DEVELOPMENT OF SEQUENCE TAGGED SITE (STS) MARKERS FOR VERTICILLIUM WILT RESISTANCE IN COTTON BASED ON RGA-AFLP ANALYSIS

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Abstract

Verticillium wilt (VW) is one of the destructive diseases in cotton. Identification of resistance gene analogs (RGAs) can provide candidate genes for marker-assisted selection (MAS) for VW resistance and cloning of VW resistance genes. The objective of this study was to identify RGA-targeted amplified fragment polymorphic (AFLP) markers (RGA-AFLP) for the conversion into sequence tagged site (STS) markers. A total of 54 RGA-AFLP markers, including 28 from a backcross inbred line (BIL) population and 26 from a recombinant inbred line (RIL) population, were cloned and sequenced. Of the 86 unique sequences, 51 were found to be homologous to genes in the Cotton Gene Index database. A total of 72 primer pairs were designed, resulting in 9 and 7 polymorphic STS markers in the BIL and RIL populations, respectively. These STS markers will be useful in linkage mapping and MAS for enhancing VW resistance in cotton.

Introduction

Cotton is the most widely cultivated fiber crop for the textile industry which plays an important role in the world economy. Verticillium wilt (VW) is one of the most destructive diseases in many crops and one of the major constraints to cotton production. Breeding and growing resistant cultivars is the most cost-effective way to control the disease. Although much effort has been made to develop VW resistant cotton cultivars, the traditional breeding method suffers low selection efficiency. Marker-assisted selection (MAS) is a promising way to facilitate selection for improvement of both quantitative and qualitative traits including VW resistance. For efficient MAS, the conversion of nonspecific molecular markers such as amplified fragment length polymorphism (AFLP) linked to VW resistance into polymorphic sequence tagged site (STS) markers is necessary for reliable genotype scoring (Meksem et al., 2001).

Resistance gene analogs (RGAs) provide targeted genes to develop useful markers for tagging disease resistance genes on a genome-wide basis. However, markers amplified by degenerate RGA primers have a lower discrimination power. AFLP has a higher level of polymorphism and throughput, but with a lower specificity (Zhang et al., 2007). In this study, a combination of RGA and AFLP, i.e., RGA-AFLP marker system (Zhang et al., 2007; Niu et al., 2011) , was performed to search for markers associated with VW resistance and to develop STS markers linked to VW resistance in a backcross inbred population (BIL) and a recombinant inbred line (RIL) population.

Materials and Methods

Mapping populations

Two permanent genetic populations were used in this study. A backcross inbred line (BIL) population of 146 lines was developed from a cross between Upland cotton SG 747 (*Gossypium hirsutum*) and Pima S-7 (*G. barbadense*) followed by two generations of backcrossing to SG 747 and three generations of selfing (Pang et al., 2012). A recombinant inbred line (RIL) population of 94 $F_{5:9}$ lines was developed from TM-1 and NM 24016 (Gore et al., 2012).

RGA-AFLP analysis

The procedure for RGA-AFLP was the same as AFLP (Vos et al., 1995) except for that one degenerate RGA primer was combined with one selective AFLP primer in the selective amplification (Zhang et al., 2007; Niu et al., 2011).Thirteen primer pairs were selected to genotype a subset of the BIL population (96 lines) and 14 primer sets were selected to genotype 94 RILs along with their parents.

Cloning and sequencing

Targeted polymorphic RGA-AFLP bands were excised from the polyacrylamide gel (PAGE), eluted in ddH_2O, and used as DNA template to reamplify with the same primer pair used for RGA-AFLP. The PCR products were then cloned into pGEM-T Easy vector system (Promega, Madison, WI). Positive clones were isolated and cultured for plasmid DNA extraction. Three clones of each targeted RGA-AFLP marker were sequenced.

Sequence alignment and analysis

Sequences of each cloned RGA-AFLP marker were first aligned using ClustalW. Unique sequences were then searched against the Cotton Gene Index database (Release 11.0, Dana-Farber Cancer Institute; http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=cotton) for homologous genes.

Designing and screening of STS primers

Sequence or gene specific primers were designed based upon the unique sequences including assembled contigs and singletons. Primers were screened using selected resistant and susceptible lines from both the BIL and RIL populations. Informative primer pairs which yielded polymorphisms were then selected to further genotype the corresponding populations. PCR products were dissolved in non-denatured PAGE for manual scoring of polymorphisms.

Results and Analysis

RGA-AFLP analysis

In the BIL population, 160 polymorphic RGA-AFLP markers were produced using 13 primer combinations. In RIL population, 114 polymorphic RGA-AFLP markers were developed with 14 primer sets.

