

## Article

# Evaluation of Crossability between *Nicotiana benthamiana* and *Nicotiana excelsior*

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**Abstract:** Wild tobacco species in the *Nicotiana* section *Suaveolentes* are promising genetic resources to introduce their disease resistance to cultivated tobacco, *Nicotiana tabacum*. However, hybrid lethality is observed in hybrid seedlings from crosses between most *Suaveolentes* species and *N. tabacum*. In particular, *N. benthamiana* belonging to the section *Suaveolentes* produces only viable hybrids after crossing with *N. tabacum*. In the present study, crossability between *N. benthamiana* and *N. excelsior* (section *Suaveolentes*) was investigated to test the possible usefulness of *N. benthamiana* as the bridge parent to transfer desirable genes of *N. excelsior* to *N. tabacum* via bridge crossing. After reciprocal crosses using three accessions of *N. benthamiana* and *N. excelsior* each, several crossing barriers such as cross-incompatibility, seed abortion, and male and female hybrid sterility were observed. Although reciprocal hybrids between *N. benthamiana* and *N. excelsior* showed a high degree of chromosome pairing in meiosis, univalents and multivalents, as well as chromosome bridges and lagging chromosomes, were observed. These meiotic abnormalities were thought to cause hybrid sterility. The possible usefulness of reciprocal hybrids between *N. benthamiana* and *N. excelsior* is discussed.

**Keywords:** chromosome pairing; cross-incompatibility; crossability; hybrid sterility; interspecific hybrid; *Nicotiana benthamiana*; *Nicotiana excelsior*; section *Suaveolentes*; seed abortion



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## 1. Introduction

An interspecific cross, regardless of whether it is naturally occurring or artificially performed, is an important evolutionary mechanism. Interspecific crosses may produce new species in nature, and several plant species are amphidiploids possessing two or more genomes that naturally arise from interspecific crosses and polyploidization [1–3]. In artificial crosses, interspecific crosses are widely used for breeding purposes or genetic analysis [4–6]. Crossability between species depends on their genotypes, ploidy levels, and environmental factors.

*Nicotiana benthamiana* is a model plant species, especially in plant virology, because genetic transformation techniques and tissue culture methods have been well established [7], and a draft genome sequence has been reported [8]. This species belongs to the *Nicotiana* section *Suaveolentes*, which consists of wild tobacco species restricted to Australasia and one species in Africa [9]. Species in the section *Suaveolentes* are allotetraploids with 30 to 48 chromosomes, excluding species that experienced a possible additional chromosome doubling event [10–14]. This section is predicted to have arisen ca. 6 Mya, and most *Suaveolentes* species have appeared within the last 2 Myr [13,15]. Goodspeed [10] presumed that crosses between early *Suaveolentes* species and subsequent backcrosses or crosses of derivatives, as well as chromosomal reorganization, were involved in the formation of the current diverse species in the section. Based on the distribution, morphological specificity,

and the somewhat low chromosome pairing in hybrids with other *Suaveolentes* species, *N. benthamiana* apparently has undergone a long period of independent differentiation [10].

*N. benthamiana* is one of the two species possessing 38 chromosomes in the section *Suaveolentes*; the other is *N. excelsior*. While *N. benthamiana* is susceptible to a wide range of pathogens and viruses [7], most other species in the section *Suaveolentes* possess disease resistance. For example, *N. excelsior* shows resistance to blue mold (*Peronospora hyoscyami* f.sp. *tabacina*), powdery mildew, and a specific strain of potato virus Y [16–18]. Therefore, *Suaveolentes* species are promising genetic resources to introduce their disease resistance to cultivated tobacco, *N. tabacum* (section *Nicotiana*). However, the transfer of desirable genes of *Suaveolentes* species into *N. tabacum* has been disrupted by reproductive isolation [19]. Hybrid lethality, a type of postzygotic reproductive isolation, is commonly observed in crosses between *N. tabacum* and most *Suaveolentes* species, including *N. excelsior* [14,19–22]. Hybrid lethality is a phenomenon that causes the death of F<sub>1</sub> hybrid seedlings. In particular, *N. benthamiana* is one of the two species yielding only viable hybrid seedlings after crossing with *N. tabacum* [23]. If *N. benthamiana* can be used as a bridge species to introduce desirable genes from *Suaveolentes* species to *N. tabacum*, tobacco breeding will be facilitated. However, while chromosome pairing in intrasectional F<sub>1</sub> hybrids of the section *Suaveolentes* has been well reported, as mentioned in the discussion, crossability between *Suaveolentes* species has been scarcely reported.

