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Identification of RAPD and ISSR markers associated with flag leaf senescence under water-stressed conditions in wheat (*Triticum aestivum* L.)

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Abstract

Time of flag leaf senescence is an important determinant of yield under stress and optimal environments. A segregating population from a cross between drought sensitive genotype (Variant-1) and drought tolerant genotype (Veery), was made to identify molecular markers linked to flag leaf senescence gene in wheat under water-stressed conditions as indicator for drought tolerance. Thirty eight RAPD and twenty-five ISSR primers were tested for polymorphism among parental genotypes and F_2 population. The present study indicated that four RAPD and two ISSR markers linked to the flag leaf senescence gene in wheat. QTLs for flag leaf senescence were associated with RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and explained 7.0%, 50.0%, 24.0% and 13.0% of the phenotypic variation, respectively. QTLs for flag leaf senescence were also associated with ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) and explained 25% and 34% of the phenotypic variation, respectively. The RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) have genetic distance of 15.6, 15.0, 13.2 and 17.4 cM from flag leaf senescence gene, respectively. The ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) also showed the genetic distance of 12.5 and 10.2 cM from flag leaf senescence gene, respectively. Therefore, these four RAPD and the two ISSR markers were linked to the QTL for the flag leaf senescence gene as indicator of drought tolerance. These markers can be used in wheat breeding programs, as a selection tool in early generations.

Keywords: flag leaf senescence; ISSR markers; RAPD markers; *Triticum aestivum* L.
Abbreviations: ISSR_inter simple sequence repeats; QTL_quantitative trait loci; RAPD_random amplified polymorphic DNA.

Introduction

Wheat (*Triticum aestivum* L.) is the most important cereal crop in the world. Wheat production under limited rain or irrigation condition has become an objective in Egypt as well as many areas worldwide due to limitation of water supply. Wheat production is adversely affected by drought in 50% of the area under production in the developing and 70% in the developed countries (Trethowan and Pfeiffer, 2000). Genetic gain for yield in these environments is estimated about half of the yield compared to well-watered environments (Rajaram et al., 1996). It is difficult to make progress for grain yield and yield components under drought as they are complex characters influenced by many environmental factors and are characterized by low heritabilities and large genotype environment interactions under drought conditions (Smith et al., 1990). Recently, Tuberosa and Saliva (2007) reported that genomics based approaches provide access to agronomically desirable alleles present at quantitative trait loci (QTLs) that affect such responses, thus enabling us to improve the drought tolerance and yield of crops under water limited conditions more effectively. Molecular markers associated with quantitative trait loci (QTL) for drought adaptive traits could greatly enhance progress in breeding for drought tolerance. Molecular markers improve the efficiency of breeding by allowing manipulation of the genome through marker-assisted selection. In wheat (*Triticum aestivum* L.), flag leaf senescence (FLS) is related to the period of reallocating resources from the source to the sink during the grain filling. Since the flag leaf photosynthesis in wheat contributes about 30–50% of the assimilates for grain filling (Sylvester-Bradley et al., 1990), the onset and rate of senescence are important factors for determining yield potential (Evans, 1993). Though, quantitative trait loci mapping for FLS as a yield determinant in winter wheat under optimal and drought-stressed environments, have been studied using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers, which revealed the genetic control of this trait and the QTLs identified on chromosome 2D associated with better performance under drought (Verma et al., 2004). To date, there has been no report of QTL mapping for flag leaf senescence using RAPD and ISSR markers. The simplicity and applicability of the RAPD and ISSR techniques have been emphasized in this study. RAPDs, involving the use of single short DNA primer to direct amplification of discrete sequences, have shown promises in cereals (D'Ovidio et al., 1990; Devos et al., 1992). ISSRs have been used successfully in genome mapping for a variety of crop species including maize, rice, barley and wheat (Ben El Maati et al., 2004; Barakat et al.,

2010). ISSR has been proposed as a new source of genetic markers which overcome the technical limitation of restriction fragment length polymorphisms (RFLP). The objectives of this investigation were to identify RAPD and ISSR markers linked to flag leaf senescence gene in wheat under water-stressed conditions and QTL mapping for flag leaf senescence gene in F_2 population, using bulked segregant analysis.

