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DREB/CBF expression in wheat and barley using the stress-inducible promoters of *HD-Zip I* genes: impact on plant development, stress tolerance and yield

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Summary

Networks of transcription factors regulate diverse physiological processes in plants to ensure that plants respond to abiotic stresses rapidly and efficiently. In this study, expression of two *DREB/CBF* genes, *TaDREB3* and *TaCBF5L*, was modulated in transgenic wheat and barley, by using stress-responsive promoters *HDZI-3* and *HDZI-4*. The promoters were derived from the durum wheat genes encoding the γ-clade TFs of the HD-Zip class I subfamily. The activities of tested promoters were induced by drought and cold in leaves of both transgenic species. Differences in sensitivity of promoters to drought strength were dependent on drought tolerance levels of cultivars used for generation of transgenic lines. Expression of the *DREB/CBF* genes under both promoters improved drought and frost tolerance of transgenic barley, and frost tolerance of transgenic wheat seedlings. Expression levels of the putative *TaCBF5L* downstream genes in leaves of transgenic wheat seedlings were up-regulated under severe drought, and up- or down-regulated under frost, compared to those of control seedlings. The application of *TaCBF5L* driven by the *HDZI-4* promoter led to the significant increase of the grain yield of transgenic wheat, compared to that of the control wild-type plants, when severe drought was applied during flowering; although no yield improvements were observed when plants grew under well-watered conditions or moderate drought. Our findings suggest that the studied *HDZI* promoters combined with the DREB/CBF factors could be used in transgenic cereal plants for improvement of abiotic stress tolerance, and the reduction of negative influence of transgenes on plant development and grain yields.

Introduction

Drought and low temperature are two significant abiotic stress factors limiting the yields of staple crops globally. To survive under harsh environments, plants need to provide rapid responses to these stress factors. The environmental stimuli are perceived by receptors and sensors such as cytoskeleton and hydroxyproline-rich and arabinogalactan glycoproteins (Humphrey *et al*., 2007; Luan, 2002; Śniegowska-Świerk *et al*., 2015; Thion *et al*., 1996). These stimuli are converted into intracellular signals by second messengers such as Ca^{2+} (Cao *et al*., 2017; Cheong *et al*., 2003; Klimecka and Muszynska, 2007; Knight *et al*., 1997; Sanders *et al*., 2002; Urao *et al*., 1994) that trigger regulatory networks through abscisic acid (ABA)-dependent and ABA-independent pathways, which guide diverse physiological changes in metabolism to provide plant adaptation and/or tolerance to detrimental influences of stresses (Heidarvand and Amiri, 2010; Kidokoro *et al*., 2017; Shinozaki *et al*., 2003; Todaka *et al*., 2017; Yang *et al*., 2011).

Two groups of genes involved in abiotic stress regulatory networks have been identified (Gong *et al*., 2015; Hu *et al*., 2007; Sazegari *et al*., 2015; Yang *et al*., 2016). The first group is represented by functional genes, whose expression is initiated or altered by stress-related transcription factors (TFs), and the final products of these genes are directly involved in biochemical and physiological changes required for stress acclimations (Nakashima *et al*., 2014; Novillo *et al*., 2011; Shinozaki *et al*., 2003). The second group comprises regulatory genes, which include

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numerous genes encoding TFs that carry out up- or down-regulation of downstream cascades of regulatory and functional genes (Harris *et al*., 2011; Pujol and Galaud, 2013; Raza *et al*., 2016; Smith, 2000). Significant changes in transcriptomes in response to environmental stresses and hence use of stress-related TFs are among effective strategies adopted by plants to deal with unfavourable growth conditions.

The APETALA2 (AP2)/ethylene-responsive element-binding (ERF) group is a superfamily of TFs with the majority of members involved in abiotic and/or biotic stress responses. The superfamily of AP2/ERF is classified into five groups, which are represented by the following subfamilies: AP2, ERF, RAV, DREB/CBF, and the subfamily of other TFs (Agarwal *et al*., 2017; Sakuma *et al*., 2002). The drought-responsive element binding (DREB) factors comprise a subfamily of the AP2/ERF family of TFs containing single AP2 DNA-binding domains, which recognize six nucleotides (A/G)CCGAC of the dehydration-responsive element/C-repeat (DRE/CRT). These *cis*-elements are located on promoter regions of target stress-responsive genes and play an important role in regulation of stress-inducible transcription (Agarwal *et al*., 2017; Bouaziz *et al*., 2015; Hrmova and Lopato, 2014; Sakuma *et al*., 2002).

Numerous TFs belonging to the DREB/CBF subfamily have been reported to enhance the stress durability of transgenic plants by regulating stress-responsive downstream genes, if overexpressed under the control of strong constitutive promoters (Agarwal *et al*., 2017; Ban *et al*., 2011; Chen *et al*., 2007; Sarkar *et al*., 2014; Xianjun *et al*., 2011). However, constitutive overexpression of stress-related regulatory genes often leads to severe growth retardation and/or a grain yield decrease under normal growth conditions (Agarwal *et al*., 2017; Kasuga *et al*., 1999; Lopato and Langridge, 2011; Morran *et al*., 2011). Several promoters of stress-inducible functional genes such as *rd29A* (Kasuga *et al*., 2004; Mallikarjuna *et al*., 2011), *HVA22* (Lee *et al*., 2003), *ZmRab17* (Morran *et al*., 2011) and *TdCor39* (Kovalchuk *et al*., 2013), and promoters of stress-inducible regulatory genes such as *LIP19* (Nakashima *et al*., 2007), *OsNAC6* (Nakashima *et al*., 2007) and *OsWRKY71* (Kovalchuk *et al*., 2013), including the promoter of the rice γ-clade *HD-Zip I* gene *Oshox24* (Nakashima *et al*., 2013), reduce the negative effects of overexpressed TFs on plant growth and/or yield. Therefore, finding and testing novel stress-inducible promoters for optimization of expression levels of transgenes is one of the critical methodologies to improve plant developmental phenotypes and yields (Agarwal *et al*., 2017; Hrmova and Lopato, 2014).

It was demonstrated that *HD-Zip I* genes from wheat, *TaHDZipI-3* and *TaHDZipI-4*, are stress-responsive and hence their promoters can be potentially used for moderate stress-inducible transgene expression in transgenic plants. It was shown that the *TaHDZipI-4* gene can be induced by ABA, drought and cold, while the *TaHDZipI-3* gene was induced by drought, but no significant responses of this gene on the elevated levels of ABA or cold were detected (Harris *et al*., 2016). Therefore, the promoters of *TaHDZipI-3* and *TaHDZipI-4* were expected to have different properties and hence could serve as the candidates of the moderate strength stress-inducible promoters for molecular breeding.

In this work, the promoters of the wheat γ-clade of *HD-Zip I* genes, *TdHDZipI-3* and *TdHDZipI-4*, were isolated from durum wheat (*Triticum turgidum* ssp. *durum*) and are designated *HDZI-3* and *HDZI-4*, respectively. *HDZI-3* and *HDZI-4* promoters were used to optimize *TaCBF5L* expression in transgenic wheat, and *TaDREB3* in transgenic barley, under two abiotic stresses drought and cold. We demonstrate that in contrast to the findings on the expression levels of *TaHDZipI-3* and *TaHDZipI-4* genes from *Triticum aestivum* (Harris *et al*., 2016), both *HDZI-3* and *HDZI-4* promoters from *T. turgidum* ssp. *durum* were induced by drought and cold. Furthermore, these two promoters had low levels of expression in unstressed wheat. Based on our study, *DREB/CBF* transgene expression under *HDZI-3* and *HDZI-4* promoters led to the improvement of drought and/or frost tolerance of transgenic barley and wheat. Aberrant development was observed in some transgenic lines, but it did not correlate with transgene expression levels. The use of the *HDZI-4* promoter in combination with the *TaCBF5L* gene significantly increased the grain yield of transgenic wheat under severe drought during flowering.

Results

Isolation of the TaCBF5L and TaDREB3 genes and phylogenetic relationships of their products to other DREB/CBF TFs

A 687-bp long cDNA of *TaCBF5L* containing a full-length coding region was isolated from roots of drought-stressed bread wheat (*T. aestivum* L. genotype RAC875), using a yeast-one-hybrid (Y1H) screen with the drought-responsive element (DRE) sequence as a bait. *TaDREB3* was isolated from the developing grain of the same wheat genotype as a bait sequence (Lopato *et al*., 2006). The phylogenetic reconstruction of phylogeny of DREB TFs at protein levels was performed with the neighbour joining algorithm in MEGA 6.06 (Tamura *et al*., 2013). The reconstruction of phylogeny showed clear subdivisions among different groups of DREB TFs from wheat, maize, rice, barley and *Arabidopsis* (Figure S1), whereby the TaCBF5L and TaDREB3 proteins allocated to the same subclade of subgroup C.

Based on the reconstruction of phylogeny (Figure S1), TaCBF5L shows a closer evolutionary relationship with TaCBF5 (84% sequence identity-SI), TdDREB3 (77% SI), HvCBF5 (76% SI), TaDREB3 (74% SI), TmCBF5 (74% SI) and ZmDBP4 (64% SI), compared to other entries in the tree (Figure S1, Table S1). The analysis of the multiple sequence alignment of six close homologous proteins with TaCBF5L revealed that both TaCBF5L and TaDREB3 contained an APETALA 2 (AP2) DNA-binding domain of 35 amino acid residues and the well-conserved PKKPAGR motifs (PKK/RPAGRxKFxETRHP), positioned at the N-termini of proteins. Additionally, the LWSY motif was identified to be a conserved motif that was positioned at the C-termini of proteins (Figure S2).

