

Silencing Proline Dehydrogenases Improves Salt and Drought Tolerance in *Gossypium hirsutum*

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ABSTRACT

Proline is a key compound that lowers cell water potential, scavenges reactive oxygen species, and stabilizes biomolecules and cell membranes, thus reducing stress-

induced damage. Proline dehydrogenase (ProDH), the first rate-limiting enzyme in proline degradation, plays a crucial role in proline accumulation. We explored the role of the GhProDH gene family in regulating physiological responses to stress conditions through transcriptome, metabolome and functional analyses. Overexpression and gene silencing lines in *Arabidopsis* and cotton revealed that *GhProDH2* plays a key role in regulating cotton's tolerance to drought and salt stress. *GhProDH2-4* improves cotton's tolerance to drought and salt stress by engaging in carbon metabolism, glyoxylate cycle, and flavonoid metabolism pathways. These findings highlight the potential role of *GhProDH2-4* to improve cotton stress resistance by genetically modifying pathways involved in proline biosynthesis and degradation.

Keywords: *Gossypium hirsutum*; Proline Dehydrogenase ; Gene overexpression; VIGS ; Transcriptome analysis; Drought and salt stress.

1. Introduction

Drought and salt stress can cause water loss in plant cells, disrupting normal physiological processes. Salt stress disturbed ion toxicity (Hanin, Ebel et al. 2016) and nutrient imbalance in plant (Verma, Jalmi et al. 2020). Dehydration not only impairs plant growth and development but also directly reduces crop yield (Krasensky et al. 2012). Exploring different mechanism in plant to resist drought and salt stress is essential to enhance crop resilience. One of the common plant responses to salt and drought stress is the production and accumulation of organic compounds called osmoprotectants or compatible solutes. These include proline (Pro), glycine betaine, choline, O-sulfate, sugars, and polyols (Silveira, Viégas Rde et al. 2003; Hoque, Okuma et al. 2007; Iqbal, Umar et al. 2015). Proline is one of the important soluble osmotic regulator in plants under stressful conditions like drought (Urmi, Islam et al. 2023), salt (Huang, Bie et al. 2009), and heavy

metals stress (Siripornadulsil, Traina et al. 2002). Some of proline's unique functions arise from its inherent properties, while others depend on the coordinated "proline cycle" involving proline synthesis in chloroplasts and cytoplasm (Verslues et al. 2010).

Proline is considered a multifunctional amino acid, which is crucial for protein biosynthesis, serving as an osmotic protective compound, stabilizing macromolecules, acting as a free radical scavenger, and maintaining redox balance (Szabados, et al. Verslues, et al.2010). Various mutants or transgenic plants with proline deficiency display stunted growth, morphological abnormalities, altered flowering time, reduced fertility, or abnormal embryo development, suggesting that well-controlled proline metabolism is critical for vegetative development as well as for sexual reproduction (Mattioli, R., et al 2012Biancucci, et al.2015 Mattioli, R., et al. 2018). The accumulation of proline in plants depends on a balance between its synthesis and degradation processes (Szabados et al. 2010). The intracellular concentration of proline is regulated through metabolic synthesis (Roosens, Thu et al. 1998). Under stress condition in plants, there are two main pathways for proline synthesis (Hu, Delauney et al. 1992). Such as Proline is primarily produced through the glutamate pathway. In this process, glutamate is first phosphorylated and then reduced by pyrroline-5-carboxylate synthase (P5CS) to form pyrroline-5-carboxylate (P5C). P5C is then further reduced to proline by P5C reductase (P5Cr) (Armengaud, Thiery et al. 2004). Proline degradation starts with its oxidation by ProDH, which converts it to P5C. P5C is then further converted to glutamic acid by P5C dehydrogenase. Alternatively, proline can be synthesized via the ornithine pathway (Verslues et al. 2010). In this pathway, ornithine is first transformed into GSA and P5C by ornithine delta-aminotransferase (OAT), and P5C is then converted into proline (Roosens, Thu et al., 1998). ProDH enzyme responsible for the first step in proline degradation, plays a critical role in controlling proline accumulation and enhancing stress resistance in plants Two

71 *ProDH1* and *ProDH2* genes was identified in *Arabidopsis harbor* (Funck, Eckard et al.
72 2010). Previous studies indicated that *ProDH1* is predominantly expressed in
73 mitochondria, while *ProDH2* is expressed in both mitochondria and plastids (Kiyosue,
74 Yoshiba et al. 1996, Van Aken, Zhang et al. 2009). The expression of the antisense *ProDH*
75 gene in *Arabidopsis* was reduced, leading to an increase in proline accumulation (Nanjo,
76 Kobayashi et al. 1999). ProDH and P5CR work together to mitigate the hypersensitive
77 response in *Arabidopsis*, while also playing a key role in strengthening the plant's defense
78 mechanisms. We found that both *ProDH1* and *ProDH2* are necessary for maximum
79 resistance against *avrpm1* and *Botrytis cinerea*. However, *ProDH22* plays a more
80 significant role in early limiting the growth of *Metarhizium anisopliae* (Monteoliva, Rizzi
81 et al. 2014).

82 The relative expression levels of proline catabolism genes (*ProDH1*, *ProDH2*)
83 increased in sensitive varieties (add varieties name here). (Rizzi, Cecchini et al. 2016).
84 The addition of proline and its analogue, thiazolidine-4-carboxylic acid (T4C), both
85 substrates of ProDH, can further intensify and expose plant responses. Under drought
86 stress, treatment with T4C in the drought-tolerant variety EC-98 leads to increase mRNA
87 levels of *ProDH* genes, which are involved in both proline production and breakdown.
88 The increase in *ProDH* gene expression may be linked to the conversion of T4C to
89 cysteine, potentially contributing to the tolerance phenotype (Furlan, Bianucci et al.
90 2020;Dietrich, Weltmeier et al. 2011) found that *bZIP1* and *bZIP53* can bind to the
91 promoter of the *ProDH* gene in *Arabidopsis*, playing a key role in reducing *ProDH* levels.
92 Similarly, (Dietmar Funck et al. 2010) observed that when *bZIP11* was inhibited by
93 sucrose, the transcription of *AtProDH2* was also suppressed. *AtProDH2* is a target gene
94 for *bZIP* in *Arabidopsis* (Hansen et al. 2008). The interaction between the unknown gene
95 *DFR1* and *AtProDH* in *Arabidopsis*, regulating drought stress. This suggests that under

96 salt and drought stress conditions, *AtProDH* affects complex regulatory network
97 mechanisms to cope with salt and drought stress.

98 Cotton is the most important fiber crop. Since it was first cultivated between 5000 and
99 10,000 years ago, cotton production has enormously influenced global economic
100 development (Stephens SG et al. 1974). Proline catabolism mediated by Proline
101 Dehydrogenases (*GhProDHs*) negatively impacts salt and drought tolerance in *Gossypium*
102 *hirsutum* by accelerating proline degradation, thereby limiting the availability of proline
103 for osmoprotection, reactive oxygen species (ROS) scavenging, and cellular homeostasis
104 under abiotic stress. It is hypothesized that the downregulation or inhibition of *GhProDH*
105 activity will enhance proline accumulation, improving the plant's physiological response
106 to salt and drought stress, resulting in better stress tolerance. Investigating the regulatory
107 role of *GhProDHs* in proline catabolism will provide insights into how proline
108 homeostasis can be manipulated to enhance cotton's resilience to harsh environmental
109 conditions. Therefore, the current study was aimed to: (1) Characterize the role of
110 *GhProDH2* in proline catabolism in *Gossypium hirsutum* under salt and drought stress
111 conditions. (2) Determine the impact of *GhProDH2* gene expression on proline
112 accumulation and stress tolerance in *Gossypium hirsutum*. (3) Explore the physiological
113 effects of manipulating *GhProDH* activity on plant growth and survival under salt and
114 drought stress. This research aims to unravel the complex role of proline catabolism in
115 *Gossypium hirsutum* and provide a foundation for developing stress-tolerant cotton
116 varieties through the manipulation of proline metabolism.

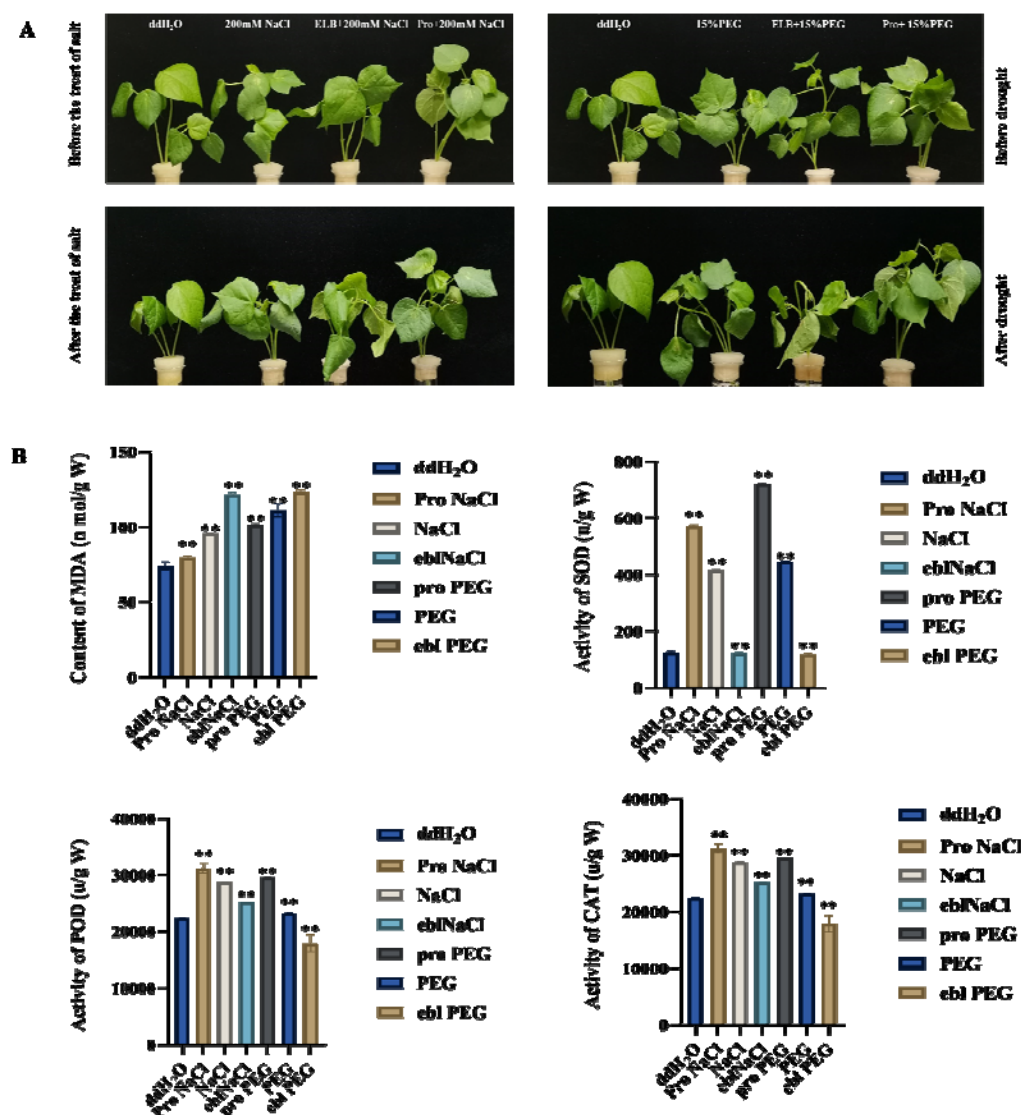
117 **2. Results**

118 **2.1. Proline enhances cotton's drought and salt tolerance**

119 In order to investigate the effects of proline on cotton under salt and drought
120 conditions, we used Proline and Proline inhibitors (ELB). We treated three leaf stage

121 cotton seedlings with ddH₂O, 200 mM NaCl, 15% PEG, Proline + 200 mM NaCl, Proline
 122 + 15% PEG, ELB + 200 mM NaCl, ELB + 15% PEG for 8 h. The results indicated that
 123 under ddH₂O conditions, plants exhibited the best growth performance while under ELB +
 124 200 mM NaCl condition, plants showed wilting on the leaf surface first, followed by leaf
 125 curling and showed wilting of all plant after 8 h. Under salinity stress, cotton plants
 126 exhibited moderate wilting. However, when salinity stress was combined with proline
 127 treatment, the wilting was less severe, indicating only mild wilting. Under ELB + 15%
 128 PEG treatment, cotton plants exhibited severe wilting while, moderate wilting was
 129 observed under PEG-only stress. When proline was applied alongside PEG, the cotton
 130 leaves showed less wilting, indicating that proline may help mitigate the negative effects
 131 of drought stress and improve the plant's tolerance to such conditions.

132 Malondialdehyde (MDA) content increased under ELB + NaCl condition while
 133 decreased under proline treatment of cotton leaves (Figure 1). ELB Under drought stress,
 134 the MDA content in cotton was highest under the ELB + 15% PEG treatment, followed by
 135 the cotton leaves treated with 15% PEG. The MDA content in cotton was found to be
 136 lowest under the Proline + 15% PEG treatment, indicating reduced oxidative damage.
 137 Under salt and drought stress conditions, the activities of antioxidant enzymes such as
 138 SOD, POD, and CAT were highest when proline was added, followed by treatments with
 139 ELB.



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142 Figure 1 The effect of proline on drought and salt tolerance of cotton (A) Growth status of
 143 three leaf cotton under control conditions of ddH₂O, salt stress (200mM NaCl), drought
 144 stress (15% PEG), and proline+salt stress (Proline+200mM NaCl), proline+ drought stress
 145 (Proline+15% PEG), Proline inhibitor+ salt stress (ELB+200mM NaCl), Proline
 146 inhibitor+ drought stress (ELB+15% PEG).(B) MDA content under various treatments,and
 147 SOD, POD, CAT content under various treatments Error bars represent ± SD of at least
 148 three biological replicates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)."

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150 2.2. Evolutionary trajectories, collinearity analysis, gene structure, cis-Acting 151 element and protein structure of ProDH in Cotton Species

152 The evolutionary relationship of *ProDH* genes in four cotton species showed a total
153 of 18 *ProDH* genes. The analysis revealed that upland cotton and Sea island cotton each
154 possess 6 *ProDH* genes, while Asian cotton and Raymond cotton have 3 *ProDH* genes
155 each. The *ProDH* genes was clustered into three groups among four different cotton
156 species. In upland cotton, *GhProDH2-1* was more closely to *GhProDH2-2*, *GhProDH2-3*
157 to *GhProDH*, and *GhProDH2-5* to *GhProDH2-6*. According to collinearity analysis,
158 *AtProDH* genes locate on chromosomes 3 and 5 (Figure 2A). *GbProDH* and *GhProDH*
159 genes are located on chromosomes A07, A08, A11, D07, D08 and D11, while *GaProDH*
160 genes were found on chromosomes 07, 08, 11 and 01, 04, 07 respectively. Among
161 *Arabidopsis* and the four cotton species has no collinearity relationship between
162 *AtProDH1* and the cotton species. The *ProDH* gene in cotton aligns only with *AtProDH2*,
163 demonstrating a linear correlation among *ProDH* genes in *G. hirsutum*, *G. arboreum*, *G.*
164 *raimondii*, and *G. barbadense* (Figure 2C). Each *ProDH* gene in *G. arboreum* and *G.*
165 *raimondii* corresponds to two *ProDH* genes in *G. hirsutum* and *G. barbadense*,
166 respectively, further confirming that *ProDH* gene duplication occurred during the
167 evolutionary process. The genome sequences and CDS sequences of *ProDH* genes in
168 *Arabidopsis thaliana*, maize, rice, cotton, and Chinese cabbage, The gene structure was
169 analyzed (Figure 2B). The vast majority of *ProDH* genes contain four exons, both
170 *Arabidopsis* and cotton have four exons.

