

# The WRKY Transcription Factor Family in *Citrus*: Valuable and Useful Candidate Genes for Citrus Breeding

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**Abstract** WRKY transcription factors belong to a large family of plant transcriptional regulators whose members have been reported to be involved in a wide range of biological roles including plant development, adaptation to environmental constraints and response to several diseases. However, little or poor information is available about WRKY's in *Citrus*. The recent release of completely assembled genomes sequences of *Citrus sinensis* and *Citrus clementina* and the availability of ESTs sequences from other citrus species allowed us to perform a genome survey for Citrus WRKY proteins. In the present study, we identified 100 WRKY members from *C. sinensis* (51), *C. clementina* (48) and *Citrus unshiu* (1), and analyzed their chromosomal distribution, gene structure, gene duplication, syntenic relation and phylogenetic analysis. A phylogenetic tree of 100 *Citrus* WRKY sequences with their orthologs from *Arabidopsis* has distinguished seven groups. The *CsWRKY* genes were distributed across all ten sweet orange chromosomes. A comprehensive approach and an integrative analysis of *Citrus* WRKY gene expression revealed variable profiles of expression within tissues and stress conditions indicating functional diversification. Thus, candidate *Citrus* WRKY genes have been proposed as potentially involved in fruit acidification, essential oil biosynthesis and abiotic/biotic stress tolerance. Our results provided essential prerequisites

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M. Ayadi and M. Hanana contributed equally to this work.

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for further *WRKY* genes cloning and functional analysis with an aim of citrus crop improvement.

**Keywords** *Citrus* · *WRKY* · Fruit acidification · Essential oil biosynthesis · Stress tolerance · Zinc finger

## Introduction

*Citrus* is one of the world's most and widest cultivated crop [1] with a worldwide production of 135 million tons in 2013 [2]. Because of their widespread geographical distribution and being thus cultivated under variable pedo-climatic conditions, citrus crops face abiotic and biotic stresses that damage final production and depreciate fruit quality. At the genomic level, *Citrus* and related genera have 18 chromosomes in diploid somatic cells [3]. Besides, there is a financial market pressure and a constant demand from the industries for better quality products in order to meet the consumers' needs and expectations. Thereby, launching *Citrus* breeding and improvement program has become a major goal and a priority for both farmer producers and industrial companies [4]. Thus, based on the fact that the only way to propagate citrus cultivars is by grafting onto rootstock, scientists from concerned countries focused their research efforts on selecting and combining rootstocks traits of abiotic (salinity and drought) and biotic (mostly *Citrus* Tristeza Virus CTV and *Phytophthora*) stresses tolerance. Such interesting and useful traits of tolerance and acclimatization could be found within *Citrus* and their relatives [5]. However, due to major constraints related to the *Citrus* biology of reproduction such as high level of heterozygosity, sterility of several varieties, apomixis or nucellar polyembryony phenomena and mostly self-incompatibility within *Citrus* cultivars, breeding of the species by conventional procedures through sexual crossing has limited success. The recent advent of new biotechnological tools has opened new insights and promising prospects for *Citrus* improvement, making those limitations bypassed [6].

*Citrus* breeding community has thus focused efforts on newly released genomic resources and transcriptomic data from international sequencing centers and programs. Interestingly, recent release of two *Citrus* species genome sequences provides an opportunity to explore molecular data and resources for *Citrus* breeding program [7]. 'Clementine' mandarin genome was the first *citrus* sequenced genome, serving as genome of reference for *citrus* [8] and was followed by 'Ridge Pineapple' sweet orange clone using next generation sequencing. These projects load a draft of high quality assemblage [4].

In the past 10 years, several work projects related to *Citrus* omics data and resources have emerged [9–13]. Yet, to reach the breeding aims and objectives, efforts still to do by combining genomic technology and genes of agricultural interest identification and manipulation [13].

Gene expression regulation is a vital mechanism for all living organisms. Transcription factors (TF) and other related components together act in the transcriptional regulating network, through binding the specific DNA sequence and activating and/or repressing the transcription of their target genes. Among these, *WRKY* proteins are considered important transcriptional regulators in defense signaling. The *WRKY* TFs family is one of the largest families of plant TFs which has been characterized for several and diverse plants species [14–16]. Usually, the *WRKY* protein has one or two *WRKY* domains of nearly 60 aa containing at its N-terminus the *WRKYGQK* conserved motif and a zinc finger motif at its C-terminus [15, 17, 18]. The *WRKYGQK* aa peptide displays a  $\beta$ -strand structure that directly

binds to DNA on the regulatory region of target genes. The WRKY binding site is made of a conserved W box: (C/T) TGAC (T/C) which is frequently found within promoter region of stress response and development associated genes. Group I WRKY TF contains two WRKY domains and is thought to be ancestral type [15] while genes containing a single WRKY domain are classified into groups II (a, b, c, d or e) or III according to their aa sequence and the structure of their zinc finger motifs [15, 19]. Rushton et al. [17] were first to report that WRKYs are involved in plant defense. In fact, WRKYs are also implicated in plant growth regulation and development such as seed dormancy and development, germination, rhizogenesis, morphogenesis of trichomes, senescence and metabolic pathways [14, 20, 21]. Moreover, WRKYs members appear to be tightly involved in the response regulation to various biotic and abiotic stresses [20, 22, 23]. The WRKY gene family has been described and studied in several plant species namely *Arabidopsis* [14, 15], rice [24, 25], cucumber [26], barley [18], maize [27], tomato [28], physic nut [29] and cotton [30]; however, little is known about *Citrus*.

Our work aims to identify all putative WRKY TFs in *Citrus* and thus to propose a list of candidate genes related to disease resistance, fruit acidification, and environmental stresses adaptation. Detailed analyses of WRKY in *Citrus* including gene classification, multiple alignment, gene phylogeny, conserved motif composition, and hierarchical classification of ESTs have been performed. The recently sequenced sweet orange genome has provided a reference genome for analyses of chromosomal locations, syntenic relationships and the expression of the *CsWRKYs* in different tissues. Since available data on *Citrus* WRKY microarray are missing, we performed a study on gene functionality and expression patterns using Genevestigator tools in comparison with the closest homologous from *Arabidopsis*. We founded that two *Citrus* WRKY genes (*CsWRKY12* and *CsWRKY14*) may be proposed as candidate genes potentially up-regulated under all abiotic stress conditions. Also, a hypothesis can then be issued on the possible involvement of *Cc53*, *Cc54*, *Cs30*, *Cc60*, *Cc67*, *Cs40*, *Cs41* and *Cs48* genes in the response of *Citrus* to drought and salinity.

Our results from the genome wide survey of *Citrus* WRKYs family provide useful basis for further and targeted molecular analysis and functional characterization of potential WRKY candidate genes.

## Methods

### Sequence Database Searches

To identify putative *WRKY* genes in *Citrus*, two strategies were adopted as follows: firstly, all *Arabidopsis thaliana* WRKY family members retrieved from Plant TFDB database (<http://planttfdb.cbi.edu.cn/>) [31] were used as queries to search homology within *Citrus* genomes using tBLASTn [32]. The e-value threshold was set to  $5 \times 10^{-06}$ ; however, only one sequence was discovered in NCBI which is *CuWRKY1*.

Secondly, query search using “WRKY & *Citrus*” keywords within ten DB: NCBI (<http://www.ncbi.nlm.nih.gov/>), TIGR (<http://plantta.jcvi.org/>), CGF (<http://cgf.ucdavis.edu/home/>), CFGP (<http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/index.html>), CGD (<http://www.Citrusgenomedb.org/>), Plant TFDB ([http://planttfdb\\_v1.cbi.pku.edu.cn](http://planttfdb_v1.cbi.pku.edu.cn)), HarvEST (<http://harvest.ucr.edu/>), Phytozome (<http://www.phytozome.net>), ICGC (<http://www.Citrusgenome.ucr.edu/>) and CBL (<http://biotecnologia.centrodecitricultura.br/index.html>). Further analyses,

based on WRKY domain presence as given by PFAM, revealed 1868 potential *WRKY* TFgenes. All these sequences were given a designation number from 1 to 1868 depending on *Citrus* species like *CsWRKY* for *Citrus sinensis*, *PtWRKY* for *Poncirus trifoliolata*, etc.

In order to increase the reliability of our results and avoid redundancy within the identified *WRKY* genes in *Citrus*, sequences were filtered using NCBI local Blast standalone (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/README.bls>) [33] and then assembled with CAP3 (Sequence Assembly Program) (<http://pbil.univ-lyon1.fr/cap3.php/>) [34]. We thus proposed a unique *WRKY* identifier and followed *Arabidopsis* classification for *WRKY* genes annotation.

### Annotation and Classification of *WRKY* Family in *Citrus*

Full-length *WRKY* sequences obtained were used for further analysis. They were blasted in Genbank database to retrieve *A. thaliana* *WRKY* homologous. Filtration of the resulting alignments was performed using a threshold e-value of  $10^{-10}$  and *WRKY*s annotation according to *Arabidopsis* classification (groups I, IIa, IIb, IIc, IId, IIe, and III) was followed. *Citrus* *WRKY* domain was aligned using Clustal X 1.83 with default settings [35]. *CsWRKY* genes were located on chromosomes in Phytozome database (Sweet Orange Genome Project 2010, <http://www.phytozome.net/orange>). Besides, *CcWRKY* genes were located on scaffold in Phytozome database (Haploid Clementine Genome, International *Citrus* Genome Consortium, 2011, <http://int-Citrusgenomics.org/>, <http://www.phytozome.net/clementine>). All gene structures (exon-intron organization) were predicted by FGENESH Softberry (<http://linux1.softberry.com/berry.phtml>) [36].

### Multiple Sequence Alignment, Structural and Phylogenetic Analysis

To gain insights into the evolutionary relationship between *Citrus* and *Arabidopsis* *WRKY* proteins, multiple alignments, motif study and phylogenetic analysis were performed. All *Citrus* *WRKY* proteins and representative members of all subgroups of *AtWRKY* protein (*AtWRKY20* (At4g26640), *AtWRKY40* (At1g80840), *AtWRKY72* (At5g15130), *AtWRKY50* (At5g26170), *AtWRKY74* (At5g28650), *AtWRKY65* (At1g29280) and *AtWRKY54* (At2g40750)) were sequences aligned by Clustal X 1.83 using default settings [35]. Phylogenetic studies of the *WRKY* proteins family were conducted using MEGA version 4 [37], Jones-Taylor Thornton (JTT) and Neighbor-Joining (bootstrap = 1000). The evolutionary distance is counted using this method [38].