In the present study, we investigated the crossability between *N. benthamiana* and *N. excelsior* to test whether *N. benthamiana* can be used as a bridge species to transfer desirable genes of *N. excelsior* into *N. tabacum*. Using three accessions of *N. benthamiana* and *N. excelsior* each, reciprocal crosses were performed. Hybrid seedlings obtained were investigated for pollen fertility, reciprocally backcrossed to both parents, and evaluated for chromosome behavior during meiosis. We discuss the possible usefulness of *N. benthamiana* as a bridge species in breeding programs.

## 2. Materials and Methods

### 2.1. Plant Materials

*N. benthamiana* ( $2n = 38$ ) accessions JT, PI 555478, and PI 555684, and *N. excelsior* ( $2n = 38$ ) accessions JT, PI 224063, and PI 555685 were used for interspecific crosses. The accessions obtained from the Leaf Tobacco Research Center (Japan Tobacco Inc., Oyama, Japan) were named JT to distinguish them from the United States Department of Agriculture (USDA) Plant Introduction (PI) accessions. All plants used as parents were cultivated in a greenhouse under natural light conditions.

### 2.2. Interspecific Crosses

*N. benthamiana* and *N. excelsior* were reciprocally crossed. Flowers of plants used as female parents were emasculated one day before anthesis and pollinated with pollen from plants used as male parents. F<sub>1</sub> seeds were sterilized with 5% sodium hypochlorite for 15 min. The sterilized seeds were sown in Petri dishes (90 mm diameter, 17 mm deep) containing 25 mL of 1/2 MS medium [24] supplemented with 1% sucrose and 0.2% Gelrite (pH 5.8) and then cultured at 25 °C (16 h light/8 h dark; approximately 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Hybrid seedlings germinated in Petri dishes were potted and cultivated in a greenhouse. The hybrid plants were reciprocally backcrossed with both parents. The number of capsules and hybrids obtained were counted for each cross.

### 2.3. Analysis of Pollen Viability

Flower buds were collected one day before anthesis, and the anthers were placed on glass slides. A drop of acetocarmine was added, and the anthers were crushed with tweezers to release pollen grains. At least 100 pollen grains were observed per anther using a light microscope (BX50; Olympus, Tokyo, Japan), and three anthers were observed per hybrid plant. Stained pollen grains were considered viable, whereas unstained pollen grains were considered inviable.

#### 2.4. Cytological Analysis of Chromosomes in Meiosis

Chromosomes in pollen mother cells during meiosis were also investigated. Anthers collected from young flower buds were fixed in chloroform/ethanol/acetic acid (6:3:1). The anthers were then placed on a glass slide and squashed in acetocarmine. Chromosome pairing during meiotic metaphase I was observed in 25 pollen mother cells per cross combination using a light microscope (Optiphot-2; Nikon, Tokyo, Japan). Chromosomes were also observed at meiotic anaphase I.

### 3. Results

#### 3.1. Reciprocal Crosses between *N. benthamiana* and *N. excelsior*

Reciprocal crosses were carried out between three *N. benthamiana* accessions and three *N. excelsior* accessions (Table 1). The percentage of capsules obtained after pollination was low for all reciprocal crosses. Although seeds were obtained from 14 of the 18 crosses, several crosses yielded a small number of seeds. In this case, all the seeds obtained were aseptically sown. No seeds germinated in crosses between *N. benthamiana* (♀) and *N. excelsior* (♂), excluding two crosses with seed germination rates of 1% and 33%. Conversely, seed germination rates in crosses between *N. excelsior* (♀) and *N. benthamiana* (♂) were high (85–96%), although the rates of the two crosses were low (18%) and moderate (55%). All hybrid seedlings obtained from the nine crosses reached maturity. Leaf and flower shapes were intermediate in appearance to those of the parents (Figure 1).

