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1 **Functional analysis of overexpressed *PtDRS1* involved in abiotic stresses enhances**
2 **growth in transgenic poplar**

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15 **Abstract**

16 Drought and salinity are the two main abiotic stressors that can disrupt plant growth and
17 survival. Various biotechnological approaches have been used to alleviate the problem of
18 drought stress by improving water stress resistance in forestry and agriculture. The
19 drought sensitive 1 *(DRS1)* gene acts as a regulator of drought stress in some model plants,
20 such as *Arabidopsis thaliana,* but there have been no reports of *DRS1* transformation in

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21 poplar plants to date. In this study, we transformed the *DRS1* gene from *Populus*
22 *trichocarpa* into *Populus deltoides × Populus euramericana* 'Nanlin895' using *Agrobacterium*
23 *tumefaciens*-mediated transformation. We confirmed that the *DRS1* gene was transformed
24 into 'Nanlin895' poplar genomes using reverse transcription polymerase chain reaction
25 (PCR), multiplex PCR, real-time PCR, and sodium dodecyl sulfate-polyacrylamide gel
26 electrophoresis. All transformed and wild-type (WT) plants were then transferred into a
27 greenhouse for complementary experiments. We analyzed the physiological and
28 biochemical responses of transgenic plants under drought and salt stresses in the
29 greenhouse, and these results were compared with control WT plants. Responses to abiotic
30 stress were greater in transgenic plants compared with WT. Based on our results,
31 introduction of the *DRS1* gene into poplar 'Nanlin895' plants significantly enhanced the
32 resistance of those plants to water deficit and high salinity, allowing higher growth rates of
33 roots and shoots in those plants. Additionally, the clawed root rate increased in
34 transformed poplars grown in culture medium or in soil, and improved survival under
35 drought and salt stress conditions.

36

37 **Key words**

38 *DRS1*; Transformation; Transgenic poplar; Abiotic stresses; Physiological and biochemical
39 responses

40 **Abbreviations:**

41 *DRS1*: Drought sensitive 1, RT-PCR: Reverse transcription polymerase chain reaction, SDS-
42 PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, MDA: Malondialdehyde,

43 SOD: Superoxide Dismutase, POD: Peroxidase, ROS: Reactive Oxygen Species, PEG: Poly
44 Ethylene Glycol, WT: Wild type

45

46 1. Introduction

47 Reduction of growth rates below their optimum levels occurs under the environmental
48 conditions referred to as abiotic stress (Cramer et al. 2011). Plants respond dynamically
49 and reversibly to abiotic stress (Skirycz and Inzé 2010), because they must adapt to new
50 conditions, and this response affects their growth and development (Santner and Estelle
51 2009). Abiotic stresses such as high salinity and drought always cause reductions in plant
52 growth (Huang et al. 2012). Drought is an abiotic environmental factor and major stressor,
53 which can decrease the plant's development rate and its productivity (Agarwal et al. 2013).
54 The nucleocytoplasmic receptors pyrabactin resistance (PYR), pyrabactin resistance-like
55 (PYL), and regulatory component of abscisic acid (ABA) receptor (RCAR) bind to ABA
56 phytohormone and inhibits type 2C protein phosphatases (PP2Cs) such as ABI1 and ABI2
57 (Park et al. 2009). Inactivation of PP2Cs leads to accumulation of active SNF1-related
58 protein kinases/SnRK2s (Park et al. 2009; Umezawa et al. 2009). SnRK2s regulate ABA-
59 responsive transcription factors such as ABA-responsive element binding proteins (AREBs),
60 leading to activation of ABA-responsive genes and ABA-related processes (Umezawa et al.
61 2009). Drought sensitive 1 (*DRS1*) is an ABA-dependent responsive gene required for ABA-
62 dependent signaling (Lee et al. 2010). ABA allows adaptation of plant cells to drought
63 stress as a phytohormone and produced under drought stress and high salinity conditions.
64 ABA promotes tolerance to drought and salt stresses by controlling stomatal closure during
65 daytime to avoid loss of water as well as activation of stress-related genes in plant cells

66 (Nakashima et al. 2012). These stress-related genes can protect plant cells through the
67 production of functional regulatory proteins and enzymes for cell signal transduction
68 (Nakashima et al. 2012). The *DRS1* gene is upregulated under water deficiency, and
69 promotes tolerance to drought stress mediated by ABA. This gene encodes a WD-40 repeat
70 family protein involved in DDB1-CUL4-mediated protein degradation during the drought
71 response, which was confirmed genetically using a genomic functional network modeling
72 system. These findings suggest a significant association between *DRS1* and ABA-mediated
73 responses to water deficiency in plants (Lee et al. 2010, 2011; Winkler et al. 2015).

74 ## 2. Material and methods

75 ### 2.1. Transformation and plant materials

76 In this study, we used wild-type (WT) 'Nanlin895' *Populus deltoides* × *P. euramericana*
77 poplar plants for the preparation of seedlings, pre-culturing, and the collection of leaf disks
78 for transformation. Total RNA was extracted from young leaves of WT *Populus trichocarpa*
79 to isolate the complete coding DNA sequence (CDS) of the *DRS1* gene. The binary vector
80 *PBI121* plasmid selected for this experiment harbored the *DRS1* gene. This vector was
81 introduced to the EHA105 strain of *Agrobacterium tumefaciens* using the freeze-thaw
82 method (Holsters et al. 1978). The cloned *PBI121:DRS1* plasmid was transformed into
83 bacteria, which were grown in Luria-Bertani (LB) medium containing 50 mg/L kanamycin
84 and 50 mg/L rifampicin in the dark at 28 °C for 72 h. After growing the bacteria, we
85 prepared an infective suspension of regenerated *Agrobacterium tumefaciens* in liquid LB
86 medium to inoculate the leaf disks over an immersion time of 60 min at 28 °C with shaking
87 at 200 rpm (OD_{600} = 0.7). After immersion, the explants were dried in a laminar airflow
88 cabinet using sterile Whatman filter paper, then the leaf disks were co-cultivated on

89 Murashige and Skoog (MS) plates containing 0.004 mg/L thidiazuron (TDZ), 0.5 mg/L N-6-
90 benzyladenine (6-BA), 25 g/L sucrose, 6 g/L agar, and 200 μM acetosyringone (AS) at pH 5,
91 followed by incubation in the dark at 28 °C for 48 h (Movahedi et al 2015a). Afterward, the
92 putative transformed explants were cultivated on selective MS medium supplemented with
93 6-BA (0.5 mg/L), TDZ (0.004 mg/L), agar (6 g/L), sucrose (25 g/L), cefotaxime (400 mg/L),
94 and kanamycin (50 mg/L) at pH 5.8, under variable phytotron conditions with 16 h light, 8
95 h dark, and average temperatures of 23–25 °C. Well-developed putative transgenic and WT
96 explants were transferred to MS medium containing agar (6 mg/L), sucrose (25 mg/L), and
97 cefotaxim (300 mg/L) at pH 5.8 under the phytotron conditions described above. *DRS1*
98 overexpression was verified in 40 lines of putative transgenic shoots using reverse
99 transcription (RT)-polymerase chain reaction (PCR), multiplex PCR, real-time PCR, and
100 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses. Verified
101 transgenic plants from 34 lines were selected and transferred to half-strength MS (Rooting)
102 media supplemented with (0, 2, 4, and 6 %) polyethylene glycol (PEG_{6000}) and (0, 25, 50,
103 and 100 mM) NaCl for evaluation of drought and salt tolerances, respectively. Well-
104 developed poplars under drought and salt stress were transferred to the greenhouse for
105 complementary physiochemical experiments.

