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RESEARCH ARTICLE

Expression analysis of *WRKY* genes from *Poncirus trifoliata* in response to pathogen infection

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The majority of WRKY transcription factors (TFs) play a role in the regulation of defense response in plants. Three *WRKY* genes, *PtrWRKY1, PtrWRKY2 and PtrWRKY3*, were previously identified in *Poncirus* and their expressions were characterized in response to cold and drought in *Poncirus* and *Citrus*. In this study, expressions of these *WRKY* genes were studied in response to infection with two major pathogens of *Citrus*, *Citrus tristeza virus* (CTV) and *Phytophthora citrophthora,* in resistant *Poncirus* and susceptible pummelo (*Citrus grandis*) plants. Northern blot analysis showed that the expression of the *PtrWRKY1* gene was induced earlier and stronger in *Poncirus* than in pummelo in response to CTV infection. On the other hand, the expression of the *PtrWRKY1* gene was not altered in response to *P. citrophthora* infection neither in *Poncirus* nor in pummelo. When the expression of the *PtrWRKY2* gene was analyzed, it was repressed by CTV inoculation in *Poncirus* plants, whereas the expression was not changed in response to CTV infection in pummelo or in response to *P. citrophthora* inoculation in *Poncirus* or pummelo. Similarly, the expression of the *PtrWRKY3* gene was repressed in *Poncirus,* but not changed in pummelo by CTV inoculation; however, the expression of the *PtrWRKY3* gene was induced in *Poncirus,* but it was repressed in pummelo in response to *P. citrophthora* inoculation. The expression analysis of three different *WRKY* genes revealed that they are differentially expressed in response to CTV and *P. citrophthora* infection in resistant *Poncirus* and susceptible pummelo suggesting that they may play a role in disease resistance in *Poncirus*.

Keywords: biotic stress; *citrus*; *citrus tristeza virus*; *phytophthora citrophthora*; WRKY transcription factor

1. Introduction

WRKY transcription factors (TFs) constitute one of the major groups of transcription factor families in plants. After the identification of the first WRKY protein, SPF1, from sweet potato (Ishiguro & Nakamura 1994), a number of *WRKY* genes were identified and characterized from different plant species, including *Arabidopsis thaliana* (Chen & Chen 2002), barley (Sun et al. 2003), tobacco (Hara et al. 2000), rice (Liu et al. 2007), parsley (Rushton et al. 1996), sugarcane (*Saccharum* hybrid cultivar) (Lambais 2001), canola (Yang et al. 2009), pepper (Oh et al. 2006), tomato (Hofmann et al. 2008), grapevine (Marchive et al. 2007) and *Poncirus trifoliata* (Şahin-Çevik 2012; Şahin-Çevik & Moore 2012; Şahin-Çevik et al. 2012). In addition, among plant species with completed genome sequences, *A. thaliana* has 74, rice (*Oryza sativa*) contains 109, and woody plant poplar (*Populus* spp.) includes 104 *WRKY* genes (Eulgem & Somssich 2007; Ross et al. 2007; Pandey & Somssich 2009).

WRKY TFs play roles in multiple developmental and physiological processes of plants, including senescence (Ulker et al. 2007), dormancy (Rohde et al. 2007), root development (Zhang et al. 2008), oxidative stress (Miller et al. 2008), metabolic pathways (Li et al. 2004), germination of seeds (Jiang & Yu 2009) and biotic and abiotic stresses (Mzid et al. 2007; Zheng et al. 2007; Zhou et al. 2008). Therefore, transcriptional regulation of *WRKY* genes in response to various biotic and abiotic stresses has been explored for a better understanding of their roles in adaptation and tolerance to these stresses and their involvement in the regulation of stress-responsive genes and pathways in *Arabidopsis* and other plants (Eulgem et al. 2000; Narusaka et al. 2004; Wu et al. 2005). Expression analysis of *WRKY* genes from different plants indicated that the majority of *WRKY* genes were regulated by abiotic stresses, such as cold, drought and salinity (Ramamoorthy et al. 2008; Jiang & Deyholos 2009) and biotic stresses, especially pathogen infection. Due to the economic importance of pathogen defense in plants, the majority of known well-characterized *WRKY* genes from *Arabidopsis* and other agricultural crops were studied in response to pathogen infection (Ryu et al. 2006). It has been shown that the expression of various *WRKY* genes from different plants were induced in response to infection with bacterial (Dellagi et al. 2000; Zheng et al. 2007), fungal (Dellagi et al. 2000; Zheng et al. 2006; Marchive et al. 2007) and viral (Yoda et al. 2002) plant pathogens. The results overall showed that *WRKY* genes are involved in the activation and regulation of

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the basal as well as pathogen-induced defense pathways in plants, and that while some are regulated by both abiotic and biotic stresses, others are only involved in biotic stress.

Citrus is one of the most widely grown and economically important fruit crops in tropical and subtropical regions of the world. Production of citrus is limited mostly by cold and diseases, such as tristeza and *Phytophthora*. The tristeza disease caused by *Citrus tristeza virus* (CTV) is one of the most economically important viral diseases of *Citrus*. CTV is a single-stranded positive-sense RNA virus belonging to *Closterovius* genus in the *Closterovridae* family (Bar-Joseph & Lee 1990). It has long thread-like, flexuous, filamentous particles about 2000 nm by 11 nm consisting of a 20 kb single-stranded positive-sense RNA molecule and the major and minor capsid proteins (Febres et al. 1996). Depending on the citrus host and scion-rootstock combinations, CTV causes various symptoms ranging from mild vein clearing to quick decline, killing all citrus varieties grafted in sour orange rootstock (Lee & Bar-Joseph 2000). CTV has caused severe epidemics in various citrus growing regions and killed millions of citrus trees in the South-American and the Mediterranean countries (Bar-Joseph et al. 1989; Moreno et al. 2008). The disease is still a major problem in all citrus growing regions of the world (Moreno et al. 2008). *Phytophthora* species are also among the most common and serious pathogens of citrus. They cause diseases in young plants, as well as in older trees and fruits. Damping-off in the seedbeds, foot and root rot and gummosis in the nurseries, and foot rot, gummosis, feeder root rot and brown rot in the orchards are the main diseases caused by *Phytophthora* species resulting in serious crop losses (Timmer & Menge 1988). *P. citrophthora* is the most important species in the *Phytophthora* complex, infecting citrus under moderate temperatures (Erwin & Ribeiro 1996).

