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

THESIS SUBMITTED TO THE ACHARYA N.G.RANGA AGRICULTURAL UNIVERSITY IN PARTIAL FULFILMENT FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN AGRICULTURE

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SEPTEMBER,2004

CERTIFICATE

Mr. R. Chandra Mouli has satisfactorily prosecuted the course of research and that the thesis entitled **"MARKER-ASSISTED BACKCROSSING OF STAY-GREEN QTLs INTO ELITE SORGHUM LINES"**, submittrd 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.

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Chairman of the Advisory Committee

Date : 1-12-2004
Place: Hyderabad

CERTIFICATE

This is to certify that the thesis entitled "**Marker-assisted backcrossing 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. R. CHANDRA MOULI** 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, **R. Chandra Mouli,** here by 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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Acknowledgement

I first thank Almighty God for his love and blessings without which I wouldn't have been able to complete my studies hitherto and present this piece of work.

It is my pride and honour to express my profound sense of gratitude for providing me an opportunity to undertake this project in ICRISAT. I immensily thank ***Dr .S. Sivaramakrishna,*** *Professor and Head of the Department, Agricultural Biotechnology for his stupendous and stimulatory guidance, moral support and valuable suggestions, during the project work. I am fortunate to have blessed with the privilege of working under his guidance.*

I humbly place on record my sincere thanks to ***Dr. K. Manorama*** *Associate Professor, Department of Agricultural Biotechnology for her keen interest and meticulous guidance during this project.*

I place my profound etiquette, deep sense of gratitude to ***Dr. C. T .Hash,*** *Principal Scientist, Molecular Breeding, ICRISAT for his constructive criticism, constant encouragement, valuable assistance and cooperation during the course of my study.*

I accolade my deep sense of honour to ***Dr. Rolf Folkertsma,Post Doc. Fellow, ICRISAT*** *for his constant support and kind back-up in planning and execution of this project.*

It gives me gratification in expressing my heartfelt gratitude to ***Dr. S. Senthilvel and Mrs. Seetha Kannan,*** *ICRISAT for their help and valuable advice rendered during the course of my project work.*

I wish to acknowledge and express sincere thanks to ***Mr.G.Soma Raju, Mr.Moss, Mr.Gafoor and Mr.Narsi Reddy*** *for their kind cooperation in the lab for successful completion of my project.*

I express deep sense of gratitude to my beloved parents ***Sri R.A. Narayana Murthy and Smt. Shakuntala Devi.*** *I also express my love and affection to my brothers* ***Suresh, Kiran, Srikanth, Gopi.***

Diction is not enough to express my unboundful gratitude and regards to my friends, class mates, seniors and my juniors for their incessant encouragement support , delightful companionship during my college life.

(R .Chandra Mouli)

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TITLE OF THE THESIS	:	MARKER ASSISTED BACKCROSSING OF STAY-GREEN QTL'S INTO ELITE SORGHUM LINES
DEGREE	:	MASTER OF SCIENCE IN AGRICULTURE
FACULTY	:	AGRICULTURE
DISCIPLINE	:	AGRICULTURAL BIOTECHNOLOGY
MAJOR ADVISOR	:	Dr. S. SIVARAMA KRISHNA
UNIVERSITY	:	ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
YEAR OF SUBMISSION	:	2004

Abstract

Sorghum [*Sorghum bicolor* (L.) Moench] is the 5th most important cereal crop globally after wheat, maize, rice and barley (FAO, 2003; FAO and ICRISAT, 1996). This C_4 grass is grown in more than 80 countries, mostly in tropical and sub-tropical regions. The average annual sorghum area cultivated amounts to 44 M ha, with an average annual grain production of 63 M tons, and average grain yield of 1.4 t ha^{-1} (FAO, 2003; FAO and ICRISAT, 1996). Sorghum was domesticated in Ethiopia and part of Congo, with secondary centers of origin in India, Sudan and Nigeria.

Production of sorghum in semi-arid regions of the world is limited by drought. Developing plants that have an advantage under water-limited conditions is a major challenge for sorghum improvement programs globally. There are three distinct stages in which drought affects sorghum: Vegetative (GS1); Pre-Flowering (GS2); and Post-Flowering (GS3). The best characterized form of drought stress tolerance in sorghum during this

post-flowering stage of growth is called "stay green." Stay-green is a drought-tolerance trait in grain sorghum. When water is limited during the grain filling period, genotypes possessing this trait maintain more photosynthetically active leaves compared with genotypes not possessing this trait.

Putative QTL for stay-green trait from B35 have been identified in five recently published studies (Tuinstra et al, 1997; Crasta et al 1999; Xu et al, 2000; Tao et al, 2000 and Subudhi et al 2000). Using the linkage map developed by Bhattramakki et al (2000) identified six genomic regions associated with stay-green trait in B35 parent. The identification of these QTL provided us an opportunity for marker assisted breeding (MAB) for introgression of QTL from B35 to recurrent parents.

We aimed at transfer of QTL from B35 to recurrent parents, S35 and ICSV111. Both recurrent parents have been advanced to BC3 and BC4 generations for introgression of QTL from donor parent using SSR marker assisted selection (MAS), targeting six QTL detected. In this study, all genotypes in two generations from each recurrent parent were screened with foreground markers to identify the genotypes for QTL of our interest and also screened with background markers to select the genotypes for all other loci from recurrent parent. Selected individuals are advanced to next generation.

CHAPTER 1

INTRODUCTION

1.1 Prologue

So far the horror scenarios foreseen by Malthus (1798) have not come true, and agricultural production has managed to keep up with the growing demands. At this moment over 6 billion people need to be fed and it is expected that in the year 2050 more than 10 billion people will inhabit the earth (FAO, 1996). Moreover, demands per capita will rise when standards of living in developing countries improve. As a consequence there will be a huge increase in the demand for food, and production will need to triple in the coming 40 years (Bindraban, 1997; WRR, 1995). History has indeed shown a continuous increase in crop yields, resulting from improved agronomy and plant breeding efforts. However, in the light of the speed at which the human population develops, and taking into account the expected reduction of available arable land due to climatic changes and human intervention, a continued effort to improve agricultural production in the future will be vital. Reduction of losses caused by abiotic and biotic stress will therefore continue to be a key issue in enhancing food security.

Scenario studies on world food security generally assume that a large increase in production will be achieved by conventional and biotechnological

genetic improvement of crop species (FAO, 1996; Agrevo, 1996). The knowledge that is obtained with the new tools of biotechnology can be used to contribute to enhanced food security throughout the world. The study presented in this thesis focuses on the use of some of the modern biological tools (Marker-Assisted Selection, MAS) for the improvement and acceleration of genetic crop improvement in sorghum.

1.2 Background information on sorghum

Sorghum [*Sorghum bicolor* (L.) Moench] is the 5th most important cereal crop globally after wheat, maize, rice and barley (FAO, 2003; FAO and ICRISAT, 1996). This C_4 grass is grown in more than 80 countries, mostly in tropical and sub-tropical regions. The average annual sorghum area cultivated amounts to 44 M ha, with an average annual grain production of 63 M tons, and average grain yield of 1.4 t ha^{-1} (FAO, 2003; FAO and ICRISAT, 1996). Sorghum was domesticated in Ethiopia and part of Congo, with secondary centers of origin in India, Sudan and Nigeria (www.africancrops.net).

Crop ecologists have described five races of cultivated sorghum that have come into common usage among sorghum breeders (www.africancrops.net).They are: durra, kafir, guinea, bicolor and caudatum. All five major races of sorghum originated and continue to be cultivated in Africa, with several races often being used for different purposes within the same agro-ecosystem. Although sorghum

cultivation has become an important component of agriculture in various industrial countries, it remains largely a developing country crop. Some 90% of the world's cultivated sorghum area is in developing countries, mainly in Africa and Asia. As a continent, Africa is the largest producer of sorghum grain with approximately 18.5 M tons produced annually. Leading producers around the world during fiscal year 2003 included the United States (9.3 M tons), India (7 M tons), Nigeria (7.8 M tons), and Mexico (5.3 M tons/208).

Production of sorghum in semi-arid regions of the world is limited by drought. Developing plants that have an advantage under water-limited conditions is a major challenge for sorghum improvement programs globally.

1.3 Sorghum usage

In many parts of the world sorghum has traditionally been used in food products and various food items; porridge, unleavened bread, cookies, cakes, couscous, and malted beverages are made from this versatile grain. Traditional food preparation of sorghum is quite varied. Boiled sorghums are one of the simplest uses and small, corneous grains are normally desired for this type of food product. The whole grain may be ground into flour or decorticated before grinding to produce either a fine particle product or flour, which is then used in various traditional foods.

Sorghum has unique properties that make it well suited for food uses. Some sorghum varieties are rich in antioxidants and all sorghum varieties are gluten-free, providing an attractive alternative for wheat allergy sufferers.

Sorghum stover is an important source of animal feed in mixed farming situations.

Sorghum grain is also an important animal feed used in countries like the U.S.A., Mexico, Brazil, Venezuela, Argentina, and Australia. Good-quality sorghums are available with a nutritional feeding value that is nearly equivalent to that of corn (such sorghums have 5% lower feed value than maize due to 1% lower fat content as a result of sorghum's relatively smaller embryo size).

As much as 12% of domestic sorghum production in India goes to produce ethanol and its various co-products.

With demand for renewable fuel sources (including ethanol) increasing, supply and demand for co-products like sorghum-DDGS (distillers dried grains with solubles, which are an excellent livestock feed) will increase as well due to sorghum grain's favorable nutritional profile.

Sorghum has distinct advantages when used in a crop rotation scheme, especially with cotton or wheat. It has a high yield potential and the highest recorded yield for the crop is 20.1 t ha^{-1}. However, sorghum grain yields in Africa and Asia, including India, remain very low.

Sorghum is one of the most drought tolerant cereal crops currently under cultivation. It offers farmers the ability to reduce costs on irrigation and other on-farm expenses (http://www.grains.org/grains/sorghum.html).

1.4 Drought tolerance

Drought is actually a meteorological event which implies the absence of rainfall for a period of time — long enough to cause moisture-depletion in the soil and water deficit with a decrease of water potential in plant tissues. But from an agricultural point of view, a working definition of drought would be the inadequacy of water availability (including precipitation, irrigation, and stored soil moisture) in quantity and distribution during the life cycle of a crop plant, which restricts the expression of full genetic potential of the plant.

Drought acts as a serious limiting factor in agricultural production by preventing a crop from reaching its genetically determined theoretical maximum yield. Most crops are sensitive to water deficits, particularly during early seedling growth and from flowering through seed development.

In agriculture, drought tolerance refers to the ability of a crop plant to produce its economic product with minimum loss in a water-deficit environment relative to a water-constraint-free production environment. An understanding of the genetic basis of drought tolerance in crop plants is a pre-requisite to evolving superior genotypes through either conventional breeding methodology or biotechnological approaches(Jiban Mitra; Current Science, vol. 80, no. 6, 25 March 2001).

1.5 Terminal drought tolerance in sorghum

There are three distinct stages in which drought affects sorghum: Vegetative (GS1); Pre-Flowering (GS2); and Post-Flowering (GS3). The plants response to stress at the two stages (GS2 and GS3) is very different, and sorghum genotypes often behave differently at the two stages, sometimes showing good drought tolerance at one stage but being drought sensitive at the other stage. Visual affects of drought sensitivity in the pre-flowering stage include leaf rolling, leaf erectness, leaf bleaching, leaf firing, delayed flowering, poor panicle exertion, saddle effect, panicle and floret blasting, and reduced panicle size. Sorghum with good pre-flowering drought tolerance exhibit the following characteristics: it resists panicle abortion, exerts panicles normally, its maturity is not delayed, and it expresses a substantial portion of its grain yield potential. Post-flowering stress is characterized by premature leaf and plant death (senescence), stalk collapse and lodging, charcoal rot, and reduced seed size. The best characterized form of

drought stress tolerance in sorghum during this post-flowering stage of growth is called "stay green." The stay-green trait can be defined as the ability to resist premature plant senescence, retain green leaves, fill grain normally, and resist lodging under conditions of post-flowering drought stress (Rosenow D.T., Breeding to enhance drought tolerance in sorghum. Proceedings of the 23rd Biennial Sorghum Industry Conference. SICNA: Lubbock, Texas, USA.).

1.6 The physiology of "Stay-green" in sorghum

1.6.1 What is stay-green?

Stay-green can be defined as extended foliar greenness during grain-filling under post-anthesis drought stress, and can be viewed as a consequence of the balance between N demand by the grain and N supply from the roots during grain filling (Borrell et al., 2001). Stay-green is a drought-tolerance trait in grain sorghum. When water is limited during the grain filling period, genotypes possessing this trait maintain more photosynthetically active leaves compared with genotypes not possessing this trait. According to Tenkouano et al., non-senescence or stay-green is a mechanism of delayed leaf and plant death that circumvents the detrimental effects of reduced soil moisture combined with high temperatures during drought stress that occurs during post-anthesis growth.

