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Seven of eight species in *Nicotiana* section *Suaveolentes* have common factors leading to hybrid lethality in crosses with *Nicotiana tabacum*

Takahiro Tezuka[1,*], Tsutomu Kuboyama[2], Toshiaki Matsuda[2] and Wataru Marubashi[3]

[1]*Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan,* [2]*School of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan and* [3]*School of Agriculture, Meiji University, Kawasaki, Kanagawa 214-8571, Japan*

** For correspondence. E-mail tezuka@plant.osakafu-u.ac.jp*

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• *Background and Aims* Reproductive isolation is a mechanism that separates species, and is classified into two types: prezygotic and postzygotic. Inviability of hybrids, or hybrid lethality, is a type of postzygotic isolation and is observed in some plant species, including *Nicotiana* species. Previous work has shown that the Q chromosome, which belongs to the S subgenome of *N. tabacum*, encodes one or more genes leading to hybrid lethality in some crosses.

• *Methods* Interspecific crosses of eight wild species were conducted in section *Suaveolentes* (which consists of species restricted to Australasia and Africa) with the cultivated species *Nicotiana tabacum*. Hybrid seedlings were cultivated at 28, 34 or 36 °C, and PCR and chromosome analysis were performed.

• *Results and Conclusions* Seven of eight wild species produced inviable hybrids after crossing. Hybrid lethality, which was observed in all crosses at 28 °C, was Type II lethality, with the characteristic symptoms of browning of hypocotyl and roots; lethality was suppressed at elevated temperatures (34 or 36 °C). Furthermore, one or more genes on the Q chromosome of *N. tabacum* were absolutely responsible for hybrid lethality, suggesting that many species of section *Suaveolentes* share the same factor that triggers hybrid lethality by interaction with the genes on the Q chromosome. Exceptionally, only one wild species, *N. fragrans*, produced 100 % viable hybrids after crossing with *N. tabacum*, suggesting that *N. fragrans* has no factor triggering hybrid lethality.

Key words: Hybrid lethality, interspecific cross, *Nicotiana* section *Suaveolentes*, Q chromosome, reproductive isolation, tobacco.

INTRODUCTION

The genus *Nicotiana* includes 76 species classified into 13 sections (Knapp *et al.*, 2004). Many researchers have attempted to reveal the origin and evolution of this complex genus. In particular, the origin of cultivated tobacco, *Nicotiana tabacum*, has been extensively studied and it has been well characterized by studies based on interspecific crosses (Lim *et al.*, 2006), chloroplast and mitochondrial DNA (Gray *et al.*, 1974; Bland *et al.*, 1985), and chromosome painting (Lim *et al.*, 2000). Furthermore, phylogenetic studies based on analysis of internal transcribed spacer (ITS) regions of rDNA (Chase *et al.*, 2003), plastid genes (Aoki and Ito, 2000; Clarkson *et al.*, 2004) and nuclear-encoded chloroplast-expressed glutamine synthetase (ncpGS; Clarkson *et al.*, 2010) have been conducted using almost all species of the genus *Nicotiana*. Phylogenetic relationships have also been inferred by random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analyses (Ren and Timko, 2001; Khan and Narayan, 2007), genome size (Leitch *et al.*, 2008) and analysis of five low-copy nuclear genes other than ncpGS (Kelly *et al.*, 2010). Because information on phylogenetic relationships has been accumulated from various angles, the genus *Nicotiana* can act as a model to understand the evolution of species.

Species of the genus *Nicotiana* are distributed mainly in the Americas and Australia. Section *Suaveolentes* includes 25 species restricted to Australasia and one African species, *N. africana*, which is the only known *Nicotiana* species in Africa (Knapp *et al.*, 2004). These 26 species in section *Suaveolentes* are geographically isolated from the majority of species in other sections, most of which are distributed in the Americas. Recent studies based on ITS region, plastid genes and ncpGS have indicated that section *Suaveolentes* is a monophyletic group (Chase *et al.*, 2003; Clarkson *et al.*, 2004, 2010). However, the relationship among species of this section and its progenitors is less well understood and requires further explanation.

Reproductive isolation is a mechanism that separates species. It is considered to play a crucial role in the evolution of animals and plants. Reproductive isolation is divided into two types of barriers, namely prezygotic and postzygotic. In plants, a typical prezygotic barrier observed after pollination is pollen–pistil incongruity. Postzygotic barriers include seed abortion and, in F_1 or later generations, weakness, inviability and sterility. Inviability of hybrids, often referred to as hybrid lethality, is observed in some plant species, including *Nicotiana* species (Yamada *et al.*, 1999), rice (Ichitani *et al.*, 2007), wheat (Chu *et al.*, 2006) and *Arabidopsis thaliana* (Bomblies *et al.*, 2007). In the genus *Nicotiana*, hybrid lethality is classified into four types based on surface symptoms as follows: Type I, browning of shoot apex and root tip; Type II, browning of hypocotyl and roots; Type III, yellowing of true

leaves; and Type IV, formation of multiple shoots (Yamada *et al.*, 1999).

Nicotiana tabacum, which belongs to section *Nicotiana*, has two subgenomes, S and T, which are similar to the genomes of its progenitors, *N. sylvestris* and *N. tomentosiformis*, respectively (Lim *et al.*, 2000, 2006). According to the classical numbering system, each chromosome of *N. tabacum* is lettered alphabetically (A–Z, excluding X and Y); chromosomes A–L belong to the T subgenome and M–Z to the S subgenome. A complete set of 24 monosomic lines of *N. tabacum* (Haplo-A to Z) has been established in the genetic background of the cultivar 'Red Russian'. They are classified mainly based on morphological characteristics and their missing chromosome has been assigned to the S or T subgenome based on observation of chromosome pairing in hybrids ($2n - 1$) from crosses of monosomic lines with *N. sylvestris* (Clausen and Cameron, 1944; Cameron, 1959). Monosomic lines are useful for locating genes on specific chromosomes and these lines, especially Haplo-Q, have been used in research on hybrid lethality (Tezuka and Marubashi, 2006*a*; Tezuka *et al.*, 2007). Involvement of the Q chromosome in the S subgenome was also confirmed using progenitors of *N. tabacum* and Q-chromosome-specific DNA markers (Tezuka and Marubashi, 2006*a*).

