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TRANSCRIPTIONAL MODIFICATIONS IN BARLEY EMBRYOS CAUSED BY SEED-BORNE PATHOGEN FUNGI INFECTION

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a mio marito Thomas, ai miei genitori e alla mia Giulietta

INDEX

RIASSUNTO

Le piante nell'ambiente si confrontano costantemente con una grande varietà di potenziali patogeni quali, batteri, funghi, virus e nematodi. Ciò nonostante lo sviluppo della malattia è un'eccezione piuttosto che la regola, dovuto alla natura altamente efficiente del coordinamento delle difese attive e passive che si sono evolute nelle piante.

Una rapida e potente risposta di difesa che blocca l'infezione del patogeno è conosciuta come gene-for-gene resistenza. Tipicamente questa resistenza dipende dallo specifico riconoscimento da parte della pianta del patogeno tramite modificazioni della parete cellulare, sintesi di fitoalessine accumulo di proteine pathogenesis related spesso inibitori delle proteasi o enzimi idrolitici che hanno come bersaglio il patogeno.

In letteratura è noto che i tessuti di riserva dei semi dei cereali sono ricchi di inibitori e idrolisi antimicrobiche. Inoltre le piante di cereali e i loro semi si difendono dalle infezioni patogene tramite enzimi e peptidi antimicrobici.

In questo studio di ricerca è stata utilizzata *Pyrenophora graminea,* l'agente causale della striatura bruna dell'orzo e l'orzo (*Hordeum vulgare*), come modello per le malattie trasmesse tramite seme.

Pyrenophora graminea è un fungo ascomicete che si trasmette tramite seme. Le ife fungine infettano i germogli di orzo attraverso la coleorizza da questa poi il fungo cresce intercellularmente negli strati del parenchima del nodo scutellare fino al coleoptile. Nelle piante suscettibili la malattia risulta in un intensa riduzione della crescita, con morta completa e prematura e perdita di granella; nelle varietà di orzo resistente invece le ife sembrano degenerare a livello della parte basale della coleorizza e nel nodo scutellare come in una sorta di reazione ipersensibile.

Lo scopo di questo lavoro di ricerca è stato quello di studiare i cambiamenti trascrizionali che avvengono livello del seme per identificare i geni che vengono attivati a livello dei tessuti embrionali durante un processo di difesa.

I materiali genetici utilizzati sono stati: isolato Dg2 di *P. graminea* (precedentemente chiamato I2) il piu virulento di una collezione di 12 isolati monocodiali testati in cultivar europeee di orzo; Mirco una varietà polistica, a cariosside vestita altamente sucscettibile all'isolato Dg2 di *P. graminea*; Thibaut varietà polistica, a cariosside vestita altamente

resistente al fungo e le NIL near isogenic lines portanti la resistenza a *P. graminea* e create dall'incrocio tra cv Thibaut e la cv Mirco dopo sei generazione di incrocio.

Per lo studio sono state utilizzate tre differenti tecniche di analisi: 1) la creazione di una libreria di sottrazione a cDNA; 2) cDNA-AFLP; 3) analisi microscopiche di istologia.

Nel primo esperimento è stata costruita una libreria di sottrazione tramite il kit PCR Select (Clontech) da campioni di orzo di controllo e trattati.

Undici cloni dei 1800 trovati sono risultati realmente espressi in modo differenziale dopo essere stati sequenziali e analizzati in banche dati non ridondanti disponibili sul web (NCBI e TIGR).

Questi cloni sono stati analizzati tramite analisi Northern e quattro sono stati trovati essere coinvolti in un probabile ruolo di scavenging dei ROS.

Nel secondo esperimento la tecnica a cDNA-AFLP è stata eseguita presso il laboratorio del prof. Massimo Delledonne dell'Università di Verona. Per l'analisi sono state utilizzate le cultivar Thibaut e Mirco e il fungo *P. graminea*. Sono stati trovati circa 1100 frammenti putativamente espressi in modo differenziale; 600 di questi frammenti, dopo essere stati sequenziati, sono stati analizzati in banca dati e suddivisi in gruppi funzionali.

Nel terzo esperimento è stata condotta un'analisi istologica dei tessuti interessati dall'infezione tramite il microscopio a fluorescenza per conoscere il preciso processo d'infezione del fungo. E' stata osservata autofluorescenza sia nella cultivar Mirco infetta che nella NIL segno di un accumulo di polifenoli a livello della parete cellulare, confermato anche dal saggio istochimico blu di toluidina.

SUMMARY

Plants are constantly confrontated with a wide variety of potential pathogens within their environment including bacteria, fungi, viruses and nematodes. Nevertheless the development of disease is the exception rather than the rule, due to the highly efficient nature of the coordinated systems of passive and active defences that have evolved in plants. A host strong and rapid defense response that blocks infection of pathogen is known as gene-for-gene resistance typically depends on specific recognition of the invanding pathogen by the plant with inducible modification of plant cell walls, the synthesis of toxic phytoalexins, and accumulation of pathogenesis related proteins, often protease inhibitors or pathogen targeted hydrolytic enzymes.

In literature it is known that the storage tissues of the cereal seeds are rich in inhibitors and microbial hydrolases. Furthermore cereal plants and their seeds defend themselves against infection by fungal pathogen by enzyme and peptide antimicrobial. In this study we have used *Pyrenophora graminea,* the agent of barley leaf stripe and barley (*Hordeum vulgare*) pathosistem as a model for seed borne diseases.

Pyrenophora graminea is a seed-borne pathogen which infects the seedling thorugh the coleorhiza from where it grows intercellulary in the parenchyma layers of the scutellur node up to the coleoptile. In susceptible plants the disease usually results in severe stunting, premature death and complete loss of grain, in resistant barley varieties, hyphae seem to degenerate in the basal part of the coleorhiza and in the scutellar node, as in a sort of hypersensitive reaction.

The aim of this work was to study early transcriptional changes which occur into the embryos in order to identify the genes which are actived these tissues during a resistant and susceptible defence reaction.

The plant genetics materials used in this work were: *P. graminea* isolate Dg2 (previously named I2), the most virulent of a collection of 12 monoconidial isolates tested on European barley cvs; Mirco a six-rowed hulled caryopsis winter cultivar highly susceptible to *P. graminea* isolate Dg2; Thibaut a six-rowed hulled caryopsis winter cultivar highly resistant

to *P. graminea* isolate Dg2; Near-isogenic lines (NILs) carrying resistance against the isolate Dg2 of *P. graminea* were generated from a cross between the resistant cv "Thibaut" and the highly susceptible cv "Mirco", followed by six backcrosses with the susceptible parent.

Three different analysis technique were employed to analyse plant pathogen interactions in defining the genetic components involved, and the transcriptional changes that occur in both the host and the pathogen: 1) A cDNA suppression subtractive library; 2) cDNA-AFLP technique; 3) histological microscopic analysis.

In the first experiments a suppression subtractive library of barley samples control and treated samples has been constructed by using the Clontech PCR Select cDNA subtraction kit.

Eleven clones of 1800 analyzed, that result really differentially expressed, have been sequenced and analysed in the non reduntant databases by using the BLAST-N, BLAST-X and TIGR algorithms, available at the NCBI and TIGR web sites. These clones where screened further by Northern blot analyses. Some this eleven clones, four have been involved on likely scavenging role of ROS during fungal infection.

In the second experiments the cDNA-AFLP TP analysis was carried out at the laboratory of prof. Massimo Delledonne in the Dipartimento Scientifico e Tecnologico holding the Agro-Biotechnology Faculty of University of Verona.

The analysis utilized the resistant cultivar Thibaut, the susceptible cultivar Mirco and the *Pyrenophora graminea* mycelium. About 1100 fragments differentially expressed are point out. 600 fragments have been sequenced. After search in data bank the fragments which have been showed an homology with known sequences have been grouped in functional groups.

In the third experiment hystologic analysis utilising the fluorescence microscope, it has allowed to known the infection procedure of fungus. The autofluorescence was observed both in cultivar Mirco infected and NIL, sign of poliphenols accumualted at level of the embryos cell wall and confirmed by blue toluidine assay.

1. INTRODUCTION

Plants are constantly confrontated with a wide variety of potential pathogens within their environment including bacteria, fungi, viruses and nematodes (fig.1).

Roots and shoots of all plants come into intimate contact with plant pathogens. Each pathogen has evolved a specific way to invade plants. Some species directly penetrate surface layers by using mechanical pressure or enzymatic attack. Others pass through natural openings (e.g., stomata or lenticels). A third group invades only tissue that has been previously wounded. Once inside the plant, three main attack strategies are deployed to utilize the host plant as a substrate: necrotrophy, in which the plant cells are killed; biotrophy, in which the plant all remain alive; and hemibiotrophy, in which the pathogen initially keeps cells alive but kills them at later stages of the infection. Nevertheless in nature the development of disease is more the exception than the rule and resistance the normality, for example less than 10% of the 100,000 known fungal species are able to colonise plants, and an even smaller fractions are able to cause disease (Haegi A. et al.).

The principal reasons which prevent a pathogen from leading the attack to plant successfully are:

- The plant species is unable to support the life-strategy requirements of the particular pathogen and thus is considered a nonhost;
- The plant possesses preformed barriers of chemical (such as phytoanticipins, that have antimicrobial activity), enzymatic or structural (cuticle, stomata and cell wall) type that allow infection only to specialized pathogen species;
- On recognition of the attacking pathogen, defence mechanism are activated such that the invasion remains localized;
- Environmental conditions change and the pathogen perishes before the infection process has reached the point at which it is no longer influenced by adverse external stresses.

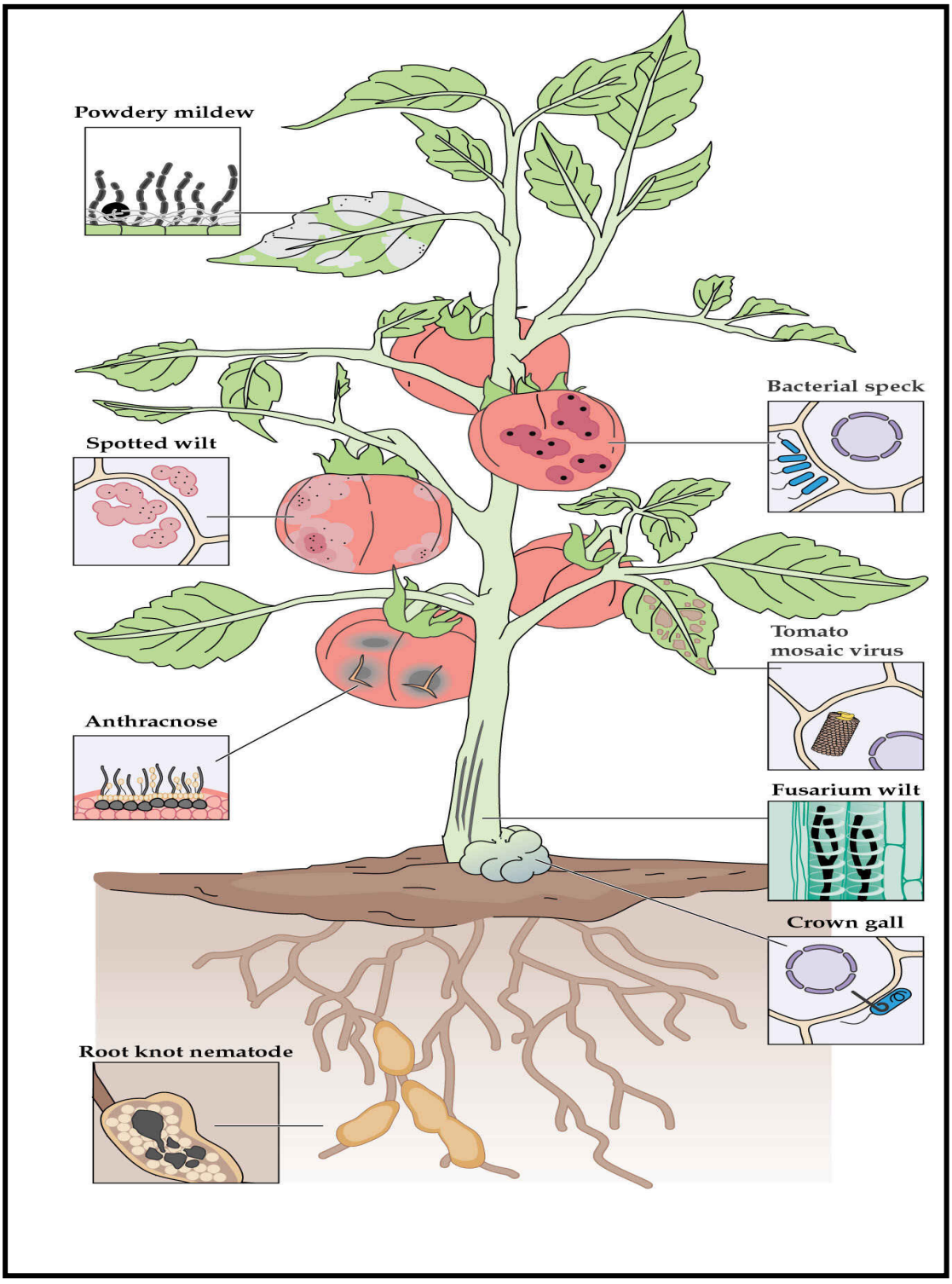


Fig. 1 : Example of plant's biotic stresses on tomato.

The first three interactions are said to represent genetic incompatibility, but only the third resistance depends exclusively on induced defence responses to limit pathogen attack.

An incompatible reaction is when the pathogen is recognized by the host which defend himself from it, preventing or delaying the growth and the diffusion in the tissues.

Successful pathogen infection and disease (compatibility) occur only if environmental conditions are favourable, if the preformed plant defences are inadequate, and if either the plant fails to detect the pathogen or the activated defence responses are ineffective.

1.1 Activation of defence responses

To defend themselves against attack from the vast array of viruses, bacteria, fungi, parasitic plants, nematodes and insects in their environment, plants are equipped both with pre-formed, constitutive chemical and mechanical barriers as well as with inducible defence systems (M. Montesano et al., 2003).

Physical barriers largely involve properties of the plant surface, that is, the cuticle, stomata, and cell walls. Chemical barriers include compounds, such as "phytoanticipins", that have antimicrobial activity and compounds that affect the vectors of plant viruses. Phenols and quinones are two classes of antimicrobial compounds produced by some plants. Inhibiting compounds may be excreted into the external environment, accumulate in dead cells or be sequestred into vacuoles in an inactive form (A. Moncrieff).

Plants are normally capable of with-standing an attack by a potential pathogen and responding with a local and systemic induction of a series of defences that prevent or limit the infection and provide enhanced resistance to subsequent infections by the same or even unrelated pathogens.

The recognition of a potential pathogen results in several early responses including rapid ion fluxes, activation of kinase cascades and the generation of reactive oxygen species (ROS).

These early events are followed by other defence responses including induction of hypersensitive response (HR), a localized form of programmed cell death (PCD) limiting pathogen spread, further reinforcement of the cell walls, and production of antimicrobial compounds such as defence proteins and phytoalexins. (M. Montesano et al., 2003; A Haegi et al., 1998).

Central to any inducible defence system is timely perception of the pathogen.

1.1.1 Elicitors

Plants are able to recognize compounds produced or released by the aggressor (so-called elicitors) and employ these to trigger defence signalling.

Originally the term elicitor was used for molecules capable of inducing the production of phytoalexins, but it is now commonly used for compounds stimulating any type of plant defence (Ebel et al., 1994; Hahn, 1996; Nürnberger, 1999). This broader definition of elicitors includes both

substances of pathogen origin (exogenous elicitors) as surface molecules (e.g chitin and glucan fragments) and compounds released from plants by the action of the pathogen (endogenous elicitors) as oligo-galacturonides which are released upon fungal polygalacturonase activity (A. Haegi et al., 1998).

Elicitors may be classified into two groups, "general elicitors" and "race specific elicitors". While general elicitors are able to trigger defence both in host and non-host plants, race specific elicitors induce defence responses leading resistance only in specific host cultivars.

Elicitors do not have any common chemical structure, but belong to a wide range of different classes of compounds including oligosaccharides, peptides, proteins and lipids. This vast array of elicitor compounds precludes the presence of a common elicitor motif and suggests that plants have the ability to recognize a number of structurally distinct molecules as signals for pathogen defences.

A single elicitor can activate plant defence responses by binding to a single receptor, and a downstream signal transduction pathway leads to the activation of different defence reactions.

Theoretically it can not be excluded that the same elicitor can bind to different receptors each triggering particular responses. However different elicitors can activate either the same defence mechanism or independently trigger different defence responses (S. Zhang et al., 1998).

1.2 Plant and pathogen recognition

1.2.1 Genetic interaction plant-pathogen

Plant-pathogen interactions, particularly those involving biotrophic parasites, are governed by the interaction of the product of pathogen A*vr* (avirulence) gene loci and alleles of the corresponding plant disease resistance (R) locus. When corresponding *R* and Avr genes are respectively present in both host and pathogen, the result is disease resistance. If either is inactive or absent, disease results. The simplest model (fig.2) that accounts for this genetic interaction requires that R products recognize avr-dependent signals and trigger the chain of signal-transduction events that culminates in activation of defence mechanism and arrest of pathogen growth (J.L. Dangl & J. Jones, 2001).

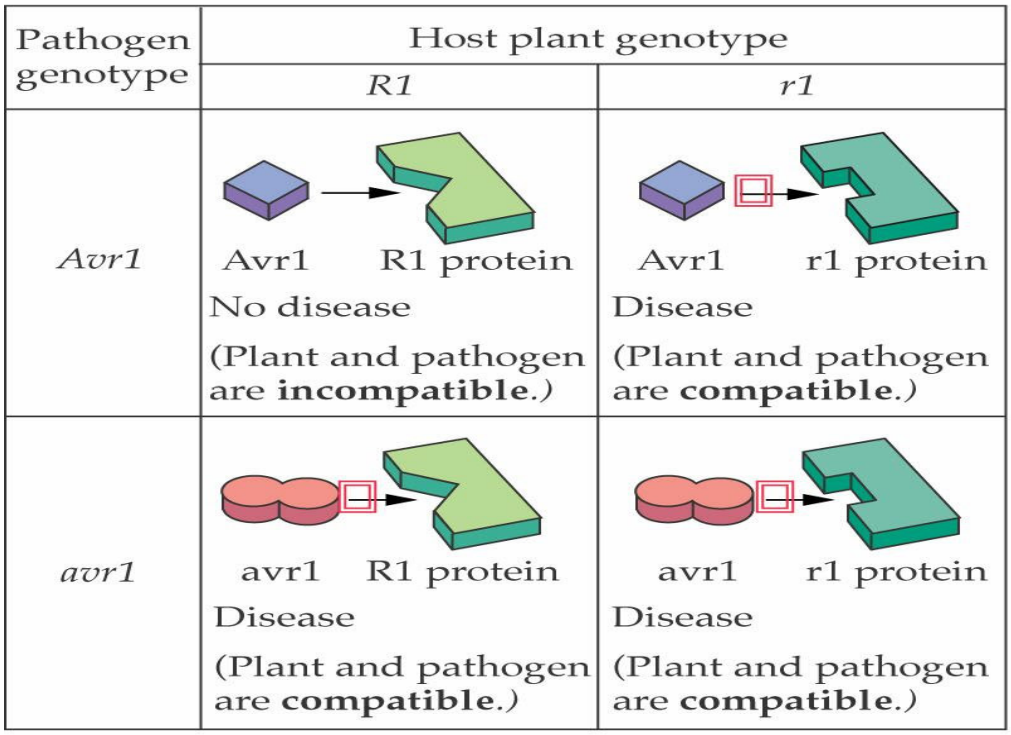


Fig 2 : Flor's gene-for-gene model

This theory was proposed more than 50 years ago by Harold Flor working with flax and the flax rust fungus, defined plant-pathogen interactions genetically producing the gene-for-gene hypotesis (E. Van Der Biezen et al., 1998; H. Flor, 1947).

1.2.2 Avirulence genes' features

Many bacterial avirulence genes have been cloned and, in general, there are no common features in the predicted products of these genes. Fewer avirulence genes have been cloned from fungal pathogens and the products of these genes are also diverse. In gene-for-gene interactions involving viruses, proteins including viral capsid proteins, RNA replication proteins and viral movement proteins have been identified as virulence gene products. A common opinion is that the primary function of the suites of avirulence gene products produced by a particular pathogen species is to assist the pathogen in both colonizing and gaining nutrition from host plants. Molecular evidence from bacterial and fungal pathogen in support of this idea is provided by observations that, in some cases, a reduction of pathogenicity occurs when avirulence genes are mutated. (J. Ellis, 2000).

1.2.3 Structure plants resistance' genes product

The isolation of R and Avr genes has been critical for understanding the underlying molecular mechanism of race-specific immunity in plants. Many R genes from monocots and dicots have been cloned during the last 10 years, encoding R proteins to bacterial, viral, fungal, oomycete, nematode and insect pathogens. Striking similarities are found in the structures of R proteins implying that fundamental modes of recognition and defence signalling have been retained through plant evolution and diversification (Dangl & Jones, 2001; Hammond-Kosack & Parker, 2003).

Most *R* genes encode proteins containing variable numbers of sequence-diversified Leucine-rich repeats (LRRs), a protein domain that is known to participate in protein protein interactions. LRR containing R proteins can be broadly divided into two classes, one with intracellular and the other with extracellular LRRs. (A. f. Bent, 1996; Hammond-Kosack & Parker, 2003).

An additional characteristic domain is central NB domain occur in diverse proteins with ATP or GTP binding activity, such as ATP synthase β subunits, Ras proteins, ribosomal elongation factors and adenylate kinase (A. F. Bent,1996). The largest class of known R genes encodes predicted intracellular proteins. These share a central nucleotide-binding (NB) site and C-terminal LRRs.

Members of this class can be further divided in two subclasses containing either N-terminal sequences predicted to form a coiled-coil (CC) structure (CC-NB-LRR subfamily) or sequences that are related to the cytoplasmic domain of the Drosophila Toll and human interleukin-1 receptor (TIR-NB-LRR subfamily). Most NB-LRR type R proteins consist of these protein modules except few containing additional domains. For example, Arabidopsis RRS1-R, confers resistance against the bacterium *Ralstonia solanacearum* and possesses an additional C-terminal WRKY domain (Hammond-Kosack & Parker, 2003).

An additional clas of R proteins contains an extracellular LRR (eLRR) which is membrane-anchored by a single transmembrane helix. Structural variations are also found within members of this class. For example, the rice Xa21 product has an additional intracellular Ser/Thr kinase module, whereas the tomato Cf gene products lack any significant intracellular domains (Ellis et al., 2000). Two more recently isolated R gene from tomato, Ve1 and Ve2, encode eLRR type proteins with a cytoplamic domain possessing sequences that in mammalian receptors stimulate their endocytosis and degradation (the ECS domain) (Hammond-Kosack & Parker, 2003).

Another classes of protein is represented by Pto host protein involved in recognition of a bacterial protein, AvrPto in tomato. Pto is a Ser/Thr kinase that interacts physically with the AvrPto protein in agreement with the involvement of protein phosphorilation in the induction of defence reaction by pathogen (A. f. Bent, 1996; Van der Biezen & J. Jones, 1998).

