"ADVANCING OF SSR BASED MARKER ASSISTED BACKCROSSING OF STAY GREEN QTLs IN TO ELITE SORGHUM LINES, S 35 AND IRAT 204"

BY

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CERTIFICATE

Mr. SRIPATHI VENKATESWARARAO, has satisfactorily prosecuted the course of research and that the thesis entitled **"ADVANCING OF SSR BASED MARKER ASSISTED BACKCROSSING OF STAY GREEN QTLs IN TO ELITE SORGHUM LINES, S 35 AND IRAT 204"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

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CERTIFICATE

This is to certify that the thesis entitled **"ADVANCING OF SSR BASED MARKER ASSISTED BACKCROSSING OF STAY GREEN QTLs IN TO ELITE SORGHUM LINES, S 35 AND IRAT 204"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN AGRICULTURE** of the Acharya N.G. Ranga Agricultural University, Hyderabad is a record of the bonafide research work carried out by **Mr. SRIPATHI.VENKATESWARARAO** under our guidance and supervision. The subject of the thesis has been approved by the Students Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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I, **SRIPATHI VENKATESWARARAO**, hereby declare that the thesis entitled **"ADVANCING OF SSR BASED MARKER ASSISTED BACKCROSSING OF STAY GREEN QTLs IN TO ELITE SORGHUM LINES, S 35 AND IRAT 204"** submitted to the Acharya N.G. Ranga Agricultural University for the degree of **MASTER OF SCIENCE IN AGRICULTURE** is a result of original research work done by me. It is further declared that the thesis or part thereof has not been published earlier in any manner.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Per cent
^{0}C	Degree Celcius
3'	Three prime
5'	Five prime
ABA	Abcisic acid
ABI	Applied Biosystems
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BAC	Bacterial Artificial Chromosome
BC	Backcross
Bp	Base Pair
cDNA	complementary DNA
CGIAR	Consultative Group of International Agriculture
Chl	Gene controlling chlorophyll content
CIMMYT	International Maize and Wheat improvement centre
cM	centimorgan
CTAB	Cetyl Trimethyl Ammonium Bromide
DDW	Double distilled water
DHLs	Double Haploid Lines
DNA	Deoxyribo Nucleic Acid
dNTPs	Dinucleotide tri-phosphates
EDTA	Ethylene Diamine Tetra Acetic acid
ESTs	Expressed Sequence Tags
et al.	and others
EtBr	Ethidium Bromide
F_1	First filial generation
F_2	Second filial generation
FAO	Food and Agricultural Organization
Fig.	Figure

FISH	Fluorescent Immuno Hybridization
g	grams
g/g	gram per gram
g/L	gram per litre
G×E	Genotype x Environment
gDNA	genomic DNA
GLA	Green Leaf Area
GLAM	Green Leaf Area at Maturity
h	hours
ha	hectare
i.e.,	which is to say, in other words
IAA	Iso-Amyl Alcohol
ICRISAT	International Crop Research Institute for Semi-Arid Tropics
kg	Kilogram
L	Litre
LD	Linkage Disequilibrium
LG	Linkage Group
LOD	Log of Odds ratio
M	Molar
M.ha	Million hectares
mA	Milli Ampere
MAB	Marker Assisted Backcrossing
MAS	Marker Assisted Selection
MAYG	Mapping As You Go approach
Mb	Million bases
mbar	Milli bar
mg	milligram
mg/L	milligram per litre
min	Minutes
ml	Milli litre
mM	Milli molar

ng	Nanogram
NIL	Near-Isogenic Line
nm	Nanometer
OD	Optical Density
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pH	Negative logarithm of hydrogen ion concentration
PIC	Polymorphism Information Content
Pmol	Picomoles
ppm	Parts per million
QPM	Quality Protein Maize
QTL	Quantitative Trait Loci
RAPD	Randomly Amplified Polymorphic DNA
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RIP	Recombinant Inbred Parent
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
RUE	Radiation Use Efficiency
SBI	*Sorghum bicolor* chromosome
SCAR	Sequence Characterized Amplified Region
SG	Stay green
SLN	Specific Leaf Nitrogen
SPAD	Soil Plant Anlysis Development
SSC	Saline Sodium Citrate
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeat
stg	Stay green QTL
STR	Short Tandem Repeat
STS	Sequence Tagged Sites

TAMU	Texas Agriculture and Mechanical University
Taq polmerase	Enzyme from *Thermus acquaticus*
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	N, N, N', N'-Tetra Methylethyline Diamine
TPLA	Total Plant Leaf Area
U	Enzyme Units
U/µl	Unit/microletre
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
V	Volts
v/v	Volume by volume
VNTR	Variable Number of Tandem Repeat
vol	volume
w/v	Weight by volume
w/w	weight per weight
λ	Lambda
µg	Microgram
µl	Microlitre
µM	Micromolar

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ABSTRACT

Sorghum bicolor (L.) Moench (2n 20) is the most important drought tolerant cereal crop and is the crop of choice in semi arid tropics. After soil nutrient deficiencies, drought stress is the major constraint limiting the sorghum production. Drought that effects during post flowering stage is often referred as terminal drought. The trait associated with terminal drought tolerance is stay green, which is complex and difficult to score with normal breeding approaches. Using MAS in breeding programs it is possible to introgress drought tolerant QTLs (*stg1, stg2, stg3, stg4, stgA, and stgB*) from the donor parent (B35) into the genetic backgrounds of elite parents like S35, ICSV 111, and IRAT 204. The homozygous QTL introgression lines

in the background of S35 and ICSV 111 were generated as a part of the present investigation by using MAB. Based on marker data obtained the seeds of self-selected plants are send to Ghana for initial phenotypic evaluation trails. In addition, the selected backcross progenies of the recurrent parent IRAT 204 were advanced to BC_3F_1 and BC_4F_1 generations. Sorghum is the logical complement of other important cereal crops and has made unique contributions in understanding the genetic basis of cereal domestication. The sequencing of sorghum will definitely help the plant breeders and molecular biologists in tracking out the unknown agronomically important genes or QTLs.

Chapter - I

Introduction

CHAPTER I

INTRODUCTION

1.1 Prologue

The production and productivity of major crop plants have reached plateaus during the past decade following adoption of green revolution technologies. However, further increases in production of cereals with improved cereal quality is imperative to feed the increasing population in spite of shrinking available land due to urbanization and industrialization. Advances in cellular and molecular biology have developed new scientific tools to help overcome some of the limitations of conventional crop breeding technologies. Now technology is in transition from the green revolution to the gene revolution (Borlaug, 2003). Developments of saturated molecular maps of many crop species and association of several genes of agronomic importance with DNA markers have increased the confidence of plant breeders for their utilization in crop improvement (Khush, 1999; Young, 1999). However, reduction of losses caused by abiotic and biotic stress will continue to be a key issue in enhancing food security.

1.2 Sorghum crop and its utility

Sorghum [*Sorghum bicolor* (L.) Moench] belongs to the family poaceae and order panicoidiacae. Sorghum is the fifth most important cereal crop globally after wheat, maize, rice and barley, and third most important cereal crop in India after wheat and rice (FAOSTAT, 2004; http://faostat.fao.org/default.jsp). In Andhra pradesh sorghum is the second most important cereal crop after rice (Survey of Indian Agriculture; The Hindu, 2005). This C_4 grass first originated in Africa and it is now grown in more than 80

countries, mostly in tropical and sub-tropical regions. Nmety percent of the world's cultivated sorghum area is in developing countries, mainly in Africa and Asia. As a continent, Africa is the largest producer of sorghum grain with approximately 18.5 M tons produced annually. World sorghum production was about 57 million metric tons during 2003-04 (FAO, 2004; Casa *et al.*, 2005). The global area occupied by the sorghum was about 61 million ha (NASS, 2004). Leading producers of sorghum around the world includes the USA, Nigeria, India, and Mexico. In India sorghum is cultivated both in kharif (rainy) and rabi (post-rainy) seasons. The area occupied by the crop during fiscal 2003-04 was around 9.49 M.ha and the total production was about 7.33 M.t with an average productivity of 772 kgs/ha, but the lower productivity during rabi was due to invariable terminal drought as the crop is grown only with residual soil moisture (Survey of Indian Agriculture; Hindu, 2005).

Sorghum is one of the most drought tolerant cereal crops currently under cultivation. It can tolerate even high temperatures above 38°C. It is often referred to as poor man's crop. Its remarkable ability to produce a crop under adverse conditions, in particular with much less water than most other grain crops, makes sorghum an important "failsafe" source of food, feed, fiber, and fuel in the global agroecosystem. Sorghum is a versatile grain, and can be used in making unleavened breads, boiled porridge or gruel, malted beverages and popped grains. From sweet sorghum jaggery and syrup is obtained, which will be useful in confectionaries. Also the stalks of sweet sorghum are used in ethanol production, which is a source of biofuel. Starch is obtained from the waxy sorghums, useful in sizing industry. The red dye is extracted from sorghum is used in the leather industry in West Africa. Some sorghum varieties are rich in antioxidants and all

sorghum varieties are gluten-free, although there are several research programs globally that are attempting to transform sorghum with wheat gluten genes in order to improve the utility of sorghum flour for making leavened bread. Sorghum has distinct advantages when used in a crop rotation scheme, especially with cotton or wheat.

Durra, kafir, guinea, bicolor and caudatum are the five races of cultivated sorghum. Sorghum (2n=2x=20) is predominantly a self-pollinated crop. Estimates of the physical size of the sorghum genome range from 700 Mb based on Cot analysis (Peterson *et al.*, 2002) to 772 Mb based on flow cytometry (Arumuganathan and Earle, 1991). This makes the sorghum genome about 60% larger than that of rice, but only about one-fourth the size of the genomes of maize or human. GC content is estimated at 37.7%. DNA renaturation kinetic analysis (Peterson *et al.*, 2002) shows the sorghum genome to be comprised of about 16% foldback DNA, 15% highly repetitive DNA (with individual families occurring at an average of 5,200 copies per genome), 41% middle-repetitive DNA (average 72 copies), and 24% low-copy DNA. The sorghum gene space is presently represented by approximately 200,000 expressed sequence tags (ESTs) that have been clustered into approximately 22,000 unisctipts, representing more than 20 diverse libraries from several genotypes.

As a model organism for tropical grasses that carry out C_4 photosynthesis, sorghum is a logical complement to the C_3 grass Oryza, the first monocot plant sequenced and the sorghum is the second cereal crop targeted for sequencing.

1.3 Terminal drought tolerance in sorghum

Drought acts as a major limiting factor in agricultural production. Drought stress does not allow the crop to reach its fullest economic yield. In agriculture, drought

tolerance refers to the ability of a crop plant to produce its economic product with minimum loss in a water-deficit environment relative to a water-constraint-free production environment. In sorghum after soil nutrient deficiencies, drought is the major abiotic constraint. Production of sorghum in semi-arid regions of the world is limited by drought. An understanding of the genetic basis of drought tolerance in crop plants is considered to be a pre-requisite to evolving superior genotypes through either conventional breeding methodology or biotechnological approaches. Developing plants that are more tolerant to drought is a major challenge for sorghum improvement programs globally.

In sorghum Pre-Flowering and Post-Flowering stages are more sensitive to drought stress than the Vegetative stage. Drought that occurs during post-flowering stage of crop growth, and is not relieved, is often referred to as ***terminal drought***. Genotypes sensitive to this type of drought are characterized by premature leaf and plant death (senescence), stalk collapse and lodging, charcoal rot, and reduced seed size. In sorghum, the best-characterized form of tolerance to drought stress during this post-flowering stage of crop growth is the so-called "***stay green***" trait. The stay-green trait is associated with terminal drought tolerance.

1.4 Stay-green trait in sorghum

The stay-green trait can be defined as the ability to resist premature plant senescence, retain green leaves, fill grain normally, and resist lodging under conditions of post-flowering drought stress (Rosenow, 1987). When water is limited during the grain-filling period, sorghum genotypes possessing this trait maintain more photosynthetically active leaves compared with genotypes not possessing this trait. According to Tenkouano

et al., (1993), non-senescence or stay-green is a mechanism of delayed leaf and plant death that circumvents the detrimental effects of reduced soil moisture combined with high temperatures during drought stress that occurs during post-anthesis crop growth. Stay-green can be defined as extended foliar greenness during grain-filling under post-anthesis drought stress, and can be viewed as a consequence of the balance between N demand by the grain and N supply from the roots during grain filling (Borrell *et al.*, 2000b).

Different types of stay-green have been recognized (Thomas and Howarth, 2000). Some are cosmetic and are not photosynthetically active, whereas others are associated with greater biomass accumulation. Grain yield is the product of grain number and grain size. As the Green Leaf Area at Maturity (GLAM) and specific leaf nitrogen (SLN) values are higher for the genotypes possessing the stay-green trait, it can potentially increase grain yield by improving both grain number and grain filling (Borrell *et al.*, 2000c).

1.5 Quantitative traits and molecular markers

Quantitative traits are governed by several genes; each gene has a small effect, which is usually cumulative although there may be interactions involving alleles at one or more of the loci concerned. These characters are often considerably affected by the environment. Genetic factors that are responsible for a part of the observed phenotypic variation for a quantitative trait can be called quantitative trait loci (QTLs). In QTL mapping the association between observed trait values and presence/absence of alleles of markers is mapped onto a linkage map and analysed. Also the size of the allelic effect of the detected QTL can be estimated.

Most of the agronomic traits are quantitative in nature. The use of DNA-based markers for the genetic analysis and manipulation of important agronomic traits has become an increasingly useful tool in plant breeding. General advantages of DNA markers include their ability to reveal the sites of variation in DNA segments, their abundance compared to phenotypic markers, and their immunity to genotype by environment interactions. The PCR-based co-dominant SSR markers have become the marker class of choice for many plant species. The wider adaptability of SSRs is that they are small repetitive DNA sequences, which are spread through out the genome of eukaryotes, are often highly polymorphic due to variation in numbers of repeated motif units.

However, the greatest potential of molecular markers appears to be in assessing genetic diversity and in accelerating the rate of gain from selection for desirable genotypes and in the manipulation of quantitative trait loci (QTLs) that condition complex economic traits. Molecular markers have been used to identify and characterize QTLs associated with several different traits in sorghum including drought tolerance (stay green trait). Several linkage maps are available in sorghum (Xu *et al.*, 1994; Chittenden *et al.*, 1994; Dufour *et al.*, 1996; Boivin *et al.*, 1999; Bhattramakki *et al.*, 2000). Recently different research groups have developed integrated functional sorghum genetic linkage map with 10 linkage groups (Menz *et al.*, 2002; Haussmann *et al.*, 2002a; Bowers *et al.*, 2003 and Kim *et al.*, 2005).

Effective use of marker-based selection or marker-assisted introgression should permit more efficient identification of genetic recombinants than is typically possible in traditional breeding based upon conventional phenotypic selection protocols.

1.6 Marker-assisted selection in Backcross breeding programs

Selection of a genotype carrying a desirable gene or gene combination via linked marker(s) is called marker-aided selection. Breeders practice marker-aided selection when a gene or genomic region controlling an important trait, that is difficult to assess, is tightly linked to another Mendelian trait, which can be easily scored. Molecular marker-aided selection involves scoring for the presence or absence of a desired plant phenotype indirectly, based on DNA banding pattern of linked markers on a gel or on a autoradiogram depending on the marker system. The rationale is that the banding pattern revealing parental origin of the bands in segregants at a given marker locus indicates presence or absence of a specific chromosomal segment that carries the desired allele. This can increase screening efficiency in the breeding program, at least for traits that are normally difficult to assess phenotypically, provided that robust associations between specific marker alleles and genomic regions controlling such traits can be established.

Markers are efficient in introgression backcross programs for simultaneously introgressing an allele and selecting for the desired genomic background. The study presented in this thesis focuses on the use of some of the modern biological tools (marker-assisted selection) for genetic crop improvement in sorghum.

Objectives of this investigation include:

1) Advancing SSR-based marker-assisted backcrossing of selected stay-green drought tolerant QTLs from donor parent B35 into the genetic backgrounds of elite sorghum recurrent parents S 35 and IRAT 204.
2) Generation of homozygous stay green QTL introgression lines in back ground of S 35.
3) Advancing from BC_2F_1 to BC_3F_1 in background of IRAT 204.

Chapter - II

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Available literature on the Marker-Assisted Backcrossing (MAB) of stay-green QTLs into elite sorghum lines is reviewed in this chapter

2.1 Stay-green trait, its physiology and gene action

Wanous *et al.,* (1991) have studied the visual ratings of stay-green trait and reported that visual ratings for the percentage of green leaf area and for the number of green leaves was highly correlated with measured green leaf values under drought stress.

Delayed leaf-senescence, or stay-green, has been regarded as a desired characteristic for the production of a number of crops including sorghum. The stay-green character in sorghum is a post-flowering drought tolerance trait, which makes plants resistant to premature senescence under drought stress during the grain filling stage (Thomas and Smart, 1993).

Tenkouano *et al.,* (1993), investigated the inheritance of charcoal rot resistance directly, by exposure of sorghum to *Macrophomina phaseolina,* and indirectly, by determination of the inheritance of non-senescence They evaluated diallel crosses between two non-senescent, charcoal rot resistant inbred lines (B35 and SC599-11E) and two senescent, charcoal rot susceptible inbreds (B1x378 and BTx623) under controlled field conditions. They determined that non-senescence was regulated by dominant and recessive epistatic interaction between two non-senescence-inducing loci and a third locus with modifying effects. They also concluded that non-senescence and

charcoal rot resistance are not different manifestations of a single trait, i.e., they are not to be evaluated with each other.

Walulu *et al.,* (1994), studied the mode of gene action for the stay-green trait in sorghum. F_1 and F_2 backcrosses obtained from a cross between B35 (stay-green trait donor) and Tx7000 (drought sensitive) were subjected to moisture stress at the grain filling period in the field and in rainout shelters. Stay-green was evaluated on an individual plant basis by visually scoring leaf and plant death. Their results suggested that a major gene influences this stay-green trait in B35, and that this gene exhibits varied levels of dominant gene action depending on the environment in which the evaluation is made. The frequency distributions of the field-grown BC_1F_1 population indicated complete dominance of this single major gene.

Van Oosterom *et al.,* (1996), based on their diallel analysis study of stay-green in sorghum, determined that the expression of heterosis for non-senescence was stable across environments/experiments. The inheritance of the timing of onset of senescence was additive, but a slow senescence rate was dominant over a fast rate.

Borrell *et al.,* (1998), suggested that genotypic variation for the stay-green trait was observed in grain sorghum hybrids when water is limiting during grain filling stage. Green leaf area at maturity (GLAM) is an excellent indicator of stay-green. The key components determining GLAM are: 1) total plant leaf area (TPLA), 2) duration of leaf senescence, and 3) rate of leaf senescence. Duration of leaf senescence is a function of the timing of the onset of senescence and the timing of physiological maturity. They examined nine hybrids from the crosses of three females varying in rate of leaf senescence (AQL39, senescent; AQL41, intermediate; A35, stay-green) and three males

similarly varying (R69264, senescent; RQL36, intermediate; RQL12, stay-green), enabling a comparison of the A35 and RQL12 sources of stay-green. They found genotypic variation for TPLA, onset of leaf senescence, duration of leaf senescence, and rate of leaf senescence. They concluded that the mechanism(s) of leaf area maintenance also varied between the A35 and RQL12 sources of stay-green.

Borrell *et al.*, (1999), found that sorghum grain size was correlated with the relative rate of leaf senescence during grain filling such that reducing the rate of leaf senescence from 3% to 1% loss of leaf area per day resulted in doubling of grain size from about 15 mg to 30 mg. Thus the stay-green trait has potential to increase sorghum grain yield by improving both grain number and grain filling ability.