171 Cis-acting elements are the binding sites of transcription factors, which regulate the
172 precise initiation and efficiency of gene transcription. 2000 upstream sequence of *ProDH*
173 genes were extracted to perform cis-acting element analysis. Cis-acting elements from

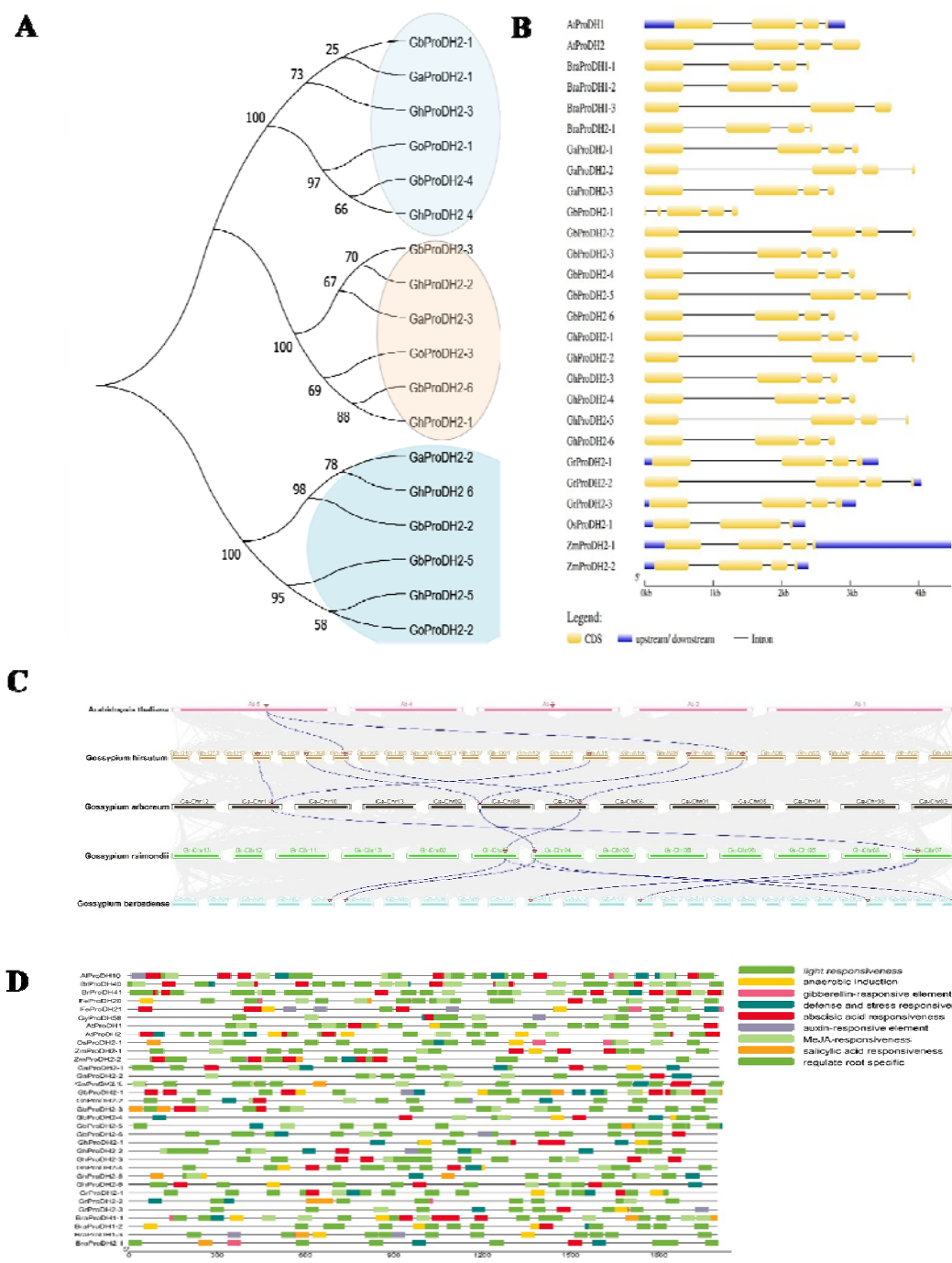
2000 upstream sequence of *ProDH* genes showed their diversified roles potential especially in various light responsiveness, anaerobic induction, gibberellin-responsive, abscisic acid responsiveness, defense and stress responses, MeJA-responsiveness, auxin-responsive element, salicylic acid responsiveness, regulate root specific nine categories (Fig. 2D). Notably, . All *ProDH* genes contain light response elements, and most of them contain stress related cis-elements except *ZmProDH 2-1*, *GbProDH 2-3*, *GhProDH 2-3* and *GrProDH 2-3*. This indicates that, *ProDH* genes are associated with stress response mechanism in plants.

We predicted the protein structure of ProDH with GMQE above 0.83 and sequence identity above 0.82, and analyzed six protein structures. We found that *GhProDH2-1* is structurally similar to *GhProDH2-2*, *GhProDH2-3* is structurally similar to *GhProDH2-4*, and *GhProDH2-5* is structurally similar to *GhProDH2-6*(appendix Figure 1).

2.3. Expression Analysis of *GhProDH* Genes in Cotton

To understand the impact of *GhProDH2* on salt and drought tolerance in cotton, RNA seq data from the database (<https://ngdc.cncb.ac.cn/>) was analyzed which showed that *GhProDH2* were significantly expressed in tissues such as roots, stems, and leaves (appendix Figure 2A). Compared to other tissues, each *GhProDH2* gene exhibits higher expression levels in leaves, with *GhProDH2-1*, *GhProDH2-4*, and *GhProDH2-6* showing more expression . Expression patterns of *GhproDH* genes under salt and drought stresses at different time intervals (1h, 3h, 6h, 12h, and 24h) illustrates the negative trend of expression level with increasing time span, indicating their possible role in stress response (appendix Figure 2B). qRT-PCR analysis was conducted for confirmation of the RNA-seq results (appendix Figure 2C D). The expression level of *GhProDH2* genes under salt and drought stress was lower than control treatment. Due to the absence of stress related cis

198 acting elements in *GhProDH2-3*, we selected five genes, *GhProDH2-1*, *GhProDH2-2*,
199 *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6* for further investigation.



201 **Figure 2 (A)** Evolutionary relationships among four cotton species (B) The gene

202 structure of *ProDH* genes in *Arabidopsis*, Maize, rice, cotton, and Chinese cabbage was
203 analyzed

204 (C) Collinearity analysis of *AtProDH*, *GhProDH*, *GaProDH*, *GrProDH* and
205 *GoProDH* genes (D) Analysis of Cis-regulatory element in *ProDH* genes.

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207 **2.4. Functional Analysis of *GhProDH* Genes in *Arabidopsis***

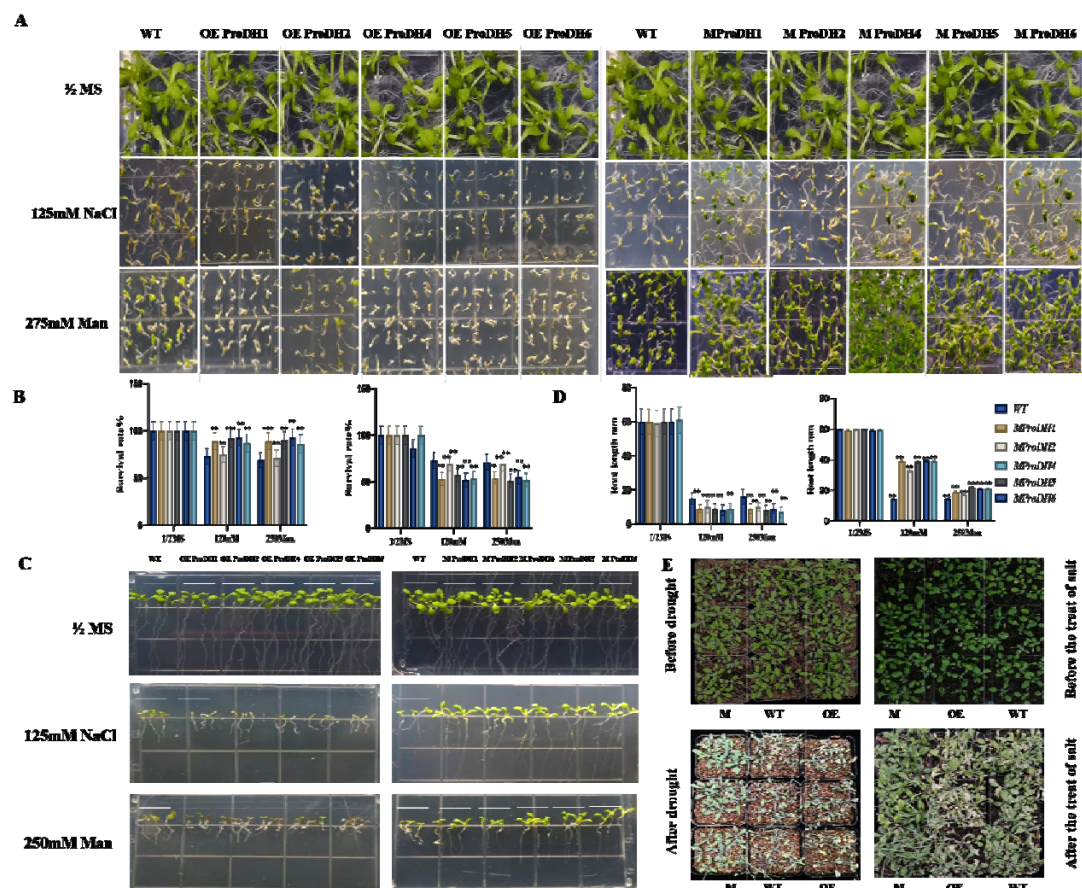
208 To investigate the biological functions of *GhProDH*s, we utilized *Agrobacterium*
209 mediated transformation to produce overexpressed lines (OE) for *GhProDH2-1*,
210 *GhProDH2-2*, *GhProDH2-4*, *GhProDH2-5* and *GhProDH2-6* as well as mutant lines
211 for *AtProDH2-1*, *AtProDH2-2*, *AtProDH2-4*, *AtProDH2-5*, *AtProDH2-6* in *Arabidopsis*
212 to explore their effects on salt and drought stress. The growth status of wilt-type (WT), OE
213 (*GhProDH2-1*, *GhProDH2-2*, *GhProDH2-4*, *GhProDH2-5*, *GhProDH2-6*), and Mutant
214 (*AtProDH2-1*, *AtProDH2-2*, *AtProDH2-4*, *AtProDH2-5*, *AtProDH2-6*) lines were
215 similar in 1/2MS medium. However, under 250mM Mannitol PEG and 125 mM NaCl
216 stress, the growth status (Figures 3A), germination rate (Figure 3B), and root growth
217 (Figures 3C and 3D) of overexpressed (*GhProDH2-1*, *GhProDH2-2*, *GhProDH2-4*,
218 *GhProDH2-5*, *GhProDH2-6*) plants were worse than WT plants. Moreover, The
219 overexpressed (*GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, *GhProDH2-6*) plants were
220 worse than The overexpressed (*GhProDH2-2*). The Mutant (*AtProDH2-1*, *AtProDH2-2*,
221 *AtProDH2-4*, *AtProDH2-5*, *AtProDH2-6*) plants were better than WT plants, Moreover,
222 The Mutant (*AtProDH2-1*, *AtProDH2-4*, *AtProDH2-5*, *AtProDH2-6*) plants were better
223 than The Mutant (*AtProDH2-2*). So, the response of gene *GhProDH2-2* to salt and

drought is not as strong as that of gene *GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*,
GhProDH2-6.

under 250 mM Mannitol PEG and 125 mM NaCl stress, the growth status (Figures
3A), germination rate (Figure 3B), and root growth (Figures 3C and 3D) of overexpressed
lines (*GhProDH2-1*, *GhProDH2-2*, *GhProDH2-4*, *GhProDH2-5*, *GhProDH2-6*) plants
were worse than WT plants. The Mutant (*AtProDH2 -1*, *AtProDH2-2*, *AtProDH2-4*,
AtProDH2-5, and *AtProDH2-6*) plants were better than WT plants. In addition the
response of gene *GhProDH2-2* to salt and drought is not as strong as that of gene
GhProDH2-1, *GhProDH2-4*, *GhProDH2-5*, *GhProDH2-6*.

To further validate the results, OE and mutant *Arabidopsis* strains of *GhProDH2-4*
were randomly selected and grown in medium for one week before being transferred to
soil.

Without treatments, there was no significant differences occurred in the growth of
WT, OE (*GhProDH2-4*), and mutant (*AtProDH2-4*) strains (Figure 3 E). To assess the
role of *GhProDH4* in drought resistance, plants were grown in well-watered soil for 10
days before being subjected to drought conditions. After 10 days without water, the OE
(*GhProDH2-4*) strain showed severe wilting, the WT strain showed moderate wilting, and
the mutant (*AtProDH2-4*) strain showed mild wilting. Similarly, after 10 days of well-
watered growth, no significant differences were observed in plant growth. However,
following 15 days of salt stress with 125 mM NaCl, the OE strain exhibited severe leaf
yellowing, the WT strain showed moderate yellowing, and the mutant strain showed only
mild yellowing.



