Genetics and Molecular Biology, 35, 1, 106-118 (2012)

www.sbg.org.br

Research Article

Genetic architecture of purple pigmentation and tagging of some loci to SSR markers in pearl millet, *Pennisetum glaucum* (L.) R. Br.

Pusapati Varalakshmi[1], Surya S Mohan Dev Tavva[1], Palakollu V Arjuna Rao[1], Muktinutalapati V Subba Rao[1] and Charles T Hash[2]

[1]*Plant Cytogenetics and Biotechnology Laboratory, Botany Department, Andhra University, Visakhapatnam, India.*
[2]*Global Theme-Biotechnology, ICRISAT, Patancheru, India.*

Abstract

This report describes the construction of integrated genetic maps in pearl millet involving certain purple phenotype and simple sequence repeat (SSR) markers. These maps provide a direct means of implementing DNA marker-assisted selection and of facilitating "map-based cloning" for engineering novel traits. The purple pigmentation of leaf sheath, midrib and leaf margin was inherited together '*en bloc*' under the control of a single dominant locus (the 'midrib complex') and was inseparably associated with the locus governing the purple coloration of the internode. The purple panicle was caused by a single dominant locus. Each of the three characters (purple lamina, purple stigma and purple seed) was governed by two complementary loci. One of the two loci governing purple seed was associated with the SSR locus *Xpsmp*2090 in linkage group 1, with a linkage value of 22 cM, while the other locus was associated with the SSR locus *Xpsmp*2270 in linkage group 6, with a linkage value of 23 cM. The locus for purple pigmentation of the midrib complex was either responsible for pigmentation of the panicle in a pleiotropic manner or was linked to it very closely and associated with the SSR locus *Xpsmp*2086 in linkage group 4, with a suggestive linkage value of 21 cM. A dominant allele at this locus seems to be a prerequisite for the development of purple pigmentation in the lamina, stigma and seed. These findings suggest that the locus for pigmentation of the midrib complex might regulate the basic steps in anthocyanin pigment development by acting as a structural gene while other loci regulate the formation of color in specific plant parts.

Key words: gene tagging, pearl millet, pigmentation genetics.

Received: May 4, 2011; Accepted: October 24, 2001.

Introduction

Integrated genetic maps involving molecular and phenotypic markers provide a direct means for investigating the number of genes influencing a trait, their location along the chromosomes and the effects of variation in their dosage. These maps also provide the information needed to implement DNA marker-assisted selection, an approach of growing importance in plant and animal improvement that facilitates "map-based cloning" for engineering novel traits. However, accurate genotyping and phenotyping of the materials is required for the successful application of this information (Zhou, 2010). The phenotypic characters related to pigmentation have been the most frequently used genetic markers in plants. These markers have been widely used to study the genetic basis of phenotypic diversity associated with evolutionary changes and with the processes of tolerance to abiotic stress and disease resistance.

Send correspondence to M.V. Subba Rao. Botany Department, Andhra University, 530003 Visakhapatnam, India. E-mail: mnpati_srao@yahoo.com.

Pearl millet [*Pennisetum glaucum* (L.) R. Br. = *P. americanum* (L.) Leeke = *P. typhoides* S&H; 2n = 14] is an important cereal of traditional farming systems in tropical and subtropical Asia and sub-Saharan Africa. This millet, which is a staple food grain and a source of feed, fodder, fuel and construction material, is grown on 29 million hectares (FAO, 2005) and supports millions of poor rural families in the hottest and driest drought-prone semi-arid regions of Africa and the Indian sub-continent, where rainfed agriculture is practiced. Despite the considerable attention that pearl millet has received from agronomists, plant breeders and cytogeneticists, and the availability of molecular maps, the tagging of genes that can be used as molecular markers for various characters in this cereal is still in its initial stages. The first RFLP-based genetic linkage map in pearl millet was reported by Liu *et al.* (1994) and has since been updated by the addition of more RFLP and SSR markers (Qi *et al.*, 2004). These markers have improved our understanding of the complex relationship between the pearl millet genome and those of other cultivated graminaceous species (Devos *et al.*, 2000), in addition to being useful in

studies of aspects such as marker-assisted breeding (Hash, 2004; Serraj *et al.*, 2005).

A review of earlier studies on the genetics of phenotypic markers in pearl millet, especially those related to pigmentation (Al-Fakhry *et al.*, 1965; Singh *et al.*, 1967; Athwal and Gill, 1966; Gill, 1969; Phul *et al.*, 1969; Gill and Athwal, 1970; Koduru and Krishna Rao, 1979; Manga *et al.*, 1988; Hanna and Burton, 1992), revealed certain apparently contradictory findings and "several of these early publications do not provide adequate descriptions of the mutant phenotypes to permit comparison by other researchers" (Anand Kumar and Andrews, 1993).

In view of these gaps in our knowledge of this crop plant, the present work was undertaken to provide a detailed description of some of the purple phenotype markers with reference to a standard color code, to report their genetic basis and to describe their linkage relationships. We also provide accurate phenotyping and genotyping of the accessions used and have tagged the loci to SSR markers. A model is suggested to explain the genetic architecture underlying the purple pigmentation; this model may also help to resolve the contradictory findings of earlier studies.

Materials and Methods

Plant material

Two inbred lines of pearl millet, *i.e.*, green dwarf (d_2) and purple (IP3128), were used in this work. Green dwarf (d_2d_2) seeds were obtained from ICRISAT, Patancheru, India. The purple plant was originally identified in 1981 in a population of 500 plants derived from combined mutagenic treatment with a 20 Kr dose of gamma rays and a 0.1% aqueous solution of ethyl methane sulphonate (EMS). Both of these accessions have been maintained through selfing and/or sib mating. Parents and F_1s were checked randomly at meiosis in pollen mother cells to eliminate those carrying chromosomal aberrations.

Seeds were germinated on moist filter paper in 4-inch diameter plastic petri dishes and after 2-3 days germinated seeds were transferred to earthenware pots filled with sterilized soil. After 25-30 days, seedlings were transplanted into the field in rows spaced 60 cm apart, with plants within each row spaced 30 cm apart. The specific color/pigmentation was described based on the Royal Horticultural Society color codes (RHS) and the IBPGR and ICRISAT (1993) descriptors for pearl millet. Gene names and symbols were defined based on the criteria established for *Arabidopsis* (Anonymous, 2005).

DNA isolation

Genomic DNA was isolated from fresh leaf tissue using the CTAB method of Saghai-Maroof *et al.* (1984). Isopropanol-precipitated DNA was washed with 70% ethanol, air dried, dissolved in 200 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at 4 °C. RNAse (Sigma-Aldrich, USA) was added to the DNA solution (final concentration: 50 µg/mL, from a stock solution of 10 mg/mL) and the mixture incubated at 37 °C for 15 min. DNA was re-precipitated with 250 µL of 7.5 M ammonium acetate and 1 mL of absolute ethanol, collected, washed in 70% ethanol, dried and re-suspended in 100 µL of TE buffer prior to storage at 4 °C.

Primers

61 simple sequence repeats (SSRs; see supplementary material Table S1 for details), expected to map across all of the seven possible linkage groups in pearl millet, were used in this work. The nucleotide sequences of the primers (PSMP for primers and *Xpsmp* for SSR loci) used to amplify these SSR markers were developed at the John Innes Institute (Qi *et al.*, 2004) and were made available to the authors by ICRISAT.

Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were done in 96-well plates using a model PTC 220 DNA engine DYAD Peltier thermal cycler (MJ Research Inc., USA). The reactions were done in a final volume of 20 µL that consisted of 2.5 µL of DNA (5 ng/mL), 2 µL of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 2 µL of 2 mM dNTP, 0.5 µL of 25 mM Mg^{2+}, 1 µL of primer (30 ng/µL), 1.5 µL of Bioline *Taq* polymerase (1 U/µL) (Bioline Reagents Ltd. UK) and 9.5 µL of distilled water. The PCR involved initial denaturation of the template DNA at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 48, 58 or 61 °C (depending on the melting temperature of the primer) for 1 min and extension at 72 °C for 1 min.

Post-PCR gel electrophoresis

PCR products were separated on 7.7% polyacrylamide gels in 10X TBE buffer. The gel was pre-run for at least 10 min at 5 V/cm (600 V, 9 W). Urea gels were used to separate the amplification products of primers PSMP 2089, PSMP 2068, PSMP 2246, PSMP 2220 and PSMP 2251. The urea gels were run at 50 °C and 100 W for 45-60 min. 4 µL of each PCR product was loaded onto the gels, along with 2 µL of 100 bp standard molecular weight marker (50 ng/µL; Qiagen) which was also loaded in the first and last lanes of the gel. The gels were run at 600-650 V in 0.5X TBE buffer for 3-3.5 h using a Bio-Rad sequencing gel apparatus. After the run, the gels were silver stained, as described by Tegelstrom (1992). Only bands in the 100-300 bp range were considered. The most anodal band was considered as band 1.

Data analysis

Data were collected from the segregating populations (F2 and Testcross) derived from a minimum of four sepa-

rate parental crosses. The corresponding data were pooled after checking the homogeneity of their variances and the pooled data are presented in the tables. The deviations between the observed and expected ratios and homogeneity of the Chi-square values for different segregating populations corresponding to a cross were checked with the Chi-square test, as described by Snedecor and Cochran (1967). The logarithm of odds (LOD) scores were calculated using the methods of Morton (1955) and Lathrop and Lalouel (1984). The odds ratio was calculated for the observed proportion of each category of data versus that expected on the basis of independent assortment of the markers. Only those cases for which the Chi-square value deviated significantly at a probability level of 0.05 and an LOD = 2.8 were taken as indicative of linkage.