Thomas and Howarth (2000), studied the stay-green trait physiologically by measuring the progress rate of senescence in several plant species. They observed that although the stay-green phenotype is superficially similar in all species and genotypes, the genetic and physiological routes to the trait are diverse. They classified the stay-green phenotype into five types (A, B, C, D and E). In Type A stay-greens, senescence is initiated late but then proceeds at a normal rate; Type B stay-greens initiate senescence on schedule, but thereafter senesce comparatively slowly; in Type C stay-green behaviour, chlorophyll may be retained more or less indefinitely; Type D confers stay-green by killing the leaf through drying or freezing; in Type E the photosynthetic capacity of an intensely green genotype follows the normal ontogenic pattern, but comparison of absolute pigment contents identifies it as a stay-green. Type A and B are the more functional stay-green types.

Borrell and Hammer (2000), observed that when sorghum hybrids were grown under terminal water deficit conditions, stay-green could be viewed as a consequence of the balance between nitrogen demand by the grain and nitrogen supply by the roots during grain filling. More nitrogen is allocated to the leaves of stay-green hybrids compared with their senescent counterparts, resulting in higher specific leaf nitrogen (SLN) levels. It is hypothesised that this higher SLN initiates a chain of responses, including enhanced radiation use efficiency (RUE) and transpiration efficiency (TE), which enable the plant to set a higher yield potential by the time of anthesis, ultimately leading to higher grain yield potential and lodging resistance under post-anthesis drought stress conditions. Earlier, Sinclair and Horie (1989), in maize and Muchow and Sinclair (1994), in sorghum demonstrated the positive correlation of photosynthetic capacity with specific leaf nitrogen (SLN).

Borrell *et al.*, (1999, 2000), reported that stay-green and grain yield were positively associated in sorghum in a range of studies conducted in both Australia and India, highlighting the value of retaining green leaf area under conditions of post-anthesis drought stress. 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).

Borrell *et al.*, (2000), observed that stay-green sorghum hybrids produced 47% more post-anthesis biomass than their senescent counterparts under terminal moisture deficit conditions.

Xu *et al.*, (2000), measured sorghum foliage chlorophyll content, non-destructively with a chlorophyll meter (SPAD values) and conventionally using a spectrophotometric method. The SPAD value had a significant linear relationship with total leaf chlorophyll (R^2=0.91) and with visual stay-green rating (R^2=0.82).

Van Oosterom *et al.*, (2001), described the effects of N-stress on development and growth of sorghum by identifying critical values for stover N content (SNC) and specific leaf nitrogen (SLN) for a range of physiological processes. Below the critical values (adjusted R^2 value for SNC is 0.98 and for SLN it is 0.78), the relative rates of processes declined linearly with declining SNC or SLN.

Payton *et al.*, (2003), suggested that the identification of genetic factors underlying the complex responses of plants to drought stress provides a solid basis for improving drought tolerance. By using B35 (drought tolerant/stay-green parent) and Tx7000 (drought sensitive/senescent parent), they have constructed two drought-induced, subtracted cDNA libraries. From these they have analyzed all sequences that are unique to specific stressed parent lines and assembled a non-reluctant collection for gene expression profiling studies. Tissue for gene expression studies was collected from field trials and greenhouse experiments from B35, Tx7000, and both recombinant inbred lines and near-isogenic lines containing specific QTLs for the stay-green trait. Physiological analyses of stress plants included gas-exchange analysis for photosynthesis, transpiration, and stomatal conductance, as well as leaf green-ness, and chlorophyll content. They have integrated the phenotypic analysis, physiological data, and expression profile data to characterize genes controlling the stay-green trait.

Jiang *et al.*, (2004), analyzed the genetic basis of the stay-green trait in rice using a population of 190 doubled haploid lines (DHLs) and defined six component traits to assess the stay-green character. They have constructed a genetic linkage map with 179 SSR (simple sequence repeat) marker loci. Also they have applied the software QTLMapper, based on a mixed linear model approach, to detect QTLs, epistatic effects and their environmental interactions for these components of the stay-green trait. A total of 46 main-effect QTLs were detected for the six component traits that can be localized to 25 chromosomal regions. The individual effects of all the QTLs were small. Fifty digenic interactions were resolved that involved 66 loci distributed on all 12 chromosomes. Environmental interactions were detected for 18 of the main-effect QTLs and 14 of the epistatic interactions. The epistatic effects and QTL interactions accounted for large proportions of the observed phenotypic variation.

Subedi *et al.*, (2005), investigated that the growth, N uptake and partitioning patterns of three contrasting maize hybrids [a conventional hybrid (Pioneer 3905), one having the stay-green trait (Pioneer 39F06Bt), and one with the leafy trait (Maizex LF 850-RR)] under controlled conditions. Their results indicated that stay-greenness in maize was exhibited only when there is an adequate supply of N in the growing medium and it is not associated with greater N acquisition.

2.2 Evolution of DNA markers

Earlier morphological markers were found to be a valuable source in varietal identification and for assessing genetic diversity, but they are having certain limitations. Later markers based on protein differences were widely used. Iso-electric variants of proteins, referred to as isozymes, were found to be important markers for specific

chromosome/chromosome regions. Many studies have aimed at assessing the genetic diversity of different crops using allozyme markers (Morden *et al.*, 1989). However, the ultimate differences between individuals lies in the nucleotide sequences of their DNA. Detection of such differences employing various molecular biology techniques has led to development of DNA-based molecular markers. Molecular markers follow simple Mendelian patterns of inheritance. They are stable and not influenced by developmental or environmental factors. DNA-based molecular markers are based on two techniques: 1) hybridization (Southern, 1975) and 2) the polymerase chain reaction (PCR, Mullis *et al.*, 1986). Restriction fragment length polymorphisms (RFLP, Wyman and White, 1980) were the first DNA-based molecular marker system, and were conceived and developed by Botstein *et al.*, (1980). Later various types of molecular markers such as random amplified length polymorphic DNA (RAPD, Williams *et al.*, 1990), amplified fragment length polymorphism (AFLP, Vos *et al.*, 1995) are used in assessing the genetic diversity of crop plants. Recently, microsatellite or SSR (Simple Sequence Repeat, Jacob *et al.*, 1991) loci, which correspond to tandemly repeated DNA with a very short repeat unit, have been identified as powerful genetic markers in plants (Morganate and Oliveri, 1993; Powell *et al.*, 1996a). Comparative studies in crop plants have shown that microsatellite markers are more variable than most other molecular markers (Powell *et al.*, 1996b; Taramino and Tingey, 1996; Pejic *et al.*, 1998) and provide a powerful methodology for discriminating between genotypes (Yang *et al.*, 1994; Russell *et al.*, 1997; Bredemeijer *et al.*, 1998).

2.3 Microsatellites or SSR's

Microsatellites are tandem arrays of short stretches of 2-6 base pairs in length, usually repeated about 15 to 30 times (Bennett, 2000). They are found both in prokaryotes and in eukaryotes. They appear scattered randomly throughout the genome. Earlier Jeffreys *et al.*, 1995 used the term minisatellites for microsatellites. Litt and Lutty (1989) introduced the term "microsatellite" to characterize the simple sequence stretches amplified by polymerase chain reaction (PCR). They were also described as simple sequence length polymorphism (SSLP) by Tautz (1989), as short tandem repeats (STRs) by Edwards *et al.*, (1991), and as variable tandem repeats (VTRs) by Nakamura (1987).

Particular characteristics of microsatellites, such as their presence in genomes of all living organisms, high levels of allelic variation, co-dominant mode of inheritance, and potential for automated analysis, make them excellent molecular markers for a number of uses, like genotyping, mapping or positional cloning of genes.

SSRs have been reported in many plant genomes such as maize, rice, sorghum, barley, soybean, brassicas, and sunflower (Tang *et al.*, 2000). The first application of microsatellite markers in plants has been in cultivar identification and now they are markers of choice in genotyping cultivars (Weising *et al.*, 1991; and Beyermann *et al.*, 1992).

The informativeness of a polymorphic marker depends upon the number of alleles and their relative frequencies. Botstein *et al.*, (1980) described the Polymorphism Information Content (PIC), which is a statistical assessment of informativeness of a marker. The greater the number of alleles at a given locus, the more informative will be

the marker for the purpose of discriminating between genotypes. However, for some purposes such as genetic diversity assessment, markers that have very large numbers of relatively rare alleles can be problematic and for such uses marker loci having a small number of relatively common alleles may be easier to use.

Haley *et al.*, (1994) demonstrated how marker information content (or polymorphism) is directly and positively related to the mean maximum test statistic in quantitative trait loci (QTL) analysis. Microsatellite information was found to be useful in assessing the genetic relationship both within and between populations (Peelman *et al.*, 1998).

2.4 Application of molecular markers in sorghum crop improvement

Molecular markers have proven to be robust and cost-effective for assessment of sorghum genetic diversity (Deu *et al.*, 1994; Oliveira *et al.*, 1996; Yang *et al.*, 1996). Genetic diversity in sorghum has been estimated utilizing several types of molecular markers (Tao *et al.*, 1993; Vierling *et al.*, 1994; Brown *et al.*, 1996; Taramino *et al.*, 1997; Uptmoor *et al.*, 2003). A set of 15 microsatellite or SSR markers has been developed for sorghum that allows a very high rate of discrimination in sorghum genetic diversity assessment (Dean *et al.*, 1999; Dje *et al.*, 2000; Grenier *et al*., 2000; Smith *et al.*, 2000). Ahnert *et al.*, (1996) used a set of 104 RFLP probes to evaluate the genetic diversity among a large set of elite proprietary sorghum inbred lines.

Studies have shown that SSR loci give good discrimination between closely related individuals in some cases even when only a few loci were employed (Powell *et al.*, 1996a; Scotti *et al.*, 2000; Kong *et al.*, 2000). The analysis of SSRs has been automated (Saghai Maroof *et al.*, 1984; Powell *et al.*, 1996b).

Pereira *et al.*, (1994) performed segregation analysis on F_2 population using 7 SSR loci in order to verify the reliability of SSR-derived polymorphism for sorghum genetic mapping.

Brown *et al.*, (1996) developed 15 SSR markers and were able to identify polymorphic loci among 17 temperately and tropically adopted lines of sorghum. Moreover the 15 SSR marker loci are widely spread on the sorghum genome and 14 of them have mapped to nine of ten sorghum linkage groups (Dean *et al*., 1999). Dean *et al.*, (1999) also assessed the genetic diversity among 95 'Orange' accessions of sorghum in the USDA germplasm collection and found 3 to 11 alleles per locus by using same set (15) of SSR markers. Taramino *et al.*, (1997), Tao *et al.*, (1998a), and Kong *et al.*, (2000) have reported 46 sorghum SSR loci. Kong *et al.*, (2000) characterized 38 sorghum SSR loci. They were followed by Bhattramakki *et al*., (2000), who reported primer sequences for 147 sorghum SSR loci, as well as genetic linkage map locations for 113 of these. More recently, Schloss *et al.*, (2002) reported nearly 70 additional sorghum SSR primer sequences derived from sorghum cDNA clones that had previously been mapped as RFLP markers.

Ghebru *et al.*, (2002) carried out an analysis to assess genetic diversity of 28 Eritrean landraces of sorghum, using a high through put SSR-based strategy developed by Kresovich and coworkers (Dean *et al.*, 1999; Smith *et al.*, 2000).

Uptmoor *et al.*, (2003 carried out comparative analysis on the genetic relatedness of 46 sorghum accessions from Southern Africa by using molecular markers like AFLPs, RAPDs, SSRs and they concluded that all of these sorghum accessions were uniquely fingerprinted by all three marker systems.

Casa *et al.*, (2005) assayed 98 simple sequence repeat (SSR) loci distributed throughout the genome in a panel of 104 accessions comprising 73 landraces and 31 wild sorghums. Evaluation of SSR polymorphisms indicated that landraces retained 86% of the diversity observed in the wild sorghums. Statistical methods (Ewens-Watterson test) for identifying genomic regions with patterns of variation consistent with selection gave significant results for 11 loci, out of which seven of these loci mapped in or near genomic regions associated with domestication-related QTL's.

Folkertsma *et al.*, (2005) reported use of 21 SSR markers to assess genetic diversity in the Guinea-race of sorghum in support of a breeding program aiming to develop F_1 hybrid cultivars within this race as a means of increasing food security in the more humid regions of Western and Central Africa with this race of sorghum find favor due to its reduced vulnerability to damage by headbugs and grain molds.

2.5 QTL and gene action

Concerning the nature of loci controlling the variation in quantitative traits, QTL can be defined in four different ways (Mackay *et al*, 1992). Quantitative trait loci (QTL) (1) are "major" genes having pleiotropic effects on other traits (see Barton, 1990 for a review); (2) are different from major genes in that alleles at QTL are constrained to having only small effects on the character (Mather, 1941); (3) are modifiers of the expression of major loci (Mukai and Cockerham, 1977); and (4) have alleles with a range of effects: alleles with large effects cause recognition of the locus as a gene with major effects, and segregation of alleles with small effects gives rise to quantitative variation (Robertson, 1985).

Gene action type (additive, dominant, overdominant [Falconer, 1981]) and gene effects have been studied extensively in many crop species like maize, sorghum, etc (Sprague, 1996; Hallauer and Miranda, 1988; Horner, 1989). The type of gene action controlling a trait is very important in decisions regarding breeding method, cultivar type (inbred, hybrid, and population, etc.), and interpretation of data from quantitative genetics experiments.

2.6 QTL mapping and QTL analysis

Most of the important agronomic traits show quantitative inheritance. They are controlled by several genes and are often considerably influenced by environment. Positions in the genome that have been found to be associated with variation in quantitatively inherited traits are referred to as quantitative trait loci (QTLs). The number of genes and their interactive effects controlling gene expression of quantitative traits are poorly understood. The first report of linkage between a marker gene and a gene that influences a quantitative trait was made by Sax *et al.*, (1923). An important subsequent attempt to map "polygenes" contributing to quantitative trait variation was subsequently reported by Thoday (1961). These early efforts contributed to the subsequent development of the concept of QTLs (Gelderman *et al*, 1975), which when combined with the modern molecular marker technology (Soller and Beckmann, 1983) and genetic linkage maps allow mapping of QTLs. QTL mapping studies have now been reported in most of crops for traits related to yield (Bezant *et al.*, 1997), quality (Teutenico and Osborn, 1994), disease and insect resistance (Chen *et al.*, 1994), and environmental adaptation (Lauric *et al.*, 1995). Putative map locations and DNA markers linked to QTLs have opened up opportunities for isolation of QTLs via map-

based cloning (Alpert and Tanksley, 1996), introgression of QTLs into elite lines or improved germplasm (Bernacchi *et al.*, 1998), and marker-assisted selection (MAS) for QTLs in applied breeding (Lande and Thompson; 1990, Tanksley; 1993). These studies help not only to elucidate the function, regulation, and expression of quantitative traits, but also provide important tools for improving crop plants.

2.6.1 Steps in QTL mapping

The first step in QTL mapping studies is to detect QTL, while minimizing the occurrence of false positives (Type I errors, that is, declaring an association between a marker and QTL when in fact one does not exist). Two distinct methods are used to detect QTL. The single marker approach, sometimes referred to as the one-way analysis of variance (ANOVA), has been used extensively, especially with isozymes (Tanksley *et al.*, 1982; Edwards *et al.*, 1987). The second approach, interval mapping, detects QTL by using flanking markers. This approach is more complicated analytically than the ANOVA approach and involves application of the maximum likelihood method, which requires sophisticated computer software (Lander and Botstein, 1989). Lander and Botstein (1989) have developed formulae for calculating significance levels appropriate for both methods when the genome size, number of chromosomes, number of marker intervals, and the overall false positive rate desired are given. Several statistical procedures have been developed for the application of both ANOVA and interval mapping (Soller and Brody, 1976; Edwards *et al.*, 1987 ; Weller, 1987; Lander and Botstein, 1989; Knapp, 1989). When the same false positive rates are used, there are few reasons to suspect that the two methods would detect substantially different QTL. Stuber *et al.*, (1992) compared the two methods and found that they identified basically the same QTL. Those researchers

reported, however, some advantages to using the interval mapping approach. Because of the increased power associated with using flanking markers, the method gives the most likely location of the QTL under the assumption of a single QTL in the interval, and the interval mapping approach allows ambiguous or missing data. Once QTL are detected, the next step is to estimate the genotypic effect of the QTL and to localize the QTL to a precise genomic region. The interval mapping approach seems superior to the ANOVA approach for both estimation of effects and localization of the QTL (Stuber *et al.*, 1992). The success of both methods depends on the linkage between marker(s) and QTL, the number and type of progeny evaluated, the heritability of the trait, and the magnitude of the effects at QTL that one desires to detect. Several methods and genetic designs have been suggested for detecting, estimating effects, and localizing QTL (Cowen, 1988; Burr *et al.*, 1988).

2.6.2 Principles of QTL analysis

QTL analysis is to look for associations between the phenotypic variation in quantitative traits and the marker alleles segregating in the mapping population. It has three essential stages: mapping of markers (genotyping), scoring of the trait (phenotyping), and association of the trait with the markers (QTL mapping). QTL analysis can be done by using statistical procedures like maximum likelihood ratio (Lander and Botstein; 1989), non-linear regression (Knapp *et al.*, 1990), linear regression (Bridges *et al.*, 1991), or a combination of maximum likelihood and multiple regression (Jensen and Stam, 1994; Zeng, 1994). Also to carry out the data analysis, the software packages like Mapmaker/QTL (Lander *et al.*,., 1987), MAPQTL (Van Oolijen and Maliepaard, 1996) Map manager QTL (Manly and Cudmore, 2001) and QGENE

(Tanksley and Nelson, 1996), QTL Cartographer (Basten *et al.*, 1994), and PlabQTL (Utz ,1995; Utz and Melchinger 1996) are available.

2.7 QTL x environment interactions:

Leon *et al.*, (2001) reported that QTLs for days to flowering in sunflower interacted with the photoperiod of the environment. A major QTL on linkage group B was not significantly associated with days to flower in a short photoperiod environment, but was significantly associated with days to flower at those environments where the photoperiod was longer. This shows that the effect of QTLs depends on the environmental conditions. Selection for the QTL on linkage group B would be expected to increase days to flower in environments with long photoperiod, but would not be effective in environments with a short photoperiod. From this experiment we can see that not only are marker-QTL associations dependent on the population evaluated, they are also dependent on the environment in which selection is conducted.

Subudhi *et al.*, (2000) reported that four major QTLs for the stay-green trait in sorghum were consistently expressed across seven environments. All the environments were manipulated with irrigation treatments so that drought stress occurred after flowering and not before flowering. The stay-green trait is known to reduce drought stress after flowering, but is not effective in reducing drought stress before flowering. Subudhi *et al.*, (2000) results demonstrated an absence of QTL x environment interaction, which is different from the results of Leon *et al.*, (2001).

Zhu *et al.*, (1999) reported that "the classification of QTL alleles as 'favorable' or 'unfavorable' may be misleading. The effect of an allele may be positive, neutral or negative depending on interactions with other loci and with environments. Therefore,

for traits such as grain yield, QTL mapping and selection experiments should place more emphasis on identifying the best multi-locus allelic combinations instead of pyramiding individual favorable QTL alleles." Their results show that using MAS will not eliminate genotype x environment interactions. Epistatic effects between QTL were also found to be important, suggesting that combinations of QTLs need to be selected for, rather than selection of individual QTLs. Due to the complications of genotype x environment interaction and epistatic effects, they suggested that phenotypic selection may be as effective as MAS for the germplasm and environments that they evaluated.

Environmental factors could have significant interactions with genetic effects on productivity and quality traits as indicated by Arora *et al.*, (1975) for sorghum genotypes. Large genotype × environment (G×E) interactions would require more extensive testing and limit average gains. However, Lodhi (1993) observed a considerable amount of genetic variability and heterosis for forage quality and yield characters, indicating that substantial gains from genetic improvement are possible.

