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Phytophthora infestans associated global gene expression profile in a late blight resistant Indian potato cv. Kufri Girdhari

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Abstract

From the past late blight is one of the most dreaded diseases of potato worldwide. In this study, microarray-based gene expression was analysed in a late blight resistant Indian potato cultivar Kufri Girdhari upon *Phytophthora infestans* infection. A comprehensive transcriptional analysis was performed using cDNA microarrays, containing 70,083 ESTs (expressed sequence tag libraries) from plant leaf tissues collected at two stages: pre-inoculation (0 h, water spray as control) and post-inoculation (72 h after inoculation) by challenge inoculation of *P. infestans*. Microarray analysis using scatter plot revealed out of total statistically significant ($P \leq 0.01$) genes (#4622), a total of 2,344 resistance genes were up-regulated at post-inoculation stage compared to pre-inoculation. These significantly genes (#2,344) were greatly expressed at post-inoculation and contribute resistance in response to the challenge inoculation of *P. infestans*. Further, hierarchical clustering classified the genes (#2,344) expressions into 8 different clusters. Study revealed that molecular chaperones were found to be mainly controlling resistance mechanism in Kufri Girdhari and acted as downstream signalling pathway for defense related genes response to various mechanisms (stress/harmone/signalling genes/transporter/metabolism). The selected highly up-regulated genes were further validated for their expression in the cultivar by real-time (RT) PCR analysis. Thus study provides many candidate genes/transcripts for future characterization and functionality assay as initial starting point for research work.

Keywords: chaperones; gene expression; late blight resistance; potato; stress response.
Abbreviations: cDNA_complementary DNA; RT-PCR_real time polymerase chain reaction; ESTs_expressed sequence tagged; MT_ million tonnes; PGSC_Potato Genome Sequencing Consortium.

Introduction

Potato is the third most important food crop in the world, after rice and wheat, with global annual production of 374 million tonnes (MT) during the year 2011. China stands the largest potato producer followed by India (88.35 and 42.33 MT, respectively). However, potato yield has been erratic across the world during the past decade ranging between 16.3 to 19.4 t/ha during the year 2000 to 2011, though showing an overall slight increase (FAOSTAT, 2011). Moreover, productivity of potato is hampered by several biotic stresses among which late blight caused by *Phytophthora infestans*, constitutes a major threat and is the most dreaded one. The pathogen spreads like a wild fire under congenial weather conditions and wipes out the entire crop within a few days. Management of late blight disease mostly depends on repeated application of fungicides. Their repeated application could cause a slow erosion of disease control due to a gradual loss of sensitivity of the targeted pathogen population to the fungicide in addition to increase production costs and environmental risk. Development of effective management strategies for late blight resistance of potato usually requires tremendous genetic resources and efforts when traditional breeding approaches are taken. Consequently, identification of new source of resistance genes is a major challenge. Breeding for resistance to *P. infestans* in potato is considered the most efficient approach to control the disease, as lot of variability exists within the genus *Solanum* and its wild relatives. Moreover, sources of resistance to late blight disease have been identified through extensive screening of germplasm. As a result of continuous breeding efforts, Central Potato Research Institute, Shimla, India has developed and released a late blight resistance cultivar Kufri Girdhari for hilly regions of India (Joseph et al., 2011).

Since resistance genes of the cv. Kufri Girdhar has not been known so far, it is essential to understand its resistance mechanism at the molecular level and to develop specific target gene products for use in breeding to develop resistant cultivars. So, a novel approach to study the resistance at the molecular scale is to survey the gene expression at phenotype level. The microarray gene expression study helps in monitoring of thousands of genes simultaneously (Shi et al., 2003). Hence, in this paper we report on identification and characterisation of the expression patterns of late blight resistance genes using microarray analysis based on 72,083 ESTs sequences and real time polymerase chain reaction (RT-PCR).

Results

Microarray analysis for gene expression

In the present study, microarray analysis revealed the sets of differentially expressed transcripts with significant differences in the gene expression levels at pre- and post-inoculation stages of *P. infestans* infections. A t-test analysis (at $P \leq 0.01$), identified a total of 4,662 genes that were statistically significant (Fig. 1a), out of which 2,344 genes

Table 1. Potato ESTs up-regulated in leaves infected by *Phytophthora infestans*, and fold change induction of the gene expression.

