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A positive regulatory role of the watermelon *ClWRKY70* gene for disease resistance in transgenic *Arabidopsis thaliana*

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Abstract

A pathogen-inducible *WRKY* cDNA was cloned from the leaves of watermelon (*Citrullus lanatus*) seedlings 24 h after inoculation with *Cladosporium cucumerinum*. The deduced protein of the gene, designated as ClWRKY70, was classified as a group III WRKY protein based on its single WRKY domain containing a Cys2HisCys zinc-finger motif. Its *Arabidopsis thaliana* sequence homologue (AtWRKY70) has been described as playing an important role in the plant defense response. *ClWRKY70* gene transcripts were highly accumulated in watermelon by salicylic acid treatment, but not by jasmonic acid. By evaluating target gene expression in transgenic *Arabidopsis* overexpressing the *ClWRKY70* gene, it is suggested that the watermelon *WRKY* gene may play a positive regulatory role in plant resistance against pathogen attack.

Additional key words: Botrytis cinerea, Citrullus lanatus, *Cladosporium cucumerinum, Erwinia carotovora*, jasmonic acid, salicylic acid.

Introduction

Cladosporium cucumerinum Ellis *et* Authur causes scab disease in cucurbit plants including watermelon (*Citrullus lanatus*) (Walker 1950, Pierson and Walker 1954, Takanashi and Iwata 1964, Kwon *et al*. 1999). Currently, there have been no watermelon cultivars completely resistant to *C. cucumerinum*. Therefore, the generation of resistant cultivars is a priority for most watermelon breeding programs.

In general, plants can reprogram their transcriptome in a highly dynamic manner in response to pathogen attack. This regulation is mainly achieved by a network of various transcription factors including *WRKY* genes, which encode a large family of regulatory proteins (Eulgem and Somssich 2007). The WRKY domain binds specifically to various W-box elements (TTGAC) that have been identified in the promoters of defense-related genes, such as pathogensis-related (PR) genes (Maleck *et al*. 2000). Therefore, it can be highly valuable for molecular crop breeding programs to identify *WRKY* genes that are involved in a complex defense response network.

The WRKY super-family consists of 74 members in *Arabidopsis*, the majority of which respond to pathogen attack, and it was demonstrated that WRKY proteins act as positive and as negative regulator in a complex defense response network (Eulgem and Somssich 2007). Among *Arabidopsis* WRKY proteins, AtWRKY70 plays a complex role, acting as a positive regulator of salicylic acid (SA) dependent defense response gene and a repressor of jasmonic acid (JA) dependent response genes (Li *et al*. 2004, 2006). AtWRKY70 displays positively-modulated systemic acquired resistance (SAR) and is dependent on NPR1 (Wang *et al*. 2006). Knoth *et al*. (2007) have provided additional data implicating that

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Abbreviations: JA - jasmonic acid; NPR1 - nonexpresser of PR genes; PDA - potato dextrose agar; PDB - potato dextrose broth; PDF1.2 - plant defensin; PR1 - pathogenesis-related protein 1; RACE-PCR - rapid amplification of cDNA end-polymerase chain reaction; SA - salicylic acid; SAR - systemic acquired resistance; SDS - sodium dodecyl sulfate; TFs - transcription factors.
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AtWRKY70 operates downstream of reactive oxygen intermediates and SA accumulation in both basal and R gene-mediated resistance against *Hyaloperonospora parasitica*. Ülker *et al*. (2007) have shown that AtWRKY70 regulates both plant defense and senescence through the SA-mediated pathway. Recently, *BnWRKY70*, a sequence homolog of *Arabidopsis AtWRKY70,* was induced in *Brassica napus* by SA treatment and in response to the fungal pathogens, *Sclerotinia sclerotiorum* and *Alternaria brassicae* (Yang *et al*. 2009). We demonstrated in this paper a positive regulatory role of the *ClWRKY70* gene, an ortholog of the *AtWRKY70* gene, in plant disease resistance against pathogen infections.

