MARKER-ASSISTED BACKCROSSING OF STAY-GREEN QTLs INTO ELITE SORGHUM LINES

By

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CERTIFICATE

Mr PUNNA RAMU has satisfactorily prosecuted the course of research and that the thesis entitled **"MARKER ASSISTED BACK-CROSSING OF STAY-GREEN QTLs INTO ELITE SORGHUM LINES"** submitted is the result of original research work done and is of sufficiently high standard to warrant its presentation to the examination I also certify that the thesis or any part thereof has not been previously submitted by him for a degree of any university

(Dr. J. H. CROUCH)
Chairman of the Advisory Committee

Date 23 02 2004

Place HYDERABAD

CERTIFICATE

This is to certify that the thesis entitled **"MARKER ASSISTED BACK-CROSSING OF STAY-GREEN QTLS INTO ELITE SORGHUM LINES"** is 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. PUNNA RAMU** under my guidance and supervision. The subject of the thesis has been approved by the student's advisory committee.

No part of the thesis has been submitted by the student 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, **PUNNA RAMU**, hereby declare that the thesis entitled "**MARKER-ASSISTED BACKCROSSING OF STAY-GREEN QTLs INTO ELITE SORGHUM LINES**", submitted to **ANGRAU** 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 any part thereof has not been published earlier in any manner.

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ABSTRACT

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop globally. This C_4 grass is grown in more than 80 countries mostly in tropical and sub-tropical regions. After soil nutrient deficiencies, drought stress is the most important abiotic constraint to sorghum production globally. Dry spells can occur at any stage of the crop growth period. In sorghum, rapid premature leaf death generally occurs when water is limiting during the grain filling stage. Therefore drought stress during the grain filling period is referred as "post-flowering drought or terminal drought". The plant character associated with post flowering drought tolerance is called "stay-green". Stay-green is associated with functional green leaf area (GLA) during and after the grain filling period. Stay-green in sorghum is associated with charcoal rot, lodging resistance and superior ruminant quality. This complex trait is difficult to score.

Genetic mapping of QTLs associated with stay-green is an important step towards developing drought tolerant hybrids. Different sources of stay-green have been identified in sorghum. The most commonly used lines in breeding program are B35 and E36-1.

Different research groups independently developed QTL maps for stay-green using different donor parents and marker systems. After identifying the consistent QTLs markers, these can be tested through introgression of QTLs from their mapped sources into sorghum elite breeding lines. This can be accomplished by cloning the genes expressing QTLs and transferring these genes to recipient breeding lines or through marker-assisted breeding (MAB) program, where QTLs are introgressed into elite

breeding lines using molecular markers. MAB is the most appropriate technique when traits are complex and difficult to score/measure like yield, abiotic stress tolerance, where the genes contributing to QTLs expression have not yet been identified, and where plant transformation systems are not well established

With the development of molecular tools and molecular genetic linkage maps for plants, marker-assisted selection (MAS) has become much more broadly applicable. From the last decade, developing ability to transfer target genomic regions using DNA markers resulted in extensive mapping experiments aimed at development of MAS

Molecular marker based genetic linkage map of sorghum has permitted the identification of six QTLs for stay-green (post-flowering drought tolerance) in sorghum line B35. This project aimed at transfer/introgression of these QTLs from B35 to recurrent parents, ISIAP Dorado and R16. BC_3 and BC_4 generations from each recurrent parent were genotyped with the markers linked to stay-green QTLs for foreground selection and evenly distributed unlinked markers for background selection to speed the recovery of recurrent parent genotype in genomic regions that are not associated with the target stay-green QTLs. Genotypes with desired marker allele profiles were selected and advanced to next generations. Further studies are necessary to confirm the introgression of QTLs and expression patterns for stay-green by phenotypic evaluation of selected genotypes.

ABBREVIATIONS

°C	degree Celsius
µl	microlitre
µg	microgram
AFLP	Amplified Fragment Length Polymorphism
BAC	Bacterial Artificial Chromosome
BC	Back Cross
bp	base pair
cM	centi Morgan
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTP	deoxy Nucleotide Tri-Phosphate
EDTA	Ethylene Diamine Tetra Acetic acid
FAO	Food and Agriculture Organization
gDNA	genomic DNA
GGT	Graphical Genotyper
GLA	Green Leaf Area
IAA	Iso-amyl Alcohol
IsDo	ISIAP Dorado
LG	Linkage Group
LOD	Log of Odds ratio
M ha	Million hectares

M	Molar
MAB	Marker-Assisted Breeding
MAS	Marker-Assisted selection
Mb	Million bases
ml	millilitre
mM	millimolar
ng	nanogram
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pmol	picomole
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RE	Relative Efficiency
RFU	Relative Fluorescence Unit
RIL	Recombinant Inbred Line
RIP	Recombinant Inbred Population
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
RP	Recurrent Parent
SCAR	Sequence Characterized Amplified Region
SLN	Specific Leaf Nitrogen
SNPs	Single Nucleotide Polymorphisms
SPAD	Soil-Plant Analysis Development

SSR	Simple Sequence Repeat
stg	stay green
TBL	Tris Borate EDTA
TE	Tris-EDTA
TEMED	N N N N Tetramethylethylenediamine
UV	Ultraviolet
V	Volt

CHAPTER I

INTRODUCTION

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INTRODUCTION

1.1 IMPORTANCE OF SORGHUM

Sorghum [*Sorghum bicolor* (L.) Moench] is the 5th most important cereal crop globally after wheat, maize, rice and barley (FAO, 2003). This C_4 grass is grown in more than 80 countries, mostly in tropical and sub-tropical regions. The average annual area sown amounts to 44 M ha. with an average annual grain production of 63 M tons and average grain yield of 1.4 ton/ha (FAO, 2003).

Sorghum (2n=20) is an important target for plant genomics due to its adaptation to harsh environments, diverse germplasm collection, and relatively small genome size. The sorghum genome contains ca. 750 Mb of DNA, which is somewhat larger than that of rice (430 Mb) but 3- to 4-fold smaller than that of maize (2400 Mb) (Arumuganathan and Earle, 1991).

1.2 USES OF SORGHUM

Sorghum can be used both as human food as well as animal feed. Unleavened bread can be prepared with flour ground from the grain. Beer is the common drink prepared from sorghum grains, especially in Africa. Stems and foliage of sorghum can be used as green chop, hay, silage,and pasture. Plant stems can be used as building material and plant remains can be used as fuel. Sorghum can be grown as a border crop for large fields.

After soil nutrient deficiency, drought stress is the most important abiotic constraint for sorghum production globally (Haussman *et al.*, 2003). Sorghum is well adapted to semi-arid environments and regarded as the model crop for studying drought tolerance among grass species. Drought spells can occur at any stage of the crop growth period. The drought spell, which occurs at the grain filling stage, is being referred to as "post-flowering drought or terminal drought". Drought stress during and after flowering may cause premature leaf senescence, which in turn may lead to stalk lodging, stalk rot disease, and significant grain yield loses.

1.4 STAY-GREEN TRAIT

The plant character associated with tolerance to terminal drought stress is called "stay-green". Leaf senescence in plants is an internally programmed degeneration process ultimately leading to plant death. It is triggered by internal and external signals. Drought and environmental stress factors can induce the onset of leaf senescence (Nooden *et al.*, 1997). A typical phenomenon of leaf senescence is the loss of chlorophyll and progressive decline in photosynthetic capacity, resulting in a deteriorated quality of vegetables, ornamental plants, and turf grasses and poor yield and grain quality of crop plants. Therefore any mechanism that postpones the onset of senescence and thus keeping the plants green for a longer time can be beneficial to plant production.

The character responsible for delayed leave senescence is referred to as "stay-green or delayed senescence or post-flowering drought tolerance" (Xu *et al.*, 2000). So far 5 types of stay-green has been reported (Cha *et al.*, 2002 and Thomas *et al.*, 2002).

- The onset of senescence is delayed; the progress of senescence is the same as in wild type
- Senescence starts on schedule but is there after comparatively slow
- Chlorophyll is retained more or less indefinitely but senescence proceeds normally
- Unlimited color retention when killing leaf by freezing, boiling or drying (herbarium specimen), and
- Chlorophyll concentration in leaves high as compared to wild type, senescence normal.

The first two classes are also referred to as "functional stay-green" and are perhaps the result of alterations of gene expression involved in the onset of senescence and the regulation of its rate of progress. Stay-green in other three classes is "cosmetic", the plants remain green for a prolonged period of time but lack photosynthetic activity.

1.4.1 Importance of stay-green in sorghum

Stay-green in sorghum is considered as a valuable trait, as it improves genotypes adaptation to post-flowering drought stress. The trait is best expressed in environments in which the crop is dependent upon stored soil moisture, where this is only sufficient to meet part of the transpiration demand (Mahalakshmi and Bidinger, 2002). Plants

possessing the stay-green trait are photosynthetically active as compared to genotypes not possessing this trait. The longevity and photosynthetic capacity of leaves are related to their N_2 content, therefore it is important to determine the role of N_2 in extending leaf greenness in stay-green genotypes (Thomas and Rogers, 1990).

Because of the dual-purpose use of sorghum and the increased drought stress in areas where sorghum is cultivated, stay-green is of increasing importance. The trait is controlled by Quantitative Trait Loci (QTL) (Walulu *et al.*, 1994). Identification of the genetic factors involved in drought stress and subsequent transfer of these factors to elite sorghum breeding lines lacking drought tolerance will provide a solid foundation to improve the drought tolerance of these breeding lines.

1.4.2 Marker-assisted breeding for stay-green

Conventional breeding for quantitative traits is often an extremely slow and laborious process and because of Genotype × Environment interactions, the results tend to be location specific. The application of DNA markers and mapping technology facilitates breeding for complex traits. After mapping QTLs for stay-green in a donor parent, markers linked to the QTLs can be employed to transfer these QTLs from a resistant/tolerant parent (donor parent) to a susceptible recipient parent (recurrent parent). This process is also referred to as foreground selection in a marker-assisted breeding (MAB) program, where the segment containing markers linked on either side of the QTLs is transferred from a donor parent to susceptible parent. In addition, selection for recurrent parent alleles at markers unlinked to the QTLs can be used during the MAB

program to hasten recovery of recurrent parent genotype in genomic regions that are not involved with the target QTLs (background selection).

1.5 DNA MARKERS IN SORGHUM

There are many types of DNA markers currently available, Restriction Fragment Length Polymorphism (RFLPs, the first generation markers), and second-generation markers using the Polymerase Chain Reaction (PCR), such as RAPDs (Randomly Amplified Polymorphic DNA, AFLP (Amplified Fragment Length Polymorphism), SSRs (Simple Sequence Repeats), SCARs (Sequence Characterized Amplified Regions), SNPs (Single Nucleotide Polymorphisms), etcetera. The use of molecular markers has become widely accepted as a valuable tool for plant breeding programs as well as for evolutionary and diversity studies.

One of the marker types widely applied in breeding programs are the SSR markers, also called microsatillites. SSR markers offer a potentially attractive combination of features that make them useful as molecular markers in breeding programs

- Highly polymorphic in plants, hence highly informative
- Require small quantities of DNA, simple and inexpensive PCR-based assay
- Show a co-dominant inheritance
- Abundant and uniformly dispersed in both human and plant genomes.

Their high information content and other favorable characteristics make them excellent genetic markers for many types of investigations including marker-assisted breeding (MAB) and DNA fingerprinting of germplasm collections. A large number of SSR loci have been genetically mapped in several agronomically important species, including rice (Cho *et al.*, 1997) soybean (Cregan *et al.*, 1999) and sorghum (Brown *et al.*, 1996; Taramino *et al.*, 1997; Bhattramakki *et al.*, 2000; Kong *et al.*, 2000).

1.6 STAY-GREEN QTLs MAPPING AND MARKER-ASSISTED SELECTION

Different research groups identified genomic regions associated with the stay-green trait in sorghum using different donor parents. Among the most commonly used donor parents for stay-green are B35 and E36-1. Using the B35 line as a donor parent, different groups independently identified six genomic regions associated with the stay-green character. Because of the importance of stay-green, we aimed at the transfer of these six stay-green QTLs into two recurrent parents, RI6 and ISIAP Dorado using marker-assisted selection. Four different research groups have contributed to identification of these stay-green QTLs from the B35 source line.

- Select ISIAP Dorado (abr. IsDo) BC_4F_1 [(((((IsDo × B35) × IsDo) × IsDo) × IsDo) × IsDo] and BC_3F_2 genotypes [selfed BC_3F_1: (((IsDo × B35) × IsDo) × IsDo) × IsDo] and R16 BC_3F_1 [(((R16 × B35) × R16) × R16)× R16)] and BC_4F_1 [(((R16 × B35) × R16) × R16)× R16) × R16)] genotypes with SSR markers linked to stay-green QTLs (foreground selection).
- Identify genotypes with maximum recovery of recurrent parent genotype among the foreground selected BC_3F_2 and BC_4F_1 genotypes using SSR markers unlinked to stay-green (background selection).
- Self selected BC_3F_2 genotypes to increase seed numbers for phenotypic evaluation during *rabi* season 2003/2004.
- Selfing the selected ISIAP Dorado BC_4F_1 genotypes.
- Backcross the selected R16 BC_3F_1 genotypes.
- Backcross the selected R16 BC_4F_1 genotypes.

