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A survey of *Nicotiana* germplasm for resistance to *Tomato spotted wilt virus* (TSWV)

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Abstract The reaction to *Tomato spotted wilt virus* (TSWV) was evaluated in 94 accessions of *Nicotiana*, originating from the Institute of Soil Science and Plant Cultivation tobacco germplasm collection in Puławy, Poland. Tests for resistance were conducted under greenhouse conditions using single TSWV isolate collected from tobacco plantation in Lublin district, Poland. The presence of the virus was verified using DAS-ELISA. SCAR markers associated with TSWV resistance gene were applied. The members of the section *Alatae,* the genus *Nicotiana*: *N. alata*, *N. forgetiana*, and *Nicotiana x sanderae* as well as *N. tabacum* cultivars: 'Polalta' and 'Wiktoria' with the TSWV resistance gene introduced from *N. alata*, displayed the hypersensitive reaction (HR) against TSWV (grade 0 on symptom intensity scale). In some of those accessions, the virus spread from the initially infected areas eliciting systemic hypersensitive reaction (SHR). Five accessions of *N. alata* and three of *Nicotianaxsanderae* were composed of 6.3–50.0 % of plants in which SHR symptoms appeared. In all of *N. forgetiana* plants HR reaction was followed by systemic infection (SHR). In *N. tabacum* 'Wiktoria' 21.1 % of plants showed HR reaction, while the remaining were susceptible (S). All of the genotypes which responded with HR or SHR reaction to TSWV infection demonstrated the presence of SCAR markers linked to the resistance gene. The remaining eighty tested accessions were identified as being susceptible upon exposure to TSWV.

Keywords Germplasm · Hypersensitive reaction (HR) · *Nicotiana* spp. · Resistance · SCAR markers · *Tomato spotted wilt virus* (TSWV)

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Introduction

The genus *Nicotiana* is one of the most numerous in the family *Solanaceae*. *Nicotiana* species are native to North and South America, Australia, some Pacific islands and Namibia in Africa. Such an extensive area of origin and evolutionary heterogeneity makes *Nicotiana* an extremely diversified group. This diversity manifests itself with variation in chromosome number, multiplicity of morphological forms, different responses to day length, presence of different alkaloids, varied resistance responses to pests and diseases (Doroszewska et al. 2009).

For many years, the most popular systematics of the genus was that by Kostoff (1943) and Goodspeed (1954) with subsequent additions made by Burbidge (1960). Recently, a revised systematics based on molecular research was proposed (Chase et al. 2003; Clarkson et al. 2004; Knapp et al. 2004). In the new

classification by Knapp et al. (2004) subgenera were dropped and only the division into sections was retained. According to the new classification, *N. trigonophylla* Dun. was renamed *N. obtusifolia* Martens et Galeotti, *N. affinis* Hort is synonymous with *N. alata* Link et Otto, and *N. bigelovii* (Torrey) Watson with *N. quadrivalvis* Pursh. *N. sanderae* Hort. is currently regarded to be hybrid between *N. alata* and *N. forgetiana* Hemsl. (*Nicotiana x sanderae*) whereas *N. eastii* Kostoff is an autotetraploid variant of *N. suaveolens* Lehm. (Chase et al. 2003; Knapp et al. 2004).

The tobacco collection maintained in the Institute of Soil Science and Plant Cultivation in Puławy, Poland, consists of 1027 accessions belonging to 64 species of the genus *Nicotiana*, including 784 botanical varieties and cultivars of *N. tabacum* and 95 forms of *N. rustica*. Collection accessions are examined for various traits but one of the most important items in the evaluation of *Nicotiana* germplasm is screening for resistance to diseases (Doroszewska and Przybyś 2007; Doroszewska and Depta 2011).

Tomato spotted wilt virus (TSWV) is an economically important pathogen which has been reported worldwide in 1,090 different host species belonging to 85 botanical families, involving such important crops as potatoes, tomatoes, tobacco, pepper, lettuce, papaya, celery, eggplant (Parella et al. 2003). In tobacco, symptoms of TSWV include chlorosis and necrosis of the leaves and stems. Oftentimes, the systemic infection is unilateral. Apical buds frequently twist and bend to about 45°. The infection may ultimately lead to stunting and death of the plants (Mumford et al. 1996).

Tomato spotted wilt virus is the member of the genus *Tospovirus*, family *Bunyaviridae* (Francki et al. 1991; Wijkamp et al. 1993), and is transmitted in nature by at least eight species of thrips (*Thripidae*: *Thysanoptera*) (Jones 2005). The occurrence of many weed hosts that harbour the pathogen and the transmission by thrips make it difficult to control the virus, therefore screening of *Nicotiana* accessions for TSWV and introgression of the resistance into commercial tobacco cultivars would be the most effective way to minimize the damage by this disease. Although resistant lines have been developed in tobacco by transforming plants with the TSWV nucleocapsid gene (Herrero et al. 2000), they are not acceptable to the tobacco market.