Most commercial *Citrus* species are susceptible to tristeza disease caused by CTV, and foot, root and brown rot diseases caused by *Phytophthora* ssp., especially *P. citrophthora*. On the other hand, *Poncirus trifoliata*, a close relative of *Citrus*, is not only cold-hardy, but also resistant to CTV and *Phytophthora ssp*. Therefore, *P. trifoliata* has been used in citrus breeding programs for improving abiotic and biotic stress tolerance of *Citrus*. Hybrids between *Citrus* and *Poncirus* enabled generation of stress-tolerant rootstocks, however, the mechanisms of abiotic and biotic stress tolerance in *Poncirus* have not been explored in depth. Although CTV resistance in *P. trifoliata* is known to be controlled by a single dominant gene (Gmitter et al. 1996; Fang et al. 1998), the resistance gene has not been cloned and the mechanisms of the resistance are not known in this species. On the other hand, resistance to *Phytophthora* ssp. has not been studied extensively and the genetic and molecular bases of *Phytophthora* resistance in *P. trifoliata* are not known.

We have previously identified and isolated three different WRKY TFs, *Ptr*WRKY1, *Ptr*WRKY2 and *Ptr*WRKY3, from two-day cold-acclimated *P. trifoliata* and the expression of these genes in response to cold and drought stresses was analyzed in *Poncirus* and pummelo (*Citrus grandis*) (Şahin-Çevik 2012; Şahin-Çevik & Moore 2012; Şahin-Çevik et al. 2012). Since, some WRKY TFs have roles in both abiotic and biotic stresses, the expression patterns of these three genes were analyzed in this study in response to infection with CTV and *P. citrophthora* in *P. trifoliata* which is resistant, and pummelo which is susceptible to both pathogens.

2. Materials and methods

2.1. Plant materials

To study the changes in the expression of three *WRKY* genes in disease-resistant and susceptible plants, we selected *P. trifoliata* cv. Rubidoux (trifoliate orange) which is resistant to both CTV and *P. citrophthora* and *C. grandis* cv. Reinking which is considered susceptible to both CTV and *P. citrophthora*. Seeds of *P. trifoliata* and *C. grandis* were obtained from the National Clonal Germplasm Repository for Citrus and Dates in Riverside, CA, USA, or extracted from fruits produced in the Çukurova University Subtropical Fruits Research and Application Center in Adana, Turkey. The seeds were planted in a soilless medium in pots and seedlings were grown and maintained in a growth chamber at 28°C and 16-h photoperiod provided by cool white fluorescent light (100 μmol m^{-2} s^{-1}).

2.2. Pathogen inoculation

2.2.1. Citrus tristeza virus (CTV) inoculation

A previously described *Citrus tristeza virus* (CTV) isolate, EG-5 (EK-1), recovered from Satsuma mandarins in the Edremit Gulf region of Turkey (Korkmaz et al. 2008), and maintained on Madam Vinous sweet orange (*Citrus sinensis* cv. Madam Vinous) was used for CTV inoculation of trifoliate orange and pummelo seedlings. Ten seedlings with a stem diameter of about 1 cm were selected from 18-month-old trifoliate orange and pummelo seedlings maintained in a growth chamber at 28°C and 16-h photoperiod. Five seedlings from each species were graft inoculated at 10 cm above the soil line with buds taken from a Madam Vinous seedling infected with CTV isolate. The remaining five seedlings were grafted with buds from a healthy sweet orange as controls for wounding (mock inoculation). At least three leaves at different positions above the inoculation sites were collected from each CTV- and mock-inoculated seedlings just before graft inoculation, and 1, 2, 4, 8 and 15 days after graft inoculation. Leaf

samples at each time point were bulked and immediately frozen in liquid nitrogen and stored at –80°C until used for total RNA isolation. CTV- and mock-inoculated *P. trifoliata* and pummelo plants were tested for the presence of CTV in 15 weeks post-inoculation (wpi) using the RT-PCR method as previously described (Korkmaz et al. 2008).

2.2.2. *Phytophthora citrophthora inoculation*

A *P. citrophthora* isolate recovered from rotting orange fruit was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures) and grown on CMA (Corn Meal Agar, Fluka Analytical, Switzerland) amended with antibiotics (pimaricin 10 μg/ml, penicillin 50 μg/ml, polymyxin 50 μg/ml). Ten seedlings with a stem diameter greater than 0.5 cm were selected from 18-month-old trifoliate orange and pummelo seedlings maintained in a growth chamber at 28°C and 16-h photoperiod. Five seedlings from each species were inoculated with mycelial inoculums of *P. citrophthora* grown on solid CMA medium (Ippolito et al. 1992). The stems of seedlings were inoculated by attaching a 4 mm diameter agar plug with mycelia removed from the edge of an actively growing 5-day-old culture into the wound made by cutting a small bark piece with a razor 10 cm above the soil line. The wounds were wrapped with parafilm to prevent desiccation (Ippolito et al. 1992). The remaining five seedlings were mock-inoculated with agar discs taken from the CMA solid medium without *P. citrophthora*. At least three leaves at different positions above the inoculation sites were collected from each *P. citrophthora* and mock-inoculated seedlings, just before inoculation, and 1, 2, 4, 8 and 15 days after inoculation. Leaf samples taken at each time point were bulked and immediately frozen in liquid nitrogen and stored at –80°C until use, for total RNA isolation.

2.3. *Total RNA isolation*

Total RNA was isolated from the leaf samples of inoculated and mock-inoculated *P. trifoliata* and pummelo seedlings using Trizol Reagents (Invitrogen, USA) according to the manufacturer's instructions. Total RNA concentration was measured by a UV-visible spectrophotometer (BioRad, USA) and the samples were stored at –80°C until use.

2.4. *Northern blot hybridizations*

The regions for gene-specific probes for the *PtrWRKY1, PtrWRKY2*, *PtrWRKY3* and *18S* ribosomal RNA (rRNA) genes were first amplified from a cDNA clone by PCR with the Advantage II DNA polymerase using a gene-specific forward primer and a gene-specific reverse primer with T7 promoter (Şahin-Çevik 2012; Şahin-Çevik & Moore 2012; Şahin-Çevik et al. 2012). The PCR-amplified gene fragments with T7 promoter sequence at 3′ end of the antisense strand were purified by Qiaquick PCR purification kit (Qiagen, Germany), denatured and used as the template for synthesis of DIG-labeled antisense riboprobes specific to each *WRKY* gene and *18S rRNA* using a DIG RNA labeling kit (Roche, Germany) according to the manufacturer's instructions.

