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Pathogenesis-related genes as tools for discovering the response of onion defence system against Iris yellow spot virus infection

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Abstract:

The Iris yellow spot virus (IYSV) is a viral disease of onions that causes significant damage and economic loss in afflicted onion crops. Pathogenesis-related (PR) genes are part of the innate immune response that onions harbour against viral infections, which to far remains unexplained. Using a sensitive and accurate real-time quantitative PCR approach, the dynamic expression of five distinct genes in infected onions were examined following biological inoculation with virulent isolate of Thrips tabaci (Lindeman) (Lindeman). The transcription levels of PR1, PR2, PR3, PR4, and PR5 genes were strongly expressed 1 day post inoculation (dpi) (dpi). Furthermore, statistical analysis demonstrated a substantial shift in peak expression levels of PR1 after 8 dpi and PR3 after 9 dpi. In contrast, the expression level change for the other genes was only mild. Further, we assessed and graded the expression stability of three reference genes (EF1- , 18S rRNA, and -actin) using geNorm and NormFinder. The entire research indicated that -actin is the most informative gene, which may be exploited as an internal control for quantitative gene expression. Our research results not only give suggestions for identification of acceptable reference genes in virally infected onions, but also significant information about immune response related genes connected with the early plant de fences against viral infection. Moreover, the PR1 gene seems to be a disease-specific gene connected to the IYSV infection in onion tissues

Key words: The defense mechanism of onions is based on the RT-PCR and PR genes of the IYSV virus.

Introduction

Onion and bulb crops face an economic danger from the Iris yellow spot virus (IYSV, a Tospovirus) in various regions of the globe (Mandal et al. 2012; Hafez et al. 2014). Onion seed harvests are particularly vulnerable to this disease, which may cause losses of up to 100%. (Mandal et al. 2012). An order of insects called Thys anoptera, which includes Thrips tabaci (Lindeman), transmits all Tospovirus viruses, which are the only plantinfecting members of Bunyaviridae (Kritzman et al. 2001; Fauquet et al. 2005). The spread of insecticide-resistant thrips over the globe has made it harder to manage this viral illness (Abe et al. 2012). For this reason, new strategies for inducing plant-viral defense systems must be developed in order to limit the harm thrips cause. Plants have developed sophisticated systems for detecting pathogen infections and initiating an efficient immune response at the cellular level (Karimi et al. 2013). Resistant (R) genes are expressed in plants, and the resulting proteins allow the pathogen's relearned virulence (Avr) proteins to be identified (Karimi et al. 2013). Onion defense responses are triggered by increased levels of the products encoded by these genes; however, this does not provide a comprehensive picture of the control of pathogen-induced defense responses in onions.. Stress and immune responses are closely resembled by the gene expression alterations caused by viral infection (Whitham et al. 2006). The elevation of pathogenesis-related (PR) genes and other genes previously linked to plant disease defense characterizes the defense-like responses (Whitham et al. 2006; Tabassum et al. 2013). Numerous genes linked with modulation of the redox state such as superoxide dismutase, glutathione S-transferases, resistance gene homologs, and additional genes of unknown function are co-induced with the PR genes in the defense response. (van Loon et al. 2006). Plant–bacterial and plant–fungal interactions have received the majority of attention in the subject of plant–pathogen interactions; plant responses to viruses, on the other hand, are still little known. The chemical mechanisms of IYSV–onion interactions are still unknown. To understand the relationship between IYSV and its host, a comprehensive gene expression study of the onion response to IYSV is required. Low levels of RNA molecules can be detected using the highly sensitive, accurate, and reproducible real-time quantitative polymerase chain reaction (RT-PCR), which has been shown to be an efficient indicator of virus gene expression in various instances, including the Tomato spotted wilt virus (Boonham et al. 2002), the Rice stripe virus (Li et al. 2012), and the IYSV (Hafez et al. 2013). In addition, the findings are dependent on transcript normalization using a gene that is expressed at a consistent level in all organs and under all circumstances (Gutierrez et al. 2008; Kozera and Rapacz 2013). Thus, housekeeping genes are attractive candidates for internal controls in the form of uni versal controls (Bustin 2002). When an

incorrect reference gene is used, RT-reliability PCR's and accuracy are compromised (Kong et al. 2014; Remans et al. 2014). An IYSV-infected onion was used to test the expression stability of three potential reference genes (EF1-, 18S rRNA, and actin) as internal controls and to evaluate the expression pattern of several PR genes (PR1, PR2, PR3, PR4, and 5) in order to better understand the onion's defense mechanism against the IYSV virus. Following an IYSV infection, this paper offers the first findings related to the discovery and description of plant defense genes.