Multiple Expectation Maximization for Motif Elicitation (MEME) program was accessed online (<http://meme.sdsc.edu/meme/intro.html>) [39] and used to highlight structural variations among *Citrus* *WRKY* proteins. The parameters were as followings: number of repetition—any, maximum number of motifs=10, and the optimum motif width was maintained to between 6 and 50 residues.

### Chromosomal Locations, Syntenic Relationships and Protein Properties of *WRKY* in Sweet Orange

In order to determine the chromosomal location of *CsWRKY2* to *CsWRKY52*, BLASTn was performed on Orange Genome [40] ([citrus.hzau.edu.cn/orange](http://citrus.hzau.edu.cn/orange)).

MapChart 2.2 [41] was used to visualize chromosome linkage on the ten *citrus* chromosomes. The alignment of genes coding sequences within individual duplicate blocks was

realized by Clustal W software. The Pamilo-Bainchi-Li substitution model, the bootstrap variance estimation method (1000 replicates) and the pairwise deletion were selected when performing codon-based *Z* test for each block [42, 43]; and only those showing significant results (*P* value <0.05) were considered.

The iTAK database (<http://bioinfo.bti.cornell.edu/cgi-bin/itak/index.cgi>) currently contains the identified TFs from sweet orange with fully sequenced genomes [44]. Synteny analysis was performed using Plant Genome Duplication Database [45]. Syntenic blocks were drawn using Circos Tool.

Physico-chemical properties, molecular weight and isoelectric point of CsWRKYs were determined on ProtParam tools website (<http://web.expasy.org/protparam/>) [46].

### **Hierarchical Classification of *Citrus* WRKY ESTs into Expression Groups**

For cluster patterns expression analysis of WRKY ESTs, HarvEST *Citrus* database (<http://www.harvest-web.org/>) was used in order to examine the distribution of 132 *Citrus*-WRKY unigenes present into the 90 cDNA libraries, with a total number of ESTs of 985. The presence/absence of WRKY ESTs in each cDNA libraries was recorded as (0 or 1) matrix and used to construct a simple model distance matrix and hierarchical dendrogram using the method of Ward [47] with appropriate functions (“dist” and “hclust”) in R language [48].

### **Expression Patterns of CsWRKY Genes**

RNA-seq data provided by Xu et al. [40] from the sweet orange genome database was employed to retrieve fragments per kilobase of transcript per million fragments mapped values (FPKM). The Multiple Array Viewer MeV v4.4.1 [49] was used to cluster the expression values of the 51 CsWRKY genes.

### **Plant Expression Database Exploitation for WRKY Gene Expression Studies in *Citrus***

For WRKY gene expression analysis in *citrus*, publicly available microarray data were obtained and consulted from Plant Expression Database (PLEXdb, <http://www.plexdb.org/>) [50]. BLASTN served to identify WRKY genes probe sets (e-value,  $4.00^{E-04}$ ). We could thus follow expression profiles of WRKY genes under several treatments of stress and different culture conditions. The GeneChip® Citrus Genome Array obtained from PLEXdb allowed us to generate expression data from seven platforms containing 69 hybridizations and from which raw data from every experimental conditions were normalized for further studies. As previously done, the Multiple Array Viewer MeV v4.4.1 [49] was also used to cluster the expression values.

### **Analysis of Expression Profiles Under Biotic and Abiotic Stresses**

Public expression data making available multiple datasets from several experiments and conditions such as Genevestigator ([www.genevestigator.ethz.ch](http://www.genevestigator.ethz.ch)) are expanding [51, 52]. Given that no microarrays data were available in *Citrus*, we used Genevestigator data with the nearest homologs in *Arabidopsis*, assuming that protein function is conserved between orthologous sequences. Genevestigator is capable of performing large assays on thousands of

microarrays giving repeatable and reliable results [51, 52]. We looked for the nearest homologous to *Arabidopsis* of the 100 full-length *WRKY* genes. We presented developmental and tissue-specific gene expressions of 20 *AtWRKY* genes found in Genevestigator and possibly involved in (a)biotic stress tolerance. We chose the hierarchical clustering tool to identify groups of genes that have similar expression profiles. Log<sub>2</sub> values for the expression of 20 genes in various stress profiles and following the application of certain stimuli were imported from Genevestigator. The hierarchical classification of 20 different transcriptomes revealed different *AtWRKY* expressions at different conditions of stress profiles.

To assess the *WRKY* co-expression network under stress conditions, Genevestigator “co-expression” tool was selected to calculate the coefficient of Pearson correlation particularly among genes proposed as candidates. Thus, a network could be drawn to show the strongly (negatively or positively) correlated genes which have a Pearson Correlation Coefficient PCC value outside the range of [−0.6, 0.6].

## Results

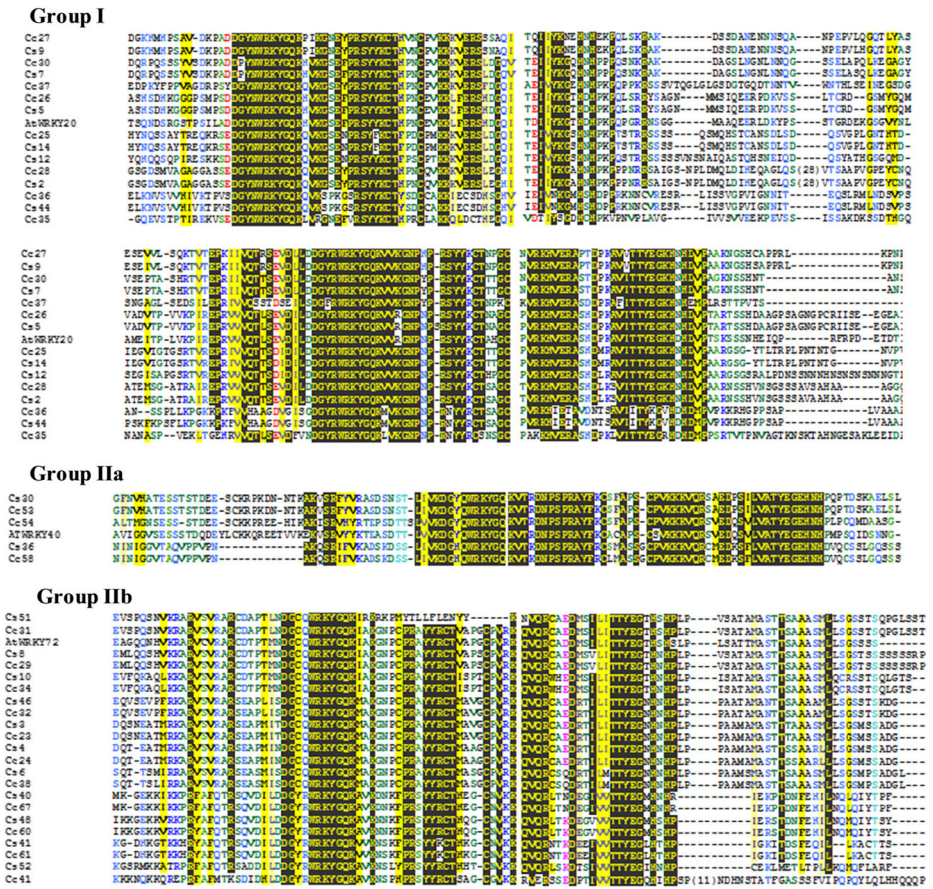
### Identification of *WRKY* Family Members in *Citrus*

As mentioned in “Methods,” several public databases were used to identify *WRKY* family genes in citrus. The homology search with *AtWRKY* representative members by tBLASTn provided one single gene from *Citrus unshiu* (AB573149.1). The keyword search within the ten DB gave a total number of 1868 putative *WRKY* genes (1768 partial and 100 full-length sequences) belonging to 19 different *citrus* species (Additional file 1), *C. sinensis* (sweet orange) being the most represented species with a frequency of 38 %. Similarly, the two *Citrus clementina* (15 %) and *Citrus reticulata* (26 %) species are strongly present in most DB. In fact, *C. sinensis* and *C. clementina* are part of the sequencing project of *Citrus* genomes [4], whereas *C. reticulata* is of commercial market interest.

Thus, 99 full-length sequences were identified within the Phytozome DB and the last and only one of *C. unshiu* was retrieved from NCBI. All other databases (TIGR, CGF, CFGP, CGD, PlantTFDB, HarvEST, ICGC, and CBL) gave only EST type sequences of which 1343 were from HarvEST (Additional file 1). In order to determine the accurate and non-redundant number of *WRKY* genes, the local alignment by BLAST at the NCBI allowed the reduction of the number of sequences from 1868 to 1395 and the program CAP3 allowed the assembly of the various EST to form contigs and reduce the number of sequences to a total of 552.

### Classification and Molecular Characterization of *Citrus WRKY* Proteins

A set of 100 full-length sequences of *WRKY* genes belonging to three *Citrus* species were identified: 1 in *C. unshiu*, 51 in *C. sinensis* (CsWRKY2 to CsWRKY52), and 48 in *C. clementina* (CcWRKY23 to CcWRKY71) (Additional file 2). Based on the *AtWRKY*s classification and *WRKY* domain alignment of these 100 *WRKY*s by Clustal X 1.83 [35] (Fig. 1), three main groups could emerge. Seventeen members harboring two *WRKY* domains were assigned to group I for whose the zinc finger motif was C<sub>2</sub>H<sub>2</sub> type [C-X<sub>4</sub>-C-X<sub>22–23</sub>-H-X<sub>1</sub>-H]. Seventy members containing only one *WRKY* domain, of which the zinc finger motif is C<sub>2</sub>H<sub>2</sub> type in the form of [C-X<sub>4–5</sub>-C-X<sub>23</sub>-H-X<sub>1</sub>-H], were assigned to



**Fig. 1** Multiple sequences alignment of the WRKY domains from *Citrus* with selected *Arabidopsis* strains WRKY transcription factors. The highly conserved WRKYGQK and the zinc finger motif sequences are highlighted in yellow, while the mismatched amino acids are marked in blue. Gaps are marked as dashes

group II including the five following subgroups: group II-a (5 members), group II-b (21 members), group II-c (23 members), group II-d (10 members), and group II-e (11 members). The group III comprises 13 members with also only one WRKY domain and where the zinc finger motif was C<sub>2</sub>HC [C-X<sub>7</sub>-C-X<sub>23-24</sub>-H-X<sub>1</sub>-C] or [C-X<sub>4</sub>-C-X<sub>23</sub>-H-X<sub>1</sub>-C] type (Additional file 2).

