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論文名	Histological and molecular biological analyses of reproductive isolation in interspecific-interploidy crosses *Nicotiana suaveolens* × *Nicotiana tabacum* （タバコ異倍数性種間交雑 *Nicotiana suaveolens* × *Nicotiana tabacum* における生殖隔離の組織学および分子生物学的解析）
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論文要旨

Reproductive isolation, which is divided into two types, prezygotic and postzygotic barriers, is a mechanism that separates species and plays a crucial role in the evolution of animals and plants (Stebbins 1966; Rieseberg and Blackman 2010). In plants, prezygotic barriers include the inhibition of pollen adhesion to the stigma, pollen germination, pollen tube growth in the style, and pollen tube penetration of the ovule micropyle (Dickinson et al. 2012). Postzygotic barriers, which occur after a successful fertilization, include seed abortion as well as the weakness, inviability, and sterility of the F_1 hybrid plants or their offspring (Li et al. 1997; Bushell et al. 2003; Ichitani et al. 2007; Kuboyama et al. 2009; Tezuka et al. 2010; Guo et al. 2016). These barriers hinder plant breeding programs, especially those involving wide hybridizations such as interspecific or intergeneric crosses.

This study aimed to investigate and overcome the postzygotic barriers in crosses between *Nicotiana suaveolens* and *N. tabacum*. In Chapter 1, I investigated what types of reproductive isolation were exhibited using several accessions of *N. suaveolens* for crosses with *N. tabacum*. In subsequent chapters, among reproductive isolation observed in Chapter 1, I focused on seed abortion and ovary abscission in interploidy-interspecific crosses. In Chapter 2, I investigated the mechanism of these reproductive barriers using ploidy manipulated lines and histological methods. In Chapter 3, I investigated whether different parental ploidy level is the only factor causing seed abortion and ovary abscission. Finally, in Chapter 4, I carried out exhaustive gene expression analysis of seed abortion in interploidy-interspecific crosses using RNA sequence.

Chapter 1. *Nicotiana suaveolens* accessions with different ploidy levels exhibit different reproductive isolation mechanisms in interspecific crosses with *Nicotiana tabacum*

I investigated whether the chromosome numbers and ploidy levels of eight *Nicotiana suaveolens* accessions are related to the reproductive isolation after crosses with *N. tabacum* by flow cytometry and chromosome analyses. Additionally, the internal transcribed spacer (ITS) regions of the eight *N. suaveolens* accessions were sequenced and compared with the previously reported sequences of 22 *Suaveolentes* species to elucidate the phylogenetic relationships in the section *Suaveolentes*. I revealed that four *N. suaveolens* accessions comprised 64 chromosomes (octoploid), while the other four accessions carried 32 chromosomes (tetraploid). Depending on the ploidy levels of *N. suaveolens*, several types of reproductive isolation were observed after crosses with *N. tabacum,* including decreases in the number of capsules and the germination rates of hybrid seeds, as well as hybrid lethality and abscission of enlarged ovaries at 12–17 days after pollination (DAP). A phylogenetic analysis involving ITS sequences divided the eight *N. suaveolens* accessions into three distinct clades. Based on the results, I confirmed that *N. suaveolens* accessions vary regarding ploidy levels and reproductive isolation mechanisms in crosses with *N. tabacum*. These accessions will be very useful for revealing and characterizing the reproductive isolation mechanisms in interspecific crosses and their relationships with ploidy levels.

