FINE MAPPING *d2,* A GENE THAT CONTROLS PLANT HEIGHT IN PEARL MILLET

by

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(Under the Direction of Katrien M. Devos)

ABSTRACT

The reduction in plant height is imparted by the gene *d2*, which is located on linkage group 4 (LG4) in pearl millet. In order to fine-map *d2*, a large F_2 mapping population was generated from the cross ICMP 451 (*D2D2)* x Tift 23DB (*d2d2*) and genotyped with markers B224C4P2 and PSM305 to identify plants that carry a recombination event in the *d2* region. Markers that mapped to the *d2* region in a previously constructed low-density map were mapped precisely using 25 recombinant plants. Additional markers were developed from the sorghum genomic sequence and mapped to the *d2* region. Based on the phenotypic and genotypic data of the ICMP 451 X Tift 23DB cross, and the genotypic data of a second cross between PT732B and P1449-2, the physical location of d_2 can be narrowed down to the interval spanned proximally by the MDH/Gene 44/ PLATZ marker cluster and distally by PSM 344 on LG4.

INDEX WORDS: Pearl millet, *Pennisetum glaucum*, *d2,* Plant height, SSCP, Comparative genomics, Sorghum

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B.S , A.N.G.R.A.U, India, 2001

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

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December 2008

ACKNOWLEDGEMENTS

This Thesis would have been impossible without the assistance of many people. With utmost gratitude, I convey my thanks to Dr. Katrien M. Devos, my major advisor for her ever untiring guidance in the conduct of research work and writing. I would also like to thank my committee members Dr. Roger Boerma and Dr. Steve Knapp for their time, valuable advice and help.

My special thanks to Ms. Trudi Thomas for her technical assistance in the lab and in the green house. I would like to extend my thanks to past and present members of our lab, Dr. Mathews Dida, Chris Papadopoulos, Dr. Xiangyang Xu and Dr. Alicia Massa for sharing their research knowledge and experience.

Finally I thank my wife and my family in India for their endless support and love throughout my educational endeavors. Also thanks to the Pioneer-GCP fellowship for funding this research.

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CHAPTER I

PURPOSE OF THE STUDY AND REVIEW OF THE LITERATURE

Several dwarfing genes have been reported in pearl millet [*Pennisetum glaucum* (L.)] that cause substantial reductions in plant height. Among them, the *d2* gene has been exploited extensively by breeders in the USA and India to develop commercial semi-dwarf hybrids. The objective of this study was to fine-map the *d2* gene. The ultimate aim is to isolate the *d2* gene in order to enhance our understanding of the function of this gene relative to other dwarfing genes in cereals.

Role of Dwarf cultivars in Global Agriculture

Since the 1960s, the world population has doubled to more than 6.4 billion people. This rapid increase together with a drastic decrease in cultivated land raised concerns, in particular in the 1960s and '70s, about a probable global food crisis. However, the development and widespread adoption of high-yielding semi-dwarf cultivars of wheat (*Triticum aestivum*) and rice (*Oryza sativa*) led to major increases in food production, known as the green revolution, and large-scale famine was averted (Sakamoto and Matsuoka, 2004). Dwarfing or reduced height genes have been a key component of this Green Revolution (Evans, 1993). Yet today, over 800 million people live a life of permanent or intermittent hunger and are chronically

undernourished. The populations of most developing countries are still increasing rapidly. To adequately feed the 9 billion people projected by 2050, food crop productivity will have to be further increased (Sakamoto and Matsuoka, 2008). This objective can be reached by introducing higher and stable yielding cultivars, in particular in developing countries. Reduced height remains a key trait for enhancing yields in many crops, including pearl millet.

Molecular Basis of the Dwarf Phenotype in Plants

Dwarf mutants in plants are crucial for elucidating regulatory mechanisms for plant growth and development. Reduced plant height is also favored as an agronomic trait in many crops. Dwarf mutants have been isolated in many species and have been extensively analyzed for their mode of inheritance and their response to plant hormones (Ashikari et al., 1999). There are various causes for dwarf phenotypes associated with, for example, gibberellins (GA) (Hooley, 1994; Reid, 1993; Ross et al., 1997), brassinosteroids (Azpiroz et al., 1998; Li and Chory, 1997), abnormal cell walls (Reiter et al., 1993), and abnormal cell elongation (Azpiroz et al., 1998; Takahashi et al., 1995). It is well known that rice and other grass mutants that are GA-deficient or GA-insensitive exhibit similar phenotypes as dwarf mutants for the same genes in dicots (Ashikari et al., 1999; Itoh et al., 2002; Itoh et al., 2001). Both the semi-dwarf cultivars of rice and wheat used in the Green Revolution are affected by GA. The rice green-revolution gene is part of the GA biosynthetic pathway (Sasaki et al., 2002; Spielmeyer et al., 2002) and the wheat green-revolution gene affects GA reception (Peng et al., 1999; Sasaki et al., 2002; Spielmeyer et al., 2002).

Molecular genetic studies using dwarf mutants of Arabidopsis (*Arabidopsis thaliana*), rice and other dicot species have revealed that the biosynthesis and perception of two hormones, gibberellins (GA) and brassinosteroids (BR) are the most important factors in determining plant height (Clouse and Sasse, 1998; Fujioka and Yokota, 2003; Mandava, 1988; Taiz L, 2002; Yamamuro et al., 2000) but the relationship between BR and dwarfism in monocot plants has not been studied as extensively as for GA.

Dwarfing Genes in Cereals

The major impact of the exploitation of height reducing genes has been in cereal crops, first in wheat and then in rice (Milach, 2001). Because of their reduced lodging vulnerability, the semi-dwarf cultivars respond positively to high levels of nitrogen application, resulting in higher yields. So, under well-managed conditions, dwarf cereal genotypes frequently yield more harvestable grain than their tall counterparts (Azhaguvel et al., 2003).

In rice, many dwarf mutants have been reported and characterized. Due to the agronomic importance of semi-dwarf mutants in rice, extensive studies have been carried out to understand the genetics of plant height reduction and to identify the genes controlling this trait (Zou et al., 2005). One of the 'Green Revolution' semi-dwarf rice cultivars, IR8, enabled dramatic yield increases and helped to avert predicted food shortages in Asia (Khush, 1999). The height reduction was caused by the recessive gene *semidwarf1* (*sd-1*), which was mapped on chromosome 1 (Cho et al., 1994). The *sd-1* gene was cloned independently by three groups (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002), and shown to impair the biosynthesis of the GA hormone through a mutation in a GA-20 oxidase. Another semi-dwarf

cultivar, *Tan-Ginbozu*, contributed to the increase in rice productivity in the 1950s in Japan. The underlying gene, *D35*, has been cloned by a homology-based approach and encodes another GA-biosynthetic enzyme, ent-kaurene synthase (Itoh et al., 2004). There are relatively few reports and studies on BR-related mutants in rice and other grass species. One of the pioneering studies on these mutants was the molecular characterization of the rice *d61* mutant, which is less sensitive to BR than wild type. Using the Arabidopsis *BRI* gene (a putative BR receptor), a homologous gene in rice called *OSBRI1* was cloned and shown to be closely linked to the *d61* locus. Complementing the *d61* mutation with the coding region of *OSBRI1* rescued the wild type phenotype (Yamamuro et al., 2000). This study revealed that BR is important for stem elongation in monocot plants, and it also served as a cue to investigate the functional role of BR in grasses. The gene underlying another rice dwarf mutant, *d2*, that showed a phenotype similar to that of *d61* has been cloned by a map-based approach and this gene was also shown to be involved in BR biosynthesis (Hong et al., 2003).

Improvement of lodging resistance is an important goal of cereal breeding and many studies have shown that lodging is strongly correlated to plant height. Wheat breeders worldwide have successfully utilized the GA-insensitive semi-dwarfing genes *Rht-B1* (*Rht1 Reduced height*) and *Rht-D1* (*Rht2*) for more than three decades to improve lodging resistance (Borner et al., 1997). To date, the *Rht-B1b* and *Rht-D1b* alleles are present in many high-yielding, semi-dwarf cultivars, where they offer simple genetic control of high harvest index and resistance to lodging. Another GA-insensitive dwarfing gene, *Rht3*, has a more extreme effect on plant height and has not been exploited in commercial cultivars (Flintham and Gale, 1982). The *Rht-1* genes of wheat have been isolated and shown to interfere with the signal transduction pathway of the GA hormone (Peng et al., 1999). Because of the impact of dwarfing genes in wheat and rice breeding

programs, extensive searches for mutants with reduced plant height have been conducted in other cereal species.

In sorghum (*Sorghum bicolor*), tall cultivars are grown in much of the world because fodder yields are almost directly proportional to plant height. However, several dwarf mutants are known and dwarf cultivars were introduced into the USA in the 1950s (Quinby and Karper, 1954). Quinby and Karper (1954) identified four recessive dwarfing genes, *dw1 (dwarf), dw2, dw3,* and d*w4* that are present in different combinations in sorghum cultivars grown in the United States. Typically three mutations are combined to develop commercial cultivars. Since the *dw3* gene improves the harvest index, it is often included in the combination (Karper, 1932; Quinby and Karper, 1954). *Dw3* alleles have pleiotropic effects on yield components (heads/plant, seeds/head, seed weight), tiller number and panicle size (Casady, 1965). One of the prominent characteristics of the *dw3* mutant is its instability. This gene has recently been cloned, and it has been shown that the instability is caused by recombination between two tandemly duplicated copies of the gene. *Dw3* is homologous to a dwarfing gene in maize (*Zea mays*), *Br2* (*brachytic*) (Multani et al., 2003) .

In barley (*Hordeum vulgare* L.), detailed comparisons of primitive and modern cultivars have established that improvements in yield have been achieved by increasing resistance to lodging, reducing time to heading, and incorporating disease resistance genes (Riggs et al., 1981). Short straw with good lodging resistance is one of the most important characters targeted in the breeding of barley. The genes that played a major role in the semi-dwarf genotypes of barley are the *sdw* gene in North America and the *denso* gene in Europe. Although the *sdw* and *denso* mutants arose from separate mutation events, they have similar effects on agronomic traits and both genes are allelic and mapped to barley chromosome 3H (Barua et al., 1993; Hellewell et

al., 2000). The *sdw1* (semi dwarf 1) gene has been used in feed barley cultivars in the United States, western Canada, and Australia, but *sdw1* appears to have negative pleiotropic effects on yield and malting quality (Hellewell et al., 2000) and it is known that feed cultivars have desirable agronomic characters but unacceptable malting and brewing qualities.

Oat (*Avena sativa* L.) is an important crop grown in temperate regions of the world. It is a valuable source of human and animal nutrition and is used for soil conservation. One of the goals of oat breeding has been to reduce plant height in order to minimize the substantial lodging problems that affect grain quality and quantity (Milach et al., 1997). There are eight dwarfing genes described and classified in oat, but only *Dw6*, *Dw7* and *Dw8* are exploited (Milach et al., 1998). The other dwarfing genes lacked utility due to extreme dwarfness or meiotic irregularities of the lines.

In foxtail millet (*Setaria italica* Beauv.), three new sources of dwarf germplasm were identified in 1992 and characterized as GA-insensitive (Dineshkumar et al., 1992).

In pearl millet [*Pennisetum glaucum* (L.)], dwarfing genes have been known for more than 20 years and several cause substantial reductions in plant height (Burton and Fortson, 1966; Gupta et al., 1985; Rao et al., 1986). A description of the pearl millet dwarfing genes is given in chapter II.

Thus, dwarfing genes have been valuable for crop improvement in several members of the grass family. In this study, the objective was to fine-map the plant height gene *d2* of pearl millet.

Pearl Millet: Taxonomy

The taxonomic history of pearl millet began in 1753 when Linnaeus included the cultigens (a plant for which a wild ancestor is unknown) into two different species, *Panicum glaucum* and *Panicum americanum* (Linnaeus, 1753). Further confusion arose when different names were given by different workers to the genus that included pearl millet, such as *Panicum*, *Holcus*, *Alopecurus*, *Cenchrus*, *Pencillaria*, and *Pennisetum* (Jauhar, 1981b). K. Schumann (1895) gave pearl millet the latin name *Pennisetum americanum* based on the name *Panicum americanum* L. that was given by Linnaeus. By the early 19th century, Chase preferred the name *Pennisetum glaucum* over *Panicum glaucum* (Chase, 1921). This name was later adopted by Hitchcock and Chase (1951) in the "Manual of the Grasses of the United States", and has been used by scientists working on pearl millet.

Pearl millet belongs to the family *Poaceae* and is an important member of the genus *Pennisetum*. The word *Pennisetum* comes from a combination of two Latin words, "*penna*," feather, and "*seta*", meaning bristle, that alludes to the plumose or feathery bristles of some species (Burton, 1968). The genus *Pennisetum* Rich. consists of about 140 species and comprises important cultivated species such as napiergrass, pearl millet, and kikuyu grass (Brunken, 1977; Kativu, 1987). The species belonging to the *Pennisetum* genus constitute a heterogeneous assemblage with different basic chromosome numbers (x = 5, 7, 8, and 9). For example, *P. ramosum* has a basic chromosome number of 5 (2n=10) while *P. glaucum* (pearl millet) (2n=2x=14) and *P. purpureum* (napiergrass) (2n=4x=28) have basic chromosome numbers of 7 (Jauhar and Hanna, 1998). Napiergrass is a perennial relative of pearl millet with an allotetraploid genome (Jauhar, 1981a).

Three germplasm pools are recognized in the genus *Pennisetum* (Harlan, 1971c). The primary pool contains all cultivated, weedy, and wild diploid (2n = 14) pearl millets which can cross freely and produce fertile offspring. The secondary pool comprises only *P. purpureum* (2n = 28), otherwise known as elephant grass or napiergrass. The cross between pearl millet and napiergrass is easily made but the progeny are sterile unless their chromosome number is artificially doubled. *P. purpureum* x *P. glaucum* hybrids are widely used as a forage propagated by cuttings (Andrews, 1993). The tertiary pool contains numerous more distantly related *Pennisetum* species of various ploidy levels which do not naturally interbreed with the primary pool, but can potentially be accessed through various wide crosses (Dujardin and Hanna, 1990; Dujardin, 1989).

