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Proteomic analysis of early responsive resistance proteins of wheat (*Triticum aestivum*) to yellow rust (*Puccinia striiformis* f. sp. *tritici*) using ProteomeLab PF2D

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Abstract

Wheat (*Triticum aestivum* L.) yellow rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most destructive diseases of wheat worldwide. To clarify the molecular details and components of the resistance response in wheat offers further possibilities to combat yellow rust. In this study, differentially regulated early response proteins in wheat leaves infected by *Pst* isolates were investigated by proteomic approaches. Total proteins extracts from leaves harvested at 24 hour post inoculation (hpi) were separated by two dimensional liquid chromatography system, ProteomeLab PF2D. Following PF2D analysis, six hundred and thirty-seven protein peaks were compared one by one between protein patterns obtained from pathogen- and mock-inoculated leaf tissue. Among those differentially expressed 33 proteins were identified in *Pst*-infected plants as compared with mock-inoculated controls by nanoLC-ESI-MS/MS. Six proteins were exhibited homology to fungal proteins. Two fungal proteins, including E3 ubiquitin protein ligase and Ubiquitin-like protein, are important members of ubiquitin-proteasome system which the importance of the its proteolytic function in regulating the virulence of pathogenic fungi has just been realized recently. Other identified 27 proteins were host proteins in response to *Pst* and classified in five groups based on their roles in diverse biological processes. The results indicated that identified defence related proteins such as pathogene related protein 1 and 4 (PR1, PR4), Glutathione S transferase (GST) are major component for systemic acquired resistance (SAR) which is one of the strong disease resistance form in plants and appears within several days following the initial pathogen attack.

Keyword: SAR; PR1; PR4; GSTs; Peroxiredoxins; Fungal proteins.
Abbreviations: 2D-LC: two dimensional-liquid chromatography; 2D-PAGE: two dimensional-gel electrophoresis; BSA: bovine serum albumin; CDP32: drought-induced protein of 32 kD; dpi: days post inoculation; eIF4E: eukaryotic translation initiation factor; ER: endoplasmic reticulum; ERp-72: endoplasmic reticulum protein 72 kDa; ETI: effector triggered immunity; GST: glutathione S transferase; HPCF: high performance chromatofocusing; hpi: hours post inoculation; HPLC: high performance liquid chromatography; HPRP: high performance reversed phase; HR: hypersensitive response; IRK: induced receptor-like kinase; IT: infection type; MS: mass spectrometry; nanoLC-ESI-MS/MS: nano flow liquid chromatography-electrospray ionization-tandem mass spectrometry; PF2D: two dimensional protein fractionation platform; pI: isoelectric point; PLGS: proteinlynx global server; PR: pathogenesis-related protein; Prx: peroxiredoxin; *Pst: puccinia striiformis* f. sp. *tritici*; PTI: pathogene triggered immunity; R: resistance; ROS: reactive oxygen species; SAR: systemic acquired resistance; SCF: skp, cullin, F-box containing complex; SOD: superoxide dismutase; Trx: thioredoxin; UPLC: ultra presssure liquid chromatography.

Introduction

Plants protect themselves against biotic and abiotic stresses by developing a wide range of strategies known as ‘defence’ or ‘stress’ responses. In plants, response to any particular stress, a subset of genes would be induced; some are early responsive and others are late responsive to cope up with the impending stress. Although both are required to defend the host against various cues, the early response genes hold the key in perceiving and amplification of different stress signals and induction of further downstream gene expression. Wheat is one of the most important and strategic cereal crops around the world. However, largest portion of its production is lost every year in many regions of the world due to rust diseases. Yellow rust (stripe rust), caused by *Puccinia striiformis* f. sp. *tritici* is one of the most widespread and yield limiting factor in wheat producing areas of the world. Identification, development and deployment of resistant genotypes are the most effective, economical and environmentally friendly approach for controlling this disease (Chen, 2009). For these reasons numerous genomic and transcriptomic studies were conducted to explain structural components and molecular

mechanism of resistance response in wheat (Wang et al., 2010). In recent years, rapidly developing proteomic technology is a very important tool to provide real insights into the extremely complex plant defense response to various stresses including pathogen attacks (Agrawal et al., 2005). In addition, integrating data from genomic, transcriptomics and proteomics will allow for a more precise knowledge of how changes in gene expression lead to changes in metabolism. But compared with DNA- or mRNA-based studies targeting yellow rust resistance response in wheat, proteomic studies are almost negligible. Success of proteomic approach is based on high-resolution separation of complex protein mixtures and its reproducibility (Suberbielle et al., 2008). Two dimensional-gel electrophoresis (2D-PAGE) is used routinely in many laboratories for this purpose. Using 2D-PAGE, 100 –10000 polypeptides can be analyzed in a single run, proteins can be separated in pure form in spots and they can not only be quantified but also analyzed by mass spectrometry (MS). However, 2D-PAGE has disadvantages such as limited loading capacity, application difficulties, inability to separate proteins with extreme isoelectric point (pI) values, or the difficulty of resolving proteins of small molecular weight (Edgar et al., 2004). Hence, alternative separation methods such as two dimensional-liquid chromatography (2D-LC) systems have been developed. Proteome-Lab^{TM} PF2D from Beckman Coulter is one of the developed 2D-LC system that separates the protein mixtures in the first dimension according to their pI using chromatofocusing, followed by a fractionation according to hydrophobicity, using reversed phase chromatography in the second dimension (McDonald et al., 2006). In addition, it has automation for fractionation processes. In this way, a protein mixture could be separated into several hundred fractions and large number of samples can be fractionated in a short time. Also 2D-LC allows the determination of a large set of proteins and novel protein discovery (Wu et al., 2007). In this study, proteomic analysis were carried out between pathogen-inoculated and mock- inoculated yellow rust-resistant Turkish bread wheat cultivar, İzgi2001, in order to identify the proteins that play important role in early resistance response to yellow rust by using PF2D system.

Results and discussion

Wheat–Pst interaction

Pathogene-host interaction is extremely important for production of accurate scientific data while studying the cereal diseases at the molecular level. The immune system enables all living organisms to protect themselves from foreign organisms and hazardous sustances. Plant immune system are examined with a four phased model supposed by Jones and Dangl (2006). According to this, phase 1 is a defence forming by effector molecules secreting by pathogene, this is also known as basal defence or " Pathogene Triggered Immunity" (PTI). In phase 2, pathogenes might overcome basal defence and thus effector triggered sussepcibility are formed. In phase 3, hypersensitive cell death (HR) that is associated with the resistant response occurs at the infection site upon activating "Effector Triggered Immunity" (ETI) as pathogene proteins and plant's resistance (R) proteins interact to each other. Death cells at the infection site have autoflorescence features. Autofluorescent material might be phytoalexin, glyceollin or related compounds, and they are accumulated in hypersensitive host cells surrounding fungal penetration sites (Ersek et al., 1982). In this study, success of pathogen inoculations was verified by observing the autofluorescence as indication of HR at 5 days post inoculation (dpi) infected leaf samples (Fig 1c-d). In addition, HR response was observed by naked eye at 15-17 dpi on leaf surfaces (Fig 1a-b). If this recognition does not form, the model enters phase 4 and pathogene overcomes ETI.

