Overexpression of *Chalcone Synthase* Improves Flavonoid Accumulation and Drought Tolerance in Tobacco

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Abstract: Flavonoids are major secondary metabolites in plants, which play important roles in maintaining the cellular redox balance in cells. Chalcone synthase (CHS) is the key enzyme in the flavonoids biosynthesis pathway, and has been proved to monitor the changes to drought stress tolerance. In this work, we overexpressed a *CHS* gene in tobacco (*Nicotiana tabacum*). The transgenic tobacco plants were more tolerant than the control plants to drought stress. The transcription levels of the key genes involved in the flavonoids pathway and the contents of seven flavonoids were also significantly raised in the transgenic tobacco plants. In addition, overexpression

 doi:10.20944/preprints201906.0103.v1

of the *CHS* gene lead to a lower concentration of the oxidative stress product malondialdehyde. Overall, the *NtCHS* gene studied in this work was considered as a candidate gene for genetic engineering to enhance drought tolerance of plants and improve response to oxidative stress.

Keywords: chalcone synthase; drought stress; flavonoids; transgenic tobacco; overexpression

 doi:10.20944/preprints201906.0103.v1

INTRODUCTION

Flavonoids are a group of plant secondary metabolites consisting of two aromatic rings linked by three carbons. Flavonoids play diverse roles in plants, such as controlling the floral pigmentation, protecting plants from ultraviolet (UV) damage, and regulating the polar transport of auxin. Moreover, flavonoids also play important roles in cellular response to biotic and abiotic stressors [1]. The structures and functions of many enzymes in the flavonoids pathway have been well characterized in plants so far, including the chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), flavonol synthase (FLS), anthocyanidin synthase (ANS), and so on [2, 3].

Recent studies have investigated the effect of drought stress on flavonoids metabolism in plants. It has been demonstrated that flavonoids accumulated rapidly in some plant species suffering the drought stress [4]. Winter wheat leafs increased the expression levels of the key flavonoid genes and the total flavonoids content in response to drought stress [5]. Total flavonoids content in the leaves of *Ziziphus jujuba var. spinosa* also increased significantly under moderate drought treatment [6]. The expression levels of several flavonoids genes were upregulated in the roots of *Scutellaria baicalensis georigi* under drought conditions [7]. In brief, all the evidences so far indicate the participation of flavonoids and their key biosynthesis genes in plant resistance to drought stress. However, little is known about the clear roles of the key flavonoids genes in the response of plant species to drought stress.

CHS gene encodes the first enzyme in the flavonoids pathway. The CHS enzyme catalyzes the synthesis of narengenin chalcone by using one molecule p-coumaryl-CoA and three malonyl-CoA, which were from the phenylpropanoid pathway[3]. CHS is believed to have evolved from primary metabolic enzymes

 doi:10.20944/preprints201906.0103.v1

involved in fatty acid biosynthesis [2]. Many *CHS* genes have been cloned from higher plants, and their structures have been well-studied [8-10]. CHS proteins exhibit diverse functions among different plants, including anti-oxidant activity, participating in floral pigment formation [11-13] and pollen fertility [14]. However, there is very little research focusing on the roles of platn *CHS* gene expression level in drought tolerance, as well as in regulating the expression patterns of the downstream genes and the metabolite concentrations.

This work aimed to investigate the role of the *Nicotiana tabacum CHS* (*NtCHS*) gene in response to drought conditions. A vector overexpressing *NtCHS* coding sequence was constructed, and then transferred into the tobacco. Transgenic plants showed increased flavonoids content, and the expression levels of relevant genes were also upregulated in the transgenic plants. All these enhanced the drought tolerance of the transgenic plants.

MATERIALS AND METHODS

Chemicals and reagents

Seven flavonoids reference standards (e.g. rutin, quercetin, kaempferol-3-rutinoside, kaempferol-glucopyranoside, naringin, naringenin, isoliquiritigenin) and the internal standard vitexin (purity≥98%) were bought from Yudingxinjie Corporation (China). Acetonitrile, methanol, ethanol and formic acid of high-performance liquid chromatography (HPLC) grade were purchased from J.T.Baker (USA). Ultra-pure water was prepared with a Milli-Q purification system (Millipore, USA). Thiobarbituric acid and titanium sulfate (both ≥98%) were obtained from Yudingxinjie Corporation (China).

