1    *Title*

2    **Characterization and responses of *Citrus* WRKY transcription factors to hormonal treatments and**

3    **abiotic stress conditions**

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9    *Abstract*

10    WRKY transcription factors (TF) family is involved in a huge variety of plant processes, including seed

11    germination, plant development, phytohormone signaling and defense against both, biotic or abiotic stress

12    conditions. In this work, WRKY TF family has been characterized in citrus. In a first experiment, the

13    relative expression of *CsWRKY*s was analyzed in shoots and roots of plants treated with abscisic acid

14    (ABA), salicylic acid (SA) and methyl jasmonate (MeJA) under *in vitro* conditions. Expression of

15    *CsWRKY*s was also determined in roots of commercial citrus rootstocks subjected to osmotic and salt

16    stress.  A total amount of 50 *Cs*WRKYs have been found and classified in the different groups of WRKY

17    family according to the WRKY domain sequences. In response to the exogenous applications of

18    phytohormones, the highest differences were observed in roots, and it was found that whereas treatments

19    with ABA and SA generally repressed *CsWRKY*s expression, exogenous application of MeJA induced

20    their overexpression. Osmotic stress repressed the expression of most of the *CsWRKY*s studied, while salt

21    stress induced their expression. Moreover, salt stress induced higher increases in *CsWRKY*s expression in

22    the tolerant rootstock *C. macrophylla*, suggesting that these TFs may play an important role in the

23    response to this stress.

28

29 *Introduction*

30 Plants respond to adverse environmental challenges by activating molecular and physiological changes to

31 minimize damage. Suitably, numerous overlapping mechanisms for coping with different stressors

32 affecting simultaneously are encoded into the plant genome (Bansal *et al.* 2016). Whereas some plant

33 responses, such as the limitation of plant growth due to photosynthetic decline is specific, to certain

34 unfavorable conditions such drought, others as the activation of signal transduction pathways, or

35 transcriptional cascades regulated by DREB or MYC transcription factors (TFs) provide tolerance to

36 several stresses (Huang *et al.* 2012).

37 Plant responses to external stimuli are mainly mediated by phytohormones. Among them, abscisic acid

38 (ABA), has been considered for a long time, the central regulator of abiotic stress resistance in plants

39 (Gómez-cadenas *et al.* 2015, Sah *et al.* 2016). However recent studies point out that salicylic acid (SA)

40 and jasmonic acid (JA) and its derivates, that have been traditionally associated to plant responses

41 against biotic stresses, can play an important role in abiotic stress-induced signaling and tolerance (de

42 Ollas *et al.* 2013, Zandalinas *et al.* 2016). Plants have developed mechanisms to face abiotic stress

43 conditions by inducing or repressing gene expression. This machinery is highly dependent on proper

44 perception and transduction of the environmental signals through a signaling cascade. Transcriptional

45 regulation of genes which expression is altered by stressful conditions plays a critical role in developing

46 stress tolerance in plants. Such regulation is mainly dependent on the temporal and spatial functioning of

47 the TFs (RoyChoudhury *et al.* 2008).

48 WRKY TFs are one of the largest families of transcriptional regulators present in higher plants although

49 have been reported in protist, slime mold, fern and pine as well (Agarwal *et al.* 2011). The WKKY family

50 includes 72 representatives in *Arabidopsis thaliana*, and more than 100 members in rice, soybean or

51 poplar, 68 in sorghum, 38 in *Physcomitrella patens*, 35 in *Sellaginella moellendorffii*, 80 in pine, and

52 about 45 in barley (reviewed in Bakshi and Oelmüller 2014).

53 WRKY factors play a key role in several biological processes, for example, leaf senescence is positive

54 regulated by *At*WRKY53 and AtWRKY6 whereas *At*WRKY54 and *At*WRKY70 negatively affect the

55 process (Robatzek and Somssich 2002, Woo *et al.* 2013).These TFs have been also reported as mediators

56 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). An example of the relevance of WRKYs in plant growth and development is the role of *At*WRKY44 which is encoded by *Atttg2* (TRANSPARENT TESTA GLABRA2), a gene involved in trichome and seed coat development (Johnson *et al.* 2002). Phytohormone signaling is also mediated by WRKYs, as it has been reported that *At*WRKY70 mediates in 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 these TFs is their involvement in plant defense against biotic and abiotic stresses. Mutants of *At*WRKY33 have an enhance susceptibility to the attack of pathoghens, including fungus as *Botrytis cinerea* and *Alternaria brassicola,* and bacteria as *Pseudomonas syringae*. Moreover, *At*WRKY33 crosstalks with *PDF1.2* and *PR-1*, which are JA and SA responsive genes respectively (Zheng *et al.* 2006). Under abiotic stress conditions WRKYs may play different roles, being induced in different plant stresses subjected to low temperatures, wounding, drought and salt stress (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 and 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 in three different groups: Group I is characterized by the presence of two different domains in the TF, the N-terminal and the C-terminal motifs. Group II is the most abundant and its members only have one WRKY motif which potential zinc ligands have the same structure that group I WRKYs ($\underline{C}–X_{4–5}–\underline{C}–X_{22–23}–\underline{H}–X_1–\underline{H}$). This group was originally subdivided into five different subgroups (IIa, IIb, IIc, IId and IIe), but recent phylogenetic analyses reveal 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 ($\underline{C}–X_7–\underline{C}–X_{23}–\underline{H}–X_1–\underline{C}$) instead of the $C_2$-$H_2$ characteristic pattern of groups I and II (Eulgem *et al.* 2000).

Citrus is the most economically important fruit tree worldwide, with more than 131 million tons of fruit produced in 2012 on more than 8.7 million ha (FAO, 2012) 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 for molecular reprogramming to enhance stress tolerance of plants, it would be very valuable to get knowledge on WRKY citrus family. However, up to date there are only a few articles

87  concerning to these TFs in the citrus relatives *Poncirus trifoliata* and *Fortunella crassifolia* (Gong *et al.*

88  2015, Şahin-Çevik 2012, Şahin-Çevik and Moore 2013) .

89  The purpose of this work was the characterization of WRKY TFs superfamily in citrus and to study their

90  relationship with abiotic stress conditions in two of the most important commercial citrus rootstocks:

91  Carrizo citrange and *Citrus macrophylla*.

92

93  ***Materials and Methods***

94  <u>Identification and classification of *C. sinensis* WRKY TFs</u>

95  *Arabidopsis thaliana* WRKY TFs transcript sequences were find in TAIR database

96  (http://www.arabidopsis.org) (Lamesch *et al.* 2012) and submitted to the *Citrus sinensis* database of

97  Phytozome (www.phytozome.org), doing a TBLASTN and obtaining the transcripts sequences of

98  *Cs*WRKY TFs (Czarnecki *et al.* 2014). Chromosome locations of the different *CsWRKY*s were obtained

99  from *Citrus sinensis* annotation project (http://citrus.hzau.edu.cn/orange/).

100  The alignment of WRKY domains was performed with Clustal Omega online application

101  (http://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was designed with MEGA6.0, using

102  the Neighbor-Joining method (Saitou and Nei 1987, Tamura *et al.* 2013). Evolutionary distances were

103  found using the p-distance method, selecting 1000 bootstrap replications (Nei and Kumar 2000). The

104  classification of *Cs*WRKYs was carried out by comparing the sequences of WRKY domains with the

105  different sequences of *At*WRKYs.

106  Searching the literature, those *At*WRKYs susceptible of being upregulated or downregulated by

107  phytohormones or abiotic stress conditions were identified and compared with *Cs*WRKYs (reviewed in

108  Chen *et al.* 2012). Primers were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-

109  bin/primer3plus/primer3plus.cgi) using the CDS sequences of *C. sinensis* obtained from Phytozome.

110  Primer size was fixed between 18 and 22 bp, the fusion temperature in the range of 57 and 63 ºC, and the

111  proportion of GC between 45 and 55 %, being their optimum values 20 pb, 60 ºC and 50 % respectively.

112  In addition, the product size was fixed between 120 and 200 bp, selecting the optimum in 150 bp.

113  Furthermore, to avoid the formation of self-dimmers and hetero-dimmers, the designed primers were

114  checked with IDT-oligoanalyzertools (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/), limiting

115    both of them to 5 bp. A list with the selected *Cs*WRKYs including the designed primers for each gene is

116    shown in Supplementary Table 1.


117    <u>Plant material and treatments</u>


118    All the experiments were performed using *in vitro* grown plants. Cultures were established and

119    maintained as described in Montoliu *et al.* (2009). Plants were maintained in an environmental chamber at

120    25ºC with a photoperiod of 16h of light during the experiments.


121    In a first set of experiments, the effect of exogenous application of different phytohormones on the

122    expression of various *CsWRKY*s genes was studied. Carrizo citrange (*Citrus sinensis* L. Osbeck x

123    *Poncirus trifoliata* L. Raf.) *in vitro* rooted shoots were cultured into 150 x 20 mm tubes on liquid MS

124    medium containing the inorganic salts of Murashige and Skoog (1962), supplemented with 0.55 mM

125    myo-inositol, 4.86 µM pyridoxine-HCl, 0.59 µM thiamine-HCl, 8.12 µM nicotinic acid and 87.64 mM

126    sucrose. 10.8 µM 1-naphthalene acetic acid and 0.3 µM gibberellic acid were added in order to induce

127    root production (Montoliu *et al.* 2010). After 30 days, these plantlets were transferred to new MS medium

128    (control treatment) or MS supplemented with different phytohormones: i) 10 µM SA, ii) 50 µM methyl

129    jasmonate (MeJA), and iii) 10 µM ABA. All phytohormones were filter-sterilized after autoclaving the

130    medium. Shoot and root samples were collected separately 24 and 72 hours after phytohormone

131    application. Samples were frozen with liquid nitrogen, ground to fine powder and stored at -80 ºC until

132    analysis.


133    Later, the relative expression of *Cs*WRKYs genes in *in vitro* cultured citrus plants subjected to different

134    abiotic stress conditions was evaluated. Osmotic stress was set by cultivating *Citrus macrophylla* plants in

135    medium supplemented with polyethylene glycol 6000 (PEG) and adjusting the osmotic potential of the

136    culture medium to -0.75 MPa (moderate stress) and -1.5 MPa (severe stress) as described in Michel and

137    Kaufmann (1973). A third group with control plants was added by transferring plants to MS medium

138    without plant growth regulators. Roots were sampled 72 h after the stress imposition for further analysis.