106 **2.2. Multiplex PCR**

107 Transformed plants were confirmed using multiplex PCR via extraction of genomic DNA
108 using the cetyl trimethylammonium bromide method (Doyle and Dickson 1987). The 731-
109 base pair (bp) fragment of the CamV35S promoter located in the transfer DNA (T-DNA)
110 region was amplified with the primers F 5'- TTACGCAGCAGGTCTCATCAA- 3' and R 5'-

111 GCGAAGGATAGTGGGATTGTG- 3'. Moreover, a 1542-bp fragment of the *DRS1* gene was
112 amplified with the specific primers F 5' –ATGGCGACTCAGAAACTAACAGAATAC -3' and R 5'-
113 CTAGCTTGACGTCCAAATGTAAACCTGG -3'. Multiplex PCR was performed with the
114 following conditions: 94 °C for 3 min; 40 cycles of 94 °C for 40 s, 60 °C for 35 s, and 72 °C
115 for 2 min; and finally 72 °C for 10 min. The PCR products were electrophoresed on 1.2 %
116 agarose G-10 with the DL-2000 DNA marker and visualized in a Bio-Rad Universal Hood-II
117 Molecular Imager Gel System.

118 **2.3. RT-PCR**

119 RT-PCR was performed to compare the expression levels of *DRS1* in the transgenic and WT
120 lines. Total RNA was extracted from fresh leaves using the Plant RNA kit (Omega Bio-tek,
121 China). Reverse transcription was performed using 4 µg of extracted RNA and oligo-dT
122 primer to synthesize the first complementary DNA (cDNA) strand of *DRS1* using the
123 Primescript™ One Step RT-PCR kit Ver. 2 (Takara Biotechnology, Dalian, China) according
124 to the manufacturer's instructions. The concentrations of extracted RNA and DNA were 660
125 and 1100 ng/µL, respectively which determined using a BioDrop (UK) spectrophotometer.
126 The forward F 5'- ATGGCGACTCAGAAACTAACAGAATAC -3' and reverse R 5' -
127 CTAGCTTGACGTCCAAATGTAAACCTGG -3' primers were designed using SnapGene
128 software and used to amplify a 1542-bp fragment of *DRS1*. We used 3 µL of cDNA template
129 and the following reaction conditions: 94 °C for 4 min; 40 cycles of 94 °C for 40 s, 60 °C for
130 35 s, and 72 °C for 120 s; and finally 72 °C for 10 min in a 50-µL PCR reaction volume. The
131 *DRS1* gene was visualized using 1.2 % agarose gels with the DL2000 DNA marker.

132 **2.4. Quantitative real-time (QRT)-PCR**

133 QRT-PCR was used to assess the copy number (based on a standard curve) and expression
134 level of the *DRS1* gene ($\Delta\Delta C_t$) in the transgenic and WT lines using a Step One-Plus PCR
135 system (Applied Biosystems, USA) and Fast Start Universal SYBR Green Master Mix (Rox;
136 No. 04913914001, Roche, USA). We used 2 μg of cDNA synthesized from the leaves, stems,
137 and roots of transformant and WT poplars, with three independent repeats of each
138 experiment. For the standard curve method, we diluted synthesized cDNA (500-, 250-, 125-,
139 62.5-, and 31.25-fold) and amplified it using the primers F 5'-
140 GGAATGTGGATAGTGAAGGGGAGAA -3' and R 5'- AGCATCCATTAAACGGAGATATCCATCA -
141 3' to separate a 149-bp fragment of *DRS1* and F 5'- GACCTTCAATGTGCCTGCAA -3' and R 5'-
142 ACCATCACCAGAATCCAGCA -3' to isolate a 100-bp fragment of β-*actin* to normalize the
143 results.

144 **2.5. SDS-PAGE**

145 SDS-PAGE analysis was performed using total protein extracted from fresh leaves of the
146 transformant and WT plants. Protein was extracted using a Solarbio plant protein
147 extraction kit (Beijing Solarbio Science & Technology, China) according to the
148 manufacturer's instructions.

149 **2.6. Phenotypic analyses in MS medium**

150 To perform phenotypic analyses of well-developed transgenic and WT poplars in MS
151 rooting medium under drought and salt stress conditions, we compared the stem height,
152 number of leaves, and number of main roots, three times during growth at 0, 15, and 30
153 days. We also calculated the clawed root rates ([main roots/total roots] × 100) of the
154 transgenic and WT lines growing under drought and salt stress in MS media containing 0
155 and 2 % PEG, and 0 and 25 mM NaCl, respectively (Movahedi et al. 2015a).

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156 ## 2.7. Physiological and biochemical analyses in greenhouse

157 To analyze the physiological and biochemical features of transformed and WT poplars
158 under drought and salt stresses, plants resistant to drought and salt stress on MS medium
159 were selected and replanted into plastic pots filled with homogenized soil, and transferred
160 to a greenhouse at 18–23 °C, 60 % humidity, and with 18 h of light and 6 h of dark daily. All
161 WT and transformant plants were watered for 45 days and acclimated completely to the
162 greenhouse conditions before the introduction of stressors. Drought and salt tolerances
163 were analyzed in the greenhouse by evaluation of biochemical (chlorophyll, carotenoid,
164 malondialdehyde [MDA], and free proline contents, and superoxide dismutase [SOD] and
165 peroxidase [POD] activities) parameters of the transgenic and WT lines under a water-
166 withholding regime and irrigation of plants with 0, 25, 50, and 100 mM NaCl for 20 days,
167 respectively. To study the physiological effects of drought stress on *DRS1* transgenic and
168 WT poplars during the stress period in the greenhouse, we evaluated the stem length, stem
169 diameter, numbers of leaves and clawed roots of all plants.

