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A Semiquantitative Rt-Pcr Assay To Evaluated The Expression Patterns Of Nbs-Lrr Family Genes In Infectedfected Citrus By Candidatus Leiberibacter Asiaticus

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ABSTRACT: Citrus has great economic importance for Iran and it is eighth citrus producer in the world. The huanglongbing (HLB) disease of citrus trees, caused by Candidatus Liberibacter asiaticus, was first reported in Iran in 2007. At present efficient strategies to control this disease is the use of plants resistant to the pathogen. In this study, expression pattern of NBS-LRR (Nucleotide Binding Site-Leucien-Rich Repeats) genes, were evaluated in different citrus by semi quantitative RT-PCR method. The results indicate that the expression of this gene in citrus trees inoculated with the citrus greening pathogen changes after inoculation.

Key Words: Candidatus Liberibacter asiaticus, HLB, Semi quantitative RT-PCR

Figures And Tables

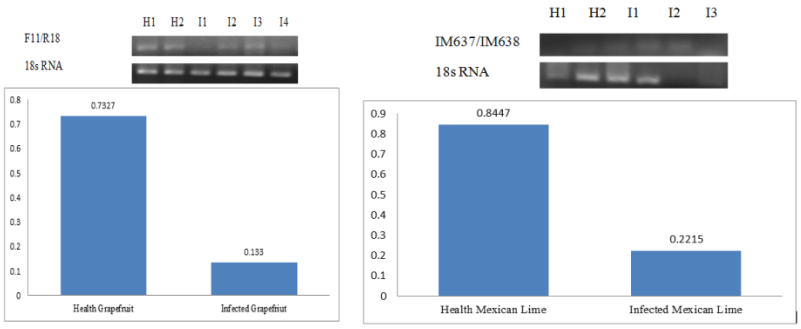
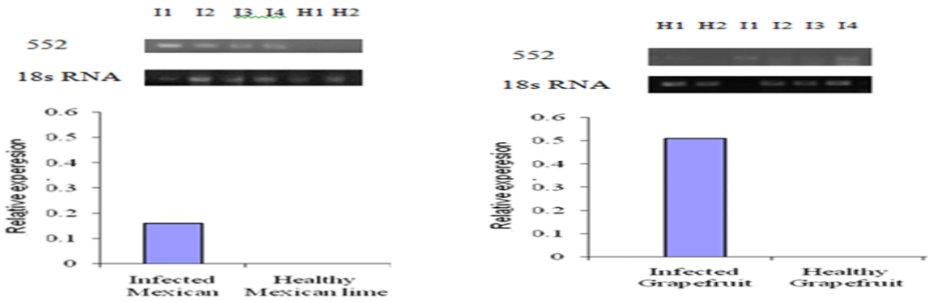


Figure 1. Semiquantitive analysis of NBS-LRR genes familly in different citrus infected by HLB pathogen using degenerate primer F11/R18 and IM637/IM638



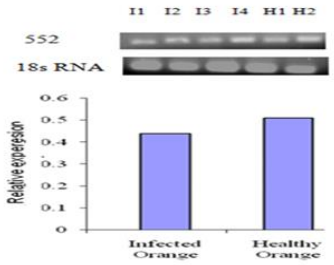


Figure 2. Semiquantitive analysis of a NBS-LRR gene in different citrus infected by HLB pathogen using spesefic primer 552.

