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REVIEW PAPER

Using genetic mapping and genomics approaches in understanding and improving drought tolerance in pearl millet

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Abstract

Drought at the reproductive stage is a major constraint to pearl millet [*Pennisetum glaucum* (L.) R. Br.] productivity. Quantitative trait locus (QTL) mapping provides a means to dissect complex traits, such as drought tolerance, into their components, each of which is controlled by QTLs. Molecular marker-supported genotypic information at the identified QTLs then enables quick and accurate accumulation of desirable alleles in plant breeding programmes. Recent genetic mapping research in pearl millet has mapped several QTLs for grain yield and its components under terminal drought stress conditions. Most importantly, a major QTL associated with grain yield and for the drought tolerance of grain yield in drought stress environments has been identified on linkage group 2 (LG 2) which accounts for up to 32% of the phenotypic variation of grain yield in mapping population testcrosses. The effect of this QTL has been validated in two independent marker-assisted backcrossing programmes, where 30% improvement in grain yield general combining ability (GCA) expected of this QTL under terminal drought stress conditions was recovered in the QTL introgression lines. To transfer effectively favourable alleles of this QTL into pearl millet varieties that otherwise are high yielding and adapted to specific agricultural zones, efforts are currently underway to develop closely spaced gene-based markers within this drought tolerance (DT)-QTL. In this review, an overview is provided of information on the genetic maps developed in pearl millet for mapping drought tolerance traits and their applications in identifying and characterizing DT-QTLs. Marker-assisted transfer of desirable QTL alleles to elite parent backgrounds, and results from introgression line validation in multiple terminal drought stress environments are discussed. Current efforts undertaken towards delimiting the interval of a major DT-QTL mapping to LG 2, and towards identifying candidate genes and physiologies underlying this QTL are presented. Highly specialized genetic stocks [QTL-near-isogenic lines (NILs), a high-resolution cross, and a germplasm population] and genomic resources (gene sequences, gene-based markers, and comparative genomics information) specifically developed for these purposes are discussed.

Key words: Fine mapping, gene-based markers, genetic mapping, germplasm population, high-resolution cross, marker-assisted backcrossing, pearl millet, QTL validation, QTLs, terminal drought tolerance.

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] ($2n=2x=14$), belonging to the family Poaceae, is the seventh most important global cereal crop. It is grown as a rainfed grain and fodder crop in the hottest, driest regions of the Sahelian zone of sub-Saharan Africa and the Indian subcontinent where dryland crop production is practised. Post-flowering drought stress is one of the most important environmental factors reducing the grain yield and yield stability of pearl

millet and increasing the incidence of crop failure in dryland production environments (Mahalakshmi *et al.*, 1987). Terminal drought stress (flowering through grain filling) is more damaging to pearl millet productivity than stress at the vegetative or pre-flowering reproductive crop growth stages. This is because pearl millet's asynchronous tillering behaviour and rapid growth rate allow it to recover rapidly from intermittent drought stress during these earlier stages of plant development, but provide no advantages under unrelieved terminal drought stress (Bidinger *et al.*, 1987; Mahalakshmi *et al.*, 1987). Improving the adaptation of pearl millet to terminal drought stress environments is, therefore, a major objective for breeding programmes aimed at improving both the crop's productivity and its yield stability (Yadav *et al.*, 2002; Bidinger and Hash, 2004).

The large variability in the timing and severity of drought stress and the inadequate understanding of its complexity have made it difficult to characterize the physiological and/or phenotypic traits required for screening and improving crop performance under drought stress. Consequently this difficulty has limited the use of a trait-based approach in plant breeding to enhance the drought tolerance of crops. Quantitative trait locus (QTL) mapping provides a means to dissect complex phenotypic characters such as drought tolerance into their component traits (QTLs), and allows the identification of molecular markers linked to desirable QTL alleles, so that they can be directly used in marker-assisted selection (MAS) (Tanksley, 1993; Mohan *et al.*, 1997; Prioul *et al.*, 1997).

Significant progress has been made in mapping a number of QTLs for components of grain and stover yield, as well as yield maintenance, under terminal drought stress conditions in pearl millet (Yadav *et al.*, 2002, 2003, 2004; Bidinger *et al.*, 2007). Most importantly, a major QTL for terminal drought tolerance has been identified and validated on pearl millet linkage group 2 (LG 2) (Bidinger *et al.*, 2005; Serraj *et al.*, 2005) using segregating populations derived from two independent crosses (H 77/833-2×PRLT 2/89-33 and ICMB 841×863B). However, the confidence interval for this LG 2 QTL is still large (~30 cM), thus complicating its applied use in MAS programmes. Hence, there is a need both for delimiting the interval of this QTL (to facilitate understanding of the traits governed by the QTL), and for identification of closely spaced gene-based markers within the drought tolerance (DT)-QTL region to facilitate efficient introgression of favourable alleles into varieties that otherwise are high yielding and adapted to specific agricultural zones. This review summarizes various genetic and genomic resources [biparental crosses, QTL maps, QTL-near-isogenic lines (NILs), a high-resolution cross (HRC)/fine-mapping population, and a germplasm population] that have been developed so far for mapping, validation, and fine mapping of this major QTL. The approaches being taken to identify gene-based markers [e.g. conserved intron-spanning primers (CISPs), expressed sequence tag-single nucleotide polymorphisms (EST-SNPs), conserved orthologous sequences (COS), and EST-simple sequence repeats (SSRs)], and their subsequent association with drought tolerance using targeted genetic and association mapping approaches are also discussed.

Genetic mapping of terminal drought tolerance

Two sets of mapping population progeny—one from a cross between two elite inbred pollinators (H 77/833-2 and PRLT 2/89-33) and the other from a cross between two elite inbred seed parents (ICMB 841 and 863B)—were used to map terminal drought tolerance of grain and stover yield and their component traits (Yadav *et al.*, 2002, 2003, 2004; Hash *et al.*, 2003; Bidinger *et al.*, 2007). In both crosses, one of the parents (PRLT 2/89-33 in the case of H 77/833-2×PRLT 2/89-33, and 863B in the case of ICMB 841×863B) was derived from the *Iniadi* landrace from Togo and Ghana, which is known for its better grain-filling ability under terminal drought stress conditions. The other parents used in each cross were elite inbred lines, the hybrids of which are widely grown by farmers in pearl millet-growing areas of north-western India. The *Iniadi* landrace differs from north-western Indian germplasm in many plant characteristics. *Iniadi* germplasm typically has fewer basal and nodal tillers, larger seeds, thicker stems and panicles, and broader leaves. Two sets of marker-assisted backcross-derived progeny were developed, the first to improve terminal drought tolerance of elite inbred pollinator H 77/833-2 using PRLT 2/89-33 as the donor parent, and the second to improve elite inbred seed parent maintainer line ICMB 841 using 863B as the donor parent, so that the future hybrids produced on them will have greater tolerance to terminal drought stress.