Several WRKYs have been described and characterized in other plant species namely *Arabidopsis* (74 *AtWRKYs*) [15], rice (102 *OsWRKYs*) [25], poplar (104 *PtWRKYs*) [53], ricin (47 *RcWRKYs*) [54], cucumber (55 *CsWRKYs*) [26], tomato (81 *SlWRKYs*) [28], barley (45 *HvWRKYs*) [18], maize (136 *ZmWRKYs*) [27] and cotton (112 *GrWRKYs* and 109 *GaWRKYs*) [55]. The different WRKY subgroups from *C. sinensis*, *C. clementina*, *A. thaliana*, *Vitis vinifera*, *Oryza sativa* and *Cucumis sativus* are described in Table 1. In our work, 51 WRKY sequences have been identified in *C. sinensis*, 48 WRKY sequences in *C. clementina* and a single sequence in *C. unshiu*. In comparison with *Arabidopsis* (125 Mb), rice (480 Mb) and cucumber (376 Mb) genomes, *Citrus* genome's size is quite average with 372 Mb and the number of WRKY sequences is approximately close to the

Group Iic

Multiple sequence alignment for Group Iic WRKY proteins. The alignment shows conserved regions across species including C. sinensis, C. clementina, A. thaliana, V. vinifera, O. sativa, and C. sativus. Conserved motifs are highlighted in yellow.

Group IId

Multiple sequence alignment for Group IId WRKY proteins. The alignment shows conserved regions across species including A. thaliana, C. sinensis, C. clementina, A. thaliana, V. vinifera, O. sativa, and C. sativus. Conserved motifs are highlighted in yellow.

Group Iie

Multiple sequence alignment for Group Iie WRKY proteins. The alignment shows conserved regions across species including C. sinensis, C. clementina, A. thaliana, V. vinifera, O. sativa, and C. sativus. Conserved motifs are highlighted in yellow.

Group III

Multiple sequence alignment for Group III WRKY proteins. The alignment shows conserved regions across species including C. sinensis, C. clementina, A. thaliana, V. vinifera, O. sativa, and C. sativus. Conserved motifs are highlighted in yellow.

Fig. 1 (continued)

Table 1 The number of subgroup WRKY genes in C. sinensis, C. clementina, A. thaliana, V. vinifera, O. sativa and C. sativus

Table with 7 columns: Group, CsWRKY, CcWRKY, AtWRKY, VvWRKY, OsWRKY, CusWRKY. Rows include subgroups I, IIa, IIb, IIc, IId, IIE, III, and Total.

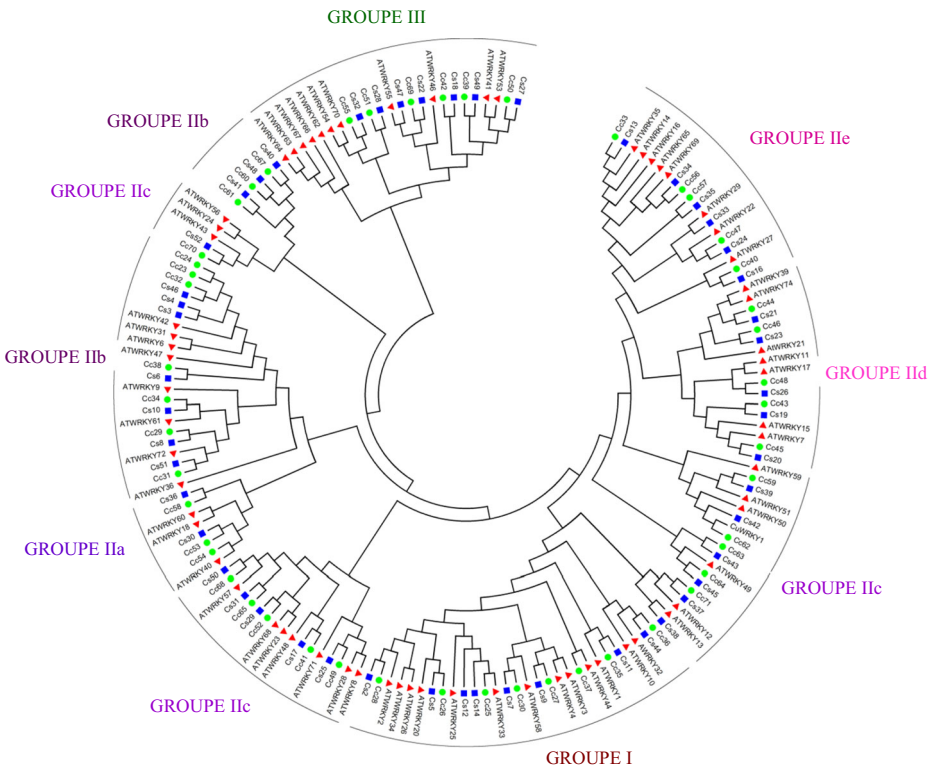


other species. Monocotyledonous plants display a higher number of WRKY proteins than dicots; indeed, maize and rice have respectively 136 and 109 WRKY proteins, while *Arabidopsis* has only 71.

The length of *Citrus WRKYs* ORF ranged from 321 bp (*CsWRKY48*) to 2166 bp (*CsWRKY2*), encoding polypeptides of 106 to 721 residues. The predicted molecular weight ranged from 12.7 kDa (*CsWRKY48*) to 77.8 kDa (*CsWRKY2*), with estimated isoelectric points from 4.7 (*CsWRKY45*) to 10.1 (*CsWRKY48*). Serine, a polar amino acid that may participate in hydrogen bonds, was the most abundant residue in 96 % of the citrus WRKY proteins. Besides, the number of exons of the WRKY sequences ranged from 2 to 7, with an average of 3 exons. A noteworthy intronic sequence was found within all WRKY domains found in the *Citrus-WRKY* genes; however, the N-terminal WRKY domain of the group I was free from intron. Even the WRKYGQK sequence was defined by Eulgem et al. [15] as the conserved heptapeptide characterizing WRKY proteins, for some species like rice, it was observed slight variations in this sequence [56, 57]. Similarly, some *Citrus WRKYs* displayed substitutions in their WRKY signature. In fact, the *CcWRKY59*, *CcWRKY62*, *CsWRKY39* and *CsWRKY42* sequences present the WRKYGKK domain. Furthermore, *CsWRKY43* and *CcWRKY63* sequences possess the WKYGGQK signature. One can assume that the variation of the WRKYGQK signature could affect its binding affinity to the W-box and thus the protein function and activity.

### Multiple Alignments and Phylogenetic Analysis Based on the WRKY Domain

Multiple alignments of amino acid residues of both *CsWRKY* and *CcWRKY* showed great divergence within each other respectively. Among the 48 *CcWRKYs* and the 51 *CsWRKYs*, both *CcWRKY27* with *CcWRKY30* and *CsWRKY7* with *CsWRKY9* showed 55 % sequences identities. Overall, identity between *Citrus WRKYs* ranged from 30 to 55 % indicating low degree of conservation. In order to examine the phylogenetic relationship among the 100 *Citrus WRKYs*, the phylogenetic tree was constructed. Sequences from *Arabidopsis WRKY* members were included in our analysis as references. An unrooted phylogenetic tree was constructed depending on alignments of amino acid sequences of 166 WRKY domains from the four species: *A. thaliana*, *C. clementina*, *C. sinensis*, and *C. unshiu*. As shown in Fig. 2, several subgroups were clearly formed on the basis of the phylogenetic analysis. Even phylogenetic trees of WRKY proteins usually display monophyletic nature [58], in our case, the attribution to defined subgroups is polyphyletic, according to the number of WRKY domains and the type of zinc finger motif. Additionally, the phylogenetic tree of WRKY domains from these species displayed a scattered distribution in all the groups indicating that the expansions of *WRKY* genes occurred before the divergence of *Citrus* and *Arabidopsis*. It has been reported that members of WRKYI group are the most ancient, with loss and/or gain of the N-terminal WRKY domain during the *WRKY* gene evolution process, and those of groups II and III are thought to be derived descendants from WRKYI group [19]. The groups IIb and IIc are divided into other groupings according to the nearest homologous with *Arabidopsis*. Subgroup IIc is closely linked to the group I with the exception of *CsWRKY52* and *CcWRKY70* and the subgroup IIb is bound to the subgroup IIa. This separation in subclades of the WRKYII group domains highlights their paraphyletic nature. On the other hand, in Fig. 2, the tree shows that group III is monophyletic.



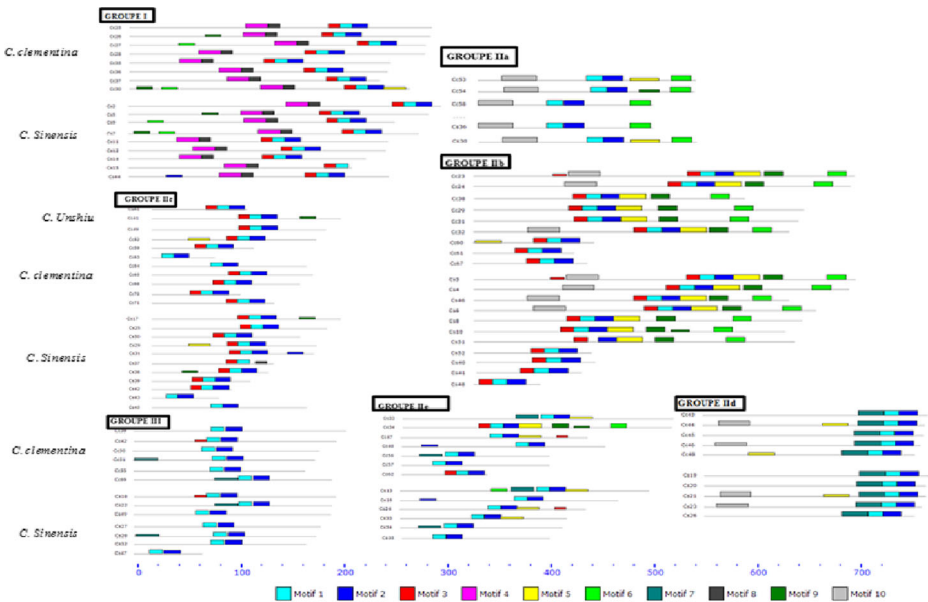
**Fig. 2** Unrooted phylogenetic tree representing relationships among *Citrus* and selected *Arabidopsis* WRKY protein. The phylogenetic tree from amino acid sequences was depicted by the MEGA v4.0 program with the neighbor-joining method. Clades of the *Citrus* WRKY domain are labeled according to the classifications of AtWRKY domains by Wu et al. [24]

As these WRKYs are involved in plants adaptation to various types of environmental stresses, duplication phenomenon occurrence within their members would contribute to the raise in acclimatization ability and rapid implementation of different signal transduction cascade of stress [27]. Based on comparative analysis among all kinds of WRKY genes plants, Zhang and Wang [19] have described an expansion of WRKY members in a more complex way towards pluricellular species.