Inventions and processes An isolated virus

Virus inoculums utilized in this investigation were obtained from a known source (Hafez et al. 2014). Datura stramonium plants were inoculated mechanically with the IYSV Egyptian isolate under greenhouse conditions to keep it alive (Hafez et al. 2013).

Sample collection and transfer of infectious organisms

It was possible to establish a virus-free culture of Thrips tabaci adult females, which were then kept in bean pods to lay eggs. According to prior research, newly born larvae were collected and raised on bean pods (Murai and Loomans 2001; Hafez et al. 2013). The top leaves of the IYSV-inoculated D developed tiny chlorotic lesions and systemic necrosis after 10–12 days. stramonium. After up to 12 hours, groups of freshly hatched larvae from virus-free thrips were collected, utilized for viral acquisition, and put on D contaminated with IYSV. After two hours, the stramonium plants were moved to another cage with healthy onion seedlings for a 24-hour period. After being sprayed with 0.01 percent Malathion, thrips died. To get RNA, we extracted three biological replicates of thrips transmission per day from 0–10 and 15 days after infection. Samples taken at the beginning of the experiment were utilized as a control.

The extraction of total RNA and the creation of cDNA

The RNeasy Mini Kit was used to extract total RNA from both healthy and diseased onion tissue (QIAGEN, Germany). With the use of an Eppendorf Biophotometer, we determined the RNA content and quality of each sample (Eppendorf, Germany). This method was used to determine the quality of the RNA samples. Total reaction volume was 25 L for the first strand of cDNA synthesis. In the reaction mixture, 2.5 L of MgCl2 buffer, 2.5 L of 2.5 mmol dNTPs, 4 L of oligo (dT) primer (20 pmol/L), 2 g RNA, and 200 U RT enzyme were used (M-MLV, Fermentas, USA). A thermal cycler (Eppendorf, Germany) was used to accomplish the reverse transcriptase process, which took place at 42°C for one hour and 72°C for ten minutes. After that, cDNA was kept at 20 °C until it could be utilized again.

Assay for RT-PCR quantification:

We used the SYBR Green SYBR Green PCR Master Mix to conduct the reverse transcription process (Fermentas, USA). An equal volume of each primer (10 pmol) was added to the 25 L reaction mixture, together with the 50 ng template cDNA (1 L), 12.5 L of 2SYBR Green PCR Master Mix, and 9.5 L of nuclease free water. There were three runs for each sample. Denaturation at 95°C for 10 minutes was followed by 40 cycles of denaturation for 15 s, annealing for 30 s, and extension at 72°C for 30 s in the amplification procedure. It was at the extension stage that the data was gathered and processed. With the aid of a Rotor-Gene 6000, the experiment was carried out (QIAGEN, ABI System, USA). Using the melting curves obtained after the 40 cycles, nonspecific products were eliminated.

Analysis of RT-PCR data

According to Livak and Schmittgen, the relative expression ratio was precisely assessed and estimated (2001). Thus, each biological sample was analyzed using the exponential function and the PCR efficiency (E) to determine the difference () in quantification cycle value (CT) between the target (CTtarget averaged from three technical replicates) and the reference (CTreference, a fixed CT value that was used for all samples). On the ABI System, we performed an automated threshold analysis to calculate the CT (threshold of cycle) value for each gene. To arrive at CTtarget, the CT value for each target gene was compared to the CTreference value.

$$\Delta C_{T_{target}} = (C_{T_{target}} - C_{T_{reference}}), \Delta C_{T_{control}} = (C_{T_{control}} - C_{T_{reference}})$$

The relative expression quantity of the target gene was determined as follows:

$$\Delta\Delta C_T = (\Delta C_{T_{target}} - \Delta C_{T_{control}}), \text{according to } 2^{-\Delta\Delta C_T} \text{ algorithm}$$