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Figure 3 Functional validation of genetically modified *Arabidopsis*. (A) Germination assays were conducted using transgenic, mutant, and wildtype *Arabidopsis* seeds on 1/2 MS medium supplemented with 125 mM NaCl and 250 mM mannitol. The untreated 1/2 MS medium served as the control. (B) Survival rates were assessed for transgenic, mutant, and wildtype seeds exposed to 125 mM NaCl and 250 mM mannitol stress conditions. (C, D) Root length comparisons were made between transgenic, mutant, and wildtype plants grown on 1/2 MS medium supplemented with 250 mM mannitol and 125 mM NaCl for 10 days. (E) Response of *GhProDH2-4* transgenic lines and wildtype plants under drought and salt stress conditions. The error bars indicate $\pm$ SD of at least three biological replicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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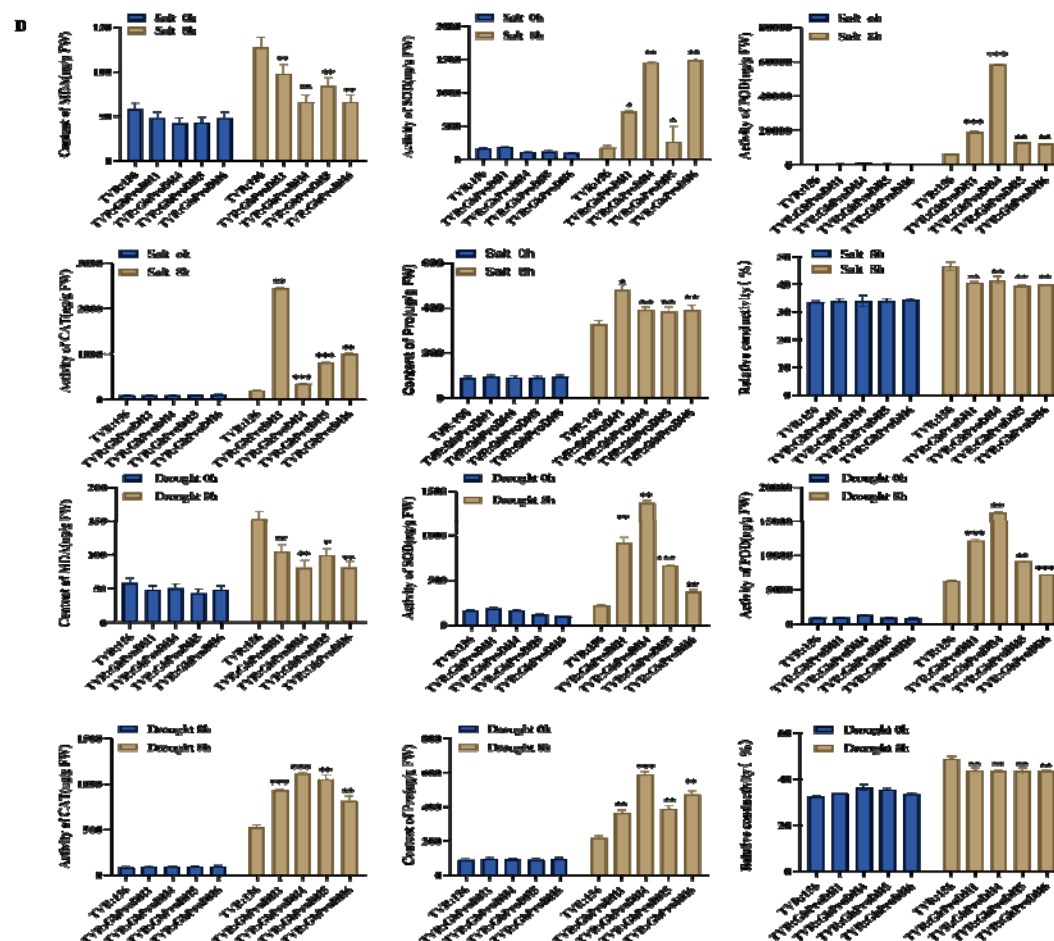
259 **2.5. Functional Characterization of *GhProDH* Genes in Cotton**

260 To investigate the function of *GhProDH2* in cotton, we employed virus-induced gene
 261 silencing (VIGS) to reduce the expression of the *GhProDH2* gene. Successful gene
 262 knockdown was confirmed by the bleaching of cotton plants infused with pYL156-PDS
 263 (Figure 4B). The expression levels of *GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, and
 264 *GhProDH2-6* in the knockdown plants were significantly lower than those in the control
 265 plants containing the empty vector (pYL156) (Figure 4 C), indicating that all four genes
 266 were effectively silenced.

267 Under 15% PEG treatment (simulated drought conditions), the control plants
268 (pYL156) exhibited leaf curling earlier than other plants. Similarly, under 200 mM NaCl
269 treatment (simulated salt stress), the control plants (pYL156) showed leaf wilting before
270 other plants. (Figure 4A). Under salt stress, the wilting degree of GhProDH2-4 stems and



271 leaves is milder than other genes (GhProDH2-1, GhProDH2-5, GhProDH2-6). Under
272 drought stress, the curling degree of GhProDH2-4 leaves is lighter than other genes
273 (GhProDH2-1, GhProDH2-5, GhProDH2-6). In addition, SOD, POD, Proline, CAT,
274 relative conductivity and MDA content were measured in VIGS mediated (*pYL156*
275 *GhProDH2*) silent plants and control plants (*pYL156*). Under normal conditions, there
276 was no significant difference in SOD, POD, Proline, relative conductivity and CAT
277 content, but under salt and drought stress, the VIGS plants exhibited higher levels of these
278 activity compared to the *pYL156* control plants. This indicates that under salt and drought
279 stress, VIGS silenced plants accumulate less ROS and increase cell osmotic pressure.
280 Conversely, the VIGS plants accumulated less MDA content (Figure 5D). These findings
281 indicate that the silencing of the *GhProDH2-1*、*GhProDH2-4*、*GhProDH2-5*、
282 *GhProDH2-6* gene improve drought and salt stress resistance of cotton .



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287 Figure 4 the impact of *GhProDH* silencing on cotton's tolerance to drought and salt stress.

288 (A) Comparison of phenotype of VIGS plants and control plants PYL156 before and after

289 and control plants PYL156 before and after CK, 200mM NaCl, 15% PEG treatment. B)

290 Representative albino marker plant used in the study. (C) Relative silencing efficiency

291 assessed by qRT-PCR, showing VIGS plants with expression reduced to less than half

292 compared to control plants. (D) Comparison of malondialdehyde (MDA) content between

293 VIGS-silenced and control plants using pYL156 under drought or salt treatment. (E)

294 Comparison of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), proline

(Pro) contents and in VIGS-silenced plants versus control plants under drought or salt treatment conditions. Error bars represent $\pm$ SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)."

2.6. Transcriptome Analysis of the impact of silencing *GhProDH* Genes on Plant Stress Responses

To explore the regulatory mechanism of *GhProDH*2s in plants, we conducted a transcriptome analysis involving (control) TRV:156 and (VIGS) TRV:*GhProDH*2-1 *GhProDH*2-4, *GhProDH*2-5 and *GhProDH*2-6 under normal (0h), drought and salt stress (8 h). The transcriptome data revealed the detection of 62,179 genes, characterized by high-quality sequencing with Q20 and Q30 base proportions averaging 97.10% and 91.17%, respectively. Alignment rates showed robust comparisons of 95.22% to the genome and 79.60% to the gene set (supplementary table1). Furthermore, we summarized the differential gene expression levels across the samples, highlighting upregulated genes in red and downregulated genes in yellow (Figure 5A). 27 DEGs were found in S156 vs *SProDH*2-1, 2154 DEGs were found in S156 vs *SProDH*2-4, 31 DEGs were found in S156 vs *SProDH*2-5, 21 DEGs were found in S156 vs *SProDH*2-6, 48 DEGs were found in D156 vs *DProDH*2-1, 9133 DEGs were found in D156 vs *DProDH*2-4, 316 DEGs were found in D156 vs *DProDH*2-5, 4701 DEGs were found in D156 vs *DProDH*2-6, it was found that S156 vs *SProDH*2-4 and D156 vs *DProDH*2-4 had the most differentially expressed genes. A total of 141 DEGs as common genes were found by Wayne diagram analysis that was performed for CK*ProDH*2-1/CK156, CK*ProDH*2-4/CK156, CK*ProDH*2-5/CK156, and CK*ProDH*2-6/CK156 (Figure 5B). KEGG enrichment analysis of these common DEGs showed that the most significant pathways were cutin, suberine and wax biosynthesis, phagosome, plant hormone signal transduction (Figure 5C), indicating that these DEGs related to common metabolic regulatory pathways. Overall,

our findings highlight the complex role of *GhProDH2* genes in plant stress responses, as revealed by comprehensive transcriptomic analyses. These analyses provide high-quality data and valuable insights into gene regulation under different environmental conditions.

2.8. Gene Ontology revealed biological functions of *GhProDH2* Genes

In order to gain a deeper insights into the differential expression of genes, we conducted Gene Ontology (GO) analysis. Under salt stress conditions, GO analysis confirmed a diverse array of biological processes (BP) affected by TRV: 156-8 h/TRV: VIGS (*GhProDH2*) -8 h. This analysis categorized findings into cellular components (CC), molecular functions (MF), and BP. Notably, the top 20 enriched pathways, based on Q values, were plotted (appendix figure3A,B).

Under salt stress, compared with the control group TRV: 156-8h, the *GhProDH2-1* silencing group significantly enriched the pathways including phosphorylation (BP), protein kinase activity (MF), sequence specific DNA binding (MF), and ATP binding (MF); The pathways significantly enriched in *GhProDH2-4* include chloroplast (CC), carbon fixation (BP), and ribose-1,5-bisphosphate carboxylase/oxygenase activator activity (MF). The pathways significantly enriched in *GhProDH2-5* include RNA polymerase II transcription factor complex (MF), fatty acid alpha hydroxylase activity (MF), L-leucine: 2-oxoglutarate aminotransferase activity (MF), and L-leucine transaminase activity (MF). The pathways significantly enriched in *GhProDH2-6* include DNA binding transcription factor activity (BP), iron ion binding (BP), and sterol metabolic process (BP).

Under drought stress, the top 20 pathways enriched in *GhProDH2-1* were ranked according to the rise and fall of Q values (Figure 5D), including glyoxylate cycle (BP),

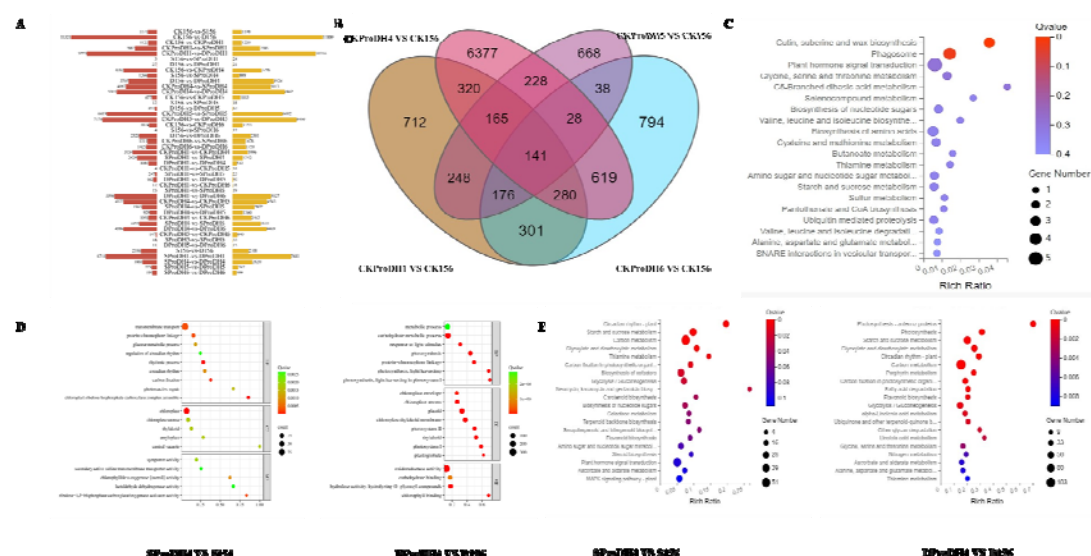
integral component of plasma membrane (CC), sucrose metabolic process (BP), and sulfate transport (BP). *GhProDH2-4* is enriched in significantly enriched pathways such as chloroplastic thylakoid membrane (CC), plastid (CC), photosynthesis (BP), and photosystem II (CC). *GhProDH2-5* was significantly enriched in the regulation of jasmonic acid mediated signaling pathway (BP), polyketide biosynthetic process (BP), and biosynthetic process (BP) pathways. *GhProDH2-6* was enriched in significantly enriched pathways such as photosystem II (CC), chloroplast thylakoid membrane (CC), chloroplast (CC), and photosystem I (CC). It can be seen that the pathways of gene enrichment under salt and drought conditions are different. And it can be summarized that under stress conditions, *GhProDH2-4* can activate more pathways.

2.9. KEGG analysis revealed Functional Enrichment pathways of *GhProDH2* Genes

KEGG analysis of *GhProDH2-1* under salt stress significantly enriched the Q value ≤ 0.05 and six pathways mainly enriched in Indole alkaloid biosynthesis Nitrogen metabolism, Betalain biosynthesis. Similarly, *GhProDH2-4* significantly enriched the Q value ≤ 0.05 in 20 pathways and it was mainly enriched in Circadian rhythm plant, Starch and sucrose metabolism, Glyoxylate and dicarboxylate metabolism. (appendix Figure3C,D) *GhProDH2-5* significantly enriched the Q value ≤ 0.05 in 4 pathways and it was mainly enriched in Glucosinolate biosynthesis Plant hormone signal transduction, Valine, leucine and isoleucine biosynthesis. Also, *GhProDH2-6* Q value ≤ 0.05 was significantly enriched in two pathways, mainly enriched in Ribosome, Brassinosteroid biosynthesis.

Under drought stress, KEGG enrichment mapping showed that *GhProDH2-1* significantly enriched the Q value ≤ 0.05 in 5 pathways and was mainly enriched in Glyoxylate and dicarboxylate metabolites, Sphingolipid metabolite carbon metabolism. *GhProDH2-4* significantly enriched the Q value ≤ 0.05 in 20 pathways and it

370 was mainly enriched in photosynthesis antenna proteins Starch and sucrose metabolism,
 371 Glyoxylate and dicarboxylate metabolism, and Circadian rhythm – plant. Under drought
 372 stress, (Figure 5E) *GhProDH2-5* significantly enriched 13 pathways, mainly in Flavonoid
 373 biosynthesis Cysteine and methionine metabolism, and Phenylpropanoid biosynthesis.
 374 *GhProDH2-6* significantly enriched 20 pathways, mainly enriched in photosynthesis
 375 Photosynthesis - antenna proteins, and Carbon metabolism. Therefore, *GhProDH2-4* is
 376 significantly enriched in both GO and KEGG pathways under these conditions, suggesting
 377 it influences more pathways than the other genes. Consequently, *GhProDH2-4* was
 378 selected for further study.



379 Figure 5: Detailed analysis of RNA-seq data depicting differentially expressed genes
 380 (DEGs) in various comparison sets. (A) Bar graph illustrating total DEGs, with red
 381 indicating upregulated and yellow-accented representing downregulated DEGs in each
 382 comparison. (B) Gene Venn diagram analysis under stress free conditions. (C) KEGG
 383 enrichment analysis of 141 genes. (D) GO enrichment analysis of *GhProDH2-4/156*, under
 384 salt stress. (E) GO enrichment analysis of *GhProDH2-4/156*, under drought stress. (F)
 385 *GhProDH5/156*, and *GhProDH6/156* under salt stress. (G) KEGG enrichment analysis of

388 GhProDH2-4/156, under drought stress. CK156= pYL156; D156= pYL156 drought 8h;
 389 S156= pYL156 salt 8h; CKProDH1= pYL156:*GhProH1*; DProDH1= pYL156:
 390 *GhProDH1* drought 8h; SProDH1= pYL156: *GhProDH1* salt 8h; CKProDH4=
 391 pYL156:*GhProH4*; DProDH4= pYL156: *GhProDH4* drought 8h; SProDH4= pYL156:
 392 *GhProDH4* salt 8h; CKProDH5= pYL156:*GhProH5*; DProDH5= pYL156: *GhProDH5*
 393 drought 8h; SProDH5= pYL156: *GhProDH5* salt 8h; CKProDH6= pYL156:*GhProH6*;
 394 DProDH6= pYL156: *GhProDH6* drought 8h; SProDH6= pYL156: *GhProDH6* salt 8h

395 **2.10. Impact of *GhProDH2-4* Silencing on Starch and Sucrose Metabolism and** 396 **Carbohydrate metabolism Pathways Under Salt and Drought Stress Conditions**

397 Silencing *GhProDH* influences pathways involved in starch and sucrose metabolism,
 398 affecting genes such as alpha, alpha trehalose phase synthesis [UDP forming] 5] [2.4.1.15],
 399 sucrose synthase [2.4.1.13, UTP glucose 1-phase uridylyltransferase [2.7.7.9] and granule
 400 bound start synthesis 1. [2.4.1.242], chloroplast/amyloplastic [3.2.1.68] glucose endo-1,3-
 401 beta-glucosidase 3 [3.2.1.39], endolucanase 17 like [3.2.1.14], and alpha glucosidase
 402 [12.4.1.1] under stress conditions as indicated by clustering heat map analysis. (appendix
 403 Figure 4A) Notably, *DProDH2-4/D156* and *D156/CK156* exhibit contrasting trends
 404 compared to *SProDH2-4/D156* and *S156/CK156*, with some genes showing
 405 downregulation such as alpha-amylase-like. ed alterations in Galactose metabolism,
 406 Neomycin, Kanamycin, and gentamicin biosynthesis, Biosynthesis of nucleotide sugars,
 407 Fructose and mannose metabolism, and Cyanamide metabolism, suggesting that
 408 *GhProDH2-4* silencing can impact various cellular sugar synthesis pathways, thereby
 409 enhancing plant drought and salt tolerance. Analysis of clustering heatmaps for
 410 *S156/SProDH2-4*, *SProDH2-4/S156*, *S156/CK156*, and *SProDH2-4/S156* after *GhProDH*
 411 silencing reveals contrasting trends in gene expression. Conversely, under salt stress,
 412 genes encoding components such as pfk (*phosphofructokinase*, , *glyceraldehyde-3-*

413 *phosphate dehydrogenase, ribulose biphosphate carboxylase large chain,*
 414 *phosphoglycerate kinase , phosphoglycerate kinase ,* show upregulation. These findings
 415 underscore the intricate regulatory role of *GhProDH2-4* in modulating gene expression
 416 patterns critical for carbohydrate metabolism, which response to adverse conditions.