Vector construction, plant transformation and confirmation

NtCHS cDNA (Accession No.: AF311783.1) was amplified using primers

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NtCHS-F 5´-AGCCATTTGAAAACCCTAG-3´ and *NtCHS*-R 5´-CAAATTTCATTATTTGCAA G-3´. Then the *NtCHS* cDNA was cloned into the pH7WG2D plasmid between attR1 and attR2 sites (Fig. 1A) and confirmed by sequencing. Thereby, a recombinant vector pH7WG2D–*NtCHS* was prepared (Fig. 1A) and then transformed into Agrobacterium strain EHA105. *Nicotiana tabacum* K326 was transformed using a leaf disk method following a standard protocol [15].

Seeds were collected from self-pollinated primary transformants. Transgenic tobacco seeds (T1 generation) were germinated with 50 mg/ml kanamycin. Seeds of transgenic and wild-type tobacco lines were all grown in pots containing manure, sand and soil (1:1:2) in a greenhouse.

The transformed tobaccos were screened to identify *Npt II* via polymerase chain reaction (PCR) with genomic DNA as the template and using primers *npt II*-F 5´-TGTCACTGAAGCGGGAAG-3´ and *npt II*-R 5´-CTTCCATCCGAGTAC GTG-3´. The PCR conditions were as follows: 1 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining and UV illumination. Genomic DNAs were isolated from the pH7WG2D-*NtCHS* transformants and control plants using a DNeasy plant mini kit (Qiagen, Netherlands) following the manufacturer's instructions.

To select positive transgenic tobaccos, we determined the *NtCHS* gene expression by fluorescent real-time quantitative PCR (RT-qPCR). Each time, 100 mg of a plant sample was ground in liquid nitrogen, and then total RNA was extracted from pH7WG2D-*NtCHS* transformants and control plants separately using a Qiagen RNeasy plant mini kit according to the manufacturer's instructions. After treatment with DNase I (GeneAnswer, China), first-strand cDNA was synthesized using avian

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105 myeloblastosis virus reverse transcriptase (Sangon, China) according to the
106 manufacturer's instructions. RT-PCR was performed on a CFX96 instrument (BIO-
107 RAD, USA). Each reaction mixture (total volume = 20 μL) contained 10 μL of SYBR
108 Premix Ex Taq (2×, TaKaRa, Japan), 1 μL of gene-specific primers *CHS*-F/*CHS*-R
109 (10 mM), and diluted cDNA (100 ng). Leaf samples of both *CHS*-overexpressing
110 tobaccos and controls were tested in triplicate. Amplification conditions were as
111 follows: 30 s at 95 °C; 40 cycles of 5 s at 95 °C, 40 s at 60 °C; one cycle of 15 s at
112 95 °C, 1 min at 60 °C, 15 s at 95 °C, 15 s at 60 °C. The internal standard gene was
113 *26S rRNA*-based gene primers [16]. No morphological difference was observed
114 between the transgenic plants and controls.

115 **Plant growth conditions and drought treatment**

116 After germination with 50 mg/ml kanamycin, seedlings of transgenic tobacco
117 seeds were transferred to culture pots in the greenhouse. The drought treatment was
118 applied for 14 days.

119 Fresh weight (FW) of leaves was measured after removal from plants. Turgid
120 weight (TW) was determined by rinsing the leaves in water at 4 °C for 12 h. Then the
121 dry weight (DW) of leaves was determined after drying at 80 °C for 48 h. Relative
122 water content (RWC) was calculated as RWC=[(FW-DW)/(TW-DW)]×100%[17].
123 H_2O_2 content in transgenic or wild-type tobaccos was measured after drought
124 treatment following [18]. Content of malondialdehyde (MDA) was detected using the
125 thiobarbituric acid method (TBA) method [19].