2.8 Construction of genetic linkage maps in sorghum

Genetic linkage maps are fundamental for the localization of genes conferring biotic and abiotic tolerance. Linkage maps of organisms are constructed to map genomic regions controlling qualitative and quantitative traits, to exercise permit indirect selection for several agronomic traits, and to isolate the genes involved based on their map position.

Nearly every agronomic trait of importance has been subjected to DNA marker mapping and QTL analyses [e.g., drought tolerance (Martin, 1999), seed hardness (Keim *et al.*, 1990), plant height (Lin *et al.*, 1995) and grain yield (Stuber *et al.*, 1987)].

Bhattramakki *et al.*, (2000) constructed an integrated SSR (113 loci) and RFLP (323 loci) marker-based genetic linkage map of sorghum using as a mapping population 137 F_8 recombinant inbred lines (RILs) derived from the cross between BTx623 and IS 3620C. Most of the SSR primer sequences reported were developed from clones isolated from two sorghum BAC libraries and three enriched sorghum genomic DNA (gDNA) libraries. Very few of the sorghum SSR primer sequences reported were developed from the sorghum DNA sequences present in public databases. Loci detected by 323 RFLP probe-enzyme combinations and 313 SSR primer pairs were mapped (LOD score ≥ 3.00). Of the SSR primers developed, 165 (53%) were found to detect polymorphism in a population composed of 18 diverse sorghum lines.

Kong *et al.*, (2000) also constructed an integrated sorghum linkage map with RFLP and SSR markers using the recombinant inbred lines derived from the cross between BTx623 and IS 3620C. The markers were distributed across the 10 sorghum linkage groups (LG), covering 1287.2 cM of the sorghum genome (based on LOD score > 5.0).

Menz *et al.*, (2002) have constructed a high-density genetic map of the sorghum genome by using AFLP technology and a recombinant inbred line population derived from the sorghum cross of BTx623 × IS3620C. The 1713 cM map encompassed 2926 loci distributed on ten linkage groups; 2454 of those loci are AFLP products generated from either the *Eco*RI/*Mse*I or *Pst*I/*Mse*I enzyme combinations. Among the non-AFLP markers, 136 are SSRs previously mapped in sorghum, and 203 are cDNA and genomic clones from rice, barley, oat, and maize. Of the nearly 3000 markers mapped, 692

comprised a LOD ≥3.0 framework map on which the remaining markers were placed with lower resolution (LOD <3 0)

Bowers *et al* (2003) constructed a high-density genetic recombination map of sequence-tagged sites for sorghum, which will be a framework for comparative, structural and evolutionary genomics of tropical grains and grasses Also they have reported a genetic recombination map for Sorghum of 2512 loci spaced at average 0 4 cM intervals based on 2050 RI LP probes, including 865 heterologous probes Mapped loci identify 61 5% of the recombination events in this progeny set and reveal strong positive crossover interference acting across intervals of ≤50 cM

Paterson *et al* (2004) have examined a sorghum-rice comparative map developed by BLAST ing sequences from 2,509 genetically mapped sorghum loci against the rice genome assembly The positions of 1,626 corresponding loci could be plotted based on the rice physical location and sorghum genetic location This revealed much colinearity, with eight sorghum linkage groups (A, D, E , F , G, H, I, and J) corresponding to single rice chromosomes (1, 4, 12, 2, 5, 11, 6, and 8), and two sorghum linkage groups (B and C) differing from rice by translocations (between chromosomes 7/9 and 3/10 respectively)

Haussmann *et al* (2004) used molecular markers for mapping resistance to the hemi-parasitic weed Striga hermonthica by using two recombinant inbred populations (RIP-1, -2) of F 3 5 lines developed from the crosses IS9830 × E 36-1 (1) and N13 × E36-1 (2) The genetic maps of RIP-1 and RIP-2 spanned 1,498 cM and 1,599 cM, respectively, with 137 and 157 markers distributed over 11 linkage groups

Levels of genetic variation and linkage disequilibrium (LD) are critical factors in association mapping methods as well as in identification of loci that have been targets of selection. Sorghum, a close relative of maize, self pollinating panicoid grass, is expected to have higher levels of LD. Hamblin *et al.*, (2004) surveyed 27 diverse *S. bicolor* accessions for estimating sequence variation at a total of 29,186 bp in 95 short regions derived from genetically mapped RFLPs located throughout the genome. They concluded that the extent of LD is at least severalfold greater in sorghum than in maize.

Nagaraj *et al.*, (2005) have mapped thirteen linkage groups (LGs) containing 60 simple sequence repeat (SSR) loci by using a set of sorghum recombinant inbred lines (RILs) obtained from the cross Ô96-4121Õ (greenbug-tolerant parent) x Redlan (greenbug-susceptible parent). The LG spanned a distance of 603.5 cM, with the number of loci per LG varying from 2 to 14. Seventeen additional SSR loci were unlinked at a log of odds value of 3.0. Composite-interval mapping identified three quantitative trait loci (QTLs) associated with biotype I and five QTLs associated with biotype K. The amount of phenotypic variation explained by these QTLs ranged from 9 to 19.6%.

Kim *et al.*, (2005a, b) integrated genetic, physical, and cytological perspectives of the *Sorghum bicolor* genome, by FISH of landed BAC's and relative lengths of metaphase chromosomes were estimated. They used elite inbred line BTx623 to estimate the molecular size of each chromosome and established the size-based nomenclature for sorghum chromosomes (SBI-01–SBI-10) and linkage groups (LG-01 to LG-10), which represents a reasonable choice for standard unified chromosome nomenclature.

Recently in pearl millet, Pittaway *et al*., (2004) developed a consensus map of 353 RFLP and 65 SSR markers using four different crosses, in which 85% of the

markers are clustered and occupied less than a third of the total map length. They have concluded that extreme localization of recombination toward the chromosome ends, resulting in gaps on the genetic map of 30 cM or more in the distal regions. The unequal distribution of recombination has consequences for the transfer of genes controlling important agronomic traits from donor to elite pearl millet germplasm.

2.9 Mapping the stay-green trait in sorghum

Most agronomically important traits of crop plants have complex inheritance patterns and are under the control of many genes. The genetic loci associated with complex traits are called quantitative trait loci (QTLs). Traits controlled by these QTLs are often strongly influenced by the environment (including the genetic background in which they are observed). Because of this, the segregation patterns observed for such polygenic traits appear to deviate from the relatively simple patterns of Mendelian inheritance, and hence the underlying genes controlling these trait are hard to trace. This limitation has been overcome by the construction of highly saturated molecular maps in many crop species. The theoretical basis of interpreting the association of marker loci with QTLs has been provided by Mather and Jinks (1971), Tanksley *et al*. (1982), Soller and Beckmann (1983), and Edwards *et al*., (1987). The theoretical basis for identification of QTLs associated with individual marker loci has also been studied by several authors (Jayakar, 1970; McMillan and Robertson, 1974; Soller and Beckmann, 1983; Edwards *et al*., 1987; and Cowen, 1988). Likewise, the use of flanking marker loci for QTL identification has been suggested by Lander and Botstein (1989) and Knapp *et al*., (1989).

The development of molecular marker technologies and the use of these markers in detecting and mapping quantitative trait loci has become a powerful approach for the studying the genetic and phenotypic basis of complex traits (Edwards *et al.*, 1987; Paterson *et al.*, 1988; Williams *et al.*, 1992). If individual genetic components associated with a complex trait can be identified, then research can focus on the function of each locus independently without the confounding effects of other segregating loci (Dorweiler *et al.*, 1993).

The complex expression of drought tolerance makes this trait difficult to study using traditional genetic and physiological methods. Use of molecular markers and QTL analysis of drought tolerance in lines grown in replicated and carefully induced drought-stressed environments has lead to a better understanding of the inheritance of this trait in sorghum (Tuinistra *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.*, 2002a).

Lander and Botstein (1989), described a set of analytical methods that modify and extend the classical theory for mapping QTLs and that are implemented in the computer software package Mapmaker/QTL. They provided explicit graphs that allow experimental geneticists to estimate, in any particular case, the number of progeny required to map QTLs underlying a quantitative trait. Detecting marker-QTL associations can be carried out by means of likelihood ratio tests that involve the use of a pair of markers bracketing a QTL, a procedure termed "interval mapping"(Jensen, 1989; Knapp *et al.*, 1990; Lander and Botstein, 1989; Weller, 1987) although simpler approaches are possible (Haley and Knott, 1992; Thoday, 1961; Weller, 1987).

Tuinstra *et al.*, (1996), identified QTLs associated with pre-flowering drought tolerance in sorghum using 98 RILs derived from a cross between inbred lines RTx7078 (pre-flowering drought tolerant, post-flowering drought sensitive) and B35 (pre-flowering drought sensitive, post-flowering drought 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 specifically associated with pre-flowering drought tolerance and also mapped eight additional regions generally associated with yield or yield components under fully-irrigated conditions.

Tuinstra *et al.*, (1997a), identified genomic regions (QTLs) associated with post-flowering drought tolerance and for potentially related components of grain development. They used the same set of 98 RILs as above, a mapping population derived from a cross between inbred lines RTx7078 (pre-flowering drought tolerant, post-flowering drought sensitive) and B35 (pre-flowering drought sensitive, post-flowering drought tolerant). 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 the "stay-green" trait under post-flowering drought stress conditions. These loci were also associated with grain yield under fully-irrigated conditions, suggesting that these drought tolerance loci have pleiotropic effects on grain yield under non-stress conditions. In this population, several different QTLs were associated with expression of the stay-grain trait. A stay-green QTL on linkage group I (= SBI-09) was positively associated with grain yield under fully-irrigated conditions and a stay-green QTL on linkage group G (= SBI-10) was weakly 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.

Tunistra *et al.*, (1998), tested in a population of near-isogenic line (NIL) pairs the phenotypic effects of three different genomic regions associated with various measures of agronomic performance in drought and/or non-drought environments. In most cases, NILs contrasting for a specific locus differed in phenotype as predicted by QTL analysis. NILs contrasting at the QTL flanked by RAPD marker loci *tM5*/75 and *tH*19/50 exhibited large differences in grain yield across a range of environments. On further analysis they concluded the differences in agronomic performance might be associated with a drought tolerance mechanism that also influences heat tolerance.

Crasta *et al.*, (1999), developed a QTL map using 142 RFLP markers from a set of RILs obtained from a cross between B35 and elite pollinator line RTx430. These RILs and their parental lines were evaluated for post-flowering drought tolerance and maturity in different environments. By using simple interval mapping they identified seven stay-green QTLs and two maturity QTLs. Out of seven stay-green QTLs detected, three major QTLs (SGA, SGD, and SGG) contributed 42% of observed phenotypic variability (LOD 9.0) and 4 minor QTLs (SGB, SGI.1, SGI.2, and SGJ) significantly contributed an additional 25% of observed phenotypic variability in stay-green ratings.

Tao *et al.*, (2000), evaluated 160 RILs, derived from a cross between QL 39 and QL 41, as a segregating population for genome mapping of the stay-green trait. They

added 118 additional markers, including 17 SSR markers and 101 RFLP markers, to a previously published linkage map (Tao *et al.*, 1998) by using same RIL population. In total they identified five genomic regions associated with the stay-green trait. They also confirmed these results by composite interval mapping with inclusion of QTL × Environment interaction.

Xu *et al.*, (2000b), mapped QTLs controlling the stay-green trait and chlorophyll content in sorghum using as a mapping population 98 F_7 RILs derived from the cross B35 × RTx7000. They identified four stay-green (*stg*) QTLs located on three linkage groups. Two stay-green QTLs, *stg*1 and *stg*2, are located on sorghum linkage group (LG) A (= SBI-03), which corresponds to chromosome 3 on the Klein bin map The other two stay-green QTLs detected are located on LG D (chromosome 2, (= SBI-02) and LG J (chromosome 10, = SBI-05), respectively. They estimated that stay-green QTLs *stg*1 and *stg*2 accounted for 13-20% and 20-30%, respectively, of the observed phenotypic variability for this trait in this RIL mapping population. They also identified three QTLs for chlorophyll content (*chl*1, *chl*2 and *chl*3) that together explained 25-30% of the observed phenotypic variability. The genomic regions corresponding to *stg*1 and *stg*2 contain an ABA-responsive 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 genetic backgrounds and environments. They evaluated the RIL mapping population from the cross B35 × RTx7000. The map of the (B35 × RTx7000)-derived RIL population (Xu *et al.*, 2000b) was expanded by the addition of 91 markers (RFLP, SSR and RAPD markers). They mapped four stay-green

QTLs and identified that there are partial similarities in case of the QTLs detected on LG A (= SBI-03) and LG B (= SBI-02) of the (B35 × RTx7000)-derived population and the (B35 × RTx7078)-derived population previously reported by Tuinstra *et al.*, (1997a). The nomenclature of the stay-green QTLs first used by Xu *et al.*, (2000b) (i.e., *stg*1, *stg*2, *stg*3 and *stg*4) was adopted as standard.

Mahalakshmi and Bidinger (2002), evaluated a set of 72 diverse genotypes of sorghum [*Sorghum bicolor* (L.) Moench] for their patterns of post-flowering leaf senescence under terminal drought stress conditions 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-adapted lines with stay-green expression equivalent to those of the best temperate-adapted lines (viz., B35 and KS19).

Kebede *et al.*, (2001), identified genomic regions associated with post-flowering drought tolerance (i.e., the stay-green trait) in sorghum using RFLP markers and an F_7 RIL population derived from cross SC56 × RTx7000. The genetic linkage map for this RIL population covers 1355 cM of the sorghum genome and consists of 144 loci. Nine QTLs, distributed across seven of the ten linkage sorghum 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 (chromosome 1 – SBI-01), LG G (chromosome 7 – SBI-10), and LG J (chromosome 10 – SBI-05) that were consistently detected across different terminal drought stress environments. They conducted comparative mapping studies, identifying that two of the sorghum stay-green QTLs identified in their study correspond to stay-green QTLs detected in maize. In

addition to this, QTLs responsible for sorghum lodging tolerance and pre-flowering drought tolerance were detected.

Cha *et al.*, (2002), mapped stay-green QTLs in rice using both phenotypic and molecular markers. They mapped the stay-green mutant [*sgr*(t)] locus to the long arm of rice chromosome 9 between RFLP markers RG662 and C985, at 1.8- and 2.1-cM intervals, respectively. They found no difference in photosynthetic activity between the stay-green mutant and yellowing wild-type leaves, indicating that senescence of the photosynthetic apparatus is proceeding normally in the mutant leaves and that the mutation only affects the rate of chlorophyll degradation during leaf senescence. Thus this rice stay-green mutant is cosmetic in nature and corresponds to the less useful category described by Thomas and Howarth (2000).

Sanchez *et al*, (2002), reported on four QTLs associated with the stay-green trait in sorghum using the RIL population derived from cross B35 × RTx7000, which were reported on previously by Xu *et al.*, (2000b) and Subudhi *et al*, (2000), and linkage maps well covered with RAPD, SSRs and RFLP markers. These four major QTLs [previously 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.

Haussmann *et al*, (2002b), developed QTL maps for the stay-green trait in sorghum using two recombinant inbred populations (RIP1 and RIP2) based on crosses of stay-green trait donor parent E36-1. The mode of gene action for the stay-green trait in their investigation ranged from purely additive to over dominance. Three QTLs on LG A (chromosome 1 – SBI-01), LG I (chromosome 5 – SBI-07) and LG G

(chromosome 7 = SBI-10) were common to both RIP1 and RIP2. These three QTLs from donor parent E36-1, along with the four QTLs from donor parent B35, are potential candidates for transfer of the stay-green trait into locally-adapted elite sorghum materials having producer and consumer-preferred grain and fodder quality traits. These findings have provided the basis for an attempt to transfer stay-green QTLs from the selected donor parents to a range of economically important recurrent parents.

2.10 Marker-assisted selection

Marker-aided selection is a new paradigm in plant breeding. It involves the selection of genotypes carrying a desirable gene, or gene combination, via linked marker(s). Breeders practice marker-aided selection when an agronomically important trait that is difficult to assess, is tightly linked to another Mendelian trait, which can be easily scored. Through marker-assisted selection (MAS) the transfer of traits from exotic donor parents to more elite locally adapted crop cultivars is possible. Morphological markers are dominant, limited in number, specific to particular genotypes, and may show stage-specific expression or pleiotropy. Due to these reasons, morphological markers have not been of much use in MAS. Molecular markers do not suffer from these limitations and thus offer advantages over the morphological markers in MAS.

Molecular marker-aided selection involves scoring segregating individuals for the presence or absence of a desired plant phenotype indirectly based on DNA banding pattern of linked markers on a gel or on autoradiogram depending on marker system. The rationale is that banding patterns reveal parental origin of the bands in segregants at a given marker locus and hence indicate presence or absence of specific chromosomal

segments that carry the desired alleles. This increases the screening efficiency in breeding programmes.

Specific breeding programmes in which marker-assisted selection has been already put to use are: 1) gene introgression and elimination of linkage drag, 2) gene pyramiding, and 3) development of heterotic hybrids.

Some of the assumptions associated with MAS are: 1)linkage relationships between markers and QTLs are consistent for progeny within the same population; 2)linkage relationships between markers and QTLs are repeatable between populations; 3)linkage relationships between markers and QTLs are real and not spurious relationships due to Type I error; 4)associations between markers and QTLs can be identified with about 100 progeny; 5)MAS will eliminate the problem of genotype x environment interactions, thus making MAS more effective than phenotypic selection.

The opportunity to manipulate complex traits via MAS/backcrossing is now available. This was not previously possible without time consuming progeny testing cycles in a conventional backcrossing system using phenotypic selection. The other issue important to QTL transfer is accuracy of selection. Two separate studies by Lande and Thompson (1990) and by Zhang and Smith (1992) showed that MAS significantly improved the accuracy of selection for traits of low heritability.

MAS has been advocated as a useful tool for rapid genetic advance in case of QTLs (Lande and Thompson, 1990; Knapp, 1994, 1998; Hospital, 1992). Gimelfarb and Lande (1995), presented a detailed analysis of the relationship between genetic markers and QTLs in the process of MAS.

Mohan *et al.*, (1997), concluded that MAS could be used to pyramid major genes including disease and insect resistance genes, with the ultimate goal of producing the crop cultivars with more desirable traits. A study conducted by Eathington *et al.*, (1997), assessed the usefulness of marker-assisted effects estimated from early generation testcross data for predicting later generations testcross performance.

MAS can be used to pyramid multiple disease resistance genes into a single crop genotype (Witcombe and Hash, 2000).

Hash *et al.*, (2003), suggested the use of 'marker-assisted selection' (MAS) as a route for the backcross transfer of previously identified stover quality traits to elite genetic backgrounds. Traits associated with improved ruminant nutritional quality of stover that were suggested for manipulation in this manner included foliar disease resistance, the stay-green component of terminal drought tolerance, and *in vivo*, *in vitro*, or NIRS-estimated dry matter digestibility.

2.11 Efficiency of marker-assisted selection

Hospital and charcosset (1997), used computer simulations to study the efficiency of MAS based on an index combining the phenotypic value and molecular score of individuals. They observed that in the first generation of selection the ratio of relative efficiency (RE) of expected efficiency of MAS over the expected efficiency of purely phenotypic selection generally increases with

1) larger population size,

2) lower heritability values of the trait, and,

3) higher type-I error risk of the regression.

In studies over successive generations of selection, higher efficiency of MAS for QTLs with large effects in early generations was balanced by a higher rate of fixation of unfavorable alleles at QTLs with small effects in later generations. This suggests that MAS may become less efficient than phenotypic selection in the long-term. MAS efficiency therefore depends on the genetic determination of the target trait.

The efficiency of MAS was generally reduced with increasing distance between the markers flanking the target QTL. The optimal distance recommended between two flanking markers is about 5–10 cM (Hospital *et al.*, 1992).