Sequence analysis of RGA-AFLP markers

A total of 54 RGA-AFLP markers (28 from BILs and 26 from RILs) were cloned and 160 clones (3 clones / marker) were sequenced except for two markers with two clones each. Of the 54 sequenced markers, 23 had one consensus sequence each; 24 had 2 different sequences each; and 7 had 3 different sequences each. This yielded a total of 86 unique sequences.

Of the 86 unique sequences amplified from RGA-AFLP DNA markers, 51 (60%) were homologous to genes in the Cotton Gene Index, indicating that the RGA-AFLP marker system was targeted to gene regions on the cotton genome. Many of these homologous gene sequences encoded products similar to those genes previously characterized in other organisms (Table 1 and 2). Of the 51 homologous gene sequences, 39 (76.5%) were similar to genes in plants including 15 sequences similar to genes from grape (*Vitis vinifera*) and 9 sequences similar to genes from *Arabidopsis thaliana*. Of the 9 sequences similar to genes in *Arabidopsis*, 5 were of particular interest because they were similar to a gene coding for an Avr9 elicitor response protein in *A. thaliana*. Seven sequences were similar to genes from different cotton species, some of which were similar to cDNA clones from bacterial-blight-resistant (BBR) cotton. Other sequences were similar to Ribonuclease F, T2, H, and Endopeptidase Clp (Table 1).

Conversion of RGA-AFLP to STS markers

A total of 72 primers were designed according to 36 unique RGA-AFLP sequences including 21 contigs and 15 singletons of interest. Ideally, only one single fragment of a specific length was amplified by the STS primers in genotypes having the target sequence. In this study, however, more than one band was produced from some of the STS primer combinations. In the BIL population, 7 primer pairs were informative and used. The polymorphism ratio of the informative primer pairs ranged from 11.1 to 82.6% with an average of 31.1%. In the RIL population, 8 primer pairs were informative and used. The polymorphism ratio of the informative primer pairs ranged from 9.1 to 89.3% with an average of 18.0%.

After genotyping, 9 STS markers were produced in the BIL population from 7 designed primer sets (Table 3, Figure 1). With 8 designed primer sets, 7 STS markers were generated in the RIL population (Table 3, Figure 1). These newly developed STS markers showed polymorphism in their respective populations and can be used for constructing a linkage map and marker-assisted selection (MAS) after they are validated to be associated with VW resistance.

Table 1. Summary of homology of cloned RGA-AFLP markers based on the Cotton Gene Index database.

Source	Organism	No. of hit	Ratio (%)	Description of hit
Plant	*Vitis vinifera*	15	29.4	Chromosome scaffolds / uncharacterized protein
	A. thaliana	9	17.6	Similar to Avr9 elicitor response protein
	Gossypium spp.	7	13.7	cDNA from BBR cotton, similar to Ribonuclease F, etc.
	Tomato / potato	5	9.8	Putative gag-pol polyprotein / retrotransposon protein
	Others	3	5.9	Retrotransposon protein / RNA-binding protein
Animal	Wolf, zebrafish, etc.	4	7.8	Hypothetical protein / synaptogyrin / open reading frame
Pathogen	Hepatitis C virus	3	5.9	Genome polyprotein
	Bacterium	2	3.9	LigA precursor / PTS family enzyme IIBC glucitol
	Fungus	2	3.9	Calcium-transporting ATPase sarcoplasmic
Alga	*Ostreococcus tauri*	1	2.0	Unknown function

Table 2. Homology of cloned RGA-AFLP markers based on the Cotton Gene Index database.

Unique sequence	Size (bp)	Unigene Accession	E value	Identity	Description of top hits	Source organism
Contig 1	167	TC243008	2.5E-27	149/159	Avr9 elicitor response protein	*A. thaliana*
Contig 5	359	TC244555	3.6E-48	165/168	Uncharacterized protein	*A. thaliana*
Contig 6	208	TC229864	5.0E-33	175/183	Chloroplast protein YCF1	*G. hirsutum*
Contig 7	471	TC236794	1.2E-12	233/388	Hypothetical protein	*Danio rerio*
Contig 11	460	TC237297	2.3E-66	192/197	Chromosome 6 scaffold_3	*Vitis vinifera*
Contig 13	578	TC243033	3.8E-98	278/290	Chromosome 2 scaffold_11	*V. vinifera*
Contig 24	323	No hit	-	-	No hit	
Contig 37	212	TC255076	2.1E-22	165/206	Chromosome 5 scaffold_2	*V. vinifera*
Contig 38	257	No hit	-	-	No hit	

