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SOMATIC HYBRIDIZATION BETWEEN *N. tabacum* (NR⁻, Km⁺) AND *N. sanderae*. HYBRID GENOME
CONSTITUTION AND EXPRESSION OF RESISTANCE TO Tomato Spotted Wilt Virus

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ABSTRACT

Leaf mesophyll protoplasts of kanamicine resistant, nitrate reductase deficient mutant Nicotiana tabacum (NR⁻, Km⁺) were fused with leaf mesophyll protoplasts of wild type Nicotiana sanderae (wt). Somatic hybrid cell colonies were selected for kanamicine resistance and nitrate reductase proficiency. Nineteen independent cell lines, capable of growth in selection medium were obtained. From 14 of them shoots were regenerated and adapted in soil. All regenerants have intermediate plant and leaf morphology. The chromosome number for regenerants from six lines was determined, as well as analyses of four isoenzymes: peroxidase, esterase, shikimate dehydrogenase and glutamate-oxaloacetate transminase were performed. Results show that all studied plants represent nuclear somatic hybrids, possessing entire genomes of both parents. Local lesion (hypersensitivity) - type resistance to TSWV has been observed in leaves of these somatic hybrid plants after virus inoculation.

Introduction
The development of protoplast culture and somatic hybridization techniques has made it possible to produce a large number of somatic hybrids between tobacco and several partly and completely sexual incompatible wild Nicotiana species (5, 6).

The major problem for application of this technique arises from the difficulty in selecting somatic hybrids originated from population that consist of unfused protoplasts, homokaryons and heterokaryons (6). One possible solution is the use of the parent as an “universal hybridizer” which carries both dominantly expressed trait (resistance) and recessive trait (auxotrophy). Such selection system can be used to assess genetic compatibility with other species lacking selectable markers (15).

The wild Nicotiana species often carry resistance to diseases (11). The transmission of resistance to tobacco mosaic virus into interspecific somatic hybrids has been reported (1, 4). *N. sanderae* is wild Nicotiana species, which is resistant to Tomato Spotted Wilt Virus (TSWV) (8). Producing of sexual hybrids between *N. tabacum* and *N. sanderae* is very difficult and most of the obtained F₁ hybrids are sterile (10). Partially fertile hybrids are obtained after using of tetraploid *N. tabacum* as mother plant (8) or by application of in vitro tissue culture technique (19). There is no report about producing of somatic hybrids between *N. tabacum* and *N. sanderae*. 

In this paper we report the obtaining of somatic hybrids between kanamicine resistant, nitrate reductase deficient mutant \textit{N.\textit{tabacum}} (NR\textsuperscript{-}, Km\textsuperscript{+}) and wild type \textit{N.\textit{sanderae}} (NR\textsuperscript{+}, Km\textsuperscript{-}). Analyses of nuclear genome composition of somatic hybrids, as well as their reaction to inoculation with TSWV are described.

**Materials and Methods**

**Plant material**
The nitrate reductase deficient mutant, possessing kanamicine resistance (after genetic transformation with NPTII gene) - \textit{Nicotiana tabacum} (NR\textsuperscript{-}, Km\textsuperscript{+}) was kindly provided by Dr.S.Kushnir, Institute of Cell Biology and Genetic Engineering, Kiev, Ukraine. Mutant plants were maintained \textit{in vitro} as shoot culture on solid AA (7) medium supplemented with 10 mM ammonium succinate and 30g/l sucrose at light (3000lx, 16h-day, 26 °C). Wild type \textit{N.\textit{sanderae}} (NR\textsuperscript{+}, Km\textsuperscript{-}) plants were maintained in vitro on solid MS medium (12) with 30g/l sucrose at light (3000lx, 16h-day, 26 °C).

**Protoplast isolation and fusion**
Leaf protoplasts from both parents were isolated from in vitro plants according to Caboche M., 1980 (3). The polyethylene glycol (PEG) fusion was performs according to the method of Menczel et all, 1981 (13).

**Cell culture, hybrid selection and regeneration**
Fused protoplasts were initially cultured for two weeks in liquid K3 medium (14) containing 0.5M glucose, 3mg/l NAA, 1mg/l BAP and 10mM ammonium succinate. Cell cultures were further diluted weekly with AA medium (7) containing 2mg/l NAA, 0.5mg/l BAP, 30-10g/l mannitol, 20g/l sucrose with gradual increasing concentrations (25-75mg/l) of kanamicine. After first week cell cultures were placed at light (1500lx, 16h-day, 26 °C). Six weeks later the obtained green cell colonies were plated for one month onto AA solid medium containing 2mg/l NAA, 0.5mg/l BAP, 30g/l sucrose and 75mg/l kanamicine in the light. Shoots were regenerated from green calli after plating on MS solid medium with 0.1mg/l NAA, 0.1mg/l BAP, 250mg/l casein hydrolysate, 30g/l sucrose at the light (3000lx, 16h-day, 26 °C). Obtained regenerants were further maintained in vitro on AA medium with 20g/l sucrose and 75mg/l kanamicine. Several propagated plants from each fusion line were transferred in soil at greenhouse.

