Overcoming Hybrid Lethality Induced by Chromosomal Instability in an Interspecific Hybrid of Genus Nicotiana

Kouki Nakata
Tokyo University of Agriculture and Technology
Hiroki Nagashima
Tokyo University of Agriculture and Technology
Haruka Yamashita
Tokyo University of Agriculture and Technology
Natsuki Inaba
Tokyo University of Agriculture and Technology
Yoshihito Shinozaki Shinozaki
Tokyo University of Agriculture and Technology
Motoki Kanekatsu
Tokyo University of Agriculture and Technology
Wataru Marubashi
Meiji University
Tetsuya Yamada (teyamada@cc.tuat.ac.jp)
Tokyo University of Agriculture and Technology

Research Article

Keywords: Hybrid lethality, Nicotiana suaveolens, Nicotiana tabacum, homoeologous chromosomes

Posted Date: January 5th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-137495/v1

Version of Record: A version of this preprint was published at Scientific Reports on April 9th, 2021. See the published version at https://doi.org/10.1038/s41598-021-87242-7.

Abstract

Hybrid lethality is a type of reproductive isolation in which hybrids die before maturation, due to the interaction between the two causative genes derived from each of the hybrid parents. The interspecific hybrid of *Nicotiana suaveolens* x *Nicotiana tabacum* is a model plant for studies of hybrid lethality. In this cross, most hybrid seedlings die, but rare individuals grow normally and mature. Separately, a technique for producing mature hybrids by artificial culture has been developed. However, the mechanism by which hybrids overcome lethality, either spontaneously or by artificial culture, remains unclear. In the present study, we found that some hybrids that overcome lethality, either spontaneously or by artificial culture, lack the distal part of the Q chromosome, a region that includes the gene responsible for lethality. Quantitative polymerase chain reaction results suggested that the distal deletion of the Q chromosome, detected in some hybrid seedlings that overcome lethality, is caused by reciprocal translocations between homoeologous chromosomes. These results indicated that the chromosomal instability during meiosis of the amphidiploid *N. tabacum* and during artificial culturing of hybrid seedlings is involved in overcoming hybrid lethality in interspecific hybrids of the genus *Nicotiana*.

Introduction

Hybrid lethality is a type of postzygotic reproductive isolation in which fertilization between different species or populations occurs, but the resulting hybrids do not mature. In higher plants that experience hybrid lethality, fertilization is successful, but the hybrid embryos die before germination, or the hybrid seedlings show lethal symptoms such as browning, withering, and yellowing after germination. Hybrid lethality has been reported in a wide range of plant species, including wheat[1], rice[2], and cotton[3]. This phenomenon is a significant obstacle that restricts the genetic resources available for use in plant cross-breeding.

It is known that hybrid lethality also occurs in various combinations of interspecific crosses in the genus *Nicotiana*[4]. Hybrid seedlings of *Nicotianasuaveolens* (2n = 2x = 32, genome constitution SuSu) and *Nicotianatabacum* (2n = 4x = 48, SSTT) germinate normally, but show type-II lethality, such that the hypocotyl browns and eventually dies a few days after germination (DAG)[5]. It has been reported that the amount of transcript of *PAL* (encoding phenylalanine ammonia-lyase), which is an immune response-related gene, is increased in hybrid seedlings that show lethality[5]. We also have confirmed that the accumulation of protein aggregates promotes programmed cell death via autophagy in cultured cells of *N.suaveolens* x *N.tabacum* hybrids that exhibit lethality[6, 7].

Hybrid lethality is caused by the interaction of two complementary genes derived from each hybrid parent[8,9]. In plants, *R* genes encoding proteins that recognize effectors derived from a pathogen during a disease response have been reported as causative genes for hybrid lethality[10-12]. In hybrid lethality within the genus *Nicotiana*, *Nt6549g30*, a kind of NBS-LRR-type *R* gene of *N. tabacum*, has been reported as the gene responsible for the type-II lethality expressed in hybrid seedlings from crosses between *N. tabacum*

and any of nine wild species, not including *N. suaveolens*, of *Nicotiana* section *Suaveolentes*[11, 13]. On the other hand, it has been confirmed that the locus responsible for lethality in the *N. suaveolens* x *N. tabacum* cross is located on the Q chromosome belonging to the S genome of *N. tabacum*[14, 15].

Previous studies have reported conflicting information regarding the chromosomal location of *Nt6549g30*. Ma[11] reported that *Nt6549g30* is present on the H chromosome. Other work has suggested that the H chromosome belongs to the T genome[16]. On the other hand, the SSR marker of Linkage group No. 11, a gene that is detected on the Q chromosome of the S genome[17], also was detected on the H chromosome[18]. Additionally, Ma[11] observed that the H chromosome belongs to the S genome. Therefore, we infer that the H chromosome in the reports of Hancock *et al.*[18] and Ma[11] is the same as the Q chromosome in the report of Tezuka *et al.*[17]. If this interpretation is valid, we consider it highly likely that *Nt6549g30* is involved in the lethality of the *N. suaveolens* x *N. tabacum* cross.

In some lethal cross combinations between *N. tabacum* and wild *Nicotiana* species, including *N. suaveolens* x *N. tabacum*, viable hybrid seedlings (i.e., hybrid seedlings that overcome lethality) appear spontaneously at a certain frequency. Hancock *et al.*[18] detected hybrid seedlings that overcame lethality at a frequency of about 1.1×10^{-3} in a *N. tabacum* x *N. africana* cross. Those authors also confirmed that the SSR marker at the distal part of Linkage group No. 11 was not detected in approximately 47% of such seedlings, suggesting the loss of the distal segment of the H chromosome (Q chromosome). This missing chromosomal end region contains *Nt6549g30* (reference 11). However, the cause of such high-frequency loss of a chromosome terminus in hybrid seedlings remains unknown.

It has been reported that reciprocal translocation can occur between homoeologous chromosomes during meiosis in allopolyploid plant species such as *Brassica napus*[19], coffee[20], and interspecific hybrids of the genus *Lilium*[21]. In *N. tabacum*, reciprocal translocation also has been reported to occur between homoeologous chromosomes, leading to loss of the *N* gene, a kind of *R* gene[22]. Based on these observations, we hypothesized that hybrid seedlings that overcome lethality following a cross between *N. tabacum* and wild species occur by formation of mutated gametes in which a distal segment of the Q chromosome, including *Nt6549g30*, is replaced with the homologous region of the homoeologous Q' chromosome by reciprocal translocation during meiosis (Hypothesis 1).

Since hybrid lethality is an obstacle to cross-breeding, various methods have been employed in attempting to produce hybrid plants that overcome lethality. In intraspecific and interspecific hybrids of wheat, hybrid plants that overcome lethality have been obtained by artificial culture of hybrid embryos[23], by proline treatment during fertilization and hybrid embryogenesis[24]. In *N. suaveolens* x *N. tabacum*, when hybrid seedlings were cultured in a medium containing a high concentration of cytokinin, vigorous shoots were formed at the stem bases of the seedlings, and regenerated plants that overcame lethality were obtained by rooting of these shoots[25]. However, the mechanism whereby plants that overcome lethality are produced by artificial culturing remains unclear.

In *in vitro* cultured explants, it is known that reactive oxygen species (ROS) are generated by oxidative stress induced by medium components (plant hormones and salts) and culture environment[26]. On the other hand, ROS are thought to cause chromosome breakage[27]. Based on the above observations, we hypothesized that the production of regenerated plants that overcome lethality by culturing hybrid seedlings of *N. suaveolens* x *N. tabacum* in cytokinin-supplemented medium results from the appearance of cells lacking the distal part of the Q chromosome during *in vitro* culture (Hypothesis 2).

Elucidating the causes of interspecific hybrids that overcome lethality is expected to reveal one aspect of the mechanism by which new species form by overcoming reproductive isolation. This information also is expected to contribute to the establishment of techniques for overcoming hybrid lethality, thereby leading to an expansion of the genetic resources available for cross-breeding. In the present study, we tested the above two hypotheses in an attempt to clarify the mechanisms whereby hybrid plants overcome lethality. First, we used the polymerase chain reaction (PCR) to assess the presence or absence of *Nt6549g30* and the distal part of the Q chromosome in seedlings and regenerated plants, derived from the *N. suaveolens* x *N. tabacum* cross, that overcame hybrid lethality. Next, we used quantitative PCR (qPCR) to confirm reciprocal translocation between the Q chromosome region where *Nt6549g30* resides and the homologous region of the homoeologous Q' chromosome.

Results

Acquisition of hybrid seedlings that overcome lethality

A total of 15,476 seeds obtained from the cross of *N. suaveolens* x *N. tabacum* were sown, from which 12,943 seeds germinated. Starting shortly after germination, most of the seedlings from this cross showed lethal symptoms, such as browning of hypocotyls and roots along with yellowing of leaves, but 16 seedlings did not show any lethal symptoms at 20 days after sowing (Fig. 1A-C). These seedlings were transferred to half-strength Murashige and Skoog medium (0.5x MS) in a plant box (Fig. 1D). The Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) method was used to evaluate the hybridity of viable seedlings; gel electrophoresis of the products showed that 12 of the 16 surviving seedlings yielded all amplicons from the two parents (i.e., RAPD bands specific to *N. suaveolens* as well as those specific to *N. tabacum*) (Fig. 2, Table S1). Among the remaining 4 viable seedlings, the s14-5 seedling lacked one of the three *N. tabacum*-specific RAPD bands, the one corresponding to the product obtained with the OPA-15 primer. The other 3 seedlings (s14-9, s14-10, s17-3) yielded only the *N. suaveolens*-specific RAPD bands.