Results

Inheritance patterns of phenotypic markers

The inheritance patterns of purple pigmentation of the leaf, panicle, stigma and seed coat were studied individually and in combinations using reciprocal crosses between plants of the two parental lines, *viz*., green dwarf (d_2) and purple (IP3128; Figure 1a,b). A common feature of inheritance noted for all of these phenotypic markers was the lack of reciprocal differences as judged from the F_1, F_2 and testcross progeny phenotypes.

Description and inheritance patterns of individual phenotypic markers

Purple foliage (purple pigmentation of leaves)

Plants of the parental purple accession showed deep purple pigmentation (purple group 79B of RHS) throughout their leaves (sheath, lamina, midrib and margin) while those of the green dwarf parental accession were green. The development of purple pigmentation was initially detected at the base of the leaf sheath in 12-15-day-old seedlings and extended over the entire leaf within 30 days after transplantation, starting at the base of the lamina and gradually spreading towards the leaf tip (Figure 1c-e). For the sake of consistency, the lamina color was recorded 55-58 days after sowing (or 30 days after seedling transplantation).

Reciprocal crosses between plants with purple leaf blades and those with green leaf blades produced F_1 plants, all of which showed purple leaves, indicating the dominant nature of the purple phenotype. These F_1 plants were selfed and also crossed to the green parental accession. The data on segregation pattern of foliage color are presented in Table 1. Three phenotypic categories were identified in the F_2 and testcross progeny, namely,

1) Category 1 – plants with purple leaves resembling those of the purple parent and F_1,

2) Category 2 – plants with a green lamina but a purple sheath, midrib and margin (Figure 1f),

3) Category 3 – plants with completely green leaves resembling those in the green parent.

The frequencies of these three phenotypic categories showed a good fit to a 9:3:4 ratio while the testcross progeny showed a good fit to a 1:1:2 ratio (Table 1). When the F_2 and testcross progeny were scored for lamina pigmentation only (purple lamina: green lamina, *i.e.*, category 1: categories 2+3), irrespective of the type of pigmentation present in the leaf sheath, midrib and leaf margin, they showed a good fit to 9:7 and 1:3 ratios, respectively ($p > 0.05$). For further genetic analysis of the category 2 phenotype, F_2 plants with a green lamina and purple pigmentation in the leaf sheath, midrib and margin were randomly selected and selfed. One third of this population (data not shown) bred true, indicating that these plants were homozygous at the loci governing this phenotype, while two-thirds segregated in a 3:1 ratio of category 2: category 3 plants.

Table 1 - Segregation patterns of purple foliage.

Progeny (ratio)	Purple lamina and purple sheath, midrib, margin and internode (Category 1)	Green lamina and purple sheath, midrib, margin and internode (Category 2)	Green lamina and green sheath, midrib, margin and internode (Category 3)	Total	χ	*p*
A) Purple parent X Green parent*						
F2 (9:3:4)	438	151	233	822	5.0284	0.08
Test cross (1:1:2)	63	49	109	221	2.9118	0.03
B) Purple lamina (Purple parent X True breeding segregants of Category 2						
F2 (3:1)	432	157	-	589	0.7748	0.68
Test cross (1:1)	66	56	-	122	0.6639	0.72
C) Purple sheath, midrib, margin and internode						
F2 (3:1)	-	500	196	696	3.7088	0.05
Test cross (1:1)	-	118	103	221	0.8869	0.64

*Ratio of purple lamina:green lamina (column 2: columns 3+4) is a good fit to 9:7 in F_2 and 1:3 in testcross progeny ($p > 0.05$).

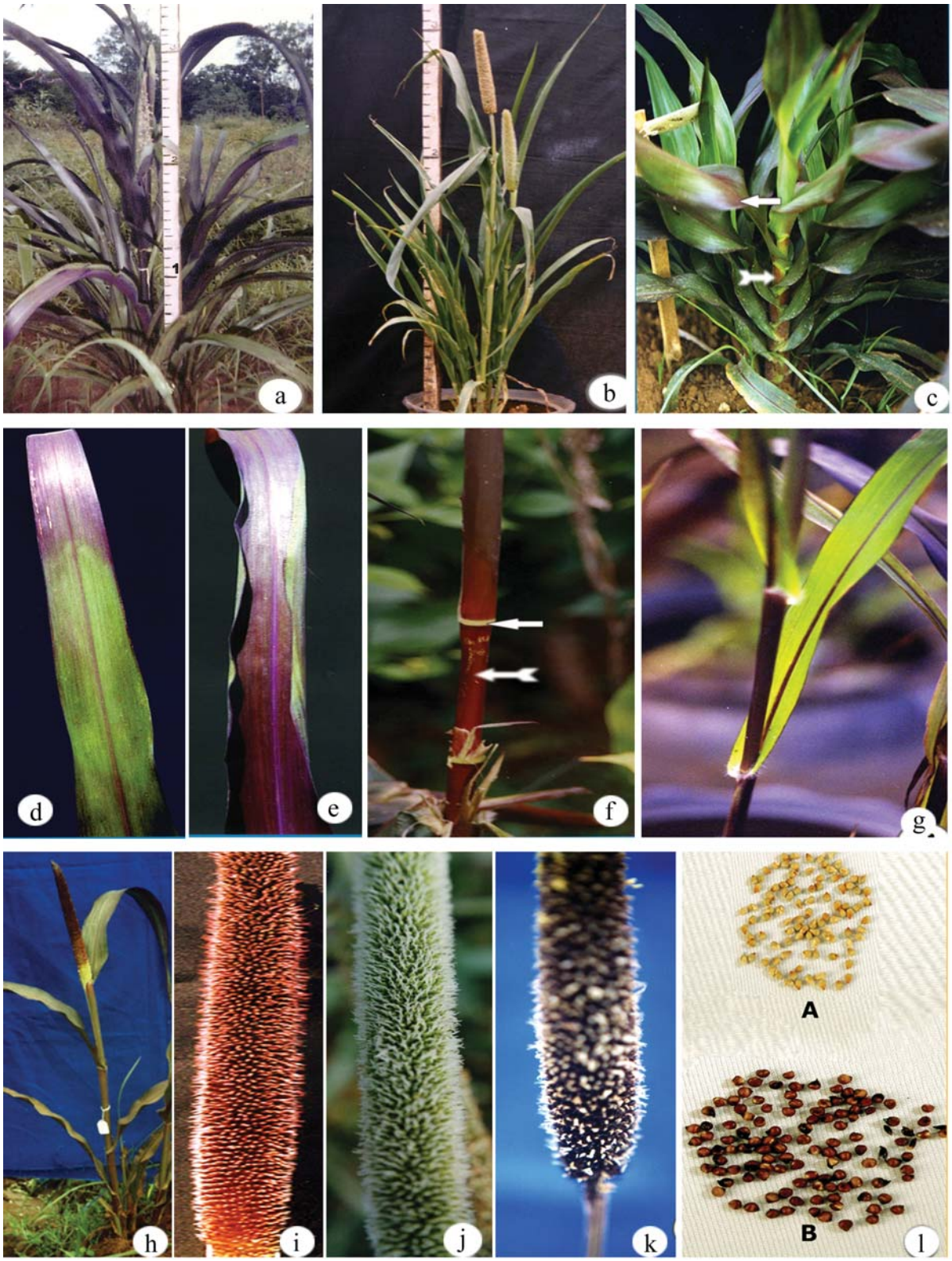

Figure 1 - Purple and non-purple phenotypes of pearl millet. a. Plant of purple parental accession. b. Plant of green dwarf (d_2) parental accession. c. Purple parent showing brachytic nature, pigmentation of leaf sheath (double arrow) and initiation of purple pigmentation in the lamina (single arrow). d. Leaf of purple parent showing initiation of purple pigmentation at the base of the lamina. e. Leaf of purple parent showing pigmentation of the entire lamina. f. Purple parent showing purple internode (double arrow) and green node (single arrow). g. F_2 segregant with green lamina and purple midrib complex (purple midrib, purple sheath and purple lamina). h. Panicle of purple parent showing development of pigmentation on the exposed part. i. Purple panicle with purple stigmas at the time of emergence. j. Green panicle with white stigmas. k. F_2 segregant with purple panicle and green stigmas at the initiation of anthesis. l. Grey (A) and purple (B) seeds from the parental accessions.

To analyze lamina pigmentation alone, plants of the purple accession were crossed to true breeding plants of category 2. Purple pigmentation of the sheath, midrib and margin was common to both of the parents. The F_1 plants showed complete purple foliage. The F_2 progeny segregated (3:1 ratio) into plants with complete purple foliage and those with a green lamina and purple sheath, midrib and margin. The test cross progeny segregated in a 1:1 ratio for these characters. These results suggested that purple pigmentation of the lamina was a monogenic dominant character.

To analyze the pigmentation of the leaf sheath, midrib and margin, the progeny of true breeding plants of category 2 that had a green lamina and purple leaf sheath, midrib and leaf margin were crossed to plants from the green parental line. The F_1 plants resulting from this cross had green lamina but a purple leaf sheath, midrib and leaf margin. These F_1 plants were selfed and also test crossed to the green dwarf. The F_2 progeny segregated in a 3:1 ratio of plants having green lamina with a purple sheath, midrib and margin and those with completely green leaf parts. In the testcross progeny, these two types segregated in a 1:1 ratio.