About 3 μg of total RNA samples isolated from inoculated plants and mock-inoculated control plants of *Poncirus* and pummelo were separated on denaturing agarose gels and transferred to nylon membranes. The membranes were pre-hybridized at 68°C for 30 min and hybridized with DIG-labeled antisense RNA probes specific to the 3′ half of the three *WRKY* genes, *PtrWRKY1, PtrWRKY2* and *PtrWRKY3* isolated from *Poncirus*, prepared using a DIG RNA labeling kit (Roche, Germany) at 68°C for 16 h. The membranes were washed two times with 2X SSC and 0.1% SDS at room temperature for 5 min, followed by two washes with 0.1X SSC and 0.1% SDS at 68°C for 15 min. The membranes were then subjected to detection of DIG-labeled RNA probes to detect RNA targets on northern blots using the DIG chemiluminescent detection kit (Roche, Germany) with the CSPD-Star substrate. After hybridizations with the gene specific probe, the membranes were re-hybridized with an 18S ribosomal RNA (rRNA) probe for loading and transfer control. The hybridization reactions were visualized by the ChemiDoc-It (UVP, England) chemiluminescent imaging system. Northern blot experiments were repeated two times for each gene. The expressions of *WRKY* and *18S rRNA* genes were measured and quantified by the LabWorks (UVP, England) image analysis software.

2.5. *Expression analysis*

To analyze the quantitative expression data for *WRKY* genes in response to pathogen infection, the measured expression values of each *WRKY* gene were first normalized by dividing the transcript amount of the 18S rRNA gene at all time points. Then, the changes in the expression analysis defined as percent induction or repression for all three *WRKY* genes were calculated by subtraction of the determined average expression value of the control (0 h) from the average expression values of different time points of stress treatment and multiplication by 100. The calculated expression level of *WRKY* genes in response to pathogen and mock inoculations were graphically presented with northern blot images. The normalized expression data were tested for significance of changes in the gene expression at different day post inoculations (dpi) for CTV, *Phytophthora* and mock inoculations by comparing the means using Duncan's multiple range test (at $P \leq 0.05$) for each gene and each application in *P. trifoliata* and *C. grandis* independently. An unpaired *t*-test was conducted to compare the expression of *WRKY* genes

in response to pathogen and mock inoculations for each time point in *P. trifoliata* and *C. grandis* separately. The differences of gene expression were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. Expression analysis of the WRKY genes in response to CTV infection

Quantification and statistical analysis of the *PtrWRKY1* gene expression data showed that the expression was significantly increased at 4–15 dpi in CTV-inoculated *Poncirus* plants. However, no significant changes in the expression of the *PtrWRKY1* gene were observed until 15 dpi in mock-inoculated *Poncirus* plants (Figure 1a, upper panel; Table S1[1]). On the other hand, the expression of this gene was induced significantly only at 8 dpi and 15 dpi in CTV-infected pummelo plants, but no significant changes were observed in the expression of this gene in mock-inoculated pummelo plants (Figure 1a, lower panel; Table S1). The results demonstrated that the *PtrWRKY1* gene is responsive to both virus infection and wounding in *Poncirus*, but only to virus infection in pummelo. These results also implied that the expression of the *PtrWRKY1* gene was induced earlier and stronger in *Poncirus* compared to pummelo.

When the expression of the *PtrWRKY2* gene was analyzed in response to CTV infection, this gene was constitutively expressed in non-inoculated *Poncirus* and pummelo control plants. While the expression of

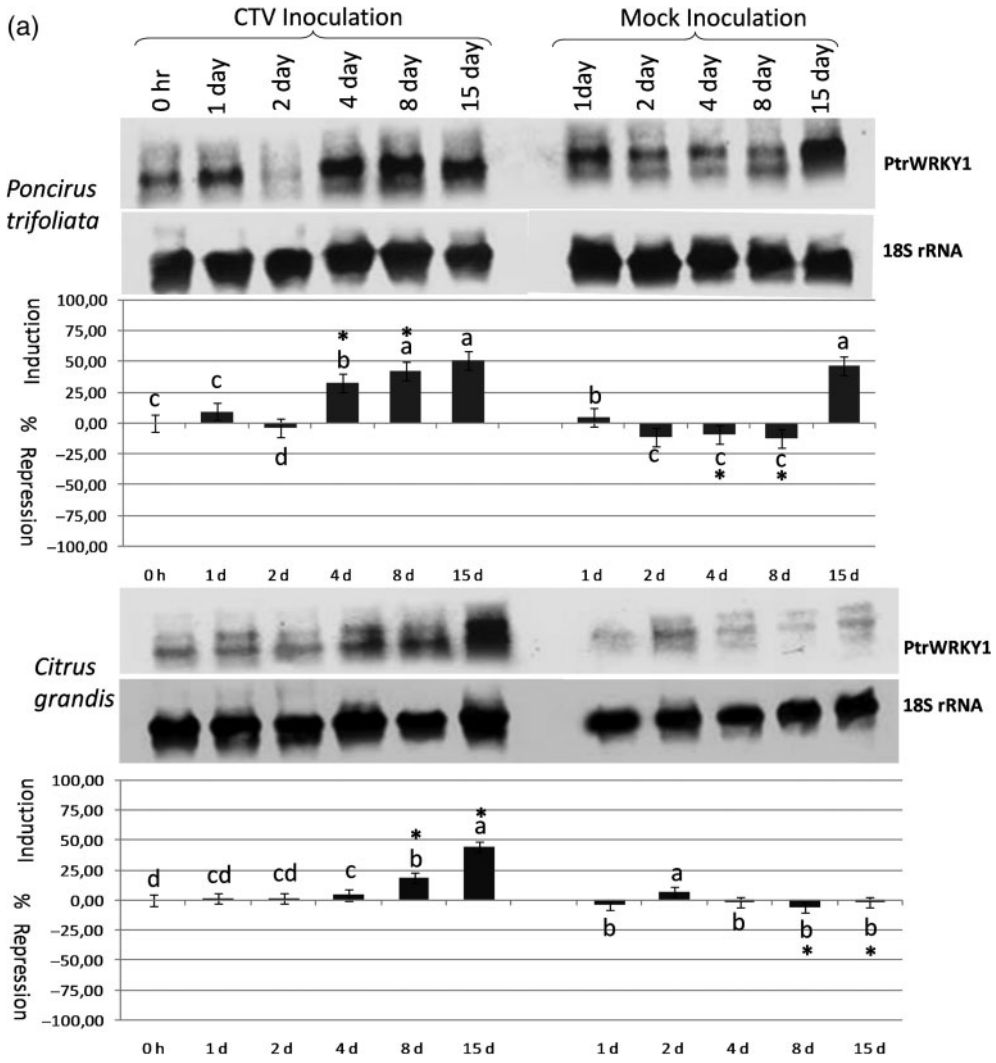

Figure 1a. The expression analysis of the *PtrWRKY1*(a), *PtrWRKY2* (b), and *PtrWRKY3* (c) gene in response to *Citrus tristeza virus* infection in *Poncirus trifoliata* and *Citrus grandis* (pummelo) detected by antisense DIG-labeled riboprobe specific to each gene shown on the right. The type and duration of inoculation are indicated on the top and inoculated plant species are shown on the left. The expression of the *18S rRNA* gene was used as a loading and transfer control and is shown below the expression of each gene. Quantification of the expression data was presented graphically under the northern blots for each gene. The error bar indicates the standard deviation of percent change in gene expression for each time point. Letters indicate groups determined by Duncan's multiple rage test (at $P \leq 0.05$) used for comparisons of the mean expression values of each gene at different time points for CTV inoculation and mock inoculation independently in *P. trifoliata* and *C. grandis*. *Statistically significant changes in the expression of the *WRKY* genes between CTV-inoculated and mock-inoculated plants.

