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Characterization of *Citrus* WRKY transcription factors and their responses to phytohormones and abiotic stresses

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Abstract

WRKY transcription factors (TF) family is involved in a huge variety of plant processes, including seed germination, plant development, phytohormone signalling, and defence against both biotic or abiotic stresses. In this work, WRKY TF family has been characterized in citrus. In a first experiment, the relative expression of *CsWRKY*s was analyzed in shoots and roots of plants treated with abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA) under *in vitro* conditions. Expression of *CsWRKY*s was also determined in roots of commercial citrus rootstocks subjected to osmotic and salt stresses. A total amount of 50 *Cs*WRKYs has been found and devided into different groups of WRKY family according to the WRKY domain sequences. In response to the applications of phytohormones, the highest differences were observed in roots, and it was found that ABA and SA generally repressed *CsWRKY*s expressions, but MeJA induced their overexpression. Osmotic stress repressed the expression of most of the *CsWRKY*s, whereas salt stress induced their expression. Moreover, salt stress induced higher increase in *CsWRKY* expressions in the tolerant rootstock *Citrus macrophylla* than in the sensitive rootstock Carrizo citrange, suggesting that these TFs may play an important role in response to this stress.

Additional key words: abscisic acid, Carrizo citrange, *Citrus macrophylla*, *Citrus sinensis* × *Poncirus trifoliata*, methyl jasmonate, osmotic stress, salicylic acid, salinity.

Introduction

Plants respond to adverse environmental challenges by activating molecular and physiological changes to minimize damage. Suitably, numerous overlapping mechanisms for coping with different stressors affecting simultaneously are encoded into the plant genome (Bansal *et al.* 2016). Whereas some plant responses are specific, the activation of signal transduction pathways, or transcriptional cascades regulated by DREB (dehydration sensitive element binding) or MYC (myelocytomatosis oncogene) transcription factors (TFs) provide tolerance to several stresses (Huang *et al.* 2012).

Plant responses to external stimuli are mainly mediated by phytohormones. Among them, abscisic acid (ABA), has been considered for a long time to be the central regulator of abiotic stress resistance in plants (Gómez-Cadenas *et al.* 2015, Sah *et al.* 2016). However, recent studies point out that salicylic acid (SA) and jasmonic acid (JA) and its derivatives can also play an important role in abiotic stress-induced signalling and tolerance (De Ollas *et al.* 2013, Zandalinas *et al.* 2016). Plants have developed mechanisms to face abiotic stress conditions by inducing or repressing gene expression. This machinery is highly dependent on proper perception and transduction of the environmental signals through a signalling cascade. Transcriptional regulation of genes whose expression is altered by stress conditions plays a critical role in developing stress tolerance in plants. Such regulation is mainly dependent on the temporal and spatial functioning of TFs (Roy Choudhury *et al.* 2008).

WRKY TFs are one of the largest families of transcriptional regulators present in higher plants (Agarwal *et al.* 2011). The WRKY family includes 72 representatives in *Arabidopsis thaliana*, and more than 100 members in rice, soybean or poplar, 68 in sorghum, 38 in *Physcomitrella patens*, 35 in *Sellaginella moellendorffii*, 80 in pine, and about 45 in barley

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Abbreviations: ABA - abscisic acid; JA - jasmonic acid; MeJA - methyl jasmonate; PEG - polyethylene glycol; RT-qPCR - reverse transcriptase quantitative polymerase chain reaction; SA - salicylic acid; TF - transcription factor.

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(reviewed in Bakshi and Oelmüller 2014). WRKY factors play a key role in several biological processes. For example, leaf senescence is positively regulated by *At*WRKY53 and *At*WRKY6 whereas *At*WRKY54 and *At*WRKY70 negatively affect this process (Robatzek and Somssich 2002, Woo *et al.* 2013). These TFs have been also reported as mediators of seed germination in rice, where *Os*WRKY51 and *Os*WRKY71 interact with abscisic acid (ABA) and gibberellins during the germination process (Xie *et al.* 2006). Another role has *At*WRKY44 which is involved in trichome and seed coat development in *Arabidopsis thaliana* (Johnson *et al.* 2002). Phytohormone signalling is also mediated by WRKYs, *e.g.*, *At*WRKY70 mediates the antagonism between SA and JA, acting simultaneously as an activator of SA-induced genes and as a repressor of JA-responsive genes, integrating signals from both pathways (Li *et al.* 2004). Moreover, one of the most studied functions of WRKY TFs is their involvement in plant defence against biotic and abiotic stresses. Mutants of *AtWRKY33* have an enhance susceptibility to the attack of pathogens, including *Botrytis cinerea*, *Alternaria brassicola,* and *Pseudomonas syringae.* Moreover, *At*WRKY33 cross-talks with *PDF1.2* and *PR-1*, which are JA and SA responsive genes, respectively, have been described (Zheng *et al.* 2006). Under abiotic stresses (low temperatures, wounding, drought, and salinity) WRKYs are induced and may play different roles (Guo *et al.* 2014, Pan and Jiang 2014).

The structure of all WRKY proteins includes the highly conserved amino acid sequence WRKYGQK and the zinc-finger-like motifs Cys(2)-His(2) or Cys(2)-HisCys that enables to bind to the DNA sequence motif TTTGACC/T, known as the W-box (Liu *et al.* 2014). According to the number of DNA binding domains and different features of the zinc-finger-like motifs, WRKYs have been classified into three different groups. Group I is characterized by the presence of two different domains, the N-terminal and the C-terminal motifs. Group II is the largest and its members have only one WRKY motif, *i.e.*, zinc-finger-like motifs with the same structure as in the members of the group I (C-X_{4-5}-C-X_{22-23}-H-X_1-H). This group was originally subdivided into five different subgroups (IIa, IIb, IIc, IId, and IIe), but recent phylogenetic analyses have revealed that subgroups IIa and IIb, and IId and IIe can be combined in IIa+b and IId+e respectively (Llorca *et al*. 2014). Finally, in group III, zinc finger motifs have a different pattern containing a C_2-HC motif (C-X_7-C-X_{23}-H-X_1-C) instead of the C_2-H_2 characteristic pattern of groups I and II (Eulgem *et al.* 2000).

Citrus is economically important fruit tree worldwide and its productivity is limited by different environmental stresses, such as high salinity, drought or heat. As WRKYs are considered as one of the master regulators of molecular reprogramming enhancing stress tolerance of plants, it would be very valuable to get knowledge on WRKY family in citrus. However, up to date there are only a few articles concerning TFs in the citrus relatives *Poncirus trifoliata* and *Fortunella crassifolia* (Gong *et al.* 2015, Şahin-Çevik 2012, Şahin-Çevik and Moore 2013).