For mapping QTLs, the two parental inbred lines were crossed and a single F_1 plant was self-pollinated to produce F_2 seeds. Leaf tissue samples were collected from each of ~150 individual F_2 plants for DNA isolation, subsequent restriction fragment length polymorphism (RFLP) genotyping (RFLP and SSR genotyping in the case of ICMB 841×863B), and genetic map construction (Fig. 1). For phenotyping the mapped progeny for grain yield and its component traits under terminal drought stress, a subset of F_3 progeny (92 from H 77/833-2×PRLT 2/89-33 and 79 from ICMB 841×863B) were test-crossed onto unrelated inbred tester(s) to produce testcross hybrids. Testcrosses were produced on an elite A-line (843A) with pollen from F_3 progeny bulks from the H 77/833-2×PRLT 2/89-33 cross, and on two different elite inbred pollinator lines (H 77/833-2 and PPMI 301) for those from ICMB 841×863B. ICMB 841×863B was evaluated using two testers to determine the influence, if any, of genetic background on the expression of the identified QTLs. The mapped progeny were phenotyped as testcross hybrids, rather than as the skeleton-mapped F_2 plants or their derived inbred progeny for the following reasons: (i) to restore heterotic vigour to partially inbred mapping progeny that might otherwise be too weak for effective screening under stress conditions (pearl millet is

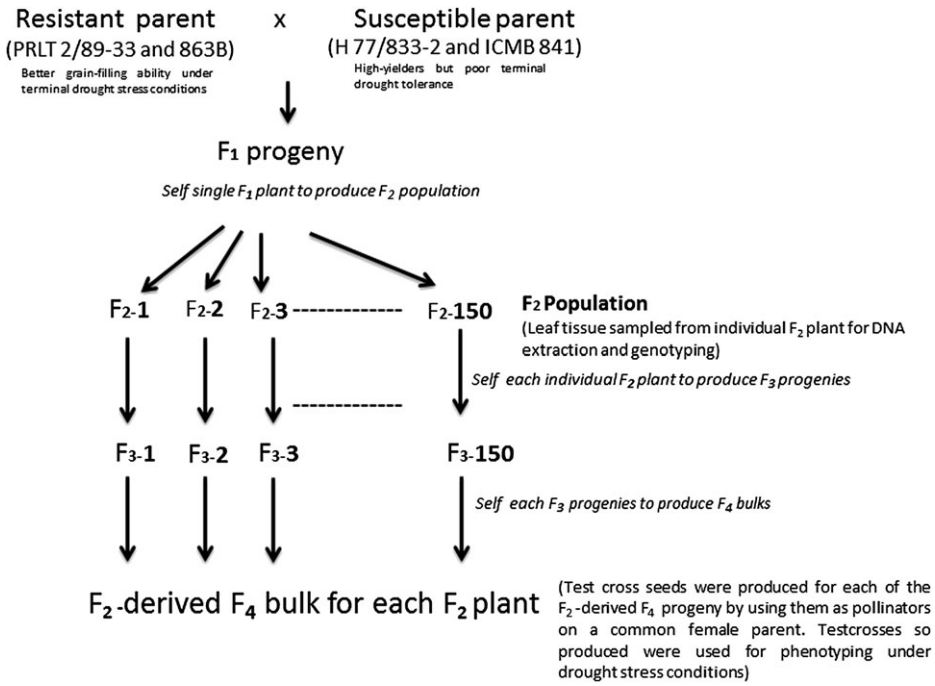

Fig. 1. Strategy followed for development of genetic linkage map and for phenotyping of traits to identify QTLs linked to traits.

highly cross-pollinated in nature and suffers considerably from inbreeding depression); (ii) to use the dominantly inherited early flowering of tester 843A and H 77/833-2 to reduce variation in flowering time among the test units, in order to focus the mapping on specific drought tolerance traits rather than traits or responses associated with variation on capacity for drought escape; and, finally, (iii) to have test units that approximate the genetic structure of the F_1 hybrids grown by farmers rather than partially inbred F_3 or F_4 lines.

In order to minimize problems with segregation distortion (due to loss of many progeny due to inbreeding depression while generating advanced inbred mapping population progeny sets) and reduce the time required to generate the mapping population progeny sets, the progeny were advanced to only F_4 self-bulks. F_4 bulks were produced by selfing random samples of 15–25 F_3 plants derived from each of the genotyped F_2 plants and their corresponding testcrosses produced.

The performance of testcrosses was characterized using managed stress environments as described in Yadav *et al.* (2002, 2004) and Bidinger *et al.* (2007). Time to flowering was recorded as the days to stigma emergence on 50% of the main stem panicles in a plot. At harvest, data were recorded on the number of plants and number of panicles per plot, dry stover yield, dry panicle yield, grain yield, and 100-seed mass. Grain yield, stover yield, total biomass at maturity, and panicle numbers were expressed on a per m^2 basis. Panicle grain number [(100×grain yield)/(panicle number× 100-seed mass)] was derived from these primary data. Harvest index (HI=grain yield/biomass yield) and panicle harvest index (PNHI=grain yield/panicle yield) were calculated on a plot basis. Drought tolerance for each trait measured was calculated by dividing the testcross entry mean trait performance in a particular stress environment by testcross entry mean trait performance in the paired fully irrigated control environment. Such a ratio reflected maintenance of individual trait performances under stress environments compared with the irrigated control environments. Drought tolerance for grain yield as well as absolute grain yield in irrigated control and stress environments (i.e. grain yield *per se*) were taken into account for assigning performance of a particular genotype in the stress environments tested.

