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Genomic factors controlling the lethality exhibited in the hybrid between *Nicotiana suaveolens* Lehm. and *N. tabacum* L.

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Abstract Interspecific hybrid plants between *Nicotiana suaveolens* and *N. tabacum* exhibit lethal symptoms at the seedling stage and cannot grow to maturity. In this investigation, an attempt was made to clarify the genomic factors responsible for this lethality. *N. suaveolens* was crossed to *N. sylvestris* (genomic constitution: SS) and *N. tomentosiformis* (TT), these latter two species being the progenitors of *N. tabacum* (SSTT). From the cross *N. suaveolens* × *N. tomentosiformis*, many seedlings were obtained through ovule culture, and these subsequently grew to maturity without exhibiting any lethality. In the reciprocal crossing between *N. sauvelons* and *N. sylvestris*, only a few hybrid seedlings were obtained through ovlue culture and all died after unfolding their cotyledons when cultured at 28 °C. This lethality could be avoided by culturing the ovules at 36 °C. These features of hybrid lethality resembled those observed in the interspecific hybrid between *N. suaveolens* and *N. tabacum*. These findings suggest that the S genome in *N. tabacum* is responsible for the lethality exhibited in the hybrid between *N. suaveolens* and *N. tabacum*.

Key words Interspecific hybrid · Lethality · Genomic factor · Progenitor

Introduction

Hybrid lethality, which has been described in some crops (Oka 1957; Stebbins 1958; Zeven 1981), restricts the utilization of wide hybridization in breeding programs. Kostoff (1930) observed hybrid lethality in some interspecific cross combinations in the genus *Nicotiana*, including the cross between *N. suaveolens* and *N. tabacum*. When *N. suaveolens* was used as the maternal parent, the hybrid seedlings developed browning at the hypocotyl and died at the cotyledonary stage even through the F_1 seeds had been easily obtained and germinated well (Lloyd 1975). The same lethal symptoms were observed on the hybrid derived from the reciprocal crossing, for which test-tube pollination was necessary to obtain the hybrids (Marubashi et al. 1988a).

Some investigators have developed effective methods to overcome the lethality exhibited in the hybrid between *N. suaveolens* and *N. tabacum* (Lloyd 1975; Marubashi et al. 1988b; Inoue et al. 1994). Lloyd (1975) cultured cotyledonary segments of the hybrid seedlings on media containing plant growth regulators and obtained viable hybrid plants that originated from the cotyledonary segments through the calli. Marubashi et al. (1988b) reported that hybrids can grow without developing any lethal symptoms at high temperature conditions (36 °C). In a recent report we described that the application of cytokinin effected the overcoming of this hybrid lethality (Inoue et al. 1994).

Although some of the physiological properties of this lethality have been investigated, its genetical basis has yet to be clarified. *N. tabacum* is an amphidiploid species [genomic constitution = SSTT] whose progenitors are *N. sylvestris* Speg. and Comes. [SS] and *N. tomentosiformis* Goodsp. [TT] (Sheen 1972; Okamuro and Goldberg 1985; Buuren et al. 1992). In the investigation reported here, we used these progenitors for conducting test crosses. We carried out reciprocal crossing between *N. suaveolens* and two progenitors of *N. tabacum* to investigate which of the genomes (S and T) was responsible for the lethality exhibited in the hybrid between *N. suaveolens* and *N. tabacum*.

Materials and methods

Plant materials

N. suaveolens (2n = 32), *N. sylvestris* (2n = 24) and *N. tomentosiformis* (2n = 24) were used in this experiment. These plants were derived from the seeds supplied by Japan Tobacco Inc. and grown in a

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greenhouse of the School of Agriculture, Ibaraki University. We performed the hybridization between *N. suaveolens* and two progenitors of *N. tabacum* in the greenhouse.

