

**MOLECULAR MAPPING OF STAY-GREEN AND
OTHER DROUGHT TOLERANCE RELATED TRIATS IN
SORGHUM [*Sorghum bicolor* (L.) Moench]**

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LIST OF ABBREVIATIONS

ANOVA	: Analysis of variance
CIM	: Composite interval mapping
CV	: Coefficient of variation
DAF	: Days after flowering
EST	: Expressed sequence tags
GA	: Genetic advance
GAM	: Genetic advance as means
GCV	: Genotypic coefficient of variation
GLA	: Green leaf area at flowering
GLA15	: Green leaf area at 15 days after flowering
GLA30	: Green leaf area at 30 days after flowering
GLA45	: Green leaf area at 45 days after flowering
GLAD	: Stay-green leaf area duration
IABT	: Institute of Agri-Biotechnology
ICRISAT	: International crops research institute for the semi-arid tropics
LAI	: Leaf area index
LG	: Linkage group
LOD	: Likelihood odd ratio
MARS	: Main agricultural research station
MAS	: Marker assisted selection
PCR	: Polymerase chain reaction
PCV	: phenotypic coefficient of variation
QTL	: Quantitative trait loci
RILs	: Recombinant inbred lines
RIPs	: Recombinant inbred populations
SIM	: Simple interval mapping
SSR	: Simple sequence repeats

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1. INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench], mostly grown in marginal soils and low moisture regimes, is an important staple food for millions of poor people and valuable cattle feed in semi-arid tropics of the world. It is the fifth most important cereal crop grown globally after wheat, maize, rice and barley production (FAO, 2006). It is an ideal crop for growing on marginal lands for food, feed, and energy needs of millions of resource poor people. Therefore, it is an important crop for food security in the semi-arid and tropical zones including East-West Africa and Asia. Global cultivation of sorghum covers an area of 43.73mha with annual production of 60mt (Sasaki. T and A. Antonio, 2009).

Sorghum is a versatile grain and it can be used in making unleavened breads, boiled porridge, malted beverages such as beer, and popped grains. Sweet sorghum can be used in jaggery and syrup preparations, which are used in confectionaries. Some sorghum varieties are rich in antioxidants and all sorghums are gluten free. Attempts to transform sorghum with gluten genes in order to improve its suitability for making leavened bread are underway (Abebe and Ejeta, 2003).

Sorghum is usually grown in areas with low rainfall, where it is difficult to grow other food crops and feed grains. Its ability to adapt to such challenging situations makes sorghum an important food and feed crop in the arid and semi-arid regions of the world (Crasta, *et al.*, 1999). It can tolerate daily maximum temperature of 38°C or more. Its remarkable ability to reasonably produce grains under adverse conditions makes sorghum important “fail-safe” sources of food, feed and fuel. Adaptation of sorghum crop to a range of environmental conditions in semi-arid regions has resulted in the evolution of extensive genetic variation for drought tolerance (Blum, 1979; Doggett, 1988), which makes it an excellent model crop of choice for studying the genetic and physiological mechanism of drought tolerance. Its relatively small genome size and extensive genome synteny with other cereals were main considerations to sequence its genome (Blum *et al.*, 2005).

In India, sorghum is primarily cultivated in two distinct seasons: June to October (rainy/*kharif*) and October to February (post-rainy/*rabi*). Though *kharif* sorghum yields better, the grain quality is severely affected due to grain mold disease, making it unsuitable for consumption. Despite the availability of a number of *kharif* sorghum hybrids and high yielding varieties, its area under *kharif* sorghum production is on declining trend; because of grain mold disease, availability of alternative commercial crops such as sunflower, Bt. cotton and maize. On the other hand, the post-rainy season crop grown mostly on stored soil moisture in deep black soils. *Rabi* sorghum grains are preferred for human consumption as they mature in rain-free and dry climate. In post-rainy season, sorghum grain and fodder yields are low as the crop is grown on residual moisture and often experiences severe terminal drought stress.

In Eastern Africa, more than 70 percent of sorghum is cultivated in dry and hot lowlands of poor soil fertility (Mutengwa *et al.*, 2005). A highly unpredictable drought stress pattern and high microclimatic variability within fields are major constraints of sorghum production. In these areas, sorghum cultivation mainly depends upon seasonal rains and post flowering moisture stress is a common feature. The situation is similar to that of *rabi* sorghum in the Indian subcontinent with respect to moisture stress is concerned, and hence the breeding objectives remain similar. Therefore, there is a need for concerted effort to improve the yield levels of sorghum under moisture stress condition. Several attempts have been made in sorghum to understand the mechanisms of drought tolerance in sorghum and to identify and improve genotypes that could tolerate moisture stress. In sorghum, the best characterized form of drought tolerance during grain filling stage of crop growth is so called “stay-green” trait which is defined as the ability of the plant to resist premature plant senescence, retain green leaf area, fill grain normally, and resist lodging under conditions of post-flowering drought stress (Rosenow, 1987). Sorghum genotypes with the stay-green trait continue to fill their grains normally and retain good fodder quality even under limited water or moisture stress conditions (Duncan *et al.* 1981, Rosenow and Clark 1981, Borrell and Hammer, 2000). Delay in the onset and reduced rate of leaf senescence (*i.e.*, two distinct components traits of stay-green) offer an effective strategy for increasing grain production, fodder quality and grain crop residues particularly under water limited conditions. Stay-green sorghum genotypes maintain photo-synthetically active leaf area better than genotypes that

do not possess this trait under limited soil moisture during grain filling stage. Stay-green has also been viewed as a consequence of balance between N demand by the grain and N supply from the roots during grain filling (Borrell *et al.*, 2000)

Breeding for improving post flowering drought tolerance in sorghum hybrids and varieties remains an important priority throughout the world. Due to difficulty in evaluating the stay-green trait *per se* under variable field conditions, unpredictable of moisture stress and large environmental interaction, the progress in improving stay-green feature in sorghum by conventional breeding methods has been slow. Consequently, study of QTLs for stay-green, a post-flowering drought and charcoal/stalk rot tolerance trait has gained increased attention. As the stay-green trait in sorghum is expressed only in those environments in which post-anthesis drought are sufficiently severe, neither efficiency nor reliability of selection is high when conventional methods of selection are followed. Hence, markers assisted selection (MAS) for stay-green trait is likely to be efficient (Borrell *et al.*, 2000; Haussmann *et al.*, 2004).

Developing appropriate mapping populations using potential and diverse parents, accurate phenotyping in multi locations and appropriate genetic algorithm to draw conclusions are the key factors in development and use of molecular markers. Evaluation of QTLs associated with yield in drought environments also provides a powerful method for identifying traits that contribute to drought tolerance. QTL mapping with Recombinant Inbred Lines (RILs) are considered more efficient and reliable than F₂ populations as RILs undergo several rounds of meiotic events, resulting in better chances for recombination between closely linked markers. RILs being genetically homozygous, they can be evaluated in replicated trials at many environments. High-resolution maps therefore can be constructed with RILs than with F₂ of similar size (Burr, 1991). Reliable estimates of genetic parameters including heritability, genetic correlation, and prediction of genetic gain and G x E interaction in this breeding material will be useful in applied breeding.

Genetic maps consisting of molecular markers have been developed in recent years for many crop plants. Saturated linkage maps are required for effective MAS. In sorghum, linkage maps (individual /integrated) have been developed using a number of RFLP (Subudhi and Nguyen, 2000), AFLP (Boivin *et al.*, 1999) and SSR markers (Reddy *et al.*, 2007). Among different types of markers available, simple sequence repeats (SSRs) satisfy the criteria of sufficient polymorphism, repeatability and cost effectiveness required for successful utilization in marker assisted selection (Brown *et al.*, 1996; Taramino *et al.*, 1997; Kong *et al.*, 2000; Bahattramakki *et al.*, 2000). These are suitable for screening the existing sorghum RIL populations to construct a genetic linkage map and to identify QTL for the target trait, stay-green and its components. Keeping the above points in view, the following objectives were set for the present study.

1. Phenotypic evaluation of two recombinant inbred populations (RIPs) for stay-green, other drought tolerance traits, yield and its component traits
2. Molecular mapping of selected EST-SSR markers
3. Identification of QTL for stay-green, other drought tolerant traits and yield components

2. REVIEW OF LITERATURE

Sorghum is one of the most drought tolerant crops and a good crop model for evaluating mechanisms of drought tolerance in cereals. Drought tolerance in sorghum is a complex trait controlled by several interacting plant and environmental factors. The growth stage (GS) at which moisture stress occurs is very important in determining the response or reaction of any plant to water stress. Two distinct drought-stress stages have been identified in sorghum a pre-flowering drought response that occurs prior to anthesis and a post-flowering drought response that is observed when water limitation occurs during the grain-filling stage. They are shown to be controlled by different genetic mechanisms and indeed sorghum hybrids containing both pre- and post-flowering drought tolerance have been developed.

A brief review of literature on various aspects relating to current work is presented below.

2.1 PRE-FLOWERING DROUGHT TOLERANCE

Sorghum lines with distinct phenotypic response to pre-flowering and post-flowering drought stress have been characterized and reliable sources of resistance to each type of stress have been identified (Rosenow, 1993). The pre-flowering response occurs when the plants are under significant moisture stress before flowering especially from panicle differentiation to flowering (GS-2). This type of stress directly affects root development, panicle size, grain number, and grain yield. Tuinstra *et al.* (1996) found six regions in the sorghum genome associated with pre-flowering drought tolerance and eight additional regions generally associated with yield or yield components under fully irrigated conditions. Four major QTLs responsible for pre-flowering drought have also been identified in linkage group C, E, and F using a SC56 × T × 7000 cross derived mapping population in sorghum (Kebede *et al.*, 2001).

2.2 POST-FLOWERING DROUGHT TOLERANCE

Post-flowering drought response in sorghum refers to moisture stress during the grain development stage (GS-3). Rapid premature leaf death generally occurs when water is limited during the grain-filling period. Premature leaf senescence, in turn, leads to charcoal rot, stalk lodging, and significant yield loss are common effects of post flowering drought stress in sorghum. Drought stress during the post-flowering period accelerates senescence in many plant species by driving many physiological processes in the same direction as normal senescence (Nooden *et al.*, 1997). Chlorophyll loss and a progressive decline in photosynthetic capacity generally characterize senescence. Early onset of senescence affects assimilation and grain filling.

2.3. STAY-GREEN TRAIT, ITS CONTRIBUTION, PHYSIOLOGY AND GENETICS

Leaf greenness depends on the concentration of chlorophyll pigment absorbing sunlight energy for photosynthesis. Leaf yellowing generally results from progressive breakdown of chlorophyll during senescence. Plants assimilate carbohydrates and nitrogen in vegetative organs (source) and remobilize them to newly developing tissues or to reproductive organs (sink) during senescence. To increase grain yield in cereal crops, source strength must be increased so that sink organs can be filled through efficient translocation. Persistence of high photosynthetic capacity and efficient nitrogen remobilization during grain filling have been considered as key factors in increasing grain yield (Abdelkhalik *et al.*, 2005; Yamaya *et al.*, 2002).

Genotypes possessing stay-green trait maintain more photosynthetically active leaves than those not possessing this trait (Rosenow *et al.*, 1983, 1996). The key issue is whether maintaining green leaf area under post-anthesis drought actually increases grain yield in stay-green genotypes compared with senescent genotypes. If this is the case, then pathways to increased green leaf area retention, such as higher maximum green leaf area, delayed onset of leaf senescence, and reduced rate of leaf senescence, should be considered to improve yield under drought. In addition to this empirical approach, the

underlying physiological mechanisms of drought-resistance should be investigated. Three mechanisms of functional expression of stay-green have been identified (Borrel *et al.*, 2000b; Thomas and Howarth, 2000) (i) delayed onset of senescence; (ii) reduced rate of senescence, and (iii) increase leaf area index at anthesis.

In sorghum the expression of stay-green character during grain filling can be viewed as a consequence of balance between demands of nitrogen (N) by the grain and supply of N through N- uptake and translocation from vegetative plant parts including stems and leaves. Leaf N-translocation occurs if grain N demands cannot be met through soil uptake and stem N-translocation (Van Oosterom *et al.*, 2005b). Increased biomass accumulation under post-anthesis drought in stay-green compared with senescent genotypes can be achieved by increasing one or both of its components: transpiration and/or transpiration efficiency. Knowledge of the extent of genetic variation in these components is required to improve yield under drought. Since the longevity and photosynthetic capacity of leaves are related to their nitrogen status (Thomas and Rogers, 1990), it is also important to determine the role of nitrogen in extending leaf greenness in stay-green genotypes.

Genome analysis provides a framework to link structural analysis of genes with the underlying physiological mechanisms, and ultimately with phenotypic performance of crop plants in a range of target environments (McCouch and Xiao, 1998). As stay-green is expressed only in those environments, in which post-anthesis drought is sufficiently severe, neither efficiency nor reliability of selection is high when conventional breeding is used to select for this trait. Hence, marker assisted selection for stay-green should greatly enhance the efficiency of selection for the trait.

Thomas and Howarth (2000) classified stay green into four types: A, B, C and D. In Type A stay-green, senescence is initiated late but then proceeds at normal rate. Type B stay-green initiate senescence on schedule, but thereafter, senescence comparatively slowly, and in type C chlorophyll may be retained more or less indefinitely but physiological functions such as photosynthesis are affected, *i.e.*, senescence is proceeding internally although the surface pigmentation is retained. There is unlimited colour retention in type D, which may be related to premature death seen in herbarium species or achieved by rapid killing as in the case of frozen foods. Therefore, type C and D are non functional form of stay-green. Generally, the first two classes are functionally stay-green: retaining both leaf greenness and photosynthetic competence much longer during senescence than the wild-type and may be a result of alteration of genes involved in the onset of senescence and the regulation of its rate of progress. Stay-green in the other two classes is cosmetic, wherein the plants are green but lack photosynthetic activity. Thomas and Howarth (2000) defined a fifth type of stay-green referred to as type E. In these stay-greens, the greater leaf greenness is related simply to higher initial chlorophyll content and thus, the decrease in chlorophyll content during senescence results in a slower reduction in leaf greenness.

Sorghum genotypes with the stay-green trait continue to fill their grain normally under drought (Rosenow and Clark, 1981) and exhibit resistance to charcoal rot (Rosenow, 1984) and lodging (Henzell *et al.*, 1984). The stay-green trait does not appear to reduce the grain yield of hybrids while at the same time it improves lodging resistance and resistance to diseases (Borrell and Hammer, 2000). Such genotypes also contain more basal stem sugars (Duncan, 1984) and cytokinins (McBee, 1984) than senescent genotypes, which may reduce the rate of drought-induced senescence (Thomas and Smart, 1993). Increased accumulation of soluble sugars found in stay-green genotypes may reduce the dependence on stored assimilates from the stem to fill the grains (McBee *et al.*, 1983). Expression of stay-green has been reported in other crops including maize (Tollenar and Daynard, 1978), tomato (Akhtar *et al.*, 1999) and oat (Helsel and Frey, 1978). A stay-green mutant of the pasture grass *Festuca pratensis* Huds, has also been identified and subsequently studied, which has led to a better understanding of the biochemistry of senescence (Kingston-Smith *et al.*, 1997; Hauck *et al.*, 1997; Thomas *et al.*, 1999).

2.3.1 Stay-green and yield

Stay-green in sorghum has been associated with a higher yield under water-limited conditions. Yield increase in stay-green type has been attributed directly to the maintenance of photosynthetic capability during grain filling period. Stay-green and yield were positively

associated in a range of studies conducted in both Australia (Borrell *et al.*, 2000) and India (Borrell, 1999), highlighting the value of retaining green leaf area under post-anthesis drought.

Grain yield is the product of grain number and grain size. Grain number is generally the main determinant of differences in grain yield, and this has also been observed for sorghum, grown under post-anthesis drought stress in southern India (Borrell *et al.*, 1999). In wheat, grain number is a function of the spike weight around anthesis (Miralles *et al.*, 1998) and similar results have been obtained for sorghum (Van Oosterom and Hammer, unpublished data). Factors, related to the stay-green mechanisms of B35 and KS19, that can potentially increase the panicle growth rate around anthesis, and hence can have a positive effect on grain number, include increased LAI (Borrell *et al.*, 1999) increased SLN, as that increases RUE (Muchow and Sinclair, 1994) and competition for assimilate from the stem. Grain size is a secondary yield determinant and is often negatively associated with grain number. Hence, grain size is independent of green leaf area at anthesis (Borrell *et al.*, 1999). However, the retention of photosynthetic capacity under water-limited conditions of stay-green hybrids ensures continued availability of new assimilates and is associated with increased N-uptake during grain filling (Borrell and Hammer, 2000) potentially improving grain size. This was illustrated a set of RILs (Borrell *et al.*, 1999) where found that grain size was found to be correlated with relative rate of leaf senescence during grain filling. Reducing rate of leaf senescence from 3 to 1% loss of leaf area per day resulted in doubling grain size from about 15 to 30 mg. Thus stay-green can potentially increase grain yield by improving grain number and grain filling ability.

2.3.2. Physiology of stay-green

Accumulation of Absciscic Acid (ABA) is the factor that may enhance survival, but it is known to reduce productivity (Mundy and Chuha, 1988). The onset of leaf senescence can be delayed by increased soil-N uptake during grain filling, as observed under terminal drought stress by Borrell and Hammer (2000). Such increased N- uptake could be associated with either increased water uptake or increased transpiration efficiency and at least one of these is required to explain enhanced biomass accumulation of stay-green types. Another mechanism to delay the onset of leaf senescence is through increased availability of stem-N for translocation, through this mechanism might compromise on the leaf-N status, if increased stem-N is not matched by increased N uptake. Higher specific leaf nitrogen (SLN) and leaf area at anthesis can also reduce the rate of senescence. Once leaf-N translocation starts, the amount of leaf area that needs to senesce in order to meet the N demand declines with improving leaf-N status (Van Oosterom *et al.*, 2005b). Genotypic variation in maximum SLN under optimum conditions has been observed in sorghum and it was associated with differences in leaf size (Van Oosterom *et al.*, 2005b). Increased leaf area at anthesis can be achieved through increased partitioning of dry matter and N to the leaves (Borrell and Hammer, 2000). However, there are indications that high leaf area index (LAI) of some stay-green hybrids is due to their tillering ability if the leaves on main shoot were smaller, particularly under low moisture (Van Oosterom *et al.*, 2005a).

This framework can explain the genotypic differences and effects of genotype-environment interaction on the expression of stay-green character. Phenotypic expression is thus the consequence of the interplay of differences in underlying traits like leaf size, leaf SLN, dry matter partitioning, N uptake and possibly transpiration efficiency. In fact, sorghum is one of the most drought-tolerant grain crops and its rich genetic diversity for stress tolerance makes it an excellent model crop for studying the genetic and physiological mechanisms of drought tolerance (Doggett, 1988)

2.3.3 Inheritance of stay-green

Although the ability of leaves to delay senescence has a genetic basis in sorghum (Van Oosterom *et al.*, 1996) its expression is strongly influenced by environmental factors such as stored soil moisture, temperature and relative humidity (RH). Varied field environments often do not offer ideal conditions for phenotypic assessment and selection. Molecular markers associated with this trait may offer a better alternative (Crasta *et al.*, 1999). Difference in patterns of senescence exist in many species that exhibit stay-green, even among genotypes that may retain same level of leaf area at maturity (Thomas and Howarth, 2000) which is influenced by both the time of onset and the rate of development of stress in an individual environment. Consistent with the hypothesis, there has to be more than one

mechanism by which leaves stay-green (Thomas and Howarth, 2000) which are likely to be controlled by different genes that are triggered by specific pattern of stress development (Dunwell, 2000).

E 36-1 and B35 stay-green types are well adapted to tropical climate. B35 (PI534133) is a selection from a converted (dwarf, early flowering) version of IS12555, an Ethiopian land race (Rosenow *et al.*, 1983). A stay-green trait in B35 is influenced by a major gene that exhibits varied levels of dominance gene action depending on the environment. It was also shown that environment had a strong influence on the mode of stay green feature in B35. It was more dominant under field conditions than the extreme water stress environment of the rainout shelter. However, Walulu *et al.* (1994) concluded that stay-green trait was a nuclear gene controlled quantitative trait.

2.4 PHENOTYPIC TRAITS ASSOCIATED WITH DROUGHT TOLERANCE

2.4.1 Plant height: Beside the genotype, plant height is often influenced by soil fertility, availability and other environmental factors. Several studies have revealed that, moisture stress generally decrease the plant height. The decrease in plant height due to water stress at boot leaf stage is because of decreased panicle extension (Esk and Musick, 1979). The terminal drought stress reduces the plant height and biomass and the average height of non-senescent sorghum genotypes was 3 to 4cm shorter than the senescent types (Duncan *et al.*, 1981). In a study with 57 sorghum recombinant inbred line (RILs) of across, k886 x cs3541, Abebe and Ejeta (2003) found that height and days to 50% flowering in non-stress environments were slightly higher than those in the respective stress environments through the differences were not significant statistically. Heritability estimates for each trait, in contrasting environments followed a similar trend of the genetic variance estimates.

2.4.2 Stem thickness: Stem stature defines plant responses to abiotic stress in a number of ways. Higher basal stem sugar content is high in stay-green genotypes, especially during post anthesis drought in sorghum. The stem reserves and grain yield are also affected by water regime. The non-senescent lines had significantly larger stem diameter and maintained a higher sugar concentration in the basal part of the stem at the time of harvest (Duncan *et al.*, 1981).

2.4.3 Days to 50 per cent flowering: The studies on changes in the phenological and physiological characters of genotypes under drought stress indicate that tolerant genotypes generally tend to flower early under drought conditions (Omanya *et al.*, 1997). The non-senescent genotypes required approximately 2 days longer to reach 50% anthesis in sorghum (Duncan *et al.*, 1981). Sorghum is known to be sensitive to water stress during flowering and early grain filling stages than at early growth period (Seetharama *et al.*, 1987). As the number of days from 50 percent flowering to physiological maturity increased, the yield also increased in hybrids, possibly due to increased number of days for dry matter accumulation in the grain (Reddy and Rao, 1978; Liong, 1965; Lonc, 1969). Early flowering, fewer tillers (or no tillers) and deeper root are known to result in escaping late –season stress (Blum, 2005). Genotypes that avoid dehydration are characterized by the maintenance of high plant water status under stress and show any of the following features; early flowering, lower plant height, low leaf area, or limited tillering in cereals (Blum, 1988; Hussmann *et al.*, 2002).

2.4.4 Seed colour: Colour of seed is one of the most evident varietal characters of the grain sorghum. Though it is not of major economical importance, yet it does influence the popularity of a variety. Naturally seed colour attracts the attention of the plant breeders during sowing. Graham (1916) of India investigated the inheritance of seed colour in the natural and artificial sorghum crosses. The colour studied was red, yellow and white. Red was found to be dominant to yellow and white, Yellow dominant to white. In the Crosses between a yellow and white seeded sorghum, the heterozygote was red seeded, segregating in the F₂ generation in the ratio of 9 red 3 yellow and 4 white. (Karper and Konner, 1919) and (Seingliner, 1921) determined the amount of cross pollination in millo by taking the advantage of the fact that the seed of F₁ of cross between white yellow millo is white. (Vinali and Corn, 1921) in reporting the inheritance certain sorghum characters, state that a close agreement to a 9:7 ratio di-hybrid ratio for brown and white seed was obtained in a crosses between

blakhull kafir and *feterita*. This colour inheritance was attributed to two factors for brown colour, carried by the *blackhull kafir*, and *s*, and spreader factor carried by the *Ferita*.

The level of tannin in sorghum is the principal concern of those employing sorghum in the poultry diet (Johnson and Brain, 2005). In this aspect, seed colour has been used in estimation of tannin content of sorghum which is often thought to be closely related to darkness of seed colour (Ralph and Waniska, 1992). For example, in the absence of confirmatory quantitative tests, the darker of two sorghum samples is suspected to have higher tannin content. Emerging technologies for generating transgenic plants provide an opportunity for enhancing existing seed traits and for adding value to seeds. For most farming community, the basic criteria to accept seed as a quality seed for food and further production is depends on seed colour. Most researchers are devoting to improve seed colour through traditional breeding. But, since this trait is controlled by oligogenic genes, it is difficult to come up with good result under conventional plant breeding. Mapping of QTL is important recent strategy to improve the quality of crop plant.

2.4.5. Glume colour: A wide array of glume colours are present within sorghum, and the genetic control of these glume colours varies depending on the germplasm in which the inheritance has been determined. The range of glume colours that can be observed on a genotype is a function of the plant colours the plant pigmentation genes, *P* and *Q*, have an indirect effect on glume coloured and purple pigmented plants almost always have dark coloured glumes than tan plans, but there are exceptions to this trend in both cases. In addition to the plant colours genes, several other genes have been reported that control glume colour. Ayyangar and Ponnaiya (1937) described the *Gep* locus, in which a dominant allele causes purple glumes at the emergence of the panicle from the leaf sheath. In addition, Ayyangar and Ponnaiya(1937) reported on two independently inherited genes that influence the production of straw of faded glumes. The recessive colour dilution gene *cd* causes glume colour to fade, while dominant *Ci* genes inhibit the development of colour in the glume except in the base of glume. Given the range of glume colours that have been observed and the role of plant colour, it is likely that there are many more genes that have not yet been described.