CHAPTER II

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

This chapter gives a detailed literature overview of the different topics dealt with in the study of marker-assisted selection of stay-green in sorghum (i.e., marker assisted backcrossing of stay-green QTLs into elite sorghum lines).

2.1 STAY-GREEN CHARACTER - CHARACTERISTICS AND GENE ACTION

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

Tenkouano *et al.* (1993) 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 by studying the populations derived from a

diallele cross between two non-senescent resistant inbred lines (B35, SC599-11E) and two senescent susceptible inbred lines (BTx378, BTx673) They also showed that the stay-green trait and charcoal rot resistance are not different manifestations of a single trait, hence they should not be equated with each other

Thomas and Howarth (2000) studied the stay green trait physiologically by measuring the progress of rate of senescence They classified the stay-green into five types (A, B, C, D and E) Type A and B are more functional stay green types

Borrell and Hammer (2000) identified that in sorghum hybrids grown under terminal water deficit, stay-green was viewed as a consequence of the balance between nitrogen demand by the grain and nitrogen supply during grain filling 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)

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

Wanous *et al* (1991) reported that visual ratings for the percentage of values under drought stress Xu *et al* (2000) measure the chlorophyll content with chlorophyll meter (SPAD values) and a spectrophotometer 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)

Akhtar *et al.* (1999) identified the marked difference in chlorophyll content between wild type and *gf* of tomato (the green flesh (gf) mutant of tomato). Based on differential expression studies using Festuca-Lollium stay-green mutants, Thomas *et al.* (2002) identified a gene that is showing sequence similarities with UDPGP gene of barley, which is tightly linked to stay-green trait. Borrell *et al.* (2000) identified that stay-green hybrids produced 47% more post-anthesis biomass than their senescent counter parts under terminal water deficit regimes.

2.2 CONSTRUCTION OF LINKAGE MAPS IN SORGHUM

Many research groups have been constructing genetic linkage maps for different crops using different DNA based markers. Widely used marker types are RFLP markers (Botstein *et al.*, 1980), RAPDs (Williams *et al.*, 1990), and SSRs (Bhattramakki *et al.*, 2000). These markers are reliable for detecting the polymorphism between the parental lines permitting construction of genetic linkage maps. Combinations of these markers are also using for construction of maps.

Kong *et al.* (2000) constructed an integrated sorghum linkage map with RFLP and SSR markers using F_8 recombinant inbred lines (RILs) derived from the cross between BTx623 and IS 3620C. The markers are distributed across the 10 linkage groups (LG) of the sorghum genome, covering 1287.2 cM [Based on LOD > 5.0].

Bhattramakki *et al.* (2000) extended this integrated SSR and RFLP linkage map of sorghum. Most of the SSRs they used were developed from clones isolated from two

sorghum BAC libraries and three enriched sorghum genomic DNA (g DNA) libraries. Very few were developed from the sorghum DNA sequences present in public databases. 323 RFLP probes and 313 SSR primer pairs were developed. Out of the SSRs, 165 (53%) of the loci found to the polymorphic in a population composed of 18 diverse sorghum lines [LOD score ≥ 3.0].

Nearly every agronomic trait imaginable has been subjected to DNA marker mapping and QTL analyses *e.g.*, drought tolerance (Martin, 1989), seed hardness (Keim *et al.*, 1990), plant height (Lin *et al.*, 1995) and yield (Stuber *et al.*, 1987).

2.3 STAY-GREEN QTL MAPPING IN SORGHUM AND RICE

The conflict between the Mendelian theory of inheritance and the observation that most traits in nature exhibit continuous variation was eventually resolved by the concept that quantitative inheritance can result from segregation of multiple genetic factors, modified by environmental factors. The theoretical basis of interpreting the association of marker loci with QTL was obtained by Mather and Jinks (1971), Tanksley *et al.* (1982), Soller and Beckmann (1983) and Edwards *et al.* (1987a,b). The theoretical basis for identification of QTLs associated with individual marker loci has been studied by several authors (Jayakar, 1970; McMillan and Robertson, 1974; Soller and Beckmann, 1990; Edwards *et al.* 1987a,b; and Cowen, 1988). Like wise the use of flanking marker loci for QTLs identification was suggested by Lander and Botstien (1989) and Knapp *et al.* (1998).

The development of molecular marker technologies and the use of these markers in quantitative trait loci has become a powerful approach for the studying the genetic and phenotypic basis of complex traits (Edward *et al.*, 1987a and b; Paterson *et al.*, 1988). 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 (Yang *et al.*, 1995). 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 environments may lead to a better understanding of this trait (Tuinistra *et at.*, 1996).

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" (Knapp *et al.*, 1990 and Lander and Botstein, 1989) although simpler approaches are possible (Haley and Knott, 1992; Thoday, 1961 and Weller, 1987).

Tuinstra *et al.* (1996) identified QTL associated with post-flowering drought tolerance in sorghum using 98 RILs derived from the cross between Tx7078 (pre-

flowering tolerant, post-flowering susceptible) and B35 (pre-flowering susceptible, post-flowering tolerant). This population was genotyped using 150 RAPD and 20 RFLP markers and its linkage map was constructed using Mapmaker/Exp. Mapmaker/QTL was then used to merge marker and phenotype data sets. They identified 6 genomic regions specifically associated with pre-flowering drought tolerance and also mapped 8 additional regions generally associated with yield or yield components under fully irrigated conditions.

Tuinstra *et al.* (1997) identified genomic regions (QTLs) associated with post-flowering drought tolerance and for potentially related components of grain development. They used 98 RILs as a mapping population derived from the cross between Tx7078 and B35. They identified 13 genomic regions associated with one or more measures of post-flowering drought tolerance. Two QTLs were identified with major effects on grain yield and "stay-green" under post-flowering drought.

Crasta *et al.* (1999) developed a linkage map using 142 RFLP markers. They used a set of 96 $F_{6:7}$ RILs obtained from the cross between B35 and Tx430. These RILs were evaluated for post-flowering drought tolerance and maturity. Simple interval mapping identified seven stay-green QTLs and two maturity QTLs. Out of seven, 3 major QTLs (SGA, SGD and SGG) contributed to 42% of phenotypic variability (LOD 9.0) and 4 minor QTLs (SGB, SGI.1, SGI.2 and SGJ) significantly contributed to an additional 25% of phenotypic variability in stay-green ratings.

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

used 118 additional markers including 17 SSRs and 101 RFLP markers which are mapped on to the same linkage map previously developed (Tao *et al.*, 1998) by using same RIL population. In total they identified 5 genomic regions associated with stay-green.

Xu *et al.* (2000) mapped the QTLs that control the stay-green and chlorophyll content in sorghum by using the F_7 RIL as a mapping population derived from the cross B35 x TX 7000. They identified the four *Stg* QTLs located on 3 linkage groups. *Stg*1 and *Stg*2 QTLs are located on LG A, other two *Stg* QTLs located on LG D and LG J, respectively. They identified that two stay-green QTLs,*Stg*1 and 2 controlled 13-20% and 20-30% of phenotypic variability for this trait. Also identified the 3 QTLs for chlorophyll content (*chl*1, *chl*2 and *chl*3) explaining 25-30% of the phenotypic variability. *Stg*1 and *Stg*2 regions also contain the genes for key photosynthetic enzymes, heat shock proteins and an ABA-responsive gene.

Subudhi *et al.* (2000) determined the consistency of QTLs controlling stay-green in sorghum across genetic backgrounds and environments. They evaluated the RIL mapping population from the cross B35 x Tx7000. The map of B35 x Tx7000 population (Xu *et al.*, 2000) was expanded by the additional 91 more markers (RFLP, SSR and RAPD markers). They mapped 4 stay-green QTLs and identified that they have partial similarities in case of LG A and LG B of B35 x Tx7000 population and B 35 x Tx7078 population (Tuinstra *et al.*, 1997). The nomenclature of that stay-green QTL such as *Stg*1, *Stg*2, *Stg*3 and *Stg*4 was adopted from Xu *et al.*, (2000).

Kebede *et al.* (2001) identified the genomic regions associated with post-flowering drought tolerance (stay-green) using F_7 RILs population derived from the cross SC56 × Tx7000 with RFLP markers. The linkage map for this RIL population covers 1355 cM of the sorghum genome and consists of 144 loci. Nine QTLs located on 7 linkage groups were detected for stay-green in several environments using the method of composite interval mapping. They also identified the 3 QTLs present on the LG A, LG G and LG J were consistence across the stress environments. They also conducted the comparative mapping studies, showing that two stay-green QTLs identified in their study corresponded to stay-green QTL regions in maize. In addition to this, 3rd and 4th QTLs are also responsible for lodging tolerance and pre-flowering drought tolerance, respectively, were detected.

Cha *et al.* (2002) mapped stay-green QTLs in rice using the phenotypic and molecular markers. They mapped the stay-green mutant [*sgr* (t)] locus to the long arm of chromosome 9 between RFLP markers RG662 and C985 at 1.8- and 2.1-cM intervals, respectively. They identified no difference on the photosynthetic activity was observed between the stay-green mutant and yellowing wild-type leaves, indicating that senescence is proceeding normally in the mutant leaves and the mutation affects only the rate of chlorophyll degradation during leaf senescence.

Sanchez *et al.* (2002) identified four QTLs associated with stay-green in sorghum using a RIL population derived from the cross B35 × Tx7000 with RAPD, SSRs and RFLP markers. Four major QTLs were consistently identified in all field trials and accounted for 53.5% of the phenotypic variance.

Haussmann *et al.* (2003) developed a QTL map for stay-green in sorghum using two recombinant inbred populations (RIP1 and RIP2) using E36-1 as a donor parent for stay-green. The mode of gene action for stay-green in their investigation ranged from purely additive to over dominance. Three QTLs on LG A, E and G were common to both RIP1 and RIP2. So these QTL could be potential candidates for transfer of stay-green into locally adapted materials. These findings provided the opportunity to transfer of QTL effectively from the selected donor parents to the recurrent parents.

Stay-green QTLs mapped by different research groups were summarized in the table 1 and 2.

2.4 MARKER-ASSISTED SELECTION (MAS)

An important area in which molecular biology is being applied to transfer of traits from donor parent to recurrent parent is marker-assisted selection (MAS). MAS has been advocated as a useful tool for rapid genetic advance in case of QTL (Lande and Thompson, 1990; Knapp, 1994,1998). Gimelfarb and Lande (1995) presented 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.

Table 1: Summary of recent stay-green mapping studies

Reference	Population	Parents	Test sites	LGs	Length in cM	Number of QTLs for stay-green
Tuinstra *et al.* (1997)	98 RIL	B35 TX7078	2 E in Mexico and Arizona, irrigated and post-flowering drought	17	1580 R	6
Crasta *et al.* (1999)	96 RIL	B35 TX430	4 E in Texas	14	1602 K	7
Xu *et al.* (2000)	98 RIL	B35 Tx7000	5 E in Texas	10	837 H	4
Subudhi *et al.* (2000)	98 RIL	B35 Tx7000	2 E in Texas added to Xu *et al.*	10	?	5
Kebede *et al.* (2000)	125 RIL	SC56 Tx7000	5 E in Texas and Kansas	10	1355 K	9
Tao *et al.* (2000)	152 RIL	QL 41 QL 39	5 E in Australia	14	1871 U	5

(E = Environments; LG = Linkage Groups; R = Recombination frequency; K, H = Kosambi and Haldane functions, respectively; U = Mapping function not indicated; ? = Map length not indicated)

Table 2: Comparison of linkage group names (Haussmann *et al.*, 2003) with other stay-green mapping studies.

Reference	Linkage Groups
Haussmann *et al.* (as Bhattramakki *et al.*, 2000)	A B C D E F G H I J
Tuinstra *et al.* (1997)	F B G ? D K E ? ? ?
Tao *et al.* (2000)	C B A F J G I E D H
Crasta *et al.* (1999), Xu *et al.* (2000), Subudhi *et al.* (2000), Kebede *et al*, (2000)	G D A C E I B H F J

MAS can be used to pyramid several resistance genes into a single host genotype. Where hybrid cultivars are possible, Witcombe and Hash (2000) have described how multiple resistance gene pyramids can be used practically to strategically deploy resistance genes in potentially more durable manner than has been previously practiced. The frequency of genotypes having resistance alleles at several loci increase greatly in both seed parent and hybrid when the overall frequency of resistance alleles in maintainer line increases.

2.4.1 Efficiency of marker-assisted selection

Hospital *et al.* (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 the ratio of relative efficiency (RE) of expected efficiency of MAS over the expected efficiency of purely phenotypic selection generally increases when considering 1) the larger population size 2) lower heritability values of the trait and 3) high type-I error risk of the regression. These studies over the successful generations of the rate of fixation of QTL shows that the higher efficiency of MAS on QTLs with large effects in early generation is balanced by a higher rate of fixation of un favorable alleles at QTLs with small effects in later generations. This explains why MAS may become less efficient than phenotypic selection in long-term process. MAS efficiency therefore depends on the genetic determination of that trait.

The efficiency of MAS was generally reduced with increasing the distance between the markers. So, optimal distance recommended between two adjacent markers

is about 5-10 cM (Hospital *et al.*, 1997). The efficiency of marker-assisted selection is less efficient than the phenotypic selection in long-term process (Hospital *et al.*, 1997).

