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# The Involvement of the *MROS2* gene in Floral Development at *Silene latifolia* ssp. *Alba*

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## Abstract

*This study is about the identifying of the gene involved in anther maturation, with autosomal location, the MROS2, through PCR , using specific primers and total genomic DNA from male and female plants of Silene latifolia ssp. alba from the Roumanian Flora.*

*Although total genomic DNA used as a template, was impurified with small quantities of proteine and polysaccharide residues, the specficity of the primers and the improvment of the PCR conditions, led to obtaining of a 785 bp PCR products specific to MROS2 gene.*

**Keywords**: *Silene latifolia*, dioecious plants, DNA, *MROS2* gene, sex determination

## Introduction

The evolution of the sex determination in dioecious plants is a very actual issue, approached by numerous groups of researchers. Sex-linkated genes and other DNA sequences there have been recently isolated in *Silene latiflolia*, using: i) cDNA or genomic library substraction methods [2, 6, 10, 15, 18, 19]; ii) microdisected and degenerate oligonucleotide-primed (DOP)-PCR amplified sex chromosomes as probes for the screening of cDNA library [5] or for FISH [3], iii) PCR-based methods such as randomly amplified poplymorphic DNA [14]. These studies resulted in the isolation of repetitive sequences [3, 6, 19] and the discovery of a four gene group called *MROS* (***m***ale ***r***eproductive ***o***rgan-***s***pecific) specifically expressed only in male reproductive organs [10, 11].

. The *MROS* genes are the correspondents of some of the ten *Men* (***m***ale ***e***nhance) genes: *MROS1* is homologue to *Men-1*, *MROS2* to *Men-4* and *MROS3* to *Men-9*. The first eight of these genes, respectively *Men-1* to *Men-8* [19] and *Men-10* [18] are expressed exclusively in the male buds and *Men-9* [15] is expressed in male flowers and is also expressed at a lower level in femele flowers.

The different names of *MROS* and *Men* of the genes involved in the development of the male flower at *Silene latifolia* were given by different research groups, which have studied them independently.

The analysis of the *Men* gene expression, was carried out at RNA level (Northern blot), extracted from male plants, female plants and female plants infected with *Ustilago violacea* [1]. The ability of the fungi to mimic the control effect of the Y chromosome and to stimulate the development of stamens in genetically female plants shows that most of the genes responsible for the organogenesis are autosomally located or on the X chromosome.

This suggests that the Y chromosome plays a role in regulating the stamen development, rather than in enconding the structural information. The stamen which are inducted by *Ustilago violacea* in the female plants appear to be normal, but they do not produce pollen and the dehiscente anthers carry smut fungus inside of pollen grains. The extraordinary biology of the interaction between *Ustilago violacea* and *Silene latifolia* has proved to be a valuable instrument in characterising the genes which express themselves during the development of the male flower [20].

These studies concluded that all ten genes are expressed in the first stages of the development of the male flower buds. Analysing the entire development of the male flower, all these genes fall into two categories, with respect to their temporal expression. Thus *Men-1, Men-4*, *Men-6* [19], *Men-10* [18] genes express themselves during the whole male flower development. However, the expression of the remaining six *Men* genes peaks in 1-2 mm flower buds and dramatically decreases as the male flowers mature. The analysis of the expression of the *Men* genes in inducted anthers in female plants infected with *Ustilago violacea* has also made possible the establishment of another difference between *Men* genes. Thus, the *Men-1, Men-4*, *Men-6* [19], *Men-9* [15] genes are expressed in the stamen from male flowers and female flowers infected with *Ustilago violacea*. The remainig six *Men* genes are not expressed in smut fungus-induced stamens [18]. This differential expression provides a molecular distinction between stamens from normal male flowers determined by the presence of Y chromosome and those induced by smut fungus in female flowers. The difference is that the *Men* genes expressed exclusively in the anthers of the flowers of normal male individuals, are the genes which are expressed until the last stages of male sex determination.

The group of *MROS* genes are present both in female and male genomes, consists of: *MROS1* (*Men-1*) involved in flowering pollen grains, *MROS2* (*Men-4*) which plays a role in anther maturation, *MROS4* [19] involved in the early male flower buds and *MROS3* (*Men-9*) which was isolated by three independent researchers groups [8, 10, 15], and plays a role in the anther tapetum maturation. Except *MROS3* (*Men-9*) gene which is located in at least two copies arranged in tandem on the X chromosomes, with additional two copies on the autosomes [12] and with a homologue pseudogene on the Y chromosome [7], all other *MROS* genes are exclusively autosomal [7]. The *MROS1* gene (1375bp) and *MROS4* gene (600bp) have introns, while *MROS2* gene (785bp) and *MROS3* gene (433bp) doesn't [9].

