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INDIRIZZO BIOTECNOLOGIE

CICLO XXII

IDENTIFICATION AND CHARACTERIZATION OF PROTEINS INVOLVED IN BIOTIC STRESS RESISTANCE OF CEREALS

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A chi mi vuol bene:
per chi sono,
per quello che sono,
per come non sono
per chi ha imparato a sognare

Se un uomo non è disposto a lottare per le sue idee, o le sue idee non valgono niente, o non vale niente lui. (Ezra Weston Loomis Pound)

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ABSTRACT

Leaf rust is one of the most important diseases of barley (*Hordeum vulgare*) and is caused by the biotrophic fungal pathogen *Puccinia hordei*. The rust fungi penetrate barley leaves through stomata and colonize cells of the mesophyll, then growing systemically through the leaf vascular tissue.

The leaf rust resistance gene *Rph15* is of outstanding interest for resistance breeding because it confers resistance to over 350 *Puccinia hordei* isolates collected from around the world (Weerasena et al. 2004).

Plant-pathogen interactions activate many cellular signalling processes and, most likely, changes on protein accumulation and phosphorylation pattern of proteins play a pivotal role in plant responses to biotic stress. In this work, a proteomic approach was undertaken to study changes in total proteins accumulation and protein phosphorylation pattern in response to the leaf rust pathogen infection in two barley near isogenic lines, *Bowman* and *Rph15*, which differ for the introgression of the leaf rust resistance gene *Rph15*. Two infection time points, 24 hours and four days, were considered for the analysis.

No statistically significant differences were identified at the early time point, 24 hours post infection, for total and phosphorylated proteins.

At four days after inoculation, total protein analysis led to the identification of twenty-one protein spots significantly up or down regulated with a fold-change equal or higher than two following pathogen infection. Most of down-regulated proteins were found in the *Rph15* near-isogenic line while no significantly differential protein abundance was recovered in the susceptible line. Nineteen out of 21 protein spots were characterized by LC-MS/MS analysis and found to be involved in photosynthesis, sugar metabolism, energy balance and defence.

Phosphoproteomics analysis was performed at four day after inoculation. A phosphoprotein enrichment methodology based on MOAC (metal oxide affinity chromatography) was optimized for subsequent 2DE analyses.

RIASSUNTO

La ruggine fogliare è una delle malattie più importanti della coltura dell'orzo (*Hordeum vulgare*) ed è causata dal patogeno fungino biotrofo *Puccinia hordei*. Il fungo penetra attraverso gli stomi delle foglie dell'orzo e colonizza le cellule del mesofillo, crescendo poi per via sistemica nei tessuti vascolari della foglia.

Il gene *Rph15* di orzo è di considerevole importanza per il miglioramento genetico della resistenza in quanto conferisce resistenza a più di 350 isolati di *P. hordei* provenienti da tutto il mondo (Weerasena et al. 2004).

L'interazione pianta-patogeno attiva numerosi processi di signalling cellulare e, molto probabilmente, l'accumulo delle proteine e i cambiamenti nel pattern di fosforilazione delle proteine giocano un ruolo centrale nella risposta della pianta in seguito a stress biotico. In questo lavoro, un approccio di tipo proteomico è stato intrapreso per studiare i cambiamenti nei pattern proteici totali e delle proteine fosforilate in seguito a risposta alla ruggine fogliare in due linee quasi isogeniche di orzo, *Bowman* e la linea *Rph15*, che differiscono per l' introgressione del gene *Rph15*. Due tempi di infezione, 24 ore e quattro giorni, sono stati presi in considerazione per le analisi.

Nessuna differenza statisticamente significativa è stata individuate nel primo tempo di infezione precoce, a 24 ore dopo l'inoculo, sia per quanto riguarda le proteine totali che per le proteine fosforilate.

A 4 giorni dall'infezione, l' analisi delle proteine totali ha consentito di identificare ventuno spot proteici significativamente up o down regolati in risposta all' infezione con un fold-change almeno di 2. La maggior parte delle proteine down-regolate sono state trovate nel campione infettato della linea isogenica contenente il gene di resistenza *Rph15*, mentre non è stata riscontrata alcuna differenza statisticamente significativa nel pattern proteico della linea isogenica suscettibile. Diciannove dei 21 spot proteici sono stati caratterizzati mediante analisi LC-MS/MS e identificati essere implicati in processi come fotosintesi, metabolismo degli zuccheri, bilancio energetico e risposte di difesa.

L'analisi del fosfoproteoma è stata condotta a quattro giorni dopo l'inoculo. Una tecnica di arricchimento in fosfoproteine basata su MOAC (cromatografia di affinità mediante ossidi metallici) che è stata ottimizzata per la successiva analisi 2DE.

ABBREVIATIONS

2DE: Bidimensional gel electrophoresis
ABA: Abscisic Acid
ACN: Acetonitrile
ADP: Adenosine diphosphate
ADPG or **ADPGlc**: ADP-glucose
AGPase: ADP glucose pyrophosphorylase
AGPPase: ADP-glucose pyrophosphatase
$Al(OH)_3$: Aluminium hydroxide
AMP: Adenosine Monophosphate
AQUA: Absolute Quantification
ATP: Adenosine triphosphate
BPH: Brown planthopper
BSA: Bovine serum albumine
CCBB: Colloidal Coomassie Brilliant Blue
CHAPS: 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate
CID: Collision-induced dissociation
Cy: Cyanine Dye
Da: Dalton
DIGE: Differential in Gel Electrophoresis
DOC: Sodium deoxycholate
dpi: Days post infection
DTT: Dithiothreitol
EC: Enzyme Commission
ECD: Electron capture dissociation
ESI: Electrospray ionization
EST: Expressed sequence tag
ETD: Electron transfer dissociation
G1P: Glucose-1-phosphate
G6P: Glucose-6-phosphate
Glc: Glucose
GPI: Glycosylphosphatidylinositol
hpi: Hours post infection
HPLC: High performance liquid chromatography
HR: Hypersensitive response
ICAT: Isotope-Coded Affinity Tag
IEF: Isoelectrofocusing
IMAC: Immobilized metal-ion affinity chromatography
IPG: Iimmobilized pH Gradient
iTRAQ: Isobaric tag for relative and absolute quantitation
JA: Jasmonic acid
LAP: Leucine aminopeptidase
LC-MS/MS: Liquid chromatography-tandem mass spectrometry
m/z: mass-to-charge ratio
MALDI-TOF: Matrix Assisted Laser Desorption Ionization- Time of flight
MAP kinases: Mitogen-activated protein kinases

ABBREVIATIONS

Mb: Mega bases
MeJA: Methyl jasmonate
MES: 2-(N-morpholino)ethanesulfonic acid
MOAC: Metal oxide affinity chromatography
MSH: β- mercaptoethanol
MudPIT: Multi-dimensional protein identification technology
MW: Molecular weight
NaF: Sodium fluoride
NILs: Near isogenic lines
NP-40: Nonidet P-40 (octyl phenoxylpolyethoxylethanol)
PAL: Phenylalanine ammonium-lyase
PEG: Polyethylene glicol
PEG: Polyethylene glycol
pI: Isoelectric point
PMSF: Phenylmethylsulfonylfluoride
P-PEPC: Phosphorylated phosphoenolpyruvate carboxylase
PR: Pathogenesis-related protein
PTM: Post-translational modification
PVPP: Polyvinylpolypyrrolidone
ROS: Reactive Oxygen Species
Rph: Resistance to *Puccinia hordei*
Rubisco LC: Rubisco Large Subunit
Rubisco: Ribulose bisphosphate carboxylase/oxygenase
RUBP: Ribulose 1,5-bisphosphate
SA: Salicylic acid
SAR: Systemic acquired resistance
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel elecrophoresis
SILAC: Stable isotope labelling with amino acid
SOD: Superoxide dismutase
Suc: Sucrose
SuSy: Sucrose synthase
TCA: Trichloroacetic acid
TLP: Thaumatin-like protein
TMT: Tandem Mass Tags
UDPG: UDP-glucose

AMINO ACID ABBREVIATIONS

Amino Acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any Amino Acid	Xaa	X

ABBREVIATIONS

INTRODUCTION

1. PLANT-PATHOGEN SYSTEM

1.1 Barley taxonomy

Barley is one of the major cereal crops worldwide. It is preferentially used for animal feeding but also as a raw material for beer production in malting industry and for human nutrition.

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Liliopsida
Order: Cyperales
Family: Poaceae
Genus: *Hordeum* (barley)
Species: *Hordeum vulgare* (common barley)

Barley is a diploid crop with 14 chromosomes. The large genome (about 5500 Mb), composed of about 80% of repetitive DNA, is presently not amenable to whole genome sequencing. In the last years a growing EST database has given more resources for transcriptional analysis in many cell state and tissues and helps to establish high-density ESTs-based molecular maps forming the basis for comparative genomic studies, trait mapping, and map-based gene isolation. The total number of barley EST in database overcomes 554691 while protein sequences accounted for 11424 in January 2010 (http://www.ncbi.nlm.nih.gov). Thus, in large genome cereal species like barley, EST sequences facilitate a comprehensive overview of gene content and represent a resource to study the evolution and organization of a genome.

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Barley genome sequencing project is in progress and informations are available at International Barley Genome Sequencing Consortium (IBGS) website.

1.2 Barley-*Puccinia hordei* interaction

Rust fungi belong to the order *Uredinales* in the Basidiomycetes and are obligate biotrophic fungi. They cause some of the most devastating plant diseases and comprise more than 7000 species in over 100 genera. The genus *Puccinia*, the largest in *Uredinales*, is considered the most economically destructive genera of biotrophic fungi (Webb CA et al. 2006). The fungus *Puccinia hordei* is a biotroph pathogen causal agent of leaf rust, a serious leaf disease of barley in worldwide. Leaf rust is also known as brown rust. It causes serious economic losses: yield reductions by up to 62% (Weerasena JS et al. 2004) and it also adversely affects grain quality by reducing grain weight and increased levels of undesirable protein in the barley growing region of the world.

The infection process of cereal rust fungi has been closely examined in several species, but the molecular mechanisms are poorly understood (Webb CA et al. 2006).

On barley, infections occur as small, orange-brown pustules mainly on the upper, but also on the lower surface of leaf blades. Pustules containing uredospores are small and circular. The aecidial stage occurs on species of *Ornithogalum* (Star of Bethlehem), which is the alternate host. Teliospores then can undergo a sexual reproduction cycle and produce aceiospores which can then re-infect barley.

Life cycle of barley leaf rust is similar to *P. graminis* one (Fig. 1.1).

Rust fungi have a complex life cycle that involves two parasitic stages, dikaryotic and monokaryotic (Heath MC 1997). The dikaryotic stage is the form of rust that causes rust disease.

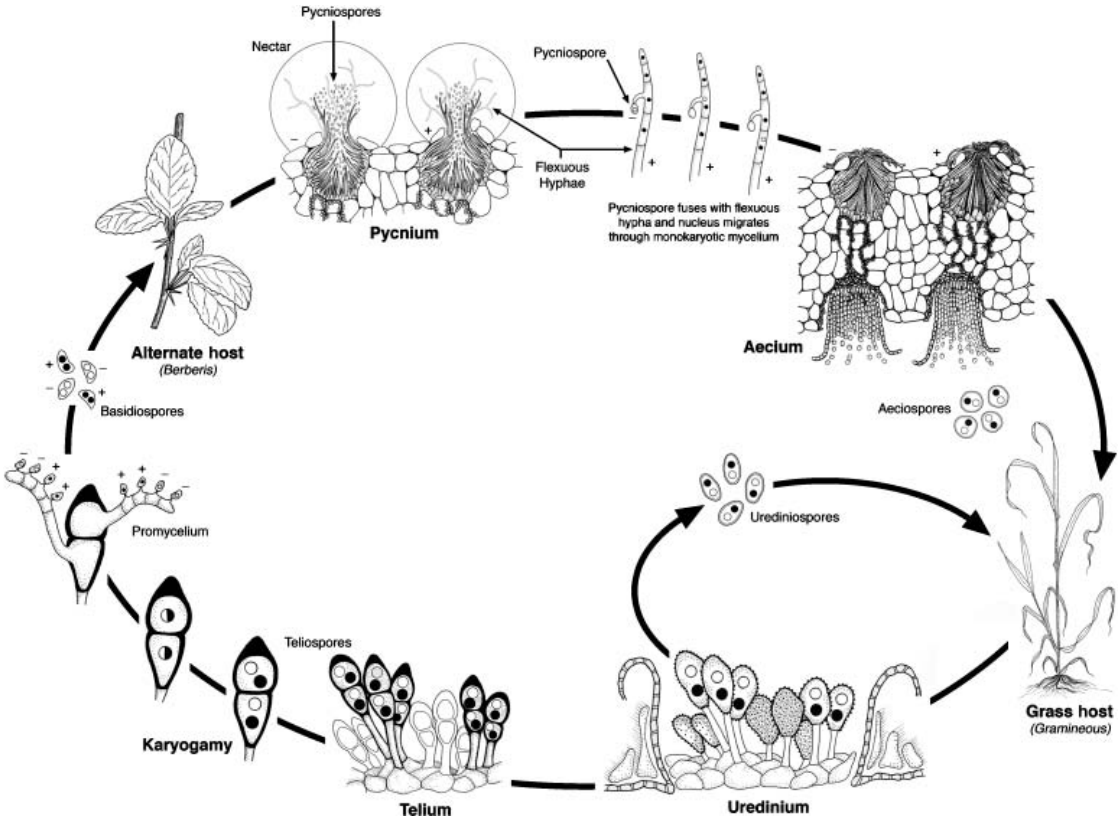

Fig. 1.1. Life cycle of basidiomycetes fungus *Puccinia graminis*. The asexual uredinial stage is repeated on the grass host with a new generation of dikaryotic urediniospores every 14–20 days under favourable conditions. Teliospores begin the sexual stage of the life cycle, and karyogamy occurs in maturing teliospores, and meiosis of the resulting diploid nucleus begins before the teliospore enters dormancy. Basidiospores infect barberry, the alternative host, on which the fungus produces haploid pycnia. Fertilization occurs with the fusion of a pycniopspore with a flexuous hypha of opposite mating type. Following fertilization, a dikaryotic aecium forms and begins producing dikaryotic aeciospores, which complete the life cycle by infecting the grass host. (Leonard KJ et al. 2005)

The life-cycle is complete when aeciospores germinate and penetrate stomata of telial/uredinial host, resulting in production of asexual urediniospores. Urediniospores are deposited by wind or rain on either side of leaf. Spores may retain viability 1-3 days after inoculation under field condition in the absence of an immediate dew period (Bolton MD et al. 2008).

The urediospore germ tube of many rust fungi responds to topology of the leaf surface to direct the germ tube to a stoma where an appressorium is produced over the stomatal aperture.

The stomata usually close promptly in response to appressorium formation, and remain closed in the presence of a mature appressorium (Bolton MD et al. 2008).

An infection peg grows between the guard cells and a vesicle is formed in the substomatal space. An infection hypha grows intercellularly between mesophyll cells and haustoria are formed from haustorial mother cells. These haustoria are generally considered the feeding structures for the fungus and are the controlling factors for host

response. They penetrate the pant cell walls but plasma membrane of the infected cells is only invaginated.

After haustorial formation, more infecting hyphae produced from the haustorial mother cell grow and come into contact with additional host cells. These also form haustorial mother cells and haustoria, resulting in a branching network of fungal mycelium (Bolton MD et al. 2008).

Leaf rust develops rapidly between 18 and 22 °C (Shitaya MJY et al. 2006; Gair R et al. 1983). Following infection, new pustules and spores may be produced within 7 days. Urediniospores can be spread across long distances. The teliospores are thick-walled and can persist in the environment. Upon germination, they develop a promycelium (basidium) with four haploid sporidia (basidiospores). The basidiospores can infect both barley and the alternate host.

General tissue chlorosis and eventual necrosis are often associated with such late infections.

Effective fungicides are available for the control of barley brown rust though severe levels of disease occur too infrequently and erratically for their routine use to be recommended (Gair R et al. 1983). The most obvious alternative to fungicide treatments is the use of resistant cultivars.

The genome of the fungus *Puccinia hordei*, the causal agent of barley leaf rust, is not sequenced but, currently, *P. graminis* and *P. triticina* sequencing projects are in progress.

1.3 Barley genetics of resistance to leaf rust

In many plant pathosystems there are two distinct forms of resistance: qualitative, race-specific resistance, and quantitative, race-non specific resistance. In general, qualitative resistance is monogenically or oligogenically inherited. This resistance is almost always based on a hypersensitive reaction of plant tissue (Jiang GL et al. 2007). Posthaustorial resistance is often associated with hypersensitive resistance, particularly in race-specific disease resistance (Neu C et al. 2003).

In contrast, quantitative resistance is polygenic, generally not based on a hypersensitive reaction (HR), and presumably more durable (Jiang GL et al. 2007).

Race-specific resistance is controlled by a gene-for-gene relationship. This resistance has been extensively studied during the last decade and a number of disease resistance genes as well as avirulence genes have been isolated from different plant-pathogen systems (Dangl JL et al. 2001).

Monogenic resistance to leaf rust pathogens in barley is governed by major genes (Resistance to *Puccinia hordei* – *Rph* genes) that are race specific. This resistance is often complete, and associated with necrosis of plant cells attacked by the pathogen sporelings. Some of the *Rph* genes have a delayed or rather weak effect on the pathogen. Such *Rph* genes confer incomplete resistance, in which the fungus forms small uredinia that are surrounded by chlorotic or necrotic plant tissue.

Some barley cultivars have a high level of non-HR polygenically inherited leaf rust resistance, which has been termed "partial resistance" by Parlevliet (1975) (Jafary H et al. 2006). The non-hypersensitivity resistance, also called "partial resistance", is not associated with plant cell necrosis. This resistance is characterized by low levels of infection than a compatible interaction where high infection levels are present (Niks RE et al. 2000). This resistance can be prehaustorial, a resistance process in which many haustorial mother cells fail to form haustoria (Martinez F et al. 2004). Thus, a reduced infection frequency and a prolonged latency period are present.

1.4 Barley *Rph 15* gene

Resistance in barley to *P. hordei* is governed by major race-specific resistance genes named *Rph*.

Several seedling resistance genes have been identified from cultivated and wild barley, of which 19 were designed *Rph1* to *Rph19*. Some mapping studies of leaf rust resistance genes in barley have shown that *Rph15* gene is located on chromosome 2HS (Weeresena JS et al. 2004) (Fig. 1.2).

The resistance provided by single *Rph* genes has often been overcome by new pathotypes, believed to have arisen via introduction of mutations. As a direct consequence, the number of effective *Rph* genes available to breeders is decreasing rapidly, suggesting the need for a new gene deployment strategy (Golegaonkar PG et al. 2009).

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Most of the reported leaf rust resistance genes have been assigned to a chromosome or specific chromosome region.

