


GmTDN1 improves wheat yields by inducing dual tolerance to both drought and low-N stress

Yongbin Zhou^{1,†}, Jun Liu^{1,†}, Jinkao Guo², Yanxia Wang², Hutai Ji³, Xiusheng Chu⁴, Kai Xiao⁵, Xueli Qi⁶, Lin Hu⁶, Hui Li⁷, Mengyun Hu⁷, Wensi Tang¹, Jiji Yan¹, Huishu Yan¹, Xinxuan Bai¹, Linhao Ge¹, Mingjie Lyu¹, Jun Chen¹, Zhaoshi Xu¹ , Ming Chen^{1,*} and Youzhi Ma^{1,*}

¹Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS)/National Key Facility for Crop Gene Resources and Genetic Improvement, Key Laboratory of Biology and Genetic Improvement of Triticeae Crops, Ministry of Agriculture, Beijing, China

²Shijiazhuang Academy of Agricultural and Forestry Sciences, Research Center of Wheat Engineering Technology of Hebei, Shijiazhuang, China

³Institute of Wheat Research, Shanxi Academy of Agricultural Sciences, Linfen, China

⁴Crop Research Institute, Shandong Academy of Agricultural Sciences, Jinan, China

⁵College of Agronomy, Agricultural University of Hebei Province, Baoding, China

⁶Wheat Research Institute, Henan Academy of Agricultural Sciences, Zhengzhou, China

⁷Hebei Laboratory of Crop Genetics and Breeding, Hebei Academy of Agriculture and Forestry Sciences, Institute for Cereal and Oil Crops, Shijiazhuang, China

Received 7 September 2021;

revised 27 April 2022;

accepted 2 May 2022.

*Correspondence (Tel +86 10-82108789;

fax +86 10-82108789; emails

mayouzhi@caas.cn(Y.M.);

chenming02@caas.cn(M.C.))

[†]These authors contributed equally to this work.

Summary

Genetically enhancing drought tolerance and nutrient use efficacy enables sustainable and stable wheat production in drought-prone areas exposed to water shortages and low soil fertility, due to global warming and declining natural resources. In this study, wheat plants, exhibiting improved drought tolerance and N-use efficacy, were developed by introducing *GmTDN1*, a gene encoding a DREB-like transcription factor, into two modern winter wheat varieties, cv Shi4185 and Jimai22. Overexpressing *GmTDN1* in wheat resulted in significantly improved drought and low-N tolerance under drought and N-deficient conditions in the greenhouse. Field trials conducted at three different locations over a period of 2–3 consecutive years showed that both Shi4185 and Jimai22 *GmTDN1* transgenic lines were agronomically superior to wild-type plants, and produced significantly higher yields under both drought and N-deficient conditions. No yield penalties were observed in these transgenic lines under normal well irrigation conditions. Overexpressing *GmTDN1* enhanced photosynthetic and osmotic adjustment capacity, antioxidant metabolism, and root mass of wheat plants, compared to those of wild-type plants, by orchestrating the expression of a set of drought stress-related genes as well as the nitrate transporter, *NRT2.5*. Furthermore, transgenic wheat with overexpressed *NRT2.5* can improve drought tolerance and nitrogen (N) absorption, suggesting that improving N absorption in *GmTDN1* transgenic wheat may contribute to drought tolerance. These findings may lead to the development of new methodologies with the capacity to simultaneously improve drought tolerance and N-use efficacy in cereal crops to ensure sustainable agriculture and global food security.

Keywords: DREB-like transcription factor, drought, nitrogen deficiency, transgenic wheat (*Triticum aestivum* L.), field trial.

Introduction

Reportedly, crop yields in drought-prone areas must be increased by 40% by 2025 to feed the ever-increasing human population (Pennisi, 2008). Wheat (*Triticum aestivum* L.), a major food crop consumed by more than 30% of the global population, is primarily grown in dry or semi-dry lands. Drought and N deficiency are major limiting factors affecting wheat production worldwide (Li *et al.*, 2021; Teixeira *et al.*, 2018). Thus, breeding wheat cultivars with synergistically improved tolerance to stressors, such as drought and low N, is vital for maintaining sustainable wheat production. However, introducing these elite agronomic traits into modern wheat varieties, via a common breeding programme that improves drought tolerance and NUE, will be challenging and time-consuming. Transgenesis, an alternative technique that improves wheat drought tolerance and N-use efficacy, has demonstrated significant potential in meeting these challenges (Mega *et al.*, 2019; Yoshida *et al.*, 2014). However, most previous studies investigating the function of

drought-related genes, including DREB-like (dehydration responsive element binding proteins) genes, have been conducted under greenhouse conditions. Thus, data on the performance of drought tolerance genes under field conditions are limited (Araus *et al.*, 2019).

Plant–water relationships and nutrient metabolism are related physiological processes (Plett *et al.*, 2020; Shangguan *et al.*, 2004). Previous physiological studies have shown that drought stress strongly affects N metabolism (Wang *et al.*, 2016). N application can enhance drought tolerance in cotton (Liu *et al.*, 2008). Given their critical importance to plant growth and productivity, understanding the mechanisms underlying N uptake and NUE during drought stress is necessary for water-scarce areas, such as semi-arid and arid regions, among others. The regulatory mechanisms linking drought stress and low-N stress are currently being investigated. Drought response transcription factor *DST* is involved in decreasing nitrogen (N) assimilation under drought stress via *OsNRT1.2* (Han *et al.*, 2022). Efforts to improve N uptake in crops must also consider the intricate ties

with limited water availability and uptake to meet the challenges of modern agriculture (Araus *et al.*, 2020; Plett *et al.*, 2020). Despite the current limited understanding regarding the connection between drought and low-N stress, studies on other stress combinations have revealed that plants have evolved many highly effective strategies to simultaneously regulate multiple stress response processes, sometimes via the same mechanism (Saijo and Loo, 2020). For example, sensitive to proton rhizotoxicity 1 (STOP1) is a master transcription factor that regulates cellular pH homeostasis and exerts pleiotropic effects on both roots and shoots, thereby enhancing tolerance to various forms of stress (Sadhukhan *et al.*, 2021). Therefore, transgenic plants have highlighted the need for strategies to improve growth in low N and drought stress environments.

The DREBs belong to a large family of transcription factors (AP2/ERF, APETALA 2/ethylene response factor). Previously, we reported that introducing a DREB-like transcription factor, GmTDN1, from soybean (*Glycine max* L. Merr.) to *Arabidopsis* significantly improved its drought tolerance (Chen *et al.*, 2009). Here, GmTDN1 was introduced to two modern winter wheat varieties, Shi4185 and Jimai22. Overexpressing GmTDN1 in wheat resulted in significantly improved drought and low-N tolerance under drought and N-deficient conditions in the greenhouse. Field trials at three locations, which were subjected to limited irrigation and N deficiency over 2–3 consecutive years, indicated that both Shi4185 and Jimai22 GmTDN1 transgenic lines were agronomically superior to wild-type (WT) plants, resulting in significantly increased yields under both drought and N deficiency stresses. Collectively, the findings from this study demonstrate that ectopic expression of GmTDN1 leads to simultaneous manipulation of both drought tolerance and low N stress tolerance in wheat, thereby illustrating a potential strategy for maintaining wheat yield stability under drought and low-N conditions.

Results

Overexpression of GmTDN1 confers wheat plants with improved drought and low N stress tolerance in the greenhouse

DREBs are known to regulate plant response to abiotic stress. We previously identified a soybean DREB, GmTDN1 (Glyma.17G047300, the *phytozome*2 database-<https://phytozome.jgi.doe.gov/pz/portal.html#>), that confers drought tolerance to *Arabidopsis* (Chen *et al.*, 2009). Phylogenetic analysis based on DREB amino acid sequences derived from different plant species revealed that GmTDN1 belongs to the DREB-A5 subfamily (Figure S1). Quantitative PCR (qPCR) of GmTDN1 expression levels in leaves and roots of soybean seedlings under stressed conditions (cold; ABA; salinity stress and two osmotic stressors: PEG and high sucrose) further confirmed that GmTDN1 is a stress-responsive gene involved in the ABA-independent stress-responsive signal pathway (Figure S2).

To assess its role in stress response, the biological functions of GmTDN1 upon ectopic expression were investigated in common wheat varieties. This gene was introduced into two of the most widely planted winter wheat varieties, Shi4185 and Jimai22 (Jm22), via bombardment. Calli for Shi4185 and Jm22 were bombarded, and PCR analysis identified that 28 and 25 T0 independent transgenic lines for Shi4185 and Jm22 were retained, respectively (Figure S3a,b). After T0 generation,

transgenic plants were identified using PCR in T1, T2, and T3 generations, and PCR negative segregation was eliminated. Based on the PCR identification and assessment of drought tolerance in greenhouse conditions, three T3 generation lines for each wheat variety (named S-OE1, S-OE2, S-OE3, and J-OE1, J-OE2, J-OE3) with the best drought tolerance and without significantly altered growth traits compared with WT, were selected for field experiments. In the T4 generation, in addition to the field experiment, copy number analysis was conducted using Southern blot, expression analysis using semi-quantitative RT-PCR, and homozygosity analysis using PCR for six transgenic lines. The result of the Southern blot showed that the copy numbers of J-OE1, J-OE2, and J-OE3 lines were 3, 1, 1 (Figure S3d), and those of S-OE1, S-OE2, and S-OE3 lines were 2, 3, 3, respectively. Meanwhile, semi-quantitative RT-PCR showed that GmTDN1 can be transcribed in transgenic lines (Figure S3e). A total of 100 T4 generation plants were randomly selected for PCR assay. Results showed that the proportion of positive transgenic plants exceeded 93% in six lines (Table S1 and Figure S3c). These results showed that GmTDN1 could be stably inherited and transcribed in six transgenic lines with a relatively low negative admixture rate.

To analyse drought tolerance, seedling development was investigated at the 3-leaf stage under four drought conditions: 7-days water-deficiency; 21-days water-deficiency; 21-days water-deficiency plus 7-days rewatering; and 28-days water-deficiency plus 7-days rewatering, using Shi4185 transgenic lines as an example. Under the 21-days water deficit, the Shi4185 transgenic lines were considerably greener than the WT Shi4185 plants, which showed obvious stress symptoms, such as decreased biomass and leaf wilting (Figure 1a). The leaves of the transgenic lines showed lower water loss rates than those of WT plants under drought stress conditions ($n = 20$; $*P < 0.05$; Figure 1b). Under the 21-days water deficit plus rewatering condition, approximately 89.34–91.96% Shi4185 transgenic lines survived, compared to 61.17% of the WT Shi4185 plants ($n = 20$; $**P < 0.01$; Figure 1c). Under severe drought conditions (28-days water deficit plus 7-days rewatering), only about 22.26% of the WT Shi4185 plants survived, whereas the respective transgenic lines exhibited 55.57–72.08% survival rates ($n = 20$; $**P < 0.01$; Figure 1c). Similar trends were observed between transgenic Jm22 lines and WT Jm22 plants (Figure S4a–c).

The downstream target genes of GmTDN1 were analysed in transgenic wheat via a ChIP assay and found that GmTDN1 regulates the expression of *NRT2.5*, a high-affinity nitrate transporter in wheat. It was, therefore, speculated that GmTDN1 may affect the traits of transgenic wheat under low-N stress. Subsequently, the behaviour of these transgenic S-OE, J-OE, and WT lines was investigated under low-N stress (2 and 0.2 mM nitrate for normal and low-N treatments, respectively) in the greenhouse. All GmTDN1 transgenic lines exhibited significant tolerance to low NO_3^- stress (Figures 1d and S4d) in the form of the significantly increased fresh shoot and root weight (Figures 1e,f and S4e,f), deeper root lengths, wider root surface area, and increased root volumes than those of WT plants under low-N conditions ($n = 20$; $*P < 0.05$; Figure S5a–d). Furthermore, the low-N treatment considerably reduced N uptake in roots of all tested WT plants, but the OE lines including S-OE3 and J-OE3, had significantly higher nitrate influx rates than those of WT plants, Shi4185 and Jm22, in a