170 ### 2.7.1. Photosynthetic pigment analysis

171 For analysis of photosynthetic pigments, we pulverized 0.1 g of fresh young leaves of WT
172 and transformant plants in liquid nitrogen and added 10 mL of 80 % acetone. After
173 incubation at 4 °C for 24 h in the dark, the mixture was centrifuged at 10,000 × g and
174 collected the supernatant. The absorption spectra were recorded at 663.8, 646.8, and 470
175 nm. Then, the contents of chlorophyll a (C_a), chlorophyll b (C_b), total chlorophyll (C_{a+b}), and
176 total carotenoid (C_{x+c}) were calculated according to the experimental equations reported by
177 Lichtenthaler et al. (1987).

178 **2.7.2. MDA, SOD, and guaiacol POD**

179 We measured the content of MDA in leaves to estimate the oxidative damage to cell
180 membrane lipids. About 0.3 g of fresh WT and transformant leaves were homogenized in
181 10 mL of 10 % trichloroacetic acid (TCA), then centrifuged at 12,000 × *g* for 10 min and
182 collected the supernatant in 2 mL microtubes. Next, 2 mL of 0.6 % thiobarbituric acid in
183 10 % TCA was added to 2 mL of the collected supernatant and heated the mixture in boiling
184 water for 30 min, then cooled it on ice. This mixture was centrifuged at 10,000 × *g* for 10
185 min and absorbance of the supernatant was measured at 440, 532, and 600 nm. The MDA
186 content was calculated according to the method of Hodges (1999). Calculation of SOD and
187 POD activities was carried out according to the methods of Satoh (1978) and Pagariya et al.
188 (2012), respectively. Activity of SOD (EC 1.15.1.1), POD (EC 1.11.1.7), and MDA was
189 determined using BioDrop spectrophotometer (Cambridge, UK).

190 **2.7.3. Free proline content**

191 To determine free proline content, we followed the method of Bates et al. (1973). We
192 collected 0.5 g of leaves from stressed and WT plants, homogenized in 8 mL of 3 % aqueous
193 sulphosalicylic acid and centrifuged at 8000 × *g* for 15 min. The reaction mixture
194 containing 2 mL of supernatant, 2 mL of acid ninhydrin, and 2 mL of glacial acetic acid was
195 boiled at 100 °C for 1 h, and the reaction was terminated by cooling in an ice bath. The free
196 proline was extracted using 4 ml of toluene, and the absorbance was determined at 520 nm
197 with a BioDrop spectrophotometer (Cambridge, UK).

198 **2.8. Statistical analysis**

199 Statistical analyses were performed using analysis of variance for all variables, with mean
200 separations determined via Duncan's test in SPSS software (Statistical Package for the

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201 Social Sciences, version 16; SPSS Inc., Chicago, IL, USA). Variables were used to test for
202 statistically significant differences, and the confidence intervals showed no overlap of mean
203 values, with an error value of 0.05.
204

205 3. Results

206 Based on our data, processed using the CLC Genomics Workbench 3 software (CLC, Taipei,
207 Taiwan), the *PtDRS1* gene contains an open reading frame with the complete 1542-bp CDS.
208 The *PtDRS1* gene of *Populus trichocarpa* encodes 513 amino acids and the protein's
209 molecular weight of 57.018 kDa, is located on the forward strand of chromosome XVIII
210 from positions 12,657,000 to 12,660,000 bp, and contains 5 exons and 4 introns. We
211 deposited our *PtDRS1* data to the National Center for Biotechnology Information online
212 system (NCBI) under accession number MF663784 (Fig. S1).

213 The pBI121 expression vector with a length of 14,758 bp genomic DNA (Genbank
214 accession number AF485783.1), was used in this study as it harbored the *DRS1* gene and
215 two restriction enzyme sites, *BamHI* and *SacI*. The complete T-DNA area of the *PBI*
216 *121/DRS1* plasmid, including the *NOS* promoter, terminator, and *CamV35S* selectable
217 marker, was transformed into plants and visualized using multiplex PCR (Fig. S2).

218 To clarify the phylogenetic relationship between DRS1 protein in poplar and other
219 plant species, we identified the DRS1 protein in the *A*. *thaliana* genome using The
220 *Arabidopsis* Information Resource (TAIR) database, and performed a search of the
221 annotated DRS1 protein using the Basic Local Alignment Search Tool (BLAST;
222 https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) to determine the similarity of this gene
223 among *Populus trichocarpa*, *Populus euphratica*, and other species of plants. We used 10

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224 open reading frame sequences from *Nicotiana tomentosiformis*, *Vitis vinifera*, *Arabidopsis*
225 *thaliana*, *Brassica rapa*, *Populus trichocarpa*, *Populus euphratica*, *Theobroma cacao*, *Ricinus*
226 *communis*, *Jatropha curcas*, and *Glycine max* obtained from Genbank which were aligned
227 using Geneious R10.3 software to construct a phylogenetic tree (Fig. 1 and Fig. S3).

228 To verify successful genetic transformation, the putative transformed leaf explants
229 were transferred to MS selective medium supplemented with 50-μg/mL kanamycin (Figs.
230 S4A, B). The developed explants were then transferred into MS shoot elongation medium,
231 which can induce shoot formation (Fig. S4C), and after 4 weeks they were moved to half-
232 strength MS medium to encourage rooting without hormones (Fig. S4D).

233 As shown in Figure 2 (B–D), the putative transformants exhibited higher and longer-
234 term rates of clawed roots than WT (Fig. 2A) in half-strength MS media. Well-grown plants
235 in rooting MS media (Fig. 2E) were replanted in homogenized soil in plastic pots and
236 transferred to a greenhouse (Fig. 2F).

237 **3.1. Multiplex PCR, RT-PCR, SDS-PAGE, and real time PCR analyses**

238 Multiplex PCR was performed to confirm transformation of *DRS1* (1542 bp) from the
239 PBI121:*DRS1* plasmid into putative transgenic poplars. Figure S5 shows that all transgenic
240 lines were integrated with the T-DNA, including the *CamV35S* promoter that was absent in
241 WT plants.

242 In addition, the results of the RT-PCR showed high expression of the *PtDRS1* gene in
243 transgenic lines compared with WT. The intensity of bands was measured using ImageJ
244 software ver. 1.5b (Fig. S6).