PF2D analysis

The extracted proteins from the *Pst*-inoculated and mock-inoculated leaf samples that belong to independent three biological replicates at 24 hpi were separated by PF2D system. For each sample, 2.0 mg protein extract was injected into the system and separated as two dimensionally by PF2D which allows the two dimensional fractionationation of the intact proteins. In the first dimension, the absorbences of pH profiles were recorded at 280 nm and 35 fractions were collected totally. As shown in Fig 2, recovered proteins were concentrated on two regions during pH gradient as follows: in pH $\geq$8.5 and pH 6.0-4.0. At the end of the pH gradient, non protein peaks (fraction 29-31) were observed because of NaCl content of washing buffer. Therefore first 23 fractions were separated in the second dimension using high performance reversed phase (HPRP) chromatraphy (Fig 2). The reproducibility of the fractionation step is completely depend on the reproducibility of the pH gradients. Freshly prepared Start and Eluent Buffer in same lot were used for separation of all samples. The graphs of the pH gradient and comparative UV chromatograms of the second dimension of the three biological replicates were matched which revealed a good reproducibility for PF2D separation among three biological replicates as shown in Fig 3. The second dimension absorbence profiles were compiled and displayed as a 2D-map using ProteoVue programme (Fig 2). In the 2D-map, each lane represents a 1st dimension fractions and shows pH interval and second dimension absorbence at 214 nm. To determine differentially expressed proteins between *Pst*-inoculated and mock-inoculated leaf samples, their 2D-maps were compared and peak-to-peak analysis (Fig 4) were carried out by DeltaVue software. Six hundred thirty-seven peaks were compared and many differentially expressed proteins were observed. The peaks which their expression differences were similar in the three independent biological replicates were selected for ESI-MS/MS analysis.

Protein identification

Protein identification was carried out by nanoLC-ESI-MS/MS. The collected data were processed by ProteinLynx Global Server (PLGS) V2.4. The results were searched using Swissprot database against to either wheat or Viridiplantae databases. In this study, thirty-three proteins were identified (Table 1) as early response-related proteins during wheat-*Pst* interaction. Six proteins within the identified 33 proteins were exhibited homology to fungal proteins. These fungi are wheat pathogenic fungi; *Phaeosphaeria nodorum*, *Gaeumannmyces graminis* and *Fusarium graminearum*. Other identified 27 plant proteins that were differentially expressed in response to the yellow rust were classified five groups based on their roles in diverse biological processes (Table 2).

Fungal proteins

Pathogenic filamentous fungi express many secretative proteins, including degradative enzymes, extracellular

Table 1. Differentially expressed proteins at 24 hpi following yellow rust inoculation.

No	Lane/ pI Range	Expr. Difference	Accesion (UniProt)	Protein Description	Function	Theo. pI	MW (Da)	*PLGS score	S.Cover. (%)	Theo. peptide	Mached peptide	Organism
1	1/4.1-4.4	up	P29557	Eukaryotic translation initiation factor	gene expression	5,2	23975	264	45	16	5	*Triticum aestivum*
2	1/4.1-4.4	induced	Q0V090	Mediator of RNA polymerase II	gene expression	4,5	30821	102	40	34	10	*Phaeosphae nodorum*
3	1/4.1-4.4	down	P26302	Phosphoribulokinase (PRK)	photosynthesis	5,6	45112	1029	56	29	18	*Triticum aestivum*
4	2/4.4-4.7	up	Q8LE52	Glutathione S transferase (GST)	oxidative stress	7,8	28495	540	10	23	2	*Arabidopsis thaliana*
5	2/4.4-4.7	up	P52589	Protein disulfide isomerase (ERp72)	protein processing	4,8	56498	311	44	49	18	*Triticum aestivum*
6	2/4.4-4.7	up	Q03033	Elongation factor	gene expression	9,4	49137	59	29	44	8	*Triticum aestivum*
7	2/4.4-4.7	down	P24065	Photosystem II cp47	photosynthesis	6,0	56056	273	19	29	6	*Triticum aestivum*
8	3/4.7-5.0	down	P12782	Phosphoglycerate kinase	photosynthesis	6,6	49808	237	42	44	15	*Triticum aestivum*
9	3/4.7-5.0	up	Q2QKB4	Splicing factor u2af large subunit	mRNA processing	7,5	60549	99	33	60	13	*Triticum aestivum*
10	3/4.7-5.0	up	Q9S7U0	Inositol 3 phosphate synthase	biosynthesis	5,2	56258	116	18	37	6	*Triticum aestivum*
11	3/4.7-5.0	induced	Q4I7N9	E3 ubiquitin protein ligase BRE1	protein turover	6,9	80160	79	32	67	23	*Fusarium graminearum*
12	3/4.7-5.0	induced	O64392	Pathogenes-related protein 4 (PR4)	defence	7,5	15624	169	62	8	4	*Triticum aestivum*
13	4/5.0-5.3	up	P62785	Histone H4	DNA folding	11,8	11402	779	28	11	2	*Triticum aestivum*
14	4/5.0-5.3	down	P00871	RuBisCo small chain	photosynthesis	8,2	13046	9818	68	17	15	*Triticum aestivum*
15	5/5.3-5.6	up	P38076	Cysteine synthase	biosynthesis	5,2	34092	1540	24	28	6	*Triticum aestivum*
16	5/5.3-5.6	up	Q9ZP21	ThioredoxinM type	electron transport	8,2	19119	510	47	18	8	*Triticum aestivum*
17	5/5.3-5.6	up	Q43199	Adenine phosphoribosyl transferase	biosynthesis	4,8	19651	151	33	13	3	*Triticum aestivum*
18	5/5.3-5.6	up	Q9LSY1	SKP1 like protein	protein turnover	4.6	17481	656	28	12	3	*Arabidopsis thaliana*
19	5/5.3-5.6	induced	Q0UNW1	Ubiquitin-like protein (ATG12)	protein turnover	5,1	14040	128	32	7	2	*Phaeosphaeria nodorum*

20	5/5.3-5.6	down	P12463	Photosystem Q B protein	photosynthesis	5,0	38895	158	12	14	3	*Triticum aestivum*
21	6/5.6-5.9	down	Q00434	Oxygen evolving enhancer protein 2	photosynthesis	9,0	27252	3349	36	21	8	*Triticum aestivum*
22	6/5.6-5.9	down	P69415	Photosystem I iron sulfur center	photosynthesis	6,6	8893	1111	43	7	3	*Triticum aestivum*
23	6/5.6-5.9	up	P69443	ATP synthase epsilon chain	biosynthesis	5,0	15208	653	42	10	6	*Triticum aestivum*
24	7/5.9-6.2	induced	Q6TCF2	Actin	structure	5.3	41580	847	22	34	8	*Gaeumanno. graminis*
25	7/5.9-6.2	down	Q7X9A6	Cytochrome b6 f complex iron sulfur	photosynthesis	8,1	23711	3627	58	19	9	*Triticum aestivum*
26	7/5.9-6.2	induced	Q94F73	Pathogenes-related protein 1 (PR1)	defence	6,8	17537	98	52	12	6	*Triticum aestivum*
27	8/6.2-6.5	down	P11383	RuBisCo large chain	photosynthesis	6,2	52817	96	13	42	5	*Triticum aestivum*
28	8/6.2-6.5	up	P02275	Histone H2A	DNA folding	11,1	15576	61	16	9	2	*Triticum aestivum*
29	8/6.2-6.5	up	Q5S1S6	Peroxiredoxin Q chloroplastic	oxidative stress	10,0	23349	769	41	19	6	*Triticum aestivum*
30	8/6.2-6.5	induced	Q0V6R0	Ubiquinone biosynthesis protein	biosynthesis	8,6	33101	120	27	31	5	*Phaeosphae. nodorum*
31	8/6.2-6.5	up	Q95H54	50s ribosomal protein	protein synthesis	12,4	14296	162	30	9	4	*Triticum aestivum*
32	11/7.1-7.4	induced	Q4I7K4	ATP dependent RNA helicase	RNA processing	8,3	71491	81	27	61	13	*Fusarium graminearum*
33	23/8.8-9.0	down	P27665	Oxygen evolving enhancer protein 1	photosynthesis	8,7	34718	1779	30	25	10	*Triticum aestivum*

*PLGS Score: it is calculated by the Protein Lynx Global Server (PLGS 2.2.5) software using a Monte Carlo algorithm to analyse all available mass spec. data and is a statistical measure of accuracy of assignation . A higher score implies greater confidence of protein identity (Wright et al. 2008).