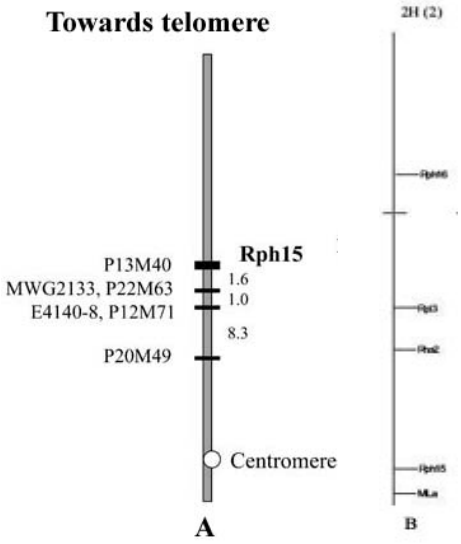

Fig. 1.2. Map of barley chromosome 2HS (A) in the area surrounding *Rph15* using Bowman (*Rph15*)xBowman mapping populations (Weeresena et al. 2004). (B) Consensus map of identified resistance genes (Chelkowski J et al. 2003).

The leaf rust resistance gene *Rph15* was derived from PI 355447, an accession of wild barley (*Hordeum vulgare* subsp. *spontaneum*) collected in Israel. It was evaluated for its reaction to a diverse collection of over 350 *P. hordei* isolates from around the world and conferred resistance to all but one of the isolates (90-3 from Israel). This gene is one of the most broadly effective resistance genes known and may therefore be useful in barley breeding programs. Thus, *Rph15* may be combined or "pyramided" with other genes as a means to expand the resistance spectrum against *P. hordei* and increase durability (Weerasena JS et al. 2004).

2. PROTEOMICS

2.1 What is Proteomics? An overview

In late 1970s researchers started to build databases of proteins using new technique of two-dimensional gel electrophoresis. In the 1990s, biological mass spectrometry emerged as powerful analytical method that removed most of the limitations of protein analysis.

Proteomics covers the global analysis of the products of gene expression, including the identification, quantification and characterization of proteins. It represents linkage among transcriptome, metabolome and genome information using bioinformatics (Fig. 2.1). The word "proteome", coined by Wilkins in 1994, represents the protein complement expressed by the genome of a cell and modified subsequent to expression. The "proteomics burst" was mainly due to an ever increasing genome sequence database (Kersten B et al. 2006).

Verification of a gene product by proteomic method is an important first step in "annotating the genome" (Pandey A et al. 2000). Modifications of the proteins that are not apparent from DNA sequence, such as different isoforms and post-translational modifications can be determined only by proteomic methodologies.

Proteomics can be divided in three major areas such as:

- ✓ **Differential Expression Proteomics** that refers to qualitative and quantitative study of the expression of total proteins under two or more different conditions. For example, proteome of a cell in two different biological conditions can be compared to investigate changes in protein expression and individualize proteins responsible to a particular state.
- ✓ **Structural Proteomics** is about the structural aspects, including the three-dimensional shape and structural complexities, of functional proteins. This includes the structural prediction of a protein when its amino acid sequence is determined directly by sequencing or from the gene with a method called homology modelling. This can be carried out by doing a homology search and computational methods of protein structural studies and predictions.

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- ✓ **Functional Proteomics** takes into account macromolecular networks involved in the life activities of a cell. Hence, it is possible to identify specific protein molecules and their role in individual metabolic activities and their contribution to the metabolic network that operates in the system.

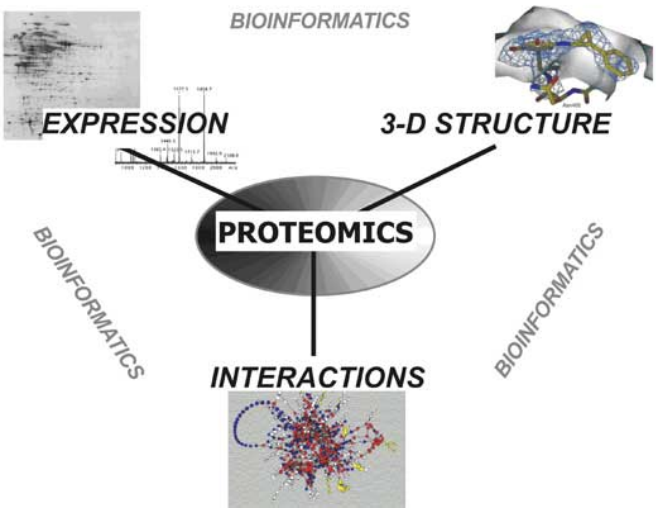


Fig. 2.1. Map of proteomics interaction: the central bridge between genomics, transcriptomics and metabolomics.

In proteomics analysis, nowadays 2DE (bidimensional sodium dodecyl sulphate polyacrylamide gel) described by O'Farrell in 1975 remain the most powerful and high throughput separation technique to provide information on protein isoforms and post-translational modifications and on protein expression level of a cell or tissue at a specific time point or at a particular biological state.

2DE consists in two orthogonal separation steps: an isoelectrofocusing where complex protein extract is separated by charge into IPG (immobilized pH gradient), thus proteins migrate according to pI (isoelectric point) into gel; followed by SDS-PAGE to resolve proteins according to their molecular mass. Two-dimensional analysis is coupled to MS for characterization of proteins in a biological process.

2DE, MS (mass spectrometry) and bioinformatics tools are the key components of an approach that has been termed "the classical proteomic methodology" (Görg A et al. 2009).

All steps in sample preparation and 2DE analysis are critical and reproducibility results must be guaranteed to provide a robustness statistical analysis. For this reason, the power of statistical test is increased adding the number of sample replicates, making it possible to draw reliable conclusions. Given the labour and cost involved in

the 2DE analysis, the number of replicates is often restricted to minimum. Technical variability can be introduced at the level of: (a) sample collection, (b) sample preparation and protein extraction, (c) sample loading and electrophoresis, (d) staining and image analysis, using different algorithm to perform spot detection and spot matching (Chich JF et al 2007; Srinark T et al. 2008).

A lot of protein extraction protocols (Isaacson T et al. 2006; Maldonado AM et al. 2008; Sheoran IS et al. 2009) are available for different tissues taking into account the subsequent analysis. Protein extraction protocol must be optimized to each tissue and to the research objectives.

The industrial production of IPG Drystrip was a milestone in proteomics in 1991 and contributed the increased reproducibility of bidimensional experiments and inter-laboratory comparisons. Other advantages of IPGs are increased resolution by the ability to generate (ultra)narrow pH gradients, reproducible separation of alkaline proteins and increased loading capacity (Görg A et al. 2004). Two type of sample application on IPG strips are possible, "cup-loading" or by "in-gel rehydratation" according to sample concentrations, kind of sample extract as particular species of proteins, and in active or passive manner.

All protocols should be modified for sample optimization. Drawbacks of 2DE are repeatedly reported with respect to analysis of very alkaline and very acidic, low abundant and/or hydrophobic proteins, moreover, it is difficult to automate. This limitations can be overtake by using IPG strips commercially available which cover overall pH range to visualized acidic or alkaline proteins. Multiple runs in parallel of second-dimension SDS-PAGE gels are preferred for large-scale proteome analysis for high throughput and maximal reproducibility. Strategies to increase analysis of low-abundant proteins are based on sub-proteome or sample prefractionation method. To date, the analysis of membrane and very hydrophobic proteins is a challenge because of their low solubility and their tendency to aggregate and precipitate in aqueous media.

In recent years, new technologies have been developed such as gel-free and label-based approaches, referred as quantitative proteomics.

The most quantitative proteomics technologies are label-based such as, SILAC (Stable isotope labelling with amino acids in cell culture), ICAT (Isotope-Coded Affinity Tag), iTRAQ (isobaric tag for relative and absolute quantitation) and DIGE

(Differential in Gel Electrophoresis) (Yang W et al. 2008). MS-based quantitative proteomics includes metabolic (*e.g.* SILAC) or chemical (*e.g.* ICAT, iTRAQ) method.

SILAC is performed in cell cultures and relies on the *in vivo* incorporation of amino acids with substituted stable (non-radioactive) isotopic nuclei (*e.g.* deuterium, ^{13}C, ^{15}N). It has been developed to detect differences in protein abundance between two (or more) samples. Two cell populations are grown in culture media that are identical except that one of them contains a 'light' and the other a 'heavy' form of a particular amino acid (*e.g.* L-arginine and ^{13}C-labeled L-arginine). The labelled amino acid is incorporated into all newly synthesized proteins.
After at least five doublings equal amounts of "light" and "heavy" cell population are combined immediately after harvesting (Fig. 2.2, A). Compared with the other quantitative proteomics techniques, SILAC is expected to have smallest technical variations (Yang W et al. 2008), high accuracy and better statistic on quantification.
Although metabolic labelling techniques are advantageous to reduce sample manipulation (*i.e.*, chemical modification or staining is not required), some drawbacks include the fact that they cannot be employed on primary tissues or bodily fluids and that they can be expensive if a large volume of growth media is required (Smith JC et al. 2008); moreover, in some cases arginine converts into proline.

ICAT is a cysteine-specific protein tagging method which contains a thiol-specific reactive group and a biotin affinity tag. This method is achieved after protein extraction, subsequently samples are combined, digested, and peptides are fractionated by biotin-avidin affinity chromatography (Fig. 2.2, B). The major advantage of ICAT is that is allows selective capture and analysis of cysteine-containing peptides, thus significantly reducing the complexity of the peptide mixture (Yang W et al. 2008; Chen S et al. 2006).

iTRAQ is performed at peptide level. An iTRAQ reagent consists of three components: (i) an amine-reactive group, which can covalently link to the N-terminal amine of peptides and the side chain of lysines; (ii) a charged reporter group with an MS/MS fragmentation site, which may give rise to diagnostic ions of m/z 114, 115, 116 or 117; and (iii) a neutral mass balance group to maintain an overall mass of 145 Da (Fig. 2.2, C). Owing to the isobaric mass design of the iTRAQ reagents,

differentially labelled peptides appear as single peaks in an MS scan, thus reducing the possibility of peak overlap and increasing detection sensitivity. When iTRAQ-tagged peptides are subjected to MS/MS analysis, the mass balance moiety is released as a neutral fragment and isotope-encoded reporter ions are liberated to provide quantitative information on proteins. Another key advantage of iTRAQ is that it is a multiplexed technology. This strategy has also limitations; first of all, high-abundance proteins are frequently identified. Secondly, since the labelling is performed at the peptide level, samples have to be processed separately until after digestion. This increases the potential for errors introduced during cell lysis, protein extraction, and/or proteolysis. Finally, since the derivatized peptides are indistinguishable in MS, MS/MS spectra have to be acquired for quantification, thus requiring more analysis time than performing result-dependent analysis on differential peptide pairs in MS (Yang W et al. 2008).

In contrast to technologies described above, **DIGE** is a gel-based quantitative technique (Fig. 2.2, D). Proteins from different samples are labelled with spectral-resolvable fluorescent dyes (Cy2, Cy3, and Cy5), pooled, and separated by 2DE. Differences in protein levels are then calculated according to spot colour and intensity. An interesting aspect of this technique is that different sample is separated in a single gel which is scanned with different lasers to detect proteins from each sample. Thus, gel to gel variations are eliminated and increased technical reproducibility, consequently, image analysis is facilitated. DIGE is a very powerful and sensitive method (Yang W et al. 2008; Görg A et al. 2004).

Many other MS-based methodology (*e.g.* AQUA, TMT) are developed and existing ones are continuously improved.

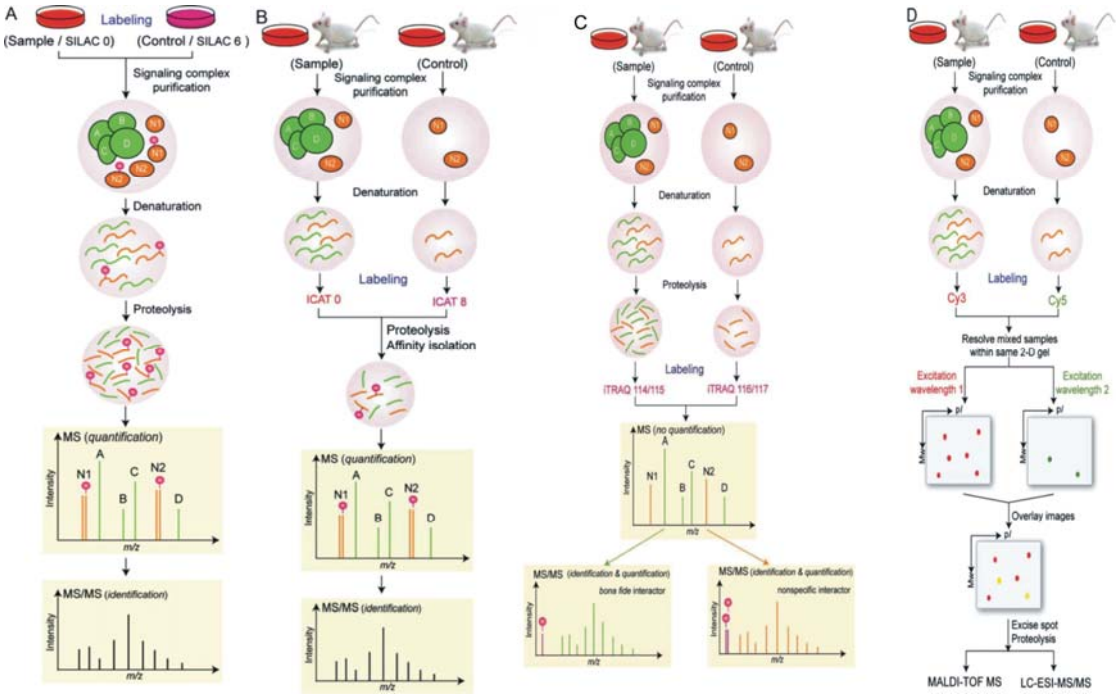


Fig. 2.2. Comparison of the most commonly used quantitative proteomic techniques: (A) SILAC; (B) ICAT; (C) iTRAQ; (D) DIGE (Yang W et al. 2008).

Metabolic labelling and chemical derivatization strategies are applied also to phosphoproteome analysis for characterization of cell signalling mechanisms (Collins MO et al. 2007; Smith JC et al. 2008; Schreiber TB et al. 2008; Thingholm TE et al. 2009).

The gel-based systems are currently the most powerful for non-model organisms (Carpentier SC et al. 2008). Despite numerous attempts to replace 2DE, which led to the development of several highly-sophisticated non-gel-based proteomics technologies, it maintains a good resolution and sensitivity. Furthermore 2DE provides information about the size and pI of the whole protein, as well as, a rough estimate of the abundance. Each proteome analysis technology currently available resembles characteristic technical advantages, but also limitations (Görg A et al. 2009).

2.2 Proteomics in plants

2DE coupled to MS has been used in plant descriptive and differential expression or comparative proteomics studies, post-translational modifications, and interactomics analysis.

Plant proteomic studies can be divided into two categories. The first, involves protein profiling of biological material with the aim of separating, sequencing and cataloging as many proteins as possible. A related and potentially complementary strategy is to target subcellular proteomes, thereby dramatically reducing the protein complexity of a particular extract and revealing important information regarding subcellular localization. The second category can be termed comparative proteomics, where the objective is not to identify the entire suite of proteins in a particular sample, but rather to characterize differences between different protein populations. This approach is thus somewhat analogous to comparative DNA microarray profiling. Example might include proteins from wild type versus mutant plants, or tissues at different stages or following responses to external stimuli (Rose JKC et al. 2004).

A typical proteomic experiment starts with protein extraction from cells, although studies with plants can be particularly challenging. This is because plant cells are not only rich in other constituents, such as cell wall polysaccharides and polyphenols that interfere with proteomics analysis, but also have a number of proteases that can degrade samples. In addition, the dominance of certain proteins can make it difficult to study other less abundant proteins. Rubisco (ribulose bisphosphate carboxylase-oxigenase) is the predominant protein in leaves (Quirino BF et al. 2009), while storage proteins are abundant in seeds.

The quality of protein extract is of importance and most unsuccessful attempts to visualize large number of well-resolved spots with minimal streaking or smearing are the results of problems with extraction and subsequent preparation. A unique extraction protocol does not exist for every proteins and each one should be evaluate what is better to obtaining results (Rose JKC et al. 2004).

Only few reports in plant proteomics are present in databases in contrast to bacteria and animal proteomics, and most of them focused in *Arabidopsis thaliana* or model plants such as rice (*Oryza sativa*), principally because they have completely sequenced genome. The existence of EST databases and orthologous sequences is very useful to identify and characterize proteins from organisms with incomplete genome sequence information. Porubleva and co-workers (2001) analyzed the proteome of maize leaves using EST databases and information from all higher plants as abundant proteins are highly conserved in plants. As results they identified proteins using homology to highly conserved proteins from other plants, especially Arabidopsis thaliana for which information about genome sequence are available.

Proteomics studies in plant biology research has increased significantly between 2006 to date (Fig. 2.3) according to development of new platforms of quantitative proteomics, as described above. SILAC technology is more often applied to microbes and mammalian cell cultures, but it seems inapplicable to plants because of the autotrophic nature of plant cells that causes difficulties in full incorporation. Recently, high percentage of ^{13}C-labeled L-arginine incorporation into Arabidopsis proteins is achieved. However chemical modification of proteins or peptides finds more applications since metabolic labelling in plants is often difficult (Chen S et al. 2006). iTRAQ strategy has been demonstrated effective in plant phosphoproteomics.

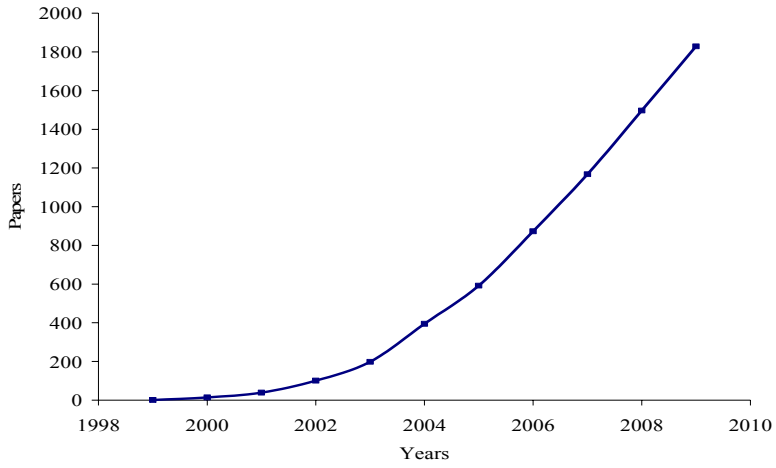


Fig. 2.3. Plant proteomics progress in scientific research until December 2009 by NCBI website.

Jorrín-Novo and colleagues (2009) summarized advances on plant proteomics about methodology as sample preparation, 2DE improvement in the area of separation of hydrophobic proteins, gel staining, analysis and automation. Looking back at previous publications it is possible to identify errors derived from incorrect experimental design, data analysis and interpretation. They reviewed also information obtained from subcellular proteomics in plants and differential expression proteomics.

Proteomic analyses of plant organs or tissues are applied to monitor developmental changes or the influence of environmental stimuli on protein patterns but are also used to compare lines with different backgrounds. Some reports published during 2002 and 2004 (Østergaard et al. 2002; Østergaard et al. 2004; Bak-Jensen KS et al. 2004) investigated proteome of seeds and malt of different barley cultivars. Even though malting is basically the germination process carried out under controlled conditions, it results in malts of different quality depending on the barley cultivar, the condition for

growth in the field, and the storage of the grains. Distinct spot pattern differences are observed between cultivar for the mature seeds and for malt. They concluded that it is possible to relate cultivar dependent differences in spot patterns to enzymes important in the malting processes. Changes in proteome seeds are visualized using two range of pH (4-7 and 6-11). Proteins identified in water-soluble fraction of mature barley seeds include housekeeping enzyme, chaperone, defence proteins and proteins related to desiccation and oxidative stress.