Nicotiana suaveolens and *N. debneyi*, which both belong to section *Suaveolentes*, produce inviable hybrids after crosses with *N. tabacum*. Previously, using monosomic lines of *N. tabacum*, it was demonstrated that the Q chromosome, which belongs to the S subgenome of *N. tabacum*, encodes one or more genes leading to hybrid lethality in these crosses (Tezuka and Marubashi, 2006*a*; Tezuka *et al.*, 2007). In addition to these two species, other species in section *Suaveolentes*, *N. gossei*, *N. megalosiphon* and *N. africana*, are reported to yield inviable hybrids after crosses with *N. tabacum*; however, hybrid lethality was not described in any detail (Tanaka, 1961; Gerstel *et al.*, 1979). Only for *N. africana* is the H chromosome presumed to be responsible for hybrid lethality through crosses with monosomic lines of *N. tabacum* (Gerstel *et al.*, 1979), as described in the Discussion. In the present study, study of hybrid lethality in crosses with *N. tabacum* was extended to another eight species of section *Suaveolentes*.

MATERIALS AND METHODS

Plant materials

Eight wild species of section *Suaveolentes*, namely *Nicotiana africana* ($2n = 46$), *N. excelsior* ($2n = 38$), *N. fragrans* ($2n = 48$), *N. goodspeedii* ($2n = 40$), *N. gossei* ($2n = 36$), *N. maritima* ($2n = 32$), *N. megalosiphon* ($2n = 40$) and *N. velutina* ($2n = 32$), were used in this study. These species were crossed with *N. tabacum* ($2n = 48$, SSTT) 'Red Russian' and 'Samsun NN' in both directions. Two other species of section *Suaveolentes*, *N. suaveolens* ($2n = 32$) and *N. debneyi* ($2n = 48$), were also crossed with *N. tabacum* 'Red Russian' as the male parent to collect hybrid seeds used as controls. *N. tabacum* monosomic lines Haplo-Q ($2n = 47$) and F_1 progeny ($2n = 47$) derived from the cross Haplo-Q × *N. tabacum* 'Samsun NN', the latter identified by Q-chromosome-specific DNA markers (Tezuka *et al.*, 2004), were used as the female parent in crosses with the other seven species. Two viable hybrids from the cross *N. tabacum* 'Red Russian' × *N. debneyi* obtained in a previous study (Tezuka and Marubashi, 2006*b*) were used to identify the presence or absence of Q-chromosome-specific DNA markers. All plants were cultivated in a greenhouse, except for *N. africana*, which was cultivated in a phytotron (natural lighting conditions, 26 °C).

Interspecific crosses

Flowers of plants used as female parents were emasculated 1 d before anthesis and pollinated with pollen of plants used as male parents. F_1 seeds were soaked in 0·5 % gibberellic acid (GA_3) solution for 30 min and sterilized with 5 % sodium hypochlorite for 15 min. The sterilized seeds were sown in Petri dishes (60 mm diameter, 17 mm deep) containing 8 mL of half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1 % sucrose and 0·2 % Gelrite (pH 5·8), and were cultured at 28 °C under continuous illumination (approx. 150 μmol m^{-2} s^{-1}).

Ovule culture was carried out as follows. Flowers of female parents were collected at 7–10 d after pollination (DAP) and their corolla, sepals and styles were removed. The ovaries were surface-sterilized with 70 % ethanol for 30 s and with 5 % sodium hypochlorite for 5 min. The ovary walls were peeled to expose the placentas with intact ovules. Fertilized and enlarged ovules were excised, placed in Petri dishes containing 8 mL of half-strength MS medium supplemented with 3 % sucrose and 0·8 % agar (pH 5·8), and cultured at 28 °C under continuous illumination.

Test-tube pollination in combination with ovule culture was carried out as previously described (Tezuka and Marubashi, 2004). Fertilized and enlarged ovules at 10–14 DAP were excised from placentas and cultured as described above.

Cultivation of hybrid seedlings

Hybrid seedlings were cultured at 28 °C under continuous illumination. Some seedlings were transferred to flat-bottomed test tubes (25 mm diameter, 100 mm length) that contained 10 mL of half-strength MS medium supplemented with 1 % sucrose and 0·2 % Gelrite (pH 5·8) immediately after germination and were cultured at 28, 34 or 36 °C under continuous illumination. Hybrid seedlings cultured at 34 or 36 °C for 30 d after germination (DAG) were transferred to 28 °C under continuous illumination. Hybrid seedlings surviving for more than 30 d after transfer were potted and cultivated in a greenhouse under natural lighting conditions.

PCR analysis

Total DNA was extracted from leaves of each plant using a cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Q-chromosome-specific DNA markers, OPB-07_{870}, OPB-13_{1400}, QCS1, QCS2, QCS3 and QCS4, were detected as previously described (Tezuka *et al.*, 2004; Tezuka and Marubashi, 2006*a*). RAPD analysis was carried out using 20 random 10-mer oligonucleotide primers

(Kit A; Operon Technologies, Inc., Alameda, CA, USA) as previously described (Tezuka *et al.*, 2007).

Chromosome analysis

Root tips were pretreated with distilled water for 24 h at 4 °C and with 2 mM 8-hydroxyquinoline for 4 h at 18 °C, then fixed in ethanol/acetic acid (3 : 1) overnight to determine chromosome numbers. The root tips were hydrolysed in 1 M HCl for 8 min at 60 °C, stained with Schiff's reagent and squashed in 45 % acetic acid. The number of chromosomes in at least five root tip cells for each plant was counted under a light microscope (Eclipse E600; Nikon, Tokyo, Japan).