Chapter 2. Type II seed abortion causes ovary abscission in interploidy-interspecific crosses between *Nicotiana suaveolens* and *Nicotiana tabacum*

When octoploid *Nicotiana suaveolens* accession PI 555565 (8x) was used as the seed parent for the cross with tetraploid *N. tabacum* (4x), enlarged ovaries were dropped at 12-17 DAP and hybrid seeds were never obtained. Meanwhile, seed abortion was observed in another interploidy-interspecific cross between *N. suaveolens* PI 555561 (8x) as the seed parent and *N. tabacum* (4x) as the pollen parent (Chapter 1). In this chapter, I investigated whether highly excess maternal genome causes the enlarged ovary abscission. Using the two interploidy-interspecific crosses, comparative analyses for the ovary abscission zone and ovule development were conducted. I revealed that distinct types of abnormalities (Type I and Type II seed abortion) were found during ovule development in the two crosses. Based on the results, I established a hypothesis that ovary abscission is a consequence of Type II seed abortion. Further cross experiments using ploidy manipulated lines added strong supports to this hypothesis: successive increase of maternal ploidy resulted in successively Type I and Type II abnormal ovule developments, and the latter was accompanied with ovary abscission. Ploidy manipulations for balance of parental ploidy levels were sufficient to restore endosperm and embryo development, and prevent the ovary abscission.

Chapter 3. Maternal genome excess causes Type II seed abortion and ovary abscission in intraspecific crosses of *Nicotiana suaveolens*

The study in Chapter 2 clearly indicated that unbalanced parental ploidy levels led to different types of seed abnormal development in interploidy-interspecific crosses. Highly excess maternal genome caused Type II seed abortion, which led to the enlarged ovary abscission. However, these experiments were conducted in interspecific crosses, and it was unclear whether the abnormal seed developments were caused only by different parental ploidy levels. To obtain further insight into the relationship between parental ploidy levels and abnormal seed development as well as ovary abscission, I carried out the intraspecific crosses using *N. suaveolens* accessions with different ploidy levels. In intraspecific crosses, normal seed development was observed in interploidy crosses (8x × 4x). However, Type I and Type II seed abortion were observed in highly maternal-excess interploidy crosses (16x × 4x), and Type II seed abortion led to ovary abscission. These results gave strong evidence to prove that the difference of parental ploidy is sufficient to cause successively Type I and Type II abnormal seed developments, and the latter led to ovary abscission.

Chapter 4. RNA sequencing analysis of seed abortion in interspecific crosses between *Nicotiana suaveolens* and *Niacotian tabacum*

To better understand the mechanisms causing the seed abortion, I conducted RNA sequencing to exhaustively identify differentially expressed genes between abnormal seed development (Type I and Type II seed abortion) and normal seed development. I identified several expression changing genes including plant hormone related genes such as *IAA*, *GID1* and *TGA*. It is reported that plant hormones are important to seed development (Figueiredo and Köhler 2018, Locascio et al 2014). Thus, my results suggested that abnormal expression patterns of plant hormone related genes were associated with seed abortion in interploidy-interspecific crosses.

In conclusion, the results obtained in this thesis showed that Type I seed abortion and Type II seed abortion with ovary abscission occur in a phased manner as maternal to paternal genome dosage increases. The expression of plant hormone related genes would be associated with seed abortion. Then, Type II seed abortion triggers several events in ovary abscission zone, such as changes of abscission related gene expression, lignin deposition, and eventually ovary abscission. Furthermore, seed abortion and ovary abscission can be overcome by ploidy manipulations for balance of parental ploidy level. Hence, the outcome of this thesis will contribute towards understanding and overcoming seed abortion and ovary abscission in interspecific and interploidy crosses in plants.

審査結果の要旨

植物育種において、新品種を作出する育種方法の主流として利用されているのは交雑育種法である。交雑親の組合せは育種目的によって様々であるが、種間交雑や属間交雑などのように、互いに遠縁な種を両親とした交雑（遠縁交雑）を行うことで、種内の品種・系統間の交雑では作り出せないような多様な品種を育成できるものと期待される。しかし、遠縁種の間には生殖隔離が発達していることが多く、遠縁交雑育種の妨げとなっている。

