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Original Paper

Tumorigenesis inheritance from the putative progenitor species of *Nicotiana rustica*

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Abstract Hybrid seedlings from crosses of *Nicotiana rustica*×*N. langsdorffii* and *N. rustica*×*N. alata* show tumors including teratomas and vitrification. In the present study, we attempted to elucidate the genetic background leading to tumorigenesis and vitrification from the viewpoint of the amphidiploidy of *N. rustica*. The species *N. undulata*, *N. paniculata*, and *N. knightiana* have been suggested to be the progenitors of *N. rustica* or closely related to its progenitors. We tested tumorigenesis in interspecific hybrids between these putative progenitors of *N. rustica* and *N. langsdorffii* or *N. alata*, which are the species in section *Alatae*. The hybrid seedlings were cultured in test tubes and their morphological characteristics were observed. According to previous reports, most of the hybrid seedlings from the crosses *N. rustica*×*N. langsdorffii* and *N. rustica*×*N. alata* formed tumors and showed vitrification. In crosses with every putative progenitor of *N. rustica*, a portion of hybrid seedlings formed tumors and showed vitrification. These observations suggested that *N. rustica* inherited the factors leading to expression of abnormal symptoms from its putative progenitors.

We also observed the influence of high temperature on the expression of abnormal symptoms of hybrid seedlings from the cross *N. rustica*×*N. alata*. While these hybrids developed teratomas and other tumors at 28°C, when cultured at 34°C, they did not show any abnormalities. This is the first report to show that phenotypic abnormalities in hybrid seedlings of *N. rustica*×*N. alata* are temperature sensitive.

Key words: interspecific hybrids, *Nicotiana rustica*, temperature sensitivity, Tumorigenesis.

Introduction

In the genus *Nicotiana*, genetic tumors are observed in some interspecific cross combinations, including the crosses *N. rustica*×*N. langsdorffii* and *N. rustica*×*N. alata* (Kostoff 1930). Some investigations of genetic tumors have been conducted. In particular, hybrids of *N. glauca*×*N. langsdorffii* were selected as interesting experimental material (Ichikawa and Syono 1991). Investigations of the relationship between tumorigenesis and internal phytohormones have also been carried out (Kung et al. 1991). In all, 17 distinct cDNA clones were isolated from genetic tumor tissues (Fujita et al. 1994). Ng*rol* genes were inferred to be related to genetic tumor formation (Nagata et al. 1996). On the other hand, very few experiments have been done using hybrids obtained from the crosses *N. rustica*×*N. langsdorffii* or *N. rustica*×*N. alata.*

N. rustica appears to have resulted from spontaneous interspecific hybridization of *N. undulata* with *N. paniculata* (Matyasek et al. 2003; Lim et al. 2005), and it has been inferred that *N. paniculata* and *N. knightiana* are closely related (Aoki and Ito 2000). Furthermore, *N. knightiana* could be a parent of *N. rustica* (Chase et al. 2003). This study investigated *N. undulata*, *N. paniculata*, and *N. knightiana*, the likely progenitors of *N. rustica*.

Tumorigenesis and hybrid lethality are known obstacles for wide hybridization that appear in *Nicotiana* hybrid seedlings (Tezuka 2012). Inoue et al. (1996) suggested that the S subgenome in *N. tabacum* possesses the factors controlling hybrid lethality expressed in crosses between *N. suaveolens* and *N. tabacum*. Marubashi and Onosato (2002) identified one chromosome (the Q chromosome) of *N. tabacum* controlling hybrid lethality in crosses between *N. tabacum* and *N. suaveolens*. Tezuka et al. (2012) reported that the Q chromosome, corresponding to linkage group 11 (Bindler et al. 2011), possesses the gene(s) responsible for hybrid lethality.

Liu and Marubashi (2013) investigated the genetic cause of hybrid lethality occurring in the cross *N. nudicaulis*×*N. tabacum* by crossing *N. nudicaulis* with two progenitors (*N. sylvestris* and *N. tomentosiformis*) of *N. tabacum*. They reported that the species origin of lethality was the S subgenome in *N. tabacum*, derived from *N. sylvestris*. Liu and Marubashi (2014)

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speculated on the relationship between vitrification and hybrid lethality, because fragmentation of nuclei during progression of lethal symptoms in hybrids was accompanied by vitrification. Liu et al. (2013) reported that the expression of hybrid lethality observed in the interspecific cross *N. nudicaulis*×*N. tabacum* was inhibited at high temperature.

We applied the analytical methods used for investigations of hybrid lethality to identify the relationship between tumors and lethality in *Nicotiana* hybrids. This report focuses on tumorigenesis including teratoma formation to reveal genetic factors inherited from *N. rustica* and its putative progenitors.