RESULTS

Production of hybrid seedlings between N. tabacum *and eight species of section* Suaveolentes

Reciprocal crosses were carried out between *N. tabacum* and eight species of section *Suaveolentes* using conventional cross-pollination. Two cultivars of *N. tabacum*, 'Red Russian' and 'Samsun NN', were used for the crosses. The results of the crosses are shown in Table 1. In general, conventional crossing was more successful in crosses using *N. tabacum* as the male parent than crosses in the opposite direction; i.e. good seeds that could germinate were obtained when six species, *N. excelsior*, *N. goodspeedii*, *N. gossei*, *N. maritima*, *N. megalosiphon* and *N. velutina*, were pollinated by *N. tabacum*. Conversely, flowers of *N. tabacum* dropped at about 7 DAP and seeds were never obtained when pollinated by *N. goodspeedii*, *N. maritima* or *N. velutina*. This suggests that fertilization did not occur in these crosses. When *N. excelsior*, *N. gossei* or *N. megalosiphon* were used as the male parent in crosses with *N. tabacum*, many *N. tabacum* flowers dropped at about 7 DAP, but some flowers produced a few capsules containing seeds. These seeds germinated poorly, suggesting that there was some kind of postzygotic barrier during seed development as well as prezygotic barriers preventing fertilization. Conventional crosses using two other species, *N. africana* and *N. fragrans*, showed different tendencies from the crosses mentioned above for the other six species of section *Suaveolentes*. Seeds could be obtained with comparative ease and the percentage of seed germination was relatively high in reciprocal crosses between *N. africana* and *N. tabacum*. In crosses using *N. fragrans*, seeds could be obtained in all cross combinations except the cross using 'Red Russian' as the female parent. Only hybrid seeds from the cross 'Samsun NN' × *N. fragrans* germinated.

Ovule culture is effective in bypassing ovule abortion in some interspecific crosses (Reed and Collins, 1978; Chung *et al.*, 1988). Ovule culture was thus carried out in two crosses that yielded few hybrid seedlings from seeds obtained through conventional crossing, i.e. *N. tabacum* × *N. excelsior* and *N. tabacum* × *N. megalosiphon*. When conventional crossing and crosses using ovule culture were compared, the

TABLE 1. *Conventional crossing between* N. tabacum *and eight species of section* Suaveolentes

Cross combination	No. of flowers pollinated	No. of capsules obtained	No. of seeds sown	No. of hybrids obtained
N. africana × 'Red Russian'	8	4	161	134
'Red Russian' × *N. africana*	20	10	200	141
N. africana × 'Samsun NN'	3	3	120	69
'Samsun NN' × *N. africana*	9	7	118	103
N. excelsior × 'Red Russian'	10	6	140	123
'Red Russian' × *N. excelsior*	14	3	120	2
N. excelsior × 'Samsun NN'	5	4	120	108
'Samsun NN' × *N. excelsior*	4	4	119	0
N. fragrans × 'Red Russian'	2	2	119	0
'Red Russian' × *N. fragrans*	9	0	–	–
N. fragrans × 'Samsun NN'	1	1	120	0
'Samsun NN' × *N. fragrans*	7	5	120	100
N. goodspeedii × 'Red Russian'	20	19	198	168
'Red Russian' × *N. goodspeedii*	20	0	–	–
N. goodspeedii × 'Samsun NN'	20	19	116	113
'Samsun NN' × *N. goodspeedii*	20	0	–	–
N. gossei × 'Red Russian'	20	20	199	196
'Red Russian' × *N. gossei*	27	2	198	6
N. gossei × 'Samsun NN'	21	15	115	93
'Samsun NN' × *N. gossei*	20	1	120	26
N. maritima × 'Red Russian'	8	6	150	142
'Red Russian' × *N. maritima*	20	0	–	–
N. maritima × 'Samsun NN'	6	6	120	117
'Samsun NN' × *N. maritima*	20	0	–	–
N. megalosiphon × 'Red Russian'	20	20	195	176
'Red Russian' × *N. megalosiphon*	20	3	313	0
N. megalosiphon × 'Samsun NN'	20	17	119	84
'Samsun NN' × *N. megalosiphon*	9	6	117	2
N. velutina × 'Red Russian'	20	17	159	123
'Red Russian' × *N. velutina*	20	0	–	–
N. velutina × 'Samsun NN'	11	10	120	109
'Samsun NN' × *N. velutina*	20	0	–	–

percentage of total hybrid seedlings obtained from the cultured seeds or ovules increased from the 0·8 % of conventional crossing to 3·5 % in the cross *N. tabacum* × *N. excelsior* (Tables 1 and 2). Ovule culture also improved the results from the cross 'Red Russian' × *N. megalosiphon*, for which no hybrid seedlings were obtained through conventional crossing.

Hybrid lethality is observed in most crosses between N. tabacum *and species of section* Suaveolentes

Hybrid seedlings obtained from crosses between *N. tabacum* and species of section *Suaveolentes* died at 28 °C, except from crosses with *N. fragrans* (Table 3). The day of appearance of first symptoms and of death varied depending on the parental species and cultivars used for the cross. Characteristic symptoms of hybrid lethality – obvious browning of hypocotyl and roots – were observed in all crosses using seven species of section *Suaveolentes* (Table 3, Fig. 1). These symptoms were identical to those in crosses between *N. suaveolens* or *N. debneyi* and *N. tabacum*, indicating that hybrid lethality in crosses between *N. tabacum* and these seven species of section *Suaveolentes* is of Type II.

