

Characterization of hybrid seedlings from crosses of *Nicotiana stocktonii* Brandegee \times *N. tabacum* L. and *N. stocktonii* \times progenitors of *N. tabacum*

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Abstract Hybrid lethality is one of the sexual barriers preventing wide hybridization. In the genus *Nicotiana*, hybrid lethality is observed in some interspecific cross combinations. Interspecific hybridization of *Nicotiana stocktonii* \times *N. tabacum* has not been carried out from the viewpoint of expression of lethality. *N. tabacum* has an S subgenome derived from *N. sylvestris* and a T subgenome derived from *N. tomentosiformis*. In this study, hybrid seedlings from the cross *N. stocktonii* \times *N. tabacum*, were obtained by ovule culture. The hybrid seedlings were classified as normal seedlings, tumorous types and vitrified plants, and they did not express lethality. Hybrid seedlings from the cross *N. stocktonii* \times *N. sylvestris* expressed lethality. Moreover, hybrid seedlings from *N. stocktonii* \times *N. tomentosiformis* also expressed lethality. This suggests that both the S genome of *N. sylvestris* and the T genome of *N. tomentosiformis* have factors causing lethality in hybrids with *N. stocktonii*. However, hybrid seedlings from *N. stocktonii* \times *N. tabacum* did not express lethal symptoms in this study. We suggested that the factors responsible for lethality in *N. tabacum* must have been lost or no longer expressed during the process of speciation, probably due to reorganization and modification of the genomes.

Key words: Hybrid lethality, *Nicotiana stocktonii*, *N. sylvestris*, *N. tabacum*, *N. tomentosiformis*.

Hybrid lethality is one of the sexual barriers preventing wide hybridization. In the genus *Nicotiana*, hybrid lethality is observed in some interspecific cross combinations, including the cross *Nicotiana suaveolens* \times *N. tabacum* (Inoue et al. 1994). Seedlings from *N. suaveolens* \times *N. tabacum* develop browning at the hypocotyl and die at the cotyledonary stage via apoptotic cell death (Yamada et al. 2000). The same lethal symptoms are observed in hybrids derived from the reciprocal cross through test-tube pollination and ovule culture (Tezuka and Marubashi 2004). *N. tabacum* is a natural amphidiploid ($2n=48$, SSTT), which presumably originated by interspecific hybridization of *N. sylvestris* ($2n=24$, SS) with *N. tomentosiformis* ($2n=24$, TT) and subsequent chromosome doubling (Gray et al. 1974; Murad et al. 2002; Sheen 1972). Inoue et al. (1996) reported that hybrids of *N. suaveolens* and *N. sylvestris* (SS) express lethality, and its features resemble those of hybrid lethality between *N. suaveolens* and *N. tabacum*. They concluded that the S subgenome in *N. tabacum* is responsible for the lethality exhibited in hybrid seedlings of *N. suaveolens* \times *N. tabacum*. Furthermore, Marubashi and Onosato (2002) crossed monosomic lines of *N. tabacum* with *N. suaveolens*. Viable hybrid seedlings were obtained when they used a monosomic line of *N.*

tabacum lacking the Q chromosome. Therefore, they proved that the Q chromosome belonging to the S subgenome of *N. tabacum* is responsible for hybrid lethality in the cross *N. tabacum* \times *N. suaveolens*.

There are four allopolyploid species in North America, *N. repanda*, *N. nudicaulis*, *N. stocktonii* and *N. nesophila*, belonging to section *Repandae* (Clarkson et al. 2004). In a previous study (Reed and Collins 1978), interspecific hybridization of *N. repanda* \times *N. tabacum* was carried out through ovule culture and the hybrid seedlings died. Furthermore, the T subgenome of *N. tabacum* is responsible for lethality in this cross, because seedlings of *N. repanda* \times *N. tomentosiformis* (TT) express lethal symptoms, whereas seedlings of *N. repanda* \times *N. sylvestris* (SS) are viable (Kobori and Marubashi 2004). Similarly, expression of hybrid lethality is observed in the cross *N. nudicaulis* \times *N. tabacum* (Liu et al. 2013). On the other hand, the S subgenome of *N. tabacum* is responsible for lethality in this cross, because seedlings of *N. sylvestris* (SS) \times *N. nudicaulis* expressed lethal symptoms, whereas seedlings of *N. nudicaulis* \times *N. tomentosiformis* (TT) were viable (Liu and Marubashi 2013).

Reed and Collins (1978) carried out interspecific hybridization in *N. stocktonii* \times *N. tabacum* through ovule culture, and obtained viable seedlings. Although

they did not describe the seedlings in detail, they did not express hybrid lethality. In the present study, we crossed *N. stocktonii* and *N. tabacum* to allow characterization of these hybrids. Furthermore, we crossed *N. stocktonii* and the two progenitors of *N. tabacum*, *N. sylvestris* (SS) and *N. tomentosiformis* (TT), to reveal the characteristics of these hybrids. We obtained different results than reported in previous studies (Inoue et al. 1996; Kobori and Marubashi 2004; Liu and Marubashi 2013) of crosses of wild species and the progenitors of allopolyploids.