Hospital and Charcosset (1997) determined the optimal position and number of marker loci for manipulating QTLs via foreground selection. Further, they investigated the combination of foreground and background selection in QTL introgression. Openshaw (1994), determined the population size and marker density required in background selection.

Knapp (1998), presented estimates of the probability of selecting one or more superior genotypes by MAS to predict its cost efficiency relative to pherotypic selection. The frequency of superior genotypes among the selected progeny increases as the selection intensity increases. Van Berloo and Stam (1998), assessed effectiveness of MAS compared to phenotypic selection, showing that MAS appears partially promising when dominant alleles are present at QTLs and linked in coupling phase. Uncertainty in the estimated map position(s) of the QTL(s) targeted for foreground selection reduces the benefits of MAS.

Young (1999), pointed out that despite innovations like better marker systems and improved genetic mapping strategies, most marker associations are not sufficiently

robust for successful MAS. Charmet *et al.*, (1999), showed that the accuracy of QTL location determination greatly affects selection efficiency of MAS. MAS for QTLs has recently started to be applied to the genetic improvement of quantitative characters in several crops such as tomato (Lawson *et al.*, 1997; Bernacchi *et al.*, 1998), maize (Graham *et al.*, 1997), barley (Han *et al.*, 1997; Toojinda *et al.*, 1998), and pearl millet (Serraj *et al.*, 2002).

Frisch *et al.*, (1999a, 1999b), determined the number of marker data points (MDP) required in background selection and the size of the segregating population required to recover desirable individuals, comparing a two-stage selection procedure (one background selection stage and one foreground selection step) with alternative selection procedures (one foreground and two or three background selection steps). They observed that as the number of selection steps increases, the total number of MDP required (and hence the operational cost to generate these) decreases.

Moreau *et al.*, (2000), evaluated the relative efficiency of MAS in the first cycle of selection through an analytical approach taking into account the effect of experimental design (population size, number of trials and replication/trial) on QTL detection. They concluded that expected economic returns of MAS compared to the phenotypic selection decreases with the cost of genotyping.

Dreher *et al.*, (2003), at CIMMYT came to some preliminary conclusions on the relative cost effectiveness of conventional breeding methods as compared to MAS for QPM (quality protein maize) lines. When phenotypic screening is simple (in other words, when it is relatively easy to determine whether a given plant variety possesses a given trait, such as a certain grain color), conventional breeding is, and will continue to

be, extremely cost-effective. Conversely, when phenotypic screening is expensive, technically difficult, or even impossible, MAS will often be advantageous. MAS offers an alternative that is simple, direct, and very reliable. Often effective selection for resistance to diseases like maize streak virus, which are strictly quarantined, can be carried out using molecular markers even at locations where the disease is not present (but with the breeding program targeting regions where the disease is currently present or is considered a significant future threat). Marker-assisted selection often allows breeders to cut down on the number of seasons needed to produce a desired product. Even a high-end MAS scheme that might run a few thousand dollars more than a conventional scheme is to the additional benefit to farmers when a variety becomes available sooner.

Podlich *et al.*, (2005), developed an effective approach for marker assisted selection of complex traits, i.e. Mapping As You Go (MAYG) approach, which continually revises estimates of QTL allele effects by remapping new elite germplasm generated over cycles of selection, thus ensuring that QTL estimates remain relevent to the current set of germplasm in the breeding program. They used Simulation to investigate the effectiveness of the MAYG approach applied to complex traits.

2.12 Marker-assisted introgression in backcross breeding programs

In several studies it has been shown that genetic markers can be used to introgress genes from one line to another (Smith *et al.*, 1987; Hillel *et al*, 1990, 1992; Groen and Timmerman, 1992; Hospital *et al.*, 1992; Groen and Smith, 1995). Markers were efficient in introgression backcross programs for simultaneously introgressing an allele and selecting for the desired genomic background. Visscher (1996), investigated

the efficiency of marker-assisted introgression in backcross populations of maize inbred lines by simulation studies.

ICRISAT has focused on initiating a large-scale high-throughput marker-assisted backcrossing program for the stay-green component of terminal drought tolerance in sorghum. As a part of this program, the present study was aimed at MABC to introgress several stay-green QTLs from donor parent B35 into the genetic backgrounds of two elite sorghum lines (IRAT 204 and S 35 – ICSV 111) of interest to the national agricultural research program in Ghana, West Africa.

Chapter - III

Materials and Methods

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3.1 Plant material used:

To advance the back cross programme, BC_4F_2 seed of S 35, BC_3F_2 seed of ICSV 111 and BC_2F_1, BC_3F_1 seed of IRAT-204 are available. S 35 and IRAT-204 are recurrent parents and B35 is the donor parent for stay green trait used in this study. [Figure 1 represents the schematic diagram of the work done at ICRISAT. Figure 2 and 3 gives the overview of the backcrossing program for S 35 and IRAT 204 parents respectively].

3.2 Description of parental lines used in the backcross programme:

3.2.1 Donor parent used:

The two major sources of stay green are B35 and E-36-1. B35 is the donor parent used in this study.

B35:

B35 is a BC1 derivative of landrace germplasm accession IS 12555, which is a durra race sorghum from Ethiopia (Rosenow et al., 2002). It is a short 3-dwarf inbred line from TAMU. It is a best-characterized source of stay-green terminal drought tolerance available in sorghum. It is a purple plant with red pericarp and thick mesocarp and awned grain. Several different research groups from the USA and Australia have identified a number of stay-green QTLs based on RIL mapping populations derived from crosses involving this line or its derivatives. Based upon a limited SSR-based genetic diversity study recently conducted at ICRISAT-Patancheru, B35 appears to be genetically quite diverged from the elite recurrent parents used in this study, which facilitates its use in

marker-assisted breeding programs. It is also phenotypically divergent from many Asian and African elite sorghum open-pollinated varieties and hybrid parental lines. It is potentially "yield resistant" due to its short plant height, small panicle size, and low grain number per panicle. B35 bred in USA by D.T.Rosenow and it was released recently as BTX 642.

3.2.2 Recurrent parents used:

S 35:

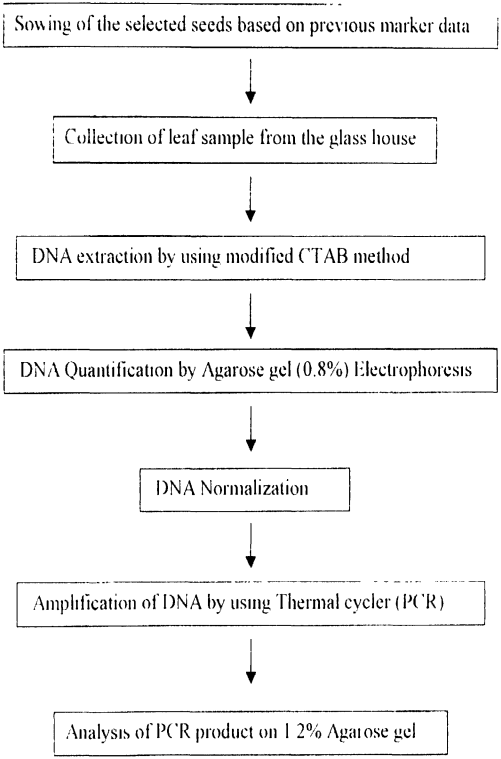
ICRISAT-derived inbred-line cultivar S35 is a selection from ICSV 111, which has been released in Cameroon and Chad. It is an open pollinated sorghum variety, which is early maturing tall, with white grain. It has achieved 10 to 15% adoption in Nigeria and Ghana.

ICSV 111:

This is a pure-line cultivar developed at ICRISAT through pedigree selection in a three-way cross (SPV 35 x E 35-1) x CS 3541. The parents SPV 35 and CS 3541 are converted photo-insensitive three-gene dwarf zerazera types originating from Ethiopia and Sudan, respectively; while E 35-1 is a zerazera type originating from Ethiopia. ICSV111 is a photo-insensitive, self-pollinated cultivar that flowers in 65-72 days and matures in 100-110 days. The green stalks are slightly sweet and juicy. It is a caudatum type, with white hard grains, thin pericarp and normal endosperm. This open-pollinated sorghum variety has been released in Ghana and Nigeria as 'Kapaala'.

Both the plants (S35 and ICSV111) can be grown as food security crops in the drought prone areas of West Central Africa including Ghana, Chad, and Cameroon.

Figure 1. Schematic representation of the Dissertation work done at ICRISAT:



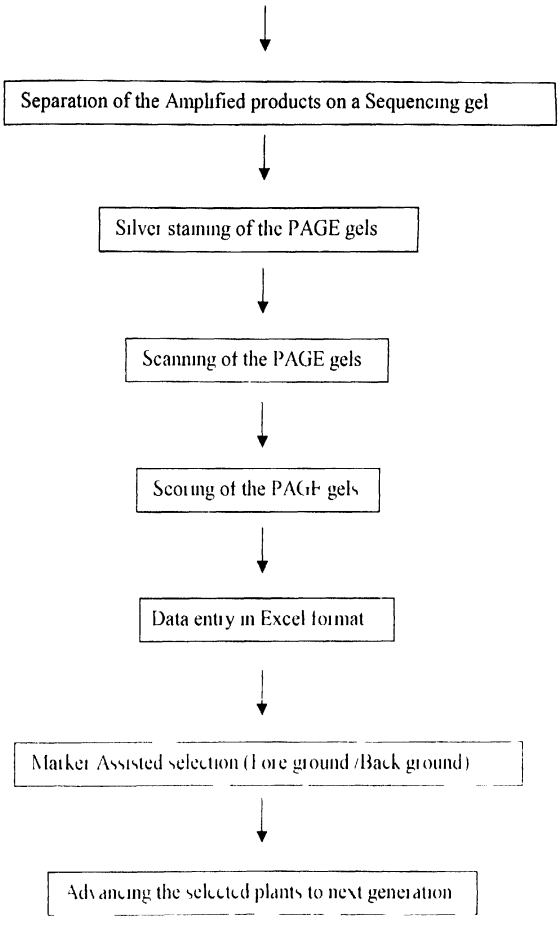

Separation of the Amplified products on a Sequencing gel
Silver staining of the PAGE gels
Scanning of the PAGE gels
Scoring of the PAGE gels
Data entry in Excel format
Market Assisted selection (Fore ground /Back ground)
Advancing the selected plants to next generation

Figure 2. Scheme for transfer of stay green QTLs into elite sorghum lines by marker-assisted selection for S 35/E 36 III population

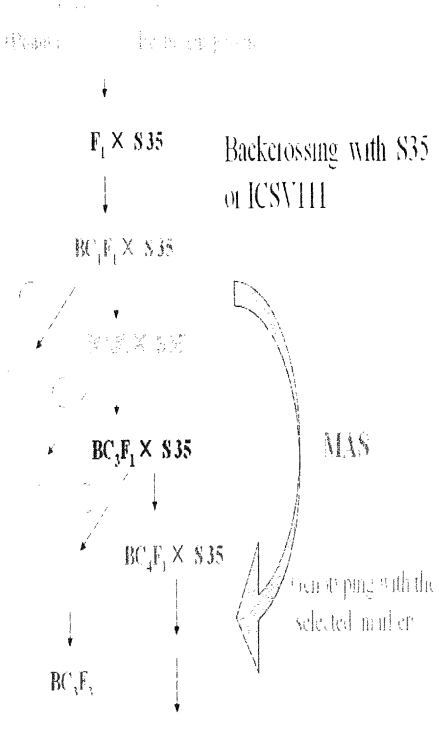
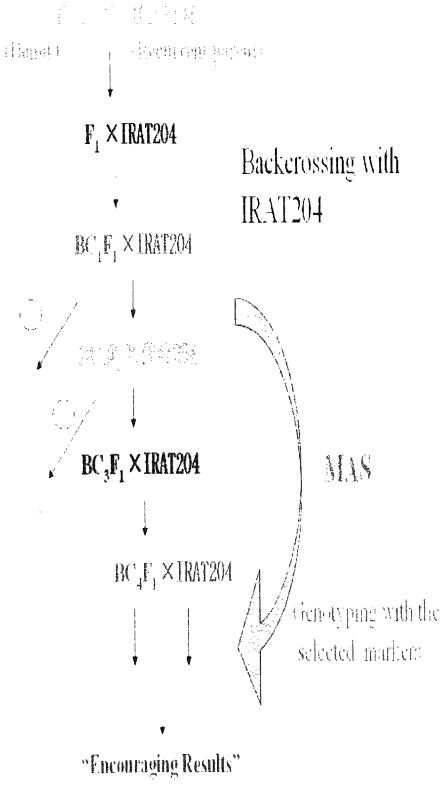


Figure 3. Scheme for transfer of stay green QTLs into elite sorghum lines by marker-assisted selection for IRAT 204 population:



IRAT-204:

IRAT-204 is an early maturing, short statured, day neutral (non photosensible) caudatum variety (Tenkouano 1993). It is one of the widely adapted varieties grown in seven drought prone areas of West East Africa. It is often cultivated in regions like Maradi (East central part of Niger; 15^0 26^0N and 8^0 33^0E), Nigeria, and Northern Ghana and Burkina Faso etc. Delayed sowing may significantly reduce the seed weight, due to lack of phonological plasticity. It has a yield potential of about 3.6 t/ha.

3.3 Methodology for DNA extraction

Extraction of DNA from plants is the starting point for a number of down stream molecular biology applications (PCR, Sequencing, etc.).

3.3.1 Sowing of the seed material:

Seeds of backcross progenies from selected individuals were sown individually in small pots in the glass house. At the same time seeds of the parental lines were sown. Staggered sowing was employed to ensure co-flowering of the recurrent parent and backcross progenies. Therefore recurrent parent seed was also sown a week before and after the sowing of backcross progenies.

3.3.2 Collection of leaf sample:

Fresh plant material is highly suitable for isolation of DNA. Six-inch long leaf strips from one-week-old seedlings are collected from individual plants of the BC_1F_1, BC_2F_2 and BC_4F_1 populations of crosses IRAT-204 X B35 and S35 (=ICSV111) XB35 respectively.

3.3.3 Extraction of DNA:

Method adopted for extraction of DNA is modified CTAB method (Saghai-Maroof et al., 1984). Murray and Thompson (1980) first developed CTAB protocol following which several modifications of protocol have been successfully developed to adopt the method to wide range of applications. CTAB is an appropriate method for extraction and purification of DNA from plants and it is particularly suitable for elimination of polysaccharides, which are major contaminants in case of Cereals. Also the quality of DNA obtained by this method is good.

The DNA thus obtained was further purified by RNase digestion followed by extraction with phenol/ chloroform/ iso-amylalcohol and ethanol precipitation.

Plant genomic DNA isolation by CTAB method involves three main steps:

1. Lysis of the cell membrane.

2. Extraction of the genomic DNA.

3. Precipitation of DNA.

A 96-well plate mini DNA extraction protocol (Mace et al., 2004) was employed for this work.

3.4 96-well plate mini DNA extraction

A. <u>Preparation and processing</u>

1. Steel balls (2 per extraction tube), pre-chilled at -20°C for about 30 minutes, and were added to the extraction tubes, which are kept on ice.

2. 3% CTAB buffer (3% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.17% β-mercaptoethonol) was pre-heated in +65°C water bath before start of sample collection.

3. Six-inch long leaf strips were collected (final weight 30 mg) from one-week-old seedlings, then cut in to pieces (1 mm in length). These strips were transferred to the extraction tubes.

B. Grinding and extraction

1. 450 µl of pre-heated 3% CTAB buffer was added to each extraction tube containing a leaf sample.

2. Grinding was carried out using Sigma GenoGrinder at 500 strokes/minute for two periods of 2 minutes each.

3. Grinding was repeated until the color of solution becomes pale green and leaf strips were sufficiently macerated.

4. After grinding, the tube box was fixed in a locking device and incubated at +65°C in a water bath for 10 minutes with occasional manual shaking.

C. Solvent extraction

1. 450 µl of chloroform: iso-amylalcohol (C: IAA=24:1) mixture was added to each tube and the samples were centrifuged at 6200 rpm for 10 minutes.

2. After centrifugation the aqueous layer was transferred to a fresh tube (approximately 300 µl).

D. Initial DNA precipitation

1. To each tube containing aqueous layer, 0.7 volume (approximately 210 µl) of cold (kept at –20°C) isopropanol was added, then solution was carefully mixed and the tubes were kept at –20°C for 10 minutes.

2. The samples were centrifuged at 6200 rpm for 15 minutes.

3. The supernatant was decanted under a fume-hood and pellets were allowed to air dry (minimum 20 minutes).

E. RNase treatment

1. In order to remove RNA 200 µl of low salt TE buffer and 30 mg of RNase (stock 10 mg/µl) were added to the each tube containing dry pellet and mixed properly.

2. The solution was incubated at 37°C for 30 minutes.

F. Solvent extraction

1. After incubation, 200 µl of phenol - C:IAA mixture (25:24:1) was added to each tube carefully mixed and centrifuged at 5000 rpm for 10 minutes.

2. The aqueous layer was transferred to the fresh tubes and the step was repeated with the C:IAA mixture.

G. DNA precipitation

1. To the tubes containing aqueous layer 15µl (approximately $1/10^{th}$ volume) 3M Sodium acetate and 300µl (2 vol) 100% ethanol was added and subsequently placed in freezer for 5 minutes.

2. Following incubation box was centrifuged at 6200 rpm for 15 minutes.

H. Ethanol wash

1. After centrifugation supernatant was carefully decanted and to the pellets add 200µl of 70% ethanol followed by centrifugation at 5000 rpm for 5 minutes.

I. Final re-suspension

1. Pellets obtained by carefully decanting the supernatant and allowed to air dry for one hour.

2. Completely dried pellets were re-suspended in 100µl of $T_{10}E_1$ buffer and kept at room temperature to dissolve completely.

3. Dissolved DNA samples were kept in 4°C.

3.5 Quantification of Genomic DNA:

It is necessary to check the quality and concentration of DNA for carrying out PCR reaction. There are three methods available for the quantification of genomic DNA in a sample.

3.5.1. Ethidium Bromide Agarose Gel Electrophoresis:

The DNA can be quantified in an agarose gel by comparing the intensity of the fluorescence emitted by an EtBr- stained DNA sample, relative to a dilution series of a DNA standard of known concentration.

The DNA quality was checked using 0.8% agarose gel. 1 µl of DNA solution was mixed with the 1 µl of orange dye and 4 µl of distilled water and loaded in to wells on 0.8% agarose gel. The gel was run for 10 min. after which the quality was checked under UV. A smear of DNA indicated poor quality whereas a clear band indicated good quality. Samples of poor quality were re-extracted.

3.5.2. Spectrophotometry:

The concentration of nucleic acids is usually determined by measuring the sample OD at 260 nm against a blank. The ratio A_{260}/A_{280} is used to estimate the purity of nucleic acid. Pure DNA should have a ratio of approximately 1.8.

3.5.3. Flourimetry:

The DNA concentration of each sample was assessed using the Spectrafluor Plus Spectrophotometer by staining DNA with Picogreen™ (1/200 dilution). Based on the Relative Fluorescence Units (RFU) values and using the standard graph, DNA concentrations were calculated.

3.6 Normalization of the DNA:

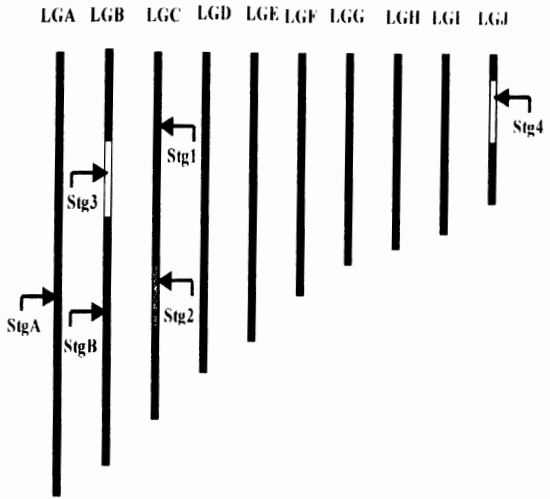
The normalization of DNA was done robotically by using Tecan liquid Handling Robotic system and the final concentration of DNA was 2.5ng/µl.