126 **RNA isolation and qRT-PCR**

127 Total RNA was extracted from leaves of *CHS*-overexpressing tobaccos or
128 control tobaccos using the RNeasy plant mini kit. First-strand cDNA was synthesized.
129 The qRT-PCR conditions and internal standard gene were the same as mentioned

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130 before. The forward and reverse primers used for qRT-PCR were as follows: CHS, 5′-
131 AGAAAAGCCTTGTGGAAGCA-3′, 5′-ACTTGGTCCAAAATT GCAGG-3′; CHI,
132 5′-GAAATCCTCCGATCCAGTGA-3′, 5′-CAACGTTGACAACATCAGGC-3′;
133 F3H, 5′-ACAGGGTGAAGTGGTCCAAG-3′, 5′-CCTTGGTTAAGGCCTCCTTC-
134 3′; F3'H, 5′-TCCAAGAATACTGGCCCAAG-3′, 5′-CTCACAACTCTCGGATG
135 CAA-3′; FLS, 5′-GAACTTGAAGGGAAAAGGGG-3′, 5′-TCCCTGTAGGAGGG
136 AGGATT-3′; DFR, 5′-TCCCATCATGCGATCATCTA-3′, 5′-ATGGCTTCTTTGT
137 CACGTCC-3′; 26S rRNA, 5′-CACGGACCAAGGAGTCTGACAT-3′, 5′-TCCCAC
138 CAATCAGCT TCCTTAC-3′.

139 **Liquid chromatography (LC) and tandem mass spectrometry (MS/MS)**

140 Flavonoids were extracted from tobaccos using a modified method [20] .
141 Freeze-dried tobacco powder (50 mg) and 2 mL of water/methanol extraction solution
142 (4:1, volume ratio) were mixed and sonicated in an ultrasonic bath at 30 °C for 20 min.
143 The extracts were then centrifuged at 4000 rpm for 20 min, and the supernatants were
144 collected.

145 Seven flavonoids (rutin, quercetin, kaempferol-3-rutinoside, kaempferol-
146 glucopyranoside, naringin, naringenin, isoliquiritigenin) were identified and
147 quantified by use of High performance liquid chromatography mass spectrometry
148 (HPLC-MS) system (LC/MS QQQ 6490, Agilent USA) and electrospray ionization
149 operated in positive mode (ESI^+). The flavonoids were separated on a zorbax sb-c18
150 column (100 × 2.1 mm^2, particle size 1.8 m; Agilent) at 30 °C. The mobile phase
151 consisted of water/ formic acid (A) (volume ratio = 99.9:0.1) and acetonitrile/formic
152 acid (B) (99.9:0.1) at a flow rate of 0.3 mL/min. Optimal separation was achieved
153 with a gradient elution: 95% A and 5% B in the beginning, maintained for 2 min,
154 changed to 75% A in 10 min, 5% A in 12 min, maintained for 3 min. The injection

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volume was 3 μL.

The optimized MS parameters were as follows: drying gas at 350 °C; capillary voltage 4.0 kV; drying gas flow 11 L/min; nebulizer pressure 45 psi. The reaction-monitoring mode with positive ESI was m/z 449.1→287.0 for kaempferol-glucopyranoside, m/z 581.2→273.1 for naringin, m/z 273.1→153.1 for naringenin, m/z 257.1→137.1 for isoliquiritigenin, and m/z 433.1→313.1 for the internal standard vitexin. Flavonoids were quantified by referring to standard curves of the seven flavonoids and vitexin dissolved in methanol/water (volume ratio = 4:1). Instrument control, data acquisition, and evaluation were finished with MassHunter Agilent 2003–2007 Data Acquisition for Triple Quad B.01.04 (B84).

RESULTS

Generation of the *NtCHS* overexpression tobacco plants

An overexpression construct pH7WG2D-*NtCHS* with *NtCHS* cDNA (AF311783.1) under the control of the CaMV35S promoter was transformed into *Nicotiana tabacum* K326 (Fig. 1A). Three independent putative positive lines were selected on the MS medium with kanamycin. The positive lines were further confirmed with genomic PCR using the primer pair *npt II*-F and *npt II*-R.

