MORPHOLOGICAL CHARACTERIZATION AND IDENTIFICATION OF MOLECULAR MARKERS FOR DWARFISM GENES IN *Sorghum bicolor* L. [Moench]

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DECLARATION

I hereby declare that the dissertation submitted by me in the fulfillment of the requirement of a Masters degree in Genetics at the University of the Free State, is my own independent work and has not previously been submitted by me at another university or faculty. I furthermore cede copyright of the dissertation to the University of the Free State.

Gertruida Martha Botha

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But to God alone, all the Glory and Praise, until the end of time.

"Now He who supplies seed to the sower and bread for food will also supply and increase your store of seed and will enlarge the harvest of your righteousness"

2 Corinthians 9:10

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LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
bp	base pairs
C	amount of DNA present in the haploid genome
cm	centimeter
DNA	deoxyribonucleic acid
DTT	dithiol tri-triol
Dw	dwarfism (dominant)
dw	dwarfism (recessive)
EDTA	ethylenediamin tetra acetic acid
et al.	et alii (and others)
g	grams
ha	hectares
HCl	hydrochloric acid
KCl	potassium chloride
kg	kilo gram
m	meter
ma	maturity gene
$MgCl_2$	magnesium chloride
ml	milliliter
ng	nano gram
NIL	near-isogenic line
nm	nano meter
NaCl	sodium chloride
OD	optical density
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	sodium dodecyl suphate
sec	seconds
SSR	simple sequence repeat
taq	*Thermus aquaticus*
TE	Tris EDTA
Tris-HCl	(Tris[hydroxymethyl]aminomethane) hydrochloric acid
ug	micro gram
UV	ultraviolet
%	percentage
°C	degree Celsius

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General Introduction

Sorghum (*Sorghum bicolor* L. [Moench]) is uniquely adapted to environmental extremes and has the ability to tolerate drought, soil toxicity and temperature extremes more efficiently than any other grain crop (Maunder, 2001). This makes sorghum an important crop in a world predicted to experience severe water scarcity by 2025 (International Water Management Institute, 1998). Current trends in sorghum production suggest a growth of 1.6% annually which will exceed one billion tons by 2008 (Frey, 1996).

The dietary role of sorghum has declined with the introduction of convenience foods like bread and pasta and the availability of maize meal. However, sorghum is an important animal grain feed. Sorghum has also retained its predominant role in the traditional beer brewing industry in most parts of Africa. In Southern Africa alone it has been estimated that about 20 million hectoliters of commercial sorghum beer are brewed annually (Cecil, 1992).

Sorghum conversion programmes are aimed at developing stable and higher yielding cultivars. This is achieved by converting tall, late maturing tropical sorghum varieties with desirable traits to short, early maturing lines, suitable for commercial combine harvesting. Conversion programmes are based on the substitution of genes that control height and maturity (Duncan *et al.*, 1991).

There are four genes associated with determining height in sorghum, dw_1, dw_2, dw_3 and dw_4, with tallness dominant to shortness (Quinby and Karper, 1954). Conversion programmes are aimed at developing three-dwarf lines that are homozygous, recessive at three of the four dwarf loci. However, four-dwarf lines, homozygous, recessive at four dwarf loci are used for backcrossing during conversion (Duncan *et al.*, 1991). The phenotypic selection of three- or four-dwarfs is complicated by environmental effects on plant height.

Photoperiodism has also been found to influence plant height. The longer a plant remains vegetative, the greater the number of nodes it makes. Late maturing sorghum plants are generally taller than early maturing sorghum. The genes controlling maturity have been shown to effect Gibberellinic Acid (GA) biosynthesis. Furthermore, GA has also been shown to alleviate dwarfism in maize mutants. Therefore, it would appear that some link exists between GA biosynthesis, maturity and dwarfism.

Genetic improvement of sorghum grain has historically been achieved through classical plant breeding. However, the advent of molecular marker technology offers great potential to contribute to the genetic improvement of sorghum. In recent years, SSRs (Simple Sequence Repeats) and AFLPs (Amplified Fragment Length Polymorphism) have been used very effectively in marker assisted breeding of different crops and are often considered the molecular markers of choice.

The objective of this study was as follows:

1. Morphologically characterize parent lines and near isogenic lines of different height classes.
2. Determine the effect of exogenous GA on plant height of different isogenic lines.
3. Identify SSR markers for the dwarf genes and deduce the genotype of the different height classes, as well as,
4. Identify AFLP markers for the different dwarf genes in the different near isogenic height classes.

Chapter 1

Sorghum history, uses and importance in Africa and the world

1.1 Origin of Sorghum

The precise origin of sorghum cultivation is lost in time. The current hypothesis outlined by Mann *et al.* (1983) suggests that cultivated sorghum arose from wild *Sorghum bicolor* subsp. *arundinaceum*. The greatest variety of cultivated and wild sorghum occurs in the northeastern region of Africa (Doggett, 1965). Wild species *S. arundinaceum*, *S. verticilliflorum* and *S. aethiopicum* are all currently found in Ethiopia (Prasada Rao, 1979). These wild sorghums are considered to be the source from which sorghum, occurring as a weed during early crop cultivation, was developed (Brhane, 1970). Furthermore, modern *Sorghum bicolor* races resemble wild varieties currently found in Ethiopia (Stemler *et al.*, 1977).

1.1.2 Sorghum distribution in Africa and Asia

Modern *S. bicolor* races are adapted to a wide range of climatic conditions, which probably occurred through habitat adaptation in Ethiopia (Stemler *et al.*, 1977). During tribal migration *S. bicolor* was further dispersed and outcrossed with other regionally adapted wild varieties (Doggett, 1965). It is currently accepted that there are five races of sorghum namely bicolor, guinea, caudatum, dura and kafir. The race bicolor is

widespread throughout Africa and Asia. Guinea is primarily found in West Africa with a secondary centre of origin in Malawi-Tanzania (de Wet *et al.*, 1970). Caudatum is most abundant from east Nigeria to eastern Sudan and southwards into Uganda. Durra is dominant in Ethiopia and westwards across drier zones (De Wet *et al.*, 1970). The durra race is cultivated in the Ethiopian-Sudan region (where it probably originated) as well as the Far East and India (Harlan, 1972). Kafir is primarily grown in East Africa, south of the equator and Southern Africa (House *et al.*, 2000).

1.2 Development of sorghum

1.2.1 Bicolor sorghum

Bicolor is thought to have originated in the Western and Southern parts of Ethiopia, which has a high rainfall and a cold and moist climate (Stemler *et al.*, 1977). The characteristic small and loose panicles of bicolor, which dry out rapidly, are adapted to these conditions. Bicolor is also not susceptible to damage by birds or grain moulds (Doggett, 1988). This type represents the earliest selection of sorghum development from wild species of *S. bicolor* including a variable complex of cultivated varieties as well as stabilised weedy derivatives representing introgression between cultivated and wild relatives (Hallpike, 1970).

1.2.2 Guinea sorghum

The guinea race is characterized by long, gaping glumes with large open inflorescences and is thought to have originated along the northern forest margins of Western Africa

and Northern Uganda (De Wet, 1978). Guinea is adapted to high rainfall (Stemler *et al.*, 1977). It is likely that guinea originated from a cross between a cultivated bicolor and the wild race *S. arundinaceum*, found in the high-rainfall areas of West Africa, introducing a harder grain, as well as adaptation to warmer, lower altitude conditions (Brhane, 1970). Guinea was historically used during the slave trade as ship and overland supplies between West Africa and the sugar estates of Southern Africa, due to the fact that the hard grains store well (De Wet, 1978).

1.2.3 Durra sorghum

Snowden (1936) suggested that durra was developed from a wild race called *S. aethiopicum*, which occurs in drier areas in Ethiopia, has above average seed size, dense panicles and is well adapted to drought. Durra was found dating back to AD 200 in Sudan (De Wet, 1977). As the climate became drier in Ethiopia, early bicolor types were probably introgressed with *S. aethiopicum* and *S. cernuum* resulting in the adaptation to drier conditions (Stemler *et al.*, 1977). The semi-nomadic people of Somalia still cultivate durra type sorghum (Doggett, 1988).

1.2.4 Caudatum sorghum

Caudatum has panicles that range in shape from compact to open and was probably developed to improve pasture characteristics for cattle (Doggett, 1988). This type is grown in the Ethiopian lowlands, Sudan and on the plains of Kenya and Tanzania by the Nuer and the Masai cattle farmers (Cranstone, 1969). It is hypothesised that caudatum originated from an introgression between *S. caudatum* and *S. nigricas* to improve tolerance to environmental conditions experienced on migratory routes (Clark, 1976).

Caudatum does not occur in India, suggesting that it is a younger race than guinea or durra (Stemler *et al.*, 1975).

1.2.5 Kafir sorghum

Kafir sorghum has panicles that are compact and cylindrical with brown or red inflorescences (Brown, 1970). In the classification of Harlan and De Wet (1972), *S. caffrorum* and *S. coriacem* form a distinct group, which was classified as the kafir group. This race is thought to have originated in Tanzania and further southwards in Southern Africa. Snowden (1936) reported kafir collections from Tanzania, Zambia, Zimbabwe, Angola and South Africa. *S. cafforum* is similar to caudatum and durra races with traces of S. *nevrosa* (Doggett, 1988). This race did not spread to West Africa or India and there is no evidence to trace it back to Ethiopia or Sudan. It is thought that kafir originated during tribal migration into Southern Africa (Brown, 1970).

1.3 Morphology of Sorghum

Sorghum is a member of the grass family Gramineae, and subfamily Panicoideae, in the tribe Andropogoneae (De Wet, 1978). Selections of improved varieties are based specifically on morphological characteristics (Stemler *et al.*, 1977). Therefore, it is important to understand the interaction between genetic, physiological and morphological characters, in order to apply selection criteria effectively. The central axis of plants in the genus Sorghum consists of a root, stem or culm, and panicle (Freeman, 1970). The stem begins at the cotyledon and ends in the growing point or terminal bud.

Leaves are lateral expansions of the stem. The flowers are modified stem structures specialised for seed production. Leaves are formed at nodes (Freeman, 1970).

1.3.1 Culm morphology

Growth of the stem is generally erect (Doggett, 1988). It is thought that the number of internodes and their length determines the height of the culm (Quinby, 1975). Three different patterns of internode elongation occur in sorghum: ever-increasing, unimodal and bimodal (Ayyangar *et al.*, 1937). Plants with ever-increasing internode development grow increasingly longer internodes from the ground upwards. Unimodal development results in shorter internodes near the base of the culm. In bimodal development, the internode nearest the base and the top of the culm are shorter than the internodes in between (Quinby, 1975). Basal stem thickness can vary between 0.5 cm and 3 cm (Doggett, 1988). Sorghum stems are solid and the texture of the centre is firm and juicy or dry and pithy (Freeman, 1970). Colour and texture of the culm centre can be associated with starch and sugar content (Freeman, 1970). A heavy coating of wax occurs on the outer stem surface almost completely masking the green colour (Freeman, 1970).

Sorghum is a typical grass with great variation in tiller capacity (Escalada and Plucknett, 1975). Certain varieties form tillers early in their growing season, while others do not tiller until after flowering (Duncan *et al.*, 1981). Sorghum is usually an annual (Freeman, 1970). However, some types can survive for several years through the generation of tillers, especially in regions where frost and seasonal drought do not occur (Duncan *et al.*, 1981).

1.3.2 Leaf morphology

The sorghum leaf consists of a sheath and leaf blade (Downes, 1968). The position where the sheath and the blade meet is the collar. The leaf blade is erect in young leaves and spreads out sideways with a gentle curve as they mature. In some cultivars, the mature leaves retain erectness. The leaf is divided by the midrib into symmetrical or slightly asymmetrical halves. The leaf sheath is attached basally around the node and encloses the culm. The sheath is thickest in the middle and progressively becomes thinner to the sides where it is membranous. The outer surface is glabrous, and the inner surface is white and glossy (Freeman, 1970).

1.3.3 Panicle morphology

The inflorescence is a compound raceme known as a panicle (Ayyangar *et al.*, 1938). The shape of the panicle varies from loose, grassy to compact. The panicle is an extension of the culm or vegetative axis. The inflorescence axis is separated into nodes and internodes although not clearly defined, especially in dense panicles (Kidd, 1952). The main axis is pointed and furrowed. The panicle branches as well as glumes and awns are hairy and are often responsible for allergic reactions in humans (Freeman, 1970). The spikelet is part of the inflorescence and consists of a short floral axis from which the upper glume, lower glume and floret arise. Panicle branches carry pedicelled and sessile spikelets the latter contains functional male and female flower parts (Cowgill, 1926). The fertile floret consists of a lemma, a palea, two lodicules and adroecium of three stamens (anthers, which bear pollen on anther stalks or filaments) and an oval ovary developing into two tiny styles each terminating in a stigma (Cowgill, 1926). After fertilization, the ovule becomes the fruit or caryopsis. The time taken for the grain to

reach its maximum weight varies with growing conditions but is usually between 25 to 55 days after blooming. The grain is generally harvested when the moisture content has dropped below 20% or more preferably below 15%, depending on climatic conditions (Pauli *et al.*, 1964).

1.3.4 Root morphology

The root system includes primary roots which develop from the radicle or first seedling root and adventitious roots which develop on the first stem internode of the young seedling below the soil (Chi, 1942). Mature plant roots are all adventitious and appear on the second node, and have abundant branched lateral roots that interweave into the soil in all directions (Artschwager, 1948).

1.4 Utilisation

1.4.1 Forage

An important consideration for the use of sorghum as fodder is the production of dhurrin (Akazawa *et al.*, 1960). Most sorghum varieties contain the cyanogenic glycoside (dhurrin) that is highly toxic because of its ability to produce prussic acid when hydrolysed (Akazawa *et al.*, 1960). As little as 0.5 g of prussic acid is enough to kill an adult cow. Dhurrin is localised in the aerial shoots of the plant, but is also found in small plants, young branches and tillers. The production of dhurrin depends on the variety of sorghum as well as environmental conditions. When the plants are about 90 cm tall, the hydrocyanic content drops below dangerous levels (Gortz *et al.*, 1982). If sorghum

fodder is cut and sun dried the prussic acid content falls rapidly while ensiling completely destroys it. Forage varieties with low prussic acid levels, are usually grown where rainfall or soil conditions are not suitable for maize production (Wall and Ross, 1970) and include Sudangrass (*Sorghum halepanese*), Columbus grass (*Sorghum almum*) and Johnson's grass (*S. miliaceum* x *S. bicolor*) (Wall and Ross, 1970). Forage sorghum varieties yield on average between 9.5 tons per ha and 11.8 tons per ha dry weight (Koller and Scholl, 1968), in comparison with forage maize yielding between 11.6 tons per ha and 14.5 tons per ha (Sprague and Dudley, 1988). Generally, less frequent harvesting result in higher yields and lower nitrogen concentrations (Sprague and Dudley, 1988). Sorghum silage gives on average lower live weight grains than maize, depending on the cultivar. In most sorghum varieties, the silage nutrient values, on a dry weight basis, are higher than that for maize in terms of protein 7.1% to 10.3% (7.5% to 8.2% in maize), crude fibre 23% to 26.7% (19.4% to 22.0% in maize), ash 6.7% to 9.1% (2.3% to 4.8% in maize) and N-free extract 50.1% to 62.0% (57.9% to 68.0% in maize) (Quinby and Marion, 1960; Sprague and Dudley, 1988). Sorghum is also better adapted to fluctuating rainfall and different soil types than maize (Doggett, 1988).

The use of grain sorghum as feed is extensive, although some races like the hegari, blackhull kafir and red kafir are used for both food and fodder (Desai, 1979). In India the stems and leaves of plants are harvested and used as fodder for small dairies in cities, and a poultry industry has developed in India where sorghum is a major feed (House *et al.*, 2000). In Sudan, the sorghum plants are cut and allowed to dry for feed. However, in many areas of Africa, cattle are allowed to forage in sorghum fields after grain harvesting (House *et al.*, 2000).

1.4.2 Sweet-stemmed Sorghum

Sweet-stemmed sorghum is used as a source of sugar, but its use is limited because processing costs are greater than that for sugarcane (Coleman, 1970). Sorghum was used in the early 1900's for the production of fuel alcohol through fermentation in Brazil, and often combined with sugarcane for this purpose (Schaffert, 1992). However, an over production of alcohol in Brazil resulted in a decline in the use of sorghum for this purpose. Sweet-stemmed sorghums are also used for stock feed and human consumption (Coleman, 1970). In the late 1920's, the annual production of syrup from sorghum fluctuated between 92 and 180 million litres per year in the USA (Doggett, 1988). However during 1954 to 1959, economic pressure altered the planting of sweet-stemmed sorghum in the USA towards larger areas of cane for sugar production and sorghum syrup production dropped to 9 million litres per year. World wide syrup production from sorghum dropped to lowest levels since the 1920's (Doggett, 1988).

1.4.3 Other plant uses

In Africa, stems from tall sorghums are used for making palisades in villages around homes (House *et al.*, 2000). Panels are made from stems and used in the construction of houses (House *et al.*, 2000). Sorghum stems and tillers of thinner varieties are used to make baskets and fish traps. Stem root bases are also used as source of fuel for cooking, particularly during the dry season in areas where trees or shrubs are sparse (Dogget, 1988). In West Africa, red dye is extracted from sorghum and used to colour leather (House *et al.*, 2000). This dye is similar to an extract from sandalwood called santalin (House *et al.*, 2000).

1.4.4 Utilisation of the grain

Sorghum is important in many African countries for human consumption. However, in some African countries, sorghum consumption has declined due to the introduction of maize and rice (Vogel and Graham, 1979). Even though the protein content of sorghum is less digestible than that of maize, rice or wheat, due to tannins (Quinsenberry and Tanksley, 1970), sorghum is an important component of the human diet in many countries, since it is well adapted to a wide range of ecological conditions that are unfavourable to most other cereals (Cecil, 1992; Gomez, 1993).

Sorghum plays a dominant role in the traditional beer brewing industry, at household and industrial levels (House *et al.*, 2000). Sorghum has become synonymous with the beer industry to the extent that brown to red seeded brewing types are widely cultivated. Most farmers, in maize growing regions cultivate a portion of land with red sorghum for household beer brewing and for the sale of excess malt to village brewers. Generally women manage the home-brewing village industry, and sales of malt and home-brew are an important resource of household earnings (Vogel and Graham, 1979). It has been estimated that in Southern Africa alone about 20 million hectolitres of commercial beer is produced annually and a quantity in excess to this is produced traditionally (Cecil, 1992). Traditional sorghum beer is typically slightly sour with an alcohol content of around 3% (House *et al.*, 2000).

In Kenya, sorghum is grown primarily for human consumption. While at least equal to maize in nutritive value, the price of sorghum is considerably higher than maize because of the availability of maize (Vogel and Graham, 1979). Sorghum is traditionally

consumed in the form of a stiff porridge (*ugali*), a thin porridge (*uji*) and a range of fermented beverages known as *busaa* (Vogel and Graham, 1979). It is estimated that 75% of all sorghum produced in Kenya is utilized in beer production (Vogel and Graham, 1979). In the more central rural areas, fermented *uji* is prepared for lactating mothers as it is thought to increase milk production (Ezama, 1979).

In Uganda, white types of sorghum are favoured for food and the red types for brewing (Vogel and Graham, 1979). Sorghum beer is highly regarded and used in ceremonies such as marriages and funerals. The by-products from beer brewing are used as food for homestead poultry production (Ezama, 1979).

In Tanzania, although many farmers have started to plant maize in the past 20 years, sorghum is still favoured, particularly in semi-arid areas where farmers often experience huge crop losses of maize due to unreliable rainfall (Vogel and Graham, 1979). Sorghum production has shown a steady increase in Tanzania, which can be attributed to the research and development done on cultivar improvement to increase yield and reduce maturity time (Mgonja *et al.*, 2001). In Tanzania, the stiff porridge "*Ugali*" is made with a mixture of sorghum and cassava flour (Olatunji *et al.*, 1992). Sorghum is generally considered as "food for the hungry or poor" but also carries a sign of status in the form of beer (Mgonja *et al.*, 2001).

In Nigeria, sorghum is by far the most significant cereal in terms of kilograms consumed per capita. Throughout Nigeria, 60% to 70% of the sorghum is grown in intercropping systems such as sorghum-millet-cowpea or sorghum-millet-groundnut (Olatunji *et al.*,

1992). Consumption is in the form of a thick porridge (*tuwo*) and served with soup or stew (Vogel and Graham, 1979). Gifts of grain sorghum are exchanged at childbirths, naming or circumcision ceremonies as well as at marriages, funerals and harvest festivals (Vogel and Graham, 1979). The use of sorghum malt in non-alcoholic products has increased greatly, particularly, that of malt cocoa as a baby weaning food (Cadbury Nigeria Food Specialities, Nigeria) as well as malt drinks with brand names like Maltina, Evamalt and Malta (House *et al.*, 2000).

Increasing urbanization and changing social and economic trends, as well as the demand for convenience foods, bread in particular, has reduced sorghum consumption in Africa and Asia (Dendy, 1992). Sorghum is currently considered a "poor mans" crop. For example, in dry regions of Africa the demand for wheat exceeds production and a dependence on imported wheat has developed (Dendy, 1992). The potential to replace wheat or to blend sorghum flour with wheat flour to produce breads, pasta, biscuits and snack foods is well researched and has even reached production in some countries (Faure, 1992). However, acceptance and commercialisation of such products is limited (Faure, 1992). One of the economic factors contributing to the decline of sorghum production in Africa is a wheat subsidy introduced by many African governments. However, in Nigeria the use of food from non-wheat products including sorghum has been encouraged by a government ban on all wheat imports (Olatunji *et al.*, 1992).

1.5 Sorghum improvement

1.5.1 Conventional Breeding

Originally farmers selected plants with desirable traits from local populations over hundreds of years (Wall and Ross, 1970). The basis of plant improvement is two-fold in that variability is introduced by outcrossing and improved varieties for specific traits are selected. Varieties with desirable traits are crossed and the segregating progeny are then screened in search of the best genetic recombination (Doggett and Majisu, 1968). For hybrid breeding the product of conventional sorghum breeding are inbred lines, that have been developed through selfing (Figure 1.1) (inbreeding) until the desirable traits are fixed in a specific line (Doggett and Jowett, 1966).

1.5.2 Hybrid Breeding

The development of male-sterility made the commercial production of F_1 hybrids possible and has become one of the most important genetic mechanisms in sorghum breeding. Male sterility is made possible through two mechanisms, genetic and cytoplasmic and both are evident by the absence of pollen production (Karper and Stephens, 1936). Genetic sterility is controlled by simple recessive genes, ms_1, ms_2, ms_3 and ms_7, which are not subject to any modifiers or restorers (Karper and Stephens, 1936; Ayyangar and Ponnaiya, 1937; Stephens *et al.*, 1952). Although genetic male-sterility has the potential to be useful for practical applications in plant breeding it has received little recognition since cytoplasmic male-sterility is considered an easier system to manipulate in hybrid development (Stephens and Quinby, 1945).

Stephens and Holland (1954) first discovered cytoplasmic male-sterility in sorghum through the crossing of milo and kafir varieties that resulted in male-sterile plants. However, the reciprocal cross did not result in male sterile progeny and it was concluded that the sterility was caused by the interaction of cytoplasm from milo with genes from kafir (Wall and Ross, 1970). The cytoplasmic male-sterile gene was later identified as ms_{c1}. In F_2 backcross populations, two recessive genes were found to control male-sterility and the two major genes causing male-sterility have been assigned as ms_{c1} and ms_{c2} (Mauder and Pickett, 1959; Erichsen and Ross, 1963; Pi and Wuu, 1963). Male sterility is hypothesised to be an interaction between the cytoplasm and nuclear genes. The presence of at least one homozygous recessive male-sterile gene results in male sterility. Cytoplasmic male-sterile parents have a genotype of ms_{c1} $Ms_{c2,}$ and 'R' lines that restore fertility are Ms_{c1} ms_{c2} (Quinby, 1981).

Economically hybrid seed can only be produced with the use of male-sterility (Doggett and Majisu, 1968). Male fertile maintainer (B) lines are interplanted with the male-sterile (A) counterpart in isolated crossing blocks. The maintainer (B) line is selfed for seed maintainance. The male-sterile (A) line sets seed through wind pollination from the maintainer line. The seed harvested from the A-line will be sterile when planted in the next generation. For hybrid seed production, male-sterile A-lines are interplanted with the restorer (R) lines. The seed harvested from the A-line is planted by farmers (Dogett, 1969).

Hybrid seed production holds the advantage of trait stability and uniformity as well as the ability to introduce new traits quickly into existing cultivars. However, the application of

male sterility is not currently feasible in Africa taking into account current agricultural practice. A long process of education will be needed to persuade communal farmers of the advantage of obtaining new seed every planting season as well as the development of maintainer and restorer lines adapted to local environmental conditions (Mangombe *et al.*, 1996).

Overwhelming evidence exist to demonstrate that hybrids improve yield (Reich and Atkins, 1970). For example, a 25% superiority of hybrids differing in maturity, over the mean yield of pure strands was reported in a study conducted in Sudan (Bebawi and Abdelaziz, 1983). Reich and Atkins (1970) also reported hybrids to be the most productive and stable cultivation type. Similarly, a trial in Texas produced mean yields of 1755 kg per ha for the male parent and 2455 kg per ha for the hybrid. When separated into dry land and irrigated trials, the hybrid yield increase was 58% over that of the best adapted inbred parent under dry land conditions and 22% under irrigation (Quinby *et al.*, 1958). There is, therefore, an important consideration of using hybrids adapted to a specific environment.

1.5.3 Yield

Low soil fertility, poor germination resulting in low stand establishment and highly variable drought stress are major production constraints in Africa and Asia. Small-holder farmers do not usually have access to fertilizers, irrigation systems or improved cultivars and have to rely on the stress tolerance and yield stability of their rain fed crops (Mangombe *et al.*, 1996). Mean grain yields of 863 kg per ha for Africa and 1157 kg per

ha for Asia in contrast to the 3994 kg per ha for the USA, emphasises these constraints (FAO, 1999).

1.5.4 Conversion programmes

Collection of exotic germplasm has become an important component of an international breeding programme (Rosenow and Dahlberg, 2000). The exotic varieties are converted for use in hybrid development (Harlan, 1972). The global aim of conversion is to develop stable and higher yielding cultivars by using exotic varieties from different climates and recombining them into more widely adapted improved cultivar types (Duncan *et al.*, 1991). This is achieved by converting tall, late-maturing tropical exotic sorghum varieties with desirable traits, to short, early-maturing lines through a crossing and backcrossing process (Rosenow and Dahlberg, 2000). The knowledge of the genetics of height and maturity has made it possible to substitute genes that control height and maturity to obtain desired genotypes (Miller, 1982). However, the specific influence of height or maturity genes in conversion programmes is unknown.

There are four associated genes which determine height (Figure 1.2) in sorghum, dw_1, dw_2, dw_3 and dw_4, with tallness (*Dw*) dominant to shortness (*dw*). A one-dwarf is homozygous, recessive at one of the four loci, the two-dwarf is homozygous, recessive at two of the four dwarfism loci, a three-dwarf is homozygous, recessive at three of the loci and a four-dwarf is recessive at all four *dw* loci (Quinby and Karper, 1954).

The non-recurrent parent in most conversion programmes is a suitable four-dwarf line, used as female in an original cross with the exotic variety (Duncan *et al.*, 1991). The F_1

is selfed and short plants from the F_2 segregating population are selected and allowed to segregate in the F_3. The shortest plants are selected in the F_3 population and backcrossed to the recurrent exotic variety, four to five times. The size of the segregating F_2 populations has to be large enough to provide several four-dwarf, early maturing genotypes for further segregation in the F_3, from which only a few plants are selected. This is a tedious process as hand emasculation is used extensively in obtaining the crosses (Miller, 1982). In most cases, two generations can be completed in a single growing season, but in some cases the F_2 generation matures too late for planting in the same season (Rosenow and Dahlberg, 2000). At least eight to ten seasons are required for the conversion of an exotic variety to a short, early maturing cultivar before it is released (Rosenow and Dahlberg, 2000).

The conversion programme has proven highly successful and the converted lines produced are excellent sources of resistance against disease, insects, drought, lodging, grain weathering and exhibit other plant and grain characteristics useful in improving the value of grain sorghum world wide (Rosenow and Dahlberg, 2000).

1.6 The genetics of dwarfism in sorghum

Wild sorghum grows to a height of up to 3 m (Doggett, 1988). However, in mechanised agriculture designed for commercial grain production, tall plants are undesirable (Morgan and Finlayson, 2000). In contrast to this, subsistence agriculture favours tall

plants to fulfil multiple needs as building material, fuel, animal feed and human consumption (Doggett, 1988; House *et al.*, 2000).

In African countries, sorghum crops are often lost due to late season drought (Mangombe *et al.*, 1996). Early maturing and shorter plants would be able to complete the vegetative cycle before the onset of drought (Mangombe *et al.,* 1996). In the event of unpredictable drought, the capacity of a short plant to remobilise and translocate nutrients to the panicle is much greater in short plants than tall plants (Mangombe *et al.,* 1996). Therefore, short plants are better adapted to drought than taller plants.

Plant height in sorghum is indirectly influenced by photoperiodism (Morgan and Finlayson, 2000). The longer the plant remains vegetative, the greater the number of nodes it makes. Late-flowering plants with longer vegetative periods are usually taller than early-flowering plants (Morgan and Finlayson, 2000). Quinby and Karper (1954) determined that the duration of growth and floral initiation is controlled by four maturity (*ma*) loci, with many alleles at each locus. The dominant Ma_1 gene must be present to enable Ma_2, Ma_3 or Ma_4 to be expressed (Quinby, 1967). Temperature and photoperiod appear to influence the *Ma* genes but only in controlling internode number. Different *ma* genes have different effects in the same genetic background (Quinby and Karper, 1954). These results suggest that although maturity contributes to height, it is not directly responsible for height determination in sorghum.

1.6.1 Dwarf genes controlling height in sorghum

There are only a few early reports on short statured sorghum (Karper, 1932; Sieglinger, 1932; Martin, 1936; Vinall *et al.,* 1936; Ayyangar *et al.,* 1937; Laubscher, 1945). However, a study by Quinby and Karper (1954) has proven that genes at different loci are directly responsible for height in sorghum. In their study four major dwarfism genes (*dw*$_1$, *dw*$_2$, *dw*$_3$ and *dw*$_4$) were found to control sorghum height alternatively referred to as dwarfism. Quinby and Karper (1954) used phenotypic classification of F_2 and F_3 populations to designate different height genotypes in various crosses (Table 1.1). The four genes were found to be unlinked and tallness partially dominant to shortness (Quinby and Karper, 1954; Quinby, 1975). It was hypothesised that the *dw* genes affected plant height by controlling internode length (Quinby and Karper, 1954; Quinby, 1975). Furthermore, it was determined that the height of a plant is dependent on the number of loci at which homozygous recessive (*dw*) alleles are present (Quinby and Karper, 1954). In theory, five height class phenotypes exist, ranging from a zero-dwarf, containing no recessive alleles (*Dw*$_1$ *Dw*$_2$ *Dw*$_3$ *Dw*$_4$) to a four-dwarf, containing only recessive alleles (*dw*$_1$ *dw*$_2$ *dw*$_3$ *dw*$_4$). However, no zero-dwarf genotypes have ever been identified, but are assumed to exist in some wild varieties (Quinby and Karper, 1954; Quinby, 1975).

Quinby and Karper (1954) determined that genotypes recessive for a single height gene ranged in size from 120 cm to 207 cm. Genotypes recessive for two height genes ranged in size from 82 cm to 126 cm and genotypes recessive for three dwarf genes ranged in size from 52 cm to 61 cm. Four-dwarf lines measured 10 cm to 15 cm shorter than corresponding three-dwarf lines (Quinby, 1975). Quinby (1975) also determined

that homozygous plants for (Dw_3) were 8 cm to 13 cm taller than heterozygous (Dw_3) plants. This supports the hypothesis that tallness is partially dominant to dwarfness (Quinby and Karper, 1954). Most commercial lines are three-dwarf for adaptation to combine harvesting (Quinby and Karper, 1954).

The height variation observed within a single height class is attributed to a modifying complex or the instability of recessive genes (Quinby and Karper, 1954). However, studies have suggested that some *dw* alleles may revert to the dominant (*Dw*) allele (Karper, 1932). For example, the dw_3 allele was found to be unstable, with reversion rates from 1:400 to 1:1800, depending on the genotype and environment (Karper, 1932; Quinby, 1963). Ross (1971) also reported instability for dw_2, again genotype or environmental dependant. Although reversion of *dw* to *Dw* alleles has been suggested to explain *dw* gene instability, it is more likely that phenotype reversion occurs through point mutations of DNA resulting in a loss of gene product functionality. The height range and effect of environment on genotype may also result in mistaken genotype identification.

1.7 Molecular development

1.7.1 Sorghum genetics

Sorghum is a diploid with 20 chromosomes (Doggett, 1988). In *Sorghum bicolor,* the estimated nuclear DNA content is 748 to 772 x 10^6 base pairs (bp) per 1C (amount of DNA present in the haploid genome). The sorghum genome is double the size of the

rice genome which is 450 x 10^6 bp per 1C, three times smaller than the maize genome estimated at 2500 x 10^6 bp per 1C and about 20 times smaller than wheat genome (Arumuganathan and Earle, 1991). Despite the ability of sorghum to grow in harsh environments and its diversity, comprehensive genetic characterization of sorghum accessions using molecular descriptors is limited (Subudhi and Nguyen, 2000).