1.6.2 Nitrogen dynamics and the stay-green phenomenon

The longevity and photosynthetic capacity of a leaf are related to its N status. During senescence, amino acids cease to be formed, existing protein is degraded and not replaced, and the resultant amino acids are translocated out of the leaf. A considerable proportion of leaf protein is bound in pigment-protein complexes of the photosynthetic apparatus. Degradation of these pigment-protein complexes results in the characteristic yellowing of the leaf as chlorophyll is released from this dissociation and subsequently broken down during senescence (Borrell et al., 2000).

From as early as 40 days after emergence, more nitrogen is allocated to the leaves of stay-green hybrids as compared with their senescent counterparts, resulting in a higher specific leaf nitrogen (SLN). It is hypothesised that this higher SLN initiates a chain of responses, including enhanced radiation use efficiency (RUE) and transpiration efficiency (TE), which enable the plant to set a higher yield potential by anthesis, ultimately leading to higher grain yield and lodging resistance under conditions of post-anthesis drought. It is further hypothesised that after anthesis, higher SLN delays the onset and reduces the rate of leaf senescence, and that this is associated with increased nitrogen uptake from the soil compared with the senescent control. Evidence to support this framework is given in the following figure (After http://www.regional.org.au/au/asa/2003):

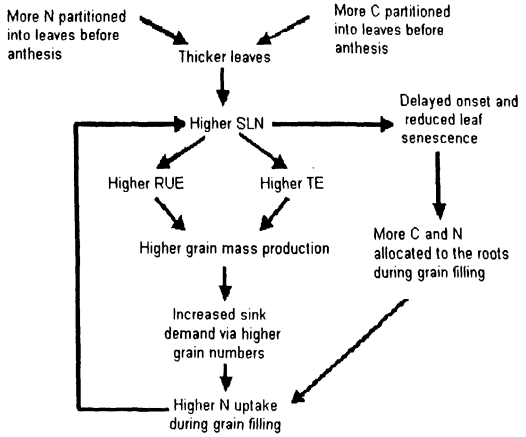


Figure 1. Nitrogen dynamics and the stay-green phenomenon in grain sorghum

The concept of a minimum SLN level, below which leaves will senesce, was proposed by Borrell and Hammer (2000). They concluded that SLN in stay-green sorghum hybrids remained above the 'threshold' senescence level for a longer period than in senescent hybrids for at least three reasons:

- The leaf N benchmark at anthesis was higher in stay-green than in senescent hybrids;

- N uptake during grain filling was higher in stay-green than senescent hybrids; and
- the remobilization of N from leaves of stay-green hybrids during grain filling was less compared with that of senescent hybrids.

1.6.3 Stay-green and yield

Grain yield is the product of grain number and grain size. Grain number is generally the main determinant of differences in grain yield, and this has also been observed for sorghum, grown under post-anthesis drought stress in southern India (Borrell et al., 1999). Factors related to the stay-green mechanisms of trait sources B35 and KS19, which can potentially increase the panicle growth rate around anthesis and hence can have a positive effect on grain number, include increased LAI (Borrell et al., 1999) and increased SLN, which increase RUE (Muchow et al., 1994) and competition for assimilate from the stem (Borrell et al., 1991, Fischer et al., 1986). Grain size is a secondary yield determinant and is often negatively associated with grain number (e.g., Bidinger et al., 2001). Hence, grain size is independent of green leaf area at anthesis (Borrell et al., 1999). However, the retention of photosynthetic capacity under water-limited conditions of stay-green hybrids ensures continued availability of new assimilates and is associated with increased N-uptake during grain filling (Borrell et al., 2000), potentially improving grain size. Borrell et al. (1999) found that grain size was correlated with relative rate of leaf senescence during grain filling such that reducing rate of

leaf senescence from 3% to 1% loss of leaf area per day resulted in doubling grain size from about 15 mg to 30 mg. Thus the stay-green trait can potentially increase grain yield by improving both grain number and grain filling ability.

1.7 Quantitative traits

Quantitative traits are typically controlled by many genes each contributing only a small part to the observed variation. Selection for quantitative traits is difficult, because the relation between observed trait values in the field (the phenotype) and the underlying genetic constitution (the genotype) is generally not straightforward. The environmental variance resulting from differences in growing conditions further obscures the relation between phenotype and genotype. Plant breeders would like to get a better grip on quantitative traits by direct selection for the genetic factors that are responsible for the observed variability in quantitative traits. This can be achieved through indirect selection: selection for other readily recognizable factors, which are associated the target trait and genes controlling it. Molecular markers, derived from recent bio-technological developments, can be used for this purpose.

1.8 Molecular markers

The discovery of restriction enzymes (Smith and Wilcox, 1970) and the polymerase chain reaction (PCR; Mullis and Faloona, 1987) have created the opportunity to visualise the composition of organisms at the DNA level, and

obtain a so-called genetic fingerprint (e.g., Kearsey and Pooni, 1996). The visualisation is routinely performed by the separation, by electrophoresis (through gels or capillaries), of DNA-fragments that result from a selective digestion with enzymes or fragments that result from a selective amplification using PCR. DNA fragment differences that result in different electrophoretic migration patterns between samples or individuals are called polymorphic markers. The visible differences of fragment banding patterns on the gel (or differences in time required to pass through the capillary) result from differences at the DNA level. Not all types of markers are the same. The information content depends on the method that was used to obtain the marker data and the population in which the markers were 'scored'. For instance, it is not always possible to distinguish genome fragments that are present in homozygous condition from heterozygous fragments. In a heterogeneous population like an F_2, co-dominant markers like RFLPs (Botstein *et al.*, 1980) and co-dominantly scored AFLPs (Vos *et al.* 1995) yield more information than dominant markers like RAPDs (Welsh and McCleland, 1990) and dominantly scored AFLPs. Advanced tools for the retrieval of marker data and the subsequent analysis have been developed and allow a quick and reliable analysis in most plant species. Important information on the genetic background of individual plants and populations can be derived from linkage that is observed between markers.

1.9 QTL analysis

Genetic factors that are responsible for a part of the observed phenotypic variation for a quantitative trait can be called quantitative trait loci (QTLs). Although similar to a gene, a QTL merely indicates a region on the genome, and could be comprised of one or more functional genes (Falconer and Mackay, 1996). In QTL-mapping the association between observed trait values and presence/absence of alleles of markers is mapped onto a linkage map and analysed. When it is significantly clear that the correlation that is observed did not result from some random process, it is proclaimed that a QTL is detected. Also the size of the allelic effect of the detected QTL can be estimated. A breeder can analyse QTL occurrences and use this knowledge to his advantage, for instance by using indirect selection. When selection is (partly) based on genetic information retrieved through the application of molecular markers this is called marker-assisted selection.

1.10 Marker-assisted selection

Marker-assisted selection (MAS), sometimes also called marker-aided selection, is a relatively new tool for plant breeders. In its simplest form it can be applied to replace evaluation of a trait that is difficult or expensive to evaluate. When a marker is found that co-segregates with a major gene for an important trait, it may be easier and cheaper to screen for the presence of the marker allele

linked to the gene, than to screen for the trait of interest. From time to time the linkage between the marker and the gene should then be verified.

When more complex, polygenic controlled traits are concerned, the breeder is faced with the problem of how to combine as many as possible beneficial alleles for the trait of interest at the QTLs that were detected. In this case the breeding material can be screened for markers that are linked to each of the QTLs. Based on such an analysis, specific crosses can be devised for the creation of an optimal genotype, combining beneficial QTL alleles from different loci. This situation, which is the main subject of this thesis, could also be called *marker-assisted breeding*.

Marker-assisted selection may be used to facilitate a controlled inflow of new genetic material into economically important, agronomically elite, genetic backgrounds. "Wild" or "Un-adapted" exotic germplasm material often carries desired components that may be missing in cultivated material. Such components can be transferred to elite cultivated material by repeated backcrossing. In a backcross program, the presence of the desired QTL alleles can be verified continuously by observing linked markers. At the same time, and with little extra effort, markers can provide information on the origin of the remainder of the genome, allowing selection within the backcross material for individual genotypes that have lost the majority of unwanted donor parent DNA in genomic regions that are not associated with the trait being introgressed. Usually the application of this *marker-assisted backcross* procedure will also result in a reduction of the number

of backcross generations that are required, thereby speeding up the breeding program if the time required for the marker data collection does not delay generation advance.

Objectives of investigation

1. Selection of S35 BC_3F1 genotypes and ICSV111 BC_3F_1 genotypes for the presence of markers linked to stay-green QTL (foreground selection) from donor B35.

2. Among the selected BC_3F_1 genotypes with donor marker alleles linked to stay-green QTL, identification of genotypes with minimal donor alleles in genomic regions more distant from these target QTL (background selection).

3. Backcrossing selected BC_3F_1 individuals to advance them to the BC_4F_1 generation.

4. Selection of S35 BC_4F_1 genotypes and ICSV111 BC_4F_1 genotypes for the presence of donor markers linked to stay-green QTL (foreground selection).

5. Among the selected BC_4F_1 genotypes with donor marker alleles linked to stay-green QTL, identification of genotypes with minimal linkage drag (background selection).

CHAPTER II

Review of Literature

This section gives an overview of the different topics dealt with in the study of marker-assisted selection of the stay-green trait in sorghum.

2.1 Stay-green character-characteristics and gene action

Tenkouano et al. (1993) investigated the inheritance of charcoal rot resistance directly, by exposure of sorghum to *Macrophomina phaseolina*, and indirectly, by determination of the inheritance of non-senescence. They evaluated diallel crosses between two non-senescent, charcoal rot resistant inbred lines (B35 and SC599-11E) and two senescent, charcoal rot susceptible inbreds (BTx378 and BTx623) under controlled field conditions. They determined that non-senescence was regulated by dominant and recessive epistatic interaction between two non-senescence-inducing loci and a third locus with modifying effects. They also concluded that non-senescence and charcoal rot resistance are not different manifestations of a single trait, i.e., they are not to be evaluated with each other.

Walulu et al. (1994) studied the mode of gene action for the stay-green trait in sorghum. F_1 and F_2 backcrosses obtained from a cross

between B35 (stay-green trait donor) and Tx7000 (drought sensitive) were subjected to moisture stress at the grain filling period in the field and rainout shelters. Stay-green was evaluated on an individual plant basis scoring visual leaf and plant death. Their results suggested that a major gene influences this stay-green trait in B35, and that this gene exhibits varied levels of dominant gene action depending on the environment in which the evaluation is made. The frequency distributions of the field-grown BC_1F_1 population indicated complete dominance of this single major gene. Van Oosterom and coworkers found similar results in 1996.

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

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

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

Borrell and Hammer (2000) observed that when sorghum hybrids were grown under terminal water deficit conditions, stay-green could be viewed as a consequence of the balance between nitrogen demand by the grain and nitrogen supply during grain filling. More nitrogen is allocated to the leaves of stay-green hybrids compared with their senescent counterparts, resulting in higher specific leaf nitrogen (SLN) levels. It is

hypothesised that this higher SLN initiates a chain of responses, including enhanced radiation use efficiency (RUE) and transpiration efficiency (TE), which enable the plant to set a higher yield potential by the time of anthesis, ultimately leading to higher grain yield potential and lodging resistance under post-anthesis drought stress conditions. Earlier, Sinclair and Horie (1989) in maize and Muchow and Sinclair (1994) in sorghum demonstrated the positive correlation of photosynthetic capacity with specific leaf nitrogen (SLN).

Borrell et al., (1999, 2000) reported that stay-green and yield were positively associated in sorghum in a range of studies conducted in both Australia and India, highlighting the value of retaining green leaf area under conditions of post-anthesis drought stress. Grain yield is the product of grain number and grain size. Grain number is generally the main determinant of differences in grain yield, and this has also been observed for sorghum, grown under post-anthesis drought stress in southern India (Borrell et al., 1999).

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

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

Wanous et al. (1991) reported that visual ratings for the percentage of values under drought stress. Xu et al. (2000) measure sorghum foliage chlorophyll content with chlorophyll meter (SPAD values) and a spectrophotometric method. The SPAD value had a significant linear relationship with total leaf chlorophyll (R^2=0.91) and with visual stay-green rating (R^2=0.82).