**Conserved Motifs and Domains in *Citrus* WRKY Proteins**

We confirmed for all the identified sequences the presence of the WRKY conserved domain referenced as PF03106 WRKY DNA binding domain [15] and annotated by the followings terms Gene Ontology: 0003700, “DNA binding Transcription Factor Activity” and Gene Ontology: 0006355, “transcriptional control” [58]. Except some sequences, *Citrus* WRKYs proteins showed the PF10533 “Zinc Plant Cluster domain.”

Using MEME software, motif distribution and domain prediction could be assessed (Fig. 3). The first and second motif correspond to WRKY domains, which were broadly distributed in the *Citrus* WRKY protein sequences. The third motif is present in all groups except for the groups IIa, IId, III and some sequences like CcWRKY63 and CcWRKY64.



**Fig. 3** Ten predicted sequence motifs were identified by the Pfam database and analyzed by Meme V4.8.1. Different motifs, numbered 1–10, are displayed in different *colored boxes*. The conserved motifs 1, 2, 3, 5, and 11 were definitely identified as WRKY domains, which are broadly distributed in the *Citrus* WRKY family. The names of all members and subgroups are displayed on the *left-hand side*, while the exact length of motif is shown at *bottom of the figure*

This pattern is generally located upstream of WRKY domain. The motifs 4 and 8 are present only in group I: pattern 4 corresponds to the second conserved domain WRKY of group I while pattern 8 corresponds to the zinc finger motif. The patterns localization of each *Citrus* WRKY sequence is close to each other. In addition, there are several patterns (motifs 1, 2, 3, 5, 6, 9, 10) in the subgroup IIb. Some motifs like 5, 10 and conserved NLS motifs were mainly found in subgroups IIa and IIb, while others like motif 6, whose function remains unknown, were only located in subgroups IIa and IIb. Motif 7, a calmodulin-binding conserved domain (CaMBD), is often present in subgroup IIc, indicating putative involvement in signal transduction via activity regulation of target proteins [59]. Additionally, this CaMBD motif was found in CcWRKY33 and CsWRKY13 of the subgroup IIe. Besides, several motifs could be likewise revealed by MEME, yet with uncharacterized function. This may indicate a high and subtle regulation of WRKY biological roles from *Citrus*.

### Chromosomal Position and Synteny Analysis of the *CsWRKY* Genes

In silico assessment of genes chromosomal location revealed a sparse distribution of the 51 *CsWRKY* genes across all ten sweet orange chromosomes (Table 2, Fig. 4). Chromosome VII harbored 11 *WRKY* genes, the highest number of *WRKY* genes located on the same chromosome. The uncharacterized and II chromosomes contained, respectively, nine and seven *WRKY* genes. In contrast, chromosomes III and VIII encompassed each only one *WRKY* gene. Besides, five gene pairs are suspected to be segmental duplicated among the sweet orange chromosomes (Fig. 4) presumably resulting from the polyploidy event in this genome:

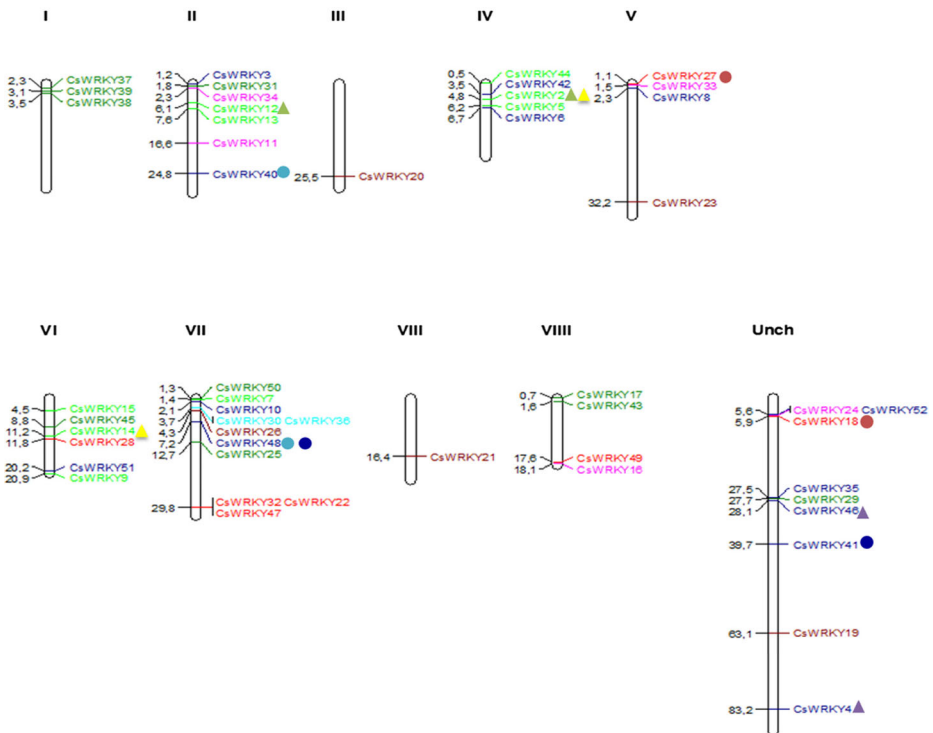
**Table 2** *CsWRKY* genes encoding WRKY proteins along with their molecular details

Gene	Gene ID (CAP)	Location	No. exons	Group	Protein		
					Length (aa)	Molecular weight	PI
CsWRKY2	Cs4g07560.2	chr4: 4,806,432..4,811,078	4	I	721	77,798.7	5.68
CsWRKY3	Cs2g02790.1	chr2: 1,166,737..1,170,265	7	IIb	618	67,180.2	5.99
CsWRKY4	orange1.1t05133.1	chrUn: 83,231,411..83,233,828	5	IIb	599	65,156.9	6.28
CsWRKY5	Cs4g09310.1	chr4: 6,195,542...6,200,799	5	I	564	61,000.6	6.47
CsWRKY6	Cs4g10020.1	chr4: 6,670,189...6,673,283	6	IIb	547	60,323.0	7.06
CsWRKY7	Cs7g03300.1	chr7: 1,410,331...1,415,689	5	I	545	59,361.4	8.78
CsWRKY8	Cs5g04160.1	chr5: 2,321,902...2,325,229	3	IIb	526	56,855.0	7.97
CsWRKY9	Cs6g21990.1	chr6: 20,895,588..20,899,292	4	I	501	55,250.6	6.84
CsWRKY10	Cs7g04260.1	chr7: 2,126,526...2,129,982	3	IIb	498	54,640.7	8.22
CsWRKY11	Cs2g19800.1	chr2: 16,597,423..16,599,756	4	I	488	53,282.5	6.45
CsWRKY12	Cs2g09020.1	chr2: 6,146,166...6,149,568	4	I	484	53,513.9	7.25
CsWRKY13	Cs2g10310.1	chr2: 7,627,245...7,630,275	3	IIe	460	49,778.6	6.08
CsWRKY14	Cs6g09420.1	chr6: 11,178,822..11,182,318	4	I	447	49,805.1	6.39
CsWRKY15	Cs6g03950.1	chr6: 4,519,015...4,524,087	3	I	421	46,662.7	8.34
CsWRKY16	Cs9g19070.1	chr9: 18,136,219..18,137,855	3	IIe	403	44,615.0	5.12
CsWRKY17	Cs9g02040.1	chr9: 671,464..673,389	3	IIc	371	40,911.6	6.00
CsWRKY18	orange1.1t00472.1	chrUn: 5,888,815...5,892,186	3	III	364	41,108.7	5.23
CsWRKY19	orange1.1t04068.1	chrUn: 63,057,402..63,060,016	3	IIId	363	40,160.0	9.23
CsWRKY20	Cs3g23190.1	chr3: 25,538,725..25,541,177	3	IIId	359	38,791.8	9.30
CsWRKY21	Cs8g13600.1	chr8: 16,355,947..16,358,822	3	IIId	359	40,539.9	9.77
CsWRKY22	Cs7g29580.1	chr7: 29,782,722..29,785,025	3	III	356	39,267.5	6.16
CsWRKY23	Cs5g30250.1	chr5: 32,172,106..32,175,238	3	IIId	352	39,494.6	9.69
CsWRKY24	orange1.1t00419.1	chrUn: 5,566,849..5,568,616	3	IIe	346	37763.5	6.32
CsWRKY25	Cs7g17180.1	chr7: 12,654,326..12,657,389	3	IIc	342	38,277.0	6.66

**Table 2** (continued)

Gene	Gene ID (CAP)	Location	No. exons	Group	Protein		
					Length (aa)	Molecular weight	PI
CsWRKY26	Cs7g07140.1	chr7: 4,260,845..4,263,354	3	IId	341	36,737.4	9.54
CsWRKY27	Cs5g02440.1	chr5: 1,080,665..1,084,277	3	III	336	37,636.4	5.20
CsWRKY28	Cs6g10120.1	chr6: 11,786,019..11,787,703	3	III	328	36,807.7	5.91
CsWRKY29	orange1.lt01713.1	chrUn: 27,672,465..27,674,620	3	IIC	322	35,539.7	8.56
CsWRKY30	Cs7g06320.1	chr7: 3,696,558..3,698,483	5	IIa	321	35,896.1	7.15
CsWRKY31	Cs2g03840.1	chr2: 1,809,564..1,810,844	3	IIC	317	35,159.0	6.80
CsWRKY32	Cs7g29570.1	chr7: 29,778,597..29,780,253	3	III	310	35,164.8	6.01
CsWRKY33	Cs5g03010.1	chr5: 1,488,895..1,490,249	3	IIe	306	34,128.2	5.76
CsWRKY34	Cs2g04520.1	chr2: 2,278,266..2,280,205	4	IIe	298	33,998.4	5.52
CsWRKY35	orange1.lt01686.2	chrUn: 27,465,432..27,466,953	3	IIe	274	31,079.3	5.12
CsWRKY36	Cs7g06330.1	chr7: 3,705,792..3,708,168	4	IIa	258	28,812.4	8.94
CsWRKY37	Cs1g03100.1	chr1: 2,270,797..2,274,197	4	IIC	240	26,956.4	6.85
CsWRKY38	Cs1g04180.1	chr1: 3,485,909..3,489,254	3	IIC	230	25,883.1	9.10
CsWRKY39	Cs1g03870.1	chr1: 3,087,282..3,091,014	3	IIC	196	22,458.4	6.54
CsWRKY40	Cs2g25560.1	chr2: 24,763,856..24,765,366	2	IIb	183	20,820.3	9.03
CsWRKY41	orange1.lt02600.1	chrUn: 39,677,835..39,678,955	2	IIb	162	18,204.4	9.56
CsWRKY42	Cs4g05760.1	chr4: 3,532,847..3,534,502	3	IIC	159	18,301.0	5.29
CsWRKY43	Cs9g03310.3	chr9: 1,610,778..1,612,866	3	IIC	135	15,569.0	8.81
CsWRKY44	Cs4g01710.1	chr4:546,928..550,996	5	I	487	53,543.4	6.06
CsWRKY45	Cs6g06940.1	chr6: 8,833,626..8,835,959	3	IIC	303	33,741.0	4.86
CsWRKY46	orange1.lt01779.1	chrUn: 28,119,497..28,123,047	6	IIb	501	54,409.9	8.58
CsWRKY47	Cs7g29580.1	chr7: 29,782,722..29,785,025	3	III	124	14,184.0	6.83
CsWRKY48	Cs7g11020.1	chr7: 7,242,159..7,244,955	2	IIb	106	12,662.4	9.89
CsWRKY49	Cs9g18480.1	chr9: 17,646,412..17,648,474	3	III	354	39,139.0	5.23
CsWRKY50	Cs7g03080.1	chr7: 1,298,173..1,303,121	3	IIC	291	32,087.3	6.19
CsWRKY51	Cs6g20850.1	chr6: 20,194,206..20,197,649	4	IIb	510	55,554.2	6.20
CsWRKY52	orange1.lt00425.1	chrUn: 5,588,061..5,589,926	2	IIb	188	21,585.4	9.30

