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Monitoring gene expression pattern in somatic hybrid of *Solanum tuberosum* and *S. pinnatisectum* for late blight resistance using microarray analysis

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Abstract

Gene expression pattern was investigated in late blight resistant potato somatic hybrid P-7 and susceptible control C-13 by microarray. cDNA microarray analysis was performed with total RNA isolated from the leaf tissues collected at disease appearance stage (72 h of post-inoculation (hpi)) after challenge inoculation with *P. infestans*. A t-test analysis identified a total of 5,810 statistically significant genes ($p \leq 0.05$), of which 2,101 genes (≥ 2-fold) were up-regulated and 3,709 genes were down-regulated. Among the up-regulated 2101 genes, the GO (gene ontology) annotation of 320 up-regulated genes (≥ 10-fold) revealed that 66 genes were GO annotated with known PGSC gene description, whereas GO annotation was not found for remaining 254 genes. Further GO analysis showed that the 66 GO-annotated genes are involved in defence response, binding function, oxidation-reduction, photosynthesis, metabolic process, tRNA processing, protein phosphorylation and methylation. The real-time polymerase chain reaction (RT-PCR) analysis of ten selected genes corroborated with the microarray results. Here, we showed an association of defence responsive genes play a key role in late blight resistance mechanism in P-7. These genes belong to disease resistance proteins (NBS-LRR class, WRKY transcription factor and MADS-box protein, zinc knuckle family protein), binding protein, patatin-01 protein, carbohydrate/lipid metabolism and oxidation-reduction/photosynthesis processes. Our findings suggested a broad spectrum of candidate genes that provide comprehensive insights into the underlying resistance mechanism against late blight in potato somatic hybrid P-7.

Keywords: Gene expression, Late blight resistance, Microarray, Potato somatic hybrid, RT-PCR, *Solanum tuberosum*, *S. pinnatisectum.*
Abbreviations: GO_gene ontology; NBS_nucleotide-binding site; LRR_leucine-rich repeats; RT-PCR_real-time polymerase chain reaction.

Introduction

Late blight, caused by the oomycete fungus *Phytophthora infestans* (Mont.) de Bary, is the most devastating disease of the potato crop. This pathogen has worldwide distribution and severe epidemic is favored by the moist and cool environment with temperature (15-20 °C) (Fry, 2008). The extensive use of fungicides has considerable success in controlling this disease. However, the rapid evolution of new strains of *P. infestans* has declined the efficacy of fungicides application. Hence, an alternate strategy to identify novel genes from the wild sources and their deployment is inevitable to provide durable resistance to the potato crop against late blight. Many late blight resistance genes have been identified/known in cultivated/semi-cultivated and wild potato species such as *Solanum demissum, S. stoloniferum*, *S. bulbocastanum, S. tuberosum* subsp. *Andigena*, *S. phureja* to name a few (Tiwari et al., 2013; 2015a). Although many interspecific potato somatic hybrids have been developed for late blight resistance using wild species such as *S. pinnatisectum* (Sarkar et al., 2011) and *S. cardiophyllum* (Chandel et al., 2015), genes conferring late blight resistance in these somatic hybrids are still unknown. Therefore, in this study we aimed to identify genes in our previously developed somatic hybrids between *S. tuberosum* dihaploid 'C-13' and wild species *S. pinnatisectum*. From the last decade, DNA microarrays constitute a robust, valuable and known high-throughput platform to study gene expression patterns on a large scale. Several microarray-based studies have been reported in potato for late blight resistance (Ros et al., 2004; Restrepo et al., 2005; Siddappa et al., 2014), tuberization (Shan et al., 2013; Tiwari et al., 2015b), flavonoid (Stushnoff et al., 2010), heat-stress (Ginzberg et al., 2009), drought (Lucca et al., 2011), starch metabolism (Ferreira et al., 2010) to name a few. In particular, transcripts expression profiles have been monitored to elucidate genes associated with late blight resistance (Wang et al., 2005). In another study, Lindqvist-Kreuze et al. (2010) compared transcript profiles in late blight-challenged *Solanum cajamarquense* and B_3C_1 potato clones and activated genes were involved in defence pathways. Recently, Ali et al. (2014) investigated compatible and incompatible interactions between *P. infestans* and potato and identified over 17000 transcripts. In this study, we aimed to analyze gene expression patterns in a known late blight resistant somatic hybrid P-7 and susceptible control 'C-13' by microarray. Pot-grown plants were sampled at disease appearance (at 72 hpi) stage after challenge inoculation by *P. infestans* under controlled conditions. Microarray analysis was performed using chips designed based on the ~39,031 protein-coding genes of the potato genome and selected genes were validated by RT-PCR analysis.