Using PCR with specific primers and genomic DNA as a template, the *MROS3* gene was also identified in other *Silene* species. Thus, in the dioecious species *Silene diclinis* and the gynodioecious species *Silene vulgaris*, the specific band of the *MROS3* gene has the same size with that one identified in *Silene latifolia* [9]. Also, in *Arabidopsis thaliana* [21] a species nonrelated with the *Silene* genus, there have been identified five homologues of the *MROS3* gene, which were named *AtMROS3a*, *AtMROS3b*, *AtMROS3c*, *AtMROS3d*, *AtMROS3e*, suggesting the ancient origin of this gene. It has been proved that at least two copies of the *MROS3* gene are tandemly arranged on the X chromosomes of *Silene latifolia*. Surprisingly, this data has shown that, in the genome of *Arabidopsis thaliana*, the homologue genes located on the autosomes are also in tandemly arranged. However, on the basis of these results, it cannot be established whether this duplication events took place in the ancestral genome or independently in *Silene latifolia* and *Arabidopsis thaliana* [9].

There are even less studies on the *MROS2* gene, which does not have introns and is expressed in the stamen of normal male flowers as well as in that of female flowers infected with *Ustilago violacea*.

## Material and Methods

**Plant materials**: *Silene latifolia* ssp. *alba* (Poiret), fam. Caryophylaceae. The plants belonging to two natural populations were sampled, from the Botanical Gardens and the Park of the Technical University, Bucharest.

**Isolation of total genomic DNA by the CTAB method** (CTAB=cetyltrimethylammonium bromide) [16], with some modifications.

Genomic DNA was extracted from leaf material collected from male and female plants of *Silene latifolia* ssp. *alba*: 300g leaves were ground into a very fine power in liquid nitrogen and transferred to an Eppendorf tube; 600 µl of hot ($65^0$C) 2X CTAB (2X CTAB w/v, 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8), 1,4 M NaCl, 1% PVP (polyvinylpyrrolidone, MW 40.000) was added. The mixture was kept in termostat bath at $65^0$C for 5 min. Then, 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The emulsion was centrifuged at 11.000 g for 5 min. The aqueous phase was transferred to another tube and 1/10 volume of hot ($65^0$C) 10X CTAB (10% CTAB w/v, 0,7 M NaCl) was added, mixed and treated with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). After centrifugation at 11.000 g for 10 min., the aqueous phase was again removed and treated with 2 µl RN-ase A (Sigma R 9009:10mg/ml) for 1 h at $37^0$C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), was added and the mixture was centrifuged at 11.000 g for 5 min. After centrifugation, the aqueous phase was again removed and treated with proteinase K to a final concentration of 2mg/ml for 1 h at $37^0$C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the mixture was centrifuged at 11.000 g for 5 min. The aqueous phase in which an equal volume of isopropanol (chilled at -$20^0$C) was added, was maintainted at -$20^0$C for 20-30 min. The DNA was pelleted again by centrifugation 10 min. and rehydrated in high salt TE buffer (10mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 1M NaCl). Two volumes of cold 100% ethanol, were added to precipitate the DNA. The DNA was pelleted by centrifugation 10 min, washed once more with 80% ethanol and air dried before being rehydrated in 0,1X TE buffer (1mM Tris (pH 8.0), 0,1mM EDTA (pH 8.0)).

**Spectrophotometrical analysis of the purity and concentration of the genomic DNA isolated.**

In order to determine the purity and concentration of the extract DNA, the samples were diluted in 0,1% and analyzed with a spectrophotometer – Specord 40-Analitikjena. The absorbance was read at λ between 200-300nm against a control sample (0,1% Tris-EDTA).

**Amplification of *MROS2* gene by Polymerase Chain Reaction.**

The amplification was conducted in a Gene Amp. PCR System 2400-Perkin Elmer. The final concentrations of the reagents were: 1X Taq polymerase activity buffer (10X PCR buffer), 2 µl of Taq polymerase (Promega), 20 mM dNTP (10mM), 0,2 µM primer MROS2-F1 and 0,2 µM primer MROS2-R1 (Sigma) (**Tabel 1**), 2 µl DNA template (1,8-3,8 µg), 9 µg BSA (Bovine Serum Albumin – GibcoBRL) 30mg/ml and distilated water until a final volume of 50µl. The first step, representing the initial denaturation was carried out at $94^0$C for 5 min. This step was followed by 30 cycles of $94^0$C for 1 min, $55^0$C for 1 min $72^0$C for 1 min. After 30 cycles, there was a final extension step at $72^0$C for 7 min [13, 22].

