domain and a chitin-binding domain, but they are smaller, due to four major deletions. They were shown to have a potent antifungal activity against several fungal pathogens [64]. Their action is based on cleavage of the fungal cell wall chitin. Their action is very similar to the one of beta-glucanase.

A constitutive expression of chitinase was found also in grape during berry growth and also in this case it was suggested that the induction of the PR proteins during fruit development was associated to embryo protection [70].

Interestingly, an harpin induced protein was found up-regulated in the **R** fruits, up-regulation which, by real time PCR analysis, was quantified as a 15-fold increase from the **S** to the **R** fruits. Harpin is a bacterial protein that can induce hypersensitive cell death and systemic acquired resistance during the non-host interactions [71]. Since many bacterial genes were found during the cDNA AFLP analysis, it could be interesting to investigate whether the interaction with a bacteria could determine the onset of resistance in the peach fruits.

Calmodulin and calcium metabolism

Variation of the cytosolic free Ca^{2+} concentration is a well known early component of signal transduction pathways involved in plant-pathogen inter-

actions. Host cells respond to pathogen attack through a rapidly increase in cytosolic Ca^{2+} concentration, which in turn initiates a cascade of reactions that leads to the defence responses. Several genes belonging to these signal transduction pathways were found differently expressed between **S** and **R** fruits. For **R** fruits, the microarray and the cDNA AFLP analysis revealed up-regulation of a *calmodulin 1* gene and an *annexin* gene. Consistently, a gene coding for a calmodulin binding protein and a cyclic nucleotide gated channel C (CNGC-c) were found up-regulated in **RI**. Annexin and CNGC are two membrane proteins, involved in Ca^{2+} uptake into plant cells [72]; calmodulin is a small Ca^{2+} binding protein that, through the interaction with other proteins (calmodulin binding proteins) allows the cellular perception and the transduction of the Ca^{2+} signal [73]. Increase in intracellular calcium is associated with apoptosis both during normal development and in hypersensitive responses to pathogens [74]. Therefore, the higher level of genes involved in the Ca^{2+} signal in **R** and in **RI** fruits, could indicated a possible association of this pathway with the resistance to *Monilinia* rot of the same fruits 4.5.

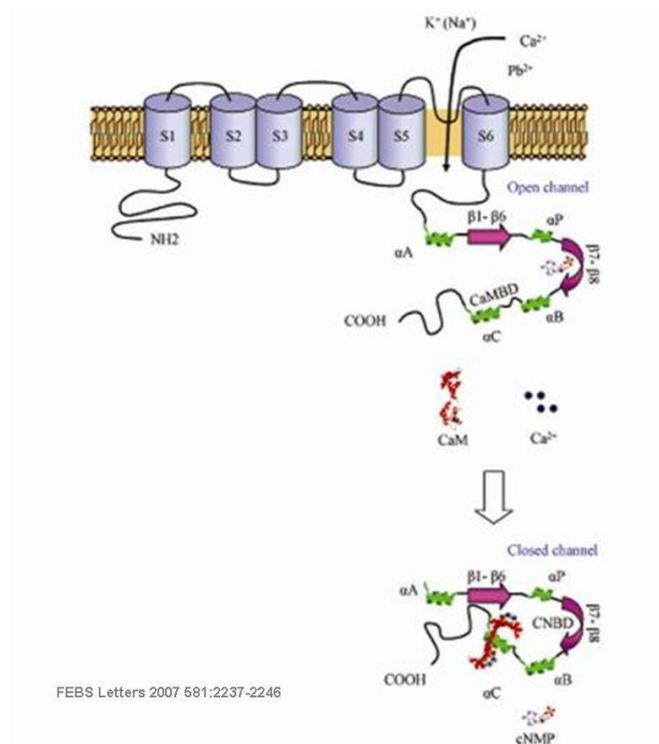


Figure 4.5: Calcium up-take and pathway.

Ferritin and fe homeostasis

In microarray analysis, *ferritin 1* gene was found down regulated in **R** fruits. This data was confirmed by real time PCR analysis. It is known that iron homeostasis is correlated with fungi pathogenesis, in particular Bostok et al.[1] referred that cutinase production in *M. fructicola* and iron availability are inversely related. The presence of high level of ferritin in the peach **S** fruits could be associated to a lower level of extracellular free iron, which lead to the activation of *Monilinia* lytic enzyme.

Iron is an essential element for most organism, however it could be deleterious toward DNA, lipids and protein, by generating free radicals in the presence of oxygen. Ferritin proteins prevent the damages holding iron in a non-reactive form in the cell. It has been showed that ferritin gene expression is transcriptionally regulated in response to oxidative stress and to microbial invasion. For example it accumulated during infection in *Arabidopsis*, suggesting an action in the basal defence mechanism [75].

Contrary to microarray, cDNA AFLP analysis revealed differences in gene expression between **H** and **I** fruits. These would be important for the early steps of interactions. TDF specifically present and missing only in the **SI** fruits (**SI+** and **SI-**, respectively) were selected as indicative of the compatible infection. Conversely, TDF specifically present and missing only in the **RI** fruits (**RI+** and **RI-**, respectively) were considered relevant for the incompatible interaction.