Materials and methods

Plant materials and population development

The sensitive (Variant-1) and tolerant genotype (Veery) of wheat used in this study. Variant-1 was derived from Sakha 69 cultivar, using somaclonal variation tool (Barakat et al., 2005). The wheat cultivars Veery is highly tolerant to drought (Rajaram et al., 1996). The two wheat genotypes that had contrasting response to drought stress were crossed to generate the F_1 seeds during winter season 2006 at the Experimental Farm Station, Faculty of Agriculture, Alexandria University, Alexandria, Egypt. The F_1 seeds population derived from the cross (Veery × Variant-1) were obtained. The F_2 seeds were obtained by selfing during winter season 2007.

Evaluation of drought tolerance

The F_2 population and their parents were planted in polyethylene bags under green house conditions in winter season 2008 to evaluate drought tolerance. The parents and the F_2 population were grown in green house in 2008 winter growing season. 100 plants from F_2 population were planted in polyethylene bag. The polyethylene bag with dimensions of (13 cm diameter, 15 cm height) was used to grow single wheat plants in a greenhouse experiment. They filled with sandy soil (3.5 kg) and were giving the total amount of daily irrigation until reaching booting stage. Drought treatment was imposed using 50% of the amount of daily irrigation. Daily irrigation water requirements were calculated by CROPWAT software (Smith, 1991) from agro-meteorological data of the studied area and Kc of wheat as follows:

$$ET\,o = \frac{0.408\,\Delta(Rn - G) + \gamma \frac{37}{Thr + 273} u2\,(e^{\circ}(\mathrm{Thr}) - ea)}{\Delta + \gamma(1 + 0.34u2)}$$

$$ETc = \mathrm{Eto} * \mathrm{Kc}$$

Where, Etc = Evapotranspiration of crop, Kc = Crop coefficient, ETo = Reference evapo-transpiration (mm h^{-1}), Rn = net radiation at the grass surface, (MJm/2hour^{-1}), G = soil heat flux density (MJm/2hour^{-1}), Thr = mean hourly air temperature (°C), Δ = Saturation slope vapor pressure curve at Thr (Kpa°C-1), γ = psychrometric constant (Kpa°C-1), eo (Thr) = saturation vapor pressure at air temperature Thr , ea = average hourly actual vapor pressure, u2 = average hourly actual wind speed (ms^{-1}). Calculated ETc, (crop evapotranspiration), which is equal to 100% of daily water consumption for the wheat was used to calculate irrigation requirements with the following equation: Daily irrigation requirements (IR) = ETc +15% (leaching requirements). The data of daily IR was adjusted to the volume of polyethylene bags used and the following (Table 1) show the volume of daily IR in cubic cm until the stage of flag leaf appearance then

Table 1. Daily IR cm^3 from 1st March to 30 April

Days	Daily IR cm^3	50% of the amount of daily irrigation cm^3
1-10 March	31.9	15.95
11-20 March	28.2	14.1
21-31 March	38.9	19.45
1-10 April	38.1	19.05
11-20 April	30.3	15.15
21-30 April	20.9	10.45

drought tests were carried out for 21 days. After 21 days from the stress condition, the flag leaf of the main tiller of each plant was selected during morning hours when leaves were fully turgid. The percentage of flag leaf area and remaining green (% GFLA) was measured using the leaf area meter (Portable Living Leaf Area Meter, Model: YMJ, Zhejiang Top Instrument Co., Ltd). These assessments were carried out by the same operator in the population to avoid any bias between operators influencing results.

DNA extraction

Genomic DNA was extracted from fresh leaves of F_2 individual plants and their parents, using the Saghai-Maroof et al. (1984) CTAB method. RNA was removed from the DNA preparation by adding 10 µl of RNAase (10 mg/ml) and then incubated for 30 min at 37°C. DNA sample concentration was quantified by a spectrophotometer (Beckman Du-65). The reagents were obtained from Pharmacia Biotech (Amersham Pharmacia Biotech Ltd., UK).

