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The wheat ABA receptor gene *TaPYL1-1B* contributes to drought tolerance and grain yield by increasing water-use efficiency

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Summary

The role of abscisic acid (ABA) receptors, PYR1/PYL/RCAR (PYLs), is well established in ABA signalling and plant drought response, but limited research has explored the regulation of wheat PYLs in this process, especially the effects of their allelic variations on drought tolerance or grain yield. Here, we found that the overexpression of a TaABFs-regulated *PYL* gene, *TaPYL1-1B*, exhibited higher ABA sensitivity, photosynthetic capacity and water-use efficiency (WUE), all contributed to higher drought tolerance than that of wild-type plants. This heightened water-saving mechanism further increased grain yield and protected productivity during water deficit. Candidate gene association analysis revealed that a favourable allele *TaPYL1-1B*$^{In\text{-}442}$, carrying an MYB recognition site insertion in the promoter, is targeted by TaMYB70 and confers enhanced expression of *TaPYL1-1B* in drought-tolerant genotypes. More importantly, an increase in frequency of the *TaPYL1-1B*$^{In\text{-}442}$ allele over decades among modern Chinese cultivars and its association with high thousand-kernel weight together demonstrated that it was artificially selected during wheat improvement efforts. Taken together, our findings illuminate the role of *TaPYL1-1B* plays in coordinating drought tolerance and grain yield. In particular, the allelic variant *TaPYL1-1B*$^{In\text{-}442}$ substantially contributes to enhanced drought tolerance while maintaining high yield, and thus represents a valuable genetic target for engineering drought-tolerant wheat germplasm.

Introduction

As an arid or semi-arid cereal crop, wheat (*Triticum aestivum* L.) provides a major source of nutrition globally, though its production is often affected by drought or water deficit, which has been worsened by climate change and a rapidly expanding of global population (Gupta *et al*., 2020; Lesk *et al*., 2016). During the past decades, considerable efforts have been devoted to research the plant survival under drought stress at the expense of grain yield (Hu and Xiong 2014; Mickelbart *et al*., 2015; Nuccio *et al*., 2018). However, widespread, severe drought do not occur that often, so the development of crops that can tolerate extreme, prolonged water deficit will not necessarily produce useful cultivars in practice. Thus, the goal for breeders, agronomists and plant geneticists is to improve the yield and viability of cultivars that produce desirable grains during less severe, but more frequent incidents of water scarcity (Hall and Richards 2013). Therefore, to breed high-yielding wheat cultivars that use water more efficiently than their present-day counterparts is an urgent objective in the development of next-generation agriculture.

To this end, advances in wheat genetics and physiology during the past decades showed that the phytohormone abscisic acid (ABA), which is produced by plants in response to drought and osmotic stresses, induces changes in gene expression that reduce water loss through transpiration via increased stomatal closure (Bailey-Serres *et al*., 2019; Munemasa *et al*., 2015; Yoshida *et al*., 2019). As ABA levels increase with drought stress, the soluble pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of the ABA receptor (RCAR) family of proteins (herein referred to as PYLs) bind ABA, leading to conformational changes in the PYLs that enable interactions with clade A type 2C protein phosphatases (PP2Cs). These interactions inhibit PP2C activity, thus releasing sucrose non-fermenting 1-related protein kinase 2 proteins (SnRK2s) from their inhibition by the PP2Cs (Fujii *et al*., 2009; Ma *et al*., 2009; Park *et al*., 2009). Activation of SnRK2s triggers their downstream phosphorylation of stress adaptation response proteins, such as ion channels that participate in stomatal closure (Brandt *et al*., 2012; Geiger *et al*., 2009; Munemasa *et al*., 2015), aquaporins (Grondin *et al*., 2015) and ABA-responsive element-binding factors (AREB/ABFs), which are master regulators of the transcriptional response to ABA (Lumba *et al*., 2014; Yoshida *et al*., 2014, 2019).

Studies aimed at enhancing plant productivity or survival under water deficit have shown that transpiration can be limited through exogenous application of ABA and ABA agonists, transgenic or ectopic *PYL* expression and down-regulation of *PP2C* expression (Cao *et al*., 2017; Mega *et al*., 2019; Okamoto *et al*., 2013; Park *et al*., 2015; Rubio *et al*., 2009; Vaidya *et al*., 2019; Yang *et al*., 2016, 2019). The *Arabidopsis* genome contains 14 genes encoding PYL receptors (Raghavendra *et al*., 2010), and

separate studies have shown that overexpression of *PYL3*, *PYL4*, *PYL5*, *PYL6*, *PYL7*, *PYL9*, *PYL11* and *PYL13* all enhanced drought tolerance in transgenic *Arabidopsis* (Pizzio *et al*., 2013; Santiago *et al*., 2009; Zhao *et al*., 2016). In rice, 13 genes have been predicted to encode the PYL receptors (Tian *et al*., 2015), and the overexpression of *OsPYL3*, *OsPYL5*, *OsPYL9* and *OsPYL11* led to higher ABA sensitivity and increased tolerance for drought stress (Kim *et al*., 2012, 2014; Tian *et al*., 2015). Moreover, recent studies have demonstrated that overexpression of ABA receptors can be used as a strategy to increase water-use efficiency (WUE) and plant productivity under water scarcity (Mega *et al*., 2019; Okamoto *et al*., 2013; Vaidya *et al*., 2017, 2019; Yang *et al*., 2016, 2019). Overall, these findings suggest that ABA receptors are excellent candidates for manipulating crop production efficiency under drought stress conditions.

Although the regulatory function of *PYLs* in drought tolerance has been demonstrated in several species, the contributions of wheat *PYLs* and their allelic variations to differences in response to drought stress remain essentially unknown. Here, we identified a wheat ABRE-binding transcription factors (TaABFs)-regulated *PYL* gene, *TaPYL1-1B*, that plays an important role in controlling ABA-mediated seed germination and seedling growth. Overexpression of *TaPYL1-1B* in transgenic wheat led to improved drought tolerance and grain yield, most likely due to enhanced WUE. Further analysis showed that a sequence variation in the *TaPYL1-1B* promoter region, specifically, InDel-442 which harboured an MYB-binding element, targeted by TaMYB70, was associated with distinct allelic differences in gene expression during drought stress. Further allele frequency analysis in hexaploid wheat accessions showed that *TaPYL1-1B* underwent strong allele-based artificial selection during modern wheat genetic improvement, resulting in an increased frequency of *TaPYL1-1B*$^{In\text{-}442}$, a variant associated with high yield and drought tolerance in modern wheat cultivars. Our results demonstrate the role of *TaPYL1-1B* in improving wheat drought tolerance and grain yield, and also shed new light on the role of allelic variation in modern breeding of wheat cultivars for higher yield and WUE, and can thus provide effective targets for engineering of WUE to find a balance between high yield and decreasing water availability.

Results

TaABFs regulate the ABA-induced expression of *TaPYL1-1B*

A phylogenetic analysis revealed that three wheat homoeologs, TaPYL1-1A, TaPYL1-1B and TaPYL1-1D, belong to the clade III PYL family and shares a close relationship with *Arabidopsis* PYL1 and PYR1, rice OsPYL10 and *B. distachyum* BdPYL1 (Figure S1a). Reciprocal BLAST analysis revealed that TaPYL1-1A, TaPYL1-1B and TaPYL1-1D share 98% ~ 99% protein sequence identity, indicating high sequence conservation among the three subgenome homoeologs (Figure S1b). Further subcellular localization of TaPYL1-GFP fusion protein under the control of the CaMV 35S promoter in wheat protoplast and tobacco leaf epidermal cells revealed that the GFP signals of TaPYL1s-GFP were localized to the cytoplasm and nucleus (Figure S2). Yeast two-hybrid assays revealed that TaPYL1-1B could interact with the known PP2C proteins, TaPP2C1, TaPP2C3, TaPP2C4 and TaPP2C6 (Mega *et al*., 2019) (Figure 1a), indicating TaPYL1 acts as a component of the core ABA signalling pathway in wheat.

After analysis of the *TaPYL1-1A*, *TaPYL1-1B* and *TaPYL1-1D* promoter sequences (~ 1.5 kb upstream of the start codon), we found that only *TaPYL1-1B* promoter carried four ABA-responsive elements (ABRE, TACGTG/CACGTA) (Figure S3). Previous studies demonstrated that ABRE-binding transcription factors (ABFs) can bind to the ABRE element in vitro and serve as master transcription factors in ABA signalling (Yoshida *et al*., 2014). In our yeast one-hybrid assay, three TaABF proteins from wheat genome IWGSC RefSeq V1.1 (IWGSC, 2018), namely TaABF1, TaABF2 and TaABF3, were found to bound the *TaPYL1-1B* promoter fragment containing ABREs in yeast (Figure 1b). An EMSA assay was then conducted to determine if the TaABF protein could bind directly to the ABRE element in *TaPYL1-1B* promoter. The TACGTG motif within a 30-bp promoter fragment (AGCCCAGCAGCTACGTGAGCTCCTCGCTGT) was used as a probe (WT), and for a control, a mutation probe (Mut) in which TACGTG was mutated to ATAAAG was used. The data indicated that the GST-TaABF2 fusion protein could bind to a WT probe although not a mutated probe, confirming that TaABF2 is able to bind directly to the *TaPYL1-1B* promoter in vitro (Figure 1c).

Subsequently, the transcriptional activation assay in tobacco leaves was completed to investigate if TaABFs directly activate the transcription of *TaPYL1-1B* (Figure 1d). Compared to results in the negative control, the LUC activities were significantly greater in tobacco leaves co-expressing the reporter carrying the *TaPYL1-1B* promoter fragment driving LUC and the effector containing TaABFs. Moreover, when the tobacco leaves were treated with exogenous ABA, the increase in the LUC activities became more apparent (Figure 1e). This indicates that ABA promotes the binding of TaABFs to the *TaPYL1-1B* promoter. Additionally, qRT-PCR analysis revealed that *TaPYL1-1B* transcript level was significantly up-regulated in wheat seedlings after ABA treatment, while *TaPYL1-1A* and *TaPYL1-1D* were only slightly up-regulated (Figure 1f). These data show that the transcription factor TaABFs have the ability to regulate *TaPYL1-1B* expression by directly binding to its promoter.

TaPYL1-1B overexpression promotes ABA-inhibited seed germination and seedling growth.

To conduct a functional analysis of *TaPYL1-1B*, three *TaPYL1-1B* transgenic overexpression lines, OE4, OE5 and OE6, were used. The expression levels of *TaPYL1-1B* were about 16–18-fold higher in OE4, OE5 and OE6 than levels found in WT of wheat *cv*. Fielder (Figure S4 a). The response to exogenous ABA in seed germination rates and shoot and root lengths of transgenic OE lines were examined to explore the role of *TaPYL1-1B* in ABA signalling. Seed germination was delayed in the OE lines compared with germination in the WT, with or without exogenously applied ABA. Moreover, this ABA-mediated inhibition of seed germination was dosage-dependent (Figure 2a,b).

