Phenotyping transgenic wheat for drought resistance

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Abstract

Realistic experimental protocols to screen for drought adaptation in controlled conditions are crucial if high throughput phenotyping is to be used for the identification of high performance lines, and is especially important in the evaluation of transgenes where stringent biosecurity measures restrict the frequency of open field trials. Transgenic DREB1A-wheat events were selected under greenhouse conditions by evaluating survival and recovery under severe drought (SURV) as well as for water use efficiency (WUE). Greenhouse experiments confirmed the advantages of transgenic events in recovery after severe water stress. Under field conditions, the group of transgenic lines did not generally outperform the controls in terms of grain yield under water deficit. However, the events selected for WUE were identified as lines that combine an acceptable yield—even higher yield (WUE-11)—under well irrigated conditions—and stable performance across the different environments generated by the experimental treatments.

Key words: Drought resistance, drought screening protocols, phenotyping, survival to severe stress, transgenic wheat, water use efficiency, yield stability.

Introduction

Drought is probably the most important abiotic stress limiting plant growth and crop productivity globally. The imperative to develop drought-resistant crops is intensifying due to increasingly limited water supplies for crop irrigation, in addition to stresses imposed by global climate change, such as elevated temperatures, changed precipitation patterns, and increased water deficit in arid and semi-arid areas (White et al., 2004). The basis of drought resistance is complex and driven by diverse drought-adaptive mechanisms (Reynolds and Tuberosa, 2008), which are normally under multigene control (Blum, 2005; Pinto et al., 2010). Plant responses to drought are also influenced by the time, intensity, duration, and frequency of the stress as well as by diverse plant–soil–atmosphere interactions. Many physiological and morphological strategies in response to water deficit have been identified ranging from dehydration avoidance to dehydration tolerance.

Dehydration avoidance refers to the capacity of the plant to maintain an adequate plant water status under the soil water deficit. The plant ‘avoids’ the stress by different strategies that include deep rooting (Lopes and Reynolds, 2010), reduced leaf area, reduced growth duration (early flowering), and mechanisms related to increased water use efficiency (WUE) (Araus et al., 2002). WUE has been defined as the ratio of total biomass or above-ground biomass or grain yield against water used during crop growth (Angus and van Herwarden, 2001). Under drought, grain yield can be expressed as a function of water use (WU), WUE, and harvest index (HI) (grain yield=WU×WUE×HI; Passioura, 1977; Salekdeh et al., 2009). For example, an improvement in WUE by crossing and selecting for high transpiration efficiency (Condon et al., 2004) led to the selection of wheat cultivars with increased yield under late-season drought conditions. Dehydration tolerance, on the other hand, refers to the ability to remain viable and grow after a dehydration–hydration cycle. Therefore, dehydration tolerance indicates the tissue capacity for withstanding desiccation. It is generally measured as the ability of the plant to recover from stress. The range of tolerance to dehydration would depend on the species and stage of development.
Further progress in breeding for drought resistance will depend on our ability to identify subtle cultivar level differences in expression of the gene networks involved in stress adaptation. Biotechnology has provided new opportunities such as the possibility of manipulating transcription factors associated with stress response which regulate a large number of genes. Such transcription factors have been used in genetic engineering for tolerance to abiotic and biotic stresses (Agarwal et al., 2006). Dehydration-responsive element-binding (DREB) transcription factors have been reported to enhance drought resistance in transgenic plants including tomato, peanuts, rice, barley, and wheat (Kasuga et al., 1999; Pellegrineschi et al., 2004; Oh et al., 2005; Bhatnagar-Mathur et al., 2007; Wang et al., 2008; Xiao et al., 2009; Morrán et al., 2010). DREB1A appeared to improve WUE in groundnut, mainly by a large modification on the root/shoot ratio under water deficit (Vadez et al., 2007). Pellegrineschi et al. (2004) placed DREB1A from Arabidopsis thaliana under the control of a stress-inducible promoter from the rd29A gene and transferred this transcription factor into Bobwhite wheat. In wheat seedlings over-expressing DREB1A transcription factor, they observed delayed leaf wilting symptoms compared with controls at 10 d after the cessation of watering. However, the association between DREB over-expression and increased wheat grain yield under water deficit has still not been demonstrated.

