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Study on epigenetic-independent control of Tam3

transposition in *Antirrhinum*

(キンギョソウのトランスポゾン Tam3 における非エピ
ジェネティックな転移制御機構に関する研究)

北海道大学 大学院農学院

生物資源科学専攻 博士後期課程

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**Study on epigenetic-independent control of Tam3
transposition in *Antirrhinum***

**Hokkaido University Graduate School of Agriculture
Division of Agrobiological Sciences Doctoral Course**

ABBREVIATIONS

Ac	Activator
CBB	Coomassie Brilliant Blue
CMT2	CHROMOMETHYLASE 2
CMT3	CHROMOMETHYLASE 3
DRM2	DOMAINS REARRANGED METHYLTRANSFERASE 2
EN	endonuclease
ER	endoplasmic reticulum
EVD	Evadé
gag	group-specific antigen
HTT	horizontal TE transfer
IN	integrase
KYP	KRYPTONITE
LINEs	long interspersed nuclear elements
LTDT	low temperature dependent transposition
LTR	long terminal repeats
MET1	METHYLTRANSFERASE 1
MITEs	miniature inverted-repeat transposable elements
NLSs	Nuclear localization signals
ORFs	open reading frames
PAGE	polyacrylamide gel electrophoresis
PBS	primer binding site
PM	plasma membrane
PPT	polypurine tract
PRO	protease
PTGS	post transcriptional gene silencing
RACE	rapid amplification of cDNA ends
RdDM	RNA-directed DNA methylation

RH	RNase H
RNAi	RNA interfering
RT	reverse transcriptase
SCAP	SREBP cleavage-activating protein
SINEs	short interspersed nuclear elements
SUVH5	SU(var)3-9 homologue 5
SUVH6	SU(var)3-9 homologue 6
TGS	transcriptional gene silencing
TIRs	terminal inverted repeats
TPase	transposase
TSD	target site duplication
VLPs	virus-like particles
Y2H	yeast two hybrid
Znf-BED	BED-zinc finger

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Chapter 1 General introduction

Transposable elements (TEs), also known as "jumping genes", are DNA fragments that move from one location on the genome to another, and often make duplicates of themselves in the process (Paul et al., 2012). Since first discovered in maize by Barbara McClintock in the 1940s (McClintock, 1948), TEs have been extensively investigated. They have already been found in the genomes of almost all present-day organisms (both prokaryotes and eukaryotes) and typically in large numbers. In plants, the proportions of TE-derived DNA vary largely among different plant species, for example, in *Arabidopsis thaliana*, TEs constitute about 20% of the genomic sequences, while over 85% of the B73 maize genome is occupied by TE-derived DNA (Schnable et al., 2009; Ahmed et al., 2011; Fiston-Lavier et al., 2012). Nowadays, the origins, roles, specificities, and regulation of these mobile elements have been subjects of great interest to scientists. We have already acquired some understanding of them, but there is still much to learn.

1.1 Classification of plant transposable elements

Based on their replication patterns, TEs are divided into two main classes: class I (RNA) and class II (DNA) TEs, which follow 'copy and paste' and 'cut and paste' mechanisms, respectively (Feschotte et al., 2002) (Figure 1.1). Both classes are abundant in many species, but some groups of organisms have a preponderance of one or the other. For instance, mammalian genomes are mainly occupied by the RNA TEs, whereas the predominant TEs in bacterial genomes are DNA TEs (Kazazian, 2004). Each group of TEs contains autonomous and non-autonomous elements (Feschotte et al., 2002). Autonomous elements themselves encode all the products required for transposition (Feschotte et al., 2002). Non-autonomous elements are usually mutated versions of their autonomous counterparts, but do not carry enough coding capacity to initiate the transposition by themselves (Feschotte et al., 2002; Slotkin and Martienssen, 2007). Hence, the transposition of autonomous elements is self-driven, while non-autonomous elements transpose relying on the enzymes coded by related autonomous

elements (Feschotte et al., 2002) (Figure 1.1).

