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Manipulation of the Expression of Tryptophan Decarboxylase Boosts Grain Functional Quality and Stress Resilience Capacity of Wheat

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ABSTRACT

Tryptophan decarboxylase (TDC), the rate-limiting enzyme of tryptophan metabolism in plants, is essential for the production of a series of bioactive molecules, such as serotonin, melatonin and the plant hormone indole-3-acetic acid and therefore plays crucial roles in various aspects of growth and development. However, its roles in determining wheat grain quality and stress resilience capacity remain unknown, limiting its utilisation in wheat improvement. In this study, we found that overexpression of *AevTDC1* (*AevTDC1*-OX) of *Aegilops variabilis*, a wheat relative, did not significantly impair yield-related traits of wheat, but remarkably elevated the content of tryptamine, serotonin, melatonin and gamma-aminobutyric acid in the grains. Additionally, *AevTDC1*-OX simultaneously improved the defensive capabilities to cereal cyst nematode (CCN) and drought stress. Besides the known role in responding to CCN infection by regulating salicylic acid biosynthesis, we further found that *AevTDC1*-OX increased the expression of *TaXTH23* and consequently enhanced cell wall thickness in the root. On the other hand, we observed significantly promoted ethylene biosynthesis in *AevTDC1*-OX wheat, which contributed to improved drought tolerance by controlling stomatal aperture. Moreover, *TaWRKY65*, upregulated by both *AevTDC1*-OX and drought stress, was unveiled to positively regulate drought tolerance. We proved that this function was achieved, at least partly, through directly modulating ethylene biosynthesis via promoting the expression of *TaACS*, a key ethylene biosynthesis gene. These results provide new knowledge on the essential roles of TDC in the functional quality of grains and the stress resilience capacity in wheat, revealing great potential for the manipulation of tryptophan metabolism for application in wheat genetic improvement.

1 | Introduction

Tryptophan (Trp) metabolism is vital for mammals. Trp derivatives, such as serotonin and melatonin, are, respectively, neurotransmitters and important hormones regulating circadian rhythm and the sleep–wake cycle (Chen et al. 2023). Additionally, they both act as antioxidants to clear free radicals (Tarocco et al. 2019; Veenstra-VanderWeele et al. 2000). The biosynthesis of these substances in the human body depends on several factors, such as the contents of Trp, trace elements

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and so on. Especially, Trp is only obtained through exogenous intake, short of which limits the synthesis of downstream derivatives. Sleep disorders and cognitive deficits caused by a shortage of serotonin and melatonin lead to a stressful, unhappy and unhealthy life, which has become a general health problem (Hardeland 2012; Štrac et al. 2016; Xie et al. 2017). Thus, regular supplementation of these substances through exogenous health-care product consumption has been a direct and efficient solution. Since tryptophan metabolism is mostly conserved in mammals and plants, it is feasible to enhance the content of these substances in the edible parts of crops by manipulating tryptophan metabolism based on plant biotechnology, which will provide a more economical and convenient way for humans to uptake these functional components through their daily diet.

The Trp metabolism is also crucial for plants as it is an important biosynthesis pathway of indole-3-acetic acid (IAA), which is an essential plant hormone involved in regulating plant growth and responses to biotic and abiotic stress, and extensively interacts with other plant hormones (Teale et al. 2006; Vidhyasekaran 2015; Waadt et al. 2022). Wheat is one of the most widely cultivated and consumed crops, feeding over two-thirds of the world's population (Tadesse et al. 2019). Hence, manipulation of tryptophan metabolism in wheat has multifaceted potential in strengthening bioactive components and biotic or abiotic stress resistance, which would be significantly valuable for ensuring both food and health security.

Tryptophan decarboxylase (TDC) mediates the transformation of Trp into tryptamine and connects the interface between primary and secondary metabolism (Chen et al. 2023; Corpas et al. 2021). In plants, TDC functions in altering the content of the downstream metabolites, such as serotonin and melatonin (Kanjanaphachoat et al. 2012; Byeon et al. 2014; Tsunoda et al. 2021), which are important to plant growth, development and stress responses (Arnao and Hernandez-Ruiz 2018; Mishra and Sarkar 2023; Zhang et al. 2014). For instance, elevated *TDC* expression contributed to the induction of melatonin during Cd treatment in rice (Lee et al. 2017). Overexpression of *HpTDC* enhanced tobacco tolerance to drought and salt stresses (Zhao et al. 2019). Ectopic expression of *PlTDC* enhanced phytomelatonin production and high-temperature stress tolerance in tobacco (Zhang et al. 2022). Besides, TDCs participated in plant resistance to insects through regulation of downstream tryptamine or serotonin (Gill and Ellis 2006; Chen et al. 2022). And serotonin was also reported to play some role in defending against *Magnaporthe oryzae* in rice (Hayashi et al. 2016). However, the detailed mechanisms of TDC in modulating stress responses are largely unclear, and its impact on grain quality and the resistance of wheat has not been reported.

Cereal cyst nematode (CCN) is a soil-borne pathogen infecting roots to seek nutrients for growth and leads to serious yield loss in wheat. Drought is a common abiotic stress in wheat production and has been becoming more severe as global warming gets aggravated. Therefore, improvement of resistance to both stresses is quite important in modern wheat breeding. *Aegilops variabilis* is a close relative of wheat and a valuable resource for excavating resistance genes. Comparative transcriptome analysis indicated that the Trp metabolism pathway was significantly promoted by CCN infection (Xu et al. 2012). Functional analysis using gene silencing technology indicated that one significantly upregulated gene, *AevTDC1*, encoding the key enzyme of Trp metabolism, was closely related to CCN resistance in *Ae. variabilis* No. 1 (Huang et al. 2018). Moreover, it could coordinate with *phenylalanine ammonia-lyase 1* (*AevPAL1*) to regulate the salicylic acid (SA) biosynthesis pathway in *Ae. variabilis* and wheat (Zhang et al. 2021). However, it remains unclear about its effects on grain metabolite content, CCN resistance, as well as drought tolerance in wheat, which is especially interesting in this hexaploid crop with a large and complicated genome.

In this study, we investigated the function of *AevTDC1* in determining the agronomic traits, CCN and drought resistance and grain metabolite content by utilising wheat lines overexpressing *AevTDC1* (*AevTDC1*-OX). The notable elevation of content of melatonin and its intermediates, as well as the antioxidant content, were detected in *AevTDC1*-OX wheat lines. The CCN resistance and drought tolerance were simultaneously enhanced. In addition, a transcription factor TaWRKY65 responding to *AevTDC1* introduction was identified to regulate drought tolerance by transcriptionally modulating ethylene (ET) biosynthesis, which further addressed the mechanism underlying the regulation of drought tolerance by *AevTDC1*. This study provides modifiable targets and effective gene resources for the coordinated improvement of grain functional bioactive ingredient and resilience capacity of wheat through biotechnology.

2 | Results

2.1 | Characteristics of Wheat Lines Overexpressing *AevTDC1*

Two independent and genetically stable lines (T_3) of *AevTDC1*-OX (Line2 and Line9) were generated and validated by PCR (Figure 1A). Further QPCR assay detected expression of *AevTDC1* in Line2 and Line9, in which the latter is strikingly higher than the former (Figure 1B). Measurements of agronomic traits indicated that the plant height (PH) and the biomass of *AevTDC1*-OX wheat were slightly lower than those of the non-transgenic wheat (Figure 1C–E). No significant changes in spike morphology and spike length (SL) were found (Figure 1F,G). Although the spikelet number per spike (SNS) of *AevTDC1*-OX wheat was slightly lower than that of non-transgenic wheat (Figure 1H), the grain number per spike (GNS) of *AevTDC1*-OX wheat was not distinguished from the non-transgenic wheat (Figure 1I). Besides, no obvious differences were found in effective spike number per plant (ESNP) (Figure 1J), and the grain-related traits, including grain length (GL), grain width (GW) and the thousand grain weight (TGW) (Figure 1K,L).