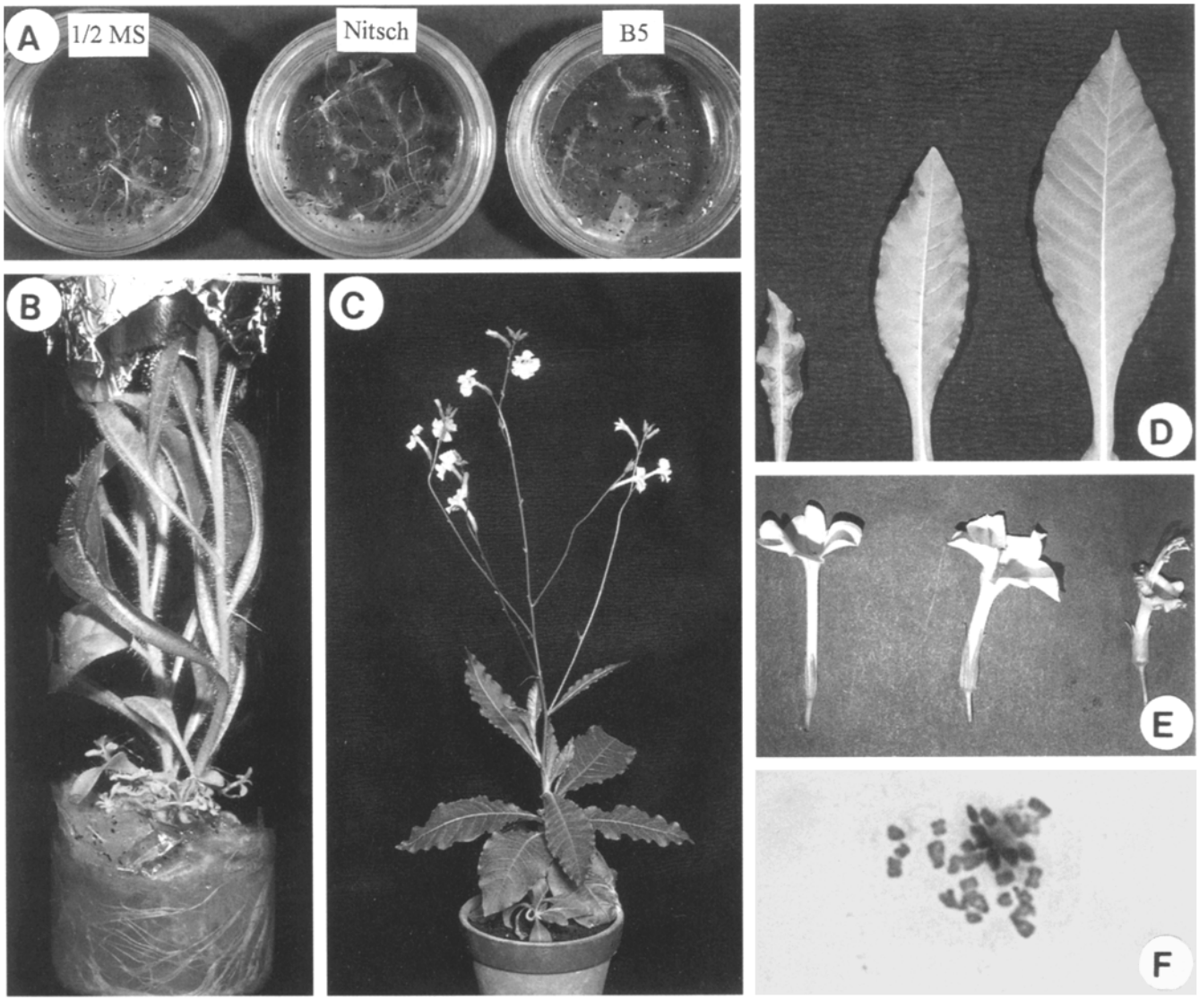
Ovule culture

In both of the two crosses, *N. suaveolens* × *N. sylvestris* and *N. suaveolens* × *N. tomentosiformis*, ovule culture was necessary to obtain hybrid seedling as no fertile seeds had been obtained in situ. Five to 10 days after the pollination with *N. sylvestris* and *N. tomentosiformis*, the ovaries of *N. suaveolens* were sterilized with 70% ethanol for 10 s and 5% sodium hypochlorite solution (0.25% liberated chloride) for 15 min, and then rinsed three times with sterile distilled water. Fertilized ovules were carefully removed from the placenta of the ovaries and placed on the three kinds of medium, i.e. MS (Murashige and Skoog 1962) with half the concentration of salts, Nitsch (Nitsch 1969) and B5 (Gamborg et al. 1968), each of which contained 6% sucrose and 0.8% agar. The ovules were cultured at 28 °C under continuous illumination (approximately 2000 lux). Three weeks after initiation of the ovule culture, the ovules were transferred to low sucrose media (1% sucrose) to stimulate germination. Hybrid seedlings derived from cultured ovules were sub-cultured on the MS medium with half the concentration of salts plus 2 mg/l indole-3-acetic acid, 3% sucrose and 0.8% agar at 28 °C under continuous illumination (approximately 2000 lux). Through all of the experiments, the pH of the culture media was adjusted to 5.8.