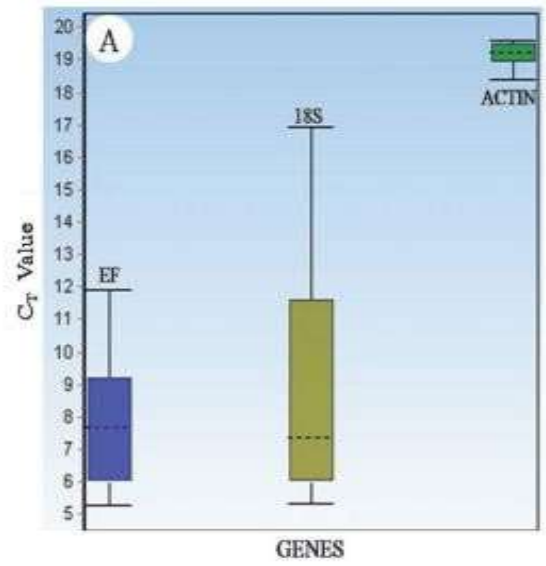
An increase (an upregulation in expression) may be shown by expression levels higher than 1, while a

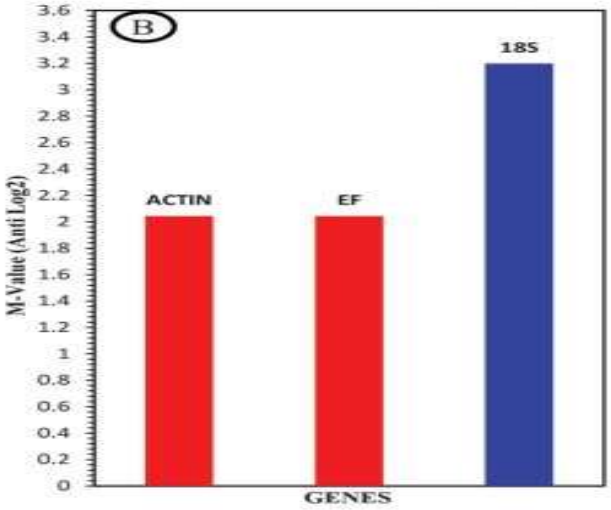
reduction (a downregulation in expression) can be demonstrated by values lower than 1.

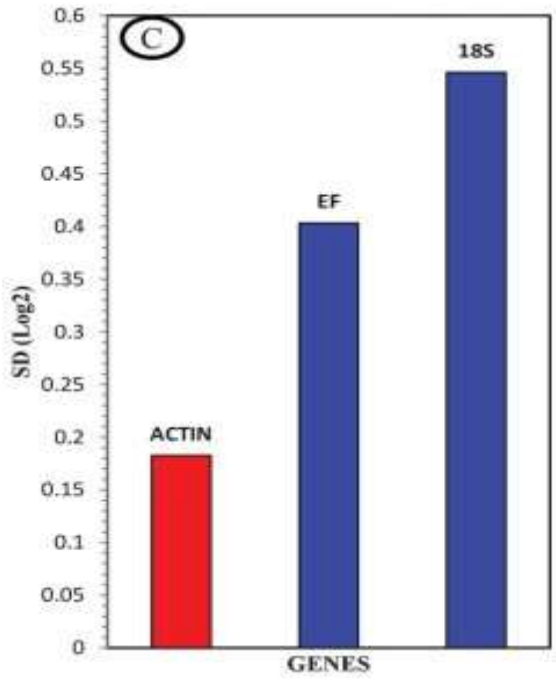
Analysis of gene expression and the stability of reference genes The CT values for all genes in onions infected with IYSV were calculated to give an overview of the relative abundance of the three onion reference genes studied. These values varied from 7.82 (EF1-) to 19.20 (-actin) in virus infected onion tissue, which represents the greatest and lowest accumulated mRNA CT values. The 18S rRNA and EF1- had CT values that fluctuated widely, with the least values being 68% and 55% of the greatest values, respectively. The least value of actin expression differed by no more than 6% from the maximum value found, representing the lowest stan dard error found in the samples despite some observed variability. An internal control for gene expression analysis of the IYSV–onion infection was found to be -actin. The expression stability of the reference genes could not be assessed by a simple comparison of the raw CT values. Because of this, the researchers used a more advanced statistical analysis to better understand how the reference genes were stable. GeNorm and NormFinder (Vandesompele et al. 2002) were used to analyze the stability of the reference genes in terms of expression stability (Andersen et al. 2004). First, the raw CT values were translated into quantities that could be compared relative to one another using the geNorm analysis. The geNorm applet was used to calculate the average gene expression stability (M value) for the three reference genes, and the genes were then sorted according to the results (Fig. 1B). In general, a lower M value means that the gene's expression is more steady. Actin and EF1, two housekeeping genes, were found to be highly expressed and stable, with M values of 2.044 for each. It is also possible to give stability values to

single candidate reference genes using the NormFinder Visual Basic tool, which is based on Microsoft Excel. Standard deviation (SD) is a measure of how stable expression is.