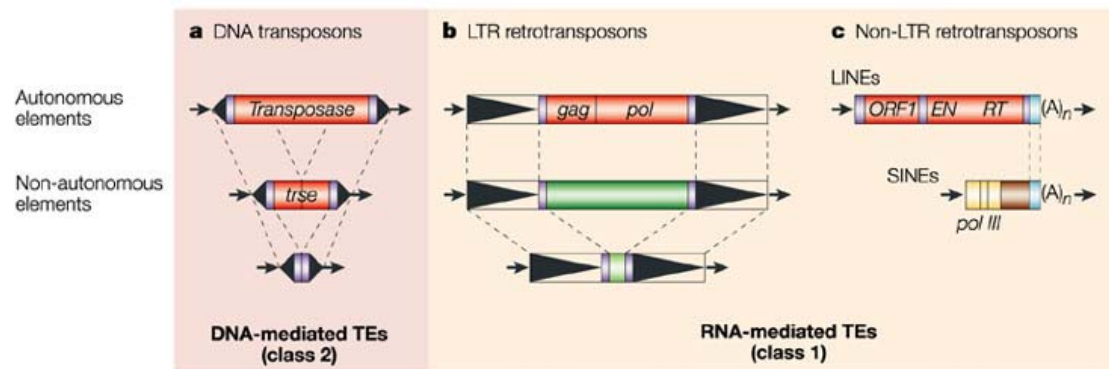


Figure 1.1 Structural features and classification of plant TEs (Feschotte et al., 2002)

TEs are divided into two main classes: class I (RNA) and class II (DNA) TEs, according to their replication patterns. Based on their abilities in encoding proteins required for transposition, each class can be subdivided into autonomous and non-autonomous elements.

1.1.1 DNA transposons

DNA transposon moves following a "cut and paste" mechanism: the transposon is cut out of its original location and inserted into another new location, using transposases and DNA polymerases to catalyze transposition (Table 1.1a). Usually, during this process, there is no increase in the copy number of DNA transposons. Hence, the mutations caused by DNA TEs are always not stable. Many DNA transposons are flanked by terminal inverted repeats (TIRs; black triangles) (Figure 1.1a). The transposase, an autonomous element-encoded enzyme, firstly recognizes and binds at or near the TIRs, and then cuts the existing copy from its original genomic location and pastes it into another new genomic location (Levin and Moran, 2011). Target-site duplication (TSD; arrows) is formed at both ends of the new copy of DNA transposon during the process of repairing the stagger gaps caused by the cleavages of the two strands at the target site (Levin and Moran, 2011). TSD is typically of 4–8bp in length as specified by the transposase (Levin and Moran, 2011) (Figure 1.1a).

In recent years, miniature inverted-repeat transposable elements (MITEs), known as a group of non-autonomous class II TEs, have become the researchers' new favorite in

the field of TEs research. MITEs are distinguished from other DNA TEs for their high-copy-number. MITEs are thought to be derived from DNA TEs in a two-step process: generating various internally deleted non-autonomous derivatives during the transposition of an autonomous element, then amplification of some derivatives (for unknown reasons) to high copy numbers (Feschotte et al., 2002). Structural features of typical MITEs include: 1) small in size (usually 50-500bp in length); 2) high-copy-number (often several hundred copies); 3) be rich in AT sequence; 4) lacking of coding capacity for transposases (Fattash et al., 2013). MITEs often locate close to or within genes, causing changes in the expression of the adjacent genes (Fattash et al., 2013).

Since the first mobile DNA element Activator (Ac) was identified in maize by Barbara McClintock, several other transposons, which share similarity to Ac, were subsequently found in different organisms (Calvi et al., 1991; Atkinson et al., 1993). Atkinson et al. (1993) named them as *hAT* transposon superfamily after hobo from *Drosophila*, Ac from maize and Tam3 from *Antirrhinum* (Calvi et al., 1991; Atkinson et al., 1993). They have been identified in most eukaryotic lineages, including plants, fungi, animals and even humans (Ladevèze et al., 2012). The general characteristics of *hAT* superfamily transposons are: 1) low-copy-number in their host genomes (usually 50-100) (Wessler, 1988); 2) short TIRs, 10–25bp in length (Feschotte and Pritham, 2007); 3) mediation of 8bp TSD upon insertion with few exceptions (Ladevèze et al., 2012); 4) the length of most *hAT* superfamily elements is not over 4kb.