Shoot and root elongation was inhibited by ABA application in both WT and transgenic OE plants (Figure 2c). Under ABA treatment in the WT, the reduction in shoot length was greater than that of root length. This resulted in a high root-to-shoot ratio of WT plants regardless of the ABA was applied. The overexpression of *TaPYL1-1B* led to an inhibition of root and shoot elongation and caused a decrease in the root-to-shoot ratio. As the ABA concentration increased, this decrease became greater (Figure 2c). These data indicate that ABA signalling in wheat is positively regulated by *TaPYL1-1B*, and in ABA-treatment conditions, this ABA hypersensitization promotes a decreased root-to-shoot ratio.

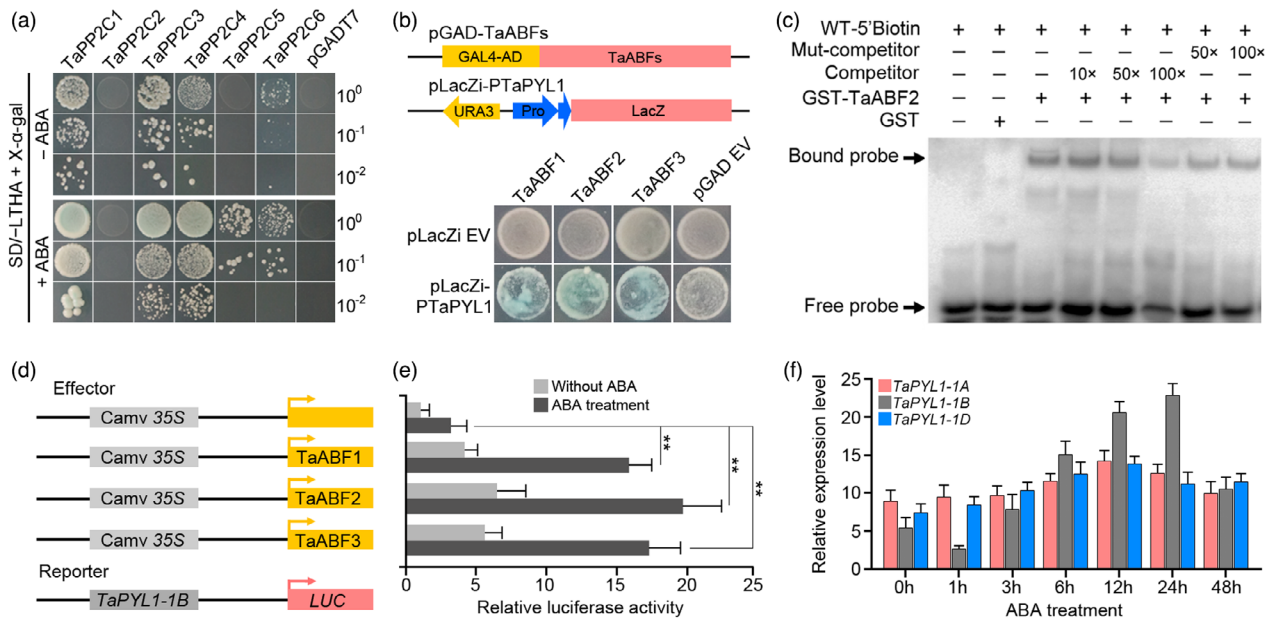


Figure 1 TaABFs regulate the *TaPYL1-1B* expression (a) Yeast two-hybrid assay to confirm the interactions of TaPYL1-1B and TaPP2Cs. The co-transfected yeast competent cells were grown on SD/−LTHA medium plus X-α-gal with or without ABA. The empty vector pGADT7 was used as a negative control. (b) Yeast one-hybrid assay to confirm the binding of TaABFs and *TaPYL1-1B* promoter. The empty vectors, pGAD EV and pLacZi EV, were used as negative controls. (c) EMSA assay to validate the binding of TaABF2 to ABRE element (TACGTG) in the *TaPYL1-1B* promoter. (d-e) TaABFs activate the transcription activity of *TaPYL1-1B* promoter. Monitoring of relative luciferase activity (e) in tobacco leaves co-transfected with the reporter and different effector constructs (d). To detect relative activity, the transfected leaves were treated either with or without 100 μM ABA for 1 h. The co-infiltration of an empty effector vector and the reporter construct served as the negative control. Data are shown as means ± SDs of at least three independent replicates. (f) qPCR analysis of *TaPYL1-1A*, *TaPYL1-1B* and *TaPYL1-1D* expression levels in 100 μM ABA treated wheat *cv*. Chinese Spring seedlings. Data are presented as means ± SD of three biological replicates. Statistical significance was determined by a Student's *t* test, ** $P < 0.01$.

TaPYL1-1B overexpression confers drought tolerance

The *TaPYL1-1B* transgenic OE lines showed a greater tolerance to drought stress than WT. To verify the drought tolerance conferred by *TaPYL1-1B*, three-leaf stage WT and transgenic plants were exposed to water deficit using a 30% PEG solution treatment. We found that after 7 days of PEG treatment, the majority of the WT plants were withered, while the leaves of OE4, OE5 and OE6 plants were only rolled. After recovery in PEG-free hydroponic solution, the majority of transgenic plants survived, while WT leaves remained fully rolled and wilted (Figure S4b). We next planted the transgenic OE lines and WT plants side-by-side in soil-containing pots, prior to water-deficit treatment at the three-leaf stage. After three weeks without watering, WT plants exhibited high water stress, with most leaves fully rolled and desiccated; in contrast, the transgenic OE plants remained green with limited rolling and wilting. Following 3 days of full watering, the majority of the WT plants failed to recover, resulting in an approximate 26%–37% survival rate, whereas > 80% of the transgenic OE plants survived intact (Figure 3a,b).

To further investigate the physiological mechanisms underlying *TaPYL1-1B*-mediated modulation of water loss, we compared differences in dehydration rate between the WT and transgenic OE lines and found that the rate of water loss among *TaPYL1-1B* overexpressing plants was lower than that of WT plants (Figure 3c). Previous work demonstrated that stomatal closure is induced to control water loss by transpiration in response to drought stress (Murata *et al.*, 2015). Thus, we measured the stomatal aperture in WT and OE4, OE5 and OE6 plants. There were no obvious differences in stomatal length and stomatal density between the WT and OE plants under well-watered conditions (Figure 3d, e). However, a significant increase in stomatal length was observed in OE plants under drought conditions (Figure 3e). Additionally, more stomata were completely closed and fewer stomata were completely open in the detached OE plant leaves than in the WT leaves under drought conditions (Figure 3f-h). Collectively, our results suggested that *TaPYL1-1B* modulates ABA signalling and positively affects drought tolerance, possibly through ABA-mediated stomatal movement.

TaPYL1-1B overexpression increases water-use efficiency

Previous studies have shown that the overexpression of ABA receptors can increase WUE in plants (Mega *et al.*, 2019; Yang *et al.*, 2016, 2019). In order to determine if *TaPYL1-1B* overexpression also affects WUE and carbon assimilation, we first measured leaf-level gas exchange and found that stomatal conductance and transpiration rates were both significantly lower in wheat plants that overexpressed *TaPYL1-1B* relative to WT, especially under water stress. In agreement with these results, CO_2 assimilation rates were increased accordingly with the steady increase in WUE for transgenic OE plants (Figure 4a-d). Furthermore, the carbon isotope discrimination ($\delta^{13}C$) analyses also revealed reduced ^{13}C fractionation in leaves of *TaPYL1-1B* overexpression plants (Figure 4e). Further examination of the relationship between the CO_2 assimilation rate and intercellular

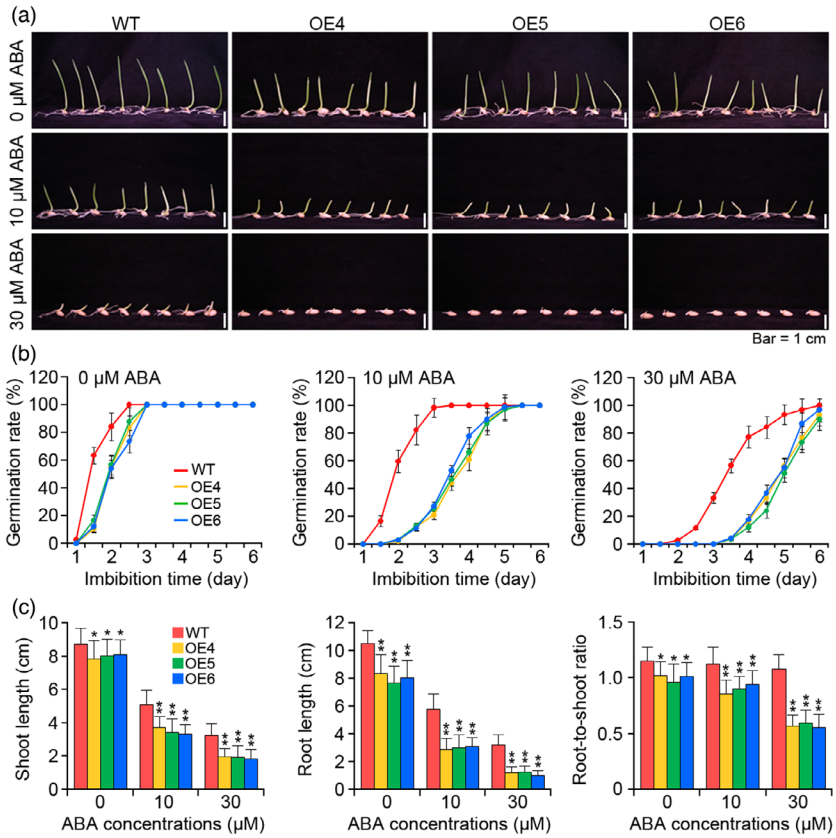


Figure 2 Effect of ABA treatments on seed germination and seedling growth of WT and *Ubi:TaPYL1-1B* transgenic wheat lines (a) Images of seed phenotypes of WT and transgenic OE lines after application of various ABA treatments for 6 days. Scale bars = 1 cm. (b) Time courses showing seed germination of WT and transgenic OE lines in the solutions containing 0, 10 and 30 μM ABA. (c) Comparisons between WT and transgenic OE lines of the shoot and root lengths and root-to-shoot ratios. Shoot and root measurements were taken 10 days after the germinated seeds of WT and transgenic OE lines were relocated to solutions of various ABA concentrations. Data are presented as the means ± SD of three replicates. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

CO_2 concentration (C_i) showed that *TaPYL1-1B* transgenic plants assimilated CO_2 more efficiently than WT, even though the C_i of *TaPYL1-1B* transgenic plants was comparable between OE lines and WT (Figure 4f,g). These results indicated that the OE lines plants possessed an increased capacity for CO_2 fixation relative to WT, even at ambient CO_2 concentrations, and taken together, clearly showed that *TaPYL1-1B* overexpression in wheat can significantly improve plant WUE and CO_2 assimilation. This increased WUE among transgenic lines ultimately resulted in slower consumption of water, and hence prolonged the retention of available soil water compared to WT plants (Figure 4h).

