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STUDIES ON THE BIOINFORMATICS ANALYSIS FOR THE PPV (PLUM POX VIRUS) MOLECULAR MARKERS

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Abstract

The plum pox virus produces an extremely damaging disease in fruit stone species with major implications in fruit production but also in phytosanitary status of fruit plantation. The genome of the virus encodes a single large polyprotein, a precursor that determines the serological properties of PPV, namely CP (Coat protein). This poly protein is proteolytically catalyzed by 3 viral encoding proteases that can synthesize up to 10 functional proteins. The capsid protein binds to the carboxyl end of the polyprotein. The in vitro properties of the viral extract vary with the strain and the plants used for propagation. Establishing the best primers for the molecular detection of the Plum pox virus is an extremely important stage, so a bioinformatics analysis it's necessary to identify potential sources of results misconduct (sources of contamination that can produce false positive results) is taken into account. The detection primers P1 5' ACCGAGACCATCACCCTCCC 3' and P2 – 5' CAGACTACACCGTCGCCAGA 3', were tested for the ability to form the hairpin secondary structure, self-dimerization capability, heterodimerization capability, Tm mismatch through the OligoAnalyzer program from IDT Company. The results have shown that the proposed primers can be used in PCR reactions and the results are not influenced by the artefacts.

Key words*: pox virus, primers, markers, bioinformatics.*

INTRODUCTION

Plum pox produces a highly damaging disease in stone fruit trees species with major implications in fruit production but also in phytosanitary status of fruit plantation.

Important economic loss and significant reduction in productive areas stimulated breeding programs aimed at enhancing resistance to the pathogen in such countries as Greece (Karayiannis I1999), France (Audergon J-M 1994), Italy (Bassi D.,1995) Spain (Egea J,1999), and the Czech Republic (Polák J 1994).

Development of molecular marker maps for segregating crosses is a significant accomplishment toward understanding the genetics of PPV resistance and developing markers that could potentially be useful in breeding programs. Four molecular genetics maps based on intraspecific crosses introducing PPV resistance from North American cultivars 'Stark Early Orange' and 'Goldrich' have been established to map a PPV resistance in apricot (Lambert P2007); (Sicard O.2007) On these maps, a major genomic region associated with PPV resistance was located on the Prunus G1 at a distance of 20–40 cm. In total, five SSR markers linked to the targeted resistance locus were identified in this region. Three of them have been already successfully tested for marker assisted selection (MAS) in a set of susceptible/resistant cultivars.

The bioinformatics analysis of molecular markers used in either PPV detection or marker assisted selection is a very important step in the process of breeding program of genetically-engineered varieties.

MATERIALS AND METHODS

This paper deals are to searching in the public data bases for sequences similar to those of primers to identify potential sources of results vitiation (sources of contamination that can produce false positive results). Activity was performed for each pair of primers with the BLAST program. The results obtained in alignment for primer pair P1 5 'ACCGAGACCATCACCCTCCC 3' and P2 - 5 'CAGACTACACCTCGCCGCCAGA 3 are shown in Figure 1. The simple way of running

the primers in a PCR is presented here; If by chance, two consecutive primers, in the neighborhood, attach themselves to the die in opposite directions, each on one of the two strands, at a distance not large enough to each other, the fragment delimited by them will be amplified, and if one of the two sites are absent in one of the individuals, amplification will not take place, highlighting a presence / absence polymorphism. In our case it refers to the presence or absence of PPV virus.

In this paper we determined: testing the capacity of the primers to form the hairpin secondary structure, the self-dimerization capability, possibility to hetero-dimerization, Tm mismatch.

The test of capacity of the primers to form the hairpin secondary structure, the self-dimerization capability, possibility to hetero-dimerization, Tm mismatch (which may prevent the obtaining of the amplification or may lead to the emergence of nonspecific alignments and non-specific PCR products) was made in silico (program OligoAnalyzer of the IDT Company) (https://eu.idtdna.com/calc/ analyzer) and were selected the following conditions:

- Type matrix – AND
- Primers concentration 0,25mM
- Concentration Na+ 50mM
- Concentration Mg++ 0,25mM
- Concentration dNTP 0,25mM

Products on target templates

>NC_006098.5 Gallus gallus breed Red Jungle Fowl isolate RJF #256 chromosome 11, GRCg6a

product length = 800
Features flanking this product:
122 bp at 5' side: LOW QUALITY PROTEIN: chymotrypsinogen A-like
120 bp at 3' side: chymotrypsinogen 2-like precursor