Table 1.1 Classes of TEs and their mobility mechanisms (Levin and Moran, 2011)

Class of TE	Structural features	Replication mechanism	Variant forms	Active examples
DNA transposons	• TIRs • Transposase	Transposase-mediated excision of donor dsDNA followed by insertion into the target site	• Some DNA transposons also mobilize via replicative mechanisms • ssDNA transposons lack TIRs: donor ssDNA is inserted into target-site ssDNA, such as for IS608 of <i>Helicobacter pylori</i>	• Tn7 in <i>Escherichia coli</i> • P elements in <i>Drosophila melanogaster</i> • Tc1 elements in <i>Caenorhabditis elegans</i>
LTR retrotransposons	• LTRs • Gag, protease, reverse transcriptase and integrase	Within virus-like particles, reverse transcriptase copies the mRNA of the TE into a full-length cDNA; integrase inserts the cDNA into target sites	• Solo LTRs are commonly found in genomes and are a result of LTR–LTR recombination	• Ty1, Ty3 and Ty5 in <i>Saccharomyces cerevisiae</i> • Tf1 and Tf2 in <i>Schizosaccharomyces pombe</i> • Tnt1 in tobacco
Non-LTR retrotransposons	• One or two ORFs • 5' truncations and inversion/deletion (for mammalian L1 elements) • Some end in poly(A) tails (for example, L1s); others do not (for example, R2)	An element-encoded endonuclease mediates TPRT. The endonuclease nicks the DNA at the target site and uses the 3' nicked end for the primer as it reverse transcribes TE mRNA	• Non-autonomous, non-LTR retrotransposons (for example, Alu and SVA elements, as well as other eukaryotic SINEs) rely on the endonuclease and reverse transcriptase of an autonomous non-LTR retrotransposon to mediate retrotransposition • The L1 retrotransposition machinery can also mobilize mRNAs (to generate processed pseudogenes) and certain non-coding RNAs (for example, the U6 snRNA)	• L1 in human, mouse, and other mammals • I factor in <i>D. melanogaster</i> • Zorro3 in <i>Candida albicans</i> • R1 and R2 in insects

L1, long interspersed element 1; LTR, long terminal repeat; SINE, short interspersed element; snRNA, small nuclear RNA; SVA, SINE-R–VNTR–Alu; TE, transposable element; TIR, terminal inverted repeat; TPRT, target-site-primed reverse transcription.

1.1.2 RNA transposons

TEs that transpose through an RNA intermediate are termed RNA transposons, also called retrotransposons. Retrotransposons move following replicative mechanisms (Table 1.1). Transposition of retrotransposons begins with transcription of an integrated copy of the element to generate an RNA copy. Then reverse transcriptase (RT) uses the RNA intermediate as template to generate double-stranded DNA, which is subsequently integrated into the host genome (Bennetzen, 2000; Smyshlyaev et al., 2013). These elements are not excised from their original genomic location when they transpose. Instead, they make a new copy that inserts elsewhere, with the potential to expand genomes. Retrotransposons can be divided into two groups on the basis of transposition mechanism and structure: long terminal repeats (LTR) retrotransposons and non-LTR retrotransposons (Figure 1.1). Both of them exist in all analyzed eukaryotic genomes. However, LTR retrotransposons are highly prevalent in the plant genomes, while in animal genomes, non-LTR retrotransposons are the major repetitive sequences (Malik and Eickbush, 1998; Jiang and Ramachandran, 2013).