TaPYL1-1B overexpression increases grain yield

To determine whether increased WUE translated to increased grain yield per liter of water consumed, *i.e.*, increased water productivity, we next compared grain production and quality between the OE4, OE5 and OE6 transgenic lines with that of WT under both well-watered and water-limited conditions in the greenhouse. Obviously, the plant height of *TaPYL1-1B* transgenic OE plants was lower than that of WT plants under well-watered conditions (Figure 5a,d). However, regardless of identical irrigation regimens, transgenic OE plants exhibited greater spike width, kernel number per plant, grain length and grain width, which led to an increased grain yield per plant than observed in WT (Figure 5b,d). It is worth noting that grain quality-related traits, such as water content, protein content, starch content and sedimentation value, were not significantly changed in transgenic OE lines (Figure S5).

We next evaluated grain yield under drought conditions and found that under identical water limitations, dramatic reductions were observed in WT grain weight and accompanying small, shrunken seeds (typical of water-stressed wheat plants), whereas the reductions of grain weight and size were substantially smaller among transgenic OE lines (Figure 5c). Similarly, the spike length, spike width, kernel number per plant, kernel length, kernel width and grain yield per plant were increased over WT in the OE lines under water deficit (Figure 5e). Collectively, these results demonstrate that *TaPYL1-1B* overexpression in wheat increases WUE and this heightened water-saving mechanism further increased grain yield and protect productivity during water deficit.

TaPYL1-1B overexpression up-regulates ABA and drought-response genes

To clarify which genes participate in the regulatory network through which *TaPYL1-1B* mediates a strongly tolerant phenotypic response to drought, we compared the transcriptomes of OE4 and OE5 lines with that of WT under both well-watered and water-deficit conditions. Under well-watered conditions, a total of 806 and 571 genes were significantly up-regulated and down-regulated (adjusted $P < 0.01$, fold change > 2 or < 0.5), respectively, in transgenic lines compared to WT (Figure 6a; Figure S6; Table S1). While under drought stress, 1386 and 706 genes (adjusted $P < 0.01$) were at least twofold up- or down-regulated, respectively, in the transgenic lines compared to WT (Figure 6a; Figure S6; Table S2). Gene ontology (GO) analysis revealed that biological pathways 'responsive to ABA', 'water deprivation' and 'osmotic stress' were greatly enriched for differentially up-regulated genes. In contrast, pathways related to 'fatty acid biosynthesis', 'response to jasmonic acid' and '"oxidation-reduction' were especially enriched for differentially down-regulated genes (Figure 6b). Genes involved in 'response to stress' were more significantly enriched in the up-regulated genes

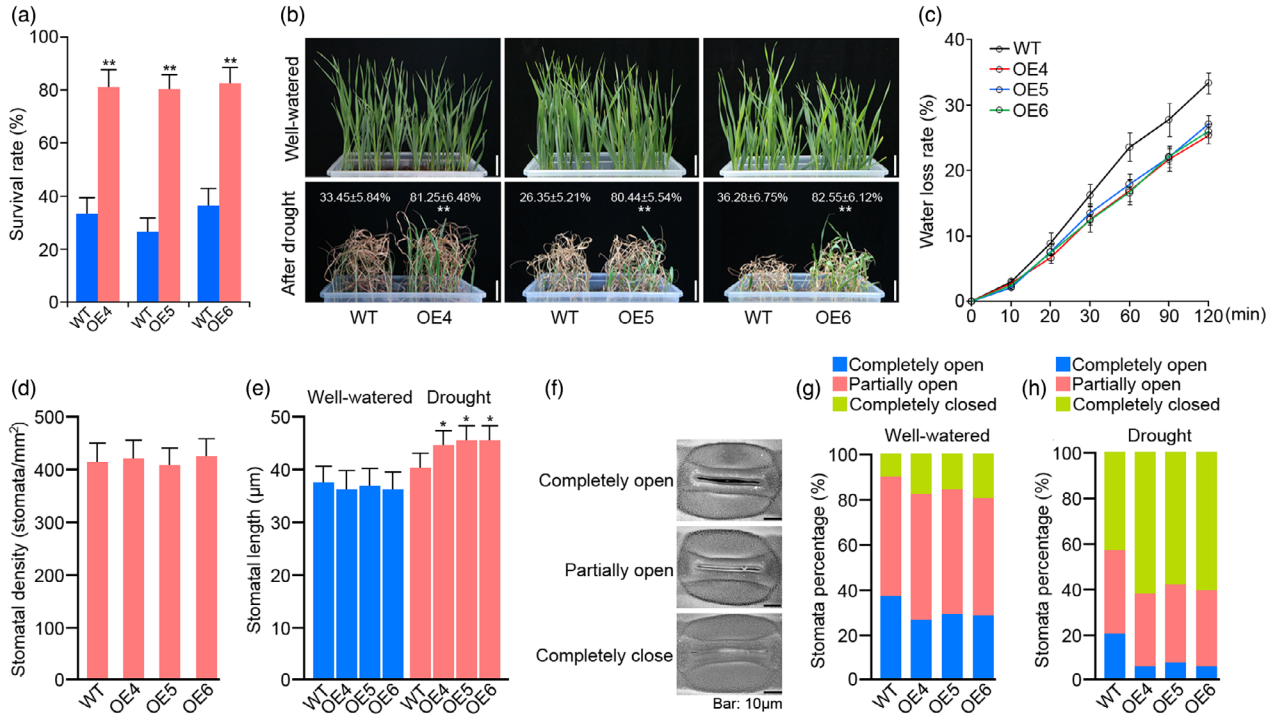


Figure 3 Drought responses of *Ubi:TaPYL1-1B* transgenic wheat lines (a) Survival rates of the WT and transgenic OE lines under drought stress. Irrigation was ceased for three-leaf-stage seedlings for ~ 30 days and then watering resumed for 3 days. (b) Plant phenotypes of the WT and transgenic OE lines before drought and after rewatering. Scale bars = 5 cm. (c) Water loss from detached leaves of WT and transgenic OE lines at different time points. Each replicate contained at least ten leaves. Water loss was expressed as a percentage of initial fresh weight. (d) Comparisons of stomatal density between WT and the transgenic OE lines. (e) Comparisons of stomatal length between WT and the transgenic OE lines. Each replicate contained at least 100 stomata. (f) Scanning electron microscope images of stomata that completely open, partially open or completely closed. Scale bar = 10 μm. (g-h) The percentage of the three levels of stomatal opening in transgenic OE lines compared to WT under well-watered (g) and drought conditions (h). WT or transgenic OE line contained at least 100 stomata in each replicate. Data are presented as means ± SD of three replicates. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

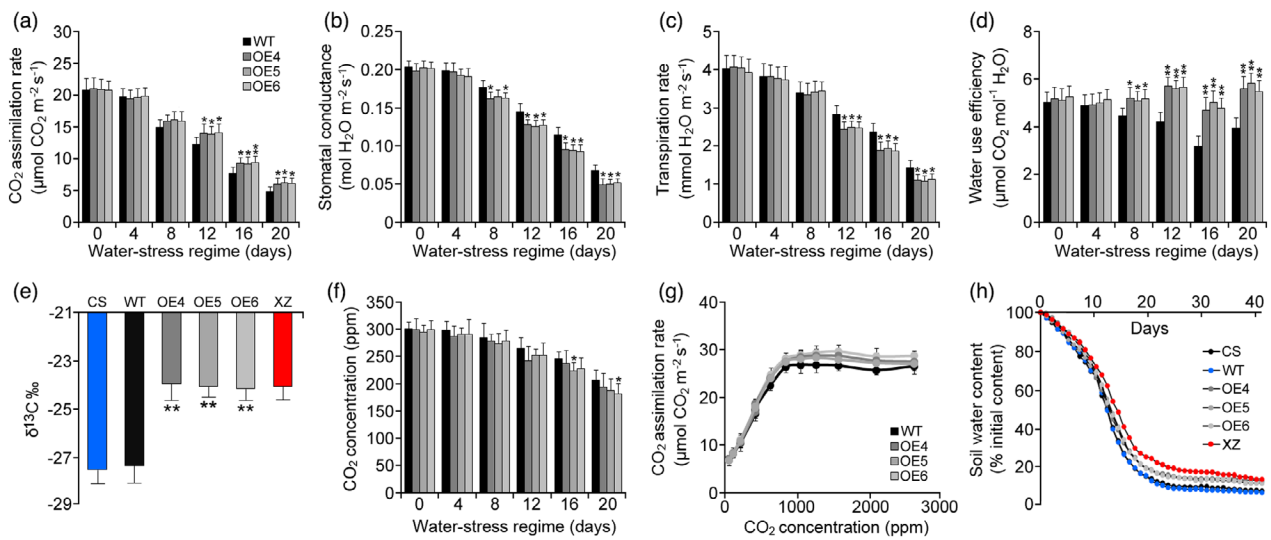


Figure 4 Improvement of photosynthetic capacity and WUE in *Ubi:TaPYL1-1B* transgenic wheat lines (a-d) Photosynthetic capacity of WT and transgenic OE lines subjected to progressive drought stress. (e) Carbon isotope composition ($\delta^{13}C$) of WT and transgenic OE lines. CS, drought-sensitive wheat *cv.* Chinese Spring; XZ, drought-tolerant wheat *cv.* Xinzi9104. (f) Intercellular CO_2 concentration (C_i) of WT and transgenic OE lines subjected to progressive drought stress. (g) Relationship between CO_2 assimilation and C_i. (h) Soil water potential changes observed in WT and transgenic OE lines. Data are presented as means ± SD of three replicates. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