Expression levels of TaHDZipI-3 and TaHDZipI-4 in different wheat tissues during different stages of plant development

The spatial expression patterns of two wheat γ-clade HD-Zip I genes, *TaHDZipI-3* and *TaHDZipI-4*, were investigated in a variety of wheat tissues (Figure 1a). The highest expression levels of both genes were seen in bract and pistil tissues suggesting that they play roles during floral development. In other tissues under well-watered conditions, the two *TaHDZipI-3* and *TaHDZipI-4* genes demonstrated a relatively low level of basal expression (Figure 1a).

It is considered that the γ-clade HD-Zip I TFs contribute to growth modulation under water deficit and that in *Arabidopsis* this translates to reduced stem elongation (Harris *et al*., 2011). As DREB/CBF TFs also contribute to growth modulation under water deficit, expression of the two *TaHDZipI-3* and *TaHDZipI-4* γ-clade genes was

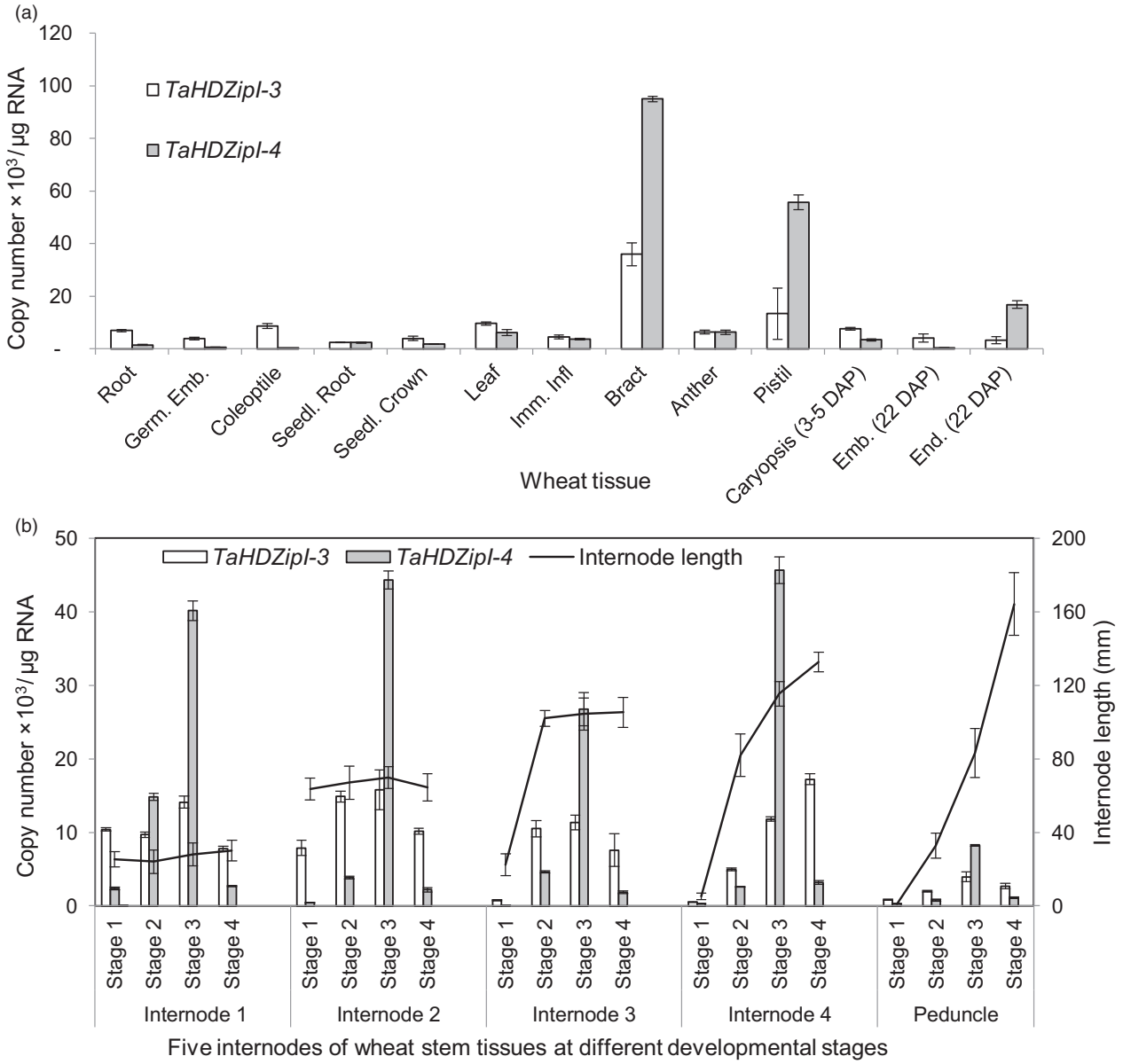

Figure 1 Levels of *TaHDZipI-3* and *TaHDZipI-4* expression in different wheat tissues and at different stages of stem development. (a) Levels of *TaHDZipI-3* and *TaHDZipI-4* expression in different tissues of wheat cv. Chinese Spring. ERF—ethylene-responsive element-binding; Emb. (22DAP)—Embryo 22 days after pollination; End. (22 DAP)—Endosperm 22 days after pollination; Germ. Emb.—Embryo in germinating seed; Imm.Infl—Immature inflorescence; Int—Internode; Seedl. crown—Seedling crown; Seedl. Root—Seedling root. (b) Spatial expression patterns of *TaHDZipI-3* and *TaHDZipI-4* expression in four stem internodes (Int) below peduncle (Ped) at four different stages of wheat (*T. aestivum* cv. RAC875) development. Internode length parameters are plotted against the secondary vertical axis. Stem stages are as follows: Stage 1 (100 mm); Stage 2 (300 mm) awns emerging; Stage 3 (400 mm) head emerging; Stage 4 (500 mm) at anthesis; peduncle emerged. Error bars represent the standard deviation of three biological replicates (a) and three technical replicates (a).

characterized in the internodes of wheat stem at different stages of development to explain any differences in the stem length that may have been observed in transgenic plants, where the expression level of DREB/CBF TFs was controlled by *HDZI* promoters. Expression was investigated in stem internodes over four different developmental stages: Stage 1 (100 mm); Stage 2 (300 mm)—awns emerging; Stage 3 (400 mm)—head emerging; Stage 4 (500 mm)—anthesis and peduncle emergence. Internodes 1–4 and the peduncle were at different stages of elongation/maturation at each stem stage development, enabling us to establish correlations between the expression levels of the two γ-clade *HD-Zip I* genes, and internode elongation and maturation. The analyses of stem developmental series revealed that the two wheat γ-clade HD-Zip I TFs were expressed differentially, both spatially and temporally, during normal stem development (Figure 1b).

The level of *TaHDZipI-3* expression was associated with the maturity of any given internode. At stage 1, internodes 1 and 2 have reached their final length and expressed *TaHDZipI-3* to relatively high levels, compared to internodes 3, 4 and the peduncle, which just started to elongate. Likewise, internode 3 reached its final length by awns emerging (stage 2) and *TaHDZipI-3* expression remained steady through stage 3 and stage 4. However, the four-time harvest points were not sufficient to determine the final length of internode 4 and the peduncle,

however, internode 4 showed increases in *TaHDZipI-3* expression as length of stem increased, whereas expression in the peduncle remained at lower levels (Figure 1b).

Expression of *TaHDZipI-4* showed a steady pattern in all five internodes, although there was an increase of gene expression from stage 1 to reach maximal expression at stage 3, followed by a dramatic decrease at stage 4 (Figure 1b).

Expression of TaCBF5L and TaDREB3 driven by HDZI-3 or HDZI-4 promoters in transgenic wheat and barley sublines, under rapid dehydration and under various drought conditions

Expression levels of *TaCBF5L* were detected in leaves of T_2 lines of transgenic wheat under well-watered and after 6-h dehydration treatments; these data were compared with those of control wild-type (WT) plants, using northern blot hybridization (Figure 2a). Expression levels of *TaDREB3* were assessed in leaves of transgenic T_1 barley lines grown under hydroponic conditions, before application of stress conditions and after 7-h incubation of seedlings without growth media components, and compared with those of control WT plants, using northern blot hybridization (Figure 2b). The results of these comparisons showed that expression of transgenes *TaCBF5L* and *TaDREB3*, controlled by either of the tested *HDZI-3* or *HDZI-4* promoters, was much stronger under dehydration than the expression levels under well-watered conditions in both wheat and barley (Figure 2). In contrast, both the *TaCBF5L* endogenous gene of WT wheat plants and *HvDREB3* endogenous gene of WT barley plants showed either very weak or undetectable hybridization signal under applied experimental conditions independently of whether RNA was isolated from leaves collected before or after dehydration.