Materials and methods

Plant materials

Seeds of *N. stocktonii* Brandege (2*n*=48), *N. tabacum* (2*n*=48, SSTT) 'Red Russian,' *N. sylvestris* Speg. & Comes (2*n*=24, SS) and *N. tomentosiformis* Goodsp. (2*n*=24, TT) were supplied by Japan Tobacco Inc. (Oyama, Japan) and used in the experiments. *N. sylvestris* and *N. tomentosiformis* are the progenitors of *N. tabacum*. Plants were grown and pollinated in a greenhouse of the School of Agriculture, Meiji University.

Interspecific hybridization of *N. stocktonii*×*N. tabacum* through ovule culture

Because it is difficult to obtain hybrid seedlings in conventional crossing between *N. stocktonii* and *N. tabacum*, ovule culture was carried out. Flowers of *N. stocktonii* used as the maternal parent were emasculated before flowering and pollinated with fresh *N. tabacum* pollen. At 6, 9 and 12 days after pollination, flowers of *N. stocktonii* were collected and the sepals, petals, and styles were removed. The ovaries were surface-sterilized with 70% ethanol for 30 s and then with 5% sodium hypochlorite for 10 min, and rinsed three times with sterilized water. The ovary walls were peeled to expose the placenta with intact ovules. All of the fertilized ovules were excised and cultured on Petri dishes containing 8 ml 1/2× MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.25% Gelrite, pH 5.8, at 28°C under continuous illumination (32 μmol photons s⁻¹ m⁻²).

Cultivation of hybrid plants

Immediately after germination, all hybrid plants of *N. stocktonii*×*N. tabacum* were separately 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, at 28°C under continuous illumination (147 μmol s⁻¹ m⁻²). Except for tumorous types, vitrified plants and normal seedlings were transferred to culture flasks that contained 60 ml of the abovementioned medium and cultured under the same conditions for 60 days after germination (DAG). At 90 DAG, normal seedlings were potted and cultured in a greenhouse under natural lighting conditions.

Interspecific crosses of *N. stocktonii*×*N. sylvestris* and of *N. stocktonii*×*N. tomentosiformis*

Conventional crossing was carried out as follows: flowers of *N. stocktonii* used as the maternal parent were emasculated before flowering and then pollinated with fresh *N. sylvestris* or *N. tomentosiformis* pollen. F1 seeds (*N. stocktonii*×*N. sylvestris* and *N. stocktonii*×*N. tomentosiformis*) were soaked in 0.05% gibberellic acid (GA3) solution for 30 min, sterilized with 5% sodium hypochlorite for 15 min and then rinsed three times with sterilized water. Sterilized F1 seeds were sown on Petri dishes containing 8 ml 1/2× MS medium supplemented with 1% sucrose and 0.25% Gelrite, pH 5.8; the plates were maintained for seed germination at 28°C under continuous illumination (32 μmol s⁻¹ m⁻²). Each hybrid seedling was transferred at 10 DAG to flat-bottomed test tubes (25 mm diameter, 100 mm length) that contained 10 ml of the abovementioned medium and cultured under continuous illumination (147 μmol s⁻¹ m⁻²). At 30 DAG, some hybrid seedlings were transferred to culture flasks that contained 60 ml of the abovementioned medium and cultured under continuous illumination (147 μmol s⁻¹ m⁻²), and the others were potted and cultivated in a greenhouse under natural lighting conditions.

Flow cytometry

For cytometric analysis, nuclei were isolated from 100 mg of leaves (except for midrib) of *N. stocktonii*×*N. tabacum*, *N. stocktonii*×*N. sylvestris*, and *N. stocktonii*×*N. tomentosiformis* hybrid seedlings and each hybrid parent; the leaves were chopped off and macerated in ice-cold buffer (Michaelson et al. 1991). The macerated tissue was filtered through a 25 mm nylon mesh. Nuclei were collected from the filtrate by centrifugation (5 min at 3000 rpm; 700×*g* and 4°C) and suspended in ice-cold buffer supplemented with 5 ml/ml DAPI for 1 min at 4°C. The DNA content of the isolated nuclei was analyzed by flow cytometry on a Cell Lab Quanta SC system (Beckman Coulter Inc., La Brea, CA, USA). Between 16,000 and 22,000 nuclei were counted.

Random amplified polymorphic DNA (RAPD) analysis

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 OPE; Operon Technologies, Inc., Alameda, CA, USA). The reaction mixture contained 5 μl 10× Ex Taq Buffer (Takara Bio Inc., Otsu, Japan), 4 μl dNTP mixture (Takara Bio Inc.), 1 μl primer, 0.25 μl TaKaRa Ex Taq (Takara Bio Inc.), and 100 ng DNA in a total volume of 50 μl. PCR amplification was performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed for 2 min at 98°C for initial denaturation, followed by 45 cycles of 10 s at 98°C, 30 s at 37°C for seedlings of *N. stocktonii*×*N. tabacum* or 39°C for seedlings of *N. stocktonii*×*N. sylvestris*, then 30 s at 72°C, and a final

extension of 1 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.

