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ORIGINAL PAPER

External application of vitexin enhances drought resistance by promoting the synthesis of flavonoids and other hormones and stabilizing the cell membrane in wheat (*Triticum aestivum* L.)

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Abstract

Drought damage has intensified through global warming, and drought stress is detrimental to wheat (*Triticum aestivum* L.) yields. Although certain compounds (such as flavonoids) are used as a convenient and effective method to improve plant drought resistance, the mechanism by which flavonoids affect plant drought resistance at the molecular level remains unclear. We found that vitexin, a flavonoid compound, significantly improved drought tolerance in the wheat variety Chang 6878 at the seedling stage. Physiological and transcriptomic analysis during the seedling stage of wheat showed that external application of vitexin increased the transcript-level expression of six genes, including phenylalanine deaminase (PAL), chalcone isomerase (CHI), chalcone synthase (CHS), flavonol synthase II (FNSII), anthocyanin reductase (ANR), and flavonoid 3′-hydroxylase (CYP75B1), which are involved in flavonoid synthesis and phenylpropanoid pathways under drought conditions. The increased expression of these enzymes increased flavonoid accumulation, a phenomenon that increases the antioxidant capacity and drought resistance of wheat. The content of plant hormones (salicylic acid, brassinolide, and ethylene) and linolenic acid increased significantly, whereas that of malondialdehyde decreased. This suggests that externally applied vitexin can improve drought tolerance in wheat through phytohormonal pathways and maintenance of cell membrane stability. Our results highlight the regulatory pathways of vitexin in wheat and suggest the future use of vitexin as a bio-stimulant to enhance drought tolerance in wheat.

Keywords Wheat · Vitexin · Flavonoids · Antioxidant · Phytohormone · Linolenic acid

Introduction

Wheat (*Triticum aestivum* L.) plays an extremely important role in China's cereal crops. However, several abiotic stressors during growth, such as drought, flooding, low temperatures, and frost (Kumar et al. 2020; Dietz et al. 2021), impede its development and yield. Drought stress on wheat exacerbated by global warming (Sallam et al. 2019; Vicente-Serrano et al. 2022). Drought stress causes stomata to close gradually, with consequent physiological and biochemical responses, including reduction of blade water potential and expansion pressure, slower cell growth, and reduced photosynthetic rates (Kumar et al. 2021). Under drought stress, the balance between reactive oxygen species (ROS) production and elimination in plant cells is disrupted, thereby affecting ROS production and metabolism in plants. ROS-mediated oxidative stress can have deleterious cytological

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effects, including peroxidation of biofilms, damage to the cell nucleus, blockage of photosynthesis, and respiratory abnormalities (Choudhury et al. 2017; Qi et al. 2018). Plants produce flavonoids for antioxidant processes (Hu et al. 2022). Flavonoids are important secondary metabolites and are representative of a class of low-molecular-weight phenolic compounds that include flavonols, isoflavones, and anthocyanins (Shen et al. 2022). They have many functions and are involved in regulating cell growth, antimicrobial activity, enhancing resistance to biotic and abiotic stresses while promoting antioxidant activity (Treutter 2005; Rodríguez De Luna et al. 2020). The antioxidant properties of flavonoids are based on the position of their hydroxyl groups and double carbon bonds (Kumar and Pandey 2013). Flavonoids can reduce ROS production by either directly scavenging or inhibiting the activity of enzymes involved in free radical production (Agrawal 2011). Flavonoids can also increase plant resistance (Zhuang et al. 2023). The flavonoid quercetin improves drought tolerance by regulating plant antioxidant and phytohormone synthesis (Singh et al. 2021). Overexpression of the key anthocyanin gene *UGT79B2/B3* increases anthocyanin accumulation, improves antioxidant capacity, and enhances plant drought tolerance (Dabravolski and Isayenkov 2023).

Phytohormones may coordinate different signaling pathways to alleviate drought stress, and there are crosslinks between different pathways (Verma et al. 2016). Abscisic acid (ABA) is the well-known factor involved in stomatal closure, although other hormones are also involved (Brodribb and McAdam 2017). Jasmonic acid interacts with ABA to positively regulate stomatal closure (Rao et al. 2023), and ethylene interacts with ABA to regulate stomatal adaptation to drought (Nazareno and Hernandez 2017). Membranes are the main substrates for most physiological and biochemical activities. Generally, when plants are under drought stress, ROS attack biofilms and cause membrane system damage and fatty acid desaturation, leading to decreased membrane fluidity (Xu et al. 2011). This also results in membrane lipid peroxidation, culminating in malondialdehyde (MDA) production. Excessive MDA accumulation leads to structural and functional changes in the cell membrane (Jogawat et al. 2021). Reduced membrane fluidity is detrimental to plant growth under drought stress because cell membranes are important for stabilizing cell metabolism and functioning (Los and Murata 2004).

In this study, we found that the external application of vitexin improved drought resistance in wheat seedlings, reduced ROS accumulation, increased the accumulation of plant hormones, such as ethylene (ET), brassinosterol (BR), and salicylic acid (SA), and reduced drought damage by maintaining cell membrane stability. Transcriptome analysis revealed that genes involved in flavonoid synthesis, antioxidant activity, plant hormone synthesis, and cell membrane stability were upregulated in wheat after vitexin treatment. The transcriptome results were consistent with the physiological results in wheat after vitexin treatment. The discovery of new drought-resistant flavonoids is beneficial for applications in field production, as the drought resistance of wheat can be quickly and effectively improved.