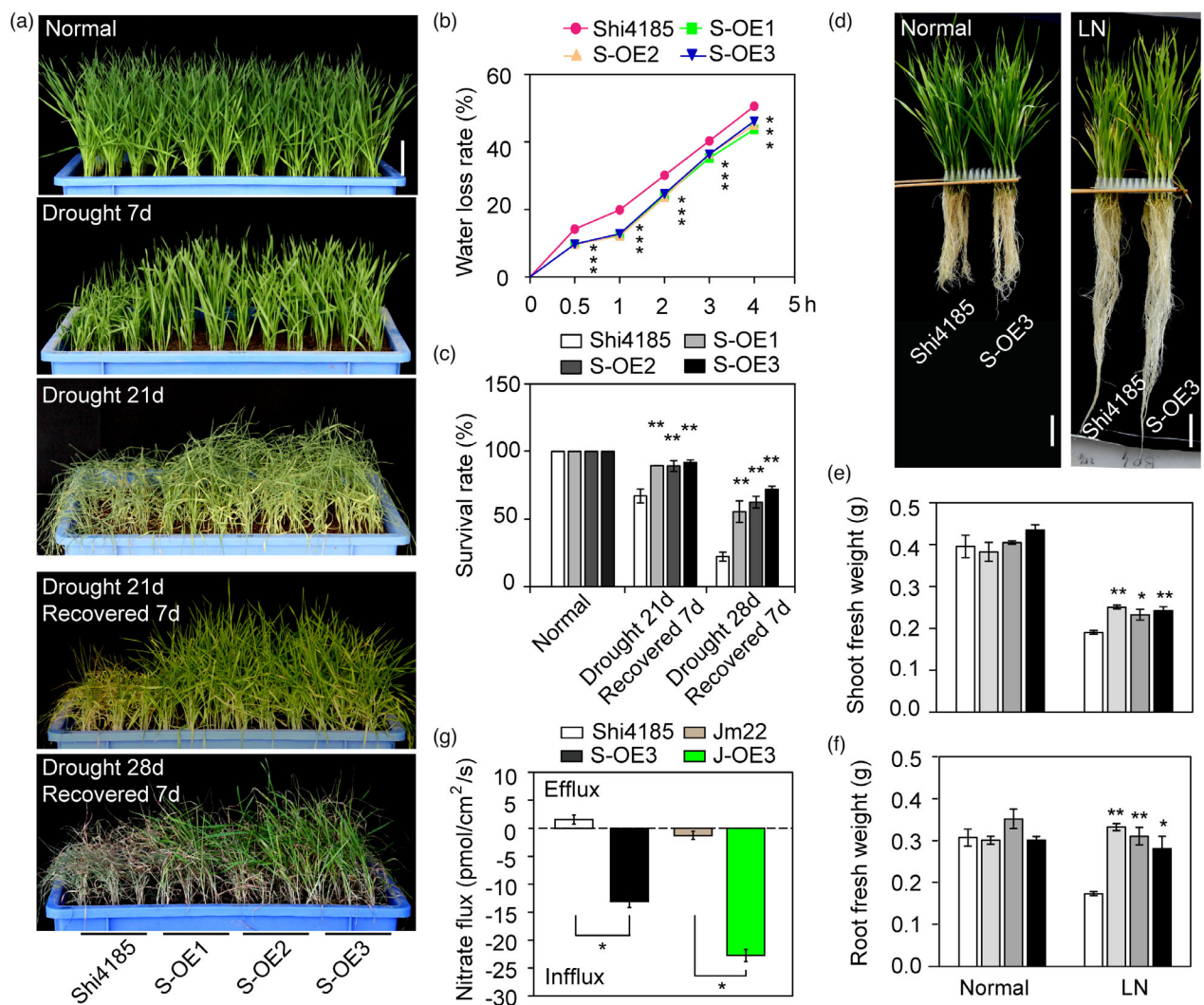


Figure 1 *GmTDN1* overexpression confers drought and low nitrogen (LN) stress tolerance. (a) Photographs of Shi4185 and S-OE lines under normal (non-stressed conditions), drought (7 and 21 days), drought (21 or 28 days) and then rehydration for 7 days. Three-leaf stage plants were subjected to drought stress (stop watering for 7 or 21 days, and then allowed to recover for 7 days) prior to capturing these images. (b) Results of a water loss assay for Shi4185 and S-OE lines. Leaves of three-leaf stage plants were detached for 5 h ($n = 4$ biologically independent samples; error bars represent means \pm SD). (c) Survival rates of wheat seedlings. (d) Seedlings of Shi4185 and the S-OE plants grown under LN and normal conditions. Three days after germination, plants were grown hydroponically for 21 days under LN and normal nutrient solution conditions. (e) Shoot fresh weight. (f) Root fresh weight. Data in e and f are presented as means \pm SD from three independent experiments (at least 20 plants in each experiment). (g) NO_3^- flux rates; NO_3^- flux rate was measured via NMT with 0.2 mM NO_3^- in the measuring solution. Here, seedlings were pretreated with 0.2 mM NO_3^- and then used to measure NO_3^- flux rates at the root surface in a measuring solution that contained 0.2 mM NO_3^- . Positive and negative flux values indicate efflux and influx, respectively. Data represent means \pm SD of at least six replicates. * $P < 0.05$ and ** $P < 0.01$ between the transgenic line and the WT control according to Student's *t*-test (two-tailed). Values are represented by the mean \pm SD.

solution containing 0.2 mM nitrate for 10 min ($n = 6$; * $P < 0.05$; Figure 1g).

To demonstrate that the improvement of N absorption and utilization of *GmTDN1* transgenic wheat contribute to the enhancement of drought tolerant, mimic pot experiments were conducted under the double stress treatment of drought and low nitrogen, using single drought treatment as a control. The results indicated that wheat seedlings grown in nitrate-deficient conditions were more tolerant to drought stress with 83–97% survival rates for OE lines compared to the 16–33% for the WT plant, whereas in nitrate-normal conditions, approximately 27–63% of

the OE line plants survived, while the WT plants showed 10–37% survival rates (Figures 2 and S6). These results indicated that the difference in survival rate between *GmTDN1* transgenic wheat and WT under low N and drought conditions is greater than that under normal and drought conditions. These results suggest that *GmTDN1* can improve N absorption and utilization in transgenic wheat compared with WT under low-N conditions, which contributes to drought tolerance of transgenic wheat. The superior traits of the transgenic lines under water deficit conditions were caused by the improved nitrate acquisition and utilization.

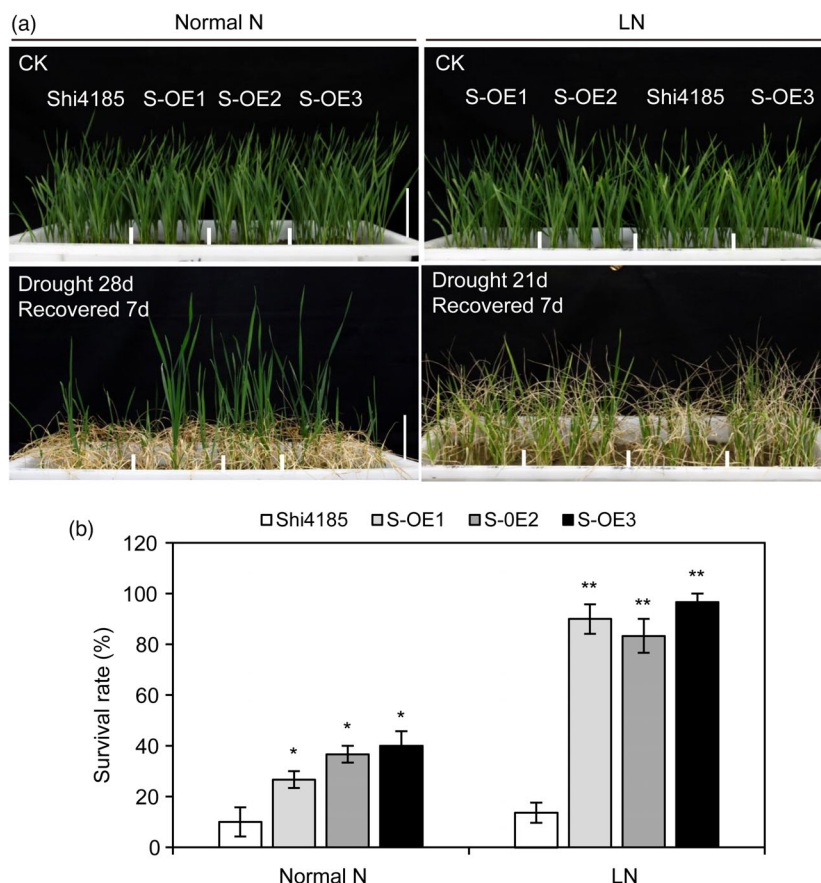


Figure 2 Drought tolerance of the Shi4185 transgenic lines assessed by improved nitrate acquisition and utilization. (a) Photographs of Shi4185 and S-OE lines under normal, drought (28 and 21 days), and rehydration for 7 days in normal and nitrogen-deficient field soil. Three-leaf stage plants were subjected to drought stress (stop watering for 28 or 21 days and then allowed to recover for 7 days) before capturing photographs. (b) Survival rates of wheat seedlings. Data represent means \pm SD of at least three replicates (at least 20 plants in each experiment). * $P < 0.05$ and ** $P < 0.01$ between the transgenic line and WT control according to Student's *t*-test (two-tailed). Values are represented by the mean \pm SD.

Overexpression of *GmTDN1* increased wheat yield potential under drought conditions in the field

Next, these transgenic lines were subjected to field trials at three locations with distinct climate conditions. The field performance of the overexpression lines, S-OE1, S-OE2, and S-OE3, and Shi4185 WT was investigated under normal (WIR), limited (LIR), or no irrigation (NIR) conditions for three consecutive wheat-growing seasons (2012–2015) across three wheat-producing areas of central China (Shijiazhuang in Hebei Province, Jinan in Shandong Province, and Beijing). These results indicated that the *GmTDN1* overexpression wheat lines had greener canopies than the WT plants under the LIR and NIR conditions (Figure 3a). All three transgenic lines showed significantly higher grain yields than the WT plants at all three locations, under both LIR and NIR conditions, across three wheat-growing seasons ($n = 9$; * $P < 0.05$), with the most significantly increased yields (~13%) observed at the Beijing site (Figure 3b and Table S2). Notably, the introduction of *GmTDN1* did not incur any yield penalties under normal irrigation conditions as no obvious yield differences were observed between WT and transgenic plants at any of the three locations for any year (Figure 3b and Table S2).

The field performance of three Jimai22 transgenic lines at three different locations (replacing Jinan with Linfen in Shanxi Province)

was further evaluated over two consecutive wheat-growing seasons (2015–2017) under the same three water regimens. Again, the wheat lines overexpressing *GmTDN1* displayed greener canopies than WT plants under LIR and NIR conditions (Figure S7). The yield of the Jimai22 transgenic lines invariably outperformed that of the WT lines under LIR or NIR conditions ($n = 6$; * $P < 0.05$), and did not differ from WT plants under WIR conditions (Figure 3c and Table S3). The most significantly increased yields (~12%) were observed under the LIR condition at the Shijiazhuang site in 2015–2017 (Table S3).

Yield composition elements were also analysed, revealing that the spike number/m², a major contributor to yield, was significantly higher compared to that of WT plants under both LIR and NIR conditions across all locations in all three years ($n = 9$; * $P < 0.05$), whereas no significant differences were detected in the grain number/spike between transgenic lines and WT plants (Tables S4 and S5). Conversely, substantial differences were observed in the extent of the increase in 1000-grain weight (TGW) that depends on differences between lines, treatment conditions, and locations across various growing seasons (Tables S4 and S5). At the Shijiazhuang site, the water use efficiency (WUE) of overexpression lines, including Shi4185 (Figure 3d) and Jm22 (Figure 3e) transgenic plants, significantly increased ($n = 3$; * $P < 0.05$) compared to that of WT plants during 2014–2016, reaching 10–20%.

