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Attempts to detect extra genomial factors in cytoplasmic male-sterile petunia lines *

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Summary. In the present study we examined the possibility that viruses, viroids or dsRNA are associated with cytoplasmic male sterile (cms) petunia. The assumption was made that if viruses or viroids were present, the treatments for elimination of viruses and viroids would produce "healthy" fertile plants. Male sterile plants were subjected to heat and cold treatments for 10 weeks and/or for 5 months, after which apical meristems were isolated and cultured with the addition of antiviral factors. The mother plants, the regenerated plants and their progeny were sterile. These treatments did not affect sterility in sterile plants or the fertility of fertile plants. No dsRNA was found in cms petunia by gel electrophoresis. Thus, our data suggest that there are no viruses, viroids or dsRNA associated with cms petunia. Our data are in agreement with recent data, which suggests that the mitochondrial DNA is the site of the cytoplasmic male sterile gene in petunia.

Key words: Viruses – Viroids – dsRNA – cms – Antiviral factors

Introduction

The viral nature of cytoplasmic male sterility (cms) in petunia has been proposed in the past because of successful transmission of cms by grafting (Frankel 1956, 1962, 1971; Edwardson and Corbett 1961; Bianchi 1963). In sugarbeet it was possible to obtain male fertile plants from cms plants (Curtis 1967; Lichter 1978) as a result of heat treatment and subsequent meristem culture. Grill and Garger (1981) showed that cms in line "447" of *Vicia faba* is associated with high molecular weight double stranded RNA (dsRNA). The authors did not succeed in transmitting the cms by grafting, but they did succeed in transmitting it by using dodder bridge (Grill and Garger 1981).

We applied Lichter's (1978) treatments with petunia but added antiviral factors (Virazole and IVR, inhibitor of virus replication) to the medium. Virazole was known to have broad spectrum antiviral activity and was effective against DNA and RNA viruses (Sidwell et al. 1972). Shepard (1977) obtained regenerated tobacco plants free of potato virus-x PVX from protoplasts infected with PVX after adding Virazole to the medium.

Inhibiting virus replication IVR is released into the medium from TMV-infected protoplasts of *Nicotiana tabacum* "Samsun NN". IVR inhibited replication of TMV, CMV and PVX in leaf disks of different hosts, indicating that it is neither host- nor virus-specific (Gera and Loebenstein 1983). We repeated the experiment of Lizarrage et al. (1980) in which they obtained viroid-free potatoes by cold treatment of the plants before isolating the meristem tip.

The specific questions we asked in the present study were: (1) Are there viruses or viroids associated with cms petunia plants; and (2) Is dsRNA associated with cms in petunia?

Materials and methods

The plant material used for all experiments was a normal fertile line 4555 of *Petunia hybrida* Hort. Vilm. cv Rosy Morn (RM), a cms line 4556 of *P. hybrida* isonuclear to 4555 (Izhar and Frankel 1976), a normal fertile line 3699 of *P. parodii* L.S.M. and a cms line 3688, having *P. parodii* nuclear background and a sterile plasmon introduced into line 3699 by repeated back-

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crosses (BC10) (Izhar et al. 1983). Seeds of "447" cms and fertile lines of *V. faba* were provided by Dr. G. Duc, I.N.R.A., Dijon, France.

Seeds were surface-sterilized and germinated in jars on Murashige and Skoog (MS) (1962) basic medium, with the addition of folic acid (0.001 mg/l), kinetin (0.03 mg/l) and IAA (0.0087 mg/l). The jars were incubated in a small growth chamber for heat and cold treatments, and in culture room as control. The heat treatment was carried out at 36 °C during 15 h of daylight and at 25 °C during 9 h of dark. The cold treatment was done at a constant temperature of 4°–6° C, under a LD 9 : 15 regime. The control consisted of LD 16 : 8, with 25 °C and 17 °C, respectively. All treatments were carried out for 10 weeks or for 5 months. At the end of treatment the plants were moved to the greenhouse and grown to flowering to determine the phenotype (fertility or sterility) and to obtain seeds by selfing or by crossing with a maintainer line. Apical meristems were isolated from the treated plants and transferred to petri dishes containing MS basic medium with zeatin (0.5 mg/l) and IAA (1.0 mg/l) with and without antiviral factors. The apical meristems were grown to plantlets in a culture room and were then moved to the greenhouse as described above.