Genetic resistance to TSWV is scarce in cultivated host species. A few sources of resistance have been identified and incorporated into commercial cultivars of tomato and pepper. *Sw-5* gene from *Solanum peruvianum* L. has been broadly utilized in tomato breeding (Stevens et al. 1992). Additionally, *Sw-6* gene was found in *S. peruvianum* and introgressed to *S. lycopersicum* L. 'UPV-1' line (Rosello et al. 2001). *Sw-7* gene from *S. chilense* (Dun.) Reiche 'LA 1938' was transferred to *S. lycopersicum* 'Y118' (Canady et al. 2001). In *Capsicum chinense* Jacq. and *C. annuum* L. the only locus to bring on TSWV resistance was named *Tsw* (Black et al. 1991; Boiteux 1995; Roggero et al. 1999).

Of the wild *Nicotiana* species, the following were the most frequently reported as potential sources for TSWV resistance: *N. alata* Link et Otto (Opoka 1969; Gajos 1978; Ivancheva-Gabrovska 1978; Jankowski 1980; Kovalenko et al. 1987; Palakarcheva and Yancheva 1989), *N. sanderae* Hort. (Ivancheva-Gabrovska 1978; Jankowski 1980; Kovalenko et al. 1987; Palakarcheva and Yancheva 1989; Shcherbatenko and Oleshchenko 2006), *N. glauca* Graham (Opoka 1969; Ivancheva-Gabrovska 1978; Pop 1979; Kovalenko et al. 1987; Shcherbatenko and Oleshchenko 2006), *N. langsdorfii* Weinm. (Gajos 1978; Pop 1979; Kovalenko et al. 1987; Palakarcheva and Yancheva 1989), *N. longiflora* Cav. (Pop 1979; Jankowski 1980), *N. trigonophylla* Dun. (Jankowski 1980; Kovalenko et al. 1987), *N. forgetiana* Hemsl. (Ivancheva-Gabrovska 1978), *N. fragrans* Hook. (Jankowski 1980), *N. noctiflora* Hook. (Opoka 1969), *N. palmeri* Gray (Kovalenko et al. 1987).

The latest host-lists of TSWV (Cho et al. 1987; Edwardson and Christie 1997; Gognalos et al. 1999; Parella et al. 2003; Scott 2012) contradict much of those earlier reports on TSWV resistance. They contain as many as 61 susceptible *Nicotiana* species, including all of the mentioned above, except *N. forgetiana*.

There are no commercially exploited TSWV-resistant tobacco cultivars either in Poland or elsewhere in the world. Some of the *Nicotiana* wild species cited above as resistant were tried as sources of resistance to TSWV in tobacco breeding. Transfer of the resistance from *N. alata* was tried by the conventional breeding method (Stoyanova 1979; Gajos 1981, 1988, 1993; Ivancheva-Gabrovska and Manolov 1982; Berbeć 1987) as well as using in vitro culture techniques

(Dorossiev et al. 1978; Patrascu and Paunescu 1997; Laskowska and Berbeć 2005; Shcherbatenko and Oleshchenko 2006) and by somatic hybridization (Nagao 1982; Flick and Evans 1983; Dimanov and Atanassov 1989). The cross-breeding between *Nicotiana x sanderae* and cultivated tobacco was conducted by Skucińska et al. (1977), Ivancheva-Gabrovska and Manolov (1982), Palakarcheva and Yancheva (1985), Dorossiev and Palakarcheva (1990), Kovalenko et al. (1989), Shcherbatenko and Oleshchenko (2006). Occasionally, the potentials of *N. glauca* and *N. noctiflora* as germplasm sources for TSWV resistance were also studied (Kovalenko et al. 1989; Dorossiev and Palakarcheva 1990; Shcherbatenko and Oleshchenko 2006).

In spite of the efforts made by numerous breeders, up to now only two tobacco (*N. tabacum* L.) cultivars, both released in Poland—'Polalta' and 'Wiktoria'—carry a confirmed TSWV resistance response of the hypersensitive type from *N. alata* (Gajos 1988, 1993; Yancheva 1990; Moon and Nicholson 2007). The gene transfer mechanism leading to the development of 'Polalta' lacks adequate documentation, but *N. otophora* seems to be involved as a bridging species in the transfer (Gajos 1981). Further multiple crossing of the interspecies hybrid (*N. tabacum* × *N. alata*) with Virginia cultivars has resulted in developing the cultivar 'Wiktoria' (Gajos 1993). However, when those resistant cultivars are crossed with other *N. tabacum* genotypes morphological deformations such as thickened and ribbon-shaped leaves, irregular venation, or tumours appear in the resulting progeny and successive segregating generations (Kennedy and Nielsen 1993, Moon and Nicholson 2007). 'Polalta' and 'Wiktoria' are not grown commercially because of poor agronomic performance caused by the introgressed DNA from *N. alata* or *N. otophora* (Kennedy and Nielsen 1993; Moon and Nicholson 2007). AFLP and SCAR markers linked with the *N. alata* DNA segment conferring resistance to TSWV were identified by Moon and Nicholson (2007) in *N. tabacum* 'Polalta'. The molecular sequence of the gene encoding TSWV resistance in that segment has not yet been established.