SN	Gene bank accession number	Putative function	Fold induction
1.	EG013368	22.7 kDa class IV heat shock protein, *Pisum sativum*	132.5
2.	SOLTU001603:Contig10	Small heat shock protein, chloroplastic OS=*Solanum lycopersicum*	123.1
3.	CN463702	17.6 kDa class I heat shock protein OS=*Solanum peruvianum*	109.9
4.	SOLTU028882:Contig1	Heat shock 70 kDa protein OS=Glycine max	91.0
5.	CV431519	Abscisic acid and environmental stress-inducible protein, *Solanum lycopersicum*	49.0
6.	CK274615	Cell division protease, ftsH homolog 6, chloroplastic *Arabidopsis thaliana*	35.1
7.	BQ511156	Zinc finger protein 609	32.8
8.	SOLTU024795:Contig1	Aspartic proteinase nepenthesin-2, Nepenthes gracilis	27.3
9.	CK861388	Dehydrodolichyl diphosphate synthase 2	21.4
10.	DN938627	Sucrose synthase OS=*Solanum tuberosum*	14.2
11.	CK272638	S-adenosylmethionine decarboxylase proenzyme OS	11.4
12.	BG890839	RNA-binding protein 42	11.4
13.	SOLTU019884:Contig1	Ammonium transporter 1 member 2, *Solanum lycopersicum*	10.7
14.	CV469808	F-box/LRR-repeat protein 14	10.6
15.	SOLTU008379:Contig2	Superoxide dismutase [Fe], chloroplastic (Fragment) OS=*Nicotiana plumbaginifolia*	10.0
16.	EG009390	Pathogenesis-related leaf protein 6 OS=*Solanum lycopersicum*	8.2
17.	AM906980	Putative late blight resistance protein homolog R1A-3 OS=*Solanum demissum*	2.5
18.	SOLTU015738:Contig1	Un Known function	115.2
19.	CV500170	Un Known function	52.1
20.	EG013050	Un Known function	57.7
21.	SOLTU032752:Contig1	Un Known function	63.4
22.	CK268164	Un Known function	64.9

Table 2. List of selected up regulated genes in *P. infestans* inoculated Kufri Girdhari cultivar validated by RT-PCR.

SN	Selected EST	Closest PGSC accession	Primer Description	Code used in RT-PCR study
1.	SOLTU015738:Contig1	PGSC0003DMG401002349	Unknown protein GCGAGGCGCGAAACAG GATCCGTCTTGGAAAAATTGCT	SOLTU Unknown
2.	BE472671	PGSC0003DMG400001442	Chaperone protein clpB 2 GTGCAAAATATCGCGGTGAA CGGATTCTGTCACTTCCTTAAGC	Chaperone
3.	BG593815	PGSC0003DMG400003530	Abscisic acid and environmental stress-inducible protein AATACGGCAACCAAGACCAAAT CAGTTTCTTGGACATGGTTTCCA	ABA
4.	BQ511156	PGSC0003DMG4000031827	Zinc finger protein 609 CGGAAATGGAGATGGCTGATA CCGCAGGCGCATCAGT	ZFR
5.	CN463702	PGSC0003DMG400030341	17.6 kDa class I heat shock protein TGGAGAAGGAAGACAAGAACGAT TCATGAATTTCCCGCTGCTT	HSP17
6.	EG013368	PGSC0003DMG400020718	22.7 kDa class IV heat shock protein GGAGAATATTGAAGGTGAGAAATGG GGAAGCTTAAACTGTCTCCAGAACTT	HSP22
7.	SOLTU001603:Contig10	PGSC0003DMG400003219	Small heat shock protein, chloroplastic TACGCACTCCTTGGGACATTC GGCATGTCGAAACGCATCT	SHSP
8.	SOLTU008379:Contig2	PGSC0003DMG400027577	Superoxide dismutase [Fe], chloroplastic (Fragment) CCTCACCATCGACGTTTGG GGTCGCCGATTCTGAAA GTC	SOD

were up-regulated ≥ 2-fold at 72 h of post-inoculation stage and statistically significant (Fig. 1b) (Supplementary file S1), while 2,318 genes were down-regulated (Supplementary file S2). These collective clusters of up-regulated genes were further used for k-means cluster analysis and functional classification of individual clusters. Expression levels of the total up-regulated (#2,344) genes were: 1845 genes by 2-4.9 fold, 317 genes by 5-10.9 folds, 151 genes by 11-50 fold, 26 genes by 51-100 fold and 5 genes at >100 fold. Our study revealed 17 candidate transcripts (mainly up-regulated) that were likely to be involved in disease resistance; out of 2,344 up regulated transcript, 951 transcripts were assigned as unknown function, in which 5 transcript showed >50 fold induction are an interesting pool for further analyses (Table 1).

Cluster analysis of microarray data

The up-regulated 2344 genes at post-inoculation stage were grouped into 8 clusters and heat map for various clusters is shown in Fig. 2. It also shows the global gene transcription profiles of potato leaf during interactions with *P. infestans.* Further, k-mean cluster analysis of each eight clusters (1 to 8) revealed expression value as presented in the form of graphical view (Fig. 3). Cluster-wise genes contents are listed in the Supplementary file S3-S10 for each corresponding cluster. Genes grouped within a cluster share a relatively similar expression profiles, however, quantitative and qualitative expression differences among members of the same cluster are likely to occur. Among the eight gene clusters (1-8), cluster 2 and 3 consisted of the maximum up-regulated genes 423 and 1471, respectively in response to *P. infestans* interactions.