4.2.2 Interactions in the susceptible fruits

Early steps in fungal infection are the adhesion to the plant surface, the germination and the differentiation of penetration structure, such as the appressorium. Afterwards colonization can start. The appressoria formation

takes place at suited places in the plant surface [76]; pathogens have to overcome the host protecting coats (waxes and cutin), using either physical forces or lytic enzymes. On the host side, the pathogen attempt leads to cellular rearrangements, such as cytoskeleton reorganization and organelle movement to the entry site of the pathogen, which results in polarization of the host cell. Furthermore, reinforcement of the cell wall and accumulation of specific proteins at the pathogen entry site can occur. In a compatible interaction the pathogen overcomes or suppress this attempt of defence. After cell wall overcome, ROS generation is a frequent plant response to pathogen attack, however, the role of ROS in mediating compatibility or resistance is controversial [77]. In fact it appears that hemi-biotrophic pathogens have evolved mechanism to cope with the oxidative stress caused by the host. In non-host interactions, if the non-adapted pathogen penetrates the cell wall, the host cell responds with the hypersensitive response and cell death. In compatible interactions, pathogens copes with the all the possible species-specific defences raised at the plant cell periphery.

From this point of view the genes with a defined role in plant defence, which were found up-regulated in the **SI** fruits, could be associated to an attempt of the host cell to protect itself from the pathogen attack.

Three **SI+** TDFs, were found to encode for proteins involved in the ubiquitination process. Proteolysis and ubiquitin- dependent lysis, play an important role in plant defence, as reported above. Furthermore, cDNA AFLP showed up-regulation of a DNAJ heat shock protein (C85, fig.3.14) in **SI+**. Heat shock proteins function as folding helper of other proteins and their expression is associated to stress response. Therefore, DNAJ induction in **SI+**, could also indicate an attempt of the host to respond to the pathogen attack.

Two homologous TDFs to transcription factors (C92, T30, fig.3.14) were also found up-regulated specifically in **SI+** fruits. They encode for proteins homolog to *A. thaliana*, whose function is not yet known. They should be investigated anyway, as interesting candidates for proteins with important functions in defence to *M.laxa* infection.

Among the **SI+** TDFs, a catalase gene was also found; catalase is implicated in the hydrogen peroxide scavenging. H_2O_2 and the other reactive oxygen intermediates accumulate in plant cell upon a pathogen attack. Induction of catalase gene in susceptible peach fruits could be explained as standard cell defence mechanism in plants which raises upon a stress and the associated H_2O_2 production [78].

4.2.3 Interactions in the resistant fruits

The genes found up-regulated only in the resistant inoculated **RI+** fruits were selected as significant of those molecular mechanisms induced by **M.laxa**, during the early phase of the interaction, mechanisms which would then establish the resistant phenotype.

With respect to the energy metabolism, cDNA AFLP analysis revealed four TDFs (C150, C152, C168, T143,fig.3.14) up-regulated in the **RI** fruits. These encode for proteins involved in either in mitochondria or chloroplast electron transport. Up-regulation of the energy pathway is known to be a host response to stress, often leading to an increase of oxygen reactive intermediates during the onset of the hypersensitive response. Consistent to this, no TDF functionally belonging to the energy metabolism class, was found up-regulated in the **SI** fruits. [44].

Among those TDFs functionally related to transcription, cDNA AFLP revealed the up-regulation of a gene named Argonaute (AGO) in **RI** fruits.

This gene encode for a protein associated with the small non-coding RNAs, such as small interference RNA sirna interference -RNAi- and microRNA -miRNA-) (fig. 4.6). In these process,the small non coding RNAs specifically silence the expression of specific genes through degradation of their homologous cognate mRNA. The role of AGO proteins is to bind the small RNA and cleave the cognate RNA [79](fig. 4.7). AGO proteins were initially functionally associated with *Arabidopsis* embryogenesis and witu leaf development [80, 81]. Recently, it was shown that they have important role in several processes associated with plant growth and development,such as the maintenance of genome integrity, the control of protein synthesis and of RNA stability and the production of a specific set of small non coding RNA [82].

Similarly to **SI+**, several TDFs functionally associated with ubiquitination and protein folding were found up-regulated in **RI**. Among these there are a small heat shock protein (C126), an armadillo protein (C128), a ubiquitin specific protease (C169) and a chaperonin CPn60 (T159). Armadillo encodes for a U-box/ARM-repeat-containing protein. These were found induced by elicitors in plants and appeared to participate in plant defence response in both basal and resistance-mediated pathways. As previously described, heat shock proteins and chaperonins are involved in protein modification processes; these proteins can have an important part in plant defense responses, by marking substrates and building specific targets in signal transduction pathways, upon pathogen attack.

Among the TDFs encoding for cellular transport genes, two ABC (ATP binding transport) like proteins (C118, C136) were found respectively down-regulated and up-regulated in **RI** samples. ABC transporters are transmembrane proteins with a cytosolic domain, involved in the ATP binding and

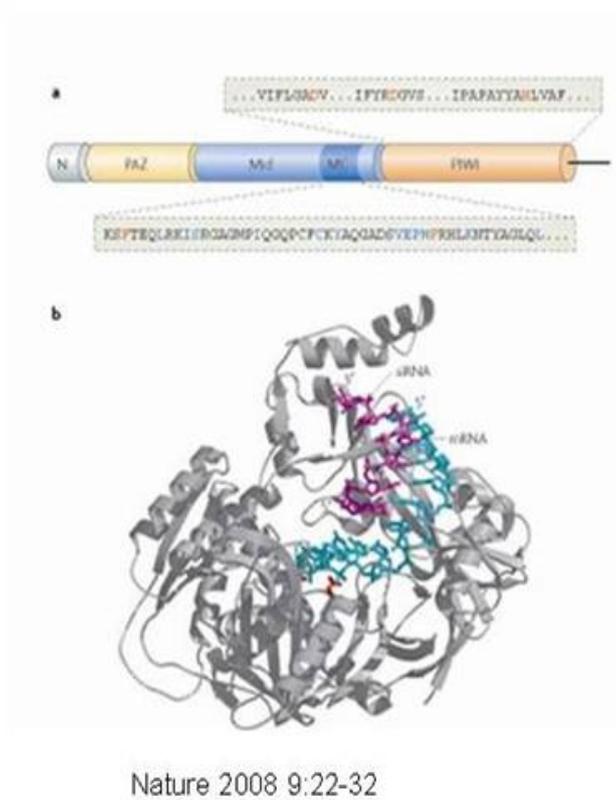


Figure 4.6: Structural features of argonaute proteins.