In none of the progeny resulting from either F_2 or testcross populations of any of the above crosses was there any separation of pigmentation in the leaf sheath, midrib and margin. In all of these cases, internode pigmentation was also associated with the pigmentation of these three parts of leaf, *i.e.,* whenever the leaf sheath, midrib and margin were purple, the internode was also purple (Figure 1g) and whenever these parts were green, the internode was also green. Thus, purple pigmentation of the internode, leaf sheath, midrib and leaf margin was apparently inherited *en bloc* and determined by a dominant allele at a single locus. For the sake of convenience, the purple pigmentation in these four plant parts (internode, leaf sheath, midrib and leaf blade margin) is denoted hereafter as pigmentation of the 'midrib complex'.

Purple panicle

Plants of the green parental accession had green panicles. In plants of the purple parental accession, the panicles were green when they were still inside the boot leaf. The purple color started developing as the panicles gradually emerged from the boot leaf, beginning first at the tip and gradually extending towards the base of the inflorescence (Figure 1h). The panicle color was recorded after complete emergence of the stigmas and corresponded to purple group 79B of RHS. After formation of the seed, the purple panicle turned purplish black (corresponding to No. 9 of pearl millet descriptors). Reciprocal crosses between plants with purple panicles and those with green panicles produced F_1 plants having purple panicles. The segregation ratios observed in the F_2 (599:222) and testcross (120:101) progeny were a good fit to purple:green ratios of 3:1 and 1:1, respectively.

Purple stigma

The stigma color was recorded after its complete emergence and before anthesis. The green parental accession had white stigmas while the purple parental accession showed purple ones (Figure 1i-k). Reciprocal crosses between plants of these two accessions produced F_1 plants, all of which had purple stigmas. The F_2 progeny segregated in a 9:7 ratio (447 purple : 305 white) and testcross progeny in a ratio of 58:162 (1:3; $p > 0.05$).

Purple pigmentation of seed coat

Seed color was recorded at the harvesting stage. In the purple parental accession, the seed was purple (purple group T9B of RHS) at earlier stages but turned purplish black at the harvesting stage (No. 9 of pearl millet descriptors; Figure 1a). The purple pigmentation was confined to the seed coat only and the endosperm was white; however, this character is referred to here as 'seed color' for the sake of presentation. The seeds of the green parental accession were grey colored (grey group 201 of RHS; Figure1 lB) with a white endosperm.

Crosses between purple-seeded and grey-seeded plants of the two parental lines revealed that the color of the F_1 seed depended on the genotype of the female parent used in the cross. For example, the F_1 seeds were grey if the female parent had grey seed and were purple when the female parent had purple seeds. Upon sowing, these two types of seeds produced F_1 plants, all of which had purple seeds after selfing or in controlled pollination using pollen from either purple- or grey-seeded parent plants. These purple-seeded F_1 plants were selfed to produce F_2 seeds. The F_2 plants produced by sowing these F_2 seeds segregated for panicles producing purple seeds (411) and those producing grey seeds (330) in a 9:7 ratio ($p > 0.05$). The testcrosses involving F_1 plants (bearing purple seeds) and the grey-seeded parental line resulted in progeny that included **62** purple seed plants and **143** grey seed plants (1:3; $p > 0.05$).

Joint segregation patterns of phenotypic markers

Purple midrib complex with purple panicle, stigma and seed

Analysis of the joint segregation pattern of the purple midrib complex with panicle pigmentation revealed the presence of only two phenotypic categories in the F_2 and testcross progeny, *viz.,* (1) plants with a purple midrib complex and purple panicle and (2) plants with a green midrib complex and green panicle. The frequencies of these two phenotypic categories showed a good fit to a 3:1 ratio in the F_2 generation and a 1:1 ratio in the testcross generation (Table 2).

The combined inheritance of the purple midrib complex and purple stigma traits revealed three phenotypic categories in the F_2 and testcross progeny that included (1) the purple midrib complex and purple stigma, (2) the purple midrib complex and white stigma, and (3) the green midrib

complex and white stigma (Table 2). The theoretically expected fourth category (green midrib complex and purple stigma) was not observed. The F_2 data showed a good fit to a 9:3:4 ratio for these three categories, while the corresponding testcross progeny segregated in a 1:1:2 ratio. The LOD scores for the F_2 and testcross progeny were < 2.

The pattern of joint segregation of the purple midrib complex and purple seed was similar to that for purple pigmentation of the stigma, *i.e.*, only three phenotypic categories were observed in the F2 and testcross progeny, namely, (1) the purple midrib complex and purple seed, (2) the purple midrib complex and grey seed, and (3) the green midrib complex and grey seed. The ratios of these three phenotypic categories were 9:3:4 and 1:1:2 in the F_2 and testcross progeny, respectively (Table 3). The LOD score values in all these cases were also < 2.

Purple panicle with purple lamina, stigma and seed

The joint segregation pattern of purple panicle (character 1) with any of the other three pigmentation traits as the second character (purple lamina or purple stigma or purple seed) was similar to that observed in the case of the midrib complex described above in that only three phenotypic categories were detected in either the F_2 or testcross progeny. These categories were: (1) both characters 1 and 2 being purple, (2) purple for character 1 and non-purple for character 2 (green for lamina, white for stigma, grey for seed), and (3) both characters green. The frequencies of these three phenotypic categories showed good fits to 9:3:4 (F_2) and 1:1:2 (testcross) ratios and the corresponding LOD scores were < 2 (Table 2).

Purple lamina with purple stigma and purple seed

The co-segregation of lamina and stigma pigmentation resulted in four phenotypic categories (Table 2), namely:

1) plants with purple lamina, purple midrib complex and purple stigma,

2) plants with purple lamina, purple midrib complex and white stigma,

3) plants with green lamina, purple midrib complex and purple stigma,

Table 2 - Joint segregation patterns of purple pigmentation in various plant parts.

Characters	Progeny	Ph 1	Ph 2	Ph 3	Ph 4	Total	Chi-square value[#]	LOD score
A) Pigmentation of midrib complex and								
i) Panicle	F2	592	0	0	222	814	1.0614 (3:1)*	-
	TC	105	0	0	116	221	0.4525 (1:1)*	-
ii) Stigma	F2	440	114	0	185	739	5.7764 (9:3:4)*	1.3099
	TC	69	42	0	108	219	6.6986 (1:1:2)*	1.4495
iii) Seed	F2	410	124	0	207	741	4.2709 (9:3:4)*	0.9203
	TC	71	52	0	92	215	7.8279 (1:1:2)*	1.6139
B) Pigmentation of panicle and								
i) Lamina	F2	428	168	0	221	817	4.9736 (9:3:4)*	1.0738
	TC	66	56	0	99	221	3.2986 (1:1:2)*	0.6989
ii) Stigma	F_2	440	114	0	185	739	5.7764 (9:3:4)*	1.3099
	TC	69	42	0	108	219	6. 6986 (1:1:2)*	1.4495
iii) Seed	F_2	410	124	0	207	741	4.2709 (9:3:4)*	0.9203
	TC	71	52	0	92	215	7. 8279 (1:1:2)*	1. 6139
C) Pigmentation of lamina and								
i) Stigma	F_2	350	93	35	260	738	316.9033(81:63:63:49)**	78.3473
	TC	99	33-	21	75	228	539.4386 (1:3:3:9)**	55.6725
ii) Seed	F_2	321	104	65	267	757	245.9433 (81:63:63:49)**	55.6449
	TC	62	23	24	105	214	192.5296 (1:3:3:9)**	25.1509
D) Pigmentation of stigma and seed								
	F_2	347	82	47	242	718	288.7104 (81:63:63:49)**	68.9312
	TC	66	20	37	92	215	223.0362 (1:3:3:9)**	27.2277

Ph = phenotype; Ph 1 = both characters purple; Ph 2 = first character purple and second character non-purple; Ph 3 = first character non-purple and second character purple; pH 4 = both characters non-purple. NB: non-purple indicates green panicle, green lamina, green midrib complex, grey seed and white stigma for the respective parts. [#]Theoretical ratios used to calculate the Chi-square values and LOD scores. *Ratio for which the data showed best fit ($p = 0.01$). **Significantly different ($p < 0.05$) from the expected theoretical ratio.

Table 3 - Segregation patterns of Simple Sequence Repeat (SSR) markers among F_2 progeny.