Results

Viable hybrid plants of N. stocktonii×*N. tabacum*

When the cross *N. stocktonii*×*N. tabacum* was carried out via conventional cross-pollination, no hybrid seedlings were obtained after fertilization. Ovule culture was necessary for obtaining hybrid seedlings. After emasculated flowers of *N. stocktonii* were pollinated with fresh *N. tabacum* pollen, the 1536 fertilized ovules obtained were excised from *N. stocktonii*×*N. tabacum* 6, 9 and 12 days after pollination. The results of ovule culture are shown in Table 1. We classified the seedlings obtained based on morphology into normal seedlings, tumorous types, and vitrified plants. After ovule culture, 31 hybrid plants had germinated by 3 weeks. Of the germinating seedlings, only 7 were normal seedlings. They did not express any lethal symptoms, and grew normally until at least the flowering stage (Figure 1A). To determine whether the normal seedlings were true hybrids, we carried out morphological observation, flow cytometry, RAPD analysis. The normal seedlings that flowered displayed uniform morphological characteristics. The shapes of their leaves intermediate in appearance between those of the parents. The flower color was intermediate in appearance between those of the parents. The flower length was longer than those of parents (Figure 1B–D).

By flow cytometric analysis, it seemed that the normal seedlings were true hybrids (Figure 1E). The DAPI fluorescence values of the G1 peaks of *N. tabacum*, the normal seedlings and *N. stocktonii* appeared mostly the same. Because we could not confirm hybridity, we carried out RAPD analysis. The RAPD patterns obtained are shown in Figure 1F. RAPD analysis using OPE-14 was carried out on two normal seedlings. Because several characteristic bands of both parents coexisted in these plants (Figure 1F, arrows), they appear to be true hybrids.

Of the hybrid plants obtained, 16 were tumorous types (Figure 2A) and 8 were vitrified plants (Figure 2B). The tumorous types rooted, but did not grow later. Vitrified plants had vigorous shoots. These tumorous types and vitrified plants did not express any lethal symptoms.

Nonviable hybrid plants of N. stocktonii×*N. sylvestris*

The results of conventional crossing between *N. stocktonii* and *N. sylvestris* are shown in Table 2. We classified the seedlings obtained based on morphology into normal seedlings, tumorous types, and vitrified plants. Hybrid seeds germinated normally. The early growth of the seedlings was normal and there were no tumorous types or vitrified plants. However, almost all normal seedlings expressed lethal symptoms and died at the vegetative stage.

Almost all normal seedlings showed normal growth and appearance until 30 DAG. After the seedlings were potted, they grew and had about 10–20 leaves in the greenhouse. By 40 DAG, the hybrid plants gradually turned yellow and brown, and died between 50 and 120 DAG (Figure 3A).

Interestingly, four of the nonviable plants with leaves that turned yellow and brown at an early stage grew new shoots. We designated these revived plants. Though the new shoots developed into green plants, all of them died before flowering (Figure 3B–D). They had both epinastic leaves (Figure 3C, arrowhead) and hyponastic leaves (Figure 3C, arrow). Based on histogram patterns from flow cytometric analysis, the epinastic leaves and hyponastic leaves were different (Figure 3E). The pattern of epinastic leaves was normal, showing a G1 peak that was higher than the G2/M peak. On the other hand, the pattern of hyponastic leaves showed a G2/M peak that was higher than the G1 peak. It seemed that epigenetic mechanisms affected the regrowth of the revived plants and the coexistence of epinastic and hyponastic leaves. Moreover, the RAPD pattern of epinastic leaves and hyponastic leaves was the same. The RAPD pattern obtained with primer OPE-14 is shown in Figure 3F.

Only one normal seedling was viable. This normal seedling did not express any lethal symptoms. It was grown to maturity and flowered in the greenhouse (Figure 4A). To determine whether this normal seedling was a true hybrid, we carried out morphological observation, flow cytometry, and RAPD analysis. The plant flowered and displayed uniform morphological characteristics. The shapes of leaves and flowers were intermediate in appearance between those of the parents. The flower color of both the viable plant and its parents was white (Figure 4B–D). Flow cytometric analysis suggested that the normal seedling was a true hybrid

Table 1. Interspecific hybridization of *N. stocktonii*×*N. tabacum* through ovule culture.

