Response of lignin and flavonoid metabolic pathways in *Capsicum annuum* to drought and waterlogging stresses

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Abstract

Water stress is a critical factor limiting the growth and development of *Capsicum annuum*. Flavonoids and lignin are important secondary metabolites that serve as signaling molecules in plant stress responses. However, the effects and regulatory mechanisms of lignin and flavonoids under water stress in *Capsicum annuum* remain unknown. The present study focused on the effects of drought and waterlogging stress on the morphology, hydrogen peroxide, and relative chlorophyll (SPAD), as well as enzyme activities, metabolite contents, and gene expression related to lignin and flavonoid metabolic pathways in *Capsicum annuum*. The results showed that drought and waterlogging stresses on the *Capsicum annuum* variety ‘Shuyu2’ significantly reduced plant height, stem thickness, and single-fruit weight, and increased fruit shape coefficients. Drought stress increased H$_2$O$_2$ and SPAD content, enhanced the activity levels of metabolic enzymes (phenylalanine deaminase, cinnamate 4-hydroxylase, coenzyme A ligase, peroxidase, and polyphenol oxidase), and up-regulated the expression of related genes, *phenylalanine deaminase (PAL)*, *trans-cinnamate monoxygenase (C4H)*, *chalcone isomerase (CHI)*, and *mangiferyl hydroxycinnamoyltransferase (HCT)*, while also promoting the accumulation of metabolites (total phenolics, flavonoids, and lignin) that have a restorative effect on drought stress. The continuous accumulation of H$_2$O$_2$ and the increase and then decrease in SPAD under waterlogging stress was also observed. Waterlogging stress also enhanced the activities of the above-mentioned metabolic enzymes, but the related genes were selectively down-regulated, e.g., *C4H*, *4CL*, and *peroxidase (POD)*, which resulted in the inhibition of the synthesis of lignin, flavonoids, and total phenols. These results indicate that the *Capsicum annuum* variety ‘Shuyu2’ is a drought-tolerant, waterlogging-sensitive variety. Meanwhile, the lignin and flavonoid pathway is a key pathway in response to drought stress in *Capsicum annuum*, which improves the theory of stress tolerance breeding in *Capsicum annuum*.

**Keywords:** *Capsicum annuum*; drought stress; waterlogging stress; flavonoid pathway; lignin pathway
Introduction

In the context of global warming, droughts and floods are becoming more frequent (Trenberth, 2005; Hirabayashi et al., 2008), and pose one of the biggest threats to future food security (Farooq et al., 2009; Ashraf, 2012). Capsicum occupies an important position in the global vegetable industry, as it is used both as food and medical treatment (Palevitch and Craker, 1996; Varghese et al., 2017). However, most common capsicum varieties are very sensitive to water stress, and, as a result, too much or too little irrigation water can cause a range of physiological responses (Moreno et al., 2003; Anstalt, 2013), leading to yield loss. Whereas studies have shown that the most sensitive periods to water stress are the flowering and fruiting periods (Katerji et al., 1992). It is a great challenge to improve the ability of capsicum to tolerate flooding stress and drought during the reproductive period.

Plants adopt and evolve various molecular mechanisms and pathways to resist water stress, including osmotic tolerance, ionic tolerance, and tissue tolerance (Munis et al., 2010; Patade et al., 2011; Abrar et al., 2020). The phenylpropane metabolic pathway mainly includes two pathways, lignin metabolism and flavonoid metabolism. Lignin biosynthesis and flavonoid biosynthesis are important pathways involved in plant response to abiotic stress (Balasundram et al., 2006; Lattanzio et al., 2006). Many studies have shown that lignin biosynthesis is enhanced under drought stress. Several plants have shown an increase in lignin content when placed under drought or water deficit conditions. For example, the gene expression of CCoAOMT (caffeoyl-CoA O-methyltransferase) was enhanced under drought stress in the root elongation region of soybean, in grape berries (Giordano et al., 2016), and in stem and leaf tissues (Liu et al., 2016) of willow jacaranda (Yamaguchi et al., 2010). In maize, drought improves drought tolerance by inducing cinnamoyl coenzyme A reductase 1 (CCR 1) and CCR 2 expression, (F3H), and flavonol synthase (FLS) genes in wheat under drought stress, accompanied by an increase in total flavonoids and anthocyanin content. Gharibi et al. (2019) found that the content of most flavonoids and the expression of related genes (phenylalanine deaminase (PAL) CHS, CHI, F3H, F5'H, and FLS) were also increased in Achillea whitehead during drought stress. Not only that, but flavonoids have been suggested as antioxidants in stressed plants. The location of their hydroxyl groups, double carbon bonds, and modifications such as glycosylation, isoprenylation, and methylation determine their antioxidant properties (Rice-Evans et al., 1997).

Waterlogging stress has different effects on lignin and flavonoid pathways. Nguyen, et al. found (Nguyen et al., 2016) that flooding reduced the lignin content in wheat internodes and was accompanied by a transcriptional repression of three genes that are highly expressed in wheat internodes, including PAL6, CCR2, and lignin synthase (F5H2), as well as a decrease in the activity of PALase. Lin et al. (2019) found that seven genes related to lignin were down-regulated and accompanied by a decrease in lignin content in soybean under waterlogged stress, which he speculated to be the reason for the softening of soybean after soaking in water. Mfarrej et al. (2022) found that the flavonoid content in the rhizomes of wheat decreased under waterlogged stress. It has been shown that genes related to CHS, CHI, dihydroflavonoid 3-hydroxylase (F3H), and colorless anthocyanin reductase (LAR) are up-regulated in the flavonoids of maceration-tolerant apple varieties, whereas these genes show a down-regulation in maceration-sensitive apple varieties (Zhang et al., 2023). This suggests that the flavonoid pathway responds selectively according to the tolerance of the plant species. Lignin is the initiation point of the phenylalanine metabolic pathway and an important substrate for the synthesis of other polyphenols such as flavonoids (Labeeuw et al., 2015). In crops such as chili peppers under water stress, flavonoids improve the physiological adaptation of plants to unfavourable environments by regulating the content of the hormone indole-3-acetic acid in the plant, which indirectly affects abscisic acid content (Swain, 1977). Moisture and high temperature stress stimulate the activities of PALase and peroxidase (POD), increase the synthesis of other phenolics, and mitigate the damage caused by stress to the plant body (Laddomada et al., 2021). A recent study conducted by Phimchan et al. (2014) showed that PALase was positively correlated with...
capsaicinoid synthesis in chili peppers under drought stress, whereas the activities of cinnamic acid 4-
hydroxylase (C4H) and POD enzymes did not follow the same trend as capsaicinoid synthesis.