Among the eight tested species of section *Suaveolentes*, only *N. fragrans* yielded 100 % viable hybrid seedlings in the cross with *N. tabacum*. All hybrid seedlings from the

TABLE 2. *Production of hybrid seedlings between* N. tabacum *and two species of section* Suaveolentes *through ovule culture*

Cross combination	Days after pollination	No. of ovaries used	No. of ovules cultured	No. of hybrids obtained
'Red Russian' × *N. excelsior*	7	1	489	6
	8	1	362	12
	9	1	512	34
	10	1	422	17
'Samsun NN' × *N. excelsior*	7	1	724	2
	8	1	758	16
	9	1	387	42
	10	1	888	28
'Red Russian' × *N. megalosiphon*	7	1	504	1
	8	1	438	1
	9	1	322	1
	10	1	418	6
'Samsun NN' × *N. megalosiphon*	7	1	358	0
	8	1	944	2
	9	1	803	3
	10	2	1413	7

TABLE 3. *Viability of hybrid seedlings between* N. tabacum *and eight species of section* Suaveolentes *at 28 °C*

Cross combination	No. of hybrids cultured	No. of hybrids		Lethality type*
		Viable	Inviable	
N. africana × 'Red Russian'	114	0	114	II
'Red Russian' × *N. africana*	115	0	115	II
N. africana × 'Samsun NN'	69	1	68	II
'Samsun NN' × *N. africana*	103	0	103	II
N. excelsior × 'Red Russian'	113	0	113	II
'Red Russian' × *N. excelsior*	61	0	61	II
N. excelsior × 'Samsun NN'	108	0	108	II
'Samsun NN' × *N. excelsior*	78	0	78	II
'Samsun NN' × *N. fragrans*	100	100	0	–
N. goodspeedii × 'Red Russian'	158	0	158	II
N. goodspeedii × 'Samsun NN'	113	0	113	II
N. gossei × 'Red Russian'	186	0	186	II
'Red Russian' × *N. gossei*	3	0	3	II
N. gossei × 'Samsun NN'	93	0	93	II
'Samsun NN' × *N. gossei*	20	0	20	II
N. maritima × 'Red Russian'	132	0	132	II
N. maritima × 'Samsun NN'	117	0	117	II
N. megalosiphon × 'Red Russian'	166	1	165	II
'Red Russian' × *N. megalosiphon*	4	0	4	II
N. megalosiphon × 'Samsun NN'	84	0	84	II
'Samsun NN' × *N. megalosiphon*	6	0	6	II
N. velutina × 'Red Russian'	113	0	113	II
N. velutina × 'Samsun NN'	109	0	109	II

*Type I, browning of shoot apex and root tip; Type II, browning of hypocotyl and roots; Type III, yellowing of true leaves; Type IV, formation of multiple shoots (Yamada *et al.*, 1999).

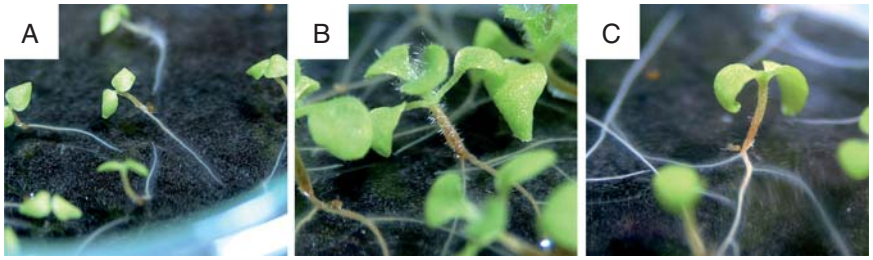

FIG. 1. Observation of the characteristic early symptoms of hybrid lethality, browning of hypocotyl and roots, in hybrid seedlings at 28 °C. Hybrid seedlings between each species of section *Suaveolentes* and *N. tabacum* 'Red Russian' were photographed. (A) Hybrids of *N. africana* at 5 DAG; (B) hybrids of *N. excelsior* at 10 DAG; (C) hybrids of *N. maritima* at 10 DAG.

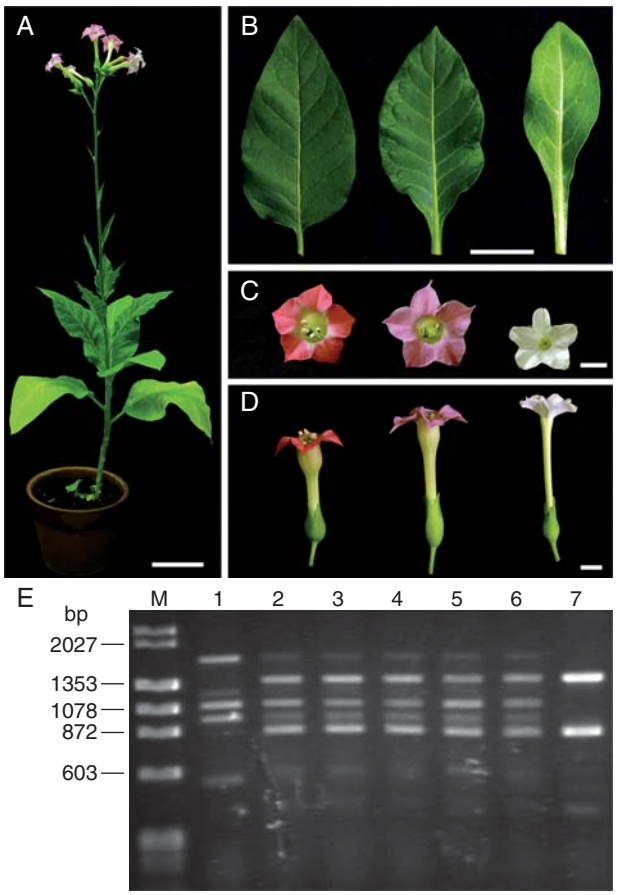

FIG. 2. Hybrids from the cross *N. tabacum* 'Samsun NN' × *N. fragrans*. (A) Shape of a hybrid plant that has grown to maturity and flowered. (B) Leaves of 'Samsun NN', a hybrid plant and *N. fragrans* (left to right). (C, D) Flowers of 'Samsun NN', a hybrid plant and *N. fragrans* (left to right). Scale bars = 5 cm (A, B) and 1 cm (C, D). (E) Confirmation of hybrid formation by RAPD analysis. M, DNA markers (λ/*Hin*d III and ϕX174/*Hae* III). Lane 1, 'Samsun NN'; lanes 2–6, hybrid plants; lane 7, *N. fragrans*.