LG	SSR locus	Primer sequence (F/R)	Band(s) scored in the purple parent (A)	Band(s) scored in the green parent (B)	Differential bands in the hybrid (H)	Number of plants in the F_2 progeny showing patterns				Chi-square[#]
						A	H	B	Total	
1	*Xpsmp* 2273	AACCCCACCAGTAAGTTGTGCTGC GATGACGACAAGACCTTCTCTCC	2	1	1, 2	52	159	73	284	7.1761*
	Xpsmp 2246	CGGATGCTAAATTAACCGAAGC CCAGCTTGCTTCTGTTGCGTTC	1	2	1, 2	71	180	70	321	4.7445
	Xpsmp 2090	AGCAGCCCAGTAATACCTCAGCTC AGCCCTAGCGCACAACACAAACTC	2	1	1, 2	81	181	66	328	4.8963
2	*Xpsmp* 2255	CATCTAAACACAACCAATCTTGAAC TGGCACTCTTAAATTGACGCAT	2	1	1, 2	91	201	109	401	1.6184
	Xpsmp 2068	CAATAACCAAACAAGCAGGCAG CTTCACTCCCACCCTTTCTAATTC	1, 2, 3, 4	3, 4, 5, 6	1, 2, 5, 6	59	217	45	321	41.0000*
	Xpsmp 2089	TTCGCCGCTGCTACATACTT TGTGCATGTTGCTGGTCATT	1, 3	2, 4	1, 2, 3, 4	66	162	83	311	2.4019
	Xpsmp 2231	TGTTGTTGGGAGAGGGTATGAG CTCTCGCCATTCTTCAAGTTCA	2, 3	1, 2	1, 3	85	179	67	331	4.1601
	Xpsmp 2237	TGGCCTTGGCCTTTCCACGCTT CAATCAGTCCGTAGTCCACACCCCA	1	2	1, 2	78	180	73	331	2.6918
	Xpsmp 2206	AGAAGAAGAGGGGGTAAGAAGGAG AGCAACATCCGTAGAGGTAGAAG	2	1	1, 2	85	161	82	328	0.1646
4	*Xpsmp* 2086	CGCTTGTTTTCCTTTCTTGCTGTT CCTTCTCAGATCCTGTGCTTTCTT	2	1	1, 2	99	210	92	401	1.1446
5	*Xpsmp* 2202	CGCTTGTTTTCCTTTCTTGCTGTT** CCTTCTCAGATCCTGTGCTTTCTT	1	2, 3	1, 2, 3	65	169	68	302	4.3509
	Xpsmp 2078	CATGCCCATGACAGTATCTTAAT** ACTGTTCGGTTCCAAAATACTT	1, 2	1, 3	2, 3	79	150	66	295	1.2305
	Xpsmp 2220	GCATCCTTCACCATTCAAGACA** TGGGAAACAGAATGGAGAAAAGAG	1, 3, 5	2, 4, 6	1, 2, 3, 4, 5, 6	84	179	56	319	9.6834*
6	*Xpsmp* 2270	AACCAGAGAAGTACATGGCCCG CGACGAACAAATTAAGGCTCTC	1, 2	3, 4	1, 2, 3, 4	74	169	74	317	1.3912
7	*Xpsmp* 2224	GGCGAATTGGAATTCAGATTG CGTAATCGTAGCGTCTCGTCTAA	1, 2, 3	3, 4,5	1, 2, 4, 5	71	163	63	297	3.2626
	Xpsmp 2263	AAAGTGAATACGATACAGGAGCTGAG CATTTCAGCCGTTAAGTGAGACAA	9, 10	1	1, 9, 10	77	160	67	304	1.5000

LG - linkage group; ** T_m = 58 °C compared to 61 °C for all other primers. [#]Chi-square tested for an expected ratio of 1:2:1 for the three phenotypes, respectively; no significant deviation at p = 0.05. *Significant deviation ($p < 0.05$).

4) plants with green lamina, green midrib complex and white stigmas.

The frequencies of these four categories deviated significantly from the expected 81:63:63:49 ratio in F_2 and 1:3:3:9 ratio in the testcross progeny based on the involvement of four independent/unlinked loci acting in two complementary pairs, with each pair segregating in a 9:7 ratio in F_2. The corresponding LOD score values were > 3 (Table 2). In the joint segregation of lamina and seed pigmentation, only four phenotypic classes similar to those described above deviated from the expected ratios in the F_2 and testcross progeny. The LOD scores were also > 3.

Purple stigma with purple seed

When the joint segregation of stigma and seed pigmentation was considered, the four phenotypic classes observed were (1) purple seed and stigma, (2) purple stigma and grey seed, (3) white stigma and purple seed, and (4) white stigma and grey seed. Their frequencies also deviated from the expected 81:63:63:49 ratio in F_2 and 1:3:3:9 in testcross progeny, with the corresponding LOD scores being > 3 (Table 2).

SSR patterns and their inheritance

When DNA samples from the two parental lines were amplified using the 61 primer pairs, only 18 pairs produced banding patterns that differed from the amplicons of the two parental lines. The remaining primers were monomorphic, *i.e.*, their amplicon banding patterns were similar to the two parents, and were not included in the present analysis. Of the 18 polymorphic primer pairs, two (PSMP2251 for LG 3 and PSMP2274 for LG 5) yielded patterns that differed from the parental lines but could not be distinguished in the F_1 hybrids, perhaps because of the occurrence of recombinant bands that made scoring difficult. For this reason, these two SSR markers were not considered for further analysis. As a result, only 16 primer pairs were used for further investigation. Table 3 summarizes the basic amplicon band patterns observed in the two parents with these 16 primers. For 13 of these 16 SSR primer pairs the segregation pattern of the amplicons in the F_2 progeny corresponded to the codominant segregation ratio of 1:2:1 ($p > 0.05$). The segregation pattern in the F_2 progeny with the remaining three primer pairs (PSMP2068, PSMP2220 and PSMP2273) deviated significantly from 1:2:1 ($p < 0.05$). Consequently, these three markers that showed skewed allelic frequencies or segregation distortion were not included in the subsequent joint segregation analysis.

Joint segregation patterns of the SSRs and purple phenotypic markers

Data on the joint segregation of pigmentation of the midrib complex with the 13 SSR markers showed a good fit to the 3:6:3:1:2:1 ratio expected based on independent assortment of these two types of markers. In all of these cases, the LOD scores were < 2. In the case of the SSR locus *Xpsmp*2086 (LG IV), the frequencies of the marker combination deviated significantly from the expected ratio ($p < 0.05$; Table 4) and the LOD score was 2.806.

The joint segregation pattern of purple pigmentation of leaf lamina with the 13 SSR loci was a good fit to the 9:18:9:7:14:7 ratio expected based on independent assortment of these markers ($p > 0.05$; data not shown). The LOD scores were < 2.4. Similarly, data for the joint segregation of panicle pigmentation with the 13 SSR markers (except *Xpsmp*2086) showed good correspondence to the 3:6:3:1:2:1 ratio ($p > 0.05$; data not shown). The corresponding LOD scores were < 2.2. The co-segregation of panicle pigmentation with *Xpsmp*2086 deviated significantly from the ratio expected for independent assortment ($p < 0.05$; Table 4). The LOD score was 2.87.

The joint segregation of purple stigma with the SSR markers showed a ratio of 9:18:9:7:14:7. Only one SSR locus (*Xpsmp*2206) deviated significantly from this ratio ($p < 0.05$; data not shown) with an LOD score of 2.613; there was no significant deviation with the other SSRs; the LOD values for joint segregation were < 2.2.

Table 4 - Linkage of the genes for pigmentation of the midrib complex, panicle and seed with SSR markers.

LG	SSR Locus	Phenotypic marker						Total	Chi-square	$P_{(Chi)}$	LOD score	$P_{(LOD)}$
		Purple			Non-purple							
		A	H	B	A	H	B					
1	*Xpsmp* 2090#	54	96	34	26	89	27	326	13.8173	0.02	3.0801	0.000083
4	*Xpsmp* 2086*	79	153	53	22	59	35	401	12.6725	0.03	2.8062	0.000103
	Xpsmp 2086**	79	152	53	22	60	35	401	13.0183	0.02	2.8746	0.000137
6	*Xpsmp* 2270#	51	94	26	46	71	21	309	18.3232	0.00	4.2857	0.000004

A – SSR pattern of parent A (purple accession), B – SSR pattern of parent B (green dwarf), H – SSR pattern of the hybrid A X B. LG – Linkage group to which SSR marker loci were previously assigned. *Joint segregation of gene for pigmentation of the midrib complex and the SSR marker locus; Chi-square and LOD values expected on the basis of a 3:6:3:1:2:1 ratio. **Joint segregation of gene for pigmentation of the panicle and the SSR marker locus; Chi-square and LOD values expected on the basis of a 3:6:3:1:2:1 ratio. #Joint segregation of genes for purple seed color and the SSR marker locus; Chi-square and LOD values expected on the basis of a 9:18:9:7:14:7 ratio.

In the case of purple seed and SSR markers, joint segregation yielded a ratio of 9:18:9:7:14:7. Chi-square analysis indicated significant deviation ($p < 0.05$; Table 4) from the expected ratio when the SSR locus *Xpsm*2090 on LG 1 was involved and the LOD value was 3.08. Another SSR locus, *Xpsmp*2270 (LG 6), also showed significant deviation ($p < 0.05$) and the LOD value was 4.29. For all of the other SSR markers used here, the observed values showed a good fit to the expected segregation ratios and gave LOD values < 2 (data not shown).

Recombination frequencies and map distances between the linked markers

The joint segregation patterns for purple seed and the SSR locus *Xpsmp*2090 (LG 1) and purple seed and the SSR locus *Xpsmp*2270 (LG 6) deviated significantly from independent assortment, as shown by Chi-square analysis and the LOD scores(see supplementary material Table S4 for details). Recombination frequencies for *Xpsmp*2090 were calculated using the Maximum Likelihood method of Allard (1956), as well as the method of Maximization of LOD scores (Snustad *et al.*, 1997); the averages of these two values are shown in Table 5. For the SSR locus *Xpsmp*2270, Allard's formula gave a linkage value of 50 that differed considerably from the results for the Chi-square and LOD analyses; hence this value was not considered here. When the LOD score threshold was set at 2.8, two more characters (purple midrib complex and purple panicle, see supplementary material Tables S2 & S3 for details) showed linkages with the SSR locus *Xpsmp*2086 (LG 4). The map distances for both of these phenotypic markers from *Xpsmp*2086 were the same (24 ± 3 cM; Table 5).