Although conventional breeding has mainly focused in selecting lines for dehydration avoidance strategies over dehydration tolerance (Blum, 2005), several reports on transgenic lines have evaluated the dehydration tolerance approach—high survival after the stress or even dehydration of seedling grown hydroponically (Nakashima et al., 2007). The success of any selection strategy would ultimately be determined by the reproductive success, thus, by the final grain yield under field conditions in the case of wheat. Results obtained under controlled conditions may not always be extrapolated to field conditions, highlighting the importance of performance confirmation under drought-stressed field environments (Ortiz et al., 2007; Bhatnagar-Mathur et al., 2008; Manavalan et al., 2009; Yang et al., 2010), in fact, impacts on yield and/or productivity have not yet been demonstrated based on selection criteria in controlled environments (non-field environments such as the greenhouse or growth cabinets). While many reports have demonstrated increased drought resistance in DREB transgenic plants under laboratory and greenhouse conditions in several crops (Dubouzet et al., 2003), very few studies have tested the performance and productivity of transgenic lines in the field (Xiao et al., 2009; Yang et al., 2010).

Field performance of 14 transgenic wheat lines previously selected under greenhouse conditions for survival to severe drought (SURV) and high water use efficiency (WUE) was evaluated here. The objective of this study was to assess biomass production (BM) and yield performance (YLD) of transgenic events relative to control lines under different water regimes in field conditions. An additional goal was to test if the selection criteria used in the greenhouse relate to BM, YLD, and the stability of those parameters when comparing the performance under the different treatments.

Materials and methods

Plant material

Bread wheat (Triticum aestivum L. var. MPB-Bobwhite26, named as ‘Bobwhite’ or BW in this paper) was transformed with the DREB1A gene from Arabidopsis thaliana under the stress-inducible promoter rd29A. The transformation method, selection, and regeneration procedures were described in Pellegrineschi et al. (2002, 2004).

The microprojectile-mediated transformation method included the use of the plasmid pAHC25 containing the gusA reporter gene and the bar selectable marker conferring resistance to 5-phosphonothyricin, both under the control of the maize ubiquitin promoter. In addition, the rd29A:DREB1A was inserted on the plasmid pBI29ApNot. Under this inducible promoter, DREB1A would only be expressed under stress conditions. A total of 447 T0 seedlings were obtained and transferred to soil in pots under greenhouse conditions. Transformed plants were selected for PPT resistance at the 5–6 leaf stage by spraying them with 0.3% (w/v) BastaTM solution. The surviving plants were then tested for the presence of the DREB1A and bar genes by Polymerase Chain Reaction (PCR). All PCR-positive plants for the DREB1A and bar genes were self-pollinated to produce the T1 generation. Results from Southern blot analysis of T1 individuals demonstrated that various copies of the DREB1A transgene were integrated into the genome of the transgenic plants (Pellegrineschi et al., 2004). In this paper, the term ‘event’ refers to the group of plants belonging to the offspring of one specific transformation episode. The events regenerated from tissue culture without the transgene or those that do not inherit the transgene due to Mendelian segregation are called ‘null’ events. ‘Conventional’ refers to the non-transformed Bobwhite.

Transgenic plants were selected by survival to severe water deficit (SL) by Pellegrineschi et al. (2004). They exposed the plants to water stress by withholding water at the 4–5 leaf stage. The selected transgenic wheat lines started to show water stress symptoms later than the control. For this study, nine lines from this group were evaluated.

Evaluation of BM and WUE under greenhouse conditions

Plants were germinated and grown to the five-leaf stage without water stress in pots under greenhouse conditions. Small pots (0.5 l) were used on the first and second dates due to greenhouse space limitations. Larger pots (13 l) were used on the third date. Polystyrene spheres were placed on top of the soil to prevent water loss. For the stress treatment, water was only applied when plants reached the permanent wilting point and repeat drought cycles were imposed until plants reached anthesis. Two pots for each genotype were kept well-watered as water treatment checks. Total water applied, above-ground biomass, and root biomass were measured and used to estimate WUE shortly after anthesis (7–10 d after anthesis).