Domestication

The earliest mention of pearl millet was in 1154 by an Arab scholar (Al-Idrisi, 1154) describing the region of Abyssinia (the Upper Nile). He mentioned that two types of millet, *durru* and *dokhn*, were commonly cultivated. The first name refers to cultivated sorghum and the second to pearl millet. The bulk of evidence on the origin of pearl millet comes from the interpretation of the present-day distribution of the crop and of its wild ancestor (*Pennisetum glaucum* ssp. *monodii*, form *mollissimum)* (Appa Rao, 1999) . In a study about the origins of agriculture in Africa, it is reported that pearl millet originated from Ntereso in northern Ghana and that cultivation dates back to 1250 B.C (Davies, 1968). Archeological remains in the form of carbonized grains indicate that pearl millet reached the northwest coast of India at Gujarat by

1000 B.C. as a result of the extensive trading that existed between Africa and west Asia (Hutchinson, 1971; Rao, 1963; Vishnu-Mittre, 1968; Vishnu-Mittre, 1977).

The wild forms of pearl millet (*P. mollissimum* and *P. violaceum* ecotypes of *P. glaucum* ssp. *monodii*) are only found in Africa, supporting the theory that pearl millet was domesticated in Africa (Sahel zone of west Africa, particularly northern Senegal-Mali and southern Mauritania) (Brunken et al., 1977; Harlan, 1971b; Harlan, 1975). Domestication has yielded genetic modifications of some original traits of the wild plants, such as seed shattering, size of the spike, intensity of branching and tillering *etc*, which define the domestication syndrome. (Harlan, 1971a). Shedding is controlled by the presence of a functional abscission layer on the rachis of the wild forms. It has been demonstrated that both shedding and seed coating have an oligogenic inheritance and that the genes involved are closely linked (Joly, 1984). This could explain the high frequency of domesticated phenotypes for these traits in backcross and F_2 progenies between wild and cultivated forms of pearl millet (Niangado, 1981).

Growing Conditions

Pearl millet is a warm season crop and planting is done in early summer in the USA. Planting in Africa and India takes place before the onset of the rainy season, which is in May in the pearl millet growing regions. Like any grain crop, pearl millet will yield best on fertile, well drained soils. However, it also performs relatively well on sandy soils under acidic soil conditions, and even when available soil moisture and soil fertility are low. This adaptation reflects pearl millet's origin in Africa, where growing conditions are adverse with extremely high temperatures. Pearl millet appears to have relatively fast root development, and the roots grow

deep into the soil to take advantage of available moisture and nutrients. In general, pearl millet overlaps with sorghum in its areas of adaptation, but it is more drought tolerant and generally matures earlier (http://www.jeffersoninstitute.org/pubs/millet.shtml, accessed on 2nd July 2008).

Distribution and Importance

Pearl millet has been used in Africa and parts of the near East as a cereal crop for nearly 3,000 years. It is cultivated on some 26 million hectare (ha) in over 40 countries, predominantly in India and Africa (Bhattacharjee et al., 2007). India is the single largest producer of this crop, both in terms of area (9.1 m ha) and production (7.3 million tons), with an average productivity of 780 kg ha^{-1} during the last 5 years. Pearl millet is also grown in some parts of the USA and in Australia, mainly as a forage and/or mulch component of minimum tillage-based cropping systems (FAOSTAT, 2005). It yields excellent forage and is said to be the best annual grazing crop in the southern USA (Burton, 1995) because of its low hydrocyanic acid content. It ranks as the fifth cereal in the world in order of economic importance (Poncet et al., 2000).

Genetic and Genomic Resources for Pearl Millet

Pearl millet is a hermaphroditic species with strong protogyny and a cross-pollination rate of up to 82% (Burton, 1974). It is a diploid species (2n=2x=14) with a DNA content of 1C= 2.36 pg (Martel et al., 1997). Over the years, a number of resources have been developed for pearl millet, including extensive germplasm collections, molecular markers and maps and a BAC (Bacterial Artificial Chromosome) library.

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has the largest collection of pearl millet germplasm (21,191 germplasm accessions from 49 countries) (Mangat, 1999). Germplasm forms an important resource for the identification of new traits that can be exploited in breeding. Knowledge of the genetic diversity present in a crop is critical to breeding programs, including the development of commercial hybrids. The genetic diversity can be assessed using both morphological characteristics and DNA markers (Kapila et al., 2008) . Being allogamous in nature, pearl millet accessions are highly heterogeneous which is reflected in the high variability found both within and among accessions (Bhattacharjee et al., 2007).

Restriction Fragment Length Polymorphism (RFLP) analyses have shown that genetic polymorphism in the pearl millet gene pool is very high, not only between species (Liu, 1992), but also within the landraces (Pilat-Andre, 1992). So far, the diversity in pearl millet has been studied using iso-enzyme loci (Tostain, 1992; Tostain, 1994), AFLP (Amplified Fragment Length Polymorphism) markers (Brocke et al., 2003), RFLPs (Bhattacharjee et al., 2002), and microsatellites (Mariac et al., 2006). In a recent study at ICRISAT, SSR (Simple Sequence Repeats) markers were used to assess and characterize the pattern of diversity in maintainer and pollinator lines (Kapila et al., 2008).

The first genetic map of pearl millet was generated by Liu et al. (1994). The map contained 181 RFLP markers covering the seven pearl millet chromosomes and spanning a genetic distance of 303 cM (Centimorgan). A subset of these markers has subsequently been transferred to a series of different crosses that segregate for agronomically important traits. Since then, there has been significant development and mapping of SSRs, of which nearly 200 are now available (Qi et al., 2001; Qi et al., 2004). Recently, single nucleotide polymorphisms (SNPs) were added to the pearl millet marker portfolio (Bertin et al., 2005). The identification of more

distally located markers has also suggested that the early pearl millet map was incomplete (Devos et al., 2000). While the overall recombination rate appears to be similar in pearl millet and other cereals, recombination is highly skewed towards the distal regions in pearl millet, making it difficult in many crosses to obtain linkage of distal markers with the centromeric marker cluster.

Other genetic resources available to millet geneticists and breeders include comparative maps, the first of which were generated with rice and foxtail millet (Devos et al., 2000). The pearl millet genome has also been incorporated into the entire grass syntenic synthesis (Devos, 2005; Devos and Gale, 2000) .

With respect to trait analyses in pearl millet, quantitative trait loci (QTL) have been mapped for downy mildew resistance (*Sclerospora graminicola*) (Jones et al., 1995; Jones et al., 2002), drought tolerance (Yadav et al., 2002; Yadav et al., 2004; Yadav et al., 2003) and for characteristics involved in domestication (Poncet et al., 2000; Poncet et al., 2002).

The development of genomic resources has also been initiated in pearl millet. Currently there are 2,854 ESTs available in the NCBI database (http://www.ncbi.nlm.nih.gov/dbEST/, accessed on 5 July 2008). A BAC library has been constructed using nuclear DNA from the pearl millet inbred line Tift 23DB (Allouis et al., 2001). The library contains a total of 159,100 clones with an average insert size of 90 kb, and corresponds to 5.8 haploid genome equivalents. With all these resources available, pearl millet is the best supported minor cereal (Bertin et al., 2005).

Comparative Genomics of Grasses

Evolutionary studies have shown that the lineage of flowering plants (Angiosperms) is about 200 million years old , while the grass family *Poaceae* diverged from a common ancestor about 50 to 60 million years ago (Kellogg, 2001). Among the members of the grass family, cereal species such as rice, wheat, maize, sorghum, and millets are the most important in terms of economic value. These species represent a great diversity in chromosome number, ploidy (*e.g.* rice 2n=2x=24, wheat 2n=6x=42) and genome size (*e.g.* 430 Mb in rice to 16,800 Mb in wheat) (Arumuganathan, 1991; Bennett et al., 2000).

Even though there is much diversity in genome size and chromosome number, comparative mapping studies have observed extensive conservation of gene content and gene order at the genetic map level (Ahn and Tanksley, 1993; Gale and Devos, 1998; Hulbert et al., 1990; Moore et al., 1995; Vandeynze et al., 1995a; Vandeynze et al., 1995b; Vandeynze et al., 1995c). Studies at the DNA sequence level have confirmed the existence of often high levels of colinearity between different genomes, but also have revealed frequent deviations in colinearity. For example, comparative sequence analysis of the *adh1* (*alcohol dehydrogenase1*) region in sorghum and maize showed the presence of mostly the same genes. However, the rice *adh1* region has no colinearity with either species (Tarchini et al., 2000; Tikhonov et al., 1999) . Similar studies involving the comparison in the grasses of other orthologous regions, *e.g.* the *sh2/a1* homologous regions of maize, sorghum, and rice (Chen et al., 1997); the *LrK* homologous regions of barley, maize, rice and wheat (Feuillet and Keller, 1999); the genomic regions near *Vrn1* and its orthologs in wheat, barley, rice, and sorghum (Ramakrishna et al., 2002), have indicated general, albeit seldom perfect, conservation of gene content and gene order between

orthologous genomic segments of grass genomes (Bennetzen and Ma, 2003). These studies also revealed that there is very little sequence homology in intergenic spaces. Although the numerous but small changes in gene content, order or orientation will complicate the use of comparative grass genetic information, comparative studies will provides many new insights into the nature of gene and genome evolution in plants (Ilic et al., 2003) .

Conserved gene content and order at the macro level gave rise to the model that individual grass species could be viewed best as manifestations of a single grass genome and that each of the strengths of studies in different grasses could be used to benefit all individual grass studies (Bennetzen and Freeling, 1993). The identification of commonly conserved segments of DNA across grass species also led to the construction of a circle model of grass genome structure, now popularly called the 'Crop Circles' (Devos, 2005; Gale and Devos, 1998; Moore et al., 1995) .

Since the discovery of colinearity among the genomes of grass species, it has been proposed that the genomics resources of model plant species can be exploited to facilitate map-based cloning in plants with large genomes. In this regard, rice has served as the primary model organism for the genomes of grass species in terms of genome research and gene isolation (Havukkala, 1996) for almost a decade. In recent years, more grass genomes are being sequenced, including sorghum, maize, and *Brachypodium distachyon*, which gives scientists access to model species more closely related to their crop of interest.

The map-based sequence of rice (Matsumoto et al., 2005) has been exploited for the identification of genes underlying diverse agronomic traits such as flowering time, plant architecture and development, fertility restoration, and disease resistance (Paterson et al., 2005). Shattering and plant height are excellent examples of traits that have been mapped to colinear

regions among grass genomes. The genes underlying these traits have been cloned (Paterson et al., 2005; Peng et al., 1999). It has been shown that the genes controlling GA-insensitive dwarfism in maize (*d8*) and wheat (*Rht-1*) are orthologous (Peng et al., 1999).

While comparative relationships hold true for many genes, disease resistance genes, however, tend to evolve quickly and are less likely to be found in conserved positions (Leister et al., 1998). Nevertheless, four QTL were recently identified in rice for quantitative resistance to blast (*Pyricularia grisea***)** that showed corresponding map positions in rice and barley (Chen et al., 2003).

The field of plant genomics has reached a stage in which large genomes have become targets for complete sequence analysis (Rabinowicz and Bennetzen, 2006). Maize was selected first because of its economic importance, its powerful genetic and genomic resources and its history as a model plant (Bennetzen et al., 2001; Bennetzen JL, 2001). Recently, a draft sequence of the maize genome was released (http://www.maizesequence.org, accessed on 5 July 2008) and the quality of the sequence will continue to be improved in the coming months. The shot-gun sequence of the sorghum genome (730 Mb) has also been released (http://www.phytozome.net/cgi-bin/gbrowse/sorghum/, accessed on 5 July 2008). Since pearl millet is more closely related to maize and sorghum than rice, the genomic sequence of these species will be more useful for the development of new markers for pearl millet compared to the rice sequence information.

Map-Based Cloning

Two different strategies can be used to elucidate the function of a gene, forward genetics (from phenotype to gene) and reverse genetics (from gene to phenotype) (Takahashi et al., 1994). A reverse genetics approach requires sequence information. To elucidate its function, a gene can be used in transformation studies to evaluate phenotypic changes due to overexpression of the transgene or a mutant phenotype can be induced by disrupting the gene function either through insertional mutagenesis like T-DNA/transposon tagging or gene silencing by RNAi (Matzke et al., 2001). Forward genetics aims to identify the sequence or the gene that underlies a specific mutant phenotype. When the mutation causing the phenotype is the result of a T-DNA or transposon insertion, identification of the gene of interest is relatively straightforward by locating the sequence tag and analyzing its neighboring sequences. The identification of the gene that underlies a trait for which chemically induced mutants or natural genetic variation exits, on the other hand, is relatively difficult and requires a laborious map-based cloning (MBC) approach (Peters et al., 2003).

Map-based cloning, also called positional cloning, is the process of identifying the molecular basis of a mutant phenotype by searching for linkage to markers whose physical location in the genome is known or that have been mapped to a specific linkage group/chromosome (Jander et al., 2002). The major strength of this approach is that it represents truly unbiased gene discovery as no preconceived idea or knowledge of the gene is required (Alonso and Ecker, 2006). MBC generally involves the following steps (Stein, 2004) :

(i) Generating a large segregating population and mapping the trait, which is inherited as a single Mendelian locus, using molecular markers.

(ii) Fine-mapping of the trait to narrow the genetic interval

(iii) Physical mapping of the target region and identification of candidate genes

(iv) Confirmation of the identity of the candidate gene(s) by complementation of the mutant phenotype with the wild-type gene by transformation, by the analysis of natural and/or induced allelic variants, or by recreating the mutant phenotype in wild-type genotypes by downregulating the expression of the candidate gene.

MBC was first carried out in mammalian systems and was successfully employed for, most notably, the isolation of the cystic fibrosis gene (Rommens et al., 1989). MBC in plants started about 10-15 years ago, and the first map-based cloning success was the isolation of the resistance gene *Pto* in tomato. *Pto* provides resistance against bacterial speck disease in tomato caused by *Pseudomonas syringae* (Martin et al., 1993).

MBC has been a powerful method for isolating genes in Arabidopsis because of the availability of large genomic resources in this species. Access to the whole-genome sequence means that there is virtually no limit on the number of polymorphic markers that can be identified. Many genes in Arabidopsis, like *SUPERMAN* which is involved in flower development (Jacobsen and Meyerowitz, 1997), *FCA* which controls flowering time (Macknight et al., 1997) and *BRX* which controls cell proliferation and elongation of the root tip (Mouchel et al., 2004) have been cloned.