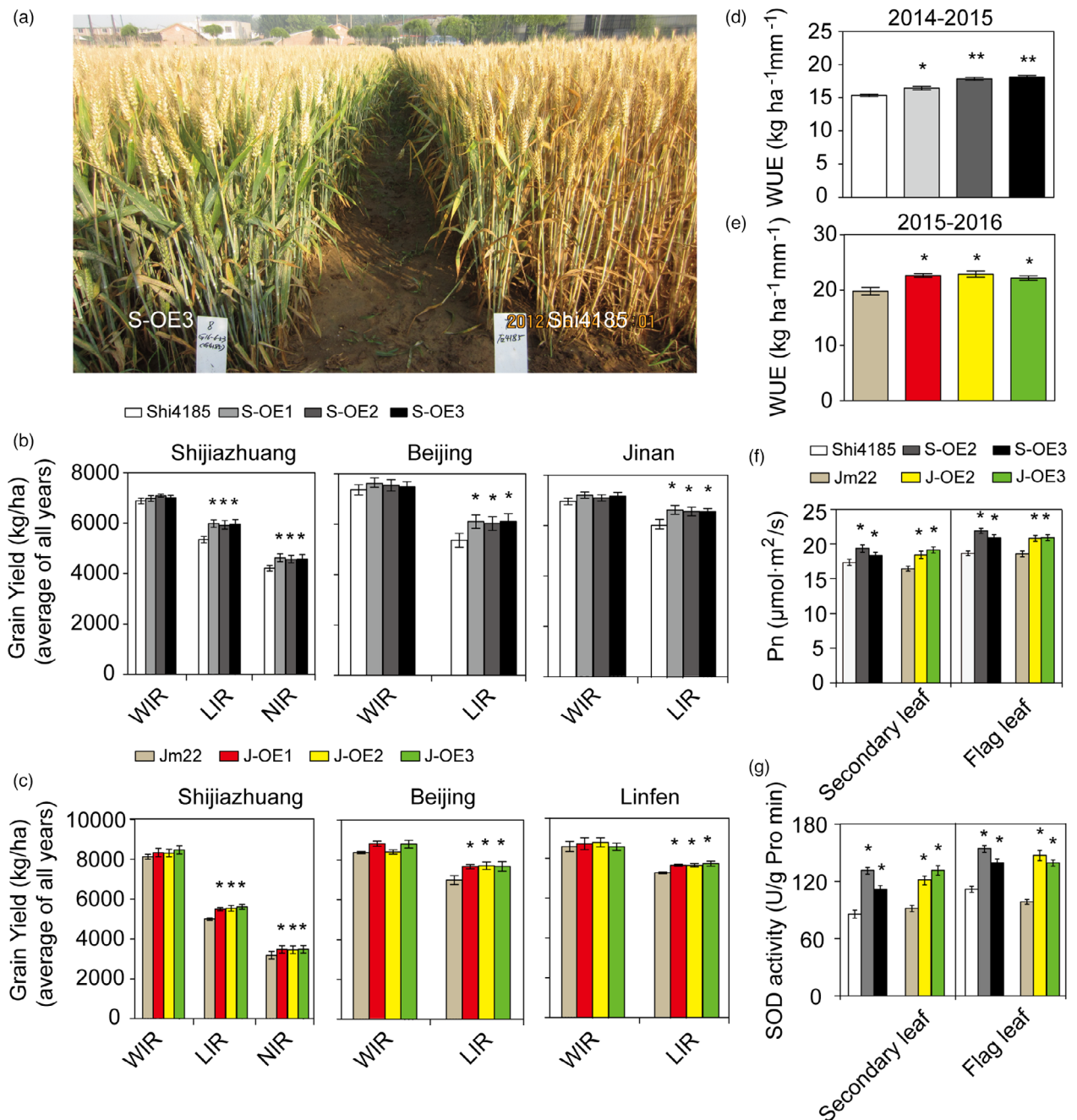


Figure 3 The effect of *GmTDN1* on grain yields in field experiments, and physiological characterization under drought stress field conditions. (a) Images of OE and Shi4185 plants during the wheat-growing season at the Shijiazhuang site under limited-irrigated conditions (LIR). (b) All year averages for grain yields of *GmTDN1* OE in the Shi4185 wheat background at all three locations. (c) All year averages for the grain yield of *GmTDN1* OE in the Jm22 wheat background at all three locations. (d) and (e) Water use efficiency (WUE) of the Shi4185 (d) and Jm22 (e) OE plants at the Shijiazhuang site. (f–g) Flag and secondary leaves were collected and examined for physiological characteristics under the LIR condition, Net photosynthesis rate (Pn) (f), SOD activity (g). * $P < 0.05$ and ** $P < 0.01$ between OE and WT plants assessed via Student's *t*-test (two-tailed). Values are represented by the mean \pm SD.

The yield was also measured under various conditions to evaluate differences in drought tolerance between transgenic wheat and WT wheat. The grain yield potentials of different transgenic lines in response to different water inputs, including rainfall and irrigation, were analysed via linear regression. Results showed that the slope of the regression curve of transgenic wheat was lower than that of WT plants, indicating that the sensitivity of OE lines to water input was reduced compared to that of WT, demonstrating that the OE lines had

advantages over WT plants across all evaluated environments (Figure S8). Some indices, such as high yield under both stressed and non-stressed conditions, may be useful for identifying drought tolerance in plants. A greater tolerance index level (TOL) was detected in WT plants (Shi4185 and Jm22), indicating that these genotypes suffer a larger grain yield (GY) reduction under stressed conditions and higher drought sensitivity compared to corresponding OE lines (Rosielle and Hambling, 1981) (Table S6). Similarly, the stress susceptibility index (SSI) was high in WT

plants and low in OE plants (Fischer, 1978). High values for indices, such as geometric mean productivity (GMP), mean productivity (MP), harmonic mean (HM), and stress tolerance index (STI), were observed in OE plants, whereas these values were low in WT plants (Fernandez, 1992; Jafari *et al.*, 2009; Rosielle and Hambling, 1981). Although these results indicated that OE lines may generally have high-stress tolerance relative to WT plants, differences were also noted between OE lines with distinct genetic backgrounds.

Biplots can be used to help identify superior genotypes associated with both stressed and non-stressed environments (Menezes *et al.*, 2014). Principal component analysis (PCA) revealed that the first two PCs, accounting for 99.99% of the total variation, were highly and positively correlated with STI and negatively correlated with TOL, respectively, thus clearly discriminating between all OE and WT wheat lines (Figure S9a). The PCA results of the OE Jm22 line revealed that the first PCA explained 98.48% of its total variation and, in addition to TOL and STI, other indices included Yp (yield under non-stress conditions), Ys (yield under stress conditions), HM, GMP, and MP, which are equally important for distinguishing between transgenic and WT lines (Figure S9b). The PCA results of the OE Shi4185 line revealed that the first PCA explained 75.70% of the total variation and that STI and TOL could discriminate between S-OE and WT wheat lines (Figure S9c).

Physiological analysis of the transgenic lines under drought conditions both in a rainout shelter and in the field

Physiological analyses were performed in a rainout shelter as well as in the field. Compared to WT plants, the OE lines showed significantly lower malondialdehyde (MDA) concentrations and relatively lower electrolyte leakage values, as well as higher proline contents, soluble protein contents, chlorophyll contents, and peroxidase (POD) activity in the flag leaves at heading and anthesis stages, during grain filling stages under drought conditions (Figure S10). No significant differences were detected between the physiological traits of the WT and *GmTDN1* OE wheat plants under normal growth conditions (Figure S10).

Exposure to drought stress after flowering exerts a greater effect on wheat yield (Guoth *et al.*, 2009; Shukla *et al.*, 2015). Therefore, the in-field physiological traits of WT and OE plants were analysed at the flowering stage during 2017–2018. Under LIR conditions, the OE lines had a significantly higher GA content in flag leaves (Figure S11), as well as higher photosynthetic capacity (higher photosynthetic rate (Pn) and yield of photosystem II (ΨPS II)), proline content, soluble sugar content, superoxide dismutase (SOD) activity, POD activity, and catalase (CAT) activity than those in WT plants (Figures 3f,g, and S12). We also detected that the malondialdehyde (MDA) content in the flag leaves as well as in the inverted second leaves of these plants was lower (Figure S12f). The expression of genes known to mediate the activity of protective enzymes and proline metabolism in plant cells (Dudziak *et al.*, 2019) were also analysed. Results show that overexpressing *GmTDN1* induced the expression of these genes in leaves (Figure S13). Collectively, the results from both the rainout shelter and in-field growth experiments indicated that wheat plants overexpressing *GmTDN1* exhibited improved drought tolerance, as well as increased photosynthetic capacity and osmotic adjustment capacity (higher content of proline and soluble sugar), and higher active antioxidant metabolism (higher activity of SOD, POD, and CAT and lower MDA content) compared to WT plants.

Overexpression of *GmTDN1* increases wheat grain yield under low-N stress in the field

Following the detection of more phenotypes showing enhanced NUE in the greenhouse, a variety of field trials were conducted (Figure 4a). The grain yields of transgenic lines were significantly increased under low-N conditions in the field, compared to those of WT plants. Field tests across two consecutive wheat-growing seasons (2015–2017) in Beijing showed that the grain yields of *GmTDN1* transgenic plants with a Jm22 background, which were subjected to low-N stress, were increased by 6.33–22.53% compared with those of WT plants ($n = 3$; $*P < 0.05$ or $**P < 0.01$; Figure 4b and Table S7). During the 2018–2019 growing season, the yield of the overexpression lines and WT were independently evaluated at Shijiazhuang in Hebei Province and Beijing. At both locations, transgenic lines invariably outperformed the yields of WT plants under low-N conditions, with more significant increases being observed in transgenic lines compared to Shi4185 and Jm22 WT plants at the Beijing site (–23.34% and –24.51% in 2018 and 2019, respectively; Table S8). The three major yield components were also analysed in more mature plants in Beijing in 2015–2016 and found that, under low-N conditions, the most obvious difference between the transgenic lines and the WT plants was the significant increase in spike number in transgenic lines. Increases in the grain number per spike and TGW depended on the type of transgenic line (Figure 4c–e and Table S9). NPK nutrient status, including NPK nutrients available in the soil before sowing and the NPK nutrients used to supplement the soil before sowing in two locations from 2015 to 2019, are shown (Table S10).

N-use-related traits following harvest were also analysed. Both the NUE (Figures 4f and S14) and grain N concentration (Figure 4g) of transgenic lines were higher than those of WT plants under low-N conditions. Conversely, no significant difference was detected in N concentration in the stem or leaf between transgenic and WT lines (Figure S15a,b). The field experiments also revealed that the transgenic expression of *GmTDN1* altered N distribution by allocating more N to grains (Figure 4h) under low-N conditions, as shown by transgenic lines that displayed lower leaf N distribution (Figure S15d) compared to those of WT plants, and no significant difference in stem N distribution (Figure S15c). These findings are similar to those previously reported for wheat expressing a glutamine synthase, *TaGS2* (Hu *et al.*, 2018).

To analyse the possible mechanism underlying the observed increases in grain yields, morphological changes were assessed, revealing that the most obvious difference between transgenic and WT plants was the greener canopies. WT leaves were yellowing under low-N conditions during the growing season (Figure 4a). The physiological traits of WT and transgenic plants were analysed at the seedling stage. The transgenic lines had significantly higher tiller numbers per m² and per plant, higher shoot and root fresh weights, and increased *Fv/Fm* values compared to WT plants under low-N conditions (Figure S16). The physiological characteristics associated with photosynthesis were analysed at the filling stage. The transgenic lines had significantly higher photosynthetic capacity (higher SPAD and *Fv/Fm* values) than WT plants under low-N conditions (Figure S15e,f).

Identification of direct *GmTDN1* protein targets when expressed ectopically in wheat

Considering that *GmTDN1* is a transcription factor, chromatin immunoprecipitation followed by sequencing (ChIP-Seq) analysis

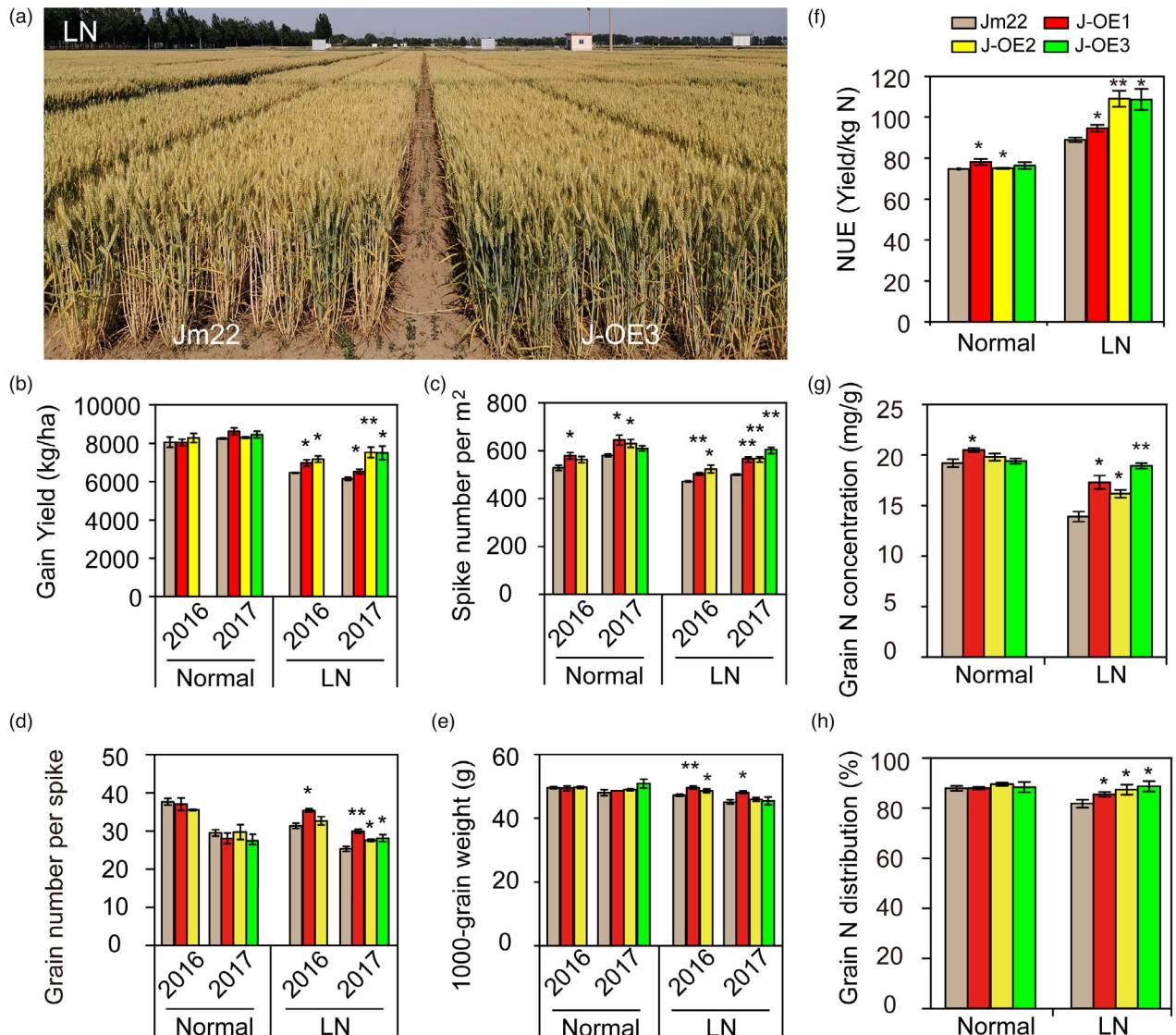


Figure 4 Grain yield and N use-related traits of the wild type and the *GmTDN1* OE lines in low nitrogen condition field experiments. (a) The images represent OE and Jm22 WT plants under low N conditions. (b) Grain yields for two consecutive years (2016–2017). (c) Spike number per m². (d) Grain number per spike. (e) 1000-grain weight. (f) NUE of lines and WT in field plots. (g) Grain N concentration. (h) Grain N distribution. NUE, grain yield/applied N fertilizer. **P* < 0.05 and ***P* < 0.01 between the transgenic line and the WT control assessed via Student's *t*-test (two-tailed). Values are represented by the mean ± SD.