Fifteen surviving seedlings (with the exception of s17-3) were potted, of which 13 flowered (Fig. 1E). Eleven of the flowered seedlings were judged to be hybrid seedlings that overcame lethality, given that these seedlings showed morphologies intermediate between those of the parental species in terms of flower color and shape, leaf size and shape, and plant posture (Fig. 1F, Table S2). The other two seedlings (s14-9 and s14-10) showed *N. suaveolens*-type flower color and plant posture, consistent with the results of the RAPD analysis. For 3 of the 15 surviving seedlings (s17-1, s17-2, and s18-1), transcripts of immune

response-related genes (Table S3) were present (in the young true leaves) at levels much lower than those detected in the cotyledons of lethal seedlings at 6 DAG (Fig. 3). Based on these results, the 11 seedlings that showed morphologies intermediate between their parents were judged to be hybrids that had overcome lethality. By this assessment, the frequency of appearance of hybrid seedlings that overcame lethality was 8.5×10^{-4}.

Acquisition of regenerated hybrid plants that overcome lethality by *in vitro* culturing of hybrid seedlings

All 35 seedlings of *N. suaveolens* x *N. tabacum* cultured in 0.5x MS medium containing 6-benzylaminopurine (BAP), a synthetic cytokinin, showed lethal symptoms shortly after germination. However, approximately one month later, many green shoots were observed to have formed at the bases of the stems of the seedlings (Fig. 4A). Shoots were cut from multiple plants; of 18 randomly selected shoots that were transplanted into 0.5x MS medium, 14 were able to root (Fig. 4B). The shoots that did not root vitrified and eventually died. When the hybridity of 12 regenerated plants that grew normally without vitrification were evaluated by the RAPD method, 10 individuals yielded all amplicons from the two parents (i.e., RAPD bands specific to *N. suaveolens* as well as those specific to *N. tabacum*) (Table S1, Fig. 2). In the remaining two regenerated plants (r18-1 and r19-4), one of the two *N. tabacum*-specific RAPD bands (that obtained with the OPA-11 primer) was not seen (Table S1).

There was no difference in morphology among the 12 regenerated plants; of 4 of these plants (r18-1, r18-4, r18-5, and r18-7) that were potted, all achieved flowering (Fig. 4C). All flowered individuals showed morphologies intermediate between those of the two parents in terms of flower color and shape, leaf size and shape, and plant posture (Fig. 4D, Table S2). As seen for the primary seedlings that overcame lethality, the abundance of transcripts of immune response-related genes in young true leaves of the individual flowered regenerated plants was notably lower than that in the cotyledons of lethal seedlings at 6 DAG (Fig. 3). Based on these results, these regenerated plants were judged to be hybrids that that had overcome lethality.

PCR confirmation of deletion of the distal part of the Q chromosome and *Nt6549g30*

PCR was performed using a primer pair capable of specifically amplifying the SSR and *Nt6549g30* markers present in the DNA of the Q chromosome belonging to the S genome of *N. tabacum* (Fig. 5). In lethal hybrid seedlings, PCR amplification products (amplicons) were detected with all SSR primer pairs and *Nt6549g30* primer pairs (Table 1). On the other hand, in the 5 of 8 individual hybrid seedlings that overcame lethality and in 3 of 12 individual viable regenerated hybrid plants that overcame lethality, no amplicon was detected for reactions using the primer pairs (PT30342, PT30365, and PT52778) targeting three SSRs in the DNA sequence of the distal part of the Q chromosome. These amplicons also were not detected from these 8 individuals when using the two primer pairs capable of amplifying *Nt6549g30*. In addition, in 6 of these 8 individuals, some other SSRs were not amplified, but the location and number of the missing SSRs differed among the individuals.

Genome analysis was performed using Genotyping by Random Amplicon Sequencing-Direct (GRAS-Di) technology[28] for the seedlings of hybrid parents (*N. suaveolens* and *N. tabacum*), two lethal hybrid seedlings (samples No. 1 and 3), and one hybrid seedling that overcame lethality (s17-2). In GRAS-Di, DNA sequence analysis of the amplicons obtained with 63 random primers and mapping to reference sequences detected 187 amplicons derived from Q-chromosome DNA in *N. tabacum* and lethal hybrid seedlings (Table S4). On the other hand, in s17-2, amplicons derived from DNA sequences located from bp 1 to 81360673 on the Q chromosome were detected. However, four amplicons (AMP0045831, AMP0067003, AMP0062028, and AMP0042233) derived from DNA sequences located between bp 81360673 and 81497158 on the Q chromosome were not detected.

Confirmation of reciprocal translocation between homoeologous chromosomes by qPCR

For amplification of the homoeologous chromosome of the Q chromosome (the Q' chromosome) belonging to the T genome of *N. tabacum*, three sets of primer pairs capable of specifically amplifying the SSRs present in the DNA sequences at both ends of the chromosome were selected (Fig. 5). Total DNA was extracted from 5 hybrid seedlings that overcame lethality and from 3 regenerated hybrid plants that overcame lethality, in which SSRs of terminal DNA of the Q chromosome were not detected, and from 3 lethal hybrid seedlings. The copy number of the PCR amplification region of each primer pair (normalized to a given amount of DNA) then was investigated by qPCR (Fig. 6). Specifically, a copy number was determined for the region amplified by two separate sets of primer pairs (NtScfTN90_54683-2 and NtScfTN90_1535-4) designed based on the Q' chromosome's terminal DNA. i.e., sequences homologous to the Q chromosome terminal DNA, including the *Nt6549g30* locus. Notably, in 4 of the 5 hybrid seedlings that overcame lethality, the copy number of these selected regions was approximately twice that in lethal hybrid seedlings. The remaining hybrid seedling (s17-1) showed a value that was similar to that seen in the lethal hybrid seedling. On the other hand, the copy numbers of the PT50790-targeted domain (corresponding to the distal part of the Q' chromosome, at the end opposite to the *Nt6549g30* locus amplification region) did not show a significant difference between lethal hybrid seedlings and hybrid seedlings that had overcome lethality. In addition, for the regenerated hybrid plants that overcame lethality, the copy number of each amplification region did not differ significantly from that of the lethal hybrid seedlings.

Discussion

This study sought to elucidate the mechanism by which individual hybrid plants overcome hybrid lethality, whether spontaneously or by artificially propagation. To achieve this goal, we formulated and tested two (non-exclusive) working hypotheses based on previous findings regarding the lethality-overcoming phenomenon of products of a *N. suaveolens* x *N. tabacum* cross. Hypothesis 1 was that seedlings that overcome lethality in the *N. suaveolens* x *N. tabacum* cross occur by formation of male gametes lacking the lethality-associated *Nt6549g30* gene, which is lost as a result of reciprocal translocations, between homoeologous chromosomes, that occur during meiosis in *N. tabacum*. Hypothesis 2 focused on the observation that culturing of *N. suaveolens* x *N. tabacum* seedlings in the

presence of cytokinin yields regenerated plants that overcome hybrid lethality at high frequency; we proposed that hybrid lethality is overcome as a result of the appearance of cells lacking the distal part of the Q chromosome during culturing.

To examine Hypothesis 1, we first identified 16 surviving seedlings from 12,943 germinated *N. suaveolens* x *N. tabacum* seedling individuals. The results of RAPD-PCR and morphological observation demonstrated that some of the viable seedlings were indeed hybrids (possessing genomes from both parents) and showed extremely low expression levels of immune response-related genes, confirming these to be hybrid seedlings that had overcome lethality (Figs. 1, 2, 3, Tables 1, S1). Specifically, 11 hybrid seedlings that had overcome lethality were obtained, and the frequency of appearance of hybrid seedlings that overcame lethality in this cross combination was calculated to be 8.5×10^{-4}. This value was close to 1.1×10^{-3}, which is the frequency of appearance of hybrid seedlings that overcame lethality in a previous study of the *N. tabacum* x *N. africana* cross[18].

PCR results showed that 5 of 8 of the lethality-overcoming seedlings lacked the distal part of the Q chromosome, a region that contains *Nt6549g30* (Table 1). When qPCR was performed using two sets of primer pairs designed to amplify DNA at the end of the Q' chromosome, the copy number of the amplified region from 4 of the 5 individuals that overcame lethality was approximately twice that of seedlings that exhibited lethality (Fig. 6). Therefore, in these 4 individuals, it appears that the end of the Q chromosome, including *Nt6549g30*, was replaced due to a reciprocal translocation between the Q chromosome and its homoeologous chromosome (Q'). These data support the existence of a lethality-overcoming mechanism consistent with our Hypothesis 1. Figure 7 shows a suggested model, based on our results, for the lethality-overcoming mechanism in *N. suaveolens* x *N. tabacum*. In this model, a hybrid seedling that overcomes lethality is produced by fertilization of a female gamete from *N. suaveolens* by a male gamete from *N. tabacum*; notably, the male gamete has lost *Nt6549g30* due to reciprocal translocation, during meiosis, between the distal regions of the Q and Q' chromosomes. This model is consistent with the overcoming of lethality in *N. tabacum* x *N. africana*[18] and in other interspecific crosses within the genus *Nicotiana* where *N. tabacum* serves as one parent.

