Title: Identification and expression of the Cucurbita WRKY transcription factors in response to water deficit and salt stress

Abstract: WRKY transcription factors (TFs) have been reported to play important roles in plant responses to various stress conditions. Although several studies on the genomic organization of the WRKY gene family in various species have been reported, the information related to the genus Cucurbita is scarce, and null in the case of Cucurbita pepo. The present study aimed to examine the response of Cucurbita pepo to water deficit and salt stress. Additionally, WRKY gene family has been identified and characterized in this species. Shoot growth was negatively affected by both adverse situations. Similarly, both salt and water stress conditions reduced transpiration and stomatal conductance in C. pepo plants. However, the quantum efficiency of PSII decreased only in those plants exposed to salt stress. The increase in proline concentration recorded in C. pepo plants subjected to salt or drought stress point out the important role of this amino acid for plant tolerance to both stress conditions.
Based on the genome sequence, 95 CmWRKY genes were found and classified into three main groups according to their orthologues in Arabidopsis. Among these, 24 and 14 CmWRKY genes were responsive to water and salt stresses, respectively. Three water stress-responsive genes were up-regulated under the adverse condition. The expression of six CmWRKY genes was induced by NaCl treatment. Therefore, a total of nine up-regulated genes related to both stresses were identified, suggesting their putative involvement in the plant response to water deficit and salt stress.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

- Proline is important for *Cucurbita pepo* tolerance to salt or drought stress.
- Ninety-five WRKY transcription factors were characterized in *C. pepo*.
- Twenty-four *CmWRKY* genes were responsive to water stress and 14 to salt stress.
- Nine up-regulated genes could be involved in plant responses to abiotic stress.

- Proline is important for *Cucurbita pepo* tolerance to salt or drought stress.
- Ninety-five WRKY transcription factors were characterized in *C. pepo*.
- Twenty-four *CmWRKY* genes were responsive to water stress and 14 to salt stress.

Identification and expression of the *Cucurbita* WRKY transcription factors in response to water deficit and salt stress.

Insaf BANKAJI[1], Noomene SLEIMI[1], Vicente VIVES-PERIS[2], Aurelio GÓMEZ-CADENAS[2], Rosa M. PÉREZ-CLEMENTE[2*].

[1] UR: Materials, Nanomaterials and Ecosystems, Faculty of Science of Bizerte, University of Carthage, Tunisia.

[2] Universitat Jaume I, Departamento de Ciencias Agrarias y del Medio Natural. Castellón, Spain.

* Corresponding author:

Rosa M. Pérez-Clemente

Departament de Ciències Agràries i del Medi Natural, Universitat Jaume I, Avda. Sos Baynat s/n, 12071, Castellón de la Plana, Spain

Phone: +34 964 729403 Fax: +34 964 7208216

e-mail: rosa.perez@uji.es

Short title: *Cucurbita pepo* WRKY TFs under abiotic stress.

Key message: Biochemical and physiological *Cucurbita pepo* responses to drought and salt stress have been studied. WRKY TFs were characterized and nine of them were up-regulated in response to these abiotic stresses.

Keywords: Cucurbita pepo, water deficit, salinity, phylogenetic analysis, *WRKY* genes.

ABSTRACT

WRKY transcription factors (TFs) have been reported to play important roles in plant responses to various stress conditions. Although several studies on the genomic organization of the *WRKY* gene family in various species have been reported, the information related to the genus Cucurbita is scarce, and null in the case of *Cucurbita pepo*. The present study aimed to examine the response of *Cucurbita pepo* to water deficit and salt stress. Additionally, *WRKY* gene family has been identified and characterized in this species. Shoot growth was negatively affected by both adverse situations. Similarly, both salt and water stress conditions reduced transpiration and stomatal conductance in *C. pepo* plants. However, the quantum efficiency of PSII decreased only in those plants exposed to salt stress. The increase in proline concentration recorded in *C. pepo* plants subjected to salt or drought stress point out the important role of this amino acid for plant tolerance to both stress conditions.

Based on the genome sequence, 95 *CmWRKY* genes were found and classified into three main groups according to their orthologues in *Arabidopsis*. Among these, 24 and 14 *CmWRKY* genes were responsive to water and salt stresses, respectively. Three water stress-responsive genes were up-regulated under the adverse condition. The expression of six *CmWRKY* genes was induced by NaCl treatment. Therefore, a total of nine up-regulated genes related to both stresses were identified, suggesting their putative involvement in the plant response to water deficit and salt stress.

55

56

57

58

59

60

61

62

63 **INTRODUCTION**

64

65 Plants have developed a wide range of strategies to mitigate the deleterious effect of various

66 biotic and abiotic stresses through physical adaption, consequence of biochemical, cellular,

67 and molecular changes (Chen et al., 2012; Finatto et al., 2018). At the molecular level, the

68 upregulation of stress-tolerance related genes contribute to the plant adaption to unfavorable

69 environmental conditions (Ke et al., 2018). In fact, transcriptomic regulation of gene

70 expression in response to developmental and environment changes, mediated by the DNA-

71 binding transcription factors (TFs), is an important regulatory mechanism in plants (Buscaill

72 and Rivas, 2014; Finatto et al., 2018).

73 WRKY proteins form a large family of transcription factors involved in plant growth

74 and development and in responses to various biotic and abiotic stresses (Wei et al., 2016).

75 They are classified into three groups based on the number of WRKY domains and nature of

76 their zinc-finger motifs. Group I contains two WRKY conserved domains and a classical zinc

77 finger motif. Group II contains single WRKY domain and a classical zinc finger motif and it

78 has been divided into five or more subgroups based on short conserved structural WRKY

79 domains. Group II WRKY TFs, containing WRKYGQK amino acid sequence with zinc

80 finger $CX_{4-5}CX_{22-23}HHX_1H$, is the largest group in most of the plants (Eulgem et al., 2000;

3

Yang et al., 2009). Group III proteins of WRKY superfamily contain a single WRKY domain and a modified zinc finger motif C_2-CH rather than classical C_2-H_2 (Kiranmai et al., 2016).

A large number of *WRKY* genes have been identified in *Arabidopsis thaliana* (Eulgem et al., 2000; Ülker and Somssich, 2004) and also in some crops such as *Oryza sativa* (Wu et al., 2005), *Hordeum vulgare* (Mangelsen et al., 2008), *Cucumis sativus* (Ling et al., 2011), and citrus (Ayadi et al., 2016; Vives-Peris et al., 2018).

During normal growth conditions, WRKY TFs regulate several developmental and physiological processes like leaf senescence, trichome development, and are involved in the regulation of biosynthetic pathways (Johnson et al., 2002), seed dormancy (Ding et al., 2014), embryogenesis (Jimmy and Babu, 2015), seed germination (Raineri et al., 2016) and hormone signaling (Vives-Peris et al., 2018). Moreover, numerous studies have demonstrated that the expression of many *WRKY* genes is highly and rapidly induced or repressed when plants are exposed to certain abiotic stresses, such as wounding, drought or salinity, pointing out that these TFs may have a regulatory function in the signaling pathways of plant response to adverse conditions (Chen et al., 2012).

Drought and salt stress are two major environmental constraints in many arid and semiarid regions (Kiranmai et al., 2016). Salinity affects almost every aspect of the physiology and biochemistry of plants and significantly reduces growth, decreases their photosynthetic capacity as a result of stomatal and/or nonstomatal limitations and has a negative impact on yield (Pilon et al., 2018). Similarly, it has been reported that drought stress negatively affects gas exchange capacity in summer squash plants growing under this adverse condition (Ors et al., 2016). To cope with these adverse culture conditions, many plants respond by overproducing compatible osmolites such as proline, altering endogenous hormonal levels (as it is the case of the absicisic acid accumulation), and promoting or repressing particular gene expression (reviewed in Arbona *et al.,* 2017).

106   *Cucurbita pepo* belongs to the Cucurbitaceae family. The "Zucchini" types rank among

107   the highest-valued vegetables worldwide, and other *C. pepo* and related *Cucurbita spp.*, are

108   food staples and rich sources of fat and vitamins (Paris, 2016).To study the response of *C.*

109   *pepo* to salt and drought stress conditions, different physiological (relative water content,

110   chlorophyll fluorescence and leaf gas exchange) and biochemical (endogenous contents of

111   malondialdehyde, proline and several phytohormones) parameters have been evaluated.

112   Although many *WRKY* genes have been recently identified in different species, the

113   identification and characterization of *WRKY* transcription factors in Cucurbitaceae family has

114   been restricted to watermelon (*Citrullus lanatus*) (Yang et al., 2018). In this study, we

115   performed a genome-wide identification of *WRKY*s in *Cucurbita pepo* and analysed their

116   classification. Moreover, we further investigated the expression profiling of *CuWRKY* genes

117   in response to two different abiotic stress conditions: high salinity and drought. This research

118   will provide insight into the possible involvement of *CmWRKY*s in abiotic stress responses in

119   *Cucurbita pepo*.

120   **MATERIAL AND METHODS**

121   **Plant material and experimental conditions**

122

123   Zucchini squash seeds were germinated in pots containing mixed soil (peat moss, perlite and

124   vermiculite in 80:10:10 ratio) and allowed to grow in a temperature-controlled greenhouse:

125    $25\pm 3.0°C$ and $18 \pm 2.0°C$ (day/night respectively) and natural photoperiod. Relative humidity

126   ranged between 60% and 85%. During this period, plants were watered three times a week

127   with a half-strength Hoagland solution (Arbona and Gómez-Cadenas, 2008). Two-week-old

128   seedlings were subjected to two stress treatments: drought and salinity. Plants were exposed

129   to drought stress by reducing the water dose to a 30% of pot-capacity. For salinity treatment,

130   plants were regularly watered (as controls) but with a nutrient solution supplemented with 90

mM NaCl. After 2 weeks, ten plants per treatment were randomly chosen for measuring shoot fresh weight and shoot length. For further analyses, leaf tissue was sampled, immediately frozen in liquid nitrogen and stored at -80 °C.

Determination of leaf relative water content

Relative water content (RWC) was determined as described in (Mahouachi et al., 2012). Briefly, leaves obtained from three different plants per treatment were collected and weighted in order to obtain fresh weight (FW) and then transferred to tubes with 50 mL of deionized water at 25 ºC. After 24 h, leaves were weighted to obtain turgid weight (TW). Finally, leaves were dried at 72 ºC and reweighed after 48 h, determining dry weight (DW). RWC was calculated according the formula RWC (%) = [(FW − DW) / (TW − DW)] * 100.

Chlorophyll fluorescence and leaf gas exchange measurements

Measurements of chlorophyll fluorescence parameters were performed with an OS 1-FL portable fluorometer (Opti-Sciences, Tyngsboro, MA, USA). Ten replicate plants per treatment were randomly chosen and the quantum yield, [$\Phi_{PSII} = (Fm' - Fs)/Fm'$], was measured in three different leaves after actinic light adaptation.

Fm' is the maximum fluorescence in leaves under regular PAR (actinic radiation) and Fs is the minimum; Φ gives information about the non-cyclic electron transport from PSII to PSI. All terminology and calculations were performed according to (López-Climent et al., 2008).

Leaf gas exchange parameters were measured with an LCpro+ portable infrared gas analyser (ADC Bioscientific Ltd, Hoddesdon, UK) under ambient CO_2 and humidity. Supplemental light was provided by a PAR lamp at 1000 µmol $m^{-2}s^{-1}$ photon flux density and air flow was set at 150 µmol s^{-1}. After instrument stabilization, measurements were taken on three leaves in each of the ten plants randomly chosen per treatment. The rate of transpiration

156 (E; mmol m^{-2} s^{-1}) and the stomatal conductance (gs; mol m^{-2} s^{-1}) were measured (López-

157 Climent et al., 2008).

158

159 **Hormone analyses**

160 Concentration of the phytohormones abscisic acid (ABA), salicylic acid (SA), jasmonic acid

161 (JA) and indole acetic acid (IAA) was determined in leaf tissue by high performance liquid

162 chromatography coupled online to a triple quadrupole mass spectrometer (Micromass,

163 Manchester, UK) through an orthogonal Z-spray electrospray ion source as described in

164 (Durgbanshi et al., 2005), with slight modifications. The extraction was performed in water

165 using 0.2 g of fresh tissue grilled to fine powder in a ball mill (MillMix20, Domel, Železniki,

166 Slovenija). [^{2}H$_6$]-ABA, [^{13}C$_6$]-SA, dihydrojasmonic acid and [^{2}H$_2$]-IAA were added as

167 internal standards. After the extraction, samples were centrifuged, and the supernatant was

168 recovered, pH adjusted to 2.8 with 30% acetic acid. Two liquid-liquid partitions were

169 performed with diethyl ether, recovering the supernatant and evaporating it in a centrifuge

170 concentrator under vacuum conditions (Speed Vac, Jouan, Saint Herblain Cedex, France).

171 Finally, the residue was resuspended in 0.5 mL of water:methanol 90:10 and filtered through

172 0.22 μM PTFE filters. 20 μL of this solution were injected to the HPLC-MS system (Acquity

173 SDS, Waters Corp., Milford, MA, USA).

174 The separation of the analytes was achieved using as a stationary phase a reversed-phase C18

175 column (Gravity, 50 × 2.1mm 1.8-μm particle size, Macherey-Nagel GmbH, Germany), and a

176 methanol:water (both with 0.1% acetic acid) gradient as mobile phase, with a flow rate of 0.3

177 mL min^{-1}. Calibration curves were performed using standards. Results were processed with

178 Masslynx v4.1 software (Waters, Barcelona, Spain).

179 **Proline analyses**

180   To quantify proline content in leaf samples, the methodology described in (Bates et al., 1973)

181   was used with some modifications. For this analysis, 50 mg of fresh material was extracted in

182   5 mL of 3% sulfosalicylic acid by sonication for 30 min. After that, samples were centrifuged

183   and the supernatant was mixed with glacial acetic acid and ninhydrin reagent (0.625 g of

184   ninhydrin in 15 mL of glacial acetic acid and 10 mL of orthotphosphoric acid 6M) in a

185   proportion 1:1:1. Samples were heated in a water bath at 100 ºC for 1h. Finally, samples were

186   centrifuged and the absorbance of the supernatant was measured at 520 nm with a

187   spectrophotometer (Thermo Spectronic Genesys 10, Waltham, MA, USA). A calibration

188   curve was performed using commercial proline as standard (Sigma-Aldrich, Madrid, Spain).

189   **Malondialdehyde analysis**

190   The concentration of malondialdehyde (MDA) was determined as described in (Hodges et al.,

191   1999). Briefly, 200 mg of fresh material was extracted with 80% absolute ethanol by

192   sonication for 30 min. After centrifugation, two aliquots of the supernatant were mixed with

193   20% trichloroacetic acid or a solution of 20% trichloroacetic acid and 0.5% thiobarbituric acid

194   in a 1:1 proportion, respectively. Both mixtures were heated in a water bath for 1h at 90 ºC.