2.2 DNA markers are used to construct genetic linkage maps in sorghum

Linkage maps of organisms, to map genomic regions controlling qualitative and quantitative traits, to exercise indirect selection for several agronomic traits and to isolate the genes involved based on their map position. Utility of molecular markers in genetics and plant breeding is depicted in the figure-2 below:

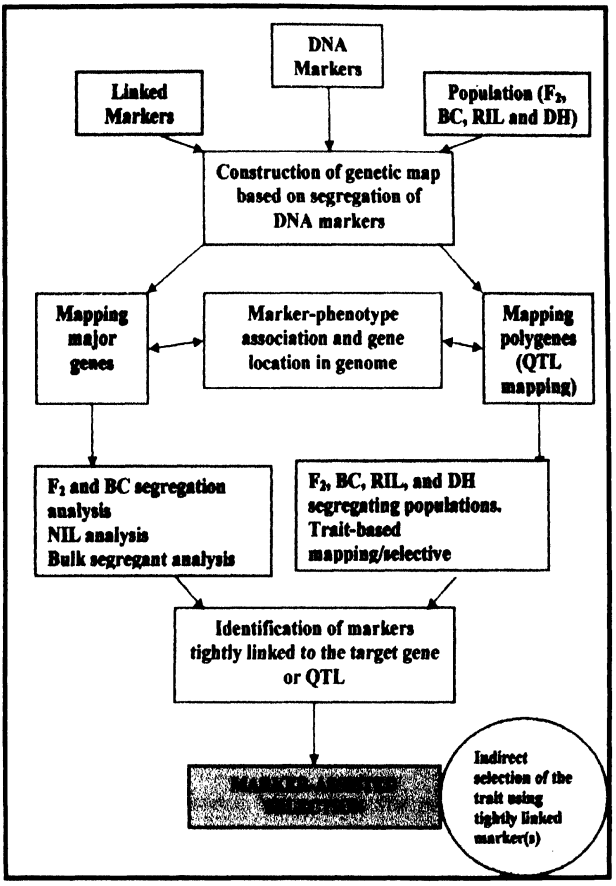


Figure 2. Stages of molecular marker utilization in genetics and plant breeding.

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

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

Kong et al. (2000) also constructed an integrated sorghum linkage map with RFLP and SSR markers using the recombinant inbred lines

derived from the cross between BTx623 and IS 3620C. The markers were distributed across the 10 sorghum linkage groups (LG), covering 1287.2 cM of the sorghum genome (based on LOD >5.0).

2.3 Mapping the stay-green trait in sorghum

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

1974; Soller and Beckmann, 1983; Edwards et al., 1987a, 1987b; and Cowen, 1988). Like wise, the use of flanking marker loci for QTL identification has suggested by Lander and Botstien (1989) and Knapp et al.

The development of molecular marker technologies and the use of these markers in detecting and mapping quantitative trait loci has become a powerful approach for the studying the genetic and phenotypic basis of complex traits (Edward et al., 1987a, 1987b; Paterson et al., 1988; Williams et al., 1992). If individual genetic components associated with a complex trait can be identified, then research can focus on the function of each locus independently without the confounding effects of other segregating loci (Dorweiler et al., 1992; Yand 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 has lead to a better understanding of the inheritance of this trait in sorghum (Tuinistra et al., 1996, 1997, 1998; Crasta et al., 1999; Tao et al., 2000; Xu et al., 2000b; Subudhi et al., 2000; Kebede et al., 2001; Sanchez et al., 2002; Haussmann et al., 2002).

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 QTL underlying a quantitative trait. Detecting marker-QTL associations can be carried out by means of likelihood ratio tests that involve the use of a pair of markers bracketing a QTL, a procedure termed "interval mapping"(Jensen, 1989; Knapp et al., 1990; Lander and Botstein, 1989; Weller, 1987) although simpler approaches are possible (Haley and Knott, 1992; Thoday, 1961; Weller, 1987).

Tuinstra et al. (1996) identified the QTL associated with post flowering drought tolerance in sorghum using 98 RILs derived from the cross between RTx7078 (pre-flowering drought tolerant, post-flowering drought sensitive) and B35 (pre-flowering drought sensitive, post-flowering drought tolerant). This population was genotyped with 150 RAPD and 20 RFLP markers and a linkage map was constructed using Mapmaker/QTL. They identified six genomic regions specifically associated with pre-flowering drought tolerance and also mapped eight additional regions

generally associated with yield or yield components under fully-irrigated conditions.

Tuinstra et al. (1997) identified the genomic regions (QTL) associated with post-flowering drought tolerance and for potentially related components of grain development. They used the same set of 98 RILs as a mapping population derived from the cross between RTx7078 (pre-flowering drought tolerant, post-flowering drought sensitive) and B35 (pre-flowering drought sensitive, post-flowering drought tolerant). They identified 13 genomic regions associated with one or more measures of post-flowering drought tolerance. Two QTLs were identified with major effects on yield and "stay-green" under post-flowering drought stress conditions. These loci were also associated with yield under fully-irrigated conditions, suggesting that these drought tolerance loci have pleiotropic effects on grain yield under non-stress conditions. In this population, several different QTL were associated with expression of the stay-grain trait. Stay-green QTL on linkage group F was positively associated with yield under fully irrigated conditions and the stay-green QTL on linkage group G was weakly associated with yield under fully irrigated conditions, suggesting the tolerance mechanism controlling yield and stay-green under

post-flowering drought also influences yield under fully irrigated conditions when the differences in stay-green were not expressed. QTL analysis also indicated an association between stay-green and rate of grain development at locus on linkage group 11. The stay-green associated with low rate of grain fill.

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

Crasta et al. (1999) developed a QTL map using 142 RFLP markers from a set of RILs obtained from the cross between B35 and elite pollinator line RTx430. These RILs and their parental lines were evaluated for post-flowering drought tolerance and maturity in different environments. By

using simple interval mapping they identified seven stay-green QTLs and two maturity QTLs. Out of seven stay-green QTLs detected, three major QTLs (SGA, SGD and SGG) contributed 42% of observed phenotypic variability (LOD 9.0) and 4 minor QTLs (SGB, SGI.1, SGI.2 and SGJ) significantly contributed an additional 25% of observed phenotypic variability in stay-green ratings.

Tao et al. (2000) evaluated 160 RILs, derived from the cross between QL 39 and QL 41, as a segregating population for genome mapping of the stay-green trait. They added 118 additional markers, including 17 SSR markers and 101 RFLP markers, to a previously published linkage map (Tao et al., 1998) by using same RIL population. In total they identified five genomic regions associated with the stay-green trait. They also confirmed these results by composite interval mapping with inclusion of QTL × Environment interaction.

Xu et al. (2000b) mapped QTLs controlling the stay-green trait and chlorophyll content in sorghum using as a mapping population 98 F_7 RILs derived from the cross B35 × RTx7000. They identified four stay-green (*Stg*) QTLs located on three linkage groups. Two stay-green QTLs, *stg*1

and *stg*2, are located on sorghum linkage group (LG) A, which corresponds to chromosome 3 on the Klein bin map The other two stay-green QTLs detected are located on LG D (chromosome 2) and LG J (chromosome 10), respectively. They estimated that stay-green QTL *stg*1 and *stg*2 account for 13-20% and 20-30%, respectively, of observed phenotypic variability for this trait in this RIL mapping population. They also identified three QTLs for chlorophyll content (*chl*1, *chl*2 and *chl*3) that together explained 25-30% of the observed phenotypic variability. The genomic regions corresponding to *stg*1 and *stg*2 contain an ABA responsive gene and genes for key photosynthetic enzymes and heat shock proteins.

Subudhi et al. (2000) assessed the consistency of QTLs controlling the stay-green trait in sorghum across several genetic backgrounds and environments. They evaluated the RIL mapping population from the cross B35 × Tx7000. The map of the (B35 × Tx7000) derived RIL population (Xu et al., 2000) was expanded by the addition of 91 markers (RFLP, SSR and RAPD markers). They mapped four stay-green QTLs and identified that there are partial similarities in case of the QTLs detected on LG A and LG B of the (B35 × RTx7000)-derived population and the (B35 × RTx7078)-derived population previously reported by Tuinstra et al. (1997).

The nomenclature of the stay-green QTLs first used by Xu et al. (2000) (i.e., *stg*1, *stg*2, *stg*3 and *stg*4) was adopted as standard.

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

Kebede et al. (2001) identified the genomic regions associated with post-flowering drought tolerance (stay-green) using RFLP markers and an F_7 RIL population derived from the cross SC56 × RTx7000. The genetic linkage map for this RIL population covers 1355 cM for the sorghum genome and consists of 144 loci. Nine QTLs, distributed across seven of the ten linkage sorghum groups, were detected for stay-green in several environments using the method of composite interval mapping. They also identified the three QTLs present on sorghum LG A (chromosome 1), LG G (chromosome 7), and LG J (chromosome 10) that were consistently

detected across different terminal drought stress environments. They also conducted comparative mapping studies, identifying that two of the sorghum stay-green QTLs identified in their study correspond to stay-green QTLs detected in maize. In addition to this, QTLs responsible for sorghum lodging tolerance and pre-flowering drought tolerance were detected.

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

Sanchez et al. (2002) reported on four QTL associated with the stay-green trait using a RIL population derived from the cross B35 × RTx7000, and linkage maps well covered with RAPD, SSRs and RFLP markers. These four major QTLs [previously reported by Crasta et al. (1999), Xu et

al. (2000), and Subudhi et al. (2000)] were consistently identified in all field trials and accounted for 53.5% of the phenotypic variance for the stay-green trait.

Haussmann et al. (2002) developed a QTL map for the stay-green trait in sorghum using two recombinant inbred populations (RIP1 and RIP2) based on donor parent E36-1. The mode of gene action for the stay-green in their investigation ranged from purely additive to over dominance. Three QTLs on LG A (chromosome 1), LG E (chromosome 5) and LG G (chromosome 7) were common to both RIP1 and RIP2. These three QTLs from donor parent E36-1, along with the four QTLs from donor parent B35, are potential candidates for transfer of the stay-green trait into locally-adapted elite sorghum materials having producer and consumer-preferred grain and fodder quality traits. These findings have provided the basis for an attempt to transfer stay-green QTLs from the selected donor parents to a range of economically important recurrent parents.

2.4 Marker-assisted selection

An important area in which molecular biology is being applied for transfer of traits from exotic donor parents to more elite locally-adapted crop cultivars is marker-assisted selection (MAS). MAS has been

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

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

MAS can be used to pyramid 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 increases greatly in both the seed parent and its hybrids when the overall frequency of resistance alleles in the maintainer line increases.

2.5 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 with

1) larger population size,

2) lower heritability values of the trait, and

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

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

The efficiency of MAS was generally reduced with increasing distance between the markers flanking the target QTL. So, the optimal distance recommended between two flanking markers is about 5–10 cM

(Hospital et al., 1997). The efficiency of marker-assisted selection may be less than that of phenotypic selection in the long-term (Hospital et al., 1997).

Knapp (1998) presented estimates of the probability of selecting one or more superior genotypes by MAS to predict 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 alleles 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 sufficiently robust for successful MAS. Charmet et al. (1999) showed that the accuracy of QTL location determination greatly affects selection efficiency. MAS for QTLs has recently started to be applied to the genetic improvement of quantitative characters 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 via foreground selection. Further, they investigated the combination of foreground and background selection in QTL introgression. Openshaw (1994) determined the population size and marker density required in background selection.

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

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

returns of MAS compared to the phenotypic selection decreases with the cost of genotyping.

Dreher et al.,(2003) at CIMMYT came to some preliminary conclusions on the relative cost effectiveness of conventional breeding methods as compared to MAS for QPM (Quality protein maize) line. When phenotypic screening is simple (in other words, when it is relatively easy to determine whether a given plant variety possesses a given trait, such as a certain grain color), conventional breeding is, and will continue to be, extremely cost-effective. Conversely, when phenotypic screening is expensive, technically difficult, or even impossible, MAS will often be advantageous. MAS offers an alternative that is simple, direct, and very reliable. Often work on diseases like maize streak virus which are strictly quarantined can be carried out using molecular markers. Marker-assisted selection often allows breeders to cut down on the number of seasons needed to produce a desired product. Even a high-end MAS scheme that might run a few thousand dollars more than a conventional scheme is to the additional benefit to farmers when a variety becomes available sooner .

CHAPTER III

Materials And Methods

3.1 Plant material

BC_3F_1 and BC_4F_1 families derived from crosses between S35 and B35: Segregating BC_3F_1 and BC_4F_1 progenies derived from a series of backcrosses between recurrent parent ICSV 111 (= S35) and stay-green trait donor parent B35 were the subject of this study. [Figure 3(a) and 3(b) for an overview of the backcrossing program].

3.2 Short description of parental lines used in the backcross program:

B35 is a BC1 derivative of landrace germplasm accession IS 12555, which is a durra race sorghum from Ethiopia (Rosenow et al., 2003). It is a well characterized source for the stay-green component of tolerance to terminal drought stress; several different research groups from the USA and Australia have identified a number of stay-green QTLs based on RIL mapping populations derived from crosses involving this line or its derivatives . Based upon a limited SSR-based genetic diversity study recently conducted at ICRISAT-Patancheru (Folkertsma et al., in preparation), B35 appears to be genetically quite diverged from the elite recurrent parents used in this study, which facilitates its use in marker-assisted breeding programs. It is also phenotypically divergent from many Asian and African elite sorghum open-pollinated varieties and hybrid parental

lines. It is potentially "yield resistant" due to its short plant height, small panicle size, and low grain number per panicle.