GRAS-Di analysis of one hybrid (s17-2) that escaped lethality suggested that lethality was overcome by reciprocal translocation. Notably, an amplicon derived from the region extending to Q chromosomal DNA bp 81360673 was detected, but no amplicon derived from the DNA region beyond bp 81497158 was detected (Table S4). This result implied that the break-point of the reciprocal translocation detected in s17-2 is located within a region of about 136 kb, positioned between bp 81360673 and bp 81497158 on the Q chromosome.

For one (s17-1) of the five seedlings that overcame lethality, qPCR analysis showed that the copy number of the amplified region was the same as that of the lethal seedlings (Fig. 6). This observation implicated deletion of the distal part of the Q chromosome in overcoming lethality in this individual. Specifically, for s17-1, products that would be amplified by three primer pairs (PT52864, PT55075, and PT60178) were not detected; these fragments would span the SSR marker located near the center of the Q chromosome

(at 62.25 cM) (Table 1). Therefore, reciprocal translocations between homoeologous chromosomes may be more likely to occur in the region near the end of the Q chromosome, while deletions of relevant loci may be more likely to occur in the region closer to the center of the chromosome. In heteroploid synthetic *Brassica* plants, both chromosomal deletions and reciprocal translocations (between homoeologous chromosomes) have been reported in synthetic progeny[29-30]. We postulate that the deletion at the distal part of the Q chromosome may be the result of aberrant segregation and cleavage of chromosomes following synapsis between homoeologous chromosomes during meiosis of *N. tabacum*, which is a heteroploid.

In 3 individuals (s14-1, s14-2, and s14-4) that overcame lethality, a number that represented approximately 38% of the 8 seedlings that overcame lethality and were subjected to PCR, amplicons were detected for all of the primer pairs (Table 1). Therefore, it appeared that these individuals did not lack the lethality-associated gene on the *N. tabacum* side (*Nt6549g30*) as a result of reciprocal translocation or deletion of the distal part of the Q chromosome end. In *N. tabacum* x *N. africana*, it has been suggested that about 37% of lethality-overcoming seedlings have intact Q chromosomes[18]. Both *N. suaveolens* and *N. africana* are included in the Suaveolentes section and are thought to be derived from amphidiploid progenitors[31]. Based on these results, we hypothesized that reciprocal translocation or deletion of the chromosome segment on which the lethality-associated gene is located also can occur in *N. suaveolens* and *N. africana*. Overcoming lethality in the seedlings of *N. suaveolens* x *N. tabacum* and *N. tabacum* x *N. africana*, in which no reciprocal translocation or deletion of the distal part of the Q chromosome was detected, may result from chromosomal mutations due to synapsis of homoeologous chromosomes. However, since the lethality-associated genes in *N. suaveolens* and *N. africana* are unknown and the genomic sequences of those species remain unpublished, this hypothesis cannot be tested at this time.

To examine our Hypothesis 2, seedlings of *N. suaveolens* x *N. tabacum* were cultured in a medium containing a high concentration of cytokinin, in an effort to obtain viable regenerated plants. The results of RAPD-PCR and morphological observation suggested the resulting regenerated plants had genomes from both parents, confirming that these individuals were indeed hybrids. These plants also showed strongly decreased expression levels of immune response-related genes. Therefore, we judged these individuals to be regenerated hybrid plants that had overcome lethality (Figs. 2, 3, 4, Tables 1, S1). In 3 (r18-1, r18-7, and r19-3) of the 12 regenerated plants that overcame lethality, PCR analysis showed that the distal part of the Q chromosome (the region containing *Nt6549g30*) had been lost, as seen for some of the seedlings that overcame lethality (Table 1). Additionally, qPCR of these three individuals showed that the copy number of the amplified region was the same as that of lethal seedlings (Fig. 6). These results suggested that the deletion of the distal part of the Q chromosome in these three individuals contributed to overcoming lethality, an inference consistent with our Hypothesis 2. Hypothesis 2 was based on the previous finding that ROS are generated in culture[26] and are involved in chromosomal cleavage[27]. In the future, it will be necessary to verify that ROS are involved in the deletion of the distal part of the Q chromosome. Such confirmatory experiments will require demonstrating ROS production in

hybrid seedlings that are cultured in a medium containing a high concentration of cytokinin, and then showing that inhibition of ROS production prevents the emergence of viable shoots.

In 9 of the 12 regenerated plants that overcame lethality, PCR analysis showed that amplicons were detected for all primer pairs (Table 1). Thus, in these individuals, it appears that lethality was overcome by a mechanism other than the deletion of the distal part of the Q chromosome. In addition to recombination-associated events that result in chromosomal lesions, mutation by DNA methylation and by DNA base substitution and deletion is known to occur in plant tissue culture[26]. It also has been suggested that the supplementation of medium with high-concentration BAP induces mutation[32]. Furthermore, certain *R* genes have been reported to show frequent DNA base substitutions and deletions during mitosis[33]. It is possible that these various genetic mutations occur in the lethality-associated genes of *N. suaveolens* and *N. tabacum*, resulting in the formation of viable shoots during tissue culture. It also is possible that ROS production under culture conditions induces deletion of the chromosomal region containing the lethality-associated gene in the *N. suaveolens* genome. To clarify these mutational mechanisms, it will be necessary to perform detailed sequence analysis of the *Nt6549g30* locus in regenerated plants that overcome lethality, and to identify the lethality-associated chromosome and gene in the *N. suaveolens* genome.

In the present study, expression analysis of immune-response-related genes (e.g., *PAL1*, *PRB1-like*, *LOX1*, and *PDF-like protein 1*) was performed in the process of detecting seedlings and regenerated plants that overcame lethality. In *A. thaliana*, the *PAL1* gene product is involved in the synthesis of salicylic acid[34], and *PRB1* encodes a PR-1-like protein expressed in response to ethylene and methyl jasmonate[35]. On the other hand, the *LOX1* gene product has been implicated in jasmonic acid synthesis[36], and *PDF1.2* encodes a jasmonic acid-responsive defensin[36]. In the present work, the expression levels of these genes in seedlings and regenerated plants that overcame lethality were much lower than those in the lethal seedlings at 6 DAG (Fig. 3). These data strongly suggested that the seedlings and regenerated plants that overcome lethality lack the function of the lethality-associated gene that triggers the immune-response signal, a deficiency that presumably is due to some genetic variation.

Hybrid lethality is a type of reproductive isolation and a mechanism for maintaining species independence. On the other hand, the results of the present study suggest that chromosomal instability permits hybrids to overcome hybrid lethality, thereby escaping reproductive isolation. We postulate that this phenomenon may contribute to the birth of new species in the process of plant evolution. In addition, hybrid lethality has been reported in a wide range of crop species[9] and is a serious obstacle in hybrid breeding of crops. The findings obtained in this study may lead to a technique for artificially breaking reproductive isolation by inducing chromosomal instability.

In summary, we found that the loss of the distal part of the Q chromosome is involved in overcoming hybrid lethality in seedlings and in regenerated plants obtained by culturing hybrid seedlings produced by interspecific *Nicotiana* crosses. We propose that loss of this factor is the result of reciprocal translocation and deletion between the Q chromosome and its homoeologous partner chromosome (Q') in hybrid

seedlings, and of a distal deletion in the Q chromosome in regenerated plants. These findings, which relate to the mechanism of breaking reproductive isolation, are likely to be important in the evolution of polyploid plant species and in the expansion of genetic resources available for cross-breeding.

Materials And Methods

Sowing hybrid seeds and obtaining viable seedlings

Hybrid seeds of *N. suaveolens* x *N. tabacum* were obtained according to the method of Yamada *et al*.[37]. The seeds were surface-sterilized by immersion in 70% ethanol for 30 seconds, followed by immersion in 5% sodium hypochlorite for 20 minutes. After washing three times with sterile water on a clean bench, seeds were sown in half-concentration MS medium (0.5x MS) that had been adjusted to pH 5.8 and supplemented with 1% sucrose and a gelling agent (0.2% GelRite® (Wako) or 0.3% Phytagel™ (Sigma)). The medium containing the seeds then was incubated at 28°C in an incubator with a photoperiod of 24 h. The number of seedlings that remained viable 20 days after sowing was determined.

Culturing hybrid seedlings in medium containing a high concentration of cytokinin, and obtaining viable shoots

After surface-sterilization as above, hybrid seeds of *N. suaveolens* x *N. tabacum* were sown in 0.5x MS that had been adjusted to pH 5.8 and supplemented with 2.0 mg/L 6-benzylaminopurine (BAP) (Sigma), 1% sucrose, and a gelling agent (0.2% GelRite® or 0.3% Phytagel™). The medium containing the seeds then was incubated at 28°C in an incubator with a photoperiod of 24 h. Viable shoots that differentiated from the stem base after incubation for more than one month from sowing were excised, transplanted to 0.5x MS without plant hormones, and rooted.

DNA extraction

Leaves or stems were collected from hybrid parents, lethal seedlings, viable seedlings, and viable regenerated plants. Total DNA was extracted from these tissues using the cetyltrimethylammonium bromide (CTAB) method according to Yamada *et al*.[37], or using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Purity and concentration of DNA samples were determined using Nanodrop (Thermo Fisher Scientific) and Qubit (Thermo Fisher Scientific) instruments.