139    To evaluate salt stress tolerance, the relative expression of several *CsWRKY*s genes was studied in Carrizo

140    citrange (salt sensitive genotype) and *C. macrophylla* (salt tolerant genotype) plants. Following the

141    approach described in Montoliu *et al.* (2009), *in vitro*-cultured plants were subjected to two different

142    treatments (culture medium supplemented with 60 or 90 mM NaCl). For further analysis, roots were

143    sampled 72 h after the stress imposition.

144    <u>Hormonal analysis</u>

145    ABA, SA and JA endogenous concentrations were determined by high performance liquid

146    chromatography with electrospray ionization tandem mass sprectrometry, using a triple quadrupole

147    (Durgbanshi *et al.* 2005). Briefly, 200 mg of fresh tissue reduced to fine powder were extracted with

148    water using a mill ball equipment (MillMix20, Domel, Železniki, Slovenija), adding [$^2$H$_6$]-ABA,

149    dehydrojasmonate and [$^{13}$C$_6$]-SA as internal standards. pH was adjusted to 3 with chlorhydric acid. The

150    extract were partitioned twice with diethyleter, the supernatant was evaporated under vacuum in a

151    centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France) at room temperature and the

152    solid residue was resuspended in 500 µL of water: methanol 90:10 and filtered through 0.22 µM PTFE

153    filters. 20 µL aliquot of this solution was directly injected into the HPLC system. (Acquity SDS, Waters

154    Corp., Milford, MA, USA). The chromatographic separation was carried out on a reversed-phase C18

155    column (Gravity, $50 \times 2.1$mm 1.8-µm particle size, Macherey-Nagel GmbH, Germany) using a

156    methanol:water, both supplemented with 0.1% acetic acid, gradient at a flow rate of 300 µL min$^{-1}$.

157    Phytohormones were quantified with a triple quadrupole mass spectrometer (Micromass, Manchester,

158    UK) connected online to the output of the column through an orthogonal Z-spray electrospray ion source.

159    Results were processed using Masslynx v4.1 software, and the phytohormone contents were quantified

160    with a standard curve prepared with commercial standards.

161    <u>cDNA obtention and qRT-PCR analysis</u>

162    RNA was extracted from frozen plant tissues with the Qiagen Kit (Qiagen, Netherlands) following

163    manufacturer instructions. After that, cDNA concentration and purity were measured with a Nanodrop

164    2000 spectrophotometer (Thermo Scientific, USA), determining 260/280 and 260/230 ratios. RNA

165    samples were reverse transcribed to cDNA with DNase I (Fermentas, USA) from 1 µg of total RNA.

166    qRT-PCR analyses were carried out with an ABI StepOne Detection System (Applied Biosystems, USA),

167    using 1 µL of cDNA, 5µL of Maxima SYBR Green/ROX qPCR (Thermo Scientific Fermentas, Spain), 1

168    µL of primers (a mix of forward and reverse 10 µM) and 3 µL of sterile water. The amplification

169    conditions were 95°C for 10 min and 40 cycles of 95°C for 10s, 60°C for 10s and 72°C for 20s.

170 Fluorescent intensity data was collected during all the extension time, and the reaction specificity was

171 trusted by melting curve analysis. Actine and tubuline were used as endogenous control genes to

172 normalize the results among samples. Relative expression of *CsWRKY*s was achieved using the Relative

173 Expression Software Tool – Multiple Condition Solver version 2 (REST-MCS) (Pfaffl *et al.* 2002, Pfaffl

174 2001). In order to facilitate the comparison and the visualization of qRT-PCR results, a hierarchical

175 cluster analysis was developed with MeV program, version 4.9.0 (Saeed et al. 2006).

176 <u>Statistical analyses</u>

177 Statistics were evaluated with the Statgraphics Plus v.5.1. software (Statistical Graphics Corp., Herndon,

178 VA, United States). Data are means of three independent determinations and were subjected to one- or

179 two-way analysis of variance (ANOVA) followed by Tukey posthoc test ($p \leq 0.05$) when a significant

180 difference was detected.

181

182 *Results*

183 All *Cs*WRKY TFs sequences were obtained and classified attending to the different groups described

184 above. A total number of 50 TFs were found and classified according to the scaffold they are located

185 according to Phytozome instead of the number of the chromosome, as this information is not available in

186 all the *CsWRKY*s identified in Citrus sinensis Anotation Project (Tab. 1).

187 As *Cs*WRKYs classified in the group I have two WRKY domains, both sequences were aligned

188 separately, distinguishing between the N-terminal and C-terminal domains (Fig. 1). Furthermore, groups

189 IIa+b and IId+e were classified separately for a better global vision of all *Cs*WRKYs distribution.

190 Thereby, these TFs were classified in the different groups, finding 9 members in the group I, 2 in the

191 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

192 III. In *Cs*WRKY21 and *Cs*WRKY47 there was found a different WRKY domain (WRKYGKK) instead

193 of the classical WRKY domain WRKYGQK.

194 In the phylogenetic tree developed with MEGA6.0, *Cs*WRKYs were located separately depending on the

195 group they belong, with the only exception of *Cs*WRKY46. However, *Cs*WRKY46 is located close to

196 *At*WRKY49 and *At*WRKY59, which also belong to the group IIc (Fig. 2).

197    Treatment with 10 µM ABA induced an increase of endogenous ABA concentration 24h after the

198    application, reaching values in shoots and roots 35.0 and 2802.8 higher than those determined in controls,

199    respectively. In the same sense, 72h after the treatment, these values were 61.0 and 1865.4 higher than

200    controls respectively (Fig. 3). This treatment also induced an increase of SA concentration in roots at 72h,

201    reaching levels 5.5 higher than control. Meanwhile, 50 µM MeJA induced endogenous JA accumulation

202    in both organs at 24h and 72h after the imposition of treatment. At 24h, shoots and roots of plants treated

203    with MeJA had values of JA 42.8 and 268.07 times higher than control, respectively. This difference

204    increased at 72h, achieving JA concentrations 46.5 and 1280.8 higher than control in shoots and roots,

205    respectively. Although 50 µM MeJA treatment also induced ABA accumulation, the increase was lower

206    than that observed for JA concentration, showing in shoots and roots concentrations 5.5 and 16.5 higher

207    than control respectively. Increased levels of endogenous SA concentration were recorded after 10 µM

208    SA application at both sampling times. 24h after the application, SA values were 20.1 and 199.3 times

209    higher than control in shoots and roots, respectively. At 72h, these values in shoots and roots were of 9.4

210    and 202.9 times higher than controls, respectively. This treatment also increased ABA and JA

211    concentrations, but these increases were not as sharp as those observed in SA levels (Fig. 3).

212    Treatment with exogenous phytohormones had also an influence on the expression of different *CsWRKY*s

213    genes (Fig. 4). ABA treatment induced the expression of *CsWRKY26* in root and shoot tissue after 24 and

214    72h respectively, and the expression of *CsWRKY28*, *CsWRKY30*, CsWRKY*31*, *CsWRKY33* and

215    *CsWRKY35* in roots at 72h. On the contrary, this treatment repressed the expression of *CsWRKY11*,

216    *CsWRKY19*, *CsWRKY33* and *CsWRKY44* in shoots at 24h and *CsWRKY19*, *CsWRKY31*, *CsWRKY35* and

217    *CsWRKY49* in shoots at 72h. In roots, ABA treatment repressed *CsWRKY19* and *CsWRKY49* expression

218    at 24h, and *CsWRKY13*, *CsWRKY18*, *CsWRKY19* and *CsWRKY41* at 72h, being *CsWRKY19* transcript

219    accumulation repressed in roots at both sampling times. In this treatment, the largest differences were

220    observed in the expression of *CsWRKY19*, *CsWRKY30* and *CsWRKY33* genes. *CsWRKY19* was highly

221    repressed in roots after 24h and 72h (decreases of 94% and 57% with respect to control, respectively). On

222    the contrary, *CsWRKY30* was up-regulated in roots, with expression values 14.7 times higher than

223    control. Finally, *CsWRKY33* showed a different expression pattern depending on the tissue, being up-

224    regulated in roots (values 8.0 times higher than the control at 72h) and down-regulated in shoots (values

225    80% lower than the control at 24h).

226    MeJA application also had a significant effect on *CsWRKY*s gene expression. In roots, depending on the

227    sampling time, different *CsWRKY*s were significantly altered. At 24h increases of transcript abundance

228    were observed in *CsWRKY22*, *CsWRKY29*, *CsWRKY30*, *CsWRKY43* and *CsWRKY49* whereas in

229    *CsWRKY2*, *CsWRKY19*, *CsWRKY26*, *CsWRKY28*, *CsWRKY29*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33*,

230    *CsWRKY35* and *CsWRKY43,* the increase in expression levels was recorded at 72h. In shoots, only

231    *CsWRKY30* and *CsWRKY35* at 24h and *CsWRKY44* at 72h were up-regulated. Contrarily, CsWRKY*33*

232    and *CsWRKY44* expression was down-regulated in roots; and CsWRKY33 in shoots at 24h, whereas

233    *CsWRKY19*, *CsWRKY30*, and *CsWRKY43* were down-regulated after 72h. Highest differences in this

234    treatment were shown in *CsWRKY19*, reaching at 72h relative expression values 93.5% lower than the

235    control, and *CsWRKY30*, with a relative expression 13.9 times higher than control in roots at 72h (Fig.4).