S 35 This is a selection from ICSV 111, which has been released in Chad and Cameroon

ICSV 111 This is a pure-line cultivar developed at ICRISAT Asia Center, Patancheru, India through pedigree selection in a three-way cross (SPV 35 x E 35-1) x CS 3541. The parents SPV 35 and CS 3541 are converted photo-insensitive three-gene dwarf zerazera types originating from Ethiopia and Sudan, respectively; while E 35-1 is a zerazera type originating from Ethiopia. ICSV111 is a photo-insensitive, self-pollinated cultivar that flowers in 65-72 days and matures in 100-110 days. The green stalks are slightly sweet and juicy. It is a caudatum type, with white hard grains, thin pericarp and normal endosperm. This open-pollinated sorghum variety has been released in Ghana as 'Kapaala'.

Figure 3(a). Scheme for transfer of stay-green QTLs into elite sorghum lines by marker-assisted selection

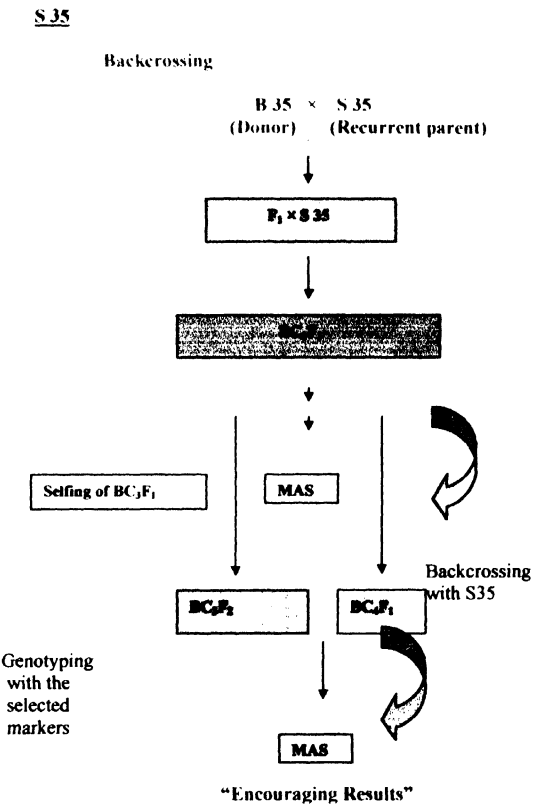
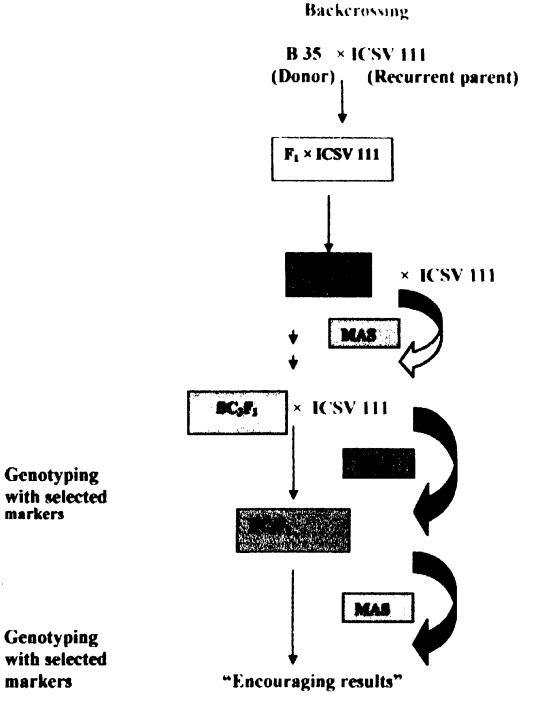


Figure 3(b). Scheme for transfer of stay-green QTL into elite sorghum lines by marker-assisted selection: BC_3F_1 and BC_4F_1 for ICSV 111

Backcrossing

Figure 3(b). Scheme for transfer of stay-green QTL into elite sorghum lines by marker-assisted selection: BC_3F_1 and BC_4F_1 for ICSV 111



3.3 DNA extraction

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

DNA from individual plants of the $BC_3F_1$and BC_4F_1 populations was extracted from one-week-old seedlings by using a modified CTAB method (Saghai-Maroof et al., 1984). 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 protocol (Mace et al., 2004) was employed.

3.4 96-well plate mini DNA extraction

A. Preparation and processing

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, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.17% β-mercaptoethonol) was pre-heated in +65°C water bath before start of sample collection.

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

B. Grinding and extraction

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

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

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

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

C. Solvent extraction

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

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

D. Initial DNA precipitation

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

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

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

E. RNase treatment

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

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

F. Solvent extraction

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

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

G. DNA precipitation

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

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

II. Ethanol wash

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

I. Final re-suspension

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

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

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

3.6 Checking DNA quality and DNA concentration

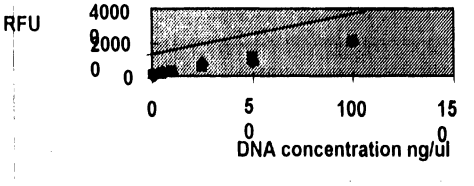
The DNA quality was checked using 0.8% agarose gel. 1 µl of DNA solution was mixed with the 1 µl of orange dye and 4 µl of distilled water and loaded in to wells on 0.8% agarose gel. The gel was run for 10 min. after which the quality was checked under UV. A smear of DNA indicated poor quality

whereas a clear band indicated good quality. Samples of poor quality were re-extracted.

The DNA concentration of each sample was assessed using the Spectrafluor Plus Spectrophotometer by staining DNA with Picogreen™ (1/200 dilution). Based on the Relative Fluorescence Units (RFU) values and using the standard graph, DNA concentrations were calculated. The DNA was diluted to a final concentration of 2.5 ng/µl. Figure 4 represents a calibration graph where

DNA concentration = -2.78273 + 0.002019*RFU

Figure 4. Standard graph expressing the correlation between RFU and DNA concentration.



3.7 Selection of the markers

SSR markers linked to QTLs for stay-green on various linkage groups [Figure-5 and Figure-6 for an overview of the staygreen QTLs in B35 parent] were used for foreground selection to select the individuals presumably having the donor allele (foreground selection) at a particular target stay-green QTL.

Figure 5: Consensus map of Stay-green QTL in B35 donor parent

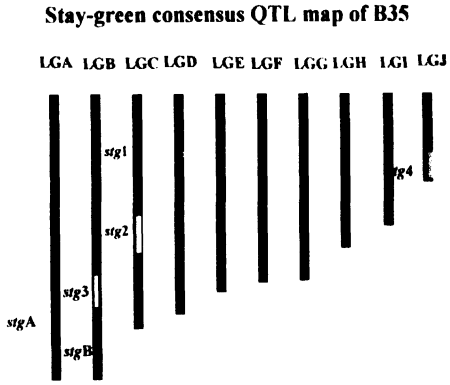
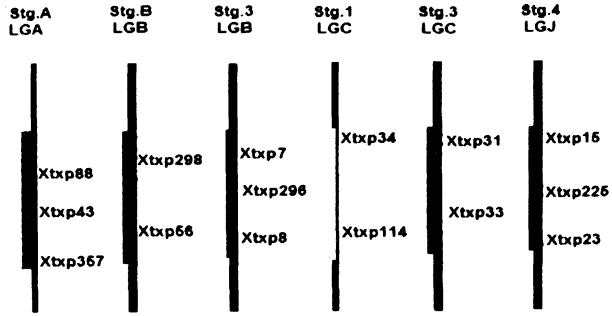


Figure 6: SSR markers linked to Consensus stay-green QTL mapped in donor parent B35



is flanked by these marker loci. The tighter the markers are linked to the QTL, the greater the chance that the QTL mapped between a pair of flanking markers has indeed been transferred (but determination of this is out side the scope of the project). Therefore, phenotypic testing of the final products of the MAB exercise need to be performed in order to confirm the transfer of stay-green QTL. At the same time selected markers unlinked to stay-green have been used to select those individuals with minimal linkage drag (background selection)

3.8 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.

- Composition of reaction mixture-
- 10 mM Tris-HCl (pH 8.3),
- 50 mM KCl, 1.25-2.5 ng of DNA,
- 2 pM of forward and reverse primer,
- 1 mM $MgCl_2$,
- 80-100 µM of each dNTP and
- 0.1 units of Taq DNA polymerase.

The touch down PCR program consisted of an initial denaturation for 15 min at +94°C and then [10 cycles of denaturation for 10 sec at +94°C, annealing at 61-52°C for 20 sec, the annealing temperature for each cycle is reduced with 1°C, and extension at +72°C for 30 sec], 35 cycles [denaturation for 10 sec at +94°C, annealing at +54°C for 20 sec and extension at +72°C for 30 sec]. The last PCR cycle is followed by a 20 min extension at +72°C to ensure amplification to equal length of both DNA strands.

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

3.9 Non-denaturing PAGE (poly acryl amide gel electrophoresis)

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

52.5 ml of doubled distilled water

7.5 ml of 10 X TBE buffer

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

450 µl of Ammonium Per Sulphate (APS) and

100 µl of TEMED.

75 ml total

Along with the samples, a standard 100 bp marker ladder (50 ng/µl) was also loaded in the first and last lane of the gel to ensure proper sizing of amplified

PCR fragments. Most of the markers used allowed clear differentiation of donor and recurrent parent alleles. The gel was run at 550 V of constant power in 0.5X TBE buffer for 3 hours using a Bio-Rad gel sequencing apparatus.

3.10 Silver staining

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

Sequential steps involved in silver staining

The gel was treated as follows:-

1. Water for 5 min.
2. 0.1% CTAB solution for 20 min (2 gm in 2 lit of water)
3. 0.3% ammonia solution for 15 min. (26 ml of 25% ammonia solution in 2 lit of water)
4. 0.1% silver nitrate solution for 15 min (2gm of silver nitrate + 8 ml of 1M NaOH in 2 lit of water and add ammonia solution up to the solution becomes colorless)
5. Developer (30 gm of Sodium carbonate + 400µl of Formaldehyde in 2 lit of water)

After developing the gels were rinsed in water for 1 min and placed in fixer (30 ml Glycerol in 2 lit of water) for less than a minute.

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

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

3.11 Data collection and analysis

3.11.1 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 (S 35, ICSV 111), "B" was defined as the presence of allele from B 35, "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.

CHAPTER IV

Results

4.1 Checking quality and quantity of DNA samples

After isolating the DNA, the samples were loaded into 0.8% agarose gels, along with the standards, for checking the DNA quality and quality. If the bands were clear, without any smear, this indicated good quality DNA. If they showed any smear, DNA was re-extracted for those samples. DNA concentrations were assessed with the Spectrafluor Plus Spectrophotometer using Picogreen™. The figure-7 below shows the quality, and gives an indication of the quantity, of DNA of samples prepared for BC_3F_1 progenies involving recurrent parent S35.

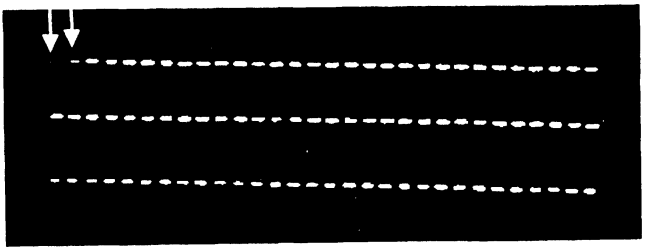


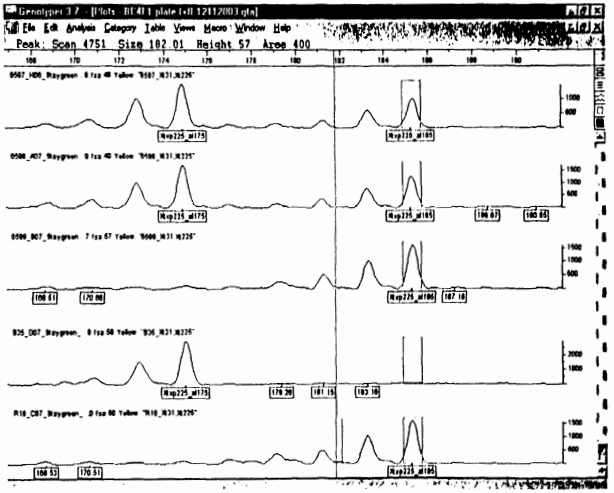
Figure7- Test of DNA quality on 0.8% agarose gel with 50 ng and 100 ng markers

Likewise DNA quality and quantity was assessed for samples of all generations and dilutions were made accordingly to reach final concentrations of 2.5 ng/µl, which was then used as template in PCR reactions.