The purpose of this work was to characterize WRKY TFs superfamily in citrus and to study their relationship and activation by different abiotic stresses in two of the most important commercial citrus rootstocks: Carrizo citrange and *Citrus macrophylla*.

Materials and methods

Identification and classification of *Citrus sinensis* WRKY TFs: *Arabidopsis thaliana* WRKY TFs transcript sequences were found in *TAIR* database (http://www.arabidopsis.org) (Lamesch *et al.* 2012) and submitted to the *C. sinensis* database of *Phytozome* (www.phytozome.org), doing a *TBLASTN* and we obtained the transcript sequences of *Cs*WRKY TFs (Czarnecki *et al.* 2014). Chromosome locations of the different *CsWRKY*s were obtained from *Citrus sinensis* annotation project (http://citrus.hzau.edu.cn/orange/).

The alignment of WRKY domains was performed with *Clustal Omega* online application (http://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was designed with *MEGA6.0,* using the neighbor-joining method (Saitou and Nei 1987, Tamura *et al.* 2013). Evolutionary distances were found using the p-distance method, selecting 1 000 bootstrap replications (Nei and Kumar 2000). The classification of *Cs*WRKYs was carried out by comparing the sequences of WRKY domains with the different sequences of *At*WRKYs.

By searching the literature, *At*WRKYs that are upregulated or downregulated by phytohormones or abiotic stresses were identified and compared with *Cs*WRKYs (reviewed in Chen *et al.* 2012). Primers were designed with *Primer3Plus* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using the coding DNA sequence (CDS) of *C. sinensis* obtained from *Phytozome*. Primer size was fixed between 18 and 22 bp, the fusion temperature ranged from 57 to 63 ºC, and the proportion of GC was between 45 and 55 %. The optimum values were 20 pb, 60 ºC, and 50 %, respectively. In addition, the product size was fixed between 120 and 200 bp, the optimum size was 150 bp. Furthermore, to avoid the formation of self-dimmers and hetero-dimmers, the designed primers were checked with *IDT-oligoanalyzer* tools (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/), limiting both of them to 5 bp. A list with the selected *Cs*WRKYs including the designed primers for each gene is shown in Table 1 Suppl.

Plants and treatments: All the experiments were performed using *in vitro* grown plants. Cultures were established and maintained as described in Montoliu *et al.*

(2009). Plants were grown in an environmental chamber at a temperature of 25 ºC, a 16-h photoperiod, and an irradiance of 150 µmol m^{-2} s^{-1}.

In a first set of experiments, the effect of application of different phytohormones on the expression of various *CsWRKY*s genes was studied. Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) *in vitro* rooted shoots were cultured into 150 × 20 mm tubes with liquid MS medium containing the inorganic salts of Murashige and Skoog (1962) medium, supplemented with 0.55 mM myo-inositol, 4.86 µM pyridoxine-HCl, 0.59 µM thiamine-HCl, 8.12 µM nicotinic acid, and 87.64 mM sucrose. In order to induce root production, 10.8 µM 1-naphthalene acetic acid and 0.3 µM gibberellic acid were added (Montoliu *et al.* 2010). After 30 d, these plantlets were transferred to new MS medium (control treatment) or MS medium supplemented with different phytohormones: 10 µM SA, or 50 µM methyl jasmonate (MeJA), or 10 µM ABA. All phytohormones were filter-sterilized after autoclaving the medium. Shoot and root samples were collected separately 24 and 72 h after phytohormone application. Samples were frozen in liquid nitrogen, ground to fine powder, and stored at -80 ºC until analyses.

Further, the relative expressions of *CsWRKYs* genes were determined in *in vitro* cultured citrus plants subjected to different abiotic stresses. Osmotic stress was induced by cultivating *Citrus macrophylla* plants in medium supplemented with polyethylene glycol (PEG_{6000}) and adjusting the osmotic potential of the culture medium to -0.75 MPa (moderate stress) and -1.5 MPa (severe stress) as described in Michel and Kaufmann (1973). Roots were sampled 72 h after the stress imposition for further analyses. To evaluate salt stress tolerance, the relative expression of several *CsWRKY*s genes was studied in Carrizo citrange (salt sensitive genotype) and *C. macrophylla* (salt tolerant genotype) plants. Following the approach described in Montoliu *et al*. (2009), *in vitro*-cultured plants were subjected to culture medium supplemented with 60 or 90 mM NaCl and roots were sampled 72 h after the stress imposition.

Analysis of phytohormones: ABA, SA, and JA content was determined by high performance liquid chromatography with electrospray ionization tandem mass spectrometry, using a triple quadrupole (Durgbanshi *et al.* 2005). Briefly, 200 mg of fresh tissue (fine powder) was extracted with water using a mill ball equipment (*MillMix20*, *Domel*, Železniki, Slovenija). As internal standards, [$^{2}H_{6}$]-ABA, dehydrojasmonate, and [$^{13}C_{6}$]-SA were added and pH was adjusted to 3 with chlorhydric acid. The extract was partitioned twice with diethyl ether, the supernatant was evaporated under vacuum in a centrifuge concentrator (*Speed Vac*, *Jouan*, Saint Herblain, France) at room temperature and the solid residue was resuspended in 0.5 cm^3 of water + methanol (90:10, v/v) and filtered through 0.22 µM *PTFE* filters. This solution (20 mm^3) was directly injected into the HPLC system (*Acquity SDS*, *Waters Corp*., Milford, MA, USA). The chromatographic separation was carried out on a reversed-phase C18 column (50 × 2.1 mm and 1.8-µm particle size; *Macherey-Nagel*, Düren, Germany) using a methanol + water mixtures, both supplemented with 0.1 % acetic acid, gradient at a flow rate of 0.3 cm^3 min^{-1}. Phytohormones were quantified with a triple quadrupole mass spectrometer (*Micromass*, Manchester, UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source. Results were processed using *Masslynx v. 4.1* software, and the phytohormone content was quantified with a standard curve prepared with commercial standards.

Reverse transcription quantitatative PCR analysis: RNA was extracted from frozen plant tissues with the *Qiagen* kit (*Qiagen*, Hilden, Germany) following the manufacturer′s instructions. After that, cDNA concentration and purity were measured with a *Nanodrop 2000* spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA), determining absorbance 260/280 and 260/230 nm ratios. RNA samples were reverse transcribed to cDNA with *DNase I* (*Fermentas*, Waltham, MA, USA) from 1 µg of total RNA. The RT-qPCR analyses were carried out with an *ABI StepOne Detection System* (*Applied Biosystems*, Foster City, CA, USA), using 1 mm^3 of cDNA, 5 mm^3 of *Maxima SYBR Green/ROX* qPCR mix (*Thermo Scientific*), 1 mm^3 of primers (a mix of forward and reverse, 10 µM), and 3 mm^3 of sterile water. The amplification conditions were 95 °C for 10 min and 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Fluorescent intensity data was collected during all the extension time, and the reaction specificity was trusted by melting curve analysis. *Actin* and *tubulin* were used as endogenous control genes to normalize the results among samples. Relative expression of *CsWRKY*s was achieved using the *Relative Expression Software Tool Solver v. 2* (REST-MCS) (Pfaffl *et al.* 2001, 2002). In order to facilitate the comparison and the visualization of RT-qPCR results, a hierarchical cluster analysis was developed with *MeV* program, *v. 4.9.0* (Saeed *et al*. 2006).