was performed for Shi4185 transgenic plants and WT seedlings at the three-leaf stage (Figure 5a). The analysis identified 7208 regions showing significantly higher enrichment of *GmTDN1* binding in the genome (i.e., enrichment peaks with *P* < 0.001) of transgenic wheat plants compared to that of WT plants; Figure 5a and Table S11. Some possible downstream genes of *GmTDN1* were further screened via gene function annotation based on observed altered physiological traits and morphological changes in transgenic lines and confirmed by qPCR analysis. *GmTDN1* significantly induced the transcription of several genes known to mediate nitrate transport, plant morphology, GA synthesis, photosynthesis, and drought-related activities (Figure 5b). Transient luciferase reporter transcriptional activation assays were then used in tobacco (*Nicotiana benthamiana*) leaves to directly confirm the ability of *GmTDN1* to activate transcription. *GmTDN1*-GFP specifically activated the expression of the *Luc*

gene driven by the native promoters of *NRT2.5* and late embryogenesis abundant (*LEA*) (Figure 5c), both of which contain DRE/CBF elements.

To determine the function of the target gene *NRT2.5*, transgenic wheat lines G3 and G4 were developed, which overexpress *NRT2.5*. Drought-tolerant analysis showed that *NRT2.5* transgenic wheat had strong drought tolerance compared to WT, while the shoot fresh weight and survival rate of transgenic wheat were significantly higher than those of WT (Figure 6a–c). Chlorate, as a nitrate analogue, shared the same pathway as that of NO₃[−] absorption and assimilation, however, chlorate absorption is toxic to plants as it inhibits growth. Chlorate sensitivity simulated nitrate uptake or assimilation in wheat. WT was significantly more resistant to chlorate than *NRT2.5* transgenic wheat (Figure 6d), which showed higher shoot length, fresh weight, and survival rate

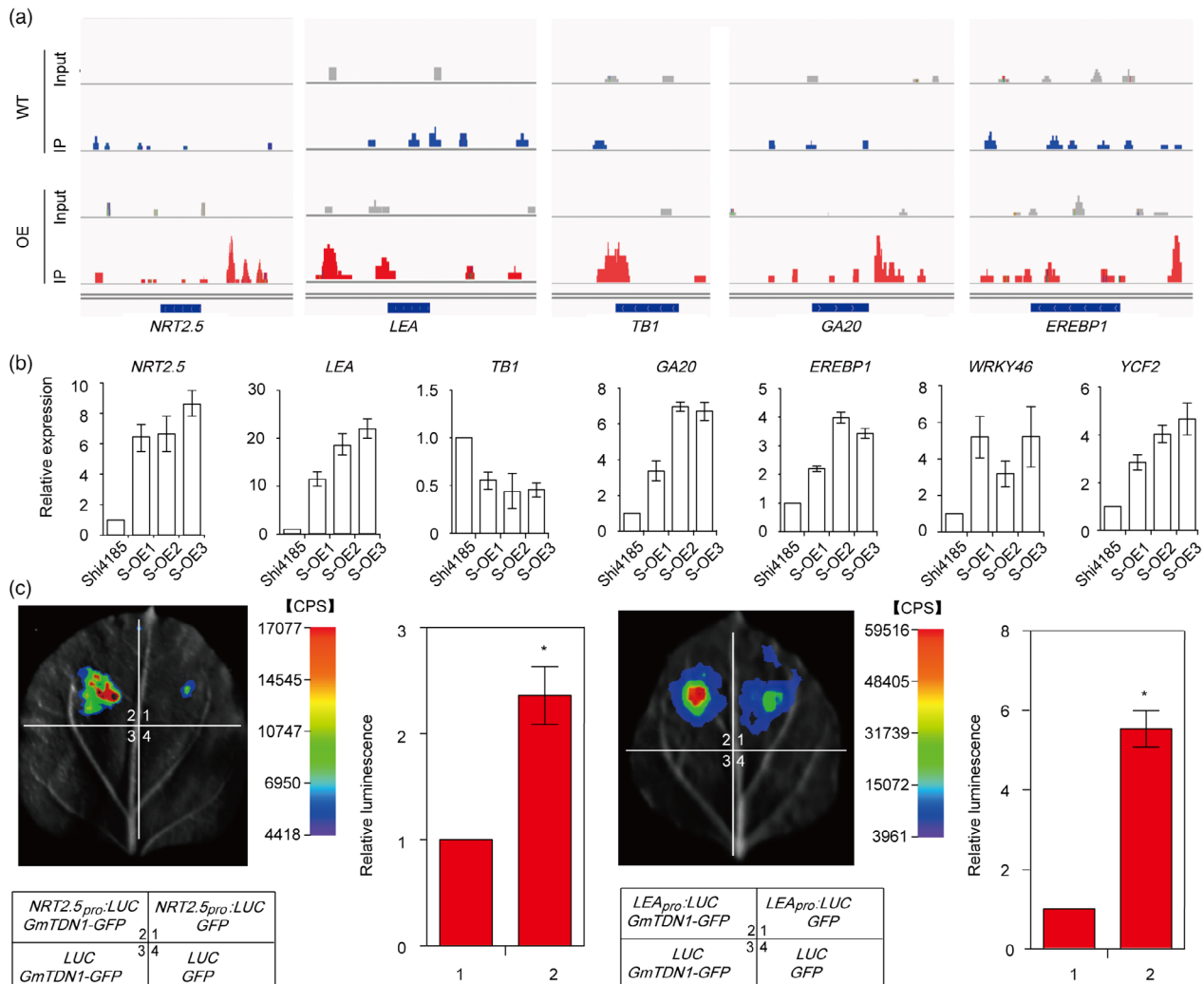


Figure 5 Identification of direct targets of transgenic GmTDN1 by ChIP-seq assay. (a) Binding GmTDN1 to promoters containing DRE cis-acting elements in GmTDN1 target genes. (b) The enrichment for occupancy of GmTDN1 at target gene loci detected in the ChIP-seq data was paired with qPCR-based analysis of potential increased transcription of these genes. (c) GmTDN1-GFP specifically activated the expression of the Luc gene as driven by the native promoters of *NRT2.5* and *LEA*.

(Figure 6e–g). These results indicated that *NRT2.5*, as a downstream target gene of GmTDN1, is involved in the promotion of nitrate absorption and assimilation under drought stress, which contributes to drought tolerance in wheat.

Our finding that transgenic plants possessed better photosynthetic capabilities (e.g., higher photosynthetic rates, SPAD values, and *Fv/Fm* values) motivated us to examine the chloroplast gene, *YCF2*, which was significantly upregulated in transgenic plants compared to WT plants (Figure 5b). Based on the observation of increased flag leaf total GA content (Figure S11) and no dwarfing phenotypes (Figure 3a), the expression of *GA20*, a gene involved in the GA signalling pathway, was then assessed. *GA20* was significantly upregulated in transgenic plants compared to WT plants (Figure 5b). Moreover, in light of these findings of increased tiller number and spike number/m² in yield composition element analysis under drought and low-N stresses, it became apparent that the ChIP-Seq included the *TB1* locus. The qPCR analysis indicated that *TB1* transcription in transgenic plants was significantly downregulated compared to that in WT plants

(Figure 5b). The function of GmTDN1 as a negative regulator of *TB1* in wheat is distinct from its transcription-enhancing activity. This data for *TB1* regulation by GmTDN1 offer a testable molecular mechanism hypothesis to explain the increase in tiller numbers observed in GmTDN1 OE wheat plants.

Discussion

In this study, wheat varieties were carefully selected for genetic transformation. Currently, Jm22, which shows high yield potential, is the most popular wheat variety in China, with an annual planting area of over 2 000 000 ha, whereas Shi4185 is a historically important drought-tolerant and water-preserving variety, albeit with a relatively low yield. The biplot results indicated that transgenic expression of *GmTDN1* in Jm22 OE lines was associated with improved drought tolerance and high grain yield potential (Figures 3c, S9 and Table S3). Furthermore, the Shi4185 OE lines exhibited both improved drought tolerance and increased yield potential (Figures 3b, S9 and Table S2). Yield potential increases were achieved by increasing tiller number and/or spike

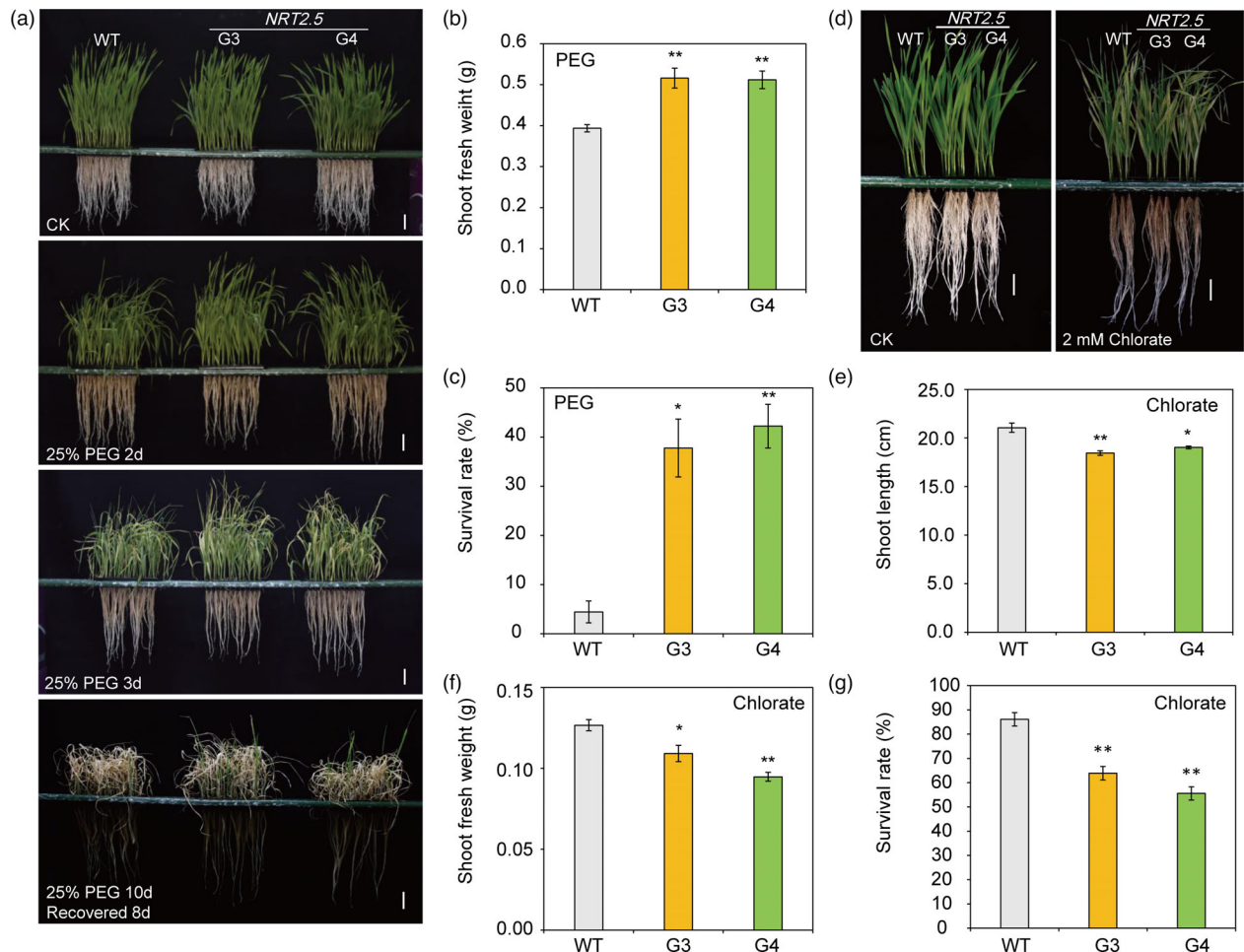


Figure 6 *NRT2.5* overexpression improved drought stress and nitrogen uptake in wheat. (a) Representative images of WT and overexpression line seedlings grown hydroponically with PEG stress. WT, G3, and G4 seedlings grown hydroponically for 3 days followed by treatment with or without 25% PEG6000 for an additional 10 days, and then recovered for 8 days. (b) Representative images showing the chlorate tolerance of WT and overexpression lines. The wheat plants were grown hydroponically with water for 3 days followed by transfer to Hoagland's liquid medium for 3 days, and then moved to the same hydroponic system supplied with 2 mM KClO_3^- for another 3 days. Wheat plants were cultured in Hoagland's liquid medium without KClO_3^- for 6 days were used as controls. The data represent the mean \pm SD from three independent experiments (at least 20 plants in each experiment). * $P < 0.05$, ** $P < 0.01$ (two-tailed Student's *t*-test).

number under both drought stress and normal conditions (Tables S4 and S5). The results of improving WUE of *GmTDN1* transgenic wheat were similar to previous findings of wheat ectopically expressing the sunflower transcription factor, *HaHB4* (Gonzalez et al., 2019). The current field experiments indicated that *GmTDN1* transgenic wheat maintained high grain yields and improved WUE or NUE under drought and low-N stress over many years compared to WT plants (Figures 3, 4 and Tables S2–S8). These results are important for ensuring wheat-related food security, especially in the arid and semi-arid areas of China. Drought is known to decrease N solubility and N utilization rates (Tezara et al., 1999), and drought and low-N stress often exist concomitantly in arid areas. Thus, the results of this study provide a useful candidate gene that may be utilized to implement new strategies that improve wheat drought tolerance and low N stress tolerance simultaneously. This work emphasizes the need to pay attention to crosstalk occurring between two stress regulation pathways rather than focusing narrowly on a single stress factor, such as either drought or low N. Moreover, this investigation of *GmTDN1* offers strong support to the recently emphasized notion

that a single gene that simultaneously confers benefits to multiple stress response traits may be used in crop improvement programmes without incurring yield penalties (Wang et al., 2018).