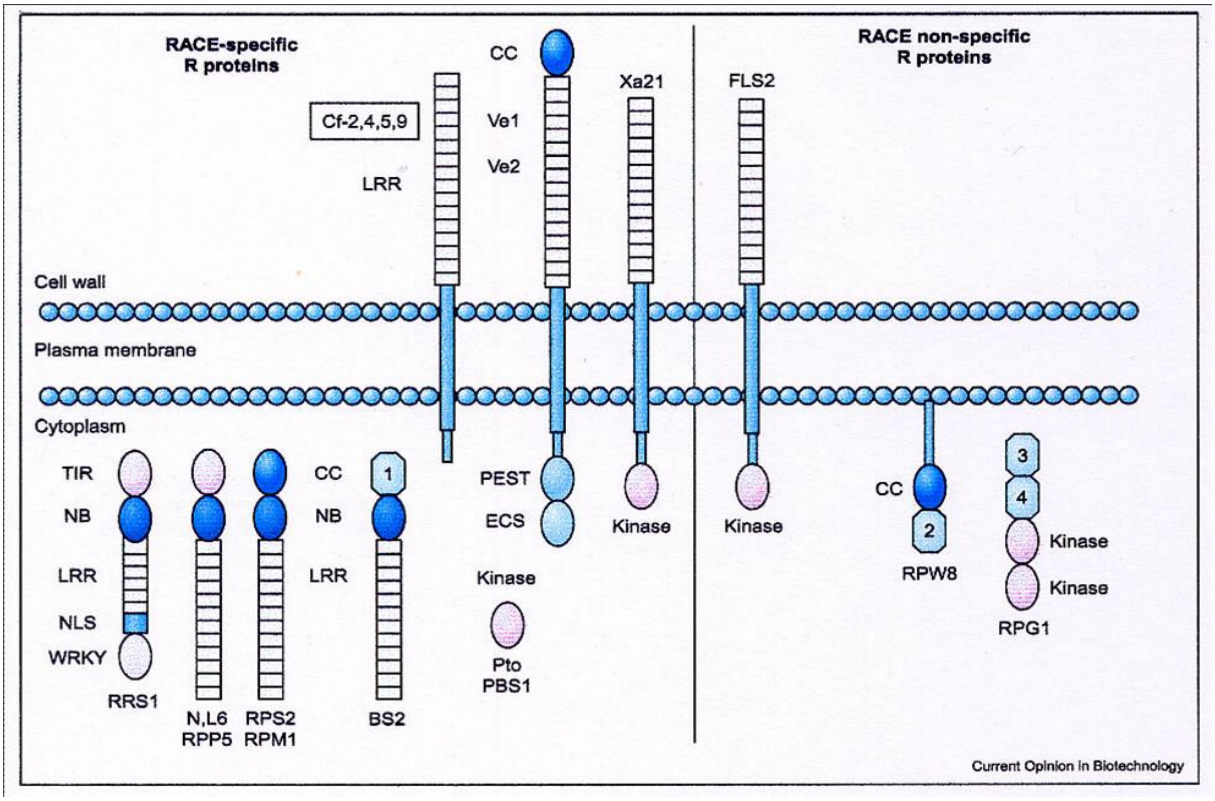


Fig 4. Schematic diagram illustranting plant LRR-containing proteins

1.2.4 Receptor-ligand model

Gene-for-gene interactions have been interpreted in terms of a receptor-ligand model in which the products of resistance genes are receptors that specifically detect the pathogen avirulence genes either directly (e.g the protein product) or indirectly (e.g. an enzyme product). The resistance receptor is envisaged to have two basic properties, specific pathogen recognition and the ability to signal to downstream response genes (J. Ellis et al., 2000). The classical receptor-ligand model (fig.3) predicts that direct interaction between an Avr protein and a matching R protein initiates the defence reaction. This model is consistent with plant-virus interactions.

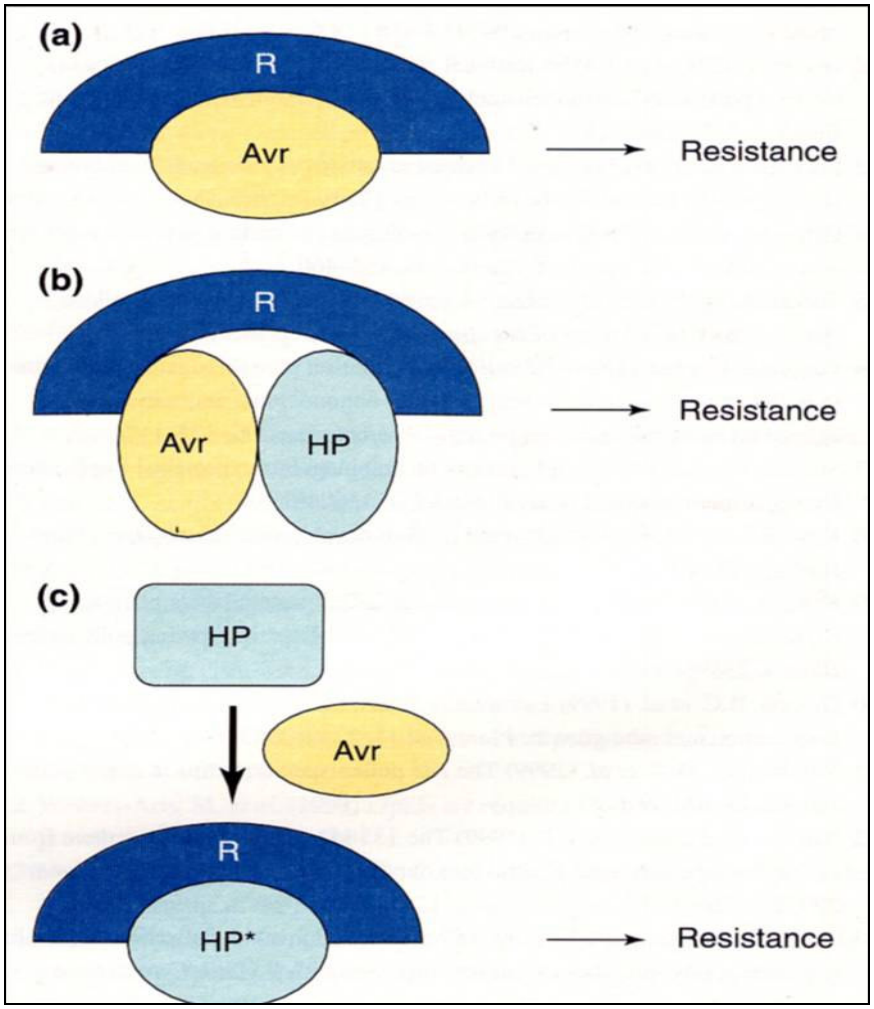


Fig 3 : Receptor – ligand model

A second receptor-ligand model envisages that the interaction of the Avr protein with one or more host proteins (HP) alters the host metabolism to the advantage of pathogen. The host receptor, R, present in resistant plants provides a surveillance mechanism (guard hypothesis) that detects Avr-HP interactions and triggers resistance. In a variation on this second mechanism, the avr protein is envisaged to modify a host protein target enzymatically to the advantage of the pathogen. The resistance receptor R recognizes the modified protein HP and trigger resistance (Pierre J. G. M. de Wit, 2002; J. Ellis et al., 2000; U. Bonas et al., 2002).

1.3 DEFENCE MECHANISMS

When a plant detects an invasion, then a set of inducible defence responses is deployed; these include programmed cell death (referred to as hypersensitive response or HR), tissue reinforcement at the infection site, production of anti-microbial metabolites and induction of "defence-associated" gene expression. Activation of "local" responses at the point of infection can be followed by establishment of secondary immunity throughout the plant (systemic acquired resistance or SAR), which is long lasting and effective against a broad spectrum of pathogens.

For inducible defences to be effective, they must be deployed rapidly; the ability of pathogens to outpace a late counterattack is well documented. On the other side, these defences cannot be unleashed with impunity, as they are resource-intensive and can inflict substantial collateral damage on host tissues. Thus, deployment must be confined to the proper place and time. These requirements suggest that complex, highly integrated regulatory network controls defence responses (J. McDowell & J.L. Dangl 2000).

The earliest detectable cellular events are ion fluxes across the plasma membrane and a burst of reactive oxygen intermediates (ROIs), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2).

Plasma-membrane depolarization in plant cells occurs in response to various physiological stimuli, including elicitors. In fact, changes in ion fluxes (Cl^- and K+ efflux, Ca^{2+} influx, alkalinization of cell culture growth medium) constitute an early inducible response, occurring within minutes after elicitor application in many plant tissues and have been correlated with the activation of typical defence responses (J. Ebel & A. Mithofer 1998). The Ca^{2+} ion is implicated in signal transduction and this is supported by a number of observations. Omission of Ca^{2+} from the culture medium blocked defence-related gene activation and/or phytoalexin formation in soybean, carrot, parsley and tobacco cells (J. Ebel & A. Mithofer 1998). Furthermore, the elicitor-induced defence responses and Ca^{2+} influx were inhibited by certain anion-channel blockers. These results indicate a position of the anion flux upstream of the Ca^{2+} flux in the putative ion-flux-mediate signal transduction chain (J. Ebel & A. Mithofer 1998).

1.3.1 Reactive oxygen species

In numerous incompatible interactions, the reactions are often associated with the death of a small number of cells at the site of infection, known as the "hypersensitive response" (HR). Initiation of resistance response include rapid and transient responses that occur mainly at the plant cell surface

and are based on the activation of pre-existing components rather than involving the biosynthetic machinery of the cell. Among the reactions identified there are: release of reactive oxygen species (ROS) termed the " oxidative burst", changes in extracellular pH and in membrane potentials, ion fluxes, changes in protein phosphorylation patterns, and the oxidative immobilization of plant cell wall proteins. Most cells posses the ability to produce and detoxify ROS. In normal conditions ROS appear in cells as inevitable by-products formed as a result of successive one-electron reductions of molecular oxygen (O_2). Most cells have also acquired the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell. In some cases, however, especially under stress conditions, these protective mechanisms are overridden by the rapid, transient, production of huge amounts of ROS, namely the oxidative burst. The term ROS is used to describe the products of the sequential reduction molecular oxygen: superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), species predominantly detected in plant-pathogen interactions (P. Wojtaszek, 1997). Plant cells have evolved three ways of generating ROS:

The first is the NADPH oxidase system analogous to that of animal phagocytes. According to this model , an elicitor molecule is recognized by an appropriate receptor located on plasma membrane this recognition involve of GTP-binding proteins, ion channels (especially Ca^{2+}), protein kinase and protein phosphatases, phospholipase A and C and possibly cyclic AMP along the signalling pathway leading to the activation of NADPH oxidase, generation of O_2 and its dismutation to H_2O_2.

The second model to generate H_2O_2 is by pH-dependent cell-wall peroxidase. When an elicitor arriving at the cell surface is recognized by the appropriate receptor molecule, and this event leads to the activation ion channels. The movement of the ions (Ca^{2+} , K^+, H^+, Cl^-) results in a transient alkalinization of matrix extracellular, which leads to an activation of pH-dependent cell-wall peroxidase. The third model is by a germin-like oxalate oxidase protein that can produce H_2O_2 from oxalic acid which has been detecte in incompatible Mla1 barley-powdery mildew interactions (P. Wojtaszek, 1997; Hammond-Kosack & J.D.G. Jones 1996).

The roles of ROS in plant is:

- H_2O_2 is directly toxic to microbes;
- H_2O_2 contribute to the structural reinforcement of plant cell walls;
- H_2O_2 is essential for the formation of lignin polymer precursors via peroxidase activity;
- H_2O_2 drive the oxidative cross-linking of cell-wall (glycol)proteins and so reduce their susceptibility to enzymic degradation;
- to induce SAR;
- the generation of ROS lead to an alteration in the redox balance in the reacting cell, which regulate the stability of specific defence-related mRNA transcripts.

Experiments with pathogens compatible and incompatible inoculation show that in both compatible and incompatible interactions, an oxidative burst occurs within the first hour after inoculation. Incompatible interactions are caraterized by a second, stronger, and more prolonged oxidative burst, and it is in these incompatible interactions that cell death occurs.

1.3.2 Hypersensitive response

Plant cell death is often the consequence of plant-pathogen interactions in both compatible and incompatible relationships (Geenberg, 1997). A notable example is localized cell collapse, called the hypersensitive response (HR), which is induced rapidly in a resistant plant at the infection site of an avirulent pathogen (Staskawicz et al., 1995; Bent,1996; Dangl et al., 1996; Hammond-Kosack and Jones, 1996). Hypersensitive cell death, which is distinct from necrosis caused by metabolic toxins or severe trauma, is programmed genetically in the plant and is a consequence of new host transcription and translation which lead to programmed cell death (PCD) phenomenon also known into animal cells (Biochemistry & Molecular biology of Plants, 2000). Those results have been confirmed by research leaded on mutant plants called “paranoid plant” because they behaved as if constantly under pathogen attack (J. L. Dangl et al., 1996).

In interactions with obligate biotrophic pathogens that form intimate haustorial associations with host cells, plant cell death would deprive the pathogen pf access to further nutrients. In interactions involving hemibiotrophic and necrotrophic pathogens, the role of the HR is less clear because these pathogens can obtain nutrients from dead plant cells. However, cellular decompartmentalization may lead to the release of harmful preformed substances that are stored in the vacuole (A.E. Osbourn, 1996). Alternatively, the levels of induced phytoalexins, which usually are rapidly turned over in plant cells, may accumulate to inhibitory concentrations because they are no longer metabolized.

ROI can have immediate effects on cell wall strengthening via peroxidase action and can be perceived by the cell to engender intracellular responses. Subsequent to the activation of defense gene transcription are other downstream effector events that include SA biosynthesis of phytoalexins and pathogenesis –related proteins, the HR, and systemic signalling.

1.3.3 Salicylic acid and SAR

Salicylic acid (SA) is derived from the phenylpropanoid pathway. SA is closely linked to the hypersensitive response. The localized cell death infect often is associated with changes in healty, distant parts of the plants that enhance resistance to secondary infection by a broad range of

pathogens. This increased resistance, which develops over a period of several days to a week after the initial pathogen invasion, is referred to as systemic acquired resistance (SAR).

SA was initially identified as long-distance signal that is translocated in the phloem from the site of pathogen invasion to uninfected leaves there initiating the development of the (SAR). Later SA was demonstrated not to be a translocated signal of SAR but its accumulation was indicated as an essential factor required for expression of multiple modes of plant disease resistance.

In Xanthi-*nc* tobacco, this signal has been proposed to be methyl salicylate, which is produced from SA in TMV-infected leaves. Unlike SA, methyl salicylate is volatile, which, it was suggested, could thus act as an airborne signal to induce PR protein accumulation and disease resistance in both neighboring plants and they healthy tissues of infected plant (Biochemistry & Molecular biology of Plants, 2000).

SA could rise ROS levels by acts binding activity to the eme group of ascorbate peroxidase .

The consequence of inhibitory activity of SA on ROS scavengers in plants would be the elevated level of H_2O_2 in the immediate vicinity of the infection site and this level has been postulated to act as a second messenger of SA in the signal-transduction pathway leading to SAR and gene activation for PR proteins (P. Wojtaszek, 1997).

1.3.4 Nitric Oxide (NO)

Nitric oxide (NO) is a signal molecule used by mammals to regulate various biological processes of the immune, nervous and vascular systems. In plant NO activity interacts with the one of ROIs and SA to induce HR and defence gene expression (M. Delledonne et al., 1998; J. Mcdowell & J.L. Dangl, 2000). The contribution in the plants of NO, SA, and H_2O_2 appear to be synergistic rather than additive, implying that they interact directly and cooperatively in a signal-amplification mechanism. Furthermore ROIs and NO stimulate SA biosynthesis, and SA in turn potentiates ROI-NO-dependent responses. These features suggest that receptor-dependent pathogen perception triggers a positive feedback loop of ROI-NO production and SA accumulation, which rapidly amplifies the initial signal and guarantees timely defence activation. Culmination of this cycle in HR cell death could release ROIs, NO and SA into intercellular spaces, and these compounds could directly inhibit pathogen growth or “warn” neighbouring cells of an imminent invasion or both (J. Mcdowell & J.L. Dangl, 2000).

1.3.5 Jasmonic acid

Jasmonic acid, methyl jasmonate and related compounds are a class of plant hormones that play an important role in regulating many cellular processes, such as wound and defence responses (E.E. Farmer et al., 1992). The production of JA is a tightly regulated process, and the concentrations of JA in unperturbed plant tissues are often very low. However JA accumulates in wounded plants or in plants and cultured cells treated with pathogen elicitors; its acts as a signal activanting the expression of various genes, such as proteinase inhibitors, thionin, and enzymes in phytoalexin metabolism (R.A. Creelman et al., 1997). JA is synthesized from linolelic acid released from the plasma membrane of wounded or pathogen-attacked, structurally damaged cells (S. Reinbothe et al., 1994). Wounding induces translocation of phospholipase D (PLD) to membranes via an influx of Ca^{2+}. PLD associated with membranes becomes active and releases free polyunsaturated fatty acids from membrane phospholipids by initianting the lipolytic process and by increasing the activities of acyl-hydrolyzing enzymes.

All of the plant responses to jasmonates, whether applied externally or released internally, appear to be correlated with alterations in gene expression. At least three major jasmonate effects have been reported: 1) the induction of novel abundant polypeptides, designated jasmonate-induced proteins (JIPs) (B. Parthier et. al., 1987) (e.g. JIP60 in barley cleaves polysome from plant, microbial, and animal origin into their ribosomal subunits (S. Reinbothe et al., 1994) only from long-term MeJa-treated leaf tissues or leaf tissues that have been exposed to stressors that cause high level jasmonate accumulation, such as harsh wounding, osmotic stress or desiccation (S. Reinbothe et al., 1994);

2) the selective repression of synthesis of several polypeptides that are present before jasmonate or stress treatment (R. A. Weidhase et al., 1987a; S. Reinbothe et al., 1993a, 1993b, 1993c), and 3) the temporally delayed general down-regulation of protein biosynthesis occurring in long-term MeJA-treated or long-term stressed leaf tissues (S. Reinbothe et al., 1994).

1.3.6 Proteins Pathogenesis Related (PR)

The term PR protein was first used to describe numerous extracellular proteins that accumulate in response to TMV infection of susceptible tobacco genotypes. Subsequently, in an array of plant-pathogen interactions, differential PR gene induction was found to be associated with incompatibility. More recently, the definition of a PR protein has been broadened to include intra- and extracellular proteins that accumulate in intact plant tissue or cultured cells after pathogen attack or elicitor treatment. Some PR proteins are chitinase and glucanases, enzymes that degrade structural polysaccharides of fungal cell walls and reduce fungal growth.

Plant defensins are a third type of defense-related genes with demonstrated antimicrobial activity. The family of basic cysteine-rich peptides with a molecular mass of less than 7 kDa, thionins, found mainly in cereals, belong to this group. Like the PR proteins, these thionins also accumulate differentially during incompatible interactions. Interestingly, a JA-mediated signal transduction pathway, distinct from the typical SA-mediated pathway leading to PR gene activation, controls thionin gene expression in Arabidopsis (K. E. Hammond-Kosack & J. D. G. Jones, 1996).

In fact genetic studies in Arabidopsis have revealed resistance responses that operate independently of SA accumulation and are mediated by JA and gaseous hormone ethylene (ET). JA and ET are also plant growth regulators, suggesting overlap between the regulatory components of development and defence. Arabidopsis mutants, compromised in their ability to respond to JA or to produce SA, have been used to elegantly demonstrate that the SA-dependent and ET-JA-dependent responses are utilized differentially against pathogens with contrasting modes of attack (B. Thomma et al., 1998). The ET-JA-dependent defence response is activated by pathogens that kill plant cells to obtain nutrients. In contrast, the SA-dependent response is triggered by pathogen that obtains nutrients from living plant tissue (X. Dong et al., 1998). This observation raises the intriguing possibility that plants can activate distinct defence responses tailored to specific types of parasites.

1.3.7 Phytoalexins

Phytoalexins are low molecular weight antibiotics produced by many plants in response to infection. There are many biotic elicitors of phytoalexin production, such as cell wall components, as well as biotic elicitors, such as heavy metals and ultraviolet light. Phytoalexins inhibit the growth of bacteria and fungi in vivo and in vitro, and production of these antibiotics during an infection can induce resistance to subsequent infections by that pathogen. Over 350 phytoalexins are known in over 100 plant species. Phytoalexins may be produced by any part of the plant, although different phytoaleins can accumulate in different organs. Generally, related plant species produce structurally-related phytoalexins, and many produce more than one, enabling the palnt present a toxic cocktail to invading pathogen. Phytoalexins are produced in cells surrounding an infection site and delivered to the infected cell packaged in lipid vesicles, creating a toxic micro-environment in the infected cell and, hopefully, preventing disease establishment.

Phytoalexin accumulation is often associated with hypersensitive cell death, although only living cell can synthesise phytoalexin. Some plants can also sequester phytoalexin into vacuoles as stores of inactive sugar-conjugates, which can be cleaved and released quickly if initial defence responses are unsuccessful (A.Mocreiff).

1.3.8 Defence from phyto-toxins

A substantial number of toxins, produced as a mixture of either related or unrelated subsatance, are produced by a large spectrum of pathogens, either to kill plant cells or to influence their metabolim (A.J. Colmenare et. al., 2002).

Fungal toxins may affect both host and non-host species (non-host selective toxins); or may be toxic only on host plants (host specific toxins). Some fungal toxins are toxic only for susceptible cultivars among host species; these host-selective toxins have been shown to be the determinats of specificity, hence of the disease (J.D. Walton, 1996).

Non-host specific phytotoxins are usually secondary metabolites, a large number of which have been identified in fungi (A. Stoessl, 1981). Non-host –specific toxins can affect a wide range of host plants, these toxins may produce all or part of the disease syndrome on host plants as well as on non-host-plant, increase the extent of the disease but are not essential for the pathogen to cause disease.

Host-selective toxins (HST) act as positive agents of virulence or pathogenicity. When an HST is involved, pathogen virulence is dominant because of the need to produce a functional toxin or enzyme, whereas avirulence is a recessive trait. HSTs are produced only by fungi, mainly by species of the fungal genera *Alternaria* and *Cochliobolus.*

Plant resistance against this kind of pathogen is acquired via the loss or alteration of the toxin target or through detoxification. Not all toxins are toxic under all conditions; some require active participation (transcription and translation) from the plant cell (J.D. Walton, 1996).

The final outcome will depend on cell type, the physiological state of the cell, the cell-cycle stage or the blend of other signals being processed by the cell at the moment the stimulus is perceived.

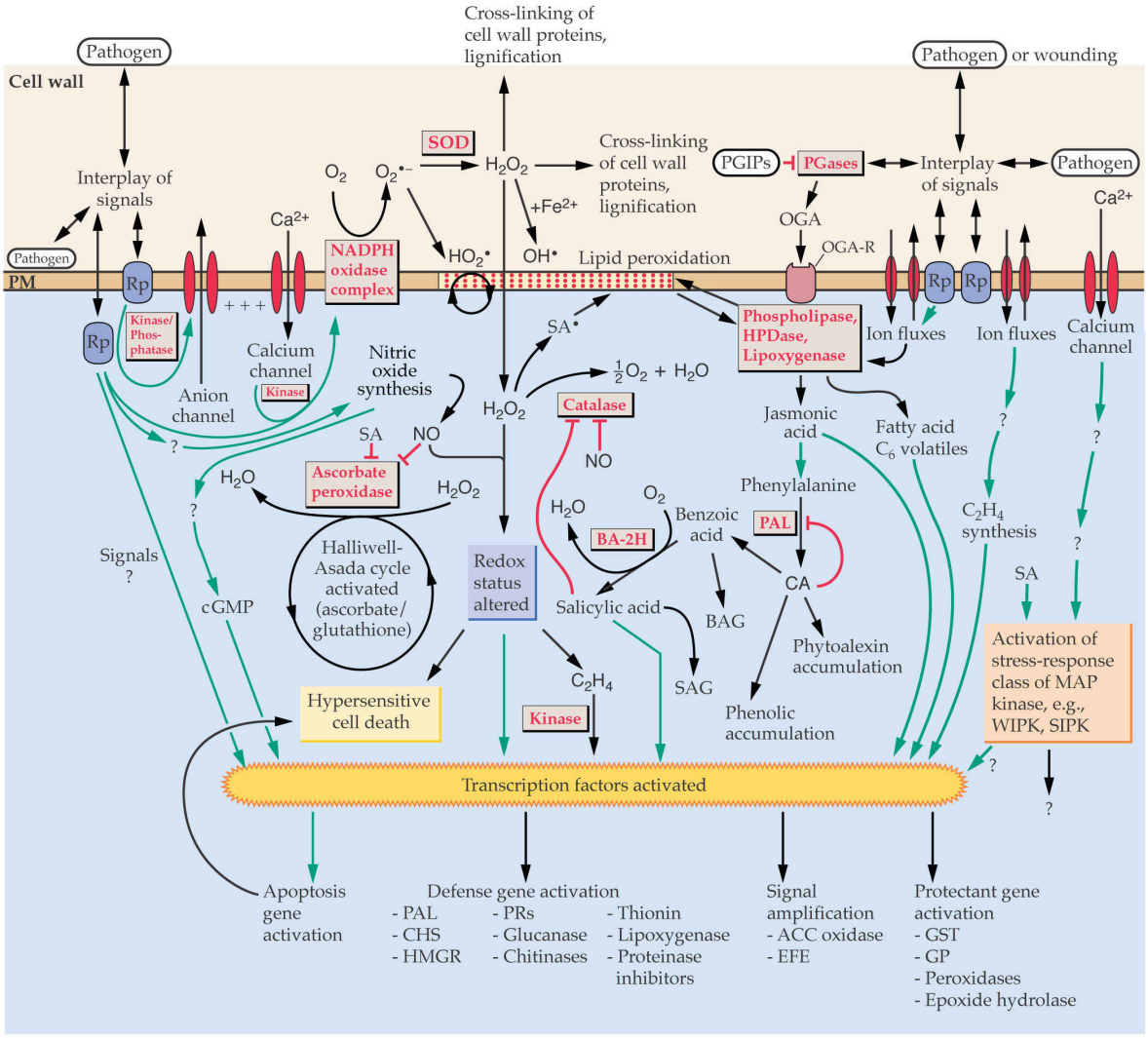


Fig. 4 Overview of signal transduction pathway activanting and coordinanting plant defense responses.