Statistical analyses: Results were evaluated with the *Statgraphics Plus v. 5.1.* software (*Statistical Graphics Corp*., Herndon, VA, USA). Data are presented as means of three independent determinations and were subjected to one- or two-way analysis of variance (*ANOVA*) followed by the Tukey posthoc test ($P \leq 0.05$) when a significant difference was detected.

Results

All *Cs*WRKY TFs sequences were obtained and devided into the different groups described above. A total number of 50 TFs were found and classified according to the scaffold (where they are located according to *Phytozome*) instead of the number of the chromosome, as this information is not available in all the *CsWRKY*s identified in *Citrus sinensis* anotation project (Table 2 Suppl.). As *Cs*WRKYs classified in the group I have two WRKY domains, both sequences were aligned separately, distinguishing between the N-terminal and C-terminal domains (Fig. 1 Suppl.). Furthermore, groups IIa+b and IId+e were classified separately for a better global vision of all *Cs*WRKYs distribution. Thereby, these TFs were classified in the different groups: 9 members in the group I, 2 in the group IIa, 8 in the group IIb, 14 in the group IIc, 5 in the group IId, 6 in the group IIe, and 6 in the group III. In *Cs*WRKY21 and *Cs*WRKY47 a different WRKY domain (WRKYGKK) instead of the classical WRKYGQK was found.

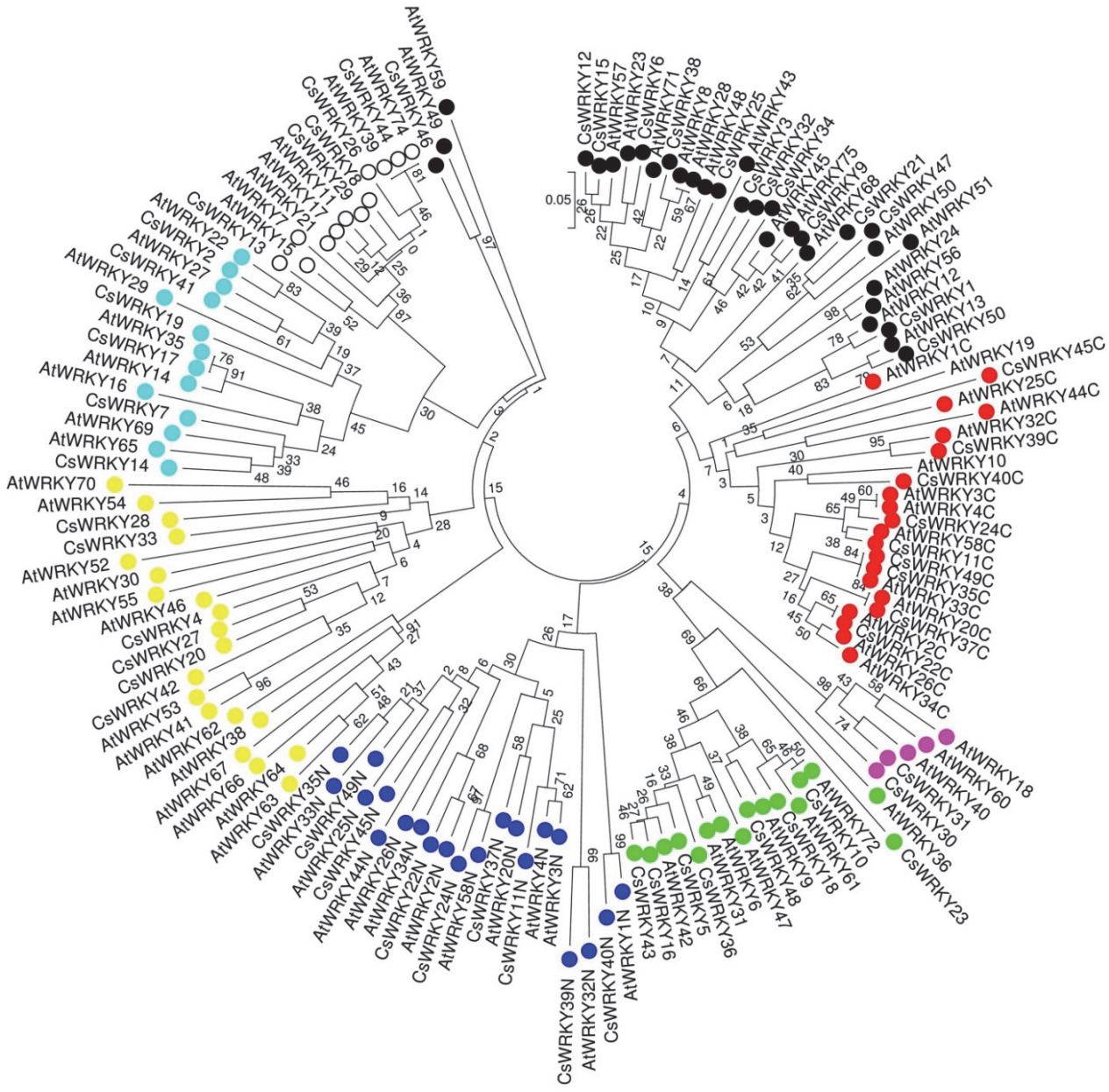

Fig. 1. Phylogenetic tree of WRKY TFs domains of *A. thaliana* and *C. sinensis*. The numbers in branches represent bootstrap values based on 1 000 replications. Different colours refer to the different groups of WRKY TFs: group I N-terminal: *dark blue*; group I C-terminal: *red*; group IIa: pink; group IIb: *green*; group IIc: *black*; group IId: *white*; group IIe: *light blue*; group III: *yellow*.

In the phylogenetic tree developed with *MEGA6.0*, *Cs*WRKYs were located separately depending on the group they belong to, with the only exception of *Cs*WRKY46. However, *Cs*WRKY46 was located closely to *At*WRKY49 and *At*WRKY59, which also belong to the group IIc (Fig. 1).