**Tabel 1:** List of primers and primers sequences used to amplify the *MROS2* gene (according E. Kejnovský et a., 2001)

| Primer | Primer sequence |
|---|---|
| MROS2-F1 | 5'-ACT AGA AAT AAT GGG GTC AC-3' |
| MROS2-R1 | 5'-GCA TGC ATT AAT CTC CCT AG-3' |

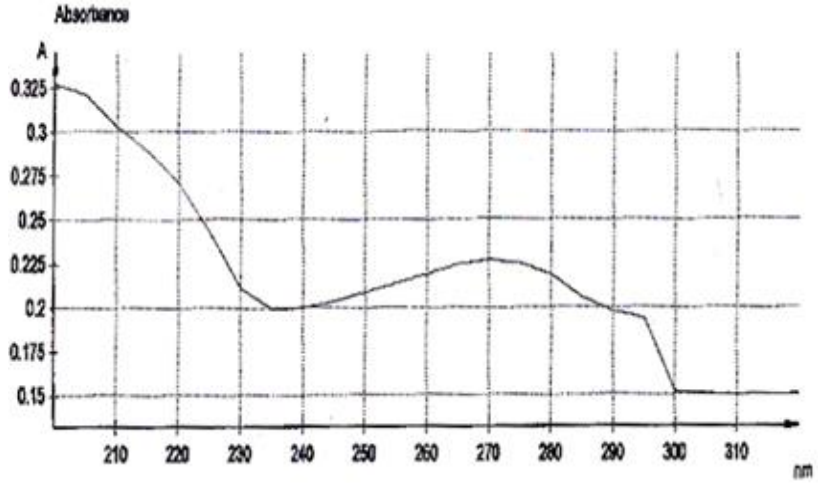
**Electrophoresis in agarose gel (1%-1,5%)** [13, 17, 22] was carried out to check the integrity of the genomic isolated DNA and of the PCR products. The agarose gel (Sigma) was solved in 1X TBE buffer pH 8,3 (0.089 M Tris, 0,0089 M acid boric, 0,002 M EDTA). The gel was stained with etidium bromide, visualised at λ=302 nm and then photographed with a Polaroid Gel Cam photocamera. Molecular weight markers used: pGEM and λDNA/BstEII (Promega)

## Results and Discussions

The goal of our research was to isolate total genomic DNA and to identify, using the PCR method, the autosomal *MROS2* gene in *Silene latifolia* ssp. *alba* in natural populations in Romania. This gene encodes for glycine-rich proteins and is specifically expressed in the flower buds of male plants.

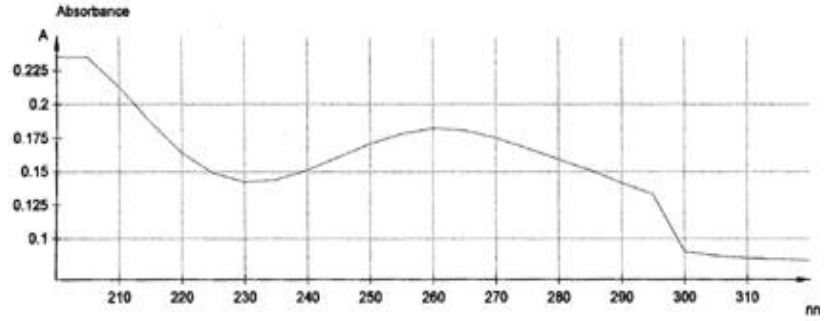
The extraction of the total genomic DNA was carried out from fresh leaves through CTAB method [16] adjusted for the particularities of this species. The protocol change concerned especially with the DNA purification, which was realized through proteine and polysaccharide precipitation. The amount of DNA which was obtained using this method is between 0,3-0,5 mg/g of leaves. The final rehydration of DNA was accomplished in 50 µl buffer 0,1X TE

The final reports $A_{260}/A_{280}$ and $A_{260}/A_{230}$ obtained after the spectrophotometrical analysis, show the presence of small quantities of proteinic and polysaccharidic residues (**Figure 1** and **2**).