1.4 METHODS TO DETECT DIFFERENTIALLY ESPRESSED GENES

Although a gene must not necessarily be up- or down-regulated to play a key role in a certain process, screening for differentially expressed genes is one of the most straightforward approaches to unravel the molecular basis of a biological system (S. Lievens et al., 2001).

Under the complexity of cell's transcriptome it's a difficult to isolate differentially expressed genes, particularly the low-abundance ones. A eukaryotic cell contains ~15,000-30,000 distinct mRNAs with a prevalence ranging from one to several thousands in a total mass of ~100,000 mRNAs.

About 50% of the transcript population is made up of a relatively small number (some hundred) of abundant transcripts, representing only 1% of the different mRNA species. The other half contains the 'rare' mRNAs (J. S. Wan et al., 1996).

Not surprisingly, the difficulty of fishing out a gene responsible for a specialized function in a certain biological program often originates from the fact that the gene is expressed at low levels whereas the bulk of a cell's mRNA is made up of highly abundant transcripts(S. Lievens et al., 2001).

A key initial step in understanding of plant pathogen interactions in defining the genetic components involved, and the transcriptional changes that occur in both the host and the pathogen (P. R. J. Brch & S. Kamoun, 2000).

1.4.1 General view of main methods

In traditional screening methods, such as differential hybridization, the hybridization pattern of the total content of cDNA libraries is compared between two samples (Maniatis et al., 1982).

The fact that the abundant transcripts are also displayed implies high redundancy of non-relevant clones and thus very low labor efficiency. This problem has been solved partly by normalization and subtraction (S. M. Hedrick et al., 1984); even then, many interesting low-aboundance differentially expressed genes are missed because of the low amplification of the hybridization signal (Z. Wang and D. D. Brown, 1991). Other drawbacks are the limitation to pairwise comparisons and the fact that the techniques are mainly qualitative because of the relative insensitivity of the hybridization (D. Bauer e al.,1993).

A major challenge in analysing plant-pathogen interaction is often the small of biological material available. This limitation has been overcome using PCR-based methods, initially developed for

DNA fingerprinting, which allow profiles of gene expression (following conversion of mRNA to cDNA) to be readily visualized (P. R. J. Brch & S. Kamoun, 2000).

One of the first differential screening methods was the **differential display (DD)** technique described by Liang and Pardee (P. Liang and A. B. Pardee, 1992).

Briefly, after cDNA synthesis using reverse transcriptase and oligo dT primer that anneals to the 3' polyA tail of mRNA, subset of cDNA populations for comparison are PCR amplified with short 5' arbitrary primers, in combination with oligo dT primers, and visualized on polyacrylamide gels (P. R. J. Brch & S. Kamoun, 2000). The expected advantages were numerous: the method would be fast, it was based on simple, well established and widely accessible techniques, making it easily applicable for most researchers; compared with previous methods the sensitivity had been increased dramatically, resulting in a good detection of low-abundance genes; both induced and repressed genes could be compared, making it highly versatile; furthermore, only a small amount of starting material was needed (P. Liang and A. B. Pardee, 1992).

However the DD reveals some drawbacks as the frequency of false positive, which may be as high as 50-75% of the excised bands. The most significant source of artefacts might be inherent to the design of the differential display method. The combination of short primers and low annealing temperatures during PCR results in non-specific and inefficient amplification (S. Zhao et al.,1995).

Another factor that may generate false positives is the competition for primers by transcripts of different abundance (C. Debouck et al., 1995).

Besides, in plants this technique tends to give a preponderance of 3'-untraslated sequences in the resulting DNA fragments, which make the data difficult to characterize further by database searches (C. W. B. Bachem et al., 1996).

In alternative protocol (J. Welsh et al., 1992) arbitrary primers are used both for cDNA synthesis and PCR amplification.

After differential display had been introduced, more methods using PCR were developed (D. H. Kozian et al.,1999; C. D. Greeen et al., 2001). A large number of techniques involved the generation of a gel profile to display differences between the different mRNA samples, but, in contrast to DD, tried to evade the use of arbitrary primers and so circumvent problems that originated from mismatch priming during amplification. Instead, restriction sites were used to generate a subset of cDNA fragments that differed in size. Amplification following restriction enzyme digestion was done with primers that matched previously ligated adaptors. Eventually, one or more selective bases were added to the 3' ends of the primers to further reduce the subset of cDNA fragments that will be displayed, such as in **cDNA-amplified fragment length polymorphism (AFLP)** (C. W. B. Bachem et al., 1996). The kinetics of gene expression revealed

by cDNA-AFLP were similar to those of northern blot analysis, rendering the displayed expression pattern quantitative (C. W. B. Bachem et al., 1996).

Two cDNA population, one coming from control plants and one from pathogen infected plants can then compared by profiling them using cDNA-AFLP; i. e. this technique has been used to identify tomato cDNAs that are up-regulated in the resistance response to *Cladosporium fulvum*, when R protein Cf-9 is activated by the Avr9 protein from pathogen (W. E Durrant et al., 2000).

Other types of techniques improved and refined the traditional technology of cDNA library construction and screening. An example is suppression **subctractive hybridization (SSH)** (L. Diatchenko et al., 1996). SSH combines the selectivity of subtractive hybridization with the sensitivity of PCR. One of its main advantages is that it allows the detection of low-abundance differentially expressed transcripts, such as many of those likely to be involved in signalling and signal transduction, and might thus identify essential regulatory components in several biological processes. The first application of SSH in the study of plant-microorganism interaction was for the isolation of potato genes that are up-regulated in the HR induced by *P. infestant* (P.R. J Birch et al., 1999).

A completely different sequence-based approach to identify differentially expressed genes is followed by **serial analysis of gene expression (SAGE)** (V.E. Valculescu et al., 1995).

In this method, very short (10-14 bp) cDNA tags are generated by restriction digestion, amplified by PCR and ligated, after which the resulting concatemers are sequenced. The tags are long enough to identify the corresponding genes unequivocally and the frequency of the tags is a measure of their expression level. This method is very fast and straightforward because it does not imply selection of mRNA to create displayable subpopulations, it does not depend on tricky procedures, such as normalization or subtraction.

Although regarded as one of the most cost-effective methods (R.A. Shimkets et al., 1999) the limitation of SAGE is that the corresponding gene can be identified only for the tags deposited in gene banks making its efficiency dependent on the complexity of available databases.

The emergence of low-cost, high-throughput DNA sequencing methods has allowed plant biology to enter the 'genomics era'. In particular, projects involving large-scale sequencing of cDNA [expressed sequence tags (ESTs)] are on-going for a wide variety of crop plants (P.R. J Birch et al., 1999). The ESTs recovered from a certain cell type indicate what kind of genes are expressed and the redundancy of a sequence gives an idea regarding the expression level. Mega-scale reverse northern approach are possible thanks to high-speed robotic printing of cDNAs. Originally, cDNAs were spotted on nylon membranes and hybridized with traditional methods (P.W. Dunne et al., 1992). With the current **microarray technology** thousand of clones are displayed on just a couple

of square centimeters of glass support and are hybridized in microvolumes with fluorescently labeled cDNA probes, resulting in improved screening sensitivity (M. Shena et al., 1995).
The power of this technology is self-evident: data can be collected for large numbers of genes in one experiment and genome-wide expression patterns can be observed. However, major limitations reside in the questionable sensitivity of the probes and in the relatively costly and time-consuming collection of the set (unique) sequences. Besides the microarray technique allow high-throughput screening, the efficiency depends on the method used to collect the arrayed sequences.

1.5 *PYRENOPHORA GRAMINEA*-BARLEY INTERACTION

Pyrenophora graminea Ito and Kuribayashi (anamorph Dresclera graminea) is a seed-borne pathogen causing barley leaf stripe. The disease is widely distributed in most barley growing areas, where it causes serious damages and yield losses (G. Delogu et al., 1989).
Infection starts during germination when the mycelium found on the pericarp of infected seeds penetrates the coleorhiza and, from there, it colonises the plant systemically. Fungal hyphae grow intercellularly from the coleorhiza up on all sides: to the scutellum and the roots, where they can remain trapped, or to the scutellar node, where they start infection of the germling (fig. 5).
In the basal part of the coleorhiza and in the scutellar node of incompatible interactions, the hyphae seem to degenerate, a fact that may be due to some kind of plant defence reaction in these regions. The establishment of plant infection is time-related. The longer it takes for the tissue to react to the invanding hyphae, the better the chances are of the fungus growing through the coleorhiza and the scutellar node (A. Haegi et al., 1998).

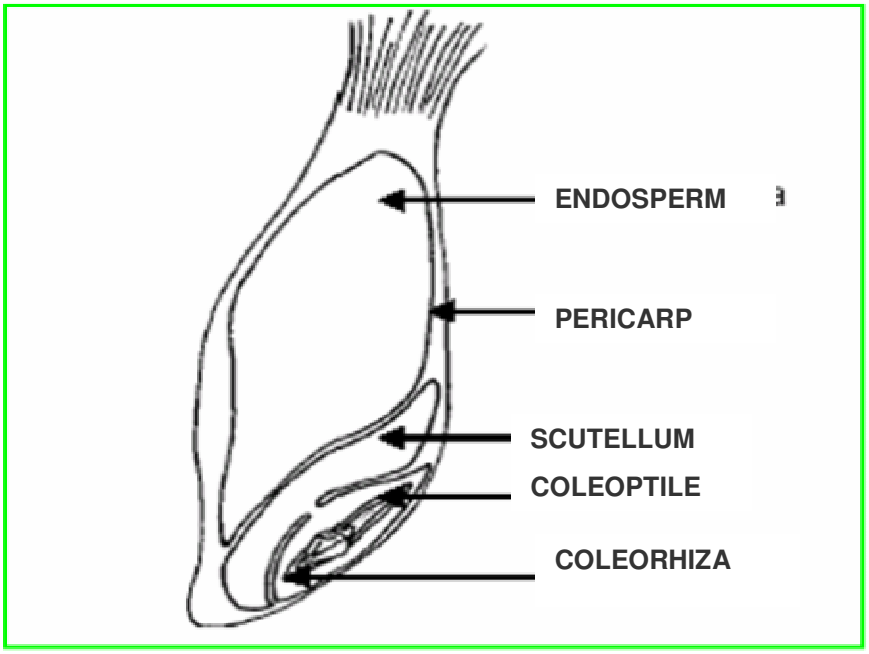


Fig. 5 Seed structure.

Fig. 6 The chlorotic stripes on barley's leaves.

In incompatible interactions, infection stops at this stage, whereas in compatible ones hyphae continue to grow into the coleoptile and later leaves and stem (R. Plantenkamp et. Al., 1976; W.R Skoropad et al., 1956).

In the latter case, symptom may even be visible at the first-leaf stage, although often they appear at the 4-5 leaf stage. Symptoms consist of chlorotic stripes that gradually extend to the full length of the leaf and finally become necrotic (fig 6). Usually infected plants stop their development, desiccate prematurely and are more or less sterile. The conidiophores of the pathogen emerge from the leaves along the necrotic stripes and conidia are able to infect seeds of healthy plants until their maturity. The fungus is unable to cause any secondary infections through leaf-to-leaf transmission.

1.5.1 Pathogen and host variability

The interaction *P. graminea*-barley provides a good model for understanding the molecular basis of diseases caused by seed-borne pathogens. Both field tests and inoculation experiments performed with monoconidial isolates suggest a broad variability in plant response to *P. graminea*, ranging from high resistance (percent of infected plants below 2%) to high susceptibility (percent of infected plants above 80%) (A. Tekauzet al.,1983; G. Delogu et a., 1989; A. Gatti et al., 1992).

This variation could be due either the genetic variability for resistance in the host or genetic variability for pathogenicity in the pathogen. In an extensive study conducted in the field with more thean 1,000 cvs and lines it has been observed that 42,4% of the barley cvs had 0-10% diseased plants; highly susceptible cvs (near 100% diseased plants) were also observed (J.P Skou & V. Haahr 1987; J.P Skou et al., 1994). Delogu et al., (1989), analysing a set of winter barley cvs of different origin for leaf stripe resistance in a replicated field trial, identified genotypes with levels of resistance ranging from highly resitant to highly susceptible.

A considerable variation in pathogenicity among different isolates was first reported by Knudsen (1986). Later, (A. Gatti et al., 1992) investigated the variation of a population of *P. graminea* collected in different Italian barley-growing areas: 12 isolates were characterized and evaluated for virulence by the artificial inoculation of 19 barley cultivars; the isolates were then classified as virulent, and some of them showed a high level of specialization. The selective pressure of the pathogen strains has led to the evolution of different resitance genes in barley.

Barley cultivar	*Pyrenophora graminea's* isolates												
	2	5	10	8	18	12	19	9	3	13	6	7	Medi
Arda	27	7	7	47	1	5	0	0	0	0	0	0	8
Aramir	56	0	61	49	0	3	5	0	0	0	0	0	15
Alpha	21	29	11	0	0	0	1	0	0	0	0	0	7
Barberousse	62	72	0	59	0	77	15	33	0	12	0	2	28
Etrusco	71	42	83	43	83	23	57	0	0	0	1	0	34
Gerbel	80	0	37	62	0	0	38	0	0	0	0	0	18
Igri	21	21	7	11	0	0	0	1	0	0	0	0	5
Jaidor	80	0	42	33	54	68	0	6	0	0	0	0	24
Kaskade	55	39	57	56	4	35	17	0	0	0	0	0	22
Mirco	90	84	89	39	31	59	56	0	24	0	0	4	40
Novo perga	87	67	40	65	0	0	3	0	0	6	0	0	22
Onice	4	0	0	8	0	0	0	2	0	0	0	0	1
Panda	73	84	10	24	35	0	6	0	0	0	3	0	20
Robur	77	62	23	6	26	5	0	0	0	2	2	0	17
Selvaggio	66	45	50	55	54	5	30	49	0	0	9	0	30
Tania	35	25	10	26	0	3	21	1	0	0	0	1	35
Thibaut	4	82	5	1	0	1	3	3	0	3	0	1	9
Tipper	9	0	0	1	0	0	0	10	0	0	1	0	2
Zita	12	8	15	5	1	1	0	0	0	0	0	1	4
Media	49	35	31	29	16	15	13	6	1	1	1	1	

Tab 1 :Barley'seedling infection ratio by artificial inoculation of P. graminea 12 isolates

1.5.2 Pyrenophora graminea pathogenicity

The only biochemical fungal factor known to be involved in this interaction is the Pg .toxin of *P. graminea;* culture filtrates and cells walls contain this phytotoxic compound which, upon infiltration in barley leaves, reproduce symptoms of barley leaf stripe (A. Haegi et al., 1994).

Gel filtration separated the phytoxic activity into two fractions of 250-350kDa and 55kDa, respectively, containing both carbohydrate and protein moieties (A. Haegi & A. Porta-puglia 1995).

The major fraction and the non-purified phytoxin had a strong tendency to aggregate as fibrilar material or crystal-like structures; this feature is lost in the minor fraction, indicated as Pg toxin, which originates from the association of the major one. This observation suggests that the toxic copmpound may be associated, at least in vitro, with hydrophobin-like molecules.

The toxic activity resides in the carbohydrate moiety: treatment with different proteases did not affect toxicity, which was instead eliminated by treatment with β-glucuronidase.These results indicate that the glycidic component of the toxic compound, which contains glucuronic acid, is responsible for the toxic activity. While the non-purified toxic compound and the major fraction also induce necrosis on non-host plants, the Pg toxin induces necrosis only on barley leaves, thus

being host-specific. This suggest that the major fraction could contain additional toxic compounds different from Pg toxin.
It was not possible to find isolates that did not produce the toxin (either naturally or by mutation), suggesting that the compound is in some way necessary for fungal viability.

1.5.3 Infection process

Skopard and Arny (1956) have observed that, in resistant barley varieties, hyphae seem to degenerate in the basal part of the coleorhiza and in the scutellar node, as in a sort of hypersensitive reaction. Many questions regarding the growth and behaviour of the fungus during the early stages of infection remain unanswered because of the difficulty of monitoring the growth of the fungus inside the pericarp first and into the plantlet later. The use of transformants expressing the β-glucoronidase gene (GUS) allowed monitoring along the infection steps.
The study of the infection process with the isolate fungi GUS (M. Aragona & A. Porta-Puglia, 1999) has shown that the fungus invades the susceptible cultivars whole embryo, while the fungus is restricted to the scutellar node in the resistant ones. The GUS gene has allowed to point out in which infection stage the resistant varieties build up a defence reaction able to stop pathogen.
In the screening by Gatti et al., 1992 the isolate Dg2 was identified as the most virulent among those tested. Isolate Dg2 has been used during recent years as a tool to select for resistance to *P. graminea* in breeding programs. Lines resistant to this isolate are also resistant to the natural field population of the pathogen, which is spread by a naturally susceptible cultivar.

1.5.4 The barley resistance to Pyrenophora graminea

Host pathogen resistance to biotic stresses can be classified as qualitative or quantitative. The former refers to traits which behaves as Mendelian genes of large effect whereas the latter decribes resistance that shows continuous variation and is usually partial (A.J. Castro et. al., 2003).
The main problem with qualitative resistance is its frequent low durability (Parlevliet, 1977) while quantitative resistance is often durable (Parlevliet, 1989). Before the advent of quantitative trait locus (QTL) mapping, analyzing the genes that control complex disease resistance traits was an overwhelming task. With DNA markers and QTL mapping, complex forms of disease resistance and their underlying genes are now more accessible (N.D. Young, 1996).
Qualitative and quantitative formes of resistance to barley leaf stripe have been identified.

A single genetic factor controlling complete resistance to *P. graminea*, derived from *Hordeum laevigatum* via cv "Vada", has been introduced into most resistant North-European two-rowed spring barley cvs (J.P Skou & V. Haahr 1987; J.P Skou et al., 1994).

This "Vada resistance" was probably introgressed into the barley genome along with the *MlLa (Laevigatum)* powdery mildew resistance, because the two factors have been found to be linked; this resistance named *Rdg1a*, has been mapped on the long arm of barley cromosome 2 (H. Giese et al., 1993; S.B Thomsen et al., 1997).

It is also known that cvs quantitatively resistant to leaf stripe are widespread in Europe (J.P Skou et al., 1994). A major QTL conferring resistance to barley leaf stripe was identified in the cv "Proctor" by means of QTL analysis; this QTL accounted for 58,5% of the variation in the trait and was mapped on the centromeric region of barley chromosome 1 (N. Pecchioni et al., 1996).

A new source of resistance, designated Rdg2a, was mapped on the telomeric region of barley chromosome 1 (7H) (G. Tacconi et. al., 2001). Plants containing Rdg2a are almost immune to the disease caused by avirulent isolates, exhibiting no brown stripes on the leaves. Rdg2a-containing breeding lines selected using Dg2 also appear to be resistant to the natural field populations of the pathogen, suggesting that Rdg2a may have a useful range of activity (L. Arru et al., 2003a).

2. AIM OF THE WORK

Plants use various defense mechanism for protecting themselves against infection by pathogens.
These mechanism include inducible modification of plant cell walls, the synthesis of toxic phytoalexins, and accumulation of pathogenesis related proteins, often protease inhibitors or pathogen targeted hydrolytic enzymes(Hahlbrock and Grisebach, 1979).
In literature it is known that the storage tissues of the cereal seeds are rich in inhibitors and microbial hydrolases (Shewry and Miflin (1985). Furthermore cereal plants and their seeds defend themselves against infection by fungal pathogen by enzyme and peptide antimicrobial (Leah R. et al.,1990).
The aim of this PhD work was to study early transcriptional changes which occur into the embryos in order to identify the genes which are actived these tissues during a resistent and susceptible defence reaction.
In this study we have used *Pyrenophora graminea,* the agent of barley leaf stripe and barley (*Hordeum vulgare*).pathosistem as a model for seed borne diseases.
Pyrenophora graminea is a seed-borne pathogen which infects the seedling thorugh the coleorhiza from where it grows intercellulary in the parenchyma layers of the scutellur node up to the coleoptile.
Skoropad and Arny (1956) have observed that, in resistant barley varieties, hyphae seem to degenerate in the basal part of the coleorhiza and in the scutellar node, as in a sort of hypersensitive reaction.
Utilising a transformed fungi isolated with the reported gene GUS it was recently demonstrated hot in isogenic lines of barley, containg a monogenetic resistant factor *Rdg2a,* the pathogen was blocked at the level of the scutellar node (Fig.7A) while in the susceptible lines the pathogen growth in the embryo tissue (Fig. 7B).

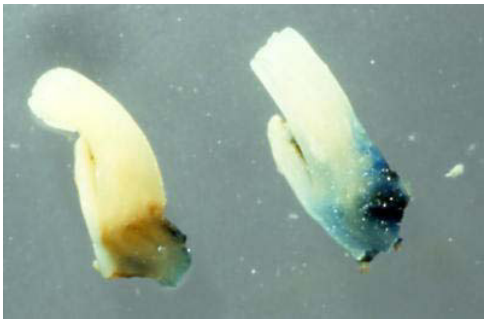


Fig.7 A B

One resistant barley NIL (Mirco-Rdg2a) and the susceptible resistent parent (Mirco).

Three different analysis technique were employed to analyse plant pathogen interactions in defining the genetic components involved, and the transcriptional changes that occur in both the host and the pathogen:

1) A cDNA suppression subtractive library;
2) cDNA-AFLP technique
3) Histological microscopic analysis

3. MATERIALS AND METHODS

3.1 Plants materials

➢ The *P. graminea* isolate used, Dg2 (previously named I2), is the most virulent of a collection of 12 monoconidial isolates tested on European barley cvs (Gatti et al., 1992).

The fungus has been grown in Petri dishes on (Potato Dextrose Agar, Difco) PDA 42g/l for 12 days in the dark at 18^0 C.

➢ Mirco is a six-rowed hulled caryopsis winter cultivar; is highly susceptible to *P. graminea* isolate Dg2. Thibaut is a six-rowed hulled caryopsis winter cultivar; is highly resistant to *P. graminea* isolate Dg2.

➢ Near-isogenic lines (NILs) carrying resistance against the isolate Dg2 of *P. graminea* were generated from a cross between the resistant cv "Thibaut" and the highly susceptible cv "Mirco", followed by six backcrosses with the susceptible parent (Fig.8).

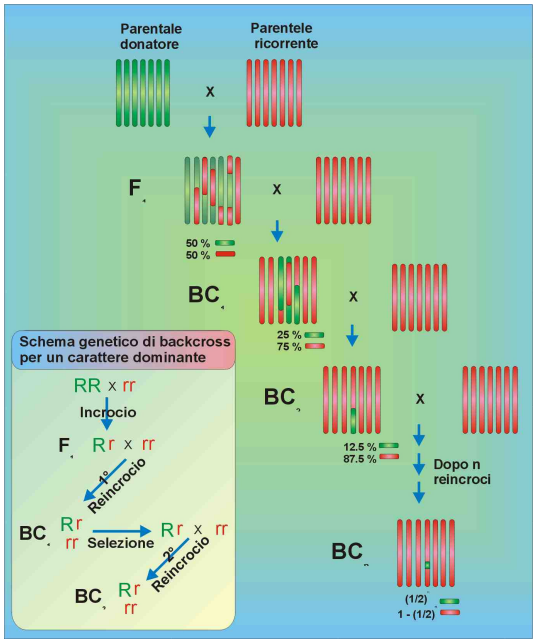


Fig. 8 Near Isogenic Line construction

3.2 Disease testing

About 150 seeds of NILs and Mirco were sterilized in 70% ethanol for 30s and 6,6% v/w NaOCl for 15 min, rinsed well in several changes of deionized water and then incubated in Petri dishes between two layers of (Potato Dextrose Agar, Difco) PDA 42g/l colonized by the actively growing mycelium of Dg2 isolate "Sandwich method" (Fig 9). The seeds were incubated for different time points of inoculation (3, 7, 11, 14 days) in the dark at 6^0 C, about 150 seeds for each time point were utilised.

The control seeds of each line were sterilized in the same manner and grown under the same conditions on 3 MM filter paper soaked in sterile water.