The PCR results showed the *npt II* gene was expressed in the three selected transgenic lines F1, F2 and F3, but not in the control tobaccos (Fig. 1B). Since the empty vector pH7WG2D contained a suicide gene ccdB between attR1 and attR2 sites, the plasmid pH7WG2D-*NtCHS* was successfully transferred into tobaccos. Transgenic lines were further selected by kanamycin resistance for subsequent analysis.

Expression patterns of the flavonoids genes in transgenic tobacco

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To investigate the expression patterns of the flavonoids genes, the leaves at the vigorous growing stage were harvested from the transgenic and control plants. The three transgenic tobacco lines (F1, F2 and F3) had significantly higher *NtCHS* transcript levels than those in the control plants (C1, C2 and C3), and thus they were chosen for subsequent experiments (Fig. 2A). Moreover, the expression levels of the *CHI, F3H, F3'H, FLS* and *DFR* genes in the transgenic tobaccos were also significantly higher than those in the wild-type tobacco plants.

Flavonoids accumulation in tobacco leafs

To investigate the effect of the NtCHS gene on the flavonoids biosynthesis in tobacco, we detected the flavonoids content in the transgenic and control leaves. As shown in Table 1, the contents of all the flavonoids detected were much hihgher in the transgenic plants than those in the control plants.

Drought tolerance of tobacco plants

To evaluate whether the *NtCHS* overexpression affects the tobacco drought tolerance, water deficit treatment was performed. As shown in Fig. 3A, the growth of the transgenic plants was significantly better than the control plants after 14 days treament.

To further characterize the performance of the transgenic tobaccos under drought stress, we monitored the changes in the concentrations of RWC, MDA and H_2O_2 before and after drought treatment. After 14 days of water shortage, the RWCs of transgenic lines F1, F2, and F3 were significantly higher than those in the control tobacco (Fig. 3B). Leaf MDA concentrations increased in the transgenic and control tobacco following drought stress (Fig. 3C).

The concentrations of the ROS were not significantly different under the drought treatment. However, the control tobacco plants had higher concentrations of

 doi:10.20944/preprints201906.0103.v1

205 H_2O_2 than the transgenic plants under drought treatment (Fig. 3D).

206

207 **DISCUSSION**

208 The flavonoids biosynthesis pathway is an important secondary metabolic
209 pathway, and the CHS is the first committed enzyme catalyzing the synthesis of
210 flavonoids branch. Previous work has suggested a role for CHSin the production of
211 flavonoids and in the improvement of plant tolerance drought stress [13, 21-24]. In
212 this work, overexpression of *NtCHS* in tobacco plants enhanced plant tolerance to
213 drought stress, and increased RWC. In addition, the transcripts of the flavonoids
214 biosynthesis genes and the flavonoids content were significantly upregulated in the
215 transgenic plants. Four of the seven measured flavonoids (rutin, quercetin,
216 kaempferol-3-rutinoside and kaempferol-glucopyranoside) showed higher contents
217 [25, 26], while the naringin, naringenin and isoliquiritigenin occupied the key
218 positions in the metabolic pathways. Overall, these results provide further evidence to
219 support the role of flavonoids in drought tolerance of plants [5, 7]

220 Exposure of plant species to drought conditions can result in increased
221 production of ROS, oxidative stress and cell membrane damage [27]. Flavonoids
222 affect plant physiology in response to external stressors [28] and play an important
223 role in the maintenance of cellular redox balance. It has been demonstrated that
224 flavonoids help prevent the formation of ROS and improve scavenging of ROS in
225 drought stressed plants [29-31]. Before exposure to the drought conditions, the
226 concentrations of MDA and H_2O_2 in the transgenic plants were similar to those in the
227 control plants. However, transgenic tobacco plants had lower concentrations of the
228 ROS and MDA than the control plants when suffering drought stress. MDA is a lipid
229 peroxidation product of plants that is produced under drought stress [32]. Therefore,

 doi:10.20944/preprints201906.0103.v1

our results further supported the role for the measured flavonoids (rutin, quercetin, kaempferol-3-rutinoside and kaempferol-glucopyranoside, aringin, naringenin and isoliquiritigenin) in maintaining the redox balance of transgenic tobacco in response to drought stress as indicated by the lower concentrations of MDA.

