

# Identification of genomic factors responsible for hybrid lethality in hybrids between *Nicotiana nudicaulis* Watson and *N. tabacum* L.

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**Abstract** Hybrid lethality was expressed in seedlings from crosses between *Nicotiana nudicaulis* and *N. tabacum* at 28°C. To clarify the subgenome responsible for this phenotype, we crossed *N. nudicaulis* with the two progenitors of *N. tabacum* (SSTT), *N. sylvestris* (SS) and *N. tomentosiformis* (TT). Seedlings of *N. nudicaulis*×*N. tomentosiformis* did not show any lethal symptoms, and these hybrid seedlings subsequently grew to maturity. On the other hand, in the cross *N. sylvestris*×*N. nudicaulis*, all seedlings cultured at 28°C died immediately after germination, and others cultured at 34°C and 36°C were nonviable or abnormal; only one hybrid plant survived. These findings suggest that the S subgenome in *N. tabacum* is responsible for the lethality occurring in hybrids between *N. nudicaulis* and *N. tabacum*. Furthermore, to determine whether the Q chromosome induces this hybrid lethality, we crossed a monosomic line of *N. tabacum* lacking the Q chromosome with *N. nudicaulis*. Subsequently, we determined the presence or absence of the Q-chromosome-specific DNA marker in hybrid seedlings. Hybrid seedlings both possessing and lacking the Q chromosome showed hybrid lethality when seedlings cultured at 34°C were transferred to 28°C. From these results, we concluded that the Q chromosome is not responsible for the hybrid lethality observed in crosses of *N. nudicaulis*×*N. tabacum*.

**Key words:** Hybrid lethality, S subgenome, Q chromosome, *N. nudicaulis*, *N. sylvestris*.

Reproductive isolation, which is classified into two types due to prezygotic and postzygotic barriers, is a mechanism playing a crucial role in the evolution of animals and plants (Stebbins 1966). In plant species, hybrid lethality, which can occur in intergeneric, interspecific, and intraspecific crosses, is a potential gene-flow postzygotic barrier. Recently, the mechanism for hybrid lethality observed in *Arabidopsis thaliana* and lettuce (*Lactuca saligna*×*L. sativa*) was concluded to be an autoimmune-like response due to the interaction between two allelic loci (Bombliès and Weigel 2007; Bombliès et al. 2007; Jeuken et al. 2009).

The genus *Nicotiana* includes 75 species that are classified into 13 sections distributed in the Americas and Australia and one species (*N. africana*) that is restricted to Africa (Knapp et al. 2004). In this genus, some interspecific cross combinations exhibit hybrid lethality. The majority of these combinations have been conducted between wild tobacco and well-studied cultivated tobacco, *Nicotiana tabacum* ( $2n=48$ , SSTT), which belongs to section *Genuinae*. *N. tabacum* is a natural allotetraploid (amphidiploid) that originated by interspecific hybridization of *N. sylvestris* ( $2n=24$ , SS) with *N. tomentosiformis* ( $2n=24$ , TT) and subsequent

chromosome doubling (Sheen 1972; Gray et al. 1974; Kenton et al. 1993; Lim et al. 2000; Murad et al. 2002, 2004; Chase et al. 2003; Clarkson et al. 2010). Each chromosome of *N. tabacum* is lettered alphabetically (A to Z, excluding X and Y); chromosomes A to L belong to the T subgenome and M to 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 'Red Russian' (Clausen and Cameron 1944; Cameron 1959) and is useful for locating genes on specific chromosomes.

In previous studies (Tezuka 2012), monosomic analyses have identified one *N. tabacum* chromosome that belongs to the S subgenome; in this chromosome, a gene or genes trigger hybrid lethality in crosses between section *Suaveolentes* and *N. tabacum*. This hybrid lethality is suppressed at elevated temperatures (32 to 36°C) and induced at 28°C. Hybrid seedlings from crosses between *N. suaveolens* and *N. tabacum* die at 28°C, but grow normally without exhibiting lethal symptoms at 36°C (Manabe et al. 1989). Hybrid seedlings of *N. suaveolens*×*N. sylvestris* die at 28°C, but not at 36°C; hybrid seedlings of *N. suaveolens*×*N. tomentosiformis* do not exhibit lethality at 28°C (Inoue

et al. 1996). Based on these results, Inoue et al. (1996) found that the S subgenome of *N. tabacum* controls hybrid lethality in these crosses. Moreover, in crosses of a monosomic line of *N. tabacum* lacking the Q chromosome (Haplo-Q) × *N. suaveolens*, viable hybrid seedlings were obtained (Marubashi and Onosato 2002).

