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SHORT NOTES

Identification of genes differentially expressed in onion infected with *Iris yellow spot virus*

Ahmed ABDELKHALEK[1], Adel ELMORSI[2], Omar ALSHEHABY[2], Neeti SANAN-MISHRA[3] and Elsayed HAFEZ[1]

[1] Plant Protection and Bimolecular Diagnosis department, ALCRI, City of Scientific Research and Technological Applications, Alexandria, Egypt
[2] Botany Department, Faculty of Science, Mansoura University, Mansoura, Egypt
[3] Plant RNAi Biology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India

Summary. *Iris yellow spot virus* (IYSV) causes severe damage and economic losses in onion production. Differential display-PCR was used to study changes in the gene expression of IYSV-infected onion plants. Representative up-regulated and down-regulated genes were selected for further study. Based on sequence analysis, the up-regulated genes were identified as retrotransposon protein, disease resistance-like proteins, chitinase, pathogenesis-related protein, cytochrome oxidase, cytochrome c, pentatricopeptide repeat-containing protein and pectin methylesterase. A DNA-binding transcriptional repressor protein gene was greatly down-regulated. . Most of the identified genes are known to play essential roles in plant defence systems, and are newly identified in onion sequences.

Key words: DD-PCR, defence system, IYSV, up/down-regulated genes.

Introduction

Onion (*Allium cepa* L., family *Alliaceae*, order *Asparagales*) is one of the most economically important crops (Vitte *et al*., 2013; ElMorsi *et al*., 2015). Onion diseases can range from insignificant amounts to more than 50%, depending on the location, environmental conditions and the causal agent (Gent and Schwartz, 2008). Among the pathogens infecting onion, *Iris yellow spot virus* (IYSV; genus *Tospovirus*, family *Bunyaviridae*) is prominent. This virus is transmitted by *Thrips tabaci* L. (Kritzman *et al*., 2001), and has spread rapidly in several onion-growing countries (Pappu *et al*., 2009; Mandal *et al*., 2012). The virus can cause up to 100% crop loss because the virus blocks the development of flower heads (Mandal *et al*., 2012).

It is well known that invading pathogens activate plant immune systems, and in many cases, this enables development of host resistance (Hafez *et al*., 2013). Virus infections cause physiological changes, symptoms and eventual yield losses in plants. Once the virus enters the host, it uses the metabolism of host cells to produce multiple copies of itself. Viruses do not always cause disease by killing cells; instead, they take over the metabolic cell processes, resulting in altering the expression of host genes (ElMorsi *et al*., 2015) and abnormal cell functioning (Pfleger and Zeyen, 1991). To understand the molecular basis of specific plant-pathogen interactions, it is important to identify the plant genes that respond to pathogen attack.

A major challenge has been to identify the transcripts of altered mRNA transcription profiles in host plants and decipher how and why the changes are initiated. The goal is to use this information to investigate the functions of genes with altered expression profiles in plant virus interactions. Knowledge of different groups of co-regulated genes will lead to hypotheses on how host cells are manipulated to

Corresponding author: A. Abdelkhalek
E-mail: abdelkhalek2@yahoo.com

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create favourable environments for replication, what defence and stress mechanisms are deployed, or how developmental defects resulting in symptoms are brought about (Teo and Chu, 2014).

The Differential Display-Polymerase Chain Reaction (DD-PCR) technique was developed by Liang and Pardee (1992), and this provides a powerful method for the rapid identification of differentially expressed genes. It has been successfully used to identify the differentially expressed mRNA in humans (de Vries *et al*., 2000) and pathogen-infected plants (Poirier *et al*., 1997; Kuno *et al*., 2000). The DD-PCR technique provides the advantage of amplifying and identifying low abundance transcripts that are differentially expressed (Hafez and Moustafa, 2011). Many studies on virus-host interactions using DD-PCR have aimed to isolate and characterize genes related to virus resistance (Andleeb *et al*., 2010). In the present study, we used DD-PCR to show that IYSV infection in onion plants induces many changes in the expression of genes involved in host defence. This has provided the candidate molecules that need to be further analysed for their structure, function and roles in symptom severity and virus propagation.