By using the 14-days drought tolerance treatment, we demonstrated that overexpression of the *NtCHS* gene in tobacco resulted in the improvement of plant drought tolerance. In addition, the transcripts of the key flavonoids pathway genes and the contents of seven flavonoids significantly increased in the transgenic plants. Furthermore, the concentrations of ROS were lower in the transgenic tobacco plants than those in the control plants, suggesting a role for flavonoids in maintaining cellular redox balance. The results in this study supported the *NtCHS* as the potential candidate gene to be targeted in genetic engineering of tobacco plants to enhance drought stress tolerance.

Acknowledgements: This work was supported by the Young Scientists Fund of Natural Science Foundation of China (31000137) and the Project of the Tobacco Genomic Program 110201601031（JY-05）

Conflict of interest disclosure: No conflict of interests is declared.

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253 **Tables**

254 **Table1** Quantification of seven flavonoids in transgenic and control tobacco lines by

255 use of HPLC-MS ESI$^+$.

Flavonoid	Dry weight (mg/g; mean±s.e.)					
	C1	C2	C3	F1	F2	F3
Rutin	1.2095 ±0.3667	0.8526 ±0.1499	0.7171 ±0.3721	9.8294 ±1.2524	24.1283 ±3.2131	42.5386 ±14.4964
Quercetin	0.4346 ±0.1447	0.7190 ±0.0708	0.5373 ±0.2265	7.5818 ±4.2555	10.2958 ±2.6337	12.138 1±5.4301
Naringenin	0.0333 ±0.0138	0.0371 ±0.0063	0.0345 ±0.0041	0.7983 ±0.3606	0.4717 ±0.0425	0.5750 ±0.0913
Kaempferol-3-rutinoside	0.0240 ±0.0034	0.2122 ±0.0268	0.0493 ±0.0197	7.4321 ±0.6083	12.1679 ±2.0946	16.0063 ±3.4738
Kaempferol-glucopyranoside	ND	0.1032 ±0.0131	ND	1.4398 ±0.3965	0.8898 ±0.1120	1.0927 ±0.3114
Naringin	ND	ND	ND	1.3620 ±0.4234	1.4423 ±0.1459	0.9575 ±0.1395
Isoliquiritigenin	0.0108 ±0.0030	0.0132 ±0.0024	0.0105 ±0.0016	0.0236 ±0.0067	0.0170 ±0.0023	0.0268 ±0.0039

256 ND, not detected (n=3)

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 doi:10.20944/preprints201906.0103.v1

FIGURE LEGENDS

Fig. 1 Schematic map of T-DNA region of pH7WG2D-NtCHS and confirmation of successful transformation. **A** Schematic representation of pH7WG2D harboring the *NtCHS* cDNA. **B** PCR using genomic DNA as the template confirms the introduction of npt II into the transgenic tobacco line. Npt II is absent in the wild-type tobacco lines C1, C2 and C3.

Fig. 2 Abundance of transcripts of flavonoid transcriptional genes for transgenic and control tobacco: (**A**) *CHS*, (**B**) *CHI*, (**C**) *F3H*, (**D**) *F3'H*, (**E**) *FLS* and (**F**) *DFR* measured by use of RT-qPCR. *: $p<0.05$; **: $p<0.01$.

Fig. 3 A Qualitative observations of drought experiment. **B** Relative water content (RWC) in transgenic and control tobacco. **C** Concentrations of MDA in transgenic and control tobacco. **D** Concentrations of H_2O_2 in transgenic and control tobacco. C1-C3: wild-type tobaccos, F1-F3: T1-generation transgenic tobaccos. *: $p<0.05$; **: $p<0.01$.