The antiviral factors were: Virazole (ICN Nutritional Biochemicals, Cavina, California) at concentrations of 0, 1, 5 and 10 mg/l designated V_0, V_1, V_5 and V_{10}, respectively. Inhibiting virus replication factor (IVR) was provided by Dr. A. Gera, ARO, The Volcani Center.

Preparation of dsRNA and gel electrophoresis was according to Bar-Joseph et al. (1983).

Table 1. The phenotypes of the regenerated plants from apical meristems taken from mother plants after 10 weeks of heat treatment and control

No. of mothers plant and phenotypes	Treatment	Medium in which apical meristems were grown	Phenotype of regenerated plants
4555-9 fertile	heat	V_0	2 fertile
4556-39 sterile	heat	V_0	6 sterile
4556-9 sterile	heat	V_0+IVR	1 sterile
4556-23 sterile	control	V_0	1 sterile
3688- sterile	control	V_0+IVR	3 sterile

Results

Heat and cold treatments

In the first experiment, 7 cms plants and 2 fertile plants were grown for 10 weeks in a high temperature regime. Two plants were grown for 10 weeks in a cold treatment regime. The plants were moved to the greenhouse and grown to flowering. In each case the plants maintained the original phenotype after heat and cold treatments.

Meristem culture

In the second experiment, 5 apical meristems were isolated from 1 fertile and 2 cms plants after 10 weeks of heat treatment. In addition, 2 meristems were isolated from 2 cms control plants. From 4 cms apical meristems – 2 from heat treatment and 2 from control – that were planted in medium with and without IVR, 11 sterile plants were regenerated. From 1 fertile apical meristem after heat treatment, 2 fertile plants were regenerated. These data are presented in Table 1.

In the third experiment, 8 apical meristems were isolated from 3 cms plants after 5 months of heat treatment and from 2 cms plants after cold treatment. In addition, 3 meristems were isolated from control plants. The apical meristems were planted in different media and grown to flowering plants. The results of this experiment are described in Table 2.

After 5 months of heat and cold treatments the mother plants were very weak and were not moved to the greenhouse for further examination.

Mother plants (after 10 weeks of treatment) and the regenerated plants maintained the original phenotype, and the same was true for their progeny. From these experiments it seems that the cytoplasmic factors causing male sterility in petunia are not of viral or viroid nature, according to the criteria used by Lizarrage et al. (1980). The heat and cold treatments with the isolation of apical meristems and the addition of antiviral factors did not

Table 2. The phenotypes of the regenerated plants from apical meristems taken from mother plants after 5 months of heat and cold treatments

No. mother plants and phenotype	Treatment	The medium in wich the apical meristem were grown	Phenotype of the regenerated plants	Phenotypes of his progeny
4556-63 sterile	heat	V_0[a]	2 sterile plants	not done
4556-63 sterile	heat	V_{10}	2 sterile plants	37 sterile plants
4555-52 fertile	heat	V_{10}	1 fertile plant	not done
4556 sterile	control	V_0	1 sterile plant	not done
4556 sterile	control	V_1	3 sterile plant	not done
4556 sterile	control	V_5	5 sterile plant	not done
4556-1 sterile	cold	V_0	5 sterile plant	64 sterile plants
4556-5 sterile	cold	V_0	2 sterile plant	16 sterile plants

[a] Virazole at different concentration; see "Materials and methods"