236    SA application also induced changes in the expression levels in 13 of the 17 *CsWRKY*s selected genes. In

237    roots, expression of *CsWRKY*13, *CsWRKY*18, *CsWRKY*19, *CsWRKY*41, *CsWRKY*43 and *CsWRKY*49

238    were down-regulated at 72h after the phytohormone application. On the contrary, *CsWRKY28*,

239    *CsWRKY30*, *CsWRKY31*, *CsWRKY33* and *CsWRKY35* were up-regulated at both sampling times. In

240    shoots, the expression levels of *CsWRKY2* and *CsWRKY44* significantly decreased at 24h, while

241    *CsWRKY13*, *CsWRKY19*, *CsWRKY35*, *CsWRKY41* and *CsWRKY49* transcript abundance was lower than

242    the control at 72h. Nevertheless, *CsWRKY31* and *CsWRKY35* were up-regulated at 24h after the

243    application of the different phytohormones, while *CsWRKY28*, *CsWRKY30* and *CsWRKY33* were up-

244    regulated either at 24 and 72h. In this treatment, the highest differences were also recorded in *CsWRKY19*

245    relative expression in shoots after 72h, reaching expression values of 92.4% lower than the control. In

246    roots, SA treatment induced an increase in *CsWRKY30* expression at 24 and 72h being 73.6 and 63.6

247    times higher than control respectively.

248    A Venn diagram (Fig. 5) reveals that most of *CsWRKY*s experienced changes in their relative expressions

249    in response to different phytohormones application. There were only three *CsWRKY*s of the 17 studied

250    which were only affected by one treatment: *CsWRKY11*, which was down-regulated in shoots of plants

251    treated with ABA, and *CsWRKY22* and *CsWRKY29*, which were up-regulated in plants treated with

252    MeJA.

253    Taken into consideration that the highest differences in gene expression were generally recorded in root

254    tissue after 72h of treatment, in the following experiments *CsWRKY*s expression was only analyzed in this

255    tissue at 72h.

256    In roots of *C. macrophylla* plants subjected to a period of 72h of osmotic stress, the relative expression of

257    this TFs was generally repressed. This was the case of *CsWRKY2*, *CsWRKY11*, *CsWRKY13*, *CsWRKY19*,

258    *CsWRKY28*, *CsWRKY29*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33*, *CsWRKY35*, *CsWRKY41* and

259    *CsWRKY44* (Fig. 6). However, *CsWRKY18*, *CsWRKY22*, *CsWRKY26*, *CsWRKY43* and *CsWRKY49* did

260    not show any difference respect to the control. Any of the *CsWRKY*s studied was up-regulated under

261    osmotic stress conditions. On the contrary, salt stress induced an over-expression either in Carrizo

262    citrange and *Citrus macrophylla* (Fig. 7). This general increase of *CsWRKY*s relative expression was

263    observed in *CsWRKY2*, *CsWRKY13*, *CsWRKY18*, *CsWRKY19*, *CsWRKY22*, *CsWRKY26*, *CsWRKY28*,

264    *CsWRKY29*, *CsWRKY30*, *CsWRKY31*, *CsWRKY33*, *CsWRKY35*, *CsWRKY41*, *CsWRKY43* and

265    *CsWRKY49*. Among the studied TFs, only *CsWRKY44* was repressed under salt stress conditions,

266    reaching values 60.8% lower than control in *C. macrophylla* plants exposed to 90 mM NaCl. Moreover,

267    roots of the salt resistant rootstock *C. macrophylla* overexpressed *CsWRKY*s in a higher extent than the

268    sensitive Carrizo citrange plants did. This higher overexpression of *CsWRKY*s in roots of *C. macrophylla*

269    respect to Carrizo citrange roots subjected to salt stress was clearly observed in *CsWRKY2*, *CsWRKY19*,

270    *CsWRKY22*, *CsWRKY28*, *CsWRKY30*, *CsWRKY31* and *CsWRKY49*.

271    For an easier visualization, a hierarchical clustering compiling all the results described above was made

272    using the program MeV4.9.0, and is presented in figure 8.

273

274    ***Discussion***

275    In this work, WRKY superfamily of TFs has been identified and characterized in citrus. As it has been

276    reported that WRKYs play pivotal roles in regulating many plant responses to stress (reviewed in Rushton

277    *et al.* 2010), the effect of the application of different stress-related phytohormones and abiotic stress

278    conditions on *CsWRKY*s relative expression was studied in two citrus genotypes, Carrizo citrange and

279    *Citrus macrophylla*, commercially used as rootstocks.

TBLASTN showed 50 *Cs*WRKYs, that were classified according to the scaffold they belong, following the procedure described for other plants where the genes chromosome location of 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 a 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).

Plant hormones are key players in regulating cell responses to external and internal stimulus; moreover, this substances interact among them (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 levels in roots of citrus plants subjected to drought (de Ollas *et al.* 2013). Positive interactions between ABA and SA have been also described in wheat (*Triticum aestivum*) 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 endogenous content of the applied phytohormone, but also induced lower accumulations of others, supporting the crosstalk among different phytohormones. Applications of MeJA and SA induced the accumulation of ABA, while ABA application increased endogenous JA and SA levels.

At the transcriptional level, hormone treatments resulted in a wide variety of changes in *CsWRKY*s genes depending on the hormone applied, the sampling time and the analyzed tissue. Most of the differences were detected in roots at 72h, probably due to the application of the different phytohormones directly to this organ. The treatment with ABA has 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 also are members of group IIa, in *A. thaliana* plants treated with ABA (Chen *et al.* 2010), suggesting that some *WRKY* genes included in group IIa are involved in ABA signaling or response.

307  MeJA caused increases on *CsWRKY* relative expression in leaves are roots, being *CsWRKY35* particularly

308  induced by MeJA. This is in concordance with results described in *Nicotiana attenuate*, where *NaWRKY3*

309  and *NaWRKY6* are also induced in the presence of JA (Skibbe *et al.* 2008).

310  The WRKY family in citrus is very sensitive to the hormonal treatments. Our data suggest specific

311  responses to the exogenous application of each hormone (whereas MeJA mostly induced over-expression

312  of *CsWRKYs*, SA and ABA downregulated these TFs). Similar findings have been described in grape

313  (*Vitis vinifera*), another woody plant, where *VvWRKY* genes down-regulated after treatments with ABA or

314  SA, and up-regulated in treatments with JA or ET (Guo *et al.* 2014). These results contrast with studies in

315  other herbaceous species as rice, where plants treated with ABA, SA and MeJA overexpressed *OsWRKY*s

316  (Ramamoorthy *et al.* 2008), or canola, where treatments with ABA, JA and ET significantly repressed

317  some *BnWRKY*s expression, while plants treated with SA overexpressed some genes of this family (Yang

318  *et al.* 2009). All these results reveal that the involvement of WRKYs in response to hormonal treatments

319  is highly dependent on the studied genotype, exhibiting high differences among species.

320  To face different abiotic stressful conditions, plants activate common mechanisms such as stomatal

321  closure, proline accumulation, enhancement of antioxidant enzymatic activities, etc. On the contrary, the

322  expression pattern of *Cs*WRKYs TFs was completely different in plants subjected to osmotic or salt

323  stress. Under osmotic stress conditions, all the *CsWRKY*s which expression was significantly altered were

324  down-regulated. Furthermore, there was a direct correlation among the relative decrease of gene

325  expression and the intensity of the osmotic stress applied. Conversely, salt stress caused an up-regulation

326  of *CsWRKYs*. This is in concordance with data reported in transgenic lines of *Arabidopsis thaliana* that

327  become more sensitive to osmotic stress when they overexpressed soybean *Gm*WRKY13 (Zhou *et al.*

328  2008). Other studies demonstrated that tobacco plants overexpressing the *Thlaspi caerulescens*

329  *TcWRKY53* are more sensitive to osmotic stress induced by PEG 6000 and sorbitol. However, this gene is

330  up-regulated in different abiotic stress conditions such as cold, salt or drought (Wei *et al.* 2008)**.** This fact

331  reveals that although in abiotic stress conditions *WRKY*s are overexpressed, they are usually

332  downregulated in osmotic stress conditions, being in agreement with the results obtained in this work.

333  The overexpression of *CsWRKYs* in citrus plants subjected to salt stress conditions described here is in

334  concordance with previous studies in other species, such as rice, populus or soybean (Jiang *et al.* 2014,

335  Ramamoorthy *et al.* 2008, Song *et al.* 2016). Although most of *CsWRKYs* were up-regulated under salt

stress conditions 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 2003). Although both

stresses applied in this work share the osmotic component, they regulated differently the expression of

*CsWRKY*s. This different regulation, therefore, seems related to the toxic component of salt stress, which

has been previously that 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 stress

conditions. Whereas osmotic stress repressed expression of most *CsWRKY*s, salt stress had the opposite

effect. Moreover, over-expression of *CsWRKYs* under salt-stress conditions in the tolerant genotype *C.*

*macrophylla* was higher than in the sensitive Carrizo citrange. 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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*Acknowledgements*

499    **Figure 1.** *Cs*WRKYs alignments by families. Common regions between the different families are marked

500    in green, while common regions inside families are marked in red. Yellow highlighted zones refer to

501    potential zinc ligands. Gaps have been inserted for an optimal alignment.

502    **Figure 2.** Phylogenetic tree of WRKY TFs domains of *A. thaliana* and *C. sinensis*. The numbers in

503    branches represent bootstrap values based on 1000 replications. Different colors refer to the different

504    groups of WRKY TFs: group I N-terminal: dark blue; group I C-terminal: red; group IIa: pink; group IIb:

505    green; group IIc: black; group IId: white; group IIe: light blue; group III: yellow.

506    **Figure 3.** Hormonal contents in shoots and roots in Carrizo citrange plants treated with 50 µM MeJA, 10

507    µM SA and 10 µM ABA. White bars represent phytohormonal contents at 24h, and grey bars after 72h.

508    Error bars refer to standard error of three replicates. Asterisks denote significant difference at $p \leq 0.05$

509    respect to control.

510    **Figure 4.** Relative expression of *CsWRKY* genes in response to MeJA, SA and ABA in shoots and roots

511    at 24 and 72h. White bars refer to shoots and grey bars refer to roots. Non-lined bars represent the relative

512    expression at 24h and lined bars at 72h. Error bars refer to standard error of three replicates. Asterisks

513    denote significant difference at $p \leq 0.05$ respect to control.

514    **Figure 5.** Venn diagram depicting the degree of overlap between the number of *CsWRKY*s which were

515    significantly regulated by exogenous applications of ABA, MeJA or SA.