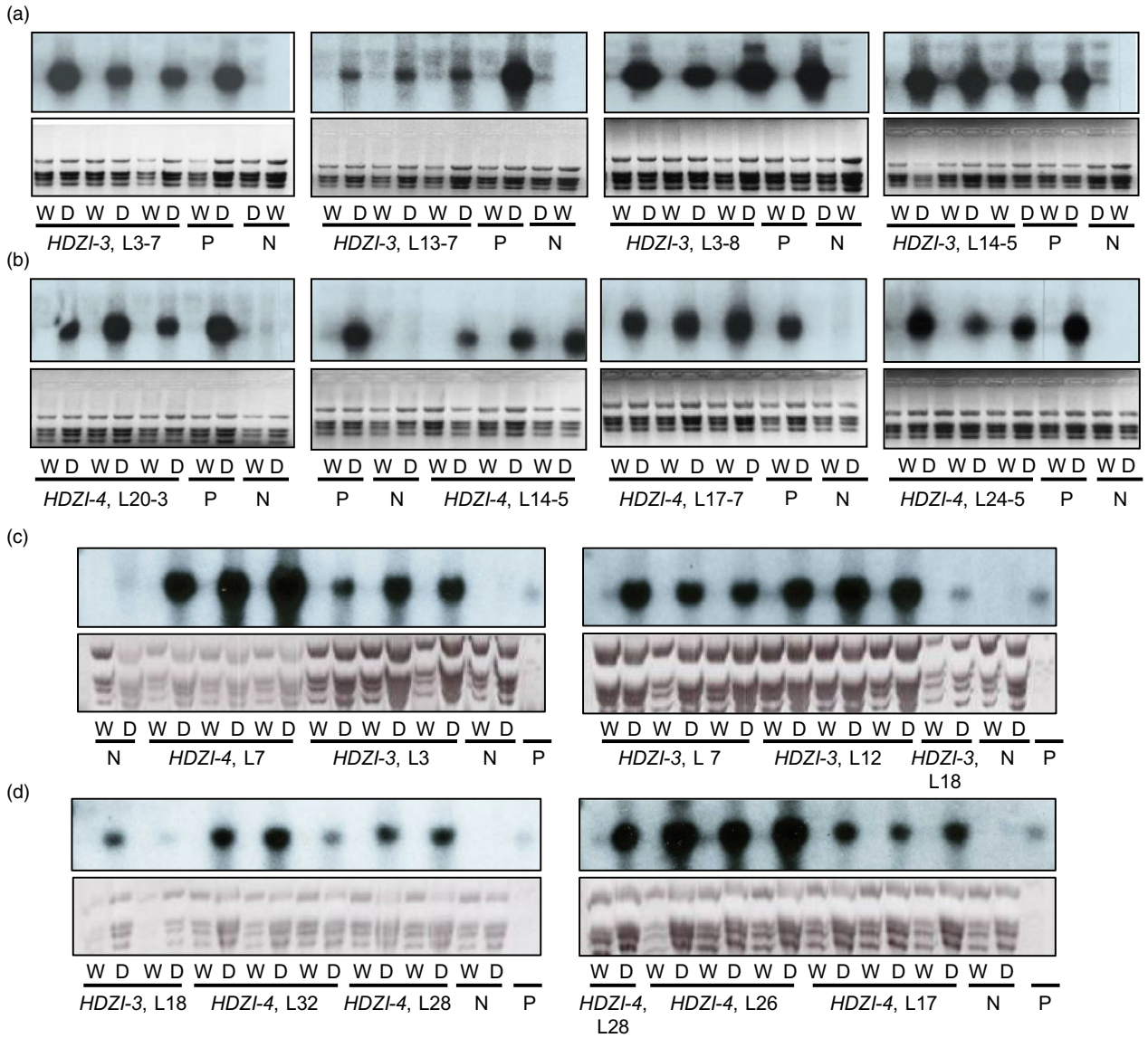

Figure 2 Induction of wheat *HDZI-3* and *HDZI-4* promoters in leaves of 3-week-old control and transgenic T_2 wheat seedlings (a) and in control and transgenic T_1 barley seedlings of the same age (b) before (W) and after 6 h of dehydration (d). N: WT plants with the endogenous *TaCBF5L or HvDREB3* genes either cannot be seen or seen as a weak band under both well-watered and dehydration conditions, and therefore were used as negative control; P: transgenic wheat plants with *TaCBF5L* transgene showing a strong band under dehydration conditions (a and b), and/or a 1000-fold diluted purified DNA fragment of the *TaDREB3* coding region (c and d) were used as positive controls; W: well-watered; D: drought.

The expression levels of *TaCBF5L* in T_4 transgenic sublines under four different drought stages were determined using the Q-PCR method. Evaluation of the data showed that the expression levels of *TaCBF5L* transgene in the drought-tolerant wheat cultivar, controlled by the *HDZI-3* or *HDZI-4* promoter, showed no or a little increase during the leaf wilting point (−1.5 to −2 MPa) or moderate drought (−2 to −3 MPa), compared to those with the basal level of expression under well-watered (−1.2 to −1.5 MPa) conditions (Figure 3a,b). However, the *TaCBF5L* expression levels were obviously up-regulated by a severe drought stress stage >−4 MPa). In contrast, in the drought-sensitive barley cultivar, the expression levels of *TaDREB3*, controlled by *HDZI-3* or *HDZI-4* promoter, increased several folds already at wilting point (−0.7 to −1.2 MPa), and in most of the tested lines expression decreased during a more severe drought stress stage >−3 MPa; Figure 3c,d).

Comparison of growth and yield characteristics of T_1 transgenic and control WT barley plants grown under well-watered conditions

Comparisons of growth and yield characteristics of selected T_1 transgenic (Figure S3) and control WT barley grown under well-watered conditions revealed that the most transgenic lines at the beginning of their reproductive stages appeared to be similar as the control WT and null-segregant plants (Figures S4 and S5). The most of transgenic lines showed similar height, number of tillers, flowering time and yield as control plants, although size and yield of a few lines had significantly decreased compared to WT plants (Figure S5). According to the northern blot hybridization data both types of lines expressed transgene, although the levels of transgene expression were not precisely quantified. Null segregants identified by PCR were removed from the experiment.

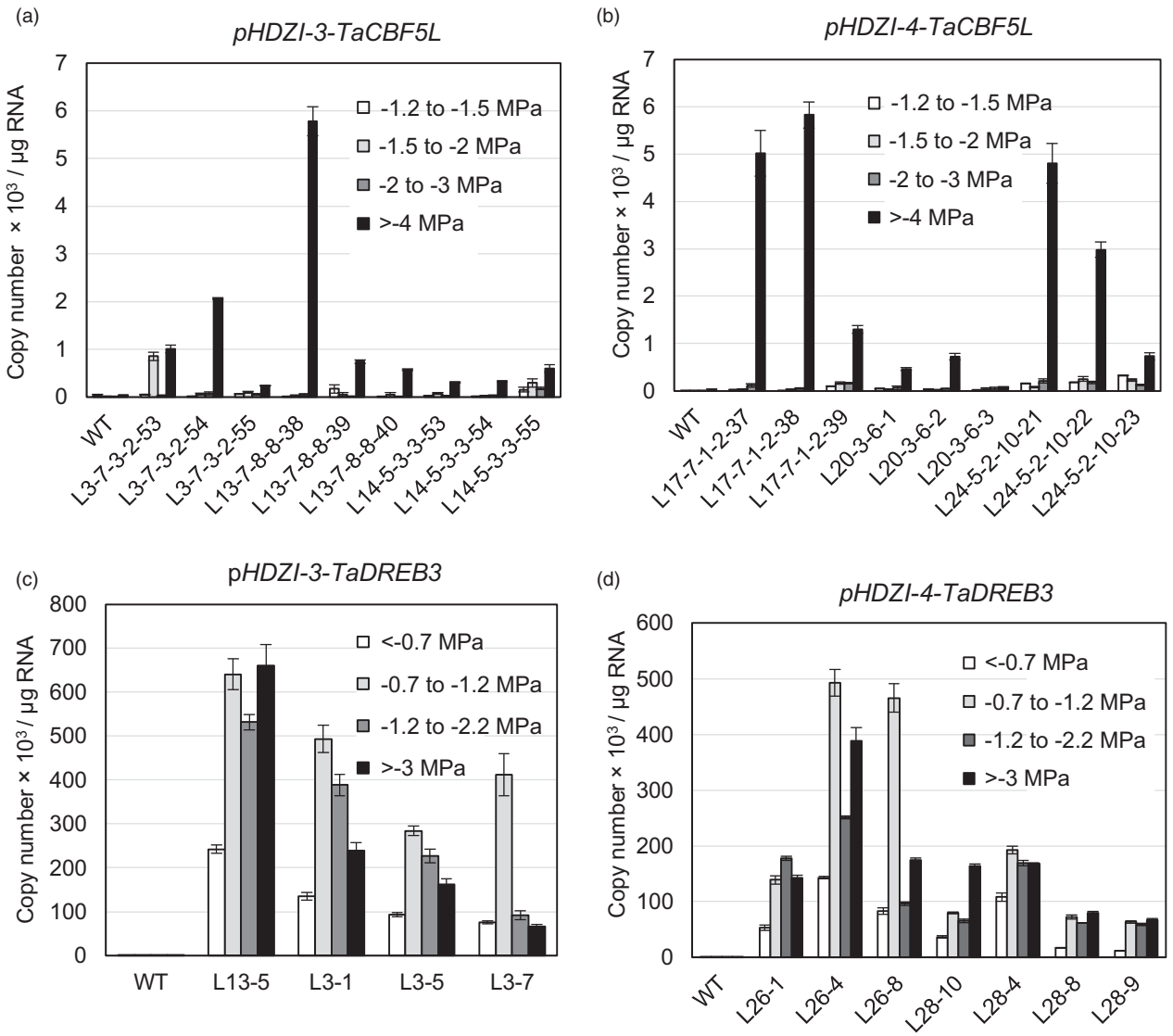

Figure 3 *TaCBF5L* or *TaDREB3* transgene expression in wheat (a and b) or barley (c and d) plants controlled by the promoter *HDZI-3* (a and c) and the promoter *HDZI-4* (b and d) under various drought stages: well-watered condition (leaf water potential with −1.2 to −1.5 MPa for wheat or 0 to −0.7 MPa for barley), the leaf wilting point (leaf water potential with −1.5 to −2 MPa for wheat or −0.7 to −1.2 MPa for barley), moderate drought (leaf water potential with −2 to −3 MPa for wheat or −1.2 to −1.5 MPa for barley) and drought condition (leaf water potential >−4 MPa for wheat or >−3 MPa for barley). The error bars represent ±SD of three technical replicates.