Materials and methods

The IYSV Egyptian isolate 2 (Acc# KC161369) was maintained continuously on *Datura stramonium* plants. Groups of newly hatched larvae from virus free *Thrips tabaci* were collected for up to 12 h, used for virus acquisition and placed on IYSV-infected *D. stramonium* plants for 2 h. They were then transferred to healthy onion seedlings in a separate cage for 24 h. Subsequently, *T. tabaci* were killed by spray application of 0.01% malathion. Three inoculated plants were collected daily from 1–10 d post infection (dpi) and then at 15 dpi, as well as non-viruliferous (mock)-inoculated plants.

Total RNA was extracted from the plants using the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN), and the first strand of cDNA synthesis was performed in a total reaction volume of 25 µL. The reaction mixture contained 2.5 µL of 5× $MgCl_2$ buffer, 2.5 µL of 2.5 mM dNTPs, 4 µL of oligo (dT) primer (20 pol μL^{-1}), 2 µg RNA, and 200 U reverse transcriptase enzyme (M-MLV; Fermentas). The reverse transcriptase reaction was performed in a thermal cycler (Eppendorf) run at 42°C for 1 h and 72°C for 10 min. One µL of cDNA was added to 2.5 µL Taq polymerase buffer 10× (Promega) containing a final concentration of 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM of primer (Table 1) and 0.2 µL Taq polymerase (5 U μL^{-1}) in a final reaction volume of 25 µL. The PCR reaction contained 40 cycles; each cycle consisted of denaturation at 95ºC for 1 min followed by annealing at 30ºC for 1 min and extension at 72ºC for 1 min. There was an initial delay for 3 min at 95ºC at the beginning of the first cycle and 10 min delay at 72ºC at the end of the last cycle as a post extension step. Amplification products were visualized using a gel documentation system (Syngene) in 1.5% agarose gel that was electrophoresed in 0.5× TBE buffer.

The resultant PCR product was excised from the gel and sequenced directly after purification with the QIA quick gel extraction kit (Qiagen Inc.). Sanger sequencing of PCR products was performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and model 3130xl Genetic Analyzer (Applied Biosystems). The obtained DNA nucleotide sequences were analysed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their identity.

Results and discussion

The plants inoculated by viruliferous *T. tabaci* showed symptoms identical to those reported on naturally IYSV-infected plants, including straw-coloured chlorotic, and necrotic lesions on the leaves. All the plant tissues inoculated with viruliferous thrips gave positive results, as indicated using a specific primer of the IYSV-NP (Pappu *et al*., 2008) gene.

To understand the molecular basis of the onion-IYSV interactions with rapid identification of the differentially expressed genes, the DD-PCR technique was performed using 11 different primers (Table 1). A total of 1,130 PCR bands (Figure 1) were common between the mock-inoculated and infected samples (monomorphic). However, 22 PCR bands were differentially expressed in all the treatments (polymorphic) (Table 1).

The comparative analyses between the mock-inoculated and infected plants were tested using statistical analysis of differential display-banding patterns. All the treatments were classified into two main clusters (Figure 2). Cluster 1 contained only mock-inoculated plants, indicating that there were genetic variations between the mock-inoculated and infected plants. Cluster 2 was divided into two groups (Figure