LTR retrotransposons are also composed of autonomous and non-autonomous groups (Figure 1.1b). The autonomous elements contain at least two open reading

frames (ORFs) in common: gag encodes a capsid-like protein that forms virus or virus-like particles (VLPs) and pol encodes four enzymes, namely protease (PRO), reverse transcriptase, integrase (IN) and RNase H (RH) (Feschotte et al., 2002) (Figure 1.2). The autonomous elements can be further subdivided into two main groups: Ty1-*copia* like retrotransposon and Ty3-*gypsy* like retrotransposon, according to the differences in the order of genes encoded by pol (Santini et al., 2002)(Figure 1.2).

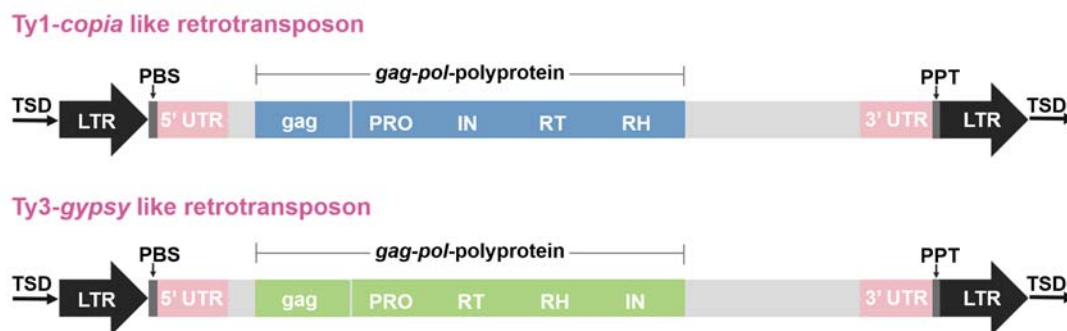


Figure 1.2 Basic structure of full-length LTR retrotransposon. Structural features include:

- 1) TSD, target site duplication, typically of 4-6bp in length, occurs upon TE integration as a result of staggered double-strand breaks at the target site flanking the TEs (Linheiro and Bergman, 2012).
- 2) LTR, long terminal repeat, are DNA sequences of hundreds or thousands base pairs. Upon insertion, the 5' and 3' LTRs are expected to be identical. However, after insertion mutations are accumulated in the LTRs at the neutral rate, so the divergence between the paired LTRs can thus be used to estimate the age of ancient retroviral insertions since insertion of that element (Cantrell et al., 2001).
- 3) PBS, primer binding site, locates 1-2bp downstream of the 5' LTR end, is the binding site of the tRNA primer required for initiation of reverse transcription in minus strand synthesis (Goldschmidt et al., 2002).
- 4) PPT, polypurine tract, locates upstream of 3' LTR, is required for initiation of reverse transcription in plus strand synthesis (Cao et al., 2015).
- 5) gag, group-specific antigen, a polypeptide, usually contains matrix, capsid, and nucleocapsid domains, forms the virus-like particle (VLP) where reverse transcription takes place (Alzohairy et al., 2014; Grau et al., 2014).
- 6) PRO, protease, processes the cleavage of gag and gag-pol polypeptide precursors (Chen et al., 2001).
- 7) RT, reverse transcriptase.
- 8) RH, RNase H.
- 9) IN, integrase.
- 10) While PRO, RT, RH and IN comprise the polypeptide pol, RT, RN and INT are responsible for retrotranscribing cDNA from RNA intermediates and inserting it into the host genome (Grau et al., 2014).

Non-LTR retrotransposons, lacking LTRs, are subdivided into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (Figure 1.1c). LINEs typically contain two coding regions: the first one is ORF1, a gag-like protein; while the second one encodes a nuclease, usually is related to an apurinic-aprimidinic repair endonuclease (EN), a RT, and in some cases an RH (Feschotte et al., 2002; Finnegan, 2012) (Figure 1.1c). At the ends of LINEs and SINEs, there is a simple sequence repeat, usually poly(A) (Feschotte et al., 2002).

1.2 Dynamic interactions between TEs and their host plant genomes

Due to being mobile elements, TEs can accumulate mutations to the plant genomes, negatively affecting the fitness of the host. Therefore, the hosts adopt diverse mechanisms to tightly control the activities of TEs. However, TEs must also maintain a certain level of activity to ensure their propagation and survival, through fighting with the host defense mechanisms. As a result, a symbiotic relationship has developed between TEs and their hosts (Labrador and Corces, 1997).