this gene was repressed at 1–15 dpi in CTV-infected *Poncirus* plants, it was induced in mock-inoculated *Poncirus* plants at all time points (Figure 1b, upper panel; Table S1). These results demonstrated that the expression of the *PtrWRKY2* gene was increased in response to wounding, whereas it was repressed in response to CTV infection in *Poncirus* (Table 1). Although expression of the *PtrWRKY2* gene was induced slightly at 2 dpi, no significant changes in the expression were observed at other time points in CTV-infected pummelo plants (Figure 1b, lower panel; Table S1). In contrast, the expression was induced very slightly at 1, 2, 8, 15 dpi, but repressed only at 4 dpi in mock-inoculated pummelo plants (Figure 1b, lower panel; Table S1) suggesting a wounding responsive gene expression. The result of the expression analyses showed a differential response of PtrWRKY2 to CTV infection and wounding in both *Poncirus* and pummel, while the expression was increased in mock-inoculated ones, but decreased in CTV-inoculated *Poncirus*.

The *PtrWRKY3* gene was weakly induced at 1, 4 and 8 dpi, but it was repressed to the same degree at 2 and 15 dpi in CTV-infected *Poncirus* plants. However, the expression of the *PtrWRKY3* gene was induced at all time points in mock-inoculated

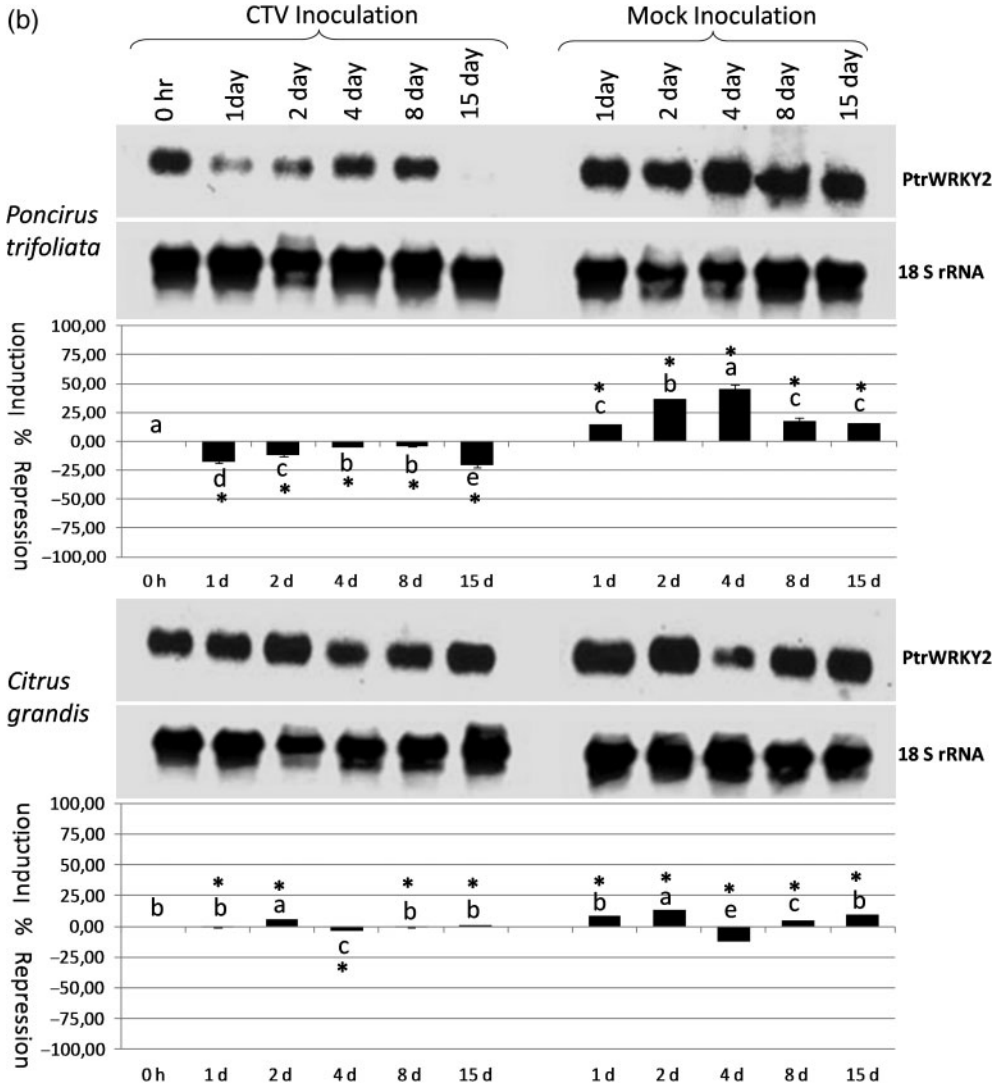

Figure 1b. (Continued)

Table 1. Summary of expressional response of three inoculations and wounding in *Poncirus trifoliata* and *Citrus grandis*.

	Poncirus trifoliata			*Citrus grandis*		
	CTV	*Phytophthora*	Wounding	CTV	*Phytophthora*	Wounding
PtrWRKY1	Induced	Repressed	Induced	Induced	Repressed	Induced
PtrWRKY2	Repressed	Repressed	Induced	Unchanged	Repressed	Repressed
PtrWRKY3	Repressed	Induced	Induced	Unchanged	Repressed	Repressed