QTLs associated with drought tolerance of grain yield

QTLs on LG 2

Using H 77/833-2×PRLT 2/89-33 (Yadav *et al.*, 1999, 2002), a QTL associated with drought tolerance of grain yield was obtained on LG 2 in two of the three stress environments, explaining up to 32% of the variation in drought tolerance response of grain yield. In the same interval on LG 2, QTLs associated with drought tolerance of 100-seed mass (three environments), HI (two environments), PNHI (two environments), and panicle number m^{-2} (one environment) also co-mapped. A QTL for drought tolerance of stover and biomass yield in the most severe stress environment also mapped to this interval on LG 2. The PRLT 2/89-33 allele at this QTL interval was associated with increased drought tolerance of grain yield and all the component traits described above. Increased HI and biomass yield conferred by the PRLT 2/89-33 allele at

this putative QTL suggested that increased drought tolerance conferred by this QTL on grain yield and its components might have been achieved by the effect of this QTL on both increased dry matter production and increased partitioning of dry matter to the grain (Yadav *et al.*, 2002). Interestingly, both the LOD score as well as the amount of variation explained by the QTL on LG 2 increased with increase in stress intensity in the screening environments used in this study, thus indicating greater importance of this QTL with increasing drought stress (Table 1).

Using a mapping population progeny set derived from cross ICMB 841×863B (Yadav *et al.*, 2004; Bidinger *et al.*, 2007), the QTL on LG 2 was again observed to be associated with drought tolerance of grain yield in early stress environments using both (H 77/833-2 and PPMI 301) testers. Its effect on drought tolerance of grain yield, however, was achieved differently depending on the tester used. In the tester 1 (high tillering line H 77/833-2) background, it exerted its effect on grain yield via maintaining biomass yield (i.e. through reduced reduction in biomass yield in stress environments) and effective panicle number m^{-2}, while in the tester 2 (large panicle line PPMI 301) background it maintained grain yield via increases in panicle grain number and HI (comparable with the effect of the PRLT 2/89-33 allele in testcrosses onto 843A). When stress was initiated at a later stage (early grain filling) on ICMB 841×863B testcrosses, the effect of this QTL on LG 2 was evident on grain yield for tester PPMI 301 but not for tester H 77/833-2. With tester PPMI 301, the 863B allele at this QTL achieved increased grain yield through its effect on increased 100-seed mass and HI. With tester H 77/833-2, although the effect of the 863B allele at this QTL was positive on panicle grain number and HI, its effect on grain yield was not detected.

In a further study, Bidinger *et al.* (2007) also identified a major grain yield (GRYLD) QTL on LG 2 with substantial LOD scores in all the three moisture environments (LOD 6.3–6.9) as well as across environments (LOD 7.9) which accounted for a significant proportion of the phenotypic variance for GRYLD in both stress (27–38%) and the stress-free irrigated control (28%) environments, as well as across these environments (25%). The GRYLD QTL co-mapped with a QTL for HI across environments and with QTLs for both grain number and grain mass under severe terminal stress.

QTLs on LG 3, 4, 5, and 6

In the (ICMB 841×863B)-based mapping population, a QTL associated with drought tolerance of grain yield was also obtained on LG 5, which explained 14.8% of the variation in reduction in grain yield (Yadav *et al.*, 2004). This QTL was detected only for the H 77/833-2 tester in environment 1, and was not expressed in the background of the PPMI 301 tester. An allele from ICMB 841 at this interval increased the drought tolerance of grain yield and the yield of stover and biomass. Similarly, a QTL associated with tolerance of grain yield in late stress environments was obtained on LG 6. The effect of this QTL was again evident only in the genetic background of tester H 77/833-2. This QTL exerted its effect on increased maintenance of grain yield via its effect on maintenance of increased HI and PNHI. The 863B allele at this QTL was associated with the maintenance of both the grain yield and HI. Bidinger *et al.* (2007) detected grain yield QTLs on LG 3 and LG 4 in late-onset stress environments using (ICMB 841×863B)-derived F_4 testcrosses, but these had lower LOD scores (2.8–3.5) and accounted for much smaller fractions (11.6–17.3%) of the phenotypic variance for grain yield, both in the late-onset stress environments and across environments, than did the LG 2 QTL.

QTLs on other linkage groups

In addition to the QTLs described above, a number of additional QTLs were detected that were associated with maintenance of grain yield-determining component traits. One on LG 1 was detected in one of the three stress environments for H 77/833-2×PRLT 2/89-33 and in only one genetic background in one of the stress environments for ICMB 841×863B. This QTL was consistently linked to increased grain filling but was apparently pleiotropic to decreased panicle number so its effect on expression of grain yield in stress environments was not evident. Similarly, a QTL on LG 6 was linked to increased grain filling in two of the three stress environments for testcrosses of the H 77/833-2×PRLT 2/89-33-derived F_3 progeny, but was pleiotropic to reduced panicle grain number. The effects conferred by these QTLs for better maintenance of grain yield-determining traits thus were compensated for by a reduction in the expression of one or more other grain yield component traits.

Table 1. LOD scores and percentage phenotypic variations explained by the LG 2 drought tolerance QTL in three post-flowering drought stress environments experiencing a different intensity of drought stress as reflected in yield reduction (unpublished data from field trials conducted during 1996 and 1997 at ICRISAT, Patancheru).

	Environment 1 (experiencing yield reduction of 27.5% compare with irrigated control)		**Environment 2 (experiencing yield reduction of 32.4% compared with irrigated control)**		**Environment 3 (experiencing yield reduction of 61.1% compared with irrigated control)**	
	LOD	**R^2**	**LOD**	**R^2**	**LOD**	**R^2**
Grain yield	2.06	10.1	2.48	13.9	5.56	24.6
Harvest index	2.32	11.0	2.73	13.3	4.00	18.2

Major QTL for MAS in pearl millet

Based on the estimated effects on target traits in terminal stress environments (Yadav *et al.*, 2002, 2004), the absence of Q×E interaction and its consistency in two different genetic backgrounds (Yadav *et al.*, 2003; Bidinger *et al.*, 2007), the grain yield DT-QTL on LG 2 was identified as a major target for MAS in pearl millet for improving grain yield and grain yield stability across variable post-flowering moisture stress environments. Bidinger *et al.* (2007) found that the projected effect of selection for this QTL included an increase in grain yield of 12 g m^{-2} across all environments, plus major gains in both individual grain mass and PNHI. For individual environments, incorporating this QTL resulted in a predicted gain of 16 g m^{-2} in the absence of stress and 13–16 g m^{-2} in the terminal stress environments. This was accompanied by predicted gains in HI (2.2%) and PNHI (3.2%) across environments (Bidinger *et al.*, 2007).