1.2.1 How the hosts defend against TEs

In plants, horizontal TE transfers (HTT) are widespread and frequent (El Baidouri et al., 2014). Hence, for the host, the first line of defense is at the entrance point to prevent the invasion of TEs. Currently, inhibition of HTT remains largely mysterious. However, there is a reason to believe that plants possibly establish anti-HTT barriers because some TEs are restricted in certain plant species, such as Tam3 in *Antirrhinum*. Although *hAT* superfamily transposons are widely found in multicellular organisms, no evidence shows that horizontal transfer happened to these elements (Rubin et al., 2001).

Once being inside a host plant genome, TEs are mainly regulated by the host through combating their activities. Epigenetic regulations are the major weapons in controlling TEs activities. At the transcriptional level, DNA methylation plays a vital role in silencing TE. In *Arabidopsis*, several mechanisms account for DNA methylation. DNA

METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3), and DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2) and/or CMT2 methylases maintain distinct DNA methylation patterns in CG, CHG and CHH contexts (where H is A, T and/or C), respectively (Cui and Cao, 2014). De novo methylation at CG, CHG and CHH sites catalyzed by DRM2 relies on an RNA-directed DNA methylation (RdDM) pathway (Cui and Cao, 2014). Moreover, histone methylation also functions at transcriptional level to confine TE activity. Accumulated data revealed that DNA methylation and specific histone modifications influence each other in both directions: histone methylation can contribute to direct the formation of DNA methylation, and DNA methylation is likely to function as a template for establishing some histone modifications after DNA replication (Cedar and Bergman, 2009). In plants, a self-reinforcing loop of DNA methylation and specific histone modifications was proposed, in which CMT3 is recruited by H3K9me2 deposited by KRYPTONITE (KYP) and its close homolog SU(var)3-9 homologue 5 (SUVH5) and SUVH6, to methylate CHG (Johnson et al., 2007; Law and Jacobsen, 2010; Du et al., 2012; Zhao and Chen, 2014). In turn, methylated CHG DNA recruits KYP to maintain methylation of H3K9 (Johnson et al., 2007; Law and Jacobsen, 2010; Du et al., 2012; Zhao and Chen, 2014). At the post-transcriptional level, TE is silenced by RNAi (RNA interfering) through mRNA degradation (Zhang et al., 2007) and translation inhibition (Iwakawa and Tomari, 2013; Li et al., 2013).

According to Voinnet and colleagues' work on the single-copy endogenous retrotransposon *Evadé* (EVD), mechanisms involved in the regulation of de novo invasion, proliferation and eventual demise of TE were partially unraveled (Mari-Ordonez et al., 2013; Cui and Cao, 2014). When EVD was reactivated, the antiviral post transcriptional gene silencing (PTGS) formed the first host defense by partially eliminating the EVD RNA to prevent EVD amplification (Mari-Ordonez et al., 2013; Cui and Cao, 2014). This initial defense was compromised when the EVD RNA was bound by its GAG capsid protein and protected from PTGS (Mari-Ordonez et al., 2013;

Cui and Cao, 2014). However, as the copy number of EVD elements increased over generations, the PTGS pathway shifted to transcriptional (TGS) pathway, inducing DNA methylation at the 5' LTR to gradually repress expression of EVD and eventually, completely silencing of EVD over several generations (Mari-Ordonez et al., 2013; Cui and Cao, 2014). Interestingly, introduction of EVD-like retrotransposons from *Arabidopsis lyrata* into *Arabidopsis thaliana*, these elements preferential target the centromere, which indicated that plants utilize additional mechanisms, such as centromere-specific localization to deal with a new invading TE (Tsukahara et al., 2012; Cui and Cao, 2014).

