Water saving traits co-map with a major terminal drought tolerance quantitative trait locus in pearl millet [Pennisetum glaucum (L.) R. Br.]

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Abstract Low transpiration rates under fully irrigated conditions decrease plant water use at vegetative stage and then increase the water availability during grain filling and then the terminal drought tolerance in pearl millet. 113 recombinant inbred lines developed from a cross between H77/833-2 and PRLT2/89-33 (terminal drought sensitive × tolerant genotype) were evaluated to map transpiration rate (Tr, a proxy for canopy conductance), organs weight, leaf area and thickness and study their interactions. Transpiration rate was increased by two H77/833-2 and two PRLT2/89-33 alleles on linkage group (LG) 2, whose importance depended on the vapor pressure deficit. The two H77/833-2 and one PRLT2/89-33 alleles co-mapped to a previously identified major terminal drought

tolerance QTL, although in a much smaller genetic interval. The other Tr allele from H77/833-2 also enhanced biomass dry weight and co-located with a formerly identified stover and tillering QTL. Leaf characteristics were linked to two loci on LG7. Plant water use was increased and decreased by different loci combinations for Tr, tillering, and leaf characteristics, whose respective importance depended on the environmental conditions. Therefore, different alleles influence plant water use, have close interactions with one another and with the environment, so that different ideotypes for plant water use exist or could be designed, from specific allele combinations conferring particular physiological characteristics for specific adaptation to a range of terminal drought conditions.

Key words Transpiration rate (Tr) · Vapor pressure deficit (VPD) · Leaf development · drought · Genotype-by-environment interaction (G×E) · QTL interaction

Introduction

Recent decades have seen an increased research interest in crops' drought tolerance improvement. Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is considered a drought tolerant crop *per se*, yet there exists considerable genotypic yield variation in drought stress environments (Bidinger and Hash 2004; Bidinger et al. 1987). Pearl millet is commonly grown on marginal lands of the semi-arid tropics where severe terminal droughts are the most yield destructive factors (Mahalakshmi et al. 1987).

The breeders' efforts to identify drought tolerant material and localize the genomic segments responsible for drought tolerance are generally based on yield performance in targeted environments, although G × E interaction effects on yield frequently hamper these selection efforts (e.g. Banziger and Cooper 2001; Tuberosa et al. 2007). G × E interactions likely reflect the relative importance of certain plant mechanisms and/or their interactions to specific environments, which can lead to specific adaptation to drought conditions. Further progress in drought tolerance breeding then depends on the identification of tolerance mechanisms and on the understanding of interactions of these mechanisms among them and with the environment (Blum 1988; Ludlow and Muchow 1990; Fussell et al. 1991). Several successful examples have been reviewed (Sinclair et al. 2004).

A major terminal drought tolerance quantitative trait locus (DT-QTL) was identified in two independent mapping populations on LG2 explaining up to 32% of pearl millet grain yield variability in severe terminal drought environments; i.e. such where crop growth depends almost entirely on moisture stored in the soil profile (Bidinger and Hash 2004; Yadav et al. 2002, 2004; Bidinger et al. 2007). A recent physiological dissection of mechanisms and traits underlying the DT-QTL has pointed to water conserving mechanisms being associated with the DT-QTL (Kholová et al. 2010a, b). This current hypothesis - that these traits, mostly expressed under non-stressed conditions (vegetative developmental stage), allow water saving in the soil profile throughout the season and make it available for the grain filling period - is in agreement with the fact that the DT-QTL confers better grain filling and seed setting (Serraj et al. 2005; Vadez et al. 2011). However, trait interaction with the environment is also critical (e.g. Tardieu et al. 2000; Raymond et al. 2003). Indeed, the lower transpiration rate of tolerant entries (Tr; g water transpired cm^{-2} leaf area) (Kholová et al. 2010a) was also sensitive to the vapor pressure deficit (VPD) (Kholová et al. 2010b). Similar mechanisms have recently been characterized in sorghum (Gholipoor et al. 2010), groundnut (Devi et al. 2010), chickpea (Zaman-Allah et al. 2011). Therefore, not only a low transpiration rate is important for water saving in pearl millet, but also how this trait responds to the environment. So, mapping of these traits requires careful consideration of the environmental conditions in which they are assessed.

Although several pearl millet near-isogenic lines containing the DT-QTL (NIL-QTLs) had lower Tr (Kholová et al. 2010 a, b), not all NIL-QTL exhibited yield advantage under terminal drought conditions (Serraj et al. 2005), suggesting that recombination event(s) in that region might have "excluded" the beneficial fragments in some of the NIL-QTL. Studies in *A. thaliana* reported a genomic region responsible for drought avoidance co-mapping with a region contributing to a constitutively lowered Tr and simultaneously leading to an enhanced TE (Masle et al. 2005; McKay et al. 2008). Following these examples, our current hypothesis is that only critical portions of the large DT-QTL, linked to specific mechanisms, matter for the terminal drought tolerance of pearl millet and these needs to be accurately mapped to enhance the precision of marker assisted introgression. In addition, Tr is only one component of plant water use, which likely

interacts with other components of plant water use (tillering, leaf area, leaf thickness). Therefore we hypothesized that Tr would be the leading trait of the DT-QTL region, and that total plant water use could be finely regulated through interactions of genomic regions involved in plant water use, including Tr.

The overall objective of this study was to map QTL for Tr and their interactions with other traits related to plant water use. Specifically, this work intended to: i) Assess whether putative Tr QTLs co-map with the DT-QTL region, ii) Identify other genomic regions related to plant water use (tillering, biomass components, leaf characteristics), iii) Cross-compare the identified QTLs of Tr and growth related traits with previous mapping studies in pearl millet, and iv) Assess how individual or interactive loci determine total plant water use and deduce linkages between traits, based on the genomic regions involved and their interaction with the environment, to design specific ideotypes.

