

TECHNICAL ARTICLE

PCR identification of *Petunia* male sterile cytoplasmNoemí Colombo^{1*}, Juan Carlos Hagiwara²**Abstract**

Petunia is a very important ornamental plant with a broad range of flower colour and size, and most of the cultivars grown are propagated through seeds. Cytoplasmic male sterility (CMS) is a maternally inherited character determined by mitochondrial genes that results in impaired pollen development. The unique and well characterized male sterile cytoplasm in *Petunia* is a valuable resource for hybrids production because it prevents self-fertilization of mother plants and ensures the purity of F1s. Introgression of the male sterile cytoplasm in elite lines of *Petunia* is achieved following a backcross scheme and can be assisted using molecular markers associated to the trait of interest. The objective of this study was to develop a molecular marker to identify the male sterile cytoplasm of *Petunia*. A PCR-based marker amplifying a region of the mitochondrial CMS-associated *urf5* only in the male sterile plants was designed. Results showed differential PCR amplification of a ≈ 600 bp product in plants carrying male sterile cytoplasm in four *Petunia* species and their F1s and BC1 generations. A multiplex PCR reaction was subsequently set up, adding specific primers amplifying a ≈ 800 bp product from the conserved region *trnT-trnL* of the chloroplast genome as a positive control in order to unambiguously identify the cytoplasm types as normal or sterile. A rapid, simple and precise molecular marker is now available for assisting breeding of F1 hybrids in *Petunia*.

Keywords: *Petunia* spp; cytoplasmic male sterility; molecular markers; ornamental plant breeding

Resumo**Identificação por PCR de citoplasma macho estéril em *Petunia***

A petúnia é uma planta ornamental muito importante, com uma ampla gama de cores e tamanhos de flores, e a maioria das cultivares são propagadas através de sementes. A esterilidade citoplasmática masculina (CMS) é um caráter hereditário da mãe, determinado por genes mitocondriais que resultam em prejuízo ao desenvolvimento do pólen. O citoplasma macho estéril único e bem caracterizado em *Petúnia* é um recurso valioso para a produção de híbridos, pois previne a autofecundação das plantas-mãe e garante a pureza do F1. A introgressão do citoplasma macho estéril nas linhas de elite de *Petúnia* é alcançada seguindo um esquema de retrocruzamento e pode ser assistida usando marcadores moleculares associados à característica de interesse. O objetivo deste estudo foi desenvolver um marcador molecular para identificar o citoplasma macho estéril de *Petunia*. Foi desenhado um marcador baseado em PCR que amplificou uma região de *urf5* associada à CMS mitocondrial apenas em plantas masculinas estéreis. Os resultados mostraram amplificação por PCR diferencial de um produto de ≈ 600 bp em plantas portadoras de citoplasma estéril masculino em quatro espécies de Petúnia e suas gerações F1s e BC1. Uma reação de PCR multiplex foi subsequentemente configurada, adicionando iniciadores específicos amplificando um produto de ≈ 800 bp da região conservada *trnT-trnL* do genoma do cloroplasto como um controle positivo, para identificar inequivocamente os tipos de citoplasma como normal ou estéril. Um marcador molecular rápido, simples e preciso está agora disponível para auxiliar no melhoramento genético de híbridos F1 em *Petunia*.

Palavras-chave: *Petunia* spp; esterilidade citoplasmática masculina; marcadores moleculares; melhoramento de plantas ornamentais

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Introduction

Cytoplasmic male sterility (CMS) is a trait resulting from the expression of mitochondrial genes which impair the normal development of pollen. As in most angiosperms mitochondria genome is maternally inherited (Corriveau and Coleman, 1988; Mogensen, 1996), CMS is maternally transmitted as well. This condition can be suppressed by the effect of nuclear genes known as restorers of fertility (*Rf*) genes. CMS/*Rf* systems have been widely used in plant breeding to increase the efficiency of hybrid production in several crops (Bohra et al., 2016; Kim and Zhang, 2018). In the case of ornamental species CMS is a desirable trait for breeding purposes to produce hybrid seed, to increase flowering duration, to avoid pollen allergens and to control invasiveness (Colombo et al., 2017).

Petunia (*Petunia hybrida*) is a very important ornamental plant in worldwide horticulture. It is characterized by its diversity in flower colour and morphology. It is the world's most popular bedding plant and in the United States, it ranks first in wholesale value among annual bedding plant crops. There are numerous commercial cultivars and most of them propagate through seeds (Ganga et al., 2011; Cao et al., 2019).