Combination of 2DE/MS and MudPIT (multi-dimensional protein identification technology) experiments for the analysis of *Arabidopsis* floral proteome, by Feng and co-workers (2009), demonstrated that results are partially overlapped. They identified 216 proteins in the flower proteome at stage when all floral organs are mature, followed by later stages with pollen germination, double fertilization, and the early embryo development. They presented also evidence for protein modification, as methylation, that is implicated in floral development and physiology also by comparison with transcript levels. In the report of Lee and colleagues (2006) the same combination of proteomic techniques was applied to detect proteins in resistant and susceptible rice challenged by the fungal pathogen *Rhizoctonia solani*. They detected differences between the resistant and susceptible lines and response to pathogen involved the recruitment of proteins from various defence pathways. The Rubisco large subunits were increased in both the resistant and the susceptible lines, where the expression ratio in the mutant line was higher than in the susceptible line. The level of Rubisco protein, crucial enzyme for photosynthesis, is known to be reduced in infected plant cells because pathogens attack chloroplasts that lead to their degradation.

Attention is paid to crop for its role in sustainable agriculture and efforts are directed to identify genes and proteins that control crop plants architecture and/or stress resistance in a wide range of environments for biotechnological improvement of crop productivity. The application of proteomics in crop breeding is usually initiated by detection of stress responsive proteins thought comparison between stressed and control plants (Salekdeh GH et al. 2007). A rice proteome database was assembled using 2DE containing proteins from different rice tissues and subcellular compartments (Komatsu S et al. 2004).

2.2.1 Plant proteomics and biotic stress

The impact of proteomics in plant biology is growing. Although proteomics can identify novel proteins, in some case, indicate that they are activate or inhibited under specific conditions, for example under stress. The presence of a pathogen during biotic stress superimposes another proteome onto that of the host and complicated analysis (Rampitsch C et al. 2006). In recent years, some reports were addressed to plant proteome modifications associated with pathogen interaction. Therefore, pathogens induce several genes, such as catalases and superoxide dismutase (SOD), which are responsible for the inactivation of H_2O_2 and O_2^-. Effector proteins expressed by the pathogen are predicted to collaborate in the suppression of basal resistance through the modification of specific host proteins. The secretion of extracellular enzymes by the pathogen, such as pectine esterase, polygalacturonases, xylanases, pectato lyases, and cellulases, is another essential process for colonization and pathogenicity (Mehta A et al. 2008). In this review, Mehta and colleagues listed plant-pathogen interactions that are investigated by proteomic approaches. With regard to plant responses, only a few proteomic studies have investigated plant-pathogen interaction. In such studies, several proteins involved in different biological processes, including defence and stress responses, signal transduction, photosynthesis, electron transport and metabolism, have been found (see Table 2.1, in the next page).

The most commonly used approach in proteomics studies of plant-pathogen interaction has been 2D gel electrophoresis followed by mass spectrometry, which aids the detection of differentially expressed proteins between pathogen-challenged and unchallenged plants.

Table 2.1. Plant proteins accumulated during different plant-fungus interactions and identified using proteomic approaches (Mehta A et al. 2008)

Protein	Studied organism	Pathogen	Accession no.[a]
Peroxidases (PR-9)	*O. sativa*	*Ma. grisea*	AAC49818
	O. sativa	*Rhizoctonia solani*	gi32879781
	Triticum aestivum	*F. graminearum*	AAL08496
	Tomato	*F. oxysporum*	–
	A. thaliana	*Fusarium* elicitor	At1g07890
β-1,3-Glucanases (PR-2)	*O. sativa*	*Ma. grisea*	BBA77783
	O. sativa	*R. solani*	gi4884530
	T. aestivum	*F. graminearum*	AAD28734
	Zea mays	*F. verticillioides*	–
	Tomato	*F. oxysporum*	AAA03617
Thaumatin-like protein (PR-5)	*O. sativa*	*Ma. grisea*	–
	O. sativa	*Ma. grisea*	T04165
	T. aestivum	*F. graminearum*	CAA66278
	Tomato	*F. oxysporum*	AAM23272
Chitinase (PR-3)	*O. sativa*	*R. solani*	gi55168113
	T. aestivum	*F. graminearum*	BAB82472
	Tomato	*F. oxysporum*	CAA78845
Glutathione *S*-transferase	*T. aestivum*	*F. graminearum*	CAC94005
	Z. mays	*F. verticillioides*	2288968
	A. thaliana	*Fusarium* elicitor	At1g02930
Glyceraldehyde 3-phosphate dehydrogenase	*O. sativa*	*R. solani*	gi166702
	T. aestivum	*F. graminearum*	XP493811
	Z. mays	*F. verticillioides*	Q09054
Pathogenesis-related class 10	*O. sativa*	*Ma. grisea*	T14817
	O. sativa	*Ma. grisea*	AF416604
	M. truncatula	*Aphanomuces euteiches*	P93333
Fructose-bisphosphate aldolase	*Z. mays*	*F. verticillioides*	P08440
	A. thaliana	Fungal elicitor	At3g52930
Probenazole-induced protein	*O. sativa*	*Ma. grisea*	T02973
	O. sativa	*Ma. grisea*	T02973
Adenosine kinase	*Z. mays*	*F. verticillioides*	AJ012281
Superoxide dismutase (Cu–Zn)	*Z. mays*	*F. verticillioides*	P23346
Glutamate dehydrogenase	*T. aestivum*	*F. graminearum*	AAB51596
Thioredoxin	*T. aestivum*	*F. graminearum*	CAA06735
Disease-resistance-response protein pi 49	*M. truncatula*	*Aphanomuces euteiches*	PI4710
20S proteasome β unit	*O. sativa*	*R. solani*	gi50933089
Chaperonin 60 β percursor	*O. sativa*	*R. solani*	gi34897924
Receptor-like protein kinase	*O. sativa*	*Ma. grisea*	
	O. sativa	*Ma. grisea*	AAL87185
14-3-3-like protein	*O. sativa*	*R. solani*	gi7271253

[a] Accession number from the organism of origin.

In rice, the blast fungus *Magnaporthe grisea* causes the most important rice disease (rice blast). Temporal changes in total proteins during this interaction were examined. After the elicitor treatment, ROS-related proteins and defence proteins were identified (Liao M et al. 2009; Ryu HS et al 2009). Comparative analysis of expression profiles of proteins in rice leaf sheaths in response to brown planthopper (*Nilaparvata lugens*, BPH), which is a serious rice pest, was performed by iTRAQ and quantitative RT-PCR in susceptible and resistant rice lines. The accumulation of 293 and 258 proteins changed significantly in the susceptible and resistant rice lines, respectively. Three

proteins involved in JA biosynthesis were induced in response to infestation, other two proteins that play a role in salicylate biosynthesis, phenylalanine ammonia-lyase and isochorismate synthase were down-regulated in response to BPH feeding. Discrepancies between mRNA and protein abundance at 96h were observed. It is evident that discrepancies could potentially be attributed to mRNA stability, splicing, translational regulation, post-translational processing, control of protein turnover, protein degradation or a combination of these. These results suggest that proteomics data are more relevant to biological responses because proteins, not RNAs, are functional products of these genes (Wei Z et al 2009).

In the report of Geddes and co-workers (2008), 43 acidic proteins spots were detected to be differentially expressed following fusarium head blight (FHB) (*Fusarium graminearum*) inoculation of tolerant and susceptible barley plants. Eighteen different protein spots were associated with metabolism, regulation and structural functions. Results of this work indicated that the induced plant defence responses following fungal infection were differentiated in resistant and susceptible barley genotypes. Different signalling pathways were activated including those leading to hypersensitive response, deposition of cell wall reinforcing materials, and the synthesis of a wide range of antimicrobial compounds including pathogenesis-related (PR) proteins and phytoalexins. Different classes of PR-proteins including PR-1, PR-2 (β-1,3-glucanases), PR-3 and PR-4 (chitinase), PR-5 (thaumatin-like protein (TLP)), and PR-9 (peroxidases) were accumulated within 6-12 h after infection. In this study, although no precursors of the JA (jasmonic acid), SA (salicylic acid), and PAL (phenylalanine ammonium-lyase) pathway were identified, a classical model of cereal defence response is reported. The SA pathway activates PR-proteins, and induces H_2O_2 accumulation as a signal for SAR. PR-3 (chitinases) and PR-5 (TLPs) are induced in cereals following treatment with either JA or SA, and PR-1 and PR-9 are only activated by the JA pathways. Resistant genotype CI4196 showed a significant increase in abundance of chitinase 2b. Chitinases are hydrolytic enzymes that inhibit the growth of many fungi in vitro by hydrolyzing the chitin of fungal cell walls. The oligomeric products of digested chitin can also act as signal molecules to stimulate further defence responses.

Other important crop cereal is wheat and a common worldwide problem resulting in yield losses is caused by the fungus *Puccinia triticina* (leaf rust). A proteomic approach was applied to investigate changes in the proteomes of both host and

pathogen during disease development in a wheat susceptible line and a near-isogenic line containing a resistance gene. The report of Rampitsch and co-workers (2006) looked at soluble leaf proteome. No proteins were found to be down-regulated. The absence of protein degradation is probably because the initial stages of the *P.triticina*-wheat interaction are more akin to symbiosis than pathogenesis, the rust being obligate biotroph, which do not kill their host. Most of the visible changes in the proteome during disease progression happened 6 days after inoculation.

3. POST-TRANSLATIONAL MODIFICATIONS

3.1 Post-Translational Modification on proteins

Post-translational modifications (PTM) of a protein are involved in several cellular processes such as protein conformation, cellular location, macromolecular interaction and activities, depending on the cell type, the tissue and the environmental conditions, enzyme regulation, signal transduction and stability.

More than 300 different types of PTMs are known and many other are regularly discovered. (Table 3.1). Modifications alter physicochemical properties of proteins. Many types of PTMs are covalently attached to amino acid residues in each protein, but they can also cleavage or delete a part of molecules. PTMs modify the primary structure of proteins in a sequence-specific way. Thus, PTMs are site specific, they are located at specific amino acid residues in proteins, usually in the context of a particular sequence motif and at specific time. Protein modification are often transient, location specific, polymorphic, substoichiometric, this means that a PTM at a given site is often present in only a small fraction of the protein molecules of a given type (Jensen ON 2006; Kwon SJ et al. 2006; Reinders J et al. 2007); or in some cases, PTMs act as molecular switches to activate, inactivate, or modify the biological activity of proteins.

Computational sequence analysis might therefore reveal potential sequence motifs for PTMs in proteins, including N-linked glycans, glycosylphosphatidylinositol (GPI) anchors and some of the most common phosphorylation sites (such as kinase-recognition motifs). However, the assignment of a PTM to a probable sequence motif in a protein by computational sequence analysis does not mean that the modifications are really present *in vivo* (Jensen ON 2006).

Table 3.1. A selection of post translational modifications, their relevant mass and biological roles (Jensen ON et al. 2006)

Post-translational modification* (modified residues)	**Change in mass (Δm, Da)**	**Example biological functions and comments**
Phosphorylation		
(Ser, Thr, Tyr)	80	Signal transduction, regulation of enzyme activity, involved in protein–protein and protein–ligand interactions; phosphorylation and sulphation have the same Δm (80 Da), but can be distinguished by positive- or negative-ion mass-spectrometry analysis
Glycosylation		
N-linked (Asn) O-linked (Ser, Thr) GPI anchor	> 800 203, > 800‡ > 1,000	Protein stability, solubility, secretion signal, regulator of interactions, extracellular recognition and interactions; modification by a GPI anchor is coupled to protein processing
Acylation		
Palmitoylation Farnesylation Myristoylation	238 204, 206§ 210	Protein localization and activity, involved in protein–protein and protein–membrane interactions
Sulphation		
(Tyr)	80	Signalling and protein localization, involved in protein–protein interactions; phosphorylation and sulphation have the same Δm (80 Da), but can be distinguished by positive- or negative-ion mass-spectrometry analysis
Ubiquitylation		
(Lys)	> 1,000	Protein degradation signal, involved in protein–protein interactions; observed as a +114-Da mass tag (Gly–Gly) after the tryptic digestion of a modified protein
Methylation		
(Lys mono-, di- and trimethylation, Arg mono- and dimethylation)	14, 28, 42	Regulates protein activity, protein–protein and protein–nucleic-acid interactions, chromatin dynamics and gene activity (histone modification); note that trimethylation and acetylation have similar mass increments
Acetylation		
(N-terminal residue, Lys)	42	Protein stability and activity, regulates protein–protein and protein–ligand interactions; note that trimethylation and acetylation have similar mass increments
Disulphide-bond formation		
(Cys)	-2	Stabilizes protein structure and activity, involved in redox processes
Oxidation		
(Met) (Trp)	16 4, 16, 32‖	Might regulate protein activity; often a chemical artefact
Deamidation		
(Asn, Gln)	1	Associated with ageing, might regulate protein activity and interactions; often a chemical artefact
Hydroxylation		
(Pro)	16	Structural stability (collagens)

*A more comprehensive list of post-translational modifications can be found on The Association of Biomolecular Resource Facilities and UNIMOD web sites (see Further information). ‡O-linked glycosylation can be a single sugar (203) or a chain of sugars (>800). §The Δm for farnesylation depends on the degree of saturation of the acyl group. ‖Different oxidized products can be generated from Trp. GPI, glycosylphosphatidylinositol.

Two-dimensional gel electrophoresis (2DE) is routinely applied to proteomic analysis of PTMs and subsequently characterized by mass spectrometry (MS) analysis

including matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, electrospray ionization (ESI) tandem mass spectrometry (MS/MS), and liquid chromatography (LC) MS/MS.

A key to improving proteomics approaches in the area of PTMs is to purify the organelles or protein species of interest prior to protein and PTM characterization by MS/MS. Association of PTMs with specific cell compartments, protein interactions or temporal windows has motivated systematic analyses of PTM changes with respect to space and time.

Post-translational modifications on plants

Plants are sessile organism, but they have an innate immune system as mammals. Plants are continually challenged by a variety of organisms (virus, fungi, bacteria, nematode, and insects) that are able to evade or suppress the primary defence responses, but they can activate secondary defence response that in most case involves a HR.

A plant defence response implies the involvement of post-translational modifications (PTMs) of pre-existing proteins in signal transduction cascades (Stulemeijer IJE et al. 2008).

A number of studies have identified that signalling cascades are activated by abiotic stresses, wounding, hormones, during plant-pathogen interaction and cell division, resulting in signal amplification. Researchers have identified different types of kinases in responding to signals. One of the most important and studied category is mitogen-activated protein kinases (MAP kinases) that convert signals from receptors to cellular responses.

MAPK cascades are composed of three protein kinases that are functionally interlinked. MAPKs are activated by dual-specificity kinases (MAPKKs) that phosphorylate at the serine/threonine and tyrosine residues in the conserved threonine-x-tyrosine (T-x-Y) motif. MAPKKs are activated when serine and serine/threonine residues present in the catalytic domain are phosphorylated by serine/threonine kinases MAPKKKs. Over the past years many components of MAPK cascade have been isolated and characterized from different plant. Only in *Arabidobsis* genome, there are 20 MAPKs, which suggest that MAP cascade in plants may be quite complex, 10 MAPKKs and 60 putative MAPKKKs (Zhang T et al. 2006; Mishra NS et al. 2006). The genome of *Arabidopsis thaliana*, with approximately the same

number of genes as that of the human genome, encodes for approximately 1100 protein kinases and 100-200 protein phosphatases, representing almost 5% of the *Arabidopsis* genome (Kersten B et al. 2006).

In contrast to the progress made in animal proteomics on PTMs, there have been few studies in plants, limited only to such field of PTMs as phosphorylation, GPI modification, and ubiquitination (Kwon SJ et al. 2006).

3.1.1 Phosphorylation of proteins

Reversible protein phosphorylation was first discovered by Krebs and Fisher in1955 and it is a highly conserved mechanism that was conserved throughout evolution.
Protein phosphorylation is considered a key event in many cellular processes as an on/off switch. This type of modification occurs in either the cytosol or the nucleus of the cell. It is estimated that one-third of eukaryotic proteins are phosphorylated at a given time (Morandell S et al. 2006), up to 50% of all proteins may be phosphorylated at some point during their life time, and over 100000 potential sites of phosphorylation exist in the human proteome (Morandell S et al. 2006; Reinders J et al. 2005; Smith JC et al. 2008).
Multi-site phosphorylation is now recognized as an important mechanism for the regulation of protein localization and functional activity and for the attenuation of protein-protein and protein-ligand interaction, although the molecular details and mechanism of regulation remains elusive (Jensen ON 2006).
Four types of phosphorylation occur, of which N-, S- and acyl-phosphorylation are very uncommon. The most common type, referred to as phosphorylation is research papers, is O-phosphorylation.
Phosphorylation mainly occurs on the hydroxyl group of hydroxyamino acid such as serine, threonine and tyrosine but can also occur on unusual residues such as hydroxyl-proline. Phosphorylation is executed by protein kinases that transfer a phosphoryl (PO_3) group from ATP to the hydroxyl group in the polar rest (R-) group of the amino acid residue, resulting in phosphoester (R-O- PO_3) bond, generating ADP (Fig. 3.1). Dephosphorylation occurs by protein phosphatases that hydrolyse the phosphoester bond, thereby releasing the phosphoryl group and restoring the hydroxyamino acid into its unphosphorylated state (Stulemeijer IJE et al. 2008).

INTRODUCTION

The covalent attachment of a phosphate group to a serine, threonine, or tyrosine residue is a reversible and dynamic process that regulates protein functions (cell division, growth, and differentiation) to cellular localization, signal transduction (Temporini C et al 2008) and half-life of proteins. In plants, protein phosphorylation plays a key role in pathogen response, for example plant-pathogen interaction, gene expression, and defence signalling.

Phosphorylation leads to heterogeneity into cells. Most phosphoproteins can be phosphorylated in different sites; this means that all the molecules of a given protein are not identically phosphorylated.

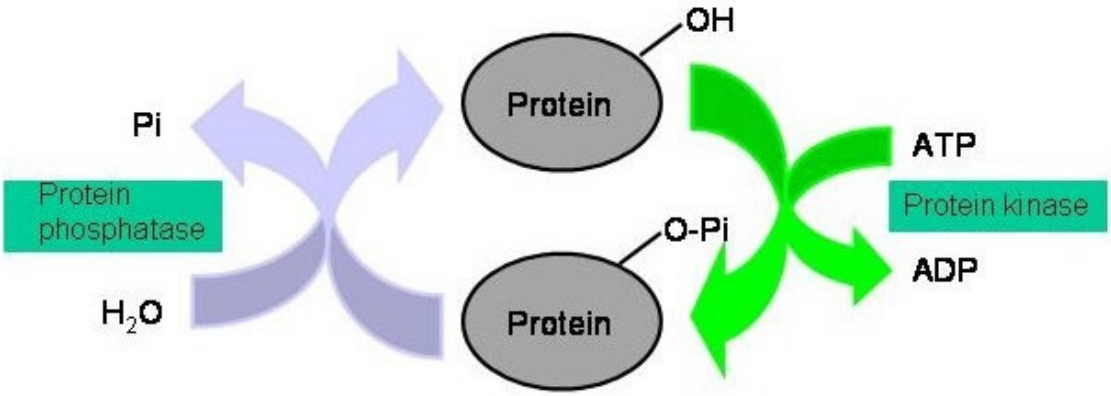


Fig 3.1. Protein phosphorylation mechanism in a cell.