Materials and methods

Plant material

The recombinant inbred line (RIL) population used in this study was based on the cross between terminal drought tolerant inbred PRLT2/89-33 and terminal drought sensitive inbred H77/833-2 (Hash et al. 1999). These parental inbred lines cross was advanced to F6 generation recombinant inbred lines (RILs) through single seed descent method. DNA was isolated from F6 inbred lines for genotyping with SSR and DArT markers (Supriya et al. 2011). Phenotypic evaluation was assessed on test-cross hybrids of these inbred parental lines, developed by crossing the inbred lines to male sterile line tester 834A (Stegmeier et al. 1998). Use of testcross hybrids is desired mainly to restore heterotic vigor of inbred lines, because pearl millet is a highly cross-pollinated species and suffers severely from inbreeding depression (Jones et al. 1995).

Phenotyping and plants growing conditions

The RIL population of 113 testcross hybrids and two parental testcross genotypes were sown into 20-cm diameter plastic pots filled with 5kg of Alfisol. Each pot was sown with several seeds in 4 separate hills, thinned to one plant per hill one week after sowing and to two homogenous plants per pot at two weeks after sowing. Adequate fertility was

provided with 300mg diammonium phosphate kg^{-1} soil at sowing and 200 mg urea kg^{-1} soil at 15 days after sowing (DAS). For logistical reasons, five experimental sets (replications) were sown sequentially on 24/11, 26/11, 28/11, 2/12 and 4/12/2009. The maximum/minimum temperature and relative humidity percentage at day/night averaged 36/15^{o}C and 35/94% during the growth period but fluctuated (Supplementary Table 1 and Figure 1). Each experimental set contained one pot of each RIL testcross genotype and three pots of each parental testcross genotype; plants were maintained well-watered during the entire duration of the experiment. Phenotyping was initiated at 18/12, 21/12, 23/12, 28/12 and 31/12 for set 1-5, so that plants had similar age in all sets at the time of measurement, (25-27 days) and all were at vegetative stage, i.e. the stage well prior the terminal drought would naturally occur and when water saving mechanisms were shown to operate (see Kholová et al. 2011a, b, c). Prior to experimentation, pots were watered to field capacity. The soil was covered with a plastic sheet and a 3 cm layer of plastic beads to limit soil evaporation. In each experimental set, six check pots containing no plants but treated similarly were kept to assess the extent of soil evaporation.

Pots were weighed three times at 7:00 a.m., 10:30 a.m. and 2:30 p.m. The weighing took typically ten minutes and the pots were weighed following the same sequence, so that the time interval between pot weighing was the same for all pots. These timings were chosen to assess plant transpiration (T) during a period with low evaporative demand in the morning hours (average VPD 1.57 kPa) and during a period with high evaporative demand in the early afternoon hours (average VPD 3.53 kPa), following previous results in similar experiments (Kholová et al. 2010b). After the third weighing, plants were re-watered to pot capacity, left to drain overnight and the same procedure was repeated the following day using the same set of plants (except of the set 5, which was assessed only once). After the last weighing on the second day of observation in a given set, the plants were harvested and leaf area (LA) measured immediately (using LA meter, LI3000 model, Li-Cor, Lincoln Nebraska, US) and later used for calculation of Tr (g water cm^{-2} leaf area h^{-1}). Other parameters were also measured; root dry weight (RDW, measured in set 3-4-5), stem dry weight (StDW), leaf dry weight (LDW), shoot dry weight (ShDW; StDW + LDW), biomass dry weight (BDW = RDW+ShDW; measured in set 3-4-5), specific leaf weight (SLW=LDW/LA). The hourly temperature and humidity were

recorded in two spots of the experimental set to calculate the average VPD within the time interval of observation. Spacing between the pots was wide so that the leaf area index was below one, avoiding mutual leaf shading.

Genotyping and linkage map development

The linkage map consisting of 321 markers (258 DArT and 63 SSR) was used to identify the QTL. The details on genotyping and map construction are available in Supriya et al. (2011). The F6 generation of that population is characterized by increased homozygosity accompanied by increase in segregation distortion, which is common in pearl millet but also in other crops (see Supriya et al., 2011). In short, about 35% of the markers showed segregation distortion, with about 10/25% in favor of H77/833-2/ PRLT2/89-33. Most of the markers showing distortion in favor of PRLT2/89-33 were concentrated on LG1 and LG6. More details on the segregation distortion can be found in Supriya et al. (2011). While segregation distortion can cause an overestimation of the recombination frequency between markers, some argue that segregation distortion has little effect on marker order and map length (Hackett and Broadfoot, 2003). This phenomenon was not overly important in two of the linkage groups (LG2 and LG7) in which many of the QTLs reported here were identified. Therefore, while we are aware that segregation distortion could be an issue for QTL detection in many crops, we assumed that much of the conclusions that are drawn in this paper, in large part from information in LG2 and LG7, would remain valid, as previously argued (Semagn et al., 2006). The earlier Qi et al. (2004) consensus linkage map using SSR and RFLP information allowed us cross-comparison of our results to previous mapping studies (Yadav et al. 2002, 2003, 2004, Nepolean et al. 2006 and Bidinger et al. 2007).

Statistical analysis

Since some traits such as the transpiration rate (Tr) depended on VPD at the time of the experiment (Kholová et al. 2010b), and others like the leaf area could be influenced by VPD conditions during plant growth (Kholová et al. 2010c), the analysis was performed both on individual experimental sets and on the best unbiased linear predicted values (BLUPs; calculated using SAS, version 9.2) which were generated for every trait and

used in mapping analysis. The variation within and between sets was assessed by the set average, standard error (SD), minimum and maximum trait values. Differences between parental genotypes across experimental sets were further evaluated using block ANOVA design with blocks defining particular observations in time. Simple correlation were analyzed between the BLUPs for each trait (CoHort software, 6.204, Monterey).

The composite interval mapping approach (CIM) was used to detect QTLs using PLABQTL, where QTLs are initially identified by simple interval mapping (SIM) and then used as co-variants for CIM, with a F-to-enter value of 8. Additive model was engaged in detecting the QTL effect for any individual loci and additive × additive model (A×A) was employed to detect interacting loci (Utz and Melchinger 1996). A threshold of 2.5 was used and 1000 bootstrap runs were performed using the same software.