195   Finally, samples were centrifuged and the absorbance was spectrophotometrically measured

196   at 440, 532 and 600 nm. MDA content was quantified as described in (Arbona et al., 2008).

197   **Identification, classification and phylogenetic analysis of *Cucurbita* WRKY TFs**

198   Sequences of *Arabidopsis thaliana WRKY* genes were downloaded from the *Arabidopsis*

199   Information Resource (TAIR, https://www.arabidopsis.org/) (Li et al., 2015) and used to

200   identify the transcript sequences of *Cucurbita maxima* WRKY TFs (Czarnecki et al., 2014)

201   obtained from the *Cucurbit Genomic Database* (http://cucurbitgenomics.org/) using a

202   TBLASTN methods. *C. maxima* genome database was used instead *C. pepo* genome database

203   since the last one was not available.

The alignment of WRKY domains was made using *Clustal Omega* online application (http://www.ebi.ac.uk/Tools/msa/clustalo/). *MEGA6.0* program was employed to construct the phylogenetic tree of identified WRKY protein domains using the neighbor-joining method (Tamura et al., 2013) with 1000 bootstrap value (Nei and Kumar, 2000). To categorize *Cm*WRKY proteins, *At*WRKY domains as query sequences to construct a phylogenetic tree were used. Based on literature (reviewed in Chen *et al.,* 2012), those *At*WRKYs being upregulated or downregulated by abiotic stresses conditions were identified and compared with *Cm*WRKYs.
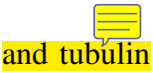
Gene-specific primers for *CmWRKY*s were designed using *Primer3Plus* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) as described in (Vives-Peris et al., 2018). Primer sequences used for the amplification are listed in Supplementary material 1.

RNA isolation, cDNA synthesis, and quantitative PCR analysis

Total RNA was isolated from zucchini leaf tissue with a commercial kit (*Qiagen*, Venlo, The Netherlands) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of 1 μg total RNA using Primescript RT Reagent Kit (*Takara*, Shiga, Japan). After that, cDNA concentration and purity were measured with a Nanodrop 2000 spectrophotometer (*Thermo Scientific*, Waltham, MA, USA), determining absorbance 260/280 and 260/230 nm ratios.

Quantitative real-time PCR was conducted with an ABI Step One detection system (*Applied Biosystems*, Foster City, CA, USA). The amplification was done in a reaction contained 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.

PCR reactions included a pre-incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10s, annealing at 60°C for 10s, and extension at 72°C for 20s. Actin

230 <mark>and tubulin</mark> were used as internal control genes. The relative expression of *CmWRKY* genes

231 was determined as previously described in (Vives-Peris et al., 2018). A hierarchical cluster

232 analysis to facilitate the visualization of RT-qPCR results was performed using the program

233 *MeV4.9.0.*

234 **Statistical analysis**

235 Data mean comparisons were performed with Statistica 8 (Statsoft, France). One-way analysis

236 of variance (ANOVA) was used to compare mean values among the different treatments. The

237 Tukey's HSD test at $p < 0.05$ was followed to assess significant differences.

238

239 **RESULTS**

240 **Effect of stress on plant growth and water status**

241 Both stress culture conditions (high salinity and drought) negatively affected the growth of

242 zucchini plants. In fact, shoot fresh weight was 56% and 65% lower in salt- and water-stressed

243 plants, respectively, when compared to control plants (Fig. 1A). Similarly, these adverse

244 conditions also had a negative impact on shoot length, exhibiting salt and water stressed plants

245 a shoot height 26% and 35% shorter than controls, respectively (Fig. 1B). Leaf RWC was not

246 affected after 2 weeks of treatment, exhibiting stressed plants values similar to those

247 obtained for controls (Fig 1C).

248 **Effect of stress on leaf gas exchange and fluorescence parameters**

249 Both, salt and water stresses reduced E and g_s values in comparison to unstressed plants

250 (Fig. 2A and 2B). The decrease in E was 63% in salt-stressed plants and 65% in plants

251 subjected to water stress. Similarly, gs was negatively affected, showing leaves from stressed

252 plants values of g_s 72% and 74% lower than controls in salt and drought stress treatments,

253 respectively.

254   Both treatments affected differently the quantum efficiency of PSII. Whereas salt stress

255   caused a decrease in Φ_{PSII} of 32% compared to control, no significant changes were recorded in

256   water stressed plants (Fig. 2C).

257   **Hormonal responses**

258   Phytohormone concentration in leaf tissue was differently altered depending on the adverse

259   condition applied and the considered hormone (Fig. 3). ABA content increased in water-

260   stressed plants, reaching values 5.7-fold higher than in controls whereas salt stress did not alter

261   the endogenous level of this hormone (Fig. 3A). Contrarily, SA concentration was not altered

262   by water stress but it was highly increased by salt treatment, reaching values 10.8–fold higher

263   in stressed plants (Fig. 3B). No differences were observed in JA and IAA endogenous content

264   in stressed plants related to controls (Fig. 3C-D).

265   **Proline and MDA contents**

266   As it is shown in figure 4A, the endogenous proline content increased in leaf tissue of stressed

267   plants, regardless of the applied stress, exhibiting values 3.16 and 2.97 times higher than

268   control in water and salt-stressed plants, respectively.

269   Leaf MDA concentration only varied in leaves from water-stressed plants, reaching values

270   2.07 times higher than controls, whereas no significant differences were observed in the

271   content of this metabolite in salt-stressed plants (Fig. 4B).

272   **Identification of WRKY genes, sequence alignment, structure and phylogenetic analysis**

273   For the identification of the WRKY gene family in zucchini genome, sequences of

274   *Arabidopsis thaliana WRKY* genes were used to identify the transcript sequences of *Cucurbita*

275   *maxima* WRKY TFs. After searching for WRKY domains and eliminating repeats, a total of

276   95 genes, named *CmWRKY1* to *CmWRKY95* were identified from the amino acid sequences

downloaded from Cucurbit Genomic Database and classified according to the chromosome they are located (Table 1).

Sequence comparisons and structural analyses showed that the WRKY domains could be classified into three large groups (I, II and III), finding 18 members belong to group I, 5 belong to group IIa, 9 belong to group IIb, 25 belong to group IIc, 13 belong to group IId, 13 belong to group IIe, and 12 belong to group III (Fig. 5).

It is worth noting that *CmWRKY*s classified in the group I have two separate WRKY domains, the N- and C-terminal domains (Fig. 5). Furthermore, the structure of the *Cm*WRKY domain clearly indicated that group II can be divided into five distinct subgroups (IIa, IIb, IIc, IId, and IIe).

In our study, *Cm*WRKY proteins contained the highly conserved sequence WRKYGQK. Except in the case of *CmWRKY17*, *CmWRKY30* and *CmWRKY62* where the atypical sequence (WRKYGKK) was identified (Fig. 5).

To examine the phylogenetic relationships of the *Cm*WRKY proteins, an unrooted phylogenetic tree was drawn with MEGA6.0 (Fig. 6). A comparison with the WRKY domains of several different *At*WRKY proteins resulted in a better separation of the different groups and subgroups.

Expression profile of *CmWRKY* genes under abiotic stress conditions

Expression profiles varied depending on the particular WRKY TF and treatment. The transcript levels of *CmWRKY43*, *CmWRKY82* and *CmWRKY90* increased after the drought stress treatment (Fig. 7). The largest difference was observed in the expression of *CmWRKY82* which was 5 times higher than that of control. On the contrary, water stress repressed the expression of the *WRKY* genes: *CmWRKY2*, *CmWRKY3 CmWRKY5*, *CmWRKY7*, *CmWRKY11*, *CmWRKY15*, *CmWRKY21*, *CmWRKY26*, *CmWRKY27*, *CmWRKY29*, *CmWRKY32*, *CmWRKY34*, *CmWRKY36*, *CmWRKY41*, *CmWRKY61*, *CmWRKY63*, *CmWRKY66*,

302 *CmWRKY67*, *CmWRKY70*, *CmWRKY72*, *CmWRKY86* and *CmWRKY88*; while *CmWRKY13*

303 and *CmWRKY15* did not show significant changes in expression in response to drought stress

304 compared with the control.

305 *CmWRKY11*, *CmWRKY15*, *CmWRKY21*, *CmWRKY26*, *CmWRKY34* and *CmWRKY66* were up-

306 regulated by salt stress. In this treatment, highest differences in gene expressions were shown

307 in *CmWRKY26* and *CmWRKY66*. Reaching values of expression 8 and 9 times higher than in

308 control, respectively

309 Among the studied genes, *CmWRKY2*, *CmWRKY5*, *CmWRKY7*, *CmWRKY27*, *CmWRKY29*,

310 *CmWRKY32*, *CmWRKY36*, and *CmWRKY41* were down-regulated after salt stress (Fig.7,

311 reaching in *CmWRKY7*, expression levels 92.5% lower than in the control. However,

312 *CmWRKY3*, *CmWRKY13*, *CmWRKY43*, *CmWRKY61*, *CmWRKY63*, *CmWRKY67*,

313 *CmWRKY70, CmWRKY72, CmWRKY82*, *CmWRKY86*, *CmWRKY88* and *CmWRKY90* did not

314 alter their expression levels respect to the control (Fig.7).

315 To analyse the general trend of WRKYs TFs expression under both stress conditions a

316 Venn diagram is represented (Fig. 8A). Results revealed that drought stress had a high impact

317 on *Cm*WRKY TFs regulation, altering the expression of 24 of 26 studied genes, being the

318 relative expression of 13 of them also affected in plants subjected to salt stress. *CmWRKY15*

319 was the unique TF exhibiting an altered expression pattern exclusively in salt stressed plants.

320 To facilitate the visualization, a heat map compiling all the results described above was made

321 (Fig. 8B).

322 **DISCUSSION**

323 The adverse impact of abiotic stress conditions on crops causes growth reduction, as

324 expressed by dry biomass production and fruit yield. Many studies have demonstrated in

325 plants cultured under saline conditions that osmotic, toxic, and nutritional factors are, in the

326 short-medium time, associated with reductions in plant performance (Neocleous and Savvas,

327   2017). Our results show that salinity negatively affected zucchini plant growth, in terms of

328   shoot weight and length, which is in agreement with other studies (Balkaya et al., 2016).

329   Similarly, (Khan et al., 2013) revealed that salinity adversely affected *Cucumis sativus* plants,

330   reducing stem length and number of leaves per plant. In addition, it has been previously

331   reported that shoot dry weight and leaf area in *Cucurbita pepo* decreased with water scarcity

332   (Sure et al., 2011). Our results show a significant decrease in vegetative growth being the

333   reduction of 65 % in weight and 35% in shoot length after 2 weeks of treatment.

334   RWC is a key indicator of the degree of cell and tissue hydration, which is critical for

335   all physiological processes. Under stress culture conditions, cell membranes can suffer

336   changes such as penetrability affecting RWC. It has been reported that varieties resistant to a

337   particular stress maintain higher RWC than susceptible ones (Sikuku et al., 2012). In the

338   present study, drought and salt stress did not reduce *Cucurbita pepo* leaf RWC, contrarily to

339   other physiological and biochemical parameters that were negatively affected by both abiotic

340   stresses. Probably more severe stress conditions or longer periods of exposure to adverse

341   growing conditions are required to detect significant alteration in RWC as a consequence of

342   the applied stresses.

343   Leaf gas exchange parameters have been often associated with plant biomass

344   accumulation and yield performance in cultivated plants (Ashraf and Harris, 2013) and the

345   growth inhibition observed in many plants subjected to salinity is often a result of decreased

346   photosynthetic capacity (Rouphael et al., 2012). Results show that both, salt and water stresses

347   caused reduction in E and g_s parameters in *Cucurbita pepo*. These results agreed with

348   (Hniličková et al., 2017) who reported that E and g_s decreased under salt stress conditions in

349   *Eruca sativa.* Similarly, values recorded for both parameters also decreased in three varieties

350   of chickpea exposed to drought stress (Mafakheri et al., 2010). The quantum efficiency of

351   PSII decreased only in plants exposed to salt stress. High salinity induces a reduction in

352   chlorophyll content, affects photosynthetic electron transport and inhibits PSII activity as a

consequence of the accumulation of salts in chloroplasts, inducing a decrease in Φ_{PSII} and an

increase in non-photochemical quenching, as it has been recorded in many species,

including barley, tobacco and even among certain halophytes, such as *Sarcocornia fruticosa*

(reviewed in Kalaji *et al.,* 2016). Our results indicate that photosynthetic ability was reduced

in *C. pepo* plants subjected to drought as E and g_s parameters were significantly lower than

in control. However, this stomatal impairment is not correlated with biochemical damage in

the photosystem II as Φ_{PSII} remained unaltered after two weeks of drought stress. It has been

previously reported (Santaniello et al., 2017) in Arabidopsis plants that the efficiency of the

photochemical apparatus is strongly limited by mild drought stress but was maintained

under more severe stress conditions, probably as consequence of metabolic adjustments, not critical PMP point

including proline accumulation. This osmolyte could play a vital role stabilizing many

functional units such as the complex II electron transport.

It has been reported in a wide range of plants that the content of proline increases under

abiotic stress conditions, contributing to the osmotic adjustment and being an indicator of

stress tolerance (Arbona et al., 2017). However, in *C. pepo* plants, the information related to

this aspect is restricted to biotic stress situations, where proline level increases after yellow

mosaic virus infection (Radwan et al., 2007). The increase in proline concentrations in

response to both abiotic stresses reported in here, confirms the role of this amino acid as a key

element for plant tolerance to salt and water stress conditions in *C. pepo*. In addition, MDA,

considered a stress marker since it is a by-product of cell membrane lipid peroxidation

induced by reactive oxygen species (Ayala et al., 2014), exhibited a significant increase in *C.*

pepo plants subjected to water stress whereas in plants cultured under high salinity MDA

levels were similar to those recorded in control plants. Similar findings have been described

in Carrizo citrange (a citrus rootstock) that responded to salt-induced oxidative stress

increasing enzymatic and non-enzymatic antioxidant defenses (Arbona et al., 2003) being this

improved oxidative stress response a mechanism to cope with this abiotic stress condition in

379   several crops. In this context, it can be argued that *C. pepo* plants were capable of inactivate

380   reactive oxygen species under salt stress conditions by the action of enzymatic and non-

381   enzymatic antioxidant compounds, avoiding oxidative damage (measured as MDA

382   accumulation).

383   Several works have previously remarked the importance of the crosstalk among

384   phytohormones and WRKY TFs to activate plant defense mechanisms against abiotic stress

385   conditions (Luo et al., 2017). In *C. pepo*, (Liu et al., 2016) reported that ABA accumulation

386   under water deficit improves plant tolerance, decreasing stomatal aperture and transpiration.