417 **2.11. Metabolic Regulation by *GhProDH2-4*: Insights into Glyoxylate Dicarboxylic** 418 **Acid Metabolism Under Salt and Drought Stresses**

419 Upon silencing *GhProDH2-4*, analysis using clustering heatmaps revealed an inverse
 420 trend between *SproDH2-4/S156* and *S156/CK156*. Similarly, under drought conditions,
 421 clustering heatmaps *DProDH2-4/D156* and *D156/CK156* also exhibited opposite trends.
 422 *aconitate hydratase, ribulose biphosphate carboxylase large chain, acetate/butyrate--*
 423 *CoA ligase AAE7 genes upregulation.*

424 These findings underscore the pivotal role of *GhProDH2-4* in regulating metabolic
 425 pathways essential for plant adaptation to salt and drought stresses, particularly through
 426 glyoxylate dicarboxylic acid metabolism and its interconnected metabolic pathways.

427 **2.12. Metabolic Regulation by *GhProDH2-4*: Insights into Flavonoid biosynthesis** 428 **Under Salt and Drought Stresses**

429 Upon silencing *GhProDH2-4*, analysis using clustering heatmaps revealed an inverse
 430 trend between *SproDH2-4/S156* and *S156/CK156*. Similarly, under drought conditions,
 431 clustering heatmaps *DProDH2-4/D156* and *D156/CK156* also exhibited opposite trends.
 432 *chalcone synthase 1, flavonol synthase/flavanone 3-hydroxylase, caffeoyl-CoA O-*
 433 *methyltransferase etc* genes upregulation.

434 **2.13. Physiological Responses of *GhProDH2-4* Suppression in Cotton Under Drought** 435 **Stress**

436 Under stress conditions, the palisade tissue in the leaves of both TRV:156 and VIGS
437 plants exhibited a transition from long columnar to short cylindrical or spherical shapes,
438 accompanied by a shift from tightly packed to loosely arranged structures, leading to
439 decreased tissue compactness over time. However, the extent of these changes in the
440 palisade tissue of VIGS plants was less pronounced compared to TRV:156 plants. This
441 observation suggests that VIGS-mediated suppression of *GhProDH2-4* may confer a
442 mitigating effect under stress conditions (Figure 6A).

443 ROS are inherently generated as byproducts of various metabolic processes such as
444 photosynthesis and respiration. ROS levels are regulated by a network of enzymatic and
445 non-enzymatic antioxidants distributed across cellular compartments. Additionally, ROS
446 can traverse through aquaporins and other transport mechanisms to different cellular
447 compartments or neighboring cells, where they function in signaling, detoxification, or
448 accumulation processes (Mittler, Zandalinas et al., 2022). Given the enrichment of ROS-
449 related processes in the extracellular domain under stress conditions, staining techniques
450 were employed to assess ROS accumulation in TRV:156 and TRV plants. The results
451 indicated that VIGS (*GhProDH2-4*) plants exhibited reduced ROS accumulation
452 compared to *TRV:156* plants (Figure 6B). Further detection of intracellular ROS levels
453 using the fluorescent probe DCFH-DA (Figure 6C) revealed that under ddH₂O treatment,
454 TRV: 156 and VIGS (*GhProDH2-4*) accumulated less fluorescence, while under salt and
455 drought conditions, TRV: 156 accumulated more fluorescence than VIGS(*GhProDH2-4*).

456 Due to drought stress, *GhProDH2-4* is significantly enriched in pathways related to
457 photosynthesis, where stomata serve as crucial regulators of plant gas exchange. Stomata
458 can dynamically adjust their aperture in response to environmental changes, thereby
459 influencing plant transpiration and photosynthetic activity (Wang, Li et al., 2024). It is
460 hypothesized that *GhProDH2-4* may influence stomatal phenotype under drought

conditions. Scanning electron microscopy analysis of both TRV:156 control plants and TRV (VIGS) plants revealed that under drought stress, the stomatal aperture of TRV plants was notably reduced compared to TRV:156 plants, indicating decreased stomatal opening in *GhProDH2-4*-suppressed plants (Figure 6D). However, we measured the net photosynthetic rate, stomatal conductance, intracellular carbon dioxide concentration, and transpiration rate of 156 and VIGS plants under ddH₂O, salt, and drought conditions using a photosynthesis analyzer 6800. We found that the stomatal conductance and transpiration rate of VIGS plants were lower than those of 156 plants, while the net photosynthetic rate and intracellular transpiration rate were higher than those of 156 plants (Figure 6E).

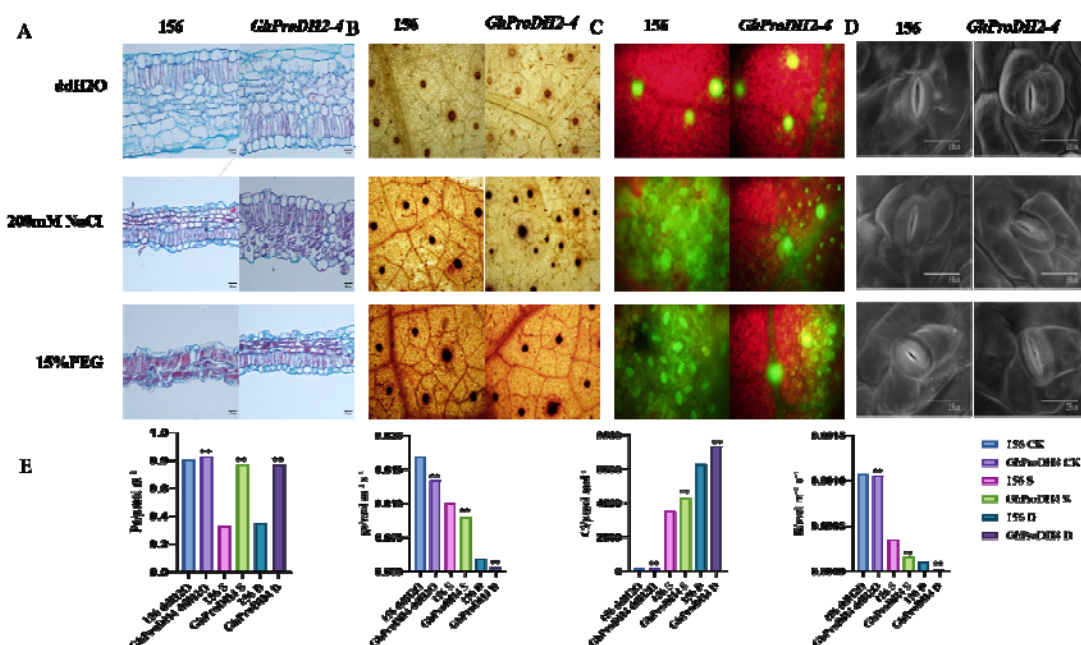


Figure 6 (A) Cotton plant TPV: 156, VIGS (*GhProDH2-4*) leaf cross-sectional condition under water, 200mM NaCl, and 15% PEG (B) DAB staining method was used to detect the accumulation status of ROS in VIGS (*GhProDH2-4*) of cotton plants with TPV: 156 under water, 200mM NaCl, and 15% PEG conditions. (C) DCFH-DA method was used to detect the accumulation status of ROS in

VIGS (GhProDH2-4) of cotton plants with TPV: 156 under water, 200mM NaCl, and 15% PEG conditions.(D) Cotton plant TPV: 156, stomatal status of VIGS (GhProDH2-4) under water, 200mM NaCl, and 15% PEG conditions. (E) Cotton plant TPV: 156, VIGS (GhProDH2-4) under water, 200mM NaCl, and 15% PEG conditions detect net photosynthetic rate (pn), intracellular carbon dioxide solubility (Ci), stomatal conductance (gs), transpiration rate (E).

483

2.14. Metabolome analysis reveals the Regulatory Role of *GhProDH* Genes in Plant Stress Responses

To further study the metabolic changes in VIGS (*GhProDH2-4*) plants and TRV-156 plants under salt and drought conditions, 819 metabolites were identified in the metabolome with CAS numbers. These included 78 amino acids and derivatives, 130 phenolic acids, 100 flavonols, 54 flavonoids, 40 nucleotides and derivatives, 54 sugars and alcohols, 43 free fatty acids, 53 organic acids, and 53 alkaloids. (appendix figure 5). Under salt stress, KEGG analysis of TRV: 156-8 h/TRV: VIGS (*GhProDH*) -8 h revealed significant enrichment in Galactose metabolism ABC transporters, Aminobenzoate degradation, Flavonoid biosynthesis, carbon metabolism, Glyoxylate and dicarboxylate metabolism (Figure 7C). Under drought stress, KEGG analysis of TRV: 156-8 h/TRV: VIGS (*GhProDH2-4*) -8 h pathway revealed significant enrichment in carbon metabolism, flavonoid metabolism, and their compound protein digestion and absorption Galactose metabolism, Pyrimidine metabolism, carbon metabolism, Glyoxylate and dicarboxylate metabolism Pathways, these pathways include both primary and secondary metabolic pathways (Figure7D). Under salt and drought stress, the correlation between the enriched of different metabolites revealed a strong and extensive interactions between sugars, alcohols, flavonoids, organic acids, and other metabolites (Figure 7E-F). Primary

metabolism can provide the necessary energy for the synthesis of secondary metabolites, such as under salt and drought conditions, which are significantly enriched in the carbon metabolism and glyoxylate dicarboxylic acid pathways. Thermal clustering analysis was conducted on flavonoids, sugars and alcohols, and organic acids, and it was found that the accumulation of these metabolites increased after gene silencing (Figure 7G).

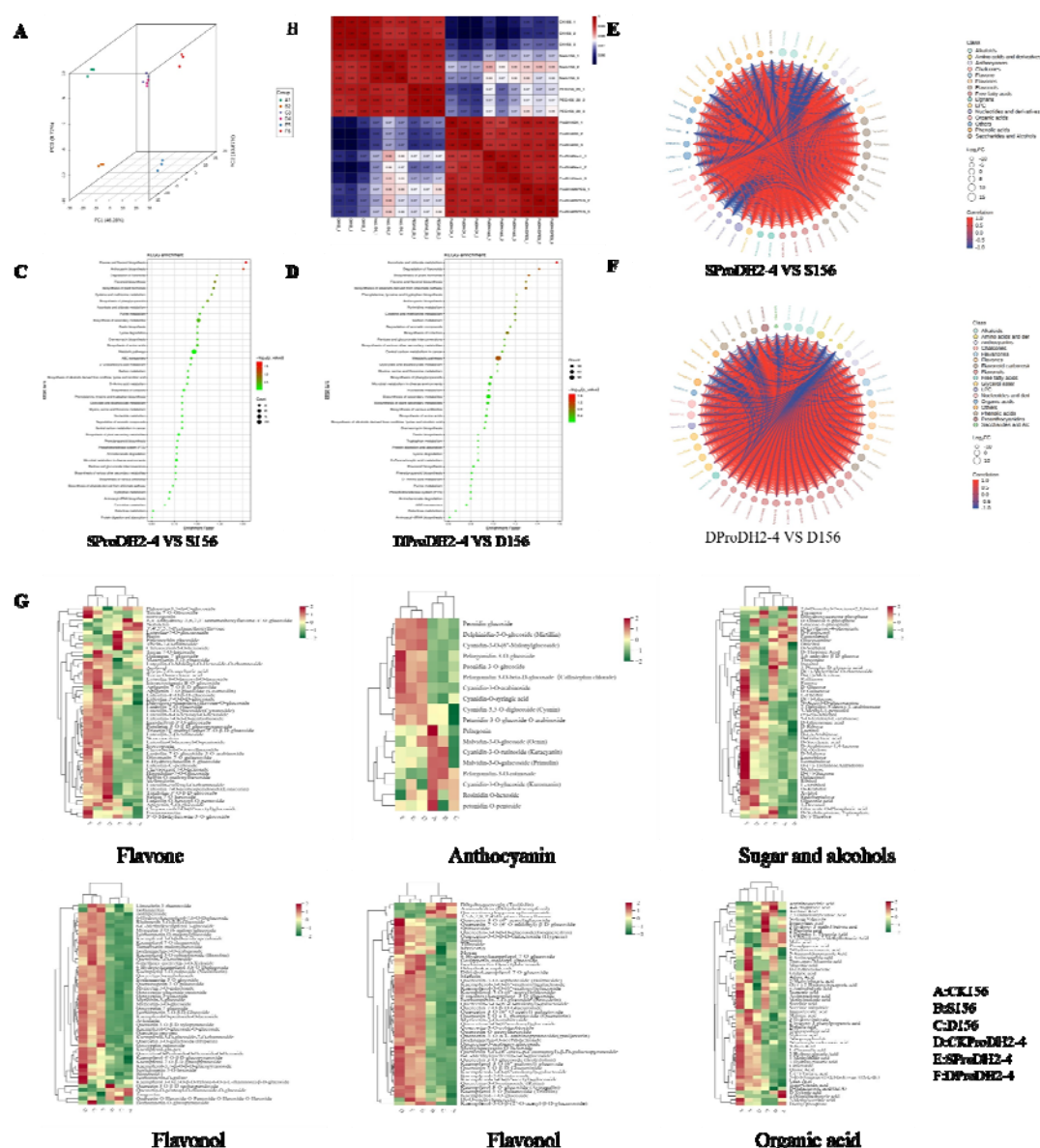


Figure 7 Principal component analysis (PCA) of metabolome profiling of 156CK, 156NaCl, 156 15%PEG, GhProDH2-4CK, GhProDH2-4NaCl, GhProDH2-

511 415%PEG.B correlation of metabolome profiling of 156CK, 156NaCl, 156
512 15%PEG, GhProDH2-4CK, GhProDH2-4NaCl, GhProDH2-415%PEG (C) ProDH2-4 VS
513 156KEGG enrichment analysis under salt stress (D) ProDH2-4 VS 156KEGG enrichment
514 analysis under drought stress (E) Correlation of differential metabolites under salt stress (F)
515 Correlation of differential metabolites under drought stress (G) Cluster heatmap analysis of
516 flavonoids, anthocyanin, flavonol, and organic acid metabolites in GhProDH2-4 and 156
517 plants under ddH₂O, salt, and drought stress

518 **2.15. Combined analysis of transcriptome and metabolome**

519 Under salt and drought stress, silencing the *GhProDH2-4* gene significantly affected
520 the carbon metabolism pathway, glyoxylate dicarboxylic acid metabolism pathway,
521 flavonoid metabolism pathway, as well as the accumulation of sugars and alcohols in both
522 transcriptome and metabolome. As a result, we concentrated on these three pathways and
523 selected key genes and metabolites to compare their expression levels between VIGS
524 (*GhProDH2-4*) plants and 156 plants under salt and drought stress conditions (Figure
525 8). We found that the metabolites of pyruvate and acetyl CoA, as well as soluble sugars
526 increase in the carbon metabolism pathway. This can reduce the accumulation of oxidative
527 active substances by altering cell osmotic pressure under salt and drought stress. In the
528 flavonoid metabolism pathway, the accumulation of substances such as kaempferol,
529 chalcone, anthocyanins, etc. can reduce the accumulation of ROS and enhance drought
530 and salt tolerance. Organic acids such as malic acid, succinic acid, and glyoxylate
531 accumulate in the dicarboxylic acid glyoxylate pathway.

