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Development of a co-dominant CMS-specific ALP marker in Tobacco (*Nicotinia tabacum*)

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ABSTRACT

One of main problems in three-line hybrid seed production of tobacco (Nicotinia tabacum) is differentiation between fertile maintainer lines and CMS counterparts that is indispensable for seed purity tests. To establish a reliable marker system namely amplified length polymorphism (ALP) for differentiating between normal fertile tobacco lines and CMS counterparts, two CMS-specific primers (designates as Ntmto1F and ntmto1R) were designed based on the alignment of the mitochondrial DNA sequence of two Gene Bank accessions (AF056245 and AF121902). Preliminary PCR tests by this primer pair on the DNA of three fertile lines and CMS counterparts from three well-known tobacco ecotypes (Burley, Basma and Virginia) and subsequent silver staining after vertical electrophoresis indicated the usefulness of the developed ALP marker system for distinguishing between two fertile and CMS counterparts. Normal fertile lines amplified a shorter fragment than the respective CMS counterparts. To validate the marker system, DNA from further fertile and CMS lines of three ecotypes were subjected to PCR amplification by the primer pair. Results showed that this marker system could discriminate two fertile and CMS groups with 100% carefulness. This marker system is highly reliable and cost-effective and can be utilized by plant breeders and hybrid seed producers.

Key words: Tobacco, CMS, Molecular marker, ALP.

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is amongst the most important industrial crops worldwide and at least in 97 countries is cultivated for economical purposes [4]. It has a large germplasme with many extra lines and types. There are many tobacco varieties and landraces and the number of new varieties in the world steadily increases. Three ecotypes famous in the word are Burley, Basma and Virginia types [19]. Therefore, distinguishing and identification of each genotype is indispensable for use in breeding programs. Currently tobacco is selected to obtain different kinds of breeding lines and then, the promising lines are adapted to different climates [2, 6, 11].

Cytoplasmic male sterility has been known for over 100 years ago. In 1943, Jones and Clarke suggested that male sterility in onion is conditioned by the interaction of the male-sterile (S) cytoplasm with the homozygous recessive genotype at a single male fertility restoration locus in the nucleus [cited by 12]. The authors also described the technique to exploit cytoplasmic-genic male sterility (CMS) for the production of hybrid seeds. The combination of a nucleus and cytoplasm from different plant species often leads to an inability to produce or release functional pollen. This developmental disorder, known as cytoplasmic male sterility (CMS), is a maternally inherited trait associated with the mitochondrial genome. Often CMS plants are characterized by abnormal floral phenotypes and alterations in mitochondrial gene expression [5, 9, 16].

The subcellular organelle mitochondrion synthesizes ATP from stored energy in the form of fats, carbohydrates and proteins. Plant mitochondrial genomes are much larger and more complex than those of other aerobic organisms so far known. They vary in size from 200 kb in *Brassica* species to 2500 kb in musk melon [14, 20]. Several actively transcribed DNA sequences having open reading frames have been identified in plant mitochondria. The functions of the products of these are not known. Genome rearrangements, recombination involving direct repeats are also implicated in the rapid and extensive rearrangements that characterize the evolution of plant mitochondrial DNA [7, 18].

Male sterile plants fail to produce functional pollen grains. CMS has been found to arise naturally as well as from intra- and interspecific crosses [5, 13]. Male sterility can arise from alterations in nuclear or cytoplasmic genes. Nuclear-cytoplasmic incompatibilities could reflect nuclear gene effects on mitochondrial gene expression from transcription, processing of RNAs to translation into amino acids.

Previously, studies of mitochondrial gene expression in male-sterile tobacco revealed that the transcriptional pattern of ATP1 was changed due to the alloplasmic condition. In the male-sterile plant a longer ATP1 transcript was found, in addition to a shorter transcript present in the male-sterile plant as well as in both parents and the fertility-restored plant [8]. Also, in mitochondria of *N. tabacum* and *N. repanda*, irrespective of nuclear background, ATP1 was co-transcribed with an upstream novel open reading frame designated as ORF274 [3].