2.2 | Analysis of Grain Metabolites

Interestingly, the grains of transgenic Line2 and Line9 exhibited obvious dark brown colour, while non-transgenic wheat

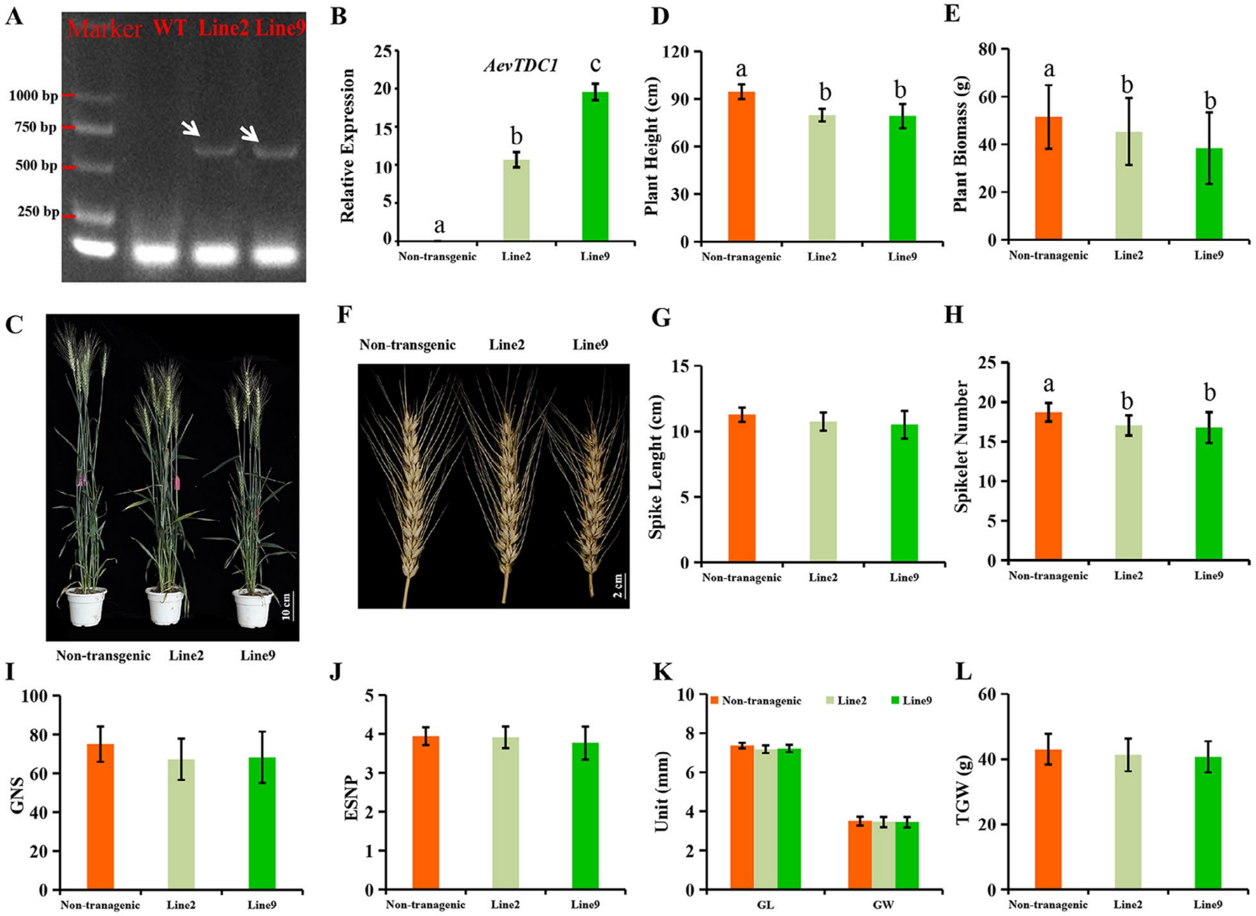

FIGURE 1 | Characteristics of *AevTDC1*-OX plant and metabolite content in the grains. (A) The PCR detection of *AevTDC1* in each line. (B) The relative expression of *AevTDC1*. (C) The morphology of the whole plant of *AevTDC1*-OX Line2, Line9 and non-transgenic plant. (D, E) PH and plant biomass comparison. (F) The spike morphology of the *AevTDC1-OX* and non-trangenic plants at the maturation stage. (G–L) Comparison of the SL, SNS, GNS, ESNP, GL, GW, and TGW, respectively. Letter a, b, c represented significant differences among three groups ($p < 0.05$) in *ANOVA* test.

grains showed normal seed colour (Figure 2A). We then analysed potential alteration of metabolite content by using widely targeted metabolome approaches. Compared to non-transgenic, nearly 31% and 34% of the detected metabolites showed significant changes in Line2 and Line9 (Figure S1A–F, Tables S2 and S3). The consistently increased metabolites in Line2 and Line9 mainly belonged to flavone, vitamins, cholines, tryptamine, and its derivatives, and the consistently decreased metabolites mainly belonged to alcohols, polyols, benzoic acid derivatives, hydroxycinnamyl derivatives, quinine acid and derivatives, and lipid-glycerolipid and lipid-glyceride (Tables S2 and S3). Besides, several well-known antioxidants, including L-ascorbate and glutathione reduced form, and γ-aminobutyric acid, an important neurotransmitter, accumulated more in Line2 and Line9 than in the non-transgenic grains (Figure 2G–I).

A dominant class of metabolites with significant differential accumulation was related to tryptophan metabolism. Analysis of the absolute content of the related metabolites in the grains by utilising HPLC–MS indicated that the content of Trp, the TDC catalytic substrate, was 12.2 μg/g in non-transgenic wheat and strikingly decreased to 0.89 and 0.37 μg/g in Line2 and Line9, respectively (Figure 2B). Consequently, tryptamine, the direct product of TDC and the sequential downstream metabolites, such as serotonin, N-hydroxy tryptamine, and melatonin, were all significantly elevated in transgenic wheat. Tryptamine content reached 1305.77 ng/g in Line2 and 2328.09 ng/g in Line9, which were almost 138-fold and 247-fold higher than those in non-transgenic grains (9.39 ng/g), respectively (Figure 2C). N-5-hydroxytryptamine content was 0.49 ng/g in non-transgenic grains and increased up to 32.43 and 27.35 ng/g in Line2 and Line9, respectively (Figure 2D). Grain serotonin content increased to 1867.84 ng/g in Line2 and 1512.89 ng/g in Line9, which were 68-fold and 55-fold higher than those of non-transgenic grains (27.16 ng/g), respectively (Figure 2E). Melatonin content was 0.04 ng/g in non-transgenic grains but increased to 1.27 ng/g and 2.45 ng/g in Line2 and Line9, respectively (Figure 2F). These results indicated that overexpression of *AevTDC1* accelerated the usage of Trp and led to a remarkable increase in the content of Trp-derived metabolites and some other bioactive substances.

2.3 | Overexpressing *AevTDC1* Enhanced CCN Resistance and Drought Tolerance

We previously found that silencing *AevTDC1* weakened CCN resistance in *Ae. variabilis* (Huang et al. 2018). To clarify whether it has a similar function in wheat, a CCN resistance assay of *AevTDC1*-OX wheat was performed and revealed that the