Lane/pI range: fractions number/pH ranges of identified proteins, Expr Difference: difference in protein expression on *Pst*- inoculated leaves compared to control, Theo. pI: theoretical pI, MW: molecular weight, S.Cover : sequence coverage, Theo. Peptide: number of theoretical peptides.

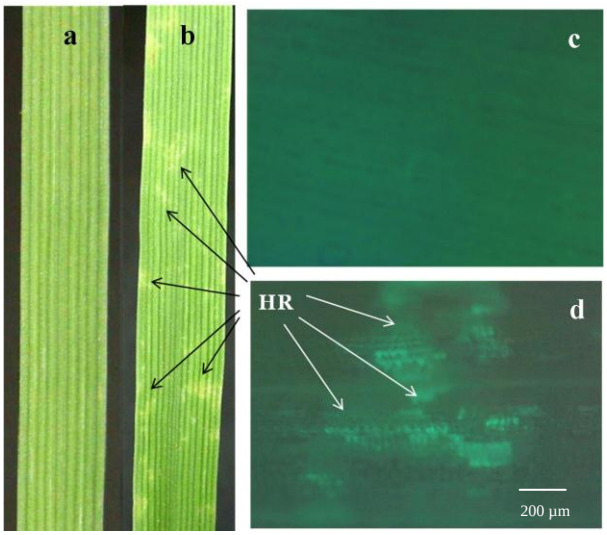

Fig 1. HR symptoms, a marker for incompatible interaction, were observed on the *Pst*-inoculated wheat leaves **(a)** mock-inoculated leaf at 17 dpi; **(b)** macroscopic appearance of HR symptoms on the infected leaf at 17 dpi; **(c)** microscopic appearance of the mock-inoculated leaf sample at 5 dpi; **(d)** microscopic appearance of HR symtoms on infected leaf at 5 dpi .

enzymes and proteins during plant-pathogen interaction. They have diverse functions in many biological reactions including nutrient aquisition, colonization, ecological interaction, plant defence interactions as elicitors and phytotoxin. Although, proteomic studies about host-pathogen interactions have generally focused on the host proteins, in recent years, researches have produced invaluable information about fungal proteins (Rampitsch et al., 2006; Zhou et al., 2006; Paper et al., 2007; Song et al., 2011). In this study, identified 6 proteins (#2, #11, #19, #24, #30 and #32, Table 1) were fungal proteins. Mediator of RNA polymerase II (#2) is component of the mediator complex, a coactivator involved in the regulated transcription of nearly all RNA polymerase II-dependent genes. Mediator functions as a bridge to convey information from gene-specific regulatory proteins to the basal RNA polymerase II transcription machinery (Hane et al., 2007). E3 ubiquitin protein ligase (#11) and Ubiquitin-like protein (#19) are ubiquitin pathway proteins which are one of the major protein turnover mechanisms that play important roles in the regulation of a variety of cellular functions including cell cycle, circadian clocks, transcription development, signal transduction and nutrient sensing (Jonkers and Rep, 2009). In addition, the importance of the proteolytic function of the ubiquitin-proteasome system in regulating the virulence of pathogenic fungi has just been realized recently. Exclusively, the studies have revealed that SCF E3 ligase mediated ubiquitin-proteasome system is essential for their virulence (Liu and Xue, 2011). Ubiqutitin system proteins have been reported in a number of plant pathogenic fungi such as *Fusarium* species, *Botryties cinerea* and *Magnaporthe oryzae* (Liu and Xue, 2011; Paper et al., 2007)

Defence and oxidative stress proteins

Identyfied four wheat proteins, PR1, PR4, GST and Peroxiredoxin Q (Prx Q) are defence response proteins which play important role in resistance against yellow rust and another pathogen infection (Zeng et al., 2010; Coram et al., 2008). Defence responses in plants against pathogen and enviromental stress are activated by their ability to sense and process stimuli. Incompatible interaction between wheat and *Pst*, ion channel gating, oxidative burst, cellular redox changes, protein kinase cascades, cell wall reinforcement, phytoalexin production and the accumulation of antimicrobial proteins are triggered as responses to the pathogen perception by plants to restrict pathogen growth and, ultimately, destroyed it (Knepper and Day, 2010). One of the earliest responses is a rapid increase in reactive oxygen species (ROS), known as oxidative burst. High concentrations of ROS play an important role as signal molecules for activation of local and systemic resistance responses (cell wall reinforcement, programmed cell death and expression of defence genes) (Mendoza, 2011). Additionally they are highly reactive and toxic for both pathogen and plant cells. ROSs are produced and detoxified in an orderly fashion to balance their toxic and defencive properties in plant cells in ideal resistance response (Bilgin, 2010). Upon infection, superoxide radicals that are converted into H_2O_2 via spontaneous dismutation or via superoxide dismutase (SOD) activity are produced. H_2O_2 has a direct antimicrobial effect to kill pathogen and triggers the HR in plants. In addition, it is a substrate for oxidative cross linking of cell wall material and a diffusible signal that induces the transcription of various resistance genes (Mellersh et al., 2002). In this study, GSTs (#4) and Prx Q (#29) proteins were identified as oxidative stress proteins. Peroxyredoxins were common thiol dependent peroxidases that have detoxification capacity for broad ranges of toxic peroxides and peroxynitrites (Baier and Dietz, 1996). Peroxiredoxins play an important role in combating the reactive oxygen species generated at the level of electron transport activities in the plant exposed to different types of biotic and abiotic stresses (Bazargani et al., 2011). In an incompatible interaction, the overexpression of Prx Q and type II Prx are observed to maintain the low peroxide concentrations outside the sites of infection and spare the uninfected cells. Peroxidases are also involved in several different defence-related processes such as suberin and lignin synthesis (Willekens et al., 1997; Rizhsky et al., 2002). Other antioxidant proteins, GST is the enzyme responsible for detoxifying xenobiotics by catalyzing their conjugation with tripeptide glutathione. GSTs play important roles in normal cellular metabolism and in detoxification of diverse ROSs. They are induced in response to oxidative stress to protect cellular components from damage caused by biotic or abiotic stresses (Liao et al., 2009; Vanacker et al., 2000). Up-regulation of these proteins were reported by different studies for self protection of plant against reactive oxygen species produced by themselves and fungus (Zhou et al., 2006; Larson et al., 2007; Liao et al., 2009; Li et al., 2011). Other earliest responses in the incompatible interactions between plant and pathogen are the expression of PR proteins and development of HR. Many PR proteins are effective as antimicrobial agents for at least certain bacterial and fungal pathogens and a defining characteristic of SAR (Knepper and Day, 2010; Zeng et al., 2010). In our study, two of the identified defence proteins are PR1 (systemic acquired resistance hallmark protein, #26) and PR4 (Wheatwin, #12). Highly induce PR1 expression at an early stage of the incompatible interaction between wheat and fungal pathogens was also observed by different researchers (VanLoon et al., 2006; Zeng et al., 2010). They reported that notably, the prominent PR1 proteins are often used as markers of the enhanced defensive state conferred by pathogen-induced SAR, but their biological activity has remained elusive.