Discussion

Plants for which the parents and F1 progeny had purple foliage also generally showed pigmentation in the leaf sheath, midrib and lamina (including the margin). However, pigmentation of the lamina segregated from that of the leaf sheath, midrib and margin in the F_2 and testcross progeny. The 9:3:4 ratio of these three phenotypic categories in the segregating progeny indicated a recessive epistatic mode of gene interaction involving two loci. Pigmentation of the midrib, margin and sheath showed no separation in any of the progeny and was inherited *en bloc* in these three parts. When this character alone was analyzed using suitable crosses, a single dominant gene control of pigmentation was inferred. Similar conclusions regarding the *en bloc* inheritance and monogenic dominant nature of pigmentation in these plant parts have been reported by Manga *et al.* (1988).

Purple sheath and leaf were analyzed separately by Athwal and Gill (1966), Singh *et al.* (1968), Gill (1969) and Gill and Athwal (1970). Based on these earlier studies, Manga *et al.* (1988) inferred that pigmentation of the midrib, margin and sheath may reflect the close linkage of separate genes and proposed the gene symbols *Pmi, Pmg* and *Psh* for purple midrib, purple margin and purple sheath, respectively. Since the results described here were similar to those obtained by Manga *et al.* (1988) we chose to use the symbols defined by these authors. However, for the sake of convenience, Pmi_{Cx} (purple) is used hereafter to denote *Pmi, Pmg* and *Psh*, whereas pmi_{Cx} (green) is used to denote *pmi, pmg* and *psh*.

Athwal and Gill (1966), Gill (1969) and Gill and Athwal (1970) reported two-loci complementary gene control for purple leaf color and described this character under the term 'purple foliage'. The purple parent in the present study showed complete purple foliage, *i.e.,* purple sheath and lamina, including the midrib and margin. When lamina color alone was considered (purple or green) irrespective of that of the midrib, margin and sheath, the segregation ratio was 9:7 in F_2 and 1:3 in the testcrosses, suggesting the role of two complementary loci, in agreement with earlier studies of purple foliage.

When the homozygotes for purple sheath, midrib, margin and green lamina (isolated from the F_2 progeny) were crossed with the original purple parent (showing purple pigmentation in all of these parts), a 3:1 ratio for purple lamina versus green lamina was obtained in the F_2 generation. Since purple pigmentation in the leaf sheath, midrib and margin was a common character of these two parents segregation of these three parts was not expected. Hence, the single gene that segregated for lamina color must have been different from the gene controlling purple pigmentation in the other three parts. We propose the gene symbol

Table 5 - Z_{max} values, recombination frequencies and map distances between linked markers.

Markers	Z_{max}	Ø	Map distance (cM)*	MLH estimate of map distance**	Average map distance
Purple midrib complex and *Xpsmp*2086 (LG 4)	36.7907	0.187	18.7	24 ± 3	21 ± 3
Purple panicle and *Xpsmp*2086 (LG 4)	36.79067	0.187	18.7	24 ± 3	21 ± 3
Purple seed and *Xpsmp*2090 (LG 1)	30.53443	0.1841	18.4	26 ± 5	22 ± 4
Purple seed and *Xpsmp*2270 (LG 6)	20.16439	0.2329	23.29	-	23.3

LG – Linkage group. Z_{max} (maximum lod score). *Calculated based on the LOD score. **Maximum likelihood (MLH) estimate calculated as described by Allard (1956). Values are shown as the mean ± SEM.

'*Pl*' for the dominant allele governing the purple color of the lamina and '*pl*' for its recessive allele (green lamina).

Plants with a purple lamina and purple midrib complex represented nine sixteenths of the F_2 population and must have involved at least one dominant allele at each of the two loci. Using the gene symbols *Pl* and $Pmi_{Cx,}$ to designate the two characters, the genotype of this complete purple foliage in F_2 could be represented as *Pl* – Pmi_{Cx} –. As such, the genotype of plants with only a purple midrib complex (the lamina being green) would be *pl pl* Pmi_{Cx} –. The remaining two genotypes, *Pl* – pmi_{Cx} pmi_{Cx} and *pl pl* pmi_{Cx} pmi_{Cx} result in completely green foliage (in the lamina and midrib complex). The recessive locus pmi_{Cx} therefore exerts epistatic control on the *Pl* locus.

The purple internode character was inferred by Manga *et al.* (1988) to be controlled by a single dominant locus that was very close (2.17 map units) to the block of genes governing purple midrib, margin and sheath. Gill (1969) also studied the inheritance of purple internode (purple stem) and described the recessive nature of the purple pigmentation and the involvement of two loci with a dominant-recessive interaction. In contrast, Koduru and Krishna Rao (1979) suggested the presence of two dominant genes with a complementary action. These various patterns of genetic control can be attributed to differences in the genotypes used in these studies and suggest a more complicated genetic control than that revealed so far in the foregoing cases and in the present study. The gene symbol *Ps* proposed by Koduru and Krishna Rao (1979) is used here to describe this genetic control.

The results described here indicate that pigmentation in the four parts (internode, leaf sheath, midrib and margin) was inherited *en bloc* and behaved as if it were under the control of a single dominant locus. Since the purple parent used here was derived from mutagenic treatment (EMS and γ-rays) of the same line as used by Manga *et al.* (1988) it is possible that this treatment may have induced an inversion involving this genomic region, thereby bringing the '*Ps*' locus still closer (< 2.17 map units) to the Pmi_{Cx} locus. The inversion may have been pericentric or so cryptic that it was not identifiable by the presence of bridges and fragments in the anaphase stage of PMC meiosis. This inversion may have also reduced or suppressed recombination in this region, resulting in linkage disequilibrium of the two loci such that only the combinations of alleles causing pigmentation (or lack of it) in these four anatomical parts were able to segregate whereas the theoretically expected recombinants were not observed.

The purple pigmentation of panicles was inferred to be governed by a single dominant gene based on the 3:1 ratio in F_2 and 1:1 ratio in the testcrosses. We propose the gene symbol '*Pp*' for this locus. Interestingly, the purple panicle character co-segregated with the purple internode complex and their joint segregation showed a 3:1 ratio in F_2 and 1:1 ratio in the testcrosses. Only the parental combinations (purple midrib complex + purple panicle and green midrib complex + green panicle) appeared in these segregating generations; no recombinants were observed. Two possible explanations could account for this type of co-segregation: (1) the gene for purple panicle may be located very close to the loci for purple pigmentation of the midrib complex or, alternately, (2) one of the loci involved in the midrib complex may also govern panicle pigmentation in a pleiotropic manner.

Joint segregation of the purple midrib complex with purple stigma or purple seed revealed the same pattern as observed between the purple midrib complex and purple lamina, *i.e.,* a two-loci recessive epistatic gene interaction. This finding suggested that the locus governing the purple midrib complex was also involved in the purple pigmentation of the stigma and seed. Hence, assuming the same logic as set out above in the case of joint segregation involving the purple midrib complex and purple lamina, the following genotypes could be deduced for plants with purple stigma and purple seeds:

Pmi_{Cx} , *Pst* Purple midrib complex and purple stigma (9/16 in F_2)

Pmi_{Cx} , *pst pst* Purple midrib complex and white stigma (3/16 in F_2)

pmi_{Cx} pmi_{Cx} , *Pst* and pmi_{Cx} pmi_{Cx} , *pst pst* Green midrib complex and white stigma (4/16 in F_2)

Pmi_{Cx} , *Psd* Purple midrib complex and purple seed (9/16 in F_2)

Pmi_{Cx} , *psd psd* Purple midrib complex and grey seed (3/16 in F_2)

pmi_{Cx} pmi_{Cx} , *Psd* , and pmi_{Cx} pmi_{Cx} , *psd psd* Green midrib complex and grey seed (4/16 in F_2)

When the F_2 population was classified into purple and non-purple categories, regardless of the color of the midrib complex, the data provided a good fit to the 9:7 ratio. As a further check, the observed frequencies of the phenotypic categories for each of the three cases of joint segregation of the purple midrib complex with (1) lamina pigmentation, (2) stigma color and (3) seed color were also tested for their conformity to the 27:21:16 ratio expected based on three independent loci (one locus for pigmentation of the midrib complex showing recessive epistasis to two independent complementary loci for each of the other three characters) and to a 27:21:9:7 ratio (without assuming recessive epistatis). The observed frequencies in all three cases deviated significantly from the expected ratios for these two segregation patterns, suggesting that either the three locus model is not applicable or that, if three or more loci are involved, then the purple midrib complex locus must be linked with at least one of the loci governing the other character. Further resolution of these linkage relationships was not possible with the present data. The two locus model, which is simpler, was used in this work.

Each of the joint segregation patterns involving (1) lamina pigmentation and stigma color, (2) lamina pigmen-

tation and seed color, and (3) stigma and seed pigmentation deviated significantly from the 81:63:63:49 ratio expected based on four independent loci (with two complementary loci for each character). If one locus is assumed to be common to the two characters, then the theoretical ratio expected based on complementary gene action involving three loci would be 27:9:9:19 (*i.e.*, both characters purple, first one purple and second green, first one green and second purple and both characters green, respectively). The observed frequencies for these character combinations also deviated from this ratio. The significant deviations from both of these independent assortment expectations, together with the high LOD scores, suggested that more than one locus was involved in the expression of purple color in these parts and that either one of these loci was common to all of these markers or that at least one locus for each might be linked to one for the other characters. The presence of gene interactions meant that it was not possible to identify which of these loci were actually linked or to determine their map distances.