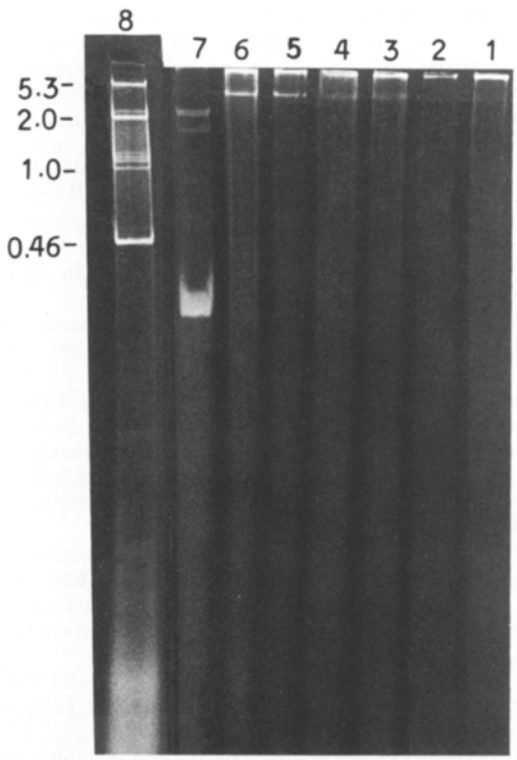

Fig. 1. Agarose gel electrophoresis of dsRNA from lines of petunia. *Lanes 1* line 4556 petunia (cms); *2* line 4555 petunia (normal); *3* line 3688 petunia (cms); *4* line 3699 petunia (normal); *5* line 3688; *6* line 3699; *7* isolated TMV; *8* marker

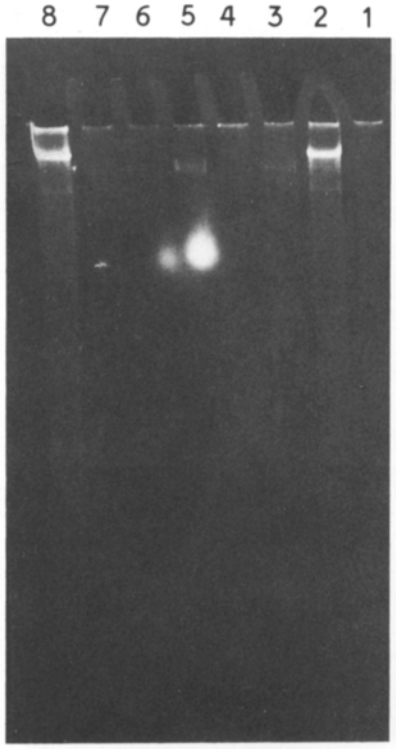

Fig. 2. Agarose gel electrophoresis of dsRNA from lines of petunia (leaves and flowers) and *Vicia faba*. *Lanes 1 Vicia faba* (normal); *2* "447" *Vicia faba* (cms); *3* 4555 petunia (leaves); *4* 4556 petunia (leaves); *5* 4555 petunia (flowers); *6* 4556 petunia (flowers); *7 Vicia faba* (normal); *8* "447" *Vicia faba* (cms)

affect the sterility of the cms plants, as would be expected if the sterility were of a viral nature.

dsRNA

In the first preparation, from 7 g of leaves of cms and normal petunia, no dsRNA was found (Fig. 1) and the typical band of dsRNA of TMV run as control is visible (Fig. 1, *lane 7*).

In the second preparation, from 7 g of leaves and flowers of cms and normal petunia and from 7 g of leaves of cms and normal *V. faba* (Fig. 2), no dsRNA was detected in the leaves or flowers of fertile and cms petunia.

The band of high molecular dsRNA typical to cms "447" *V. faba* is visible in *lanes 2* and *8* (Fig. 2).