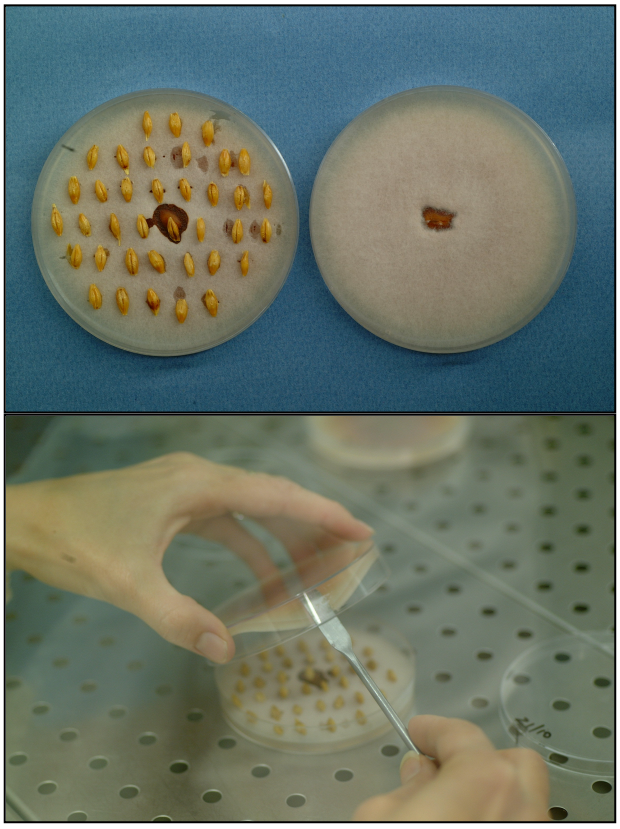


Fig 9. Sandwich method

3.3 mRNA isolation from embryos seeds

Frozen NIL and Mirco's embryos were ground in liquid nitrogen to a fine powder, suspended in 50 mM Tris pH 9, 10mM-EDTA, 0,1 M NaCl, 2% (w/v) SDS and subjected to three phenol-chloroform (1:1, v/v) extractions. Then the poly(A) RNAs were isolated by affinity chromatography

on oligo(dT)-cellulose (Boerhinger Mannheim) according to published methods (Sambrook et al., 1989).

The RNA poly(A) extracted has been used to produce and screening a subtractive library by suppression-subtractive hybridization by using the CLONTECH PCR-Select kit.

3.4 Generation of a subtractive library

Genetic materials utilized have been seeds of Near Isogenic Lines carrying resistance against the isolate Dg2 of *P. graminea* and seeds of susceptible cultivar Mirco. The seeds were incubated for different time points of inoculation (3, 7, 11, 14 days) in the dark at 6^0 C, about 150 seeds for each time point were utilised.

The control seeds of each line were sterilized in the same manner and grown under the same conditions on 3 MM filter paper soaked in sterile water.

Subtractive hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. First, both mRNA populations are converted into cDNA. The cDNA in which specific differentially accumulated transcripts are to be found is called “tester” (seeds infected), and the reference cDNA is called “driver” (seeds control). The tester and driver cDNAs (fig. 10) are digested with a four-base-cutting restriction enzyme (RsaI) that yields blunt ends. The tester cDNA is then subdivided into two portions, each of which is ligated to a different ds cDNA adaptor. The ends of the adaptor do not have a phosphate group, so only one strand of each adaptor attaches to the 5’ ends of the cDNA.

The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in.

Two hybridizations are the performed. In the first, an excess of driver cDNA is added to each portion of tester cDNA. The samples are then heat denatured and allowed to anneal. Generating the type **a, b, c,** and d molecules in each sample.Type **a** *ss* molecules include equal concentrations of high- and low-abundance sequences bcause reannealing is faster for the more abundant molecules due to the second-order kinetics of hybridization. At the same time, type **a** molecules are significantly enriched for differentially expressed sequences, as common nontarget cDNAs form type **c** molecules with the driver. During the second hybridization, the two primary hybridization samples are mixed together. Now the type **a** cDNAs from each tester sample are able to associate and form new type **e** hybrids, *ds* tester molecules with different *ss* adaptor sequences on each end. Fresh denatured driver cDNA is added to further enrich fraction **e** for differentially expressed

sequences. The entire population of molecules is then subjected to PCR to amplify the desired differentially expressed sequences. During PCR, type **a** and **d** molecules are missing primer annealing sites, and thus cannot be amplified. Due to the suppression PCR effect, most type b molecules form a pan-like structure that prevents their exponential amplification. Type **c** molecules have only one primer annealing site and can only be amplified linearly. Only type **e** molecules, which have two different primer annealing sites, can be amplified exponentially. These differentially expressed sequences are greatly enriched in the final subtracted cDNA pool, which can be cloned to create a subtractive library.

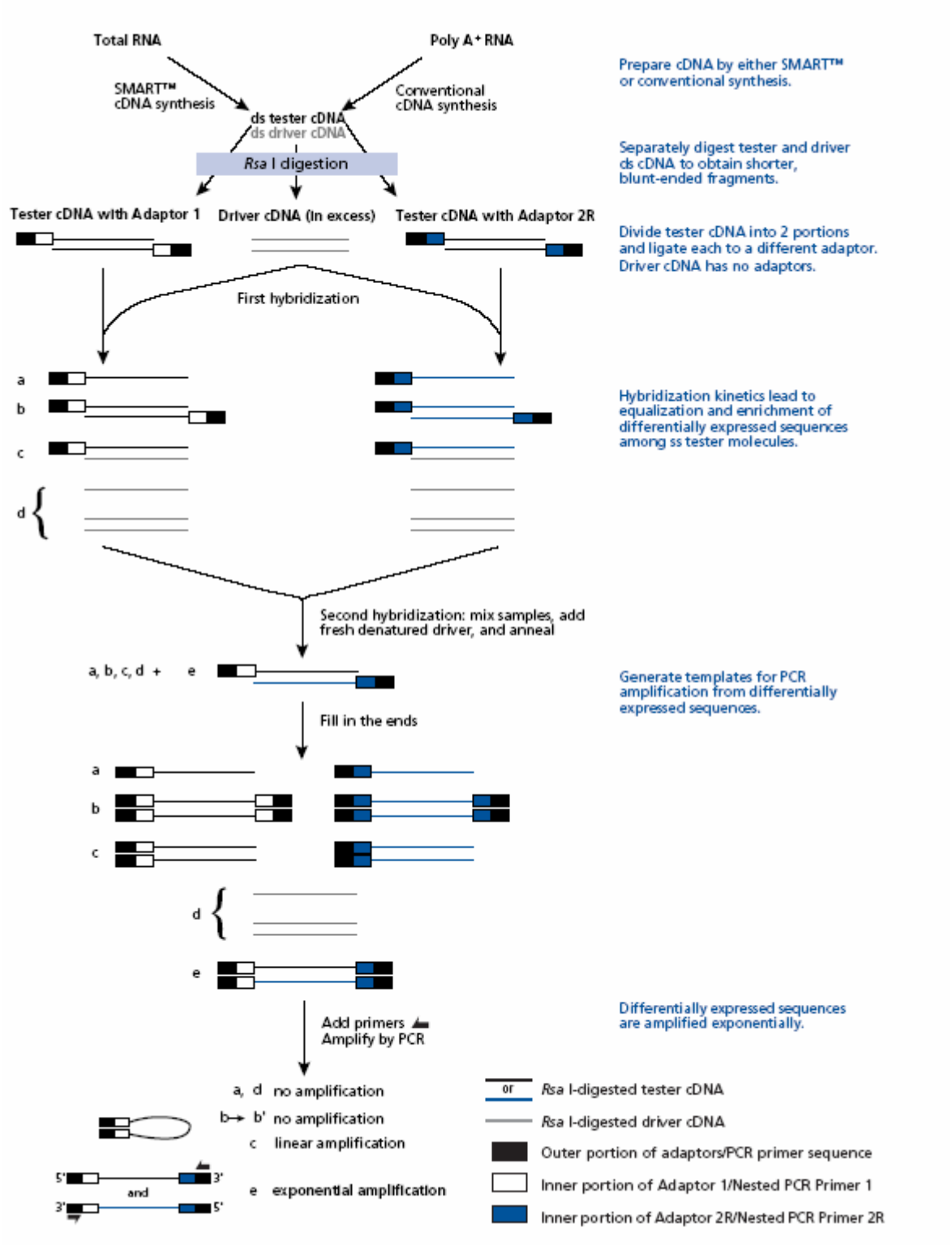


Fig 10. PCR-Select scheme

3.4.1 Adaptor and Primer Sequences

cDna synthesis primer 5'-TTTTGTACAATT30-3'

Primer 1 (P1): 5'-CTAATACGACTCACTATAGGGC-3'

Nested primer 1 (NP1): 5'-TCGAGCGGCCGCCCGGGCAGGT-3'

Nested primer 2r (NP2R): 5'-AGCGTGGTCGCGGCCAGGT-3'

Adaptors sequences:

Ad1:

5'-CTAATACGACTCATATAGGGCTCGAGCGGCCGCCCGGGCAGGTGGCCCGTCCA-3'

Ad2:

5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGTGCCGGCTCCA-3'

3.5 Cloning in TA vector

The subtractive library has been cloned in pGEM-T Easay vector (pGEM-T Easy Vector System, Promega). Before the ligation, the cDNA subtracted mixture was subjected to one additional incubation of 1h at 72^0 C with addition of dATP and Taq DNA polimerase (Invitrogen) to assure that most fragments contain A protruding. Approximately 100ng PCR-amplified cDNA was ligated without further purification into 50ng vector and the reaction mixture was used to transform in E. coli Competent Cells DHα (Invitrogen). The library was plated on LB agar plates containg 100μg/ml ampicillin and 50μg/ml X-gal. The plates were incubated at 37^0 C until the colonies were identified by a blue/white screening system.

3.6 Hight density reverse northern blot and screening

Each individual colon as picked and inoculated in 20 sterile 96-well culture plate containing 150 μl per well of "Freezing Broth" culture and ampicillin 100μg/ml.

After incubation overnight at 37^0 C the plates were stored at -80^0C. At the same time each colonies was picked in a 96-well plate and then a colony PCR was performed. The product of each colony PCR was loaded onto agarose gel and denatured and blotted with NaOH 0,4N onto nylon filters

Millipore Immobilion ^{TM} –Ny^{+} Transfer Membrane. To maximize the sensitivity of the PCR-Select Differential Screening Kit probes for hybridization were derived from the forward- and reverse-subtractive libraries in which tester serves as the driver and the driver as tester.

Probes forward and reverse, before their utilization, were digested with RsaI enzyme to remove the adaptors which could cause an high background.

About 200ηg for each probe was radiolabeled with [α-^{32}P] dCTP by random-priming reaction.

The hybridization was performed at 65°C in 6× SSC, 2× Denhardt's solution (Sambrook *et al*., 1989), 0.1% SDS, and 100 μg ml^{-1} of denatured herring-sperm DNA. The filters were washed for 20 min at 65°C two times with 2× and 1× SSC containing 0.1% SDS.

After exposure to film (Kodak) putative positive clones were selected.

Candidate clones were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit on an ABI PRISM 310 Genetic Analyzer automated sequencing machine (PE Applied Biosystem).

DNA sequences were compared with those in the non-redundant databases by using the BLAST-N and BLAST-X algorithms, available at the NCBI (http://www.ncbi.nlm.nih.gov) and TIGR (http://www.tigr.org) sites.

3.7 Northern blotting analysis

Equal amounts (2μg) of poly(A) RNAs for each sample were separated on an 0,8% agarose formaldehyde gel and transferred and blotted onto a Hybond-N^{+} membrane (Amersham Biosciences).

3.8 cDNA-AFLP TP

The cDNA-AFLP TP analysis as carried out at the laboratory of prof. Massimo Delledonne in the Dipartimento Scientifico e Tecnologico holding the Agro-Biotechnology Faculty of University of Verona.

The analysis utilized the resistant cultivar Thibaut, the susceptible cultivar Mirco and the *Pyrenophora graminea* mycelium. Seeds were incubated for three different time points of inoculation (7, 10, 15 days) in the dark at 6^{0} C.

The system is based on the use of highly stringent PCR conditions, facilitated by adding double-stranded adaptors on the ends of restriction fragments which serve as primer sites during amplification. Selective fragment amplification is achieved by adding one or more bases on to the

PCR primers which will only then be successfully extended if the complementary sequence is present in the fragment flaking the restriction site, thereby reducing the number of visualized bands (Bachem et al., 1996).

AFLP-TP approach to differ from cDNA-AFLP only to one supplementary step interposed between two digestions (Fig. 11). That allow to isolate only cDNA *ds* terminal shares which contain oligo dT.

The main features of this technique are: 1) high sensitivity 2) unique fragment for each cDNA 3) proportionality between band intensity and relative amount of different cDNA 4) lower number of selective amplifications needed (32 instead of 256).

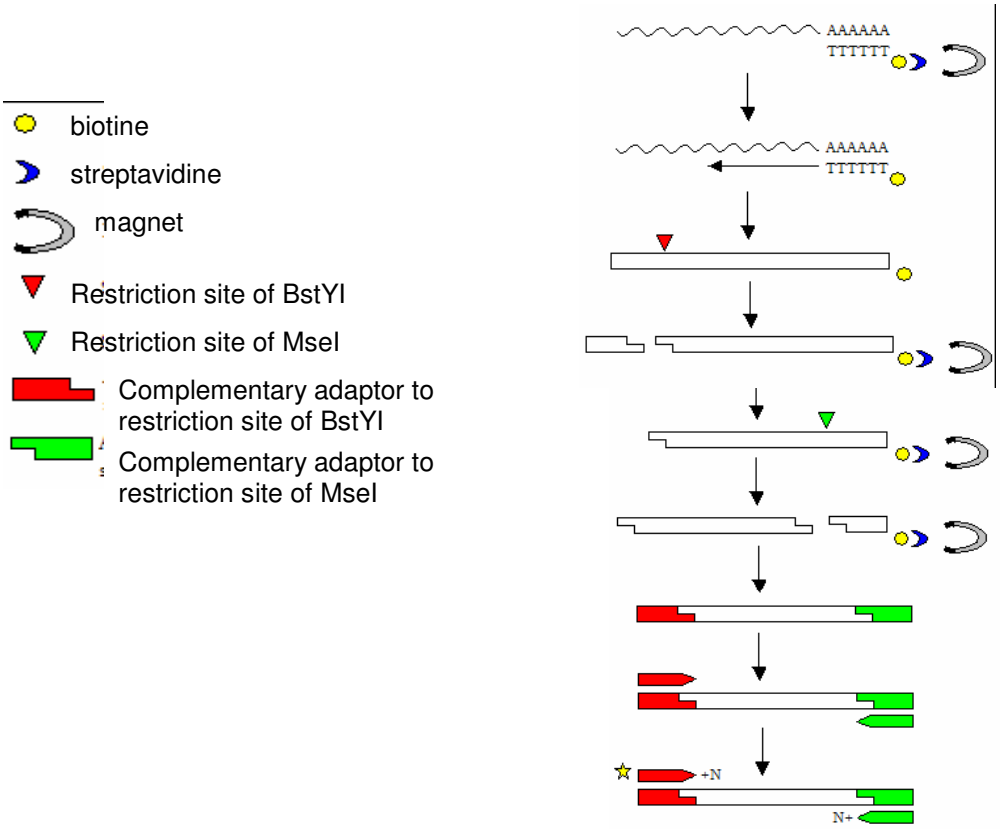


Fig 11. Description of cDNA-AFLP TP

1100 fragments differentially expressed were obtained. 400 fragments were cloned in pGEM-T Easay vector (pGEM-T Easy Vector System, Promega).

In the laboratory of Istituto Sperimentale for the Cerealicoltura of Fiorenzuola d'Arda each individual colony as picked and used to inoculate 96-well culture plate containing 150 µl per well of "Freezing Broth" and ampicillin 100µg/ml.

After incubation overnight at 37^0 C the plates were stored at -80^0C. At the same time each colony was inoculated in 5 ml of LB culture, growth overnight at 37^0 C, then plasmid DNA was extracted with Wizard Plus SV Minipreps DNA Purification System (PROMEGA).

Each miniprep as digested with EcoRV enzyme and controlled on an agarose gel 1%.

Each cloned fragment as sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit on an ABI PRISM 310 Genetic Analyzer automated sequencing machine (PE Applied Biosystem).

The remaining 700 fragments were directly amplified by PCR (Tab. 2,3):

PCR reaction conditions		
	stock	**µl**
H_2O	20µl	
PCR buffer	10X	5
dNTPs	10mM	0,8 µl
MgCl2	50mM	1,5 µl
Primer MseAI	1µM	1,2 µl
Primer BstYI	1µM	1,2 µl
Taq Pol.	1u/ µl	0,2 µl

Tab 2. PCR reaction condition

Thermal cycle		
Temp ^{0}C	**Time sec**	**n^0 cycle**
94^0C	4min	1
94^0C	40	35
52^0C	60	
72^0C	40	
72^0C	5min	1

Tab 3. Thermal cycle

Each amplified fragment as controlled on an agarose gel 1%.

Each amplified fragment as purified to (Istituto Sperimentale for Patologia Vegetale, Rome) with Nucleospin 1 and 2 Macherey-Nagel and then sequenced to Gene Lab ENEA of Casaccia - Rome.

3.9 Histochemistry

Sections of fresh embryo material were cut with a razor-blade, mounted in water, examined with a epifluorescence microscope using a UV filter set with a 335- to 380-nm excitation and a 420-nm barrier filter, then immersed in sodium hypochlorite (4% active chlorine) for 10 min, rinsed with water and stained. After staining, sections were mounted in glycerine/water (15%, v/v) and observed with a light microscope.

Phenolic compounds were detected with Toluidine Blue. Sections were immersed in 0.01% (w/v) Toluidine Blue in 0.1 M potassium dihydrogen phosphate-NaOH buffer, pH 6, for 2 min.

Suberin was stained by immersing sections in 50% ethanol for a few seconds, then in 0.07% (w/v) Sudan III in 70% ethanol for 5-10 min and then in 50% ethanol for about 1 min.

Weisner test (phloroglucinol-HCl) was employed for analysis of lignins. Sections were immersed in 3 volumes of ethanol mixed with 1 volume of concentrated HCl, for about 30 min.

4. RESULTS AND DISCUSSION

4.1 Realization of a cDNA subtracted library with PCR-Select

The subtractive library was obtained utilising as tester (treated) a RNA poly(A) sample obtained from Mirco and NIL embryos treated as following: germinating seeds were grown for different timing (3, 7, 11 and 14 days) on Petri dishes between two layers of Potato Dextrose Agar colonized by actively growing mycelium of Dg2 the isolate. As driver sample (control) the seeds of each line were grown on 3 MM filter paper soaked in sterile water. The samples, driver and tester, were grown in the dark at 6^0 C under controlled condition. From mRNA tester and driver mRNAs were synthesized double stranded cDNAs which subsequently were digested with the restriction enzyme RsaI .The digested tester cDNA were subdivided in two portions. For each portions a ligation reaction with different adaptors asconducted and the subsequent steps were carried out as described in materials and methods..

Each fragments was cloned into a plasmid vector. A Total of 1800 clones were analyzed. The bacteria colony were ordered in a total of 20 96-well plate. The fragments were blotted in double onto nylon filters; 32P dCTP labelled subtracted tester and driver cDNA were used as probe for hybridization.hybridized. The use of radiolabelled subtractive probes was performed in order to allow the identification also of low abundance. Filters were exposed to imaging film.

The subctracted library was therefore sceened with a “reverse Northern” approach. By comparing hybridization signals of the filters hybridized with the driver probe with those hybridized with the driver tester, quantatively differentially expressed genes were therefore identified (Fig. 11).

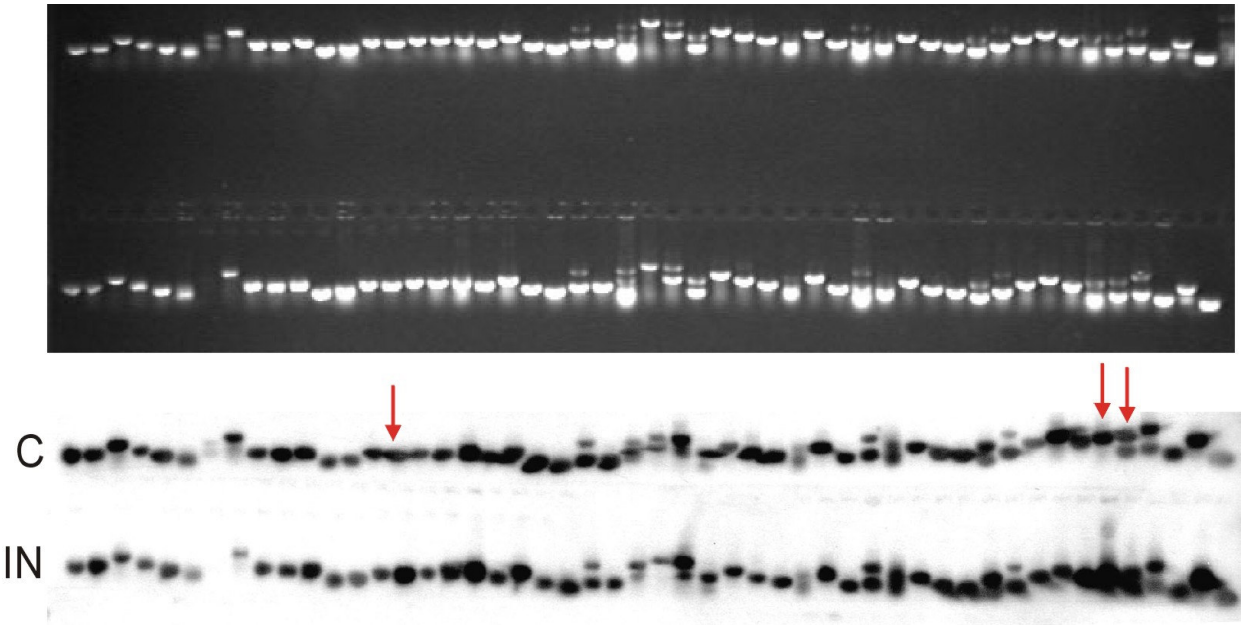


Fig. 11 Comparison hybridization signals of the filters hybridized with the driver probe with those hybridized with the driver tester

During a first screening, 30 putatively differentially expressed clones were selected.

The colonies corresponding to the 30 clones were inoculated in 5 ml of LB culture, and the plasmid DNAs was extracted. Each miniprep was sequenced using T7and T3 primers.

The same clones were subjected to PCR-colony amplification, agarose gel separated and blotted onto nylon filters.

A second round of "reverse Northern" screening was therefore carried out following the some procedure as the first one.

In this second screening were selected 11 clones putatively differentially expressed (table 4).

DNA sequences were compared with those in the non-redundant database by using the BLAST-N and BLAST-X algorithms, available at the NCBI (http://www.ncni.nlm.nih.gov) and TIGR (http://www.tigr.org) web sites.

Some clones were analysed with Northerns in which RNAs (2µg) were obtained from control tissues and from tissues subjected at differentially time points of inoculation.

Clone	Length, bp	Homology	Blast program	GenBank accession no of sequence homolog	E-value
XIXA6	385	H.vulgare mRNA for jasmonate induced protein.	N	X82937	1e-09
XIXE8	443	Oryza sativa (japonica cultivar-group) mdr8 gene for MDR-like ABC transporter, exons 1-12.	X	AJ535064	5e-33
VIE2	493	Triticum aestivum cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPDH mRNA,	N	AF251217.	2e-141
XIXD7	285	H.vulgare gene encoding jasmonate-induced protein.	N	X98124	e-101
XIXG11	402	Oryza sativa (japonica cultivar-group) ribosomal protein L28-like	N	NM_191665	2e-41
XIXA8	350	Hordeum vulgare mRNA for vacuolar membrane proton-translocating	N	D13472.2	3e-78
XVIIIA3	404	Barley glyceraldehyde-3-phosphate dehydrogenase mRNA, 3' end.	N	M36650	0.0
XIXB7	388	RuBisCO subunit binding-protein beta subunit, chloroplast (60 kDa chaperonin beta subunit) (CPN-60 beta)	X	Q43831	1e-13
E3	260	Zea mays ATP-sulfurylase complete cds	N	AF016305	8e-57
E7	290	Triticum aestivum glutathione-s-transferase Cla47 mRNA	N	AY064480.1	1e-44
E9	580	Zea mays mRNA for ferrodoxin-sulfite reductase precursor	N	D50679.1	5e-93

Table 4 Putatively differentially expressed clones

Some clones were instead analysed by “mininorthern”. The “mini-northern” were realized with 2µg of mRNA amounts mRNA at different time extracted from NIL infected and control tissues.