PCR amplification

Thirty-eight RAPD and 25 ISSR primers (Barakat et al., 2010) were used in the present investigation to amplify the template DNA. The PCR reaction mixture consisted of 20-50 ng genomic DNA, 1×PCR buffer, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 0.1 µM primer and 1U *Taq* polymerase in a 25 µL volume. Template DNA was initially denatured at 94°C for 4 min, followed by 45 cycles of 94°C for 1 min, 36°C (for RAPD analysis) or 50°C (for ISSR analysis) for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. Amplification products were fractionated on 1% (for RAPD analysis) or 2% (for ISSR analysis) agarose gel.

Bulked segregant analysis

Bulked–segregant analysis (BSA) was used in conjunction with RAPD and ISSR analysis (Michelmore et al., 1991) to find markers linked to flag leaf senescence gene as indicator for drought tolerance. Tolerant and sensitive bulks were prepared from F_2 individuals by pooling aliquots, containing equivalent amounts of total DNA, approximately, 50 ng/µl from each of ten sensitive and ten tolerant F_2 plants selected, based on initial phenotypic assessments. RAPD and ISSR primers were, then, screened on the parents and the two bulk DNA samples, from which some primer combinations revealed bands that were polymorphic, not only among parental genotypes, but also between the pair of the bulk DNA. Based on the evaluations of DNA bulks, individual F_2 plants were analyzed with co-

Table 2. QTL analysis and significant association between drought tolerance and markers (RAPD and ISSR) in the 100 F_2 plants population of Veery ×Variant-1, using Chi – square (χ^2), correlation (r) and coefficient of determination (R^2) analysis

	Markers	Tested of plant			Expected Ratio	χ^2	r	R^2	QTL cM	LOD
		Total	T	S						
RAPD	$Pr11_{230bp}$	100	80	20	3 : 1	1.33^{ns}	0.26	0.07**	18.6	16.4
	$Pr19_{240bp}$	100	67	33	3 : 1	3.41^{ns}	-0.71	0.50**	15.0	19.0
	$OPU06_{340bp}$	100	67	33	3 : 1	3.41^{ns}	0.49	0.24**	13.2	20.7
	$OPH13_{450bp}$	100	64	36	3 : 1	6.4**	-0.36	0.13**	17.4	16.0
ISSR	$M1_{1100bp}$	100	67	33	3 : 1	3.41^{ns}	0.50	0.25**	12.5	21.6
	$AD2_{300bp}$	100	76	24	3 : 1	0.05^{ns}	-0.58	0.34**	10.2	26.0

T=Tolerant plants , S= sensitive plants; Ns , ** non significant and significant at .01 level of probability

Table 3. Analysis of variance of the difference for flag leaf senescence as determinant for drought tolerance in F_2 population

Marker	Source	DF	SS	MS	F	P
$Pr11_{230bp}$	Genotypes	1	520.75	520.75	4.578	0.034
	Error	98	11146.5	113.73		
	Total	99	11667.25			
$Pr19_{240bp}$	Genotypes	1	4057.45	4057.45	52.25	0.0001
	Error	98	7609.81	77.65		
	Total	99	11667.25			
$OPU06_{340bp}$	Genotypes	1	3029.35	3029.35	34.37	0.0001
	Error	98	8637.91	88.14		
	Total	99	11667.25			
$OPH13_{450bp}$	Genotypes	1	3208.85	3208.85	37.18	0.0001
	Error	98	8458.41	86.31		
	Total	99	11667.25			
$M1_{1100bp}$	Genotypes	1	3089.19	3089.19	35.29	0.0001
	Error	98	8578.07	87.53		
	Total	99	11667.25			
$AD2_{300bp}$	Genotypes	1	4073.52	4073.52	52.57	0.0001
	Error	98	7593.74	77.49		
	Total	99	11667.25			

segregating primers (Barakat et al., 2010) to confirm linkage of RAPD and ISSR markers to the drought tolerant genes.