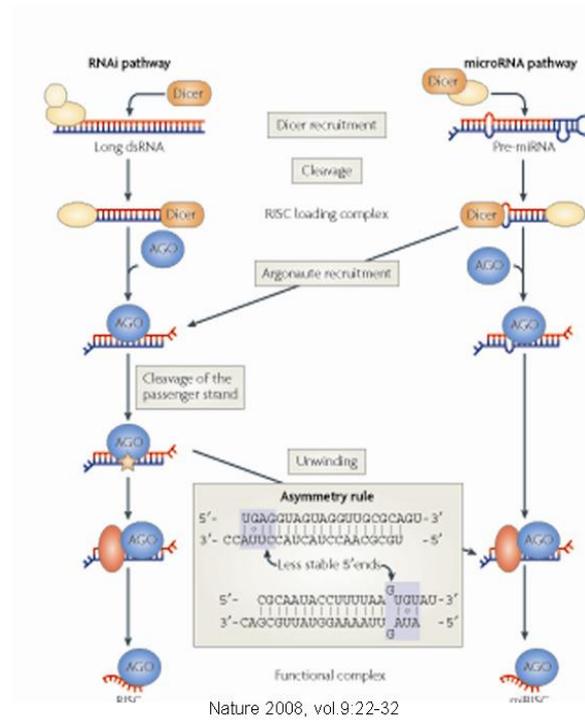


Figure 4.7: Assembly of the argonaute-small RNA complex.

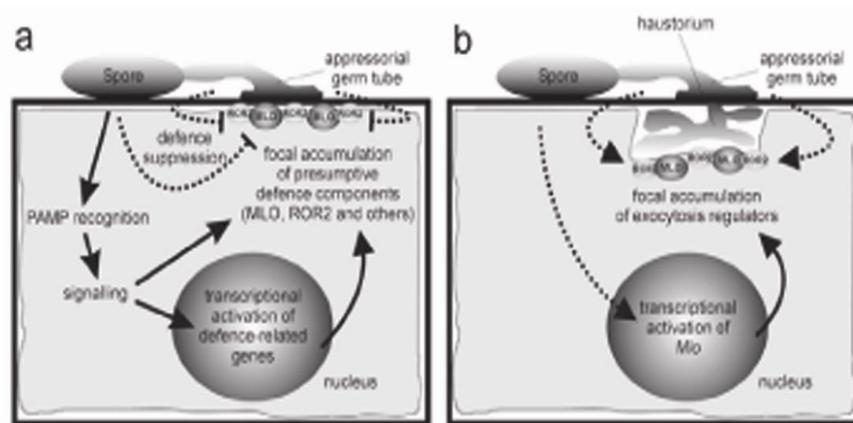
hydrolysis. They are known to be involved in the transport of several substances, including secondary metabolites that act as defence compounds [83]. As an example the ABC transporter AtPDR12 of *A. thaliana* gene expression was induced after inoculation with compatible and incompatible pathogens and after exposure to some stress factors, such as salicylic acid, ethylene and methyl-jasmonate. These data suggested an involvement of ABC transporters in plant defence [84]

A phospholipase C (C149) was up-regulated in the infected resistant fruits. In plants, phospholipase C produces phosphatidic acid (PA), a second messenger involved in the activation of NADPH oxidase complex, MAP kinase cascade and ion channel leading to the oxidative burst [85].

Among the TDFs up-regulated in RI samples, two fragments are known to have a direct relationship with stress and defence: C164, encoding for a universal stress protein and C177, encoding for an MLO transmembrane protein. The universal stress protein domain binds ATP and it is believed to be involved in signal transduction. Its function has been well studied in prokaryotic systems, such as *E. coli*, where it mediates survival of cells starved for a wide variety of nutrients, or exposed to toxic chemicals, or osmotic stress or UV light damage. Recently, this domain was also found as the N-term domain of a receptor-like cytoplasmic kinases of *Arabidopsis* (RLCK class VI)[86]. However the precise biological function of the bacterial UspA protein and the UspA domain of eukaryotic proteins is not known.

MLO transmembrane protein is a well studied component of the plant innate immunity response. It is believed that MLO is necessary for building of the plant delivery system for toxic cargo at the infection site. Consistently to this MLO is accumulated on the plasma membrane at the pathogen penetration site. However recent data have reported on the importance of MLO for

successful compatible interaction. In the compatible powdery mildew-barley is required for successful penetration of the host cell wall by the powdery mildew ascomycota: barley lacking functional MLO are resistant against the pathogen. This suggests a sort of exploitation mechanism of the MLO system by the pathogen for its successful penetration 4.8. Barley MLO is an integral plasma membrane-localized protein, possessing seven hydrophobic membrane-spanning helices [87]. The cytoplasmic C-terminus harbours an amphiphilic α -helix that serves as a calmodulin binding domain Calcium-dependent calmodulin binding to this peptide domain preliminary evidence suggests that MLO may form homooligomers and requires interplay of cytoplasmic domains for functionality. Recently, a gene Ror2 (required for mlo resistance 2) that, when mutated, suppresses mlo-mediated resistance was found to encode a plasma membrane-resident syntaxin, which in our cDNA AFLP analysis also appeared as differentially expressed gene in R fruits (RH +RI)[88].



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Figure 4.8: Model of assumed MLO corruption by the powdery mildew fungus in barley.