Map-Based Gene Cloning in Cereal Species

MBC in rice has become relatively easy because of the availability of a complete genome sequence (Matsumoto et al., 2005) and the development of extensive genomic resources such as

high density transcript linkage maps (Wu et al., 2002; Yamamoto and Sasaki, 1997), nearly 1.2 million ESTs (Zhu and Buell, 2007), more than 30,000 full length cDNA sequences (Kikuchi et al., 2003; Xie et al., 2005) and a sequence-ready BAC/PAC physical map (Chen et al., 2002; Sasaki et al., 2005). As in tomato, the first gene that was cloned in rice using a map-based approach was a disease resistant gene, *Xa21*. *Xa21* confers resistance against the bacterial pathogen *Xanthomonas oryzae pv. oryzae* (Song et al., 1995). Since then, many agronomically important genes including the *d1* (*Dwarf1*) gene that controls culm length (Ashikari et al., 1999), the *sd-1* gene that drove the green revolution (Monna et al., 2002), *dwarf11* (*d11*) that regulates seed length (Tanabe et al., 2005) and a novel cytochrome P450 *CYP81A6* which confers resistance to two different classes of herbicides (Pan et al., 2006) have been cloned. In addition to genes underlying traits that are inherited in a Mendelian fashion, genes underlying quantitative trait loci (QTL) have also been cloned. In rice, the genes controlling heading time (*Hd1, Hd3a, Hd6*) have been isolated by positional cloning (Kojima et al., 2002; Takahashi et al., 2001; Yano et al., 2000).

MBC has been less frequently used in other grass species like maize, sorghum, and millets. In maize, the MBC approach could generally be avoided because of the availability of well characterized endogenous transposon tagging systems (Walbot, 2000). Furthermore, chromosome walking is extremely difficult in a large genome that contains vast amounts of repetitive DNA. But conservation of synteny across cereal genomes in combination with the available genomic resources for maize makes a MBC approach feasible. It has even been stated that chromosomal walking could be much faster than traditional transposon tagging (Bortiri et al., 2006). The first gene isolated in maize by positional cloning underlay a QTL, *tga1* (teosinte glume architecture) (Wang et al., 2005). Recently, the *ra3* (*RAMOSA3*) gene that controls the

inflorescence architecture in maize was positionally cloned (Satoh-Nagasawa et al., 2006). Since QTL are not amenable to transposon tagging and not every gene can be cloned using this approach, and with the near-completion of maize sequence, the future of MBC in maize has brightened.

Barley is a temperate cereal with a genome twice the size of the maize genome. One of the first, and also one of the most comprehensive applications of colinearity between rice and barley was the attempt to clone the barley disease resistance gene *Rpg1* by chromosome walking in rice (Han et al., 1999; Kilian et al., 1997). The targeted region in barley was highly colinear with the orthologous rice region for most genes but a homolog of the barley *Rpg1* disease resistance gene was not present in the orthologous region of the rice genome (Brueggeman et al., 2002). The *Rpg1* gene was eventually isolated by map-based cloning in barley and was facilitated by the availability of rice markers targeted to the *Rpg1* region. *Rpg1* was shown to be a novel disease resistance gene with homology to receptor kinases.

Chromosomal walking in wheat is even more cumbersome because of polyploidy in addition to a large genome size and the abundance of repetitive elements (hexaploid wheat, 2n=6x=42, 1C=16,800 Mb). Exploitation of comparative information with smaller-genome model species can facilitate the gene isolation process. The wheat *Vrn-1* (vernalization response gene) has been isolated by MBC and this process was assisted by the colinearity that exists between the target region in wheat and rice chromosome 3 (Yan et al., 2003). Other examples of genes isolated by MBC in wheat include the vernalization response genes *Vrn-2* (Yan et al., 2004), *Vrn-3* (Yan et al., 2006), the domestication gene *Q* (Simons et al., 2006), the homoeologous pairing control gene *Ph1* (Griffiths et al., 2006), the leaf rust resistance genes

Lr21 (Huang et al., 2003), *Lr10* (Feuillet et al., 2003) and *Lr1* (Cloutier et al., 2007) and powdery mildew resistance gene *Pm3b* (Yahiaoui et al., 2004).

Although MBC is becoming a routine task in model crops (rice, Arabidopsis) and species with small genomes, it remains challenging in crops that have larger genomes like wheat, barley, maize and pearl millet. In this study, we initiated the map-based cloning of a dwarfing gene, *d2*, in pearl millet. The first step, the development of a high density genetic map has been completed, and work has started towards the construction of a physical contig of the *d2* region.

CHAPTER II

FINE-MAPPING *d2,* A GENE THAT CONTROLS PLANT HEIGHT IN PEARL MILLET

Abstract

All pearl millet hybrids grown in the US and India are semi-dwarfs. The reduction in plant height is imparted by the gene *d2*, which is located on linkage group 4 in pearl millet. In order to fine-map *d2*, we have generated a large F_2 mapping population from the cross ICMP 451 (*D2D2)* x Tift 23DB (*d2d2*). Two PCR-based markers, B224C4P2 and PSM305 that had previously been shown to flank *d2* and spanned a genetic distance of ~4.0 cM were selected to genotype the F_2 population to identify plants that carry a recombination event in the *d2* region. We have genotyped 915 plants using the single-strand-conformation polymorphism (SSCP) technique and 29 recombinant plants were found. This gives a new estimate of 1.6 cM for the B224C4P2 – PSM305 interval that spans *d2*. Markers that mapped to the *d2* region in a previously constructed low-density map were then mapped precisely using 25 of the 29 recombinant plants. Based on comparative information, additional markers were developed from the sorghum genomic sequence and mapped to the *d2* region. No recombination events were present between the markers that mapped between B224C4P2 and PSM344, resulting in the formation of a marker cluster of seven markers. The *d2* gene also cosegregated with this marker cluster. Mapping in a second mapping population generated from the cross PT 732B (*d2d2*) x

P1449-2 (*D2D2*) resulted in the marker cluster being split into two clusters that were separated by two recombination events. Based on the phenotypic and genotypic data obtained in the two mapping populations, the physical location of d_2 can be narrowed down to the interval spanned by the MDH/ Gene 44/PLATZ marker cluster (proximal side) and PSM 344 (distal end) on LG4.

Introduction

Landraces of pearl millet (*Pennisetum glaucum* (L.) R. Br.) from the Indian subcontinent and Africa grow very tall and can measure up to 3 m (Rai and Rao, 1991). Research on dwarfing genes has indicated that several major genes can cause substantial reductions in plant height in pearl millet (Burton and Fortson, 1966; Gupta et al., 1985; Rao et al., 1986). Burton and Fortson (1966) reported five different naturally occurring sources of reduced plant height in pearl millet, named *D1* to *D*5. Later, a further eight non-allelic dwarf lines were identified from the world collection of pearl millet germplasm maintained at ICRISAT (Rao et al., 1986). Dwarfness in *D1* and *D2* was controlled largely by one or two recessive genes and in lines *D3*, *D4,* and *D5* by more than two recessive genes. Even though several dwarfs were reported in pearl millet (Koduru and Rao, 1983), initially only two dwarfs were assigned gene symbols, *d1* (present in Tift 238) and *d2* (present in Tift 23DB) (Burton and Fortson, 1966). In continuation of these gene symbols, two newly identified dwarfing genes (Rao et al., 1986) were designated as *d3* (IP 10401) and *d4* (IP 10402).

Dwarf lines having *d1* and *d2* are classified as dwarfs as the culm and peduncle measure about 50 cm and the mutants are indistinguishable from the tall lines until flowering. The *d3* and *d4* mutants are classified as extreme dwarfs because of their tufted growth habit due to

shortening of the internodes and leaf sheaths near the base of the plant (Rao et al., 1986). Devi et al. (1994) tested gibberellin sensitivity in five non-allelic dwarf mutants of pearl millet and showed that *d1*, *d2* and *d4* are GA insensitive while *d3* is GA-deficient and can be turned into a wild-type phenotype by the application of exogenous GA.

Among the reported dwarfing genes in pearl millet, the *d2* dwarfing gene has been most widely used by breeders because it was among the first ones to be discovered and was reported to have no adverse effect on general combining ability and several developmental traits (Thakare and Murty, 1972). It has several pleiotropic effects. Primarily, *d2* reduces plant height by about 50% through a reduction in the length of all stem internodes except the peduncle (Burton and Fortson, 1966), leading to a higher proportion of leaves (Rai and Hanna, 1990). Based on the composition and digestibility of dwarf pearl millet forage, it is reported that the *d2* gene could be used to improve the nutritive value of pearl millet forage (Johnson et al., 1968). Green forage from *d2* dwarf plants has significantly higher *in vitro* dry matter digestibility (IVDMD) than that from tall plants (Hanna et al., 1979). However, *d2* has a negative effect on yield in some backgrounds (Bidinger and Raju, 1990; Rai and Rao, 1991). The reduced yield is associated with a poorer ability to fill the grain (Bidinger, 1993). In another study, it has been shown that the dwarf lines have longer heads and more tillers and these characteristics can be effectively exploited by manipulating the genetic background to negate the poorer grain filling ability of *d2* mutants (Rai and Rao, 1991). The *d2* dwarfing gene has been mapped to pearl millet linkage group 4 (LG 4) using an F_2 mapping population derived from the cross IP 18293 X Tift 238D1 (Azhaguvel et al., 2003) and an F_4 segregating population obtained by selfing an F_3 plant from the cross PT 732B X P1449-2 that was heterozygous for most of linkage group 4 (F.K. Padi and K.M. Devos, unpublished).

The objective of this study was to fine-map the *d2* gene and we will report the detailed molecular mapping of *d2* on linkage group 4.

Materials and Methods

Plant Material

Two pearl millet inbred lines ICMP 451 (developed at ICRISAT, India) and Tift 23DB (Coastal Plain Experiment Station, Tifton, GA) were used as parents (Figure 2.1). A cross was made using ICMP 451, the tall parent (*D2D2)*, as male and the dwarf parent, Tift 23DB (*d2d2*), as female in the Plant Biology green houses of the University of Georgia, Athens. Because the *d2* dwarfing gene is recessive, true F_1 plants were selected based on their tall phenotype and hybrid genotype. A single F_1 plant that produced a large number of panicles was selected and selfed to generate F_2 seed. To identify recombination events in the target interval, F_2 plants were grown and analyzed in batches of 100 to 200 plants (Figure 2.2). A total of 1000 plants were grown and 915 plants were genotyped. Recombinant F_2 plants were grown to maturity and selfed, and seeds were harvested. Of 29 recombinant F_2 plants, four did not survive. To confirm the F_2 phenotype at the *d2* locus, F_3 progeny (25 plants/F_2 recombinant) were grown and scored for plant height at maturity (Figure 2.3).

We have also used a second mapping population derived from the cross PT 732B (*d2d2*) x P1449-2 (*D2D2*), which was initially developed to map the pearl millet downy mildew resistance gene, dm2, which is also located on LG4 (Padi, 2002). The mapping population was generated by selfing a single F3 plant that was heterozygous for most of LG4, including the d2 region.

Genomic DNA Extraction

DNA extractions were done from the parental genotypes and from individual F_2 plants using an ultra-quick genomic DNA extraction method (Steiner et al., 1995). Fifty mg of young leaves (3.wk. old) (Figure 2.2) were homogenized in a 1.5 ml eppendorf tube using a power drill equipped with a disposable pestle. The ground leaf material was suspended in 400 µL of pre-warmed (65^{o}C) extraction buffer (200 mM TrisHCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The suspension was mixed with 135 µL of 5 M potassium acetate, incubated on ice for 10 min, and centrifuged at 13,000 rpm for 10 min at 4 $^{\circ}$C. The supernatant was mixed with 0.8 volume of cold isopropanol, incubated at -20 $^{\circ}$C for 30 min, and centrifuged at 13,000 rpm for 15 min. After performing an ethanol (70%) wash, the pellet was dried in a vacuum centrifuge for 4 to 5 min and resuspended in TE buffer (pH 8.0).

Marker Development from the Sorghum Genome Sequence

Comparative mapping has shown that the *d2* region in pearl millet, which is located on LG 4, is orthologous to the distal end of the long arm of rice chromosome 8 (Devos et al., 2000). Taking advantage of the high level of colinearity that exists between cereal genomes, Padi (2002) and Faure (2004) developed PCR-based markers from the distal region of rice chromosome 8 and mapped these to the pearl millet *d2* region. Phylogenetically, pearl millet is more closely related to sorghum and maize than to rice. With the availability of the sorghum genomic sequence, we initiated the development of conserved sets of markers for pearl millet based on the sorghum sequence (http://www.phytozome.net/cgi-bin/gbrowse/sorghum, accessed

on 5 July, 2008). A BLASTn analysis was conducted with the sequence of rice BACs (AP004623) and PACs (P1 Artificial Chromosome) (AP004587, AP005544) (nearly ~ 450 kb from chromosome 8) that were located in a region orthologous to the pearl millet *d2* region against the sorghum database (http://www.phytozome.net/cgi-bin/gbrowse/sorghum). The corresponding sorghum sequence (~ 800 kb region from chromosome 7) was annotated for putative genes using the program FGENESH (http://www.softberry.com/berry.phtml, accessed on 5 July, 2008). All predicted gene models were screened against the TIGR *Poaceae* repeat database (http://www.tigr.org/tdb/e2k1/plant.repeats, accessed on 5 July, 2008) to eliminate known transposable elements. The remaining putative genes were used in a BLASTn search (NCBI) against expressed sequence tags (ESTs) from the Panicoideae members of the grass family (sorghum, sugarcane, maize, pearl millet). Homologous ESTs along with the putative genes were aligned using the multiple sequence alignment program Multalin (Corpet, 1988).

Primer Design

Primer sets were designed to span introns and located within the conserved exons (regions that showed 0-3 nucleotide mismatch between the different *Panicoideae* species) (Figure 2.4). Primer design criteria included the size of the intron (200-1000 bp), melting temperature (58°C -62°C), length of the primers (18-22 bp) and GC content (50-60%).