DNA-based molecular markers have acted as valuable tools and have found their own position in various scientific fields like characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics,

taxonomy, plant breeding, and diagnostics. Species-specific markers identified in studies are useful in identification of the true hybrids and monitoring introgression of useful genes from the wild relatives [22]. Comparison of mitochondrial DNA of normal fertile and CMS plants provides a good opportunity to design a desirable marker system for differentiating the fertile lines from CMS counterparts. In the present work an ALP marker was developed based on tobacco ATP1 gene for distinguishing the CMS and fertile normal lines of tobacco.

MATERIAL AND METHODS

Plant Material

Seventeen lines of tobacco distributed in three well-known ecotypes including Burley, Basma and Viginia, were used in this study. Eight of them had normal cytoplasm and 9 other carried cytoplasmic male sterility (CMS) (Table 1). The lines were obtained from tobacco research institute, Tirtash, Northern Iran.

Table 1. Tobacco lines used in the study

#	Cultivar	Cytoplasm	Ecotype
1	Burley 21	Normal	Burley
2	CMS Burley21	CMS	Burley
3	Basma	Normal	Basma
4	CMS Basma	CMS	Basma
5	Coker347	Normal	Virginia
6	CMS Coker347 (RGH4×C347)	CMS	Virginia
7	CMS Coker347 (NC100×C347)	CMS	Virginia
8	Orumia205	Normal	Basma
9	CMS Orumia205	CMS	Basma
10	Coker176	Normal	Virginia
11	CMS Coker176	CMS	Virginia
12	MN994	Normal	Virginia
13	CMS MN994 (NC100×MN994)	CMS	Virginia
14	Izmir	Normal	Basma
15	CMS Izmir	CMS	Basma
16	Tmv2	Normal	Virginia
17	CMS Tmv2	CMS	Virginia

DNA extraction, primer designing and PCR conditions

DNA was extracted using CTAB method [17] with some modifications [1]. For differentiation of normal and CMS-carrying lines, one pair of CMS-specific primers (designated as Ntmt01) was designed on the sequence of ATP1 gene of tobacco [Ntmt01F: 5′-GGACCACCTTAAGCAAATAG-3′; Ntmt01R: 5′-CTACCAGTCTCTCCTTTTTTTATTCC-3′]. For this, two Gene Bank accessions (AF056245 and AF121902) were aligned to identify regions with more probable mutations. Expected size of PCR-product amplified by these two primers was of ~130 bp length (from nucleotide numbers 261106 to 261235 on mitochondrial reference genome (AF121902). Thus, these primer pair was used to develop an amplified length polymorphism (ALP) molecular marker system [10].

For PCR reaction, Cinnagen Master Mix was used in 12.5 microliter volumes according to manufacture recommendations. Electrophoresis was conducted in a 6% acrylamid gel on Appelex electrophoresis set and then, the gel was treated by silver staining.

RESULTS AND DISCUSSION

Preliminary test

For establishment of a CMS-specific marker, initially were amplified the DNA of two representatives (one CMS and one normal fertile counterpart) of three known tobacco ecotypes (including Burley, Basma and Virginia) using Ntmt01 primer pair. Result of this preliminary test obviously could differentiate the two fertile and CMS groups (Figure 1). As seen in the figure, normal fertile lines amplified a shorter band than the respective CMS counterparts. Allele size in normal fertile and CMS lines was 130-131 and 134-135 bp, respectively.

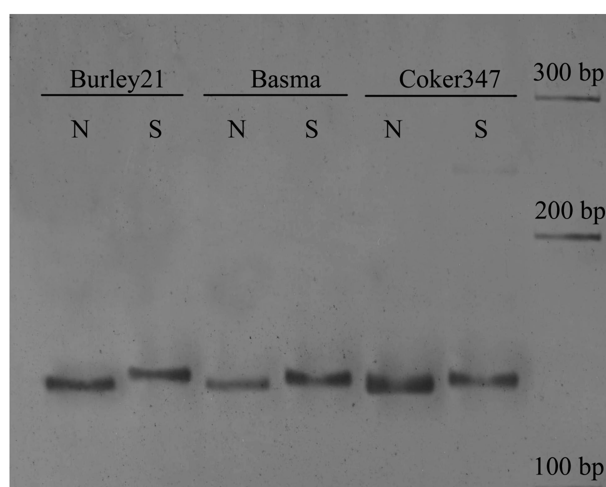

Figure 1. Preliminary PCR test with three normal fertile and three CMS counterparts from three well-known tobacco ecotypes