Confirmation of hybridity by RAPD-PCR

Hybridity was tested by RAPD-PCR with reference to Marubashi & Onosato[14] and Tezuka *et al.*[38]. Six PCR primers were used, including OPA-1, OPA-5, OPA-9, OPA-11, OPA-12, and OPA-15 (Operon). KAPA Taq Extra Hotstart Ready mix with dye (KAPA Biosystems) was used for PCR. The PCR reaction solution was prepared by combining 10 µL of 2x KAPA Taq Extra Hotstart Ready mix with dye, 2 µL of OPA primer at 10 µM, and 20 ng of DNA; the solution then was adjusted to 20 µL with PCR-grade water and mixed. The PCR reaction solution was subjected to initial denaturation at 95°C for 15 min, followed by 45 cycles of

denaturation at 94°C for 1 min, annealing at 35°C for 2 min, and extension at 72°C for 3 min, using an Applied Biosystems 2720 Thermal Cycler (Life Technologies). The PCR products were electrophoresed (along with a commercial DNA size marker) on a 2% or 3% agarose gel supplemented with 0.01% Gel Red (Biotium) using TAE buffer as the electrophoresis buffer, and the gel was photographed with UV light to detect the PCR products.

Confirmation of hybridity by morphological observation

Viable seedlings and regenerated plants were transplanted into pots containing Super Soil Mix A (Sakata Seed), acclimated in a constant temperature room at 25°C for 16 h, and allowed to flower. Leaf and flower morphology and plant posture were compared with those of the parent strains.

Expression analysis

Total RNA was extracted from cotyledons of lethal seedlings, true leaves of viable seedlings, and regenerated *N. suaveolens* x *N. tabacum* plants using RNAiso Plus (Takara) according to the method of Shinozaki *et al.*[39]. Lethal seedlings were cultivated according to the method described above for “Sowing hybrid seeds and obtaining viable seedlings”; the sample consisted of the cotyledons collected from 30 to 50 individuals at the 6-DAG stage. Seedlings and regenerated plants that overcame lethality were cultivated according to the method described above for “Confirmation of hybridity by morphological observation”, and young true leaves were sampled. Purity and concentration of RNA samples were determined using Nanodrop and Qubit instruments. A PrimerScript RT reagent Kit with gDNA Eraser (Takara) was used for synthesis of cDNA, and the initial template cDNA was adjusted to 10 ng/μL. The immune-response genes for *N. tabacum*, which have high homology with those of *A. thaliana*, were used as the targets for expression analysis (Table S5). Primer pairs targeting each gene were designed using Primer3Plus (https://primer3plus.com/) and NetPrimer (PREMIER Biosoft International; http://www.premierbiosoft.com/netprimer/) software programs (Table S5). For real-time quantitative reverse transcription (qRT) -PCR, a mixture corresponding to 5 μL of KAPA SYBR FAST Universal 2x qPCR Master Mix (KAPA Biosystems), 0.2 μL of 10 μM Forward primer, 0.2 μL of 10 μM Reverse primer, 3.6 μL of PCR-grade water, and 1 μL of cDNA solution was generated and distributed to each well of a 0.2-mL 48-well PCR plate. The PCR reaction solution was subjected to initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 94°C for 10 s, and annealing and extension at 60°C for 30 s using an Eco Real-Time PCR System (Illumina). Finally, melting curve analysis was performed from 60°C to 95°C and the plate then was maintained at 4°C. The mRNA copy number of each gene in 10 ng of total RNA of each sample was determined by comparison to a standard curve. The mRNA copy number of *RFC3*, a housekeeping gene, was determined as an internal standard and used for normalization to determine relative transcript amounts. qRT-PCR analysis was performed in triplicate; the resulting data were used to calculate mean transcript levels.

Detection of *Nt6549g30* and SSR markers on the Q chromosome

The nucleotide sequence of *Nt6549g30* was obtained from Ma[11], and gene-specific primers *Nt6549g30*-1 and *Nt6549g30*-3 were designed using NetPrimer software (Fig. 5, Table S5). The SSR markers on the Q chromosome (Linkage group No. 11) were obtained from Bindler *et al.* [40, 41], and correspond to primer pairs that amplify products from *N. tabacum* but not from *N. suaveolens*, or those that generate amplicons of distinct sizes in the two species (Fig. 1, Table S5). These PCR amplifications used the KAPA Taq Extra PCR Kit (KAPA Biosystems), with each 10-μL reaction comprising 2.0 μL of 5x KAPA Taq Extra Buffer, 0.6 μL of 25 mM $MgCl_2$, 0.2 μL of 10 mM dNTP Mix, 0.5 μL of 10 μM Forward primer, 0.5 μL of 10 μM Reverse primer, 0.5 μL of 5 U/μL KAPA Taq Extra DNA Polymerase, 20 ng of DNA, and PCR-grade water to volume. Alternatively, the reactions used the KAPA TaqExtra Hotstart Ready mix with dye, with each 10-μL reaction comprising 5 μL of 2x KAPA TaqExtra Hotstart Ready mix with dye, 0.5 μL of 10 μM Forward primer, 0.5 μL of 10 μM Reverse primer, 20 ng of DNA, and PCR-grade water to volume. For detection of *Nt6549g30*, the PCR reaction solutions were subjected to initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min. For detection of SSR markers, the PCR reaction solutions were subjected to initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min. The PCR products were electrophoresed and visualized as described above for RAPD-PCR products.

GRAS-Di

GRAS-Di analysis was performed under contract at Gene Bay Co., Ltd. Among the obtained GRAS-Di amplicons, those specific to *N. tabacum* reference genomic sequence according to Edwards *et al*.[42] (ftp://ftp.solgenomics.net/genomes/Nicotiana_tabacum/edwards_et_al_2017/assembly/Nitab-v4.5_genome_Chr_Edwards2017.fasta), those specific to the scaffolds included in the Q chromosome (Linkage group No. 11), and those with no mismatch were selected. For each individual, the marker with the number of sequenced reads judged to be 0 was defined as “no detection”, and the marker for which the number of sequenced reads was judged to be greater than 0 was defined as “detection”.

Analysis of the copy number of the SSR marker on the Q homoeologous chromosome

The SSR marker on the homoeologous chromosome of the Q chromosome (Linkage group No. 13, Edwards *et al*.[42]) from Bindler *et al.* [41] that failed to exhibit PCR amplification in *N. suaveolens* was selected and used for qPCR (Fig. 5, Table S5). In addition, Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to identify the scaffold (Sierro *et al*. [43]) that mapped to the homoeologous partner of the Q chromosome and contained the SSR marker. Flanking sequences from this scaffold were used to design primers (thereby defining DNA markers) that generated products from *N. tabacum* but not from *N. suaveolens* (Fig. 5, Table S5). Real-time q-PCR was performed according to the method described above in “Expression analysis” using (as templates) the total DNA of lethal seedlings, viable seedlings, and regenerated plants in which no SSR marker corresponding to the distal part of the Q chromosome was detected. These measurements were repeated three times; the resulting data were used to calculate mean values.

Declarations

Contributions

K.N., H.N., and T.Y. designed the study; K.N. and T.Y. wrote the manuscript; K.N., H.N., and H.Y. performed experiments and collected and analyzed data; N.I., Y.S., W.M., and M.K. provided technical support and conceptual advice.

Acknowledgements

This work was partly supported by a Grant-in Aid for Challenging Exploratory Research (No. 246580004) to T. Yamada from the Japan Society for the Promotion of Science (JSPS).

Competing interests

The authors declare no competing interests.

Data Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

References

1. Tsunewaki, K. Monosomic and Conventional Analyses in Common wheat. III Lethality. *The Japanese Journal of Genetics*, 35(3), 71-75 (1960).
2. Oka, H. I. Genic analysis for the sterility of hybrids between distantly related varieties of cultivated rice. *Journal of Genetics*, 55(3), 397-409 (1957).
3. Song, L. et al. Interaction of novel Dobzhansky–Muller type genes for the induction of hybrid lethality between *Gossypium hirsutum* and *G. barbadensecv*. Coastland R4-4. *Theoretical and applied genetics*, 119(1), 33-41 (2009).
4. Yamada, T. et al. Detection of four lethality types in interspecific crosses among *Nicotiana* species through the use of three rescue methods for lethality. *Breeding science*, 49(3), 203-210 (1999).
5. Shiragaki, K. et al. Phenylalanine ammonia-lyase and phenolic compounds are related to hybrid lethality in the cross *Nicotiana suaveolens* × *N. tabacum.Plant Biotechnology*, 20-0606 (2020).
6. Ueno, N. et al. Accumulation of protein aggregates induces autolytic programmed cell death in hybrid tobacco cells expressing hybrid lethality. *Scientific reports*, 9(1), 1-10 (2019).
7. Ueno, N. et al. Time course of programmed cell death, which included autophagic features, in hybrid tobacco cells expressing hybrid lethality. *Plant cell reports*, 35(12), 2475-2488 (2016).
8. Mallet, J. What does *Drosophila* genetics tell us about speciation?. *Trends in ecology & evolution*, 21(7), 386-393 (2006).