4.3 Pru P1 and Pru P2 expression and activity test

Among the sequences found up-regulated in the **R** fruits, two genes, Pru P1 and Pru P2, having high similarity to members of the PR10 proteins, were isolated. Interestingly, during the development of the μ PEACH1.0 microarray [20, ?], PRU P2 was classified as the most represented EST (5% of the total EST number) found in peach fruit. PR-10 proteins are known to be pollen or food allergens. For several PR10 protein the biological function is still yet unknown, however, many of them have shown to be involved in activities related to disease resistance and for this reason they have been included in the PR protein super family. Members of PR10 were found to be developmentally regulated in different plant tissue [89]. Particularly, expression of the *M. domestica* Mal-d1 (major allergen) was found ripening differentially regulated during fruit growth [90]. With respect to this, it is interesting to note that Pru P2 as been found to be the most represented transcript in peach fruits of the S3 - S4 growth phase, the phased in which ethylene accumulation occurs [20]. PR-10 proteins were also shown to be induced upon pathogen infection [36]; The induction of a wide range of PR-10 proteins was characterized in various plant species in response to viruses, bacteria and fungi [89].

As the Pr-10 proteins are widely spread in plants and a putative role in defence, biological characterization of these kind of proteins has a great importance for their potential application in a biomedical field as well as in plant protection. Peach PR10 were first classified as storage proteins, found in large amount in bark, xylem and root of peach tree during the dormancy period, suggesting a role in defence against pathogens and abiotic stress [91].

Similar features were found for another peach PR protein, defensin [66], which were also found differentially regulated in R vs S fruit in our study.

Sequences analysis revealed that Pru P1 and Pru P2 protein sequences share 98% of similarity to the cherry major allergen (*P. avium*), 88% to apple Mal d1 protein and 76% to birch Bet v1, which is the first plant PR-10 to be characterized [92] (fig.4.9). However, differently from the other pr protein, PRU P1 and 2, like the PR-10 proteins, do not contain a signal peptide sequence, . This suggests that they are intracellular proteins, in contrast to most of other PR proteins which are generally extracellular.

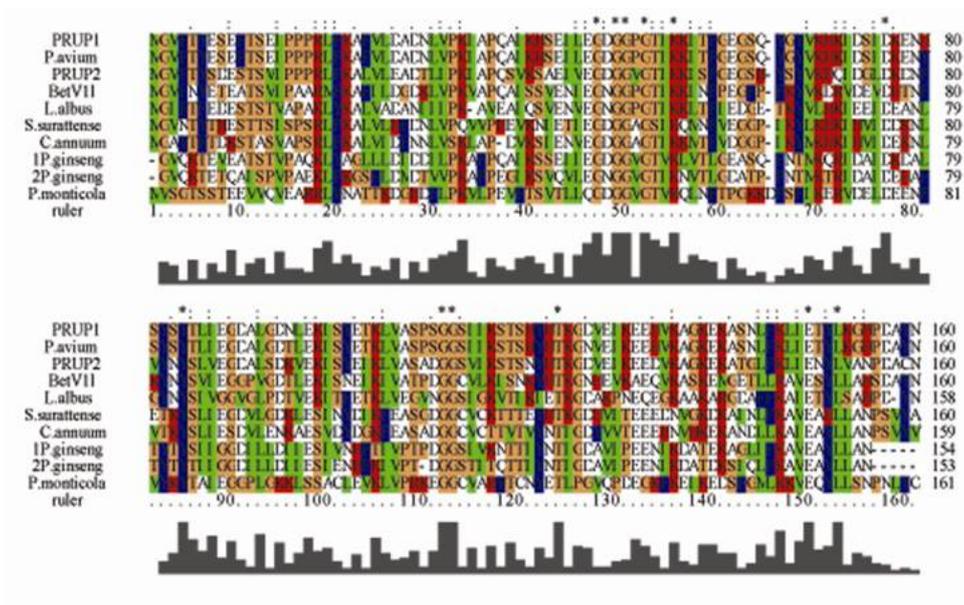


Figure 4.9: Multiple sequences alignment of PR 10 protein from different plant species.

Despite the relatively low sequence similarity, PR10 share a common tertiary structure as shown by the NMR and X-ray structure of Betv1 and Pru av1. Interestingly, the protein structure revealed the presence of a P-loop motive (GXGGXG), which is well conserved even in the peach allergens and

in other PR-10 proteins. This P-loop motif is typical of nucleotide binding proteins functioning as nucleotide binding site in enzymes such as kinases or RNases [93]. Consistently, a ribonuclease activity has been shown for several PR-10 proteins, such as the ginseng [94], the birch bet v1 [95], the white lupin [96], the hot pepper [97] and the yellow-fruit nightshade [98]. In this study the ribonuclease activity of the recombinant Pru P1 and Pru P2 proteins was assayed against peach total RNA. Both proteins displayed RNase activity, despite Pru P2 with higher efficiency. It was suggested that the ribonucleolytic activity of PR-10 proteins could be important for the host cells to contrast pathogen penetration [89]. The up-regulation of peach Pru proteins in **R** fruits could then be determinant in the decrease of susceptibility.