**Table 1.** Reciprocal crosses between *N. benthamiana* and *N. excelsior*.

Cross Combination	No. of Flowers Pollinated	No. of Capsules Obtained	No. of Seeds Sown	No. of Hybrids Obtained
<i>N. benthamiana</i> JT × <i>N. excelsior</i> JT	40	10 (25% <sup>1</sup> )	178	2 (1% <sup>2</sup> )
<i>N. benthamiana</i> JT × <i>N. excelsior</i> PI 224063	11	2 (18%)	48	0 (0%)
<i>N. benthamiana</i> JT × <i>N. excelsior</i> PI 555685	5	1 (20%)	2	0 (0%)
<i>N. benthamiana</i> PI 555478 × <i>N. excelsior</i> JT	20	0 (0%)	–	–
<i>N. benthamiana</i> PI 555478 × <i>N. excelsior</i> PI 224063	13	1 (8%)	22	0 (0%)
<i>N. benthamiana</i> PI 555478 × <i>N. excelsior</i> PI 555685	20	1 (5%)	143	0 (0%)
<i>N. benthamiana</i> PI 555684 × <i>N. excelsior</i> JT	20	2 (%)	27	9 (33%)
<i>N. benthamiana</i> PI 555684 × <i>N. excelsior</i> PI 224063	5	1 (20%)	48	0 (0%)
<i>N. benthamiana</i> PI 555684 × <i>N. excelsior</i> PI 555685	20	0 (0%)	–	–
<i>N. excelsior</i> JT × <i>N. benthamiana</i> JT	18	1 (6%)	32	28 (88%)
<i>N. excelsior</i> JT × <i>N. benthamiana</i> PI 555478	20	0 (0%)	–	–
<i>N. excelsior</i> JT × <i>N. benthamiana</i> PI 555684	9	1 (11%)	17	3 (18%)
<i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> JT	17	2 (12%)	23	22 (96%)
<i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555478	17	2 (12%)	20	11 (55%)
<i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555684	6	2 (33%)	20	17 (85%)
<i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> JT	3	1 (33%)	31	29 (94%)
<i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555478	22	0 (0%)	–	–
<i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555684	2	1 (50%)	34	32 (94%)

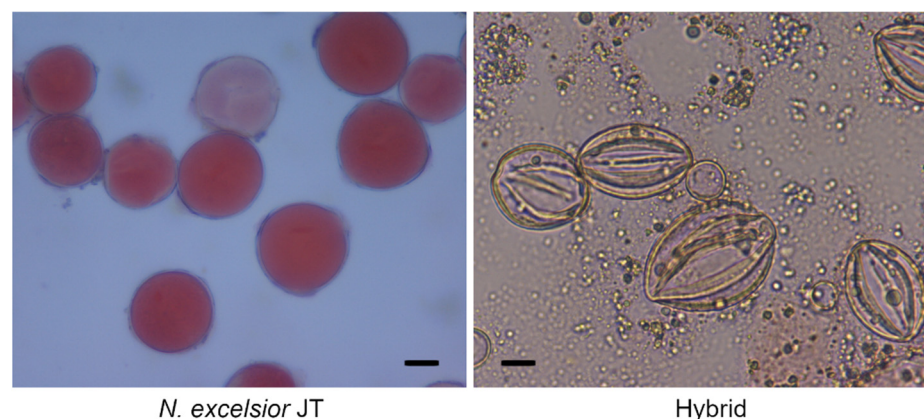
<sup>1</sup> Percentage of capsules obtained. <sup>2</sup> Percentage of seed germination.



**Figure 1.** Leaves and flowers of reciprocal hybrids between *N. benthamiana* PI 555684 and *N. excelsior* JT. Scale bars = 1 cm.