Materials and methods

Plant materials and growing conditions

Full, uniformly sized, wheat seeds (C6878) were put into a dish and soaked overnight in warm water. The soaked seeds were placed evenly on a moistened filter paper spread on a Petri dish, which was incubated at 20 °C in a ventilated place. Water was added to ensure that the seeds absorbed water. Seed germination was observed by taking young shoots that grew to half the length of the seeds as the standard for seedling transplantation. They were moved to a hydroponic tank for the experiment.

Experimental treatment and design

The treatment group was divided into normal treatment (W; Hoagland nutrient solution), normal treatment externally applied vitexin (WV; Hoagland nutrient solution + 50 μM vitexin), drought treatment (D; Hoagland nutrient solution + 25% PEG), and drought treatment externally applied vitexin (DV; Hoagland nutrient solution + 25% PEG + 50 μM vitexin) groups. Vitexin was dissolved in a mixture of Hoagland nutrient solution and PEG. Hoagland nutrient solution was used to treat the plants after rehydration.

Determination of leaf relative water content

Water was removed from the surface of the leaves by blotting paper (water absorption) and weighed as fresh weight W0. The leaves were placed in water for a few hours, water was removed from the surface by blotting paper and the leaves were weighed until the weight remained stable and saturated fresh weight (W1) was measured. The leaves were subjected to heat treatment at 105 °C for 10 min and the samples were dried continuously at 80 °C until the weight remained stable and the dry weight (W2) was measured. The determination formula is as follows,

$$Relative\ water\ content\ (RWC)\,\% = (\mathrm{W0} - \mathrm{W2})/(\mathrm{W1} - \mathrm{W2}) \times 100\%.$$

Determination of chlorophyll content

The leaves were cut into pieces and placed in 10 mL of acetone-ethanol (1:1) mixture and incubated in the dark for 24 h until all leaves turned white. The solution was filtered, and the absorbance was measured at 645 nm and 663 nm using an enzyme marker. Total chlorophyll content was assessed as follows,

$$C_{\text{total}}(mg.l) = (20D645 + 8.02D663) \times V/(1000W).$$

Methods used to determine biochemical indicators

Hydrogen peroxide (H_2O_2), MDA, superoxide anion (O_2^-), catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) activities were measured when 0.1 g of leaves were sampled from each experimental group for three days of treatment. MDA, SOD, POD, H_2O_2, and CAT kits were purchased from Beijing Solarbio Technology Co., Ltd.; PRO and O_2^- kits were purchased from Suzhou Keming Biotechnology Co., Ltd.; soluble protein was purchased from Jiangsu Addison Biotechnology Co., Ltd.

Levels of the plant hormones SA, BR, and ET were measured. The ABA kits were purchased from Abmart Shanghai Co., Ltd. SA, BR, and ET were obtained from Jiangsu Yutong Biotechnology Co. Ltd.

Transcriptome analysis

In this study, 4 treatments were set up with 3 replicates per treatment, and a total of 12 samples were sent for transcriptome sequencing (W, WV, D, DV). Transcriptome data were obtained from Beijing Qingke Biotechnology Co. Ltd.

The raw data in the fastq format (raw reads) were initially processed with an in-house Perl script to acquire clean data. Hisat2 software was employed to map the libraries, which had been sequenced on Illumina, to the reference genome. EBseq was employed to identify the differences between the processed samples and the differentially expressed genes (DEGs) (FDR < 0.05&| log2(foldchange)|≥1). Finally, GO and KEGG analyses were performed based on the results of the differential gene screen.

qRT-PCR

The total RNA extraction kit was procured from Zolman Biotechnology, Beijing, China, and the reverse into first-strand cDNA kit was procured from Beijing Full-type Gold Bio Co., Ltd. qRT-PCR was performed using the SuperReal PreMix kit (Beijing Quantype Gold Biological Co., Ltd.).

Primers used for quantification are listed in the online resource Supplementary Table 1. The experiment was repeated three times for each gene.

Statistical analysis

Each sample was repeated three times in each experiment. Statistical analyses were performed using SPSS software and t-test was used for comparison between two groups. Treatment effects were assessed using Duncan's test ($p < 0.05$).

Results

Effect of exogenous vitexin on morphological and physiological characteristics of wheat seedlings under drought stress

To investigate the effect of vitexin on wheat growth, four treatment groups (W, WV, D, DV) were organized. The results showed that the leaves of wheat seedlings under external vitexin were yellowish under normal water treatment (W and WV). There were significant differences in drought tolerance of wheat seedlings under D and DV treatment groups. Greater wilting of seedlings was observed under the D treatment than under DV treatment, and the plants were less able to recover growth after rewatering. Under the water treatment (W and WV), there was no change in the aboveground fresh weight, aboveground dry weight, relative water content, and survival rate of plants, but the chlorophyll content in the W treatment was higher than that in the WV treatment. The shoot fresh weight, shoot dry weight, underground fresh weight, underground dry weight and relative water content of DV treatment were increased by 75%, 35.5%, 30%, 97%, and 51.9%, respectively, compared with D treatment. The survival of the DV treatment (76%) was 36% higher than that of the D treatment (40%). The highest chlorophyll content was observed in the DV treatment group (Fig. 1).