Results:







licates. The geNorm and Norm Finder findings showed that the -actin gene was more stable in virusinfected onion tissues than the other two genes, with SD values of 0.18 (Fig. 1C).

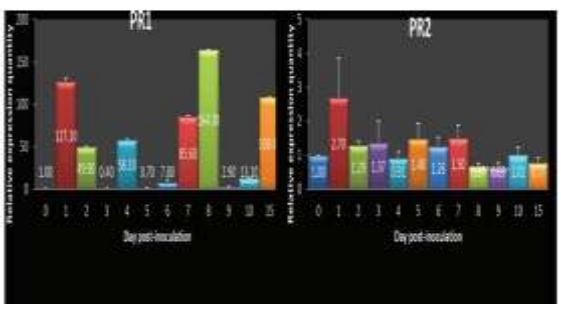
PR gene expression in relation to time:

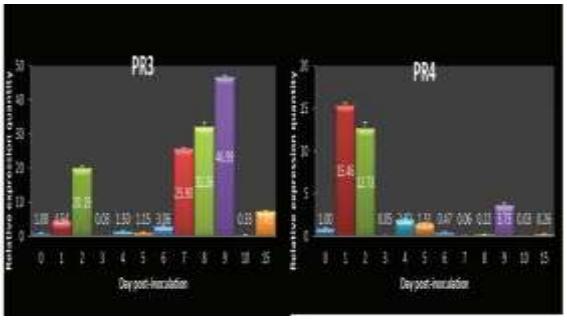
In the quantification analysis, the RT-PCR comparative approach was applied, which mathematically transforms the CT value into the relative expression levels of genes. Once the onion plant has been infected with IYSV, this research found that PR1 is immediately increased or upregulated (Fig. 2). At 1 dpi, the expression level was substantially higher than in the control (127.10). Furthermore, by 8 dpi, the expression levels were even more distinct, with PR1 expressing 164.30 times more than in the control. Furthermore, PR1 expression was downregulated at 3 and 5 dpi, with values of 0.40 and 0.70 compared to the control. PR1 was shown to be related with the IYSV–onion viral infection in a time-course analysis. As seen in Figure 2, the viral infection slowed the expression of PR2 compared to PR1. First three days after infection, the gene was marginally upregulated with greater expression levels than in the control plants. At 7 dpi, the expression level dropped to 1.50 compared to the control, and at 5 dpi, it dropped to 1.48. Compared to the control, the expression of PR2 was significantly reduced at 4, 8, 9, and 15 dpi by 0.91, 0.67, 0.65, and 0.77, respectively. As a consequence of these findings, it seemed that the virus first stimulated PR2 expression before going on to silence the gene altogether. At 1 dpi, IYSV infection produces a 4.54-fold increase in the PR3 gene expression compared to the control. At 2 dpi, the gene was interestingly still stimulated (20.19). (Fig. 2). During the course of the experiment, PR3's expression level rose to 25.90% of the control, and peaked at 9 dpi (46.79%). At 10 dpi, the expression level was reduced. At 1 dpi, the PR4 gene's expression level was 15.46 ng/ml, which fell to 12.73 ng/ml at 2 dpi, according to (Fig. 2). 3 dpi (0.05) showed a dramatic drop in expression compared to the control. Proliferation of the protein PR4 grew to 2.02 times its normal level at four days after injection (dpi) before entirely shutting down at 6, 7, 8, 10, and 15. Thaumatin-like proteins make up the PR5 family. The virus had a mild effect on PR5, according to the results of this investigation (Fig. 2). When compared to control plants, an expression rate of 5.38 was used to activate the gene. Finally, at 5 dpi, the greatest expressiveness level was 4.72 dpi at 2 dpi, 1.13 dpi at 3 dpi, and 1.16 at 4 dpi. PR5 gene expression rose fast after 9 days of pregnancy, with a 2.56-fold rise in expression at 10 and 3.58-fold increase in expression at 15 days of pregnancy. We were able to summarize the ratio of the relative expression levels of the PR genes thanks to the research done here. At both 1 and 2 dpi, the PR genes were all expressed. At 1 dpi, PR1 and PR4 were the two most highly expressed genes, respectively. In addition, at 2 dpi, PR1 was the most highly expressed gene, followed by PR3 in expression. A three-day post-irradiation analysis revealed that the PR1-PR4 pathway was completely shut down, but PR2 and PR5 remained hardly expressed. Aside from PR2, which had its expression reduced, all other PR genes were upregulated at 4 dpi except for PR1. In addition, except for PR1, all PR genes had higher expression levels at 5 dpi than the controls. PR1 and PR3 showed significant levels of expression between 6 and 10 dpi, as well as at 15 dpi, with the exception of 10 dpi, when PR3 was completely repressed.