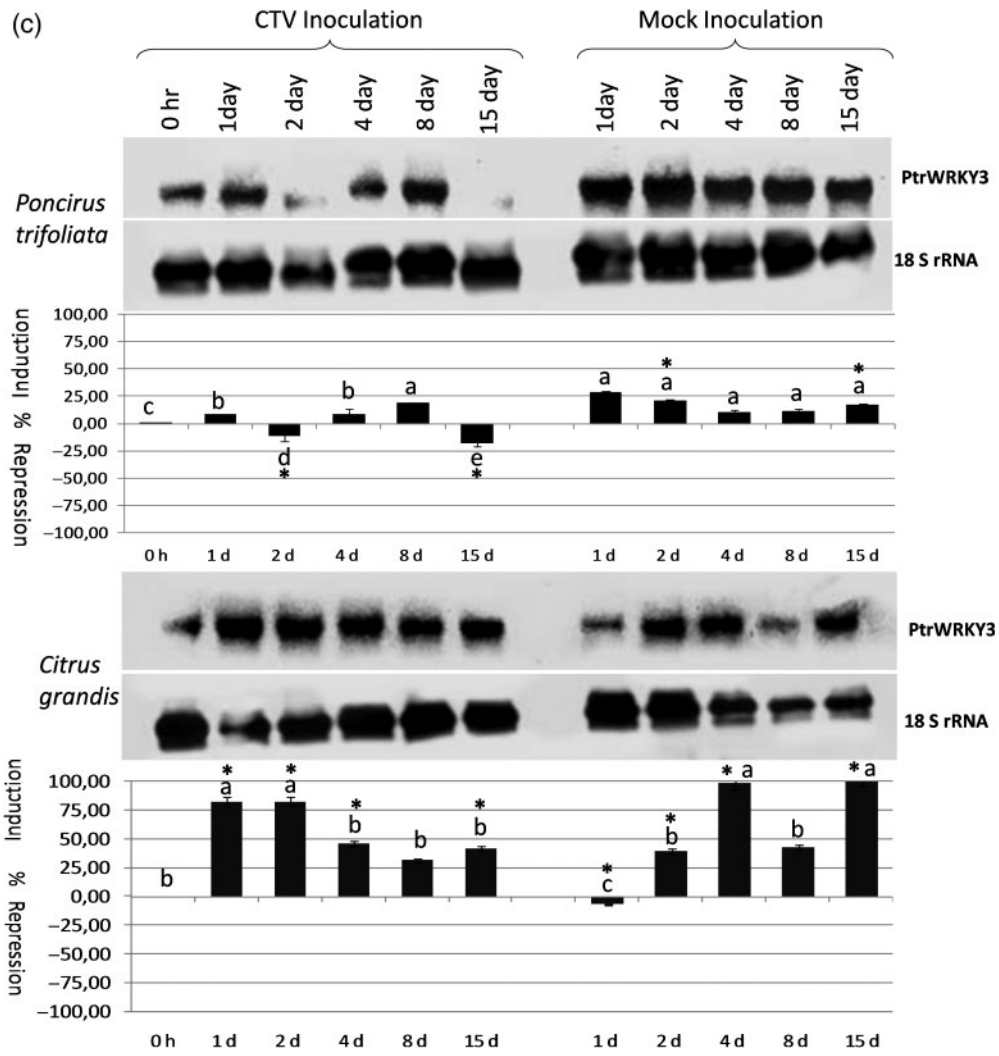

Figure 1c. (Continued)

Poncirus plants (Figure 1c, upper panel; Table S1) suggesting that this gene was induced in response to wounding in *Poncirus*. The statistical analysis of the expression in CTV and mock-inoculated *Poncirus* plants showed that changes were significant only at 2 and 15 dpi where the expression was repressed by CTV inoculation. On the other hand, it was significantly induced throughout the experiment in both CTV and mock-inoculated pummelo plants, except 1 d after mock inoculation (Figure 1c, lower panel; Table S1), suggesting that induction of the expression was not in response to CTV inoculation, but in response to wounding in pummelo. Overall expression analyses showed that the *PtrWRKY3* gene was induced in response to wounding in both *Poncirus* and pummelo plants, but it was repressed by CTV inoculation only in CTV-resistant *Poncirus*.

To determine the occurrence of CTV inoculation in the grafted plants, mock- and CTV-inoculated plants were tested for the presence of CTV at 15 dpi and 15 wpi by RT-PCR. CTV was not detected in any of the mock- or CTV-inoculated *Poncirus* and pummelo plants tested at 15 dpi, which is considered as the early stage of infection. However, while CTV was detected only in CTV-inoculated pummelo plants, none of the mock-inoculated pummelo plants and none of the mock- and CTV-inoculated *Poncirus* plants were infected with CTV 15 wpi, which is considered as the late stage of infection (data not shown).

3.2. Expression of the PtrWRKY genes in response to P. citrophthora infection

Quantification and statistical analyses of the *PtrWRKY1* gene expression in response to *P. citrophthora* infection showed repression at 4–15 dpi in *P. citrophthora*-inoculated *Poncirus*. However, it was induced at 4–15 dpi in mock-inoculated *Poncirus* plants (Figure 2a, upper panel; Table S2). The expression of the *PtrWRKY1* gene was decreased significantly at 4 and 15 dpi in response to *P. citrophthora* infection in pummelo. On the other hand, while the expression of this gene was significantly repressed at 4 dpi, it was significantly induced at 8 and 15 dpi in mock-inoculated pummelo plants. The results suggested that the expression of the *PtrWRKY1* gene is repressed by *P. citrophthora* infection, but it is induced in response to wounding.