3.7 Selection of the markers:

SSRs are small repetitive DNA sequences, which are spread through out the genome of eukaryotes, are often highly polymorphic due to variation in number of repeat units, provide the basis of PCR based multi-allelic, co-dominant marker system.

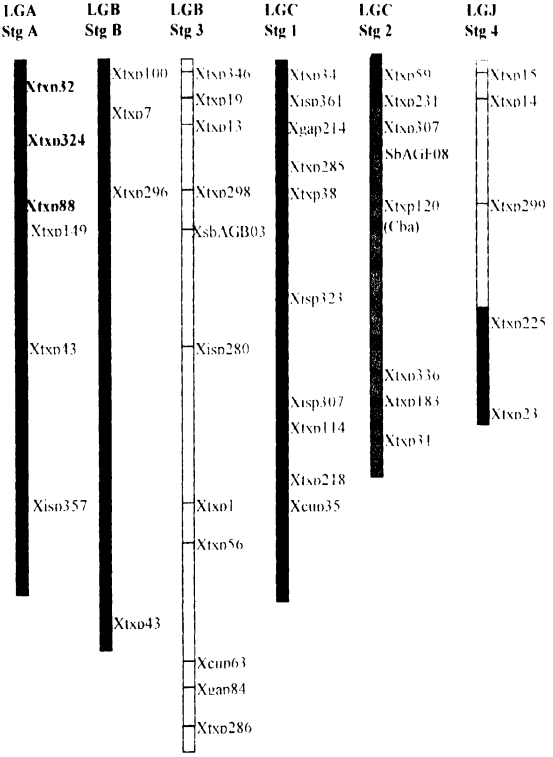
SSR markers linked to QTLs for stay-green on various linkage groups [Figure-4 and Figure-5 for an overview of the stay green QTLs in B35 parent] were used for foreground selection to select the individuals presumably having the donor allele (foreground selection) at a particular target stay-green QTL. Donor parent alleles at foreground marker loci indicate the presence of the target stay-green QTL that is flanked by these marker loci. The tighter the markers are linked to the QTL, the greater the chance that the QTL mapped between a pair of flanking markers has indeed been transferred (but determination of this is out side the scope of the project). Therefore, phenotypic testing of the final products of the MAB exercise need to be performed in order to confirm the transfer of stay-green QTL. At the same time selected markers

Figure 4: Consensus map of Stay-green QTL in B35 donor parent



Stay-green consensus QTL map of B35 [After Bhattramakki *et al.*, 2000].

Figure 5: SSR markers linked to Consensus stay-green QTL mapped in donor parent B35



unlinked to stay-green have been used to select those individuals with minimal linkage drag (background selection).

3.8 PCR Amplification of SSR markers:

PCR is a technique that enables the amplification of specific sequences of nucleic acids. The development of PCR technique is a milestone in genome analysis. Kary Mullis (1986) of Cetus Corporation invented this technique, which was originally described by Saiki (1985).

In PCR two primers (short single stranded DNA sequences) are used that are complementary to opposite strand of DNA sequence to be amplified. After heat mediated denaturation of the template DNA the primers bind to their respective sequence (annealing) on the template DNA and a DNA polymerase synthesizes a complementary strand in 5'-3' direction (extension). Each round of denaturation, annealing and extension is known as a cycle. With each cycle the amount of the template DNA sequence amplified doubles.

PCR reactions were conducted in 384 wells plates in a PE 9700 Perkin Elmer (Norwalk Conn.) DNA thermocycler. The reactions were performed in volumes of 5µl using four different PCR protocols (appendix IV) and a touchdown PCR program.

PCR amplification protocols used for microsatellites are generally standard and can be carried out in a total volume of 5µl.depending on which of the possible strategies for electrophoresis and subsequent scoring of alleles is used, PCR amplification employs either unlabelled primer pairs or primer pairs with one of the primers being radiolabelled or fluorolabelled. Electrophoresis of unlabelled PCR products can be carried out on smaller 20cm vertical PAGE gels or on horizontal agorose gels. Small electrophoresis systems have

certain advantages in that immersion staining with silver or EtBr is possible which may be the most desirable methods in non-radioactive laboratories. The one disadvantage of small gel systems is that alleles differing by one or two base pairs are sometimes difficult to resolve where the total allele length exceeds 200bp. Radio-labelling is either by direct incorporation of labeled dNTP's or by end-labelling of the PCR primers. Automated systems such as the Pharmia ALF and the Applied Biosystems 373 and 377 Automated DNA sequencers require one of the primers to be labelled with fluorescent dye. Electrophoresis of radiolabelled and fluorolabelled PCR products is normally carried out on sequencing-length gels.

Loci that can be amplified with minimum non-specific annealing and which have non-overlapping allele size ranges can be separated simultaneously in both the radioactive and fluorescent approach. Several loci can be co-amplified during PCR (i.e. multiplexing). Multiplexing allows the rapid genotyping of large sample sizes across several loci. A major advantage of automated systems is the availability of dyes that fluoresce at different wavelengths (FAM, HEX, NED, TET: Perkin Elmer/ABI) enabling highly efficient, simultaneous electrophoresis of several loci with over lapping allele sizes.

3.8.1 Composition of reaction mixture:

The composition of polymerase chain reaction mixture used for the amplification of the targeted QTL was given in table 5.

Table 1: List of Markers used for foreground selection of BC_3F_2 population for recurrent parent S 35

	Marker	Sizes	
		S 35	B 35
Stg A	*Xtxp88*	120	102
	Xtxp32	128	130
	Xtxp357	274	276
	Xtxp149	172	168
	Xtxp43	152	180
Stg B	*Xtxp7*	233	221
	Xtxp207	184	201
	Xtxp296	168	164
Stg 3	*Xtxp19*	288	326
	Xtxp298	209	203
	Xtxp56	null	323
	Xtxp286	193	216
Stg 4	*Xtxp225*	163	161
	Xtxp23	174	183
	Xtxp257	302	240

Note: A total of 15 foreground markers were screened for targeting 4 QTLs controlling the stay-green trait for the backcross population of S 35 x B 35.

Table 2: List of Markers used for foreground selection of BC_3F_2 population for recurrent parent ICSV 111

	Marker	Sizes	
		ICSV 111	B 35
Stg A	*Xtxp32*	128	130
	Xtxp357	274	276
	Xtxp149	172	168
Stg 1	*Xtxp34*	233	221
	Xtxp285	184	201
	Xtxp114	168	164
	Xtxp361	288	326
	Xtxp307	209	203
	Xtxp323	null	323
Stg 2	*Xtxp114*	193	216
	Xtxp307	349	355
	XsbAgF08	140/170/190	150/180/200
Stg 3	*Xtxp19*	288	326
	Xtxp298	209	203
	Xtxp56	null	323
	Xtxp286	193	216

Note: A total of 16 foreground markers were screened for targeting 4 QTLs controlling the stay-green trait for the backcross population of ICSV 111 x B 35.

Table 3: List of Markers used for foreground selection of BC_1F_1 and BC_2F_1 populations for recurrent parent IRAT 204

	Marker	Sizes	
		IRAT 204	B 35
Stg A	*Xtxp34*	328	331
	Xtxp285	222	237
	Xtxp114	233	230
	Xisp361	207	195
	Xisp323	159.84	161.58
	Xisp307	349	355
	Xgap214	182	184
Stg 2	*Xtxp59*	210	205
	Xtxp231	189/209	189
	Xtxp336	160	164
	Xtxp183	196	212
	Xtxp31	209	231/233
	Xtxp205	202	205
Stg 3	*Xtxp1*	206	180
	Xtxp298	209	203
	Xtxp286	193	216
	Xtxp56	354	323
Stg 4	*Xtxp15*	212	220
	Xtxp14	148	135
	Xtxp23	175	183
	Xtxp225	162/177	175
	Xtxp299	165	170
	Xisp257	306	240

Note: A total of 23 foreground markers were screened for targeting 4 QTLs controlling the stay-green trait for the backcross population of IRAT 204 x B 35.

Table 4: List of Markers used for foreground selection of BC_3F_1 population for recurrent parent IRAT 204

	Marker	Sizes	
		IRAT 204	B 35
Stg A			
	Xtxp88	135	102
	Xtxp32	142	130
	Xtxp43	165	144
	Xisp357	274	276
	Xisp149	168	172
Stg B			
	Xtxp207	184	174
	Xtxp296	168	164
	Xtxp7	233	222
	Bl139914	205	210
	Xcup26	220	226

Note: A total of 10 foreground markers were screened for targeting 2 QTLs controlling the stay-green trait for the backcross population of IRAT 204 x B 35.

- ***XBl*139914** is a SSR marker mapping to the bottom of sorghum linkage group SBI-02, near stay-green QTL *stg* B that is detected by primers provided from the lab of Dr. john mullet at Texas A and M University. This marker was used for screening the BC_3F_1 population.

Table 5. Composition of PCR mixture:

Reagents	Stock	Final concentration
DNA	X	1.25-2.5ng/µl
PCR buffer	10X	1X
KCL	500mM	50mM
Tris HCL (P^H8.3)	100mM	10mM
$MgCl_2$	10mM	1mM
Primer	10pm	2pM
dNTP mix	2mM each	80-100µM each
Taq polymerase	1U/µl	0.1U/µl
Milli Q water	-	Complete to 5µl
Total reaction volume:	5µl	

[Table 5]

3.8.2 Touch down PCR program used:

Initial denaturation for 15 min at +94°C

1. Denaturation: for 10 sec at +94°C
2. Annealing: at 61-52°C for 20 sec (the annealing temperature for each cycle is reduced by 1°C) } For 10 cycles
3. Extension: at +72°C for 30 sec.
4. Denaturation: for 10 sec at +94°C
5. Annealing: at +54°C for 20 sec } For 35 cycles
6. Extension at +72°C for 30 sec.

The last PCR cycle is followed by a final extension of 20 min at +72°C to ensure amplification to equal length of both DNA strands.

Hold: 4°C forever.

Note:

If the parents showing the polymorphism differ in product size by more than 5bp, then PCR products were separated on 6% non-denaturing PAGE (Poly Acrylamide Gel Electrophoresis) gels and silver stained using the procedure of Fritz et al (1999). If the polymorphism detected between the parents is less than 5 bp, then PCR products were separated by capillary electrophoresis using ABI Prism 3700 (Perkin Elmer) DNA Sequencer. For capillary electrophoresis purpose fluorescent-labeled primes are used in the PCR reactions.

3.9 Non-denaturing PAGE (polyacrylamide gel electrophoresis)

1 µl of loading dye (orange red + EDTA + NaCl + glycerol) was added to 3 µl of PCR product. From this mixture, 2 µl of sample is loaded into 6% non-denaturing PAGE gel. The gel was prepared using:

52.5 ml of doubled distilled water

7.5 ml of 10 X TBE buffer

15 ml of Acrylamide: Bis-acrylamide (29:1) solution

450 µl of Ammonium Per-Sulphate (APS) and

100 µl of TEMED.

75 ml total

Along with the samples, a standard 100 bp marker ladder (50 ng/µl) was also loaded in the first and last lane of the gel to ensure proper sizing of amplified PCR fragments. Most of the markers used allowed clear differentiation of donor and recurrent parent alleles. The gel was run at 550-600 V of constant power in 0.5X TBE buffer for 3 hours using a Bio-Rad gel sequencing unit.

3.10 Silver staining

After running of PAGE gels for required time, the gels were developed by silver staining.

Sequential steps involved in silver staining

The gel was treated as follows -

1. Water for 5 min.
2. 0.1% CTAB solution for 20 min (1.5 gm in 1.5 lit of water)
3. 0.3% ammonia solution for 15 min. (19.5 ml of 25% ammonia solution in 1.5 lit of water)
4. 0.1% silver nitrate solution for 15 min (1.5 gm of silver nitrate + 6 ml of 1M NaOH in 1.5 lit of water, then solution gives cloudy appearance to that add ammonia solution till the solution becomes colorless)
5. Developer (22.5 gm of Sodium carbonate + 400µl of Formaldehyde in 1.5 lit of water)

After developing the gels were rinsed in water for 1 min and placed in fixer (22.5 ml Glycerol in 1.5 lit of water) for less than a minute.

Note: - Continuous shaking is required throughout the silver staining procedure.

After silver staining of the PAGE gels, the size (base pair) of the intensely amplified specific bands or alleles for each SSR marker was estimated based on its migration relative to the 100bp DNA ladder (fragments ranging from 100bp to 1000bp) and presence or absence of parental alleles were scored.

3.11 Data collection and analysis:

3.11.1 Scanning of gels:

After silver staining the gel is cleaned and allowed to dry for some time. Later the gel is scanned under UMAX scanner. The gel image was saved in the computer connected to UMAX scanner, which will be useful for further reference.

3.11.2 Scoring of the gels:

Scoring of microsatellites is relatively a simple process as the electrophoresis systems used for this purpose have high resolution (to a single base pair). The accurate sizing of alleles is achieved by running size markers, such as known DNA sequence, alongside the system, and in automated systems, internal size markers using a unique fluorescent label results in the sizing of the alleles in each individual of even greater accuracy. One difficulty in scoring microsatellite gels is that with mono- and di-nucleotide repeat unit microsatellites, replication slippage during amplification can lead to the presence of confusing products on the gel. Some times these slippage products are present as less intense bands of usually 1 - 5 repeat units smaller (and occasionally, greater) than actual allele. The slippage band becomes relatively less intense, the more it deviates in size from the native allele; the more it is easy to identify and ignore the band. In case of heterozygous individual scoring of such bands is much more difficult.

Automatic systems detect fluorolabelled PCR products using a laser and capillary electrophoresis. The results are transmitted directly in to a computer database where they are available for analysis using software's such as GENESCAN and GENOTYPER (Applied Biosystems/ABI). These analysis programs provide algorithms that separate native alleles automatically from slippage products.

The bands appeared on the gels were scored as A, B, H, OFF and "-" based on their pattern compared with those of the parents. "A" is referred to as the presence of allele from the recurrent parent (S 35- IC SV 111, IRAI-204), "B" is referred to as the presence of allele from B 35, "H" is referred as the heterozygous (i.e. presence of both recurrent and donor parent alleles), "OFF" was defined as an allele neither from donor parent nor from the recurrent parent and "-" is referred as a missing sample.

3.11.3 Preparation of Score sheet of the gels:

After scoring, the data is entered in to Excel spread sheet and type of the Backcross population of the crosses is entered.

3.11.4 Score sheet from the Sequencer:

For the samples whose product size is less than 5bp, labeled primers (FAM, HEX, NED) are used for keeping a PCR reaction. Then ABI plate is prepared by using Rox and Formamide.It is submitted to ABI prism 3700 (Perkin Elmer) DNA sequencer. The Data is directly obtained from the Sequencer based on the peaks obtained from the graph.

Chapter - IV

Results

CHAPTER IV

RESULTS

4.1 Checking quality and quantity of DNA samples

Genomic DNA was isolated from fresh tender leaves of sorghum seedlings. After isolating the DNA, the samples were loaded onto 0.8% ready to run agarose gel, along with a set of standards, for checking their DNA quality and quantity. If the bands were clear, without any smear, this indicated good quality DNA. If they showed any smear, DNA was re-extracted for those samples. It was estimated that the quantity of the extracted DNA was approximately 260 ng/µl when compared with HindIII digested λ DNA standards (50 ng, 100 ng and 200 ng), which indicates that the DNA obtained was in good quantity. For further confirmation DNA quantification can be done using a spectrophotometer. If the absorbance ratio at wavelengths of 260 and 280 nm exceeds 1.80, it indicates that the isolated DNA is of high quality and free of organic contaminants. Figure 6 shows the quality, and gives an indication of the quantity, of DNA in samples prepared for BC_1F_1 progenies involving recurrent parent IRAT 204.

Likewise DNA quality and quantity was assessed for samples of all generations and dilutions were made accordingly to prepare working solutions with final DNA concentrations of 2.5 ng/µl, which were then used as templates for polymerase chain reactions.

Polymerase chain reactions were performed with selected primers to generate the marker data sets required for foreground selection for all segregating generations with the three recurrent parents (ICSV 111, its sub-selection S 35, and IRAT 204).

After a set of PCR reactions was completed, PCR products were loaded onto 1.2% agarose gel along with 100 bp marker ladder DNA to confirm that PCR amplification of the product had been successful. Figure 7 shows such amplified products on a 1.2% agarose gel. PCR reactions were repeated for DNA sample × SSR primer pair combinations for which amplification had initially failed.

Finally the amplified products are loaded on 6% PAGE gels and separated electrophoretically. Following silver staining of these PAGE gels, the bands were scored for each segregant being genotype, and compared with the parental alleles along with the 100 bp marker ladder used as a reference for allele size.

For the SSR primers whose product size differences were below 5 bp, scoring was done employing the ICRISAT Applied Genomics Laboratory's ABI Prism 3700 (Perkin Elmer) automated DNA sequencer, which is based on the principle of capillary electrophoresis. For this purpose fluorescent dye-labeled primers were used for the PCR reaction. Figures 8 and 9 are examples of the output files from the ABI sequencer for different marker loci for different backcross generations with different recurrent parents.

4.2 Recurrent parent S 35

For recurrent parent S 35, a total of 15 foreground markers were used targeting four QTLs controlling the stay-green trait (see Table 1 for a list of foreground markers used for the S 35 crosses). In the BC_4F_2 generation, a total of 16 populations were genotyped and selected individuals advanced by selfing to obtain the BC_4F_3 generation. Samples of BC_4F_3 seeds produced on the selected BC_4F_2 plants have been sent to Ghana for initial phenotypic evaluation trials. Figure 11 shows a silver-stained PAGE gel for the SSR marker alleles of B35 and S 35 at locus Xtxp43.

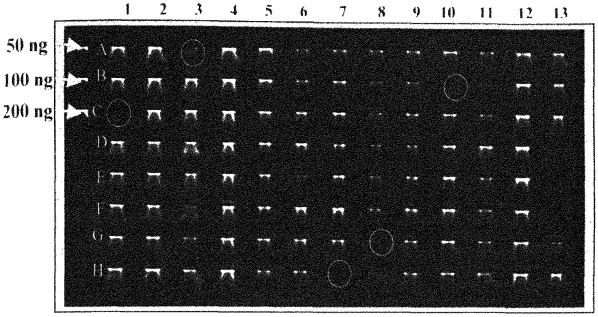
4.3 Recurrent parent ICSV 111

For recurrent parent ICSV 111, a total of 16 foreground markers were used for screening QTLs controlling the targeted stay-green trait (see Table 2 for a list of foreground markers used for ICSV 111 crosses). In the BC_3F_2 generation, a total of 13 populations were screened and selected individuals advanced by selfing to obtain the BC_3F_3 generation. Samples of BC_3F_3 seeds produced on the selected BC_3F_2 plants have been sent to Ghana for initial phenotypic evaluation trials. Figure 11 shows a silver-stained PAGE gel for the SSR marker alleles of B35 and ICSV 111 at locus Xtxp285.

4.4 Recurrent parent IRAT 204

For recurrent parent IRAT 204, a total of 23 foreground markers were used in the BC_1F_1 and BC_2F_1 generations for screening the four targeted QTLs controlling the stay-green trait. For the BC_3F_1 generation a total of 10 foreground markers were used for screening two targeted QTLs (see Tables 3 and 4 for lists of foreground markers used for IRAT 204 crosses). In the BC_3F_1 generation, a total of 10 populations were screened and selected individuals advanced by selfing and by backcrossing emasculated florets with pollen from recurrent parent IRAT 204. Figure 10 shows a silver-stained PAGE gel for the SSR marker alleles of B35 and IRAT 204 at locus Xcup26.