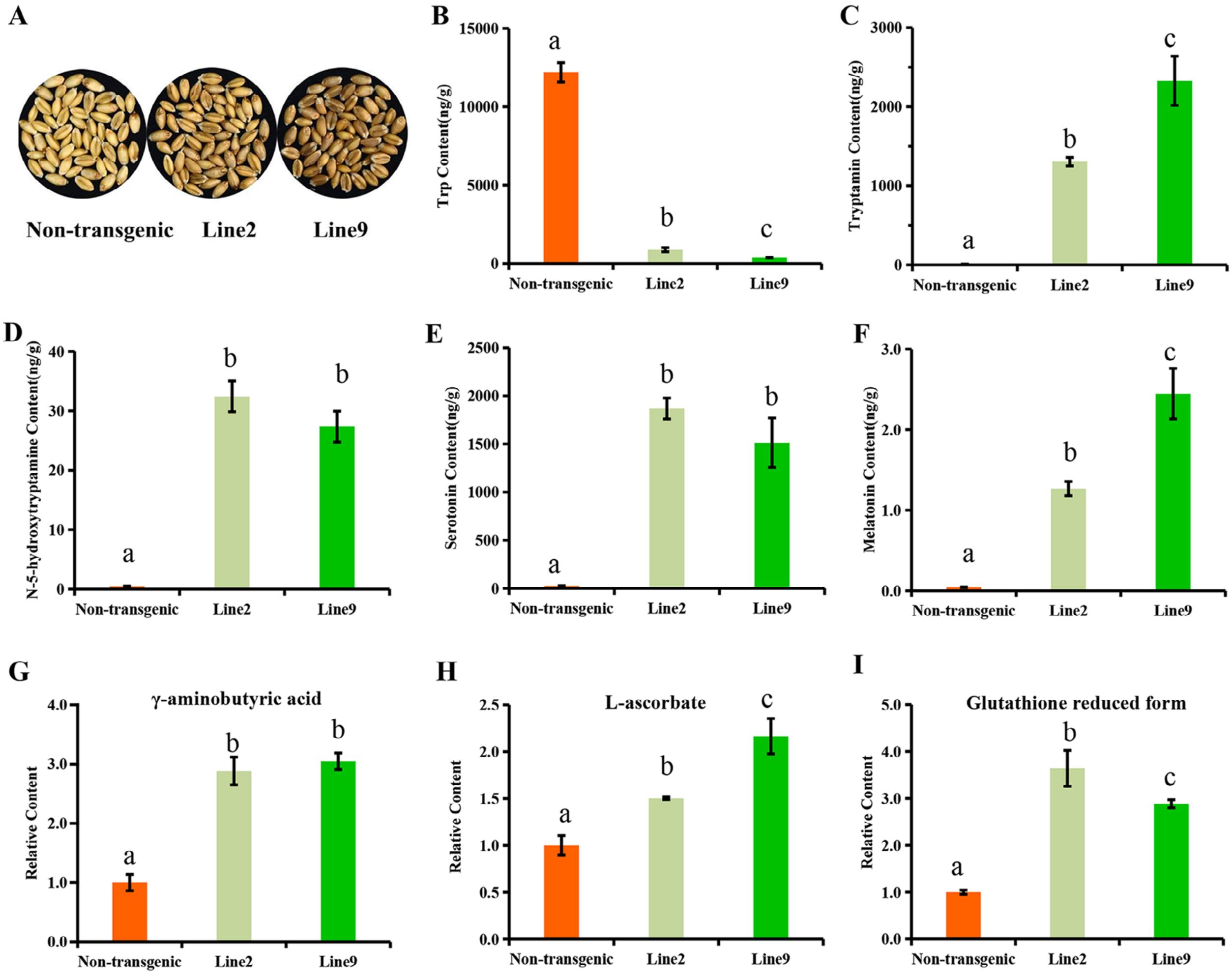

FIGURE 2 | Content of tryptophan, tryptophan-derivatives and several bioactive compound in the grains. (A) The grains phenotype from non-transgenic and *AevTDC1*-OX wheat. (B–F) The quantitative content of Trp, tryptamine, N-5-hydroxytryptamine, serotonin, and melatonin in the grains of each line. (G–I) The relative content of γ-aminobutyric acid, L-ascorbate, and glutathione reduction form in the grains of each line. Letter a, b, c represented significant differences among three groups ($p < 0.05$).

number of newly formed cereal cysts in the planting soil of Line2 (about 11 cysts/plant in average) and Line9 (about 5 cysts/plant in average) was significantly less than that in the non-transgenic plant (about 16 cysts/plant in average) (Figure 3A). This result suggested that *AevTDC1* maintains its function in regulating CCN resistance in wheat.

We previously revealed that overexpression of *AevTDC1* elevated SA content, which was positively correlated to CCN resistance (Zhang et al. 2021). To get more insight into the molecular mechanism of *AevTDC1*-regulated CCN resistance, we explored if the resistance benefited from the enhancement of the cell wall, the first layer of plant defence to pathogens. We detected that the expression of the *Xyloglucan Endotransglucosylase/Hydrolase 23* (*XTH23*) gene, which is responsible for cell wall loosening and plant cell expansion (Jian et al. 2022), was almost threefold in Line9 root compared to non-transgenic wheat root (Figure 3B). Moreover, transmission electron microscope (TEM) observation showed that the cell wall thickness of *AevTDC1*-OX root (462 nm) was significantly increased, which was approximately 33% thicker than that of non-transgenic wheat (345 nm) (Figure 3C,D).

Additionally, we testify the function of *AevTDC1* in regulating drought tolerance. Under well-watering conditions, the whole plant status showcased no obvious difference, and the leaf water content (LWC) was all nearly 87% in non-transgenic and *AevTDC1*-OX lines (Figure 4A,B). At 5 days post-water limitation (dpwl), the LWC decreased to 80.3% in non-transgenic wheat, and the leaves exhibited obviously water-loss status. Meanwhile, LWC of Line2 and Line9 was 83% and 85.4%, respectively, and *AevTDC1*-OX wheat maintained a good growth condition (Figure 4A,C). At 22 dpwl, non-transgenic plants were totally wilted, but a portion of *AevTDC1*-OX plants stayed green (Figure 4A). At 3 days after re-watering, the survival rate (SR) of two *AevTDC1*-OX lines was about 36.0% and 57.7%, respectively, whereas that of the non-transgenic wheat was only 25.9% (Figure 4D). Interestingly, we observed that the stomatal aperture of non-transgenic plants was statistically larger than that of Line2 and Line9 under well-watering conditions (Figure 4E,G), while stomatal density had no obvious difference (Figure 4E,F). These results indicated that overexpressing *AevTDC1* could decrease stomatal aperture and improve drought tolerance in wheat.

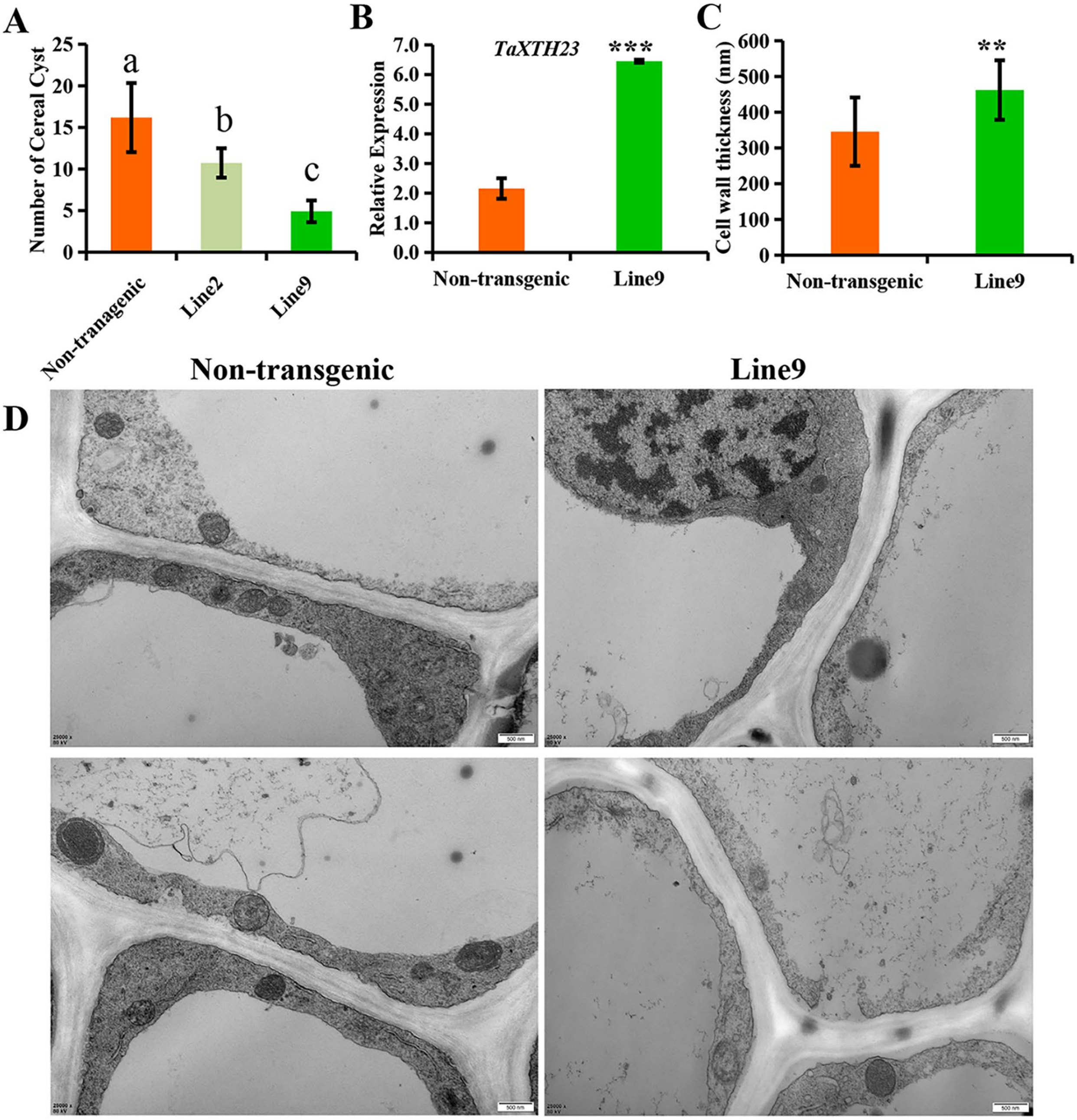

FIGURE 3 | CCN resistance and cell wall thickness observation. (A) The number of newly-formed cereal cysts in the soil of each line. (B) Expression of *TaXTH23* in non-transgenic and Line9. (C) Statistical analysis of root cell wall thickness. (D) Transmission electron microscope (TEM) images of root cell wall. Letter a, b, c represented significant differences among three groups ($p < 0.05$). Asterisk *** and **, respectively, represented $p < 0.001$ and $p < 0.01$.