543 3. DISCUSSION

544 Cotton is one of the most important fiber crops, accounting for 35% of global fiber
545 (Abdelraheem et al. 2019). Drought alone is currently reported to affect about 45% of
546 agricultural land worldwide; likewise, about 19.5% of the cultivable agricultural lands are
547 under salinity stress (Dos Reis et al. 2012) which have a significant impact on the yield of
548 cotton. Proline is an amino acid which in primary metabolism in plants, serves as one of
549 the most widely distributed and effective organic compatible osmolytes (Agami et al.
550 2016; Mohamed et al. 2020). It accumulates in plant cells under stress conditions and
551 plays an important role in plant stress resistance (Ben Rejeb et al.2012). Role of proline in
552 enhancing the salt and drought tolerance in plants has been studied as it can participate in
553 regulation of cell division, cell elongation, cell wall self-assembly to sustain a fast
554 elongation for the hairy roots and other organs including reproductive system (Per, Tasir
555 S. et al.2017; Singh et al.2015Duan et al. 2024; Trovato et al. 2008) . The growth
556 performance of cotton plants was investigated with exogenous application of proline and
557 ELB (proline inhibitor) under salt and drought stress conditions. Under salt and drought
558 conditions, the growth performance of cotton plants was better under exogenous proline
559 treatment than that under exogenous treatment of proline inhibitor (Figure 1.) The use
560 of proline inhibitor (EBL) to treat transgenic lemons resulted in a decrease in salt
561 tolerance, while the supply of exogenous proline partially restored the salt tolerance of the
562 VIGS strain (Dai, W.et al.2018).ProDH , the rate-limiting enzyme in the first step of
563 proline degradation pathway, has a certain impact on proline content. To investigate the
564 regulatory mechanism of ProDH on salt and drought tolerance in cotton plants, we studied
565 the function of *GhProDH2* gene. This study identified 18 *ProDH* genes from four cotton

species and distributed them into three groups. Structural analyses of the *ProDH* gene revealed the similarities between motif and structure of *ProDH* genes. Specifically, the six *GhProDH2* gene structures in upland cotton contain four exons (Figure 2A).

A collinearity analysis was performed between *Arabidopsis* and four cotton species, revealing that the *ProDH* gene in cotton does not have a homologous pair with *AtProDH1*, but shows a certain linear correlation with *AtProDH2* (Figure 2C). This suggests a possible divergence in the evolutionary paths of these genes between cotton and *Arabidopsis*. To further understand the regulatory mechanisms of *ProDH* genes, a cis-acting element analysis was conducted on the 2000 upstream sequences of *ProDH* genes from various developmental stages of plants. The results identified nine categories of cis-acting elements: photo reactivity, anaerobic inducibility, gibberellin responsive elements, abscisic acid responsiveness, defense and stress responsiveness, MeJA responsiveness, auxin responsive elements, salicylic acid responsiveness, and regulatory root specificity. However, *GhProDH2-3* was found to have no stress cis acting elements in upland cotton (Figure 2D). This comprehensive analysis highlights the conserved structural features of *ProDH* genes in upland cotton and their potential regulatory elements, providing insights into their roles in stress responses and primary metabolism. Through protein structure analysis, it was found that *GhProDH2-1* is structurally similar to *GhProDH2-2*, while *GhProDH2-3* is structurally similar to *GhProDH2-4*, and *GhProDH2-5* is structurally similar to *GhProDH2-6* (Attach Figure 1). Transcriptome data showed that *GhProDH2-1*, *GhProDH2-2*, *GhProDH2-3*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6* were all expressed in the plant leaves.

Through overexpression and silencing of *GhProDH2-1*, *GhProDH2-2*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6*, The *GhProDH2* gene was found to have a negative

regulatory effect on salt and drought tolerance. Due to the weaker response of *GhProDH2-2* to salt and drought stress compared to other genes. We next studied the drought and salt tolerance of *GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6* in cotton. *GhProDH2-4* has been found to have a strong response to salt and drought than other genes (*GhProDH2-1*, *GhProDH2-5*, and *GhProDH2-6*). Salt and drought stress generate higher ROS molecules which the antioxidant enzymes can effectively eliminate its production by metabolic activities (Meng, Y.C. et al. 2021). ROS (Uzilday, B. et al.). The activity levels of superoxide dismutase, peroxidase, and catalase were higher in the silenced VIGS *GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6* of cotton than that in the control group TRV: 156. These results are in agreement with several previous studies (Schekaleva, O. et al. 2024 ;Liu, C. et al. 2023). MDA, as one of the end products of lipid peroxidation reaction in organisms, is an important parameter reflecting the potential antioxidant capacity of the plant cells and can indirectly reflect the degree of tissue peroxidation damage (Xu, Y. et al. 2020; Tsikas, D. et al.2017). Transcriptome analysis was conducted on silenced plants (VIGS *GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, *GhProDH2-6*) and the control (TRV: 156) in upland cotton. Under stress-free conditions, all silenced plants exhibited enrichment in cellular components and shared the same GO pathway, confirming their homology. However, each gene also displayed unique enriched pathways, highlighting their distinct and synergistic roles. GO and KEGG enrichment analyses under salt and drought stress revealed that VIGS (*GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6*) plants had significantly enriched pathways compared to TRV: 156. Despite *GhProDH2-5* and *GhProDH2-6* being more closely related, their significantly enriched pathways were not more similar to each other than to those of the other genes. This indicates a complex

614 interaction and distinct functional contributions of each *GhProDH* gene under stress
 615 conditions. The various genes of the *GhProDH* family (*GhProDH2-1*, *GhProDH2-4*,
 616 *GhProDH2-5*, and *GhProDH2-6*) regulate different pathways to collectively combat salt
 617 and drought stress. Notably, *GhProDH2-4* is significantly enriched in both GO and KEGG
 618 pathways under these conditions, suggesting it influences more pathways than the other
 619 genes. Consequently, *GhProDH2-4* was selected for further study. It was found that
 620 silencing *GhProDH2-4* significantly affects the starch sucrose pathway, carbon
 621 metabolism pathway, glyoxylate dicarboxylic acid pathway, and flavonoid metabolism
 622 pathway under salt and drought stress.

623 Anatomical observations of cotton leaves under salt and drought conditions revealed
 624 that silencing *GhProDH2-4* mitigates the tightening of sponge and palisade tissues (Figure
 625 7). The *GhProDH2-4* gene is enriched in the photosynthesis pathway, where stomatal
 626 movement is essential for regulating photosynthesis rate, water balance, and plant
 627 immunity (Rodrigues et al. 2022). This research indicates that *GhProDH2-4* gene
 628 enhances resistance to salt and drought stress by modulating stomatal opening (Figure 7).
 629 ROS can damage proteins, lipids, and nucleic acids and can alter protein properties by
 630 forming covalent bonds, thus regulating their activity and function (Møller, Jensen et al.
 631 2007). The production of ROS is a direct defense mechanism against pathogens, as they
 632 are toxic to pathogens and signal responses to biotic and abiotic stress stimuli or
 633 developmental cues (Wrzaczek, Brosché et al. 2013, Baxter, Mittler et al. 2014). Net
 634 photosynthetic rate can be used to characterize the health status of plants and their ability
 635 to accumulate organic matter (Zhang, Sun et al. 2022). Silencing *GhProDH2-4* reduces
 636 ROS accumulation, thereby enhancing resistance to salt and drought stress. Transcriptome
 637 analysis of silenced *GhProDH2-4* under stress conditions showed significant enrichment
 638 in starch and sucrose metabolism, carbohydrate metabolism, glyoxylate, and dicarboxylate

639 metabolism pathways, flavonoid biosynthesis indicating that salt and drought stress share
640 common signaling pathways.

641 Metabolomics study revealed that under salt and drought stress, VIGS (*GhProDH2-4*)
642 was significantly enriched in carbon metabolism, flavonoid metabolism, and glyoxylate
643 dicarboxylic acid pathway compared to TRV: 156. Flavonoids are essential antioxidants
644 for plant growth, development, and reproduction, and can enhance plant stress resistance
645 (Zhang, Huang et al. 2022; W. Yang Yang, et al. 2021). Carbon metabolism is not only
646 crucial for plant growth and development, but also plays a key regulatory role in stress
647 response (Li, Li et al. 2024; Fang, S. et al. 2023). Glyoxylate dicarboxylic acid metabolism
648 plays a critical role in carbohydrate synthesis, serving as both an energy source and
649 signaling molecules in response to abiotic stresses in plants (Zeb, Liu et al. 2022; Lv, H. et
650 al. 2024). The enrichment of glyoxylate dicarboxylic acid metabolism is advantageous for
651 accumulating osmotic substances, initiating osmotic regulation, and clearing ROS (Huang,
652 Peng et al. 2021). It was found that there is a certain accumulation of metabolic sugars,
653 alcohols, acetone metabolites, and organic acids in the metabolome. Through joint
654 analysis of transcriptome, metabolome, and physiological phenotype, it was found that
655 silencing the *GhProDH2-4* gene upregulates carbon metabolism pathway related genes to
656 provide plants with certain energy, increase net photosynthetic rate, and accumulate
657 soluble sugars. Increased concentrations of soluble sugars have been linked to enhanced
658 drought (O'Brien, Valtat et al. 2020) and salt tolerance (Li, Meng et al. 2022) in plants,
659 through controlling cell osmotic pressure. Silencing of *GhProDH2-4* gene also increases
660 proline content, cell osmotic pressure and affects the glyoxylate dicarboxylic acid pathway,
661 leading to the accumulation of organic acids and reducing ROS accumulation (Figure 9).
662 Moreover, silencing the *GhProDH2-4* gene increases gene expression in acetone related
663 pathways, leading to the accumulation of secondary metabolites such as flavonoids which

depriving ROS accumulation, thereby jointly regulating cotton's drought and salt tolerance (Figure 9).

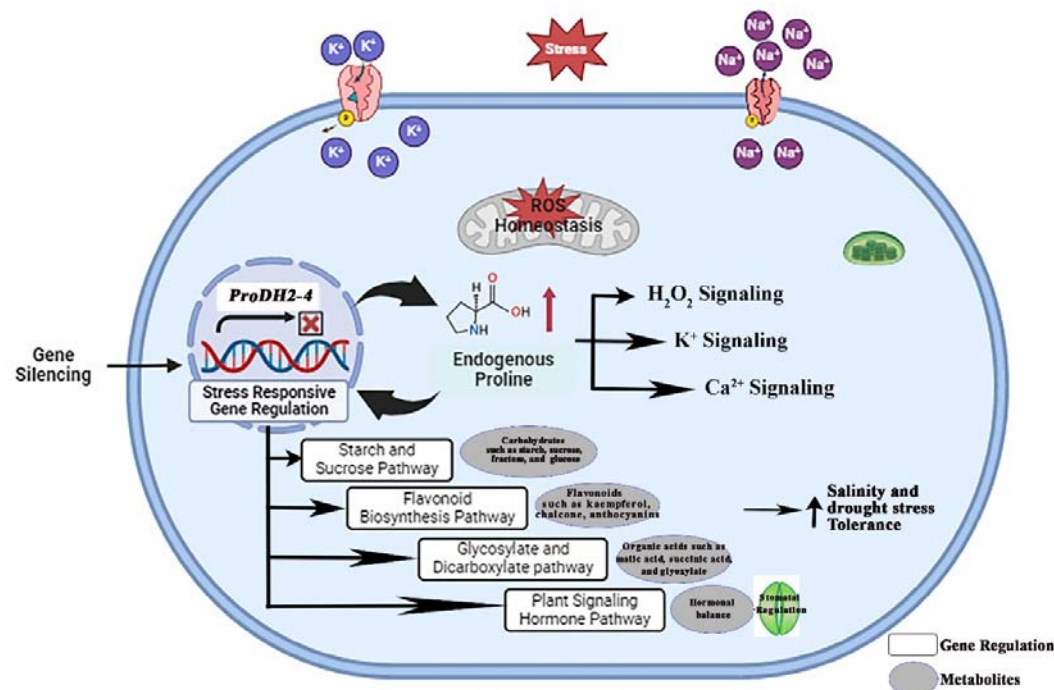


Figure 9 Regulatory network model of ProDH2-4 role in cotton plant under drought stress and salt stress.

4. CONCLUSION

This study explores how proline enhances the drought and salt tolerance of cotton. The ProDH gene family, as the rate limiting enzyme for proline degradation, has a certain impact on plant drought and salt tolerance. There was only *GhProDH2* gene in cotton, which has a certain degree of conservation. Evolutionary tree and protein structure prediction revealed that *GhProDH2-1*, *GhProDH2-3* and *GhProDH2-5* were more closely related to *GhProDH2-2*, *GhProDH2-4*, and *GhProDH2-6*, respectively. Overexpression

and silencing of *GhProDH2-1*, *GhProDH2-2*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6* genes in *Arabidopsis thaliana* tolerate the toxicity of drought and salt stress. In addition, the response of *GhProDH2-2* gene to salt and drought stress is weaker than other genes(*GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6*). In cotton, VIGS silencing of *GhProDH2-1*, *GhProDH2-4*, *GhProDH2-5*, and *GhProDH2-6* genes enhanced cotton drought and salt tolerance. Under salt and drought stress, The wilting status of cotton leaves after silencing *GhProDH2-4* is milder than other genes(*GhProDH2-1*, *GhProDH2-5*, and *GhProDH2-6*) Transcriptome analysis revealed that under salt and drought conditions, *GhProDH2-4* had the most significant impact on DEGs. Through microstructure analysis, it was found that silencing *GhProDH2-4* alleviates leaf wrinkling, reduces ROS accumulation, decreases stomatal opening, and enhances net photosynthetic rate. Through transcriptome, metabolome and physiological phenotype joint analyses, it was found that silencing of *GhProDH2-4* enhances cotton's drought and salt tolerance by affecting carbon metabolism, flavonoid metabolism, glyoxylate dicarboxylic acid pathway accumulation of soluble sugars, flavonoid metabolites, organic acids, and other substances. This study provides a theoretical basis for exploring role of ProDH in the drought and salt tolerance of plants. At the same time. provides valuable genetic targets for engineering crop varieties with highly stress resistant.