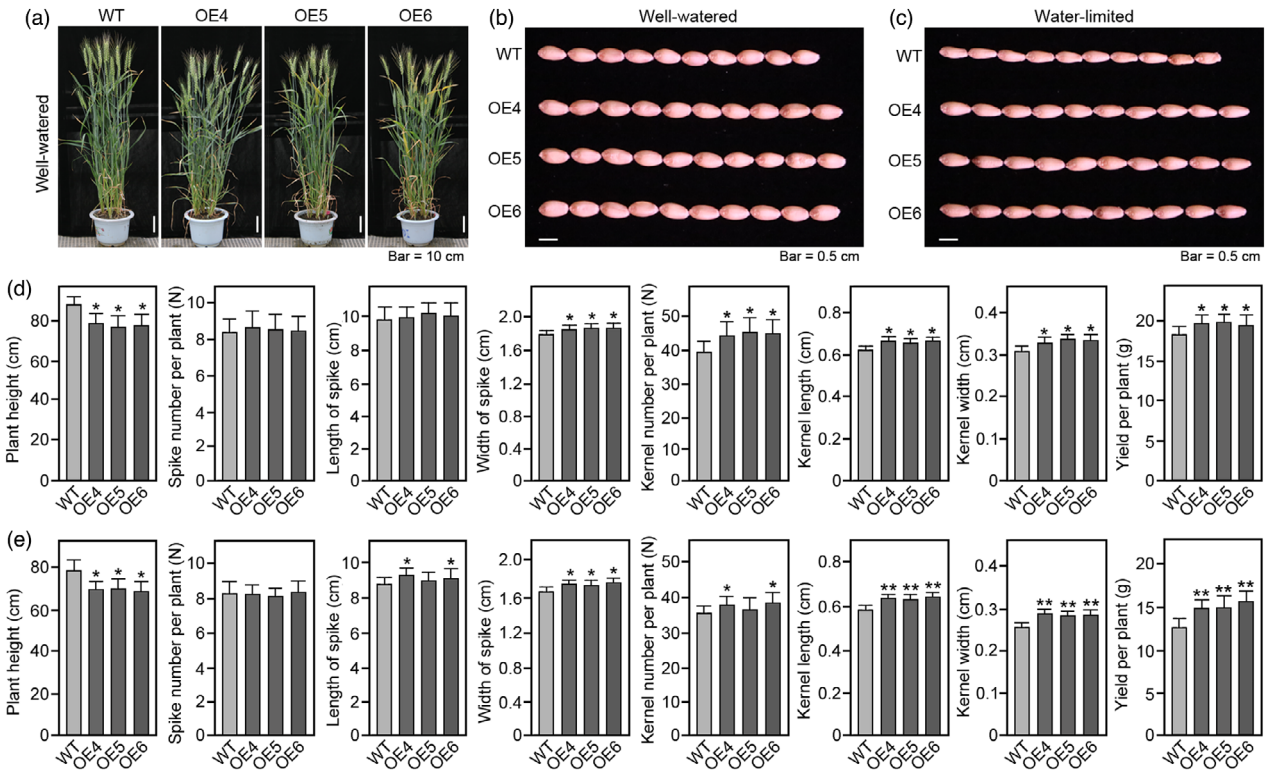


Figure 5 Improvement of grain yield in *Ubi:TaPYL1-1B* transgenic wheat lines (a) Images of representative WT and transgenic OE plants under well-watered conditions. Scale bars = 10 cm. (b) Seed shape of WT and transgenic OE lines under well-watered conditions. Scale bars = 0.5 cm. (c) Seed shape of WT and transgenic OE lines under water-limited conditions. Scale bars = 0.5 cm. (d-e) Agronomic traits of WT and transgenic OE lines under well-watered (d) and water-limited conditions (e). A minimum of 12 plants were measured in each replicate. Data represent the mean ± SD of two replicates. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

among the drought-treated samples, as compared with the well-watered ones (Figure 6b).

Considering the elevated sensitivity to ABA among the *TaPYL1-1B* overexpression lines, we also compared the transcriptomes of WT and transgenic OE4 and OE5 lines under ABA treatment and found 1179 and 1566 differentially up- and down-regulated genes (adjusted $P < 0.01$, fold change > 2 or < 0.5), respectively, in OE lines compared to WT (Figure 6a; Figure S6; Table S3). GO analysis showed that up-regulated genes were mainly enriched in 'protein phosphorylation', 'response to ABA', 'response to osmotic stress', 'response to water deprivation', 'plant-type hypersensitive response', 'transcription regulation' and 'leaf senescence' pathways. In contrast, down-regulated genes were found in 'fatty acid biosynthetic process', 'response to jasmonic acid' and 'flavonoid biosynthetic process', among others (Figure 6b).

Notably, up-regulated genes were enriched in 'response to ABA', 'response to osmotic stress' and 'response to water deprivation' biological pathways under both well-watered and stress treatment conditions. Increased expression of several well-characterized drought- or ABA-responsive genes was verified by qPCR in samples from the OE transgenic lines (Figure S7). We thus hypothesized that these transcriptomic alterations in *TaPYL1-1B* overexpressing plants collectively lead to a rapid drought response entailing an increased stomatal closure, reduced transpiration rate and heightened protection of photosynthesis machinery, thereby resulting in enhanced WUE.

TaPYL1-1B$^{In\text{-}442}$ allele is associated with higher drought tolerance

To further investigate whether genetic variation in *TaPYL1-1B* was associated with phenotypic differences in seedling drought tolerance among wheat varieties, we first interrogated GWAS results obtained in our previous work (Mao *et al.*, 2020) and found that 7 SNP markers around *TaPYL1-1B* were significantly associated with drought tolerance under the MLM model ($P < 0.01$) (Figure S8 a). The leading SNP was located in the 2854 bp upstream of *TaPYL1-1B* (Figure S8b). Furthermore, *TaPYL1-1B* expression was up-regulated under drought stress conditions in the drought-tolerant wheat *cv*. Pubing202 and drought-sensitive *cv*. Chinese Spring (Figure S8c). Thus, *TaPYL1-1B* was revealed to be a potentially important candidate gene significantly associated with drought tolerance among wheat accessions. To accurately identify genetic variations, *TaPYL1-1B* was re-sequenced in a wheat variation panel composed of 120 representative selected varieties from worldwide. In total, seven SNPs/indels were identified in 2.8-kb genomic region of *TaPYL1-1B* with minor allele frequency (MAF) equal to or more than 0.05 (Table S4). In order to trace the variants significantly associated with seedling SR, we classified the 120 wheat genotypes into five haplotype groups, based on these seven SNP/indel variants (Figure 7a,b). Hap 2 formed the largest group (n = 57), whereas Hap1 comprised the second largest group (n = 56), while Hap 3, Hap 4 and Hap 5 all formed minor groups consisting of only a few

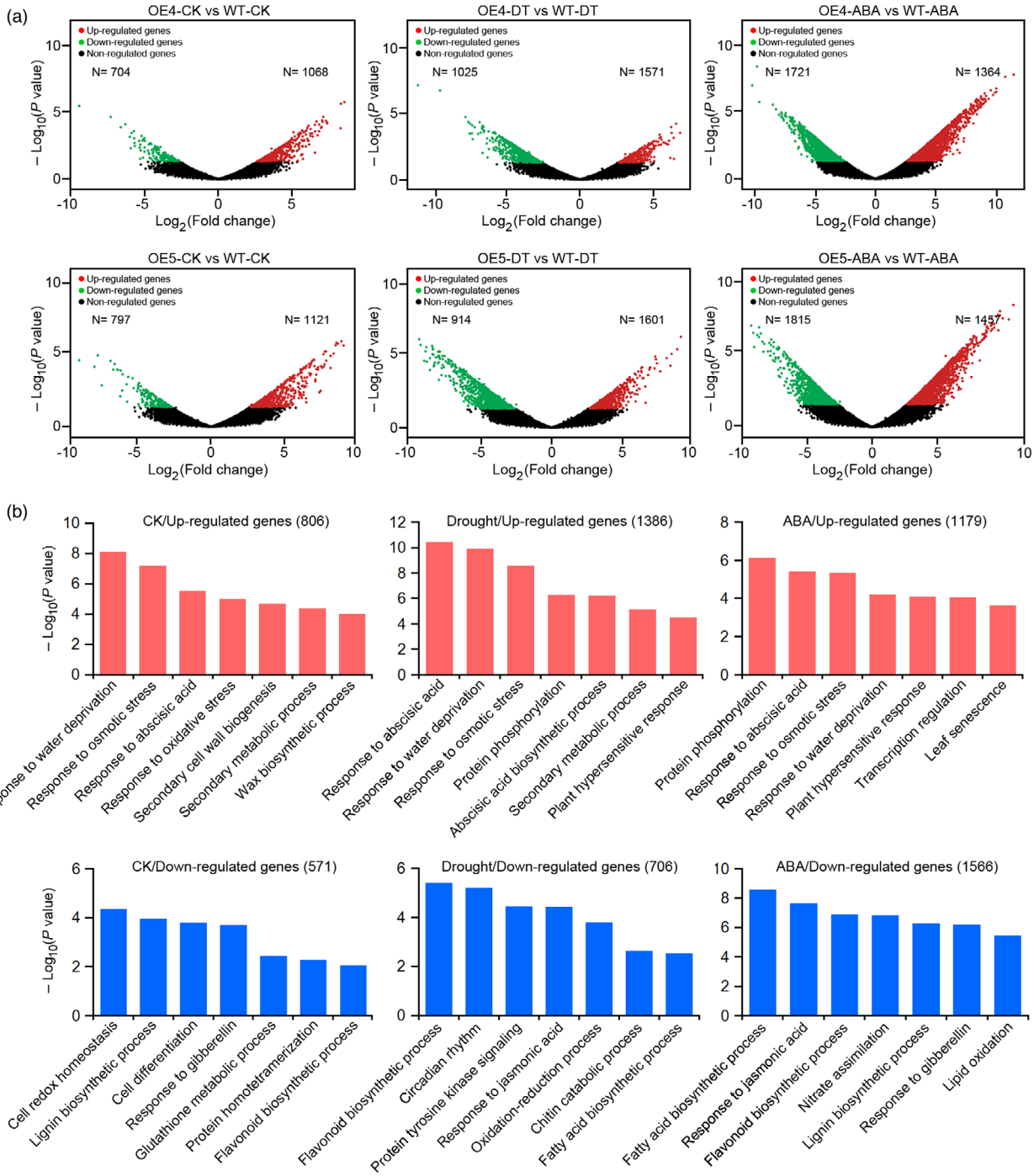


Figure 6 Transcriptomic analysis of *Ubi:TaPYL1-1B* transgenic wheat lines (a) Volcano plots of up- and down-regulated genes in transgenic OE4 and OE5 lines under non-stress (CK), drought (DT) and ABA treatment conditions. The significantly up-regulated or down-regulated genes in transgenic OE lines were defined as the differentially expressed gene with fold change > 2 or < 0.5 and adjusted $P < 0.01$. (b) Gene ontology of biological pathways enriched in the transgenic OE lines based on the significantly up- or down-regulated genes under CK, DT and ABA conditions.

varieties. Statistically, wheat varieties with Hap1 exhibited significantly higher SR than those with Hap 2 ($P = 1.56 \times 10^{-16}$), and two varieties carrying Hap 5 also showed higher SR than the five varieties carrying either Hap 3 or Hap 4 (Figure 7b,c). Therefore, we designated Hap 1/Hap 5 as the tolerant alleles and Hap 2/Hap 3/Hap 4 as sensitive alleles of *TaPYL1-1B*. Furthermore, using the 20-bp indel (InDel-442) as a polymorphism marker, two alleles *TaPYL1-1B*$^{\text{In-442}}$ and *TaPYL1-1B*$^{\text{Del-442}}$ were distinguished (Figure S9).