Functional categorization of identified genes

The up-regulated (#2,344) and down-regulated (#2,318) genes were used to identify and/or assign putative functions for *P. infestans* regulated genes. The differentially expressed up-regulated genes were grouped into 10 functional categories, excluding 951 unknown-functional genes. These genes were grouped into categories like: i) metabolism (26%), ii) stress (14%), iii) protein with binding function (13%), iv) transcription related (10%), v) signal transduction (10%), vi) protein synthesis (9%), vii) cellular transport (8%), viii) development (4%), ix) defence (4%) and cellular organization (2%) (Fig. 4). Majority of up-regulated genes seem to be related to five major biological changes, including metabolism, stress, protein with binding function, transcription and signalling. Specifically, up-regulated genes in the stress category included small heat shock protein family (17.7 Kda, 22.2 Kda, 70 Kda, 80 Kda and 90 Kda), chaperones, co-chaperone molecule protein phosphatase 2C, ABA and stress inducible protein and glutathione S transferase, and glycine rich protein. Transcription related genes included zinc finger (MYND type) and (C3HC4-type) RING finger family proteins, heat shock protein transcription factor, NAC domain as well as WRKY DNA-binding protein, MYB domain protein 144, NAC domain containing protein 22, basic helix-loop-helix (bHLH) protein- related, and ethylene-response factor 1. Genes involved in signal category included calcium, calcyclin and F-BOX/LRR domain containing protein, diacylglycerol kinase, serine/threonine protein kinase and ethylene response sensor 1. Whereas, genes related to cellular transport category involved ABC family transporter, sodium and hydrogen exchanger. Genes involved to pathogenesis related family proteins, acidic

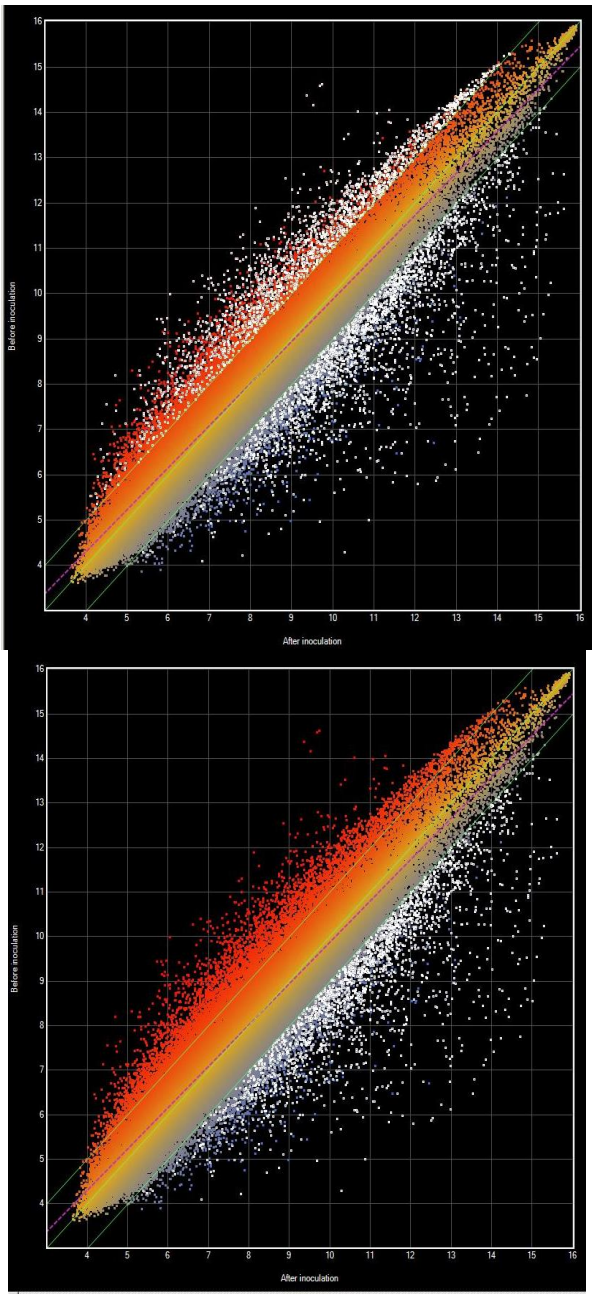

Fig 1. Scatter plots of gene differentially expressed in potato cv. Kufri Girdhari at pre- and post-inoculation stages. A total of 4,662 genes were statistically significant ($p \leq 0.01$) at 2 fold change (a), of which 2,344 genes were up-regulated genes (b) at the same statistical significance.