5. MATERIALS AND METHODS

5.1. Research design and treatments

To promote seed germination in the upland cotton variety "TM-1", seeds were surface sterilized by using 95% concentrated sulfuric acid, and dried. The seeds were transplanted into pots containing a 2:1 mixture of nutrient soil and vermiculite. The growth conditions included a temperature of 25°C, humidity of 37%, a light cycle of 16/8 h, and a light

701 intensity of 2000 lx in a constant temperature incubator. Seedlings were subjected to
702 drought and salt treatments by using 15% PEG and 15% NaCl, respectively. Samples were
703 collected at 1h, 3h, 6h, 12h, and 24h intervals, immediately frozen in liquid nitrogen, and
704 stored at -80°C for RNA extraction.

705 For *Arabidopsis thaliana*, seeds were sterilized with mercury chloride and placed on 1/2
706 MS culture medium. They were kept in the dark at 4°C for 24 h, then transferred to a
707 constant temperature incubator for about a week. Once the seedlings were growing well,
708 they were moved to pots filled with a 2:1 mixture of nutrient soil and vermiculite. After 3
709 to 5 days of drought treatment, physiological indicators were measured. Seven days after
710 treatment, the plants' morphology was observed, and survival rates were recorded after 3
711 days of rehydration..

712 **5.2.RNA extraction and qRT PCR analysis**

713 To extract RNA from the prepared samples, the samples were grinded in liquid
714 nitrogen and use the RNAPrep Pure Plant Plus Kit. Measure the RNA concentration, and
715 then use the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit to
716 reverse transcribe the RNA into cDNA. Dilute the cDNA five times and use it as a
717 template for fluorescence quantitative PCR (q-PCR). The reaction system and program
718 details are provided in Tables 1-1 and 1-2. q-PCR primers are designed using the Real-
719 Time PCR (TaqMan) Primer and Probes Design Tool website, with Ghactin serving as the
720 internal reference gene. The relative gene expression levels are calculated using the $2^{-\Delta\Delta C_t}$
721 $\Delta\Delta C_t$ method by three replicates.

722 **5.3.Cloning of *GhProDH* gene**

723 Coding sequence (CDS) were used for clone the *GhProDH* gene with specific
724 primers(appendix table S2) . Using cDNA as the template for amplification the complete

725 CDS sequence. Run the PCR product on a 1% agarose gel electrophoresis to obtain the
726 electrophoresis product. Use the Megan Agarose Gel Recovery Test Kit to cut and recover
727 the bands of the target fragment size, then purify them to obtain the target gene. Using the
728 ClonExpress II One-Step Cloning Kit, ligate the target gene into the T vector. Transform
729 the Vazyme #C502 competent cells through heat shock. Plate the transformed *Escherichia*
730 *coli* onto LB medium containing kanamycin and incubate overnight at 37°C in an inverted
731 position

732 **5.4.Construction of VIGS and Overexpression Vectors for *GhProDH* Gene**

733 Specific primers were used for overexpression and VIGS of the *GhProDH* gene.
734 Using the extracted RNA Reverse transcript into cDNA as a template, the gene fragments
735 were amplified and purified, then stored at -20 °C for future use. The overexpression
736 vector PBI121 was cleaved at Hind III and BamHI sites, while the VIGS vector Pyl156
737 utilized BamHI and SacI sites. Following gel electrophoresis and recovery, the linearized
738 vectors underwent Fusion Ligation technology to form complete constructs. Subsequently,
739 these vectors were transformed into *Escherichia coli*, where single clones were selected
740 and sequenced for verification. Plasmids from confirmed sequences were extracted and
741 transformed into *Agrobacterium tumefaciens*. After cultivation on LB agar supplemented
742 with kanamycin and rifampicin, monoclonal colonies were selected and re-validated to
743 ensure correct insertion of the *GhProDH* gene. Finally, the validated bacteria were
744 preserved at -80°C for future experiments.

745 **5.5.Agrobacterium-Mediated Transformation and Infiltration Process**

746 The recombinant plasmids were transformed into *Agrobacterium* using the GV3101
747 Chemical Competent Cell reagent kit. Cultures of PYL156 PDS (positive control),
748 PYL192 (auxiliary vector), PYL156 *GhProDH2-1* (positive control), PYL156

749 *GhProDH2-4* (positive control), PYL156 *GhProDH2-5* (positive control), PYL156
 750 *GhProDH2-6* (positive control), and PYL156 (empty vector) were grown in liquid LB
 751 medium supplemented with rifampicin and kanamycin at 28°C with agitation at 200 RPM.
 752 They were shaken overnight until reaching an OD600 value of 1.0-1.5, then centrifuged
 753 and resuspended in a heavy suspension solution (MES, magnesium chloride, AS, water) at
 754 25°C in darkness for 3-4 h. The PYL156 (EV), PYL156 *GhProDH2-1* (VIGS), PYL156
 755 *GhProDH2-4* (VIGS), PYL156 *GhProDH2-5* (VIGS), PYL156 *GhProDH2-6* (VIGS),
 756 and PYL PDS suspensions were mixed with PYL192 in a 1:1 ratio. Each cotyledon's
 757 underside was injected with the mixed solution, followed by incubation in a 25°C
 758 incubator in darkness for 12 h, then transferred to a light cycle of 16 h light/8 h dark.

759 5.6. Infection of Arabidopsis and Screening of Transgenic Arabidopsis:

760 *Agrobacterium tumefaciens* carrying overexpression vectors was added to LB liquid
 761 medium containing kanamycin and rifampicin, and shaken at 28°C and 200 rpm until
 762 reaching an OD value of 1.0-1.5. After centrifugation, the bacterial pellet was resuspended
 763 in an equal volume of resuspension buffer (1/2MS, 5% sucrose, pH 5.8), adjusting the OD
 764 value of the suspension to 0.8. The suspension was treated in darkness at 25°C for 2 hours.
 765 Then, 0.02% Silwet L-77 was added to the suspension for infection. Flower buds were
 766 immersed in the suspension for about 1 minute, laid flat, and incubated in darkness for 24
 767 hours before returning to normal growth conditions. This infection process was repeated
 768 after one week.

769 Following infection, seeds of *Arabidopsis thaliana* were designated as the T0 generation.
 770 These seeds underwent a 5-minute wash in mercuric chloride, followed by 7-8 washes in
 771 sterile water. They were then plated on 1/2MS medium containing kanamycin for 24 h of
 772 vernalization. Positive seedlings were selected and transplanted into small pots. The seeds

obtained from these plants were labeled as the T1 generation, and the process was repeated until obtaining seeds from the T3 generation.

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Author Contributions

Suli Bai: performed research, tools, analyzed data, wrote the paper and editing. Suli Bai and Pei Zhao: resources, investigation. Fei Su , Shuai Wang, Junjuan Wang and Yan Li: tools, visualization, computational. Ibrahim A. A. Mohamed and Zujun Yin: designed the research, contributed new analytic, writing-reviewing and editing. All authors have read and approved the final manuscript.

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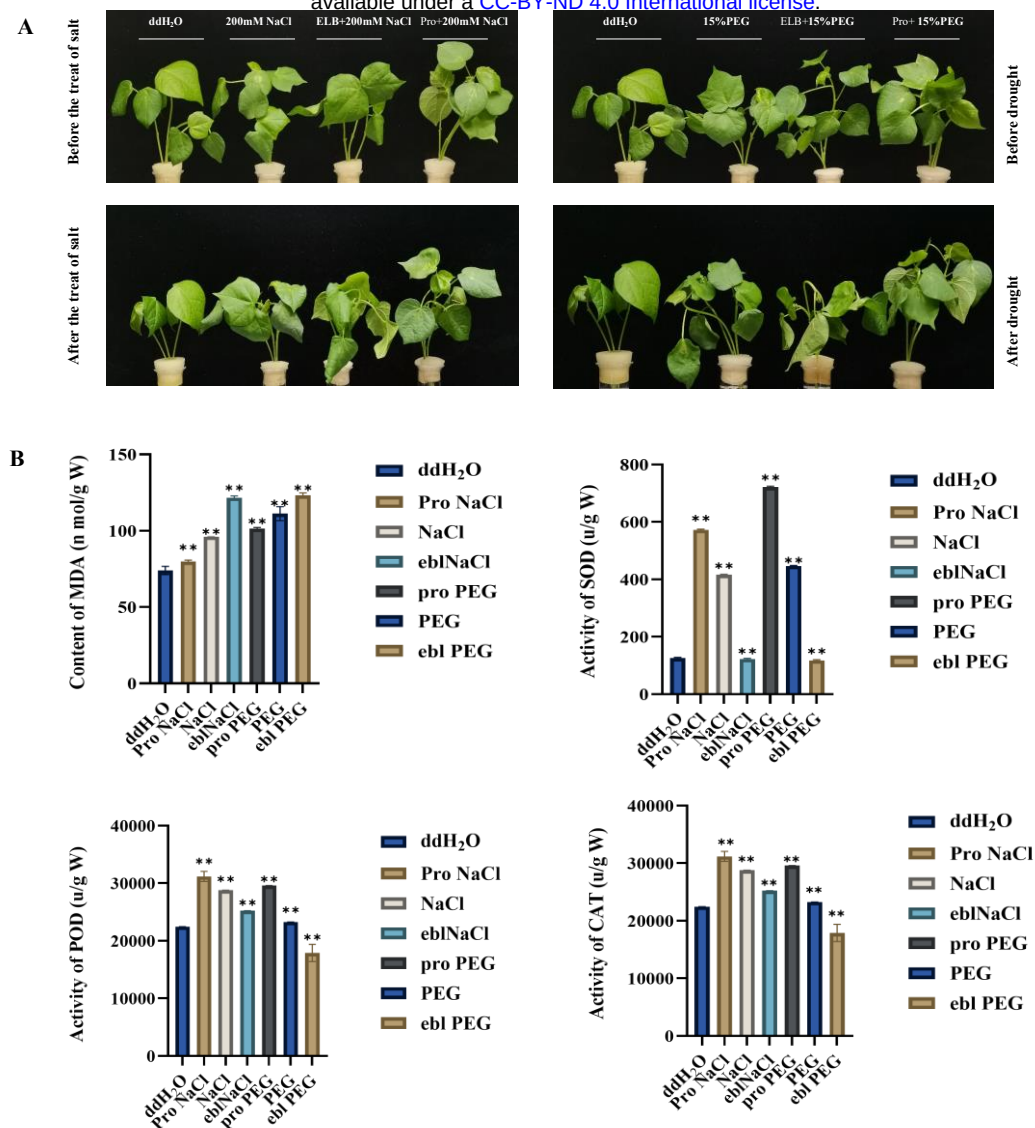


Figure 1 The effect of proline on drought and salt tolerance of cotton (A) Growth status of three leaf cotton under control conditions of ddH₂O, salt stress (200mM NaCl), drought stress (20% PEG), and proline+salt stress (Proline+200mM NaCl), proline+drought stress (Proline+20% PEG), Proline inhibitor+ salt stress (ELB+200mM NaCl), Proline inhibitor+ drought stress (ELB+20% PEG).(B) MDA content under various treatments, and SOD, POD, CAT content under various treatments Error bars represent $\pm$ SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

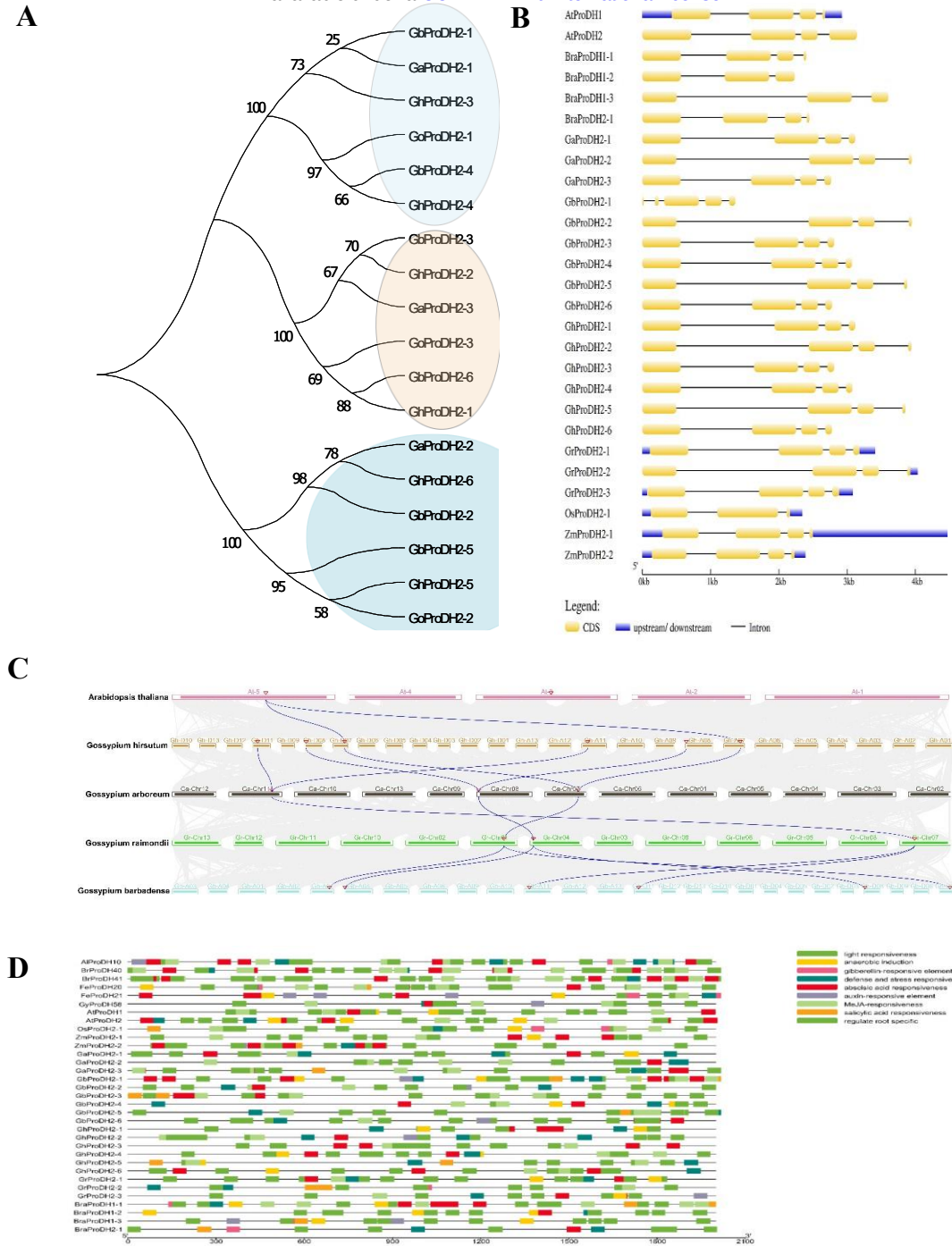


Figure 2 (A) Evolutionary relationships among four cotton species (B) The gene structure of *ProDH* genes in *Arabidopsis*, Maize, rice, cotton, and Chinese cabbage was analyzed (C) Collinearity analysis of *AtProDH*, *GhProDH*, *GaProDH*, *GrProDH* and *GoProDH* genes (D) Analysis of Cis-regulatory element in *ProDH* genes.