Materials and methods

An inbred line of watermelon [*Citrullus lanatus* (Thunb.) Matsum.] susceptible to *Cladosporium cucumerinum* was selected. Watermelon plantlets were placed in plastic pots containing sterilized soil, and then grown for 3 additional weeks prior to pathogen inoculation or chemical treatment. The seedlings were grown under a 16-h photoperiod with irradiance of 80 μmol(photons) m^{-2} s^{-1} (using 40 W fluorescent tubes), temperature of 22 ± 1 °C and relative humidity of 50 - 60 %. Hormone treatments were performed by spraying an aqueous solution containing 0.015 % (v/v) *Silwet L-77* (*Van Meeuwen Chemicals*, Weesp, The Netherlands), supplemented with 5 mM SA (*Sigma*, St. Louis, MO, USA) or with 100 μM methyl jasmonate (MeJA; *Sigma-Aldrich*, Milwaukee, WI, USA) onto plant leaves. Control plants were treated with 0.015 % (v/v) *Silwet L-77* only.

Inoculation of watermelon plants was performed by spraying a suspension of C. *cucumerinum* spores (5×10^6 cm^{-3}). The pathogen which causes scab disease was obtained from watermelon seedlings (Kwon *et al*. 1999). Inoculated plants were maintained in a growth chamber for 24 h at 100 % relative humidity. To produce enough spores, the fungus was grown on a potato dextrose agar (PDA; *Difco*, USA) plates for 2 weeks at 25 °C in an incubator.

The pathogenic fungus, *Botrytis cinerea* 4709 (obtained from Dr. S. Zhang, University of Missouri, Columbia, USA), was grown on PDA plates for 2 weeks at 25 °C. The inoculum was then transferred to a ½-strength PDA plate, after which it was incubated for an additional 1 week at 25 °C. To produce spores, the plates were again incubated for 2 d at 25 °C under irradiance of 80 μmol m^{-2} s^{-1}). Three-week-old *Arabidopsis* plants were inoculated dropwise with a 0.01 cm^3 suspension of the spores in potato dextrose broth (PDB) (8×10^5 cm^{-3}). Inoculated plants were maintained in a growth chamber for 24 h at 100 % relative humidity. Disease progression was evaluated 5 d after inoculation by measuring the lesion area using *Image Inside* software program *v. 2.32* (*Ehwa Optical Company*, Seoul, Korea). Three independent experiments were performed with at least 50 plants per experiment.

The pathogenic bacteria, *Erwinia carotovora* subsp. *carotovora* SCC1 obtained from Dr. E. Tapio Palva (University of Helsinki, Finland), were streaked onto a 2 % King's medium B (KB) agar plate followed by incubation at 28 °C for 2 d. To prepare inocula, bacteria were grown overnight in KB broth, pelleted by centrifugation at 10 000 *g* for 10 min, washed once with sterile water, and resuspended to 1×10^8 cfu cm^{-3}. Three-week-old *Arabidopsis* plants cultivated in plates were inoculated dropwise with 0.005 cm^3 suspension of the bacteria. *Arabidopsis* seeds were placed on ½-strength MS agar supplemented with 3 % sucrose in each well of a 12-well microtiter plate (*SPL*, Pocheon, Korea). Three days after inoculation, soft rot disease was rated by counting the number of symptomatic plants per plate. Three independent experiments were performed with at least 100 plants per experiment.