The purple midrib complex in the recessive homozygous state (pmi_{Cx} pmi_{Cx}) was inferred to prevent lamina pigmentation by acting as a recessive loss-of-function allele. Several dominant inhibitor loci associated with anthocyanin pigmentation have been reported in rice (see Reddy 1996). For example, the dominant allele *Ilb* in rice inhibits purple color development in the leaf blade. Pmi_{Cx} of the present work is comparable to *Ilb* of rice, with the difference that pmi_{Cx} is inhibitory in a recessive state while *Ilb* is inhibitory in a dominant state. Another inhibitory gene that inhibits gene color is *C1* in maize (Cone *et al.*, 1986). This inhibitory locus encodes a DNA-binding protein that is associated with regulation of the anthocyanin biosynthetic pathway. Such inhibitor alleles may be very useful tools in aiding our understanding of the genetic regulatory mechanisms involved in biosynthetic pathways such as those leading to anthocyanin production (Reddy, 1996).

Based on the foregoing discussion of the joint segregation of pigmentation markers, the following model is suggested for the genetic architecture of purple pigmentation in pearl millet. This model assumes that a functional allele at the Pmi_{Cx} locus is a prerequisite for the development of purple pigmentation in at least four other parts (lamina, panicle, stigma and seed). With the exception of panicle, pigment development in the remaining plant parts requires additional loci, at least one for each part. Thus, Pmi_{Cx} may regulate the basic steps in anthocyanin pigment development while other loci control the formation of color in specific plant parts. In rice, the genes associated with anthocyanin pigment development form three groups, namely, (1) structural genes responsible for pigment development, (2) regulatory genes responsible for the distribution of pigment in various plant parts, and (3) inhibitory genes responsible for the suppression of pigmentation in specific plant parts.

A similar classification of structural and regulatory genes is also possible in maize, where about 20 genes are reported to be involved in the development of pigmentation in various plant parts (reviewed by Mol *et al.*, 1998). About seven structural genes encode the biosynthetic enzymes that are coordinately controlled at the transcriptional level by the products of at least two groups of regulatory genes; these genes are responsible for the development and tissue-specific pigmentation of plant and seed tissues in maize. By analogy with the pigment system in rice and maize, the Pmi_{Cx} gene in pearl millet is a structural gene responsible for the production of anthocyanin and pmi_{Cx} is its recessive allele. The remaining loci (*Ps, Pl, Pp, Pst* and *Psd*) may have a regulatory role and control the development of pigment in the internode, lamina, panicle, stigma and seed respectively.

SSR analysis and gene tagging

The pigmentation of lamina did not deviate from the expected values for independent assortment with any of the 13 SSR loci used in this study; indeed, the corresponding LOD scores were < 2, indicating that genes controlling these morphological markers sorted independently of the 13 SSR marker loci. Purple pigmentation of the midrib complex, panicle, stigma and seeded deviated significantly from independent assortment with some of the SSR marker loci, as shown by the Chi-square values. When the threshold value for the LOD score was held at 3, only purple seeds showed linkage with *Xpsmp*2090 (LG 1) and *Xpsmp*2270 (LG 6).

While a LOD score threshold of 3 is widely considered as a definite indication of a "significant association" among traits, this threshold is nevertheless somewhat arbitrary (Nyholt, 2000). Lander and Kruglyak (1995) proposed the term 'suggestive linkage' for cases that are not significant (< 3) but point to a certain level of association between the markers and the trait, based on other considerations. Van Ooijen (1999) used the LOD threshold of 2.7 to establish suggestive linkage in F_2 populations. With reference to characters involving epistasis, quantitative trait loci (QTL) analysis in maize used LOD scores > 2.2 as indications of linkage (Schön *et al.*, 1993; Lübberstedt *et al.*, 1998; Cardinal *et al.*, 2001; Krakowsky *et al.*, 2002). Casley *et al.* (1999) used LOD threshold values of 2-2.8 in their detection of QTLs that affected caffeine metabolism in mice.

In view of these suggestions, when the threshold limit for a significant LOD score was kept at 2.8, two more phenotypic markers could be considered as linked to SSR markers, namely, (1) the purple midrib complex with *Xpsmp*2086 (LG 4) and (2) purple panicle with *Xpsmp*2086 (LG 4). Interestingly, genes controlling both of these morphological traits were associated with the same SSR marker. As already discussed above, the locus for purple pigmentation of the midrib complex might either be closely linked to the single locus controlling panicle pigmentation

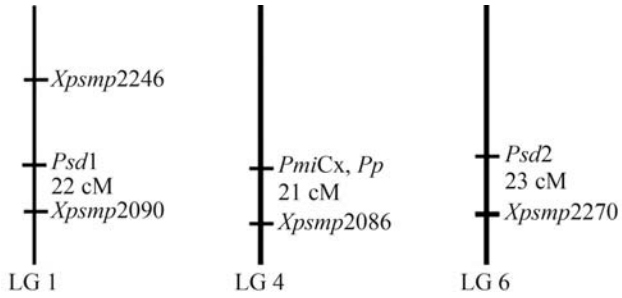

Figure 2 - Diagrammatic representation of the tentative locations (not drawn to scale) of the loci for phenotypic markers in relation to the SSR loci.

or might also govern the pigmentation of earheads in a pleiotropic manner. In either case, this assumption is supported by the inference that genes associated with both of these morphological markers are associated with the same SSR marker and with the same degree of suggestive linkage. However, even this SSR analysis could not clarify whether these two morphological traits involved close linkage or pleiotropism. The location *of Pmi*Cx on LG 4 agreed with the findings of Azhaguvel *et al.* (2003) in pearl millet who inferred 'loose linkage' between the purple foliage locus and RFLP markers on LG 4.

The linkage between genes controlling the purple seed trait and two SSR loci mapping on two independent linkage groups (1 and 2) indicated that two independent loci were likely to be involved in the expression of this trait. This conclusion supports the observation that more than one locus (in addition to the purple midrib complex locus) is involved in controlling seed pigmentation.

The apparent independent assortment observed between some of the markers may indicate either that the locus is not present in the same linkage group, or that its position in the linkage group may be located = 50 recombination units (Ø = 0.5) from other loci. The tentative map locations of the four phenotypic marker loci are depicted in Figure 2.

Acknowledgments

The authors thank ICRISAT for providing access to the generous facilities of the Plant Sciences Research Programme R8183 funded by funded by the UK Department for International Development (DFID) and administered by CAZS Natural Resources for the benefit of developing countries. The general facilities provided by the Department of Botany under the auspices of UGC SAP and COSIST are also gratefully acknowledged. P.V. thanks CSIR, New Delhi, for providing a research fellowship.

References

Al-Fakhry AK, Grogan CO and Sarvella P (1965) Some genetic studies on babala, *Pennisetum typhoideum* L.(Rich.). Z Pflanzenzuecht 54:182-187.

Allard RW (1956) Formulas and tables to facilitate the calculation of recombination values. Hilgardia 24:235-279.

Allouis S, Qi X, Lindup S, Gale MD and Devos KM (2001) Construction of a BAC library of pearl millet, *Pennisetum glaucum*. Theor Appl Genet 102:1200-1205.

Anand Kumar K and Andrews DJ (1993) Genetics of qualitative traits in pearl millet: A review. Crop Sci 33:1-19.

Athwal DS and Gill BS (1966) Inheritance of bristling and purple pigmentation in *Pennisetum typhoides* (Burm.) S & H J Res Punjab Agric Univ 3:253-259.

Azhaguvel P, Hash CT, Rangasamy P and Sharma A (2003) Mapping the d_1 and d_2 dwarfing genes and purple foliage colour locus P in pearl millet. J Hered 94:155-159.

Cardinal AJ, Lee M, Sharpova N, Woodman-Clikeman WL and Long MJ (2001) Genetics, mapping and analysis of quantitative trait loci for resistance to stalk tunneling by the European corn borer in maize. Crop Sci 41:835-845.

Casley WL, Menzies JA, Whitehouse LW and Moon TW (1999) Detection of quantitative trait loci affecting caffeine metabolism by internal mapping in a genome-wide scan of C_3H/He J X APN F_2 mice. Drug Metab Dispos 27:1375-1380.

Cone KC, Burr FA and Burr B (1986) Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc Natl Acad Sci USA 83:9631-9635.

Devos KM, Pittaway TS, Renolds A and Gale MD (2000) Comparative mapping reveals a complex relationship between the pearl millet genome and those of foxmail millet and rice. Theor Appl Genet 100:190-198.

Gill BS (1969) Inheritance of pigmentation in some plant parts in pearl millet. Ind J Genet 29:468-472.

Gill BS and Athwal DS (1970) Genetics of some ear characters and some plant pigmentation in pearl millet. Ind J Genet 30:519-525.

Hanna WW and Burton GW (1992) Genetics of red and purple plant color in pearl millet. J Hered 83:386-388.

Hash CT (2004) Molecular markers and marker-assisted selection for pearl millet in India. In: Raney T (ed) The State of Food and Agriculture 2003-04. Part I. Agricultural Biotechnology: Meeting the Needs of the Poor? FAO, Rome, pp 14.

IBPGR and ICRISAT (1993) Descriptors for pearl millet, *Pennisetum glaucum* (L.) R. Br. IBPGR, Rome, and ICRISAT, Patancheru, pp 1-48.

Koduru PRK and Krishna Rao M (1979) Inheritance of anthocyanin and waxy stem in *Pennisetum*. J Cytol Genet 14:18-21.

Krakowsky MD, Brinkman MJ, Woodman-Clikeman WL and Lee M (2002) Genetic components of resistance to stalk tunneling by the European corn borer in maize. Crop Sci. 42:1309-1315.

