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High potential sources of resistance to spot blotch in barley supported by AFLP markers

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Abstract

Spot blotch (SB) caused by *Cochliobolus sativus* presents a serious problem for barley production worldwide, and identification new sources of resistance is a key objective for many breeders. Breeding for resistance is an economically and environmentally friendly approach to reduce negative effects of the disease, and understanding the genetic basis of this resistance can enhance the development of resistant varieties. With that goal, F2 barley recombinant lines obtained from a cross of highly barley resistant cv. Banteng and the susceptible cv. WI2291 were tested for SB resistance which supported by AFLP markers. Following greenhouse evaluations under SB artificial infection conditions, results demonstrated different significant levels among barley recombinant lines ranged from highly susceptible to resistant. However, out of the 54 recombinant lines, 25 were resistant than the others which had 7 AFLP unique bands at different regions in the genome, this distribution and resistance or susceptibility can indicate that some positive or negative markers are common either to resistant or susceptible lines. The unique and common AFLP bands might be of considerable interest for enhancing effective resistance to SB.

Keywords: Barley, spot blotch, recombinant lines, AFLP polymorphism, resistance

1. Introduction

Barley (*Hordeum vulgare* L.) is a major cereal grain grown in Syria and worldwide. Grain yield of barley is continuously challenged by several limiting factors including some fungal diseases that have the potential to induce significant losses. Spot blotch (SB) caused by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* (Sacc.) Shoemaker) is an economically important fungal disease of barley that affects wheat and several other small grains worldwide [1,2].

SB management strategies consist of fungicide application, inoculum reducing cultural practices, and the introgression of resistance into popular cultivars [3]. Development of resistant cultivars is considered to be the most practical way to control this disease [4], however, sources of complete resistance to SB have not been identified, and current Syrian barley cultivars are considered to be only moderately resistant toward *C. sativus* [5].

Complex quantitative inheritance of SB has presented the progress in barley breeding programs for its resistance [6,7]. Several SB resistance QTLs have been reported on different barley chromosomes using methods of both bi-parental mapping and association mapping, and various major genes conferring race-specific resistance to *C. sativus* have been determined [8,9].

Major difficulties in previous SB breeding efforts have been concentrated on screening plants for this disease resistance and transferring of resistance genes. Since, breeding for SB resistance is hindered by several causes, especially the rapid emerging new *C. sativus* pathotypes and partial effectiveness in elite varieties due to linkage drag [3,10]. Therefore, barley breeders need a continuous supply of new sources of resistance and improved knowledge about the reaction of barley genotypes to keep up with the pace imposed by the challenges of *C. sativus* pathogen and the demands of producers.

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The use of molecular markers for barley genetic studies has recently received a great deal of attention from breeders. However, amplified fragment length polymorphism (AFLP) markers have proved to be an effective and highly reproducible markers that permit inspection of polymorphism at a big number of loci throughout a genome within a short time [11].

The purpose of this work was to study the SB resistance in the 54 barley recombinant lines, and to detect AFLP molecular polymorphism between the two parents, Banteng 'resistant' and WI2291 'susceptible' and their progeny with a view to use these polymorphisms as genetic markers of resistance in future searches for resistant plants.

2. Materials and Method

2.1. Plant material

A total of 54 F2 barley recombinant lines were produced through crossing between cv. Banteng x WI2291 possessing different SB reactions. Banteng was received from Germany and has proved highly resistant to all SB isolates available so far over fifteen years [12], therefore, it was chosen and used in this work. WI2291, a universal susceptible cultivar received from Australia was chosen to be used in the experiments. Seeds of parental cultivars and F2 lines were grown in individual pots filled with sterilized peat moss with three replicates. Pots were placed in a growth chamber under temperature 22°C/18°C (day/night) at 90% R.H.

2.2. C. sativus inoculation

One of the *C. sativus* isolates, Pt4, was used as inoculum in all experiments. Pt4 is one of the most virulent isolate predominantly found in Syria and routinely used to screen breeding materials [13]. The isolate was grown on potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) and incubated in darkness under 23 °C for 10 days. Then, conidia were collected with 10 mL of sterile distilled water, and the conidial suspension was adjusted to 2×10^4 conidia/mL. Plants were inoculated at a fully expanded third-leaf of all the seedlings by uniformly spraying each plant with approximately 0.5 mL of inoculum suspension using a hand held sprayer. Inoculated plants were placed in darkness for 18 h at 20°C in a mist chamber at 95-100% R.H, and then were transferred to the growth chamber at 24°C/16°C (day/night) until the plants were evaluated for SB symptom development 14 days post-inoculation. The disease reaction of each genotype was assessed based on a scale of 1 to 9 as described by Fetch and Steffenson [14] where: 1 = highly resistant and 9 = highly susceptible.