*CsWRKY12* and *CsWRKY2*, *CsWRKY14* and *CsWRKY2*, *CsWRKY4* and *CsWRKY46*, *CsWRKY40* and *CsWRKY48*, *CsWRKY41* and *CsWRKY48*, *CsWRKY27* and *CsWRKY18*; assuming thus functional divergence between duplicated genes. Each of those



**Fig. 4** Localization of the identified *CsWRKY* genes on the sweet orange chromosomes. The chromosome number is indicated at the top of each chromosome. *Identical colored circles or squares* indicate potential segmental duplicated WRKY gene pairs. The genes marked in different color indicate the group of the WRKY family (I, *pistachio green*; IIa, *burgundy*; IIb, *blue*; IIc, *green*; IId, *blue sky*; IIe, *pink*; III, *red*)

genes pairs belongs to the same group of WRKY. Moreover, the phylogenetic analysis performed by MEGA 6 software (Additional file 3) let assume very close relationships among *CsWRKYs*.

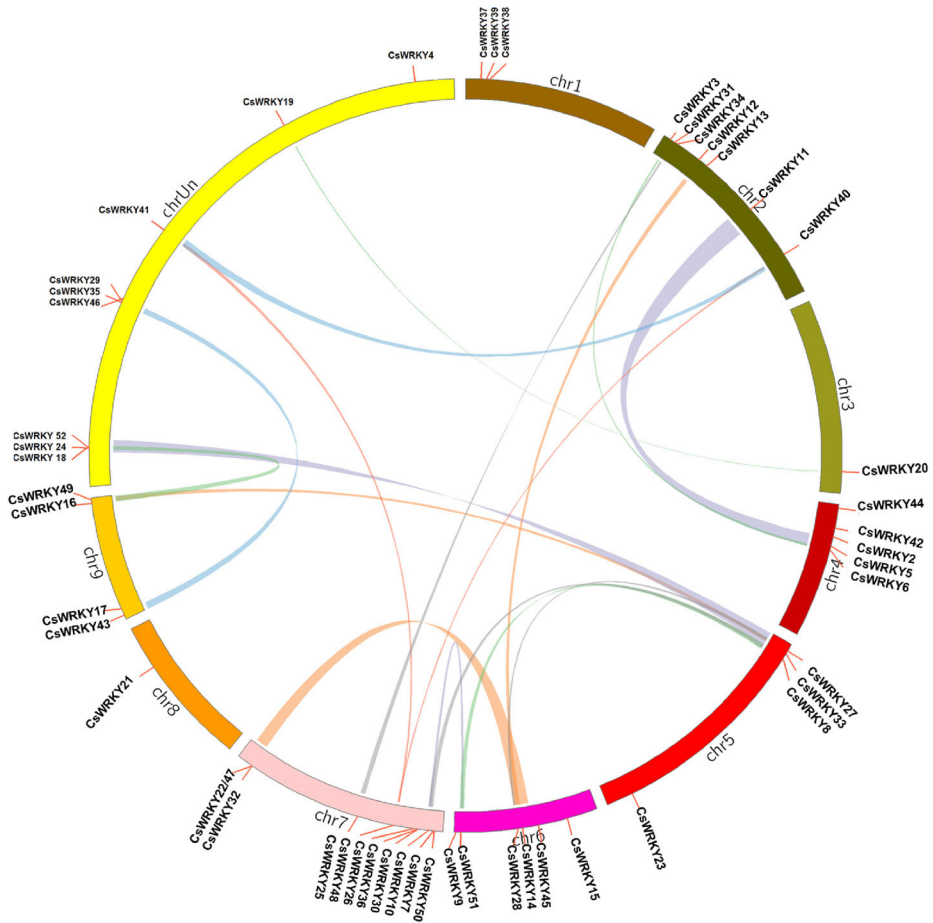
An extensive number of *Arabidopsis* model plant *WRKYs* have been widely investigated [15, 60] and thus we performed for *Citrus WRKYs* an analysis of synteny (Fig. 5) that led to the identification of 17 pairs of syntenic relations.

Several citrus orthologs namely *CsWRKY11*, *CsWRKY27*, and *CsWRKY33* displayed synteny location with *CsWRKY5* and *CsWRKY2*, *CsWRKY 24* and *CsWRKY18*, respectively.

All these results together suggest a tight conservation of WRKYs family and therefore its involvement and crucial role in plant biology and development.

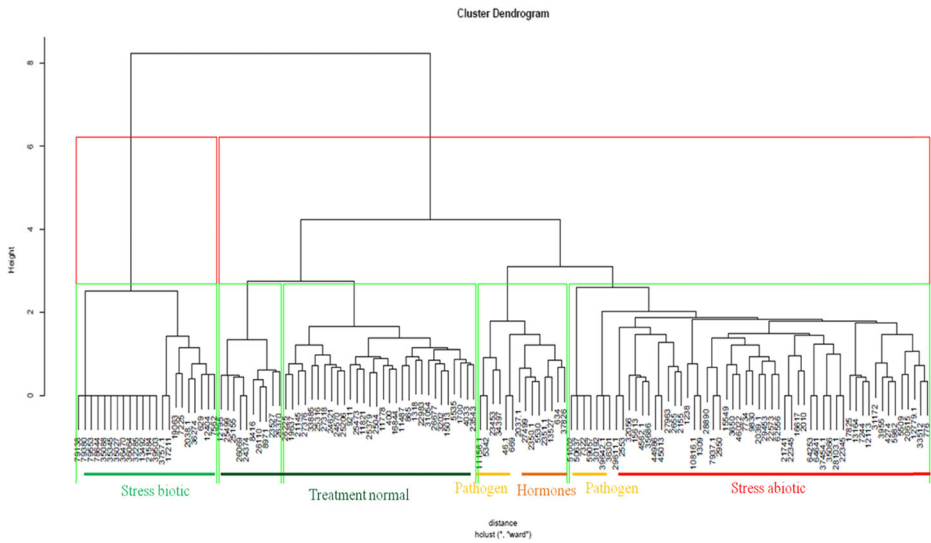
### Hierarchical Classification of *WRKY* ESTs into Expression Groups

In order to elucidate the possible role of the *WRKYs* in citrus adaptation to stressful conditions, the expression profile of *WRKYs* was analyzed under several stimuli such as biotic or abiotic stresses [61] or hormonal treatments [62]. To identify *Citrus WRKY* ESTs putatively associated with important physiological mechanisms, expression data of 132 unigenes were clustered. It would become thus possible to distinguish through



**Fig. 5** Chromosome distribution and synteny analysis of sweet orange *WRKY* genes. Chromosomes 1–Unch are shown with *different colors and in a circular form*. The approximate distribution of each *CsWRKY* gene is marked with a *short red line on the circle*. *Colored curves* denote the details of syntenic regions between *CsWRKY* genes

several kinds of treatments diverse and specific patterns of gene expressions. *Citrus* unigenes can be separated into five main groups based on their library distribution (Fig. 6). The first cluster (biotic stress) grouped unigenes only from UCRPT01 library that displays induced *WRKY ESTs* in response to Tristeza Virus infection, the most damaging virus affecting *Citrus* [63]. The second cluster (no treatment) containing *ESTs* from various libraries is divided into two subclusters: the first, grouped unigenes only from CitNFL library which concern mixed tissue of *C. reticulata* and the second grouped unigenes from various libraries. The third cluster contains unigenes from libraries involved in plant development by ethylene (AbsAov1). The remaining unigenes (group of pathogen) were distributed libraries of UCRCS11 (red scale), CS00-C1-401, and CM30-C1-401 (*Citrus leprosis virus CiLV*). The biggest and last cluster grouped unigenes from libraries involved mainly in abiotic stress. The most represented library in this cluster were FLAVEDO4 (heat) and UCRCS06 (storage).



**Fig. 6** Dendrogram showing relationships among *Citrus* WRKY unigenes based on their expression data

### Expressions of *Citrus* WRKYs in Different Tissues

Transcriptome sequencing data of several citrus tissues and organs (i.e., callus, fruit, flower, and leaf) [40], publicly available, were surveyed and exploited in order to retrieve and study expression profiles variations of *CsWRKY* genes (Table 2), and FPKM value was employed to determine the levels of genes expression. Thirty-eight genes at least of the 51 checked *CsWRKY* were significantly induced in one of the four organs. All the top five highly expressed *CsWRKY* genes, including *CsWRKY28*, *CsWRKY39*, *CsWRKY25*, *CsWRKY14* and *CsWRKY12*, were detected in callus, suggesting a probable role in meristematic cell growth and development.

A constitutive expression was reported in all tissues and organs for *CsWRKY2*, *CsWRKY7*, *CsWRKY9*, *CsWRKY18*, *CsWRKY19*, *CsWRKY23*, *CsWRKY26*, *CsWRKY28* and *CsWRKY32* (Fig. 7), callus being the tissue displaying the highest level of gene expression. Meanwhile, *CsWRKY52* is down-regulated in all the organs.