Total RNA was isolated from watermelon leaves 24 h after inoculation with *C. cucumerinum* by phenol/ sodiumdodecyl sulphate/LiCl extraction (Zhu *et al.* 1998). A full-length cDNA library was then constructed using a cDNA synthesis kit, *ZAP*-cDNA® synthesis kit and *ZAP*-cDNA® *Gigapack® II Gold* cloning kit (*Stratagene*, USA). WRKY domain-specific PCR was performed using a synthetic degenerated primer (5'TGGCGNAARTAYGGNCARAAR3'), an oligo d(T) primer (*Invitrogen*, USA), and the newly constructed cDNAs as templates. PCR amplification was performed at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s for 30 cycles. A 0.7 kb partial cDNA fragment was produced, which was then cloned into *pGEM T-easy* vector (*Promega*, USA). The full-length cDNA was obtained from the 0.7 kb partial cDNA fragment by 3',5'-RACE-PCR (*Invitrogen*). Total RNA (2 μg) were reverse-transcribed and used as a template for RACE-PCR. For the 5'-RACE, the gene specific primers 5'-GAGAGTGTGTTGACCTTCGTAC-3' (GSP1), 5'-GTCCTGCAAAAGCCCATGGTC-3' (GSP2) and 5'-TCCTTCCTCATTTGCAATGGC-3' (GSP3) were used in combination with the universal primers, AAP,UAP and AUAP, in the 1st PCR, 1st nested PCR and 2nd nested PCR amplification, respectively. For 3'-RACE, the gene specific primer 5'-TATGGGCAGAAGGCG GTGAAG-3' (GSP4) and universal primers, UAP and AUAP, were used. The 5',3'-RACE-PCR products were cloned into *pGEM T-easy* vector and subjected to direct sequencing. The full length cDNA was amplified using cDNA templates and two gene specific primers, 5'-TTCATAGACATGGAGCCCGCG-3' and 5'-AGG GTGTTATTTTGATTCTATTG-3'.

The overexpression cassette *35S-ClWRKY70* was constructed as follows: a 915 bp fragment of the *ClWRKY70* cDNA coding region including the ATG start site and TGA stop codon, was amplified by PCR using

synthetic primers. For sense-oriented insertion of cDNA into the predigested binary vector pBI121, a set of synthetic primers, [left 5'-GCTCTAGAGCATGGA GCCCGCG-3' (*XbaI* restriction site underlined) and right 5'-GGGGTACCCCGTGTTATTTTGATTCTAT-3' (*KpnI* restriction site underlined)], was used.

Arabidopsis thaliana L. ecotype Columbia (Col-0) was transformed according to the vacuum infiltration method (Bechtold and Pelletier 1998) using *Agrobacterium tumefaciens* LBA4404. T_1 seeds were plated on MS medium containing kanamycin (50 mg dm^{-3}), and surviving transgenic plants were selected after 1 week.

For Northern blot analysis, 20 µg of total RNA were separated on a 1.2 % formaldehyde/agarose gel and transferred onto a nylon membrane. The RNA blot was probed with P^{32}-labeled cDNA probe and washed at high stringency. Hybridization and P^{32}-labeling of cDNA were performed according to standard procedures (Sambrook *et al*. 1989).

Semiquantative RT-PCR was performed using a *ClWRKY70* gene (GQ453670) specific primer (forward, 5′-TATGGGCAGAAGGCGGTGAAG-3′ and reverse, 5′-TCCTTCCTCATTTGCAATGGC-3′). *actin1* gene (AT2G37620) (primer set: forward, 5'-CATCAG GAAGGACTTGTACGG-3' and reverse, 5'-GATGGA CCTGACTCGTCATAC-3') of *Arabidopsis* plant is used as an internal standard. Sequences of primers for *PR1* (AT2G14610) and *PDF1.2* (AT5G44420) genes are 5'-GGCCTTACGGGGAAAACTTA-3' and 5'-ACTTTG GCACATCCGAGTCT-3', and 5'-AATGGATCCATG GCTAAGTTTGCTTCCATC-3' and 5'-AATGAATTC AATACACACGATTTAGCACC 3', respectively. The reaction mixture (0.02 cm^3) contained 2 µg of total RNA, 0.5 µg of each primer, and appropriate amounts of enzymes in an *AccuPower*^{TM} *RT Premix* tube (*Bioneer*; Daejeon, Korea). For the control reaction, no RNA was added in the reaction mixture. The mixture was then incubated at 42 °C for 1 h to create the cDNA. About 200 ng of the cDNA in 0.002 cm^3 of reaction mixture was then transferred to *AccuPowerTM* PCR *Premix* tube (*Bioneer*). The gene was amplified via 35 PCR cycles at 94 °C for 35 s, 58 °C for 35 s, and 72 °C for 1 min. The PCR products were analyzed on 0.7 % agarose gel and stained by ethidium bromide.