245 To compare the *DRS1* expression levels in WT and transgenic lines using SDS-PAGE,
246 we selected six independent transgenic plants: T-4-4, T-4-2, T-4-3, T-2-2, T-4-1, T-2-4.
247 Total DRS1 protein was separated using 10 % SDS-PAGE and Coomassie Blue R-250 (Bio-
248 Rad Mini-Protein Tetra System, USA). As expected, the DRS1 protein showed thicker bands
249 in transgenic lines compared to WT, with a molecular weight of 57.018 kDa (Fig. S7).
250 The transgene copy numbers of *DRS1* were determined via Real time PCR based on
251 the formula $X = Y$-intercept/slope degrees (X = *copy number*, $Y = C_t$), (Movahedi et al.
252 2015b). The results revealed that the average gene copy number of *DRS1* in transgenic
253 plants is 10.86 with a slope of –3.70 and an R^2 value of 0.996 (Fig. S8A), whereas the
254 average *DRS1* gene copy number in WT poplars is 8.63, with a slope of –3.83 and R^2 value
255 of 0.992 (Fig. S8C). In addition, C_t values for the *DRS1* gene of transgenic and WT lines
256 during the exponential phase of amplification were 1.84 (Fig. S8B) and 1.12 (Fig. S8D)
257 respectively.
258 Real-time PCR comparative analysis using the $\Delta\Delta C_t$ method was performed with
259 expression of the *β-actin* housekeeping gene as an internal reference. The results of this
260 analysis revealed higher expression levels of the *DRS1* gene in the leaves, roots, and stems
261 of eight independent transgenic lines (T-2-4, T-4-1, T-4-3, T-4-4, T-6-O, T-N-A, T-2-2, and T-
262 4-2) compared to the WT (Fig. 3A–C). Furthermore, the average expression levels of the
263 *DRS1* gene in the leaf, stem, and root tissues of transformants revealed that *DRS1* is
264 predominantly expressed in leaves, with 1.21-fold higher expression than WT poplars. In
265 addition, the average expression levels of root and stem tissues of transgenic plants were
266 1.11- and 1.18-fold higher than those of the WT (Fig. 3D).

267 **3.2. Evaluation of drought and salt tolerance in MS culture media**

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268 We studied the effects of *DRS1* overexpression on the growing shoots, roots, and leaves of
269 transgenic poplars growing in MS culture media containing NaCl and PEG_{6000} to investigate
270 salt and drought stresses, respectively. Evaluation of salt tolerances of WT and transgenic
271 poplars in MS media containing 0, 25, 50, and 100 mM NaCl revealed that the growth of
272 stem in WT plants was completely stopped, also the number of leaves were reduced and
273 their leaves turned yellow and withered (Figs. S9 A, B).

274 Transgenic and WT plants exhibited equal rates of main root growth in medium
275 lacking NaCl over 30 days of stress. In contrast, *DRS1* transgenic poplars remained
276 unaffected and exhibited normal growth of roots, stems, and leaves in medium containing
277 25 mM NaCl, and were able to root with 50 and 100 mM NaCl (Fig. 4 A and B).

278 Evaluation of drought tolerances of WT and transgenic plants was carried out
279 through the culture of plants in MS media containing 0, 2, 4, and 6 % PEG_{6000}. Based on our
280 data (Fig. 5A and B), transgenic poplars showed an increase by 1.14-fold higher rate of root
281 growth than WT on the last day of testing in media lacking PEG. On the 15th day of drought
282 stress, the transformed plants were rooted in MS media containing 0, 2, and 4 % PEG at 2.3-,
283 2-, and 1.5-fold higher rates than WT lines, respectively.

284 Moreover, on the 30th day in medium containing 2 % PEG, transgenic plants rooted at
285 a 2-fold higher rate than WT. At day 15, the WT plants had not rooted in medium
286 containing 4 % PEG, but by the last day of drought stress, all WT and transgenic plants
287 exhibited the same rates of root growth in medium with 4 % PEG. Comparison of plant
288 roots in medium containing 6 % PEG revealed that WT plants were unable to root in this
289 medium, while transgenic plants exhibited a 2-fold higher rate of rooting than WT during
290 the stress period (Fig. 5B).

291 Calculation of stem growth rate under drought stress in medium lacking PEG revealed
292 that all of the WT and transgenic plants maintained normal growth of shoots during the
293 stress period. Comparison of stem length between WT plants in media lacking PEG and
294 with 2 % PEG revealed a 1.36-fold reduction of stem length in the plants grown under
295 drought conditions, whereas transgenic poplars grown in MS media containing 0 and 2 %
296 PEG indicated normal rates of stem growth under drought stress. Treatment of WT plants
297 with higher PEG concentrations, such as 4 % and 6 %, suppressed stem growth in those
298 plants over 30 days, whereas all transgenic lines maintained normal stem growth rates in
299 media containing 4 and 6 % PEG during the stress period (Fig. S10A).

300 Over 30 days of drought stress, the leaves on WT plants withered and decreased in
301 number when grown on MS media containing 4 and 6 % PEG. The calculation of leaf
302 numbers in WT and transgenic lines also revealed more leaves in transgenic poplars
303 compared to WT at various concentrations of PEG. The transgenic poplars not only
304 maintained their growth rates in media containing 0, 2, and 4 % PEG, but also retained
305 their leaves in medium containing 6 % PEG over 30 days of drought stress (Fig. S10B).

306 We analyzed the clawed root rate of the WT and transgenic lines under drought and
307 salt stresses in MS medium. Our results revealed that when grown in medium lacking PEG,
308 the clawed root rate of transgenic plants was 2.8-fold higher than that of WT plants,
309 whereas in MS medium containing 2 % PEG, clawing roots increased dramatically to 16-
310 fold higher than WT (Fig. 6A). Evaluation of the clawed root rate under salt stress was
311 performed by growing transgenic and WT plants in MS media containing 0 and 25 mM NaCl.
312 Our results indicated that after 15 days, the transgenic plants exhibited 1.83-fold higher
313 clawed root rates than WT in medium lacking NaCl. The rate of clawed roots in transgenic

314 plants increased, reaching 2.2-fold higher in transformants than WT on the last day of
315 stress. In MS medium containing 25 mM NaCl, WT plants did not root, but transgenic plants
316 rooted during the 30-day stress period (Fig. 6B).

317 3.3. Evaluation of drought and salt tolerances of transgenic poplars in a 318 greenhouse

319 Evaluation of the physiological changes in transgenic and WT plants was performed after
320 45 days of well-irrigated acclimatization of plants to the greenhouse. We compared
321 transgenic and WT plants after 20 days of drought or salt stress to determine the
322 physiological (shoot length, stem diameter, and leaf number) and biochemical (total
323 chlorophyll, carotenoid, MDA, free proline contents; SOD and POD activities) differences.
324 For the 45 days prior to drought stress, the plants were irrigated with 150 mL of water in a
325 greenhouse, and during this time the lengths of the stems of WT plants were slightly
326 shorter than those of transgenic plants, although the differences between WT and
327 transformants increased dramatically after the stress period. Evaluation of drought and salt
328 tolerances of three independent transgenic (T-4-2-1, T-4-4, and T-4-2-2) and WT lines
329 revealed that over 20 days of drought stress, the transgenic plants exhibited greater stem
330 lengths and diameters in addition to a greater number of leaves and clawed roots than WT
331 (Fig. 7A–D).

332 Our study of three independent transgenic and WT lines revealed that the average
333 growth rates of stem length, stem diameter, and number of leaves in transgenic lines under
334 drought stress were 1.29-, 1.13-, and 1.45-fold higher than those of the WT, respectively
335 (Fig. 8A–C).