2.4 | Comparative Transcriptome Analysis of *AevTDC1*-OX Wheat and Non-Transgenic Wheat Under Drought Stress

In order to explore the molecular mechanisms of drought tolerance enhancement, comparative transcriptomic analysis was performed to identify differentially expressed genes (DEG) in response to drought stress between Line9 and wheat cultivar Fielder at three time points of drought treatment, representing well-watering status and non-dehydration (Nd) treatment (LWC≈87%), mild dehydration (Md) treatment (LWC≈80%), and severe dehydration (Sd) treatment (LWC≈74%).

Under Nd condition, there were 311 DEGs (Fold Change ≥2 and FDR < 0.01) between non-transgenic wheat (CK) and Line9 (OX) (Figure S2A,B). These DEGs were mainly enriched in pathways of plant hormone signal transduction, linoleic acid metabolism, phenylpropanoid biosynthesis, phenylalanine metabolism, plant-pathogen interaction, tryptophan metabolism, isoquinoline alkaloid biosynthesis and tyrosine metabolism (Figure S2D). In particular, the genes related to biosynthesis and signalling of salicylic acid and ethylene showed obviously increased expressions in Line9. Meanwhile, some transcription factors, such as WRKY, HSF and bHLH, also showed differential expressions (Table S4).

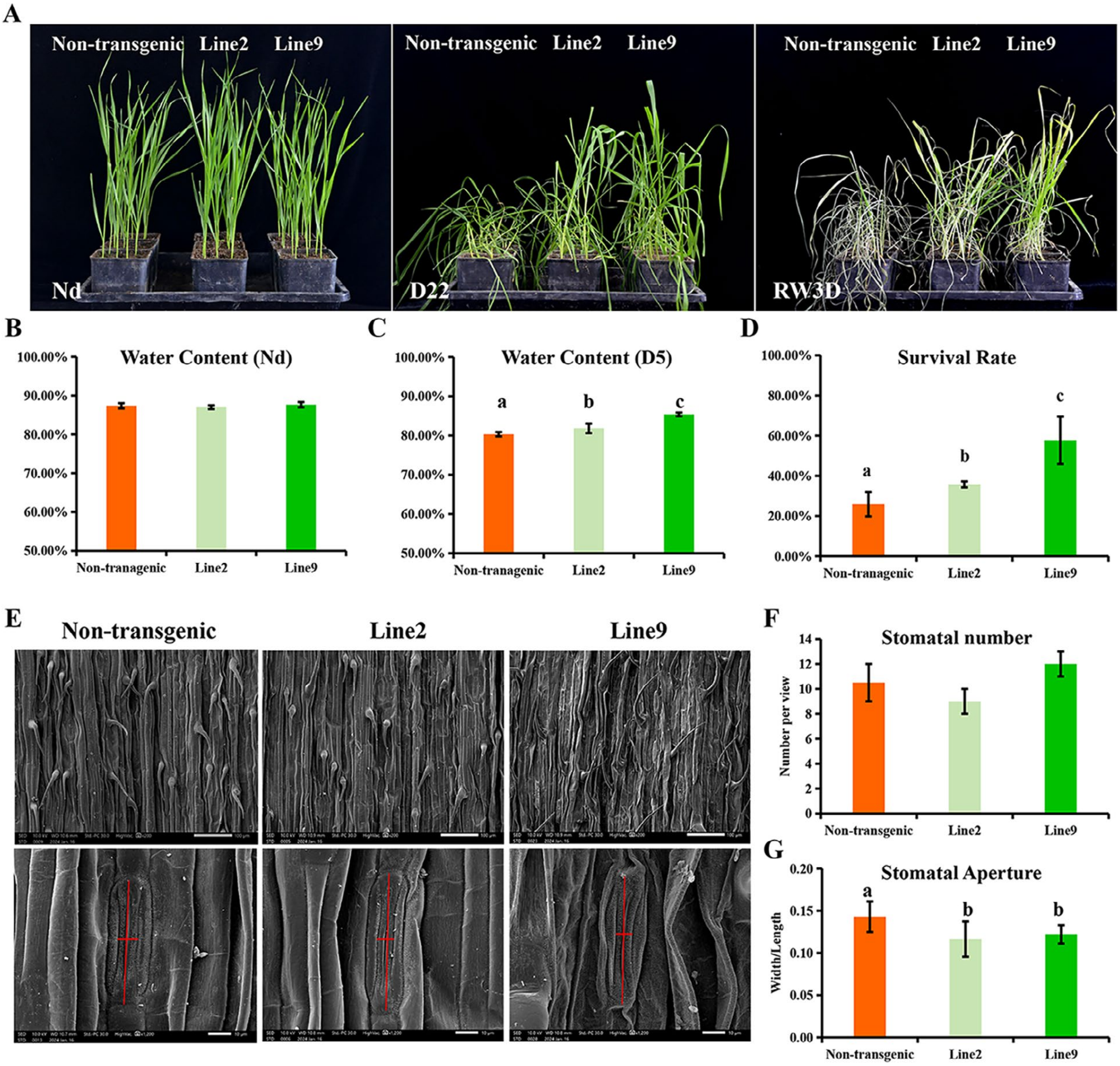

FIGURE 4 | Stomatal observation and phenotypes of non-transgenic and *AevTDC1*-OX wheat under drought stress. (A) The phenotypes of non-transgenic, Line2, and Line9 wheat under well-watering (Nd), drought treatment for 22 days (D22) and 3 days after recovering watering following drought treatment for 4 weeks (RW3D). (B, C) The leaf water content of non-transgenic, Line2, and Line9 under Nd and drought treatment for 5 days (D5). (D) The survival rate at RW3D. (E) The SEM graphs of stomatals of each line. The red line marked the length and width of one stomatal. (F) The number of stomatal per view of each line. (G) The stomatal aperture of each line. Letter a, b, c represented significant differences among three groups ($p < 0.05$).

A large amount of DEGs responsive to drought treatments (both Md and Sd) were identified in CK and OX lines, which were involved in plant hormone signal transduction, plant-pathogen interaction, starch and sucrose metabolism, phenylpropanoid biosynthesis and ubiquitin mediated proteolysis, etc. As expected, the ABA biosynthesis pathway was strikingly induced by drought in all lines. However, the changes in expression levels are more significant in the *AevTDC1*-OX line. For example, *9-cis-epoxycarotenoid dioxygenase* (*NCED*), encoding a rate-limiting enzyme for ABA biosynthesis, expressed higher in Line9 than in the non-transgenic plant under Md (Figure S3A). Further, 126 key DEGs were identified through crossing comparisons, which showed similar expression changes in groups of Nd-OX, Md-CK and Sd-CK compared to the Nd-CK group (Figure S2D), representing a set of genes responsive to both *AevTDC1* overexpression and drought stress. They were mainly classified into plant-pathogen interaction, plant hormone signal transduction, linoleic acid metabolism and flavonoid biosynthesis pathways, etc.