Protein phosphorylation events are detected by increases in amino acid residues mass of +80 kDa, which report the addition of HPO_3. Sites of phosphorylation can be identified from mass shifts in fragment ions generated by gas-phase fragmentation (MS/MS) of phosphopeptides. Typically, MS/MS is performed using collision-induced dissociation (CID) in positive ion mode in which ions commonly acquired positive charge by addition of protons, but it produces limited or weak fragment ion spectra for certain phosphopeptides. Thus, alternative fragmentation methods are developed for PTM identification such as electron capture dissociation (ECD) and electron transfer dissociation (ETD). ECD and ETD are complementary to CID but are gentler fragmentation techniques, which preferentially cleave along the peptide backbone while retaining the labile phosphate moiety, so they have advantages for detecting phosphorylation and other PTMs unstable to MS/MS, because peptide fragmentation is less influenced by peptide sequence, and neutral loss reaction are reduced (Witze ES et al. 2007; Yang W et al. 2008).

For proteomic analysis of protein phosphorylation, western blotting and phosphor-specific staining are the most frequently used method to detect phosphorylated proteins. The advent of phosphosensor dye, ProQ Diamond (Molecular Probes, Inc.), facilitated analysis permitting sensitive detection of phosphoproteins on 2D gels (Schulenberg B et al. 2003; Agrawal GK et al. 2005) without the use of antibodies or radioactive isotopes and following by staining and imaging to reveal protein expression levels using a fluorescent total protein dye, SYPRO Ruby (Molecular Probes, Inc.), on the same gel. High quality anti-pTyr antibodies are commercially available, with which tyrosine phosphorylated proteins can be detected by high sensitivity. However, anti-pSer/pThr antibodies that can universally detect Ser/Thr phosphorylation are sensitive to amino acid sequence context (Yang W et al. 2008).

3.2 Phosphoproteomics in plants and stress response

Phosphoproteomics is still one of the most challenging tasks in contemporary proteomics. A number of reviews summarized the methods for separation and detection of phosphoproteins and phosphopeptides (Collins MO et al. 2007; Delom F et al. 2006; Mumby et al. 2005). Phosphoproteomics may have a benefit of identifying signalling components that would not be revealed by transcriptome analysis or two-hybrid system.

The completion of genome sequencing in both *Arabidopsis* and rice and advances in genomic and proteomic technologies has significantly facilitated the study of plant response to stress. Chitteti and co-workers (2007) focused their studies on protein differential regulation within the first 24 hours of salinity stress in rice. The majority of proteins stained heavily by ProQ Diamond (Molecular Probes, Inc.) dye had a relative low pI. This observation is consistent with the fact that protein phosphorylation results in acidification of the protein. Seven proteins displayed differential expression whether the gel was stained by ProQ Diamond (Molecular Probes, Inc.) dye or SYPRO Ruby (Molecular Probes, Inc.) stain. The other differentially regulated proteins were specific either to ProQ Diamond stain or SYPRO Ruby stain, 3 and 9 proteins, respectively. These results suggested that most proteins with quantity change during salinity stress were not phosphoproteins or have no change in phosphorylation level. On the other hand, most differentially regulated

phosphoproteins have modification level change instead of quantity change during salinity stress.

Plant mitochondria play a central role in cellular energy production, metabolism and stress responses. A proteomic approach employing 2DE, phosphoprotein enrichment and the phosphospecific stain ProQ Diamond (Molecular Probes, Inc.) was applied to mitochondria samples to determine the role of phosphorylation in *Arabidopsis* mitochondria (Ito J et al. 2009). A series of studies in plants over nearly 30 years have reported a total 22 plant mitochondrial proteins with some evidence of phosphorylation. Only nine of confirmed *Arabidopsis* mitochondrial phosphoproteins were identified in this study.

Jones and colleagues (2006) described changes to the phosphoproteome of *Arabidopsis* during the defence response to *Pseudomonas syringae* pv. *tomato*. In this study, they enriched for phosphorylated proteins by affinity chromatography from total soluble protein extracted from green leaves inoculated with bacteria. Although such *in planta* studies may suffer from increased biological variability, it is nevertheless important to study the pathogen infection response in the environment of the apoplast where the continued presence of the pathogen contributes significantly to the host defence response and effector proteins are correctly delivered into the plant cell. Phosphoproteins were extracted from total soluble protein using affinity columns (Qiagen) coupled to iTRAQ. This study identified six proteins robustly changing between challenges, corresponding to most abundant proteins in the phosphoproteome.

3.3 Enrichment method

A very small amount of proteins in a certain modification state is present in a heterogeneous population of proteins. This requires more material than needed for identification of an abundant protein and specific enrichment methods can be applied to separate modified proteins or peptides from unmodified species.

Rubisco is considered as the most abundant protein in the world, and it is estimate as more than half of the total leaf protein content. Rubisco can deplete by a commercial columns or using differential precipitation by PEG. Widjaja and colleagues (2009) asserted that the more labor-intensive Rubisco depletion *via* PEG fractionation is superior to the commercial antibody-based depletion.

Abundant proteins mask the presence of low abundant proteins. A lot of efforts have been made to remove the abundant proteins by affinity-based fractionation. The most powerful approaches dealing with this issue are sample pre-fractionation and zooming techniques that reduce sample complexity to enrich for low abundant proteins. A wide variety of fractionation tools is available which are based on electrophoretic or chromatographic separation of a specific subset of proteins. (Carpentier SC et al. 2008). Some methods use chemical reactions to capture PTMs, or use combinations of protein-digestion (Jensen ON 2006).

The work of Meimoun et al. (2007) describes the use of a commercial phosphoprotein affinity matrix (Qiagen) on sorghum and *Arabidopsis* extract. In this work authors showed the column efficiency to recover P-PEPC with no contamination by non-P-PEPC. The same chromatographic matrix was used to obtain a phosphoprotein-enriched fraction from two model plant, *A. thaliana* and *Medicago truncatula* by Laugesen and co-workers (2006).

These approaches in combination with an advance in MS technology, would improve the sensitivity and accuracy for the determination of PTMs.

High sensitivity is desirable in PTM proteomics to detect substrate proteins that exist in low abundance in cells. The detection sensitivity of a PTM proteomics screening depends on four factors: (i) yield of affinity enrichment, (ii) level of contamination from irrelevant peptides, (iii) sensitivity of HPLC/MS/MS system, and (iv) complexity of the peptide mixture (Zhao Y et al. 2009).

At present, there are several techniques for the analysis of phosphoproteins, and no one method supersedes the others in terms of the rapid and accurate identification of phosphoproteins.

Most common enrichment methods of phosphorylated proteins and/or peptides are based on immunoreactivity, charge and chemical reactivity of phosphate group (Yang W et al.2008). A survey of the different strategies for phosphoprotein enrichment is shown in Fig. 3.2.

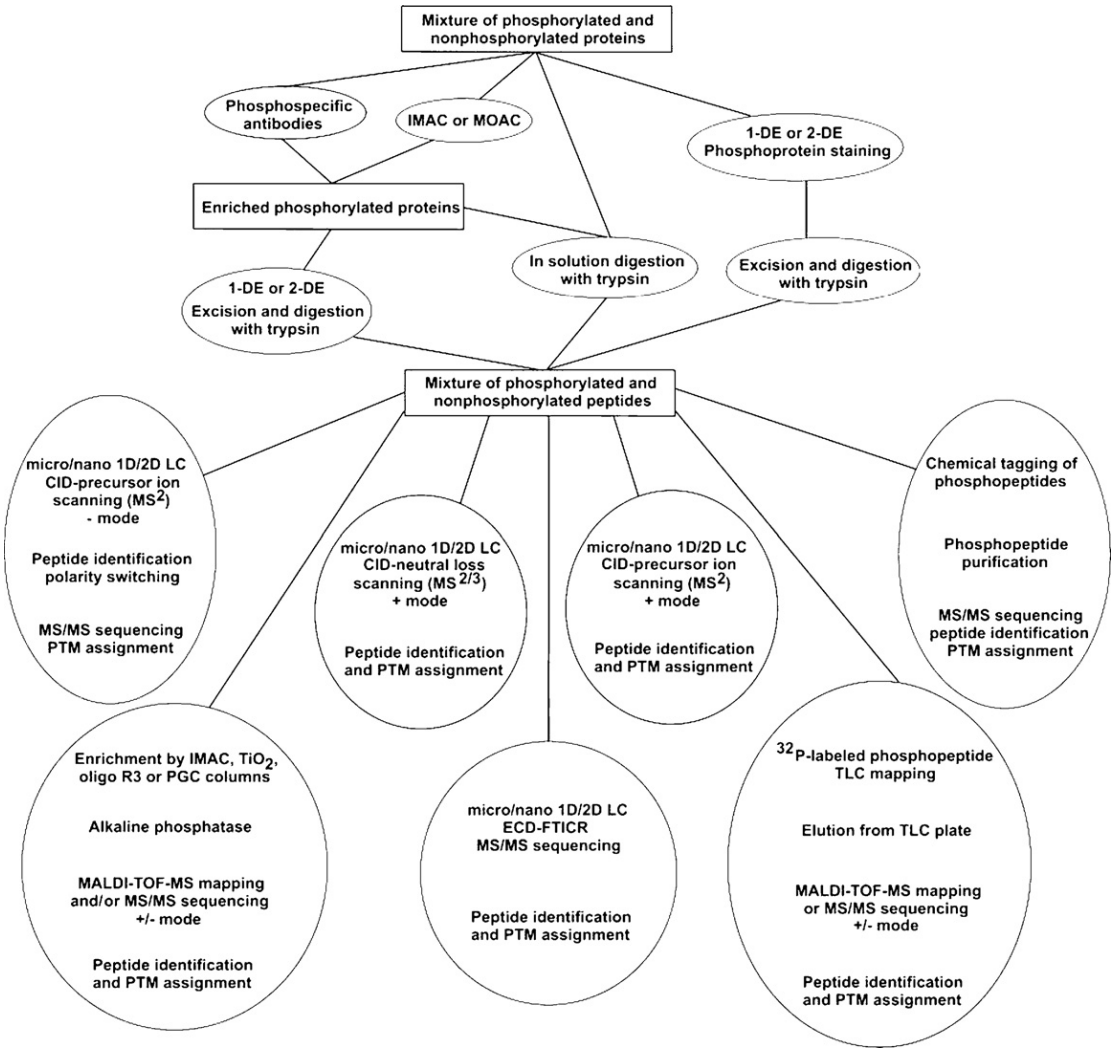


Fig. 3.2. Different techniques for the enrichment and the analysis of phosphoproteins based on MS procedures (D'Ambrosio C et al. 2007).

In a large scale study of phosphorylation, such as in a phosphoproteome, good phosphopeptides/phosphoproteins enrichment methods are applied. Most strategies applied to phosphoprotein enrichment can apply to phosphopeptide enrichment as well. Some of the most used methods are described below.

IMAC (immobilized metal-ion affinity chromatography) uses strong positive charge of transition metals, such as Fe^{3+} and Ga^{3+} that are immobilized on a polymeric supports. These metal cations bind negatively charged phosphate group with electrostatic interaction in stationary phase, and thus enrich phosphorylated peptides from a complex mixture. Non-phosphorylated species can be washed away and the phosphoproteins may be eluted by salt and/or pH-gradients. The coordination site on the metal ion may also interact with carboxylic groups of the peptides, resulting in non-specific binding.

An IMAC (Fe3+)-based technique was allowed recovery up to 90% of phosphoproteins and compatible with 2DE.

Metal oxide affinity chromatography (MOAC) is an emerging method of phosphopeptide enrichment based on different kinds of MOAC nanoparticles (TiO_2, ZrO_2, Al_2O_3). In Leitner (2009) is possible to give a brief overview of all different metal oxides that has been used to date. Weckwerth W and co-workers have proposed using aluminium hydroxide ($Al(OH)_3$) as an economic alternative to other metal oxide for phosphoprotein/phosphopeptide enrichment. $Al(OH)_3$ is applied on plant phosphoproteome analysis by Wolschin et al. (2005) who compared the method to commercial phosphoprotein enrichment kits but also applied to *Arabidopsis* leaf and seeds material. The following staining with a phosphospecific fluorescent dye to detect protein phosphorylation and its changes on gels facilitate to evaluate the yield of method. The utility of this methodology was demonstrated by the identification of phosphorylation sites in *Arabidopsis thaliana* seed proteins by 1D SDS-PAGE and MS. An improvement of this approach was carried out by Röhrig and co-worker (2008) to analyze phosphoproteins derived from various plant tissues. They have modified MOAC protocol to adapt with 2D SDS-PAGE. This method was also effective in circumventing problems associated with IMAC-based technique, such as non-specific binding of acidic proteins and peptides (Kersten B et al. 2006).

Immunoprecipitation. This strategy involves the use of antibodies specific for phosphorylated amino acid. It represents a powerful strategy for isolation and purification of phosphorylated peptides or proteins. In this approach, the antibody, directed against phosphorylated residues is often immobilized on a resin.

This approach has the advantage that it has the strong selectivity of an antigen-antibody system. A prerequisite for this approach is a good working antibody. For phosphotyrosine a set of good-working antibodies is commercially available. Although antibodies against serine/threonine-phosphorylated proteins have become available in recent years, they do not always work in immunoprecipitation experiments (Pinkse MWH et al. 2006).

Derivatization methods contain different chemical modification reactions to tag phosphate group (Fig. 3.3). Most of these approaches exploit the fact that phosphate esters on serine and threonine β-eliminates under alkaline conditions, with the formation of α,β-unsaturated double-bonds. Phosphotyrosine does not β-eliminate, making this approach specific for serine and threonine phosphorylated peptides. However, these chemical modification strategies can often lead to unwanted side reaction and they often require tailor-made bioinformatics tools to analyze MS data (Pinkse MWH et al. 2006).

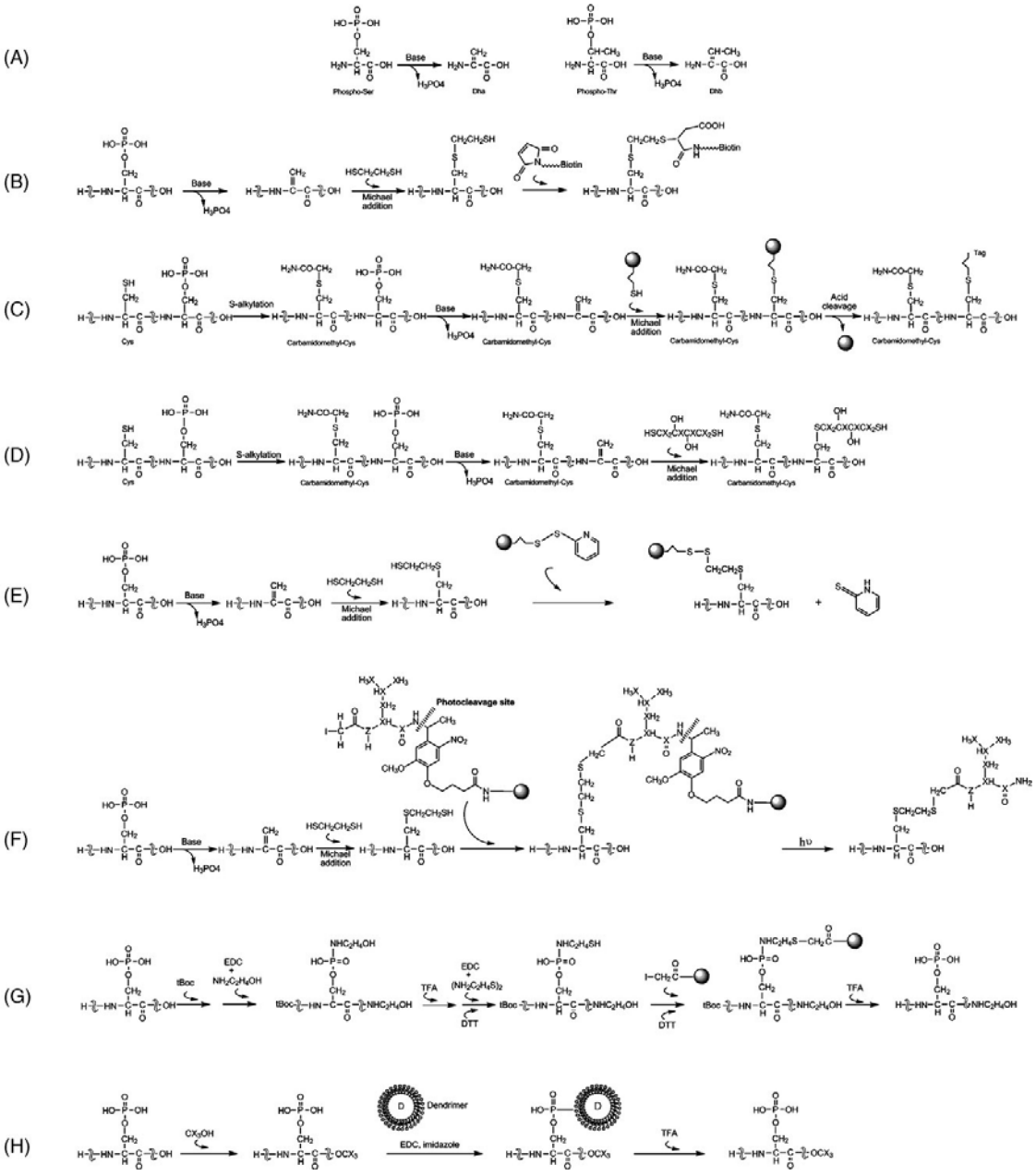


Fig. 3.3. Various strategies for the enrichment of phosphopeptides based on different chemical modification reactions. (Panel A) Base-catalyzed β-elimination of pSer and pThr yielding Dha and Dhb, respectively. Enrichment approaches by chemical tagging of the phosphate group based on a β-elimination/Michael addition chemistry modification (panel B, C, D, E, F). Enrichment approaches by chemical tagging of the phosphate group based on a carbodiimide condensation chemistry (panel G, H). Depending on the possibility to perform quantitative measurements, X corresponds to H or D (panel D and H); similarly X and Z correspond to ^{12}C or ^{13}C and ^{14}N or ^{15}N, respectively (Panel F) (D'Ambrosio C et al. 2007).

Choosing the most appropriate analytical method should depend on the sample quantity, complexity and availability and the type of phosphorylation involved. Pinkse et al. (2006) provides a summary of some enrichment strategies used in phosphoproteomics (Table 3.2).

Table 3.2. Comparison summery table for phosphorylated enrichment strategies (Pinkse MWH et al. 2006).