cross *N. tabacum* × *N. fragrans* were still viable at 30 DAG at 28 °C (Table 3). Twenty hybrid seedlings were selected at random and cultivated in a greenhouse. These seedlings grew to maturity and flowered (Fig. 2A). The morphological characteristics of hybrid plants were uniform. The leaf shape and flower shape of hybrid plants were intermediate in appearance between those of parents (Fig. 2B–D). Five hybrid plants were analysed and all had 48 chromosomes, which is the sum of the number of haploid chromosomes of the parents. RAPD analysis was carried out with 20 random primers on five hybrid plants to confirm that these plants were true hybrids. All 20 random primers gave RAPD patterns showing clear polymorphisms between the parents; 32 bands were detected only in 'Samsun NN' and 41 bands were detected only in *N. fragrans*. Hybrid plants had all 73 bands characteristic of both parents, indicating that they were true hybrids. RAPD patterns obtained with the primer OPA-19 are shown in Fig. 2E. Hybrid plants from the cross *N. tabacum* × *N. fragrans* were self-sterile and cross-sterile with their parents.

Hybrid lethality is suppressed at elevated temperatures

Hybrid lethality in crosses of three species, *N. debneyi*, *N. suaveolens* and *N. gossei*, with *N. tabacum* was reported to be suppressed at 34, 36 and 37 °C, respectively (Mino *et al.*, 2002; Yamada and Marubashi, 2003; Tezuka *et al.*, 2007). Whether hybrid lethality in crosses between *N. tabacum* and the seven species of section *Suaveolentes*, *N. africana*, *N. excelsior*, *N. goodspeedii*, *N. gossei*, *N. maritima*, *N. megalosiphon* and *N. velutina*, is also suppressed at elevated temperatures was assessed. Hybrid lethality in all crosses was completely suppressed at 34 °C for 30 DAG except in crosses using *N. africana* as a parent (Table 4). Hybrid seedlings from reciprocal crosses between *N. africana* and *N. tabacum* did not die but the base of the stem frequently turned brown at 34 °C at 30 DAG. Hybrid lethality in these crosses was completely suppressed for 30 DAG by culture at 36 °C (Table 4).

Hybrid seedlings cultured at 34 or 36 °C were transferred to 28 °C to confirm that hybrid lethality was induced at 28 °C. Hybrid seedlings from all crosses died after transfer (Table 4).

Production of hybrid seedlings between N. tabacum *monosomic lines lacking the Q chromosome and seven species of section* Suaveolentes

Next, two monosomic lines of *N. tabacum* lacking the Q chromosome, namely Haplo-Q and F_1 progeny derived from the cross Haplo-Q × *N. tabacum* 'Samsun NN' (Tezuka *et al.*, 2004), were used for crosses with seven species of section *Suaveolentes*, *N. africana*, *N. excelsior*, *N. goodspeedii*, *N. gossei*, *N. maritima*, *N. megalosiphon* and *N. velutina*. Monosomic lines were used as female parents

TABLE 4. *Effects of elevated temperatures on hybrid lethality observed in hybrid seedlings between* N. tabacum *and seven species of section* Suaveolentes

Cross combination	Temperature (°C)	No. of hybrids cultured	No. of hybrids showing lethal symptoms	
			By 30 DAG	After transfer to 28 °C*
N. africana × 'Red Russian'	34	10	6	4
	36	10	0	10
'Red Russian' × *N. africana*	34	16	16	–
	36	10	0	10
N. excelsior × 'Red Russian'	34	10	0	10
'Red Russian' × *N. excelsior*	34	10	0	10
'Samsun NN' × *N. excelsior*	34	10	0	10
N. goodspeedii × 'Red Russian'	34	10	0	10
N. gossei × 'Red Russian'	34	10	0	10
'Red Russian' × *N. gossei*	34	3	0	3
'Samsun NN' × *N. gossei*	34	6	0	6
N. maritima × 'Red Russian'	34	10	0	10
N. megalosiphon × 'Red Russian'	34	10	0	10
'Red Russian' × *N. megalosiphon*	34	5	0	5
'Samsun NN' × *N. megalosiphon*	34	8	0	8
N. velutina × 'Red Russian'	34	10	0	10

*Hybrid seedlings that did not show lethal symptoms at 30 DAG were transferred from the elevated temperature to 28 °C.

for the crosses, as pollen of Haplo-Q aborts at a high frequency (Cameron, 1959) and the transmission of the monosomic condition through pollen is very low (Olmo, 1935). Methods to produce hybrid seedlings were determined based on results of crosses using disomic *N. tabacum* (Tables 1 and 2). Conventional crossing was conducted for crosses using *N. africana* or *N. gossei*. Ovule culture was carried out after conventional crossing using *N. excelsior* or *N. megalosiphon*. For *N. goodspeedii*, *N. maritima* and *N. velutina*, as mentioned above, fertilization did not occur on pollination by *N. tabacum*. In such cases, hybrid seedlings could be obtained by test-tube pollination in combination with ovule culture (Tezuka and Marubashi, 2004; Tezuka *et al.*, 2007). Thus, test-tube pollination and ovule culture were carried out in crosses of *N. tabacum* to these three species. As a result, hybrid seedlings between monosomic lines of *N. tabacum* and all seven species of section *Suaveolentes* were obtained (Tables 5–7). Hybrid seedlings were cultured at 34 or 36 °C, temperatures at which hybrid lethality was suppressed (Table 4).