Table 2. Functional category of the identified wheat proteins.

Class
Disease resistance and oxidative stress
GSTs
PR4
PR1
Prx Q
Photosynthesis
phosphoribulokinase chloroplastic
phosphoglycerate kinase chloroplastic
cytochrome b6 f complex iron sulfur subunit
oxygen evolving enhancer protein 1 chloroplastic
oxygen evolving enhancer protein 2 chloroplastic
photosystem I iron sulfur center
photosystem II cp47
photosystem Q B protein
RuBisCo small chain
RuBisCo large chain
Gene expression and regulation
eukaryotic translation initiation factor
elongation factor
splicing factor U2af
histone H4
histone H2A
Protein metabolism
Protein disulfide isomerase (ERp72)
SPK1-like
50S ribosomal protein
Metabolism
inositol 3 phosphate synthase
cysteine synthase
thioredoxin M type
adenine phosphoribosyl transferase
ATP synthase subunit

PR4 possess antifungal activity against several pathogenic fungi and specifically induced in wheat upon fungal infection. It binds to chitin and xylanase inhibitor protein which inhibits a fungal enzyme that degrades plant cell walls (Hurkman et al., 2009). Its inhibitor effect for hyphal growth and spore germination of pathogens in vitro was shown Caruso et al. (2001) and Bertini et al. (2003) reported that the pathogen-induced wheat PR4 genes are activated by SAR. Therefore, they considered that PR4 is useful markers of SAR. In addition, PR4 plays important role in HR which is characteristics for resistance response in wheat against yellow rust (Guevara et al., 2010; Wang et al., 2010).

Photosynthesis

Ten of the 27 wheat proteins are down regulated proteins (#3, #7, #8, #14, #20, #21, #22, #25, #27, #33, Table 1) playing role in photosynthesis and electron transport. They are related energy production as would be expected from leaf tissue. However, expression levels of these proteins were reduced. It is not surprising because there are many examples for repressing of photosynthetic genes expression following attack by insects or pathogens and abiotic stresses (Li et al., 2011; Bilgin et al., 2010; Nabity et al., 2009; Zhou et al., 2006; Bazargani et al., 20011). This situation was termed as "hidden cost" of defence because photosyntetic capacity is reduced to allocate resources from growth to defence (Bilgin et al., 2008).

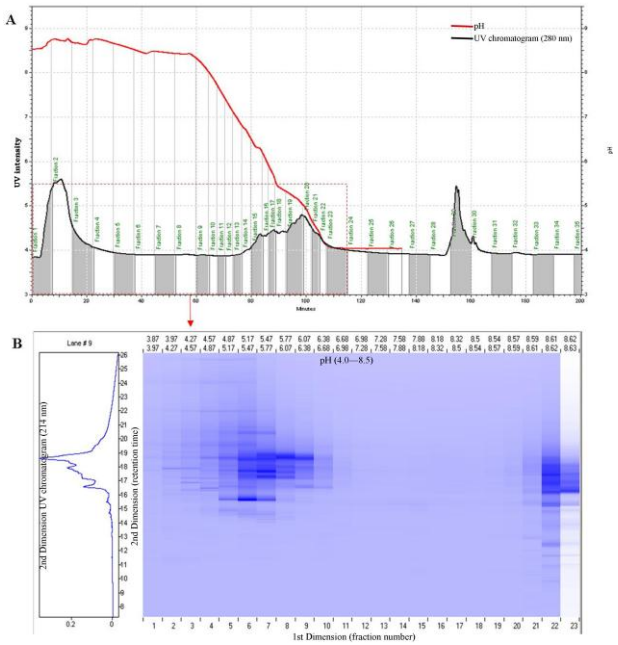

Fig 2. Two dimensional seperation of protein extract by PF2D. **(A)** Figure shows pH gradient, UV absorbance of proteins at 280 nm and collected fractions in the 1st dimension. The X-axis corresponds to the retention time in the column, and the left Y-axis to the pH and UV intensity; **(B)** Shown is PF2D ProteoVue virtual 2D-map of representative leaf samples. The Y-axis corresponds to the column retention time in minutes, the upper X-axis indicates the pH interwals and the lower X-axis corresponds to the fraction number.

It is suggested that HR might be another reason of this reduction. As known, HR is a highly effective, rapid and intense activation of numerous defence reactions that function to isolate and thus limit colonization by biotrophic pathogens. Researches support that photosynthetic activity was decreased and photosynthesis-related genes were repressed during the HR (Li et al., 2011; Zou et al., 2005; Lisa et al., 1999). However there are example reports about the inducible expression of the photosynthetic genes following attack by pathogens (Trumble et al., 1993; Shen et al., 2003). This upregulation was explained that chloroplasts of the some leaf zone were not yet affected directly pathogen attack and photosynthetic activity was increased to compensate the loss in adjacent infected cells.

Gene expression and regulation

A number of biochemical changes were contributed to the early response at the hosts following pathogen perception. In host, one of the first reactions following pathogen attack is rearragement of gene expression and regulation for trancriptional and/or posttranslational activation of defence related genes (Zhu et al., 1996). We identified up-regulated eight proteins (#1, #6, #9, #13, #28, Table 1) involved in gene expression and regulation however some of these proteins were associated with defence response by other researchers. One of them, eIF4E (#1) is an eukaryotic translation initiation factor but its key role in plant resistance to potiviruses was detailly rewieved by Robaglia and Caranta (2006) and reported that translation initiation factors, particularly the eIF4E and eIF4G protein families, were found to be essential determinants in the outcome of RNA virus infections. Another protein is splicing factor Uaf2(#9).

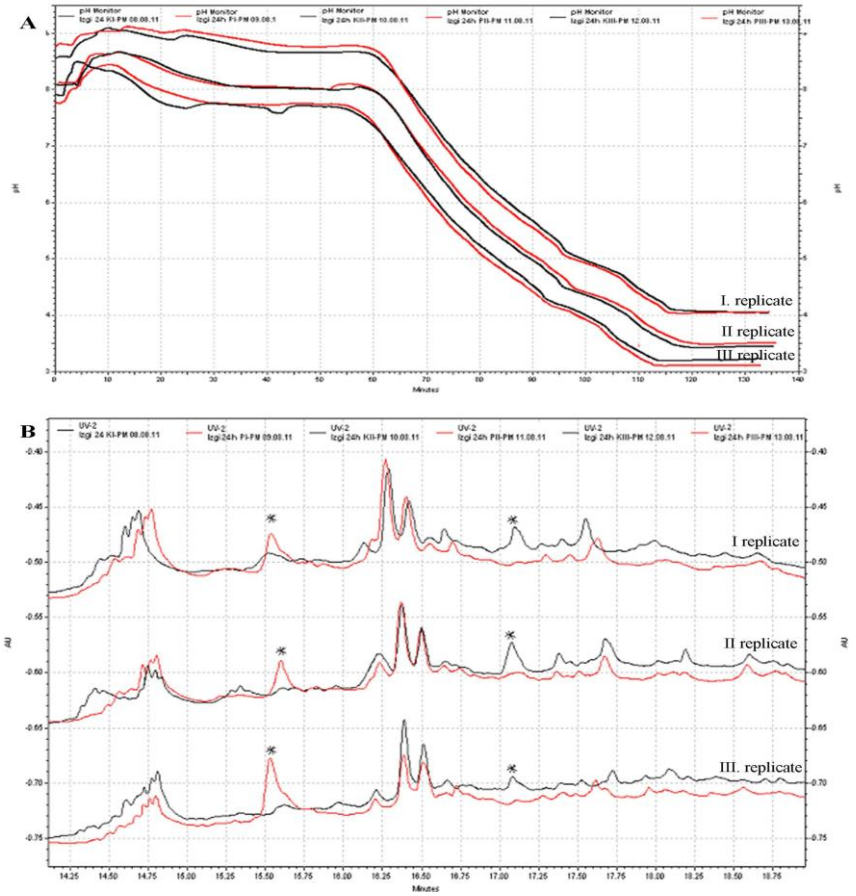

Fig 3. High reproducibility of two dimensional seperation of ProteomeLab PF2D. **A**. Comparison of the 1st dimension pH gradients which were obtained from 1st dimension seperation of the *Pst*- and mock inoculated leaf protein extracts between three independent biological replicates; **B.** Representative examples of the repeatability of PF2D 2nd dimension fractionation of three biological replicates. Compared protein UV (214 nm) profiles obtained after injection of the identical 1st dimension fractions of *Pst*- and mock inoculated leaf protein extract of the three independent biological replicates. (black lines represent mock-inoculated samples, red lines represent *Pst*-inoculated samples, stars represent differentially expressed proteins).