Treatment with 10 μM ABA induced an increase of ABA content 24 h after the application, reaching the values in shoots and roots 35.0 and 2802.8 times higher than those determined in controls, respectively, and 72 h after the treatment, these values were 61.0 and 1865.4 times higher than those in controls, respectively (Fig. 2*A*). This treatment also induced an increase of SA content in roots after 72 h, reaching the highest SA content 5.5 times higher than that in control. Meanwhile, 50 μM MeJA induced JA accumulation in both organs 24 and 72 h after the imposition of treatment. After 24 h, shoots and roots of plants treated with MeJA had content of JA 42.8 and 268.1 times higher than in control, respectively. This difference increased 72 h after application, achieving JA content 46.5 and 1280.8 higher than in control shoots and roots, respectively (Fig. 2*B*). Although 50 μM MeJA treatment also induced ABA accumulation, the increase was lower than that observed for JA content, showing ABA content in shoots and roots 5.5 and 16.5 higher times than in control. Increased content of SA was recorded after 10 μM SA application at both sampling times: 24 h after the application, SA content in shoots and roots was 20.1 and 199.3 times higher than in control and 72 h after the application, these values in shoots and roots were of 9.4 and 202.9 times higher than in controls, respectively. This treatment also increased ABA and JA content, but the increases were lower than those observed in SA content (Fig. 2*C*).

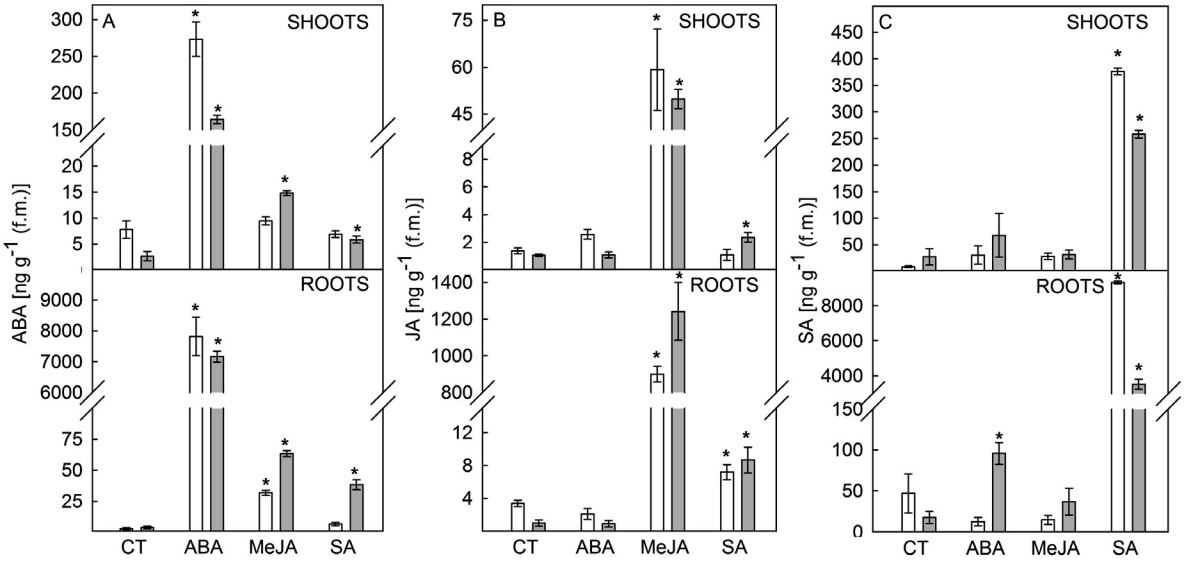

Fig. 2. The content of abscisic acid (ABA; *A*), jasmonic acid (JA; *B*), and salicylic acid (SA; *C*) in shoots and roots of Carrizo citrange plants treated with 10 μM ABA, 50 μM MeJA, or 10 μM SA for 24 h (w*hite bars*) or 72 h (*grey bars)*. Means ± SEs, $n = 3$. *Asterisks* denote significant difference at $P \leq 0.05$ with respect to control (CT, without phytohormones).

Treatments with phytohormones influenced also the expression of different *CsWRKY*s genes (Fig. 3). ABA treatment induced the expression of *CsWRKY26* in root and shoot tissue after 24 and 72 h, and the expression of *CsWRKY28*, *CsWRKY30*, CsWRKY*31*, *CsWRKY33*, and *CsWRKY35* in roots after 72 h. On the contrary, this treatment repressed the expression of *CsWRKY11*, *CsWRKY19*, *CsWRKY33*, and *CsWRKY44* in shoots after 24 h and *CsWRKY19*, *CsWRKY31*, *CsWRKY35*, and *CsWRKY49* in shoots after 72 h. In roots, ABA treatment repressed *CsWRKY19* and *CsWRKY49* expression after 24 h, and *CsWRKY13*, *CsWRKY18*, *CsWRKY19*, and *CsWRKY41* after 72 h. In this treatment, the largest differences were observed in the expression of *CsWRKY19*, *CsWRKY30*, and *CsWRKY33* genes. After 24 and 72 h, *CsWRKY19* expression was decreased by 94 and 57 % with respect to control. On the contrary, *CsWRKY30* expression in roots was 14.7 times higher than that of the control. Finally, *CsWRKY33* showed a different expression pattern depending on the tissue, being up-regulated in roots (values 8.0 times higher than in the control at 72 h) and down-regulated in shoots (values 80 % lower than in the control at 24 h).

Application of MeJA had also a significant effect on *CsWRKY*s gene expression. In roots, the increase of transcript abundance was observed for *CsWRKY22*, *CsWRKY29*, *CsWRKY30*, *CsWRKY43* and *CsWRKY49* after 24 h, whereas for *CsWRKY2*, *CsWRKY19*, *CsWRKY26*, *CsWRKY28*, *CsWRKY29*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33*, *CsWRKY35* and *CsWRKY43* it was after 72 h. In shoots, only *CsWRKY30* and *CsWRKY35* after 24 h and *CsWRKY44* after 72 h were up-regulated. Contrarily, CsWRKY*33* and *CsWRKY44* expressions were down-regulated in roots and CsWRKY33 in shoots after 24 h, whereas the expressions of *CsWRKY19*, *CsWRKY30*, and *CsWRKY43* were down-

regulated after 72 h. The highest differences in expressions were recorded in roots after 72 h, where *CsWRKY19* reached 93.5 % lower expression than in the control, and *CsWRKY30* 13.9 times higher expression than in control (Fig. 3).