2.4.6 Awn: The genetic control of awning is variable and the result depends up on which germplasms are being evaluated. Vinal and Corn (1921) reported that awn- less was dominant to awned. Singlinger *et al.* (1934) reported that awn less (*A*) was dominant to absence of *AA* (awn less), additional alleles determined the specific type of awn present ranging from *aa*(strong awn) to *a'a'* (tip awn) or *a'a*(weak awn). Vinal and Corn (1921) reported a serious of crossing using shallu as a source of strong awns, the presence of awns always dominant to awn less, tip awn, and weak awn. In two crosses, the traits segregated as a single dominant gene, while in the other populations the distribution of awns and their size could not be placed into discrete classes. In summary it appears that both dominant and recessive forms of strong awn exist and several genes are involved in the determining additional types of awns.

2.5 BIOCHEMISTRY OF DROUGHT RESPONSE

In response to various environmental stresses, plants have developed different physiological and biochemical strategies to adopt and tolerate water stress conditions; accumulation of low molecular weight osmolytes such as sugar, alcohols, amino acids and increased activity of certain enzymes have been suggested as major mechanisms responsible for the adaption or tolerance of plants to osmotic stresses (Yancey *et al.*, 1982). In addition to metabolic changes and accumulation of low molecular weight protective compounds, a large set of plant genes are transcriptionally activated, which leads to accumulation of new proteins in vegetative tissues under osmotic stress conditions (Skriver and Mundy, 1990; Chandler and Roobertson, 1994). Recent studies have demonstrated that genetic manipulation for accumulation of low molecular weight osmolytes resulted in increased tolerance to water stress in transgenic plants (Rathinasabapathi *et al.*, 1994; Saneok *et al.*, 1995).

The changes in primary metabolism are a general response to drought and the enzymes of sugar metabolism are critical in desiccation tolerance. In the resurrection plant *Craterostigma plantagineum*, the overall levels of sucrose phosphate synthesis and sucrose synthesis increased immediately in response to drought which is considered to be general

response of plants to drought (Quick *et al.*, 1989; Pareek *et al.*, 1997). Various soluble sugars and carbohydrates serve as solutes for osmotic adjustment and sugar such as sucrose, maltose and trehalose are also known to stabilize the activity of enzymes during senescence (Carpenter *et al.*, 1987).

Enhanced protease activities may also be an important feature of stress metabolism, releasing amino acid from stored polypeptides for the synthesis of new proteins (Gurrero *et al.*, 1990; Ezechia *et al.*, 1977). The enzymes involved in the pathways of proline biosynthesis (Yoshoda *et al.*, 1995) and glycine-betaine biosynthesis (Ishitani *et al.*, 1995) are found to show increased activity during drought. Apart from this, the hydrophilic LEA proteins are consistently expressed with increased level during drought (Mundy and Chua, 1988). These proteins have been shown to have a functional role in stress tolerance (HVA1 a group 3 LEA protein from barley and LEA 25, group 4 LEA protein from tomato). Xu *et al.* (1996) and Pelah *et al.* (1997) reported the differential accumulation of dehydrins, sucrose synthase and soluble sugars in *populus tremula* during water stress.

In addition to metabolic changes and accumulation of protective compounds, a large number of plant genes are transcriptionally activated, which leads to the accumulation of new proteins in plants under osmotic stress conditions (Skriver and Mudy, 1990; Chandler and Robertson, 1994) which might have a role in stress tolerance. Several molecular studies have been conducted to characterize the pattern of gene expression in plant under water deficit conditions. The metabolism of carbohydrates, fats, proteins and nucleic acids is adversely affected upon exposure to stress. The expression of gene regulating the synthesis of these compounds is altered in response to stress (Ingram and Bartels, 1996).

Drought stress has been shown to cause alterations in the chemical composition and physical properties of the cell wall and the changes such as lignifications of cell wall are associated with the expression of genes encoding S-adenosyl methionine synthetase (Espartero *et al.*, 1994). Genes encoding proteins with sequence similarity to proteases are reported to be induced by drought in pea (Gurrero *et al.*, 1990) and Arabidopsis (Koizumi *et al.*, 1993). Function of these enzymes might be degrading proteins irreparably damaged by drought (Gurrero *et al.*, 1990; Singla *et al.*, 1997). During drought stress, protein residues might be modified by chemical processes such as deamination, isomerization or oxidation and it is thus likely that enzymes with functions in protein repair are up-regulated or induced during drought. Mudgett and Clark (1994) reported increased levels of L-isoaspartyl methyl transferases which have a role in converting the drought damaged L-isoaspartyl residues back to L-aspartyl residues.

Decreasing leaf water content and consequent stomata closure resulted in reduced CO₂ availability, of active oxygen species and increased photo-respiratory activity, which resulted in the production of H₂O₂ (Mittler and Zilinskas, 1994). To scavenge these toxins, genes encoding detoxifying enzymes such as ascorbate peroxidase (Mittler and Zilinskas, 1994) are found to be up-regulated. The genes encoding LEA proteins are also up regulated during drought and these proteins accumulate in abundance during seed maturation and drought.

The key to delay aging in sorghum is the stay-green character, which reduces the loss of protein from sorghum leaves and allows greater accumulation of starch during the latter stage of grain filling. The inheritance of stay-green leaf area duration (GLAD) showed that individual hybrids had higher GLAD values than their best parent's additive genetic effects. Thus the cultivation of stay-green varieties, once available, can benefit farmers by reducing the risk of crop failure due to more drought tolerance and provide better quality fodder.

2.6 MOLECULAR MARKERS

One of the most important uses of molecular biology in plant breeding is the development of genetic maps and markers based on cloned DNA sequences. With the advent of new molecular techniques, the use of molecular marker as a tool for rapid, directed and compressive genetic manipulation of crop plants has become important. The genotypes scored at molecular level provide a large number of markers for analysis of characters. For almost all the major crops and several minor crops, genetic maps based on restriction fragment length polymorphism (RFLP) have been or are being developed and useful traits, including quantitative traits, are being tagged. Genetic polymorphisms analyzed by the study

of RFLPs reveal variations and provide a potentially useful molecular approach to tagging qualitative or quantitative trait loci for selection (Tanksley *et al.*, 1989; Agramaha *et al.*, 2002). The sorghum genome has been extensively studied by RFLPs (Hulbert *et al.*, 1990; Peng *et al.*, 1999; Katsar *et al.*, 2002). Attempts to develop a complete genetic linkage map in sorghum using DNA clones are reported by Pereira and Lee (1995); Pereira *et al.* (1999); Klein *et al.* (2001); Boivin *et al.* (1999) obtained a complete RFLP-based genetic linkage map using recombinant sorghum inbred lines. Further, the new technologies to study DNA polymorphism like Randomly Amplified Polymorphic DNA (RAPD) Williams *et al.* (1990) and polymerase chain reaction (PCR)-mediated genetic polymorphisms are also being applied to study agronomically important traits in sorghum. RAPD analysis of sorghum lines also has revealed significant genetic diversity (Vierling *et al.*, 1994; Gowda *et al.*, 1995; Mankir *et al.*, 1997).

2.6.1 EST-SSR markers: Microsatellites or SSR markers are widely used in cultivar fingerprinting, genetic diversity assessment, molecular mapping, and marker-assisted breeding. Genic SSRs have the potential of being functional markers in cases where polymorphism in the repeat motifs affect the function of the gene (Anderson and Lubberstwdt, 2003) permitting 'direct allele selection' for the target trait (Sorrelles and Wilson, 1997; Zhang *et al.*, 2002). Recently, a 'Dof' homolog (DAG1 gene) that showed a strong effect on seed germination in Arabidopsis (Papi *et al.*, 2000 ; Albin and Jones 1987) was mapped on chromosome 1B of wheat by using wheat EST-SSR primers (Gao *et al.*, 2004). Similarly, Yu *et al.* (2004) identified two EST-SSR markers linked to photoperiod response gene in wheat. Mapping candidate genes and genic SSRs will also facilitate genome alignment across related species. (Yu *et al.*, 2004; Varshney *et al.*, 2005).

EST-SSRs have been integrated and genome wide genetic maps have been prepared in many crops viz. Wheat (Yu *et al.*, 2004; Gao *et al.*, 2004), barley (Thiel *et al.*, 2003), rye (Khlestkina *et al.*, 2004) and rye grass (Warnke *et al.*, 2004), cotton (Zhiguo *et al.*, 2006; Han *et al.*, 2004), soybean (Zhang *et al.*, 2004), potato (Feingold *et al.*, 2005), kiwi fruit (Fraser *et al.*, 2004), raspberry (Graham *et al.*, 2004) etc. Genic SSRs show a characteristic distribution throughout the genome and get concentrated in gene rich regions, unlike genomic SSRs, which are clustered around the centromere (Thiel *et al.*, 2003; Yu *et al.* 2004; Gao *et al.*, 2004). Distribution of genic SSRs on the genetic map will show the distribution of genes in the genome.

High rates of transferability of SSR loci across species (>50%) within a genus have been reported (Thiel *et al.*, 2003; Eujayl *et al.*, 2004; Saha *et al.*, 2004). However, the transferability of SSR loci across genera and beyond appears to be low (Thiel *et al.*, 2003). EST-SSR markers are derived from transcribed regions of DNA and are expected to be more conserved and have a higher rate of transferability across species than genomic SSR markers (Scott *et al.*, 2000). As reviewed in Elis *et al.* (2006) the ability to effectively transfer polymorphic EST-SSRs across taxa has now been demonstrated in a number of cases, most commonly in studies involving economically important crops species. Scott *et al.* (2000) tested the transferability of 10 *vitis* EST-SSR markers among grape cultivars, other grape species and related genera, and found high level of transferability, with over 60% of marker tested working across taxa. Moreover, all of the transferable markers provide to be polymorphic at the level of cultivar, *vitis* species and between related genera. In cereals, Gupta *et al.* (2003) demonstrated extensive transferability of *Triticum aestivum* L. (bread wheat) EST-SSR to 18 related wild species in the *Triticum aestivum* complex and five species of barley, oats, rye, rice and maize. Over 80% of primer pairs of tested were transferable to the 18 related species, while nearly 60% showed transferability to one or more of the more distantly related cereal species. Because of this transferability, EST-SSR markers are well suited for application in cross-species genetic studies (Kantety *et al.*, 2002; Thiel *et al.*, 2003; Eujayl *et al.*, 2004; Saha *et al.*, 2004).

Though EST-SSRs are expensive to develop, they are serving as useful as additional markers in mapping and MAS and have the advantage of providing functional relevance to the marked region of the genome.

2.6.2 Nuclear SSR in sorghum: Nuclear SSRs markers have been used in a variety of plant species and in genetic mapping initiatives for cereals including wheat, barley (Roder *et al.*, 1998), and rice (Temnykh *et al.*, 2000) and sorghum (reviewed in Sanchez *et al.*, 2002).

Microsatellites are highly reproducible co-dominant markers that offer the potential for automation, facilitating the rapid screening of plant lines review in (Gupta *et al.*, 1996). The process of SSR-marker development from genomic libraries is justified for major crop species, but it is too expensive and labor intensive for related species that lack significant agricultural importance. Studies have explored the possibilities of using genomic SSRs generated from one species for use in related species. Rarely, however, has a significant number of SSR markers been transferred across species and used in alien introgression studies (Roder *et al.*, 1998). An alternative is to use the increasing availability of other sequencing resources from crop species for developing SSR markers in related species (Mullan *et al.*, 2004).

SSR-containing clones isolated from both BAC (Bacterial artificial chromosome) and enriched gDNA libraries and database sequences that contain SSRs were the sources for the sorghum SSRs mapped by Bhatramakki *et al.* (2000). Targeted isolation of SSR loci using BAC clones as proposed by Cregan *et al.* (1999) is likely to be the most efficient method for placing SSR loci in the segments. BT x 623 is the reference genotype used for sorghum molecular marker genotyping. It was the source of DNA used to construct the enriched libraries and the two sorghum BAC libraries that are currently available (Bhatramakki *et al.*, 2000). PCR primers for the amplification of DNA fragments containing SSRs from sorghum were successfully developed through three different approaches by Brown *et al.* (1996) and it was reported that sorghum fragments can be amplified using at least some maize SSR primers (Brown *et al.*, 1996).

Map location of 46 SSR loci (Taramino *et al.*, 1997) and (Kong *et al.*, 2000) and 113 novel SSR loci (including four SSR containing gene loci) (Bhatramakki *et al.*, 2000) have been reported to date. SSR markers have been incorporated in to an existing RFLP based map of (Xu *et al.*, 1994; Kong *et al.*, 1997) and the map of Peng *et al.* (1999) and Bhatramakki *et al.* (2000). Out of 85 polymorphic primers 62 nuclear and 4 geneic SSR (Reddy *et al.*, 2007) were used for map construction and localization of QTL for charcoal rot disease.

2.6.3 Parental polymorphism by SSR markers

The most important step in the construction of a linkage map is to identify DNA markers that reveal differences between parents (*i.e.* polymorphic markers). It is critical that sufficient polymorphism exists between parents in order to construct a linkage map (Young, 2001). In general, cross pollinating species possess higher levels of DNA polymorphism compared to inbreeding species; mapping in inbreeding species generally requires the selection of parents that are distinctly related. In many cases, parents that provide adequate polymorphism are selected on the basis of the level of genetic diversity between parents (Anderson *et al.*, 1993) in sorghum most of SSR loci are sufficiently polymorphic to be use-full in the MAS program (Kong *et al.*, 2000). The probability that two accessions in any working group will have different alleles at a locus range from 0.67 to 0.88 depending upon the group they belong.

2.6.4 QTL mapping

QTL mapping is the statistical study of the alleles that occur in a locus and the phenotypes (physical forms or traits) that they produce. Because most traits of interest are governed by more than one gene, defining and studying the entire locus of genes related to a trait gives hope of understanding what effect the genotype of an individual might have in the real world. Most agronomically important traits of crop plants have a complex inheritance pattern and are under the control of many genes. The genetic loci associated with complex traits are called quantitative trait loci (QTLs). QTLs identify a particular region of the genome as containing a gene that is associated with the trait being assayed or measured. They are shown as intervals across a chromosome, where the probability of association is plotted for each marker used in the mapping experiment.

The QTL techniques were developed in the late 1980s and can be performed on inbred strains of any species. The basic concept associating genetic marker with quantitative traits was first proposed by Sax (1923). Genetics have recognized the potential use of linkage between quantitative traits (QTLs) for studying the nature of quantitative genetic variation (Sax, 1923; Waxelson, 1923; Simth, 1937; Everson and Schaller, 1955; Thoday, 1961).

Traits controlled by these QTL are often strongly affected by environment. Because of this, the segregation pattern observed for such polygenic traits appear to deviate from the relatively simple patterns of Mendelian inheritance and hence the underlying gene controlling these traits are hard to trace. This limitation has been overcome by construction of highly saturated molecular map in many crop plants. The theoretical basis of interpreting the association of marker loci with QTLs has been provided by (Mather and Jinks, 1971)

The first report of linkage between a marker gene and a gene that influence quantitative trait loci was made by Sax (1923). An important subsequent attempt to map polygenes contributing to quantitative trait variation was reported by Thoday (1961). These early efforts contributed to the subsequent developments of the concepts of QTLs, which when combined with modern molecular marker technology (Soller and Beckman, 1983) and genetic linkage maps allowing mapping of QTLs.

QTL analysis is to look for association between the phenotypic variation in quantitative traits and the marker alleles segregating in the mapping population. Identifying a gene or QTL within a plant genome is like finding the proverbial needle in a haystack. However, QTL analysis can be used to divide the haystack in manageable piles and systematically search them. In simple terms, QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured (Tanksley, 1993). It has three essential stages: mapping of markers (genotyping), scoring of traits (phenotyping), and association of the traits with markers (QTL mapping). QTL analysis can be done by using statistical procedure like maximum likelihood ratio (Lander and Botstein, 1989) non-linear regression (Knap *et al.*, 1990) linear regression (Bridges *et al.*, 1991) or a combination of maximum likelihood and multiple regressions (Jansen and Stam, 1994). The first step in QTL mapping studies is to detect QTL, while minimizing the occurrence of false positive (type I error). Two distinct methods are used to detect QTLs; the single-marker analysis (Tanksley *et al.*, 1982) and interval mapping (Lander and Botstein, 1989). Once the QTLs are detected, the second step is to the genotypic effect of the QTL and to localize the QTLs to a precise genomic region. The development of molecular marker technologies and the use of these markers in detecting and mapping quantitative traits loci has become powerful approaches for studying the genetic and phenotypic bases of complex traits (Edwards *et al.*, 1987; Paterson *et al.*, 1988). If the individual genetic components associated with complex traits can be identified, then research can be focus on the function of each locus independently without the confounding effects of other segregating loci (Dorweiler *et al.*, 1993).

2.6.5 QTL mapping in sorghum

Tolerance to abiotic stress is genetically complex and highly influenced by environmental factors. Because of this, selection for improved abiotic stress tolerance is often difficult, and gains from selection are usually quite low. Drought tolerance is one such trait, and is especially important in sorghum, given its exceptional drought tolerance relative to other crops, and its importance in semi-arid parts of the world. The genetic and physiological mechanisms that condition the expression of drought tolerance in sorghum and other crops are still poorly understood, Controlled by many genes and dependent on the timing and severity of moisture stress, drought tolerance has been difficult to study and characterize. Identification of QTL and associated molecular markers, based on carefully managed replicated tests, should facilitate improvement of drought tolerance in sorghum, and also help to increase our understanding of the underlying physiological and genetic mechanisms controlling this complex trait in sorghum, with possible application in other related crops

Molecular markers have been used to identify and characterize QTL associated with many different traits in sorghum, including plant height and maturity (Pereira and Lee, 1995; Henzell *et al.*, 1992) traits associated with domestication (Paterson *et al.*, 1995), disease resistance (Gowda *et al.*, 1995; Wang *et al.*, 2006), insect resistance (Nagaraj *et al.*, 2005), and drought tolerance (Tuinstra *et al.*, 1996; 1997b; Wen *et al.*, 2002). Identification of QTL often leads to further investigations to identify the underlying gene or genes through fine mapping and map-based cloning. When successfully implemented, such studies provide valuable insight into the genetic mechanisms controlling complex, and often economically

important traits. However, from a practical plant breeding standpoint, QTL are usually identified for the purpose of finding linked molecular markers that can be utilized in trait introgression for crop improvement, and often the specific underlying genes are not identified.

Genetic linkage maps are fundamental for the localization of genes conferring biotic and abiotic stress tolerance. Linkage mapping in sorghum takes advantage of the plant's straightforward diploid genetics, amenability to inbreeding, and high levels of polymorphism between *Sorghum* species and adequate levels within *S. bicolor*. High-density reference maps of one intraspecific *S. bicolor* (Xu *et al.*, 1994; Bhattaramakki *et al.*, 2000; Klein *et al.*, 2000; Menz *et al.*, 2002) and one inter-specific *S. bicolor* x *S. propinquum* (Chittenden *et al.*, 1994; Bowers *et al.*, 2003) cross provide about 2,600 sequence tagged- sites (based on low-copy probes that have been sequenced), 2,454 AFLP and 1,375 sequence-scanned (based on sequences of genetically anchored BAC clones) loci. The two maps share one common parent (*S. bicolor* 'BTx623') and are essentially collinear (Feltus *et al.*, 2006). Cytological characterization of the individual sorghum chromosomes has provided a generally adopted numbering system (Kim *et al.*, 2005). More than 800 markers mapped in sorghum are derived from other taxa (hence serve as comparative anchors), and additional sorghum markers have been mapped directly in other taxa or can be plotted based on sequence similarity. Anchoring of the sorghum maps to those of rice (Paterson *et al.*, 1995a; Paterson *et al.*, 2004), maize (Whitkus *et al.*, 1992; Bowers *et al.*, 2003), sugarcane (Dufour *et al.*, 1997; Ming *et al.*, 1998), millet (Jessup *et al.*, 2003), switch grass (Missaoui *et al.*, 2005), Bermuda grass (Bethel *et al.*, 2006), and others provides for the cross-utilization of results to simultaneously advance knowledge of many important crops.

The linkage maps of sorghum have been employed in the "tagging" (mapping) of genes for a large number of diverse traits. The interspecific population has been especially useful for characterization of genes related to domestication, such as seed size, shattering (Paterson *et al.*, 1995a), tillering, and rhizomatousness (Paterson *et al.*, 1995b). Plant height and flowering time (Lin *et al.*, 1995; Ulanich *et al.*, 1996) have been a high priority. Resistance genes have been tagged for numerous diseases (Tao *et al.*, 1998; Singh *et al.*, 2006; reviewed in Sanchez , 2002), key insect pests (Tao *et al.*, 2003; Nagaraj *et al.*, 2005), and also the parasitic weed, striga (Hausmann *et al.*, 2004; Mutengwa *et al.*, 2005). Genes and QTLs have been identified that are related to abiotic stresses including post-reproductive stage drought tolerance (Stay-green) (Crasta *et al.*, 1999; Subudhi *et al.*, 2000; Xu *et al.*, 2000; Hausmann *et al.*, 2002), pre-harvest sprouting, (Lijavetzky *et al.*, 2000; Carrari *et al.*, 2003), and aluminum tolerance (Magalhaes *et al.*, 2004). Numerous additional morphological characteristics have also been mapped in interspecific and/or intraspecific populations (Feltus *et al.*, 2006).

Xu *et al.* (2000b) mapped QTLs controlling chlorophyll content in sorghum using a mapping population of 98 RILs derived from the cross B35 xTRx7000. They identified three QTLs for leaf chlorophyll content (ch11, ch12, and ch13) that together explained 25-30% of the observed phenotypic variability. The QTLs for chlorophyll content, were observed to overlap the above three stay-green QTLs present on the C and B linkage groups. The genomic regions corresponding to stg1 and stg2 contain an ABA-responding gene and genes for key photosynthetic enzymes and heat shock proteins.

QTLs conferring resistance to sorghum downy mildew (*Peronosclerospora sorghi*) and Rajasthan downy mildew (*P. heteropogoni*) have been mapped (Nair *et al.*, 2005). Sorghum midge resistance and green bug resistance mechanisms have been identified through QTL (Tao *et al.*, 2003). Comparative mapping between sorghum and sugarcane suggested the identification of useful markers for stalk number and suckering in sugarcane would be facilitated by focusing on sorghum QTLs associated with related traits (Jordan *et al.*, 2004). Similarly, resistance gene analysis in sugarcane and sorghum were studied and their association with QTLs for rust resistance was estimated (Mcintyre *et al.*, 2005).

QTLs affecting flooding tolerance and other physiological and morphological traits in *Echinochloa crusgalli* were analyzed. As a part of comparative mapping study between sugarcane and sorghum, a sugarcane cDNA clone with homology to maize Rp1-D rust resistance gene was mapped in sorghum (Mcintyre *et al.*, 2004). Molecular markers for resistance of sorghum to *Striga hermonthica* were mapped in two Recombinant Inbred Populations (RIP I and II) of F (3:5) lines developed from the crosses IS9830 x E36-1 (I) and N 13 x E36-1 (II). The genetic maps of RIP-I and RIP-II spanned 1498 cM and 1599 cM

respectively, with 137 and 157 markers distributed over 11 linkage groups. Composite interval mapping, detected 11 and 9 QTLs in sets 1 and 2 of RIP-I explaining 77.6 and 80 per cent of genetic variance respectively (Haussmann *et al.*, 2004).

Reddy *et al.* (2007) identify QTLs associated with stalk rot resistance in sorghum using 93 RILs derived from a cross between inbred lines IS22380 (susceptible) x E36-1(resistance). This population was genotyped with 62 nuclear and 4 genic SSR and 19 RAPD markers and linkage group was constructed using mapmaker/QTL software. They identify, for three parameters (percentage lodging, length of infection and number of internodes crossed), five QTL at Dharwad and 4 QTL at Bjapur locations on the linkage group A, B, D and I.

2.6.6 QTL mapping in stay-green

A number of physiological and biochemical traits have been hypothesized to enhance drought tolerance. Yet, only a few of these mechanisms have been demonstrated to be causally related to the expression of tolerance to drought under field conditions. As with *Striga* resistance, they have made a slow but significant progress via empirical breeding of sorghum for drought tolerance by breaking this complex trait into specific phenological stages of plant development, namely early-season (seedling), midseason (pre-flowering), and late-season (post-flowering) drought responses. Using this approach, scientist have identified sorghum germplasm that are uniquely pre-flowering or post-flowering drought tolerant and a few that combine tolerance at both stages. They have developed new improved drought tolerant sorghum lines in diverse and elite germplasm backgrounds.