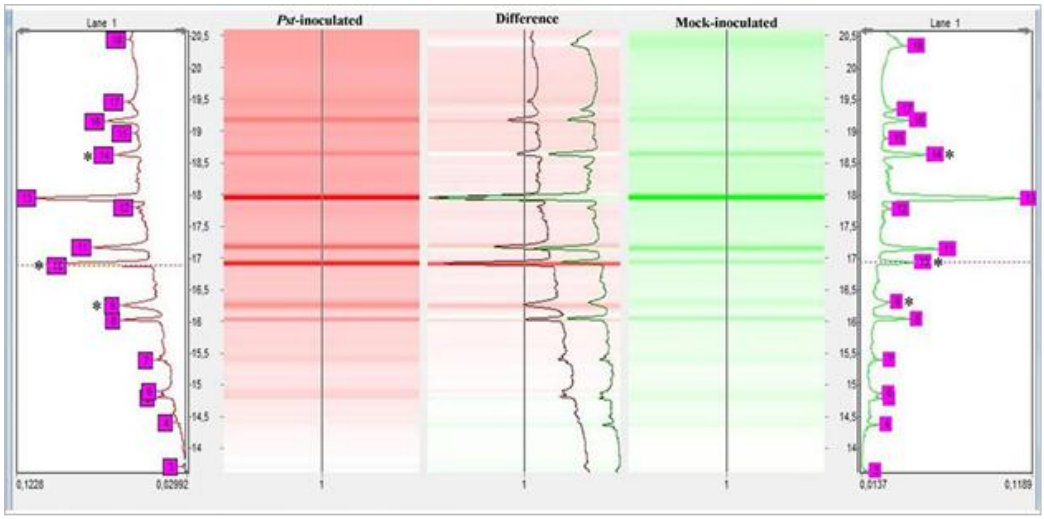

Fig 4. DeltaVue provides more accurate comparison between two 2D-maps obtained from *Pst*- (red) and mock-inoculated (green) leaf samples. Differences between two chromatograms are displayed in the middle column showing with both UV peaks and dominant protein bands. Each numbered peak represents a protein (stars represent selected differentially expressed proteins for MS analysis).

Alternative splicing in plants is an important posttranscriptional regulatory mechanism in modulating gene expression. Additionally, some researches indicated that alternative splicing of pre-mRNAs of R genes plays an important role in plant defense responses (Reddy, 2001). Identified two wheat proteins were well known histone proteins, [H4 (#13) and H2A (#28)] that are crucial to the condensed packing of DNA and also involved in transcription regulation, DNA replication, repair, and condensation. Additionally, their potential antimicrobial activities, both intact proteins and cleaved peptides were also reported by Kawasaki and Iwamoto (2008). Also, wheat (*Triticum* spp.) histones were tested to determine of their antifungal properties and H1-H4 showed significant viability reduction of several *Fusarium* species, especially *F. graminearum* by De Lucca et al. (2011).

Protein metabolism

Three up-regulated proteins were identified including ER-72 (#5), SPK1-like (#18) and 50S ribosomal protein (#33) at 24 hpi and known to be related to protein metabolism such as translation, processing and degredation. 50S ribosomal protein was involved in the protein biosynthesis. Second identified metabolism related protein was protein disulfide isomerase (#5), it is also known as ERp-72 or endoplasmic reticulum protein 72 kDa which acts as a molecular ER chaperone. ER chaperones are critical not only for quality control of proteins processed in the ER, but also for regulation of ER signalling in response to ER stress. ERp-72 catalyses the rearrangement of -S-S- bonds in proteins. It is part of a large chaperone multiprotein complex. ERp-72 identified as a component that is differentially regulated directly following pathogen recognition. The function of cytoplasmic chaperones during innate immunity has been studied in detail by several research groups. Conversely, very little is known about the function of ER chaperones during innate immunity. However, they suggest that ER chaperones are upregulated during innate immunity to aid accumulation of an induced receptor-like kinase (IRK) required for a successful immune response (Caplan et al., 2009). Most plant metabolic pathways were regulated by a balanced synthesis and degradation of enzymes controlling them. In plants, ubiquitination was modulated environmental and endogenous signals, including responses to pathogen attack (Hare et al., 2003; Delaure et al., 2008). SPK1 (#18) is an important member of the ubiquitination system and proteasomal degradation of target proteins. The SPK1, Cullin and F-box protein were formed SCF complex (E3 ligases) which is essential to determine target protein. Results from various research groups indicate that E3 ligases and the related protein breakdown play an important role in the signal transduction pathways leading to disease resistance (Devoto et al., 2003; Zeng et al., 2006; Dreher and Callis, 2007)

Metabolism

We also identified five proteins (#10, #15, #16, #17, #23, Table 1) are related to primeary metabolic pathways included biosynthesis and energy metabolism, these proteins were up-regulated as well. Adenine phosporibosyl transferase (#17), cysteine synthase (#15) are enzymes involved in amino acid biosynthesis. Myo-Inositol-1-phosphate synthase (#10) catalyzes the conversion of D-glucose 6-phosphate to 1L-myo-inositol-1-phosphate. It is the first committed step in the production of all inositol-containing compounds, including phospholipids, Inositol phosphates also play an important role in signal transduction (Brandon et al., 2012). ATP synthase (#23) is an important enzyme of energy metabolism which produces ATP from ADP in the presence of a proton gradient across the membrane. Thioredoxin (Trx) M type (#16) protein is one of the four Trx proteins and chloroplast drought-induced protein of 32 kD (CDP32), a thioredoxin-like protein that is Trx reductase as an electron donor for the reaction, which is not a physiological reductant (Kong et al., 2000).