To determine whether other members of the A5 subgroup belonging to the DREB family respond to drought and low N stress in wheat and soybean, the expression patterns of A5 subgroup members were analysed in different wheat plants including the drought-tolerant wheat variety Hanxuan10, drought-sensitive wheat variety Chinese Spring, *GmTDN1* transgenic lines, and two WT wheat varieties under drought and low-N conditions. The results showed that certain A5 subgroup members (TraesCS3A02G313900 and TraesCS3B02G158000) in wheat responded to both drought and low-N conditions to varying degrees while having a different expression pattern (Figures S17 and S18). In addition, the expression patterns of A5 subgroup members, including *GmTDN1* (GLYMA17G047300), were analysed in drought-tolerant soybean Jindu21 and drought-sensitive soybean Zhonghuang35 under drought and low-N conditions, respectively. The results showed that *GmTDN1* was only induced in drought-tolerant soybean Jindu21 by both

stresses, while other members responded to single stress (Figure S19). These results showed that A5 subgroup members may play an important role in the simultaneous regulation of crop tolerance to drought and low N stress. In addition, the degree, and the number of A5 subgroup members in response to the two stresses in wheat were higher than that in soybean, which might be related to the stronger stress tolerance of wheat than soybean.

Previous research has established that *DREB1A/CBF*-like transcription factors affect drought-related physiological processes (Maruyama *et al.*, 2009). However, the downstream network of *GmTDN1*, when ectopically expressed in wheat, was distinct from that of other *DREB1A/CBF*-like transcription factors. The most interesting result in this respect is the finding that *GmTDN1* activates the transcription of the nitrate transporter gene *NRT2.5* (Lezhneva *et al.*, 2014). Furthermore, this study findings indicated that *GmTDN1* positively regulates GA synthesis by activating the transcription of *GA20ox3* (Qin *et al.*, 2013) (Figures 5b and S11), which clarifies the absence of a plant dwarfing phenotype among the *GmTDN1* transgenic wheat plants, whereas such a phenotype has been reported in *DREB1A/CBF* transgenic wheat plants. These differences between growth-related effects resulting from the overexpression of *GmTDN1* and *DREB1A/CBF* in wheat may be associated with these genes arising from different *DREB* subgroups. *GmTDN1* is of the A5 subgroup (Chen *et al.*, 2009), whereas most characterized *DREB1A/CBF* genes are of the A1 subgroup (Agarwal *et al.*, 2017) of the *DREB* family. In addition, overexpression of *GmDREB1*, another member of the soybean A5 subgroup of the AP2/ERF family, did not affect the normal growth of transgenic wheat (Zhou *et al.*, 2020). Certain cases are showing that overexpression of other AP2/ERF members did not cause plant dwarfing (Jisha *et al.*, 2015).

Our ChIP results showed that *Ycf2* is a direct downstream gene of *GmTDN1* in transgenic wheat (Figure 5b). *Ycfs* encode a chloroplast protein that is only expressed in response to limited water availability; *Ycf1* and *Ycf2* are involved in maintaining proper CO₂ assimilation via a high photosynthesis rate under limited conditions (Ruiz-Nieto *et al.*, 2015). The ChIP finding of *GmTDN1* binding to the *Ycf2* locus as well as the qPCR analysis showing that the *GmTDN1* overexpressing plants displayed higher *Ycf2* transcription than WT plants are per our observation that *GmTDN1* transgenic wheat has higher photosynthetic efficiency under drought conditions (Figure 3f). Whether this physiological characteristic is directly related to the function of the *Ycfs* product is unclear and requires further study. Moreover, *GmTDN1* was found to bind to, and promote transcriptional activation of some known drought-related genes, such as an AP2/ERF-type transcription factor *EREBP1* which regulate important functions of plant growth and development as well as responses to environmental stimuli (Jisha *et al.*, 2015), WRKY transcription factor *WRKY46* is vital in improving drought tolerance through ABA-dependent and ABA-independent pathways (Li *et al.*, 2020); *LEA* genes also play important roles in plant growth and development, especially the cellular dehydration tolerance (Liu *et al.*, 2019) (Figures S5 and S7).

GmTDN1 was found to bind to the promoter of *TB1* (Figure 5), a transcription factor that regulates the strigolactone pathway (Guo *et al.*, 2013), and downregulates its expression. This putative negative regulatory function of *GmTDN1* binding, ostensibly at the DRE of the *TB1* promoter, apparently opposes the transcriptional activation function of *GmTDN1* that was observed in relation to *GA20*, *Ycf2*, and *LEA*, among others. Such

seemingly simultaneous positive/negative activities have been reported for other TFs, including *AtYY1* (Li *et al.*, 2016b) and *AtWRKY53* (Zheng *et al.*, 2019). In such cases, the apparent paradox was resolved by the subsequent discovery of distinct regulatory protein recruitment functions of the TFs in question. Co-immunoprecipitation experiments with *GmTDN1* and protein samples from wheat may help identify the various interacting partners, which help mediate the effect exerted by *GmTDN1*'s on transcription. The increase in *GmTDN1* transgenic wheat yield under drought was found to be primarily due to the increase in spike number (Tables S4, S5, S9). This is substantiated by the fact that *TB1* is known to negatively regulate plant branching (Guo *et al.*, 2013). This phenotype seems to be consistent with the function of *TB1*, suggesting that *GmTDN1* may affect the tillering of OE wheat by regulating the strigolactones pathway. Further studies are needed to investigate the relationship between *TB1* promoter binding, *TB1* transcription levels, strigolactone biosynthesis, and signalling, and the tillering number in *GmTDN1* transgenic wheat.

Our work shows that, upon its ectopic overexpression in wheat, *GmTDN1* simultaneously regulates plant tolerance to both drought and low-N stress. In this study, overexpression of *NRT2.5* can improve drought tolerance and N absorption efficiency of transgenic wheat (Figure 6). In addition, results of pots experiments under the double stress treatment of drought and low N indicated that the difference in survival rate between *GmTDN1* transgenic wheat and WT under low N plus drought conditions is greater than that under normal plus drought conditions (Figures 2 and S6), suggesting that *GmTDN1* further increased N absorption and utilization compared to WT under low-N conditions, which contributed to drought tolerance of transgenic wheat. These results suggest that the *GmTDN1*-*NRT2.5* regulatory module might be involved in the promotion of nitrate absorption and assimilation under drought stress, which contributes to drought tolerance. Other studies have shown that drought tolerance is associated with the uptake and utilization of N in plants at the molecular level. A transcription regulation network analysis showed that 21 core transcription factors were involved in N-associated metabolism and growth in *Arabidopsis*. Among these, two *DREB*-like genes, *DREB2A* and *DREB26*, were found to affect N assimilation (Gaudinier *et al.*, 2018). The high-affinity nitrate transporter *NRT2.1* positively regulates water transport capacity of roots, which, in turn, is associated with the regulation of transcriptional and translational levels of plasma membrane aquaporins, thereby revealing a link between *NRT2.1* expression, NO₃⁻ use, and water stress, which suggests that *NRT2.1* plays a functional role in drought tolerance (Li *et al.*, 2016a). Conversely, exposure to salt and osmotic stresses for 24 h downregulated the expression of *NRT1.1*, *NRT2.1*, and *NRT1.5* in *Brassica juncea* and *Arabidopsis* (Goel and Singh, 2015; Taochy *et al.*, 2015). The ABA pathway regulates N uptake through the nitrate transporter *TaNRT2s* in wheat (Wang *et al.*, 2020). These studies indicated the presence of an interaction between drought stress response and low-N stress response at the molecular level. To counter simultaneous drought and low-N stress environments, plants have evolved certain regulatory pathways that simultaneously regulate their responses to low-N and drought stresses. Deepening the current understanding of the functioning of proteins, such as *GmTDN1*, will enable us to better regulate these response pathways, resulting in significant improvements in the environmental adaptability of wheat and other crops (Figure 7).

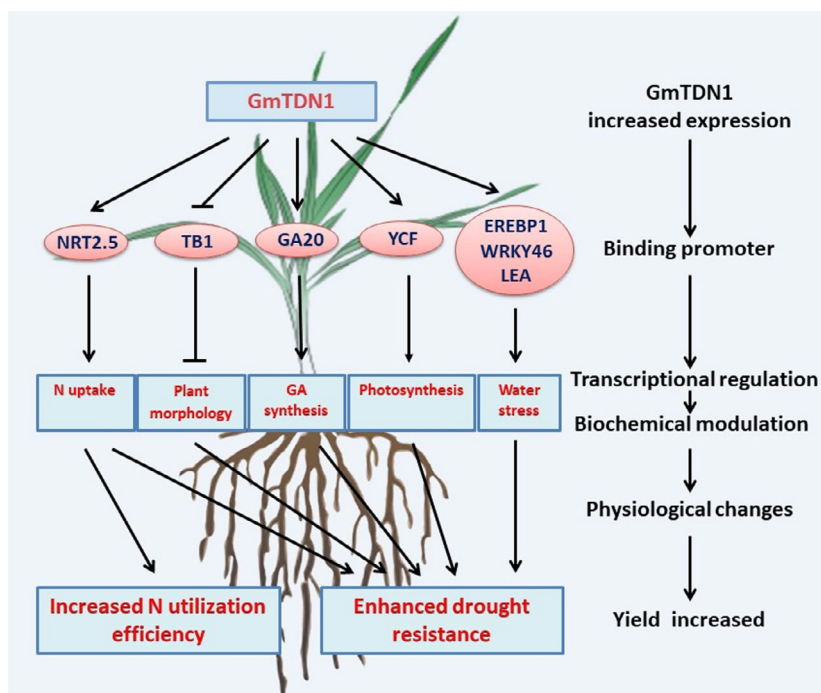


Figure 7 Proposed model for GmTDN1 action in wheat. GmTDN1 binds to and promotes the transcription of target genes (in blue), with downstream impacts on biochemical and physiological processes including N uptake, plant morphology, GA synthesis, photosynthesis, and water stress (in red), leading to enhancement of drought tolerance, N utilization efficiency, and increased grain yield.

Experimental procedures

Hierarchical analysis

A hierarchical analysis of the AP2/ERF family was performed by downloading the protein sequences of wheat, soybean, and *Arabidopsis* AP2 transcription factors (Pfam Identifier PF00847) from the Ensemble Plants database (release 45, <http://sep2019-plants.ensembl.org/index.html>). ClustalW was used to align sequences (Larkin *et al.*, 2007). The DREB-A5 subfamilies in the AP2/ERF family were further classified in *Arabidopsis*, soybean, and wheat (Figure S1) (Nakano *et al.*, 2006).