2.5 | Ethylene Content Was Positively Associated With Drought Tolerance

Among the 126 key DEGs, we discovered that the expression of genes related to ethylene biosynthesis and signalling was elevated in Line9 compared with non-transgenic plants and was also induced by drought stress. QPCR assays revalidated that

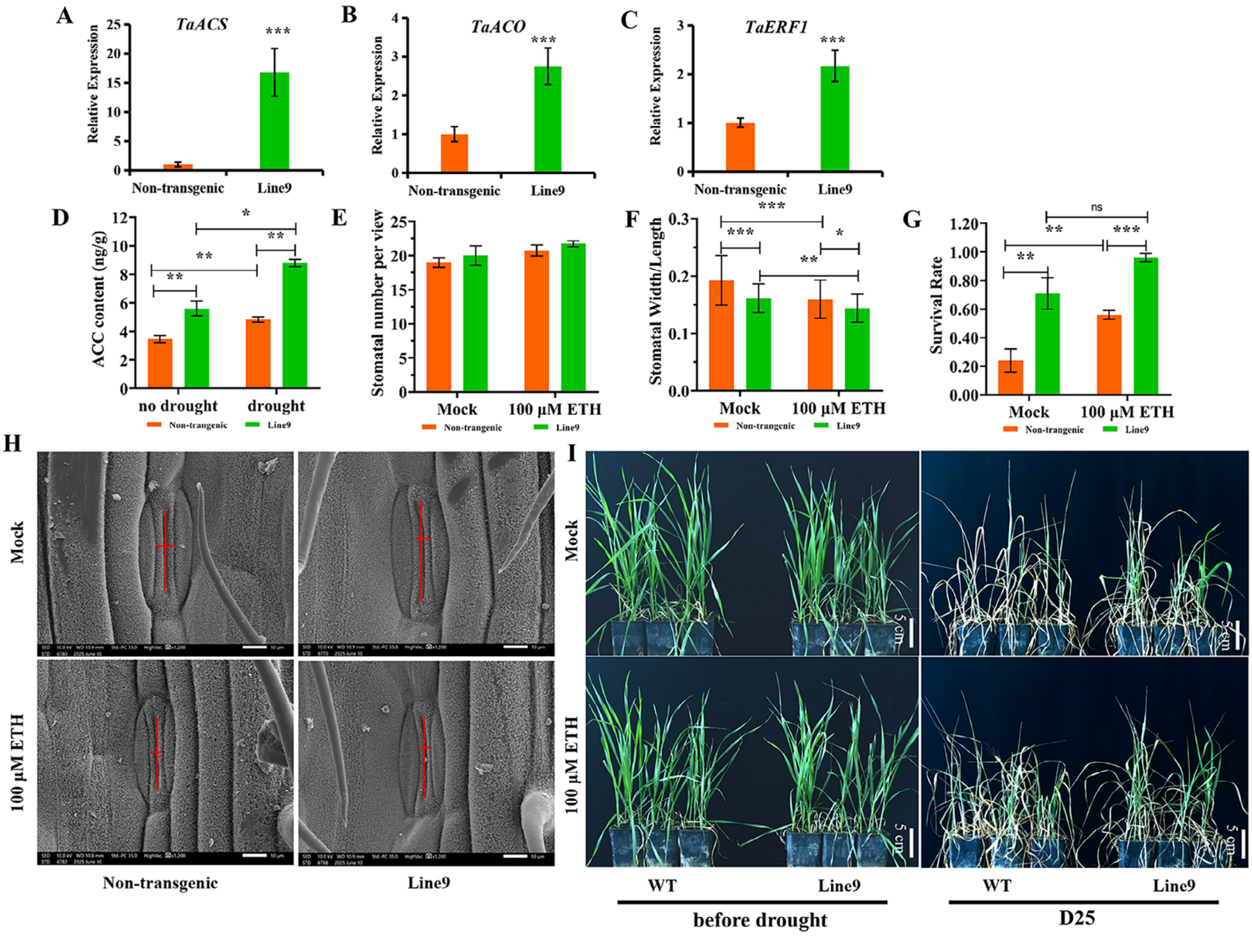

FIGURE 5 | Comparison of ethylene related genes expression and ACC content, and the effect of exogenous ETH on drought tolerance. (A–C) The relative expression of *TaACS*, *TaACO* and *TaERF1* in non-transgenic and Line9 under well-watering condition. (D) The content of ACC in non-transgenic and Line9 under Nd and drought treatment for 3 days. (E) The number of stomatal per view of each treatment. (F) The stomatal aperture of each treatment. (G) SR of Mock and ETH treated wheat. (H) SEM graphs of stomatals of plants from each treatment. (I) The phenotype of mock and ETH treated wheat under well-watering (Nd), and 3 days after recovering watering following drought treatment for 4 weeks (RW3D). ***, ** and *, respectively, represented $p < 0.001$, $p < 0.01$ and $p < 0.05$.

the expression of *1-aminocyclopropane-1-carboxylate synthase* (*ACS*) and *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*), the key biosynthesis genes of ethylene, was more than twofold in Line9 than in non-transgenic wheat (Figure 5A,B). The expression of *Ethylene-responsive transcription factor* 1 (*ERF1*) was also elevated (Figure 5C). Furthermore, we measured the content of endogenous ACC, as well as the ABA, a key phytohormone for drought tolerance. As expected, ACC content was obviously higher in Line9 than that in the non-transgenic, while ABA content showed no obvious difference under well-watering conditions (Figures 5D and S3B). At 5 dpwl, ACC content was significantly elevated by drought compared to that in WT and Line9 before drought, appearing to parallel changes with the expression of its biosynthetic genes. Meanwhile, ACC content was notably higher in Line9 than in WT, no matter under well-watering conditions or drought stress (Figure 5D).

To unravel the relationship between ethylene content and drought tolerance, non-transgenic and Line9 were pre-treated by exogenous ethephon (ETH) to simulate ethylene effects, and then subjected to drought tolerance test. After ETH treatment, we observed and calculated the stomatal density and aperture through SEM. The data showed that there was no difference in stomatal density between WT and Line9 in the mock and ETH groups (Figures 5E and S3C). But ETH treatment reduced the stomatal aperture of both materials compared to mock treatment (Figure 5F,H). Meanwhile, stomatal aperture was also statistically smaller in Line9 than in WT, no matter under mock treatment or ETH treatment (Figure 5F).

Ultimately, ETH treatment significantly improved SR of non-transgenic wheat from 24% to 56%, and slightly affected SR of Line9 from 71% to 95% (Figure 5G,I). Therefore, we inferred that higher ACC content of *AevTDC1*-OX lines contributes, at least partially, to the enhanced drought tolerance by regulating stomatal aperture.

2.6 | TaWRKY65 Positively Regulated Ethylene Biosynthesis and Drought Tolerance in Wheat

To further explore the regulatory mechanism of the enhanced drought tolerance, we focused on transcription factor genes among the 126 DEGs. Thereinto, *TaWRKY65* showed a