516    **Figure 6.** Relative expression of *CsWRKY* genes in response to osmotic stress in *C. macrophylla* roots at

517    72h, using treatments of 0 (Control), -0.75 and -1.5 MPa with polyethylene glycol 6000. Error bars refer

518    to standard error of three replicates. Asterisks denote significant difference at $p \leq 0.05$ between control
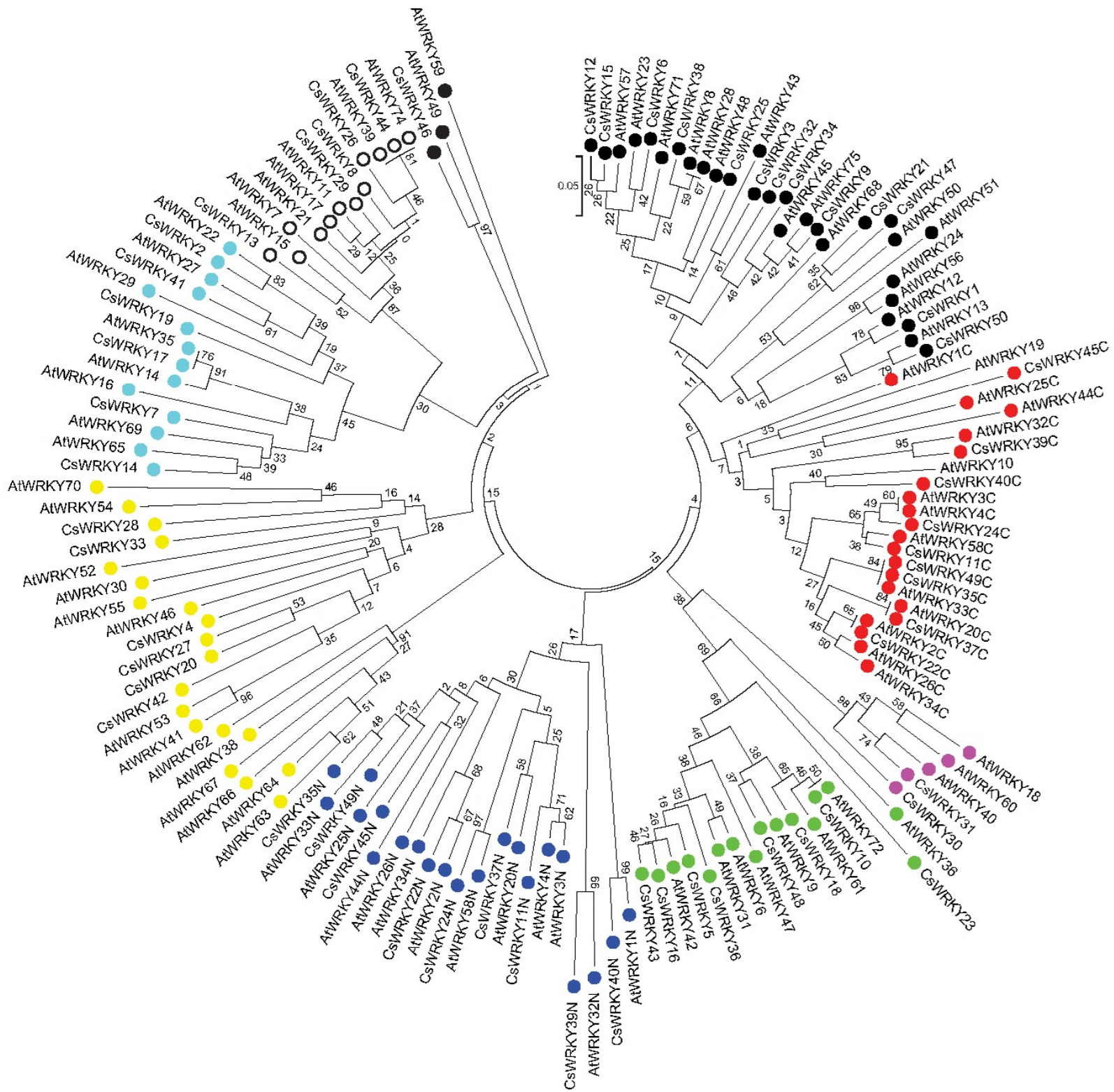
519    and stressed samples.

520    **Figure 7.** Relative expression of CsWRKY genes in response to salt stress in Carrizo citrange (CC) and

521    *C. macrophylla* (CM) roots at 72h. White bars refer to control, light grey bars refer to 60mM and dark

522    grey bars refer to 90 mM. Error bars refer to standard error of three replicates. Asterisks denote

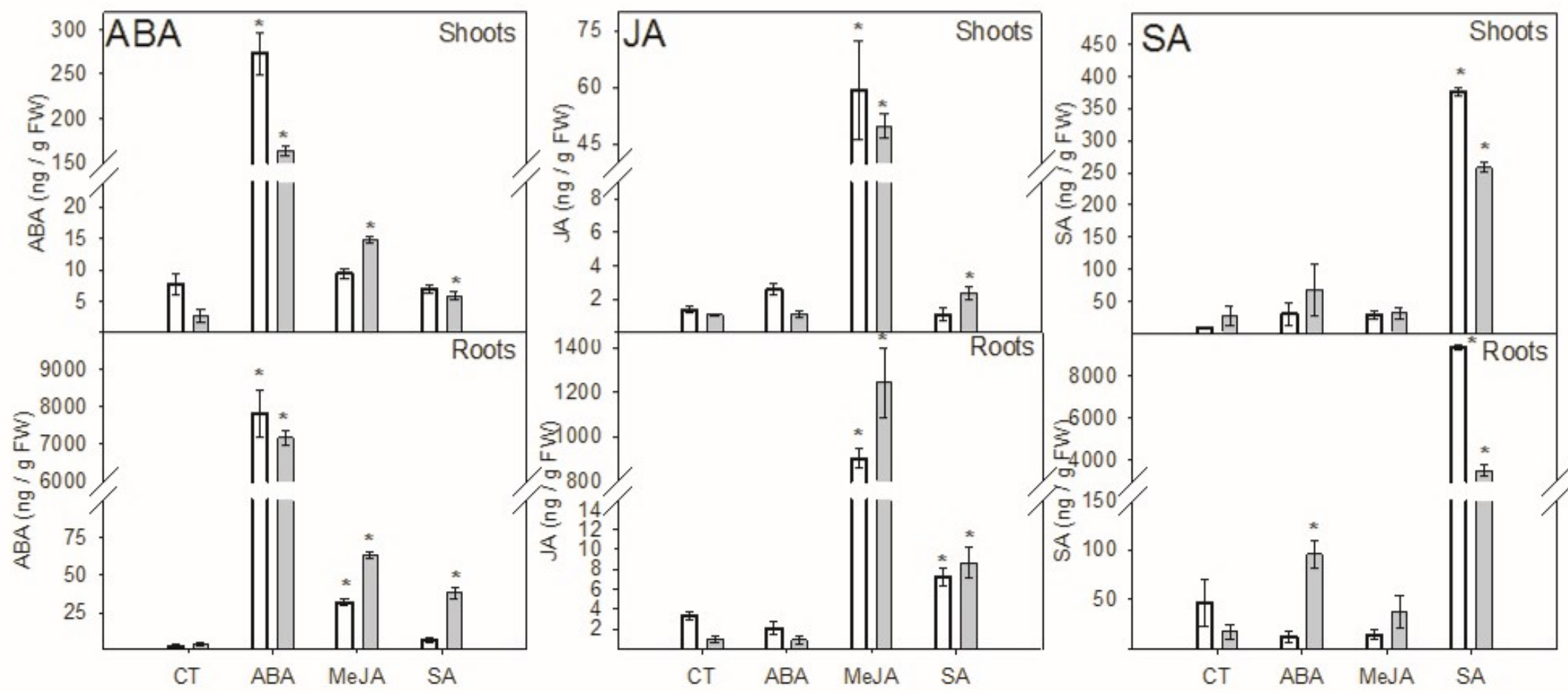523    significant difference at $p \leq 0.05$ between control and stressed samples.

524    **Figure 8.** Hierarchical clustering of relative expression profiles of selected CsWRKYs. The color scale

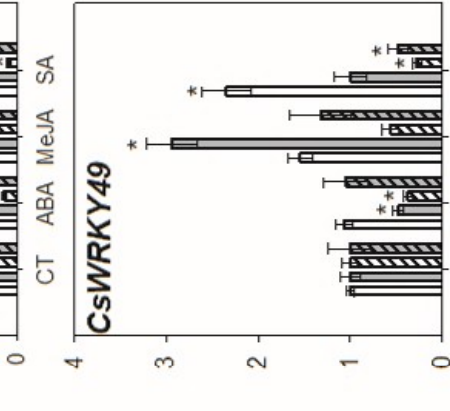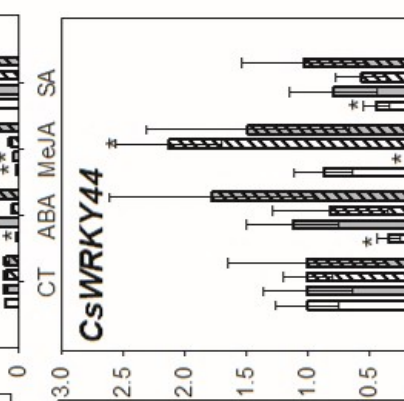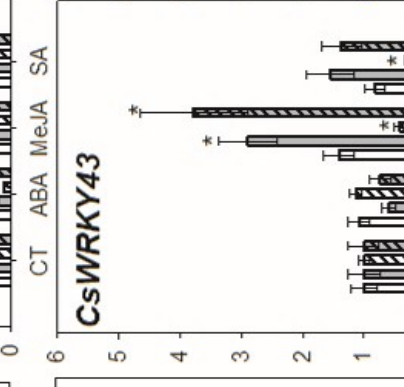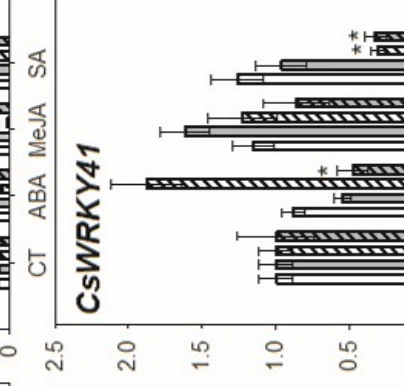525    represents relative expression levels. Green and red represent decreasing and increasing transcripts

526    concentrations respectively. A: Phytohormone application experiment at 24 and 72h in shoots (S) and

527    roots (R). B: Osmotic stress experiment with osmotic potentials of -0.75 and -1.50 MPa in *C.*

528    *macrophylla*. C: Salt stress experiment in concentrations of 60 and 90 mM NaCl in Carrizo citrange and

529    *C. macrophylla*.