PCR was done with selected primers for both foreground and background selection for all generations. After a set of PCR reactions was completed, PCR products were loaded onto 6% PAGE gels and separated electrophoretically. Following silver staining of the PAGE gels, the bands were scored and compared with the parental alleles and with a 100 bp marker ladder as a reference for allele size.

For the primers whose product size difference is below 5 bp, scoring was done employing the ICRISAT Applied Genomics Laboratory's ABI Prism 3700 (Perkin Elmer) automated DNA sequencer, which is based on the principle of capillary electrophoresis. PCR is done using primers that are fluorescently-labeled. Figure-8 gives an insight about the scoring of output from the ABI sequencer for the marker *Xtxp*225 in the S 35 BC_4F_1 generation.

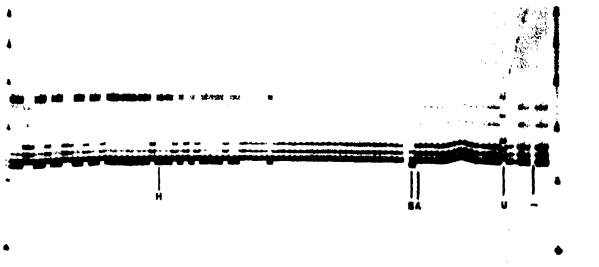
Figure 8-Graphical representation (ABI Prism 3700 chromatogram) of the PCR products of the primer pair for sorghum SSR marker locus *Xtxp225* for the BC4F1 generation on introgression of stay-green QTLs from donor parent B35 and recurrent parent S 35.



4.2 RECURRENT PARENT S35

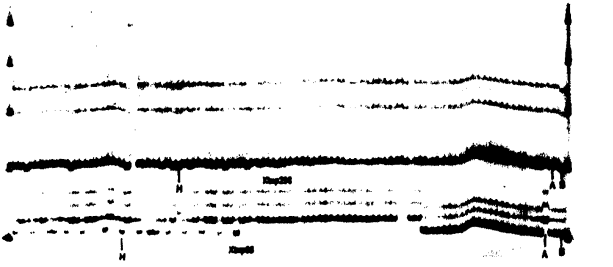
For recurrent parent S35, a total of 14 foreground markers and 16 background markers were screened for targeting QTLs controlling the stay-green trait (Table-1 for foreground and Table-1a for background markers used for the S35 crosses). In the BC_3F_1 generation, a total of 18 populations and in the BC_4F_1 generation a total of 11 populations were genotyped and selected individuals advanced by selfing and backcrossing with pollen from recurrent parent S 35. Figure-9 shows the PAGE gel for the SSR marker alleles of B35 and S 35 at locus *Xtxp*07.

Figure 9. PAGE gel for SSR locus *Xtxp07* with 100 bp ladder for a set of BC_3F_1 progenies based on donor parent B35 and recurrent parent S 35.



Marker *Xtxp07*; Allele sizes: B35/S 35 222/233; A = S 35 homozygote; B = B35 homozygote; H = heterozygote

Figure 10. PAGE gel for the SSR loci *Xtxp88* and *Xtxp298* for a set of BC_3F_1 progenies based on donor parent B35 and recurrent parent ICSV 111 (post-PCR multiplexing).



Markers *Xtxp88Xtxp298*; Allele sizes for *Xtxp88*: B35 = 102, ICSV 111 = 139

Table 1. List of markers used for screening backcross populations based on donor parent B35 and recurrent parent S 35.

Marker	Target QTL	Product size for B35	Product size for S 35
*Xtxp*88	*stg*A	102	120
*Xtxp*43	*stg*A	144	162
*Xtxp*357	*stg*A	276	274
*Xtxp*7	*stg*B	222	233
*Xtxp*296	*stg*B	164	168
*Xtxp*114	*stg*1	230	233
*Xtxp*34	*stg*1	331	328
*Xtxp*31	*stg*2	233	209
*Xtxp*33	*stg*2	223	242
*Xtxp*298	*stg*3	203	209
*Xtxp*56	*stg*3	323	–
*Xtxp*15	*stg*4	220	215
*Xtxp*23	*stg*4	183	174
*Xtxp*225	*stg*4	161	163

TABLE-1(a) List of background markers used for selection of recurrent parent S35

Linkage group	Marker used	Size for B35	Size for S35
A	Xtxp248	212	200
B	Xtxp19	284	330
C	Xtxp9	116	145
D	Xtxp12	195	175
	Xtxp21	168	174
E	Xtxp40	131	137
	Xtxp295	167	175
F	Xtxp289	280	330
	Xtxp230	195	208
G	Xtxp141	151	133
	Xtxp20	204	181
H	Xtxp210	200	184

	Xtxp47	300	290
I	Xtxp57	246	241
	Xtxp06	130	125
J	Xtxp65	140	135

4.3 Recurrent parent ICSV 111

For recurrent parent ICSV 111, a total of 8 foreground markers and 13 background markers were selected for screening the targeted QTLs controlling the stay-green trait (Table-2 for list of foreground markers and Table-2a for list of background markers used for ICSV111 crosses). In the BC_3F_1 generation, a total of 7 populations were screened and selected individuals advanced by selfing and by backcrossing emasculated florets with pollen from recurrent parent ICSV 111. Figure-10 below shows the PAGE gel for the post-PCR multiplexing of *Xtxp*298 and *Xtxp*88.

Table 2. List of markers used for screening backcross populations based on donor parent B35 and recurrent parent ICSV 111.

Marker	Target QTL	Product size for B35	Product size for ICSV 111
*Xtxp*88	*Stg*A	102	139
*Xtxp*43	*Stg*A	144	162
*Xtxp*114	*stg*1	230	233
*Xtxp*34	*stg*1	331	328
*Xtxp*31	*stg*2	233	209
*Xtxp*33	*stg*2	223	215
*Xtxp*298	*stg*3	203	209
*Xtxp*56	*stg*3	323	330

Table- 2(a) List of markers used for background selection of the recurrent parent ICSV111

Linkage group	Marker used	Size for B35	Size for S35
A	Xtxp248	212	257
B	Xtxp25	132	150
C	Xtxp9	116	160
D	Xtxp12	132	150
	Xtxp21	168	174
E	Xtxp295	167	173
F	Xtxp289	280	300
	Xtxp230	195	210
G	Xtxp141	151	145
	Xtxp20	204	217
H	Xtxp210	200	208
I	Xtxp57	246	254
J	Xtxp65	140	130

Table-3 Part of scoring sheet for the S 35-BC_3F_1 generation

	*stg*A			*stg*B		*stg*3		*stg*4		
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*357	*Xtxp*7	*Xtxp*296	*Xtxp* 56	*Xtxp* 298	*Xtxp* 15	*Xtxp* 225	*Xtxp* 23
6601	H	H	H	H	H	A	A	–	H	H
6602	H	H	H	A	A	A	H	H	H	H
6603	H	H	H	A	A	–	A	H	H	H
6604	B	B	B	H	H	A	A	–	B	A
6605	H	H	H	H	H	–	H	H	H	H
6606	B	B	B	H	B	–	A	H	H	H
6607	H	B	H	H	A	A	H	H	H	H
6608	H	H	B	A	A	–	A	A	A	H
6609	H	B	H	H	H	–	H	–	A	A
6610	H	B	H	H	H	A	A	A	A	A
6611	H	B	B	H	A	A	A	H	H	H
6612	H	H	H	A	A	A	H	–	A	A
6613	H	H	H	H	A	–	H	H	H	–
6625	A	U	A	H	A	A	H	H	A	A
6626	A	A	A	H	A	H	H	A	A	A
6627	A	A	A	H	H	B	H	H	H	–
6628	A	A	A	A	A	A	A	H	H	A
6629	A	A	A	H	A	H	H	H	H	A
6630	A	A	A	H	H	H	H	A	A	A
6631	A	A	A	A	A	H	A	–	A	A
6632	A	A	A	H	A	H	H	A	A	A
6633	A	A	A	H	H	A	H	H	H	A
6634	A	A	A	A	H	–	A	H	H	H

* Note: H= Heterozygote; A = S 35 allele homozygote; B = B35 allele homozygote; U = Off-type; – = Missing datapoint

Contd.. Table-3 Part of scoring sheet for the S 35-BC_3F_1 generation

	*stg*A			*stg*B		*stg*3		*stg*4		
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*357	*Xtxp*7	*Xtxp*296	*Xtxp* 56	*Xtxp* 298	*Xtxp* 15	*Xtxp* 225	*Xtxp* 23
6635	A	U	A	A	A	A	H	H	H	A
6636	A	A	–	A	A	A	H	–	A	A
6637	A	A	A	A	H	A	H	A	A	A
6638	A	A	A	H	A	A	H	A	A	A
6639	A	A	A	H	A	B	H	–	H	A
6640	A	A	A	A	H	B	A	H	H	A
6649	H	H	H	A	H	B	H	H	H	A
6650	H	H	H	A	H	A	H	H	H	H
6651	H	H	H	A	H	A	H	A	A	A
6652	H	H	H	H	H	A	H	–	H	H
6653	H	H	H	H	H	H	H	H	H	H
6654	H	H	H	A	H	A	H	A	A	A
6655	H	H	H	A	H	A	H	A	A	H
6656	H	H	H	A	H	A	H	A	H	A
6657	H	H	H	H	H	A	H	A	A	A
6658	H	H	H	A	H	A	H	H	H	A
6659	H	H	H	A	H	A	H	H	H	H
6660	H	H	H	–	H	–	H	–	A	H
6661	H	H	H	A	H	A	H	H	H	H
6662	H	H	H	A	H	B	H	H	H	H
6663	H	H	H	–	H	–	?	–	A	A
6664	H	H	H	–	H	–	H	H	H	H
6665	H	H	H	H	H	B	A	H	H	H

Contd.. Table-3 Part of scoring sheet for the S 35-BC_3F_1 generation

	*stg*A			*stg*B		*stg*3		*stg*4		
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*357	*Xtxp*7	*Xtxp*296	*Xtxp* 56	*Xtxp* 298	*Xtxp* 15	*Xtxp* 225	*Xtxp* 23
6672	A	A	H	H	A	–	H	H	A	A
6673	H	H	H	A	A	A	H	H	A	H
6674	A	A	A	A	A	A	H	A	A	H
6675	A	A	H	A	A	A	?	H	A	A
6676	A	A	A	A	A	B	A	A	A	A
6677	A	A	A	A	A	A	A	–	A	H
6678	H	U	H	A	A	A	–	A	A	H
6679	A	A	A	A	A	A	H	H	A	H
6680	H	A	H	A	A	U	H	A	A	A
6681	H	H	B	A	A	A	A	H	A	H
6682	H	H	H	A	A	H	A	–	A	H
6683	H	H	H	A	A	A	H	A	A	A
6684	A	A	A	A	A	A	?	H	A	A
6685	B	B	B	A	A	A	A	–	A	H
6686	H	A	A	A	A	A	H	H	A	H
6687	A	A	A	A	A	B	A	H	A	H
6688	U	U	U	A	U	H	B	A	U	B
6697	A	A	A	A	A	A	H	A	A	H
6698	H	H	A	A	A	A	H	–	A	H
6699	A	A	A	A	A	A	?	H	A	H
6700	H	H	H	A	A	A	A	A	A	H
6701	H	H	A	–	A	U	A	–	A	H
6702	A	A	A	A	A	A	A	H	A	H

Contd.. Table-3 Part of scoring sheet for the S 35-BC_3F_1 generation

	*stg*A			*stg*B		*stg*3		*stg*4		
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*357	*Xtxp*7	*Xtxp*296	*Xtxp* 56	*Xtxp* 298	*Xtxp* 15	*Xtxp* 225	*Xtxp* 23
6703	A	A	A	A	A	A	?	A	A	H
6704	A	A	H	A	A	–	H	A	A	H
6705	A	A	A	A	A	A	H	A	A	A
6706	A	A	A	A	A	A	A	–	A	H
6708	H	H	H	A	A	A	H	A	A	H
6709	H	H	H	A	A	A	U	A	A	A
6710	H	H	H	A	A	A	B	–	B	B
6711	A	A	H	A	A	A	B	H	B	H
6732	A	A	A	A	A	A	H	H	H	H
6733	–	H	H	A	A	A	H	A	A	A
6734	H	H	H	A	A	A	A	H	A	H
6735	H	–	H	A	A	A	A	–	A	H
6736	A	A	A	A	A	A	A	A	H	A
6737	A	A	H	A	A	A	H	H	H	H
6738	A	A	H	A	A	A	H	–	H	H
6739	H	H	H	A	A	A	–	H	H	H
6740	H	H	H	A	A	A	A	A	A	A
6741	H	H	H	A	A	A	H	A	A	A
6742	H	H	H	A	A	A	A	H	A	H
6743	A	A	A	A	A	A	B	A	A	A
6744	A	A	A	A	A	A	A	A	H	A
6745	A	A	A	A	A	A	H	A	A	A
6746	A	A	A	A	–	A	H	–	H	H