1.7.2 Marker assisted selections

Molecular markers are pieces of DNA that serve as markers for genes at specific loci (regions) on a chromosome, specifically associated with a particular trait (Subudhi and Nguyen, 2000). Several different DNA marker techniques such as restriction fragment length polymorphisms (RFLPs) (Helentjaris *et al.*, 1986; Hulbert *et al.*, 1990; Chittenden *et al.,* 1994; Pereira *et al.*, 1994; Xu *et al.*, 1994) and PCR based approaches such as random amplified polymorphic DNA (RAPDs) (Williams *et al.*, 1990; Tao *et al.*, 1993; Jaiswal *et al.*, 1988), simple sequence repeats (SSRs) (Lit and Lutty, 1989; Lagercrantz *et al.*, 1993; Brown *et al.*, 1996; Taramino *et al.*, 1997) or microsattellites and amplified fragment length polymorphisms (AFLPs) (Zabeau and Vos, 1993; Vos *et al.*, 1995; Maheswaran *et al.*, 1997; Boivin *et al.*, 1999) have been used successfully to assess genetic relationships in sorghum.

Molecular markers have the advantage of improving the effectiveness of conventional breeding through the selection of desirable characteristics based on the presence of molecular markers, which are linked to the particular trait in question (Lee, 1996). Molecular markers are discrete, co-dominant or dominant non-deleterious characters

that are not environmentally affected and free of epistatic interaction (McIntyre *et al.*, 2001). In contrast, some morphological characteristics are only expressed during specific stages of plant growth and development or specific environmental conditions. Marker selection can be applied on seed, seedlings or mature plants and on any type of tissue (McIntyre *et al.*, 2001). Therefore, molecular markers can be used to speed up the incorporation of desirable genes into existing cultivars, develop new cultivars through the assessment of genetic diversity and comparative mapping. It contributes to the understanding of the genetic basis of complex traits for crop improvement (Pereira and Lee, 1995). Molecular markers have been used on sorghum with great effect, especially RFLPs, SSRs and AFLPs (Chittenden *et al.,* 1994; Lin *et al.*, 1995; Brown *et al.*, 1996; Boivin *et al.,* 1999).

1.8 Molecular techniques

1.8.1 RFLPs

Restriction fragment length polymorphism (RFLP) is a hybridisation-based technique that requires the use of a library of cloned DNA fragments (Helentjaris *et al.*, 1986; Hulbert *et al.*, 1990; Chittenden *et al.,* 1994; Pereira *et al.*, 1994; Xu *et al.*, 1994). In RFLPs, the DNA is digested with one or more restriction endonucleases. The resulting restriction fragments are resolved electrophoretically according to size and transferred to a nylon membrane and hybridised with probes (Jones *et al.*, 1997). Probe hybridisation is visualised radioactively or with the use of fluorescence labelling (Helentjaris *et al.*,

1986). Most RFLP markers are inherited in a co-dominant manner, and are useful as to anchor map loci (Jones *et al.*, 1997).

Sorghum genome mapping based on RFLPs, began in 1990 (Hulbert *et al.*, 1990). Numerous genetic maps have been constructed for sorghum (Hulbert *et al.*, 1990; Binelli *et al.*, 1992; Whitkus *et al.*, 1992; Chittenden *et al.*, 1994; Pereira *et al.*, 1994; Ragab *et al.*, 1994; Xu *et al.*, 1994; Dufour *et al.*, 1997; Tao *et al.*, 1998; Crasta *et al.*, 1999; Peng *et al.*, 1999; Subudhi and Nguyen, 2000). Originally, maize probes were used to map the sorghum genome (Hulbert *et al.*, 1990; Binelli *et al.*, 1992; Whitkus *et al.*, 1992; Pereira *et al.*, 1994). Initial maps identified between five and 15 linkage groups (Hulbert *et al.*, 1990; Binelli *et al.*, 1992; Whitkus *et al.*, 1992). Pereira *et al.* (1994) produced the first map with ten linkage groups using 201 mapped loci. A number of maps with 10 linkage groups have subsequently been developed (Chittenden *et al.*, 1994; Ragab *et al.*, 1994; Xu *et al.*, 1994) using recombinant inbred lines (RILs) with a variety of probes, including sorghum, maize, sugarcane and other cereal anchor probes (Dufour *et al.*, 1997; Tao *et al.*, 1998). Peng *et al.* (1999) constructed a comprehensive RFLP genomic sorghum map using sorghum, maize, oats, barley and rice probes, placing 323 mapped loci into 10 linkage groups. Subudhi and Nguyen (2000) took this further by incorporating all the major sorghum RFLP maps into one using RIL mapping populations and probes from Chittenden *et al.* (1994), Ragab *et al.* (1994) and Xu *et al.* (1994) as well as other cereal and maize anchor probes.

1.8.2 RAPDs

RAPDs is a PCR based technique and does not require cloning or sequencing (Williams *et al.*, 1990). This technique allows the simultaneous detection of several loci, using short 8 to 12 base oligonucleotide primers (Williams *et al.*, 1990). However, although seemingly easy, inexpensive and quick, assay reproducibility is problematic due to the effect of PCR temperature conditions on the binding of the short oligonucleotide primers (Jones *et al.*, 1997). RAPDs has not been used extensively in sorghum. Jaiswal *et al.* (1998) used RAPDs to differentiate between male sterile lines, maintainer lines, restorer lines as well as partial restorers, and is currently the only published study on sorghum using RAPDs.

1.8.3 SSRs

Simple sequence repeats (SSRs) or microsatellites are heritable tandem repeats of short DNA sequence motifs (2 to 5 basis) that occur universally in eukaryotic genomes (Litt and Lutty, 1989). The copy number of tandem repeats is used to express the variation among taxa and between individual genomes (Litt and Lutty, 1989). Microsatellites are analysed by PCR using the microsatellite sequence as primer generating inter simple sequence repeats (ISSRs) or by amplifying the simple sequence repeat (SSR) with primers that flank the microsatellite repeat region (Litt and Lutty, 1989; De Oliveira *et al.*, 1996). The SSR or ISSR fragments are resolved using gel electrophoresis. The application of ISSRs is similar to RAPDs and less informative than SSRs. The DNA sequences flanking SSRs are usually conserved in and between species (Akkaya *et al.*, 1992; Sanghai-Maroof *et al.*, 1994). Variation in the number of tandem repeats, results in PCR products of different length (Litt and Lutty, 1989; De

Oliveira *et al.*, 1996). SSRs are highly polymorphic, due to mutations causing variation in the number of repeat units, and different alleles can be detected at a single locus (Akkaya *et al.*, 1992; Sanghai-Maroof *et al.*, 1994). SSRs is one of the preferred molecular techniques for detailed mapping of genomes, diversity studies and DNA fingerprinting (Akkaya *et al.*, 1992). The SSR technique is rapid, reproducible, technically simple, and reasonably inexpensive and requires only small amounts of DNA (Lit and Lutty, 1989). SSR markers are codominant and consistently distributed through plant genomes (Akkaya *et al.*, 1992; Lagercrantz *et al.*, 1993). SSRs are currently the marker of choice for breeding programs as they combine the convenience of a PCR marker with the cross-transferability of a RFLP marker (Mclyntyre *et al.*, 2001).

The first report of SSRs in sorghum was by Brown *et al.* (1996) to identify SSRs in sorghum and develop PCR primers that could be used to screen sorghum germplasm collections. A total of 47 sorghum SSR-specific primers were developed through a strategy of constructing genomic libraries, screening by hybridisation with SSR probes and sequencing positive clones (Brown *et al.*, 1996). Since library screening and sequencing are costly and labour intensive two other methods for SSR identification were also investigated. Firstly, a search was conducted for SSRs in sorghum sequence databases and secondly, SSR primers from maize and paspalum (*Paspalum vaginatum*) were also tested on sorghum. Currently a total of 62 sorghum SSR specific primers have been developed for the sorghum genome (Brown *et al.,* 1996; Taramino *et al.*, 1997).

1.8.4 AFLPs

Amplified fragment length polymorphism (AFLP) is a PCR based technique, incorporating the reproducibility of RFLPs with the ease of RAPDs (Vos *et al.*, 1995). Oligonucleotide adaptors are ligated to the end of restriction fragments. Adaptor complimentary primers with 3' bases added are used during pre-selective amplification of ligated fragments (Zabeau and Vos, 1993; Vos *et al.*, 1995). This is followed by a process of selective amplification, which uses primers with adaptor sequences (Vos *et al.*, 1995). Different loci can easily be detected by the manipulation of the 3' end extension of the primer sequence (Vos *et al.*, 1995). AFLP markers are generally dominant but can also be codominant. AFLP markers are often inherited in tightly linked clusters, but randomly distributed markers also occur outside these cluster (Vos *et al.*, 1995). AFLPs is more effective than RFLPs to fill in gaps in genetic linkage maps. AFLP data also allow the estimation of genetic distances among inbred lines and DNA fingerprinting for genotype identification (Zabeau and Vos, 1993). To date AFLPs has only been used in sorghum to saturate RFLP maps, but has had no further application (Boivin *et al.*, 1999; Klein *et al.*, 2000).

1.8.5 QTLs for height

QTL analysis is a method for studying polygenic traits and provides valuable information of the genetic basis of the traits of interest (Falconer and Mackay, 1996). Experimental designs or estimating map positions of QTLs are extensions of standard methods for mapping single genes, and are based on the linkage between loci and polygenic traits (Falconer and Mackay, 1996).

QTL analysis using RFLPs has been used to study the loci associated with height in sorghum. Pereira and Lee (1995) mapped QTLs influencing plant height using a F_2 population from a cross between an inbred three-dwarf line (dw_1 Dw_2 dw_3 dw_4) and tall parent of undetermined genotype thought to be a zero-dwarf (262 cm) but probably a one-dwarf. QTLs in four different linkage groups A, B, E and H accounted for 63.4% of the total phenotypic variation in height (Pereira and Lee, 1995). Lee (1996) identified 6 QTLs in a F_3 population derived from the F_2 mapping population used by Pereira and Lee (1996). Four of the QTLs map to plant height QTL regions in maize (Lee, 1996). However, other studies have identified QTLs in different linkage groups (A, C, D, G and H) (Chittenden *et al.*, 1994; Lin *et al.*, 1995). QTL analyses of height in sorghum confirm that few loci are involved in controlling height. However, the dwarfism genes cannot currently be associated with specific chromosomal loci and further study is needed. The ability to identify different dwarfism genes will be of utmost importance in mapping the position of the different *dw* loci in sorghum. Therefore, the identification of molecular markers for the different *dw* genes is of utmost importance.

1.9 Conclusion

Sorghum remains an important crop for human consumption in Africa, especially in terms of nutritional value as well as environmental adaptation. Despite this, sorghum is not produced intensively throughout the world. The reason for this is that in first world crop production, with set agricultural practice, drought and environmental adaptation is not of primary concern and most other cultivated crops are well researched. In contrast

to this, most developing countries have a slow rate of economic growth and lag behind in terms of technology development. Furthermore, sorghum is not a primary first world crop and thus variety improvement is not a priority. However, despite a decline in sorghum production and consumption as food in the world, the sorghum beer industry in Africa is a growing one.

Height and maturity characteristics form the cornerstone of all conversion programmes for improved commercial sorghum varieties. Exotic tall and late maturing sorghum with desirable traits, can easily be converted to short, early maturing forms which can be used throughout of the world. Important economic characteristics introduced through conversion include disease resistance, insect resistance, drought tolerance, heat tolerance, lodging and salinity tolerance as well as improved kernel characteristics (Miller, 1982). Even though the needs of communal farmers differ from that of commercial farmers, shorter plant stature and early grain maturity are higher yielding and drought tolerant and could be grown very effectively in Africa (Mangombe *et al.*, 1996).

Manipulating height is an important factor in conversion programmes. Since height is influenced by genotype and environmental interactions mistaken genotype identification can result from only phenotypic characterisation. Phenotype selection of height class in conversion programmes is tedious and costly. The ability to identify specific dwarfism genotypes with the use of molecular markers would facilitate sorghum conversion and breeding.

Table 1.1. The height and genotype of sorghum varieties used in the study of Quinby and Karper (1954).

Variety	Genotype	Height (cm)
Recessive for one gene		
Durra	$Dw_1\ Dw_2\ Dw_3\ dw_4$	159
Sumac	$Dw_1\ Dw_2\ Dw_3\ dw_4$	166
Shallu	$Dw_1\ Dw_2\ Dw_3\ dw_4$	157
Spur Feterita	$Dw_1\ Dw_2\ Dw_3\ dw_4$	120
Tall white sooner milo	$Dw_1\ Dw_2\ Dw_3\ dw_4$	127
Standard yellow milo	$Dw_1\ Dw_2\ Dw_3\ dw_4$	173
Standard broomcorn	$Dw_1\ Dw_2\ dw_3\ Dw_4$	207
Recessive for two genes		
Texas blackhull kafir	$Dw_1\ Dw_2\ dw_3\ dw_4$	100
Bonita	$Dw_1\ dw_2\ Dw_3\ dw_4$	82
Early hegari	$Dw_1\ dw_2\ Dw_3\ dw_4$	105
Hegari	$Dw_1\ dw_2\ Dw_3\ dw_4$	126
Dwarf sooner milo	$Dw_1\ Dw_2\ Dw_3\ dw_4$	94
Dwarf yellow milo	$dw_1\ Dw_2\ Dw_3\ dw_4$	106
Acme broomcorn	$Dw_1\ dw_2\ dw_3\ Dw_4$	112
Japanese dwarf broomcorn	$dw_1\ Dw_2\ dw_3\ Dw_4$	92
Recessive for three genes		
Martin	$dw_1\ Dw_2\ dw_3\ dw_4$	61
Plainsman	$dw_1\ Dw_2\ dw_3\ dw_4$	52
Double dwarf white sooner milo	$Dw_1\ dw_2\ Dw_3\ dw_4$	53
Double dwarf yellow milo	$dw_1\ dw_2\ dw_3\ dw_4$	60

Figure 1.1. Sorghum ears covered with paper bags during the production of selfed-seed.

Figure 1.2. Typical different plant heights for sorghum.

Chapter 2

Morphological characterisation of near-isogenic *Sorghum bicolor* (L.) Moench lines for different height classes

Abstract

Sorghum is grown, mainly in the tropics and semi-arid areas of the subtropics by subsistence farmers. These farmers rely on sorghum as a food supply throughout the year as well as for a cash income. Sorghum is adapted to different environmental conditions and produces relatively high yields under drought stress or in poor soil types. The discovery of dwarfism (*dw*) genes made the development of varieties suited to combine harvesting. However, little research has been done on the dwarfism genes since the 1960's when the first combine height cultivars were developed. Four dwarfism genes are involved in determining height namely, dw_1, dw_2, dw_3 and dw_4, where tallness is dominant to dwarfness. However, the interaction of *dw* genes and their effect on other morphological characters is uncertain. In this study, near-isogenic lines (NILs) for the dwarfism genes were phenotypically characterised. Significant differences were observed in plant height, internode length, number of nodes, flag leaf width, panicle weight and number of panicle branches. Furthermore, internode length and flag leaf length were found to correlate with plant height. However, in contrast to previous reports a correlation was found between leaf sheath length, panicle length and panicle weight. No correlation was found between number of internodes and plant height.

2.1 Introduction

North West Africa (Ethiopia) is considered to be the centre of origin of *Sorghum bicolor* (L.) Moench, especially the races bicolor and dura, with guina from West Africa,

caudatum from Nigeria and Sudan, while kafir is primarily from East and Southern Africa (Harlan, 1972; House *et al.*, 2000). The slave trade was responsible for the introduction of sorghum to the New World at the end of the nineteenth century (Doggett, 1988). The first introduced cultivars were tall and late maturing. However, North American farmers selected and propagated natural occurring mutations for reduced height, maturity and increased production. The development of the combine harvester in the late 1920`s influenced the selection of combine height sorghum resulting in the eventual release of the Beaver variety in 1931 which originated from a kafir x milo cross (Martin, 1932). Unfortunately, this cultivar proved vulnerable to root and stalk rot (*Periconia circinata*). Martin (1936) developed a resistant Beaver variety, which was subsequently multiplied and released as Martin's Milo in 1941, and which is generally known today as Martin. This variety was considered to have excellent combine qualities and was very popular during 1942 to 1954 (Doggett, 1988). The discovery and use of male-sterility resulted in the development of hybrids that are currently commercially planted (Morgan and Finlayson, 2000).

Miller (1982) describes a detailed conversion programme to obtain three-dwarf lines from tropical collections, for the use of commercial hybrid development. Most of advances in sorghum breeding and development have occurred in the USA where African lines are converted for combine height (Duncan *et al.*, 1991).

Sorghum is grown in Africa by communal farmers using traditional farming techniques in semi-arid conditions, with a mean grain yield of 0.86 tons per ha compared to 1.16 tons per ha in Asia (FAO, 1999). Drought stress, poor stand establishment and low soil

fertility are the main reasons for low yields. Communal farmers depend on the adaptability of their crops to moisture stress and poor soil conditions, since they generally do not have access to fertilizers and irrigation. Therefore, the needs of communal farmers in terms of trait requirements are different to those of commercial farmers. Communal farmers harvest by hand and combine height is thus not an important feature for rural sorghum production. The main considerations among communal farmers are adaptability of cultivars to low and poorly distributed rainfall and high soil salinity (Harwood, 1979). Small-scale farmers cannot afford fertilizer or hybrid seed (Mangombe *et al.*, 1996).

In contrast to its predominant use as animal feed in first world countries such as the United States, smallholder farmers use sorghum almost entirely for own consumption. Sorghum grain serves as an ingredient of many unique indigenous foods in the form of steamed, boiled, baked, fried or parched foodstuffs as well as beer and even wine production in China (Yan, 1993). Sorghum stems are often used to make palisades around traditional houses and also in the construction of houses. Sorghum dry matter is also used for fuel where trees and shrubs are sparse (Wall and Ross, 1970). This explains the preference for tall plants among subsistence framers. Although short plants may be more suited to drought conditions, they do not meet the overall required characteristics of communal farmers. It is only in areas where the crop is mechanically harvested that the shorter stature of plants is preferred (Quinby, 1975).

The discovery of dwarfism genes made it possible to reduce plant height, for combine harvesting as well as limit the impact of wind lodging. Quinby and Karper (1954)

showed that the genes influencing flower initiation time also affect plant height in sorghum. They observed that tall genotypes with longer flowering and maturing periods exhibit poor grain yield, due to end of growing season drought conditions. Quinby and Karper (1954) identified four loci, dw_1, dw_2, dw_3, and dw_4, which mediate plant height in sorghum, with tallness (*Dw*) dominant to dwarfness (*dw*). Although the four *dw* genes mediate five different height classes, no commercial cultivars are known to exist for the tallest height class (zero-*dw*) containing all four dominant (*Dw*) genes. The 1*dw* height class contains a single homozygous *dw* gene at any of the four dwarfism loci. The 2*dw* height class is homozygous, recessive at any two of the four, dwarfism loci and the 3*dw* height class homozygous, recessive at any three of the four, dwarfism loci. The 4*dw* height class, shortest of the five height classes, is homozygous, recessive at all four the dwarfism loci. A statistical quantitative study of height in sorghum by Hadley (1957) suggested that instead of dwarfism genes at four loci, two major and eight minor genes could also explain dwarfism in sorghum. However, no further research was published to confirm this. Furthermore, all the subsequent reports, on dwarfism genes are based on the assignments of genotypes to different varieties made by Quinby and Karper (1954).

The effect of recessive alleles at any of the four *dw* loci can decrease tallness by 50 cm or more (Quinby, 1975). However, if other recessive dwarfism genes are already present, the effect at additional loci is decreased. Thus, the height difference between a 3*dw* and a 4*dw* (homozygous, recessive at three and four height loci, respectively) can be as little as 10 cm (Quinby and Karper, 1954). Quinby and Karper (1954) attributed the length differences between height classes, to the inhibiting effect of *dw* genes on cell elongation as well as the possible influence of modifying genes. In addition to this,

homozygous tall plants were 8 cm to 13 cm taller than the heterozygous tall plants. From these findings it was assumed that tallness is partially dominant to dwarfness (Quinby and Karper, 1954). However, the effect of heterozygous dwarf genes on height has not been studied extensively.

Martin, Westland and Redbine sorghum varieties are reported to possess an unstable *dw* gene (Quinby and Karper, 1954). It is assumed that the dw_3 allele is unstable as this is the only gene common to these varieties (Quinby and Karper, 1954). However, Karper (1932) also reported instability of the dw_4 allele and Ross (1971) described a mutant Dw_2^a allele. The effect of allele instability is the reversion of the recessive allele to the dominant form. Therefore, a point mutation may be responsible for the loss of gene functionality and explain the effect of gene reversion.

The dwarfism genes are reported to be brachytic in nature, having no effect on leaf size, tiller number, culm and panicle size (Quinby and Karper, 1954; Quinby, 1973; Morgan and Finalayson, 2000). To date, all the information on dwarfism in sorghum breeding is based on findings made during 1950 to 1973 (Hadley, 1957; Casady, 1965; Maunder and Weddige, 1966; Shertz, 1973), which is based on the assignment of genotype to height class by Quinby and Karper (1954).

The aim of this study was to study the effect of dwarfism genes on plant height and other plant and ear traits in grain sorghum, using four near-isogenic lines involving dw_1, dw_2, dw_3 and dw_4 loci.

2.2 Materials and Methods

2.2.1 Plant material

Near-isogenic lines, 1*dw*, 2*dw*, 3*dw*, and 4*dw* for the different dwarfism genes were developed at the Grain Crops Institute of the Agricultural Research Council at Potchefstroom, South Africa. The lines were developed through a backcross process in which a tall tropical sorghum cultivar (SA748) was crossed with a four-dwarf Martin. The F_1 population was selfed, and subsequent short plants selected from segregating F_2 populations and backcrossed to the tall SA748 for eight generations. The non-recurrent parent line was an early maturing 4dw (dw_1 dw_2 dw_3 dw_4) Martin B-line, Btx406 of US origin. The genotype of the recurrent parent (SA748) is unknown and assumed to be homozygous, recessive (*dw*) at one of the dwarfing loci.

2.2.2 Evaluation of plant and ear characteristics

Ten plants of each near-isogenic line (NIL) and parent line were planted under shade cloth and irrigation at Potchefstoom (ARC Grain Crops Institute) and evaluated for plant height. Five plants of each near-isogenic line (NIL) and parent line were evaluated for internode length, number of nodes, flag leaf length and width, panicle length, number of panicle primary branches and total panicle weight. Measurements were taken after the seeds had reached physiological maturity. Plant height was measured from ground level to top of the panicle. Stalk lengths were measured from ground level to the base of panicle. Data was analysed using Agrobase for Windows 98 (Agronomix software INC.).

Linear regression was performed to determine the relationship between plant height and other morphological characteristics using Microsoft Excel (Microsoft 2000).

2.3 Results

The average height of the recurrent parent line SA748 was 217 cm and 74 cm for the donor Btx406 parent. The 1*dw* NIL plants measured an average of 199 cm, the 2*dw* 171 cm, the 3*dw* 127 cm and the 4*dw* 86 cm (Table 2.1). The difference in height between the 1*dw*, 2*dw*, 3*dw* and 4*dw* NIL genotypes was 28 cm, 44 cm and 41 cm, respectively. The LSD for height class was 8.2 with a R-square (repeatability) of 0.97 (Table 2.1).

The average number of nodes for SA748 was 11 and 8 for Btx406. The average number of nodes was 9.8 for the 1*dw* NIL, 9.6 for the 2*dw* NIL, 10.6 for the 3*dw* NIL and 10 for the 4*dw* NIL. The R-square for average number of nodes was 0.57 and the LSD was 1.13. The average internode length was 18.28 cm for the recurrent parent SA748, 16.56 cm for the 1*dw* NIL, 16.08 cm for the 2*dw* NIL, 14 cm for the 3*dw* NIL, 11.24 cm for the 4*dw* NIL and 10.68 cm for Btx406. The average internode length was significantly different with a 1.82 LSD and R-square of 0.81 (Table 2.1).

Leaf sheath length ranged from 16.49 cm to 20.22 cm among all the genotypes and did not differ significantly (R-square of 0.24). Flag leaf width ranged from 6.24 cm to 8.64 cm among genotypes and differed significantly with a 3.63 LSD and R-square of 0.57.

Flag leaf length ranged between 43.76 cm and 64.64 cm among genotypes and differed significantly with a 1.02 LSD and R-square of 0.78 (Table 2.1).

Panicle length ranged from 21.60 cm to 25.26 cm and mean values were not significantly different. The number of panicle branches was statistically significantly different and ranged from 88.2 for the 1*dw* parent to 69.8 for the 4*dw* NIL. The LSD for the number of panicle branches was 7.52 and R-square was 0.86. Panicle weight ranged from 28.70 g to 112,99 g over all the genotypes and differences were highly significant with an R-square of 0.68 and an LSD of 26.60 (Table 2.1).

Plant height was significantly correlated to average internode length (correlation R-square of regression of 0.97) (Figure 2.1) and flag leaf length (correlation R-square of regression of 0.90) (Figure 2.2). A significant binomial correlation was found between plant height and flag leaf width (correlation R-square of regression of 0.91) (Figure 2.3), panicle weight (correlation R-square of regression of 0.90) (Figure 2.4), leaf sheath length (correlation R-square of regression of 0.96) (Figure 2.5) and panicle length (correlation R-square of regression of 0.98) (Figure 2.6). No correlation was observed between plant height and the number of panicle branches (Figure 2.7) as well as the number of nodes (Figure 2.8).

2.4 Discussion

The different near-isogenic lines for dwarfism differed significantly for plant height, being the most important phenotypic characteristic in the study of dwarfism genes. Differences between plant height classes decreased with increases in the number of *dw* genes present (Table 2.1). Therefore, the different dwarfism genes do not contribute equally to plant height, supporting the findings of Wall and Ross (1970). The reason for the reduction in height difference is not known. It has been proposed that the difference in length is a result of a modifying complex whereby the plant stature is shortened by the amount comparable to the effect of one homozygous recessive *dw* gene present (Quinby and Karper, 1954). However, the difference between subsequent height classes is not consistent. These data suggest that different *dw* genes each have a different effect on height or that each locus is responsible for equal amounts of GA_3, but its GA_3 effect on growth diminishes with increased availability of GA_3.

In the comparison of morphological characteristics in the different dwarf NILs it was found that plant height was significantly correlated to internode length (Figure 2.1) as reported by Quinby and Karper (1954). However, the number of nodes per plant showed no correlation towards plant height (Figure 2.7).

Significant correlation was also found between plant height and flag leaf length (Figure 2.2) supporting previous reports (Casady, 1965; Freeman and Javier, 1965; Maunder and Weddige, 1966; Brooks, 1967). Significant binomial correlations were found to exist

between plant height and flag leaf width (Figure 2.3) and panicle weight (Figure 2.4). Significant binomial correlations were also found between plant height and leaf sheath length (Figure 2.5) and panicle length (Figure 2.6) even though these characteristics were not significantly different between the different height classes. These results are in contrast to previous reports that tall plants have longer leaf sheaths, leaf blades, have greater yields of grain and heavier kernels (Casady, 1965; Freeman and Javier, 1965; Maunder and Weddige, 1966; Brooks, 1967). However, in all previous reports where correlations were made between plant height and other characteristics, results were based on only two consecutive height classes. The use of near-isogenic lines for all four different height classes has thus made a more comprehensive comparative study of morphological characters possible. Furthermore, we hypothesise that the photosynthetic surface area of the 3*dw* plants is optimal since larger flag leaves may result in optimum yield potential.

In previous studies to determine the correlation between plant height and other morphological characteristics, comparisons were made between different sorghum dwarf varieties with different genetic backgrounds, usually only between 2*dw* and 3*dw* height classes (Casady, 1965; Freeman and Javier, 1965; Maunder and Weddige, 1966; Brooks, 1967). Consequently no real conclusions can be drawn from these data, which are influenced by the interaction of genotype and the environment. The current study clearly demonstrates the significant correlation between plant height and flag leaf length, flag leaf width and panicle weight using near-isogenic dwarf lines. The correlation of plant height to leaf sheath length and panicle length further demonstrates the benefit of the selection of the 3*dw* height class for hybrid development.

The deduction of height genotype is problematic as the height of different genotypes is not always consistent and is environmentally influenced (Quinby and Karper, 1954; Hadley, 1957; Ross, 1971; Shertz, 1973; Ross and Kofoid, 1979). For example, in a study by Quinby and Karper (1954) the shortest cultivar in the 1*dw* height class was 120 cm and the tallest cultivar in the 2*dw* height class was 126 cm tall differing only by 6 cm. Furthermore, the height of the 1*dw* or 2*dw* plants in the study of Quinby and Karper (1954) would place them in the 1*dw*, 2*dw* or 3*dw* height classes of the present study, respectively. It is clear that height genotype identification is difficult and would be facilitated by the development of DNA markers for the different height genes.

It is important to note that despite more than 50 years of active research, very little is known on the precise genetic effect of the dwarfism genes in sorghum. In a conversion programme (Miller, 1982), the identification of genotypes is performed phenotypically. Line conversion is aimed at developing three-dwarfs (dw_1, Dw_2, dw_3 and dw_4) from tall exotic plants that provides new sources of disease resistance, insect resistance, improved plant characteristics like drought tolerance, salinity tolerance, lodging resistance, improved grain yield, yield stability under diverse conditions and increased grain filling rates as well as outstanding kernel characteristics like thin pericarps, increased protein content and improved flavour. DNA markers for dwarfism genes would assist in the selection of desired three-dwarfs developed in conversion programmes. Therefore, the development of DNA markers for the *dw* genes would be of great benefit to identify specific genotypes but also to study the effect of the different *dw* genes.

Table 2.1. Averages and analysis of variance of nine leaf and ear characteristics evaluated for four near-isogenic and parental sorghum lines.

	Recurrent parent SA 748	Near-isogenic lines				Donor parent Btx406			
Characteristic	**1 *dw***	**1 *dw***	**2 *dw***	**3 *dw***	**4 *dw***	**4 *dw***	**MS**	**R-square**	**LSD**
Plant height (cm)	217	199	171	127	86	74	35238.33**	0.97	8.20
Internode length (cm)	18.28	16.65	16.08	14.00	11.24	10.68	46.49**	0.81	1.82
Number of nodes	11	9.8	9.6	10.6	10	8	5.39**	0.57	1.13
Leaf sheath length (cm)	18.57	18.8	18.65	18.48	16.49	20.22	7.08	0.24	3.63
Flag leaf width (cm)	7.40	6.34	7.40	8.64	6.24	7.14	3.81**	0.57	1.02
Flag leaf length (cm)	58.77	64.64	58.96	56.84	43.76	44.15	369.02**	0.79	5.53
Panicle weight (g)	99.24	93.93	101.13	112.99	61.16	28.7	5037.33**	0.68	26.6
Panicle length (cm)	22.26	23.02	24.00	25.26	24.74	21.6	10.26	0.29	3.08
Number of panicle branches	88.4	66.6	62.2	97.6	69.8	59.8	1177.57**	0.86	7.52

** Significant at p=0.01

Figure 2.1. The relationship between plant height and internode length in near-isogenic lines of sorghum.

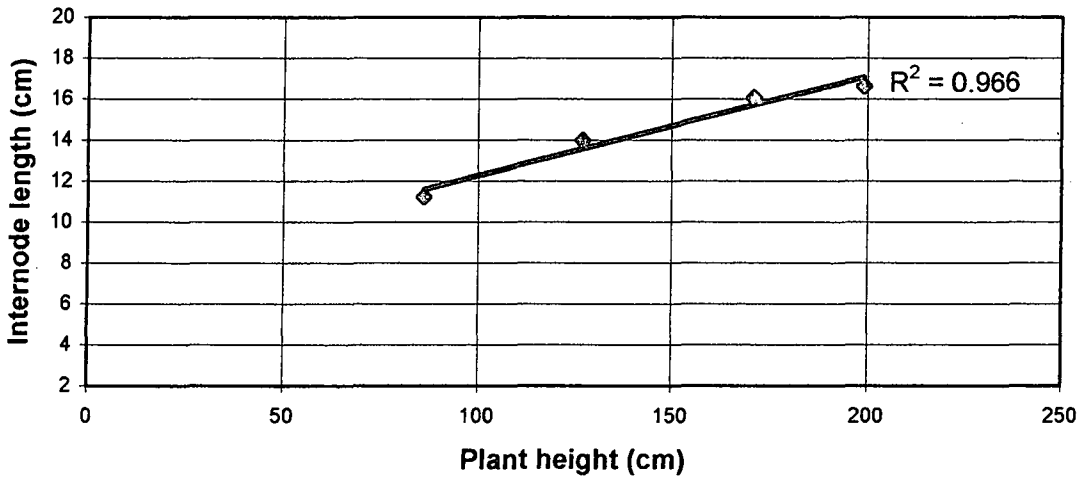


Figure 2.2. The relationship between plant height and flag leaf length in near-isogenic lines of sorghum.