The types of drought tolerance mechanisms are categorized as avoidance, avoidance and tolerance. According to the results of our previous year’s experiments, the yield and other indicators of the ‘Shuyu2’ variety show that this variety has a certain degree of drought tolerance (Figure S1), but the molecular mechanism of drought tolerance has not been studied. At the same time, the tolerance of this variety to waterlogging stress is not clear. In order to investigate the molecular mechanism of flavonoid and lignin metabolism in chili peppers under drought and waterlogging stress, the present study utilized RNA-Seq to investigate the molecular mechanism of flavonoid and lignin metabolism in peppers under drought and waterlogging stress. In this study, we conducted drought and waterlogging experiments for 7 and 14 days, respectively, using RNA-Seq technology and the Capsicum annuum cultivar ‘Shuyu2’ as experimental materials, and sequenced the two treatment groups according to the results of physiological and biochemical indexes. In this study, epigenetic traits, physiological and biochemical indexes and RNA-Seq were combined to investigate the metabolic mechanisms of flavonoids and lignin in Capsicum annuum under water stress, which provided a theoretical basis for subsequent research on Capsicum annuum resistance.

Materials and Methods

Raw material and growing conditions of Capsicum annuum

The test material for this experiment was ‘Shuyu2’, and the experiment was carried out in the greenhouse of the Hunan Agricultural University. Firstly, we chose capsicum seeds with full grains and uniform size, sowed them in plastic nutrient pots in the greenhouse (the substrate was charcoal soil/vermiculite = 2:1), and managed them using the conventional seedling nursery method. Seedlings grew to four leaves and were then transplanted in the rain net room in plastic pots (pot height 23 cm, diameter 22 cm). The soil used in the pots for the substrate soil had the following characteristics: total organic matter + total humic acid ≥ 50%, total nutrients ≥ 3.5%, and a pondus hydrogenii (pH) value of 5.5-6.5; the natural soil was mixed with a mixture at a ratio of 1:1. The soil should be sterilized with carbendazim and trichlorfon in advance. Each pot was filled with 4.5 kg of soil to 90% full, and 8 g of nitrogen, phosphorus, and potassium compound fertilizer was applied. Regular field management was carried out after planting. In order to mimic extreme disaster weather, we chose two stresses, extreme drought and waterlogged stress, to treat the plants. The experimental Capsicum annuum variety was ‘Shuyu2’, and the following three treatments were implemented 10 days after flowering: CK, control treatment (natural state, soil relative water content of 70-85%); SD, severe drought stress (soil relative water content of 25-40%); and, waterlogging stress (ZS) treatment. The method of waterlogging stress treatment involved placing the Capsicum annuum plants, which had been planted in plastic pots, into large plastic buckets containing water, and the treatment process required maintaining the depth of water in the outer buckets at the same level as the soil layer (which did not come up to the rim of the plastic pots). Four pots were planted per treatment with three replicates, for a total of thirty-six pots.

Determination of plant height, stem thickness, and single-fruit indicators

After the water stress treatments reached the set field water holding capacity, nine plants with uniform growth were first selected and labelled. After that, plant height at the flowering and fruiting stages was determined every 7 days with a straightedge, and stem thickness was determined with a vernier caliper. Mature (dark green) fruits were harvested from Capsicum annuum plants cultivated under control and treatment conditions. Mature fruits were harvested after 7 and 14 days of water stress. Individual fruit fresh weight (g) and dry fruit weight (g) (i.e., oven-dried weight) were measured using a balance. Fruit length was measured in
terms of fruit diameter using vernier calipers, and fruit length was divided by fruit diameter to calculate the fruit shape coefficient.

**Determination of total phenols, lignin, and flavonoids in Capsicum annuum**

The total phenol content of the samples was measured using an assay kit (BC1340, Solarbio, Beijing, China) according to the manufacturer’s instructions. The total phenolic content of the fruit was recorded in mg. Gallic acid was used as a standard (Singleton et al., 1999). The lignin content of the samples was measured using an assay kit (BC4200, Solarbio, Beijing, China) according to the manufacturer’s instructions. The determination of flavonoid content was performed using a colorimetric assay (Molina-Quijada et al., 2010; Pérez-Hernández et al., 2012).

**Hydrogen peroxide content and (SPAD) relative chlorophyll content in chili peppers and determination of enzyme activities related to polyphenol metabolism**

The relative content of chlorophyll (SPAD) was measured using a SPAD-502PLUS chlorophyll meter at 8.00-9.30 in the morning, 7 and 14 days after stress. Hydrogen peroxide (H₂O₂) was detected using the CHEKINETM H₂O₂ micro method. The kit was from Abbkine, California, USA. Enzyme-linked immunosorbent assay (ELISA) was performed for the determination of contents of phenylalanine (PAL), cinnamate 4-hydroxylase (C4H), and peroxidase (POD). The kits utilized were obtained from Beijing Solarbio Biotechnology Co., and all measurements were performed according to the manufacturer’s reference manual.

**RNA extraction, library construction, and transcriptome sequencing**

RNA extracted from mature fruits of *Capsicum annuum* treated with SD for 7 days and ZS for 7 days was collected for transcriptome sequencing. Total RNA extraction, cDNA library construction, and transcriptome sequencing were performed by Wuhan IlaiBio (Wuhan, China). TRIzol reagent was used for RNA extraction, followed by treatment with RNase-free DNase I to remove genomic DNA contamination. Nine cDNA libraries, CK (CK1, CK2, CK3), SD7 (SD1, SD2, SD3), and ZS7 (ZS1, ZS2, ZS3), were constructed from CK, SD-treated, and ZS-treated fruits samples, respectively. The Illumina Paired End Sample Prep Kit was used for library construction, including mRNA enrichment, fragmentation, and double-stranded cDNA synthesis. Finally, the libraries were sequenced using the Illumina HiSeq™ 2000 platform. After filtering the raw sequence reads using the Illumina pipeline, the 3’ adaptor sequence was removed. Additionally, low-quality reads, including short reads (<25 nucleotides), empty reads, and reads with only one copy number, were eliminated. The Trinity program was then used for de novo transcriptome assembly, generating unigenes (Grabherr et al., 2011; Wang et al., 2013). For conservative and accurate annotation, only sequences with perfect homology or no more than two nucleotide mismatches were considered. The assembled transcripts were used as query sequences to search against the NCBI non-redundant (nr) protein and COG (Clusters of Orthologous Groups of proteins) databases using BLASTX. The Blast2GO program was employed to obtain gene ontology (GO) annotations for the assembled transcripts (Conesa et al., 2005). Following GO annotation, WEGO software was used for GO functional classification (Ye et al., 2006).