Materials and methods

Plant materials and pathogen inoculation

Yellow rust resistant wheat cultivar "Izgi2001" was developed by Anatolian Agricultural Research Institute (AARI) and registered in Turkey (Variety Registration and Seed Certification Center of Turkey, http://www.ttsm.gov.tr /TR/belge/1-248/tescilli-cesitler-listesi.html). *Pst* isolates were obtained from Central Research Institute for Field Crops (Turkey) and virulent to Yr2, Yr6, Yr7, Yr8, Yr9, Yr11, Yr12, Yr17, Yr18, Yr27, YrA+ and avirulent to Yr1, Yr5, Yr10, Yr15, Yr24, YrSP, YrCv. Eighty seeds of İzgi2001 were planted in pots (7 cm diameters) filled with sterile peat. Each pot contained 4 seeds and then incubated in a greenhouse at 20°C in 16/8 h day/night photoperiod. Thirteen days after germination, at the two-leaf stage, half of the seedlings were inoculated by spraying with a 10 mg/mL suspension of urediniospores in light mineral oil (Soltrol 170, ChemPoint, Limburg, Netherlands). Remaining half were mock-inoculated (control) with equivalent volume (0,1 ml per plant) of spore-free mineral oil. Fifteen minutes later following inoculation, plants were transferred to dark dew chambers with humidity of 95-100% at 9^{o}C for 24h. After this period they were transferred to a greenhouse adjusted to 15 ^{o}C for dark cycle and 25^{o}C for light cycle. First leaves of the *Pst*-inoculated and mock-inoculated seedlings were harvested at 24 hpi and immediately placed in liquid nitrogene. They were stored in a –80^{o}C freezer until protein extraction. Three independent biological replicates were analyzed in this study. Resistance response was monitored by flourescence microscope (Leica DMI6000 B) at 5th dpi on the second leaves. In addition, infection type (IT) was recorded on a 0- to-9 scale (McNeal et al., 1971) about 15 dpi on second leaves. ITs 0 to 6 were considered low ITs while 7 to 9 were high ITs. Leaf samples of the seedling that their ITs were 0 or 1 were used for proteomic analysis.

Protein extraction

Protein extraction was performed by using combined protocols of Kim et al. (2001) and Rampitsch et al. (2006). Two grams of leaf samples were ground to fine powder in liquid nitrogene by using the Retsch MM301 system. The powder was homogenized in 20 mL of ice-cold Mg/NP-40 extraction buffer containing 0.5 M Tris.HCl (Roche 122010) pH 8.3, 2% (v/v) NP-40 (Sigma I7771), 20 mM MgCl2 (Sigma M8266), 2% (v/v) beta-mercaptoethanol (Applichem A1108), 1 mM PMSF (Sigma P7626) and 1% (w/v) PVPP (Fluka 77627). After centrifugation at 12 000×g for 15 min at +4^{o}C, proteins in the supernatant were fractionated with 15% PEG 4000 (Sigma 95904). Samples were incubated on ice for 30 min and then centrifuged at 15000×g for 10 min. The supernatant were precipitated by adding four volumes of cold acetone and put at -20^{o}C overnight. After centrifugation at

12000×g for 20 min at +4°C, pellet was washed 6 times with cold acetone. The pellets were dissolved in 2 ml Solubilization Buffer [7.5 M urea (Sigma U0631), 2.5 M thiourea (Sigma T7875), 12.5% (v/v) glycerol (Sigma C3023) , 62.5 mM tris (Roche 122010), 2.5% (w/v) 1 n-octylglucopranoside (Sigma 08001), 6.25 mM TCEP (Sigma C4706) and 1.25 mM protease inhibitor coctail (Sigma P2714)]. Solubilized sample was sonicated five times for 5 sec and centrifuged at 30.000×g for 30 min and then supernatant was centrifuged at 90.000×g for 1 h respectively. The final protein content was determined using a Bradford Microassay Procedure (Bio-Rad Laboratories) by using BSA as a standard. Samples were stored in aliquots (500 µg/500 µl) at -80℃.

First dimension fractionation by PF2D

ProteomeLab™ PF2D system is a 2D-liquid chromatography system for two dimensional seperation of protein mixtures. Proteins were seperated in first dimension according to their isoelectric point (pI) by chromatofocusing method. The first dimension separation was performed at room temperature with two buffers (Start Buffer pH 8.5 and Eluent Buffer pH 4.0). These buffers were freshly prepared according to Barre and Solioz (2006). The high performance chromatofocusing (HPCF) column was kept according to the manufacturer's instructions (Eprogen-A51680). The column was washed with water with 0.2 ml/min flow rate for 45 min and then Start Buffer began to flow during 130th min with 0.2 ml/min flow rate in order to equilibrate the coloumn. At the same time, extracted protein samples were desalted on a PD-10 SephadexTM G-25 gel filtration column and eluted with 3.5 ml the chromatofocusing Start Buffer. Protein quantification was performed by using micro BCA Protein Assay Kit (Sigma, 020M617.1). After column equilibration, 2 mg desalted protein sample was loaded into the column with a manual injector. In the first dimension, proteins bind to a strong anion exchanger and the pH began to decrease after 60 min from 8.5 to 4.0. Proteins were eluted with a continuous decreasing and fractions were collected at 0.3 pH interval in a 96 deepwell plate.

Second dimension fractionation by PF2D

PF2D second dimension separation utilizes reverse phase high performance liquid chromatography (HPLC) fractionation. Eluted proteins during pH gradient in the 1st dimension were separated in a second dimension by their hydrophobic properties. Two solvents were used for hydrophobicity gradient: 0.1% TFA (v/v) in HPLC water (Solvent A) and 0.08% TFA (v/v) in acetonitrile (ACN) (Solvent B). Separation was performed at 50°C with 0.75 ml/min flow rate and protein absorbence was detected by UV2 detector at 214 nm for each fraction. HPRP column equilibration was achieved with Solvent A for 10 min followed by Solvent B for 5 min to each injection. From selected each first dimension fractions, 0,2 ml sample was injected to second dimension module, run for two min, and the column was eluted with a linear gradient of 0–100% Solvent B for 25 min. Proteins were collected starting at 5 min and ending at 25 min in 0.75 min intervals. Thereafter, Solvent B was continued for 5 min, followed by re-equilibration with 100% Solvent A for 10 min. The 32 Karat™ Software (Beckman Coulter) was used for data processing, calculation of peak areas and heights. Protein profile for each sample was genarated by ProteoVue software as a 2D-Map. The *Pst*-inoculated and mock-inoculated protein profiles were compared and peak-to-peak analysis of their chromatograms were carried out by using DeltaVue software. This software was specifically developed to detect quantitative differences among compared proteins peaks. In our previous study (unpublished data), three technical replicates were performed and area of 200 peaks were statistically analysed by t-test to calculate for the minimum fold-change values for selection of the differentially expressed proteins. According to this, peaks that expression levels are more than 2 fold-between *Pst* and mock-inoculated samples were considered to be significant and selected for identification.

NanoLC-ESI-MS/MS analysis

Selected fractions were dried under vacuum, and resuspended by adding 10 µL 50 mM NH_4HCO_3 (Fluka, 09830). Disulphite bonds were reduced by adding 1 µL of 100 mM DTT (Sigma, 43815) and incubated at 60°C for 1 h. The reduced cysteine side chains were modified by the addition of 1 µL of 200 mM IAA (Sigma, I1149) and incubated in the dark at room temperature for 30 min. Proteolytical digestion was done by the addition of 0,2 µg of proteomics grade trypsin (Sigma, T6567) in 50 mM NH_4HCO_3 and incubated at 37°C overnight. Peptides were purified using C18 ZipTip® (Millipore) according to manufacturer's recommendation. Eluates were dried under vacuum and resuspended in 5 µL of HPLC grade water with 0.1% formic acid (Sigma, 94318) and 50 fmol calibrant (ADH1_YEAST- Waters MassPrep Enolase Digestion Standart, 186002325) for mass spectrometer analysis. Two microliter of sample was loaded on the system [nanoACQUITY ultra presssure liquid chromatography (UPLC) and SYNAPT high definition mass spectrometer] with nanolockspray ion source. Prior to the injection, the columns were equilibrated with 97% mobile phase A (water with 0.1% FA) and 3% mobile phase B (ACN containing 0.1% FA). The column temperature was set to 35°C. First, peptides were trapped on a nanoACQUITY UPLC Symmetry C18 Trap column (5 µm particle size, 180 µm i.d. x 20 mm length) at 5 µl/min flow rate for 5 min. Peptides were separated from the trap column by gradient elution onto an analytical column (nanoACQUITY UPLC BEH C18 Column, 1.7 µm particle size, 75 µm i.d. x 250 mm length), at 300 nl/min flow rate with a linear gradient from 5 to 40% ACN over 90 min. Data independent acquisition mode (MSE) was carried out by operating the instrument at positive ion V mode, applying the MS and MS/MS functions over 1.5 sec intervals with 6 V low energy and 15-40 V high energy collision to collect the peptide mass to charge ratio (m/z) and the product ion information to deduce the amino acid sequence. To correct for the mass drift the internal mass calibrant Glu-fibrinopeptide was infused every 45 sec through the nanolockspray ion source at 300 nl/min flow rate. Peptide signal data between 50-1600 m/z values were collected. Tandem mass spectra extraction, charge state deconvolution and deisotoping steps were processed with ProteinLynx Global Server V2.4 (Waters Corp, Milford, MA) and searched with the IDENTITY^E algorithm against wheat reviewed protein database from Uniprot. Identity^E was set up to search null assuming the digestion enzyme trypsin and searched with a fragment ion mass tolerance of 0.028 Da and a parent ion tolerance of 0.011 Da. The Apex3D data preparation parameters were set to 0.2 min chromatographic peak width, 10.000 MS TOF resolution, 150 counts for low energy threshold, 50 counts for elevated energy threshold,

