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Identification of microsatellite markers associated with staygreen trait in wheat RILs

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Abstract

The Recombinant Inbred Lines in F_7 generation derived from two crosses (Ning8201 x Sonalika and Yangmai6 x Sonalika) along with parents were evaluated for varying degree of expression for staygreen, for two consecutive years in two replications. LAUG values ranged from 73.87 to 75.62 in staygreen parents, Ning8201 and Yangmai6, as compared to non-staygreen parent Sonalika (10.38). F_1 showed intermediate behaviour for staygreen expression indicating additive nature of inheritance for the trait. The distribution of RILs was normal indicating polygenic nature of the trait. Analysis of variance for LAUG displayed significant GxE interaction. Of the 119 SSR markers used in two crosses, 22 were polymorphic on parents. In Ning8201 x Sonalika, SSR markers WMC-10, WMC-74, and WMC-76 were significantly ($p<0.001$) linked with staygreen trait. In the other cross (Yangmai6 x Sonalika) the markers BARC-109, BARC-1120, BARC-04 and BARC-74 were significantly linked. The polymorphic markers for both crosses grouped under one linkage group (MAPMAKER/ EXP 3.0b). Two QTLs were detected in Ning8201 x Sonalika cross (R^2=8.28) and (R^2=18.11). A major QTL was detected in Yangmai6 x Sonalika, which alone explained 44.34% of the phenotypic variation. The rest of the loci contributed on an average 12.9% to the total phenotypic variability. The linked markers obtained in the present study for staygreen trait can be used further for fine mapping and cloning of gene that can be used further for molecular assisted breeding in wheat.

Key words: Wheat, staygreen, LAUG, microsatellite markers, RILs

Introduction

Staygreen generally refers to delay in senescence in crops [1] and is considered an important trait. This trait, is believed to affect radiation use efficiency, nutrient mobilization and also proved important under heat stress [2-4]. Occurrence of staygreen trait reported in different crops has been widely used in breeding for tolerance to heat and draught, disease resistance such as stem rot [5, 6]. Positive correlation of staygreen trait with high grain yield has been reported in wheat [2], sorghum [7] and maize [8]. Positive correlation between staygreen and spot blotch resistance [9] and heat tolerance under delayed sowing [2] has also been reported. In durum wheat, a staygreen mutant is associated with increase in leaf area, rate and duration of grain filling and photosynthetic competence [10]. Staygreen duration of flag leaf and harvest index showed positive correlation with water use efficiency during grain formation of wheat [11]. Since there is a strong association between the duration of photosynthetically active leaf area and grain yield [12], selection for staygreen trait is expected to have a significant implication in productivity of wheat particularly under harsh environments [4]. The genetic basis of staygreen trait has been studied in some of the crops like rice, soybean, sorghum and sunflower. In wheat, only one gene with two alleles was reported to control staygreen trait; this gene had high heritability and showed partial dominance with additive effects [13]. However the staygreen trait has been reported under the control of polygens having additive effects [9].

Traditional methods of plant breeding have made a significant contribution to crop improvement, but they have been rather slow in targeting polygenic traits [14]. Molecular markers can serve as a potential tool for

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tagging of genes polygenic in nature and of economic importance. Microsatellites, that are simple sequence repeats of only a few base pairs (<6), have emerged as an important source of ubiquitous genetic marker system. Tagging a gene with molecular marker requires a mapping population. Of the various populations, RILs are more rewarding if the purpose is to tag or map the QTL since RILs provide maximum opportunity of recombination to take place between gene and marker [14]. Genetic mapping of the QTL conferring staygreen has been undertaken in different crop species [15-17]. Malone *et al*. [18] used co-dominant primers (SCAR) developed from AFLP markers to map staygreen gene in wheat using 6 NILs.

Assay at molecular level shows that microsatellite markers are abundant in wheat [19]. Reports on the use of molecular markers to characterize staygreen gene in wheat are scanty. In view of absence of knowledge about any SSR markers for staygreen in wheat, the present investigation was undertaken with the objective to find SSR markers for this trait.

Materials and methods

The experiment was conducted during *rabi* 2005 and 2006, at the Research Farm, of Banaras Hindu University, Varanasi. Off-season facility at IARI Regional Station, Wellington, Tamil Nadu (India) was utilized to advance generations of the crosses. The RILs were derived from the crosses between two parents Ning 8201 and Yangmai 6 with stay green traits and a non-stay green parent Sonalika, and advanced through single plant descent from individual F_2 plants. Random F_2 derived F_3 populations of the two crosses were advanced to F_4 following the pedigree method where a random plant in each generation from each line was harvested [9]. Before making the cross, one generation of the parents were subjected to selfing by bagging to avoid any out crossing. Only selfed seeds were used for making the crosses.