**Quantification of gene expression levels**

HTSeq v0.6.1 was used to count the number of read segments mapped to each gene (ftp://ftp.ensemblgenomes.org:21/pub/plants/release-47/fasta/Capsicum annuum_annuum/). In addition, fragments per kilobase of exon model per million mapped read (FPKM) values were calculated for each gene, based on the length of the gene and the number of read segments mapped to that gene.
**Differential expression analysis**

Differential expression analysis between treatments was performed using the DESeq R package (1.18.0). P-values were adjusted using the Benjamini method to control for the false discovery rate. Genes with adjusted P-values < 0.05 identified using DESeq were designated as differentially expressed.

**GO enrichment analysis and KEGG pathway enrichment analysis**

A Gene Ontology (GO) enrichment analysis of DEGs was conducted using the GOseq R package. GO terms with a corrected P-value < 0.05 were considered significantly enriched by DEGs. The statistical enrichment of DEGs in KEGG pathways was tested using KOBAS software. According to the enrichment factor and Q values, the larger the value of enrichment factor, the greater the degree of enrichment; the Q-value (range 0-1) is the P-value after correction for multiple hypothesis testing, and the closer to zero the Q-value is, the more significant the enrichment is.

**qRT-PCR analysis**

The transcript levels of 11 genes involved in the lignin and flavonoid synthesis pathway were analyzed using quantitative real-time PCR (qRT-PCR). The screening conditions for differential expression of genes were as follows: fold-change differences > 2, false discovery rate (FDR) < 0.05. The gene-specific primers utilized (Table S1) were designed using Primer Premier 6.0 software. All qRT-PCRs were performed on a Light Cycler 96 system (Roche Diagnostics, GmbH, Mannheim, Germany). Reactions were completed in 20-μL volumes with SYBR Green qPCR Master Mix according to the manufacturer’s manual. The reaction was carried out at 95 °C for 2 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Three biological replicates were assayed. Actin was used as the reference gene (ubiquitin-conjugating gene). The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta CT}$ method, and the data were normalized to the geometric mean of the reference control genes.

**Statistical analysis**

All statistical analyses were performed with Origin2022 (OriginLab, Northampton), and significance of between- and intragroup differences was tested with the LSD range test, P < 0.05.)

**Results**

**Effect of water stress on plant height, stem thickness, and single-fruit index of Capsicum annuum**

In order to compare the growth status of Capsicum annuum under drought and waterlogging stress, we recorded the agronomic traits of Capsicum annuum plants at different stress time points (Figure 1). As shown in Figure 1, the height of Capsicum annuum plants at 7 days of severe drought (SD) stress was slightly lower than that of the control (CK), but there was no significant difference. At 7 days of waterlogging (ZS) stress, the height of Capsicum annuum plants was significantly lower than that of the control; at the same time, they were also shorter than those at 7 days of SD, and the whole plant showed a wilted state. As can be seen from Figure 1, the effects of drought and waterlogging stress on the plant height and stem thickness of pepper plants were mainly concentrated on day 7 (Figure 1A, 1B). On the 14th day of the stress, plant height and stem thickness decreased, but the amount of change was not significantly different from that on the 7th day. Therefore, we concluded that the effects of drought and waterlogging stress on the growth of pepper plants were mainly present at the early stage of the stress. According to Table 1, the fresh weight of a single Capsicum annuum fruit was gradually decreased with continued drought stress, and there was no significant difference between the fresh weight of a single fruit at 7 and 14 days of SD stress. In contrast, under ZS stress, the fresh fruit weight of Capsicum annuum was initially lower and then slightly increased, but there was no significant difference in the...
single-fruit fresh weight between 7 and 14 days of ZS stress. ZS stress and SD stress for 7 days reduced the single-fruit weight by 76.33% and 77.59%, respectively, relative to CK conditions; thus, both waterlogging and drought stress severely damaged *Capsicum annuum*.

**Figure 1.** Growth of *Capsicum annuum* plants under drought and waterlogging stress: control (CK), waterlogging (ZS), and severe drought (SD). Bars show standard deviation (SD, n = 9). Letters indicate significant differences at P < 0.05 among the treatments. All the data in the figure are average values, and if there is one same lowercase letter in the same group of data, the difference in 0.05 level is not significant (two-factor ANOVA LSD method for multiple comparison).

**Table 1.** Effect of drought and waterlogging stress on single-fruit indexes of *Capsicum annuum*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (d)</th>
<th>Fresh weight per fruit (g)</th>
<th>Dry weight per fruit (g)</th>
<th>Fruit length (mm)</th>
<th>Fruit stem (mm)</th>
<th>Fruit shape factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>7</td>
<td>11.11±1.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>110.48±7.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.74±0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.10±0.79&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>CK</td>
<td>14</td>
<td>10.01±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>131.65±7.06&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.18±0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.12±1.03&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZS</td>
<td>7</td>
<td>2.63±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97.68±14.12&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.66±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.11±1.16&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZS</td>
<td>14</td>
<td>3.64±0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.95±10.75&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.89±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.86±1.69&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>7</td>
<td>2.49±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>81.52±4.34&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.14±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.39±0.79&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>14</td>
<td>2.32±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80.56±8.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.38±0.32&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.77±1.10&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means followed by different letters indicate significant differences at p < 0.05 according to LSD test (n = 9).