The Q chromosome encodes gene(s) leading to hybrid lethality in all crosses between N. tabacum *and seven species of section* Suaveolentes

Two Q-chromosome-specific DNA markers, QCS2 and QCS3 (Tezuka *et al.*, 2004; Tezuka and Marubashi, 2006*a*), were used to determine whether the Q chromosome was present in hybrid seedlings between monosomic lines of *N. tabacum* and seven species of section *Suaveolentes*. Hybrid seedlings from respective crosses were divided into two types, hybrids possessing the Q chromosome and those lacking the Q chromosome, as summarized in Table 8. Some ovules yielded primary roots without shoots. These hybrids were excluded from analysis as genomic DNA could not be isolated.

If the Q chromosome is responsible for hybrid lethality, hybrid seedlings possessing the Q chromosome will die and those lacking the Q chromosome will live at 28 °C. To verify this assumption, hybrid seedlings cultured at 34 or 36 °C were transferred to 28 °C. In all crosses, hybrid seedlings possessing the Q chromosome died and those lacking the Q chromosome survived (Table 8). The hybrid seedlings lacking the Q chromosome grew to maturity and flowered without symptoms of hybrid lethality when they were potted and cultivated in a greenhouse. Hybrid plants were morphologically uniform within each cross combination and were intermediate between those of the parents.

TABLE 5. *Production of hybrid seedlings in conventional crosses between monosomic lines of* N. tabacum *lacking the Q chromosome and two species of section* Suaveolentes

Cross combination	No. of flowers pollinated	No. of capsules obtained	No. of seeds sown	No. of hybrids obtained
Haplo-Q × *N. africana*	7	3	80	72
(Haplo-Q × 'Samsun NN') × *N. africana*	1	1	80	72
Haplo-Q × *N. gossei*	18	4	259	12
(Haplo-Q × 'Samsun NN') × *N. gossei*	27	21	196	30

Analysis of rare viable hybrids obtained from crosses with disomic N. tabacum

One of 68 hybrid seedlings from the cross *N. africana* × *N. tabacum* 'Samsun NN' and one of 166 hybrid seedlings from the cross *N. megalosiphon* × *N. tabacum* 'Red Russian' were viable at 28 °C (Table 3). When these two viable hybrids were potted and cultivated in a greenhouse, they grew to maturity and flowered. Hybrid plants were morphologically intermediate between those of the parents. The hybrid plant from the cross *N. africana* × *N. tabacum* had

TABLE 6. *Production of hybrid seedlings between monosomic lines of* N. tabacum *lacking the Q chromosome and two species of section* Suaveolentes *by ovule culture*

Cross combination	Days after pollination	No. of ovaries used	No. of ovules cultured	No. of hybrids obtained
(Haplo-Q × 'Samsun NN') × *N. excelsior*	8	3	253	27
	9	1	59	16
(Haplo-Q × 'Samsun NN') × *N. megalosiphon*	7	1	605	23
	8	2	922	19
	9	1	898	54
	10	2	795	47

TABLE 7. *Production of hybrid seedlings between monosomic lines of* N. tabacum *lacking the Q chromosome and three species of section* Suaveolentes *by test-tube pollination and ovule culture*

Cross combination	No. of placentas pollinated	No. of ovules cultured	No. of hybrids obtained
(Haplo-Q × 'Samsun NN') × *N. goodspeedii*	14	565	37
(Haplo-Q × 'Samsun NN') × *N. maritima*	7	423	32
(Haplo-Q × 'Samsun NN') × *N. velutina*	48	576	8

TABLE 8. *Relationship between the Q chromosome and hybrid lethality observed in crosses between monosomic lines of* N. tabacum *lacking the Q chromosome and seven species of section* Suaveolentes

Cross combination	DNA markers*	No. of hybrids		
		Total	Viable	Inviable
Haplo-Q × *N. africana*	+	16	0	16
	−	56	56	0
(Haplo-Q × 'Samsun NN') × *N. africana*	+	13	0	13
	−	59	59	0
(Haplo-Q × 'Samsun NN') × *N. excelsior*	+	5	0	5
	−	19	19	0
(Haplo-Q × 'Samsun NN') × *N. goodspeedii*	+	12	0	12
	−	18	18	0
Haplo-Q × *N. gossei*	+	4	0	4
	−	8	8	0
(Haplo-Q × 'Samsun NN') × *N. gossei*	+	2	0	2
	−	28	28	0
(Haplo-Q × 'Samsun NN') × *N. maritima*	+	10	0	10
	−	21	21	0
(Haplo-Q × 'Samsun NN') × *N. megalosiphon*	+	7	0	7
	−	125	125	0
(Haplo-Q × 'Samsun NN') × *N. velutina*	+	3	0	3
	−	5	5	0

*DNA markers used were QCS2 and QCS3 (Tezuka and Marubashi, 2004, 2006*a*). A '+' indicates that Q-chromosome-specific DNA markers were detected and a '−' indicates that they were not.

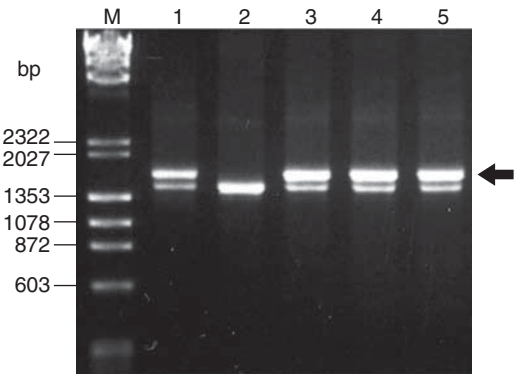

FIG. 3. Detection of the marker QCS2 in viable hybrid seedlings from crosses between wild species of section *Suaveolentes* and *N. tabacum*. The marker QCS2, indicated by an arrow, was not detected in hybrid seedlings from the cross *N. africana* × *N. tabacum*. M, DNA markers (λ/*Hin*d III and ϕX174/*Hae* III). Lane 1, *N. tabacum* 'Red Russian'; lane 2, hybrid seedling from the cross *N. africana* × *N. tabacum* 'Samsun NN'; lane 3, hybrid seedling from the cross *N. megalosiphon* × *N. tabacum* 'Red Russian'; lanes 4, 5, hybrid seedlings from the cross *N. tabacum* 'Red Russian' × *N. debneyi* (Tezuka and Marubashi, 2006*b*).