The evaluated lines included a group of five events (WUE-11 to WUE-15) which was selected from the original group of 170 lines generated by Pellegrineschi et al. (2002, 2004) based on high WUE and PCR analyses confirming the presence of the transgene and no segregation (H Wu, unpublished data). The nine transgenic events selected by survival (SL) were also evaluated. Comparisons were made to both the null-event and the non-transformed Bobwhite.

Evaluation of survival to severe water deficit of the lines selected by WUE

Seeds were imbibed in filter-paper-lined Petri dishes to ensure uniform germination. Germinated seeds were transplanted to pots (37×32×13 cm) in a controlled greenhouse. The five events selected by WUE (WUE-11 to WUE-15) plus BW were planted in the same pot, in
a randomized design with two replicates per pot (first date) and three replicates per plot (date two). Pots were well irrigated until plants reached the 2–3 leaf stage. At this stage, the watering was stopped. Five water stress treatments were then randomly applied to a total of ten pots (two replicates per water stress treatment). Stress treatments consisted in periods of 400, 450, 500, 525, and 550 accumulated growing degree days (GDD) without watering (approximately 21, 23, 25, 27, and 29 d, respectively). After the stress treatment the pots were irrigated to saturation. Plants were afterwards fully irrigated until maturity. Plant survival for each transformation event was recorded as the number of plants that recovered growth after the stress over the total number of plants exposed to the stress. Figure 1, for example, shows plant recovery after a period of 21 d without irrigation (400 accumulated GDD) and 23 d (450 accumulated GDD).

Growing degree days (GDD) was calculated as:

\[
\text{GDD accumulated} = \frac{[(\text{maximum} + \text{minimum daily temperature})/2] - \text{base temperature}}{C}
\]

where wheat base temperature was estimated as 5.5 °C.

Evaluation of plant performance under field conditions

Experiments were conducted at CIMMYT’s subtropical experimental station in Tlaltizapan, Morelos, Mexico (18°41’ N, 99°10’ W, 940 m asl) during one growing season (2009–2010). Mean temperatures varied from 21 °C (November) to 23.3 °C (March), while the plots received a total of 89.4 mm of rain from November to March. The soil was a calcareous Vertisol. Soil properties in the 0–30 cm depth were a pH of 8.2, 2.6% organic matter, 154 ppm of inorganic-N, 7.8 ppm of available Olsen P, and 214 ppm of K. This 0–30 layer had an apparent density of 1.055 g cm⁻³, a saturation point of 64%, a field capacity of 34%, and a permanent wilting point of 20.5%.

Seed of nine events selected by SURV, five events selected by WUE, the null-event, and the non-transformed line Bobwhite were planted on 12 November 2009. Plots consisted in two raised beds (2 m long) with two rows per bed (15 cm inter-row spacing) according to the bed-planting system described by Limón-Ortega et al. (2000). Seeding rate was ~150 plants m⁻². The experiment followed an α-lattice design (Falconer, 1989) with two replicates. All plots received 80 kg N ha⁻¹ and 80 kg P ha⁻¹ at sowing using a combination of urea and diammonium phosphate. In addition, plots received 80 kg N ha⁻¹ (urea) at tillering (21 December 2009). Chemical control was regularly applied to prevent insect proliferation. Weeds were chemically and manually controlled.

Three water deficit treatments (DEF, ANT, and GF) were applied by reducing the irrigation starting at different phenological stages (Table 1). The well-irrigated plots (IRR) received a total of 260 mm of irrigation water throughout the growing season. These well-irrigated plots were watered before the visual symptoms of water stress were observed. The plots under the most severe water deficit treatment (DEF) received a total of 68 mm from irrigation, which represented 26% of the amount applied in the IRR treatment. Plots under the ANT treatments were well-irrigated to the booting stage, receiving a total irrigation of 121 mm. The effect of a terminal stress starting in grainfill was evaluated in the GF group by stopping the irrigation at the early grainfill stage. Gravimetric soil water content was determined by taking soil samples at 0–30, 30–60, 60–90, and 90–120 cm. Total soil water ranged from 251 mm (DEF) to 477 mm (IRR).