336 On the first day of drought stress, the average number of leaves in the transgenic lines
337 was smaller than in the WT, but those leaves withered and decreased in a number of WT
338 plants during the 20-day stress period (Fig. 8C). Moreover, the rate of clawed roots in
339 transgenic lines T-1, T-2, and T-3 was 2.19-, 1.71-, and 1.68-fold higher than WT on the last
340 day of drought stress (Fig. 7D). The photosynthetic pigment levels under drought and salt
341 stresses were calculated based on the method of Lichtenthaler (1987). Our data revealed
342 that under normal conditions, the transgenic poplars had slightly higher levels of total
343 chlorophyll and carotenoids than WT (Fig. 9A and B). Under different levels of salt stress
344 with 25, 50, and 100 mM NaCl, the total chlorophyll content in WT and transgenic plants
345 decreased, in contrast with carotenoid content, which increased in those plants. The
346 transformed plants grown under salt stress exhibited higher contents of chlorophyll and
347 carotenoids, which were 2.33- and 2.31- fold higher than WT, respectively (Fig. 9A). Under
348 drought stress, the total chlorophyll and carotenoid content in the transformants increased
349 slightly after 10 days, but this increase was not observed in the WT. The total chlorophyll
350 content was decreased under drought stress in both the transformants and WT on day 30,
351 but it was dramatically higher in the transformants at 5.29-fold more than in the WT (Fig.
352 9B). Evaluation of carotenoid content on the last day of drought stress also revealed
353 increasing carotenoid levels in both the WT and transformants, with a 2-fold higher
354 carotenoid level in transformants (Fig. 9B).

355 The transgenic and WT plants grown under normal conditions exhibited similar SOD
356 activity, whereas the transgenic plants exhibited higher POD activity than WT under
357 normal conditions. Under drought and salt stresses, the transgenic plants exhibited 1.2-
358 and 1.4-fold higher SOD activity than WT, respectively (Fig. 9C and D). POD activity under

359 drought and salt stresses in transgenic plants was 1.71- and 1.69-fold higher than in the
360 WT, respectively (Fig. 9E and F).

361 Evaluation of proline content in the absence of drought or salt stress indicated a
362 negligible difference between WT and transgenic plants, whereas comparison of proline
363 revealed 1.75- and 1.95-fold higher levels in transgenic plants under drought and salt
364 stresses, respectively (Fig. 9G and H). Our data revealed that under normal conditions, the
365 transgenic lines had slightly higher MDA than the WT but, under drought and salt stresses,
366 MDA decreased by 1.11- and 1.31-fold, respectively, in transgenic plants compared with the
367 WT (Fig. 9I and J).
368

369 ## 4. Discussion

370 Reduction of water content due to water stress can decrease cell size and growth rates in
371 plants (Shao et al. 2008). Drought avoidance is achieved through a variety of adaptive traits,
372 such as minimization of water loss and optimization of water uptake via reduced
373 transpiration and increased rooting, respectively (Basu et al. 2016). An accepted
374 physiological definition of stress in the plant sciences refers to a plant's responses to
375 various environmental conditions. Plants exhibit physiological, biochemical, and molecular
376 reactions to different environmental conditions to minimize the destructive effects of both
377 abiotic and biotic stresses, such as drought and salinity (Shao et al. 2008). Plants avoid
378 water deficiency by modifying their root and stem growth rates as well as leaf biomass
379 production (Wang et al. 2012). Our observations revealed that overexpression of *DRS1*
380 gene in transgenic poplar 'Nanlin895' plants can modify these physiological reactions and
381 improve water uptake through larger numbers of roots under both drought and salt
382 stresses. The root characteristics, such as root length, root length density, and the number

383 of main roots, are essential for plants to have well-established above ground parts by
384 capture more water from soil (Manivannan et al. 2007). One adaptation mechanism to
385 drought stress in plants is enhancing water uptake ability by using a deep root system to
386 escape from drought (Paez-Garcia et al. 2015). Some *Populus* species have been shown to
387 exhibit a significant decrease in root length under drought stress (Nautiyal et al. 2002). We
388 compared the roots of transformed and WT plants after drought stress in a greenhouse on
389 MS medium, and the results revealed that overexpression of *DRS1* affected the roots of
390 transformants, and thus improved water uptake by those plants during drought stress via
391 growth of longer and thicker roots in transformed poplars. The stem length of populus
392 species decreased under drought stress (Smirnoff 1998). According to Nautiyal et al.
393 (2002), a significant reduction of shoot height was observed in *Populus cathayana* under
394 drought stress. Mohsenzadeh et al. (2006) reported that the level of tolerance or sensitivity
395 to water stress depends on the length and severity of water loss as well as the
396 developmental stage of the plants. We revealed that WT plants were significantly affected
397 by PEG concentrations of 2, 4, and 6 % in MS medium and stem elongation completely
398 suppressed under drought stress for 30 days, whereas in transgenic poplar plants
399 overexpressed *DRS1*, the stem length increased normally at all PEG concentrations during
400 drought stress period. Water deficits reduce the number of leaves in many species of plants,
401 including *Populus* (Shao et al. 2008). Based on our observations, the number of leaves on
402 WT plants under drought stress decreased in MS media containing 4 and 6 % PEG, whereas
403 transgenic plants exhibited normal leaf growth in 4 % PEG, and retained their leaves in
404 medium containing 6 % PEG. Hence, drought stress did not affect leaf number in transgenic
405 poplars after expression of the *DRS1* gene. ABA is a major phytohormone that regulates

406 various processes, and enables plant adaptation to drought stress (Wilkinson et al. 2012).
407 ABA is synthesized in roots and translocated to leaves to initiate adaptation in plants
408 encountering drought stress through stomatal closure and reduction of plant growth
409 (Wilkinson and Davies 2010). ABA also plays a major role in plant responses to drought,
410 cold, and osmotic stresses (Movahedi et al. 2015a). The *DRS1* gene is directly upregulated
411 upon drought stress in an ABA-dependent system (Winkler et al. 2015; Zhu et al. 2015),
412 and causes increased drought tolerance mediated by ABA in response to environmental
413 stresses (Lee et al. 2010, 2011). Reactive oxygen species (ROS), including singlet oxygen
414 ($^1O_2$), hydroxyl radicals (OH^-), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), play
415 essential roles in plant immune responses and control plant adaptations to biotic and
416 abiotic stresses (Qi et al. 2017). The exposure of plants to abiotic stresses such as drought
417 and salt causes increased ROS levels and the accumulation of ROS leads to an increase in
418 the concentration of ABA. High levels of ROS are harmful to plants and can damage proteins,
419 nucleic acids, and chlorophyll (Choudhury et al. 2017; Movahedi et al. 2015a; Ma et al.
420 2013). The oxidative damage that results from the accumulation of ROS in plants is reduced
421 by a complex defense mechanism that includes enzymatic and nonenzymatic antioxidant
422 systems such as SOD, POD, catalase, and glutathione reductase. Carotenoids are
423 nonenzymatic antioxidants, which can inhibit lipid peroxidation, quench chlorophyll and O_2,
424 and stabilize membranes to scavenge ROS in plants (Prochazkova et al. 2001; Yuan et al.
425 2016). Environmental stressors have been shown to increase carotenoid content in
426 addition to enhancing light-protection mechanisms, leading to increased contents of
427 chlorophyll and carotenoids. SOD is a major scavenger of O_2^-, which it converts into O_2 and
428 H_2O_2. H_2O_2 is then converted into H_2O and O_2 by catalase, while POD decomposes H_2O_2 by