endochitinase, TIR-NBS class disease resistance proteins, stress response proteins, peroxidases, and cell wall-associated protein, cell division protease, cysteine protease were also up regulated. Those down-regulated genes belonged to the protein with binding functions includes auxin binding protein which was found to be highly suppressed during pathogen interaction, whereas stress related protein such as glycine rich binding protein, defense molecule such as osmotin, patatin, proline rich protein and some of the pathogenesis related proteins and defense with protein synthesis molecule such as aspartic proteinase inhibitor, cysteine protease inhibitor and some forms of aspartic protease were also suppressed during pathogen interaction.

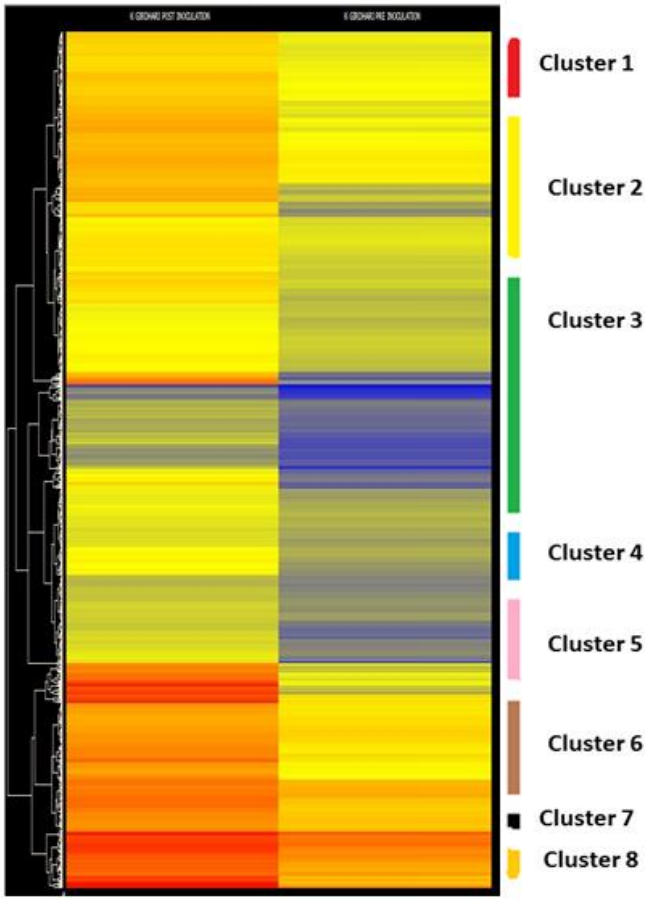

Fig 2. Heat map profiles of statistically significant genes (#4,662) using cluster analysis showing 8 clusters containing expression profile of various genes for each gene.

Microarray data validation through RT-PCR

To evaluate the validity of the microarray results in an independent manner, the expression patterns of selected eight ESTs genes were examined by RT-PCR. These genes represented different functional categories and regulation patterns by *P. infestans* infection. For example, the gene encoding chaperone activity i.e., heat shock protein family was induced >60 fold at-post inoculation compared to pre-inoculation, whereas ABA and stress inducible protein and superoxide dismutase (SOD) showed up-regulation at 10-30 fold by *P. infestans* interaction. Selected closest accession of potato gene from PGSC (Potato Genome Sequencing Consortium) database and the designed primer detail and gene accession ID are mentioned in Table 2. Our results indicated that the overall profiles of gene expression in RT-PCR analysis were similar to that revealed by the microarray data for the selected genes (Fig. 5).