The genotype matrix mapping (GMM, software, version 2.1) approach was used to assess putative interactions between loci (Isobe et al. 2007). In our case the number of interacting loci was limited to two and three because of the modest RIL population size. Nevertheless, the GMM approach uses a different method of QTL identification (GMM uses F-measures algorithm of QTL detection) than PLABQTL software does (CIM), and the single QTL estimation cannot be rigorously compared between these two methods.

Principal component analysis (PCA) was used to visualize the investigated traits' relations in multidimensional space using R software (version 2.11.1). PCA output was further used to map common genetic background (pleiotropy) of all three major components, where each major component clubbed together related traits (using PLABQTL). For this PCA analysis only the BLUP means were used. The broad sense heritability (h^2) was calculated as $h^2=\sigma^2G/(\sigma^2G+\sigma^2E)$ with GenSTAT (version 12)

Results

Individual QTLs determining transpiration rate (Tr)

Tr at low VPD (morning hours) was about half than Tr at high VPD (noon hours) but, as expected, there was also variability between sets within particular VPD regime (details in Suppl. Table 1). The Tr of tolerant PRLT2/89-33 was among the lowest values of the Tr distribution (morning-noon Tr; 0.011-0.025 g cm^{-2} h^{-1}), and was 40-45% lower at low-

high VPD than sensitive H77/833-2 which tended to the opposite end of the Tr distribution (morning-noon Tr; 0.016-0.035 g cm^{-2} h^{-1}), with several transgressive segregants on both sides of the distribution (Suppl. Table 2). There was highly significant genotypic effect for Tr under both low and high VPD.

Three QTL for the transpiration rate mapped in the major DT-QTL interval on linkage group 2: **(i)** one QTL under low VPD conditions only, around 260 cM (between 258 and 264 cM) and hereafter referred to LG2, 260 for simplicity, where '260' is used as a short name for the full confidence interval. As expected, the terminal drought sensitive H77/833-2 provided the allele for the LG2, 260 QTL for Tr under low VPD, which was responsible for up to 26% of the Tr variation; **(ii)** One QTL explaining up to 16% of Tr variation under low VPD, located at LG2, 322 cM, with a positive effect from H77/833-2 (Suppl. Table 3, Fig. 1b). This QTL controlled Tr under both low and high VPD conditions, although the QTL accounted only for maximum of 13% of the phenotypic variations explained (PVE) in high VPD conditions. Interestingly, the VPD condition in which this QTL was identified under low VPD was relatively higher (2.19 kPa) compared to the average morning VPD of the other experimental sets. **(iii)** One QTL explaining up to 14% of Tr variation under low VPD only, located on LG2, 315, with a positive effect from PRLT2/89-33 (Suppl. Table 3, Fig. 1b). A fourth QTL for Tr, explaining up to 25% of the Tr variation, was found on LG2, 10, away from the major DT-QTL region, under low VPD conditions only, with a positive allele contributed by PRLT2/89-33 parent. Interestingly, the very same genome position was also found controlling biomass accumulation and its components (LG2, 10; see below). Additionally, few minor loci affecting Tr were located on LG3 and LG7 (Suppl. Table 3).

In sum, the transpiration rate (Tr, g water cm^{-2} leaf area h^{-1}) was strongly linked to several major QTL regions across the range of VPD conditions, in particular three different regions co-mapping with the DT-QTL, two of which with a positive allele from the sensitive parent and one of these specific to high VPD conditions, but also another region unreported before with positive loci being contributed by the PRLT2/89-33 allele, suggesting the complexity of the transpiration rate trait, but also its rather simple genetic determination (Suppl. Table 3, Fig. 1).

Individual QTL determining biomass and its components

Variation in biomass and components was found both across the RIL population and experimental sets (Suppl. Table 2). PRLT2/89-33 developed about 60% larger LA and thinner leaves at this early developmental stage than H77/833-2 (Suppl. Table 2), in agreement with earlier report (Kholová et al. 2010c).

Biomass accumulation was influenced by multiple genomic regions across LG1, LG2, LG4, LG6, LG7 (Suppl. Table 4, Fig. 1, Suppl. Fig. 2). A major QTL explaining up to 22% variability in the BDW and its components (LDW, SDW, RDW, StDW and ShDW) was located on LG2, 10, with a positive effect from drought sensitive H77/833-2. Another QTL explaining more than 10% of the variation, was found on LG7, 110, with positive allele from terminal drought tolerant PRLT2/89-33. Several smaller QTLs were found on LG1, explaining usually less than 10% of the variability. Few other alleles were also identified on LG1, 54, LG4, 100 and LG6, 20-50.

Interestingly, the positive PRLT2/89-33 allele for biomass dry weight on LG7, 110 also explained 29% of the leaf area (LA) variations and 21% of the leaf dry weight (LDW) variations. Another locus on LG7, 75, explained up to 15% of the variation in the specific leaf weight (SLW) with positive allele from H77/833-2, and up to 12% LA variation with positive allele from PRLT2/89-33. So, this locus, distinct from LG7, 110, appeared to play a specific role on balancing leaf area and leaf thickening (high SLW indicating thick leaves). The above discussed positive PRLT2/89-33 allele on LG 2, 10 also explained up to 18% of the SLW variation (Suppl. Table 4; Fig.1). None of QTL interactions detected for biomass traits using the A×A model in PLABQTL software was further considered because these interactions couldn't explain higher portion of variation compared to single detected QTL.

Individual QTLs determining transpiration (T)

The RIL population segregated widely for transpiration related traits. Despite its 60% higher leaf area PRLT2/89-33 had only 25-20% higher transpiration than H77/833-2 at this developmental stage under low and high VPD (Suppl. Table 2), which was related to a 40-45% lower Tr at low-high VPD in PRLT2/89-33 than in H77/833-2.