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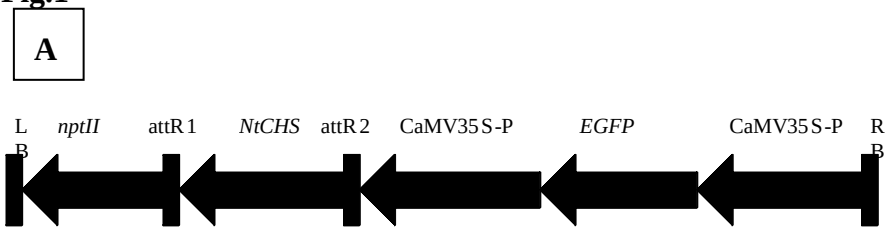
273 **Fig.1**



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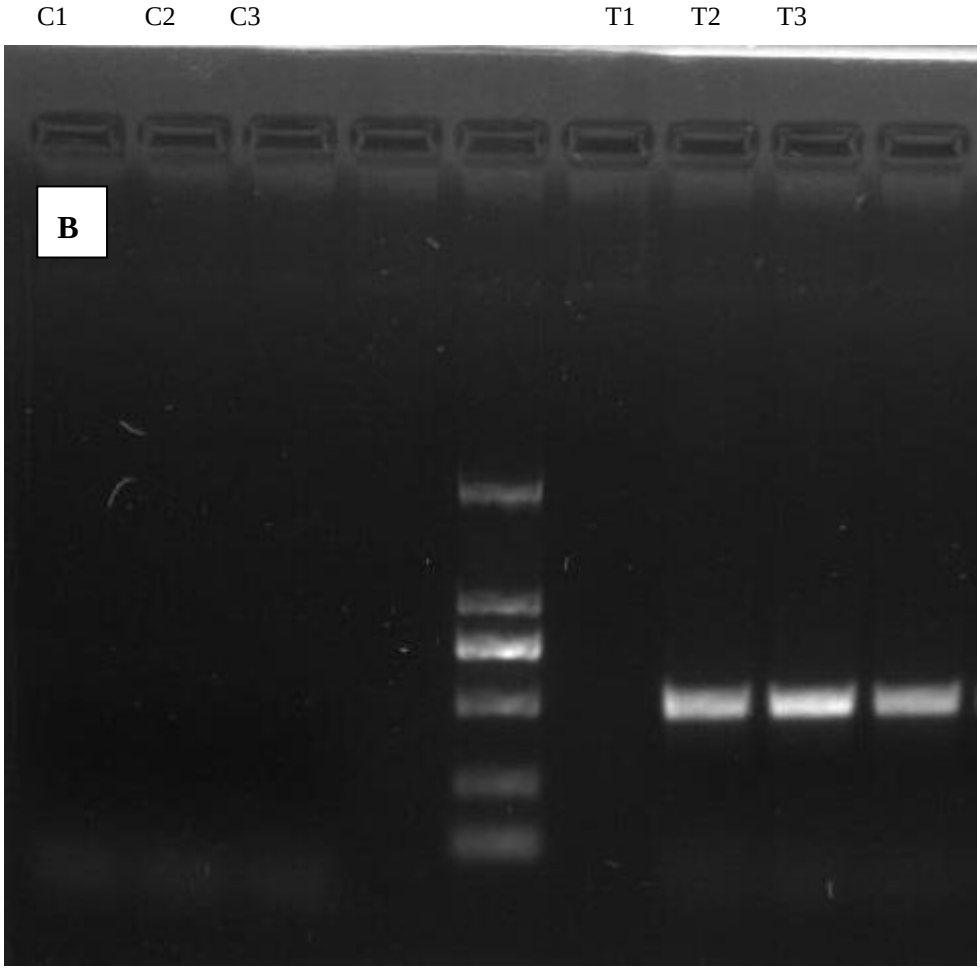