The F_4 lines were obtained from around 140-150 randomly chosen space planted F_3 plants. Half of the seed of F_4 plants was advanced to F_5 at the off-season nursery, Wellington. Following similar approach, F_6, F_7, and F_8 lines were obtained. Thus 130-150 lines of F_6, F_7, and F_8 generations were raised for three consecutive years. For crop protection tilt (propiconazole; [1-{[2-(2,4-dichlophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl}-1H-1,2,4-triazole]) @ 625 g a.i./hectare was sprayed at two growth stages [20], GS 54 and GS 69 to prevent spot blotch and leaf rust, the two most important diseases of eastern India. The material was planted in three rows of 3m with 30cm distance between the rows with approximately 40-50 plants per row. Staygreen and non-staygreen parents were planted after every 20 rows for comparison.

Staygreen assessment

Staygreen trait was measured using two approaches (i) difference of leaf and spike greenness scores in 0-9 scale (modified version of 1-10 scale of Silva *et al*. [13], and (ii) a new parameter "Leaf Area Under Greenness" (LAUG; [9]). In the LAUG approach scores for green area of the flag leaf and that of spikes were estimated visually on a 0-9 scale at four day intervals starting from late milk till physiological maturity marked by complete loss of green colour in both flag leaf and spike.

The difference of green area under spike and flag leaf at time t_i was used as Y_i. The formula used for calculating LAUG was:

$$LAUG = \sum_{i=1}^{a} [\{(Y_i + Y_{(i+1)}) / 2\} \times (t_{(i+1)} - t_i)]$$

Where, Y_i = difference of green area under spike and flag leaf (0-9 scale) at time t_i

$t_{(i+1)} - t_i$ = time (days) between two consecutive readings

Sample collection and DNA isolation

DNA was isolated from leaf samples collected from 15-21 days old seedlings using CTAB method with minor modifications. Two DNA bulks were prepared from each cross taking the DNA of 5 rows from both the extremes (staygreen and non-staygreen lines). These bulks were prepared based on the phenotypic data of all the RILs.

PCR analysis

A total of 119 primer pairs including 24 pairs of BARC series (http://www.scabusa.org) and 30 pairs of WMC series (http://www.wheat.pw.usda.gov) of SSR were used to search for polymorphism between the parents and the RILs. The fragment size of all the polymorphic markers ranged from 250 to 100 bp. Most of the markers obtained were already mapped on 5B and 7B chromosomes [21, 22]. For some of the markers the chromosome location was unknown.

SSR primers synthesized by Operon Technologies CA (Almanda, USA) were used. The PCR reactions were performed in a volume of 25 ml in

TECHNE (UK) Thermocycler and 10ml volume for PAGE. The PCR reaction mixture contained 250nM of each primer, 200mM of each dNTPs, 2mM $MgCl_2$, 1.5 Unit of *Taq* DNA polymerase and 50-100ng of template DNA. PCR cycling was performed as: initial denaturation for 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 50, 55, or 60°C (depending on the denaturation temperature of primers), 2 min at 72°C and a final extension step of 10 min at 72°C. All the PCR products were separated on 2% agarose gel and on 8% polyacrylamide non-denaturing gel. Silver staining was done following standard protocol to visualize the bands. Photographs of PAGE gels were taken through digital camera, under white light.

Data analysis

Statistical analysis was done using MS Excel 5.0 software. Analysis of variance (ANOVA) was done for staygreen trait. For each segregating marker, a Chi-square analysis (a=0.001) was performed to test for deviation from the expected segregation ratio (1:1). Linkage analysis was performed with MAPMAKER/EXP var 3.0b [23]. A threshold log likelihood ratio (LOD) of 2.0 or greater was used. The mean LAUG values of RILs over two years were analyzed by simple interval mapping using software MAPMAKER/QTL 1.1b version [24].