Materials and methods

Plant materials

Seeds of *N. rustica* L. (2n=48), *N. undulata* Ruiz & Pavon (2n=24), *N. paniculata* L. (2n=24), *N. knightiana* Goodsp. (2n=24), *N. langsdorffii* Weinmann (2n=18), and *N. alata* Link & Otto (2n=18) used in the experiments were supplied by Japan Tobacco Inc. (Oyama, Japan). Plants were grown and pollinated in a greenhouse of the School of Agriculture, Meiji University.

Interspecific crosses

Conventional crossing and sowing were carried out as follows: flowers of *N. rustica* and the putative progenitors of *N. rustica* used as maternal parents were emasculated immediately before pollination with pollen of *N. langsdorffii* or *N. alata*. F1 seeds were soaked in 0.05% gibberellic acid (GA_3) 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 (Murashige and Skoog 1962), pH 5.8, supplemented with 1% sucrose and 0.25% Gelrite; the petri dishes were maintained at 28°C under continuous illumination (30 μmol m^{-2}s^{-1}) to germinate seeds.

Cultivation and classification of hybrid seedlings

Immediately after germination, hybrid seedlings were transferred to test tubes that contained 10 ml 1/2 MS medium (described above), and cultured at 28°C for 50 days under continuous illumination (147–160 μmol m^{-2} s^{-1}). The culture medium used for the hybrid seedlings contained no plant growth regulators. Because seedlings of parental species grow normally on this medium, we did not set up control groups using parental species. Hybrid seedlings were classified as phenotypically normal or abnormal based on observation. Hybrid seedlings that formed tumors including teratomas (tumors with malformed leaf-like and stem-like structures), exhibited vitrification or developed symptoms of hybrid lethality were regarded as abnormal. A symptom that was observed frequently in a cross combination was regarded as the main reaction of the cross.

Morphological observation

Phenotypically normal plants cultured in test tubes for 50 days at 28°C under continuous illumination (147–160 μmol m^{-2} s^{-1}) were potted and cultured in a greenhouse under natural lighting conditions. To confirm hybridity, the morphology of flowers and leaves of normal plants and their parent species were compared.

Chromosome analysis

Root tips of hybrid seedlings 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 for 1 h to determine chromosome number. The root tips were hydrolyzed in 0.94 mol l^{-1} HCl for 8 min at 60°C, stained with Schiff's reagent and squashed in 45% acetic acid. The number of chromosomes for each plant was counted under a BX51 light microscope (Olympus, Tokyo, Japan), and photographed using a DP70 automatic photomicrography system (Olympus).

Flow cytometry

For cytometric analysis, nuclei were isolated from 200 to 300 mg of leaves (except midrib), petals, tumors, or vitrified tissue of hybrid seedlings and each hybrid parent; these materials were macerated in ice-cold buffer (Michaelson et al. 1991). The macerated tissue was filtered through a 25 μm nylon mesh. Nuclei were collected from the filtrate by centrifugation (5 min at 700 *g* and 4°C) and suspended in ice-cold buffer supplemented with 150 μl DAPI (Nuclear isolation and staining solution-10; NPE Systems, Pembroke Pines, FL, USA) for 5 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., Brea, CA, USA). At most 20,000 nuclei were counted.

Table 1. Interspecific hybridization between *N. rustica* and the putative progenitors of *N. rustica* and *N. langsdorffii*.

Cross combination		No. of hybrids germinating	No. of hybrids obtained	
Maternal	Paternal		Normal	Abnormal
N. rustica	*N. langsdorffii*	41	1	40 (0.976)
N. undulata		39	30	9 (0.231)
N. paniculata		51	23	28 (0.549)
N. knightiana		37	6	31 (0.838)

Values in parentheses are the rate of abnormal plants. Abnormal: plants forming teratomas or other tumors, showing vitrification or malformation, or dying. Normal: plants not categorized as abnormal.