387   Therefore, the increase of ABA concentration observed in plants subjected to drought could

388   suppose a plant strategy to tolerate water deprivation, as it is correlated to the decrease of

389   transpiration and stomatal conductance, and quantum yield maintenance.

390   The exogenous application of SA has been also reported as a salt stress mitigator,

391   promoting photosynthesis and the biosynthesis of antioxidant enzymes (Ma et al., 2017). In

392   addition, exogenous SA treatment in *C. pepo* plants under control conditions is beneficial for

393   this crop, promoting nutrient uptake and increasing its productivity (AL-Rubaye and Atia,

394   2016). Consequently, the higher SA levels in salt-stressed plants observed in this work, could

395   also induce salt stress tolerance in *C. pepo* plants.

396   Previous studies conducted to get knowledge on the interaction of WRKY TFs with

397   phytohormones under abiotic stress conditions have reported that *At*WRKY33 expression is

398   induced by salt stress, but not by drought stress or ABA treatment (Jiang and Deyholos,

399   2009). Similar results were found in this work, the homologous *Cm*WRKY11 and

400   *Cm*WRKY66 TFs were overexpressed in salt stressed plants, but its expression remained in

401   control levels under drought conditions.

402   In the present study, WRKY superfamily of TFs has been identified and characterized

403   in *C. maxima* since *C. pepo* genome was not available. Gene expression of *CmWRKY*s was

404   studied in leaves of *C. pepo* under optimal growth conditions and in response to two different

405   abiotic stresses: drought and high salinity. Genes of this superfamily play critical roles in the

406   adaptation of plants to various abiotic stresses (Wei et al., 2016), and it has been reported that

407   several WRKY proteins are involved in plant drought and salinity stress responses (Golldack

408   et al., 2011).

409   Ninety-five *Cm*WRKYs were classified according to the chromosome they belong. In

410   our study, besides the highly conserved WRKYGQK motifs, we found atypical sequence

411   (WRKYGKK) in *CmWRKY17*, *CmWRKY30* and *CmWRKY62* (Fig. 5). (Vives-Peris et al.,

412   2018)observed the same results in the case of *Cs*WRKY21 and *Cs*WRKY47 in *Citrus*. In

413   addition, three variants were observed in *Triticum aestivum* WRKYs, namely WRKYGKK,

414   WRKYGEK, and WSKYGQK besides the highly conserved WRKYGQK motifs (Ning et al.,

415   2017). According to (Yang et al., 2009), WRKY TFs that do not contain the canonical

416   WRKYGQK motif, a binding sequence other than the W-box element ((C/T)TGAC(C/T))

417   exist.

418   In the present ~~work~~, some *CmWRKY* genes were up-regulated by drought and NaCl

419   treatments. In contrast, other *CmWRKY* genes were down-regulated. During water stress,

420   three *CmWRKY* genes were up-regulated in *C. pepo*. This is the case of *CmWRKY43*,

421   *CmWRKY82* and *CmWRKY90*. Whereas, six *CmWRKY* genes were up-regulated in *C. pepo*

422   plants exposed to salt stress. Indeed, *CmWRKY11*, *CmWRKY15*, *CmWRKY21*, *CmWRKY26*,

423   *CmWRKY34* and *CmWRKY66* were over-expressed in response to salinity. Numerous studies

424   have demonstrated that many *WRKY* genes are strongly and rapidly up-regulated a response to

425   certain abiotic stresses indicating their regulatory function in these signaling pathways (Chen

426   et al., 2012). In *Cucumis sativus* 23 *WRKY* genes were differentially expressed in response to

427   abiotic stresses (cold, drought or salinity) (Ling et al., 2011). In *Fragaria vesca*, 11 *FvWRKY*

428   genes responded dramatically to various stimuli at the transcriptional level, indicating

429   versatile roles in responses to abiotic stresses (Wei et al., 2016). A relatively large group of

genes were significantly up-regulated in *Triticum aestivum* under water-deficit condition (Ning et al., 2017). Twenty-five *CsWRKY* genes showed differential expression in response to drought, NaCl, and Cd stress in *Cannabis sativa* (Xin et al., 2016). In rice, under abiotic stresses (cold, drought and salinity) or various phytohormone treatments, 54 *WRKY* genes showed significant differences in their transcript abundance (Ramamoorthy et al., 2008). In wheat, 8 of 15 *WRKY* genes were also responsive to NaCl or polyethylene glycol treatment (Wu et al., 2008). A *WRKY* gene, *HvWRKY38*, is expressed in response to cold and drought stress response in barley (Marè et al., 2004) while in soybean at least nine *WRKY* genes are found to be differentially expressed under abiotic stress (Zhou et al., 2008). Over-expression of either *AtWRKY25* or *AtWRKY33* increases salt tolerance in *Arabidopsis* (Jiang and Deyholos, 2009). Similarly, over-expression of *GmWRKY54* from *Glycine max* in transgenic lines enhanced the salt and drought tolerance, possibly through the regulation of transcription factor gene, salt tolerance Zn finger (STZ/Zat10), and DREB2A (Banerjee and Roychoudhury, 2015).

These data provide evidence that different WRKY proteins play differential roles in specific abiotic stress responses. The rapid up-regulation of WRKY genes assure the successful transduction of the signals to activate adaptive responses and regulation of stress-related genes, and finally result in plant stress tolerance (Chen et al., 2012).

In this work, the responses of zucchini squash plants to salt or drought stress conditions were studied. Both adverse conditions negatively affected plant growth (in terms of fresh weight and shoot length). Although there was a reduction in plant growth under stress conditions, some other parameters were not negatively affected and it can be considered that plants could tolerate the imposed conditions. To this relative tolerance contributed the decrease of E and gs, probably induced by the concomitant increase of ABA, the maintenance of the ΦPSII, which accounts for a photosynthetic system relatively tolerant to adverse

455 conditions. This relative tolerance could be related to the accumulation of proline and the

456 efficiency of the antioxidant system that prevents the accumulation of reactive species and

457 oxidative damage, as evidenced by the absence of MDA accumulation under salt stress

458 conditions. Ninety-five *WRKY* genes were identified in this species and expression of some of

459 these genes in response to water deficit and salt stress conditions analysed. Twenty-five

460 *CmWRKY* genes were associated to abiotic stresses and nine *CmWRKY* genes were up-

461 regulated in response to both abiotic stresses. The gene expression profiles obtained revealed

462 that *CmWRKY*s are involved in zucchini responses to both stresses. These results provide a

463 platform for further investigations on the function of the *WRKY* gene family and improvement

464 of tolerance to abiotic stress.

465

466 **ACKNOWLEDGEMENTS**

469
470

REFERENCES

AL-Rubaye, B.C.H., Atia, E.A., 2016. The Influence of foliar sprays on the growth and yield of summer squash. Int. J. Sci. Eng. Res. 7, 664–669.

Arbona, V., Flors, V., Jacas, J., García-Agustín, P., Gómez-Cadenas, A., 2003. Enzymatic and non-enzymatic antioxidant responses of Carrizo citrange, a salt-sensitive citrus rootstock, to different levels of salinity. Plant Cell Physiol. 44, 388–394. https://doi.org/10.1093/pcp/pcg059

Arbona, V., Gómez-Cadenas, A., 2008. Hormonal modulation of citrus responses to flooding. J. Plant Growth Regul. 27, 241–250. https://doi.org/10.1007/s00344-008-9051-x

Arbona, V., Hossain, Z., López-Climent, M.F., Pérez-Clemente, R.M., Gómez-Cadenas, A., 2008. Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. Physiol. Plant. 132, 452–466. https://doi.org/10.1111/j.1399-3054.2007.01029.x

Arbona, V., Manzi, M., Zandalinas, S.I., Vives-Peris, V., Pérez-Clemente, R.M., Gómez-Cadenas, A., 2017. Physiological, metabolic, and molecular responses of plants to abiotic stress, in: Stress Signaling in Plants: Genomics and Proteomics Perspective, Volume 2. pp. 1–35. https://doi.org/10.1007/978-3-319-42183-4_1

Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: An overview. Photosynthetica 51, 163–190. https://doi.org/10.1007/s11099-013-0021-6

Ayadi, M., Hanana, M., Kharrat, N., Merchaoui, H., Marzoug, R. Ben, Lauvergeat, V., Rebaï, A., Mzid, R., 2016. The WRKY transcription factor family in *Citrus*: valuable and useful candidate genes for citrus breeding. Appl. Biochem. Biotechnol. 180, 516–543. https://doi.org/10.1007/s12010-016-2114-8

Ayala, A., Muñoz, M.F., Argüelles, S., 2014. Lipid peroxidation: production, metabolism, and

494   signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid. Med. Cell.

495   Longev. 2014, 360438. https://doi.org/10.1155/2014/360438

496 Balkaya, A., Yildiz, S., Horuz, A., Doğru, S.M., 2016. Effects of salt stress on vegetative

497   growth parameters and ion accumulations in cucurbit rootstock genotypes. J. Crop

498   Breed. Genet. 2, 11–24.

499 Banerjee, A., Roychoudhury, A., 2015. WRKY proteins: Signaling and regulation of

500   expression during abiotic stress responses. Sci. World J. 2015, 807560.

501   https://doi.org/10.1155/2015/807560

502 Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-

503   stress studies. Plant Soil 39, 205–207. https://doi.org/10.1007/BF00018060

504 Buscaill, P., Rivas, S., 2014. Transcriptional control of plant defence responses. Curr. Opin.

505   Plant Biol. 20, 35–46. https://doi.org/10.1016/j.pbi.2014.04.004

506 Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., Yu, D., 2012. The role of WRKY transcription

507   factors in plant abiotic stresses. Biochim. Biophys. Acta 1819, 120–128.

508   https://doi.org/10.1016/j.bbagrm.2011.09.002

509 Czarnecki, O., Yang, J., Wang, X., Wang, S., Muchero, W., Tuskan, G. a, Chen, J.-G., 2014.

510   Characterization of *MORE AXILLARY GROWTH* genes in *Populus*. PLoS One 9,

511   e102757. https://doi.org/10.1371/journal.pone.0102757

512 Ding, Z.J., Yan, J.Y., Li, G.X., Wu, Z.C., Zhang, S.Q., Zheng, S.J., 2014. WRKY41 controls

513   Arabidopsis seed dormancy via direct regulation of *ABI3* transcript levels not

514   downstream of ABA. Plant J. 79, 810–823. https://doi.org/10.1111/tpj.12597

515 Durgbanshi, A., Arbona, V., Pozo, O., Miersch, O., Sancho, J. V., Gómez-Cadenas, A., 2005.

516   Simultaneous determination of multiple phytohormones in plant extracts by liquid

517   chromatography-electrospray tandem mass spectrometry. J. Agric. Food Chem. 53,

518   8437–8442. https://doi.org/10.1021/jf050884b

519 Eulgem, T., Rushton, P.J., Robatzek, S., Somssich, I.E., 2000. The WRKY superfamily of

520   plant transcription factors. Trends Plant Sci. 5, 199–206.

521 Finatto, T., Viana, V.E., Woyann, L.G., Busanello, C., da Maia, L.C., de Oliveira, A.C., 2018.

522   Can WRKY transcription factors help plants to overcome environmental challenges?

523   Genet. Mol. Biol. 41, 533–544. https://doi.org/10.1590/1678-4685-gmb-2017-0232

524 Golldack, D., Lüking, I., Yang, O., 2011. Plant tolerance to drought and salinity: Stress

525   regulating transcription factors and their functional significance in the cellular

526   transcriptional network. Plant Cell Rep. 30, 1383–1391. https://doi.org/10.1007/s00299-

527   011-1068-0

528 Hniličková, H., Hnilička, F., Martinková, J., Kraus, K., 2017. Effects of salt stress on water

529   status, photosynthesis and chlorophyll fluorescence of rocket. Plant, Soil Environ. 63,

530   362–367. https://doi.org/10.17221/398/2017-PSE

531 Hodges, D.M., Delong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric

532   acid-reactive-substances assay for estimating lipid peroxidation in plant tissues

533   containing anthocyanin and other interfering compounds. Planta 207, 604–611.

534 Jiang, Y., Deyholos, M.K., 2009. Functional characterization of Arabidopsis NaCl-inducible

535   *WRKY25* and *WRKY33* transcription factors in abiotic stresses. Plant Mol. Biol. 69, 91–

536   105. https://doi.org/10.1007/s11103-008-9408-3

537 Jimmy, J.L., Babu, S., 2015. Role of *Os*WRKY transcription factors in rice disease resistance.

538   Trop. Plant Pathol. 40, 355–361. https://doi.org/10.1007/s40858-015-0058-0

539 Johnson, C.S., Kolevski, B., Smyth, D.R., 2002. *TRANSPARENT TESTA GLABRA2*, a

540   trichome and seed coat development gene of Arabidopsis, encodes a WRKY

541   transcription factor. Plant Cell 14, 1359–1375. https://doi.org/10.1105/tpc.001404

542   Kalaji, H.M., Jajoo, A., Oukarroum, A., Brestic, M., Zivcak, M., Samborska, I.A., Cetner,

543   M.D., Łukasik, I., Goltsev, V., Ladle, R.J., 2016. Chlorophyll a fluorescence as a tool to

544   monitor physiological status of plants under abiotic stress conditions. Acta Physiol.

545   Plant. 38, 102. https://doi.org/10.1007/s11738-016-2113-y

546   Ke, Q., Park, S.C., Ji, C.Y., Kim, H.S., Wang, Z., Wang, S., Li, H., Xu, B., Deng, X., Kwak,

547   S.S., 2018. Stress-induced expression of the sweetpotato gene *IbLEA14* in poplar confers

548   enhanced tolerance to multiple abiotic stresses. Environ. Exp. Bot. 156, 261–270.

549   https://doi.org/10.1016/j.envexpbot.2018.09.014

550   Khan, M.M., Al-Mas'oudi, R.S.M., Al-Said, F., Khan, I., 2013. Salinity effects on growth,

551   electrolyte leakage, chlorophyll content and lipid peroxidation in cucumber (*Cucumis*

552   *sativus* L.), in: 2013 International Conference on Food and Agricultural Sciences. pp.

553   28–32. https://doi.org/10.7763/IPCBEE.2013.V55.6

554   Kiranmai, K., Gunupuru, L.R., Nareshkumar, A., Reddy, V.A., Lokesh, U., Pandurangaiah,

555   M., Venkatesh, B., Kirankumar, T.V., Sudhakar, C., 2016. Expression analysis of

556   WRKY transcription factor genes in response to abiotic stresses in horsegram

557   (*Macrotyloma uniflorum* (Lam.) Verdc.). Am. J. Mol. Biol. 06, 125–137.

558   https://doi.org/10.4236/ajmb.2016.64013

559   Li, C., Li, D., Shao, F., Lu, S., 2015. Molecular cloning and expression analysis of WRKY

560   transcription factor genes in *Salvia miltiorrhiza*. BMC Genomics 16, 200.