Results

Gene expressions analysis

Microarray analysis in somatic hybrid P-7 and control C-13 revealed a set of differentially expressed genes for late blight resistance (Table1, Supplementary Tables S1 and S2). A t-test analysis identified a total of 5,810 genes that were differentially expressed and statistically significant (at $p \leq 0.05$), of which 2,101 genes were up-regulated (≥ 2-fold), whereas 3,709 genes were down-regulated (< 2-fold) in P-7 vs. control C-13. Furthermore, 320 genes were highly up-regulated (at ≥10-fold) in P-7 (Fig. 1) and therefore selected for further analysis. A few important genes identified in this study are presented in Table 1.

GO annotation

In general, the GO terms are classified at three levels namely biological process (P), molecular function (F) and cellular component (C). Out of 320 up-regulated genes (at ≥10-fold), 66 genes were GO annotated (Supplementary Table S1), whereas no annotation was found for 254 genes (Supplementary Table S2). Accordingly, based on the GO annotations, the GO-annotated 66 genes were grouped into seven sets namely: (1) defence response (9), (2) binding function (12), (3) oxidation-reduction and photosynthesis (14), (4) metabolic process (16), (5) tRNA processing (5), (6) protein phosphorylation (8), and (7) methylation (2). Besides, the GO-unannotated 254 genes with known/unknown PGSC descriptions and Uniprot ID are presented in Supplementary Table S2. A few selected genes of are briefly described here.

Set 1 was enriched with 9 up-regulated genes (10 to 49-fold) dedicated to defence response in P-7 against late blight (Supplementary Tables S1). Maximum expression value was observed in pathogenesis-related protein PR1 (PGSC0003DMG400005111; 49.54-fold) followed by SNKR2GH5 protein (PGSC0003DMG400011920; 26.89-fold). Many other defence responsive genes conferred resistance to late blight in P-7 were Sn-1 protein (PGSC0003DMG400002874), disease resistance protein BS2 (PGSC0003DMG 401002242), Hcr2-0A (e.g. PGSC0003DMG402025507), Hcr9-OR2A (PGSC0003DMG 400006652), Hcr2-5D (PGSC0003DMG401025507), Hcr2-0B (e.g. PGSC0003DMG 400023864), late blight resistance proteins (e.g. PGSC0003DMG400022876), disease resistance proteins (e.g. PGSC0003DMG 400015349), NB-ARC domain containing proteins (e.g. PGSC0003DMG 400046783). In addition, several transcription factors namely WRKY domain (PGSC0003DMG400025481), MADS-box protein (PGSC0003DMG 400042019) and transcription factor hy5 (PGSC0003DMG400042745); zinc knuckle family protein (e.g. PGSC0003DMG 400041706); and elongation factor 1-alpha (PGSC0003DMG 400016556) were involved during host-pathogen interaction to confer resistance in P-7. In Set 2, twelve genes were identified for metal ion/nucleic acid binding functions and implicated to late blight resistance mechanism in P-7 (Table 1). A few selected genes included histone H4 (PGSC0003DMG400023522), ATP-binding protein (PGSC0003DMG400026433), 'chromo' domain containing proteins (e.g. PGSC0003DMG400027834), gag-pol polyproteins (e.g. PGSC0003DMG400045596), Bcl-2-associated athanogene (PGSC0003DMG400010462), DNA-directed RNA polymerase subunit alpha (PGSC0003DMG400003335) to name a few (Supplementary Table S1). Besides, several genes were involved in lipid and carbohydrate metabolism to provide late blight resistance in P-7. They were associated with oxidation-reduction and photosynthesis (Set 3) and carbohydrate/lipid metabolism (Set 4) (Supplementary Table S1). A few of selected genes in Set 3 were lipoxygenase (PGSC0003DMG400019709), chlorophyll a/b binding proteins (e.g. PGSC0003DMG 400013414), cytochrome P450 (PGSC0003DMG 400009814), ferredoxin II (PGSC0003DMG400037286) and NADH dehydrogenase subunit 5 (PGSC0003DMG 400000928) (Supplementary Table S1). Set 4 included 16 up-regulated genes (10-23 fold) were few selected genes were phospholipase A1 (PGSC0003DMG401010218), beta-glucosidase (PGSC0003DMG 400038064), proline-rich protein 1 (PGSC0003DMG400029700) and patatin-01 (PGSC0003DMG 400017091) (Supplementary Table S1). Besides these, several genes associated in tRNA processing (Set 5), protein phosphorylation (Set 6) and methylation (Set 7) processes that played an important role during defense response of P-7 (Supplementary Table S1).