Knapp (1998) presented the estimates of probability of selecting one or more superior genotypes by MAS to estimate its cost efficiency relative to phenotypic 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 allele are present at QTLs and linked in coupling phase. Uncertainty in estimated QTL map positions 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 successfully robust for successful MAS. Charmet *et al.* (1999) studied the accuracy of QTLs location determination greatly affects selection efficiency. MAS for QTLs have recently started to be applied to the genetic improvement of quantitative character in several crops such as tomato (Lowson *et al.*, 1997; Bernaclhi *et al.*, 1998), maize (Graham *et al.*, 1997) and barley (Han *et al.*, 1997; Toojinda *et al.*, 1998).

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

Frisch *et al.* (1999) determined the number of marker data points (MDP) required in background selection, size of the population and compared a two-stage selection procedure (one background and one foreground selection), with alternative selection procedures (one foreground and two or three background selection steps). They

concluded that as the number of selection processes increases, the number of MDP required 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 QTLs detection. They concluded that expected economic returns of MAS compared to the phenotypic selection decreases with the cost of genotyping.

Because of the benefits of MAS, we aimed the transfer the stay-green QTLs into elite sorghum lines (R16 and ISIAP Dorado) through MAS using SSR markers (Bhattramakki *et al.*, 2000).

CHAPTER

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 PLANT MATERIAL

BC_3F_2 (7 populations) and BC_4F_1 (7 populations) derived from crosses between ISIAP Dorado and B35; BC_3F_1 (9 populations) and BC_4F_1 (8 populations) population derived from crosses between R16 and B35. In all these populations, B35 is the donor parent for stay-green QTLs and ISIAP Dorado and R16 are the recurrent parents. [Figure 1(a) and 1(b) showing an overview of the backcrossing program].

3.2 SHORT DESCRIPTION OF PARENTAL LINES USED IN THE BACKCROSS PROGRAM

3.2.1 B35

BC1 derivative of IS 12555, a durra race sorghum germplasm accession of Ethiopian origin. It is well characterized for "stay-green" drought tolerance; different research groups have identified a number of QTLs in mapping populations derived from crosses involving this source of the stay-green trait (Crasta *et al.*, 1999; Xu *et al.*, 2000; Subudhi *et al.*, 2000, Sanchez *et al* 2002). Genetically quite divergent from recurrent parents, which facilitates its to use in marker-assisted breeding programs. Agronomically

Figure 1(a). Scheme for transfer of stay-green QTLs into elite sorghum lines by marker-assisted backcrossing

Recurrent parent – ISIAP Dorado

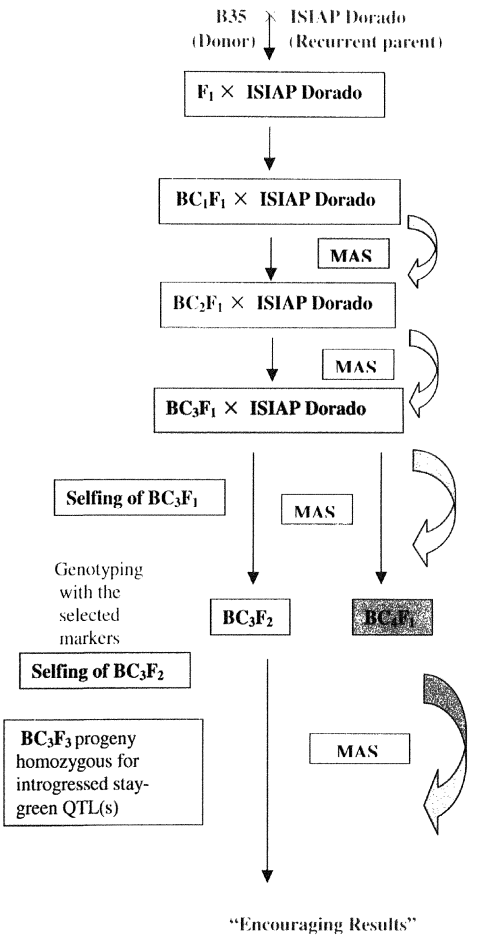
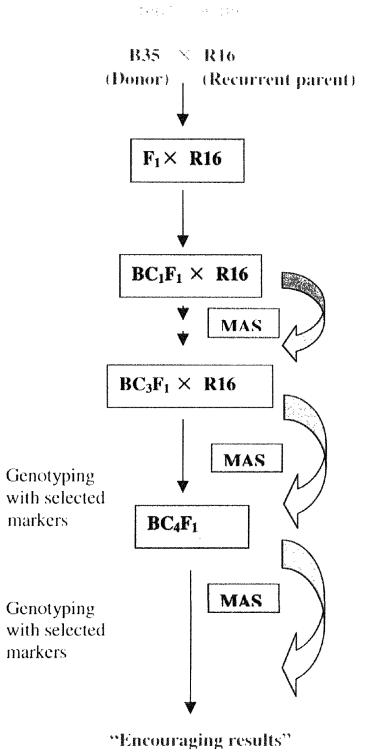


Figure 1 (b): Scheme for transfer of stay-green QTLs into elite sorghum lines by marker-assisted selection:

Recurrent parent – R16



diverged from elite Asian African and Latin American sorghum lines Potentially yield resistant

3.2.2 ISIAP Dorado

Dwarf plant drought-sensitive improved cultivar of ICRISAT origin having large hard white grain that has been released and/or cultivated in El Salvador Mexico Paraguay, and Egypt

3.2.3 R16

This is a highly senescent *rabi*-adapted breeding line of Indian origin

3.3 DNA EXTRACTION

Seeds of backcross progeny from selected individuals were sown individually in small pots At the same time seeds of the recurrent 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

DNA from the BC_3F_2 BC_3F_1 and BC_4F_1 populations was extracted from one-week-old seedlings by using a modified CTAB method (Mace *et al* 2004) DNA was further purified by RNase digestion followed by extraction with phenol/chloroform/iso-

amylalcohol and ethanol precipitation. A 96 well plate mini DNA extraction was employed.

3.3.1 96 well plate mini DNA extraction

A. Preparation

1. Steel balls (2 per extraction tube), pre-chilled at -20°C for about 30 minutes, were added to the extraction tubes which are kept on ice.
2. 3% CTAB buffer (3%w/v CTAB, 1.4 M NaCl, 20mM EDTA, 100mM Tris-Hcl, pH 8.0, 0.17% β-mercaptoethonol) was pre-heated in 65°C water bath before start of sample collection.
3. Six inches long leaf strips were collected (final weight 30mg) from one-week-old seedlings cut in to pieces (1mm in length). These strips were transferred to the extraction tubes.

B. Grinding and extraction

1. 450µl of pre-heated 3% CTAB buffer was added to each extraction tube containing leaf sample.
2. Grinding was carried out using Sigma GenoGrinder at 500 strokes/minute for 2 minutes.
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 450µl of Chloroform Iso-amyl alcohol (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 300µl)

D Initial DNA precipitation

1 To each tube containing aqueous layer, 0 7 volume (approximately 210µl) 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 200µl of low salt TE buffer and 30µg of RNase (stock 10mg/ml) 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, 200µl of Phenol Chlorofom IAA mixture (25 24 1) was added to each tube carefully mixed and centrifuged at 5000rpm for 10 minutes

2 The aqueous layer was transferred to the fresh tubes and the step was repeated with the chloroform 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 tubes were 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 resuspended 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.

3.4 CHECKING DNA QUALITY AND DNA CONCENTRATION

The DNA quality was checked using 1.2% ready-made agarose gels (Amersham Biosciences). 1 µl of DNA solution was mixed with the 1 µl of orange dye and 8 µl of distilled water and loaded in to 1.2% ready to run agarose gel. The gel was run for 10

minutes 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 (Figure 4)

The DNA concentration was assessed using Spectrafluor Plus Spectrophotometer by staining DNA with Picogreen™ (1/200 dilution) Based on the Relative Fluorescence Units (RFU) values and using the standard graph (Figure 2), DNA concentrations were calculated The DNA was diluted to a final concentration of 2 5ng/µl

DNA concentration = 2 78273+0 002019*RFU

3.5 SELECTION OF THE MARKERS

SSR markers linked to QTLs for stay green [Figure 3(b)] were used for foreground selection to select the individuals presumably having the donor allele (foreground selection) Foreground markers indicate the presence of stay green QTLs However, the tighter the markers are linked to the QTLs, the greater the chance that the QTLs in between both markers has indeed been transferred (assessment outside the scope of this project, planned for *rabi* season 2003/2004) Therefore, phenotypic testing of the final products of this MAB exercise needs to be performed in order to confirm the transfer of the target stay-green QTLs. At the same time selected the markers unlinked to stay-green have been used to select those individuals with minimal drag of non target genomic regions from the stay-green donor parent B35 (background selection) [Table 3(a), (b) and 4] Figure 3(a) showing the consensus map of stay-green QTLs in B35 donor parent

Figure 2. Standard graph showing the correlation between RFU and DNA concentration.

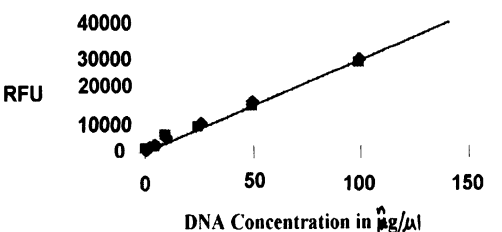


Figure 3(a): Consensus map of Stay-green QTLs in B35 donor parent.

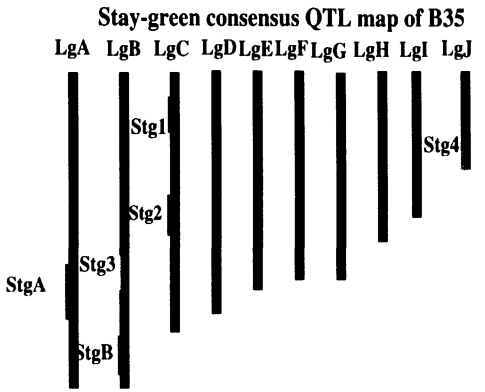


Figure 3(b): SSR markers linked to consensus stay-green QTLs mapped in donor parent B35.

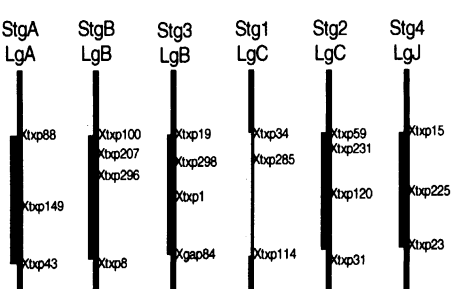


Table 3: Markers selected for screening

(a) Recurrent Parent R16

Generation BC3F1 R16

Generation	Population	No. of individuals	Targeted QTL	Markers Screened
BC3F1	2885	14	StgB	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2812	1	StgC	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2839	8	Stg3, StgB	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2884	17	Stg3, StgB	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2820	15	Stg1, Stg2, StgC	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2835	20	Stg1, Stg2, Stg3?, Stg4?, StgB	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2834	6	Stg1, Stg2, Stg3?, Sg4?, StgB	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2829	15	Stg2, Stg3?, Stg4?, StgB	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*
BC3F1	2850	18	Stg1, Stg2, Stg3?, StgB, StgC	*Xtxp88, 357, 248, 19, 298, 100, 07, 296, 34, 285, 31, 33, 21, 40, 295, 289, 230, 141, 20, 210, 47, 57, 06, 65, 225, 23*

Generation BC4F1 R16

Generation	Population	No. of individuals	Targeted QTL	Markers Screened
BC4F1	4061	22	StgB	*Xtxp43, 100, 07, 296, 47,*
BC4F1	4123	8	Stg3, StgB	*Xgap84, Xtxp100, 07, 296, 285, 225*
BC4F1	4390	10	Stg1, Stg2, StgC	*Xtxp43, 34, 285, 31, 33, 20, 57, 225*
BC4F1	4431	50	Stg1, Stg2, Stg3?, Stg4?, StgB	*Xtxp43, 248, 298, 34, 285, 31, 33, 289, 20, 225, 23.*
BC4F1	4101	4	Stg1, Stg2, Stg3?, Stg4?, StgB	*Xtxp43, 298, Xgap84, Xtxp07, 296, 34, 285, 40, 230, 225, 23.*
BC4F1	4448	13	Stg2?, Stg3?, Stg4?, StgB	*Xtxp43, 298, Xgap84, Xtxp34, 285, 295, 289, 141, 20*
BC4F1	4462	8	Stg1, Stg2, Stg3?, StgB, StgC	*Xtxp43, 248, Xgap84, Xtxp100, 07, 296, 34, 285, 31, 33, 230, 20, 57*
BC4F1	4465	14	Stg1, Stg2, Stg3?, StgB, StgC	*Xtxp43, 248, Xgap84, Xtxp34, 285, 31, 33, 230, 141, 20, 47*