Because of the poor breeding progress, the contradictory data available in the literature and the necessity to diversify available sources of resistance, there is a continued need to search for possible new resistant accessions. Our objective was to re-examine the reaction of *Nicotiana* germplasm accessions mechanically inoculated with TSWV in combination with the use of SCAR markers (Moon and Nicholson 2007) to detect the presence of the DNA segment bearing the TSWV resistance-conferring gene.

Materials and methods

Plant materials

Ninety-four entries, belonging to 62 species of the genus *Nicotiana*, including seven botanical varieties and two cultivars of *N. tabacum*, twelve varieties of *N. rustica*, five of *N. quadrivalvis*, seven populations of *N. alata* of diverse origin, and four of *Nicotiana x sanderae* (Table 1), were used in this study. All of them came from the collection of the Institute of Soil Science and Plant Cultivation—State Research Institute in Puławy, Poland.

Species and section names were given according to the current classification by Knapp et al. (2004).

Resistance tests

The TSWV isolate used for inoculation tests was collected from one plant that showed typical systemic symptoms of TSWV infection on a tobacco plantation in Łąkoć, Lublin district, eastern Poland. The isolate was multiplied on the *N. tabacum* 'Samsun' plants, resistant to *Tobacco mosaic virus,* according to the method given below. In order to avoid possible changes in the inoculum after repeated mechanical inoculations, the isolate was multiplied only three times. Serological methods were used to confirm that the TSWV isolate was free from the contamination with common tobacco viruses (*Potato virus Y*, *Tobacco mosaic virus*). Infected plant tissues from 'Samsun' leaves prepared in 2009 were deep-freezed (−80 °C) and thawed prior to resistance tests. Inoculum was prepared as follows: thawed tissues were ground in phosphate buffer (9.078 g/l KH_2PO_4 and 11.867 g/l Na_2HPO_4) with the addition of 0.5 % mercapthoethanol and 1 % carborundum (400 mesh) (Tsakiridis and Gooding 1972). The proportion was 1 g tissue to 2 ml of buffer.

The isolates were multiplied, collection accessions were grown, and resistance tests were conducted in a cabinet in constant conditions of 25 °C under a 15/9 h light/dark cycle at a light intensity of 12,000 Lux.

Table 1 Evaluation of *Nicotiana* germplasm accesions to mechanical TSWV infection

Species	Symptoms	ELISA positive plants/total tested plants	Symptom severity index
Section: *Alatae*			
N. alata var. *alba* (from Oslo)	HR/SHR	03/10	0/3
N. alata (from Oslo)	HR	00/15	0
N. alata (from Warsaw)	HR	00/16	0
N. alata var. *grandiflora* (from Vilnius)	HR/SHR	08/16	0/2
N. alata var. *grandiflora* (from Bergerac)	HR/SHR	05/16	0/2
N. alata (from Scafati)	HR/SHR	03/15	0/2
N. alata 'Biały Narcyz' (breeding in Warsaw)	HR/SHR	01/16	0/3
N. forgetiana	SHR	10/10	3
N. langsdorfii	S	06/06	3
N. longiflora	S	06/06	2
N. plumbaginifolia	S	06/06	3
Nicotiana x sanderae (from Warsaw)[a]	HR/SHR	06/12	0/2
Nicotiana x sanderae—lilac (from Oslo)	HR/SHR	04/12	0/1
Nicotiana x sanderae—white (from Oslo)	HR	00/11	0
Nicotiana x sanderae—red (from Oslo)	HR/SHR	02/12	0/2
Section: *Nicotiana*			
N. tabacum var. *atropurpurea grandiflora*	S	06/06	2
N. tabacum var. *auriculata*	S	06/06	2
N. tabacum var. *fructicosa*	S	06/06	2
N. tabacum var. *gigantea*	S	06/06	2
N. tabacum var. *havanensis*	S	06/06	2
N. tabacum var. *petiolaris*	S	06/06	2
N. tabacum var. *macrophylla purpurea*	S	06/06	2
N. tabacum 'Polalta'	HR	00/16	0
N. tabacum 'Wiktoria'	HR/S	15/19	0/3
Section: *Noctiflorae*			
N. glauca	S	06/06	2
N. noctiflora	S	06/06	3
N. petunioides	S	06/06	1
Section: *Paniculatae*			
N. benavidesii	S	06/06	1
N. cordifolia	S	06/06	3
N. knightiana	S	06/06	2
N. paniculata	S	06/06	2
N. raimondii	S	06/06	3
N. solanifolia	S	06/06	3
Section: *Petunioides*			
N. acuminata	S	06/06	3
N. attenuata	S	06/06	3
N. corymbosa	S	06/06	3
N. linearis	S	06/06	2
N. miersii	S	06/06	2