The breeding and selection efforts were based on reliable phenotypic markers associated with morphological and yield-related symptoms that occur at pre-flowering and post flowering stages of plant development as long as moisture stress treatments are induced in a timely manner. Some of these marker traits are simply inherited, while others appear quantitative, rendering them amenable to QTL marker analysis and marker-assisted introgression. Molecular markers linked to QTL for drought tolerance could be used to increase the efficiency of breeding efforts to select sorghum germplasm with enhanced drought tolerance. Scientific Studies have been conducted using a number of RI population derived from a cross between T x 7078, which shows pre-flowering drought tolerance, and B35, which shows post-flowering tolerance. A genetic map was constructed using RAPD and RFLP markers and the RI lines were carefully evaluated for response to drought in a series of pre-flowering or post-flowering stress environments, both of which were in turn compared to fully irrigated controls. Pre-flowering drought tolerance was assessed by measuring grain yield under drought stress, yield stability (drought versus irrigated), seed set stability, and plant height stability. Single marker analysis identified six QTL associated specifically with pre-flowering drought tolerance.

Despite the fact that drought stress was much more severe in the first growing season, QTL by environmental effects were relatively small, suggesting that these QTL are stable across varying levels of drought stress or other confounding climatic factors. In addition, some markers showing significant association with yield or seed set under irrigated conditions also showed significance for drought tolerance (Tuinstra *et al.*, 1996). Assessment of post-flowering drought tolerance in sorghum by measuring grain yield under drought stress, yield stability, seed weight stability, and stay-green score was established in a study conducted under carefully managed drought stress conditions (Wanous *et al.*, 1999; Can and Yoshida (1999). The term 'stay-green' refers to delayed senescence which is associated with post-flowering drought tolerance irrespective of the maturity of the genotype. Twelve QTL for post-flowering drought tolerance were identified by single marker analysis (Tuinstra *et al.*, 1997b). Six QTL for stay-green were identified on five linkage groups. QTL on three of these linkages groups were also positively associated with grain yield under irrigated conditions. This indicates that there may be a physiological link between the expressions of Stay-green under post-flowering drought and grain yield under non-drought conditions. Because of the importance given to the stay-green trait as a readily visible and heritable drought response trait, several additional studies have been conducted to characterize and map this trait in various populations. Three of these studies (Crasta *et al.*, 1999; Xu *et al.*, 2000; Subudhi *et al.*, 2000) also used B35 as the source of the stay-green trait. The complex expression of drought tolerance makes stay-green trait difficult to study using traditional genetic and physiological methods. Use of molecular markers and QTL analysis of drought tolerance line grown in replicated and carefully induced drought stressed environments has led to a better

understanding of inheritance of this trait in sorghum (Tuinstra *et al.*, 1996, 1997a, 1998; Crasta *et al.*, 1999; Tao *et al.*, 2000; Xu *et al.*, 2000b; Subudhi *et al.*, 2000; Kebede *et al.*, 2001; Sanchez *et al.*, 2002; Haussmann *et al.*, 2002b). QTL studies for stay-green traits resulted in the identification of several genomic regions associated with post-flowering stress resistance.

In a recent review comparing these studies, Sanchez *et al.* (2002) named four major stay-green QTL derived from B35 that were consistently identified: *Stg1* and *Stg2* (chromosome 3), *Stg3* (chromosome 2) and *Stg4* (chromosome 5). Two of these, *Stg1* and *Stg3*, correspond to two QTL identified by Tuinstra *et al.* (1997b). A QTL for stay-green was also identified by Tao *et al.* (2000) at the same location as *Stg3* in a RI population derived from a cross between QL41 and QL39. QL41 is a stay-green line derived from B35 so this discovery is not surprising, however the other three QTL were not identified in this study, but several additional stay-green loci were found, some of which were associated with QL39. Using the stay-green line SC56, which is unrelated to B35, Kebede *et al.* (2001) found stay-green QTL at the same locations as *Stg1* and *Stg4*. They also detected a stay-green QTL on chromosome 1, which appears to correspond to a QTL found by Tuinstra *et al.* (1997b) and Crasta *et al.* (1999). These results indicate possible commonalities between stay-green mechanisms from diverse sources.

More recently Haussmann *et al.* (2002) analyzed the stay-green phenotype at various phenological stages by measuring the percentage of leaf area remaining green at weekly intervals (extrapolated to 15 d, 30 d, and 45 d) after flowering during drought conditions in two RI populations. The stay-green source line E36-1, which is unrelated to B35 and SC56, was common to both populations. Five to nine QTL were identified for each of the three time points in each population, and several of these overlapped, suggesting that some aspects of stay-green may be regulated in a stage-specific manner, while others are not. Three QTL derived from E36-1 were common to both populations. One QTL, which was previously identified in other studies with B35 (Tuinstra *et al.*, 1997b; Crasta *et al.*, 1999; Subudhi *et al.*, 2000) was detected on chromosome 2, though it is different from *Stg3*. Most other stay-green QTL found by Haussmann *et al.* (2002) appear to be unique to E36-1. From the results of multiple studies it is clear that there are several sources of this trait and likely several mechanisms by which sorghum can remain green and photosynthetically active during post-flowering drought stress. Some of these mechanisms may be common among the germplasm while others seem to be unique to a particular source. Thus, there appears to be great potential for the use of marker-assisted selection to pyramid multiple stay-green genes from these various sources into drought-tolerant breeding lines and cultivars.

Tuinstra *et al.* (1996) identified QTLs associated with pre-flowering drought tolerance in sorghum using 98 RILs derived from a cross between inbred lines RT x7078(pre-flowering drought tolerant, post-flowering drought sensitive) and B35 (pre-flowering drought sensitive, post-flowering tolerant). This population was genotyped with 150 RAPD and 20 RFLP markers and a linkage map was constructed using mapmaker/QTL. They identified six genomic regions associated with pre-flowering drought tolerance and also mapped eight additional regions generally associated with yield or yield components under fully irrigated condition. Using the same mapping population as above Tuinstra *et al.* (1997a) ; Bhat (1975) , identified components of grain development and post-flowering drought tolerance in sorghum. They identified 13 genomic regions associated with one or more measures of post-flowering drought tolerance. Two QTLs were identified with major effects on grain yield and stay-green under post-flowering drought stress condition. In this population, several different QTLs were associated with expression of stay-green trait. A stay-green QTL on linkage group F (SBI-09) was positively associated with grain yield under fully irrigated conditions and a stay-green QTL on linkage group G (SBI-10) was associated with grain yield under fully irrigated conditions, suggesting the tolerance mechanism controlling grain yield and stay green under post-flowering drought also influences grain yield under fully irrigated conditions when the differences in stay-green were not expressed. QTL analysis also indicated an association between the stay-green trait and the rate of grain development at a locus on linkage group H (SBI-08). The stay-green trait was associated with a lower rate of grain filling.

Crasta *et al.* (1999) using RILs derived from B35 x (Tx430), identified seven QTLs for stay-green trait. Three major stay-green QTLs (*StgA*, *StgD*, and *StgG*) contributed to 42% of the phenotypic variability (LOD 9.0) and the remaining four minor QTLs (*StgB*, *StgI.1*,

Stgl.2, and StgJ) contributed an additional 25% of the phenotypic variability in stay-green ratings.

Tao *et al.* (2000) identified three regions on separate linkage groups that were found to be associated with stay-green by using 160 RILs derived from a cross between QL39 and QL41, as a segregating population for genome mapping of stay-green trait. They added 118 additional markers to previously published linkage map (Tao *et al.*, 1998; Totad, 2005) by using same RILs population and identified a total of five genomic regions associated with the stay-green traits.

Xu *et al.* (2000b) mapped QTLs controlling stay-green and chlorophyll content in sorghum using a mapping population of 98 RILs derived from the cross B35 x TRx7000. They identified four stay-green (stg) QTLs located on three linkage groups. Two QTLs, Stg1 are located on linkage group C (SBI-03). The other two QTLs Stg3 and Stg 4 are located on linkage group B(SBI-02) and linkage group J (SBI-05) ,respectively of these QTLs Stg1 and Stg2 accounted for 13-20% and 20-30% , respectively, of the observed phenotypic variability for this trait. They also identified three QTLs for leaf chlorophyll content (ch11, ch12, and ch13) that together explained 25-30% of the observed phenotypic variability. The QTLs for chlorophyll content, were observed to overlap the above three stay-green QTLs present on the C and B linkage groups. The genomic regions corresponding to stg1 and stg2 contain an ABA-responding gene and genes for key photosynthetic enzymes and heat shock proteins.

Subudhi *et al.* (2000) assessed the consistency of QTLs controlling the stay-green trait in sorghum across several environments and genetic backgrounds. They evaluated the B35 x RT x 7000 derived RIL population. The earlier map Xu *et al.*, 2000b was expanded by the addition of 91 markers. They mapped four stay green QTLs and identified that there are partial similarities in case of the QTLs detected on LGC (SBI-03) and (LGB-02) of the B35 x RTx7000- derived population and B35 x Rt x 7078-derived population previously reported by Tuinstra *et al.* (1997a).

Kebede *et al.* (2001) identified QTLs associated with using post-flowering drought tolerance in sorghum using an F7 RIL population derived from cross SC56 x RT x 7000. Nine QTLs distributed across seven of the ten linkage groups, were detected for the stay-green trait in several environments using the composite interval mapping method. They also identified three stay green QTLs present on sorghum LG A (SBI-01), LG G (SBI-10) and LG J (SBI-05) that were consistently detected across different terminal drought stress environments. They conducted comparative mapping studies, identifying two of the sorghum stay-green QTLs identified in their study correspond to stay green QTLs detected in maize. In addition to these, QTLs responsible for sorghum lodging tolerance and pre-flowering drought tolerance were detected.

Sanchez *et al.* (2002) reported on four QTLs associated with the stay-green trait in sorghum using the RIL population derived from cross B35 x RT x 7000, which were reported on previously by Xu *et al.* (2000b) and Subudhi *et al.* (2000) ,and linkage maps were covered with RAPD, SSRs and RFLP markers. These four major QTLs reported by Crasta *et al.* (1999); Xu *et al.* (2000b), and Subudhi *et al.* (2000) were consistently identified in all field trials and accounted for 53.5% of the observed phenotypic variance for the stay-green trait.

Mahalakshmi and Bidinger (2002) evaluated a set of 72 diverse genotypes of sorghum for their Pattern of post-flowering leaf senescence under terminal drought condition to identify superior sources of the stay-green trait. Leaf senescence patterns were determined by fitting logistics or linear functions to the percentage of green leaf area (%GLA). They identified several tropically- adopted lines with stay-green expression equivalent to those of the best temperate –adopted lines (*viz.*, B35 and KS19).

Hausmann *et al.* (2002b) using two recombinant inbred population (RIPs); IS9830 x E36-1 and N13 x E36-1 (RIP2), identified QTLs, explaining 31% to 42% of the phenotypic variation for green leaf area percentage at 15, 30 and 45 days after flowering (E36-1 is stay-green source). The mode of action of QTLs for stay-green trait in their investigation ranged from purely additive to over dominance. Three QTLs on LG A (SBI-01), LG E (SBI-07) and LG G (SBI-10) were common to both RIP1 and RIP2. These three QTLs from donor parent E36-1, along with the QTLs from donor parent B35, are potential candidates for transfer of the stay green trait in to locally adopted elite sorghum materials.

In sorghum research, efforts are currently focused on fine mapping and transfer of the stay-green QTLs to elite non-stay-green sorghum lines through marker-assisted selection (MAS) (Subudhi *et al.*, 1999 and Hash *et al.*, 2003). Subudhi *et al.* (2000) reported that Stg2 and Stg3 interacted together most favorably explaining 49.8% of phenotypic variation, incorporation of which should improve stay-green expression.

2.7 STATISTICAL TECHNIQUES FOR QTL ANALYSIS

QTL analysis is to look for associations between the quantitative trait and the marked allele segregating in population. It has three essential stages: mapping of the markers (genotyping), scoring of the trait (phenotyping) and association of trait with markers (QTL-mapping).

The traditional approach for detecting a QTL in the vicinity of a marker involves studying single genetic markers one at a time (Soller and Brody, 1976; Tanksley *et al.*, 1982) and (Edwards *et al.*, 1987). However, if the QTL does not lie at the marker locus, its phenotypic effect diminishes relative to the true effect of the QTL as the distance (recombination frequency) increases between the marker locus and the QTL (Edwards *et al.*, 1987) and (Lander and Botstein, 1989). To overcome this, Knapp (1989) developed an approach, which utilizes pairs of markers in a sequential manner and estimates the phenotypic effect of the QTL and its significance in the region bracketed by the two markers in each pair. Lander and Botstein (1989) reported development of interval mapping method for QTLs, using 'Log of Odd ratios' (LOD) scores. An alternative approach using multiple regressions was developed by Haley and Kontt, (1992). It often produces very similar results and employs simple programming. Tests of significance and confidence intervals can be obtained by boot strapping approaches (Visscher *et al.*, 1996). However, it is reported by Tanksley *et al.*, (1982) that the statistical detection of QTLs is likely to depend not only on the type of population utilized, but also be on the intralocus and interlocus interactions of the segregating QTL.

For simple interval mapping the most widely used software is MAPMAKER. MAPMAKER is based on the concept of the LOD score (Morton, 1955). MAPMAKER/EXP is an interactive linkage analysis package initially developed by Lander *et al.* (1987) for constructing primary linkage maps of markers segregating in experimental crosses. MAPMAKER/EXP performs full multipoint linkage analysis for dominant, recessive (such as qualitative phenotypic marker) and co-dominant (such as RFLP) markers in BC, F₂ and F₃ (self) intercrosses, and recombinant inbred (RI) lines. MAPMAKER/QTL is a companion program to MAPMAKER/EXP. To apply a linkage map to QTL analysis, MAPMAKER has been modified to carry out QTL analysis using mathematical models and interfaces very much like the original program (Lander and Botstein, 1989). It uses the technique of interval mapping and simultaneous search. Interval mapping uses the power of a complete genetic map to discern weak effects from genetic distance between marker locus and putative QTL, while simultaneous search allows to fit multiple QTL across the genome at once, thus reducing overall non-genetic variance in the trait. Intervals between adjacent pairs of markers along a chromosome are scanned and the likelihood profile of a QTL being at any particular point in each interval is determined or to be more precise, the log of the ratio of the likelihood (LOD) of there being one QTL vs. no QTL at a particular point (Lander and Botstein, 1989). LOD score must exceed a value of threshold LOD value to be considered indicative of the presence of a QTL.

QTL Cartographer is a suite of programs for mapping quantitative trait loci onto a genetic linkage map. The general experimental paradigm begins with a pair of inbred parental lines that differ in the trait of interest and in the set of marker genotypes. The programs use linear regression, interval mapping (Lander and Botstein, 1989) composite interval mapping (CIM) (Zeng, 1993; Zeng, 1994) or multiple interval mapping methods to dissect the underlying genetics of the quantitative traits. Mapping is done onto a set of linked genetic markers with known recombination frequencies. Genetic linkage maps and data files can be imported from MAPMAKER. The mapping program uses a dynamic algorithm that allows a host of statistical models to be fitted and compared, including various gene actions (additive and dominance), QTL-environment interactions, and close linkage. QTL Cartographer mapping programs can handle data from backcrosses, intercrosses, double haploids and recombinant inbreds, as well as a few other experimental designs. It is a powerful package

that can be customizable for its various mapping functions (such as centimorgan, morgan and recombination distances) and statistical thresholds (such as permutation times and significance levels). The inclusion of appropriate markers as cofactors in multiple regression models removes the confounding effects of QTL placed outside the intervals being tested (Jansen, 1993). Compared to simple interval mapping (SIM; without cofactors), CIM provides an increased power to detect linked QTL (Zeng, 1994). Dao-hua he *et al.* (2005) did QTL analysis by QTL Cartographer software v2.0 using composite interval mapping method ($LOD \geq 3.0$). A total of 21 QTLs were identified for fiber yield and yield components in cotton, which were located on 15 linkage groups.

PLABQTL software, as described by Rami *et al.* (1998) can also perform CIM, but it differs from QTL Cartographer in by using regression-based methods for estimating QTL effects. QTL Cartographer uses a combination of maximum likelihood estimation similar to interval mapping and regression to control genetic background (Beavis, 1994).

For intended use of linkage information in marker assisted breeding, a program like Mapmanager (Manley and Cudmore, 1998) helps to keep of marker data in a population of interest. While, Hyper gene helps to display graphical genotypes (Young and Tanksley, 1989). The program qGENE seeks to bring all of these important DNA marker tools together into a single package (Nelson, 1997). Despite considerable effort by statisticians, all methods of analysis yield essentially similar QTL locations and gene effects, while there is some slight variation in the confidence intervals.

3. MATERIAL AND METHODS

The present investigations were carried out at the Institute of Agri-Biotechnology (IABT) and Main Agricultural Research Station, UAS, Dharwad. The details of material used and the methodology adopted in the present investigation are described below.

3.1 PLANT MATERIAL

Two recombinant inbred populations (RIPs) of sorghum, each consisting of 226 F_8 lines, were developed from the crosses IS9830 x E36-1 (RIP1) and N13 x E36-1 (RIP2). The line E36-1 is a donor for stay-green traits in these RIPs, is a high yielding breeding line assigned to guinea-caudatum hybrid race with Ethiopian origin. Line IS9830 is a tall Sudanese feteria belonging to the caudatum race. The line N13 is durra sorghum from India. The populations were developed at ICRISAT, Hyderabad, Patancheru, India and maintained by IABT, consisted of 226 RILs each in F_8 generation. Of the three parents, IS9830 and N-13 are sensitive to moisture stress, susceptible to charcoal rot and non-stay-green type while E36-1, a donor for stay-green, origin of Ethiopia, well adapted to tropics and also resistant to charcoal rot and lodging (Hausmann, 2004). The mapping population was phenotyped in the field for stay green and other drought related traits at the Main Agricultural Research Station (MARS), Dharwad during *rabi* season, 2007/08. Previously mapped markers in IABT laboratory were used as sources of genotypic data in the same RIP, to anchor the new markers.

3.2 EVALUATION OF RILs FOR STAY-GREEN AND OTHER DROUGHT RELATED TRAITS

Two recombinant inbred populations (RIPs) of sorghum, each consisting of 226 RILs in F_8 generations were utilized for phenotyping. The seed materials sown on October 25/2007 as *rabi* season crop with spacing of 45 x 15 cm and all other recommended agronomic practices were followed. Each RIPs sown together with the two respective parental lines in a 19 x 12 simple lattice design with four replications. Each entry had a single row with 15 plants. To eliminate the border effect three rows of M35-1 and SPV86 were sown on all sides of the field. Plots were thinned at 10 days after seedling emergence (DAE) to a spacing of 15 cm between plants within rows when the seedlings were at 4 leaf stage. The crops were protected from both leaf feeding insect pests and stem borers using appropriate pesticides.

3.3 OBSERVATIONS OF COMPONENTS TRAITS OF DROUGHT RESISTANCE

3.3.1 Estimation of senescence

At the time of emergence of flag leaf, three representative plants in each plot were tagged, the length and width of the upper six leaves were measured, and the area of each estimated as: leaf length x leaf width x 0.7 (The 0.7 factor was determined by measuring the leaf length, breadth and, actual area of 50 randomly chosen leaves). Beginning of at flag-leaf emergence, the percentage of each of the upper six leaves of each tagged plant remaining green was visually estimated at weekly intervals according to Mahalakshmi and Bidinger (2002) with little modification. The green leaf area (GLA) of each tagged plant was computed by multiplying the percent green-leaf area by measured area of each leaf and summing across the six measured leaves. The percentage of green leaf area (%GLA) for each plant, for each week, was calculated by dividing the estimated GLA for that week by its measured leaf area at flowering. Plot value for %GLA was derived by averaging the three individual plant values for each plot. The weekly % GLA data were used to fit an appropriate equation to describe the pattern of leaf senescence during the period of observations. The fitted equations for each individual plot were used to estimate the %GL at 15, 30 and 45 days after flowering of the individual entries (%GLA15, %GLA30 and %GLA45) respectively.

3.3.2 Observations of other drought related traits

Other than stay-green traits, data for different phenotypic characteristics were collected as per the research plan during 2007/2008 post rainy *rabi* season. Traits assessed

in the two trials included the plant height at full maturity, days to 50% flowering, head length, panicle exertion, stem thickness, number of spikelet per head, seed colour, 100 seed weight, glum colour, glume cover, number of leaves per plant at full maturity stage, inflorescence type(panicle type), own type, and yield per plant, assessed as described below.

Plant height: Plant height was measured at fully plant maturity time from the surface of the soil to the tip of plant as described in descriptor for sorghum (IBPGR and ICRISAT, 1993).

Days to 50% flowering: The time interval (days) from sowing to anthesis in 50 percent of the plants was recorded as days to 50 % flowering.

Stem thickness: This was measured at the middle of first inter node above the ground level at the time of anthesis with a thread and expressed as circumference in cm.

Seed colour: The distributions of different seed colour *per se* were recorded based on MUNSULE colour chart after harvesting. The colour chart has a range of different colour and the seed colour was recorded when the colour falls in the given chart range out of the total colour range.

Seed yield per plant: Weight of dried seeds in grams (g) from each plant was recorded at the time of harvest.

3.4 MOLECULAR MARKER ANALYSIS

3.4.1 Genomic DNA preparation from sorghum leaves

DNA was extracted from a bulk of 6 to 7 individual plants of each entry. Three to four week old seedling were collected from field and frozen in liquid nitrogen and stored at -80°C till use. DNA was prepared following Krishna and Jawali (1997) method with minor modifications as follows:

1. About 2 g of frozen tissue sample was ground to fine powder in liquid nitrogen, using autoclaved mortar and pestle.
2. The ground tissue was transferred to 2ml eppendorff tube containing 900µl of extraction buffer.
3. The contents were mixed well and incubated at 65°C for 40 min.
4. The contents were then cooled on room temperature for 10 min.
5. Equal volume of Phenol: chlorophorm: iso-amylalchol (PCI) was added in the ratio of 25:24:1 and mixed thoroughly.
6. The contents were spin for 15 min at 13000rpm at 4°C.
7. About 600 µl of supernatant was transferred to fresh tube and remaining was discarded.
8. Equal volume of phenol-chlorophorm was added to the content in the ratio of 24:1
9. About 600 µl of supernatant was transferred to fresh tube and remaining was discarded.
10. About 600 µl of isopropanol ammonium acetate mixture was added to supernatant to precipitate nucleic acids and kept in -20°C refrigerator overnight.
11. The contents were mixed thoroughly and centrifuged for 20 min. at 1300 rpm to pellet the nucleic acids.
12. The supernatant was discarded and the pellet was washed with 70 percent alcohol, tubes were inverted on blotting paper to dry the pellet.
13. Pellet was dissolved in 100 µl of T₁₀E₁.
14. RNAase (5 µl) (10mg/ml) was added to each tube. DNA was re-dissolved by tapping the pellet suspended in RNAase and incubated at room temperature (37°C) for 30 min.
15. The DNA was precipitated by adding 1/10th volume of 3M sodium acetate and 2.5 volumes of absolute ethanol.
16. The contents were mixed gently and incubated at 4°C for 30 min.
17. The contents were centrifuged for 1 min at 3000rpm.

18. DNA pellet was rinsed with 70 percent ethanol twice and centrifuged at 3000 rpm for 1 min.
19. Supernatant was discarded and tubes were put in drier to get rid of excess ethanol.
20. Pellet was dissolved in 100 µl of T₁₀E₁.

3.4.2 Genomic DNA Purification

1. Equal volume (100 µl) of phenol: chloroform: iso-amylalcohol (25:24:1) mixture was added to each tube and the content were mixed by inverting.
2. The contents were spun at 2500 rpm for 10 min and supernatant was transferred to fresh tubes.

Step 1 and 2 were repeated.

3. To the supernatant above , 10 µl of 3M NaOH, (1/10 volume of aqueous layer) and 2.5 volume of chilled absolute ethanol was added, mixed gently and incubated at -20°C for 15-20min.
4. The DNA was spooled in 1.5 ml eppendorf tubes using glass hook, washed with 70% alcohol and dried in vacuum.
5. DNA was dissolved in 100 µl T₁₀E₁ and kept -20° C till its further use.

The details on preparation of solutions and buffers are presented in Appendix.

3.4.3 Determination of quantity and quality of isolated genomic DNA by Nano-drop spectrophotometer

1. Initialization of the machine was done by the help of NANO- pure water.
2. The machine was set to blank by loading 3 µl T₁₀E₁ with which the DNA sample was dissolved already.
3. The quantity of the DNA was measured by Loading 3 µl DNA sample on NANO drop spectrophotometer.
4. The machine gives the DNA quantity in ng/ µl and OD value. Accordingly, using quantified DNA sample, dilution of DNA sample was done to the required DNA concentration for further use. In each step the machine was cleaned with help of tissue paper.