Discussion

The up-regulated genes during pathogen and host interactions represent a prime component to understand the molecular events of host defense/resistance mechanism. Based on the global expression changes of 2,344 up-regulated genes after *P. infestans* infection, the distinct stages of expression changes could be discriminated. Our results classified the up-regulated genes into 8 clusters according to their functionality expression patterns. Most of the genes in cluster 2 and 3 are associated with defense signalling pathway and which mainly includes stress, binding protein genes, transcription and signalling genes. Their strong increase at 72 h post-infection may be accompanied by a highly destructive necrotrophic phase and huge metabolic changes. Other set or cluster covered all catalogues of genes identified and their gradually increased expression pattern reflected a defense process in potato to the infection of *P. infestans*, which obviously employed comprehensive mechanisms. Most of the genes assigned to the clusters were either disease-defense-related or stress related or involved in the signal transduction metabolic pathways, which strongly expressed after pathogen interactions, indicating a strong response of the plant defense system. The plant defense response comprises a multitude of reactions that are activated upon infection (Hammond-Kosack and Jones, 1996). Not surprisingly, host-transcriptional activity is substantially modulated and redirected over the course of such defense responses (Scheideler et al., 2002). During the plant-pathogen interaction stage, the genes with putative functions of transcription-related, such as heat shock protein transcription factor (SOLTU003634: Contig3, CK245936), zinc finger ring-box protein-like (CO267872) and NAC domain containing protein NAC22 (SOLTU000015: Contig20), transcription factor MYB44 (BI178347), transcriptional activator TAF-1 (SOLTU013388:Contig1) were induced by two to six fold gene expression. This suggests the importance of the transcription elements in activating the defense system during host-pathogen interactions. Our results showed that many genes in potato leaves had been up-regulated by the infection of *P. infestans* that are involved in the whole process of plant defense, from signal transduction to the synthesis of defense related genes and cell wall modification. Resistance (R) proteins in plants mediate recognition of specific pathogen-derived factors called Avirulence (Avr) proteins. Upon Avr perception, R proteins initiate defence responses that limit further pathogen ingress. These responses often result in macroscopically visible cell death, referred to as the hypersensitive response (HR). The majority of R proteins are NB-ARC-LRR proteins, which contain a central nucleotide-binding and hydrolysing domain (NB-ARC) and a C-terminal leucine-rich repeat (LRR) domain (Martin et al., 2003; Tameling and Takken, 2008; Bozkurt et al. 2011). In our study, 14% of the gene encoded stress related genes, among which 17.7 Kda, 22.4 Kda, 70 Kda, 80 Kda and 90 Kda Hsp chaperones (Hubert et al., 2003; Lu et al., 2003; Liu et al., 2004; Fuente et al., 2005) and its co-chaperone molecules like protein phosphatase 1 and 2 (PP1 and PP2 by 2.5 fold) (Pearl and Prodromou, 2006). This has been well demonstrated in tomato (van Ooijen et al., 2010). It is reported that during *Phytophthora* infection process, many of the defense related R gene as well as signalling related genes were also up-regulated, which is noticed in our experimental studies also. Moreover, in the present study, the harmone ABA and stress induced protein were up regulated 9-38 fold by the *P. infestans* interaction. Long known only for its role in abiotic stress tolerance, recent evidence shows that abscisic acid (ABA) also has a prominent role in biotic stress. Although it acts as a negative regulator of disease resistance, ABA can also promote plant defense and is involved in a complicated network of synergistic and antagonistic interactions. ABA hormonal metabolism gene Probable indole-3-acetic acid-amido synthetase (CK861064), was found 4.7 fold change, therefore it is very important to find the importance of hormonal metabolism gene in synthesising or regulation of ABA in response to *Phytophthtora* interactions. Our data demonstrated that a number of defense-