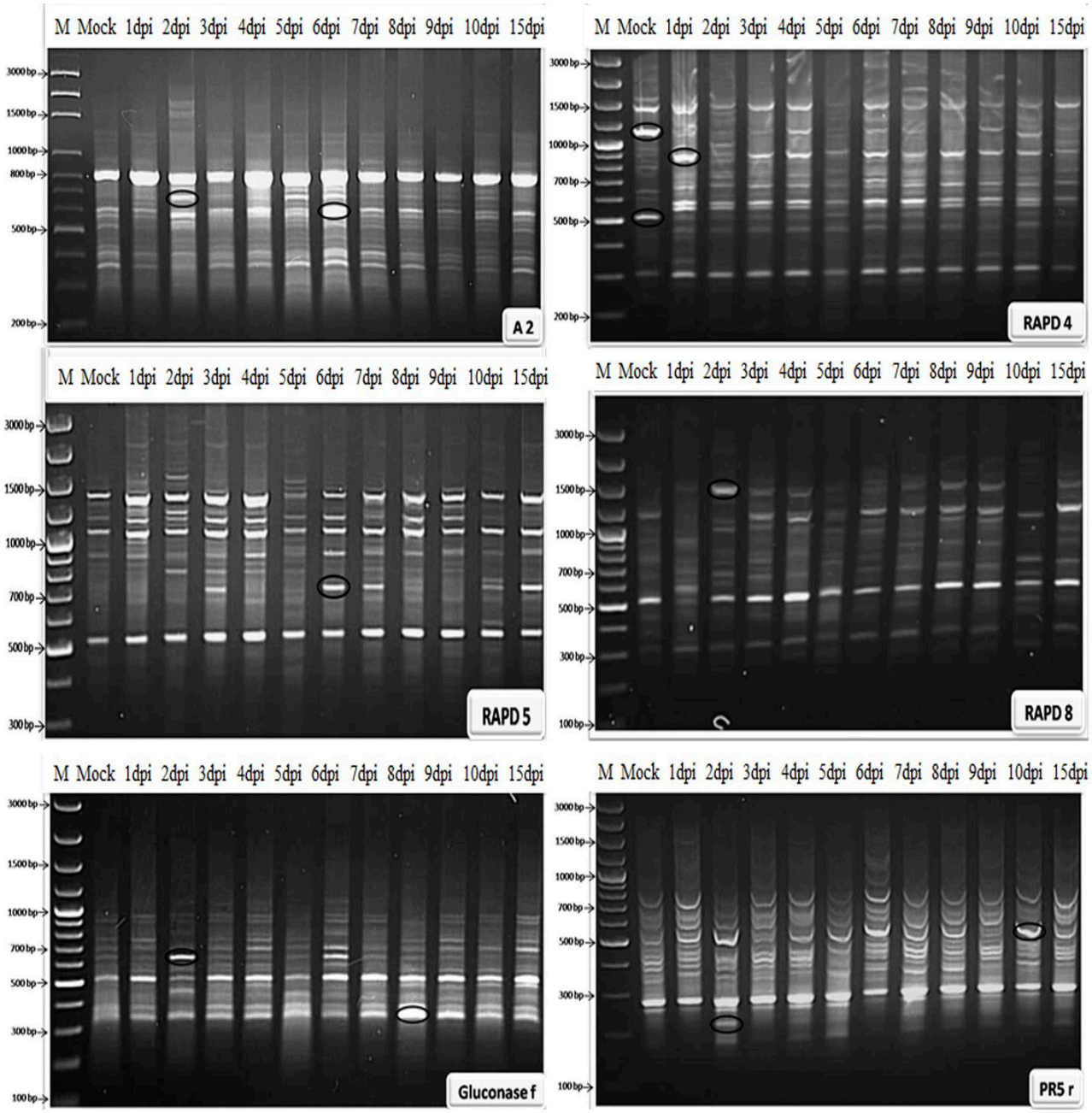

Figure 1. Agarose gel electrophoresis (1.5%) in TBE buffer stained with ethidium bromide, showing differential display PCR using primers A2, RAPD 4, RAPD 5, RAPD 8, Gluconase-f and PR5-r. Lane M, DNA marker 100 bp ladder; lane "Mock" control (mock-inoculated plant tissues); lanes 1 dpi to 15 dpi, plant tissues collected at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 15 d post inoculation with *Iris yellow spot virus*. Black ovals indicate the bands that were sequenced.

2). Group 1 contained almost all the dpi (1, 3, 4, 5, 6, 7, 8, 9, 10 and 15 dpi) samples. Group 2 included only 2 dpi samples, containing many polymorphic bands, in a distinguished separate group. The dendrogram results revealed that IYSV induced variation in gene expression within onion tissues as early as 1 dpi.

Table 1. Primers used in DD-PCR, and the total numbers of up or down-regulated bands.