429 oxidation of substrates (Yuan et al. 2016). We observed approximately the same levels of
430 total carotenoid and chlorophyll under normal conditions, but the transformed plants
431 exhibited higher carotenoid and chlorophyll contents under drought and salt stresses. In
432 the present study, comparison of SOD and POD between transformants and WT showed
433 higher activities in transgenic plants. Movahedi et al. (2015a) reported that ROS could
434 oxidize unsaturated fatty acids such as MDA to form lipid hydroperoxides in cell
435 membranes. MDA is widely used as a marker of lipid oxidation in the plant response to
436 environmental stresses, and its concentration is increased under both abiotic and biotic
437 stresses, reflecting free radical-induced oxidative damage at the cellular level in plants
438 (Nouairi et al. 2009). Our results revealed that the MDA level under salt stress in transgenic
439 plants is much lower than in the WT, and this decrease was observed at all salt
440 concentrations. Under drought stress, this marker increased up to 10 days in all plants,
441 then decreased dramatically in transformants due to expression of *DRS1*. The accumulation
442 of osmolytes can affect the physiological machinery in plants (Zhou et al. 2014). Proline is
443 an amino acid that is essential for the protection of the cell membrane from ROS damage
444 (Ajithkumar and Panneerselvam 2014). Proline also plays a role as an osmoprotectant of
445 cells against salt-stress, as it accumulates in stressed plants and counteracts the damaging
446 effects of stress by affecting uptake and accumulation of inorganic nutrients, reducing
447 dehydration damage, and increasing antioxidant defense systems to mitigate the harmful
448 effects of abiotic stresses such as salt, drought, and cold (Ábrahám et al. 2010). In the
449 present study, the proline level increased in both WT and transgenic plants under drought
450 and salt stresses, but the transgenic plants exhibited significantly higher proline levels.

451 In conclusion, we extracted the *PtDRS1* gene, overexpressed it in 'Nanlin895' poplars, and
452 confirm complete integration of this gene in the plant genome through various molecular
453 analyses in different growing stages of plants. All transgenic plants showed resistance to
454 drought and salt stresses and improved their physiological and biochemical responses to
455 these stresses in comparison with WT plants.

456

457 **Conflict of interest**

458 The authors declare no conflicts of interest.

459

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463 **Author's contributions**

464 In this study, Kourosh Mohammadi directed the research group and prepared the
465 manuscript. All experiments were carried out by Kourosh Mohammadi, Ali Movahedi, and
466 Samaneh sadat Maleki. Bioinformatics were designed and analyzed by Kourosh
467 Mohammadi, Ali Movahedi, Samaneh sadat Maleki, Weibo Sun, Jiaxin Zhang, Amir Almasi
468 Zadeh Yaghuti and Saeed Nourmohammadi. Qiang Zhuge supervised the research.

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583

584 # Figure legends

585 **Fig. 1.** Cladogram of the drought sensitive 1 (*DRS1*) gene from different plant species generated through open
586 reading frame sequence alignment. Phylogenic analysis of plants, including names and National Center for
587 Biotechnology Information accession numbers, was performed using Geneious R10.3 software. Bootstrap
588 analysis was carried out with 1,000 replicates to determine the maximum confidence values of the
589 phylogenetic tree.

590 **Fig. 2.** Comparison of clawed roots between Nanlin895 poplars indicated longer and thicker roots in
591 transgenic plants compared with wild type (WT). (A) Root length of WT in 'Nanlin895' poplar. (B–D)
592 Transgenic Nanlin895 plants with longer and thicker clawed roots. (E) Well growing poplars on half strength
593 Murashige and Skoog (MS) root media. (F) Transgenic Nanlin895 poplar in homogenized soil in a greenhouse.

594 **Fig. 3.** Expression of the *DRS1* gene in different tissues of transgenic and WT poplars, carried out by
595 quantitative real-time reverse transcription (RT) polymerase chain reaction (PCR). (A–C) Expression levels of

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596 the *DRS1* gene in the leaves, roots, and stems of eight independent transgenic and WT plants. (D) Comparison
597 of the average expression level of leaf, root, and stem of WT and transgenic lines. The statistical significance
598 was set at $P \leq 0.05$. Error bars represent standard error (SE). Significant differences are indicated with
599 asterisks.

600 **Fig. 4.** Comparison of growth in WT and transgenic independent lines influenced by salt stress in MS culture
601 media. (A) Poplar plants treated with 0, 25, 50, and 100 mM NaCl indicated that WT poplars could not survive
602 under stress conditions but the transgenic plants maintained their growth rate when treated with a high
603 concentration of salt over a period of 30 days. (B) Number of main roots compared between WT and
604 transgenic lines treated with different concentrations of salt. Scale bar 1 cm; including SE. Significant
605 differences are indicated with asterisks.

606 **Fig. 5.** Comparison of growth in WT and transgenic lines influenced by poly ethylene glycol (PEG) MW 6000
607 in MS culture media. (A) Poplar plants were treated with 0, 2, 4, and 6 % PEG. The WT poplars could not
608 survive in MS culture medium containing 6 % PEG, unlike transgenic poplars, which maintained their growth
609 rate and rooted under high concentrations of PEG. (B) Number of main roots in WT and transgenic lines
610 under different concentrations of PEG. Scale bar 1 cm; including SE. Significant differences are indicated with
611 asterisks.

612 **Fig. 6.** Comparison of clawed root rate (%) in WT and transgenic lines treated with PEG and NaCl in MS
613 culture medium for 30 days. (A) Compared means between WT and transgenic poplars treated with 0 and
614 2 % PEG. (B) Compared means between WT and transgenic poplars treated with 0 and 25 mM NaCl. The
615 significant differences are shown with asterisks. SE bars are shown.