Fig. 1. Plant recovery after a period without irrigation of (A) 21 d (400 accumulated GDD) and (B) 23 d (450 accumulated GDD).
Table 1. Date, wheat growth stages, and millimetres of water applied by drip irrigation for treatments in the open-field trial, Tlaltizapan, Mexico, 2009–2010

Plants were grown under severe stress (DEF), terminal water deficit starting at anthesis (ANT), terminal water deficit starting in grain filling (GF), and well irrigated conditions (IRR) in an open field trial in Mexico, 2009–2010.

| Irrigation date   | Growth stage | Severe stress (DEF) | Stress starting at anthesis (ANT) | Terminal stress (GF) | Well-irrigated conditions (IRR) |
|-------------------|--------------|---------------------|-----------------------------------|----------------------|--------------------------------|
| November 2009     | Germination  | 58                  | 58                                | 58                   | 58                             |
| December 2009     | Tillering-Booting | 10                | 63                                | 63                   | 63                             |
| January 2010      | Heading      | –                   | –                                 | 40                   | 71                             |
| February 2010     | Grain fill   | (*)                 | (*)                               | (*)                  | 34(*)                          |
| March 2010        | Grain fill   | –                   | –                                 | –                    | 34                             |
| Total             |              | 68                  | 121                               | 161                  | 260                            |


*(*) 56 mm from rain on 4 February 2010.

Biomass (BM) was estimated from a sample of all above-ground tissue from a 25 cm length of the bed, starting at least 50 cm from the end of the plot to avoid border effects. Samples were taken approximately 10 d after heading. Fresh biomass was oven dried at 70 °C for 48 h for dry weight measurement. Total biomass was then expressed as grams per square metre. Canopy temperature (CT) measurements were taken using a hand-held infrared thermometer in clear days, not earlier than 11.00 h and not later than 15.00 h to avoid dew and to evaluate the plants when air water demand is maximum (Reynolds et al., 1998).

Grain yield (YLD) was estimated by harvesting 1 m × 1.5 m (2 bed) plots, excluding borders in each plot. Harvest was conducted in March 2010.

Statistical analysis

Variance components and their errors were obtained by mixed effects analysis using PROC GLM (greenhouse experiments) and PROC MIXED (field experiment) in SAS (SAS Institute Inc., 2004). For the field experiment, the effects of genotypes, replicates, and blocks within replicates were considered as random, and irrigation treatments, genotypes, and genotype × irrigation interactions were analysed as fixed effects. Tests of significance of fixed effects were conducted using the appropriate mean squares (Steel et al., 1997). Phenotypic correlation analysis was conducted using SAS.

Both grain yield and above ground biomass data from the field experiment were subjected to site regression (SREG) model analysis (Crossa and Cornelius, 1997) in SAS version 9.1 (SAS Institute Inc., 2004). The SREG model estimates the genotype main effects (G) in combination with the GE (GGE) model:

\[
\hat{y}_{ij} = \mu + \delta_{i} + \sum_{m=1}^{n} \lambda_{m} \gamma_{im} + \epsilon_{ij}
\]

where \(\gamma_{ij}\) is the mean of the \(i^{th}\) genotype in the \(j^{th}\) environment with \(n\) replications in each of the \(I \times J\) cells; \(\mu\) is the grand mean over all genotypes and environments; \(\delta_{i}\) is the additive effect of the \(i^{th}\) genotype. In addition, \(\lambda_{m}\), \(\gamma_{im}\), and \(\gamma_{im}\) are estimated from the singular value decomposition of the term that includes genotypes and genotype × environment interaction \(\tau_{i}^{*}+\langle\delta_{i}\rangle\) where \(\tau_{i}\) is the additive effect of the \(i^{th}\) genotype and \(\langle\delta_{i}\rangle\) is the non-additive effect of the \(i^{th}\) genotype in the \(j^{th}\) environment. \(\epsilon_{ij}\) is the average residual error assumed to be NID (0, \(\sigma^{2}/n\)) where \(\sigma^{2}\) is the within-environment error variance (assumed to be constant across locations and genotypes) and \(n\) is the number of replications. The biplots from the SREG model were constructed from the first two principal components (PC1 and PC2) and were used to compare the performance of the different genotypes at different environments.