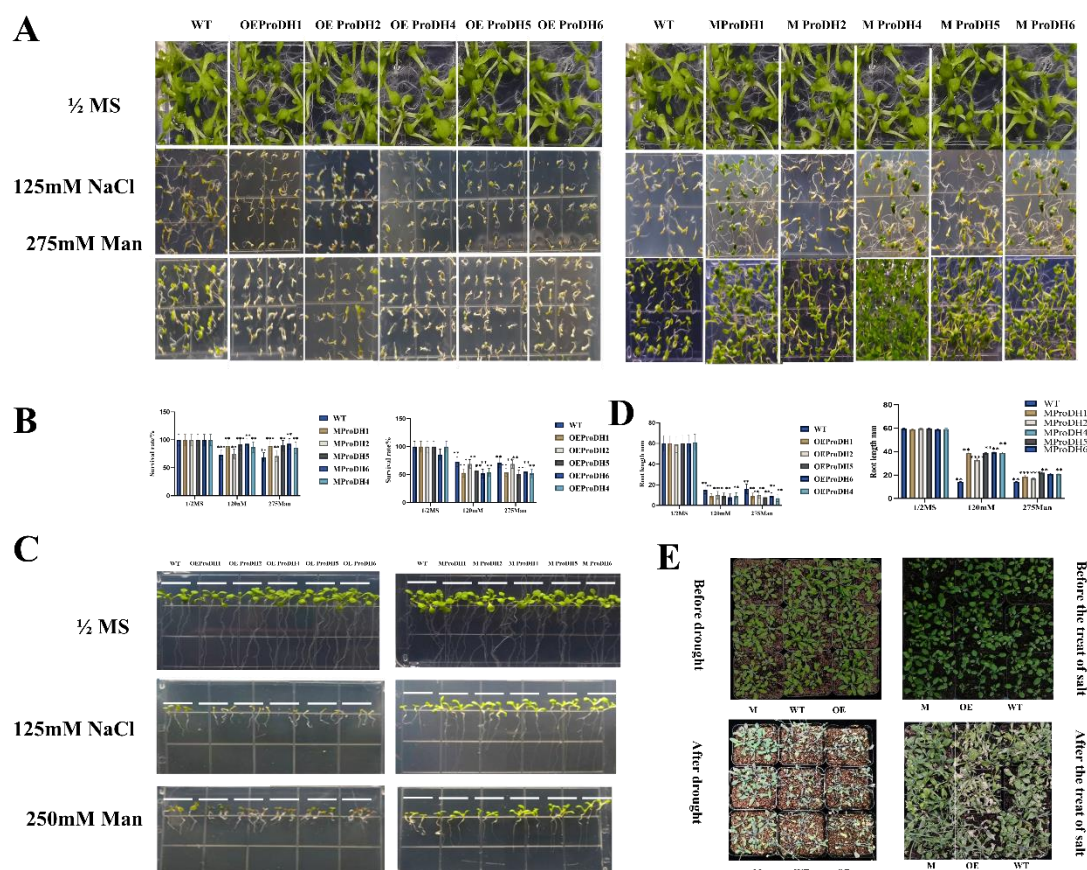


Figure 3 Functional validation of genetically modified *Arabidopsis*. (A) Germination assays were conducted using transgenic, mutant, and wildtype *Arabidopsis* seeds on $\frac{1}{2}$ MS medium supplemented with 125 mM NaCl and 250 mM mannitol. The untreated $\frac{1}{2}$ MS medium served as the control. (B) Survival rates were assessed for transgenic, mutant, and wildtype seeds exposed to 125 mM NaCl and 250 mM mannitol stress conditions. (C, D) Root length comparisons were made between transgenic, mutant, and wildtype plants grown on $\frac{1}{2}$ MS medium supplemented with 250 mM mannitol and 125 mM NaCl for 10 days. (E) Response of *GhProDH4* transgenic lines and wildtype plants under drought and salt stress conditions. The error bars indicate $\pm$ SD of at least three biological replicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

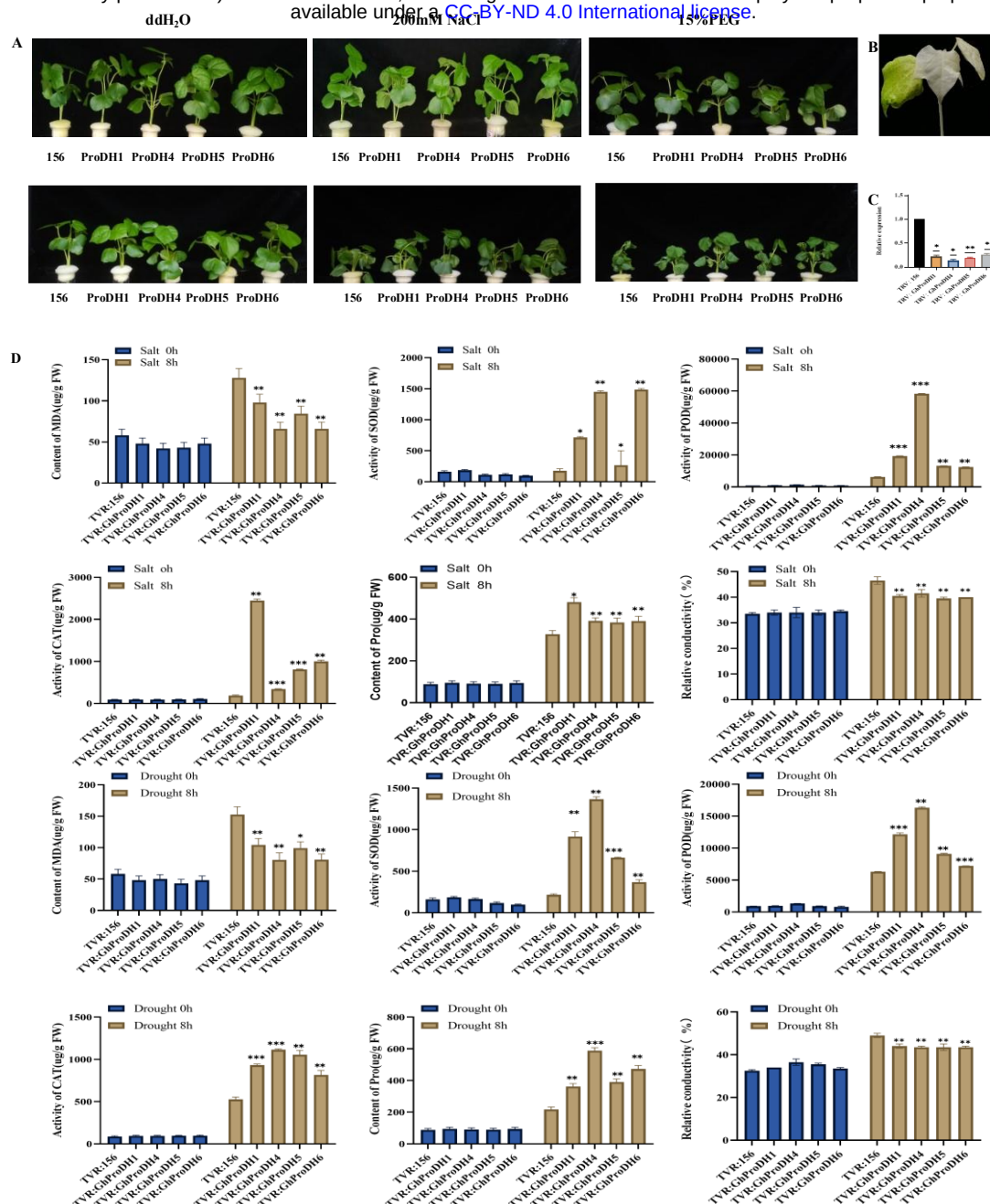


Figure 4 the impact of *GhProDH* silencing on cotton's tolerance to drought and salt stress. (A) Comparison of phenotype of VIGS plants and control plants PYL156 before and after and control plants PYL156 before and after CK, 200mM NaCl, 20%PEG treatment. (B) Representative albino marker plant used in the study. (C) Relative silencing efficiency assessed by qRT-PCR, showing VIGS plants with expression reduced to less than half compared to control plants. (D) Comparison of malondialdehyde (MDA) content between VIGS-silenced and control plants using pYL156 under drought or salt treatment. (E) Comparison of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), proline (Pro) contents and in VIGS-silenced plants versus control plants under drought or salt treatment conditions. Error bars represent $\pm$ SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

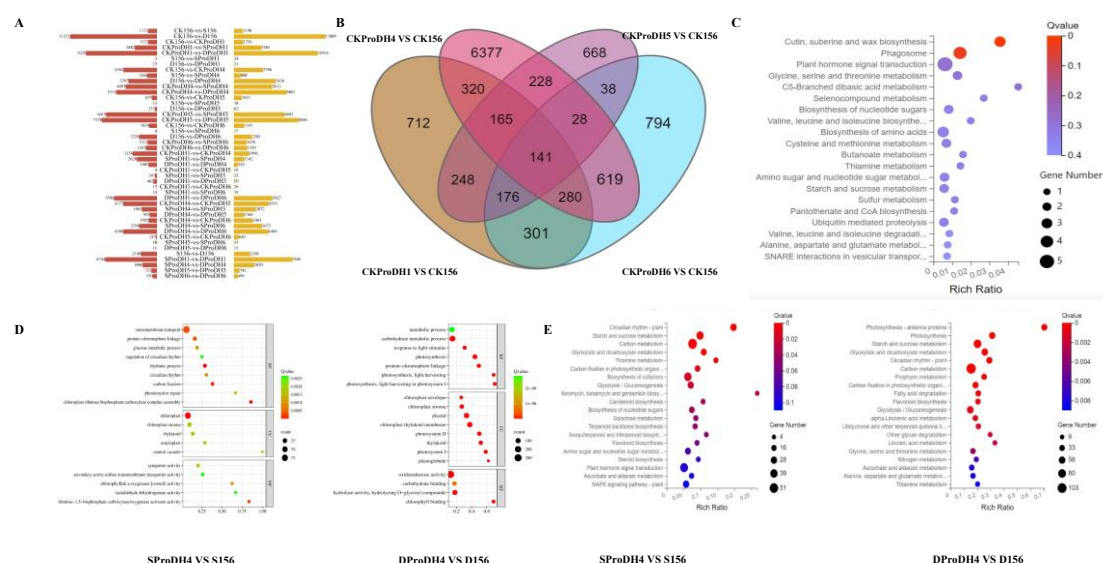


Figure 5: Detailed analysis of RNA-seq data depicting differentially expressed genes (DEGs) in various comparison sets. (A) Bar graph illustrating total DEGs, with red indicating upregulated and yellow-accented representing downregulated DEGs in each comparison. (B) Gene Venn diagram analysis under stress free conditions. (C) KEGG enrichment analysis of 141 genes. (D) GO enrichment analysis of GhProDH1/156, GhProDH4/156, GhProDH5/156, and GhProDH6/156 under salt stress. (E) GO enrichment analysis of GhProDH1/156, GhProDH4/156, GhProDH5/156, and GhProDH6/156 under drought stress. (F) KEGG enrichment analysis of GhProDH1/156, GhProDH4/156, GhProDH5/156, and GhProDH6/156 under salt stress. (G) KEGG enrichment analysis of GhProDH1/156, GhProDH4/156, GhProDH5/156, and GhProDH6/156 under drought stress. CK156= pYL156; D156= pYL156 drought 8h; S156= pYL156 salt 8h; CKProDH1= pYL156: *GhProH1*; DProDH1= pYL156: *GhProDH1* drought 8h; SProDH1= pYL156: *GhProDH1* salt 8h; CKProDH4= pYL156: *GhProH4*; DProDH4= pYL156: *GhProDH4* drought 8h; SProDH4= pYL156: *GhProDH4* salt 8h; CKProDH5= pYL156: *GhProH5*; DProDH5= pYL156: *GhProDH5* drought 8h; SProDH5= pYL156: *GhProDH5* salt 8h; CKProDH6= pYL156: *GhProH6*; DProDH6= pYL156: *GhProDH6* drought 8h; SProDH6= pYL156: *GhProDH6* salt 8h

Figure 6: (A) Clustering heatmaps illustrating starch and sucrose pathway analysis for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156. (B) Clustering heatmaps depicting starch and sucrose pathway analysis for SProDH4/S156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156. (C) Heat map illustrating glyoxylate dicarboxylic acid pathway clustering for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156. (D) Heat map depicting glyoxylate dicarboxylic acid pathway clustering for SProDH4/S156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156. (E) Heat map depicting Carbohydrate

metabolism for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156. (F) Heat map illustrating Carbohydrate metabolism for SProDH4/S156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156. (G) Heat map depicting Flavonoid biosynthesis for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156. (H) Heat map depicting Flavonoid biosynthesis for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156.

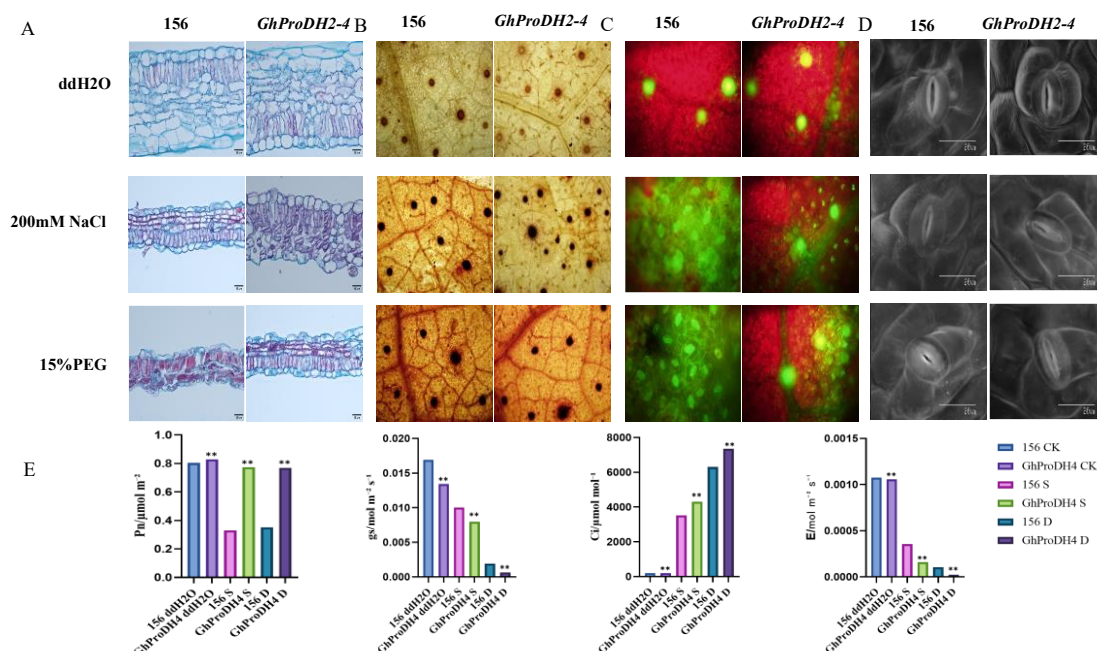


Figure 6 (A) Cotton plant TPV: 156, VIGS (GhProDH2-4) leaf cross-sectional condition under water, 200mM NaCl, and 20% PEG (B) DAB staining method was used to detect the accumulation status of ROS in VIGS (GhProDH2-4) of cotton plants with TPV: 156 under water, 200mM NaCl, and 20% PEG conditions. (C) DCFH-DA method was used to detect the accumulation status of ROS in VIGS (GhProDH2-4) of cotton plants with TPV: 156 under water, 200mM NaCl, and 20% PEG conditions. (D) Cotton plant TPV: 156, stomatal status of VIGS (GhProDH2-4) under water, 200mM NaCl, and 20% PEG conditions. (E) Cotton plant TPV: 156, VIGS (GhProDH2-4) under water, 200mM NaCl, and 20% PEG conditions detect net photosynthetic rate (pn), intracellular carbon dioxide solubility (Ci), stomatal conductance (gs), transpiration rate (E).