561   https://doi.org/10.1186/s12864-015-1411-x

562   Ling, J., Jiang, W., Zhang, Y., Yu, H., Mao, Z., Gu, X., Huang, S., Xie, B., 2011. Genome-

563   wide analysis of WRKY gene family in *Cucumis sativus*. BMC Genomics 12, 471.

564   https://doi.org/10.1186/1471-2164-12-471

565   Liu, S., Li, H., Lv, X., Ahammed, G.J., Xia, X., Zhou, J., Shi, K., Asami, T., Yu, J., Zhou, Y.,

566   2016. Grafting cucumber onto luffa improves drought tolerance by increasing ABA

567   biosynthesis and sensitivity. Sci. Rep. 6, 20212. https://doi.org/10.1038/srep20212

568   López-Climent, M.F., Arbona, V., Pérez-Clemente, R.M., Gómez-Cadenas, A., 2008.

569   Relationship between salt tolerance and photosynthetic machinery performance in citrus.

570   Environ. Exp. Bot. 62, 176–184. https://doi.org/10.1016/j.envexpbot.2007.08.002

571   Luo, D.L., Ba, L.J., Shan, W., Kuang, J.F., Lu, W.J., Chen, J.Y., 2017. Involvement of

572   WRKY transcription factors in abscisic-acid-induced cold tolerance of banana fruit. J.

573   Agric. Food Chem. 65, 3627–3635. https://doi.org/10.1021/acs.jafc.7b00915

574   Ma, X., Zheng, J., Zhang, X., Hu, Q., Qian, R., 2017. Salicylic acid alleviates the adverse

575   effects of salt stress on *Dianthus superbus* (Caryophyllaceae) by activating

576   photosynthesis, protecting morphological structure, and enhancing the antioxidant

577   system. Front. Plant Sci. 8, 600. https://doi.org/10.3389/fpls.2017.00600

578   Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P.C., Sohrabi, E., 2010. Effect of

579   drought stress on yield, proline and chlorophyll contents in three chickpea cultivars.

580   Aust. J. Crop Sci. 4, 580–585.

581   Mahouachi, J., Argamasilla, R., Gómez-Cadenas, A., 2012. Influence of exogenous glycine

582   betaine and abscisic acid on papaya in responses to water-deficit stress. J. Plant Growth

583   Regul. 31, 1–10. https://doi.org/10.1007/s00344-011-9214-z

584   Mangelsen, E., Kilian, J., Berendzen, K.W., Kolukisaoglu, Ü.H., Harter, K., Jansson, C.,

585   Wanke, D., 2008. Phylogenetic and comparative gene expression analysis of barley

586   (*Hordeum vulgare*) WRKY transcription factor family reveals putatively retained

24

587   functions between monocots and dicots. BMC Genomics 9, 194.

588   https://doi.org/10.1186/1471-2164-9-194

589   Marè, C., Mazzucotelli, E., Crosatti, C., Francia, E., Stanca, A.M., Cattivelli, L., 2004. Hv-

590   WRKY38: A new transcription factor involved in cold- and drought-response in barley.

591   Plant Mol. Biol. 55, 399–416. https://doi.org/10.1007/s11103-004-0906-7

592   Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press,

593   New York.

594   Neocleous, D., Savvas, D., 2017. Simulating NaCl accumulation in a closed hydroponic crop

595   of zucchini: Impact on macronutrient uptake, growth, yield, and photosynthesis. Jounal

596   Plant Nutr. Soil Sicience 180, 283–293. https://doi.org/10.1002/jpln.201600338

597   Ning, P., Liu, C., Kang, J., Lv, J., 2017. Genome-wide analysis of WRKY transcription

598   factors in wheat (*Triticum aestivum* L.) and differential expression under water deficit

599   condition. PeerJ 5, e3232. https://doi.org/10.7717/peerj.3232

600   Ors, S., Ekinci, M., Yildirim, E., Sahin, U., 2016. Changes in gas exchange capacity and

601   selected physiological properties of squash seedlings (*Cucurbita pepo* L.) under well-

602   watered and drought stress conditions. Arch. Agron. Soil Sci. 62, 1700–1710.

603   https://doi.org/10.1080/03650340.2016.1168517

604   Paris, H.S., 2016. Germplasm enhancement of *Cucurbita pepo* (pumpkin, squash, gourd:

605   Cucurbitaceae): progress and challenges. Euphytica 208, 415–438.

606   https://doi.org/10.1007/s10681-015-1605-y

607   Pilon, C., Snider, J.L., Sobolev, V., Chastain, D.R., Sorensen, R.B., Meeks, C.D., Massa,

608   A.N., Walk, T., Singh, B., Earl, H.J., 2018. Assessing stomatal and non-stomatal

609   limitations to carbon assimilation under progressive drought in peanut (*Arachis*

610   *hypogaea* L.). J. Plant Physiol. 231, 124–134.

611   https://doi.org/10.1016/j.jplph.2018.09.007

612 Radwan, D.E.M., Fayez, K.A., Mahmoud, S.Y., Hamad, A., Lu, G., 2007. Physiological and

613   metabolic changes of *Cucurbita pepo* leaves in response to zucchini yellow mosaic virus

614   (ZYMV) infection and salicylic acid treatments. Plant Physiol. Biochem. 45, 480–489.

615   https://doi.org/10.1016/j.plaphy.2007.03.002

616 Raineri, J., Hartman, M.D., Chan, R.L., Iglesias, A.A., Ribichich, K.F., 2016. A sunflower

617   WRKY transcription factor stimulates the mobilization of seed-stored reserves during

618   germination and post-germination growth. Plant Cell Rep. 35, 1875–1890.

619   https://doi.org/10.1007/s00299-016-2002-2

620 Ramamoorthy, R., Jiang, S.Y., Kumar, N., Venkatesh, P.N., Ramachandran, S., 2008. A

621   comprehensive transcriptional profiling of the WRKY gene family in rice under various

622   abiotic and phytohormone treatments. Plant Cell Physiol. 49, 865–879.

623   https://doi.org/10.1093/pcp/pcn061

624 Rouphael, Y., Cardarelli, M., Lucini, L., Rea, E., Colla, G., 2012. Improving melon and

625   cucumber photosynthetic activity, mineral composition, and growth performance under

626   salinity stress by grafting onto *Cucurbita* hybrid rootstocks. Photosynthetica 50, 180–

627   188. https://doi.org/10.1007/s11099-012-0002-1

628 Santaniello, A., Scartazza, A., Gresta, F., Loreti, E., Biasone, A., Di Tommaso, D., Piaggesi,

629   A., Perata, P., 2017. *Ascophyllum nodosum* seaweed extract alleviates drought stress in

630   arabidopsis by affecting photosynthetic performance and related gene expression. Front.

631   Plant Sci. 8, 1362. https://doi.org/10.3389/fpls.2017.01362

632 Sikuku, P.A., Onyango, J.C., Netondo, G.W., 2012. Physiological and biochemical responses

633   of five nerica rice varieties (*Oryza sativa* L.) to water deficit at vegetative and

634   reproductive stage. Agric. Biol. J. North Am. 3, 93–104.

635   https://doi.org/10.5251/abjna.2012.3.3.93.104

636   Sure, S., Arooie, H., Moghadam, R.D., 2011. Influence of drought stress and its interaction

637   with salicylic acid on medicinal pumpkin (*Cucurbita pepo* L.) seedling growth. Bot. Res.

638   J. 4, 35–40.

639   Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular

640   Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30, 2725–2729.

641   https://doi.org/10.1093/molbev/mst197

642   Ülker, B., Somssich, I.E., 2004. WRKY transcription factors: From DNA binding towards

643   biological function. Curr. Opin. Plant Biol. 7, 491–498.

644   https://doi.org/10.1016/j.pbi.2004.07.012

645   Vives-Peris, V., Marmaneu, D., Gómez-Cadenas, A., Pérez-Clemente, R.M., 2018.

646   Characterization of *Citrus* WRKY transcription factors and their responses to

647   phytohormones and abiotic stresses. Biol. Plant. 62, 33–44.

648   https://doi.org/10.1007/s10535-017-0737-4

649   Wei, W., Hu, Y., Han, Y.T., Zhang, K., Zhao, F.L., Feng, J.Y., 2016. The WRKY

650   transcription factors in the diploid woodland strawberry Fragaria vesca: Identification

651   and expression analysis under biotic and abiotic stresses. Plant Physiol. Biochem. 105,

652   129–144. https://doi.org/10.1016/j.plaphy.2016.04.014

653   Wu, H., Ni, Z., Yao, Y., Guo, G., Sun, Q., 2008. Cloning and expression profiles of 15 genes

654   encoding WRKY transcription factor in wheat (*Triticum aestivem* L.). Prog. Nat. Sci. 18,

655   697–705. https://doi.org/10.1016/j.pnsc.2007.12.006

656   Wu, K.L., Guo, Z.J., Wang, H.H., Li, J., 2005. The WRKY Family of transcription factors in

657   rice and their origins. DNA Res. 12, 9–26.

658   Xin, P.F., Gao, C.S., Cheng, C.H., Tang, Q., Dong, Z.X., Zhao, L.N., Zang, G.G., 2016.

659   Identification and characterization of hemp WRKY transcription factors in response to

660   abiotic stresses. Biol. Plant. 60, 489–495. https://doi.org/10.1007/s10535-016-0621-7

661   Yang, B., Jiang, Y., Rahman, M.H., Deyholos, M.K., Kav, N.N. V, 2009. Identification and

662   expression analysis of WRKY transcription factor genes in canola (*Brassica napus* L.) in

663   response to fungal pathogens and hormone treatments. BMC Plant Biol. 9, 68.

664   https://doi.org/10.1186/1471-2229-9-68

665   Yang, X., Li, H., Yang, Y., Wang, Y., Mo, Y., Zhang, R., Zhang, Y., Ma, J., Wei, C., Zhang,

666   X., 2018. Identification and expression analyses of WRKYgenes reveal their

667   involvement in growth and abiotic stress response in watermelon (*Citrullus lanatus*).

668   PLoS One 13, e0191308. https://doi.org/10.1371/journal.pone.0191308

669   Zhou, Q.Y., Tian, A.G., Zou, H.F., Xie, Z.M., Lei, G., Huang, J., Wang, C.M., Wang, H.W.,

670   Zhang, J.S., Chen, S.Y., 2008. Soybean WRKY-type transcription factor genes,

671   *GmWRKY13*, *GmWRKY21*, and *GmWRKY54*, confer differential tolerance to abiotic

672   stresses in transgenic *Arabidopsis* plants. Plant Biotechnol. J. 6, 486–503.

673   https://doi.org/10.1111/j.1467-7652.2008.00336.x

674

675

677　　　**Figure captions**

678　**Figure 1.** Shoot fresh weight (A), shoot length (B) and RWC (C) in *Cucurbita pepo* plants

679　under water deficit and salt stress. Data represent mean values of ten independent plants per

680　treatment ± standard error. Different letters denote statistical differences at $p \leq 0.05$.

681　**Figure 2.** Transpiration (A), stomatal conductance and Quantum yield (C) in *Cucurbita pepo*

682　plants under water deficit and salt stress. Data represent mean values of ten independent

683　plants per treatment ± standard error. Different letters denote statistical differences at $p \leq 0.05$

684　**Figure 3**. Hormonal content in *Cucurbita pepo* plants under water deficit and salt stress. A:

685　Abscisic acid B: Salicylic acid C: Jasmonic and D: Indol-acetic acid. Data are mean values of

686　3 independent determinations ± standard error. Different letters denote statistical differences

687　at $p \leq 0.05$.

688　**Figure 4.** Proline and MDA content in *Cucurbita pepo* plants under water deficit and salt

689　stress. Data are mean values of 3 independent determinations ± standard error. Different

690　letters denote statistical differences at $p \leq 0.05$.

691　**Figure 5.** *CmWRKYs* alignments by families. Common regions between the different families

692　are marked in green, while common regions inside families are marked in red. Yellow

693　highlighted zones refer to potential zinc ligands. Gaps have been inserted for an optimal

694　alignment.

695　**Figure 6.** Phylogenetic tree of WRKY TFs domains of *A. thaliana* and *C. maxima*. The

696　numbers in branches represent bootstrap values based on 1 000 replications. Different

697　symbols refer to the different groups of WRKY TFs: group I N-terminal: ○; group I C-

698　terminal: ●; group IIa: ◁; group IIb: ◇ group IIc: ◆; group IId: □ ; group IIe: ■; group

699　III:

700　**Figure 7.** Relative expression of *CmWRKY* genes in response to water and salt stresses in the

701　leaves of zucchini. Asterisks denote statistical differences at $p \leq 0.05$ between control and

702　stressed plants.

703　**Figure 8.** Expression profiles of *WRKY* genes presented in response to the experimental

704　treatments relative to the control samples and visualized as heat maps (A). The colour scale

705　represents relative expression levels. Green and red represent decreasing and increasing

706　transcript content, respectively B: Venn diagram depicting the degree of overlap between the

707　number of *CmWRKYs* which were significantly regulated by water deficit and salt stress.

708

709

711 **Table1**. List of the *CmWRKY* genes, classified according to the group and chromosome they are
712 located (start and end refer to the gen position in the chromosome).