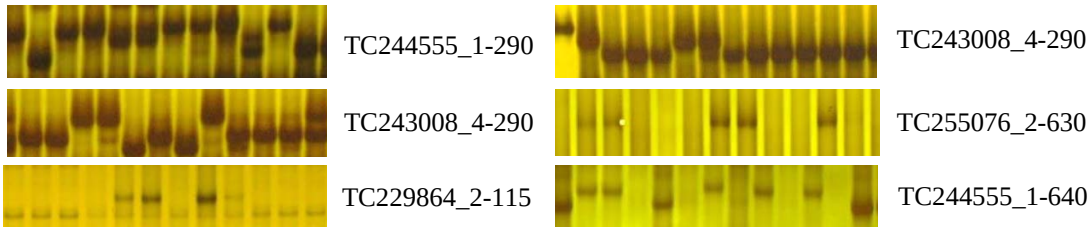

Figure 1. Selected STS markers showed polymorphisms in respective population. The left three gel photos showed polymorphisms in BILs, and the right three gel photos showed polymorphisms in RILs.

Table 3. STS markers and corresponding primer information in BIL and RIL population.

STS marker ID	Clone ID	Size (bp)	Primer sequences (5′ → 3′)	Tm (℃)	GC (%)	Length (bp)
BIL population						
TC243008_4-290	NLRRF4-180	167	F: TTATGCCATCCTCTCCCAAG	60.0	50.0	20
			R: CGGATCTAGGGTCGGAGTT	60.0	55.0	20
TC244555_1-290	RLKF2-470	359	F: AACCACTCCGCCATTAACAC	59.9	50.0	20
			R: ACGACATCGCGTTCTCTTTC	60.4	50.0	20
TC244555_1-280	RLKF2-470	359	F: AACCACTCCGCCATTAACAC	59.9	50.0	20
			R: ACGACATCGCGTTCTCTTTC	60.4	50.0	20
TC229864_1-780	PtoKin1E-225	208	F: CAAGCAGAAGGTGAAGAG	51.6	50.0	18
			R: TTGCTATCTGTGCCCATC	55.5	50.0	18
TC229864_2-1150	PtoKin1E-225	208	F: AGCAAAGCATCTACCTGG	53.8	50.0	18
			R: CTTGGACTTGATGGTGTTG	54.8	47.4	19
TC236794_3-205	RLKR-E-500	471	F: CTCCAACACTTCCCAGAACC	59.6	55.0	20
			R: CGAGAATGCAATGCGATAG	59.9	45.0	20
TC236794_3-200	RLKR-E-500	471	F: CTCCAACACTTCCCAGAACC	59.6	55.0	20
			R: CGAGAATGCAATGCGATAG	59.9	45.0	20
TC237297_3-460	Ptokin1E-495	460	F: TCACAATCTCAGCACGAG	51.9	50.0	18
			R: AACACCAATGACAAAGACC	51.7	45.0	20
TC243033_1-1490	RLKR-E -640	578	F: TAACAATGACGGCTGGTG	56.5	50.0	18
			R: TCAGGGAAGAGAGTAGCAG	53.0	53.0	19
RIL population						
TC243008_4-300	NLRRF4-180	167	F: TTATGCCATCCTCTCCCAAG	60.0	50.0	20
			R: CGGATCTAGGGTCGGAGTT	60.0	55.0	20
TC243008_4-290	RLKF2-470	167	F: TTATGCCATCCTCTCCCAAG	60.0	50.0	20
			R: CGGATCTAGGGTCGGAGTT	60.0	55.0	20
9_1-310	NLRRF4-350	323	F: ATCGTGTTGAGCCATTCC	56.9	50.0	18
			R: GACTCAGTTTGGCGCATAC	56.8	52.6	19
9_1-300	NLRRF4-350	323	F: ATCGTGTTGAGCCATTCC	56.9	50.0	18
			R: GACTCAGTTTGGCGCATAC	56.8	52.6	19
TC255076_2-630	RLRRF3-280	212	F: AGCATTTGAGAAGTTAGTCC	54.3	42.9	21
			R: AAGCAAGCAACAAGGTATC	56.1	45.0	20
TC244555_1-640	RLKF2-470	359	F: AACCACTCCGCCATTAACAC	59.9	50.0	20
			R: ACGACATCGCGTTCTCTTTC	60.4	50.0	20
TC244555_1-290	RLKF2-470	359	F: AACCACTCCGCCATTAACAC	59.9	50.0	20
			R: ACGACATCGCGTTCTCTTTC	60.4	50.0	20

Summary

Based on the RGA-AFLP analysis, out of 160 RGA-AFLP markers in the BIL population, 9 were successfully converted into STS markers. In the RIL population, of 114 RGA-AFLP markers, 7 were converted into STS markers. These STS markers will be useful for gene mapping and MAS for disease resistance including VW resistance.

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