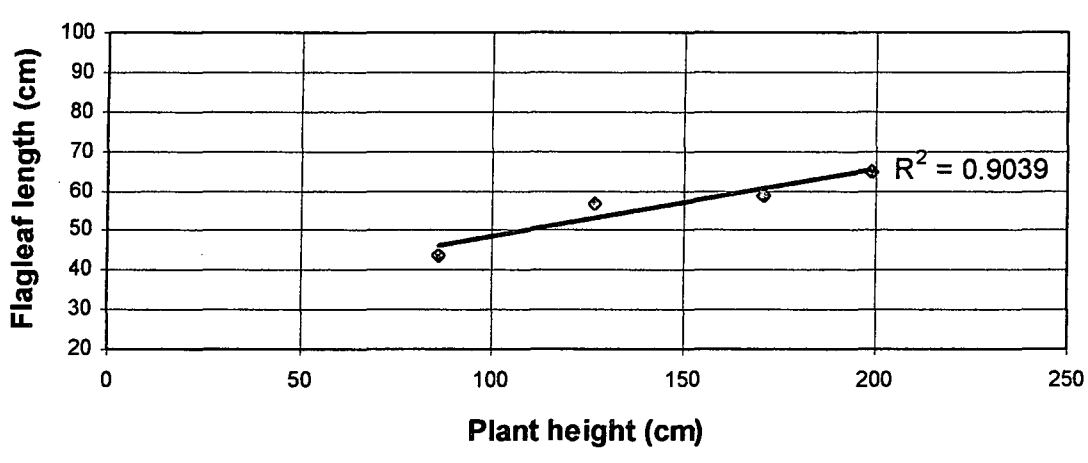


Figure 2.3. The relationship betwee plant height and flag leaf width in near-isogenic lines of sorghum.

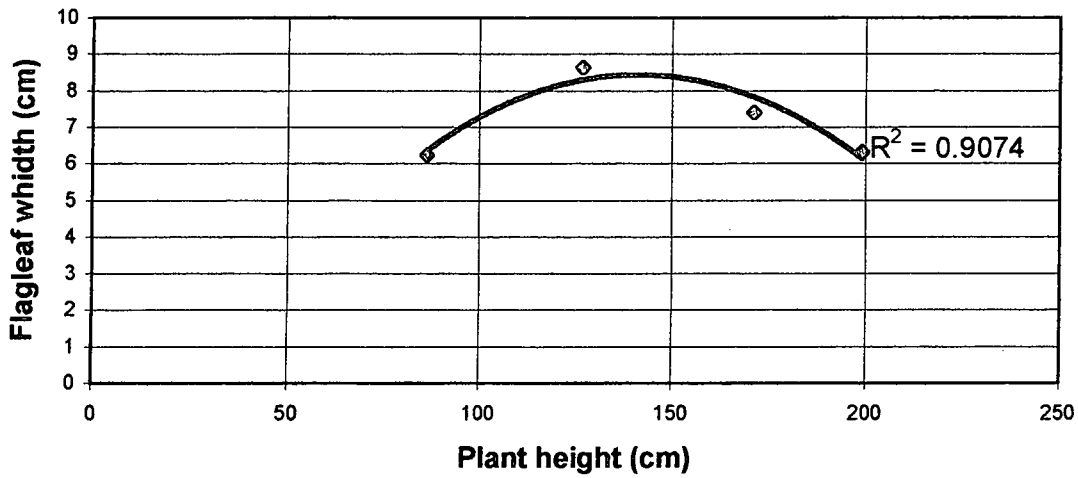


Figure 2.4. The relationship between plant height and panicle weight in near-isogenic lines of sorghum.

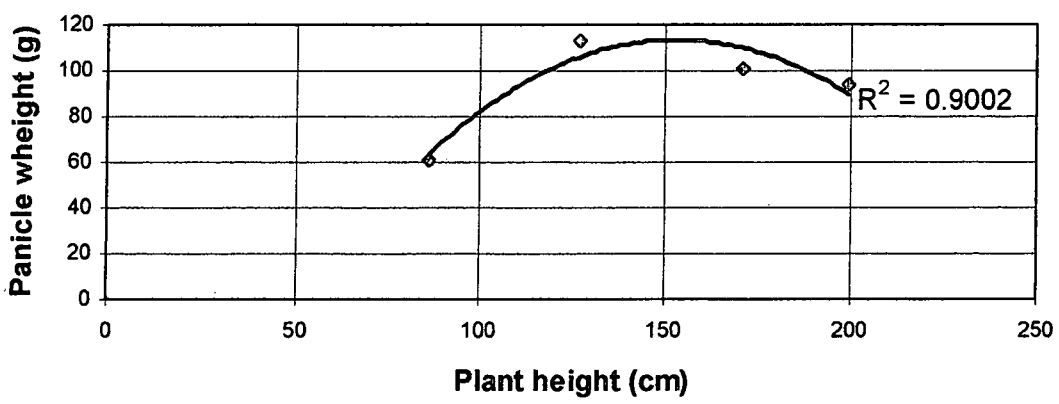


Figure 2.5. The relationship between plant height and leaf sheath length in near-isogenic lines of sorghum.

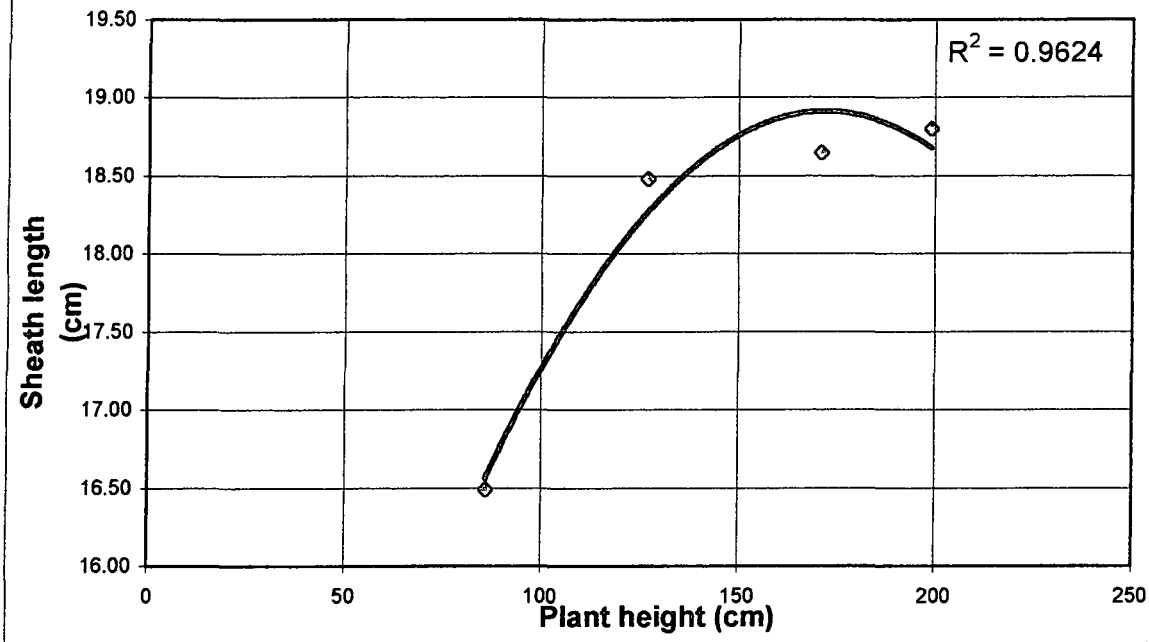


Figure 2.6. The relationship between plant height and panicle length in near-isogenic lines of sorghum.

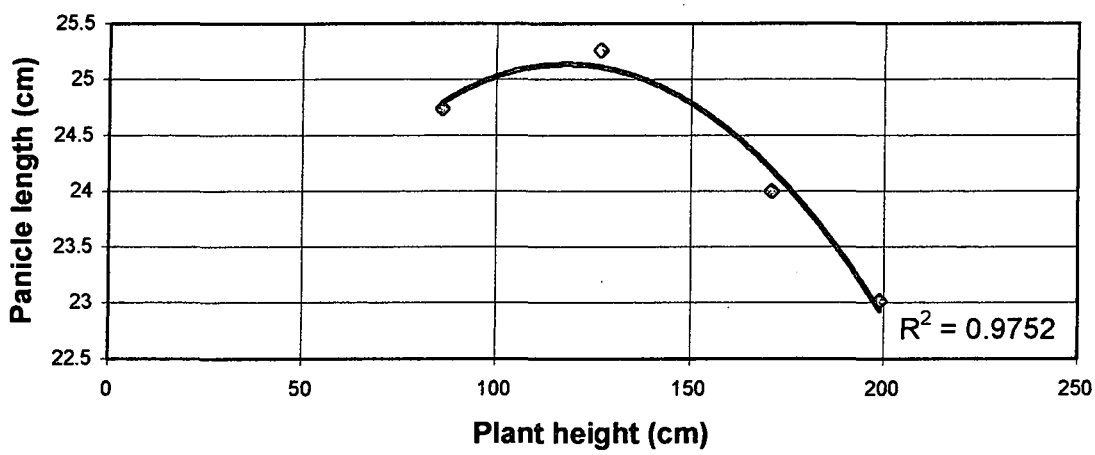


Figure 2.7. The relationship between plant height and number of nodes in near-isogenic lines of sorghum.

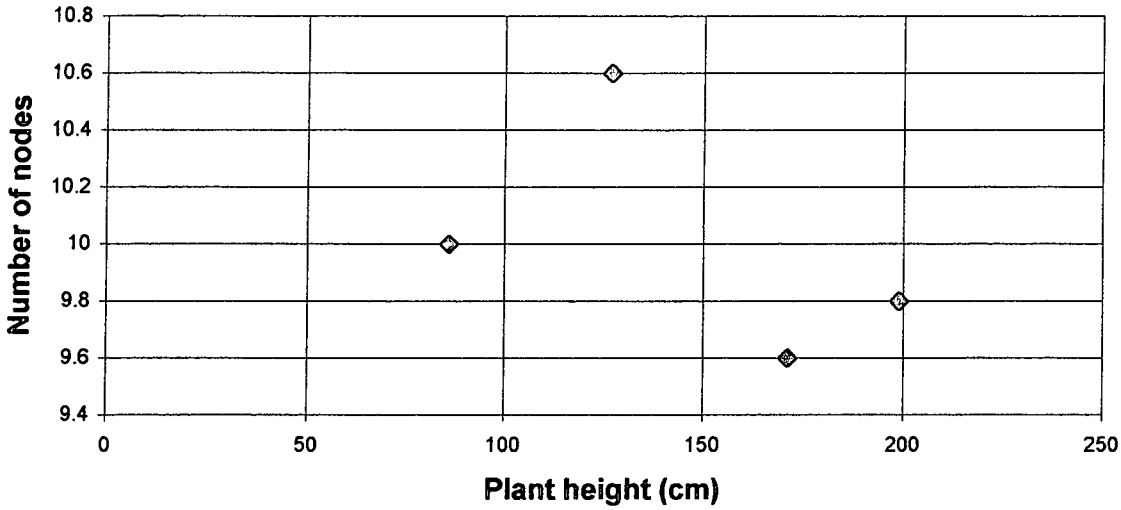
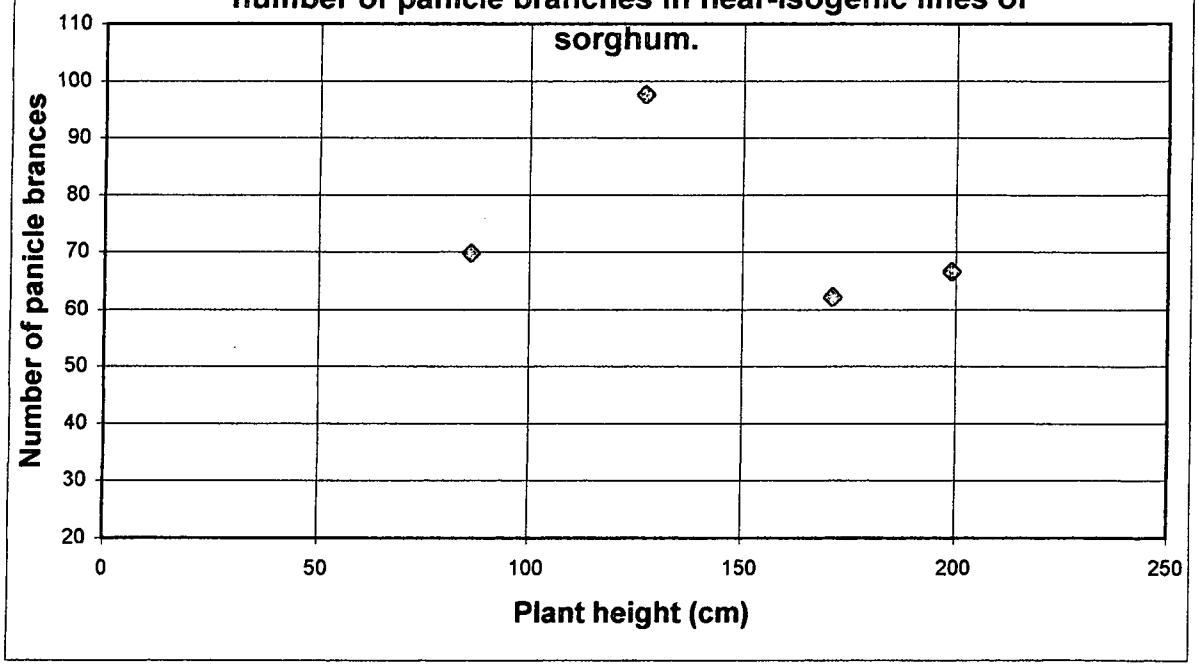


Figure 2.8. The relationship between plant height and number of panicle branches in near-isogenic lines of sorghum.



Chapter 3

The influence of Gibberellic Acid on Plant Height in sorghum

Abstract

Different sorghum varieties respond differently to changes in photoperiod. The internode length increases with the time a sorghum plant remains in its vegetative life cycle. It is known that four dwarf loci mediate different height classes in sorghum. Mutant dwarf genes in maize have been found to reduce the concentration of enzymes in the gibberellin (GA) biosynthetic pathway. The application of exogenous GA has been found to increase the height of maize and sorghum plants. To determine the interaction of dwarf genes with GA or GA synthesis, exogenous GA_3 was applied to near-isogenic lines for different height classes as well as the donor and recurrent parents. The application of exogenous GA_3 did not affect plant height at maturity. However, different growth stage specific effects were observed which may have induced an earlier onset of internode elongation. Four maturity (*ma*) genes control plant growth and floral initiation in sorghum. These data suggest that the genes controlling plant height in sorghum do not affect GA_3 synthesis or perception directly but rather the response to GA_3 and that other genes, possibly *ma* genes, further regulate the synthesis of GA_3 during the active growth phase. Therefore, the product(s) of the dwarf genes may act as negative regulator(s) in response to GA.

3.1 Introduction

The longer a sorghum plant remains vegetative, the greater number of leaves and nodes it develops (Quinby, 1974). Thus, photoperiodism has an indirect influence on height in sorghum. Late maturing plants are generally taller than early flowering

sorghum plants (Morgan and Finlayson, 2000). Four dwarf loci mediate plant height in sorghum (Quinby and Karper, 1954). The dwarf genes are thought to affect plant height by controlling internode length (Quinby and Karper, 1954; Quinby, 1974). The height of a sorghum plant is conditional to the number of loci at which recessive alleles are present. Therefore, theoretically five classes of height genotype exist, ranging from a zero-dwarf (tallest) to four-dwarf (shortest). However, no zero-dwarfs have yet been identified (Quinby and Karper 1954). Four-dwarf cultivars are used in the first cross of conversion programmes to produce three-dwarfs that are suited to combine harvesting (Miller, 1982).

Studies on height physiology in grasses have focussed on shoot elongation in maize (Thimann, 1980; Phinney, 1983; McMillan and Phinney, 1987; Rood *et al.,* 1988), rice (Reid, 1987; Hedden and Lenton, 1988), oats (Adams *et al.*, 1973) and wheat (Reid, 1990). These studies have focused on enzymes encoded in the Gibberellin (GA) biosynthetic pathway.

3.1.1 Metabolism of Gibberellins (GA)

Gibberellins are synthesised from acetate units of acetyl coenzyme A via the mevalonic acid pathway (Salisbury and Ross, 1991). *Geranylgeranyl pyrophosphate,* a 20-carbon molecule serves as a carbon donor for GA synthesis and is converted to *copalyl pyrophosphate*, consisting of two benzene rings, which is converted to *kaurene.* The conversion of *kaurene* to GA involves the oxidation of intermediate compounds (Salisbury and Ross, 1991). The first GA compound synthesised is GA_{12}, a 20-carbon molecule. GA_{12} gives rise to 20- and 19-carbon GAs, in the endoplasmic reticulum

(Hedden and Lenton, 1988; Salisbury and Ross, 1991). Different plant species follow different pathways from this point to form different gibberellins (Salisbury and Ross, 1991).

In studying the effect of GA on height, GA sensitive mutants and GA insensitive mutants have been identified in maize (Phinney, 1983). These mutants are pleiotrophic, reducing the size of all organs as well as affecting sexual development (Phinney, 1983). GA sensitive mutants are involved in GA synthesis and GA perception in the cell while GA insensitive mutants are involved in the response pathway to GA (Salisbury and Ross, 1991).

Only GA_1 is known to control stem elongation in maize plants even though many variants of GA are present (MacMillan and Phinney, 1987). Different mutant genes have been identified in maize that result in a dwarf phenotype (MacMillan and Phinney, 1987). These genes control GA synthesis and the phenotype can be restored to the wild type by the application of GA (MacMillan and Phinney, 1987).

Although GA is required for stem elongation, it is not a general stem elongation enzyme. GA insensitive mutants have also been identified in wheat and maize (Reid, 1990). These mutant plants produce adequate levels of GA_1, but are not able to respond to it, possibly due to a lack of receptor protein (Salisbury and Ross, 1991).

3.1.2 Internode elongation in sorghum

While dwarfism genes in maize are pleiotrophic, dwarfism genes in sorghum are brachytic (Quinby and Karper, 1954). Dwarfism genes in sorghum do not regulate organ size as in the case of maize (Phinney and Spray, 1987). In sorghum, the presence of recessive genes reduces plant height (Quinby and Karper, 1954). Furthermore, the dwarfism genes result in a proportional reduction of internode length (Quinby and Karper, 1954; Shertz, 1970).

Sorghum exhibits three different internode elongation patterns: ever-increasing, unimodal and bimodal (Ayyangar *et al.*, 1937). Plants with the ever-increasing pattern develop increasingly longer internodes from the soil level upward with the shortest internode found nearest the base. In the unimodal pattern, the shortest internode is found nearest the panicle and for the bimodal pattern, the internodes nearest the top and bottom of the culm are shorter than the intermediate internodes. The different internode elongation patterns are associated with early, medium and late maturing varieties (Ayyangar *et al.*, 1937). It has been suggested that the maturity genes also influence internode elongation (Quinby, 1975).

Maturity (ma) genes are responsible for shoot elongation after floral initiation (Quinby, 1995). For example, plants with the ma_3 gene exhibit earlier and more rapid shoot elongation after floral initiation than plants with the Ma_3 gene (Childs *et al.*, 1997). The Ma_3 gene encodes the phytochrome B apoprotein that is not detectable in plants with the ma_3 genotype (Foster *et al.*, 1994; Childs *et al.*, 1997). Phytochrome B influence internode elongation and may result in the regulation of GA biosynthesis.

3.1.3 Effect of GA

Exogenous application of GA_3 on sorghum and maize seedlings, has been shown to affect leaf blade length, leaf sheath length as well as internode length in sorghum (Morgan *et al.,* 1977). However, it is thought that GA_3 is cultivar specific with no response in certain cultivars and a doubling of height in others (Morgan *et al.,* 1977). Morgan *et al.* (1977) reported that the application of GA_3 at saturating levels (136 ug) on 6-week old plants, at two-week intervals, increased the height of plants between 20% and 60%. Increasing the dosage of GA_3 above 136 ug did not have any additional height increase effect (Morgan *et al.,* 1977). It appears that exogenous application of GA_3 acts to remove the effect of one recessive *dw* gene (Morgan *et al.,* 1977). However, this study was performed on different genotypes and direct comparison of results is difficult. The difference in variety response suggests the influence of other genes, possibly maturity genes on the effect of GA_3.

The aim of this study was to determine the effect of exogenous GA application on plant height of near-isogenic lines differing in plant height as well as of the donor and recurrent parental lines.

3.2 Materials and Methods

3.2.1 Plant material

Near-isogenic lines, for the different height classes, 1*dw*, 2*dw*, 3*dw*, and 4*dw* were developed at the Grain Crops Institute of the Agricultural Research Council at

Potchefstroom, South Africa. The lines were developed through a backcross process in which a tall tropical sorghum cultivar (SA748), of unknown genotype but assumed to be 1*dw*, was crossed with a four-dwarf Martin (*dw*$_1$ *dw*$_2$ *dw*$_3$ *dw*$_4$) of US origin. The F_1 population was selfed, short plants selected from the segregating F_2 population and backcrossed to the tall SA748 parent for eight generations and the heterozygous types were selfed after each successive backcross. The present study was conducted in a greenhouse at the University of the Free State, Department of Plant Sciences, Bloemfontein, South Africa.

Two replicates consisted of two seeds from each height class were planted in 23 cm diameter pots. Two plants were randomly selected in each height class for treatment with GA_3 and two to serve as controls. The two replicates consisted of four height classes including parental lines were placed in separate adjacent cubicles, two weeks after germination. GA_3 (300 ug/ml) was applied with a spray bottle directly on the shoots, stems and leaves every second day on the experimental set, according to the maximum dose response suggested by Morgan *et al.* (1977). Height of control plants and treated plants were measured once a week from the ground to the tip of the most recently formed leaf. At the 11th week after germination, measurements were taken from the ground to the top of panicle.

3.3 Results

At the onset of the treatments, the control and GA_3 treated plants differed by at most 7 cm two weeks after germination (WAG) (Table 3.1). At four WAG, the control and GA_3 treated plants had increased similarly in height. The GA_3 treated plants of the 1*dw* NIL were 21 cm, the 2*dw* NIL plants were 5 cm and the 3*dw* NIL plants were 19 cm taller than the control. The 4*dw* NIL GA_3 treated plants were 15 cm shorter than the control plants.

After five weeks, the GA_3 treated plants were on average 4 cm to 29 cm taller than the control plants with the exception of the 1dw parent (SA748), which did not show response (Table 3.1). After eight weeks, the difference in height between GA_3 treated and control plants of the 1*dw* NILs was 74 cm, the 2*dw* NILs 18 cm, the 3*dw* NILs 12 cm and the 4*dw* NILs 1 cm. The 1*dw* parent control was 1 cm taller compared to the GA_3 treated plants and the 4*dw* GA_3 treated parent (Btx406) was 15 cm taller than the control (Table 3.1).

After ten weeks the presence of ears was visible in the GA_3 treated plants of the 4*dw* parent (Btx406), the 4*dw* NIL, the 3*dw* NIL and the 1*dw* NIL as well as in the control plants of the 4*dw* NIL. The difference in height between GA_3 treated and control plants was 24 cm in the 4*dw* parent (Btx406), 1 cm in the 4*dw* NIL, the 3*dw* NIL and the 1*dw* parent (SA748), respectively, 2 cm in the 2*dw* NIL and 45 cm in the 1*dw* NIL (Table 3.1).

After eleven weeks, the difference in height between GA_3 treated and control plants was from 1 cm to 4 cm. Height ranged from 77.5 cm (4*dw* NIL) to 168 cm (1dw NIL). There were 5 distinct height groups with an LSD of 4.387 and p = 0.001 (Table 3.2).

3.4 Discussion

The application of GA_3 to NILs for different height classes and parental lines produced no significant difference in final height between the treated and control plants (Table 3.2). This contradicts a previous report, which found height increase of up to 20% and 60% in some genotypes after exogenous GA application (Morgan *et al.*, 1977). Data from the present study suggests that the different dwarf genes do not respond to GA treatment.

The greatest effect of GA_3 application was observed by increased height and differed for the different genotypes. The greatest effect between weeks 2 to 5 was observed for the 4*dw* NIL, between weeks 6 to 11 for the 4*dw* parent (Btx406), the 1*dw* NIL and the 2*dw* NIL, as well as between weeks 2 to 11 for the 3*dw* NIL. These data suggest that the effect of exogenous GA_3 is growth stage dependent for the different lines studied. It is known that the maturity genes are associated with the regulation of GA_3 synthesis (Childs *et al.*, 1997). Furthermore, different maturity genes appear to have different effects on stem elongation (Foster *et al.*, 1994; Childs *et al.*, 1997). Therefore, the growth stage effect of exogenous GA_3 could be a result of the effect of different *ma* genes.

The application of GA_3 had no effect on the 1dw recurrent parent (SA748) in comparison to the control (Figure 3.6). It is known that ma_3 must be present before the maturity genes have any effect on elongation (Childs *et al.*, 1997). These data indicate that the 1*dw* parent does not posses ma_3 as no response to GA_3 application was observed. This also suggests that ma_3 and a combination of other ma genes is present in the other lines.

The application of GA_3 did not act by increasing a height similar to the effect of adding a dominant (*Dw*) gene, as previously suggested (Morgan *et al.*, 1977). These data support the hypothesis that the *dw* genes have an additive effect on height (Quinby and Karper, 1954; Ross, 1971). These data suggest that an increase in the number of *dw* genes present decreases the response to GA_3 in terms of height increase of sorghum plants. It is also evident that other genes, possibly maturity (*ma*) genes further modulate the response of GA_3 by increasing height. This excludes the effect of *dw* gene products in GA synthesis or GA perception and suggests that the recessive gene products may act as a negative regulator in response to GA_3. The small population size used in this study makes statistical comparison difficult and more plants should be included in further studies.

Table 3.1. The response in growth of GA_3 treated plants and control plants in NIL dwarf sorghum lines as well as parental lines.

Excession	Treatment	Plant length at weekly interval (cm)									
		Week 2	Week3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
Btx406 4*dw* parent	GA_3	18	26	39	56	52	85	82	86	102*	103*
Btx406 4*dw* parent	Control	12	31	45	45	55	56	67	74	78	102*
4*dw* NIL	GA_3	14	19	25	59	53	58	66	70	76*	77*
4*dw* NIL	Control	20	32	40	50	59	62	67	75	77*	78*
3*dw* NIL	GA_3	10	24	38	55	60	62	80	102	102*	102*
3*dw* NIL	Control	17	19	19	26	36	48	68	83	101	101*
2*dw* NIL	GA_3	19	28	40	61	61	72	103	107	106	120*
2*dw* NIL	Control	19	26	35	57	64	62	85	99	108	121*
1dw NIL	GA_3	13	30	50	60	77	107	167	185	170*	170*
1dw NIL	Control	13	25	29	51	68	70	93	115	125	166*
SA748 1*dw* parent	GA_3	12	26	33	61	65	77	103	129	133	152*
SA748 1*dw* parent	Control	17	29	40	61	70	77	104	123	132	154*

* presence of ear

Table 3.2. Plant height of GA_3 and control teated plants for four near-isogenic lines and two parental lines at 11 weeks after germination (LSD 4.387).

Excession	Plant height (cm)		
	GA_3	Control	*
4dw parent (Btx406)	103	102	b
4dw NIL	77	78	a
3dw NIL	102	101	b
2dw NIL	120	121	c
1dw NIL	170	166	e
1dw parent (SA748)	152	154	d

* means followed by the same letter do not differ significantly

Figure 3.1. Response in growth of GA3 treated and control plants in the 4dw parent (Btx406) line over time.

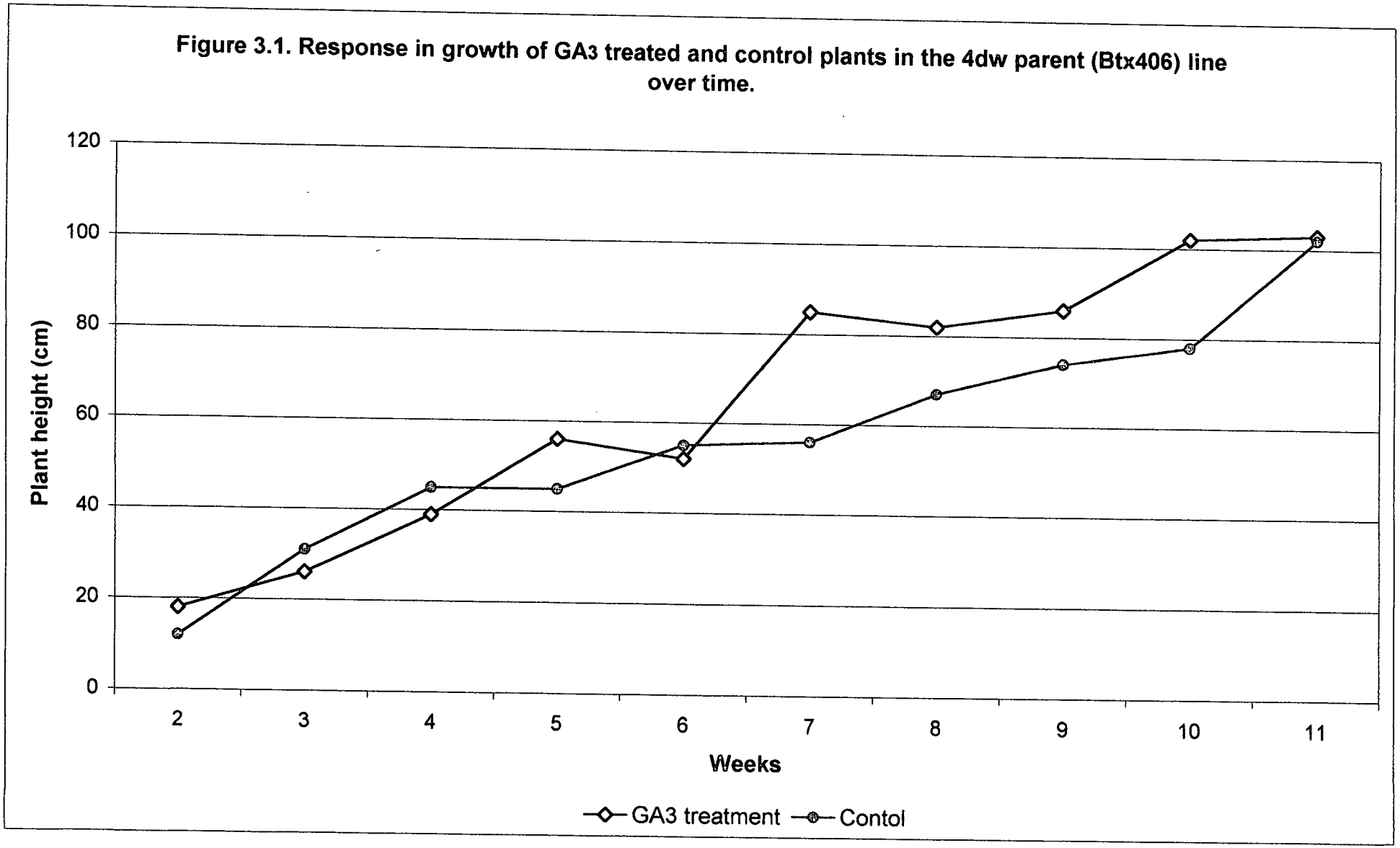


Figure 3.2. Response in growth of GA3 treated and control plants in the 4dw NILs over time.

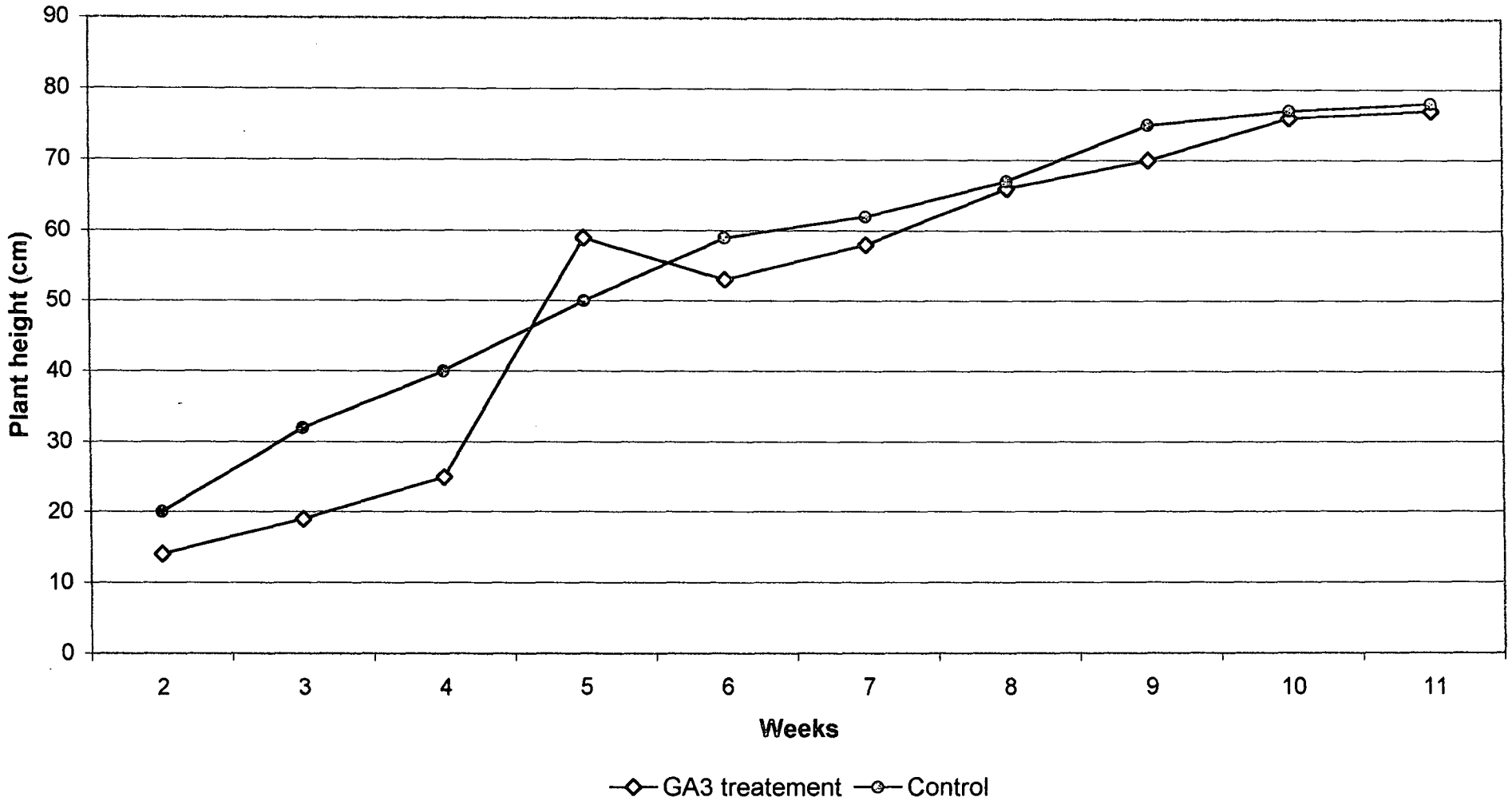


Figure 3.3. Response in growth of GA3 treated and control plants in 3dwNILs over time.