Next, the effects of *TaPYL1-1B*$^{\text{In-442}}$ and *TaPYL1-1B*$^{\text{Del-442}}$ alleles on drought tolerance in wheat seedlings were compared by crossing drought-tolerant variety Pubing202 with two

drought-sensitive varieties, GLUYAS EARLY and Wanmai33, to construct two bi-parental $F_{3:4}$ populations (Figure 7d). In these crosses, the *TaPYL1-1B*$^{Del\text{-}442}$ allele was carried by both GLUYAS EARLY and Wanmai33, and Pubing202 had the *TaPYL1-1B*$^{In\text{-}442}$ allele. Then, the two $F_{3:4}$ segregation populations were genotyped by 20-bp polymorphism marker, the homozygous progenies were subjected to drought conditions and the plant survival rates after stress and re-watering were calculated to quantitatively measure the phenotypic contributions of the *TaPYL1-1B*$^{In\text{-}442}$ and *TaPYL1-1B*$^{Del\text{-}442}$ alleles to drought tolerance. The results showed that survival rate of *TaPYL1-1B*$^{Del\text{-}442}$ homozygous plants was lower than that of *TaPYL1-1B*$^{In\text{-}442}$ homozygous in each segregation population (Figure 7e).

To further investigate whether the 20-bp indel was responsible for the observed phenotypic differences in drought tolerance and yield-related traits, we used a binary vector expressing Cas9 and two guide RNAs (gRNAs) to generate mutations in immature embryos of wheat *cv*. Fielder carrying the *TaPYL1-1B*$^{In\text{-}442}$ 20-bp insertion allele (Figure 7f). We obtained three independent mutant lines with deletions of the 20-bp insertion or its flanking sequences. Subsequent qPCR analysis showed that, compared to WT, *TaPYL1-1B* expression was significantly reduced in the edited 20-bp deletion line (C3) under both well-watered and water-deficit conditions (Figure 7g,h). Phenotypic evaluation of drought tolerance confirmed that the C3 mutant was exhibited significantly greater sensitivity to drought stress than WT (Figure 7g,i; Figure S10). As a whole, these data contribute to the premise that the drought-tolerant phenotype impacted by genetic variation in *TaPYL1-1B* and the *TaPYL1-1B*$^{In\text{-}442}$ allele is associated with higher drought tolerance in wheat seedlings.

TaPYL1-1B$^{In\text{-}442}$ allele is associated with significantly increased kernel size and TKW

Previous studies have shown that favoured alleles progressively accumulate through breeding (Barrero *et al*., 2011). To determine whether the *TaPYL1-1B*$^{In\text{-}442}$ allele was selected and promulgated through Chinese wheat breeding programmes, we investigated the geographic distribution of *TaPYL1-1B*$^{In\text{-}442}$ and *TaPYL1-1B*$^{Del\text{-}442}$ alleles among wheat landraces and current modern cultivars (MC) across Chinese wheat production areas. The results surprisingly showed that the *TaPYL1-1B*$^{In\text{-}442}$ allele underwent strong positive selection through modern Chinese wheat breeding (Figure 8a), and the frequency of *TaPYL1-1B*$^{In\text{-}442}$ showed a continuous increase consistent with increasing TKW since the 1940s (Figure 8b; Ma *et al*., 2016). Haplotyping analysis showed that *TaPYL1-1B*$^{In\text{-}442}$ was positively selected in breeding among landraces (Figure 8c) and MC (Figure 8d), especially in production zones I, II and III, the main production zones in China (Zhang *et al*., 2002). These results demonstrated that *TaPYL1-1B*$^{In\text{-}442}$ has undergone strong artificial selection commensurate with the improvement of wheat for high yield.

We inferred that *TaPYL1-1B*$^{In\text{-}442}$ is potentially associated with high grain yield, since this allele has increased in prevalence commensurately with increasing wheat production resulting from artificial selection/breeding. After genotyping the Chinese mini-core collection (MCC), we compared phenotypes and genotypes accessions carrying either the *TaPYL1-1B*$^{In\text{-}442}$ or *TaPYL1-1B*$^{Del\text{-}442}$ alleles in wheat landraces. This analysis revealed significant differences in grain traits ($P < 0.05$), with larger kernel length and kernel width, and higher thousand kernel weight in *TaPYL1-1B*$^{In\text{-}442}$ genotypes across two years and two growing regions (Ma *et al*., 2016) (Figure 8e). We next conducted association analysis using a larger panel of MC to confirm the relationship between kernel traits and InDel-442 alleles using the same growing season data. We again found that kernel length, kernel width and thousand kernel weight were significantly different between genotypes carrying the drought-tolerant or sensitive alleles ($P < 0.05$) and further found that *TaPYL1-1B*$^{In\text{-}442}$ lines in the MC panel exhibited significantly higher kernel number than *TaPYL1-1B*$^{Del\text{-}442}$ in both growing seasons ($P < 0.05$), though other spike-related traits did not (Figure 8f). Furthermore, comparison of kernel traits between C3 and WT revealed that the CRISPR-Cas9-mediated 20-bp deletion (Del-442) also resulted in decreased kernel size (Figure S11). Collectively, these results confirmed that the *TaPYL1-1B*$^{In\text{-}442}$ allele was significantly associated with larger grain size and higher TKW.

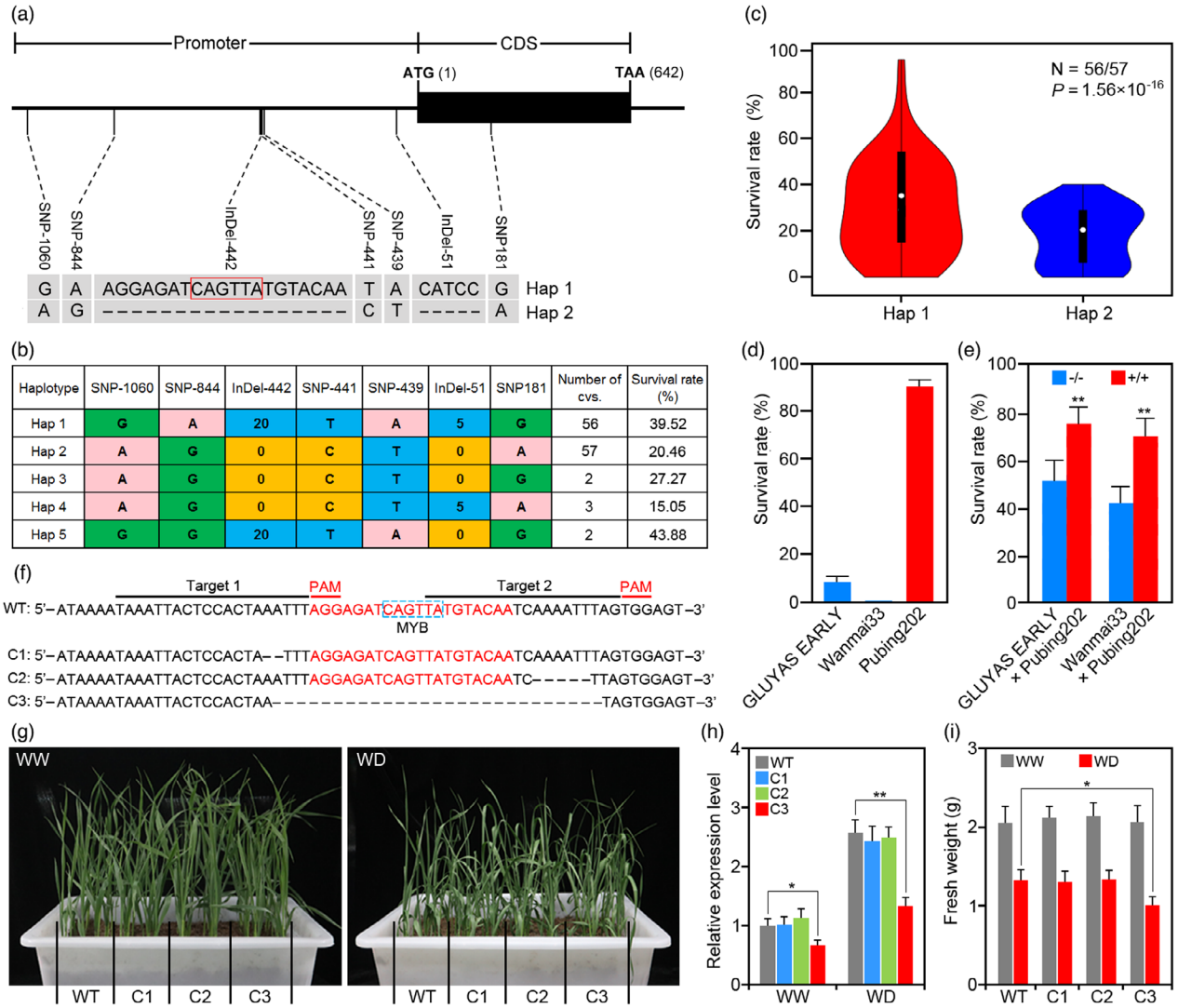
TaMYB70 targets In-442 variant to enhance *TaPYL1-1B* expression

Since the majority of the genetic variations were found to be located in the promoter, we quantified the expression of *TaPYL1-1B* using qPCR under well-watered and drought conditions for 21 wheat varieties harbouring the tolerant allele (*TaPYL1-1B*$^{In\text{-}442}$) and 23 varieties with the sensitive one (*TaPYL1-1B*$^{Del\text{-}442}$). We found that expression of the *TaPYL1-1B* tolerant alleles was significantly higher than that of the sensitive alleles under drought stress ($P < 0.01$), whereas no significant differences were observed under well-watered conditions (Figure 9a). To further explore how SNPs/indels potentially alter *TaPYL1-1B* promoter activity, we scrutinized the location of the six SNPs/indels to determine if they disrupted or altered the sequence of promoter *cis*-elements. Notably, we identified an MYB recognition site (MYBR, CAGTTA) located within a 20-bp insertion variant of InDel-442, while other variants were not located within *cis*-elements, nor led to any clear changes in *cis*-element function (Figure 7a; Figure 9b). Importantly, promoter sequence analysis revealed two distinct MITE insertions belonging to the *Tc1/Mariner* superfamily that harboured Del-442 and In-442 alleles, respectively (Figure S12), indicating that InDel-442 is most likely derived from transposon insertions.