RT-PCR analysis

Ten highly up-regulated genes were selected and validated through RT-PCR analysis. The RT-PCR experimental data showed that 10 genes were differentially expressed during the *P. infestans* infection. Our results showed similar gene expression patterns like the microarray results (Table 2 and Fig. 2).

Discussion

To understand host-pathogen interaction, transcripts expression was analyzed by microarray in a late blight resistant somatic hybrid P-7 and a susceptible control C-13 after challenge inoculation with *P. infestans*. A set of differential expression of 5,810 genes showed that 320 genes (≥ 10-fold) were highly up-regulated in P-7 and provides a new insight into understanding the late blight resistance mechanism in P-7. Given that after initial pathogen recognition, there are various signaling events, which led to the activation of the plant defence mechanism by recognizing the pathogen-derived effector molecules on the gene-for-gene concept. Moreover, it is known that plant metabolism and development are one of the largest groups of genes activated during the pathogen infection (Schenk et al., 2000) and a wide array of multiple reactions is activated during the pathogen attack (Dangl and Jones, 2001). Here, we discuss some selected up-regulated genes for future implications.

First, pathogenesis-related PR1 protein (PGSC0003DMG 400005111) showed the highest expression (49.54-fold) in P-7 for late blight resistance. Importantly, the PR1 protein is characterized as the highly up-regulated gene in potato leaves after challenge inoculation with *P. infestans* (Avrova et al., 2004). Moreover, pathogen-related proteins are often used as the SA marker gene and are abundantly induced in the host plant as a defence response to biotic and abiotic stresses (Loon et al., 2006). Furtherr, Vleeshouwers et al. (2000) have demonstrated a basal expression correlation level of SAR marker genes result in enhanced resistance to late blight. In addition, the resistance genes (SNKR2GH5 protein: PGSC0003DMG400011920; Sn-1 protein: PGSC0003DMG 400002874) activate the hypersensitive response and result in localized cell and tissue death around the site of infection (Hammond-Kosack and Jones, 1997). In plants, the nucleo-tide-binding site (NBS)- leucine-rich repeats (LRR) domains resistance proteins form the largest class of genes (Bozkurt et al., 2011) and play a key role in P-7 for defense response

Table 1. A few selected up-regulated genes identified in somatic hybrid P-7 for late blight resistance by microarray analysis.