### 3.2. Fertility of Hybrids between *N. benthamiana* and *N. excelsior*

The pollen viability of the hybrid plants was investigated by staining with acetocarmine. More than 90% of the pollen grains were stained and considered viable in both parents (Figure 2). However, pollen grains with irregular shapes were observed, and none of the pollen grains were stained in all hybrid plants from the nine crosses, suggesting pollen inviability (Figure 2 and Table 2).



**Figure 2.** Pollen grains stained with acetocarmine in *N. excelsior* JT and a hybrid from the cross *N. excelsior* JT × *N. benthamiana* JT. Scale bars = 10 µm.



**Table 2.** Pollen viability of hybrids between *N. benthamiana* and *N. excelsior*.

Cross Combination	Total no. of Pollens Observed	Percentage of Pollen Viability
<i>N. benthamiana</i> JT × <i>N. excelsior</i> JT	368	0
<i>N. benthamiana</i> PI 555684 × <i>N. excelsior</i> JT	326	0
<i>N. excelsior</i> JT × <i>N. benthamiana</i> JT	348	0
<i>N. excelsior</i> JT × <i>N. benthamiana</i> PI 555684	335	0
<i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> JT	331	0
<i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555478	331	0
<i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555684	360	0
<i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> JT	313	0
<i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555684	318	0

Hybrid plants from all nine crosses were backcrossed to both parents as male parents. However, seeds could not be obtained from all crosses (Table 3). Seeds were also not obtained in backcrosses of hybrid plants from four crosses to both parents as female parents, as predicted by pollen staining (Table 4).

**Table 3.** Backcrosses of hybrids between *N. benthamiana* and *N. excelsior* to both parents as male parents.

Cross Combination	No. of Flowers Pollinated	No. of Capsules Obtained
( <i>N. benthamiana</i> JT × <i>N. excelsior</i> JT) × <i>N. benthamiana</i> JT	20	0
( <i>N. benthamiana</i> JT × <i>N. excelsior</i> JT) × <i>N. excelsior</i> JT	20	0
( <i>N. benthamiana</i> PI 555684 × <i>N. excelsior</i> JT) × <i>N. benthamiana</i> PI 555684	20	0
( <i>N. benthamiana</i> PI 555684 × <i>N. excelsior</i> JT) × <i>N. excelsior</i> JT	20	0
( <i>N. excelsior</i> JT × <i>N. benthamiana</i> JT) × <i>N. benthamiana</i> JT	20	0
( <i>N. excelsior</i> JT × <i>N. benthamiana</i> JT) × <i>N. excelsior</i> JT	20	0
( <i>N. excelsior</i> JT × <i>N. benthamiana</i> PI 555684) × <i>N. benthamiana</i> PI 555684	20	0
( <i>N. excelsior</i> JT × <i>N. benthamiana</i> PI 555684) × <i>N. excelsior</i> JT	20	0
( <i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> JT) × <i>N. benthamiana</i> JT	20	0
( <i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> JT) × <i>N. excelsior</i> PI 224063	20	0
( <i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555478) × <i>N. benthamiana</i> PI 555478	20	0
( <i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555478) × <i>N. excelsior</i> PI 224063	20	0
( <i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555684) × <i>N. benthamiana</i> PI 555684	23	0
( <i>N. excelsior</i> PI 224063 × <i>N. benthamiana</i> PI 555684) × <i>N. excelsior</i> PI 224063	20	0
( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> JT) × <i>N. benthamiana</i> JT	20	0
( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> JT) × <i>N. excelsior</i> PI 555685	20	0
( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555684) × <i>N. benthamiana</i> PI 555684	20	0
( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555684) × <i>N. excelsior</i> PI 555685	20	0