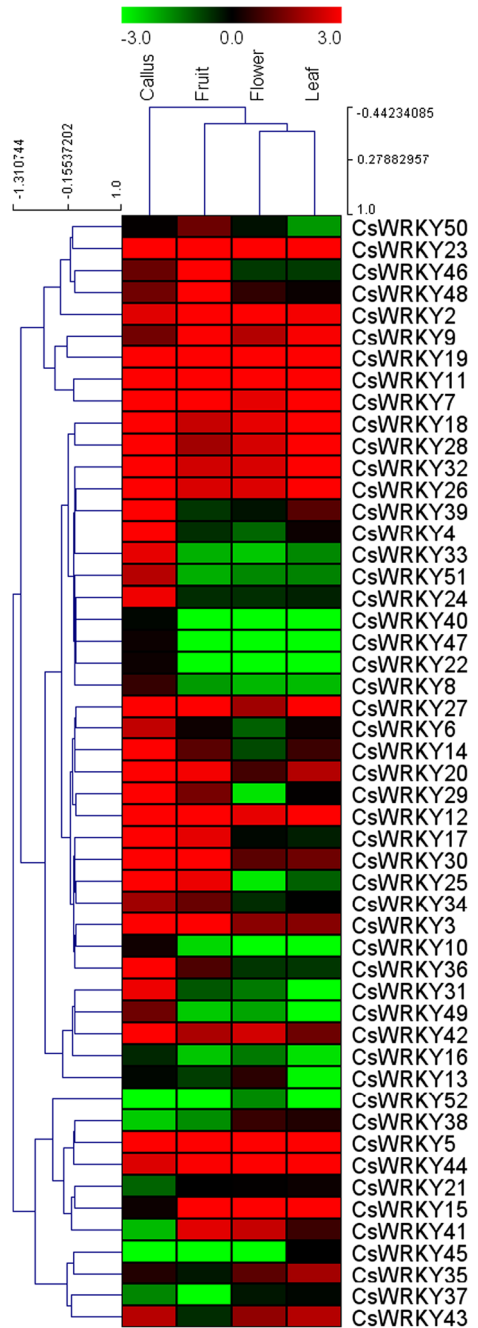
Callus displayed genes exclusively up-regulated in this tissue, namely *CsWRKY8*, *CsWRKY10*, *CsWRKY22*, *CsWRKY24*, *CsWRKY31*, *CsWRKY33*, *CsWRKY40*, *CsWRKY49* and *CsWRKY51*. *CsWRKY15* and *CsWRKY41* were both expressed in fruit, flower, and leaf, and *CsWRKY38* was specifically and solely transcribed in flower.

The transcriptional analysis of 31 *CsWRKY* and 28 *CcWRKY* genes within citrus organs and fruit ripening stages showed variations in expression patterns, indicating their putative role in fruit maturation. Particularly, a transcriptional analysis of epithelial cells in grapefruit pee, where it is assumed that secretory glands synthesize citrus essential oil, showed that *CsWRKY10–51* and *CcWRKY34–58* were up-regulated in comparison with the parenchyma cells, the non-oil-biosynthesizing cells, as the control cell type. These groups of WRKY genes highly expressed in epithelial cells could be involved in the essential oil biosynthetic pathway.

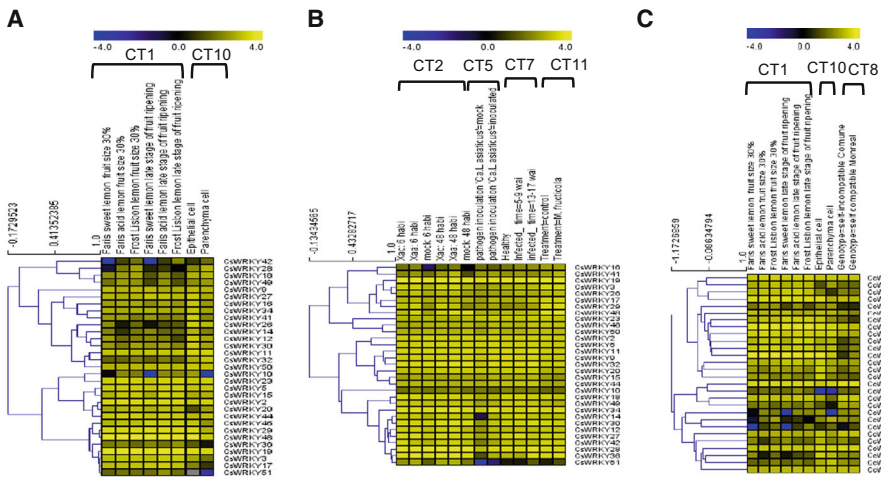
As citrus pulps contain a wide range of acidity content depending on genotypes, we made a comparison at the transcriptional level between three lemon genotypes differing in their acidity



**Fig. 7** Expression analysis and hierarchical clustering of all *WRKY* genes in *C. sinensis* in different organs (leaf, callus, flower, fruit). Heat map representation and hierarchical clustering of the *CsWRKY* genes. The FPKM values retrieved from published RNA-seq data were log<sub>2</sub> transformed and the heat map generated using TIGR MeV v4.1.1. Color scale at the end of the dendrogram represents relative expression levels: green represents low level and red indicates high level; asterisk indicates *WRKY* genes



(standard, sweet, and acid). We found that *CcWRKY34–62* and *CsWRKY42–10* displayed higher transcript levels in acidic fruits than sweetness fruits, indicating their possible role in fruit acidification during ripening (Fig. 8).



**Fig. 8** Expression analysis and hierarchical clustering of WRKY genes in *C. sinensis* and *C. clementina* (a) and under different conditions: CT1 (Lemon acidity) and CT10 (Transcriptional analysis of epithelial cells in grapefruit peel) in *C. sinensis* (b) under different biotic stress: CT2 (Transcriptional analysis of the sweet orange interaction with the citrus canker pathogens), CT5 (Expression data of sweet orange in response to *Candidatus Liberibacter asiaticus* infection at advanced stage), CT7 (Gene expression in *C. sinensis* following infection with the bacterial pathogen *Candidatus Liberibacter asiaticus* causing Huanglongbing in Florida), and CT11 (Global changes in gene expression of grapefruit peel tissue in response to the yeast biocontrol agent *Monilinia fruticicola* in *citrus sinensis*) (c) under different conditions: CT1 (Lemon acidity) and CT10 (Transcriptional analysis of epithelial cells in grapefruit peel) and CT8 (Comparative analysis of styler canal cells transcriptome in clementine mandarin) in *C. clementina*. Color scale at the end of each dendrogram represents relative expression levels: blue represents low level and yellow indicates high level; asterisk indicates WRKY genes

Microarray data (CT8) related to self-incompatibility in clementine did not reveal any interesting candidate for this biological mechanism (Additional file 4).

Transcription factors (i.e., WRKYs) whose expression is elevated in particular tissues regulate in all likelihood transcription activity of genes involved in plant growth and developmental processes [64–66].

### In Silico Analysis of Expression Profiles Under Abiotic Stress

A preliminary way to elucidate genes function is to analyze their spatio-temporal expression levels. Abiotic and biotic stresses are known to induce the expression of a multitude of WRKYs [67], meaning their role in regulatory transcription activity under such constraints. Some *AtWRKYs* (Additional file 3) were selected for the study of their expression under abiotic stress within five different tissues: root culture, seedling, rosette, adult leaf, and roots. In normal conditions, almost all WRKY genes are expressed especially in roots, where perception of various environmental stresses is proceeded.

In contrast, some genes such as *AtWRKY70* reveal a difference in expression in adult leaf. Indeed, the root system is an organ of perception of various environmental stresses.

To define the specificity and the interaction between the transcriptomic responses to the different treatments studied, we analyzed the over-expressed and under-expressed genes. The data set shows that the same TF can be induced or repressed under different stress states (Fig. 9). Our data matrix included 44 different abiotic stresses (anoxia, cold,

drought, dark, heat, hypoxia, etc.). *CsWRKY12* and *CsWRKY14* displayed high levels of transcripts under all stress conditions and thus may be proposed as candidate genes potentially involved in abiotic stress response, particularly drought and heat adaptation. Moreover, these latter were found to be co-expressed with a salt inducible zinc finger gene (Fig. 9e) in a highly correlated manner, confirming thus their involvement in abiotic stress tolerance.

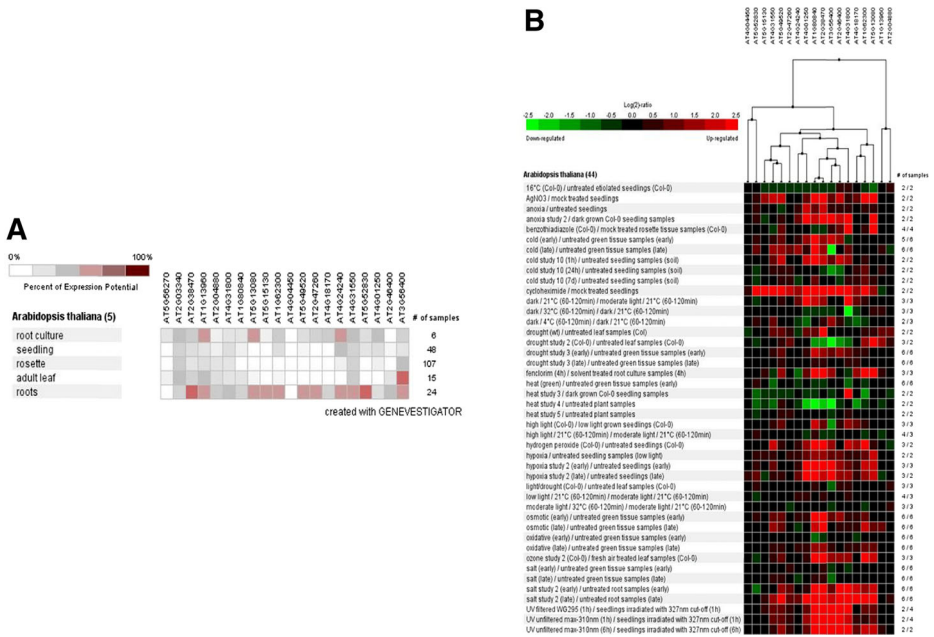
In our bicluster data matrix, nine *AtWRKY* genes (*W33*, *W18*, *W40*, *W75*, *W6*, *W48*, *W28*, *W11*, *W22* and *W46*) were expressed as specific mediators in salt response (150 mM NaCl). For example, *WRKY33* (At2g38470), the nearest homologous to *CcWRKY52* and *CsWRKY29*, was induced in roots treated by NaCl [68], which corroborates their mediation in salinity adaptation. The pathway analysis shows that induction of *WRKY33* expression depends in part on the Abscisic acid (ABA), but is independent of the SOS system (Salt Overly-Sensitive) [68]. Transgenic plants over-expressing either *WRKY25* or *WRKY33* better behave under salinity conditions than the sensitive double mutant *wrky25-wrky33* [69]. Drought stress which is often linked to salinity generally reduces plant growth and crop productivity [70]. A hypothesis can then be issued on the possible involvement of *Cc53*, *Cc54*, *Cs30*, *Cc60*, *Cc67*, *Cs40*, *Cs41* and *Cs48* genes in the response of *Citrus* to drought and salinity.