46 chromosomes, which is less than the 47 chromosomes expected for a true hybrid. The hybrid plant from the cross *N. megalosiphon* × *N. tabacum* had 44 chromosomes, which is the sum of the number of haploid chromosomes of the parents.

Whether the Q chromosome was present in the hybrid plant from the cross *N. africana* × *N. tabacum* and from the cross *N. megalosiphon* × *N. tabacum* was investigated. In addition, two viable hybrids from the cross *N. tabacum* 'Red Russian' × *N. debneyi* (Tezuka and Marubashi, 2006*b*), for which hybrid seedlings are usually inviable, were investigated. When six Q-chromosome-specific DNA markers, OPB-07_{870}, OPB-13_{1400}, QCS1, QCS2, QCS3 and QCS4, were tested, all markers were detected in hybrid plants from crosses *N. megalosiphon* × *N. tabacum* and *N. tabacum* × *N. debneyi*. Conversely, none of the markers was detected in the hybrid plant from the cross *N. africana* × *N. tabacum* (Fig. 3).

DISCUSSION

Among the eight species of section *Suaveolentes* used in this study, seven yielded inviable hybrids after crosses with *N. tabacum*. In reciprocal crosses of *N. tabacum* with four species, *N. africana*, *N. excelsior*, *N. gossei* and *N. megalosiphon*, inviable hybrids could be obtained in both

directions through conventional crossing or ovule culture. Conversely, in crosses of *N. tabacum* with *N. goodspeedii*, *N. maritima* or *N. velutina*, inviable hybrids were obtained when these wild species were used as the female parent, whereas no hybrids were obtained in crosses using them as the male parent. However, we confirmed that hybrid lethality was observed in hybrids between *N. tabacum* and these three wild species through crosses using monosomic lines of *N. tabacum* lacking the Q chromosome. Hybrid lethality was observed in reciprocal crosses between these seven wild species and *N. tabacum*, suggesting that hybrid lethality is due to the interaction of coexisting heterologous genomes, and not to a cytoplasmic effect.

Hybrid lethality observed in crosses between *N. tabacum* and seven species of section *Suaveolentes* was of Type II, for which characteristic symptoms are browning of hypocotyl and roots. The present observation that hybrid lethality in these crosses was suppressed at 34 or 36 °C supported the suggestion that Type II lethality in all crosses might be suppressed at elevated temperatures by Yamada *et al.* (1999). In the present study, hybrid lethality in reciprocal crosses between *N. tabacum* and *N. africana* was not completely suppressed at 34 °C, whereas hybrid lethality in other crosses was completely suppressed at this temperature. Although the mechanism of suppression of hybrid lethality at elevated temperatures is not clear, it may be common for each cross. It is interesting that more elevated temperatures than 34 °C were required to suppress hybrid lethality only in crosses using *N. africana* as the parent. Hamada and Inoue (1999) also reported that hybrid lethality in the cross between *N. tabacum* and *N. africana* was suppressed at 36 °C but not at 32 °C.

In crosses between monosomic lines of *N. tabacum* lacking the Q chromosome and seven species of section *Suaveolentes*, hybrid seedlings possessing the Q chromosome were inviable and those lacking the Q chromosome were viable at 28 °C. Therefore, we concluded that the Q chromosome, which belongs to the S subgenome in *N. tabacum*, encodes gene(s) leading to hybrid lethality in crosses with seven species of section *Suaveolentes*. This is the same conclusion as that arrived at in crosses between *N. tabacum* and two species of section *Suaveolentes*, *N. suaveolens* and *N. debneyi* (Tezuka and Marubashi, 2006*a*; Tezuka *et al.*, 2007). For *N. africana*, however, other interesting results were reported; when *N. africana* was crossed with all 24 monosomic lines of *N. tabacum* (Haplo-A to Z), only Haplo-H produced a great number of viable hybrids (Gerstel *et al.*, 1979). Based on these results, the H chromosome, which belongs to the T subgenome in *N. tabacum*, is considered to be related to hybrid lethality. As it was not clear whether the viable hybrids from the cross Haplo-H × *N. africana* lacked the H chromosome in their study, further studies will be needed to verify H chromosome involvement in hybrid lethality.

Programmed cell death (PCD) accompanied by apoptotic features is observed in Type II lethality in crosses of *N. suaveolens* or *N. debneyi* with *N. tabacum* (Yamada *et al.*, 2000; Marubashi and Kobayashi, 2002; Tezuka and Marubashi, 2004, 2006*b*). In these crosses, gene(s) on the Q chromosome cause hybrid lethality (Tezuka and Marubashi, 2006*a*; Tezuka *et al.*, 2007). Hybrid lethality observed in crosses between seven species of section *Suaveolentes* and *N. tabacum* was also Type II and was caused by the interaction of one or more genes on the Q chromosome and some factor in wild species. Considering the common points, hybrid lethality observed in crosses using the seven section *Suaveolentes* species would also be expected to involve PCD accompanied by apoptotic features. However, a different type of PCD is observed in hybrid lethality in the cross *N. gossei* × *N. tabacum* (Mino *et al.*, 2005). PCD in this cross is accompanied by vacuolar collapse and apoptotic features are never observed. Therefore, these observations suggest that, in hybrid lethality caused by the interaction of a gene on the Q chromosome and an interacting factor in wild species, (1) both PCD pathways are present within a cross combination or (2) a particular type of PCD is involved depending on the cross combination.

