TRANSFERRING DROUGHT TOLERANCE OF THE STAY-GREEN TRAIT IN SORGHUM FROM E36-1 AN ETHIOPIAN LINE INTO OCHUTI, A FARMER PREFERRED KENYAN VARIERY

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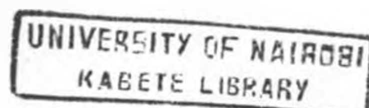

Declaration

This thesis is my original work and has not been presented for the award of a degree in any other university or for any other award.

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Dedication

I dedicate this research to God and to my parents who taught me that even the largest task can be accomplished if it is done one step at a time.

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List of abbreviations

°C	Degree (s) Celsius
BC	Backcross generation
bp	Base pairs
cM	Centimorgan
CTAB	Cetyl trimethyl-ammonium bromide
ddH_20	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EW	Epicuticular wax
FAO	Food and Agriculture Organisation
GCP	Generation Challenge Program
HCl	Hydrochloric acid
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
LG	Linkage group
M	Molar
MAB	Marker assisted backcrossing
MAS	Marker assisted selection
ml	Millilitre
mm	Millimeter
mM	Millimolar
ng	Nanogram
PCR	Polymerase chain reaction
PPB	Participatory plant breeding
QTL	Quantitative trait locus
RP	Recurrrent parent
rpm	Rotations per minute
SBI	Sorghum bicolor
SLN	Specific leaf nitrogen
SSR	Simple sequence repeat
TE	Tris EDTA
UN	United Nations
µg	Microgram
µl	Microlitre

Abstract

In this study marker-assisted backcross breeding was utilized to transfer stay-green QTL from the Ethiopian donor parental line E36-1 into a Kenyan Farmer-preferred variety, Ochuti as the recurrent parental line. E36-1 has 3 stay green QTL located at three linkage groups of sorghum genome (SBI-01, SBI-07 and SBI-10). Five foreground markers that were polymorphic between the two parental genotypes were used to identify true F_1 individuals. Only two of the possible three QTL namely, SBI-07 and SBI-10 were identified as having been transferred into three individual genotypes. This is because there were no polymorphic markers available flanking stay-green QTL at linkage group SBI-01. The identified F_1 genotypes were used as the female parents in the generation of 128 BC_1F_1 individuals. About 25% of the BC_1F_1 progenies that were genotyped had at least one QTL introgressed. As is the case in all marker-assisted back-cross breeding, the rate of success in introgressing QTL from donor to recurrent parental lines, depends on the number of plants screened. Thirty polymorphic background SSR markers were used to select twenty BC_1F_1 individuals that had a higher proportion of recurrent parent genome. These were backcrossed to recurrent parent to generate 157 BC_2F_1 family lines. These were screened with the five foreground SSR markers where 45 individuals were confirmed to be having among them single and double introgressions of stay-green QTL. The results of this study showed that, it is possible to introgress stay-green QTL that govern drought tolerance in from an exotic source to a locally preferred variety in sorghum successfully.

CHAPTER ONE

INTRODUCTION

1.1 Background

Sorghum bicolor (L) Moench is globally the fifth most important cereal crop after wheat (*Triticum sp.*), rice (*Oryza sativa*), maize (*Zea mays*), and barley (*Hordeum vulgare*) according to FAOSTAT (2009).

The maximum recorded yield for sorghum is 21t ha^{-1}, but the average yield worldwide is only 1.28 t ha^{-1} (Wittwer, 1980; FAO, 2001). Even in the USA average yields are only 3.2 t ha^{-1} (FAO, 2001). Based on these statistics, sorghum production averages between 5 and 15% of its maximum recorded yield potential in a typical environment. While biotic stresses reduce yield potential in specific environments, most of the reduction in sorghum yield is attributed to abiotic stress, primarily drought (Kramer and Boyer, 1995). However, there are other serious and widespread constraints such as soil resource and soil fertility management including soil physical degradation, fertility depletion and nitrogen deficiency.

Sorghum is a drought resistant, low input cereal grain grown throughout the world. It is adapted to a wide range of tropical as well as temperate climates, although it is best known for its adaptation to drought-prone semi-arid tropical regions. It is mostly a plant of hot, dry regions and can survive in cool weather as well as waterlogged habitats (Dogget, 1988; House, 1995). Over 80% of the area devoted to sorghum production lies within Africa and Asia.

Sorghum probably originated in Ethiopia and has spread from there to other parts of Africa, India, Southeast Asia, Australia and the United States (Dogget and Rao, 1995). Major producers of sorghum are the USA, India, Nigeria, China, Mexico, Sudan and Argentina. The bulk of African sorghum production is centered in the savanna zone of east, west and central

Africa, where the grain is a major component of the daily menu for millions of people. Sorghum is made into unleavened breads, boiled porridge or gruel, malted beverages including beer and specialty foods such as popped grain and syrup from sweet sorghum (De Vries and Toenniessen, 2001). The straw of traditional tall sorghums is used to make palisades in villages or around homesteads. The plant bases are an important source of fuel for cooking and the stems of wild varieties are used to make baskets or fish traps. Dye extracted from sorghum is used in West Africa to color leather red (Singh *et al.*, 2006). In western Africa, Nigeria has emerged as a pioneer in the industrial utilization of sorghum. Sorghum is a principle feed ingredient for both cattle and poultry (De Vries and Toenniessen, 2001) and in recent years, it has become an important source of biofuel (Laopaiboon *et al.*, 2007; ICRISAT, 2007a; 2007b). In the United States the sorghum grain is used for livestock feed and stems and foliage for hay, silage and pastures (House, 1995).

Fifty nine percent of world sorghum growing area is in Africa. Asian countries occupy 25% of world sorghum area. North and Central America covers 11% of sorghum area and 4% is in South America. The developing countries in Asia and Africa contribute more than 70% of total sorghum production in the world. Asia alone contributes 45% of world sorghum production. North and Central America produces 21% of sorghum and 6% is in South America(FAOSTAT,2009).

Production of sorghum is in the hands of small scale farmers covering a wide range of different ecological zones and production systems. Yields are low and highly variable. Low soil fertility, heat and low and highly variable rainfall are among the major production constraints. A particular challenge is the great variability of the start of the rainy season, which leads to great uncertainty of the date of sowing (Niangado, 2001).

Grain sorghum yields are very low in eastern Africa as compared to yields in the USA and well below the genetic potential. The Kenyan mean yields range from 0.6 to 1.5 t ha^{-1} as

compared to 4.3 t ha^{-1} in the USA (FAOSTAT, 2009). High yield losses are due to numerous biotic and abiotic stresses. Soil water deficits and *Striga* are the most important constraints in Kenyan sorghum production (Wortmann *et al*., 2007).

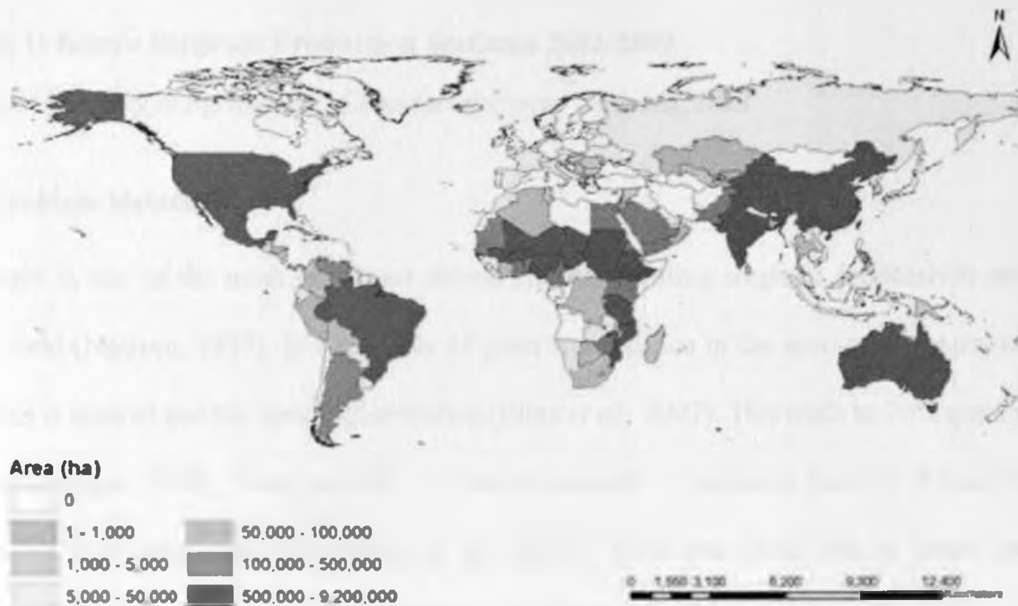


Figure 1: Sorghum cultivating areas.

Source:http://www.icrisat.org/vasat/learning_resources/crops/sorghum/sorghum_prodpractices/html/m2l1/index.html

As a traditional crop in Kenya, sorghum provides food security and is becoming a suitable alternative for maize in many places where this crop fails and in Kenya is predominantly grown in Eastern, Western and Nyanza provinces.

Province	production (metric Tonnes)		value (Billion Kshs.)	
	2002	2003	2002	2003
Central	156	136	2.618.600	2.071.395
North Eastern	2.963	5.282	59.268.000	105.640.000
Nyanza	69.597	67.655	670.000.000	783.432.800
Western	11.505	20.245	204.532.800	244.988.000
Coast	377	322	16.963.300	7.923.601
Eastern	26.829	25.951	305.904.000	415.920.000
Rift Valley	67.799	6.892	77.522.132	789.582.517
Total	118,227	126,433	1,336,433	2,349,558,313

Table 1: Kenya Sorghum Production Statistics 2002-2003

Source: Ministry of Agriculture; Crop Development Division, 2003

1.2 Problem statement

Drought is one of the most important abiotic stresses limiting sorghum productivity around the world (Nguyen, 1997). It's certainly of great significance in the semi-arid tropics, where rainfall is limited and has erratic distribution (Ejeta *et al.*, 2007). This leads to 70% grain yield losses (Pocket, 2009). Food security is a major concern in semi-arid parts of Kenya which cover 75% of land mass (Kanyanjua *et al.*, 2002). This low yields due to losses due to drought leads to hunger, poverty, malnutrition and high expenditure through imports.

Decreased water availability and increased food demand worldwide require development of more water efficient crops (Balota *et al.*, 2008). Sorghum is one of the crops that tolerate environments subjected to high temperatures and limited water or precipitation since it has a C4 photosynthesis specialization like maize or sugarcane (Edwards *et al.*, 2004). However, drought and especially at post-flowering stage, highly affects grain yields in sorghum. This is because at this stage sorghum becomes susceptible to charcoal rot and stalk lodging leading to poor grain filling. Delayed senescence (stay green) improves adaptation to post-flowering drought stress (Blum, 1989). Plants with stay green resist drought-induced premature plant senescence (Borrell *et al.*, 2000). Post-flowering drought can be alleviated by breeding for improved drought tolerance traits, such as stay green. Stay green is characterized by the

plant's ability to retain photosynthetic capacity longer under water stress conditions and produce normal grain (Rosenow 1987, Walulu *et al*., 1994).

Stay green trait is heritable and progress from selection can be attained (Subudhi *et al*., 2000). However, the progress in improving drought tolerance in sorghum with conventional breeding methods has been slow. This has prompted the need to identify markers linked to stay green QTL in order to accelerate breeding activities for the incorporation of drought tolerance into elite genotypes (Crasta *et al*., 1999; Xu *et al*., 2000).

1.2 Justification of the study

The adaptability of sorghum to drought gives it a greater potential to provide food security especially in Kenya where 70% of maize growing areas are turning into semi arid areas conducive for growing sorghum, a drought tolerant plant (The East African 2010). Extremely high temperatures and low amounts precipitation reduce yields in sorghum especially the post flowering drought (Subudhi *et al*., 2002). Therefore, most sorghum breeding programmes are working towards development of improved varieties which are drought tolerant, with better quality, disease resistance, and agronomic traits (Klein *et al*., 2008, Knoll and Ejeta, 2008). Breeding for drought tolerance in sorghum is important because the plants will often die as a result of excessive water stress during grain filling (Stout and Simpson 1978; Rosenow and Clark 1981). In sorghum, rapid premature leaf death occurs as a result of water-stress during grain filling. Premature leaf senescence, in turn, leads to charcoal rot, stalk lodging, and significant yield loss (Subudhi *et al*., 2002).

Stay green is a drought resistance mechanism that enables the plant to continue to fill their grains normally under water limited conditions (Rosenow and Clark 1981; Duncan *et al*. 1981). This results in increased yields (Henzell 1992; Borell and Douglas 1997), resistance to charcoal rot and lodging (Rosenow 1983; Duncan 1984; Woodfin *et al*, 1988).