Data analysis

Goodness of fit to a 3:1 ratio was calculated for RAPD and ISSR markers by Chi-square test. The association between molecular markers (RAPD and ISSR) and the flag leaf senescence as indicator for drought tolerance genes was assessed with correlation and simple regression analysis, using PROC REG of SAS version 9.1 software packages (SAS Institute, Cary, NC, 2007). Magnitude of the marker associated phenotypic effect was described by simple correlation (r) and the coefficient of determination (R^2), which represented the fraction of variance explained by the polymorphism of the marker. Single-marker analysis to detect main effect of QTL was performed by the method of Liu (1998). Significant association of a tested marker with a QTL for flag leaf senescence gene was detected by single-factor ANOVA.

Linkage analysis

Map manager QTX Version 0.22 (Meer et al., 2001) was used to analyze the linkage relationship of RAPD and ISSR markers detected from bulked segregate analysis. Linkage was detected when a log of the likelihood ratio (LOD) threshold of 3.0 and maximum distance was 50 cM. The Kosambi's mapping function was used. The chromosomal location of the QTLs for flag leaf senescence was further confirmed by the simple interval mapping method using QGENE programe (Nelson, 1997).

Results

RAPD markers analysis

Out of 38 arbitrary primers screened for polymorphisms between (Veery and Variant-1), 24 RAPD primers (63.2 %), with higher polymorphic bands were suitable to differentiate between the two parents. Of these 24 RAPD primers, Pr_{11}

primer (5' CAATCGCCGT 3'), produced one strong polymorphic band at 230 bp, that was present only in the tolerant parent (Veery), as shown in Figure (1). The Pr11 primer was selected for screening DNA bulks and their parental DNA. The Pr_{11} primer, generated one polymorphic fragments at 230 bp, which was presented only in tolerant bulk and Veery (tolerant parent) whereas missing in sensitive bulk and Variant-1 (sensitive parent), as shown in Fig 1. In addition primer Pr19 (5` CAAACGTCGG 3'), produced a strong polymorphic band at 240 bp that was present only in the sensitive bulked DNA, but not in the tolerant bulked DNA. Primer OPU06 (5` ACCTTTGCGG 3'), produced a strong polymorphic band at 340 bp that was present only in the sensitive bulked DNA, but not in the tolerant bulked DNA. Also, primer OPH13 (5` GACGCCACAC 3'), produced a strong polymorphic band at 450 bp that was present only in the sensitive bulked DNA, but not in the tolerant bulked DNA. These RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) were regarded as candidate markers, linked to the flag leaf senescence gene as indicator for drought tolerance. These polymorphic markers; viz, $Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$, were further used to check their linkage to the flag leaf senescence gene, using a segregating F_2 population, derived from the cross between the tolerant parent (Veery) and the sensitive parent (Variant-1). When analyzing the individual plants of F_2 population, the $Pr11_{230bp}$ and $OPU06_{340bp}$ fragments were amplified and obtained only in F_2 tolerant individuals. In addition $Pr19_{240bp}$ and $OPH13_{450bp}$ fragments were amplified in the DNA, obtained only in F_2 sensitive plants. For the RAPD markers, $Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$, 80, 33, 67 and 36 of 100 individuals exhibited the amplified polymorphic fragments, in the F_2 population, respectively (230, 240, 340 and 450bp), while the remaining did not. The ratio fitted the expected Mendalian ratio (3:1) for all markers except for $OPH13_{450bp}$ which did not fit accordingly (Table 2). To check for potential co-segregantion of DNA fragments and drought tolerant phenotypes, correlation and simple regression analysis were carried out in order to confirm association among the $Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$ markers and the flag leaf senescence gene as indicator for tolerant to drought in all 100 F_2 progenies. The results showed that the correlation and the coefficient of determination (R^2) for the relationship between the four markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and the phenotypes of F_2 individuals were significant and r = 0.26, -0.71, 0.49 and -0.36 were recorded, respectively. The coefficients of determination (R^2) were also 0.07, 0.50, 0.24 and 0.13, for these markers, respectively (Table 2) which indicates that the four markers were associated with the flag leaf senescence gene as indicator for drought tolerance.