Tezuka et al. (2004) developed seven Q-chromosome-specific DNA markers. Since one marker, OPC-11<sub>922</sub>, was not detected on the Q chromosome of the cultivar Samsun NN but was detected on the Q chromosome of Haplo-Q, F<sub>1</sub> hybrids of Haplo-Q × Samsun NN were classified into two groups: monosomic plants without OPC-11<sub>922</sub> and disomic plants with OPC-11<sub>922</sub>. In these hybrids, the number of monosomic plants was almost the same as disomic plants. Using this approach, Tezuka et al. (2006) carried out crosses of Haplo-Q × *N. suaveolens* and found by using four Q-chromosome-specific DNA markers that hybrid seedlings in which Q-chromosome-specific DNA markers were detected expressed hybrid lethality, while hybrid seedlings in which Q-chromosome-specific DNA markers were not detected did not. These results confirmed that the Q chromosome of the S subgenome is a causal chromosome for hybrid lethality. On the other hand, genes controlling hybrid lethality in the cross *N. occidentalis* × *N. tabacum* appear in both the S and T subgenomes of *N. tabacum* (Tezuka and Marubashi 2012).

Recently, the first linkage map for *N. tabacum* was constructed by Bindler et al. (2011) with SSR markers. Tezuka et al. (2012) used this map and interspecific crosses between *N. africana* and an *N. tabacum* monosomic line lacking the Q chromosome to determine that linkage group 11 corresponded to the Q chromosome and that the gene(s) responsible for hybrid lethality mapped to the region of linkage group 11 that included SSR markers PT30342 and PT30365.

Section *Repandae* exists in North America and consists of four allopolyploid species: *N. nudicaulis*, *N. repanda*, *N. stocktonii* and *N. nesophila* (Clarkson et al. 2004). In previous studies (Reed and Collins 1978), hybrid lethality was observed in crosses of *N. repanda* × *N. tabacum*, *N. stocktonii* × *N. tabacum* and *N. nesophila* × *N. tabacum*. Moreover, crosses between *N. repanda* and *N. tomentosiformis* or *N. sylvestris* were performed to determine which subgenome (S or T) is responsible for hybrid lethality expressed in the cross *N. repanda* × *N. tabacum*. Crosses between *N. repanda* and *N. tomentosiformis* resulted in hybrid lethality, but crosses between *N. repanda* and *N. sylvestris* did not. Based on these results, Kobori and Marubashi (2004) concluded that the T subgenome included the causative gene(s) for hybrid lethality in this cross.

*Nicotiana nudicaulis*, which belongs in the section *Repandae*, is closely related to *N. repanda* (Clarkson et al. 2005; 2010). In reciprocal interspecific crosses between

*N. nudicaulis* and *N. tabacum*, hybrid lethality was observed at 28°C, but not at 34°C (Yamada et al. 1999; Liu et al. 2013). In this study, we crossed *N. nudicaulis* with two progenitors of *N. tabacum*, *N. sylvestris* (SS) and *N. tomentosiformis* (TT), to reveal which subgenome of *N. tabacum* contains the genomic factors responsible for hybrid lethality in hybrids between *N. nudicaulis* and *N. tabacum*.

## Materials and methods

### Plant materials

The seeds of *N. nudicaulis* Watson ( $2n=48$ ), *N. sylvestris* Speg. & Comes. ( $2n=24$ , SS) and *N. tomentosiformis* Goodsp. ( $2n=24$ , TT) were used in these experiments and were supplied by Japan Tobacco, Inc. (Oyama, Japan). The *N. tabacum* monosomic line Haplo-Q ( $2n=47$ ) was supplied by Dr. T. Kubo, Japan Tobacco, Inc. These monosomics were originally produced at the Department of Genetics, University of California, Berkeley (Cameron 1959). Plants were grown and pollinated in the greenhouse of the School of Agriculture, Meiji University.

### Interspecific crosses of *N. nudicaulis* × *N. tomentosiformis*

Conventional crossing and sowing were carried out as follows: flowers of *N. nudicaulis* used as maternal parents were emasculated immediately before pollination with pollen of *N. tomentosiformis*. F<sub>1</sub> seeds (*N. nudicaulis* × *N. tomentosiformis*) were soaked in 0.05% gibberellic acid (GA<sub>3</sub>) solution for 30 min, sterilized with 5% sodium hypochlorite for 15 min, and then rinsed three times with sterilized water. Sterilized F<sub>1</sub> seeds were sown on Petri dishes containing 8 ml of 1/2 MS medium (Murashige and Skoog 1962) supplemented with 1% sucrose and 0.25% Gelrite, pH 5.8; the plates were maintained at 28°C under continuous illumination ( $32 \mu\text{mol s}^{-1} \text{m}^{-2}$ ) for seed germination. Immediately after germination, hybrid seedlings were cultured at 28°C and 34°C. At 20 days after germination (DAG), hybrid seedlings cultured at 28°C and 34°C were all viable. Hybrid seedlings surviving for more than 20 DAG were potted and cultivated in a greenhouse under natural lighting conditions.