A QTL explaining 9% and 5% of the T variation under high and low VPD was found on LG7, 110, with positive allele from PRLT2/89-33. Under low VPD conditions there was a significant positive effect of loci LG2, 260 (up to 10%) and LG2, 322 (up to 8%) on T, both from sensitive parent H77/833-2, co-located in loci controlling Tr. Under high VPD conditions, only the locus LG2, 10 explained up to 18% of the variation. Again, any detected A×A QTL interactions could not explain higher portion of variation than single detected QTL. In sum, the total amount of water transpired (T) in low and high VPD conditions were controlled by loci that were also linked to Tr and biomass components, and to a QTL on LG3, 25, explaining 13% of the variation, with positive allele from H77/833-2 (Suppl. Fig.2; Suppl. Table 3 and 4).

Analysis of trait relationships and mapping of PCA

A simple correlation analysis (Suppl. Table 5) and a principal components analysis (PCA, Fig 2 and Suppl. Table 6,) were performed to decipher the relationships between parameters. As expected, simple correlation analysis showed that the majority of investigated traits were inter-related. The purpose of the PCA was to group individual parameters in a more comprehensive manner. Three principal components (PC) explained 86% of the variability. The PC1 (60%) had strong positive loading from all shoot biomass traits and transpiration (T), and relatively strong negative loading from Tr and SLW which agrees well with the strong negative correlations between these traits (Suppl. Table 5). The PC2 (20%) had strong negative loading from Tr, SLW, and somewhat T under low VPD. Finally, the PC3 (6%) had a strong negative loading for root dry weight (RDW) and a strong positive loading from the transpiration under low VPD (TM) (Suppl. Table 6). Interestingly, both analyses showed that Tr was significantly, though less strongly, related to traits corresponding to the biomass and/or absolute values of T under high VPD while the Tr-TM relationship was non-significant. Simultaneously, all biomass traits and T were positively related (Fig. 2, Suppl. Table 5).

To assess the pleiotropy of putative QTL regions the coordinate values of RIL lines on each of the three main principal vectors and of each PC clusters were used in a CIM analysis. A single QTL for PC2 on LG2, 322 (14%) was found, with a positive allele from tolerant parent PRLT2/89-33 (Suppl. Table 4). Considering the strongest loadings

of this PC2 were Tr and SLW, we interpret that both these traits are probably cross-regulated from this genome portion.

Interaction of QTLs affecting transpiration rate (Tr)

GMM analysis showed that the interaction of two and three loci could in many cases increase the PVE compared to single locus PVE (Table 1). Although the RIL population exhibited some level of segregation distortion preferring male alleles (Supriya et al. 2011), a majority of the interaction estimates were supported by a reasonable number of RILs allowing reliable analysis (Table 1). The major loci found by GMM were identical to those found using CIM approach, although GMM output could not be rigorously compared to CIM results from PLABQTL. In the following text symbols A, B, H stand for alleles originated from sensitive parent (H77/833-2), tolerant parent (PRLT2/89-33) and their heterozygous alleles combination (AB) respectively. Only the strongest QTLs or QTL interactions are discussed.

The major loci for Tr identified through CIM (namely LG2, 260 and LG2, 315) had large interactions with other loci and consequently explained more variation than the sum of their single locus effects (Table 1). Tr under low VPD was increased by the combination of alleles on LG2, 315(B)-LG2, 261(A) (positive effect 26%) and/or from the LG7, 75 (A)–LG2, 216 (B)–LG1, 115(A) (positive effect 29%) (Fig.3a). Similarly, Tr under high VPD was increased by the interaction of LG7, 75 (A)-LG2, 315 (B)-LG2, 258 (A) (positive effect 23%). Surprisingly, the allele on LG2, 322 (A), though identified as single strong QTL enhancing Tr in the CIM analysis, did not interact with other loci of similar effect on Tr (note: LG2, 315 (B) and LG2, 322 (A) appear to be two distinct antagonistic QTLs).

Interaction of QTLs affecting biomass and biomass components

Total biomass (BDW) was positively influenced by the single effect of LG2, 0 (A, 20%), a locus that was also identified in the CIM analysis. No allele combinations could explain a higher proportion of the variation than this single region on LG2, 0 (A) (Table 1).

Biomass increases were mostly explained by a higher leaf biomass (LDW), which was linked to a single locus on LG7, 113 (B, 11%) and in particular to the allele combination

of LG7, 107 (B)-LG5, 13 (B)-LG2, 0 (A) (17%). Leaf area was also strongly influenced by this LG7 region (around 110, B, 15%) but also by a different locus on LG7, 75 (A). The combination of alleles on LG7, 71 (A)-LG2, 205 (A) and on LG7, 107 (A)-LG2, 323 (A)-LG2, 260 (A) decreased LA (-19%). By contrast, combination of alleles on LG7, 71 (B)-LG7, 113 (B)-LG2, 0 (A), and on LG7, 71 (B)-LG2, 0 (A) increased LA, explaining 21% and 22% of the variations. Finally, most of SLW variation was also dependent on three alleles combination on LG7, 75 (A)-LG1, 296 (A)-LG1, 115 (A) and LG7, 75 (A)-LG1, 296 (A)-LG1, 131 (A) explaining 17 and 19% of SLW variation. The remaining biomass part, root dry weight, was strongly influenced by the combinations of alleles on LG7, 81(B)-LG4, 18 (B)-LG2, 346 (A), explaining 36% of the RDW variation, where PVE estimates were based on 10 RILs carrying that marker combination (Table 1).

Interaction of QTLs affecting transpiration (T)

The percentage variation in absolute transpiration (T) also increased with allele combinations, although many of these loci were not identified by the CIM algorithm (Suppl. Table 3 and Table 1). The most effective allele combination positively influencing T in low VPD conditions (TM) was: LG5, 10 (B)-LG2, 319 (A)-LG1, 75(B) (17%) (Fig. 3b). In contrast, T at high VPD regime (TA) was strongly and positively influenced by single effect of QTL on LG2, 0 (A, 13%), this loci being the same influencing biomass in the CIM analysis (Suppl. Table 3 and Table 1, Fig. 3c). Under high VPD also, the following combination of three alleles from loci on LG7, 122 (A)-LG5, 23 (A)-LG7, 173 (A) (17%) and LG4, 98 (A)-LG1, 194 (A)-LG1, 46 (B) (18%) had a negative effect on TA. In sum, a majority of strong alleles' combinations participating in T regulation combined "biomass" QTLs effect with the effects of loci playing a part in Tr regulation (Suppl. Tables 3 and 4, Fig.1).