Ovule culture at high temperature condition

The effects of high temperature on the growth of hybrid plants were observed by culturing the F_1 ovules of *N. suaveolens* × *N. sylvestirs* at 28 °C (ordinary temperature) and 36 °C (high temperature) on MS medium with half the concentration of salts plus 3% sucrose and 0.8% agar under continuous illumination (approximately 2000 lux). To investigate the development of embroys at high temperature, we sectioned cultured ovules at 10-μm thickness and stained them with Delafield's hematoxylin for microscopic examination.

Fig. 1A–F Development, morphological characters and somatic chromosome number of hybrid plants between *N. suaveolens* and *N. tomentosiformis* obtained by ovule culture. **A** In vitro germination of hybrid seeds through ovule culture, **B** a hybrid seedling 2 months after the initiation of ovule culture, **C** a hybrid plant at flowering in a greenhouse, **D** leaves of *N. suaveolens* (*left*), *N. tomentosiformis* (*right*) and a hybrid plant (*center*), **E** flowers of *N. suaveolens* (*left*), *N. tomentosiformis* (*right*) and a hybrid plant (*center*), **F** a somatic cell containing 28 chromosomes



Test-tube pollination

As zygotic embryos were not obtained through the conventional crossing of *N. sylvestris* × *N. suaveolens*, test-tube pollination and ovule culture were performed following Marubashi and Nakajima (1985). To promote the growth of fertilized ovules, 1 week after test-tube pollination we transferred the swollen ovules to MS medium with half the concentration of salts plus 3% sucrose and 0.8% agar and cultured them at 28 °C under continuous illumination (approximately 2000 lux).

Cytological observation

Chromosomes were observed by the squash method (Burns 1964). The petals of flower buds were hydrolyzed and stained by acetic carmine to which iron had been added.

Results

Hybridization of *N. suaveolens* × *N. tomentosiformis*

As fertile seeds were not obtained in situ in this cross combination, we tried to produce the hybrid plants through ovule culture. Out of about 10 000 ovules cultured, a total of 295 plants were produced at 28 °C (Table 1, Fig. 1A). These plants did not exhibit lethality and grew well at 28 °C (Fig. 1B). Fifteen of these plants were potted and transferred to a greenhouse where the lowest temperature was 15 °C, and these grew vigorously without lethal symptoms, maturing within 5 months after the initiation of ovule culture. In this experiment, the highest germination rate was obtained on the MS medium (Table 1). None of the hybrids obtained through ovule culture at 28 °C exhibited lethality (Table 2), and they all grew vigorously.

Morphological characteristics were observed in the mature plants derived from the ovule culture (Fig. 1C). Their plant height was intermediate between that of *N. suaveolens* and *N. tomentosiformis*, they had normal leaves, which were similar in shape to the leaves of *N. tomentosiformis*, leaf size was intermediate between that of the parents (Fig. 1D), as were flower shape and color (pale pink) (Fig. 1E). There was no significant variation in morphological characters among the mature plants. The somatic chromosome number of 5 mature plants was 28 (Fig. 1F), which corresponded to the combined number of *N. suaveolens* (n = 16) and *N. tomentosiformis* (n = 12). These observations indicate that the mature plants obtained by the ovule culture were complete hybrids between *N. suaveolens* and *N. tomentosiformis*.