Forward primer	1	ACCGAGACCATCACCCTCCC	20
Template	1968868	....C.G.....A...	1968887
Forward primer	1	ACCGAGACCATCACCCTCCC	20
Template	1969667	....C.G.....A...	1969648

Figure 1. BLAST analyses for P1 and P2 primers

RESULTS AND DISCUSSIONS

The genome of the virus encodes a single large polyprotein, a precursor that determines the serological properties of PPV, namely CP (Coat protein). This polyprotein is proteolytically catalyzed by 3 viral coding proteins that can synthesize up to 10 functional proteins. The capsid protein binds to the carboxy terminal of the polyprotein. The *in vitro* properties of the viral extract vary with the strain and the plants used for propagation.

Establishing the best primers for the molecular detection of Plum pox virus is an extremely important stage, so bioinformatics analysis to identify potential sources of outcome (sources of contamination that can produce false positive results) is taken into account.

The development of SSR markers aimed at defining genes of interest involved in PPV resistance, using the Primer 0.5 software for this purpose. (Figures 2)

The PCR was performed with 10 ng of genomic DNA, 2 mM MgCl2, 0.1 mM dNTP, 1x buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl] 0.4 μM for each sense primer and antisense, and 0.5 U of Taq DNA Polymerase (Qbiogen).

The annealing temperature was set at 57.5 °C and the elongation time was 90 seconds. The amplified fragments were highlighted in a 1.5% electrophoresis gel.

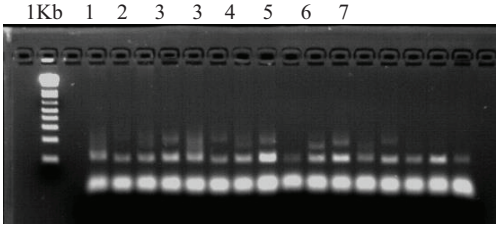

Figure 2 Electrophoresis for PCR with SSR markers in apricot progenies

The detection primers P1 5 'ACCGAGACCATCACCCTCCC 3' and P2 - 5 'CAGACTACACCGTCGCCAGA 3' were tested for ability to form hairpin secondary structures, self-dimerization capability, heterodimerization possibility, Tm mismatch through OligoAnalyzer program from IDT Company. The results have shown that the proposed primers can be used in PCR reactions and the results are not influenced by the artifacts.

The results obtained from the BLAST analysis indicated that the primers used in this study can produce nonspecific amplification reactions using genetic material from Gallus gallus, Bos taurus, Mus musculus, Pan troglodytes or Canis lupus as template. Taking in account the way when the biological samples used in this study were taken, we consider that the obtained

results only reflect the characteristics of the biological material used, the possibility of being influenced by the cross-contamination with biological material from the aforementioned species being null. ***Tm mismatch*** (figure 3).

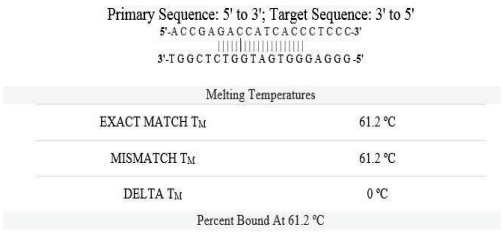

Figure 3: Exact and Single Base Mismatch DNA Thermodynamics, Primer 1

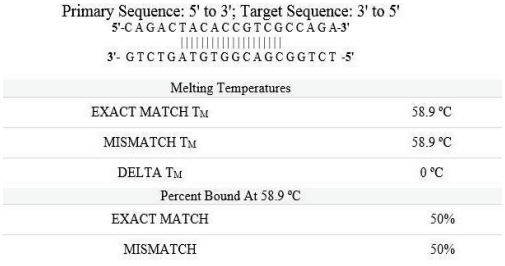

Figure 4: Primer pair I, Primer 2. CAGACTACACCGTCGCCAGA

The results obtained show that for the primer pairs tested:

- estimated alignment temperatures are less than 20 ° C; - the GC account is between 50 and 65%;

- theoretically, primers can form a small number of secondary structures; the structures are formed by the complementarity of 1-3 pairs of nitrogen bases and are stable at temperatures lower than 27^0C.