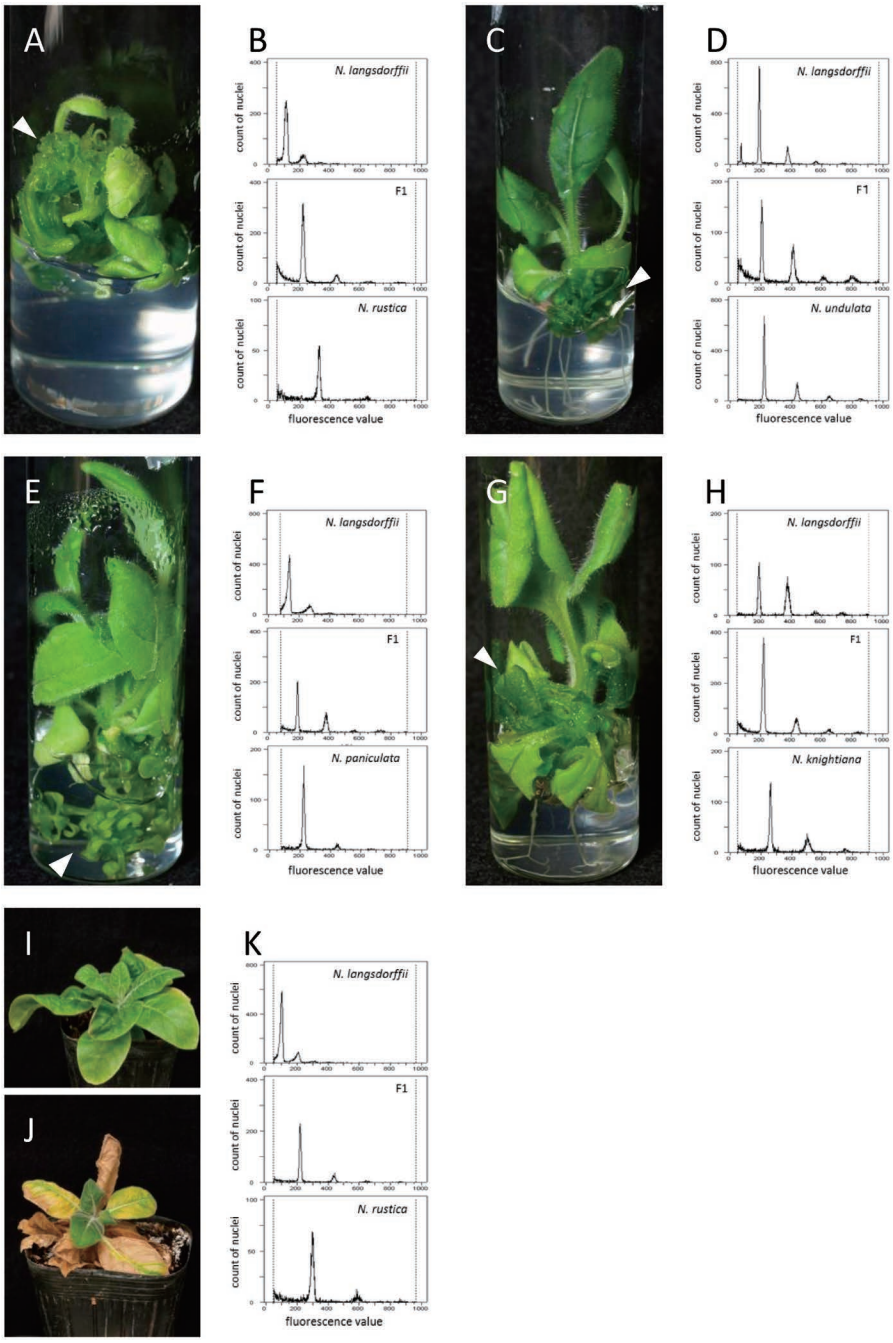

Figure 1. Hybrid of *N. rustica*×*N. langsdorffii* (abnormal); (A) Morphology of abnormal plant that showed vitrification. The arrowhead indicates vitrification. (B) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. rustica*. Hybrid of *N. undulata*×*N. langsdorffii* (abnormal); (C) Morphology of abnormal plant that showed vitrification. The arrowhead indicates vitrification. (D) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. undulata*. Hybrid of *N. paniculata*×*N. langsdorffii* (abnormal); (E) Morphology of abnormal plant that formed teratomas (arrowhead). (F) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. paniculata*. Hybrid of *N. knightiana*×*N. langsdorffii* (abnormal); (G) Morphology of abnormal plant that showed vitrification. The arrowhead indicates vitrification. (H) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. knightiana*. Hybrid of *N. rustica*×*N. langsdorffii* (normal) (I) Morphology of a normal plant. (J) Hybrid seedling that withered and died. (K) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. rustica*.

Cultivation and classification of hybrid seedlings from crosses between N. rustica and N. alata at different temperatures (28°C or 34°C)

After germination at 28°C, hybrid seedlings were transferred to test tubes that contained 10 ml 1/2 MS medium (described above) and cultured at 34°C for 30 days under continuous illumination (32 μmol m^{-2} s^{-1}). Subsequently, approximately half of the phenotypically normal hybrid seedlings were transferred from 34°C to 28°C or left at 34°C and cultured for 50 days under continuous illumination (30 μmol m^{-2} s^{-1}).

