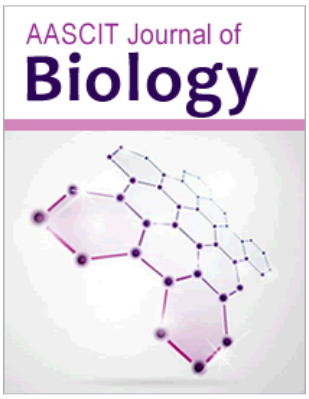
AASCIT Journal of Biology
2015; 1(3): 34-37
Published online July 20, 2015 (http://www.aascit.org/journal/biology)

AASCIT American Association for Science and Technology



Keywords
ALP,
CMS-Specific,
Molecular Marker,
Purity,
Tobacco

Received: June 29, 2015
Revised: July 6, 2015
Accepted: July 7, 2015

Discrimination of Fertile Normal and CMS Counterparts in Tobacco (*Nicotinia tabacum*) Using a Mitochondrial-Specific Amplified Length Polymorphism (ALP) Marker

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Citation
Asadollah Ahmadikhah. Discrimination of Fertile Normal and CMS Counterparts in Tobacco (*Nicotinia tabacum*) Using a Mitochondrial-Specific Amplified Length Polymorphism (ALP) Marker. *AASCIT Journal of Biology.* Vol. 1, No. 3, 2015, pp. 34-37.

Abstract
The discrimination of fertile maintainer lines and CMS counterparts is indispensable for seed purity tests which is one of the main problems in three-line hybrid seed production of tobacco (*Nicotinia tabacum*). To develop a CMS-specific, reliable marker system for discriminating the 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 (viz. Burley, Basma and Virginia) and subsequent silver staining after vertical electrophoresis indicated the usefulness of the developed amplified length polymorphism (ALP) marker system for distinguishing between two fertile and CMS counterparts. A shorter fragment of 130 bp length was amplified in normal fertile lines, compared to that of the respective CMS counterparts of 134 bp length. PCR with further fertile and CMS lines of three ecotypes by the primer pair validated the reliability of the developed marker system. Results showed that this marker system could discriminate two fertile and CMS groups with 100% carefulness. The marker system is highly reliable and cost-effective, and can be utilized by plant breeders and hybrid seed producers for different purposes (e.g. marker-assisted selection and hybrid seed purity tests).

1. Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the most important industrial crops worldwide and at least in 97 countries is cultivated for economical purposes (Goodman 2005). It has a large germplasm 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 well-known ecotypes in the word are Burley, Basma and Virginia types (Stoyanova 2004, Kilian and Graner 2012). Currently tobacco is selected to obtain different kinds of breeding lines and then, the promising lines are adapted to different climates (Daniell et al 2005, Julio et al 2006).

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 Havey2000). The authors

also described the technique to exploit cytoplasmic-genic male sterility (CMS) for the production of hybrid seeds. This developmental disorder, known as cytoplasmic male sterility (CMS), is a maternally inherited trait (Sato and Sato 2013). Cytoplasmic male sterility (CMS) in plants caused by lesion or rearrangement of the mitochondrial genome is unable to produce functional pollens (Jing et al 2001). Nuclear genes are required to restore pollen fertility to CMS lines. In many plant species, male fertility of CMS plants is restored by nuclear-encoded fertility restorer genes referred to as *Rf* (*Restorer of fertility*) or *Fr* (*Fertility restorer*) (Hanson and Bentolila 2004). Therefore, the CMS systems are widely used for hybrid seed production. Often CMS plants are characterized by abnormal floral phenotypes and alterations in mitochondrial gene expression (Hanson and Bentolila 2004, Sato and Sato 2013). The subcellular organelle mitochondrion synthesizes ATP from stored energy in the form of fats, carbohydrates and proteins (Sabar et al 2003, Chase 2007). 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 (Alverson et al 2010). 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 (Fauron et al 2004).

Male sterile plants fail to produce functional pollen grains. CMS has been found to arise naturally as well as from intra- and interspecific crosses (Sato and Sato 2013). 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 (Bergman et al 2000, Sugiyama et al 2005). 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 (Bergman et al 2000).

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 (Zhang et al 2006). When introducing a gene or a trait, markers linked to the gene can be used to select plants possessing the desired trait, and markers throughout the genome can be used to select plants that are genetically similar to the recurrent parent (Semgan et al 2006, Singh et al 2012). 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.