SN	Gene ID	PGSC description	Fold change[a]	*p* value	Uniprot ID	GO annotation[b]	Gene set/category
1.	PGSC0003DMG400005111	PR1 protein	49.54	0.05	M1A2A4	GO:0005576 (extracellular region) (C)	Defense response
2.	PGSC0003DMG400011920	SNKR2GH5 protein	26.89	0.05	M1AV76	GO:0043531 (ADP binding) (P)	Defense response
3.	PGSC0003DMG400002217	NBS-LRR resistance protein	15.42	0.05	M0ZQ85	GO:0043531 (ADP binding) (F)	Defense response
4.	PGSC0003DMG400002874	Sn-1 protein	10.39	0.05	M0ZT12	GO:0006952 (defense response) (P)	Defense response
5.	PGSC0003DMG401002242	Disease resistance protein BS2	10.00	0.05	M0ZQC7	GO:0043531 (ADP binding) (F)	Defense response
6.	PGSC0003DMG402025507	Hcr2-0A	25.87	0.05	M1CE91	NA	NA
7.	PGSC0003DMG400041706	Zinc knuckle family protein	10.84	0.05	M1DP59	GO:0003676 (F)	Defense response
8.	PGSC0003DMG400016556	Elongation factor 1-alpha	11.32	0.05	M1BDH0	GO:0005525 (GTP binding) (F)	Defense response
9.	PGSC0003DMG400025481	WRKY domain class transcription factor	10.30	0.05	M1CE46	GO:0003700 (sequence-specific DNA binding transcription factor activity) (F)	Defense response
10.	PGSC0003DMG400042019	MADS-box protein	17.82	0.05	M1DPU0	NA	NA
11.	PGSC0003DMG400026433	ATP binding protein	15.26	0.05	M1CI84	GO:0000166 (nucleotide binding) (F)	Binding function
12.	PGSC0003DMG400027834	'chromo' domain containing protein	14.60	0.05	M1CNY4	GO:0003676 (nucleic acid binding) (F)	Binding function
13.	PGSC0003DMG400045596	Gag-pol polyprotein	11.42	0.05	M1DXJ8	GO:0003676 (nucleic acid binding) (F)	Binding function
14.	PGSC0003DMG400023522	Histone H4	17.81	0.05	M1B9C1	GO:0003677 (DNA binding) (F)	Binding function
15.	PGSC0003DMG400017091	Patatin-01	12.22	0.05	M1BFJ1	GO:0016787 (hydrolase activity) (F)	Metabolic process

Note: This table indicates only a few selected genes from a total of 2101 up-regulated genes differentially expressed in leaves of P-7 and C-13. Due to space limitation all up-regulated genes list are mentioned in the Supplementary file Tables S1 and S2. [a] Fold change represents genes that were differentially expressed between P-7 and C-13. [b] GO, Gene Ontology; the GO ID/category is given in parenthesis: (P), biological process; (F), molecular function; (C), cellular component. NA: not available.

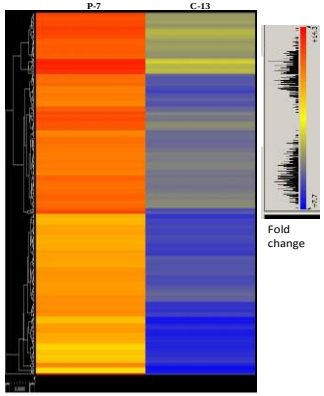

Fig 1. Heat map profiles of differentially expressed and statistically significant ($p \leq 0.05$) 320 gene up-regulated at ≥ 10 fold change between late blight resistant P-7 and susceptible control C-13 after challenge inoculation with the pathogen *P. infestans*.

Table 2. Details of selected up-regulated genes ($p \leq 0.05$) and primer sequences for validation through RT-PCR analysis.