Backcross breeding can be used to introgress these stay green QTL which had earlier been identified and mapped in some varieties (E36-1, B35 and SC56) into sorghum varieties that does not possess the trait. Conventional backcross breeding utilizes phenotypic selection criteria which is tedious and time consuming. This has prompted researchers to develop a more effective and efficient method of selection for the genes that controls the trait of interest using DNA markers, that is, marker-assisted backcrossing (MAB) or simply, marker-assisted selection (MAS) (Young and Tanksley, 1989; Michelmore, 1995; Ribaut and Hoisington, 1998 and Xu *et al.*, 2000). This method saves time compared to conventional backcrossing since the trait of interest can be introgressed more accurately and across fewer generations (Frisch *et al.* 1999). It also ensures minimum linkage drag whereby there is less of the donor parent genome fragment other than the target locus (Hospital 2005; Frisch *et al.*, 1999). It is through implementation of MAB that drought tolerant sorghum varieties will be realized in Kenya.

1.3 Objectives

1.4.1 General objective

Improving sorghum productivity in Kenya through use of marker-assisted backcrossing for drought tolerant trait, stay-green

1.4.2 Specific objective

1. Identification of polymorphic markers and confirmation of heterozygous F_1 generation.
2. Genotyping BC_1F_1 and BC_2F_1 populations for stay-green QTL from a generation of a cross between Ochuti and E36-1

CHAPTER 2

LITERATURE REVIEW

2.1 Drought resistance in plants

Drought is a major abiotic factor limiting agriculture in the world today and a major cause of significant yield reduction in crop plants (Boyer, 1982). The possible global climate change scenerios suggest a future increase in the risk of drought (Tahir and Mehdi, 2001).

In most plants response to water deficit is a combination of survival strategies and this makes drought resistance complex. Water deficit causes both physical and physiological events to take place and this makes the determination of the function of an observed response a difficult task (Bray, 1997)

The response mechanism in plants that allows them to survive, grow and yield satisfactorily under water stress conditions is referred to as drought resistance. In sorghum, the adaptations leading to drought resistance can be divided into: drought escape, avoidance and tolerance (Ludlow and Muchow, 1990).

2.1.1 Drought escape

Drought escape enables plants like desert ephemerals and short season annuals like sorghum germinate, grow rapidly and complete the whole life cycle before the soil water is exhausted in arid environments with low and variable rainfall. This mechanism involves rapid physiological development such as, early flowering and early maturity, developmental plasticity (variation in duration of growth period depending on the extent of water deficit) and remobilization of pre-anthesis assimilates to grain (Turner, 1980). Although drought escape is a desirable method of reducing yield losses due to water stress, it is not a feasible method in many areas of the world because of inconsistent weather patterns (Dalton, 1967). In such a situation, the plant must have the morphological or genetic capability to tolerate water stress.

2.1.2 Drought avoidance

Drought avoidance is the ability of the plants to prevent reduction of tissue water potential during water deficit by increasing water uptake through the roots and by increasing stomatal resistance (O' Toole & Chang, 1979). Drought avoidance mechanisms can be classified into water conserving mechanisms such as the specialized C4 photosynthetic pathway and water collecting mechanisms like the formation of deep roots which allows better access to groundwater resources (Yambao *et al.*, 1992). Water conserving mechanisms decrease water loss and reduce leaf growth, increase stomatal and cuticular resistance and accelerate leaf senescence (Jones, 1980). Water-collecting mechanisms are extended root growth, and decreased resistance for water uptake through the roots. However, this adaptation to drought has a disadvantage whereby reduction of water loss through stomatal closure and reduced leaf area usually result in reduced assimilation of carbon dioxide.

2.1.3 Drought tolerance

This is the ability of the cells and tissues to withstand reduced water potentials during water deficit. Drought tolerant plants can cope with the stress factor and tolerate desiccation to a greater extent and therefore survive longer when water is limiting (Hsiao, 1982). Mechanisms of drought tolerance are osmotic adjustment and antioxidant capacity. Osmotic adjustment results from the accumulation of compatible solutes within cells, which lower the osmotic potential and helps, maintain turgor of both shoots and roots as plants experience water stress (Nguyen *et al.*, 1997). Osmotic adjustment enables water uptake to continue under increasing stress in many species and, in some cases, is associated with maintenance of growth and stable yield under drought (Gunasekera and Berkowitz, 1992). On the other hand, plants vary greatly in their capability to tolerate stress conditions; hence some of them are unable to endure stress so wilt and die. Tolerant plants can endure stress by undergoing certain physiological changes

in their tissues thus maintain their cell water potential turgidity at normal level, in spite of soil water deficiency (Simmons *et al.*, 1989).

Antioxidant capacity is the ability of the sorghum plant to detoxify reactive oxygen radicals that cause cellular injury such as lipid peroxidation and protein modification (Mckersie and Lesham, 1994). These radicals result from several environmental stresses including drought (Smirnoff, 1993; Zang and Kirkham, 1994).

Crop plants have developed other different drought tolerance mechanisms during various stages of development, for instance in sorghum drought tolerance has been grouped in two distinct stages namely; pre-flowering and post-flowering (Tuinstra *et al.*, 1997). Pre-flowering response in sorghum occurs when the plants are under significant moisture stress prior to anthesis. Water stress during this period directly affects the development of panicles, the grain numbers and consequently the grain yield (Subudhi *et al.*, 2000; Subudhi *et al.*, 2002; Tuberosa *et al.*, 2003; Ramesh *et al.*, 2006). Post-flowering drought tolerance is found important in sorghum plants since it ensures increased grain yields (Rosenow *et al.*, 1983). It is expressed when moisture stress occurs during the grain filling stage.

Other traits associated with drought resistance are: heat tolerance (Basnayake *et al.*, 1995), transpiration efficiency (Muchow *et al.*, 1996), rooting depth and patterns (Jordan and Miller, 1980), and epicuticular wax (Maiti *et al.*, 1984).

2.2 Drought response in sorghum

Early research in sorghum indicated that the most effective way to reduce loss due to water stress was through the use of early maturing genotypes to avoid late season water stress (Blum, 1979). Even though it was not technically a drought resistance mechanism, sorghum production and its growth as a crop in the Midwestern United States was based on the

development of early maturing genotypes that avoided late season drought stress (Smith and Frederiksen, 2001). In many region of the world, the use of specific maturity types to utilize seasonal rainfall is still a common practice and an important mechanism for controlling losses due to water stress.

Drought stress response in sorghum depends on the stage of growth in which the drought stress occurs (Agboma *et al.*, 1997). Pauli *et al.*, (1964), divided sorghum growth into three stages. Growth stage 1 (GS1) is the vegetative stage that begins with germination and ends at panicle differentiation. Growth stage 2 (GS2) is the pre-flowering or reproductive phase of growth ranging from panicle differentiation until the end of anthesis. Growth stage 3 (GS3) is post-flowering or grain filling phase, and begins immediately after anthesis and continues until physiological maturity of the grain. The drought resistance reaction is controlled by different genetic mechanisms at every stage of growth (Rosenow *et al.*, 1997).

Drought stress tolerance in GS1 is an important trait since interaction between genotype and environment begins at planting with the germination process. Sorghum germination is influenced by the amount of available soil water, genotype of the seedling and the environment in which the seed was produced (Evans and Stickler, 1961; Howarth *et al.*, 1997) In GS2 and GS3, two distinct reactions to water stress have been identified and characterized. Both reactions are based on growth stage and have distinct and different phenotypic expressions (Rosenow and Clark, 1981; Rosenow *et al.*, 1983). The pre-flowering stress response occurs when a plant encounters significant drought stress during GS2 prior to anthesis.

Sorghum susceptible to pre-flowering drought stress will exhibit symptoms such as leaf rolling, leaf tip burn, delayed flowering, poor panicle exertion, panicle blasting, and reduced

panicle size (Rosenow *et al*., 1997). Pre-flowering water limitation affects yield potential by influencing the panicle size and seed number.

With the help of molecular markers, sorghum breeders have managed to dissect the inheritance of pre-flowering drought tolerance. Tuinstura *et al*., (1996) evaluated a recombinant inbred line population and found six distinct genomic regions that were specifically associated with pre-flowering drought tolerance. These loci accounted for approximately 40 per cent of the total phenotypic variation for yield under drought stress and most of these regions were detectable across environments. Kebede *et al*., (2001) identified four QTL that controlled pre-flowering drought tolerance in sorghum but none of the QTL identified were consistent across all environments.

Post-flowering water stress results from drought stress that is encountered at GS3 during grain fill. Symptoms of post-flowering drought stress susceptibility include premature leaf and plant senescence, stalk lodging and charcoal rot, and a reduction in grain size (Rosenow and Clark 1997).Water stress during GS3 can result in significant reduced yields as the plant is unable to complete grain filling. Lodging is due to remobilization of carbohydrates from the stem in an attempt to complete the grain filling process. This leads to weakening of the stem and if charcoal rot (*Macrophomina phaseolina*) subsequently invades, it further weakens the stem causing significant lodging (Moghogho and Pande, 1984). Reduction in grain size occurs because the plant is unable to obtain enough assimilate (nitrogen and carbohydrate) to fill the developing grains (Borrell *et al*., 1999).

Sources of genetic resistance to post-flowering drought stress have been successfully identified by breeders. The resistance enables plants to retain chlorophyll in their leaves and maintain the ability to carry out photosynthesis hence the name 'staygreen' (Rosenow 1983).

Stay green genotypes are less susceptible to stalk lodging (Woodfin *et al*., 1998) and resistant to charcoal rot (Rosenow, 1983). They also retain higher levels of stem carbohydrates than non-stay green genotypes. Stay green genotypes have also been found to contain higher cytokine levels (McBee 1984, Ambler *et al*., 1987) and more stem sugars (Duncan *et al*., 1981; McBee and Miller, 1982; Dahlberg, 1992) than their non-stay green counterparts.

Studies done in Australia, (Borrell *et al*., 2001) and in India (Borrell *et al*., 1999), illustrated the importance of retaining green leaf area with respect to yields. The stay green trait is controlled by several genes, each contributing to the expression of the trait (Rosenow and Clark, 1981). Several sorghum genotypes have been identified that exhibits the stay green trait such as: B35, E36-1 and SC56 (Rosenow, 1983; Kebede *et al*., 2001, Haussmann *et al*., 2002). B35 is an Ethiopian durra sorghum, E36-1 is a high yielding breeding line from Ethiopia assigned to the Guinea-Caudatum race (Haussmann *et al*., 2002), while SC56, (Kebede *et al*., 2001) has been derived from Sudanese Caudatum-Nigricans sorghum. All these lines have been used as a source of the stay green trait for research (Tuinstra *et al*., 1997, 1998, Crasta *et al*., 1999; Subudhi *et al*., 2000; Tao *et al*., 2000, Xu *et al*., 2000).

2.3 Physiology of stay green trait

Stay green is a nuclear-gene-controlled quantitative trait that confers post-flowering drought resistance that allows plants to resist premature senescence under severe soil moisture stress (Walulu *et al*., 1994). Stay green is a consequence of the balance between the nitrogen (N) demand by the grain and the N supply during grain filling (Borrell *et al*., 2001). Other than N dynamics, the other component of stay green is transpiration efficiency (TE), which is a measure of the amount of biomass the plant produces per unit of water it captures for transpiration.

Borell and Hammer (2000) observed that stay green genotypes allocate more N to the leaves compared to non-stay green genotypes, hence their higher specific leaf nitrogen (SLN). They hypothesized that this higher SLN enhanced radiation use efficiency (RUE) and transpiration efficiency (TE). These enabled the plant to set a higher yield potential by anthesis, resulting in higher grain yield and lodging resistance under post-flowering water stress. The onset of leaf senescence can be delayed by increased soil-N uptake during grain filling, as observed under terminal drought stress by Borrell and Hammer (2000). Such increased N uptake can be associated with either increased water uptake (transpiration, T) or increased TE as at least one of these is required to explain the enhanced biomass accumulation and yield of stay green types (Borrell and Hammer, 2000). The other mechanism believed to delay the onset of leaf senescence is the availability of stem-N for translocation, although this mechanism might compromise the leaf-N status if increased stem-N is not matched with increased total N-uptake (Borrell and Hammer, 2000).

According to Thomas and Rogers (1990), longevity of a leaf is intimately related to its nitrogen status. During leaf 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, resulting in the characteristic yellowing of the leaves as chlorophyll is broken down. It is likely that the senescence of the leaves is triggered by an increased demand for nitrogen elsewhere. In a senescing leaf, there is competition for nitrogen between developing grain and leaves, which is usually to the advantage of grains.