Table 1. primers designed or used in this study

Sequence	Primer
5'- GCGGTAATTCCAGCTCCAA-3'	18s forward
5´-TAATTTCTTCAAAGTAACAGCG-3´	18s reveres
5´-CGGGGCGTCTAACTCATAGA-3´	CG forward
5´-GGGGAAAGACAACCTTGGA-3´	CG reveres
5´-CCATTgCAGgggATTCATCTT-3´	63 forward
5´-TACgTATCgAACgCAAACAC-3´	63 reveres
5´-GGDGTDGGNAARACWAC-3´	F11
5´-AANGCHAGNGGYAANCC-3´	R18
5´-ARIGCTARIGGIARICC-3´	LM637
5´-GGIGGIGTIGGIAAIACIAC-3´	LM638

INTRODUCTION

Candidatus Leiberibacter asiaticus is an obligate biotrophic plant pathogen that causes citrus greening disease, also called Huanglongbing or yellow dragon disease, a devastating disease resulting in significant economic losses (Zhao, 1981; Capoor, 1963). Ca. Leiberibacter asiaticus (Las) is phloem-inhabiting prokaryotes, transmitted by Asian citrus pysllid, Diaphorina citri in Asia and America (Bové, 2006).

There are no curative methods to control HLB. The most effective way to reduce the severity of greening disease is using HLB free seedlings (Ferguson, 2003). In some cases, the occurrence of the HLB disease symptoms could be affected by rootstock (Kapur et al., 1982). It is hoped that the efforts in pathogen and host sequencing and bioinformatics will point to resistant genes or pathogen vulnerabilities thus paving the way for future incorporation of disease resistance mechanisms. At the present time, the citrus industry is composed of numerous commercial cultivars and species, a vast majority of which are moderately to highly susceptible to HLB (F.F.T.C., 2003).

In response to microbial attack plants activate a lot of defense responses leading to hypersensitive reaction, production of reactive oxygen species, cell wall modifications, synthesis of pathogenesis-related proteins, and accumulation of phytoalexins (Hammond-Kosack and Jones, 1996). Disease symptom is the result of molecular, cellular and physiological changes in a susceptible host and is part of a defense response which limits the pathogen in the plant, but does not restrict its spread (O'Donnell et al., 2003). A number of disease resistance genes (R genes) have been cloned from several plant species (Baker et al.1997). Recent cloning of R-genes from a variety of plants has identified four structural classes of proteins (Hammond-Kosack and Jones, 1997). Most R-genes contain leucine-rich repeat (LRR) domains attached to either nucleotide binding site (NBS). These classes share structural similarity with proteins also involved in development. Through the amino acid sequence analysis of these resistance genes, it has been found that most plant disease resistance genes encode NBS-LRR resistance protein (Ellis et al., 2000). Numerous disease resistance gene-like DNA sequences were cloned from C. grandis and P. trifoliata, using a PCR approach with degenerate primers designed from conserved NBS (nucleotidebinding site) motifs found in a number of plant resistance genes. These results indicate that resistance-gene candidate sequences amplified with the NBS-derived degenerate primers are valuable sources for developing markers in disease resistance gene tagging, mapping, and cloning (Deng et al., 2000).

Using similar technologies, markers are being sought to tag other resistance genes in Citrus and its relatives that responsible for the citrus disease such as citrus greening and lime witches broom. In this study, we report a semiquantitative assay for quantization of these genes in infected citrus by HLB pathogen and evaluation of possible influence of them on the citrus reaction to greening.

METHODS

PLANT MATERIAL AND INOCULATION

Six healthy greenhouse-grown 1-year-old grapefruit (Citrus paradisi), C. grandis, C. sciences var. Valencia, Mexican lime (C. aurantifolia) used in this experiment. The healthy grapefruits and healthy grandis were Jiroft and Sari native type respectively and they have a health certificate from the Plant Protection Organization (PPO) of Iran. The spacemen of Las infected orange were grafted to four healthy grapefruit. Purity of the inoculum has been tested using OI1/OI2c (Jagoueix et al. 1996) and A2/J5 (Hocquellet et al., 1999) primers sets (table 1) for confirming single infection. All plant material used for inoculation was confirmed to be free of Citrus tristeza virus by RT–PCR analysis. The grafted plants covered for one month with plastic bags to increase humidity and were arranged randomly on the greenhouse bench. They kept under natural light conditions at a temperature of 25-28°C.