530    **Table 1.** List of the *Cs*WRKY TFs family, classified according to the group and scaffold they belong.

531    Start and End columns refer to the location of the gene in the respective scaffold. Full length column

532    indicates genes length in base pairs.

533    **Sup. Table 1.** *CsWRKY*s studied in phytohormones application and abiotic stress conditions and primers

534    used.

Relative expression

CsWRKY2, CsWRKY11, CsWRKY13, CsWRKY18, CsWRKY19, CsWRKY22, CsWRKY26, CsWRKY28, CsWRKY29, CsWRKY30, CsWRKY31, CsWRKY33, CsWRKY35, CsWRKY41, CsWRKY43, CsWRKY44, CsWRKY49

CT, ABA, MeJA, SA

Relative expression

A

B

C
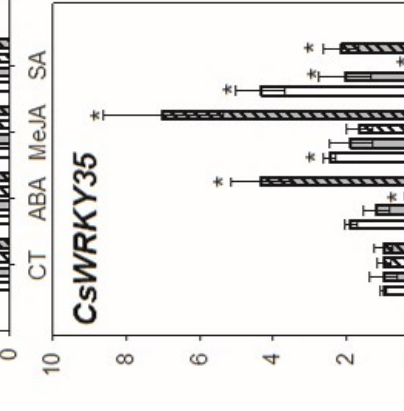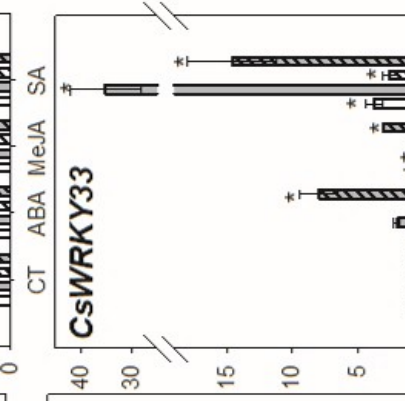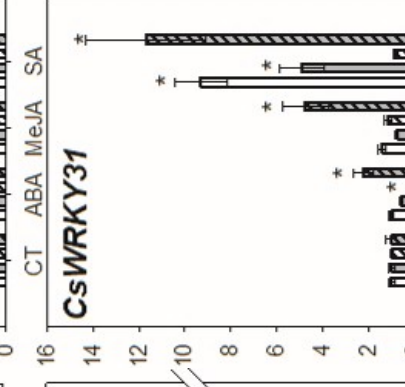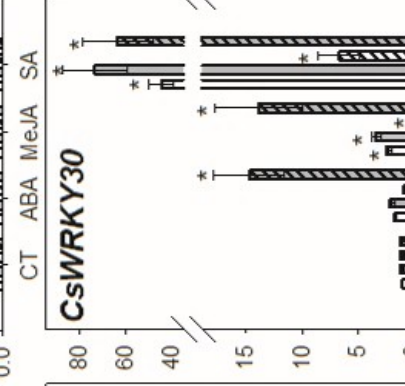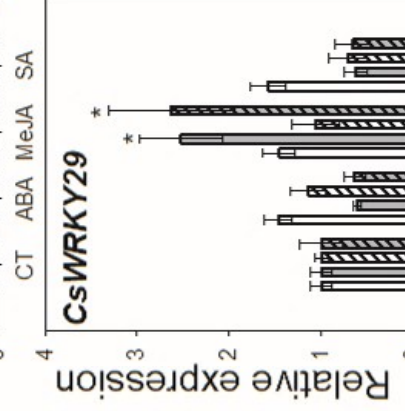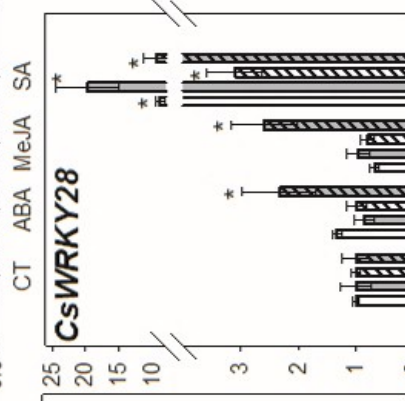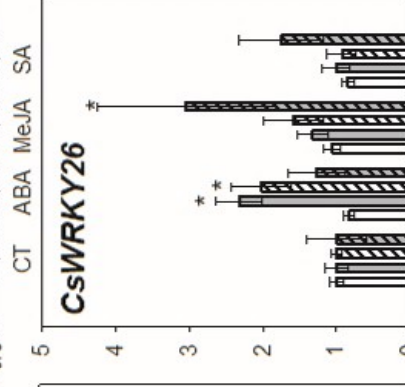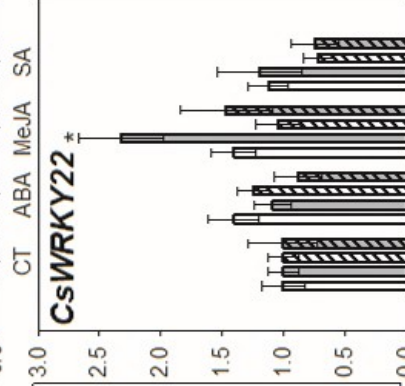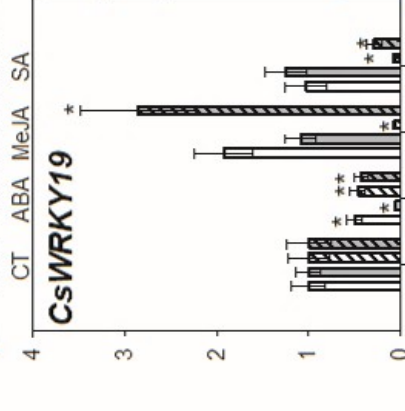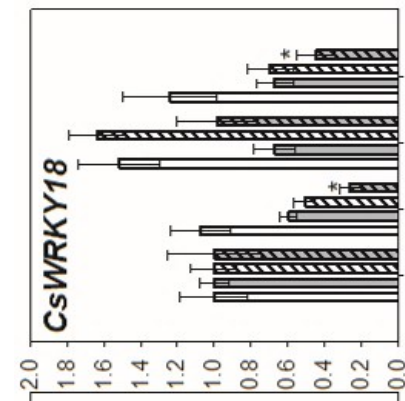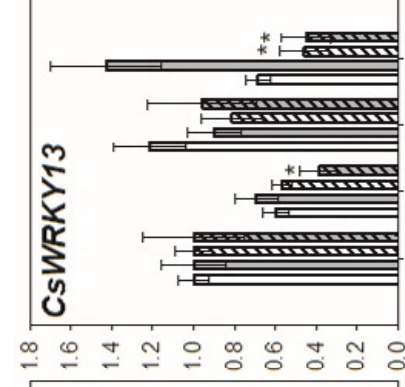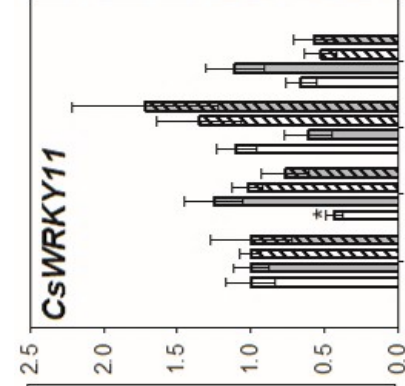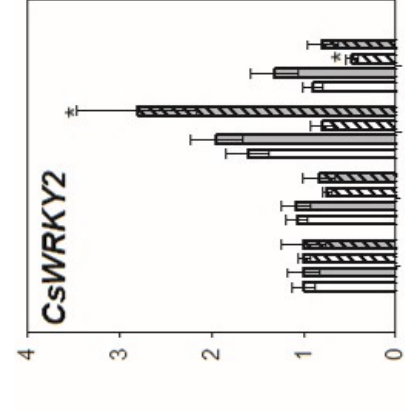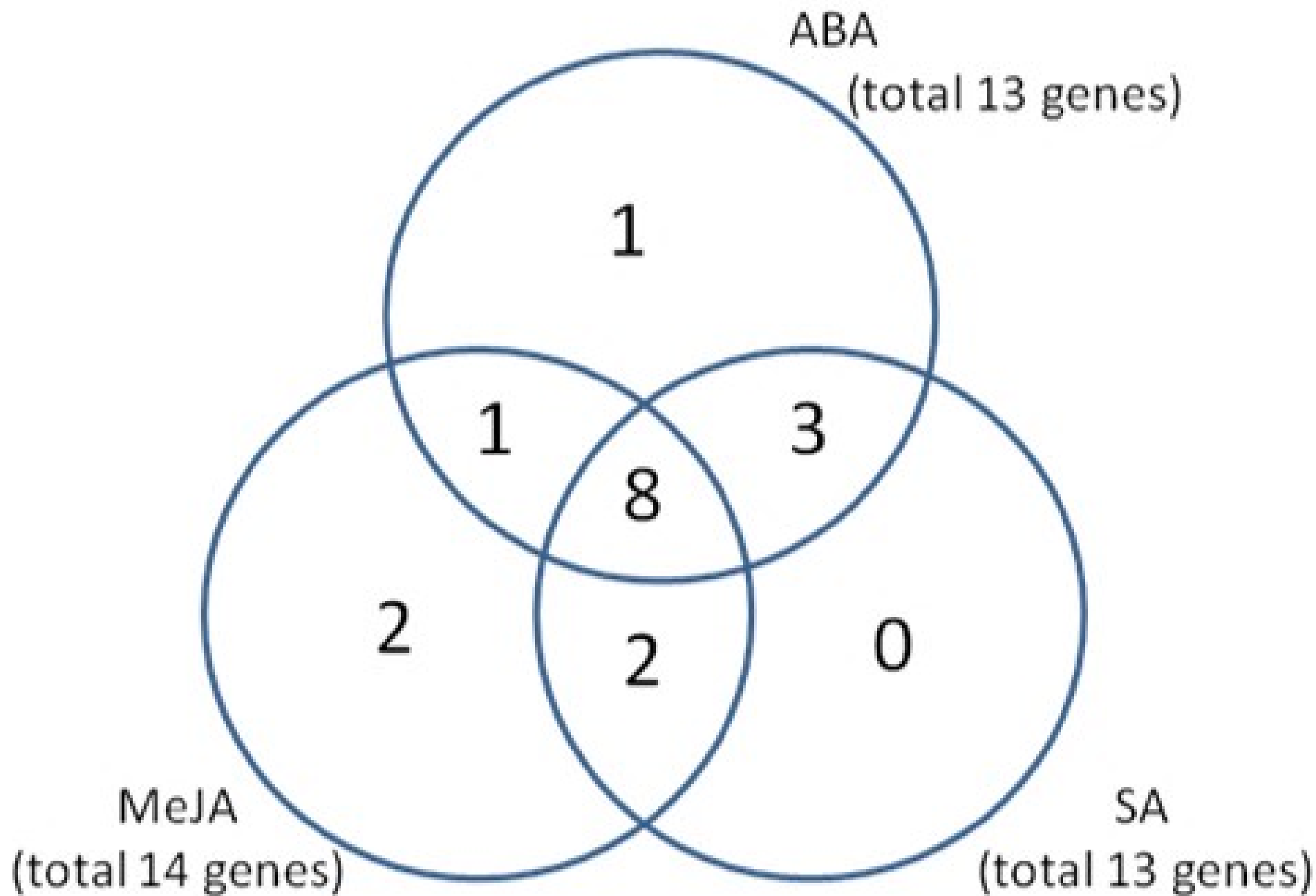
**Group I N-terminal**