Contd.. Table-3 Part of scoring sheet for the S 35-BC_3F_1 generation										
	*stg*A			*stg*B		*stg*3		*stg*4		
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*357	*Xtxp*7	*Xtxp*296	*Xtxp* 56	*Xtxp* 298	*Xtxp* 15	*Xtxp* 225	*Xtxp* 23
6747	A	H	A	A	A	A	A	A	A	A
6748	A	A	A	A	A	A	H	H	H	H
B35-1	B	B	B	B	B	B	B	B	B	B
S 35-1	A	A	A	A	A	A	A	A	A	A

Table-4 Part of scoring sheet for the S 35-BC3F1 generation								
	*stg*B		*stg*1		*stg*2		*stg*4	
Plant No.	*Xtxp*7	*Xtxp*296	*Xtxp* 34	*Xtxp* 114	*Xtxp*31	*Xtxp*33	*Xtxp*15	*Xtxp*23
7282	A	A	H	H	H	H	A	–
7283	H	H	H	H	H	H	A	H
7284	A	U	A	A	A	A	B	B
7285	A	A	H	H	H	H	A	H
7286	A	A	H	H	A	A	A	A
7287	H	–	–	–	H	A	A	A
7288	H	H	A	A	A	A	A	A
7289	H	H	A	A	H	H	A	H
7290	H	H	H	H	H	H	A	A
7291	H	H	A	H	A	A	A	A
7292	H	H	A	A	A	A	A	A
7293	H	A	H	A	A	A	A	H
7294	A	A	A	A	A	A	A	–
7295	A	–	–	–	A	A	A	A
7296	A	A	H	A	H	H	A	H
7297	H	H	A	B	A	A	A	A
7298	H	H	A	A	A	A	A	A
7299	A	A	H	H	H	H	A	H
7300	H	H	A	A	A	A	A	H
7301	H	H	H	A	A	A	A	H
7302	H	A	A	A	A	H	A	A

Contd. Table-4 Part of scoring sheet for the S 55-BC3F1 generation

	*stg*B		*stg*1		stg2		*stg*4	
Plant No.	*Xtxp*7	*Xtxp*296	*Xtxp* 34	*Xtxp* 114	*Xtxp*31	*Xtxp*33	*Xtxp*15	*Xtxp*23
7303	H	H	H	H	H	H	A	H
7304	H	H	A	A	A	A	A	A
7305	H	H	H	B	H	H	A	H
7327	H	H	A	A	H	H	A	A
7328	H	H	A	A	H	H	A	A
7329	H	H	A	A	A	A	A	A
7330	H	A	A	A	A	H	A	A
7331	A	A	A	A	A	A	A	A
7332	H	H	A	A	A	A	A	A
7333	H	H	A	A	A	H	A	A
7334	A	H	A	A	H	H	A	A
7335	A	A	A	A	A	H	A	A
7336	A	A	A	A	A	A	A	A
7337	H	H	A	A	H	–	A	A
7338	A	H	A	A	H	H	A	A
7339	H	H	A	A	A	A	A	A
7340	H	H	A	A	H	H	A	A
7341	H	H	A	A	H	?	A	A
7342	A	A	A	A	H	H	A	A
7343	H	H	A	A	H	H	A	H
7344	H	H	A	A	B	U	A	A
7345	A	A	A	A	A	A	A	A
7346	H	H	A	A	A	H	A	A
7347	H	H	A	A	A	H	A	A
7348	A	A	A	A	H	H	A	A

Contd.. Table-4 Part of scoring sheet for the S 35-BC3F1 generation

	*stg*B		*stg*1		*stg*2		*stg*4	
Plant No.	*Xtxp*7	*Xtxp*296	*Xtxp* 34	*Xtxp* 114	*Xtxp*31	*Xtxp*33	*Xtxp*15	*Xtxp*23
7351	A	A	H	A	A	U	H	H
7352	A	A	H	H	H	H	H	B
7353	A	A	H	H	H	H	H	–
7354	A	A	H	H	A	A	A	A
7355	A	A	A	A	A	A	A	A
7356	A	A	H	H	H	A	H	H
7357	A	A	H	H	H	H	H	H
7358	A	A	A	A	A	A	B	B
7359	A	A	A	A	A	A	A	A
7360	A	A	H	A	A	A	H	H
7361	A	A	A	A	H	H	H	H
7362	A	A	A	A	A	A	A	A
7363	A	A	A	H	A	A	H	H
7364	A	A	H	H	H	H	A	A
7365	A	A	B	A	A	A	H	H
7366	A	A	H	A	A	A	H	H
7367	A	A	A	A	A	A	H	H
7368	A	A	A	A	A	H	H	H
7369	–	A	A	–	–	–	–	–
7370	A	A	A	A	A	A	A	A
7371	A	–	–	–	H	H	H	H
7372	A	A	A	A	H	A	A	A
7375	A	A	A	A	H	H	A	A
7376	A	A	A	A	H	H	A	A
7377	A	A	A	A	A	A	A	A

Contd.. Table-4 Part of scoring sheet for the S 35-BC3F1 generation

	*stg*B		*stg*1		*stg*2		*stg*4	
Plant No.	*Xtxp*7	*Xtxp*296	*Xtxp* 34	*Xtxp* 114	*Xtxp*31	*Xtxp*33	*Xtxp*15	*Xtxp*23
7378	A	A	A	A	H	H	A	A
7379	U	U	A	A	B	U	B	B
7380	A	A	A	A	H	H	A	A
7381	A	A	A	A	A	A	A	A
7382	A	A	A	A	H	A	A	A
7383	A	A	A	A	A	A	A	A
7384	A	A	A	A	H	A	A	A
7385	A	A	A	A	A	A	A	A
7386	A	A	A	A	A	A	A	A
7387	A	A	A	A	H	A	A	A
7388	A	A	A	A	A	A	A	A
7389	A	A	A	A	–	A	A	A
7390	A	A	A	A	H	H	A	A
7391	A	A	A	A	A	A	A	A
7392	A	A	A	A	A	A	A	A
7393	A	A	A	A	A	A	A	A
7394	A	A	A	A	A	A	A	A
7395	A	A	A	A	A	A	A	A
7396	A	A	A	A	H	H	A	H
7397	A	A	A	A	H	H	A	A
B35-5	B	B	B	B	B	B	B	B
S 35-5	A	A	A	A	A	A	A	A

Table-5 Part of scoring sheet for the S 35-BC4F1 generation						
	*stg*A		*stg*B	*stg*3	*stg*4	
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*07	*Xtxp*298	*Xtxp*15	*Xtxp*23
8601	A	H	A	H	H	–
8602	H	B	A	H	A	–
8603	H	B	A	H	A	A
8604	A	A	A	H	A	A
8605	A	A	A	A	H	H
8606	H	A	A	A	H	–
8607	H	A	A	A	B	–
8608	H	A	A	A	H	B
8609	A	A	A	A	H	B
8610	A	A	A	A	H	H
8611	A	A	A	A	B	A
8612	B	A	A	A	A	–
8613	A	A	A	A	B	A
8614	H	A	A	A	H	–
8615	H	A	A	A	H	A
8616	H	–	A	A	H	A
8617	A	A	A	A	B	A
8618	A	A	A	A	A	A
8619	A	A	A	A	H	A
8620	A	A	A	A	A	–
8621	H	H	A	H	H	H
8622	B	A	A	B	H	H

Contd.. Table-5 Part of scoring sheet for the S 35-BC4F1 generation						
	*stg*A		*stg*B	*stg*3	*stg*4	
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*07	*Xtxp*298	*Xtxp*15	*Xtxp*23
8623	B	H	A	H	H	H
8624	B	A	A	H	H	B
8625	A	H	A	B	H	–
8626	H	–	A	B	H	H
8627	H	H	A	H	H	–
8628	A	A	A	H	A	–
8629	A	A	A	H	A	A
8630	A	A	A	A	A	H
8631	A	A	B	A	A	H
8632	A	A	A	A	A	H
8633	A	A	A	A	A	A
8634	A	A	H	A	A	A
8635	A	A	H	A	A	A
8636	A	A	H	A	A	H
8637	A	A	H	A	A	A
8638	A	A	H	A	A	A
8639	A	A	A	A	A	A
8640	A	A	H	A	A	A
8641	A	A	A	A	A	A
8642	A	A	H	A	A	A
8643	A	A	A	A	A	–
8644	A	A	B	A	A	–
8645	A	A	H	A	A	–
8646	A	A	B	A	A	B
8647	A	A	H	A	A	A

Contd.. Table-5 Part of scoring sheet for the S 35-BC4F1 generation						
	*stg*A		*stg*B	*stg*3	*stg*4	
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*07	*Xtxp*298	*Xtxp*15	*Xtxp*23
8648	A	A	H	A	A	H
8649	A	A	–	A	A	B
8650	A	A	H	A	A	–
8651	A	A	A	A	A	A
8652	A	A	A	A	A	H
8653	B	A	A	A	A	H
8654	B	A	B	A	A	A
8655	B	A	B	A	A	A
8656	A	A	H	A	A	A
8657	B	A	A	A	A	–
8658	B	A	H	A	A	–
8659	A	A	A	A	A	–
8660	A	A	A	A	A	A
8661	B	A	A	A	A	A
8662	B	A	A	A	A	A
8663	H	A	A	A	A	A
8664	A	A	H	A	A	–
8665	B	A	H	A	A	A
8666	A	A	A	A	A	A
8667	H	A	A	A	A	A
8668	B	A	B	A	A	A
8669	H	A	A	A	A	–
8670	B	A	–	A	A	A
8671	H	A	A	A	A	A
8672	A	A	A	A	A	A

Contd.. Table-5 Part of scoring sheet for the S 35-BC4F1 generation						
	*stg*A		*stg*B	*stg*3	*stg*4	
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*07	*Xtxp*298	*Xtxp*15	*Xtxp*23
8673	B	A	A	A	A	–
8674	B	A	A	A	A	A
8675	B	A	A	A	A	A
8676	B	A	H	A	A	A
8683	A	A	H	B	A	A
8684	B	A	H	B	A	A
8685	A	A	A	H	A	–
8686	B	H	B	B	A	A
8687	A	H	H	A	A	A
8688	A	A	A	H	A	A
8689	A	–	A	H	H	A
8690	A	A	B	A	A	A
8691	A	A	H	A	A	–
8692	H	A	A	A	A	–
B35	B	B	B	B	B	B
S 35	A	A	A	A	A	A

Table-6 Part of scoring sheet for the ICSV111-BC3F1					
	stg.A	*stg.2*		*stg.3*	
Marker	*Xtxp88*	*Xtxp31*	*Xtxp33*	*Xtxp298*	*Xtxp56*
7711	A	A	A	A	A
7712	A	H	A	A	A
7713	A	A	A	A	H
7714	A	A	A	A	A
7715	H	A	A	A	A
7716	A	A	B	-	A
7717	B	A	A	A	A
7718	A	A	A	A	B
7719	H	B	A	H	A
7720	A	B	A	A	A
7721	A	A	H	A	A
7722	A	A	A	A	A
7723	A	A	-	H	A
7724	H	A	A	A	A
7725	H	A	A	A	A
7736	H	H	H	H	A
7737	A	A	A	A	A
7738	A	A	H	A	A
7759	H	H	H	A	A
7760	A	H	-	A	B
7761	H	H	H	A	A
7762	A	A	A	H	A
7781	A	A	H	H	A
7782	-	-	-	-	A

Contd.. Table-6 Part of scoring sheet for the ICSV111-BC3F1					
	stg.A	*stg.2*		*stg.3*	
Marker	*Xtxp88*	*Xtxp31*	*Xtxp33*	*Xtxp298*	*Xtxp56*
7783	A	A	A	A	A
7784	A	-	A	A	A
7785	H	A	H	A	H
7786	A	A	A	A	A
7817	A	H	H	A	-
7818	A	B	A	A	A
7819	H	A	-	H	A
7820	A	A	A	A	A
7822	A	A	A	A	A
7823	H	A	A	A	A
7824	A	H	H	H	A
7829	-	A	A	A	H
7830	H	H	A	A	H
7831	-	H	H	H	H
7832	A	B	A	A	H
7833	H	H	A	-	-
7834	A	A	H	H	A
7835	A	-	H	-	H
7836	A	A	A	A	A
7837	-	A	-	H	A
7838	-	A	A	A	A
7839	A	H	A	A	-
7840	A	A	B	A	-
7841	-	H	A	-	B

Marker	*Xtxp88*	*Xtxp31*	*Xtxp33*	*Xtxp298*	*Xtxp56*
7842	A	A	A	-	A
7843	A	H	A	A	A
7844	A	A	A	A	H
7845	H	H	-	-	B
7846	B	A	-	A	A
7847	H	A	H	A	H
7848	A	A	A	A	B
7853	A	H	A	H	A
7854	A	A	A	B	A
7855	A	A	A	H	A
7856	A	-	A	A	H
7857	A	A	A	A	H
7858	A	A	A	A	A
7859	A	A	A	H	H
7860	A	A	A	A	B
7861	A	A	A	A	A
7862	A	A	A	H	H
7863	A	H	-	A	A
7864	B	-	A	H	A
7865	A	A	A	-	B
7866	A	A	B	B	-
B35	B	B	B	B	B
ICSV111	A	A	A	A	A