Results

Evaluation of BM and WUE under greenhouse conditions

Differences in above-ground biomass and WUE were observed when those parameters were evaluated shortly after anthesis on drought-stressed and well-irrigated plants grown in small pots under greenhouse conditions (Table 2). On average, drought reduced biomass by 42% after repeated drought cycles imposed from the 5-leaf stage to anthesis. The transgenic line WUE-12 had the lowest biomass reduction (19%) between the well-irrigated and the water-deficit treatments. The line SL2 showed the largest reduction in biomass (48%), while the control (Bobwhite) had a biomass reduction of 46%.

Significant genotypic differences were observed for biomass under both well-irrigated and water-deficit conditions (Table 2). The events selected by survival rate did not differ from the null and non-transformed control. However, significant differences were observed for the events selected as having high WUE. Under water deficit, all of the events selected for high WUE had higher biomass production than the null and control line. Similarly, three high WUE-selected events had higher biomass than non-transformed Bobwhite under well-irrigated conditions.

Following the same pattern described for plant biomass, significant differences were also observed for WUE under water deficit (Table 2). As expected, four of the events selected for higher WUE outperformed the WUE values for both the null and BW under water deficit. All WUE-selected events tended to have higher WUE than controls under well-irrigated conditions, although the differences was only significant (>\(P=0.05\)) for one of the five events. A significant and positive correlation was observed between BM and WUE both under well-irrigated conditions \((r=0.72, P < 0.001)\) and water deficit \((r=0.88, P < 0.001)\).

No significant differences were detected between the null event and BW, even though the null events tended to have higher biomass and WUE values than the control.
Evaluation of survival to severe water deficit of the lines selected by WUE

Wilting was equally observed for transgenic and non-transgenic plants at 17 d after the last uniform irrigation, which was equivalent to 350 GDD. On average, the treatment that was lethal to 50% of the plants was observed after 23 d from the last irrigation (450 GDD). The treatment that was lethal to 100% of the plants was observed after 29 d from the last irrigation (550 GDD).

Results obtained in greenhouse experiments confirmed the advantage of DREB1A-transgenic events on plant recovery after severe water stress when compared with non-transformed Bobwhite (Pellegrineschi et al., 2004). Average survival rates after 23 d from the last irrigation are shown in Table 3. The highest survival rate was observed in transgenic line WUE-13 where 67% of the tested plants survived when only 19% of BW survived. Comparing only the plants that survived the severe stress treatment, no advantages in height, number of tillers per plant, biomass, number of green leaves per plant, or leaf growth per day were observed in transgenic survival-selected lines compared with Bobwhite.

Evaluation of plant performance under field conditions

The irrigation treatments varied from 260 mm of water (IRR) to 68 mm (DEF) applied throughout the growing season, while total soil water to the crop ranged from 251 mm (DEF) to 477 mm (IRR). These irrigation regimens had a significant main effect (P < 0.01) for both BM and YLD. The total biomass was reduced from 504 g m⁻² at IRR to 325 g m⁻² at DEF. The mean for YLD (averaged across genotypes) ranged from 318 g m⁻² at IRR to 199 g m⁻² at DEF. A significant negative correlation was observed between CT and grain yield (r = −0.75; P < 0.0001).

Significant differences were also observed when comparing the genotype BM and YLD performance (Table 4). The transgenic line SL-5 had a significantly higher BM compared with both BW and the null event in GF. One of the transgenic events selected by WUE (WUE-11) outperformed the control lines for YLD under IRR conditions. This