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CsWRKY22N  AGASSEDGYNWRKYGQKVKGSEYP-RSYYKCTHPN-CQVKKKVERS-LEGHITEIIYKGAHNHPKP
CsWRKY45N  AGDRPSYDGYNWRKYGQKVKGSEYP-RSYYKCTHPN-CPVKKKVERS-EDGQIAEIVKGEHNHPKP
CsWRKY49N  RESKKSDDGYNWRKYGQKVKGSENP-RSYYKCTEPS-CPTKKKVERS-LDGQITEIVKGSNNHPKP
CsWRKY35N  REQKRSEDGYNWRKYGQKVKGSENP-RSYFKCTFPD-CPMKKKVERS-LDGQITEIVKGKHNHPKP
CsWRKY11N  VSDKPADDPYNWRKYGQKHVKGSEF-RSYFKCTHPN-CPVKKKVERS-LDGQVTEIIYKGQHNHPPP
CsWRKY24N  AVDKPADDPYNWRKYGQKPIKGNEYP-RSYYKCTHVN-CPVKKKVERS-SNAQITQIIYKNEHHEKP
CsWRKY37N  GPSMPSDGYNWRKYGQKVKGSEF-RSYYKCTHPN-CEVKKLFERS-HDGQITEIIYKGTHDHPKP
CsWRKY40N  IREKVSEDGYNWRKYGQKLVRGNEFV-RSYYKCTHPR-CLAKKQLDCT-HEGQIVDTIYSGDHCHPKV
CsWRKY39N  IVKTPVSDGYNWRKYGQKVKSPKGS-RSYYKCTYSD-CCAKKIECSD-HSGHVIEIVNKGMHSHDPP
```

**Group I C-terminal**

```
CsWRKY22C  SEVDILDDGYRWRKYGQKVVKGNPNP-RSYYKCTSA-G--CTVRKHVERASHDLKSVITTYEGKHNHDVP
CsWRKY37C  SEVDILDGYRWRKYGQKVVKGNPNP-RSYYKCTNA-G--CPVRKHVERASHDPKAVITTYEGKHNHDVP
CsWRKY11C  SEVDLLDDGYRWRKYGQKVVKGNPYP-RSYYKCTTT-G--CNVRKHVERASTDPKAVITTYEGKHNHDVP
CsWRKY24C  SEVDLLDDGYRWRKYGQKVVKGNPHP-RSYYKCTNP-G--CNVRKHVERAPTDPKAVVTTYEGKHNHDVP
CsWRKY49C  SDIDILDGYRWRKYGQKVVKGNPNP-RSYYKCTHP-G--CPVRKHVERASHDLRAVITTYEGKHNHDVP
CsWRKY35C  SDIDILDDGYRWRKYGQKVVKGNPNP-RSYYKCTTT-G--CPVRKHVERASHDMRAVITTYEGKHNHDVP
CsWRKY40C  SEVDFVNDGYRWRKYGQKLVKGNPNP-RNYYRCSNS-G--CPAKKHVERASHDPKLVITTYEGRHDHDMP
CsWRKY39C  GDVGISGDGYRWRKYGQKMVKGNPNP-RNYYRCTSA-G--CPVRKHIETAVDNTSAVIITYKGVTHDMP
CsWRKY45C  TDSEILSDGFRWRKYGQKVVKGNPYP------RLRLD---NNLTSNITS------MYTLLFLEN---
```
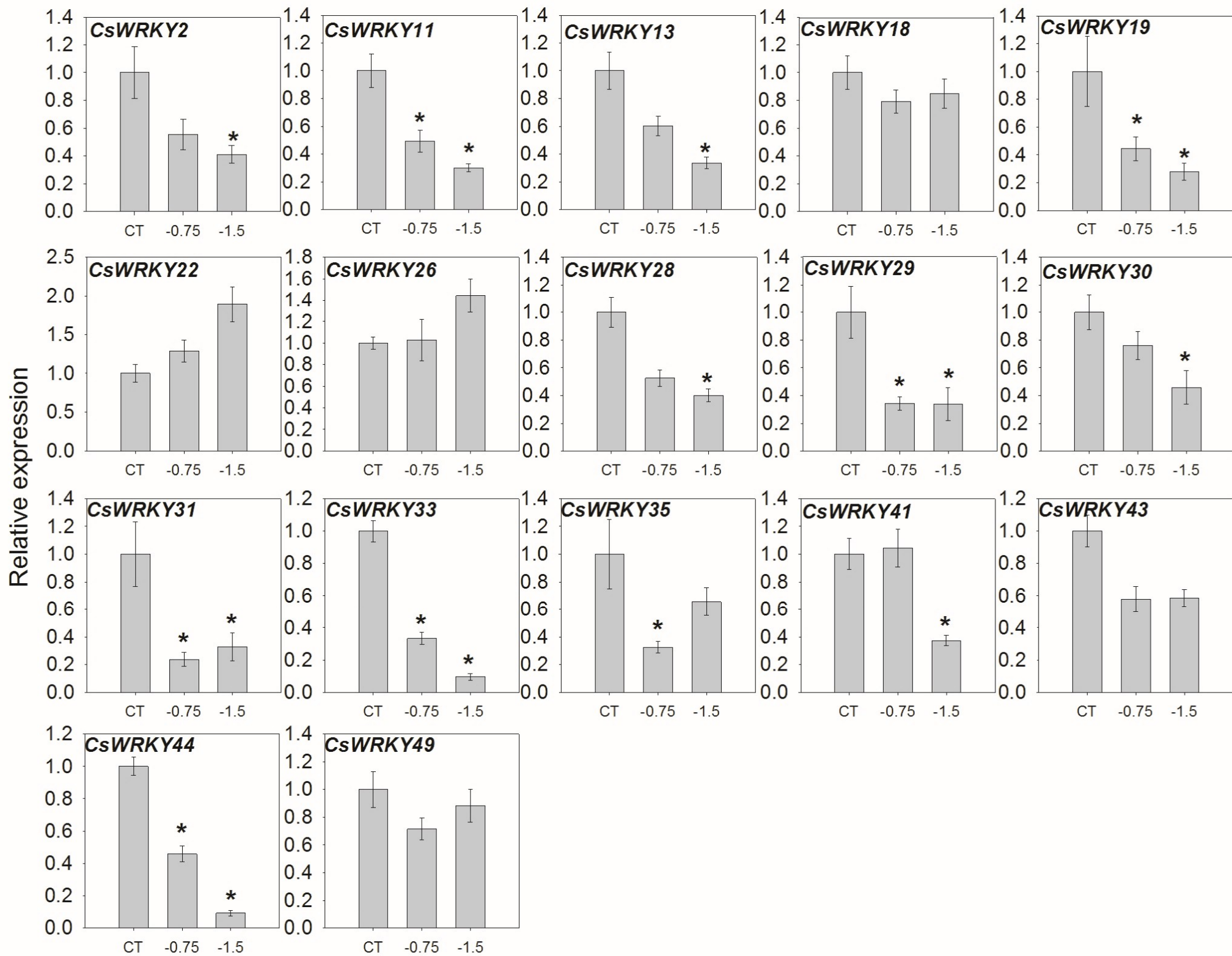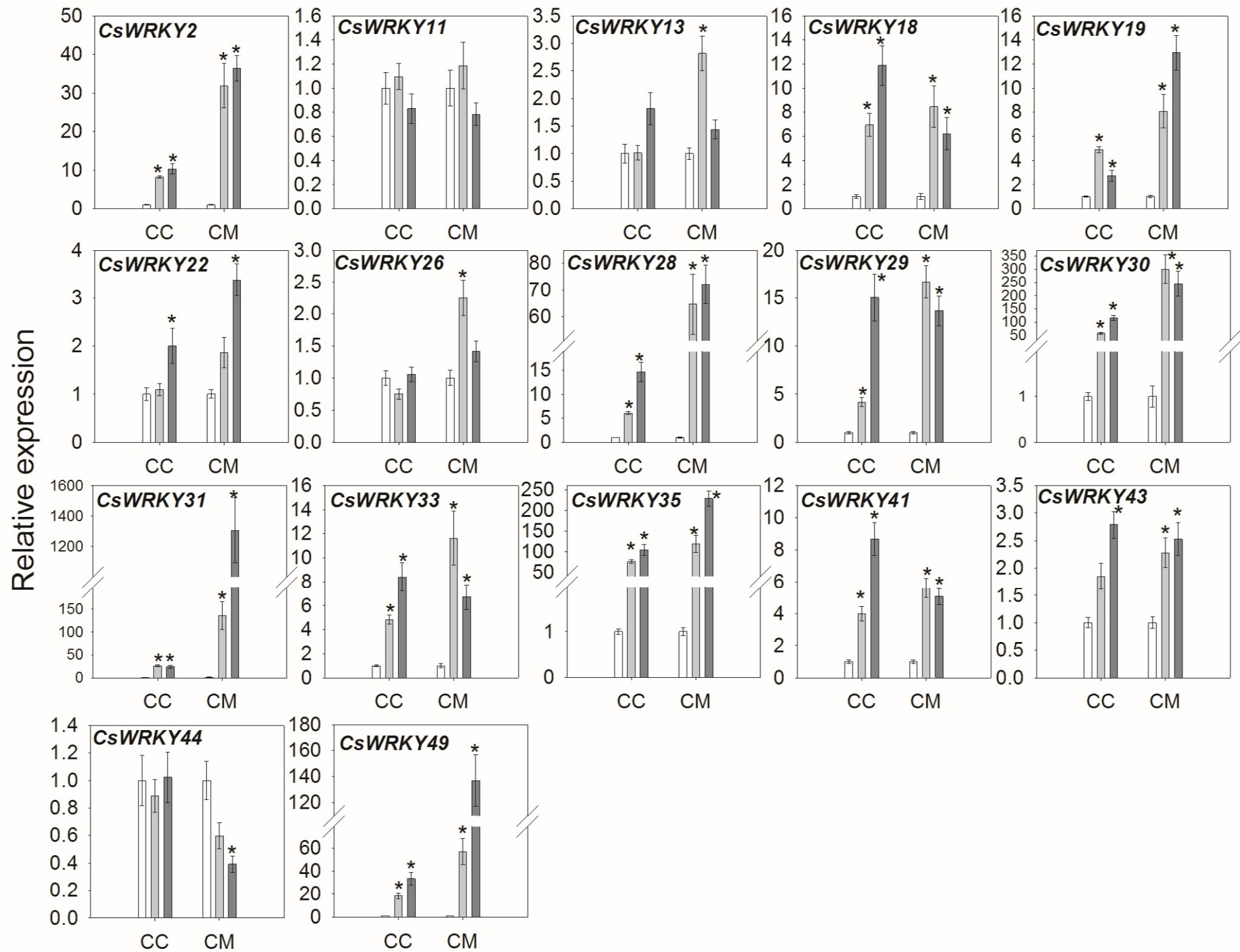
**Group IIa**

