PCR identification of Petunia male sterile cytoplasm

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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 urfS 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-trn-L 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 Petunia 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 Petunia 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 urfS 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 Petunia 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-trn-L 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*, *coxll* 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, 1991). In this case, 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’-CATACCTAATGCGATGCTCT-3’ and R: 5’-TCTACCAGTTTCGCCCATAAT-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.

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’-AGACTTGGACCAGCTTTTTT-3’ and *urfS*-R: 5’-TCTTTTCATTCAGCCATCC-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 MgCl2; 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. An additional 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.

PCR IDENTIFICATION OF PETUNIA MALE STERILE CYTOPLASM
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.

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

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

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 chloroplastic intergenic region trnT-trnL and the mitochondrial urfS target (Figure 3).
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-compatible; others, like *P. hybrida* are predominantly self-compatible (Robbins et al., 2000). In *Petunia axillaris* the ssp. *axillaris* is mostly self-compatible 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 cytoplasms 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 *urfS*, 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 cytoplasms undoubtedly. Amplification of the positive control ensures that the absence of product observed when amplifying the region of the CMS-associated *urfS* 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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