Table-7 Part of scoring sheet for ICSV 111 BC3F1 generation					
	*stg*A		*stg*2		*stg*3
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*31	*Xtxp*33	*Xtxp*298
8701	H	H	A	A	A
8702	H	H	A	A	A
8703	H	H	A	A	A
8704	H	B	A	A	A
8705	H	A	A	A	A
8706	A	A	A	A	A
8707	H	A	A	–	A
8708	A	B	A	A	–
8709	A	A	A	A	A
8710	A	A	A	–	A
8711	H	H	A	A	A
8712	A	A	A	A	H
8713	A	A	A	A	A
8714	H	H	A	A	A
8715	A	B	A	A	A
8716	B	B	A	A	A
8717	A	A	A	A	H
8718	A	A	A	A	A
8719	A	A	A	A	A
8720	A	A	A	A	A
8721	A	A	A	A	A
8722	A	A	A	A	A
8723	A	A	A	A	H
8724	–	B	H	A	A

Contd..Table-7 Part of scoring sheet for ICSV 111 BC3F1 generation					
	*stg*A		*stg*2		*stg*3
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*31	*Xtxp*33	*Xtxp*298
8725	B	B	H	H	A
8726	B	B	H	A	A
8727	B	B	H	A	A
8728	B	H	H	H	A
8729	B	B	H	H	A
8730	B	B	A	A	A
8731	A	A	H	H	A
8732	A	A	A	A	A
8733	B	H	H	A	A
8734	A	A	A	A	H
8735	A	A	A	A	B
8736	A	A	A	A	B
8737	A	A	A	A	H
8738	A	A	A	–	A
8739	A	A	A	A	H
8740	A	A	A	A	B
8741	A	A	A	A	B
8742	U	A	A	A	H
8743	A	A	A	A	A
8744	A	A	A	A	B
8745	A	A	A	A	A
8746	A	A	A	A	H
8747	A	A	H	–	A
8748	A	A	A	A	A
8749	A	A	H	A	A
8750	A	A	A	A	A

Contd..Table-7 Part of scoring sheet for ICSV 111 BC3F1 generation					
	*stg*A		*stg*2		*stg*3
Plant No.	*Xtxp*88	*Xtxp*43	*Xtxp*31	*Xtxp*33	*Xtxp*298
8751	A	A	–	–	A
8752	A	A	A	B	A
8753	A	A	A	A	A
8754	A	A	A	A	A
8755	A	A	A	A	A
8756	A	A	A	A	A
8757	A	A	B	A	A
8758	A	A	H	H	A
8759	A	A	A	–	A
8760	A	A	H	–	A
8761	A	A	H	A	A
8762	A	A	A	A	A
8763	–	A	H	H	A
8764	A	A	A	A	A
8765	A	A	H	A	A
8766	A	A	H	A	A
8767	A	A	A	A	A
8768	A	A	A	–	A
8769	A	A	A	A	A
8770	A	A	H	A	A
8771	–	B	A	A	A
8772	B	B	A	A	A
8773	B	B	H	–	A
8774	B	B	B	–	A
8775	B	B	A	A	A
8776	B	B	H	A	A

Contd. Table-7 Part of scoring sheet for ICSV 111 BC3F1 generation					
	stgA		*stg2*		*stg3*
Plant No.	*Xtxp88*	*Xtxp43*	*Xtxp31*	*Xtxp33*	*Xtxp298*
8777	B	B	A	–	A
8778	B	B	A	A	A
8779	B	B	A	A	A
8780	B	B	A	A	A
8781	B	B	B	B	A
8782	B	B	B	A	A
8783	B	B	A	–	A
8784	B	B	H	A	A
8785	B	B	A	H	A
8786	B	B	H	A	A
8787	B	B	B	A	A
8788	B	B	A	A	A
8789	B	B	H	A	A
8790	B	B	B	A	A
8791	B	B	B	H	A
8792	B	B	B	–	A
8793	B	B	H	A	A
8794	B	B	A	–	A
8795	B	B	H	H	A
B35	B	B	B	B	B
ICSV 111	A	A	A	A	A

CHAPTER V

Discussion

Using marker-assisted selection, we are able to introgress genomic regions from exotic stay-green donor parent B35 into the genetic backgrounds of elite recurrent parents (S 35 and ICSV 111) over two generations. Markers linked to stay-green QTL regions to be transferred from donor B35 to recurrent parents S 35 and ICSV 111 were used for foreground selection whereas unlinked markers, evenly distributed over genomic regions of the recurrent parent that were to be recovered, were used for background selection. Based on the genotype data, individuals heterozygous (in the BC_3F_1 and BC_4F_1 generation) for markers spanning stay-green QTL regions were selected during the first step of selection (foreground selection). From among the individuals selected in this first step, those with minimal presence of donor alleles at marker loci unlinked to stay-green QTLs were selected during the second step (background selection).

5.1 Selection criteria

Markers, especially foreground markers, were taken into consideration for selection of the individual segregants to be advanced. The

individuals scored as 'A', 'H' or 'B' for markers used in foreground selection and 'A' allele for background markers were selected for generation advance. Individuals scored 'H' at a particular, are expected to produce progeny segregating 1:2:1 for homozygosity for the recurrent parent allele (scored 'A'), heterozygosity (scored 'H'), and homozygosity for the donor parent allele (scored 'B') if they are advanced by selfing, or segregating 1:1 for homozygosity for the recurrent parent allele (scored 'A') and heterozygosity (scored 'H') if they are advanced by backcrossing to the recurrent parent. Presence of the 'A' genotype for background markers and the 'H' genotype for foreground markers flanking a particular stay-green QTL ensures the recovery of the recurrent parent genome (S 35 or ICSV 111) while advancing introgression of a genomic region contributing to the stay-green trait. Individuals meeting these criteria were selected and advanced to next the generation by selfing and backcrossing. Individuals scored 'A' for all (foreground and background) markers should be very similar to the recurrent parent (in fact, they should be identical to the recurrent parent except for small introgressions that were not detected due to the limited genomic coverage possible with the small total number of marker used in this study) and could be selected as controls entries for use in field trials to assess the efficacy of marker-assisted selection for the stay-green trait.

For selected individuals, the markers scored as 'H' or 'B' and those that didn't amplify during the BC_3F_1 generation were screened again in next generation. The markers scored 'A' (i.e., homozygous for the allele of the recurrent parent) are not tested further in more advanced generations because recovery of the recurrent parent genotype at these loci has been completed and their genetic constitution is not expected to change further assuming a negligible rate of mutation and no outcrossing to non-recurrent parent genotypes. Once the recurrent parent genome has been recovered for all the background markers, a generation of selfing and selection for donor parent marker allele homozygotes at loci flanking specific target stay-green QTLs will be conducted, and the selected genotypes then multiplied by selfing and tested multilocationally to evaluate them phenotypically for the stay-green character and other agronomic traits. After testing, if the progeny with the stay-green trait are found to be significantly superior compared with the recurrent parent controls, they can be released as improved versions of that variety with improved potential to tolerate terminal drought stress due to introgression of the stay-green character.

5.2 BC_3F_1 generation for recurrent parent S 35

Out of 19 BC_3F_1 individuals (6601–6619) screened in population 3001 (as an example), 4 individuals were selected and advanced by backcrossing to the BC_4F_1. These 4 selected individuals were targeted for

QTLs *stg*A, and *stg*A + *stg*4. Based on the genotyping results from 18 BC_3F_1 populations (367 individuals), 100 individuals were selected and advanced to the next generation by selfing and backcrossing [Table 8].

Generation BC_3F_1 for recurrent parent S 35			
Population	Individuals	Selected individuals	Targeted QTL(s)
3001	6601–6619	6612 and 6613	*stg*A
		6602 and 6603	*stg* A + *stg* 4
3002	6625–6648	6633, 6627, and 6630	*stg*B?
		6626, 6629, 6632, and 6630	*stg*3?
		6628 and 6634	*stg* 4
3003	6649–6672	6651, 6654, 6655, 6658, 6660, and 6663	*stg* A
		6652, 6657, and 6665	*stg* B?
		6650, 6659, 6661, and 6664	*stg* A + *stg*4
		6653	*stg*A + *stg*4 + *stg*3? + *stg*B?
3004	6673–6696	6683, 6673, 6682, and 6685	*stg*A
3005	6697–6707	6700	*stg*A
3006	6732–6755	6734, 6740, and 6741	*stg*A
		6732, 6737, and 6748	*stg*4
		6739	*stg*A + *stg*4
3007	6756–6776	6758	*stg*3? + *stg*4
		6760 and 6771	*stg*4
		6773	*stg*3?
3008	6708–6714	6708 and 6709	*stg*A
3009	7877–7881	7877, 7879, and7880	*stg*B?
3010	6778–6801	6783	*stg*4

3011	6802–6825	6807, 6811, and 6815	*stg*3? + *stg*B?
3012	6826–6849	6827, 6829, 6831, and 6832	*stg*3? + *stg*B? + *stg*4
		6828, 6839, and 6842	*stg*3? + *stg*.B?
3013	6850–6857	None	
3031	7282–7305	7282,7283,7285,7299	*stg* 1 + *stg*2
		7291, 7292, and 7304	*stg*B?
		7289	*stg*2 + *stg*B?
		7283, 7290, and 7303	*stg*1 + *stg*2 + *stg*B?
3032	7306–7326	None	
3033	7327–7350	7327, 7328, 7340, and 7343	*stg*2 + *stg*B?
		7329, 7332, 7339, 7346, and 7347	*stg*B?
		7338, 7342, and 7348	*stg*2
3034	7351–7374	7353	*stg*1 + *stg*2
		7356, 7357, 7361, and 7371	*stg*2 + *stg* 4
		7360, 7363, 7366, and 7368	*stg*4
		7364	*stg*2
3035	7375–7398	7375, 7376, 7378, 7380, 7390, 7396, and 7397	*stg*2

5.3 BC_4F_1 generation for recurrent parent S 35

Out of 88 BC_4F_1 individuals (8601-8692) screened in population 3001, 3002, 3006, 3008, 3010, 3011, 3031 about 26 individuals were selected for advancement for further generation based on the genotyping results. These selected individuals were targeted for QTLs *stg*A, and *stg*A + *stg*4, stg.3?+stg.4, stg.3?, stg.B+stg.4 etc., [Table 9].

Table-9. GENERATION BC_4F_1 OF S35

Population	Genotyopes	Selected genotypes	Targeted QTL
3001	8601-8603	8602	stg.A+stg.3?
		8603	stg.A+stg.3?
3006	8604-8605	8604	stg.3?
		8605	stg.4
3010	8606-8610	8608	stg.4
		8610	stg.4
3008	8621-8627	8621, 8627	stg.A+stg.3?+stg.4
		8622, 8625	stg.4
		8623	stg.3?+stg.4
		8624	stg.3?
		8626	stg.A+stg.4
3031	8630-8636	8635	stg.B
	8637-8643	8638,8640	stg.B
	8644-8649	8645, 8647,8648	stg.B
	8650-8656	8650	stg.B
3011	8676-8682	8676	stg.B
	8683-8685	8683, 8684, 8685	stg.B
3002	8687-8689	8688	stg.3
	8690-8692	8691	stg.B+stg.4

5.4 BC_3F_1 generation for recurrent parent ICSV 111

Out of 58 BC_3F_1 individuals screened in population 3050-3056 about 13 individuals were selected for advancement for further generation based on the genotyping results. These selected individuals were targeted for QTLs *stg*A, stg.B, stg.1, stg.2 etc., [Table -10].

Table-10. GENERATION BC_3F_1 OF ICSV111

Population	Genotyopes	Selected genotypes	Targeted QTL
3050	7711-7734	7715,7719,7725,7726	Stg.A
3051	7735-7758	7736	Stg.A+ Stg.2
3052	7759-7780	7759,7761	Stg.A+stg.1+ Stg.2
3053	7781-7804	7785	Stg.A
3054	7805-7828	7817,7824	Stg.2
3055	7829-7852	7831	Stg.3
3056	7853-7876	7862,7859	Stg.3

5.4 BC_4F_1 generation for recurrent parent ICSV 111

Out of 120 BC_4F_1 individuals screened in population 3050-3056 about 24 individuals were selected for advancement for further generation based on the genotyping results. These selected individuals were targeted for QTLs *stg*A, stg.B, stg.1, stg.2 etc., [Table 11].

