American Journal of Plant Sciences, 2013, 4, 2039-2042
http://dx.doi.org/10.4236/ajps.2013.410255 Published Online October 2013 (http://www.scirp.org/journal/ajps)

Search for a Microsatellite Marker Linked with Resistance Gene to *Xanthomonas axonopodis* pv. *malvacearum* in Brazilian Cotton

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Received August 1st, 2013; revised September 1st, 2013; accepted October 1st, 2013

ABSTRACT

The cotton cultivar DELTAOPAL is resistant under field as well as under glasshouse conditions to the Brazilian isolates of *Xanthomonas axonopodis* pv. *malvacearum* (Xam). Segregating populations derived from the cross between this cultivar and one susceptible cv. BRS ITA 90, were utilized to identify molecular marker linked with the resistance gene to Xam by "Bulk Segregant Analysis (BSA)". Two hundred and twenty microsatellite (Single Sequence Repeat—SSR) primers were tested. The amplification products were visualized in polyacrylamide gels stained with silver nitrate. Only one primer was informative and showed polymorphism between the DNA of the parents and their respective bulks of homozygous F_2 populations contrasting for resistance and susceptibility, and hence was used to analyze DNA of 120 F_2 populations. The microsatellite primer yielded one band of 80 bp linked with the resistance locus, which was absent in the susceptible parent as well as in the bulk of the homozygous susceptible plants of the cross. The segregation ratio as determined by phenotypic analysis was 3R:1S. It is believed that the microsatellite marker was linked with the resistance locus and hence may offer new perspectives for marker assisted selection against the angular leaf spot disease of cotton. It is however, felt necessary to repeat the microsatellite analysis and make sure that the primer is tightly linked with the resistance locus and at the same time verify the genetic distance between the marker and the resistance locus.

Keywords: *Gossypium hirsutum* L.; *Xanthomonas axonopodis* pv. *Malvacearum*; Genetic Markers; Marker Assisted Selection

1. Introduction

The angular leaf spot of cotton, also known as "bacterial blight" and "black arm" of cotton, caused by *Xanthomonas axonopodis* pv. *malvacearum* (Xam) is economically important in several cotton growing areas of Asia, Africa, USA and the Latin America, including Brazil. The pathogen is seed transmitted and can cause appreciable yield losses. In the USA the estimated annual losses could be between 0.1% and 2.3% [1]. In Brazil, the disease is reported to cause heavy yield losses under favorable weather conditions [2]. So far, there is no practical method to eradicate the pathogen from the seed. Many commercial cultivars are susceptible and the disease is not controlled by fungicidal applications. The angular leaf spot disease however, can be controlled through varietal resistance.

Resistance has been attributed to the combination of two or more major resistance genes and a modifier complex. Nineteen races of the pathogen currently are recognized in the USA [1,3]. In Brazil, although sources of resistance like cvs. DELTAOPAL, EPAMIG LIÇA and FIBERMAX 986, are available, breeding for resistance against this disease is difficult. Conventional breeding strategies for disease resistance which involve pyramiding of resistance genes are time consuming, expensive and require artificial inoculations under glasshouse and field conditions. Invariably, the artificial inoculations with bacteria give a margin for misinterpretation of the results because of the inconsistent reactions influenced by unsuccessful inoculations especially under field conditions [4]. Marker assisted selection (MAS) is being advocated

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to aid selection of resistant plants in early generations in some plant species [5-12]. The MAS technique allows us to identify the presence of resistance gene(s) in segregating populations of early generations within a few days and avoids repeated inoculations under field conditions. It is based on the genotypic reaction and not on the phenotypic reaction of the plant. The rapid identification of segregating plants carrying resistance genes drastically reduces the number of segregating plants to be evaluated in subsequent generations. Besides, the MAS offers complete reliability in identification of plants carrying the resistance gene. For this purpose, however, prior knowledge about the molecular marker linked with the gene of interest is a prerequisite.

Attempts were made in the present investigation to identify microsatellite markers tightly linked with the resistance genes in the cv. DELTAOPAL so that they can be useful in marker assisted breeding programs.