2.3. DNA extraction and AFLP analysis

Genomic DNA was extracted from each genotype using a modified CTAB (hexadecyltrimethyl ammonium bromide) procedure [15]. Extracted DNA was quantified by spectrophotometer and then diluted to 50ng/µL for AFLP analysis. The AFLP protocol described by Vos et al. [11] was used, and a total of 64 EcoR1/MseI primer combinations were tested (Table 2). DNA was double digested with *Eco*RI and *Mse*RI at 37ºC for 3 hrs and then samples were incubated at 70ºC for 15 min to inactivate the restriction endonucleases. *Eco*RI and *Mse*RI adapters were ligated to the digested DNA samples to generate template DNA for amplification. Pre-amplification was performed with +1-primers each carrying one selective nucleotide (*Eco*RI + A, *Mse*RI + C) in a Gene Amp 9700 Thermocycler (Applied Biosystems, USA) for 20 cycles set at 94ºC denaturation (30 sec), 56ºC annealing (40 sec), and 72ºC extension (50 sec). The initial denaturation was done at 94ºC for 30 sec and the final extension at 72ºC for 8 min. The amplification products were diluted 10-folds in H_2O and stored at -20ºC. Selective AFLP amplification was done with *Eco*RI + 3 and *Mse*RI +3 primers and 5 µL of the diluted PCR products from the pre-amplification.

Each *Eco*RI, *Mse*I primer pairs were screened on barley parents and derived lines to assess the ability of these primer pairs to detect molecular variation. The PCR amplifications were carried out as follows: one cycle at 94ºC for 30 sec, 68ºC for 30 sec, and 72ºC for 60 sec; followed by 12 cycles of touchdown PCR in which the annealing temperature was decreased by 0.7ºC every cycle until a touchdown annealing temperature of 59ºC was reached. An additional 20 cycles were carried out as described above for pre-amplification.

The amount 8 µL of reaction product was mixed with 4µL of formamide loading buffer (98% [v/v] formamide, 10 mM EDTA,0.005% [v/v] of each of xylene cyanol and bromophenol blue) incubated at 90ºC for 3 min for and cooled on ice. Products were separated on a 6% (w/v) denaturing polyacrylamide gels. The gel was run at 60 W until the xylene cyanol was about two-thirds down the length of the gel. The experiments were repeated twice.

2.4. Statistical analysis

Only distinct, major, reproducible AFLP bands were scored. Monomorphic bands were excluded from data analyses. Percentage of polymorphism was calculated as the ratio of polymorphic bands over the total band numbers. The level of polymorphism was analyzed by counting the number of the selected polymorphic bands [16].

3. Results and Discussion

In the present work, two barley parents with different resistance levels to SB isolate Pt4 were used. Figure 1 showed that SB caused more severe infection on the susceptible parent 'WI2291' as compared with the resistant one 'Banteng'. Results demonstrated that barley seedlings had a range of infection response, progeny that produced small necrotic lesions with very slight diffuse marginal chlorosis in the 1 to 3 range were classified as high resistance, whereas those that produced medium-sized necrotic lesions with restricted chlorotic margin in the 4 to 5 range were classified as moderately susceptible, and that produced large necrotic lesions with distinct chlorotic margins in the 6 to 9 range were classified as susceptible (Table 1). However, based on this scale the recombinant lines were evaluated and classified as 25 resistant, 14 moderately susceptible and 15 susceptible lines (Table 1).

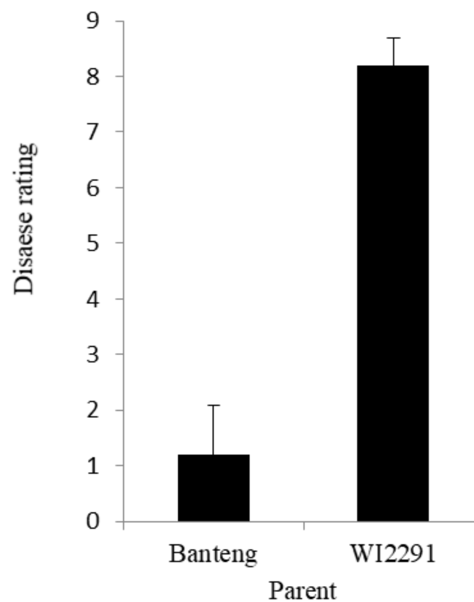

Figure 1. Frequency of disease reactions incited on the barley resistant cv. Banteng and susceptible cv. WI2291, 14 days after spot blotch infection

Table 1. SB infection response of the 54 derived progeny tested in this study.