This temperature is much lower compared to the lowest calculated primer temperature (57.5 ° C). Consequently, the theoretical secondary theorems that the primers can form cannot be found within the temperature range in which the PCR program is running (57.5-95^0C) and, therefore, they cannot influence the amplification reaction; possibilities for self-dimerization and heterodimerisation are reduced

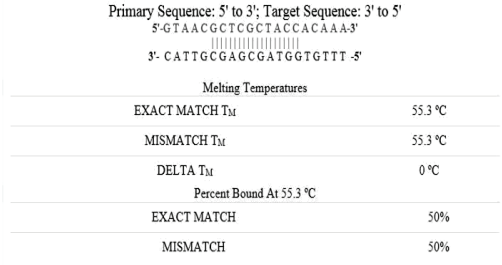

Figure 5: Primer pair II, Primer 1. GTAACGCTCGCTACCACAAA

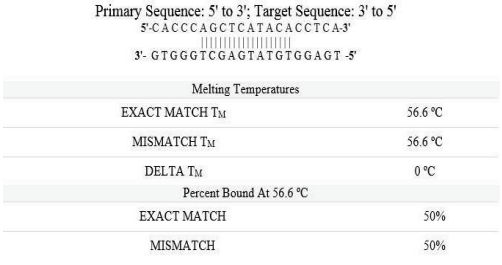

Figure 6: Primer pair II, Primer 2. CACCCAGCTCATACACCTCA

These results show that the proposed primers can be used in PCR reactions and the results are not influenced by the artifacts.

In addition, SSR markers (PGS1_24) - F: CTCTTCTCGCCTCCCAATTT with R: GCTTAGCCCTGGGTACAAG and F: ATCTGCTCTTTCCCTCACCT with R: GATTATCCCTCAACCCATCC were significant for the first screening in a large population and may be useful to start a MAS (Marked Assisted Selection) in the breeding program of natural genetic resistance to PPV.

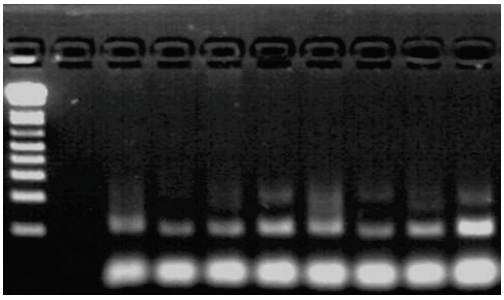

Figure 7 Electrophoresis for PCR with SSR markers in apricot progenies

Further evaluation of these loci will be required to characterize the genetic control of the resistance to PPV. Due to the co-dominant

nature of SSR markers, together with their high genetic transportability, the development of SSR analysis associated with apricot PPV resistance can facilitate the use of MAS in strategies aimed at improving natural resistance to PPV.

CONCLUSIONS

PCR reactions are characterized by a logarithmic amplification of the sequence of interest, the amount of PCR product at the end of the reaction being uncorrelated with the initial amount of the template. Although the PCR reaction does not provide information on the biologically active product of genes (i.e., proteins), functional genomics studies have demonstrated the existence of a close correlation between protein function and gene expression.

Confirmation of amplicon identity is required to verify whether amplified DNA matches the target sequence chosen. Gel electrophoresis is a simple way to check the size of PCR products. However, sequencing the amplicons and comparing the results with those in the databases is the most effective method of checking the results obtained by PCR. The amplicons obtained by conventional PCR were sequenced after purification with Wizard PCR Preps DNA Purification System (Promega). Primers used for PCR amplification were BigDye (ABI PRISM® BigDye TM Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems). The reaction products were separated by agarose gel migration and analyzed using ABI PRISM 310 (Applied Biosystems). Alignment of sequences was done with the BioEdit software (CLUSTAL W application). The analyzes were performed in comparison to gene sequences in the GenBank data bank.

The sequencing was performed with the ABI PRISM 310 genetic analyzer, whose working principle consists in determining the sequence of the nucleotide bases in the DNA sample: the fluorescent markers are attached to the ACGT extension products that appear in the sequencing reaction, the markers being incorporated by using dideoxynucleotides labeled in position 3; extension of primers carried out under the action of Ampli-Taq polymerase. Although the reaction mixtures and temperature cycles are similar for PCR and sequencing, the two techniques differ by their very purpose: Amplification of an unknown sequence by the use of two primers and a double stranded DNA chain in the PCR test; Establishing the nucleotide sequence at the level of a segment of DNA of interest by using one or both primers and a double or single stranded DNA sequence in case of sequencing.

Under these conditions, the molecular marker screening is extremely effective to identify genotypes with natural genetic resistance to PPV without the need for special infection conditions. Of course, in order to correlate the results, it is also necessary to screen for artificial infection conditions, at least for the most important genotypes for validation of marker assisted selection.

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