The third preparation consisted of a mixture of 7 g of equal amounts of leaves of petunia and *V. faba* (Fig. 3). In *lanes 2, 5* and *6* the typical band of cms "447" *V. faba* is shown. A mixture of leaves of petunia and *V. faba* was used as a control to show that during preparation there is no degradation of the dsRNA by the petunia leaves.

Discussion

In the present study we examined the possibility that viruses or viroids are associated with male sterility in petunia. Similarly, we looked for possible dsRNA association with male sterility. We assumed that if viruses or viroids are present, the conventional treatments for elimination of virus or viroids will produce "healthy" fertile plants. We used Lichter's (1978) treatment with the addition of antiviral factors virazole and IVR.

In our experiments only a small number of plants developed to flowering (5 from the cold treatment and 19 from the heat treatment). However, even with that small number of plants we should expect some positive results (some fertile plants out of cms plants) as compared with Lizarrage et al. (1980) and with Lichter (1978). No dsRNA was found in petunia correlated with sterility as was found for "447" cms *V. faba*. The existence of dsRNA was described in mitochondria of cms-S maize (Finnegan and Brown 1986), in sugarbeet mitochondria (Powling 1981), and in fertile and cms sunflower cytoplasm (Brown et al. 1986). In all those cases the existence of dsRNA was not correlated with sterility. The case of "447" cms *V. faba* is a unique one: the dsRNA is located in particles (cytoplasmic spherical bodies) that are virus-like entities. In the other cases the dsRNA was free in cytoplasm or mitochondria. Our data suggest that there are no viruses viroids or dsRNA associated with cms petunia. These findings were also confirmed by data pro-

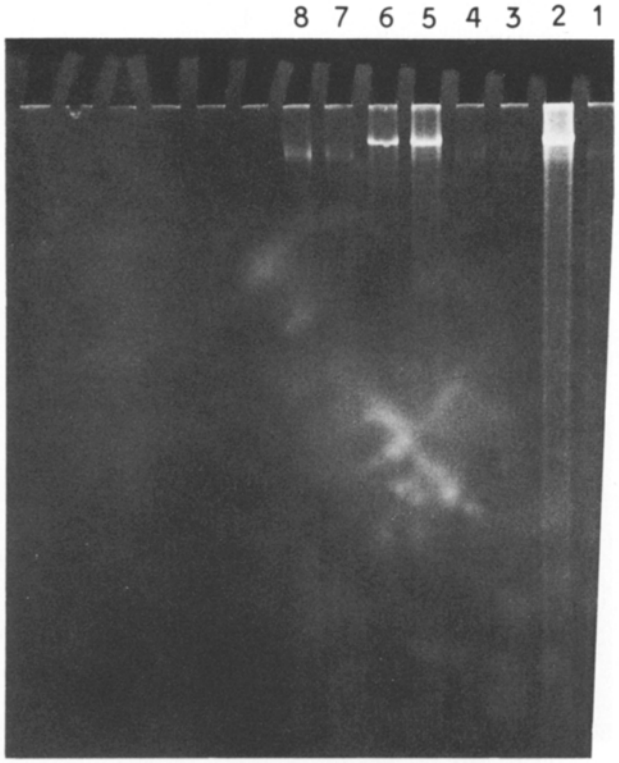

Fig. 3. Agarose gel electrophoretic of dsRNA from a mixture of petunia and *Vicia faba* leaves. *Lanes 1 Vicia faba* (normal); *2 Vicia faba* (cms); *3* 4555 petunia; *4* 4556 petunia; *5* 4556 petunia + *Vicia faba* (cms); *6* 4555 petunia + *Vicia faba* (cms); *7* 4556 petunia + *Vicia faba* (normal); *8* 4555 petunia + *Vicia faba* (normal)

duced in our laboratory using an electron microscope (Evenor 1987).

Our data are in agreement with recent work showing that male sterility in petunia is associated with the specific arrangement of mitochondrial DNA (Boeshore et al. 1985; Young and Hanson 1987; Clark et al. 1985, 1988).

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