PCR Conditions

In order to reduce non-specific amplification, touch-down PCR (Don et al., 1991) was used to amplify the templates. Touch-down PCR involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles. The annealing temperature is decreased by 1 °C or by 0.5 °C in every cycle until a specified annealing temperature is reached. This temperature is then used for the remaining number of cycles. This allows for the enrichment of the correct product over any non-specific product.

PCRs were performed using a touchdown (59 °C-52 °C) PCR program with a total of 42 cycles and the following conditions: 94ºC for 30 sec, 59-52 °C for 30 sec (-1 °C/cycle), and 72 °C for 1 minute. Each reaction had a total volume of 20 µL and comprised 2 µL of 50 ng/µL template DNA, 4 µL of 5.0X PCR buffer (Promega), 1.2 µL (1.5-2.0 mM) of $MgCl_2$ (Promega), 0.16 µL (0.2 mM) of dNTP's (exACT Gene) and 0.16 µL 5 U/µL of Promega Taq DNA polymerase.

Size and sequence variation in PCR products were identified by running the products on 3% MetaPhor agarose gels (Lonza) and by using the single strand conformation polymorphism (SSCP) technique, respectively.

SSCP Analysis: Gel Mixture

In order to look for polymorphisms in DNA composition as well as small variations in the size of the amplicons, the samples were run on a MDE™ (Mutation Detection Enhancement) acrylamide gel which displays single strand conformation polymorphisms (SSCP) (Martins-

Lopes et al., 2001). MDE™ gel solution is a polyacrylamide-like matrix that has a high sensitivity for DNA conformational differences. DNA is separated on the basis of both size and conformation thus increasing the potential to detect variation compared to standard polyacrylamide gels (Soto and Sukumar, 1992), The gel mix consists of 15 ml of MDE^{TM} gel solution (LONZA), 3 ml of 10X TBE, 24 µL of TEMED (tetramethylethylenediamine) (SIGMA), 240 µL of 10% APS (ammonium persulphate) in a total volume of 60 ml. PCR products were mixed with formamide dye (24% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol) in the ratio of 2:1 (PCR product: formamide dye). The mixture was denatured for 5 minutes, the samples were loaded and the gels (43 x 35 cm) were run overnight under non-denaturing conditions at room temperature at 8W.

Staining Procedure

When the loading dye has migrated over the desired distance, the gel system is taken apart. The gel is fixed (200 ml of acetic acid and 1800 ml of water) for about 30 to 45 min and then rinsed with distilled water (2 litres) for 15 min. Fixation is important to enhance stain sensitivity as it immobilizes the DNA molecules in the gel matrix to avoid diffusion and subsequent image blurring. Fixing neutralizes buffer and other chemical residues, which can interfere with staining (Bassam and Gresshoff, 2007).

The second step involves the staining of the DNA with silver stain solution. It consists of 12 ml of 1 N silver nitrate and 3 ml of formaldehyde in 2 L of distilled H_2O. The stain is added to the gel and the gel is gently shaken for 30 min. The gel is rinsed with distilled water for 10 sec before developing. The developing solution consists of 60 g of anhydrous sodium carbonate in 2

L of distilled H_2O, cooled to 4°C to which 300 µL of sodium thiosulphate (0.1 N) and 3 ml of formaldehyde (40%) are added immediately prior to adding the developer to the gel. The gel is developed until the DNA fragments are clearly visible. The gel is rinsed in H_2O, dried and scored.

Mapping

Initially, all markers were screened on the parents of the mapping population and the markers that were polymorphic were employed on the entire population. The genotype of each progeny was recorded. The frequency of recombination events was used to calculate the genetic distance between markers.

Phenotyping

Dwarf *d2d2* plants were indistinguishable from tall *D2D2* and *D2d2* plants until flowering and the difference in height can be observed only after anthesis. So, the phenotypic data was collected only after the plants had matured. Plant height and other parameters like height of the top node, peduncle length and panicle length were measured for each of the 25 recombinant F_2 plants using a 1 m ruler. The parents, ICMP 451 and Tift 23DB, differ also in the length of the bristles present on the panicles, and the presence or absence of long bristles was recorded for all the informative recombinants. As it is not possible to differentiate F_2 plants that are homozygous for the tall *D2* allele from those that are heterozygous *D2/d2*, the allelic composition at the *D2* locus for each F_2 was confirmed by growing 25 F_3 seeds per recombinant

F_2 plant. The same phenotypic data were collected for the F_3 plants as for the F_2 plants (see appendix-1).

Statistical Data Analyses

The data were statistically analyzed using the analysis of variance procedures of the statistical analysis system (SAS version 9.1). Duncan's multiple range test was used to do the mean comparisons.

Results

In an effort to fine-map the *d2* locus and to precisely determine the genetic distance between the molecular markers that most closely flanked *d2*, the PCR-based markers, B224C4P2 (BAC end sequence) and PSM305 that had previously been shown to flank *d2* (Figure 2.5), were selected to genotype a larger F_2 population that was generated from the cross ICMP 451 (*D2D2)* x Tift 23DB (*d2d2*). Although PSM344 was more closely linked to *d2* than PSM305 (Figure 2.4) and detected variation between the parents of the mapping population, the PSM344 polymorphism was dominant. So the use of the more distantly located PSM305 marker was preferred for the identification of plants that had undergone recombination in the *d2* region. Genotyping was done for 915 plants using the SSCP technique (Figure 2.6) and 29 plants were found to carry a recombination event in the target region (Table 2.1). This gave us an estimate of 1.6 cM for the B224C4P2 – PSM305 interval that spans *d2*.

Fine Mapping of Markers Previously Mapped to the d2 Region

Markers R1963, 364H23-4, R8-1-5, and PSM344 that had previously been shown to be located in the B224C4P2 – PSM305 interval (Figure 2.5) were polymorphic in the ICMP 451 x Tift 23DB population and mapped using the selected set of informative plants. R1963 co-segregated with 364H23-4. A total of three recombination events were identified between markers B224C4P2 and R1963/364H23, and an additional recombination event was identified between the latter two markers and R8-1-5. R8-1-5 was separated from PSM344 by 6 recombination events, and PSM344 and PSM305 were separated by 13 recombination events. The corresponding map is presented in Figure 2.7.

It had previously been reported that the markers PSR492 and R1963 were present on BAC 293B22 (Padi, 2002) (Figure 2.5). BAC 293B22 has since been sequenced (A.C. Pontaroli and J.L. Bennetzen, unpublished data), which has revealed that this BAC contains three genes including R1963 but not PSR492. R1963 is an amino peptidase N, and the other two genes encode a putative ripening regulated protein (RRP) and a putative C2 domain-containing protein. The BAC end clone B224C4P2 was also present on this BAC. None of the genes present on BAC 293B22 are likely candidates for *d2*. RRP was mapped as a dominant marker and cosegregated with R1963 (Figure 2.7). From the BAC sequence, we know that the order of the markers and genes present on BAC 293B22 is B224C4P2, RRP, R1963, and the putative C2 domain-containing protein. In order to further saturate the target region with markers, new markers were developed from the sorghum sequence.

Utilizing the Synteny between Pearl Millet and Sorghum to Develop New Markers for the d2 Region

Comparative maps have been established between pearl millet and rice (Devos et al., 2000) but so far no markers have been mapped in pearl millet using the sorghum sequence. A BLASTn search with markers R1963, 364H23-4, R8-1-5 on LG4 in pearl millet against the sorghum genomic sequence was conducted and all the markers gave significant hits to a region of chromosome 7 (Table 2.2), identified as being orthologous to the *d2* region. As we know that high levels of colinearity exist among the grass species, several primers were designed against 40 putative genes annotated in this region of the sorghum sequence (see materials and methods). Among the markers developed, 30 (75%) amplified well with the genotypes ICMP 451 and Tift 23DB, 10 were polymorphic (25%) (Table 2.3) and, of these, five markers (MDH, *Br2/Dw3*, 8567, 1264, and Gene44) (Table 2.4) mapped to the *d2* region (Figure 2.8). Even though the other six markers were polymorphic, the genotypic scores indicated that they were located elsewhere in the genome The *Br2/Dw3* locus was mapped as a dominant marker (Figure 2.9). Several markers (*Br2*, Gene14, R8-1-5) gave a spurious band in line 184, one of the 25 informative F_2 plants, and this line was not further considered in the analysis. Since the recombination event between R8-1-5, and R1963 and 364H23-4 was identified in line 184 (Figure 2.7), R8-1-5, R1963 and 364H23-4 were now considered to cosegregate (Figure 3.0. a). No recombination events were noticed between these markers and the newly developed sorghum markers, Gene 44, MDH, Br2/Dw3, 8567, and 1264 (Figure 3.0 b). Because of lack of recombination in the target region, a second available population derived from the cross PT 732B X P1449-2, was employed.

Fine Mapping of d2 Using a Second Mapping Population

As no recombination events were identified between the markers R1963, RRP, MDH, R8-1-5, 364H23-4, Gene44, 1264, 8567, and Br2/dw3 in the ICMP 451 x Tift 23DB fine-mapping population, we attempted to map these markers on a set of 14 F_4 genotypes selected from a mapping population of 554 individuals from a cross between PT 732B (d_2d_2) and P1449-2 (D_2D_2) that were recombinant for the B224C4P2 – PSM344 interval (Padi, 2002; Faure, 2004) (Table 2.5). The markers fell into two groups. Gene44, R1963, MDH, and PLATZ, a marker that was monomorphic in the ICMP 451 X Tift 23DB population, cosegregated with B224C4P2. The markers 1264, 364H23-4 and R8-1-5 also cosegregated. The remaining markers were monomorphic in the PT 732B X P1449-2 population. The two groups of markers were separated by two recombination events, which translated into a genetic distance of 0.2 cM (Figure 3.1).

Colinearity of the d2 Region in Pearl Millet with the Orthologous Regions in Sorghum and Rice

Despite the lack of recombination, combining the mapping data from the two crosses with the sequence information from BAC clone 293B22 indicates that the order of the markers is PSR492 - B224C4P2 - RRP - R1963 - putative C2 domain-containing protein – MDH & Gene44 & PLATZ – 364H23-4 & R8-1-5 & 1264 – PSM344 – PSM305. Markers separated by '&' cannot be ordered based on the current information. Markers 8567 and Br2/dw3 belong to either the MDH/Gene44/PLATZ or the 364H23-4/R8-1-5/1264 cluster. No sorghum or rice homologs

were identified for markers B224C4P2, PSM344 and PSM305. Comparing the order of the remaining markers in the pearl millet d_2 region and in the corresponding sorghum and rice regions indicates the presence of a rearrangement that differentiates the pearl millet genome from those of sorghum and rice (Figure 3.2). Considering that sorghum is more closely related to pearl millet than to rice, we can conclude that this inversion must have taken place in pearl millet or in the lineage that give rise to pearl millet. Previous comparative studies have indicated that the sorghum genome is less duplicated and less disturbed by local rearrangements relative to rice compared to maize (Ahn and Tanksley, 1993; Wilson et al., 1999) or sugarcane (Dufour et al., 1997). The rice genome has also been demonstrated to be a relatively stable genome (Ilic et al., 2003). Comparison of the organization of the genomes of rice, foxtail millet, sugarcane, sorghum, pearl millet, maize, wheat and oat have shown that pearl millet, on the other hand, has undergone many chromosomal rearrangements (Devos et al., 2000). The rearrangement in the *d2* region involves a relatively small region and had not previously been observed in the comparative studies carried out at the map level.

Precise Mapping of d2

To place *d2* on the molecular map, phenotypic data is essential. As it is not possible to differentiate F_2 plants that are homozygous for the tall *D2* allele from those that are heterozygous *d2/D2*, plant height was measured in F_3 families to establish the genotype at the *d2* locus in the informative F_2 plants (25 plants per recombinant, derived by selfing each recombinant F_2 plants) (Table 2.6 and Appendix-1). One F_2 line (line 184) had already been discarded because of the amplification of non-parental alleles with some markers. A second line, plant 417, had purple

leaves and stems, a trait that was not present in either of the parents. Because some purple millet genotypes (Tift 23Red) were growing in the glasshouse at the time the informative F_2 plants were selfed, the logical explanation is that the F_2 progeny was cross-pollinated by Tift 23Red rather than self-pollinated. Panicles for selfing were bagged prior to emergence of the stigma, but bagging may have happened too late to prevent cross-pollination in the case of line 417. All 'F_3' progeny from plant 417 were tall, while the other markers in the *d2* region were heterozygous. Assuming that the plant 417 was heterozygous at the *d2* locus, outcrossing with a *D2D2* Tift 23Red plant would result in progeny that are either homozygous *D2D2* or heterozygous *D2d2* tall. The data thus fits with the hypothesis that the progeny derived from 417 are the result of outcrossing of line 417 with Tift 23Red, which is tall. Therefore, out of 25 F_2 recombinants, phenotypic data of two recombinants, lines 184 and 417, were eliminated and 23 recombinants were considered for the final analysis. The plant height for each of the $F_{2:3}$ plants, with the exception of those plants that were weak and did not grown well, and the derived genotype at the *d2* locus for the corresponding F_2 plants are given in Table 2.6. The ratio of tall and dwarf F_2 recombinants ($\chi^2 = 0.36$, $P>0.7$) and $F_{2:3}$ progeny ($\chi^2 = 2.02$, $P>0.2$) fits the expected 3:1 ratio for a single locus (Table 2.7 a) and also the genotypic ratio of 1:2:1 ($X^2 = 0.12$, *P> 0.9)* (Table 2.7 b).

Integration of the height phenotypic data with the genotypic data showed that the *d2* locus cosegregated with the marker cluster comprising B364H23-4, R8-1-5, and 1264. The physical location of d_2 can thus be narrowed down to the interval spanned proximally by the markers MDH/Gene 44/ PLATZ marker cluster and distally by PSM 344 on LG4. At this stage, it is not possible to further refine the map position of *d2*.