Evaluation of phenotypes of T_3 transgenic wheat lines grown under well-watered conditions and under moderate drought applied during the flowering stage

Four sublines of T_3 transgenic and WT wheat plants were planted in two deep containers and subjected to constant well-watered conditions in the first container, and to moderate drought during the flowering stage in the second container. Plant growth characteristics and yield components of these plants were evaluated and compared at the end of their reproduction stages.

Transgenic sublines L13-7-8 and L14-5-3 transformed with the pHDZI-3-TaCBF5L construct showed similar phenotypic features such as tiller, spike, seed number, single grain weight, plant height, grain weight per plant and total dry biomass, compared to those of WT plants (Figure 4a). However, two other transgenic sublines L3-7-3 and L3-8-8, derived from the same L3 line, showed significantly smaller sizes of plants, fewer seeds, less biomass and grain yield than those of WT under moderate drought (Figure 4a). In addition, all sublines were subjected to well-watered conditions, and the subline L14-5-3 that was exposed to moderate drought conditions, flowered between 2 and 3 days earlier than WT plants.

From the four transgenic sublines transformed with the pHDZI-4-TaCBF5L construct, two sublines grown under well-watered conditions, and one subline exposed to mild drought, showed lower spike numbers and grain yields than WT plants. Three

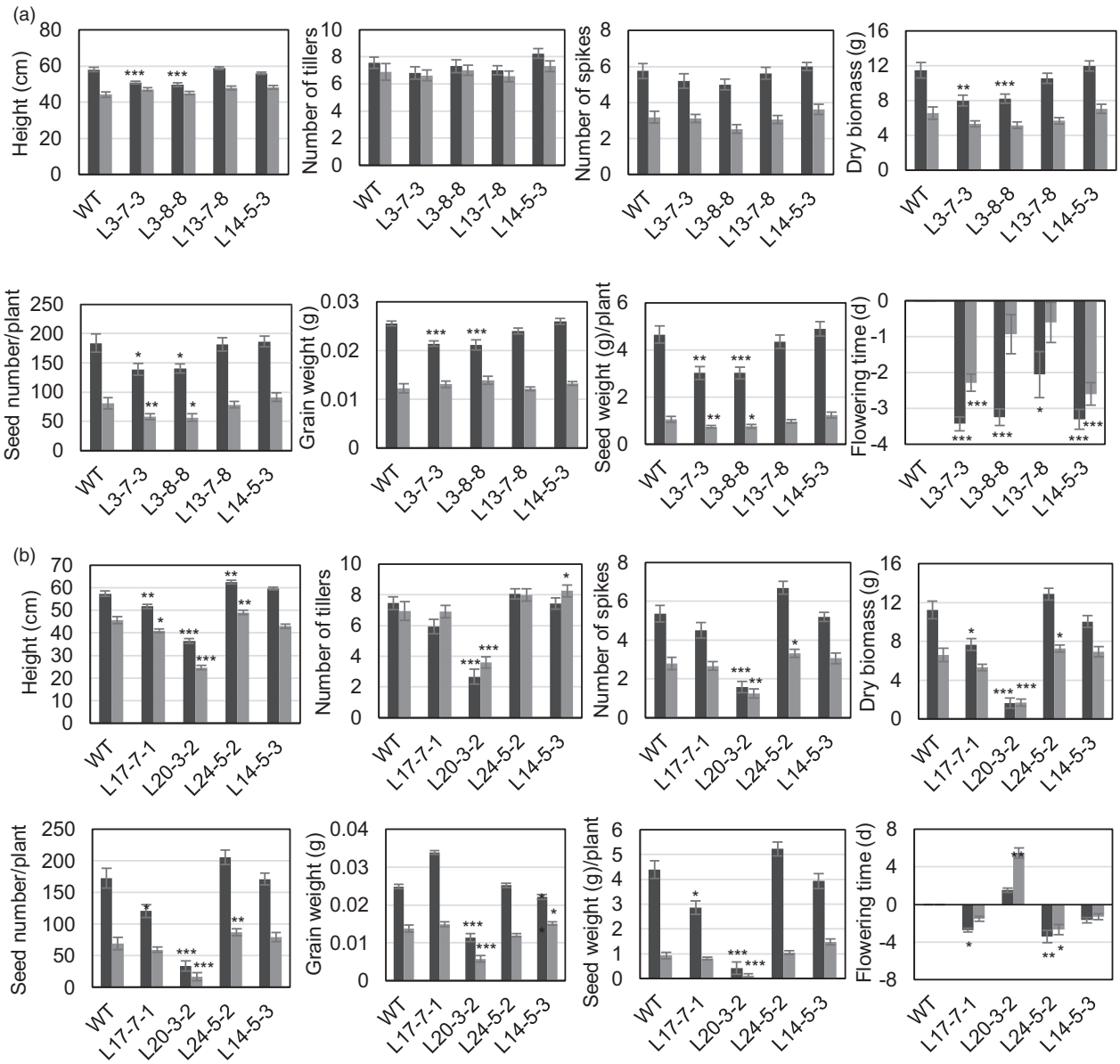

Figure 4 Growth characteristics and yield components of control wild-type (WT) and transgenic wheat (*Triticum aestivum* cv. Gladius) transformed with pHDZI-3-TaCBF5L (a) and pHDZI-4-TaCBF5L (b) under well-watered (black boxes) and moderate drought (grey boxes) conditions. Flowering time of transgenic plants was compared with the average flowering time of 16 control WT plants, which is represented as day 0. Values represent means ± SE (*n* varies for each column and is shown in each case directly on the graphs) at '*' $P < 0.05$, '**' for $P < 0.01$ and '***' for $P < 0.001$, which were calculated by the Student's *t*-test (unpaired, two-tailed).

sublines of transgenic plants transformed with the pHDZI-4-TaCBF5L construct flowered 2–3 days earlier than WT plants, although the L20-3-2 subline was significantly delayed in growth and flowered 5 days later than WT plants (Figure 4b). In addition, L20-3-2 subline had a lower plant height, lower tiller and seed numbers, and produced less biomass compared to the WT plants. However, the rest of sublines had similar number of tillers than the WT plants (Figure 4b).

Evaluation of phenotypes of T_4 transgenic wheat lines grown under severe drought during the flowering stage

Two independent lines of transgenic wheat transformed with the pHDZI-3-TaCBF5L or pHDZI-4-TaCBF5L constructs were grown alongside WT plants in pots with water-saturated soil for 3–4 weeks, then the plant watering was withheld, and the phenotypic evaluation was performed at the end of the reproductive stage. Over 95% of the transgenic and WT wheat plants survived seedling stages and proceeded to reproductive stages. The soil water content curves indicated that the plants were exposed to severe drought (25%–35% of soil water content) during flowering time (Figures S6 and S7).

Transgenic sublines L14-5-3-3 and L13-7-8-11 with transgene driven by the *HDZI-3* promoter had similar numbers of spikes and tillers compared to those of WT plants (Figure 5a). However, both sublines showed slightly delayed flowering, in addition, the L13-7-8-11 subline plants had smaller size, fewer seeds, less biomass and lower grain yield than the control WT plants (Figure 5a). Consistently, transgenic L14-5-3-1 and L24-5-2-1 sublines with a transgene driven by *HDZI-4* promoter showed similar spike and tiller numbers as the control WT plants (Figure 5b). However, both types of transgenic lines had significantly larger size, higher biomass and seed numbers, and, therefore, higher grain yields compared to the control WT plants (Figure 5b). In addition, both transgenic sublines with *TaCBF5L* driven by the *HDZI-4* promoter flowered 3–4 days earlier than the control WT plants (Figure 5b).

Stress-inducible expression of TaDREB3 gene driven by the HDZI-3 or HDZI-4 promoters improves drought tolerance of transgenic barley seedlings

The comparison of drought tolerance of transgenic wheat and barley was performed at the vegetative stage of plant development. It was measured as a recovery rate of seedlings subjected to stringent (lethal effect for the most control plants) drought conditions. Control and transgenic plants of the similar size were selected for the experiment. Three consecutive experiments using wheat seedlings revealed no significant improvement of transgenic seedlings' survival rates compared to the control WT seedlings for both promoter-transgene constructs (data not shown). In contrast, improvement of drought tolerance of transgenic barley seedlings was obvious in every experiment, where the transgene driven by either *HDZI-3* or *HDZI-4* promoter in barley yielded positive results (Figure S8).

Expression levels of putative downstream genes of TaCBF5L in leaves in control WT and transgenic wheat plants under well-watered conditions and under drought

The levels of expression of the *TaCBF5L* transgene and those of stress-inducible *LEA/COR/DHN* genes, *TaRab17*, *TaCor410*, *TaCor18*, *TaRab15* and *Wlt10*, in control WT and transgenic wheat plants were very different under well-watered condition (leaf water potential −1.2 to −1.5 MPa) and severe drought (leaf water potential >−4 MPa; Figure 6). In all cases except *Wlt10*, the levels of transgene expression under severe drought increased compared to those under well-watered conditions with the *HDZI-3* promoter application. In most cases, the increase of expression levels of most tested genes was higher in transgenic than in the control WT lines. As an exception, *TaRab15* gene expression in most transgenic lines was lower than that of the control WT plants.

Stress-inducible expression of DREB/CBF genes driven by HDZI-3 or HDZI-4 promoter improves frost tolerance of transgenic wheat and barley seedlings

Wheat seedling frost tolerance of three T_4 lines transformed with the pHDZI-3-TaCBF5L construct was compared with that of control WT plants. Based on the evaluation of survival rates (Figure 7a), control WT plants did not grow well and only no more than 6% of them survived the harsh conditions of frost. However, all examined transgenic lines showed strong tolerance to frost, with a survival rate that was three- to fourfold higher than that of the control WT plants (Figure 7a). Moreover, survival rates of each two transgenic L3-8-8-11 and L14-5-3-3 lines were significantly higher than those of the control WT plants (Figure 7a).