Results and discussion

Phenotypic analysis for LAUG in parents and RILs

Mean LAUG values exhibited by staygreen parents Ning 8201 and Yangmai 6 ranged between 73-82, whereas for RILs it ranged between 0.37 to 73 (Tables 1and 2). Staygreen response for most of the RILs in the two crosses fell in the intermediate range (Tables 1 and 2). The normal distribution in the two crosses suggested polygenic control. Based on LAUG values RILs were grouped in to four categories, homozygous parental type staygreen, homozygous parental type non-staygreen and intermediate types as moderately staygreen and moderately non-staygreen. Analysis of variance using LAUG as a parameter for staygreen for RILs of Yamgmai6 x Sonalika (CV 3.18; SE 1.09) and Ning8201 x Sonalika (CV 3.22; SE 1.28) crosses in spring wheat revealed significance for replication, trait, year, lines and displayed that the G x E interaction was significant at 0.05 LS. Staygreen response of the F_1's generated from the two crosses Ning 8201 x Sonalika and Yangmai6 x Sonalika, displayed intermediate response indicating that staygreen is controlled by more than one gene. Normal distribution for LAUG displayed by RILs also suggested that staygreen was polygenically controlled. There are some reports on different crops on the genetics and inheritance of staygreen trait, which indicates a complex pattern of inheritance [25, 26] and influence of environment on the expression of staygreen trait. In the present study also GxE interaction for staygreen trait was significant indicating the environmental influence on the expression of this trait. Silva *et al*. [13] in wheat suggested one gene with two alleles and high predominance of additive effect for staygreen expression. However our recent work [9] reveals polygenic control with around four genes showing additive effect in spring wheat.

Table 1. Staygreen response of parents used to generate RIL mapping population

Parents	Pedigree	Response	Mean LAUG
Yangmai6	CIMMYT, CID 239288	Staygreen	73.87
Ning8201	CIMMYT, CID 95659	Staygreen	75.62
Sonalika	II54-368/AN/3/YT54/ N10B//LR64 (II18427-4R-1M)	Non-staygreen	10.38
		SE	±14.51

Table 2. Range, mean LAUG and LSD for staygreen response in the two crosses

Cross	Range	Mean LAUG	*LSD (1%)
Yangmai6 x Sonalika	0.87-68.87	34.29±1.09	2.807
Ning8201x Sonalika	0.37-68.63	39.95±1.28	3.308

*Significant at 0.01 probability

Microsatellite marker analysis

Microsatellite marker analysis was done in the RILs of the two cross, Ning 8201 x Sonalika and Yangmai 6 x Sonalika. The distribution for mean LAUG values was normal, indicating the presence of more than one gene control for staygreen trait. Out of the 119 markers used, 22 were polymorphic in parents (staygreen and non staygreen). Only those primers which were polymorphic for both the parents were further used. In the 83 RILs of Ning 8201 x Sonalika, 3 markers WMC 10, WMC 74 and WMC 76 were significantly linked with staygreen trait (P<0.001), as estimated by single marker ANOVA (P=0.05) (Table 3). In the 85 RILs of Yangmai 6 x Sonalika, markers BARC 109, BARC 1120, BARC 04,

BARC 74 and BARC 1172 displayed linkage with staygreen trait. Regression of LAUG on all the markers was highly significant (Table 3) for both the crosses. The linked markers showed polymorphism among parents and also in the two extreme bulks of staygreen and non-staygreen lines. The polymorphic markers for Ning 8201 x Sonalika and Yangmai 6 x Sonalika were grouped in one linkage group (Fig. 1). In Ning 8201 x Sonalika, markers WMC 76, WMC 74 and WMC 10 were closely linked with the trait (Fig. 1). In Yangmai 6 x Sonalika cross, markers BARC 1120 and BARC 109 were closely linked (Fig. 1). Rest of the markers BARC 04, BARC 74 and BARC 1172, though were significantly linked with the trait but did not show close linkage.

Genetic mapping of QTL conferring staygreen has already been undertaken in number of plant species using different marker systems [8, 17]. Lot of work has been done on sorghum crop for mapping staygreen conferring drought tolerance [27, 28]. There are very few reports on the use of molecular markers to map staygreen trait in wheat [18]. In the present study SSR markers were used to map the staygreen gene in two RIL populations as they are highly polymorphic and genome specific [21]. The BSA approach was used to detect the QTL (s) controlling staygreen trait. However, BSA is generally not regarded a useful approach for either detection of QTLs for quantitative traits which may be controlled by several genes with small effect or when QTL is loosely linked to the marker [14, 21]. This is because the two bulks are frequently contaminated with alternative alleles if mischaracterization occurs [29]. However, this study demonstrates that with a large population and precise phenotyping, BSA approach is fruitful if bulks are made carefully from extreme ends, in detecting QTL with large effect.