Figure 6: Checking the quality of DNA samples (IRAT 204 × B35) on a ready-to-run agarose gel with 50 ng, 100 ng and 200 ng markers.



Note: Red circles indicate the missing samples. These samples were re-extracted and loaded on the 13th column of the gel.

Figure 7: Checking for PCR amplified products on 1.2% agarose gel with 100 bp ladder.

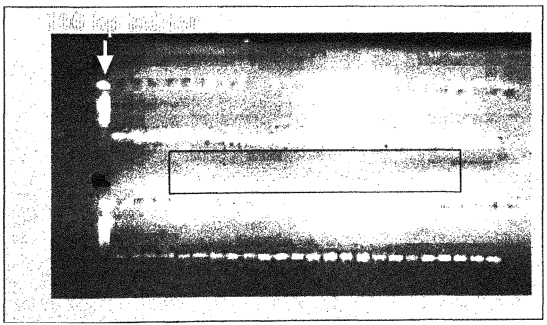


Figure 8: Annotated graphical representation (ABI Prism 3700 chromatogram) of the PCR products of the primer pair for sorghum SSR marker locus *Xtxp147* for the BC$_4$F$_3$ generation of introgression of stay-green QTLs from donor parent B35 into the background of recurrent parent IS 9830.

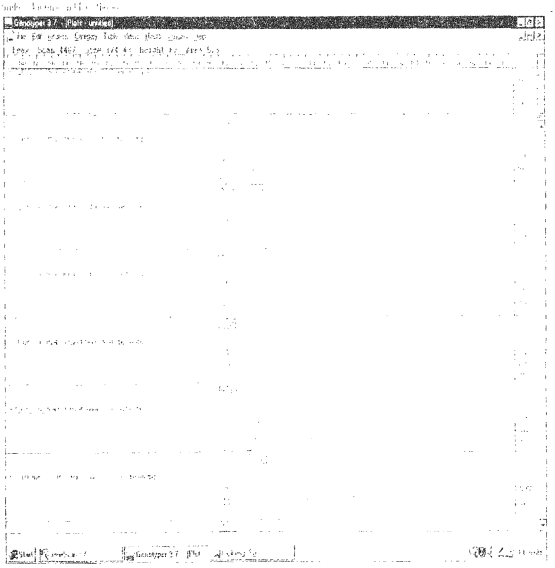


Figure 9: Annotated graphical representation (ABI Prism 3700 chromatogram) of the PCR products of the primer pair for sorghum SSR marker locus *Xtxp*298 for the BC_3F_1 generation of introgression of stay-green QTLs from donor parent B35 into the background of recurrent parent IRAT 204.

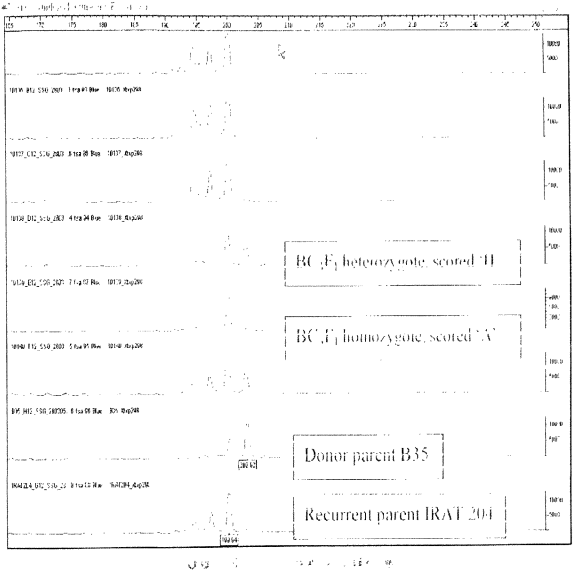
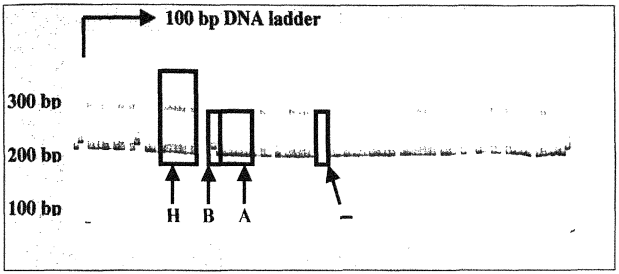
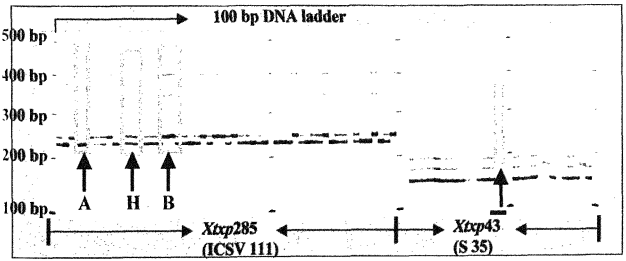


Figure 10: Scoring of SSR marker locus *Xcup*26 on a silver-stained PAGE gel for the BC_2F_1 generation (IRAT 204 X B 35).



Marker ***Xcup*26;** Allele sizes: IRAT 204/B35=222/233 bp; A = IRAT 204 homozygote; B = B35 homozygote; H = heterozygote; and " " = missing sample.

Figure 11: Scoring of SSR marker loci *Xtxp*285 and *Xtxp*43 on a silver-stained PAGE gel PAGE gel for the BC_3F_2 generation.



Markers ***Xtxp*285, *Xtxp*43**; Allele sizes for ***Xtxp*285**: B35 = 232 bp, S 35 = 222 bp
Allele sizes for ***Xtxp*43**: B35 = 152 bp, S 35 = 180 bp
A =S 35, homozygote; B = B35, homozygote; H = heterozygote, and " " missing sample.

4.5 Entering the genotyping data in to score sheets

After scoring the gels, the genotype data was entered into scoring sheets by using MS Excel spreadsheet software. Scoring is given as A/B/H/-/O, where A represents the homozygote for the recurrent parent allele; B represents the homozygote for the donor parent allele; H represents the heterozygote for both of the parent alleles; '-' represents the missing data point (due to PCR failure or DNA isolation failure); and 'O' represents the presence of an unexpected, off-type allele. Representative genotype scoring sheets for various populations of recurrent parents S 35 (BC_4F_2), ICSV 111 (BC_3F_2) and IRAT 204 (BC_1F_1, BC_2F_1, and BC_3F_1) are given in Tables 6, 7, 8, 9 and 10.

Representative score sheets for different segregating populations are given in the following pages:

Table 6: Score sheet for BC_4F_2 population of cross S 35 × B35 for target QTL *stg4* genotyped with different SSR markers.

stg4				*stg4*			
Plant no	***Xisp257***	***Xtxp225***	***Xtxp23***	**Plant no**	***Xisp257***	***Xtxp225***	***Xtxp23***
8932	B	H	H	8994	B	A	B
8933	H	B	H	8995	B	A	H
8934	H	B	H	8996	A	B	A
8935	H	-	H	8997	-	A	B
8936	B	B	H	8998	H	H	H
8937	H	-	H	8999	A	B	B
8938	-	A	A	9000	H	H	A
8939	-	H	-	9001	H	B	H
8940	B	H	H	9002	H	B	H
8941	H	H	H	9003	H	B	B
8942	B	B	B	9004	A	B	A
8943	H	B	B	9005	A	B	A
8944	B	B	B	9006	B	A	B
8945	A	B	H	9007	B	A	H
8946	-	B	A	9008	H	B	A
8947	-	H	H	9009	B	A	H
8948	H	A	H	9010	B	B	B
8949	H	H	A	9011	B	H	H
8950	B	B	H	9012	B	A	H
8951	A	B	H	9013	B	B	H
8952	B	B	H	9014	B	A	H
8953	-	B	B	9015	B	A	B
8954	-	H	-	9016	B	A	H
8955	H	A	A	9017	B	A	B
8608	1	H	1	9036	B	H	H
8956	B	H	A	9037	B	A	B
8957	H	A	B	9038	B	A	B
8958	A	B	B	9039	A	B	B
8959	A	B	B	9040	B	A	B
8960	A	B	B	9041	B	B	B
8961	B	B	B	9042	B	B	B
8962	B	B	B	9043	B	-	B

'ontinuation of Table 6:

*stg*4				*stg*4			
Plant. no.	***Xisp*257**	***Xtxp*225**	***Xtxp*23**	**Plant. no.**	***Xisp*257**	***Xtxp*225**	***Xtxp*23**
8963	B	H	B	9044	B	B	B
8964	H	A	B	9045	B	H	B
8965	-	B	B	9046	A	B	B
8966	B	H	B	9047	B	H	B
8967	A	B	B	9048	B	H	B
8968	A	B	B	9049	B	A	B
8969	H	A	B	9050	B	H	B
8970	H	B	B	9051	-	H	B
8971	H	H	B	9052	B	A	B
8972	H	H	B	9053	B	H	B
8973	H	A	B	9054	B	B	B
8974	B	H	B	9055	A	H	B
8975	B	A	B	9056	H	H	B
8976	B	A	B	9057	B	A	B
8977	B	A	B	9058	H	H	B
8978	B	A	B	9059	A	H	B
8979	B	A	B	9060	A	A	B
8980	B	H	A	9061	B	H	B
8981	A	B	H	9062	B	H	-
8982	B	H	A	9063	B	H	B
8983	B	H	H	9064	A	B	B
8984	A	H	A	9065	B	A	B
8985	B	B	H	9066	B	B	B
8986	B	B	H	9067	B	B	B
8987	A	B	B	9068	-	B	B
8988	B	H	B	9069	B	A	B
8989	H	B	A	9070	B	A	B
8990	B	B	B	9071	B	H	B
8991	B	-	H	9072	A	B	B
8992	A	B	A	9073	B	A	B
8993	H	B	H	9074	A	B	B
S 35	A	A	A	B35	B	B	B

Table 7: Score sheet for BC_3F_2 population of cross ICSV 111 × B35 for target QTL *stg*A with three SSR markers.

*stg*A				*stg*A			
Plant. no.	*Xtxp*32	*Xtxp*149	*Xtxp*357	Plant. no.	*Xtxp*32	*Xtxp*149	*Xtxp*357
9298	A	A	A	9345	A	A	A
9299	A	A	A	9346	A	A	A
9300	A	A	A	9347	A	A	A
9301	-	-	A	9348	A	A	A
9302	A	A	A	9349	H	A	A
9303	A	A	A	9350	A	A	A
9304	A	A	A	9351	A	A	A
9305	A	-	A	9352	H	A	A
9306	A	-	A	9353	A	A	A
9307	A	A	A	9354	H	A	A
9308	A	A	A	9355	B	A	A
9309	A	A	A	9356	B	A	A
9310	A	A	A	9357	B	A	A
9311	A	A	A	9358	-	A	A
9312	A	A	A	9359	B	-	A
9313	H	A	A	9360	A	A	A
9314	A	-	A	9361	B	A	A
9315	A	A	A	9362	H	A	A
9316	A	A	A	9363	H	A	A
9317	A	-	A	9364	B	A	A
9318	A	A	A	9365	H	A	A
9319	A	A	A	9366	-	A	A
9320	A	A	A	9367	B	A	A
9321	A	A	A	9368	H	A	A
9322	A	A	A	9369	-	A	A
9323	A	A	A	9370	H	A	A
9324	A	A	A	9371	H	A	A
9325	A	A	A	9372	A	A	A
9326	-	A	A	9373	A	A	A
9327	A	A	A	9374	A	A	A
9328	-	A	A	9375	A	A	A
9329	A	A	A	9376	A	A	A
9330	A	A	A	9377	A	-	A
9331	A	A	A	9378	A	A	A
9332	A	A	A	9379	A	A	A
9333	A	A	A	9380	A	A	A
9334	A	A	A	9381	A	A	A
9335	A	A	A	9382	A	A	A
9336	A	A	A	9383	A	A	A
9337	A	A	A	9384	-	A	A
9338	A	A	A	9385	A	-	A

Continuation of Table 7:

	*stg*A				*stg*A		
Plant. no.	***Xtxp*32**	***Xtxp*149**	***Xtxp*357**	**Plant. no.**	***Xtxp*32**	***Xtxp*149**	***Xtxp*357**
9339	A	A	A	9386	A	A	A
9340	A	A	A	9387	A	A	A
9341	A	A	A	9388	B	H	A
9342	A	A	A	9389	A	A	A
9343	A	A	A	9390	A	A	A
9344	A	A	A	9391	-	A	A
ICSV 111	A	A	A	B35	B	B	B

Table 8: Score sheet for BC_3F_2 population of cross ICSV 111 × B35 for targeted QTL *stg*2 with three SSR markers.

	*stg*2				*stg*2		
Plant no.	***Xtxp*114**	***Xisp*307**	***XSbAGF*08**	**Plant no.**	***Xtxp*114**	***Xisp*307**	***XSbAGF*08**
9408	B	B	B	9463	H	A	A
9409	-	B	B	9464	H	H	H
9410	*A*	H	A	9465	H	H	B
9411	H	H	H	9466	-	-	-
9412	*A*	A	H	9467	H	-	H/B
9413	H	B	A	9468	H	-	B
9414	H	A	B	9469	-	-	-
9415	-	A	A	8816	A	-	-
9416	H	-	B	9520	-	A	-
9417	A	-	B	9521	H	A	B
9418	A	A	B	9522	-	A	B
9419	A	H	B	9523	H	A	B
9420	H	H	A	9524	B	A	H
9421	-	H	B	9525	-	-	A
9422	H	A	A	9526	H	-	B
8758	-	-	-	9527	B	A	B
9455	A	A	B	9528	B	A	B
9456	H	H	B	9529	-	-	B
9457	H	-	H	9530	H	A	B
9458	B	B	H	9531	B	A	B
9459	B	H	H/B?	9532	H	A	H
9460	H	H	B	9533	B	A	B
9461	A	-	B	9534	H	A	H
9462	A	A	H	9535	H	A	B
ICSV 111	A	A	A	B35	B	B	B

Table 9: Score sheet for BC_3F_1 population of cross IRAT 204 × B35 for targeted QTL *stg*B with three different SSR markers.

	stgB				*stgB*		
Plant no.	***Xtxp*7**	***Xtxp*296**	***XBI*139914**	**Plant no.**	***Xtxp*7**	***Xtxp*296**	***XBI*139914**
9185	A	H	H	9231	A	A	A
9186	H	H	H	9232	A	H	A
9187	A	B	-	9233	A	H	A
9188	H	H	-	9234	A	H	A
9189	H	-	H	9235	-	B	A
9190	H	A	B	9236	A	H	A
9191	A	A	A	9237	A	A	A
9192	H	A	A	9238	A	A	A
9193	A	H	A	9239	A	B	A
9194	A	A	A	9240	A	B	A
9195	B	A	B	9241	A	H	A
9196	H	A	A	9242	A	H	A
9197	H	A	-	9243	A	B	A
9198	B	A	A	9244	A	A	A
9199	H	A	B	9245	A	-	A
9200	-	A	B	9246	A	A	A
9201	B	A	A	9247	A	A	A
9202	A	A	A	9248	A	A	A
9203	H	A	A	9249	A	A	A
9204	H	A	A	9250	A	A	A
9205	A	A	B	9251	A	A	A
9206	A	A	A	9252	A	A	A
9207	H	A	A	9253	A	A	A
9208	B	A	H	9254	A	A	A
9209	H	A	A	9255	A	A	A
9210	H	A	A	9256	-	A	A
9211	A	A	B	9257	A	A	A
9212	A	A	A	9258	A	A	A
9213	B	A	B	9259	A	A	A
9214	A	B	H	9260	A	B	A
9215	B	B	A	9261	A	B	A
9216	H	B	A	9262	A	B	A
9217	B	B	-	9263	A	B	A
9218	B	B	B	9264	A	B	A
9219	A	B	A	9265	A	B	A
9220	A	B	A	9266	A	B	A
9221	A	B	A	9267	A	B	A
9222	A	H	A	9268	A	B	A
9223	A	H	A	9269	A	-	-
9224	A	H	A	9270	A	H	A
9225	A	A	A	9271	A	A	A
9226	A	H	A	9272	A	A	A

Continuation of table 9:

stgB				*stgB*			
Plant no.	***Xtxp*7**	***Xtxp*296**	***XBI*139914**	**Plant no.**	***Xtxp*7**	***Xtxp*296**	***XBI*139914**
9227	-	A	-	9273	A	B	A
9228	A	H	A	9274	A	H	A
9229	A	B	A	9275	A	A	A
9230	A	A	A	9276	A	H	A
IRAT204	A	A	A	9277	A	A	A
B35	B	B	B	-	-	-	-

Table 10: Score sheet for BC_1F_1 and BC_2F_1 populations of cross IRAT 204 × B35 for the targeted QTL *stg*1 with seven different SSR markers.

	*stg*1						
Plant no.	***Xgap*214**	***Xtxp*34**	***Xtxp*285**	***Xisp*361**	***Xisp*323**	***Xtxp*114**	***Xisp*307**
8501	H	H	H	H	A	A	B
8502	A	A	A	A	A	A	B
8503	A	A	A	A	A	H	B
8504	H	H	H	H	H	H	B
8505	-	H	H	H	A	H	-
8506	H	H	H	H	H	H	B
8507	A	H	H	H	H	H	B
8508	-	A	A	A	A	A	-
8509	-	H	-	H	O	A	B
8510	A	A	H	H	H	A	B
8511	A	H	H	H	H	H	B
8512	A	H	H	H	O	H	B
8513	A	A	A	A	A	-	-
8514	A	H	H	H	H	H	B
8515	A	H	H	H	H	A	B
8516	H	H	H	H	H	H	-
8517	A	A	A	A	H	A	B
8518	A	A	A	A	A	A	B
8519	A	A	A	A	A	H	B
8520	A	A	-	A	A	H	B
8521	A	A	A	A	H	H	-
8522	O	A	A	A	O	H	B
8523	A	H	H	H	H	H	B
8524	H	H	H	-	A	A	B
8525	A	A	H	H	H	H	B
8526	A	A	H	H	A	A	B
8527	A	A	H	H	H	H	B
8528	A	H	H	H	O	A	B
8529	A	H	A	H	H	A	B

Continuation of Table 10:

	*stg*1						
Plant no.	*Xgap*214	*Xtxp*34	*Xtxp*285	*Xisp*361	*Xisp*323	*Xtxp*114	*Xisp*307
8530	A	H	H	H	H	H	B
8531	A	H	H	H	A	H	-
8532	-	A	A	A	A	A	B
8533	A	A	-	A	A	H	B
8534	A	H	H	H	H	A	B
8535	A	H	H	H	H	H	B
8536	-	A	-	H	H	H	B
8537	A	A	U	A	A	H	-
8538	A	A	A	A	H	A	B
8539	A	A	A	A	A	A	B
8540	O	H	H	H	B	H	B
8541	B	A	A	A	A	-	B
8542	A	H	H	H	A	A	B
8543	H	A	-	A	A	A	-
8544	H	H	H	H	-	A	B
8545	-	A	-	-	A	-	-
8546	-	A	-	A	A	H	B
8547	-	H	-	H	H	A	B
8548	-	A	-	A	A	H	-
8549	O	A	A	A	A	A	-
8550	A	A	A	A	A	A	B
8551	B	H	H	H	H	A	B
8552	B	A	A	A	H	-	-
8553	-	H	H	H	H	A	A
8554	B	H	H	H	H	A	-
8555	B	H	H	H	A	A	B
8556	A	A	A	A	A	A	A
8557	A	A	A	A	A	A	-
8558	A	A	A	A	A	H	B
8559	A	O	U	H	B	A	B
8560	A	A	A	A	H	-	-
8561	A	H	-	H	A	A	B
8562	A	A	A	A	A	A	-
8563	A	H	H	H	H	A	A
8564	A	A	A	A	H	A	-
8565	A	A	A	A	A	A	-
8566	B	A	A	A	H	A	B
8567	A	A	A	A	A	-	A
8568	H	A	A	A	A	A	-
8569	A	H	A	H	-	A	B
8570	A	A	H	H	H	A	-
8571	A	A	A	A	A	A	A
8572	B	A	A	A	A	H	
8573	A	A	A	A	-	A	A

Continuation of Table 10:

	*stg*1						
Plant no.	*Xgap*214	*Xtxp*34	*Xtxp*285	*Xisp*361	*Xisp*323	*Xtxp*114	*Xisp*307
8574	A	A	A	A	-	H	A
8575	A	A	A	A	-	A	A
8576	A	H	H	H	-	A	A
8577	A	A	A	A	A	A	A
8578	A	A	A	A	A	A	A
8579	A	H	B	H	B	A	A
8580	A	A	A	A	A	A	A
8581	A	A	A	A	A	H	A
8582	A	B	A	A	A	A	B
8583	-	A	A	A	A	H	B
8584	A	A	H	H	A	A	B
8585	H	A	A	A	A	A	B
8586	H	B	A	-	H	B	-
8587	A	B	A	A	H	A	B
8588	H	A	A	A	A	A	B
8589	A	A	H	H	H	A	B
8590	B	B	-	A	A	H	B
8591	H	H	H	H	H	A	B
8592	-	B	H	-	-	-	-
8593	-	A	A	A	A	A	
8594	H	A	A	A	A	A	B
IRAT 204	A	A	A	A	A	A	A
B35	B	B	B	B	B	B	B

4.6 Phenotypic evaluation of the population

Seed of the selected BC_4F_3 progenies of recurrent parent S 35 and the BC_3F_3 progenies of recurrent parent ICSV 111 has been sent to Ghana for initial phenotypic evaluation. In total, 42 homozygous introgression lines, targeting six stay-green QTLs (*stg*A, *stg*B, *stg*1, *stg*2, *stg*3, and *stg*4) were selected for phenotypic evaluation. Tables 11a and 11b list selected materials for which seed was sent to Ghana for undertaking the initial phenotypic evaluation trials during the rainy season of 2005.