Table-11. Generation BC_4F_1 ICSV111

Population	Genotyopes	Selected genotypes	Targeted QTL
3050	8701-8709	8701,8702, 8703	stg.A
3053	8710-8713	8711	stg.A
		8712	stg.3+stg.1
3056	8714-8723	8714	stg.A
		8717	stg.3+stg.1
		8723	stg.3
3051	8724-8733	8725,8729,8731	stg.2
		8728	stg.2+stg.1
3055	8734-8746	8742	stg.3
3054	8747-8770	8758	stg.2
		8763	stg.2+stg.1
3052	8771-8818	8790	stg.1
		8793	stg.2+stg.1
		8795, 8798, 8799, 8802, 8811, 8812, 8816	stg.2

5.5 Backcrossing Attempted for the Selected BC_3F_1 Individuals

Advance of selected individuals from the BC_3F_1 progeny involving parent S 35 and BC_3F_1 progeny involving recurrent parent ICSV 111

Out of the 100 individuals selected for advancement only 45 individuals were backcrossed with recurrent parent S 35 and 10 individuals were backcrossed with recurrent parent ICSV 111. BC_4F_1 seed numbers harvested from these 55 individuals are listed in Table 12. The reasons for comparatively low number of crosses made are as follows:

- The individual segregants in the BC_3F_1 populations showed different times of panicle initiation than their recurrent parents (which were sown on multiple sowing dates in order to minimize this common problem in backcross generations).

- Due to slight drizzle during crossing season, which is quite abnormal in the summer season, collection of pollen became a difficult task. Pollen collection should always be carried in dry weather conditions as pollen sticks to the wet sides of pollen collection bags from which it cannot be shaken onto the stigmas of emasculated florets.
- Re-sowing of some pots where the BC3F1 seed initially sown had failed to germinate was not accompanied by additional sowing of the recurrent parent. Plants produced by this later BC_3F_1 sowing came to flowering late—during the peak of the summer season's high temperatures, which adversely affected pollen production and ultimately resulted in poor crossing and selfed seed set.
- The short period of sorghum pollen viability, and lack of an effective pollen storage protocol for soghum is another major limitation.
- Even in the crossed individuals there was poor seed set due to the relatively high temperatures during fertilization and early grain filling stages of growth.
- Finally, a major problem was that the foreground marker data generation was not completed before the segregating generation individuals started to flower, so that the number of plants that needed to be emasculated for backcrossing was greater than the capacity of the team of ICRISAT support staff assisting with this activity so that choice of plants to be emasculated was in fact random rather than being based

on the data for marker alleles at loci flanking the target stay-green QTLs.

Table-12. BC_4F_1 seeds produced during attempted backcrossing for selected individuals from the BC_3F_1 generation for recurrent parent S 35.

Serial Number	Cross	Number of BC_4F_1 seeds produced
1	6602 × S 35-1	3
2	6603 × 8005	-
3	6628 × 6983	6
4	6630 × 6954	3
5	6665 × 6951	5
6	6682 × 8006	7
7	6683 × 6949	-
8	6685 × 6948	-
9	6708 × 6972	2
10	6732 × 6596	2
11	6737 × 6952	1
12	6748 × 6950	8
13	6758 × 6983	1
14	6760 × 6960	6
15	6783 × 6962	4
16	6805 × 6967	3
17	6811 × 6985	1
18	6815 × 6947	3
19	6832 × 6956	12
20	6889 × S 35-1	3
21	6905 × 6584	3
22	6908 × 6564	1
23	7289 × 6956	4
24	7290 × 6983	8

25	7297 x 6967	29
26	7298 x 6967	11
27	7303 x 8007	24
28	7305 x 8043	17
29	7327 x 6967	4
30	7328 x 6985	6
31	7329 x 6972	7
32	7338 x 6956	12
33	7343 x 8050	15
34	7348 x 8010	5
35	7351 x 6973	1
36	7353 x S 35-2	2
37	7356 x 6983	1
38	7357 x 6985	9
39	7360 x 6967	4
40	7361 x 6966	2
41	7363 x 6967	3
42	7365 x 6976	2
43	7366 x 6985	2
44	7368 x 6971	20
45	7390 x S 35-2	2

Table 13. BC_4F_1 seeds produced during attempted backcrossing for selected individuals from the BC_3F_1 generation for recurrent parent ICSV 111.

Serial Number	Cross	Number of BC_4F_1 seeds produced
1	7715 x 6951	1
2	7719 x 6947	1
3	7725 x 6967	4
4	7726 x 6971	1
5	7736 x 8001	5
6	7759 x 6967	1
7	7761 x 6956	4
8	7785 x S 35-1	11
9	7817 x 6956	4
10	7824 x S 35-1	2
11	7862 x 6963	4

5.6 Efficiency of marker-assisted selection

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

cultivars (or the rate of gain achieved between cultivar releases if the frequency of releases in time remains the same), MAS generates large additional economic benefits (Morris et al., 2003).

The efficiency of MAS is generally reduced with increasing genetic distance between the flanking markers used for each QTL for which MAS is being applied. Hospital et al. (1997), based on the simulation studies, recommended an optimal distance between two adjacent flanking markers of about 5–10 cM. We observed that the frequency of recurrent genotypes among the selected progeny increased as the selection intensity for recurrent genotypes increased, as predicted by Knapp (1998). Practically speaking, the number of markers that must be used decreases in each successive backcross generation, because once the recurrent parent allele has been fixed at any given non-target locus, it is not necessary to continue screening at that locus in subsequent generations as the locus will remain homozygous for the rest of the generations of selection regardless of whether these involve selfing or backcrossing to the recurrent parent (Morris et al., 2003). The decreasing number of markers required in each successive generation reflects the increasing percentage of the recurrent parent genome that is recovered in homozygous form, and hence fixed, in each backcross generation.

The fact that MAS technology is so challenging should not be a reason for discouragement, but instead, should provide a wake-up call for

more ingenuity, better planning and execution of marker-assisted breeding programs. MAS for quantitative traits is in an important transition phase, and the field is on the verge of producing convincing results. Technology development, including automation, allele-specific diagnostics and DNA chips, will make marker-assisted selection approaches based on large-scale screening much more powerful and effective (Young, 1999) in future.

5.7 Recommendations for the future:

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

- Fine mapping of the individual stay-green QTLs can be initiated using BC_5F_1 progeny produced by backcrossing selected BC_4F_1 individuals heterozygous for markers flanking single stay-green QTLs and homozygous for recurrent parent alleles at all other marker loci tested, following field evaluation of the corresponding BC_4F_2 progenies

to confirm that they are segregating in the expected Mendelian manner for the stay-green phenotype..

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

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

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

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

- Comparative genome mapping of potentially related traits in other related cereals (especially rice and maize) should be performed

5.8 Focus areas for more advanced studies:

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

Based on progress to date, it is very much clear that a multidisciplinary approach-conbining physiology, breeding and biotechnology is required for an effective understanding of a plant response to drought stress (Ribaut et al., 2002; Jones et al., 1997, Prioul et al., 1997). The QTLs characterized provide a powerful base of information and germplasm for the genetic dissection of physiological drought. This

approach can be combined with functional genomics and proteomics to identify the key pathways involved in drought stress tolerance and sensitivity, and further provide an insight of how these pathways interact. This in turn, may lead to more efficient and effective strategies for developing cereals with higher levels of sustainable productivity under water-limited conditions.

CHAPTER VI

Summary

Sorghum [*Sorghum bicolor* (L.) Moench] is the 5th most important cereal crop globally after wheat, maize, rice and barley (FAO, 2003; FAO and ICRISAT, 1996). This C_4 grass is grown in more than 80 countries, mostly in tropical and sub-tropical regions. The average annual sorghum area cultivated amounts to 44 M ha, with an average annual grain production of 63 M tons, and average grain yield of 1.4 t ha^{-1} (FAO, 2003; FAO and ICRISAT, 1996). Sorghum was domesticated in Ethiopia and part of Congo, with secondary centers of origin in India, Sudan and Nigeria.

Production of sorghum in semi-arid regions of the world is limited by drought. Developing plants that have an advantage under water-limited conditions is a major challenge for sorghum improvement programs globally. There are three distinct stages in which drought affects sorghum: Vegetative (GS1); Pre-Flowering (GS2); and Post-Flowering (GS3). The best characterized form of drought stress tolerance in sorghum during this post-flowering stage of growth is called "stay green." Stay-green is a drought-tolerance trait in grain sorghum. When water is limited during the

grain filling period, genotypes possessing this trait maintain more photosynthetically active leaves compared with genotypes not possessing this trait.

Putative QTL for stay-green trait from B35 have been identified in five recently published studies (Tuinstra et al, 1997: Crasta et al 1999; Xu et al, 2000; Tao et al, 2000 and Subudhi et al 2000). Using the linkage map developed by Bhattramakki et al (2000) identified six genomic regions associated with stay-green trait in B35 parent. The identification of these QTL provided us an opportunity for marker assisted breeding (MAB) for introgression of QTL from B35 to recurrent parents.

We aimed at transfer of QTL from B35 to recurrent parents, S35 and ICSV111. Both recurrent parents have been advanced to BC3 and BC4 generations for introgression of QTL from donor parent using SSR marker assisted selection (MAS), targeting six QTL detected. In this study, all genotypes in two generations from each recurrent parent were screened with foreground markers to identify the genotypes for QTL of our interest and also screened with background markers to select the genotypes for all other loci from recurrent parent. Selected individuals are advanced to next generation.

Field evaluation(phenotyping) of the stay green behaviour is required to enhance the selection potential. Nature of the dominance and epistatic properties of these QTL's should also be studied. Whether the QTL's are structural or

regulatory is to studied in detail. Feasibility of generating EST's, to have better understanding of this complex trait and Comparative genome mapping to study the trait in other related cereals will make marker assisted selection approaches based on large scale screening much more powerful and effective.

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APPENDIX

Marker details

Marker	Sequence		Annealing temperature	Linkage group
	Forward	Reverse		
Xtxp65	CACGTCGTCACCAACCAA	GTTAAACGAAAGGGAAATGGC	55	J
Xtxp141	TGTATGGCCTAGCTTATCT	CAACAAGCCAACCTAAA	55	G
Xtxp149	AGCCTTGCATGATGTTCC	GCTATGCTTGGTGTGGG	55	A
Xtxp7	ACATCTACTACCCTCTCACC	ACACATCGAGACCAGTTG	53.7	B
Xtxp15	CACAAACACTAGTGCCTTATC	CATAGACACCTAGGCCATC	56.7	J
Xtxp34	TGGTTCGTATCCTTCTCTACAG	CATATACCTCCTCGTCGCTC	59.4	C
Xtxp43	AGTCACAGCACACTGCTTGTC	AATTTACCTGGCGCTCTGC	56.7	A
Xtxp31	TGCGAGGCTGCCCTACTAG	TGGACGTACCTATTGGTGC	56.7	C
Xtxp33	GAGCTACACAGGGTTCAAC	CCTAGCTATTCCTTGGTTG	54.5	C
Xtxp23	AATCAACAAGAGCGGGAAAG	TTGAGATTCGCTCCACTCC	56.7	J
Xtxp225	TTGTTGCATGTTGGTTATAG	CAAACAAGTTCAGAAGCTC	55	J
Xtxp248	GGGTGTCCAATGTTGTCTGC	GGCCGTTACTGTCCCTTACTCA	50	A
Xtxp296	CAGAAATAACATATAATGATGG GGTGAA	ATGCTGTTATGATTTAGAGCCTG TAGAGTT	55	B
Xtxp298	GCATGTGTCAGATGATCTGGT GA	GCTGTTAGCTTCTTCTAATCGTC GGT	55	B
Xtxp57	GGAACTTTTGACGGGTAGTGC	CGATCGTGATGTCCCAATC	55	I
Xtxp88	CGTGAATCAGCGAGTGTTGG	TGCGTAATGTTCCTGCTC	53	A

Xtxp114	CGTCTTCTACCGCGTCCT	CATAATCCCACTCAACAATCC	50	C
Xtxp210	CGCTTTTCTGAAAATATTAAGGAC	GATGAGCGATGGAGGAGAG	55	H
Xtxp230	GCTACCGCTGCTGCTCT	AGGGGGCATCCAAGAAAT	55	F
Xtxp289	AAGTGGGGTGAAGAGATA	CTGCCTTTCCGACTC	55	F
Xtxp295	AAATCATGCATCCATGTTCGTCTTC	CTCCCGCTACAAGAGTACATTCATAGCTTA	55	E
Xtxp56	TGTCTTCGTAGTTGCGTGTTG	CCGAAGGAGTGCTTTGGAC	55	B
Xtxp357	CGCAGAAATACGATTG	GCTATCTGGAGTAACTGTGT	55	A

(Bhattramaki 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 5ul reaction

Number	Primer (2pM/ul)	$MgCl_2$ (10mM)	dNTP's (2mM)	DNA (2.5ng)	Enzyme (0.5U/ul)	Buffer (10x)	Distilled Water
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	.5	1.550
7	1.0	1.00	0.375	0.5	0.20	0.5	1.425