Cloning and characterization of WRKY gene homologs in Chieh-qua (*Benincasa hispida* Cogn. var. Chieh-qua How) and their expression in response to fusaric acid treatment

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**Abstract** The WRKY transcription factors play an important role in plant resistance for biotic and abiotic stresses. In the present study, we cloned 10 WRKY gene homologs (*CqWRKY*) in Chieh-qua (*Benincasa hispida* Cogn. var. Chieh-qua) using the rapid-amplification of cDNA ends (RACE) or homology-based cloning methods. We characterized the structure of these *CqWRKY* genes. Phylogenetic analysis of these sequences with cucumber homologs suggested possible structural conservation of these genes among cucurbit crops. We examined the expression levels of these genes in response to fusaric acid (FA) treatment between resistant and susceptible Chieh-qua lines with quantitative real-time PCR. All genes could be upregulated upon FA treatment, but four *CqWRKY* genes exhibited differential expression between resistant and susceptible lines before and after FA application. *CqWRKY31* seemed to be a positive regulator while *CqWRKY1*, *CqWRKY23* and *CqWRKY53* were negative regulators of fusaric resistance. This is the first report of characterization of WRKY family genes in Chieh-qua. The results may also be useful in breeding Chieh-qua for Fusarium wilt resistance.

**Keywords** Chieh-qua · *Benincasa hispida* · WRKY transcription factor · Fusarium resistance · Gene expression

**Introduction**

Chieh-qua, *Benincasa hispida* Cogn. var. *Chieh-qua* How., is an important vegetable crop in the Cucurbitaceae family, which is widely cultivated all over South China and Southeast Asian countries (He et al. 2007). It is a subspecies of the wax gourd (*Benincasa hispida* (Thunb.) Cogn.). Chieh-qua has a vigorous annual vine and often grown with a trellis support (Cantwell et al. 1996). Its immature fruit is consumed that can be baked, fried, boiled or pickled. Fusarium wilt (FW herein after), caused by *Fusarium oxysporum* Sch., is one of the most serious diseases in Chieh-qua production (He et al. 2007). Fusaric acid (5-butylpyridine-2-carboxylic acid, FA) is the major toxin which is produced by *F. oxysporum* pathogen. Several studies have shown that FA plays a critical role in boosting FW symptom development in plants (Löfller and Mouris 1992; Liu et al. 2011). After *F. oxysporum* infection, FA was present in the whole banana plant and there was a positive correlation between its concentration and the incidence rate of disease symptoms (Dong et al. 2012). Similar correlation was also found in Chieh-qua, and FA treatment can be used as an effective method in screening of FW resistance (He et al. 2009). Breeding of resistant varieties is an effective and environmental friendly approach to solve the problem of Fusarium wilt in Chieh-qua.
qua whereas identifying resistance-related genes presents an important significance in breeding of resistant variety.

The WRKY protein family is named after the characteristic amino acid sequence WRKYGQK (Rushton et al. 2010). According to the quantity of WRKY domains and the type of zinc-finger motifs in the sequence, WRKY proteins can be classified into three major groups and five subgroups (Eulgem et al. 2000). Through combining its conserved WRKY domain to the cognate cis-acting elements, W box, WRKY protein performs its functions specifically (Eulgem et al. 2000). W box contains an invariable TGAC bases core and whichever base replaced will decline the DNA-binding activities (Dong et al. 2003). It is well known that WRKY transcription factors act on a variety of plant biochemical reactions with a rapid and instant feature, such as growth and senescence, metabolism, biotic and abiotic stresses (Zhang et al. 2008; Miao et al. 2004; Suttipanta et al. 2011; Wang et al. 2013; Li et al. 2013). In Arabidopsis thaliana, for instance, WRKY53 is implicated in leaf senescence process (Miao et al. 2004), while WRKY25, WRKY26 and WRKY33 are coordinately in response to heat stress (Li et al. 2011). Sun and Yu (2015) found that AtWRKY53 controls stomata open and close by regulating H2O2 contents and starch degradation.

There are many studies on the resistant functions of WRKY transcription factors in response to pathogen infection. For example, Lai et al. (2008) found that the Arabidopsis AtWRKY3 and AtWRKY4 played a positive role in resistance to necrotrophic pathogens, and AtWRKY4 played a negative role in resistance to biotrophic pathogens. In rice, OsWRKY71 overexpression plant exhibited enhanced resistance to Xanthomonas oryzaepv. oryzae (Xoo), a virulent bacterial pathogen (Liu et al. 2007). The Arabidopsis transgenic mutant overexpressing CIWRKY70 (cloned from Citrullus lanatus) showed a significantly decreased impaired area upon Botrytis cinerea or Erwinia carotovora (Cho et al. 2012).