Heritability analysis

Broad sense heritability coefficient ($h^2=\sigma G/(\sigma G+\sigma E)$) of Tr was high (0.84 and 0.80 in low and high VPD, respectively). The heritability of other investigated traits was; 0.70 (TM), 0.74 (TA), 0.46 (StDW), 0.53 (ShDW), 0.27 (RDW), 0.45 (BDW), 0.50 (LDW), 0.63 (LA), 0.44 (SLW).

Discussion

Several major loci were related to transpiration rate (a proxy for leaf conductance), leaf characteristics (area, thickness) and biomass. Transpiration rate (Tr) was linked to four major loci on LG2, 10; LG2, 260; LG2, 315 and LG2, 322, three of them co-mapping with a previously identified major terminal drought tolerance QTL interval on LG2. The relative importance of these regions for Tr determination was dependent on current vapor pressure deficit. The locations of these major QTLs were consistent with previous studies on similar plant material. The majority of QTLs identified by GMM were consistent with those detected by PLABQTL and showed that the combined action of some strong loci and/or loci of negligible individual effect (e.g. for biomass components) could explain a far higher portion of trait variation than single locus effects. The PCA clearly indicated that, depending on PC dimension, Tr and transpiration (T) vectors had either same or opposite direction. The genetic analysis then also pointed out that T depended mostly on loci interactions involving Tr-related loci and biomass-related loci. The heritability of the traits was high and the loci linked to them were usually very small, which opens the possibility to breed, for example by marker-assisted recurrent selection (MARS), lines having specific allele combinations leading to set levels of plant water use, towards adaptation to specific drought conditions.

Transpiration rate (Tr)

The major QTLs for the investigated traits were obtained consistently, thought not always across experimental sets. Such situation was expected because both the environmental conditions in which the plants developed and in which Tr was assessed varied, and these have been reported to greatly affect water use traits, in particular leaf area and Tr (Kholova et al., 2011c). In this report, it was shown for instance that leaf area development of PRLT2/89-33 was decreased under high VPD conditions. In the case of Tr, QTL explaining large percentage of the Tr variation were found in the region on LG2 previously identified as the major QTL determining drought tolerance (DT-QTL) in two populations (Yadav et al. 2002, 2004; Bidinger 2007). In this region, two major positive alleles from sensitive parent H77/833-2 (LG2, 260 and LG2, 322) spanned a genetic

interval of 6 and 8 cM which is much smaller than interval of the DT-QTL between markers *Xpsmp2237*, 193cM and *Xpsmp2059*, 328cM. This is in agreement with the lower Tr under fully-irrigated conditions of near-isogenic lines (NILs) introgressed with the DT-QTL (Kholová et al. 2010a, b). Yet, two other strong regions controlling Tr, both with positive alleles contributed by tolerant PRLT2/89-33 were identified on LG2 (LG2, 10cM and LG2, 315). The LG2, 10 locus with allele from H77/833-2 also enhanced biomass probably through tillering, whereas the allele from PRLT2/89-33 at this locus might be related to leaf expansion and thickening processes. The LG2, 315 allele from PRLT2/89-33 was also an important region interacting with other Tr influencing loci.

Interestingly, all four QTL on LG2 affected Tr under low VPD, whereas only LG2, 322 from sensitive H77/833-2 had a strong effect on Tr under high VPD. In view of our previous results showing a close involvement of the DT-QTL on a reduction of Tr under high VPD in lines introgressed with the DT-QTL but cultivated under low VPD conditions of the glasshouse (Kholova et al., 2011b), we could have expected to find more QTL for Tr under high VPD. Here, the plants were cultivated outdoors, under higher VPD conditions than the glasshouse. Our recent work also shows that the Tr differences between parental lines are smaller when the plants developed under high VPD conditions (Kholova et al., 2011c), and this is related to a lower leaf area development of PRLT2/89-33. Such results indicate the existence of different, but inter-linked, physiological mechanisms to regulate Tr, in which the environmental conditions play an important role (similarly in Kholová et al. 2011 c). From our data we interpret that two loci (LG2, 322 and, to a lesser extent LG2, 260) may increase Tr synergistically across wide VPD range while others may modulate this "Tr tuning" when environment changes. For example, there may be an antagonistic influence of the PRLT2/89-33 alleles on LG2, 10 and LG2, 315 on Tr under low VPD that may neutralize the effects of LG2, 260 and LG2, 322. This observation is in complete agreement with Yadav et al. (2002) where the very same regions were described and suggested to counteract each other effect on grain yield and panicle harvest drought tolerance indexes depending on the onset of drought and its severity. Therefore, from the physiological point of view, the balance of several interacting mechanisms determine Tr, depending on the prevailing environmental conditions: (i) mechanisms determined by LG2, 260, LG2, 322 (H77/833-2 allele

increasing Tr); (ii) mechanisms related to leaf area and may be leaf thickening influenced by presence of PRLT2/89-33 positive allele on LG2, 315, their interaction with loci on LG7, 75 (allele influencing leaves thickening from H77/833-2) and LG2, 10 (PRLT2/89-33 allele influencing leaves mass, area, and thickening), This simple genetic determination of Tr, an important trait contributing to terminal drought tolerance of pearl millet, opens the possibility to recombine different sets of alleles towards desired Tr level for fitness under specific drought situations.

Leaf characteristics

Leaf area and leaf thickness are other critical factors influencing plant's transpiration and one of the objectives of the study was to compare their genetic regulation to that of Tr. Leaf weight and leaf area were influenced by a major regions on LG7, 110 (positive allele from PRLT2/89-33) and a smaller region on LG2, 10 (positive allele from H77/833-2). The LA QTL on LG7 from PRLT2/89-33 agrees with our recent results of a higher leaf area in PRLT2/89-33 than in H77/833-2 under low VPD conditions (Kholová et al. 2010c). This QTL is also in line with a stover yield QTL found in the same genomic region on LG7 (Yadav et al. 2002; Bidinger et al. 2007). This "stover yield" QTL was found under low VPD only, which also agree with an enhanced leaf area development under low VPD in tolerant genotype (PRLT2/89-33; Kholová et al. 2010c). In synthesis, we suggest the action of the LG7, 110 regions may be specific to leaf expansion and is probably prevalent under low VPD conditions of growth.