9. Bomblies, K., & Weigel, D. Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. *Nature Reviews Genetics*, 8(5), 382. (2007)
10. Jeuken, M. J. et al. *Rin4* causes hybrid necrosis and race-specific resistance in an interspecific lettuce hybrid. *The Plant Cell*, 21(10), 3368-3378 (2009).
11. Ma, J. M. Y. The Fine Mapping of Two Black Shank Resistance Loci and Identification of a Hybrid Lethality Gene in Tobacco (2017). *PhD thesis, North Carolina state university*.
12. Deng, J. et al. A CC-NBS-LRR gene induces hybrid lethality in cotton. *Journal of experimental botany*, 70(19), 5145-5156 (2019).
13. Ma, J. et al. Identification and editing of a hybrid lethality gene expands the range of interspecific hybridization potential in *Nicotiana*. *Theoretical and Applied Genetics*, 1-11(2020).
14. Marubashi, W., & Onosato, K. Q chromosome controls the lethality of interspecific hybrids between *Nicotiana tabacum* and *N. suaveolens*. *Breeding science*, 52(2), 137-142 (2002).
15. Tezuka, T., & Marubashi, W. Hybrid lethality in interspecific hybrids between *Nicotiana tabacum* and *N. suaveolens*: evidence that the Q chromosome causes hybrid lethality based on Q-chromosome-specific DNA markers. *Theoretical and applied genetics*, 112(6), 1172-1178 (2006).
16. Clausen, R. E., & Cameron, D. R. Inheritance in *Nicotiana tabacum*. XVIII. Monosomic analysis. *Genetics*, 29(5), 447 (1944).
17. Tezuka, T. et al. Identification of *Nicotiana tabacum* linkage group corresponding to the Q chromosome gene (s) involved in hybrid lethality. *PloS one*, 7(5), e37822 (2012).
18. Hancock, W. G. et al. Identification of maternal haploids of *Nicotiana tabacum* aided by transgenic expression of green fluorescent protein: Evidence for chromosome elimination in the *N. tabacum* x *N. africana* interspecific cross. *Molecular breeding*, 35(9), 179 (2015).
19. Higgins, E. E. et al. Detecting de novo homoeologous recombination events in cultivated *Brassica napus* using a genome-wide SNP array. *G3: Genes, Genomes, Genetics*, 8(8), 2673-2683 (2018).
20. Lashermes, P. Genome rearrangements derived from homoeologous recombination following allopolyploidy speciation in coffee. *The Plant Journal*, 78(4), 674-685 (2014).
21. Lim, K. B. et al. Homoeologous recombination in interspecific hybrids of *Lilium*. *Korean J Breed*, 35, 8-12 (2003).
22. Chen, S. et al. Unstable Allotetraploid Tobacco Genome due to Frequent Homeologous Recombination, Segmental Deletion, and Chromosome Loss. *Molecular plant*, 11(7), 914-927 (2018).
23. Chen, Z. et al. Use of tissue culture to bypass wheat hybrid necrosis. *Theor. Appl. Genet*. 78, 57-60 (1989).
24. Sinha, A. K. et al. Novel technique for precluding hybrid necrosis in bread wheat. *Int J Tro Agr*, 34(3), 761-765 (2016).
25. Inoue, E. Improvement of the method for overcoming the hybrid lethality between *Nicotiana suaveolens* and *N. tabacum* by culture of F1 seeds in liquid media containing cytokinins. *Japanese Journal of Breeding*, 47(3), 211-216 (1997).

26. Krishna, H. et al. Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech*, 6(1), 54 (2016).
27. Czene, S., & Harms-Ringdahl, M. Detection of single-strand breaks and formamidopyrimidine-DNA glycosylase-sensitive sites in DNA of cultured human fibroblasts. *Mutation Research/DNA Repair*, 336(3), 235-242 (1995).
28. Enoki, H., & Takeuchi, Y. New genotyping technology, GRAS-Di, using next generation sequencer. Proceedings of the Plant and Animal genome conference XXVI. San Diego, CA (2018). Available online: https://pag.confex.com/pag/xxvi/meetingapp.cgi/Paper/29067 (accessed on 12 December 2020)
29. Xiong, Z. et al. Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus*. *Proceedings of the National Academy of Sciences*, 108(19), 7908-7913 (2011).
30. Gaebelein, R. et al. Inherited allelic variants and novel karyotype changes influence fertility and genome stability in *Brassica* allohexaploids. *New Phytologist (2019) 223: 965–978* (2019).
31. Clarkson, J. J. et al. Nuclear glutamine synthetase evolution in *Nicotiana*: phylogenetics and the origins of allotetraploid and homoploid (diploid) hybrids. *Molecular Phylogenetics and Evolution*, 55(1), 99-112 (2010).
32. Sun, S. et al. Tissue culture-induced somaclonal variation of decreased pollen viability in torenia (*Torenia fournieri* Lind.). *Botanical studies*, 54(1), 36 (2013).
33. Ogawa, T. et al. Efficient In Planta Detection and Dissection of De Novo Mutation Events in the *Arabidopsis thaliana* Disease Resistance Gene *UNI*. *Plant and Cell Physiology*, 57(6), 1123-1132 (2016).
34. Mauch-Mani, B., & Slusarenko, A. J. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to *Peronospora parasitica*. *The Plant Cell*, *8*(2), 203-212 (1996).
35. Santamaria, M. et al. The promoter of a basic PR1-like gene, AtPRB1, from Arabidopsis establishes an organ-specific expression pattern and responsiveness to ethylene and methyl jasmonate. *Plant Molecular Biology*, *47*(5), 641-652 (2001).
36. Reymond, P., & Farmer, E. E. Jasmonate and salicylate as global signals for defense gene expression. *Current opinion in plant biology*, 1(5), 404-411 (1998).
37. Yamada, T. et al. Apoptotic cell death induces temperature-sensitive lethality in hybrid seedlings and calli derived from the cross of *Nicotiana suaveolens*× *N. tabacum*. *Planta*, *211*(5), 614-622 (2000).
38. Tezuka, T. et al. Possible involvement of genes on the Q chromosome of Nicotiana tabacum in expression of hybrid lethality and programmed cell death during interspecific hybridization to *Nicotiana debneyi*. *Planta*, *226*(3), 753-764 (2007).
39. Shinozaki, Y. et al. Expression of an *AtNAP* gene homolog in senescing morning glory (*Ipomoea nil*) petals of two cultivars with a different flower life span. *Journal of plant physiology*, *171*(8), 633-638 (2014).

40. Bindler, G. et al. A microsatellite marker based linkage map of tobacco. *Theoretical and Applied Genetics*, 114(2), 341-349 (2007).
41. Bindler, G. et al. A high density genetic map of tobacco (*Nicotiana tabacum* L.) obtained from large scale microsatellite marker development. *Theoretical and Applied Genetics*, 123(2), 219 (2011).
42. Edwards, K. D. et al. A reference genome for *Nicotiana tabacum* enables map-based cloning of homeologous loci implicated in nitrogen utilization efficiency. *BMC genomics*, 18(1), 448 (2017).
43. Sierro, N. et al. The tobacco genome sequence and its comparison with those of tomato and potato. *Nature communications*, 5, 3833 (2014).

Tables

Table 1. Detection of products, amplified by SSR and *Nt6549g30* primer pairs, from the Q chromosome (Linkage group No. 11) of *N. tabacum* in lethal seedlings, viable seedlings, and viable regenerated plants of the *N. suaveolens* x *N. tabacum* cross.

Type of primer pair	Name of primer pair	Distance (cM)[c]	*N. suaveolens*	*N. tabacum*	lethal seedlings			viable seedlings							
					1	2	3	s14-1	s14-2	s14-4	s14-7	s14-8	s17-1	s17-2	s18-1
SSR[a]	PT20383	38.6	-	+	+	+	+	+	+	+	+	+	+	+	+
	PT1348	49.0	-	+	+	+	+	+	+	+	+	+	+	+	+
	PT54570	75.9	-	+	+	+	+	+	+	+	+	+	+	+	+
	PT52864	99.5	-	+	+	+	+	+	+	+	+	+	-	+	+
	PT55075	105.7	-	+	+	+	+	+	+	+	+	+	-	+	+
	PT60178	109.6	-	+	+	+	+	+	+	+	+	+	-	+	+
	PT53094	117.6	-	+	+	+	+	+	+	+	+	-	-	-	-
	PT30342	122.0	-	+	+	+	+	+	+	+	-	-	-	-	-
	PT30365	122.8	-	+	+	+	+	+	+	+	-	-	-	-	-
	PT52778	124.5	-	+	+	+	+	+	+	+	-	-	-	-	-
Gene	*Nt6549g30* -1[b]	N.A.	-	+	+	+	+	+	+	+	-	-	-	-	-
	Nt6549g30 -3[b]		-	+	+	+	+	+	+	+	-	-	-	-	-

Type of primer pair	Name of primer pair	Distance (cM)[c]	viable regenerated plants											
			r18-1	r18-4	r18-5	r18-7	r19-1	r19-2	r19-3	r19-4	r19-5	r19-6	r19-7	r19-8
SSR[a]	PT20383	38.6	+	+	+	+	+	+	+	+	+	+	+	+
	PT1348	49.0	+	+	+	-	+	+	+	+	+	+	+	+
	PT54570	75.9	+	+	+	+	+	+	+	+	+	+	+	+
	PT52864	99.5	-	+	+	-	+	+	+	+	+	+	+	+
	PT55075	105.7	-	+	+	-	+	+	+	+	+	+	+	+
	PT60178	109.6	-	+	+	-	+	+	+	+	+	+	+	+
	PT53094	117.6	-	+	+	-	+	+	+	+	+	+	+	+
	PT30342	122.0	-	+	+	-	+	+	-	+	+	+	+	+
	PT30365	122.8	-	+	+	-	+	+	-	+	+	+	+	+
	PT52778	124.5	-	+	+	-	+	+	-	+	+	+	+	+
Gene	*Nt6549g30* -1[b]	N.A.	-	+	+	-	+	+	-	+	+	+	+	+
	Nt6549g30 -3[b]		-	+	+	-	+	+	-	+	+	+	+	+

'+' indicates the presence of allele of *N. tabacum*, '-' indicates the absence of allele of *N. tabacum*. N.A., not applicable.

a: Bindler *et al.* [40, 41]

b: Closely linked to PT30342, as confirmed by linkage analysis (Ma[11])

c: Genetic distance from the marker amplified by primer pair PT54560.