In the present study, we cloned 10 Chieh-qua WRKY sequences using RACE (rapid-amplification of cDNA ends) and homology-based cloning. We characterized these CqWRKY sequences and investigated their expression in response to FA treatment in FW resistant and susceptible Chieh-qua lines. Four CqWRKY genes were found to be potentially associated with FW resistance.

Materials and methods

Materials

The Fusarium-resistant A39FA and susceptible H5 Chieh-qua (Benincasa hispida Cogn. var. Chieh-qua How) inbred lines used in the research were provided by the Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, China. Chieh-qua seedlings were growing in 1:1 mixture of sterile soil and vermiculite at 25 ± 2 °C and 60% relative humidity under a 16-h light/8-h dark cycle (Xie et al. 2006). Twenty-day seedlings were used for fusaric acid (FA) treatment. The fusaric acid was purchased from Tokyo Chemical Industry, Japan.

Cloning of full-length cDNA of CqWRKY gene homologs

Total RNA was isolated from A39FA leaves with the TransZol™ Plant reagent (TRANSGEN, China) following the manufacturer’s instructions. The first cDNA strand was synthesized from 1 μg total RNA using PrimeScript™ RT kit followed with treatment of gDNA Eraser (TaKaRa, Japan). Primer pairs (Table 1) used for amplification of Chieh-qua WRKY gene homologs (CqWRKY) were designed with Oligo v7.37 based on sequences of the wax gourd transcriptome developed in our lab (Jiang et al. 2013). Homolog-based cloning of the CqWRKY genes followed Dubé et al. (2008).

Bioinformatics analysis of CqWRKY sequences

The ORF (open reading frame) sequences of these cloned CqWRKY genes were searched with the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Arabidopsis WRKY sequences were downloaded from TAIR (http://www.arabidopsis.org/). Cucumber WRKY sequences were obtained from cucumber genome database (http://www.cucumber.genomics.org.cn/). Multiple sequence alignment of cucumber and Chieh-qua WRKY sequences was performed with ClustalW v2.1 (Larkin et al. 2007) and GeneDoc (Nicholas et al. 1995). A phylogenetic tree was constructed with MEGA5.0 software using the Neighbor-Joining method with 1000 bootstrap replicates (Tamura et al. 2011).

The motifs presented in 10 CqWRKY proteins were identified with MEME v4.11.2 (http://meme-suite.org/tools/meme/) with the following parameters: number of repetitions: any; maximum number of motifs: 50; optimum width of each motif: 6–300 residues (Huang et al. 2012).

Fusaric acid treatments

Twenty-day-old resistant (A39FA) and susceptible (H5) Chieh-qua seedlings with two fully expanded leaves were treated with FA solution (200 mg/L) using the root-soaking method with sterile water as the blank control (Zhang et al. 2014). Briefly, the roots of seedlings were cut into ~5 cm length and submerged in the FA solution. The treated plants were maintained in the culturing room at 25 ± 2 °C.
Leaf samples were collected from each seedling at 0, 6, 12 and 24 h after FA treatment. There were three replications per treatment. All samples were flash frozen in liquid nitrogen and stored at -70 °C until total RNA extraction.

CqWRKY gene expression analysis

Quantitative real-time PCR (qRT-PCR) was performed for selected CqWRKY genes in both resistant and susceptible Chieh-qua lines before and after FA treatments. The qRT-PCR primers are provided in Table 2, which were designed with Oligo v7.37. The qRT-PCR reactions were performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad, Germany). Each 10 µL mixture contained 5 µL of SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan), 0.25 µl each of forward and reverse primer (10 µM), 4 µL of cDNA template (50 ng/µL) and 0.5 µL of sterile water. The PCR program included an initial denaturation (95 °C/30 s), then followed by 40 cycles of 95 °C/5 s, 60 °C/20 s, 72 °C/30 s. A melting curve was obtained from 65 to 95 °C, increment of 0.5 °C for 5 s. There were three biological and technical replicates for each sample. The actin gene was used as the internal standard to normalize each sample (Zhang et al. 2014). Relative gene expression was calculated with the 2−ΔΔCt method (Livak and Schmittgen 2001).

Data analysis

All statistical analyses were performed in R (v3.2.0). The standard deviation of fold changes of expression levels was calculated from three replicates; significance tests were performed with the Tukey’s multiple-range test (P < 0.05). Origin Pro 8 (v8.0724) was used for visualization of relative transcription levels of CqWRKY genes.