Table 1 continued

Species	Symptoms	ELISA positive plants/total tested plants	Symptom severity index
N. pauciflora	S	06/06	3
Section: *Polydicliae*			
N. quadrivalvis var. *bigelovii*	S	06/06	2
N. quadrivalvis var. *quadrivalvis*	S	06/06	2
N. quadrivalvis var. *multivalvis*	S	06/06	3
N. quadrivalvis var. *wallacei*	S	06/06	2
N. quadrivalvis	S	06/06	2
Section: *Repandae*			
N. nesophila	S	06/06	3
N. nudicaulis	S	06/06	3
N. repanda	S	06/06	2
N. stoctonii	S	06/06	2
Section: *Rusticae*			
N. rustica var. *argentea*	S	06/06	2
N. rustica var. *brasilia*	S	06/06	2
N. rustica var. *cerinthoides*	S	06/06	2
N. rustica var. *chlorotica*	S	06/06	2
N. rustica var. *eischfeldii*	S	06/06	2
N. rustica var. *humilis*	S	06/06	2
N. rustica var. *neuchestii*	S	06/06	2
N. rustica var. *ovata*	S	06/06	2
N. rustica var. *oviformis*	S	06/06	2
N. rustica var. *pavonii*	S	06/06	2
N. rustica var. *pumila*	S	06/06	2
N. rustica var. *texana*	S	06/06	2
Section: *Suaveolentes*			
N. africana	S	06/06	2
N. amplexicaulis	S	06/06	2
N. benthamiana	S	06/06	2
N. cavicola	S	06/06	2
N. debney	S	06/06	3
N. eastii[a]	S	06/06	2
N. excelsior	S	06/06	3
N. exigua	S	06/06	3
N. goodspeedii	S	06/06	2
N. gossei	S	06/06	2
N. hesperis	S	06/06	3
N. ingulba	S	06/06	3
N. maritima	S	06/06	2
N. megalosiphon	S	06/06	2
N. occidentalis	S	06/06	3
N. rosulata	S	06/06	3
N. rotundifolia	S	06/06	3

Table 1 continued

Species	Symptoms	ELISA positive plants/total tested plants	Symptom severity index
N. simulans	S	06/06	2
N. suaveolens	S	06/06	2
N. umbratica	S	06/06	2
N. velutina	S	06/06	1
N. wuttkei	S	06/06	2
Section: *Sylvestres*			
N. sylvestris	S	06/06	3
Section: *Tomentosae*			
N. kawakamii	S	06/06	2
N. otophora	S	06/06	1
N. setchelli	S	06/06	1
N. tomentosiformis	S	06/06	2
Section: *Trigonophyllae*			
N. obtusifolia	S	06/06	1
N. palmeri	S	06/06	1
Section: *Undulatae*			
N. arentsii	S	06/06	1
N. glutinosa	S	06/06	3
N. thyrsiflora	S	06/06	3
N. undulata	S	06/06	2
N. wigandioides	S	06/06	1

The origin of *Alatae* section accessions, given in brackets, mean: from Oslo—included to the Pulawy collection in 1948 from Oslo Botanical Garden; from Warsaw—included from Warsaw Botanical Garden in 1969; breeding in Warsaw—breeding in unknown institution of Warsaw, aquired in 1956; from Vilnius—comes from unknown Vilnius institution, 1935; from Bergerac—aquired from The Tobacco Institute of Bergerac, France, 1938; from Scafati—from Scafati Tobacco Institute, Italy, 1968

[a] According to Knapp et al. (2004) classification was not given the status of separate species

Plants were germinated in a sterilized peat soil mixture in small pots (7.0 cm × 6.0 cm × 6.0 cm). One-week-old seedlings were transplanted to 160 plastic cell trays, ten cells for each accession. Four to eight weeks after sowing, when the seedlings developed at least three pairs of true leaves, they were individually planted to pots of 16 cm in diameter, containing steamed soil.

Resistance tests were conducted in 2010 and 2011. About 200 plants were inoculated in one series. Eight plants were prepared per accession, from which two plants were used as the blank controls. In each resistance test, eight plants of standard control cultivars: susceptible 'Wiślica' and resistant 'Polalta' were included. In *N. alata*, *N. sanderae*, *N. forgetiana*, *N. tabacum*'Polalta' and *N. tabacum* 'Wiktoria' accessions, six to fifteen plants were additionally tested, depending on availability of seeds.

Virus was transmitted to six-leaved plants using mechanical inoculation. Blank controls were inoculated only with phosphate buffer and carborundum. These controls were used as negatives for the ELISA test. Plants were monitored for the progress of disease symptoms 5, 7, 14, 21 and 35 days after inoculation. The symptoms were determined as: HR—hypersensitive reaction—score 0 on the symptoms intensity scale, SHR—systemic hypersensitive reaction, S—susceptible. The intensity of the symptoms in plants which developed systemic infection was scored on the following scale: 1—chlorotic spots, vein clearing, 2—

necrotic spots, stem and leaves necrosis, curving of the apex, flower deformation, wrinkled upper leaves, 3—inhibited growth, plants die out. TSWV infection was confirmed by a standard DAS-ELISA as described by Clark and Adams (1977), using a commercial polyclonal antiserum against TSWV proteins (Bioreba AG, Switzerland). Absorbance after serological reaction was measured at the optical density of 405 nm with a Tecan microplate reader. Samples were taken from the top leaves of each plant. In all *N. alata*, *N. sanderae*, *N. forgetiana*, *N. tabacum* 'Polalta' and *N. tabacum* 'Wiktoria' plants, samples were taken from two stalk positions—top leaves (without or with symptoms) and bottom leaves with necrotic local lesions. All leaf samplings for ELISA tests were taken 1 week after development of the TSWV symptoms. Plants were considered as susceptible when samples thereof showed an absorbance value of at least twice that of the absorbance reading in the negative controls.