Field evaluation of drought tolerance QTL effects

Initial assessment of the putative DT-QTL on LG 2 of pearl millet was made by comparing hybrids made with a top-cross pollinator (TCP) bred from progeny selected from the original mapping population (H 77/833-2×PRLT 2/89-33) for the presence of the tolerant allele at the target QTL versus hybrids made with a TCP bred from progeny selected for field performance in the field trials used to phenotype the original mapping population (Bidinger *et al.*, 2005). A set of 24 top-cross hybrids (12 male-sterile lines×2 TCPs) was evaluated in 21 field environments, which included both non-stressed and drought-stressed treatments during the flowering and grain-filling stages. The hybrids of the QTL-based pollinator were significantly, but modestly, higher yielding in a series of both absolute and partial terminal stress environments, but at the cost of a lower yield in the non-stressed evaluation environments. This particular pattern of adaptation in hybrids of the QTL-based pollinator was consistent with their general phenotype—earlier flowering, limited effective basal tillering, lower biomass, and a higher HI—which resembled the phenotype of the drought-tolerant parent of the mapping population, and which appeared to provide advantages under a post-flowering stress. The results confirmed the effectiveness of the putative DT-QTL on LG 2, and suggested that it enhances drought tolerance by favouring a phenotype with adaptation to terminal stress, rather than by improving drought tolerance at a more basic physiological level, at least when used as a direct selection criterion.

Validation of the major QTL using NILs

A more rigorous evaluation of the putative DT-QTL was done by using NILs of H 77/833-2, into which various putative DT-QTL segments have been introgressed from the donor parent PRLT 2/89-33 by marker-assisted backcrossing (Serraj *et al.*, 2005). BC_4F_3 progeny from selected BC_4F_2 plants homozygous for various portions of the LG 2 target region were crossed to five different seed parents, and the resulting hybrids were evaluated under a range of moisture regimes (non-stressed control, early-onset, medium-onset, and late-onset terminal drought stress). The hybrids exhibited a large variation in yield component expression and yield response to the moisture regimes, but there was a consistent yield advantage in hybrids carrying alleles from the drought-tolerant donor parent PRLT 2/89-33 in the vicinity of the target QTL. Several of the introgression lines had a significant, positive general combining ability (GCA) (across all testcrosses) for grain yield under terminal stress, which was associated with a higher PNHI. This superior grain yield performance of the introgression line hybrids was accompanied by increased biomass yields and reduced grain HIs instead of the reduced biomass yield and increased grain HI that contribute to the higher grain yield potential and superior terminal drought tolerance for grain yield of hybrids of the donor parent.

To assess further the performance of selected QTL-NIL pollinator lines from the GCA trial, a line-source gradient stress experiment was conducted using 20 trial entries comprising testcrosses (on four seed parents) of the two pollinators with the highest GCA values for grain yield under stress (ICMR 01029 and ICMR 01031), one pollinator with a negative GCA for grain yield under stress, and two mapping population parental lines from which the introgression lines were bred (Serraj *et al.*, 2005). The results confirmed the previous findings of yield advantage of ICMR 01029 and ICMR 01031 compared with parents H 77/833-2 and PRLT 2/89-33.

Physiological effects associated with the DT-QTL on LG 2

Having established the effects of a major DT-QTL of LG 2 on yield and yield-contributing traits, efforts currently are underway to dissect the physiological processes underlying this QTL. As it stands, this QTL covers a large part of LG 2 and may harbour genes controlling a number of physiological mechanisms that together contribute to its effect on grain yield. To cope with drought, plants essentially follow two strategies: (i) make optimum use of the available water; and (ii) extract more water from the soil profile. Physiological and biochemical traits associated with these strategies are being explored in the drought-tolerant and -sensitive parents and in QTL-NILs to understand their relationship to the DT-QTL on LG 2. NILs introgressed with the DT-QTL are preferred for these studies because they offer unique opportunities to focus the analysis on the effects of a specific genomic region containing the QTL in an otherwise uniform genetic background (Giuliani *et al.*, 2005).

Significant differences in transpiration rate (Tr; g water cm^{-2} d^{-1}) were observed between the drought-tolerant

(PRLT 2/89-33 and 863B) and drought-sensitive parents (H 77/833-2 and ICMB 841) of the mapping families used in mapping the DT-QTL on LG 2, and among the NILs (ICMR 01029, ICMR 01031, and ICMR 02041) introgressed with this DT-QTL (Kholova *et al.*, 2010*a*, *b*). Tr is a proxy for stomatal conductance and integrates the behaviour of stomata over a substantial period of time. Moreover compared with instantaneous stomatal conductance measurements, gravimetric measurements of Tr are less variable, making it more convenient to measure in large numbers of entries. Compared with drought-sensitive parents, lower Tr values were found in the drought-tolerant parents at both pre- and post-flowering crop growth stages. Interestingly, as in the drought-tolerant parents, the lower Tr values were also evident in the QTL-NILs introgressed with the DT-QTL (Kholova *et al.*, 2010*a*), thus indicating that this DT-QTL interval was conferring low stomatal conductance to the QTL-NILs.

In the work reported above, it was also established that the soil moisture threshold (assessed using the fraction of volumetric soil water available; Ritchie, 1981) was lower in both drought-tolerant parents and the QTL-NILs (ICMR 1029 and 1031). To address whether the lower Tr observed for the drought-tolerant genotypes was due to high leaf abscisic acid (ABA,) and whether a lower Tr in these genotypes is the consequence of lower Tr at high vapour pressure deficit (VPD), some follow-up experiments were conducted. The drought-tolerant parent PRLT 2/89-33 was found to have a higher ABA concentration in leaves than the drought-sensitive parent H 77/833-2 under both well-watered and water-stressed conditions (Kholova *et al.*, 2010*b*). It was also found that tolerant parents had transpiration sensitivity to increasing VPD. Indeed, the slope of transpiration response to VPD decreased by about half above a VPD of 145–190 kPa in both the drought-tolerant parent PRLT 2/89-33 and the two QTL-NILs (ICMR 1029 and 1031) introgressed with the LG 2 DT-QTL. On the other hand, the slope of the transpiration response to VPD was unchanged in the drought-sensitive (H 77/833-2) parent. Similar studies in other crops have indicated high genetic variability of the transpiration response to VPD (Sadok and Sinclair, 2009; Devi *et al.*, 2010). Thompson *et al.* (2007) have shown that overproduction of ABA leads to enhanced transpiration efficiency and root hydraulic conductivity, and influences leaf expansion in tomato. In pearl millet, it needs to be tested whether high leaf ABA content and the lower Tr and the Tr sensitivity to VPD are linked. The highly specialized fine-mapping population (described below), segregating only in the DT-QTL region on LG 2, is being used to resolve these questions. These studies will also confirm the causal role, if any, of these physiological traits in expression of this DT-QTL.