Table 11a: List of selected BC_4F_3 progenies of S 35 x B35 cross

Selected BC_4F_3 progenies of S 35 x B35					
Targeted QTL	Ser. no.	Plant no.	Targeted QTL	Ser. no.	Plant no.
*stg*A			*stg*3		
	1	8858		14	8881
	2	8873		15	8888
	3	9105		16	8890
	4	9122		17	8897
	5	9127		18	9289
	6	9142		19	9290
	7	9144		20	8908
	8	9147	*stg*4		
	9	9151		21	8942
	10	9162		22	8944
*stg*B				23	8990
	11	9128		24	9010
	12	9127		-	-
	13	9125		-	-

Table 11b: List of selected BC_3F_3 progenies of ICSV 111 x B35 cross

Selected BC_3F_3 progenies of ICSV 111 x B35		
Targeted QTL	Ser. no.	Plant no.
*stg*1		
	25	9408
	26	9409
	27	9458
	28	9528
	29	9531
	30	9533
*stg*2		
	31	9472
	32	9478
	33	9487
	34	9493
	35	9495
	36	9496
	37	9498
	38	9505
	39	9510
	40	9518
*stg*3		
	41	9444
	42	9451

Chapter - V

Discussion

CHAPTER V

Discussion

This work represents the advancement of the marker-assisted backcrossing of stay-green QTLs into two elite sorghum lines, S 35 and IRAT 204. Marker-assisted introgression of desirable alleles may have several advantages over introgression using phenotypic information only. For the allele to be introgressed, marked chromosome segments ensure that the correct segment of donor genome is incorporated in to the recipient line. For alleles acting in a non-additive manner, especially those involved in inter-locus (epistatic) interactions, using markers may be the only way to ensure a successful trait introgression programme.

Using marker-assisted selection, within less than one year we are able to advance introgression of genomic regions from exotic stay-green donor parent B35 into the genetic backgrounds of the two elite recurrent parents (S 35 and IRAT 204) by two generations. Markers linked to stay-green QTL regions to be transferred from donor B35 to recurrent parents S 35 and IRAT 204 were used for foreground selection, whereas unlinked markers evenly distributed over genomic regions of the recurrent parent that were to be recovered, were used for background selection. Based on the genotype data, individuals heterozygous (in the BC_3F_1 generation) or homozygous (BC_3F_2 generation) for markers spanning stay-green QTL regions were selected during the first step of selection (foreground selection). From among the individuals selected in this first step, those with minimal presence of donor alleles at marker loci unlinked to stay-green QTLs were selected during the second step of selection (background selection).

5.1 Criteria for the Selection of Individuals for Generation Advance

Foreground markers used for the selection of the individual segregants that are to be advanced. The individuals scored as 'H' or 'B' for markers used in foreground selection and 'A' allele for background markers were selected for generation advance. Individuals scored 'H' at a particular locus, are expected to produce progeny segregating 1:2:1 for homozygosity for the recurrent parent allele (scored 'A'), heterozygosity (scored 'H'), and homozygosity for the donor parent allele (scored 'B') if they are advanced by selfing, or segregating 1:1 for homozygosity for the recurrent parent allele (scored 'A') and heterozygosity (scored 'H') if they are advanced by backcrossing to the recurrent parent. Presence of the 'A' genotype for background markers and the 'H' genotype for foreground markers flanking a particular stay-green QTL ensures the recovery of the recurrent parent genome (S 35 or IRAT 204) while advancing introgression of a genomic region contributing to the stay-green trait. Individuals meeting these criteria were selected and advanced to next the generation by selfing and backcrossing. Individuals scored 'A' for all (foreground and background) markers should be very similar to the recurrent parent (in fact, they should be identical to the recurrent parent except for small introgressions that were not detected due to the limited genomic coverage possible with the small total number of marker used in this study) and could be selected as controls entries for use in field trials to assess the efficacy of marker-assisted selection for the stay-green trait.

For selected individuals, the markers scored as 'H' or 'B' and those that didn't amplify during the BC_nF_1 generation were screened again in next ($BC_{n+1}F_1$ or BC_nF_2) generation. The markers scored 'A' (i.e., homozygous for the allele of the recurrent parent) are not tested further in more advanced generations because recovery of the recurrent parent genotype at these loci has been completed and their genetic

constitution is not expected to change further assuming a negligible rate of mutation and no outcrossing to non-recurrent parent genotypes. Once the recurrent parent genome has been recovered for all the background markers, a generation of selfing and selection for donor parent marker allele homozygotes at loci flanking specific target stay-green QTLs will be conducted, and the selected genotypes then multiplied by selfing. The resulting stay-green QTL introgression lines can then be tested multilocationally, along with their recurrent parents, to evaluate them phenotypically for the stay-green character and other agronomic traits. After testing, if the progeny with the stay-green trait are found to be significantly superior compared with the recurrent parent controls, they can be released as improved versions of that variety with improved potential to tolerate terminal drought stress due to introgression of genes conferring the stay-green character.

5.2 Advancing the BC_4F_2 generation for recurrent parents S 35 and BC_3F_2 generation for the recurrent parent ICSV 111

Selected BC_4F_2 progenies were sown along with their recurrent parent S 35, and BC_3F_2 progenies for recurrent parent ICSV 111 (multiple sowing dates for recurrent parents is advisable), and donor parent B35. Tissue samples for individual BC_nF_2 plants in each progeny were collected. DNA was isolated and quantified and working samples of uniform DNA concentration were prepared. PCR amplification of the BC_4F_2 population progenies was done with primers for SSR markers that flank the targeted stay-green QTLs. By using PAGE gels or the ABI 3700 sequencer, SSR marker data for foreground selection (at least three marker loci per targeted stay-green QTL) was generated.

By using foreground marker data, individual BC_4F_2 and BC_3F_2 plants were selected and selfed to obtain homozygous stay-green QTL introgression lines. Selfed selected BC_nF_2 individuals were harvested on a single-plant basis, noting those that most closely resembled the recurrent parent. The BC_4F_3 and BC_3F_3 seed thus obtained will be multiplied and the phenotypic evaluation(s) of the homozygous stay-green QTL introgression lines need to be conducted. Based on the marker data generated for BC_4F_2 progenies for recurrent parent, S 35 and BC_3F_2 progenies for recurrent parent, ICSV 111, 42 BC_nF_3 introgression lines homozygous for various stay-green QTLs were identified and sent to Ghana for undertaking field evaluation trails during the rainy season of 2005.

(P.T.O)

5.3 BC_4F_2 generation for recurrent parent S 35

Out of 271 BC_4F_2 individuals (8825-8674; 8675-8700) screened in populations 3001, 3006, 3008, 3031, 3029, 3033, and 3011, a total of 16 individuals were selected for advancement, based on the genotyping results. These selected individuals were targeted for stay-green QTLs *stg*A, *stg*A + *stg*3, *stg*B, *stg*3, and *stg*4 (Table 12). The selected BC_4F_2 individuals were selfed to obtain BC_4F_3 seed.

Table 12: Selected BC_4F_2 generation individuals for recurrent parent S 35

Generation	Populations	Selected individuals	Targeted QTLs
BC_4F_2	3001	8601	*stg*3
BC_4F_2	3001	8602	*stg*A+*stg*3
BC_4F_2	3001	8603	*stg*A+*stg*3
BC_4F_2	3006	8604	*stg*3
BC_4F_2	3006	8605	*stg*4
BC_4F_2	3006	8608	*stg*4
BC_4F_2	3006	8610	*stg*4
BC_4F_2	3008	8624	*stg*3
BC_4F_2	3008	8625	*stg*4
BC_4F_2	3008	8626	*stg*A
BC_4F_2	3031	8629	*stg*3
BC_4F_2	3031	8640	*stg*B
BC_4F_2	3029	8645	*stg*B
BC_4F_2	3033	8664	*stg*B
BC_4F_2	3031	8683	*stg*B
BC_4F_2	3011	8688	*stg*3

5.4 BC_3F_2 generation for recurrent parent ICSV 111

Out of 237 BC_3F_2 individuals (8701-8938) screened in population 3050, 3053, 3056, 3055, 3054, and 3052, a total of 13 individuals were selected for advancement based on the genotyping results. The selected BC_3F_2 individuals were selfed to obtain BC_3F_3 seed. These selected individuals were targeted for stay-green QTLs *stg*A, *stg*1, *stg*2, and *stg*3, etc (Table 13).

Table 13: Selected BC_3F_2 generation individuals for recurrent parent ICSV 111

Generation	Populations	Selected individuals	Targeted QTLs
BC_3F_2	3050	8701	*stg*A
BC_3F_2	3050	8702	*stg*A
BC_3F_2	3050	8703	*stg*A
BC_3F_2	3053	8711	*stg*A
BC_3F_2	3053	8712	*stg*3
BC_3F_2	3056	8714	*stg*A
BC_3F_2	3056	8617	*stg*3
BC_3F_2	3056	8729	*stg*2
BC_3F_2	3055	8737	*stg*3
BC_3F_2	3055	8742	*stg*3
BC_3F_2	3054	8758	*stg*2
BC_3F_2	3052	8793	*stg*1
BC_3F_2	3052	8816	*stg*2

Seed of the selected BC_4F_3 progenies for recurrent parent S 35 and the selected BC_3F_3 progenies for recurrent parent ICSV 111 have been sent to Ghana for phenotypic evaluation. Out of 42 homozygous introgression lines selected for phenotypic testing in Ghana, 24 samples are of BC_4F_3 progenies targeting 4 QTLs (*stg*A, *stg*B, *stg*3, and *stg*4) and 18 samples are of BC_3F_3 progenies targeting 3 QTLs (*stg*1, *stg*2 and *stg*3). Table 14 shows the list of selected seed material that has been sent to Ghana for the phenotypic evaluation in the rainy season of 2005.

Table 14: Selected BC_4F_3 and BC_3F_3 stay-green QTL introgression lines in the genetic background of S 35 and ICSV 111

Selected BC_4F_3 progenies of S 35			Selected BC_3F_3 progenies of ICSV 111		
Targeted QTL	**Ser. no.**	**Plant no.**	**Targeted QTL**	**Ser. no.**	**Plant no.**
***stg*A**	1	8858	***stg*1**	25	9408
	2	8873		26	9409
	3	9105		27	9458
	4	9122		28	9528
	5	9127		29	9531
	6	9142		30	9533
	7	9144	***stg*2**	31	9472
	8	9147		32	9478
	9	9151		33	9487
	10	9162		34	9493
***stg*B**	11	9128		35	9495
	12	9127		36	9496
	13	9125		37	9498
***stg*3**	14	8881		38	9505
	15	8888		39	9510
	16	8890		40	9518
	17	8897	***stg*3**	41	9444
	18	9289		42	9451
	19	9290	-	-	-
	20	8908	-	-	-
***stg*4**	21	8942	-	-	-
	22	8944	-	-	-
	23	8990	-	-	-
	24	9010	-	-	-

5.5 Advancing the backcross generations for recurrent parent IRAT 204

Selected BC_1F_1, BC_2F_1, and BC_3F_1 progenies were sown along with recurrent parent IRAT 204 (multiple sowing dates for recurrent parent is advisable) and donor parent B35. Tissue samples for individual BC_nF_1 plants were collected. DNA was isolated and quantified and working samples of uniform DNA concentration were prepared. PCR amplification of DNA samples for all the BC_nF_1 individuals was done with primers for SSR markers flanking the targeted stay-green QTLs. By using PAGE gels or the ABI 3700 sequencer, SSR marker data for foreground selection (at least three marker loci per targeted stay-green QTL) was generated.

By using foreground marker data, individual BC_nF_1 plants heterozygous at all marker loci associated with one or more of the targeted stay-green QTLs were selected. Selected plants were emasculated for an additional backcross to their recurrent parent. About 100 florets on a panicle of each selected BC_nF_1 plants were emasculated and backcrossed to produce $BC_{n+1}F_1$ seed. Some of the florets were retained for selfing to produce BC_nF_2 seed from each selected BC_nF_1 plant. The information on foreground marker genotype of the BC_nF_1 plants is given in Tables 15, 16, and 17.

5.6 BC_1F_1 generation for recurrent parent IRAT 204

Out of 40 BC_1F_1 individuals (8501-8540) screened, 13 individuals were selected for generation advancement based on the genotyping results. The selected BC_1F_1 plants were advanced to BC_2F_1 by backcrossing emasculated florets with pollen from recurrent parent IRAT 204. These selected individuals were targeted for QTLs *stg*A + *stg*B, *stg*3+*stg*A+*stg*B, *stg*A, *stg*B, and *stg*3(?)+*stg*B, etc (Table 15).

Table 15: Selected BC_1F_1 generation individuals for recurrent parent IRAT 204

Generation	Pedigree	No. of BC_2F_1individuals sown	Targeted QTLs
BC_1F_1	8503	7	*stg*A + *stg*B
BC_1F_1	8506	3	*stg*3 + *stg*A + *stg*B
BC_1F_1	8508	3	*stg*A
BC_1F_1	8509	3	*stg*B
BC_1F_1	8510	3	*stg*3 + *stg*A + *stg*B
BC_1F_1	8511	18	*stg*3 + *stg*B
BC_1F_1	8512	3	*stg*A
BC_1F_1	8513	17	*stg*A
BC_1F_1	8519	14	*stg*A + *stg*B
BC_1F_1	8523	18	*stg*A + *stg*B
BC_1F_1	8524	1	*stg*A + *stg*B
BC_1F_1	8533	12	*stg*3(?) + *stg*B
BC_1F_1	8536	14	*stg*B
-	Total	116	-

5.7 BC_2F_1 generation for recurrent parent IRAT 204

Out of 53 BC_2F_1 individuals (8541-8594) screened, a total of 13 individuals were selected for generation advancement based on the genotyping results. The selected BC_2F_1 individuals were selfed and backcrossed to advance to the next generation. These selected individuals were targeted for stay-green QTLs *stg*A, *stg*B, and *stg*A + *stg*B, (Table 16).

Table 16: Selected BC_2F_1 generation individuals for recurrent parent IRAT 204

Generation	Pedigree	No. of BC_2F_1 individuals sown	Targeted QTLs
BC_2F_1	8543	6	*stg*B
BC_2F_1	8545	16	*stg*B
BC_2F_1	8547	16	*stg*A
BC_2F_1	8562	10	*stg*A + *stg*B
BC_2F_1	8565	12	*stg*A
BC_2F_1	8566	6	*stg*A
BC_2F_1	8568	5	*stg*A
BC_2F_1	8571	13	*stg*A + *stg*B
BC_2F_1	8573	13	*stg*B
BC_2F_1	8585	5	*stg*A
BC_2F_1	8586	5	*stg*A
BC_2F_1	8592	15	*stg*A + *stg*B
BC_2F_1	8594	14	*stg*A + *stg*B
-	Total	136	-

5.8 BC_3F_1 generation for recurrent parent IRAT 204

Out of 182 BC_3F_1 individuals (9958-10140) screened, a total of sixteen individuals were selected for advancement to the next generation based on the genotyping results. These sixteen selected BC_3F_1 individuals were selfed and backcrossed to advance them to the next generation. These selected individuals were targeted for stay-green QTLs *stg*A, *stg*A + *stg*B, and *stg*B (Table 17).

Table 17: Selected BC_3F_1 generation individuals for recurrent parent IRAT 204

Generation	Pedigree	No. of Individuals sown	No. of individuals selected for advancement	Targeted QTL
BC_3F_1	8868	11	-	*stg*A
BC_3F_1	8874	11	-	*stg*A
BC_3F_1	8878	11	3	*stg*A
BC_3F_1	8882	11	1	*stg*A
BC_3F_1	8893	16	3	*stg*B
BC_3F_1	8901	16	1	*stg*B
BC_3F_1	9096	16	3	*stg*B
BC_3F_1	9100	30	2	*stg*A + *stg*B
BC_3F_1	9108	30	3	*stg*A + *stg*B
BC_3F_1	9114	30	-	*stg*A + *stg*B
-	TOTAL	182	16	

As all of the BC_3F_1 individuals selected for advancement could be backcrossed with recurrent parent (IRAT 204) for BC_nF_1 populations, only a few backcrosses were made. The reasons for comparatively low number of backcrosses being made are as follows:

- ✓ The major problem was that the foreground marker data generation was not completed before the segregating generation individuals started to flower, so

that the number of plants that needed to be emasculated for backcrossing was greater than the capacity of the team of ICRISAT support staff assisting with this activity so that choice of plants to be emasculated was in fact random rather than being based on the data for marker alleles at loci flanking the target stay-green QTLs.

The individual segregants in the BC_nF_1 populations showed different times of panicle initiation than their recurrent parent (which was sown on multiple sowing dates in order to minimize this common problem in advancing backcross generations).

Due to slight drizzle during the crossing season, which is quite abnormal in the summer season, collection of pollen became a difficult task. Pollen collection should always be carried in dry weather conditions as otherwise the collected pollen sticks to the wet sides of pollen collection bags from which it cannot be shaken onto the stigmas of emasculated florets.

Re-sowing of some pots where the BC_nF_1 seed initially sown had failed to germinate was not accompanied by additional sowing of the recurrent parent. Plants produced by this later BC_nF_1 sowing came to flowering late during the peak of the summer season's high temperatures, which adversely affected pollen production and ultimately resulted in poor crossed seed set and poor selfed seed set.

The short period of sorghum pollen viability, and lack of an effective pollen storage protocol for sorghum is another major limitation.

Even in the crossed individuals there was poor seed set due to the relatively high temperatures during fertilization and early grain filling stages of growth.