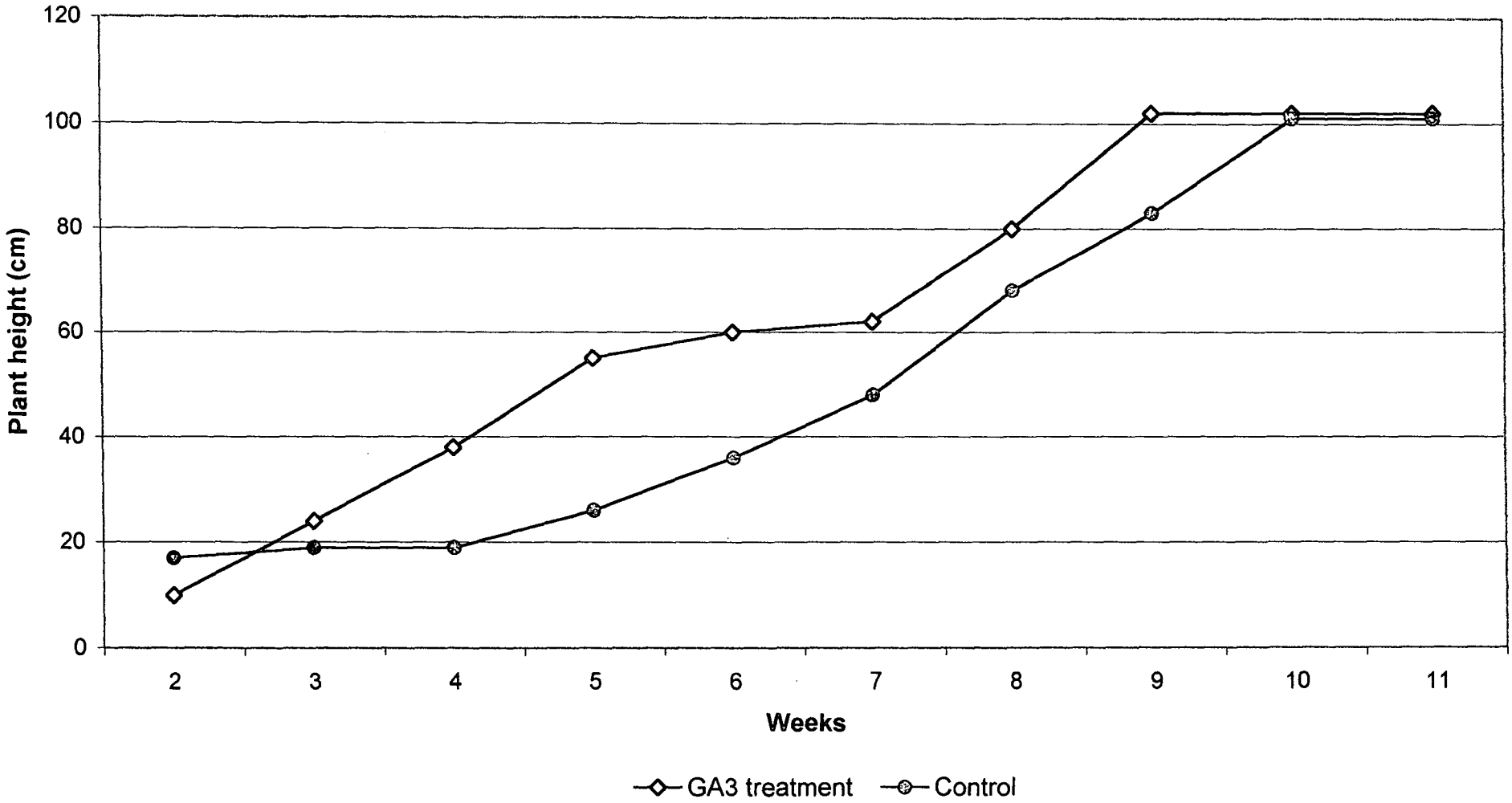


Figure 3.4. Response in growth of GA3 treated and control plants in 2dw NILs over time.

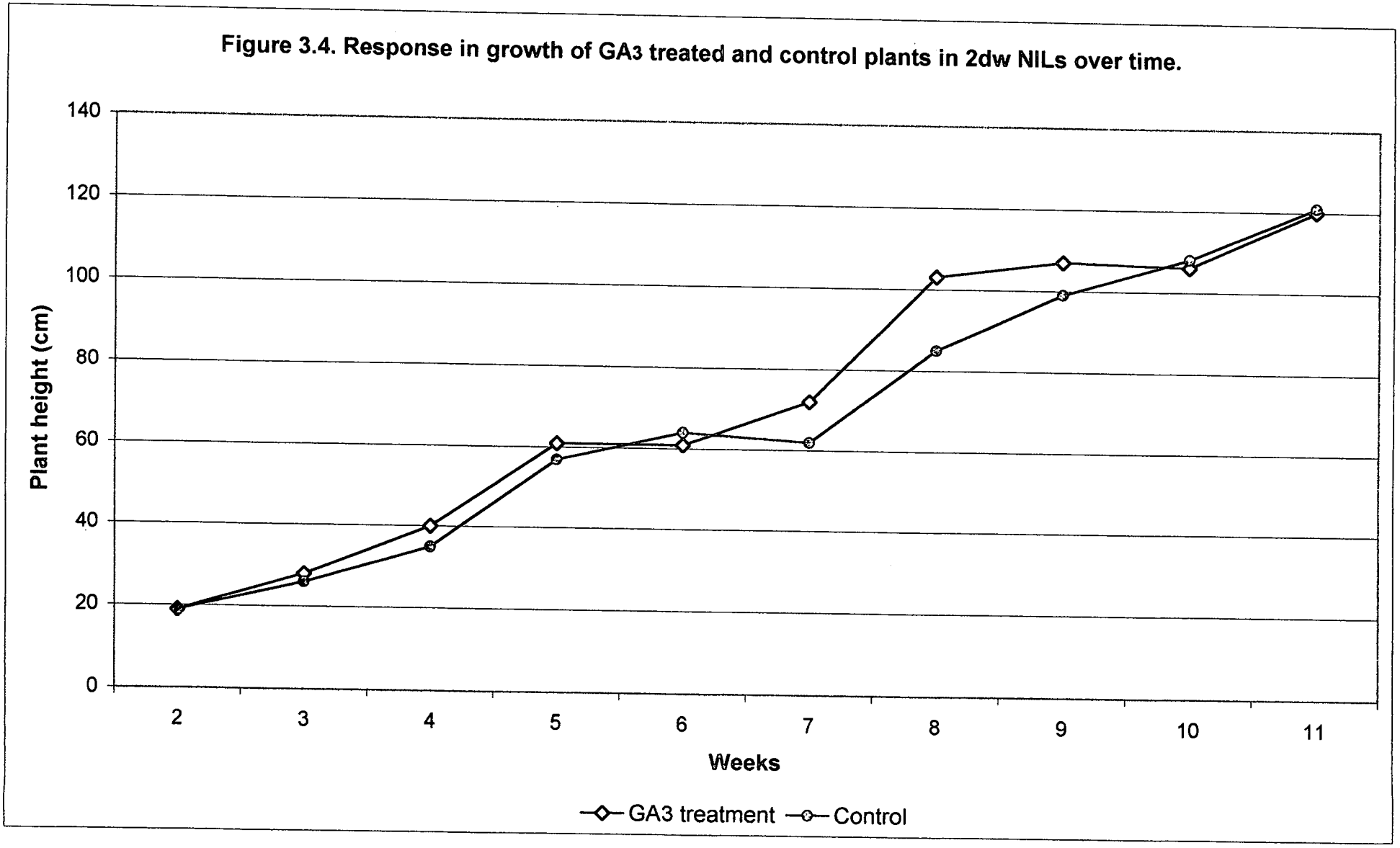


Figure 3.5. Response in growth of GA3 treated and control plants in 1*dw* NILs over time.

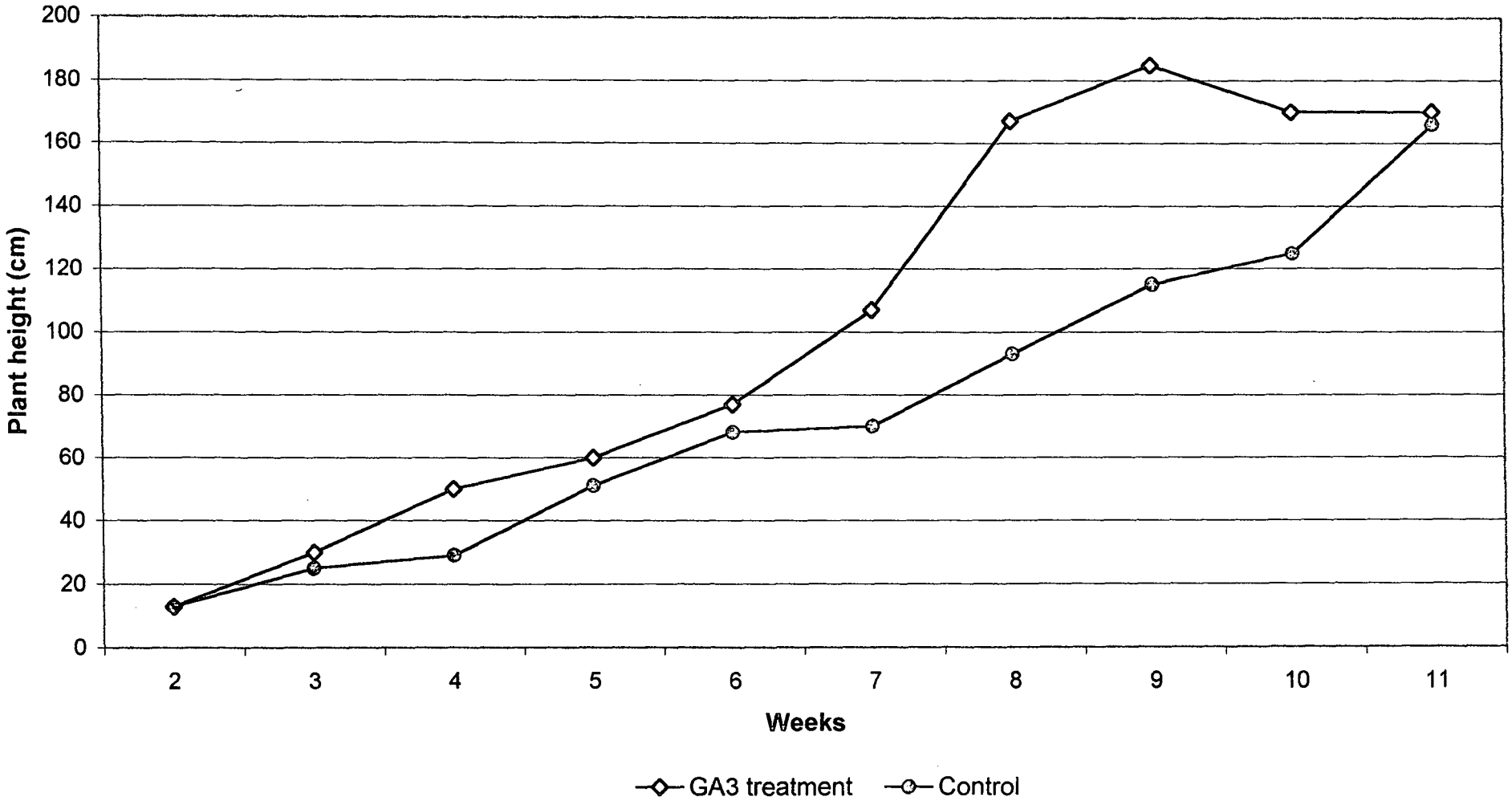
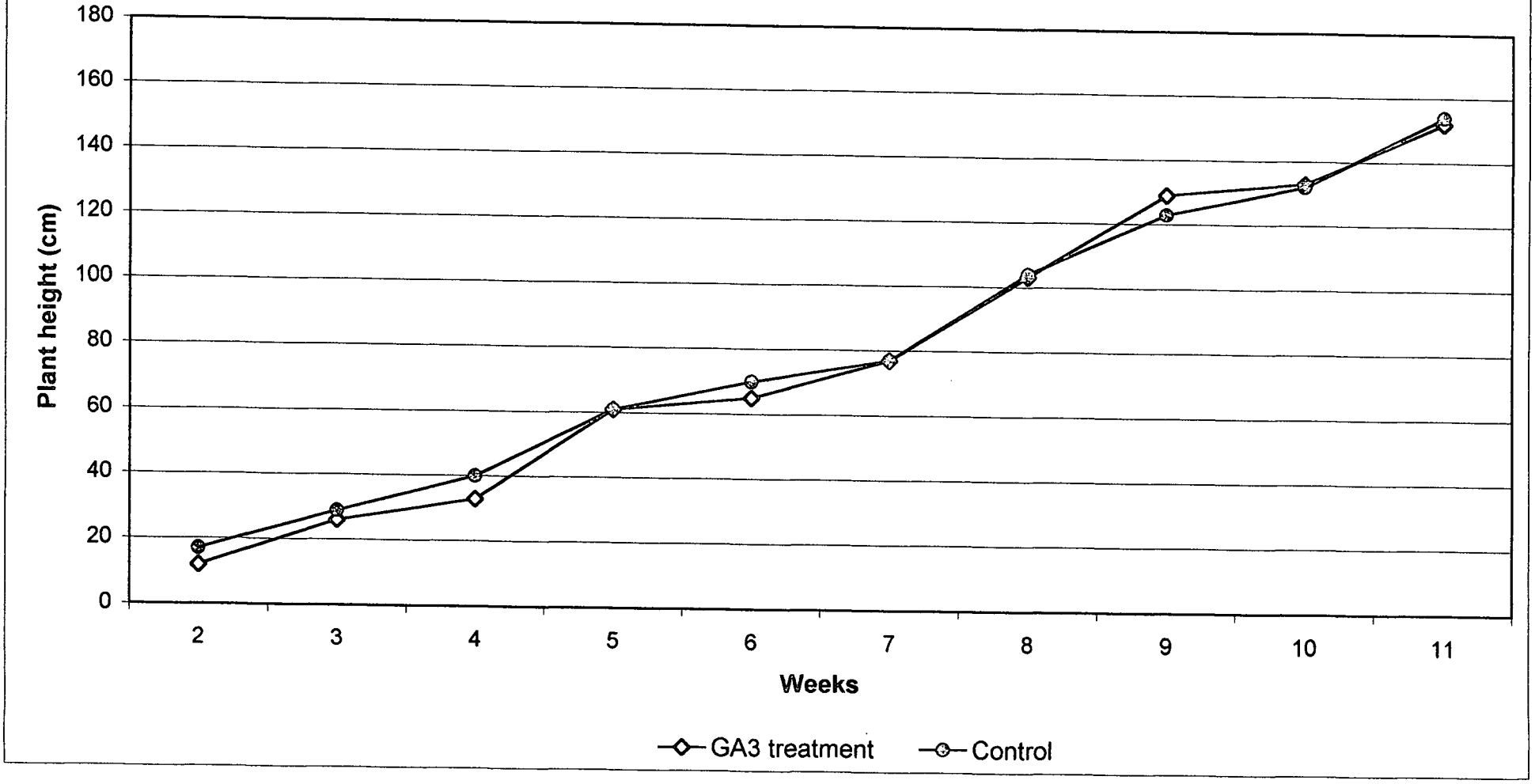


Figure 3.6. Response in growth of GA3 treated and control plants in the 1*dw* parent (SA748) line over time.



Chapter 4

Identification of Simple Sequence Repeats (SSR) linked to dwarfism genes in Sorghum

Abstract

Exotic or tropical sorghum is an important source of variation for traits such as improved yield, insect, disease and drought resistance and improved grain quality. Sorghum conversion programmes are aimed at converting tall, late maturing exotic varieties into short statured, early maturing cultivars. Phenotypic traits of grain sorghum are often difficult to measure, have low heritability, are genetically complex or are influenced by environmental conditions. Furthermore, the process of line conversion is tedious and time consuming. Plant height is controlled by dwarfism genes at four loci namely, dw_1, dw_2, dw_3 and dw_4. The use of molecular markers to select the correct height variant would greatly facilitate the conversion process. SSR (Simple Sequence Repeats) markers are increasingly being used in crop development as a tool in marker-assisted breeding. A PCR based approach was used to study SSRs in near-isogenic lines for the different height classes in sorghum. Four out of eighteen SSR loci were found to be associated with height in sorghum. SSR markers were identified for dw_1, dw_2 and Dw_2. This is the first study to deduce the height genotypes for the different near-isogenic lines and parental lines using SSR markers.

4.1 Introduction

Sorghum grain crop improvement is achieved through conventional plant breeding (Doggett, 1988). However, desirable traits of grain sorghum are often difficult to measure phenotypically, have low heritability, are genetically complex and are

influenced by environmental conditions. These factors thus restrain the rate of sorghum variety improvement (McIntyre *et al.*, 2001). Molecular marker technology has great potential to contribute to the genetic improvement of sorghum, especially in conversion programmes like those undertaken by the International Crops Research Institute of the Semi-Arid Tropics (ICRISAT), the Texas Experimental Station Agricultural Research Service (ARS-TEAS) and the Tropical Agricultural Research Station (TARS) (Stephens *et al.*, 1967; Miller, 1982).

Sorghum conversion involves backcross breeding, where an exotic plant with desired traits is crossed with short, early-maturing lines (Rosenow and Dahlberg, 2000). The F_1 progeny are selfed and short, early maturing plants selected from F_2 progeny. The F_2 progeny are again selfed to minimise segregation and plants in the F_3 are backcrossed to the exotic line to ensure that the desired traits are maintained (Miller, 1982). Conversion projects are aimed at transforming tall, late or non-flowering sorghum with unique properties into commercially acceptable short, early maturing forms that can be planted anywhere in the world (Duncan *et al.*, 1991).

Plant height in sorghum is conditioned at four dwarfism loci dw_1, dw_2, dw_3, and dw_4, with tallness (*Dw*) dominant to dwarfness (*dw*) (Quinby and Karper, 1954). Although the four *dw* genes mediate five different height classes, no cultivars are known to exist for the tallest height class (zero-*dw*), dominant (*Dw*) at all four dwarfism loci. The 1*dw* height class is homozygous, recessive at a single *dw* locus. The 2*dw* height class is homozygous, recessive at two of the four *dw* loci and the 3*dw* height class is homozygous, recessive at any three of the four *dw* loci. The 4*dw* height class, shortest

of the height classes, is homozygous, recessive at all four the *dw* loci (Quinby and Karper, 1954). The effect of recessive alleles at any of the four *dw* loci can decrease tallness by 50 cm or more (Quinby and Karper, 1954). However, if other recessive *dw* genes are already present then the effect of additional loci is decreased. Thus, the height difference between a 3*dw* and a 4*dw* (recessive at three and four height loci, respectively) can be as little as 10 cm (Quinby and Karper, 1954). In addition to this, homozygous tall plants are taller than heterozygous tall plants (Quinby and Karper, 1954). Therefore, the difficulty of identifying the genotype of a specific phenotype makes height selection during line conversion difficult (McIntyre *et al.*, 2001).

The introduction of molecular markers to plant breeding has proven to be a valuable tool for germplasm conservation and diversity studies (McIntyre *et al.*, 2001). Several markers have been identified in sorghum with great effect, using RFLPs, SSRs and AFLPs (Chittenden *et al.,* 1994; Lin *et al.*, 1995; Brown *et al.*, 1996; Boivin *et al.,* 1999).

SSR marker technology has successfully been developed and used for DNA fingerprinting and genome mapping in sorghum, rice, barley, wheat, maize and soybean and other crops (Senior *et al.*, 1993; Wu and Tanksley, 1993; Cregan *et al.*, 1994; Saghai Maroof *et al.*, 1994; Yang *et al.*, 1994; Röder *et al.*, 1998; Brown *et al.*, 1996; Taramino *et al.*, 1997). This relatively new class of molecular markers is derived from tandemly repeated oligonucleotide repeats such as $(AG)_n$ or $(ATT)_n$, usually two to five nucleotide bases repeated n times (Litt and Lutty, 1989). These heritable repeats are highly polymorphic even among closely related individuals (Akkaya *et al.*, 1992). Such markers are especially useful for molecular analysis in organisms with low levels of

diversity (Dietrich *et al.*, 1994). Allozyme studies on sorghum have shown that genetic diversity is lower in sorghum than in cereals like maize and barley (Moredent *et al.*, 1989; Ollitrault *et al.*, 1989). This makes SSRs an ideal marker type in sorghum, especially for use in near-isogenic lines.

The initial cost and time required to develop SSRs is high (Taramino *et al.*, 1997). SSRs are developed by constructing genomic libraries and screening with SSR probes and sequencing positive clones, by searching for SSRs in sorghum sequence databases and testing primers from maize and paspalum on sorghum (Smith *et al.*, 2000). However, since the advent of SSR markers, 62 sorghum specific SSR markers have been developed (Brown *et al.,* 1996; Taramino *et al.*, 1997). The first development of SSRs in sorghum was by Brown *et al.* (1996) who published a total of 49 sorghum SSR specific PCR primers. In a similar study, Taramino *et al.* (1997) reported on the analysis of 13 SSR loci in sorghum, as well as the only incidence of SSR sorghum specific mapping.

Since the development of SSR sorghum specific primers, six studies have incorporated SSRs or microsatellites in genetic diversity studies (Wenzel *et al.*, 1998; Dean *et al.*, 1999; Djè *et al.*, 1999; Djè *et al.*, 2000; Smith *et al.*, 2000; Klein *et al.*, 2001; Ghebru *et al.*, 2002). In all of these studies, the genetic diversity between germplasm accessions was studied. Smith *et al.* (2000) used SSRs to determine the potential of hybrid seed production as well as genetic resource conservation and management of sorghum.

Taramino *et al.* (1997) mapped seven SSRs in five linkage groups A, E, F, H and J and identified two height QTLs in A and H using 2*dw* and 3*dw* lines. Using RFLP QTLs,

Pereira and Lee (1995) identified four linkage groups, A, B, E and H with significant effects on plant height. For all the QTL studies thus far, only linkage groups A and H have commonly been associated with height (Lin *et al.* 1995; Pereira and Lee, 1995; Taramino *et al.*, 1997). Pereira and Lee (1995) suggested that the Dw_2 locus is located on linkage group H and Dw_3 on linkage group A based on pleiotropic effects on panicle and tiller characteristics (Dw_3) and panicle characteristics (Dw_2).

The aim of this study was to identify useful polymorphisms in near-isogenic lines for plant height using SSRs in an attempt to find associations with *dw* genes as present in the different near-isogenic lines. Previously mapped SSRs were included in this study in an attempt to associate the *dw* genes to different linkage groups.

4.2 Materials and Methods

4.2.1 Plant material

Near-isogenic lines for the different height classes 1*dw*, 2*dw*, 3*dw* and 4*dw* were used in this study. The lines were developed at the Grain Crops Institute of the Agricultural Research Council at Potchefstroom, South Africa, through a backcross process in which a tall tropical sorghum cultivar (SA748) was crossed with a four-dwarf Martin (Btx406). The F_1 population was selfed, single short plants selected and backcrossed to the tall parent (SA748). This procedure was repeated for eight generations. The donor parent line, Btx406 of US origin, is an early maturing 4*dw* (dw_1 dw_2 dw_3 dw_4) Martin B-line. The

genotype of the recurrent parent, SA748, is unknown, but is assumed homozygous, recessive at one *dw* locus due to its specific plant height (230 cm).

4.2.2 DNA extraction

Ten plants from each NIL and parental lines, Btx406 and SA748 were grown from seed for two weeks. DNA was extracted according to a modified method of Edwards *et al.* (1991). Leaf material was collected and ground to a fine powder in liquid nitrogen. The ground plant material was incubated at 65°C, with periodic shaking for one hour in 10 ml extraction buffer (0.01 M EDTA [pH 8.0], 0.05 M Tris-HCl [pH 8.0], 0.1 M NaCl and 1% SDS) with the addition of 8 M Urea. After incubation, the cellular debris and proteins were extracted by the addition of chloroform:iso-amylalcohol (24:1) in a 1:1 ratio followed by centrifugation for 10 min at 8000 rpm. Chloroform extractions were repeated until the interface was visually clear of debris. This was followed by the addition of 2 volumes 100% cold ethanol to the supernatant to precipitate the DNA. The precipitated DNA was spooled using a sterile Pasteur pipette and washed twice in 70% ethanol. The DNA was resuspended in 200 ul sterile distilled water and stored at –20°C.

4.2.3 DNA concentration determination

The concentration of DNA was determined spectrophotometrically at 260 nm using the formula: [DNA] = Optical density (OD_{260}) x dilution x constant (50 ug/ml). The DNA was diluted to 100 ng/ul. Bulked DNA was used and 100 ng DNA from ten plants in each height class was bulked. Individual plants in each height class were also tested to ensure genotype uniformity.

4.2.4 SSR amplification

PCR amplification was used to detect SSR loci using 18 primer pairs (Table 4.1). PCR was performed in 25 ul reactions containing: 50 ng of bulked DNA, 10 pmoles each of 'forward' and 'reverse' primer, 2.5 ul 10 x PCR buffer (100 mM Tris-HCl [pH 8.4], 500 mM KCl and 15 mM $MgCl_2$) and 0.5 U ampli *Taq* DNA polymerase (Roche). Temperature cycling was performed in the Applied Biosystems GeneAmp^R PCR system 2700. The amplification profile consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of 95°C for 1 min, 45°C or 50°C (annealing temperature) (Table 4.1) for 2 min and 72°C for 2 min. The extension time at 72°C was increased to 10 min in the final PCR cycle.

4.2.5 SSR visualisation

The PCR products were analysed on a 2% agarose gel (Molecular Screening agarose Roche) at 75V for 2 hours in 0.5 TAE (0.438 g/L Tris, 0.09 ml/L Acetic acid and 0.022 g/L EDTA). SSR fragments were visualised using ethidium bromide under UV light. DNA fragments were sized using a 100 bp ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500) (Promega) and the Gel Doc 1000^{TM} image analysis system (Biorad).

4.2.6 Data analysis

Fragment data was tabulated in Microsoft Excel and genetic distance analysis and dendrograms determined using the Hierarchical clustering method (NCSS 2000). Percentage polymorphism was calculated manually over all the primer combinations used and the diversity index calculated using the formula: $[1-\Sigma(pi)^2]$ (N/N –1) were N is

the population size and pi is frequency of the ith allele in the population studied (Pereira *et al.*, 1994).

Genotypes were assigned according to the presence of loci assumed to be associated with height based on the following criteria for genotype deduction:

1. The four-dwarf height class contains only recessive genes (dw_1 dw_2 dw_3 dw_4).
2. The one-dwarf height class contains only one homozygous, recessive gene and is assumed to be dw_4 as this gene is the predominant recessive gene in all commercial sorghum lines (Quinby and Karper, 1954; Schertz, 1973; Ross and Kofoid, 1979).
3. The two-dwarf height class, must contain two homozygous, recessive genes. The three-dwarf height class must contain three homozygous, recessive genes.
4. Dominance at a locus can include both the homozygous (*DwDw*) and heterozygous (*Dwdw*) condition, but the presence of the recessive trait implies homozygosity at a locus (*dwdw*).

4.3 Results

Fifteen SSR primers amplified a total of 31 bands, on average 2.1 per primer pair (Figure 4.1). There was a 90% polymorphism over all the bulked lines analysed for the different SSR primers. Individual plants in each height class produced the same amplification products as bulked lines. The polymorphic information content (diversity index) over all the primers ranged from 0.17 to 0.73 (Table 4.2).

Three SSR primers for $(AC)_{14}$, $(AG)_{27}$ and $(ACGAC)_4(AG)_6$ produced no amplification product. A single non-polymorphic amplification product was produced for $(AG)_{14}$ (188 bp) and $(AG)_{39}$ (120 bp). A single polymorphic SSR was amplified for $(AG)_{19}$ (190 bp), $(AG)_{20}$ (202 bp), $(AG)_{38}$ (120 bp), $(AG)_{41}$ (96 bp) and $(ACA)_9$ (245 bp) (Table 4.3).

Two alleles were amplified for $(AG)_{12}$ (70 bp and 200 bp), $(AG)_{16}$ (74 bp and 260 bp), $(AG)_{33}$ (101 bp and 580 bp), $(AG)_{35a}$ (70 and 100 bp) and $(AG)_{34}GA(CA)_4$ (88 bp and 150 bp) (Table 4.2).

Four SSR primer pairs identified multilocus fragments for $(AG)_{16}$ (80 bp, 160 bp and 195 bp), $(AG)_{18}$ (300 bp, 600 bp and 660 bp), $(AC)_{25}$ (377 bp, 571 bp, 664 bp, 1523 bp and 1887 bp), $(AG)_{35b}$ (116 bp, 195 bp, 338 bp and 520 bp) (Table 4.2).

Four SSR markers conformed to the genotype deduction criteria and were used to determine the genotypes for parent and NIL lines (Table 4.4; 4.5). Potential markers were identified for dw_1 $(AG)_{16}$ (70 bp) and $(AG)_{20}$ (202 bp) present in all the lines except the 1*dw* parent (SA748). The markers for Dw_2 $(AG)_{35b}$ (116 bp) was present in the 1*dw* parent, the 1*dw* NIL and the 2*dw* NIL and dw_2 $(AG)_{35b}$ (195 bp) was present in the 4*dw* parent, the 3*dw* NIL and the 4*dw* NIL (Table 4.5).

The genetic distance among all the NILs was an average of 0.578 and the genetic distance between the 1dw parent (SA748) and 4dw parent (Btx406) was 0.681. The genetic distance between the NILs and 4dw parent (Btx406) and 1dw parent (SA748) was 0.705 and 0.735, respectively (Table 4.6).

Clustering analysis using genetic distances determined by SSR data identified two main clusters A and B. Cluster A contained the near-isogenic lines and was further sub divided into sub-cluster 1 and 2. Sub-cluster 1 contained the 2dw and 3dw NILs, while sub-cluster 2 contained the 1dw and 4dw NILs. Cluster B contained the parental lines Btx406 and SA748 (Figure 4.2).

4.4 Discussion

Of the fifteen primers successfully used to amplify SSR loci in the near-isogenic lines for plant height, twelve were polymorphic (Table 4.2) and four identified potential markers for specific *dw* genes based on the genotype deduction criteria (Table 4.3). The identification of SSR loci associated with height enabled the assignment of genotypes to the different near-isogenic as well as parental lines (Table 4.5). SSR markers were identified for dw_1, dw_2 and Dw_3. The identification of *dw* genes using SSR markers will reduce the time needed for line conversion considerably. This is the first study to deduce height genotype by assigning potential markers to specific dwarf genes or their alleles (Table 5.5).

Taramino *et al.* (1997) determined that locus $(AG)_{35b}$ was in linkage group A and $(AG)_{33}$ in linkage group H. Pereira and Lee (1995) suggested that Dw_3 was in linkage group A and Dw_2 in linkage group H based on RFLP QTLs. However, $(AG)_{33}$ was not found to be associated with the dwarfism genes in the present study. Therefore, it was assumed that dw_3 was in linkage group A (Pereira and Lee, 1995; Taramino *et al.*, 1997).

A number of SSR loci were polymorphic for the different near-isogenic lines but could not be specifically associated with dwarfism genes. Quinby and Karper (1954) reported the effect of maturity genes on sorghum plant height. Therefore, it is possible that the polymorphic loci not associated with *dw* genes may be associated to maturity genes or other height associated genes.

The genetic distances and resulting dendrogram indicate that as expected the NILs are more closely related to each other than the recurrent 1dw parent (SA748) and the donor 4dw parent (Btx406). However, the NILs are almost equally related to each parent line, at 0.705 and 0.735 respectively. This suggests that the introgressed segment from the 4*dw* parent (Btx406) is large and has been maintained in backcross populations. The selection of short, early maturing plants during backcrossing may have introduced a bias for the presence of dwarfism as well as maturity genes. This supports the hypothesis that other genes are also associated with plant height in sorghum.