Effect of exogenous vitexin on antioxidant osmoregulation processes, and phytohormone biosynthesis under drought stress in wheat

To investigate the effects of vitexin on antioxidant and osmotic regulation processes in wheat, we analyzed the contents of oxidation products (H_2O_2, O_2^-, and MDA), antioxidant enzymes (SOD, CAT, and POD) activities, and osmotic substances (proline and soluble protein) in wheat under different treatments. Under normal conditions (W), vitexin treatment (WV) did not significantly alter the parameters, except for an increase in O_2^- content and SOD

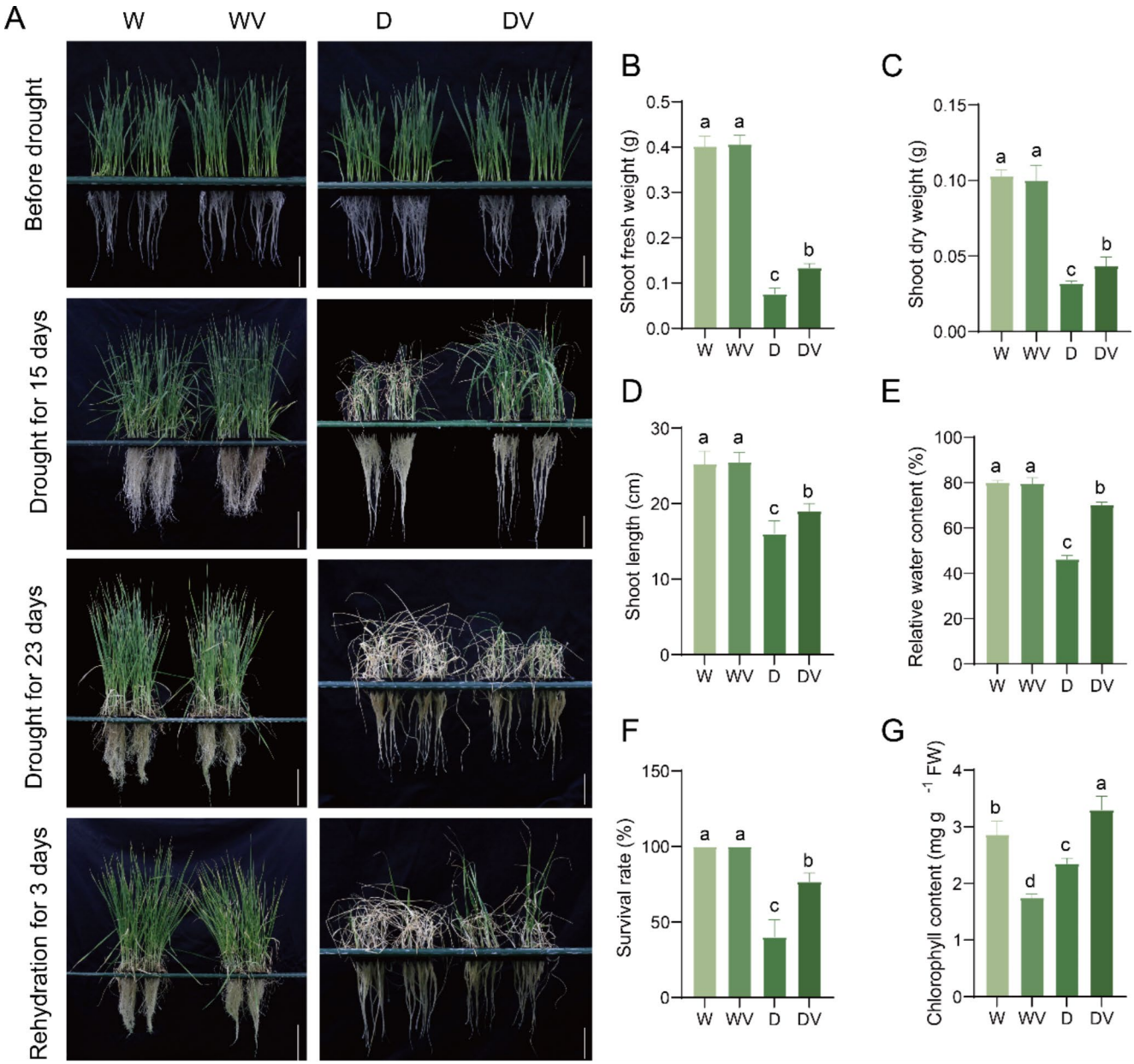

Fig. 1 Effect of vitexin application on morphology and physiology of wheat (*Triticum aestivum* L.) under drought stress. The selected wheat variety was Chang 6878. Group W (Hoagland nutrient solution), group WV (Hoagland nutrient solution + 50 μM vitexin treatment), Group D (Hoagland nutrient solution + 25% PEG) and DV (Hoagland nutrient solution + 25% PEG + 50 μM vitexin). (**A**) Photos of wheat grown at different times. (**B**) Shoot fresh weight. (**C**) Shoot dry weight. (**D**) Shoot length. (**E**) Survival rate. (**F**) Relative water content. (**G**) Chlorophyll content. Bars represent the mean ± SD of three replicates; different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range test

activity. In the DV group, vitexin significantly reduced the accumulation of MDA, O_2^-, and H_2O_2 in wheat leaves, and further increased CAT, SOD, and POD activities (Fig. 2). In addition, vitexin (DV) increased proline and soluble protein accumulation in wheat compared with the drought treatment (D) (Fig. 2). Therefore, vitexin treatment of wheat seedlings increased wheat resistance by improving the antioxidant capacity and maintaining cell membrane stability.