Figures

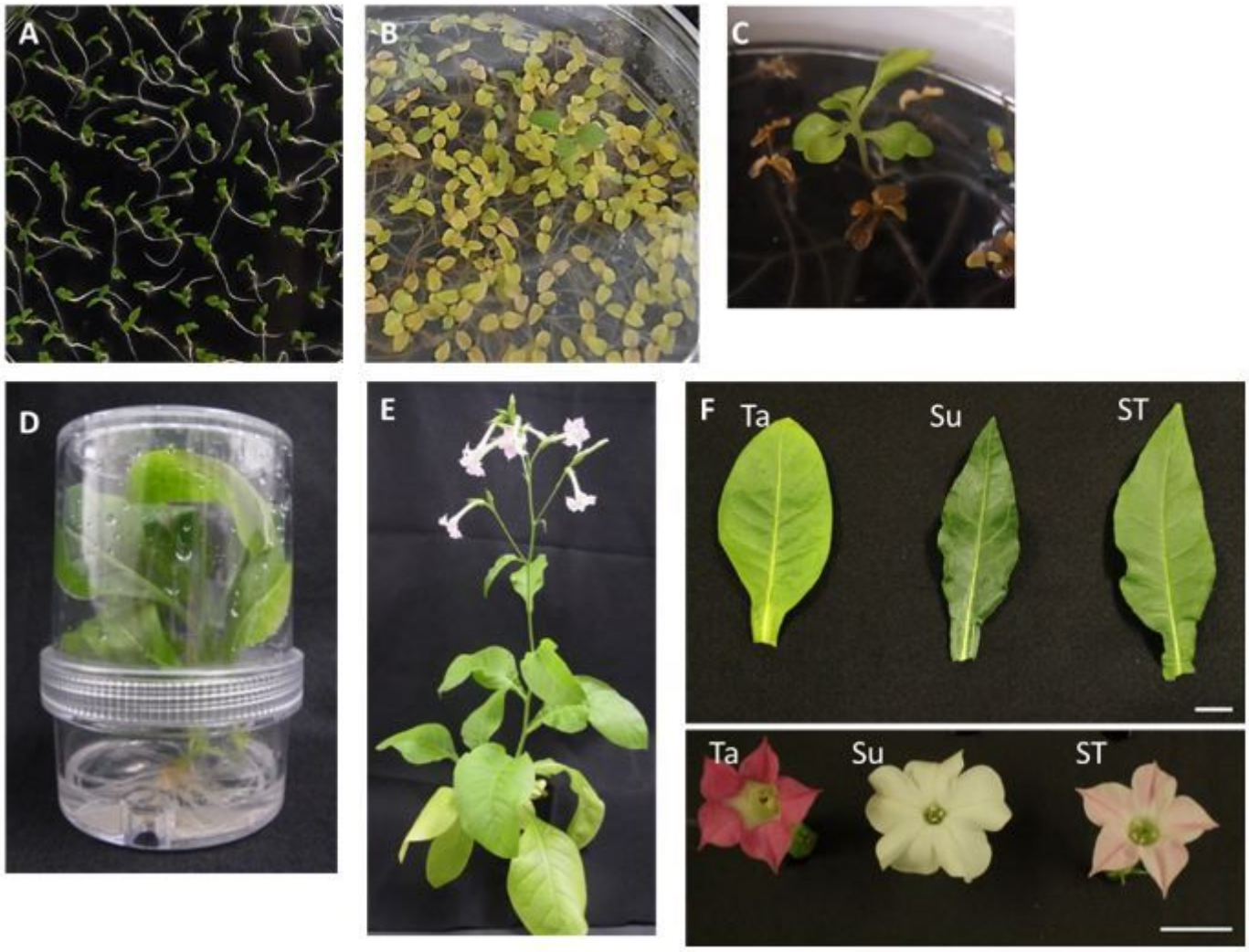

Figure 1

Process of obtaining hybrid viable seedlings. (A) Hybrid seedlings at 3 days after germination (DAG). (B) Seedlings at 14 DAG. (C) Seedling not showing lethality at 20 DAG. (D) Viable hybrid seedling cultured and propagated in a plant box. (E) Matured viable plant. (F) Leaf and flower phenotype of parents and hybrid plant. Ta: N. tabacum, Su: N. suaveolens, ST: N. suaveolens x N. tabacum. Scale bar: 2 cm.

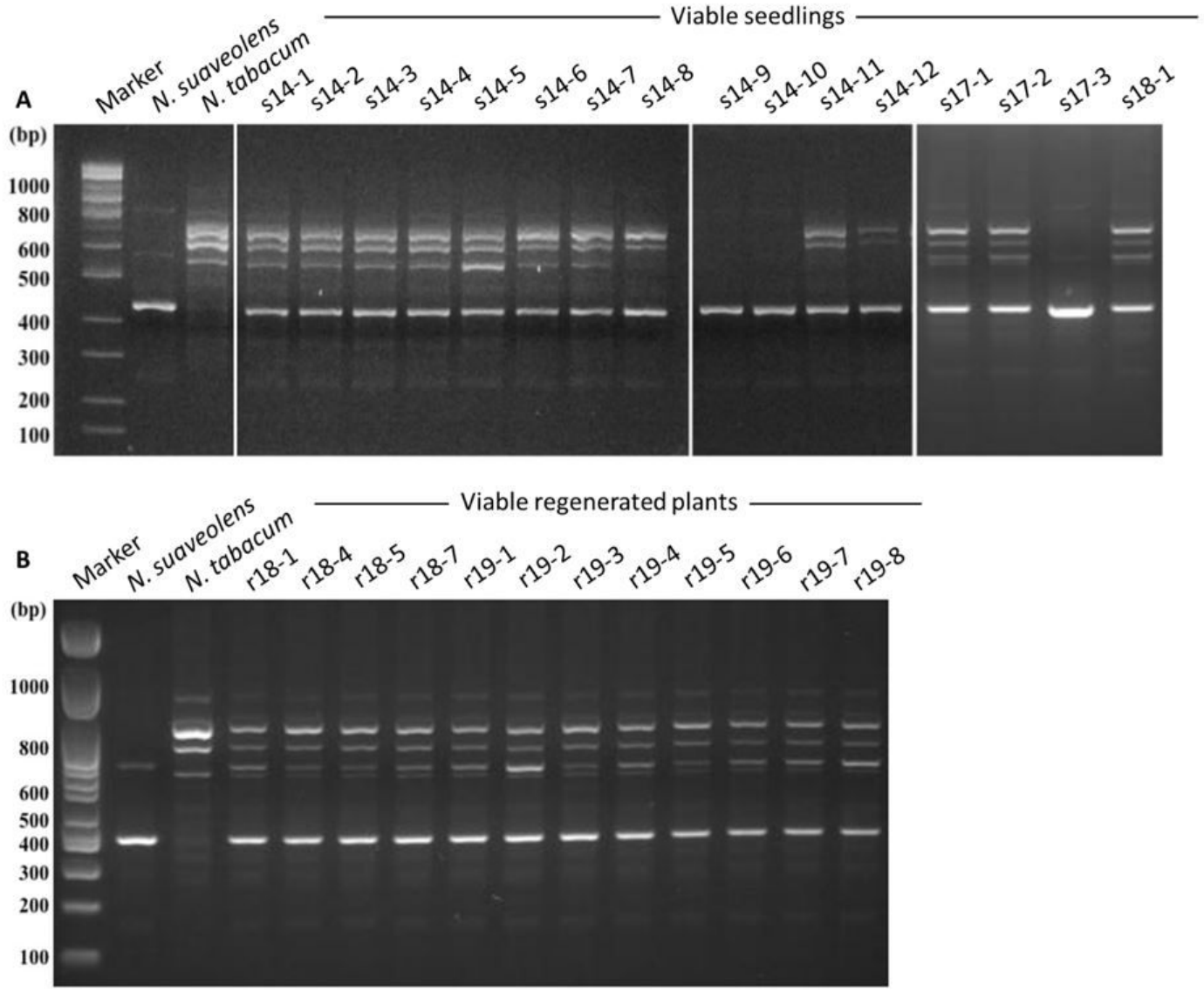

Figure 2

Confirmation of hybridity of viable seedlings and viable regenerated plants of the N. suaveolens x N. tabacum cross. Products of Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) performed with primer OPA-12 were separated by agarose gel electrophoresis to assess hybridity of viable seedlings (A) and viable regenerated plants (B). See also Table S1.