2. Material and Methods

2.1. Plant Material

Seventeen tobacco lines of 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. *Plant material used in this study.*

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

2.2. DNA Extraction, Primer Designing and PCR Conditions

DNA was extracted using CTAB method (Saghai-Maroof et al 1984) with some modifications (Ahmadikhah 2009). For difscrimination of normal and CMS lines, one pair of CMS-specific primers (designated as Ntmt01) was designed on the basis of sequence of ATP1 gene of tobacco [Ntmt01F: 5`-GGACCACCTTAAGCAAATAG-3`; Ntmt01R: 5`-CTACCAGTCTCTCCTTTTTTTATTCC3´]. 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 (Hongtrakul 1997). 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.

3. Results

3.1. Establishment of the Marker System

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

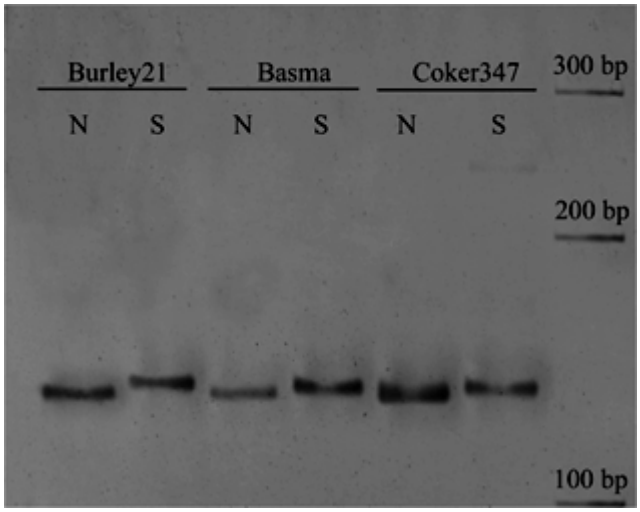

Figure 1. *Preliminary PCR test with three normal fertile and three CMS counterparts from three well-known tobacco ecotypes. PCR products were run on a 6% polyacrylamide gel and then were silver stained. N: normal fertile lines; S: CMS lines.*

3.2. Validation of the Marker System

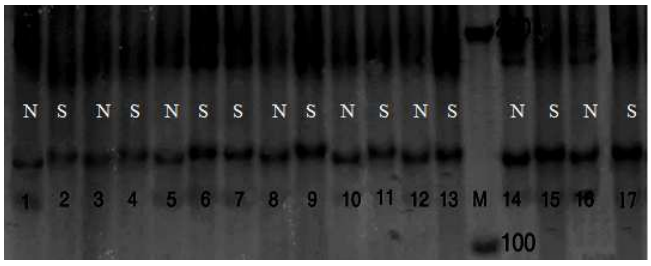

Figure 2. *Electrophoresis pattern obtained using Ntmt01 primer pair. Lanes 1, 3, 5, 8, 10, 12, 14 and 16 are normal fertile lines, and lanes 2, 4, 6, 7, 9, 11, 13, 15 and 17 are male sterile lines, respectively. Lanes 6 and 7 are alloplasmic CMS lines of Virgunia ecotype. M: size marker (100 bp ladder). PCR products were run on a 6% polyacrylamide gel and then were silver stained. N: normal cytoplasm, S: sterile cytoplasm. For further information refer to Table 1.*

For validation of the marker system, further CMS and fertile counterparts, including 6 CMS and 5 fertile lines were selected and amplified using Ntmt01 primer pair. Again we observed the same banding pattern for CMS and their fertile counterparts; so that all the fertile lines produced a shorter band than the respective CMS counterparts (Figure 2). Of course, this result was reliable each time we examined the marker system (at least three times) with partial or complete set of the studied genotypes.

Diversity analysis showed that there was a high genetic variation in the studied population at ATP1 locus (Table 2). As seen, number of effective alleles (ne) was perfect (2 out of 2) and mean gene diversity was also perfect (0.50), indicating that the marker system could well differentiate normal cytoplasm line from CMS lines.

Table 2. *Summary of genic variation statistics at the studied locus.*

Locus	na	ne	h	I
ATP1	2	2	0.50	0.69
S.D	0.00	0.00	0.00	0.00

na = Observed number of alleles; ne = Effective number of alleles; h = Nei's (1973) gene diversity; I = Shannon's Information index.

4. Discussion

Discrimination of 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 (Merilä and Crnokrak 2001, Ahmadikhah and Alavi 2009). 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 (Lu et al 2009). 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 (Wang et al 2010, Muthamilarasan et al 2014). 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, and also for seed purity tests; for instance it can be utilized for evaluation of the mixing rate of two seed batches of normal fertile and CMS lines. The marker system is applicable for monitoring introgression of a CMS character from donor line into recipient one (Zeng et al 2009, Gupta et al 2010). As the difference in allele sizes in the developed marker in this study is relatively low (4-5 bp), the use of silver staining is inevitable for obtaining desirable results (Byun et al 2009).

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