Primer	Sequence 5′……3′	Total number of bands	Monomorphic bands	Polymorphic bands	Time of up/down-regulation	Size of band	Range of band patterns
A2	GAAACGGGTGGTGATCGC	96	94	2	2 dpi 6 dpi	650 600	280 :1700
RAPD1	TGCCGAGCTG	135	135	-	-	-	180 : 800
RAPD4	CCTTGACGCA	123	118	5	Mock-inoculated 1 dpi	500, 1050 800	300 : 1600
RAPD5	TTCGACCCAG	75	71	4	6 dpi	700	520 : 2200
RAPD6	AAAGCTGCGG	61	59	2	2 dpi 10 dpi	600, 600	350 : 900
RAPD8	ACCTGAACGG	111	107	4	2 dpi	1500	290 : 2000
RAPD9	GGTCTACACC	132	132	-	-	-	200 : 1500
Gluconase-r	ACAACCTTCACATTTGGTGCC	78	78	-	-	-	450 : 1500
Gluconase-f	CGGCGTCTGTTATGGAGGAAA	133	131	2	2 dpi 8 dpi	650 350	300 : 1100
PR5-f	ATGGGCTACTTGACATCTTCTT	60	60	-	-	-	200 : 500
PR5-r	TTATGGGCAAAAAACAACCCT	148	145	3	2 dpi 10 dpi	250 550	200 : 1200

These results agree with the observations of ElMorsi *et al.* (2015). Compared to mock-inoculated plants, transcriptional changes with more than 6,000 potato genes were differentially expressed at 1 and 3 dpi for *Potato virus Y* (Baebler *et al.*, 2014). Furthermore, some genes coding for pathogenesis-related (PR) proteins, cell wall rearrangements and secondary metabolite synthesis were induced at 1 dpi.

All the polymorphic bands were excised from the agarose gel, purified and submitted for sequencing. During the sequencing process, some genes were missed, and others had low-quality sequence values. Ten up-regulated bands and one down-regulated band were successfully sequenced and characterized. Sequence analysis revealed that the up-regulated transcripts were involved in host defence mechanisms. Among these were genes coding for Retrotransposon protein (Acc# KM114292, KM114293), Disease resistance-like protein (Acc# KM114294, KM114295), Chitinase (Acc# KM114296), Pathogenesis-related protein (Acc# KM114297), Cytochrome oxidase (Acc # KM114298), Cytochrome C (Acc# KM114299), Pentatricopeptide repeat-containing protein (Acc# KM114300), Pectin methylesterase (Acc# KM114301), which were up- regulated at 1, 2, 6, 8 and 10 dpi. The down-regulated transcripts coded for the DNA-binding transcriptional repressor protein (Acc# KM114302).

Retrotransposons are major genomic components of most eukaryotic organisms (Jurka *et al*., 2007). In plants, they constitute 15% of the nuclear DNA in *Arabidopsis thaliana*, 50–80% of some grass genomes and more than 90% in some *Liliaceae* (Feschotte *et al*., 2002; Sabot and Schulman, 2006; Huang *et al*., 2012). Retrotransposons play important roles in the evolution and function of plant genomes through gene copying or gene transfer within the genomes. Retrotransposons are closely related to the infectious and endogenous retroviruses (Wilhelm and Wilhelm, 2001; Todorovska, 2007). In the present study, the

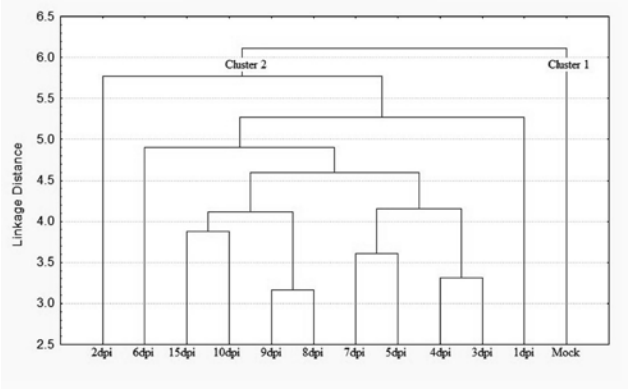

Figure 2. Dendrogram showing the genetic relationships between mock-inoculated and *Iris yellow spot virus*-infected onion samples at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 15dpi, based on DD-PCR banding patterns with 11 primers. Pairwise genetic comparisons and UPGMA cluster analysis of the data matrix was performed by the unweighted pair-group method using the STATICA 5 program.

transcription of retrotransposon in the IYSV-infected onion plants agrees with the results of Todorovska (2007), who reported that most retrotransposons were activated by stress and environmental factors. In addition, the activation of retrotransposons due to virus infection has been observed in maize (Johns *et al*., 1985) and tobacco (Hirochika *et al*., 1996). Furthermore, Karijolich *et al*. (2015) showed that virus-induced retrotransposon expression contributes to the activation of innate immune signalling during infection. Gurkok (2017) reported that *Panicum mosaic virus* and its satellite virus induced the transcriptional activity of several transposons in infected host tissues.