Detection of Las infection by PCR

The greening inoculated branches in grapefruit, C. grandis, C. sciences var. Valencia, C. aurantifolia) were sampled every month after inoculation. Total DNA was extracted from leaf samples (vascular tissues of leaf veins and petioles) using the method originally described by Daire et al (1997) with some modifications. DNA amplification was performed in 25 ml total reaction volume in an Applied Biosystem thermal cycler. PCR primers was A2 and J5(table 1) to fine the time that Las was detectable in trees by PCR (Jagoueix et al., 1996).

RNA isolation and cDNA synthesis

For semiquantitative analysis samples were collected a week, a month after inoculation and in symptomatic stage of sweet orange. Sampling done from leaves above grafting region. Total RNAs of infected and non-infected were extracted from all replicates using Trizol reagent (Invitrogen, life technologies, USA) according to the manufacturer's instructions. cDNA synthesis and cDNA-AFLP analysis were performed in 4 replicates. 2 µg of total RNA was used to synthesize from cDNA using RevertAid H Minus First Strand cDNA Synthesis kit (Fermentase, Germany) according to the manufacturer's instructions. Second strand cDNA synthesis was performed by adding the first strand mix to 150 µl total volume of 15 µl 10 x cDNAII buffer, 35 U DNA Polymerase I (Fermentase, Germany), 3 U RNase H (Fermentase, Germany), 1.00 µl dNTPs (25 mM) and incubating for 2 h at 16°C. The resulting double-stranded cDNA was purified according to powell and Gannon (2002). The concentration of cDNAs was determined using spectrophotometer (Bio-rad) and their quality was determined by electrophoresis on 1.2% agaros gel.

Reverse transcription standardization using the reference gene

As the yields of the reverse transcription step were variable, each reverse transcription product was normalized before co-amplification. For each sample tested, reverse transcription efficiency was first evaluated by amplifying the reference gene. For this study, 36 PCR rounds were carried out with reference to the PCR amplification curve. Peak height values were compared with the reference gene PCR amplification curve determined previously and the number of co amplification PCR rounds were then adjusted for sample in order to obtain PCR efficiencies where NBS-LRRgene/18s rRNA gene ratios were reproducible.

Comparative-Quantitative RT-PCR by degenerate and specific primers

Four primer pairs were used along with 18s rRNA primers for the same PCR reactions. Degenerate primers were designed Degenerate primers were designed from highly conserved motifs, kinase-1a (K) and hydrophobic domain (HD) of NBS-LRR type resistance genes (Table 1). Primer F11 was designed in the sense direction corresponding to the amino-acid sequence GVGKTT found in the P-loop of different R genes. Reverse primers include R11, R16 and R18 that target conserved motifs (Deng et al., 1997). PCR reactions were performed in a reaction consisting of 50 mM KCl, 15 mM Tris-HCl (pH 8.4), 2 mM MgCl2 , 0.25 mM dNTP mix, 0.25-0.5 µM of each primer, 0.1-1 µl of cDNA synthesis products and 1unit Taq DNA polymerase (CinnaGen, Tehran, Iran). The PCR reactions were performed on a thermocycler (Perkin-Elmer, GeneAmp 9600) with the following program: 4 min incubation at 94°C and then 36 cycles of 45 second, 94°C; 45 second, 52°C; and 45second, 72°C; before final extension at 72°C for 5 min. Ethidium bromide-stained gel photos were scanned to obtain numeric data representing the band intensities. All calculations and chart drawings were performed on Microsoft Excel softwares. Relative band intensities were calculated through dividing the intensity of each NBS-LRR gene band by that of the 18s rRNA gene band and standardized with respect to the length of DNA fragments.

For confirmation comparative-Quantitative RT-PCR analysis by degenerate primers, specific primers have been designed (table 1) from NBS-LRR fragment produced from degenerate primers. Comparative-Quantitative RT-PCR analysis have been performed according previous step but specific primers have been used instead of degenerate primers.