Days after pollination	No. of ovaries cultured	No. of ovules cultured	No. of hybrids germinating	Normal seedlings		Tumorous		Vitrified	
				Nonviable	Viable	Nonviable	Viable	Nonviable	Viable
6	3	525	28	0	5	0	15	0	8
9	3	496	2	0	1	0	1	0	0
12	3	515	1	0	1	0	0	0	0

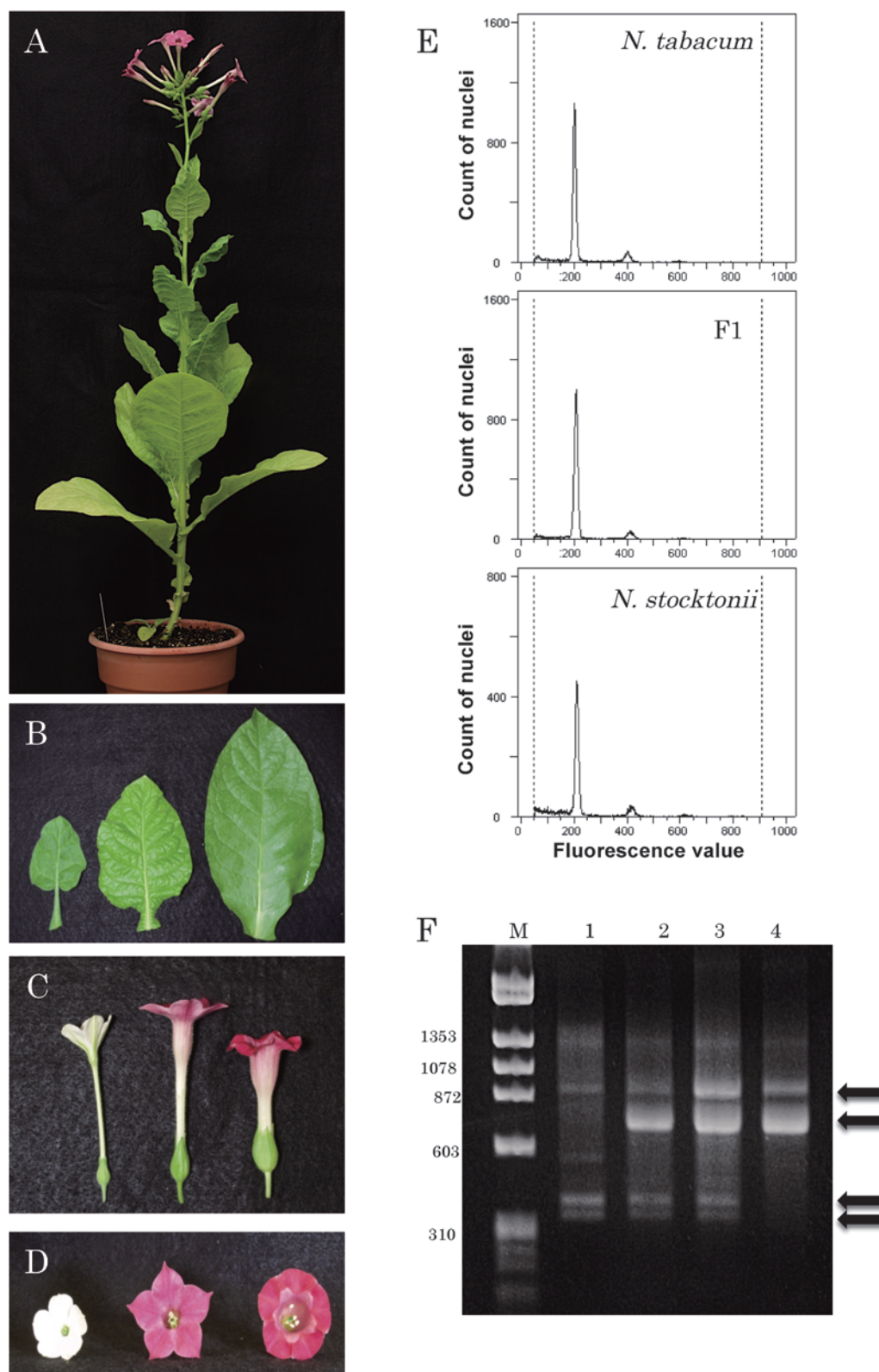


Figure 1. Hybrid *N. stocktonii* × *N. tabacum* obtained through ovule culture. (A) Shape of a viable plant that has grown to maturity and flowered. (B) Leaves of *N. stocktonii*, a hybrid seedling and *N. tabacum* (left to right). (C and D) Flowers of *N. stocktonii*, a hybrid seedling and *N. tabacum* (left to right). (E) Nuclear DNA contents of *N. tabacum*, a hybrid seedling and *N. stocktonii*. (F) Confirmation of hybrid formation by RAPD analysis using primer OPE-14. M: DNA ladder marker, Lane 1: *N. stocktonii*, Lanes 2 and 3: hybrid seedling from a *N. stocktonii* × *N. tabacum* cross, Lane 4: *N. tabacum*.

Table 2. Interspecific hybridization of *N. stocktonii* × progenitors of *N. tabacum* through conventional crossing.