Detection of markers linked to TSWV resistance

Detections of markers were preceded by isolation of DNA carried out according to the methodology described by Czubacka and Doroszewska (2010). DNA was amplified by polymerase chain reaction (PCR). Four AFLP markers: AAC/CCC172, ACT/CTA169, AAG/CGA228, ACT/CTA268, previously determined to be closely associated in coupling with the TSWV resistance gene present in cultivar Polalta (Moon and Nicholson 2007) were used in this study. Those markers use pairs of SCAR primers: AGCTTC TTTTCTCTTTCCATTTTT and CAGAAGAAAAA CTGCTGGAGCTAT, ACTTTTCACACCAAAAAC TCACG and GTAGATGATAAAGATTGAAGAAA ACAA, TAGATGTCATGAATGGAACTACGG and TTTTGATCGAAAAACCCAACC, CTGATCGTT CCAGCAGGTTCTTAT and GGAGCTATTTCCA GACACGAA, respectively. The size of the PCR-amplified products of the first three markers were 117, 105 and 117 bp. The ATC/CTA268 marker generates two PCR-amplified products (161 and 200 bp) in genotypes carrying the TSWV resistance gene and one product (200 bp) in genotypes that do not possess that gene. The amount of 1 µl plant DNA (approximately 20 ng/µl) was added to the mixture containing 1.9 units of Taq polymerase (Fermentas) and 4.5 pmol of each primer per 20 µl reaction, 1.5 × PCR buffer, 2 mM $MgCl_2$, 312.5 µM dNTPs. The PCR was done in a termocycler using the following amplification procedure: for 2 min at 94 °C followed by 30 cycles: 30 s at 94 °C, 30 s at 53.5, 50, 53.5 and 55 °C for individual markers, 40 s at 72 °C and a final elongation step 5 min at 72 °C. The products of PCR were analysed by electrophoresis in 2 % agarose gels in 1 × TBE buffer (100 mM Tris, 90 Mm H_3BO_3, 1 mM EDTA, pH 8.5) at 7–10 V/cm.

PCR analysis was done on all TSWV tested plants of *N. alata*, *N. forgetiana*, *Nicotiana x sanderae*, *N. langsdorfii*, *N. longiflora*, *N. plumbaginifolia*, *N. tabacum* 'Polalta', *N. tabacum* 'Wiktoria' and on susceptible plants of *N. tabacum* 'Wiślica' as a control.

Results

The symptoms of TSWV varied greatly depending on the species involved. *N. alata*, *Nicotiana x sanderae, N. forgetiana, N. tabacum* 'Polalta' and some plants of *N. tabacum* 'Wiktoria' showed the typical hypersensitive reaction (HR). In those accessions local yellow discolorations which evolved into concentric necrotic local spots appeared in 5–7 days after inoculation. In *N. alata*, *Nicotiana x sanderae* and *N. forgetiana* necrotic spots were 6–7 mm in diameter and in 'Polalta' and 'Wiktoria'—2–3 mm. In some plants of those accessions local lesions were subsequently accompanied by chlorotic spots, and in 14 days the systemic infection set in, at first manifested as fast-growing and coalescing chlorotic necrotic spots, which ultimately led to leaf and apex distortion (systemic hypersensitive reaction—SHR) (Table 1).

In plants with HR reaction TSWV was detected only in the inoculated leaves that showed localized concentric lesions but not in the newly developed, non-inoculated leaves. No systemic symptoms developed in these plants within 5 weeks after inoculation. Two of the seven *N. alata* populations, one of the four *Nicotiana x sanderae* as well as *N. tabacum* 'Polalta' were found to be composed entirely of plants that showed HR response. In the remaining *N. alata* and *Nicotiana x sanderae* accessions listed in the Table 1, plants with SHR symptoms appeared. In those plants, TSWV spread out of the necrotic lesions into the inoculated leaves and was able to bring about systemic infection. SHR plants accounted for 6.3—50.0 % of *N. alata* populations under study and from 16.7 to 50.0 % of *Nicotiana x sanderae.* Four out of 19 'Wiktoria'

plants screened for the reaction to TSWV showed HR response, the remaining were susceptible (S). In *N. forgetiana* TSWV induced a hypersensitive response, shown by the appearance of necrotic local lesions 5 days post inoculation, but severe systemic symptoms developed in all of the tested plants within the next 10 days (SHR reaction). Virus accumulation in the newly developed, non-inoculated leaves of all of the SHR plants showed ELISA absorbance values within a range of 0.369–2.747.