SN	PGSC gene ID	PGSC Description	Fold change*	Sequence (5'→3')
1.	PGSC0003DMG400005111	PR1 protein	49.54	F: GTGTGGCGTAACTCGGTACGT
				R: AAAATACCACCCGTTGTTGCA
2.	PGSC0003DMG400011920	SNKR2GH5 protein	26.89	F: CCGTGATCTTCCGTCTTCCA
				R: TCCACCCGTATCGACAACAA
3.	PGSC0003DMG402025507	Hcr2-0A	25.87	F: TCAAACAACAAATTCCAAGGACATA
				R: CAGTACCCGAATCGCAATGA
4.	PGSC0003DMG400020533	Protein ycf2	25.36	F: GGGCAAGCGGATCATTTATG
				R: TGCAAGAACCCCGAATCATT
5.	PGSC0003DMG400014738	unknown function	25.15	F: AGCTTTGATCCCACGAGTTTTC
				R: CTGCAGTTCACCAAGAGTGGAA
6.	PGSC0003DMG400025595	Kinase	23.56	F: AGTATTTGCATCGAGGCTGTGA
				R: AAAGGAGTGTTGTGTGGCTTGA
7.	PGSC0003DMG400020534	Protein ycf2	23.47	F: CCTTCTTCTTGTTGCTGGATATCTC
				R: CACTAGAGGCCCGGGAAAC
8.	PGSC0003DMG401010218	Phospholipase A1	22.95	F: GAGAACCTTTCAGCTGGCATTT
				R: CTAAACCTTGAGTGCCAGCAACT
9.	PGSC0003DMG400015345	unknown function	22.95	F: TGACTCGTATGGCGGTAACCT
				R: CACTCGTTCCACATTGCTCTGA
10.	PGSC0003DMG400015347	unknown function	22.29	F: GCTCACAATGCCTTCTTTTCTTC
				R: TGCCTCAAATTCGTCAATCG

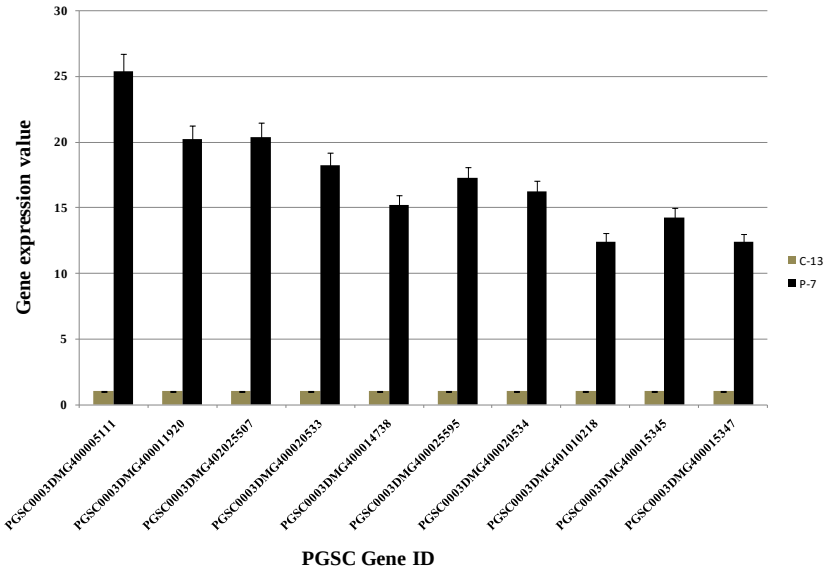

Fig 2. Real-time PCR validation of the ten up-regulated genes showed conformity of microarray results for the gene expression in late blight resistant P-7 and susceptible control C-13.