The only male sterile cytoplasm described in the genus *Petunia* (Izhar, 1978) has been associated with a mitochondrial chimeric gene termed *pcf*, which includes portions of the coding regions of *atp9*, *coxII* and an unidentified ORF named *urfS*. In the presence of the dominant nuclear gene *Rf* the *pcf* transcript profile is altered and the amount of the PCF protein is greatly reduced resulting in male fertility restoration (Young and Hanson, 1987; Bentolila et al., 2002; Gillman et al., 2009).

The line 3688 of *Petunia axillaris* (Lam.) Britton, Stern & Poggenb. ssp. *Parodii* (Steere) Cabrera, carrying the male sterile cytoplasm, has been used in intra and interspecific crosses by different genotypes of the *Petunia* breeding program at INTA, in order to start the development of different cytoplasmic male sterile lines. The introgression of the male sterile cytoplasm is achieved following a backcross scheme, using the pollen donor of the crosses as the recurrent parent. Molecular identification of cytoplasm types has proved helpful to assist the development of male sterile lines as well as to determine hybrid seed purity in different crops (Dwivedi et al., 2008; Bohra et al., 2016). Although marker assisted selection has been applied for different characters in several ornamental crops (Debener, 2001; Aswath and Thaneshwari, 2017) no molecular

marker associated with *Petunia* male sterile cytoplasm is available so far. The objective of this work was to develop a molecular marker to identify *Petunia* male sterile cytoplasm.

Materials and methods

Plant material

The germplasm used in this study includes the line 3688 of *Petunia axillaris* ssp. *parodii* carrying the male sterile cytoplasm (S) and 5 male fertile lines of *P. axillaris* ssp. *parodii*, *P. interior*, *P. hybrida* and *P. integrifolia*, carrying normal cytoplasm (N). Crosses were made between 3688 as the female parent and 4 pollen donors producing 4 F1s and one BC1 generations; all the progenies carry the male sterile cytoplasm due to the maternal inheritance of the character. In Table 1 the genotypes used and their cytoplasm types are shown.

Molecular analysis

Genomic DNA was extracted according to Doyle and Doyle (1987) from young leaves of plants of all the genotypes described in Table 1 grown in the greenhouse. The specific mitochondrial primers *urfS*-F: 5'-AGACTTGGACCCGAGCTTTT-3' and *urfS*-R: 5'-TCTTTTCCCATTTCAGCCATC-3' were designed based on the sequence of *urfS* (Nivison et al., 1994) using the program Primer 3. The expected PCR product was 588 bp. PCR amplification was carried out in a final volume of 25 µl, with 20 ng of genomic DNA; 20 mM Tris-HCl pH 8.4, 50 mM KCl; 1.5 mM Mg Cl₂; 0.2 mM each dNTPs; 0.12 µM of each primer and 1 U *Taq* polymerase (Invitrogen™). The thermal profile was 94 °C for 3 min; 35 cycles at 94 °C for 30 s; 58 °C for 15 s; 72 °C for 30 s and a final extension at 72 °C for 10 min. Another PCR was performed using the universal primers F: 5'-CATTACAAATGCGATGCTCT-3' and R: 5'-TCTACCGATTTTCGCCATATC-3' to amplify the chloroplast intergenic region *trnT-trnL* (Taberlet et al., 1991). In this case, the thermal profile was 94 °C for 3 min; 35 cycles at 94 °C for 1 min; 55 °C for 30 s; 72 °C for 1 min and a final extension at 72 °C for 10 min. A multiplex PCR was made including both pairs of primers -0.2 mM each- with the following thermal profile: 94 °C for 3 min; 35 cycles at 94 °C for 30 s; 58 °C for 30 s; 72 °C for 30 s and a final extension at 72 °C for 10 min. PCR products were visualized under UV light after electrophoresis in 1.5 % agarose gels stained with ethidium bromide.

Table 1. List of analysed genotypes and their cytoplasm type: male fertile (F) or male sterile (S).