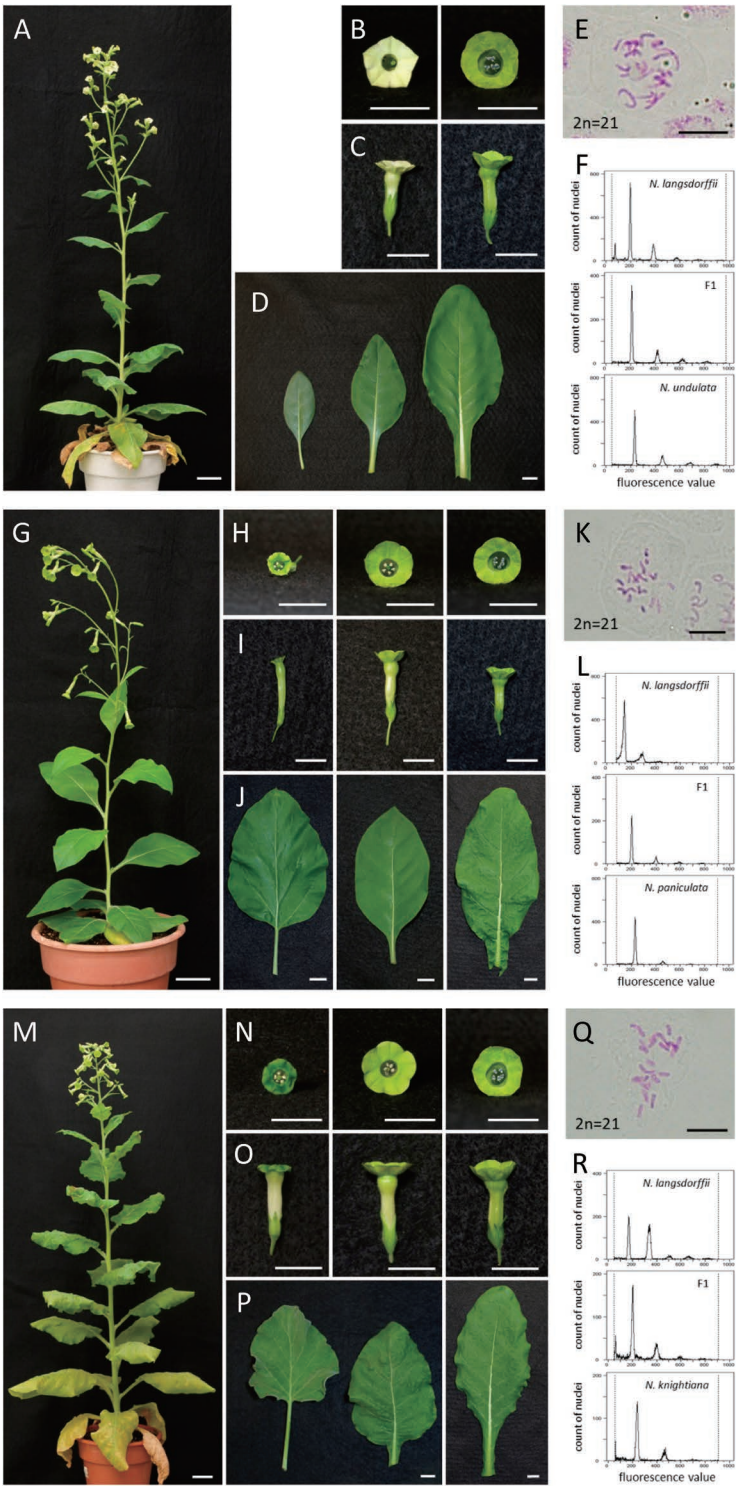

Figure 2. Hybrid of *N. undulata*×*N. langsdorffii* (normal); (A) Morphology of a normal plant that has grown to maturity and flowered. (B, C) Flowers of a hybrid seedling and *N. langsdorffii* (left to right). (D) Leaves of *N. undulata*, a hybrid seedling, and *N. langsdorffii* (left to right). (E) Image of root tip cell of a hybrid plant, showing the number of chromosomes. (F) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. undulata*. Scale bars are 4 cm (A), 1.5 cm (B, C, D) and 10 μm (E). Hybrid of *N. paniculata*×*N. langsdorffii* (normal); (G) Morphology of a normal plant that has grown to maturity and flowered. (H, I) Flowers of *N. paniculata*, a hybrid seedling, and *N. langsdorffii* (left to right). (J) Leaves of *N. paniculata*, a hybrid seedling, and *N. langsdorffii* (left to right). (K) Image of root tip cell of a hybrid plant, showing the number of chromosomes. (L) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. paniculata*. Scale bars are 5 cm (G), 1.5 cm (H, I, J) and 10 μm (K). Hybrid of *N. knightiana*×*N. langsdorffii* (normal); (M) Morphology of a normal plant that has grown to maturity and flowered. (N, O) Flowers of *N. knightiana*, a hybrid seedling, and *N. langsdorffii* (left to right). (P) Leaves of *N. knightiana*, a hybrid seedling, and *N. langsdorffii* (left to right). (Q) Image of root tip cell of a hybrid plant, showing the number of chromosomes. (R) Nuclear DNA content of *N. langsdorffii*, a hybrid seedling, and *N. knightiana*. Scale bars are 5 cm (M), 1.5 cm (N, O, P) and 10 μm (Q).