Group	Gene	*Cucurbita* locus	Chromosome	Begin	End	Protein length
I	*CmWRKY5*	Cma_018501	1	3726739	3729055	548
	CmWRKY11	Cma_024068	2	4293272	4296213	452
	CmWRKY27	Cma_001683	4	8477538	8480743	482
	CmWRKY28	Cma_013360	4	16197407	16202326	559
	CmWRKY33	Cma_031066	5	5706455	5712086	1112
	CmWRKY34	Cma_028110	6	940762	942398	290
	CmWRKY49	Cma_028567	9	8997284	9008422	1609
	CmWRKY50	Cma_028568	9	9010912	9052610	2096
	CmWRKY53	Cma_011408	10	2113706	2118074	506
	CmWRKY54	Cma_011043	10	3895566	3902781	744
	CmWRKY55	Cma_003034	11	830454	834259	507
	CmWRKY63	Cma_011895	11	12851050	12852281	266
	CmWRKY66	Cma_021280	12	3467830	3468691	145
	CmWRKY67	Cma_031116	13	373134	375249	564
	CmWRKY75	Cma_008911	14	5555368	5556989	288
	CmWRKY81	Cma_014760	15	3435856	3440958	564
	CmWRKY82	Cma_030563	15	6759134	6762945	740
	CmWRKY88	Cma_024550	16	7656778	7660177	708
IIa	*CmWRKY3*	Cma_017975	1	822636	824640	302
	CmWRKY7	Cma_007461	1	11362729	11365303	345
	CmWRKY29	Cma_012901	4	18688997	18698230	1305
	CmWRKY61	Cma_012041	11	12117559	12120379	535
	CmWRKY70	Cma_007890	14	149735	152271	475
IIb	*CmWRKY15*	Cma_005326	3	5107884	5110515	618
	CmWRKY22	Cma_000508	4	2585081	2587490	468
	CmWRKY41	Cma_021396	7	284916	288237	453
	CmWRKY47	Cma_004112	9	1067795	1069450	385
	CmWRKY51	Cma_011695	10	826059	829561	489
	CmWRKY57	Cma_002737	11	2235976	2240549	502
	CmWRKY65	Cma_020990	12	1860926	1862820	310
	CmWRKY78	Cma_006686	14	14511317	14513110	324
	CmWRKY91	Cma_014030	17	7412849	7415141	291
IIc	*CmWRKY4*	Cma_018069	1	1325486	1327427	297
	CmWRKY10	Cma_024185	2	3501164	3503758	209
	CmWRKY14	Cma_005455	3	4495889	4497565	292
	CmWRKY17	Cma_004829	3	7238698	7239405	165
	CmWRKY18	Cma_004824	3	7258119	7259224	228
	CmWRKY19	Cma_004498	3	8745541	8746898	266
	CmWRKY24	Cma_000723	4	3680441	3681595	185
	CmWRKY30	Cma_017440	5	1548177	1550124	308
	CmWRKY31	Cma_017373	5	1889454	1892865	203
	CmWRKY37	Cma_029454	6	7070890	7072101	347
	CmWRKY38	Cma_029417	6	7334632	7335810	322
	CmWRKY40	Cma_031895	6	10315006	10316678	206
	CmWRKY44	Cma_022694	8	617192	618506	290
	CmWRKY56	Cma_002845	11	1730598	1732013	277
	CmWRKY59	Cma_012570	11	8583212	8586172	424
	CmWRKY60	Cma_012340	11	9902931	9905220	175
	CmWRKY62	Cma_011953	11	12604487	12606828	275
	CmWRKY68	Cma_020055	13	5832711	5834151	275
	CmWRKY69	Cma_020402	13	7382978	7385739	599
	CmWRKY71	Cma_008041	14	761708	768956	926
	CmWRKY74	Cma_008797	14	4878868	4880895	305
	CmWRKY83	Cma_009005	16	303081	304939	456
	CmWRKY85	Cma_009486	16	2839755	2841702	353

Group	Gene	*Cucurbita* locus	Chromosome	Begin	End	Protein length
	CmWRKY93	Cma_013787	17	8604589	8609002	1136
	CmWRKY95	Cma_015538	18	3291663	3293888	389
IId	*CmWRKY6*	Cma_007375	1	10882048	10884101	346
	CmWRKY8	Cma_007620	1	12075582	12078149	380
	CmWRKY9	Cma_024288	2	2585531	2586851	278
	CmWRKY13	Cma_016956	2	9870246	9873833	303
	CmWRKY16	Cma_004913	3	6836165	6838258	314
	CmWRKY43	Cma_021673	7	1412905	1415697	619
	CmWRKY45	Cma_022498	8	1790125	1792536	339
	CmWRKY46	Cma_019202	8	7800804	7804554	264
	CmWRKY48	Cma_003836	9	2358729	2360437	342
	CmWRKY52	Cma_011511	10	1677276	1678709	269
	CmWRKY86	Cma_009513	16	2998775	2999747	233
	CmWRKY90	Cma_014489	17	4553753	4555295	279
	CmWRKY94	Cma_015639	18	2364208	2366027	263
IIe	*CmWRKY2*	Cma_030215	0	58251834	58253294	332
	CmWRKY12	Cma_016271	2	6451561	6452816	334
	CmWRKY20	Cma_004411	3	9131488	9140753	922
	CmWRKY25	Cma_000724	4	3682891	3684179	255
	CmWRKY36	Cma_022985	6	3513185	3516840	740
	CmWRKY39	Cma_032056	6	9833175	9834847	323
	CmWRKY58	Cma_002256	11	4927593	4930117	534
	CmWRKY64	Cma_020719	12	370354	373398	281
	CmWRKY72	Cma_008439	14	2887228	2894814	1357
	CmWRKY76	Cma_006170	14	11876011	11877554	323
	CmWRKY80	Cma_015225	15	1187701	1197353	1280
	CmWRKY87	Cma_024498	16	7269003	7274618	552
	CmWRKY92	Cma_013832	17	8415383	8418312	328
III	*CmWRKY1*	Cma_029652	0	22348382	22349741	323
	CmWRKY21	Cma_000098	4	487467	489639	292
	CmWRKY23	Cma_000700	4	3544000	3547509	356
	CmWRKY26	Cma_001059	4	5470832	5472142	260
	CmWRKY32	Cma_017350	5	2013965	2025563	811
	CmWRKY35	Cma_023027	6	3263898	3268590	578
	CmWRKY42	Cma_021481	7	683658	685147	249
	CmWRKY73	Cma_008584	14	3641445	3643996	172
	CmWRKY77	Cma_006655	14	14362158	14364009	329
	CmWRKY79	Cma_006753	14	14852671	14854096	213
	CmWRKY84	Cma_009302	16	1747075	1749663	422
	CmWRKY89	Cma_023751	17	2109793	2110780	270

713

714

715

716

Table 1

1   **Table1**. List of the *CmWRKY* genes, classified according to the group and chromosome they are
2   located (start and end refer to the gen position in the chromosome).

Group	Gene	*Cucurbita* locus	Chromosome	Begin	End	Protein length
I	*CmWRKY5*	Cma_018501	1	3726739	3729055	548
	CmWRKY11	Cma_024068	2	4293272	4296213	452
	CmWRKY27	Cma_001683	4	8477538	8480743	482
	CmWRKY28	Cma_013360	4	16197407	16202326	559
	CmWRKY33	Cma_031066	5	5706455	5712086	1112
	CmWRKY34	Cma_028110	6	940762	942398	290
	CmWRKY49	Cma_028567	9	8997284	9008422	1609
	CmWRKY50	Cma_028568	9	9010912	9052610	2096
	CmWRKY53	Cma_011408	10	2113706	2118074	506
	CmWRKY54	Cma_011043	10	3895566	3902781	744
	CmWRKY55	Cma_003034	11	830454	834259	507
	CmWRKY63	Cma_011895	11	12851050	12852281	266
	CmWRKY66	Cma_021280	12	3467830	3468691	145
	CmWRKY67	Cma_031116	13	373134	375249	564
	CmWRKY75	Cma_008911	14	5555368	5556989	288
	CmWRKY81	Cma_014760	15	3435856	3440958	564
	CmWRKY82	Cma_030563	15	6759134	6762945	740
	CmWRKY88	Cma_024550	16	7656778	7660177	708
IIa	*CmWRKY3*	Cma_017975	1	822636	824640	302
	CmWRKY7	Cma_007461	1	11362729	11365303	345
	CmWRKY29	Cma_012901	4	18688997	18698230	1305
	CmWRKY61	Cma_012041	11	12117559	12120379	535
	CmWRKY70	Cma_007890	14	149735	152271	475
IIb	*CmWRKY15*	Cma_005326	3	5107884	5110515	618
	CmWRKY22	Cma_000508	4	2585081	2587490	468
	CmWRKY41	Cma_021396	7	284916	288237	453
	CmWRKY47	Cma_004112	9	1067795	1069450	385
	CmWRKY51	Cma_011695	10	826059	829561	489
	CmWRKY57	Cma_002737	11	2235976	2240549	502
	CmWRKY65	Cma_020990	12	1860926	1862820	310
	CmWRKY78	Cma_006686	14	14511317	14513110	324
	CmWRKY91	Cma_014030	17	7412849	7415141	291
IIc	*CmWRKY4*	Cma_018069	1	1325486	1327427	297
	CmWRKY10	Cma_024185	2	3501164	3503758	209
	CmWRKY14	Cma_005455	3	4495889	4497565	292
	CmWRKY17	Cma_004829	3	7238698	7239405	165
	CmWRKY18	Cma_004824	3	7258119	7259224	228
	CmWRKY19	Cma_004498	3	8745541	8746898	266
	CmWRKY24	Cma_000723	4	3680441	3681595	185
	CmWRKY30	Cma_017440	5	1548177	1550124	308
	CmWRKY31	Cma_017373	5	1889454	1892865	203
	CmWRKY37	Cma_029454	6	7070890	7072101	347
	CmWRKY38	Cma_029417	6	7334632	7335810	322
	CmWRKY40	Cma_031895	6	10315006	10316678	206
	CmWRKY44	Cma_022694	8	617192	618506	290
	CmWRKY56	Cma_002845	11	1730598	1732013	277
	CmWRKY59	Cma_012570	11	8583212	8586172	424
	CmWRKY60	Cma_012340	11	9902931	9905220	175
	CmWRKY62	Cma_011953	11	12604487	12606828	275
	CmWRKY68	Cma_020055	13	5832711	5834151	275
	CmWRKY69	Cma_020402	13	7382978	7385739	599
	CmWRKY71	Cma_008041	14	761708	768956	926
	CmWRKY74	Cma_008797	14	4878868	4880895	305
	CmWRKY83	Cma_009005	16	303081	304939	456
	CmWRKY85	Cma_009486	16	2839755	2841702	353
	CmWRKY93	Cma_013787	17	8604589	8609002	1136
	CmWRKY95	Cma_015538	18	3291663	3293888	389

| Group	Gene	*Cucurbita* locus	Chromosome	Begin	End	Protein length
IId	*CmWRKY6*	Cma_007375	1	10882048	10884101	346
	CmWRKY8	Cma_007620	1	12075582	12078149	380
	CmWRKY9	Cma_024288	2	2585531	2586851	278
	CmWRKY13	Cma_016956	2	9870246	9873833	303
	CmWRKY16	Cma_004913	3	6836165	6838258	314
	CmWRKY43	Cma_021673	7	1412905	1415697	619
	CmWRKY45	Cma_022498	8	1790125	1792536	339
	CmWRKY46	Cma_019202	8	7800804	7804554	264
	CmWRKY48	Cma_003836	9	2358729	2360437	342
	CmWRKY52	Cma_011511	10	1677276	1678709	269
	CmWRKY86	Cma_009513	16	2998775	2999747	233
	CmWRKY90	Cma_014489	17	4553753	4555295	279
	CmWRKY94	Cma_015639	18	2364208	2366027	263
IIe	*CmWRKY2*	Cma_030215	0	58251834	58253294	332
	CmWRKY12	Cma_016271	2	6451561	6452816	334
	CmWRKY20	Cma_004411	3	9131488	9140753	922
	CmWRKY25	Cma_000724	4	3682891	3684179	255
	CmWRKY36	Cma_022985	6	3513185	3516840	740
	CmWRKY39	Cma_032056	6	9833175	9834847	323
	CmWRKY58	Cma_002256	11	4927593	4930117	534
	CmWRKY64	Cma_020719	12	370354	373398	281
	CmWRKY72	Cma_008439	14	2887228	2894814	1357
	CmWRKY76	Cma_006170	14	11876011	11877554	323
	CmWRKY80	Cma_015225	15	1187701	1197353	1280
	CmWRKY87	Cma_024498	16	7269003	7274618	552
	CmWRKY92	Cma_013832	17	8415383	8418312	328
III	*CmWRKY1*	Cma_029652	0	22348382	22349741	323
	CmWRKY21	Cma_000098	4	487467	489639	292
	CmWRKY23	Cma_000700	4	3544000	3547509	356
	CmWRKY26	Cma_001059	4	5470832	5472142	260
	CmWRKY32	Cma_017350	5	2013965	2025563	811
	CmWRKY35	Cma_023027	6	3263898	3268590	578
	CmWRKY42	Cma_021481	7	683658	685147	249
	CmWRKY73	Cma_008584	14	3641445	3643996	172
	CmWRKY77	Cma_006655	14	14362158	14364009	329
	CmWRKY79	Cma_006753	14	14852671	14854096	213
	CmWRKY84	Cma_009302	16	1747075	1749663	422
	CmWRKY89	Cma_023751	17	2109793	2110780	270