The clone **E3** was high homology with Zea mays cDNA which encode for **ATP-sulfurilase** enzyme

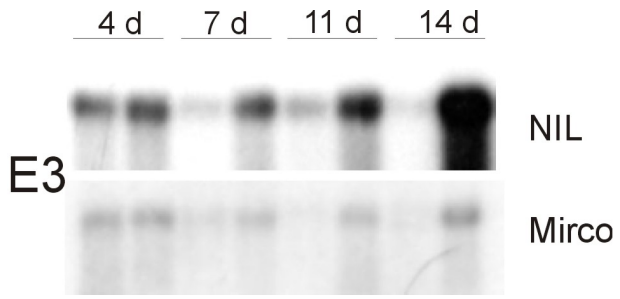


Fig 12 Clone E3 Northern analysis

Transcriptional activity of this gene (fig 12) is higher in the resistant genotype in comparison to the susceptible one.

The clone **E9** is homolog to a Zea mays cDNA which encode for a **ferrodoxin-sulfite reductase**.

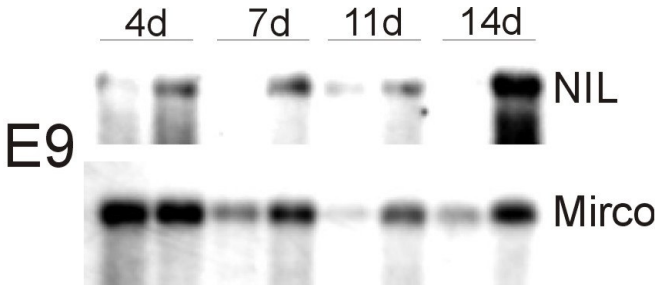


Fig. 13 Clone E9 northern analysis

Northern analysis with this clone shows that NIL sample has a lower expression level of this gene during first timing of infection (4 to 11 days) in compareson to the susceptible Mirco). The expression level increases in the NIL 14 days of infection (fig 13).

The clone **E7** has an high homology with a *Triticum aestivum* cDNA encoding for a **glutathione-S-transferase enzyme.**

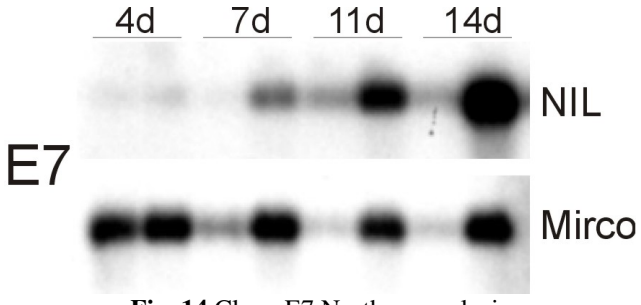


Fig. 14 Clone E7 Northern analysis

The Northern analysis shows that E7 has an high expression level in the susceptible parent Mirco at the first time points of inoculation, while at late timing the expression level was higher in the resistant Mirco (fig 14).

4.1.1 Clones E3 and E9

The clones E3 and E9 homologs respectively with ATP-sulfurylase and ferrodoxin sulfite reductase enzyme encoding genes (table 1) are involved in cysteine (Cys) pathway (Fig 15).

The biosynthesis of Cys constitutes the final step of the sulphur reduction patways in plant.

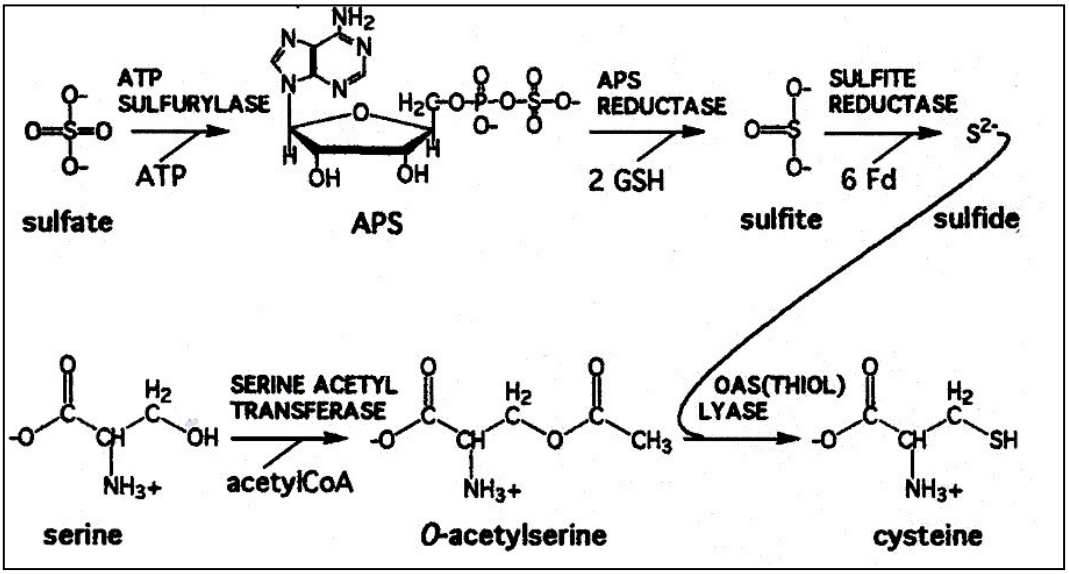


Fig. 15 Cysteine synthesis

The Cys serves as a precursor for the synthesis of various sulfur-containing metabolites, of which glutathione (GSH) represents the major storage and transport form of reduced sulfur (Rennenberg, 1997; Noctor et al., 1998).

4.1.2 Clone E7

This cDNAs is homologs to a Glutathione S-transferase encoding gene (table 4).

Glutathione (GSH) a tripeptide (-L-glutamyl-L-cisteinyl-glycine), distributed in the intracellular space of plants, animals, and microorganisms has two general functions: to remove toxic metabolites from the cell and to maintain cellular sulfhydril groups in their reduced form.

GSH can function as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore it functions directly as a free radical scavenger. GSH may stabilise membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price *et al.*, 1990).

Oxidative stress is a term commonly used to describe plants adverse effects of ROS on plants. A variety of enzymatic and non enzymatic mechanisms exist to metabolize ROS into less harmful chemical species.

The term antioxidant metabolism describes the detoxification of ROS, and the chemicals involved are generally referred to as antioxidants. Glutathione is one of several chemical compounds in plants that are involved in the detoxification of ROS. Glutathione can be oxidized directly by oxidants and also as a component of the Halliwell-Asada cycle that maintains the cellular ascorbate pool in a reduced state (Noctor et al., 1998) (Fig 16).

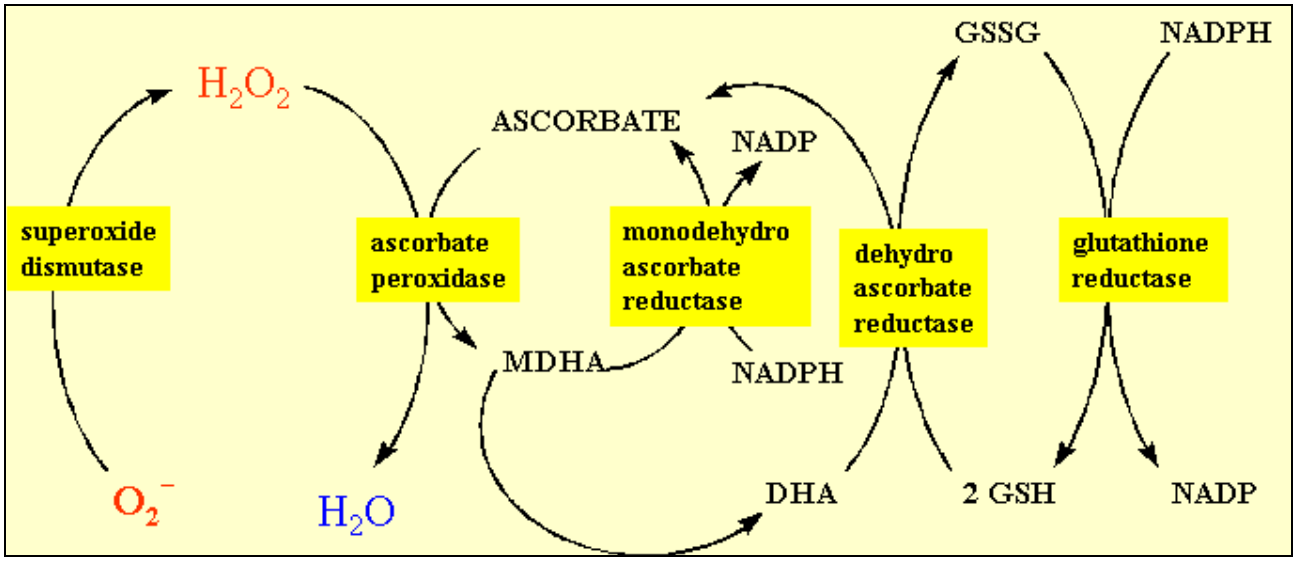


Fig. 16 Halliwell-Asada cycle

The clone E7 is homologs to glutathione-S-transferase, an enzyme which may to be involved in conjuganting electrophiles generated from the reaction of hydroxyl radicals with lipids and DNA, and thus contribute to oxidative stress resistance (Marss K.A, 1996).

Glutathione *S*-transferases has been shown to be induced by a wide range of chemical agents (Ulmasov et al, 1995), wounding, heavy metals, ethylene, and ozone (Marrs, 1996).

Glutathione S-transferases (GSTs) are ubiquitous enzymes catalysing the addition of reduced glutathione (GSH) to electrophilic substrates, which tags them for vacuolar sequestration (Edwards et al. 2000) (Fig 17). GSTs have direct cytoprotective activities and they might be essential for the preservation of plants during environmental stress and disease, as well as for the support of normal development (Marrs 1996). In addition to catalyzing GSH conjugation, GSTs also exhibit glutathione peroxidase (GSH-PX) activity, which suggests a role in protection against oxidative stress.

Sulfate uptake and assimilation
Glutathione conjugation

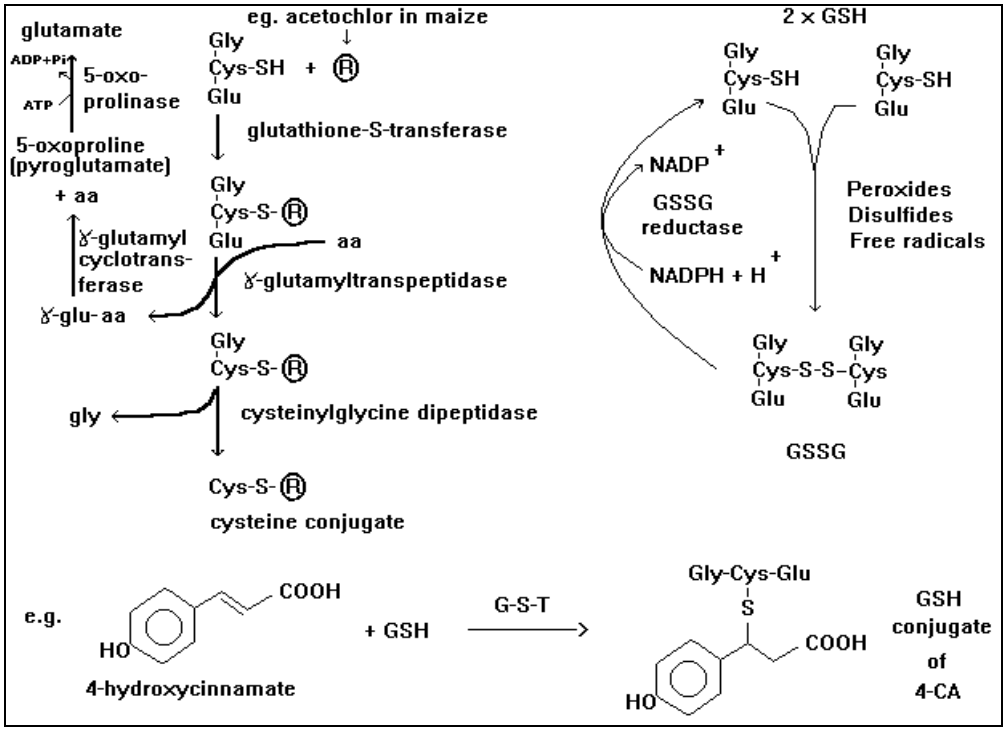


Fig 17. Sulfate uptake and assimilation; glutathione conjugation.

4.1.3 Clone XIXE8

Fig. 18 mininorthern XIXE8

CloneXIXE8 is homolog to a **MDR-like ABC transporter** gene**,** (mini-northern analysis figure 18). *Mdr* genes are genes associated with multi-drug resistance in mammalian tumor cells, and are inducible by a class of compounds known to inhibit chloride ion channels. *Mdr* genes encode a family of P-glycoproteins that belong to the super family of ATP-binding transport proteins. In

plants, these transporters likely play the general role of sequestering, secreting or otherwise detoxifying various xenobiotics.

The ATP-binding cassette (ABC) transporters are members of a large family of active transport proteins energized directly by ATP hydrolysis. Originally identified in microbial and animal cells, ABC transporters are able to use the energy of ATP hydrolysis directly to pump organic molecules (especially large anionic molecules) across a membrane. Like the P-type H^+-ATP synthases, ABC transporters form a phosphorylated intermediate during catalysis, and they are therefore inhibited by vanadate.

Commonly, ABC transporters are found at the tonoplast, where they are sometimes referred to as glutathione conjugate pumps, or GS-X pumps, since they often transport molecules that have been covalently attached to glutathione. The vacuolar GS-X pumps of plant cells function in herbicide detoxification, protection against oxidative damage, pigment accumulation, and the storage of antimicrobial compounds.

These results lead to hypothesis that an increased glutathione synthesis could be connected to a possible scavenging role of ROS produced during fungi infection. This hypothesis is strengthened by strong induction of glutathione- S-transferase gene and by the presence of MDR like ABC-transporter.

This hypothesis induced us to verify if oxalate oxidase gene expression occur. Oxalate oxidase catalyses the conversion of oxalate to CO2 and H_2O_2 and the activity of this enzyme may also be important in certain plant pathogen interaction (Wojtszek, 1997).

Northern analysis was carried out utilising resistant NIL in order to verify if the oxalate oxidase gene expression results following *Pyrenophora graminea* infection (Fig 19).

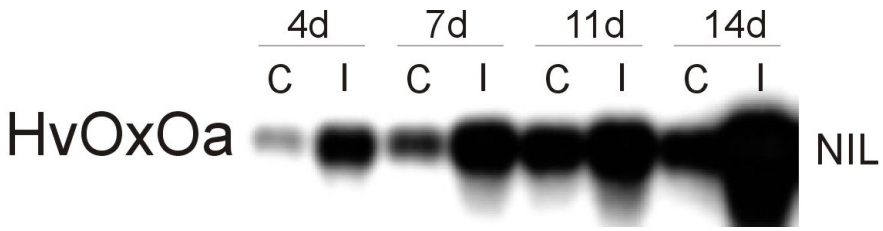


Fig 19. Hordeum vulgare clone that codify to oxalate oxidase enzyme Northern analysis

The northern analysis shows a considerable induction of the oxalate-oxidase gene. The induction is remarkable evident since four day from infection.

The result shows that ROS could be involved in the barley embryo defence response against leaf stripe. The GSTs and ABC pump identified as involved in this study could therefore have a

protection role against toxic compounds produced following reaction with ROS during the embryo defense response.

For the remaining 7 clones present in table 2, only mini-northern analysis is shown because the Northern analysis with different time points to see a likely increase differentially expression level is not still carried out.
For the clones XIXA6 and XIXD7, homologs with H.vulgare mRNA for jasmonate induced protein, are a slight increase of mRNAs was detected in the inoculated samples (mini-northern analysis fig. 20; fig. 21).

Fig. 20 mininorthern XIXA6

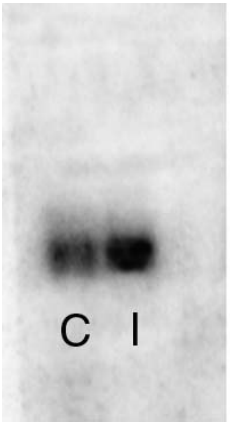


Fig. 21 mininorthern XIXD7

In literature it is known that Jasmonic acid and related compounds are a class of plant hormones that play an important role in regulating many cellular processes, such as wound and defence responses (see introduction pag.).

The clone XVIIIA3 (fig.22) and clone VIE2 (fig.23) are homologs to glyceraldehyde-3-phosphate dehydrogenase ezyme involved in many metabolic process. Clone XVIIIA3 in data bank search show an 00e-value with the enzyme. Also for these clones only a slight increase in the accumulated of the corresponding mRNAs was observed in the inoculated samples.

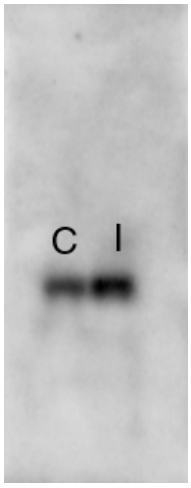


Fig. 22 mininorthern XVIIIA3

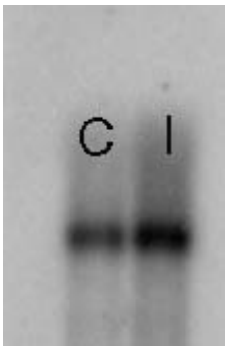


Fig 23 mininorthern VIE2

The Clone XIXG11 (fig. 24) is hmologs with Oryza sativa (japonica cultivar-group) ribosomal protein L28-like a protein involved in post-transcriptional process.

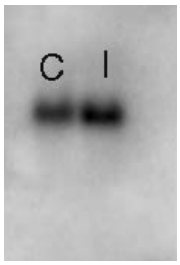


Fig. 24 minorthern XIXG11

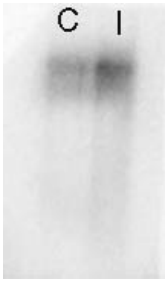


Fig 25 mininorthern XIXB7

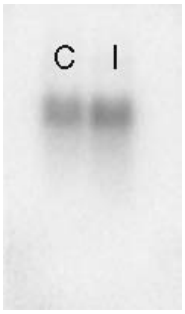


Fig 26 minorthern XIXA8

The clone XIXB7 (fig. 25) RuBisCO subunit binding-protein beta subunit, chloroplast (60 kDa chaperonin beta subunit) (CPN-60 beta). The cpn60s are a group of ubiquitous proteins with a subunit size of approximately 60 kD that a share a functional and structural similarity to the tetradecameric *Escherichia coli* GroEL complex (Gatenby, 1992). Eukariotic representatives of this group include the chloroplast Rubisco subunit-binding protein (Hemmingsen and Ellis, 1986; Hemmingsen et al., 1988) and the mitochondrial cpn60 protein (Prasad and Hallberg, 1989; Tsugeki et al., 1992).

Clone XIXA8 (fig.26) is homolog with Hordeum vulgare mRNA for vacuolar membrane proton-translocating; for this clone the Northern analysis evidentiate assence of differential expression between control and inoculated samples.

4.2 cDNA-AFLP TP

The cDNA-AFLP TP analysis was carried out at the laboratory of prof. Massimo Delledonne in the Dipartimento Scientifico e Tecnologico holding in the Agro-Biotechnology Faculty of University of Verona. The analysis utilised the resistant cultivar Thibaut the susceptible cultivar Mirco and the *Pyrenophora graminea* mycelium. 1100 differentially expressed fragments from seeds were incubated for different time points of inoculation (7, 10, 15 days) in the dark at 6^0 C. A cluster analysis was carried out and 8 expression clusters were obtained.

- Cluster 1: It assembles all *Phyrenophora graminea* genes detected during interaction with the barley plant.
- Cluster 2: It assembles all genes that are induced during interaction in the susceptible line and that are constitutively expressed in the resistant line.
- Cluster 3: It assembles all transcripts expressed in both resistant and susceptible plant only later on infection.
- Cluster 4: It assembles all transcripts induced in response to fungi infection only in the resistant plants.
- Cluster 5: It assembles all transcripts induced in response to fungi infection only in the susceptible plants.
- Cluster 6: It assembles all transcripts induced in response to fungi infection only in the susceptible plants after 15 days from the infection (late phases).
- Cluster 7: It assembles all transcripts induced in the resistant healthy plant and repressed during infection.
- Cluster 8: It assemble all specific genes present inthe healthy susceptible plant whose transcription has been repressed.

About 600 sequences of 1100 have been successfully sequenced and compared with sequences present in the non-reduntant database by using the BLAST-N and BLAST-X algorithms, available at the NCBI (http://www.ncni.nlm.nih.gov) and TIGR (http://www.tigr.org) web site.

The sequences analyzed have been assembled in functional groups. This allowed identification of several groups related to defense response, metabolism and biosynthesis, signal transduction, post-transcriptional processes and many other (Table 5).