Data were analyzed by *ANOVA* using *JMP 4.0* software (SAS Institute; Cary, NC, USA). The significance of observed values was determined by Duncan's multiple range test ($P = 0.05$).

Results and discussion

Identification of the *ClWRKY70* gene: The *C. cucumerinum* infection-inducible *ClWRKY70* cDNA is 915 base pairs long, and encodes a polypeptide consisting of 304 amino acids sharing a high similarity to group III WRKY proteins of higher plants containing a single WRKY domain and Cys2HisCys zinc-finger motif (Eulgem *et al*. 2000). The amino acid sequence of ClWRKY70 (GenBank accession GQ453670) is moderately similar to *Brassica napus* BnWRKY70 (31 % identity), *Glycine max* GmWRKY58 (27 % identity) and *Arabidopsis* AtWRKY70 (28 % identity) (Fig. 1*A*), but increases in similarity to 65, 63 and 66 %, respectively, when only the WRKY domain is aligned (Fig. 1*B*). Among group III *Arabidopsis* WRKY proteins, AtWRKY70 is the most similar to ClWRKY70 (Fig. 1*C*). This newly characterized gene is the first WRKY factor described in watermelon and presents structural hallmarks that allow its classification within group III of WRKY TFs (Eulgem *et al*. 2000). *AtWRKY70* (the *ClWRKY70 Arabidopsis* homolog) has been described as playing an important role in the plant defense response as well (Li *et al*. 2004, 2006, Wang *et al*. 2006, Knoth *et al*. 2007, Ülker *et al.* 2007, Yang *et al*. 2009).
ClWRKY70 mRNA transcripts gradually accumulated in watermelon 6 to 48 h after inoculation with *C. cucumerium* (Fig. 2*A*). Increase of *ClWRKY70* gene expression was found to be apparent 2 to 4 h after treatment of watermelon leaves with 5 mM SA, but then gradually diminished after 6 h (Fig. 2*B*). JA treatment decreased the expression of *ClWRKY70* in watermelon leaves (Fig. 2*B*). Many WRKYs have also been found to act as transcriptional modulators of the SA- and JA-mediated responses in *Arabidopsis* and other species (Li *et al.* 2006, Marchive *et al.* 2007). ClWRKY70 appears to be a common element that functions in SA-dependent signaling in watermelon, and its expression is modulated by fungal infection (Fig. 2*A*). Several studies have reported the induced expression of *AtWRKY70* or its homolog in response to SA (Knoth *et al.* 2007, Li *et al.* 2004, 2006, Ülker *et al.* 2007, Yang *et al.* 2009). Currently, the molecular mechanisms underlying pathogen resistance in watermelon are unknown, as are the elements involved in their regulation. SA could be involved in signaling in watermelon similar to other well-known plant-pathogen interactions. The idea is strongly supported by the data, which can lead one to speculate that SA has a role in the transcriptional activation of defense-related genes during defense response by modulating *ClWRKY70* expression under *C. cucumerinum* infection.

Function of the *ClWRKY70* gene: To examine ClWRKY70 protein function during the regulation of plant defense response, several *Arabidopsis* lines overexpressing *ClWRKY70* in response to *B. cinerea* or *E. carotovora* challenge were analyzed. Overexpression of *ClWRKY70* significantly reduced the lesion area developed by transgenic *Arabidopsis* compared to wild-type pants upon *B. cinerea* infection (Fig. 3*A*). All plants developed similar symptoms upon *B. cinerea* infection