Table 2. Interspecific hybridization between *N. rustica* and the putative progenitors of *N. rustica* and *N. alata*.

Cross combination		No. of hybrids germinating	No. of hybrids obtained	
Maternal	Paternal		Normal	Abnormal
N. rustica	*N. alata*	72	0	72 (1.00)
N. undulata		—	—	—
N. paniculata		36	18	18 (0.50)
N. knightiana		39	23	16 (0.41)

— No cross combination obtained due to poor development of *N. undulata*.

Results

Characteristics of interspecific hybrids between N. rustica, the putative progenitors of N. rustica, and N. langsdorffii

Hybrid seeds obtained from the crosses of *N. rustica*×*N. langsdorffii*, and hybrid seeds of putative progenitors of *N. rustica*×*N. langsdorffii* were germinated at 28°C. Hybrid seedlings were cultured for 50 days at 28°C, then classified as normal or abnormal plants based on their observed characteristics.

Normal and abnormal plants were obtained from all crosses (Table 1). To confirm hybridity of the normal plants, morphological observation, chromosome analysis and flow cytometry were carried out. For the abnormal plants, only flow cytometry was carried out.

Almost all of the hybrid seedlings (97.6%) from the cross *N. rustica*×*N. langsdorffii* were classified as abnormal and most showed vitrification (Figure 1A). Flow cytometry was performed to clarify hybridity of vitrified abnormal plants (Figure 1B). The areas of the G1 peaks during the cell cycle of vitrified seedlings were intermediate between those of their parents. Based on this result, we confirmed that the vitrified seedlings were true hybrids. In the crosses of *N. undulata*×*N. langsdorffii*, *N. paniculata*×*N. langsdorffii*, and *N. knightiana*×*N. langsdorffii*, respectively 23.1%, 54.9%, and 83.8% of hybrid seedlings showed morphological abnormalities, such as teratomas, other tumors, or vitrification (Table 1 and Figures 1C–H). Flow cytometric analysis indicated that abnormal seedlings showed DNA content intermediate between the parental species, indicative of hybrids.

One normal plant obtained from the cross *N. rustica*×*N. langsdorffii* withered and died before flowering (Figure 1I, J, K), while normal plants obtained from the crosses *N. undulata*×*N. langsdorffii*, *N. paniculata*×*N. langsdorffii*, and *N. knightiana*×*N. langsdorffii* grew to maturity. These plants were presumed to be true hybrids based on morphological observation, chromosome analysis and flow cytometry (Figure 2). The flowers of *N. undulata* are not shown because there were very few and all were used for cross-pollination.

Characteristics of interspecific hybrids between N. rustica, the putative progenitors of N. rustica (except N. undulata), and N. alata

Hybrid seeds obtained from the cross *N. rustica*×*N. alata*, and hybrid seeds of putative progenitors (except for *N. undulata*) of *N. rustica*×*N. alata* were germinated at 28°C. Hybrid seedlings were cultured for 50 days at 28°C, and classified as normal or abnormal based on observed characteristics.

In the cross *N. rustica*×*N. alata*, all hybrid seedlings (100%) were abnormal (Table 2), and most formed tumors or showed vitrification (Figure 3A). The vitrified seedlings were presumed to be true hybrids based on flow cytometry (Figure 3B). In the crosses of *N. paniculata*×*N. alata* and of *N. knightiana*×*N. alata*, respectively 50.0% and 41.0% of hybrid seedlings showed morphological abnormalities, such as tumors, teratomas, or vitrification (Table 2 and Figures 3C–F). Flow cytometric analysis indicated that abnormal seedlings showed DNA content intermediate between the parental species, indicative of hybrids.

In both crosses, *N. paniculata*×*N. alata* and *N. knightiana*×*N. alata*, mature normal plants were presumed to be true hybrids based on morphological observation, chromosome analysis and flow cytometry (Figures 3G–R).

Temperature-sensitive lethality in N. rustica×N. alata hybrid seedlings

Hybrid seeds obtained from the cross *N. rustica*×*N. alata* were germinated at 28°C. Hybrid seedlings were cultured for 30 days at 28°C or 34°C (Table 3). All hybrid seedlings cultured at 28°C were abnormal (Figure 4A). Both normal and abnormal plants were obtained from hybrid seedlings cultured at 34°C (Figure 4B).