A delayed remobilization of N from the leaves or a remobilization of N from leaves having a larger pool of N in stay green genotypes would indeed maintain photosynthetic capacity longer, and therefore carbohydrate supply to the developing grain, possibly resulting in higher

grain yield. Indeed, prolonged accumulation of dry matter and N by above-ground plant parts of maize during grain filling has been reported as an important characteristic associated with high yields (Moll *et al.*, 1994).

2.4 Mapping of stay green QTL in sorghum

Genome mapping in sorghum using DNA markers began in the 1990s. Tuinstra *et al.* (1997) identified components of grain development and post-flowering drought tolerance in sorghum. An effective approach for high-resolution mapping and characterization of individual loci governing drought resistance QTL is through analysis of near-isogenic lines (NILs) differing at respective QTL. The approaches employed in the mapping of the stay green trait generally involved generation of a mapping population followed by its analysis. Examples of mapping populations included recombinant inbred lines (RILs), F_2 and heterogeneous inbred families (Subudhi *et al.*, 2002; Tuinstra *et al.*, 1997). For example, fine mapping of stay green QTL in the B35 sorghum variety was achieved through the analysis of a RIL mapping population developed from the cross B35 × 7000 and resulted in the identification of four loci: *Stg 1, Stg 2, Stg 3* and *Stg 4* (Xu *et al.*, 2000; Subudhi *et al.*, 2002). Using similar approaches, stay green QTL have been mapped in several other sorghum varieties including SC56 and E36-1 (Haussmann *et al.*, 2002; Kebede *et al.*, 2001; Tuinstra *et al.*, 1997). The stay green QTL resulting from a mapping study can be targeted for MAS depending on their relative effects and position thus providing opportunities to accelerate drought tolerance in breeding programmes (Subudhi *et al.*, 2002).

2.5 Field evaluation of stay green trait

Several methods have been described in evaluation of stay green trait, for example; in an experiment done in India (Reddy *et al.*, 2001) the data that were recorded for agronomic traits included time to 50% flowering, plant height, plant agronomic score (1 = most desirable and 5

= least desirable) and stay-green score (1 = 0 to 10% leaves dried and 5 = >75% leaves dried) at maturity. In another study the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru during the 2002 and 2003 post-rainy seasons, evaluated some genotypes for stay green trait with E 36-1as one of the controls of stay green cultivar in a randomized complete block design (RCBD). Irrigation was given at sowing and during early crop growth stages, but stopped two weeks prior to flowering so that the moisture stress could develop after flowering. The data recorded were for: time to 50% flowering, plant height, plant agronomic score (1 = most desirable and 5= least desirable), grain yield, 100-grain weight and stay-green score at maturity based on visual ratings (Wanous *et al*., 1991) using 1 to 5 scale (1 = 0 to 10% leaves dried and 5 = >75% leaves dried) based on the proportion of leaf area of normal sized leaves that had senesced and dried. The rate of senescence determines the maintenance of quality flowers and seed set (Xu *et al*., 2000).

2.6 Molecular markers used in sorghum breeding

Morphological markers used to be the source of varietal identification and genetic diversity assessment. Later biochemical markers were widely used to assess genetic diversity of crops (Morden *et al*., 1989). These morphological and biochemical markers were found to have some limitations. However, the ultimate difference between individuals lies in the nucleotide sequences of their DNA. This incite has led to development of DNA-based molecular markers which follow simple Mendelian genetics. Unlike morphological markers, molecular markers are stable and not influenced by developmental or environmental factors (Jaccoud *et al*., 2001).

A molecular marker is a chromosomal landmark or allele that allows for the tracing of a specific region of DNA. The markers and the genes they probe are close together on the same chromosome hence they tend to stay together as each generation of plant is produced.

Molecular markers are based on two major techniques: hybridization (Southern, 1975; Jaccoud *et al.*, 2001) and polymerase chain reaction (PCR) (Mullis *et al.*, 1986). Hybridization-based restriction fragment length polymorphism (RFLP) was the first DNA-based molecular marker system (Wyman and White, 1980; Botstein *et al.*, 1980). Later, various types of molecular markers based on the PCR, such as randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), sequence-tagged site (STS) (Edwards *et al*, 1991), and simple sequence repeat (SSR) (Litt and Lutty, 1989; Tautz *et al.*, 1989; Weber and May, 1989; Jacob *et al.*, 1991) markers have been developed. These markers have been applied in various genetic studies including: assessment of genetic variability and characterization of germplasm, fingerprinting of genotypes, marker-assisted selection and identification of sequences of useful candidate genes (Korzun *et al.*, 2003).

SSRs are DNA sequences with repeat lengths of few base pairs (Tautz, 1989) and short tandem repeats (STRs) (Edwards *et al.*, 1991). The repeat units are generally mono-, di-, tri-, tetra- or penta- nucleotide repeat types like AA--, AG--, CGA short stretches of DNA that are variable (Haussmann *et al.*, 2002). If these repeats are long enough and uninterrupted, they are excellent genetic markers due to their high level of polymorphism (Powel *et al.*, 1996). SSRs are generally assumed to be evenly distributed over genomes (Dietrich *et al.*, 1996) but rare within coding regions (Hancock, 1995).

As molecular markers, SSRs combine several desirable properties including high levels of polymorphism and informativeness, selective neutrality, high reproducibility, rapid and simple genotyping assays (Powel *et al.*, 1996). They are also said to be co-dominant that is, they can allow scoring of heterozygous individuals.

2.7 Marker-assisted backcrossing

Backcross breeding is a procedure of transferring favorable alleles from a donor genotype (either an unadapted line or a wild relative), which has mostly poor agronomic properties in to a recipient elite genotype of farmer preferred variety (Allard, 1960). The recipient parent contains a large number of desirable attributes but is deficient in only a few characteristics. In this procedure there is introgression of a target gene or chromosomal region from a donor line into the genomic background of a recipient line. The objective is to reduce the donor genome content of the progenies by repeated back-crosses to the recipient line. Backcross breeding can be done in two ways: conventional backcrossing and marker-assisted backcrossing (MAB) (Semagn *et al.*, 2006b). Conventional backcross breeding is primarily based on phenotypic selection of superior individuals among segregating progenies resulting from hybridization. This process of selection is usually time consuming and tedious and depend on in the traits being selectable by phenotype (Babu *et al.*, 2004; Tuberosa *et al.*, 2008).

Molecular markers are tools that can be used as chromosome landmarks to facilitate the introgression of genes associated with economically important agronomic traits. Molecular markers are not affected by the environment and are detectable at all stages of plant growth (Jaccoud *et al.*, 2001).

In MAB results of DNA tests assist in the selection of individuals to become the parents in the next generation of a genetic improvement program (Semagn *et al.*, 2009). Molecular markers are directly used in the breeding program to follow the introgression of target gene(s) (Gupta *et al.*, 1999; Babu *et al.*, 2004; Francia *et al.*, 2005).

MAB can only be successful if there is a genetic map with an adequate number of uniformly-spaced polymorphic markers to accurately locate desired gene(s) or QTL or to locate, close

linkage between the QTL or a major gene of interest and adjacent markers and if there is adequate recombination between the markers and rest of the genome (Babu *et al.*, 2004). According to previous studies (Young and Tanksley, 1989; Ribaut and Hoisington, 1998; Xu, 2002; Xu *et al.*, 2005) MAB improves the efficiency of backcross if the trait is difficult to manage through conventional phenotypic selection because it is expensive or time consuming to measure, or have low penetrance or complex inheritance or if traits are those whose selection depends on specific environments or developmental stages that influence the expression of the target phenotype. MAB also hastens selection of recessive alleles during backcrossing since transfer of these genes through conventional breeding requires additional selfing generations after every backcross making it a time consuming procedure. Foreground selection is the use of markers to assess the presence of the desirable QTL allele at a genetic locus (Tanksley 1983; Melchinger, 1990) while background selection is the use of markers that are not in close vicinity with the trait of interest. Marker-assisted background selection was initially proposed by Young and Tanksley (1989) and was later described as background selection by Hospital and Charcosset (1997). It accelerates the recovery of the recurrent parent genome by selecting the individuals that are double homozygotes (Hospital and Decoux, 2002) that is, individuals that are heterozygous at the target locus but homozygous for recurrent parent alleles at two markers flanking the target locus on each side. The markers used for foreground selection are tightly linked markers flanking the target QTL. It is less possible for the selected marker allele to be separated from the desired trait by a recombination event since they are closely linked. The markers for background selection are those that are evenly spaced along other chromosomes (i.e. unlinked to QTL) of the recurrent parent (Semagn *et al.*, 2006b).

CHAPTER THREE

IDENTIFICATION OF POLYMORPHIC MARKERS AND CONFIRMATION OF HETEROZYGOUS F_1 GENERATION

Abstract

Marker-assisted selection (MAS) is implemented on the basis of polymorphic molecular markers flanking the target QTL. Foreground molecular markers are those that help identify the individuals that carry the target gene(s). On the other hand background markers are those that accelerate the return to recipient parent genome outside the target gene. The markers chosen for background selection are those that are far from the target locus and in non-target chromosomes.In this study a set of 24 foreground and 128 background SSR markers were screened for polymorphism. The foreground markers selected were those that flank the QTL that confer the stay green trait in variety E36-1. The stay green QTL of E36-1 are in chromosome 1 (SBI-01), chromosome 7 (SBI-07) and chromosome 10 (SBI-10). However, the background markers were those that are a distance away from the stay green QTL especially on the chromosomes where there no stay green QTL on chromosomes 2, 3, 4, 5, 6, 8 and 9. Out of 24 foreground markers, only five showed polymorphism between the two parental genotypes E36-1 and Ochuti and out of 128 background markers, 30 were polymorphic. The five polymorphic markers were used to screen 96 samples for heterozygozity in a cross between E36-1 and Ochuti. Out of 96 samples, only samples 17, 28 and 32 were true breeding F_1 genotypes.

3.1 Introduction

3.1.1 Marker-assisted selection of F_1 generation

In marker-assisted backcrossing, foreground selection is the use of molecular markers associated to select individuals that possess the trait after a cross between the donor parent and recipient parents (Semagn *et al.*, 2006a). In foreground selection, the closer the markers that flank the target locus, the less the linkage drag (Hospital, 2001). Linkage drag is the transfer of undesirable donor alleles on the carrier chromosomes (Young and Tanksley, 1989).

Early generation selection in a breeding programme can be achieved when polymorphic molecular markers between two parental genotypes are used to select true F_1 plants by analyzing co-dominant markers having the alleles of both parental genotypes. True F_1s are selected and advanced to generate BC_1F_1 generation. The advantage of foreground selection is that, prior to field evaluation of large number of plants, breeders can considerably reduce the number of plants by the use of tightly linked markers as diagnostic tool to identify the plant carrying the genes or QTL of interest (Tanksley, 1983). However, this selection method can be limited by lack of equipment, skilled manpower and lack or limited funding for research (De Villiers and Semagn, 2009)

3.1.2 Polymerase chain reaction (PCR) using M-13 tailed SSR markers

Most genotyping with SSRs is performed by PCR with defined oligonucletide primers. Genotyping with SSRs was first described by Litt and Luty in 1989. After amplification of the DNA fragments their lengths need to be analyzed with either agarose or polyacrilamide gel electrophoresis or laser detection systems (Schuelke, 2000). For analysis with laser detection system, one of the primers has to carry a florescent dye label, which can either be FAM, VIC, PET or NED.

The procedure for fluorescent labelling of PCR fragments can be performed with 3 primers; a sequence-specific forward primer with M-13 (-21) tail at its 5' end, a sequence-specific reverse primer and the universal fluorescent-labeled M-13 (-21) primer (Schuelke, 2000). The forward primer is used in one fourth the amount of the reverse primer, so that the M-13 (-21) universal primer can take over when the forward primer is used up therefore incorporating the fluorescent dye in the PCR product (Schuelke, 2000).