Section *Suaveolentes* is a monophyletic group in analyses of the ITS region, plastid genes and ncpGS (Chase *et al.*, 2003; Clarkson *et al.*, 2004, 2010). This section is considered to have originated from a single polyploid event some 10 Mya, followed by speciation to produce the species known today (Leitch *et al.*, 2008). Furthermore, two progenitors of the section are proposed: *N. sylvestris* would be the maternal progenitor and section *Trigonophyllae* would be the paternal progenitor (Clarkson *et al.*, 2010). However, the data obtained from these analyses were insufficient to reveal the relationship among species of section *Suaveolentes* and its progenitors, as incongruously genomic DNA of *N. sylvestris* failed to show hybridization with species from section *Suaveolentes* in genomic *in situ* hybridization experiments (Chase *et al.*, 2003). Hybrid lethality, a type of reproductive isolation, might be useful for the classification of section *Suaveolentes* species. Based on the results of the present study and previous studies (Tezuka and Marubashi, 2006*a*; Tezuka *et al.*, 2007), ten species of section *Suaveolentes* can be divided into two groups: species yielding inviable hybrids and species yielding viable hybrids on crosses with *N. tabacum*. The former group consists of nine species: *N. africana*, *N. debneyi*, *N. excelsior*, *N. goodspeedii*, *N. gossei*, *N. maritima*, *N. megalosiphon*, *N. suaveolens* and *N. velutina*. This group may be further divided into two subgroups based on type of PCD observed, as mentioned above. The latter group consists of only one species, *N. fragrans*, which is distributed in islands of the South Pacific. *N. fragrans* is geographically isolated from Australian species and is extremely far from *N. suaveolens*, which approximates the morphological mean of section *Suaveolentes* species (Wheeler, 1945).

It is interesting how and why only *N. fragrans* yields 100 % viable hybrids in crosses with *N. tabacum*. F_1 hybrids or allopolyploids with genomes from more than one species present the nucleus with a totally different situation. This can result in an irreversible burst of reorganization and modification of the genomes involved, leading to stability of F_1 hybrids or allopolyploids (Jones and Hegarty, 2009). Because both *N. fragrans* and *N. tabacum* have 48 chromosomes and are polyploids, such reorganization and modification might be related to the production of 100 % viable hybrids. However, *N. debneyi*, which also has 48 chromosomes, yielded inviable hybrids after crosses with *N. tabacum* (Marubashi and Kobayashi, 2002; Tezuka and Marubashi, 2006*b*; Tezuka *et al.*, 2007). Alternatively, *N. fragrans* may not possess any

factor triggering hybrid lethality by interaction with gene(s) on the Q chromosome. Considering that the common factor triggering hybrid lethality is widely distributed in species of section *Suaveolentes* and the presumed single origin of the section as mentioned above, an ancestral species involved in the formation of the section might already have had the factor triggering hybrid lethality. The factor might have been lost through speciation, probably including reorganization and modification of the genomes, leading to the formation of *N. fragrans*. We are currently carrying out studies on hybrid lethality using species in sections other than *Suaveolentes*. These studies will provide additional results to clarify how section *Suaveolentes* evolved.

Many hybrid seedlings lacking the Q chromosome were obtained compared with those possessing the Q chromosome in certain crosses between monosomic lines of *N. tabacum* and species of section *Suaveolentes*. For example, seven hybrids possessed the Q chromosome and 125 hybrids lacked the Q chromosome in crosses using *N. megalosiphon*. A small number of hybrids were obtained from crosses between *N. tabacum* disomic and *N. megalosiphon* through conventional crossing or ovule culture. These results suggest that hybrid lethality observed in seedlings is also likely to be involved in embryonic development after fertilization. However, this idea is not applicable to crosses using *N. africana*. In the cross *N. tabacum* × *N. africana*, hybrid seeds germinated well and many hybrid seedlings were obtained. Because these hybrid seedlings were considered to have the Q chromosome, hybrid lethality is quite unlikely to be involved in embryonic development in this case. Therefore, it seems that $n - 1$ gametes lacking the Q chromosome are more likely to be fertilized than n gametes in this interspecific cross using monosomic lines of *N. tabacum* lacking the Q chromosome.

A few viable hybrids are occasionally obtained from several hundred hybrid seedlings that are usually inviable in some interspecific crosses in the genus *Nicotiana* (Tezuka and Marubashi, 2006*b*). A viable hybrid from the cross *N. africana* × *N. tabacum* and another from the cross *N. megalosiphon* × *N. tabacum* are apparent examples of such infrequent viable hybrids. Previously, it was shown that PCD related to hybrid lethality does not occur in viable hybrids of *N. tabacum* × *N. debneyi* (Tezuka and Marubashi, 2006*b*). However, the reason why PCD does not occur in viable hybrids is still unclear. The present study has shown that a viable hybrid from the cross *N. africana* × *N. tabacum* lacked the Q chromosome. Therefore, deletion of the chromosome encoding genes leading to hybrid lethality contributes to some of the cases in which a few viable hybrids are occasionally obtained.

Climate change, including climate warming, can affect ecosystems (Thomas *et al.*, 2004). As revealed in our present and previous studies (Yamada *et al.*, 1999; Marubashi and Kobayashi, 2002), hybrid lethality in interspecific crosses in the genus *Nicotiana* is temperature sensitive. In other plant taxa such as *Gossypium* (Phillips, 1977) and *Arabidopsis thaliana* (Bomblies *et al.*, 2007), hybrid lethality could be suppressed at higher temperatures than typical temperatures for their growth. Therefore, climate warming could produce hybrid-lethality-suppressed hybrids from species now separated by hybrid lethality. If such hybrids produce progeny, they may ultimately give rise to new species. Special attention might be needed for changes in ecosystems triggering such a mechanism breaking down hybrid lethality.

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