3.5 PARENTAL POLYMORPHISM WITH GENIC(EST-SSR) AND NUCLEAR SSRS

Initial screening of the parents was carried out for parental polymorphism before genotyping RILs. For this, parental DNA from IS9830 (P1) and E36-1 (P2) were subjected to PCR amplification with genic and nuclear SSR. Thus, parents were screened with 530 genic and 75 nuclear SSRs marker, of which 37(7.35%) genic and 8(11.11%) nuclear SSR were detected polymorphism on 2.5% agarose gel and 6% polyacridmine gel (PAGE). The information sources of genic SSRs marker were from Taramino *et al.*, (1997) and Bhatramakki *et al.*, (2000) set of SSR marker for sorghum. Genic SSRs used in the present investigation are a subset derived from sorghum expressed sequence tag (ESTs) database, recently developed in IABT laboratory. Further an in-house annotated ESTs containing SSRs database was searched for the ESTs having similarity to the known drought and resistance related genes. About 10 such sequences were available in the database and the primers were designed for the repeats present in them with an optimal T_m of 60°C (57-65°C); GC content of 30-70% with low chance of dimer or hair loop formation and with 100-300bp amplicon size. The primers so designed were used for parental polymorphism and then to RILs genotyping.

The experimental protocol used for these studies were;

3.5.1 PCR reaction conditions for the amplification of marker loci

Amplification reaction mixture for genic and genomic SSRs was prepared in 0.2ml thin walled PCR strips or tubes containing following components in a total volume of 25µl for parental polymorphism and 12µl for genotyping.

Genic SSR

Components	Quantity (µl/reaction)
Genomic DNA	2.0
dNTPs mix (2.5mM each)	1.0
Primer (10pM/µl)(Forward and Reverses)	2.0(one each for F & R)
10X assay buffer	2.5
Taq DNA polymerase (in-house)	0.5
Sterile water	17.0
Total	25µl

PCR reactions were carried out using Master cycler gradient 5331-Eppendorf version 2.30.

31-09, Germany. The cycler was programmed as follows, for genomic and genic SSRs amplification

Sl. No.	Step	Temperature (°C)	Duration (min)	Number of cycles
1.	Initial denaturation	94	4	1
2.	Final denaturation	94	1	36
3.	Annealing	42-62	2	
4.	Extension	72	2	
5.	Final Extension	72	10	1
6	Store	4	Until removed	

Annealing temperatures were adjusted depending on the primer used. After the completion of PCR, the products were stored at 4 °C until the gel electrophoresis was done.

3.5.2 Polyacrylmide gel electrophoresis (PAGE)

Six per cent denaturing polyacrylmide gels were used for separation and visualization of PCR amplified microsatellite products. Denaturing gels were cast in sequin-Gen GT nucleic acid electrophoresis cell (Biorad Ltd) as per the protocol in manual published by MRF Hyderabad. Glass plates were prepared before making the gel solution. Both outer (large) glass (IPC unit) and inner (Small) glass plate were cleaned thoroughly with warm water and detergent. Second wash was given with deionised water.

Fresh binding solution was prepared in fume hood by adding 4µl of bind silane to 1ml of 0.5 percent acetic acid in 95 per cent ethanol in a 1.5ml micro-centrifuge tube. Mixture was poured to notched (inner glass plate) and spread with tissue paper over the entire surface of the plate. The treated side was demarcated to distinguish between untreated and treated side. Repel silane of about 250µl was added to 750µl of 0.5 acetic acid in 95 per cent ethanol

in a 1.5 ml micro centrifuge tube. Mixture was poured on to large glass plates and spread using tissue paper.

Spacers of 0.4 mm thickness were placed along the side edges of the bind silane-treated surface (small glass plates). Large plate was put on small plate so that treated surface faced each other and they were clamped on both sides and the assembly was placed in a precision caster base for sealing both sides to ensure no leakage from bottom or sides. For casting each gel, 80ml of 6 per cent acrylmide gel was prepared, just prior to pouring. For each 80ml of solution, 60 μ l of TEMED (N, N, N' N'-teteramethylethylenediamine) and 600 μ l of 10 per cent APS (ammonium per sulphate) was added to initiate the polymerization process. The contents were mixed gently by swirling and bubbles were avoided before pouring, the assembly was kept on the bench top so that it made 45-degreeangle. Assembly was tilted so that one of the bottom corners was raised above the other and then the solution was carefully poured in to the space between the glass plates, starting at the lower corner. As the acrylmidine filled the space; gel assembly was lowered so that both bottom corners were on the bench, parallel to the bench top.

Shark toothcomb of 0.4mm thickness (49wells) was inserted with straight side facing the gel at the top of the gel. Comb was put straight across the top moving not more than 5mm of notched plate. If bubbles were formed during pouring, they were dislodged by tapping. Gel was left for 20 to 40 minutes for complete polymerization.

Electrophoresis: After the polymerization process, assembly was detached from the clamp and precision caster base and it was then placed in universal base against the back wall. IPC was locked to the base in vertical position by fitting stabilizer bar.

1. Tris free base, boric acid and EDTA (TBE) (5x) was poured in the upper tank with IPC unit and the rest was poured in the bottom chamber (1.8L of buffer was prepared fresh each time).
2. Comb was removed and excess polyacrylmide gel was removed with razor blade. Tissue paper was used to clean the glass with buffer.
3. Air bubbles and unpolymerised acrylmidine on the top of gel were removed by squirting with 5 per cent TBE by using syringe.
4. Pre-run was (50-60min) given to chive gel surface temperature of 45 to 50°C with following conditions. Temperature 45-50° C, voltage 2000V, 50mA, constant power of 80W were used.
5. SSR loading dye (3x STR dye) was added to PCR products to final of 1% and samples were denatured by heating to 95°C for 4 min and immediately cooled on ice.
6. After the pre-run, urea was flushed from the well area using a transfer pipette and carefully inserted the shark tooth comb in to the gel so that teeth were just touching the surface of gel. Care was taken to avoid the piercing of gel too deeply.
7. 3 μ l of each sample was loaded in to the wells for separation of various alleles. Reference DNA ladder (100bp) was loaded in the first or last well after denaturing.
8. Gel was run using the same conditions as in step 1. It was electrophoresed till the tracking dye reached bottom of the gel.

Visualization of SSR bands

After electrophoresis, clamps were loosened and buffer was removed. Glass plates were separated using plastic wedge at the right corner. The gel was affixed to small glass plate. DNA fragments separated were detected following silver staining. Each solution was prepared in separate containers. The same solutions were used four times over a period of 48hrs. except for developer; which was freshly prepared during the staining process.

Silver staining

1. Gel was rinsed with distilled water for 3 to 5 min and placed in a suitable shallow depth plastic tray.
2. Gel was soaked in 2L of one per cent acetic acid (fix solution) for 20 min.

3. The gel was rinsed with water 2 times, each with 2-3min.
4. The gel was kept for staining with 2 L of one per cent silver nitrate for 20 min.
5. A quick water wash was given for 10-15sec.
6. Developer solution was added to the tray and gently rocked until the bands appeared.
7. Developer was removed and the plate was placed in fixer of the stop solution for 5min.
8. Gel was placed in 2L of impregnate solution for 15 min.
9. Lastly, gel was washed with water for 5 min and kept for drying overnight.

All the steps were carried out under constant shaking conditions.

3.6 GENOTYPING RILs WITH GENIC AND NUCLEAR SSRs:

Mapping population consisting of 226 RILs and parents were screened with polymorphic genic SSR markers based on parental survey.

Scoring SSRs alleles and genotyping

The banding pattern obtained after silver staining were scored as follows.

1. Homozygote for the allele from the parental strain P1 (N-13) at locus as A=1
2. Homozygote for the allele for parental strain P2 (E36-1) at the locus as B=3
3. Heterozygote carrying the alleles from both P1 and P2 strains H=2
4. Off types showing banding pattern different from their parents and missing data for the individual at the locus =0

After scoring the individual progeny the data was entered in Microsoft Excel spread sheet in format suitable for linkage analysis by MAPMAKER/EXP (3.0b) and mapmaker/ QTL (1.1b) software.

3.7 STATISTICAL ANALYSIS

3.7.1 Phenotypic characters

Analysis of variance (ANOVA): the analysis of variance for different characters was performed as in Burton and Devane (1953).

Phenotypic and genotypic coefficient of variation: the estimates of phenotypic and genotypic coefficient of variation were obtained by using the following formula (Singh and Chudhary, 1977).

$$PCV (\%) = \frac{\sqrt{VP}}{\bar{X}} \times 100$$

$$GCV (\%) = \frac{\sqrt{Vg}}{\bar{X}} \times 100$$

Where,

PCV - Phenotypic Coefficient of Variation

VP- Phenotypic Variance

GCV- Genotypic Coefficient of Variance

Vg - Genotypic variance

$\bar{X}$ - Mean of RIL's

GCV and PCV values were categorized as low, moderate and high values as indicated by Sivasubramanian and Menon (1973) as follows.

0-10% - Low

10-20% - Moderate

20% and above High

Phenotypic correlation: phenotypic correlation coefficients were estimated by using the following formula (Singh and Choudhary, 1977).

$$r_p = \frac{\text{CoVp}(X,Y)}{\sqrt{\text{Varp}(x) \text{Varp}(y)}}$$

Where,

r_p -Phenotypic correlation coefficient

CoVp (x, y) - Phenotypic covariance between characters x, y

$\text{Var}_p(x)$ - Phenotypic variance in character x

$\text{Var}_p(y)$ -Phenotypic variance in character y

The observed value of correlation coefficient was compared with the tabulated value for (n-2) degree of freedom for test of significance.

Heritability: it was estimated in RILs for all resistance components as well as the ratio of total genotypic variance to the phenotypic variance, Falconer (1989).

$$H^2 = \frac{V_g}{V_p} \times 100$$

Where, H - %Heritability coefficient

V_g -Genotypic variance

V_p - Phenotypic variance

The heritability percentage categorized as low, moderate and high as given by Robinson *et al.* (1949).

0-30- low

30-60% -moderate

60% and above high

Genetic advance: The extent of genetic advance expected through selection for each of the character was calculated as in Johnson *et al.* (1955).

$$GA (\%) = H \times P \times K$$

Where, H- Heritability

P-Phenotypic standard deviation

K-selection differential (2.06 at 5%)

Genetic advance as per cent of mean (GAM)

$$GA \text{ as per cent mean} = (GA/\bar{X}) \times 100$$

Where, GA-Genetic advance

$\bar{X}$ - General mean

Genetic advance as per cent of mean was categorized as low, moderate and high by following. Johnson *et al.* (1995) as follows.

0-10% - Low

10-20%- Moderate

Above 20%- High

3.8 MARKER DATA ANALYSIS

3.8.1 Marker segregation

Chi-square test was performed to check for segregation of marker alleles of each locus. The calculated χ^2 values were compared with table values at one degree of freedom for each marker locus. The marker status of any of the 226 RILs and parents for each locus was scored either as P_1 or P_2 allele.

3.8.2 Construction of genetic linkage map

A computer package, MAPMAKER/ EXP version 3.0b (Lander and Botstein, 1989) was used for construction of linkage frame. The 'group' command with a minimum LOD scores 2.5 and maximum distance of 30 per cent recombination was used. Multipoint linkage analysis on loci with groups was subsequently performed. For each group, three-point linkage analysis was conducted followed by order command. After all scores were checked, the framework command for each declared linkage group was used to construct the final map. The SSR marker (40) map in IS980 x E36-1 RILs population made by Haussmann (2002) was used as reference map for assigning markers to definite linkage groups using computer package MAPMAKER /EXP version 3.0b, as described above. Thus the data for total of 16 genic and 4 nuclear SSRs markers were used to construct a genetic map for the RILs population IS9830 x E36-1.

Linkage distance in terms of centi- morgan (cM) values was calculated using Kosambi's mapping function. The genic SSR markers used in this study were designed at IABT and used for genotyping RIL populations IS9830 x E36-1. As the genic –SSR markers used in this study are new and not mapped previously, set of markers was kindly provided by Hash (ICRISAT). Accordingly, interval-mapping technique was adopted for the construction of linkage groups and QTL analysis (Haussman *et al.*, 2002).

3.8.3 QTL analysis

MAPMAKER/EXP performs full multipoint linkage analysis for dominant and co-dominant markers in a mapping population. The output of MAPMAKER/EXP contains information on linkage groups and linkage distances between the markers on each linkage group are included as input file for QTL cartographer (2.5b). QTL cartographer is suit of programs for mapping quantitative trait loci (QTLs) on to a genetic linkage map. The QTLs identified at a cut off LOD of 2.5 for components of resistance were assigned groups based on linkage positions of markers on respective linkage maps.

4. EXPERIMENTAL RESULTS

The results of the present investigation are presented related to under the following headings

1. Phenotyping of two recombinant inbred populations (RIPs) for stay-green, other drought tolerance traits, yield and yield components
2. Molecular mapping of selected EST-SSR markers
3. Identification of QTLs for stay-green, other drought tolerant traits, yield and yield components

4.1 PHENOTYPING OF RIPs FOR STAY-GREEN AND OTHER DROUGHT RELATED TRAITS, YIELD AND YIELD COMPONENTS

The recombinant inbred lines developed from two crosses viz. IS9830 x E36-1(RIP I) and N13 x E36-1(RIP II) along with their parental lines were evaluated during 2007/08 post rainy (*rabi*) season at Main Agricultural Research Station (MARS), Dharwad. The analysis of variance (ANOVA), summaries of mean performance and correlation of stay-green and other related traits were computed. Analysis of variance showed significant ($P \leq 0.05$) and highly significant differences among the RILs ($P \leq 0.001$) for all 13 characters considered in this study. The present investigation revealed considerable amount of variation for all the 13 characters studied. The extent of variability in respect of range, mean, phenotypic coefficients of variation (PCV), genotypic coefficients of variation (GCV), heritability and genetic advance are presented in the Tables (1-8). The details of frequency distribution, patterns of RILs with respect to stay-green and other drought tolerance traits are graphically presented in fig. 1 to 7, for both the populations.

Quantitative characteristics considered for evaluation of stay-green and other related traits were green leaf area percentage 15, 30 and 45 days after flowering (%GLA15, %GLA30, and %GLA45), number of spikelets per plant, head extension length (cm), plant height(cm), head length(cm), number of leaves per plant, stem thickness (cm) and yield/plant (g). Similarly high degrees of variability was observed in qualitative characteristics viz, seed colour, panicle type, glume colour and glume covers among the RILs of both population and their results of present investigations are presented graphically.

4.1.1 Stay-green

The frequency distribution of the three stay-green traits in RIP I and RIP II were unimodal and approximately normal fig 1 and 2 and showed highly significant differences among RILs in both populations (Table 1 & 2). Transgressive segregation occurred in both RIPs for all measured stay-green traits. During early stage of the plant it may be noticed that on average, RIP I flowered relatively about 2 weeks earlier than RIP II. The mean percentage green leaf area decreased from 84% at 15 days after flowering to 38-44% at 45 days after flowering in both RIPs. Genetically the variation among F_8 lines within each RIPs was highly significant for all the 13 traits, studied.

In both RIPs, PCV and GCV were high for %GLA30 and %GLA45. Significant positive correlation was observed between stay-green traits and yield in both RIPs at genotypic level. Per cent of green leaf area at 15 days after flowering (%GLA15) was positive and significantly correlated to all 13 traits studied except for panicle exertion and days to 50% flowering. Per cent GLA30 (%GLA30) was negatively correlated with panicle exertion and days to 50% flowering, and positive and significantly correlated to the all other traits (Tables 5&6 for RIP I and Tables 7&8 for RIP II). Percentage green leaf area at 15, 30, and 45 days after flowering, were highly heritable in both RIPs. In addition to stay-green character, plant height, head exertion, stem thickness, head length, spikelets number per plant, leaves number per plant and 100 seed weight were highly heritable while yield was moderately heritable.

Table 1. ANOVA for stay-green, yield and yield component traits in RILs derived from IS9830 x E36-1(RIP I) cross at MARS, Dharwad

Sources of variation	Mean sum of squares													
Component s	DF	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13
Treatments	224	104*	710.6**	439.4**	2406.9**	1.42**	103.18*	473.2**	275.2**	3.9**	0.36**	60.14**	501.2**	253461.75**
Error	448	11.75	12.4	23.04	124.12	0.84	3.478	6.17	3.1	0.31	0.16	5.13	18.73	48215.84

Table 2. ANOVA for stay-green, yield and yield component traits in RILs derived from N13 x E36-1(RIP II) cross at MARS, Dharwad

Sources of variation	Mean sum of squares													
Component s	DF	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13
Treatments	224	290.49**	540**	143.6**	2810.5**	29.1**	82.7**	29.8**	290.4**	2.97**	0.73**	109.8**	318. **	251461**
Error	448	31.4	16.3	25.3	800.4	6.2	1.3	6.2	23.4	0.62	0.31	3.5	68.8	48215

X1: %GLA15
X2: %GLA30
X3: %GLA45
X4: Plant height (cm)
X5: Stem thickness (cm)

X 6: Panicle exertion (cm)
X7: Head length (cm)
X8: No. of spikelets/ plant
X9: No. of leaves /plant

X10: 100 seed weight (g)
X11: Days to 50 % flowering
X12: Grain yield (g)
X13: Green leaf area (cm²)

Table 3. Genetic variability for stay-green, yield and yield component traits in RILs derived from IS9830 x E36-1(RIP I) cross

Sl. No	Characters	P1	P2	Range	Mean $\pm$ S.Em.	GCV (%)	PCV (%)	Heritability (%)	GA (%)
1	%GLA15	81.00	95.00	76.0-95.0	83.17 $\pm$ 0.29	5.72	7.01	66.77	6.86
2	%GLA30	44.00	84.00	22.2-89.50	64.78 $\pm$ 0.98	20.71	21.43	93.4	41.91
3	%GLA45	30.98	64.00	12.2-70.0	36.6 $\pm$ 0.78	22.87	25.27	81.87	39.55
4	GLA at flowering(cm ²)	569.22	1511.50	515-1953.68	1158.3 $\pm$ 11.69	19.56	27.24	51.55	22.29
5	Plant height(cm)	160.00	174.00	117.0-257.0	174.0 $\pm$ 1.82	13.74	15.16	82.87	23.86
6	Stem thickness(cm)	3.30	6.00	2.2-7.2	4.65 $\pm$ 514	11.96	15.10	62.75	18.89
7	Panicle exertion(cm)	24.80	7.00	3.1-29.0	14.78 $\pm$ 0.38	33.79	36.05	87.55	58.71
8	Head length (cm)	24.22	25.27	11.4-27.0	18.0 $\pm$ 0.12	12.4	16 .89	94.9	65.06
9	No. of spikelets/head	34.40	53.00	19.5-59.0	37.1 $\pm$ 0.62	22.35	22.76	95.42	44.00
10	No. of leaves/plant	7.00	8.00	3.8-9.0	7 $\pm$ 0.07	14.4	16.75	73.88	22.64
11	100 seed weight(g)	2.67	3.87	2.6-4.8	3.71 $\pm$ 0.03	15.99	18.58	72.65	23.77
12	Days to 50% flowering	67.00	59.00	47.0-70.0	58.0 $\pm$ 0.45	6.39	7.49	52.78	15.80
13	Yield/plant(g)	34.10	148.1	34.7 -180	105.4 $\pm$ 2.11	30.28	32.550	56.55	26.3

%GLA15: Per cent of green leaf area at 15 days after flowering

%GLA30: Per cent of green leaf area at 30 days after flowering

%GLA45: Per cent of green leaf area at 45 days after flowering

P1=IS9830, P2=E36-1

Table 4. Genetic variability for stay-green, yield and yield component trait in RILs derived from N13 x E36-1(RIP II) cross

Sl. No	Characters	P1	P2	Range	Mean $\pm$ S.Em.	GCV (%)	PCV (%)	Heritability (%)	GA (%)
1	%GLA15	82.20	95.00	57.0-99.00	84.3 $\pm$ 0.43	9.71	11.82	67.35	10.24
2	%GLA30	62.00	84.00	22.0-90.00	73.0 $\pm$ 0.67	14.62	15.50	88.93	25.17
3	%GLA45	44.60	65.20	7.7-81.50	44 $\pm$ 1.30	30.17	31.23	93.31	33.00
4	GLA at flowering(cm ²)	569.30	1512.4	675.1-1993.40	1282.01 $\pm$ 17.7	18.3	22.30	67.20	26.7
5	Plant height(cm)	178.00	174.0	117-267	183 $\pm$ 1.90	12.36	13.90	61.56	26.00
6	Stem thickness(cm)	45.00	6.70	2.8-6.20	3.2.05 $\pm$ 0.06	13.46	19.26	48.87	17.00
7	Panicle exertion(cm)	12.700	7.50	2.7-23.40	12.2 $\pm$ 0.30	36.72	37.90	93.86	20.70
8	No. of leaves/plant	6.80	8.50	5.2-10.22	7.3 $\pm$ 0.60	10.46	11.95	48.99	8.80
9	No. of spikelets/head	45.00	53.10	19.0-66.00	41.7 $\pm$ 0.50	19.69	22.88	74.06	11.21
10	100seed weight(g)	3.10	4.10	2.5-4.70	3.6 $\pm$ 0.01	10.35	11.26	76.24	23.00
11	Head length (cm)	22.60	25.26	9.7-27.60	17.8 $\pm$ 0.13	30.22	32.33	94.98	35.00
12	Days to 50% flowering	68.00	62.00	50-72	62.1 $\pm$ 0.40	8.22	8.75	58.27	16.49
13	Yield/plant(g)	60.00	150.00	44.0-181.0	108.1 $\pm$ 0.60	20.7	22.99	47.65	22.00

%GLA15: Per cent of green leaf area at 15 days after flowering

%GLA30: Per cent of green leaf area at 30 days after flowering

%GLA45: Per cent of green leaf area at 45 days after flowering

P1=IS9830, P2=E36-1

4.1.1.1 Per cent of green leaf area at 15 days after flowering (%GLA 15)

Wide variation was observed for percentage green leaf area at 15 days after flowering (%GLA15) where the mean value among the RILs ranged between 76 and 95 percent with over all mean 83.30 per cent. The genotypic and phenotypic coefficients of variation were 5.72 and 7.01 per cent respectively. The heritability estimate was high at 66.77 per cent with an expected genetic advance of 6.89 per cent. At this stage, slight difference was observed between the two parents, IS9830 (81%) and E36-1(95%) in green leaf area. In RIP II, the pattern of variations observed were similar to that of RIP I ; However, more variations were observed in RIP II as compared to RIP I in per cent green leaf area at 15 days after flowering (%GLA15), where the mean value ranged from 57 to 99 percent with over all mean value of 84.3 per cent. The genotypic and phenotypic coefficient of variation was 9.71 and 11.82 per cent respectively. Estimated heritability value for this trait in RIP II was high at 67.35 per cent with an expected genetic advance of 8.48 percent.

4.1.1.2 Per cent of green leaf area at 30 days after flowering (% GLA30)

In RIPI the mean value ranged between 22.22 and 89.5 per cent. The phenotypic coefficients (PCV) and genotypic coefficient of variation (GCV) recorded for this trait was 21.43 and 20.71 per cent respectively having very high heritability of 93.4 per cent with expected genetic advance of 41.9 per cent. At 30 days after flowering, there was great variation for weighted per cent green leaf area among parents of IS9830 (44%) and E36-1(82%). In RIP II, high variation ranged from 22 to 90 per cent of green leaf area at 30 days at flowering (GLA30) with over all mean of 73 per cent was recorded. The GCV and PCV were 14.62 and 15.5 respectively, with high heritability of 88.93 per cent and expected genetic advance of 19.69 per cent.

4.1.1.3 Per cent of green leaf area 45 days after flowering (%GLA45)

A very wide range of variation from 30.18 to 70.6 percent for %GLA among the RILs with a mean of 44.per cent was recorded. The PCV of 25.30 per cent, GCV of 22.87 per cent and heritability was high (81.87%). During 45 days after flowering, the variation between the parents of IS9830 (30.98) and E36-1(64.0) in per cent of green leaf area were highly significant. In RIP II, similar to that of RIPI, very wide range of variation from 7.7 to 81.5 per cent with a total mean of 44 per cent green leaf area was recorded. Very high estimated heritability (93.3%), with a per cent of 30.73 and 31.23 GCV and PCV respectively were recorded.

4.1.2 Evaluation of other drought tolerance traits

4.1.2.1 Plant height (cm)

A range of variation from 117-257cm with a mean value of 174 cm for plant height was observed. The PCV and GCV for this trait were 12.36 and 13.90 per cent respectively, with high heritability of 82.87 per cent and an expected genetic advance of 23.86 per cent over mean. In RIP II, a similar trend of variation was observed for plant height where the mean value among the RILs ranged from 117 to 267 cm with overall mean of 183 cm. The phenotypic and genotypic coefficients of variability were 13.9 and 12.36 per cent respectively. The estimate of heritability was 61.56 per cent with expected genetic advance of 21.05 per cent.

4.1.2.2. Stem thickness (cm)

The mean value of this trait was 3.6 cm with a wide range of from 2.20 to 6 cm. The PCV and GCV for traits were 15.10 per cent and 11.96 per cent respectively with high heritability 62.75 per cent and GAM 18 .89. In RIP II, the mean value for this trait was 3.70 cm ranging from 1.10 to 6.4 cm. The PCV and GCV for this trait were 19.26 and 13.46 per cent respectively and moderate heritability of 48.87 per cent with genetic advance of 11.78 per cent.