Wheat materials and genetic transformation

Two common commercial winter wheat (*T. aestivum*) varieties, Shi4185 and Jimai22 (Jm22), were used as transformed genotypes. GmTDN1 (GenBank accession no. DQ208969) from *G. max* was inserted into the pSHGmTDN1 plasmid vector under the control of the ubiquitin (Ubi) promoter, following which the circular plasmids were digested with *KpnI* and *SpeI*; the linear minimal expression cassette-only containing the promoter (Ubi), target gene (GmTDN1), and terminator (Nos) was transformed into immature embryos of Shi4185 and Jimai22 wheat (Figure S20) using particle bombardment (Xu *et al.*, 2007). The *bar* herbicide-resistance gene was used as the plant selection marker gene and co-transformed into immature wheat embryos. After a two-round selection using an herbicide, 130 regenerated plantlets occurred on 1450 calli of Shi4185, and 110 regenerated plantlets occurred on 1600 calli of Jimai22. Genomic DNA was extracted using the CTAB method (Murray and Thompson, 1980). The T0 to T3 generations of the transgenic plants were tracked using PCR-based genotyping with primer 1; 100 random samples from T4

generation in the field were detected with primer 2. Primers specific for *GmTDN1* are listed in Table S12 (*GmTDN1*-F, *Nos*-R form the terminator). *GmTDN1* copy number was examined via Southern blotting (Southern, 1975) using two endonucleases, *KpnI* and *EcoRV* (Figure S20) to generate a single cut point in the linear minimal expression cassette for the transgenic Jm22 lines. After drought tolerance screening, T4 transgenic Shi4185 lines S-OE1, S-OE2, and S-OE3, as well as transgenic Jm22 lines J-OE1, J-OE2, and J-OE3 with strong drought tolerance were selected for further study in the greenhouse. The transcript levels of *GmTDN1* in the T4 generation field plant of the transgenic lines leaves were measured using semi-quantitative RT-PCR (Figure S3e) (Shafi *et al.*, 2015), with the primers listed in Table S12.

Wheat growth in greenhouse

T4 generation overexpressing *GmTDN1* lines (S-OE1-3 and J-OE1-3) and WT (Shi4185 and Jimai22), and overexpressing *NRT2.5* lines (G3 and G4) and WT (Zhengmai7698) were used for further analysis. Seeds were soaked in water for 24 h at 4°C, and germinated for 48 h at room temperature (20 °C). Greenhouse growth condition was a 16:8 L:D photoperiod at 20 ± 2 °C, 50–70% relative humidity, and a photon fluence rate of 300 μmol photons m⁻² s⁻¹.

For mimic pot experiments, the most uniformly germinated seeds were sown in boxes (65 × 35 × 15 cm) filled with mixed soil (nutrient soil and vermiculite in 1:1 ratio; Figure 1a). The overexpressed lines and WT seeds were planted in a box with three rows for each line. Each experiment was repeated thrice. Soil moisture was maintained at levels adequate for normal growth (75–80%) and relative soil water content was calculated using the Drying and Weighing Method (Boyer *et al.*, 2008).

When the third leaf became visible, watering was stopped for 21 days until the relative soil water content reached 40–45%, and then rehydrated for 7 days until a relative soil water content of 75–80% was reached. Water was also stopped for 28 days until the soil moisture reached <30% and then rehydrated for 7 days until a relative soil water content of 75–80% was reached. For the controls, plants were grown with the relative soil water content maintained at 75–80%. The degree of leaf curling was observed after cessation of watering for 7 days and determined the survival rates of all lines after cessation of watering for 21 or 28 days, and rehydration for 7 days. The water loss rates of leaves from the plants were determined in relation to untreated control leaves at the same treatment time point, as previously described (Yu *et al.*, 2017). Twenty plants of each line in each repetition were randomly collected to measure and analyse. In addition, drought stress experiments were performed in normal and N-deficient field soil (Figures 2 and S6). Briefly, seedlings grown for 7 days were subjected to drought stress for 21 or 28 days; almost all WT plants had died and then were allowed to recover for 7 days.

For the hydroponic culture experiment of the low-N tolerance, a 96-well plate with the bottom removed was used to sow the most uniformly germinated seeds and then floated on water in a greenhouse. Three days later, the seedlings were cultured in Hoagland's liquid medium with different nitrate concentrations. The culture solution used for these hydroponic culture experiments has been previously described (Qu *et al.*, 2015; Ren *et al.*, 2012), and contained $\text{Ca}(\text{NO}_3)_2$ (2 mM for normal N treatment and 0.2 mM for low-N treatment, respectively). The nutrient solution was refreshed every day and its pH was adjusted to 6.0 with dilute HCl and KOH before refreshing. Three replicates were performed for each treatment. After 21 days, morphological parameters and total plant N concentrations of 20 randomly selected plants were measured. Root morphological analyses were conducted via computerized scanning with Delta-T Scan (Delta-T Devices Ltd., UK) and WinRHIZO (Regent Instrument Inc., Ottawa, ON, Canada).

For polyethylene glycol (PEG) treatment, seedlings in the 96-well plate with the bottom removed were first cultured in water for 3 days and moved to Hoagland's liquid medium in a greenhouse. Three days later, three-leaf stage seedlings were treated with 25% PEG6000 for 10 days and rewatered 8 days. Drought tolerant was viewed at different stages.

For chlorate sensitivity assays, seedlings in the 96-well plate with the bottom removed were first cultured in water for 3 days and moved to Hoagland's liquid medium. Seven days later, three-leaf stage seedlings were treated with 2 mM KClO_3^- for 3 days. The seedlings were cultivated in the greenhouse. Chlorate sensitivity was viewed after KClO_3^- treatment for 2 days.

Measurements of root nitrate flux rate

WT and transgenic line plants wheat seedlings (7 days after germination) were grown for 7 days in a nutrient solution that contained 0.2 mM nitrate was used to measure net nitrate fluxes. The roots of these plants were then transferred to a measuring solution containing 0.1 mM of NH_4KNO_3 , 0.1 mM of KCL, 0.1 mM of CaCl_2 , and 0.3 mM of MES (pH6.0), and allowed to balance for 10 min, then the maximum nitrate flux rates along the root were recorded using an NMT (MA01002; Younger USA LLC), at Xuyue BioFunction Institute in Beijing as previously described (He *et al.*, 2015).

Field experiments

Large-scale field drought-tolerance field trials: field experiments were conducted for five consecutive years (2012–2017) in three major wheat-growing areas in the North China Plain (NCP) as follows: Shijiazhuang in Hebei Province (37°24'11"N, 114°25'53"E); Beijing (40°13'49"N, 116°33'28"E); Jinan in Shandong Province (36°42'13"N, 117°04'23"E); and Linfen in Shanxi Province (36°14'48"N, 111°58'09"E). The three drought conditions involved (i) no irrigation (NIR) from sowing to harvest, (ii) limited irrigation (LIR) with only one irrigation being applied at jointing stage, and (iii) ample irrigation/well irrigated (WIR) with one irrigation applied at the jointing stage and additional irrigation applied at the grain filling stage. Each experiment was laid out in a randomized block (12 m²/plot) design with three replicates. A precipitation survey indicated that rainfall in the four NCP regions of the current experiment was very limited during the wheat-growing season (Figure S17). To conduct these field experiments, *GmTDN1* transgenic and WT plants were sown in October each year (2012–2016) and harvested in the following June (2013–2017). Details of the experimental plots are provided in the Supplemental Methods. Three different watering schedules were applied via furrow irrigation following previous field research protocols for drought stress conditions (Reza *et al.*, 2009). Details are provided in the Supplemental Methods. Other crop management practices were consistent with local cultivation practices used for wheat varieties in the field. The yield was calculated using the actual grain weight of each plot via conversion of the plot area. The major agronomic traits of transgenic wheat and WT were investigated based on 20 randomly sampled plants from the plots, including grain number per spike and TGW. Spike number in two 1-m long rows of each plot was recorded. Grain WUE (kg ha⁻¹ mm⁻¹) was defined as per Zhou *et al.* (2020).

Drought tolerance of transgenic wheat was evaluated under various environmental conditions according to several indices (Supplementary methods). Biplot display was performed using the R Statistical Software.

Low-N-tolerant field trials: Two consecutive field experiments were conducted at the ShunYi experimental station of the Institute of Crop Sciences of the Chinese Academy of Agricultural Sciences (40°13'49"N, 116°33'28"E) in Beijing in 2015–2017. Again, two field experiments were conducted during the 2018–2019 growing season in two locations: Beijing and Shijiazhuang (at the Dishang Experimental Station, Institute of Grain and Oil Crops, Hebei Academy of Agriculture and Forestry Sciences; 37°55'58"N, 114°43'30"E). All field experiments contained two treatments, each replicated thrice. The nutrient content in the soil and fertilized fields are shown in Table S9. Both treatments were treated with 7.5 g/m² P as calcium superphosphate at the Beijing site, and 8 g/m² P and 10 g/m² K as potassium sulphate at the Shijiazhuang site. At the Beijing site, the plot size for each genotype in each replicate was 6 m² (4 m × 1.5 m), which included six rows spaced 25 cm apart; the sowing density was 270 germinating seeds per m² during 2015–2016 and 2018–2019, and 330 germinating seeds per m² during 2016–2017. At the Shijiazhuang site, the plot size was 12 m² (8 m × 1.5 m) and each plot included eight rows spaced 20 cm apart, with a sowing density of 270 germinating seeds per m². At maturity, 20 plants in each plot were randomly sampled to measure total N concentration, grain number per spike, and TGW. Spike numbers

in two 1-m long rows of each plot were recorded. The yield was calculated using the actual grain weight of each plot via conversion of the plot area.

Measurement of total N concentration

The total N concentration in plant samples, including leaves, stems, and grains was measured at the Beijing site during the 2016–2017 growing season. Plant samples were dried at 80°C before measuring dry weight. Dried samples were milled and subsequently digested using concentrated H₂SO₄ to determine total N using the semi-automated Kjeldahl method (TecatorKjeltec Auto 1030 Analyzer; Tecator, Hoganas, Sweden).

Measurements of drought-related physiological characteristics in the field, and under rainproof shelters

Following the flowering stage in the field, 10 individual plants from each of the transgenic and WT lines were randomly sampled for each of two irrigation treatments (WIR and LIR) at the Shijiazhuang location. All indexes for transgenic and WT plants were determined using flag leaves and secondary leaves.

To conduct rainproof shelter experiments, post-emergent seedlings were transferred into square boxes (1.2 × 0.6 × 0.6 m) filled with surface soil in the field, each with 12 rows spaced 10 cm apart and 30 seeds sown per row. Pot experiments were divided into two groups: (i) drought treatment, in which the plants of OE and WT lines were subjected to drought treatment (relative soil water content of 40–45%) at either the heading stage, anthesis, or grain filling stage; and (ii) normal treatment, in which plants of transgenic and WT lines were provided with an adequate water supply (relative soil water content of 75–80%) at all stages. All physiological characteristics of the transgenic and WT plants were determined using flag leaves following soil water measurements.

Each sample was frozen immediately with liquid nitrogen in the field or rainproof shelter and transferred to the laboratory on dry ice. For physiological characteristics analysis, the samples were ground with a mortar and pestle in liquid nitrogen, after which the powdered sample was immediately aliquoted into 2-mL pre-chilled bullet tubes and stored frozen until analysis. For GA measurements for plants overexpressing *GmTDN1*, the flag leaves of plants that had just begun to flower were collected, the powders (approximately 20 g) of lyophilized plant materials were extracted with 300 mL of pre-cooled aqueous methanol (80% MeOH [vol/vol]) for each measurement. Endogenous GA extract and quantitative analysis were performed via high-pressure liquid chromatography as described previously (Varbanova *et al.*, 2007; Zhang *et al.*, 2012). MDA content as the level of lipid peroxidation in the plant was determined as previously described (Jan *et al.*, 2017). Approximately 0.1 g of a wheat leaf was used and absorbance values at 450, 532, and 600 nm were determined with a spectrometer (Perkin-Elmer Lambda 25, Boston, MA). Total soluble protein was measured using Coomassie Brilliant Blue according to standard methods (Bradford, 1976). The proline content of fresh leaves was determined as previously described (Hosseini *et al.*, 2017). Wheat leaf samples (~0.1 g) for the measurement of proline were treated with 3% (w/v) sulphosalicylic acid and boiled for 1 h. The amount of proline was measured with ninhydrin and detected at 520 nm; a proline standard liquid was used as a reference. The activities of SOD, POD, and CAT in the leaves were assessed using enzyme activity analysis kits (KEMINGBIOTECH, Jiangsu, China) according to the manufacturer's protocol. Soluble sugars were determined via

colorimetric hydrolysing assay (anthrone procedure), as previously described (Maness, 2010).

From 10 randomly selected plants' fresh flag leaves, relative electrolyte leakage in fresh leaves was measured based on the relative conductivity of the leaves as previously described (Zhang and Huang, 2010). The total chlorophyll content of fresh, fully expanded leaves was determined according to previous methods (Hosseini *et al.*, 2017). The flag leaf net photosynthetic rate (Pn) and Ψ PSII were measured using an LI-COR 6400XT photosynthesis system in full sunlight at 09:00–11:00 on clear days (Lang *et al.*, 2018). The SPAD values of flag leaves were determined using a SPAD-502 chlorophyll meter (Japan, KONICA MINOLTA). Chlorophyll fluorescence from leaf tissue was measured using a PocketPea Fluorometer (Hansatech®, Norfolk, UK). The ratio of variable fluorescence to maximal fluorescence (*Fv/Fm*) was used to assess the maximum quantum efficiency of PSII in dark-adapted plants for 20 min, following manufacturer settings at 09:00–10:00 and 14:00–15:00 on a clear day. All values were measured from the middle of the leaf.