In our analysis, *AtWRKY40* was expressed in response to cold (4 °C). According to the results of Genevestigator, *Cc53*, *Cc54*, *Cs30*, *Cs28*, *Cs32*, *Cc51* and *Cc55* TFs could be potentially implicated in cold stress tolerance. In contrast, the heat stress inhibits the expression of the following genes: *Cs7*, *Cs9*, *Cs12*, *Cs14*, *Cc60*, *Cc67*, *Cs40*, *Cs41*, *Cs48*, *Cs20*, *Cs19*, *Cc45*, *Cs24*, *Cs33*, *Cc47*, *Cc40*, *Cs28*, *Cs32*, *Cc51* and *Cc55S*.

## Expression Studies of *WRKYs* in Response to Biotic Stress

To assess and confirm *Citrus* *WRKY* TFs involvement in the response to biotic stress and the regulation of target genes transcription, *AtWRKY* orthologs were analyzed for their expression profiles under pathogens infestation like *Alternaria brassicicola*, *Botrytis cinerea*, *Erysiphe orontii*, *Golovinomyces cichoracearum*, *Pseudomonas syringae*, *Phytophthora infestans* and *Phytophthora parasitica* (Fig. 10). For *Arabidopsis* microarray analysis, root, adult leaf and rosette were selected. Almost all *WRKY* genes are expressed, especially in roots under biotic stress, except *AtWRKY70* which reveals a difference in expression in adult leaf and rosette.

Microarray data analysis showed that globally 47 *Citrus* *WRKY* genes were differentially expressed, among them 24 were up-regulated and 23 were down-regulated under the above-mentioned biotic aggression (Fig. 10). Interestingly, five *WRKY* genes (*Cc60*, *Cc67*, *Cs40*, *Cs41* and *Cs48*) were always up-regulated by these pathogens infections which make them good candidates for stress adaptation improvement. *AtWRKY75*, which has been shown as a positive regulator of defense against diseases [71], represents the closely related orthologs of *Arabidopsis* to these latter (*Cc60*, *Cc67*, *Cs40*, *Cs41* and *Cs48*). In our bicluster data matrix, *AtWRKY33*, *AtWRKY40* and *AtWRKY75* genes were up-regulated under *Pseudomonas syringae*, *Phytophthora infestans* and *Phytophthora parasitica* contamination. In contrast, these genes were down-regulated in the case of *A. brassicicola* and *G. cichoracearum* infestation. For example, the insert mutant *AtW33* (*Cs12*, *Cs14* orthologs) has increased sensitivity to the *B. cinerea* necrotrophic fungi. The susceptibility of the plant is correlated with weak levels of PR-1 transcripts [72].



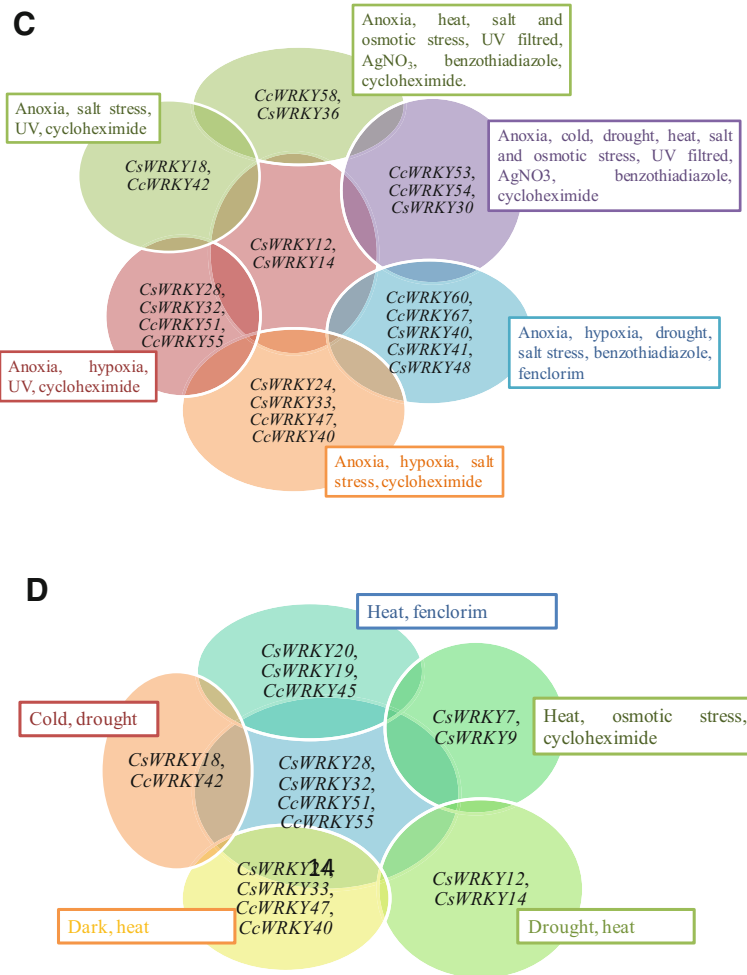
**Fig. 9** Profile of WRKY gene expression under abiotic stress by the Genevestigator database. **a** Percentage of potential WRKY gene expression in different tissues of *A. thaliana*. **b** Hierarchical clustering of 20 *AtWRKY* under various abiotic stress conditions in *A. thaliana* (log 2 scale). The genes up-regulated are marked in red, while the down-regulated genes are marked in green. Summary of the expression analyses of WRKY genes up-regulated (c) and down-regulated (d). **e** The dendrogram indicates the degree of similarity (Pearson’s correlation) between gene expression profiles and WRKY conditions tested. Pearson Correlation Coefficient of *AtWRKY33* (potentially *CsWRKY12* and *CsWRKY14*)

Some TFs have been known to be associated in a complex interaction with redundant roles, antagonists or distinct in plant-microbe interactions. This has been demonstrated, for example, for *AtW18*, *AtW40* and *AtW60* which act as regulators of basal resistance in plants [73]. The most correlated gene with *AtWRKY75* is glutathione S transferase which is implicated in oxidative stress (Fig. 10e).

Citrus GeneChip array related to canker disease (GPL5731) was prospected to survey the transcription patterns of sweet orange WRKYs (CT2) (Fig. 8, Additional file 4). *CsWRKY16* was the only one to be up-regulated by these pathogens, suggesting its putative role in canker disease resistance. Similarly, the Affymetrix GeneChip® citrus genome array (CT5) analysis demonstrated that *CsWRKY14* could be associated with defense response against Huanglongbing infection (HLB, Yellow Dragon Disease), the most severe disease of citrus.

**Expression Analysis of WRKYs in Response to Hormonal Treatments**

In our bicluster data matrix, the most important variation of expression after hormonal stimulus is observed after methyl jasmonate (MeJa) treatment. This treatment represses at the same time *AtW3*, *AtW75*, *AtW6*, *AtW23*, *AtW28*, *AtW22* and *AtW70*, and induces *AtW40* at the root and aerial parts levels (Fig. 11). The Meja, derived from jasmonic acid, is a plant hormone acting as cell regulator in several of aspects of plant biology, such as

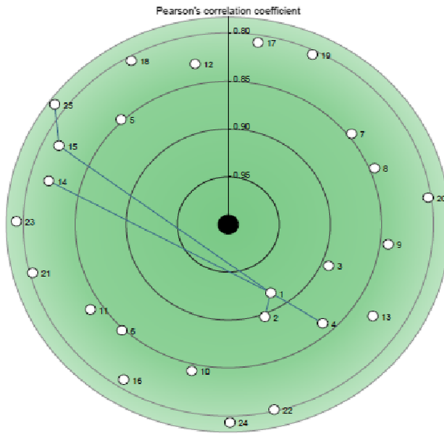


**Fig. 9** (continued)

flowering, rhizogenesis, seed germination, fruit ripening and senescence [74]. So and by homology, these *Cs7*, *Cs9*, *Cc60*, *Cc67*, *Cs40*, *Cs41*, *Cs48*, *Cc49*, *Cs31*, *Cs20*, *Cs19*, *Cc45*, *Cs24*, *Cs33*, *Cc47*, *Cc40*, *Cs28*, *Cs32*, *Cc51* and *Cc55* genes would be suppressed after treatment with Meja; however, *Cc53*, *Cc54* and *CS30* genes are induced by the same treatment (Fig. 11). ABA plays role in several plant developmental and adaptation processes including stomatal closure, seed germination, embryo maturation, flowering, leaf senescence and stress signal transduction [75]. According to the results of the Genevestigator database, *Cs12*, *Cs14*, *Cc53*, *Cc54* and *Cs30* are potentially involved in the stress response pathway which is ABA dependent (Fig. 11). Jasmonic acid (JA) and salicylic acid (SA) are phytohormones that function as signaling molecules involved in plant responses to abiotic and biotic stresses as well as plant growth and development namely photosynthesis, transpiration, ion uptake and transport and plant defense

**E**

Dataset: 210 perturbations (sample selection: STRESS)  
 Target gene: AT2G38470 (267028\_at in gene selection AT-GENES-0)



Gene	Score	Description
1 AT3G55680	0.92	salt-inducible zinc finger 1
2 AT1G08040	0.90	WRKY DNA-binding protein 40
3 AT1G05575	0.89	unknown protein, FUNCTIONS IN: molecular_funct...
4 AT4G17400	0.86	ethylene responsive element binding factor 8
5 AT2G40140	0.85	zinc finger (CCH4-type) family protein
6 AT3G52400	0.85	syntaxin of plants 122
7 AT5G27420	0.85	carbon/nitrogen insensitive 1
8 AT1G23710	0.85	Protein of unknown function (DUF1645)
9 AT2G26530	0.84	Protein of unknown function (DUF1645)
10 AT2G33810	0.84	laccasein B
11 AT4G34150	0.84	Calcium-dependent lipid-binding (CaLB domain) fa...
12 AT5G27200	0.83	Heavy metal transport/detoxification superfamily pro...
13 AT5G54400	0.83	pinoid-binding protein 1
14 AT3G54540	0.82	glycine-rich protein
15 AT4G22750	0.82	unknown protein, BEST Arabidopsis thaliana protei...
16 AT5G08320	0.81	NDR1/HIN1-like 3
17 AT3G45640	0.81	mitogen-activated protein kinase 3
18 AT3G00930	0.80	cytochrome BCl synthesis
19 AT1G16020	0.80	unknown protein, BEST Arabidopsis thaliana protei...
20 AT1G68660	0.80	Protein of unknown function (DUF556)
21 AT3G49530	0.80	NAC domain containing protein 62
22 AT5G51160	0.80	Integrase-type DNA-binding superfamily protein
23 AT1G27730	0.79	salt tolerance zinc finger
24 AT4G27280	0.79	Calcium-binding EF-hand family protein
25 AT4G24570	0.79	dicarboxylate carrier 2

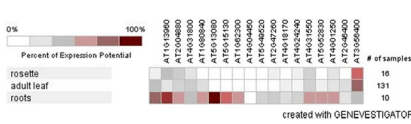
Show only genes with correlation above: 0.759

Connect genes with mutual correlation at least: 0.871

Fig. 9 (continued)

responses against pathogens [76, 77]. *AtW70* (*Cs28*, *Cs32*, *Cc51* and *Cc55 orthologs*) modulates interference in signaling pathways between SA and JA [78].