There was a consistency between the dry weight and fresh weight of *Capsicum annuum* under waterlogging and drought stress. Fruit length was decreased by both ZS stress and SD stress, by 11.59% and 26.21%, respectively, relative to CK conditions at 7 days. Based on the longitudinal comparison of the same stress, there was no significant change in fruit length under the same stress at 14 days. The fruit length at 7 days of SD stress was 16.54% less than that at 7 days of ZS stress. The effects of water stress on fruit stems were slightly different from such effects on fruit length; additionally, there was no significant change in *Capsicum annuum* fruit stems at 7 days of ZS stress compared to control conditions, but *Capsicum annuum* fruit stems were already significantly (29.75%) thinner relative to CK conditions at 7 days of drought. At 14 days of ZS stress, the *Capsicum annuum* fruiting stems started to change compared to CK conditions, but the difference was not significant compared to that at 7 days of ZS stress. However, after 14 days of SD stress, *Capsicum annuum* fruiting stems became thicker than at 7 days, but there was no significant change compared to the control. The change in the fruit shape coefficient showed opposite trends between the control and treatment groups. The coefficient of fruit shape was progressively larger in the treatment groups compared to the control group, and the stems were progressively smaller in both waterlogged and drought-stressed fruit.

**Effect of drought and waterlogging stress on hydrogen peroxide (H₂O₂) content and relative chlorophyll content of Capsicum annuum**

According to Figure 2A, it can be seen that the content of H₂O₂ in *Capsicum annuum* under drought stress was significantly decreased on day 7 in comparison with CK by about 1%. The content of H₂O₂ under
waterlogged stress was not significantly different from that of CK. On the 14th day of stress, the results showed that the H$_2$O$_2$ content under drought stress slightly increased compared with that of drought at 14 days, but there was no significant difference. The content of H$_2$O$_2$ in Capsicum annuum on the 14th day of waterlogging increased sharply by about 1% and 11% compared to CK and SD, respectively. According to Figure 2B, the SPAD value of capsicum annuum showed a gradual increase under drought stress, while the same value showed an increase and then a decrease under waterlogged stress. Based on the above results, we guessed that this Capsicum annuum variety has some tolerance to drought stress, but is waterlogging-sensitive.

**Figure 2.** Effects of drought and waterlogging stress on the hydrogen peroxide content of Capsicum annuum (A). Effect of drought and waterlogging stress on the relative chlorophyll content of Capsicum annuum (B)

Bars show standard deviation (SD, n = 9). Letters indicate significant differences at P < 0.05 among the treatments. All the data in the figure are average values, and if there is one same lowercase letter in the same group of data, the difference in 0.05 level is not significant (two-factor ANOVA LSD method for multiple comparison).

**Effect of drought and waterlogging stress on lignin, flavonoid, and total phenol contents of Capsicum annuum**

Water deficit severely affects the metabolism of secondary metabolites in Capsicum annuum. As shown in Figure 2A, at 7 days of SD stress, the lignin content of Capsicum annuum fruits increased very significantly (52.27% mg/g) compared to the control, whereas at 14 days of SD stress, lignin slightly decreased in comparison with 7 days of SD stress, but was still elevated, with an increase of 30.33% compared to the control. The lignin content of Capsicum annuum under SD stress conditions was consistently high, whereas the ZS stress induced the opposite response. At 7 days of ZS stress, the lignin content of Capsicum annuum was slightly decreased (11.82% mg/g) compared to the control. It was then reduced by 24.18% mg/g at 14 days of ZS stress compared to CK conditions (Figure 3A). Flavonoid content and total phenolic content showed the same trend as lignin. Capsicum annuum flavonoid content increased gradually during SD stress (Figure 3B); however, there was no significant difference between 7 and 14 days of SD stress, and flavonoid content increased by 36.49% mg/g at 7 days of SD stress compared to CK stress. The flavonoid content of Capsicum annuum was reduced by 14.21% mg/g at 7 days and 49.47% mg/g at 14 days of ZS stress (Figure 3B). Total phenolic content increased gradually with SD stress; specifically, it increased by 11.99% mg/g at 7 days and 21.73% mg/g at 14 days of SD stress compared to CK conditions (Figure 3C). Total phenolic content under ZS stress first increased and then decreased, but there was no significant difference in the amount of increase at 7 days of ZS as compared to CK conditions. Total phenols increased by 5.12% mg/g compared to the control at 7 days of ZS stress and then decreased by 31.84% mg/g compared to the control at 14 days of ZS stress. Thus, SD stress increased and ZS stress gradually decreased the content of total phenols, lignin, and flavonoids in Capsicum annuum fruits.
**Figure 3.** Effect of drought and waterlogging stress on lignin (A), flavonoids (B), and total phenolic (C) contents of *Capsicum annuum*

Bars show standard deviation (SD, n = 9). Letters indicate significant differences at P < 0.05 among the treatments.

All the data in the figure are average values, and if there is one same lowercase letter in the same group of data, the difference in 0.05 level is not significant (two-factor ANOVA LSD method for multiple comparison).

**Effect of drought and waterlogging stress on C4H, PAL, and POD activities of Capsicum annuum**

The activity of C4H under SD stress was gradually increased (Figure 4A). C4H activity was 12.82% higher than the control at 7 days of SD stress, and 82.30% higher than the control, reaching its maximum, when the SD stress reached 14 days. In contrast, under ZS stress, the activity of C4H initially increased and then decreased. However, on day 14, there was no significant difference in C4H activity compared to the control. Specifically, on day 14, the C4H activity of ZS-treated samples was reduced by 2.1% compared to the CK samples, but this difference was not statistically significant. The activity of PAL is shown in Figure 3B. Changes under SD stress and ZS stress were consistent, and the highest activity of PAL was reached at 7 days of SD stress, when it was 15.52% higher than that of the control. PAL activity under ZS stress first increased and then decreased, and it reached a maximum of 38.57 U/g at 7 days of ZS stress, which was 37.98% higher than that of the CK activity; thereafter, PAL activity gradually decreased, but it was always higher than that of the control (Figure 4B). At 14 days of ZS stress, PAL activity was 14.12% higher than under control conditions. The highest POD activity was observed at 7 days of SD stress, which was 49.71% higher than the control. At 14 days of ZS stress, POD activity was higher than that of the SD group, which was higher than the CK activity. Thus, PAL activity first increased and then decreased under both SD and ZS stress, whereas C4H enzyme activity gradually increased under SD stress, showing the same trend as that of PAL under ZS stress. POD enzyme activity first increased and then decreased under SD stress, whereas it constantly increased under ZS stress (Figure 4C).
Figure 4. Effect of drought and waterlogging stress on cinnamic acid 4-hydroxylase (C4H), phenylalanine ammonia-lyase (PAL), and peroxidase (POD) activities of *Capsicum annuum*. Bars show standard deviations (SD, n = 9). Labelling with different letters indicates significant differences at P < 0.05 among the treatments.