Table 2 Lethality observed in the interspecific hybrids between *N. suaveolens* and two progenitors of *N. tabacum* at 28 °C

Cross combination[a]	Number of ovules cultured	Number of plants obtained	Number of plants exhibiting lethality
N. sua. × *N. tom.*	10 550	295	0
N. sua × *N. syl.*	10 408	7	7
N. syl. × *N. sua.*	63[b]	1	1

[a] *N. sua, N. suaveolens; N. tom, N. tomentosiformis; N. sel, N. sylvesris*
[b] The ovules were fertilized by test-tube pollination

Table 1 Number of the plants derived from fertilized ovules cultured at various ages, after the cross of *N. suaveolens* × *N. tomentosiformis* on three different media at 28 °C

Culture media[a]	Days after pollination	Number of ovules cultured	Number of plants obtained[b]
1/2 MS	5	674	1
	6	774	6
	7	484	18
	8	677	33
	9	654	47
	10	533	35
	Total	**3796**	**140**
Nitsch	5	358	1
	6	633	0
	7	534	4
	8	645	8
	9	537	16
	10	501	27
	Total	**3208**	**56**
B5	5	674	3
	6	774	6
	7	484	16
	8	677	4
	9	654	25
	10	533	45
	Total	**3796**	**99**

[a] MS, Murashige and Skoog (1962); Nitsch, Nitsch (1969); B5, Gamborg et al. (1968)
[b] The data were obtained 60 days after the initiation of culture

Table 3 Number of the plants derived from fertilized ovules cultured at various ages, after the cross of *N. suaveolens* × *N. sylvestris*, on three different media at 28 °C

Culture media[a]	Days after Pollination	Number of ovules cultured	Number of plants obtained[b]
1/2 MS	5	688	0
	6	748	0
	7	602	0
	8	545	0
	9	514	1
	10	395	0
	Total	**3492**	**1**
Nitsch	5	914	0
	6	703	0
	7	451	0
	8	447	0
	9	423	0
	10	303	0
	Total	**3241**	**0**
B5	5	924	0
	6	409	0
	7	714	1
	8	543	0
	9	569	4
	10	516	1
	Total	**3675**	**6**

[a] MS, Murashige and Skoog (1962); Nitsch, Nitsch (1969); B5, Gamborg et al. (1968)
[b] The data were obtained 60 days after the initiation of culture

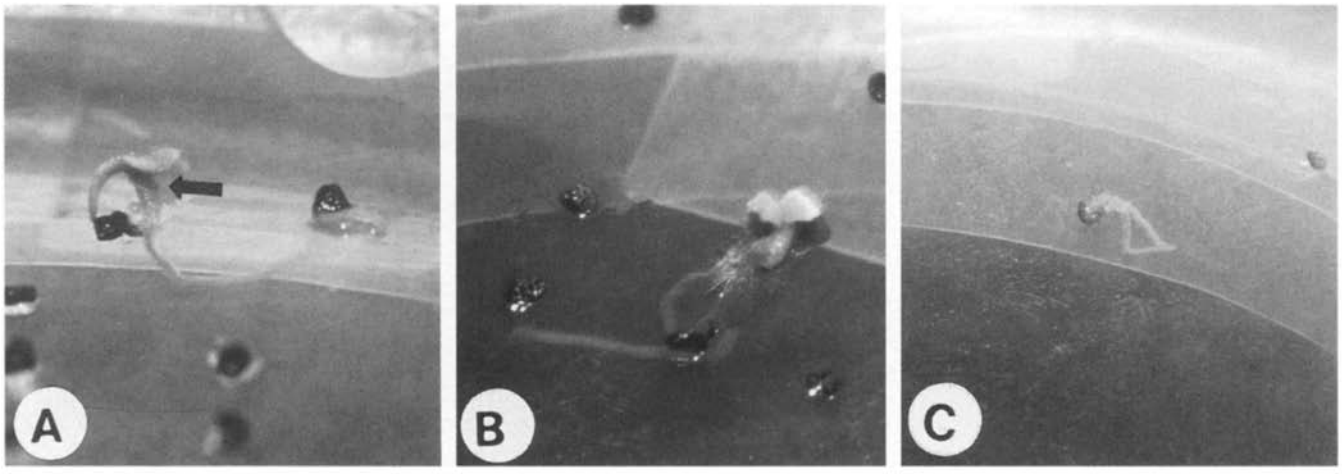


Fig. 2A–C Lethality of hybrid plants between *N. suaveolens* and *N. sylvestris*. **A** A seedling of *N. suaveolens* × *N. sylvestris* exhibiting browning in the hypocotyl (*arrow*), **B** a dying seedling of *N. suaveolens* × *N. sylevestris*, **C** a dying seedling of *N. sylvestris* × *N. suaveolens*, which was obtained through test-tube pollination

