博 士 論 文 の 要 約

博士の専攻分野の名称： 博　士（農学）　　氏名　Wang Shasha

学位論文題名

Novel inactivation mechanisms of transposon uncovered by the *Old* and *New Stabiliser* loci in Tam3 system of *Antirrhinum*

(キンギョソウの Tam3 システムにおける *Old Stabiliser* と *New Stabiliser* 遺伝子座による新規のトランスポゾン抑制機構**)**

Background

Snapdragon (*Antirrhinum majus*) is known to exhibit occasional genetic instabilities that are manifested as variegations and morphological chimeras. In the 1980s and 1990s, several transposon-induced mutants in *Antirrhinum* affecting flower color, morphology, chlorophyll and other changes were identified. Of these mutants, Tam3 (the third transposon of *A. majus*) has been associated with the most of the gene discoveries involved in the mutants. Tam3 belongs to the hAT superfamily (Ac, hobo and Tam3) and is activated at low temperatures (around 15 ℃) while remaining stable at high temperatures (around 25 ℃). It exhibits low-temperature-dependent transposition (LTDT). Several flower variegation lines have been caused by Tam3 insertion into pigmentation loci responsible for anthocyanin synthesis. This variegation arises at 15 ℃ due to LTDT of Tam3; however, an inactive Tam3 at 25℃ displays no variegation in white or pale-colored petals. Here I studied Tam3 inactivation independent of LTDT due to genetic factors that immobilize Tam3 even at 15℃. It is known that certain *Antirrhinum* lines possess the Tam3 suppression factors named *Old Stabiliser* (*OSt*) and *New Stabiliser* (*NSt*).

Research objectives

In this study, I explored the mysterious field of Tam3 suppression by *OSt* and *NSt*, at low temperature. Firstly, I identified the two suppressor elements and analyzed their gene structures, chromosomal positions and linkage with Tam3 inactivation trait. Secondly, the mechanism(s) by which *OSt* and *NSt* suppress Tam3 transposition were investigated. Finally, I found an epigenetic phenomenon, paramutation, occurring at *NSt* locus in *Antirrhinum*.

Methodology

1) Identification of *OSt* and *NSt* elements and possible mechanisms by which both *Sts* inactivate Tam3 transposition

OSt and *NSt* are semi-dominant and dominant loci for Tam3 suppression, respectively, and are unlinked. A possible reason for the *Stabiliser* phenotypes has long been proposed to be related to alterations in Tam3 itself. To investigate the relationship between Tam3 and petal variegation phenotypes, Southern hybridizations were performed for both F_2 segregations in the *OSt* and *NSt* populations using the Tam3 sequence as a probe. The association of isolated *OSt* and *NSt* candidates with *Stabiliser* phenotypes was identified by Southern hybridization and PCR amplifications. And sequence alignment and dot plot analyses were carried out to determine the structures and genomic positions of *OSt* and *NSt*.

I then examined how the two *Stabiliser* loci inhibit Tam3 transposition. Tam3 transposition requires four steps involving the *Tam3 TPase* gene: transcription, translation, nuclear import, and binding to Tam3 elements. Firstly, I performed qRT-PCR assay, immunoblot analysis and transient assay using *Antirrhinum* protoplasts to investigate the production and nuclear import of Tam3 TPase. Following these experiments, I concluded that the two *Stabilisers* are probably involved in the final step of Tam3 transposition, the binding of Tam3 TPase to Tam3 DNA sequences, to limit Tam3 transposition. I therefore investigated the effect of *OSt* and *NSt* on the binding affinity of Tam3 TPase to its target sites by electrophoretic mobility shift assay (EMSA). However, neither *OSt* and *NSt* altered the binding affinity of Tam3

TPase to its target motifs.