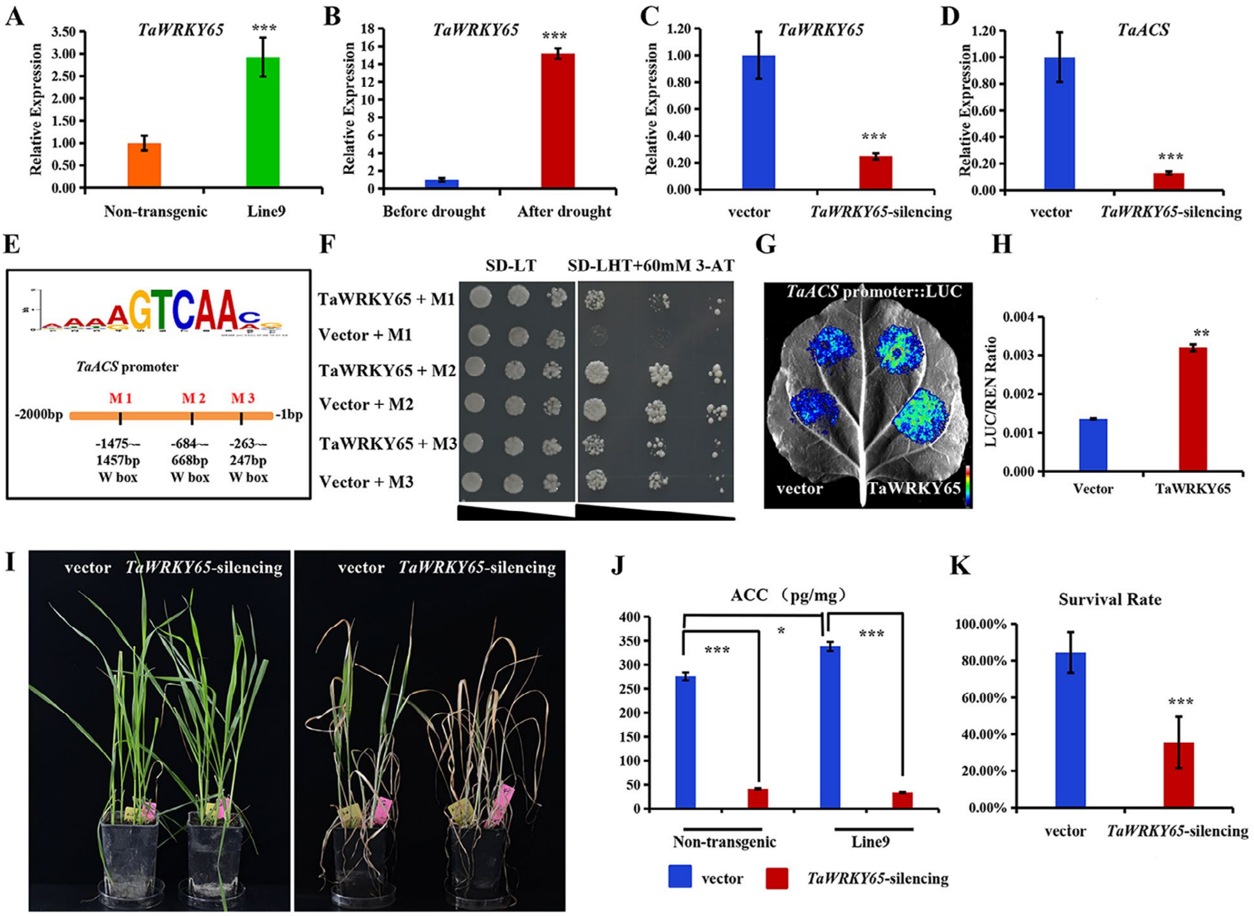

FIGURE 6 | *TaWRKY65* regulated ethylene biosynthesis and drought tolerance. (A) Expression comparison of *TaWRKY65* between non-transgenic and Line9 wheat. (B) Expression comparison of *TaWRKY65* under well-watering (Nd) and drought treatment. (C, D) The relative expression of *TaWRKY65* and *TaACS* in vector-control and *TaWRKY65*-silencing wheat in Line9. (E) Predicted W-box motifs in the promoter region of *TaACS*. (F) Y1H assay tested the direct binding of TaWRKY65 to W-box of *TaACS* promoter. (G) Dual-luciferase assay verified that TaWRKY65 could improve luc expression by binding to *TaACS* promoter. (H) Luc fluoresence/Ren fluoresence ratio of different treatment. (I) Phenotype of *TaWRKY65*-silencing plants under drought stress. (J) Endogenous ACC content were measured in vector-control and *TaWRKY65*-silencing plants in non-transgenic and Line9 background. (K) SR of vector-control and *TaWRKY65*-silencing plants under drought stress. ***, ** and *, respectively, represented $p < 0.001$, $p < 0.01$ and $p < 0.05$.

similar expression pattern to *TaACS* according to transcriptome data. Validation of expression level via QPCR indicated that before drought treatment, the expression of *TaWRKY65* in *AevTDC1*-OX wheat was nearly three folds that in non-transgenic wheat (Figure 6A). After drought treatment, the expression of *TaWRKY65* was obviously induced compared to before drought treatment (Figure 6B). In addition, the promoter region of *TaACS* was predicted to contain three W-box motifs with the typical binding sites (TTGACC/T) of WRKY transcription factors (Figure 6E). Therefore, we explored whether there was a correlation between *TaWRKY65* and *TaACS*. Firstly, we used barley stripe mosaic virus (BSMV) induced gene silencing to knock down the expression of *TaWRKY65*. QPCR results showed that silencing of *TaWRKY65* decreased the expression of both *TaWRKY65* and *TaACS* (Figure 6C,D). Meanwhile, the content of endogenous ACC was remarkably downregulated (Figure 6J). Secondly, we utilised yeast one-hybrid and luciferase (LUC) reporter assay to detect whether TaWRKY65 was able to directly bind to the promoter of *TaACS* and then activate its expression. Three predicted W-box motifs (M1 ~ M3) of the *TaACS* promoter were, respectively, tested in the yeast one-hybrid system and showed that *TaWRKY65* could bind to M1. Yeast with *TaWRKY65* and M1 could grow on selection medium, while yeast with YFP and M1 could not. Due to the strong auto-activation of M2 and M3, it could not distinguish whether TaWRKY65 is able to bind to M2 and M3 or not (Figure 6F). Moreover, we found that TaWRKY65 significantly activated the expression of *LUC* driven by the *TaACS* promoter (Figure 6G,H). These evidences confirmed that TaWRKY65 functions in the transcriptional activation of *TaACS*.

We further investigated the functions of *TaWRKY65* in regulating drought tolerance. Plants of Line9 with silenced *TaWRKY65* showed less tolerant to drought than vector-control plants (Figure 6I). SR of *TaWRKY65*-silencing plants was only about 34%, which was obviously lower than the SR of vector-control plants (almost 85%) (Figure 6K).

3 | Discussion

3.1 | Overexpression of *AevTDC1* Dramatically Increased the Trp-Derived Metabolite Content in Wheat Grains

TDC is the key enzyme in tryptophan metabolism and is essential for the content of its downstream metabolites, such as serotonin and melatonin, which are quite important bioactive molecules for human health. Previous studies achieved an increase in the content of these metabolites through manipulating the expression of catalytic enzyme genes. For example, heterologous expression of the sheep serotonin *N-acetyltransferase* (*NAT*) gene in rice produced more melatonin than the WT plants (Park and Back 2012). *OsTDC3* transgenic rice elevated the production of tryptamine, serotonin, N-acetylserotonin and melatonin in the grains (Byeon et al. 2014). However, the effect of *TDC* genes on the content of the Trp-derived bioactive metabolites in wheat is unclear. In our study, by overexpressing *AevTDC1* in wheat, we found similar results as reported in rice (Kanjanaphachoat et al. 2012), including a significant increase of Trp-derived metabolites, such as serotonin and melatonin, in the grains, as well as a deepened grain colour (Figure 2A,C–F). It is worthy to mention that the content of melatonin in *AevTDC1*-OX grains was almost two folds that in the *OsTDC3* transgenic rice. Additionally, the content of L-ascorbate, glutathione reduced form and γ-aminobutyric acid were elevated significantly as well (Figure 2G–I). These results suggested that *AevTDC1* could improve the functional quality of wheat grain and has the potential to be utilised in food supplements.

3.2 | *AevTDC1* Simultaneously Promoted Resistance to Biotic and Abiotic Stresses in Wheat

CCN infection causes serious yield loss. However, genetic resources and functional genes for CCN resistance are extremely scarce in wheat breeding. In previous work, we found that silencing of *AevTDC1* compromised resistance to CCN in *Ae. variabilis* (Huang et al. 2018). Meanwhile, it worked together with *AevPAL1* to coordinate SA biosynthesis and downstream metabolism in *Ae. variabilis* and wheat (Zhang et al. 2021). In this study, we further found that overexpressing *AevTDC1* significantly reduced CCN cyst formation and exhibited a dosage effect on CCN resistance (Figure 3A), more strongly supporting its positive role in regulating CCN resistance in wheat. In addition, we detected higher expression of *TaXTH23* in the root of *AevTDC1*-OX plants than in the non-transgenic plants (Figure 3B). In soybean, *GmTINY*-mediated induction of *GmXTH* genes altered cell wall thickness, which contributed to enhanced resistance against soybean cyst nematode (He et al. 2025). We also found a consequential cell wall thickening in the root along with the upregulation of *TaXTH23* (Figure 3C,D). This result suggested that *AevTDC1* can also restrict the infection and development of CCN by enhancing the cellular physical barrier, and thus provided further insight into the mechanisms underlying the promoted CCN resistance observed in the present study.

Drought has become a serious abiotic stress, largely limiting wheat production. It is quite important to elevate the drought tolerance of wheat varieties in breeding. Although several genes were reported playing important roles in drought tolerance in wheat (Mao et al. 2022; Mei et al. 2022; Tian et al. 2023; Wang et al. 2021; Yang et al. 2024), the number of these genes is far from sufficient for breeding applications. Here, we found that overexpression of *AevTDC1* significantly enhanced wheat drought tolerance (Figure 4), providing an effective gene resource for improving drought tolerance. A previous work reported that *PlTDC1* of *Paeonia lactiflora* could enhance drought and high-temperature tolerance in tobacco (Zhao et al. 2019), revealing a similar regulating role of *TDC* in both dicots and monocots.

More importantly, the improved dual resistance to CCN and drought was achieved by overexpression of the same catalytic enzyme gene, *AevTDC1*, which could efficiently help plants resist the negative effects caused by CCN and drought and simultaneously improve the grain qualities. Although overexpression of *AevTDC1* resulted in a slight adverse effect on PH, SNS and plant biomass (Figure 1D,E,H), the three key yield-determining traits, that is, GNS, ESNP and TGW, had not been impaired (Figure 1I,J,L). Therefore, *AevTDC1* might have only a very weak impact on yield and is a potentially favourable gene for wheat breeding. The tryptophan metabolism may also be considered a target for modification to optimise or extend environmental adaptability. As TDC functions very upstream, it will have a wide impact on the production of metabolites and signalling pathways beneath it. Therefore, dissection of regulators, such as transcription factors, downstream of the TDC may have greater potential and applicability for improving wheat functional quality and environmental resilience.