4.1.2.3. Days to 50% flowering

In RIP I, the mean value of days to 50 % flowering was 58 days with a range of 47 to 70 days. The PCV and GCV were low at 7.46 and 6.4 per cent respectively, but the heritability estimate was 72.7 per cent. In RIP II, the mean of days to 50% flowering was 62.10 with a

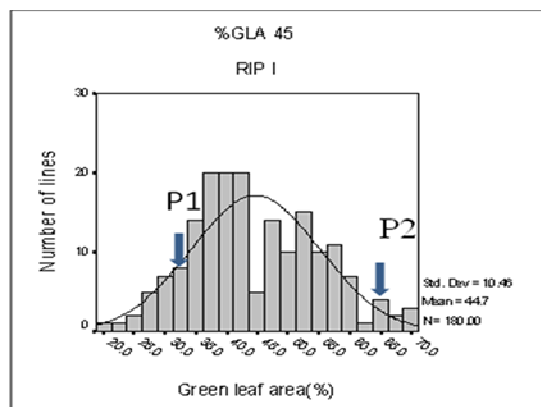
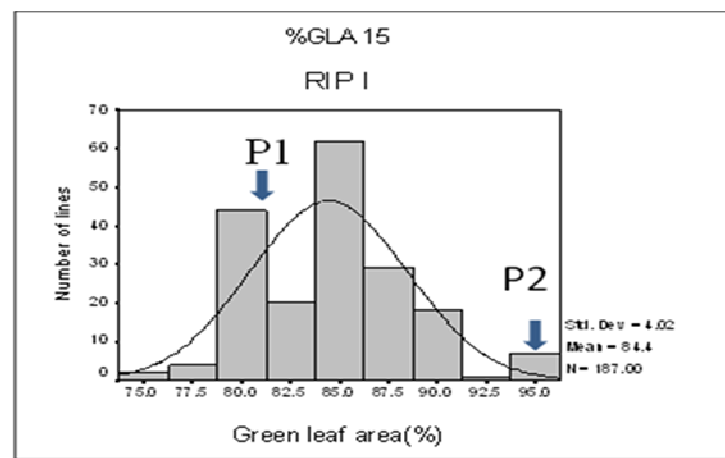
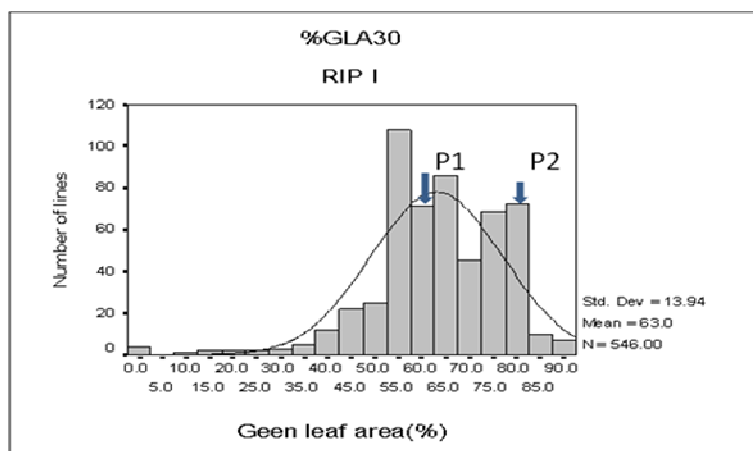


Fig 1. Frequency distribution of the F_8 RILs of RIP I derived from IS9830 x E36-1 for the per cent of green leaf area of A) 15, B) 30 and C) 45 days after flowering (%GLA15, % GLA30, and % GLA45, respectively)

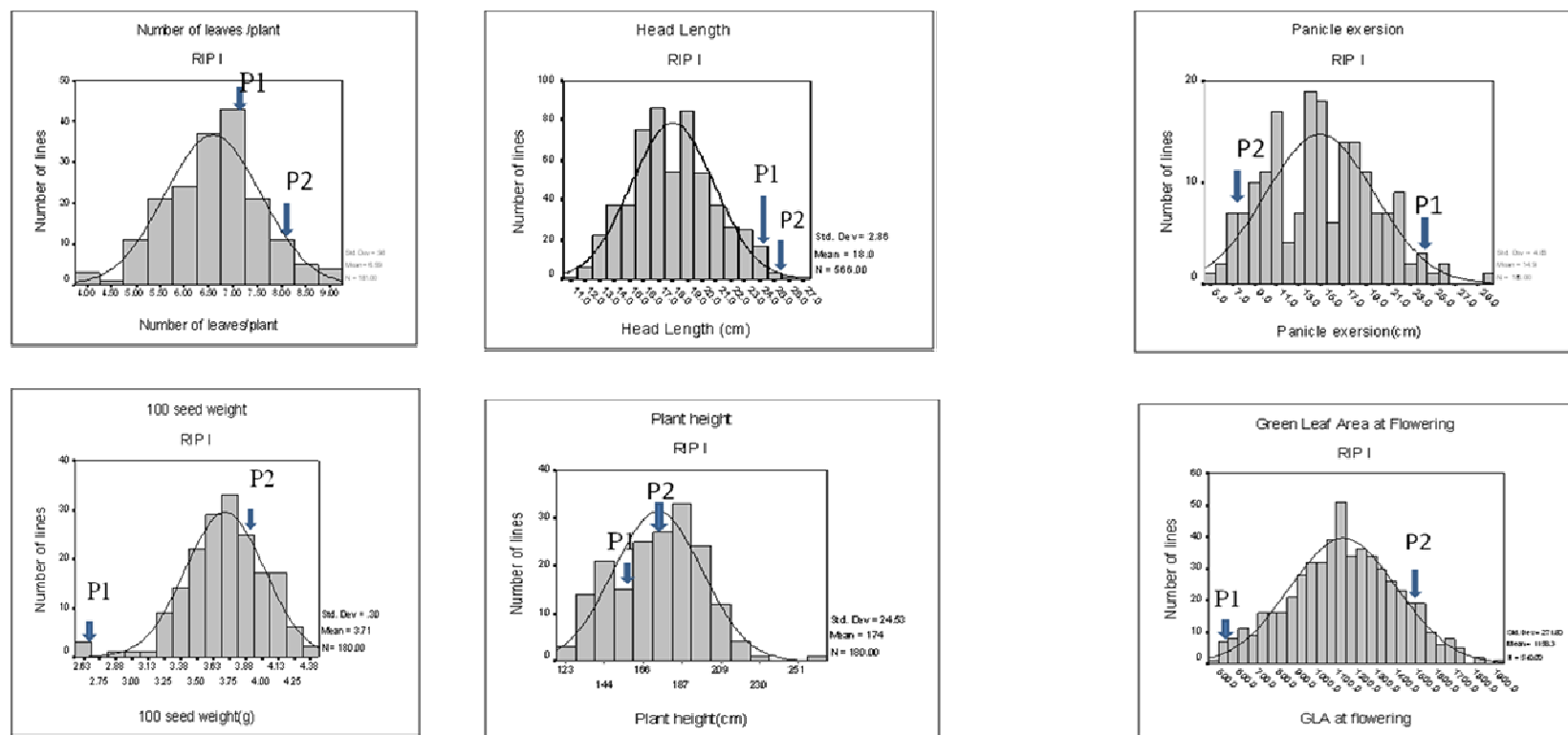


Fig. 2. Frequency distribution pattern of drought related traits viz number of leaves/plant, head length, panicle exertion 100 seed weight, plant height, and green leaf area at flowering in F₈ RILs derived from IS9830 x E36-1

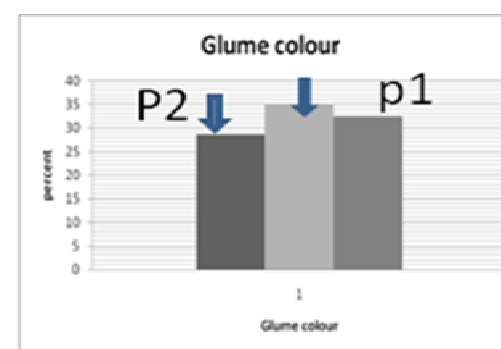
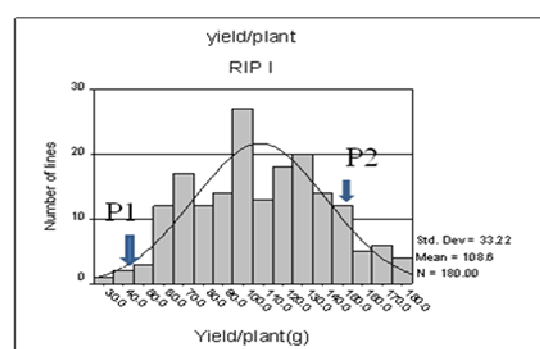
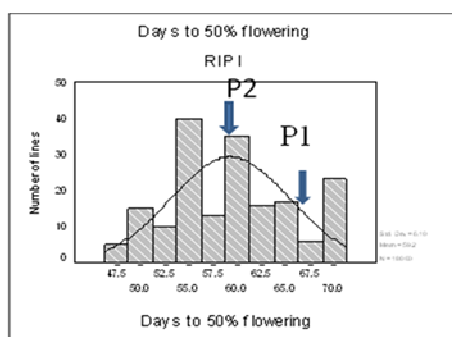
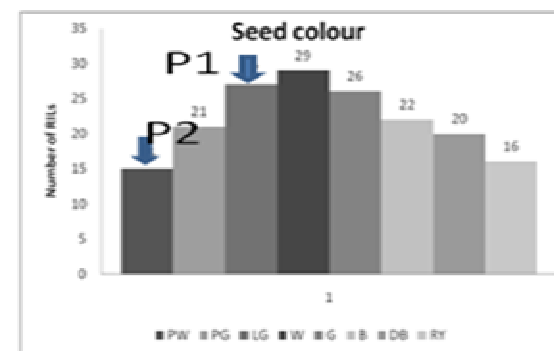
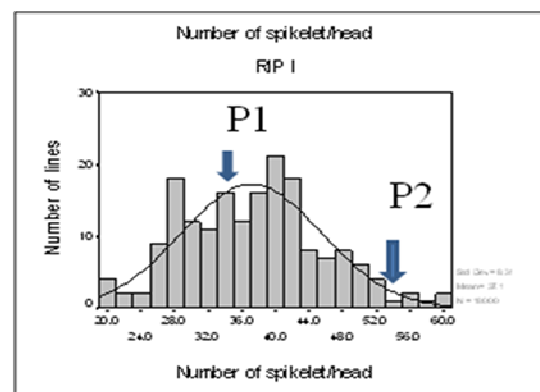
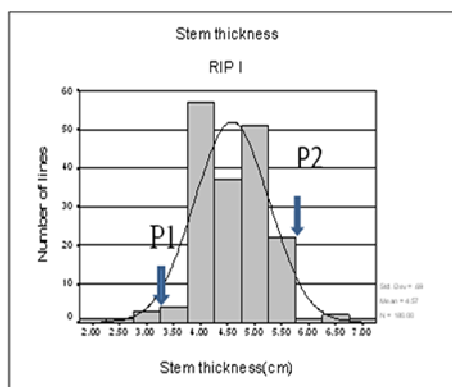


Fig. 3. Frequency distribution pattern of drought related traits viz, stem thickness, No, of spikelets/head, seed colour, days to 50% flowering, yield/plant and glume colour in F_8 RILs derived from IS9830 x E36-1

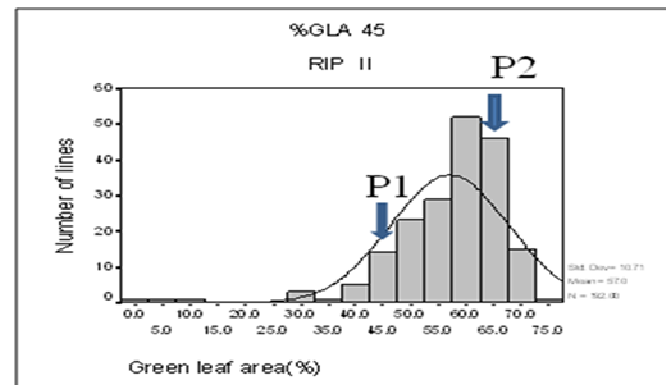
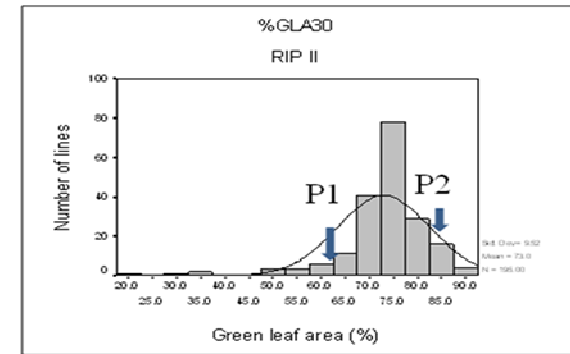
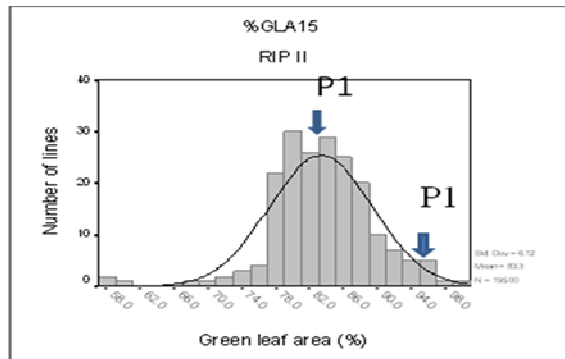


Fig. 4. Frequency distribution of the F_8 lines of RIP II derived from N13 x E36-1 for the percentage of green leaf area at A) 15, B) 30 and C) 45 days after flowering (%GLA15, %GLA15, %GLA30 and %GLA45, respectively)

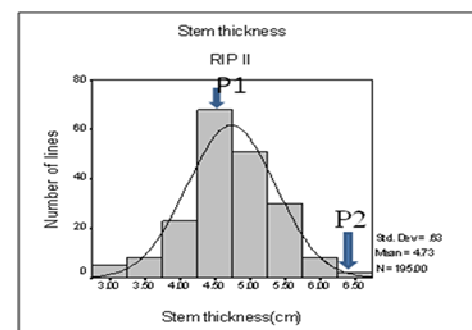
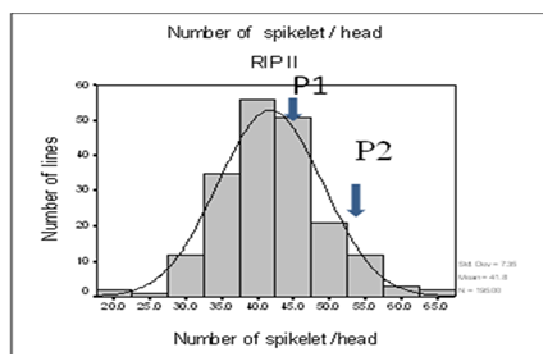
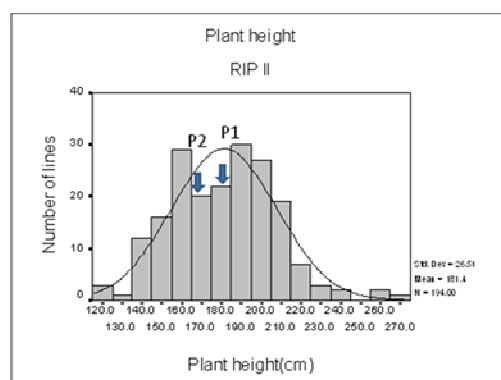
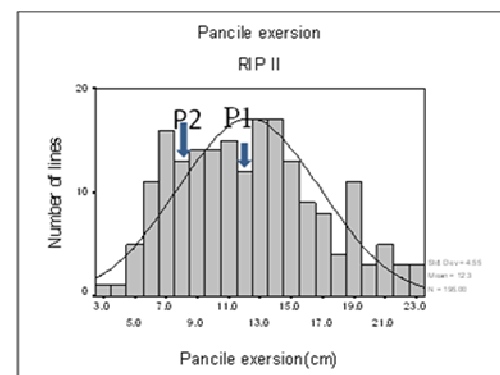
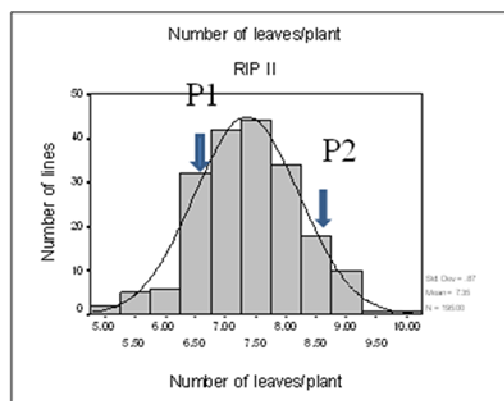
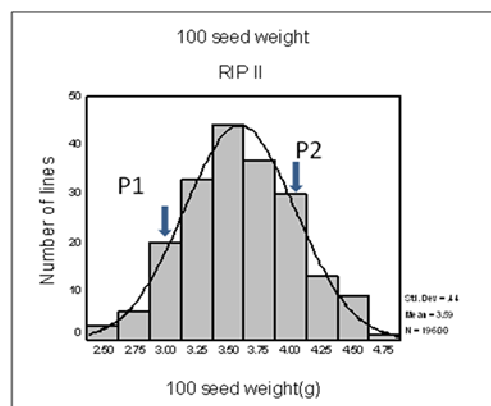


Fig. 5. Frequency distribution pattern of drought related traits viz, No. of Leaves/plant, panicle exertion, stem thickness, 100 seed weight No. of spikelets/head and plant height in F_8 RILs derived from N13 x E36-1

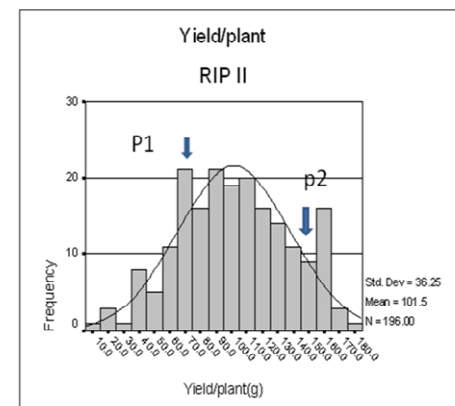
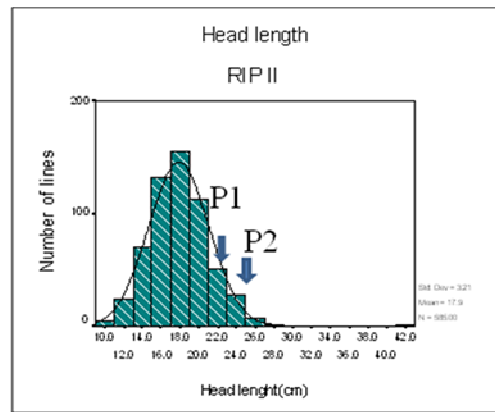
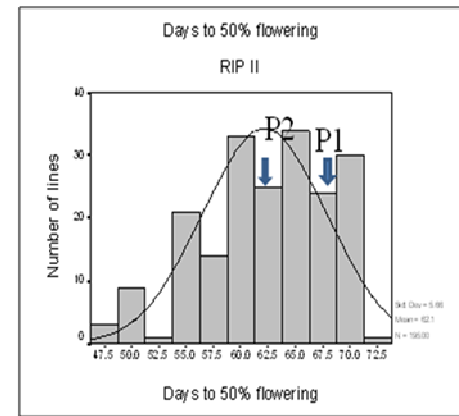
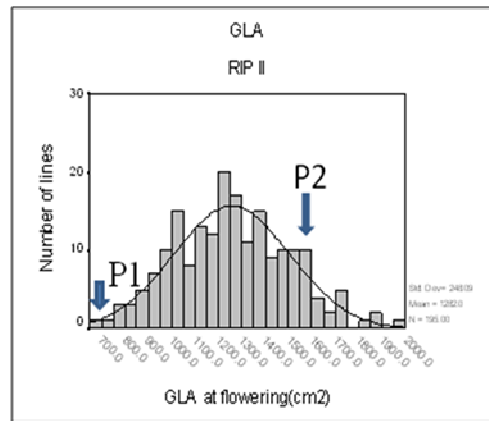


Fig. 6. Frequency distribution pattern of drought related traits viz green leaf area at flowering (GLA), days to 50% flowering, head length and yield/plant in F_8 RILs derived from N13 x E36-1

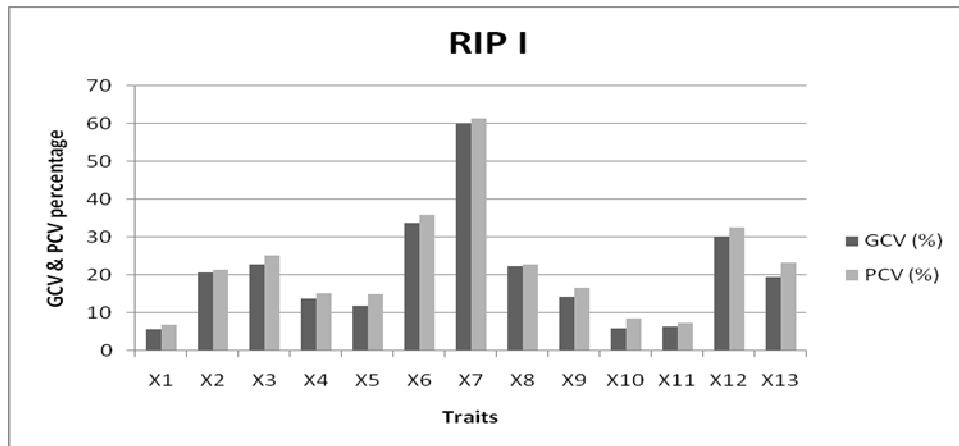


Fig. 7. PCV and GCV for 13 drought tolerant and yield related traits (pooled) in RILs of IS9830 x E36-1

Fig. 7. PCV and GCV for 13 drought tolerant and yield related traits (pooled) in RILs of IS9830 x E36-1

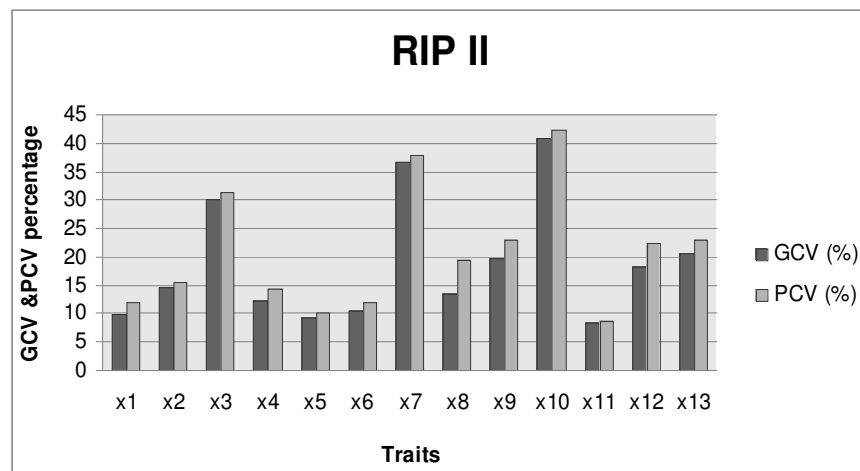


Fig.8. PCV and GCV for 13 drought tolerant and yield related traits (pooled) in RILs of N13 x E36-1

Fig. 8. PCV and GCV for 13 drought tolerant and yield related traits (pooled) in RILs of N13 x E26-1

range of 50 to 72 days. The per cent phenotypic and genotypic coefficient of variability for this trait was 8.75 and 8.22 respectively. The heritability estimate was 88.27 per cent with an expected genetic advance of 10.29 per cent.

4.1.2.4 Number of spikelets per head

The average number of spikelets per head was 37 ranging from 19.5 to 59. PCV and GCV were 22.76 and 22.35 per cent respectively and high heritability of 95.4 per cent and high expected genetic variation was observed. In RIP II, high variation was observed for this trait as the mean values ranges between 19 and 66 with over all mean value of 41.7. The PCV and GCV are 22.8 and 19.6 per cent respectively, with estimate heritability of 74.06 per cent and expected genetic advance of 11.21per cent.

4.1.2.5 Panicle exertions (cm)

The mean of panicle exertion length was 14.78 ranging from 3.1 to 29, with 36.05 and 33.79 PCV and GCV values, respectively. The heritability of this trait was high 87.55 per cent with expected genetic advance of 58 per cent. In RIP II, high variation was observed in panicle exertion. The mean value of panicle exertion was 12.7 and ranged from 2.7 to 23.4. High PCV and GCV of 37.9 and 36.72 per cent respectively, were observed with very high heritability of 65.5 per cent and GAM 70.86.

4.1.2.6. Head length (cm)

The range of ear head length varies from 11.4 to 27 cm with an average of 18 cm. The PCV and GCV of this trait were 12.4 and 16 .89 per cent respectively, and with heritability of 94.98 per cent, and with expected genetic advance of 65 per cent. In RIP II, the range of variation was from 9.7 to 27.6 cm. The overall mean being 17.8 cm .Coefficients of variability for phenotypic and genotypic observations were 32.33 and 30.22 per cent respectively. Heritability estimation was found to be high with GA (6.26%) predicted over per cent mean.