and 1200 counts for the intensity threshold. Databank search query was set to minimum 3 fragment ion matches per peptide, minimum 7 fragment ion matches per protein, minimum 1 peptide matches per protein and 1 missed cleavage. Carbamidomethyl-cysteine fixed modification and Acetyl N-TERM, deamidation of asparagine and glutamine, oxidation of methionine variable modifications were set.

Conclusion

Although many studies about wheat-*Pst* interactions at the genomic and transcriptomic level have been carried out, our overall understanding of the molecular mechanism of the resistance is still very superficial. On the other hand, the early response events hold the key in perceiving and amplification of different stress signals and induction of further downstream gene expression. Therefore identification of the early response-related proteins are key points to elucidate of the complex plant-pathogen interaction and plant defense system. In recent, proteomic analysis is accepted as a very useful tool for providing more informative solid data to elucidate of molecular mechanism of the biological procces. The research reported here is one of the very few proteomic studies concerning resistance response in wheat to yellow rust (Li et al., 2011; Ma et al., 2009). Unlike the others, significant proteins which have role in early defence responses were identified in this study. We expect that our results make a significant contribution to understanding of the wheat resistance to yellow rust and to develop new strategies for combating this disease. However, additional study including various time points will be necessary to elucidate this complex response.

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References

Agrawal GK, Yonekura MIY, Iwahashi H, Rakwal R (2005) System, trends and perspectives of proteomics in dicot plants Part III: Unraveling the proteomes influenced by the environment, and at the levels of function and genetic relationships. J Chromatogr B. 815:137-145

Baier M, Dietz KJ (1996) Primary structure and expression of plant homologues of animal and fungal thioredoxin-dependent peroxide reductases and bacterial alkyl hydroperoxide reductases. Plant Mol Biol. 31:553-564

Barre O, Solioz M (2006) Improved protocol for chromatofocusing on the ProteomeLab PF2D. Proteomics. 6:5096-5098

Bazargani MM, Sarhadi E, Bushehri AA, Matros A, Mock HP, Naghavi MR, Hajihoseini V, Mardi M, Hajirezaei MR, Moradi F, Ehdaie B, Salekdeh GH (2011) A proteomics view on the role of drought-induced senescence and oxidative stress defense in enhanced stem reserves remobilization in wheat. J Prot. 74(10):1959-73

Bertini L, Leonardi L, Caporale C, Tucci M, Cascone N, Di Berardino I, Buonocore V, Caruso C (2003) Pathogen-responsive wheat PR4 genes are induced by activators of systemic acquired resistance and wounding. Plant Sci. 164:1067-1078

Bilgin DD, Zavala JA, Zhu J, Clough SJ, Ort DR, DeLucia EH (2010) Biotic stress globally downregulates photosynthesis genes. Plant Cell Environ. 33:1597-1613

Bilgin DD, Aldea M, O'Neill BF, Benitez M, Li M, Clough SJ, DeLucia EH (2008) Elevated ozone alters soybean-virus interaction. Mol Plant Microbe Int. 21:1297-1308

Brandon SG, Urs FB, Susanne HB (2012) Identification of lipids and lipid-binding proteins in phloem exudates from *Arabidopsis thaliana*. J Exp Bot. 63(10): 3603-3616

Caplan JL, Zhu X, Mamillapalli P, Marathe R, Anandalakshmi R, Dinesh-Kumar SP (2009) Induced ER chaperones regulate a receptor-like kinase to mediate antiviral innate immune response in plants. Cell Host Microbe. 6:457-469

Caruso C, Bertini L, Tucci M, Caporale C, Nobile M, Leonardi L, Buonocore V (2001) Recombinant wheat antifungal PR4 proteins expressed in *Escherichia coli*. Protein Expres Purif. 23:380-388

Chen WQ, Wu LR, Liu TG, Xu SC, Jin SL, Peng YL, Wang BT (2009) Race dynamics, diversity, and virulence evolution in *Puccinia striiformis f.* sp. *tritici*, the causal agent of wheat stripe rust in China from 2003 to 2007. Plant Dis. 93:1093-1101

Coram TE, Wang M, Chen XM (2008) Transcriptome analysis of the wheat–*Puccinia striiformis* f. sp. *tritici* interaction. Mol Plant Path. 9(2):157-169

De Lucca AJ, Heden LO, Ingber B, Bhatnagar D (2011) Antifungal properties of wheat histones (H1_H4) and purified wheat Histone H1. Agric Food Chem. 59:6933-6939

Delauré S, Van Hemelrijck W, De bolle M, Cammue B, De Coninck B (2008) Building up plant defenses by breaking down proteins. Plant Sci. 174:375-385

Devoto A, Muskett PR, Shirashu K (2003) Role of ubiquitination in the regulation of plant defence against pathogens. Curr Opin Plant Biol. 6:307-311

Dreher K, Callis J (2007) Ubiquitin, hormones and biotic stress in plants. Ann Bot. 99:787-822

Edgar N, Vollmer M, Hörth P, Vad C (2004) 2D-LC/MS techniques for the identification of proteins in highly complex mixtures. Expert Rev Proteomics. 1 (1):37-46

Ersek T, Holliday M, and Keen NT (1982) Association of hypersensitive host cell death and autofluorescence with a gene for resistance to *Peronospora manshurica* in soybean. Phytopathol. 72(6):628-631

Guevara-Morato MA, Garcia de Lacoba M, Garcia-Luque I, Serra MT (2010) Characterization of a pathogenesis-related protein 4 (PR-4) induced in Capsicum chinense L3 plants with dual RNase and DNase activities. J Exp Bot. 61(12):3259-3271

Hane JK, Lowe RGT, Solomon PS, Tan KC, Schoch CL, Spatafora JW, Crous PW, Kodira CD, Birren BW, Galagan JE, Torriani SFF, McDonald BA, Oliver RP (2007) Dothideomycete-plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. Plant Cell. 19:3347-3368

Hare PD, Seo HS, Yang JY, and Chua NH (2003) Modulation of sensitivity and selectivity in plant signaling by proteasomal destabilization. Curr Opin Plant Biol. 6:453-462

Hurkman WJ, Vensel WH, Tanaka CK, Whitehand L, Altenbach SB (2009) Effect of high temperature on albumin and globulin accumulation in the endosperm proteome of the developing wheat grain. J Cereal Sci. 49:12-23