Potential Candidate Genes for d2

Although we have not yet fully defined the boundaries of the pearl millet *d2* region in sorghum, the syntenic sorghum region used for marker development in our study was analyzed for potential candidate genes for *d2*. The best candidate gene is *dw3*, which encodes a class of P-glycoprotein and has been shown to affect plant height in both maize (*Br2,* the maize *dw3* homolog) and sorghum (Multani et al., 2003). This candidate gene cosegregated with *d2* (Figure 2.9). Dwarfness in *Br2* and *dw3* mutants results from the loss of a P-glycoprotein that alters polar auxin transport in the maize stalk. Interestingly, the mutant phenotype could not be reversed by hormones like GAs, brassinosteroids or auxins, which shows that the mutation is not involved in the biosynthesis of these growth regulators. The *Br2* mutation results in shortening of the lower stalk internodes and no other plant parts (coleoptile, leaves, ear) are significantly affected. *d2* in pearl millet also reduces the internodal length without affecting the other plant parts like leaves. In a study by Devi et al., (1994), an effect of *d2* on coleoptile length was not observed as there was no significant difference in coleoptile length between dwarfs and tall plants. The phenotypic similarities and the orthology of the *dw3* region in sorghum with the *d2* region in pearl millet make *dw3* a potential candidate for *d2*. No other obvious candidate genes were found in the sorghum genomic sequence analyzed.

PCR Screening of a BAC Library with Markers that are Linked to d2

It was mentioned in the previous sections that a BAC library from the pearl millet variety Tift 23DB is available (Allouis et al., 2001). A partial BAC contig had previously been established for the d_2 region (Figure 2.5). We were interested to screen the BAC library with the markers that were newly mapped to the d_2 region so that we could establish their physical location in or in relation to the existing contig. In first instance, we screened the BAC super pools (each super pool contains 3840 individual BAC clones) with the markers R1963, B364H23-4, PSM344 and 1264 to potentially find overlapping clones. R1963, B364H23-4 and PSM344 had previously been hybridized to the BAC library (Faure, 2004) and the positive clones identified by hybridization were contained within the positive superpools identified by PCR. Unfortunately, none of these BACs spanned *d2* as none carried a marker from each side of *d2* (Figure 3.3). Marker 1264 identified superpools 16, 21, 23, 24, 27, 33, 35 and 42), when further tested on the pools, pools 201, 228, 263, 349, 414 and 417 were shown to contain marker 1264. None of these pools had previously been identified with any of the markers that mapped to the *d2* region. Additional markers are needed to generate a contig for the *d2* region. Alternatively, we can skim the sequence of the BAC clones and develop new markers from the BAC sequence for genetic mapping.

Effect of Bristle Length, Peduncle Length, and Panicle Length on Plant Height

In addition to plant height, we have also measured peduncle length, panicle length and recorded the presence or absence of long bristles on the panicle. The height of plants was

significantly affected by the presence of long bristles (Table 2.8). The difference in plant height between plants with long and short bristles is significant at $P<0.01$ and was evident in both the D2D2 and d2d2 genotypes. Tall plants with long bristles are 17.5% (batch I) and 9.5% (batch II) taller than the plants with short bristles. A similar effect was noticed on dwarf plants, which are 23% (batch I) when they have long compared to short bristles(Table 2.8). A similar pleiotropic effect of bristle length on plant height had previously been observed in homozygous dwarfs carrying the *d4* dwarfing gene (FK Padi and KM Devos, unpublished). The gene that controls bristle length is located on pearl millet LG 1 and it has been shown that this gene significantly ($P<0.001$) enhances the height of pearl millet plants that are homozygous for the *d4* dwarfing gene, but not of tall plants (FK Padi and KM Devos, unpublished). Our current data suggest that this gene has a pleiotropic affect on plant height, which warrants further study.

It is always interesting to see how traits such as plant height, peduncle length, and panicle length are associated with each other. Our preliminary phenotypic data suggest that there is a positive correlation ($P<0.0001$) between these parameters at the maximum growth stage of the plant (Table 2.9). We have also analyzed the data for the peduncle and panicle length. The mean peduncle length was significantly different ($P<0.01$) between the tall and dwarf plants (Table 3.0). Our data also show that dwarf plants (*d2d2*) with bristles have significantly (*P<0.01)* longer peduncles than dwarf plants shorter or no bristles (Table 3.1). With respect to panicle length, irrespective of tallness or dwarf, the presence of long bristles as a significant affect on panicle length (Table 3.1). So this data suggest that the bristle gene has pleiotropic effects on height, peduncle length and panicle length. All the analyses were done separately for the two sets of plants that were grown in different conditions (Batch I and II). No significant difference was observed for the dwarf plants in Batch II between long bristle and short bristle plants regarding

height, peduncle, and panicle length (Table 2.8 and Table 3.1). This could be attributed to the small sample size of dwarfs in Batch II. Thus our preliminary results show that the bristle gene could pleiotropically affect many characters.

Discussion

Even though plant height is generally a simply inherited trait in pearl millet, so far not many studies have been reported that involved the mapping of the genes that regulate plant height in this species. In 2003, it was reported that the *d2* gene mapped between two RFLP markers that were separated from *d2* by a genetic distance of 6.8 cM and 16.4 cM (Azhaguvel et al., 2003). In a study by Faure (2004), *d2* had been mapped to a region of ~2 cM and a partial contig was developed for the region. Unfortunately, the gene could not be mapped precisely because *d2* was scored as a dominant marker and phenotypic data for the critical recombinant plants was not available.

In this study, *d2* has been shown to cosegregate with the marker cluster of R1963, MDH, Gene 44, 364H23, R8-1-5, and 1264. With the data from the the two crosses, the physical location of d_2 can be narrowed down to the interval spanned by the MDH/Gene 44/PLATZ marker cluster (proximal side) and PSM 344 (distal end) on LG4. Marker development was facilitated by the availability of the complete genome sequence of the major cereal species rice and sorghum, the partial sequence of maize (~80%) and the availability of hundreds of thousands of grass EST sequences.

Comparative analyses of cereal genomes at the macro (map) level, the results of which have been synthesized in the form of the "Crop Circles" (Devos, 2005), have demonstrated that

LG 4 in pearl millet is orthologous to rice chromosome 8 and sorghum chromosome 7. Orthology between pearl millet LG 4, rice chromosome 8 and sorghum chromosome 7 was confirmed in our analysis. However, the *d2* region corresponds to the distal region of the long arm of rice chromosome 8, and synteny with rice chromosome 8 breaks down at the end of the chromosome. Colinearity at the microlevel can be investigated by sequencing the target region (*d2*) in pearl millet and comparing the orthologous *d2* region in sorghum (chromosome 7) and rice (chromosome 8). This will provide information on the conservation of gene content and order and may also uncover deviations like deletions, duplications and other rearrangements that may have taken place in pearl millet relative to sorghum and rice in addition to the inversion we have described earlier. .

All markers that were mapped between 224C4P2 and PSM344 in the progeny of our first cross, ICMP 451 x Tift 23DB, cosegregated (Figure 3.0 b). Recombination was identified only between 224C4P2 and the marker cluster, and between the marker cluster and PSM344. These markers were found in a region comprising around 800 kb in the distal region of sorghum chromosome 7. Nearly 40 confirmed genes (based on a BLASTx search) were found in this region. This suggests a gene density of around one gene per 20 kb in the orthologous region of *d2* in sorghum. For small genomes like arabidopsis (~125 Mb) and rice (~430 Mb) , the average gene density is 1 gene/ 4-5 kb (TheArabidopsisInitiative, 2000) and 1 gene/10 kb (Matsumoto et al., 2005) . Since the sorghum genome (~735Mb) (Bedell et al., 2005) is nearly two times bigger than the rice genome and the length of the intergenic regions is correlated with genome size (Bennetzen et al., 1998), the gene density for sorghum in the *d2* orthologous region may not be unexpected. Considering that the pearl millet genome is larger than the sorghum genome, and that *d2* is located interstitially on pearl millet LG 4, one would expect the physical size of the

pearl millet region to be larger than the orthologous sorghum region. However, because we do not have a contig spanning the *d2* region, at this point we cannot predict the gene density or recombination rate in this region of pearl millet. It is known that gene density and recombination rate vary from region to region and from species to species. For example in the *Adh1* / *yu22* region, the genes were separated by more than 120 kb in maize, whereas the orthologous genes in sorghum were only 50 kb apart (Bennetzen et al., 1998). Repeated elements accumulate in the intergenic regions in large genome species, and pearl millet has a genome size similar to that of maize. It would be interesting to look at the organization of the *d2* region in pearl millet and its orthologous regions in rice, sorghum and maize.

Although no recombination was observed in the *d2* region between the newly developed markers (Figure 3.0 b) in the ICMP 451 x Tift 23DB mapping population, it is likely that critical cross-overs may be found in this region if the size of the mapping population is increased. Since *d2* is located interstitially on LG 4, but outside the centromeric marker cluster, one would not expect extensive suppression of recombination in this region. In pearl millet, the ratio of physical distance (haploid genome size ~2400 Mb) to genetic distance (~473 cM) is approximately 5 Mb/cM. Suppressed recombination can be a major limiting factor for isolating a gene because crossovers play a major role in delimiting the target region (Alpert and Tanksley, 1996). For example in tomato, the gene *Tm-2a* which confers resistance to tobacco mosaic virus is located in the centromeric region and has a very high ratio of physical to genetic distance of about 4 Mb/ cM (Ganal et al., 1989). A similar problem was encountered in the *Mla* region in barley where the lack of recombination resulted in a physical to genetic distance ratio of 5 Mb/ cM (Wei et al., 1999).

Future Work to be Done

Based on our results, future work should be initiated in two directions. Firstly, the number of progeny that are to be analyzed needs to be extended so that recombinants can be obtained in the *d2* region. The development of a genetic map with a resolution of 0.05 cM (~250 kb) could possibly delimit the physical size of the *d2* region to two to three BAC clones. Secondly, it would be interesting to study the expression of the genes that were mapped in the *d2* region, in particular of the *Br/dw3* gene that is a good candidate for *d2*. RT-PCR can be done to look at the level of transcript of these genes in the wild type and dwarf mutant at specific development stages of the plant. It has been reported that the dwarf phenotype in *d2* lines is expressed only during a short span in the life cycle of the plants, and is observed only in the stem internodes at the time of flowering (Devi et al., 1994).

Summary

The research work reported in the previous chapter had the objective of developing a high-density genetic map of the *d2* region in pearl millet. The project was initiated with a low density map and markers developed from the sorghum genomic sequence allowed the fine-mapping of the *d2* region on pearl millet LG 4. We have identified a potential candidate gene, *Br2* / *dw3,* for *d2* from the sorghum genomic sequence. Mutations in *dw3* and *Br2* cause altered growth and a dwarf phenotype in sorghum and maize, respectively. In the present study, the physical location of d_2 can be narrowed down to the interval spanned by the MDH/ Gene

44/PLATZ marker cluster (proximal side) and PSM 344 (distal end) on LG4. It also demonstrates the application of the completed sequence of sorghum as a tool for marker development in pearl millet. A similar approach can be utilized for other species like foxtail millet (*Setaria italica)* and tef (*Eragrostis tef*) as sorghum is more closely related to the genomes of these crops than to that of rice. Our data based on the position of markers from the sorghum sequence shows that rearrangements exist between the genomes of pearl millet and sorghum. Our preliminary data also suggests that the gene that controls bristle length has some pleiotropic effect on plant height, peduncle length, and panicle length, which requires further study.

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Table 2.1. F_2 progeny (ICMP 451 x Tift 23DB) that carried a recombination event in the *d2* region between markers B224C4P2 and PSM305

Plant ID	B224C4P2	PSM305
1	B	H
22*	A	H
55	B	H
177	B	H
184	B	H
188*	B	H
263	A	H
310	A	H
320	A	H
344	B	H
349	A	H
374	B	H
417	H	B
429*	A	H
435*	B	H
477	B	H
479	H	A
486	H	A
496	B	H
514	H	A
612	A	H
778	A	H
787	H	A
900	A	H
914	H	A
924	A	H
930	A	H
701	A	H
812	A	H

* Plants did not survive

Table 2.2. BLASTn hits against the sorghum genomic sequence using pearl millet markers as query sequences

Marker	Homologous hits from sorghum genome	Score
R1963	Chromosome-7	137.3
364H23-4	Chromosome-7	65.9
R8-1-5	Chromosome-7	230.4

All hits were selected based on a e-value <e-8

Table 2.3. Primers designed based on genes annotated in the sorghum sequence and used for marker development in pearl millet

Name of the oligo	Putative gene description	Forward primer	Reverse primer
ABC	ABC transporter protein	GTCAGGCTCAGAGCAAAGAG	ACTGGTGGTGGAAGTTTGCC
Br2/Dw3	P-glycoprotein	ACTTCACCGTCTTCTGCTGC	TCCACCCGCCCCGTCACC
CKFP	Protein kinase family protein	GCTTTAGACGGTGCAGAC	GAAGCTCATCCAATCCCTC
PGRP	Proton gradient regulatory protein	AGCTTCAGCTCCGTGCTTC	GGAATCCGAGCTTCTCTCC
PMP	Membrane protein	GCATCTGTCCAACTGACAGC	ATCTTGCTGCCTTGAGGTTC
Gene14	Antimicrobial protein	AGCCCTTCATCGGCCACC	CCCAGCATGTGGTGCTTC
8567 *		CGCCTTCGACATGGACGAC	CTTGTCGTGGCGGTAGTCG
1264 *		TGATAAACCCAGCAACCTTGG	GAATCAAGTACACAGCTATGC
PLATZ	Transcription factor	CCACGCGCTTCTTCCTCC	TCCTCCACCTCGGACACG
MDH	Malate dehydrogenase	CATGCACTGACGAGGTTGG	TGCCCCATTTTTGGATGAGC
Gene 44*		CTTGGCTCACCATGTGGTG	GACTCTAGTTCTGCTTGCAG

*Hypothetical protein (no significant putative proteins are available in the NCBI protein database)
Only the primers/genes that are polymorphic are listed
The marker PLATZ, which is highlighted, is polymorphic only in the cross PT 732B x P1449-2. The remaining markers are polymorphic in the cross ICMP 451 x Tift23DB.