4.1.2.7. Number of leaves per plant

The average number of leaves per plant was 7 ranging from 4 to 9. The PCV and GCV were 16.75 and 14.4 per cent respectively and heritability was 73.88 per cent. In RIP II, the mean value for number of leaves per plant was 7.3 and less variation has been observed with range of 5 to 10. Moderate PCV of 11.95 and GCV of 10.46 per cent were recorded for this trait. The estimated heritability also moderate (48.99%) with GAM of 11.61.

4.1.2.8 100 seed weight (g)

The 100 seed weight was significantly varied from 2.6 to 4.48 g across RILs with a mean of 3.17 g. The percentage of phenotypic and genotypic coefficient of variability for this trait was 12.5 and 5.99 per cent respectively. In this trait both heritability (72.65%) and expected genetic advance (13.77) were high. In RIP II, the mean value of 100 grain mass across the entries was 3.6g. The PCV and GCV were 11.26 and 10.35 percent respectively. The estimated heritability value and genetic advance also high.

4.1.2.9 Grain yield per plant (g)

In RIP, grain yield per plant was significantly varied from 34.7 to180 g across RILs with a mean 105g. The per cent phenotypic and genotypic variations for yield traits were 30.58 per cent and 32.55 per cent respectively. Moderate heritability of 56 .55 with an expected genetic advance of 3.6 per cent was recorded. In RIP II, grain yield (g) per plant was significantly varied from 30 to 180 across RILs with the mean value of 150g. Phenotypic and genotypic coefficient of variability for this trait was 22.99 and 20.7 per cent respectively. Moderate heritability of 47.65 with expected genetic advance of 10.73 per cent was recorded.

4.2 EVALUATION OF QUALITATIVE CHARACTERS RELATED TO DROUGHT TOLERANCE TRAITS

4.2.1 Seed colour

High sorghum seed colour distributions were observed in RIP I as compared to RIP II. As per munsell colour chart, eight types of seed colour, viz light gray (LG), gray (G), white (W), brown (B), pinkish gray (PG), dark brown (DB), red yellow (RY) and pinkish white (PW)

Parental seed colour



RILs seed colour



**Plate 1. Morphological variation for seed colours among RILs derived from
IS9830 x E36-1(RIP I) cross**

Plate 1. Morphological variation for seed colours among RILs derived form IS9830 x E36-1(RIP I) cross

were identified. The highest frequency in RIP I seed colour distribution was light gray, which is similar to one of the parent, IS9830. The lowest frequencies in this population were dark brown and red yellow. The second parental type in this colour distribution was pinkish white (PW) i.e. similar to E36-1. The other six were different from both parents and categorized to recombinant inbred line types. Similarly in RIP II, the result of munsell colour chart showed that four types of seed colour were identified including to the parental type, viz brown dark, brown, red yellow and gray.

4.2.2 Glume Colour

Wide ranges of glume colour distribution were observed in RIP I as compared to RIP II. Light brown, dark brown, black, yellow, and white are the most common glume colour observed in RIP I. Light brown and dark brown are common glume colour for both populations. In both populations, light brown glume colour was highly frequented followed by dark brown. Glume colour derived from N13 x E36-1 (RIP II) was limited to two type of colour light brown and dark brown with approximately equal frequency. Therefore, glume colour distribution of RILs derived from IS9830 x E36-1 was more diversified as compared to RIP II RILs.

4.2.3 Glume cover

The general extent of glume cover in sorghum is indicated by percentage in sorghum discriminator as 25%, 50% and 75%. In RIP I, parents were significantly different for glum colour, while there is no difference in RIP II parents. Therefore data for glum cover of RILs derived from IS9830 x E36-1 (RIP I) cross revealed that fifty per cent of the population was 50% glume cover and 49% of the population was 75% glume cover which were similar to the parent IS9830 and E36-1 respectively. Almost the population was segregated for glume cover in to 1:1 ratio.

4.3 CORRELATION ANALYSIS

Data was subjected to SPAR1 software and correlation analysis was computed for the stay-green parameter and other drought related traits. Stay-green, drought tolerance trait is a complex character and its expression depends on interplay of several component characters. A correlation study can provide the role of component characters to the target phenotype. In the present investigation, both genotypic and phenotypic correlations were worked out for stay-green parameters and yield attributing traits, to assess the extent and nature of association between component traits of drought tolerance and yield.

The results are presented in Table 5 and 6 for RIP I and Table 7 & 8 for RIP II, respectively for genotypic and phenotypic correlations.

Per cent of green leaf area at 15 days after flowering (%GLA15)

Among the stay- green parameter, highly significant and positive association were found between %GLA15 and %GLA45, leaves number per plant, stem thickness, green leaf area at flowering (GLA), at both phenotypic and genotypic level. But %GLA30, plant height, and head length were significant and positively correlated at genetic level only. In contrast, highly significant negative correlations were recorded against panicle exertion and days to 50% flowering to the same trait at both phenotypic and genotypic level. In RIP II, %GLA15 showed highly significant positive association between GLA 30, GLA45 and plant height at both phenotypic and genotypic level.

Per cent of green leaf area at 30 days after flowering (%GLA30)

Highly significant and positive association were found between GLA30 and GLA45, leaves number per head, stem thickness, head length and GLA at both phenotypic and genotypic level. GLA30 showed highly significant negative association between days to 50% flowering and panicle exertion at both phenotypic and genotypic level. Highly significant positive association at both phenotypic and genotypic level were observed between %GLA30 and %GLA45, plant height, number of leaves per plant and yield in RIP II.

Per cent of green leaf area at 45 days after flowering (%GLA45)

In RIP I, a highly significant ($p \leq 0.001$) and positive associations were found between %GLA45 and leaves number per plant, stem thickness, number of spikelets per head and

Table 5. Coefficients of phenotypic correlation among the investigated traits in Recombinant Inbred Population derived from IS9830 x E36-1 (RIP I) cross

Traits	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13
X1	1	0.425*	0.277**	0.091*	0.051*	0.196**	-0.130**	0.113**	0.70*	0.061*	-0.279**	0.075*	0.135**
X2		1	0.530**	0.031	0.042	0.261**	-0.049	0.130**	0.020	0.131**	-0.257**	0.234	0.287**
X3			1	0.036	0.014	0.103**	-0.124**	0.113**	0.111**	0.089*	-0.252**	0.209	0.245**
X4				1	0.062	0.214**	0.247**	0.114**	0.047	0.121**	-0.047	-0.034	-0.020
X5					1	0.051	-0.054	0.034	0.019	0.059	0.048	0.015**	-0.080
X6						1	-0.114**	0.285**	0.028	0.026	0.277**	0.223**	0.211**
X7							1	-0.162**	-0.044	0.033	-0.071	-0.074	-0.156**
X8								1	0.074*	-0.006**	0.112**	0.157*	0.161**
X9									1	0.019	0.173**	0.457**	0.323
X10										1	0.087*	0.020	0.037
X11											1	-0.321**	-0.239**
X12												1	0.560**

X1: Per cent of green leaf area at 15 days after flowering (%GLA15) flowering

X2: Per cent of green leaf area at 30 days after flowering (%GLA30) (cm²)

X3: Per cent of green leaf area at 45 days after flowering (%GLA45)

X4: Plant height (cm)

X5: 100 seed weight (g)

*=significant at 0.05 probability level. **= Highly significant at 0.01 probability level.

X6: Leaves number per plant

X7: Panicle exertion (cm)

X8: Stem thickness (cm)

X9: Number of spikelets per head

X10: Head length (cm)

X11: Days to 50%

X12: Green leaf area at flowering

X13: Yield/ plant (g)

Table 6. Coefficients of genotypic correlation among the investigated traits in Recombinant Inbred Population derived from IS9830 x E36-1 (RIP I) cross

Traits	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13
X1	1	0.532**	0.342**	0.129**	0.095	0.288**	-0.165**	0.138**	0.091*	0.120**	-0.149**	0.175	0.133
X2		1	0.599**	0.043	0.063	0.312**	-0.052	0.157**	0.214**	0.210**	-0.191**	0.127	0.261*
X3			1	-0.030	0.037	0.134**	-0.151**	0.149**	0.126**	0.159**	-0.277**	0.349*	0.243*
X4				1	0.124**	0.268**	0.292**	0.136**	0.051	0.179**	-0.055	-0.069	-0.039
X5					1	0.128**	-0.118**	0.174**	0.030	-0.056	0.109**	0.066	0.077*
X6						1	-0.124**	0.349**	0.030	0.021	0.266**	0.303**	0.278**
X7							1	-0.185**	-0.042	0.078*	-0.075*	-0.214**	-0.086*
X8								1	0.090**	0.024	0.128**	0.212**	0.191**
X9									1	0.015	0.178**	0.458**	0.494**
X10										1	0.124**	0.119**	0.023
X11											1	0.335**	-0.348*
X12												1	0.772**

X1: Per cent of green leaf area at 15 days after flowering (%GLA15)

X2: Per cent of green leaf area at 30 days after flowering (%GLA30)

X3: Per cent of green leaf area at 45 days after flowering (%GLA45)

X4: Plant height (cm)

X5: 100 seed weight (g)

*=significant at 0.05 probability level. **= Highly significant at 0.01 probability level.

X6: Leaves number per plant

X7: Panicle exertion (cm)

X8: Stem thickness (cm)

X9: Number of spikelets per head

X10: Head length (cm)

X11: Days to 50% flowering

X12: Green leaf area at flowering (cm²)

X13: Yield/ plant (g)

Table 7. Coefficients of Phenotypic correlation among the investigated traits in Recombinant Inbred Population derived from N13 x E36-1 (RIP II) cross

Traits	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13
X1	1	0.293**	0.183**	0.094*	0.012	0.018	-0.067	0.013	0.033	0.047	-0.034	0.025**	0.031**
X2		1	0.628**	0.116**	0.020	0.155**	-0.014	-0.025	0.008	-0.003	-0.0161	0.01**	0.189**
X3			1	0.058	0.025	0.268**	-0.121**	0.020	0.141**	-0.084	-0.207**	0.017**	0.230**
X4				1	0.008	0.126**	0.329**	-0.042	0.032	0.131**	-0.064	0.083	0.048
X5					1	-0.017	-0.062	0.013	-0.054	-0.050	-0.041	0.007	0.021
X6						1	-0.048	0.095*	0.073	-0.085	0.033	0.232**	0.234**
X7							1	-0.118**	-0.076	0.232**	0.025	-0.052	-0.092*
X8								1	0.012	0.038	0.004	0.175**	0.151**
X9									1	0.073	0.042	0.064	0.249**
X10										1	0.084*	0.011	0.049*
X11											1	-0.017	-0.066
X12												1	0.118

X1: Per cent of green leaf area at 15 days after flowering (%GLA15)

X2: Per cent of green leaf area at 30 days after flowering (%GLA30)

X3: Per cent of green leaf area at 45 days after flowering (%GLA45)

X4: Plant height (cm)

X5: 100 seed weight (g)

X6: Leaves number per plant

X7: Panicle exertion (cm)

X8: Stem thickness (cm)

X9: Number of spikelets per head

X10: Head length (cm)

X11: Days to 50% flowering

X12: Green leaf area at flowering (cm²)

X13: Yield/ plant (g)

*=significant at 0.05 probability level. **= Highly significant at 0.01 probability level.

Table 8. Coefficients of genotypic correlation among the investigated traits in Recombinant Inbred Population derived from N13 x E36-1 (RIP II) cross

Traits	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13
X1	1	0.429**	0.239**	0.201**	-0.002	0.031	-0.091	0.039	0.042	0.117**	-0.029	0.063**	0.039
X2		1	0.730**	0.194**	0.016	0.274	-0.016**	-0.073	0.045	0.013	-0.203**	0.18**	0.319**
X3			1	0.110*	0.021	0.382**	-0.135**	0.059	0.256**	-0.123**	-0.227**	0.12**	0.328**
X4				1	-0.116**	0.181**	0.541**	-0.218**	0.218**	0.020	0.276**	0.163**	0.149**
X5					1	0.027	-0.121**	-0.04	-0.154*	-0.189**	-0.062	0.034	0.016
X6						1	-0.057	0.238**	0.079*	-0.088*	0.067	0.352**	0.387**
X7							1	-0.237**	-0.122**	0.237**	0.025	-0.051	-0.119**
X8								1	0.110**	-0.122**	0.090	0.391**	0.215**
X9									1	0.051	0.086	0.144**	0.349**
X10										1	0.120	-0.012*	0.006
X11											1	-0.029	-0.090*
X12												1	0.279**

X1: Per cent of green leaf area at 15 days after flowering (%GLA15)

X2: Per cent of green leaf area at 30 days after flowering (%GLA30)

X3: Per cent of green leaf area at 45 days after flowering (%GLA45)

X4: Plant height (cm)

X5: 100 seed weight (g)

X6: Leaves number per plant

X7: Panicle exertion (cm)

X8: Stem thickness (cm)

X9: Number of spikelets per head

X10: Head length (cm)

X11: Days to 50% flowering

X12: Green leaf area at flowering (cm²)

X13: Yield/ plant (g)

*=significant at 0.05 probability level. **= Highly significant at 0.01 probability level.

GLA at both phenotypic and genotypic level. But highly positive association was recorded with head length at genetic level only. Per cent of green leaf area at 45 days after flowering (%GLA45) showed moderate association between plant height, 100 seed weight and yield. In RIP II, %GLA45 showed highly and significant positive association with number of leaves per plant, number of spikelets per head and yield per plant.

Yield: In RIP I, yield showed significant positive association with 100 seed weight, leaves number/plant, stem thickness and GLA at both phenotypic and genotypic level. But highly significant negative association observed against days to 50 per cent flowering and panicle exertion to this trait and non-significant positive association was observed between yield and GLA30, GLA45, panicle exertion and head length. In RIP II, this trait showed highly and significant positive association with GLA30, 45, number of leaves per plant and number of spikelets per head. Similarly, negative correlations were recorded between days to 50% flowering and panicle exertion.

4.4 MOLECULAR MAPPING

4.4.1 Parental polymorphism

The parental lines, IS9830 and E36-1 were surveyed against nuclear and genic SSR markers to detect polymorphism and identify useful markers in order to screen across RILs to generate genotypic data. In this study, a total of 530 EST-SSR and 73 nuclear SSR markers were used to identify the parental polymorphism, of which 45 markers could detect polymorphism between the two parents, IS9830 and E36-1. Among 45 polymorphic primers screened, 31 primers were detected on 2.5% agarose gel and 14 primers were detected on 6% PAGE (Poly Acrylimidine Gel Electrophoresis). Out of 45 polymorphic markers, 37(7.0%) and 8 (11.11%) were genic and nuclear SSR, respectively. A total of 20 polymorphic markers were used to genotype individual RILs of mapping population.

4.4.2 Marker Segregation

Marker segregation pattern of all 20 polymorphic markers in RILs was checked for fitness to expected 1:1 ratio. Calculated χ^2 values at one degree of freedom were compared with tabled value for each marker locus (Table 9). All polymorphic 20 SSR markers loci segregated into 1:1 ratio.

4.4.3 Construction of linkage map

The size of mapping population used for construction of linkage map was 226 RILs derived from a cross IS9830 x E36-1(RIP I) and N13 x E36-1 (RIP II). Mapping of markers was accomplished by using MAPMAKER/EXP (3.b) software (Lander *et.al*, 1987). Linkage distance in terms of centimorgan (cM) value was calculated using kosambi mapping function. A total of 66 markers were found associated among themselves with varied distances on ten linkage groups. Based on the anchor marker and that of the order of SSR markers in different groups as per the map of Haussmann *et al.* (2004) all the EST-SSR markers (20) were assigned to ten different linkage groups. The genetic map of IS9830 x E36-1(RIP I) and N13 x E36-1(RIP II) had a total of 1558.1 cM and 1602 cM respectively.

The details of linkage groups in which these markers belonged and linkage distance in cM are given (Table 9) and linkage maps are presented in plate 4, 5 and 6 for both population.

All the markers were assigned to different linkage groups on the basis of anchor markers in the linkage map. The marker in the present study covered to all the ten linkage group A to J (plate 5 and 6). The number of marker mapped per linkage group ranges from four (LGF) to nine (LGA) and four (LGJ) and nine (LGI) in RIP I and II respectively. The linkage map thus constructed had a total coverage of 1558.9 cM and 1605.8 cM and the markers were distributed unevenly through the linkage group with an average distance of 31.176 cM and 31.77 cM in RIP I&II respectively. The highest and the lowest distance observed were between xisp230-xtxp41 (154 cM) on linkage group D and xtxp105-xtxp 18(1.4 cM) on linkage group H, respectively. The number of markers assigned to linkage group A,B,C,D,E,F,G,H,I, and J were 9,6,7,7,5,4,5,6,5, and 6 respectively and other six are not mapped.



Plate 2. Parental survey to identify polymorphic SSR Marker on 6% PAGE

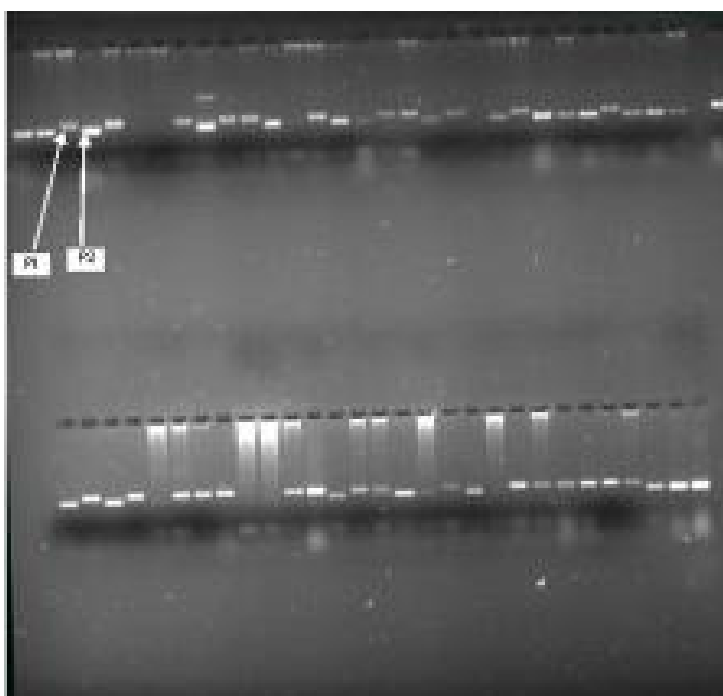


Plate 3. Segregation patterns of xiabt332 marker across RIL's (IS9830 x 36-1) and parent on 2.5% gel

Table 9. Test of significance for expected segregation ratio of genic and nuclear SSR markers in RILs of IS9830 x E36-1

Sl. No.	Marker	P1	P2	χ^2 table value <P>0.001=3.81
1	<i>xiabt184</i>	118	108	0.442
2	<i>xiabt358</i>	115	109	0.177
3	<i>xiabt54</i>	110	106	0.513
4	<i>xiabt455</i>	109	117	0.283
5	<i>xiabt332</i>	118	108	0.442
6	<i>xiabt364</i>	105	117	0.708
7	<i>xiabt342</i>	126	100	2.991
8	<i>txtp96</i>	122	104	1.434
9	<i>xiabt356</i>	116	110	0.159
10	<i>txtp58</i>	117	111	0.177
11	<i>xiabt147</i>	121	105	1.133
12	<i>xiabt439</i>	120	106	0.867
13	<i>xiabt176</i>	111	115	0.071
14	<i>xiabt95</i>	104	122	1.434
15	<i>xiabt445</i>	119	107	0.637
16	<i>xiabt32</i>	112	114	0.018
17	<i>txtp94</i>	126	100	2.991
18	<i>xiabt343</i>	119	107	0.637
19	<i>txtp113</i>	112	114	0.018
20	<i>xiabt352</i>	117	109	0.283

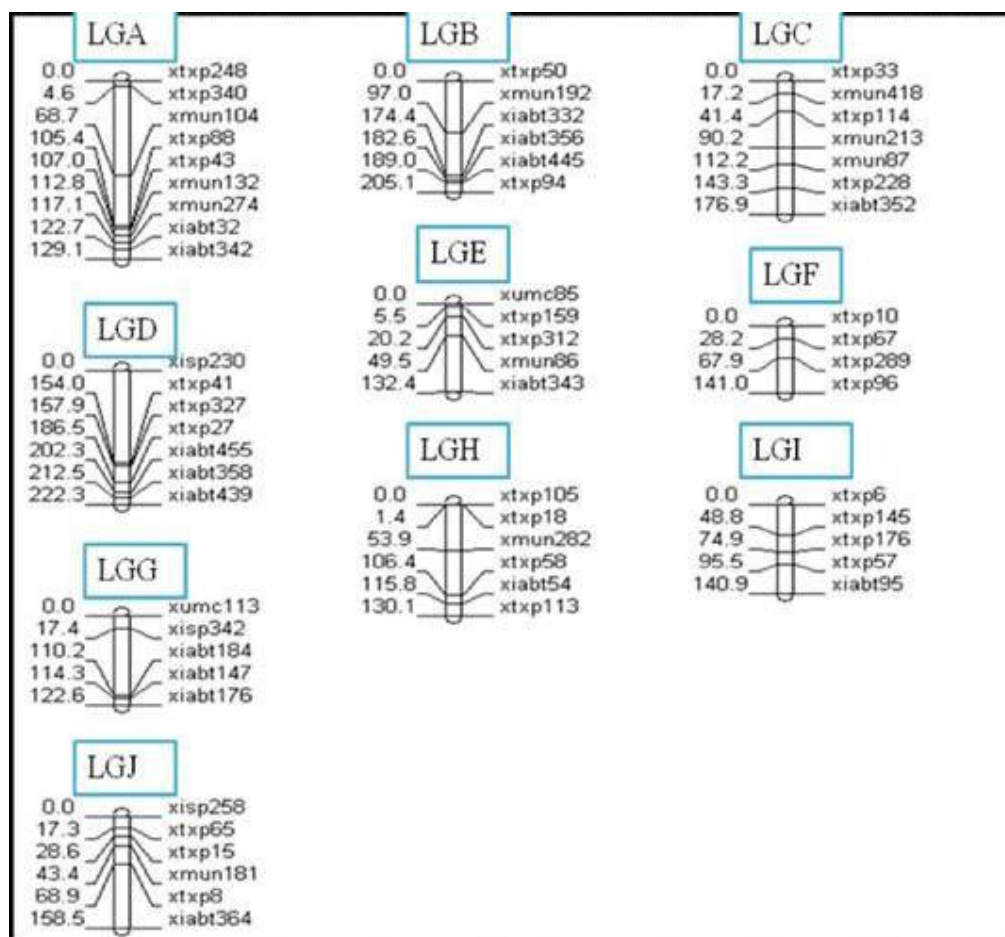
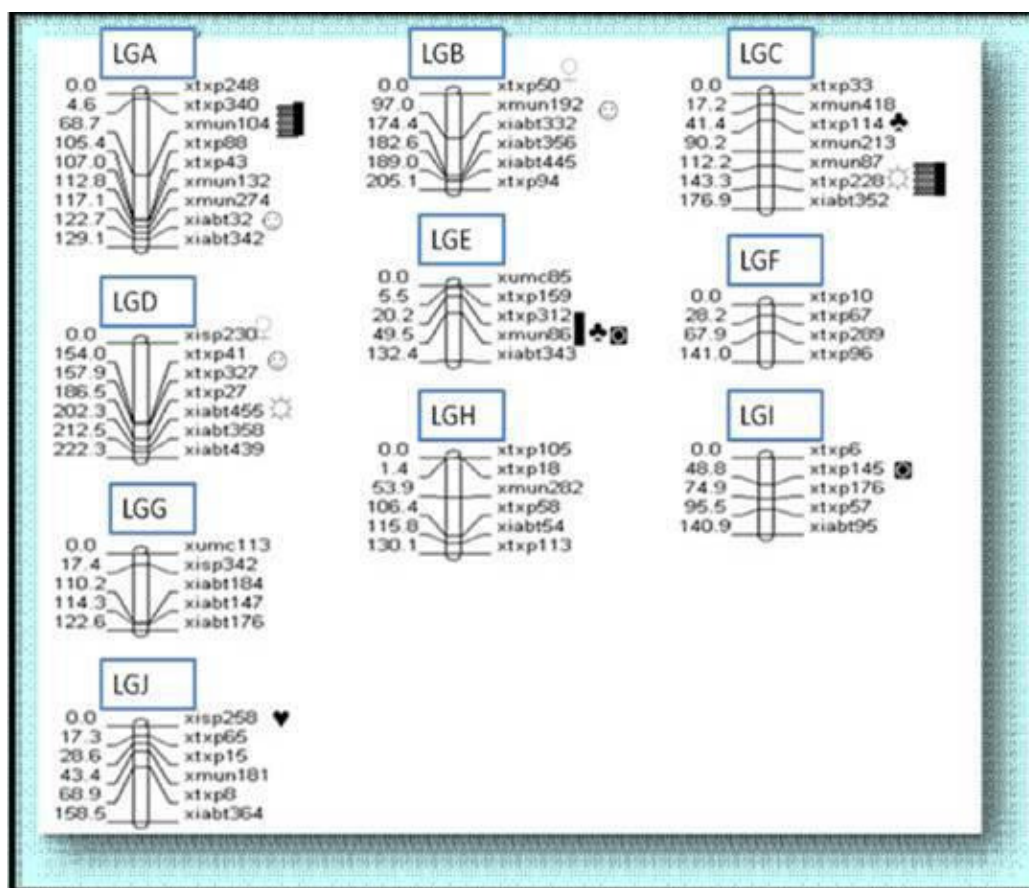