According to my results and those of others, it was assumed that *OSt* and *NSt* interfere with the ability of the Tam3 TPase to bind to its target sequences on the chromosome, without compromising Tam3 TPase production. Firstly, I investigated whether the *OSt*- and *NSt*- factors might be RNA products. And RNA can be classified into three types: messenger RNA (mRNA), long non-coding RNA (lncRNA) and small RNA (sRNA), so I performed poly(A)-enriched RNA-seq, rRNA-removed RNA-seq and sRNA-seq analyses to explore the transcription of the *Tam3 TPase* gene in mRNA, lncRNA and sRNA, respectively, from *OSt*, *NSt* and *ost*/*nst* plants. Specific sRNAs, accumulated in the 5′ region of Tam3 sequence in *OSt* and *NSt* line compared to the *ost*/*nst* line, could be responsible for Tam3 suppression. And TRV-mediated virus-induced gene silencing was performed to confirm the suppressive effect of these specific sRNAs on the transposition of Tam3.

According to my results, the two *Stabiliser* might generate sRNAs to repress Tam3 transposition by two possible mechanisms at the binding step of Tam3 TPase to Tam3 DNA: one is that the sRNAs bind to Tam3 DNA, and the other is that the sRNAs interact with Tam3 TPase. Here, I focused on investigating which mechanism is most possible in *NSt* line. I performed S1-RNaseH-S1 digestion and RNA-EMSA analyses to examine the binding of *NSt*-derived sRNAs to Tam3 DNA and Tam3 TPase, respectively.

2) Finding of paramutation phenomenon at *NSt* locus in *Antirrhinum*

HAM3 line (*NSt* line) was crossed to two different *nst* lines: HAM5 and HAM22. In the F2 population of H-3×22 cross, the segregation was abnormal and exhibited paramutation phenomenon. *NSt* and *nst-22* alleles derived from HAM3 and HAM22 lines were identified as paramutagenic and paramutable alelles, respectively, based on the segregation result. Then, PCR analysis was performed to determine the structure of *nst-22* allele. To investigate the maintenance of paramutated *nst-22* allele, segregation was examined in the self-crossed population of paramutated *nst-22* plants

in F3, F4 and F5 generations. Additionally, Tam3 TPase transcription and translation were investigated in paramutated *nst-22* plants by qRT-PCR and immunoblot analyses. Finally, I performed sRNA-seq analyses to confirm sRNA profiles in Tam3 in paramutated plants in H-3×22 cross.

Results

1) Identification of *OSt* and *NSt* elements and possible mechanisms by which both *Sts* inactivate Tam3 transposition

Transposon tagging identified two *St* loci involving derivatives of Tam3 with unique structures: *OSt* has a pseudo-Tam3 copy whose 5′ terminal region has been arranged compared to the cognate Tam3 element, and *NSt* consists of two intact copies of Tam3 in head-to-head orientation. Neither locus interferes with the production of the intact Tam3 TPase or the nuclear import of TPase. Both *OSt* and *NSt* produce specific sRNAs from their 5′ terminal regions containing multiple TPase binding motifs. I performed sRNA induction experiments by TRV-mediated virus induced gene silencing targeting the 5′ terminal region in the Tam3 sequence, resulted in the suppressed Tam3 transposition of the *ost*/*nst* plants Additionally, I employed S1-RNase H-S1 digestion system to detect whether *NSt*-derived sRNAs can target Tam3 DNA in *NSt* plants grown at 15 ℃, but the sRNA-DNA-interacting triplexes cannot be observed. Instead, the sRNAs generated by *NSt* harbored several TPase binding motifs and can interact with synthesized Tam3 TPase *in vitro*. These results suggested that the specific sRNAs could repress Tam3 transposition by targeting the Tam3 TPase binding motifs or interacting with the TPase itself in *OSt* line. And *NSt* might suppress Tam3 transposition by generate the sRNAs to interact with the TPase itself instead of targeting Tam3 DNA.