All the data in the figure are average values, and if there is one same lowercase letter in the same group of data, the difference in 0.05 level is not significant (two-factor ANOVA LSD method for multiple comparison).

Transcriptome sequencing and assembly

The total length of raw reads of the nine samples ranged from 40,611,338 bp to 49,497,736 bp, and the total length of clean reads after filtering ranged from 4,035,946 bp to 4,927,552 bp; the mean Q20 and Q30 values of the samples were 97.73% and 93.40%, respectively, and the mean value of the GC base content in the samples was 42.72%. Thus, the proportion of low-quality bases was low, and the sequencing quality was sufficient for the resulting data to be used for subsequent analyses. For details, please see S1.

Differentially expressed genes

The total number of differentially expressed genes (DEGs) between SD and CK treatments was 6800, with 3864 up-regulated and a total of 2936 genes down-regulated (Figure 5A). The total number of DEGs between ZS and CK was 9084, with 4744 up-regulated and 4340 down-regulated. The total number of DEGs in response to ZS stress was greater than the number of DEGs in response to SD stress. As shown in the Venn diagram in Figure 5B, a total of 9084 DEGs were obtained from the comparison of ZS and CK treatments, and 6800 obtained from the comparison of SD and CK treatments. Of all these DEGs, 3150 were common to both groups; 5934 DEGs responded to the ZS group alone, and 3650 DEGs responded to the SD group alone. The cluster heatmap of the nine samples (Figure 5C) shows that the expression within the sample groups was similar, and the gene expression between the groups showed large differences.
Gene Ontology functional enrichment analysis

The Gene Ontology (GO) enrichment histogram of DEGs can intuitively show the distribution of DEGs enriched in GO terms corresponding to biological processes, cellular components, and molecular functions. We determined the 30 most enriched GO terms. According to the database annotation results, the total number of nodes mapped to the SD/CK comparison in the biological process, molecular function, and cellular component categories was 3875 (Figure 6A). The numbers of enriched terms for biological processes, molecular functions, and cellular components were 2272 (59%), 1161 (30%), and 442 (11%). The total number of relevant DEGs annotations obtained was 128,270 due to the presence of the same transcript mapped to different GO terms. Of these, 66,859 were for biological processes, 42,844 for molecular functions, and 18,567 for cellular components. Based on sorting the DEGS enrichment entries according to DEG enrichment, enriched terms corresponding to biological processes mainly focused on bioregulation, bioprocess regulation, cellular process modulation molecular process regulation, etc.; those corresponding to cellular components mainly focused on membrane-bound organelles; and those corresponding to molecular functions mainly focused on enzyme binding, exopeptidases, and serine-type endopeptidase activities.

The total number of enriched GO terms in the biological process, cellular component, and molecular function categories for the ZS/CK treatment comparison (Figure 6B) was 4386, with 2536 (58%), 1320 (30%), and 530 (12%) corresponding to biological processes, molecular functions, and cellular components, respectively. The total number of DEG annotations was 180,843, with 92,437, 59,180, and 27,426 corresponding to biological processes, molecular functions, and cellular components, respectively. DEG
enrichment for biological process terms was mainly focused on cellular processes, cellular nitride metabolism processes, biosynthetic processes, organic biosynthetic processes, and cellular biosynthetic processes; those corresponding to cellular components were mainly focused on the cytoplasm, organelle membrane, endomembrane system, envelope, and organelle membrane; and those corresponding to molecular functions were mainly focused on the three aspects of isomerase activity, cofactor cis-trans isomerase activity, and cis-trans isomerase activity.

![Gene Ontology (GO) functional enrichment analysis of differentially expressed genes (DEGs) compared with the drought group (A) and waterlogged group (B)](image)

**KEGG pathway analysis**

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, a total of 130 KEGG pathways were enriched in the SD/CK comparison (Figure 7A). Figure 7A shows the 20 most significant metabolic pathways. The SD/CK comparison was mainly enriched for secondary metabolite biosynthesis, RNA transcription, flavonoid biosynthesis, pentose phosphate pathway, and galactose metabolism, among other pathways. The KEGG pathway enrichment analysis revealed that a total of 129 KEGG pathways were enriched in the ZS/CK comparison (Figure 7B). Figure 7B shows the top 20 most
significant metabolic pathways filtered by enrichment factor and Q-value. The most significantly enriched pathways were mainly alanine, aspartate, and glutamate metabolism; galactose metabolism; starch and sucrose metabolism; and amino acid biosynthesis. According to Figure 7, it can be found that both the drought group and the waterlogged group were enriched in the biosynthetic process at the same time, and the first five metabolic pathways enriched in the drought group included secondary metabolite biosynthesis and flavonoid biosynthesis pathways. Moreover, lignin, total phenols, and flavonoids belong to the category of secondary metabolites. This echoes the results of the previous study (Figure 4), where drought increased lignin, total phenols, and flavonoid content. However, the five significantly enriched pathways before waterlogging stress did not have these two pathways. This also indicates that secondary metabolism biosynthesis and flavonoid biosynthesis were not significantly enriched under waterlogged conditions. This tends to be consistent with the results of lignin and flavonoids tested and analyzed under waterlogging stress in the previous period.

**Figure 7.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis of differentially expressed genes in comparisons of the drought group (A) and waterlogged group (B) with the control conditions

**Real-time fluorescence quantitative PCR validation**

Based on the transcriptome data, 39 differential genes of the lignin synthesis pathway in the SD group were identified. Based on volcano plots, 23 genes were up-regulated, 9 genes were down-regulated, and 7 genes did not obviously differ in their expression (Figure 8A). A total of 47 DEGs were screened for the lignin synthesis pathway in the ZS group relative to the control, of which the number of down-regulated genes was 27, the number of up-regulated genes was 10, and the expression of 7 genes was not significantly different (Figure 8B). A total of 30 genes related to the flavonoid synthesis pathway were identified in the two
treatments, and the screening conditions for differential expression of genes were as follows: fold-change differences > 2, FDR < 0.05. A total of 17 DEGs related to the flavonoid pathway were screened in the SD group, of which 14 genes were up-regulated, 1 gene was down-regulated, and the differential expression of 2 genes was not obvious (Figure 8C). A total of twelve DEGs related to the flavonoid pathway were identified in the ZS group relative to the control, of which four were up-regulated, six were down-regulated, and one had a non-significant difference in gene expression (Figure 8D). Eleven genes with high fold-change differences and relatively high expression were identified from among the above genes. Seven genes, namely, encoding chalcone synthase 2 (CHS2), chalcone isomerase (CHI), cytochromeP45093B2 (CYP45093B2), mangiferyl hydroxycinnamoyltransferase (HCT), phenylalanine ammonia lyase 1 (PAL1), B-glucosidase 12, and peroxidase (POD12), had high fold-change differences under SD stress relative to the control. In contrast, four genes with significant differences were identified in the ZS treatment relative to the control, namely, trans-cinnamate monoxygenase (C4H), 4-coumarate coenzyme A ligase (4CL), peroxidase3 (POD3), and caffeic acid 3-O-methyltransferase (COMT). These results based on RNA-Seq were confirmed by qRT-PCR. As shown in Figure 9, the qRT-PCR results were consistent with the sequencing results, and the fold-change differences were basically the same, indicating that the transcriptome data were reliable.