ISSR marker analysis

Out of 33 ISSR primers, screened for polymorphisms between the two tested parents, thirteen ISSR primers (34.2 %) with higher polymorphic bands were suitably recognized to differentiate between the two parents. Of these thirteen ISSR primers, M1 and AD2 (5'(AC) 8CG3' and 5'(AGC) 6G3', respectively), which produced two strong polymorphic bands at 1100 and 300 bp, respectively. The M1 primer produced one

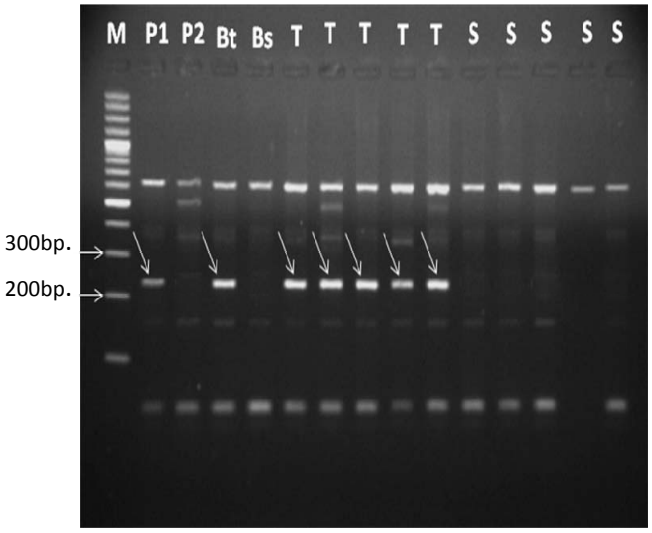

Fig 1. RAPD fragments, produced by primer 11 (5` CAATCGCCGT 3'), M: Molecular weight followed by P_I and P_2 parents Veery and Variant-1, respectively. Bt, bulk tolerant; Bs, bulk sensitive, F_2 individuals in the cross (T: Tolerant; S: sensitive). Arrow points to polymorphic bands of the $Pr11_{230bp}$ marker.

strong polymorphic band at 1100 bp, that was present only in the tolerant parent (Veery), as shown in Fig 2. The M1 primer was selected for screening DNA bulks and their parental DNA. The M1 primer , generated one polymorphic fragments at 1100 bp, which was present only in tolerant bulk and Veery (tolerant parent) and were missing in sensitive bulk and Variant-1 (sensitive parent), as shown in Fig 2. In addition, AD2 primer was generated one polymorphic fragment at 300 bp that were present only in the sensitive parent (Variant-1). AD2 ISSR primer was selected for screening DNA bulks and their parental DNA. The primer AD2, generated the polymorphic fragments with 300 bp length, which were present only in the sensitive bulk and Variant-1 (sensitive parent) and missing in tolerant bulk and Veery (tolerant parent). These ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) were regarded as candidate markers linked to the flag leaf senescence gene as indicator for drought tolerance in wheat.

These polymorphic markers, $M1_{1100bp}$ and $AD2_{300bp}$, were further used to check their linkage to the flag leaf senescence gene on the segregating F_2 population, derived from the cross between the tolerant parent, Veery, and the sensitive, Variant-1. Analysis of F_2 population showed that the $M1_{1100bp}$ fragments were only amplified in tolerant and $AD2_{300bp}$ in sensitive individuals. In the F_2 population, the ISSR markers, of $M1_{1100bp}$ and $AD2_{300bp}$, exhibited the amplified polymorphic fragments of 1100 and 300 bp in 76 and 24 of 100 individuals respectively, while, the remaining did not. The ratio fitted the expected Mendalian ratio (3:1) (χ^2= 3.41 and 0.05, P < 0.1 respectively) (Table 2). To check for potential co-segregantion of DNA fragments and drought tolerant phenotypes, correlation and simple regression analysis were carried out in order to confirm an association between the each $M1_{1100bp}$ and $AD2_{300bp}$ markers and the flag leaf senescence gene as indicator for the tolerance to drought in all 100 F_2 progenies.