3

4

Figure 1
Click here to download high resolution image
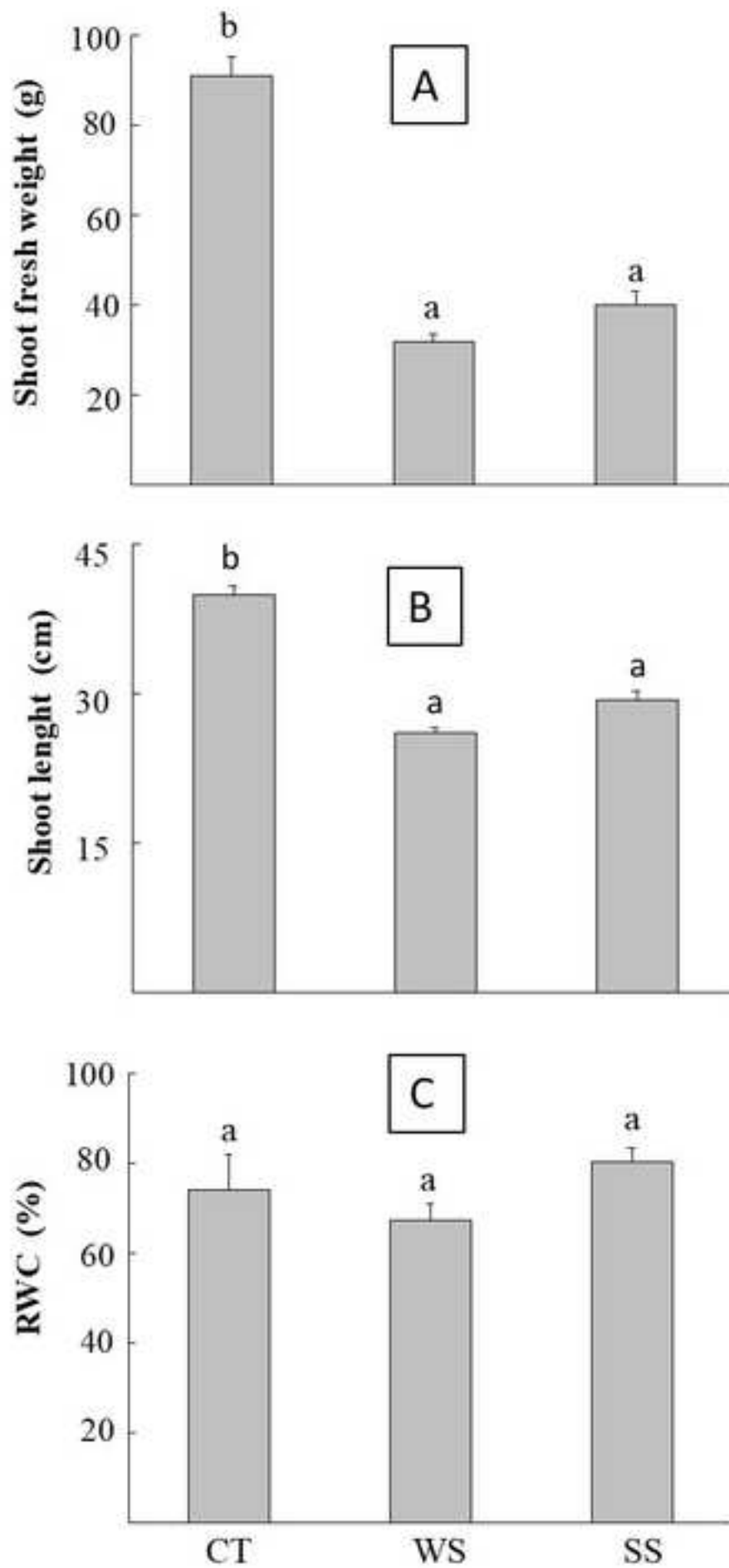
Figure 2
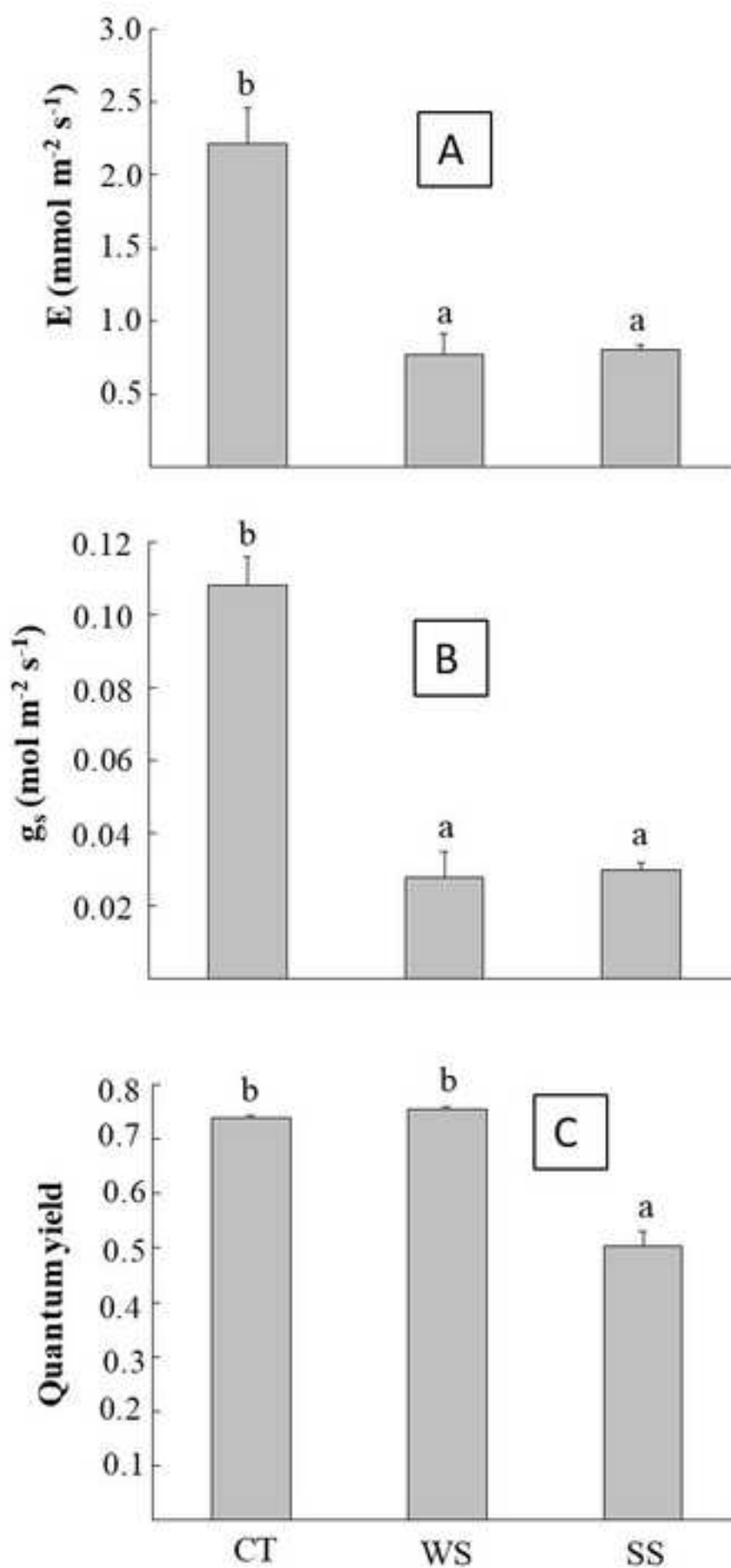**Click here to download high resolution image**
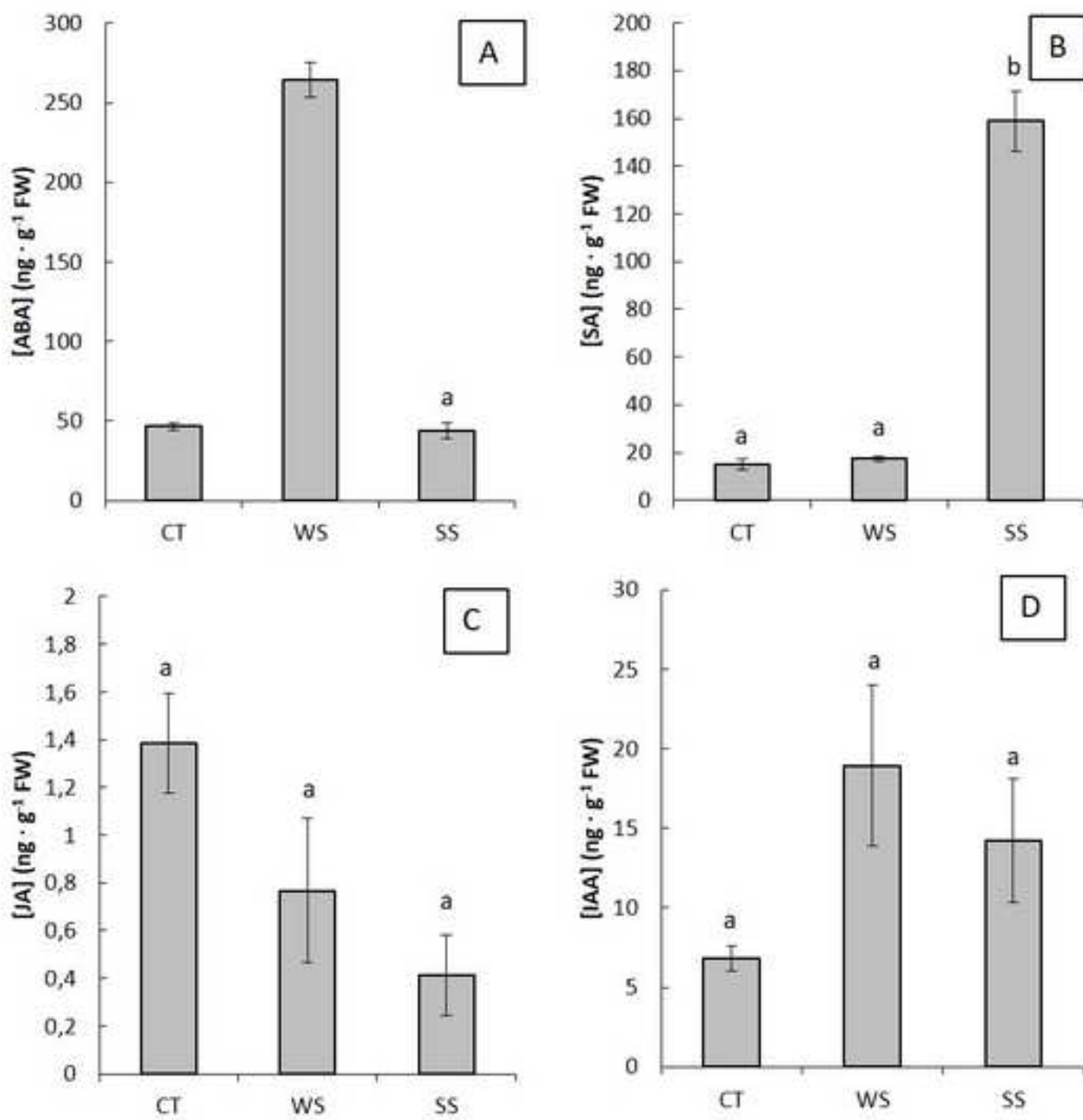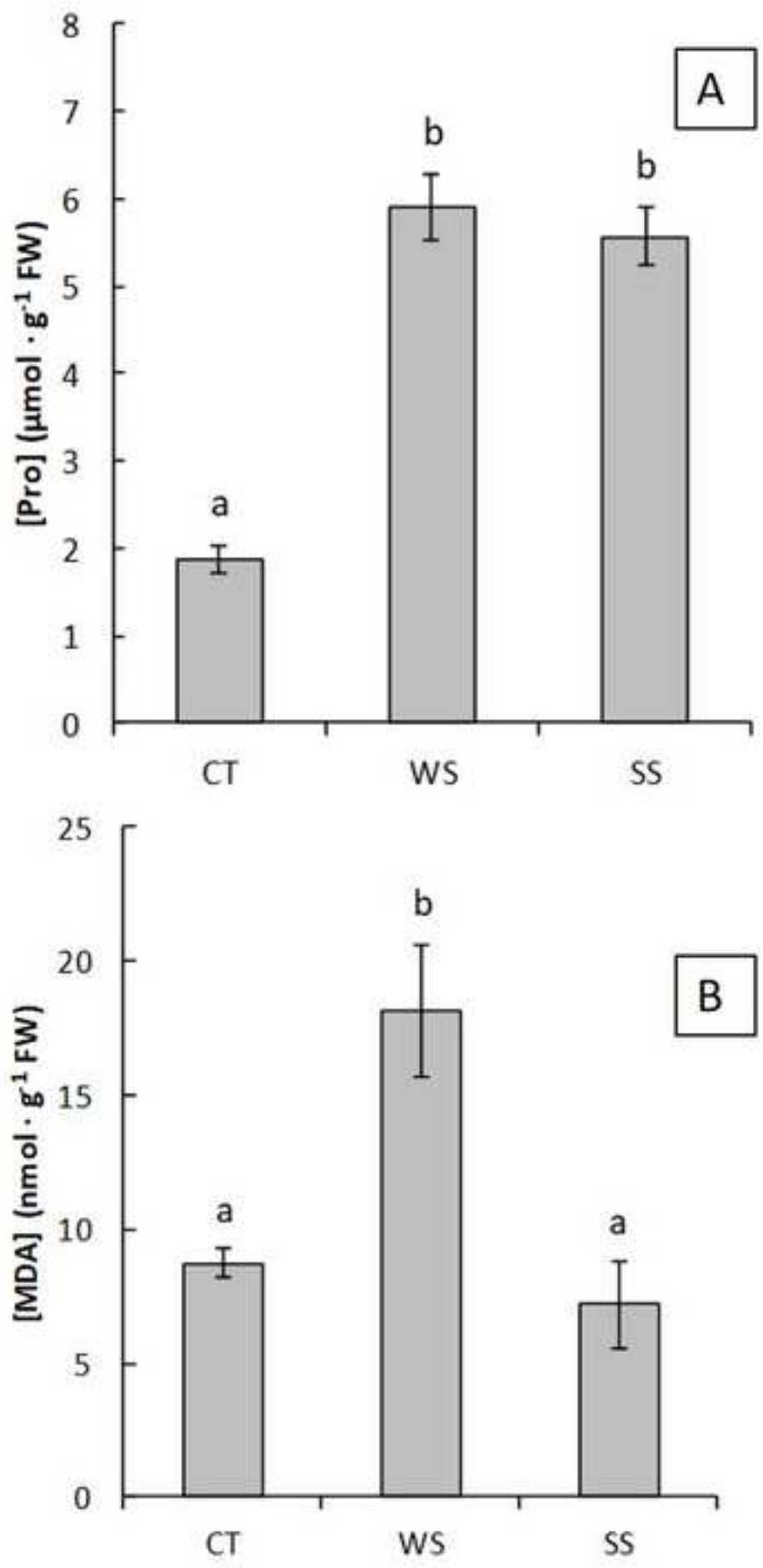

Figure 2

Figure 3

Figure 4
[Click here to download high resolution image](#)