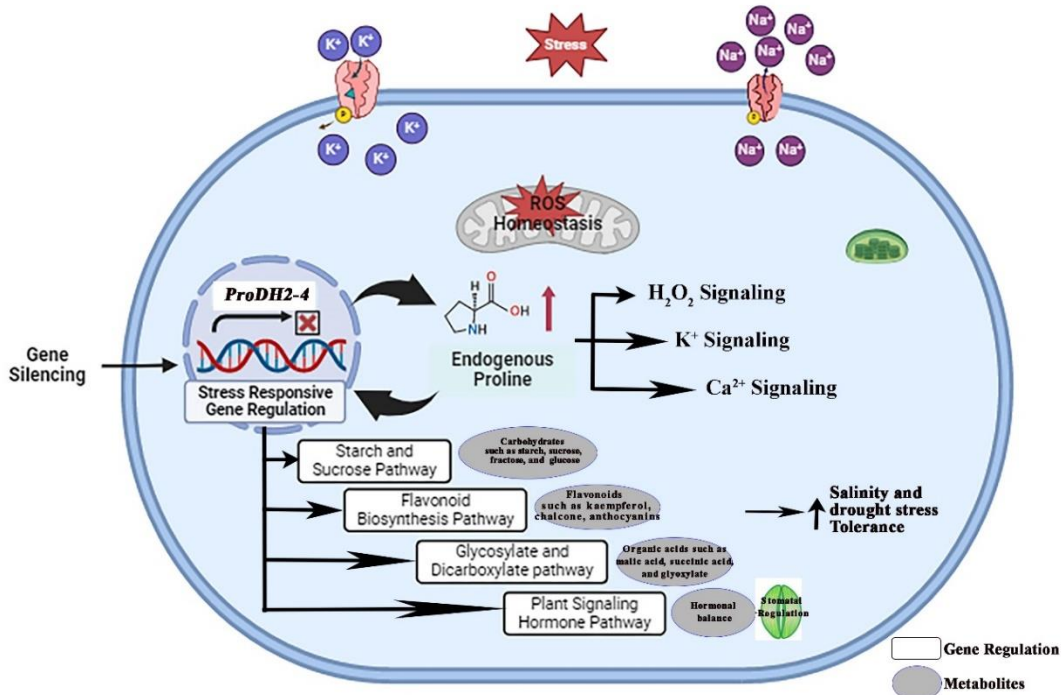
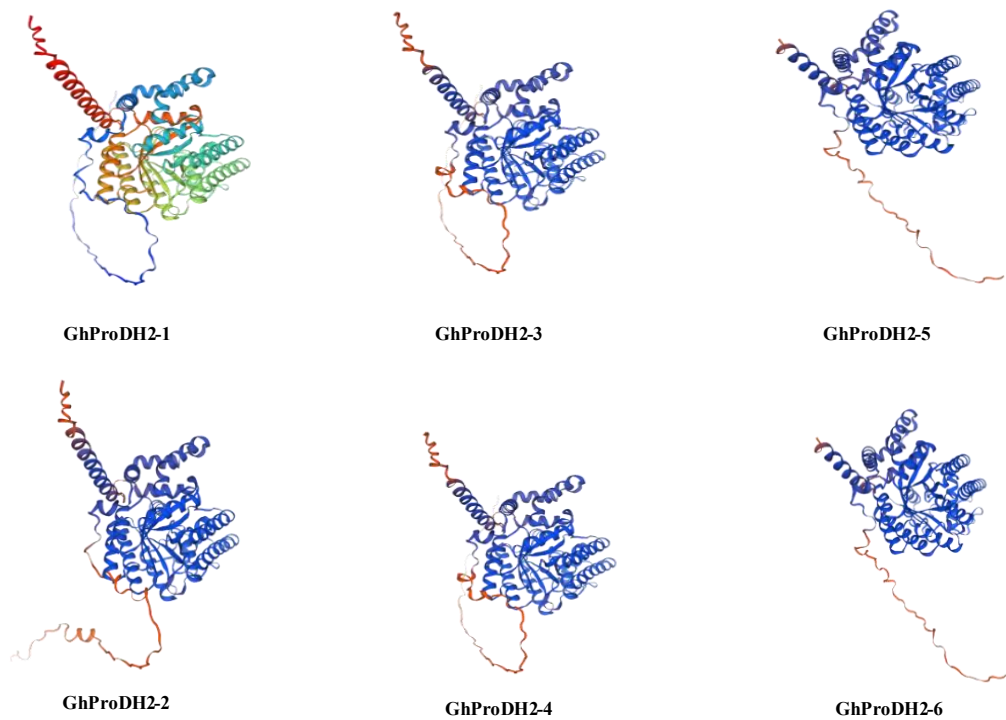
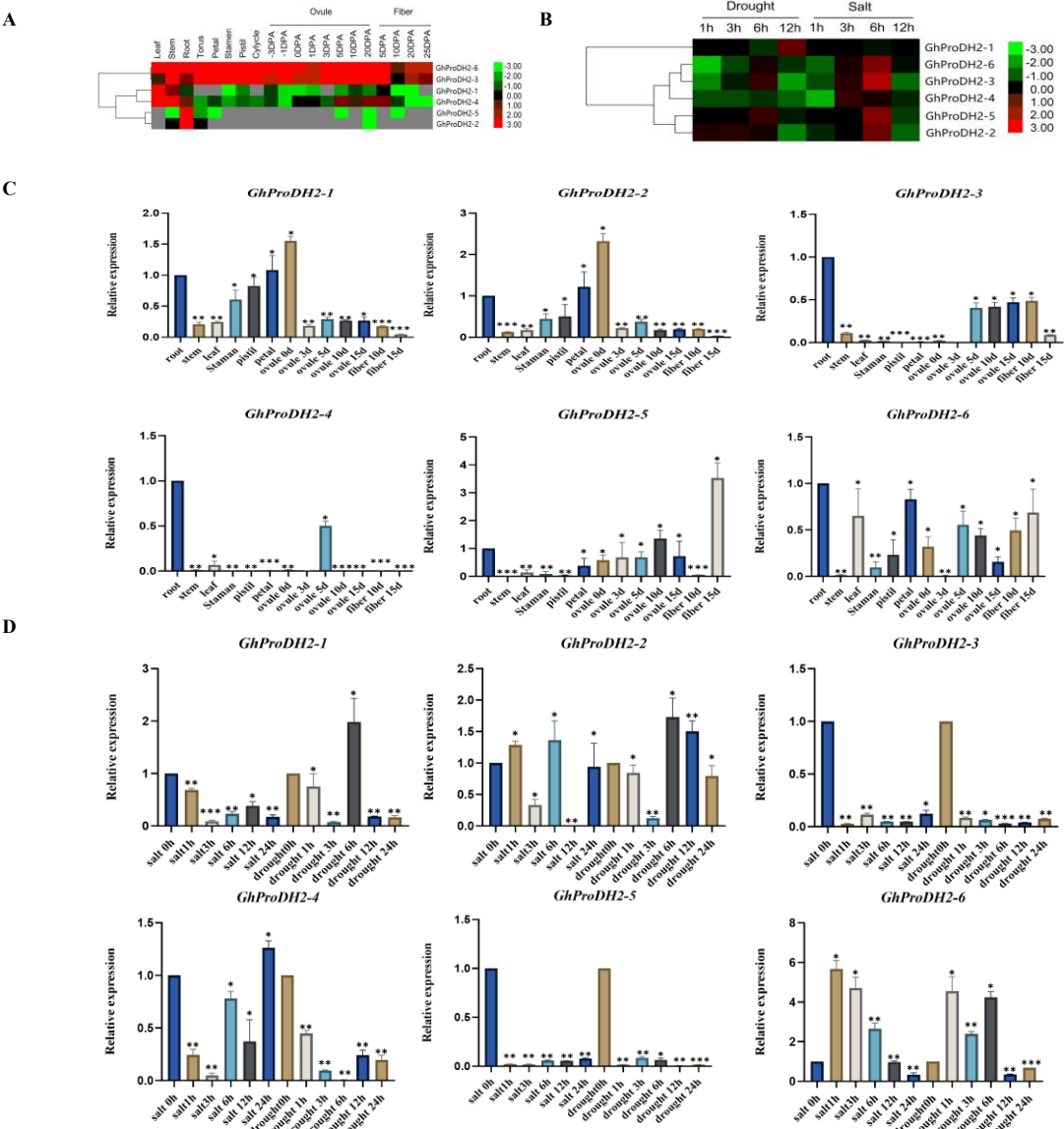


Figure 9 Regulatory network model of ProDH2-4 role in cotton plant under drought stress and salt stress.



appendix figure 1: Prediction of *GhProDH2* protein structure in upland cotton



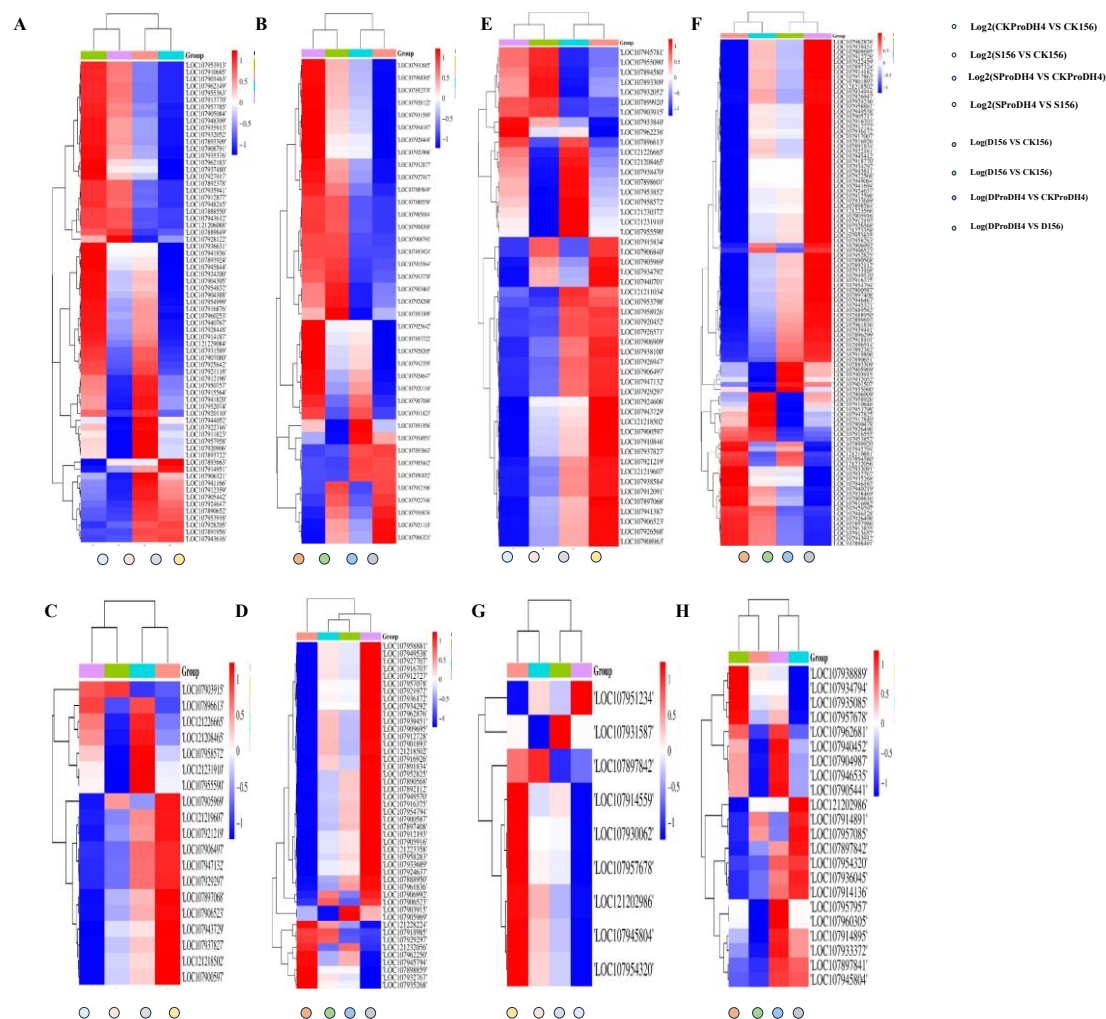
appendix figure 2 (A) Expression patterns of *GhProDH* genes in various tissues, (B) Expression patterns of *GhProDH* genes under different stresses. (C) Expression patterns of *GhProDH* genes in various tissues, qRT-PCR analysis verifying the expression pattern of *GhProDH* genes in tissues while (D) Expression patterns of *GhProDH* genes under different stresses, qRT-PCR based verification of expression pattern of *GhProDH* gene under stress The error bars represent $\pm$ SD of at least three biological replicates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

pYL156: *GhProDH4* drought 8h; SProDH4= pYL156: *GhProDH4* salt 8h;

CKProDH5= pYL156:*GhProH5*; DProDH5= pYL156: *GhProDH5* drought 8h;

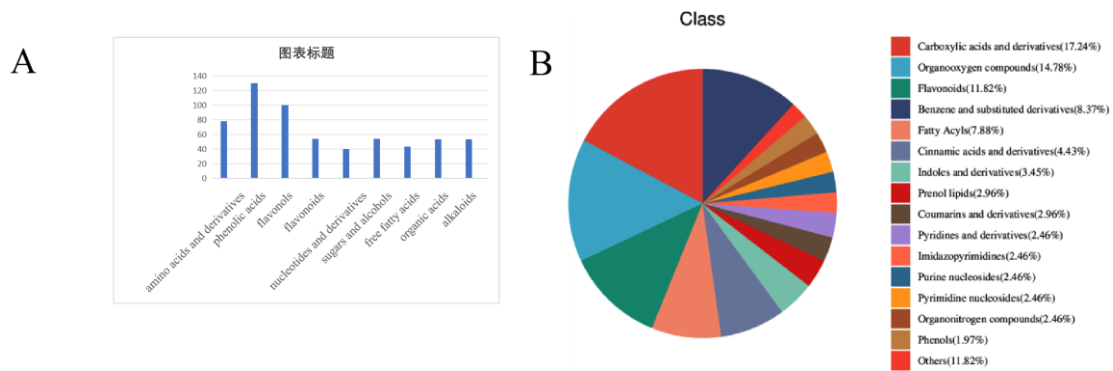
SProDH5= pYL156: *GhProDH5* salt 8h; CKProDH6= pYL156:*GhProH6*; DProDH6=

pYL156: *GhProDH6* drought 8h; SProDH6= pYL156: *GhProDH6* salt 8h



appendix figure 4: (A) Heat map depicting starch and sucrose pathway clustering for SProDH4/S156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156(B) Heat map illustrating starch and sucrose pathway glyoxylate dicarboxylic acid pathway clustering for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156.(C) Clustering heatmaps illustrating glyoxylate dicarboxylic acid pathway analysis for SProDH4/S156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156. (D) Clustering heatmaps illustrating glyoxylate dicarboxylic acid

pathway analysis for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156. (E) Heat map illustrating Carbohydrate metabolism for SProDH4/S156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156.(F) Heat map depicting Carbohydrate metabolism for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156.(G) Heat map depicting Flavonoid biosynthesis for SProDH4/D156, S156/CK156, SProDH4/CKProDH4, and CKProDH4/CK156. (H) Heat map depicting Flavonoid biosynthesis for DProDH4/D156, D156/CK156, DProDH4/CKProDH4, and CKProDH4/CK156.



appendix figure 5 (A) The content of various substances in the metabolome. (B) The proportion of each substance content in the metabolome.