Fifteen phenotypically normal hybrid seedlings obtained at 34°C were transferred to 28°C and cultured for 50 days. The remaining normal plants were kept at 34°C for 50 days (Table 4); they continued to grow normally (Figure 4C). However, after apparently normal plants were transferred to 28°C, abnormal tissues arose at their shoot apices (Figures 4D–F). All of the normal plants from this cross had become abnormal by 50 days after transfer to 28°C (Figure 4G).

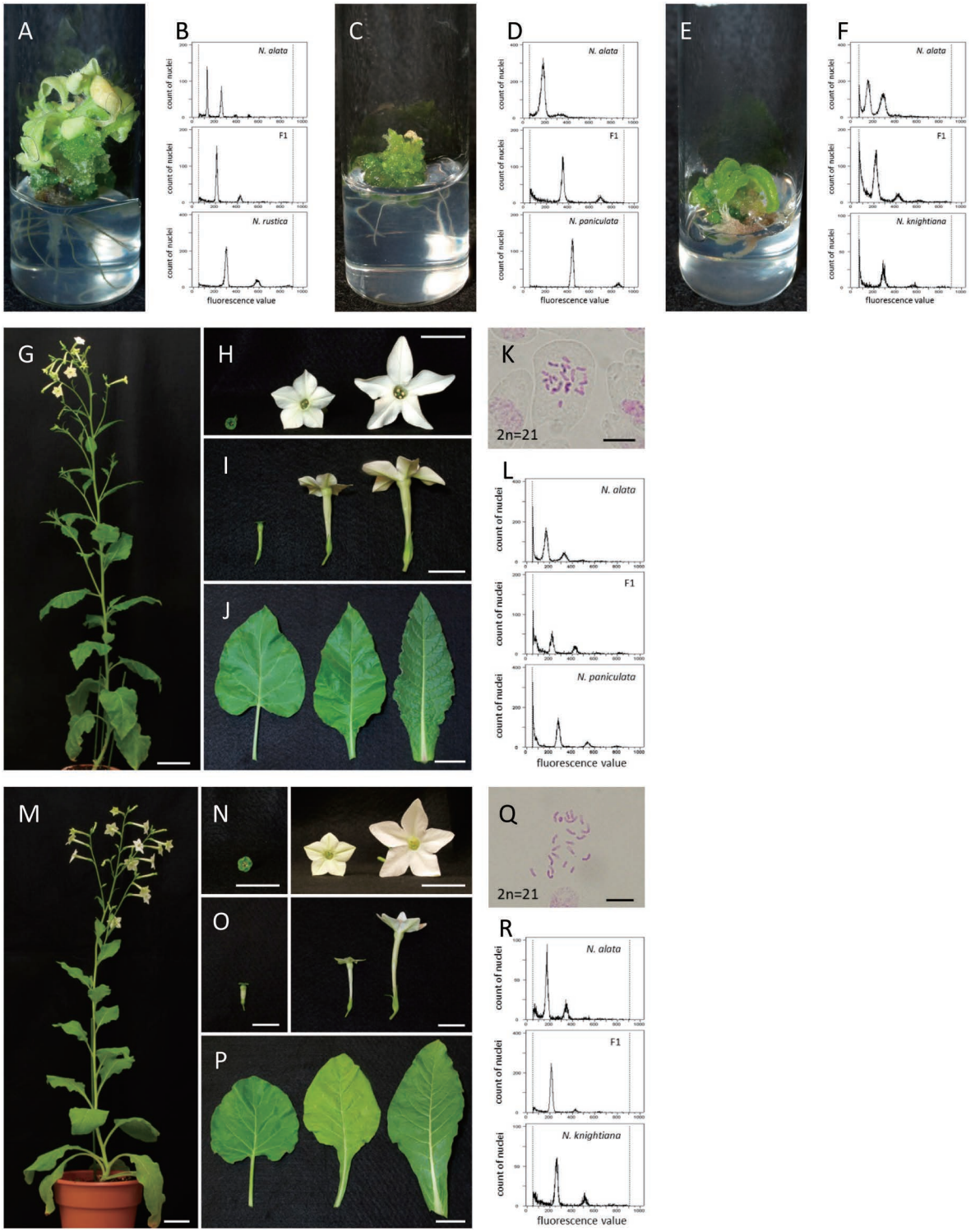

Figure 3. Hybrid of *N. rustica*×*N. alata* (abnormal); (A) Morphology of abnormal plant that formed a tumor. (B) Nuclear DNA content of *N. alata*, a hybrid seedling, and *N. rustica*. Hybrid of *N. paniculata*×*N. alata* (abnormal); (C) Morphology of abnormal plant that formed a tumor. (D) Nuclear DNA content of *N. alata*, a hybrid seedling, and *N. paniculata*. Hybrid of *N. knightiana*×*N. alata* (abnormal); (E) Morphology of abnormal plant that showed vitrification. (F) Nuclear DNA content of *N. alata*, a hybrid seedling, and *N. knightiana*. Hybrid of *N. paniculata*×*N. alata* (normal); (G) Morphology of a normal plant that has grown to maturity and flowered. (H, I) Flowers of *N. paniculata*, a hybrid seedling, and *N. alata* (left to right). (J) Leaves of *N. paniculata*, a hybrid seedling, and *N. alata* (left to right). (K) Image of root tip cell of a hybrid plant, showing the number of chromosomes. (L) Nuclear DNA content of *N. alata*, a hybrid seedling, and *N. paniculata*. Scale bars are 10 cm (G), 3 cm (H, I, J) and 10 μm (K). Hybrid of *N. knightiana*×*N. alata* (normal); (M) Morphology of a normal plant that has grown to maturity and flowered. (N, O) Flowers of *N. knightiana*, a hybrid seedling, and *N. alata* (left to right). (P) Leaves of *N. knightiana*, a hybrid seedling, and *N. alata* (left to right). (Q) Image of root tip cell of a hybrid plant, showing the number of chromosomes. (R) Nuclear DNA content of *N. alata*, a hybrid seedling, and *N. knightiana*. Scale bars are 5 cm (M), 3 cm (N, O, P) and 10 μm (Q).

Table 3. Classification of hybrid seedlings of *N. rustica*×*N. alata* cultured for 30 days.

Temperature	No. of hybrids germinating	No. of hybrids obtained[1]	
		Normal	Abnormal
28°C	16	0	16
34°C	45	34	11

[1] Cultured in test tube for 30 days at 28°C or 34°C.

Table 4. Occurrence of abnormal phenomena inhibited by high temperature.

Temperature	Total[1]	No. of hybrids obtained[2]	
		Normal	Abnormal
(34°C→) 28°C	15	0	15
(34°C→) 34°C	19	19	0

[1] Plants were identical to normal plants represented in Table 3. [2] Cultured in test tube for 50 days at 28°C or 34°C.

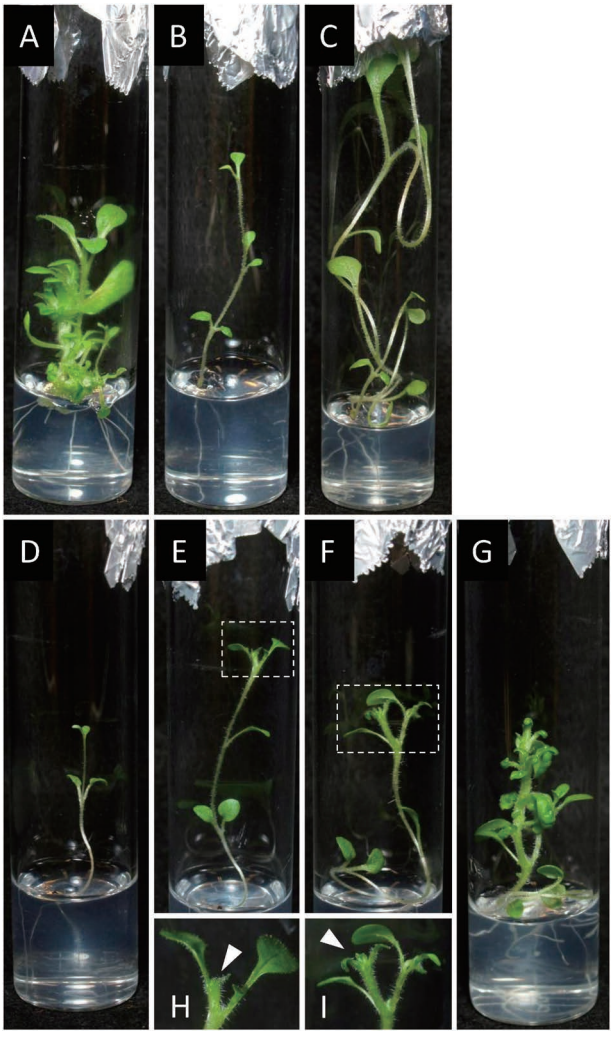

Figure 4. Phenotypically normal *N. rustica*×*N. alata* hybrid plant transferred from 34°C to 28°C that became abnormal (A) A hybrid plant cultured at 28°C for 30 days after the plant was transferred to a test tube. (B, C) Phenotypically normal hybrid plants cultured respectively at 34°C for 30 and 80 days in test tubes. (D, E, F, G) Plants transferred from 34°C to 28°C. (D) A normal hybrid plant cultured at 34°C for 30 days in a test tube. (E) A hybrid seedling making abnormal tissue 12 days after transfer to 28°C. (F) An abnormal hybrid seedling 20 days after transfer to 28°C. (G) An abnormal hybrid seedling 50 days after transfer to 28°C. (H) and (I) enlarged views of E and F, respectively. Arrowheads indicate teratomas.