Disease reaction[a]	Infection class	No. lines	Disease development
1-3	R	25	Small round to oblong dark brown necrotic lesions
4-5	MS	14	light brown lesions with whitish gray centers and chlorotic margins
6-9	S	15	Solid dark brown necrotic lesions with expanding chlorosis and necrosis
Total		54	

[a]Disease reaction was assessed uasing a scale 1-9 (Fetch and Steffenson 1999).

R: resistant; MS: Moderatley susceptible and S (susceptible)

Table 2. Primers used for selective amplification

Code	Name	Primers sequence (5′–3′)
E1	*Eco*RI adapter	5′-AATTGGTACGCAGTCTAC-3′ 3′- CCATGCGTCAGATGCTC-5′
E2	*Mse*I adapter	5′-TACTCAGGACTCAT-3′ 3′-GAGTCCTGAGTAGCAG-5′
E3	*Eco*RI + A	5′-GACTGCGTACCAATTC**A**-3′
M1	*Mse*I + C	5′-GATGAGTCCTGAGTAA**C**-3′
E4	*Eco*RI +ACG	5′-GACTGCGTACCAATTC**ACG**-3′
E5	*Eco*RI +ACT	5′-GACTGCGTACCAATTC**ACT**-3′
E6	*Eco*RI +AAG	5′-GACTGCGTACCAATTC**AAG**-3′
E7	*Eco*RI+ AAC	5′-GACTGCGTACCAATTC**AAC**-3′
E8	*Eco*RI+ ACA	5′-GACTGCGTACCAATTC**ACA**-3′
E9	*Eco*RI+ AGG	5′-GACTGCGTACCAATTC**AGG**-3′
E10	*Eco*RI+ ACC	5′-GACTGCGTACCAATTC**ACC**-3′
E11	*Eco*RI+ AGC	5′-GACTGCGTACCAATTC**AGC**-3′
M12	*Mse*I + CAG	5′-GATGAGTCCTGAGTAA**CAG**-3'
M13	*Mse*I + CTG	5′-GATGAGTCCTGAGTAA**CTG**-3'
M14	*Mse*I + CAT	5′-GATGAGTCCTGAGTAA**CAT**-3'
M15	*Mse*I + CTA	5′-GATGAGTCCTGAGTAA**CTA**-3'
M16	*Mse*I + CTC	5′-GATGAGTCCTGAGTAA**CTC**-3'
M17	*Mse*I + CAC	5′-GATGAGTCCTGAGTAA**CAC**-3'
M18	*Mse*I + CAA	5′-GATGAGTCCTGAGTAA**CAA**-3'
M19	*Mse*I + CTT	5′-GATGAGTCCTGAGTAA**CTT**-3'

This continuous distribution of SB reactions from slower of 1 to 9 found among barley lines suggests a lack of complete resistance and the potential involvement of a number of major genes [17,18]. This will likely make breeding for resistance more difficult than if the resistance had been controlled by single genes having major effects. Results of classical genetic analyses indicate that resistance is controlled by 1-3 loci, depending on the barley accession and the SB isolate used for testing [19,20]. However, differences in resistance gene numbers and loci may be attributed to the effects of the various environmental conditions and pathogen isolates used [21].

AFLP primer combinations gave a number of polymorphic bands with an average number of alleles per combination 19.1 allele/primer pair. The percentage of polymorphic bands over all primer pair was 71 % (Table 3). They amplified a total 1419 AFLP bands (for the 54 barley genotypes assayed) with 20 private and 1388 common bands. However, analysis of the distribution of polymorphic bands between recombinant lines and parents revealed unique genotype-specific markers, since, a total of 394 common bands were detected in the 25 resistance recombinant lines, 7 of which were unique. Whereas, the 15 susceptible lines yielded a bigger number of unique bands (495) with 5 of which were unique (Tables 4 and 5).