This is the first study to use SSRs in identifying genes associated with height and to deduce the genotypes of different height classes in sorghum. Possible SSR markers associated with genes influencing height will facilitate conversion programmes for exotic varieties.

Table 4.1. Sorghum SSR primers used for the amplification of SSR loci.

SSR repeat	Primer	Flanking sequence (5'-3')	Tm°C	Product Size bp**	Diversity Index	Linkage Group*
$(AG)_{12}$	Sb 5-85a	F:AGACGCTTTTCTCTCTCTCTCTCTCTCT R:TAGCCCTGCCGCATACTGAATG	60	200-225	0.8	nd
$(AC)_{14}$	Sb 4-12a	F:GAAAAATCTCCGTCAATCCCAAAATA R:CGCTGAACAACGAAAGGAATAAGTG	60	200-225	0.45	nd
$(AG)_{14}$	Sb 6-84a	F:CGCTCTCGGGATGAATGA R:TAACGGACCACTAACAAATGATT	58	170-190	0.69	nd
$(AG)_{15}$	Sb 4-32a	F:CTCGGCGGTTAGCACAGTCAC R:GCCCATAGACAGACAGCAAAGCC	59	160-180	0.7	nd
$(AG)_{16}$	Sb 4-15a	F:GCTGCTAAGCCGTGCTGA R:TTATTTGGGTGAAGTAGAGGTGAACA	57	120-130	0.62	nd
$(AG)_{18}$	Sb 6-57a	F:ACAGGGCTTTAGGGAAATCG R:CCATCACCGTCGGCATCT	60	285-305	0.46	nd
$(AG)_{19}$	Sb 6-36a	F:GCATAATGACGGCGTGCTC R:CTTCCAAGTGAAAGAAACCATCA	60	155-190	0.73	nd
$(AG)_{20}$	Sb 5-236a	F:GCCAAGAGAAACACAAACAA R:AGCAATGTATTTAGGCAACACA	57	165-185	0.86	nd
$(AC)_{25}$	Sb 6-342a	F:TGCTTGTGAGAGTGCCTCCCT R:GTGAACCTGCTGCTTTAGTCGATG	50	250-320	0.31	nd
$(AG)_{27}$	Sb 1-10a	F:GTGCCGCTTTGCTCGCA R:TGCTATGTTGTTTGCTTCTCCCTCTC	65	350-400	0.51	nd
$(AG)_{33}$	SbAGA01b	F:CGAACCATGATAAATGACTG R:ATCCGTTTCACAAAAAAAGT	54	88-106	0.89	H
$(AG)_{35a}$	SbAGB02b	F:CTCTGATATGTCGTTGTGCT R:ATAGAGAGGATAGCTTATAGCTCA	54	101-121	0.75	A
$(AG)_{35b}$	SbAGF06b	F:GTTAAACGACCAATCACCC R:TAGAGGTGTCACTGATGAGC	54	110-180	0.69	A
$(AG)_{39}$	SbAGH04b	F:GGCACTCATGGAGTCACA R:TTTATCCAAATCAAACCGG	54	110-170	0.98	F
$(AG)_{41}$	SbAGB03b	F:GTGTGTGTAGCTTCTTGGG R:ACGTAGGAGTAGTTTCTAGGATT	54	94-158	0.72	F
$(ACA)_9$	SbKAFGK11	F:AGCATCTTACAACAACCAAT R:CTAGTGCACTGAGTGATGAC	54	142-166	0.8	J
$(AG)_{34}GA(CA)_4$	SbAGE03b	F:AGCTCTCAGCCTTTCACAAT R:GGAAGAAAGGAATGACTTGA	54	81-151	0.46	E
$(ACGAC)_4$/ $(AG)_6$	Sb 4-22a	F:TGAGCCGAAAACCGTGAG R:CCCAAAACCAAGAGGAAGG	59	270-300	0.66	nd

nd - not determined

* QTL linkage group (Taramino *et al*., 1997)

** Expected product size in base pairs (bp) (Brown *et al*., 1996; Taramino *et al*., 1997)

a Brown *et al*. (1996)

b Taramino *et al*. (1997)

Table 4.2. SSR polymophism, product size and diversity index for near-isogenic 1*dw*, 2*dw*,3*dw*, 4*dw* and parental lines SA748 and Btx406.

SSR repeat	Primer	Repeat result					Tm°C	Polymophism %	Product Size bp	Diversity Index
$(AG)_{12}$	Sb 5-85	$(AG)_{10}$	$(AG)_{75}$				45	50	70-200	0.583
$(AG)_{14}$	Sb 6-84	$(AG)_{73}$					45	0	188	0.17
$(AG)_{15}$	Sb 4-32	$(AG)_{18}$	$(AG)_{58}$	$(AG)_{76}$			50	25	80-195	0.630
$(AG)_{16}$	Sb 4-15	$(AG)_{13}$	$(AG)_{108}$				45	50	70-260	0.58
$(AG)_{18}$	Sb 6-57	$(AG)_{131}$	$(AG)_{281}$	$(AG)_{311}$			45	50	300-660	0.73
$(AG)_{19}$	Sb 6-36	$(AG)_{74}$					45	83	190	0.31
$(AG)_{20}$	Sb 5-236	$(AG)_{80}$					45	83	202	0.31
$(AC)_{25}$	Sb 6-342	$(AG)_{332}$	$(AG)_{526}$	$(AG)_{619}$	$(AG)_{1478}$	$(AG)_{1842}$	50	51	377-1887	0.5
$(AG)_{33}$	SbAGA01	$(AG)_{31}$	$(AG)_{270}$				50	50	101-580	0.59
$(AG)_{35a}$	SbAGB02	$(AG)_{13}$	$(AG)_{28}$				50	50	70-100	0.58
$(AG)_{35b}$	SbAGF06	$(AG)_{39}$	$(AG)_{241}$	$(AG)_{150}$	$(AG)_{241}$		50	83	116-520	0.51
$(AG)_{39}$	SbAGH04	$(AG)_{42}$					50	0	120	0.167
$(AG)_{41}$	SbAGB03	$(AG)_{27}$					50	83	96	0.31
$(ACA)_{9}$	SbKAFGK1	$(ACA)_{71}$					50	83	245	0.31
$(AG)_{34}GA(CA)_{4}$	SbAGE03	$(AG)_{18}GA(CA)_{2}$		$(AG)_{37}GA(CA)_{8}$			50	42	88-150	0.51

Table 4.3. Amplified SSR fragments (bp) for bulked DNA of near-isogenic sorghum height lines.

SSR repeat	$(AG)_{12}$		$(AG)_{14}$	$(AG)_{15}$			$(AG)_{16}$		$(AG)_{18}$			$(AG)_{19}$	$(AG)_{20}$	$(AC)_{25}$				
Fragment size*	70	200	188	80	160	195	70	260	300	600	660	190	202	377	571	664	1523	1887
Genotype																		
4*dw* parent	0	1	1	0	1	0	1	0	0	1	1	1	1	0	0	0	0	0
4*dw* NIL	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	1	1	1
3*dw* NIL	0	1	1	1	0	1	1	0	0	0	0	1	1	0	0	1	1	1
2*dw* NIL	0	1	1	1	0	1	1	0	0	0	0	0	1	1	1	1	0	1
1*dw* NIL	1	0	1	1	0	1	1	0	1	0	0	1	1	0	0	1	1	1
1*dw* parent	0	1	1	0	1	0	0	1	0	1	1	1	0	1	1	1	1	1

*1 = presence
0 = absence

Table 4.3. Continued.

	$(AG)_{33}$		$(AG)_{35a}$		$(AG)_{41}$	$(AG)_{35b}$				$(AG)_{39}$	$(ACA)_9$	$(AG)_{34}GA(CA)_4$	
Genotype	101	580	70	100	96	116	195	338	520	120	245	88	150
4*dw* parent	1	0	0	1	0	0	1	1	0	1	1	0	0
4*dw* NIL	1	0	1	0	1	0	1	1	1	1	1	1	0
3*dw* NIL	0	1	0	1	1	0	1	1	0	1	1	0	1
2*dw* NIL	1	0	0	1	1	1	0	1	0	1	1	1	0
1*dw* NIL	1	0	1	0	1	1	0	1	0	1	0	1	0
1*dw* parent	1	0	0	1	1	1	0	1	1	1	1	0	1

*1 = presence
0 = absence

Table 4.4. Amplified SSR markers conforming to the genotype deduction criteria.

Repeat	Size (bp)	dwarf gene/s
(AG)16	70	*dw*$_1$
(AG)20	202	*dw*$_1$
(AG)35b	116	*Dw3*
(AG)35b	195	*dw*$_2$

Table 4.5. Deduced height genotypes for near-isogenic, donor parent (Btx406) and recurrent parent (SA748) based on SSR markers.

Height class	Genotype							
4*dw* donor parent (Btx406)	dw_1	dw_1	dw_2	dw_2	dw_3	dw_3	dw_4	dw_4
4*dw* NIL	dw_1	dw_1	dw_2	dw_2	dw_3	dw_3	dw_4	dw_4
3*dw* NIL	dw_1	dw_1	Dw_2	dw_2	dw_3	dw_3	dw_4	dw_4
2*dw* NIL	dw_1	dw_1	Dw_2	Dw_2	Dw_3	Dw_3	dw_4	dw_4
1*dw* NIL	Dw_1	dw_1	Dw_2	Dw_2	Dw_3	Dw_3	dw_4	dw_4
1*dw* recurrent parent (SA748)	Dw_1	Dw_1	Dw_2	Dw_2	Dw_3	Dw_3	dw_4	dw_4

Table 4.6. Genetic distances for the Btx406 4*dw* parent, the 4*dw* NIL, the 3*dw* NIL, the 2*dw* NIL and the SA748 1*dw* parent.

0.681				
0.655	0.681			
0.707	0.732	0.598		
0.802	0.500	0.655	0.598	
0.681	0.756	0.732	0.681	0.824

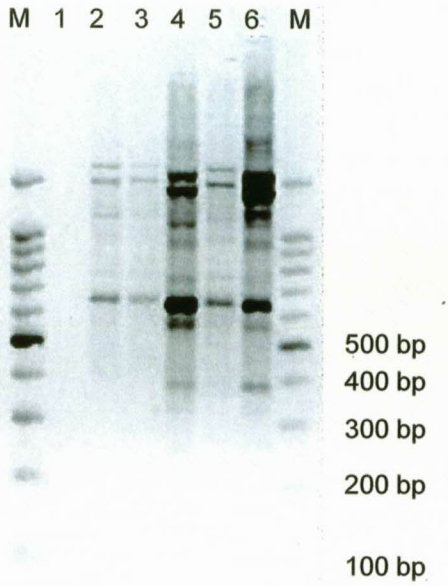


Figure 4.1. SSR amplification products visualized on a 2% agarose gel, stained with ethidium bromide and visualized under UV light for SSR primer Sb6-342a $(AC)_{25}$. Lanes M represents a molecular size standard (100 bp ladder), lane 1 the 4 dw parent, lane 2 the 4 dw NIL, lane 3 the 3 dw NIL, lane 4 the 2 dw NIL, lane 5 the 1 dw NIL and lane 6 the 1 dw parent.

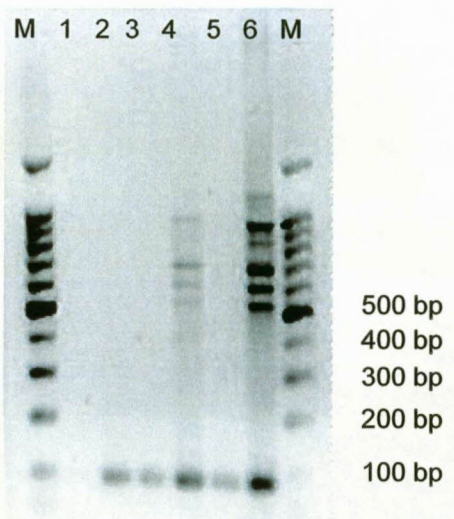


Figure 4.3. SSR amplification products visualized on a 2% agarose gel, stained with ethidium bromide and visualized under UV light for SSR primer SbAGB03b $(AG)_{41}$. Lanes M represents a molecular size standard (100 bp ladder), lane 1 the 4 dw parent, lane 2 the 4 dw NIL, lane 3 the 3 dw NIL, lane 4 the 2 dw NIL, lane 5 the 1 dw NIL and lane 6 the 1 dw parent.

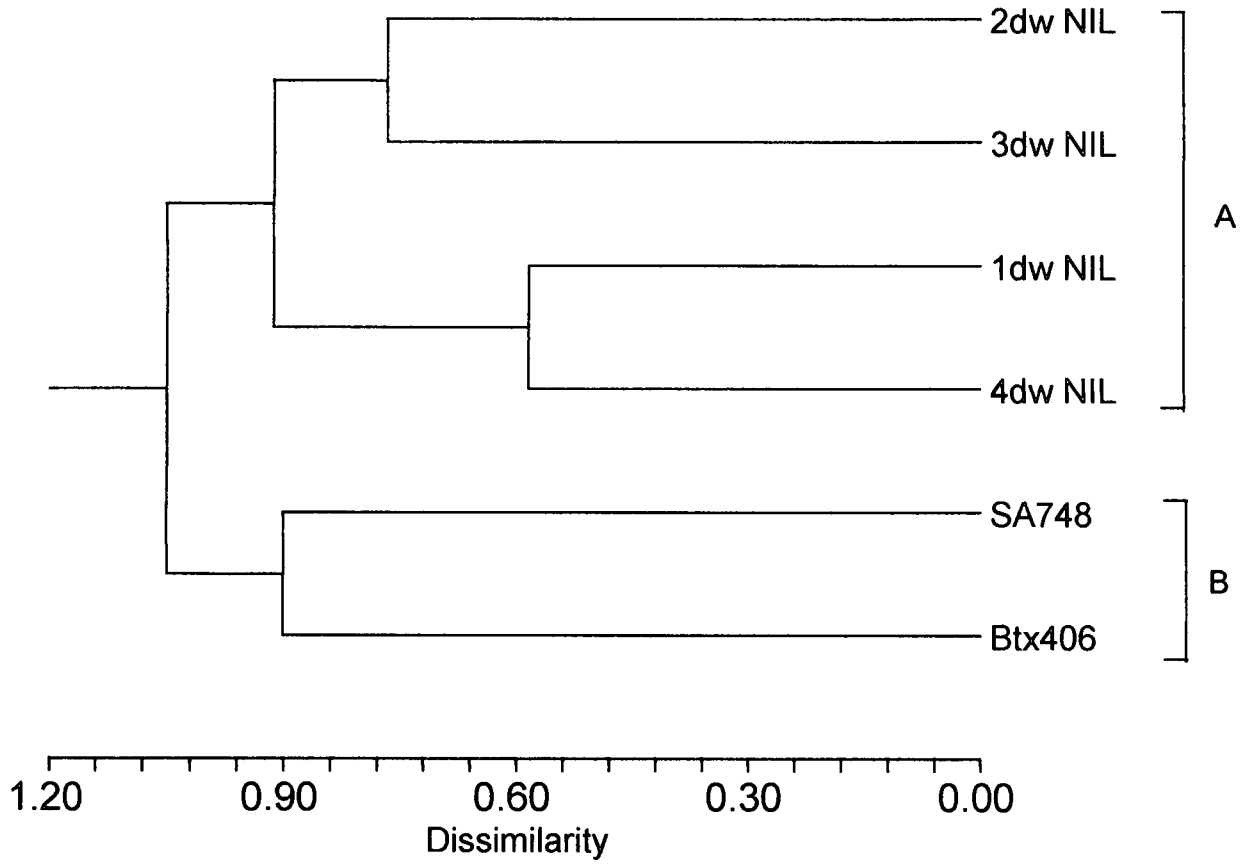


Figure 4.2. Dendrogram based on genetic distances using SSR data.

Chapter 5

The identification of AFLP markers for dwarf genes in near-isogenic and parental lines of sorghum

Abstract

The conversion of exotic varieties in terms of height and maturity has largely contributed to the improvement of sorghum cultivars for yield, drought tolerance, disease and insect resistance, improved grain quality and other traits. Height or dwarfism and maturity are manipulated in the conversion process. Dwarfism is controlled by four different loci which in theory result in five height classes: 0*dw* (not identified), 1*dw* (tall), 2*dw* (intermediate to tall), 3*dw* (intermediate to short) and 4*dw* (short). Marker assisted breeding has great potential to contribute to breeding programmes and consequently to the genetic improvement of sorghum. Amplified fragment length polymorphism (AFLP) analysis is a PCR-based molecular marker technique that detects at least 10 times more genetic loci than other PCR-based molecular techniques. The AFLP technique has proven effective in diversity studies and linkage mapping. AFLPs was used in this study to distinguish between different near isogenic lines for the different height classes as well as parental lines to identify possible markers for specific height genes as well as assign height genotypes to the different height classes in sorghum. In total, 164 possible markers for the dwarfism genes were identified using 16 different primer combinations. AFLP markers were identified for dw_1, Dw_1, dw_2, Dw_2, dw_3 and Dw_3. This is the first application of AFLPs, to identify markers for the dwarfism genes, on near-isogenic lines for the four height classes in sorghum.

5.1 Introduction

Most of the approximately 40 000 accessions in the World Sorghum Collection are from tropical regions of the world and are tall, late maturing and not useful in commercial sorghum production (Rosenow and Dahlberg, 2000). Consequently, only a small portion of the total genetic variation available for sorghum improvement is used in breeding programmes and hybrid development (Rosenow and Dhalberg, 2000). The development of higher yield, drought tolerance, disease and insect resistance, improved grain quality and other desirable traits is accomplished by using varieties that have been converted in terms of height and maturity (Miller, 1982). Material from conversion programmes is very useful in providing new sources of germplasm for sorghum breeders throughout the world (Duncan *et al.*, 1991).

Quinby and Karper (1954) determined that four, recessive, non-linked, brachytic dwarfing genes control plant height in sorghum. These genes were designated dw_1, dw_2, dw_3 and dw_4. The height of a plant is contiguous to the number of loci at which recessive (*dw*) alleles are present (Quinby and Karper, 1954; Hadley, 1957). Thus, plants containing no recessive alleles will theoretically grow in excess of 3 to 4 meters in height, although no such varieties are known to exist. All sorghum lines, are recessive at one locus (one-dwarf), and range between 120 cm to 207 cm in size. Two-dwarf lines, recessive at two loci, range in size between 80 cm and 126 cm, three-dwarf varieties, recessive at three loci, range between 52 cm and 80 cm and four-dwarf plants, recessive at all four loci, are approximately 50 cm tall (Quinby and Karper, 1954; Hadley,

1957; Quinby, 1974). Three-dwarf lines are favoured for commercial farming to accommodate combine harvesting of grain (Harlan, 1972).

Additional factors besides dwarfism such as photoperiodism or maturity also indirectly affect plant height (Quinby, 1967). The longer a sorghum plant remains vegetative, the greater the number of leaves and nodes it makes (Morgan and Finlayson, 2000). Since maturity genes (*ma*) affect the number of internodes and leaves produced before floral initiation, maturity genes also influence plant height (Quinby, 1967). However, the influence of these factors on height is difficult to quantify and is variety dependent.

The objective of conversion programmes is to "convert" tall and late maturing types to short and early maturing lines that can be used in all areas of the world in commercial grain production (Miller, 1982; Duncan *et al.*, 1991). The conversion of a tall, topical, exotic sorghum variety to a three-dwarf is done by the initial crossing of the exotic variety with a suitable four-dwarf line, as female parent. The F_1 progeny were selfed and short, early maturing plants were selected from the F_2 progeny and selfed again to minimise segregation (Miller, 1982). The short F_3 progeny are then backcrossed to the exotic line four to five times to complete the conversion (Rosenow and Dahlberg, 2000). The conversion of a tall or late-maturing variety is achieved over the duration of approximately ten growing seasons (Rosenow and Dahlberg, 2000). Therefore, this process is very time consuming and costly.

Molecular markers have great potential to contribute to conversion programmes and consequently to the genetic improvement of sorghum, especially to reduce the time

required for conversion (McIntyre *et al.*, 2001). The identification of molecular markers for specific dwarfism genes will reduce the number of backcross populations required by excluding tall plants. Molecular markers will also contribute to the understanding of gene interactions in the sorghum genome (McIntyre *et al.*, 2001).

Molecular marker techniques such as RFLPs (Helentjaris *et al.*, 1986; Hulbert *et al.*, 1990; Chittenden *et al.,* 1994; Pereira *et al.*, 1994; Xu *et al.*, 1994), RAPDs (Williams *et al.*, 1990; Tao *et al.*, 1993; Jaiswal *et al.*, 1988), SSRs (Lit and Lutty, 1989; Lagercrantz *et al.*, 1993; Brown *et al.*, 1996; Taramino *et al.*, 1997) and AFLPs (Zabeau and Vos, 1993; Vos *et al.*, 1995; Maheswaran *et al.*, 1997; Boivin *et al.*, 1999) have proven to be powerful tools for the assessment of genetic relationships in sorghum plant populations (Mclyntyre *et al.*, 2001). These techniques are used to identify DNA markers that are distributed throughout the genome and are not subject to environmental change (Subudhi and Nguyen, 2000). Therefore, the use of molecular markers, associated with specific genes, is superior to traditional selection methods and is an important breeding tool in marker-assisted selection.

Vos *et al.* (1995) developed a novel PCR-based fingerprinting technique called amplified fragment length polymorphism (AFLP) that combines the advantages of PCR based markers in terms of time efficiency and the reliability of RFLP markers. The AFLP technique involves the restriction of genomic DNA with two endonucleases followed by the ligation of site-specific adaptors onto the restricted DNA fragments (Zabeau and Vos, 1993; Vos *et al.*, 1995). PCR primers complementary to the adaptor sequences, with additional bases at the 3' ends, are used to amplify the DNA fragments. During

PCR, only DNA fragments with complimentary nucleotides matching the selective nucleotides of the primer are amplified (Vos *et al.*, 1995). The amplified fragments are resolved according to size on sequence gels or using capillary electrophoresis (Zabeau and Vos, 1993; Vos *et al.*, 1995).

AFLP assays require no prior sequence knowledge and detect much greater numbers of loci than any other marker technique currently available (Vos *et al.*, 1995; Maheswaran *et al.*, 1997; Boivin *et al.*, 1999). The capacity to rapidly screen large numbers of loci using AFLPs has specific application in linkage mapping. Smith *et al.* (2000) used AFLP mapping to correlate yield and heterosis with similar results using RFLP data, among maize inbred lines. In another study on soybean by VanToal *et al.* (1997), genetic parental contributions to progeny was revealed using 10 to 14 polymorphic DNA fragments per primer pair. It has also been suggested that AFLP markers may allow breeders to follow changes that result from selection, genetic drift and seed mixtures (VanToal *et al.*, 1997).

Two studies have been published on the use of AFLPs in the construction of sorghum genetic linkage maps (Boivin *et al.*, 1999; Klein *et al.*, 2000). Both studies employ the mapping of AFLP markers onto RFLP maps (Chittenden *et al.,* 1994; Dufour *et al.*, 1997).

The objective of this study was to identify potential AFLP markers for the dwarfism genes in parental lines as well as near-isogenic lines for the four different height classes.

5.2 Materials and Methods

5.2.1 Plant material

Near-isogenic lines, for the different height classes 1*dw*, 2*dw*, 3*dw* and 4*dw* were used in this study. The lines were developed at the Grain Crops Institute of the Agricultural Research Council at Potchefstroom, South Africa, through a backcross process in which a tall tropical sorghum cultivar (SA748) was crossed with an early maturing four-dwarf Martin (Btx406) of US origin. The genotype of the recurrent parent, SA748, is unknown but is assumed to be a 1*dw* and is 230 cm tall. The F_1 population was selfed, short plants selected and backcrossed to the tall SA748 parent. This procedure was repeated for eight generations.

5.2.2 DNA extraction

DNA was extracted from leaf tissue two weeks after germination from ten plants of each NIL height class as well as Btx406 and SA748. DNA was extracted according to a modified method of Edwards *et al.* (1991). Leaf material was collected and ground to a fine powder in liquid nitrogen. The homogenised plant material was incubated at 65°C, with periodic shaking, for one hour in 10 ml extraction buffer (0.01 M EDTA [pH 8.0], 0.05 M Tris-HCl [pH 8.0], 0.1 M NaCl and 1% SDS) containing 8 M Urea. After incubation, the cellular debris and proteins were extracted by the addition of chloroform:iso-amylalcohol (24:1) in a 1:1 ratio and centrifuged for 10 min at 8000 rpm. Chloroform extractions were repeated until the interface was visually clear of cellular debris. The DNA was precipitated by the addition of 2 volumes of 100% cold ethanol to

the retained supernatant. The DNA precipitate was spooled using a sterile Pasteur pipette and washed twice in 70% ethanol. The DNA was re-suspended in 200 ul sterile distilled water and stored at –20°C.

5.2.3 DNA concentration and bulked DNA

The concentration of DNA was determined spectrophotometrically at 260 nm using the formula: [DNA] = Optical density (OD_{260}) x dilution x constant (50 ug/ml). The DNA was diluted to 100 ng/ul. Bulk segregate analysis was used and 100 ng DNA from plants in each height class was bulked.

5.2.4 DNA digestion and ligation

Bulked DNA (250 ng) was digested with 1.25 units of *Mse*I and *EcoRI* endonuclease, respectively, in 5x reaction buffer (50 mM Tris-HCl (pH 7.5), 50 mM Mg-Acetate, 250 mM K-Acetate) at 37°C for 3 hours according to manufacturer's recommendations (Invitrogen). *EcoR*I and *Mse*I adaptors were ligated to the ends of restriction fragments in 25 ul reactions containing: 24 ul adapter ligation solution and 1 ul T4 DNA ligase (1 unit/ul in 10 mM Tris-HCl [pH 7.5], 1 mM DTT, 50 mM KCl and 50% glycerol (v/v) for 3 h at 20°C (Table 5.1). Ligated DNA was diluted 10 times in TE buffer (10 mM Tris-HCl [pH 8] and 0.1 nM EDTA) and stored at –20°C.

5.2.5 AFLP fragment amplification and visualisation

Pre-selective PCR was performed in 25 ul reactions containing: 5 ul (1:10) ligation product, 2.5 ul 10x PCR buffer (200 mM Tris-HCl [pH 8.4], 1.5 mM $MgCl_2$, 500 mM KCl and 1 U ampli *Taq* DNA polymerase (Roche)). Pre-selective PCR amplification was

performed for 20 cycles at 94°C for 30 sec, 56°C for 60 sec and 72°C for 120 sec (Table 5.1). Pre-selective PCR amplification was confirmed by gel electrophoresis and the amplified product diluted 1:50 in TE buffer (10 mM Tris-HCl [pH 8] and 0.1 nM EDTA).

Selective PCR amplification was performed in 20 ul reactions containing: 5 ul pre-selective template DNA (1:50 dilution), 6.7 ng/ul Mse primer with selected nucleotide extensions, 6.7 ng/ul Eco primer with selected nucleotide extensions (Eco-ACA and Eco-AAC labelled with Fam and Ned, respectively) (PE Biosystems), 2 ul 10x PCR buffer (200 mM Tris-HCl [pH 8.4], 15 mM $MgCl_2$, 500 mM KCl) and 1 U ampli *Taq* DNA polymerase (Roche). Selective PCR amplification was performed for 35 cycles at 94°C for 30 sec, 65°C for 60 sec (with a temperature reduction of 0.7°C per cycle for 12 cycles to 56°C) and 72°C for 120 sec.

Following selective amplification, 5 ul of amplification product was mixed with 24 ul formamide and 1 ul GeneScan™ 1000 Rox™ size standard marker (PE Biosystems). The mixture was denatured at 94°C for 5 min and quick cooled in ice slurry. Fragments were resolved using an ABI Prism 310 Automated capillary sequencer (PE Biosysems).

5.2.6 Data analysis

AFLP fragments larger than 40 bp with a peak height above 50 were tabulated using Microsoft Excel. Distance analysis and dendrograms were determined using the hierarchical clustering method (NCSS 2000).

Genotypes were assigned for the near isogenic lines for different height classes and parental lines according to the presence of AFLP fragments based on the following

criteria for genotype deduction (as well as from deductions made in chapter 4 based on SSR data):

1. The four-dwarf height class contains only recessive genes (dw_1 dw_2 dw_3 dw_4).
2. The one-dwarf height class contains only one homozygous recessive gene and is assumed to be dw_4 as this gene is the predominant recessive gene in all commercial sorghum lines (Quinby and Karper, 1954; Schertz, 1973; Ross and Kofoid, 1979).
3. The two-dwarf height class contains two homozygous recessive genes. The three-dwarf height class contains three homozygous recessive genes.
4. Dominance at a locus can include both the homozygous (*DwDw*) and heterozygous (*Dwdw*) condition, but the presence of the recessive trait implies homozygosity at a locus (*dwdw*).

5.3 Results

A total of 16 AFLP primer combinations were tested on dwarf NIL and parental lines (Figure 5.1). Of these, three primer combinations (M-CTG and E-AAC, M-CAC and E-AAC as well as M-CTC and E-AAC) did not amplify any AFLP fragments. A total of 953 fragments were amplified for all the 13 primer combinations with an average of 73 fragments per primer combination (Appendix B). The fragments were 67.3% polymorphic over all the lines and 47.8% polymorphic among the near-isogenic lines (Table 5.2).

Primer combination M-CAA and E-ACA amplified 71 fragments with a total of 74.6% polymorphism and 69.0% polymorphism among the NILs. Primer combination M-CAT and E-ACA amplified 91 fragments with a total of 55.5% polymorphism and 44.0% polymorphism among the NILs. Primer combination M-CTG and E-ACA amplified 85 fragments with a total of 41.2% polymorphism and 27.1% polymorphism among the NILs. Primer combination M-CTT and E-ACA amplified 111 fragments with a total of 78.4% polymorphism and 60.4% polymorphism among the NILs. Primer combination M-CTA and E-ACA amplified 111 fragments with a total of 62.3% polymorphism and 53.2% polymorphism among the NILs. Primer combination M-CAG and E-ACA amplified 105 fragments with a total of 79.0% polymorphism and 65.7% polymorphism among the NILs. Primer combination M-CAC and E-ACA amplified 52 fragments with a total of 78.8% polymorphism and 57.7% polymorphism among the NILs. Primer combination M-CTC and E-ACA amplified 16 fragments with a total of 75.0% polymorphism and 43.8% polymorphism among the NILs. Primer combination M-CAA and E-AAC amplified 74 fragments with a total of 79.7% polymorphism and 47.3% polymorphism among the NILs. Primer combination M-CAT and E-AAC amplified 49 fragments with a total of 28.6% polymorphism and 8.2% polymorphism among the NILs. Primer combination M-CTT and E-AAC amplified 78 fragments with a total of 87.2% polymorphism and 60.3% polymorphism among the NILs. Primer combination M-CTA and E-AAC amplified 54 fragments with a total of 83.3% polymorphism and 26.0% polymorphism among the NILs. Primer combination M-CAG and E-AAC amplified 56 fragments with a total of 51.8% polymorphism and 58.9% polymorphism among the NILs (Table 5.2).

Polymorphic fragment data was tabulated in terms of presence in the 4*dw* donor (Btx406), 1dw recurrent (SA748) parent and near-isogenic lines (Table 5.3), presence in only the 4dw donor (Btx406) parent and NILs (Table 5.4), as well as presence in only the 1*dw* recurrent (SA748) parent and NILs (Table 5.5).

Based on the genotype deduction criteria and genotypes deduced from SSR data (Chapter 4), 72 possible markers for dw_1 were identified using 10 primer combinations (M-CAT and E-ACA, M-CAT and E-AAC, M-CTG and E-ACA, M-CTT and E-ACA, M-CTA and E-ACA, M-CTA and E-AAC, M-CAG and E-ACA, M-CAG and E-AAC as well as M-CAC and E-ACA) (Table 5.6). For dw_2, 52 possible markers were identified using 8 primer combinations (M-CTA and AAC, M-CAG and E-ACA, M-CAT and ACA, M-CTG and E-ACA, M-CAG and E-AAC, M-CTA and E-ACA, M-CTT and E-ACA as well as M-CTT and E-AAC) (Table 5.7). For dw_3, 12 possible markers were identified using primer combination (M-CAT and E-ACA, M-CTT and E-ACA, M-CTT and E-AAC, M-CTA and E-ACA, M-CAG and E-ACA as well as M-CAG and E-ACA (Table 5.8). For Dw_1, 17 possible markers were identified using primer combination (M-CAA and E-ACA, M-CAA and E-AAC, M-CTG and E-ACA, M-CTT and E-ACA, M-CTT and E-AAC, M-CAG and E-ACA, M-CTC and E-AAC) (Table 5.9). For Dw_2, 5 possible markers were identified using primer combination (M-CAA and E-AAC, M-CAT and E-ACA, M-CTC and E-AAC) (Table 5.9). For Dw_3, 6 potential markers were identified using primer combination (M-CAA and E-AAC, M-CTT and E-ACA, M-CAG and E-ACA, M-CTC and E-AAC) (Table 5.9).

The allocation of markers for *dw2* was complicated by more than one possible genotype with regard to the *dw2* locus in all the different lines. The genotype deduced for *dw2* was based on the most probable combination supported by 52 markers for the 1*dw* recurrent parent (SA748) not to contain any recessive *dw2* alleles (Table 5.7). The next possible combination for *dw2* was supported by 25 markers the 1*dw* NIL not to contain any recessive *dw2* alleles. Other combinations were supported by 13 fragments or less (data not shown).