Application of SA induced changes in the expression in 13 of 17 *CsWRKY*s selected genes. In roots, the expressions of *CsWRKY13*, *CsWRKY18*, *CsWRKY19*, *CsWRKY41*, *CsWRKY43*, and *CsWRKY49* were down-regulated after 72 h. On the contrary, *CsWRKY28*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33,* and *CsWRKY35* expressions were up-regulated at both sampling times. In

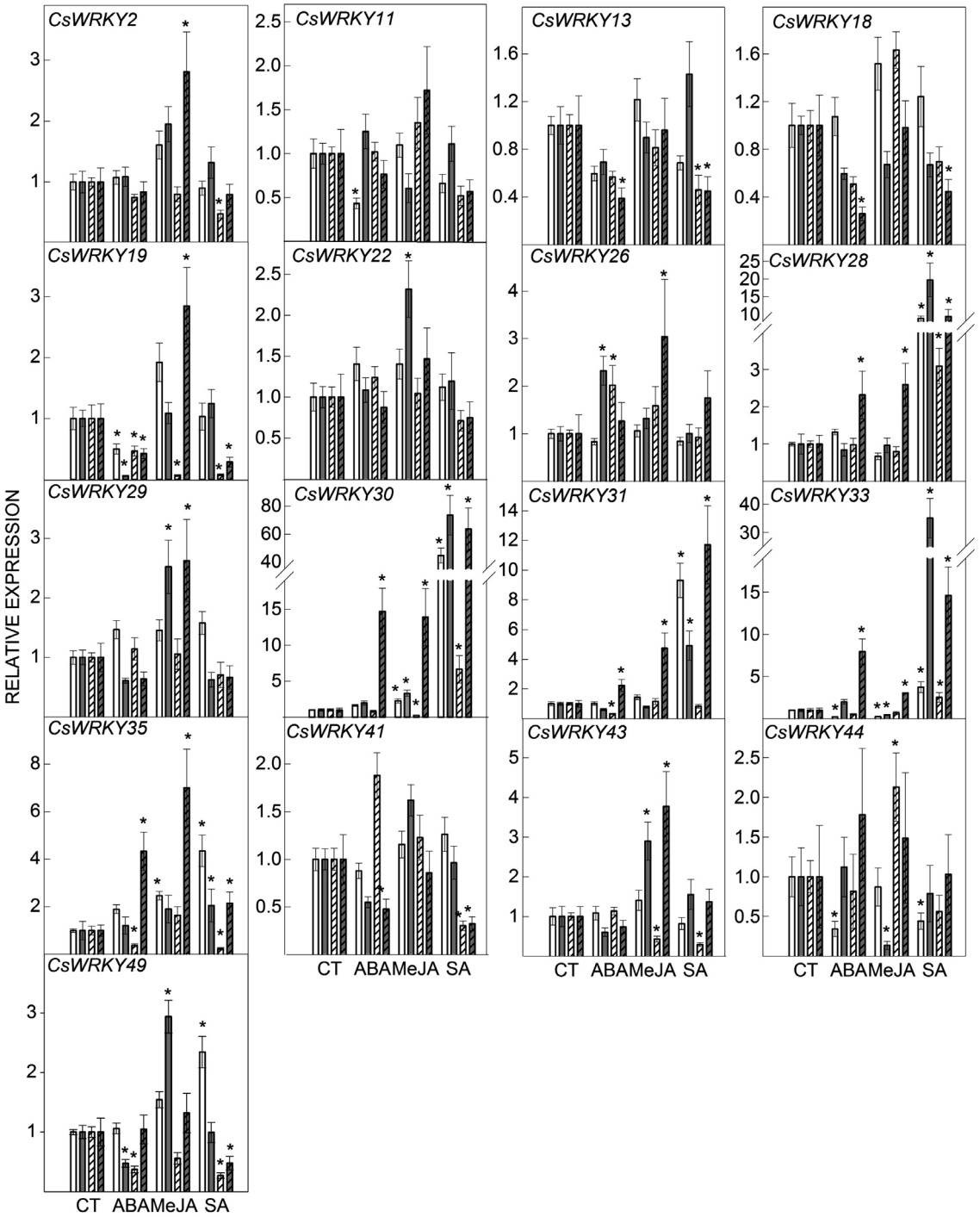

Fig. 3. Relative expressions of different *CsWRKY* genes in response to 10 µM ABA, 50 µM MeJA, or 10 µM SA in shoots and roots 24 and 72 h after the treatments. *White bars* refer to shoots and *grey bars* refer to roots. *Plain bars* represent the relative expression after 24 h and *lined bars* after 72 h. Means ± SEs, $n = 3$. *Asterisks* denote significant difference at $P \leq 0.05$ with respect to control (CT, without phytohormones).

shoots, the expressions of *CsWRKY2* and *CsWRKY44* significantly decreased after 24 h, while *CsWRKY13*, *CsWRKY19*, *CsWRKY35*, *CsWRKY41*, and *CsWRKY49* transcript abundance was lower than in the control after 72 h. In roots, SA treatment induced the highest increase in *CsWRKY30* expression after 24 and 72 h being 73.6 and 63.6 times higher than in control, respectively.

Nevertheless, expressions of *CsWRKY31* and *CsWRKY35* were up-regulated 24 h after the application of different phytohormones and expressions of *CsWRKY28*, *CsWRKY30*, and *CsWRKY33* were up-regulated after either 24 or 72 h. A Venn diagram (Fig. 4) reveals that most of *CsWRKY*s experienced changes in relative expressions in response to different phytohormones. There were only three *CsWRKY*s of the 17 studied which were only affected by one treatment: *CsWRKY11*, which was down-regulated in shoots of plants treated with ABA, and *CsWRKY22* and *CsWRKY29*, which were up-regulated in plants treated with MeJA.

Taken into consideration that the highest differences in gene expression were generally recorded in root tissue 72 h after the treatment, in the following experiments *CsWRKY*s expression was only analyzed in this tissue after 72 h.

In roots of *C. macrophylla* plants subjected to osmotic stress for 72 h, the relative expressions of these TFs were generally repressed. This was the case of *CsWRKY2*, *CsWRKY11*, *CsWRKY13*, *CsWRKY19*, *CsWRKY28*, *CsWRKY29*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33*, *CsWRKY35*, *CsWRKY41*, and *CsWRKY44* (Fig. 5). However, *CsWRKY18*, *CsWRKY22*, *CsWRKY26*, *CsWRKY43*, and *CsWRKY49* did not show any difference in respect to the control. None of the *CsWRKY*s studied in *C. macrophylla* was up-regulated under osmotic stress.

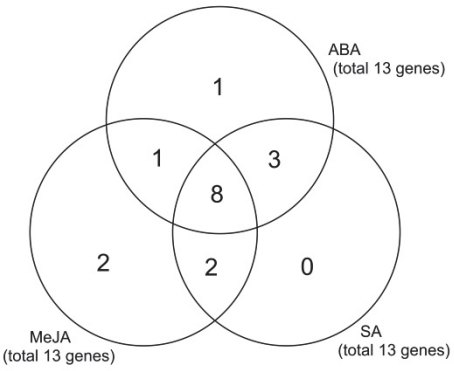

Fig. 4. Venn diagram depicting the degree of overlap between the number of *CsWRKY*s which were significantly regulated by applications of 10 μM ABA, 50 μM MeJA, or 10 μM SA .