Cross combination	No. of seeds sown	No. of hybrids germinating	Normal seedlings		Tumorous		Vitrified	
			Nonviable	Viable	Nonviable	Viable	Nonviable	Viable
<i>N. stocktonii</i> × <i>N. sylvestris</i>	102	58	57 (4)*	1	0	0	0	0
<i>N. stocktonii</i> × <i>N. tomentosiformis</i>	96	60	60	0	0	0	0	0

* 4 plants revived. They had lost their shoots at an early stage and revived but then died before flowering.

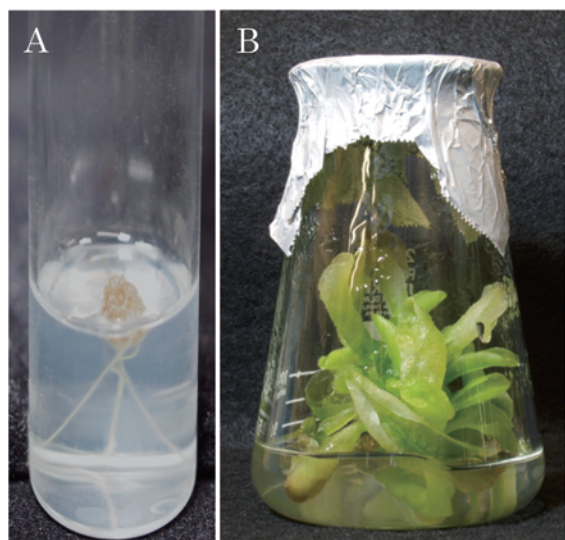


Figure 2. Other types of *N. stocktonii* × *N. tabacum* hybrids obtained through ovule culture. (A) A tumorous plant. (B) A vitrified plant.

(Figure 4E). The DAPI fluorescence value of the G1 peak of the viable plant was intermediate between those of the parents. The RAPD patterns obtained are shown in Figure 4F. RAPD analysis using OPE-14 was carried out on the normal seedling. Because several characteristic bands of both parents coexisted in the plant (Figure 4F, arrows), it appears to be a true hybrid. Because this normal seedling was an exceptional phenomenon in this study, we did not include it in the subsequent discussion.

Nonviable hybrid plants of *N. stocktonii* × *N. tomentosiformis*

The results of conventional crossing between *N. stocktonii* and *N. tomentosiformis* are shown in Table 2. As before, based on their morphology, we classified the seedlings obtained into normal seedlings, tumorous types, and vitrified plants. The hybrid seeds obtained germinated normally. The growth of the seedlings was normal and there were no tumorous types or vitrified plants. All normal seedlings expressed lethal symptoms. After the roots of the seedlings turned brown and the leaves turned yellow, all of the normal seedlings died by 50 DAG (Figure 5A, B). To determine whether the normal seedlings were true hybrids, we carried out flow cytometry while they were still alive. Flow cytometric analysis suggested that the normal seedlings were true

hybrids (Figure 5C). The DAPI fluorescence values of the G1 peak of the seedlings were intermediate between those of the parents.

Discussion

When *N. repanda* and *N. nudicaulis* (section *Repandae*) were crossed with *N. tabacum*, the hybrid seedlings expressed lethal symptoms. Zhou et al. (1991) reported that hybrid seedlings of *N. repanda* × *N. tabacum* obtained through ovule culture had lethal symptoms, which they called hybrid inviability, including poor root systems, retarded growth, and eventual death. Liu et al. (2013) reported that hybrid seedlings of *N. nudicaulis* × *N. tabacum*, also obtained through ovule culture, developed browning at the shoot apex and the root tip at an early stage, and died by 4 DAG at 28°C, again showing hybrid lethality. In contrast, Reed and Collins (1978) obtained viable seedlings from *N. stocktonii* × *N. tabacum*. Moreover, when we crossed *N. stocktonii* (which also belongs to section *Repandae*) and *N. tabacum* through ovule culture, we obtained normal seedlings, tumorous types and vitrified plants. These hybrid plants grew without lethal symptoms at 28°C and the normal seedlings eventually flowered in the greenhouse. In the present study, we showed that hybrid lethality was not expressed in the cross *N. stocktonii* × *N. tabacum*, including in tumorous types and vitrified plants. It seems that epigenetic mechanisms affect the outcome of the various types of offspring.