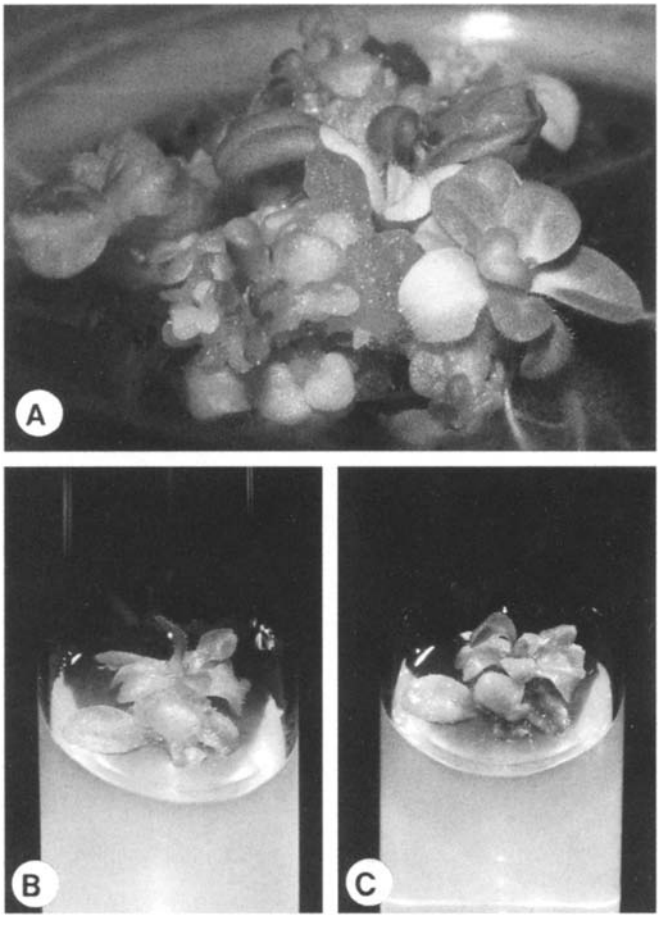
Hybridization of *N. suaveolens* × *N. sylvestris*

As fertile seeds were not obtained in situ in this cross combination, we also tried to produce hybrid plants through ovule culture. However, only 7 plants were grown from about 10 000 ovules cultured at 28 °C (Table 3, Fig. 2). The highest germination rate was obtained in ovules cultured 9 days after pollination. B5 was most effective medium for ovule culture of the three basal media used in this experiment. All of the seedlings exhibited browning at the hypocotyl within 2 or 3 days after the germination (Fig. 2A). The symptoms quickly spread to the whole body of the plants (Fig. 2B), so that all of them died after unfolding their cotyledons when cultured at 28 °C. The feature of lethality observed here resembled that of the hybrids between *N. suaveolens* and *N. tabacum* (Lloyd 1975; Marubashi et al. 1988a). All of the hybrids obtained through ovule culture at 28 °C exhibited lethality (Table 2).

Ovule culture at 36 °C was performed to observe the effects of high temperature on the growth of the hybrid plant. Fifty-four plants germinated from 2730 ovules at 36 °C (Table 4, Fig. 3A), whereas no hybrid plants germinated at the normal temperature (28 °C). The plants grew vigorously without any lethal symptoms at 36 °C (Fig. 3B). When the temperature was changed from 36 °C to 28 °C, the plants developed browning within 3 days (Fig. 3C) and died within 2 weeks. Because the acclimatization was very difficult in these plants at 36 °C, mature plants could not be obtained in this cross combination.

To observe the growth of the zygotic embryo, histological treatments were carried out on cultured ovules developed from the cross of *N. suaveolens* × *N. sylvestris*. The embryos in situ ceased to grow at about 1 week after the pollination and consequently degenerated 1 week later in the greenhouse (Fig. 4). No organ formation was observed in the embryo sacs of ovules cultured at 28 °C. Callus induction was observed in some of the embryo sacs of ovules cultured at 36 °C (Fig. 4F).