### Test tube pollination and ovule culture

Because no fertile seeds were obtained *in situ* from either the cross *N. nudicaulis* × *N. sylvestris* or *N. nudicaulis* × *N. tabacum* monosomic line Haplo-Q, test tube pollination and ovule culture, carried out as described by Marubashi and Nakajima (1985), were necessary to obtain hybrid seedlings. Anthers of *N. nudicaulis* were aseptically excised from still-closed flowers and stimulated to dehisce in an incubator (28°C). Flowers of *N. sylvestris* or Haplo-Q were emasculated 1 day before anthesis; the next day, flowers of *N. sylvestris* or Haplo-Q were collected and their sepals, petals, and styles were removed. Their ovaries were surface-sterilized with 70% ethanol for 30 s

and then with 5% sodium hypochlorite for 10 min. The ovary walls were peeled to expose the placenta with intact ovules and then the placentas were placed on 1/2 MS medium with 3% sucrose. Pollen of *N. nudicaulis* was spread on the surface of the placentas. Pollinated placentas were maintained at 28°C under continuous illumination ( $32 \mu\text{mol s}^{-1} \text{m}^{-2}$ ). Fertilized, enlarged ovules were excised 8 days after test tube pollination and cultured on 1/2 MS medium at 28°C under continuous illumination ( $32 \mu\text{mol s}^{-1} \text{m}^{-2}$ ).

### Cultivation of hybrid seedlings

Immediately after germination, hybrid seedlings of *N. sylvestris* × *N. nudicaulis* were transferred to flat-bottomed test tubes that contained 10 ml of 1/2 MS medium supplemented with 1% sucrose and 0.25% Gelrite, pH 5.8, and cultured at 28°C, 34°C, or 36°C. In a previous report (Liu et al. 2013), the desired results were obtained in a cross of *N. nudicaulis* × *N. tabacum* at 34°C. In a preliminary experiment, it was difficult to obtain normal hybrid seedlings of *N. sylvestris* × *N. nudicaulis* at 34°C, so the 36°C condition was added.

The hybrid seedlings of Haplo-Q × *N. nudicaulis* were transferred to flat-bottomed test tubes that contained 10 ml of 1/2 MS medium supplemented with 1% sucrose and 0.25% Gelrite, pH 5.8, and cultured at 34°C immediately after germination. Enlarging seedlings of Haplo-Q × *N. nudicaulis* were transferred to flat-bottomed test tubes (25-mm diameter, 100-mm length) that contained 10 ml 1/2 MS medium supplemented with 1% sucrose and 0.25% Gelrite, pH 5.8, 10 DAG. Seedlings cultured at 34°C for 30–40 DAG were then transferred to 28°C under continuous illumination ( $32 \mu\text{mol s}^{-1} \text{m}^{-2}$ ) to induce hybrid lethality after analysis using Q-chromosome-specific DNA markers.

### Random amplified polymorphic DNA (RAPD) analysis and detection of Q-chromosome-specific DNA markers

Total DNA was extracted from the leaves of the hybrid seedlings using the cetyltrimethylammonium bromide method of Murray and Thompson (1980). RAPD analysis was carried out as described by Williams et al. (1990) using 20 random 10-mer oligonucleotide primers (Kit A; Operon Technologies, Inc., Alameda, CA, USA). The reaction mixture contained 10  $\mu\text{l}$  SapphireAmp® Fast PCR Master Mix (Takara Bio Inc., Otsu, Japan), 0.5  $\mu\text{M}$  primer, and 120 ng DNA in a total volume of 20  $\mu\text{l}$ . PCR amplification was performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed for 3 min at 94°C for initial denaturation, followed by 50 cycles of 30 s at 94°C, 1 min at 25°C, 2 min at 72°C, and a final extension of 5 min at 72°C. PCR products were separated by electrophoresis on 1.5% agarose gels in TAE buffer and stained with ethidium bromide to visualize DNA bands. The Q-chromosome-specific DNA marker QCS1 was detected as described by Tezuka and Marubashi (2006).

### Chromosome analysis of hybrid seedlings from crosses between *N. nudicaulis* and *N. tomentosiformis*

Root tips were pretreated with distilled water for 24 h at 4°C and with 2 mM 8-hydroxyquinoline for 4 h at 4°C, then fixed in a 3:1 mixture of ethanol and acetic acid overnight to determine chromosome numbers. The root tips were hydrolyzed in 1 N 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 BX51 light microscope (Olympus, Tokyo, Japan), and photographed using a DP70 automatic photomicrography system (Olympus).

## Results

### Viable hybrid seedlings of *N. nudicaulis* × *N. tomentosiformis*

The crosses of *N. nudicaulis* × *N. tomentosiformis* were carried out via conventional cross-pollination; hybrid seeds were obtained approximately 3 weeks after pollination. Hybrid seeds from *N. nudicaulis* × *N. tomentosiformis* crosses were sown in 1/2 MS medium and germinated at 28°C. The hybrid seedlings germinated well, and all of the hybrid seedlings were viable at 20 DAG.