Genetic distances were calculated based on fragments for all the individual AFLP primer combinations (Appendix C) and for the combined data set from all the primer combinations (Table 5.11). The genetic distance ranged from 0.595 to 0.687 among the different NILs and was 0.62 between the NILs and the 4*dw* parent (Btx406), and 0.566 between the NILs and the 1*dw* parent (SA748).

Dendrograms were based on the genetic distances for all the individual primer combinations used (Appendix C) and for the combined data set (Figure 5.2). The two parental lines clustered furthest apart from each other. The NILs grouped between parental lines with the 1*dw* NIL closest to the 1*dw* parent (SA748) and the 4*dw* NIL closest to the 4*dw* parent (Btx406).

5.4 Discussion

A total of 164 potential AFLP markers were identified for dw_1, Dw_1, dw_2, Dw_2, dw_3 and Dw_3. AFLP markers were assigned to specific loci, based on the genotypes deduced from SSR data (Chapter 4). According to the SSR deduced genotypes (Chapter 4), markers for dw_1 had to be present in all the lines except the 1*dw* recurrent parent (SA748) line, as this line contained dw_4. In total 72, potential markers were identified for dw_1 (Table 5.6). Similarly, AFLP markers for dw_2 had to be present in the 3*dw* NIL, 4*dw* NIL and 4*dw* donor parent (Btx406) line, but not the 1*dw* parent (SA748) and 1*dw* NIL. Fifty-two possible markers were identified for dw_2 (Table 5.7). Based on SSR data AFLP markers for dw_3 could only be present in the 3*dw* NIL, the 4*dw* NIL and the 4*dw* parent (Btx406). A total of 12 potential markers were identified for dw_3 (Table 5.8). The identification of markers for the dominant dwarf genes was based on the presence or absence of recessive genes in each genotype. A total of 28 possible markers were identified for Dw_1, Dw_2 and Dw_3 (Table 5.9). Although, the presence of markers in all the different lines could possibly be associated with dw_4 or Dw_4, it was impossible to exclude non-dwarf associated markers and no further deductions were made for these genes. Of the 164 AFLP markers that conformed to the genotype deduction criteria, the presence of 126 of these were in agreement with the genotypes deduced using SSR data. The other 38 polymorphisms did not conform to the genotype deduction criteria and were possibly associated with the different maturity loci. This study is the first application of the AFLP technique in the identification of possible markers for specific

height genes in sorghum as well as the confirmation of SSR deduced genotypes to different NIL height classes (Chapter 4) (Table 4.5; 5.11).

The AFLP data were unexpectedly highly polymorphic (47.8%) among the different NILs for the different height classes, especially after 8 backcross generations (Table 5.2) (Van Eck *et al.*, 1995; Maughan *et al.*, 1996). This indicates that large donor segments are present in the backcrossed 4*dw* line resulting in segregation in the selfed F_2 lines after backcrossing. It is known that the dwarfism genes are associated with at least four different loci, while the maturity genes account for eight different loci. The maturity genotypes for the different parental lines are unknown but thought to be different based on field observations. The selection of short, early maturing plants during backcrossing may have resulted in the introgression of large donor segments, which would segregate in the selfed F_2 plants. This would account for the presence of large donor segments in the near isogenic lines, which is supported by genetic distance data. A 47.8% polymorphism among the NILs suggests that the genes associated directly or indirectly with height and maturity are spread over at least 50% of the genome, resulting in a bias towards retaining larger introgressed segments even after eight backcrossed generations. Furthermore, two QTL studies have in total associated 7 linkage groups to height with only two of these, A and H, commonly identified in both (Lin *et al.*, 1995; Pereira and Lee, 1995). This suggests that a complex of genes is associated with internode elongation and height determination.

The dendrogram based on genetic distance data indicates a closer relationship between the 4*dw* NIL and the 4*dw* parent (Btx406), and the 1*dw* NIL and the 1*dw* recurrent

parent (SA748). The 2*dw* NIL and 3*dw* NIL lines grouped between the1 *dw* parent and 1*dw* NIL and the4 *dw* parent and 4*dw* NIL (Figure 5.2).

The AFLP technique has proven highly successful in identifying 164 potential markers for the dwarfism genes in different height classes. Furthermore, 126 of these markers add additional support to all the genotype deductions hypothesised from SSR data (Chapter 4). The high incidence of polymorphisms detected using AFLPs emphasises the complex interaction between dwarfism and maturity or other genes. The possible AFLP markers identified for the different height loci require further verification on genotypes deduced by phenotype. The use of AFLP markers to identify dwarf genes will allow the acceleration of conversion programmes and allow a greater manipulation of height in sorghum.

Table 5.1. AFLP adapters and primers used for ligation, pre-selective and selective amplification reactions.

Adapter/Primer	**Sequence**
Adapter	
EcoRI adaper	5'-CTCGRAGACTCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
MseI adapter	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'

Primer	Sequence (5'-3')
MseI primer	GATGAGTCCTGAGTAA
M-CAA	GATGAGTCCTGAGTAACAA
M-CAT	GATGAGTCCTGAGTAACAT
M-CTG	GATGAGTCCTGAGTAACTG
M-CTT	GATGAGTCCTGAGTAACTT
M-CTA	GATGAGTCCTGAGTAACTA
M-CAG	GATGAGTCCTGAGTAACAG
M-CAC	GATGAGTCCTGAGTAACAC
M-CTC	GATGAGTCCTGAGTAACTC
EcoRI primer	GATCTGCGTACCAATTC
E-ACA	GATCTGCGTACCAATTCACA (Fam labelled)
E-AAC	GATCTGCGTACCAATTCAAC (Ned labelled)

Table 5.2. Polymorphism % of AFLP fragments obtained for the different primer combinations in NIL and parent lines.

Primer combination	Total number of fragments	Total percentage polymophism %	Percentage polymorphism among NILs %
M-CAA and E-ACA	71	74.6	69.0
M-CAT and E-ACA	91	55.5	44.0
M-CTG and E-ACA	85	41.2	27.1
M-CTT and E-ACA	111	78.4	60.4
M-CTA and E-ACA	111	62.3	53.2
M-CAG and E-ACA	105	79.0	65.7
M-CAC and E-ACA	52	78.8	57.7
M-CTC and E-ACA	16	75.0	43.8
M-CAA and E-AAC	74	79.7	47.3
M-CAT and E-AAC	49	28.6	8.2
M-CTT and E-AAC	78	87.2	60.3
M-CTA and E-AAC	54	83.3	26.0
M-CAG and E-AAC	56	51.8	58.9
Total	953	67.3	47.8

Table 5.3. Polymorphic fragments present in both donor parent (Btx406), recurrent parent (SA748) and one or more NILs.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CAA and E-ACA						
49	1	0	0	1	1	1
179	1	0	0	1	1	1
200	1	0	1	1	1	1
231	1	0	0	1	0	1
241	1	0	0	1	1	1
252	1	1	0	1	1	1
309	1	0	0	1	1	1
351	1	1	0	1	1	1
373	1	0	0	1	0	1
M-CAA and E-AAC						
61	1	1	0	1	1	1
76	1	0	1	1	0	1
95	1	1	1	0	0	1
M-CAT and E-ACA						
43	1	1	1	1	0	1
68	1	1	1	0	1	1
74	1	1	0	1	1	1
76	1	1	1	0	1	1
88	1	0	1	1	1	1
97	1	1	0	1	1	1
98	1	0	1	0	1	1
121	1	1	1	0	1	1
141	1	1	1	0	1	1
145	1	1	1	1	0	1
151	1	1	0	1	1	1
156	1	0	0	1	1	1
255	1	1	1	0	1	1
M-CAT and E-AAC						
82	1	1	0	0	1	1
161	1	0	1	1	1	1
M-CTG and E-ACA						
140	1	1	0	1	1	1
152	1	1	1	1	0	1

Table 5.3. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CTT and E-ACA						
92	1	1	1	1	0	1
95	1	0	1	0	1	1
97	1	0	1	0	1	1
117	1	1	0	1	1	1
121	1	0	1	1	1	1
123	1	1	0	1	1	1
134	1	1	0	1	1	1
151	1	0	0	1	1	1
155	1	1	1	1	0	1
167	1	1	1	0	1	1
205	1	1	1	1	0	1
229	1	1	0	1	0	1
232	1	1	0	1	1	1
260	1	1	0	1	0	1
M-CTT and E-AAC						
87	1	1	1	1	0	1
94	1	1	1	1	0	1
108	1	0	1	1	1	1
109	1	1	1	1	0	1
112	1	1	1	1	0	1
118	1	1	1	1	0	1
120	1	1	1	1	0	1
126	1	1	1	1	0	1
129	1	1	1	1	0	1
162	1	1	1	1	0	1
205	1	1	1	1	0	1
213	1	1	1	1	0	1
M-CTA and E-ACA						
62	1	1	1	0	1	1
89	1	1	1	0	0	1
109	1	0	1	1	1	1
M-CTA and E-AAC						
100	1	1	1	0	1	1
110	1	1	1	1	0	1
112	1	1	1	0	1	1
122	1	1	1	1	0	1
152	1	1	1	0	0	1
M-CAG and E-ACA						
66	1	1	0	1	0	1
69	1	1	0	0	1	1
76	1	1	0	0	1	1
95	1	1	0	0	0	1
117	1	1	0	1	1	1
119	1	1	0	0	1	1
139	1	1	0	0	1	1
141	1	1	0	1	0	1

Table 5.3. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CAG and E-AAC						
60	1	1	1	0	1	1
92	1	1	1	1	0	1
93	1	1	1	1	0	1
94	1	1	1	1	0	1
177	1	1	1	0	0	1
196	1	1	1	0	1	1
225	1	1	1	0	1	1
234	1	1	1	0	1	1
292	1	1	1	0	1	1
362	1	1	1	1	0	1
M-CAC and E-ACA						
56	1	1	1	1	0	1
60	1	0	1	1	0	1
61	1	0	0	1	0	1
72	1	1	1	0	0	1
74	1	0	0	1	0	1
92	1	0	1	0	1	1
99	1	1	1	0	0	1
104	1	0	1	1	1	1
107	1	0	1	0	1	1
123	1	1	1	0	1	1
153	1	0	1	1	1	1
156	1	1	0	0	1	1

Table 5.4. Polymorphic fragments present in the donor parent (Btx406) and one or more NILs but not in the recurrent parent (SA748).

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CAA and E-ACA						
59	1	1	0	0	0	0
132	1	0	0	1	0	0
228	1	1	0	1	0	0
M-CAA and E-AAC						
139	1	1	0	0	0	0
208	1	1	0	0	0	0
M-CAT and E-ACA						
49	1	1	1	0	0	0
50	1	1	1	1	0	0
51	1	1	1	0	0	0
52	1	1	1	1	1	0
53	1	1	1	0	0	0
55	1	1	1	1	0	0
84	1	1	1	0	0	0
96	1	1	1	0	0	0
105	1	1	1	1	0	0
136	1	1	1	1	1	0
164	1	1	1	1	1	0
168	1	0	0	1	0	0
183	1	1	1	1	1	0
199	1	1	1	0	1	0
218	1	1	1	1	1	0
226	1	1	1	1	1	0
228	1	1	1	0	1	0
231	1	0	1	0	1	0
271	1	1	1	0	0	0
M-CAT and E-AAC						
65	1	0	1	1	1	0
72	1	0	0	1	1	0
74	1	1	1	1	1	0
86	1	1	1	1	1	0
89	1	1	1	1	1	0
96	1	1	1	1	1	0
131	1	1	1	1	1	0
137	1	1	1	1	1	0
164	1	1	1	1	1	0
185	1	1	1	1	1	0
476	1	1	1	1	1	0
M-CTG and E-ACA						
72	1	1	1	1	0	0
80	1	1	1	1	1	0
97	1	1	1	1	0	0
98	1	1	1	1	1	0
115	1	0	0	1	0	0
120	1	1	1	1	1	0

Table 5.4. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
122	1	1	0	1	0	0
130	1	1	1	1	1	0
133	1	1	1	1	1	0
144	1	0	0	1	0	0
146	1	0	0	1	0	0
150	1	1	0	1	1	0
154	1	0	1	1	0	0
158	1	1	1	1	1	0
187	1	1	1	0	1	0
234	1	1	1	1	1	0
237	1	1	0	1	1	0
253	1	1	0	1	1	0
397	1	1	1	1	1	0
411	1	1	0	0	1	0
M-CTT and E-ACA						
40	1	1	1	1	1	0
43	1	0	1	0	1	0
44	1	1	1	1	1	0
51	1	1	1	1	1	0
53	1	1	1	0	1	0
66	1	1	1	1	1	0
67	1	1	1	1	1	0
71	1	1	1	1	1	0
74	1	1	1	1	1	0
75	1	1	1	1	0	0
76	1	1	1	1	0	0
82	1	1	1	1	1	0
86	1	1	1	1	0	0
106	1	1	0	1	0	0
110	1	1	1	1	1	0
115	1	0	1	1	0	0
119	1	1	1	1	0	0
120	1	0	1	1	0	0
125	1	1	1	1	0	0
128	1	1	1	1	1	0
133	1	1	1	0	1	0
147	1	0	1	1	1	0
148	1	1	1	1	0	0
152	1	1	1	1	0	0
163	1	1	1	1	0	0
175	1	1	1	0	0	0
176	1	1	1	1	0	0
180	1	1	1	1	0	0
189	1	1	1	1	0	0
191	1	1	0	1	1	0
202	1	1	0	0	0	0
212	1	1	0	1	0	0
221	1	1	0	1	0	0
235	1	1	1	1	1	0
245	1	0	1	1	0	0
255	1	1	1	1	1	0

Table 5.4. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CTT and E-AAC						
58	1	1	1	1	0	0
66	1	1	1	1	0	0
67	1	1	1	1	0	0
70	1	1	1	1	0	0
71	1	1	1	1	0	0
78	1	1	1	1	1	0
81	1	1	1	1	0	0
86	1	1	1	1	0	0
92	1	1	1	1	0	0
93	1	1	1	1	1	0
99	1	1	1	1	0	0
103	1	1	0	1	1	0
106	1	1	1	1	0	0
113	1	1	1	1	0	0
115	1	1	1	1	1	0
116	1	1	1	1	0	0
122	1	1	1	1	0	0
125	1	1	1	1	0	0
136	1	1	1	1	0	0
152	1	1	1	1	0	0
169	1	1	1	1	1	0
181	1	1	1	1	0	0
184	1	1	1	1	0	0
185	1	1	1	1	0	0
202	1	1	1	1	0	0
208	1	1	1	1	0	0
233	1	1	1	1	1	0
243	1	1	1	1	0	0
284	1	1	1	0	0	0
290	1	1	1	1	1	0
304	1	1	1	1	1	0
415	1	1	1	1	0	0
M-CTA and E-ACA						
82	1	1	1	1	1	0
99	1	1	1	1	1	0
120	1	0	1	0	0	0
124	1	1	1	1	1	0
135	1	1	1	1	0	0
143	1	1	1	1	1	0
145	1	1	1	1	0	0
155	1	1	0	1	0	0
162	1	1	1	1	1	0
179	1	1	1	1	1	0
191	1	1	1	1	1	0
193	1	1	1	1	0	0
200	1	1	0	0	0	0
204	1	1	0	1	0	0
210	1	1	1	1	1	0
212	1	1	1	1	0	0
232	1	1	1	1	0	0

Table 5.4. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
237	1	1	1	1	1	0
245	1	1	0	0	0	0
248	1	1	1	1	0	0
256	1	1	1	0	0	0
260	1	1	1	1	0	0
277	1	1	1	1	1	0
286	1	1	1	0	0	0
295	1	1	0	0	0	0
327	1	1	0	1	0	0
362	1	0	0	1	0	0
389	1	1	1	1	1	0
418	1	1	1	1	0	0
439	1	1	1	1	0	0
459	1	1	1	1	0	0
484	1	1	0	1	0	0
M-CTA and E-AAC						
62	1	1	1	1	0	0
68	1	0	0	1	0	0
69	1	1	1	1	1	0
70	1	1	1	1	1	0
72	1	1	1	1	1	0
73	1	1	1	1	1	0
82	1	0	1	1	0	0
93	1	1	1	0	0	0
97	1	0	0	1	1	0
102	1	1	1	1	0	0
103	1	1	1	1	1	0
108	1	0	1	1	1	0
109	1	1	1	1	1	0
114	1	1	1	0	0	0
132	1	1	1	1	1	0
155	1	1	1	1	1	0
161	1	0	1	0	0	0
M-CAG and E-ACA						
53	1	0	0	1	0	0
93	1	1	0	1	0	0
101	1	0	0	1	0	0
112	1	0	0	0	1	0
114	1	1	0	1	1	0
129	1	1	0	0	0	0
132	1	1	0	1	0	0
135	1	1	0	1	0	0
137	1	1	0	0	0	0
144	1	0	0	0	1	0
149	1	1	1	1	1	0
156	1	0	0	1	1	0
158	1	1	0	0	0	0
165	1	0	0	1	0	0
171	1	0	0	1	0	0
187	1	1	0	1	1	0

Table 5.4. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
198	1	1	0	1	1	0
206	1	1	1	1	1	0
247	1	0	1	1	1	0
254	1	1	1	0	0	0
273	1	1	1	1	0	0
290	1	0	1	0	1	0
333	1	0	0	0	1	0
M-CAG and E-AAC						
81	1	1	1	1	1	0
86	1	1	1	1	0	0
113	1	1	1	0	0	0
121	1	1	1	1	1	0
133	1	0	1	0	0	0
135	1	1	1	0	1	0
161	1	1	1	1	1	0
203	1	1	1	1	0	0
M-CAC and E-ACA						
63	1	1	0	0	0	0
78	1	1	1	1	1	0
118	1	0	1	0	1	0
141	1	0	1	1	0	0
164	1	1	1	1	1	0
206	1	1	1	1	1	0
209	1	1	1	1	1	0
248	1	1	1	1	1	0
290	1	1	1	1	1	0

Table 5.5. Polymorphic fragments present in the recurrent parent (SA748) and NILs but not in the donor parent (Btx406).

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CAA and E-ACA						
50	0	1	0	0	0	1
53	0	0	0	1	0	1
58	0	0	1	1	0	1
62	0	1	0	1	0	1
69	0	1	0	1	1	1
71	0	1	0	1	1	1
73	0	0	0	1	0	1
77	0	1	1	1	0	1
80	0	1	0	1	0	1
81	0	1	0	1	0	1
93	0	1	1	1	1	1
100	0	1	1	0	0	1
112	0	1	1	0	0	1
133	0	0	0	1	0	1
137	0	1	0	1	1	1
140	0	1	0	1	0	1
161	0	0	1	1	0	1
164	0	1	0	0	0	1
173	0	1	1	1	1	1
192	0	1	0	1	0	1
206	0	1	0	1	1	1
221	0	1	1	1	0	1
224	0	1	0	1	1	1
273	0	0	0	0	1	1
M-AAC and E-AAC						
58	0	1	0	1	0	1
60	0	0	0	1	0	1
62	0	1	1	1	0	1
64	0	0	1	0	1	1
66	0	0	0	1	0	1
78	0	1	0	1	0	1
81	0	1	1	1	1	1
85	0	1	1	0	0	1
86	0	1	0	1	1	1
90	0	1	1	1	1	1
91	0	1	1	1	1	1
94	0	1	1	1	1	1
97	0	1	1	1	1	1
98	0	1	0	1	1	1
100	0	0	0	1	1	1
101	0	1	1	1	1	1
106	0	0	0	1	0	1
112	0	1	0	1	0	1
114	0	1	0	1	1	1
126	0	1	0	1	1	1
131	0	1	1	1	1	1
135	0	0	0	1	0	1
143	0	0	1	1	1	1

Table 5.5. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
145	0	0	1	1	1	1
152	0	1	0	0	0	1
157	0	1	1	1	0	1
165	0	1	0	1	1	1
176	0	0	0	0	1	1
229	0	1	0	1	1	1
256	0	1	0	1	1	1
314	0	1	1	1	1	1
384	0	1	0	1	1	1
417	0	1	0	1	1	1
453	0	1	1	1	1	1
M-CAT and E-ACA						
123	0	0	1	1	1	1
153	0	1	1	1	1	1
171	0	1	1	0	1	1
202	0	1	1	0	1	1
M-CAT and E-AAC						
58	0	1	0	1	0	1
117	0	1	1	1	1	1
M-CTG and E-ACA						
79	0	1	0	0	1	1
84	0	1	1	1	1	1
100	0	0	0	0	1	1
174	0	1	1	1	1	1
M-CTT and E-ACA						
50	0	0	0	0	1	1
56	0	0	0	0	1	1
58	0	0	0	0	1	1
100	0	0	1	0	1	1
102	0	0	0	1	0	1
107	0	0	0	0	1	1
109	0	1	1	0	1	1
127	0	0	1	0	0	1
130	0	0	0	0	1	1
132	0	0	0	1	0	1
146	0	0	0	0	1	1
161	0	0	0	1	1	1
165	0	0	0	0	1	1
172	0	0	0	1	1	1
182	0	1	0	0	1	1
194	0	0	0	1	0	1
M-CTT and E-AAC						
90	0	0	0	0	1	1
96	0	1	0	1	0	1
132	0	0	1	1	0	1
138	0	0	0	0	1	1
178	0	0	0	0	1	1
192	0	0	0	0	1	1

Table 5.5. Continued.

Size (bp)	4*dw* parent (Btx406)	4*dw* NIL	3*dw* NIL	2*dw* NIL	1*dw* NIL	1*dw* parent (SA748)
M-CTA and E-ACA						
66	0	1	1	1	0	1
72	0	1	1	0	1	1
128	0	1	1	1	1	1
M-CAG and E-ACA						
50	0	1	1	0	1	1
62	0	1	0	1	0	1
64	0	1	0	0	0	1
71	0	0	0	1	0	1
75	0	0	0	1	0	1
79	0	1	0	1	1	1
80	0	1	0	1	1	1
89	0	1	0	1	0	1
103	0	1	0	1	0	1
104	0	1	1	1	1	1
111	0	0	0	0	1	1
125	0	1	0	0	0	1
128	0	1	1	1	1	1
142	0	1	0	1	0	1
153	0	1	1	1	1	1
160	0	1	1	1	1	1
168	0	1	0	1	1	1
217	0	1	0	0	1	1
252	0	0	0	1	1	1
260	0	0	0	1	1	1
348	0	1	1	1	1	1
M-CAG and E-AAC						
63	0	1	1	0	1	1
97	0	1	0	0	0	1
115	0	1	0	0	0	1
116	0	1	0	0	0	1
192	0	0	1	0	0	1
471	0	0	1	0	0	1
M-ACA and E-ACA						
45	0	1	1	1	1	1
70	0	0	0	0	1	1
85	0	1	0	0	1	1
100	0	1	1	1	0	1
106	0	0	1	1	0	1
184	0	0	1	1	0	1
198	0	0	0	0	1	1
296	0	1	1	1	1	1
M-CTC and E-ACA						
79	0	0	0	1	0	1
80	0	0	0	1	0	1
86	0	0	1	1	1	1
102	0	1	1	1	1	1
106	0	1	1	1	1	1
142	0	0	1	1	1	1
153	0	0	0	0	1	1
163	0	0	0	0	1	1
166	0	0	0	1	1	1

Table 5.6. AFLP markers for dw_1 (Table 5.4).

Primer combination	Size (bp)
M-CAT and E-ACA	52, 136, 164, 183, 218, 226
M-CAT and E-AAC	74, 86, 89, 96, 131, 137, 164, 185, 476
M-CTG and E-ACA	80, 120, 130, 133, 158, 234, 397
M-CTT and E-ACA	40, 44, 66, 67, 71, 74, 82, 110, 128, 235, 255
M-CTT and E-AAC	78, 93, 115, 169, 233, 290, 304
M-CTA and E-ACA	82, 99, 124, 143, 162, 179, 191, 210, 237, 277, 389
M-CTA and E-AAC	62, 69, 70, 72, 73, 103, 109, 132, 155
M-CAG and E-ACA	149, 206
M-CAG and E-AAC	81, 121, 161
M-CAC and E-ACA	78, 164, 206, 209, 248, 290

Table 5.7. AFLP markers for dw_2 (Table 5.4).

Primer combination	Size (bp)
M-CAT and E-ACA	50, 105
M-CTG and E-ACA	72, 97
M-CTT and E-ACA	75, 86, 119, 125, 148, 152, 163, 176, 180, 189
M-CTT and E-AAC	58, 66, 70, 71, 81, 86, 92, 99, 106, 113, 116, 122, 125, 136, 181, 184, 185, 202, 208, 243, 415
M-CTA and E-ACA	135, 145, 193, 212, 232, 248, 260, 418, 439, 459
M-CTA and E-AAC	62
M-CAG and E-ACA	273
M-CAG and E-AAC	86, 203

Table 5.8. AFLP markers for dw_3.

Primer combination	Size (bp)
M-CAT and E-ACA	49
	51
	53
	84
	96
	271
M-CTT and E-ACA	175
M-CTT and E-AAC	284
M-CTA and E-ACA	256
	286
M-CAG and E-ACA	254
M-CAG and E-ACA	113

Table 5.9. AFLP markers for dominant alleles (Table 5.5).

Primer combination	Size (bp)	Gene
M-CAA and E-ACA	273	*Dw1*
M-CAA and E-AAC	100	*Dw3*
	143	*Dw2*
	145	*Dw2*
	176	*Dw1*
M-CAT and E-ACA	123	*Dw2*
M-CTG and E-ACA	100	*Dw1*
M-CTT and E-ACA	50	*Dw1*
	56	*Dw1*
	58	*Dw1*
	107	*Dw1*
	130	*Dw1*
	146	*Dw1*
	161	*Dw3*
	165	*Dw1*
	172	*Dw3*
M-CTT and E-AAC	90	*Dw1*
	138	*Dw1*
	178	*Dw1*
	192	*Dw1*
M-CAG and E-ACA	111	*Dw1*
	252	*Dw3*
	260	*Dw3*
M-CTC and E-AAC	86	*Dw2*
	142	*Dw2*
	153	*Dw1*
	163	*Dw1*
	166	*Dw3*

Table 5.10. Deduced genotypes for near-isogenic, donor parent (Btx406) and recurrent parent (SA748) using AFLP markers.

Height class	Genotype							
4*dw* donor parent (Btx406)	*dw*1	*dw*1	*dw*2	*dw*2	*dw*3	*dw*3	*dw*4	*dw*4
4*dw* NIL	*dw*1	*dw*1	*dw*2	*dw*2	*dw*3	*dw*3	*dw*4	*dw*4
3*dw* NIL	*dw*1	*dw*1	*Dw*2	*dw*2	*dw*3	*dw*3	*dw*4	*dw*4
2*dw* NIL	*dw*1	*dw*1	*Dw*2	*dw*2	*Dw*3	*Dw*3	*dw*4	*dw*4
1*dw* NIL	*Dw*1	*dw*1	*Dw*2	*Dw*2	*Dw*3	*Dw*3	*dw*4	*dw*4
1*dw* recurrent parent (SA748)	*Dw*1	*Dw*1	*Dw*2	*Dw*2	*Dw*3	*Dw*3	*dw*4	*dw*4

Table 5.11. Genetic distances determined using AFLP data for the Btx406 4*dw* parent, the 4*dw* NIL, the 3*dw* NIL, the 1*dw* NIL and the SA748 parent.

0.595				
0.565	0.564			
0.641	0.591	0.631		
0.687	0.677	0.632	0.690	
0.813	0.763	0.760	0.763	0.666

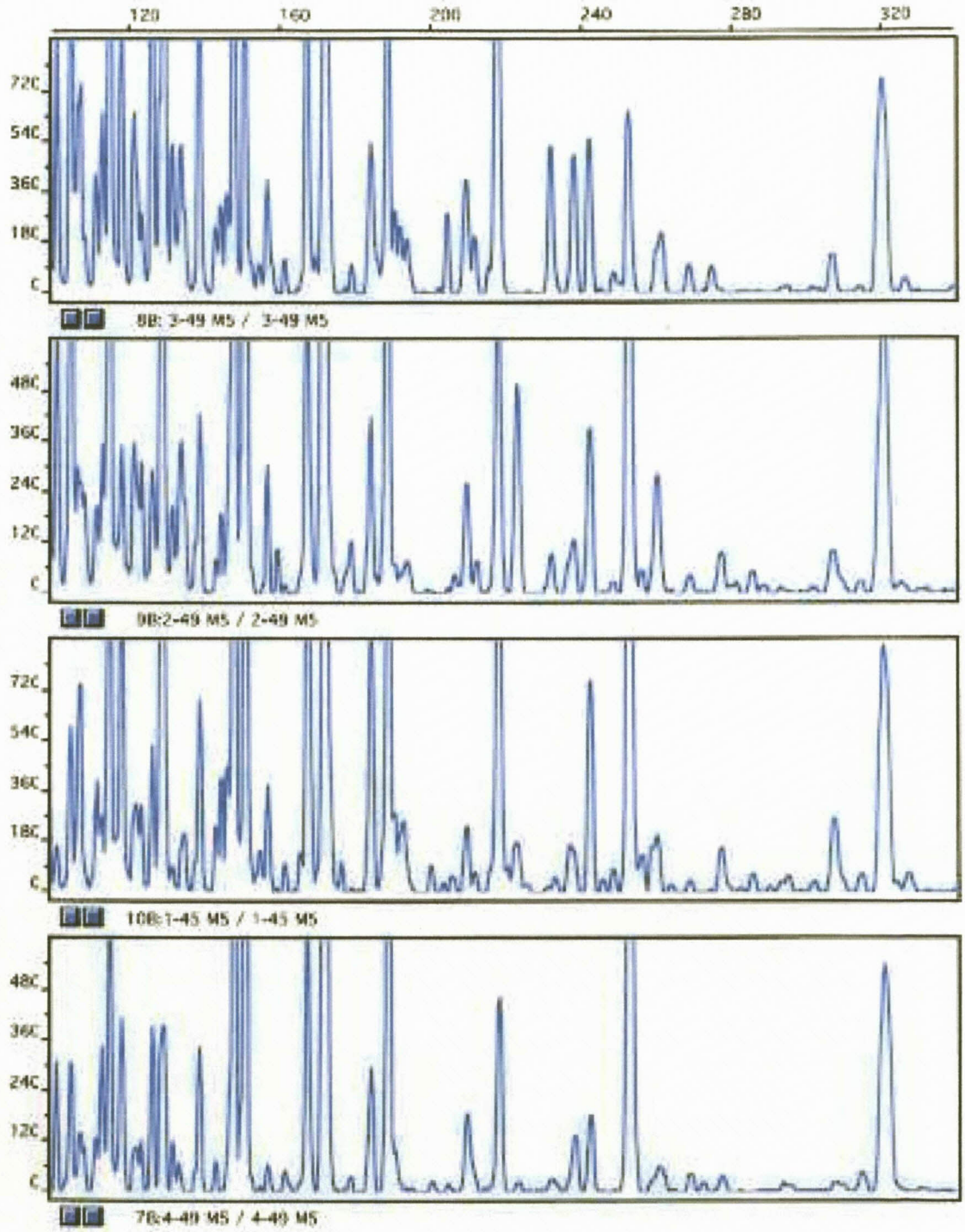


Figure 5.1. A typical AFLP electropherogram for primer combination M-CTA and E-ACA for the 4 *dw* NIL, 3 *dw* NIL, 2 *dw* NIL and 1 *dw* NIL height classes.

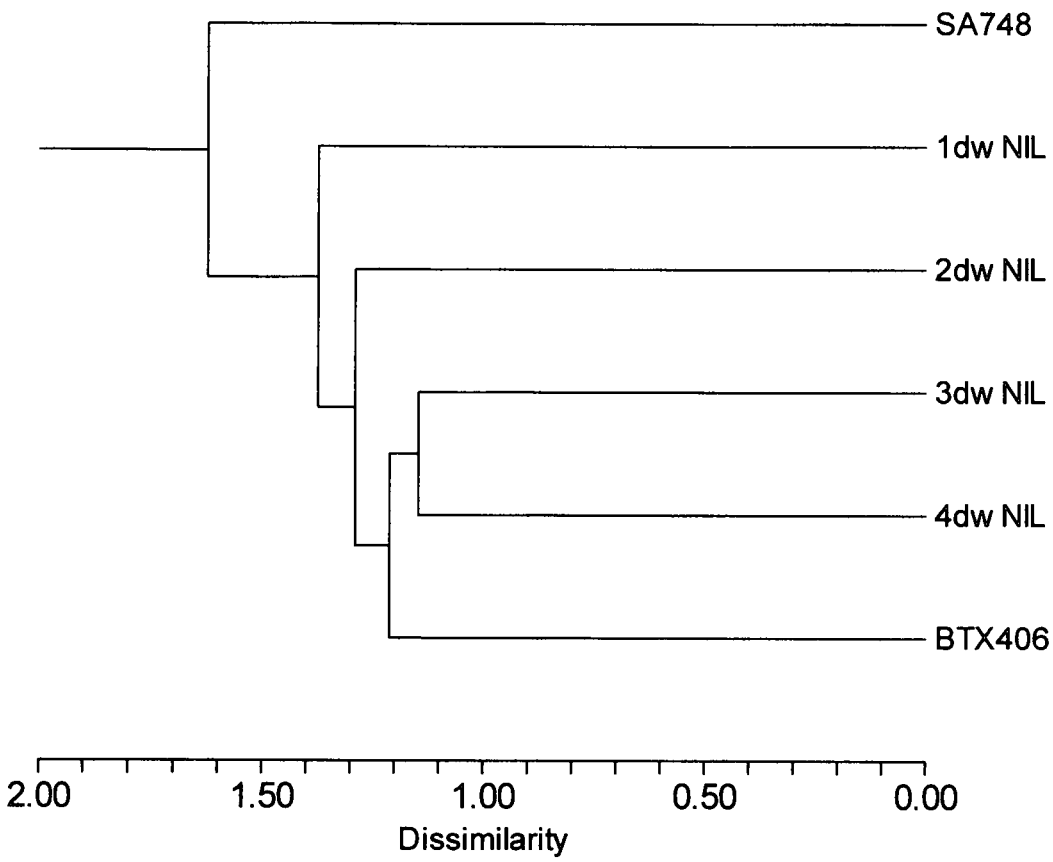


Figure 5.2. Dendrogram based on genetic distances using AFLP data.