Various PR-10 proteins were also found to have a direct antimicrobial activity against fungal pathogens. Ocatin, a PR-10 protein from *O. tuberosa*, has direct antifungal effect against *Nectria hematococcus* and *Fusarium oxysporum*[99]; the SsPR10 protein of *Solanum surattense* inhibits the growth of *Pycularia oryzae*, but did not have effects on the growth of *S. sclerotium* and *Gibberella zeae*[98]. Pru P1 and Pru P2 recombinant protein were tested for inhibition of hyphal growth of *M. laxa*, *M. fructigena*, *B. cinerea*, *C. acutatum*, *Rhizopus* and *P. expansum*, however any inhibition was observed. This is the first characterization of the biological function of PR-10 proteins in peach. The peach Pru P1 and P2 displayed RNase activity, but with different efficiency; after 2 hours of incubation, Pru P1 partially degraded peach RNA, whereas Pru P2 showed complete degradation. Recent data on other PR-10, suggest that the RNase function is not the primary function of PR-10 [89]. Several PR10 homologs, in fact, were shown to bind a large number of physiological ligands, such as cytokinins, fatty acids, flavonoids and brassinosteroids. Pru av 1, a PR 10 cherry allergen, which share strong

sequence similarity to the peach Pru P proteins, is able to bind phytosteroid and brassinosteroid [100]. Brassinosteroids have important functions in the regulation of plant growth and development and they have been found to induce disease resistance in plants [89]. PR-10 proteins interacting with the brassinolide may have an important role in the steroid hormone-mediate resistance. Whether peach Pru P1 and 2 also have binding activity is not yet known and must be investigated in order to define their role in pathogenesis.

Bibliography

- [1] R. M. Bostock, S. M. Wilcox, G. Wang and J. E. Adaskkaveg, 1999. Suppression of *Monilinia fructicola* cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology* 54:37-50
- [2] R. J. W. Byrde and H.J. Willetts, 1977. *The Brown Rot Fungi of Fruit. Their Biology and Control*. Pergamon Press, Oxford.
- [3] M. Mari, L. Casalini, E. Baraldi, P. Bertlini and G. C. Pratella, 2003. Susceptibility of apricot and peach fruit to *Monilinia laxa*. *Postharvest Biology and Technology* 30:105-109. during phenological stages
- [4] P. H. Fourie and G. Holz, 2003. Germination of dry, airborne conidia of *Monilinia laxa* and disease expression on nectarine fruit. *Australasian Plant pathology* 32:9-18
- [5] J. Northover and A. R. Biggs, 1990. Susceptibility of immature and mature sweet and sour cherries to *Monilinia fructicola*. *Plant Disease* 74:280-284.
- [6] R. J. W. Byrde, A. H. Fielding and A. H. Williams, 1960. The role of oxidized polyphenols in the varietal resistance of apples to brown

- rot. In: J. B. Pridham ed. Phenolics in plants in health and disease. Pergamon Press, London 95-99.
- [7] L. R. Beuchat and D. A. Golden , 1989. Antimicrobials occurring naturally in foods. *Food technology* 43:134-142.
- [8] A. Aharoni and O. Vorst, 2001. DNA microarray for functional plant genomics. *Plant Molecular biology* 48:99/118.
- [9] R. A. Dixon, L. Achnine, P. Kota, C. J. Liu, M. S. Srinivasa and L. Wang, 2002. The phenylpropanoid pathway and plant defence- a genomics perspective. *Molecular Plant Pathology* 3:371-390.
- [10] K. M. Davies and K. E. Schwinn, 2003. Transcriptional regulation of secondary metabolism. *Functional Plant Biology* 30:913-925.
- [11] J. Shah, 2005. Lipids, lipases and lipid modifying enzymes in plant disease resistance. *Annual review phytopathology* 43:8.1-8.32.
- [12] R. Alba, Z. Fei, P. Payton, Y. Liu, S. L. Moore, J. Jovannoni *et. al* , 2004. ESTs, cDNA microarrays, and gene expression profiling:tools for dissecting plant physiology and development. *Plant Physiology* 39:697-714.
- [13] K. Malek, A. Levine, T. Eulgem, A. Morgan, J. Schimd, K.A. Lawton, J. L. Dangl, R. A. Dietrich, 2000. The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nature Genetics* 26:403-410.
- [14] P. reymond, H. Weber, M. Damond, E.E. Farmer, 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant cell* 12:707-720.

- [15] P. M. Schenk, K. Kazan, I. Wilson, J.P. Anderson, T. Richmond, S. C. Somerville and J. M. Manners, 2000. Coordinated plant defense responses in arabidopsis revealed by microarray analysis. PNAS 97:11655-11660.
- [16] A. Aharoni and A. O Connell, 2002. Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. Journal of experimental botany 53:2073-2087.
- [17] S. Fonseca, L. Hackelr, A. Zvara, S. Ferreira, A. Balde, D. Dudits, M. S. pais and L. G. Puskas, 2005. Monitoring gene expression along pear fruit development, ripening and senescence using cDNA microarrays. Plant science 167:457-469.
- [18] T. Shimada, H. Fuiii, T. Endo, J. Yakazi, N. Kishimoto, K. Shimbo, S. Kikuchi and M. Omura, 2005. Toward comprehensive expression profiling by microarray analysis in citrus: monitoring the expression profiles of 2213 genes during fruit development. Plant science 168:1383-1385.
- [19] D. L. E. Waters, T. A. Holton, E. M. Ablett, L. S. Lee and R. J. Henry, 2005. cDNA microarrays analysis of developing grape(*Vitis vinifera* cv shiraz) berry skin. Functional and integrative genomics 5:40-58.
- [20] L. Trainotti, C. Boghi, F. Ziliotto, D. Zanin, A. RAsori, G. Casadoro, A. Ramina and P. Tonutti, 2006. The use of microarray μ PEACH1.0 to investigate transcriptome changes during transition from pre-climacteric to climacteric phase in peach fruit. Plant Science 170: 606-613.