$A_{230}$= 0,2110 $A_{260}$= 0,2189 $A_{280}$= 0,2186

**Figure 1**. The spectrum of absorbtion in UV of the DNA extract from female plant of *Silene latifolia* ssp. *alba*




$A_{230}$= 0,1425 $A_{260}$= 0,1824 $A_{280}$= 0,1592

**Figure 2.** The spectrum of absorbtion in UV of the DNA extract from male plant of *Silene latifolia* ssp. *alba*

The electrophoretic analysis of isolated genomic DNA from *Silene latifolia* ssp. *alba*, revealed that our method provides unfragmented DNA which is sufficiently concentrated to be used as a template in PCR (**Figure 3**).

**Figure 3**. Agarose gel electrophoresis in (1%) of the genomic DNA extracted from *Silene latifolia* ssp. *alba* plants:
1. DNA female plant;
2. DNA male plant

In order to identify the presence of the gene with autosomal location (*MROS2*), in *Silene latifolia* ssp. *alba*, through PCR, two sample of total genomic DNA, isolated from leaves of female and male plants have been selected.

Using the specific primers MROS2-F1 and MROS2-R1, PCR products from both DNA samples, with a molecular weight of 785 bp, specific to the *MROS2* gene were obtained. The weight assessement of the products was done in comparison with molecular markers λDNA/BstEII and pGEM (**Figure 4**).

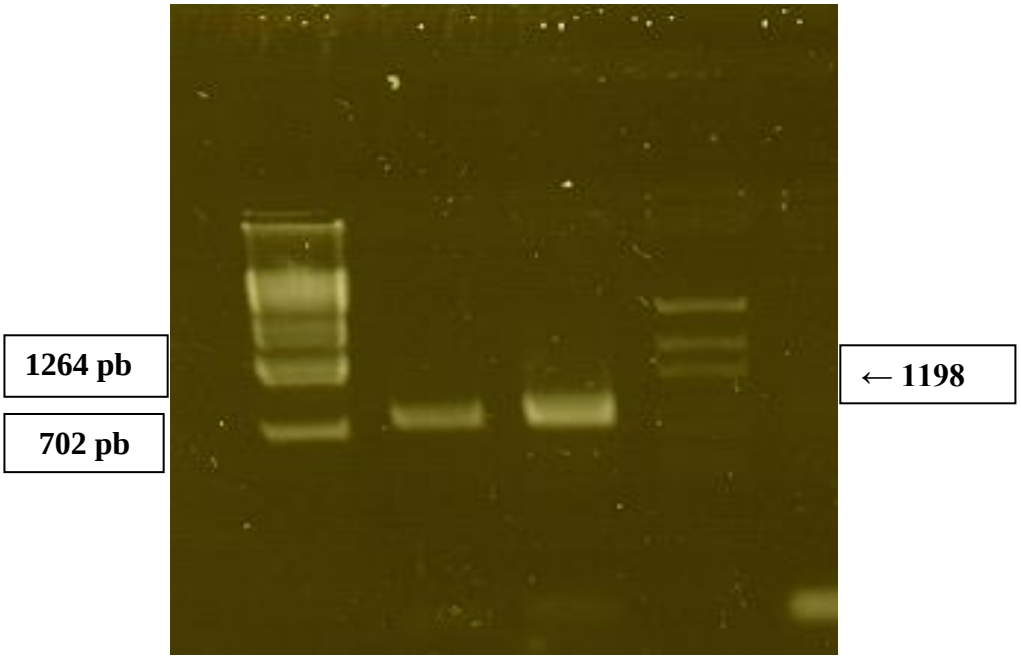



**Figure 4**. Agarose gel electrophoresis (1,5%) of the PCR reaction products for *MROS2* gene (785bp). 1. molecular weight marker λDNA/BstEII; 2. amplicons obtained from DNA isolated from the female plant; 3. amplicons obtained from DNA isolated from the male plant; 4. molecular weight marker pGEM; 5. negativ marker.

The specificity of the choosen primers and the establishment of the proper conditions for the DNA amplification, allowed us to obtain specific PCR products of the autosomal *MROS2* gene in both cases (DNA isolated from female and male plants).

## Prospects:

The identifying of MROS2 gene at *Silene latifolia* represents a first step from a series of future studies concerning the dioecious species as *Silene heuffelli*, *Humulus lupulus* and *Rumex acetosa* and other uncertain dioecious species as *Silene otites* or *Spinacia sp.*. Thus, these researches will try to establish the presence of this gene involved in the development of the flower buds, expressed only in male plants.

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