To investigate the effect of vitexin on plant hormone synthesis, the effects of vitexin on SA, BR and ET contents were measured under different treatments in this study. The results showed that the contents of all hormones increased under normal treatment with external application of vitexin. However, the SA, BR, and ET contents were significantly increased in the DV group (Fig. 2). The above results indicate that the application of vitexin under drought stress

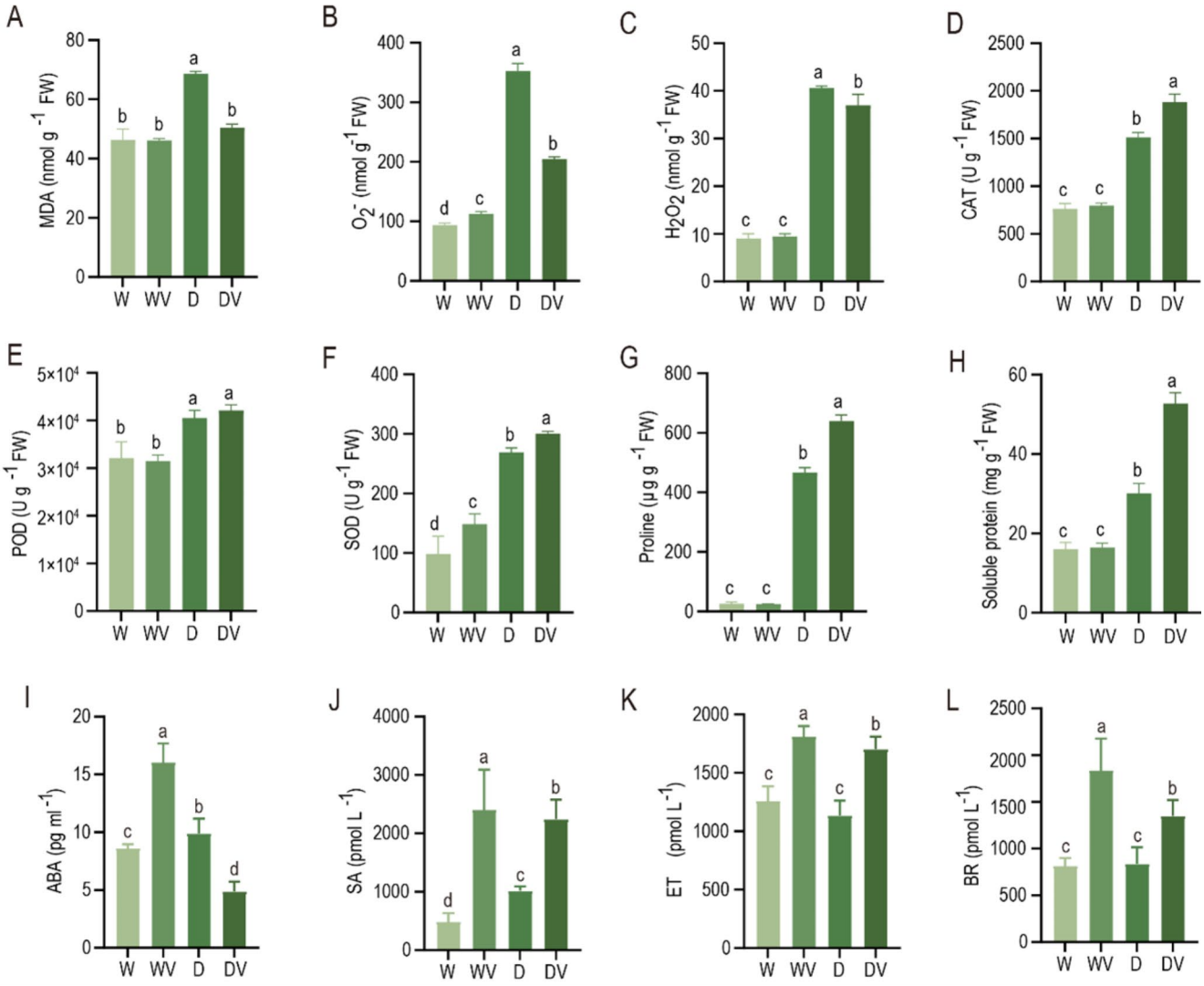

Fig. 2 Effect of vitexin application on (**A**) malondialdehyde (MDA) content, (**B**) O_2^- content, (**C**) H_2O_2 content, (**D**) catalase (CAT) activity, (**E**) superoxide dismutase (SOD) activity, (**F**) peroxidase (POD) activity, (**G**) proline content, (**H**) soluble protein content, (**I**)ABA content, (**J**) salicylic acid (SA) content, (**K**) brassinolide (BR) content, and (**L**) ethylene (ET) contents of wheat seedlings under drought stress. Group W (Hoagland nutrient solution), group WV (Hoagland nutrient solution + 50 μM vitexin treatment), Group D (Hoagland nutrient solution + 25% PEG) and DV (Hoagland nutrient solution + 25% PEG + 50 μM vitexin). Bars represent the mean ± SD of three replicates; different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

can improve the drought tolerance of wheat seedlings by increasing the content of phytohormones.

Transcriptome analysis of wheat treated with vitexin

To reveal the effects of vitexin application on gene transcription in wheat seedlings under drought stress, each treatment (W, WV, D, DV) was replicated three times when conducting transcriptome sequencing analyses. According to the sequencing requirements, the average Q30 ratio was above 85%, and a total of twelve RNA-seq libraries were constructed in this study. The average clean data obtained from each library was approximately 17 GB (Q30 > 95.55%) (Online Resource Table 2). The quality of the data meets the requirements of the analyses.

Differential gene (DEG) screening was based on fold change ≥ 2 and $P < 0.05$ based on sequencing results of 12 libraries. Venn diagrams were plotted for each group of differential genes as required. The figure shows the number of differential genes specific to each comparison group, and the number of differential genes shared between comparison groups (Online Resource Fig. 1A). A total of 1,785 DEGs, including 721 upregulated and 1,064 downregulated genes, were identified between the drought-applied vitexin treatment and drought treatment (D vs. DV) (Online Resource

Fig. 1B). A cluster analysis revealed a clear separation between the samples, with replicates from each treatment clustered together (Online Resource Fig. 1C).