In a previous study (Kobori and Marubashi 2004), hybrid seedlings of *N. repanda* × *N. sylvestris* did not express hybrid lethality and grew normally. Hybrid seedlings of *N. repanda* × *N. tomentosiformis* grew normally until 30 DAG. The shoot and the roots of the seedlings turned brown by 90 DAG, and then they died. Kobori and Marubashi (2004) concluded that the T subgenome of *N. tabacum* is responsible for lethality in the cross *N. repanda* × *N. tabacum*. Furthermore, Liu and Marubashi (2013) carried out crosses of *N. sylvestris* × *N. nudicaulis* by test tube pollination and ovule culture. The hybrid seedlings developed browning at the shoot apex and the root tip at an early stage, and died by 2 DAG at 28°C. On the other hand, hybrid seedlings of *N. nudicaulis* × *N. tomentosiformis* were viable. They concluded that the S subgenome of *N. tabacum* is

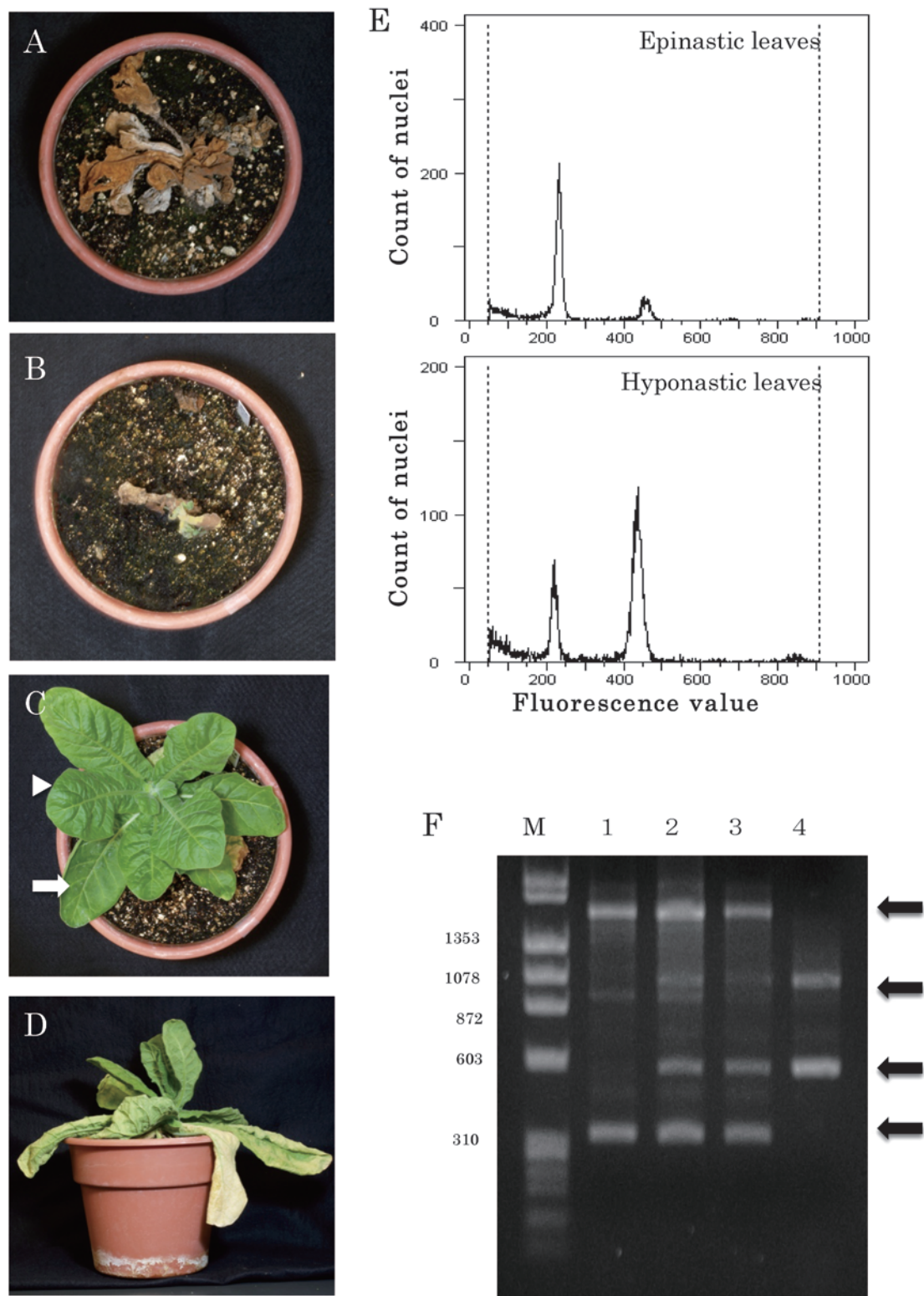


Figure 3. Other types of *N. stocktonii* × *N. sylvestris* hybrids. (A) A nonviable plant. (B) A revived plant that lost its shoots at an early stage. (C) A revived plant derived from (B) that has new shoots. This plant had both epinastic leaves (arrowhead) and hyponastic leaves (arrow). (D) A plant derived from (C) that expressed lethal symptoms and died. (E) Nuclear DNA contents of epinastic leaves of the revived plant and hyponastic leaves of the revived plant. (F) Confirmation of hybrid formation by RAPD analysis using primer OPE-14. M: DNA ladder marker, Lane 1: *N. stocktonii*, Lane 2: Revived plant that had epinastic leaves, Lane 3: Revived plant that had hyponastic leaves, Lane 4: *N. sylvestris*.