Three T_4 lines transformed with the pHDZI-4-TaCBF5L construct after frost treatment showed a tendency to recover stronger than the control WT plants. Survival rates of transgenic wheat plants were 1.2- to 2.0-fold higher than that of the control WT plants, suggesting that the *TaCBF5L* under *HDZI-4* promoter provides a bit lower enhancement of the wheat frost tolerance than the *HDZI-3* promoter (Figure 7b). Frost tolerance data obtained in similar experiments for T_1 barley seedlings revealed a similar picture (Figure 7c,d). Frost tolerance improvement was delivered by both pHDZI-3-TaDREB3 and pHDZI-4-TaDREB3 constructs; however, in the case of the *HDZI-3* promoter, the frost tolerance enhancement was clearly stronger than that of the *HDZI-4* promoter (Figure 7c,d).

The expression levels of the *TaCBF5L* transgene in most wheat sublines with *HDZI-3* or *HDZI-4* promoters increased up to several folds after cold treatment. In some plants, however, no significant activation of the promoter was observed, although the basal levels of the promoter activity were high (Figure 8a,b). In contrast, when examining barley transgenic plants, the picture was slightly different. Firstly, the basal activities of both *HDZI-3* and *HDZI-4* promoters (relatively to stress-induced activities) were overall stronger in transgenic barley lines than those in transgenic wheat plants, and, secondly, the activation of the *HDZI-4* promoter under the low temperature of 4 °C was in general stronger than the activation of the *HDZI-3* promoter (Figure 8c,d).

Activation of stress-inducible genes by overexpression of TaCBF5L under low temperature

Expression of five *LEA/COR/DHN* genes, *TaRab17*, *TaCor410*, *TaCor18*, *TaRab15* and *Wlt10*, as the putative downstream stress-inducible genes of *TaCBF5L*, was examined in the leaves of transgenic wheat and control WT plants in the absence of stress and after the exposure to 4 °C (Figure 9). Nearly all tested genes in all transgenic lines transformed with either pHDZI-3-TaCBF5L or pHDZI-4-TaCBF5L constructs demonstrated stronger expression than the control WT plants under normal growth temperatures. Some of the tested downstream genes in the transgenic lines were up-regulated, while the others were clearly repressed, and other genes kept their expression levels unchanged, when

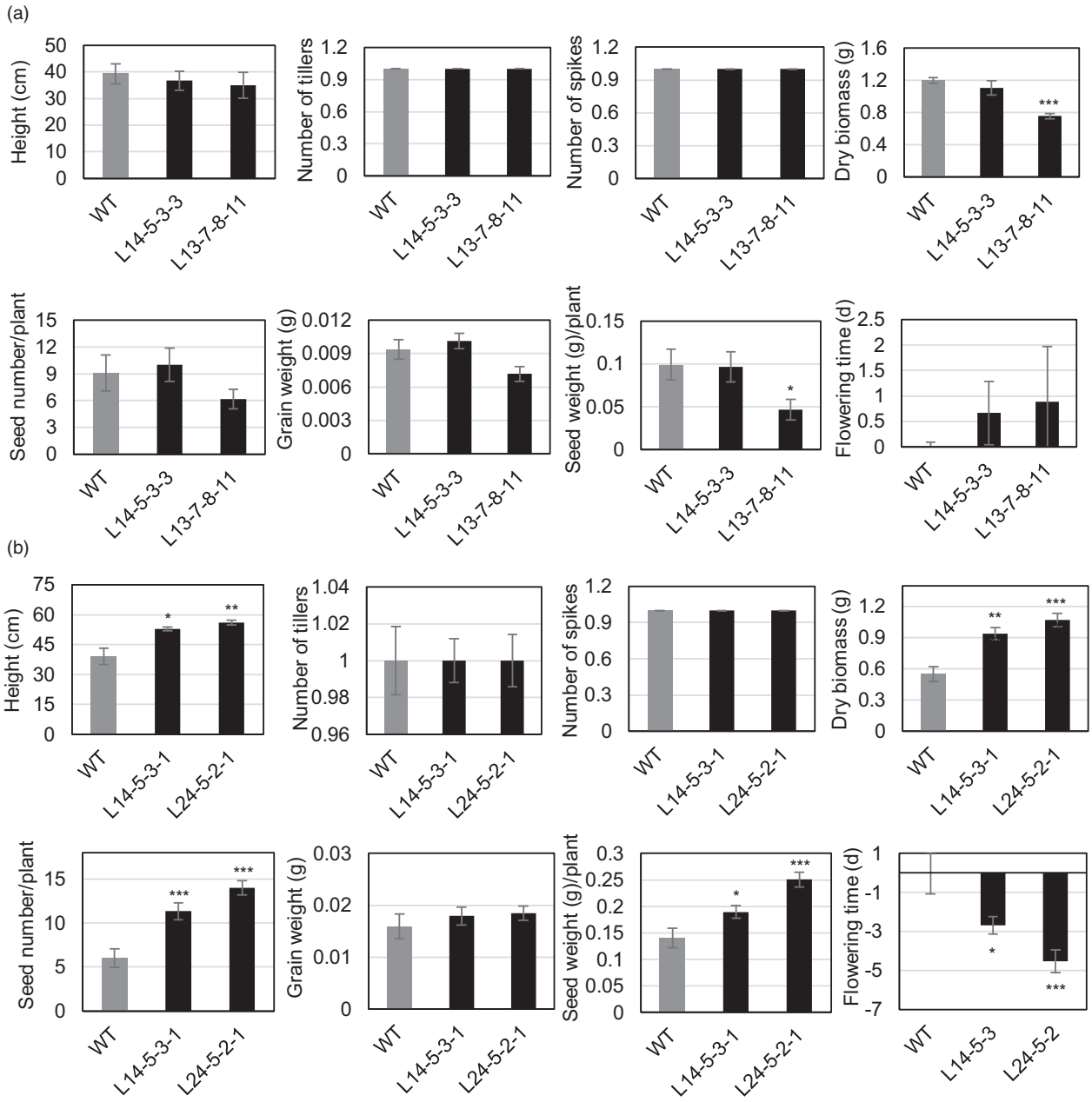

Figure 5 Growth characteristics and yield components of control wild-type (WT) and transgenic wheat (*T. aestivum* cv. Gladius) transformed with pHDZI-3-TaCBF5L (a), and pHDZI-4-TaCBF5L (b) under severe drought. Flowering time of transgenic plants was compared to average flowering times of 16 control WT plants, which is represented as day 0. Values represent means ± SE (*n* varies for each column and is shown in each case directly on the graphs) at $*P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$, which were calculated by Student's *t*-test (unpaired, two-tailed).

compared to the expression levels of the same genes in the WT plants. Overall the expression patterns of downstream genes in *HDZI-3* and *HDZI-4* wheat transgenic lines were comparable. However, in *HDZI-3* transgenic lines these patterns were more consistent than those in *HDZI*-4 transgenic lines.

GUS expression pattern under different stresses in the T_1 transgenic wheat transformed with the pHDZI-3-GUS or HDZI-4-GUS construct

To analyse the spatial and temporal activity of the *pHDZI-3* and *pHDZI-4* promoters, wheat was transformed with pHDZI-3-GUS and pHDZI-4-GUS fusion constructs. Twenty-two independent T_1 transgenic wheat lines (six lines with the pHDZI-3-GUS reporter construct and sixteen lines with the pHDZI-4-GUS reporter construct; Tables S2 and S3) were generated and analysed in the pilot experiment using hydroponic conditions. Plants from each subline were treated with cold, high salinity, increased ABA levels and dehydration, respectively (Figure S9). All analysed transgenic wheat plants transformed with either pHDZI-3-GUS or pHDZI-4-GUS constructs showed no *GUS* expression under salinity and ABA. Three transgenic T_1 pHDZI-3-GUS lines (Lines 3, 5 and 7) showed *GUS* expression in the coleoptiles and roots of