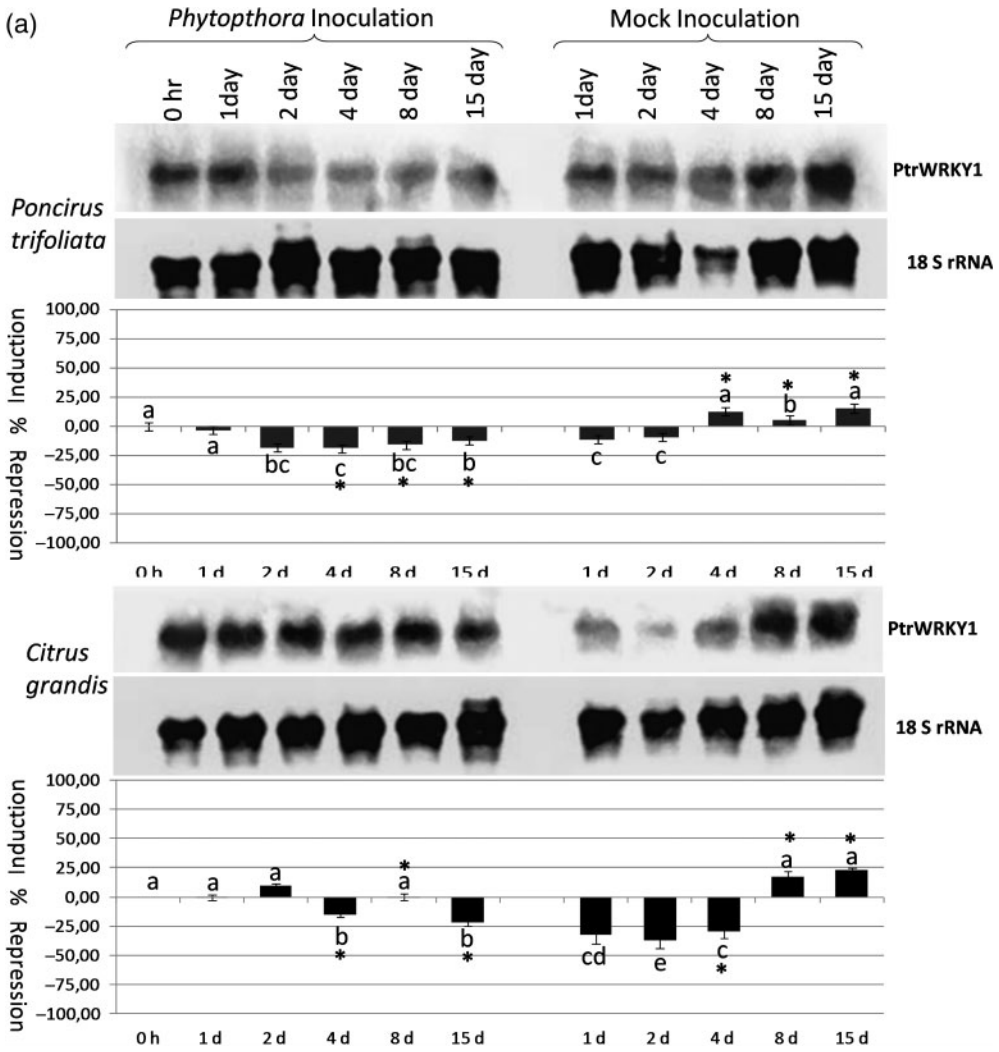

Figure 2a. The expression analysis of the *PtrWRKY1* (a), *PtrWRKY2* (b), and *PtrWRKY3* (c) gene in response to *Phytophthora citrophthora* infection in *Poncirus trifoliata* and *Citrus grandis* (pummelo) detected by antisense DIG-labeled riboprobe specific to each gene shown on the right. The type and duration of inoculation are indicated on the top and inoculated plant species are shown on the left. The expression of *18S rRNA* gene was used as a loading and transfer control and is shown below the expression of each gene. Quantification of the expression data was presented under the northern blot. The error bar indicates the standard deviation of percent change in gene expression for each time point. Letters indicate groups determined by Duncan's multiple rage test (at $P \leq 0.05$) used for comparisons of the mean expression values of each gene at different time points for *P. citrophthora* inoculation and mock inoculation independently in *P. trifoliata* and *C. grandis*. *Statistically significant changes in the expression of *WRKY* genes in *Phytophthora*-inoculated and mock-inoculated plants.

The expression analyses of the *PtrWRKY2* gene in *P. citrophthora*- and mock-inoculated *Poncirus* plants revealed no significant changes except at 4 dpi, when the expression was repressed in mock-inoculated *Poncirus* plants (Figure 2b, upper panel; Table S2). In contrast, the expression of this gene was significantly changed at all time points except at 4 dpi in *P. citrophthora* and mock-inoculated pummelo plants. The expression was only repressed at 2 and 4 dpi in *P. citrophthora*-inoculated pummelo plants, whereas it was significantly repressed at 1–15 dpi in mock-inoculated pummelo plants (Figure 2b, lower panel; Table S2). These results suggested that changes in the expression of the *PtrWRKY2* gene were mostly due to wounding rather than *P. citrophthora* infection in *Phytophthora*-susceptible pummelo plants.

The expression of the *PtrWRKY3* gene was induced gradually from 1 to 15 dpi in *P. citrophthora*-inoculated *Poncirus* plants; however, it was induced only at 8 and 15 dpi in mock-inoculated *Poncirus* (Figure 2c, upper panel; Table S2). Statistical analysis showed the *PtrWRKY3* gene was induced significantly at 1–8 dpi in response to *P. citrophthora* inoculation in *Poncirus*, but it was induced by wounding at 15 dpi in both *P. citrophthora* and mock-inoculated plants. On the other hand, while the expression of the *PtrWRKY3* gene was repressed significantly at 15 dpi in *P. citrophthora*-inoculated pummelo plants, it was repressed at all time points at