Five of the hybrid seedlings were cultivated in a greenhouse and three were randomly selected. All of the selected seedlings grew to maturity and came into flower (Figure 1A). The mature hybrid plants displayed uniform morphological characteristics, with leaf and flower shapes that were intermediate in appearance between those of the parents (Figure 1B–D). Chromosomal analyses of the hybrid plants revealed that each possessed 36 chromosomes, which is the sum of the number of haploid chromosomes of the parents (Figure 1E).

RAPD analysis using the primer OPC-16 was carried out on three hybrid plants to confirm that they were true hybrids (Figure 1F). These plants had several bands characteristic of both parents (arrows); these patterns indicated that they are true hybrids. Similar results, shown in Figure 1F, were also obtained using several other random primers (data not shown). Hybrid plants from the cross *N. nudicaulis* × *N. tomentosiformis* were sterile.

### Nonviable hybrid seedlings of *N. sylvestris* × *N. nudicaulis*

When the *N. nudicaulis* × *N. sylvestris* crosses were carried out via conventional cross-pollination, the diploid seeds of *N. nudicaulis* but no hybrid seeds were obtained. We inferred that fertilization between *N. nudicaulis* and *N. sylvestris* did not occur because of cleistogamy in *N. nudicaulis*. To obtain hybrid seedlings, we carried out crosses of *N. sylvestris* × *N. nudicaulis* by test tube pollination and ovule culture. Placentas



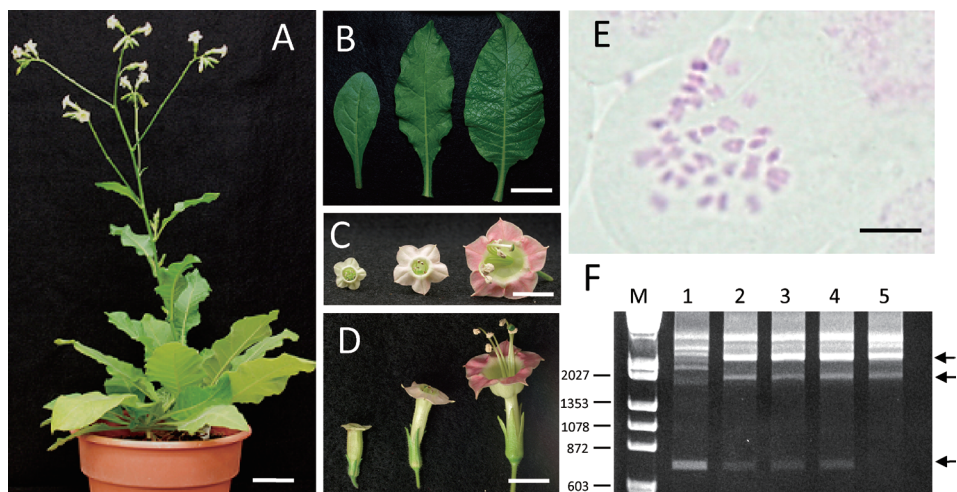


Figure 1. The hybrid of *N. nudicaulis*×*N. tomentosiformis*. (A) Shape of a hybrid plant that has grown to maturity and flowered. (B) Leaves of *N. nudicaulis*, a hybrid and *N. tomentosiformis* (left to right). (C and D) Flowers of *N. nudicaulis*, a hybrid and *N. tomentosiformis* (left to right). (E) Image of root tip cell of a hybrid plant showing the number of chromosomes. (F) Confirmation of hybrid formation by RAPD analysis using primer OPC-16. M: DNA ladder maker ( $\lambda$ /HindIII and  $\psi$ X174/HaeIII), Lane 1: *N. nudicaulis*, Lanes 2–4: hybrid seedlings from a *N. nudicaulis*×*N. tomentosiformis* cross, Lane 5: *N. tomentosiformis*. The bands characteristic of both parents are indicated by arrows. Scale bars are 10 cm (A), 3 cm (B), 1 cm (C and D) and 100  $\mu$ m (E).



Figure 2. Hybrid seedlings from a *N. sylvestris*×*N. nudicaulis* cross cultured at 28°C. (A) A hybrid seedling without any lethal symptoms immediately after germination. (B) Lethal symptoms observed in hybrid seedlings cultured at 28°C for 2 DAG. (C) A seedling of *N. nudicaulis* kept at 28°C for 2 DAG. Scale bars are 1 mm.

( $n=563$ ) were pollinated. Thereafter, 456 enlarged ovules were obtained 8 days after pollination and 54 of them began to germinate 3 weeks after ovule culture (Table 1). To confirm the expression of hybrid lethality, 20 hybrid seedlings were left at 28°C (Table 1); all of these seedlings died within 2 DAG. Symptoms of lethality, including browning of the shoot apex and the root tip, were observed in the hybrid seedlings at an early stage (Figure 2).