The measurement of root systems and water extraction under water-stressed conditions in drought-tolerant and -sensitive parents, and in the QTL-NILs, is also underway. Preliminary data indicate that the tolerant parent PRLT 2/89-33 and the QTL-NIL ICMR 01029 have more profuse root systems in deeper soil layers than does the drought-sensitive parent H 77/833-2 (Vadez *et al.*, 2007). In these experiments, rooting was measured in plants exposed to well-watered and water-stressed conditions in deep and large PVC tubes (3 m long, 16 cm diameter). These rooting data obtained using PVC tubes were in agreement with the neutron probe measurements under field conditions, thus confirming that testcrosses of terminal drought-tolerant genotypes extracted significantly larger amounts of soil water from deeper soil layers than the drought-sensitive H 77/833-2. Notably, such differences were more conspicuous at the grain-filling stages of crop growth. Measuring root characteristics in these ways, however, is time-consuming and is prone to large experimental errors. To overcome these limitations, a lysimetric system has recently been set up at ICRISAT-Patancheru that makes use of large PVC tubes to mimic field conditions. In this system, plant population density and soil volume available are maintained similar to the field conditions (Vadez *et al.*, 2008). Testcrosses of the drought-tolerant and -sensitive parents, as well as of the NILs introgressed with the DT-QTL, are currently being assessed for their capacity to extract water from the soil profiles using this system.

Tests are also being carried out to determine whether grain filling stops at different levels of soil moisture in the drought-tolerant and -sensitive parents, and in the NILs differing for the QTL. This trait is particularly important to investigate because under residual moisture there would be less and less water available for extraction by the plants even when root morphologies are favourable.

Added advantage of the major DT-QTL under salt stress

Apart from drought, large parts of arid and semi-arid regions of the world where pearl millet is grown are characterized by saline underground waters and in-land salinity. Dual tolerance to both drought and salt stress is therefore a desirable trait to have in pearl millet cultivars. Drought and salt stress are physiologically linked because both result in limiting the crop's physiological access to water. This prompted the testing of whether the major DT-QTL mapping to LG 2 of pearl millet would have a pleiotropic effect on salt tolerance under a range of salinity and alkalinity stress treatments. Drought-tolerant (PRLT 2/89-33) and -sensitive (H 77/833-2) parents, and NILs introgressed with the DT-QTL were tested for a range of traits under salinity and alkalinity stress treatments (Sharma *et al.*, 2010). These genotypes were evaluated for germination and seedling emergence, and for vegetative growth and yield-related traits. Na^+ and K^+ accumulation, their compartmentation in different plant parts, and their effects on growth and/or yield parameters were also measured in these treatments. Reduction in seedling emergence was much higher (80%) in the hybrid of the drought-sensitive parent H 77/833-2 than in the hybrids of the drought-tolerant parent PRLT 2/89-33 (50%) and QTL-NIL

ICMR 01029 (64%). At the vegetative stage (24 d after sowing), the drought-tolerant parent and the QTL-NIL also recorded greater shoot growth than the drought-sensitive parent under both salinity and alkalinity stress treatments. Similarly, compared with the sensitive parent, lesser reductions in yield and yield-related parameters were evident in the drought-tolerant parent and in the QTL-NIL. Further, the drought-sensitive parent H 77/833-2, irrespective of the growth stage, accumulated significantly higher concentrations of toxic Na^+ in shoot parts than the drought-tolerant parent PRLT 2/89-33 and QTL-NIL ICMR 1029 in all salinity and alkalinity stress treatments (Fig. 2). Moreover, differences in Na^+ accumulation became more conspicuous in these genotypes as the salinity and alkalinity concentration in the medium increased. For example, at the highest salinity (ECiw 12 dS/m) and alkalinity (pH 9.4) levels, the drought-sensitive parent accumulated more than twice the concentration of Na^+ in leaves compared with the drought-tolerant parent and the QTL-NIL. These observations led us to conclude that the DT-QTL on LG 2 is also associated with reduced transport of Na^+ to photosynthesizing leaves under saline and alkaline stress conditions. The detailed physiological mechanism(s) contributing to the reduced accumulation of Na^+ in leaves of drought-tolerant parents and in the DT-QTL introgression lines was not investigated in these studies but currently is a subject of investigation in our laboratory. Recent physiological studies on these genotypes have elegantly established that they differ in transpiration rate and ABA concentration (Kholová *et al.*, 2010*a*, *b*). A low transpiration rate (Yadav *et al.*, 1996; Munns and Richards, 2007) and a high ABA concentration (Voisin *et al.*, 2006) also play an important role in reduced salt uptake. Possible roles of these physiological traits as well as of other factors underpinning drought and salt tolerance associated with this QTL are being examined in greater detail.