5.9 Efficiency of marker-assisted selection (MAS)

Lande and Thompson (1990) and Lande (1992) investigated the efficiency of MAS for both individual selection and mass selection in random-mating populations. There are three approaches to applying MAS to plant breeding: (1) selection on markers alone with no measurement of phenotype; (2) simultaneous selection based on both markers and phenotype; and (3) two-stage selection, the first stage involving use of markers to select among seedlings and the second involving phenotypic selection among surviving adults. For selection of individuals, the efficiency of MAS relative to that of phenotypic selection of the same intensity is $(p/h^2)^{1/2}$, where p is the proportion of the additive genetic variance for the target trait accounted for by markers, and h^2 is the trait heritability. Selection on markers alone will be more efficient than phenotypic selection only when the proportion of the genetic variance explained by markers exceeds the heritability of the trait. Therefore, selection on markers alone will be most useful for traits with low heritabilities when large proportions of their variability have been explained by markers.

Lande and Thompson (1990) concluded that molecular marker loci can be used to enhance the efficiency of artificial selection for quantitative traits. The potential efficiency of MAS depends upon the heritability of the trait, the proportion of the genetic variance explained by the markers, and the selection method. A major practical problem in using MAS is that recombination will reduce linkage disequilibrium between the markers and the target QTL, thus diminishing selection effectiveness. The successful application of MAS will require very tight linkages between markers and QTL, especially if very few marker loci are used for foreground selection.

Dudley (1993) showed that the additive genetic variance explained by the marker decreases as the recombination between the marker and QTL increases and stated that the marker-assisted selection (MAS) will by more effective than phenotypic selection when the proportion of additive variance accounted for by the marker loci is greater than the heritability of the trait.

MAS is predicted to be more effective than phenotypic selection for traits of low heritabililty, which suggests that the additive effects of QTLs detected would be of small magnitude. The results from Beavis et al. (1994) suggested that the MAS can be effectively used to evaluate a large number of progeny from a small number of crosses.

Moreau et al. (1998), based on computer simulation studies, concluded that MAS can be more efficient than selection based only on phenotype in a large range of situations as long as the size of the population is at least 200, the heritability of the trait is between 0.05 and 0.50, and the markers are relatively close to the QTLs. They also explained that heritability could be increased by increasing the number of replicates used in phenotyping. They stated that phenotypic selection based on three replicates would be as effective as MAS based on one replicate.

Yousef and Juvik (2001) compared MAS to phenotypic selection in sweet corn for one cycle. They found that MAS was more effective than phenotypic selection for the majority of traits evaluated. They used relatively small population sizes to identify marker-QTL associations. The population used for selection was the same population used to map the QTLs. Their results are encouraging because one mapping population consisted of 214 families and the other population consisted of 117 families. They suggested that the effectiveness of MAS would likely decrease

with further cycles of selection as marker-QTL associations are reduced due to crossing-over.

Marker-assisted selection has the potential to greatly reduce the time required for selecting desirable genotypes with traits of interest (Morris et al., 2003). Marker-assisted selection is more efficient and cost-effective than conventional selection for traits with low heritabilities and large phenotypic effects (Hospital et al., 1997). Through MAS, we advanced two backcross generations within one year (2004-/05). When conventional breeding strategies are applied, the advancement of two backcross generations with selection for the stay-green character would take two years, and it might be very difficult to differentiate reliably between individuals heterozygous for more than one of the genomic regions contributing to the trait. Conventional breeding schemes feature lower short-term operational costs during the research stage, but take longer times to complete, whereas MAB features higher short-term operational costs during the research stage, but takes less time to complete. The varietal testing prior to release stage and adoption stages are assumed to be identical in terms of cost as well as duration in case of MAS and conventional selection. From an economic point of view, the advantage of MAS thus derives from the fact that the varietal testing prior to release and adoption stages move forward in time. This suggests that MAS needs more initial investment but is worthwhile because by accelerating the rate of release of improved cultivars (or the rate of gain achieved between cultivar releases if the frequency of releases in time remains the same), MAS generates large additional economic benefits (Morris et al., 2003).

The efficiency of MAS is generally reduced with increasing genetic distance between the flanking markers used for each QTL for which MAS is being applied. Hospital et al. (1997), based on the simulation studies, recommended an optimal

distance between two adjacent flanking markers of about 5–10 cM. We observed that the frequency of recurrent parent genotypes among the selected progeny increased as the selection intensity for recurrent genotypes increased, as predicted by Knapp (1998). Practically speaking, the number of markers that must be used decreases in each successive backcross generation, because once the recurrent parent allele has been fixed at any given non-target locus, it is not necessary to continue screening at that locus in subsequent generations as the locus will remain homozygous for the rest of the generations of selection regardless of whether these involve selfing or backcrossing to the recurrent parent (Morris et al., 2003). The decreasing number of markers required in each successive generation reflects the increasing percentage of the recurrent parent genome that is recovered in homozygous form, and hence fixed, in each backcross generation.

The fact that MAS technology is so challenging should not be a reason for discouragement, but instead, should provide a wake-up call for more ingenuity, better planning and execution of marker-assisted breeding programs. MAS for quantitative traits is in an important transition phase, and the field is on the verge of producing convincing results. Technology development, including automation, allele-specific diagnostics and DNA chips, will make marker-assisted selection approaches based on large-scale screening much more powerful and effective in future (Young, 1999).

5.10 Conclusion:

From the data obtained, SSRs seem to be a very useful tool in backcross breeding programs of cultivated species such as sorghum. They provide reproducible results and are fairly simple to obtain. Overall, marker assisted selection has proven to be a very useful technique in plant breeding. Through these techniques, we

(ICRISAT-Molecular breeding team) are able to produce cultivars of agriculturally important traits with QTLs for drought tolerance that were not possible before the advent of DNA technology. By adopting high throughput protocols and automation techniques in MAS, one can able to produce improved cultivars within short span of time. Unlike transgenic technology, MAS is simply an improvement on an age-old method of crop breeding. No foreign DNA is introduced into the plant, and no environmentally harmful genes have been incorporated. MAS is simply the transfer of useful traits among already potential mating population.

In future research, the genetic maps that have been developed by MAS will become more and more saturated as more techniques are developed and more markers uncovered and mapped. That will be easier for scoring complex traits like stay green as in sorghum.

Chapter - VI

Summary

CHAPTER VI

Summary

Sorghum [*Sorghum bicolor* (L.) Moench] is the 5th most important cereal crop globally (FAO, 2004). This C_4 grass is grown as 'failsafe' crop in tropical and sub tropical regions. Production of sorghum in semi-arid regions of the world is limited by drought. Developing drought tolerant plants is a major challenge for sorghum improvement programs globally.

Post-Flowering stage is mostly vulnerable to drought in grain sorghum and it is often referred to as Terminal drought. The character that is associated with terminal drought tolerance is "stay green." The stay green trait is associated with functional Green Leaf Area and the genotypes possessing this complex trait maintain more photosynthetically active leaves compared with genotypes not possessing it.

Genetic mapping of QTLs associated with stay green is an important step towards developing drought tolerant hybrids. Best-characterized source of stay green are B 35 and E 36-1. Different research groups independently developed QTL maps for stay green using different donor parents and marker systems.

Putative QTL for stay-green trait from B35 have been identified in five recently published studies (Tuinstra *et al*, 1997; Crasta *et al* 1999; Xu *et al*, 2000;

Tao *et al*, 2000 and Subudhi *et al* 2000). Using the linkage map developed by Bhattramakki *et al* (2000) identified six genomic regions associated with stay-green trait in B35 parent. The identification of these QTL provided us an opportunity for marker-assisted breeding (MAB) for introgression of QTL from B35 to recurrent parents.

At ICRISAT we aimed at transfer of QTL from donor parent B35 to recurrent parents, S35 and IRAT 204. For the recurrent parent S35 selected BC_4F_2 seed is sown in the glass house and selfed to obtain BC_4F_3 seed. Based up on the SSR marker data generated the selected BC_4F_3 seed was sent to Ghana for phenotypic evaluation for checking stability of homozygous QTL introgression lines, targeting six QTLs. For the recurrent parent IRAT 204 selected BC_2F_1 and BC_3F_1 plants are advanced to BC_3F_1 and BC_4 F_1 generations respectively. In this Marker Assisted Back crossing (MAB) program foreground markers were used for screening the genotypes possessing targeted QTLs.

Field evaluation (phenotype) of the stay green behaviour is required to enhance the selection potential. Nature of the dominance and epistatic properties of these QTL's should also be studied. Whether the QTLs are structural or regulatory is to studied in detail. Feasibility of generating ESTs, to have better understanding of this complex trait and Comparative genome mapping to study the trait in other related cereals will make marker assisted selection approaches based on large scale screening much more powerful and effective.

Future Goals

FUTURE STRATEGIES

Field evaluation (phenotyping) to assess the success or failure of the stay-green QTL introgression attempted in this study will be required for both single- and multiple-QTL introgression homozygotes (with the non-stay-green recurrent parent as a negative control for this trait and a positive control for grain and stover yield, and other agronomic and product quality traits of potential interest to farmers and consumers) once these have been developed by a generation of selfing and identified by a further generation of marker-assisted selection.

Fine mapping of the individual stay-green QTLs can be initiated using BC_3F_1 progeny produced by backcrossing selected BC_4F_1 individuals heterozygous for markers flanking single stay-green QTLs and homozygous for recurrent parent alleles at all other marker loci tested, following field evaluation of the corresponding BC_4F_2 progenies to confirm that they are segregating in the expected Mendelian manner for the stay-green phenotype.

Nature of the dominance and epistatic properties of these QTLs should be studied in selected BC_4F_2 families developed by selfing BC_4F_1 individuals heterozygous for various pair-wise combinations of the six target stay-green QTLs from donor parent B35.

Interaction(s) of the QTL(s) with environment can be studied only once sufficient seed of individual QTL introgression homozygotes can be multiplied by selfing to permit their evaluation in multi-locational replicated trials

Whether the QTLs are structural or regulatory in nature needs to be determined. If they are regulatory in nature, the breadth of their sphere of influence will need to be assessed.

Feasibility of generating ESTs, to have better understanding of this complex trait, should be explored in the QTL introgression lines under both stress and non-stress conditions and in various plant tissues (roots, leaves, and panicles).

Comparative genome mapping of potentially related traits in other related cereals (especially rice and maize) should be performed. Moreover, sorghum genome analysis offers novel learning opportunities relevant to weed biology as well.

Protein expression studies need to be done to confirm the stable single QTL introgression lines and development of a more integrated sorghum linkage map is essential for the exploitation of the maximum diversity present in the sorghum crop.

Focus areas for more advanced studies

During the past decade, the development of molecular genetics and QTL analysis has allowed us to identify genomic regions involved in drought tolerance in several crop species including sorghum. The weakness of this quantitative-genetic approach is that it provides very little information about the mechanisms and pathways involved in drought tolerance (or) about the multitude of genes involved in the plant's response to drought. The recent development of functional genomics should help to overcome this limitation, because it can allow us to study simultaneously the expression of several thousand genes. Use of near-isogenic QTL introgression lines in such studies will help to focus attention on the variation in expression of genes that are at least physically linked to the genomic region that is contributing most to the phenotypic expression of the trait of interest.

Based on progress to date, it is very much clear that a multidisciplinary approach-combining physiology, breeding and biotechnology is required for an effective understanding of a plant response to drought stress (Ribaut et al., 2002;

Jones et al., 1997, Prioul et al., 1997). The QTLs characterized provide a powerful base of information and germplasm for the genetic dissection of physiological drought. This approach can be combined with functional genomics and proteomics to identify the key pathways involved in drought stress tolerance and sensitivity, and further provide an insight of how these pathways interact. This in turn, may lead to more efficient and effective strategies for developing cereals (sorghum) with higher levels of sustainable productivity under water-limited conditions.

As a model organism for tropical grasses that carry out C_4 photosynthesis, sorghum is a logical complement to the C_3 grass Oryza, the first monocot plant that was sequenced. The relatively small genome of sorghum is likely to be appreciably less complex to assemble than the larger and more repetitive genomes of other major C_4 crops, such as maize and sugarcane. Sorghum is the second cereal crop targeted for sequence.

Finally, sorghum fills a key gap in biogeography, with the African origin of S. bicolor complementing the Asian origin of rice, American origin of maize, and Middle Eastern origin of the Triticeae (wheat, barley, and others). Sorghum has made unique contributions to understanding the genetic basis of cereal domestication, which appears to have occurred independently on these different continents by the imposition of many parallel selective pressures to divergent taxa (Paterson et al., 1995). In addition, a finished sorghum sequence will be valuable for determination of the provenance of differences between rice and maize in sequence repertoire and organization. Specifically, phylogenetic "triangulation" using parsimony-based approaches to compare sorghum, maize, and rice will permit one to infer whether polymorphisms among these species are recent or ancient.

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Appendices

APPENDIX I
LIST OF CHEMICALS USED

- λ DNA uncut (Invitrogen)
- λ DNA-100bp ladder (Invitrogen)
- Acryl amide (Sigma)
- Agarose (USB)
- Ammonium per sulphate (Sigma)
- Bind silane (Sigma)
- Bis-acryl amide (Sigma)
- Boric acid (Merk)
- Bromophenol blue (Sigma)
- Chloroform (Qualigens)
- CTAB (Cetyl Trimethyl Ammonium Bromide) (USB)
- dNTP's (Di nucleotide triphosphates) (Amersham)
- EDTA (Ethylene Diamino Tetra Acetic acid) (USB)
- Ethidium bromide (USB)
- Ethyl alcohol (Les Alcohol De Commerce Inc.)
- Formaldehyde (Qualigens)
- Glucose (Qualigens)
- Glycerol (Qualigens)
- Isoamyl alcohol (Merk)
- Isopropanol (Qualigens)
- KCL (Qualigens)
- Liquid NH_3 (Qualigens)
- Mercaptoethanol (Merk)

- $MgCl_2$ (Ampli)
- Na_2CO_3 (Qualigens)
- NaCl (Sodium chloride) (Qualigens)
- NaOH (Sodiun hydroxide) (Qualigens)
- Orange dye (Sigma)
- PCR Buffer (Ampli)
- Phenol (Bangalore Genei)
- Potassium acetate(Qualigens)
- Proteinase 'K' (Sigma)
- Ready to run agarose gel (QIAGEN)
- Repel silane (Amersham)
- RNase A (Ribonucleases) (Sigma)
- Silver nitrate (Sigma)
- *Taq* polymerase (Ampli / Bioline)
- TEMED (Sigma)
- Tris-HCl (Sigma)
- Trizma base (Sigma)
- Xylene cynol (Sigma)

APPENDIX II

BUFFERS AND STOCK SOLUTIONS

CTAB buffer (3%)

3% w/v CTAB

1.4 M NaCl

20 mM EDTA

100 mM Tris-HCl

Adjust the P^H to 8.0

0.17% β-mercaptoethonol

- The buffer is preheated at 60° C in a water bath before DNA extraction.

Chloroform : Iso Ammyl Alcohol [C:IAA]

Chloroform and Iso amyl alcohol are mixed in the ratio of 24:1 and can be used for solvent extaction. The transfer of C: IAA is done in the fume-hood.

EDTA (0.5M, pH 8.0) [Stock]

73.05 g of disodium EDTA .2 H_2O is dissolved in 400 ml of H_2O and stirred using magnetic stirrer and adjusted pH to 8.0 with NaOH (1N), autoclaved and storedat room temperature.

Ethidium Bromide

Stock 20 mg/ml can be prepared by dissolving 1 gm of ethidium bromide in 50 ml of water. EtBr has to handled with care as it is an intercalating agent.

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Equal parts of equilibrated phenol and Chloroform: Isoamyl alcohol (24:1) were mixed and stored at 4°C. Transfer is carried out in the fume-hood.

Tris-HCl (1 M)

121.1 g of tris base was dissolved in 800 ml of H_2O and the pH was adjusted to 8.0 and made up to 1 litre with sterile water.

3 M Sodium Acetate

24.6 gm of sodium acetate dissolved in 60 ml double distilled water. The pH was adjusted to 5.2 using glacial acetic acid. The final volume was made up to 100 ml with distilled water and autoclaved.

10X Loading Dye –50 ml

Sucrose	33.35g
Bromophenol Blue	0.2g
Distilled Water	Up to 50 ml

40% Acrylamide/bis-acrylamide (19:1) –100 ml

Acrylamide	38
Bis-Acrylamide	2
Distilled Water	Up to 100ml

Can be stored at 4° C

10X Tris Borate EDTA [TBE Buffer]

Tris base	54g
Boric Acid	27.5g
EDTA (0.5M, pH 8.0)	20ml
Distilled Water	make up to 500ml

Can be stored at room temperature

Ammonium per-sulphate (10%)

1 g of Ammonium per-sulphate (APS) was dissolved in 10 ml of double distilled water. It should be stored at -20^{0}C .If the APS will be used up with in a few weeks, it can be stored at -4^{0}C.

Composition of 6% PAGE gels:

Distilled water	52.5ml
TBE (10X)	7.5ml
Acryl amide: Bis-acrylamide	15.0ml
APS	450µl
TEMED	100µl
	about 75ml

Silver staining solutions (I-VIII) and their composition was given in the chapter III.

APPENDIX - III

EQUIPMENT

- 384 well PCR plate (Axygen Scientific)
- 96 tube-DNA mini extraction kit (Axygen Scientific)
- 96 well micro titre plate (*NUNC*)
- 96 well PCR plate (Axygen Scientific)
- ABI Prism automated DNA sequencer (ABI 3100/ABI 3700) (Perkin Elmer)
- Agarose Gel Electrophoresis system (Bio-Rad)
- Autoclave (Sanyo)
- Conical flasks (Tarsons)
- DNA speed vac concentrator (Vaccum dryer) (Thermo electron corp.)
- DNA thermal cycler (Perkin Elmer)
- Electronic metteler balance (Toledo)
- Freezer - 30°C (Sanyo)
- Freezer and refrigerator of 4° C and -20° C (LG)
- Fume hood (Fisher Scientific)
- Gel imaging system (*Uvi Doc*)
- Gel scanner (*UMAX*)
- Geno grinder (*Geno2000*)
- Gloves (Microflex)
- Ice maker (Hoshizaki)
- Incubator (37°C) (Sanyo)
- Magnetic Stirrer (Janke and Kunkel)

- Measuring cylinders (plastic) (Tarsons)
- Measuring cylinders (glass) (BOROSIL)
- Microcentrifuge (Sigma)
- Microwave oven (LG)
- Mini centrifuge (Bangalore Genei)
- Milli 'Q' Water (MILLIPORE)
- Mini steel balls (Toledo)
- Peltier thermal cycler (PTC-200) (MJ Research)
- pH meter (Banglore Genei)
- Pipetteman (Finnpipette)
- Pipetteman (multi channel) (Finnpipette)
- Sequencing gel (Bio-Rad)
- Tecan Liquid Handling Robotic System (Tecan)
- Tip boxes (Axygen Scientific)
- UV- absorbance spectrophotometer (Unicam)
- UV- transilluminator (UVP)
- Vortex mixer (Banglore Genei)
- Water bath with thermostat (30° C to 100° C) (Julabo)
- Water bath (60° C) (Precision Scientific)
- Weighing boats (Imperial)

APPENDIX IV

PCR PROTOCOLS USED

Protocol No:	Primer (2pM/µl)	$MgCl_2$ (10mM)	Buffer (10x)	*Taq* Enzyme (0.5U/µl)	DNTPs (2mM)	DNA (2.5ng)	DDW
4	0.50	0.75	0.50	0.25	0.500	0.50	2.000
5	0.50	1.00	0.50	0.20	0.250	1.00	1.550
7	1.00	1.00	0.50	0.20	0.375	0.5	1.425

(Total reaction volume for all the above protocols is 5µl)

APPENDIX V

SOFT WARE & WEB SITES USED

1) Microsoft Excel spread sheet.
2) http faostat.fao.org
3) http www.ncbi.nlm.nih.gov
4) http://www.graminae.com
5) http://www.google.com