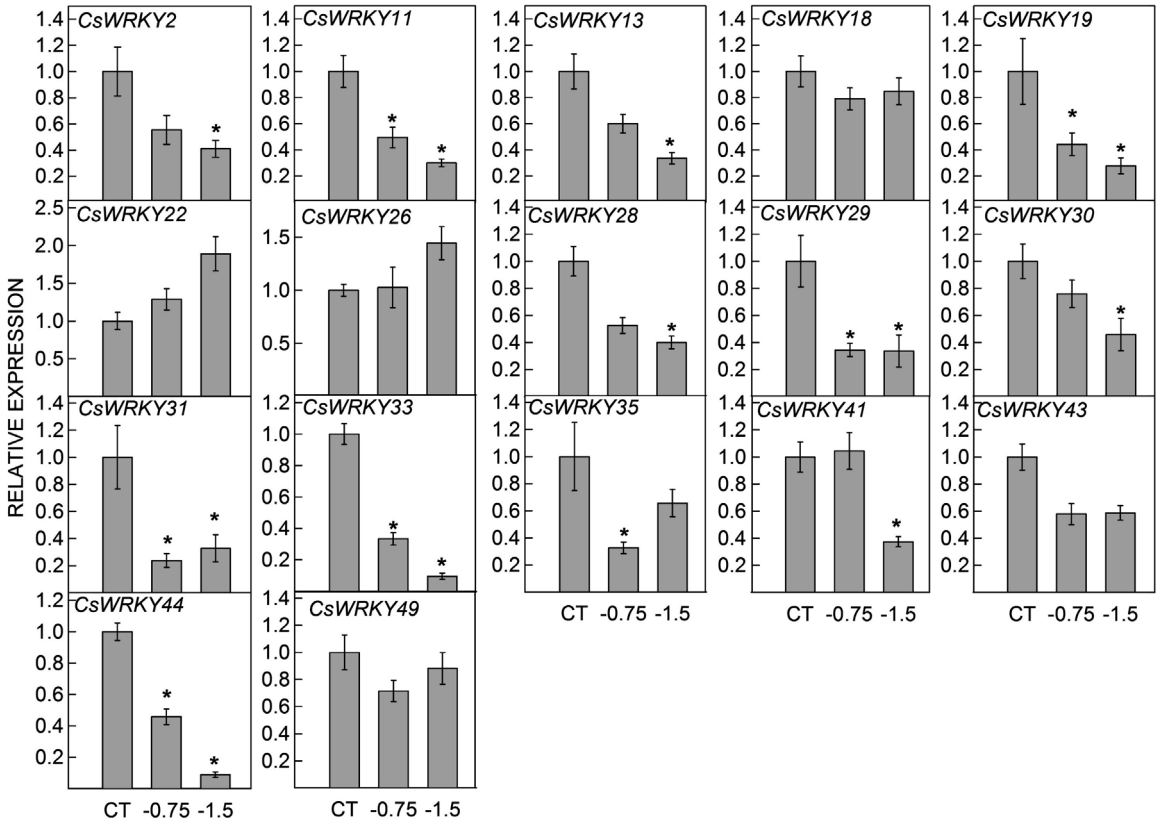

Fig. 5. Relative expressions of *CsWRKY* genes in response to osmotic stress (-0.75 and -1.5 MPa; polyethylene glycol 6000) in *Citrus macrophylla* roots after 72 h, using treatment at 0 h as control (CT). Means ± SEs, $n = 3$. *Asterisks* denote significant difference at $P \leq 0.05$ between control and stressed plants.

On the contrary, salt stress induced an over-expression of some *CsWRKY*s either in Carrizo citrange or in *Citrus macrophylla* (Fig. 6). This general increase in *CsWRKY*s expression was observed in *CsWRKY2*, *CsWRKY13*, *CsWRKY18*, *CsWRKY19*, *CsWRKY22*, *CsWRKY26*, *CsWRKY28*, *CsWRKY29*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33*, *CsWRKY35*, *CsWRKY41*, *CsWRKY43* and *CsWRKY49*. Among the studied TFs, only *CsWRKY44* was repressed under salt stress, reaching 60.8 % of the control value in *C. macrophylla* plants exposed to 90 mM NaCl. Moreover, roots of the salt resistant rootstock *C. macrophylla* overexpressed *CsWRKY*s in a higher extent than the sensitive Carrizo citrange plants did. This higher overexpression of *CsWRKY*s in roots of *C. macrophylla* in comparison to Carrizo citrange roots subjected to salt stress was clearly observed in *CsWRKY2*, *CsWRKY19*, *CsWRKY22*, *CsWRKY28*, *CsWRKY30*, *CsWRKY31* and *CsWRKY49*.

For easier visualization, a hierarchical clustering compiling all the results described above was made using the program *MeV4.9.0* (Fig. 7).