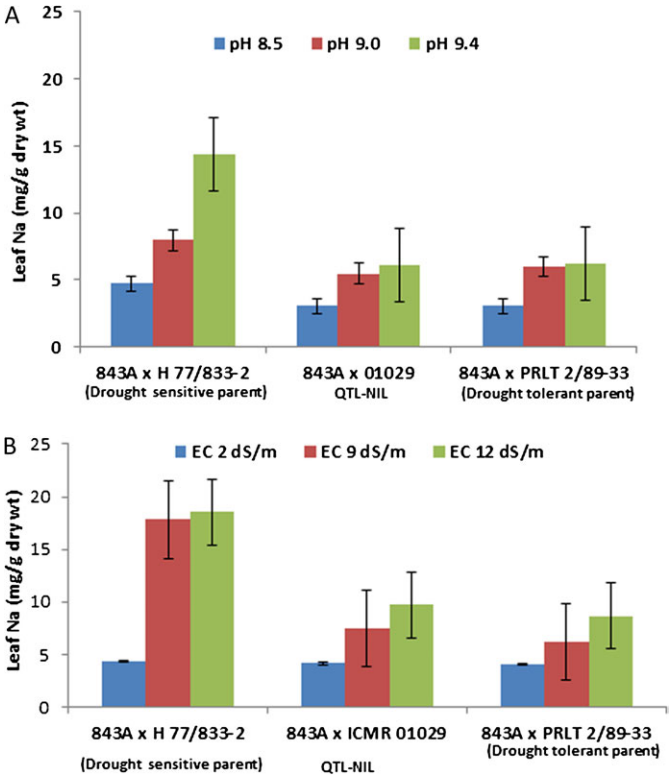

Fig. 2. Na^+ accumulation recorded in the leaves of drought-sensitive (H 77/833-2) and drought-tolerant (PRLT 2/89-33) parents, and in the QTL-NIL (ICMR 01029) introgressed with the drought tolerance QTL at three alkalinity (A) and salinity (B) levels.

Fine mapping of the major DT-QTL on LG 2

Development of a high-resolution cross/fine-mapping population

Due to the limited marker density currently available in pearl millet maps and the modest size of the mapping populations used in QTL mapping studies, the confidence interval of the DT-QTL mapping on LG 2 is still quite large. Reducing the interval of its mapping will enhance both the speed and the precision of introgression of this QTL in elite pearl millet cultivars. Delimiting the interval of the QTL will also minimize linkage drag of agronomically deleterious genes likely to be present in such a large interval.

To achieve this, a HRC segregating specifically for the DT-QTL interval on LG 2 has been developed, as outlined in Fig. 3. The HRC was developed initially with two objectives in mind—to fine-map the DT-QTL interval on LG 2 and to pyramid this DT-QTL with the downy mildew resistance (DMR) QTLs on LGs 1 and 4. An NIL of the H 77/833-2 parent introgressed with the DT-QTL on LG 2, ICMR 01029 (Fig. 3), was crossed with another NIL of the H 77/833-2 parent, ICMR 1004, introgressed with DMR QTLs on LGs 1 and 4. F_1 plants of each of the seven plant-by-plant crosses made between ICMR 1029 and ICMR 1004 were selfed to produce F_2 seeds. Seeds from two F_2 families were taken forward for development of a high-resolution fine-mapping population of ~2500 individuals segregating for the DT-QTL interval on LG 2.

Identification of informative recombinants in the DT-QTL region

Phenotyping a population of size as large as 2500 individuals under different moisture regimes (e.g. control, early-, and late-onset stress environments) in a replicated field trial is difficult to accomplish. Therefore, a smaller subset of the 300 most informative recombinants, representing all probable parental combinations, was identified from within this fine-mapping population. The fine-mapping population was genotyped with six SSR markers bracketing the entire DT-QTL region on LG 2 to identify high value recombinants representing the maximum probable parental combinations at the six marker locus alleles. These selected recombinants were subsequently selfed to generate the corresponding true breeding recombinant lines for each of the six individual marker locus alleles and also to produce a sufficient quantity of testcross seeds. The informative recombinants so identified are currently being phenotyped as testcrosses (onto 843A) for yield and yield-contributing traits under

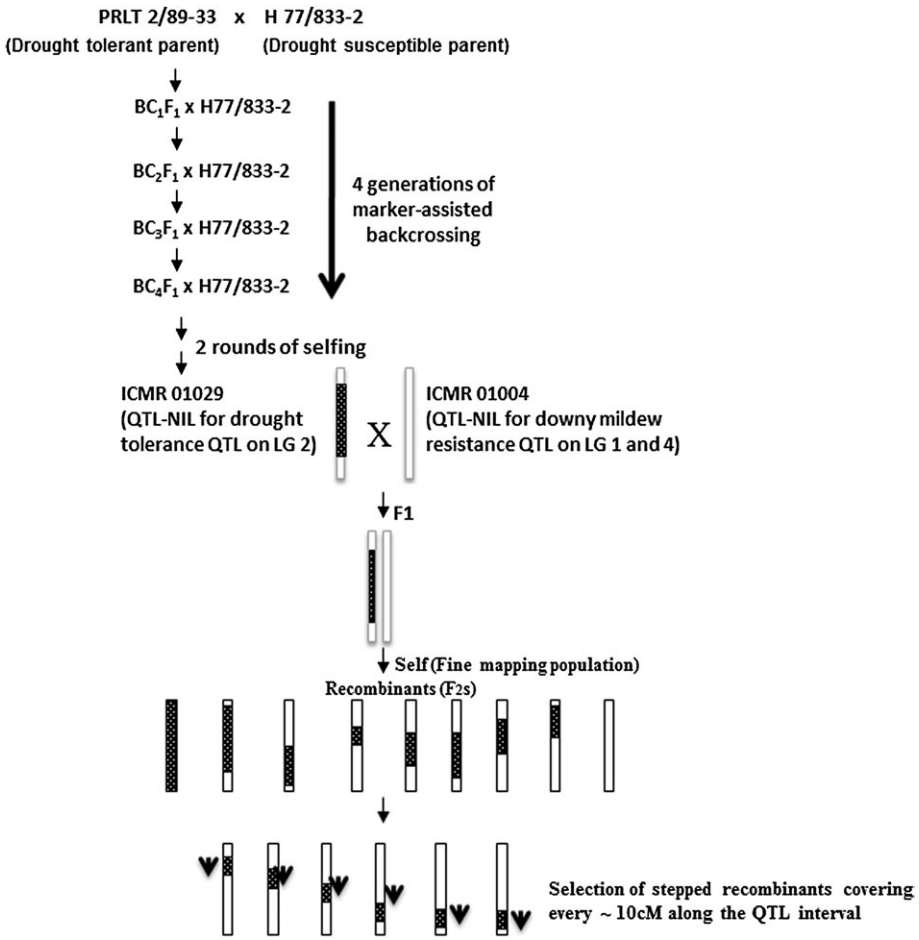

Fig. 3. Diagrammatic representation of the genetic strategy undertaken for development of the QTL-NIL (ICMR 01029), the high-resolution cross (HRC; fine-mapping population), and selection of recombinants, segregating for DT-QTL region, from within the HRC.

three moisture levels. They are also being genotyped using newly developed gene-based markers (EST-SNPs and CISPs), as described below.