3.1.3 Allele's detection methods

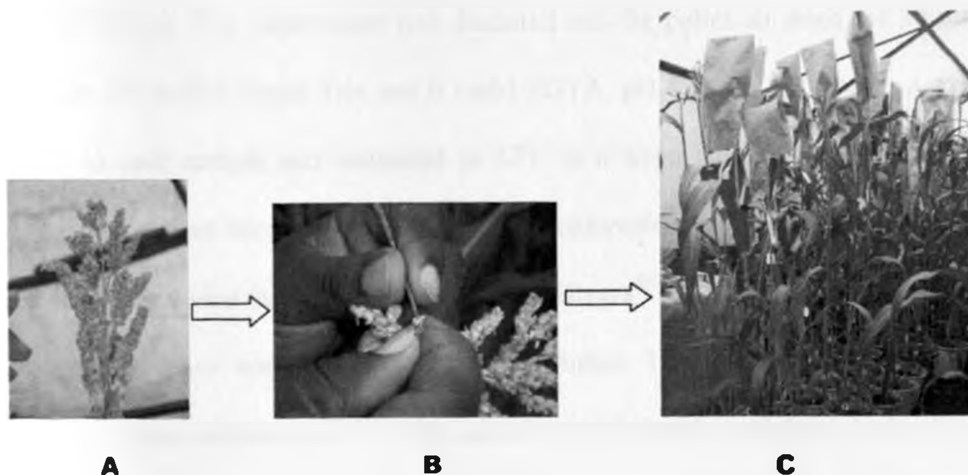
Agarose and denaturing/ non-denaturing polyacrylamide gel electrophoresis are the two common methods for manual analysis of fragments generated using molecular marker systems. Separated fragments are detected either by ethidium bromide, $GelRed_{TM}$ (Biotium) or silver staining of the gels. However, accurate sizing is difficult with both agarose and polyacrylamide gels and these matrices do not allow resolution to within a single base pair unit. Moreover the mobility is also affected by sequence composition so that repeat unit confounds migration of complementary strands in a gel-based system. For example, CA strands moves faster on denaturing polyacrylamide gels than GT strands and this can result in two bands instead of one for the same allele (Saitoh *et al.*, 1998).

In marker-assisted selection using SSRs, accurate sizing of alleles is crucial and detection system need to have a high resolution power to even differentiate a single base pair difference. This can now be achieved through automated fluorescent-based capillary detection system such as ABI 3130 and ABI 3730 (Applied Biosytems). These systems allow co-loading, whereby there is individual amplification of multiple SSR loci and thereafter pooling the PCR products. This takes place if the PCR products are labeled with different fluorescent labels or if the allele sizes are different to avoid overlapping of the alleles.

3.2 Materials and methods

3.2.1 Plant material: Parents and F_1 generation

Seeds of inbred lines of Ochuti (recurrent parent) and E36-1(donor parent) were sown in a greenhouse at the College of Agriculture and Veterinary Sciences (CAVS), University of Nairobi, in May 2009 and two leaves of each parent were harvested 14 days after sowing. The leaves were preserved in 70% ethanol in eppendorf tubes and transferred to Biosciences east and central Africa (BecA) laboratories at the International Livestock Research Institute (ILRI), Nairobi, for DNA extraction and genotyping. The plants continued growing and at flowering, artificial hybridization was done by hand emasculation and pollination. Normal bisexual florets of Ochuti were hand emasculated and pollen from E36-1 transferred to the stigma of the emasculated florets as shown in fig.2 below. The resulting F_1 seeds were sown and the leaves of 96 F_1 progenies harvested and stored in 70% ethanol in eppendorf tubes for genotyping to confirm the heterozygous F_1 genotypes. At flowering of F_1 genotypes, heterozygous individuals were back-crossed to Ochuti (Recurrent parent) to generate BC_1F_1 genotypes.



A-sorghum panicle ready for emasculation; **B**-hand emasculation using a toothpick; **C**-cross pollinated sorghum plants

Figure 2: Emasculation of the sorghum panicle and cross-pollination

3.2.2 DNA extraction

The sampled leaf from each plant was used for DNA extraction using the Cetyl-trimethyl Ammonium Bromide (CTAB) mini-prep method as developed by Mace *et al.* (2004). Two steel beads were inserted in each well of a strip tube (Green tree Scientific, USA) together with the leaf samples cut into small pieces to enable easy maceration of the samples and to increase the surface area for detergent activity. Pre-heated (65°C) extraction buffer 450μl containing 3% (w/v) CTAB, 1.4M NaCl, 0.2 % (v/v) β-Mercapto-ethanol and 20 mM EDTA was added to the leaf samples and macerated using a SPEX Sample Prep 2000 Geno/Grinder®. The macerated substance was incubated for 15 minutes at 65°C with occasional mixing. Solvent extraction was done by adding 450μl chloroform: isoamyalcohol (24:1) to each sample and mixed thoroughly by inversion. The tubes were centrifuged at 4000 rpm for 10 minutes at 24°C using Allegra™ 25R centrifuge (BECKMAN $COULTER_{TM}$) and the upper portion transferred into clean tubes (about 400μl). Isopropanol (0.7 volume) was added and inverted to mix and the tubes were centrifuged after 20-30 minutes incubation at -20 °C at 4000rpm for 15 minutes using Allegra™ 25R centrifuge (BECKMAN $COULTER_{TM}$). The supernatant was decanted and the pellet air dried for 30 minutes. 200μl low salt TE buffer (1mM Tris and 0.1mM EDTA, pH 8) with 3μl RNase A (10mg/ml) was added to each sample and incubated at 37°C in a water bath to digest the RNA. A second solvent extraction was done by adding 200μl chloroform: isoamyalcohol (24:1) to each tube and inverting twice to mix and centrifuged (Allegra™ 25R centrifuge). After centrifugation the aqueous layer was transferred into clean tubes. 315μl ethanol and 1/10 volume of 3M sodium acetate solution (pH 5.2) was added to each sample and then placing the samples at -20°C for 5 minutes to allow precipitation. The tubes were then centrifuged at 4000rpm (Allegra™ 25R centrifuge) for 5 minutes and the supernatant discarded. To wash the DNA pellet, 200μl of 70% ethanol was added and centrifuged at 4000 rpm for 5 minutes. The DNA

pellet was air-dried for one hour and then re-suspended in 100µl low salt TE (10mM Tris, 1mM EDTA pH 8) buffer and stored at 4°C.

3.2.3 DNA Quality and purity check

DNA quality was determined using electrophoresis with 0.8% (w/v) agarose gel stained with GelRed$_{TM}$ (Biotium) 5µl/100ml TBE (Tris base, boric acid and 0.5M EDTA pH8) buffer. This was a critical step since high quality genomic DNA was required for PCR during genotyping. 2µl of DNA was mixed with loading buffer (25mg bromophenol blue (0.25%), 25mg xylene xyanol (0.25%), 4g sucrose (40%)), were electrophoresed for 1 hour at 100volts in a 1X TBE buffer (Tris base, boric acid and 0.5M EDTA pH8). The fragments were visualized under UV light and photographed using a Scion camera (Scion Corporation). The DNA quantity was determined using a Nanodrop spectrophotometer (Themo scientific). The minimum required concentration for PCR reaction was 10 ng/µl.

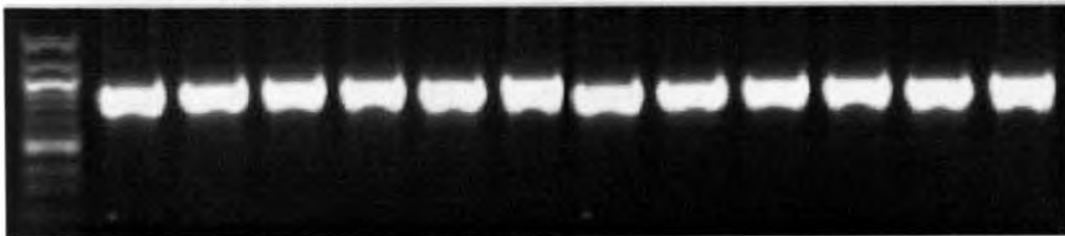


Figure 3: Agarose gel (0.8% w/v) image showing good quality, intact genomic DNA

3.2.4 PCR and capillary electrophoresis

A set of 24 foreground SSR markers (table 2) were used for genotyping. The markers used were M-13 (-21) tailed (5'CAC GAC GTT GTA AAA CGA C 3') forward primers. These foreground markers selected were those that flank the QTL that confer the stay green trait in variety E36-1.

The PCR components for a 10 µl reaction were: 2 mM $MgCl_2$, 1x PCR buffer, 0.20 µM reverse primer, 0.04 µM forward primer, 0.16 µM florescent dye label, which was either FAM, VIC, PET or NED, 0.04 mM of each of the four dNTPs and 0.2 U DNA polymerase (Sibenzyme®), 30ng template DNA and top up to 10 µl reaction volume, double distilled water was added. Temperature cycling was carried out using a GeneAmp® PCR system 9600 (PE-Applied Biosystems) with the following protocol: 15 min at 94°C , 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, with a final extension of 20 min at 72°C. Following PCR, a few reaction products from each SSR marker were randomly selected to confirm proper amplification and product concentration on a 2% (w/v) agarose gel. Samples that amplified well were subjected to capillary electrophoresis to determine their sizes.

Table 2: Foreground markers used to screen 96 F1 samples generated from Ochuti x E36-1 cross

Marker name	Flourescent dye	Allele sizes		Chromosome with stay-green QTL
		E36-1	Ochuti	
Xcup24	6-Fam	188	188	SBI-01
Xcup33	Ned	284	284	SBI-01
Xcup032	Vic	155	155	SBI-01
Xtxp043	Pet	152	152	SBI-01
Xtxp088	6-Fam	114	114	SBI-01
Xtxp149	Ned	196	196	SBI-01
Xtxp357	Vic	120	120	SBI-01
Xisep1028	6-Fam	223	223	SBI-01
Xisp0276	Pet			SBI-01
Xisp0324	6-Fam	135	135	SBI-01
Xcup057	Ned	200	200	SBI-07
Xgap342	Pet	304	260	SBI-07
Xisep0328				SBI-07
Xtxp159	Ned	193	195	SBI-07
Xtxp227	Vic	120	120	SBI-07
Xtxp278	Pet	153	153	SBI-07
Xtxp312	6-Fam	235	160	SBI-07
Xgap001	Ned	259	266	SBI-10
Xtxp141	Vic	170	170	SBI-10
Xcup16	6-Fam	252	262	SBI-10
Xisep0639	6-Fam	95	95	SBI-10
Xcup07	Ned			SBI-10
Xcup66	Vic	105	105	SBI-10
Xcup43	Pet	243	243	SBI-10

Allele detection method was by use of capillary electrophoresis on an ABI 3730 DNA sequencer (Applied Biosystems), a fluorescent based detection system that uses a highly optimized polymer as the separation matrix. This facilitated accurate sizing of the microsatellite alleles to within ±0.3 base pairs (Buhariwalla and Crouch, 2004). PCR products of 3-4 individual primer pairs were co-loaded post-PCR based on the florescent dye, fragment size and dye florescence strength; to reduce the unit cost of high throughput genotyping. 1.5 - 2.5 µl labeled PCR products (depending on the intensity of the bands on the agarose gel) were loaded and mixed with 7.84µl Hi-Di formamide (PE-Applied Biosystems) helped to keep the double strands of DNA apart together with 0.16 µl GeneScan Liz 500 internal molecular weight size standard (orange) (Applied Biosystems) before denaturing at 94°C for 5 minutes on a GeneAmp® PCR system 9600 (PE-Applied Biosystems).

3.2.5 Data analysis

Sizing of the PCR products of 35-500 base pairs was facilitated by GeneScan Liz 500 internal lane size standard which had fragment sizes of 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 base pairs. Analysis was done using GeneMapper® Software v4.0 where the allele(s) of each genotype in form of peaks were inspected and to ensure correct sizing. To ensure reproducibility of the results in each electrophoresis run, the two parents were included in every run as control.

3.3 Results

3.3.1 DNA quality and purity check

Most of the samples had high molecular weight ranging from 16.45ng/µl in sample ID no.89 to 663.26 ng/µl in sample ID no.50 (Appendix I). The optical density (OD) ratio (260nm/280nm) which helps assess the DNA quality ranged between and 1.79 to 2.04 which

indicates that the quality of DNA was ideal for PCR reactions. The pure DNA OD ratio 260nm/280nm range is 1.8-2.0. On the agarose gel electrophoresis the good quality DNA showed a clear band while a low quality DNA showed a smear.

3.3.2 PCR and capillary electrophoresis

Agarose electrophoresis of PCR products showed successful amplification of the foreground markers as shown in Figure 4 below.

Figure 4: Agarose (2% w/v) gel showing successful amplification for Xgap342 used in foreground screening

The amplicons were then separated through capillary electrophoresis to generate raw data that was later analyzed using GeneMapper® Software v4.0. Allele calling using GeneMapper helped identify the polymorphic markers and the heterozygous genotypes at F_1 generation as shown in Figure 5 below.

3.3.4 Screening for polymorphism

Screening for polymorphism was done for both foreground markers and background markers. A total of 24 foreground SSRs and 128 background SSR primer pairs were screened for polymorphism with the two parents. Among these, five foreground (Table 3) and 30 background markers (Table 4) were found to be polymorphic.