GO classification and KEGG analyses of DEGs after vitexin treatment

GO classification and KEGG analysis of applying vitexin to wheat seedlings under drought stress were performed using transcriptomic data. In the GO analysis, the comparison between the D treatment and the DV treatment was dominated by 37 GOs terms (Fig. 3). Among the identified biological processes, cellular and metabolic processes were associated with the largest number of genes. Among the cellular components, the terms associated with the DEGs were mainly membrane parts, membranes, and organelles. Molecular function analysis revealed that antioxidant, signal transduction, and transport activities were the terms most strongly associated with these genes.

To identify the major pathways of action of vitexin in drought resistance, a KEGG pathway enrichment analysis was performed on the DEGs between the drought-treated vitexin treatment and drought treatment (D vs. DV). The results of DEG annotations were categorized according to the pathway types in the KEGG, and significant pathways were identified. The DEGs were enriched in 89 KEGG pathways. According to the specific analysis of each pathway, we mainly focused on the top 15 pathways that predominantly included "plant hormone signal transduction" (ko04075), "MAPK signaling pathway-plant" (ko04016), "Cutin, suberin, and wax biosynthesis" (ko00073), "Fatty acid degradation" (ko00071), "Peroxisome" (ko04146), "Flavonoid biosynthesis" (ko00941), "α-Linolenic acid metabolism" (ko00592), and "linoleic acid metabolism" (ko00591) (Fig. 3). These regulatory pathways were not present in the comparison group with the normal water treatment (W) (Online Resource Fig. 2), suggesting that externally applied vitexin can increase drought resistance through several pathways. The results showed that several genes are involved in the synthesis of key enzymes involved in the phenylpropanoid and flavonoid pathways, and several genes (including those for SA, BR, and ethylene biosynthesis) are involved in phytohormone biosynthesis and signaling; all these genes are related to cell membrane stabilization (Fig. 4). In summary, these results show that vitexin increases the drought tolerance of wheat seedlings under drought stress mainly through the regulation of key enzymes in the phenylpropanoid and flavonoid pathways that play an antioxidant role, as well as through regulatory plant hormone and the maintenance of cell membrane stability.

Validation of sequencing data by qRT-PCR analysis

To verify the quality of the transcriptome data between the drought-treated vitexin treatment (DV group) and drought treatment (D group), we randomly selected nine genes for qRT-PCR analysis. The selected genes included those related to key enzymes in the phenylpropanoid and flavonoid pathways, genes related to phytohormones, and genes related to cell membrane stabilization responses. The flavonoid pathway genes included chalcone isomerase (*CHI*), flavonol synthase II (*FNSII*), flavonoid 3′-hydroxylase (*CYP75B1*), phenylalanine deaminase (*PAL*), and peroxidase; phytohormone-related genes included pathogenesis-related proteins and *Ein3*; and genes associated with membrane stability included lipoxygenase and fatty acyl-CoA reductase. The expression trend of DEGs determined by RNA-seq analysis was consistent with the results of qRT-PCR analysis. These results show the reliability of the RNA-seq data (Fig. 5).

Discussion

Effect of vitexin on the phenylpropanoid pathway and flavonoid pathway in wheat seedlings under drought stress conditions

In plants, flavonoid metabolism is a subpathway of the phenylpropane pathway. In the phenylpropanoid pathway, the precursor L-phenylalanine produces cinnamic acid via PAL. Cinnamic acid is converted to cinnamyl-CoA by cinnamic acid 4-hydroxylase (C4H) and 4-coumaric acid CoA ligase (4CL), and it is subsequently channeled into the flavonoid pathway. Trans-cinnamic acid 4-mono oxidase (CYP73A) converts cinnamoyl-CoA to *p*-coumaroyl-CoA, naringenin, liquiritigenin, and eriodictyol under the effect of chalcone synthase (CHS) and CHI. Naringenin produces luteolin under the action of FNSII and CYP75B1. Eriodictyol produces dihydroquercetin under the action of naringenin 3-dioxygenase (F3H), and dihydroquercetin and cyanidin under the action of CYP75B1, flavanone 4-reductase (DFR), and anthocyanidin synthase (ANS). Finally, epicatechin is generated by cyanidin in the presence of anthocyanidin reductase (ANR), and cyanidin can also enter the anthocyanin synthesis pathway (He et al. 2008; Petrussa et al. 2013; Liu et al. 2021) (Online Resource Fig. 3). Upon treating wheat seedlings with vitexin under drought stress (DV group), the transcriptome revealed increased expressions of genes encoding key synthases involved in the phenylpropanoid and flavonoid pathways, PAL, CHS, CHI, CYP75B1, FNSII, and ANR, which can lead to an increase in flavonoid levels (Liu et al. 2022; Wang et al. 2022; Lam and Wang 2022). Flavonoid compounds have good oxidizing properties (Sarker and Oba 2020), which is