Discussion

Kostoff (1930) and Näf (1958) reported that specific progeny obtained in the genus *Nicotiana* formed tumors. Hybrid seedlings from the cross *N. rustica*×*N. langsdorffii* were some of these progeny. In this study, we cultured hybrid seedlings from the cross *N. rustica*×*N. langsdorffii*, and found that these hybrids formed tumors including teratomas and showed vitrification. In previous studies, *N. rustica* was found to be amphidiploid (Goodspeed 1954), and it was inferred that the progenitors of *N. rustica* were *N. undulata* and *N. paniculata* (Matyasek et al. 2003; Lim et al. 2005). We presumed that abnormal symptoms arose in hybrid seedlings of *N. rustica*×*N. langsdorffii* as a result of factors originating from one of the progenitors. We grew hybrid seedlings under controlled media conditions, so that our experiments were able to identify genetic reactions of hybrid seedlings and clarify the relationship between an amphidiploid and its putative progenitors in terms of tumorigenesis or vitrification.

We crossed *N. rustica* and each of its presumptive progenitors (*N. undulata*, *N. paniculata*, *N. knightiana*) with *N. langsdorffii*. All but one hybrid seedling obtained from the cross *N. rustica*×*N. langsdorffii* showed abnormal symptoms. A portion of the hybrid seedlings obtained from the crosses of putative progenitors of *N. rustica* (*N. undulata*, *N. paniculata*, *N. knightiana*) with *N. langsdorffii* were classified as abnormal. Previous studies also reported tumor formation in hybrid seedlings from the cross *N. paniculata*×*N. langsdorffii* (Dremljug 1936; Kostoff 1930). The finding that seedlings from the cross *N. undulata*×*N. langsdorffii* showed tumorigenesis or vitrification is new. Almost all of the hybrids from the cross *N. rustica*×*N. langsdorffii* showed abnormal symptoms, while some of the hybrids of the other crosses did. These results suggested that the factor inducing abnormal symptoms is present not in a specific progenitor but in all putative progenitors of *N. rustica*, and that the ability of genes to induce morphological abnormalities derived from each the progenitors differed. In consequence, morphological abnormalities in *N. rustica* possibly occurred due to accumulated effects of genetic factors derived from its progenitors, *N. undulata*, *N. paniculata* or *N. knightiana* (Table 1).

We investigated the genetic factors inherited from *N. rustica* and its putative progenitors responsible for abnormal symptoms in seedlings from the cross *N. rustica*×*N. alata* similarly to the cross *N. rustica*×*N. langsdorffii*. Only *N. paniculata* and *N. knightiana*

were used as the male parent in crosses because *N. undulata* had very few flowers. All of the hybrid seedlings obtained from the cross *N. rustica*×*N. alata* presented morphological abnormalities including tumor formation. Kostoff (1930) and Näf (1958) also reported that seedlings obtained from this cross formed tumors. A portion of hybrid seedlings from the crosses *N. paniculata*×*N. alata* and *N. knightiana*×*N. alata* were also classified as abnormal. Yamada et al. (1999) reported that seedlings from the cross *N. paniculata*×*N. alata* expressed hybrid lethality, but their observations did not agree with ours. The results might depend on the genetic background of *N. alata*, which is self-incompatible. Thus, both *N. paniculata* and *N. knightiana* apparently possess factors that lead to abnormal symptoms in hybrid seedlings.

A factor or gene from a single parent is responsible for hybrid lethality in specific crosses of *Nicotiana* species (Kobori and Marubashi 2004; Liu and Marubashi 2013; Tezuka et al. 2007). On the other hand, in this study, hybrid seedlings obtained from the cross *N. rustica*×*N. langsdorffii* and putative progenitors of *N. rustica*×*N. langsdorffii* showed abnormalities. This suggests that *N. rustica* inherited factors that produce tumors from both progenitors.

Hybrid lethality in the genus *Nicotiana* is generally inhibited by culturing plants at high temperature (Tezuka and Marubashi 2012; Yamada et al. 1999). The temperature sensitivity of hybrid lethality enables physiological studies. Masuda et al. (2003) revealed that the factor triggering hybrid lethality was expressed 3 h after induction by a shift to a low temperature (from 36°C to 28°C). Yamada and Marubashi (2003) reported a close relationship between hybrid lethality and an increase in ethylene. The present study revealed that the formation of tumors in the cross combination *N. rustica*×*N. alata* is temperature sensitive, like hybrid lethality, facilitating physiological study of tumorigenesis.

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