616 **Fig. 7.** Phenotypic differences between three transgenic lines and WT 'Nanlin895' poplars over 20 days of
617 drought stress in a greenhouse. Overexpression of *DRS1* in transgenic plants increased drought tolerance and
618 resulted in a higher growth rate of clawed roots compared with WT. (A) WT and three independent
619 transgenic plants, T-4-2-1, T-4-4, and T-4-2-2, after 10 days of drought stress. (B) WT and three transgenic
620 plants after 20 days of drought stress. (C) Comparison of clawed roots between WT and transgenic poplars
621 after 20 days of a water-withholding regime. (D) Significant differences in clawed root rates between the WT
622 and three transgenic lines, T-1, T-2, and T-3, after 20 days of drought stress are indicated with asterisks. The
623 data were collected from the average results of three replicates. SE bars are shown. Scale bar is 1 cm.

624 **Fig. 8.** Comparison of growth in WT and transgenic lines. (A, B) Comparison of means for stem length and
625 diameter. (C) Comparison of means for number of leaves. Significant differences are indicated with asterisks.
626 The data were selected from average results between transformant and WT lines of 'Nanlin895' over 20 days
627 of a water-withholding regime in a greenhouse. SE bars are shown.
628 **Fig. 9.** Biochemical activity levels in transgenic and WT lines under abiotic (salt and drought) stresses. (A, B)
629 Comparison of photosynthetic pigment levels (total chlorophyll and carotenoid). Comparison of antioxidant
630 activities in WT and transgenic lines, represented by (C, D) superoxide dismutase, (E, F) guaiacol peroxidase,
631 (G, H) free proline content, and (I, J) malondialdehyde activities under salinity and drought stresses,
632 respectively. Significant differences are indicated with asterisks. SE bars are shown.

633 ## Supplementary legends

634 **Fig. S1.** Schematic diagram of the *DRS1* gene and its location on chromosome number XVIII of *Populus*
635 *trichocarpa*. The bold and thin lines with numbers above them represent the lengths of exons and introns
636 (base pairs [bp]), respectively.
637 **Fig. S2.** Transfer DNA regions of the *PBI121* binary vector, which contain the *DRS1* complementary DNA
638 (cDNA) sequence and two restriction enzyme sites, *BamHI* and *SacI,* with expression driven by the *CaMV35S*
639 promoter.
640 **Fig. S3.** Alignment of *DRS1* gene to identify the similarity among *PtDRS1* and other species of plants using
641 Geneious ver.10.3.
642 **Fig. S4.** Regeneration of leaf explants on MS culture media. (A, B) Leaf explants after co-cultivation with *A.*
643 *tumefaciens* on selective MS medium supplemented with 50-μg/mL kanamycin. (C) Well-grown shoots on MS
644 shoot elongation medium. (D) The transformed poplar was rooted on half-strength MS medium. Scale bar is 1
645 cm.
646 **Fig. S5.** Multiplex PCR analysis of the *DRS1* gene. Lanes 1 and 14, Trans 2K DNA marker II. Lane 2, *CamV35S*
647 isolated from the *PBI121* plasmid used as a control lane. Lane 3, *DRS1* and *CamV35S* isolated from the
648 *PBI121:DRS1* plasmid as a positive control. Lanes 4–11 represent independent positive transgenic lines with
649 *DRS1* and *CAMV35*. Lane 12, WT. Lane 13, negative control.

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650 **Fig. S6.** RT-PCR analysis of overexpression of the *DRS1* gene in WT and transgenic poplars. Lanes 1 and 12,
651 Trans 2K DNA marker II. Lane 2, positive control of the 1542-bp *DRS1* gene isolated from the *PBI121:DRS1*
652 plasmid. Lane 3 represents the *DRS1* gene of WT poplar. Lanes 4–10, *DRS1* gene in seven transgenic poplars.
653 Lane 11, negative control. The quantification of PCR band intensities for WT and transgenic poplars were
654 carried out using ImageJ software (ver. 1.5 b), and is shown below each line of PCR bands.
655 **Fig. S7.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of transgenic and WT plants.
656 Total protein was separated using 10 % SDS-PAGE for the WT and six selected transgenic plants (T-4-4, T-4-2,
657 T-4-3, T-2-2, T-4-1, and T-2-4). The first lane (M) contains unstained protein molecular weight marker (Lot
658 Number: 00311586, Thermo Scientific). Lanes 2–7, transgenic poplars. Lane 8, WT.
659 **Fig. S8.** Amplification of full-length *DRS1* cDNA. RT-PCR was performed on five-fold serial dilutions of cDNA
660 prepared from transformed and WT poplar plants using Fast Start Universal SYBR Master Mix (ROX). (A, C)
661 Transgene and WT standard curves of *DRS1* with slopes of –3.707 and –3.837 and R^2 values of 0.996 and
662 0.992, respectively. (B, D) Amplification plots of template copy number vs. threshold cycle (C_t) of *DRS1*
663 transgenic and WT lines with values of 1.843 and 1.124, respectively.
664 **Fig. S9.** Influence of salt stress on stem and leaf growth in plants treated with 0, 25, 50, and 100 mM NaCl in
665 MS culture media. (A, B) Comparison of means for stems height and number of leaves between WT and
666 transgenic poplars. Significant differences for stem height and number of leaves of transgenic plants
667 compared to WT in media containing 0, 25, 50, and 100 mM NaCl are denoted with asterisks.
668 **Fig. S10.** Influence of drought stress on stem and leaf growth in plants treated with 0, 2, 4, and 6 % PEG in MS
669 culture media. (A, B) Comparison of means for stems height and number of leaves of WT and independent
670 transgenic lines. Significant differences are indicated by asterisks which showed higher growth rates in
671 transgenic lines than the WT over 1 month. Bars represent SE.
672