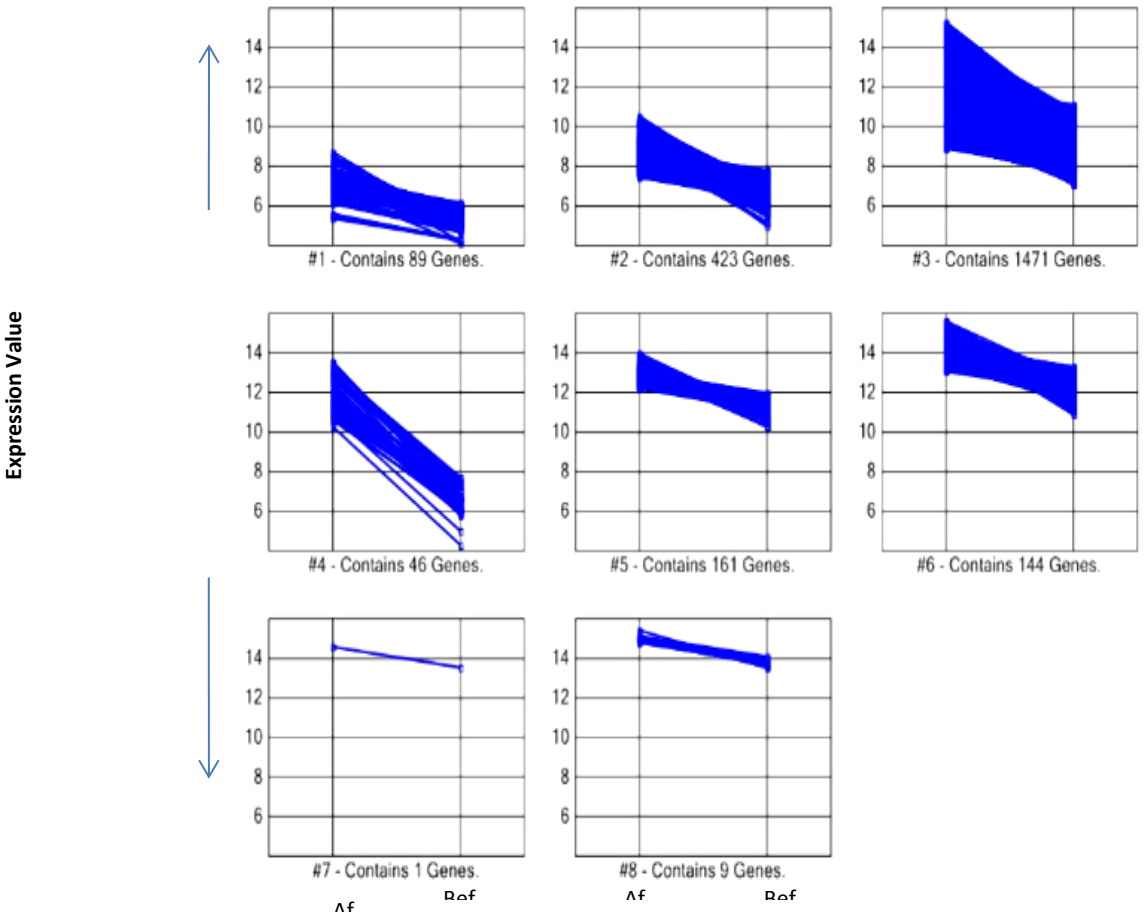

Fig 3. Hierarchical clustering using k-mean analysis of up-regulated genes (#2,344) showing accurate genes counts in each corresponding cluster at post-inoculation stage of *Phytophthora* infection.

related genes of potatoes were up-regulated after infection with *P. infestans*. Some are associated with systemic acquired resistance (SAR), including those encoding 3-8 fold change expression of pathogenesis-related leaf protein 4 (SOLTU009146:Contig4), pathogenesis-related leaf protein 6 (EG009390), basic form of pathogenesis related protein (SOLTU000081:Contig1) and basic endochititnase (CX162048) grouped in cluster 3. Apart from these genes, R genes like putative disease resistance protein (SOLTU017524:Contig1) and putative late blight resistance protein homolog R1A-3 (AM906980) suggesting their function during the host–pathogen interaction, or reached a maximum expression (2 to 5 fold) at post-inoculation stage. This was considered as the strong point of the necrotrophic phase of the infection (Vleeshouwers et al. 2000) and similar results were also obtained from the similar compatible reaction between potato and *P. infestans* (Avrova et al., 2004) and many other pathosystems (Heath, 2000; Boava et al., 2011; Sarowar et al., 2011). Interestingly, Aspartic proteinase, nepenthesin 1 gene (SOLTU016977, SOLTU011402, SOLTU024795: Contig1) was also mainly up regulated in defense related gene category by 4-13 fold than compared to other PR genes. Recent intriguing results suggest possible roles for these enzymes in programmed cell-death of tissues and in pathogen resistance (Simoes et al. 2004; Xia et al. 2004; Muñoz et al. 2010). Interestingly, some of the aspartic proteinase nephenthesin and its inhibitor (CN216774) were also down regulated by > 10 fold. As far we know, this protease activity is mainly involved in protein secretion and cleavage in to specific fold for proper establishment in the host system, which includes family of protein genes to perform the specific functions. Signal transduction genes such as calcium-binding protein gene (SOLTU004975: Contig2), calcyclin-binding protein (9-10 fold) (SOLTU007888:Contig1), ankyrin repeat domain containing protein (2-3 fold) (CV504133), F-box/LRR-repeat protein 14 (CV469808) (4.5-6.5 fold) and homeobox-leucine zipper protein (3.5 fold) (DN907481) were identified as up-regulated genes. Calcium-binding protein may play a role in defense response signaling pathways, because the signal from Ca^{2+} is one of the key signals that initiate general stress and resistance reactions (Wang et al., 2009). Ankyrin repeat family protein gene has been found in a number of eukaryotic proteins covering a wide variety of functions including adaptor/regulatory modules in antioxidation metabolism pathogen defense signal transduction (Wu et al., 2009). Our results confirmed, at least partially, the involvement of these two signals and signal transduction genes in activating and maintaining a defense response to *P. infestans*. The regulation of defense gene expression is largely governed by specific transcription factors. The present research revealed that five genes presumed as transcription factors were strongly up-regulated during pathogen interaction. A gene encoding the NAC-domain protein, NAC22 protein (SOLTU000015: Contig20) NAC 17, 18, 19 was found to be up-regulated by 2.3-4.7 fold by *P. infestans* infection in the present research. This is in accordance with the previous finding showing that NAC domain protein is a family of plant specific transcription factors involved in several aspects of plant development (Kikuchi et al. 2000). Zinc finger protein