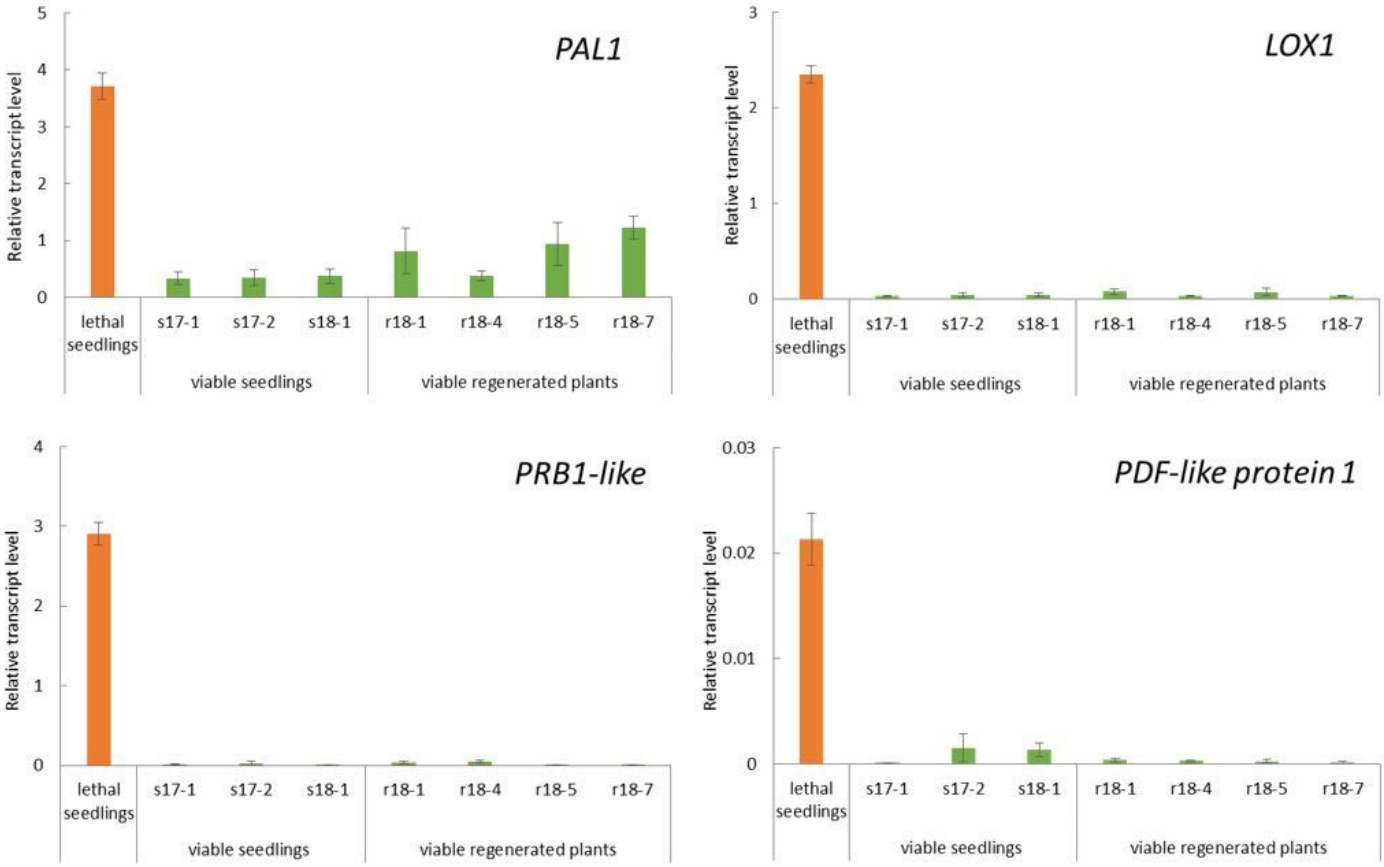

Figure 3

Relative transcript levels of immune response-related genes in cotyledons of lethal seedlings (at 6 days after germination (DAG)) and in young true leaves of viable seedlings (s17-1, s17-2, and s18-1) and viable regenerated plants (r18-1, r18-4, r18-5, and r18-7) of the N. suaveolens x N. tabacum cross. Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was used to determine transcript levels of PAL (Phenylalanine ammonia lyase) 1, PRB (Basic form of Pathogenesis-related protein) -1 like, LOX (Lipoxygenase) 1, PDF (Plant defensin) -like protein 1 relative to those of RFC3 (a housekeeping gene). Data are shown as the mean ± SE from triplicate samples (n=3) for each treatment.

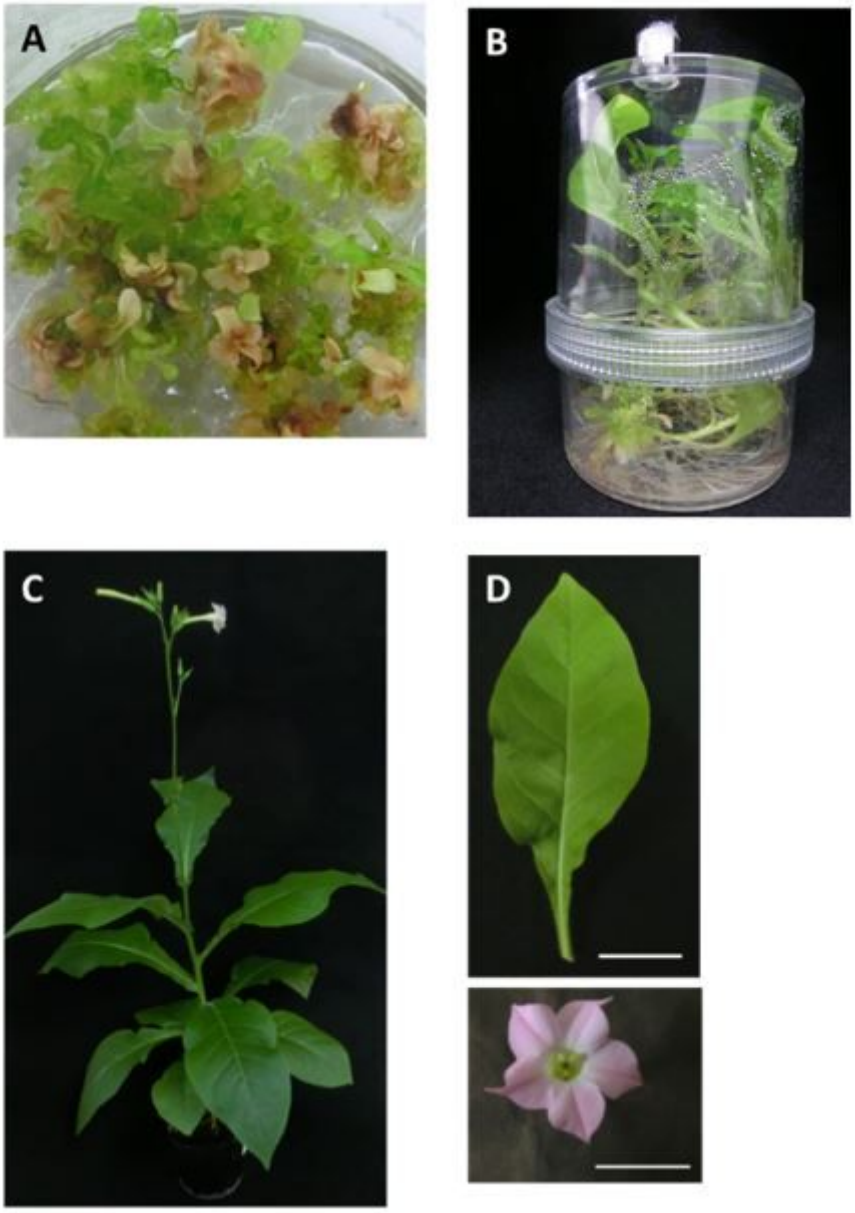

Figure 4

Process of obtaining hybrid regenerated plants not showing lethality. (A) Viable shoots formed on hybrid seedlings cultured on medium supplemented with 2.0 mg/L 6-benzylaminopurine (BAP). (B) Regenerated plants from viable shoots propagated in a plant box. (C) Matured regenerated plant. (D) Leaf and flower phenotype of one regenerated plant. Scale bar: 2 cm.