	Technology 1	Technology 2	Technology 3	Technology 4	Technology 5
Name of specific technology	Phosphoprotein enrichment kits	Phosphospecific antibodies	Chemical derivatization	IMAC	TiO_2, ZrO_2
Name of specific technologies with associated companies and company websites	Different formats available from various companies and suppliers.	Various companies and suppliers.		IDA or NTA resin available from various suppliers.	Titansphere or MonoTip TiO (GlSciences), and Sachtopore (Sachtleben Chemie GmbH and ZirChrom Separations)
Pros	High reduction of sample complexity.	Selectivity and sensitivity of antibody/antigen system.	Taylor made chemistry offers user a wide range of different affinity enrichment protocols.	Proven to be useful in large-scale analysis.	Straightforward enrichment method. Robust and chemically inert material.
Cons	Minimal information is usually supplied by manufactures on principle of purification.	Mainly works best for tyrosine phosphorylated proteins or specific antibodies directed against specific phosphopeptides.	Often multiple reaction steps are needed, leading to potential loss of sample and side reactions.	Limited specificity and capacity, biased toward multiple phosphorylated peptides, slow binding characteristic, affinity for acidic peptides.	Although high capacity and fast absorption, desorption is considerably slower.

AIMS OF WORK

Plant responses to pathogen infection have been deeply characterized from a transcriptomic point of view while the proteomic characterization of such responses has only recently started. In this work, a proteomic approach was applied to gain knowledge on protein accumulation changes in response to a biotic stress with the final objective of identifying pathways involved in the defence responses. Two near-isogenic lines were considered for the study: *Bowman*, susceptible to leaf rust and the resistant line *Rph15* containing the *Rph15* gene that confers resistance to over 350 *Puccinia hordei* isolates. This study was therefore aimed also in identifying changes in protein accumulation in a barley genotype carrying an extremely efficient rust resistance gene in comparison to a susceptible near-isogenic genotype. In this project a "classical" proteomic approach based on 2D/MS was performed to detect differentially accumulated proteins between infected and non-infected barley leaves. Even if barley genome sequencing has just started, the availability of a growing barley EST database and of the rice genome sequence aided the identification of proteins characterized by LC-MS/MS analysis.

In addition, a phosphoprotein enrichment technique was implemented on our experimental system with the aim of identifying pathogen infection-related changes in protein phosphorylation.

AIMS OF WORK

MATERIALS AND METHODS

1. MATERIALS

1.1 Chemical reagents

Reagents employed in the study were obtained from GE Healthcare (acrylamide, bis-acrylamide, IPG buffer, Immobiline DryStrip, cover fluid, CHAPS, iodoacetamide), Sigma (DTT, Phosphatase Inhibitor Cocktail, methanol, Pefabloc), Baker (ACN), Molecular Probes (ProQ Diamond stain, SYPRO Ruby, Rinohide, Peppermint stick MW marker). All other chemicals were standard reagent grade laboratory chemicals.

1.2 Barley leaf rust

The study was performed with barley leaf rust causal agent *Puccinia hordei* isolate 4 was kindly provided by Prof. Brian J. Steffenson, Department of Plant Pathology, University of Minnesota, USA. The fungus urediniospores were propagated on the appropriate susceptible host species and stored at -80°C. Inoculation was carried out after spores activation by shortly thermal shock at 37°C. The active spores were implemented on barley leaves and then were collected to infect barley seedlings.

1.3 Plant materials

Two near-isogenic lines (NILs) of barley (*Hordeum vulgare*) were used in this study. The line Bowman was susceptible to fungus infection while the NIL Rph15 was a resistant line. These barley genotypes were kindly provided by Prof. Jerry Franckowiak, North Dakota State University, USA. The line Rph15 contains the leaf rust resistance gene *Rph15* (Weerasena JS et al. 2004).

All barley seedlings were grown at 16 h (21°C):8 h (16°C) light:dark photoperiod in growth chamber or in greenhouse. At the first-leaf stage, barley seedlings were

inoculated with the fungus spores. A control experiments were also conducted without fungus infection.

The rust spores were mixed with talcum powder (1:10 v/v) before barley leaves infection. Spore density was of about 200 spores per cm^2.

Seedlings growth condition after incubation with *Puccinia hordei* were: for 24h at 20°C in complete darkness and 100% relative humidity, then plants were transferred at 20°C under a 14 h:10 h light:dark photoperiod and 59% relative humidity (Martinez F et al. 2004).

Each experiment was performed in triplicate. At the end of the infection times, the leaf samples were collected and stored at -80°C.

2. METHODS

2.1 Protein extraction

Barley leaf tissues were transferred into a pre-chilled mortar where they were ground to a fine powder with a pestle in liquid nitrogen and homogenized in an extraction buffer (0.5 M Tris-HCl pH 8.0, 0.7 M sucrose, 10 mM NaF, 1 mM PMSF, 0.1 mg mL^{-1} Pefabloc, 0.2% (v/v) Triton X-100, 2 μL mL^{-1} Phosphatase Inhibitor Cocktail (Sigma), 0.2 (v/v) β- mercaptoethanol) and PVPP 1% on ice. When powder was melted liquid, the suspension was transferred to centrifuge tube on ice and incubating for 30 minutes with regular mixing.

An identical volume of phenol saturated with TRIS-HCl 1 M, pH 8.0 was added and shaked.

The suspension was left at 4°C temperature for 2 hours.

Phenolic phase was separated by 30 minutes centrifugation at 4°C temperature at 4500 x g and it was collected in centrifugation tube.

5 volumes of cold 0.1 M ammonium acetate in methanol were added to the collected phenol phase and it was stored at -20°C overnight (Hurkman WJ et al. 1986).

The second day, samples were centrifuged at 4500 g for 30 min at 4°C. The supernatant was discarded and the pellet was solubilized with cold ammonium acetate, and it was stored at -20°C for 2 h.

Proteins were precipitated by centrifugation at 4500 g at 4°C for 30 min.
Three washing steps were performed with acetone 80% and the last one was stored at -20°C overnight.
This phenol extraction method is according to Isaacson and co-workers (2006), with some modifications.
In the last day, samples were centrifuged at 4500 g at 4°C for 40 min and the pellet was dried. The final pellet was solubilized in isoelectrofocusing buffer containing 7 M Urea, 2 M Thiourea, 3% w/v CHAPS, 1% w/v NP40, 50 mM DTT, 2% v/v ampholytes. Following incubation at room temperature for 1 h and centrifugation the supernatant at 10000 rpm for 10 minutes at room temperature. Then, samples were stored at -80°C until further use.
Proteins concentration was determined by 2D Quant Kit (GE Healthcare) and Bradford method, according to manufacturer's instructions (Biorad protein assay kit), with BSA as the standard.
For each sample two different extractions were performed.

2.2 Protein Quantification

Protein quantification was performed by Bradford dye assay which was based on Coomassie blue dye. This assay gives a colorimetric response protein dependent
This technique is simpler, faster, and it is subject to less interference by common reagents and nonprotein components of biological samples.
Coomassie blue is a hydrophobic dye used in the Bradford method to bind to the hydrophobic portions of proteins (Fig.2.1). Coomassie blue, in its protonated form, has a maximum light absorption at 465 nm. However when placed in a dilute acidic solution its anionic form, it binds preferentially to arginine residues and the complex absorbs light maximally at 595 nm. The amount of dye bound can be detected by a spectrophotometer.
BSA was used as the standard curve to determined the concentration of samples
The Biorad protein assay dye was diluited 1:5 in water before use. 1 µl of sample to be determined was added to 200 µl of the dye and absorbance at 595 nm was read after incubation of 5 min. Spectrophotometric reads of samples was in triplicate and serial dilutions of standard solution (0.5 µg, 1 µg, 1.5 µg, 2 µg, 2.5 µg, 3 µg) was in duplicate to give the calibration curve. A "blank" absorbance was subtracted to

absorbance raw data of unknown samples. Using the Lambert-Beer law is feasible to relate the amount of light absorbed to the concentration of the absorbing molecule, in this case proteins.

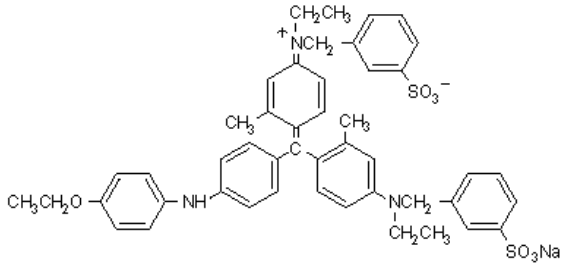


Fig. 2.1. Coomassie blue chemical structure of Bradford method

Absorbance reads were measured by Multimode Detection DTX instrumentation.

2.3 Tris-Tricine SDS-PAGE

Quality of proteins extracted was analyzed by SDS-PAGE according to Schägger and colleagues (1987). Protein mixture composition was also evaluated with this procedure because it is possible a resolution of protein below 10 kDa.

Before sample loading, solubilized proteins were mixed with Schägger sample buffer (50 mM Tris/HCl pH 6.8, 12% (w/v) Glycerol, 4% (w/v) SDS, 2% (w/v) MSH, trace of bromophenol blue) and boiled for 5 min.

2.4 2D electrophoresis

2-DE was performed by loading 300µg of proteins on 24 cm IPG strips.

IEF (Isoelectrofocusing)

Fig 2.2. Apparatus for 2D electrophoresis

A solubilized protein sample was loaded on a 24 cm strips (GE Healthcare) with a linear pH gradient pH 3-10 and then with pH 4-7 to gain the highest possible resolution.

The first dimension run was performed on IPGphor3 Manifold (GE Healthcare) and dry IPG strips were hydrated until 10 hours by a hydration solution (see below) before use in reswelling tray under mineral oil.

Sample in isoelectrofocusing buffer (7 M Urea, 2 M Thiourea, 3% w/v CHAPS, 1% w/v NP40, 50 mM DTT, 2% v/v ampholytes, traces of Bromophenol Blue) was applied after gel rehydration by cup loading immediately prior to IEF starting.

IEF hydration buffer

	Final concentration
Urea	7 M
Thiourea	2 M
CHAPS	3 % w/v
NP40	1 % w/v
Ampholytes	0.5 % v/v
DTT	10 mM
Orange G	0.002 % w/v (trace)

IEF was conducted at 20°C with current limit of 75µA/strip for about 60kVh.

Running IEF parameters:

Step	500V	1 h
Gradient	1000V	6 h
Gradient	10000V	3 h
Step	10000V	4 h 30 min

SDS-PAGE

A second dimension run in 2D electrophoresis according to molecular weight of protein was performed after reduction and alkylation of IPG strips. After IEF, strips were equilibrated by gentle stirring for 15 minutes in an equilibration buffer (100 mM Tris-HCl pH 6.8, 7 M Urea, 2 M Thiourea, 30% (w/v) glycerol, 2% (w/v) SDS added with 0.5% (w/v) DTT for disulfide bridges reduction, and for additional 15 minutes in the same equilibration buffer added with 4.5% (w/v) iodoacetamide for cysteine alkylation and 0.002% (w/v) bromophenol blue.
Second-dimensional SDS-PAGE (Laemmli UK 1970) was run in 12.5% acrylamide gels using ETTAN DALT*six* apparatus (GE Healthcare). IPG strip was blocked with an hot agarose solution on running gel.
Running was conducted for 30 min at 5 W/gel followed by 15 W/gel, until the bromophenol blue dye front left the gel.
For each sample four or six different technical replicates were performed for phosphospecific or total staining, respectively.

Running buffer 10X SDS-PAGE

	Final concentration
Tris	250 mM
Glycine	1.92 M
SDS	2% w/v
H_20	To final volume

2.5 Staining

Silver stain (Heukeshoven and Dernick 1986)

After SDS-PAGE Tris-Tricine, gel were fixed in ethanol/acetic acid solution and stained with silver nitrate with modifications of Heukeshoven and Dernick procedure. This is probably the most sensitive and reproducible silver stain method. It has a sensitivity about 0.1 ng of protein/spot as low as fluorescence SYPRO Ruby stain (Molecular Probes, Inc.). Stained gels were scanned in an EPSON ScanMaker i900 MicroTek Scanner

Fluorescent stainig

A number of commercially fluorescence dyes are available and they exhibit a dynamic range of about three-four order of magnitude. In this work two kinds of stains were applied to each gel. A fluorescent phosphospecific staining, ProQ Diamond (Molecular Probes) was used as first, and consequently a fluorescent stain for total proteins, SYPRO Ruby (Molecular Probes).

ProQ Diamond has a detection sensitivity of 1-8 ng of phosphoprotein/spot (Liu J et al. 2007) while SYPRO Ruby has a sensitivity of 0.5-5 ng of protein (Miller I et al. 2006).

Fluorescent staining are reversible and highly compatible with downstream analysis like mass spectrometry (Berggren et al. 2000). Staining protocol was modified according Agrawal (2005) and a diluted ProQ Diamond staining was used. All steps were performed in gentle shaking.

After 2-DE gels were fixed overnight in 50% (v/v) methanol and 10% (v/v) acetic acid. In the second day gels were washed twice in deionized water for 15 minutes and a diluted 1:2 ProQ Diamond stain was employed for 2 hour. Destain step was executed four times with 20% (v/v) acetonitrile and 50mM sodium acetate, pH 4.0. Before scan gels were washed in deionized water.

After scanning gels were rinsed in deionized water and each single gel were immersed overnight in SYPRO Ruby. Gels were destained in 10% (v/v) methanol and 7% (v/v) acetic acid and before scan they were washed in deionized water.

Images were acquired on Typhoon 9210 laser scanner (GE Healthcare) following ProQ Diamond, with 532 nm excitation and 560 nm longpass emission filter, and SYPRO Ruby stain, with two excitation peaks at 280 nm and 450 nm; and 610 nm bandpass emission filter.

Coomassie Stain (Neuhoff 1988)

Proteins were visualized using colloidal Coomassie Blue G-250(CCBB) procedure, as previously described by Neuhoff and co-workers (1988). Gel were fixed overnight in a solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid. Subsequently, they were stained for five days using the solution made up of 8% (w/v) ammonium sulphate, 0.8% (v/v) phosphoric acid, 20% (v/v) methanol and 0.08% (w/v) Coomassie Blue G-250.
The gels were scanned in an EPSON ScanMaker i900 MicroTek Scanner and analyzed with ImageMaster 2D Platinum Software (GE Healthcare), after being washed in deionized water.

2.6 Image acquisition and data analysis

Gels were acquired with a phosphoimager scanner Typhoon 9210 (GE Healthacare).
Spot detection, matching and image analysis were performed by ImageMaster 2D Platinum Software (version 6.0, GE Healthcare). Automatic matching was complemented by manual matching. Normalized spot volumes, as the percentage of the total volume of all the spots present in gel, of six replicate gels for total proteins analysis and four replicate gels for phosphorylated proteins analysis, for each sample condition, were compared both for total and phosphorylated proteins.

Matches which showed al least two-fold change in their relative volumes were subjected to two-way ANOVA test in order to verify if their changes in expression were statistically significant ($p<0.05$). Three different software were used for statistical analysis: Systat Software, R language and STATISTICA.

2.7 Protein in-gel digestion, mass spectrometry analysis and protein characterization

Spots were excised from CCBB-stained 2-DE gels and in-gel digested with trypsin [Sequencing grade modified Trypsin V5111, Promega, Madison] as described by Prinsi and co-workers (2009).

The LC-ESI-MS/MS experiments were conducted using a Surveyor (MS pump Plus) HPLC system directly connected to the Nano-ESI source of a Finnigan LCQ DECA XP MAX ion trap mass spectrometer (ThermoFisher Scientific Inc., Waltham, USA). Chromatography separations were carried out on a ZORBAX 300SB-C18 column (75 μm I.D × 150 mm length, 3.5 μm particle size, Agilent Tecnologies ®), using a linear gradient from 5 to 60% solvent B [solvent A: 0.1% (v/v) formic acid; solvent B: ACN containing 0.1% (v/v) formic acid] with a flow of 300 nl/min. Nano-ESI was performed in positive ionization mode with spray voltage and capillary temperature set to 1.7 kV and 180°C, respectively. Data were collected in the full-scan and data dependent MS/MS mode with collision energy of 35% and a dynamic exclusion window of 3 min.

Spectra were searched by TurboSEQUEST® incorporated in BioworksBrowser 3.2 software (ThermoFisher Scientific Inc., Waltham, USA) against the *Hordeum vulgare* protein subset (*7825 entries*) and against the *Hordeum vulgare* EST subset (*525775 entries*), both downloaded from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The searches were carried out assuming parent ion and fragment ion mass tolerance of ± 2 Da and ± 1 Da, respectively, one possible missed cleavage *per* peptide, fixed carboxyamidomethylation of cysteine and variable methionine oxidation. Positive hits were filtered on the basis of peptides scores [Xcorr ≥ 1.5 (+1 charge state), ≥ 2.0 (+2 charge state), ≥ 2.5 (≥ 3 charge state), ΔCn ≥ 0.1, peptide probability < 1×10^{-3} and Sf ≥ 0.70] (Eng JK et al.1994). If needed, identified peptides were used in protein similarity search performed against the *Viridiplantae* subset of the NCBI-nr database using the FASTS algorithm (Mackey AJ et al.2002). Physical properties of the characterized proteins were predicted by *in silico* tools at ExPASy (www.expasy.org).

2.8 Phosphoprotein enrichment method: metal oxide affinity chromatography (MOAC)

Protein extraction and separation from DNA/RNA with phenol

Resuspension on IEF buffer and enrichment of phosphorylated proteins

Precipitation of proteins with DOC-TCA and separation of proteins (SDS-PAGE and 2D electrophoresis)

Detection of phosphoproteins and verification of enrichment using phosphate specific fluorescent labelling

Buffer:

Incubation buffer 1 (IB1): 30 mM MES
0.1 M sodium glutamate
0.1 M potassium aspartate
20 mM Imidazole
0.12% (w/v) CHAPS
Adjust at pH 6.1 with HCl

Incubation buffer 2 (IB2): 30 mM MES
0.1 M sodium glutamate
0.1 M potassium aspartate
20 mM Imidazole
0.12% (w/v) CHAPS
Adjust at pH 6.1 with HCl

Elution buffer: 8 M Urea
0.2 M Potassium pyrophosphate

Up to 2 g of leaf sample was extracted as described above an resuspended in IEF buffer (7 M Urea, 2 M Thiourea, 3% w/v CHAPS, 1% w/v NP40, 50 mM DTT, 2% v/v ampholytes). Sample solutions, corresponding to 4 mg of proteins, were mixed with 6 ml of IB1 and put in shaking rotator for 30 min at 10°C.

Aluminum hydroxide (Sigma A-1577) was preequilibrated twice with IB1 by a washing step: 320 mg matrix was mixed with 6 ml IB1, centrifuged at 12.000 g for 10 min at 10°C.

Up to 4 mg of protein in 6 ml of IB1 was loaded onto 320 mg of Al(OH)3 matrix. The mixture was rotated at 10°C for one hour and 30 min and consecutively centrifuged for 10 min at 12.000 g. The supernatant was discarded or saved for the analysis of unbound protein and the matrix was washed four times by adding 6 ml IB1 and incubated for 10 min in each step, subsequently centrifuging at 12.000 g for 10 minute. The matrix was washed twice by adding 6 ml IB2 and incubated for 10 min in each step and centrifuging at 12.000 g for 10 min. Proteins are eluted from the matrix by adding 4 ml of elution buffer and incubating the mixture for 30 min at room temperature in rotator followed by centrifugation at 15.000 g for 10 min. The supernatant containing putative phosphoproteins was repeatedly centrifuged to eliminate matrix. Phosphoproteins in supernatant were recovered and concentrated by DOC-TCA precipitation modified protocol.