To suppress lethality, 17 hybrid seedlings each were transferred to 34°C or 36°C immediately after germination. Of the 17 hybrid seedlings cultured at 34°C (Table 1), only one was viable (Figure 3A); this seedling was cultured at 34°C for 3 months after germination and then transferred to 28°C, where it died 10 days after transfer (Figure 3B). Among the other hybrid seedlings grown at 34°C, 11 out of 17 were nonviable (Figure 4A) and 4 of the 17 hybrid plants showed vitrification (Figure 4B). One of the 17 hybrid plants was a tumorous hybrid; a tumor developed at the shoot apex of the nonviable plant (Figure 4C). Among the 17 hybrid plants, various changes were observed, including changes from

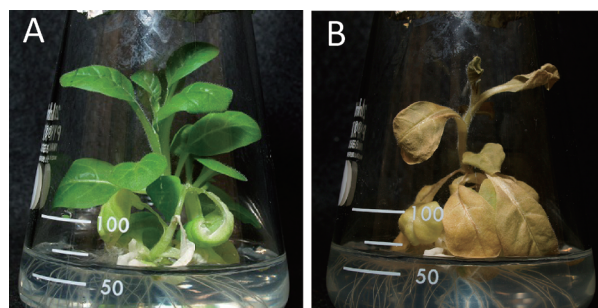


Figure 3. One hybrid plant from a *N. sylvestris*×*N. nudicaulis* cross that was cultured at 34°C. (A) A hybrid seedling without any lethal symptoms at 34°C for 3 months after germination. (B) Lethal symptoms observed in this hybrid plant after transfer from 34 to 28°C; it died 10 days after transfer.

tumorous growth to vitrification or death (Figure 4E and F), and changes from a nonviable hybrid plant to one showing vitrification (Figure 4D).

Of the 17 hybrid seedlings cultured at 36°C (Table 1), 10 showed albinism; 4 became albino before the cotyledons developed, and the others began to display



Table 1. Interspecific hybridization of *N. sylvestris* × *N. nudicaulis*.

Temperature	No. of placentas pollinated	No. of ovules cultured	No. of hybrids obtained					
			Total	Nonviable	Vitrified	Albino	Tumorous	Viable
28°C	563	456	20	20	0	0	0	0
34°C	—	—	17	11(1 <sup>a</sup> )	4(2 <sup>b</sup> ,1 <sup>c</sup> )	0	1 <sup>d</sup>	1
36°C	—	—	17	5	1 <sup>e</sup>	10	1 <sup>f</sup>	0

a: A tumorous hybrid plant died within 100 DAG (Figure 4E). b: Vitrification occurred on a tumorous hybrid plants (Figure 4F). c: Vitrification occurred on a nonviable hybrid plant (Figure 4D). d: Tumor developed on a nonviable hybrid plant (Figure 4C). e: Vitrification occurred on a tumorous hybrid plant (Figure 5C). f: Tumor developed on an albino hybrid plant (Figure 5D).

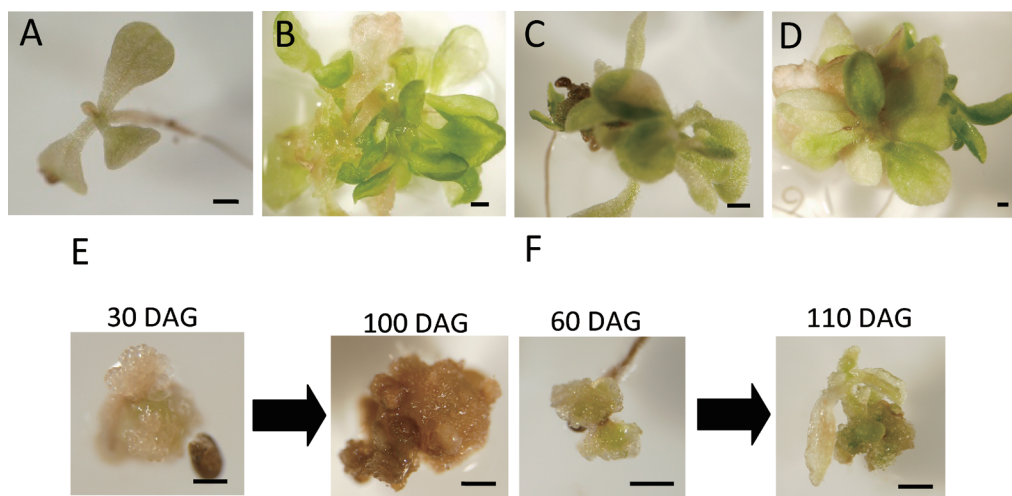


Figure 4. Several types of abnormal hybrid plants from a *N. sylvestris* × *N. nudicaulis* cross cultured at 34°C. (A) A hybrid plant cultured at 34°C showing lethal symptoms; it died at 30 DAG. (B) A hybrid plant that showed vitrification. (C) A tumor that developed at the shoot apex of a nonviable hybrid plant. (D) Vitrification that occurred at the shoot apex of a nonviable hybrid plant. (E) A tumorous hybrid plant that died within 100 DAG. (F) Vitrification that occurred on a tumorous hybrid plant. Scale bars are 1 mm.