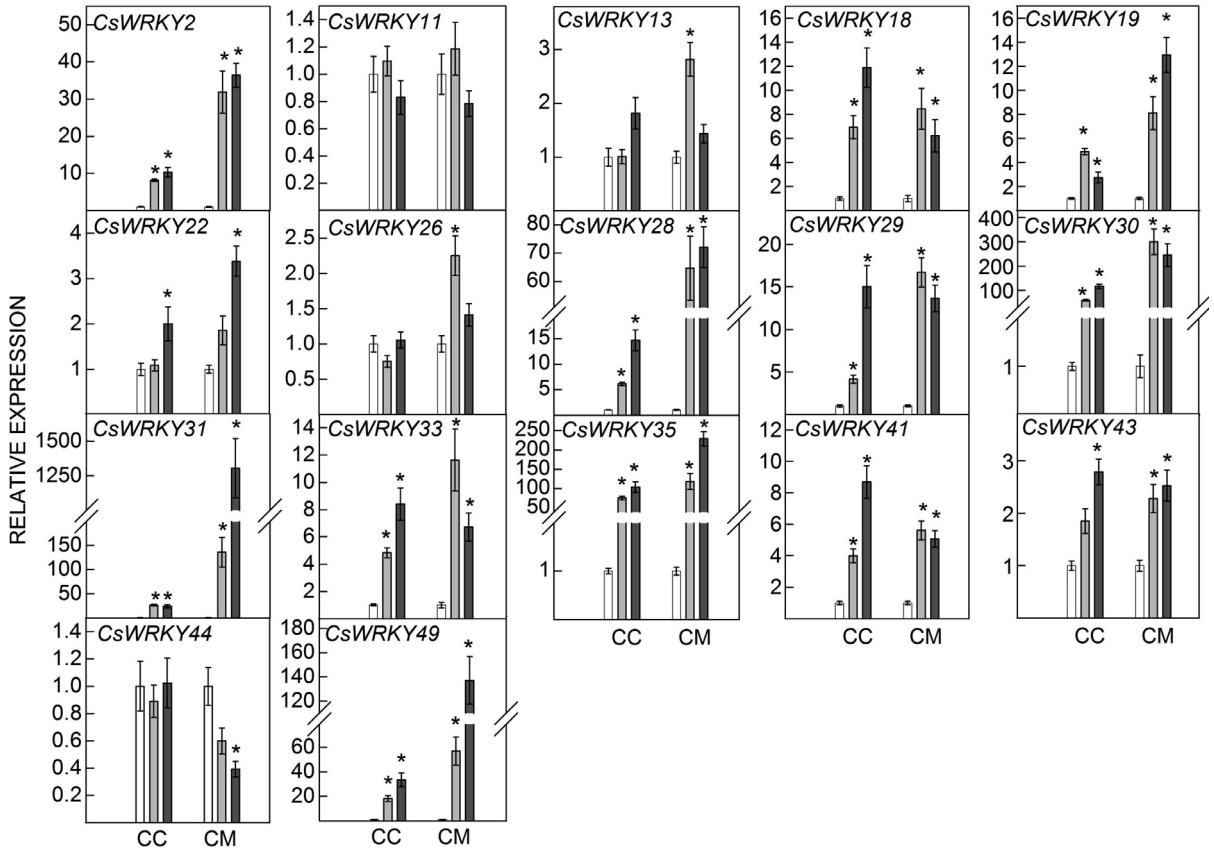

Fig. 6. Relative expressions of *CsWRKY* genes in Carrizo citrange (CC) and *Citrus macrophylla* (CM) roots in response to salt stress for 72 h. *White bars* refer to control, *light grey bars* refer to 60 mM NaCl and *dark grey bars* refer to 90 mM NaCl. Means ± SEs, $n = 3$. *Asterisks* denote significant difference at $P \leq 0.05$ between control and stressed plants.

Discussion

In this work, WRKY superfamily of TFs has been identified and characterized in citrus. As it has been reported that WRKYs play pivotal roles in regulating many plant responses to stresses (reviewed in Rushton *et al.* 2010), the effects of different stress-related phytohormones and two abiotic stresses on *CsWRKY*s relative expression were studied in two citrus genotypes, Carrizo citrange and *Citrus macrophylla*, commercially used rootstocks.

TBLASTN showed 50 *Cs*WRKYs, that were classified according to the scaffold they belong, following the procedure described for other plants where the chromosome location of the genes is not available (Chen *et al.* 2015). However, not all *Cs*WRKY TFs contain the classical WRKYGQK domain. This is the case of *Cs*WRKY21 and *Cs*WRKY47 which have a different WRKY domain (WRKYGKK). This was also found in other species, such as *Hordeum vulgare*, that has other WRKY domains in addition to the classical WRKYGQK, such as WRKYGKK, WQKYGQK, WRKYGEK, and

WSKYGQM (Mangelsen *et al.* 2008). This difference in the classical WRKYGQK motif causes different binding with the W-box in tobacco plants, where *Nt*WRKY12 contains the same WRKYGKK motif, which binds to the sequence TTTTCCAC, instead of the classical W-box (Van Verk *et al.* 2008).

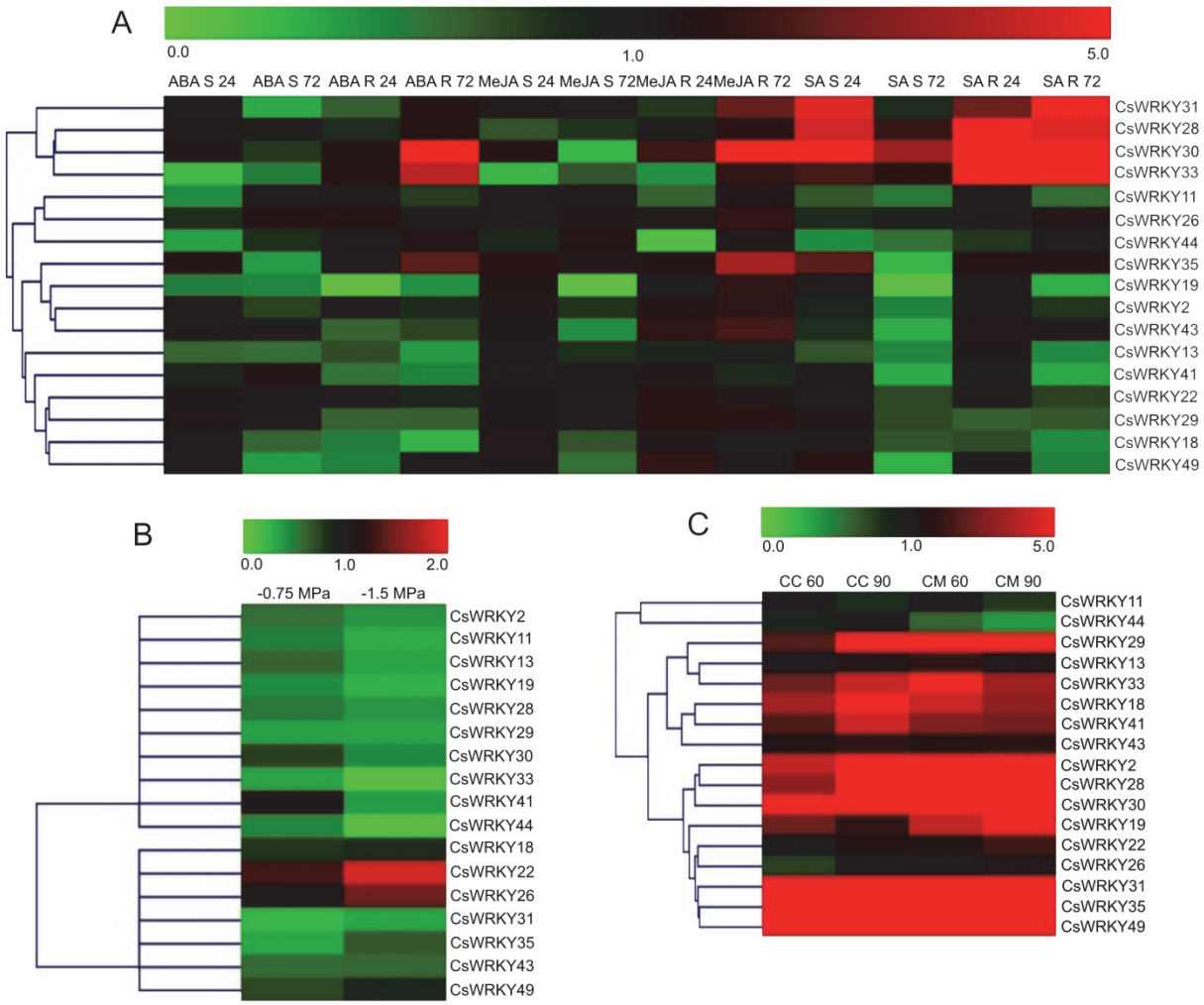

Fig. 7. Hierarchical clustering of relative expression profiles of selected CsWRKYs. The colour scale represents relative expression levels. *Green* and *red* represent decreasing and increasing transcript content, respectively. *A* - Expression in shoots (S) and roots (R) 24 and 72 h after phytohormone application. *B* - Expression in *C. macrophylla* treated with PEG-induced osmotic stress of -0.75 and -1.50 MPa for 72 h. *C* - Expression in Carrizo citrange (CC) and *C. macrophylla* (CM) under *s*alt stress (60 and 90 mM NaCl) for 72 h.