Transcript analysis

Total RNA was extracted from leaves of wheat and leaves and roots of soybean using a Trizol kit (Takara, Dalian, China) according to the manufacturer's instructions. For each sample, 4 µg of total RNA was digested in a 25 µL with Turbo DNA-free DNaseI to remove genomic DNA contamination (Takara, Dalian, China). First-strand cDNA was synthesized using 1 µg of DNaseI-treated RNA with the PrimeScript First-Strand cDNA Synthesis kit (Takara) in a 20-µL reaction volume according to the manufacturer's protocol. All samples were brought to 100 µL in volume with approximately 10 ng of cDNA per microlitre.

For RT-PCR analysis of the OE plants, PCR was performed with 1 µL of the first-strand cDNA reaction and amplification reaction mixtures contained 0.2 mM of each deoxyribonucleotide triphosphate, 1.2 mM of MgSO₄, and 0.4 mM of each primer. Specific forward and reverse primers for *GmTDN1* were 5'-CATGACGCCGTCGAGAT-3' and 5'-CCGTTACTGAACCGTTAAGAGTG-3', respectively, and *TaActin* as an internal reference, were 5'-CTCCCTCACAACAACCGC-3' and 5'-TACCAGGAAGTTCATACCAAC-3', respectively. PCR amplification was performed in a DNA thermal cycler (Bio-Rad, USA), with the following conditions: 95 °C for 3 min, 45 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s, followed by 4 °C forever. The PCR products were fractionated on a 1.2% (w/v) agarose gel and visualized via ethidium bromide staining.

For qRT-PCR analysis, 1 µL cDNA was used in a 20 µL of qPCR reaction volume using SYBR Green PCR MasterMix (TIANGEN-BIOTECH, Beijing, China) according to the manufacturer's instructions. Specific forward and reverse primers were designed for genes shown in Table S11, amplification of the *TaActin* (TraesCS1B02G283900.1) using the forward primer 5'-CTCCCTCACAACAACCGC-3' and the reverse primer 5'-TACCAGGAAGTTCATACCAAC-3', the *Tatubulin* (U76558) using the forward primer 5'-CATGCRATCCRCGTCTCGACCT-3' and the reverse primer 5'-CGCACTTCATGATGGAGTTGTAT-3' were used as the internal control. Specific forward and reverse primers for *GmTDN1* in soybean were 5'-CATGACGCCGTCGAGAT-3' and 5'-CCGTTACTGAACCGTTAAGAGTG-3', respectively. Amplification of the *GmActin* gene (U60506) using the forward primer 5'-ACATTGTTCTTAGTGGTGGCT-3' and the reverse primer 5'-CTGTTGGAAGGTGCTGAG-3' was used as the internal control. Primer sets of 0.4 µM final concentrations for

each primer were used in a final volume of 20 μL well⁻¹. Quantitative real-time PCR was performed in 96-well plates on an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA) as previously described (Le *et al.*, 2011) following the manufacturer's instructions. Three biological replications for each line were performed in each test. Relative transcript levels of genes were calculated using the $2^{-\Delta\Delta C_T}$ method (Li *et al.*, 2011). Amplicon-based fluorescence thresholds were used to obtain the C_T values, which were used, together with the amplicon-based mean efficiency, to calculate the initial quantity of mRNA transcripts. Finally, the mRNA levels of each transcript were normalized with those of the corresponding reference gene transcript.

Chromatin immunoprecipitation and sequencing (ChIP-Seq)

Chromatin immunoprecipitation (ChIP) was performed in duplicate on the youngest fully expanded leaf from WT Shi4185 and OE line S-OE2 plants using an anti-GmTDN1 antibody (Youlong Biotech, Shanghai China). ChIP without antibody was included as a control for each sample. These assays were performed as previously described (Bowler *et al.*, 2004; Wamstad *et al.*, 2012). Construction of sequencing libraries, sequencing, and basic data analyses was conducted by Shanghai Cloud-seq Biotech Co. Ltd (Shanghai, China). In the ChIP-Seq experiment, DNA fragments associated with GmTDN1 protein are enriched using GmTDN1 specific antibody, and all enriched DNA fragments were sequenced using the Illumina instrument following the manufacturer's instructions. Based on sequence results, all peaks were annotated by their nearest gene. The candidate downstream genes could be further verified using qRT-PCR and ChIP-qPCR. The primers used in qRT-PCR and ChIP-qPCR are provided in Table S12.

Transient luciferase expression assays

The leaf ChIP-Seq data were screened for potential direct target genes (*i.e.*, loci potentially directly bound by the GmTDN1 transcription factor) that contain a DRE (DNA binding element of DREB-like transcription factors) within 2 kb upstream of the coding sequence (<https://www.dna.affrc.go.jp/PLACE/>). Through gene function annotation and prediction of DREs in the DNA enrichment region, two genes, *NRT2.5* (high-affinity nitrate transporter 2.5, TraesCS6A01G032900) and *LEA* TraesCS1A01G125700, were identified as putative direct target genes. The reporter constructs *Pro LEA::LUC* and *Pro NRT2.5::LUC* and the effector construct *GmTDN1-GFP* were used to complete the Luciferase Transient Expression Assays according to a previous method (Cheng *et al.*, 2018).

Statistical analysis

Significant differences between the yields and physiological characteristics of WT and GmTDN1 samples were determined via two-tailed Student's *t*-tests. Error bars represent SD in all figures and tables. For each treatment, the SD of the mean was calculated based on at least three biological replicates.

Acknowledgements

This research was financially supported by the National Key R & D Program of China (2020YFE0202300), the National Key Research and Development Program of China, the Agricultural Science and Technology Innovation Program (CAAS-ZDRW202109 and CAAS-ZDRW202002), and the Central Public-interest Scientific Institution Basal Research Fund.

We would like to thank Editage (www.editage.cn) for English language editing.

Conflict of interest

The authors declare no competing interests.

Author contributions

Y.M. and M.C. conceived this project and designed the experiments. Y.Z. performed most of the experimental work. J.L. and L.M. contributed to ChIP data analysis. J.G., Y.W., H.J., X.C. X.Q., L.H., H.L., M.H. and X.B. contributed to field experimental work. K.X., J.Y., and H.Y. contributed to physiological traits and gene expression analysis. W.T. contributed to principal component analysis. J.C. and Z.X. provided experimental suggestions. Y.Z. wrote the manuscript. Y.M. and M.C. revised the manuscript. All authors read and approved the final manuscript.

References

- Agarwal, P.K., Gupta, K., Lopato, S. and Agarwal, P. (2017) Dehydration responsive element binding transcription factors and their applications for the engineering of stress tolerance. *J. Exp. Bot.* **68**, 2135–2148.
- Araus, J.L., Serret, M.D. and Lopes, M.S. (2019) Transgenic solutions to increase yield and stability in wheat: shining hope or flash in the pan? *J. Exp. Bot.* **70**, 1419–1424.
- Araus, V., Swift, J., Alvarez, J.M., Henry, A. and Coruzzi, G.M. (2020) A balancing act: how plants integrate nitrogen and water signals. *J. Exp. Bot.* **71**, 4442–4451.
- Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M. and Paszkowski, J. (2004) Chromatin techniques for plant cells. *Plant J.* **39**, 776–789.
- Boyer, J.S., James, R.A., Munns, R., Condon, T.A.G. and Passioura, J.B. (2008) Osmotic adjustment leads to anomalously low estimates of relative water content in wheat and barley. *Funct. Plant Biol.* **35**, 1172–1182.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Chen, M., Xu, Z.S., Xia, L.Q., Li, L.C., Cheng, X.G., Dong, J.H., Wang, Q.Y. *et al.* (2009) Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, GmDREB3, in soybean (*Glycine max* L.). *J. Exp. Bot.* **60**, 121–135.
- Cheng, X., Zhang, S., Tao, W., Zhang, X., Liu, J., Sun, J., Zhang, H. *et al.* (2018) INDETERMINATE SPIKELET1 recruits histone deacetylase and a transcriptional repression complex to regulate rice salt tolerance. *Plant Physiol.* **178**, 824–837.
- Dudziak, K., Zapalska, M., Borner, A., Szczerba, H., Kowalczyk, K. and Nowak, M. (2019) Analysis of wheat gene expression related to the oxidative stress response and signal transduction under short-term osmotic stress. *Sci Rep.* **9**, 2743.
- Fernandez, G.C.J. (1992) Effective selection criteria for assessing stress tolerance. In *Proceedings of the International Symposium on Adaptation of Vegetables and Other Food Crops in Temperature and Water Stress* (Kuo, C.G., ed.), pp. 257–270. Tainan, Taiwan: Publication.
- Fischer, R.A.M.R. (1978) Drought resistance in spring wheat cultivars. Part I: grain yield response. *J. Agric. Res.* **29**, 897–912.
- Gaudinier, A., Rodriguez-Medina, J., Zhang, L., Olson, A., Liseron-Monfils, C., Bagman, A.M., Foret, J. *et al.* (2018) Transcriptional regulation of nitrogen-associated metabolism and growth. *Nature*, **563**, 259–264.
- Goel, P. and Singh, A.K. (2015) Abiotic stresses downregulate key genes involved in nitrogen uptake and assimilation in *Brassica juncea* L. *PLoS One*, **10**, e0143645.
- Gonzalez, F.G., Capella, M., Ribichich, K.F., Curin, F., Giacomelli, J.I., Ayala, F., Watson, G. *et al.* (2019) Field-grown transgenic wheat expressing the sunflower gene HaHB4 significantly outyields the wild type. *J. Exp. Bot.* **70**, 1669–1681.