| Strategy for selection | Code | Well irrigated | Water deficit |
|------------------------|------|----------------|--------------|
|                        |      | WU (ml)         | Biomass (g plant⁻¹) | WUE (mg ml⁻¹ plant⁻¹) | WU (ml)         | Biomass (g plant⁻¹) | WUE (mg ml⁻¹ plant⁻¹) |
| Water use efficiency   | WUE-11 | 1407 | 3.97 | 2.93 | 843 | 2.55 | 3.16 |
|                        | WUE-12 | 1180 | 2.90 | 2.50 | 710 | 2.34 | 3.26 |
|                        | WUE-13 | 1336 | 3.26 | 2.52 | 697 | 1.97 | 2.78 |
|                        | WUE-14 | 1314 | 3.55 | 2.77 | 806 | 2.64 | 3.39 |
|                        | WUE-15 | 1309 | 3.37 | 2.66 | 777 | 2.61 | 3.41 |
| Survival               | SL-1   | 1130 | 2.64 | 2.36 | 853 | 1.45 | 1.88 |
|                        | SL-2   | 1334 | 3.21 | 2.45 | 902 | 1.67 | 1.91 |
|                        | SL-4   | 1484 | 3.08 | 2.13 | 977 | 1.70 | 1.79 |
|                        | SL-5   | 1087 | 2.43 | 2.25 | 783 | 1.43 | 1.88 |
|                        | SL-6   | 1135 | 2.67 | 2.38 | 783 | 1.30 | 1.74 |
|                        | SL-7   | 1229 | 2.76 | 2.30 | 868 | 1.47 | 1.78 |
|                        | SL-8   | 1210 | 2.82 | 2.41 | 818 | 1.46 | 1.86 |
|                        | SL-9   | 1171 | 2.71 | 2.32 | 770 | 1.35 | 1.80 |
|                        | SL-10  | 1114 | 2.46 | 2.29 | 880 | 1.55 | 1.81 |
| Null-event             | Null   | 1252 | 2.94 | 2.40 | 733 | 1.59 | 2.37 |
| Bobwhite               | BW     | 1221 | 2.74 | 2.25 | 729 | 1.47 | 2.06 |

Table 3. Survival rate (%) and standard error from four (first date) or six (second date) transgenic plants and control plants (non-transformed Bobwhite) at 23 d after the last irrigation

| Group      | Survival rate (%) | SE |
|------------|-------------------|----|
| WUE-11     | 48                | 12 |
| WUE-12     | 48                | 12 |
| WUE-13     | 67                | 10 |
| WUE-14     | 45                | 5  |
| WUE-15     | 45                | 9  |
| Bobwhite   | 19                | 9  |

Mean 45
CV 17
P>F 0.047
LSD (5%) 28

a SE, standard error.
transgenic line had a yield of 366 g m\(^{-2}\) under IRR while yields of 297 g m\(^{-2}\) and 310 g m\(^{-2}\) were observed for BW and null event, respectively. Even when significant differences were observed under IRR, no significant increases in YLD were identified for the transgenic events compared with the null and the conventional line in any of reduced irrigation treatments.

Differential ranking of events was observed for both BM and YLD across water deficit treatments. The SREG analysis for BM and YLD in this set of 14 transgenic lines, a null event, and a BW in four irrigation treatments are presented in Fig. 2. In both graphs, there was a clear separation between responses (treatment axes) observed under the three reduced irrigation treatments and responses in the well-irrigated treatment.

For BM, the two first components explained 46.8% and 36.7% of the total genotype\(\times\)treatment interaction. The SREG analysis for BM demonstrated that the event SL-5 was the top ranking at the DEF treatments, while SL-6, WUE-13, and WUE-11 were the best at IRR.

Considering the same group of lines, the two first components of the SREG biplot for YLD explained 72% and 17% of the total genotype\(\times\)treatment interaction. As described before, the transgenic event WUE-11 was the highest ranked line under the well irrigated treatment. The rest of the WUE lines are grouped at the centre of Fig. 2.

Even though none of the transgenic events outperformed BW or the null event for YLD, the events selected by WUE tended to be the most stable lines, being grouped in the centre in both SREG biplot graphs. These WUE events were then identified by the SREG biplots as lines that combine an acceptable BM and YLD and stable performance across different environments generated by the water-deficit treatment.