Nº	Identification	Description	Cytoplasm type
1	3688	<i>Petunia axillaris</i> (Lam.) Britton, Stern & Poggenb. ssp. <i>parodii</i> (Steere) Cabrera. Dr. M. Hanson, Cornell University, Ithaca, N.Y., EEUU	S
2	20111128C1	<i>P. axillaris</i> (Lam.) Britton, Stern & Poggenb. ssp. <i>parodii</i> (Steere) Cabrera.(Mercedes, Corrientes, Argentina)	F
3	20131025A1	<i>P. interior</i> Ando & Hashim..(Gral.Manuel Belgrano, Misiones, Argentina)	F
4	15-206#2@	<i>P. hybrida</i>	F
5	15-232#1@	<i>P. hybrida</i>	F
6	20151125A1	<i>P. axillaris</i> (Lam.) Britton, Stern & Poggenb. ssp. <i>parodii</i> (Steere) Cabrera. (San Jerónimo, Santa Fe, Argentina)	F
7	20151129A1	<i>P. integrifolia</i> (Hook.) Schinz & Thell. ssp. <i>Integrifolia</i> . (Gral. San Martín, Misiones, Argentina)	F
8	3688 x 20131025A1	<i>Petunia axillaris</i> ssp. <i>parodii</i> 3688 x <i>P. interior</i>	S
9	3688 x 15-206#2@	<i>Petunia axillaris</i> ssp. <i>parodii</i> 3688 x <i>P. hybrida</i>	S
10	16-164 x 15-232#1@	(<i>Petunia axillaris</i> ssp. <i>parodii</i> 3688 x 15-232#1@) x <i>P. hybrida</i>	S
11	3688 x 20151125A1	<i>Petunia axillaris</i> ssp. <i>parodii</i> 3688 x <i>P. axillaris</i> ssp. <i>parodii</i>	S
12	3688 x 20151129A1	<i>Petunia axillaris</i> ssp. <i>parodii</i> 3688 x <i>P. integrifolia</i>	S

Results

Amplification of the *urfS* region

Specific primers were designed on the *urfS* region of the mitochondrial chimeric gene *pcf* associated to CMS in *Petunia*. As expected, plants of the line 3688 carrying the male sterile cytoplasm amplified a product of ≈ 600 bp corresponding to a region of *urfS*. F1 and BC1 plants

from crosses using 3688 as the mother plant inherited the male sterile cytoplasm maternally and therefore they amplified the same ≈ 600 bp product. On the contrary, no amplification product was observed in plants from the lines carrying the fertile cytoplasm (Figure 1). The lack of PCR product in plants with fertile cytoplasm suggests the need of a positive control in the same PCR reaction to discard any PCR failure.



Figure 1. PCR identification of cytoplasm types in *Petunia*. L: 100 bp DNA ladder; Lanes 1 to 12 correspond to genotypes mentioned in Table 1; lane 13: negative control

Amplification of the chloroplast intergenic region *trnT-trnL*

In order to test for the suitability of the chloroplast intergenic region *trnT-trnL* as a positive control, universal

primers were first tried separately. Plants carrying both cytoplasm types amplified a product of ≈ 800 bp (Figure 2), thus proving appropriate as a positive control in a multiplex PCR reaction.

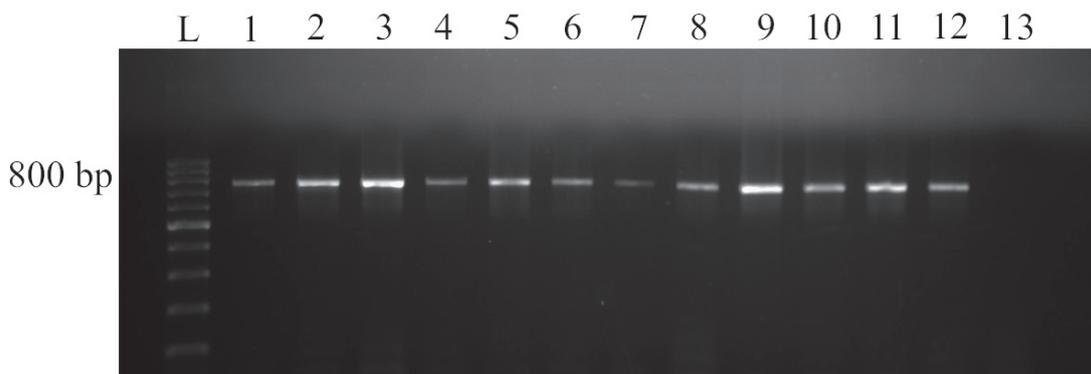


Figure 2. PCR identification of the chloroplast intergenic region *trnT-trnL* in *Petunia*. L: 100 bp DNA ladder; Lanes 1 to 12 correspond to genotypes mentioned in Table 1; lane 13: negative control

Multiplex PCR

A multiplex PCR including both *urf-S* and *trnT-trnL* primers in the same reaction was set up. As expected, plants of all the analysed genotypes amplified the chloroplast

intergenic region as a product of ≈ 800 bp. Besides, plants carrying the male sterile cytoplasm amplified both the chloroplast intergenic region *trnT-trnL* and the mitochondrial *urfS* target (Figure 3).