**Figure 8.** Volcano plots of lignin-related differentially expressed genes (DEGs) under drought (A) and waterlogging stress (B) relative to control conditions. Drought (C) and waterlogging stress (C) flavonoid-related DEG volcano plots.
Correlation analysis of differential genes with secondary metabolites

Based on the results of Figure 10A, it can be seen that the expression of genes CHS2, CHI, CYP45093B2, HCT, C4H, PAL1, β-Glucosidase12, POD12, and POD3 was positively correlated with the total phenolic content, lignin content, and flavonoid content of Capsicum annuum fruits at 7 days of SD stress. On the other hand, the expression of 4CL and COMT genes was negatively correlated with these three metabolites. Based on the results of Figure 10B, it can be seen that the expression of genes CHS2, CHI, HCT, β-Glucosidase 12, POD12, and 4CL showed a positive correlation with the total phenol content at seven days of ZS stress. The expression of genes CYP45093B2, C4H, PAL1, POD3, and COMT had a negative correlation with the total phenol content. Genes CYP45093B2, C4H, PAL1, POD3, and COMT had a negative correlation with total phenol content. The expression of genes CHS2, CHI, CYP45093B2, HCT, PAL1, β-Glucosidase12, POD12, and 4CL revealed a negative correlation with the content of flavonoids and lignin. The expression of genes HCT, POD3, and COMT was positively correlated with lignin and flavonoid contents. We can further analyze this based on Figure 11. This metabolic process begins with phenylalanine, which is catalyzed by PAL to generate trans-cinnamic acid. Furthermore, trans-cinnamic acid is transformed into coumaric acid, ferulic acid, erucic acid, caffeic acid, and other compounds through the action of C4H (Wu et al., 2021). In the final stages, 4CL, cinnamyl-alcohol dehydrogenase (CAD), caffeate 3-O-methyltransferase (COMT), cinnamyl CoA 3-O-methyltransferase (COMT), caffeoyl CCoAOMT, and CCR collectively contribute to the production of phenylpropanoid metabolites such as lignin, flavones, and polyphenols. The POD enzyme catalyzes the formation of lignin and many other oxidized phenols, which helps to enhance the defense barrier of cell structure and has the function of clearing oxygen free radicals (Abassi et al., 1998; Lin et al., 2005; Sharma et al., 2019). The PAL enzyme is the switch to catalyze the transformation of L-phenylalanine to cinnamic acid. Cinnamic acid is a well-known and common key substrate for lignin and flavonoid synthesis pathways. Under drought stress, the increase in PAL1 expression promotes the synthesis of the PAL enzyme, which further catalyzes the transformation of L-phenylalanine to cinnamic acid, increasing the substrate for the next reaction. The POD enzyme is the catalyst for the last step of lignin synthesis. P-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol need the catalytic reaction of the POD enzyme to finally form lignin. The
increase in POD12 and POD3 expression in Figure 10A increases the content of the POD enzyme, speeding up the formation of lignin. CHI and CHS are key enzymes in the flavonoid metabolism pathway (Dao et al., 2011; Tong et al., 2021). The CHS enzyme uses acetyl-CoA and 4-coumaric acid CoA as substrates, further catalyzing flavonoid compounds. The CHI enzyme is responsible for catalyzing trihydroxylchalcone and tetrahydroxylchalcone into glycyrrhizin and hesperidin, providing upstream substrates for the synthesis of flavonoids. The increase in CHI and CHS expression in Figure 9A increases the content of the CHI enzyme and CHS enzyme, positively regulating the content of flavonoids. However, in Figure 10B, under waterlogging conditions, the above-mentioned genes are mostly down-regulated, thus inhibiting the synthesis of lignin and flavonoids. Naturally, there are genes with negative correlations in the drought group, such as COMT, and there are genes with positive correlations in the waterlogged group. However, fewer genes with different relationships cannot change the result of the whole pathway being promoted or inhibited (Table S2).

**Figure 10.** Correlation analysis between differential genes and total phenols, flavonoids, and lignin under drought stress (A). Correlation analysis between differential genes and total phenols, flavonoids, and lignin under waterlogged stress (B)

**Figure 11.** A scheme of phenylpropanoid metabolism in plants. This figure is adopted from Dong et al. (Dong and Lin, 2021)
Discussion

_Capsicum annuum_ defends itself against abiotic stresses by producing secondary metabolites (Zamljen _et al._, 2020); the _Capsicum annuum_ variety ‘Shuyu2’ was the subject of this study. The present research focused on plant height, stem thickness, single-fruit weight, three enzymes (PAL, C4H, and POD) related to flavonoid and lignin metabolism in _Capsicum annuum_, and the metabolic patterns of total phenols, flavonoids, and lignin. The changes and molecular mechanisms of secondary metabolites in _Capsicum annuum_ under drought and waterlogging stress were also investigated at the transcriptome level.

**Effect of drought and waterlogging stress on agronomic and yield traits of Capsicum annuum**

The effects of both drought and waterlogging stress on _Capsicum annuum_ plants and various single-fruit indexes were mainly concentrated in the early stage (Figure 1, Table 1). As demonstrated in Figure 1B, a significant decrease in the height of pepper plants was observed after 14 days of SD stress compared to ZS stress. This reduction could potentially be attributed to the increased energy expenditure required for the production of antioxidants for self-defense under stressful conditions, which consequently limits plant growth (Mahmood _et al._, 2021). We have already mentioned that ‘Shuyu2’ has a certain drought resistance, which indicates that under drought conditions, the ability of Capsicum itself to scavenge oxygen radicals is greater than the ability to produce oxygen radicals. This also explains the accelerated synthesis of the POD enzyme under drought stress, and it is precisely because the more enzymes synthesized, the faster the chemical reaction produced and the more energy consumed (Arnold and Nikoloski, 2014; Godoy _et al._, 2021), and therefore the nutrient supply response is impeded (Hu and Schmidhalter, 2005), leading to growth inhibition. Moreover, Table 1 indicates that fruit development was also negatively impacted. The fruit shape coefficient at 14 days of SD stress was lower than that at 14 days of ZS stress, implying that the water deficit had a more pronounced effect on the longitudinal growth of the fruit.