```
CsWRKY30  DSSLIVKDGHQWRKYGQKVTKDNPSP-RAYFRCSMASSG--CPVKKKVQRCMEDKSFLVATYEGEHNHDVQ
CsWRKY31  NSTLIVKDGYQWRKYGQKVTRDNPSP-RAYFRCSFAPS--CPVKKKVQRSAEDPSILVATYEGEHNHPQP
```

**Group IIb**

```
CsWRKY10  CDTPTMNDGCQWRKYGQKIAKGNPCP-RAYYRCTISPT--CPVRKQVQRWHEDMSILITTYEGTHNHPLP
CsWRKY18  CDTPTMNDGCQWRKYGQKIAKGNPCP-RAYYRCTVAPS--CPVRKQVQRCAEDMSVLITTYEGTHNHPLP
CsWRKY5   SEAPLISDGCQWRKYGQKMAKGNPCP-RAYYRCTMAVG--CPVRKQVQRCAEDRTILITTYEGNHNHPLP
CsWRKY16  SEAPMITDGCQWRKYGQKMAKGNPCP-RAYYRCTMAVG--CPVRKQVQRCAEDRTILITTYEGNHNHPLP
CsWRKY36  SEASMISDGCQWRKYGQKMAKGNPCP-RAYYRCTMASG--CPVRKQVQRCSQDRTILMTTYEGNHNHPLP
CsWRKY43  SEAPMINDGCQWRKYGQKMAKGNPCP-RAYYRCTMAAG--CPVRKQVQRCAEDRTILITTYEGNHNHPLP
CsWRKY48  CQAATINDGCQWRKYGQKIAKGNPCP-RAYYRCTVAPG--CPVRKQVQRCLEDMSILIT----
CsWRKY23  CDAPTLNDGCQWRKYGQKIRRKP-------YYKNVQRCAEDMSILITTYEGTHSHPLP
```

**Group IIc**

```
CsWRKY21  SDVEILDDGFKWRKYGQKMVKNSPNP-RNYYKCSVDG--CPVKKRVERDRDDPSYVITTYEGFHTHQSN
CsWRKY47  SELEVMDDGFKWRKYGKKSVKNSPNP-RNYYKCSTGG--CQVKKRVERDREDSSYVITTYEGTHNHESP
CsWRKY1   SDVDVLDDGYKWRKYGQKIVKNSLHP-RSYYRCTHNN--CRVKKRVERLSEDCRMVITTYEGRHNHSPC
CsWRKY50  SDIDVLDDGYKWRKYGQKVVKNTQHP-RSYYRCTQDN--CRVKKRVERLAEDPRMVITTYEGRHVHSPS
CsWRKY12  SEVDHLEDGYRWRKYGQKAVKNSPFP-RSYYRCTNSK--CTVKKRVERSSEDPTIVITTYEGQHCHHTV
CsWRKY38  SEIDHLEDGYRWRKYGQKAVKNSPYP-RSYYRCTSQK--CTVKKRVERSYQDPTVVITTYEGQHNHQCP
CsWRKY15  SEVDHLEDGYRWRKYGQKAVKNSPFP-RSYYRCTTQK--CGVKKRVERSYEDPSIVITTYEGQHSHPLP
CsWRKY6   SEVDHLEDGYRWRKYGQKAVKDSPFP-RSYYRCTSAS--CNVKKRVERSYTDPSIVVTTYEGQHNHPSP
CsWRKY25  SDIDHLDDGYRWRKYGQKAVKNSPHP-RSYYRCTSAG--CGVKKRVERSSEDPTIVVTTYEGQIHPSP
CsWRKY9   SQVDILDDGYRWRKYGQKAVKNNKFP-RSYYRCTHQG--CNVKKQVQRLTKDEGVVTTYEGMHSHPIE
CsWRKY34  SQVDILDDGYRWRKYGQKAVKDNKFP-RSYYRCTHEG--CNVKKQVQRLTNDEGIVVTTYEGMHNHRIE
CsWRKY32  SQVDILDDGYRWRKYGQKAVKNSKFP-RSYYRCTHKG--CNVKKQVQRNTKDEEIVVTTYEGLHTHPIG
CsWRKY3   SADDILDDGYRWRKYGQKAVKNSLYP-RSYYRCTHHT--CNVKKQVQRLSKDTSIVVTTYEGIHNHPCE
CsWRKY46  WGNGMADDGYKWRKYGQKSIKNSPNP-RSYYKCTNPR--CSAKKQVERSCDDPDTLTITYEGLHLHFAY
```

**Group IId**

```
CsWRKY26  KLADIPPDDYSWRKYGQKPIKGSPHP-RGYYKCSSMRG--CPARKHVERCLEEPTMLIVTYEGHNHPRL
CsWRKY44  KVADIPPDEYTWRKYGQKPIKGSPHP-RGYYKCSSVRG--CPARKHVERCPEEPSMLIVTYEGHNHSRI
CsWRKY29  KIADIPPDEYSWRKYGQKPIKGSPYP-RGYYKCSTMRG--CPARKHVERAPDDPTMLIVTYEGHRHSQA
CsWRKY8   KMADIPPDDYSWRKYGQKPIKGSPHP-RGYYKCSSVRG--CPARKHVERALDDPMMLIVTYEGDHNHAFA
CsWRKY13  RLSDIPPDDFSWRKYGQKPIKGSPHP-RGYYKCSSVRG--CPARKHVERALDDPSMLVVTYEGHNHSLS
```

**Group IIe**

```
CsWRKY2   PAEGLSDVWAWRKYGQKPIKGSPYP-RGYYRCSSSKG--CLARKQVERNRSDPGMFIVTYTAEHNHPAP
CsWRKY19  TADCLACDKWAWRKYGQKPIKGSPYP-RGYYRCSSSKG--CLARKQVERSSADPGVFIITYGAEHNHGHP
CsWRKY7   KNEGPPSDFWSWRKYGQKPIKGSPYP-RGYYRCSTSKG--CSAKKQVERCRTDASMLIITYSSHNHPCP
CsWRKY17  SGEVVPSDLWAWRKYGQKPIKGSPYP-RGYYRCSSSKG--CSARKQVERSRTDPNMLVITYSEHNHPWP
CsWRKY14  GESAPPSDSWAWRKYGQKPIKGSPYP-RGYYRCSSSKG--CSARKQVERSSVDPSMLITYSCEHNHPWP
CsWRKY41  TEEKLSADLWAWRKYGQKPIKGSPYP-RNYYRCSSSKG--CAARKQVERSNTDPNIYIVSYTGDHTHPRP
```

**Group III**