MATERIALS AND METHODS

RESULTS

1. Comparative analysis of barley-leaf rust interaction

Plant proteomic studies represent an interesting challenge in plant biology, mostly because few reports are present in literature and only few plant genomes are sequenced to provide information on expressed genes. Finally, there are more difficulties in plant protein extraction compared to mammals cells.

Near-isogenic barley lines, *Bowman* (susceptible) and *Rph15* (leaf rust-resistant) were grown in controlled conditions and the first leaves were harvested at two time point after artificial inoculation with *Puccinia hordei* spores (24 hours post inoculation – hpi - and 4 days post inoculation - dpi).
Plants were inoculated by spraying the leaves with a talcum powder-fungus spores mixture. The control plants were physically separated from the inoculated ones in different boxes. After harvesting, plants were maintained in controlled conditions in order to ascertain the successful infection of the plants that where subsequently used for proteomic analysis. At 4 days post infection barley seedlings of both resistant and susceptible lines didn't show any phenotypic feature of infection and sporulation was clearly visible on *Bowman* lines leaves only 7-8 days post infection.(Fig 1.1).

Fig. 1.1. Time course of the infection process in both Bowman and Rph15 isogenic lines.

A phenol-based method, instead of the TCA-acetone one, was chosen to obtain total protein extracts from barley leaves because it gives a better resolution of spots in gel electrophoresis and also provides more soluble protein pellet. A proteomic approach

based on 2D gel electrophoresis was carried out to compare the two barley lines during fungal infection in control and infected condition.

2D gel electrophoresis is a powerful proteomics technique for separating complex mixtures of proteins (Fig. 1.2) also coupled with a sensitive gel staining.

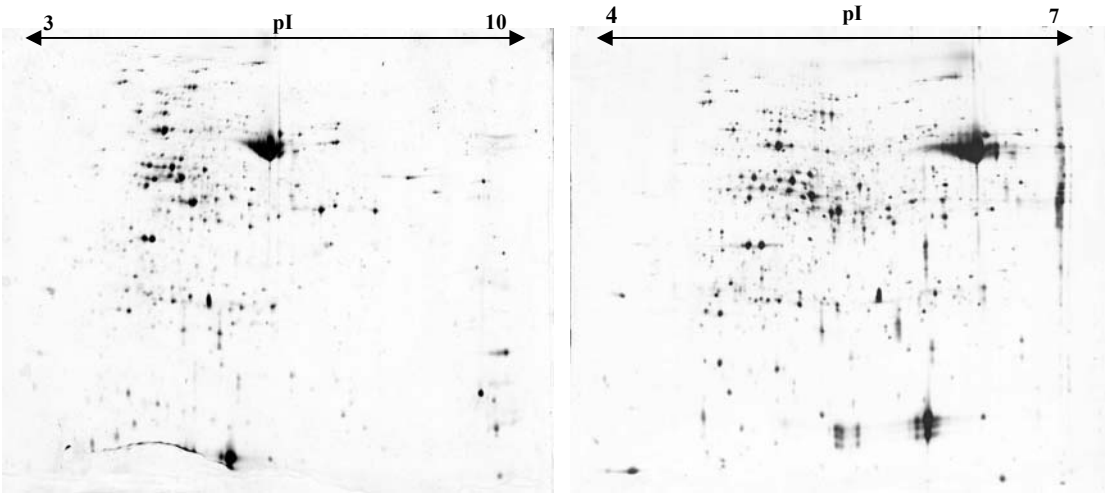


Fig. 2. Comparison of two different pH range of 24 cm strips to visualize protein pattern. Gels were stained with silver stain methodology.

The resolution of the protein extract was compared on 24 cm IPG strips at two different pH range (pH 3-10 and pH 4-7) (Fig. 1.2). We observed that most of the proteins were present at the acidic end, thus a pH range of 4-7 was chosen to further analyses. In the 2D map for the total protein analysis, an average of 800 protein spots were visualized with fluorescent staining. The number of spots visualized in a 2DE map, their resolution and reproducibility, depends to a great extent on the tissue sample and the protein extraction protocol, as most of the technical problems of 2D electrophoresis are related to protein solubilization and separation, which are often associated with the co-extraction of non-proteic components.

A sensitive silver stain protocol was also adopted to check quality of the samples and if any troubles occurred during the separation.

A comparative analysis was first performed at 24 hpi both for phosphoproteins and total proteins by multiplexing proteomic analysis with fluorescent stains for total and phosphoproteins of both susceptible and resistant lines. Two biological replicates and six technical replicates were performed for the analysis of total proteins in leaves extracts from controls and infected plant materials. Four technical replicates were

instead performed for the analysis of phosphorylated proteins of the same leaf extracts.

Proteins (300 µg) were subjected to IEF in a pH range of 4-7. After 2D gel electrophoresis each gel was stained with ProQ Diamond (Molecular probes, Inc.), a phosphospecific fluorescence staining (Fig. 1.3), according to a modified and optimized protocol in which a diluted stain solution was used (Agrawal GK et al. 2005).

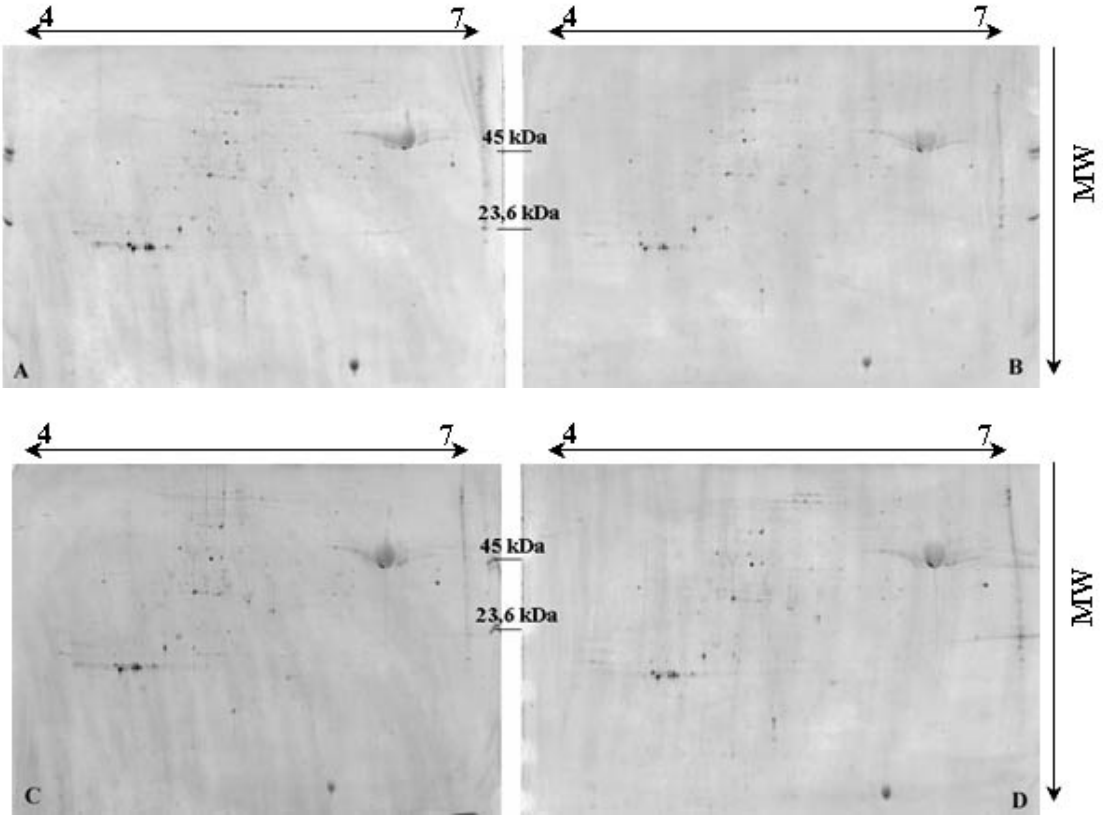


Fig. 1.3. Proteins (300 µg) were analyzed by IEF at pH 4-7, followed by 12.5% SDS PAGE and visualization by phosphospecific staining, ProQ Diamond (molecular Probes, Inc.). In (A) *Bowman* in control condition at 24 hpi; (B) *Bowman* inoculated at 24 hpi; (C) *Rph15* line at 24 hpi non-inoculated and (D) *Rph15* line inoculated by pathogen.

All glasses were extensively cleaned before use in order to reduce the bound of the phosphospecific dye to aspecific residuals of phosphate groups. A total of 16 2D gels were subjected to comparative phosphoproteomic analysis. Fluorescent staining was carried out with a solution of ProQ Diamond diluted 1:2 for 2 h and background noise was reduced. Small and large subunit of Rubisco was partially phosphorylated. Gels were scanned with a phosphoimager scanner (Typhoon 9210, GE Healthcare) and the average number of detected spots was about 200. The gels were analyzed with the image analysis software ImageMaster 2D Platinum (GE Healthcare) to detected differential spot pattern among studied condition.

After scanning, gels were rinsed in deionized water and stained with a total protein fluorescent stain (SYPRO Ruby, Molecular Probes, Inc.) over night; the day after the gels were scanned (Fig. 1.4).

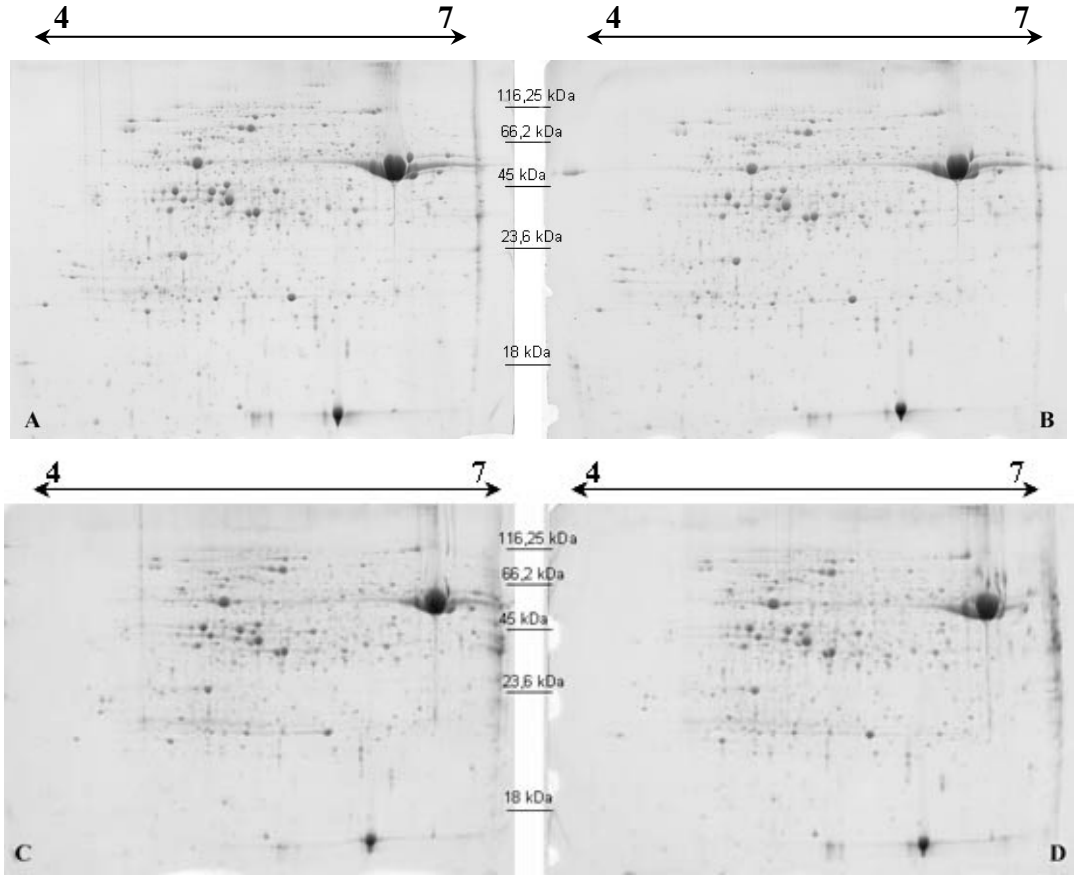


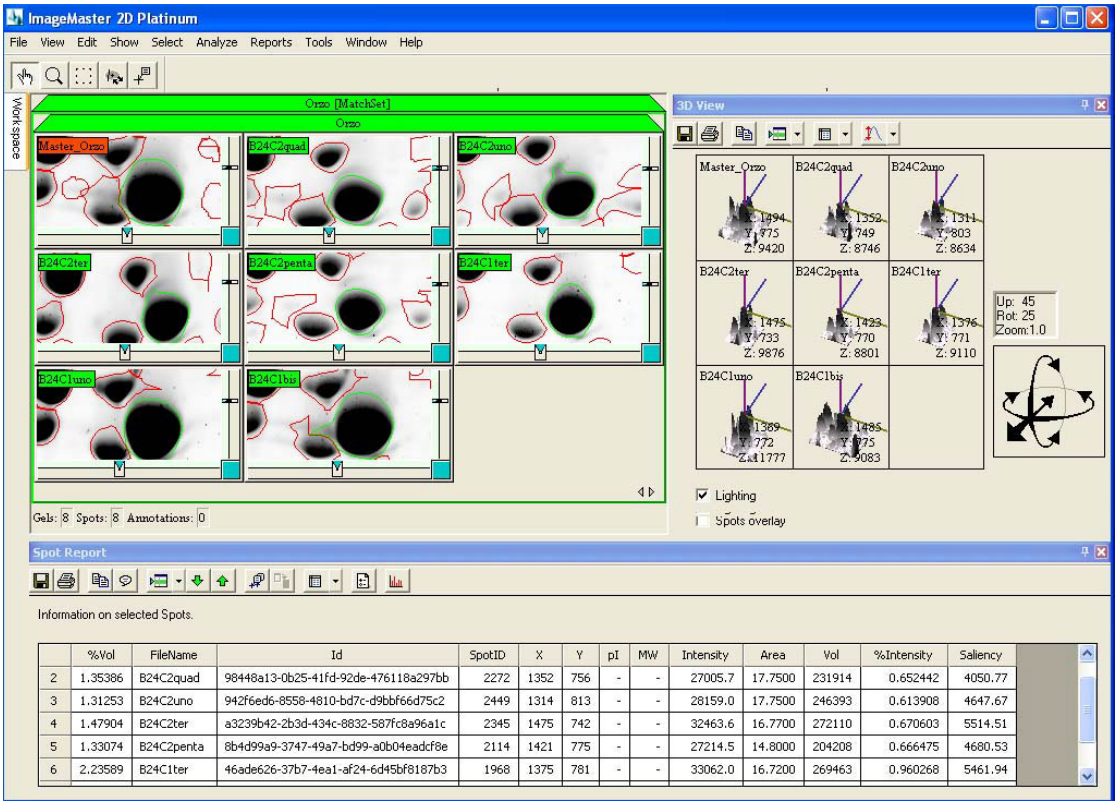
Fig. 1.4. Proteins (300 µg) were analyzed by IEF at pH 4-7, followed by 12.5% SDS PAGE and visualization by a total fluorescence staining, SYPRO Ruby (Molecular Probes, Inc.) at 24 hpi in (A) *Bowman* control sample; (B) *Bowman* inoculated (C) *Rph15* (resistant) mock condition and (D) *Rph15* inoculated.

A total of 24 gel images for total proteins and 16 gel images were used for statistical analysis of differential protein accumulation at 24 hpi. The average number of detected spots in 2D gels was about 850. No statistically significant differences were identified at this time point both for total and phosphorylated proteins.

A second time point of infection of 4 dpi was therefore analysed to detect differential protein expression after plant-pathogen interaction. The same experimental design was carried out as described above and barley leaves were harvested at 4 dpi and the same number of gels was investigated. The average number of detected spots was about 850. Spots were analyzed with ImageMaster 2D Platinum software (GE Healthcare) and manual spot editing and matching was performed automatically and manually checked (Fig. 1.5). To ascertain quantitative changes in the proteomic maps, the

relative spot volumes *(%Vol)* were evaluated by software-assisted analysis. The *%volume* value is the useful parameter to quantify and compare gel spots, corresponding to the volume of spot in a gel containing "n" spots. This parameter takes into account variations due to protein loading and staining.

Two-way ANOVA test ($p<0.05$), coupled with a threshold of two-fold change in accumulation, revealed 21 differentially accumulated spots in response to pathogen interaction in the two barley lines (Fig. 1.6).



	%Vol	FileName	Id	SpotID	X	Y	pI	MW	Intensity	Area	Vol	%Intensity	Saliency
2	1.35386	B24C2quad	98448a13-0b25-41fd-92de-476118a297bb	2272	1352	756	-	-	27005.7	17.7500	231914	0.652442	4050.77
3	1.31253	B24C2uno	942f6ed6-8558-4810-bd7c-d9bbf66d75c2	2449	1314	813	-	-	28159.0	17.7500	246393	0.613908	4647.67
4	1.47904	B24C2ter	a3239b42-2b3d-434c-8832-587fc8a96a1c	2345	1475	742	-	-	32463.6	16.7700	272110	0.670603	5514.51
5	1.33074	B24C2penta	8b4d99a9-3747-49a7-bd99-a0b04eadcf8e	2114	1421	775	-	-	27214.5	14.8000	204208	0.666475	4680.53
6	2.23589	B24C1ter	46ade626-37b7-4ea1-af24-6d45bf8187b3	1968	1375	781	-	-	33062.0	16.7200	269463	0.960268	5461.94

Fig. 1.5. Screenshot of ImageMaster 2D Platinum software (GE Healtcare) that was used for image analysis, to detect, edit and match spots of different gels inserted into MatchSet. Comparative analysis was performed into class set of gels. Analysis of gel matching produced a list of putative differentially expressed proteins, according to *%Vol* that was exported and statistically analysed with statistic software.

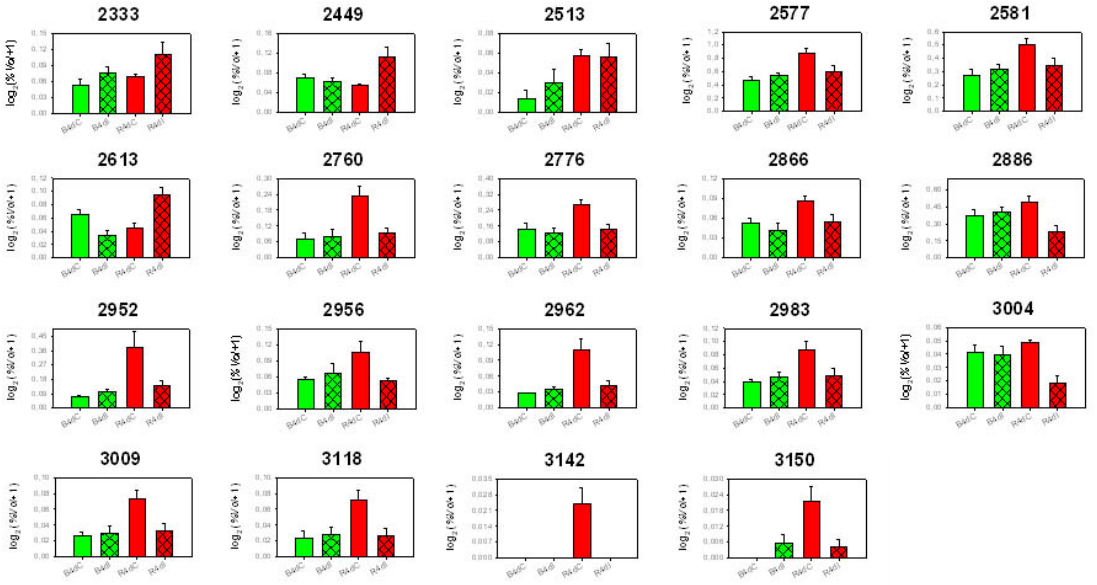


Fig. 1.6. Differential spot expression expressed in log (*%Vol*) and their SD after two-way ANOVA test of spot identified by LC-MS/MS. Green bar were referred to *Bowman* and red one to *Rph15* line, cross bars were referred to infected condition.