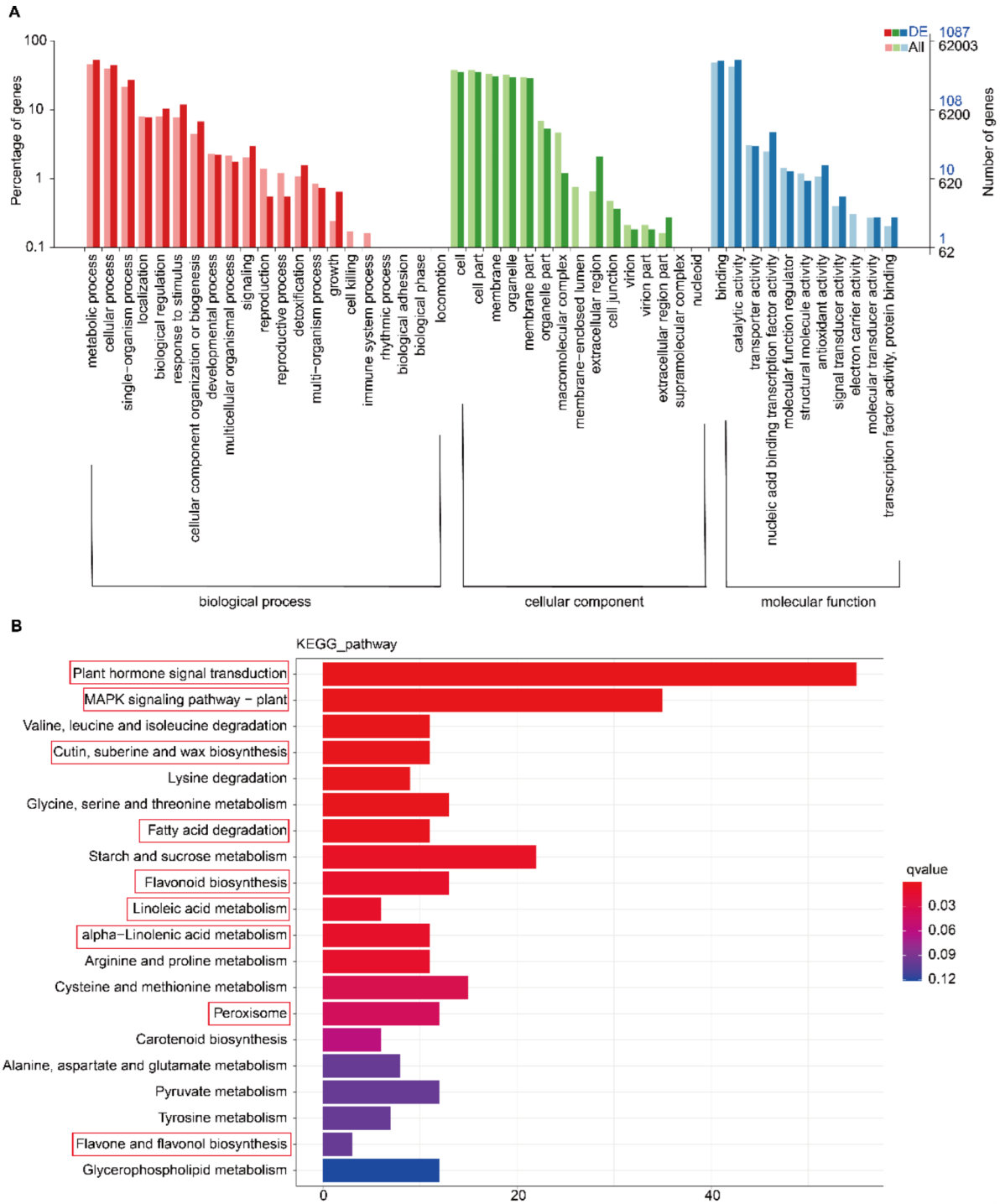

Fig. 3 GO and KEGG maps resulting from analysis of differentially expressed genes. (**A**) Functional classification of GO term enrichment analysis of DEGs in the comparison between wheat seedlings under drought stress conditions treated with 50 µM vitexin (vitexin) (DV group) and seedlings only under drought stress conditions (drought) (D group). (**B**) Annotation results of KEGG; differentially expressed genes for vitexin vs. drought, were categorized by pathway type in KEGG

one of the reasons why vitexin reduced the ROS content in this study. In addition, our results showed a significant increase in the proline and soluble protein contents. Flavonoids (quercetin, coumarin, naringenin, and kaempferol) significantly enhanced antioxidant properties and drought damage in Si-treated seedlings of GG79 (fast-growing genotype) under drought stress conditions (Patel et al. 2021). In addition, oxidative activity analysis showed that excessive