Tab.5 Functional groups. N= blastn; X=blastX; T= TIGR

Clone	Length, bp	Cluster	Homology	Blast program	GenBank accession no of sequence homolog	E-value
Metabolism & Biosynthsesis						
TCA19	191		Triticum aestivum putative S-locus receptor kinase	N	gi|62131094|gb|AY963808.1|	2e-10
CAA21	262	5	putative aminotransferase [Oryza sativa (japonica cultivar-group)]	X	gi|31433398|gb|AAP54917.1|	5e-14
CAC23	91	6	Hordeum vulgare putative high-affinity potassium transporter HvHAK1)	N	gi|2746085|gb|AF025292.1|AF025292	6e-14
CAC34	199	6	ALM beta-like [Oryza sativa (japonica cultivar-group)]	X	gi|55773826|dbj|BAD72364.1|	1.9
CAG15	466	1	putative oligopeptidase B [Oryza sativa (japonica cultivar-group)]	X	gi|54291559|dbj|BAD62483.1|	7e-56
CAT8	340	5	putative uracil phosphoribosyltransferase [Oryza sativa (japonica	X	gi|53749356|gb|AAU90215.1|	1e-38
CCA54	177	8	putative LRR-containing F-box protein [Oryza sativa (japonica cultivar-group)]	X	gi|53749250|gb|AAU90110.1|	6e-13
CCA42	420		Hordeum vulgare subsp. vulgare cDNA clone HC108E03 similar to NP_201189.1| (NM_125779) glucosidase II alpha subunit [Arabidopsis thaliana]	N	gi|21885133|gb|BQ740126.1|	0.0
CCA57	300		envelope glycoprotein [Human immunodeficiency virus 1]	X	gi|13661769|gb|AF321868.1|AF321868	1e-06
CCG12	259	5	putative N-carbamyl-L-amino acid amidohydrolase [Oryza sativa (japonica cultivar-group)]	X	gi|52076511|dbj|BAD45389.1|	9e-14
CCG9	350	4	Chain F, Structure Of The Kainate Receptor Subunit Glur6 Agonist Binding Domain Complexed With Domoic Acid	X	gi|60594194|pdb|1YAE|F	5.5
TTC7	449	7	cell division cycle protein 48, putative / CDC48, putative [Arabidopsis thaliana]	X	gi|15231775|ref|NP_190891.1|	4e-20
TTC8	425	7	non-ribosomal peptide synthetase [Methylococcus capsulatus str. Bath]	X	gi|53757566|gb|AAU91857.1|	1.1
TCC17	420	1	large polymerase protein [Isfahan virus]	X	gi|57834043|emb|CAH17548.1|	2.5
CCC1	215		Phosphoglycerate dehydrogenase-like protein, partial (90%)	T	barley|TC139914	4.7e-33
TCC7	455	7	putative cytochrome B5 [Oryza sativa (japonica cultivar-group)]	X	gi|34905998|ref|NP_914346.1|	0.001

TCC84	198	7	BNEL109f9 Barley EST endosperm library Hordeum vulgare subsp. vulgare cDNA clone BNEL109f9 5' similar to phosphatidylinositolglycan-related, mRNA sequence.	N	gi|51517387|gb|CV054366.1|	0.13
TGA23	197	3	quinone oxidoreductase-like protein [Arabidopsis thaliana]	X	gi|21536967|gb|AAM61308.1|	7.2
TGA52	182	8	Hordeum vulgare subsp. vulgare cDNA clone HB107H04_SK.ab1 similar to Hydrogen dehydrogenase, Aminomethyltransferase, mRNA sequence.	N	gi|19152318|gb|BM816304.1|	1e-72
TGT11	339	5	Human DNA sequence from clone RP3-420J14 on chromosome 6p24.1-24.3Contains an S-adenosylmethionine decarboxylase 1 (AMD1) (EC4.1.1.50, ADOMETDC) pseudogene and a CpG island	N	gi|4581351|emb|AL035671.5|HS420J14	0.18
TGT26	470	5	putative endoplasmic reticulum oxidoreductin [Oryza sativa (japonica cultivar-group)]	X	gi|50582733|gb|AAT78803.1|	2e-31
TGT31	284	5	prolyl 4-hydroxylase [Acanthamoeba polyphaga mimivirus]	X	gi|55417206|gb|AAV50856.1|	9.5
TGT34	266	5	Oryza sativa (japonica cultivar-group), mRNA translation initiation factor 5A [Oryza sativa (japonica cultivar-group)]	X	gi|50882443|ref|NM_194115.2|	1e-36
TTA16	287		Oryza sativa (japonica cultivar-group) aspartate aminotransferase	N	NM_191327	e-100
TGG15	200		Hordeum vulgare catalase (Cat2) mRNA, complete	N	gi|684947|gb|U20778.1|HVU20778	1.9
CTA22	351	6	H.vulgare mRNA for alpha-amylase	N	gi|2213471|emb|Y11276.1|HVAA24AMY	3e-130
TCA31	258		Pyrus communis putative starch branching enzyme I-like mRNA,	N	gi|47059615|gb|AY435423.1|	0.19
TGC41	140		H.vulgare mRNA for UDP-glucose pyrophosphorylase	N	gi|1212995|emb|X91347.1|HVUDPGPP	8e-64
TTA11	297		Hordeum vulgare subsp. vulgare mRNA for ADP-ribosylation factor	N	gi|23304412|emb|AJ508228.2|HVU508228	1e-135
TGG15	200		Hordeum vulgare catalase (Cat2)	N	gi|684947|gb|U20778.1|HVU20778	1.9
Metabolism						
CGG16	391	6	putative microtubule associated protein [Oryza sativa (japonica cultivar-group)]	X	gi|50508631|dbj|BAD31027.1|	7e-08
CGG38	576	7	putative Aconitate hydratase [Oryza sativa (japonica cultivar-group)]	X	gi|50941891|ref|XP_480473.1|	1e-41

CGG39	437	7	putative acyl-activating enzyme [Oryza sativa (japonica cultivar-group)]	X	gi|50919229|ref|XP_470011.1|	1e-08
CGG8	407	6	dissimilatory sulfite reductase alpha subunit [Archaeoglobus veneficus]	X	gi|33320441|gb|AAQ05933.1|	1.9
CGT1	545	6	putative Vacuolar ATP synthase subunit F [Oryza sativa (japonica cultivar-group)]	X	gi|50916028|ref|XP_468478.1|	1e-61
CGT17	400	1	putative DNA damage binding protein 1 [Oryza sativa (japonica cultivar-group)]	X	gi|50932983|ref|XP_476019.1|	1e-59
CGT18	370	1	Arabidopsis thaliana clone U20464 putative adenine phosphoribosyltransferase (At4g22570) mRNA, complete cds	N	gi|28393996|gb|BT004412.1|	0.0
CGT2	407	1	Triticum aestivum RUB1-conjugating enzyme mRNA, partial cds	N	gi|32400751|gb|AF469491.1|	4e-09
TAC16	110	1	Hordeum vulgare partial mRNA for monodehydroascorbate reductase	N	gi|15626360|emb|AJ345035.1|HVU345035	1e-24
TCC35	505		Vulgare cDNA clone baak44j08 3'. Similar Lolium perenne partial mRNA for ice recrystallisation inhibition protein	N	gi|21139863|dbj|BJ461352.1|	0.0
TCC83	283	8	trehalose-6-phosphate phosphatase [Oryza sativa (japonica cultivar-group)]	X	gi|50911509|ref|XP_467162.1|	1.1
TGC11	422	6	26S proteasome regulatory subunit-like protein [Oryza sativa (japonica cultivar-group)]	X	gi|50725969|dbj|BAD33496.1|	5e-30
TGC40	520	6	putative actin depolymerizing factor [Sorghum bicolor]	X	gi|48374972|gb|AAT42170.1|	2e-41
TGC47	421	6	putative alpha-galactosidase [Oryza sativa (japonic cultivar-group)]	X	gi|56202255|dbj|BAD73696.1|	1e-17
TGC5	546	8	putative HAK2 [Oryza sativa (japonica cultivar-group)]	X	gi|50899924|ref|XP_450750.1|	1e-26
TGC53	449	6	putative HAK2 (K+ transporter) [Oryza sativa (japonica cultivar-group)]	X	gi|34907198|ref|NP_914946.1|	5e-30
TGC63	168	6	pyrrolidone carboxyl peptidase-like protein [Oryza sativa (japonic cultivar-group)]	X	gi|50939513|ref|XP_479284.1|	5e-11
TGC89	281	6	COG0078: Ornithine carbamoyltransferase [Magnetococcus sp. MC-1]	X	gi|48833164|ref|ZP_00290186.1|	5.6

TGT2	644	2	putative vacuolar protein sorting-associated protein [Oryza sativa (japonica cultivar-group)]	X	gi|50919153|ref|XP_469973.1|	2e-19
TGT35	240	5	Hordeum vulgare partial mRNA for putative thioredoxin reductase	X	gi|38174810|emb|AJ495771.1|HVU495771	2e-99
TGT45	178	8	H.vulgare mRNA for serine carboxypeptidase I, CP-MI	N	gi|1731987|emb|Y09603.1|HVCPI	7e-06
TGT50	261	5	Hordeum vulgare partial mRNA for putative thioredoxin reductase	N	gi|38174810|emb|AJ495771.1|HVU495771	2e-89
TTC12	404	8	putative NIC2 [Oryza sativa (japonica cultivar-group)]	X	gi|56201647|dbj|BAD73111.1|	5e-48
TTC31	195	7	PP1/PP2A phosphatases pleiotropic regulator PRL1	X	gi|3122638|sp|Q42384|PRL1_ARATH	0.13
TTC61	244	7	putative mannose-6-phosphate isomerase [Oryza sativa (japonica cultivar-group)]	X	gi|34895502|ref|NP_909094.1|	2e-18
TGC54	378		Triticum aestivum serine/threonine protein kinase gene	N	gi|60393198|gb|AY924304.1|	0.68
CCC34	424		putative gamma-carbonic anhydrase [Oryza sativa (japonica cultivar-group)]	X	gi|56785028|dbj|BAD82610.1|	2e-12
Postranscriptional Process						
TGC51	249		Z.mays mRNA for acidic ribosomal protein P0	N	gi|1550813|emb|Y07959.1|ZMRPP0	3e-37
TGC6	392		60S acidic ribosomal protein P0 [Zea mays]	N	gi|1550814|emb|CAA69256.1|	6e-26
TTA12	320	1	Hordeum vulgare L41 ribosomal protein.	N	AJ001160.	1e-94
CCC81	367	4	Triticum aestivum 40S ribosomal protein mRNA, partial cds	N	gi|32400860|gb|AF479043.1|	9e-40
TCA35	155		H.vulgare mRNA for elongation factor 1-alpha	N	gi|949877|emb|Z50789.1|HVEF1ALFA	2e-71
CAA12	221	8	Triticum aestivum cytoplasmatic ribosomal protein S13 mRNA,	N	gi|52548253|gb|AY736126.1|	2e-19
CAA4	434	5	putative translation initiation factor [Oryza sativa (japonic cultivar-group)]	N	gi|53791638|dbj|BAD53005.1|	0.13
CAC9	119	6	Barley EST endosperm library Hordeum similar to putative 40Sribosomal protein S2, mRNA sequence.	N	gi|51525014|gb|CV061875.1|	5e-16
CAG1	511	3	putative elongation factor [Solanum demissum]	N	gi|48209911|gb|AAT40505.1|	1e-09
CAT5	267	5	putative 40S ribosomal protein S2 [Oryza sativa (japonica cultivar-group)]	X	gi|50919281|ref|XP_470037.1|	3e-08

CAT6	260	8	Barley EST endosperm library Hordeum vulgare subsp.vulgare cDNA clone BNEL9G12 5' similar to putative 40S ribosomal protein S2, mRNA sequence.	N	gi|51527511|gb|CV064334.1|	e-125
CGG13	140	6	Barley histone H3 mRNA,	N	gi|167060|gb|M34928.1|BLYHISH3PA	7e-30
CCC24	157		H.vulgare mRNA for elongation factor 1-alpha		gi|949877|emb|Z50789.1|HVEF1ALFA	2e-09
CCC48	232		H.vulgare mRNA for ribosomal protein L17-1	N	gi|19101|emb|X62724.1|HVRNPL171	1e-104
CGG7	240	6	putative aminotransferase [Oryza sativa]	X	gi|33340007|gb|AAQ14479.1|	0.001
TAA21	486	8	calcium-binding EF hand-like protein [Oryza sativa (japonica	X	gi|50905997|ref|XP_464487.1|	2e-51
TCC8	422	1	glutamyl-tRNA synthetase (gltX) [Mycoplasma genitalium G-37]	X	gi|12045321|ref|NP_073132.1|	7.2
TGA54		3	H.vulgare mRNA for elongation factor 1-alpha	N	gi|949877|emb|Z50789.1|HVEF1ALFA	e-116
TGC14	400	6	elongation factor 1 gamma-like protein [Oryza sativa (japonica cultivar-group)]	X	gi|29367381|gb|AAO72563.1|	2e-26
TGC35	380	6	H.vulgare mRNA for elongation factor 1-alpha	N	gi|949877|emb|Z50789.1|HVEF1ALFA	4e-83
TGC45	316	6	endoribonuclease L-PSP family protein [Arabidopsis thaliana]	X	gi|15229304|ref|NP_187098.1|	9.5
TGC48	624	6	Oryza sativa (japonica cultivar-group) isolate 22944 elongation factor 1 gamma-like protein mRNA, partial cds	N	gi|29367380|gb|AY224444.1|	9e-27
TGT14	490	5	small nuclear ribonucleoprotein-like [Oryza sativa (japonica cultivar-group)]	X	gi|56785201|dbj|BAD81919.1|	6e-25
TGT18	420	5	H.vulgare mRNA for elongation factor 1-alpha	N	gi|949877|emb|Z50789.1|HVEF1ALFA	e-116
TGT7	417	4	BNEL80d7 Barley EST endosperm library Hordeum vulgare subsp. vulgare cDNA clone BNEL80d7 5' similar to ELONGATION FACTOR 1-ALPHA, mRNA sequence.	N	gi|51525701|gb|CV062562.1|	0.0
TTC18	316	8	putative chloroplast 50S ribosomal protein L31 [Oryza sativa (japonica cultivar-group)]	X	gi|34912982|ref|NP_917838.1|	2e-35
TTA12	320	1	Hordeum vulgare L41 ribosomal protein.	N	AJ001160.	1e-94
CGC11	408	1	Wheat histone H3 gene	N	X00937.	e-129
TTC50	363	5	60S ribosomal protein L7A [Oryza sativa (japonica cultivar-group)]	X	gi|50944205|ref|XP_481630.1|	1e-18

TTC57	318	5	chloroplast 50S ribosomal protein L31-like [Oryza sativa (japonica cultivar-group)]	X	gi|20161807|dbj|BAB90722.1|	1e-40
CTA20	261	4	Triticum aestivum 40S ribosomal protein mRNA,	N	gi|32400860|gb|AF479043.1|	5e-21
CTC38	341	8	putative ATP/GTP nucleotide-binding protein [Oryza sativa (japonica cultivar-group)]	X	gi|50936969|ref|XP_478012.1|	1e-35
CTC28	800	6	putative initiation factor 3g [Oryza sativa (japonica cultivar-group)]	X	gi|50915436|ref|XP_468182.1|	2e-48
CTG3	616	6	Hordeum vulgare subsp. vulgare cDNA clone HX10H09 EST similar Triticum aestivum ribosomal protein L19 mRNA	X	gi|30078142|gb|CB876156.1|	0.0
CTG5	500	1	putative ribosomal protein L19 [Oryza sativa (japonica cultivar-group)]	X	gi|29837764|gb|AAP05800.1|	8e-25
TTC46	475	5	60S ribosomal protein L7A	X	50944205|ref|XP_481630.1|	6e-19
CCA7	229	8	Triticum aestivum ribosomal protein L18 mRNA	N	gi|57471707|gb|AY846824.1|	6e-66
TGC6	392		Z.mays mRNA for acidic ribosomal protein P0	N	gi|1550813|emb|Y07959.1|ZMRPP0	3e-38
CTA23	229		Hordeum vulgare partial eIF(iso)4E gene for putative eukaryotic	N	gi|63024872|emb|AJ704455.1|	3e-15
CCC33	281		H.vulgare mRNA for elongation factor 1-alpha	N	gi|949877|emb|Z50789.1|HVEF1ALFA	4e-09
TTC69	260		H.vulgare mRNA for ribosomal protein L17-1	N	gi|19101|emb|X62724.1|HVRNPL171	4e-23
CGA55	313		40S ribosomal protein S24	T	barley|TC109412	1.7e-35
CCA26	359	7	Triticum aestivum cyclophilin mRNA	N	gi|32401383|gb|AF542973.1|	8e-60
TCC13	236	1	Hordeum vulgare mRNA for Ribosomal protein S7 (rps7 gene)	N	gi|14787423|emb|AJ315794.1|HVU315794	2e-106
TTC76	322		60S ribosomal protein, putative [Arabidopsis thaliana]	X	gi|17369176|sp|Q9LSA3|RL30_ARATH	0.30
CTC13	175		similar to 60S ribosomal protein L23a	X	gi|82999964|ref|XP_898815.1|	3e-05
TTC39	120	7	ribosomal protein L18a,	X	gi|51517998|gb|CV054977.1|	7e-45
TTC76	322		60S ribosomal protein, putative	X	gi|21593857|gb|AAM65824.1|	0.30
CTC27	802	6	putative translation initiation factor	X	gi|34896594|ref|NP_909641.1|	3e-92
Lipidic metabolism						
CCA44	365	8	COG0332: 3-oxoacyl-[acyl-carrier-protein] synthase III [Moorella thermoacetica ATCC 39073]	X	gi|49235507|ref|ZP_00329575.1|	4.1
CGG5	545	6	type II inositol polyphosphate 5-phosphatase [Arabidopsis thaliana]	X	gi|56405854|gb|AAV87317.1|	3e-44

CTA1	646	8	putative Lipid-A-disaccharide synthase [Oryza sativa (japonica cultivar-group)]	X	gi|57899604|dbj|BAD87183.1|	4e-10
TTC60	247	7	putative 3-hydroxybutyryl-CoA dehydrogenase [Oryza sativa (japonica cultivar-group)]	X	gi|34908888|ref|NP_915791.1|	2e-13
CGA34	319		delta-9 fatty acid desaturase; stearoyl-CoA desaturase	X	gi|757862|emb|CAA59938.1|	1e-09
Metabolism & photosynthesis						
TGA47	198	8	Triticum aestivum putative cytochrome P450-like mRNA, partial sequence	N	gi|32351000|gb|AY290727.1|	6e-22
TGA20	229	3	putative cytochrome P450 [Oryza sativa (japonica cultivar-group)]	N	gi|34912888|ref|NP_917791.1|	5e-04
TGT33	255	5	Zea mays cytochrome P450 monooxygenase CYP72A26 gene	N	gi|21805633|gb|AY071866.1|	0.003
CCA29	328		Lolium rigidum clone Lol-79 putative cytochrome P450 mRNA	N	gi|13661771|gb|AF321869.1|AF321869	2e-57
CCA8	342	1	Lolium rigidum clone Lol-83 putative cytochrome P450 Mrna	N	gi|13661773|gb|AF321870.1|AF321870	1e-06
TGT48	274	5	Zea mays cytochrome P450 monooxygenase CYP72A26 gene	N	gi|21805633|gb|AY071866.1|	7e-04
CGG20	161	7	Pyrus communis cytochrome P450 monooxygenase mRNA, partial cds	X	gi|38640721|gb|AY436774.1|	1.6
TGT4	284	8	chloroplast protein-translocon-like protein	X	gi|50918167|ref|XP_469480.1|	1.1
TAA17	550	7	Lolium rigidum clone Lol-62 putative cytochrome P450 mRNA, complete cds	N	gi|13661767|gb|AF321867.1|AF321867	7e-52
Aminoacidic Metabolism						
CCA43	263	8	putative N-carbamyl-L-amino acid amidohydrolase [Oryza sativa(japonica cultivar-group)]	X	gi|52076511|dbj|BAD45389.1|	1e-28
CCC32	148		Pyrus communis ferredoxin-dependent glutamate synthase-like mRNA,	N	gi|38640726|gb|AY436777.1|	6.3
CCC46	263		Hordeum vulgare subsp. vulgare glutamine-dependent asparagine synthetase 1 mRNA, complete cds	N	gi|13925885|gb|AF307145.1|	1e-125
TCC10	386	1	putative tryptophan synthase beta-subunit	X	gi|50940893|ref|XP_479974.1|	2e-21
CTC11	367		signal peptidase	X	gi|15240934|ref|NP_198095.1|	2e-11
CAC17	169	6	hypothetical protein, proline- and glutamine-rich	X	gi|51856466|dbj|BAD40624.1|	5.6
CGG3	618	4	protein N-terminal asparagine amidohydrolase protein-like	X	gi|51090455|dbj|BAD35425.1|	1e-72
Proteolytic machinary						
CAA16	534	5	Barley ubiquitin (mub1) gene	N	gi|167072|gb|M60175.1|BLYMUB1	e-156

CAA6	371	5	Oryza sativa (japonica cultivar-group) mRNA for ubiquitin/ribosomal polyprotein, ubiquitin, ribosomal protein,	N	gi|303856|dbj|D12629.1|RICRPOLYP	6e-70
CAC12	295	6	Hordeum vulgare Barke developing caryopsis A.fatua mRNA for tetraubiquitin	N	gi|12038637|emb|AL512134.1|	6e-54
CCA16	605	3	Hordeum vulgare subsp. vulgare cDNA clone HB106A01_SK.ab1 similar to 1,4-alpha-glucan branching enzyme,Ubiquitin—protein ligase,4-alpha-glucanotransferase.	N	gi|19152282|gb|BM816268.1|	0.0
CCA5	499	8	Wheat chloroplast clpP gene for proteolytic subunit of ATP-dependent protease	N	gi|12334|emb|X54484.1|CHTACLPP	4e-28
CGT3	405	1	putative RAD23 protein [Oryza sativa (japonica cultivar-group)]	X	gi|50252077|dbj|BAD28007.1|	7e-24
TAC10	285	1	polyubiquitin 2	X	gi|20562909|gb|AAM22748.1|	4e-04
CAA26	290	1	ubiquitin	X	gi|30523391|gb|AAP31578.1|	9e-14
Defense response						
CAA7	322	8	Zea mays sulfur starvation induced isoflavone reductase-like	N	gi|1205985|gb|U33318.1|ZMU33318	6e-08
TCA14	255		pathogenesis-related protein 10b [Sorghum bicolor]	X	gi|58978057|gb|AAW83210.1|	1e-04
CCA55	171	8	Triticum aestivum phytochelatin synthase (PCS1) mRNA, complete cds	N	gi|5757803|gb|AF093752.1|AF093752	8e-20
CGA24	234	4	Triticum aestivum heat shock protein 70 (HSP70) mRNA, HSP70-S allele	N	gi|6670930|gb|AF074969.1|AF074969	5e-23
CGA26	212	7	Pyrus communis putative heat shock protein 90-like mRNA	N	gi|47059612|gb|AY435421.1|	0.033
CGG6	522	4	Barley mRNA for leaf-specific thionin (clone DB4)	N	gi|19112|emb|X05576.1|HVTHIOR1	0.0
CGT33	262	1	Hordeum vulgare cytosolic heat shock protein 90 (HSP90) mRNA, complete cds	N	gi|32765548|gb|AY325266.1|	6e-90
CTA6	404	4	Hordeum vulgare NADPH-dependent HC-toxin reductase mRNA	N	gi|1679776|gb|U77463.1|HVU77463	e-159
TGA41	250	3	Hordeum vulgare glutathione transferase (GST6) mRNA, complete cds.	N	gi|18479037|gb|AF430069.1|	2e-86
TAC14	133	7	23 kDa jasmonate-induced protein	X	gi|400094|sp|P32024|JI23_HORVU	0.29
TAC3	320	7	Wheat gstA1 gene for glutathione-S-transferase	N	gi|21787|emb|X56012.1|TAGSTA1	8e-19
TGA16	221	3	Hordeum vulgare glutathione transferase (GST6) mRNA, complete cds	N	gi|18479037|gb|AF430069.1|	e-112

TGA17	360	7	Hordeum vulgare glutathione transferase (GST6) mRNA, complete cds	N	gi|18479037|gb|AF430069.1|	e-102
TGT23	340	8	Triticum aestivum heat shock protein 70 (HSP70) mRNA, HSP70-S allele,complete cds	N	gi|6670930|gb|AF07969.1|AF074969	2e-41
TGT32	255	4	Hordeum vulgare glutathione transferase (GST6) mRNA,	N	gi|18479037|gb|AF430069.1|	2e-78
TTC46	295	5	H.vulgare gene encoding jasmonate-induced protein	X	gi|1561617|emb|X98124.1|HVJIP	5e-110
TGC44	290		T.aestivum mRNA for heat shock protein	N	gi|21806|emb|X58279.1|TAHSP173	2e-37
TGC6	290		Z.mays mRNA for annexin p33	N	gi|6272284|emb|X98244.2|ZMANNP33	1e-45
TGA33	213	2	annexin p33	X	gi|6272285|emb|CAA66900.2|	2e-43
Transcriptional process						
TAA10	241	1	replication protein A1 large subunit [Cryptosporidium parvum]	X	gi|46228369|gb|EAK89268.1|	9.3
TCC40	385	1	putative cleavage and polyadenylation specifity factor [Oryza sativa (japonica cultivar-group)]	X	gi|29126360|gb|AAO66552.1|	9e-27
TGC67	151	6	CRS2-associated factor 2 [Zea mays]	X	gi|30349369|gb|AAP22136.1|	6e-04
TGC88	321	6	putative DNA helicase [Oryza sativa (japonica cultivar-group)]	X	gi|55773868|dbj|BAD72453.1|	0.005
Protein transporter						
TAA25	323	8	putative VEF family protein [Oryza sativa (japonica cultivar-group)]	X	gi|51091709|dbj|BAD36510.1|	6e-21
CGA40	146	3	Hordeum vulgare vacuolar proton-translocating	N	gi|4099149|gb|U84269.1|HVU84269	2e-71
TAA28	270	7	putative organic solute transporter [Oryza sativa]	X	gi|34897328|ref|NP_910010.1|	5e-24
TAA6	248	8	DOT1-like, histone H3 methyltransferase [Homo sapiens]	X	gi|22094135|ref|NP_115871.1|	7.1
TGC85	424	6	similar to UP|Q8GU84 (Q8GU84) PDR-like ABC transporter,partial (41%)	T	barley|TC141870	2.4e-19
TTC48	348	7	importin-beta2 [Oryza sativa (japonica cultivar-group)]	X	gi|3983665|dbj|BAA34862.1|	2e-06
CCC43	333		Hordeum vulgare peptide transporter (ptr1	N	gi|2655097|gb|AF023472.1|AF023472	1e-27
CAC14	244	6	COG0683: ABC-type branched-chain amino acid transport systems, periplasmic component [Ralstonia eutropha JMP134]	X	gi|46132463|ref|ZP_00170960.2|	4.3
CAG4	412	1	putative amino acid transport protein [Oryza sativa (japonica cultivar-group)]	X	gi|34907364|ref|NP_915029.1|	1e-06