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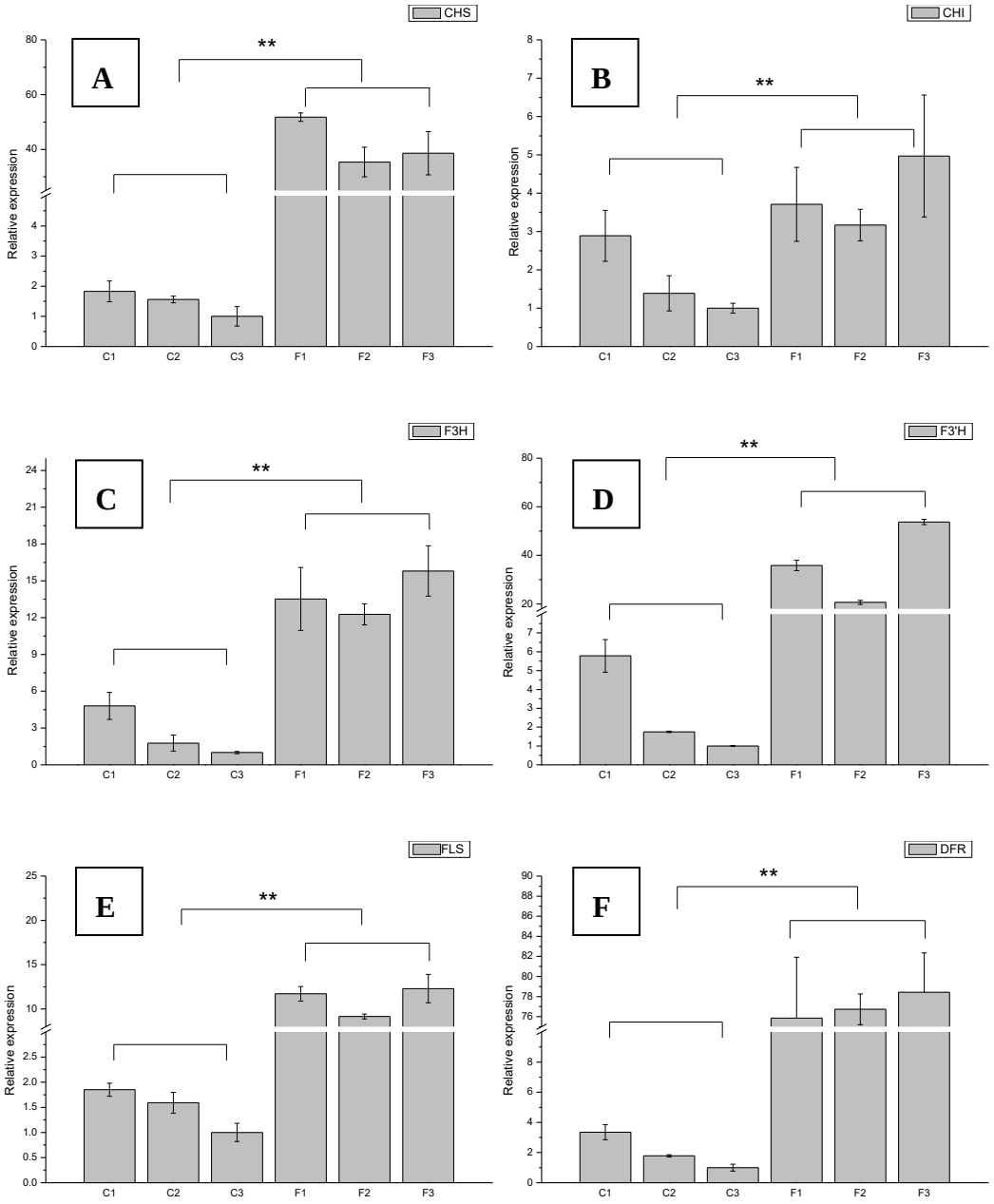
278 **Fig. 2**