In the resistant NIL *Rph15*, a total of 9 spots were significantly down regulated, while three protein spots were accumulated at a fold change ratio above two in the inoculated sample with respect to the control condition. No statistically significant changes in protein accumulation were identified in the susceptible NIL *Bowman* at 4 dpi.

Nineteen out of 21 differentially expressed spots were identified using LC-ESI-MS/MS and are listed in table 1.

Twelve of the identified proteins corresponded to Rubisco or Rubisco activase, two proteins were related to sugar metabolism, four spots were related to proteins involved in plant defence responce and one spot was correspondent to an ATP synthase protein.

Table 1. List of the spots identified by LC-ESI-MS/MS and bioinformatics analysis.

Spot ID	Accession number	Species	Protein description	Abb.	M_r^a/ pI^a	M_r^b/ pI^b	Cov. (%)c
2333	**CAZ64535**	*Hordeum vulgare*	**Sucrose synthase**	Susy	73.1/ 6.2	92.2/ 5.8	18.4
2449	**XP_002454700**	*Sorghum bicolor*	**Leucine aminopeptidase 2, chloroplastic**[d]	LAP 2	49.8/ 5.9	61.8/ 7.6	20.0
2513	**Q40073**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic**	RA A	42.0/ 5.1	46.1/ 5.6[e]	28.1[e]
2577	**Q40073**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic**	RA A	35.4/ 5.4	46.1 / 5.6[e]	43.5[e]
2581	**Q40073**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic**	RA A	36.0/ 5.3	46.1/ 5.6[e]	34.6[e]
2613	**CAA54616**	*Hordeum vulgare*	**Flavonoid 7-O-methyltransferase**	n.	34.6/ 5.6	42.3/ 5.4	29.5
2760	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	23.4/ 6.9	52.9/ 6.2[e]	16.3[e]
2776	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	23.2/ 6.8	52.9/ 6.2[e]	22.6[e]
2866	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	22.0/ 6.8	52.9/ 6.2[e]	8.0[e]
2886	**CAA55345**	*Hordeum vulgare*	**chitinase**	CHT	21.8/ 5.3	26.6/ 6.1	29.4
2952	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	20.8/ 5.2	52.9/ 6.2[e]	9.0[e]
2956	**BAD31057**	*Oryza sativa*	**Chitinase III-like protein**	CHT III	20.7/ 5.4	18.9/ 6.5	12.7
2962	**CAC32847**	*Hordeum vulgare*	**Adenosine diphosphate glucose pyrophosphatase**		20.6/ 5.2	19.5/ 5.7[e]	17.4[e]

Table 1 (*continued*)

Spot ID	Accession number	Species	Protein description	Abb.	M_r[a]/ pI[a]	M_r[b]/ pI[b]	*Cov. (%)*[c]
2983	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	19.7/ 5.4	52.9/ 6.2[e]	4.6[e]
3004	**XP_002465461**	*Sorghum bicolor*	**ATP synthase B chain** [d]	ATPase	19.0/ 4.8	22.8/ 5.3	6.1
3009	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	18.8/ 5.4	52.9/ 6.2[e]	4.2[e]
3118	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	23.4/ 5.5	52.9/ 6.2[e]	6.9[e]
3142	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	23.2/ 5.7	52.9/ 6.2[e]	4.4[e]
3150	**P05698**	*Hordeum vulgare*	**Ribulose bisphosphate carboxylase large chain**	RuBisCO LC	23.1/ 4.8	52.9/ 6.2[e]	4.8[e]

Abb.: abbreviation.
a: experimental molecular weight and p*I*.
b: theoretical molecular weight and p*I*.
c: amino acid coverage (%).
d: annotation obtained by the meaning of the BLASTP alignment analysis against the v*iridiplantae* subset of the nr-database at the NCBI.
e: value referred to the mature form of the protein.

2. Phosphoprotein enrichment methodology

A phosphoprotein enrichment methodology was applied before the 2D SDS-PAGE in order to gain in sensitivity and resolution of phosphorylated spots.

Metal oxide affinity chromatography (MOAC), using aluminium hydroxide was chosen for enrichment due to the higher selectivity for phosphate group, and the lower non-specific binding of acidic proteins and peptides of this technique. The amount of protein necessary for successful enrichment is high, mainly because of considerable sample loss during the procedure. Approximately 4 mg of total protein are required to obtain at least 300 μg of protein in the enriched fraction.

The enrichment procedure was performed under denaturing conditions in order to decrease the risk of dephosphorylation and degradation events respectively caused by endogenous proteases and phosphatases. To that purpose proteins were extracted using a phenol-based method with sodium fluoride (a phosphatase inhibitor) and a phosphatase inhibitor cocktail (Sigma) and then solubilized in isoelectrofocusing buffer. Proteins and $Al(OH)_3$ matrix were previously equilibrated in an optimized buffer and putative phosphoproteins were eluted by pyrophosphate salt. The efficiency of the method was checked by SDS-PAGE (Fig. 2.1), where total proteins (S) were compared with MOAC eluted fraction (E). After Pro-Q staining it can be observed that even if some low molecular proteins were lost during MOAC, the pattern of phosprilated proteins of the MOAC eluate was almost totally coincident with the one obtained for the total proteins.

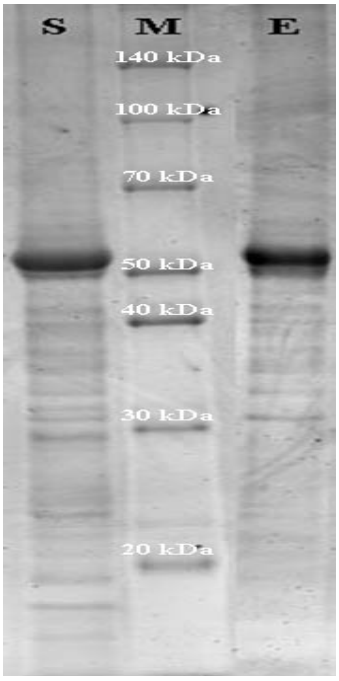


Fig. 2.1. Comparison of phosphoproteins in a crude total protein extract (30 µg) and a phosphoprotein enriched sample by the MOAC method (30 µg) in a 10% SDS-PAGE, visualization by a phosphospecific stain (ProQ Diamond). S, crude total extract; E, phosphoprotein eluted sample after MOAC enrichment.

Putative eluted phosphoproteins were precipitated by TCA/Acetone method and the pellet was re-suspended in IEF buffer before further processing. An isoelectrofocusing run was performed to test the applicability of the enrichment method to 2DE, but proteins were not focalized, probably due to the presence of matrix particles or the high salt concentration in the eluate that interferred with the run, as demonstrated by

the high current applied to the strip (Fig. 2.2A); therefore the 2D SDS-PAGE was not run.

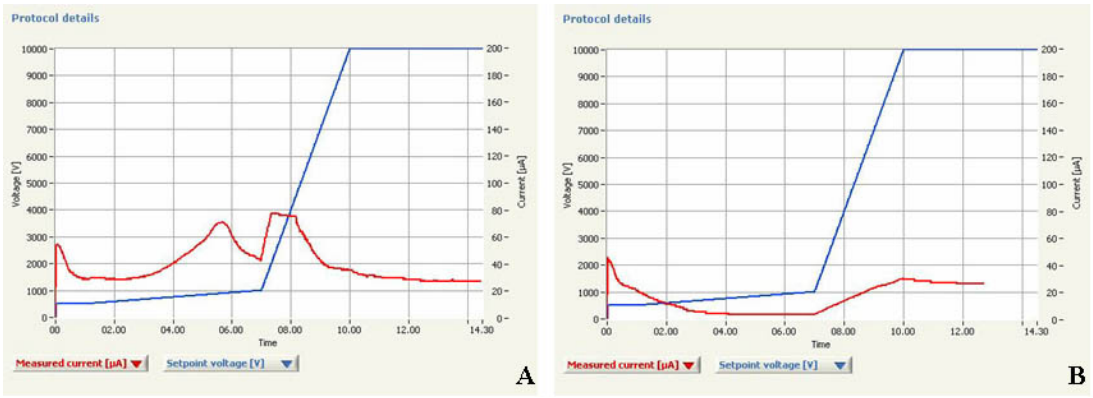


Fig. 2.2. Graphs of isoelectrofocusing run. In A) a bad graph of isoelectrofocusing and in B) a good isoelectrofocusing after MOAC procedure.

Changes to the precipitation procedure after MOAC were applied and a TCA-DOC/Acetone protocol was modified by the introduction of a solubilization step in Tris/HCl pH 7.5 .Graphs of isoelectrofocusing runs are shown on Fig. 2.2 B: with the modified procedure, interfering molecules were eliminated and proteins migrated in gel according to their pI, as shown by the low current transfer. Phosphoprotein enrichment of MOAC was clearly highlighted on the 2D maps shown in Fig. 2.3 where MOAC sample was compared to non–MOAC sample. The same experiment also allows an evaluation of the reproducibility of the MOAC methodology. An obvious overlapping of the protein spots was observed in the two MOAC samples (Fig. 2.3 A and B) demonstrating the high level of reproducibility of the MOAC processed samples.

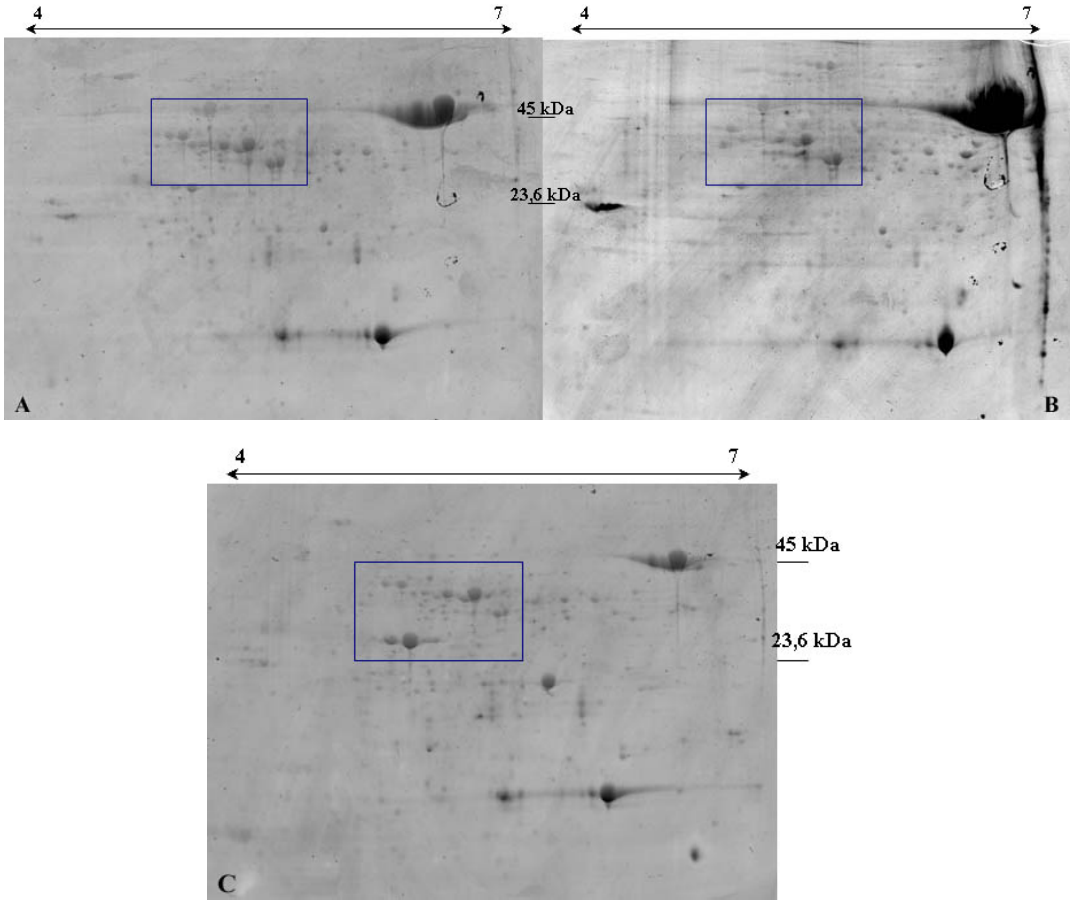


Fig 2.3. Reproducibility and enrichment in MOAC on 2D map. 300 µg of putative phosphoproteins obtained from two independent MOAC experiments were loaded on 12,5% SDS-PAGE (A and B) and were compared to a non-MOAC total proteins sample (C). A section of enriched proteins was pointed with blue rectangle in all three images. Gels were staining using ProQ Diamond stain.

RESULTS

DISCUSSION

In this work a proteomic approach was utilized to analyze changes in protein accumulation during an incompatible and a compatible interactions between barley and the leaf rust pathogen *Puccinia hordei* with the aim of identifying defence pathways involved in barley-leaf rust interaction and differentially regulated in resistant or susceptible interactions. An extremely efficient leaf rust resistance gene, *Rph15*, governed the incompatible interaction, as this gene demonstrated to confers resistance towards more than 300 pathogen isolates. Two infection time points were subjected to the proteomic study, an early time point at 24 hpi and a late time point at 4 dpi. As at the time points used for the analyses symptoms were still not visible, during all the artificial inoculation experiments the outcome of the inoculations were verified by leaving in the inoculation box sample leaves of the susceptible genotype until symptom development (sporulation of the pathogen at 7-8 dpi). Two dimensional gel electrophoresis and fluorescence staining were used to identify differentially expressed proteins in response to the fungus attack.

Both total and phosphorylated protein accumulation in the two isogenic lines were compared, in control and inoculated condition. No significant differentially expressed proteins were found in the two NILs after comparisons at 24 hpi both for total and phosphorylated proteins. Most likely, at this infection time still there are not changes in protein accumulation into infected cells that can be detected using the procedures we have adopted in the present work, although bibliographic data report that at 24 hpi intercellular hyphal growth is already been established in compatible interactions (Lin KC et al. 1998; Sellam MA et al. 1976). In agreement with our results, no changes in protein accumulation were detected at 3 dpi in the interaction between wheat and the leaf rust pathogen *Puccinia triticina* (Rampitsch et al. 2006). Phosphoproteins were visualized by ProQ Diamond phosphospecific staining at the same time point after infection but, also this analysis revealed no differential protein accumulation in all the comparisons performed. This was an unexpected result because being the phosphorylation a post translational modification implied in signalling transduction, such a post-translational event should represent an early stress response that occurs to modulate responses and to balance protein functions. A possible reason for these

results could be that only one third of the total proteins loaded in 2DE are normally phosphorylated at a given time (Morandell S et al. 2006) and although the fluorescent staining is very sensitive, differentially regulated phosphoproteins were probably too diluted for efficient detection.

A possible solution to this problem was proposed in this work of thesis by the developing of an enrichment methodology based on metal oxide affinity chromatography using $Al(OH)_3$. Few publications with applications of this new technique in phosphoproteomic analysis were found with exhaustive information (Wolschin F et al. 2005; Röhring H et al. 2008). Indeed, MOAC protocols are still in improvement, mainly because the subsequent application of 2D SDS-PAGE is difficult. Part of the efforts of this thesis were therefore directed to optimize a protocol for 2D SDS-PAGE phosphoproteomic analyses. To date, a standardized protocol of MOAC is available and differential phosphoprotein accumulation analysis at 4 dpi is in progress.

A later time point of inoculation (4 dpi) was investigated to search for differentially accumulated proteins in response to pathogen infection. This experimental time point was chosen because it was in the middle of the whole disease progression (eight days are normally required for pathogen sporulation in compatible interactions). The total protein patterns were analyzed after gel staining with SYPRO Ruby staining. Image analysis was performed with ImageMaster 2D Platinum software (GE Healthcare) and twenty-one protein spots displaying at least a two-fold change were identified as differentially accumulated in the different conditions analysed. After LC-MS/MS analysis, nineteen out of 21 differentially expressed spots were characterized.

Several of the differential spots identified corresponded to enzymes related to the carbohydrate metabolism, Calvin cycle and defence-related proteins.

It was previously reported that the ability of photosynthetic assimilation is severely reduced during the plant response to biotic stress and this is connected to the fact that many pathogens suppress photosynthesis process in the host. Rubisco represents the most expressed proteins in leaves, representing about 30% of total leaf proteins (Feller U et al. 2008; Kang SG et al. 2007) and its amount generally drops after infection (Zhou W et al. 2006). As expected, many spots were identified in the present work as degradation products of Rubisco. A total of eight down accumulated protein spots

were identified as Rubisco large chain (Rubisco LC) (spot 2760, 2776, 2866, 2952, 2983, 3009, 3118, 3142, 3150) with different M_r values (about 20-25 kDa) supporting that this Rubisco subunit was subjected to proteolytic degradation during the infection process. Degradation of photosynthetic apparatus may contribute to stop pathogen growth into barley cells and promotes the activation of defences in non infected cells (Boccara M et al. 2007; Yu CL et al. 2008). This assumption can be supported by the observation that turnover of Rubisco was markedly reduced in the *Rph15* genotype, probably because these subunits are rapidly degraded to small peptide and amino acids. No statistically significant differences between conditions were observed in *Bowman* probably because in the susceptible line proteolytic pathways were not readily activated to assist Rubisco fragments degradation. The same Rubisco LC degradation trend was observed in rice as induced by bacterial blight infection and in wheat after *F. graminearum* infection (Kang SG et al. 2007; Zhou W et al 2006). Furthermore, the degradation of Rubisco was observed that can be initiated or accelerated by reactive oxygen species (Feller U et al. 2008).

The same behaviour of Rubisco LC was observed for protein spots corresponding to chloroplastic Rubisco activase A (spots 2513, 2581, 2577). Rubisco activase catalyzes the activation of Rubisco and involves the ATP-dependent carboxylation of the epsilon-amino group of lysine of ribulose-1,5-bisphosphate carboxylase/oxygenase leading to a carbamate structure. Activase is required because the ribulose 1,5-bisphosphate (RuBP) substrate also binds to an inactive form of the enzyme and blocks the binding of the ‘‘activating’’ CO_2 on a Lys present in the active site to form a carbamate. It can be hypothesized that Rubisco activase accumulation is down regulated because of the lack of substrate (Rubisco is in fact degraded). Activity of this enzyme is also regulated by ATP/ADP ratio in the cytosol. When photosynthetic activity is repressed, the levels of cytosolic ADP increases and, in turn, inactivates Rubisco activase. It is therefore plausible that a reduction in photosynthetic activity in the resistant genotype *Rph15* can led to the observed reduction of Rubisco activase accumulation. In the susceptible genotype *Bowman*, no substantial reduction of Rubisco LC was observed; in agreement with this result we also observed that Rubisco activase abundance was basically not affected by the infection process.