Table 2.4. Markers developed from sorghum chromosome 7 and mapped in pearl millet LG 4

Marker	Sorghum chromosome	Contig	Position in sorghum genome	EST matches
Br2/Dw3	7	108	58614142 - 58618660	Maize- EE175874 Sugarcane- CA212954
8567	7	108	58373734 - 58374233	Maize- EE172898 Switch grass-DN151153
1264	7	108	58372032- 58372570	Sorghum - CN124670 Sugarcane- CA281489 Maize- DT535275
PLATZ *	7	185	59039335-59040429	Maize-C0444334 Switch grass-FE621230 Sorghum-BG053672
MDH	7	185	589408226 - 58942027	Sorghum- CX612351 Sugarcane- CA243590 Maize- BG321073
Gene44	7	185	58783308- 58784010	Sugarcane - CA065035 Maize - CF630212 Sorghum - CN139107

*Marker mapped in second mapping population of PT 732B x P144-2

Table 2.5. Progeny from the cross PT 732B x P1449-2 that carried a recombination event in the *d2* region between markers B224C4P2 and PSM344

Plant ID	B224C4P2	PSM344
18	H	B
20	H	A
24	H	A
134	H	A
384	A	H
388	H	B
390	A	H
494	B	H
510	A	H
519	H	A
602	H	A
666	B	H
679	H	A
695	H	A

Table 2.6. Mean plant height of $F_{2:3}$ progeny (see Appendix 1 for the height measurements of all F2:3 plants)

Plant ID	*D2* Genotype	Height (cm)	Height range	Std.Err
1	*d2d2*	87.8 (n=24)	64-109	2.9
55	*D2d2*	134.6 (n=20)	70-156	6.7
177	*d2d2*	63.9 (n=24)	37-89	2.6
263	*D2D2*	108.5 (n=25)	83-136	3.3
310	*D2D2*	155 (n=25)	117-194	4.6
320	*D2D2*	151 (n=24)	120-188	4.3
344	*d2d2*	86.3 (n=22)	64-115	3.6
349	*D2D2*	100.8 (n=25)	81-132	2.9
374	*d2d2*	82.8 (n=24)	50-108	3.7
477	*d2d2*	64.8 (n=25)	40-102	3.2
479	*D2D2*	153 (n=22)	95-204	6.3
486	*D2d2*	109.5 (n=24)	56-168	6.2
496	*D2d2*	136.1 (n=23)	73-171	5.7
514	*D2d2*	129.8 (n=21)	79-200	7.7
612	*D2D2*	150.3 (n=23)	108-200	5.1
778	*D2D2*	150.1 (n=17)	125-201	5.9
787	*D2d2*	160.2 (n=15)	124-200	4.2
900	*D2D2*	180.4 (n=13)	145-215	7.4
914	*D2d2*	201 (n=15)	127-260	11
924	*D2D2*	186.4 (n=22)	151-200	5.3
930	*D2D2*	204.5 (n=18)	130-260	8.6
701	*D2D2*	179.8 (n=15)	158-224	6.3
812	*D2D2*	203.1 (n=17)	150-230	5.6

Plant ID = F_2 recombinant plant

Table 2.7. Determining the goodness of fit of the segregation data to Mendelian ratios using a chi-square test

(a) Based on phenotypic data (tesing for 3:1 segregation of talls:dwarfs) of the $F_{2:3}$ plants

Cross	Population	Number of Talls (*D2D2* and *D2d2*)[1]	Number of Dwarfs (*d2d2*)[1]	Total	X^2 *
ICMP451 X Tift23DB	F_2 recombinants	16	7	23	0.36
	F_2:F_3	334	129	463	2.02

[1]Weak plants were not included in the analysis.
*The observed chi-square test statistic with 1 df (2 classes -1) is well within the range of expected 3:1 segregation (tall: dwarf) ratio

(b) Based on genotypic data (testing for 1:2:1 segregation of *D2D2:D2d2:d2d2*) of the F_2 plants

Cross	Population	*D2D2*	*D2d2*	*d2d2*	Total	X^2 *
ICMP 451 X Tift23DB	F_2	12	6	5	23	0.128

*The observed chi-square test statistic with 2 df (3 classes -1) is well within the range of expected 1:2:1 segregation (DD: Dd: dd) ratio in the genetic cross

Table 2.8. Mean plant height (cm) of the tall and dwarf plants with long and short bristles (BB= Genotypes with long bristles, bb=Genotypes with short bristles)

	Tall (*D2D2* and *D2d2*)		**Dwarf (*d2d2*)**	
	BB/Bb	**bb**	**BB/Bb**	**bb**
***Batch I**	149 [a] (n=106)	123 [b] (n=50)	85.5 [a] (n=52)	66.2 [b] (n=59)
Batch II	179.3 [a] (n=84)	163 [b] (n=69)	80.7 [a] (n=9)	74.1 [a] (n=9)

[a]Means for the plant height within a row for tall or dwarf indicated with different letters are significantly different at the 0.01 level.

*Plants were grown in different batches meaning at different times and different conditions

Table 2.9. Correlation coefficients (r) for plant height, peduncle length, and panicle length at the maximum growth stage (before maturity) of pearl millet

Trait	**Peduncle length**	**Panicle length**
Height	0.53 ***	0.34***
Peduncle length		0.31***

Pearson correlation coefficients, N=477
*** Correlation Coefficient (r) is significant at < 0.0001 level

Table 3.0. Mean peduncle length (cm) and panicle length (cm) of tall and dwarf lines

Character	**Mean**	
	Tall (*D2D2/D2d2*) (n= 316)	**Dwarf** (*d2d2*) (n=161)
Peduncle length, cm	25.4 [a]	21.4 [b]
Panicle length, cm	15.3 [a]	12.7 [b]

[a] Means for the peduncle length and panicle length within a row for tall and dwarf indicated with different letters are significantly different at the 0.01 level.

Table 3.1. Mean peduncle length (cm) and panicle length (cm) of tall and dwarf lines with long and short bristles (BB= Genotypes with long bristles, bb=Genotypes with short bristles)

	Trait/ Character	Tall (*D2D2/D2d2*)		Dwarf (*d2d2*)	
		BB/Bb	**bb**	**BB/Bb**	**bb**
Batch I	Peduncle length	24.4 [a] (n=106)	23.5 [a] (n=50)	22.5 [a] (n=52)	18.9 [b] (n=59)
Batch II	Peduncle length	28.0 [a] (n=84)	26.6 [a] (n=69)	22.3 [a] (n=8)	19.0 [a] (n=9)
Batch I	Panicle length	15.5 [a] (n=106)	13.0 [b] (n=50)	14.7 [a] (n=52)	11.1 [b] (n=59)
Batch II	Panicle length	16.1 [a] (n=84)	14.4 [a] (n=69)	11.0 [a] (n=8)	11.3 [a] (n=9)

[a] Means for the peduncle length and panicle length within a row for tall or dwarf and batch I or batch II indicated with different letters are significantly different at the 0.01 level.

Figure 2.1. Dwarf (Tift 23DB) and tall parents (ICMP 451) used to establish the mapping population

Figure 2.2. F_2 progeny derived from the cross ICMP 451 X Tift 23DB (two weeks old plants used for DNA extractions)

Figure 2.3. $F_{2:3}$ progeny that were phenotyped for plant height

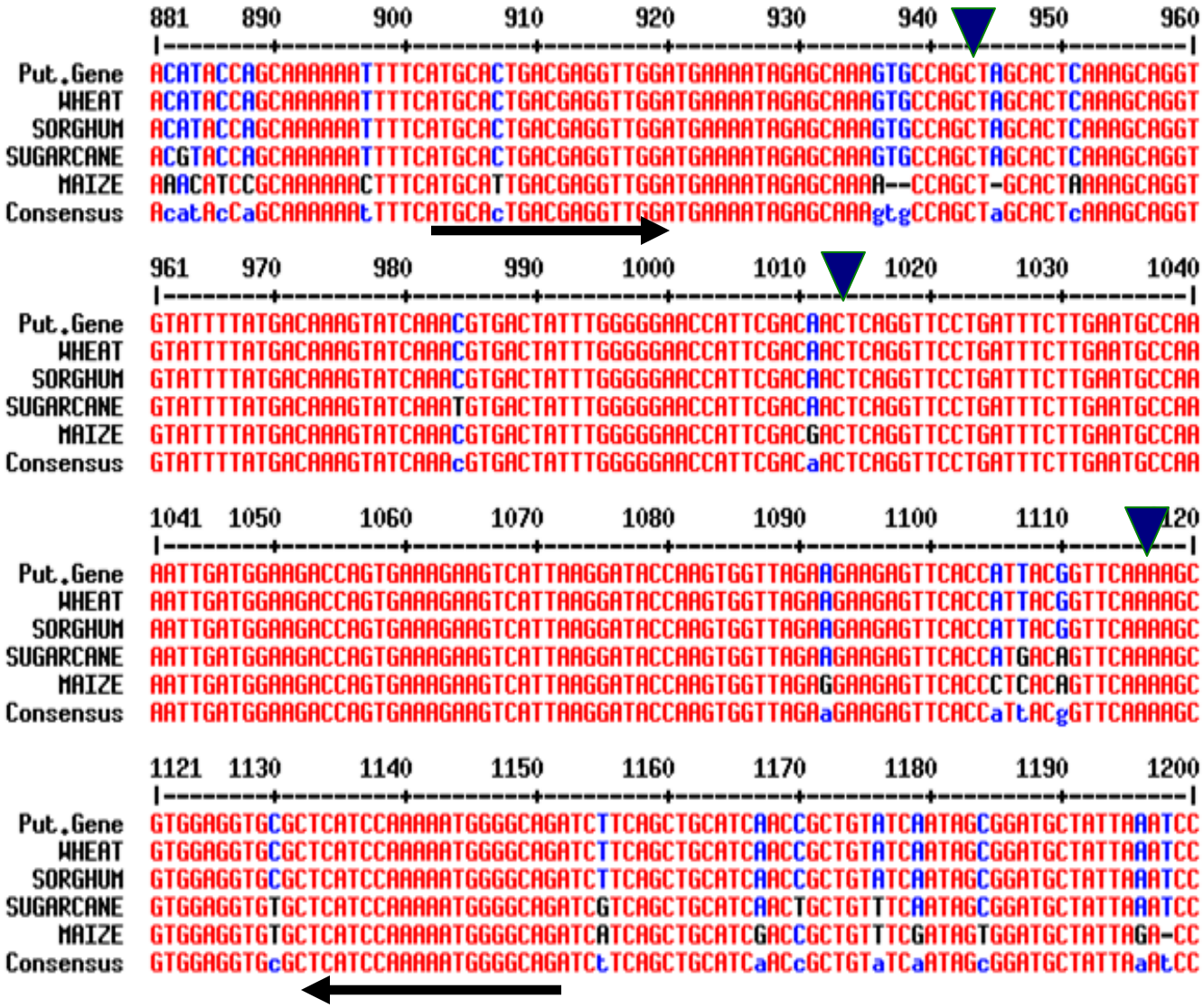


Figure 2.4. Primer sets were designed against conserved regions in exons and spanned, where possible, one or more introns. Highly conserved regions were identified by aligning homologous ESTs of several cereal species using the program Multalin (Corpet, 1988)

▼ Position of Introns

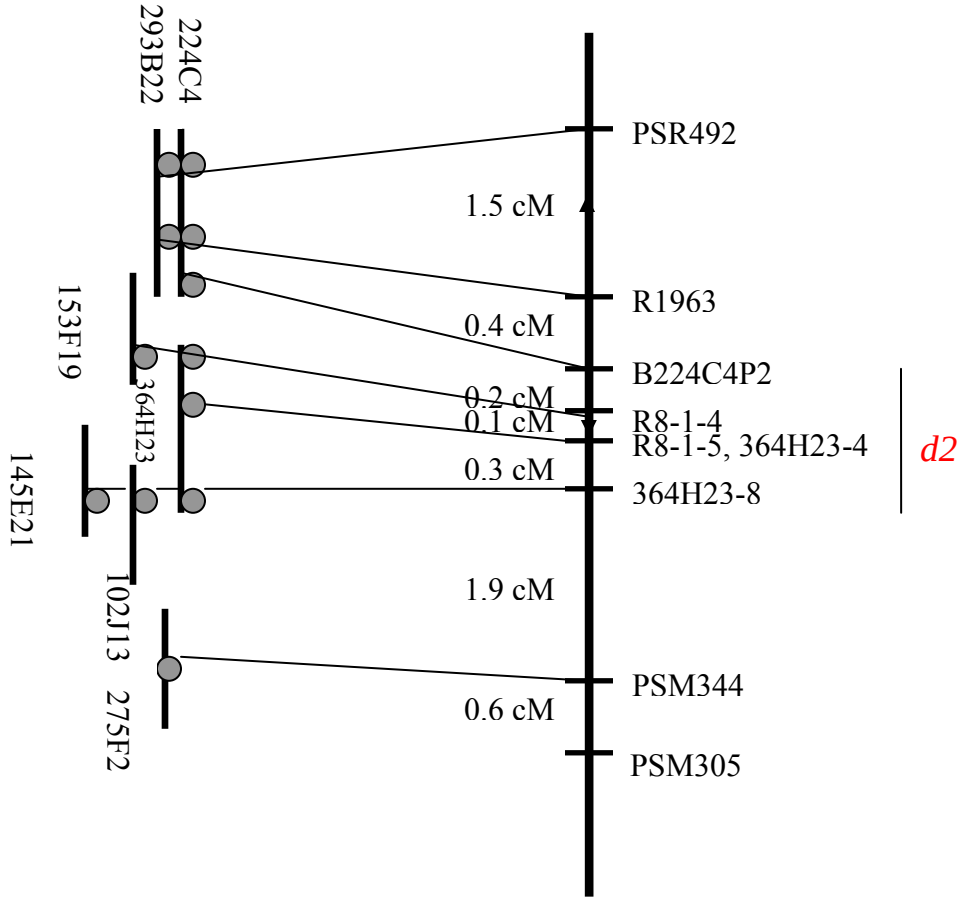
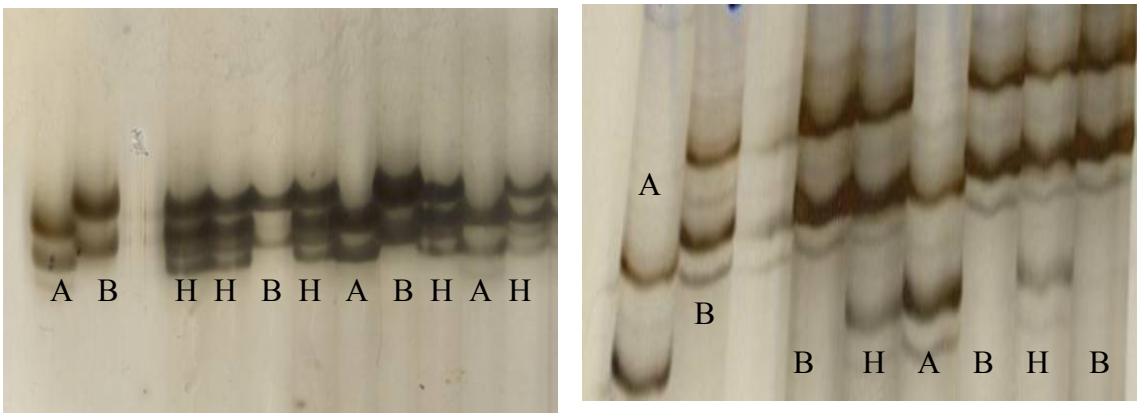