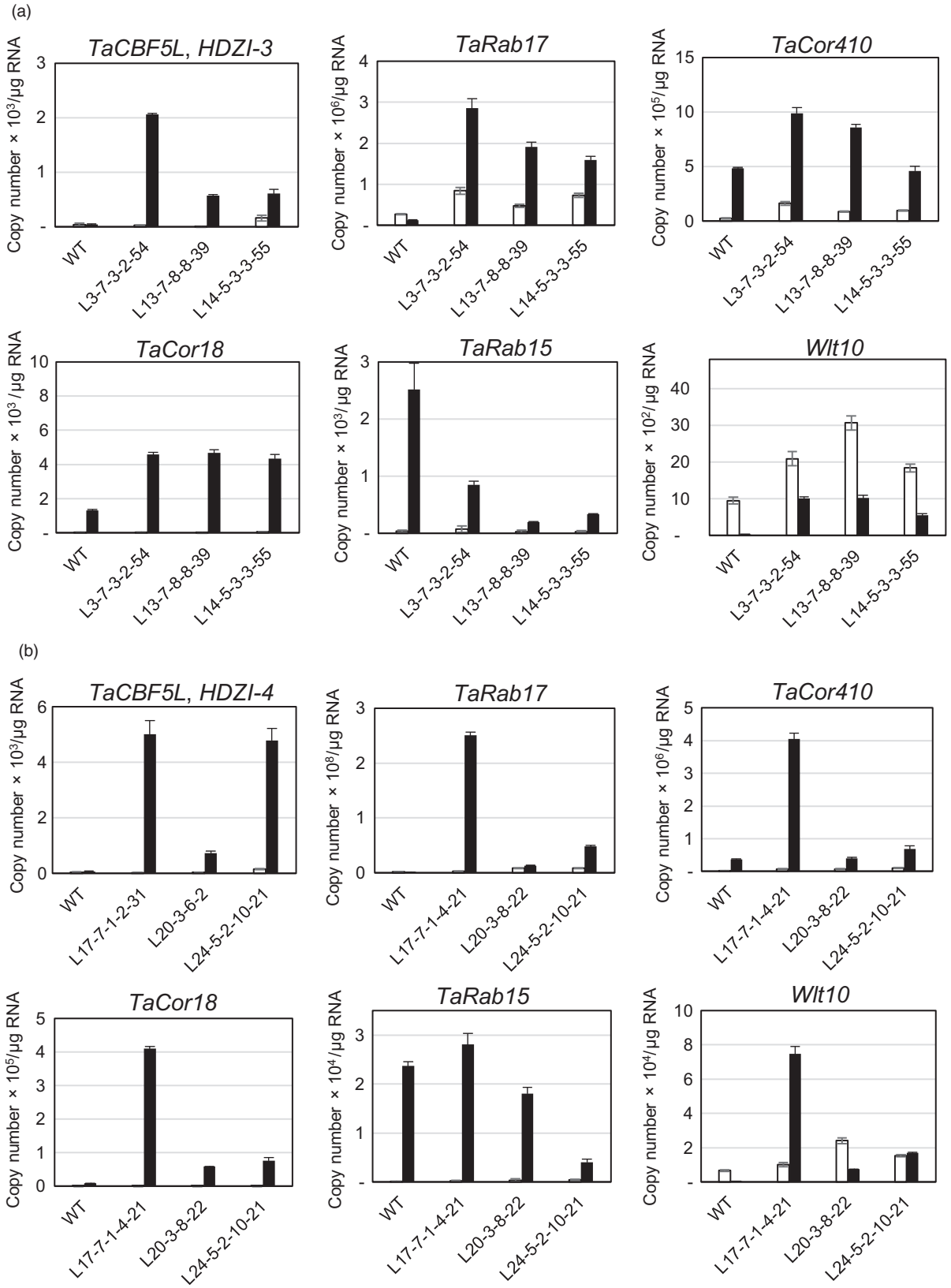

Figure 6 Expression of the *TaCBF5L* transgene and stress-inducible *LEA/COR/DHN* genes in control WT and transgenic wheat plants with inducible overexpression of *TaCBF5L* controlled by *HDZI-3* (a) and *HDZI-4* (b) promoters. Expression levels of the *TaCBF5L* transgene and selected stress-inducible genes were estimated under well-watered conditions (white boxes) and severe drought (leaf water potential >−4 MPa; black boxes).

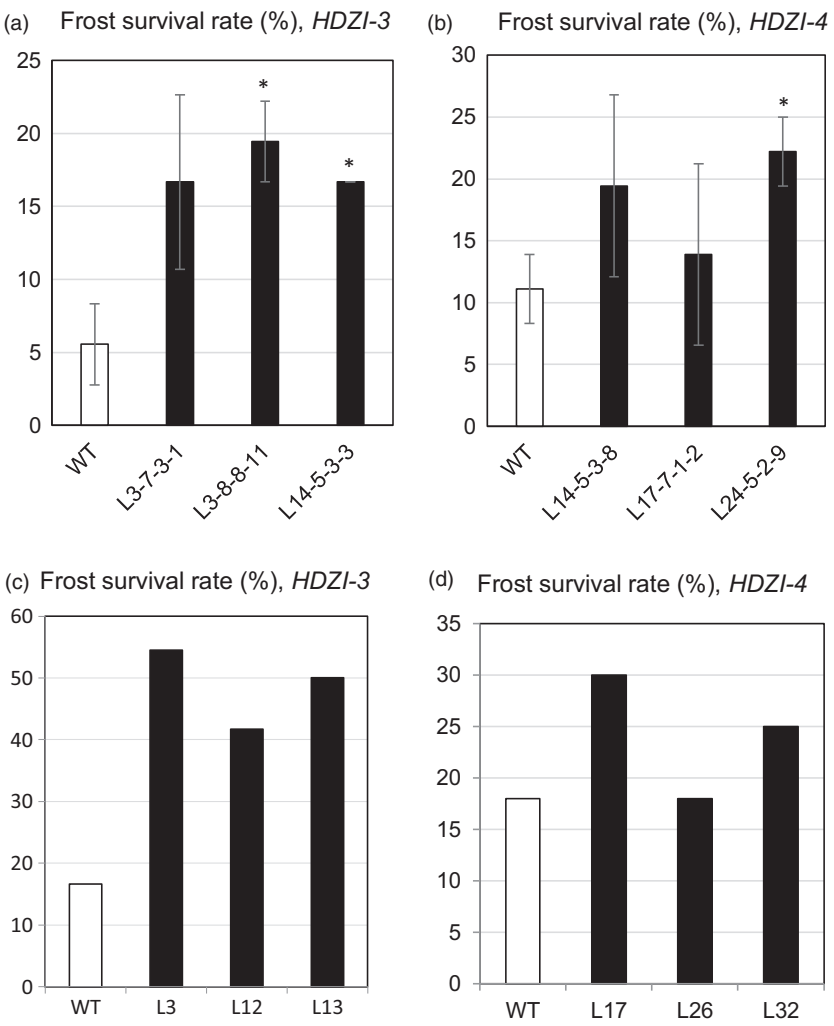

Figure 7 Frost survival rates of control WT and transgenic T_3 wheat plants transformed with pHDZI-3-TaCBF5L (a) and pHDZI-4-TaCBF5L (b) constructs. Error bars represent ±SD of three technical replicates. Differences between transgenic and WT plants were tested in the unpaired Student's *t*-test (*$P < 0.05$). Frost survival rate of control WT and transgenic T_1 barley seedlings transformed with pHDZI-3-TaDREB3 (c) and pHDZI-4-TaDREB3 (d); data in panels (c) and (d) are based on a single experiment, thus no ±SD values are included.

seedlings under cold stress, and one line (Line 3) showed a weak *HDZI-3* promoter activity in the roots under dehydration (Figure S9a,b). Weak staining of coleoptiles was observed in eight T_1 pHDZI-4-GUS transgenic lines (Lines 1a, 2, 4, 6, 8, 10, 12 and 13) after cold treatment (Figure S9a,c). However, the detected GUS activity was too weak to proceed with histochemical analysis of the spatial pattern of the promoter activity. No GUS activity was detected in other plant tissues.

Discussion

Drought and frost may impair plant growth and development at any time point of a plant life cycle. However, the sensitivity to drought and frost is especially acute during reproductive stages. In the case of drought this is because of the plant–water status changes, leading to a high transpiration rate and the declining reserves of soil moisture towards the end of a vegetation season (Saini and Westgate, 1999). The exact reasons of high sensitivity of wheat and barley to night frosts at flowering are unknown. It is noteworthy that a particularly strong sensitivity of one or both gametophytes to below-zero temperatures occurs a short time before, during and/or short time after fertilization.

In this work, we used two representatives of the wheat DREB/CBF family of TFs to investigate stress-inducible expression in transgenic wheat and barley, and to study the impact of transgenes driven by two distinct stress-inducible durum wheat promoters on growth characteristics, yield components and tolerance of transgenic plants to drought and frost at reproductive and/or vegetative stages of plant development. Additional details regarding the selection of donor plants and transgenes can be found in Supporting Discussion.

Q-PCR analyses of *TaHDZipI-3* and *TaHDZipI-4* expression in a variety of plant tissues in the absence of stress revealed relatively low levels of expression of both genes in all examined tissues except for the floral tissues, suggesting that the *TaHDZipI-3* and *TaHDZipI-4* promoters could elevate expression of target genes in frost vulnerable florets and initiate accumulation of protective proteins before stress (Figure 1a). The analysis of *TaHDZipI-3* and *TaHDZipI-4* expression in expanding parts of the stem at different stages of development revealed that expression of the *TaHDZipI-3* gene was low, while the expression levels of *TaHDZipI-4* were more variable and relatively high during head emergence. Although the DREB/CBF proteins may suppress the growth of transgenic plants (Kasuga *et al.*, 1999; Kovalchuk *et al.*, 2013; Morran *et al.*, 2011), relatively low basal levels of both *HDZI* promoters (particularly low in wheat) applied through expression of the *DREB/CBF* transgenes may not significantly affect stem elongation under the optimal growth conditions, except during transitioning to flowering. Based on *TaHDZipI-3* and *TaHDZipI-4* expression data under stress (Harris *et al.*, 2016), we expected