against late blight. Besides, several up-regulated disease resistance genes such as BS2 (PGSC0003DMG401002242) and late blight resistance proteins (e.g. PGSC0003DMG400022876) containing NBS-LRR domains restrict pathogen growth by hypersensitive response (van der Vossen et al., 2003). These resistance proteins contain NBS-LRR domains, NB-ARC (PGSC0003DMG400046783), P-loop containing nucleoside triphosphate hydrolase (P-loop NTPase) that confer resistance to bacterial, viral and fungal pathogens. It is observed that P-loop NTPases plays various roles in programmed cell death, disease and stress response in plants (Leipe et al., 2004). Most resistance proteins contain a central nucleotide-binding domain called NB-ARC consists of three sub-domains: NB, ARC1, and ARC2. The NB-ARC domain is a functional ATPase domain, and its nucleotide-binding state is proposed to regulate the activity of the R protein (van Ooijen et al., 2008). Another *R* genes class that encodes cytoplasmically localized proteins containing a predicted nucleotide binding site and multiple LRRs near the C-terminus. In this study, several *Hcr* genes (e.g. PGSC0003DMG402025507) were highly expressed in P-7 in response to the pathogen. The *Hcr* genes (for homologs of *Cladosporium* resistance gene *Cf* of tomato) confer race-specific resistance to the leaf mold pathogen and encode membrane-anchored proteins largely composed of extracytoplasmic LRRs (Dixon et al., 2008).

Second, many genes were involved in transcription factors during pathogen interaction for resistance mechanism in P-7. For example, the WRKY domain (PGSC0003DMG400025481) constitutes a major family of plant transcription factors and regulates various processes like plant development, senescence and resistance against biotic and abiotic stresses (Huang et al., 2012). In general, the WRKY transcription factor plays a key role as repressors as well as activators in plant developmental process (Rushton et al., 2010). Higher expression of transcription related WRKY genes has been reported during the compatible interaction of potato and *P. infestans* (Dellagi et al., 2000). In addition, zinc finger proteins (PGSC0003DMG400041706) are among the most abundant proteins in eukaryotic genomes. Zinc finger structures are diverse in functions that include DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (Laity et al., 2002). The MADS-box transcription factors (PGSC0003DMG400042019) play important in plant growth and developmental process. Recent studies on MADS-box family genes showed their involvement in stress resistance in addition to their growth and developmental functions in *Brassica rapa* (Saha et al., 2015).

Last, numerous genes were involved in binding functions during host-pathogen interaction provide resistance to P-7, such as PGSC0003DMG400026433, PGSC0003DMG400027834 and PGSC0003DMG400045596. In potato, most of the chromo domains and gag-pol polyproteins containing proteins have a CCHC zinc finger domain playing broader functions in zinc ion and nucleic acid binding (Lawrencea et al., 2014). The chromo domain containing proteins (e.g. PGSC0003DMG400027834) are highly conserved in plants that include a chromo box motif (Exner et al., 2009). The integrase core domain containing protein contains the domain P-loop NTPase fold, which plays an essential role in biological processes such as programmed cell death, disease and stress response in plants. The histone H4 (PGSC0003DMG400023522) is a core histone protein present in the nucleus and plays an essential role in DNA binding. Importantly, patatin-01 (PGSC0003DMG400017091) is the family of a glycoprotein having a patatin domain playing an important role in various metabolic processes. The patatin proteins encode a major storage protein in potato and posses enzymatic function as lipid acyl hydrolases, which play essential roles in the plant defence and antioxidant activities (Liu et al., 2003).

Materials and Methods

Plant materials

In this study, late blight resistant potato somatic hybrid P-7 and susceptible control C-13 were used. P-7 was developed earlier by protoplast fusion between *Solanum tuberosum* dihaploid 'C-13' and diploid wild species *S. pinnatisectum* (Sarkar et al. 2011). This highly resistant clone P-7 was selected based on the previous study, as registered to the National Bureau of Plant Genetic Resources (NBPGR), New Delhi (Sarkar et al., 2013). In vitro propagated plants were multiplied and maintained in the Division of Crop Improvement of ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh, India.