Development and mapping of new gene-based markers in the DT-QTL region of LG 2

Hitherto, genetic maps in pearl millet have been based on markers such as RFLPs and amplified fragment length polymorphism (AFLPs) (Liu *et al.*, 1994; Devos and Gale, 1997; Devos *et al.*, 2000; Yadav *et al.*, 2002, 2004), with the SSRs-based maps (Qi *et al.*, 2001, Sehthilvel *et al.*, 2008) now being in ascendancy. These markers not only have proven very useful in the development of skeleton maps and in the identification of genomic regions associated with agronomic traits but have also improved our understanding of complex relationships between the pearl millet genome and those of other graminaceous species (Devos *et al.*, 2000) and also in assessing the diversity and understanding the complex dynamics of gene flow in pearl millet (Mariac *et al.*, 2006*a*, *b*; Allinne *et al.*, 2008). However, to link important traits to functional sequence variations, there is a need to develop functional genic markers of which currently there is a paucity in pearl millet. Efforts are currently concentrating on a targeted mapping approach to saturate the DT-QTL region of LG 2 with gene-based markers. Sequence information available from rice and other better studied genomes are utilized to identify pearl millet ESTs that would potentially map in the DT-QTL genomic region. CISP markers have been developed from these ESTs and are being mapped using available pearl millet mapping families (Fig. 4a, b). CISPs mapping in the DT-QTL regions are also being studied in the HRC segregating specifically for the DT-QTL interval of LG 2.

For designing EST-SNPs, an EST resequencing approach is utilized. Seven hundred primers have been designed from the drought-related ESTs using Primer 3 software to amplify an average region of 400 bp that covers part of the 3' untranslated region (3'UTR). Theoretically, 3'UTRs are expected to have greater sequence polymorphisms. Amplified products from these regions have been sequenced using the ABI Prism 3130 sequencer and aligned (using the Clustal X software package) in the parental genotypes (H 77/833-2, PRLT 2/89-33, ICMR 01029, and ICMR 1004) to identify informative SNPs and insertions–deletions (indels) for mapping in the DT-QTL region.

The candidate gene approach which links the genes of the relevant biochemical pathway to the trait of interest is becoming another powerful tool in QTL studies. To identify

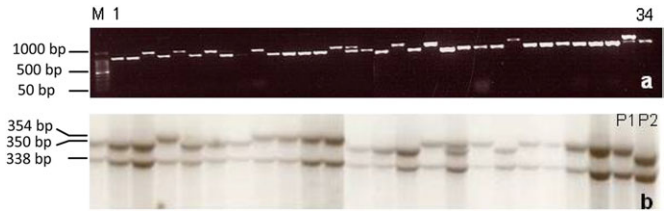

Fig. 4. (a) Polymorphism patterns of a CISP primer in 34 genotypes that have served as parents in the available pearl millet mapping populations, and (b) a representative profile of a CISP marker in the mapping population of the cross between PRLT 2/89-33 (P1)×H 77/833-2 (P2). M represents a 100 bp ladder.

genes underlying the DT-QTL on LG 2, an *in silico* analysis of the candidate region of LG 2 is being conducted. The pearl millet LG 2 DT-QTL region is aligned to equivalent genomic regions within *Gramineae* using GRAMENE portal (www.gramene.org). Pearl millet LG 2 shows synteny with rice chromosomes 2S, 3L, 6S, and 10L (Fig. 5), LG I of foxtail millet, 7H of barley, and LG 10 of sorghum. Corresponding genes (~200) within the rice bacterial artificial chromosome (BAC) contigs from each of the four syntenic rice chromosome regions have been retrieved using the TIGR rice database. The majority of these genes are associated with cellular metabolism, signal transduction, and transcriptional regulation. Primer pairs from these sequences are designed to amplify orthologous genes/genic sequences from the pearl millet genome and are being mapped to saturate the DT-QTL region on LG 2. Currently, the genotyping of the original mapping population (PRLT 2/89-33×H 77/833-2) is continuing with gene-based markers. Preliminary analysis of the genotypic data obtained has revealed 17 genes mapping in the DT-QTL region on LG 2.

The vast amount of sequence information and/or mutants available for drought-related genes mapped/cloned in model and (or) non-model species is another resource that is being used to identify candidate genes in the DT-QTL region. A number of genes known to influence drought response in model and other crops, for instance the reactive oxygen species (ROS)-scavenging protein Mn-SOD (McKersie *et al.*, 1996), the Ca-dependent protein kinase OsCDPK7 (Saijo *et al.*, 2000), the ion transport protein AVP1 (Gaxiola *et al.*, 2001), cell elongation proteins EXP15, EXP2, and EXP13 (Lee *et al.*, 2001), the ABA signalling factor CBF4 (Haake *et al.*, 2002), the syntaxin protein OSM1/SYP61 (Zhu *et al.*, 2002), *DREB* (Hsieh *et al.*, 2002), the AREB JEREF1 (Zhang *et al.*, 2004), protein phosphorylation, SnRK2 (Umezawa *et al.*, 2004), protein kinase NPK1 (Shou *et al.*, 2004), transpiration efficiency *ERECTA* (Masle *et al.*, 2005), and transcription factors MYB and MYC (Cominelli *et al.*, 2005) are also being used to identify candidate genes in the DT-QTL region on LG 2.

Association mapping analysis

Natural populations which are the products of many cycles of recombination offer additional potential to fine-map and resolve QTL location by association mapping and (or) linkage disequilibrium analysis. A composite germplasm set of 2000 diverse pearl millet breeding lines and accessions collected from Africa and Asia is therefore being utilized for these purposes. Initially a small set of 24 SSR markers, distributed over the seven LGs of pearl millet, was analysed in these accessions to identify a 'diversity panel' of 288 genotypes representing the whole breadth of genetic variation in the pearl millet germplasm pool. In addition to SSR diversity, differences in flowering time were also taken into account while arriving at the decision to select the subset of genotypes for the 'diversity panel'. To reduce any confounding effect of flowering time (i.e. drought escape) in post-flowering drought stress phenotyping experiments, it is essential that the test entries do not have a wide range in flowering time (Bidinger *et al.*, 1987; Yadav *et al.*, 2002). Four maturity groups were assembled from within the 288 genotypes included in the 'diversity panel' with differences in flowering time as small as 3–4 d among the groups. Currently, these four maturity groups are being subjected to field evaluation under three post-flowering drought stress regimes (control, early stress, and late stress). These group of genotypes are also being genotyped using candidate gene-based CISPs and/or EST-SNPs mapping in the QTL region to identify gene-based markers that are tightly linked to the DT-QTL on LG 2. It is hoped that this complementary approach will identify 'generic markers' associated with the DT-QTL, thus facilitating effective transfer of favourable alleles of this important QTL in elite pearl millet backgrounds. Identification of such markers will also facilitate estimation of allelic identity and diversity for the DT-QTL alleles in pearl millet germplasm and gene pools. Using a similar association mapping approach, Saïdou *et al.* (2009) detected significant associations between the light perception gene *PHYC* and flowering time, spike length, and stem diameter in pearl millet.