Summary

Sorghum is a grain crop with enormous potential in meeting the nutritional needs of people in developing countries, as well as optimising current meat and poultry production in the rest of the world. Sorghum has been overlooked in recent years and is not utilised to its full potential, especially in African countries. The conversion of exotic lines in terms of height and maturity holds great potential for the development of improved varieties. The genes controlling height or dwarfism (dw) have also been found to enhance drought tolerance making it an important trait to be incorporated for sorghum improvement in semi-arid areas. However, little information is available on the genetic and physiological basis of height.

In this study, near-isogenic lines developed for different height classes were characterised (Chapter 2). Significant differences were observed in plant height, internode length and flag leaf length between different height classes as previously hypothesised. However, the current study also identified significant differences in flag leaf width, panicle weight and number of panicle branches between the different height classes, in contrast to previous reports comparing only two height classes. Furthermore, this is the first report that plant height is correlated to flag leaf length, flag leaf width, panicle weight, leaf sheath length and panicle length.

The exogenous application of gibberellic acid (GA) did not result in a final height increase, as expected from previous reports (Chapter 3). However, it was observed that

GA application may affected the growth response in the different height classes. This suggests that dwarf genes probably do not affect gibberellic acid biosynthesis but rather may act as a negative regulator in response to GA and that other genes, possibly the maturity genes determine the response to GA.

This is the first report of using SSRs on near-isogenic lines for different height classes to identify specific dwarfism genes (Chapter 4). Potential markers were identified for the dw_1, dw_2 and Dw_3 genes in sorghum. These markers also allowed the deduction of genotypes for the different height classes. Linkage group A was confirmed to contain loci affecting plant height. These markers will facilitate sorghum breeding and reduce the time needed for line conversion.

The AFLP technique was effective in identifying a total of 164 possible markers for the different dwarfism genes (Chapter 5). Markers were identified for recessive and dominant dwarfism alleles at dw_1, dw_2 as well as dw_3. Furthermore, the genetic distance observed between the different near-isogenic height classes and the parental lines indicate that a bias exists in the retention of an unexpectedly large donor segment, possibly introduced through the selection of height and maturity characteristics.

Future research resulting from the present study includes further investigation of the interaction between the dwarfism and maturity genes, as well as the verification of SSR and AFLP markers in genetic material with confirmed genotypes in order to incorporate these markers into international breeding programmes. The use of these markers in

conversion programmes will allow more cost effective and accelerated sorghum improvement.

Opsomming

Sorghum is 'n graangewas met geweldige potensiaal om te voldoen aan voedings behoeftes in ontwikkelende lande asook vee en pluimvee produksie in die wêreld. Die volle potensiaal van sorghum in Afrika is nie die afgelope paar jaar verwesenlik nie. Eksotiese sorghum lyne hou groot potensiaal in vir die ontwikkeling van verbeterde sorghum lyne. Droogte-toleransie in sorghum kan verbeter word vir droër gebiede met die inkorporering van dwerg-gene (dw) wat planthoogte beheer. Daar bestaan egter nie voldoende inligting oor die fisiologiese beheer en interaksie van dwerg-gene nie.

In hierdie studie is naby-isogeniese-lyne vir verskillende planthoogtes gekarakteriseer (Hoofstuk 2). Betekenisvolle verskille is waargeneem tussen planthoogte en internodale-lengtes en vlagblaarlengtes, soos in vorige studies aangedui. In teenstelling met vorige studies waar slegs twee planthoogte-lyne gebruik is, is betekenisvolle verskille tussen planthoogte en vlagblaarbreedtes, aargewig en die hoeveelheid aarvertakkings waargeneem. In hierdie studie word planthoogte vir die eerste keer gekorreleer met vlagblaarlengte, vlagblaarbreedte, blaarskede-lengte, aargewig en aarlengte.

In teenstelling met wat verwag is, het die eksterne toedinging van gibberelliensuur (GA_3) nie die finale lengte van naby-isogeniese-dwerglyne vehoog nie (Hoofstuk 3). 'n moontlikke reaksie op GA_3 toedining is egter waargeneem in die groei van die verskillende hoogte-lyne. Daar mag dus afgelei word dat die dwerggene nie die

produksie van die plantgroeihormoon GA_3 beheer nie, maar eerder optree as 'n negatiewe reguleerder tydens vrystelling van GA. Resultate in die studie dui ook op die rol van ander gene, moontlik volwassenheidsgene (*ma*) in die regulering van GA_3.

Hierdie is die eerste studie wat gebruik maak van SSRe (kort DNA basis opeenvolging herhalings) op naby-isogeniese-lyne vir verskillende hoogte-lyne (Hoofstuk 4). Moontlikke merkers is geïdentifieseer vir die dw_1, dw_2 en Dw_2 gene in sorghum. Die afleiding van genotipe vir die naby-isogeniese-lyne is moontlik gemaak deur SSR merkers. Koppelingsgroep A is wel geassosieer met 'n invloed op planthooghte in sorghum. Met die gebruik van hierdie merkers kan sorghumteling bevoordeel word in terme van tydvermindering en plantlynveranderinge.

Die AFLP (geamplifieseerde fragmentlengte polymorfisme) tegniek is effektief gebruik om 164 moontlikke merkers vir die verskillende dwerggene asook vir dw_3 te ientifiseer (Hoofstuk 5). Genetiese afstande tussen die naby-isogeniese-lyne en die ouerlyne dui op 'n bo verwagte oordraging van chromosomale DNA deur die skenker ouer, waarskynlik as gevolg van seleksie vir beide hoogte- en volwassenheidsgene.

Die interaksie tussen dwerggene (*dw*) en volwassenheids gene (*ma*) dien as uitvloeisel van hierdie studie vir toekomstige navorsing. Bevestiging van die merkers met bestaande genotipes wat in hierdie studie geïdentifiseer is, is nodig. Die gebuik van hierdie merkers sal die verbetering van sorghum van groot waarde wees.

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Appendix A

Table of morhological characteristics evaluated for the 4*dw* parent (Btx406), the 4*dw* NIL, the 3*dw* NIL, the 2*dw* NIL, the1*dw* NIL and the1*dw* parent (SA748).

Height class	1*dw* parent(Btx40€	4*dw* NIL	3*dw* NIL	2*dw* NIL	1 *dw* NIL	*dw* parent (SA748
Characteristics of individual plants						
Plant height (cm)						
1	80	90	130	155	200	210
2	80	90	140	165	200	210
3	80	85	140	180	190	230
4	70	85	130	180	190	220
5	70	80	120	170	200	230
6	70	85	120	160	230	220
7	65	85	80	180	190	210
8	70	85	140	180	190	220
9	80	90	130	170	210	220
10	75	85	140	170	190	200
Flag leaf length (cm)						
1	38.31	45.00	57.08	68.32	60.00	59.77
2	44.78	48.40	54.97	47.04	64.79	62.00
3	45.22	45.33	60.00	60.11	68.21	57.07
4	52.66	39.12	62.06	62.22	64.89	55.00
5	39.76	40.95	50.10	57.10	65.33	60.00
Flag leaf width (cm)						
1	8.7	6.1	8.9	6.0	6.1	6.7
2	7.0	7.9	9.1	5.9	6.9	8.3
3	6.1	5.2	8.2	8.0	5.7	7.0
4	7.1	5.9	7.8	9.1	6.0	6.9
5	6.8	6.1	9.2	8.0	7.0	8.1
Sheath length (cm)						
1	29.89	13.22	23.11	19.78	20.00	16.59
2	17.31	17.95	15.01	18.21	17.22	23.30
3	15.91	20.11	17.87	20.00	19.58	18.02
4	18.00	15.00	16.33	19.03	20.10	15.00
5	20.10	16.21	20.06	16.23	17.11	19.95
Number of nodes						
1	8	8	11	8	10	10
2	8	11	10	9	9	11
3	8	8	10	10	9	12
4	9	9	11	10	11	10
5	9	11	10	11	10	11
Panicle weight (g)						
1	15.14	77.22	138.35	128.00	125.08	101.88
2	44.23	73.08	146.91	119.93	81.21	95.80
3	18.07	54.07	84.78	136.26	70.91	110.52
4	20.38	48.63	95.00	58.69	81.54	90.78
5	45.66	52.79	99.93	62.78	125.08	97.21
Panicle length (cm)						
1	22.10	23.90	26.30	19.80	22.30	21.30
2	24.00	26.20	25.10	21.00	25.00	20.80
3	15.30	25.70	20.80	27.10	20.10	25.00
4	20.60	23.10	26.90	25.80	23.00	21.60
5	26.10	24.80	27.20	26.30	24.60	22.60
Number of panicle branches						
1	61	76	110	88	58	91
2	70	73	95	55	66	83
3	53	63	93	63	67	93
4	55	75	98	62	77	90
5	60	62	92	73	65	85

Appendix B

AFLP Primer combination M-CAA and E-ACA

Fragment size	56	58	60	61	62	64	65	66	68	70	71	72	73	76	77	78	81	83	85	86	88	89	90	91	92	94	95	97	98	100
BTX406 4dwP	1	0	0	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0
4dw NIL 1-45	1	1	0	1	1	0	0	0	0	1	0	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0
3dw NIL 2-49	1	0	0	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	0	0
2dw NIL 3-49	1	1	1	1	1	0	0	1	0	1	0	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1
1dw NIL 4-50	1	0	0	1	0	1	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	1
SA748 1dwP	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Fragment size	101	106	107	108	111	112	114	116	124	125	126	130	131	133	135	139	141	143	144	145	147	151	152	153	155	157	158	165	175	176
BTX406 4dwP	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0
4dw NIL 1-45	1	0	1	1	0	1	1	0	0	0	1	1	1	1	0	1	1	0	0	0	1	1	1	0	1	1	0	1	0	0
3dw NIL 2-49	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	1	0	0	1	1	0	0	1	0
2dw NIL 3-49	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0	0	1	1	1	1	1	0
1dw NIL 4-50	1	0	1	1	0	0	1	0	0	0	1	0	1	1	0	0	1	1	0	1	0	1	0	0	1	0	1	1	0	1
SA748 1dwP	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1

Fragment size	181	200	201	208	210	223	229	252	256	266	314	384	417	453
BTX406 4dwP	0	0	0	1	1	1	0	0	0	1	0	0	0	0
4dw NIL 1-45	0	0	1	1	1	1	1	0	1	1	1	1	1	1
3dw NIL 2-49	0	0	1	0	1	1	0	0	0	1	1	0	0	1
2dw NIL 3-49	0	0	1	0	1	1	1	0	1	1	1	1	1	1
1dw NIL 4-50	0	0	1	0	1	1	1	0	1	1	1	1	1	1
SA748 1dwP	1	1	1	0	1	1	1	1	1	1	1	1	1	1

AFLP Primer combination M-CAT and E-ACA

Fragment size	40	43	44	46	49	50	51	52	53	54	55	62	63	66	68	72	74	75	76	78	80	82	84	87	88	91	92	94	96	97
BTX406 4dwP	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1
4dw NIL 1-45	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1
3dw NIL 2-49	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0
2dw NIL 3-49	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	1	1	1	0	1	0	1
1dw NIL 4-50	1	0	0	1	0	0	0	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	0	1
SA748 1dwP	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	0	1

Fragment size	98	100	102	105	108	111	112	113	114	116	118	119	120	121	123	124	126	131	136	138	141	145	146	151	153	156	158	164	166	167
BTX406 4dwP	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
4dw NIL 1-45	0	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1
3dw NIL 2-49	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1
2dw NIL 3-49	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
1dw NIL 4-50	1	1	1	0	1	1	1	0	1	1	0	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1
SA748 1dwP	1	1	1	0	1	1	1	0	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1

Fragment size	168	171	173	175	178	181	183	186	191	199	202	205	218	220	222	226	228	231	235	240	251	255	257	263	267	271	285	387	418	428	443
BTX406 4dwP	1	0	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
4dw NIL 1-45	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0
3dw NIL 2-49	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0
2dw NIL 3-49	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1	1	0
1dw NIL 4-50	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0
SA748 1dwP	0	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0

AFLP Primer combination M-CTG and E-ACA

Fragment size	40	46	49	59	62	64	69	71	72	75	77	79	80	81	84	68	90	92	95	96	97	98	100	104	107	109	111	112	113	115
BTX406 4dwP	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1
4dw NIL 1-45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	1	1	1	0
3dw NIL 2-49	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0
2dw NIL 3-49	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1
1dw NIL 4-50	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0
SA748 1dwP	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1	0

Fragment size	117	120	122	125	128	130	131	133	139	140	141	144	146	148	150	152	154	158	160	163	169	174	176	180	185	187	192	201	211	216
BTX406 4dwP	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1
4dw NIL 1-45	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1
3dw NIL 2-49	1	1	0	1	1	1	1	1	1	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
2dw NIL 3-49	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1
1dw NIL 4-50	1	1	0	1	1	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1
SA748 1dwP	1	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1

Fragment size	219	223	230	234	235	237	246	253	259	269	286	292	309	314	328	355	363	374	386	397	411	428	460	477	492
BTX406 4dwP	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
4dw NIL 1-45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
3dw NIL 2-49	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1
2dw NIL 3-49	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
1dw NIL 4-50	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
SA748 1dwP	1	1	1	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1

AFLP Primer combination M-CTT and E-ACA

Fragment size	40	43	44	46	47	50	51	53	55	56	58	62	63	66	67	68	70	71	74	75	76	82	83	86	87	90	92	95	96	97
BTX406 4dwP	1	1	1	1	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4dw NIL 1-45	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
3dw NIL 2-49	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2dw NIL 3-49	1	0	1	1	0	0	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
1dw NIL 4-50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1
SA748 1dwP	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0	1	1	0	0	0	0	0	1	0	1	1	1	1	1	1

Fragment size	99	100	101	102	103	104	106	107	108	109	110	112	114	115	117	119	120	121	122	123	124	125	127	128	130	132	133	134	138	140
BTX406 4dwP	1	0	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1
4dw NIL 1-45	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1	1	0	0	1	1	0	1	0	1	0	0	1	1	1	1
3dw NIL 2-49	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	0	0	1	0	1	1
2dw NIL 3-49	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1
1dw NIL 4-50	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	0	0	0	1	1	0	1	1	1	1
SA748 1dwP	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	1	0	1	1	0	1	1	1

Fragment size	141	144	145	146	147	148	150	151	152	154	155	158	161	163	165	167	169	170	172	175	176	178	180	182	183	186	189	191	194	195
BTX406 4dwP	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0	1	0	1	1	1	1	0	0	1	1	1	0	0
4dw NIL 1-45	0	0	1	0	0	1	0	0	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0
3dw NIL 2-49	0	1	1	0	1	1	0	0	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	0	0	1	1	0	0	0
2dw NIL 3-49	0	0	1	0	1	1	0	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	0	0	1	1	1	1	1
1dw NIL 4-50	1	1	1	1	1	0	0	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	0	1	0	1	0	1	0	1
SA748 1dwP	0	0	1	1	0	0	1	1	0	0	1	1	1	0	1	1	0	1	1	0	0	1	0	1	1	1	0	0	1	0

Fragment size	197	200	202	205	208	218	212	218	221	223	225	226	229	232	235	241	245	251	255	258	260	264	269	273	274	276	280	287	292	296
BTX406 4dwP	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	0	1
4dw NIL 1-45	1	0	1	1	0	1	1	1	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	1	0	1	0	1	0	1
3dw NIL 2-49	1	0	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	1	0	1
2dw NIL 3-49	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1
1dw NIL 4-50	1	1	0	0	1	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	0	1
SA748 1dwP	1	0	0	1	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	1	0	1	0	0	1

Fragment size	299	304	321	323	326	327	333	334	344	362	365	370	379	382	419	427	488
BTX406 4dwP	0	1	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1
4dw NIL 1-45	0	0	0	0	0	1	1	0	1	0	1	1	0	1	1	1	1
3dw NIL 2-49	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1
2dw NIL 3-49	0	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
1dw NIL 4-50	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	0	1
SA748 1dwP	1	0	1	0	0	1	1	0	0	0	0	0	0	1	1	1	1

AFLP Primer combination M-CTA and E-ACA

Fragment size	41	42	47	50	51	61	62	63	64	66	68	69	72	73	75	76	78	80	81	82	88	89	90	94	95	97	99	101	105	107
BTX406 4dwP	1	1	1	0	1	0	1	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
4dw NIL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
3dw NIL	1	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
2dw NIL	1	0	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
1dw NIL	1	0	1	0	1	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1
SA748 1dwP	1	0	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1

Fragment size	108	109	112	113	115	117	118	120	122	124	126	127	128	129	132	134	135	139	143	145	147	148	151	155	157	162	163	166	168	170
BTX406 4dwP	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
4dw NIL	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0
3dw NIL	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0
2dw NIL	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
1dw NIL	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	0	1	1	1	0	1	1	0	0	1	0
SA748 1dwP	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	0	0	0	1	0

Fragment size	172	176	179	185	189	191	192	193	200	204	206	210	212	218	220	223	232	237	243	245	248	253	256	260	269	277	286	295	307	315
BTX406 4dwP	1	0	1	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	0	0
4dw NIL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3dw NIL	1	0	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1
2dw NIL	1	0	1	1	1	1	1	1	0	1	0	1	1	1	0	0	1	1	1	0	1	1	0	1	1	1	0	0	1	1
1dw NIL	1	0	1	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0	1	1	0	0	0	1
SA748 1dwP	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0

Fragment size	321	327	349	352	355	362	363	373	389	395	408	418	424	436	439	443	449	459	465	479	484
BTX406 4dwP	1	1	1	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	0	1	1
4dw NIL	1	1	1	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1
3dw NIL	1	0	1	0	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0
2dw NIL	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1	1
1dw NIL	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	1	0
SA748 1dwP	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0

AFLP Primer combination M-CAG and E-ACA

Fragment size	50	53	59	62	64	66	68	69	71	73	75	76	77	79	80	81	83	84	85	87	89	93	94	95	97	99	101	103	104	107
BTX406 4dwP	0	1	1	0	0	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	0	0
4dw NIL	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1
3dw NIL	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0
2dw NIL	0	1	0	1	0	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1
1dw NIL	1	0	0	0	0	0	1	1	0	1	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0
SA748 1dwP	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0

Fragment size	108	109	111	112	113	114	116	117	118	119	120	123	125	127	128	129	132	135	137	139	141	142	144	149	153	154	156	158	160	164
BTX406 4dwP	1	1	0	1	0	1	1	1	0	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1
4dw NIL	1	1	0	0	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1
3dw NIL	1	1	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1
2dw NIL	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	0	0	1	1	0	1	1	0	1	0	1	1
1dw NIL	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	1	1	1	1	1	0	1	1
SA748 1dwP	1	1	1	0	0	0	1	1	0	1	1	0	1	1	1	0	0	0	0	1	1	1	0	0	1	0	0	0	1	1

Fragment size	165	168	169	171	173	176	178	180	181	184	187	198	202	206	217	218	220	222	224	231	235	243	247	252	254	260	263	273	276	281
BTX406 4dwP	1	0	0	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0
4dw NIL	0	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	0	1	0	1	1	0	0
3dw NIL	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	0	1
2dw NIL	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	0
1dw NIL	0	1	0	0	0	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0	1	1	0	0	0
SA748 1dwP	0	1	0	0	0	1	1	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	1	1	0	0	0

Fragment size	282	286	290	292	297	316	326	333	341	344	348	402	424	437	471
BTX406 4dwP	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0
4dw NIL	1	1	0	1	1	1	0	0	1	0	1	0	0	0	0
3dw NIL	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
2dw NIL	1	1	0	0	0	1	1	0	1	0	1	0	0	0	0
1dw NIL	1	1	1	1	0	1	0	1	1	0	1	0	0	0	0
SA748 1dwP	0	0	0	0	0	1	0	0	1	1	1	0	0	1	0

AFLP Primer combination M-CAC and E-ACA

Fragment size	42	45	48	56	59	60	61	63	70	72	74	75	77	78	81	85	87	92	96	99	100	103	104	105	106	107	112	118	119	121
Btx406 4*dw*P	1	0	1	1	0	1	1	1	0	1	1	0	1	1	1	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1
4*dw* NIL	1	1	1	1	0	0	0	1	0	1	0	0	1	1	1	1	1	0	0	1	1	0	0	0	0	0	1	0	1	1
3*dw* NIL	1	1	1	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0	1	1	0	1	0	1	1	0	1	1	1
2*dw* NIL	1	1	1	1	1	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1
1*dw* NIL	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1
SA748 1*dw*P	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	0	1	0	1	1	0	0	1	1

Fragment size	123	134	141	151	153	156	164	165	171	184	198	206	209	213	226	238	248	253	268	278	290	296
Btx406 4*dw*P	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	0	0	1	1	0
4*dw* NIL	1	1	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1
3*dw* NIL	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1
2*dw* NIL	0	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1
1*dw* NIL	1	1	0	1	1	1	1	1	0	0	1	1	1	1	0	1	1	0	0	1	1	1
SA748 1*dw*P	1	1	0	1	1	1	0	1	0	1	1	0	0	1	0	1	0	0	0	1	0	1

AFLP Primer combination M-CTC and E-ACA

Fragment size	42	48	64	66	72	79	80	86	97	102	106	118	142	153	163	166
Btx406 4*dw*P	1	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0
4*dw* NIL	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0
3*dw* NIL	1	1	1	0	1	0	0	1	0	1	1	0	1	0	0	0
2*dw* NIL	1	1	1	0	1	1	1	1	0	1	1	0	1	0	0	1
1*dw* NIL	1	1	1	0	1	0	0	1	0	1	1	0	1	1	1	1
SA748 1*dw*P	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

AFLP Primer combination M-CAA and E-AAC

Fragment size	56	58	60	61	62	64	65	66	68	70	71	72	73	76	77	78	81	83	85	86	88	89	90	91	92	94	95	97	98	100
BTX406 4dwP	1	0	0	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0
4dw NIL 1-45	1	1	0	1	1	0	0	0	0	1	0	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0
3dw NIL 2-49	1	0	0	0	1	1	0	0	0	1	0	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	0	0
2dw NIL 3-49	1	1	1	1	1	0	0	1	0	1	0	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1
1dw NIL 4-50	1	0	0	1	0	1	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	1
SA748 1dwP	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Fragment size	101	106	107	108	111	112	114	116	124	125	126	130	131	133	135	139	141	143	144	145	147	151	152	153	155	157	158	165	175	176
BTX406 4dwP	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0
4dw NIL 1-45	1	0	1	1	0	1	1	0	0	0	1	1	1	1	0	1	1	0	0	0	1	1	1	0	1	1	0	1	0	0
3dw NIL 2-49	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	1	0	0	1	1	0	0	1	0
2dw NIL 3-49	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0	0	1	1	1	1	1	0
1dw NIL 4-50	1	0	1	1	0	0	1	0	0	0	1	0	1	1	0	0	1	1	0	1	0	1	0	0	1	0	1	1	0	1
SA748 1dwP	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1

Fragment size	181	200	201	208	210	223	229	252	256	266	314	384	417	453
BTX406 4dwP	0	0	0	1	1	1	0	0	0	1	0	0	0	0
4dw NIL 1-45	0	0	1	1	1	1	1	0	1	1	1	1	1	1
3dw NIL 2-49	0	0	1	0	1	1	0	0	0	1	1	0	0	1
2dw NIL 3-49	0	0	1	0	1	1	1	0	1	1	1	1	1	1
1dw NIL 4-50	0	0	1	0	1	1	1	0	1	1	1	1	1	1
SA748 1dwP	1	1	1	0	1	1	1	1	1	1	1	1	1	1

AFLP Primer combination M-CAT and E-AAC

Fragment size	57	62	65	58	72	74	80	82	84	86	89	92	94	96	97	98	103	107	108	110	111	114	117	120	126	131	134	137	138	139
BTX406 4dwP	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
4dw NIL 1-45	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
3dw NIL 2-49	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
2dw NIL 3-49	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
1dw NIL 4-50	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
SA748 1dwP	1	1	0	1	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1

Fragment size	158	161	164	166	169	172	185	193	209	212	224	249	256	262	270	296	304	476	480
BTX406 4dwP	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4dw NIL 1-45	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3dw NIL 2-49	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2dw NIL 3-49	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1dw NIL 4-50	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
SA748 1dwP	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1

AFLP Primer combination M-CTT and E-AAC

Fragment size	58	61	66	67	70	71	75	76	78	81	84	86	87	90	92	93	94	95	96	99	101	103	106	108	109	112	113	114	115	116
BTX406 4dwP	1	0	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1
4dw NIL 1-45	1	0	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
3dw NIL 2-49	1	0	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1
2dw NIL 3-49	1	0	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
1dw NIL 4-50	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	0	1	1	0
SA748 1dwP	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	1	1	1	0	1	0	0

Fragment size	118	120	122	125	126	129	132	136	138	143	147	149	152	160	162	169	170	178	181	184	185	189	192	193	202	205	208	210	213	233
BTX406 4dwP	1	1	1	1	1	1	0	1	0	1	0	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1	1	0	1	1
4dw NIL 1-45	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0	1	1
3dw NIL 2-49	1	1	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0	1	1
2dw NIL 3-49	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0	1	1
1dw NIL 4-50	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	1
SA748 1dwP	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	0	0	0	1	1	1	0	1	0	1	1	0

Fragment size	243	249	257	274	278	284	287	290	294	299	304	307	323	336	370	381	415	466
BTX406 4dwP	1	0	0	1	0	1	0	1	0	0	1	0	1	1	1	1	1	1
4dw NIL 1-45	1	0	0	1	0	1	0	1	0	0	1	0	1	1	1	1	1	1
3dw NIL 2-49	1	0	0	1	0	1	0	1	0	0	1	0	1	1	1	1	1	1
2dw NIL 3-49	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	1
1dw NIL 4-50	0	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1
SA748 1dwP	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	0	1

AFLP Primer combination M-CTA and E-AAC

Fragment size	54	158	62	64	66	68	69	70	72	73	75	76	78	79	80	81	82	86	89	93	97	100	101	102	103	108	109	110	112	114
BTX406 4dwP	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4dw NIL	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1
3dw NIL	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
2dw NIL	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0
1dw NIL	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	0	1	0
SA748 1dwP	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	0

Fragment size	116	119	122	125	128	132	137	143	145	146	152	155	159	161	172	178	189	218	221	238	247	264	295	453
BTX406 4dwP	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
4dw NIL	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1
3dw NIL	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1
2dw NIL	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1
1dw NIL	1	1	0	1	1	1	1	1	0	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1
SA748 1dwP	1	1	1	1	1	0	1	1	0	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1

AFLP Primer combination M-CAG and E-AAC

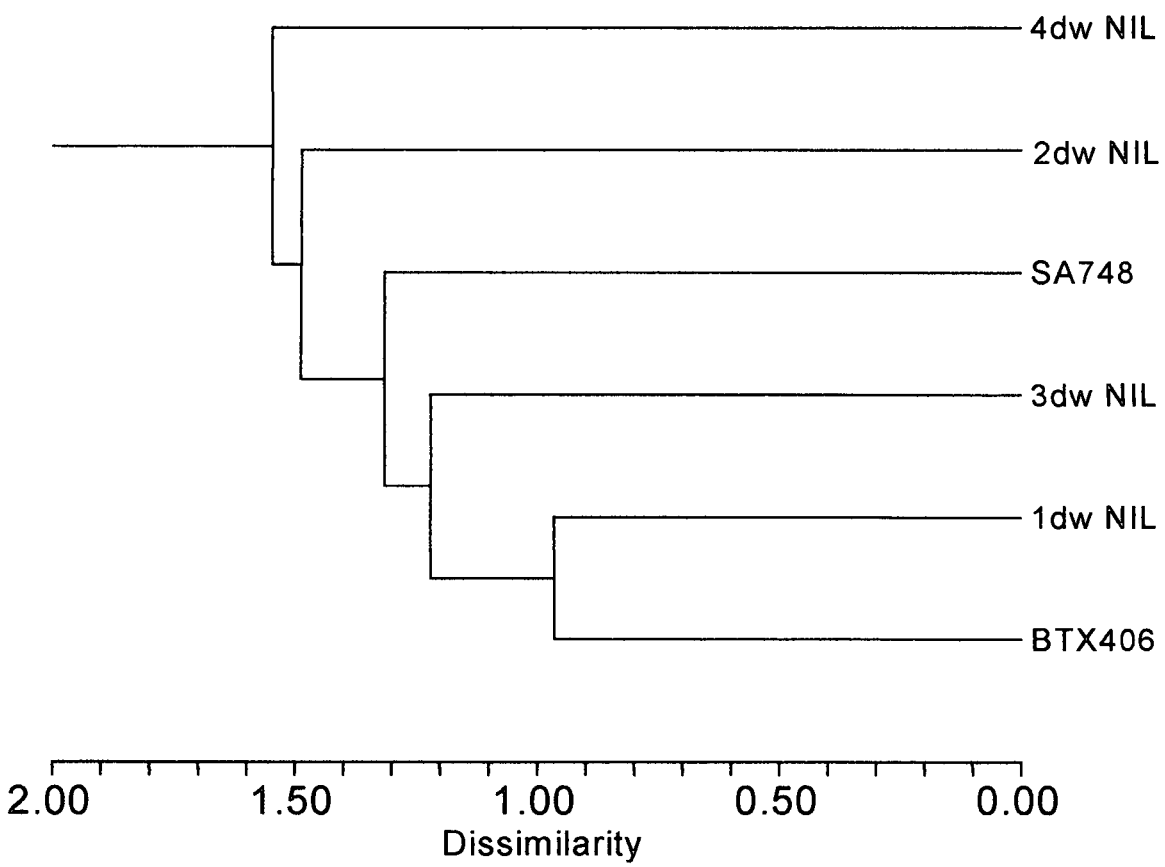
Fragment size	51	60	62	63	65	67	68	70	71	73	74	81	83	86	87	92	93	94	97	99	104	109	113	114	115	116	121	123	126	133
Btx406 4dwP	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	0	1
4dw NIL	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
3dw NIL	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	1	1	1
2dw NIL	1	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1	0	0	1	0	0	1	1	0	0
1dw NIL	1	1	0	1	1	1	1	1	1	0	1	1	1	0	1	0	0	0	0	1	1	0	0	1	0	0	1	1	0	0
SA748 1dwP	0	1	0	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0

Fragment size	134	135	137	145	146	153	160	161	177	184	187	192	196	203	207	209	225	234	260	280	287	292	309	364	391	471
Btx406 4dwP	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0
4dw NIL	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	0	0	0	1	1	1	0	0
3dw NIL	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1
2dw NIL	0	0	1	1	1	1	1	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0
1dw NIL	0	1	1	1	1	1	1	1	0	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	0	0
SA748 1dwP	0	0	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1

Appendix C

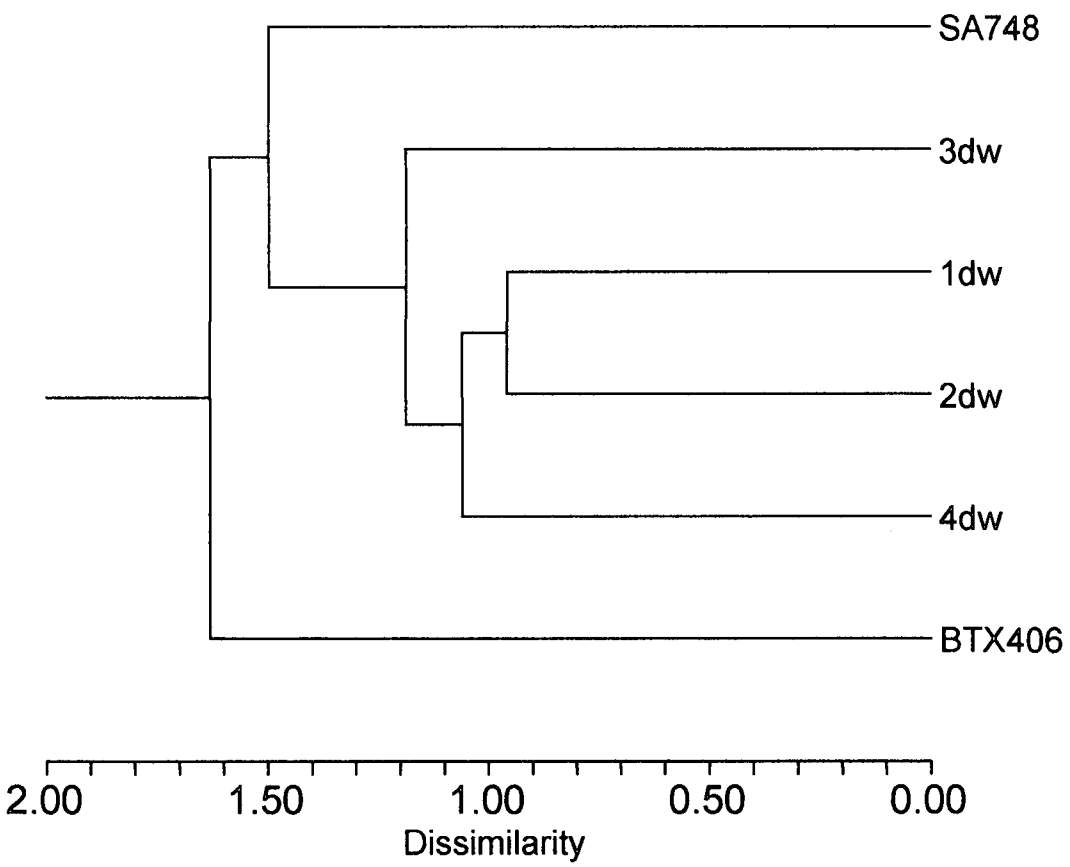
Genetic distances and dendrogram based on AFLP data using primer combination M-CAA and E-ACA.