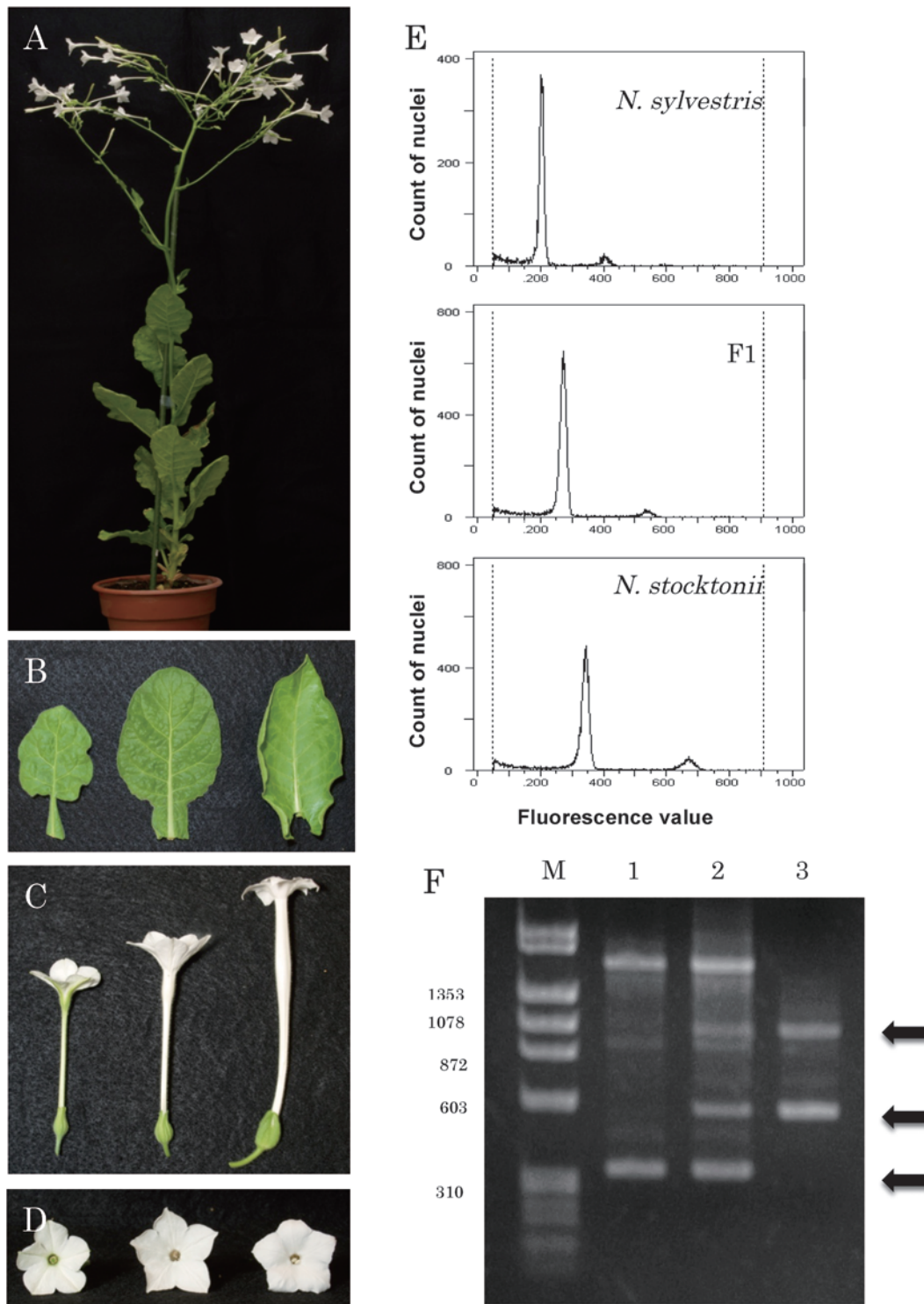


Figure 4. Hybrid of *N. stocktonii* × *N. sylvestris*. (A) Shape of a hybrid plant that has grown to maturity and flowered. (B) Leaves of *N. stocktonii*, a hybrid seedling and *N. sylvestris* (left to right). (C and D) Flowers of *N. stocktonii*, a hybrid seedling and *N. sylvestris* (left to right). (E) Nuclear DNA contents of *N. sylvestris*, a hybrid seedling and *N. stocktonii*. (F) Confirmation of hybrid formation by RAPD analysis using primer OPE-14. M: DNA ladder marker, Lane 1: *N. stocktonii*, Lane 2: hybrid seedling from a *N. stocktonii* × *N. sylvestris* cross, Lane 3: *N. sylvestris*.