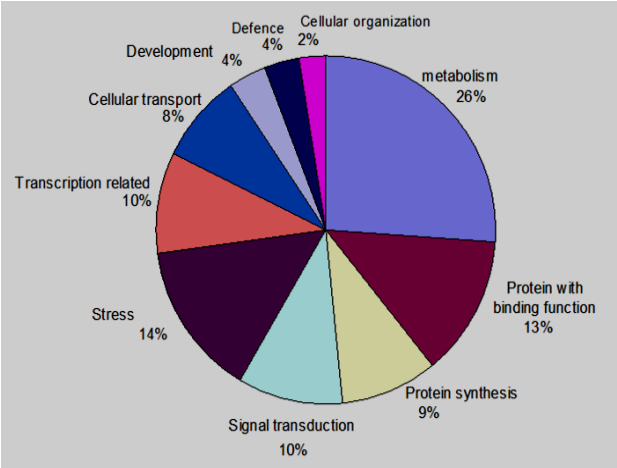

Fig 4. Pie chart showing proportion of *P. infestans*-induced up-regulated genes (#2,344) into ten functional categories based on the Gene Ontology and the uniprot website.

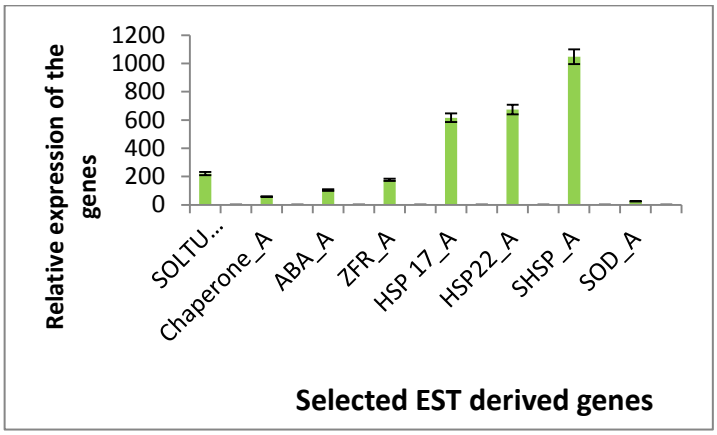

Fig 5. Microarray data validation for selected up regulated genes through RT-PCR showing relative expression of eight selected genes as codes are listed in Table 2. (A: post-inoculation and B: pre-inoculation)

609 (> 30 fold) (BQ511156), CONSTANS-LIKE 1 and CONSTANS-LIKE 2 gene (2-13.7 fold) (BG890762 and SOLTU030260: Contig1) were identified as gradually increasing after inoculation in the present research and this has a pivotal role in regulation of plant defense through chitin and NADPH oxidase mediated pathway. Although assignment of a defensive function to this kind of gene in potato still needs further attention, together with the previous finding that this gene was up-regulated in potato leaves challenged by an incompatible *P. infestans* strain (Ronning et al., 2003), it implicated that the zinc finger/binding-domain gene plays a role in horizontal resistance of potato against *P. infestans*. As far, we know for inducing disease resistance pathway, HR play a major role and act as downstream signalling pathway for enhancing the systemic resistance in crop plants, HR related genes like sodium/hydrogen exchanger (SOLTU006001: Contig2,) superoxide dismutase (Fe), chloroplastic (Fragment) (SOLTU008379: Contig2) were found mainly up regulated by 5.2-10 fold. This has been associated with the bacterial tobacco interaction studies, demonstrated the scavenging of reactive oxygen species (ROS) in cell fate for HR response (Cvetkovska et al., 2012). In this study using potato microarray comprised of 70,083 genes, global expression of transcripts regulated by the fungal pathogen *P. infestans* in potato cv. Kufri Girdhari provided a more comprehensive assessment of gene expression profiles by following infection. These findings provide an overview of the underlying mechanisms related to modulation and regulation of various biochemical pathways in response to *Phytophthora* interactions. However, it should be noted out that some of the genes investigated in this microarray study lack annotation, and therefore their roles in the late blight disease resistance response are yet to be elucidated, which, in turn, may provide further insights into these findings.

Conclusions

Based on distinct expression profiles and functionality assays of late blight resistant potato cultivar Kufri Girdhari, chaperones mediated distinct expression profiles of various defense related genes information were observed. These chaperone signalling was a significantly overpresented pathway among differentially expressed genes in response to *Phytophthora* interactions. Since stressfull stimuli, chaperone receptor controls the stability and functionality of R genes through regulation of protein holding or folding pathway, these genes may act as downstream signalling for defense related pathway to confer plant adaptation to late blight resistance. We have identified the transcript level of numerous ABA and stress inducible protein, zinc finger transcription factor, heat stress transcription factor, defense related protease and pathogenesis related genes which were found to be highly up-regulated. In contrary to up-regulated genes such as protease inhibitor, auxin binding protein and other RNA binding protein were found to be highly suppressed at post-inoculation stage of *Phytophthora* interactions. However, these results should be followed up by functional studies of differentially expressed candidate gene as well as mapping of late blight resistance in other cultivar of potato.