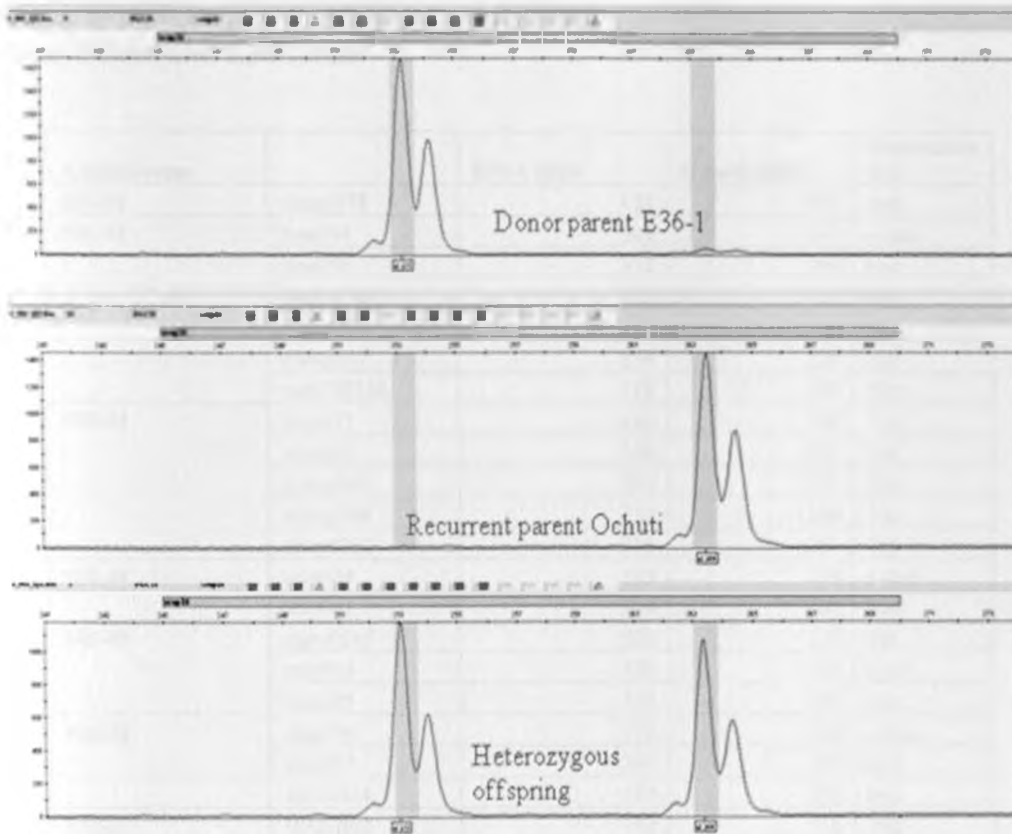


Figure 5: Electropherograms from ABI3730 capillary sequencer showing the donor parent allele, recurrent parent allele and the heterozygous offspring

Table 3: Polymorphic foreground markers for genotyping F_1 genotypes from Ochuti x E36-1 cross and subsequent generations

Marker	Ochuti	E36-1	Linkage Group
xtxp159	195	193	SBI-07
xtxp312	160	235	SBI-07
xgap342	260	304	SBI-07
xcup016	262	252	SBI-10
xgap001	266	259	SBI-10

Table 4: Polymorphic background markers for genotyping BC_1F_1 and BC_2F_1 arising from Ochuti and E36-1cross

Chromosome		E36-1 allele	Ochuti allele	Fluorescent dye
SBI-01	xiabtp378	173	191	Ned
SBI-02	xtxp304	264	213	6-Fam
	xtxp096	152	196	Ned
	Xiabtp168	102	115	Vic
	xiabtp346	190	194	Ned
	xiabtp247	250	238	Ned
	msbCIR238	112	107	Vic
SBI-03	xtxp033	246	249	Vic
	xiabtp29	219	251	Pet
	xiabtp369	269	271	Pet
	xiabtp386	239	249	Vic
	xisep0101	233	231	Pet
SBI-04	xtxp024	169	174	6-Fam
	xtxp021	194	188	Ned
SBI-05	xisp10215	223	220	Pet
	xtxp015	228	238	6-Fam
	xtxp225	195	187	Ned
SBI-06	xtxp176	178	180	6-Fam
	xtxp057	264	266	Ned
	xiabtp424	189	191	Ned
SBI-07	sbAGB02	120	116	Vic
	xiabtp26	115	111	6-Fam
	xiabtp361	289	291	Vic
SBI-08	msbCIR240	126	128	6-Fam
	xiabtp310	230	228	Pet
SBI-09	xtxp258	238	212	6-Fam
	xisep0506	234	227	'
	Xiabtp103	273	248	6-Fam
	xisep0550	204	209	Pet
SBI-10	msbCIR283	132	138	Ned

3.3.5 Confirmation of heterozygous F_1 individuals

Out of ninety six F_1 genotypes, only five that had at least one stay green QTL. Genotypes ID no. 17, 28 and 32 had two stay green QTL; SBI-07 and SBI-10 QTL. Genotypes ID no. 8 and 10 had one QTL; SBI-10 QTL introgressed (Table 5). The true F_1 individuals are those that are heterozygous, that is, there are two different alleles for each marker: Recurrent parent (Ochuti) allele and donor parent (E36-1) allele. For example, sample 17 has alleles of Ochuti and E36-1, 262 and 252 respectively at locus represented by marker Xcup16, 266 allele for

Ochuti and 259 allele for E36-1 at locus represented by marker Xgap001, thus the sample is heterozygous for stay green QTL at chromosome 10.

Table 5: Heterozygous F1s at Chromosome 7 and Chromosome 10 QTL

Sample ID No.	SBI-10 QTL				SBI-07 QTL						Introgression
	XCUP16		Xgap001		Xtxp159		Xtxp312		Xgap342		
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	
E36-1	252		259		193		235		303		
Ochuti		262		266		195		160		305	
8	252	262	259	266	193	195	235	160			Single introgression SBI-10 QTL
10	252	262	259	266			235	160			Single introgression SBI-10 QTL
17	252	262	259	266	193	195	235	160	303	305	Double introgression SBI- 7 and SBI-10 QTL
28	252	262	259	266	193	195	235	160	303	305	Double introgression SBI- 7 and SBI-10 QTL
32	252	262	259	266	193	195	235	160	303	305	Double introgression SBI- 7 and SBI-10 QTL

The rest of the samples screened for heterozygosity are shown in Appendix II.

3.4 Discussion

In this study, the DNA isolated amplified well with the SSR primers used. The quality was characterized by non-smeared bands on the 0.8% (w/v) agarose gel (Figure 5). This signifies intact non-degraded DNA. The concentrations of the DNA were enough for PCRs required as was measured by spectrophotometer which shows the absorbance of DNA solution at 260 nm and 280 nm wavelengths. The spectrophotometer also indicates the presence of protein contaminants but does not indicate whether the DNA is degraded or not (Semagn 2006a). If the 260nm/280nm ratio is lower than 1.8, it is an indication of presence of protein, phenol or other contaminants that absorbs strongly at 280nm wavelength. A ratio of >2.0 it indicates

RNA contamination (Thermo Scientific, 2008). According to Powel *et al* (1996) as reviewed by Mital and Kumar (2009), SSRs require small amount of DNA as a starting material.

Most of the foreground and background markers screened for polymorphism did not reveal the differences between E36-1 and Ochuti. This means that they were homozygous and therefore it would be difficult to discriminate between each of the parents' allele in the progeny. Adequate polymorphism is critical in construction of linkage maps as well as marker-assisted selection (MAS) (Young, 1994). For instance, in this study, marker xcup 24 was monomorphic with allele size of 188 base pairs in both E36-1 and Ochuti. This reveals that the two parents are closely related at this locus.

Offspring of a cross between the two distantly related parents should normally be expected to be heterozygous. The markers used to confirm this should be co-dominant as is the case of SSRs (Semagn *et al.*, 2006a). Out of 96 genotypes screened for heterozygosity, only three genotypes were confirmed to be heterozygous meaning that they had the alleles for donor and recipient parents. Theoretically, an F_1 should possess 50% of each of the parent genome (Frisch *et al.*, 1999a).

CHAPTER 4

GENOTYPING BC_1F_1 AND BC_2F_1 POPULATIONS FOR STAY GREEN QTL FROM A GENERATION OF A CROSS OF OCHUTI X E36-1

Abstract

The objective of this study was to introgress the stay green QTL into Ochuti recurrent parent from donor E36-1 while recovering the recurrent parent genome. This was done by backcrossing the five heterozygous F_1 genotypes to Ochuti to generate BC_1 F_1. 128 samples of BC_1F_1 were genotyped with 5 polymorphic SSR markers for stay green trait targeting two possible QTL from the previous F_1 generation. 32 genotypes were found to be possessing one or two stay green QTL. 22 of these genotypes had more that 70% of the Ochuti genome after genotyping them with 30 polymorphic background SSR markers. These genotypes were advanced to back cross two (BC_2) with introgression of donor segments flanked by SSR markers for single or double stay green QTL from donor E36-1. 157 samples of BC_2F_1 were genotyped and 45 samples had either a single or double introgression of stay green QTL. Upon flowering these progenies were selfed to generate BC_2F_2. These results confirm that it is possible to introgress stay-green QTL into a preferred local genetic background and be able to generate drought tolerant sorghum progenies in two back crosses.

4.1 Introduction

4.1.1 Backcross breeding

Backcross breeding aims at introgression and fixing of the favourable alleles in the farmer-preferred line with as little as possible of the remainder of genome from the donor line (Allard 1999). The proposed route is through a number of generations of backcrossing a line that carries the allele to be introgressed to a recipient line followed by a selfing to make the desired allele homozygous (Hospital *et al.*, 1992). The goal of doing so is to obtain a line as identical as possible to the recurrent parent with the addition of the gene of interest. With each succeeding backcross generation, a greater proportion of the recurrent parent genes are obtained along with the donor line's gene(s) of interest.

Conventional backcrossing involves selection based on the phenotypic scoring of the trait of interest. Introgression of a single gene is usually accomplished by six generations of backcrossing (Allard, 1960). If the gene is recessive, the process requires more generations of selfing thus nine or more seasons are needed for the phenotypic identification of the homozygous recessive plant to be used in the next backcross generation. Using markers, this can be achieved by the forth backcross (BC_4), third (BC_3) or even BC_2 (Visscher *et al.*, 1996; Hospital and Charcosset 1997; Frisch *et al.*, 1999). This saves two to four back cross generations.

MAB is the efficient method of backcrossing that accelerates recovery of the recurrent parent genome (Frisch *et al.*, 1999). DNA-based markers are used to select genotypes in every backcross generation, that carry the introgressed gene, then among these those carrying the lowest proportion of donor genes at other loci are chosen. MAB aims at reducing the length of the donor-type segment carried along with the introgressed gene to an acceptable size and recovery of the composition of the recipient genome as quickly as possible (Frisch *et al.*,

1999). Compared to conventional backcross approach, MAB can save two to three backcrosses and considerably reduces the time required for the release of an improved variety (Servin and Hospital, 2002).

Additional advantages of MAB include: avoidance of vagaries in phenotyping due to abiotic and biotic stresses when the targeted locus controls a trait whose expression is influenced by such factors, reduction in the number of individuals to be screened in each selection cycle and identification of individuals with a shorter segment introgressed from the donor parental line.

4.2 Materials and methods

4.2.1 Plant materials: BC_1F_1 and BC_2F_1

Seeds of presumed BC_1F_1 generated from the cross of F_1 individuals and Ochuti were sown in a greenhouse of the College of Agriculture and Veterinary Sciences (CAVS), University of Nairobi. Two leaves of each of the 128 plants were harvested 14 days after sowing at two-leaf stage. The leaves were preserved in 70% ethanol in eppendorf tubes for DNA extraction and genotyping. Preliminary genotyping was done in the BecA laboratories (ILRI Nairobi) to identify the samples possessing stay green QTL. Based on genotyping information BC_1F_1 plants were selected and backcrossed to the recurrent parent to generate BC_2F_1. The resulting BC_2F_1 seeds were sown and the leaves of 157 BC_2F_1 progenies harvested for genotyping. The 157 BC_2F_1 were screened using QTL-flanking markers for donor parent alleles (foreground selection) and using markers located away from the target stay green QTL regions to identify individuals possessing the highest proportion of the recurrent parent genome (background selection). The selected individuals were selfed to produce BC_2F_2 or BC_2S_1.

4.2.2 DNA extraction, quantification and normalization

Genomic DNA was isolated from leaf samples of BC_1F_1 and BC_2F_1 using the CTAB mini-prep method of Mace *et al.* (2004) as described in section 3.2.2.