Future prospects and challenges

The identification of a major QTL for drought tolerance on LG 2 in pearl millet and its validation in two independent MAS programmes provides a strong impetus to pursue this key genomic region further to answer the remaining challenging questions. Is the drought tolerance phenotype associated with this QTL region characterized by the action of several genes independently or in combinations, or is one gene acting by pleiotropic effects on a number of traits? What physiological mechanism(s) are associated with the identified locus? What specific gene(s) underlie the QTL? Gathering this information would enhance both our understanding and the deployment of this high value QTL effectively in crop improvement programmes.

To address these challenges we have equipped ourselves with necessary genetic stocks (QTL-NILs, an HRC, and a germplasm population) and genomic resources (gene sequences, gene-based markers, and comparative genomics information). Once gene-level genetic dissection of the

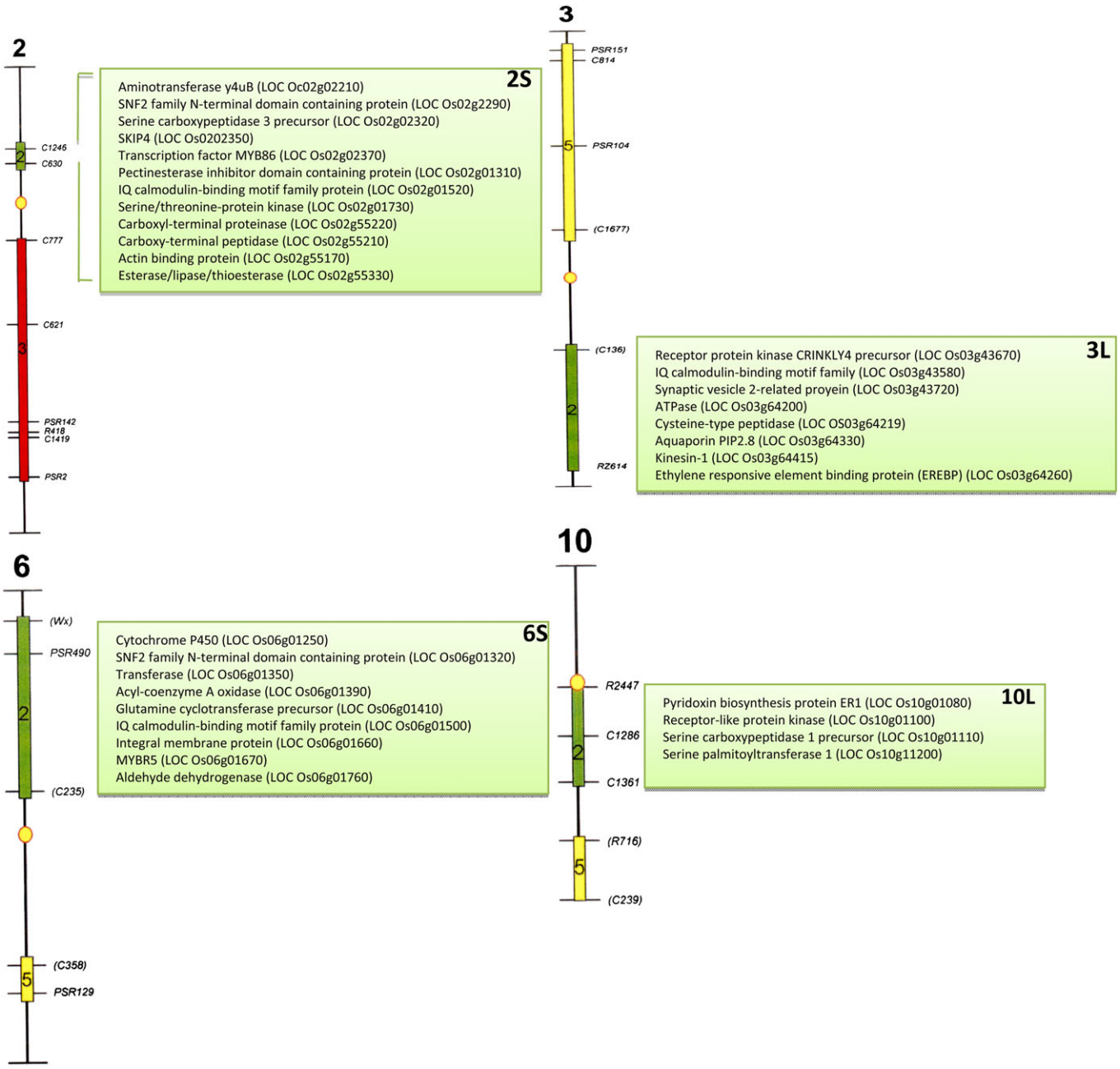

Fig. 5. Comparative alignment of pearl millet LG 2 with that of the rice genome based on information obtained from Devos *et al.* (2000). A subset of the retrieved genes, for developing markers for the DT-QTL region, mapping on the rice chromosome regions are indicated by a rectangular box on the right side of each chromosome.

different components of the drought stress response is achieved, and their allelic identity and diversity in the germplasm is better understood, using them in selection and pyramiding of favourable QTL alleles in a trait-based drought tolerance improvement programme will be straightforward.

It is envisaged that the specialized genetic resources discussed in this review not only will resolve the necessary information about this QTL but also will serve as a platform for new research opportunities in the future. For instance, they will open up vistas for map-based cloning of this major QTL. Whilst QTL cloning represents a huge challenge in terms of the technology, time, and the resources required, the advantages derived from its success will be directly applicable not only to pearl millet but across the Poaceae.

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