In the remaining entries listed in Table 1, typical systemic symptoms (S) of the TSWV infection were observed with varying intensity. In *N. petunioides, N. benavidesii, N. velutina, N. otophora, N. setchelli, N. obtusifolia, N. palmeri, N. arentsii, N. wigandioides,* vein clearing as well as individual chlorotic spots were observed, without necrotic symptoms. Frequently unilateral vein clearing occurred, involving only one half of the leaf (a score of 1 on the symptoms scale). DAS-ELISA absorbance values in the non-inoculated leaves (from 0.490 to 2.890) confirm their susceptibility. The remaining 71 accessions listed in Table 1 developed more severe symptoms. In the typical course of the disease, chlorotic and necrotic rings and irregular spots on the inoculated leaves appeared seven days after infection and grew into large chlorotic and necrotic areas. During the next 14 days the infection symptoms, including vein clearing, leaf distortions, and the characteristic curving of the apex, spread to the upper portion of the plant. This systemic infection was often unilateral (score of 2 on the symptom intensity scale). Stunting and death, (score of 3) followed in some of those accessions. The content of TSWV in the upper leaves of the accessions with scores of 2 or 3, resulted in DAS-ELISA absorbance values from 0.299 to 3.194 and was not correlated with symptoms manifested by the plants.

Plants of *N. alata*, *N. forgetiana*, *Nicotiana x sanderae* and *N. tabacum* 'Polalta' responded to all four TSWV resistance-linked markers (Table 2; Fig. 1). Of the 19 *N. tabacum* 'Wiktoria' tested plants, four (21.1 %) which showed HR reaction to TSWV responded to all four markers and the others failed to show response to any. The remaining entries listen in

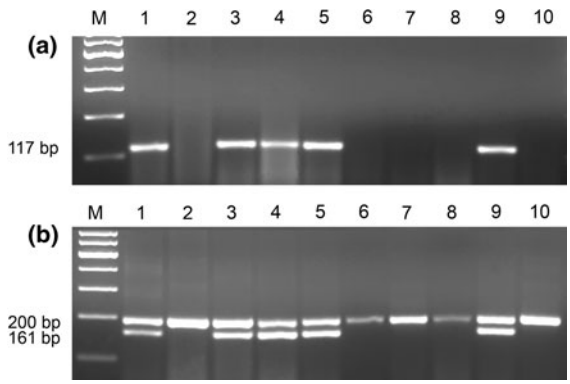

Fig. 1 Amplification profiles generated by SCAR markers S-AAC/CCC172 (**a**) and S-ACT/CTA268 (**b**), linked with TSWV resistance gene. *Lane M* 100 bp DNA ladder, *lane 1 N. tabacum* 'Polalta', *lane 2* susceptible control *N. tabacum* 'Wiślica', *lanes 3–10 N. alata*, *N. forgetiana*, *Nicotiana x sanderae*, *N. langsdorfii*, *N. longiflora*, *N. plumbaginifolia*, *N. tabacum'Wiktoria'* resistant individual, *N. tabacum'Wiktoria'* susceptible individual

Table 2 Response to SCAR markers (Moon and Nicholson 2007) linked to TSWV resistance in some species of the section *Alatae, N. tabacum* cultivars with the resistance introduced from *N. alata* and *N. tabacum* 'Wiślica' as a negative control

Genotype	Markers			
	AAC/CCC172	ACT/CTA268	ACG/CCG169	AAG/CGA228
N. tabacum 'Polalta'	+	+	+	+
N. tabacum 'Wiślica'	−	−	−	−
N. alata	+	+	+	+
N. forgetiana	+	+	+	+
Nicotiana x sanderae	+	+	+	+
N. langsdorfii	−	−	−	−
N. longiflora	−	–	−	−
N. plumbaginifolia	−	−	−	−
N. tabacum 'Wiktoria'[a]	±	±	±	±

[a] Four out of 19 'Wiktoria' plants responded to all four SCAR markers

Table 2 did not respond to any of the markers. All plants of the genotypes which showed the HR or SHR reactions responded to TSWV resistance-linked molecular markers.

Discussion

In this study, only the members of the section *Alatae*: *N. alata*, *N. forgetiana*, and *Nicotiana x sanderae* as well as cultivars of *N. tabacum*: 'Polalta' and 'Wiktoria', with the resistance gene introduced from *N. alata* (Gajos 1988, 1993; Yancheva 1990; Moon and Nicholson 2007) showed localized induced cell death at the site of TSWV infection.

The resistance to TSWV is in various plant species associated with a hypersensitivity response (HR). This type of reaction was detected in tomato (*Solanum lycopersicum* L. 'Steven') carried by the *Sw-5* gene of resistance (Stevens et al. 1992) as well as in peanut (*Arachis hypogaea* L.), cultivar 'C11-2-39' (Mandal et al. 2002). The same symptoms followed by abscising of cotyledons and leaves developed on resistant plants of *Capsicum chinense* Jacq. and *C. annuum* L. ('PI 152225' and 'PI 159236') which carried the *Tsw* gene (Black et al. 1991).

A hypersensitive response (HR) is defined as rapid death of plant cells at the site of pathogen ingress in association with the restraint on pathogen growth, which confines the pathogen to the initial infection area (Goodman and Novacky 1994). This kind of "gene-for-gene" resistance in plants, in which resistant host and the avirulent pathogen are incompatible, is termed as *R-Avr*-mediated defense response (Flor 1971). The arrest of an avirulent virus is associated with induction of a variety of defense-related genes including pathogenesis-related (PR) proteins and a wide range of physiological changes (Hammond-Kosack and Jones 1996).