## Discussion

Realistic greenhouse experimental protocols for screening for drought resistance are crucial for the identification of high performance lines, especially in studies trying to demonstrate the potential benefit of a specific transgene due to the restrictions on open field trials of transgenic material. Several studies conducted in transgenic crops, while having success in assessing transgene expression, may give misleading conclusions from an agronomic or physiological perspective (Bhatnagar-Mathur et al., 2008), mainly due to the fact that testing transgenic lines is generally conducted under artificial stress conditions, small pots, and at early seedling growth stages (Yang et al., 2010). In this study, the effect of two different selection criteria were tested by evaluating the field performance of 14 transgenic wheat lines previously selected by high survival after severe water deficit and high WUE under greenhouse conditions. Fourteen genetically modified events, one null event, and one non-transformed control line were exposed to three levels of water deficit and to well-irrigated conditions in the field. The treatments affected plant productivity as measured by above-ground biomass and...
Increasing the number of plants evaluated per event (De et al., 1995; Arun et al., 2002). The resulting transformed wheat lines could, therefore, be considered nearly isogenic to SH 98 26 Bobwhite. Nevertheless, it is still possible that some variability within line SH 98 26 may have confounded the effect of the transgene, explaining some of the differences observed between lines. Variation between the transformed lines could also be due to position effects of the DREB1A insertions (i.e. undetermined genomic location may cause variations in gene expression; Xu, 2010) as well as related to the number of copies of the transgene in each transformed event. In addition, somaclonal variation is not unknown when wheat plants are regenerated from an embryogenic calli, which may result in agronomic differences between lines coming from tissue culture (Cheng et al., 1992; Karp, 1995; Arun et al., 2007). Based on theoretical relationships between variance components, it has been suggested that priority should be given to testing more events, rather than increasing the number of plants evaluated per event (De Wolf et al., 2010). Consequently, even when the lines are considered to be isolines, the significant variability observed in the performance of transformed lines, both under greenhouse and field conditions is not unexpected, reinforcing the need for reliable and relevant selection methods to identify superior material.

The results from pot experiments confirmed the higher survival and recovery after severe water deficit which had previously been reported for the DREB gene in wheat (Pellegrineschi et al., 2004; Gao et al., 2009) and other crops such as tobacco (Kasuga et al., 2004), rice (Dubouzet et al., 2003), maize (Qin et al., 2004), chickpea (Bhatnagar-Mathur et al., 2004), and soybean (Li et al., 2005). Experiments previously conducted under greenhouse conditions by Pellegrineschi et al. (2004) exposed AtDREB1A transgenic wheat plants to water stress by withholding water at the 4–5 leaf stage. In these evaluations, the selected transgenic wheat lines started to show water-stress symptoms to severe stress later than the controls. The presence of the transgene was always associated with higher levels of drought resistance. Similarly, higher survival rates after depriving water for 20 d at the stem elongation stage were observed in GhDREB wheat transgenic lines grown in pots (Gao et al., 2009). It has been suggested that this improved survival after severe drought conditions in the genetically modified plants relative to controls could be associated with either the activation of genes related to drought resistance or to a reduced consumption of water resulting from smaller plant sizes, i.e. a more conservative growth pattern in the transgenics compared with controls (Bhatnagar-Mathur et al., 2004; Morran et al., 2010). Therefore, whenever the final objective of transgenic crops is to increase grain yield, conservative growth patterns should be avoided. The use of drought-inducible promoters has been proposed as a way to minimize the stunted growth patterns observed under constitutive transgene expression (Kasuga et al., 1999; Morran et al., 2010). In greenhouse experiments, the expression of DREB1A from Arabidopsis thaliana driven by the stress-inducible promoter rd29A increased the survival rate of transgenic plants without growth retardation. In addition, the positive association between WUE and total biomass suggests that an increase in grain yield is possible through increasing WUE in...
transgenic plants, assuming that HI is maintained (Wright, 1996).

Results from this work suggest that the use of WUE as a mechanism of dehydration avoidance may increase the efficiency for selection of more stable lines. An improvement in WUE should be associated with a faster crop growth rate (Condon et al., 2002) especially for Mediterranean-type environments or within-season rainfall environments. If selection by WUE is only driven by variations in water use rather than biomass per given amount of water use, the selected plants could be the smaller ones, the ones that used less water over the growing season (Blum, 2005). A more ‘conservative’ water use and reduced total biomass may work for some particular environments, but are probably not the best criteria for selecting high-performance, high-yielding lines for Mediterranean-type environments, where factors such as fast crop growth rate, ability to remobilize stored reserves, earlier flowering, better water extraction, and stomatal insensitivity to water deficit have been found to be correlated with higher and more stable yields (Condon et al., 2002, 2004).