Results

The WRKY genes of Chieh-qua

In total, we identified 10 CqWRKY sequences through rapid-amplification of cDNA ends (RACE) (CqWRKY1) or homology-based cloning method (9). Based on sequence homology with the cucumber WRKY genes, the 9 CqWRKY genes were designated as CqWRKY21, CqWRKY23, CqWRKY31, CqWRKY34, CqWRKY37, CqWRKY45, CqWRKY50, CqWRKY53 and CqWRKY54, respectively. The full-length cDNA sequences varied from 612 to 1746 bp and the predicted proteins ranged from 204 to 582 amino acid residues. The domain features of these WRKY genes are provided in Table 3.
Phylogenetic relationships of Chieh-qua and cucumber WRKY sequences

We examined the phylogenetic relationship of 10 CqWRKY and 57 cucumber CsWRKY genes, and the unrooted phylogenetic tree is presented in Fig. 1. All branching nodes were supported with high bootstrap values. From Fig. 1, it can be seen that all 10 CqWRKYs belonged to Groups I, IIa, IIc, IIId and III, each of which contained 3, 1, 2, 1, 3 genes, respectively. Group I members were closer to those in Group II, especially Group IIc, and Group IIId seemed more distant to other groups and subgroups except for Group IIe.

CqWRKY motif structure analysis

WRKY proteins are characterized by the conserved polypeptide WRKYGQK (Eulgem et al. 2000), and variations of this heptapeptide have been rarely demonstrated.
among large number of WRKY proteins (Zhang and Wang 2005; Xie et al. 2005). We compared the WRKY domains in 10 CqWRKY proteins with those from Arabidopsis (AtWRKY) and cucumber (CsWRKY). The representative alignment is presented in Fig. 2. As shown in Fig. 2, sequences in these WRKY domains of different species were highly conserved. Most heptapeptide sequences in the CqWRKY genes contained were WRKYGQK, and only CqWRKY54 had WRKYGKK. Compared with other groups and subgroups, the WRKY domains in Group III were less conserved.

We compared the amino acid motifs presented in CqWRKY and AtWRKY proteins, which is illustrated in Fig. 3. The number of location of motifs among these proteins was largely consistent. Except for CqWRKY34, motif 2 presented in the remaining 9 CqWRKY proteins. The CqWRKYs in Groups IIId and III shared common conserved motifs with motifs 5 and 7 located downstream of the WRKY domain and motif 2 upstream of it. However, in Group 2a, motif 7 was located in before the WRKY domain. Motif 3 only appeared in Group 2a.

Expression of CqWRKY genes in response to FA treatment

We examined expression of CqWRKY genes in A39FA (FW resistant) and H5 (FW susceptible) before and after FA treatment with qPCR, and results are presented in Fig. 4. The expression of all 10 CqWRKY genes was strongly induced by FA application. The expression of CqWRKY1, CqWRKY23, CqWRKY31 and CqWRKY53 did not show significant differences between the resistant and susceptible lines before treatment ($P > 0.05$); all of them showed significant increase in expression after FA treatment. Among them, CqWRKY31 had a higher expression in A39FA than H5, and the transcription level reached to the peak at 12 h after treatment. For CqWRKY1, CqWRKY23 and CqWRKY53, the expression level was higher in H5 than in A39FA. Both CqWRKY1 and CqWRKY53 reached their peak expression at 12 h after treatment whereas that of CqWRKY23 peaked at 6 h after FA application. Although CqWRKY34 and CqWRKY45 responded positively to FA stress, their expressions did not show
significant differences between these two cultivars. Besides, the expression levels of CqWRKY21, CqWRKY37 and CqWRKY54 did not show consistent change across different time points between A39FA and H5. Lastly, CqWRKY50 had significant differential expression between the two lines even before FA treatment.

**Fig. 2** Alignment of WRKY domain amino acid sequences among CqWRKY, selected AtWRKY and CsWRKY proteins. Alignment was performed with Clustal W and GeneDoc. The suffix ‘N’ and ‘C’ of Group 1 indicates the N-terminal and C-terminal WRKY domain, respectively. The conserved residues are highlighted in black and gray boxes. The conserved WRKY amino acid signatures are underlined in red and the zinc-finger motifs are underlined in blue.

**Fig. 3** The amino acid motifs presented in CqWRKY and AtWRKY proteins characterized with the MEME 4112 Program. The grey solid line represents the corresponding WRKY protein and the red boxes represent WRKY motifs. The various colored boxes represent different motifs and their location in each WRKY sequence.
Discussion

A number of studies have shown that WRKY transcription factors participate in defense responses against various abiotic and biotic stresses (Eulgem et al. 2000; Dong et al. 2003; Xie et al. 2005; Wu et al. 2005; Eulgem and Somssich 2007; Yang et al. 2009; Rushton et al. 2010; Dang et al. 2013, 2014; Liu et al. 2014). For example, in Arabidopsis, AtWRKY28 and AtWRKY75 overexpression mutants exhibited increased resistance to oxalic acid and Sclerotinia sclerotiorum stresses (Chen et al. 2013). Kim et al. (2016) found that the expression of two cold-responsive genes, OsTGFR and WSI76, were elevated in rice OsWRKY71-overexpressing lines under cold stress suggesting OsWRKY71 as a positive regulator in cold tolerance. In potato, StWRKY1-overexpressing lines showed an enhanced resistance to Phytophthora infestans and an elevated tolerance to dehydration (Shahzad et al. 2016). In addition, broccoli BoWRKY6-overexpressing mutant exhibited a highly resistance to downy mildew (Hyaloperonospora parasitica) (Jiang et al. 2016).