Further yeast one-hybrid assay and EMSA assays revealed that a stress-induced TaMYB70 can bind directly to the MYBR element (CAGTTA) in a 20-bp insertion variant of InDel-442 (Figure S13; Figure 9c,d). To verify whether the 20-bp indel in the promoter region affected the *TaPYL1-1B* gene expression, we constructed a series of tolerant and sensitive allele promoter deletion fragments to remove other *cis*-elements, with the smallest fragment carrying InDel-442 but excluding other elements to emphasize its effect on transcription (Figure 9b). These truncated fragments were then inserted upstream of a *LUC* reporter vector construct to compare differences in transcriptional activation in tobacco leaves (Figure 9e). The LUC activities were significantly greater in tobacco leaves co-expressing the reporter carrying the *TaPYL1-1B*$^{In\text{-}442}$ promoter fragments driving LUC and the effector containing TaMYB70 than in those carrying the *TaPYL1-1B*$^{Del\text{-}442}$ promoter fragments (Figure 9f), suggesting that the 20-bp insertion containing the MYB-binding site possibly induces allelic differences in *TaPYL1-1B* promoter activity.

Discussion

There are many factors involved in ABA signalling, and the precise expression of which is controlled at the transcriptional level. For instance, exogenous ABA treatments can significantly induce the



Haplotype	SNP-1060	SNP-844	InDel-442	SNP-441	SNP-439	InDel-51	SNP181	Number of cvs.	Survival rate (%)
Hap 1	G	A	20	T	A	5	G	56	39.52
Hap 2	A	G	0	C	T	0	A	57	20.46
Hap 3	A	G	0	C	T	0	G	2	27.27
Hap 4	A	G	0	C	T	5	A	3	15.05
Hap 5	G	G	20	T	A	0	G	2	43.88

Figure 7 Genetic variations in *TaPYL1-1B* and their association with wheat drought tolerance (a) Distribution of DNA polymorphisms within the *TaPYL1-1B* promoter and the coding sequence region. The red frame indicates an MYB-binding sequence. (b) Haplotype analysis of *TaPYL1-1B* genotypes among 120 wheat varieties based on seven SNPs/indels. (c) Comparison of drought tolerance between wheat varieties carrying Hap 1 and Hap 2 genotypes. (d) Survival rates of wheat *cv*. Pubing202, Wanmai33 and GLUYAS EARLY plants under severe drought stress. (e) The survival rates of the F_4 individuals carrying either the homozygous tolerant (+/+) or sensitive (-/-) allele of *TaPYL1-1B* in response to drought conditions. (f) Targeted mutagenesis of the 20-bp insertion via CRISPR-Cas9. Red labels indicate protospacer adjacent motif (PAM) sequences. Three independent lines were obtained harbouring deletions of the 20-bp insertion or its flanking sequence. (g) Phenotypic analysis of drought tolerance and (h) *TaPYL1-1B* relative expression levels in deletion mutants and WT plants under well-watered (WW) and water-deficit (WD) conditions. (i) Fresh weight of mutant and WT plants under WW and WD conditions. Data represent the mean ± SD of three replicates. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

expression of group A *PP2C* genes, of which the promoters were directly bound by the ABF transcription factors, causing negative feedback regulation of ABA signalling (Wang, Hou *et al*., 2019; Zhang Xu, *et al*., 2017). Drought, salt and ABA stressors can also induce the expression of many *PYLs* (He *et al*., 2018; Li *et al*., 2018). However, it is still not clear how *PYLs* are regulated at the transcriptional level. In our study, we found that wheat TaABFs function as positive regulators in the maintenance of the expression of *TaPYL1-1B* by directly binding to their promoters (Figure 1b–e), indicating the *PYLs* expression is also precisely regulated at the transcriptional level.

Due to their pivotal role in modulating transpiration, ABA receptors have been previously targeted as candidate genes for engineering increased drought tolerance and water productivity in *Arabidopsis* and wheat (Kim *et al*., 2014; Mega *et al*., 2019; Santiago *et al*., 2009; Yang *et al*., 2016, 2019; Yu *et al*., 2017). Based on this established function of *TaPYL1-1B* in transpiration, we extended the current understanding of *PYR1/PYL/RCAR* gene activity during response to drought in wheat. Through close examination of the effects *TaPYL1-1B* overexpression, particularly on traits related to drought and ABA response, we found that this gene heightens sensitivity to ABA (Figure 2), in agreement with observations of *PYL* gene impacts on ABA perception in other species (González-Guzmán *et al*., 2014; He *et al*., 2018; Ma *et al*., 2009; Park *et al*., 2009; Pri-Tal *et al*., 2017; Tian *et al*., 2015). We also found that higher CO_2 assimilation rates and lower transpiration rates, and hence elevated WUE (Figure 4a–d), were correlated with lower water consumption and higher soil water

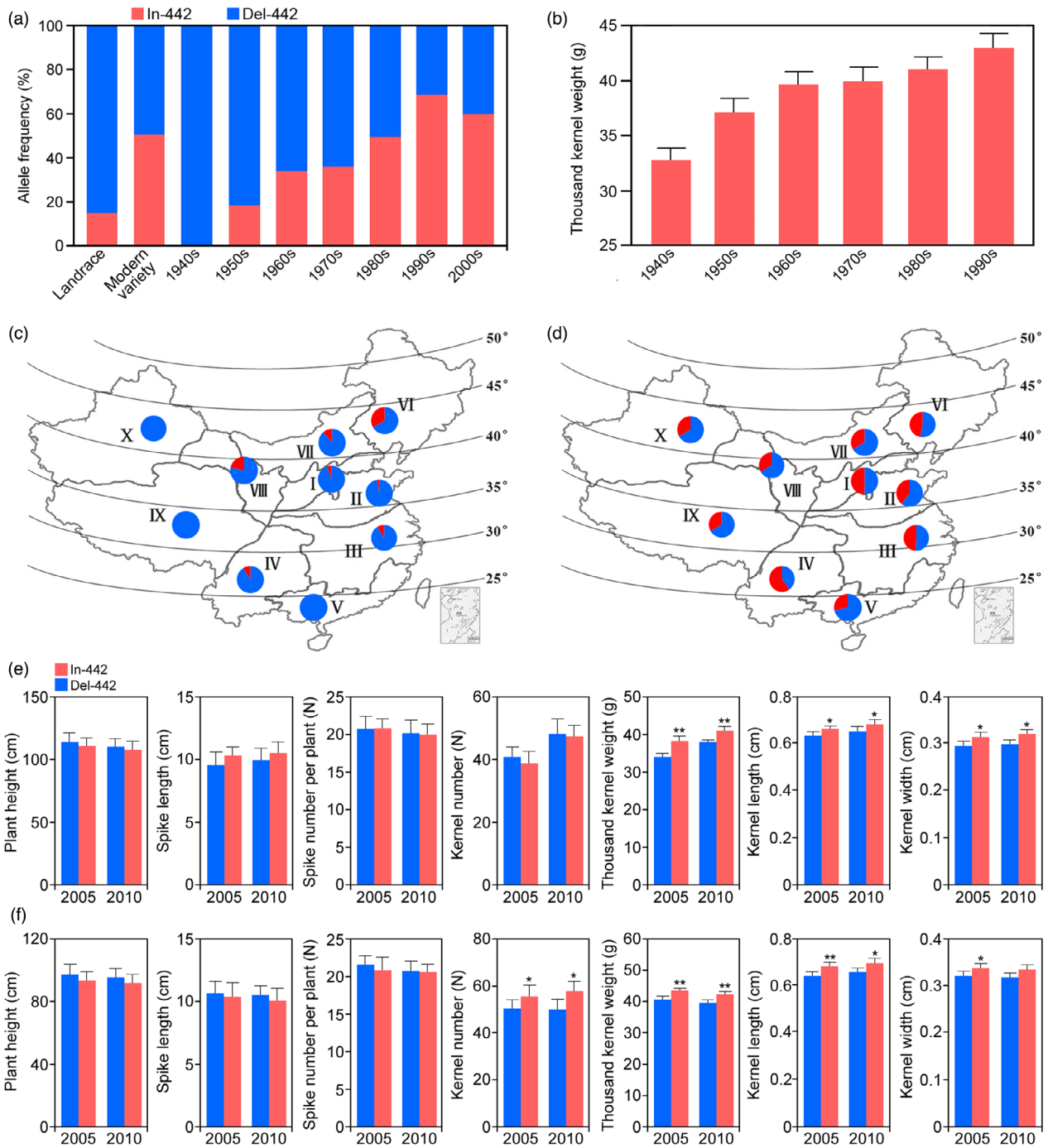


Figure 8 Distribution of *TaPYL1-1B*$^{In\text{-}442}$ allele in Chinese wheat cultivars and landraces and comparison of grain-related traits among *TaPYL1-1B* alleles (a) Frequencies of *TaPYL1-1B*$^{In\text{-}442}$ and *TaPYL1-1B*$^{Del\text{-}442}$ alleles in Chinese modern cultivars from the 1940s to 2000s. (b) Changes in TKW over decades in Chinese modern cultivars from the 1940s to 1990s. (c-d) Distribution of *TaPYL1-1B*$^{In\text{-}442}$ and *TaPYL1-1B*$^{Del\text{-}442}$ alleles in 157 landraces (c) and 348 modern cultivars (d) from 10 Chinese ecological zones. (e-f) Comparison of grain-related traits between *TaPYL1-1B*$^{In\text{-}442}$ and *TaPYL1-1B*$^{Del\text{-}442}$ alleles in 154 landraces (e) and 344 modern cultivars (f) in two growing regions. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

retention among *TaPYL1-1B* transgenic lines (Figure 4h). These phenotypic changes result from differential up-regulation of water stress response- and ABA response-associated genes (Figure 6b) and physiological changes including reduced stomatal aperture and water loss (Figure 3c–h) compared to changes observed in WT plants. This rapid stomatal closure correlated with reduced dehydration and transpiration rates was supported by previous studies showing a relationship between stomatal closure and rates of water consumption (Murata *et al.*, 2015). Collectively, these findings suggest a positive regulatory role for *TaPYL1-1B* in adaptation to drought stress in wheat.