Water use efficiency measured under greenhouse drought conditions is therefore proposed as an adequate trait to select for stable wheat yields under water deficit. The use of traits with higher heritability than yield per se, has been proposed as an alternative for selecting towards high yield potential, especially under severe stress conditions (Araus et al., 2002). The available data indicate that, in this set of lines where the transcription factor DREB1A from *Arabidopsis thaliana* was driven by the stress-inducible promoter from the rd29A gene, high WUE is not associated with conservative growth in the absence of water stress, WUE being positively associated with BM. The increment in WUE was mainly driven by increasing plant biomass rather than by reducing water use, reduced growth rate or plant size. The events selected by WUE tended to be the most stable lines, being grouped in the centre in both SREG biplot graphs. According to the results observed in the biplot, the selections made using the WUE criteria identified the best genotypes across different environments (wide adaptation).

A cultivar with the capacity to use more water during its growth would have lower CT, mainly as a result of more open stomata. In this study, the better yielding cultivars showed cooler canopies than drought-susceptible genotypes. The significant correlations found between CT and grain yield, in addition to the low cost and easy procedure, reinforces the potential of CT for predicting high yields in rainfed environments (Araus et al., 2002; Olivares-Villegas et al., 2007).

While no increases in grain yield under stress are reported, it is generally accepted that before having an elite, high-yielding, genetically modified crop a ‘first phase’ of evaluation is necessary, which involves the screening of a large number of events, typically more than 30 (De Wolf et al., 2010), in an easily transformed background to identify potential lines having the transgene active and integrated in a suitable insertion location. A reduction in grain yields has been recognized as a ‘yield penalty’ associated with *Agrobacterium*-mediated transformation in rice (Xiao et al., 2007), which could be due to (i) detrimental effects caused by the tissue-culture regeneration, (ii) random insertion of T-DNA, (iii) disruption of genes related to yield, and/or (iv) genetic or physiological incompatibilities. To overcome these difficulties, it has been proposed that a high number of transformed lines should be screened for drought resistance, morphological and phenotypic traits, as well as yield performance under field conditions. Considering that the 14 transgenic lines evaluated in this study showed no pleiotropic effect, nor unpredictable unwanted effects when compared with control lines, it is possible to assume that development of high-yielding transgenic wheat lines is achievable if appropriate transformation and screening protocols are implemented.

Although to date no transgenic wheat has yet been commercialized, it has the potential to contribute to a ‘major leap forward in grain yield potential’ that wheat breeders are demanding (Graybosch and Peterson, 2010), considering the challenge of increasing food production to feed a growing world population under a warming climate. A concurrent effort in the identification of appropriate gene and gene–promoter combinations, the insertion of the proper transgene in appropriate backgrounds, and correct selection methods would need to be implemented. This must be part of an integrated, multidisciplinary approach spanning plant molecular biology, physiology, and breeding (Manavalan et al., 2009) to achieve the needed improvement in the genetic potential for grain yield. An example of this venture is the current evaluation of performance under drought of new transgenic wheat lines with different promoter–gene combinations. Performance of the most promising lines will be validated under both greenhouse and field conditions.

**Main conclusions**

(i) From greenhouse experiments, DREB1A from *Arabidopsis thaliana*, driven by the stress-inducible promoter rd29A increased the survival rate of transgenic plants without growth retardation.

(ii) The positive association between WUE and total biomass suggests that an increase in grain yield may be possible by increasing WUE in transgenic plants, assuming that HI is maintained.

(iii) Under field conditions, the events selected by WUE in greenhouse screens were found to combine an acceptable yield—an even higher yield for WUE-11 under well-irrigated conditions—and stable performance across the different environments generated by the treatments.

(iv) While no increases in grain yield under stress are reported, the 14 transgenic lines evaluated in the field showed no pleiotropic effects, nor unpredictable unwanted effects when compared with control lines. Therefore, it is possible to assume that high yielding wheat transgenic lines would be achievable if adequate transformation and screening protocols are implemented.
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