3(b): Recurrent Parent ISIAP Dorado

Generation BC3F2 ISIAP Dorado

Generation	Population	No. of individuals	Targeted QTL	Markers screened
BC3F2	3317	24	Stg3	*Xbxp298, 56, Xgap84, Xtxp100, 25, 21, 12, 40, 295, 289, 230, 141, 20, 210*
BC3F2	3369	48	Stg1, Stg2, Stg3, Stg4?, StgB	*Xbxp88, 56, 100, Xgap84, Xtxp207, 296, 34, 285, 114, 31, 33, 12, 40, 295, 230, 141, 20, 6, 65, 15*
BC3F2	3379	38	Stg1, Stg2, Stg3, Stg4?, StgB	*Xbxp56, Xgap84, Xbxp100, 207, 296, 285, 114, 31, 33, 12, 40, 295, 20, 6*
BC3F2	3750	46	Stg1, Stg2, Stg3?, Stg4, StgB	*Xbxp56, Xgap84, Xbxp100, 207, 296, 31, 21, 40, 210, 65, 15, 23*
BC3F2	3754	152	Stg1, Stg2, Stg3?, Stg4, StgB	*Xbxp56, Xgap84, Xbxp100, 207, 296, 34, 285, 114, 33, 40, 295, 210, 15, 23*
BC3F2	3756	24	Stg1, Stg2, Stg3?, Stg4, StgB	*Xbxp296, 34, 285, 114, 65, 23*
BC3F2	3758	24	Stg1, Stg2, Stg3?, Stg4, StgB	*Xbxp100, 207, 296, 34, 285, 114, 31, 40, 295, 210, 65, 15, 23*

Generation BC4F1 ISIAP Dorado

Generation	Population	No. of individuals	Targeted QTL	Markers screened
BC4F1	3629	25	Stg2	*Xbxp88, 149, 43, 298, 56, Xgap84, Xtxp100, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*
BC4F1	3689	6	Stg3	*Xbxp88, 149, 43, 298, 56, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*
BC4F1	3726	8	Stg4	*Xbxp88, 149, 43, 298, 56, 100, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*
BC4F1	3371	3	Stg1, Stg2, Stg3, Stg4, StgB	*Xbxp88, 149, 43, 298, 56, 100, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*
BC4F1	3786	4	Stg1, Stg2, Stg3, Stg4, StgB	*Xbxp88, 149, 43, 298, 56, 100, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*
BC4F1	3399	18	Stg1, Stg2, Stg3?, Stg4, StgB	*Xbxp88, 149, 43, 298, 56, 100, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*
BC4F1	3805	4	Stg1, Stg2, Stg3?, Stg4, StgB	*Xbxp88, 149, 43, 298, 56, 100, 207, 296, 285, 38, 114, 31, 33, 15, 225, 23*

Table 4: Marker details

Marker details for recurrent parent R16

Foreground markers

Linkage group	QTL associated	SSR markers
A	Stg A	*Xtxp88, Xtxp43, Xtxp357*
B	Stg 3, B	*Xtxp298, Xgap84, Xtxp100, Xtxp207, Xtxp296*
C	Stg 1, 2	*Xtxp34, Xtxp285, Xtxp114, Xtxp31, Xtxp33*
J	Stg 4	*Xtxp225, Xtxp23*

Background markers that are unlinked to stay-green QTL

Linkage group	SSR markers
A	*Xtxp248*
B	*Xtxp19*
D	*Xtxp12, Xtxp21*
E	*Xtxp40, Xtxp295*
F	*Xtxp289, Xtxp230*
G	*Xtxp141, Xtxp20*
H	*Xtxp210, Xtxp47*
I	*Xtxp57, Xtxp06*
J	*Xtxp65*

Marker details for recurrent parent ISIAP Dorado

Foreground markers

Linkage group	QTL associated	SSR markers
A	StgA	Xtxp88, Xtxp149, Xtxp43
B	Stg3, StgB	Xtxp298, Xtxp56, Xgap84, Xtxp100, Xtxp207, Xtxp296
C	Stg1, Stg2	Xtxp34, Xtxp285, Xtxp114, Xtxp31, Xtxp33
J	Stg4	Xtxp15,Xtxp225, Xtxp23

Background markers that are unlinked to stay-green QTL

Linkage group	SSR markers
A	*Xtxp248*
B	*Xtxp25*
D	*Xtxp12, Xtxp21*
E	*Xtxp40, Xtxp295*
F	*Xtxp289, Xtxp230*
G	*Xtxp141, Xtxp20*
H	*Xtxp210*
I	*Xtxp57, Xtxp06*
J	*Xtxp65*

3.6 AMPLIFICATION OF SSR MARKERS

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 1) and a touchdown PCR program. Reaction mixture contains 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.25-2.5 ng of DNA, 2pm of forward and reverse primer, 1mm $MgCl_2$, 80-100µm of each dNTP and 0.1 units of Taq DNA polymerase. The touch down PCR program consisted of initial denaturation for 15 minutes at 94°C and then [10 cycles of denaturation for 10 seconds at 94°C, annealing at 61-52°C for 20 seconds, the annealing temperature for each cycle is reduced with 1°C, and extension at 72°C for 30 seconds], 35 cycles [denaturation for 10 seconds at 94°C, annealing at 54°C for 20 seconds and extension at 72°C for 30 seconds]. The last PCR cycle is followed by a 20 minutes extension at 72°C to ensure amplification to equal length of both DNA strands.

If the parents showing the polymorphism more than 5 bp, then PCR products were separated on 6% non-denaturing PAGE (Poly Acrylamide Gel Electrophoresis) gels and silver stained using the modified procedure developed by Kolodny (1984). If the polymorphism between the parents is less than 5 bp, then PCR products were separated in capillary electrophoresis using ABI Prism 3700 (Perkin Elmer). For this purpose fluorescent-labeled primes were used [Figure 5]. (Table 5(a) and (b)).

Table 5(a): List of markers screened on PAGE and ABI Prism Recurrent Parent R16

Marker	Product size		PAGE/ABI
	B35	R16	
Xt-7	221	219	ABI
Xt-57	246	241	PAGE
Xt-47	300	290	PAGE
Xt-43	144	162	PAGE
Xt-40	131	137	PAGE
Xt-34	330	369	PAGE
Xt-33	223	229	ABI
Xt-31	220	218	ABI
Xt-298	202	222	PAGE
Xt-296	262	264	ABI
Xt-295	167	173	PAGE
Xt-289	280	330	PAGE
Xt-285	237	240	ABI
Xt-248	212	200	PAGE
Xt-230	195	208	PAGE
Xt-23	183	181	PAGE
Xt-225	173	184	PAGE
Xt-21	168	172	ABI
Xt-20	204	181	PAGE
Xt-141	151	133	PAGE
Xt-100	103	106	ABI
Xg-84	210	193	PAGE

Table 5(b): List of markers screened on PAGE and ABI Prism Recurrent Parent ISIAP Dorado

Marker	Product size		PAGE/ABI
	B35	ISIAP Dorado	
Xtxt296	170	172	ABI
Xtxp34	330	365	PAGE
Xtxp285	235	220	PAGE
Xtxp114	150	145	PAGE
Xtxp65	140	130	PAGE
Xtxp100	96	100	ABI
Xtxp207	201	235	PAGE
Xtxp31	220	200	PAGE
Xtxp40	160	162	ABI
Xtxp295	165	175	PAGE
Xtxp210	200	210	PAGE
Xtxp15	220	215	PAGE
Xtxp23	183	174	PAGE
Xtxp56	374	543	ABI
Xgap84	196	217	PAGE
Xtxp21	170	175	PAGE
Xtxp88	103	122	PAGE
Xtxp33	225	230	PAGE
Xtxp12	130	150	PAGE
Xtxp230	195	210	PAGE
Xtxp20	205	215	PAGE
Xtxp06	130	105	PAGE
Xtxp298	202	184	PAGE
Xtxp25	132	150	PAGE
Xtxp289	280	300	PAGE
Xtxp248	210	260	PAGE
Xtxp57	245	255	PAGE
Xtxp141	150	145	PAGE
Xtxp149	171	167	ABI

3.7 NON-DENATURING PAGE

(POLY ACRYLAMIDE GEL ELECTROPHORESIS)

1 µl of loading dye (orange red + EDTA + NaCl + Glycerol) was added to 3 µl of PCR product. From these mixture, loaded the 2 µl of sample into the 6% non-denaturing PAGE gel.

The gel was prepared using

52.5ml of doubled distilled water

7.5ml of 10 X TBE buffer

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

450µl of Ammonium Per Sulphate (APS) and

100µl of TEMED.

75ml total

Along with the samples, 100 bp marker (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 V of constant power in 0.5 × TBE buffer for 3 hours using a Bio Rad gel sequencing apparatus.

3.7.1 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 placed in

- water for 5 minutes.
- 0.1% CTAB solution for 20 minutes (2 g in 2 lit of water)
- 0.3% ammonia solution for 15 minutes. (26 ml of 25% ammonia solution in 2 lit of water)
- 0.1% silver nitrate solution for 15 minutes (2g of silver nitrate + 8 ml of 1M NaOH in 2 lit of water and add ammonia solution up to the solution becomes colorless)
- water for few seconds
- Developer (30 g of Sodium carbonate + 400µl of Formaldehyde in 2 lit of water)

After developing the bands gels were rinsed in water for 1 minutes and placed it in fixer (30 ml Glycerol in 2 lit of water) for a few seconds. 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 100 bp DNA ladder (fragments ranging from 100 bp to 1000 bp) and presence or absence of parental alleles were scored.

3.8 DATA COLLECTION AND ANALYSIS

3.8.1 Scoring of the gels

The bands in the gels were scored as A, B, H, OFF and "---" based on their pattern compared with those of the parents. "A" was defined as the presence of allele from the recurrent parent (SIAP Dorado/R16), "B" was defined as the presence of allele from B35, "H" was defined as the heterozygous (presence of both recurrent and donor parent alleles), "OFF" was defined as an allele from neither from donor parent nor from the recurrent parent allele and "—" was a missing sample.

3.8.2 Data analysis

Data was analyzed by Graphical Genotyper (GGT)

A graphical representation of molecular marker data is an important tool in the process of selection and evaluation of plant material. The GGT computer program (Ralph van Berloo, 2001) enables representation of molecular marker data by simple chromosome drawings in several ways.

3.8.3 Viewing the graphical genotypes

When a GGT data set has been constructed it can be opened by GGT. Several linkage groups can be opened simultaneously. GGT draws a graphical representation of the data in two ways

A) Arranged by linkage group and

B) Arranged by individual.

The differences between these two drawing modes are schematically drawn. The 'by linkage group' viewing mode is selected by default. This will show the first linkage group of all individuals next to each other. It is possible to display only a subset of the population.

The other mode for viewing graphical genotypes is 'by individual'. This mode is useful when several linkage groups of one plant or line need to be viewed together. Per individual, all linkage groups that have been opened are displayed next to each other, resulting in a partial or complete overview of a particular individual genome.

When the data indicate that a crossover event has occurred in between two markers, the exact location of the crossover is unknown. GGT uses the most probable position for the crossover, exactly in the middle of the two markers, to change the color-coding. Caution should be taken when interpreting crossover events, especially crossovers occurring between markers that are positioned at large distances from each other.

3.8.4 Selection

In 'Linkage Group' viewing mode, it is possible to specify a desired (marker) genotype for several linkage groups. Only the graphical genotypes of individuals that match this genotype are drawn and a list of these individuals is presented. Selection is done by demanding specific markers to carry an allele of the desired genotypes. In this way a population can be screened very efficiently for regions of interest.

3.9 RECOMBINATION FREQUENCIES

Kosambi's map function (Kosambi., 1944)

It is possible to calculate the recombination fraction if we know the map distance between the markers.

3.9.1 Expected recombination fraction

$$p = 1/2(1-e^{-2d})$$

Where

p = observed recombination fraction

d = map distance between two markers in Morgans

3.9.2 Observed recombination fraction

$$\text{Crossing over \%} = \frac{\text{Number of recombinants}}{\text{Total number of individuals screened}}$$

1% of crossing over = 1 map unit in centi Morgans

After calculating the crossing over %, it is converted to map units and recombination fractions were calculated using Kosambi's function.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

4.1 CHECKING THE DNA CONCENTRATION

After isolating the DNA, the samples were loaded into 1.2% ready-made agarose gel for testing the DNA quantity and quality, along with the standards. If the bands were clear, this indicate the good quality of the DNA. If they showed a smear, DNA was re-extracted from those individuals. DNA concentrations were assessed with the Spectrafluor Plus Spectrophotometer using Picogreen™. Figure 4 shows the quality, and gives an indication of the quantity, of DNA of BC_4F_1 R16 plate 1.

Likewise DNA quality and quantity was assessed for all generations and dilutions were made accordingly to reach final concentrations of 2.5 ng/µl.

PCR was done with selected primers for both foreground and background selection for allgenerations [Table 3 (a)&(b)]. After the PCR reaction was completed, PCR products were loaded onto 6% PAGE gels. Following electrophoresis and silver staining, the bands were scored as compared to the parental alleles.

Fig 4: DNA quality testing in 1.2% ready to run agarose gel of BC_4F_1 R16

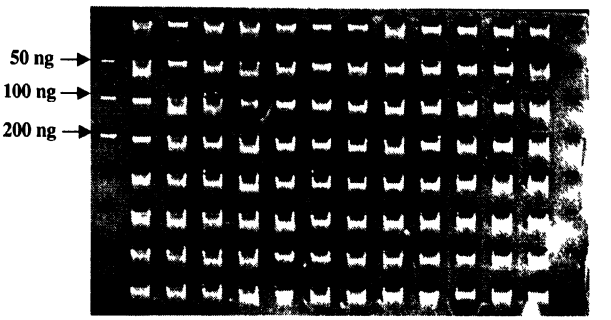
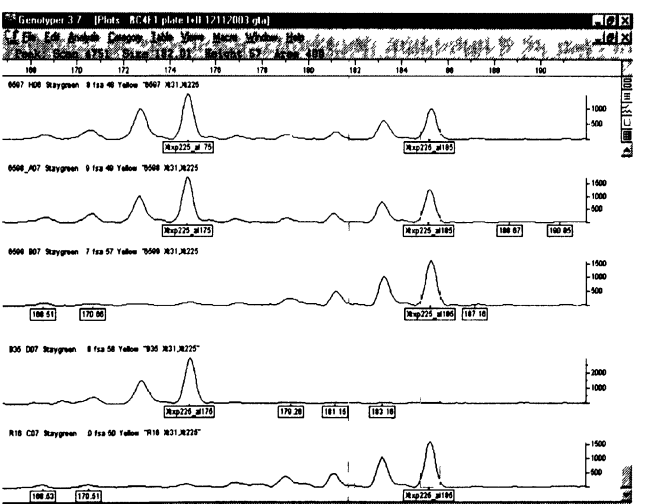


Figure 5: Graphical representation (Chromatogram) of the marker *Xtxp225* for BC_4F_1 R16 analysed through ABI Prism 3700.