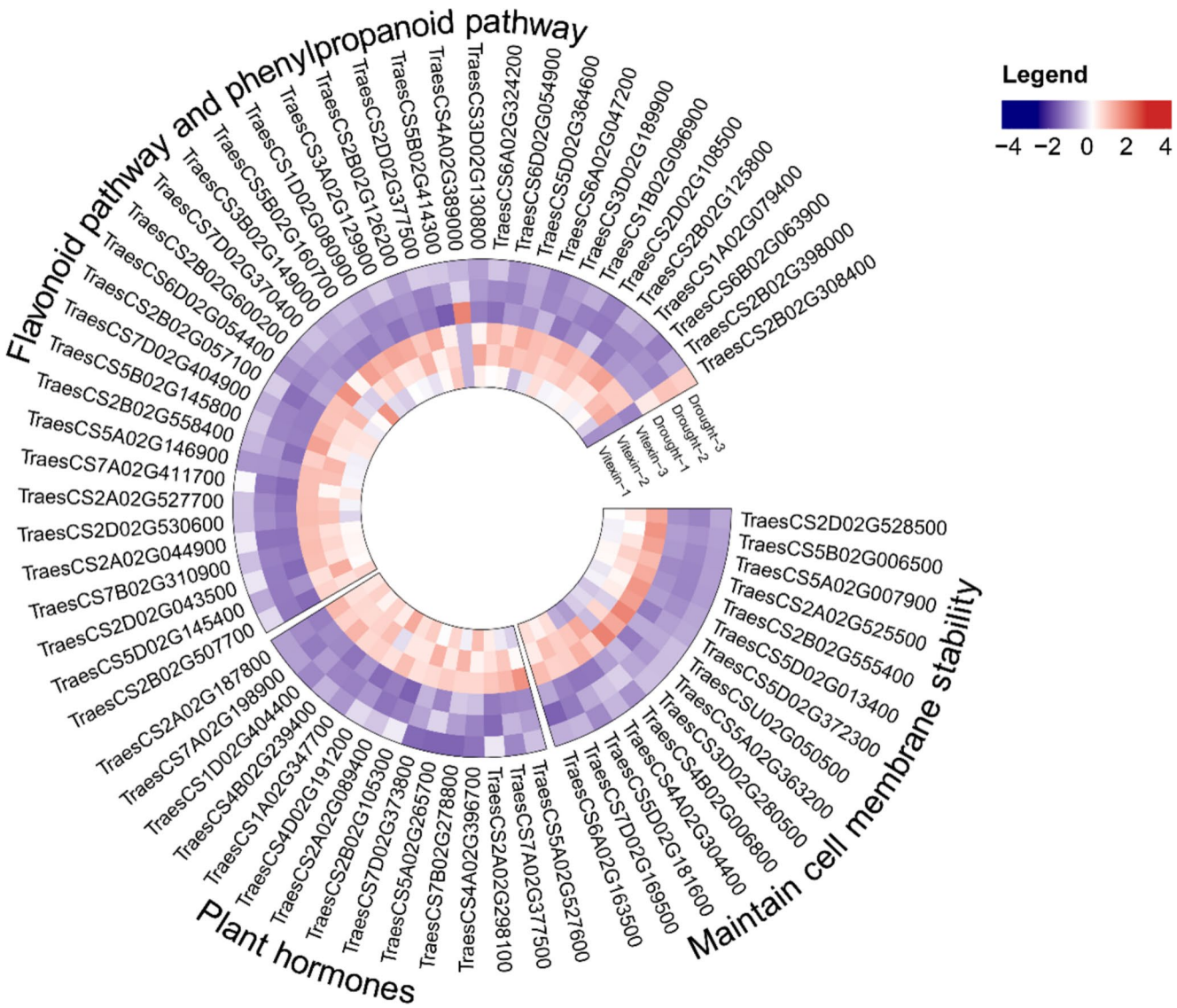

Fig. 4 Major KEGG pathways are associated with the regulation of drought tolerance by vitexin. DEGs are associated with the phenylpropanoid pathway and the flavonoid pathway; phytohormone synthesis and transduction; and cell membrane stabilization. The drought group (D group) was exposed to drought stress, and the vitexin group was exposed to drought stress and 50 μM vitexin treatment was applied (DV group)

accumulation of kaempferol and quercetin led to powerful non-enzymatic antioxidant activities, which could mitigate ROS accumulation under drought stress conditions (Jan et al. 2022). The anthocyanin-rich genotype showed robust resistance to drought stress, the ROS content was significantly reduced, and the antioxidant enzyme activities were significantly increased. In the current study, expression of genes encoding key enzymes in the phenylpropanoid and flavonoid pathways was significantly increased, further increasing the accumulation of flavonoids and anthocyanins and resulting in enhanced antioxidant activity and reduced drought-induced damage.

Effect of vitexin on phytohormones in wheat seedlings under drought stress conditions

Plant hormones play a vital role in regulating plant adaptation to various environments (Aerts et al. 2021). In the current study, we found that the BR, SA, and ETA levels increased when seedlings subjected to drought stress were treated with vitexin (DV group), and this result was consistent with that of transcriptome analysis. In a previous study, the antioxidant enzyme activities and proline and chlorophyll contents were increased in citrus plants under drought stress conditions subjected to SA treatment (Khan et al. 2021). It is also known that the exogenous manipulation of BR or alteration