In contrast, SLW, was positively influenced by the PRLT2/89-33 allele LG2, 10, the H77/833-2 alleles on LG2, 204 and LG7, 75, and few minor alleles on LG1. Therefore it appears that leaf thickening processes (proxied by SLW) might be mostly related to the H77/833-2 positive alleles on LG2, 204 and LG7, 75, which are also distinct from those affecting LA and LDW. This is in line with observations on *A. thaliana* showing independent genetic control of meristematic cell division and proliferation (Tsuge et al. 1996; Tsukaya 2005). The allele from PRLT2/89-33 at the LG2, 10 loci increased SLW, whereas the H77/833-2 allele at that locus increased stem weight / tillering (as discussed further). We hypothesized this locus to pre-determine the carbon assimilates allocation towards leaves or stems. Similar principle was recently emphasized for sorghum where

the "tillering" *versus* "leafy" phenotype was discussed as result of internal plant competition for carbon sink (Kim et al. 2010a, b). The plants directing their carbon sources towards leaf mass were also hypothesized to be more capable of withstanding harsh drought conditions, however genotypes investing in tillering were hypothesized to succeed in environments where water is plentiful (Hammer et al. 1996; Kim et al. 2010 a), which fits our case.

Interestingly, H77/833-2 allele LG7, 75 interacted closely with loci LG2, 260 (H77/833-2)–LG2, 315 (PRLT2/89-33) and increased Tr under high VPD. Therefore it appears that the plant possessing thick leaves and both Tr enhancing mechanisms on LG2, 260 and 315 loci would have higher Tr values. This fact fits with the PCA showing Tr and SLW being closely related and mapping results of PC2 where a common QTL was found for both these traits combination (LG2, 320).

Biomass – total biomass, root, stem and shoot

The phenotypic expression of biomass and its components relied largely on the presence of one allele from H77/833-2 on LG2, 10 locus and one allele from PRLT2/89-33 on LG7, 110 (Suppl. Table 4). The presence of LG2, 10 alleles from H77/833-2 explained also 12% of root biomass variation and 19% of stem weight variation. These results agree with previous reports (Bidinger 2004, 2007; Yadav 2002, 2003, 2004). A region close to LG2, 10 was previously identified to contribute to increased biomass and stover yield under optimal conditions (Yadav et al. 2002). This positive H77/833-2 allele was also responsible for an increased number of panicles, likely related to the higher tillering habit of this genotype (Yadav et al. 2002). This region on LG2 also increased tillering in other work (Poncet et al. 2002; Nepolean 2002). The GMM analysis of biomass and its components confirmed these results by showing a single locus on LG2, 0 increasing total biomass and stem dry weight, whereas no combination of effect explained a notably larger percentage of the variation for these traits.

Absolute transpiration (T)

Water saving mechanisms under non-stress conditions are hypothesized to keep water in the soil profile to sustain grain filling and are therefore critical for the terminal drought

tolerance of crops (Vadez et al. 2011). Therefore, the ultimate purpose of this study was to dissect the genetic regulation of absolute transpiration. The GMM approach strengthened the idea (Isobe et al. 2007; Ravi et al. 2010) that synergistic action of several QTLs loci can add up effects on complex quantitative traits, like T here. However, these previous studies limited themselves to listing the possible effects of multiple loci interaction and highlighting the need for consideration of these interactions when employing markers in breeding programs, but did not explain the important physiological implications of specific trait combinations for adapting to variable drought scenario.

CIM showed that water used (T) depended on several loci – in most cases similar to those determining Tr (Suppl. Table 3, Fig 1), biomass accumulation and its partitioning (LG2, 10, LG7, 110), and also on independent QTL on LG3 (LG3, 28cM). However, none of these loci had large individual effect on T, except LG2, 10. The outcome of the GMM analysis validated the hypothesis that larger proportion of the variation in plant's transpiration could be explained by loci interaction (Fig. 3 band c). Interestingly, under low VPD, all interactions involved loci related to Tr with contribution from the sensitive H77/833-2 allele, and all interactions increased plant transpiration. By contrast, under high VPD conditions, none of the interaction involved Tr loci but rather leaf and tillering characteristics loci, with both tolerant and sensitive allele being involved in positive and negative interaction effects on T. Plant’s transpiration could be tuned up by alleles enhancing biomass accumulation (LG2, 10 and possibly few minor QTLs on LG1, LG4, LG5, and LG6) and tuned up or down by mechanisms decreasing Tr, depending on allele presence (LG2, 260; LG2, 315), themselves closely interacting with LA- and SLW-related loci on LG7. For instance, ideotypes with thicker leaves lower LA and high biomass (high tillering) had higher transpiration. This knowledge considerably deepens the understanding of G×E interactions and opens the possibility to design plant ideotypes with desired levels of transpiration, then suited for specific target environment. For example, in environments where water is intermittently available through the season, the focus would likely be on ideotypes capable of maximizing water uptake. In environments where incoming rainfall is limited and water supply restricted to soil moisture (terminal drought situation), the focus would be on ideotypes capable of using water slowly and

leaving some for the grain filling period (e.g. low T). This latter ideotype would consist of genotypes having alleles responsible for low tillering phenotype, higher LA and thin leaves (lower SLW). Similar concept was previously presented for sorghum using simulation modeling (Hammer et al. 1996; Hammer et al. 2006; Kim et al. 2010 a, b).

Conclusion

Four QTLs for Tr were found, two from H77/833-2 (sensitive genotype) and two PRLT2/89-33 (tolerant genotype) alleles on chromosome (LG) 2, the importance of each region being dependent on the environmental conditions at the time of measurement. Three of these Tr QTLs mapped to a region previously identified as the major terminal drought tolerance QTL on LG2, explained large variation of the Tr phenotypic variance, and spanned small genome portions. Two QTL were specific to low VPD conditions. Absolute transpiration was closely linked to interaction of Tr loci from sensitive H77/833-2, with plant biomass and leaf characteristics (from LG7) under low VPD whereas transpiration under high VPD conditions resulted from interaction of loci for biomass and leaf characteristics. This study revealed the genetic basis of different traits influencing plant water use under non-stressed conditions, their genetic basis, their interactions with the environment (G×E), and opens the possibility to engineer successful ideotypes with set level of water use from specific combinations of alleles for location specific requirements.

