Original Article

MOLECULAR MARKER LINKED TO THE RB1 GENE AND ASSOCIATION WITH PRE-HARVEST SPROUTING TOLERANCE IN WHEAT

MARCADOR MOLECULAR LIGADO AO GENE RB1 EM TRIGO E ASSOCIAÇÃO COM A TOLERÂNCIA À GERMINAÇÃO PRÉ-COLHEITA

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ABSTRACT: The objective of this study was to identify microsatellites molecular markers linked to R genes, that confer the wheat grain color and evaluate the association with pre-harvest sprouting tolerance. This study was conducted with a F_2 population with 203 plants, derived from the cross between PFAU x CD0666 genotypes, which segregates for one grain color gene. Were analyzed 43 molecular markers located in regions that containing R genes on 3B and 3D chromosomes. The Xbarc344 molecular marker was identified as linked to the R gene at a distance of 26.1 cM with 83.25% of selection efficiency. From the phenotypic analysis was confirmed the association between grain color with Xbarc344 molecular marker, however, there was no association between Xbarc344 with pre-harvest sprouting tolerance. Also, there was no association between grain color with pre-harvest sprouting tolerance. Although the grain color in wheat is a trait easy to select, it is known that not all R genes are involved with pre-harvest sprouting tolerance. Therefore, it is important the genetic mapping and specific evaluation of each R gene with pre-harvest sprouting tolerance. Thus, the molecular markers identified as associated with each R gene can be used to select plants that contain such genes, since it is not possible to identify phenotypically which R gene each plant has.

KEYWORDS: *Triticum aestivum* L. Microsatellite. Grain color. Dormancy

INTRODUCTION

The pre-harvest sprouting (PHS) affects wheat growing fields of several producing regions in the world, causing loss in yield and drastic reduction of hectolitre weight (HW) and flour quality for the industry. The damages to flour quality are caused by triggering initial enzymatic reaction of the germination process. The PHS occurs by the contribution of many factors, external and internal. Most of them are controlled by genetic factors, which determine spike morphology, hormonal sensitivity and alpha-amylase inhibitors (BERNARD et al., 2005). In addition, weather conditions such as temperature, rainfall during harvesting, drought during the grain maturation period and the interaction of all these with physiological and genetic factors are directly related with the wheat pre-harvest sprouting (FLINTHAM, 2000).

Usually white grain wheat have been reported as more PHS susceptible than red grain wheat, however there is variation in both. The grain red color is used as a marker for PHS tolerance in the wheat breeding programs (BASSOI; FLINTHAM, 2005). This association existing between PHS and grain color can be due to either a strong linkage between the genes or to the pleiotropic effects of the grain color genes (McCAIG; DePAUW, 1992).

The red pigmentation of the seeds coat in hexaploid wheat is a trait controlled by a set of three R genes, located on the 3A, 3B, and 3D chromosomes (FLINTHAM, 2000). A single R gene is enough for expressing red seeds of wheat. There are numerous wheat cultivars with red grains highly PHS susceptible, therefore it is possible that not all R genes are associated with the wheat seeds dormancy. This would explain the association of the red color with the PHS tolerance, while would also explain why many cultivars are susceptible even having red grains (BASSOI; FLINTHAM, 2005).

The R genes were mapped close to the xgwm155, xgwm4010, and xgwm4306 molecular markers in the 3A, 3B, and 3D chromosomes, respectively (SHERMAN et al., 2008). However, the molecular markers of 3B and 3D chromosomes are private and can not be used freely for selection or identification of these genes, therefore it's necessary the identification of new molecular markers for these regions. Once that it's necessary

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just one copy of any R genes to produce the red grain phenotype, molecular markers linked to this gene can be used to identify which R gene or genes is/are present in each plant or cultivar.

The aim of this study was to identify public microsatellite molecular markers that are linked to R gene, which controls the wheat grain color trait, and to evaluate the association of grain color with PHS tolerance.

MATERIAL AND METHODS

It was used a F_2 population with 203 plants, derived from crossing CD0666/3/PFAU/SERI.1B//AMAD, which segregates to one grain color gene. The parents in this work are denominated as PFAU x CD0666. The CD0666 lineage has red grains and PFAU/SERI.1B//AMAD has white grains. The phenotypic information of this population (note of germination and grain color) was obtained previously for each F_2 plant (SANTOS et al., 2010). Were evaluated 43 markers and the polymorphic molecular markers were used to amplify all the F_2 population.