```
CsWRKY20  EGPFE--DGYSWRKYGQKDILGAKYP-RSYYRCTYRNT--CWATKQVQRSDEDPSTFEVTYRGTHTCFNG
CsWRKY42  KDPQEDVSKKSWRKYGQKDILGAKYP-RSYYRCTYRNGQ--CLATKQVQRSDEDPTIFEITYRGNHTCAQA
CsWRKY4   EGTLD--DGYCWRKYGQKDILGRNEF-RGYYRCTHRHARG--CLATKQVQRSDDPSMFEVTYGRHKCSQN
CsWRKY27  EIPPE--DGYTWRKYGQKEILNSKYP-RSYYRCTHQKLYK--CPAKKQVQRLDDPSTFEVAYGDHTCHMS
CsWRKY28  SSTSN--DGHAWRKYGQKEILNTKHP-RSYFRCTHKYVQG--CRATKQVQRRDDPQMYDTTYIGHTCRDI
CsWRKY33  STLTD--DGHAWRKYGQKVILNAREF-RNYERCTHKFDQG--CQASKQVQRIQEEPLHRTTYGRHTCKSL
```

| Group | Gene | | Phytozome | | | | Citrus sinensis Anotation Project | |
| | | Locus | Scaffold | Start | End | Full length | Locus | Chromosome |
|---|---|---|---|---|---|---|---|---|
| I | CsWRKY11 | orange1.1g009051m.g | 3 | 3348929 | 3352620 | 3692 | Cs1g03100 | 1 |
| I | CsWRKY22 | orange1.1g004963m.g | 13 | 1838851 | 1843181 | 4331 | Cs7g04260 | 7 |
| I | CsWRKY24 | orange1.1g010802m.g | 15 | 1591146 | 1594814 | 3669 | Cs7g03300 | 7 |
| I | CsWRKY35 | orange1.1g013222m.g | 42 | 689265 | 692549 | 3285 | Cs7g03080 | 7 |
| I | CsWRKY37 | orange1.1g008458m.g | 49 | 532438 | 537337 | 4900 | orange1.1t04068 | - |
| I | CsWRKY39 | orange1.1g036653m.g | 68 | 482854 | 486663 | 3810 | Cs2g04520 | 2 |
| I | CsWRKY40 | orange1.1g011340m.g | 77 | 307046 | 310334 | 3289 | Cs2g03840 | 2 |
| I | CsWRKY45 | orange1.1g014629m.g | 172 | 174342 | 178393 | 4052 | Cs2g02790 | 2 |
| I | CsWRKY49 | orange1.1g011483m.g | 626 | 42514 | 46157 | 3644 | Cs2g10310 | 2 |
| IIa | CsWRKY30 | orange1.1g025097m.g | 25 | 996904 | 999151 | 2248 | Cs5g04160 | 5 |
| IIa | CsWRKY31 | orange1.1g020831m.g | 25 | 1006589 | 1008372 | 1784 | Cs5g03010 | 5 |
| IIb | CsWRKY5 | orange1.1g040711m.g | 1 | 3810277 | 3813167 | 2891 | orange1.1t00419 | - |
| IIb | CsWRKY10 | orange1.1g010903m.g | 3 | 2727603 | 2730035 | 2433 | Cs5g02450 | 5 |
| IIb | CsWRKY16 | orange1.1g007099m.g | 7 | 1958471 | 1961762 | 3292 | Cs4g05760 | 4 |
| IIb | CsWRKY18 | orange1.1g009794m.g | 9 | 279247 | 281128 | 1882 | Cs4g07560 | 4 |
| IIb | CsWRKY23 | orange1.1g045987m.g | 15 | 942103 | 943915 | 1813 | Cs6g20850 | 6 |
| IIb | CsWRKY36 | orange1.1g008964m.g | 49 | 126215 | 128926 | 2712 | Cs6g21990 | 6 |
| IIb | CsWRKY43 | orange1.1g007546m.g | 121 | 389458 | 391889 | 2432 | Cs9g02040 | 9 |
| IIb | CsWRKY48 | orange1.1g036819m.g | 568 | 96439 | 97905 | 1467 | Cs5g30250 | 5 |
| IIc | CsWRKY1 | orange1.1g026216m.g | 1 | 354623 | 357157 | 2535 | Cs7g29580 | 7 |
| IIc | CsWRKY3 | orange1.1g046286m.g | 1 | 2355401 | 2357338 | 1938 | Cs7g29570 | 7 |
| IIc | CsWRKY6 | orange1.1g020713m.g | 1 | 4236944 | 4238415 | 1472 | Cs7g07140 | 7 |
| IIc | CsWRKY9 | orange1.1g043122m.g | 3 | 164635 | 166589 | 1955 | orange1.1t00425 | - |
| IIc | CsWRKY12 | orange1.1g045509m.g | 3 | 3456550 | 3459372 | 2823 | Cs7g06330 | 7 |
| IIc | CsWRKY15 | orange1.1g021142m.g | 7 | 1347875 | 1349215 | 1341 | Cs7g06320 | 7 |
| IIc | CsWRKY21 | orange1.1g031482m.g | 13 | 646034 | 647324 | 1291 | orange1.1t02600 | - |
| IIc | CsWRKY25 | orange1.1g017479m.g | 16 | 1122184 | 1123697 | 1514 | Cs6g10120 | 6 |
| IIc | CsWRKY32 | orange1.1g031298m.g | 35 | 211943 | 212982 | 1040 | Cs2g25560 | 2 |
| IIc | CsWRKY34 | orange1.1g030050m.g | 38 | 626400 | 630108 | 3709 | Cs6g09420 | 6 |
| IIc | CsWRKY38 | orange1.1g019375m.g | 57 | 464133 | 466331 | 2199 | Cs4g10020 | 4 |
| IIc | CsWRKY46 | orange1.1g038951m.g | 189 | 257377 | 258688 | 1312 | Cs4g09310 | 4 |
| IIc | CsWRKY47 | orange1.1g029257m.g | 362 | 80427 | 84896 | 4470 | Cs7g17180 | 7 |
| IIc | CsWRKY50 | orange1.1g026950m.g | 1166 | 10456 | 13713 | 3258 | Cs4g01710 | 4 |
| IId | CsWRKY8 | orange1.1g018215m.g | 2 | 1863592 | 1865359 | 1768 | orange1.1t00472 | - |
| IId | CsWRKY13 | orange1.1g017930m.g | 6 | 1346375 | 1348315 | 1941 | Cs2g19800 | 2 |
| IId | CsWRKY26 | orange1.1g018659m.g | 22 | 289543 | 292094 | 2552 | Cs9g19070 | 9 |
| IId | CsWRKY29 | orange1.1g019404m.g | 25 | 483188 | 485204 | 2017 | Cs9g18480 | 9 |
| IId | CsWRKY44 | orange1.1g018255m.g | 124 | 381097 | 383496 | 2400 | orange1.1t05133 | - |
| IIe | CsWRKY2 | orange1.1g019126m.g | 1 | 2333743 | 2335147 | 1405 | Cs8g13600 | 8 |
| IIe | CsWRKY7 | orange1.1g023982m.g | 1 | 4402815 | 4404405 | 1591 | Cs6g03950 | 6 |
| IIe | CsWRKY14 | orange1.1g022353m.g | 7 | 901284 | 905441 | 4158 | Cs6g06940 | 6 |
| IIe | CsWRKY17 | orange1.1g012605m.g | 8 | 2345601 | 2348134 | 2534 | Cs1g03870 | 1 |
| IIe | CsWRKY19 | orange1.1g021896m.g | 9 | 1025605 | 1026760 | 1156 | orange1.1t01175 | - |
| IIe | CsWRKY41 | orange1.1g015616m.g | 97 | 230834 | 232265 | 1432 | Cs2g09020 | 2 |
| III | CsWRKY4 | orange1.1g017895m.g | 1 | 2647888 | 2651028 | 3141 | orange1.1t01779 | - |
| III | CsWRKY20 | orange1.1g019737m.g | 9 | 1409048 | 1410893 | 1846 | Cs1g04180 | 1 |
| III | CsWRKY27 | orange1.1g018407m.g | 24 | 82311 | 84401 | 2091 | orange1.1t01713 | - |
| III | CsWRKY28 | orange1.1g021598m.g | 24 | 87173 | 88697 | 1525 | orange1.1t01686 | - |
| III | CsWRKY33 | orange1.1g020291m.g | 36 | 115494 | 117084 | 1591 | Cs3g23190 | 3 |
| III | CsWRKY42 | orange1.1g045032m.g | 104 | 283550 | 285259 | 1710 | Cs7g11020 | 7 |

| *Cs*WRKY | Left Primer | Right Primer | Product size (bp) |
| --- | --- | --- | --- |
| *Cs*WRKY19 | GGAACGAAGCAGTGCAGATC | CAAGTGGTTTGTTCTGGGCG | 158 |
| *Cs*WRKY22 | CTCTGGCTCCTCAAGTGCCG | CTGCTGCCTTCCAGGTACTC | 145 |
| *Cs*WRKY28 | GTTCCTCGTCTAGCATCCCG | GCTTCTGGCTCGTCAGATGA | 145 |
| *Cs*WRKY31 | CAAGTGTTCTTTCGCCCCAA | GATGACGCCGCAGAAACATG | 195 |
| *Cs*WRKY35 | GCCATATACAGCCGCAATGC | GTGGCTCGTCTTTGGCTCTA | 134 |
| *Cs*WRKY49 | GCTCTGCCGGATAATAGCAG | CGAGGGTATAGGGTGCTTGG | 178 |
| *Cs*WRKY2 | GCAGTTCAAAGGGGTGCTTG | CTTCCGGCGAGTGAGTTTCT | 130 |
| *Cs*WRKY11 | CAGAAGCATGTGAAGGGCAG | GCATCCTTGGCACGTTTATT | 173 |
| *Cs*WRKY13 | GCCTCTGACAACTTGGCTTC | CTCGGATGAGGAGATCCTTT | 197 |
| *Cs*WRKY18 | CTGCACTGTTGCACCTTCAT | GATGTTGAGCCGGACAATAG | 177 |
| *Cs*WRKY26 | CAATCAAGGGTTCTCCCCAT | GTGGTTATGTTCGCCTTCGT | 137 |
| *Cs*WRKY29 | CAAGAAGCTGCAACGCAAGG | GAGACGGTGAGATCGGTGAG | 113 |
| *Cs*WRKY30 | GCATCTGGTGAAAGCAATGA | GTGCTGTGACTCCTCCAATA | 131 |
| *Cs*WRKY33 | GCGGAGTCATGGACAGAACA | CTTACTTGCTTGGCAGCCTT | 150 |
| *Cs*WRKY41 | GGCAGCACCCGAAACAAATT | CTTCATCCACTGCGGGAGTT | 154 |
| *Cs*WRKY43 | CATTGCAGGCAAGAACAAGA | GATTAAGGCAGACGGGGAAC | 164 |
| *Cs*WRKY44 | CAACAGCAACACATGGGCAT | CTGCGGCACACCAATCAAAT | 192 |