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Table 1 Genotype matrix mapping (GMM) mapping analysis of BLUP means of investigated traits; i.e. leaf dry weight (LDW), root dry weight (RDW), shoot dry weight (ShDW), stem dry weight (StDW), biomass dry weight (BDW), leaf area (LA) specific leaf weight (SLW). Transpiration rate (Tr) and absolute transpiration (T) are presented with suffix M which stands for measurements under low VPD (morning) and with suffix A representing measurements under high VPD (noon hours). F represents the significance level for particular QTL or QTL combination. Following column shows the number of lines carrying this particular marker combination in the RIL population. In the column locus (allele) is presented the QTL peak marker along with the origin of alleles; A, B, H stands for alleles originated from H77 (sensitive), PRLT (tolerant) and H means heterozygous locus (A and B present in genome). For any particular QTL or their combination, the percentage of variation explained (PVE) is shown (positive value signify the QTL combination increases the trait value and *vice versa*). Table shows exact QTL peak marker position with regards to linkage group (LG) and the interval identified by CIM to which this marker probably belongs

Trait name	No. of lines	F	Number of QTLs	Locus(allele)	**PVE**	Peak QTL position (LG, cM)	Probable CIM QTL corresponding interval (LG, interval)
TrM	17	10.46	1	P13113(A)	**11.38**	6, 32	6, 40
	42	12.55	1	P12608(A)	**9.98**	4, 97	4, 100
	69	15.63	1	P8443(A)	**11.17**	2, 322	2, 322
	63	16.68	1	P8464(A)	**13.19**	2, 258	2, 260
	67	14.24	1	P6013(A)	**11.20**	2, 204	2, 205
	19	12.20	1	P8984(B)	**11.62**	2, 0	2, 10
	40	14.08	1	P10103(A)	**10.82**	1, 115	X
	10	41.76	2	P11469(B) P8139(A)	**24.60**	2, 315-2, 261	2, 315 – 2, 260
	10	64.81	3	P9089(A) P9529(B) P10103(A)	**29.27**	7, 75-2, 216-1, 115	7, 75 –X-X
TrA	35	7.86	1	P7046(A)	**6.30**	7, 71	7, 75
	43	7.62	1	P10653(A)	**5.58**	1, 138	1, 130
	40	8.07	1	P10103(A)	**6.26**	1, 115	X
	10	26.15	2	P11469(B) P8139(A)	**15.50**	2, 315-2, 261	2, 315-2, 260
	7	50.85	3	P9089(A) P11469(B) P8464(A)	**22.85**	7, 75-2, 315- 2, 258	7, 75-2, 315-2, 260
TM	56	13.84	1	Xipes0154(B)	**7.33**	7, 113	7, 110
	58	13.07	1	P9391(B)	**7.76**	7, 87	7, 75
	16	24.56	2	P7046(A) P12228(A)	**-12.44**	7, 71-5, 17	7, 75-X
	18	32.38	3	P9391(B) Xctm21(A) P9306(A)	**13.15**	7, 87-2, 319-2, 308	7,75-2, 322-2,315
	7	33.25	3	Xipes0105(B) Xctm21(A) P10902(A)	**15.47**	2, 26-2, 319-2, 156	X-2,322-X
	12	32.97	3	Xipes0015(B) P6931(B) Xctm21(A)	**15.34**	7, 21-6, 37-2, 319	X-6,40-2,322
	12	32.70	3	P11397(B) P7861(B) P12641(A)	**15.04**	6, 88-2, 356-2, 240	X-X-2, 260
	13	33.41	3	P11628(B) Xctm21(A) P12077(B)	**15.31**	5, 42-2, 319-1, 75	X-2,322-1,80
	9	32.68	3	P13090(B) Xctm21(A) P12077(B)	**16.99**	5, 10-2, 319-1, 75	X-2,322-1,80
TA	74	19.91	1	P8984(A)	**13.49**	2,0	2, 10
	21	28.86	2	P12604(A) P11369(A)	**-12.24**	7, 122-1, 173	7, 110-1, 200
	29	26.17	2	P11960(B) P8182(B)	**12.12**	7, 107-1, 72	7, 110 – 1,80
	12	36.11	3	P12604(A) P11502(A) P11369(A)	**-16.63**	7, 122-5, 23-7, 173	7,110-X-1, 200
	21	35.13	3	P9391(B) P13090(B) P11990(B)	**13.35**	7, 87-5, 10-1, 77	7,75-X-1,80
	23	35.60	3	P9391(B) P8984(A) P10013(B)	**14.48**	7, 87-2, 0-1, 161	7,75-2, 10-X
	22	33.99	3	Xipes0145(B) P8984(A) P11369(B)	**14.51**	7, 113-2, 0-7, 173	7, 110-2, 10-1, 200
	10	34.97	3	P12608(A) P7542(A) P11403(B)	**-17.86**	4, 98-1, 194-1, 46	4, 100-1, 200-1, 52
LA	47	29.29	1	P11960(B)	**15.01**	7, 107	7, 110
	20	49.44	2	P6612(A) P6013(A)	**-19.45**	7, 71-2, 205	7, 75-2, 205
	36	52.28	2	P7046(B) P8984(A)	**21.77**	7, 71-2, 0	7, 75-2, 10
	27	53.35	3	Xipes0154(B) P7046(B) P8984(A)	**21.05**	7, 113-7-71-2,0	7, 110-7, 75-2, 10
	22	54.38	3	P11960(A) P6665(A) P11702(A)	**-18.85**	7, 107-2, 323-2, 264	7, 110-2, 322-2, 260
LDW	56	26.62	1	Xipes0154(B)	**10.78**	7, 113	7, 110
	41	43.81	2	Xipes0154(B) P8984(A)	**14.14**	7, 113-2,0	7, 110-2, 10
	22	51.81	3	P11960(B) P12052(B) P8984(A)	**17.16**	7, 107-5,13-2,0	7, 110-X-2, 10
SLW	67	15.91	1	P6013(A)	**7.13**	2, 205	2, 205
	18	35.34	2	P9089(A) P9529(B)	**11.51**	7, 75-1,216	7, 75-X
	19	35.77	2	P9089(A) Xicmp4010(B)	**11.20**	7, 75-1,216	7, 75-1, 200