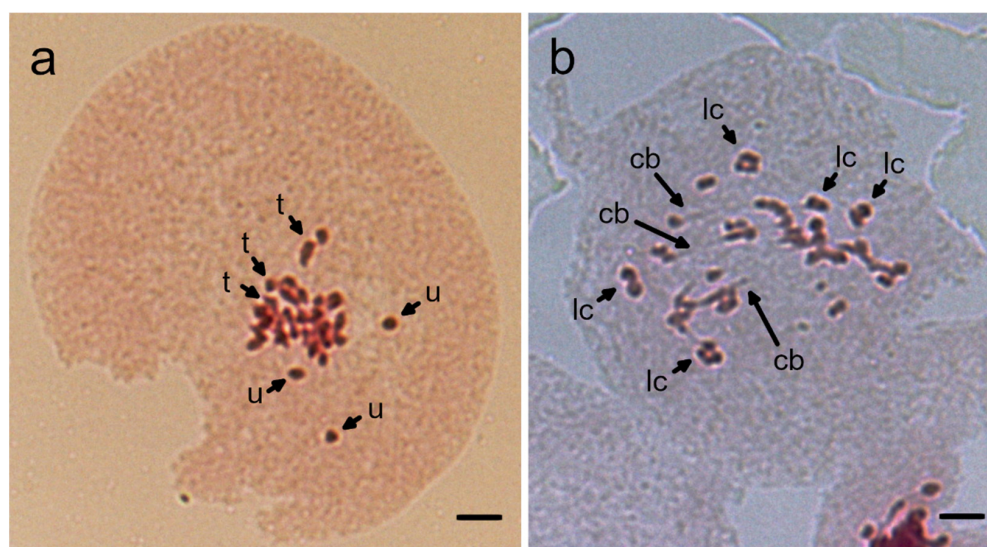
**Table 4.** Backcrosses of hybrids between *N. benthamiana* and *N. excelsior* to both parents as female parents.

Cross Combination	No. of Flowers Pollinated	No. of Capsules Obtained
<i>N. benthamiana</i> JT × ( <i>N. benthamiana</i> JT × <i>N. excelsior</i> JT)	20	0
<i>N. excelsior</i> JT × ( <i>N. benthamiana</i> JT × <i>N. excelsior</i> JT)	14	0
<i>N. benthamiana</i> JT × ( <i>N. excelsior</i> JT × <i>N. benthamiana</i> JT)	20	0
<i>N. excelsior</i> JT × ( <i>N. excelsior</i> JT × <i>N. benthamiana</i> JT)	20	0
<i>N. benthamiana</i> JT × ( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> JT)	20	0
<i>N. excelsior</i> PI 555685 × ( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> JT)	20	0
<i>N. benthamiana</i> PI 555684 × ( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555684)	10	0
<i>N. excelsior</i> PI 555685 × ( <i>N. excelsior</i> PI 555685 × <i>N. benthamiana</i> PI 555684)	20	0

### 3.3. Chromosomal Instability in Reciprocal Hybrids between *N. benthamiana* and *N. excelsior*

Chromosomes of reciprocal hybrids between *N. benthamiana* JT and *N. excelsior* JT were examined during meiosis. At meiotic metaphase I, chromosome pairing with an average of 3.5 univalents, 12.8 bivalents, 2.4 trivalents, and 0.1 tetravalents per pollen mother cell was observed in hybrid plants from the cross *N. benthamiana* JT × *N. excelsior* JT (Figure 3a and Table 5). In hybrid plants with the opposite direction of hybridization, chromosome pairing with an average of 2.8 univalents, 14.0 bivalents, and 2.4 trivalents was observed per pollen mother cell (Table 5). When chromosomes at meiotic anaphase I were observed

in hybrids from the cross *N. benthamiana* JT × *N. excelsior* JT, chromosome bridges and lagging chromosomes, which are aberration phenomena, were observed (Figure 3b).



**Figure 3.** Chromosome instability in hybrids between *N. benthamiana* JT and *N. excelsior* JT. (a) Chromosome pairing in meiotic metaphase I. u, univalent; t, trivalent. (b) Chromosomes at meiotic anaphase I. cb, chromosome bridge; lc, lagging chromosome. Scale bars = 10  $\mu$ m.