Figure 5

```
Group I N-terminal
CmWRKY49N NVRTPASDGYNWRKYGQKQVKSPKGSRSYYKCTY--S-ECCAKKIECCDHSG-H-RTEIVYRSQHSHDPP
CmWRKY53N NVRTPASDGYNWRKYGQKQVKIPKGSRSYYKCTY--S-GCCAKKIECCDHSG-L-VTEVVYKSQHSHDPP
CmWRKY55N IREKVSEDGYNWRKYGQKLVKGNVFVRSYYRCTH--P-TCMVKKQLERTHDG-K-ITDIIYFGPHDHPRP
CmWRKY33N VSDRLSDDGYNWRKYGQKHVKGSEFPRSYYKCTH--P-NCEVKKLFERSHDG-Q-IVDIIYKGTHDHPKP
CmWRKY81N VSDRISDDGYNWRKYGQKQVKGSEFPRSYYKCTH--P-NCEVKKLFERSHDG-Q-ITDIVYKGTHDHPKL
CmWRKY34N GSGAPSEDGYNWRKYGQKQVKGSEYPRSYYKCTH--P-NCQVKKKVERSNEG-H-ITEIIYKGTHNHPKP
CmWRKY82N GCGAPSEDGYNWRKYGQKQVKGSEYPRSYYKCTH--P-NCQVKKKVERSHEG-H-ITEIIYKGTHNHVKP
CmWRKY50N GMLRTSEDGYNWRKYGQKQVKGSEYPRSYYKCTH--P-NCQVKKKVERSLDG-Q-ITEIIYKGAHIHAKP
CmWRKY54N GMLKTSEDGYNWRKYGQKQVKGSEYPRSYYKCTH--P-NCIVKKKVERSLDG-Q-ITEIIYTGAHNHSKP
CmWRKY28N ASDKPADGYNWRKYGQKLVKGSEYPRSYYKCTH--L-NCPVKKKIERSPDG-Q-ITEIIYKGQHNHEPP
CmWRKY75N ASDKPADGYNWRKYGQKLVKGSEYPRSYYKCTH--L-NCPVKKKIERSPDG-Q-ITEIIYKGQHNHERP
CmWRKY27N TVGRPADGYNWRKYGQKQVKGGEFPRSYYKCTH--P-NCPVRKKVERSLEG-Q-VTEIIYKGEHNHKQP
CmWRKY88N NADRPADGYNWRKYGQKQVKGSEFPRSYYKCTY--P-NCPVKKKVERSLEG-H-ITEIIYKGEHNHERP
CmWRKY67N SCAQPSYDGYNWRKYGQKKVKGSKYPRSYYKCTH--P-NCPVKKKVERSLDG-K-ITEIVYKGEHDHPKP
CmWRKY11N PKNRASDDGYNWRKYGQKLVKGSENPRSYYKCTH--P-NCPVRKQVEKSLNG-Q-ITEIVYKSKHNHPKP
CmWRKY66N EQKKSENDGYNWRKYGQKQVKGSENPRSYYKCTF--P-NCPTKKKVERSLDG-Q-ITEIVYKGSHNHAKP
CmWRKY5N  TVNRRSDGYNWRKYGQKQVKGSENPRSYYKCTF--P-NCPTKKKVERSLDG-Q-ITEIVYKGGHNHPKP
CmWRKY63N TVNRRSDGYNWRKYGQKQVKGSENPRSYYKCTF--P-SCSTKKKVERSLDG-Q-ITEIVYKGSHNHPKP
Group I C-terminal
CmWRKY53C GDVGISGDGYRWRKYGQKMVKGNPHPRNYYRCTS--A-GCPVRKHIESAVEN-PSVVIITYKGVHDHDMP
CmWRKY55C SEVDIVNDGYRWRKYGQKLVKGNPNPRSYYRCSS--P-GCPVKKHVERASHD-PKVVLTTYEGHHDHDMP
CmWRKY11C SSVDKLDDGYWWRKYGQKVVKGNPNPRSYYKCTY--A-GCGVRKHIERASHD-LKAVMTTYEGKHNHEIP
CmWRKY27C SEVDLLDDGYRWRKYGQKIVKGNPYPRSYYKCTT--P-GCNVRKHVERASTD-PKAVITTYEGKHNHDVP
CmWRKY88C SEVDLLDDGYRWRKYGQKTVKGNPYPRSYYKCTT--L-GCNVRKHVERASTD-QKAVITTYEGKHNHDVP
CmWRKY28C SEVDLLDDGYRWRKYGQKVVKGNPNPRSYYKCTS--A-GCNVRKHVERSSTD-SKAVVTTYEGKHNHDVP
CmWRKY75C SEVDLLDDGYRWRKYGQKVVKGNPNPRSYYKCTS--A-GCNVRKHVERSSTD-SKAVVTTYEGKHNHDVP
CmWRKY50C TDVDILEDGYRWRKYGQKVVKGNPNPRSYYKCTS--A-GCAVRKHVERASHD-LKCVITTYEGKHNHEVP
CmWRKY54C TEFDILEDGYRWRKYGQKVVKGNPNPRSYYKCTS--T-GCSVRKHVERASHD-LKCVITTYEGKHNHLVP
CmWRKY66C SEIDILPDGYRWRKYGQKVVKGNPNPRSYYKCTS--L-GCPVRKHIERAAND-TRAVITTYEGKHNHEVP
CmWRKY5C  SDIDILDDGYRWRKYGQKVVKGNPNPRSYYKCTN--P-GCPVRKHVERASHD-LRAVITTYEGKHNHEVP
CmWRKY63C SDIDILDDGYRWRKYGQKVVKGNPNPRSYYKCTN--P-GCPVRKHVERASHD-QRAVITTYEGKHNHDVP
CmWRKY33C SEVDILDDGYRWRKYGQKVVRGNPNPRSYYKCTN--V-GCPVRKHVERASHD-PKAVITTYEGKHNHDVP
CmWRKY81C SEVDILDDGYRWRKYGQKVVRGNPNPRSYYKCTN--V-GCPVRKHVERASHD-PKAVITTYEGKHNHDVP
CmWRKY34C SEVDILDDGYRWRKYGQKVVKGNPNPRSYYKCTN--P-GCTVRKHVERASHD-LKSVITTYEGKHNHDVP
CmWRKY82C SEVDILDDGYRWRKYGQKVVKGNPNPRSYYKCTN--P-GCTVRKHVERASHD-LKSVITTYEGKHNHDVP
CmWRKY49C GDVGISGDGYRWRKYGQKMVKGSPHPRNYYRCTS--A-GCPVRKHIESAVEN-PYAVIITYEGVHNHDMP
CmWRKY67C ADIEISGKGIRWRKYGQKVVKGNLYPRSYYRCTG--L-KCKARKYVERASEV-PDSFITTYEGKHNHDIS
Group IIa
CmWRKY7  DPSLVVKDGYQWRKYGQKVTRDNPSPRAYFKCSS--APNCPVKKKVQRSLED-PTILVATYEGEHSHASH
CmWRKY3  DSNLVVKDGYQWRKYGQKVTRDNPCPRAYFKCSF--APTCPVKKKVQRSVED-QSILVATYEGEHNHPQS
CmWRKY70 DSNLVVKDGYQWRKYGQKVTRDNPCPRAYFKCSF--APTCPVKKKVQRSVED-QSVLVATYEGEHNHPQA
CmWRKY29 DSSLVVKDGYQWRKYGQKVTKDNPSPRAYYKCSF--APSCPVKRKVQRSVED-PSYLVATYEGEHNHPKP
CmWRKY61 DSSLAVKDGYQWRKYGQKVTKDNPSPRAYYKCSF--APSCPVKKKVQRSVQD-SSYLVATYEGEHNHKKP
Group IIb
CmWRKY15 SEAPMISDGCQWRKYGQKMAKGNPCPRAYYRCTM--AVGCPVRKQVQRCAED-RTILITTYEGNHNHPLP
CmWRKY41 SEAPMISDGCQWRKYGQKMAKGNPCPRAYYRCTM--AVGCPVRKQVQRCAED-TTILITTYEGNHNHPLP
CmWRKY57 SEAPMITDGCQWRKYGQKMAKGNPCPRAYYRCTM--AAGCPVRKQVQRCAED-KTILITTYEGNHNHPLP
CmWRKY91 SEAPMITDGCQWRKYGQKMAKGNPCPRAYYRCTM--AAGCPVRKQVQRCAED-KTILITTYEGNHNHPLP
CmWRKY47 APLVQMNDGCQWRKYGQKTAKGNPCPRAYYRCTG--APSCPVRKQVQRSVDD-ISILITTYEGTHNHPLP
CmWRKY51 CDTPTLNDGCQWRKYGQKIAKGNPCPRAYYRCTG--APSCPVRKQVQRSVDD-ISILITTYEGTHNHPLP
CmWRKY22 CEAATMNDGCQWRKYGQKIAKGNPCPRAYYRCTV--APGCPVRKQVQRCLED-MSILITTYEGTHNHPLP
CmWRKY78 CESATMNDGCQWRKYGQKIAKGNPCPRAYYRCTV--APGCPVRKQVQRCLED-MSILITTYEGTHNHPLP
CmWRKY65 CETTTMNDGCQWRKYGQKIAKGNPCPRAYYRCTG--SPTCPVRKQVQRCADD-MSILITTYEGNHNHPLP
Group IIc
CmWRKY17 SVLEILDDGFKWRKYGKKSVKNTSHLRNYYKCLN--G-GCGVKKRVERDRDD-SSYVITTYEGIHNHASP
CmWRKY30 SKVEVLDDGFKWRKYGKKMVKNSPNPRNYYKCSV--E-GCPVKKRVERDRED-PKYVITTYEGVHTHASQ
CmWRKY62 SEVEILDDGFKWRKYGKKMVKNSPNPRNYYKCSV--E-GCPVKKRVERDRDD-PKYVITTYEGVHTHESS
CmWRKY74 SDVDVLDDGYKWRKYGQKVVKNSLHPRSYYRCTH--S-NCRVKKRVERLSED-CRMVITTYEGRHNHSPC
CmWRKY10 TDVDVLDDGYKWRKYGQKVVKNTHHPRSYYRCTQ--D-DCRVKKRVERLDED-PRMVITTYEGRHIHSPS
CmWRKY95 SDVDVLDDGYKWRKYGQKVVKNTQHPRSYYRCTQ--D-HCRVKKRVERLAED-PRMVITTYEGRHVHSPS
CmWRKY18 TDVDVLDDGYKWRKYGQKVVKNTLHPRSYYRCTE--E-NCKVKKRVERLAED-PRMVITTYEGRHAHSPS
CmWRKY38 SQVDILDDGYRWRKYGQKGVKNNKFPRSYYKCTH--Q-GCKVKKQVQRLTRD-EGVVVTTYEGIHSHPIQ
CmWRKY56 SQVDILDDGYRWRKYGQKAVKNNKFPRSYYKCTH--Q-GCNVKKQVQRLTRD-EGVVVTTYEGMHTHSID
CmWRKY68 SQVDILDDGYRWRKYGQKAVKNNKFPRSYYKCTH--Q-GCNVKKQVQRLTKD-EGVVVTTYEGMHTHSID
CmWRKY24 SAEDVLDDGYRWRKYGQKAVKHSTHPRSYYRCTH--H-TCNVKKQIQRHSKD-PTIVVTTYEGIHNHPSE
CmWRKY40 TQIDHLEDGYRWRKYGQKTVKNSVFPRSYYRCTT--P-NCGVKKRVERSFQD-PSIVITTYEGQHNHPIA
CmWRKY19 SEVDHLEDGYRWRKYGQKAVKNSAYPRSYYRCTT--Q-KCGVKKRVERSYED-PSIVITTYEGQHNHPIP
```

```
CmWRKY59  TEIDQLEDGYRWRKYGQKAVKNSPYPRSYYRCTS--Q-KCVVKKRVERSYQD-PSLVITTYEGQHIHHCP
CmWRKY93  SEIDHLEDGYRWRKYGQKAVKNSPYPRSYYRCTS--Q-KCLVKKRVERSYQD-PSVVITTYEGQHNHHCP
CmWRKY44  TEMDHLDDGYRWRKYGQKAVKNSPYPRSYYRCTT--A-GCGVKKRVERSSHD-PSVVVTTYEGQHNHQSP
CmWRKY83  SEVDHLDDGYRWRKYGQKAVKNSPYPRSYYRCTT--A-GCGVKKRVERSSDD-PSVVVTTYEGQHIHQSP
CmWRKY37  SDIDHLDDGYRWRKYGQKTVKNSPYPRSYYRCTT--S-QCGVKKRVERSSSD-HSIVITTYEGQHTHQSP
CmWRKY71  SDIDHLDDGYRWRKYGQKAVKNSPYPRSYYRCTT--V-GCGVKKRVERLSND-QSTVVTTYEGQHTHQSP
CmWRKY31  SEVDHLEDGYRWRKYGQKAVKYSPFPRSYYRCTN--S-KCTVKKRVERSSED-PSVVITTYEGQHCHHTV
CmWRKY60  SEVDHLEDGYRWRKYGQKAVKNSPFP----RCTN--S-KCTVKKRVERSSED-PTVVITTYEGQHCHHTV
CmWRKY85  SEVDHLEDGYRWRKYGQKAVKNSPYPRSYYRCTN--V-ACNVKKRVERYLKD-SSIVVTTYEGQHTHSSP
CmWRKY4   SEVDHLEDGYRWRKYGQKAVKNSPFPRSYYRCTN--A-SCNVKKRVERSFVD-PTVVVTTYEGQHTHPSP
CmWRKY69  SEVDHLEDGYRWRKYGQKAVKNSPFPRSYYRCTT--P-TCNVKKRVERCSND-PTILVTTYEGQHTHPTP
CmWRKY14  DGNSLADDGYKWRKYGQKSIKNSPNPRSYYRCSN--P-RCSAKKQVERSMED-PDTFVTTYEGLHLHFAY
Group IId
CmWRKY48  KIADIPPDEYSWRKYGQKPIKGSPYPRGYYRCST--VKGCPARKKVERVRDD-PTMLLVTYDGDHRHPQP
CmWRKY52  KIAEIPSDEYSWRKYGQKPIKGSPYPRGYYRCSA--VKGCPARKKVERARDD-PAMLVVTYDGDHRHPPA
CmWRKY6   KIADIPPDEYSWRKYGQKPIKGSPYPRGYYKCSS--MRGCPARKHVERDPND-PAMLIVTYEGEHRHTQS
CmWRKY46  KIADIPTDEFSWRKYGQKPIKGSPYPRAYYKCST--MRGCPARKHVERNPKD-PAMLIITYEGEHRHTPS
CmWRKY13  KLADIPSDEYSWRKYGQKPIKGSPHPRGYYKCSS--MRGCPARKHVERCLQQ-PSMLIVTYEGEHSHPTI
CmWRKY43  KLADIPPDDYSWRKYGQKPIKGSPHPRGYYKCSS--MRGCPARKHVERCVEE-PSMLIVTYEGEHNHPRI
CmWRKY86  KLADIPPDDFSWRKYGQKPIKGSPHPRGYYKCSS--MRGCPARKHVERCLEE-PSMLIVTYEGEHNHPRI
CmWRKY90  KLADIPSDDYSWRKYGQKPIKGSPHPRGYYKCSS--MRGCPARKHVERCLED-PSMLIVTYEGEHNHPKM
CmWRKY16  KMADIPPDDYSWRKYGQKPIKGSPHPRGYYKCSS--VRGCPARKHVERAGDD-PAMLVVTYEGEHNHTLS
CmWRKY8   KLADIPPDDYSWRKYGQKPIKGSPHPRGYYKCSS--LRGCPARKHVERALDD-PTMLIVTYENDHNHALS
CmWRKY45  KLADIPPDDYSWRKYGQKPIKGSPHPRGYYKCSS--LRGCPARKHVERALDD-PTMLIVTYENDHNHAHS
CmWRKY9   KNADIPPDDYSWRKYGQKPIKGSPYPRGYYKCSS--LRGCPARKHVERASDD-PSMLIVTYEGDHNHSQS
CmWRKY94  KNADIPPDDYSWRKYGQKPIKGSPYPRGYYKCSS--LRGCPARKHVERASDD-PSMLIVTYEGDHNHSQS
Group IIe
CmWRKY12  TADNLSTDMWAWRKYGQKPIKGSPYPRNYYRCSS--SKGCGARKQVERSTAD-PETFIVTYTGDHTHPRP
CmWRKY76  TADNLSTDMWAWRKYGQKPIKGSPYPRNYYRCSS--SKGCGARKQVERSNAD-PDSFIITYTGEHIHPRP
CmWRKY64  GSTTPPSDSWAWRKYGQKPIKGSPYPRAYYRCSS--SKGCPARKQVERNRLD-PTMLLITYSCEHNHSGP
CmWRKY58  GEAYPPSDSWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCPARKQVERSRVD-PTKLVITYSFDHNHQLP
CmWRKY92  GEAYPPSDSWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCPARKQVERSRVD-PTKLVITYAFDHNHQLP
CmWRKY25  KADSVCSDSWGWRKYGQKPIKGSPYPRSYYRCSS--SKGCSARKQVERSFSD-PDIFVVTYTAEHNHAEP
CmWRKY80  KAEGVCSDSWGWRKYGQKPIKGSPYPRSYYRCSS--SKGCSARKQVERSLSD-PGAFVITYTAEHNHAEP
CmWRKY2   PAEALSSDIWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCMARKQVERNRSD-PGMFIVTYTAEHNHPAP
CmWRKY36  PAEALSSDIWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCMARKQVERNRSD-PGMFIVTYTAEHNHPAP
CmWRKY72  PAESLSSDIWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCMARKQVERNRSD-PGMFIVTYTAEHNHPAP
CmWRKY20  NGEVIPSDLWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCSARKQVERSRTD-PNMLVITYTSEHNHPWP
CmWRKY39  NGEVIPSDLWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCSARKQVERSRTD-PNMLVITYTSEHNHPWP
CmWRKY87  SGEVVPSDLWAWRKYGQKPIKGSPYPRGYYRCSS--SKGCSARKQVERSRTD-PNMLVITYTSEHNHPWP
Group III
CmWRKY26  VTAATAEDGRAWRKYGQKAIQNKTYPKSYYRCTHKYDQSCPAVKHVQRIEDNSKIMYEITYISDHTCAPA
CmWRKY32  DSSSLVDDGHAWRKYGQKSIQNAKFPRNYYRCTHKFDQGCQASKQVQQVEEH-PPKFRTTYYGHHTCTNF
CmWRKY21  NTELPPDDGFTWRKYGQKEILGSRFPRGYFRCTHQKLYHCPAKKHVQRLDHD-PHTFEVAYLGDHTCHMS
CmWRKY23  GFEGPHEDGYSWRKYGQKDILGATYPRSYYRCTFRNTQNCWAVKQVQRSDED-PSVFEITYRGKHTCSQG
CmWRKY79  GFDGPHEDGYSWRKYGQKDILGATFPRSYYRCTFRNTQNCWAIKQVQRSDED-HSVFDITYRGKHTCSQG
CmWRKY77  ALEGSLDDGFCWRKYGQKGILGAKLPRGYYRCTHRNLQGCLATKQVQQSDHD-PNVFEITYRGTHSCTQV
CmWRKY89  ALEGSLDDGFSWRKYGQKGIFGAKHPRGYYRCTHRNLQGCVATKQVQRSDDD-PTIFKITYRGNHTCSQV
CmWRKY1   AAEGPLNDGHSWRKYGQKDIHGANFPRCYYRCTHRNVRGCLATKQVQKSDND-PNIFEVTYRGQHTCNQS
CmWRKY35  AAEGPLNDGHSWRKYGQKDIHGANFPRCYYRCTHRNVRGCLATKQVQKSDND-PNIFEVTYRGQHTCNQS
CmWRKY73  APEGPLNDGYSWRKYGQKDIHGANFPRCYYRCSHRHERGCLATKQVQRSDND-PNIFDVTYRGRHTCNQS
CmWRKY42  AIGALPDDGFSWRKYGQKDILGSKFPRGYFRCSHRFAQGCSATKLVQRSDND-PSMYEITYRGKHTCNKP
CmWRKY84  AVEGPLHDGFSWRKYGQKDILGSKFPRGYFRCSHRFTLGCKATKQVQKSDND-PTIYEVTYKGTHTCNRP
```
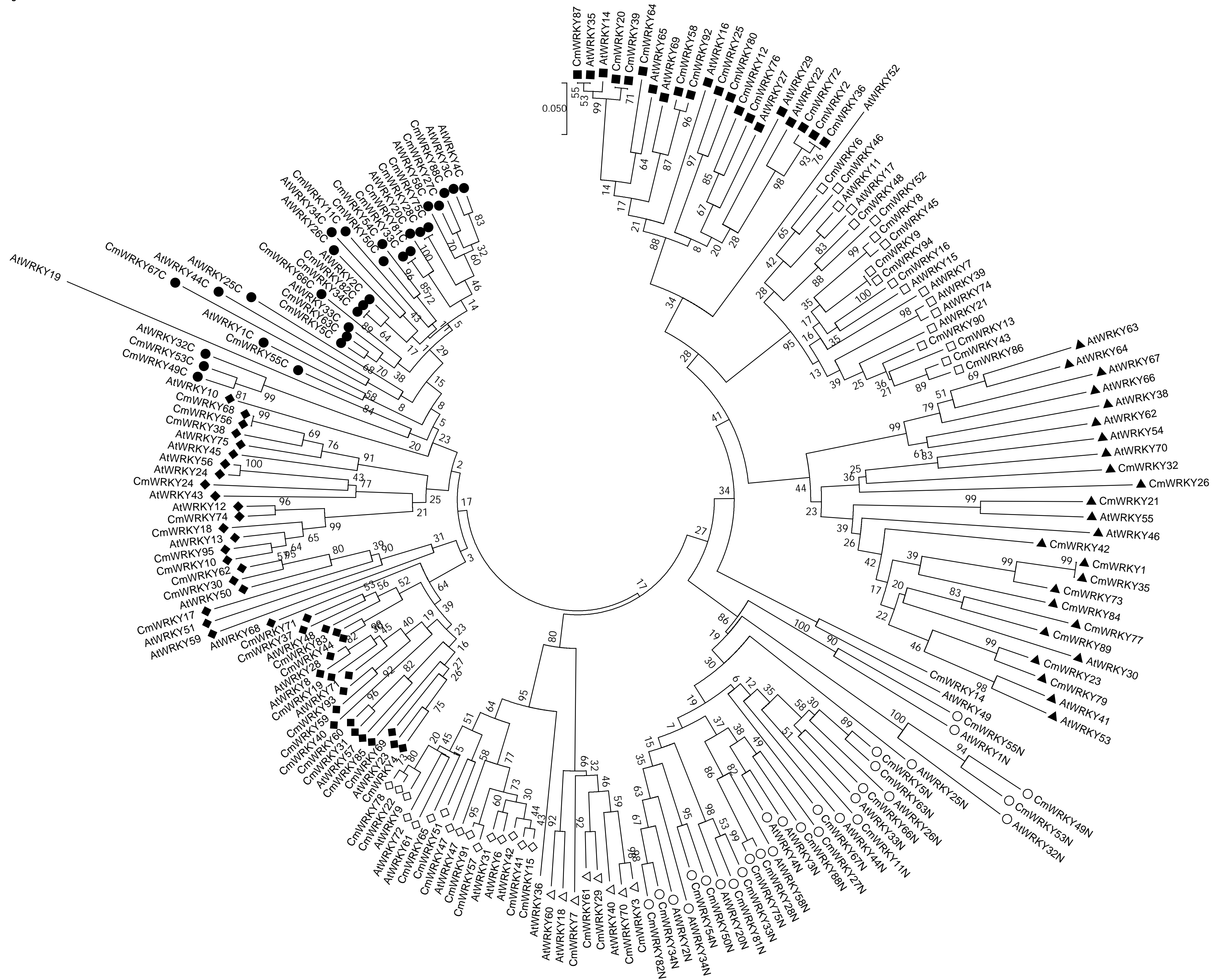
Figure 6