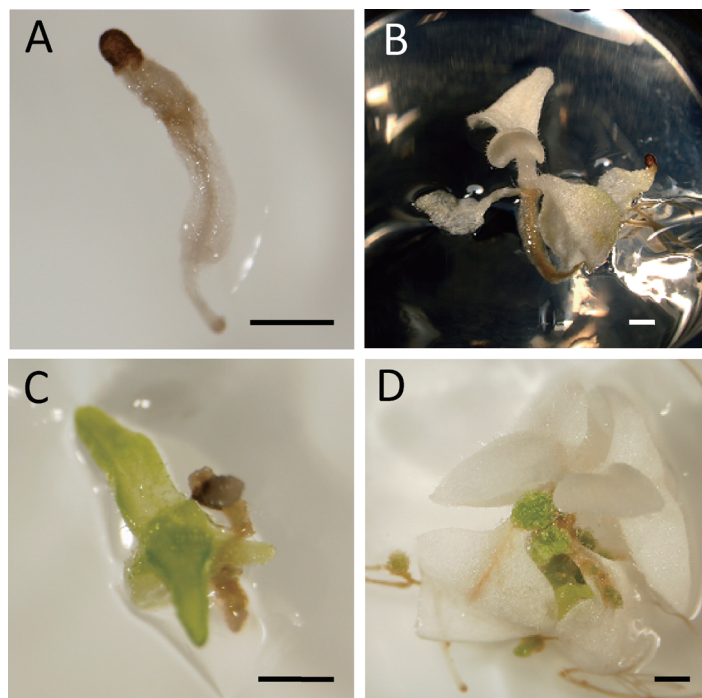


Figure 5. Several types of abnormal hybrid plants from a *N. sylvestris* × *N. nudicaulis* cross cultured at 36°C. (A) A hybrid plant that showed albinism before the cotyledons developed. (B) A hybrid plant that showed albinism after the cotyledons developed. (C) Vitrification occurring on a tumorous hybrid plant. (D) Tumors developing at the shoot apex and the roots of an albino hybrid plant. Scale bars are 1 mm.

Table 2. Production of hybrid seedlings and phenotypic ratio for the two types ( $\pm$ DNA marker) from the cross of Haplo-Q $\times$ *N. nudicaulis*.

Cross combination	No. of placentas pollinated	No. of ovules cultured	No. of hybrids <sup>a</sup> obtained	DNA marker <sup>c</sup>		$\chi^2$ (1:1)	P value
				+	-		
Haplo-Q $\times$ <i>N. nudicaulis</i>	12	319	70 <sup>b</sup>	8	24	8.0	0.005–0.001

a: Counted 60 days after ovules were cultured. b: 32 of 70 hybrid seedlings were used for detecting the DNA marker. c: The DNA marker used was QCS1 (Tezuka et al. 2004). “+” indicates the Q-chromosome-specific DNA marker was detected and “-” indicates that it was not.

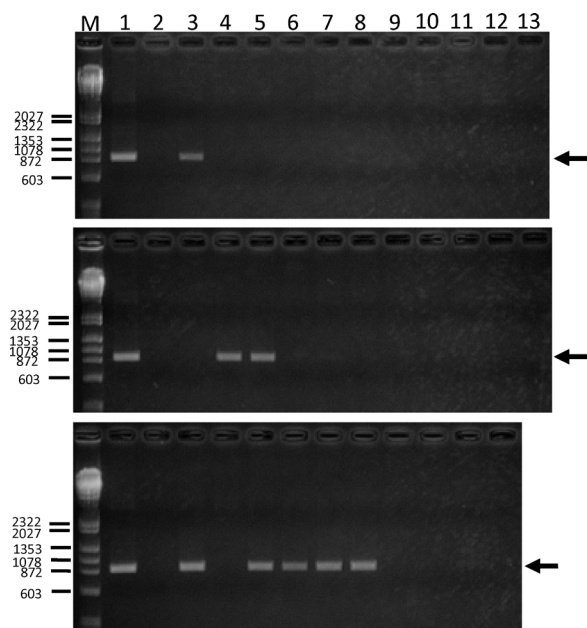


Figure 6. Detection of the marker QCS1 in hybrid seedlings from a Haplo-Q $\times$ *N. nudicaulis* cross. The marker QCS1, indicated by arrows, was detected in hybrid seedlings. M: DNA ladder maker ( $\lambda$ /HindIII and  $\psi$ X174/HaeIII), Lane 1: Haplo-Q, Lane 2: *N. nudicaulis*, Lanes 3–13: hybrid seedlings from a Haplo-Q $\times$ *N. nudicaulis* cross.

albinism after the cotyledons developed (Figure 5A and B). Five of the 17 hybrid plants were nonviable; lethality symptoms were the same as those exhibited by the nonviable hybrid plants at 34°C. One of the 17 hybrid plants showed vitrification (Figure 5C) and one hybrid plant was tumorous; tumors developed at the shoot apex and the roots of the albino plant at 70 DAG (Figure 5D).