Lander ES and Kruglyak L (1995) Genetic dissection of complex traits: Guidelines for interpreting and reporting linkage results. Nat Genet 11:241-247.

Lathrop GM and Lalouel JM (1984) Easy calculation of LOD scores and genetic risks on small computers. Am J Hum Genet 198:460-465.

Liu CJ, Witcombe JR, Pittaway TS, Nash M, Hash CT, Busso CS and Gale MD (1994) An RFLP-based genetic map of pearl millet (*Pennisetum glaucum*). Theor Appl Genet 89:481-487.

Lübberstedt T, Klein D and Melchinger AE (1998) Comparative QTL mapping of resistance to *Ustilago maydes* across four populations of European flint maize. Theor Appl Genet 97:1321-1330.

Manga V, Saideswara Rao Y, Raju NSN and Subba Rao MV (1988) Inheritance and linkage of genes controlling some purple phenotypes in pearl millet, *Pennisetum americanum* (L.) leeke. Genet Iberica 40:133-141.

Mol J, Grotewold E and Koes R (1998) How genes paint flowers and seeds. Trends Plant Sci 3:212-217.

Morton NE (1955) Sequential tests for the detection of linkage. Am J Hum Genet 7:277-318.

Nyholt DR (2000) All LODs are not created equal. Am J Hum Genet 67:282-288.

Phul PS, Athwal BS and Gill BS (1969) Genetics of grain color in pearl millet. Ind J Genet Plant Breed 29:438-445.

Qi X, Pittaway TS, Lindup S, Liu H, Waterman E, Padi FK, Hash CT, Zhu J, Gale MD and Devos KM (2004) An integrated genetic map and a new set of simple sequence repeat marker for pearl millet, *Pennisetum glaucum.* Theor Appl Genet 109:1485-1493.

Reddy AR (1996) Genetic and molecular analysis of the anthocyanin pigmentation pathway in rice. Rice genetics III. Proceedings of the Third International Rice Genetics Symposium,16-20 October, 1995. Manila, pp 341-352.

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW (1984) Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014-8018.

Schön CC, Lee M, Melchinger AE, Guthriew D and Woodman WL (1993) Mapping and characterization of quantitative trait loci affecting resistance against second-generation European corn borer in maize with the aid of RFLPs. Heredity 70:648-659.

Serraj R, Hash CT, Rizvi SMH, Sharma A, Yadav RS and Bidinger FR (2005) Recent advances in marker-assisted selection for drought tolerance in pearl millet. (Proceedings of the Fifth Asian Crop Science Conference). Plant Prod Sci 8:334-337.

Singh D, Mishra SW, Singh AB and Singh SP (1967) Inheritance in pearl millet. Ind J Genet 27:426-428.

Singh D, Lal S and Yadav HR (1968) Inheritance of certain qualitative characters in pearl millet (*Pennisetum typhoides* S&H). J Indian Bot Soc 47:388-395.

Snedecor GW and Cochran WG (1967) Statistical Methods. Oxford and IBH Publishing Company, New Delhi, 503 pp.

Snustad DP, Simmons MJ and Jenkins JB (1997) Principles of Genetics. John Wiley and Sons Inc., New York, 740 pp.

Tegelstrom H (1992) Detection of mitochondrial DNA fragments. In: Hoelzel AR (ed) Molecular Genetic Analysis of Populations: A Practical Approach. IRL Press, Oxford, pp 89-114.

Van Ooijen JW (1999) LOD significance thresholds for QTL analysis in experimental populations of diploid species. Heredity 83:613-624.

Zhou M (2010) Genotyping and phenotyping errors are the two major reasons that reduce the accuracy of QTL results. Plant Breed 130:203-208.

Internet Resources

Anonymous (2005) *Arabidopsis* nomenclature. http://www.arabidopsis.org/info/guidelines.jsp. (November 10, 2007).

FAO (2005) Global network on integrated soil management for sustainable use of salt-affected soils. Rome, Italy: FAO Land and Plant Nutrition Management Service. http://www.fao.org/ag/agl/agll/spush. (November 10, 2007).

Supplementary Material

The following online material is available for this article:

Table S1: List of 61 SSR primers.

Table S2: Linkage assessment of the gene for midrib complex color and pearl millet SSR marker loci.

Table S3: Linkage assessment of the gene for panicle pigmentation and SSR marker loci.

Table S4: Linkage assessment of the genes for purple seed color with SSR marker loci.

This material is available as part of the online article from http://www.scielo.br/gmb.

Associate Editor: Everaldo Gonçalves de Barros

Table S1 - List of 61 SSR primers of pearl millet and their characteristics.

Linkage group	SSR marker tried	Primer sequence (F/R)	PCR product (bp)	Remark
LG 1	PSMP2006	GACTTATAGTCACTGGGAAAGCTC GCTTTAATAACTTTGTGCGTATT	253	L
	PSMP2030	ACCAGAGCTTGGAAATCAGCAC CATAATGCTTCAAATCTGCCACAC	107	M
	PSMP2069	CCCATCTGAAATCTGGCTGAGAA CCGTGTTCGTACAAGGTTTTGC	225	M
	PSMP2090	AGCAGCCCAGTAATACCTCAGCTC AGCCCTAGCGCACAACACAAACTC	178	P
	PSMP2232	TGTTGTTGGGAGAGGGTATGAG CTCTCGCCATTCTTCAAGTTCA	233	M
	PSMP2246	CGGATGCTAAATTAACCGAAGC CCAGCTTGCTTCTGTT**G**CGTTC	262	P
	PSMP2273	AACCCCACCAGTAAGTTGTGCTGC GATGACGACAAGACCTTCTCTCC	169	P
LG 2	PSMP2050	ATCAAACGGCATCAGACAAC GGATCTCTTAGTGTGGTGGAGAGC	102	M
	PSMP2059	GGGGAGATGAGAAAACACAATCAC TCGAGAGAGGAACCTGATCCTAA	119	M
	PSMP2066	ATATTAGAGCATTGCATCGC GCATAGCAGCATACAGCAGCAACTAA	267	M
	PSMP2068	CAATAACCAAACAAGCAGGCAG CTTCACTCCCACCCTTTCTAATTC	105	P
	PSMP2072	GAAATCTACACAAGGGTCTCCA GTACGGCAGAATGACATCTGAA	165	M
	PSMP2077	GCCAATATTATTCCCAAGTGAACA CTCTTGGTTGCATATCTTTCTTTT	180	M
	PSMP2088	AAGAAGCCACCAGCACAAAA TGCATGAAAGTAGAGGATGGTAAA	149	M
	PSMP2089	TTCGCCGCTGCTACATACTT TGTGCATGTTGCTGGTCATT	127	P
	PSMP2201	CCCGACGTTATGCGTTAAGTT A TCCATCCATCCATTAATCCAC	364	M
	PSMP2206	AGAAGAAGAGGGGGTAAGAAGGAG AGCAACATCCGTAGAGGTAGAAG	203	P
	PSMP2211	CTGCATGACGTGTGAACCAATACC AACAAATCAGCACCAGCCTCC	256	L
	PSMP2231	TGTTGTTGGGAGAGGGTATGAG CTCTCGCCATTCTTCAAGTTCA	226	P
	PSMP2232	TGTTGTTGGGAGAGGGTATGAG CTCTCGCCATTCTTCAAGTTCA	230	M
	PSMP2237	TGGCCTTGGCCTTTCCACGCTT CAATCAGTCCGTAGTCCACACCCCA	233	P

	PSMP2255	CATCTAAACACAACCAATCTTGAAC TGGCACTCTTAAATTGACGCAT	264	P
LG 3	PSMP2056	ACCTGTAGCTTCAAAATTCAAAAA AATTCAGTGTGATTTCGATGTTGC	213	L
	PSMP2070	ACAGAAAAAGAGAGGCACAGGAGA GCCACTCGATGGAAATGTGAAA	226	M
	PSMP2071	TTGCAGTCCCACGAATTATTTG CTTTGAATTTATAATCCTCATACT	181	M
	PSMP2214	CGCACAGTACGTGTGAGTGAAG GATTGAGCAGCAAAAACCAGC	246	M
	PSMP2227	ACACCAAACACCAACCATAAAG TCGTCAGCAATCACTAATGACC	197	M
	PSMP2251	TCAAACATAGATATGCCGTGCCTCC CAGCAAGTCGTGAGGTTCGGATA	162	P
LG 4	PSMP2267	GGAAGGCGTAGGGATCAATCTCAC ATCCACCCGACGAAGGAAACGA	241	M
	PSMP2008	GATCATGTTGTCATGAATCACC ACACTACACCTACATACGCTCC	238	L
	PSMP2076	GGAATAGTATATTGGCAAAATGTG ATACTACACACTGTAAGCATTGTC	161	M
	PSMP2081	CTGTGCTGTCATTGTTACCA TCAGATCACCTATTACTTTCCCT	167	M
	PSMP2084	AATCTAGTGATCTAGTGTGCTTCC GGTTAGTTTGTTTGAGGCAAATGC	245	M
	PSMP2085	GCACATCATCTCTATAGTATGCAG GCATCCGTCATCAGGAAATAA	176	M
	PSMP2086	CGCTTGTTTTCCTTTCTTGCTGTT CCTTCTCAGATCCTGTGCTTTCTT	122	P
LG 5	PSMP2001	CATGAAGCCAATTAGGTCTC ACCATCTGACTTGTTCTTATCC	304	M
	PSMP2064	ACCGAATTAAAGTCATGGATCG TTGATCTTCTGACACAAATGAG	190	M
	PSMP2078	CATGCCCATGACAGTATCTTAAT ACTGTTCGGTTCCAAAATACTT	172	P
	PSMP2202*	CTGCCTGTTGAGAATAATGAG GTTCCGAATATAGAGCCCAAG	161	P
	PSMP2208*	GAAAGAGCAAACTGAACAATCCC ACTTTGCCCTGGATGATCCTC	246	M
	PSMP2220*	GCATCCTTCACCATTCAAGACA TGGGAAACAGAATGGAGAAAAGAG	128	P
	PSMP2233	TGTTTTCTCCTCTTAGGCTTCGTTC ACCTTCTCCGCCACTAAACAACT	258	M
	PSMP2274*	CACCTAGACTCTACACAATGCAAC AATATCAAGTGATCCACCTCCCAA	265	P
LG 6	PSMP2018	CGCAAGACATTTTAGTATCACC ACAGTCATCCTCAGTCGTCC	203	L