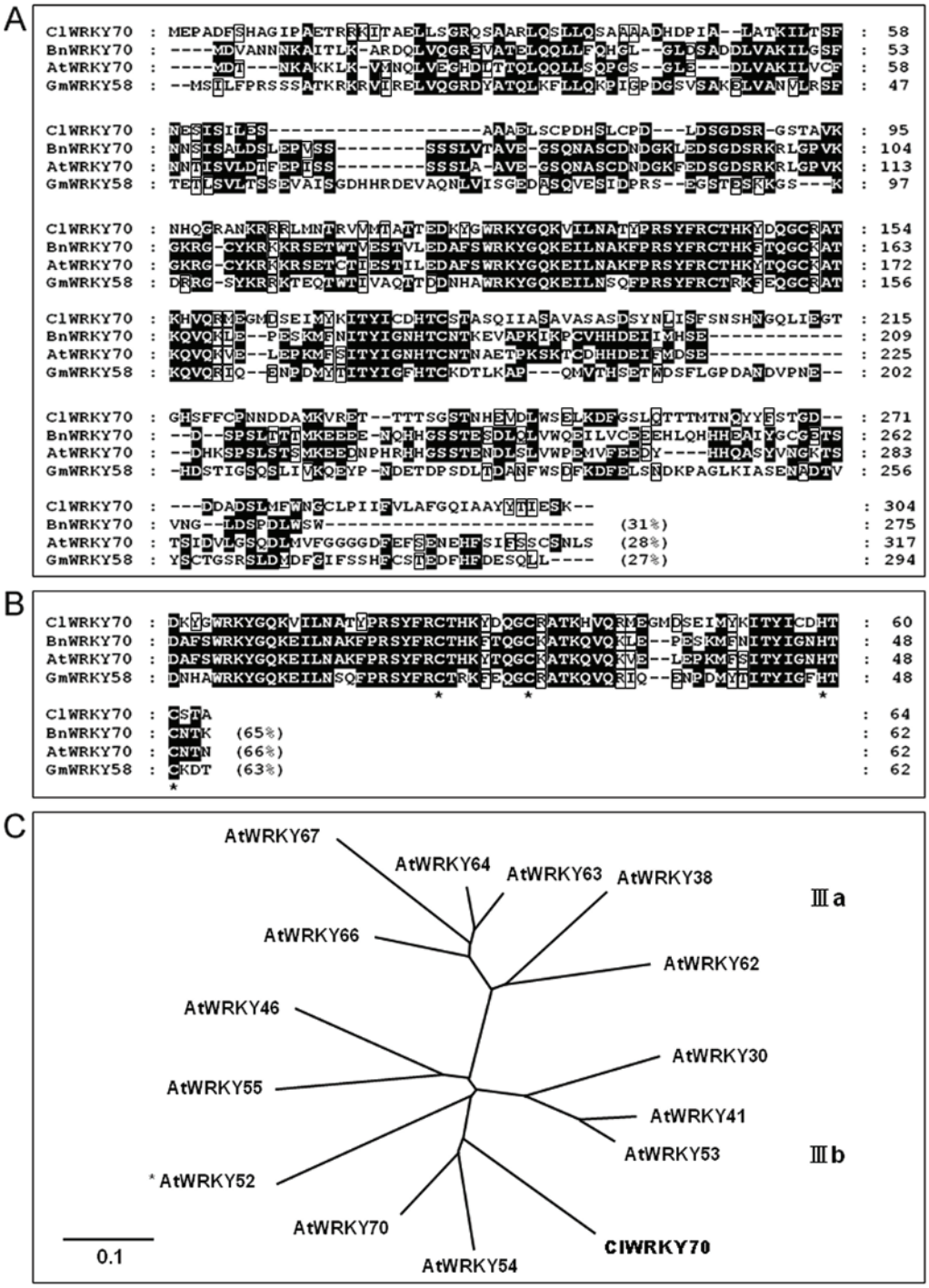

Fig. 1. *A* - Alignment of ClWRKY70 with related WRKY proteins from other plant species. The deduced amino acid sequence was aligned with BnWRKY70 (ACQ76810) from canola, GmWRKY58 (ABY84662) from soybean and AtWRKY70 (AF421157) from *Arabidopsis* using *Clustal W* program with default parameters through *EMBnet* (http://www.ch.embnet.org/software/ClustalW.html). *Black shadings* and *rectangular squares* completed with *BOXSHADE 3.21* (http://www.ch.embnet.org/software/BOX_form.html), indicate conserved amino acid residues. *B* - Comparison of the WRKY domain of ClWRKY70 with other plant WRKY domains. The four variant cysteine and histidine residues that could serve as a tetrahedral coordination site for zinc ion are marked by an *asterisk*. *C* - Phylogenic tree of the ClWRKY70 with group III WRKY proteins from *Arabidopsis*. Amino acid sequence of WRKY domain of each protein is compared. The diagram shows an unrooted tree constructed with the *TreeView* program (*v.1.6.6*). Schematic representations of typical members of each subgroup are shown above or underneath the tree. But, AtWRKY52 marked by an *asterisk*, is classified neither IIIa nor IIIb subgroup.