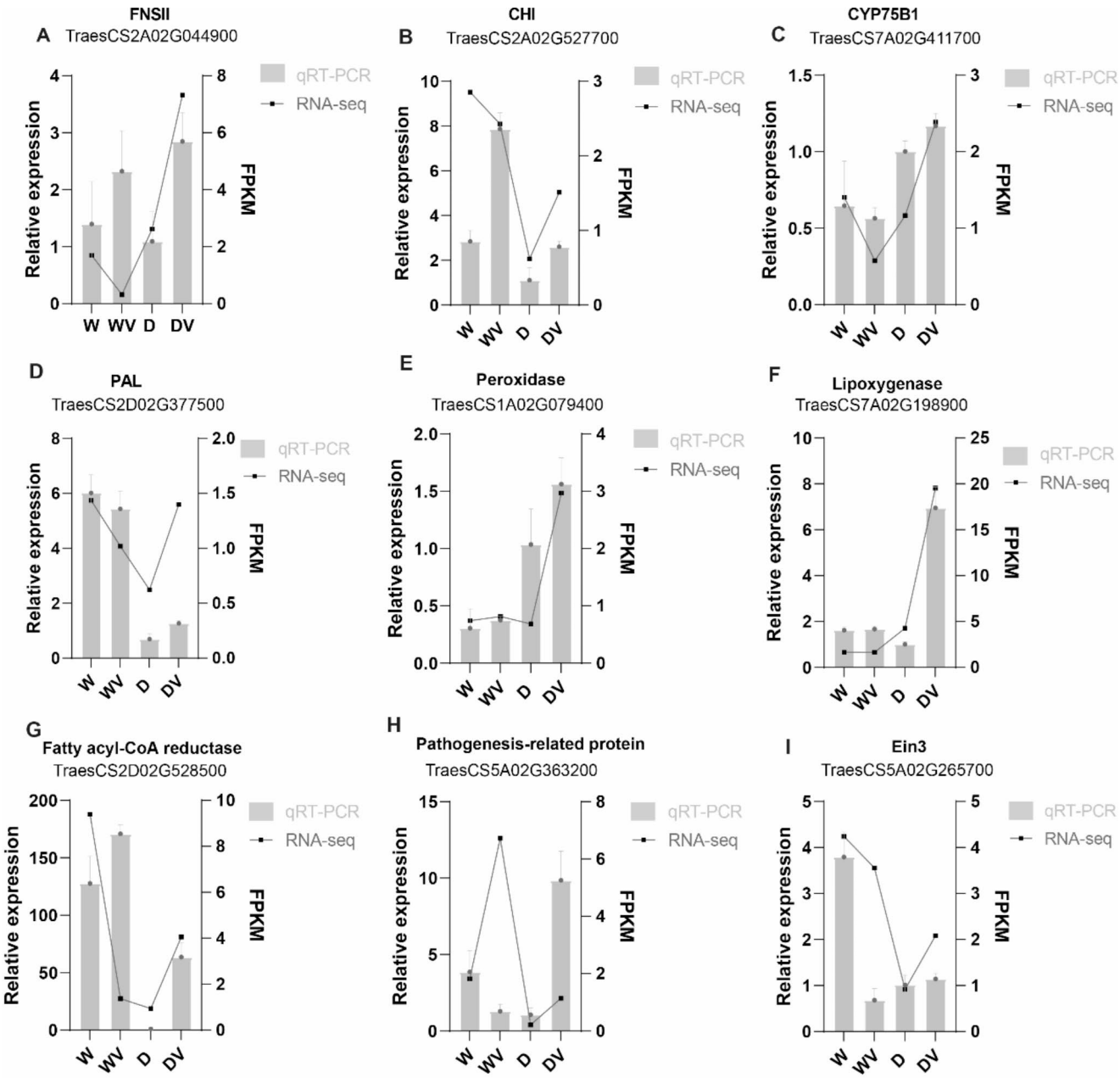

Fig. 5 Validation of DEGs in key pathways associated with vitexin-regulated drought tolerance in wheat by qRT-PCR. (**A**–**E**) The expression of genes related to the phenylpropanoid pathway and flavonoid pathway was significantly upregulated by vitexin under drought stress. (**F, G**) The expression of cell wall and cell membrane-related genes was significantly increased in seedlings treated with vitexin under drought stress. (**H**, **I**) The expression of phytohormone-related genes in seedlings treated with vitexin under drought stress. Group W (Hoagland nutrient solution), group WV (Hoagland nutrient solution + 50 μM vitexin treatment), Group D (Hoagland nutrient solution + 25% PEG) and DV (Hoagland nutrient solution + 25% PEG + 50 μM vitexin). Bars represent the mean ± SD of three replicates; different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

of its control genes can increase drought resistance (Nolan et al. 2020). Furthermore, transgenic *Arabidopsis thaliana* harboring the ethylene response factor AtERF019, which exhibits a lower transpiration rate and smaller stomata, also exhibits greater drought tolerance (Scarpeci et al. 2017). Our study showed that phytohormones increase drought tolerance following vitexin treatment.

Vitexin increases membrane stability to increase drought tolerance

The layer of water molecules tightly bound to the cell membrane is an important factor in stabilizing the cell membrane. Therefore, when drought causes extreme dehydration, the cell membrane loses its water layer, a phenomenon that

results in the altered arrangement of the membrane lipid molecules and causes the membrane to lose its biological activity by changing its position and conformation (Qi et al. 2018). In this study, the transcriptome analysis revealed an increase in the expression of fatty acid-related genes, linolenic acid, and wax extract after vitexin treatment of seedlings under drought stress conditions. The results showed that the reduced MDA content indicated reduced ROS damage to the cell membrane. In a previous study, the transcriptome revealed the expression of genes associated with increased fruit fatty acid synthesis and drought stress in tomato under drought conditions (Asakura et al. 2021). In addition, wax ester coverage in leaves and stems of WSD1 mutants was reduced under drought conditions, demonstrating the relationship between wax lipids and drought (Patwari et al. 2019). The current study shows that vitexin treatment under conditions of drought stress (DV treatment) improved the drought tolerance by increasing their water retention capacity and restoring cell membrane stability.

Conclusions

Physiological and biochemical analyses showed that vitexin increases the dry weight, fresh weight, relative water content, survival rate, antioxidant activity, and plant hormone content, and reduces the MDA content of wheat seedlings. Transcriptome analysis and quantification showed increased expression of key enzyme genes in the phenylalanine and flavonoid pathways (Online resource Fig. 4); therefore, vitexin improves the drought tolerance of wheat seedlings through these pathways.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10725-024-01266-3.

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Author contributions LMZ provided experimental ideas; YJJ and ZMS assisted with experimental methods and equipment; WQY and WYL assisted with analyzing the data; and TWS and CK assisted in reviewing the manuscript and providing revisions. The manuscript was proofread and approved by all authors.

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Data availability The corresponding author can provide data upon request.

Declarations

Competing interests All the authors mentioned in the manuscript declare no conflicts of interest.

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