Determination of quality, quantity and purity of the isolated DNA was essential and were determined using agarose (0.8% (w/v) gel and Nanodrop (Thermo Fisher Scientific) spectrophotometer. All DNA samples were diluted to the required concentration (10 ng/µl). This was necessary to ensure uniformity in results.

4.2.3 PCR and capillary electrophoresis

4.2.3.1 Foreground selection

A set of five polymorphic foreground SSR markers which were previously used to confirm heterozygosity of F_1s were run with 128 and 157 samples of BC_1F_1 and BC_1F_2 respectively. The PCR reactions were set in 10 µl volume in 384-well PCR plates with the PCR mix containing 30ng template DNA, 2 mM $MgCl_2$, 1x PCR buffer, 0.20 µM reverse primer, 0.04 µM forward primer, 0.16 µM florescent dye label, which was either FAM, VIC, PET or NED, 0.04 mM of each of the four dNTPs and 0.2 U DNA polymerase (Sibenzyme®). The final volume of the reaction mixture was topped up to 10µl with doubled distilled water. Temperature cycling was carried out using GeneAmp® PCR system 9600 (PE-Applied Biosystems) under the following conditions: 15 min at 94°C , 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, with a final extension of 20 min at 72°C. The PCR products were ran on 2 %(w/v) agarose gel to confirm amplification and the allele sizes were determined in a DNA analyzer.

Genotyping was carried out by capillary electrophoresis using the ABI 3730 DNA sequencer (Applied Biosystems), a fluorescent based detection system. PCR products were co-loaded post-PCR based on dye label and expected fragment size to reduce the unit cost of high

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throughput genotyping. 1.5 -2.5µl labeled PCR products (depending on the intensity of the bands on the agarose gel) were loaded and mixed with 7.84µl Hi-Di formamide (PE-Applied Biosystems) and 0.16µl GeneScan Liz 500 internal molecular weight size standard (orange) (Applied Biosystems) before denaturing at 94°C for 5 minutes on a GeneAmp® PCR system 9600 (PE-Applied Biosystems). Formamide (PE-Applied Biosystems) helped to keep the double strands of DNA apart.

4.2.3.2 Background selection

A set of thirty polymorphic SSR background markers screened in section 3.3.4. were run with 32 and 45 samples of BC_1F_1 and BC_1F_2 respectively. These genotypes were those that possessed at least one stay green QTL after foreground selection. PCRs and capillary electrophoresis were done as described in foreground selection of section 3.3.2.

4.2.4 Data analysis

Analysis of fragments from ABI 3730 DNA sequencer (Applied Biosystems) was done using GeneMapper® Software v4.0. Sizing of the PCR products of 35-500 base pairs was facilitated by GeneScan Liz 500 internal lane size standard. To verify the repeatability of PCR and capillary electrophoresis run of each SSR marker, a control of the two parents (Ochuti and E36-1) were included.

4.3Results

4.3.1 Quality and Quantity of the isolated DNA

The genomic DNA isolated showed high purity according to the nanodrop spectrophotometer reading as shown in Figure 6 below. Most of the samples had 260/280 ratio of between 1.8 and 2.0 although there are some that had < 1.8 and > 2.0 (Appendix 1).

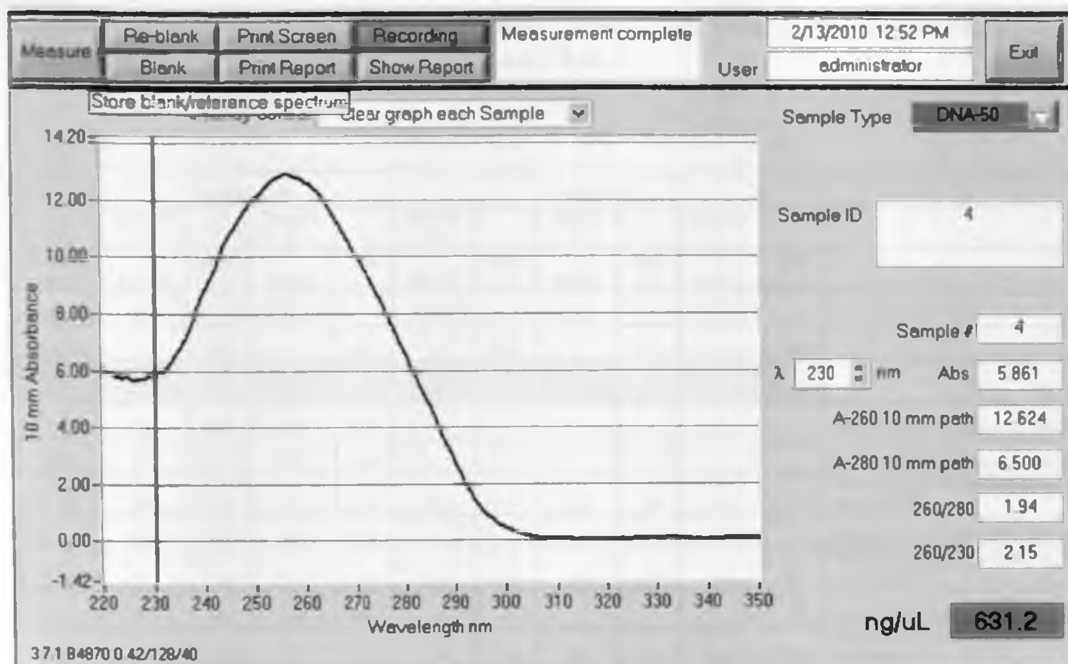

Figure 6: Graphical representation of good quality DNA demonstrated by ratio of absorbances (260/280nm) and DNA concentration (ng/µl)

4.3.2 Foreground selection of BC_1F_1

This was done to identify the samples that had the stay green QTL using five polymorphic SSR markers that are tightly linked to the QTL. Of 128 samples genotyped, 32 were found to have introgressed one or two stay green QTL. Among these, one sample was found to have introgressed SB1-07 stay green QTL, 11 SBI-10 whereas twenty others had introgressed two stay green QTL at SBI-07 and SBI-10 (Table 6). Single and double introgression means that one and two QTL were successfully introgressed respectively. In Table 6 below, the highlighted genotype for example sample 17, is the F_1 genotype which was heterozygous for stay green QTL have introgressed two QTL and was the female parent during backcrossing. The successive samples for example 17_04 and 17_19 are the BC_1F_1 genotypes derived from a cross between sample 17 and Ochuti.

Table 6: Genotypes with introgressed stay- green QTL from E36-1 in the BC_1F_1 generation for a cross between Ochuti and E36-1

	SBI-10 QTL						SBI-07 QTL				
	XCUP1 6		Xgap00 1		Xtxp15 9		Xtxp31 2		Xgap34 2		
sample name	allele 1	allel e 2	allele 1	allel e 2	allele 1	allel e 2	allele 1	allel e 2	allele 1	allel e 2	
E36_1	252		259		193		238		303		
Ochuti		262		266		195		160		305	
Sample 17	252	262	259	266	193	195	238	160	303	305	
17_04	251	262	259	266							Single QTL introgression for SBI-10
17_19	251	262	259	266							Single QTL introgression for SBI-10
17_20	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_24	251	262	259	266							Single QTL introgression for SBI-10
17_26	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_27	251	262	259	266							Single QTL introgression for SBI-10
17_29	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_30	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_31	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_32	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_33	251	262	259	266							Single QTL introgression for SBI-10
17_36	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
17_37	251	262	259	266							Single QTL introgression for SBI-10
Sample 28	[illegible]	262	259	266	193	195	238	160	303	305	
28_04	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
28_13	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
28_17	251	262	259	266							Single QTL introgression for SBI-10
28_19	251	262	259	266							Double QTL introgression for SBI-07 and SBI-10
28_23	251	262	259	266	193	195	238	160	303	305	Double QTL introgression for SBI-07 and SBI-10
28_28					193	195	235	160	303	305	Single QTL introgression for SBI-07
28_29	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
Sample 32	252	262	[illegible]	266	193	195	235	160	303	305	
32_01	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_04	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_05	251	262	259	266							Single QTL introgression for SBI-10
32_07	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_08	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_09	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_11	251	262	259	266	193	195					Single QTL introgression for SBI-10
32_14	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_15	251	262	259	266							Single QTL introgression for SBI-10
32_19	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_22	251	262	259	266	193	195	235	160	303	305	Double QTL introgression for SBI-07 and SBI-10
32_26	251	262	259	266							Single QTL introgression for SBI-10

4.3.3 Background screening BC_1F_1

This was done to identify individuals advanced from foreground selection that had the highest proportion of Ochuti with use of polymorphic SSR markers distributed across the 10 sorghum chromosomes. 30 markers were used.

Out of 32 samples identified to be having stay green QTL in foreground selection, 22 were selected to be advanced to the next backcross generation after background selection. This selection was done in preference to samples that had 70% and above of the homozygous recurrent parent loci combined with heterozygous loci (Table 7).

Table 7: Marker-assisted background selection of BC_1F_1 progenies from Ochuti x E36-1 generation of a cross

Genotype	No. Of homozygous RP loci	No. Of heterozygous loci	No. of homozygous DP loci	No Data	% of ochuti recovered	% heterozygous loci	%RP+% heterozygoous loci
17 20	25	3	1	1	83	10	93
17 24	15	13	2	0	50	43	93
17 26	12	9	6	3	40	30	70
17 27	9	9	5	7	30	30	60
17 29	13	8	3	6	43	27	70
17 30	13	11	4	2	43	37	80
17 31	5	5	1	19	17	17	33
17 32	14	9	5	2	47	30	77
17 33	15	6	3	6	50	20	70
17 36	16	8	5	1	53	27	80
17 37	15	5	7	3	50	17	67
28 04	12	8	7	3	40	27	67
28 13	11	10	2	7	37	33	70
28 17	3	2	1	24	10	7	17
28 19	2	2	1	25	7	7	13
28 23	17	12	1	0	57	40	97
28 28	14	10	4	2	47	33	80
28 29	10	12	5	3	33	40	73
32 01	15	10	3	2	50	33	83
32 04	17	10	1	2	57	33	90
32 05	7	10	9	4	23	33	57
32 07	12	10	6	2	40	33	73
32 08	16	7	5	2	53	23	77
32 09	13	10	6	1	43	33	77
32 11	12	8	2	8	40	27	67
32 14	14	9	2	5	47	30	77
32 15	8	15	3	4	27	50	77
32 19	13	11	2	4	43	37	80
32 22	10	11	6	3	33	37	70
32 26	11	11	5	3	37	37	73

4.3.4 Foreground screening BC_2F_1

A total of 157 samples were genotyped for the possible introgression of stay green QTL at chromosomes SBI-07 and SBI-10. Among these, 46 samples were found to have introgressed at least one stay green QTL and others having double introgression as shown in Table 8. However there are those that did not introgress any of these QTL and this is shown in Appendix III.

Table 8 below shows the parents that generated F_1s and the progenies of a cross between F_1s and recurrent parent, Ochuti. The highlighted samples are the parents of the successive genotypes. For example, E36-1 and Ochuti are the parents of sample 17_20; sample 17_20 is the female parent of sample 17_20_2 with Ochuti being the male parent.