Materials and methods

Plant material

In the present study, a late blight resistant India potato cv. Kufri Girdhari was used for microarray-based gene expression/transcriptome analysis. Tubers were planted in earthen pots (20 × 25 cm^2) containing a sterile mixture of soil: FYM-based compost (1:1, v/v) in a greenhouse under an average of 14 h day length, with a mean temperature of 20 ºC in day and 15 ºC in night. Six weeks old potato plants were utilized for the study. Plants were sprayed with zoospore suspension of *P. infestans* (4×10^4 zoosporangia/ml) in artificial *in vitro* screening chamber. Leaf samples were collected at pre- and post-inoculation stages at 0 h (water sprayed plant as a control) and 72 h after inoculation of *P. infestans*, respectively. Samples were snap frozen in liquid nitrogen and stored at -80^{o}C till further use.

Oligonucleotide microarray construction

Whole genome EST array for potato was custom designed and synthesized by Roche NimbleGen, Inc., Madison, USA. The 1,38,938 probes were designed targeting 72,083 ESTs derived from SolEST database (http://biosrv.cab.unina.it/solestdb/; D'Agostino et al., 2009). The 72,083 cDNA clones for microarray fabrication were selected as unique EST sequences with function of adversity resistance to biotic or abiotic stresses.The arrays were of 12x135K format, which contained 12 arrays on a single slide and each array could typically contain 1,35,000 probe features.

RNA preparation and fluorescent labelling of probes

Total RNA was extracted from leaf tissues (pre- and post-inoculation samples) using RNeasy Plant Mini Kit (Qiagen). Total RNA was reverse transcribed into double stranded cDNA by using the cDNA synthesis system (Roche). The cDNA was further labelled with Cy3 before hybridization using One-Color DNA labeling Kit (Roche NimbleGen) following the manufacturer's instructions.

Microarray hybridization, washing, and scanning

Using random assignment, each Cy3-labeled cDNA sample was applied to custom expression microarray. The samples were hybridized to the array for 16 hours at 42°C on a Hybridization System (Roche NimbleGen) followed by washing and subsequent drying on MicroArray dryer (Roche NimbleGen) as per the manufacturer's recommendation. Hybridized microarray slides were scanned at 2 µm resolution, for Cy3 at 532 nm with NimbleGen MS 200 microarray scanner (Roche NimbleGen).

Microarray data analysis

Microarray expression data was extracted from the scanned images of the arrays using NimblScan software v 1.0. A Robust Multiarray Analysis (RMA) was performed as described by Irizarry et al. (2003a; 2003b). In the same software involving background adjustment, quantile normalization and median polish summarization. The resultant calls files contained normalized expression value for each EST interrogated by the array**.**

Functional analysis

Functional analysis of differentially expressing ESTs were performed using ArrayStar software version 4.0.2 The selected ESTs were assigned to different functional groups based on the information gathered from the Gene Ontology website (http://www.geneontology.org).

Real time-PCR

Reverse transcription real-time PCR (RT-PCR) was performed with eight selected genes to validate the microarray results (Table 1). The selected genes were biologically verified by detecting the differences in expression between inoculated and uninoculated plants. Closest homology of selected EST sequences for potato genes were retrieved from Potato Genome Sequencing Consortium (PGSC) database based on sequence homology and used to design the primer for RT-PCR analysis (Table 1). Oligonucleotide primers were designed using Primer Express 2.0 software (Applied Biosystems, California, USA). cDNAs were synthesized from 3.0 µg of total RNA using Multiscribe reverse transcriptase (50 U/µL) with an oligo (dT) primer (High capacity cDNA synthesis reverse transcriptase kit, USA) according to the manufacturer's instructions. cDNA was treated with RNAse H (1µl) for 20 min at 37°C to remove any contaminating RNA. RT-PCR was performed using Power SYBR® Green PCR Master Mix reagent (ABI). The reaction consisted of 150 ng of cDNA and 120 nM of each gene-specific primer in a final volume of 15µL. Amplification was carried out for three technical replicates for each sample, including negative controls. An ABI PRISM HT7900 (ABI) was used for the following thermal cycles: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min. Expression levels were assessed based on the number of amplification cycles needed to reach a common fixed threshold (cycle threshold - Ct) in the exponential phase of PCR. For relative quantification, the $2^{-\Delta\Delta CT}$ method between conditions in RT-PCR was applied.

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