2. Material and Methods

Genetic seed material and crosses: Seeds of DELTAOPAL as resistant and BRS ITA 90 as susceptible, were obtained from the germplasm collection of IAPAR and were multiplied by selfing a typical representative plant of each cultivar. Crosses were made between the resistant and susceptible cultivars and F_2 seeds were obtained. One hundred and twenty segregating F_2 plants were evaluated for disease reaction by artificial inoculation under glasshouse conditions. DNA of F_2 plants was extracted and stored for further use. Twenty segregating plants classified as highly resistant and another 20 classified as highly susceptible were selfed to produce F_3 seeds. Four hundred plants derived from the 20 $F_{2:3}$ families (20 plants of each F2 plant), were inoculated in the same way as the F_2 plants in order to identify the F_2 plants homozygous for resistance and homozygous for susceptibility and the DNA of only such F_2 plants was used for constructing homozygous "bulks". Two contrasting "bulks" composed of equal quantities of DNA of five homozygous F_2 plants for resistance and four homozygous F_2 plants for susceptibility were formed and used for "Bulk Segregant Analysis"—BSA [9]. All populations were conducted in the glasshouse.

Inoculations and evaluation of disease symptoms: The inoculation technique, the incubation conditions and the disease assessment scale were basically the same as reported earlier [13]. Inoculations were made using the tooth pick method and an aggressive isolate (N˚ 13403) of Xam from the culture collection of IAPAR, representing race 18. Inoculated plants were incubated in a mist chamber for 24 h, adjusted at 22˚C - 24˚C and about 100% relative humidity, and later were transferred onto the glasshouse bench. Plants were evaluated for the disease intensity 20 days after inoculation using a disease severity scale of 0 - 3 [13], where 0 = resistant (incompatible reaction) and 1 - 3 = susceptible (compatible reaction).

DNA extraction: DNA was extracted before inoculation using a protocol as described by Doyle & Doyle [14]. The amount of DNA was quantified by electrophoresis gels as well as by a DyNa Quant 200 Fluorometer (Pharmacia). The DNA samples were diluted in 100 μL TE buffer, adjusted to 20 $ng \cdot \mu L^{-1}$ and stored at −20˚C for further use. Samples which showed degradation of DNA in electrophoresis gels were discarded and DNA extraction was repeated. RNA was eliminated by adding 3 μL of RNAase (10 $mg \cdot mL^{-1}$).

Bulked segregant analysis and PCR protocols: PCR reactions were performed in 15 μL volumes containing 1.8 μL of 1 M $MgCl_2$, 1.5 μL of 100 mM/500 mM Tris-KCl (pH 8.3), 1.5 μL of 0.25 mM dNTP, 0.8 μL of BSA 1%, 1.5 μL of each one of the 0.2 μM of primer Forward and primer Reverse, 3.0 μL (30 ng) DNA, 1.0 μL (1 unit) Taq DNA polimerase (Pharmacia, USA), and 3.2 μL of autoclaved distilled water. Negative controls without DNA were maintained in all the reactions. Two hundred and twenty microsatellite primers (Invitrogen) were tested against the DNA of two bulks of F_2 populations contrasting for resistance and susceptibility and their respective parents. Amplification was performed in a thermal cycler (MJ Research, Inc. Watertown, MA, USA), according to the following program: 94˚C for 4 min followed by 60 cycles of 94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 45 sec, and a final extension at 72˚C for 7 min. Amplification products (15 μL) were electrophoresed in polyacrylamide gels (10%) with TBE running buffer, stained with silver nitrate, and were scanned into a computer imaging file using a Kodak EDAS 120 digital camera. After the identification of the molecular marker, segregation ratio for the resistance and susceptibility was determined using DNA of all the F_2 plants.

3. Results & Discussion

Analysis of the F_2 populations derived from the cross DELTAOPAL × BRS ITA 90, yielded a segregation ratio of 3R:1S suggesting the presence of a single dominant gene for resistance to Xam [3]. Out of the 220 microsatellite primers tested, only one (Primer BNL 2634. Reverse, Sequence 5' to 3' CCCAGCTGCTTATTGG-TTTC; Forward, Sequence 5' to 3' AACAACATT-GAAAGTCGGGG), showed polymorphism between the parents and between the two F_2 homozygous bulks contrasting for resistance and susceptibility.

Figure 1 shows the banding pattern obtained in BSA with one microsatellite primer. The microsatellite primer yielded one band of 80 bp linked with the resistance lo-

cus which was absent in the susceptible parent as well as in the bulk of the homozygous susceptible plants. The segregation ratio as determined by phenotypic analysis was 1:2:1 (**Table 1**). It can be observed that all the F2 plants which composed the susceptible bulk have the same allele as the susceptible progenitor. The plants which composed the resistant bulk have the same allele as the resistant progenitor, in homozygous or in heterozygous form. Since the plants which composed the bulks were selected by analyzing their respective progenies as being homozygous, the heterozygous genotype for the marker may be resultant of the recombination. It is believed that the microsatellite marker was linked with the resistance locus and hence may offer new perspectives for marker assisted selection against angular leaf spot disease of cotton in Brazil.