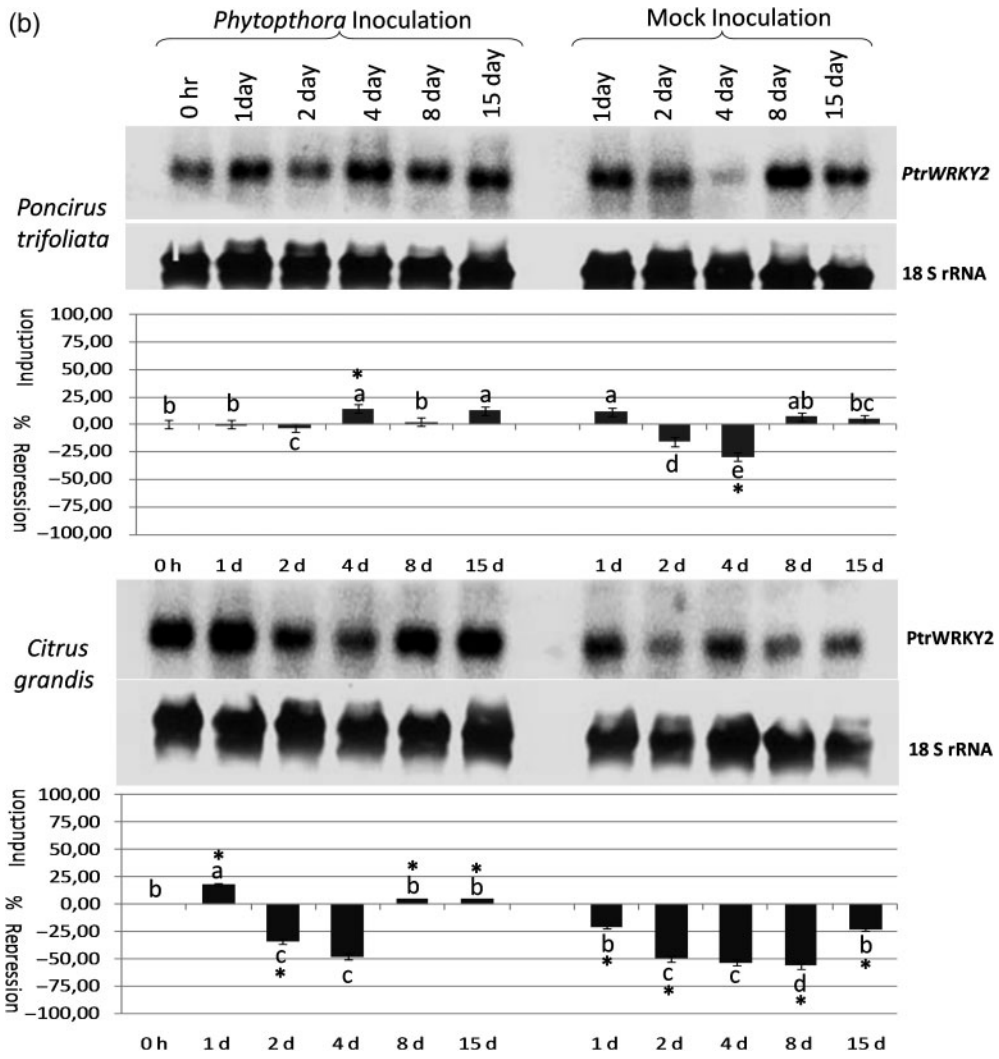

Figure 2b. (Continued)

different degrees in mock-inoculated pummelo plants (Figure 2C, lower panel; Table S2). Statistical analysis revealed that the *PtrWRKY3* gene was repressed significantly at 1–8 dpi only by wounding in pummelo plants (Table S2). These results showed that the *PtrWRKY3* gene was induced in response to *P. citrophthora* inoculation in *Phytophthora*-resistant *Poncirus*, but it was repressed by wounding in *Phytophthora*-susceptible pummelo plants.

4. Discussion

To understand the roles of WRKY TFs during pathogen infection in *Poncirus* and *Citrus*, the expressions of three *WRKY* genes, *PtrWRKY1, PtrWRKY2* and *PtrWRKY3* from *Poncirus* were analyzed in response to CTV, and *P. citrophthora* inoculation in *P. trifoliata* cv. Rubidoux which is resistant to both pathogens, and *Citrus grandis* cv. Reinking which is considered susceptible to both pathogens, were selected in this study. Although Mexican lime and sweet orange are the most susceptible *Citrus* species to CTV, pummelo was selected as susceptible host due to its relative sensitivity to both pathogens, as well as cold and drought stresses used for initial characterization of these *WRKY* genes. It was previously reported that some *Citrus grandis* cv. Chandler was resistant to CTV (Fang & Roose 1999), but other pummelo cultivars showed differential susceptibility to different CTV isolates (Garnsey et al. 1996). Other reports indicated that CTV is able to infect most pummelo cultivars (Xueyuan et al. 2002), and our RT-PCR results also showed that the CTV isolate used in this study was able to infect and replicate in pummelo.

The expression analyses of three different *WRKY* genes showed differential responses to these pathogens in two different hosts (Table 1). While the *PtrWRKY1* gene was induced in CTV-resistant *Poncirus*, the *PtrWRKY2* and the *PtrWRKY3* genes were repressed by CTV infection and induced by wounding in both *Poncirus* and pummelo plants. The expressions of the *PtrWRKY1* and *PtrWRKY2* genes were repressed or not changed in response to *P. citrophthora* inoculation in both *Poncirus* and pummelo plants, but they were repressed or induced in response to wounding in both *Poncirus* and pummelo plants. However, the *PtrWRKY3* gene was

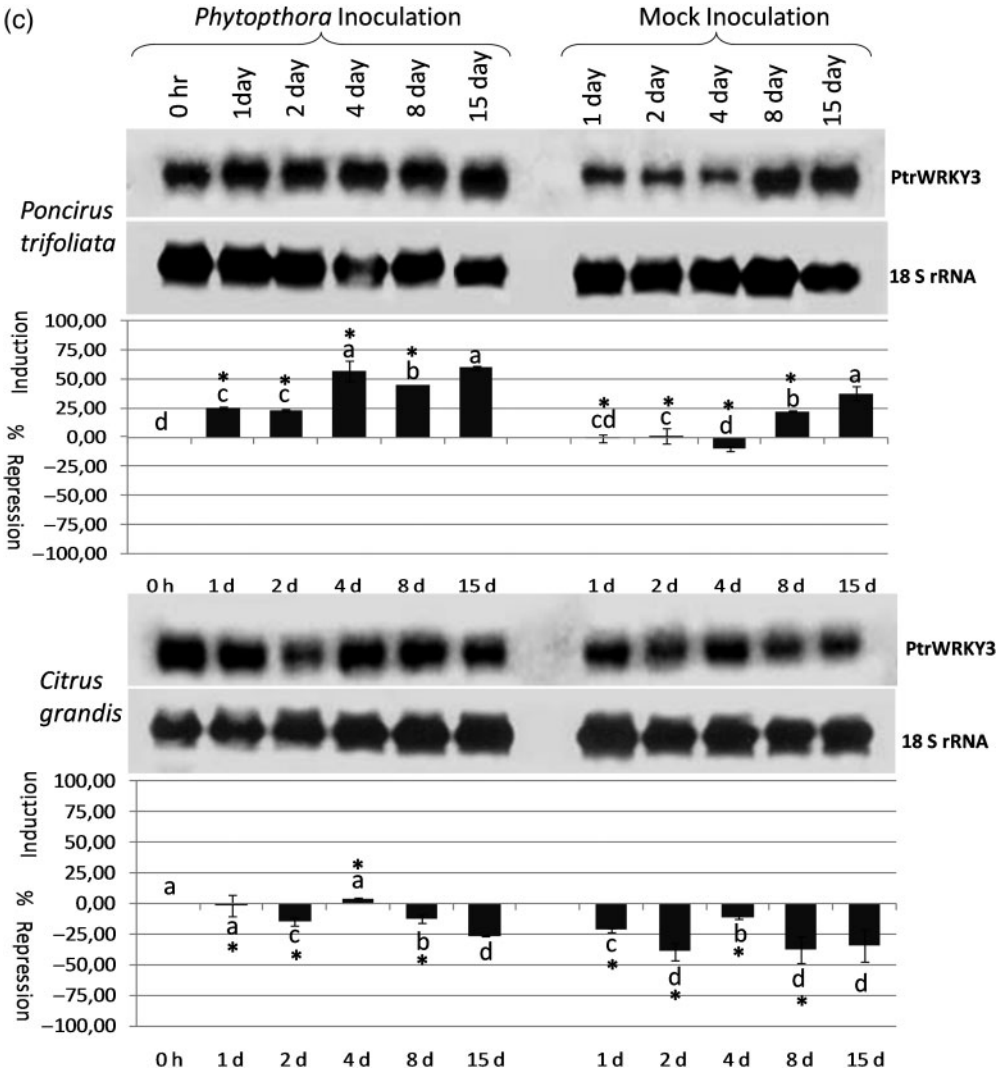

Figure 2c. (Continued)

induced in response to *P. citrophthora* inoculation in resistant *Poncirus*, but was repressed in response to *P. citrophthora* infection in susceptible pummelo.