	PSMP2048	TGAATTGGGAATAAAGGAGACC ACGTGTGCCTGCTTTTAGTAAC	252	L
	PSMP2213*	CCCAAAAGAACCACACCCAC GTTGATGCTACTGCTCGTTTTG	193	M
	PSMP2248	TCTGTTTIGTTTGGGTCAGGTCCTTC CGAATACGTATGGAGAACTGCGCATC	167	M
	PSMP2270	AACCAGAGAAGTACATGGCCCG CGACGAACAAATTAAGGCTCTC	153	P
LG 7	PSMP2013	GTAACCCACTAACCCTTACC GTCGCACAGAAAAAGAATAG	153	M
	PSMP2019	TGTGCCACAGCTTGTTCCTC CAAGCAGCCAGTTCCTCATC	248	M
	PSMP2027	AGCAATCCGATAACAAGGAC AGCTTTGGAAAAGGTGATCC	273	M
	PSMP2033	CTATACCATTGAATTGAAAGGTC CAATCTTTAGCTTTTTCAAGAGAC	200	M
	PSMP2040	CATTACACGTTTCTTCAAACGC TCTTCGGCCTAATAGCTCTAAC	163	M
	PSMP2043	TCATATTCTCCTGTCTAAAACGTC ACAAATCGTACAAGTTCCACTC	192	L
	PSMP2063	GAGCACATGAAATAGGAAGCAG AAGGTAGTTATAGTTAGCTTGATC	166	M
	PSMP2079	AGCCGAAGGCTAATCAACAA GTGGTCAGCAGCAGATGTAA	165	L
	PSMP2087	GGAACAGACTCCATACCTGAAA TACCTGCCTGTGCTGTTAGT	126	L
	PSMP2203*	GAACTTGATGAGTGCCACTAGC TTGTGTAGGGAGCAACCTTGAT	357	M
	PSMP2224	GGCGAATTGGAATTCAGATTG CGTAATCGTAGCGTCTCGTCTAA	157	P
	PSMP2263	AAAGTGAATACGATACAGGAGCTGAG CATTTCAGCCGTTAAGTGAGACAA	238	P
	PSMP2266	CAAGGATGGCTGAAGGGCTATG TTTCCAGCCCACACCAGTAATC	181	L

L – low resolution, M – monomorphic, P – polymorphic. *T_m = 58 °C while for all other primers, it was 61 °C.

Table S2 - Linkage assessment of the gene for midrib complex color and pearl millet SSR marker loci.

LG	SSR locus	Midrib complex color						Total	Chi-square	$P_{(Chi)}$	LOD score*	$P_{(LOD)}$
		Purple			Green							
		A	H	B	A	H	B					
1	*Xpsmp* 2246	54	136	51	27	46	15	329	8.1996	0.15	1.7960	0.002014
	Xpsmp 2090	65	126	49	15	55	17	327	9.6932	0.08	2.0792	0.000986
2	*Xpsmp* 2255	62	150	82	28	50	29	401	3.8944	0.56	0.8647	0.022990
	Xpsmp 2089	47	119	61	19	43	22	311	3.1522	0.68	0.7120	0.035087
	Xpsmp 2231	64	130	48	21	49	19	331	5.0725	0.41	1.1432	0.010881
	Xpsmp 2237	56	124	61	22	49	19	331	2.2367	0.81	0.4734	0.069903
	Xpsmp 2206	61	121	58	25	40	24	329	1.8369	0.87	0.3800	0.092930
4	*Xpsmp* 2086	79	153	53	22	59	35	401	12.6725	0.03	2.8062	0.000103
5	*Xpsmp* 2202	50	119	50	15	44	16	294	4.0045	0.55	0.8660	0.022911
	Xpsmp 2078	56	109	50	24	39	17	295	2.4554	0.78	0.5051	0.063606
6	*Xpsmp* 2270	57	120	53	18	47	22	317	2.5878	0.76	0.5504	0.055686
7	*Xpsmp* 2224	54	128	46	17	45	18	308	5.4286	0.37	1.1996	0.009377
	Xpsmp 2263	61	107	51	16	53	10	304	8.2105	0.14	1.6583	0.002860

A - SSR pattern of parent A (purple accession), B - SSR pattern of parent B (green dwarf), H - SSR pattern of the hybrid A X B. LG – Linkage group to which SSR marker loci were previously assigned. *Chi-square of LOD values expected on the basis of a 3:6:3:1:2:1 ratio for the joint segregation of the genes for pigmentation of the midrib complex and the SSR marker locus.

Table S3 – Linkage assessment of the gene for panicle color and pearl millet SSR marker loci.

LG	SSR locus	Panicle color						Total	Chi-square	$P_{(Chi)}$	LOD score*	$P_{(LOD)}$
		Purple			Green							
		A	H	B	A	H	B					
1	*Xpsmp* 2246	54	135	51	27	47	15	329	8.2644	0.14	1.8086	0.001951
	Xpsmp 2090	65	125	49	15	56	17	327	10.362	0.07	2.2020	0.000725
2	*Xpsmp* 2255	63	152	77	289	49	32	401	4.3267	0.50	0.9281	0.019350
	Xpsmp 2089	47	118	61	19	44	22	311	3.3580	0.64	0.7532	0.031211
	Xpsmp 2231	64	129	48	21	50	19	331	5.3786	0.37	1.2026	0.009302
	Xpsmp 2237	56	123	61	22	50	19	331	2.6395	0.75	0.5534	0.055191
	Xpsmp 2206	61	120	58	25	41	24	329	1.8531	0.87	0.3836	0.091901
4	*Xpsmp* 2086	79	152	53	22	60	35	401	13.0183	0.02	2.8746	0.000137
5	*Xpsmp* 2202	52	119	49	13	44	16	293	4.8749	0.43	1.0813	0.012823
	Xpsmp 2078	56	109	49	23	41	17	295	2.4554	0.78	0.5186	0.061128
6	*Xpsmp* 2270	57	119	53	20	48	22	319	2.7806	0.73	0.5873	0.050025
7	*Xpsmp* 2224	54	128	46	17	25	18	288	8.3056	0.14	1.8603	0.001711
	Xpsmp 2263	61	106	51	16	54	16	304	9.1579	0.10	1.8364	0.0018018

A - SSR pattern of parent A (purple accession), B - SSR pattern of parent B (green dwarf), H - SSR pattern of the hybrid A X B. LG – Linkage group to which SSR marker loci were previously assigned. *Chi-square of LOD values expected on the basis of a 3:6:3:1:2:1 ratio for the joint segregation of the genes for pigmentation of the panicle and the SSR marker locus.

Table S4 - Tagging of the genes for purple seed color with pearl millet SSR marker loci.

LG	SSR locus	Seed color						Total	Chi-square	$P_{(Chi)}$	LOD score*	$P_{(LOD)}$
		Purple			Pearly							
		A	H	B	A	H	B					
1	*Xpsmp* 2246	43	98	40	35	82	24	322	6.8148	0.24	1.5601	0.054133
	Xpsmp 2090	54	96	34	26	89	27	326	13.8173	0.02	3.0801	0.000083
2	*Xpsmp* 2255	52	95	59	39	88	45	378	2.6688	0.75	0.5816	0.05086
	Xpsmp 2089	26	92	47	33	58	35	291	8.4661	0.13	1.9972	0.001212
	Xpsmp 2231	55	96	35	30	80	31	326	7.1352	0.21	1.5879	0.003424
	Xpsmp 2237	44	100	43	32	80	28	327	4.0659	0.54	0.9047	0.020618
	Xpsmp 2206	51	80	53	32	74	31	321	4.2597	0.51	0.9180	0.019886
4	*Xpsmp* 2086	59	103	41	34	87	41	365	4.7750	0.44	1.0652	0.013386
5	*Xpsmp* 2202	37	82	35	26	83	31	294	8.0857	0.15	1.6894	0.002641
	Xpsmp 2078	46	74	29	33	68	33	283	5.1597	0.40	1.1739	0.010034
6	*Xpsmp* 2270	51	94	26	46	71	21	309	18.3232	0.00	4.2857	0.000004
7	*Xpsmp* 2224	38	78	34	29	73	28	280	3.3542	0.64	0.7095	0.035335
	Xpsmp 2263	46	77	32	30	73	31	289	4.3764	0.50	0.9612	0.017693

A - SSR pattern of parent A (purple accession), B - SSR pattern of parent B (green dwarf), H - SSR pattern of the hybrid A X B. LG – Linkage group to which SSR marker loci were previously assigned. *Chi-square of LOD values expected on the basis of a 9:18:9:7:14:7 ratio for the joint segregation of the genes for purple seed color and the SSR marker locus.