except for the lesion area, which was decreased in the overexpression lines compared with that of wild-type. Increased resistance to the pathogen *E. carotovora* was also observed upon overexpression of *ClWRKY70* compared to control plants (Fig. 3*B*). Therefore, a positive regulatory role of the *ClWRKY70* gene for disease resistance in transgenic *Arabidopsis* is demonstrated against fungal and bacterial pathogens.

Based on the observation that the *ClWRKY70* gene acted as positive regulator in a complex defense response network, the expression of defense marker genes such as *PR1* and *PDF1.2* were analyzed. Constitutive expression of the *ClWRKY70* gene in T_4 line increased the accumulation *PR1* and *PDF1.2* gene transcrips even before fungal or bacterial infection (0 h). After inoculation with fungus or bacteria, *PR1* and *PDF1.2* gene expression was higher in transgenic *Arabidopsis* compared to wild type (Fig. 4). Gene expression also experienced priming, as evidenced by high accumulation as early as 12 h and 6 h in response to *B. cinerea* and *E. carotovora* infection, respectively (Fig. 4). The capacity for augmented defense expression is referred to as priming, and this phenomenon has been demonstrated in various plant species in response to pathogen

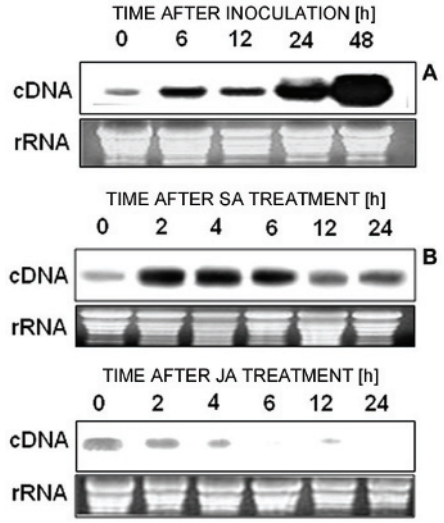

Fig. 2. Differential induction of *ClWRKY70* gene expression following *C. cucumerium* infection (*A*), and 5 mM SA or 100 μM JA treatment (*B*). Northern blot analysis was performed using P^{32}-labeled *ClWRKY70* cDNA probe. Equal loading was verified before blotting by visualization of rRNA on the gel with ethidium bromide (*bottom*).