**TSWV-inoculation test**
Yong greenhouse adapted parents and regenerants were tested for TSWV-resistance as previously described by Jankulova et al.1992 (9). Three in vitro propagated plants from each fusion line were tested.

**Results and Discussion**

**Somatic hybrid selection and regeneration**
After PEG-fusion the protoplasts from kanamicine resistant, nitrate reductase mutant \textit{N.\textit{tabacum}} (NR\textsuperscript{-}, Km\textsuperscript{+}) and wild type \textit{N.\textit{sanderae}} (NR\textsuperscript{+}, Km\textsuperscript{-}) grew in succinate supplemented medium. After 2-3 dilitions with selection medium the NR\textsuperscript{-} and Km\textsuperscript{-} colonies stopped growing and only colonies derived from fusion products remained green and continued to grow. No green growing colonies were detected in control cultures for which the PEG-fusion procedure was not applied (Fig. 1). Totally 19 green NR\textsuperscript{+}Km\textsuperscript{+}
colonies were obtained from fusion experiments. Shoots were regenerated from 14 of them. All regenerated shoots formed roots on minimal medium with 75mg/l kanamicine. One shoot from each colony was further propagated.

Obtained results confirm the effective use of universal hybridizers for selection of somatic hybrids between them and lacking selectable markers shown by Brunold et al 1987 (2) and Ye et al. 1987 (18). All regenerated plants possess nuclear encoded trait (NR-proficiency, Km-resistance) from both parents what is an indication of the transfer of part or entire parental nuclear genomes into obtained somatic hybrids.

Morphology of regenerated plants
All regenerated plants have morphology intermediate between both parental species. These plants have a partially rosette growth habit, smaller, thicker and more oval leaves than *N.tabacum*. Similar to *N.sanderae* the plants have anthocian pigmentation on stem base. Plants from five lines formed flowers with altered morphology: flowers without stamens or formation of secondary petaloids instead of stamens. Pollen viability of rest of the plants was between 2 % and 21 %.

Nuclear constitution of the somatic hybrids
The nuclear constitution of the hybrids was investigated on six plants, each of which was
which qualify them as true nuclear hybrids (Fig. 2, Table). Both \textit{N.tabacum} and \textit{N.sanderae} specific bands were present in isocyanide patterns of peroxidase, esterase, shikimate- dehydrogenase and glutamate-oxaloacetate transminase for these plants (Fig. 3, Table).

Results of these analyses show that entire nuclear genomes of both parents are transferred into the studied hybrids. Parental genomes function normally together within hybrid cells without spontaneously elimination of parental nuclear genetic material. A similar genome constitution was reported also for other interspecific somatic hybrids in \textit{Nicotiana}, obtained after selection by using nuclear encoded traits (4, 5).

**Test for resistance to Tomato Spotted Wilt Virus**

In vitro propagated and greenhouse adapted plants from 13 fusion lines were tested for resistance to \textit{Tomato Spotted Wilt Virus}. All tested plants were found to be \textit{TSWV}-resistant with formation of local necrotic lesions, similar to the hypersensitive reaction of \textit{N.sanderae} (Fig. 4). This indicates that the \textit{TSWV}-resistance of \textit{N.sanderae} is being fully expressed in somatic hybrids when its entire nuclear genome is present. These results confirm results of \textit{TSWV}-resistance transfer
from *N. sanderae* in sexual hybrids *N. tabacum* × *N. sanderae* reported by Ivancheva-Gabrovska 1978 (8) and Zagorska et al. 1993 (19) and demonstrate the possible application of somatic hybridization: *N. tabacum* + *N. sanderae* for obtaining of TSWV-resistant tobacco cultivars.

**Conclusions**

Somatic hybrids possessing entire nuclear genomes of both parents were obtained after protoplast fusion between kanamicine resistant, nitrate reductase deficient mutant *N. tabacum* (NR⁻, Km⁺) and wild type *N. sanderae* (wt) plants. The TSWV resistance of *N. sanderae* is being fully expressed in studied somatic hybrids.

**REFERENCES**