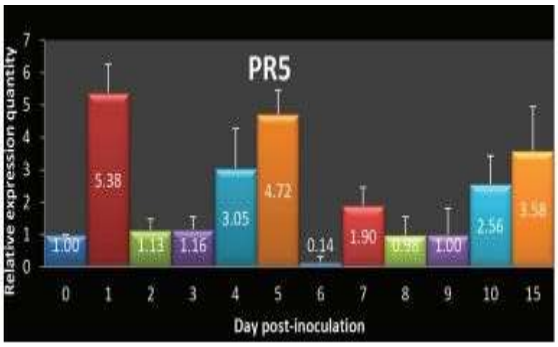
Discussion

Rapid quantitative polymerase chain reaction is a powerful tool for gene expression investigation and the identification of plant viruses. RT-PCR may also be used to uncover the mechanisms by which viruses attack plant tissues (Peters et al. 2004; Varga and James 2005; Hafez et al. 2013). Experiments involving normalization by the most and least stable

reference genes need selecting appropriate internal controls (reference genes) (Liu et al. 2012). Data may be interpreted incorrectly if reference genes that have not been validated are used in the study incorrectly (Mafra et al. 2012). In order to get accurate results from target gene expression analysis, researchers must use stable reference genes in a variety of different physiological and biological states (Gutierrez et al. 2008; Maltseva et al. 2013). If an incorrect reference gene is used, RT-reliability PCR's and accuracy are compromised (Ferguson et al. 2010). Because of this, it is imperative that all reference genes be validated prior to the experiment in order to ensure that the stability of their expression under certain experimental conditions is confirmed and to prevent inaccurate data interpretation and subsequent incorrect conclusions from being drawn (Bustin 2002; Gutierrez et al. 2008). Thus, two software tools, geNorm and NormFinder, were used to analyze the expression of three well-known reference genes. To establish the optimum number of stable housekeeping genes required for accurate normalization (Vandesompele et al., 2002), an analysis using geNorm was carried out, while an evaluation of the quality of the data generated by geNorm was carried out using NormFinder (Andersen et al. 2004; Pfaffl et al. 2004). GeNorm's best reference housekeeping genes (-actin and EF1-) both met high stability criteria with the same M value when used as housekeeping genes (2.044). EF1- and the 18S rRNA had SD values of 0.40 and 0.54, respectively, according to the NormFinder study, which placed -actin as the most stable gene. This gene, long utilized as an internal control for gene expression investigations (Nicot et al. 2005), has recently been employed to investigate induced and differential gene expression during Wheat germplasm development.A diagram like this one is shown in Figure 2. Pathogenesis-related (PR) gene expression levels at different time points after immunization were studied (dpi). For each of the three plants, the mean readings are presented and the error bars reflect the standard deviations of the means.







Wheat tissues infected with the yellow mosaic virus (WYMV) (Jaroová and Kundu, 2010). TSWV quantification using individualized thrip vectors has also used -actin gene as an internal control as an additional measure of success (Rotenberg et al. 2009). 18S rRNA and EF1- are often employed as reference genes in real-time PCR (RT-PCR) investigations. Potatoes have been shown to have stable expression of EF1-, whereas the 18S rRNA has been found to be acceptable for normalization of Barley yellow dwarf virus-infected cereals (Wood et al. 2000). EF1- and 18S rRNA, on the other hand, performed poorly in this investigation, despite the geNorm analysis placing EF1- higher and higher. EF1- and 18S rRNA expression levels were shown to be very variable in virus-infected tomatoes (Mascia et al. 2010; Castro et al. 2012), indicating that these frequently used reference genes may not be the best options for assessing gene expression in onions during viral infection. For proper normalization of a target gene in virus-infected onion, we found that -actin is the most stable reference gene. Systemic acquired resistance and pathogen development, multiplication, and/or dissemination may be inhibited by the PR gene set, according to certain theories (van Loon and van Strien 2002). The PR1 gene was shown to be related with the IYSV–onion viral infection based on a time-course expression investigation. One day postinjury (dpi) saw a significant increase in PR1 transcript levels, which peaked at eight days after injury (dpi). Although some data revealed that tobacco and tomato PR1 proteins have antifungal action, the biological function of PR1 family proteins is still