The results showed that the correlation and the coefficient of determination (R^2) for the relationship between presence of the two markers, $M1_{1100bp}$ and $AD2_{300bp}$, and the phenotypes of F_2 individuals were significant and they recorded r = 0.5 and -0.58, respectively whereas, R^2 = 0.25 and 0.34, respectively (Table 2). This indicates that these two markers were linked to the flag leaf senescence gene as well.

QTL analysis

The linkage relationship between the RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and the flag leaf senescence gene as indicator for drought tolerance was estimated, using F_2 population, derived from the cross, Veery X Variant-1. The genetic distance between RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and the flag leaf senescence gene were determined to be 15.6, 15.0, 13.2 and 17.4 cM with LOD scores of 16.4, 19.0, 20.7 and 16.0, respectively (Table 2 and Fig. 3). Therefore, RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$ primers) were linked to the quantitative trait loci (QTL) for the flag leaf senescence gene as indicator for drought tolerance. After mapmaker linkage analysis on the F_2 population, the genetic distance between ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) and the flag leaf senescence gene was determined to be 12.5 and 10.2 cM, respectively, with LOD scores of 21.6 and 26.0 respectively (Table 2 and Fig. 3). Therefore, ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) were linked to the quantitative trait loci (QTL) for the flag leaf senescence gene as well. One-way ANOVA was carried out using marker genotypes as groups. The ANOVA on RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) genotypes as groups for flag leaf senescence established significant association between marker (RAPD markers and ISSR markers) and phenotype (flag leaf senescence) (Tables 2 and 3). The single marker ANOVA analysis revealed that RAPD markers-linked QTL ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) accounted for 7.0%, 50.0%, 24.0% and 13.0% of the total variation of flag leaf senescence in F2 population, respectively. While, ISSR markers-linked QTL ($M1_{1100bp}$ and $AD2_{300bp}$) accounted for about 25% and 34% of the phenotypic variation of leaf senescence in F_2 population, respectively, in wheat under water-stressed conditions.

Discussion

Water-stress tolerance in wheat is a quantitatively inherited trait controlled by several genetic loci which their genetic components are difficult to measure (Forster et al., 2000). Identification of associated molecular markers at a major locus contributing to water-stress tolerance would be useful for the indirect selection of wheat plants for water-stress tolerance (Visser, 1994). However, identifying molecular markers associated with important genes or traits in most instances requires screening of a relatively large number of individuals in the population (Lawson et al., 1994). Bulked segregant analysis (BSA) was originally developed to overcome this difficulty, because comparing bulk samples is easier than evaluating many individuals in different populations (Sweeney and Dannebeger, 1994). In this study, mapping quantitative trait loci for flag leaf senescence gene as indicator for drought tolerance in wheat

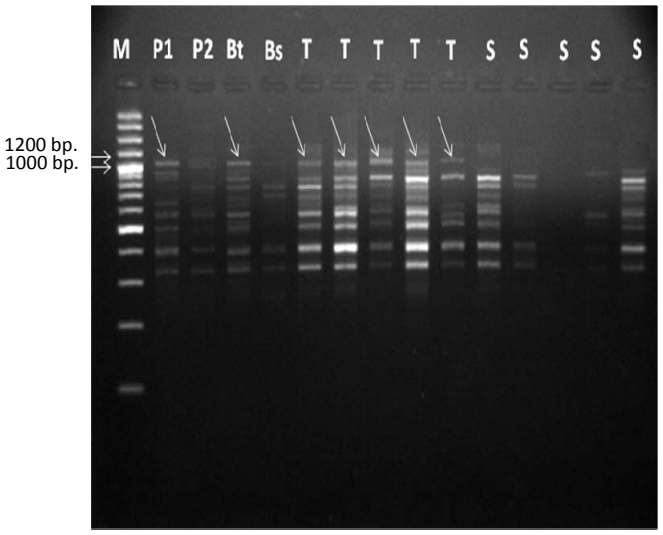

Fig 2. ISSR fragments, produced by M1 (5` (AC)8CG 3'), M: Molecular weight, followed by P_1 and P_2 parents Veery and Variant-1, respectively. Bt, bulk tolerant; Bs, bulk sensitive, F_2 individuals in the cross (T: Tolerant; S: sensitive). Arrow points to polymorphic bands of the $M1_{1100bp}$ marker.