In this study, all of the hybrid seedlings died at 28°C; furthermore, half of the hybrid plants were nonviable and only one hybrid plant was viable at 34°C. At 36°C, more plants were viable at 34°C but most were albino. Based on these results, in *N. sylvestris* $\times$ *N. nudicaulis* crosses, 34°C was more effective than 36°C for obtaining normal hybrid plants.

#### Nonviable hybrid seedlings of Haplo-Q $\times$ *N. nudicaulis*

*N. tabacum* monosomic plant lacking the Q chromosome was crossed with *N. nudicaulis* to determine whether the Q chromosome is responsible for hybrid lethality. Since the fertility of pollen of Haplo-Q is very low, the monosomic line was used as a maternal parent. A total

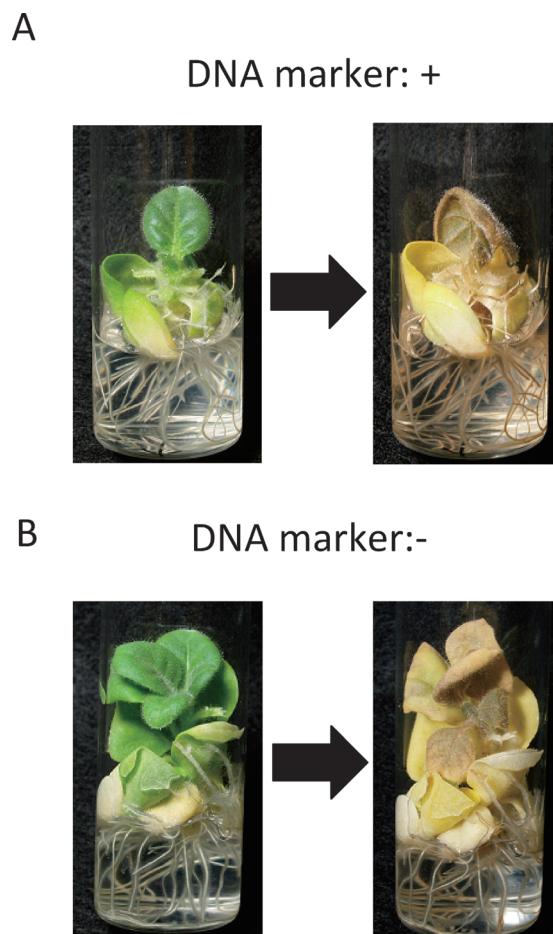


Figure 7. Appearance of hybrid seedlings from a Haplo-Q $\times$ *N. nudicaulis* cross after transfer from 34 to 28°C. Hybrid seedlings were cultured at 34°C for 40–50 DAG and then transferred to 28°C. (A) When a hybrid seedling in which a Q-chromosome-specific DNA marker was detected was transferred to 28°C, the seedling expressed hybrid lethality and died 10 days after transfer from 34 to 28°C. (B) When a hybrid seedling in which the Q-chromosome-specific DNA marker was not detected was transferred to 28°C, the seedling also expressed hybrid lethality and died 10 days after transfer from 34 to 28°C.

of 12 placentas of Haplo-Q were pollinated in test tubes. Thereafter, 319 enlarged ovules were obtained 8 days later, and 70 began to germinate 3 weeks after ovule culture (Table 2). These hybrid seedlings were then grown at 34°C, and 32 of them were assessed for the presence or absence of the Q-chromosome using a Q-chromosome-specific DNA marker, QCS1 (Figure 6). Among the hybrid plants, 8 possessed the Q chromosome and 24

Table 3. Absence of the relationship between the Q chromosome and hybrid lethality observed in the cross of Haplo-Q×*N. nudicaulis*.

Cross combination	DNA marker <sup>a</sup>	No. of hybrids		
		Total	Viable	Nonviable
Haplo-Q× <i>N. nudicaulis</i>	+	4	0	4
	–	6	0	6

a: The DNA marker used was QCS1 (Tezuka et al. 2004). “+” indicates that the Q-chromosome-specific DNA marker was detected and “–” indicates that it was not.

lacked it (Table 2); the segregation ratio did not fit a 1:1 ratio ( $p < 0.005$ ).