3.3 | The Role of Plant Hormones in the *AevTDC1*-Regulated Responses to Stresses

Plant hormones are very crucial for plant growth, development, as well as the responses to biotic and abiotic stresses. The growing evidence shows that plant hormone crosstalk forms a complicated orchestrated regulatory network. In this study, we found that overexpressing *AevTDC1* indeed enhanced CCN resistance, which might at least partially be attributed to elevated SA content (Zhang et al. 2021) and thickened cell wall in *AevTDC1*-OX root (Figure 3). On the other hand, we unexpectedly detected obviously increased ACC content (Figure 5D) and enhanced drought tolerance in *AevTDC1*-OX wheat (Figure 4). Transcriptomic analysis, hormone content measurement and exogenous ETH treatment assay together indicated that ethylene participates in the drought tolerance enhancement by regulating stomatal aperture (Figure 5).

ABA is well known for controlling stomatal movement in plant responses to drought stress (Hsu et al. 2021; Waadt et al. 2022). Ethylene has been reported to affect stomatal closure/opening antagonistically or synergistically with other hormones (Song et al. 2023; Wilkinson and Davies 2010). Comprehensively, ABA and ethylene antagonistically affect each other's synthesis under drought stress and regulate stomatal movement (Müller 2021; Tanaka et al. 2005). In the present study, the expression alteration of *TaNCED3* was greater in *AevTDC1*-OX plants than in non-transgenic plants under the drought treatment, which showed consistent changes to ethylene (Figure S3A). Thus, we inferred that ethylene and ABA might synergistically regulate stomatal aperture in wheat in response to drought stress. These results revealed that the enhanced drought tolerance might be ascribed to a smaller stomatal aperture caused by more ethylene accumulation and a finely tuned hormone network in *AevTDC1*-OX wheat.

The SA has been reported to promote stomatal closure under drought stress or plant immunity in previous studies (Elsisi et al. 2024; Wang et al. 2023). On the other hand, ET was also proved to play positive effects on soybean cyst nematode resistance (He et al. 2025). Thus, the simultaneous promotion of SA and ET biosynthesis by *AevTDC1* overexpression may have less or no antagonistic effects on responses to CCN and drought stress and therefore facilitate the resilience of wheat to both stresses.

In addition, WRKY family members were found to be involved in plant hormones pathway to regulate biotic and abiotic stress responses (Chen et al. 2020; Ge et al. 2024; Li et al. 2012; Lv et al. 2022). However, few studies built a direct connection between WRKYs and ethylene biosynthesis in drought tolerance. In this study, *TaWRKY65* was identified to positively regulate drought tolerance in wheat (Figure 6) and proved to be a novel transcriptional activator of ethylene biosynthesis functioning through directly binding to *TaACS* promoter (Figure 6). Since WRKYs play a core role in plant hormones crosstalk (Javed and Gao 2023; Jiang et al. 2014), it is worth further investigating whether *TaWRKY65* is or is not a node gene of crosstalking between ETH and other plant hormones, such as ABA and SA.

Combing these results, we concluded that manipulation of tryptophan metabolism through overexpressing *AevTDC1* significantly altered grain secondary metabolites and hormone biosynthesis in wheat, which consequently fortified grain functional quality, and bio- and abiotic stress resistance (Figure 7).

4 | Experimental Procedures

4.1 | Plant Materials and Growth Conditions

The *AevTDC1*-OX wheat was obtained as described previously (Zhang et al. 2021). Seeds of *AevTDC1*-OX and non-transgenic wheat were kept in our laboratory. In the greenhouse, seeds were soaked in sterilised water and kept at 4°C for 1 day, then germinated on wet filter paper under 23°C. About 2 days later, little seedlings were transplanted into soil for cultivation at 23°C under a 16 h light/8 h dark photoperiod.

4.2 | Agronomic Traits Investigation

For agronomic phenotype investigation, all plants were planted in the field in Shifang, Sichuan province, China (N 31°06′37.07″, E 104°09′19.70″, altitude 521 m). PH, ESNP, GNS, SL, SNS, GL, GW, TGW and plant biomass were measured. At least thirty individuals of each line were used for analysis. PH, ESNP, SL, SNS, and the plant biomass were manually measured at the maturity stage. The grains were manually threshed according to individual spikes, and the GNS, GL, GW and TGW were measured with automatic seed testing software V2.0 (Wan Shen).

4.3 | Metabolomic Analysis

To profile the components of grains, five gram whole grains of each line were collected. Three replicates were used in this test. As previously described, metabolite profiling was carried out using a widely targeted metabolome method by Wuhan Metware Biotechnology Co. Ltd. (Wuhan, China) (http://www.metware.cn/) (Zhang et al. 2021).

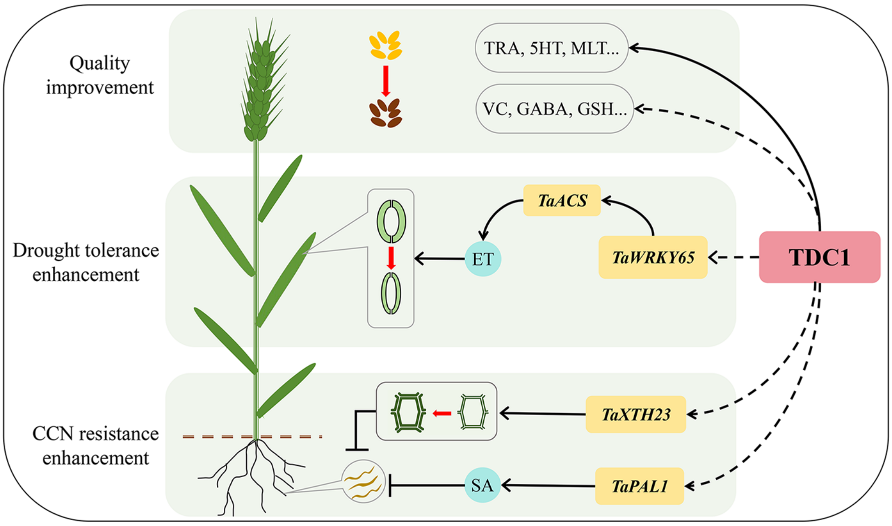

FIGURE 7 | The sketch map of the role of *AevTDC1* in improving grain health benefits and resistance to stress in wheat. Overexpressing *AevTDC1* promoted the conversion of Trp into tryptamine (TRA), serotonin (5HT), N-hydroxytryptamine (N5HT) and melatonin (MLT). Meanwhile, it elevated accumulation of antioxidants, such as L-ascorbate (VC), glutathione reduced form (GSH) and γ-aminobutyric acid (GABA) in grains. On the other hand, *AevTDC1* overexpression simultaneously enhanced resistance to biotic and abiotic stress. *AevTDC1* enhanced CCN resistance by mediating salicylic acid (SA) synthesis via *TaPAL* and thickening root cell wall via *TaXTH23*. And it improved drought tolerance through upregulating expression of TaWRKY65-TaACS module, which sequentially led to higher ethylene (ET) accumulation, smaller stomatal aperture and more tolerant to drought. Besides, these antioxidants might help improving stress resistance by scavenging free radicals. Arrow end represents promotion. Blunt end represents suppression. The solid line stands for known modes of action. The dashed line stands for unknown modes of action.