4.2 R16 RECURRENT PARENT

For recurrent parent R16, a total of 15 foreground and 15 background markers were screened (Table 4). In the BC_3F_1 generation a total of 9 populations and in the BC_4F_1 generation a total of 8 populations were genotyped. Figure 6(a & b) shows the amplified PCR products of *Xtxp210* and *Xtxp298/Xtxp34* on 6% PAGE gel for the generations BC_3F_1 R16 and BC_4F_1 R16, respectively.

4.3 ISIAP DORADO RECURRENT PARENT

For recurrent parent ISIAP Dorado, a total of 17 foreground markers and 14 background markers were selected for screening (Table 4). In both BC_3F_2 and BC_4F_1 generations, a total of 7 populations were screened. Figure 7(a and b) shows the amplified PCR products of the markers *Xtxp210/Xtxp34* and *Xtxp230/Xtxp289* for generations BC_3F_2 and BC_4F_1, respectively.

Tables 6, 7, 8 and 9 show the results (partial scoring sheet) obtained for the SSR markers present on linkage group A, B and C (foreground and background markers). Figures 8 (a) and (b), 9 (a) and (b), 10 (a) and (b) and 11 (a) and (b) showing the Graphical Genotyper overview of segregation of SSR markers and selected individuals in respective generation.

4.4 RECOMBINATION FREQUENCIES

Recombination frequencies were calculated according to Kosambi's function (Kosambi, 1944). Table 10 showing the recombination frequencies across the generations.

Table 6: Part of scoring sheet for the markers present on LG A, B and C.

BC3F1 R16 Stg 27, 37, 47, B

			Stg4, LGA, foreground			LGA, bg	LGB, bg	*Stg3* and *Stg8*, LGB, foreground					*Stg1*, *Stg2* and *StgC*, LGC, foreground			
		Individual	*Xtxp88*	*Xtxp43*	*Xtxp357*	*Xtxp248*	*Xtxp19*	*Xtxp298*	*Xgap84*	*Xtxp100*	*Xtxp7*	*Xtxp296*	*Xtxp34*	*Xtxp285*	*Xtxp31*	*Xtxp33*
BC1F1		**122**		**A**	**A**			**H**	**H**	**A**	**H**	**H**	**H**		**H**	**H**
BC2F1		**2829**	**A**	**A**	**A**			**H**	**H**	-	**H**	**H**	**B**	**B**	**A**	**A**
1	**BC3F1**	**4087**	A		A	B		B	B	B	H	H	B	B	A	A
2	**BC3F1**	**4088**	A		A	A	A	H	H	A	A	A	H	H	A	A
3	BC3F1	4090	A		A	A	A	H	A	B	H	H	H	H	A	A
4	BC3F1	4092	A		A	A	A	A	A	B	A	A	B	B	A	A
5	**BC3F1**	**4093**	A		A	A	A	H	A	A	A	A	H	H	A	A
6	BC3F1	4438	A		A	H	A	A	A	B	B	H	B	B	A	A
7	BC3F1	4439	A		A	H	A	A	H	B	H	A	B	B	H?	A
8	**BC3F1**	**4440**	A		A	A		B	B	B	A		B	B	A	A
9	BC3F1	4442	A		A	B	A	H	H	B	H	A	B	B	H?	A
10	BC3F1	4443	A		A	H		H	H	A	A	A	H	H	H?	A
11	BC3F1	4444	A		A		A	H	H	A	A	A	H	H	H?	A
12	BC3F1	4445	A		A	H	A	H	H	A	A	A	H	H	H?	A
13	BC3F1	4447	A		A	B	A	H	H	B	H	H	B	B	A	A
14	**BC3F1**	**4448**	A		A	A	A	H	H	A	A	A	H	H	A	A
15	BC3F1	4449	A		A		A	A	H	B	H	H	H	B	A	A

Bold genotypes refers to selected individuals in respective generation.

Table 7: Part of scoring sheet for the markers present on LG A, B and C.
R16 BC4F1
Stg 1, 2, 37, 47, B

			Stg4, LGA, foreground			LGA, bg	LGB, bg	*Stg3* and *Stg8*, LGB, foreground					*Stg1* and *Stg2*, LGC, foreground			
		Individual	*Xbp88*	*Xbp43*	*Xbp357*	*Xbp248*	*Xbp19*	*Xbp298*	*Xgap84*	*Xbp100*	*Xbp7*	*Xbp296*	*Xbp34*	*Xbp285*	*Xbp31*	*Xbp33*
BC3F1		**4431**	**A**		**A**	**H**	**A**	**H**	**A**	**A**	**A**	**A**	**H**	**H**	**H**	**A**
1	**BC4F1**	**6550**		**A**		**H**							**A**	**A**	**A**	
2	BC4F1	6551						**A**					**H**	**H**	**A**	
3	BC4F1	6552											**H**	**A**	**B**	
4	**BC4F1**	**6553**		**A**		**A**		**A**					**H**	**H**	**B**	
5	**BC4F1**	**6554**		**A**		**A**		**H**					**A**	**A**	**A**	
6	BC4F1	6555						**A**					**A**	**A**	**A**	
7	**BC4F1**	**6556**		**A**		**H**		**H**					**H**	**H**	**A**	
8	**BC4F1**	**6557**		**A**		**A**		**H**					**A**	**A**	**A**	
9	BC4F1	6558											**A**	**A**	**B**	
10	BC4F1	6559						**A**					**H**	**H**	**A**	
11	BC4F1	6560		**A**		**A**							**A**	**A**	**A**	
12	BC4F1	6561						**H**					**H**	**H**	**A**	
13	BC4F1	6562						**B**					**A**	**A**	**A**	
14	BC4F1	6563						**A**					**A**	**A**	**A**	
15	**BC4F1**	**6564**		**A**		**A**		**H**					**H**	**H**	**B**	
16	BC4F1	6565		**A**		**H**		**B**					**H**	**H**	**A**	
17	BC4F1	6566						**A**					**H**	**H**	**A**	
18	BC4F1	6567		**A**		**A**		**H**					**H**	**H**	**B**	
19	**BC4F1**	**6568**		**A**		**A**		**H**					**H**	**H**	**B**	
20	BC4F1	6569		**A**		**A**		**A**					**H**	**H**	**B**	
21	BC4F1	6570		**A**		**A**		**H**					**H**	**H**	**A**	
22	BC4F1	6571		**A**		**A**		**H**					**H**	**H**	**B**	
23	BC4F1	6572		**A**		**A**		**A**					**H**	**H**	**A**	
24	**BC4F1**	**6573**		**A**		**H**		**A**					**A**	**A**	**A**	
25	BC4F1	6574						**H**					**A**	-	-	
26	BC4F1	6575		**A**		**H**		**A**					**H**	**H**	**A**	
27	BC4F1	6576						**A**					**H**	-	-	
28	BC4F1	6577						**A**					**A**	**A**	**B**	
29	BC4F1	6578						**A**					**A**	**A**	**B**	
30	BC4F1	6579		**A**		**H**		**H**					**A**	**A**	**B**	
31	**BC4F1**	**6580**		**A**		**A**		**H**					**A**	**A**	**B**	
32	BC4F1	6581						**H**					**A**	**A**	**B**	
33	**BC4F1**	**6582**		**A**		**A**		**H**					**A**	**A**	**A**	
34	BC4F1	6583						**H**					**A**	**A**	**A**	
35	BC4F1	6584						**B**					**A**	**A**	**A**	
36	BC4F1	6585											**A**	**A**	**A**	
37	**BC4F1**	**6586**		**A**		**A**		**A**					**A**	**A**	**B**	
38	BC4F1	6587						**A**					**A**	**A**	**B**	
39	**BC4F1**	**6588**		**A**		**A**		**A**					**H**	**H**	**B**	
40	BC4F1	6589		**A**		**H**		**A**					**H**	**H**	**B**	
41	BC4F1	6590						**H**					**A**	**A**	**A**	
42	BC4F1	6591						**A**					**H**	**H**	**A**	
43	BC4F1	6592		**A**		**H**		**A**					**A**	**A**	**B**	
44	BC4F1	6593						**A**					**A**	**A**	**A**	
45	BC4F1	6594		**A**		**A**		**H**					**H**	**H**	**B**	
46	BC4F1	6595		**A**		**H**		**A**					**H**	**H**	**A**	
47	BC4F1	6596						**H**					**A**	**A**	**A**	
48	BC4F1	6597						**H**					**A**	**H**	**B**	
49	**BC4F1**	**6598**		**A**		**A**		**A**					**H**	**H**	**B**	
50	BC4F1	6599						**A**					**H**	**H**	**A**	

Bold genotypes refers to selected individuals in respective generation.

Table 8: Part of scoring sheet for the markers present on LG A, B and C.
ISIAP Dorado BC3F2

Stg 1, 2, 3?, 4, B

			Stg4, LGA, foreground			LGA, bg	LGB, bg	*Stg3* and *Stg8*, LGB, foreground						
		Individual	*Xtxp88*	*Xtxp149*	*Xtxp43*	*Xtxp248*	*Xtxp25*	*Xtxp298*	*Xtxp56*	*Xgap84*	*Xtxp100*	*Xtxp207*	*Xtxp296*	*Xtxp8*
		522	H		A			A		H	H			H
BC2F1		**1451**	**A**	**A**	**A**			**A**	**H**				**H**	**H**	
BC3F1-II		**3750**	**A**	**A**	**A**	**A**	**A**	**A**	**H**	**H**	**H**	**H**	**H**	
1	BC3F2	5179							A	A	H		H	
2	BC3F2	5180							A	A	H		A	
3	BC3F2	5181							A	A	B		B	
4	BC3F2	5182							A	A	A		A	
5	BC3F2	5183							A	A	H	B	H	
6	BC3F2	5184							A	A	A		A	
7	BC3F2	5187							A	A	H	H	H	
8	BC3F2	5188							A	A	H	B	H	
9	BC3F2	5189							A	A	B	H	H	
10	BC3F2	5190							A	A	B	B	H	
11	BC3F2	5191							A	A	A	A	H	
12	BC3F2	5192							A	A	B	?	B	
13	BC3F2	5193							A	A	A	B	A	
14	BC3F2	5194							A	A		H		
15	BC3F2	5195							A	A	B	H	H	
16	BC3F2	5195							A	A	A	B	A	
17	BC3F2	5196							A	A	H	A	H	
18	BC3F2	5196							A	A	A	H	A	
19	BC3F2	5197							A	A	H	H	H	
20	BC3F2	5197							A	A	B	H	H	
21	BC3F2	5198							A	A	A		H	
22	BC3F2	5198							A	A	B	A	B	
23	BC3F2	5199							A	A	H	H	H	
24	BC3F2	5199							A	A	H	H	H	
25	BC3F2	5203							A	A	H	H	H	
26	BC3F2	5204							A	A	A	A	A	
27	BC3F2	5205							A	A	A	A		
28	BC3F2	5206							A	A	A	A	A	
29	BC3F2	5207							A	A	A	A	A	
30	BC3F2	**5208**							A	A	B	B	B	
31	BC3F2	5209							A	A	A	H	H	
32	BC3F2	5210							A	A	A	A	A	
33	BC3F2	5211							A	A	H		H	
34	BC3F2	5212							A	A	H	H	H	
35	BC3F2	5213							A	A	H	H	H	
36	BC3F2	5214							A	A	H	H		
37	BC3F2	5215							A	A	B	H	H	
38	BC3F2	**5216**							A	A	A	A	A	
39	BC3F2	5217							A	A	B	B	B	
40	BC3F2	5218							A	A	H	H	H	
41	BC3F2	5219							A	A	H	H	H	
42	BC3F2	5220							A	A	H	B	B	
43	BC3F2	5221							A	A	H	H	H	
44	BC3F2	5222							A	A	H	H	H	
45	BC3F2	**5223**							A	A	B	B	B	
46	BC3F2	5224							A	A	B	B	B	

Bold genotypes refers to selected individuals in respective generation.

Table 9: Part of scoring sheet for the markers present on LG A, B and C.