Table 8: Genotypes introgressing stay green QTL in BC_2F_1

	SBI-10 QTL						SBI-07 QTL				
Sample Name	XCUP16		Xgap001		Xtxp159		Xtxp312		Xgap342		
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	
E36 1	252		259		193		238		303		
Ochuti		262		266		195		163		305	
17 20	251	262	259	266	193	195	238	163	303	305	
17 20 2	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
17 26	251	262	259	266	193	195	238	163	303	305	
17 26 3	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
17 27	251	262	259	266	193		238	163	303	305	
17 27 1	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
17 29	251	262	259	266	193	195	238	163	303	305	
17 29 1	252	262	259	266			237	163	303	305	Single QTL introgression for SBI-10
17 29 6	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
17 32	251	262	259	266	193	195	238	163	303	305	
17 32 3	252	262	259	266	193	195		163	303		Single QTL introgression for SBI-10
17 32 6	252		259		193	195	237	163	303	305	Single QTL introgression for SBI-07
17 33	251	262	259	266	193		238	163	303		
17 33 4	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
17 36	251	262	259	266	193	195	238	163	303	305	
17 36 4	252	262	259	266	193	195		163	303	305	Single QTL introgression for SBI-10
17 36 5	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
17 36 1	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10

Sample name	Allele 1	Allele 2	Allele1	Allele2	Allele 1	Allele2	Allele 1	Alelle 2	Allele 1	Allele 2	
28_13	251	262	259	266	193	195	238	163	303	305	
28_13_2	252	262	259		193	195	237	163	303	305	Single QTL introgression for SBI-07
28_13_3	252	262	259	266			237	163	303	305	Single QTL introgression for SBI-10
28_13_4	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
28_23	251	262	259	266	193	195	238	163	303	305	
28_23_1		262	259	266	193	195	237	163	303	305	Single QTL introgression for SBI-07
28_23_2	252		259	266	193	195	237	163	303	305	Single QTL introgression for SBI-07
28_23_4	252	262	259		193	195	237	163	303	305	Single QTL introgression for SBI-10
28_23_5	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
28_23_7	252	262	259	266				163	303	305	Single QTL introgression for SBI-10
28_29	251	262	259	266	193	195	238	163	303	305	
28_29_10	252	262	259	266	193		237	163	303	305	Single QTL introgression for SBI-10
28_29_12	252	262	259		193	195	237	163	303	305	Single QTL introgression for SBI-07
28_29_13	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
28_29_4	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
28_29_5	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
28_29_9	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
28_04	251	262	259	266	193	195	238	163	303	305	
28_4_1	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
32_01	251	262	259	266	193	195	238	163	303	305	
32_1_2		262	259		193	195	237	163	303	305	Single QTL introgression for SBI-07
32_1_7	252	262	259	266					303	305	Single QTL introgression for SBI-10
32_11	251	262	259	266	193	195		163	303	305	
32_11_1	252	262	259	266	193	195		163	303	305	Single QTL introgression for SBI-10
32_15	251	262	259	266	193	195		163	303	305	
32_15_1	252	262	259	266	193	195		163	303	305	Single QTL introgression for SBI-10
32_15_3	252	262	259	266	193	195		163	303	305	Single QTL introgression for SBI-10
32_19	251	262	259	266	193	195	238	163	303	305	
32_19_1		262	259	266	193	195	237	163	303	305	Single QTL introgression for SBI-07
32_22	251	262	259	266	193	195	238	163	303	305	
32_22_1	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
32_22_3	252	262		266	193	195	237	163	303	305	Single QTL introgression for SBI-07
32_22_4	252	262	259	266	193	195		163	303	305	Single QTL introgression for SBI-10
32_26	251	262	259	266	193	195	238	163		305	
32_26_2	252	262	259	266	193	195	237	163	305		Single QTL introgression for SBI-10
32_07	251	262	259	266	193	195	238	163	303	305	
32_7_3	252		259		193	195	237	163	303	305	
32_08	251	262	259	266	193	195	238	163	303	305	
32_8_1	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
32_8_10	252	262	259	266	193	195			303	305	Single QTL introgression for SBI-10
32_8_2	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
32_8_5	252	262	259	266	193	195		163	303	305	Single QTL introgression for SBI-10
32_8_6	252	262	259	266	193	195		237	303	305	Single QTL introgression for SBI-10
32_8_8	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10
32_8_9		262	259	266	193	195	237	163	303	305	Single QTL introgression for SBI-07

Sample name	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	
32_09	251	262	259	266	193	195	238	163	[illegible]	305	
32_9_1	252	262	259	266	193	195	237	163	303	305	Double QTL introgression for SBI-07 and SBI-10

4.3.5 Background selection

Forty five samples identified to be having stay green QTL in the foreground selection were advanced for background screening. The percentage of the recurrent parent genome recovered for each of these samples is as shown in Table 9.

Table 9: background screening for BC_2F_1

Sample Name	No. of RP loci	No. Of Heterozygous loci	No. of DP loci	% RP recovered	% heterozygous loci	%RP+% heterozygous loci
17_20	25	3	1	83	1	84
17_20_2	27	1	0	90	0.3	90
17_26	12	9	6	40	3	43
17_26_3	22	1	4	73	0.3	74
17_27	9	9	5	30	3.0	33
17_27_1	17	8	3	57	2.7	59
17_29	13	8	3	43	2.7	46
17_29_1	20	9	0	67	3.0	70
17_29_6	19	8	1	63	2.7	66
17_32	14	9	5	47	3	50
17_32_3	18	9	1	60	3.0	63
17_32_6	17	8	2	57	2.7	59
17_33	15	6	3	50	2	52
17_33_4	19	5	2	63	1.7	65
17_36	16	8	5	53	2.7	56
17_36_4	17	5	6	57	1.7	58
17_36_5	22	5	6	73	1.7	75
17_36_1	22	8	0	73	2.7	76
28_13	11	10	2	37	3.3	40
28_13_2	16	3	2	53	1.0	54
28_13_3	18	6	3	60	2	62
28_13_4	19	6	2	63	2	65
28_23	17	12	1	57	4.0	61
28_23_1	17	2	2	57	0.7	57
28_23_2	15	8	3	50	2.7	53
28_23_4	20	3	3	67	1.0	68
28_23_5	19	4	3	63	1.3	65
28_23_7	14	5	10	47	1.7	48
28_29	10	12	5	33	4.0	37
28_29_10	21	1	2	70	0.3	70
28_29_12	15	7	3	50	2.3	52
28_29_13	15	6	3	50	2.0	52
28_29_4	17	7	3	57	2.3	59
28_29_5	20	4	3	67	1.3	68

Sample Name	No. of RP loci	No. Of Heterozygous loci	No. of DP loci	% RP recovered	% heterozygous loci	%RP+% heterozygoous loci
28_29_9	18	6	2	60	2.0	62
28_04	12	8	7	40	2.7	43
28_4_1	22	5	2	73	1.7	75
32_01	15	10	3	50	3.3	53
32_1_2	16	1	6	53	0.3	54
32_1_7	19	3	3	63	1	64
32_11	12	8	2	40	2.7	43
32_11_1	16	5	5	53	1.7	55
32_15	8	15	3	27	5	32
32_15_1	18	5	2	60	1.7	62
32_15_3	20	2	5	67	0.7	67
32_19	13	11	2	43	3.7	47
32_19_1	19	2	5	63	0.7	64
32_22	10	11	6	33	3.7	37
32_22_1	21	2	3	70	0.7	71
32_22_3	18	9	1	60	3.0	63
32_22_4	22	5	1	73	1.7	75
32_26	11	11	5	37	3.7	40
32_26_2	20	6	1	67	2.0	69
32_07	12	10	6	40	3.3	43
32_7_3	19	4	4	63	1.3	65
32_08	16	7	5	53	2.3	56
32_8_1	22	4	2	73	1.3	75
32_8_10	22	4	2	73	1.3	75
32_8_2	21	3	1	70	1.0	71
32_8_5	18	7	1	60	2.3	62
32_8_6	20	5	1	67	1.7	68
32_8_8	21	4	2	70	1.3	71
32_8_9	17	6	3	57	2	59
32_09	13	10	6	43	3.3	47
32_9_1	19	6	3	63	2.0	65

4.3.6 Selection of individuals to be advanced to the next generation

This was done based on the presence of at least one stay green QTL in the foreground selection with priority given to individuals with higher proportion of genome of the recipient parent in background selection.

4.4 Discussion

The DNA isolated for BC_1F_1 and BC_2F_1 samples was as appropriate for genotypic analysis with SSR markers as that of parents and F_1s in chapter 3 .The qualities was high as characterized by absorbance ratios (260nm/280nm).

Five SSR markers that were polymorphic for the parental lines and linked to QTL for stay green on various linkage groups (SBI-07 and SBI-10) were used to select individuals presumably having the donor allele at a particular target stay green QTL. These markers were selected from the linkage map constructed by Bhattramakki *et al.* (2000). Donor parent alleles at foreground marker loci indicate the presence of the target stay green QTL that is flanked by these marker loci (Tanksley 1983; Melchinger, 1990). The markers must be tightly-linked to the target loci; ideally <5cM from the QTL (Collard *et al.* 2005).

The three F_1 genotypes possessing stay green QTL were crossed with Ochuti, the recurrent parent to generate the 32 BC_1F_1 genotypes that were used to generate 157 presumed BC_2F_1 genotypes.

128 BC_1F_1 and 157 BC_2F_1 genotypes presumed to possess a maximum of two putative stay green QTL were genotyped with five SSR markers that map in the vicinity of these QTL . From this 32 BC_1F_1 and 45 BC_2F_1 plants were found to possess the target QTL. The BC_1F_1 generation had twenty plants with double introgressions, sample 28_28 with single introgression of QTL at linkage group SBI-07 and 11 with single introgression of stay green QTL at SBI-10 (Table 6). 20 BC_1F_1 families were selected based on the background markers information. Priority was given to the families which had the most of the recurrent parent genome recovered. In BC_2F_1 there were 19 plants which showed double introgressions, 10 and 16 with single introgressions of stay green QTL located at SBI-07 and SBI-10 respectively. Single introgressions in Tables 6 and 7 are those that are heterozygous with regard to each marker linked to either QTL at SBI-07 or SBI 10 while double introgressions are shown by heterozygosity of all the markers linked to the two QTL at Chromosome 7 and Chromosome 10.

Hospital, (2005) quoted that; the number of samples genotyped per family was determined by the number of QTL introgressed in each family of the previous family. Mugambi, (2009) indicated that for each QTL introgression targeted in a back-cross generation, it is

recommended that 11 to 22 individuals be screened to capture each QTL with a 95% ($p \leq 0.05$) confidence.

Frisch *et al.*, (1999) through simulations concluded that marker-assisted selection is, within certain limits, more efficient for large populations than for higher marker densities.

Plants displaying heterozygous genotypes at these markers were selected before pollination and this reduced the population size by four-fold. The genotypes that were not hererozygous were discarded. In a similar study by Ribaut and Ragot (2007) the population size was reduced by eight-fold where they were introgressing drought tolerance trait in maize.

Thirty SSR markers were distributed in the non-QTL regions across the ten linkage groups were used for background selection in BC_1F_1 and BC_2F_1 generations. This was to ensure that most, if not all, of the genome of the recurrent parent had been recovered by successive backcrosses.

In BC_1F_1, 32 individuals were screened with these markers of these, 20 families with single and double introgressions were selected based on the foreground and background genotyping data. These were those that were heterozygous at all marker loci in the vicinity of target QTL regions (foreground selection) and homozygous for the recurrent parent alleles (Ochuti) in the non-QTL regions (background selection). The selected BC_1F_1 plants were backcrossed to Ochuti to produce BC_2F_1 progenies.

A total of 45 BC_2F_1 progenies were subjected to background screening after confirmation that they posses either one or two possible stay green QTL introgressed. The background was necessary to identify introgression lines having the highest level recovery of the recurrent parent genome in regions not linked to the stay green QTL(s). According to the experiments done by Stam and Zeven (1981), Young and Tanksley (1989a) and Frisch and Melchinger (2000), its shown that without background selection, the introgressed segment(s) could remain fairly long even after several generations of backcrossing hence contributing to the presence of non-target parts of the donor genome in the final breeding product. This is referred to as

linkage drag. For example, young and Tanksley (1989a) reported linkage drag that accompanies backcross breeding programmes when they genotyped the chromosome carrying the *Tm2* disease resistance gene in several tomato cultivars that were developed by introgressing the gene from a wild relative, *Lycopersicon peruvianum* via backcross breeding. They found that even cultivars developed after 20 backcrosses contained introgressed segments as large as 4 cM and one cultivar developed after 11 backcrosses still contained the entire chromosome arm carrying the gene from the donor parent.

The selected BC_2F_1 plants were selfed and BC_2F_2 seeds were produced for further evaluation in terms of their stay green phenotype and/or further backcrossing depending on the level of recurrent parent recovery. From visual observation of the backcross progenies in the greenhouse, there are indications that characters such as the panicle size and plant height of the recurrent parent have been recovered in most of the backcross progenies. However, their performance will be known after a proper evaluation of the progenies in a replicated field trial to assess expression of the stay-green trait and its effects on terminal drought tolerance.

The success story of MAB has been reported by scientists at CIMMYT (International Center for Wheat and Maize Improvement) as they developed quality protein maize (QPM) (Dreher *et al.*, 2001). QPM refers to maize genotypes in which the *opaque2* gene is introgressed along with endosperm modifiers that provide the hard kernel texture. Scientists have successfully developed an innovative combination based on SSR markers for *opaque2* allele and phenotypic selection for kernel vitreousness (through the accumulation of endosperm modifiers) for conversion of normal maize lines into QPM. Three SSR markers, *umc1066*, *phi057* and *phi112* are being utilized in PCR-based assays to select individuals carrying the *opaque2* gene in successive backcross generations, and hence, the time required is reduced to half. In addition, marker-aided background selection can help recover the same level of recurrent parent genome in three generations as would be achieved by six generations of

conventional selections. In this study up to 90% of the recurrent parent was recovered at BC_2F_1 (Table 9).