unclear (Riviere et al. 2008; Urano et al. 2013). (Niderman et al. 1995). Thus, plants that overexpressed PR1 from Capsicum annuum were more resistant to phyto pathogenic bacteria and oomycetes in both Arabidopsis thaliana and Nicotiana tabacum (Hong and Hwang 2005;
Sarowar et al. 2005). When an onion plant is infected with the IYSV, PR1 is promptly activated or up regulated, and it is expressed throughout the plant's life cycle. Research conducted by Cutt and colleagues discovered that PR1 proteins from tobacco are implicated in viral resistance (Cutt et al. 1989). It is clear that the PR1 protein of tobacco is not adequate for Tobacco mosaic virus (TMV) resistance, and this means that the PR1 proteins may not operate as distinct antiviral factors in tobacco. Nitric oxide has also been shown to boost PR1 expression after tobacco virus infection, as reported by Li and colleagues in 2014. (Li et al. 2014). Various plant diseases are harmful to different PR1 proteins that plants generate (Niderman et al. 1995). When onion cells were infected with IYSV at 1 day post infection (dpi), PR1 expression increased and peaked at 8 days post infection (dpi), suggesting that this protein's overexpression is one of the initial defensive responses. Using our results and those of Abe and colleagues (2012), we conclude that the PR1 gene expression is the consequence of a viral infection and not a result of thrips feeding, which supports our findings and Abe and colleagues' (2012) findings. To their surprise, they observed that thrips feeding had no effect on TSWV accumulation or gene expression triggered by TSWV infection (Abe et al. 2012). Many genes were not affected by the bug (Pieris rapae) eating on Arabidopsis, according to Reymond & colleagues (2000). (Reymond et al. 2000). Glucan endo- 1,3glucosidases, which are monomeric enzymes with molecular weights ranging from around 20 to 23 kDa, are members of the PR2 protein family. The hydrolytic cleavage of -1,3-glucanase in plant cell walls is carried out by these carefully controlled enzymes (Doxey et al. 2007; Sinha et al. 2014). Numerous herbaceous plants' -1,3 glucanase has been shown to be protective against infections in research studies (Zemanek et al. 2002; Eboigbe et al. 2014). It has been reported that inoculating soybeans with the bacterial pathogen P. syringaepv. glycinea increased the activity of -1,3-glucanase (Cheong et al. 2000). Soybean seedlings infected with Phytophthora megasperma f. sp. Glycinea or exposed to chemical stress release endo—1,3glucosidase, which releases elicitor-active fragments from the fungus's cell wall preparations, stimulating defense responses in adjacent cells as well as inducing acquired resistance to future infection. In this investigation, we found that the expression of -1,3-glucanase was downregulated after bacterial and fungal infection, which is consistent with the findings of Ahn and colleagues (Ahn et al. 2014). After the first two days of infection, we hypothesize that the viral infection increases expression of this gene, but thereafter totally shuts down gene expression. There are chitinases that hydrolyze chitin (a linear homopolymer of N-acetylglucosamine) in the PR3 family of proteins (van Loon 1999; RomoDumaresq et al. 2014). Chitinases are primarily involved in the organism's defense against pathogens in animals and plants (Singh and Subudhi 2014). After infection with IYSV, the PR3 gene was rapidly activated, with expression levels peaking at 4.54 dpi and rising to 30.19 dpi at 2 dpi. To test this hypothesis, we looked at the PR3 expression level at the seven-day postinfection (dpi) and at the ten-day postinfection (dpi). According to the findings, viral infections induce chitinases (Lawton et al. 1992; Busam Pagination not final (cite DOI) / Pagination provisoire), which is consistent with previous investigations (citer le DOI) ElMorsi and co-authors 7

Conclusions

RT-PCR was used to normalize gene expression in onions for the first time, as far as we know. This paper covers the process used to verify a set of regularly used candi date reference genes. GeNorm and NormFinder evaluations of the findings identified onion -actin as the best appropriate reference gene. As a consequence of biostress on the onion, new patterns of genes for pathogenesisrelated proteins were discovered (either increased or decreased). For the first time, molecular characterization has been done of onion defense responses to the host–parasite interaction involving IYSV, an essential plant disease. However, additional characterization and functional examination of the genes discovered in this work will lead to a more complete knowledge of onion–pathogen interactions.

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