DNA extraction was performed according to the extraction protocol described by Schuster et al. (2004). DNA concentration of each sample was estimated spectrophotometrically by absorbance at 260 nm using Nanodrop 1000, with each absorbance unit corresponding to the concentration of 50 µg mg L^{-1} of DNA.

Polimerase chain reactions (PCR) were performed at a total volume of 20 uL, containing: 99 ng of DNA, 1.5 mM of $MgCl_2$, 1x buffer (2 mM of Tris and 5 mM of KCl), 300 µM of each deoxynucleotide (dATP, dTTP, dGTP, and dCTP), 0,8 µM of each primer (forward and reverse), and one unit of Taq DNA Polimerase. Amplifications were performed in a Thermo Hybaid thermocycler (Ashford, Middlesex, United Kingdom), programmed for one initial cycle of 94ºC for 3 min., 45 intermediate cycles of 94ºC for 1 min., 55ºC for 1 min. and 72ºC for 2 min. and, a final extension of 72ºC for 10 min.

After the amplification, DNA fragments were separated in 7% denaturing polyacrylamide gel, using SB buffer 0.5X (5 mmol L^{-1} of sodium hydroxide, adjusted to pH 8 with boric acid). After electrophoresis, the gels were stained with silver nitrate and for displaying the DNA fragments it was used sodium hydroxide and later it was scanned using scanner for storage of results. The molecular markers used were obtained from wheat genetic map (SONG et al., 2005) and are located on 3B and 3D chromosomes, which have grain color genes.

The linkage analysis between molecular markers and wheat grain color loci was performed using GQMOL software (CRUZ; SCHUSTER, 2006), using the Kosambi mapping function (KOSAMBI, 1943). The analysis of molecular markers segregation was performed using the chi-square test (χ^2). The associations between molecular markers and grain color, molecular markers and PHS, and grain color with PHS were analyzed by contingency table, using GENES software (CRUZ, 2001). The selection efficiency (SE) of the molecular markers, expressed in percentage, was obtained by the expression:

$$SE = \frac{\text{Number of plants correctly selected with the marker}}{\text{Total number of evaluated plants}} \text{x} 100$$

RESULTS AND DISCUSSION

On previous study (SANTOS et al., 2010), has identified that the F_2 population, derived from the crossing between CD0666/3/PFAU/SERI.1B//AMAD segregated for one R gene. In order to identify molecular markers linked to the R gene which is present in the CD0666 lineage, which have grain color genes and were previously mapped (SHERMAN et al., 2008), were used molecular markers located on 3B and 3D chromosomes. Five polymorphic molecular markers were identified between the parental genotypes: Xbarc229, Xgwm108, and Xbarc344 located on 3B chromosome and Xwmc631 and Xbarc323 on 3D chromosome. Chi-square test (χ^2) confirmed the Mendelian segregation for Xbarc344, Xwmc631, and Xbarc323 markers. For Xbarc229 and Xgwm108 the Mendelian segregation can not be confirmed (Table 1).

Among the polymorphic markers of 3B group, Xbarc229 needed to be evaluated as a dominant marker, because it was not possible to distinguish heterozygous individuals from homozygous ones, with genotype similar to PFAU. Furthermore, Xbarc229 and Xgwm108 markers showed segregation distortion and were not considered in the cosegregation analysis.

The Xbarc344 molecular marker segregated at the expected ratio. According to cosegregation analysis, this molecular marker is linked to R gene, which gives color to grain in wheat, at a distance of 26.3 cM with LOD score of 8.41. The Figure 1 shows the amplification pattern of Xbarc344 marker. Similar results were reported by Sherman et al. (2008) in a population with 96 plants derived from the cross between DOLLAR x MTHW9904. In

this study it was demonstrated that Xbarc344 marker is linked to RB1 gene at a genetic distance of 8.3 cM. This difference in the estimation of genetic distance between them found in both studies may be explained by the size of population, that in the current study was 203 F_2 plants.

Table 1. Segregation of molecular markers used for mapping grain color gene on wheat F_2 population derived from PFAU x CD0666.