Here, the AFLP-generated unique bands at different regions in the barley genome might be explained by the fact that there is more than one gene involved in SB resistance [9]. Accordingly, it could be postulated that this marker may be linked to the SB resistance gene; however, we can correlate or link between this marker and resistance, since the susceptible parent ‘WI2291’ lacks this marker. The most interesting observation is that some positive AFLP markers are present in both resistant genotypes (Banteng and resistant recombinant lines) but are absent in the susceptible parent WI2291 (Table 5).

On the other hand, this work revealed that AFLP was very sensitive for detecting markers in genetic studies of barley recombinant lines, and the banding patterns attained using AFLP were found to be highly reproducible when the same DNA sample was used in independent experiments. However, silver staining was used in this study because silver-stained AFLP gels have been reported to produce a larger number of better-defined bands than phosphorous-32-labeled gels [22,23].

4. Conclusions

Collectively, this research illustrates that AFLP markers clearly revealed the differences between the barley recombinant lines and their parents. The distribution of AFLP markers and SB resistance or susceptibility levels among the parents and progeny indicated that some positive or negative markers are common either to resistant or susceptible genotypes. The common polymorphic bands for either susceptible or resistant genotypes were highlighted in the present study. These bands can be employed to identify molecular marker(s) tightly linked to SB resistance gene(s) and to map those genes in the F2 population of a cross between the extremely resistant and susceptible barley genotypes characterized herein. On the other hand, out of the 54 resistant genotypes, 25 were highly resistant which could be considered as possible donors for SB resistance in further breeding programs.

Table 3. AFLP band numbers and polymorphic bands revealed in 54 barley lines.

Primer combination	Total No. of bands	No. of polymorphic bands	% Polymorphism
AAC x CTG	15	13	86.7
ACG x CAG	20	11	55.0
ACG x CAT	16	11	68.8
ACT x CAT	21	12	57.1
ACT x CTG	18	15	83.3
AGG x CTG	20	11	55.0
AGG x CAG	18	12	66.7
AGC x CAG	18	14	77.8
CAT x CTG	22	15	68.2
AAC x CTG	23	21	91.3
Mean	19.1	13.5	71.0

Table 4. AFLP bands dected within 54 barley lines used in the study.

Class	No. lines	AFLP bands		
		Common	Private	Total bands
R	25	394	7	401
MS	14	510	8	518
S	15	495	5	500
Total	54	1399	20	1419

Table 5. SB reaction of the parental barley cultivars ' Banteng and WI2291', and the 54 derived progeny and AFLP unique bands

Parent/Progeny	SB reaction [a]	AFLP unique bands[b]			
		700bp	600bp	500bp	400bp
Banteng	R	+	+	+	+
WI2291	S	-	-	-	-
1	S	-	-	-	-
2	S	-	-	-	-
3	R	-	+	+	+
4	R	-	-	+	+
5	S	-	-	-	-
6	S	-	-	-	-
7	S	-	-	-	-
8	MS	+	-	-	-
9	R	+	+	+	+
10	MS	-	+	+	+
11	MS	-	+	+	+
12	MS	+	-	-	+
13	S	-	-	-	-
14	S	-	-	-	-
15	MS	-	+	+	+
16	R	+	+	+	-
17	MS	-	+	+	-
18	MS	-	+	-	-
19	R	-	+	+	+
20	MS	-	+	+	+
21	S	-	-	-	-
22	R	+	+	+	+
23	R	+	+	+	+
24	R	+	+	+	+
25	R	+	+	+	+
26	S	-	-	-	-
27	S	-	-	-	-
28	R	+	+	+	+
29	S	-	-	-	-
30	MS	+	-	-	-
31	R	+	+	+	+
32	R	-	+	-	+
33	R	+	+	+	+
34	MS	+	-	+	-
35	S	-	-	-	-
36	S	-	-	-	-
37	R	+	+	+	+
38	R	-	+	-	+
39	R	+	+	+	+
40	R	+	+	+	+
41	R	+	+	+	+
42	R	+	+	+	+
43	R	+	-	+	+
44	R	-	+	-	+
45	S	-	-	-	-
46	MS	+	-	-	+
47	R	+	-	-	+
48	R	-	-	-	-
49	S	-	-	-	-
50	MS	+	-	+	+
51	MS	-	-	+	-
52	MS	-	+	+	-
53	R	+	+	+	+
54	R	+	+	+	+

[a]Disease reaction was assessed uasing a scale 1-9 according to Fetch and Steffenson (1999). R: resistant; MS: Moderatley susceptible and S (susceptible). [b] Presence (+) or absence (–) of an AFLP unique band.

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