Sampling for late blight resistance

In vitro-raised plants (in triplicates) of P-7 and C-13 were grown in the pots (20 × 25 cm^2) containing a sterile mixture of soil:farm yard manure-based compost (1:1, w/w) in a glasshouse at ICAR-CPRI, Shimla. Six-weeks-old plants were used for late blight resistant assay under the controlled conditions (18±2 °C temperature and 80–90 % relative humidity) after challenge inoculation with *P. infestans* isolate HP09/40 (A2 mating type and races 1.2.3.4.5.6.7.8.9.10.11) as standard procedures described by Tiwari et al. (2015a). The leaf samples were collected at 72 h on disease appearance. These samples were snap frozen in liquid nitrogen and stored at -80 °C until use.

RNA isolation and microarray analysis

Total RNA was isolated from the leaf tissues of P-7 and C-13 using RNeasy plant mini kit (Qiagen, Venlo, Limburg, Netherlands). The RNA quality was verified by agarose gel electrophoresis and further concentration and purity (260/280 nm >1.8) was checked by a ND-1000 NanoDrop (Wilmington, USA). RNA was checked for quality by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A total of 39,031 genes from the potato genome sequence (Potato Genome Sequencing Consortium, 2011) (http://potato.plantbiology.msu.edu/ index.shtml) were used for cDNA microarray analysis on NimbleGen Systems (NimbleGen, Madison, WI) platform following detailed procedures are described elsewhere by Tiwari et al (2015b). For each of the 39,031 genes, 60-mer oligonucleotides probes with a minimum of three probes per gene were synthesized on the microarray slide. The array used in the experiment was 12 × 135K format, which contained 12 arrays on a single slide (25 × 76 mm) and each array typically included 135,000 probes (remaining feature were filled with more replicates of the probes in a random manner). The feature size of the microarrays was 13 × 13 μm with an array size of 8.9 × 6.5 mm. Three technical replicates (same RNA samples onto 3 arrays) of each sample (P-7 and C-13) were used for microarray analysis.

Data analysis and GO characterization

Microarray image analysis was performed with NimbleScan software v1.0 as described by Tiwari et al (2015b). A *t*-test analysis was performed at $p \leq 0.05$ to analyze the differentially expressed genes in the leaf tissues of P-7 and control C-13 using ArrayStar software version 4.0.2. Gene description was recorded from PGSC (http://potato.plantbiology.msu.edu/ index.shtml), Uniprot (http://www.uniprot.org/), and GO functional characterization by the QuickGO search (http://www.ebi.ac.uk/QuickGO/).

RT-PCR analysis

Ten selected up-regulated candidate genes identified by microarray were validated through RT-PCR. The selected potato gene sequences were retrieved from PGSC database to design gene-specific primer pairs (Table 2). Total RNA, as in microarray, were processed for RT-PCR analysis using Power SYBR Green PCR Master Mix (Part No. 4367659, Applied Biosystems Warrington, UK) on the ABI PRISM HT7900 following thermal cycler profiles 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, 60 °C for 1 min, 72 °C for 30 s as described in Tiwari et al. (2015b). RT-PCR data were also analyzed as described elsewhere Tiwari et al. (2015b). An internal standard constitutively expressed gene *β-tubulin* in potato (Taylor et al., 1994) as used in RT-PCR.

Conclusion

In summary, we analyzed microarray-based gene expression patterns, which provide valuable knowledge on the genes controlling late blight resistance in an interspecific potato somatic hybrid. Our study showed an association of up-regulated defence response genes belonged to disease resistance proteins (NBS-LRR class, WRKY transcription factor and MADS-box protein, zinc knuckle family protein), binding proteins and patatin-01 protein play key roles in late blight resistance mechanism in P-7. In addition, several genes dedicated to carbohydrate/lipid metabolism and oxidation-reduction/photosynthesis process might be involved in resistance. Thus, a comprehensive analysis of differentially expresses genes in P-7 led to a better understanding of the molecular processes involved in late blight resistance. The identified late blight resistance genes in this study would deepen our knowledge to uncover the resistance mechanism in potato.

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