Apart from epigenetic regulation, TEs are also subjected to other host defense mechanisms which are epigenetic-independent. TE is under purifying selection from hosts, which accumulates mutations in the TE to repress its activity. TE can be removed from the plant genomes by recombination (Tenailon et al., 2010). In addition, different organism may have developed additional mechanism in silencing TE. Fujino et al (2011) found the transposition of Tam3 is associated with the nuclear localization of Tam3 transpose (TPase), which implies that the nuclear localization of TPase might be subject to regulation of Tam3 silencing in *Antirrhinum* (Fujino et al., 2011).

1.2.2 TE activation and amplification: escape from control

Although hosts adopt a series of mechanisms in eliminating TE activity, a variety of conditions can trigger transposon activation. In plants, tissue culture is the most common condition that is associated with transposon activation (Lisch, 2009). The normally epigenetically silent TEs become active and can increase their copy number in tissue culture process (Lisch, 2009). Lullaby, an active copia-like retrotransposon in rice, can transpose in the calli and regenerated seedlings (Picault et al., 2009). The transposition of LINE type retrotransposon Karma was only detected in the next generation of regenerated plants as well as in later generations, while in cultured cells or in the first generation of regenerated plants, Karma was confined to transcriptional

activation (Komatsu et al., 2003). It is suggested that the post-transcriptional regulation of Karma retrotransposition is development dependent (Komatsu et al., 2003). Biotic and abiotic stresses are also known to reverse transposon silencing. The transposition of Tam3 is temperature dependent: Tam3 exhibits an unusual and remarkable feature of activation at low growth temperatures (15°C) and inhibition at high temperatures above 25°C (Harrison and Fincham, 1964; Carpenter et al., 1987). In *Arabidopsis*, heat shock not only activated ONSEN transcription but also synthesized extrachromosomal DNA copies (Ito et al., 2011). Retrotransposition of ONSEN was observed in the progeny of heat stressed plants deficient in siRNAs (Ito et al., 2011). Transposons can be transcriptionally activated through hybridization, which can clearly lead to global changes in patterns of DNA methylation and activation of transposon expression (Kashkush et al., 2002; Liu et al., 2004; Josefsson et al., 2006; Lisch, 2009)

There exists at least two main ways utilized by TEs to escape extinction: the first one is to minimize the harmful effects on hosts (e.g. low replication rate), in order to escape the eyes of selection in their current hosts and the second is to horizontally transfer to a new host genome ahead of inactivation (Schaack et al., 2010). The lifecycle of a TE is similar to a cradle-to-grave process: from the birth of a new TE when an active copy colonizes a novel host genome to its death when all copies in a lineage are lost (by chance or negative selection) or inactivated (Schaack et al., 2010). Horizontal transfer is likely to allow the TE to colonize a new genome in which host suppression mechanisms are inefficient (Hartl et al., 1997; Feschotte and Pritham, 2007), either because they have not had time to co-evolve or are copy number-dependent (Jensen et al., 1999; Zhou and Eickbush, 2009).

1.2.3 Coevolution between TEs and their hosts

During long term evolution, massive copies of TEs have been tolerated by their hosts. Since first discovered by McClintock, TEs have been considered “junk DNA” or “selfish DNA”, parasites which have no direct selective benefit to the host (Schaack et

al., 2010). As also called mobile elements, they have the potential for gene disruption, chromosome breakage, illegitimate recombination, and genome rearrangement (Slotkin and Martienssen, 2007). However, accumulating data reveal that the presence of TE sequences in a genome is of overall benefit to the population. TE benefits the host organisms through their functions in genome organization, gene expression regulation and new gene creation (Levin and Moran, 2011; Rebollo et al., 2012; Tsuchiya and Eulgem, 2013; Kabelitz et al., 2014). Importantly, they form a source of genetic variation that can be utilized by natural selection (Lisch, 2013; Kabelitz et al., 2014). In addition, TEs can be co-opted for host functions. Bundock et al (2005) found that DAYSLEEPER, a member of the *hAT* superfamily transposons, is essential for plant development in *Arabidopsis* (Bundock and Hooykaas, 2005). Either knockout or overexpression of DAYSLEEPER in plants showed growth defect phenotypes (Bundock and Hooykaas, 2005). All in all, the relationship between TEs and their hosts is very complicated. Although TEs are deleterious to hosts, they are an important component of the host genomes and become a major players in evolution.