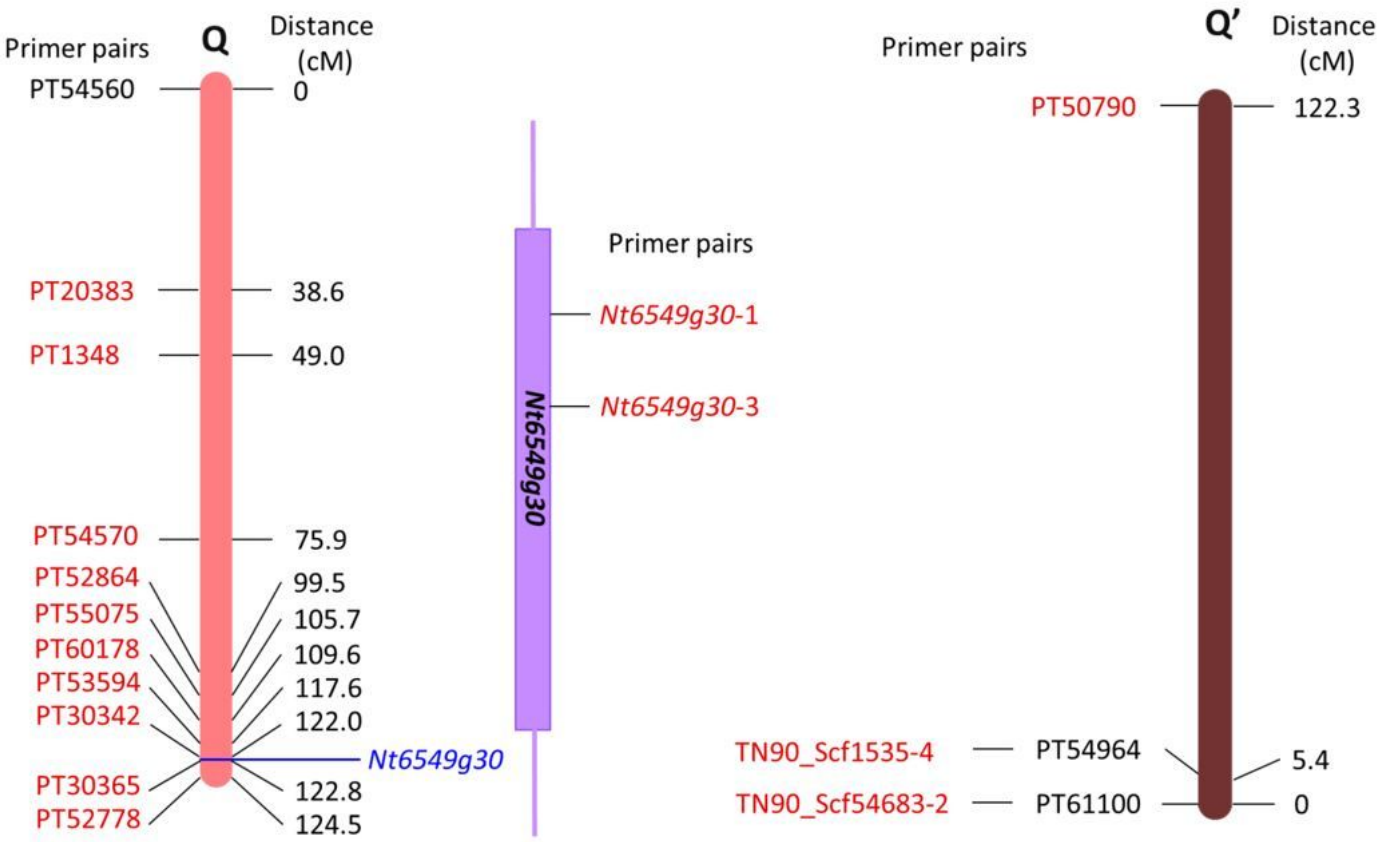

Figure 5

Locations of primer pairs on the Q chromosome (Linkage group No. 11), Nt6549g30, and the Q' (Q homologous) chromosome (Linkage group No. 13). The primer pairs used in this study are indicated in red, whereas others are indicated in black. Nt6549g30 is closely linked to PT30342, as confirmed by linkage analysis11. Distance (cM) indicates genetic distance from the primer pair PT54560 on the Q chromosome, and from the primer pair PT61100 on the Q' chromosome. TN90_Scf1535-4 and TN90_Scf54683-2 are located close to PT54964 and PT61100, respectively (within ± 31 kbp). See also Table S5 for primer list.

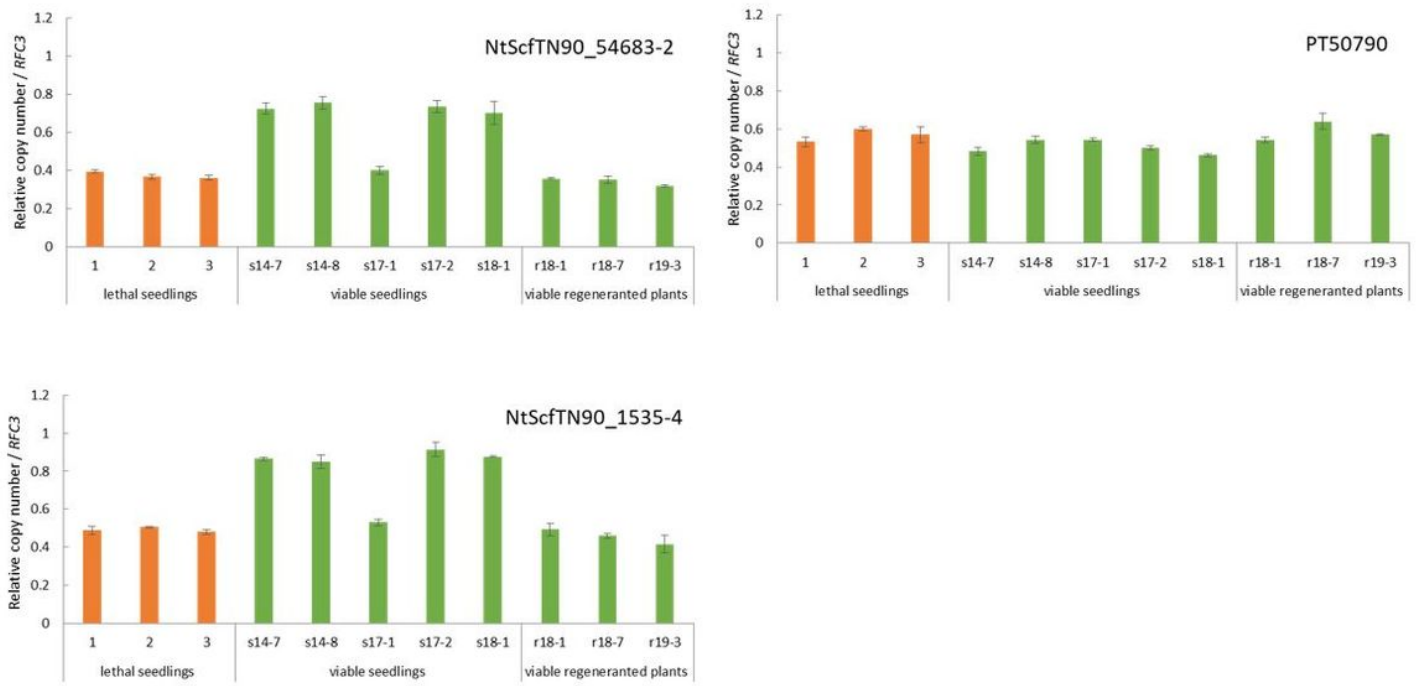

Figure 6

Mean (±SE) of relative copy number of each marker on the Q' chromosome in lethal seedlings (No. 1-No. 3), viable seedlings lacking distal part of the Q chromosome (s14-7~8, s17-1~2, and s18-1), and viable regenerated plants lacking distal part of the Q chromosome (r18-1, r18-7, and r19-3). Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was used to determine copy number of each region on the Q' chromosome relative to that of the RFC3 gene. Data are shown as the mean ± SE from triplicate experiments for each DNA sample.

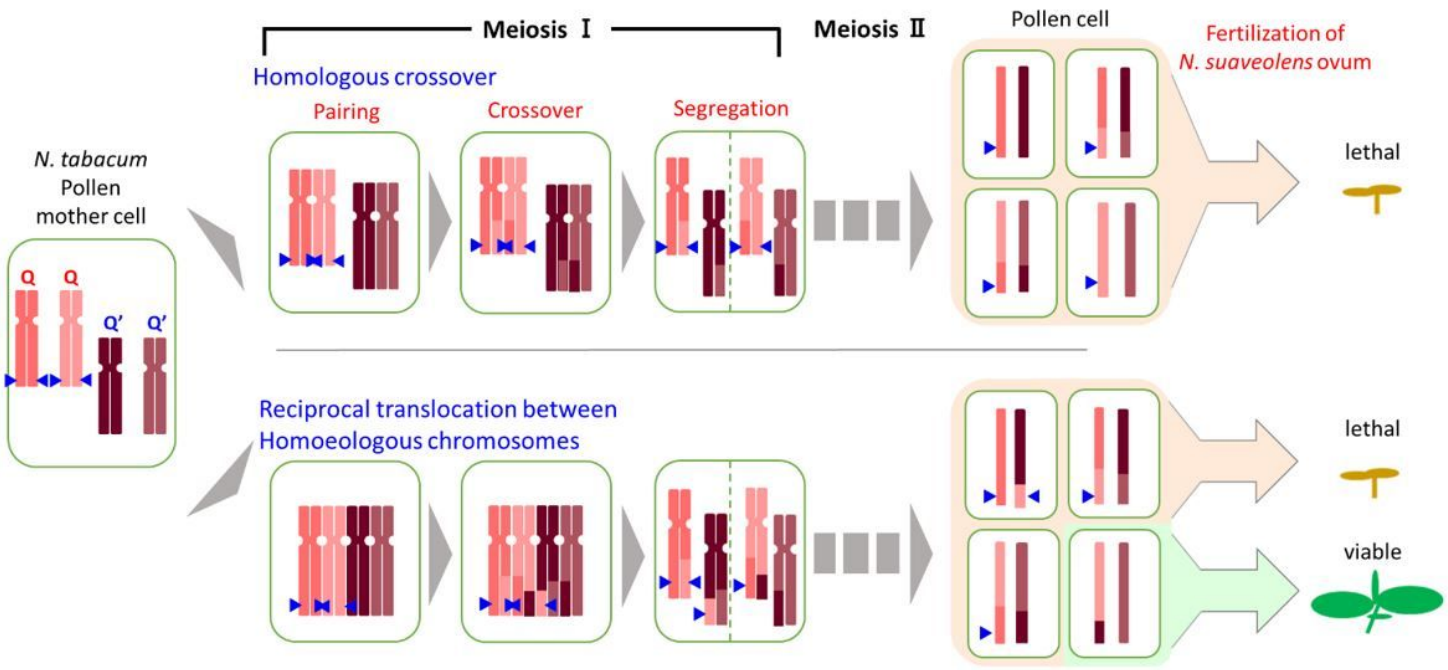

Figure 7

A proposed model for overcoming hybrid lethality by reciprocal translocation between homoeologous chromosomes in hybrid seedlings from the N. suaveolens x N. tabacum cross. Blue arrowhead indicates hybrid lethality-associated gene of N. tabacum.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupportingInformation.docx