Plate 4. Genetic linkage map for stay-green in sorghum RIL's of IS9830 x E36-1

Table 10. Linkage distance between pairs of markers in RILs of IS9830 x E36-1

Sl. No.	Linkage group	Distance	Distance (cM)
1	A	<i>txp248 – txp340</i>	4.6
2		<i>txp340 – xmun104</i>	64.1
3		<i>xmun104 – txp88</i>	36.7
4		<i>txp88 – txp43</i>	1.6
5		<i>txp43 – xmun132</i>	5.8
6		<i>xmun132 – xmun274</i>	4.3
7		<i>xmun274 – xiabt32</i>	5.6
8		<i>xiabt32 – xiabt342</i>	6.4
9	B	<i>txp50 – xmun192</i>	97.0
10		<i>xmun192 – xiabt332</i>	77.4
11		<i>xiabt332 – xiabt356</i>	8.2
12		<i>xiabt356 – xiabt445</i>	6.4
13		<i>xiabt445 – txp94</i>	16.1
14	C	<i>txp33 – xmun418</i>	17.2
15		<i>xmun418 – txp114</i>	24.2
16		<i>txp114 – xmun213</i>	48.8
17		<i>xmun213 – xmun87</i>	22.0
18		<i>xmun87 – txp228</i>	31.1
19		<i>txp228 – xiabt352</i>	33.6
20	D	<i>xisp230 – txp41</i>	154.0
21		<i>txp41 – txp327</i>	3.9
22		<i>txp327 – txp27</i>	28.6
23		<i>txp27 – xiabt455</i>	15.8
24		<i>xiabt455 – xiabt358</i>	10.2
25		<i>xiabt358 – xiabt439</i>	9.8
26	E	<i>xumc85 – txp159</i>	5.5
27		<i>txp159 – txp312</i>	14.7
28		<i>txp312 – xmun86</i>	29.3
29		<i>xmun86 – xiabt343</i>	82.9
30	F	<i>txp10 – txp67</i>	28.2
31		<i>txp67 – txp289</i>	39.7
32		<i>txp289 – txp96</i>	73.1
33	G	<i>xumc113 – xisp342</i>	17.4
34		<i>xisp342 – xiabt184</i>	92.8
35		<i>xiabt184 – xiabt147</i>	4.1
36		<i>xiabt147 – xiabt176</i>	8.3
37	H	<i>txp105 – txp18</i>	1.4
38		<i>txp18 – xmun282</i>	52.5
39		<i>xmun282 – txp58</i>	52.5
40		<i>txp58 – xiabt54</i>	9.4
41		<i>xiabt54 – txp113</i>	14.3
42	I	<i>txp6 – txp145</i>	48.8
43		<i>txp145 – txp176</i>	26.1
44		<i>txp176 – txp57</i>	20.6
45		<i>txp57 – xiabt95</i>	45.4
46	J	<i>xisp258 – txp65</i>	17.3
47		<i>txp65 – txp15</i>	11.3
48		<i>txp15 – xmun181</i>	14.8
49		<i>xmun181 – txp8</i>	25.5
50		<i>txp8 – xiabt364</i>	89.6



? =GIA15
 ? =GLA30
 ? =GLA45
 ? =plant height (cm)
 ? =yield/plant (g)
 ? =Panicle exertion (cm)
 ? =seed color
 ? =Days to 50% flowering

Plate 5. Localization of QTLs associated with stay-green and other drought tolerant traits in sorghum RIL's of IS9830 x E36-1, identified by CIM (Threshold LOD>2.50) at Dharwad

Table 11. Characteristics of QTLs associated with different traits in RILs derived from IS9830 x E36-1(RIP I) at MARS, Dharwad

Traits	Markers	LG	QTL position(cM)	Peak LOD	Additive effect	Phenotypic variance
1	<i>txtp340</i>	J	22.18	2.05	5.40	22.30
3	<i>xmun104</i>	A	106.6	2.51	2.82	10.10
	<i>txtp228</i>	C	175.27	2.58	3.70	9.62
4	<i>txtp228</i>	C	175.27	3.70	2.60	14.90
	<i>xiabt455</i>	D	203.00	3.20	2.80	11.00
5	<i>txtp228</i>	C	175.27	2.59	1.66	14.8
	<i>xmun104</i>	A	106.5	3.30	2.01	20.00
	<i>txtp312</i>	E	44.00	2.60	0.94	6.21
6	<i>xmun86</i>	E	127.47	2.13	2.35	15.8
	<i>txtp176</i>	I	28.65	2.20	-0.146	24.08
8	<i>txtp114</i>	C	57.40	2.16	1.73	12.50
	<i>xmun86</i>	E	57.40	2.58	-1.72	11.3
12	<i>txtp50</i>	B	52.01	2.59	-4.44	30
	<i>xisp230</i>	D	58.01	2.41	-4.71	44.00
13	<i>xiabt32</i>	A	124.75	2.93	0.64	16.00
	<i>xmun192</i>	B	141.05	2.23	1.64	33.09
	<i>xisp230</i>	D	68.00	2.68	1.65	34.40

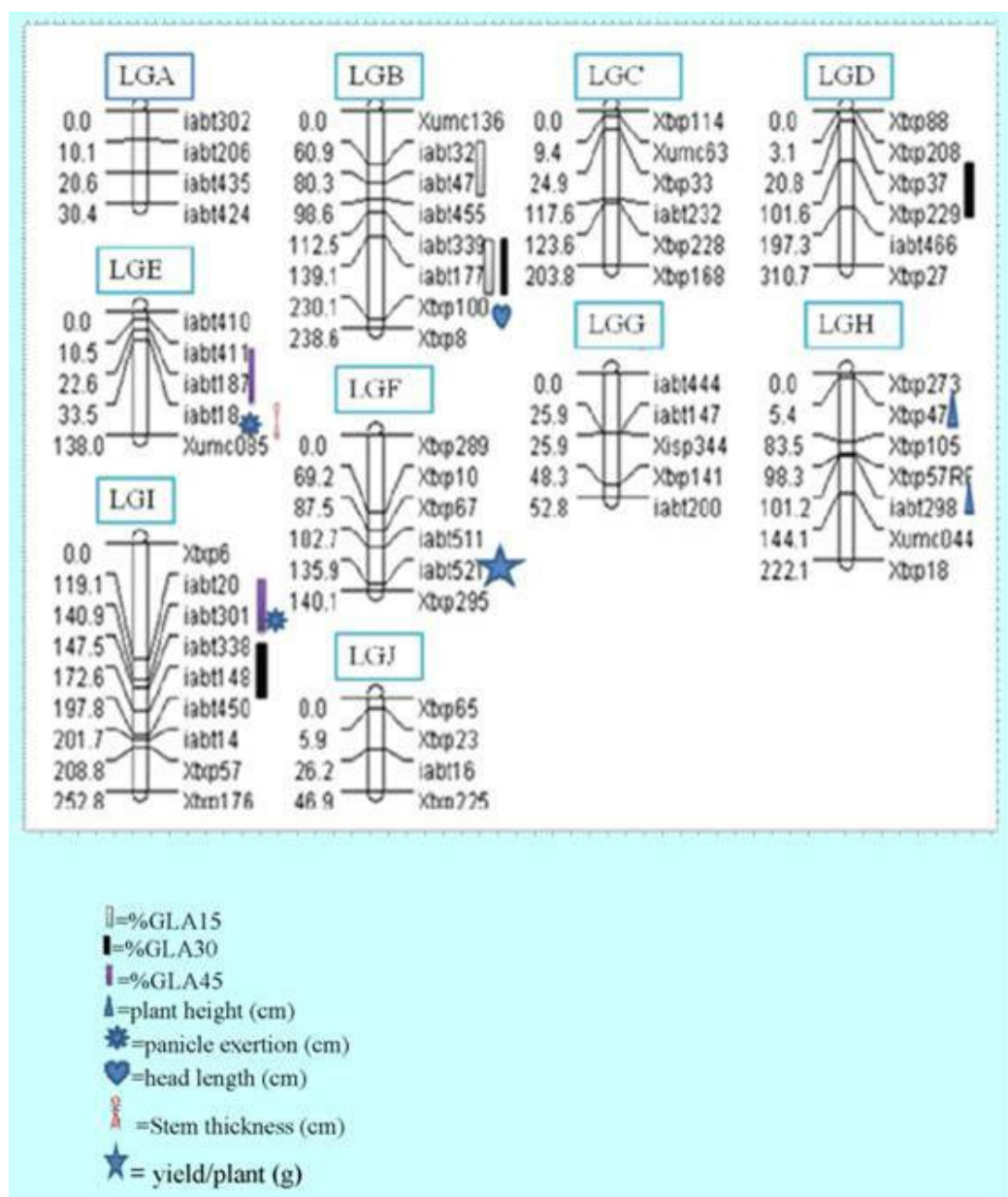


Plate 6. Localization of QTLs associated with stay-green and other drought tolerant related traits and yield components in sorghum RIL's of N13 x E36-1 composite interval mapping (threshold LOD>2.5) at Dharwad

Table 12. Characteristics of QTLs associated with different traits in RILs derived from N13 x E36-1 (RIP II) at MARS, Dharwad

Traits	Markers	LG	QTL position(cM)	Peak LOD	Additive effect	Phenotypic variance
1	<i>iabt321</i>	B	62.20	2.5	-0.92	12.0
2	<i>iabt339</i>	B	123.00	2.52	-1.65	5.0
	<i>txtp37</i>	D	98.00	3.2	1.92	4.2
	<i>iabt338</i>	I	148.00	2.55	1.70	7.9
3	<i>iabt187</i>	E	20.80	3.12	-11.31	39.0
	<i>iabt301</i>	I	140.88	2.63	3.85	5.1
4	<i>iabt521</i>	F	135.90	2.06	1.64	14.8
5	<i>txtp47</i>	H	71.37	2.85	-12.73	20.20
	<i>txtp57RF</i>	H	98.27	3.00	11.22	12.9
8	<i>iabt18</i>	E	97.00	2.08	2.40	31.9
	<i>iabt301</i>	I	141.00	2.90	-2.50	43.39
9	<i>iabt18</i>	E	135.00	3.06	-0.17	8.6
10	<i>txtp100</i>	B	232.00	2.6	-1.64	9.00

*Composite interval mapping with QTL cartographer 2.5b

For RILs derived from N13 x E36-1 (RIP II), linkage map construction was done using the basic file on already available mapped marker for QTL identification using present phenotypic data.

4.5. IDENTIFICATION OF QTLs

The linkage map constructed for two recombinant inbred population (RIPs) IS9830 x E36-1 (RIP I) and N13 x E36-1 (RIP II) each consist of 226 RILs were used for QTL localization. The software QTL Cartographer (2.5b) was used to analyze data by Composite Interval Mapping (CIM) procedure, implemented with a LOD score of 2.5 as threshold value for QTL significance. The additive effect and the estimates of the portion of phenotypic variation explained by individual QTL were computed. All the QTLs associated with 13 phenotypic recorded at MARS, Dharwad are presented in Tables 11&12 for RIP I and RIP II respectively. Graphical representation of chromosomal position of QTLs and flanking markers are presented in plate 5 &6 for both populations. In these mapping populations, the female parent (marker allele A) IS9830 and/or N13 is non stay-green type and male parent (marker allele B) E36-1 is stay-green. The results are described below from CIM analysis for identifications of QTLs for stay-green, other drought tolerant traits and yield components.

4.5.1 QTL for stay-green

QTLs for per cent of green leaf area at 15 days after flowering (%GLA15)

Two major QTLs detected for %GLA15 on linkage group A and were flanked by Xmun104-xtxp88 and xtp-xiabt352 respectively. They accounts for 10.10 and 9.6 per cent phenotypic variance with 2.82 and 2.64 per cent additive effect of an allele from E36-1. QTL for GLA30 overlaps with QTL GLA45 on the linkage group A and C of 98 cM and 175.25 cM respectively.

In RIP II for the same trait, GLA15, CIM detected two QTLs on same chromosome (B) on different positions. One QTL was bracketed by iabt321 - iabt477 at distance of 62.2 cM explaining a phenotypic variation of 12% and the allele from E36-1 had a negative effect (-0.92). The second QTL was bracketed by iabt339- iabt177 at a distance of 123 cM explaining the phenotypic variation of 10.10 % and the allele from E36-1 had additive effect (1.32) at 2.5 LOD score.

QTLs for Per cent of green leaf area at 30 days after flowering (%GLA30)

In RIP I, composite interval mapping (CIM) detected two QTLs for percentage green leaf area 30 days after flowering in two linkage groups, at C and D which explained phenotypic variation of 14.9 and 11.0 per cent respectively, with an additive effect from E36-1 at MARS, Dharwad location. QTL located on linkage group C is bracketed by xtp228-xiabt352, at a distance of 175.25 cM, while QTL located on the linkage group D is bracketed by xiabt455-xiabt358 at a distance of 87.41 cM.

In RIP II, for GLA30 three QTLs on linkage group B (iabt339), D (xtxp37) and I (iabt338) were detected. They explain phenotypic variance of 5%, 4.2% and 7.9 % with 2.1, 3.2, and 2.55 LOD score with -1.65, 1.9, 1.7 additive effect on allele from E36-1.

QTL for per cent of green leaf area at 45 days after flowering (%GLA45)

Three major QTLs are detected on a linkage group A, C and E were flanked by xtp88-xumn104, xtp228-xiabt352, and xtp312-xmun86, respectively and accounted 14.8%, 20%, 6.21% phenotypic variance. They had 1.66, 2.01, 0.94 per cent additive effect from male parents. QTL at linkage group C at a distance of 175.27 cM was common for all three stay-green traits (%GLA15, %GLA30 and %GLA45). One QTL on the Linkage group A at a distance of 98.6 cM was common between two stay-green traits (%GLA30 and %GLA45). In RIP II two major QTLs detected on linkage group E and I bracketed by iabt187-iabt411 and iabt301-iabt20 respectively. They accounted for 39 % and 5.1 % of phenotypic variance having 3.12 and 2.63 LOD score with -11.31 and 3.85 per cent additive effects from male parent respectively.

4.5.2 QTL for Yield and other components

One QTL for plant height was detected on the linkage group J was bracketed by xisp258-xtxp65 a distance of 19.29 cM it accounts 4.4 per cent phenotypic variance and had an additive effect of 5.48 per cent at 2.05 LOD value. In RIP II, two major QTLs were detected for plant height on the linkage group H flanked by iabt47-xtxp105 and xtp57RF-iabt298 at map distance of 71.37cM and 98.27 cM. They account for 20.20% and 12.5 % phenotypic variation with additive effect of -12.73 and 11.22 at 2.85 and 3.01 LOD score respectively.

Three major QTLs for seed colour detected on the linkage group A, B, D were bracketed by xiabt32-xmun274, xiabt332-xmun192, xtp27-xtxp327, at a distance of 105.39, 141.05, 68 cM respectively. They accounts 16.0, 53.65, 54.40 per cent phenotypic variance with additive effect of 0.64, 1.64 and 1.65 from male parent at LOD value of 2.92, 2.23, 2.68 respectively.

Two QTLs detected for grain yield on the linkage groups E and I were bracketed by xumc86-xibt343 and xtp6-xtxp145 at map distance of 127.47 and 28.65 cM explaining 15.8 % and 24.08% for phenotypic variance and had an additive effect of -0.15 per cent from male parent at LOD value of 2.2. In RIP II, QTL for yield detected on linkage group F was bracketed by iabt521-xtxp295 at a genetic distance of 135.9 cM. It had 1.64 additive effects from male parent and accounts 4.8 per cent phenotypic variance.

Two QTLs detected for panicle exertion on the linkage group C and E were between xtp114-xmun418 and xmun86-xtxp312 each at a distance of 57.4 cM which explain a total of 23.8 per cent phenotypic variance. They had 1.73 and -1.712 additive effect at LOD score of 2.16 and 2.58 respectively. Similarly in RIP II, two QTLs detected for panicle exertion on linkage group E and I were bracketed by iabt187-xumc085 and iabt301-iabt338 at map distance of 87 cM and 141 cM explaining 31.91% and 43.39 % phenotypic variance .They had 2.4 and -2.5 additive effect from male parent at LOD score of 2.08 and 2.9 respectively

Two QTLs for days to 50% flowering detected on the linkage group B and D were bracketed by xtp50-xmun192 and xisp230-xtxp41 at a distance of 52.01 and 58 cM, which accounts 30 and 44 per cent for phenotypic variance, with additive effect value of -4.4 and 4.7 at LOD score of 2.59 and 2.41 respectively.

In RIP II, one QTL for head length was detected on linkage group B flanked by xtp8-xtxp100 at a position of 232 cM which accounted 9.00 per cent phenotypic variance with additive effect value of -1.64 % at LOD score of 2.6. Similarly, in this population one major QTL was detected for stem thickness on linkage group E at map distance of 135 cM which explained 8.6 per cent phenotypic variance with additive effect of -0.17% at 3.05 LOD score.

5. DISCUSSION

Sorghum is one of the most important food and feed crops in the arid and semi-arid and regions of the world. Development and utilization of crop cultivars adapted to drought-prone conditions is a long term solution for improving and stabilizing crop productivity. Little progress has been documented on the use of specific physiological traits to enhance drought tolerance, partly because of the poor understanding of physiological mechanism and their inheritance (Rosenow *et al.*, 1981). In sorghum, moisture stress during and after flowering stage causes premature leaf senescence, which in turn leads to stalk lodging, stalk rot disease and significant yield loss (Rosenow and Clark, 1995). The present investigation revealed considerable amount of variation for all the 13 characters studied, which indicates that there is scope for improvement. Stay-green is a term used to describe resistance to premature senescence during post flowering stage under severe soil moisture stress. It is a nuclear gene-controlled quantitative trait (Walulu *et al.*, 1994). In many crops species, stay-green plants have shown increased resistance to drought, diseases and have better quality forage for animals. The high in chlorophyll content is an ideal source of the pigments for food industry (Thomas and Smart, 1993).

There are a number of factors that progress under selection for stay-green trait, using traditional method of breeding; i) the timing and intensity of moisture stress ii) large interaction between stress response and growth stage, iii) interaction of drought with heat and other factors and inability to accurately evaluate stay-green features until plants reach physiological maturity. But selection based on markers could facilitate manipulation of quantitative traits without affecting other important agronomic traits by allowing evaluation of breeding progeny for the presence of target gene/genes. Genetic mapping of QTLs (quantitative trait loci) conferring stay-green is an important step towards understanding and utilizing drought- resistant varieties and hybrids.

Molecular markers allow breeders to track genetic loci controlling drought tolerance even under non-target environments. This will reduce the cost for field evaluation, increase breeding efficiency and allows simultaneous selection for drought tolerance and other agronomic characters. Mapping and identification of genomic regions associated with drought and yield would serve as the basis to initiate marker assisted transfer of relevant genome regions into elite genotypes. Use of saturated genetic map especially with functional marker wherever possible would help to identification and understanding of genomic regions responsible for a complex quantitative trait and pave way for positional cloning of desired gene in sorghum. In the present study, RILs derived from two crosses; IS9830 (senescence genotype) x E36-1 (stay-green source) and N13 x E36-1 were characterized for drought tolerance, yield and yield components. To construct a genetic linkage map for these populations, parental polymorphism followed by genotyping with genic and nuclear SSR markers were done.

5.1 GENETIC VARIABILITY AND CHARACTER ASSOCIATION FOR STAY-GREEN AND OTHER TRAITS

Accurate phenotyping of the test population is critical to understand the genetic nature of any quantitative trait. The interaction of environment with genetic factors, whose individual effect is difficult to determine leads to the expression of any quantitative trait. Through quantitative genetic theory, analysis of such traits for separating genetic effect from the environmental effect is possible; however, it is important that the test populations have sufficient variability to make proper inference. In both populations selected for the study, RILs displayed considerable differences in their mean phenotypic value for all 13 drought tolerant and yield related traits studied. This is also obvious as the RILs were derived from phenotypically distinct parents for several traits including stay-green, charcoal rot resistance and other drought related components.

The mean green leaf area decreased from 84% 15 DAF to 44% at 45 days after flowering. The presence of genetic variation was low in case of GLA15 DAF, moderate in case of GLA30 DAF and high in case of GLA45 DAF in both RIPs evaluated under field condition. According to Haussmann *et al.* (2002), the mean GLA decreased from 74% at 15 DAF to 36-38% at 45 DAF in both RIPs which were studied in both India and Africa post rainy

season. Current evaluation of these populations was at F_8 and the genetic variation among the RILs within each RIP was highly significant for all traits studied. Coefficients of error variation were low or moderate for all the 13 traits in both populations. The frequency distribution of the stay-green traits in both RIPs was unimodal and approximately normal. The parental lines were highly significant for all stages of stay-green. These results are in accordance with Haussmann *et al.* (2002) who evaluated same RIPs for two years in $F_3:F_5$ stage.

The wide variation among the RILs indicates the utility of RILs for further genetic investigations. The PCV was higher than GCV for all the 13 traits studied indicating their polygenic inheritance.

High values of PCV and GCV in both RIPs were evident for %GLA45, panicle exertion, head length, number of spikelets per head, yield and green leaf area at flowering indicated that variation for these characters contributed markedly to the total variability. These results are in accordance with results obtained by Haussmann *et al.* (2002) of the current population. A number of reports in the sorghum were available involving different crosses (B35 x TR x 700; RT x 7078; B35 x Rt x 7078; SC56 Xrt x 700; IS2238 x E36-1) in an independent field evaluation similar to the present investigation (Xu *et al.*, 2000b; Kebede *et al.*, 2001; Subudhi *et al.*, 2000; Berenje, 1990; Rao and Patil, 1996); Reddy *et al.*, 1996; Sundaresha, 2006).

Moderate PCV and GCV were observed for GLA, plant height, and stem thickness number of leaves per plant. The range of variability was comparatively low for days to 50 per cent flowering in RIP II and 100 seed weight in RIP I. Similar observation were also made by Raja and Parikh, (1980); Patel *et al.* (1980); Asthana *et al.*., 1995; Nimbalkar *et al.* (1988) ; Biradar *et al.* (1996), in sorghum germplasms and RIPs.

High variability is necessary for improvement of traits through selections. The coefficient of variation reveals only the extent of variability present for different characters in a population. The effectiveness of selection for a trait depends on the relative level of genetic and non-genetic variation. Heritability is a useful quantitative parameter, which considers the role of heredity and environment determining the expression of a character (Allard, 1960; Kukadia *et al.*, 1983; kumar and Singh, 1986). Effective selection can be achieved only when additive effects are substantial and environmental effects are small. In the present investigation exceptionally, high estimates of broad sense heritability were noticed for character viz., %GLA15, %GLA30 and %GLA45 and, stem thickness, head length, number of spikelets per head and 100 seed weight, in RIP I. Several researchers made similar observation in different crosses, (Reddy *et al.*, 1996; Punhuri, 2004; Mahalakshmi and Brider, 2002). Sundaresha (2006) reported similar observations in RIP II from 2005-06 field evaluation. The high heritability observed for these traits indicates that it is less subjected to environmental influences.

Moderate heritability estimates were noticed for GLA at flowering and yield in RIP I and plant height, number leaves per plant, stem thickness and yield in RIP II. Presence of variability in the population is an important factor and prerequisite for selection to be effective, while heritability indicate how well this can be done. Expected genetic advance is a parameter that considers both variability and heritability and provides a realistic estimate of gain achievable following selection. Hence both populations are useful in simple selection for target traits.

Heritability coupled with high genetic advance over per cent mean was observed for the %GLA30, %GLA45, 100 seed weight, plant height and green leaf area at flowering in both RIP I and RIP II as common traits. In addition, panicle exertion, head length, number of spikelets per head, number of leaves per plant and yield showed high heritability coupled with high genetic advance in RIP I. Days to 50% flowering showed, moderate heritability coupled with, moderate genetic advance over per cent mean in both populations. Likewise, stem thickness and number of spikelets per head showed moderate genetic advance over per cent mean in RIP I and RIP II, respectively. Selection made through these characters would be effective as they are more likely to be controlled by additive gene effects.

Among the stay-green parameters %GLA15 was high and significantly correlated to %GLA45, leaves number per plant, stem thickness, green leaf area at flowering (GLA), at both phenotypic and genotypic level. But GLA30, plant height, and head length are significant and positively correlated at genetic level only. In RIP II, %GLA15 showed highly significant

positive association between %GLA30, %GLA45 and plant height at both phenotypic and genotypic level. Similar results were also obtained by Haussmann *et al.* (2002); Sundaresha (2006).

Highly significant and positive associations were found between %GLA30 DAF and %GLA45 DAF, leaves number per head, stem thickness, head length, GLA and yield at both phenotypic and genotypic level. GLA30 showed highly significant negative association between days to 50% flowering and panicle exertion in both RIPs. In RIP II, %GLA 45DAF and plant height, number of leaves per plant and yield were showed highly significant positive association at both phenotypic and genotypic level.