Some *N. alata* and *Nicotiana x sanderae* populations investigated in this study were composed entirely of HR plants whereas some other segregated for the HR and SHR plants, in which virus-induced HR was followed by SHR. In *N. forgetiana* all plants developed SHR. This could be the reason for some discrepancies in the literature regarding the resistance of these species and their suitability as potential sources of resistance to TSWV in tobacco improvement (Ivancheva-Gabrovska 1978; Jankowski 1980; Kovalenko et al. 1987; Palakarcheva and Yancheva 1989) or susceptibility (Opoka 1969; Ruter and Gitaitis 1993). Elicitation of SHR, instead of localized HR, is quite frequent in the interactions involving avirulent viruses and resistant plants. Similar to our findings, Mandal et. al. (2002) observed, that in 17.6–46.7 % plants of the peanut 'C11-2-39' initial HR was followed by systemic infection. In *Solanum lycopersicum* up to 8.0 % of plants were found to be infected with TSWV (Stevens et al. 1992; Rosello et al. 1998). Also in *Capsicum* a small but significant proportion of plants in resistant 'PI 152225' and 'PI 159236' lines showed systemic TSWV infection (Soler et al. 1999).

Currently, the underlying molecular and biochemical mechanisms leading to SHR are not understood (Hajimorad et al. 2005). Dinesh-Kumar et al. (2000) have postulated that SHR is a consequence of delayed occurrence of biochemical and physiological events that are associated with localized HR. They found, that the HR induced by TMV in a tobacco plant which carried an in vitro-constructed variant of *N*-resistance gene, occasionally spread to the entire plant, resulting in SHR. The authors suggest that some loss-off-function *N* alleles such as the TIR (Toll/interleukin-1 receptor homology region) deletion and the point mutation in the NBS (nucleotide-binding site) may cause a systemic hypersensitive response (SHR).

The mechanism of the HR reaction is strongly influenced by such conditions as high temperatures or the development stage of the plants at virus inoculation (Roggero et al. 1996; Moury et al. 1997; Soler et al. 1998; Mandal et al. 2002). The resistance could be also lost by a mutation in the corresponding pathogen *Avr* gene (Ellis et al. 2000). In our study, ambient conditions, plant development stage at inoculation, and the isolate used were constant, so that there is some chance that this resistance response was genotype-dependent.

One of such factors that can result in virus escape to distant tissues, provoking SHR in resistant genotypes could be heterozygosity rather than homozygosity of the *R*-genotype plant (Moury et al. 1998; Chen et al. 1994). In *N. alata* and *Nicotiana x sanderae* the self-incompatibility and large polymorphism of the entries may be the reason of the incomplete homozygosity and segregation of the resistance trait. Small outcrossing populations such as those maintained in germplasm collections are particularly prone to genetic drift and consequent changes in allele frequencies. Gajos

(1981) and Yancheva (1990) report for *N. tabacum* 'Polalta' that heterozygous generations were resistant, but when resistant 'Polalta' × 'Wiślica' DH breeding lines were backcrossed to 'Wiślica' (Laskowska and Berbeć 2010), in the resulting heterozygous hybrid populations the proportion of resistant to susceptible plants differed slightly from the expected ratios in favor of susceptible plants.

The observed ratio of resistant to susceptible haploid plants obtained through androgenesis from the F_1 'Polalta' with susceptible 'Wiślica' hybrid was skewed, resulting in an excess of susceptible genotypes (Laskowska and Berbeć 2010). The changes in the genetic background of an R gene-carrying plant can influence the HR reaction (Hajimorad et al. 2005; Lanfermeijer et al. 2005), which fits with the results presented here. The resistance in different populations of *N. alata* ranged from 50 to 100 %. The resistance factor transferred from *N. alata* into *N. tabacum* gave a good protection against TSWV when employed in the genetic milieu of 'Polalta' (100.0 % of resistant plants) and was poorly expressed and erratic in 'Wiktoria' (21.1 % of resistant plants).

'Polalta' is recognized as the best source of resistance to TSWV in tobacco. The cultivar is used in tobacco breeding and as the resistance check in testing for resistance (Herrero et al. 2000; Moon and Nicholson 2007; Laskowska and Berbeć 2010). Gajos (1981) formulated a hypothesis of monogenic dominant resistance to TSWV introgressed from *N. alata* to the *N. tabacum* 'Polalta'. This model of inheritance was confirmed by Yancheva (1990) and Moon and Nicholson (2007). Likewise TSWV resistance in breeding lines of tobacco obtained from 'Polalta' by Laskowska and Berbeć (2010) depended on one dominant gene. The presence of minor resistance-modifying genes cannot be entirely excluded as a factor affecting the expression of resistance. Kennedy and Nielsen (1993) investigated F_1, F_2 and backcross generations and assumed 'Polalta' to possess digenic resistance. According to Fraser (1990) virus localization is a phenomenon associated with resistance alleles which are phenotypically dominant and are usually controlled by one or a few major genes. The genes controlling resistance to TSWV in tomato (*Sw-5*) and pepper (*Tsw*) are inherited as a single dominant character (Stevens et al. 1992; Boiteux and de Avila 1994).