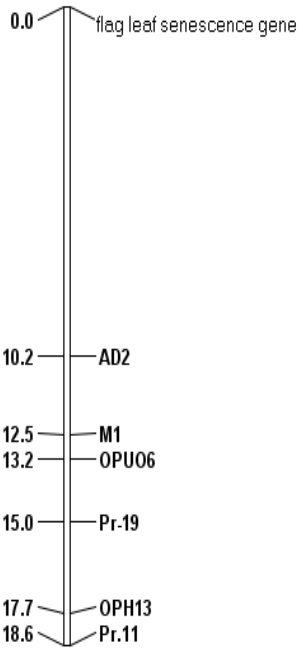

Fig 3. RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$), ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) and the flag leaf senescence gene were located through the MAPMAKER-QTL analysis. All distances are given in centi-Morgan, using Kosambi's mapping function.

under drought stress are described in a segregating population (Veery × Variant-1). The plant population were genotyped by RAPD and ISSR markers. The timing of flag leaf senescence (FLS) is an important determinant of yield under stress and optimal environments. Cereal genotypes have been shown to exhibit differences in flag leaf senescence under drought, which affect yields such as sorghum (Rosenow and Clarke, 1981), maize (Baenziger et al., 1999) and durum wheat (Hafsi et al., 2000). QTL analysis based on a genetic map derived from 48 doubled haploid lines using (SSR) markers, revealed the genetic control of this trait (Verma et al., 2004). The location of quantitative trait genes using QTL analysis is a labour and time consuming process since all the polymorphic markers are supposed to be screened on the segregating population. In recent years, some markers tightly linked to genes were found by using BSA (Xu et al., 1995; Mackay and Caligari, 2000; Zheng et al., 2002; Altinkue et al., 2003; Podlich et al., 2004; Govindaraj et al., 2005, Babiker et al., 2009). BSA was firstly reported by Michelmore et al. (1991) to identify RAPD markers tightly linked to genes for resistance to lettuce downy mildew. Using a method inspired by BSA, we are able to identify several types of molecular markers associated with flag leaf senescence in wheat under water-stressed conditions. These markers should be useful for marker-assisted selection. The present results support the idea that BSA can provide fast detection of molecular markers linked to genes of interest. Using BSA combining RAPD and ISSR markers analysis, we identified four RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and two ISSR ($M1_{1100bp}$ and $AD2_{300bp}$) linked to the flag leaf senescence gene as indicator for drought tolerance in wheat. In this study, QTLs for flag leaf senescence gene in wheat under water-stressed conditions were associated with RAPD markers ($Pr11_{230bp}$, $Pr19_{240bp}$, $OPU06_{340bp}$ and $OPH13_{450bp}$) and explained 7%, 50%, 24% and 13% of the phenotypic variation, respectively. While, QTLs for flag leaf senescence were associated with ISSR markers ($M1_{1100bp}$ and $AD2_{300bp}$) and explained 25% and 34% of the phenotypic variation, respectively. The use of molecular markers can increase the efficiency of conventional plant breeding by identifying markers linked to the trait of interest, which are difficult to evaluate and/or are largely affected by the environment. Hence, there is a need to develop a rapid screening method to select the drought tolerant individuals. Tight linkage between molecular markers and gene for flag leaf senescence can be of great benefit to drought tolerance breeding programs by allowing the investigator to follow the DNA markers (PCR-based markers) through early generations rather than waiting for phenotypic expression of the tolerant genes. Molecular markers that are closely linked with target alleles present a useful tool in plant breeding since they can help to detect the tolerant genes of interest without the need of carrying out field evaluations. Also, it allows for screening big number of breeding materials at early growth stages and in short time. The present study indicated that RAPD and ISSR markers, combined with bulked segregant analysis, could be used to identify molecular markers linked to the flag leaf senescence gene as indicator for drought tolerance in wheat. Once these markers are identified, they can be used in wheat breeding programs as a selection tool in early generations.

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