Fig. 3A–C Growth of hybrids obtained from *N. suaveolens* × *N. sylvestris* through ovule culture at 36 °C: **A** germination of hybrid seedlings 1 month after initiation of the culture at 36 °C, **B** hybrid seedlings cultured for 2 months at 36 °C, **C** hybrid seedlings cultured for 2 months at 36 °C and then grown for 3 days at 28 °C



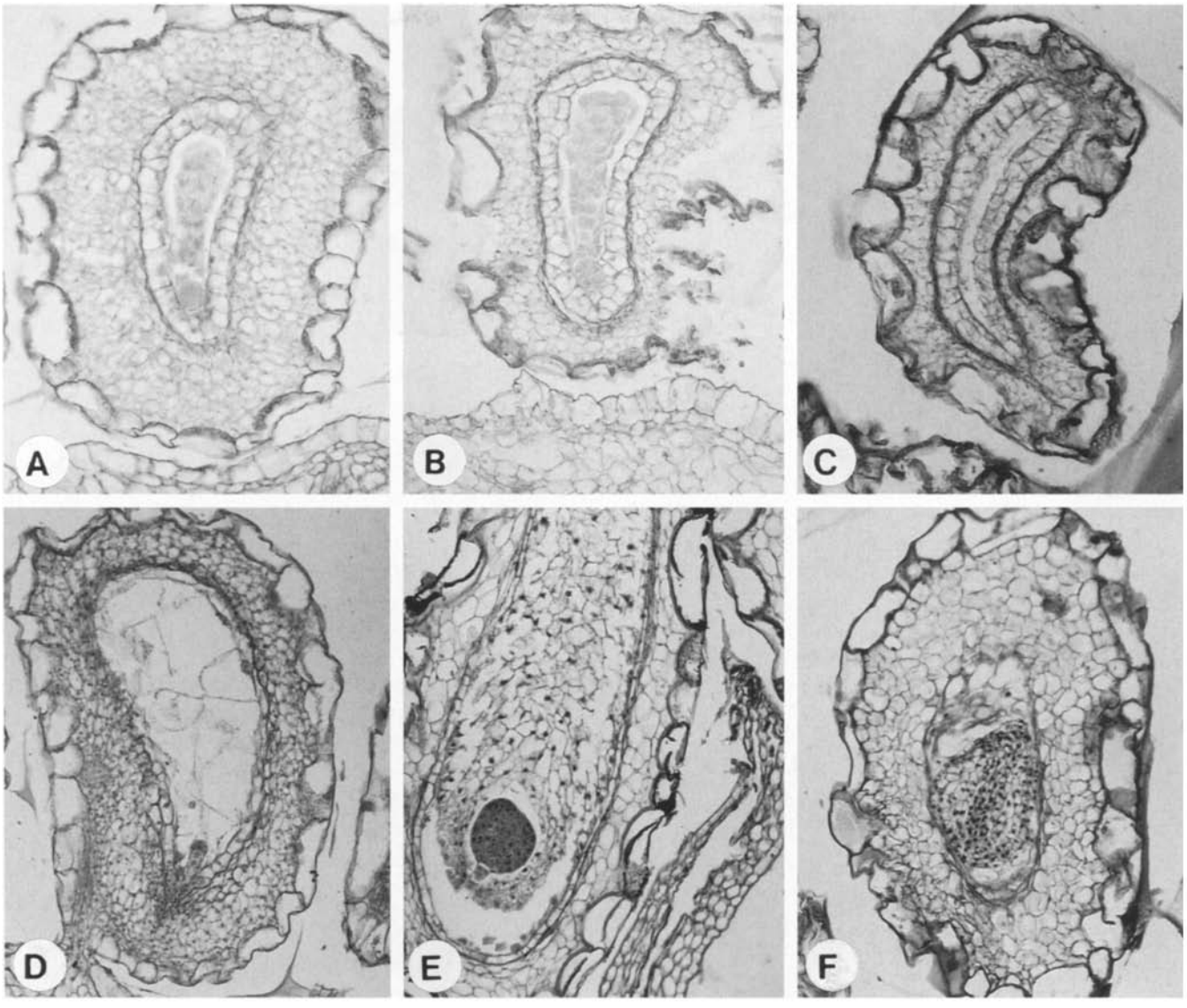


Fig. 4A–F Features of embryo sacs of greenhouse-grown *N. suaveolens*, after crossing with *N. sylvestris* pollen or self-pollination. **A** A globular hybrid embryo (8 days after crossing), **B** a globular hybrid embryo (14 days after crossing), **C** an embyro sac without any embryo (14 days after crossing), **D** an early globular embryo of *N. suaveolens* (7 days after self-pollination), **E** a heart-shaped embyro of *N. suaveolens* (14 days after self-pollination), **F** callus-like structure that appeared in a embryo sac cultured at 36 °C