Single marker analysis

One way ANOVA for each marker indicated that all the markers had significant (P<0.05) effect on the expression of staygreen in RIL population (Table 3). Default LOD threshold value was 3.0 and the distance

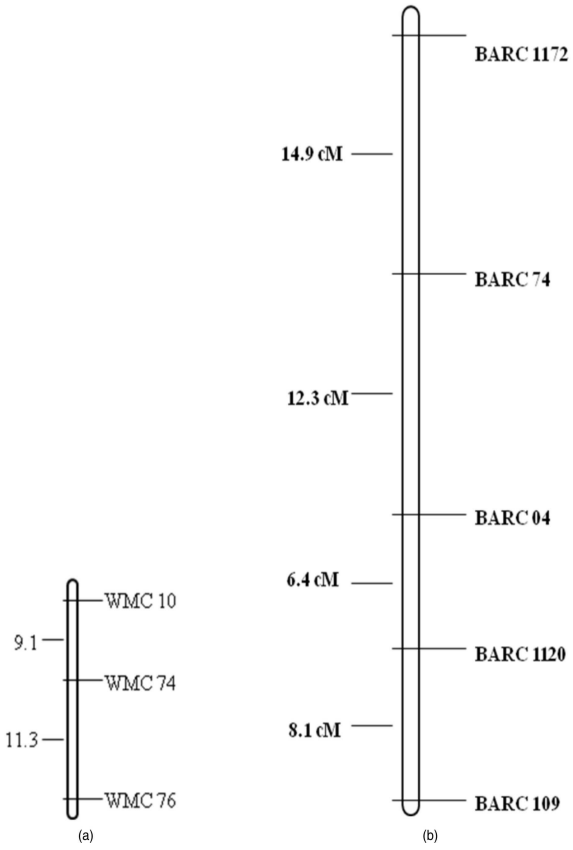

Fig. 1. Genetic linkage map for staygreen in wheat RIL's (A) Yangmai 6 x Sonalika (B) **Ning 8201 x Sonalika**

Table 3. Linkage distance between pairs of markers in RILs, Map distance, R^2 values, and F-value from one way ANOVA for markers linked to staygreen gene in two crosses of spring wheat

Markers	Map distance	Linkage group	R^2 value	F value	P value	Error prob
Ning 8012 x Sonalika						
WMC 10-WMC74	9.1cM	7B	2.15	1.47**	0.002	1.00%
WMC 74-WMC76	11.3cM	7B	8.28	7.14**	0.009	1.00%
Yangmai 6 x Sonalika						
BARC 109-BARC 1120	8.1cM	5B	11.31	10.56**	0.002	1.00%
BARC 1120-BARC04	6.4cM	4B	44.34	58.37**	$7.76x10^{-11}$	1.00%
BARC 04-BARC74	12.3cM	5B	15.26	14.51**	0.0003	1.00%
BARC 74-BARC112	14.9cM	7B	11.57	10.55**	0.0017	1.00%

threshold was 80 Haldane cM. The markers were considered to be linked when LOD score was greater than the threshold, and the map distance lesser than distance threshold. Single marker QTL analysis revealed two QTLs in Ning 8201 x Sonalika, near the markers WMC 74 (R^2=8.28) and WMC 10 (R^2=18.11) (Table 3). In Yangmai 6 x Sonalika, a major QTL was detected near BARC 1120 marker, which alone explained 44.34 % of the phenotypic variation. The rest of the loci contributed on an average 12.9% of the total phenotypic variation for staygreen trait suggesting that these markers were either tightly linked to QTL that had a small undetectable effect or were loosely linked to QTLs that had large effect [30]. WMC 10 and WMC 76 were located on 7B chromosome but the location of WMC 74 was unknown. BARC 109, BARC 04, BARC 74, BARC 1120 and BARC 1172 were located on chromosome 5B. Three polymorphic markers WMC 10, WMC 74 and WMC 76 located on 7B chromosome were mapped in Ning 8201 x Sonalika. In Yangmai6 x Sonalika five polymorphic markers were mapped on 5B chromosome. The marker information generated here can be used as a potential tool to clone staygreen gene through map based cloning or positional cloning approach. The linked markers obtained in the present study for major staygreen QTL, suggests a promising application of molecular assisted techniques for crop improvement using staygreen trait as a measure for biotic and abiotic stresses.

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