**Effects of drought and waterlogging stress on SPAD and H2O2 in Capsicum annuum**

The effects of drought and waterlogging stress on H2O2 and SPAD of _Capsicum annuum_ are very interesting. Under drought stress, these two indicators do not show the trend of H2O2 accumulation and SPAD decline in conventional studies (Basal and Szabó, 2020; Sohag _et al._, 2020). On the contrary, H2O2 decreased first and then increased, and SPAD value gradually increased under drought stress. A review of the literature shows that this phenomenon is not uncommon in plants. Fotovat _et al._ (2007) found that wheat varieties with high transpiration rate were associated with higher SPAD under drought stress. Hasheminasab _et al._ (2012) found that drought-tolerant wheat varieties have lower H2O2 under drought stress, which may be related to plant resistance. Nahar _et al._ (2018) found that under drought stress, the proline content of drought-tolerant varieties increased, while the H2O2 content decreased to resist drought stress. This indicates that the variety ‘Shuyu2’ has relatively strong antioxidant properties, which are closely related to lignin, flavonoids and POD enzymes that we will discuss next (Bhattacharjee, 2010; Khursheed _et al._, 2022; Liu _et al._, 2015). All three have the function of clearing reactive oxygen species. Under waterlogging stress, H2O2 gradually accumulated, SPAD content increased slightly in the early stage and decreased in the later stage, which also indicated that this _Capsicum annuum_ variety was sensitive to waterlogging.

**Effects of drought and waterlogging stress on total phenols, flavonoids, and lignin in Capsicum annuum**

Total phenols, flavonoids, and lignin have long been recognized as important components of plant stress tolerance. In the present study, a significant increase in lignin content was found in _Capsicum annuum_ on day 7 of drought stress, which was suggested to be consistent with the lignin deposition that occurs in plants exposed to drought stress in order to ensure the effective axial and radial transport of water in the plant (Wen
et al., 2021). This is consistent with the finding that the overexpression of OsNAC1k in alfalfa promotes the accumulation of lignin in the leaves and roots, thus improving drought tolerance (Jung et al., 2022). Meanwhile, the total phenolic and flavonoid contents were also increased under drought stress (Figure 3). Kumar et al. (2020) similarly showed that phenolics, especially flavonoids, play an important role in the neutralization of reactive oxygen species and the repair of damaged cells, which supports the results of the present study. Phenolic compounds can protect chloroplasts located deep within leaf tissue by absorbing light penetrating the leaf and converting it to blue fluorescence. In this way, phenolic compounds can act as photoprotectors, limiting the excitation of chlorophyll in the event of leaf dehydration (Hura et al., 2008). This is consistent with the elevated SPAD values under drought stress in this experiment. The present results indicate that although the flavonoid content of Capsicum annuum decreased under waterlogging stress conditions, the total phenols initially increased, agreeing with the findings Alhdad et al. (2013), who found that the damage caused by excess water in Suaeda maritima was accompanied by an increase in the concentration of phenolics under waterlogging stress conditions. Naikoo et al. (2019) similarly found that drought and flooding stresses elevated phenolic accumulation in Brassica napus.

**Effects of drought and waterlogging on PAL, POD, and C4H enzyme activities in Capsicum annuum**

PAL, C4H, and POD have been reported as important enzymes involved in secondary compound biosynthesis in Capsicum annuum (Blum et al., 2003; Curry et al., 1999). Naikoo et al. (2019) also found a strong correlation between PAL activity and phenolics, and notably, the results of the present experiment also revealed that PAL activity was positively associated with C4H enzyme activity and POD enzyme activity, as well as the changes in total phenolics, lignin, and flavonoids under drought stress. These findings contrast with those of Gu et al. (2020) in tea tree, who found that enzymes related to lignin, flavonoids, and long-chain fatty acid biosynthesis were all down-regulated under drought stress. The enzyme activity was gradually increased under waterlogged stress, which is in agreement with the study of Hu et al. (2024).

**Effects of drought and waterlogging on differential genes of flavonoid and lignin metabolism pathways in Capsicum annuum**

PAL has been shown to be a key enzyme in the regulation of the metabolic flow from primary metabolism to phenylpropanoid metabolism, and the gene encoding PAL is regulated by environmental and developmental cues (Khan et al., 2003); genes CHI, CHS, CAH, and 4CL all encode proteins that play important roles in the lignin and flavonoid synthesis pathway (Anguelova-Merhar et al., 2001; Chen et al., 2000). Cheng et al. (2018) showed that these four genes are significantly up-regulated in drought-stressed Huang Fuling to promote flavonoid synthesis. CHI enzymes are key enzymes in the plant stress response to pathogenic microorganisms, and the accumulation of CHI enzymes has also been suggested to be part of the multicomponent defense response. Additionally, PAL, POD, and CHI were verified through qRT-PCR to be up-regulated in the drought stress group, consistent with the trends in both metabolism-related enzyme activities tested and analyzed as well as flavonoid and lignin contents. The photosynthesis of plants under drought stress conditions is inhibited, while the membrane lipid peroxidation reaction is intensified to produce many oxygen radicals (Gill et al., 2012; Nawaz et al., 2020). This is undoubtedly harmful to plant growth. Flavonoids also come into play as an important secondary metabolite. There are many types of flavonoids, one of which is anthocyanins (Landi et al., 2015). Anthocyanins promote photosynthesis by absorbing sunlight and converting it into chemical energy (Neill, 2002). They also clear oxygen free radicals and bind to proteins to prevent peroxidation (Riaz et al., 2016). Under water deficit conditions, cellular osmotic pressure increases and more water enters the cell interior by means of free diffusion (Vadiga et al., 1961). Lignin is an important component of the cell wall, and an increase in lignin can give more mechanical support to the cell wall and adapt to changes in intracellular pressure (Kumar et al., 2021). Moreover, in the waterlogging stress group,
C4H, 4CL, POD, and COMT were down-regulated in both the transcriptomic results and the qRT-PCR validation, consistent with the findings of Kreuzwieser et al. (2009), showing that the molecular response of grey poplar after flooding included a significant down-regulation of lignin biosynthesis genes (PAL, C4H, 4CL, COMT, and CCoAOMT) in roots. Additionally, the contents of lignin and flavonoids, which are regulated by the above genes, were decreased; the reduction of cell wall protein biosynthesis and lignin deposition in seedling roots and hypocotyls under flooding stress in soybean found by Komatsu et al. (2010) is consistent with the results of the present study. This may be due to the fact that under waterlogged conditions plants are in a state of hypoxia and the supply of energy is limited. The catalytic reaction of flavonoids and lignin synthesis could not be successfully carried out as a result. The process of glycolysis is accelerated and ATP is released during the production of pyruvate from glucose, thus providing energy for the active transport of plant nutrients (Givan, 1999).