There are also several studies about WRKY transcription factors in the Cucurbitaceae family. For example, in cucumber, Ling et al. (2011) identified 55 WRKY sequences in the cucumber genome and found that 23 of them displayed differential expression under at least one abiotic stress (drought, cold or salinity). Xu et al. (2015) investigated expression of these cucumber WRKY genes in response to inoculation of Phytophthora melonis and found that only six of them showed significant differential expression. Besides, the expression levels of CsWRKY30 and CsWRKY6 were elevated after infection with the powdery mildew pathogen (Alfandi et al. 2012). In watermelon, Yang et al. (2014) found that the expression of

Fig. 4 Differential expression patterns of CqWRKY genes in leaves under FA treatment. Y-axis is the relative gene expression level. The Chieh-qua Actin gene was used as an internal control for data normalization. X-axis is the hours after FA treatment (0, 6, 12 and 24 h). F FA treatment, H H2O control. Error bars are calculated according to three replicates.
CIWRKY1 was elevated after infection with *Fusarium oxysporum* f. sp. *niveum*.

In the present study, for the first time, we cloned 10 *CqWRKY* genes, and found that they shared high-degree homology with the cucumber homologs (Fig. 1). We show that some of these *CqWRKY* genes exhibited differential expression in FW-resistant and -susceptible lines before and after FA treatment (Fig. 4). A large number of WRKY genes have been found to be involved in plant resistances against pathogen infection (Zheng et al. 2006; Zhang et al. 2012). For example, transgenic *AtWRKY18* *Arabidopsis* showed an outstanding resistance to the bacterial pathogen *Pseudomonas syringae* (Chen and Chen, 2002) while overexpressing *BnWRKY33* transgenic *Brassica napus* showed remarkable enhanced resistance to *Sclerotinia sclerotiorum* (Wang et al., 2014). In cotton, Guo et al. (2011) found that the transcription level of *GhWRKY3* dramatically increased when inoculated with *Fusarium oxysporum* f. sp. *vasinfectum*. Also in cotton, Yu et al. (2012) detected the accumulation of the *GhWRKY15* transcripts when treated with *Fusarium oxysporum* f. sp. *vasinfectum*. In this study, we found four *CqWRKY* genes with response to FA treatment and their expression was significantly different between resistant and susceptible lines (Fig. 4). *CqWRKY31* showed a higher level of expression in A39FA than H5, which may suggest that this gene might be a positive regulator of fusaric resistance. On the contrary, *CqWRKY1*, 23 and 53 were strongly induced by FA in the susceptible H5, implying that they might play a negative role for regulating resistant to fusaric wilt. In other studies, the expression patterns of WRKY family genes presented different trends under the same treatment. Ling et al. (2011) found that *CsWRKY2* was upregulated in response to cold and salt, while *CsWRKY28* was down-regulated in cold and *CsWRKY23* was down-regulated in salt in cucumber. Similarly, Yang et al. (2009) found that *BnWRKY20* and 32 were suppressed by *S. sclerotiorum* infection while other several *BnWRKY*s were highly induced after the inoculation in canola. These results showed that WRKY family genes responded to biotic and abiotic stresses in both positive and negative way. In summary, our results indicated that *CqWRKY31* was positively related, while *CqWRKY1*, 23 and 53 were negatively correlated to fusaric resistance. These results may have potential application in breeding of resistance to Fusarium wilt in Chieh-qua.

**Conclusions**

For the first time, we cloned 10 *CqWRKY* family genes in Chieh-qua including *CqWRKY1*, 21, 23, 31, 34, 37, 45, 50, 53 and 54. Four of them exhibited differential expression in resistant and susceptible Chieh-qua varieties in response to FA treatment. *CqWRKY31* may be a positive regulator whereas *CqWRKY1*, *CqWRKY23* and *CqWRKY53* may be negative regulators of fusaric resistance. Results from this study may be useful in breeding Chieh-qua for Fusarium wilt resistance.

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**Compliance with ethical standards**

**Conflict of interest** The authors declared that they have no conflicts of interest to this work.

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