Another up-regulated gene codes for PR protein. It is already known that PR genes play many important roles in plant defence responses against pathogen attack (Zhang *et al*., 2010). The induction of the PR at 2 dpi indicated that there is a relationship between IYSV infection and PR transcription. van Loon *et al*. (2006) reported that induction of PR proteins is associated with viral infection. In addition, Durrant and Dong (2004) confirmed that the accumulation of PR proteins is very often associated with systemic acquired resistance against a wide range of pathogens. Furthermore, many disease resistance (R) genes have been isolated from solanaceous species (tomato, potato, pepper, and tobacco), and from barley, rice, and flax. These genes can confer the resistance to pathogens such as viruses, bacteria, fungi and nematodes (Martin *et al*., 2003). The up-regulation of the R and PR genes at 2, 8 and 10 dpi suggests that these genes play roles in the onion defence system against IYSV infection.

At 6 dpi, the transcript of pentatricopeptide repeat (PPR) gene was upregulated. The PPR family is one of the largest gene families in higher plants, and it plays a central and broad role in modulating the expression of organelle genes. Most of the PPR proteins are predicted to be targeted to chloroplasts and/or mitochondria (Williams and Barkan, 2003). The PPR proteins have been proposed as participating in post-transcriptional regulation of gene expression, including RNA cleavage, splicing, editing, and translation (Grennan, 2011). The molecular functions of most PPR proteins characterized so far fall into these functional categories (Andres *et al*., 2007; Schmitz-Linneweber and Small, 2008). Our results agree with those of Wu *et al*., (2016), who reported that PPR genes may play important roles in plant anti-viral defence.

Plant cell walls give the cells shape and control intercellular transport. Cell walls are also the first host defence that encounters invading organisms (Carpita and Gibeaut, 1993). Many of the recognition events during the plant/pathogen interactions occur at the cell wall level. Consequently, invasion of plant tissues usually results in degradation of the cell walls (Cooper, 1984). Pectin is one of the main components of plant cell walls. In onions, pectin constitutes 11 to 12% of the cell wall dry weight. This substance is secreted in a methylesterified form and is demethylesterified by pectin methylesterase (PME) to make cell walls rigid (Micheli, 2001). The action of PME is important to plant development and defence as it makes pectin susceptible to hydrolysis by enzymes such as endopolygalacturonases. Plant PMEs are involved in the systemic spread of *Tobacco mosaic virus* within plants by binding to virus-encoded movement proteins (Chen *et al*., 2000; Chen and Citovsky, 2003; Lionetti *et al*., 2014). Silencing of PMEs in *Nicotiana* spp. delays systemic movement of *Turnip vein clearing virus* and reduces host susceptibility to virus infection (Bubici *et al*., 2015).

The mitochondrial cytochrome gene suppresses virus infection and prevents systemic spread in plant tissues. The cytochrome oxidase gene plays an important defence role against pathogens, especially viruses (Hafez and Moustafa, 2011). These enzymes blocking virus-silencing processes during infection of

plant cells. Mutation in the cytochrome oxidase gene may increase plant sensitivity to virus infections.

The IYSV down-regulated gene was found to code for the transcriptional repressors. This protein binds to specific sites on DNA to prevent transcription of nearby genes. Most repressors inhibit the initiation of transcription. A DNA-binding transcriptional repressor gene blocks the attachment of RNA polymerase to the promoter, thus preventing transcription of the genes through preventing RNA polymerase from initiating the transcription process and activating host gene expression to enhance pathogen virulence (Dangl *et al*., 2013). In nature, plants use DNA/RNA binding proteins for defence against virus infections (Huh and Paek, 2013).

Upon virus infection, plant defence systems activate to induce many immune system genes. The analysis presented here has identified onion genes expressed after IYSV infection. Studies of the pathways in which these genes are involved will give more information about the physiology of early onset of disease caused by IYSV, and may elucidate the mechanisms of host tolerance to the pathogen. Further characterization and functional analysis of the genes, identified in this study, will provide increased understanding of onion-IYSV interactions.

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Author contributions

E.H conceived and designed the study; A.A carried out the experiments; A.E, O.E. wrote the draft of manuscript, N.S. analysed the data and edited the manuscript. All authors read, revised, and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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