Hybridization of *N. sylvestris* × *N. suaveolens*

In this cross combination, test-tube pollination was necessary to obtain zygotic embryos because fertilization was not observed in the conventional pollination. Sixty-three ovules enlarged on 18 placentas, and only 1 seed germinated at 28 °C. The plant exhibited browning at the hypocotyl about 2 days after germination and the symptoms quickly spread to the whole body of the plant (Fig. 2C). The plant died within a few weeks after germination in a similar manner as the hybrids of *N. suaveolens* × *N. sylvestris* (Fig. 2). No viable plants were obtained from this cross (Table 2).

Discussion

Upon hybridizing *N. suaveolens* with two progenitor species of *N. tabacum* we obtained hybrids from three cross combinations. The reciprocal hybrids between *N. suaveolens* and *N. sylvestris* exhibited lethality, while the hybrids between *N. suaveolens* and *N. tomentosiformis* as the male parent did not exhibit the lethality and grew to maturity (Table 2). Figure 5 illustrates the effects of the genomic constitution on the expression of lethality in the interspecific hybrids obtained here. The hybrids originating from *N. suaveolens* × *N. tabacum* and *N. suaveolens* × *N. sylvestris* exhibited lethality in a similar manner. This observation suggests that all of the hybrids containing a genome from *N. suaveolens* (Su) and that

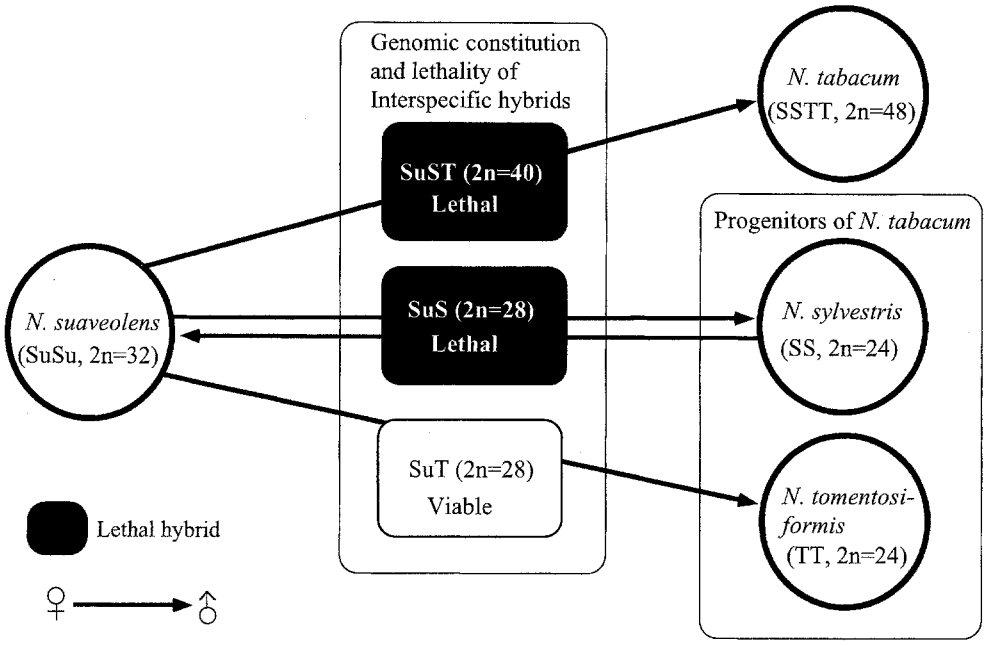


Fig. 5 Scheme showing genomic factors responsible for hybrid lethality in the interspecific cross involving *N. suaveolens*

Table 4 Effect of temperature on the effeciency of ovule culture in the cross of *N. suaveolens* × *N. sylvestis*

Culture temperature	Days after Pollination	Number of ovules cultured	Number of plants obtained[a]
36°C	7	768	14
	8	664	18
	9	660	22
	10	638	0
	Total	**2730**	**54**
36°C	7	609	0
	8	823	0
	9	612	0
	10	644	0
	Total	**2688**	**0**

[a] The data were obtained 30 days after the initation of culture

from *N. sylvestris* or *N. tabacum* (S) exhibit the same type of lethality. These findings suggest that the S genome in *N. tabacum* is responsible for the lethality of the hybrids between *N. suaveolens* and *N. tabacum*.