RESULTS

Grafting

HLB symptoms normally appeared on the new shoots of sweet orange 4 to 5 months after inoculation. No symptoms observed in grandis and grape fruit and Mexican lime 6 month after inoculation but HLB pathogen could been detected in grape fruit and Mexican lime trees and in grandis a year after inoculation by PCR.

Internal controls as reference genes

To assess gene expression by relative semiquantitative assay, co-amplified internal controls might be established as reference genes. Expression levels of 18s rRNA housekeeping genes were evaluated using cDNA obtained from the infected citrus by Las, which is considered to be highly expressed, and the NBS LRR family genes, which is moderately expressed in infected citrus by Las as compared with other housekeeping genes. The amplification efficiencies of these genes were determined in different PCR rounds. To evaluate the relative expression of each gene of interest as compared with 18s rRNA gene, co-amplifications were performed using cDNA obtained from infected citrus by Las. PCR efficiencies were determined in different rounds when 18s rRNA gene were used as reference genes. As the expression level of the studied genes was low to moderate, 18s rRNA gene was the most useful reference gene for this study.

Repeatability and reproducibility

Several control experiments were performed using the infected citrus by Las RNA to validate this semi-quantitative RT-PCR technique. The geldoc apparatus was evaluated by loading 10 aliquots prepared from one PCR co-amplification. The signal detected was found to be proportional to several cDNA dilutions added to the PCR mixture after each co-amplification with 18s rRNA gene (data not shown). Reproducibility of relative semi-quantification of gene expression level using 18s rRNA gene as the reference gene (genes/18s rRNA gene ratio) was evaluated at different PCR rounds. The genes/18s rRNA gene ratios were maintained during the co-amplification for a greater number of cycles than the range of log linear amplification phase. All subsequent analyses were thus performed in this range of PCR co-amplification efficiencies. The intra-assay reproducibility of PCR coamplifications performed with the same PCR round was evaluated and showed minimal variations in the genes/18s rRNA gene ratios. The between-assay reproducibility of RT-PCR co-amplifications was evaluated using different RT and PCR mixes. The determination of the PCR co-amplification round was determined after each RT as described in Materials and Methods. The coefficient of variation was evaluated for each ratio after co-amplifications in 3 to 5 independent RTPCRs.

NBS-LRR family gene mRNA expression levels in infected citrus by Las RNA

The expression pattern differences between different species (Figs 1) a week and a month after inoculation. As expected, NBS-LRR family gene was expressed in infected Mexican lime fourth time more than healthy one a month after inoculation. NBS-LRR family gene was detected at a low level in sweet orange a month after inoculation (Fig 1). These expression results were consistent with those has been established by grafting analysis, highlighting its over-expression in grapefruit and its low expression in sweet orange. These results have been confirmed by comparative analysis of these genes by specific primers (Fig 2). The results indicate that the expression of this gene in citrus fruits inoculated with the citrus greening pathogen changes after inoculation. NBS-LRR 552 expression increased 2, 3, 5 times in infected plant relative to healthy ones respectively in grandis, Mexican lime and grapefruit, but no modulate in sweet orange. These results in agreement with grafting analysis.

DISCUSSION

Recent technological advances in the detection of protein–protein interactions has led to the discovery of numerous new proteins involved in the plant resistance pathway. The preponderance of some proteins as compared with others should be important for determining resistance or tolerance mechanism (Ellis et al., 2000). To test the physiological and pathological potential of these proteins, molecular techniques are useful for evaluating their expression level in healthy and infected plant. We describe here a relative semiquantitative RT-PCR method

suitable for the detection of marked variations in the expression levels of NBS LRR family genes (Deng et al., 2000). It's a simple technique for screening many resistance genes in numerous samples.