生殖隔離は種を隔てる機構であり、種分化の過程で大きな役割を果たしてきたと考えられている。交配後に認められる生殖隔離は、受精前隔離と受精後隔離の 2 つに分けられる。植物では、柱頭における花粉付着阻害および花粉発芽阻害、花柱における花粉管伸長阻害、胚珠の珠孔への花粉管侵入阻害が受精前隔離に含まれる。一方、受精後隔離には、種子発育不全、雑種弱勢、雑種致死、雑種不稔、雑種崩壊が含まれる。このように植物では多様な生殖隔離が認められるが、その機構については未だ十分に解明されていない。

本研究では、*Nicotiana* 属（タバコ属）の野生種 *N. suaveolens* および栽培種 *N. tabacum* を用いて生殖隔離の解析を行った。主として種子発育不全および子房落下の 2 つの生殖隔離を対象とし、組織学的および分子生物学的手法を基盤として、それらの機構を解明することを目的とした。

第 1 章では、8 系統の *N. suaveolens* を *N. tabacum* と交雑すると、親にした *N. suaveolens* の系統によっていくつかの生殖隔離（低結実率、種子発育不全、子房落下、雑種致死）が生じることを明らかにした。染色体解析とフローサイトメトリー解析の結果、*N.*

suaveolens 8 系統のうち、4 系統は 32 本の染色体をもつ 4 倍体、残りの 4 系統は 64 本の染色体をもつ 8 倍体であった。加えて、rDNA の ITS 領域を用いた分子系統解析の結果、系統樹上で *N. suaveolens* 8 系統が 3 つの分岐群に分類された。以上の結果から、*N. suaveolens* × *N. tabacum* の交雑における生殖隔離は、*N. suaveolens* の倍数性に依存する隔離と倍数性に依存しない隔離に分けられることが示唆された。次章以降では、倍数性依存と考えられた種子発育不全と子房落下に焦点を絞り解析を行った。

第 2 章では、*N. tabacum* との交雑において、種子発育不全を生じる *N. suaveolens* PI 555561 および子房落下を生じる *N. suaveolens* PI 555565 を用いて比較解析を行った。胚珠の組織学的観察により､2 つの種間交雑組合せでは異なるタイプの発育異常が生じることが明らかとなり、それぞれをタイプ I およびタイプ II とした。さらに、親系統の倍数性を操作して交雑実験を行うことで、父親の倍数性に対して母親の倍数性が増加するにつれて、タイプ I およびタイプ II 種子発育不全が段階的に生じること、タイプ II 種子発育不全は子房落下を伴うことを明らかにした。また、両親の倍数性を適切に調節することでこれらの生殖隔離が克服できることも示した。

第 3 章では、第 2 章で得られた結論をさらに検証するために、倍数性を操作した一連の *N. suaveolens* 系統を用いて系統間交雑を行った。その結果、母親の倍数性を増加させることで､系統間交雑においても 2 タイプの種子発育不全および子房落下が再現できた。したがって、①父親の倍数性に対して母親の倍数性が増加することで種子発育不全が生じること、②両親の倍数性の差がさらに広がることで種子発育不全の症状が重くなること、③タイプ II 種子発育不全が子房落下を引き起こすことが示された。

第 4 章では、RNA-seq 解析を用いて、種子発育不全に関与する遺伝子の網羅的な同定を行った。その結果、オーキシンやジベレリンなどの植物ホルモンに関連する遺伝子を含む多数の遺伝子が種子発育不全に関与することが示唆された。

以上のように、本研究では、種子発育不全と子房落下が交雑親間の倍数性差に起因しており、タイプ II 種子発育不全が子房落下を引き起こすことを明らかにした。また、種子発育不全に関連する多数の遺伝子を明らかにした。これらの知見は、植物の交雑育種において障害となっている生殖隔離の機構解明、並びに克服法開発に寄与するものであり、植物育種学の発展にも大きく貢献するものである。よって、本論文の審査ならびに最終試験の結果と併せて、博士（応用生命科学）の学位を授与することを適当と認める。