Taking a comprehensive view, we believe that under drought stress, the osmoregulatory function of pepper cells is turned on after receiving the water deficit signal. At the same time, the plant self-defense system is turned on (Koc et al., 2024). When plants are stressed, there is a balance between the production of reactive oxygen radicals in the body and the antioxidant defense system, which leads to the accumulation of H2O2 (Hasanuzzaman et al., 2021). However, at the same time, the plant self-defense mechanism—self-contained antioxidants: lignin and flavonoids—starts to work (Sharifi-Rad et al., 2020). Drought stress signals stimulate the expression of key genes for lignin and flavonoid synthesis such as PAL, 4CH, COMT, CHI, CHS, etc., which further promotes the synthesis of key enzymes encoded by these genes in response to the drought stress, and ultimately provides as many flavonoids and lignin as possible to the plant body under drought stress. As mentioned above, some special structures of flavonoids and lignin, such as hydroxyl groups, are destined to have certain oxygen radical scavenging functions (Dizhbi et al., 2004; Gharibi et al., 2019). This has been demonstrated in several other studies (Khursheid et al., 2022; Liu et al., 2015). According to our results, drought stress SPAD value is increased, hydrogen peroxide content is decreased, and POD enzyme activity is increased. This suggests that the increased flavonoids and lignin synergize with peroxidases in the plant to act on oxygen free radicals, while anthocyanins exercise their mission to protect chlorophyll. As for the experiment on waterlogging stress, our results showed that 'Shuyu2' variety is a waterlogging-sensitive variety, since it showed a decrease in flavonoid and lignin content, and a down-regulation of genes C4H, POD, and COMT under waterlogged conditions. Although PAL and POD enzyme activities were higher than those of the control group, according to related studies, PAL and POD enzymes are encoded by multiple genes (Wang et al., 2021), and at the same time, these two enzymes perform multiple functions (Karimzadeh et al., 2019; Zhao et al., 2021). It has been reported that PALase is not only a key enzyme in the lignin and flavonoid synthesis pathway, but also an important step in alkaloid synthesis (Karimzadeh et al., 2019). Moreover, the roles of POD enzymes include the well-known scavenging of oxygen radicals. Because of the sensitivity of the variety itself to waterlogging, which leads to excessive H2O2 accumulation in the pepper itself, despite the increasing activity of the POD enzyme, H2O2 continues to accumulate. Although the results showed that SPAD content was increased early, this may be due to the fact that photosynthesis is less affected in the early stages of waterlogging stress. We found that the POD enzyme gene was down-regulated under waterlogging stress, but the enzyme activity was up-regulated. The previous statement mentioned that enzymes are encoded by multiple genes. In this study, only POD differential genes enriched in flavonoid and lignin metabolic pathways were studied. Although it is down-regulated, there are many genes in the POD enzyme family (Xiao et al., 2020), and many related reaction pathways are involved (Zhu et al., 2013). Whether more genes are up-regulated in other metabolic pathways needs to be further explored in subsequent studies. Studies have shown that POD gene is highly expressed in pumpkin at the early stage of waterlogging stress, which indicates that other metabolic pathways may enrich genes with high POD enzyme expression in pepper under waterlogging stress, ultimately leading to an increase in POD enzyme activity. At the same time, we all know that enzymes are encoded by
genes, but enzyme activity is not only regulated by genes, but also affected by temperature, pH (Boo et al., 2011) and so on. In addition to this, we found that the accumulation of lignin and flavonoids under drought stress is a common feature of Solanaceae. Hasanagić et al. (2020) found that in small tomato after prolonged drought stress, there is a large accumulation of total phenols, as well as an increase in flavonoids and lignin. A study by Wang et al. (2017) showed that overexpression of the C4H gene in potato increased the lignin and flavonoid content of tobacco under drought, thereby increasing the drought tolerance of tobacco. However, there are fewer studies in this area under flooding stress.

Conclusions

The present experiment was conducted to investigate the molecular mechanism of lignin and flavonoid gene expression regulation in Capsicum annuum cultivar ‘Shuyu2’ under drought and waterlogging stresses. We found that ‘Shuyu2’ has a certain degree of drought tolerance and is more sensitive to waterlogging stress. The results showed that both drought and waterlogging stresses severely affected plant height, stem thickness, and single-fruit growth. Under drought stress, H₂O₂ in Capsicum annuum fruits was maintained at the normal level as compared with CK, and the SPAD content showed an upward trend. The lignin and flavonoid pathway-related genes (PAL, CHS, CHI) were up-regulated, and the enzyme activities were enhanced, which promoted the synthesis of lignin and flavonoids. H₂O₂ continued to accumulate in Capsicum annuum under waterlogging stress, and SPAD showed a tendency of increasing and then decreasing. Most genes of the flavonoid and lignin metabolic pathways were downregulated, the related enzyme activities had non-uniformity, and the flavonoid and lignin contents decreased. The results of this study provide key candidate genes CHS and CHI for molecular breeding of Capsicum annuum varieties, as well as a molecular basis for our next step of gene cloning and functional validation of the flavonoid pathway.

Authors’ Contributions

XL designed the experiments; XL and XQ performed material harvesting and measurements; XL and XQ extracted RNA and prepared sequencing libraries; XL, HJ, HP, YM, HX, and ZL analyzed the data; XL wrote the manuscript; AR edited the manuscript. The authors read and approved the final manuscript. J.Y supervised the study and provided funding. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References


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