CGG37	311	7	putative clathrin coat assembly protein AP17 [Oryza sativa (japonica cultivar-group)]	X	gi|50945381|ref|XP_482218.1|	8e-39
TGC5	423	4	putative potassium transporter	X	gi|18138061|emb|CAD20577.1|	3e-21
TGT49	250	1	putative sodium-dicarboxylate cotransporter	X	gi|50947385|ref|XP_483220.1|	8e-09
Lipidic metabolism						
CCA30	317	8	putative acyl carrier protein [Oryza sativa (japonica cultivar-group)]	X	gi|34900336|ref|NP_911514.1|	0.66
CCA44	365	8	COG0332: 3-oxoacyl-[acyl-carrier-protein] synthase III [Moorella thermoacetica ATCC 39073]	X	gi|49235507|ref|ZP_00329575.1|	4.1
CAG14	550	1	lipase-like [Oryza sativa (japonica cultivar-group)]	N	gi|56784453|dbj|BAD82546.1|	4e-30
CGG5	545		type II inositol polyphosphate 5-phosphatase [Arabidopsis thaliana]	X	gi|56405854|gb|AAV87317.1|	3e-44
CTA1	646		putative Lipid-A-disaccharide synthase [Oryza sativa (japonica cultivar-group)]	X	gi|57899604|dbj|BAD87183.1|	4e-10
TTC60	247	7	putative Lipid-A-disaccharide synthase [Oryza sativa (japonica cultivar-group)]	X	gi|34908888|ref|NP_915791.1|	4e-10
Signal trasduction						
CCA55	209	5	Zinc finger protein 36 homolog)	T	barley|TC131819	6.6e-33
TAC11	211	7	Serine/threonine-protein kinase KIN2	X	gi|3915745|sp|P13186|KIN2_YEAST	9.5
TCC5	440	1	Similar Triticum aestivum serine/threonine protein kinase mRNA, complete cds	N	gi|40952500|gb|CK566926.1|	0.0
TGA49	190	8	Hordeum vulgare subsp. vulgare GAMYB-binding protein mRNA, complete cds	N	gi|48093486|gb|AY587552.1|	4e-26
TGC68	210	6	putative protein serine/threonine kinase [Dictyostelium discoideum]	N	gi|60467030|gb|EAL65072.1|	0.37
TGT29	280	5	cdc2-related protein kinase 1 [Plasmodium falciparum 3D7]	X	gi|23498244|emb|CAD49215.1|	0.66
TGT37	170	5	putative zinc finger and C2 domain protein [Oryza sativa (japonic cultivar-group)]	X	gi|45735988|dbj|BAD13017.1|	7e-05
TTC59	300	5	zinc finger (C2H2 type) family protein [Arabidopsis thaliana]	X	gi|18424689|ref|NP_568969.1|	3e-08
CCA28	358		CCHC-type zinc finger protein-like [Oryza sativa (japonica cultivar-group)]	X	gi|50913041|ref|XP_467928.1|	0.17
CGG24	289	7	Mus musculus cyclin-dependent kinase-like 3 (Cdkl3), mRNA	X	|gi|24418888|ref|NM_153785.1|	0.15
CTT20	245	5	zinc finger (C3HC4-type RING finger) family protein [Arabidopsis thaliana]	X	gi|18404810|ref|NP_564653.1|	2e-04

CTC24	118	1	Triticum aestivum calmodulin TaCaM2-2mRNA	N	gi|1754998|gb|U48691.1|TAU48691	2e-23
CAA23	500	1	putative calmodulin-binding transcription factor	N	gi|37991923|gb|AAR06369.1|	2e-23
Cell growth						
CTT8	217		Triticum aestivum expansin EXPB10 mRNA, complete cds	N	gi|44894811|gb|AY543544.1|	7e-84
CCC2	343		Triticum aestivum beta-tubulin 6 (Tubb6) mRNA	N	gi|4098332|gb|U76897.1|TAU76897	2e-125
CCA37	245	4	Microtubule-associated protein MAP65-1a, partial (66%)	T	barley|TC148881	6.1e-19
Stress						
TTA27	263	7	putative oxidative-stress responsive [Oryza sativa (japonica cultivar-group)]	X	gi|51535448|dbj|BAD37346.1|	0.38
TTC65	181	7	Hordeum vulgare subsp. vulgare dehydrin 13 (Dhn13) gene, complete cds	N	gi|50812721|gb|AY681974.1|	1e-41
Membrane channel						
CGA45	165		Hordeum vulgare vacuolar proton-translocating ATPase subunit	N	gi|4099149|gb|U84269.1|HVU84269	3e-67
CAC6	327	6	PREDICTED: similar to inversin isoform b, partial [Bos taurus]	X	gi|61839425|ref|XP_612465.1|	3.2
TTA17	250	6	aquaporin	X	gi|29650729|gb|AAO86708.1|	1e-10
Fungi						
CAA10	241	8	germinating Phytophthora infestans cDNA	N	gi|58108022|gb|CV918473.1|	6e-32
CAC15	200	6	Pyrus communis phosphatidyl glycerol specific phospholipase C-like mRNA, partial sequence	N	gi|38640729|gb|AY436779.1|	2.0
CAT1	236	5	Per Giberella= simile a polyubiquitin	N	gi|46128780|ref|XM_388944.1|	4e-17
CGG10	454	6	Mycosphaerella graminicola cDNA	N	gi|47032455|emb|AJ639398.1|	9e-51
TCC33	601	1	adenosine deaminase, putative [Aspergillus fumigatus]	X	gi|42820728|emb|CAF32041.1|	5e-45
TCC38	432	1	Alternaria alternata putative mannitol dehydrogenase gene	N	gi|23344706|gb|AF541874.1|	e-106
TCG24	262		Fusobacterium nucleatum subsp. nucleatum ATCC 25586	N	gi|20095250|gb|AE009951.2|	3.3
TGC57	230		Cochliobolus spicifer partial xyn11A gene for putative beta-1,4-xylanase,	N	gi|11691646|emb|AJ297247.1|CSP297247	3e-37

As shown in table some of sequenceted fragments does not belong to any cluster group because those groups were made only for the most representatives classes of differential gene expressed detected. The biggest functional groups are "Metabolism and Biosynthesis" and "Post-transcriptional process. In this study the embryos barley response to pathogen lead to increase

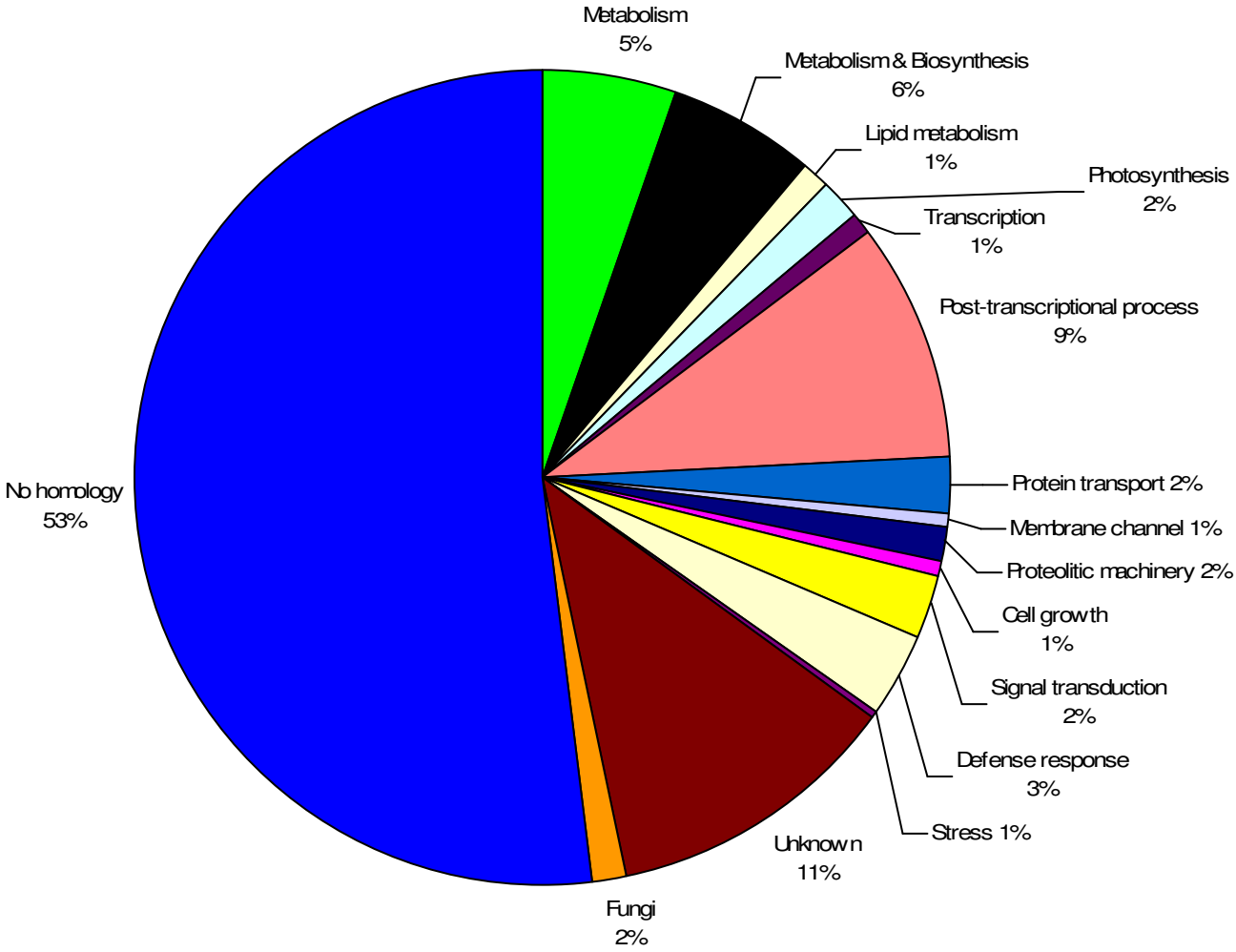
of most transcripts tag coding for elongation factor EF-1α, ribosomal genes 40S and 60S presents in the functional group "Postranscriptional Process".

In the functional group "Defense response" the clusters 7, 3 and 4 there are most fragment. In this functional group, some clones as CGG6 homologs with thionin barley, CGA24 homologs to heat shock protein of *Triticum aestivum* (before known in literature as specific defence genes) belong to the cluster in which are present transcripts accumulated in response to fungi infection only in the resistant plants. Besides in the group "Defense response" there are also four clones homologs to glutathione S-transferase.

In the functional group "Metabolism & photosynthesis" seven fragments encoding to cytochrome P450 that in higher plants, is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase).

In this study all fragments present in the "Proteolitic machinery" coding to ubiquitin. The major pathway utilized by eukaryotic cells to degrade proteins is ubiquitin-dependent proteolysis and has emerged as being fundamentally important in many aspects of development such as hormone signalling, light perception and cicardian rhythm, as well as in plant defence signalling (Callis and Vierstra, 2000; Liu et al, 2002).

The data bank research (NCBI blast-N, blast-X and TIGR) for most of sequenceted fragments shows homologies with clones of unknown function or never isolated (graphic 1).



Graphic 1: Functional groups

4. 3 Hystological microscopic analysis

Mycroscope fluorescence analysis was utilized to verify physiological and molecular responses in the embryo of barley tissues subjected to defense response after *P. graminea* inoculation.
The genetic materials used have been:

- Resistant Near Isogenic Line (Rdg2a gene introgression Mirco-Rdg2a)
- Susceptible cultivar Mirco;
- *Pyrenophora graminea* fungus transformed with the reporter gene GUS.

The observations have been done by Olympus Microscope Model BX51. The images capture has been obtained by model DP-50 Digital Camera.

4.3.1 GUS Test and Autofluorescence

The analysis have been carried out utilising a *Pyrenophora graminea* Dg2 isolate transformed with the reporter gene GUS, utilising this approach was possible to better follow the colonization pattern of the host by the hyphae pathogen..
By photos (fig 26 and 27) of Dg2-GUS it is evident the intercellular fungal growth. No appressoria are formed during the colonization process and the fungus seems to growth by systematically degrading the cell walls of the host cell.

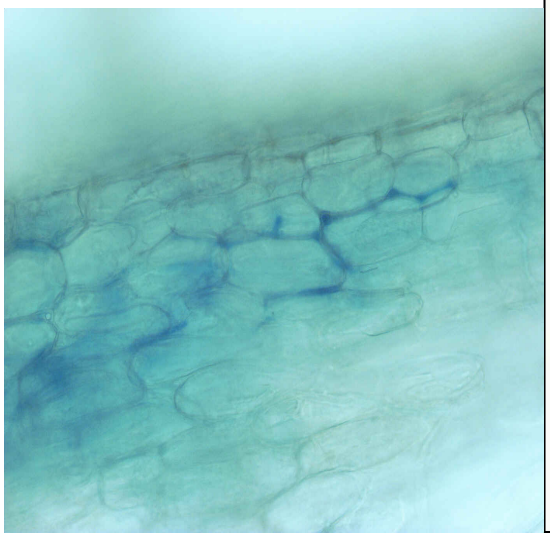


Fig. 26 Intercellular growth of hyphae

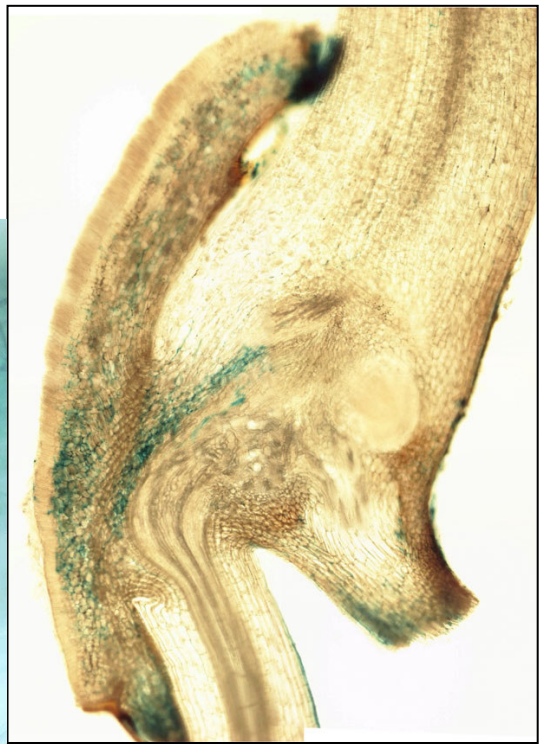


Fig.27 Intercellular growth of hyphae

The histological evidence show that the scutellar node seems to be site in which the resistant response is manifested. In fact Mirco (the susceptible genotype) allows a strong scutellum colonization and hyphaee continue until meristematic apex (Fig.28a); in the NIL only coleorhiza and basal part of scutellum are infected and the fungal growth become stopped at the level of the scutellar node (Fig. 28b).

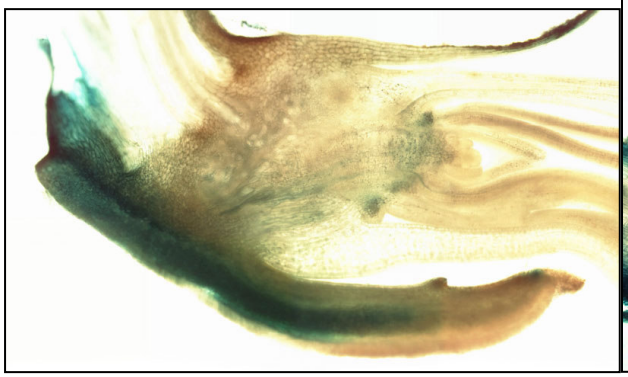


Fig28a Mirco 20days after infection (d.a.i) GUS test

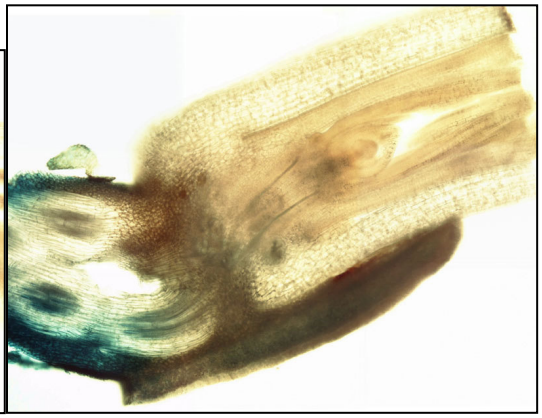


Fig28b NIL 20 (d.a.i) Gus test

The autofluorescence analysis and GUS test have been carried out onto the same sample sections concomitantly evidentiate fluorescent area and mycelium growth.

Autofluorescence is common to Mirco and NIL as response to infection (Fig.29a; 29b and fig.30a; 30b).

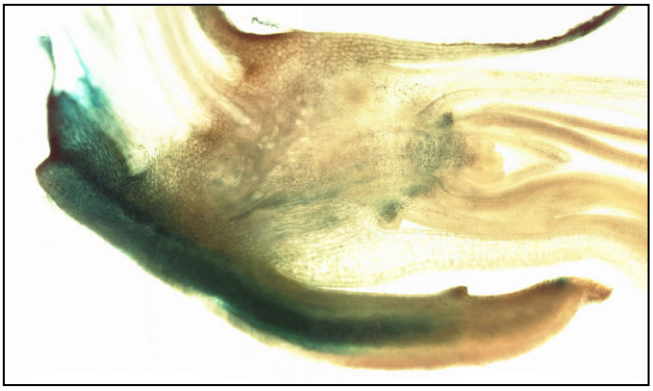


Fig 29a: Mirco 20 d.a.i GUS test.

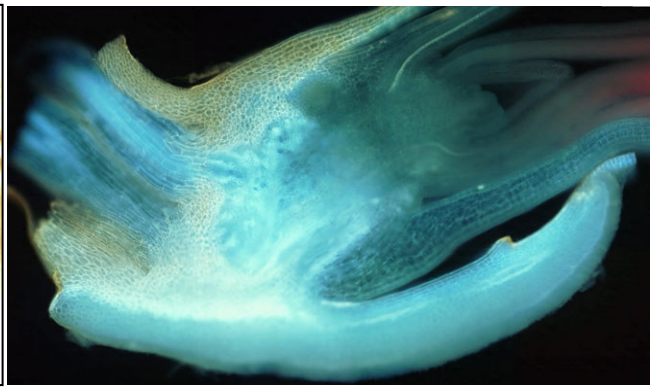


Fig 29b: Mirco 20 d.a.i autofluorescence analysis.

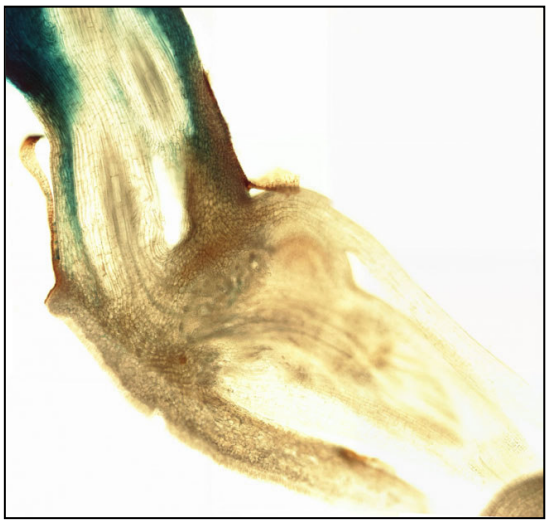


Fig.30a NIL 20 d.a.i Gus test

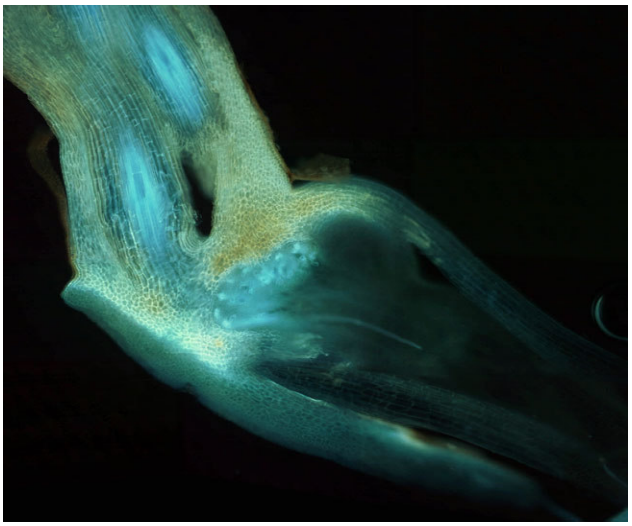
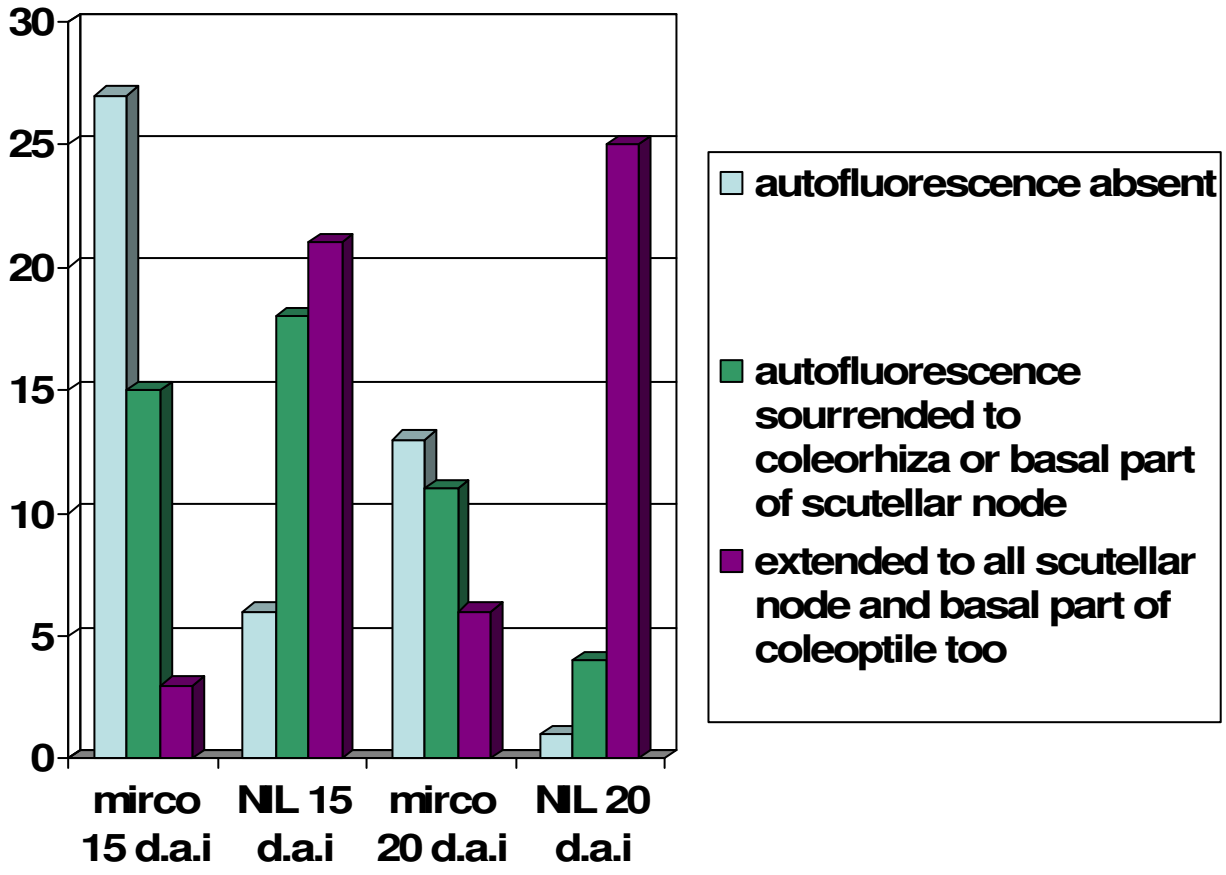


Fig. 30b NIL 20 d.a.i autofluorescence analysis

By magnification of the autofluorescence analysis areas it has been observed that issued fluorecent light come from intercellular spaces rather than and not from the whole cell (fig.31a; fig. 31b; fig.

32). This support that the autofluorescence generanting substances (likely polyphenols) are deposited at the level of the cell wall of infected embryos.

We have observed that there are not qualitative difference comparing Mirco and NIL in fact both resistant and susceptible genotype shared this timing of response. A more subtile analysis of the time point of inoculation pointed out that the autofluorescence is anticipated in the resistant genotype in comparison to the susceptible one. In addition, analysis carried out on a large number of embryos from both susceptible and resistant genotypes (about 40 embryos for each genotype) (graphic 2) allowed the indentification of a quantitative response: the intensity of autofluorescence was higher at all the time points analysed, in the resistant genotype with respect to the susceptible one.



Graphic2: Quantitative evaluation of embryo cells showing pathogen-induced autofluorescence.