**Table 5.** Metaphase I chromosome pairing in reciprocal hybrids between *N. benthamiana* JT and *N. excelsior* JT.

Cross Combination	Mean Chromosome Configuration Per Cell			
	Univalent	Bivalent	Trivalent	Tetraploid
<i>N. benthamiana</i> JT × <i>N. excelsior</i> JT	3.50 ± 0.32	12.80 ± 0.25	2.80 ± 0.18	0.12 ± 0.07
<i>N. excelsior</i> JT × <i>N. benthamiana</i> JT	2.80 ± 0.21	14.04 ± 0.24	2.44 ± 0.15	0

#### 4. Discussion

Crossing results in the present study indicate that several isolation barriers exist between *N. benthamiana* and *N. excelsior*. In reciprocal crosses, the percentage of capsules obtained was low, suggesting the presence of prezygotic cross-incompatibility (Table 1). Excluding reciprocal crosses between *N. benthamiana* PI 555684 and *N. excelsior* JT, seed germination percentages were generally lower in crosses using *N. benthamiana* as maternal parents than in the opposite crosses (Table 1). These differences between reciprocal crosses are caused by seed abortion, which is observed unilaterally. Although all obtained hybrid seedlings matured, they were both male and female sterile (Figure 2 and Tables 2–4).

In the *Nicotiana* section *Suaveolentes*, a high degree of chromosome pairing in meiosis has been reported in several interspecific hybrids [10,22,25–32]. Reciprocal hybrids between *N. benthamiana* JT and *N. excelsior* JT also showed a high degree of chromosome pairing, suggesting high homology of the genomes. However, in addition to bivalents, univalents, trivalents, and tetravalents, as well as chromosome bridges and lagging chromosomes, were observed (Figure 3 and Table 5). Hybrid sterility can be caused by meiotic irregularities, chromosomal aberrations, or interactions between nuclear genes or between the cytoplasm and nuclear genes [33–37]. Although formation of the multivalents suggested some homologies between corresponding chromosomes as inferred from the monophyly of *Suaveolentes* species and a presumption that the species are allotetraploids derived from common ancestors [10,13,15,38], the univalents and multivalents, as well as chromosome

bridges and lagging chromosomes, would lead to imbalanced chromosome segregation, resulting in hybrid sterility in crosses between *N. benthamiana* and *N. excelsior*. It was also reported that because amphiploids produced by chromosome doubling of sterile F<sub>1</sub> hybrids between *Suaveolentes* species showed full fertility, sterility in the F<sub>1</sub> hybrids can be attributed to chromosomal causes [39].

In crosses between *N. excelsior* and *N. tabacum*, the following several crossing barriers were observed: the prezygotic barrier preventing fertilization and the postzygotic barrier during seed development in the cross using *N. excelsior* as the male parent, and hybrid lethality in reciprocal crosses [19]. Meanwhile, *N. benthamiana* produced viable hybrid plants when crossed using *N. tabacum* as the male parent, although the reciprocal cross was unsuccessful because of the prezygotic barrier [23]. These facts promise to transfer desirable genes of *N. excelsior* into *N. tabacum* using *N. benthamiana* as a bridge species. However, both male and female sterility in reciprocal hybrids between *N. benthamiana* and *N. excelsior* would disturb the bridge cross. Several reports have provided useful information to address this problem. Hybrid sterility can often be overcome by chromosome doubling methods using colchicine treatment or tissue culture [40–42]. As mentioned above, amphiploids derived from several sterile intrasectional hybrids in the section *Suaveolentes* also showed regular chromosome pairing in a majority of the pollen mother cells and full fertility [39]. Each chromosome is considered to have exact copy and chromosomal homology by chromosome doubling, leading to fertility restoration [42]. Therefore, fertility can be restored through the production of amphiploids from reciprocal hybrids between *N. benthamiana* and *N. excelsior*. If this is accomplished, hybrids obtained in the present study would be useful for transferring the desirable genes of *N. excelsior* to *N. tabacum*.

## 5. Conclusions

Several crossing barriers such as cross-incompatibility, seed abortion, and male and female hybrid sterility were observed in reciprocal crosses using three accessions of *N. benthamiana* and *N. excelsior* each. Although reciprocal hybrids between *N. benthamiana* and *N. excelsior* showed a high degree of chromosome pairing in meiosis, univalents and multivalents, as well as chromosome bridges and lagging chromosomes, were observed. These meiotic abnormalities were thought to cause hybrid sterility.

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