1.646706				
1.241372	1.511420			
1.555839	1.615074	1.641476		
0.964996	1.493372	1.195782	1.430026	
1.362285	1.466107	1.417950	1.318870	1.160984



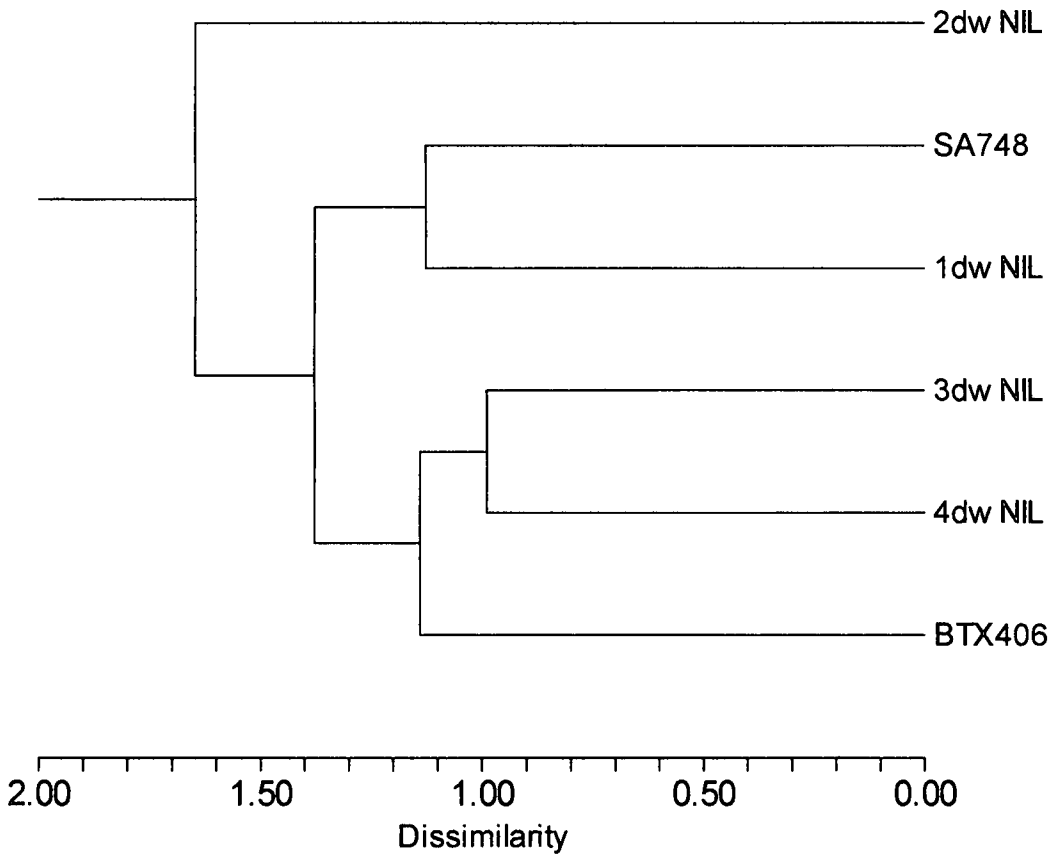
Genetic distances and dendrogram based on AFLP data using primer combination M-CAA and E-AAC.

1.522815				
1.342283	1.212957			
1.698969	1.071442	1.233512		
1.531283	1.046340	1.112882	0.956676	
2.059810	1.545758	1.627264	1.349755	1.468540



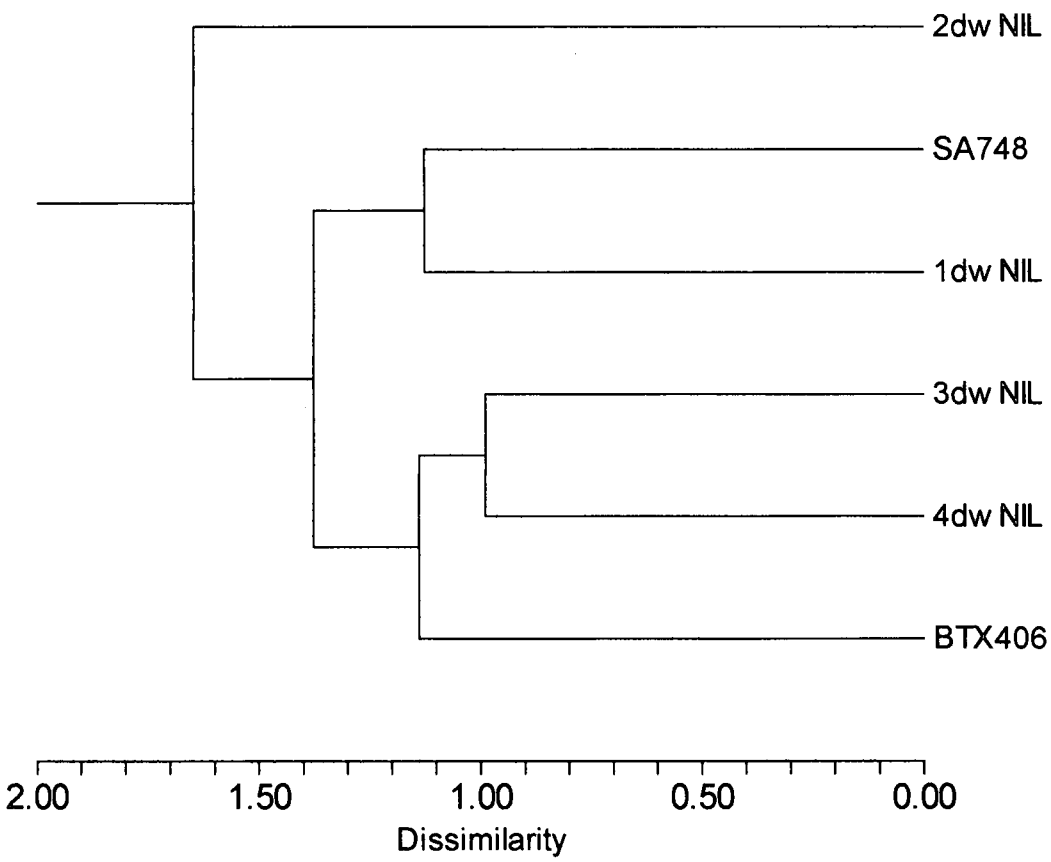
Genetic distances and dendrogram based on AFLP data using primer combination M-CAT and E-ACA.

1.041650				
1.236492	0.988884			
1.645960	1.623069	1.710313		
1.225439	1.180194	1.244033	1.539723	
1.545786	1.464431	1.603567	1.718249	1.126369



Genetic distances and dendrogram based on AFLP data using primer combination M-CAT and E-AAC.

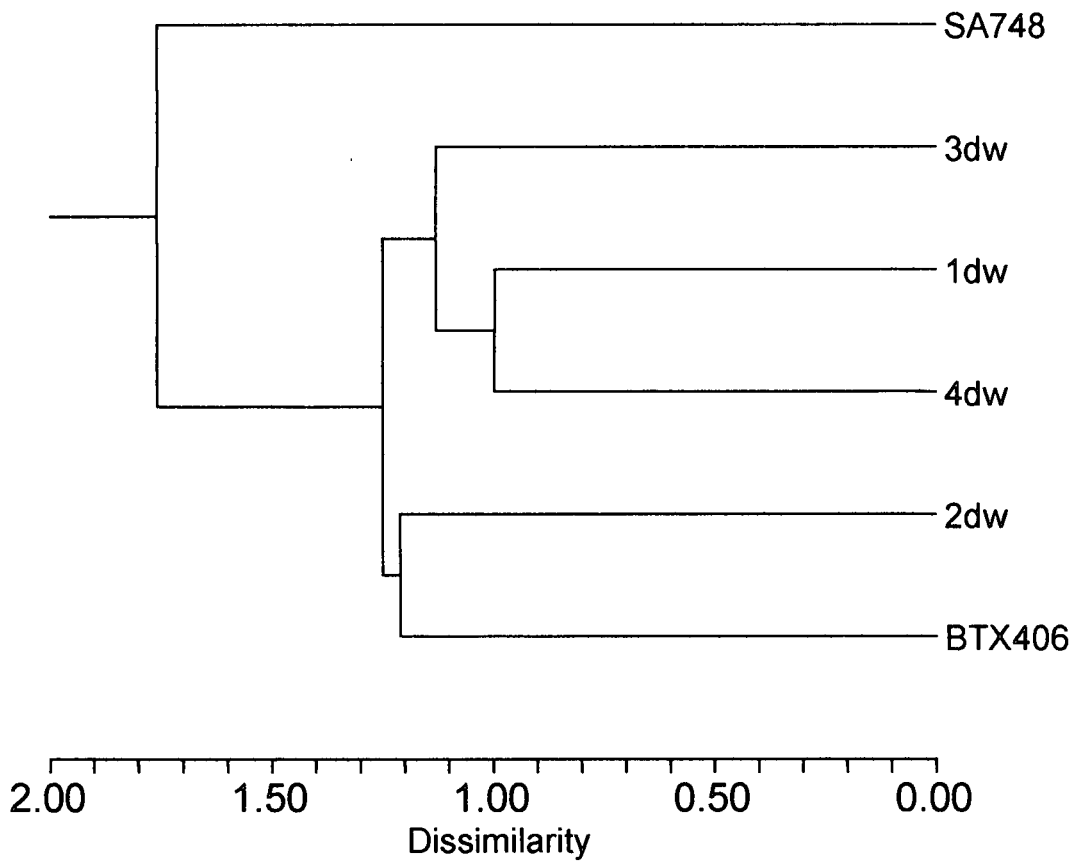
1.422853				
1.214698	0.995086			
1.214698	0.995086	0.626224		
1.028992	0.982693	0.645497	0.645497	
2.201826	1.878673	1.952876	1.952876	1.946591



Genetic distances and dendrogram based on AFLP data using primer combination M-CAA and E-AAC.

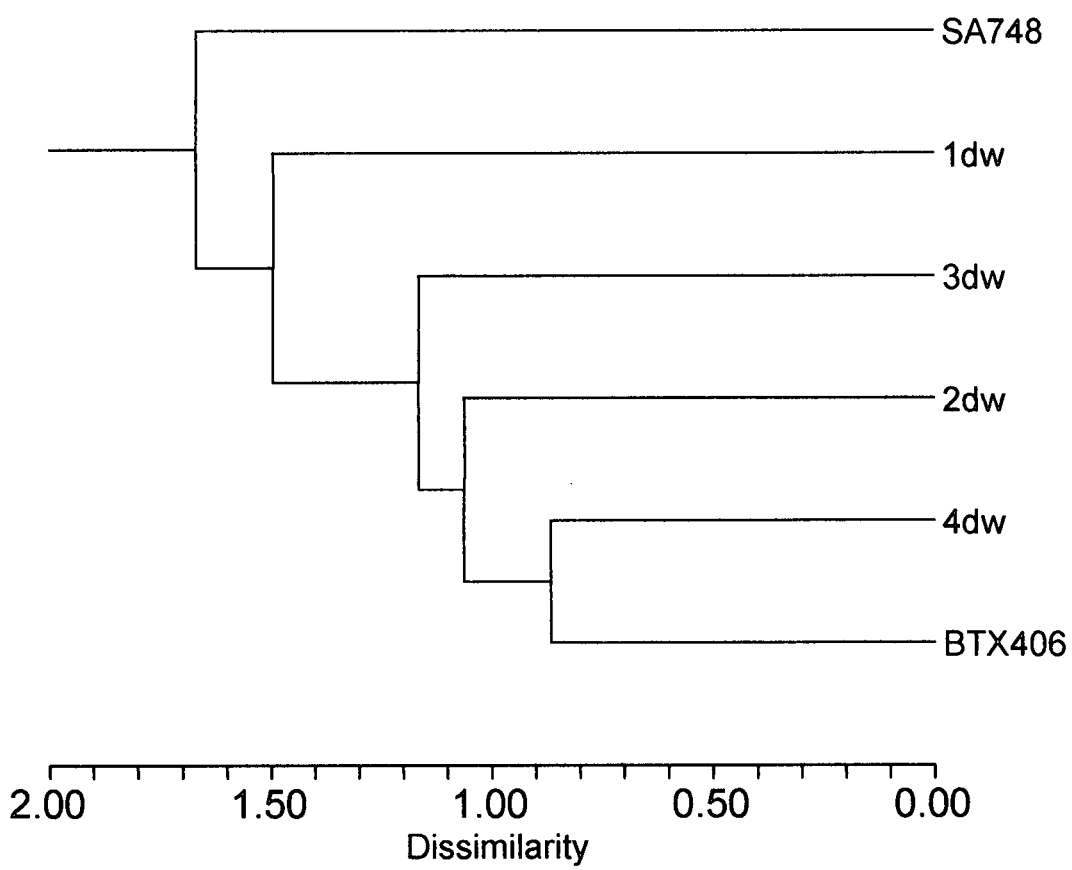
Genetic distances and dendrogram based on AFLP data using primer combination M-CTG and E-ACA.

1.155731				
1.307488	1.087811			
1.210077	1.073490	1.254516		
1.307488	0.996422	1.169045	1.398128	
1.918581	1.721710	1.641718	1.926630	1.582644



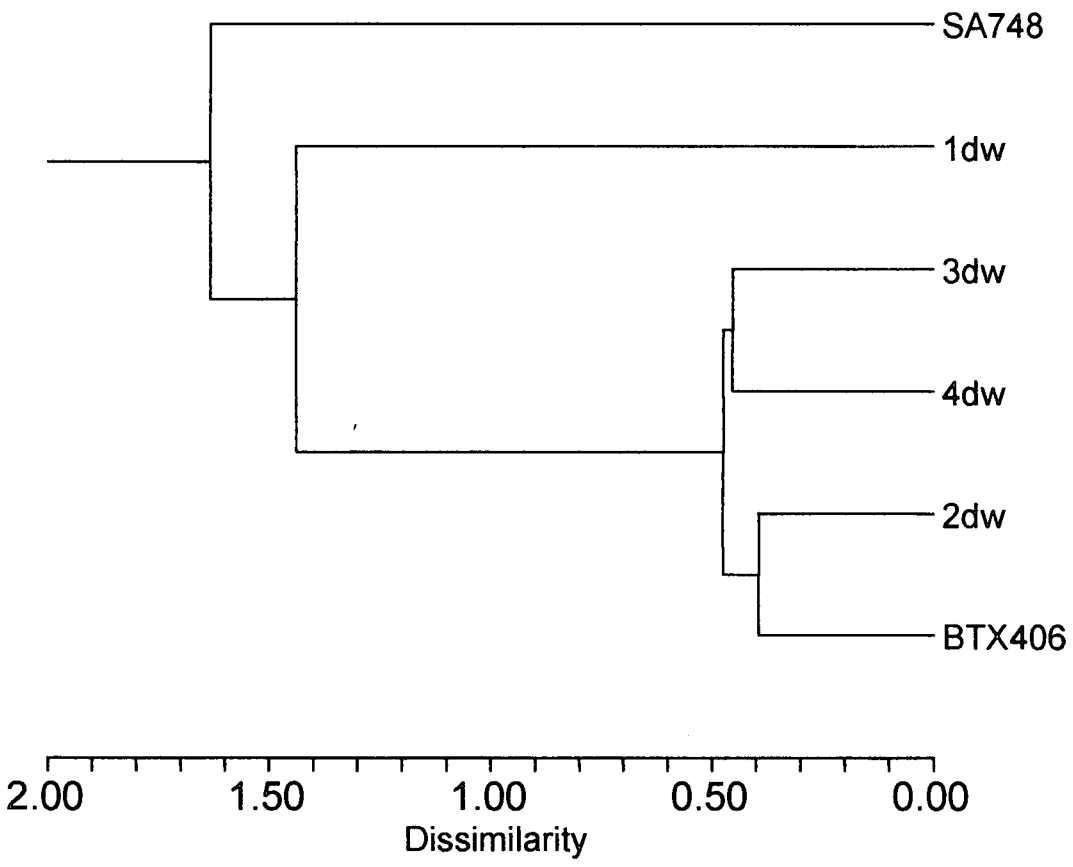
Genetic distances and dendrogram based on AFLP data using primer combination M-CTT and E-ACA.

0.864688				
1.097837	1.078336			
1.056651	1.065740	1.316151		
1.507440	1.477715	1.400782	1.592808	
1.668286	1.680957	1.717961	1.725803	1.551085



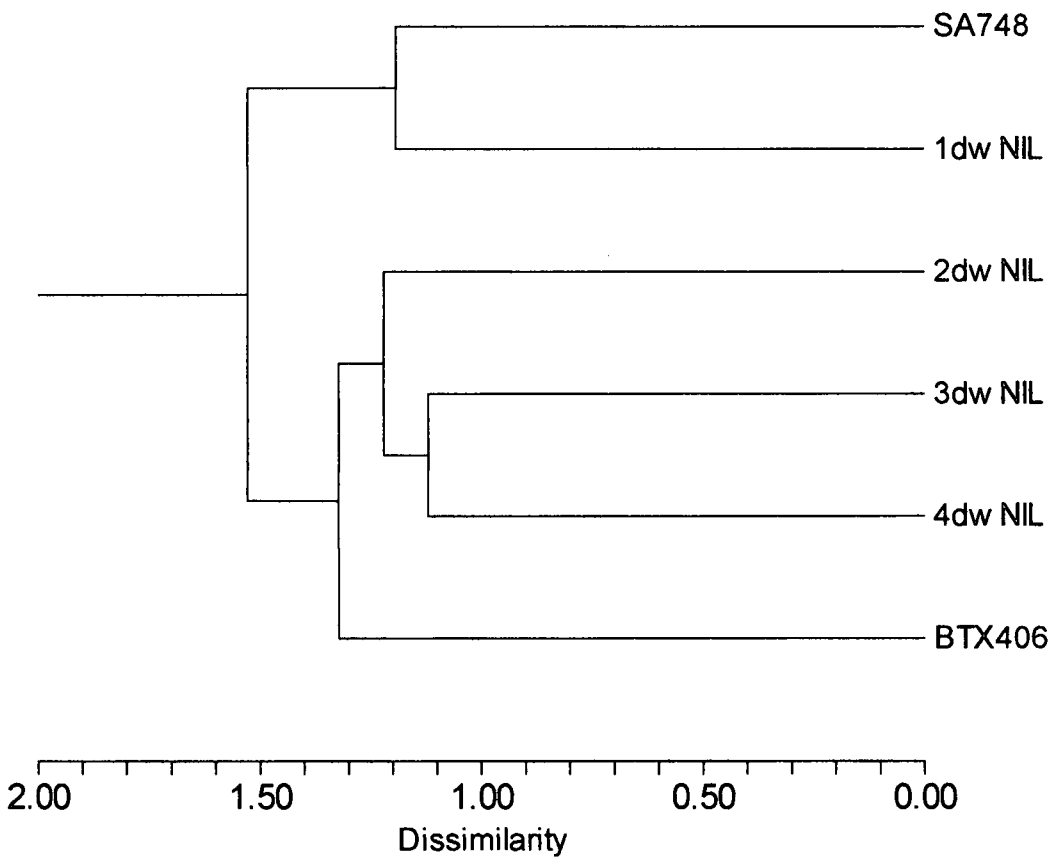
Genetic distances and dendrogram based on AFLP data using primer combination M-CTT and E-AAC.

0.493821				
0.457875	0.452091			
0.394034	0.478484	0.464531		
1.442464	1.440638	1.428716	1.444591	
1.660691	1.682730	1.648763	1.637553	1.537257



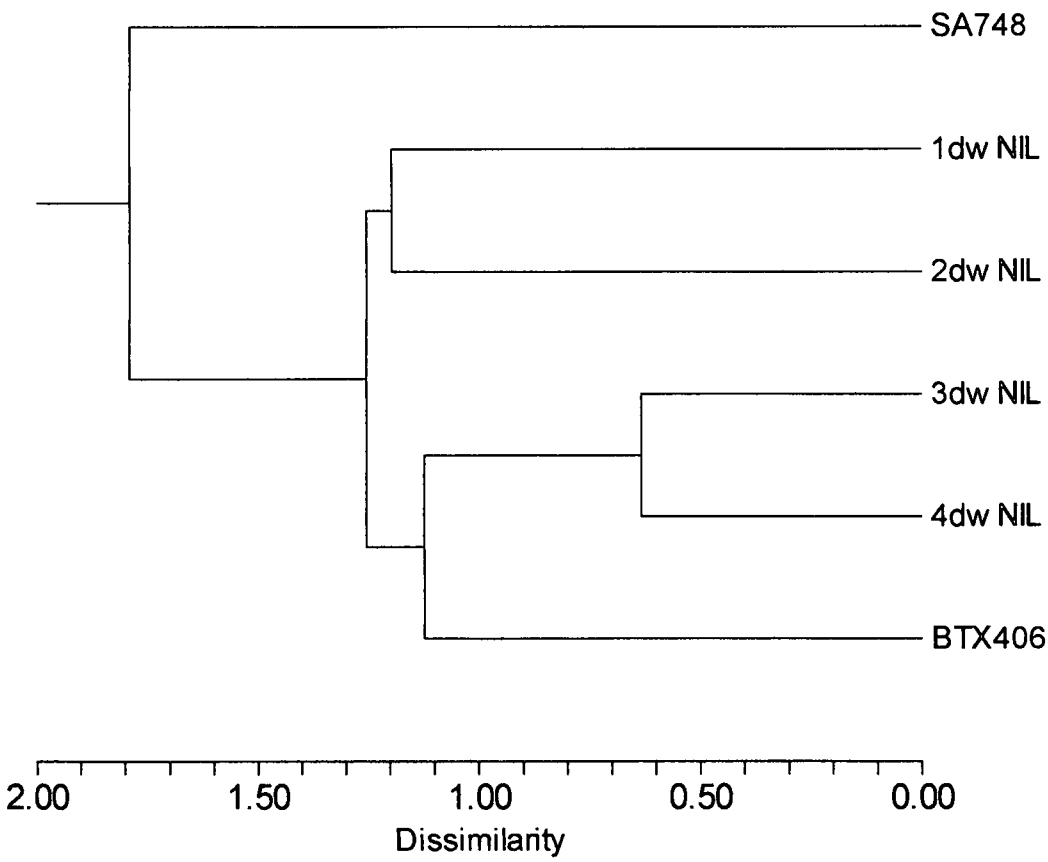
Genetic distances and dendrogram based on AFLP data using primer combination M-CTA and E-ACA.

1.386649				
1.225245	1.120224			
1.358073	1.307032	1.134357		
1.427153	1.554169	1.127312	1.345381	
1.685797	1.855041	1.515443	1.716414	1.192809



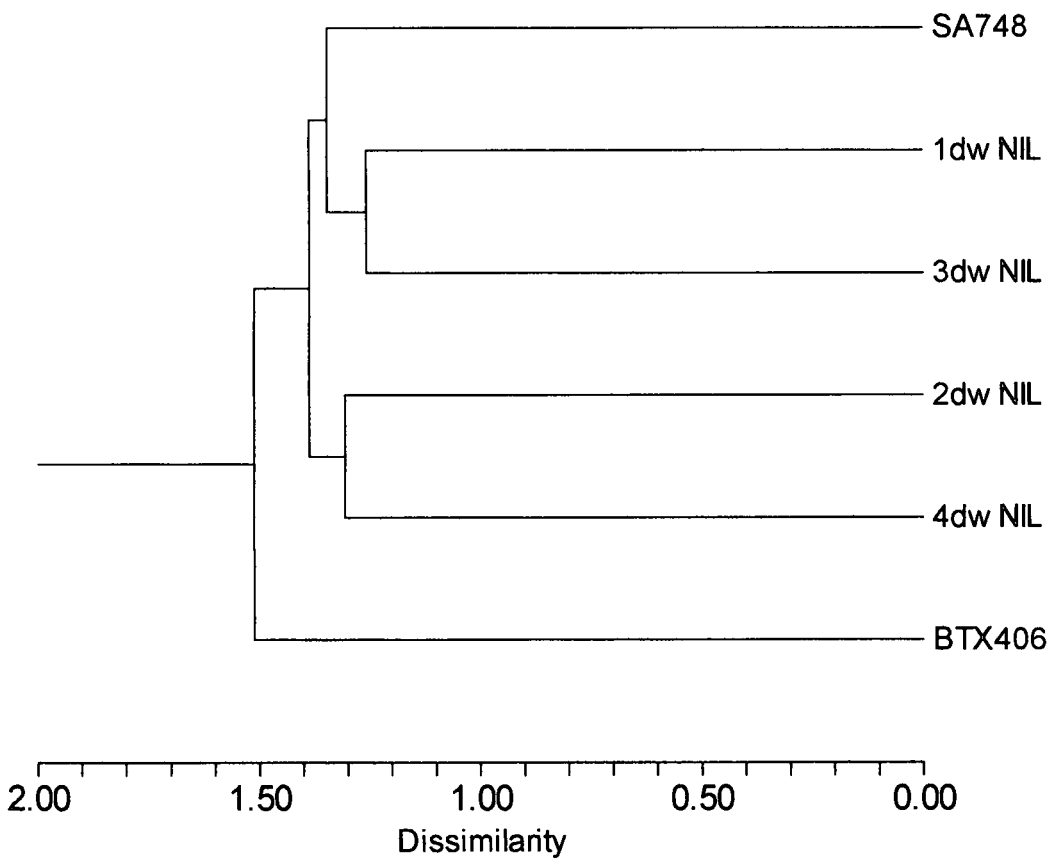
Genetic distances and dendrogram based on AFLP data using primer combination M-CTA and E-AAC.

1.212079				
1.033393	0.633431			
1.332175	1.164018	1.109721		
1.521452	1.170628	1.222222	1.195413	
1.963400	1.632048	1.750661	1.877416	1.727590



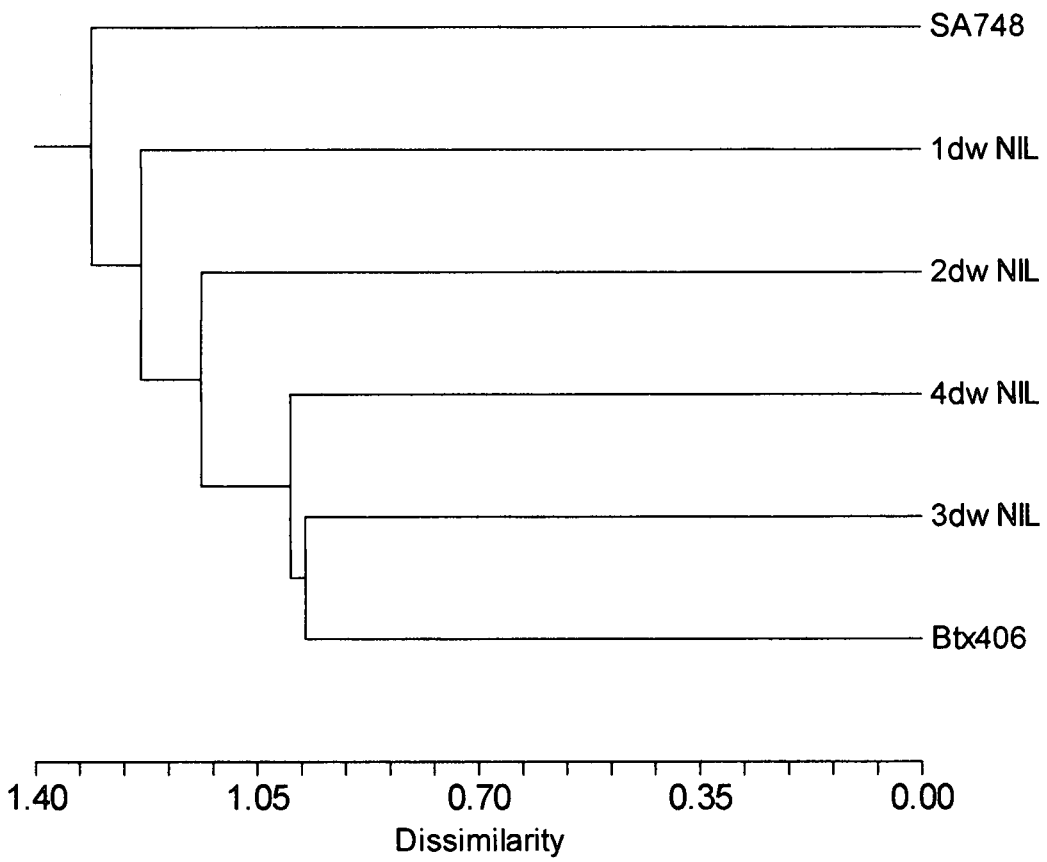
Genetic distances and dendrogram based on AFLP data using primer combination M-CAG and E-ACA.

1.463066				
1.483849	1.437800			
1.552624	1.305303	1.525552		
1.409235	1.269032	1.257508	1.315264	
1.652551	1.320217	1.430098	1.457221	1.264276



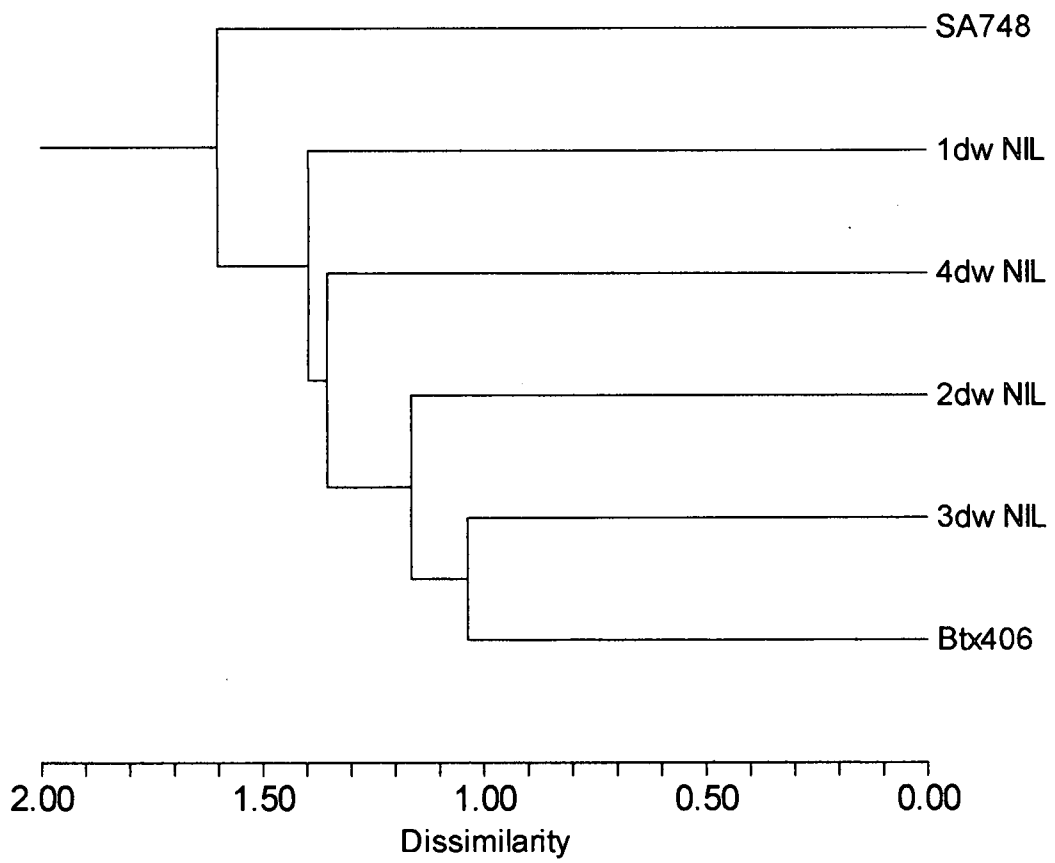
Genetic distances and dendrogram based on AFLP data using primer combination M-CAG and E-AAC.

1.020967				
0.974245	0.974245			
1.152252	1.080123	1.181304		
1.305681	1.175912	1.204394	1.247030	
1.417705	1.097635	1.289352	1.363880	1.383927



Genetic distances and dendrogram based on AFLP data using primer combination M-CAC and E-AAC.

1.333587				
1.036920	1.326711			
1.289419	1.400494	1.036920		
1.367450	1.365218	1.299625	1.548668	
1.543410	1.704251	1.476785	1.663819	1.621753



Genetic distances and dendrogram based on AFLP data using primer combination M-CTC and E-ACA.

1.145644				
1.391941	0.790569			
1.685312	1.236033	0.950146		
1.685312	1.236033	0.950146	1.118034	
2.037837	1.861526	1.685312	1.391941	1.391941