- [21] W. E. Durrant, O. Rowland, P. Piedras, K. E. Hammond-Kosack and J. D.G. Jones, 2000. cDNA-AFLP Reveals a Striking Overlap in Race-Specific Resistance and Wound Response Gene Expression Profiles. *The Plant cell* 12:963/977.
- [22] T. Qubbaj, A. Reineke and C. P. W. Zebitz, 2004. Molecular interactions between rosy apple aphids, *Dysaphis plantaginea*, and resistant and susceptible cultivars of its primary host *Malus domestica*. *Entomologia experimentalis et applicata* 115:145-152.
- [23] V. Schurdi-Levraud Escalettes, C. Hullot, D. Wawrzynczak, E. Mathieu, J-P. Eyquard, O. Le Gall and V. Decroocq, 2006. *Plum pox virus* induces differential gene expression in the partially resistant stone fruit tree *Prunus armeniaca* cv. Goldrich. *Gene* 374:96-103.
- [24] B. R. Sarosh and J. Meijer, 2007. Transcriptional profiling by cDNA-AFLP reveals novel insights during methyl jasmonate, wounding and insect attack in *Brassica napus*. *Plant molecular biology* 64:425-438.
- [25] J. G de Diego, F. D. Rodriguez, J. L. R. Lorenzo, P. Grappin and E. Cerventes, 2005. cDNA-AFLP analysis of seed germination in *Arabidopsis thaliana* identifies transposons and new genomic sequences. *Journal of plant pathology* 163:452-462.
- [26] J. Bove, P. Lucas, B. Godin, L. Oge, M. Jullien and P. Grappin, 2005. Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant molecular biology* 57:593-612.

- [27] Breyne P., Dreesen R., 2003. Quantitative cDNA-AFLP analysis for genome-wide expression studies. *Molecular and Genetics Genomics*. 269: 173-179
- [28] C. W. B. Bachem, R.S. van der Hoeven, S. M. de Bruijn, D. Vreugdenhil, M. Zabeau and R. G. F. Visser, 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. *The plant journal* 9:745-753.
- [29] J. Peranen, M. Rikonen, M. Hyvonen and L. Kaarlainen, 1996. T7 vectors with a modified T7lac promoter for expression of protein in *Escherichia coli*. *Analytical biochemistry* 236:371-373.
- [30] E. F. Fritsch, J. Sambrook and T. Maniatis, 1989. *Molecular cloning*.
- [31] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Willer and D. J. Lipman, 1997. Gapped BLAST and PSI-BLAST a new generation of protein database search program. *Nucleic acids research* 24:3389-3402.
- [32] Estree Consortium, Development of an oligo-based microarray (μ PEACH1.0) for genomics studies in peach fruit, *Acta Horticulturae* 682 (2005) 263 - 268.
- [33] P. Vos, R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijeters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau, 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic acid research* 23:4407-4414.
- [34] *Current protocols in molecular biology* Ausubel et al. ed. Wiley.

-
- [35] G. A. Churchill, 2002. Fundamentals of experiment design for cDNA microarray. *Nature genetics* 32:490-495.
- [36] H. Puhringer, D. Moll, K. Hoffmann-Sommergruber, B. watillon, H. Katinger and M. Laimer da Camara Machado, 2000. The promoter of an apple Ypr10 gene, encoding the major allergen Mal d 1, is stress- and pathogen-inducible. *Plant science* 152:35-50.
- [37] V. Balbi and A. Devoto, 2007. Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. *New Phytologist* 177:301-318.
- [38] D. R. Walters, 2000. Polyamines in plant-microbe interactions. *Physiological and Molecular Plant Pathology* 57:137-146
- [39] D. R. Walters, 2003. Polyamines and plant disease. *Phytochemistry* 64:97-107.
- [40] V. Ziosi, S. Scaramagli, A.M. Bregoli, S. Biondi and P. Torregiani, 2003. Peach (*Prunus persica* L.) fruit growth and ripening: transcript levels and activity of polyamine biosynthetic enzymes in the mesocarp. *Journal of plant physiology* 160:1109-1115.
- [41] S. Kopriva, 2006. Regulation of Sulfate Assimilation in *Arabidopsis* and Beyond. *Annals of Botany* 97:479-495.
- [42] F. B. Abeles and C. L. Biles, 1991. Characterization of Peroxidases in Lignifying Peach Fruit Endocarp. *Plant physiology* 95: 269-273.
- [43] D. Walters, A. Newton and G. D. Lyon, 2007. *Induced Resistance for Plant Defence: A Sustainable Approach*. Blackwell Publishing.

- [44] D. P. Maxwell, R. Nickels and L. McIntosh, 2002. Evidence of mitochondrial involvement in the transduction of signals required for the induction of genes associated with pathogen attack and senescence. *The Plant Journal* 29(3): 269-279.
- [45] G. Noctro, R. De Paere and C. H. Foyer, 2007. Mitochondrial redox biology and homeostasis in plants. *Trends in Plant Science* 12:125-134.
- [46] N. Gutterson and T. L. Reuber, 2004. Regulation of disease resistance pathways by AP2/ERF transcription factors. *Current opinion in plant biology* 7:465-471.
- [47] R. Takai, N. Matsuda, A. Nakano, K. Hasegawa and N. Shibuya et al. 2002. EL5, a rice N-acetylchitooligosaccharide elicitor-responsive RING-H2 finger protein, is a ubiquitin ligase which functions in vitro in co-operation with an elicitor-responsive ubiquitin-conjugating enzyme, OsUBC5b. *Plant Journal* 30:447-455.
- [48] A. Levine, R. Tenhaken, R. Dixon and C. Lamb, 1994. H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79:583-593.
- [49] K. Dreher and J. Callis, 2007. Ubiquitin, hormones and biotic stress in plants. *Annals of Botany* 99:787-822.
- [50] S. Robatzek, 2007. Vesicle trafficking in plant immune responses. *Cellular microbiology* 9:1-8.
- [51] E. Schemlzer, 2002. Cell polarization, a crucial process in fungal defence. *Trends in plant science* 7:411-415.