The molecular analysis with the use of SCAR markers described by Moon and Nicholson (2007) as tightly linked to TSWV resistance gene, confirmed its presence in all tested plants of both HR and SHR genotypes shown in Table 2. The lack of the HR/SHR responses and the absence of reaction to resistance-linked molecular markers in some 'Wiktoria' plants suggest the loss of resistance gene rather than changes to the gene's molecular structure. In Poland, the resistance conferred by the R gene in 'Wiktoria' was overcome by TSWV soon after it was introgressed into that cultivar. In the study of Moon and Nicholson (2007) the plants of 'Wiktoria' responded to 17 markers linked to the resistance gene.

This is the first report which provides the reaction of *N. forgetiana* to the resistance-linked molecular markers described by Moon and Nicholson (2007). The species, closely related to *N. alata* (Clarkson et al. 2004) was found completely susceptible (SHR) to TSWV. Minor differences in genomic sequences at the amino acid level, related to resistance responses, may exist between these two species.

The hypersensitive reaction (HR) is the only mechanism responsible for resistance to TSWV known in *Nicotiana.* In natural conditions it seems to be confined to only one species—*N. alata* section *Alatae*, if we take into account *Nicotiana x sanderae*'s origin as artificial hybrid species of *N. alata* × *N. forgetiana*. The majority of wild *Nicotiana* accessions showed systemic symptoms following mechanical inoculation with TSWV.

Most of the *Nicotiana* species named in early reports as potential sources for TSWV resistance, including *N. glauca*, *N. langsdorfii*, *N. longiflora*, *N. trigonophylla* (syn. *N. obtusifolia*), *N. noctiflora*, *N. palmeri*, turned out to be susceptible in our study. One of the reasons for this difference can be that those early investigations (Opoka 1969; Jankowski 1980) relied on natural vector-mediated infection rather than artificial inoculations and their data reflected the feeding preferences of the insect vector rather than intrinsic resistance mechanisms of the host plant. In the other early investigations, in which mechanical inoculation was applied, the reason can be impermanence of the virus which rapidly deactivates once inoculum is prepared and the lack of serological and molecular methods. From the species listed above, *N. trigonophylla* (syn. *N. obtusifolia*), *N. palmeri*, *N. glauca* and *N. noctiflora* are slow-growing perennials and the slow increase of tissue leaves can result in poor symptom development.

In our study most of the species with the lower symptom intensities (score 1): *N. obtusifolia*, *N. palmeri* (*Trigonophyllae*), *N. otophora*, *N. setchelli* (*Tomentosae*), *N. benavidesii* (*Paniculatae*), *N. arentsii*, *N. wigandioides* (*Undulatae*) are slow-growing perennials or woody shrubs, which could affect the symptom development. Only *N. petunioides* (*Noctiflorae*) and *N. velutina* (*Suaveolentes*) are annual herbs.

The group of collection accessions found as susceptible in this study generally agrees with the lists of susceptible hosts reported by other investigators, of which the most extensive is the one compiled by Parella et al. (2003). Among the entries not evaluated in this study but listed by that investigator are: *Nicotiana x calyciflora*, *N. clevelandii*, *N. cycliflora*, *Nicotiana x edwardsonii*, *N. fragrans*, *N. bonariensis*, *N. tomentosa*. Other species listed by Parella et al. (2003)—*N. angustifolia, N. caudigera, N. macrophylla and N. multivalvis*—are synonymous to *N. acuminata*, *N. pauciflora, N. tabacum* var. *macrophylla purpurea* and *N. quadrivalvis* var. *multivalvis* of this study. The species *N. wuttkei* was previously evaluated as susceptible by Laskowska and Berbeć (2003).

This study encompassed 12 entries the reaction of which to TSWV had not been reported in literature: *N. africana*, *N. amplexicaulis*, *N. cavicola*, *N. corymbosa*, *N. eastii* (autotetraploid of *N. suaveolens*), *N. gossei*, *N. kawakamii*, *N. otophora*, *N. pauciflora, N. setchelli, N. simulans*, *N. umbratica* and many botanical varieties within *N. tabacum*, *N. rustica* and *N. quadrivalvis* (Table 1). All those entries were found to be susceptible to TSWV.

In conclusion, it can be stated with a fair degree of probability that the *N. alata*-derived gene which we propose to name *RTSW-al* and which, apart from *N. alata*, gives resistance to TSWV in *Nicotiana x sanderae*, *N. tabacum* 'Polalta' and, to a certain degree, in 'Wiktoria' is the only source of resistance to TSWV in the genus *Nicotiana*. That source of resistance is difficult to employ in tobacco breeding because of genetic distance between the donors of resistance and the cultivated *N. tabacum*, the expected linkage drag in the hybrids and the unstable expression shown by *RTSW-al* gene. In the future, it will be important to study the molecular structure of the resistance factor in different genotypes of *Nicotiana* as well as to look into the biochemical mechanisms underlying the control of HR/SHR reaction.

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