A highly significant and positive association was found between %GLA45 and leaves number per plant, stem thickness, number of spikelets per head and GLA at both phenotypic and genotypic level. But %GLA45 showed highly positive association with head length at genetic level. GLA 45 showed moderate association between plant height, 100 seed weight and yield in RIP I. In RIP II, %GLA45 showed high significant positive association with number of leaves per plant, number of spikelets per head and yield. Differences among genotypes in % GLA were significantly affected by time to flowering: in the present investigation, highly significant negative association was observed between %GLA at different stages and time of flowering. According to Mahalakshmi and Bidinger (2002), difference among genotypes in %GLA were significantly affected by time of flowering. Later flowering genotypes had a significantly lower %GLA at 30DAF($r=-0, P<0.003$), similar %GLA at 30DAF($r=0; P>0.003$), but significantly higher %GLA at 45 DAF($r=0.43, p>0.001$). Apparently, grain yields were more affected (directly) by the stress in the latter flowering genotypes, and as a result there was less translocation from the leaves. Thus differences among the genotype in the escape from stress, which result in differences in grain number or grain growth under grain filling stress, and consequently in the amount of nitrogen translocation from the leaves, affected leaf senescence in anticipated ways.

Highly significant positive associations were observed between yield and stay-green at different stages in the case of RIP II, while moderate or less correlation was recorded for the same trait in RIP I. In both populations highly significant negative association was observed between yield and stay-green traits at different stage and days to 50 per cent flowering. Similar observation was made by Haussmann *et al.*, (2002) and Mahalakshmi and Bidinger (2002).

Among yield components plant height was highly associated to panicle exertion and head length while negative association was observed against yield. This may be because when the plant posses more than expected height, the food allocation from source to sink of different part will be more and less amount processed food reaches to seed development and consequently resulted in yield reduction.

5.2 MOLECULAR MAPPING AND QTL ANALYSIS

Parental polymorphism

The development of molecular markers has resulted in renewed interest in genetic mapping. Genetic map construction effort is greatly helped by availability of good permanent mapping population such as RILs. It is necessary to enrich genetic maps as more markers become available. Such mapping population could be used by many researchers, simultaneously, so that the mapping process is cumulative, towards the development of saturated molecular genetics map for any species. A variety of DNA markers such as SSR have been used as genetic markers for mapping. EST-SSR unlike the genomic markers, show the position of functional part of the genome in the relation to others. Such markers are useful in providing functional association to the linked marker. Though EST-SSRs are expensive to develop, they are serving as useful as additional markers in mapping and MAS and have the advantage of providing functional relevance to the marked region of the genome

In this study, a total of 530 EST-SSR and 73 nuclear SSR markers were used to identify the parental polymorphism, of which 45 markers could detect polymorphism between the two parents (IS9830 and E36-1). Among 45 polymorphic primers screened, 31 primers were detected on 2.5% agarose gel and 14 primers were detected on 6% PAGE (Poly Acrylimidine Gel Electrophoresis). Out of 45 polymorphic markers, 37(7.0%) and 8 (11.11%) were genic and nuclear SSR, respectively. The polymorphic primers detected between

IS9830 x E36-1 were less as compared to parent N13 and E36-1. This is because i) the genetic distance between IS9830 and E36-1 is less (Hausmann *et al.*, 2002) ii) EST-SSR primers have been reported to be less polymorphic compared with genomic SSR in crop plants because of greater DNA sequence conservation in transcribed region (Scott *et al.*, 2000; Rungis *et al.*, 2004).

In RIP I, a total of 20 genic SSR and 46 anchor markers were used for linkage map construction with the help of mapmaker/EXP (3.0b) software. All 66 markers could be mapped on to the ten linkage group viz A,B,C,D,E,F,G,H,I and J. In RIP II, a total of 44 genic SSR already available genotypic data were used to construct linkage map for QTL analysis. The genetic map of IS9830 x E36-1 (RIP I) and N13 x E36-1 (RIP II) had a total of 1558.1 cM and 1602 with 66 and 44 markers distributed over ten linkage groups in both RIP I and II respectively. All the markers were assigned to different linkage groups on the basis of anchor markers in the linkage map. The marker in the present study covered to all linkage group A to J (plate 3). The number of marker mapped per linkage group ranges from four (LGF) to nine (LGA) and four (LGJ) and nine in (LGI) respectively, for RIP I and II. The markers were distributed unevenly through the linkage group with an average distance of 31.176 cM and 31.77 cM in RIP I & II respectively. The highest and the lowest distance observed were between xisp230-xtxp41 (154 cM) on linkage group D and xtxp105-xtxp 18(1.4 cM) on H, respectively. The number of markers assigned to linkage group A,B,C,D,E,F,G,H,I, and J of different chromosome were 9,6,7,7,5,4,5,6,5, and 6 respectively. Similarly, in Hausmann *et al.*, 2002, the genetic map constructed for RIP I had a total length of 1291.2 cM, with 128 markers (AFLPs, RFLPs, SSRs and RAPDs) distributed over ten linkage groups. For RIP II, they have constructed a genetic distance of 1,438.1 cM which contain 146 markers and distributed over 12 linkage groups. Maximum numbers of markers were assigned to the linkage group I.

In the current investigation, for RILs derived from N13 x E36-1 (RIP II) linkage map construction was done using the basic file on already available mapped markers for QTL identification using present phenotypic data. A total of 44 markers were used to construct the genetic linkage map. Most of SSR markers available at present in sorghum have been mapped in linkage map construction. Selection of some of SSR marker in the present study was based on the linkage map constructed by (Hausmann *et al.* 2002, 2004) and Tuinstra *et al.* (1997)) and from Cornel set of SSR marker for sorghum. Further, through in house effort, several genic SSRs were identified in EST database of sorghum; subsets of these were also used in the present study.

5.3 QTL MAPPING

To locate a QTL between flanking markers, analysis of co-segregation of marker and phenotypic information of traits is important. If the proportion of recombinants with higher or/ and lower phenotypic values is less, tight linkage between flanking markers and QTL is indicated. Theoretically, it is possible to gather desired QTLs into a genotype by marker-assisted selection. QTL mapping is likely to provide information for better and efficient selection strategies for any trait or traits in question. Besides, QTL analysis is the only molecular tool available to dissect a complex quantitative character, governed by many genes with small individual effects, into recognizable genetic units or blocks. Xu *et al.* (2000) reported that the extreme phenotypes of quantitative traits comes from the association of favorable or unfavorable QTL alleles, while a preponderance of intermediate phenotype usually indicate allelic dispersion. The genetic stocks with dispersed QTL alleles usually show similar phenotype, making it difficult to identify genetic differences only by phenotypic evaluation. Given to complex genetic nature of most of economic traits, the knowledge of QTL allelic status in breeding population and the genomic marker associated with them can greatly increase the efficiency of selection by picking individuals by their markers status. Therefore, MAS is a powerful tool applicable to any simple or complex character such as resistance to disease, abiotic stresses, yield, etc.

Linkage map constructed in the present study in populations, RIP1 and RIP II, each consisting 226 individuals of the RIL population. Both RILs were used for QTL mapping. The software QTL cartographer (2.5b) was used for QTL analysis by composite interval mapping (CIM) procedure implemented with a LOD score of 2.5 as threshold value for QTL significance. The additive effect and the estimates of the phenotypic variation were computed.

). In RIP I of the present study QTLs for stay-green were detected on linkage group A, C, D, and E while, in RIP II the QTLs were detected on B, E, and I linkage group. In the present investigation, the highest numbers of QTLs for different traits were detected on the linkage group B and I of RIP II. QTLs with significant effects are likely to be detected when the alleles are in the association than dispersed. Stay-green QTL have been detected and assigned to genetic map of sorghum over the linkage group (Biovine *et al.*, 1999; Peng *et al.*, 1999; Bahatramakki *et al.*, 2000; Haussmann *et al.*, 2000; khannur, 1993).

QTL for stay- green and other traits

Linkage maps are available in sorghum for striga resistance, stay-green of (Haussmann *et al.*, 2002, 2004; Bhatramakki *et al.*, 2000) targeted population. It has been reported that the QTL for the stay-green, the traits that keeps the plant to remain green for the extended period after anthesis is also known to impart terminal drought tolerance and charcoal resistance. It has been reported that QTLs for the stay-green located in the linkage groups; A, B, D, E, G, and H (Tuinstra *et al.*, 1997).

In the present investigation of RIP I, Composite interval mapping detected two major QTLs for % GLA15, two for %GLA30 and three for %GLA45 which explain 19.7%, 25.9% and 31.01% of phenotypic variation respectively. One major QTL on linkage group C is common for three traits of %GLA (15, 30 and 45) at a distance of 175.25 cM. The maximum QTLs were detected on linkage group A and C with a wide distance of 1.4 to 154 cM. Individual coefficient of determination i.e. the percentage of phenotypic variance explained by the individual QTL ranged from 4.9 to 44 per cent. Similar report was made by Haussman *et al.* (2002) on the same population based on phenotypic evaluation of F₃:F₅ families at both India and Africa.

In RIP II of the present investigation, seven QTLs for stay-green trait were detected on linkage group B, D, E, I, which corresponds to report made Kebede *et al.* (2001) and Poornima (2006). One QTL on the linkage group A was common for GLA30 and GLA45, and the other QTL on linkage group C was common for all stay-green traits. Haussman *et al.* (2002) and (2006) reported similar observation on the same population.

Two QTLs for grain yield detected on the linkage group E and I were bracketed by xumc86-xibt343 and xtxp6-xtxp145 at map distance of 127.47 and 28.65 cM explaining 24.08% and 15.8 % for phenotypic variation respectively. Similar report was made by Tuinstra *et al.* (1997) in different population where, they identify three QTLs, associated with grain yield on linkage groups E, F, and G. The yield QTL on linkage group E was associated with long duration of grain fill; the QTL on linkage group F was associated with stay-green. These QTLs accounted for 24% of the variability in grain yield. Two QTLs for panicle exertion detected on the linkage group E and C each explained 28% phenotypic variation.

In RIP II, one QTL for GL15 detected on linkage group B at the position of 62.2 cM was explain a phenotypic variation of 12 % with additive value of -0.92 at LOD score of 2.5. Three QTLs for GLA30 on linkage group B, D and I were explained a total of 17.1% phenotypic variation. Similarly two QTLs detected on the linkage groups E and I were explain 44.1 % of phenotypic variation. In RIP II, Surprisingly, the two major QTLs for high % GLA30, on the linkage group B and on the linkage group E for GLA45 were contributed by non- stay-green line N13. In the same population, Haussmann *et al.* (2002) detected five QTLs for high %GLA15 and eight each for GLA30 and GLA45, which explain the total coverage of 31%, 37% and 38% genetic variation, respectively. In RIP II, at its F₃:F₅ stages, where they found both parents contribute alleles and major QTLs for high percentage of stay-green derived from non-stay-green parent, line N13. At IABT, similar report was made by Sundaresha (2006) on the same population.

Three major QTLs for seed colour detected on the linkage group A, B, D were bracketed by xiabt32-xmun274, xiabt332-xmun192, xtxp27-xtxp327, at a distance of 105.39, 141.05, 68 cM respectively. They accounts 16.0, 33.09, 34.40 per cent phenotypic variance with additive effect of 0.64, 1.64 and 1.65 from male parent at LOD value of 2.92, 2.23, 2.68 respectively. QTL detected on the linkage group A share same chromosomal region with stay-green traits, GLA30 and GLA45. Similarly, the QTL detected on linkage group D was located on the vicinity of one of the stay-green trait (%GLA 15) QTL. The QTLs detected for seed colour on linkage group A and D were associated with stay-green, while, QTL on linkage group B was associated with days to 50% flowering.

FUTURE PLAN OF WORK

- Further saturation of genetic linkage map with additional informative markers is necessary for both QTL analysis and to possibly pin point genes involved in regulating stay green traits.
- Well resolved map will facilitate map based cloning

6. SUMMARY AND CONCLUSIONS

Sorghum grown principally in the semi-arid regions of the tropics and subtropics and has numerous characteristics, including drought tolerance and resistance to various diseases and pests that adapt it to the harsh environments of these regions. Susceptibility to drought can occur during the early vegetative seedling stage, during the period of panicle development prior to flowering and during the post-flowering stage of grain development. Susceptibility during the post-flowering stage is characterized by reduced seed size and grain yield, premature plant and leaf senescence, and increased stalk lodging. Stay-green trait is reported to be associated with post-flowering drought tolerance. The result of this study on evaluation of phenotypic performance of RILs for stay-green and drought related traits, mapping of EST-SSR markers and identification of QTLs for stay-green and other drought related traits are summarized here.

1. Phenotypic evaluation of two recombinant inbred population (RIPs) each consisting of 226 RILs derived from IS9830 x E36-1(RIP I) and N13 x E36-1(RIP II) under post-rainy season (*rabi*), at Main Agricultural Research Station (MARS), Dharwad revealed significant difference between individual RILs and parents for all traits studied.
2. High values of PCV and GCV in both RIPs were observed for %GLA30%, %GLA45, panicle exertion, and head length, number of spikelet per head, yield and green leaf area at flowering indicating that variation for these characters contributed markedly to the total variability.
3. Moderate PCV and GCV were observed for green leaf area at flowering (GLA), plant height, and stem thickness number of leaves per plant
4. In the present investigation, both RIP I & RIP II recorded exceptionally high estimates of broad sense heritability were noticed for character viz., %GLA15, %GLA30 and %GLA45, stem thickness, head length, number of spikelets per head and seed yield per plant
5. Among the stay- green parameter %GLA15 was high and significantly correlated to %GLA45, number of leaves per plant, stem thickness, GLA, at both phenotypic and genotypic level. Significant and positive association were found between GLA30DAF and GLA45DAF, leaves number per plant, stem thickness, head length, GLA and yield at both phenotypic and genotypic level for both populations.
6. In both populations, there was significant negative associations were observed between yield and stay-green traits at different stages against days to 50 % flowering.
7. A total of 530 EST-SSR and 73 Nuclear SSR markers were used to identify parental polymorphism, of which 45 markers could detect polymorphism between the two parents, IS9830 and E36-1. Among 45 polymorphic primers screened, 31 primers were detected on 2.5% agarose gel and 14 primers were detected on 6% PAGE (Poly Acrylimidine Gel Electrophoresis). Out of 45 polymorphic markers, 37(7.35%) and 8 (11.11%) were genic and nuclear SSR, respectively.
8. Construction of linkage map was carried out for both populations by using MAPMAKER/EXP, to ten linkage groups A, B, C, D, F, G, H, I and J in relation to previous maps.
9. QTL analysis indicated that the presence of two major QTLs for GLA15, two QTLs for GLA30 and three QTLs for GLA45 which explain 19.7%, 25.9% and 31.01% of phenotypic variation. One major QTL at linkage group C is common for three traits of GLA (15, 30 and 45) at a distance of 175.25 cM .The maximum QTLs were detected on linkage group A and C with a wide distance of 1.4 to 154 cM.
10. In RIP I, out of seventeen QTLs, seven for stay-green trait, three QTLs for seed colour, and two QTLs for yield, two QTLs each for panicle exertion and days to 50% flowering and one QTL for plant height were identified.
11. In RIP II, out of thirteen QTLs, six QTLs for stay-green trait, two QTLs each for panicle exertion and plant height, one QTL each for stem thickness, head length and yield were identified.

From present investigation we can conclude that Putative QTLs and the corresponding flanking markers for stay-green and other drought tolerance traits have been identified for RIP I, at Dharwad location. In RIP II, QTLs for stay-green have been identified and verified second time for Dharwad location. But further verification is required in across

environment and year for both populations. However, it is necessary to add many more markers to the genetic map, especially the functional marker such as EST-SSR. Saturated genetic map along with complete genome sequence that is available in sorghum will help in the understanding the genetic architecture of qualitative characters.

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APPENDIX

APPENDIX I:

Preparation of stock solutions

Extraction buffer	Tris HCL, PH 8.0	100mM
	EDTA, PH 8.0	50mM
	NaCl	500mM
20%SDS	20g of sodium doedycyle sulphate (sodium lauryl Sulphate) dissolved in 100ml of d H ₂ O. Should not Be autoclaved	
5M potassium acetate	5M potassium acetate	60.0ml
	Glacial acetic acid	11.5ml
	H ₂ O	28.5ml
Isopropanol Ammonium Acetate Mixture	Three volumes of 10M Isopropanol and 1volume of 10M ammonium acetate	
T ₁₀ E ₁	Tris 10mM containing 1mM EDTA	
RNase(10mg/ml)	Dissolve RNase in water, place in a tube in a Boiling water bath for 10min. Allow this to cool on a Bench and store at -20°C	
Chloroform isoamylalcohol (24:1)	Chloroform 240ml isoamyle alchol 10ml. store in a dark room temperature. Make up and dispense the Solution in fumed cupboard.	
Ethanol (70%)	Absolute alchol	70ml
	Distill water	30ml
NaCl 5M	Dissolve 292.2g NaCl in 750ml water. Make up to 1liter with water, filter and autoclave.	
Phenol chloroform (24:1)	Mix equal volumes of the buffered phenol and Chloroform isoamyl alcohol (24:1) store at 4°C	
Sodium acetate (2.5M, PH 5.2)	Dissolve 340.2g sodium acetate in 500ml of water Adjust PH to 5.2 with glacial acetic acid and make Up the volume to 1liter and autoclave.	
Tris HCl (1M, PH 8.0)	Dissolve 212.1g Tris in 800ml of water. Adjust PH 8.0 With conc.HCl. Make up the volume to 1L and Autoclave	
Ethidium bromide (10mg/ml)	Dissolve 100mg ethidium bromide in 10ml of Distil water, wrap tube in aluminum foil and Store at 4°C	

4% acrylamide solution (1000ml)	double distilled water	450ml
	5X TBE	100ml
	Urea	420g
	40% Acrylamide/ bisacrylamide	100ml
	(19:1) (W/W)	
	Combine water and TBE buffer in beaker, heat	
	Using a microwave, add urea and stir until dissolved.	
5x TBE	Adjust the volume any large particles. Then	
	Acrylamide Solution is combined with other	
	Ingredients in a storage container.	
	Tris base	
	EDTA	46g
	Boric acid	
	dH2O to 10L (store at room temperature, if left for long period of time , some of salts will precipitate. It may be advisable to discard this buffer and make fresh buffer	
0.5% acetic acid in 95% ethanol	glacial acetic acid	1ml
	95% ethanol	199ml
	Aliquot and store at room temperature	
10% APS (Ammonium Persulphate)	Ammonium persulphate	1ml
	dH2O	199ml
	Should be store at -20°C. If the APS will be used	
	With in the few weeks, store in light-tight bottle at 4°C.	
TEMED(N,N',N' –tetra		
Methyl ethelen diamine)	store at 4°C	
Binding solution	Bind silane (γ- metacryloxy profile trimethoxy Silane) M-6514 sigma industries	
	4μl of bind silane + 1000μl of 0.5% acetic acid in 95% ehanol	
Repel solution	Repel silane (Dichloro dimethyl silane 99%) M-440272 sigma industries	
	250μg repel silane + 750μg of 0.5% acetic acid in 95% ethanol (should be done in fume hood)	
Loading dye (3X STR loading solution)	5M NaOH	0.2ml
	95% formamide	95ml
	Bromophenol blue	50mg

	Xylene cyonol	50mg
	Sd water make upto	100ml
100bp DNA ladder	100bpmarker (Genetics)	10µl of
	3X SSR dye	95µl
	Sterile dH2O	95µl
Fix/ stop solution (10% acetic acid)	glacial acetic acid in 1800ml dH2O	200ml
Impregnant solution	glycerol	10%
	Glacial acetic acid	10%
Staining solution	siliver nitrate	2g
	37& formaldehyde	3ml
	ddH2O	2000ml
or	Should be stored at room temperature in cabinet other dark storage space	
Developer solution	sodium carbonate	60g
	37% formaldehyde	3ml
	Sodium thiosulphate (10mg/ml)	400µl
	dH2O	2000ml
	Sodium carbonate	60g
use.	This solution must be prepared fresh for each	
carbonate	Prepare the solution by dissolving sodium In water and chilling to about 10°C by placing on Ice or in freezer. Just before use add the formaldehyde Sodium thiosulphate	
Loading Dye (6X)	Bromophenol blue	0.25g
	Sucrose in water	40 %(W/V)
	Stored at 4°C	
50% TAE (Tris- Acetate EDTA)	Tris base	242g/l
	Glacial acetic acid	57.1ml
	0.5 EDTA (PH 8.0)	100ml
	Distilled water	1000ml

APPENDIX II

List of primer used in the present investigation for genetic map construction of RILs derived from IS9830 x E36-1 (RIP II)

Sl. No	Primer name	Forward Sequence	Reverse sequence
1	<i>xiabt439</i>	AAGATTCCCAAAGCTGCTGA	GAGTACCCCTCCGTCGAGA
2	<i>xiabt445</i>	GGGGAGGTTTCAGGAACAAGA	CGGTGGTTCAGTGTGTTTTG
3	<i>xiabt455</i>	GTGCCAGCTTATGTTGAACG	GGAGGAGCATACAGCCACTG
4	<i>xiabt356</i>	AGGAGTCATGTGGCTCCATC	GCATCAACAACTGGACTGG
5	<i>xiabt332</i>	CTGCCATGCTGCTGTACATTA	CTTCCCCACGTCTTCTTCTG
6	<i>xiabt342</i>	CTCAGCCACCATCGCATATC	CTCGGCTACACCTCTGGAAC
8	<i>xiabt343</i>	GGAAGAGTGCCTGAGTGGAG	GGTCCAAGGAATGCTGACATA
9	<i>xiabt364</i>	GCACTAATGCCATGCAAATG	CAGCAGGCAGAGTGTAGGTG
11	<i>xiabt352</i>	GCTACCACGGTTTTTGCCTA	TGAACAGAGAGATTTTCACATGC
12	<i>xiabt358</i>	ACGGCTCAAACACCAACTTTG	CTCCAACGTGGAGAGCACACA
13	<i>xiabt184</i>	CCACCAGGGGGTTGTATATG	GAGCTGCAACACAAGGACAA
14	<i>xiabt176</i>	GCAAGGAAGAACCCCTTCTC	GCAATGGAAGTCGACGTAGG
15	<i>xiabt147</i>	GTCGTCGTCTTCCTCCAAGA	AAACGTCAAATTCTAGCTCCTATCA
16	<i>xiabt32</i>	GCTACCACGGTTTTTGCCTA	TGAACAGAGAGATTTTCACATGC
17	<i>xiabt54</i>	ACGGCTCAAACACCAACTTTG	CTCCAACGTGGAGAGCACAC
18	<i>txtp96</i>	CCACCAGGGGGTTGTATATG	GAGCTGCAACACAAGGACAAGC
19	<i>txtp94</i>	GCAAGGAAGAACCCCTTCTC	GCAATGGAAGTCGACGTAGG
20	<i>txtp113</i>	GTCGTCGTCTTCCTCCAAGA	AAACGTCAAATTCTAGCTCCTAT

MOLECULAR MAPPING OF STAY-GREEN AND OTHER DROUGHT TOLERANCE RELATED TRAITS IN SORGHUM [*Sorghum bicolor* (L.) Moench]

ADDISSU GEBRE 2008

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ABSTRACT

The stay-green trait reported component of tolerance to terminal drought stress in sorghum. To map quantitative trait loci (QTLs) for stay-green and other drought tolerance related traits two recombinant inbred populations (RIPs) of F_8 lines developed from the crosses IS9830 x E36-1 (RIP I) and N13 x E36-1 (RIP II) were used. The stay-green source line was E36-1 while IS9830 and N13 were non-stay-green lines. A total of 530 genic SSR and 75 nuclear SSR were used for parental survey of which 45 polymorphic SSR were identified with using both 6% PAGE and 2.5 agarose gel. From the total 45 polymorphic primers 20 were genotyped across RILs and used for genetic map construction together with 46 anchor primers. The genetic maps of RIP I and RIP II spanned 1559.1 cM and 1608 cM with 66 and 44 markers distributed over 10 linkage groups, respectively.

The two RIPs were phenotyped during post-rainy season at Main Agricultural Research Station (MARS), Dharwad. The measures of stay-green were the green leaf area percentage at 15, 30 and 45 days after flowering (%GLA 15, %GLA30 and %GLA45, respectively). The QTLs detected for % GLA15, %GLA30 and %GLA45 days after flowering explained a phenotypic variations of 34.3, 15.8 and 49.1 in RIP I, and 17, 44.1, and 14 percent in RIP II, respectively. Putative QTLs for other drought tolerance related traits viz. plant height; panicle exertion, head length, and grain yield were detected. Estimated broad sense heritability for %GLA15, %GLA30 and %GLA45 amounted to 0.68, 0.93 and 0.81 in RIP I and 0.67, 0.88, 0.93 in RIP II, respectively. Across the three measures of stay-green, one QTL on linkage group D was common for both RIPs with the stay-green alleles originated from E36-1. The QTLs detected for both population for stay-green and drought tolerance related traits needs validation across year and environment.