- [52] M. Ron and A. Avni, 2004. The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant cell* 16:1604-1615.
- [53] L. M. Kawchuk, J. Hachey, D.R. Lynch, F. Kulcsar, G. van Rooijen and D. R. Waterer, 2001. Tomato Ve disease resistance genes encode cell surface-like receptor. *PNAS* 98:6511-6515.
- [54] N. C. Collins, H. Thordal-Christensen, V. Lipka, S. Bau E. Kombrick, J. L. Qiu, 2003. SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425:973-977.
- [55] A. Driouich, A. Jauneau and L. A. Staehelin, 1997. 7-dehydrobrefeldin A, a naturally occurring brefeldin A derivative, inhibits secretory transport and causes a cis-to-trans breakdown of Golgi stack in plant cell. *Plant physiology* 113:487-492.
- [56] A.H. West and A.M. Stock, 2001. Histidine kinases and response regulator proteins in two-component signaling systems. *Trends in Biochemical Science* 26:369-376.
- [57] B. Kobe and A. v. Kajava, 2001. The leucine-rich repeats as a protein recognition motif. *Current opinion in structural Biology* 11:725-732.
- [58] J. M. McDowell and S. A. Simon, 2006. Recent insights into R gene evolution. *Molecular Plant Pathology* 7:437-448.
- [59] T. Montoya, T. Nomura, K. Farrar, T. Kaneta, T. Yokota and G. J. Bishop, 2002. Cloning the Tomato Curl3 Gene Highlights the Putative Dual Role of the Leucine-Rich Repeat Receptor Kinase tBRI1/SR160 in Plant Steroid Hormone and Peptide Hormone Signaling. *The Plant cell* 14:3163-3176.

- [60] S. E. Clark, R. W. Williams and E. M. Meyerowitz, 1997. The *CLAVATA1* Gene Encodes a Putative Receptor Kinase That Controls Shoot and Floral Meristem Size in Arabidopsis. *Cell* 89:575-585.
- [61] E. D. Shpak, C. T. Berthiaume, E.J. Hill and K. U. Torii, 2003. Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by promoting cell proliferation. *Development* 131:1491-1501.
- [62] C. He, S.H. Fong, D. Yang, G. L. Wang, 1999. BMWMK1 a novel map kinase induce by fungal infection and mechanical wounding in rice. *Molecular Plant Microbe Interactions* 12:1064-1073.
- [63] H. Nakagami, A. Pitzschke and H. Hirt, 2005. Emerging MAP kinase pathways in plant stress signaling. *Trends in Plant Science* 10:339-346.
- [64] C. P. Selitrennikoff, 2001. Antifungal proteins. *Applied and Environmental Microbiology* 67:2883-2894.
- [65] B. P.H. J. Thomma, B. P. A. Cammue and K. Thevissen, 2002. Plant defensins 216:193-202.
- [66] M.E. Wisniewski, C. L. Bassett, T. S. Artlip, R. P. Webb, W. J. Janisiewicz, J. L. Norelli, M. Goldway and S. Droby, 2003. Characterization of a defensin in bark and fruit tissues of peach and antimicrobial activity of a recombinant defensin in the yeast, *Pichia pastoris* *Physiologia Plantarum* 11:563-572.
- [67] J. Thimmapuram, T. S. Ko, S. S. Korban, 2001. Characterization and expression of beta-1,3-glucanase in peach. *Molecular genetics and genomics* 265:469-479.

- [68] A. B. zemanek, T. S. Ko, J. Thimmapuram, F. A. Hammerschlang, S. S. Korban, 2002. Changes in beta-1,3-glucanase mRNA levels in peach in response to treatment with pathogen culture filtrates, wounding, and other elicitors. *Journal of Plant Physiology*, 159:877-889.
- [69] T. S. Ko, S. Lee, S. C. Schaefer and S. S. korban, 2003. Characterization of a tissue-specific and developmentally regulated beta-1,3-glucanase gene family in *Prunus persica*. *Plant Physiology and Biochemistry* 41:955-963.
- [70] S. P. Robinson, A. K. Jacobs and I. B. Dry, 1996. A class IV chitinase is highly expressed in grape berries during ripening. *Plant Physiology* 11:771-778.
- [71] J. L. Peng, H.S. Dong, H. P. Dong, T.P. Delaney, J. M. Bonasera and S. V. Beer, 2003. Harpin-elicited hypersensitive cell death and pathogen resistance require the NDR1 and EDS1 genes. *Physiological and Molecular Plant Pathology* 62:317-326.
- [72] P. J. White, H. C. Bowen, V. Demidchik, C. Nichols and J. M. Davies, 2002. Genes for calcium-permeable channels in the plasma membrane of plant root cells. *Biochimica et biophysica acta* 1564:299-309.
- [73] P. J. white and M. R, Broadley, 2003. Calcium in plants. *Annals of botany* 92:487-511.
- [74] A. Levine, R.I. Pennell, M.E. Alvarez, R. Palmer and C. Lamb, 1996. Calcium mediated apoptosis in a plant hypersensitive diseases reistance response. *Current Biology* 6:427-437.