Our results showed that semi-quantitative RT-PCR provides a rapid and reliable way of quantitating the amounts of the NBS-LRR mRNA present in small quantity using degenerate and specific primers.. Due to the resolution limit of ethidium bromide staining detection, a biorad apparatus was used and permitted the detection of fragments that did not appear after runs on 2% agarose or 5% acrylamide gels. Moreover, this method was easily reproducible and sensitive for detecting low mRNA expression. Despite the low expression of some genes, marked variations could be detected.

The results obtained in this study were largely in agreement with those previously obtained using grafting analysis (Hajivand et al. 2009 and 2011). However, in our hands the NBS-LRR genes, showed high mRNA expression in grapefruit, contrasting with the low resistance previously obtained by grafting analysis (Hajivand et al. 2009 and 2011). In sweet orange samples, NBS LRR semiquantitive results obtained at the mRNA level were consistent with those at the grafting.

This technique might be useful for detecting wide variations in NBS-LRR gene expression in resistance genes studies, and might lead to further experiments at the protein level for developing new biological markers.

REFERENCES

Barkley P.2004. Citrus diseases and disorders. 44 pp. ISBN 0 7313 1519 7.

Bove JM.2006. Huanglongbing: a destructive, newly-emerging, century-old disease of Citrus, J. Plant Pathol., 88, 7–37.

Capoor SP.1963. Decline of citrus trees in India. Bulletin National Institute of Science India, 24, 48-64.

Daire X, Clair D, Larrue JW, Boudon-Padieu E. 1997. Survey for grapevine yellows in diverse European countries and Israel. Vitis, 36, 53–54.

Deng Z, Huang S, Xiao S, Gmitter FG. Jr. 1997. Development and characterization of SCAR markers linked to the citrus tristiza virus resistance gene from Poncirus trifoliata. Genome, 40,697–704.

Ellis J, Dodds P, Pryor T. 2000. Structure, function and evolution of plant disease resistance genes. Curr. Opin. Plant Biol. 3, 278–284.

Ferguson WS, Castle SC. 2003. Considerations for Choosing the Right Rootstocks. Florida, Gainesville, Institute of Food and Agricultural Sciences, University of Florida.

FFTC.2003. Food & Fertilizer Technology Center for the Asian and pacific Region, Citrus Greening and Virus Diseases of Citrus, 2003-06-01 ed.

Hajivand S., Thohirah LA, Kamaruzaman S, Siti Nor Akmar A, Nur Ashikin PA. 2009. Differential reacation species in Malaysia to huanglongbing (HLB) disease grafting method, American Journal of Agricultural and Biological Sciences, 4(1), 32-38.

Hajivand Sh, Thohirah L, Kamaruzaman S, Siti Nor Akmar A.2011. Potential use of selected citrus rootstocks and interstocks against HLB disease in Malaysia, Crop Protection, 30, 521-525.

Hammond-Kosack KE, Jones JDG.1996. Resistance gene-dependent plant defense responses, Plant Cell, 8,1773–1791.

Hocquellet A, Toorawa P, Bove JM, Garnier M. 1999. Detection and identification of the two Candidatus Liberobacter species associated with citrus huanglongbing by PCR amplification of ribosomal protein genes of the h operon. Mol. Cell. Probes 13, 373-379.

Jagoueix S, Bové JM, Garnier M.1996. PCR detection of the two Candidatus Liberobacter species associated with greening disease of citrus. Mol. Cell. Probes, 10, 43– 50.

Kapur SP, Cheema SS, Chohan JS.1982. Evaluation of rough lemon strains and other rootstocks against greening-disease of citrus. Scientia Horticulturae, 18(1), 71-75.

O'Donnell PJ, Schmelz EA, Moussatche P, Lund ST, Jones JB, Klee HJ.2003. Susceptible to in tolerance range of hormonal actions in a susceptible Arabidopsis pathogen response, Plant J. 33, 245–257.

Zhao XY.1981. Citrus yellow shoot disease(huanglongbing)-a review. Int. Soc. of .Citricult., pages 466–469.