Figure 2.5. Genetic map and partial physical map of the *d2* region on pearl millet LG4 (Faure, 2004)



P1 P2 F_2 progeny P1 P2 F_2 progeny

Marker B224C4P2 Marker PSM305

P1= ICMP 451, P2= Tift 23DB

Figure 2.6. Genotyping of the mapping population with markers B224C4P2 and PSM305 using the SSCP technique

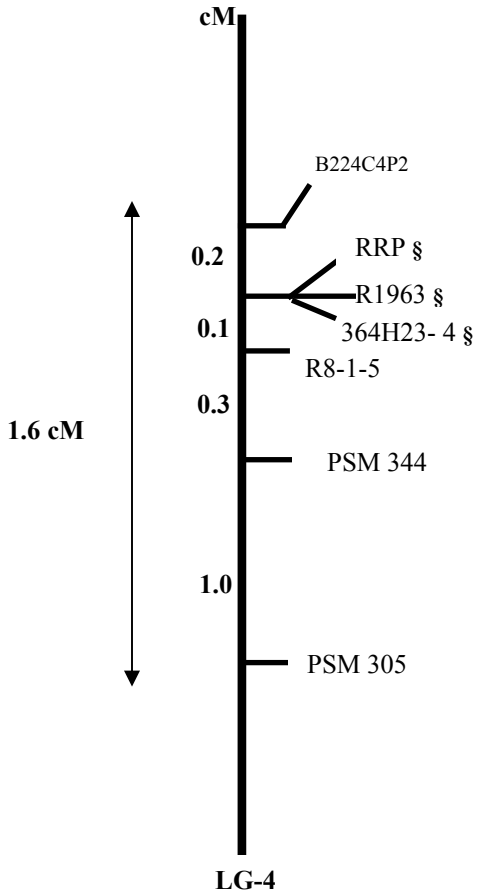


Figure 2.7. Genetic map of the *d2* region on linkage group 4 showing the location of markers that had previously been allocated to the region using low density mapping

§ No recombination events were observed between these markers

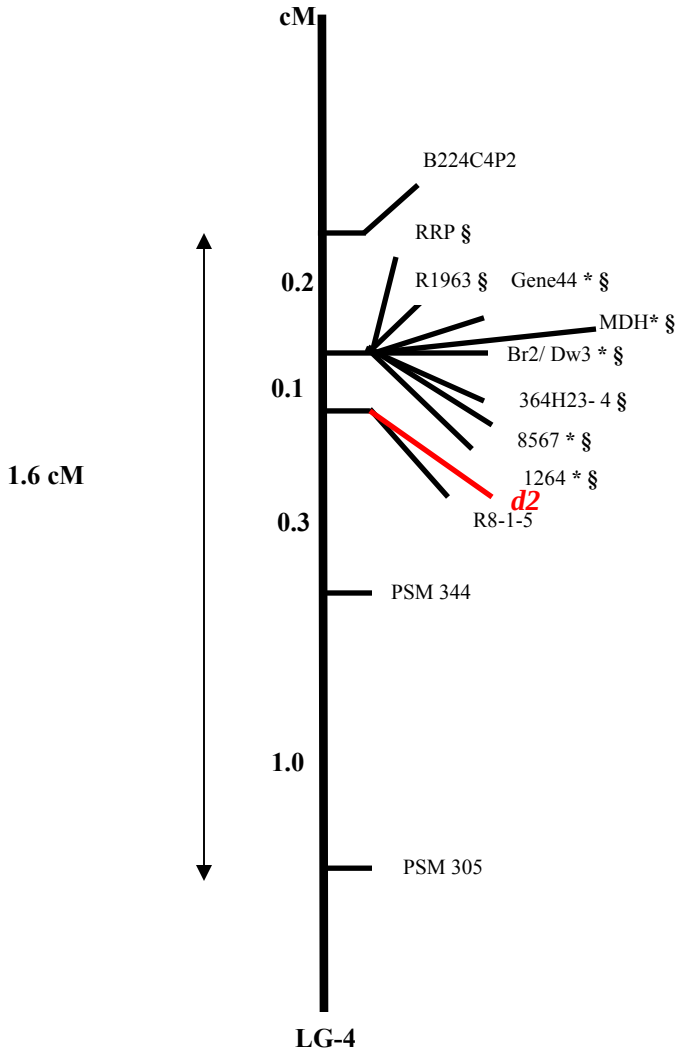


Figure 2.8. High density genetic map of *d2* developed from the cross ICMP 451 x Tift 23DB on LG 4 following the mapping of markers developed from the sorghum sequence.

* Markers developed from the sorghum sequence

§ No recombination was observed between these markers

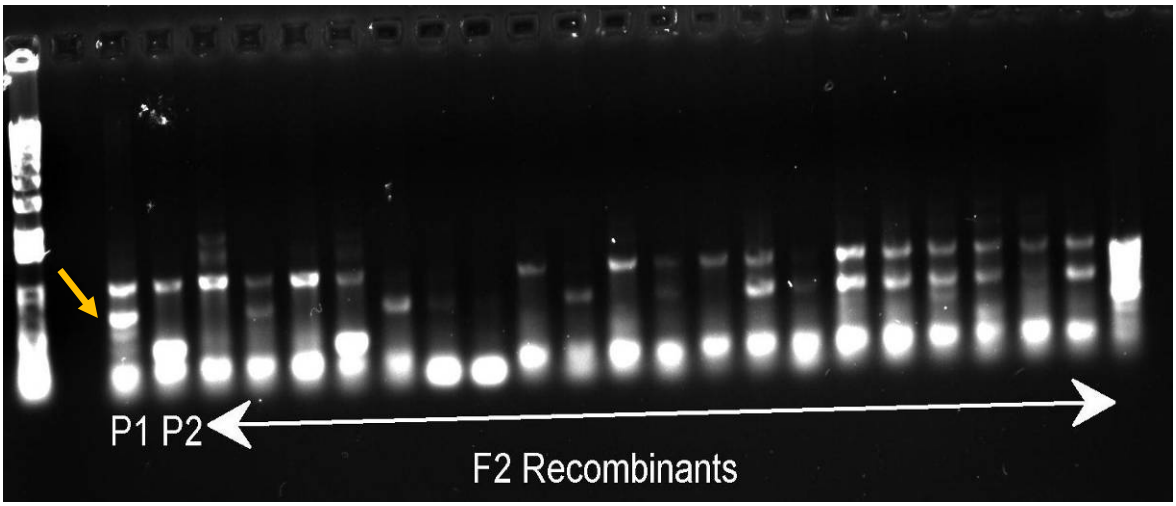


Figure 2.9. Mapping of *Br2*/*Dw3* on F_2 recombinants on a 3% Metaphor agarose gel
P1= ICMP 451 (Tall parent)
P2= Tift 23DB (Dwarf parent)
The arrow indicates the band that cosegregates with *d2*

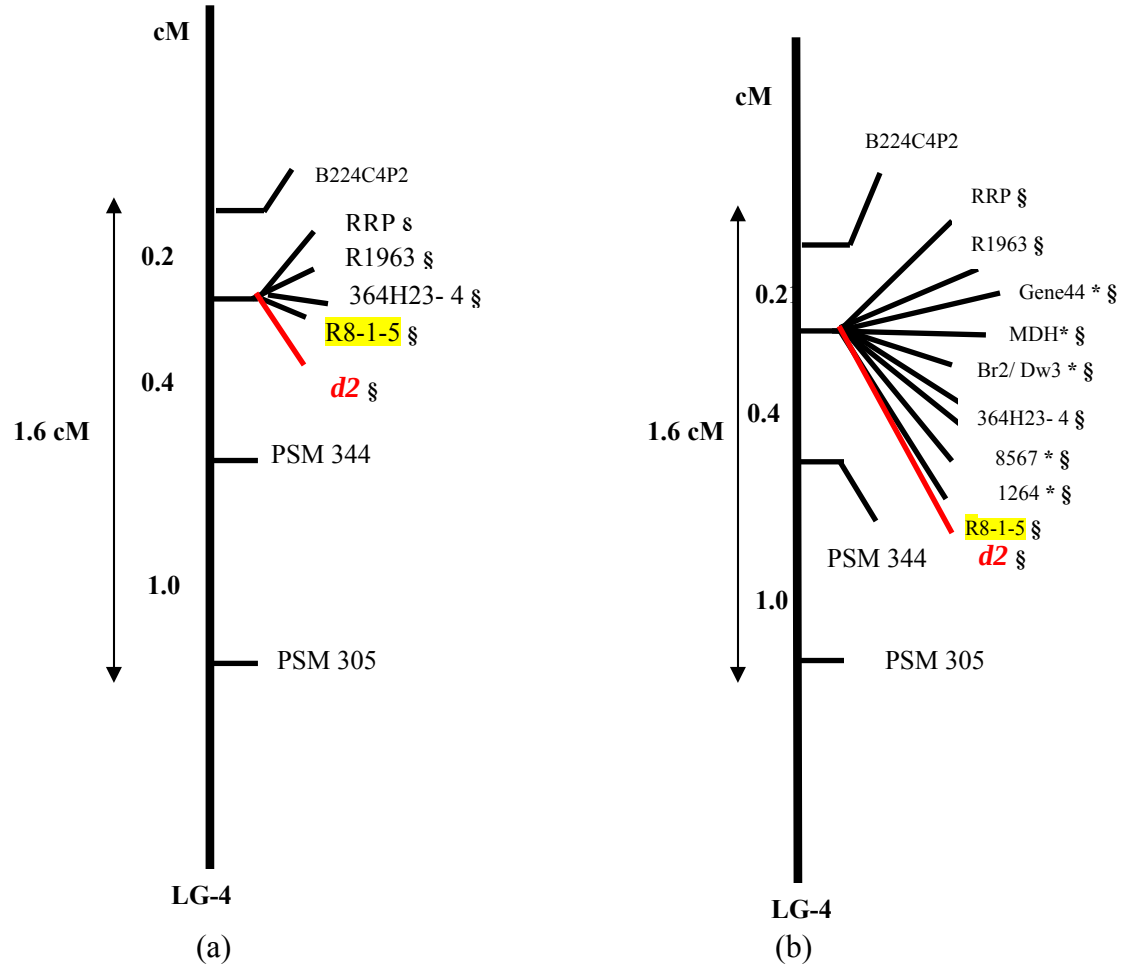


Figure 3.0. Genetic map of *d2* after plant 184 was excluded from the analysis resulting in marker R8-1-5 and *d2* to cosegregate with other markers as shown in the figure

(a) Genetic map before mapping of the sorghum markers

(b) High density genetic map after mapping of the new markers developed from the sorghum sequence

* Markers developed from the sorghum sequence

§ No recombination was observed between these markers

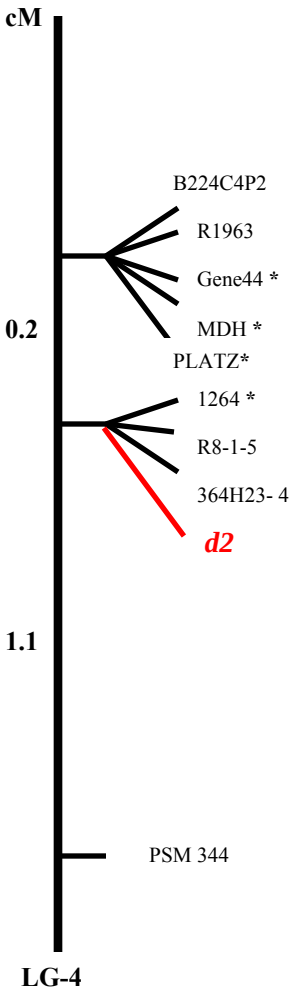


Figure 3.1. Genetic map of the *d2* region on linkage group 4 based on a second mapping population developed from the cross PT 732B X P1449-2 **α**

* Markers developed from the sorghum sequence

α Phenotypic data is not available for the recombinant plants

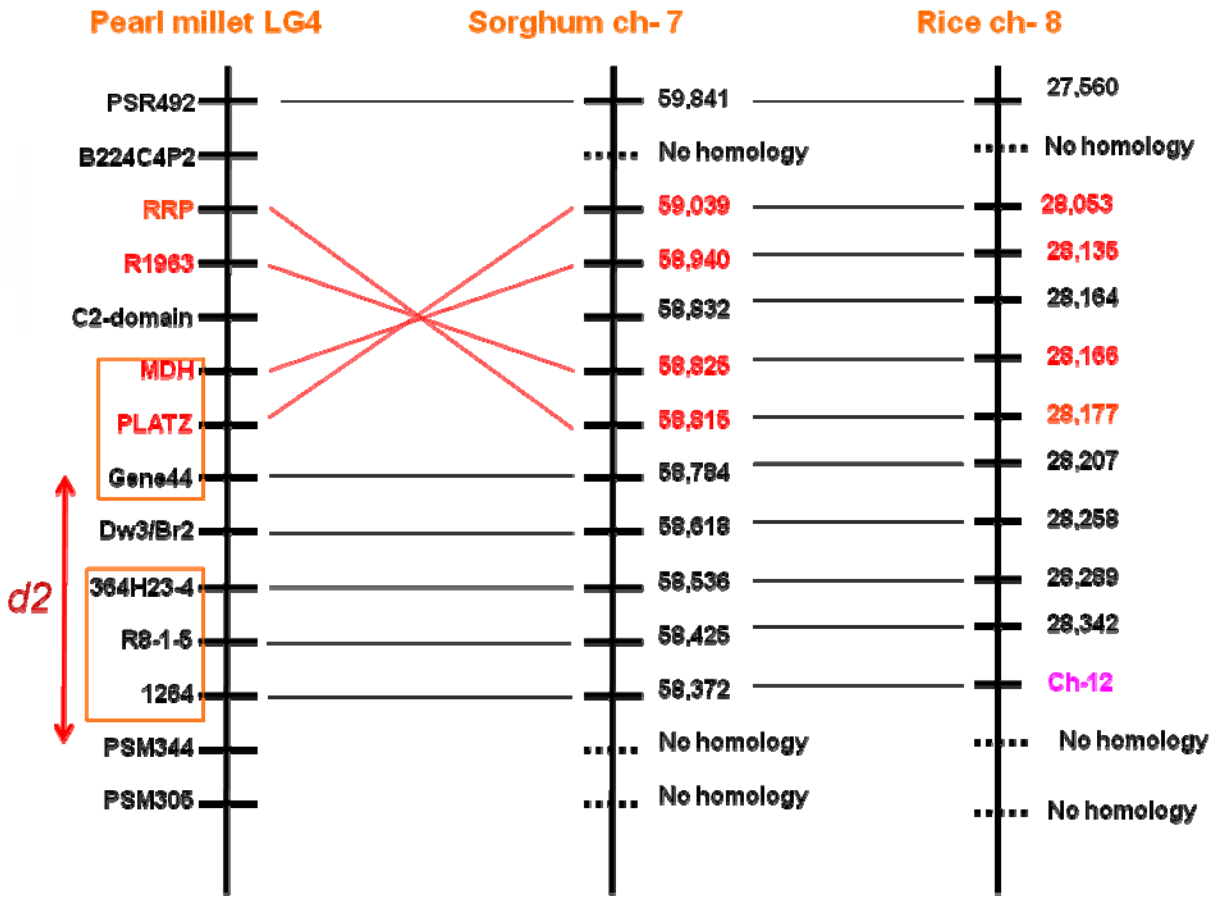


Figure 3.2. Rearrangement in the *d2* region in pearl millet relative to the orthologous regions in sorghum chromosome 7 and rice chromosome 8.
The physical location of *d2* is indicated with an arrow.