- [75] A. Dellagi, M. Rigault, D. Segond, C. Roux, Y. Kraepiel, F. cellier, J. Briat, F. Gaymard and D. Expert, 2005. Siderophore-mediated upregulation of *Arabidopsis* ferritin. *The plant journal* 43: 262-272.
- [76] N. D. Read, L. J. Kellock, T. J. Collins, A. M. Gundlach, 1997. Role of topography sensing for infection structure differentiation in cereal rust fungi. *Planta* 202: 163-170.
- [77] R. J. O Connell and R. Panstruga, 2006. Tête à tête inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. *New Phytologist* 171 : 699-718.
- [78] R. Hckelhoven and K. H. Kogel, 2003. Reactive oxygen intermediates in plant - microbe interactions: Who is who in powdery mildew resistance? *Planta* 216: 891 . 902.
- [79] G. Hutvagner and M. J. Simard, 2008. Argonaute proteins: key players in RNA silencing. *Nature* 9:22-32.
- [80] B. Moussian, H. Schoof, A. Haecker, G. Jurgens and T. Laux, 1998. Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. *EMBO Journal* 17: 1799-1809.
- [81] K. Bohmert, 1998. AGO1 defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO Journal* 17: 170-180.
- [82] M. W. Jones-Rhoades, D. P. Bartel and B. Bartel, 2006. MicroRNAs and Their Regulatory Roles in Plants. *Annual review in plant biology* 57:19-53.

- [83] K. Yazaki, 2006. ABC transporter involved in the transport of plant secondary metabolites. *FEBS Letters* 580: 1183-1191.
- [84] E. J. Campbell, P. M. Schenk, L. Kazan, I. Pennincks, J.P. Anderson, D. J. Maclean, B. P. cammue, P. R. Ebert and J. M. Manners, 2003. Pathogen-Responsive Expression of a Putative ATP-Binding Cassette Transporter Gene Conferring Resistance to the Diterpenoid Sclareol Is Regulated by Multiple Defense Signaling Pathways in Arabidopsis. *Plant Physiology* 133:1-13.
- [85] A. M. Laxalt and T. Munnik, 2002. Phospholipid signalling in plant defence. *Current opinion in plant biology* 5: 1-7.
- [86] M. E. Jurca, S. Bottka and A. Feher, 2007. Characterization of a family of Arabidopsis receptor-like cytoplasmic kinases (RLCK class VI). *Plant cell reporter* .
- [87] R. Panstruga, 2005. Serpentine plant MLO proteins as entry portals for powdery mildew fungi. *Biochem Soc Trans* 33: 389-392
- [88] A. Devoto, P. Piffanelli, I. Nilsson, E. Wallin, R. Panstruga, G. von Heijne and P. Schulze-Lefert 1999. Topology, subcellular localization, and sequence diversity of the Mlo family in plants. *Journal of Biological Chemistry* 274: 34993-35004.
- [89] J-J. Liu and A. K. M. Ekramoddoullah, 2006. The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. *Physiological and Molecular Plant Pathology* 68:3-13.
- [90] R.G. Atkinson, J. Perry, T. Matsui, G. S. Ross, E.A. Macrea, 1996. A stress-, pathogenesis-, and allergen-related cDNA in apple fruit is also

- ripening-related. New Zealand Journal of Crop Horticulturae Science 24:103-107.
- [91] M. Wisniewski, C. Bassett and R. Arora, 2004. Distribution and partial characterization of seasonally expressed proteins in different aged shoots and roots of Loring peach (*Prunus persica*). Tree physiology 24:339-345.
- [92] I. Swoboda, A. Jilek, F. Ferreira, K. Hoffmann-Sommergruber, O. Scheiner, D. Kraft, et al, 1995. Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. Journal of biological chemistry 270:2607-2613.
- [93] Saraste, 1990. The P-loop a common motif in ATP- and GTP-binding protein. Trends in Biochemical Sciences. 15:430-434.
- [94] G. P. Moiseyev, L. I. Fedoreyeva, Y.N. Zhuravlev, E. Yasnetskaya, P. A. Jekel and J. Beintema, 1997. Primary structures of two ribonucleases from ginseng calluses. FEBS letters 407:207-210.
- [95] A. Bufe, M. D. Spangfort, H. Kahlert, M. Schlaak, W. M. Becker, 1996. The major birch pollen allergen, Betv 1, shows ribonuclease activity. Planta 199:413-415.
- [96] B. Bantignies, J. Seguin, I. Muzac, F. Dedaldechamp, P. Gulick et al., 2000. Direct evidence for ribonucleolytic activity of a PR-10 like protein from white lupin root. Plant Molecular biology 42:871-881.
- [97] C. J. Park, K. J. Kim, R. Shin, J. M. Park, Y. C. Shin, K. H. Paek, 2004. Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant journal 37:186-198.

-
- [98] X. Liu, B. Huang, J. Lin, J. Fei, Z. Chen, Y. Pang, X. Sun and K. Tang, 2005. A novel pathogenesis-related protein (SsPR10) from *Solanum surattense* with ribonucleolytic and antimicrobial activity is stress- and pathogen-inducible. *Journal of plant physiology* 163:546-556.
- [99] T. Flores, A. Alape-Giron, M. Flores-Diaz and H. E. Flores, 2002. Ocatin. A Novel Tuber Storage Protein from the Andean Tuber Crop Oca with Antibacterial and Antifungal Activities. *Plant physiology* 128:1291-1302.
- [100] P. Neudecker, K. Scweimwe, J. Nerkamp, S. Scheurer, S. Vieths, H. Sticht and P. Rosch, 2001. Allergic cross-reactivity made visible.
- [101] T.Z. Berardini, S. Mundodi, R. Reiser, E. Huala, M. Garcia-Hernandez, P. Zhang, L.M. Mueller, J. Yoon, A. Doyle, G. Lander, N. Moseyko, D. Yoo, I. Xu, B. Zoeckler, M. Montoya, N. Miller, D. Weems, S.Y. Rhee, 2004. Functional annotation of the *Arabidopsis* genome using controlled vocabularies, *Plant Physiology* 135:745-755.