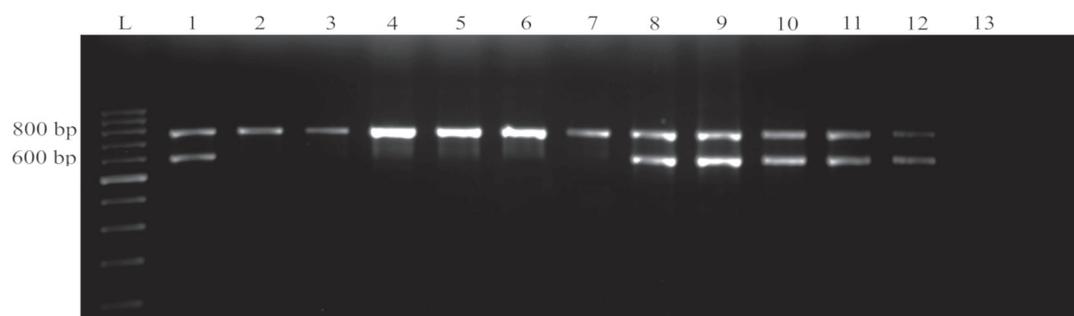


Figure 3. Multiplex PCR identification of cytoplasm types in *Petunia*. L: 100 bp DNA ladder; Lanes 1 to 12 correspond to genotypes mentioned in Table 1; lane 13: negative control

Discussion

Petunia F1 hybrids lead the commercial offer of cultivars due to their superior vigour, uniformity, and performance (Ganga et al., 2011). A cost-efficient hybrid production system requires a reliable pollination control to avoid self-fertilization of female plants. Some *Petunia* species, like *P. integrifolia*, are invariably self-incompatible; others, like *P. hybrida* are predominantly self-compatible (Robbins et al., 2000). In *Petunia axillaris* the ssp. *axillaris* is mostly self-incompatible but the ssp. *parodii* and *subandina* are entirely self-compatible (Tsukamoto et al., 2003). A well-characterized male sterile cytoplasm which prevents pollen development is available in *Petunia* (Gillman et al., 2009) and can be transferred by backcross to elite female lines in a breeding program. In this context, molecular markers associated to cytoplasm types are of value to trace sterile cytoplasm during their introgression into elite lines and to determine genetic purity of F1s (Shu et al., 2016; Swamy et al., 2017; Ferreira and Santos, 2018).

The differential PCR amplification of a ≈ 600 bp product, corresponding to a region of the CMS-associated *urf5*, allowed the identification of male sterile and male fertile cytoplasm types in agreement with plant phenotypes in all the analysed *Petunia* lines, the F1s and the BC1 generations. However, due to the dominant nature of this marker, a positive control was later included in a multiplex PCR reaction to identify the normal cytoplasm undoubtedly. Amplification of the positive control ensures that the absence of product observed when amplifying the region of the CMS-associated *urf5* is not due to a failure in the PCR reaction. The highly conserved chloroplast intergenic region *trnT-trnL* previously amplified in a wide range of taxa (Taberlett, 1991) served as a positive control in the PCR reaction and permitted the precise identification of both cytoplasm types.

Conclusions

A simple and rapid PCR-based marker was developed to clearly identify the male sterile cytoplasm of *Petunia*.

This marker will be of help in *Petunia* hybrid breeding to ensure the purity of male sterile lines and F1 hybrids.

Author Contribution

N.C. ⁰⁰⁰⁰⁻⁰⁰⁰²⁻⁸²⁶⁹⁻⁶⁰³⁴ and **J.C.H.** ⁰⁰⁰⁰⁻⁰⁰⁰³⁻¹⁴⁷²⁻⁹⁵¹⁶: planning the experiments, obtaining and analyzing data and writing the article.

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