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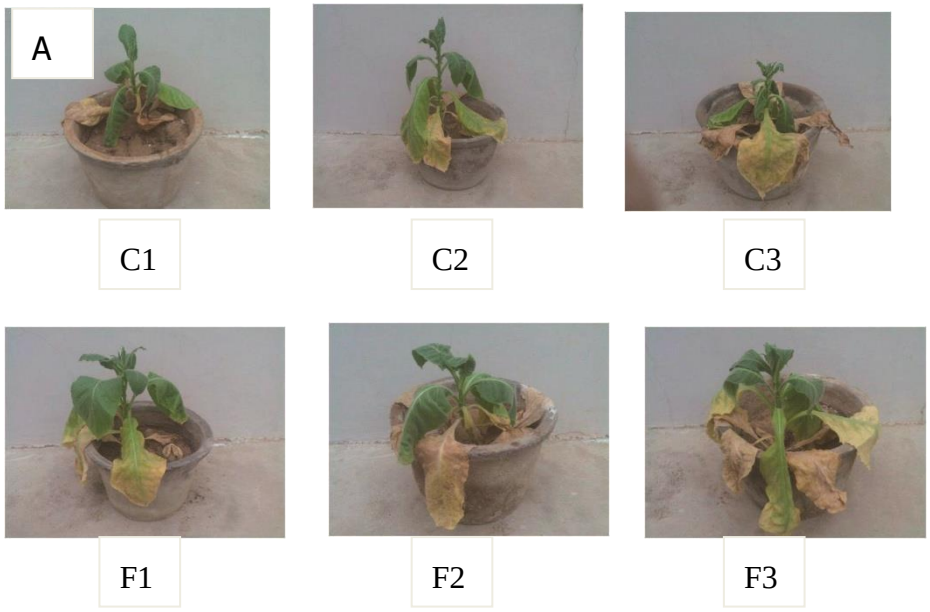
283 **Fig. 3 Fig.3**



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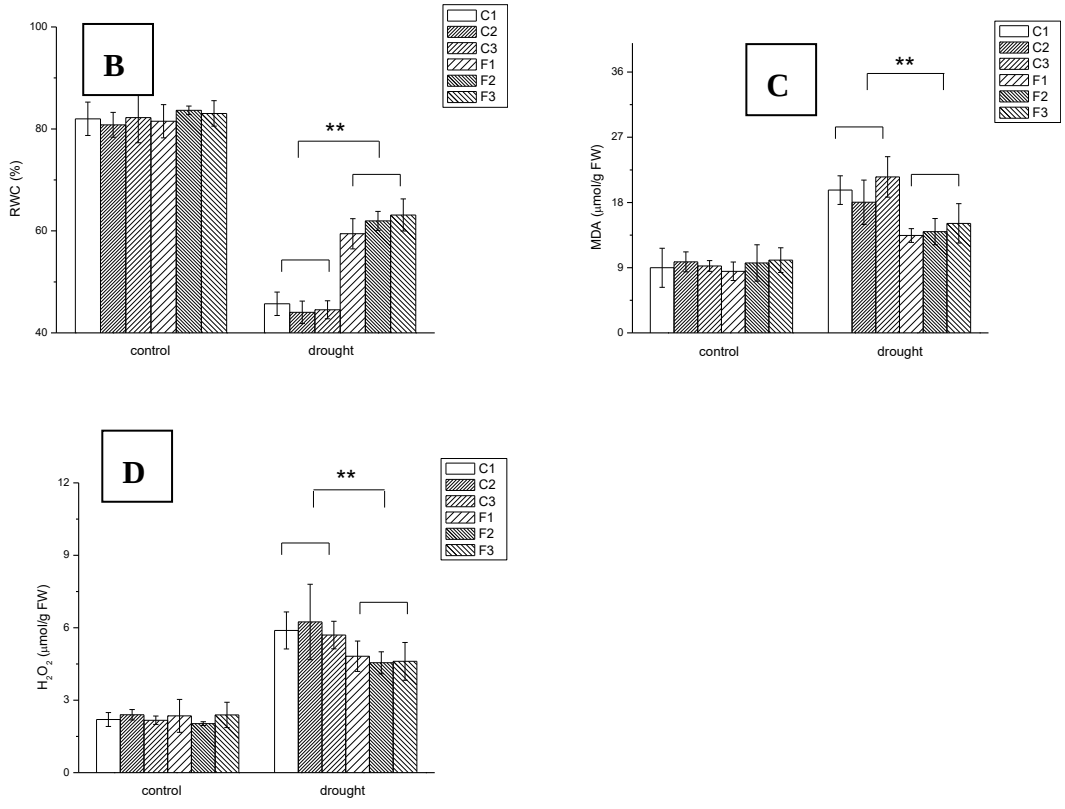

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