4.4 | Quantification of Endogenous Trp, Serotonin, N-5-Hydroxytryptamine and Melatonin Levels From Plant Tissues

Plant samples (100 mg FW) and spiked isotope-labelled internal standard (13C1115C2-Trp, 2H4-5HT, 2H4-MLT) were extracted with ethyl acetate (EA) overnight at 4°C. After centrifuging, the supernatant was collected and the same volume of n-hexane (Hex) was added. Then, the sample was loaded onto a Silica SPE cartridge (Waters) activated and equilibrated with MeOH, EA and 50% EA/Hex (v/v) in turn. After washing with 50% EA/Hex, MLT was eluted with EA, then Trp and 5HT were eluted with 1% formic acid in EA/MeOH (1/3, v/v). After dried in N2 stream, the eluent was redissolved in 20% MeOH for LC–MS/MS analysis. MLT analysis was performed on a CSH C18 column (50 × 2.1 mm, 1.7 μm) with UPLC instrument (Waters) combined with 6500 Qtrap Mass Spectrometry (AB SCIEX). The inlet method was set as follows: mobile phase A: 0.05% formic acid in water, B: 0.05% formic acid in ACN. Gradient: 0–1.5 min, 1% B; 1.5–10.5 min, 1% B to 30% B; 10.5–11.5 min, 30% B to 80% B; 11.5–12.5 min, 80% B; 12.5–13.5 min, 80% B to 1% B; 13.5–15.5 min, 1% B. The optimised electrospray ionisation (ESI) operating parameters for positive mode were: IS, 5000 V; CUR, 35 psi; TEM, 500°C; GS1, 45 psi; GS2, 55 psi. The MRM transitions were as follows: MLT 233.1 > 174.1, 233.1 > 130.1; 2H4-MLT 237.1 > 178.1, 237.1 > 134.1; Trp 205.1 > 188.1, 205.1 > 146.1; 13C1115C2-Trp 218.1 > 200.1, 218.1 > 156.1; 5HT 177.1 > 160.1, 177.1 > 115.1; 2H4-5HT 181.1 > 164.1, 181.1 > 118.1; three biological replicates were analysed for each treatment.

4.5 | Measurement of Endogenous ACC and ABA Content Measurement

Approximately 100 mg (fresh weight, FW) plant tissue were homogenised under liquid nitrogen, weighted and extracted with 90% methanol (MeOH) overnight at 4°C containing 2H6-ABA and 2H4-ACC as internal standards. Endogenous ABA and ACC were purified and measured as previously described (Xin et al. 2020) with some modifications in detection conditions. LC–MS/MS analysis was performed on a UPLC system (Waters) coupled to the 5500 Qtrap system (AB SCIEX). For the analysis of ABA, LC separation used a BEH C18 column (1.7 μm, 100 × 2.1 mm; Waters) with mobile phase A, 0.05% acetic acid in water and B, acetonitrile. The gradient was set with initial 10% B and increased to 90% B within 6 min. ABA was detected in negative multiple reaction monitoring (MRM) mode with transition and the transitions were as follows: ABA 263.0 > 153.1, 263.0 > 219.1; 2H6-ABA 269.2 > 159.2, 269.2 > 225.2. The source parameters were set as: IS, −4500 V; CUR, 35 psi; TEM, 400°C; GS1, 45 psi; GS2, 55 psi. ACC analysis was performed on a BEH Amide column (1.7 μm, 100 × 2.1 mm; Waters) with mobile phase A, 0.1% formic acid in water and B, 0.1% formic acid in acetonitrile. The gradient was set with initial 90% B and decreased to 65% B within 6 min. ACC was detected in positive MRM mode with transition and the transitions were as follows: ACC 102.1 > 56.0, 102.1 > 30.0; 2H4-ACC 106.1 > 60.0, 106.1 > 32.0. The source parameters were set as: IS, 5500 V; CUR, 30 psi; TEM, 550°C; GS1, 50 psi; GS2, 55 psi. Three biological replicates were analysed for each treatment.

4.6 | CCN Inoculation and Resistance Assay

CCN J2 hatching, inoculation and new-formed cyst calculation were performed as previously described (Zhang et al. 2021). At least fifteen individual plants were counted for analysis. This experiment was performed three times. The average number of new-formed cysts per plant was shown.

4.7 | Drought Tolerance Assay

For ethephon treatment, plants were first cultured in hydroponics with or without 100 μM ETH for 3 days and then transplanted into soil for drought treatment.

For drought tolerance test, at least 15 4-week seedlings were used. All plants were well-watered before stopping watering. The LWC was, respectively, calculated under well-watering and drought treatment conditions. Plants were treated for about 3 weeks under drought and then recovered watering. The SR was calculated at 3 days post recovering watering. The experiment was repeated five times and results displayed the average SR ± standard error.

4.8 | Comparative Transcriptome Analysis

For transcriptome analysis, three groups with different LWC were separately sampled. Leaves under well-watering (LWC≈87%) were sampled as Nd group. After drought treatment, the leaves with LWC≈80% and leaves with LWC≈74% were separately collected into Md group and Sd group. At least ten plants were sampled and four replicates were used for sequencing. Transcriptome sequencing and analysis were operated by Biomarker Technologies Co., LTD.

4.9 | BSMV Induced Gene Silencing

The 384 ~ 555 bp of coding sequence (CDS) of *TaWRKY65* was cloned and ligated into BSMV γ plasmid for VIGS vector generation. BSMV γ empty vector was used as the negative vector control. The infectious virus was synthesised and inoculated according to the methods described previously (Zhang et al. 2021).

4.10 | RNA Extraction and QPCR

The leaves were collected and ground into powder using liquid nitrogen. Total RNA was extracted with a kit (Foregene) and cDNA was synthesised using a reverse transcription kit (TransGen Biotech). QPCR was performed as described previously. *Elongation factor 1α* gene was used as the internal reference. The primers used were listed in Table S1.

4.11 | Stomatal and Cell Wall Observation

To inspect the stomata, we prepared samples and adopted a scanning electron microscope (SEM) according to the method described (Yuan et al. 2024). About 3-week-old plants with the same developmental status were used for sampling. The newly unfolded leaves were cut and firstly fixed with 3% glutaraldehyde buffer stored at 4°C. Then samples were fixed with 1% osmium acid and underwent progressive dehydration with ethanol. The sample holder was placed into the ion sputtering instrument for spraying treatment. The images were captured with a scanning electron microscope (JSM-IT700HR, Japan). Stomatal density was calculated by the stomatal number per view, and the results showed the mean number of at least five reviews. Stomatal aperture was calculated using the formula (stomatal width/stomatal length). No less than thirty stomata were measured, and results showed the average of the data. The experiment was performed three times.

To measure cell wall thickness of root, sample preparation and TEM observation were performed as described by He et al. (2025). Primary root segments, about 1 cm distance from the root tip, were sampled for fixation. Images were captured using a transmission electron microscope (JEM-1400FLASH, Japan). No less than sixteen cell walls were measured and results expressed as means ± SEs.

4.12 | Yeast One-Hybrid

The binding motifs were predicted on the website (https://plantregmap.gao-lab.org/). Two repeats of M1 (−1475 ~ −1457 bp, AGTTGACTACTCATGTCAT), M2 (−684 ~ −668 bp, CTCGTTTTGACCAACTC) and M3 (−263 ~ −247 bp, TGTCCCTTGACCTGCGT) of the *TaACS* promoter were, respectively, ligated into the pHIS2 plasmid to construct the bait vectors. The CDS sequence of *TaWRKY65* was ligated into the pRec2 plasmid to generate the prey vector. The YFP sequence ligated into pRec2 was used as the negative prey vector. The prey and bait plasmids were co-transformed into the yeast strain Y187. The diluted transformants yeast droplets with the same OD were cultured on SD-LT and SD-LTH with different concentrations of 3-AT.

4.13 | Luciferase Reporter Assay

The ~1960 bp promoter sequence of *TaACS* was amplified from genomic DNA and ligated into the pGreenII 0800-LUC vector to generate the reporter plasmid 35S::REN-*TaACS* Pro::LUC. The full-length CDS of *TaWRKY65* was amplified and inserted to Pcambia1300-35s-YFP to generate the effector plasmid 35S::TaWRKY65. The effector and reporter plasmids were co-transformed into *Agrobacterium* strain *GV3101* containing the pSoup plasmid (Biomed). Dual-luciferase reporter assay was carried out in *N. benthamiana* plant leaves as described (Sun et al. 2023).

4.14 | Statistics Analysis

Student's t-test was performed between two groups, and ANOVA analysis was performed among more than two groups using the SPSS 16.0 software.

4.15 | Accession Numbers

TaWRKY65 (TraesCS3D02G289500), *TaACS* (TraesCS2D02G394200), *TaACO* (TraesCS5A02G234200), *TaERF1* (TraesCS4D02G298600), *TaNCED3* (TraesCS5A02G374000), *TaXTH23* (TraesCS7B02G327200).

Author Contributions

H.Z. and H.L. conceived the experiment. H.Z. performed most of the experiments and data analysis. H.L. supervised the experiments. H.Z. wrote the manuscript. H.L. revised the manuscript. Q.H., L.Y., X.S., Q.L., J.Z., G.D., J.L., T.L. and M.Y. assisted the experiments. S.C., P.X. and J.C. helped the measurement of hormones. All authors approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.