DISCUSSION

Cytosolic aminopeptidases and oligopeptidases cleave peptides released from plastids to free amino acids; in addition, the proteasome and other endoproteolytic systems are proteolytic machineries also located in the cytosol (Yang L et al. 2004). The protease-controlled pathways are critical for plant development and stress response. The role of the ubiquitin-mediated proteolysis in wound and defence responses was suggested by the observation of up-regulation of genes encoding ubiquitin in response to infection of potato by the pathogen *Phytophthora infestans* (Basso M et al. 1996). Peptidases, such as aminopeptidases, carboxypeptidases, endo- and exopeptidases represent plant responses to wound and pathogens (Yang L et al. 2004; Ogiwara N et al.2005; Chao WS 2000). The turnover of unfolded or damaged proteins that accumulate as a result of oxidative burst associated with wound or pathogen response would require recycling by the action of these peptidases. Spot 2449 was characterized as a LAP (Leucine aminopeptidase) protein. This enzyme (EC 3.4.11.1) is a widespread enzyme in plant and animal species and in both it has a defence role. This enzyme hydrolyzes the N-terminal amino acid residues from peptides and proteins. Many LAPs from plant species have generally hexameric structure and they were detected in floral organs and fruits, and it was observed that these proteins are induced by wounding, MeJA, ABA, pathogens and pests, salinity, and water deficit. LAPs are the most characterized enzymes in tomato for their localisation and activities (Narváez-Vasquez J et al. 2008; Fowler JH et al. 2009). Ogiwara and colleagues (2005) partially purified three LAP isoforms named LAP 1, LAP 2, LAP 3 from barley seedlings and a monomeric structure was suggested in barley leaves. In their study, the induction profile of LAP was observed in response to several stimuli and the enzyme activity observed in the study can be linked to the data produced in this thesis where in resistant *Rph15* line the LAP 2 protein was more abundant in infected condition with respect to control samples. Conversely, in the susceptible line no significant differences were noted when inoculated and control samples were compared. The increased amount of LAP in the infected leaves is supposed to stimulate proteolytic pathways to recycle nutrients and to eliminate damaged proteins from infected cells.

Spot 3004 represents a ATP synthase B chain that is involved in photosynthesis and oxidative respiration This enzyme uses a transmembrane electrochemical proton gradient to drive synthesis of ATP in chloroplast and mitochondria. This protein was down-regulated in the resistant line after infection while no significant changes were

observed in the *Bowman* line. This assumption is supported by results of Kang SG and colleagues (2007) in which the reduction of ATP synthase protein in infected resistant cells may results from mitochondrial and/or thylakoid membrane damage caused by ROS during the oxidative burst. ROS contribute to hypersensitive cell death pathogen-induced of infected cells or serve as a signal to activate defence responses in distant uninfected cells.

The spot 2333 was identified as a sucrose synthase (SuSy) enzyme, a soluble enzyme localized in the cytosol In this work SuSy accumulation was significantly increased in infected leaves of both susceptible and resistant isogenic lines. Plant defence against pathogens is costly in terms of energy and carbohydrate consumption. Carbohydrate metabolism has been found to be modulated by fungus colonization of barley leaves (Wright D et al. 1995) and activation of sugar metabolism has been found to contribute in resistance induction in infected plant (Shoresh M et al 2008). The down-regulation of photosynthesis and the simultaneous increased demand for assimilates very often leads to sugar mobilization during plant-pathogen interaction (Berger S et al. 2007) (Fig. 1).

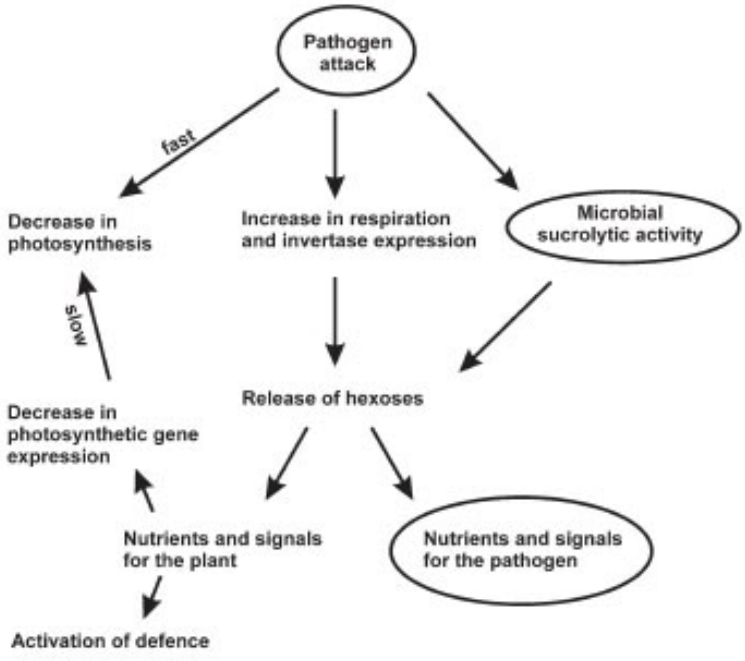


Fig. 1. Model of changes in carbohydrate metabolism in response to infection with biothropic pathogen (Berger S et al. 2007).

DISCUSSION

Rolland and colleagues (2002) reviewed sugar metabolism and signalling induction in plants (Fig. 2).

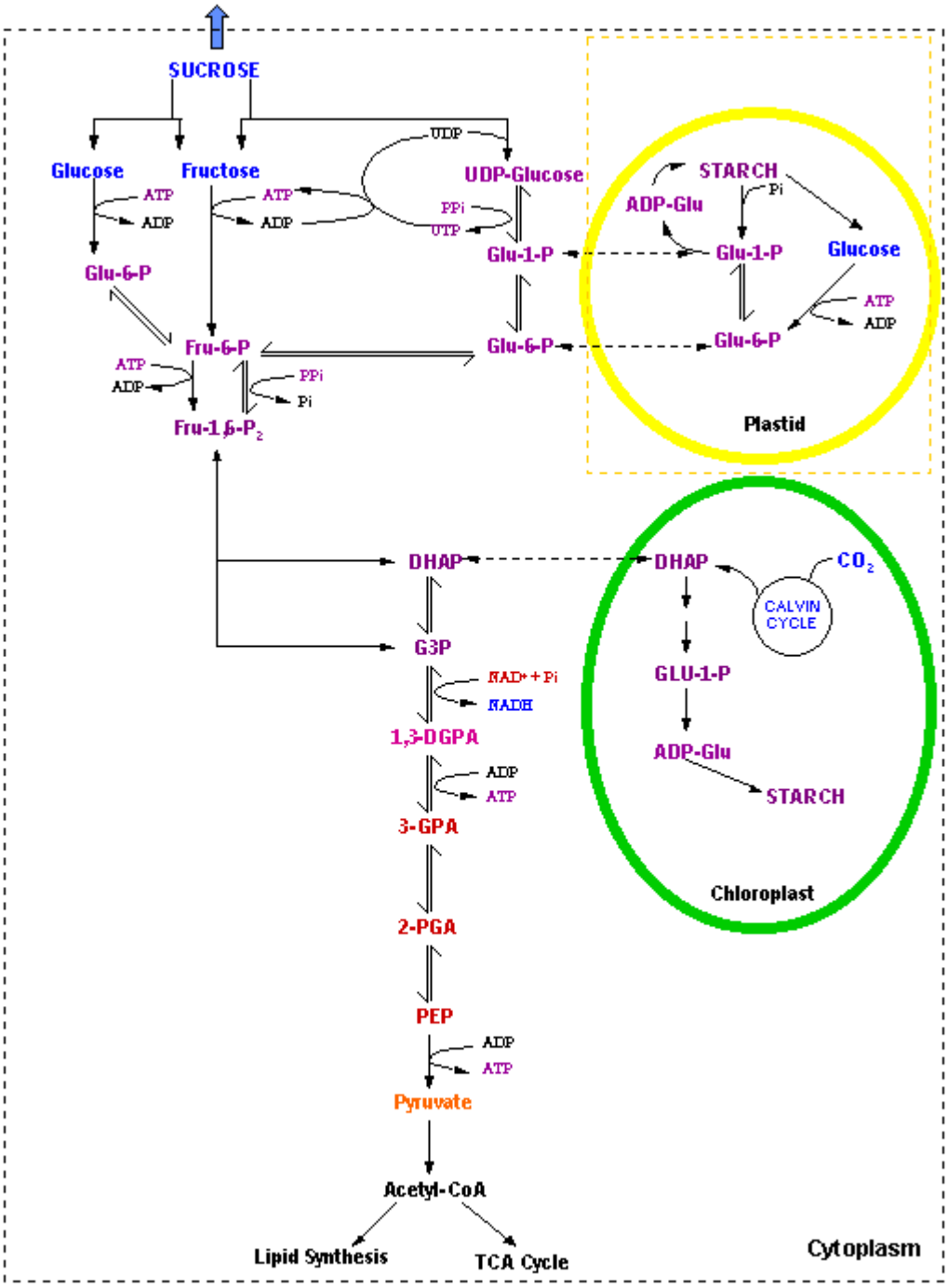


Fig. 2. Carbohydrate metabolism was linked to energy metabolism.

In most plants, sucrose is both the primary product of photosynthesis and the transported form of assimilated carbon. It is synthesized in mesophyll cells of photosynthetically active parts of plants, such as mature leaves (source), and

traslocated via the phloem to the sink tissue, such as young leaves and seeds. In this work of thesis, SuSy (EC. 2.4.1.13) accumulation was increased in infected leaves both in susceptible and resistant isogenic line. SuSy cleaves sucrose into fructose and UDP-glucose and an increased SuSy activity would result in decreased starch biosynthesis. This assumption is supported also by the observed decreased abundance of spot 2962 in infected tissues of the resistant genotype representing an ADP-glucose pyrophosphatase (AGPPase) enzyme. AGPPase catalyzes ADPG breakdown to produce AMP and G1P, and its reduced accumulation/activity drastically lead to a reduction in starch biosynthesis (Baroja-Fernández E et al. 2004; Rodríguez-López M et al 2000). SuSy has a dual role in producing both cytosolic ADPG, necessary to starch biosynthesis, and UDPG, necessary for cell wall and glycoprotein biosynthesis (Shoresh M et al 2008). The AGPPase enzyme catalyzes the hydrolytic breakdown of ADPG and plays a physiological significant role in cleaving excess of substrates. AGPPase acts as "housecleaning" enzymes whose role is to cleanse the cell of potentially deleterious endogenous sugar nucleotides and to modulate the accumulation of metabolic intermediates by diverting them into alternative pathway in response to biochemical need (Rodríguez-López et al. 2000).
Studies in which the SuSy gene was over-expressed resulted in increased cellulose synthesis, providing evidence for a direct connection between sucrose supply (sink strength), its breakdown, and cellulose deposition through the activity of this enzyme (Coleman HD et al. 2009; Essmann J et al. 2008). Carbohydrate metabolism contributes to synthesis of various defence compounds such as phenolic molecules, phytoalexins and lignin, which is involved to cell wall reinforcement. An hypothesis of AGPPase involvement in biosynthesis of cell wall polysaccharides, glycoproteins, and glycolipids was suggested as a defence response of barley infected by *Blumeria graminis* f.sp. *hordei* (Shetty NP et al. 2009).

Pathogenesis-related proteins were also identified by mass spectrometry analysis in our barley leaves samples infected with leaf rust. In particular, spot 2886 was identified as a chitinase of *Hordeum vulgare* and spot 2956 as a chitinase III-like protein of *Oryza sativa*. Chitinases (EC 3.2.1.14) are present in various organisms and catalyze the hydrolytic cleavage of the β-1, 4-glycosidic bond in biopolymers N-acetylglucosamine (chitin) found in fungal cell walls (Punja and Zhang 1993). These enzymes have a role in plant defence by degrading the fungal cell walls. Chitinases

can generally be divided into two categories, endochitinases and exochitinases, with respect to their hydrolytic sites. Plant chitinases, however, are classified into seven classes (I-VII), on the basis of their structure, substrate specificity, mechanisms of catalysis, and sensitivity to inhibitors. Chitinase activity is induced by various factors like pathogen attacks, treatment with elicitors, abiotic factors like drought, cold, salinity. These pathogenesis-related proteins play a dual role, both by inhibiting fungal growth by cell wall digestion and by releasing pathogen elicitors to induce further defence reactions. More researches attempts to elucidate how chitinases function in plant defence mechanism (Dana M et al. 2006; Fujita K et al. 2004; Zareie R et al. 2002; Liu JJ et al. 2005; Anguelova-Merhar VS et al. 2001). In many cases, wounding results in the accumulation of both protein and mRNAs. Even if in some cases chitinase gene expression was found to be higher at constitutive level in resistant cultivars with respect to the susceptible ones. In several studies resistance was associated with an early accumulation of β-1,3-glucanase and chitinase transcripts followed by subsequent reduction in level. Data obtained in this work are only in partial agreement with the above statements, because chitinase and chitinase III-like protein abundance was significantly decreased in infected resistant line while in the susceptible line no significant differences were observed. For both the chitinases identified, the level of accumulated proteins was however constitutively higher in un-inoculated tissues of the resistant NIL with respect to the susceptible one. This behaviour supports the possibility that an higher constitutive level of defence proteins are present in the resistant genotype that can operate as an early defence barriers against the pathogen infection. Additionally it can also be hypothesized that resistance is associated with chitinase activity in apoplastic fluid through the accumulation of these defence proteins in the apoplast near pathogen infection sites. Accordingly, it is reported that chitinases are secreted in apoplast to limit pathogen growth (Joosten MH et al. 1989; Shetty NP et al. 2009). Chitinase corresponding to spot 2886 have a secretory signal at the N-terminal end thus supporting the hypothesis above. This secretory signal was identified by using the software iPSORT and TargetP 1.1. The protein extraction method used in this work was not specific for separation of apoplastic proteins that could therefore be absent or reduced in our 2D gel electrophoresis analyses.

A flavonoid 7-*O*-methyltransferase enzyme corresponding to spot 2613 was also identified during this study. This enzyme is involved in isoflavonoid biosynthesis and, consequently, in the production of phytoalexins, important antimicrobial compounds for disease resistance that rapidly accumulate during incompatible plant-pathogen interactions. Christensen and co-workers (1998) studied a flavonoid *O*-methyltransferase in barley leaves and found that the enzyme activity is readily enhanced following barley infection with the powdery mildew biotrophic pathogen. The authors suggested that the flavonoid 7-*O*-methyltransferase defence function is related to the production of lignin precursors. In agreement with this last research, we have observed that flavonoid 7-*O*-methyltransferase protein was accumulated in infected leaves of the resistant NIL *Rph15* while in the susceptible NIL *Bowman* infection resulted in a down accumulation of this protein. In several plant pathogen interactions it was observed that successful pathogen colonization during compatible interactions is associated with the suppression of the plant defence responses by molecules secreted in the host cells by the invading fungal pathogen (Houterman PM et al. 2007). It is therefore possible that in the resistant NIL the resistance gene-mediated pathogen recognition lead to the activation of defence responses while in the susceptible NIL *Bowman*, successful leaf rust colonization is associated to suppression of defence responses. Further investigations of flavonoid 7-*O*-methyltransferase gene transcription and protein accumulation at different inoculation time points will be necessary to clarify the role of this protein in barley resistance to leaf rust.

DISCUSSION

CONCLUSIONS

The proteomic work discussed in this thesis was focused on the comparative analysis of protein accumulation during leaf rust infection in two barley near-isogenic lines, *Bowman* and *Rph15*. *Rph15* was know as resistant to over 350 isolates of *Puccinia hordei*, while *Bowman* is a susceptible genotype. No information about which defence mechanisms are activated during the pathogen attack were available for this experimental system. In this work, two aspects have been pursued with proteomic analyses: first, to highlight which metabolisms are affected in barley leaves as a response to leaf rust infection; second, to verify possible differences among compatible and incompatible interactions.

Changes in differential accumulated proteins were detected in infected condition of resistant and susceptible line only at 4 dpi, while at 24 hpi, no significant differences in protein accumulation were highlighted both in compatible and incompatible interactions.

Accumulation of proteins involved in the photosynthetic process, like Rubisco LC and Rubisco activase, was found to be down regulated in the resistant line but is generally not affected in the susceptible near-isogenic genotype. Reduction in photosynthesis was previously observed in barley leaves infected with biotrophic pathogens, thus this protein change was somewhat expected. However, it is interesting to note that the increased degradation (or reduced turnover) of these proteins is mainly associated to the resistant near-isogenic line. It can be hypothesized that an increased requirement of proteins specifically devoted to defence processes in the resistant genetic background can be supported with amino acids derived from degradation or reduced turnover of the abundant photosynthesis-related proteins. The delayed or reduced activation of such defence reactions in the susceptible genotype would lead to the observed differences between resistant and susceptible near-isogenic lines. An increased proteolytic requirement in the resistant genotype is also supported by the observed higher accumulation of Leu-aminopeptidase with respect to the susceptible *Bowman* line.

A redirection of sugar metabolism towards defence responses, like cell wall reinforcement, would explain the increased accumulation of SuSy and reduction of the

AGPPase protein. Both these responses were observed in the resistant genotype only. Such modifications would lead to a reduction in starch accumulation, a response that has been previously observed in leaves infected by pathogens (Horst RJ et al. 2008).

Cell death-associated hypersensitive response represents the main resistance response of cereals to leaf rust pathogens and is associated to irreversible damage to biological membranes. This defence response is activated in barley by the *Rph15* rust resistance gene as resulted from the observation of necrotic spots on infected leaves in the *Rph15* resistant line thus providing a possible explanation to the observed reduction of the membrane associated ATP synthase B chain in the resistant near isogenic line only.

Finally, when resistant and susceptible near-isogenic lines are compare, a differential behaviour was observed also for pathogenesis-related proteins like chitinases and flavonoid 7-O-methyltransferase. Deployment in chitinases of the resistant NIL was proposed to be due to their secretion into apoplastic spaces to limit fungus growth through degradation of the pathogen cell wall. Increased accumulation of flavonoid 7-O-methyltransferase would lead to increased production of lignin precursors involved in cell wall reinforcement, a well documented defence response towards biotrophic pathogens like the leaf rust fungus.

In conclusion, several leaf rust resistance-associated modifications in protein accumulation have been identified in the present work. Additional analyses (i.e. transcriptional analyses and western blot analyses) will better clarify the role of the identified proteins in the *Rph15*-governed leaf rust resistance.

As an ultimate goal, optimized MOAC protocols were established to gain an increased detection of phosphoprotein spots. This methodology is being applied to identify differential expressed phosphoproteins in the same experimental system in which total proteins have been characterized and will provide further information about the biological function of the barley *Rph15* broad effective leaf rust resistance gene.

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