While we closely examined the contributions of WUE and soil water retention in drought response, we also systematically explored the effects of *TaPYL1-1B* overexpression on yield-

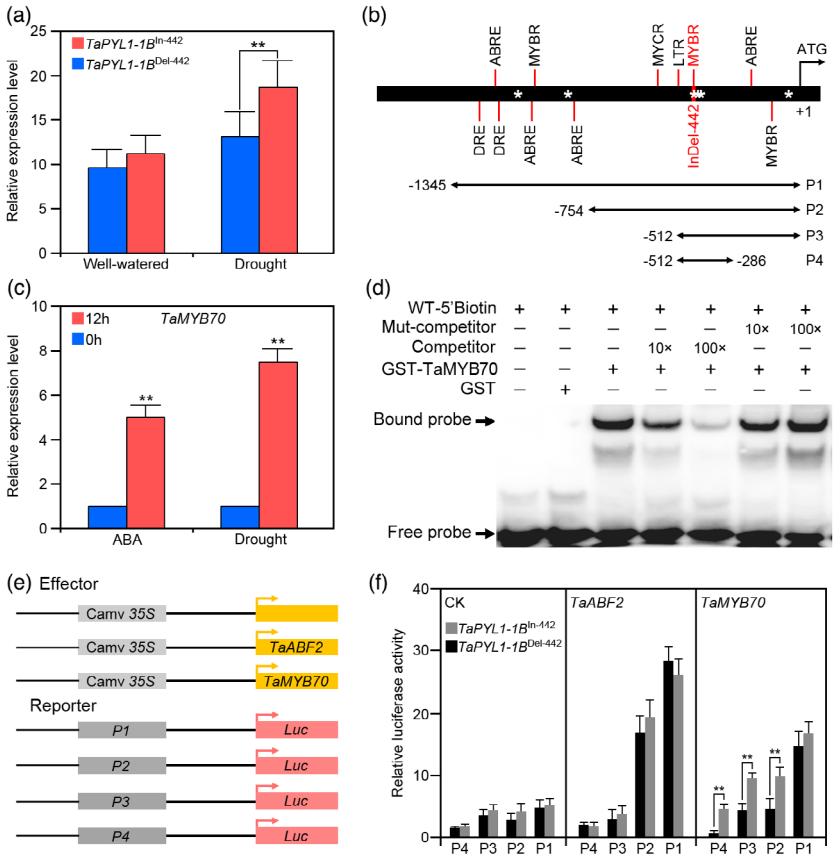


Figure 9 TaMYB70 target In-442 allele to enchance *TaPYL1-1B* expression (a) Comparison of *TaPYL1-1B* expression between the wheat varieties carrying *TaPYL1-1B*$^{In-442}$ and *TaPYL1-1B*$^{Del-442}$ genotypes. The gene expression level was determined among 44 wheat varieties under well-watered and drought stress conditions. Drought stress was estimated by the decrease in the relative leaf water content (RLWC) from 98% (well-watered) to 58% (severe drought). (b) Schematic diagram of the promoter fragment constructions. Six polymorphic markers in the promoter of *TaPYL1-1B* are indicated by white asterisks. (c) Analysis of *TaMYB70* expression levels by qPCR in 20% PEG and 100 μM ABA treated wheat *cv*. Chinese Spring seedlings. (d) EMSA assay to confirm the binding of TaMYB70 to MYBR element (CAGTTA) within InDel-442 in the *TaPYL1-1B* promoter in vitro. (e-f) TaMYB70 activates the transcription of *TaPYL1-1B* promoter. (e) A diagram showing the construction of reporter and different effector vectors. (f) Quantify the relative luciferase activity in tobacco leaves co-transfected with the reporter and different effector constructs. The co-infiltration of an empty effector vector and the reporter construct served as the negative control. Data represent the mean ± SD of three replicates. Statistical significance was determined by a Student's *t* test, * $P < 0.05$; ** $P < 0.01$.

associated traits. We found that *TaPYL1-1B* transgenic lines had slightly higher grain yields under well-watered conditions (Figure 5b,d) and significantly higher yields under water deficit (Figure 5c,e) than WT, in agreement with observations of *PYL* genes impact on grain yields in other species (Mega *et al*., 2019; Yang *et al*., 2016, 2019). We speculated that these yield traits were related to significant morphological differences, such as increased spike width, kernel number, kernel length and kernel width (Figure 5d,e), and that these quantitative grain traits were closely related to the increased WUE mediated by activation of ABA signalling. Similar effects of 'water-banking' on increased yield have been reported in both genetically engineered drought-tolerant maize and wheat lines (Cooper *et al*., 2014; Mega *et al*., 2019; Nemali *et al*., 2015; Wang *et al*., 2016), supporting our approach of ABA signal modulation as a viable strategy for crop yield improvement in water-limited environments.

Although many studies have investigated the plant molecular response to drought stress (Hu and Xiong 2014; Gong *et al*., 2020; Gupta *et al*., 2020; Zhu, 2016), the impacts of allelic sequence variation on differences in drought-tolerant phenotypes remain largely unexplored. To date, relatively few quantitative trait loci (QTLs) responsible for drought tolerance in cereal crops have been revealed through association analysis (Liu *et al*., 2013; Mao *et al*., 2015; Singh *et al*., 2015; Wang *et al*., 2016; Xiang *et al*., 2017; Wang and Qin 2017; Xiong *et al*., 2018; Mao *et al*., 2020; Zhang *et al*., 2020). In this study, candidate gene association analyses helped identify polymorphic SNPs/indels in the *TaPYL1-1B* promoter that were significantly associated with drought tolerance in wheat seedlings, the presence of which enabled classification of *TaPYL1-1B* variants into five haplotypes (Figure 7a,b). Among them, the two major haplotypes consisted of drought-tolerant (Hap 1) and drought-sensitive (Hap 2) alleles (Figure 7a–c). Thus, according to the polymorphism marker InDel-442 (Figure S9), two alleles of *TaPYL1-1B* were distinguishable and supporting our findings that varieties carrying *TaPYL1-1B*$^{In-442}$ exhibited relatively higher drought tolerance than those carrying *TaPYL1-1B*$^{Del-442}$ (Figure 7).

Moreover, our sequence analyses revealed that the *TaPYL1-1B*$^{In-442}$ favourable allele was present in hexaploid wheat landraces with lower allele frequency, while in MC with higher

frequency (Figure 8a). This finding suggests that *TaPYL1-1B*$^{In\text{-}442}$ has been strongly selected during modern wheat improvement (Figure 8b), a hypothesis supported by the increasing frequency of the *TaPYL1-1B*$^{In\text{-}442}$ allele among MC over time across 10 Chinese ecological zones compared to its lower frequency among landrace populations (Figure 8c,d). This expansion in *TaPYL1-1B*$^{In\text{-}442}$ distribution was further supported by meta-analyses of MC and CRISPR-Cas9 edited 20-bp deletion mutant which showed that *TaPYL1-1B*$^{In\text{-}442}$ allele was associated with significantly larger kernel size and higher TKW than for cultivars carrying *TaPYL1-1B*$^{Del\text{-}442}$ (Figure 8e,f; Figure S11), strongly reflecting a breeding history of selection for higher yield (Barrero *et al*., 2011). Similarly, several yield-related genes in wheat have reportedly undergone strong artificial selection during wheat polyploidization and genetic improvement, such as *TaSUS1*, *TaBT1*, *TaGS5*, *TaSPL20*, *TaSPL21* and *TaGW2* (Hou *et al*., 2014; Ma *et al*., 2016; Qin *et al*., 2017; Su *et al*., 2011; Wang Guo, *et al*., 2019; Zhang, Li *et al*., 2017).

In addition to identify the drought-tolerant allele of *TaPYL1-1B*, our study revealed a regulatory mechanism associated with the natural variation of wheat drought tolerance. Further expression analysis showed that the *TaPYL1-1B*$^{In\text{-}442}$ allele exhibited significantly higher expression in response to drought stress than those carrying *TaPYL1-1B*$^{Del\text{-}442}$ (Figure 9a). Searches of the PlantCARE database, in conjunction with EMSA and promoter activity assays, revealed a 20-bp promoter insertion allele, InDel-442, which carries a putative MYB TF recognition site, targeted by TaMYB70, that induces higher expression of *TaPYL1-1B* (Figure 7a; Figure 9). In addition, promoter sequence analysis revealed that the functional variation InDel-442 is harboured in two MITEs (Figure S12), indicating *TaPYL1-1B*$^{In\text{-}442}$ is likely a derived allele from transposon insertion. Similar to this finding in *TaPYL1-1B*, variation in the expression of several genes due to *cis*-elements or transposon insertion/deletions have also been reported to affect drought tolerance in maize in a dose-dependent manner, such as an ERSE (endoplasmic reticulum stress response element) deletion in *ZmPP2C-A10*, an 82-bp transposable element insertion in *ZmNAC111*, and a 366-bp insertion in *ZmVPP1* (Mao *et al*., 2015; Wang *et al*., 2016; Xiang *et al*., 2017).

The data presented herein allow for the proposal of a working model illustrating that a component in the core ABA signalling pathway, *TaPYL1-1B*, acts as a critical regulator in drought tolerance, and its allelic variation in the promoter sequence contributes to drought tolerance and grain yield in wheat (Figure 10). In light of our results, we propose that stacking the *TaPYL1-1B*$^{In\text{-}442}$ favourable allele with other genes related to high yield and drought tolerance can potentially lead to elite wheat breeding lines with highly desirable grain traits as well as a decrease in the usage of water.

Materials and Methods

Plant materials and growth conditions

To obtain *TaPYL1-1B* overexpression (OE) wheat lines, the *TaPYL1-1B* (*TraesCS1B02G206600*) coding region was amplified from the wheat *cv*. Chinese Spring and the sequencing confirmed PCR product was cloned into a plant binary vector pCAMBIA3301 under the control of the ubiquitin promoter (Mao *et al*., 2020). The wild-type (WT) wheat *cv*. Fielder plants were then transformed by *Agrobacterium tumefaciens* EHA105 carrying the expression vector and ultimately constructed 12 independent positive OE lines. Similar phenotypes were exhibited by all transgenic lines, and further analyses were conducted on three of them (OE4, OE5 and OE6).

For analysis of promoter mutants, a CRISPR-Cas9 expression vector driven by the TaU3 wheat promoter was used to generate edited mutants (Li *et al*., 2021). The plasmid was introduced by *A. tumefaciens*-mediated transformation into wild-type (WT) wheat cv. Fielder plants and resulted in construction of three independent mutant lines.

Other *Triticum aestivum* accessions utilized in this study included 120 hexaploid wheat global varieties (Mao *et al*., 2020), 154 wheat landraces from the Chinese wheat minicore collection (MCC) and 344 wheat MC released since the 1940s (Hao *et al*., 2008). All of these accessions were used to conduct the association analysis of phenotypic traits and markers. The drought tolerance of 120 wheat varieties was phenotyped in the cultivation pool according to the methods previously described (Mao *et al*., 2020). Agronomic traits, including kernel number per spike (KN), kernel length (KL), kernel width (KW), and thousand-kernel weight (TKW), were investigated for the MCC and MC populations at Luoyang, Henan Province, in 2005, and at Shunyi, Beijing, in 2010 (Ma *et al*., 2016).

Gene expression analysis by qRT-PCR

Wheat *cv*. Chinese Spring seeds were germinated on wet filter paper for 3 days at 20°C. The germinated seeds were then transferred to a hydroponic solution containing nutrients. Seedlings at the three-leaf-stage were cultivated at 20% PEG-6000 for drought treatment and in 100 μM ABA solution for ABA treatment. Total RNA was extracted using RNAiso Plus (Takara, Beijing, China) and reverse transcribed using M-MLV reverse transcriptase (Takara, Beijing, China) following the manufacturer's instructions. qRT-PCR was performed as described previously (Mao *et al*., 2020). Primers used in this study are listed in Supporting Information Table S5.