SSR Marker	L.G	Observed values				Hypothesis	χ^2	P %
		CD0666 allele	Het.	PFAU + Het.	PFAU allele			
Xbarc344	3B	66	99		44	1:2:1	5.21	7.39
Xbarc229	3B	43		88		1:3	4.28	3.86
Xwmc631	3D	39	68		51	1:2:1	4.89	8.69
Xbarc323	3D	27	31		22	1:2:1	4.68	9.66
Xgwm108	3B	123	10		25	1:2:1	242.10	0

L.G: Linkage Group; CD0666: Homozygous with genotype of red grain parental (CD0666); Het: Heterozygous; PFAU: Homozygous with genotype of white grain parental (PFAU); PFAU + Het.: Dominant marker. Homozygous genotype for PFAU and heterozygous genotype can not be distinguished; P: Probability

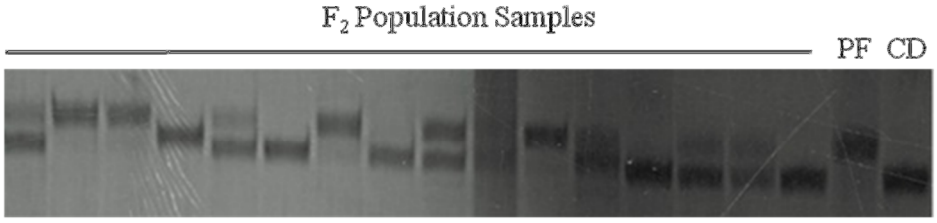

Figure 1. DNA amplification standard of plants in the PFAU x CD0666 population with Xbarc 344 microsatellite primer. PF=PFAU; CD=CD0666.

The F_2 population derived from the cross between CD0666/3/PFAU/SERI.1B//AMAD segregates to a single R gene, it can be concluded that CD0666 lineage has the RB1 gene, since the associated marker is located on 3B chromosome. Previously, other markers were reported in this chromosome and linked to the RB1 gene (SHERMAN et al., 2008). However, some of them are private ownership and could not be used in this study. Another 19 molecular markers on 3B chromosome were also evaluated, however only Xgwm229 showed polymorphism (Figure 2).

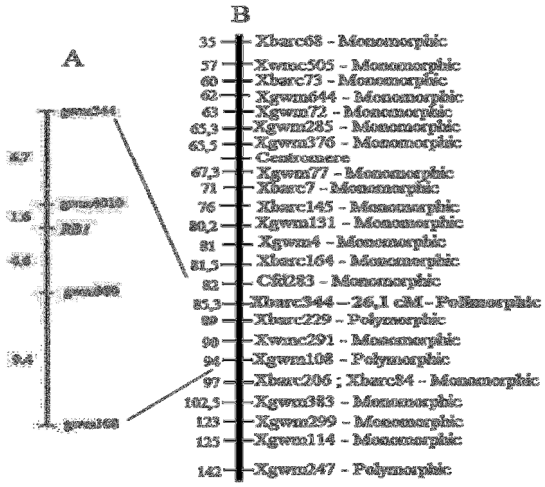

Figure 2. A - 3B chromosome fragment, containing RB1 gene (SHERMAN et al., 2008). Although this figure shows the Xgwm344 marker, the correct is Xbarc344, as shown in table 1 of the original article. **B** - Localization of molecular markers on 3B chromosome used in the mapping study of wheat grain color gene in PFAU x CD0666 population.

The large number of monomorphic markers among parental genotypes of the crossing used in this study reinforces the recommendation of the validation of molecular markers in different populations from these. This is because not always the marker identified in a population or in a genetic background is informative for the other populations.

With the phenotypic analysis of grains it was possible to confirm the association between wheat grain color and Xbarc344 marker ($\chi^2 = 59.77$, P = 0%) (Table 2). The red color is dominant and one can observe that among the 153 plants with red grain 90.85% showed genotype that confirms this characteristic, i.e., alleles of CD0666 parental in homozygous or heterozygous. Among the 50 plants with white grain phenotype, 60% are homozygous genotype with alleles of PFAU parental. For this population, the selection efficiency (SE) of Xbarc344 marker to differentiate white from red grain genotypes was 83.25%. The difference to 100% is due to the recombination between molecular marker and gene.

Grain color is important because it has a strong and significant relation with the expression of PHS tolerance (BASSOI; FLINTHAM, 2005). However, according to the results of the contingency test ($\chi^2 = 0.265$, P = 60.65%), there is not any association between Xbarc344 molecular marker and the PHS tolerance (Table 2).

Table 2. Contingency table comparing grain color phenotypic analysis with Xbarc344 marker genotypic analysis and PHS phenotypic analysis with Xbarc344 marker in F_2 wheat population PFAU x CD0666.