2) Finding of paramutation phenomenon at *NSt* locus in *Antirrhinum*

In the progenies of *NSt* crossed with *nst-22* (HAM3 × HAM22), I observed

paramutation where Tam3 transposition was suppressed even if *NSt* allele was absent. The *NSt*, *nst-22*, and *nst-5* alleles originated from HAM3, HAM22 and HAM5 lines, respectively. When crossing between HAM3 and HAM22, *NSt* stabilized Tam3 with *nst-22*, resulting in *NSt* becoming a paramutagenic allele and *nst-22* becoming a paramutable allele. The crossing between *NSt* and *nst-5* produced an active Tam3 linked to a homozygous *nst-5* allele, designated the neutral allele. After the *NSt* allele paramutates the *nst-22* allele, the Tam3-stabilized state of paramutated *nst-22* allele can be inherited to progeny and be maintained at a high level in successive generations. Moreover, the structure of *nst-22* is distinct from *NSt* and *nst-5* because no Tam3 reside in the *nst-22* allele. I expected that the *NSt*-specific sRNAs could function in paramutated *nst-22*/*nst-22* plants, and thus sRNA-seq was performed to examine these sRNAs. However, I cannot detect these sRNAs in the paramutated *nst-22*/*nst-22* plants. The reason for the difficulty in the sRNA detection might be that the amount of *NSt*-specific sRNAs decreases during inheritance. The mechanism involved in the paramutation may provide new insight to how transposons are inactivated in the plant genomes.

Discussion

1) Identification of *OSt* and *NSt* elements and possible mechanisms by which both *Sts* inactivate Tam3 transposition

In general, DNA methylation and histone modification could create barriers on the DNA sequence to prevent recognition and binding of DNA-binding proteins, resulting in transcriptional inhibition. However, my results do not support the involvement of DNA methylation or histone modification, as the transcription of *Tam3 TPase* occurs normally. And I found that sRNAs from the Tam3 5′ terminal sequence exists in *OSt* and *NSt* lines, and thus the sRNAs could restrict the binding of Tam3 TPase to Tam3 sequences via two possible ways. One is that the recognition of Tam3 TPase are covered by the sRNAs, and the other is that the sRNAs compete for Tam3 TPase preventing it from binding. For the above reasons, the function of these sRNAs is likely different from known TEs-derived sRNAs.

In plants, sRNAs are commonly generated from dsRNAs derived from hairpin RNAs or pairing of sense and antisense RNAs by cleavage by dicer proteins. Transcription of the 5′ rearranged end of Tam3 in *OSt* in sense and antisense strands could generate paired dsRNAs, and a one-way transcription product of the sense strand from Tam3-L to Tam3-R could form a hairpin-shaped dsRNAs. Digestion of these dsRNAs by DICER-LIKE (DCL) proteins was responsible for sRNAs formation in *OSt* and *NSt*. And different DCL proteins could be dominant in the both *Stabiliser* lines: DCL3 could be functional for the generation of 24 nt sRNAs in *OSt* line, and DCL1-2 could result in the production of 21-22 nt sRNAs in *NSt* line.

3) Finding of paramutation phenomenon at *NSt* locus in *Antirrhinum*

Abnormal trait segregation can be caused by different factors including cytoplasmic inheritance, gametic selection, zygotic lethality and paramutation. In H-3×22 cross, F_2 genotype segregation ratio follows the Mendelian segregation first law, but the most plants in the population carrying recessive *nst-22*/*nst-22* genotype exhibits the phenotype similar to the dominant *NSt*/*NSt* population showing low-spotted phenotype. Thus, the abnormal phenotype segregation in the H-3×22 cross is the result of paramutation. At a locus participating in paramutation, three types of alleles can be found: paramutagenic, paramutable and neutral. In the H-3×22 paramutation cross, the *NSt* and *nst-22* alleles originated from HAM3 and HAM22 at *NSt* locus, respectively, are paramutagenic and paramutable. Additionally, *nst-5* allele stemmed from HAM5 is neutral.

In F2 population of H-3×22 cross, the penetrance of *NSt* allele to *nst-22* allele is incomplete because a proportion of F2 *nst-22*/*nst-22* plants were low-spotted. Although the expression state of paramutated *nst-22* allele is unstable, the paramutated *nst-22* still exhibited high heritability. Paramutation does not act on DNA sequence itself but epigenetically alters the expression state of paramutable alleles by interacting with paramutagenic alleles. *NSt*-derived sRNAs could inhibit the binding of Tam3 TPase to Tam3 DNA, resulting in Tam3 suppression. However, the specific sRNAs were not observed in the paramutated *nst-22*/*nst-22* plants. And some possible

factors would be required for the failure of these sRNAs detection.