- Guo, S.Y., Xu, Y.Y., Liu, H.H., Mao, Z.W., Zhang, C., Ma, Y., Zhang, Q.R. et al. (2013) The interaction between OsMADS57 and OsTB1 modulates rice tillering via DWARF14. *Nat. Commun.* **4**, 1566.
- Guoth, A., Tari, I., Galle, A., Csizsar, J., Pecsvaradi, A., Cseuz, L. and Erdei, L. (2009) Comparison of the drought stress responses of tolerant and sensitive wheat cultivars during grain filling: changes in flag leaf photosynthetic activity, ABA levels, and grain yield. *J. Plant Growth Regul.* **28**, 167–176.
- Han, M.L., Lv, Q.Y., Zhang, J., Wang, T., Zhang, C.X., Tan, R.J., Wang, Y.L. et al. (2022) Decreasing nitrogen assimilation under drought stress by suppressing DST-mediated activation of Nitrate Reductase 1.2 in rice. *Mol. Plant*, **15**, 167–178.
- He, X., Qu, B., Li, W., Zhao, X., Teng, W., Ma, W., Ren, Y. et al. (2015) The nitrate-inducible NAC transcription factor TaNAC2-5A controls nitrate response and increases wheat yield. *Plant Physiol.* **169**, 1991–2005.
- Hosseini, F., Mosaddeghi, M.R. and Dexter, A.R. (2017) Effect of the fungus *Piriformospora indica* on physiological characteristics and root morphology of wheat under combined drought and mechanical stresses. *Plant Physiol. Biochem.* **118**, 107–120.
- Hu, M., Zhao, X., Liu, Q., Hong, X., Zhang, W., Zhang, Y., Sun, L. et al. (2018) Transgenic expression of plastidic glutamine synthetase increases nitrogen uptake and yield in wheat. *Plant Biotechnol. J.* **16**, 1858–1867.
- Jafari, A., Paknejad, F. and Al-Ahmadi, M.J. (2009) Evaluation of selection indices for drought tolerance of corn (*Zea mays* L.) hybrids. *Int. J. Plant Prod.* **3**, 33–38.
- Jan, A.U., Hadi, F., Midrarullah, Nawaz, M.A. and Rahman, K. (2017) Potassium and zinc increase tolerance to salt stress in wheat (*Triticum aestivum* L.). *Plant Physiol. Biochem.* **116**, 139–149.
- Jisha, V., Dampanaboina, L., Vadassery, J., Mithofer, A., Kappara, S. and Ramanan, R. (2015) Overexpression of an AP2/ERF Type transcription factor OsEREBP1 confers biotic and abiotic stress tolerance in rice. *PLoS One*, **10**, e0127831.
- Lang, Y., Wang, M., Xia, J.B. and Zhao, Q.K. (2018) Effects of soil drought stress on photosynthetic gas exchange traits and chlorophyll fluorescence in *Forsythia suspensa*. *J. For. Res.* **29**, 45–53.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F. et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947–2948.
- Le, D.T., Nishiyama, R., Watanabe, Y., Mochida, K., Yamaguchi-Shinozaki, K., Shinozaki, K. and Tran, L.S.P. (2011) Genome-wide expression profiling of soybean two-component system genes in soybean root and shoot tissues under dehydration stress. *DNA Res.* **18**, 17–29.
- Lezhneva, L., Kiba, T., Feria-Bourrellier, A.B., Lafouge, F., Boutet-Mercey, S., Zoufan, P., Sakakibara, H. et al. (2014) The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. *Plant J.* **80**, 230–241.
- Li, C.-W., Su, R.-C., Cheng, C.-P., Sanjaya, You, S.-J., Hsieh, T.-H., Chao, T.-C. et al. (2011) Tomato RAV transcription factor is a pivotal modulator involved in the AP2/EREBP-mediated defense pathway. *Plant Physiol.* **156**, 213–227.
- Li, G.W., Tillard, P., Gojon, A. and Maurel, C. (2016a) Dual regulation of root hydraulic conductivity and plasma membrane aquaporins by plant nitrate accumulation and high-affinity nitrate transporter NRT2.1. *Plant Cell Physiol.* **57**, 733–742.
- Li, M.X., Wu, P.L., Sexton, D.M.H. and Ma, Z.G. (2021) Potential shifts in climate zones under a future global warming scenario using soil moisture classification. *Clim. Dynam.* **56**, 2071–2092.
- Li, T., Wu, X.Y., Li, H., Song, J.H. and Liu, J.Y. (2016b) A dual-function transcription factor, AtYY1, is a novel negative regulator of the Arabidopsis ABA response network. *Mol. Plant*, **9**, 650–661.
- Li, X., Tang, Y., Zhou, C., Zhang, L. and Lv, J. (2020) A wheat WRKY transcription factor TaWRKY46 enhances tolerance to osmotic stress in transgenic Arabidopsis plants. *Int. J. Mol. Sci.* **21**, 1321.
- Liu, D., Sun, J., Zhu, D., Lyu, G., Zhang, C., Liu, J., Wang, H. et al. (2019) Genome-Wide identification and expression profiles of late embryogenesis-abundant (LEA) genes during grain maturation in wheat (*Triticum aestivum* L.). *Genes*, **10**, 696.
- Liu, R.X., Zhou, Z.G., Guo, W.Q., Chen, B.L. and Osterbuis, D.M. (2008) Effects of N fertilization on root development and activity of water-stressed cotton (*Gossypium hirsutum* L.) plants. *Agric. Water Manag.* **95**, 1261–1270.
- Maness, N. (2010) Extraction and analysis of soluble carbohydrates. *Methods Mol. Biol.* **639**, 341–370.
- Maruyama, K., Takeda, M., Kidokoro, S., Yamada, K., Sakuma, Y., Urano, K., Fujita, M. et al. (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant Physiol.* **150**, 1972–1980.
- Mega, R., Abe, F., Kim, J.S., Tsuboi, Y., Tanaka, K., Kobayashi, H., Sakata, Y. et al. (2019) Tuning water-use efficiency and drought tolerance in wheat using abscisic acid receptors. *Nat. Plants*, **5**, 153–159.
- Menezes, C.B., Ticona-Benavente, C.A., Tardin, F.D., Cardoso, M.J., Bastos, E.A., Nogueira, D.W., Portugal, A.F. et al. (2014) Selection indices to identify drought-tolerant grain sorghum cultivars. *Genet. Mol. Res.* **13**, 9817–9827.
- Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.
- Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H. (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* **140**, 411–432.
- Pennisi, E. (2008) Plant genetics. The blue revolution, drop by drop, gene by gene. *Science*, **320**, 171–173.
- Plett, D.C., Ranathunge, K., Melino, V.J., Kuya, N., Uga, Y. and Kronzucker, H.J. (2020) The intersection of nitrogen nutrition and water use in plants: new paths toward improved crop productivity. *J. Exp. Bot.* **71**, 4452–4468.
- Qin, X., Liu, J.H., Zhao, W.S., Chen, X.J., Guo, Z.J. and Peng, Y.L. (2013) Gibberellin 20-oxidase gene OsGA20ox3 regulates plant stature and disease development in rice. *Mol. Plant Microbe Interact.* **26**, 227–239.
- Qu, B., He, X., Wang, J., Zhao, Y., Teng, W., Shao, A., Zhao, X. et al. (2015) A wheat CCAAT box-binding transcription factor increases the grain yield of wheat with less fertilizer input. *Plant Physiol.* **167**, 411–423.
- Ren, Y.Z., He, X., Liu, D.C., Li, J.J., Zhao, X.Q., Li, B., Tong, Y.P. et al. (2012) Major quantitative trait loci for seminal root morphology of wheat seedlings. *Mol. Breeding*, **30**, 139–148.
- Reza, T., Farzad, F. and Amir, M.N. (2009) Effective selection criteria for assessing drought stress tolerance in durum wheat (*Triticum durum* Desf.). *Gen Appl. Plant Physiol.* **35**, 64–74.
- Rosielle, A.A. and Hamblin, J. (1981) Theoretical aspects of selection for yield in stress and non stress environments. *Crop. Sci.* **21**, 943–946.
- Ruiz-Nieto, J.E., Aguirre-Mancilla, C.L., Acosta-Gallegos, J.A., Raya-Perez, J.C., Piedra-Ibarra, E., Vazquez-Medrano, J. and Montero-Tavera, V. (2015) Photosynthesis and chloroplast genes are involved in water-use efficiency in common bean. *Plant Physiol. Biochem.* **86**, 166–173.
- Sadhukhan, A., Kobayashi, Y., Iuchi, S., and Koyama, H. (2021) Synergistic and antagonistic pleiotropy of STOP1 in stress tolerance. *Trends Plant Sci.* **26**, 1014–1022.
- Saijo, Y. and Loo, E.P.I. (2020) Plant immunity in signal integration between biotic and abiotic stress responses. *New Phytol.* **225**, 87–104.
- Shafi, A., Chauhan, R., Gill, T., Swarnkar, M.K., Sreenivasulu, Y., Kumar, S., Kumar, N. et al. (2015) Expression of SOD and APX genes positively regulates secondary cell wall biosynthesis and promotes plant growth and yield in Arabidopsis under salt stress. *Plant Mol. Biol.* **87**, 615–631.
- Shangguan, Z.P., Shao, M.A., Ren, S.J., Zhang, L.M. and Xue, Q. (2004) Effect of nitrogen on root and shoot relations and gas exchange in winter wheat. *Bot. Bull. Acad. Sin.* **45**, 49–54.
- Shukla, S., Singh, K., Patil, R.V., Kadam, S., Bharti, S., Prasad, P., Singh, N.K. et al. (2015) Genomic regions associated with grain yield under drought stress in wheat (*Triticum aestivum* L.). *Euphytica*, **203**, 449–467.
- Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503–517.
- Taochy, C., Gaillard, I., Ipotesi, E., Oomen, R., Leonhardt, N., Zimmermann, S., Peltier, J.B. et al. (2015) The Arabidopsis root stele transporter NPF2.3 contributes to nitrate translocation to shoots under salt stress. *Plant J.* **83**, 466–479.
- Teixeira, E.I., de Ruiter, J., Ausseil, A.G., Daigneault, A., Johnstone, P., Holmes, A., Tait, A. et al. (2018) Adapting crop rotations to climate change in regional impact modelling assessments. *Sci. Total Environ.* **616**, 785–795.

- Tezara, W., Mitchell, V.J., Driscoll, S.D. and Lawlor, D.W. (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. *Nature*, **401**, 914–917.
- Varbanova, M., Yamaguchi, S., Yang, Y., McKelvey, K., Hanada, A., Borochov, R., Yu, F. *et al.* (2007) Methylation of gibberellins by Arabidopsis GAMT1 and GAMT2. *Plant Cell*, **19**, 32–45.
- Wamstad, J.A., Alexander, J.M., Truty, R.M., Shrikumar, A., Li, F., Eilertson, K.E., Ding, H. *et al.* (2012) Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. *Cell*, **151**, 206–220.
- Wang, J., Zhou, L., Shi, H., Chern, M., Yu, H., Yi, H., He, M. *et al.* (2018) A single transcription factor promotes both yield and immunity in rice. *Science*, **361**, 1026–1028.
- Wang, M., Zhang, P., Liu, Q., Li, G., Di, D., Xia, G., Kronzucker, H. *et al.* (2020) TaANR1-TaBG1 and TaWabi5-TaNRT2s/NARs link ABA metabolism and nitrate acquisition in wheat roots. *Plant Physiol.* **182**, 1440–1453.
- Wang, X.B., Wang, L.F. and Shangguan, Z. (2016) Leaf gas exchange and fluorescence of two winter wheat varieties in response to drought stress and nitrogen supply. *PLoS One*, **11**, e0165733.
- Xu, Z.S., Xia, L.Q., Chen, M., Cheng, X.G., Zhang, R.Y., Li, L.C., Zhao, Y.X. *et al.* (2007) Isolation and molecular characterization of the *Triticum aestivum* L. ethylene-responsive factor 1 (TaERF1) that increases multiple stress tolerance. *Plant Mol. Biol.* **65**, 719–732.
- Yoshida, T., Mogami, J. and Yamaguchi-Shinozaki, K. (2014) ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr. Opin. Plant Biol.* **21**, 133–139.
- Yu, T.F., Xu, Z.S., Guo, J.K., Wang, Y.X., Abernathy, B., Fu, J.D., Chen, X. *et al.* (2017) Improved drought tolerance in wheat plants overexpressing a synthetic bacterial cold shock protein gene SeCspA. *Sci. Rep.* **7**, 44050.
- Zhang, X., Teixeira da Silva, J.A., Duan, J., Deng, R., Xu, X. and Ma, G. (2012) Endogenous hormone levels and anatomical characters of haustoria in *Santalum album* L. seedlings before and after attachment to the host. *J. Plant Physiol.* **169**, 859–866.
- Zhang, Z. and Huang, R. (2010) Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor TERF2/LeERF2 is modulated by ethylene biosynthesis. *Plant Mol. Biol.* **73**, 241–249.
- Zheng, Y., Ge, J., Bao, C., Chang, W., Liu, J., Shao, J., Liu, X. *et al.* (2019) Histone deacetylase HDA9 and WRKY53 transcription factor are mutual antagonists in regulation of plant stress response. *Mol. Plant*, **13**, 598–611.
- Zhou, Y.B., Chen, M., Guo, J.K., Wang, Y.X., Min, D.H., Jiang, Q.Y., Ji, H.T. *et al.* (2020) Overexpression of soybean DREB1 enhances drought stress tolerance of transgenic wheat in the field. *J. Exp. Bot.* **71**, 1842–1857.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic analysis of *GmTDN1*.

Figure S2 Expression of *GmTDN1* in leaves and roots of soybean seedlings under various stressors.

Figure S3 Analysis of *GmTDN1* expression and Southern blotting for the *GmTDN1* locus in transgenic wheat.

Figure S4 Drought and low-N tolerance of *GmTDN1* transgenic Jm22 at the seedling stage.

Figure S5 Growth parameters of the WT and OE lines hydroponically grown under control and low-N conditions.

Figure S6 Drought tolerance of the Jm22 transgenic lines assessed by improved nitrate acquisition and utilization.

Figure S7 OE and Jm22 lines at the Shijiazhuang site under drought stress field conditions.

Figure S8 Grain yield response of individual genotypes to water input including rainfall and irrigation.

Figure S9 Biplot diagram of wheat genotypes and drought indices.

Figure S10 *GmTDN1* transgenic wheat exhibits improvements in a variety of physiological traits in rainproof shelter experiments.

Figure S11 Flag leaf total GA content under WIR and LIR conditions.

Figure S12 *GmTDN1* transgenic wheat exhibits improvements in a variety of physiological traits when exposed to drought stress under field conditions.

Figure S13 Expression level of genes related to the activity of protective enzymes and proline metabolism in OE lines.

Figure S14 NUE of OE lines and WT in field plots for 2018–2019 years.

Figure S15 Agronomic traits and N use-related traits of wild-type and transgenic lines in field experiments during the 2016–2017 growing season.

Figure S16 *GmTDN1* improves physiological traits in OE and WT lines at the seedling stage at the Beijing site.

Figure S17 Expression patterns of A5 subgroup members in wheat under drought stress.

Figure S18 Expression patterns of A5 subgroup members in wheat under low N stress.

Figure S19 Expression patterns of A5 subgroup members in soybean under drought and low-N stresses.

Figure S20 *GmTDN1* expression vector map and minimal expression frame DNA fragment.

Figure S21 Cumulative precipitation for the relevant five years at the four experimental stations.

Table S1 Results of PCR and copy number analysis in six T4 generation transgenic lines.

Table S2 Yield results of OE lines and WT Shi4185 at three locations, including Shijiazhuang, Beijing, and Jinan from 2012 to 2015.

Table S3 Yield results of OE lines and WT Jm22 at three field experiment locations.

Table S4 Three yield factors for OE lines and WT Shi4185 at three locations from 2012 to 2015.

Table S5 Three yield factors for OE lines and WT Jm22 at three locations from 2015 to 2017.

Table S6 Drought tolerance indices of wheat genotypes under limited-irrigated and well-irrigated conditions.

Table S7 Yield results of OE lines and WT Jm22 in Beijing from 2015 to 2017 under low N and normal treatments.

Table S8 Yield results of OE lines and WT at two locations, including Beijing and Shijiazhuang during 2018–2019 under low N and normal N treatments.

Table S9 Three yield factors for OE lines and WT Jm22 under low N and normal N treatments in Beijing.

Table S10 NPK nutrient status in the soil before sowing at two locations from 2015 to 2019.

Table S11 Differentially Enriched Regions.

Table S12 Primers used in this study.