Markers in the colored box cannot be ordered based on the current information

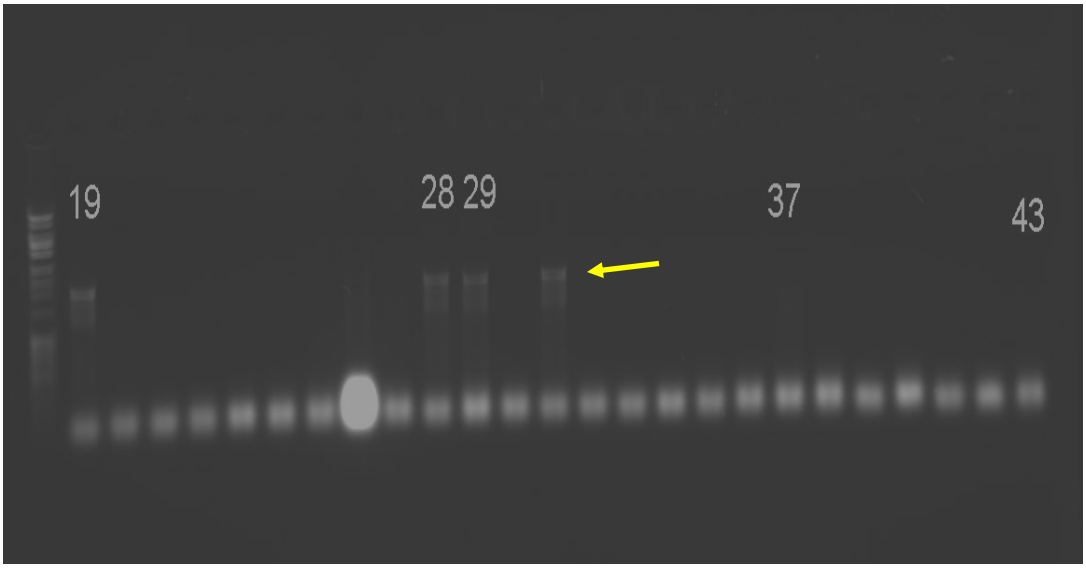


Fig 3.3. PCR of BAC super pools with the marker PSM344
(numbers indicate the super pools that amplified with this marker)

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APPENDIX-1

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
1	1	70	Present	55	1	156	Present
	2	64	Present		2	146	Present
	3	94	Present		3	124	Present
	4	90	Present		4	138	Present
	5	85	Present		5	124	Present
	6	90	Present		6	140	Present
	7	84	Present		7	156	Present
	8	83	Present		8	105	Present
	9	83	Present		9	144	Present
	10	81	Present		10	143	Present
	11	103	Present		11	166	Present
	12	108	Present		12	101	Present
	13	104	Present		13	70	Present
	14	85	Present		14	175	Present
	15	89	Present		15	169	Present
	16	51	Present		16	173	Present
	17	86	Present		17	120	Present
	18	84	Present		18	119	Present
	19	97	Present		19	128	Present
	20	109	Present		20	81	Present
	21	70	Present		*		
	22	85	Present		*		
	23	106	Present		*		
	24	107	Present		*		

Plant ID = F_2 recombinant plant
Bristles: Present= Long bristles
Absent= Either absent or short bristles
* Plants that did not survive

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
177	1	60	Absent	263	1	85	Absent
	2	61	Absent		2	133	Absent
	3	73	Absent		3	105	Absent
	4	70	Absent		4	86	Absent
	5	70	Absent		5	104	Absent
	6	60	Absent		6	104	Absent
	7	55	Absent		7	110	Absent
	8	68	Absent		8	110	Absent
	9	55	Absent		9	102	Absent
	10	64	Absent		10	104	Absent
	11	70	Absent		11	131	Absent
	12	58	Absent		12	104	Absent
	13	44	Absent		13	104	Absent
	14	79	Absent		14	121	Absent
	15	89	Absent		15	102	Absent
	16	82	Absent		16	118	Absent
	17	62	Absent		17	103	Absent
	18	63	Absent		18	134	Absent
	19	60	Absent		19	131	Absent
	20	46	Absent		20	83	Absent
	21	37	Absent		21	136	Absent
	22	53	Absent		22	124	Absent
	23	69	Absent		23	106	Absent
	24	87	Absent		24	102	Absent
	*				25	71	Absent

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
310	1	160	Present	320	1	160	Present
	2	157	Present		2	165	Present
	3	194	Present		3	164	Present
	4	160	Present		4	137	Present
	5	137	Present		5	131	Present
	6	147	Present		6	188	Present
	7	125	Present		7	148	Present
	8	130	Present		8	120	Present
	9	168	Present		9	178	Present
	10	160	Present		10	120	Present
	11	162	Present		11	154	Present
	12	165	Present		12	158	Present
	13	129	Present		13	163	Present
	14	117	Present		14	146	Present
	15	175	Present		15	120	Present
	16	191	Present		16	186	Present
	17	173	Present		17	149	Present
	18	203	Present		18	180	Present
	19	155	Present		19	126	Present
	20	158	Present		20	136	Present
	21	120	Present		21	152	Present
	22	160	Present		22	177	Present
	23	147	Present		23	120	Present
	24	170	Present		24	134	Present
	25	123	Present		*		

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
344	1	74	Present	349	1	95	Absent
	2	96	Present		2	110	Absent
	3	67	Present		3	81	Absent
	4	68	Present		4	84	Absent
	5	100	Present		5	85	Absent
	6	85	Present		6	92	Absent
	7	64	Present		7	93	Absent
	8	115	Present		8	102	Absent
	9	103	Present		9	90	Absent
	10	121	Present		10	85	Absent
	11	95	Present		11	87	Absent
	12	68	Present		12	102	Absent
	13	55	Present		13	101	Absent
	14	92	Present		14	96	Absent
	15	82	Present		15	91	Absent
	16	78	Present		16	98	Absent
	17	98	Present		17	87	Absent
	18	103	Present		18	104	Absent
	19	77	Present		19	110	Absent
	20	96	Present		20	128	Absent
	21	64	Present		21	132	Absent
	22	100	Present		22	110	Absent
	23	84			23	108	Absent
	*				24	126	Absent
	*				25	125	Absent

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
374	1	95	Present	477	1	71	Absent
	2	90	Present		2	102	Absent
	3	56	Present		3	68	Absent
	4	89	Present		4	61	Absent
	5	91	Present		5	60	Absent
	6	90	Present		6	91	Absent
	7	77	Present		7	87	Absent
	8	95	Present		8	46	Absent
	9	107	Present		9	70	Absent
	10	92	Present		10	59	Absent
	11	76	Present		11	50	Absent
	12	61	Present		12	61	Absent
	13	97	Present		13	75	Absent
	14	57	Present		14	37	Absent
	15	108	Present		15	59	Absent
	16	90	Present		16	92	Absent
	17	108	Present		17	71	Absent
	18	69	Present		18	40	Absent
	19	50	Absent		19	68	Absent
	20	80	Absent		20	40	Absent
	21	83	Absent		21	59	Absent
	22	107	Absent		22	54	Absent
	23	70	Absent		23	61	Absent
	24	50	Absent		24	69	Absent
	*				25	70	Absent

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
479	1	161	Present	486	1	129	Present
	2	180	Present		2	56	Present
	3	167	Present		3	84	Present
	4	170	Present		4	83	Absent
	5	154	Present		5	129	Absent
	6	204	Present		6	113	Absent
	7	117	Present		7	78	Absent
	8	187	Present		8	75	Absent
	9	165	Present		9	100	Absent
	10	204	Present		10	141	Absent
	11	170	Present		11	79	Absent
	12	131	Present		12	76	Absent
	13	156	Present		13	129	Absent
	14	95	Present		14	101	Absent
	15	119	Present		15	145	Absent
	16	148	Present		16	168	Absent
	17	151	Present		17	150	Absent
	18	187	Present		18	97	Absent
	19	134	Absent		19	130	Absent
	20	116	Absent		20	79	Absent
	21	121	Absent		21	116	Absent
	22	130	Absent		22	84	Absent
	*				23	150	Absent
	*				24	138	Absent

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
496	1	161	Present	514	1	131	Present
	2	145	Present		2	118	Present
	3	145	Present		3	107	Present
	4	149	Present		4	83	Present
	5	142	Present		5	140	Present
	6	171	Present		6	162	Present
	7	139	Present		7	93	Present
	8	127	Present		8	79	Present
	9	143	Present		9	190	Present
	10	137	Present		10	121	Present
	11	148	Present		11	158	Present
	12	77	Present		12	108	Present
	13	155	Present		13	149	Present
	14	143	Present		14	80	Present
	15	108	Present		15	111	Present
	16	138	Present		16	169	Present
	17	166	Present		17	169	Present
	18	166	Present		18	108	Present
	19	73	Present		19	108	Present
	20	111	Present		20	143	Present
	21	115	Present		21	200	Present
	22	59	Absent		*		
	23	92	Absent		*		

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
612	1	144	Absent	778	1	153	Present
	2	162	Absent		2	140	Present
	3	136	Absent		3	179	Present
	4	147	Absent		4	136	Present
	5	200	Absent		5	115	Present
	6	134	Absent		6	145	Present
	7	189	Absent		7	125	Present
	8	162	Absent		8	201	Present
	9	170	Absent		9	138	Present
	10	120	Absent		10	110	Present
	11	162	Absent		11	178	Present
	12	173	Absent		12	152	Present
	13	113	Absent		13	160	Present
	14	106	Absent		14	144	Present
	15	157	Absent		15	140	Present
	16	140	Absent		16	187	Present
	17	168	Absent		17	150	Present
	18	187	Absent		*		
	19	154	Absent		*		
	20	167	Absent		*		
	21	140	Absent		*		
	22	108	Absent		*		
	23	129	Absent		*		

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
787	1	170	Present	900	1	189	Present
	2	173	Present		2	192	Present
	3	171	Present		3	142	Present
	4	140	Absent		4	215	Present
	5	168	Absent		5	150	Present
	6	151	Absent		6	200	Present
	7	157	Absent		7	194	Present
	8	165	Absent		8	190	Present
	9	131	Absent		9	187	Absent
	10	147	Absent		10	224	Absent
	11	124	Absent		11	158	Absent
	12	168	Absent		12	160	Absent
	13	158	Absent		13	145	Absent
	14	148	Absent		*		
	15	200	Absent		*		

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
914	1	127	Absent	924	1	185	Absent
	2	175	Absent		2	180	Absent
	3	231	Absent		3	187	Absent
	4	205	Absent		4	198	Absent
	5	183	Absent		5	170	Absent
	6	130	Absent		6	167	Absent
	7	204	Absent		7	180	Absent
	8	240	Absent		8	160	Absent
	9	202	Absent		9	210	Absent
	10	270	Absent		10	146	Absent
	11	260	Absent		11	159	Absent
	12	180	Absent		12	179	Absent
	13	220	Absent		13	210	Absent
	14	160	Present		14	152	Absent
	15	229	Present		15	227	Absent
	*				16	200	Absent
	*				17	210	Absent
	*				18	240	Absent
	*				19	151	Absent
	*				20	200	Absent
	*				21	190	Absent
	*				22	200	Absent

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles	Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
930	1	150	Absent	701	1	186	Present
	2	220	Absent		2	210	Present
	3	200	Absent		3	224	Present
	4	197	Absent		4	157	Present
	5	207	Absent		5	181	Present
	6	205	Absent		6	157	Present
	7	200	Absent		7	200	Present
	8	180	Absent		8	158	Present
	9	194	Absent		9	200	Present
	10	210	Absent		10	136	Present
	11	258	Absent		11	184	Present
	12	260	Absent		12	180	Present
	13	110	Absent		13	162	Present
	14	220	Absent		14	160	Present
	15	260	Absent		15	202	Absent
	16	220	Absent		*		
	17	181	Absent		*		
	18	210	Absent		*		

Plant ID	$F_{2:3}$ Progeny	Height (cm)	Bristles
812	1	150	Present
	2	190	Present
	3	210	Present
	4	210	Present
	5	210	Present
	6	221	Present
	7	230	Present
	8	235	Present
	9	203	Absent
	10	210	Absent
	11	197	Absent
	12	180	Absent
	13	189	Absent
	14	230	Absent
	15	200	Absent
	16	224	Absent
	17	165	Absent