Phenotype	Xbarc 344		Total	χ^2	P
	PFAU allele	CD0666 allele and heterozygous			
Red Grain	14	139	153		
White Grain	30	20	50		
Total	44	166	203	59.77	0%
Resistant	1	2	3		
Susceptible	43	161	204		
Total	44	163	207	0.265	60.65%

P: Probability

The most important loci, which control PHS tolerance, are located on three homologous chromosomes, 3A, 3B, and 3D (JAISLWAL et al., 2012), but 4A chromosome was also mentioned for containing the *Phs* gene (KUWAL et al., 2012). However, *viviparous-1* gene family is associated with PHS tolerance in several cereals species (XIA et al., 2008; CHANG et al., 2011). On wheat the R genes, which are responsible for grain color, are also the biggest contributors to PHS tolerance (FOLEY; FENNIMORE, 1998; FLINTHAM et al., 2002). The Xbarc 344 marker is located on 3B chromosome and, even having linkage with the grain color feature, it didn't show association with PHS tolerance.

According to the contingency test ($\chi^2 = 0.128$, P = 72.04%), no association was observed between grain color and PHS tolerance (Table 3). Among the 154 plants with red grain color, only 2 showed PHS tolerance. Among the 201 PHS susceptible plants, 152 had red grain color. Although it is common the association between grain color and PHS tolerance, not all R genes are associated with this trait. The RB1 gene, located on 3B chromosome, has no association with PHS tolerance.

Table 3. Contingency table comparing PHS phenotypic analysis with grain color phenotipic analysis in PFAU x CD0666 wheat population.

Phenotype	Red	White	Total
Resistant	2	1	3
Susceptible	152	49	201
Total	154	50	204

$\chi^2 = 0.128$; P = 72.04%

Several studies suggest that there is genetic linkage or pleiotropy between grain color and PHS tolerance (FLINTHAM; GALE, 1988; FLINTHAM; HUMPHREY, 1993). On the other hand, it was also suggested that dormancy and PHS tolerance control extends beyond grain color genes. The R genes are dominant and PHS tolerance (dormancy) inheritance studies identified recessive genes controlling this trait (CUNHA et al., 2004).

The identification of molecular markers associated with grain color is a promising tool for marker-assisted selection in wheat breeding, since they can be used for identifying which R genes are associated with PHS resistance. Grain color is a trait easy to select for wheat, however, not all R genes are associated with PHS tolerance. This explains the fact that some red grain cultivars are more PHS tolerant than others. Therefore, it is necessary to evaluate the specific association of R genes with PHS tolerance and this must be done by mapping of the three R genes and by the specific evaluation of each gene with PHS tolerance.

For this reason, new contrasting populations for others R genes should be evaluated. If there is specific association of a R gene with PHS resistance, molecular markers linked to this gene can be used to select plants that contain the specific gene, since it is not possible to differentiate phenotypically plants containing different R genes.

CONCLUSIONS

CD0666 parental lineage has the RB1 coding gene for the grain red color phenotype.

Xbarc344 marker is linked at 26.1 cM from the RB1 gene in the population from the PFAU x CD0666 cross.

There was no association of Xbarc344 marker with PHS tolerance and it was not observed grain color association with the PHS tolerance.

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RESUMO: O objetivo deste trabalho foi identificar marcadores moleculares microssatélites ligados aos genes R, que conferem a cor do grão em trigo e avaliar a associação com a tolerância à germinação na espiga. O estudo foi realizado em uma população com 203 plantas F_2, derivadas do cruzamento entre os genótipos PFAU x CD0666, que segregam para um gene da cor do grão. Foram analisados 43 marcadores moleculares localizados nas regiões que contêm os genes R nos cromossomos 3B e 3D. Foi identificado que o marcador Xbarc344 está ligado ao gene R a uma distância de 26,1 cM e apresentou eficiência de seleção de 83,25%. A partir da análise fenotípica foi possível confirmar a associação da cor do grão com o marcador Xbarc344, no entanto, não houve associação deste marcador e da cor do grão com a tolerância à germinação na espiga. Embora a cor do grão seja uma característica fácil de selecionar em trigo, é notório que nem todos os genes R estão envolvidos com a tolerância à germinação na espiga. Sendo assim, é importante o mapeamento genético e a avaliação específica da associação de cada gene R com esta característica. Assim, os marcadores moleculares identificados como associados a cada gene R podem ser utilizados para selecionar plantas que contenham tais genes, uma vez que não é possível identificar fenotipicamente qual gene R cada planta possuí.

PALAVRAS-CHAVE: *Triticum aestivum* L.. Microssatélites. Cor do grão. Dormência

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