	9	55.23	3	P9089(A) P8244(A) P10103(A)	**17.05**	7, 75-1,296-1,115	7, 75-X-1,100
	7	55.87	3	P9089(A) P11894(A) P10103(A)	**19.05**	7, 75-1,296-1,131	7, 75-X-X
RDW	74	14.25	1	P8984(A)	**22.20**	2,0	2, 10
	22	19.59	2	Xpsmp2203(B) P10184(B)	**22.36**	7, 113-4,0	7, 110-X
	3	19.93	2	P6932(B) Xipes0101(H)	**53.65**	5,3-1,108	X-1,80
	11	20.21	2	P10768(A) P8984(B)	**-24.26**	4,106-2,0	4, 100-2, 10
	23	20.33	2	P7958(B) P7330(A)	**24.02**	4, 96-2, 336	4, 100-2,322
	27	19.49	2	P7958(B) P8694(A)	**22.52**	4, 96-2, 20	4, 100-2, 10
	10	38.16	3	P9936(B) P11711(B) Xipes0218(A)	**36.37**	7, 81-4,18-2, 346	7, 75-X-X
BDW	74	26.16	1	P8984(A)	**20.25**	2,0	2, 10
	50	35.22	2	P9721(B) P8984(A)	**17.67**	7,67-2,0	7, 75-2, 10
	16	40.68	3	Xpsmp2203(B) P8694(A) Xctm12(B)	**22.75**	7, 113-2, 332-1,202	7, 110-2, 322-1, 200
	36	43.07	3	P9721(B) P7494(B) P8984(A)	**18.97**	7,67-5,13-2,0	7, 75-X-2, 10
	19	41.46	3	P8694(A) P10594(A) Xctm12(B)	**20.54**	2, 332-2,14-1,202	2, 322-2, 10-1, 200
LDW	56	26.62	1	Xipes0154(B)	**10.78**	7, 113	7, 110
	41	43.81	2	Xipes0154(B) P8984(A)	**14.14**	7, 113-2, 0	7, 110
	22	51.81	3	P11960(B) P12052(B) P8984(A)	**17.16**	7, 107-5, 13-2,0	7, 110-X-2, 10
StDW	74	24.83	1	P8984(A)	**20.95**	2,0	2, 10
	21	31.08	2	Xipes0154(A) P9529(B)	**-17.61**	7, 113-1, 216	7, 110-X
	20	31.72	2	P6478(A) P7387(A)	**-17.51**	4, 98-1,183	4, 100-1, 200
	27	33.29	2	P10110(A) P8268(B)	**-16.66**	4, 95-2, 249	4, 100-2,260
	15	31.08	2	P10110(A) P8984(B)	**-20.74**	4, 95-2, 0	4, 100-2,10
	31	30.94	2	P6665(A) P8268(B)	**-15.51**	4, 95-2, 323	4, 100-2, 322
	21	55.58	3	P12608(A) P6665(A) P8268(B)	**-21.85**	4, 98-2, 323-2, 249	4, 100-2,322-2,260
ShDW	56	24.56	1	Xipes0154(B)	**10.90**	7, 113	7, 110
	74	24.68	1	P8984(A)	**15.78**	2,0	2, 10
	41	45.25	2	Xipes0154(B) P8984(A)	**15.15**	7, 113-2, 0	7, 110-2, 10
	22	55.19	3	P11960(B) P12052(B) P8984(A)	**18.30**	7, 107-5, 13-2,0	7, 110-X-2, 10

Figure captions

Fig. 1 Visualization of approximate QTLs positions within the linkage groups of major importance (LG; chromosomes 1, 2, and 7, letter a to c) consisting of SSR and DArT markers and their positions in cM. QTLs were detected for all investigated traits, i.e. leaf dry weight (LDW), root dry weight (RDW), shoot dry weight (ShD W), stem dry weight (StDW), biomass dry weight (BDW), leaf area (LA) specific leaf weight (SLW) and principal components (PCA 1, 2, 3). Transpiration rate (Tr) and absolute transpiration (T) are presented with suffix M which stands for measurements under low VPD (morning hours) and with suffix A representing measurements under high VPD (noon hours). QTLs are shown for particular replications (suffix of trait name indicates day of measurement in December 2009) and for replication based BLUP means (indicated as BLUP suffix of the trait). The content of the brackets behind the trait name stands for the 95% confidence interval of particular QTL. Red or blue colored font indicates that the positive effect comes from PRLT (tolerant) or H77 (sensitive) allele. Approximate positions of QTLs previously detected are also visualized on the sides of the chromosomes

Fig. 2 Graphical output of principal component analysis (PCA). In the bi-plot the values of traits vectors are presented in red arrows (i.e. transpiration rate (Tr), absolute transpiration (T), leaf dry weight (LDW), root dry weight (RDW), shoot dry weight (ShDW), stem dry weight (StDW), biomass dry weight (BDW), leaf area (LA) specific leaf weight (SLW); Traits with suffix M were measured during 7:30-10:30 a.m. and traits with suffix A were measured during 10:30 a.m.-2:30 p.m)) based on two major principal components (PC1-x axis; PC2 – y axis). The numbers represents recombinant inbred lines numbers (RIL numbers) and its position shows the particular RIL traits loadings with regards to PC1 and PC2

Fig. 3 QTL interactions from the GMM analysis for the transpiration rate in the morning under low VPD conditions (TrM) and in the afternoon under high VPD conditions (TrA) (a), for the transpiration in the morning (TM) (b), and for the transpiration in the afternoon (TA) (c)