Germination and seedling growth assay

Seed germination assay was conducted using fully filled and uniform seeds of WT and transgenic OE lines. The seeds underwent sterilization for 1 min in 70% (v/v) ethanol solution and washed three times with sterile water, then were sown on sterile filter paper in Petri dishes containing various concentrations of ABA. The petri dishes were then subsequently transferred to a growth chamber at 20°C with a 16-h light/8-h dark cycle. Seed germination was considered as the coleoptile was occurred. For the seedling growth assay, solutions containing various ABA concentrations were prepared, and the germinated seeds of WT and transgenic OE lines were placed. Shoot and root lengths were measured after 10 days.

Drought tolerance and water loss assay

Drought tolerance assay was performed in individual plastic box (35.0 cm × 20.0 cm × 15.0 cm) filled with a mixture of soil: vermiculite (2:1 ratio). Each T_3 transgenic OE line was planted alongside WT (32 plants in each line) in the same box and grown at 16-h light/8-h dark, 14/12°C conditions. Three-leaf-stage seedlings grown in soil were subjected to ~ 30 days of drought conditions, after which they were watered for 3 days to allow recovery. The surviving plants were then counted and photographed. Three independent biological replicates were completed for this analysis. For the leaf water loss assay, the leaves of 30-day-old plants were detached and dehydrated at room temperature (~ 20°C) with 40% relative humidity, which was

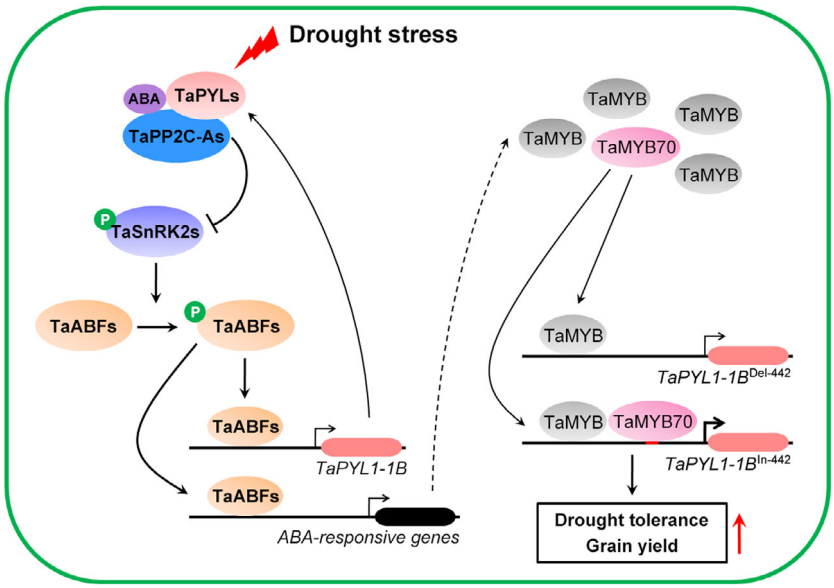


Figure 10 A proposed working model depicts the critical role of *TaPYL1-1B* in regulating ABA signalling and drought tolerance, and its favourable allele *TaPYL1-1B*$^{In-442}$ contributes to higher drought tolerance and grain yield in wheat. P, phosphorylation. Arrows, promotion; bars, inhibition. Dashed line depicts predicted regulation.

weighed at predetermined times. The water loss rate was calculated as the percentage of total weight lost at each dehydration time point compared with the initial weight, using 10 leaves from each transgenic OE line or WT.

Soil water content and photosynthesis measurement

WT plants and *TaPYL1-1B* transgenic OE lines were grown in plastic boxes (0.6 kg of soil:vermiculite mixture; 35.0 cm × 20.0 cm × 15.0 cm). The boxes were kept at a relative humidity of 60%, day/night temperatures of 14/12°C and a light/dark photoperiod of 16/8 h, and they did not have holes for drainage. The surface of the soil was covered with aluminium foil to mitigate evaporative water loss and openings through which plants could grow were created. The WT plants and transgenic OE plants received equal amounts of water until the three-leaf stage, at which time irrigation was stopped. Once water withholding began, soil water content (SWC) was documented every other day using a 10HS soil moisture sensor. Photosynthesis was measured using a LiCor-6800 portable photosynthesis system (LI-COR, Lincoln, NB, USA) on the third fully expanded leaves from *TaPYL1-1B* transgenic and WT plants. To analyse the CO_2 response curves, the leaves of the three-leaf stage plants were first acclimatized for 1 h at 2000 μmol m^{-2} s^{-1} of red and blue light and 400 ppm CO_2, and then slowly adjusted from 0 to 100, 200, 400, 600, 800, 1000, 1200, 1500, 2000 and finally 2500 ppm. Statistical data were based on 10 seedlings for each line, and the experiment was repeated for three times.

RNA-seq analysis

Transcriptome analysis on the leaves from hydroponically cultivated 20-day-old WT and *TaPYL1-1B* transgenic plants was performed after exposing them to each of three conditions, that is under 20% PEG-6000 incubated for 6 h, 100 μM ABA treated for 6 h, and under well-watered conditions. Total RNA of each sample was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions and was evaluated for quality and quantity with Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). Library preparation and RNA sequencing were performed according to the experimental manual. RNA-seq data were analysed as previously described (Ramírez-González *et al.*, 2018). Genes with corrected-$P < 0.01$ and an absolute fold change > 2 were considered significantly differentially expressed. Gene ontology (GO) categories with genes that were significantly up- or down-regulated were identified using GOseq R package (Young *et al.*, 2010).

TaPYL1-1B gene association analysis

One hundred twenty representative wheat varieties were used to conduct association analysis based on corresponding phenotypic drought tolerance (SR) data. The genetic variations of *TaPYL-1B* were obtained by amplifying and sequencing the 5′ - and 3′-UTR sequences, coding region (including introns) and the ~ 1.5 kb promoter of *TaPYL1-1B*. These sequences were assembled using DNAMAN and aligned using MEGA version 7.0. Then, nucleotide polymorphisms, such as InDels and SNPs, from these genotypes were identified (MAF ≥ 0.05), and their association with SR was analysed using the MLM model and the TASSEL 5.0 program (Bradbury *et al.*, 2007).

Yeast-one-hybrid assay

Effectors were generated by amplifying the coding sequences of *TaABFs* and *TaMYBs* from wheat *cv.* Chinese Spring and independently fusing them with the activation domain of the pB42AD vector (Clontech, Mountain View, CA, USA). The *TaPYL-1B* promoter fragments were amplified and cloned into the pLacZi vector (Promega, Madison, WI, USA), driving *LacZ* reporter expression. The yeast strain EGY48 was created by the co-transformation of effector and reporter plasmids, and it was then cultured on SD/−Trp/−Ura dropout medium containing X-gal at 30°C for blue colour development. Empty pB42AD and pLacZi served as negative controls.

Gel mobility shift assay

Using wheat *cv.* Chinese Spring, the coding regions of *TaABF2* and *TaMYB70* were amplified and cloned into the pGEX4T-1 vector, then transformed into the *E. coli* strain BL21 (Promega, Madison, WI, USA). GST as well as GST-TaABF2 and GST-TaMYB70 fusion proteins were purified following the

manufacturer's protocol. A biotin labelling kit (Invitrogen, Waltham, MA, USA) was used to amplify and label DNA probes, and following the manufacturer's instructions, a LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA) was used to perform shift assays of gel mobility.

Relative luciferase (LUC) activity assay

Using wheat *cv*. Chinese Spring, approximately 1.5 kb of the *TaPYL-1B* promoter and truncated promoter fragments were amplified, then cloned into pGreenII 0800-LUC (Promega, Madison, WI, USA) to generate the reporter construct and drive the *LUC* gene expression. The coding regions of *TaABFs* and *TaMYB70* were amplified from Chinese Spring and cloned into pGreenII 62-SK (Promega, Madison, WI, USA) driven by the CaMV 35S promoter for generation of the effector constructs. The effector and reporter constructs were then co-transfected into tobacco leaves, which were used to measure the relative LUC activity levels by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manual.

Accession numbers

The raw reads of the RNA-seq have been deposited in the National Center for Biotechnology Information (NCBI) under the accession number SRR11573018-SRR11573026.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

H.M., X.Z. and Z.K. designed the research; H.M., C.J., X.C., B.C., F.M., F.L., Y.Z., S.L., L.D., T.L., C.H. and X.W. performed the experiments; H.M., C.J., X.C. and B.C. analysed the data; H.M., X.Z. and Z.K. wrote the manuscript with contributions from all authors.

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Supporting information

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

Figure S1 Phylogeny and protein sequences of TaPYL1-1A, TaPYL1-1B and TaPYL1-1D.

Figure S2 Subcellular localization of TaPYL1 homeologs.

Figure S3 Screening for ABRE and MYB cis-elements in the TaPYL1-1B promoter.

Figure S4 Drought tolerance assessment of WT and Ubi:TaPYL1-1B transgenic lines cultivated in 30% PEG solution.

Figure S5 Comparation of grain-quality related traits between WT and transgenic lines under well-watered conditions.

Figure S6 Venn diagrams of up- or down-regulated genes in TaPYL1-1B transgenic OE4 and OE5 lines relative to WT plants under normal, drought and ABA conditions using a significance cutoff of $P < 0.01$ and a fold change (FC) > 2.

Figure S7 qRT-PCR verification of increased expression of eight genes involved in drought and ABA response in TaPYL1-1B transgenic wheat lines.

Figure S8 Identification and molecular characterization of drought tolerance gene TaPYL1-1B in wheat.

Figure S9 Molecular marker development based on InDel-442 variant.

Figure S10 Phenotypic analysis of drought tolerance of CRISPR-Cas9 mutant lines and WT plants under well-watered, water-deficit and re-watering conditions.

Figure S11 Agronomic traits of WT and CRISPR-Cas9 edited C3 mutant under well-watered conditions.

Figure S12 The DNA sequence and structure of MITE insertions in the TaPYL1-1BIn-442 and TaPYL1-1BDel-442 promoters.

Figure S13 Identification of drought-responsive MYB genes in wheat and validate their binding ability to TaPYL1-1B promoter fragment.

Table S1 Significantly down-regulated genes in Ubi:TaPYL1-1B transgenic wheat grown under well-watered conditions.

Table S2 Significantly down-regulated genes in Ubi:TaPYL1-1B transgenic wheat grown under drought conditions.

Table S3 Significantly down-regulated genes in Ubi:TaPYL1-1B transgenic wheat grown under ABA treated conditions.

Table S4 Variations in the TaPYL1-1B genomic region and their association with wheat drought tolerance.

Table S5 Primers used in this research.