As to the effect of temperature on lethality, the hypocotyl of the hybrids between *N. suaveolens* and *N. sylvestris* exhibited browning at 28 °C but not at 36 °C. The hybrids grown at 36 °C survived for longer periods than those at 28 °C. A similar situation was reported in the hybrid of *N. suaveolens* × *N. tabacum* which also did not exhibit the lethality at 36 °C (Marubashi et al. 1988b).

Using the entire series of monosomics of *N. tabacum* cv 'Red Russian', Gerstel et al. (1979) analyzed the lethality of the hybrid between *N. africana* and *N. tabacum*. Among the 24 monosomic lines crossed with *N. africana* as the pollen parent, a monosomic line lacking a H chromosome (haplo H) produced viable offsprings. This result suggests that the lethality of the hybrid between *N. africana* and *N. tabacum* is controlled by a gene(s) on the H chromosome, which is a constituent of the T genome in *N. tabacum*. A similar monosomic analysis will reveal the chromosome, which is responsible for the lethality of *N. suaveolens* × *N. tabacum* hybrids.

References

Burns JA (1964) A technique for making preparations of mitotic chromosomes from *Nicotiana* flowers. Tob Sci 1:22–23

Buuren M, Neuhaus J-M, Shinshi H, Ryals J, Meins F-Jr (1992) The sturcture and regulation of homeologous tabacco endochitinase genes of *Nicotiana sylvestris* and *N. tomentosiformis* origin. Mol Gen Genet 232:460–469

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gerstel GU, Burns JA, Burk LG (1979) Interspecific hybridizations with an African tobacco, *Nicotiana africana* Merxm. J. Hered 70:342–344

Inoue E, Marubashi W, Niwa M (1994) Simple method for overcoming the lethality observed in the hybrid between *Nicotiana suaveolens* and *N. tabacum*. Breed Sci 44:333–336

Kostoff D (1930) Ontogeny, genetics and cytology of *Nicotiana* hybrids. Genetica 12:33–139

Lloyd R (1975) Tissue culture as means of circumventing lethality in an interspecific *Nicotiana* hybrid. Tob Sci 19:4–6

Marubashi W, Nakajima T (1985) Overcoming cross-incompatibility between *Nicotiana tabacum* L. and *N. rustica* L. by test-tube pollination and ovule culture. Jpn J Breed 35:429–437

Marubashi W, Nakamura N, Onozawa Y (1988a) Production of interspecific seedlings between *Nicotiana tabacum* L. and *N. sauveolens* Lehm. via test-tube pollination and exhibition of seedling lethality. Jpn J Breed 37[Suppl 1]:116–117

Marubashi W, Tatsuno I, Onozawa Y (1988b) Effect of high temperature on the growth of hybrid lethal seedlings between *Nicotiana sauveolens* Lehm. × *N. tabacum* L. Jpn J Breed 38[Suppl 1]: 66–67

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physical Plant 15:473–497

Nitsch JP (1969) Experimental androgenesis in *Nicotiana*. Phytomorphology 19:389–404

Oka H-I (1957) Phylogenetic differentiation of cultivated rice. XV. Complementary lethal genes in rice. Jpn J Genet 32 [Suppl 3]: 83–87

Okamuro JK, Goldberg RB (1985) Tobacco single-copy DNA is highly homologous to sequences present in the genomes of its diploid progenitors. Mol Gen Genet 198:290–298

Sheen SJ (1972) Isozymic evidence bearing on the origin of *Nicotiana tabacum* L. Evolution 26:143–154

Stebbins GL (1958) The inviability, weakness, and sterility of interspecific hybrids. Adv Genet 9:147–215

Zeven AC (1981) Eighth supplementary list of wheat varieties classified according to their genotype for hybrid necrosis. Euphytica 30:521–539