WRKY proteins are grouped into three classes based on the number of WRKY domains. A number of WRKY TFs with a single WRKY domain were classified into Group II, including *Arabidopsis* WRKY33 (Zheng et al. 2006). This group contains a single WRKY domain followed by Cys_2His_2 motif, as the *PtrWRKY1* gene used in this study. For this reason, the *PtrWRKY1* gene belongs to the Group II proteins, but phylogenetic analysis showed that the WRKY domain is closely related to N-terminal WRKY domain Group I WRKY TFs (Şahin-Çevik & Moore 2012). Unlike PtrWRKY1, PtrWRKY2 and PtrWRKY3 which contain two WRKY DNA binding domains are classified as Group I WRKY TFs (Şahin-Çevik 2012; Şahin-Çevik et al. 2012). The majority of defense-related WRKY proteins isolated from crop plants, including parsley WRKY1, *Arabidopsis* ZAP1, pepper *Ca*WRKY2 and tomato *Lp*WRKY1 contain two WRKY domains and belong to Group I WRKY proteins (de Pater et al. 1996; Eulgem et al. 1999; Oh et al. 2006; Hofmann et al. 2008). The expression data showing responsiveness of the *WRKY* genes of the present study to pathogen infection in *Poncirus* and *Citrus*, supports our previous findings, indicating that these *WRKY* genes are phylogenetically related to defense-responsive WRKY proteins from other plants.

The *PtrWRKY1* gene was induced upon infection with CTV in *Poncirus* and to some extent, in pummelo plants. However, the expression was first induced at 4 dpi in CTV-resistant *Poncirus* plants, but at 8 dpi in CTV-susceptible pummelo plants. In addition, the expression of the *PtrWRKY1* gene was also increased in response to wounding in *Poncirus*, but this change was not detected until 15 dpi. These results indicated that the expression of the *PtrWRKY1* gene was induced earlier in the infection period to a greater extent in CTV-resistant *Poncirus* than in pummelo. On the other hand, the expression of the *PtrWRKY3* gene was induced in response to *P. citrophthora* infection and wounding in *Poncirus*. Many WRKY TFs, including WRKY3, WRKY4, WRKY18, WRKY22, WRKY29, WRKY25,

WRKY33, WRKY40 and WRKY60 from *Arabidopsis* (Asai et al. 2002; Zheng et al. 2006; Xu et al. 2006; Zheng et al. 2007; Lai et al. 2008), TIZZ from tobacco (Yoda et al. 2002), *Ca*WRKY-a (Park et al. 2006) and *Ca*WRKY2 (Oh et al. 2006) from pepper, and *Vv*WRKY1 and *Vv*WRKY2 from grapevine (Marchive et. al. 2007) are induced in response to pathogen infections. The involvement of some of the *WRKY* genes in plant defense and resistance to specific pathogens was experimentally demonstrated, suggesting that the *PtrWRKY1* gene induced in response to CTV infection and the *PtrWRKY3* gene induced in response to *P. citrophthora* infection may contribute resistance to these pathogens in *Poncirus*.

The *PtrWRKY2* and *PtrWRKY3* genes were both induced in response to wounding in both *Poncirus* and pummelo, but did not change or were repressed in response to CTV infection in *Poncirus* and pummelo. However, the timing of repression was much earlier and the degree of repression was much higher in CTV-resistant *Poncirus* than the CTV-susceptible pummelo, suggesting that they may be negative regulators of CTV resistance in *Poncirus*. While some WRKY proteins are induced in response to pathogen infection and act as a positive regulator of pathogen defense, others including WRKY11, WRKY17 and WRKY48 from *Arabidopsis* and OsWRKY62 from rice were repressed, and their involvement in negative regulation of disease resistance in respective plants was demonstrated (Journot-Catalino et al. 2006; Xu et al. 2006; Zheng et al. 2007; Xing et al. 2008). Based on the expression data showing repression of the *PtrWRKY2* and *PtrWRKY3* genes in CTV-resistant *Poncirus*, but not in CTV-susceptible pummelo, it may be speculated that three TFs may be involved in the negative regulation of CTV resistance in *Poncirus*.

Most of *Arabidopsis WRKY* genes having a role in the regulation of the defense response were induced as early as 2 or 4 h after pathogen-infection or application of defense-related hormones, such as salicylic acid (SA), methyl jasmonic acid (JA) or ethylene (ET). Their expression was decreased by 24 h after infection or elicitor treatment suggesting that they are involved in early transcriptional activation (Yoda et al. 2002; Dong et al. 2003; Oh et al. 2006). In this study, the first transcriptional response of *Poncirus WRKY* genes was measured starting at 1 d after inoculation with pathogens or wounding in *Poncirus* and pummelo plants, and this induction continued over 15 d. In this aspect, this study is the first report examining the expression of *WRKY* genes in response to wounding and pathogen infection for an extended period. Studying the earlier response of *WRKY* genes of *Poncirus* to pathogen infection may provide a better understanding of the physiological function of the corresponding proteins. The difference in induction pattern in response to wounding and pathogen infection in *Arabidopsis* and *Citrus* could simply result from the functions of different *WRKY* genes or differences in the host-pathogen systems.

The expression of *Arabidopsis* WRKY3 and WRKY4 TFs were rapidly induced after infection with the fungal pathogen *Botrytis cinerea*, as well as treatment with signal molecules, such as H_2O_2, SA, JA or 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ET biosynthesis (Lai et al. 2008). Therefore, the expression of the *PtrWRKY1*, *PtrWRKY2* and *PtrWRKY3* genes should also be explored after elicitor treatments.

In this study, changes in the expression of the *PtrWRKY1*, *PtrWRKY2* and *PtrWRKY3* genes were determined in response to two different pathogens in *Poncirus* and *Citrus* species, pummelo. This is the first report of the expression analysis of WRKY TFs in response to pathogen infection in an economically important perennial crop plant. Since the involvement of these three WRKY TFs in cold and drought stress response has previously been shown, this study extended to the disease response of these genes. It provides a more comprehensive understanding of the involvement of *Poncirus* WRKY TFs in environmental stress response in *Poncirus* and *Citrus*. However, further studies are needed for a better understanding of the functions and specific involvements of each *WRKY* gene in abiotic and biotic stress response in *Poncirus*, and their possible use for improving biotic and abiotic stress tolerance in commercial *Citrus* species.

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Note

1. Supplemental Content may be viewed online at http://dx.doi.org/10.1080/17429145.2013.796596

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