**A**



**B**

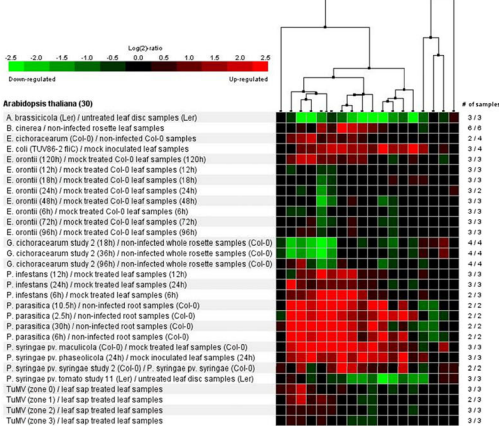
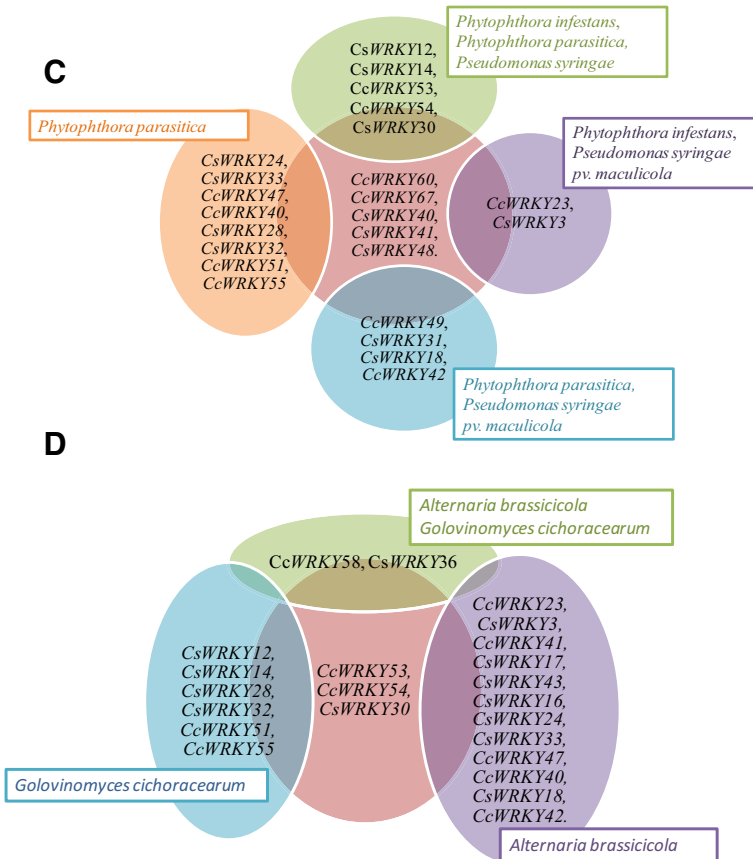


Fig. 10 Profiles of *WRKY* gene expression under biotic stress by the database Genevestigator: a percentage of potential *WRKY* gene expression in different tissues of *A. thaliana*. b Hierarchical clustering of 20 *AtWRKY* under various biotic stress conditions in *A. thaliana* (log 2 scale). The genes up-regulated are marked in red, while the down-regulated genes are marked in green. Summary of the expression analyses of *WRKY* genes up-regulated (c) and down-regulated (d). e Pearson correlation coefficient of *AtWRKY75* (potentially *CcWRKY60*, *CcWRKY67*, *CsWRKY40*, *CsWRKY41* and *CsWRKY48*)



**Fig. 10** (continued)

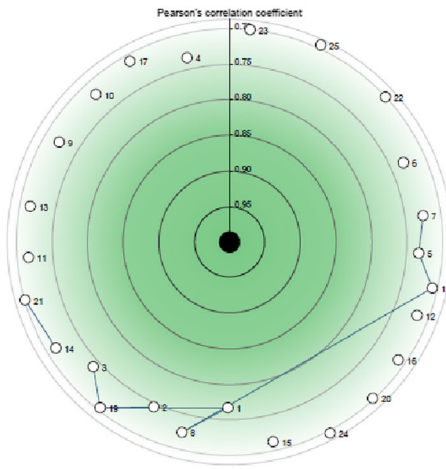
## Conclusion

The public availability of citrus genomic and transcriptomic data led us to the identification and characterization of 100 WRKY genes from *C. sinensis* (51), *C. clementina* (48) and *C. unshiu* (1). Our approach allowed us to select interesting citrus WRKY members that play crucial role in plant development and stress responses, and whose further functional characterization would lead to a better understanding of their biological impact in pivotal biological pathways. A combined phylogenetic analysis with WRKY orthologous from *Arabidopsis* showed that the *Citrus* WRKY superfamily could be classified in seven groups. We noted that *AtWRKYs*, *CsWRKYs* and *CcWRKYs* characterized by similar functions in stress resistance showed an ability to be grouped. Thus, *AtWRKY3* and *AtWRKY4*, contributing to the defense against *B. cinerea* [79] were clustered together on the phylogenetic tree with *CcWRKY34* and *CsWRKY7*, both belonging to group I. Similarly, *AtWRKY72*, the representative gene of the group IIb, involved in basal immunity in *Arabidopsis* [80], clustered together with *CsWRKY51* and *CcWRKY31* within the phylogenetic tree. In group IIc, *AtWRKY11* and *AtWRKY17*, characterized by their involvement in response to pathogens

**E**

Dataset: 2213 perturbations (sample selection: AT-SAMPLES-0)

Target gene: AT5G13080 (245676\_at in gene selection AT-GENES-0)



Description of the most correlated genes (top 25)

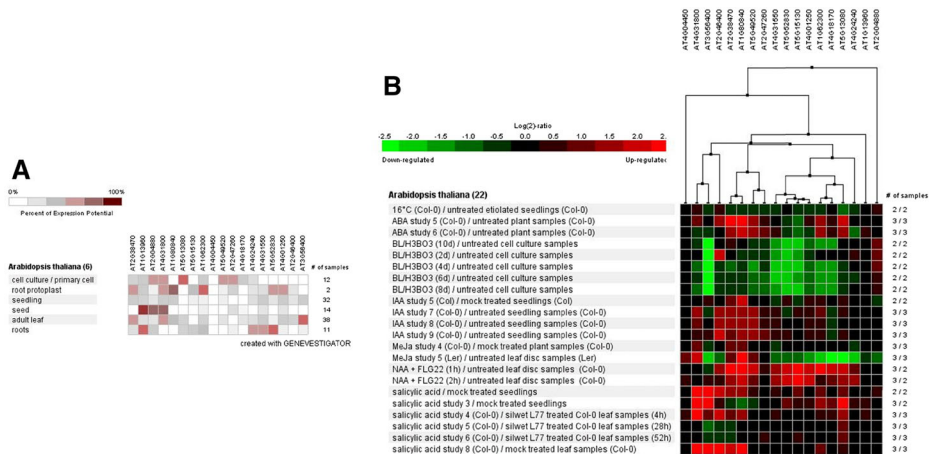
Gene	Score	Description
1	AT3G13650	0.77 unknown protein: BEST Arabidopsis thaliana protei...
2	AT1G74600	0.74 glutathione S-transferase TAU 10
3	AT1G33030	0.74 O-methyltransferase family protein
4	AT1G69620	0.73 alpha/beta-hydrolases superfamily protein
5	AT1G14360	0.73 Leucine-rich repeat protein kinase family protein
6	AT1G28100	0.73 unknown protein: BEST Arabidopsis thaliana protei...
7	AT3G64150	0.72 S-adenosyl-L-methionine-dependent methyltransfe...
8	AT1G66680	0.72 Late embryogenesis abundant (LEA) hydroxyprolin...
9	AT3G48820	0.72 phosphate transporter 3.2
10	AT1G32350	0.72 alternative oxidase 1D
11	AT1G67950	0.72 calloseglucanase 3-O-methyltransferase
12	AT1G62300	0.72 WRKY family transcription factor
13	AT1G69630	0.71 glutathione S-transferase TAU 11
14	AT1G15520	0.71 pleiotropic drug resistance 12
15	AT1G13340	0.71 Regulator of Vps4 activity in the MVB pathway protein
16	AT4G39670	0.71 Glycolipid transfer protein (GLTP) family protein
17	AT2G34650	0.71 cytochrome P450, family 710, subfamily A, polysep...
18	AT5G07340	0.71 ARM repeat superfamily protein
19	AT5G38000	0.70 Thionin domain superfamily protein
20	AT3G22160	0.70 VG motif-containing protein
21	AT2G35980	0.70 Late embryogenesis abundant (LEA) hydroxyprolin...
22	AT1G69840	0.70 Phosphoglycerate mutase family protein
23	AT1G10500	0.70 NAC domain containing protein 53
24	AT1G64610	0.70 TransducinWD40 repeat-like superfamily protein
25	AT5G42150	0.69 peptidoglycan-binding LysM domain-containing pro...

Show only genes with correlation above: 0.695

Connect genes with mutual correlation at least: 0.804

**Fig. 10** (continued)

or nematode [81], clustered together with *CcWRKY48* and *CsWRKY28*. The analysis of several *Citrus* gene expression data demonstrated the putative implication of *WRKY* groups or lonely genes in diseases resistance (CTV, CiLV, Canker and yellow dragon diseases and miscellaneous pathogens infestations), environmental stress adaptation (salinity, drought, heat and cold) and various plant biological mechanisms (plant development, cell growth, fruit acidification and essential oil biosynthesis) via hormonal mediation (ABA, ethylene, JA and



**Fig. 11** Profiles of WRKY gene expression in different tissues (a) and under various hormonal treatments (b)



SA). In conclusion, *Citrus WRKYs* family is potentially involved in several plant developmental processes, abiotic and biotic stress responses, and thus, members of this family could be suitable candidates for further *Citrus* breeding and improvement program.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.

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