ISIAP Dorado BC4F1

Stg 1, 2, 37, 4, 8

			Stg4, LGA, foreground			LGA, bg	LGB, bg	*Stg3* and *Stg8*, LGB, foreground							*Stg1*, *Stg2*, and *Stg*C, LGC, foreground					
		Individual	*Xtxp88*	*Xtxp149*	*Xtxp43*	*Xtxp248*	*Xtxp25*	*Xtxp298*	*Xtxp56*	*Xgap84*	*Xtxp100*	*Xtxp207*	*Xtxp296*	*Xtxp8*	*Xtxp34*	*Xtxp265*	*Xtxp38*	*Xtxp114*	*Xtxp31*	*Xtxp33*
BC3F1		**3399**	A	A	A	A	A	A	A	A	A	A	A		H	H		B	H	A
1	**BC4F1**	**5700**	A	A	A	B	A	A	A	A	A	A	A		A	A	A	A	H	A
2	**BC4F1**	5701	A	A	A	A	A	A	H	H	B	B	B		A	A	A	A	B	A
3	**BC4F1**	5702	A	A	A	A	A	A	H	B	H	B	H		A	A	A	A	A	-
4	**BC4F1**	5703	A	A	A	A	A	A	B	B	H	B	H		A	A	A	A	B	-
5	**BC4F1**	5704	A	A	A	A	A	A	A	A	H	B	H		A	A	A	A	B	A
6	**BC4F1**	5705	U	A	A	A	A	A	B	H	H	B	H			A	A	A	B	A
7	**BC4F1**	**5706**	A	A	A	A	A	A	H	H	H	B	H		B	A	A	A	H	A
8	**BC4F1**	**5707**	A	A	A	A	A	A	A	A	H	B	H		A	A	A	A	H	A
9	**BC4F1**	**5708**	A	A	A	A	A	A	B	A	B	B	B		A	A	A	A	B	A
10	**BC4F1**	5709	A	A	A	B	A	A	A	A	B	B	U		A	A	A	A	A	A
11	**BC4F1**	**5710**	A	A	A	B	A	A	H	H	H	A	H		A	A	A	A	H	A
12	**BC4F1**	5711	A	A	A	A	A	A	H	H	H	B	H		A	A	A	A	H	A
13	**BC4F1**	**5712**	U	A	A	A	A	A	-	A	A	B	H		A	A	A	A	A	A
14	**BC4F1**	5713	U	A	A	A	A	A	H	H	B	B	B		A	A	A	A	B	A
15	**BC4F1**	**5714**	U	A	A	A	A	A	H	H	A	B	B			A	A	A	H	A
16	**BC4F1**	**5715**	-	A	A	A	A	A	A	A	A	A	B		A	A	A	A	A	A
17	**BC4F1**	**5716**	A	A	A	A	A	A	H	H	H	B	H		A	A	A	A	H	A
18	**BC4F1**	**5717**	A	A	A	A	A	A	H	B	B	B	H		A	A	A	A	H	A

Bold genotypes refers to selected individuals in respective generation.

Figure 6(a): PAGE gel for SSR marker *Xtxp210* for BC_4F_1 R16 (96 genotypes)

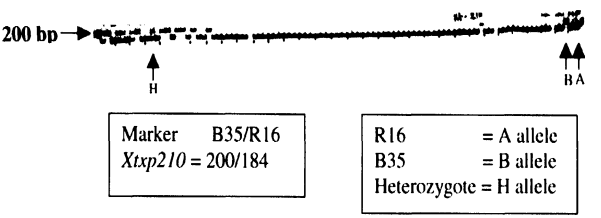


Figure 6(b): PAGE gel for the primer *Xtxp298*, *Xtxp34* for BC_4F_1 R16 (52 genotypes)

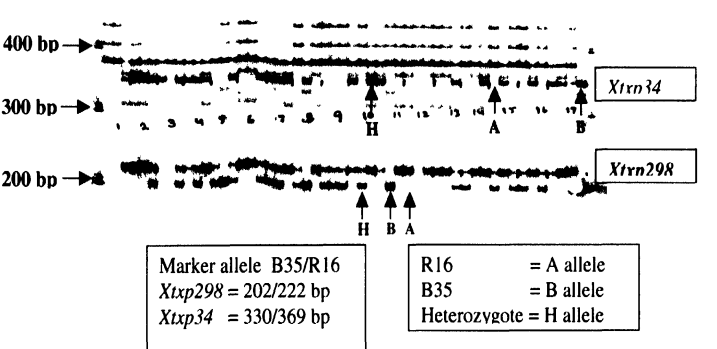
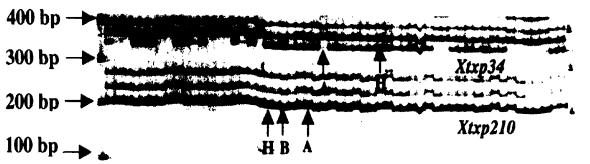


Figure 7(a): PAGE gel for SSR marker pair *Xtxp210/Xtxp34* for BC_3F_2 ISIAP Dorado (52 genotypes)



Marker alleles B35/ISIAP Dorado	ISIAP Dorado = A allele
Xtxp210 = 200/205 bp	B35 = B allele
Xtxp34 = 330/369 bp	Heterozygote =H allele

Figure 7(b): PAGE gel for SSR marker pair *Xtxp230/Xtxp289* for BC_4F_1 ISIAP Dorado (96 genotypes)

Marker alleles B35/I Dorado	ISIAP Dorado = A allele
Xtxp230 = 190/195 bp	B35 = B allele
Xtxp289 = 290/295 bp	Heterozygote = H allele

Figure 8(a): Graphical Genotyper overview of segregation of foreground and background SSR markers in BC_3F_1 R16. Indicated are selected genotypes for backcrossing [Population 2829]

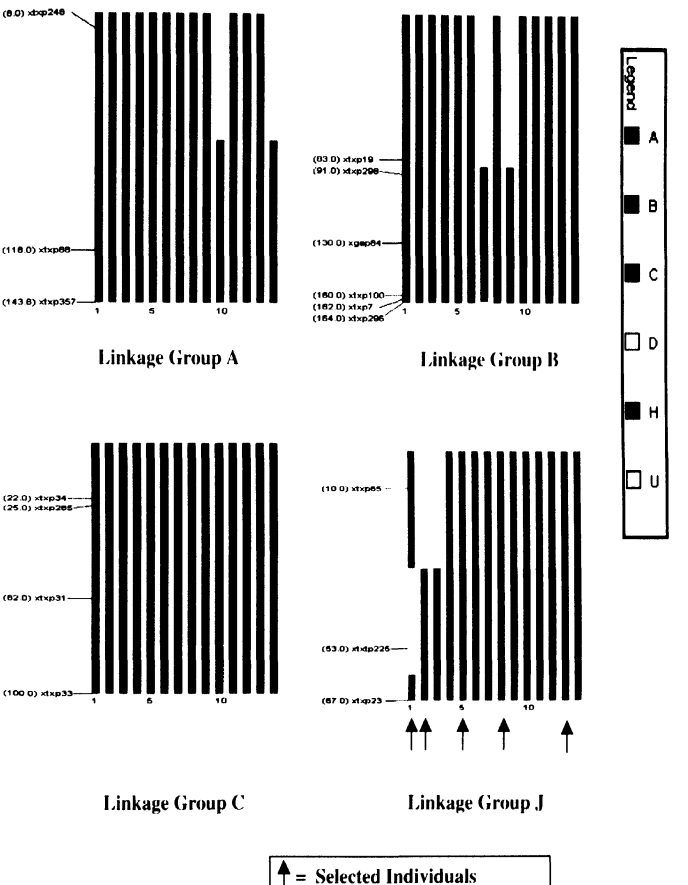


Figure 8(b): GGT overview of the segregation of SSR marker of four selected BC_3F_1 R16 individuals

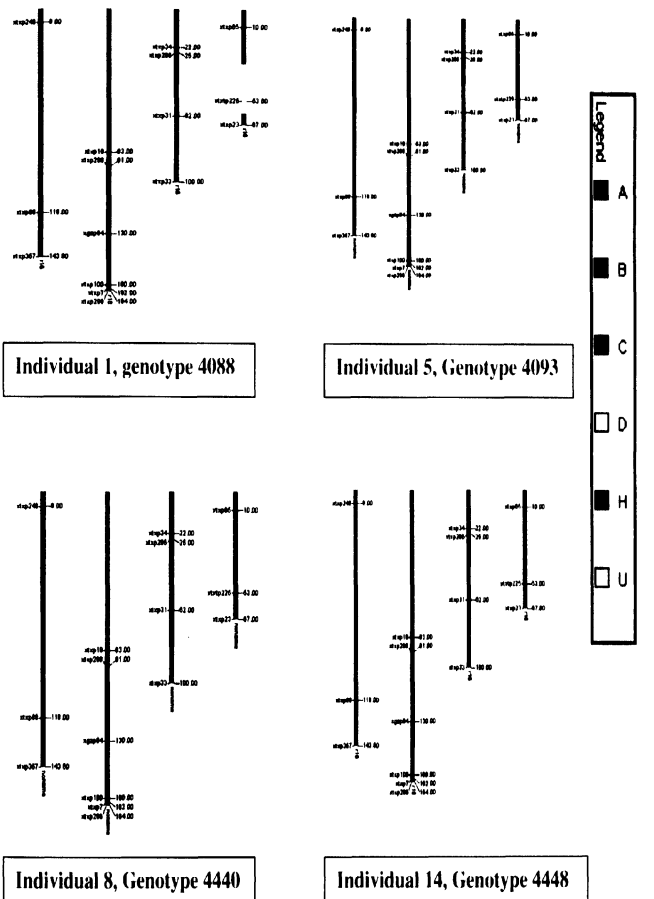


Figure 9(a): Graphical Genotyper overview of segregation of foreground and background SSR markers in BC_4F_1 R16. Indicated are selected genotypes for back-crossing [Population 4431]

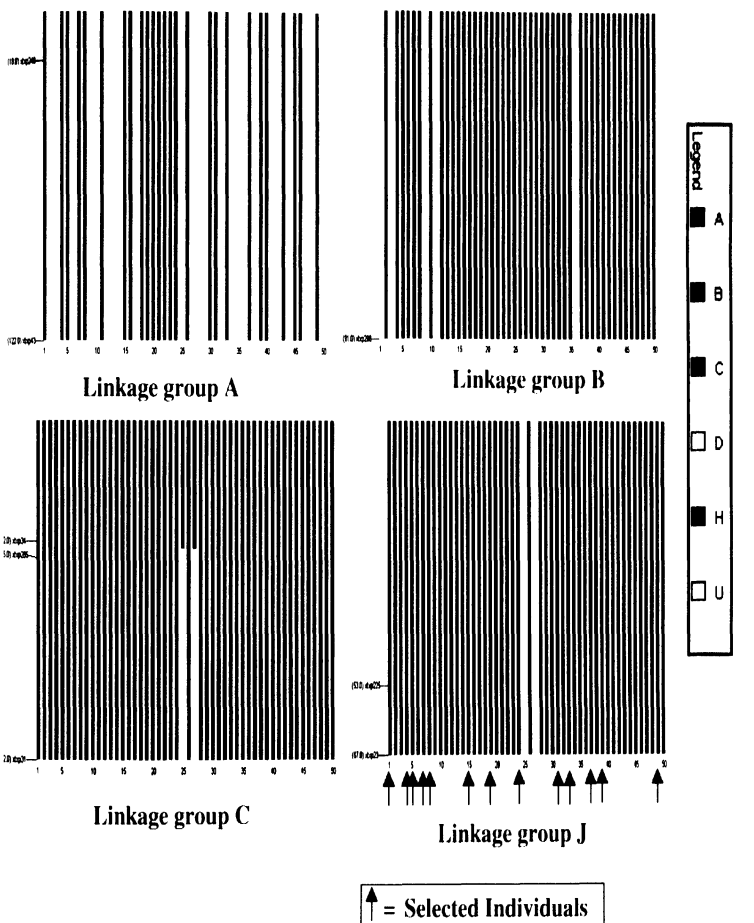


Figure 9(b): GGT overview of the segregation of SSR marker of four selected BC_4F_1 R16 individuals (individual mode).