Jones JDG, Dangl JL (2006) The plant immune system. Nature. 444:323-329

Jonkers W, Rep M (2009) Lessons from fungal F-box proteins. Eukaryot Cell. 8:677-95

Kawasaki H, Iwamoto S (2008) Potential roles of histones in host defense as antimicrobial agents. Infect Disord Drug Targets. 8:195-205

Kim ST, Cho KS, Jang YS, Kang YK (2001) Two-dimensional electrophoretic analysis of rice proteins by polyethylene glycol fractionation for protein arrays. Electrophoresis. 22:2103-2109

Knepper C, Day B (2010) From perception to activation: the molecular-genetic and biochemical land scape of disease resistance signalling in plants. In: Last R (ed) The Arabidopsis Book 8, 1st edn. NW, Washington

Kong W, Shiota S, Shi Y, Nakayama H, Nakayama K (2000) A novel peroxiredoxin of the plant *Sedum lineare* is a homologue of *Escherichia coli* bacterioferritin comigratory protein (Bcp). Biochem J. 351:107-114

Larson RL, Hill AL, Nuñez A (2007) Characterization of Protein Changes Associated with Sugar Beet (*Beta vulgaris*) Resistance and Susceptibility to Fusarium oxysporum. J Agric Food Chem. 55:7905-7915

Li H, Wei G, Xu J, Huang L, Zhensheng K (2011) Identification of wheat proteins with altered expression levels in leaves infected by the stripe rust pathogen. Acta Physiol Plant. 33(6):2423-2435

Liao M, Li Y, Wang Z (2009) Identification of elicitor-responsive proteins in rice leaves by a proteomic approach. Proteomics. 9:2809-2819

Lisa JA, Kennaway B, MacGregor, Randall SK, Doug HB, Julie K, Alan WB (1999) The relationship between photosynthesis and a mastoparan-ınduced hypersensitive response in ısolated mesophyll cells. Plant Physiol. 119:1233-1241

Liu TB, Xue C (2011) The ubiquitin-proteasome system and F-box proteins in pathogenic fungi. Mycobiology. 39(4):243-248

Ma C, Xu SC, Xu Q, Zhang ZH, Pan YH (2009) Proteomic analysis of stripe rust resistance wheat line Taichung29*6/Yr5 inoculated with stripe rust race CYR32. Scientia Agricultura Sinica 42:1616-1623

McDonald T, Sheng S, Stanley B, Chen D, Ko Y, Cole RN, Pedersen P, Van Eyk JE (2006) Expanding the subproteome of the inner mitochondria using protein separation technologies: one- and two-dimensional liquid chromatography and two-dimensional gel electrophoresis. Mol Cell Proteomics. 5:2392-2411

McNeal FH, Konzak CF, Smith EP, Tate WS, Russell TS (1971) A uniform system for recording and processing creal research data.,Agricultural Research Service Bulletin 34-121. USDA, Washington

Mehta A, Brasileiro AC, Souza DS, Romano E, Campos MA, Grossi-de-Sá MF, Silva MS, Franco OL, Fragoso RR, Bevitori R, Rocha TL (2008) Plant-pathogen interactions: what is proteomics telling us? FEBS J. 275(15):3731-46

Mellersh DG, Foulds IV, Higgins VJ, Heath MC (2002) H_2O_2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. Plant J. 29:257-268

Mendoza M (2011) Oxidative burst in plant-pathogen interaction. Biotecnología Vegetal. 11(2):67-75

Nabity PD, Zavala JA, DeLucia EH (2009) Indirect suppression of photosynthesis on individual leaves by arthropod herbivory. Ann Bot. 103:655-663

Paper JM, Scott-Craig JS, Adhikari ND, Cuomo CA, Walton JD (2007) Comparative proteomics of extracellular proteins in vitro and in planta from the pathogenic fungus *Fusarium graminearum.* Proteomics. 7:3171-3183

Rampitsch C, Bykova NV, Mccallum B, Beimicik E, Ens W (2006) Analysis of the wheat and *Puccinia triticina* (leaf rust) proteomes during a susceptible host-pathogen interaction. Proteomics. 6:1897-1907

Reddy AS (2001) Calcium: silver bullet in signaling. Plant Sci. 160:381-404

Rizhsky L, Hallak-Herr E, Van Breusegem F, Rachmilevitch S, Barr JE, Rodermel S, Inze D, Mittler R (2002) Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. Plant J. 32:329-342

Robaglia C, Caranta C (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends Plant Sci. 11:40-45

Shen S, Jing Y, Kuang T (2003) Proteomics approach to identify wound-response related proteins from rice leaf sheath. Proteomics. 3:527-535

Song X, Rampitsch C, Soltani B, Mauthe W, Linning R, Banks T, McCallum B, Bakkeren G (2011) Proteome analysis of wheat leaf rust fungus, *Puccinia triticina*, infection structures enriched for haustoria. Proteomics. 11:944-963

Suberbielle E, Gonzalez-Dunia D, Pont F (2008) High reproducibility of two-dimensional liquid chromatography using pH-driven fractionation with a pressure-resistant electrode. J Chromatog B. 871:125-129

Trumble JT, Kolondy-Hirsch DM, Ting IP (1993) Plant compensation for arthropod herbivory. Annu Rev Entomol. 38:93-119

Vanacker H, Carver TL, Foyer CH (2000) Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction. Plant Physiol. 123:1289-1300

VanLooN LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol. 44:135-162

Wang X, Liu W, Chen X, Tang C, Dong Y, Ma J, Huang X, Wei G, Han Q, Huang L, Kang Z (2010) Differential gene expression in incompatible interaction between wheat and stripe rust fungus revealed by cDNA-AFLP and comparison to compatible interaction. BMC Plant Biol. 10:9-15

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inze D, Van Camp W (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. EMBO J. 16:4806-4816

Wright CA, Edelmann M, Digleria K, Kollnberger S, Kramer H, McGowan S, McHugh K, Taylor S, Kessler BM, Bowness P (2008) Ankylosing spondylitis monocytes show upregulation of proteins involved in inflammation and the Ubiquitin Proteasome pathway. Ann Rheum Dis. 68(10):1626-32

Wu WW, Wang G, Yu M, Knepper MA, Shen RF (2007) Identification and quantification of basic and acidic proteins using solution-based two dimensional protein fractionation and label-free or 180-labeling mass spectrometry. J Proteome Res. 6:2447-59

Zeng X, Wang , Ali M., Zhang H, Liu X, Li W, Ji W (2010). Profiling gene expression patterns of stripe rust (Puccinia striiformis f. sp. tritici) resistance gene in new wheat germplasm. Pak J Bot. 42(6):425-4266

Zeng LR, Vega-Sanchez ME, Zhu T, Wang GL (2006) Ubiquitination mediated protein degradation and modification: an emerging theme in plant-microbe interactions. Cell Res. 16:413-426

Zhou W, Eudes F and Laroche A (2006) Identification of differentially regulated proteins in response to a compatible interaction between the pathogen *Fusarium graminearum* and its host, *Triticum aestivum*. Proteomics. 6:4599-4609

Zhu Q, Dröge-Laser W, Dixon RA, Lamb C (1996) Transcriptional activation of plant defense genes. Currt Opin Genet Dev. 6: 624-630

Zou J, Rodriguez-Zas S, Aldea M, Li M, Zhu J, Gonzalez DO, Vodkin LO, DeLucia E, Clough SJ (2005) Expression profiling soybean response to Pseudomonas syringae reveals new defense-related genes and rapid HR-specific downregulation of photosynthesis. Mol Plant Microbe In. 18:1161-1174