Plant hormones are key players in regulating cell responses to external and internal stimuli; moreover, these substances interact with each other (Gómez-Cadenas *et al.* 2014) to fine-tune cell responses. In this way, it has been described that a temporary accumulation of JA is needed for a further increase of ABA content in roots of citrus plants subjected to drought (De Ollas *et al.* 2013). Positive correlation between ABA and SA has been also described in wheat where treatments with SA induced a transient accumulation of ABA (Shakirova *et al.* 2016). In this work, treatments with ABA, JA, and SA not only caused an increase in the content of the applied phytohormone, but also induced slight accumulations of others, supporting the crosstalk among different phytohormones. Applications of MeJA and SA induced the accumulation of ABA, whereas ABA application increased endogenous JA and SA content.

At the transcriptional level, hormone treatments resulted in a wide variety of changes in *CsWRKY*s genes expression depending on the hormone applied, the sampling time, and the analyzed tissue. Most of the differences were detected in roots 72 h after the treatments, probably due to the application of the different phytohormones directly to this organ. The treatment with ABA had different effects depending on the *CsWRKY* analyzed. Thus, it induced the expression of *CsWRKY30* and *CsWRKY31,* belonging to group IIa. These results are consistent with the expression profile of *AtWRKY18*, *AtWRKY40* and *AtWRKY60*, which are also members of group IIa, in *A. thaliana* plants treated with

ABA (Chen *et al.* 2010), suggesting that some *WRKY* genes including those in group IIa are involved in ABA signalling or response. MeJA caused increases on *CsWRKY* relative expression in leaves and roots, particularly *CsWRKY35* was induced by MeJA. This is in concordance with the results described in *Nicotiana attenuate*, where *NaWRKY3* and *NaWRKY6* are also induced in the presence of JA (Skibbe *et al.* 2008).

The WRKY family in citrus is very sensitive to hormonal treatments. Our data suggest specific responses to the application of each hormone, whereas MeJA mostly induced over-expression of *CsWRKYs*, but SA and ABA downregulated these TFs. Similar findings have been described in grape, where *VvWRKY* genes are down-regulated after the treatments with ABA or SA, and up-regulated after treatments with JA or ethylene (Guo *et al.* 2014). These results contrast with studies in herbaceous species as rice, where plants treated with ABA, SA, and MeJA overexpressed *OsWRKY*s (Ramamoorthy *et al.* 2008), or canola, where treatments with ABA, JA, and ethylene significantly repressed some *BnWRKY*s expression, while plants treated with SA overexpressed some genes of this family (Yang *et al.* 2009). All these results reveal that the involvement of WRKYs in response to hormonal treatments is highly dependent on the studied genotype, exhibiting high differences among species.

To face different abiotic stresses, plants activate common mechanisms such as stomatal closure, proline accumulation, enhancement of antioxidant enzymatic activities, *etc*. On the contrary, the expression pattern of *Cs*WRKYs TFs was completely different in plants subjected to osmotic or salt stress. Under osmotic stress, all the *CsWRKY*s which expression was significantly altered by hormone treatment were down-regulated. Furthermore, there was a direct correlation among the relative decrease of gene expression and the duration of the osmotic stress applied. Conversely, salt stress caused an up-regulation of *CsWRKYs*. Other studies demonstrated that tobacco plants overexpressing the *Thlaspi caerulescens TcWRKY53* are more sensitive to osmotic stress induced by PEG_{6000} and sorbitol. However, this gene is up-regulated under other abiotic stresses such as cold, salt, or drought (Wei *et al.* 2008). This fact reveals that although *WRKY*s are overexpressed under some abiotic stresses, they are usually downregulated under osmotic stress, which is in agreement with the results obtained in this work.

The overexpression of *CsWRKY*s in citrus plants subjected to salt stress described here is in concordance with previous studies in other species, such as rice, poplar, or soybean (Ramamoorthy *et al.* 2008, Jiang *et al.* 2014, Song *et al.* 2016). Although most of *CsWRKY*s were up-regulated under salt stress in both, salt sensitive and tolerant citrus genotypes, this up-regulation was higher in the salt tolerant genotype *C. macrophylla,* suggesting that *CsWRKYs* might play an important role in mediating the plant response to salt stress (Iglesias *et al.* 2004). Compatible results indicate that over-expressing cotton *GhWRKY34* in *Arabidopsis thaliana* plants enhances their tolerance to salt stress (Zhou *et al.* 2015).

Salt stress has a double negative effect on plant performance. It induces an initial osmotic stress followed by ion toxicity due to the absorption of Cl^- and Na^+ ions by plant tissues (Moya *et al*. 2003). Although both stresses applied in this work share the osmotic component, they differently regulated the expression of *CsWRKY*s. This different regulation, therefore, seems to be related to the toxic component of salt stress, which induced specific responses in citrus (Gomez-Cadenas *et al.* 1998).

In conclusion, in this work 50 putative *Cs*WRKYs have been identified and classified according to the scaffold they are located. The gene expression profiles obtained after different phytohormone treatments and abiotic stress situations revealed that *CsWRKYs* are involved in citrus responses to abiotic stress. In general terms, ABA and SA repressed *Cs*WRKYs expression, whereas MeJA induced it. Differences in the expression of *CsWRKY*s were observed in plants subjected to different abiotic stresses. Whereas osmotic stress repressed the expression of most *CsWRKY*s, salt stress had the opposite effect. Moreover, over-expression of *CsWRKYs* under salt-stress was higher in the salt tolerant genotype *C. macrophylla* than in the sensitive Carrizo citrange. This is in agreement with other studies, where plants overexpressing *WRKYs* have a higher tolerance to salt stress, as occurs with *A. thaliana* plants overexpressing soybean *GmWRKY54* (Zhou *et al*. 2008). The present investigation demonstrates that a number of *CsWRKY* genes are involved in abiotic stress responses, and provides clues for the selection of candidate genes to be used in future breeding programs.

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