PCR products were run on a 6% polyacrylamide gel and then were silver stained. N: normal fertile lines; S: CMS lines

Validation of the marker system

For validation of the above result, we selected further CMS and fertile counterparts, including 6 CMS and 5 fertile lines and amplified those using Ntmt01 primer pair. Again we observed the same banding pattern for CMS and their fertile counterparts; all the fertile lines produced a shorter band than the respective CMS counterparts (Figure 2).

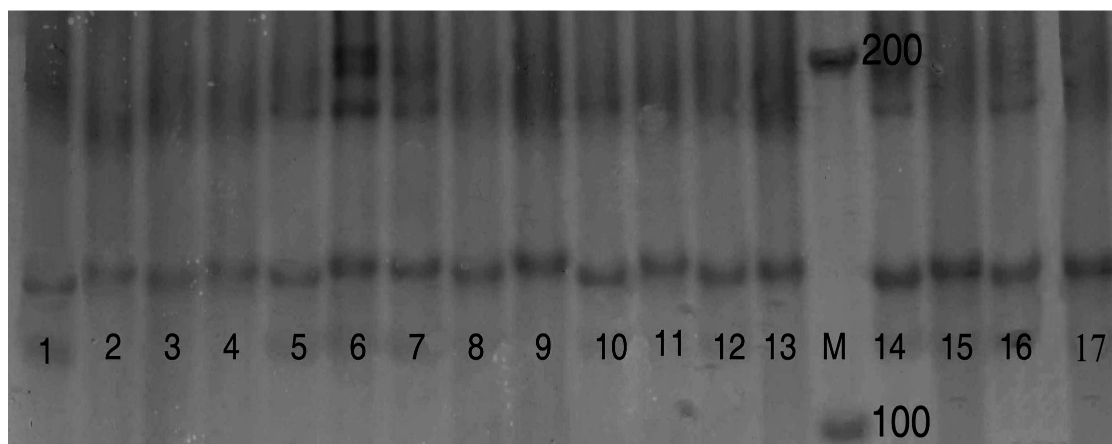

Figure 2. Electrophoresis pattern obtained using Ntmt01 primer pair

Lanes 1, 3, 5, 8, 10, 12, 14 and 16 are normal fertile lines, and lanes 2, 4, 6, 7, 9, 11, 13, 15 and 17 are male sterile lines, respectively. Lanes 6 and 7 are alloplasmic CMS lines of Virgunia ecotype. M: size marker (100 bp ladder). PCR products were run on a 6% polyacrylamide gel and then were silver stained. For further information refer to table 1.

Differentiating the normal fertile and CMS counterparts of tobacco is an important issue in breeding programs and also to prevent mixing the two seed batches in fields of hybrid seed producers. Morphological characters usually vary with environments and are very unreliable. The number of karyotypical characters is limited, and the use of isozyme markers is restricted to a few polymorphic enzyme systems encoded by a small number of loci [15]. However, the use of molecular markers for this purpose is more reliable and provides more information on genetic variation within cultivated tobacco lines. Trait-specific markers identified in most studies are useful in identification of the true hybrids and monitoring introgression of useful genes from the wild relatives [21]. The marker system developed in this study could successfully differentiate the normal fertile and CMS counterparts of tobacco that is needed for identification of true hybrids, for seed purity tests (evaluation of the mixing rate of two seed batches of normal fertile and CMS lines), and for monitoring introgression of a CMS character from donor line into recipient one. As the allele difference in the studied locus is relatively low (4-5 bp), the use of silver staining is inevitable for obtaining desirable results.

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