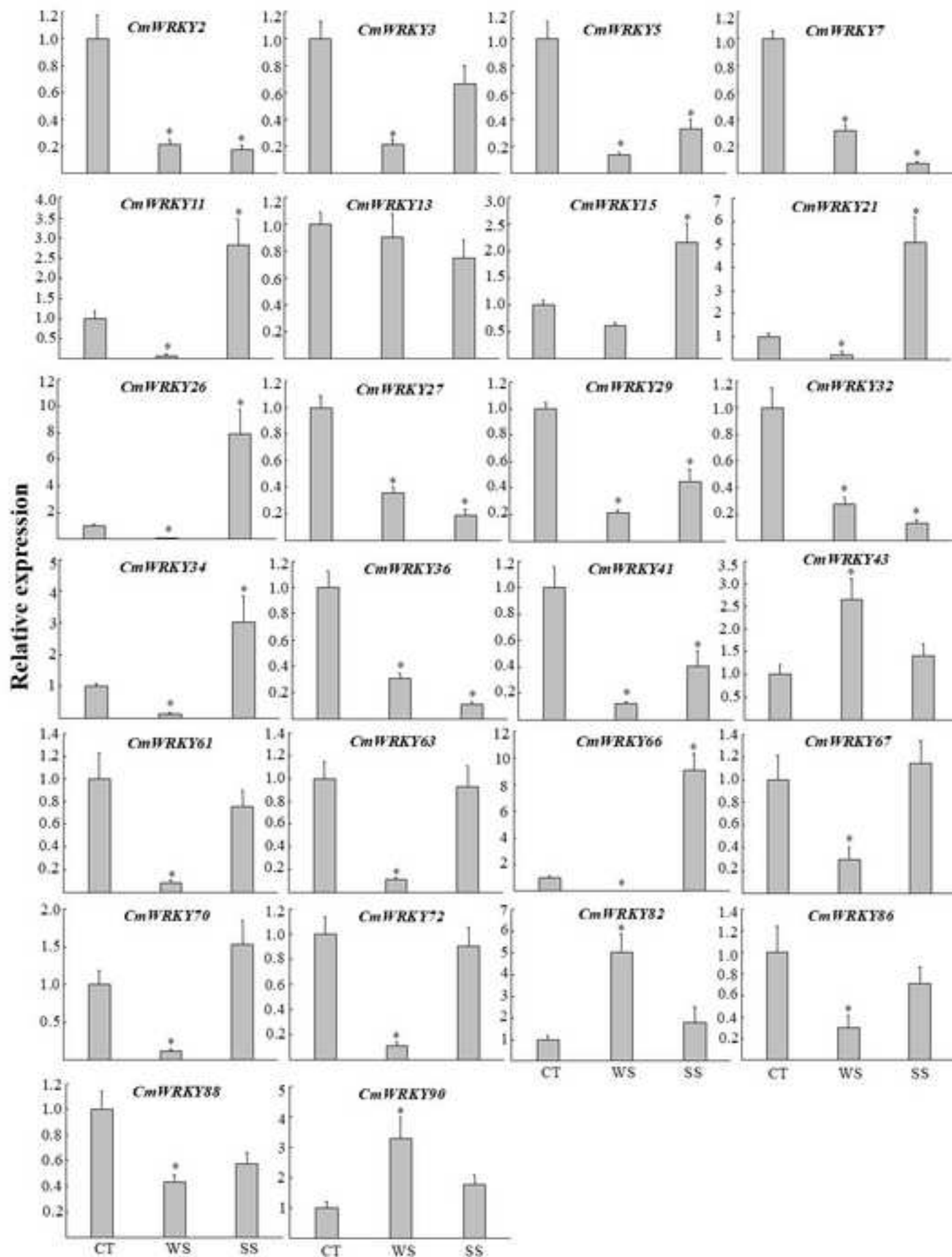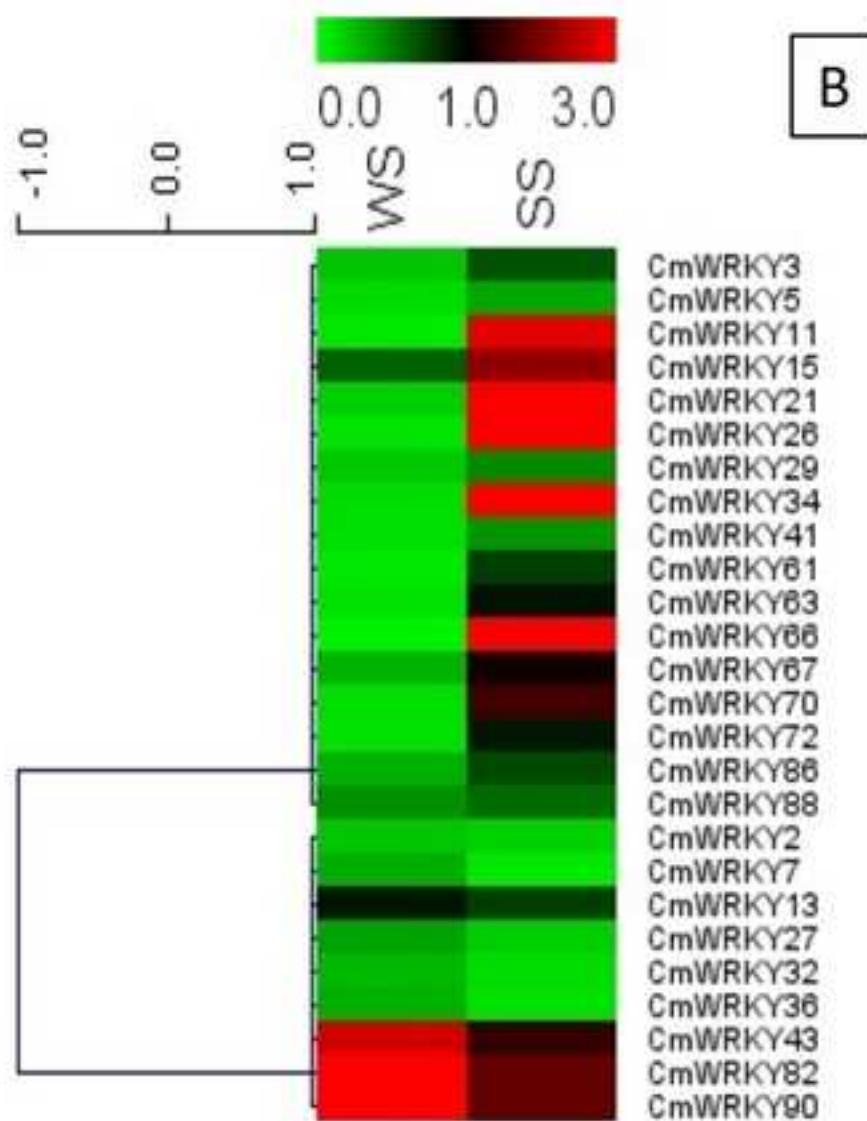**Figure 7**
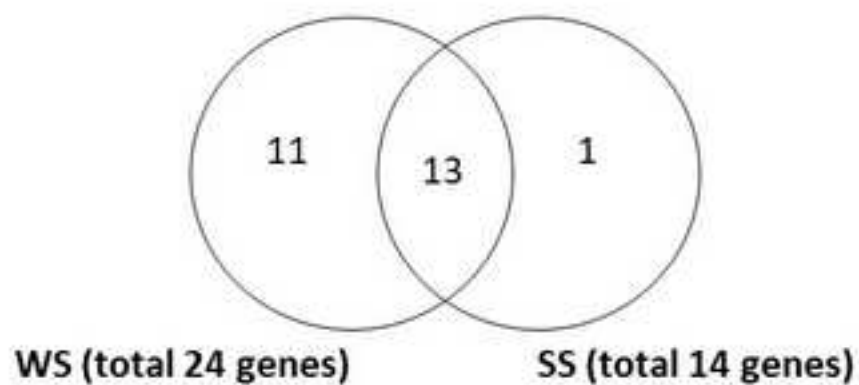[Click here to download high resolution image](#)

Supplementary Material