Sundaram *et al.* (2008) used PCR-based molecular markers in a backcross breeding program to introgress three major bacterial blight resistance genes (*Xa21, xa13 and xa5*) into Samba Mahsuri an indica rice variety from a donor line (SS1113). They conducted both foreground and background selections in their experiment to select for the plants possessing these resistance genes and that have maximum contribution from the recurrent parent genome.

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

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

The efficiency of MAS is generally increased by reducing genetic distance between the flanking markers used for each target QTL. Hospital *et al.* (1997), based on simulation studies, recommended an optimal distance between two adjacent markers of about 5–10 cM. Knapp (1998) predicted that the frequency of recurrent parent genotypes among the selected progenies increased as the selection intensity for the recurrent parent increased as evident in this study (Table 9). Practically, the number of markers that must be used decreases in each successive backcross generation, as once the recurrent parent allele has been fixed at any given non-target region, it is not necessary to continue screening at that locus in subsequent

generations as the locus will remain homozygous for the remaining generations of selection regardless of whether this involves 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.

Marker-assisted selection has the potential to greatly reduce the time required for selecting desirable genotypes with traits of interest (Morris *et al*., 2003). In this study selection was done in the early backcross generations hence reducing the time that would have been taken to do field evaluations in case of conventional breeding methods. Also, with conventional breeding it might have been difficult to differentiate with equal reliability between individuals heterozygous for more than one of the genomic regions contributing to the trait. For instance, in this study it was with precision that the true F_1, BC_1F_1 and BC_2F_1 genotypes were identified. Moreover, the population sizes were drastically reduced comparing with conventional breeding. MAB features higher short-term operational costs during the researched stage but takes less time to complete and provides a better understanding of which genomic regions contain donor parent introgressions. This initial investment is worthwhile since it accelerates the rate of release of improved varieties which generates additional economic benefits (Morris *et al*., 2003).

CHAPTER 5

Summary, conclusion and recommendations

5.1 Summary of the study

This study aimed at introgressing three possible stay green QTL from the donor parent E36-1 into Kenyan farmers'-preferred variety Ochuti by the use of PCR-based molecular markers. The study provides evidence that genotyping is a core component in Marker-assisted backcrossing since it enables selection before anthesis and this reduces on the number of plants handled at every backcross generation.

Of the three possible stay green QTL namely, SBI-07 and SBI-10 were transferred into three individuals out of 96 samples. These three F_1 genotypes were used to generate 128 presumed BC_1F_1 individuals. Five polymorphic foreground markers and thirty background markers were used to select 20 BC_1F_1 genotypes. These 20 individuals were backcrossed to Ochuti to generate 157 BC_2F_1 family lines. From the 157 BC_2F_1 individuals, 45 individuals were found to have introgressed at least one stay green QTL(s)

5.2 Conclusion

Marker-assisted backcrossing can successfully and accurately transfer genomic regions contributing to the stay green trait into a genetically diverse sorghum variety grown in areas prone to drought. The two stay-green QTL introgressed into Ochuti will enhance productivity under drought conditions. Although MAB is an expensive affair it enables breeders to release new varieties within a short time hence it becomes economically viable in the long run.

5.3 Recommendations

The material developed needs to be evaluated in replicated field trials to assess the potential usefulness of the introgressed stay green QTL and the stability of the introgressed genes.

Since there were no polymorphic markers available for this study to confirm the presence of stay-green QTL at linkage group SBI-01, it is recommendable for screening of more markers linked to this QTL for polymorphism and hence heterozygosity of the BC_2S_1 genotypes.

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APPENDIX

Appendix I: DNA concentrations and absorbance ratio (260nm/280nm) for 1211 individuals used in this study

DNA concentrations and absorbance ratio (260nm/280nm)

F.T NANODROP READINGS

Sample ID	ng/ul	260/280
1	78.05	1.89
2	267.93	1.85
3	77.06	1.93
4	130.51	1.84
5	275.48	1.84
6	222.79	1.86
7	139.79	1.84
8	157.44	1.88
9	220.85	1.85
10	360.01	1.85
11	318.68	1.86
12	607.25	1.9
13	152.69	1.86
14	139.67	1.88
15	302.34	1.87
16	248.76	1.84
17	157.46	1.85
18	228.96	1.87
19	218.48	1.87
20	295.08	1.86
21	360.58	1.86
22	256.78	1.84
23	401.55	1.85
24	341.06	1.84
25	292.97	1.86
26	307.32	1.86
27	264.15	1.81
28	379.74	1.86
29	300.53	1.86
30	384.22	1.86
31	587.74	1.84
32	226.54	1.86
33	92.85	1.79
34	133.32	1.84
36	182.8	1.83
37	159.85	1.83
37	255.52	1.84
38	144.14	1.79
39	320.31	1.86
40	140.2	1.85
41	198.51	1.83
42	366.18	1.85
43	496.28	1.87
44	276.7	1.9
45	357.47	1.92
46	328.09	1.9
47	173.59	1.87
48	161.32	1.91
49	225.73	1.89
50	663.26	1.93
51	504.24	1.9
52	423.45	1.9
53	494.93	1.84
54	371	1.9
55	443.26	1.91
56	310.65	1.9
57	111.04	1.85
58	306.35	1.9
59	346.94	1.87
60	357.91	1.89
61	485.38	1.88
62	368.4	1.89
63	427.83	1.89
64	230.28	1.9
65	342.61	1.91
67	501.88	1.88
68	641.51	1.9
69	227.58	1.93
70	189.09	1.9
71	213.61	1.91
72	167.51	1.92
73	282.21	1.9
74	289.8	1.89
75	148.14	1.86
76	221.75	1.86

Sample ID	ng/ul	260/280
87	275.2	1.87
88	201.1	1.84
89	16.45	2.04
90	302.5	1.89
91	228.5	1.88
92	205.7	1.89
93	170.6	1.86
94	158.7	1.86
95	230.8	1.89
96	167	1.87

BC.F. NANODROP READINGS

Sample ID	ng/ul	260/280
8.1	41.19	2.04
8.2	66.06	2.07
8.3	58.66	2.08
8.4	93.8	2.02
8.5	117.5	1.98
8.6	100.3	1.98
8.7	113.1	1.96
8.8	129.7	1.99
8.9	61.95	1.98
8.1	152.9	1.93
8.11	74.27	1.93
8.12	121.9	2.02
8.13	78.83	1.98
8.14	80.03	2.04
8.15	85.11	1.97
8.16	59.55	2
8.17	60.43	1.87
8.18	97.19	1.96
8.19	104.5	1.8
8.2	58.07	1.95
17.1	131.6	1.64
17.2	75.63	1.91
17.3	85.94	1.94
17.4	72.3	1.83
17.5	64.33	1.71
17.6	107.7	1.98
17.7	47.57	1.91
17.8	161.3	1.9
17.9	51.33	1.96
17.1	69.64	2.01
17.11	66.45	2
17.12	94.3	1.63
17.13	90.46	1.63
17.14	37.64	1.77
17.15	147.6	1.8
17.16	59.12	1.89
17.17	71.21	1.8
17.18	106.5	1.86
17.19	70.56	1.82
17.2	37.26	2.02
17.21	81.85	1.92
17.22	48.19	1.88
17.23	231.3	1.54
17.24	155.1	1.68
17.25	212.2	1.6
17.26	134.6	1.65
17.27	80.29	1.95
17.28	95.6	1.76
17.29	66.73	1.88
17.3	75.03	1.85
17.31	6.82	2.4
17.32	92.2	1.83
17.33	9.19	2.34
17.34	111.5	1.74
17.35	171.3	1.63
17.36	65.62	1.92
17.37	46.39	1.95
17.38	107.8	1.72
28.1	84.54	1.7
28.2	143.9	1.64
28.3	139.8	1.77
28.4	128	1.66
28.5	106.4	1.72
28.6	77.6	1.82
28.7	76.64	1.79
28.9	70.93	1.78
28.1	96.8	1.72
28.11	105.8	1.63
28.12	72.3	1.79
28.13	86.21	1.81
28.14	124.9	1.69
28.15	116	1.69
28.16	71.86	1.85
28.17	88.68	1.87
28.18	47.95	1.78

Sample ID	ng/ul	260/280
28.3	46.8	2.21
28.31	42.6	2.07
28.32	71.8	2.1
28.33	49.1	2.08
32.1	64.2	2.14
32.2	15.7	3.2
32.3	17.9	1.61
32.4	68.1	1.84
32.5	84.3	1.9
32.6	57.8	1.86
32.7	47.9	1.87
32.8	17.8	1.85
32.9	16.8	1.99
32.1	19.4	1.75
32.11	33.1	1.93
32.12	90.5	1.9
32.13	139	1.95
32.14	134	1.93
32.15	185	1.96
32.16	33.6	1.85
32.17	32.1	1.9
32.18	18.6	2.34
32.19	67.4	1.71
32.2	119	1.87
32.21	69.9	1.85
32.22	169	1.78
32.23	50.2	1.78
32.24	20	1.92
32.25	40.8	1.89
32.26	9.86	1.66
32.27	34.3	1.87
32.28	84.8	1.89
32.29	128	1.92
32.3	97.5	1.9
32.31	77.4	1.94
32.32	81.6	1.95
32.33	26.1	1.99
32.34	15.1	2.23
32.35	1059	1.95
32.36	1267	1.97
32.37	705	1.98

Appendix II: Allelic data for F_1 progeny of E36-1 x Ochuti genotyped

Sample	SBI-10 QTL				SBI-07 QTL						Introgression
ID No.	XCUP16 allele 1	allele 2	Xgap001 allele 1	allele 2	Xtxp159 allele 1	allele 2	Xtxp312 allele 1	allele 2	Xgap342 allele 1	allele 2	
E36-1	252		259		193		235		303		
Ochuti		262		266		195		160		305	
1		262		266			235	160			
2		262		266		195		160			
3		262		266		195		160			
4		262		266		195		160			
5		262		266		195		160			
6		262		266		195		160			
7		262		266		195		160			
8	**252**	**262**	**259**	**266**	**193**	**195**	**235**	**160**			**Single introgression SBI-10 QTL**
9				266				160			
10	**252**	**262**	**259**	**266**		**195**		**160**			**Single introgression SBI-10 QTL**
11		262		266		195		160			
12		262		266		195		160			
13						195					
14		262		266		195		160			
15		262		266		195		160			
16		262		266		195		160			
17	**252**	**262**	**259**	**266**	**193**	**195**	**235**	**160**	**303**	**305**	**Double introgression SBI- 7 and SBI-10 QTL**
18		262		266				160			
19		262		266		195		160			
20		262		266		195		160			
21		262		266		195		160			
22		262		266		195		160			
23						195					
24		262		266		195		160			
25		262		266		195		160			
26		262		266		195					
27		262		266		195		160			
28	**252**	**262**	**259**	**266**	**193**	**195**	**235**	**160**	**303**	**305**	**Double introgression SBI- 7 and SBI-10 QTL**
29		262		266		195		160			
30		262		266		195		160			
31		262		266		195		160			
32	**252**	**262**	**259**	**266**	**193**	**195**	**235**	**160**	**303**	**305**	**Double introgression SBI- 7 and SBI-10 QTL**
33		262		266		195		160			
34		262		266		195		160			
35		262						160			
36		262		266		195		160			
37		262		266		195		160			
38		262		266		195		160			
39		262		266		195		160			
40		262		266				160			
41		262		266		195		160			
42		262		266		195		160			
43		262		266		195		160			
44		262		266		195		160			
45		262		266		195		160			
46				266		195		160			
47		262		266		195	235				
48		262		266		195		160			
49		262		266		195		160			
50		262		266		195		160			
51		262		266		195		160			
52		262				195		160			
53		262		266		195		160			
54		262		266		195		160			
55		262		266		195		160			
56		262		266		195		160			
57		262		266		195		160			
58		262		266		195		160			
59		262		266		195		160			
60		262		266		195		160			
61				266		195		160			
62		262		266		195					
63		262		266		195		160			
64		262		266		195		160			
65		262		266		195		160			
66		262		266		195		160			
67		262				195		160			
68		262		266		195		160			
69		262		266		195		160			
70		262		266		195		160			
71		262		266		195		160			

ID No.	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	
72		262		266		195		160			
73		262		266		195		160			
74		262				195		160			
75		262		266		195		160			
76		262		266		195		160			
77		262				195	235				
78		262		266		195		160			
79		262		266		195		160			
80		262		266		195		160			
81				266		195		160			
82		262		266		195		160			
83		262				195		160			
84		262		266				160			
85		262		266		195		160			
86		262		266		195		160			
87		262				195		160			
88		262		266		195		160			
89				266		195		160			
90				266		195		160			
91		262		266		195					
92		262				195		160			
93		262		266		195		160			
94		262		266		195		160			
95		262		266		195		160			
96		262		266		195		160			

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