The cv. DELTAOPAL is resistant to the populations of Xam occurring in Brazil, including the race 18. So far, there is no report of angular leaf spot symptoms on this cultivar under the diversified cotton growing areas of Brazil. Dominance for resistance in this cultivar was complete. The number of F2 individuals used in constructing two contrasting bulks was small. According to Michelmore *et al*. [9], loci not segregating in the population, whether linked or not, will not distinguish the bulks.

The search for molecular markers tightly linked with the resistance gene is a random process. We tested 220 SSR primers which represent only a very small portion of the cotton genome. Since the resistance to *R. areola* is governed by one gene, only one locus of the whole genome would have this gene. SSR primers which do not amplify regions near this locus would always be segregating irrespective of the resistance indicating that there is no genetic linkage. Only the primers which amplify regions near the locus which has the resistance gene would segregate with it and would indicate the existence of genetic linkage. It is for this reason that several SSR primers encompassing different regions of the genome need to be tested to increase the chances of identifying primers tightly linked with the resistance gene.

Further work is necessary to verify whether the microsatellite marker identified in the present investigation is tightly linked with the resistance locus in cv. DELTAOPAL. It would also be interesting to verify whether the microsatellite marker is linked with the resistance gene(s) in other cotton cultivars with different genetic background including cultivars of *G. barbadense*. Other than marker-assisted selection in breeding populations, resistant lines or accessions from germplasm collection can also be screened once a molecular marker tightly linked with the resistance gene is identified. Molecular marker has an additional advantage in the sense that the

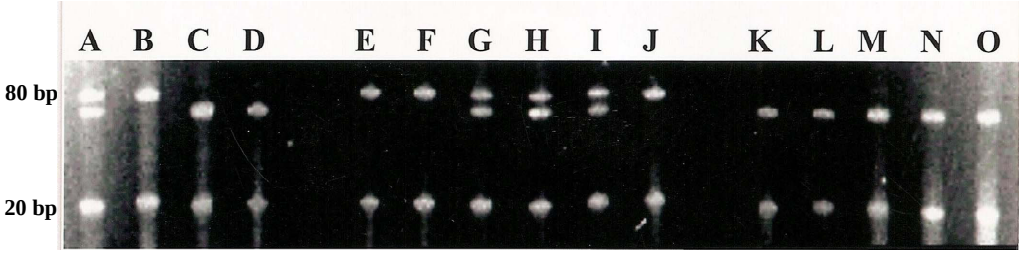

Figure 1. Bulk segregant analysis showing banding pattern obtain in polyacrylamide gel with microsatellite primer BNL 2643. Lane A = resistant bulk; B = resistant parent (DELTAOPAL); C = susceptible bulk; D = susceptible parent (ITA 90). Lanes E-J = F2 homozygous resistant; Lanes G-I = heterozygous resistant individuals. Lane K-N = F2 homozygous susceptible and O = susceptible parent.

Table 1. Segregation of disease reactions for the leaf area infected by *Xanthomonas axonopodis* pv. *malvacearum* (Xam), among parental cotton cultivars and their segregating populations of the cross DELTAOPAL X BRS ITA 90.

Parents and progeny	Leaf reaction**	Total No. of indivíduals	Theoretical ratio (R:S)	Number of individuals in each category of infection*				χ^2	Prob. %
				Observed		Expected			
				R	S	R	S		
DELTAOPAL	R	23	1:0	23	0	23	0		
BRS ITA 90	S	18	0:1	0	18	0	18		
F_1	R	20	1:0	20	0	20	0		
F_2	Segreg.	127	3:1	95	32	95.25	31.75	0.002	94.91

*Inoculations were performed in the glasshouse, on 25 days old plants, using an aggressive isolate of Xam and disease reactions were observed 20 days after inoculation; **R = Resistant; S = Susceptible; Segreg = Segregating. Source: Zandoná *et al*. (2006).

expression of the marker is not masked by epistatic interactions that occur between resistance genes, permitting thereby, pyramiding of resistance genes in agronomically desirable cultivars [5,15].

4. Conclusion

Out of 220 SSR primers screened, only one primer showed polymorphism between resistant and susceptible parents and their bulks contrasting for resistance and susceptibility for angular leaf spot of cotton. The results indicate that the marker is linked with the resistance gene and could be used in breeding programs aimed at marker assisted selection in Brazil. However, further research is needed to verify if the marker is tightly linked with the resistance loci and the distance between the marker and the resistance locus.

5. Acknowledgements

The present research was conducted under the financial support of IMA, MT, Brazil. Thanks are also due to Luceleine P. Lopes, Carla Zandoná, and Priscila Alves, for the technical support.

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