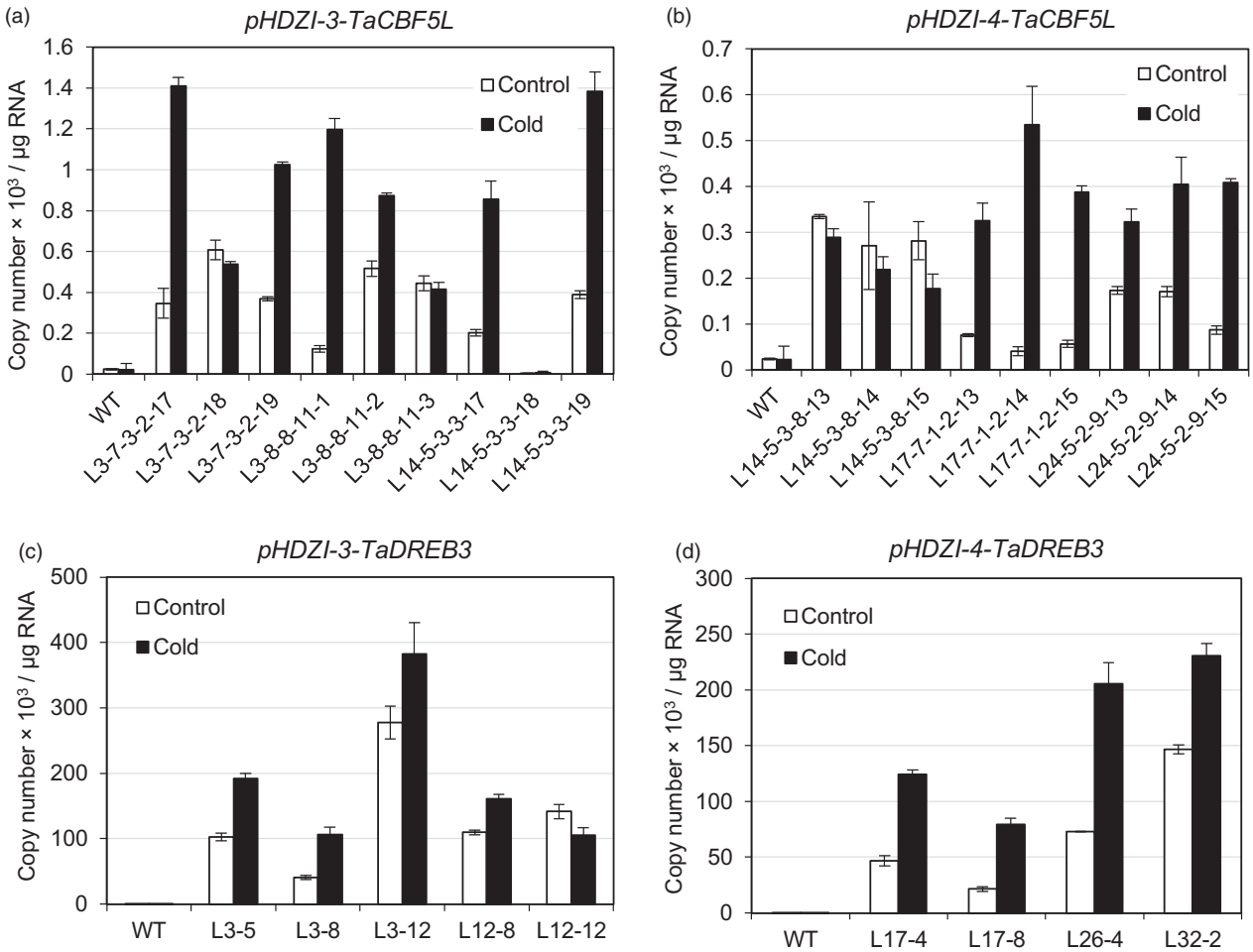

Figure 8 *TaCBF5L* (a and b) or *TaDREB3* (c and d) transgene expression levels controlled by the *HDZI-3* (a and c) and *HDZI-4* (b and d) promoters in leaves of control WT and transgenic T4 wheat (a and b) or T1 barley plants (c and d) grown at 24 °C (control) and subjected to cold treatment at 4 °C. The error bars represent ±SD of three technical replicates.

that the promoters would exert different properties: the *HDZI-3* would be induced only by drought/dehydration, while *HDZI-4* would be induced by both drought/dehydration and low temperatures. However, our study shows that both promoters in wheat and barley were induced by drought and cold, albeit with different strength. The reason for this is unclear, although we suggest that the minor differences in promoter sequences of genes from bread and durum wheat, or the absence of the distal repressor sequences from the *HDZI-3* promoter fragment controlling cold response, could play roles (Figures S10–S13). In addition to different strength of promoters, we identified promoter-dependent differences in phenotypes, stress tolerance and downstream gene expression in both transgenic wheat and barley plants. Further discussion of the promoter activity studies using transgenic plants transformed with promoter-GUS fusion constructs can be found in Supporting Discussion.

Based on the statement above and the results of our previous works (Kovalchuk *et al*., 2013; Morran *et al*., 2011; Shavrukov *et al*., 2016), we conclude that: (i) accurate selection of lines; (ii) use of untransformed donor plants and two or three backcrosses of selected homozygous transgenic lines with acceptable phenotypes and (iii) accurate selection of backcrossed plants for transgene presence and/or expression could provide stable lines with low detrimental effects on genomic DNA (that occur during the process of plant transformation) and enhance stress tolerance and decrease or abolish the negative influence of the transgene on plant development.

The analysis of plant phenotypes and yield was performed under well-watered and drought conditions. In addition, the molecular analysis of regulation of several stress-responsive genes, which are potential downstream genes of DREB/CBF TFs showed that this analysis (Figures 7 and 8) supported the observed enhancement of stress tolerance.

Drought tolerance improvement was not observed in transgenic wheat seedlings in three consecutive experiments. This result was not unexpected if one considered the relatively high tolerance of the wheat Gladius cultivar to drought, achieved through breeding programmes for the Australian environment. Our previous attempts to enhance drought tolerance in the drought-tolerant Gladius cultivar by overexpression of the *DREB/CBF* and *bZIP* encoding genes resulted in minor or no improvements of tolerance (Amalraj *et al*., 2016; Luang *et al*., 2018). However, the significant improvement was achieved by using HD-Zip I and ERF-like (SHN1) TFs (Bi *et al*., 2018; Yang *et al*., 2018), which most likely regulate different aspects of drought response. These improvements suggested the reasons for a high

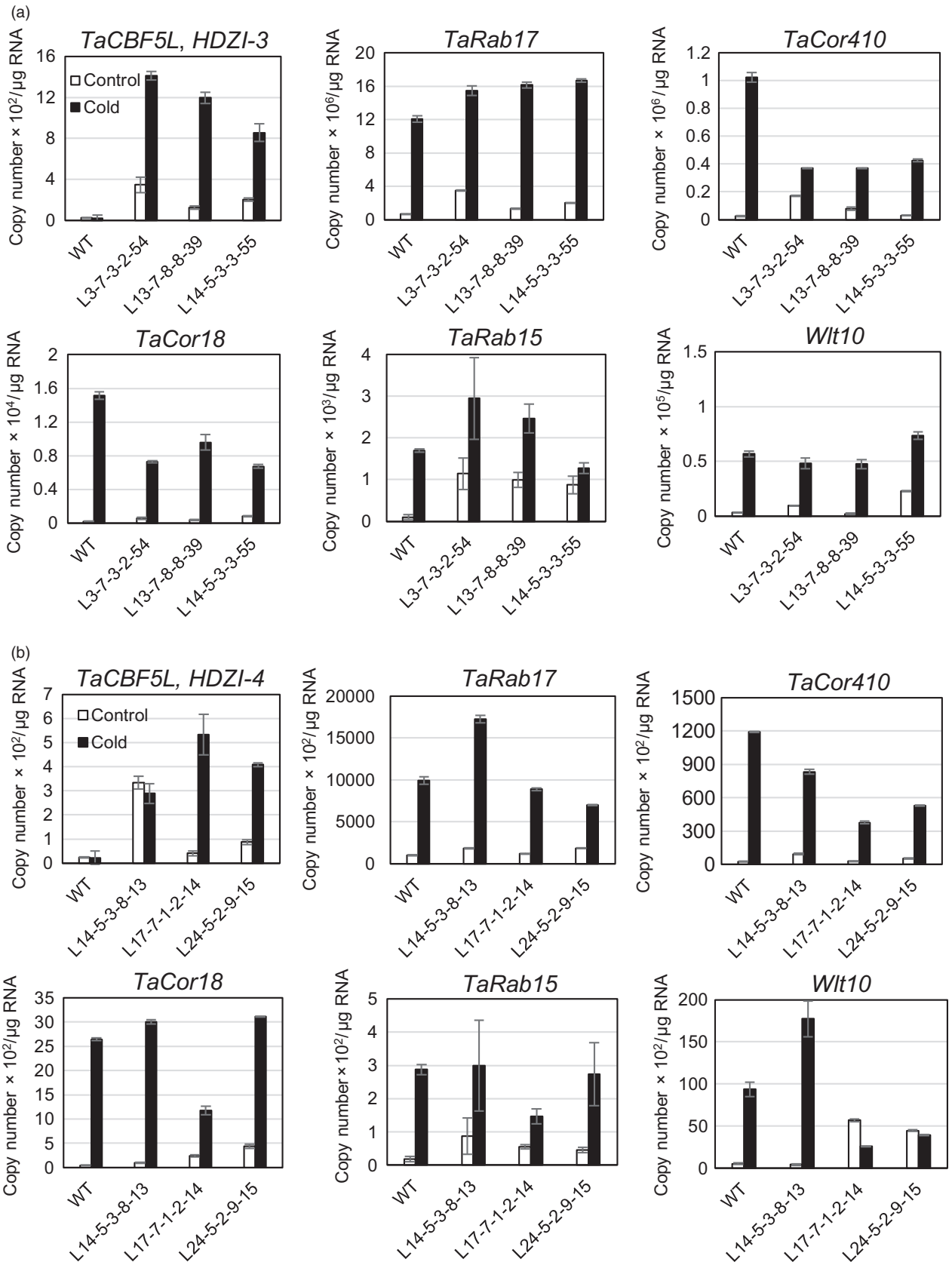

Figure 9 Expression of the *TaCBF5L* transgene and stress-inducible *LEA/COR/DHN* genes in transgenic wheat plants with overexpression of *TaCBF5L* controlled by *HDZI-3* (a) and *HDZI-4* (b) promoters in control WT and transgenic T_4 lines at 23 °C (Control) and under the cold treatment at 4 °C (Cold).

tolerance of the Gladius cultivar to drought, which was introgressed during breeding of this cultivar. In contrast, the drought tolerance of all the tested transgenic barley lines was higher than that of the control WT plants. Thus, the sensitivity of the barley Golden Promise cultivar to drought provided us with the opportunity to improve its drought tolerance through DREB/CBF overexpression.