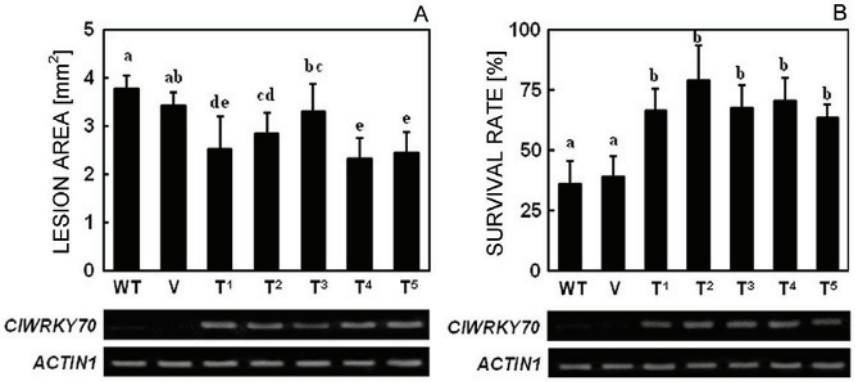

Fig. 3. Induced resistance appeared in ClWRKY70-overexpressing transgenic *Arabidopsis* upon infection with the fungal pathogen *B. cinerea* (*A*) and with the bacterial pathogen *E. carotovora* (*B*). Enhanced expressions of the watermelon *ClWRKY70* and *actin1* gene in transgenic *Arabidopsis* were measured by semiquantitative RT-PCR method. *A* - Each leaf of 3-week-old seedling was inoculated by dropping a 0.01 cm^3 of the fungal conidia suspension (8×10^5 spores cm^{-3}), and then developed lesion area was measured 5 d after inoculation. *B* - Each leaf of 3-week-old seedling was inoculated by dropping a 0.005 cm^3 of the bacterial suspension (1×10^8 cfu cm^{-3}), and then survival rate was measured 2 d after inoculation. WT - wild-type, V - vector only, T^1, T^2, T^3, T^4 and T^5 - five independent T_1 lines.

infection (Conrath *et al*. 2002). These results suggest that *ClWRKY70* gene expression is involved in disease resistance in plants to fungal and bacterial pathogens. The data coincide well with previous findings in which the *Arabidopsis* sequence homolog, AtWRKY70, was found to play an important role in plant defense response (Knoth *et al*. 2007, Wang *et al.* 2006).

A question may arise why both transcripts (*PR1* and *PDF1.2*) were accumulated in transgenic *Arabidopsis* overexpressing *ClWRKY70*, since expression of *ClWRKY70* was SA- and not JA-dependent (Fig. 2). Contrary to expectations, resistance to both pathogens in ClWRKY70-overexpressing *Arabidopsis* plants was coupled with both *PR1* and *PDF1.2* gene induction (Fig. 4). This can be explained by the knowledge that pathways involving JA and ethylene (ET) are considered effective against necrotrophic pathogens, insects and wounding, whereas those involving SA are more effective against biotrophs (Kunkel and Brooks 2002). Therefore, we can expect that the main defense response in watermelon may involve JA and ET-dependent pathways in response to *C. cucumerinum* infection, and also that SA-dependent *ClWRKY70* gene expression can contribute to the total defense reaction. However, a direct conclusion may be difficult to make based solely on *ClWRKY70* function due to differences between the plants.

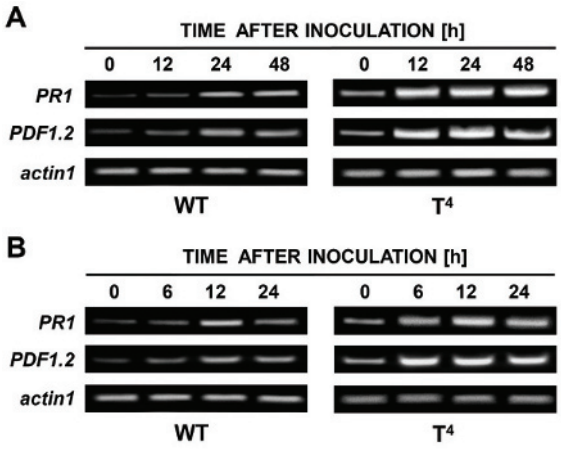

Fig. 4. Expression kinetics of defense-related genes (*PR1* and *PDF1.2*) in wild-type and ClWRKY70-overexpressing transgenic *Arabidopsis* upon *B. cinerea* (*A*) or *E. carotovora* (*B*) infection. 3-week-old seedlings were inoculated by spraying a conidial suspension of *B. cinerea* (8×10^5 spores cm^{-3}) or a bacterial suspension of *E. carotovora* (1×10^8 cfu cm^{-3}). Gene expression was analyzed in time course by semiquantitative RT-PCR after inoculation with pathogen. The specific primer set was used to detect the expression pattern of each gene. WT - wild-type, T^4 - a T_1 line.

The gene is cloned from watermelon, but is transformed into *Arabidopsis* where its function is analyzed. Since it is still unclear how ClWRKY70 interacts with *Arabidopsis* defense response proteins in the network regulating disease resistance, additional approaches like *ClWRKY70* gene-silencing in watermelon are desirable.

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