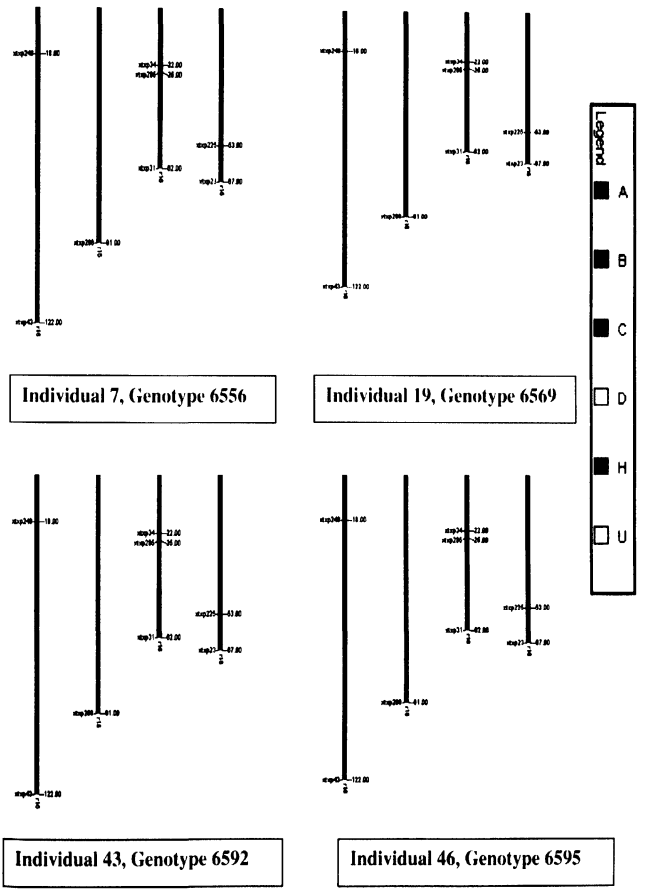


Figure 10(a): Graphical Genotyper overview of segregation of foreground and background SSR markers in BC_3F_2 Isiap Dorado. Indicated are selected genotypes for back-crossing [Population 3750]

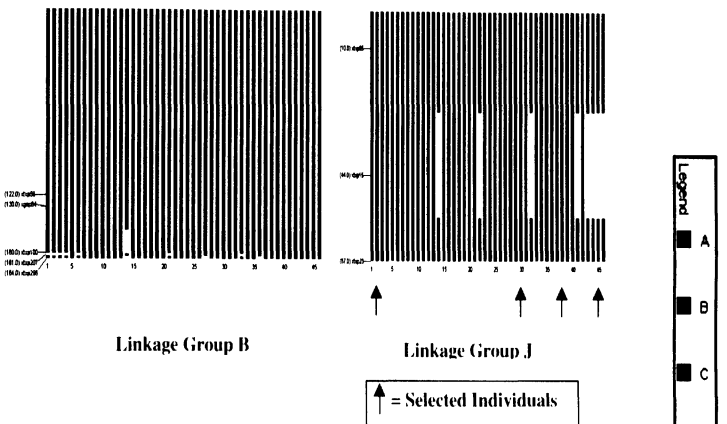
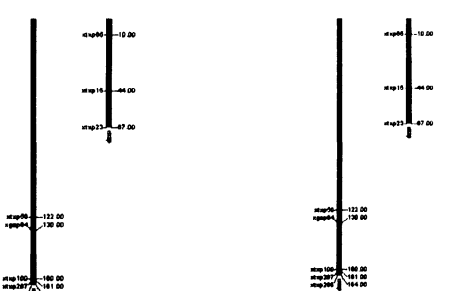


Figure 10 (b): GGT overview of the segregation of SSR marker of two selected BC_3F_2 Isiap Dorado individuals [Population 3750]



Individual 30, Genotype 5208 Individual 38, Genotype 5216

Figure 11(a): Graphical Genotyper overview of segregation of foreground and background SSR markers in BC_4F_1 Isiap Dorado. Indicated are selected genotypes for back-crossing [Population 3399]

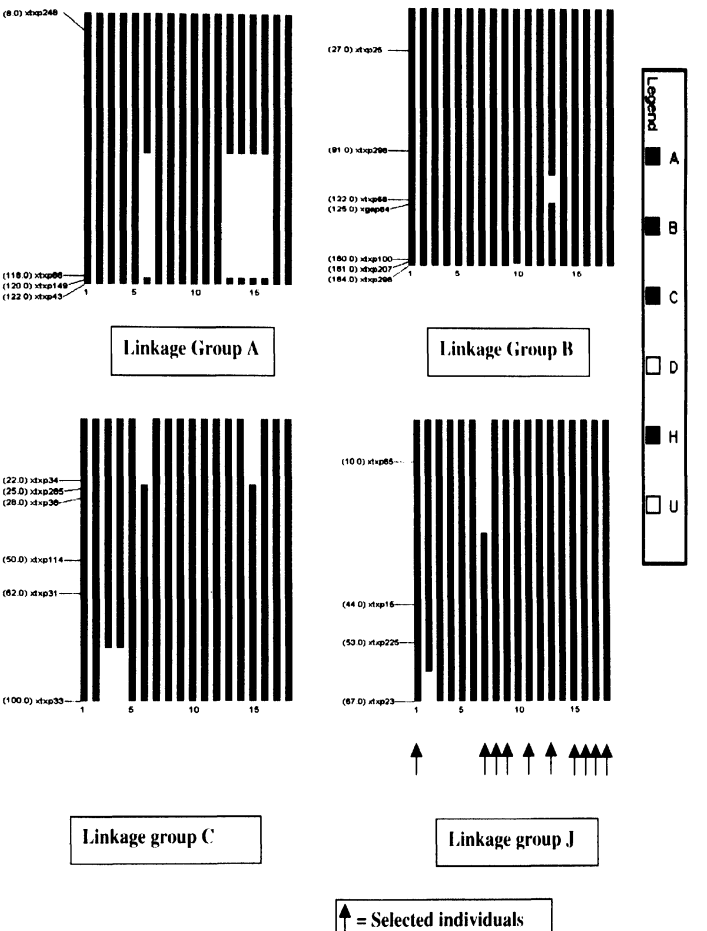
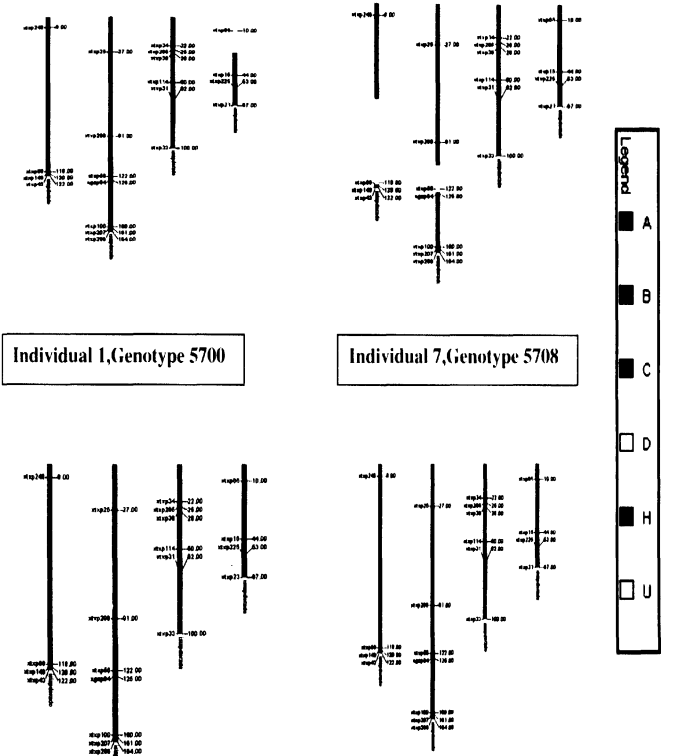


Figure 11(b): GGT overview of the segregation of SSR marker of four selected BC4F1 Isiap Dorado individuals [Population 3399]



Individual 1,Genotype 5700

Individual 7,Genotype 5708

Individual 17,Genotype 5716

Individual 18,Genotype 5717

Table10: Recombinarion frequencies calculated across the generations using Kosambi's map function

Recurrent parent R16

Recombination frequencies for marker pairs present on sorghum linkage groups C and J

Marker interval	Expected recombination frequency	Observed recombination frequency	
		BC3F1	BC4F1
Linkage group C			
Xtx 34- Xtxp285	8.07	6.23	3.85
Xtxp34-Xtxp31	37.145	24.157	29.265
Xtxp34-Xtxp33	39.26	32.8	-
Xtxp285-Xtxp31	34.67	30.35	25.7
Xtxp285-Xtxp33	37.2	30.35	-
Xtxp31-Xtxp33	8.25	24.157	-
Linkage group J			
Xtxp225-Xtxp23	12.85	32.8	17.8

Recurrent parent ISIAP Dorado

Recombination frequencies for marker pairs present on sorghum linkage groups B and J

Marker interval	Expected recombination frequency	Observed recombination frequency	
		BC3F2	BC4F1
Linkage group B			
Xtxp56-Xgap84	8.25	-	14.05
Xtxp56-Xtxp100	26.62	28.15	27
Xtxp56-Xtxp207	27.55	28.15	31.65
Xtxp56-Xtxp296	28.415	31.65	24.3
Xgap84-Xtxp100	22.01	28.15	27
Xgap84-Xtxp207	23.01	28.15	29.44
Xgap84-Xtxp296	24.15	31.65	31.65
Xtxp100-Xtxp207	1.965	17.6	31.65
Xtxp100-Xtxp296	3.845	14.7	9.57
Xtxp207-Xtxp296	1.965	11.45	35.238
Linkage group J			
Xtxp15-Xtxp225	7.565	-	21.29
Xtxp15-Xtxp23	18.44	18.99	33.53
Xtxp225-Xtxp23	12.85	-	29.426

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

Using marker-assisted selection, we are able to introgress genomic regions from stay-green donor B35 to recurrent parents (ISIAP Dorado and R16) over two generations. Markers linked to stay-green QTL regions to be transferred from donor B35 to recurrent parents, ISIAP Dorado or R16 were used for foreground selection whereas unlinked markers, evenly distributed over genomic regions to be retained of the recurrent parent, were used for background selection. Based on the genotype data, individuals heterozygous (BC_3F_1, BC_4F_1 generation) or homozygous (BC_3F_2 generation) for markers spanning stay-green QTLs were selected during the first step of selection (foreground selection). Among the selected individuals, those genotypes with minimal presence of donor alleles unlinked to stay-green QTLs were selected during the second step (background selection).

5.1 CRITERIA FOR SELECTING THE INDIVIDUALS

Markers, especially foreground markers, are taken into consideration for selection of genotypes. The genotypes having 'B/H' allele for foreground markers and 'A' allele for background markers are selected. 'H' allele, in future, because of recombination it may recover 'A/B' allele. Presence of 'A' allele for background markers and 'B/H' allele for foreground markers ensures the recovery of recurrent parent genome (R16/ISIAP Dorado) with stay-green trait. Genotypes meeting above criteria were selected and

advanced to next generations. Genotypes showing 'A' allele for all (foreground and background) markers were selected as control. In a population if a marker showing same allele for all genotypes, one or two individuals meeting the selection criteria were selected as representatives of the population.

For selected individuals, the markers showing 'B/H' allele and not amplified markers during current generation were screened in the next generation. The markers showing the 'A' allele for the markers are not tested in more advanced generations as neither selfing or backcrossing are expected to change the genomic composition of these regions in which recovery of recurrent parent genome has been accomplished with respect to the particular marker. Once achieving the recurrent parent genome recovery for all markers outside the targeted stay-green QTLs introgression regions, selected QTLs introgression heterozygotes are selfed and the resulting $BCnF_2$ individuals marker genotyped to identify QTLs introgression homozygotes, that are then multiplied by selfing to provide seed for field-testing to evaluate the stay-green character phenotypically. After testing, if the stay-green QTLs introgression line is found significantly better than the controls, it can be released as a new variety or itself used as an elite donor of the terminal drought tolerance (stay-green) character.

5.2 RECURRENT PERENT R16

5.2.1 BC_3F_1 R16

Out of 15 BC_3F_1 individuals screened in population 2829 (as e.g.), 5 individuals were selected and advanced to BC_4F_1 [Figure8 (a) and (b)]. These genotypes were targeted for QTLs *Stg2*, *Stg3*? *Stg4*? and *Stg*B. Based on the genotyping results from 9

Table11(a): Selected individuals in BC3F1 and BC4F1 generations for recurrent parent R16

Generation BC3F1 R16

Generation	Population	No. of individuals	No. of selected individuals	Targeted QTL
BC3F1	2885	14	1	Stg B
BC3F1	2812	1	1	Stg C
BC3F1	2839	8	5	Stg3, StgB
BC3F1	2884	17	2	Stg3, StgB
BC3F1	2820	15	3	Stg1, Stg2
BC3F1	2835	20	6	Stg1, Stg2, Stg3?, Stg4?, StgB
BC3F1	2834	6	3	Stg1, Stg2, Stg3?, Sg4?, StgB
BC3F1	2829	15	5	Stg2, Stg3?, Stg4?, StgB
BC3F1	2850	18	7	Stg1, Stg2, Stg3?, StgB, StgC

Generation BC4F1 R16

Generation	Population	No. of individuals	No. of selected individuals	Targeted QTL
BC4F1	4061	22	2	StgB
BC4F1	4123	8	1	Stg3, StgB
BC4F1	4390	10	4	Stg1, Stg2
BC4F1	4431	50	13	Stg1, Stg2, Stg3?, Stg4?, StgB
BC4F1	4101	4	2	Stg1, Stg2, Stg3?, Stg4?, StgB
BC4F1	4448	13	9	Stg2?, Stg3?, Stg4?, StgB
BC4F1	4462	8	2	Stg1, Stg2, Stg3?, StgB, StgC
BC4F1	4465	14	1	Stg1, Stg2, Stg3?, StgB, StgC

populations (114 individuals), 33 individuals were selected and advanced to the next generation [Table 11(a)]

5.2.2 BC_4F_1 R16

Out of 50 BC_4F_1 individuals screened in population 4431 (as e.g.), 13 individuals were selected and advanced to the next generation [Figure 9 (a) and (b)]. The genotypes were targeted for QTLs *Stg*1, *Stg*2, *Stg*3? *Stg*4? and *Stg*B. It is observed that markers linked to the QTLs present on linkage group C are still segregating. So the selected individuals were also screened for markers, which are not linked to the QTL regions to increase recovery of the recurrent parent genome in regions that do not contribute to the stay-green trait. Likewise the individuals from all eight BC_4F_1populations (129 individuals) were screened and 34 selected individuals from all populations are advanced to BC_5F_1 [Table 11(a)].