The analysis of transgenic wheat and barley growth characteristics, and yield components under well-watered conditions and mild drought (wheat only) revealed some lines had the same or very similar phenotypes as the control WT plants and some lines had worsened. However, both types of lines expressed transgenes and demonstrated their function of the significant improvements of frost tolerance. It is notable that all except one of the tested transgenic lines flowered a few days earlier than the control WT plants; this is very unusual for overexpression DREB/CBF TFs, which typically lead to significant delays in flowering due to a slower growth of transgenic plants.

Taking into consideration that both tested promoters in transgenic wheat were activated only under strong drought, we performed the 'drought-during-flowering' experiment under harsh drought conditions (Figures S6 and S7). In this case, the behaviour of transgenic wheat lines was dependent on the HDZI-3 and HDZI-4 promoters used in each transgenic plant. The pHDZI-3-TaCBF5L lines showed a decline in most yield components. In contrast, both transgenic wheat lines transformed with the pHDZI-4-TaCBF5L construct significantly increased plant biomass and seed number per spike, resulting in the significant increase of the grain weight per plant (yield). Transition to flowering in these two lines occurred 3–5 days earlier than that in the control WT plants. We have no explanation for differences in yield and their dependence upon the promoter used except a possibility that the differences in the drought-induced spatial expression pattern could be attributed to each promoter regulating *TaCBF5L* overexpression.

The analysis of potential downstream stress-responsive genes directly or indirectly regulated by TaCBF5L in transgenic wheat plants revealed that all five tested genes were up-regulated in transgenic wheat lines compared to the control WT plants under well-watered conditions (obviously because of the basal levels of transgene expression), while the behaviour of the tested genes under strong drought was different (Figure 6). The results obtained for the *HDZI-3* promoter were more consistent than those for the *HDZI-4* promoter, which likely point out to differences in spatial patterns of promoter activities.

Vegetative frost tolerance enhancement was observed in both transgenic species and through the application of both *HDZI* promoters. However, while with the *HDZI-3* promoter a significant improvement of frost tolerance was achieved in both transgenic wheat and barley compared to control WT plants, the *HDZI-4* promoter performance in both transgenic plants was less convincing. The possible explanation could be the higher overall basal activity level of the *HDZI-3* promoter. This could provide slightly higher transgene expression, and hence higher basal levels of the target stress-responsive genes and their products prior to stress, which could lead to better pre-adaptation of *HDZI-3* transgenic plants to cold. The other explanation could be in the differences of spatial expression of transgenes under two tested promoters. These differences could lead to the diverse levels of transgene product accumulation in the most vulnerable to stress plant tissues that in turn could provide various levels of the transgene-produced advantages under stress.

The analysis of expression of downstream genes perhaps confirms the role of overall higher basal levels of transgene expression in transgenic lines, when the *HDZI-3* promoter was applied, and consequently a better preparation of plants to a cold stress during growth under the optimal for plant temperatures. Notably, similar downstream genes may be regulated by the same *TaCBF5L* transgene under drought and cold conditions in different ways. For instance, the stress-inducible *TaCor410* gene was up-regulated under drought and down-regulated under low temperature independently ~~of~~ whether either *HDZI-3* or *HDZI-4* promoters were used. On the other hand, *TaCor18* gene expression was up-regulated by drought but down-regulated by cold, only when the *HDZI-3* promoter was used. The *Wlt10* expression level was up-regulated by drought and but it was not affected by cold by the *HDZI-3* promoter application. Notably, in the absence of stress, all tested downstream genes were up-regulated by the basal levels of the *TaCBF5L* transgene.

By summarizing our data, we conclude that the application of each of two tested *HDZI-3* and *HDZI-4* promoters has its own advantages and disadvantages. Transgenic lines with developmental phenotypes similar to those of the WT donor plants can be selected for both promoters. In barley, both promoters were effective tools to increase drought tolerance at a vegetative stage by overexpression of the DREB/CBF TFs, and therefore could be used for drought tolerance enhancements of the drought-sensitive crop species. However, both promoters in combination with *TaCBF5L* failed to improve the survival rates of the drought-tolerant wheat under water deficit. The *HDZI-3* promoter provided better frost tolerance than the *HDZI-4* promoter in both wheat and barley, most likely due to higher basal activity levels, which lead to a better provision for upcoming stress. On the contrary, the application of *HDZI-4* promoter delivered yield improvements in wheat, providing flowering occurred under strong drought, while the application of the *HDZI-3* promoter provided no gains in a grain yield under the same conditions.

In conclusion, we suggest that both tested wheat *HDZI-3* and *HDZI-4* promoters could be used in transgenic crop plants in combination with DREB/CBF TFs for the improvement of the abiotic stress tolerance, and for the concurrent retention of original phenotypes and yields.

Experimental procedures

Isolation and identification of the TaCBF5L and TaDREB3 genes

A full-length cDNA of *TaCBF5L* was isolated from roots of the drought-stressed *T. aestivum* L. genotype RAC875, using a modified yeast-one hybrid approach (Lopato *et al.*, 2006; Pyvovarenko and Lopato, 2011) with DRE *cis*-element TACCGAC as a bait. Isolation of *TaDREB3* cDNA and characterization of the gene in transgenic wheat and barley was described earlier (Kovalchuk *et al.*, 2013; Lopato *et al.*, 2006; Morran *et al.*, 2011). The homologous to TaCBF5L and TaDREB3 proteins from a variety of species such as *Arabidopsis*, wheat, rice, maize and barley were found using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990), and a non-redundant protein sequence database of the National Center for Biotechnology Information (NCBI). The multiple protein sequence alignment of the homologous proteins to TaCBF5L and TaDREB5 was conducted using MAFFT version 7 (Katoh and Standley, 2013). A phylogenetic tree was reconstructed based on the alignment results using Molecular Evolutionary Genetics Analysis (MEGA 6.06; Tamura *et al.*,

2013) with neighbour joining and p-distance specifications and 1000 bootstrap replications.

Supporting Experimental procedures contain protocols for plasmid construction and plant transformation, determination of transgene copy number and expression levels by quantitative real-time PCR (Q-PCR) and northern blot hybridization, selection of transgenic wheat and barley sublines, comparison of growth and yield components of selected sublines with control WT wheat plants grown under different drought conditions and well-watered conditions, survival rates comparison of wheat and barley seedlings under terminal drought and frost and analysis of promoter activation in transgenic wheat seedlings by checking *GUS* expression.

GenBank accession numbers

TaCBF5L—MF406152, *TdHDZipI-3* promoter (*HDZI-3*)—MG063277, *TdHDZipI-4* promoter (*HDZI-4*)—MG063278.

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Conflict of interest

Authors declare no conflict of interest.

Author contributions

Y.Y. performed experiments and analysed data. H.H.J.B., J.H., M.R., Y.L., I.M., N.B., L.C. and S.S.H. assisted with experiments and analysed data. N.K., S.L. and S.H. conceived the project, designed experiments and analysed data. Y.Y., S.H., N.K. and S.L. wrote the manuscript. H.H.J.B. and M.H. contributed to writing. M.H. and S.L. edited manuscript. All authors commented on the manuscript.

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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 The phylogenetic tree of DREB TFs from a representative dicot *Arabidopsis* and monocots wheat and barley.

Figure S2 Multiple sequence alignment of TaCBF5L and six homologous proteins with a close evolutionary relationship to TaCBF5L from wheat (Ta—*T. aestivum*; Tm—*T. monoccocum*), maize (Zm—*Zea mais*), and barley (Hv—*Hordeum vulgare*).

Figure S3 Copy numbers of the *TaCBF5L* transgene estimated by Q-PCR in T_1 transgenic wheat (a) and barley (b).

Figure S4 Control WT barley (*H. vulgare* cv. Golden promise) and transgenic T_1 barley lines transformed with the pHDZI-3-TaDREB3 and pHDZI-4-TaDREB3 constructs.

Figure S5 Growth characteristics and yield components of control WT and transgenic barley transformed with pHDZI-3-TaDREB3 (a), and pHDZI-4-TaDREB3 (b) constructs grown under well-watered conditions.

Figure S6 Details of the flowering-under-severe-drought experiment of wheat plants.

Figure S7 Pot soil water content for plant flowering experiment under severe drought.

Figure S8 Drought survival rates of control WT and transgenic T_1 barley seedlings transformed with the pHDZI-3-TaDREB3 (a) and pHDZI-4-TaDREB3 (b) constructs, subjected to severe drought.

Figure S9 The results of the promoter-GUS activity studies using T_1 transgenic wheat plants transformed with pHDZI-3-GUS and pHDZI-4-GUS constructs.

Figure S10 Soil water tension monitored at 10 cm and 30 cm depths in large containers used for wheat growth under well-watered conditions or gradually increasing drought.

Figure S11 Details of drought tolerance experiments.

Figure S12 Details of frost tolerance experiments.

Figure S13 Alignments of *TdHDZipl-3* and *TdHDZipl-4* promoter sequences and sequences of corresponding genes of *Triticum aestivum* cv. Chinese Spring, identified in the Whole Genome Reference Assembly Pseudomolecules v1.0 databases of the International Wheat Genome Sequencing Consortium, using the BLAST software (Altschul *et al*.,).

Table S1 List of PCR primers, and Q-PCR and Northern hybridisation primers and probes, used in this study for investigated genes

Table S2 Proteins, homologous to TaCBF5L from wheat, maize, rice, barley and *Arabidopsis* were searched using the BLAST tool (Altschul *et al*.,)

Table S3 The list of T_1 transgenic wheat lines transformed with promoter-GUS constructs and tested for the GUS activity

Data S1 Supporting Experimental procedures, Supporting Discussion, Supporting References