Using the Q-chromosome-specific DNA marker, the remaining 10 hybrid seedlings were divided into two types: 4 hybrid seedlings possessing the Q chromosome and 6 hybrid seedlings lacking the Q chromosome (Table 3). Both types of hybrid seedling were cultured for 40–50 DAG and then transferred from 34 to 28°C. Subsequently, 10 days after transfer to 28°C, both types of seedlings showed lethal symptoms and died (Figure 7A and B). These results indicated that the Q chromosome does not control the lethality of hybrids between *N. nudicaulis* and *N. tabacum*.

## Discussion

*N. repanda* and *N. nudicaulis* belong to the same section (Leitch et al. 2008). When *N. repanda* is crossed with the two progenitor species of *N. tabacum*, *N. sylvestris* and *N. tomentosiformis*, hybrid lethality is expressed in seedlings of *N. repanda*×*N. tomentosiformis*, but not in seedlings of *N. repanda*×*N. sylvestris*. Based on these results, Kobori and Marubashi (2004) concluded that the T subgenome is the cause of hybrid lethality occurring in the cross *N. repanda*×*N. tabacum*. In the present study, we confirmed expression of hybrid lethality in the seedlings of *N. sylvestris*×*N. nudicaulis*, and not in hybrid seedlings of *N. nudicaulis*×*N. tomentosiformis*. These results indicate that the S subgenome controls hybrid lethality in *N. nudicaulis*×*N. tabacum* and clarify that in *N. nudicaulis* and *N. repanda*, different subgenomes (S or T) interact to lead to hybrid lethality.

In a previous study (Inoue et al. 2000), hybrid lethality was observed and suppressed by high temperature in the cross *N. suaveolens*×*N. sylvestris*. However, in the cross *N. sylvestris*×*N. nudicaulis*, hybrid plants were nonviable or abnormal, even at high temperatures (34°C and 36°C). The number of plants obtained was greater at 36°C than at 34°C but most of them were albino. Based on these results, it is very difficult to obtain normal hybrid plants of *N. sylvestris*×*N. nudicaulis*. In other words, the hybrid lethality of *N. sylvestris*×*N. nudicaulis* is very severe. The shape of the hybrid plants of this cross are nonuniform, unlike crosses of wild species (section *Suaveolentes*)×*N. tabacum* (Tezuka et al. 2010).

In another study (Tezuka et al. 2004), in hybrid

seedlings from the cross Haplo-Q×*N. tabacum* cv. Samsun NN, both monosomic and disomic plants were identified using the Q-chromosome-specific DNA marker OPC-11<sub>922</sub>, which was not detected in Samsun NN ( $2n=48$ ), but was detected in Haplo-Q. According to this DNA marker, the number of monosomic plants and disomic plants was almost the same. These observations indicate that the absence of the Q chromosome has no effect on the process of embryo sac development or embryogenesis. However, in seedlings of Haplo-Q×*N. nudicaulis*, the ratio of hybrids possessing the Q chromosome to hybrids lacking the Q chromosome was 1:3. These results indicated that when Haplo-Q is crossed with *N. nudicaulis*, embryogenesis is more favorable for hybrid plants lacking the Q chromosome than for hybrid plants possessing the Q chromosome. We concluded that the Q chromosome might have gene(s) that inhibit embryogenesis when Haplo-Q is crossed with *N. nudicaulis*.

Marubashi and Onosato (2002) carried out crosses of 10 monosomic lines ( $2n=47$ ; Haplo-M to Z, except for Haplo-P and Haplo-V) of *N. tabacum* and *N. suaveolens*. Only when Haplo-Q was crossed with *N. suaveolens* could viable plants be obtained. Furthermore, when Haplo-Q is crossed with *N. suaveolens* and the hybrid seedlings cultured at 36°C are transferred to 28°C, those possessing the Q chromosome die and those lacking the Q chromosome do not (Tezuka and Marubashi 2006). In the present study, hybrid seedlings both possessing and lacking the Q chromosome died within 10 days of transfer from 34 to 28°C. Based on these results, the Q chromosome is not the cause of hybrid lethality in *N. nudicaulis*×*N. tabacum*.

In this study, we inferred that both the inhibition of hybrid embryogenesis by the interaction of the Q chromosome with the genome of *N. nudicaulis* and hybrid lethality in the seedlings due to the interaction of the chromosome(s) of the S subgenome with the genome of *N. nudicaulis* existed in the cross *N. nudicaulis*×*N. tabacum*. In other words, there are two reproductive barriers acting by different mechanisms in the cross *N. nudicaulis*×*N. tabacum*. These results suggested that the gene or genes inhibiting hybrid embryogenesis is on the Q chromosome and the gene or genes causing hybrid lethality are on chromosomes other than the Q chromosome.



Currently, we are attempting to perform crosses of other monosomic lines of *N. tabacum* and *N. nudicaulis* to determine the chromosome of *N. tabacum* that is responsible for hybrid lethality of *N. nudicaulis* × *N. tabacum* crosses.

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