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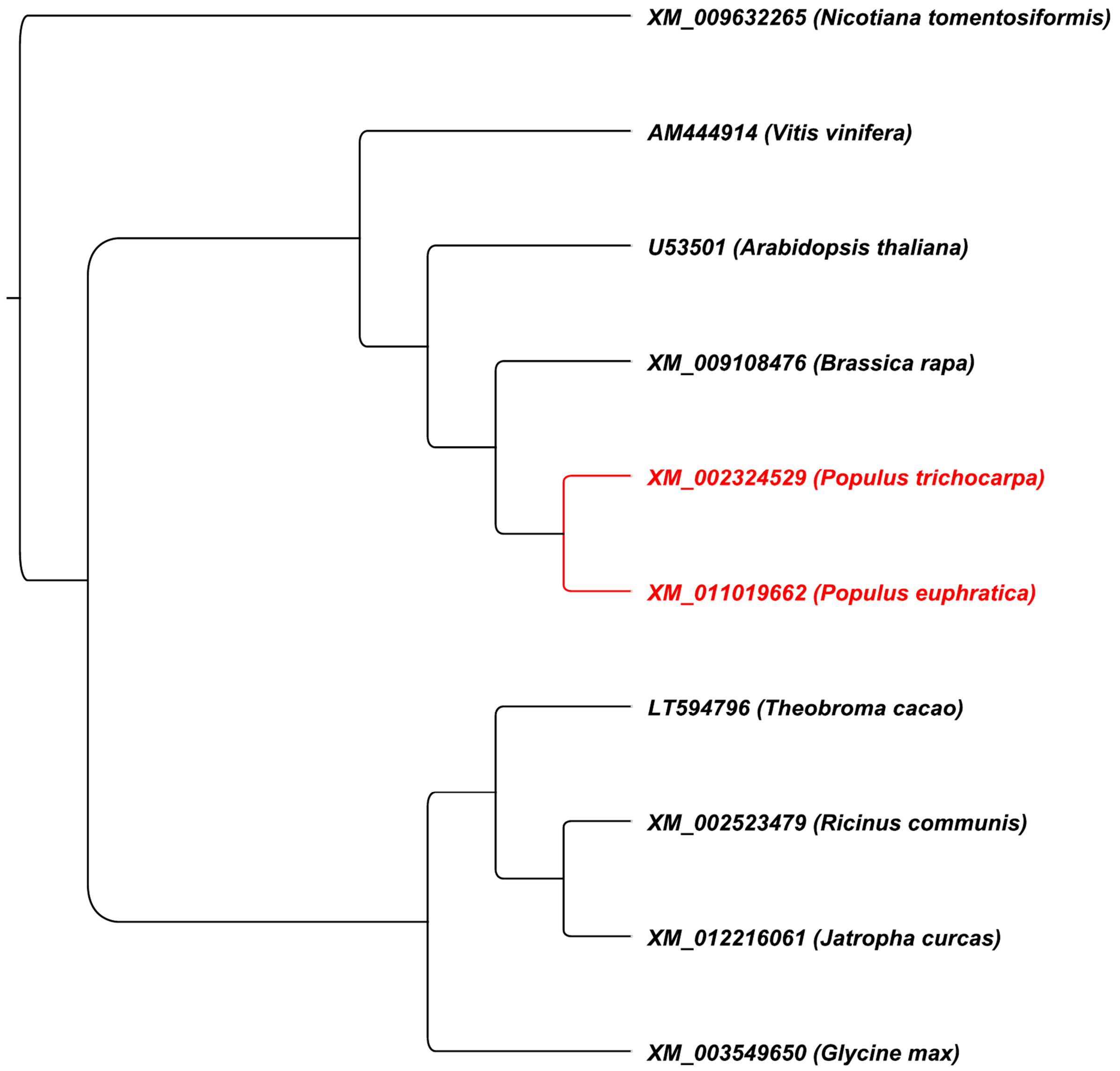

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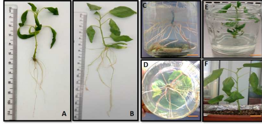

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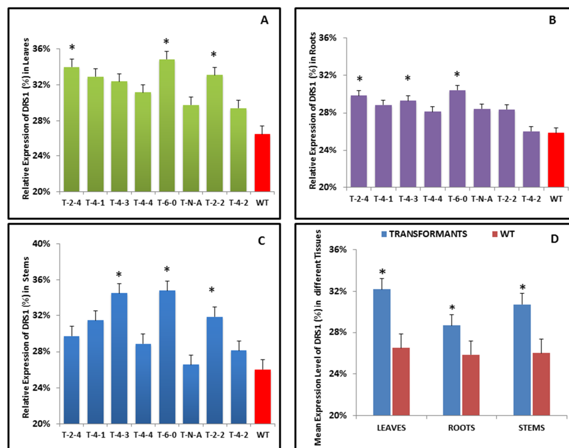

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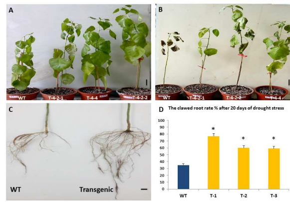

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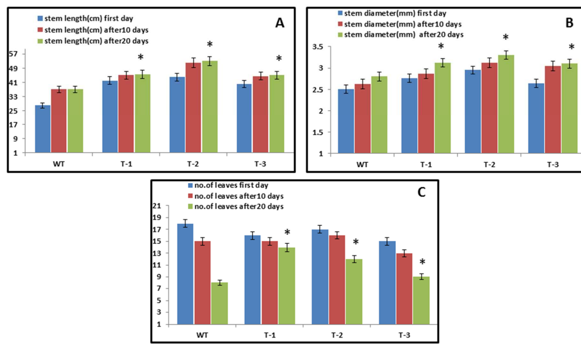

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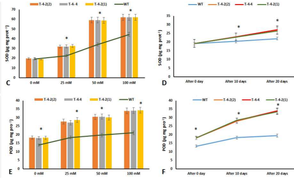

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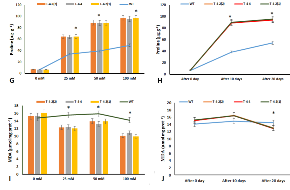

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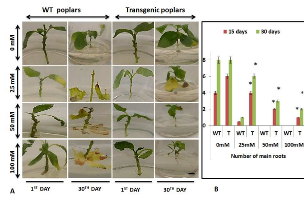

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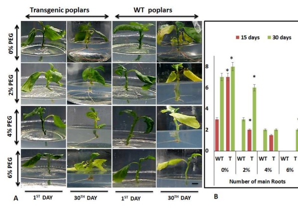

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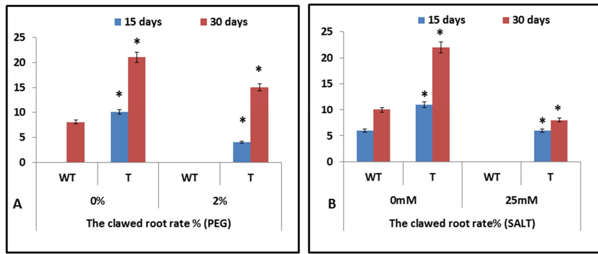

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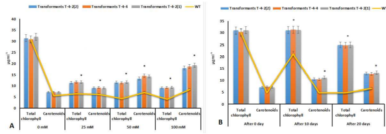

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'Highlights'

- *PtDRS1* identified and cloned for the first time from poplar plants.
- Transgenic *DRS1* poplars revealed increase resistance to salt and drought stresses.
- The overexpression of *PtDRS1* enhances growth of leaf, root and stem in poplar plants.
- Leaves showed more expression of *PtDRS1* than stems and roots in transgenic poplars.

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Contributions

In this study, Kourosh Mohammadi directed the research group and prepared the manuscript. All experiments were carried out by Kourosh Mohammadi, Ali Movahedi, and Samaneh sadat Maleki. Bioinformatics were designed and analyzed by Kourosh Mohammadi, Ali Movahedi, Samaneh sadat Maleki, Weibo Sun, Jiaxin Zhang, Amir Almasi Zadeh Yaghuti and Saeed Nourmohammadi. Qiang Zhuge supervised the research.

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