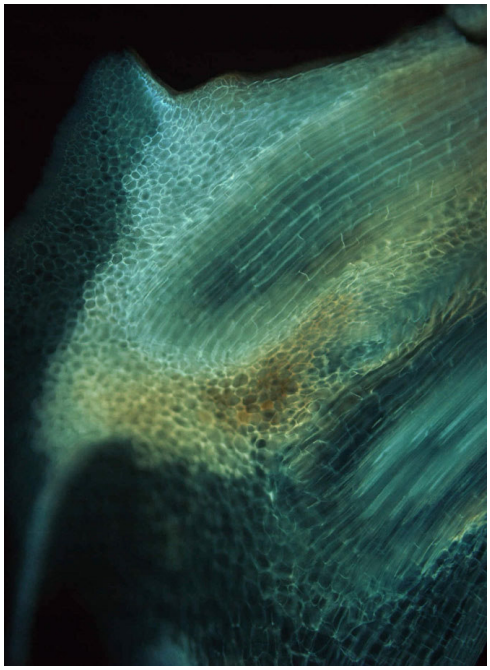


Fig 31a Mirco 20 d.a.i

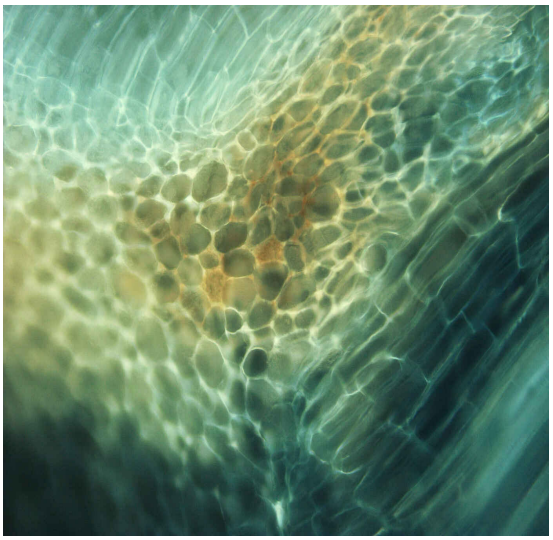


Fig 31b Magnification of section fig 31a

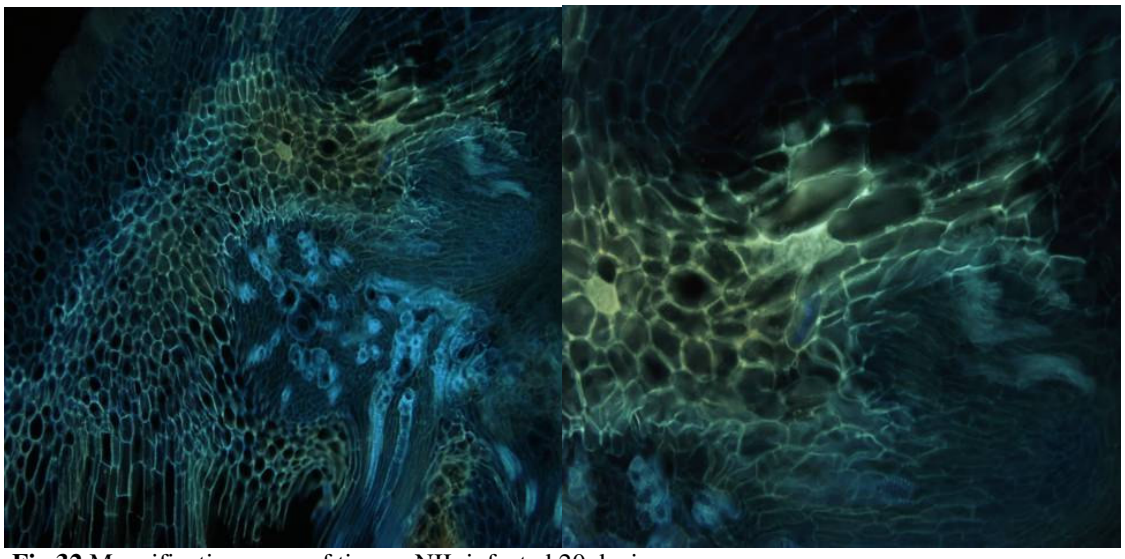


Fig.32 Magnification areas of tissues NIL infected 20 d.a.i

4.3.2 Histochemical toluidine blue assay

Histochemical toluidine blue assay evidentiate the deposit of polyphenol compounds by a green/green-blue colouring. The microscopic observation has been carried out onto both NIL and Mirco infected samples.

These observations show a positive blue staining indicating that polyphenol compounds are accumulated at the level of the cell wall during the defence response. In addition we have verified that the positive toluidine blue staining sites coincide with the autofluorescent areas indicating a causal relationship between the two kind of responses. Those observations have been possible because the same section has been subjected to both kind of analyses (Fig. 33a; 33b).

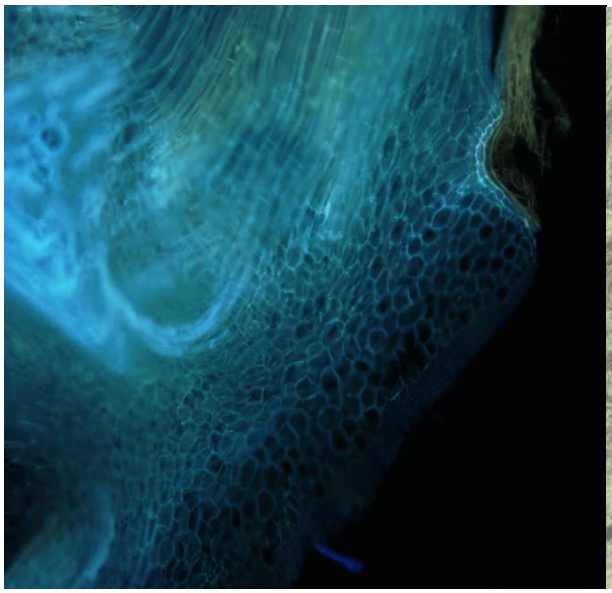


Fig.33a Autofluorescence Mirco 20 d.a.i

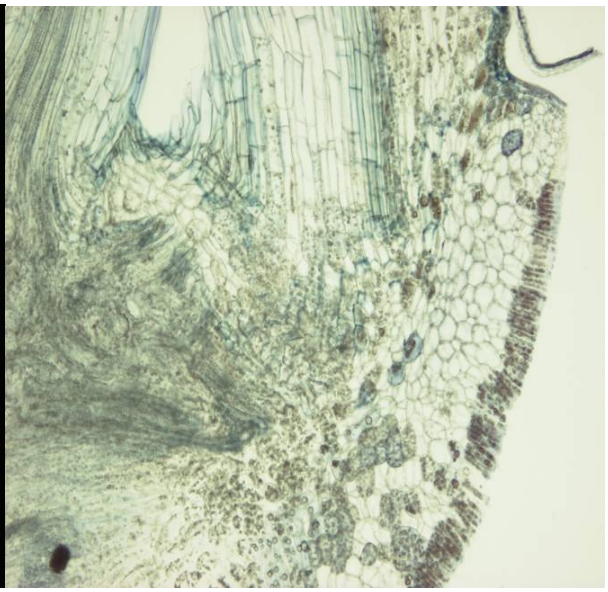


Fig.33b Toluidine blu assay Mirco 20 d.a.i

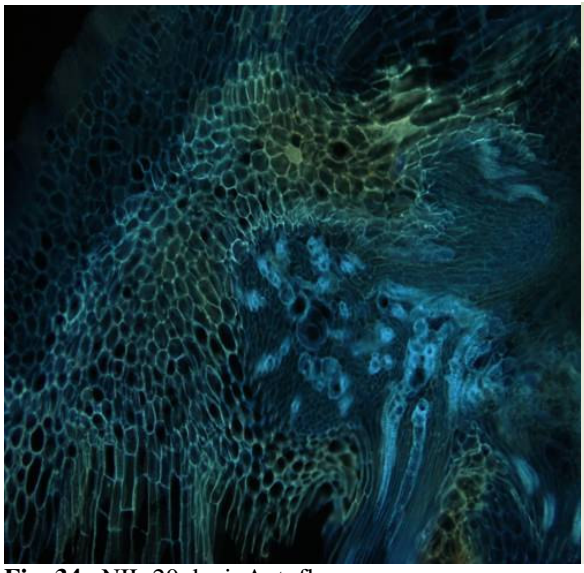


Fig. 34a NIL 20 d.a.i. Autofluorescence

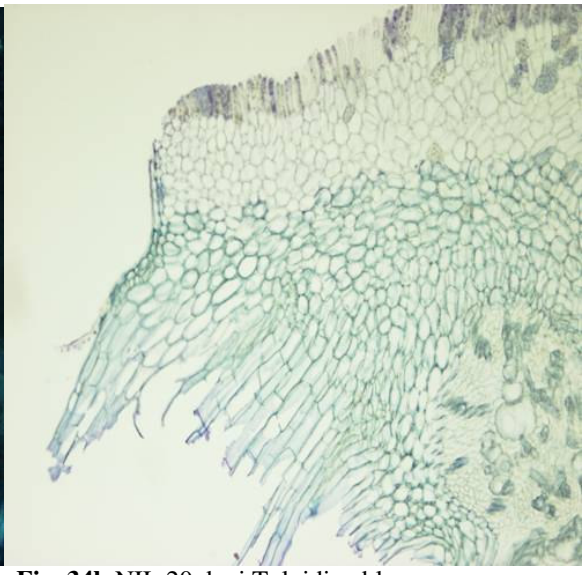


Fig. 34b NIL 20 d.a.i Toluidine blue assay

To understand whose polyphenols are involved a staining with SUDAN III and phoruglucinol have been carried out

4.3.3 SUDAN III staining

SUDAN III staining is specific to point out the deposit of suberine in cell-wall by red colouring. The results of the staining in both genotypes have been negative (fig. 35a/b; 36a/b).

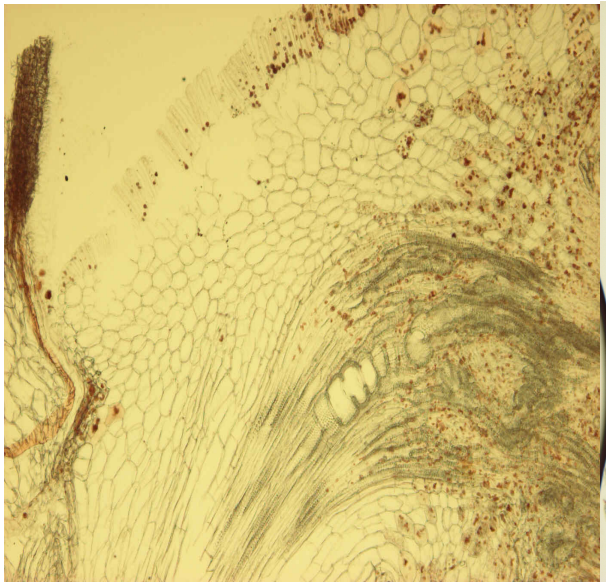


Fig. 35a Mirco control 20d

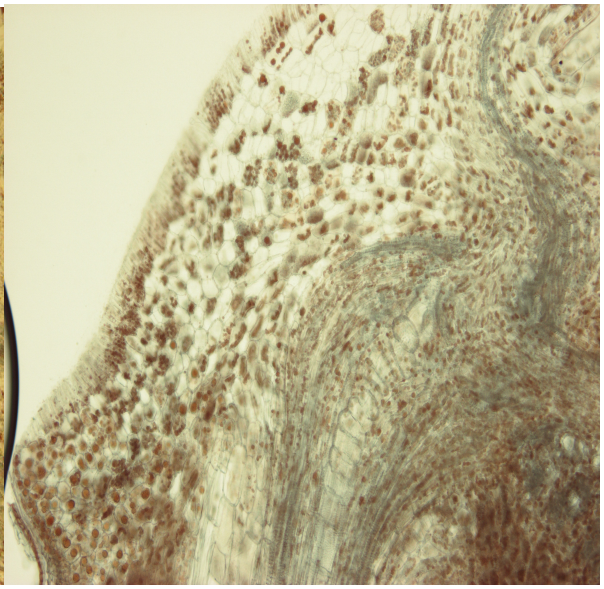


Fig 35b NIL control 20d

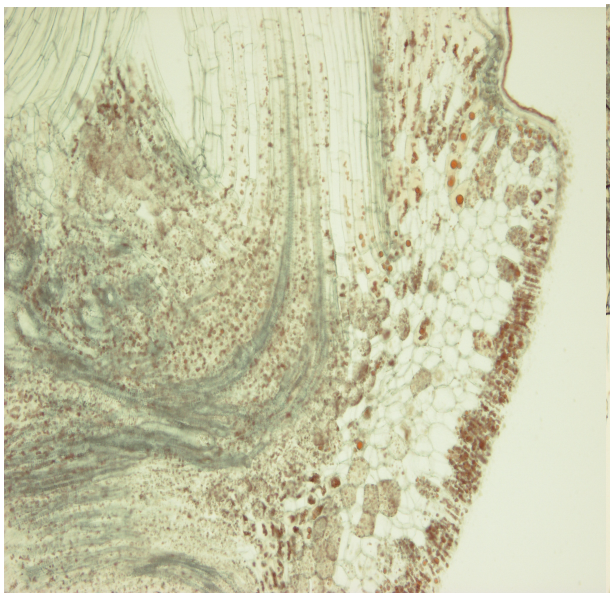


Fig. 36a Mirco 20 d.a.i

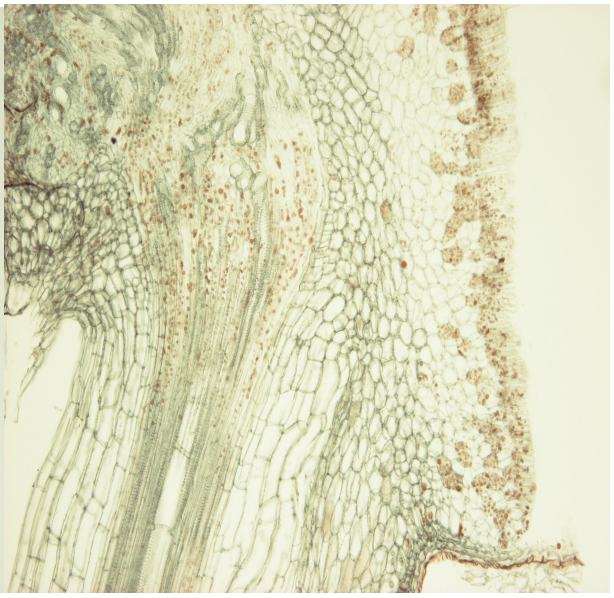


Fig. 36b NIL 20 d.a.i

4.3.4 Phuoroglucinol stains

Phluoroglucinol stains is specific to point out the deposit of lignin and suberine with red/orange colouring in the cell-wall. The analysis has carried out on infected samples of the resistant NILs at 21 days post infection (fig. 37a/b; fig.38a/b). The results of the staining have been negative.

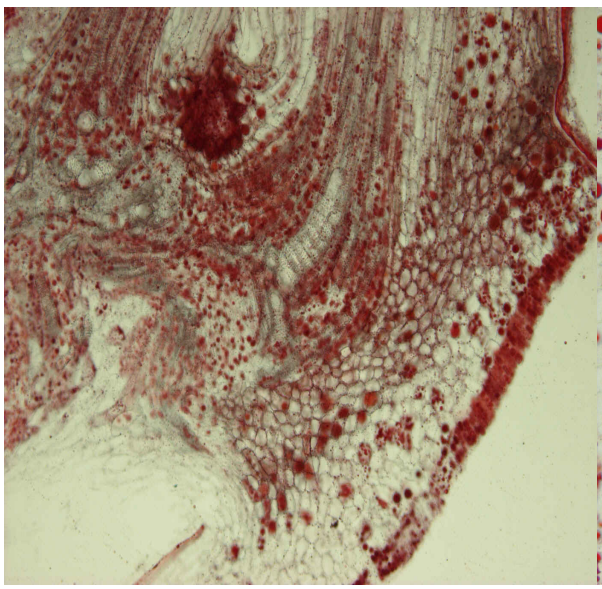


Fig.37a NIL 20 d.a.i.100X magnifying

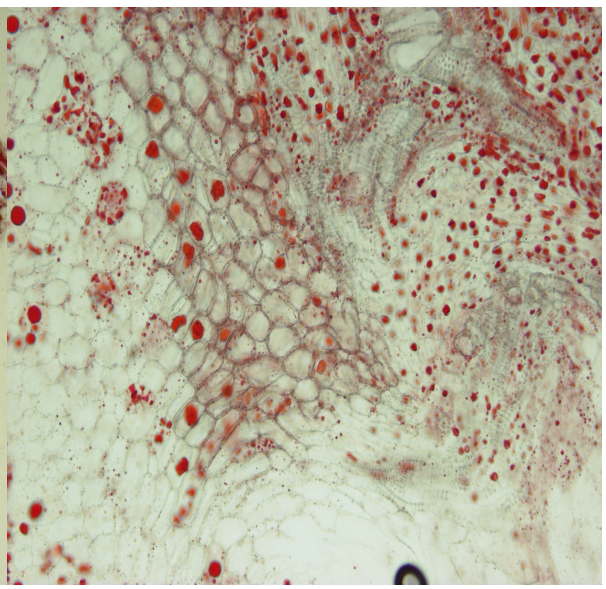


Fig.37b NIL 20 d.a.i. 200X magnifying

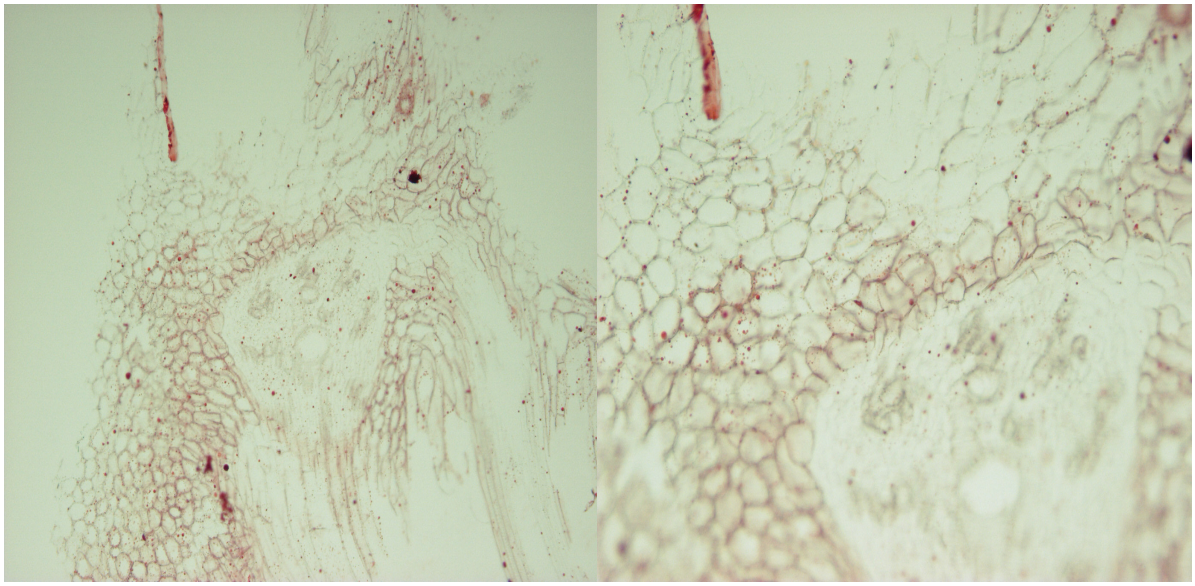


Fig. 38a NIL 20 d.a.i. 100X magnifying

Fig. 38b 20 d.a.i NIL 200X magnifying

The results show therefore an accumulation of polyphenols in the cell-wall which can be evidentiated by auto-fluorescence analysis and by toluidine blue staining. Even if a qualitative difference does not take place in this kind of response between resistant and susceptible genotypes, analysis of a large numbers of embryos allowed us to verify that different response between resistant and susceptible does exists in terms of the timing of induction of the autofluorescence (earlier induction in the resistant genotype) and in terms of intensity of the autofluorescence (more intensity observed in the resistant genotype). The polyphenols detected does not seem to belong the lignine or suberine production back (SUDAN III and phluoroglucinol stains). Finally, because we have observe the whole cell autofluorescence does not occur during the embryos defence response, we suppose that barley resistant response against *P.graminea* does not involve programmed cell death.

5. CONCLUSION AND PERSPECTIVES

Barley leaf stripe is caused by fungal seed-borne pathogen *Pyrenophora graminea*.

In susceptible plants the disease usually results in severe stunting, premature and complete loss of grain. A single genetic factor Rdg2a conferring complete resistance to the highly virulent isolate Dg2 the most virulent among 12 tested of *Pyrenophora graminea* has been identified in the six-rowed barley cultivar Thibaut.

The resistance pattern show that at the level of embryos fungal hyphae degenerate in the basal part of the coleorhiza and in the scutellar node with the formation of brown tissue which block fungal colonization.

With this PhD's work we have study early transcriptional changes which occur into the barley embryos during defence's response.

Three different analysis technique were employed to study host-pathogen interaction.

Creation of subctractive cDNA library with PCR-Select techinique;

cDNA-AFLP TP;

Histological microscopic analysis.

5.1 Creation of subctractive cDNA library with PCR-Select techinique

From a cDNA library of 1800 clones, only 11 clones have been found to be differentially expressed (tab. 4 pag. 40 Result and Discussion) of these eleven clones, four are putatively involved on scavenging of ROS during fungal infection. In fact for two clones an high homology with enzymes belonging to pathway of Cysteine synthesis (fig. 15), essential aminoacid for Glutathione synthesis,were found; one clone has homology with glutathione-S-transferase (fig. 14), an enzyme which catalyze the addition of reduced glutathione to electrophilic substrates activities and it might be essential for the preservation of plants during environmental stress and disease; one clone was homolg to MDR-like ABC transporter (fig. 18) that often transport molecules that have been covalently attached to glutathione. The vacuolar GS-X pumps of plant cells function in herbicide detoxification, protection against oxidative damage, pigment accumulation, and the storage of antimicrobial compounds.

The remaining clones found with PCR-Select will be analysed in an future works more detailed with Northern analysis realized with genetic materials inoculated at different time points (3, 7, 11, 14 days) to analyse the regulation expression at different inoculation time points.
With the PCR-Select technique we realized a very low yield expressed differentially genes. differentially expressed, in fact only 11 clones over 1800 (with a ratio of 0,6%) are really differentially expressed.
This is probably due to the fact that most of defense genes in embryos level, during germinating, have a basal expression level which has made the difficult the isolation these genes classes.

5.2 cDNA-AFLP TP

cDNA-AFLP TP is a powerful technique to study the transcripts belonging to both barley and Pyrenophora graminea expressed during their interaction. About 1100 putatively fragments differentially expressed have been isolated. After search in gene bank 600 fragments of 1100 showing homology with known sequences have been grouped in functional groups (see tab 5). The data bank research (NCBI blast-N, blast-X and TIGR) for most of sequenced fragments shows homologies with clones of unknown function or never isolated. Thus the construction of a microarray with these 600 cDNA-AFLP fragments is foreseen to investigate then thoroughly. This microarray wil be hybridized with mRNA's probes from susceptible Mirco's cultivar and resistant near isogenic line (Rdg2a introgression) at early and late infection times. These additional data will allow identidification of truly induced or repressed genes involved in during the interaction *Pyrenophora graminea*-barley and will contribute to improve knowledge in plant biotic stress response.

5.3 Histological Microscopic analysis

The hystologic analysis utilising the fluorescence microscope, has allowed to better define the infection pattern of *Pyrenophora graminea* on barley. The observations, carried out utilising fungal isolate transformed with gene reporter GUS, confirmed that the fungus colonization is intercellular (fig. 26; fig 27). The hyphae in fact advance only in the apoplast way. First the hyphae colonyze the coleorhiza tissues, later the basal part of the scutellum and finally the meristematic area and the apex (Fig. 28a; fig. 28b).
The autofluorescence analysis confirmed that the scutellar node is the area in which the resistant response is deployed of infection, in fact the autofluorescence come from this histological zone (Fig. 29a and 29b; fig. 30a and 30b).

The autofluorescence light come from intercellular spaces rather than from the whole cell; this support that a programmed cell death is not involved in this resistance response. To confirm this hypothesis we are utilizing the TUNEL analysis which will allow to point out present/absence of nuclear DNA degradation a clear sign of apoptotic process.

The autofluorescence was observed both in cultivar Mirco infected and NIL infected. To verify if exist a qualitative difference response between resistant and susceptible genotypes analysis of a large numbers of embryos allowed us to verify that different response does exists in terms of the timing of induction of the autofluorescence (earlier induction in the resistant genotype) and in terms of intensity of the autofluorescence (more intensity observed in the resistant genotype). By blue toluidine assay deposit of poliphenols has been confirmed the positive staining sites corresponding to the cell wall of Mirco and NIL embryos and was coincident with autofluorescence tissues (Fig. 33a and 33b; fig. 34a and 34b). However by a SUDAN III and Phluoroglucinol staining it was not possible to point out rispectively suberine and lignine accumulate. Therefore we have to hypothesize that a different kind of phenolic compounds are accumulated in the cell wall of barley embryos.

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