5.3 RECURRENT PARENT ISIAP DORADO

5.3.1 BC_3F_2 ISIAP Dorado

A total of 46 BC_3F_2 individuals were screened from population 3750 (as e.g.), but only three individuals were selected [Figure10 (a) and (b)]. And these were targeted for QTLs *Stg*1, *Stg*2, *Stg*3? *Stg*4, and *Stg*B. A total 7 populations consisting 356 individuals were genotyped and advanced to the next generation by selfing (Table 11(b)). The selfed seeds from selected BC_3F_2 individuals were sown in the field for phenotypic evaluation of the stay-green character during *rabi* season 2003/2004. This phenotypic evaluation is expected to confirm the proper introgression of the target QTLs from the donor parent.

Table 11(b): Selected individuals in BC3F2 and BC4F1 generations for recurrent parent ISIAP Dorado

Generation BC3F2 ISIAP Dorado

Generation	Population	No. of individuals	No. of selected individuals	Targeted QTL
BC3F2	3317	24	4	Stg3
BC3F2	3369	48	4	Stg1, Stg2, Stg3, Stg4?, StgB
BC3F2	3379	38	4	Stg1, Stg2, Stg3, Stg4?, StgB
BC3F2	3750	46	3	Stg1, Stg2, Stg3?, Stg4, StgB
BC3F2	3754	152	9	Stg1, Stg2, Stg3?, Stg4, StgB
BC3F2	3756	24	2	Stg1, Stg2, Stg3?, Stg4, StgB
BC3F2	3758	24	3	Stg1, Stg2, Stg3?, Stg4, StgB

Generation BC4F1 ISIAP Dorado

Generation	Population	No. of individuals	No. of selected individuals	Targeted QTL
BC4F1	3629	25	5	Stg2
BC4F1	3689	6	2	Stg3
BC4F1	3726	8	2	Stg4
BC4F1	3371	3	1	Stg1, Stg2, Stg3, StgB, Stg4
BC4F1	3786	4	4	Stg1, Stg2, Stg3, StgB, Stg4
BC4F1	3399	18	10	Stg1, Stg2, Stg3?, Stg4, StgB
BC4F1	3805	4	4	Stg1, Stg2, Stg3?, Stg4, StgB

5.3.2 BC_4F_1 ISIAP Dorado

Out of 18 BC_4F_1 individuals genotyped, 10 genotypes were selected from the population 3399 (as e.g.), for QTLs *Stg*1, *Stg*2, *Stg*3? *Stg*4, and *Stg*B [Figure11 (a) and (b)]. Total 7 populations consisting 68 individuals were genotyped and 28 individuals were selected and advanced to the next generation for selfing [Table 11(b)].

5.4 RECOMBINATION FREQUENCIES

Recombination frequencies were calculated for two backcross generations of recurrent parent R16 with selected genotypes (BC_3F_1 and BC_4F_1) and recurrent parent ISIAP Dorado with selected genotypes (BC_3F_2 and BC_4F_1). For each generation, the number of recombinants present was determined, the crossing-over percentage calculated and the percentage converted into Kosambi's map units (in cM) (Kosambi, 1944). The observed recombination frequencies were compared with the expected recombination fractions. Expected recombination frequencies were calculated based on the distance between the respective markers, using the linkage map developed by Bhattramakki et al (2000), with the help of Kosambi's map function (Table 10).

When the expected and calculated recombination frequencies are compared, it is noticed that in general they are very similar. However there are a few differences; for instance the distance between the markers *Xtxp100* and *Xtxp207* present on the linkage group B according to Bhattramakki et al. (2000) is 1 cM whereas the calculated distance between these two markers based on the recombination frequencies is 20 cM for back-cross generation BC_3F_2 of ISIAP Dorado with selected genotypes. The reasons for

deviations between expected and observed frequencies might be the small population size analyzed (causing sample bias) or the parents used in the breeding program (Bhatttramakki et al. used the F_7 population derived from the BTx623 × IS3620C for developing the linkage map whereas we used BC_3 and BC_4 populations derived from the cross B35 × ISIAP Dorado/R16).

5.5 EFFICIENCY OF MARKER ASSISTED SELECTION

Marker-assisted selection has the potential to greatly reduce the cost and time 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 a low heritability and a high phenotypic trait effect (Hospital *et al.*, 1997). Through MAS, we advanced four generations within two years. When conventional breeding strategies are applied, the advancement of four backcross generations with phenotypic selection for the stay-green trait will take at least four years. Conventional breeding schemes feature low costs during the research stage, but require longer time to complete, whereas MAB features high cost during the research stage, but it takes less time to complete. Release stage and adoption stages are assumed to be identical to those of conventional selection in terms of cost as well as duration in case of MAS. From an economic point of view, the advantage of MAS thus derives from the fact that the 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 varietal release, MAS generates large additional economic benefits (Morris et al., 2003).

The efficiency of MAS is generally reduced with increasing genetic distance between the markers applied. Hospital et al. (1997), based on the simulation studies, recommended an optimal distance between two adjacent markers of about 5-10 cM. We observed that the frequency of recurrent genotypes among the selected progeny increases as the selection intensity for recurrent genotypes increases, as reported by Knapp (1998). Practically speaking, the number of markers that must be used decreases in each successive backcross generation (Table 12), because once the recurrent parent allele has been fixed at any given non-target locus, it is not necessary to continue screening at the locus in subsequent generations since the locus will remain homozygous for the rest of the selection (Morris et al., 2003). Therefore in BC_3F_1 R16 on an average 27 markers/population were tested and in BC_4F_1 R16 the number of markers were reduced to 10 markers/population. In BC_3F_2 ISIAP Dorado, on an average 20 markers were screened and reduced to 13 in BC_4F_1 generation (Table 12). The decreasing number of markers reflects the increasing percentage of recurrent parent that is fixed at each backcross generation.

Table 12: List of no. of individuals and markers tested in each generation

	R16		ISIAP Dorado	
Generation	No. of markers/ population	No. of individuals	No. of markers/population	No. of individuals
BC_1F_1	13	19	13	33
BC_2F_1	15	140	16	190
BC_3F_1	27	114	30	93
BC_3F_2	-	-	20	356
BC_4F_1	10	129	13	68

The fact that MAS technology is so challenging should not be a reason for discouragement, but instead, 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 (Young, 1999).

5.6 RECOMMENDATIONS FOR THE FUTURE STUDY

Phenotyping of the stay-green behaviour for the selected individuals is needed. Introgression of any trait should be confirmed phenotypically after several generations of genotyping. In this context, the selected genotypes in BC_3F_2 ISIAP Dorado and R16 are being evaluated for stay-green QTLs during *rabi* season 2003/2004. Fine mapping for these stay-green QTLs is possible, once the presence of different stay-green QTLs is phenotypically confirmed. ESTs can be generated from this work for better understanding of this complex trait. Comparative genome mapping will help us to study this trait in other related cereals.

CHAPTER VI

SUMMARY

SUMMARY

Sorghum is the fifth most important cereal crop globally after wheat, maize, rice and barley. Sorghum, a C_4 grass, is grown in more than 80 countries mostly in tropical and sub-tropical regions. In these regions, drought stress is perhaps the most important abiotic constraint to sorghum production.

The stay-green trait allows the plant to literally stay green for a longer period during unrelieved drought stress that occurs after flowering. The genotypes not having this trait start senescence when exposed to drought stress. Stay-green allows the continued accumulation of assimilates, resulting in higher yields under conditions of post-flowering drought stress. Stay-green is the best-characterized component of terminal drought tolerance available in sorghum. It has also been reported to be associated with other traits like charcoal rot resistance (Tenkouano *et al.*, 1993) and superior ruminant quality of grain crop residues produced under conditions of terminal drought stress.

Putative QTLs for the stay-green trait, derived from source B35, have been identified in six recently published studies (Tuinstra *et al.*, 1997; Crasta *et al.*, 1999; Xu *et al.*, 2000; Tao *et al.*, 2000; Subudhi *et al.*, 2000; and Sanchez *et al.*, 2002). Using the linkage map developed by Bhattramakki *et al.* (2000) as a standard reference, the ICRISAT sorghum breeding team identified six consensus genomic regions associated with the stay-green trait from the B35 source. The identification of these consensus QTL regions provided an opportunity for marker-assisted breeding (MAB) for introgression of these stay-green QTLs from B35 to elite recurrent parents.

We aimed at transfer of QTLs from B35 to recurrent parents, ISIAP Dorado (originally released in Central America) and R16 (a *rabi*-adapted breeding line of Indian origin). Both recurrent parents have been advanced to BC_3 and BC_4 generations for introgression of QTLs from the donor parent using SSR marker-assisted selection (MAS) targeting the six consensus stay-green QTLs. In this study, all genotypes in two generations from each recurrent parent were screened with foreground markers to identify the individuals having the QTL alleles of our interest and also screened with background markers to further select among these individuals at all other loci for recurrent parent alleles. Selected individuals are advanced to the next generation.

Based on the number of recombinants observed between each pair of linked markers, recombination frequencies were calculated. The expected and calculated recombination frequencies were slightly different. Possible reasons for these deviations are small population sizes of the segregating generations, small numbers of marker data points studied, and mapping population specificity of recombination rates and map distance (calculated map distance according to the number of recombinants observed) varies greatly when compared with the distance reported between the respective markers in the linkage map of Bhattramakki *et al.* (2000). Phenotyping studies are required to confirm the introgression of the targeted stay-green QTLs from the B35 donor parent. Further, fine mapping, ESTs generation from near-isogenic pairs, and comparative genome mapping is possible for better understanding of the stay-green trait. These studies could facilitate more efficiently exploiting QTLs for stay-green in a MAB program to benefit global sorghum breeding efforts.

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The pattern of "literature cited" is in accordance with the "guidelines" for thesis presentation for Acharya N. G. Ranga Agricultural University, Hyderabad.

APPENDIX

Appendix 1

Marker details

Locus name	Primer sequence		SSR repeat motif	Annealing temperature	Linkage group	Protocol
	Forward primer	Reverse primer				
Xtxp65	CACGTCGTCACCAACCAA	GTTAAACGAAAGGGAAATGGC	(ACC)4 (CCA)3 CG(CT)8	55	J	5
Xtxp141	TGTATGGCCTAGCTTATCT	CAACAAGCCAACCTAAA	(GA)23	55	G	5
Xtxp149	AGCCTTGCATGATGTTCC	GCTATGCTTGGTGTGGG	*(CT)10*	55	A	5
Xtxp207	ACACATCTACTACCCTCTCACCCT	TGATAGACTTGTGAGCAGCTCC	(CT)14	55	B	4
Xtxp225	TTGTTGCATGTTGGTTATAG	CAAACAAGTTCAGAAGCTC	(CT)8 (CA)8 CCC(CA)6	55	J	5
Xtxp248	GGGTGTCCAATGTTGTCTGC	GGCCGTTACTGTCCCTTACTCA	*(AG)5 (GA)28*	50	A	5
Xtxp296	CAGAAATAACATATAATGATGGGGTGAA	ATGCTGTTATGATTTAGAGCCTGTAGAGTT	(CA)18	55	B	5
Xtxp298	GCATGTGTCAGATGATCTGGTGA	GCTGTTAGCTTCTTCTAATCGTCGGT	(AGA)23	55	B	5
Xtxp57	GGAACTTTTGACGGGTAGTGC	CGATCGTGATGTCCCAATC	*(GT)21*	55	I	7
Xtxp88	CGTGAATCAGCGAGTGTTGG	TGCGTAATGTTCCTGCTC	(AG)31	53	A	4
Xtxp100 (Kaf)	CCGGCCGGCCAACCAACCAC	TGCCCCAACGCTCACGGTCCC	(CT)19	55	B	5
Xtxp114	CGTCTTCTACCCGCGTCCT	CATAATCCCACTCAACAATCC	*(AGG)8*	50	C	7
Xtxp210	CGCTTTTCTGAAAATATTAAGGAC	GATGAGCGATGGAGGAGAG	(CT)10	55	H	7
Xtxp230	GCTACCGCTGCTGCTCT	AGGGGGCATCCAAGAAAT	(GA)28	55	F	5
Xtxp265	ATTTGATTCTTCTTGCTTTGCCTTGT	TTGTCATTTCCCCCTTCTTTCTTTT	*(CTT)11 CTC(CTT)16*	55	C	7
Xtxp289	AAGTGGGGTGAAGAGATA	CTGCCTTTCCGACTC	(CTT)16 (AGG)6	55	F	5
Xtxp295	AAATCATGCATCCATGTTCGTCTTC	CTCCCGCTACAAGAGTACATTCATAGCTTA	(TC)19	55	E	7
Xtxp56	TGTCTTCGTAGTTGCGTGTTG	CCGAAGGAGTGCTTTGGAC	(GA)39	55	B	5
Xtxp357	CGCAGAAATACGATTG	GCTATCTGGAGTAACTGTGT	(GT)10	55	A	5

(Bhattramakki D, Dong J, Chhabra A K, and Hart G E. 2000. An integrated SSR and RFLP linkage map of *Sorghum bicolor* (L.) Moench. Genome 43: 988-1002)

Protocols

PCR conditions for 5 ul reaction

Number	Primer (2 pM/ul) in ul	$MgCl_2$ (10 mM) in ul	dNTPs (2 mM) in ul	DNA (2.5 ng) in ul	Enzyme (0.5 U/ul) in ul	Buffer (10X) in ul	Distilled water in ul
4	0.5	0.75	0.500	0.5	0.25	0.5	2.000
5	0.5	1.00	0.250	1.0	0.20	0.5	1.550
7	1.0	1.00	0.375	0.5	0.20	0.5	1.425