responsible for lethality in the cross *N. nudicaulis* × *N. tabacum*. In the present study, we carried out crosses of *N. stocktonii* with the progenitors of *N. tabacum*. In the cross *N. stocktonii* × *N. sylvestris*, almost all normal seedlings grew at an early stage, but they expressed

lethal symptoms and died. In addition, we obtained revived plants that eventually died. Moreover, all normal seedlings of *N. stocktonii* × *N. tomentosiformis* showed lethal symptoms and died. These results suggest that both the S genome of *N. sylvestris* and the T genome of

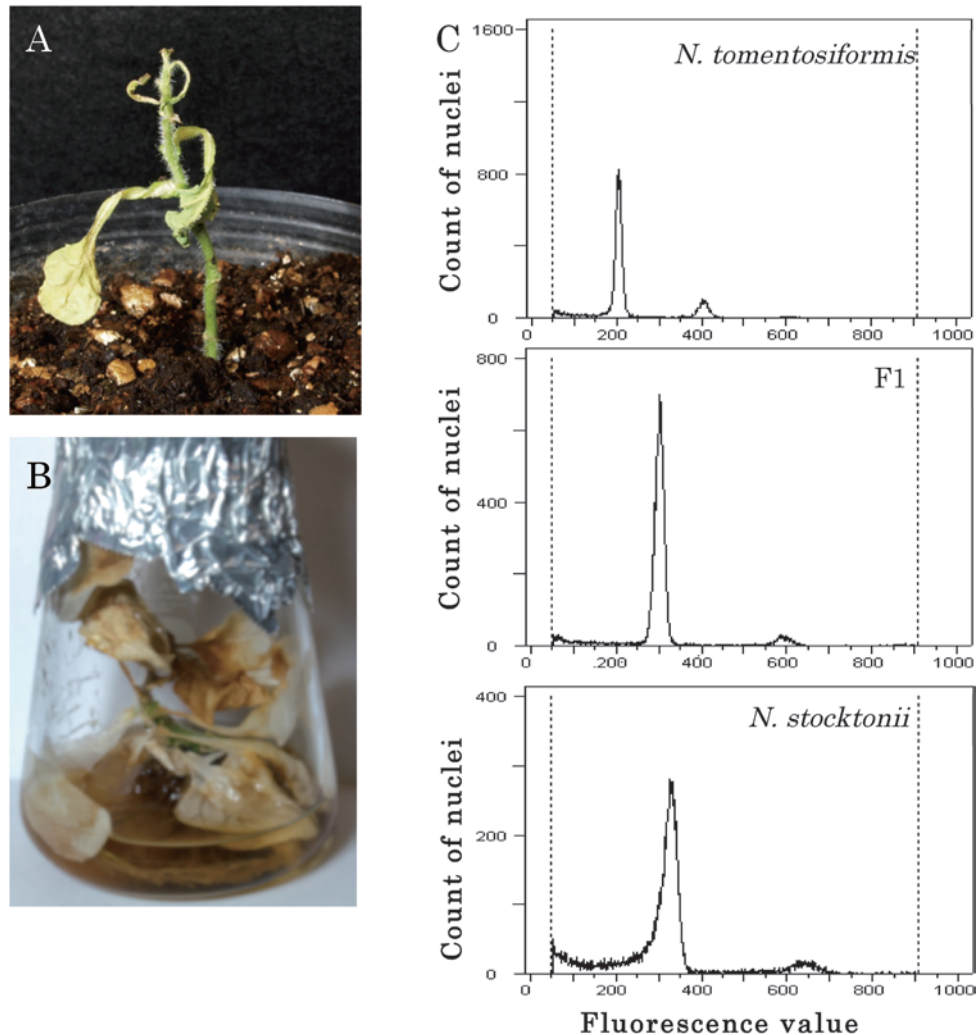


Figure 5. Hybrids of *N. stocktonii* × *N. tomentosiformis*. (A) A nonviable plant. (B) A nonviable plant in 1/2 MS medium. (C) Nuclear DNA contents of *N. tomentosiformis*, a hybrid seedling from *N. stocktonii* × *N. tomentosiformis* and *N. stocktonii*.

N. tomentosiformis have factors that can lead to hybrid lethality.

However, hybrid seedlings from *N. stocktonii* × *N. tabacum* did not express lethal symptoms in this study. Recently, the first linkage map for *N. tabacum* was constructed by Bindler et al. (2011) with SSR markers. In the *N. tabacum* genome, the S genome and T genome did not double simply and several types of variation occurred. Among the 24 linkage groups, they reported that four contain genome-specific markers from both linkage groups. We hypothesize that the factors in *N. tabacum* must have been lost or no longer expressed during the process of speciation, probably due to reorganization and modification of the genomes.

The probability of simultaneous mutation of two loci is generally very small. To resolve whether the factors leading to lethality occurred during genome reorganization and modification, we suggest crossing *N. stocktonii* with synthesized *N. tabacum* that has an S genome and T genome without the variation. This will

clarify whether simultaneous mutations occurred during the process of reorganization of the genomes.

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