1.3 Prospection

In the past few decades, TEs have attracted the attention of more and more scientists and become a hot area of research. Increasing awareness of TE has been acquired. At the beginning, TEs were once thought as “junk” DNA, with no influence on their host genomes. But nowadays, one cannot ignore the notion that TEs have played an vital role in the evolution of their hosts through structuring genomes and generating genetic diversity (Levin and Moran, 2011). By understanding how, when and where TEs integrate, how the host responds to this ever-present threat, and how the two sides fight with each other, we might be able to uncover the dynamic forces that shape the genomes and the driving forces in promoting co-evolution between TEs and hosts (Levin and Moran, 2011).

1.4 My study on DNA transposon Tam3 in *Antirrhinum*

Plant genomes contain a number of active TEs reported as entities that perturb genome integrity. These have the potential for gene disruption, chromosome breakage, illegitimate recombination, and genome rearrangement (Slotkin and Martienssen, 2007). To maintain the stability and integrity of plant genomes, TE activities are under rigorous control of the hosts.

The mechanisms of silencing TEs in plants include processes such as DNA methylation (Pikaard et al., 2008; Matzke and Mosher, 2014), histone modification (Saze et al., 2012), mRNA degradation (Zhang et al., 2007), and translation inhibition (Iwakawa and Tomari, 2013; Li et al., 2013). These epigenetic regulations range from complete transcriptional silencing by DNA methylation to the elimination of transcripts and translation inhibition by RNA-induced silencing complexes.

Several active transposons have been identified in *Antirrhinum*, of which Tam3 is a DNA transposon belonging to the *hAT* (hobo, Ac, Tam3) superfamily (Calvi et al., 1991). *hAT* family members are widely distributed in multicellular organisms, including plants, animals, and fungi (Rubin et al., 2001). Unlike most other transposons, Tam3 exhibits the unusual feature of activation at low growth temperatures (around 15°C) and inhibition at high temperatures (above 25°C) (Harrison and Fincham, 1964; Carpenter et al., 1987). Tam3 has been associated with several loci responsible for anthocyanin pigmentation in *Antirrhinum*, whose alleles might have caused the flower petal variegations described by Darwin and de Vries (Galun, 2003; Schwarz-Sommer et al., 2003; Hudson et al., 2008). However, the underlying mechanism controlling Tam3 activity is not fully understood. In *Antirrhinum*, Tam3 transposase (TPase) can be transcribed into mRNA and then translated into protein, which demonstrate identical expression patterns at both low and high temperatures (Uchiyama et al., 2008; Fujino et al., 2011). Thus, TGS and PTGS do not seem to be involved in the suppression of Tam3. Epigenetic regulation can be transmitted to daughter cells through mitotic cell

division (Probst et al., 2009). However, when *Antirrhinum* plants initially grown under high temperatures are transferred to low temperatures, newly formed flowers show variegations in the petals owing to the transposition of Tam3. These indicate that epigenetic control is not the cause of Tam3 inactivation.

The life cycle of a DNA transposon consists of both nuclear and cytoplasmic stages. A DNA transposon in the genome is transcribed into mRNA, which is then exported to the cytoplasm and translated into TPase protein. The TPase is imported back into the nucleus to bind the DNA target site and excise the DNA transposon, which is then ligated into a new target site. In *Antirrhinum*, temperature controls the sublocalization of Tam3 TPase, which is severely restricted to the plasma membrane (PM) under high temperatures, along with silencing of Tam3 (Fujino et al., 2011). This suggests that mediation of TPase localization is a possible way to limit DNA transposon activity.

My data indicate that certain *Antirrhinum*-specific factor(s) are involved in this process. These proteins can interact with the BED-zinc finger (Znf-BED) domain of Tam3 TPase, and fully detain the TPase at PM at the high temperature, which abolishes nuclear import of the TPase. However, at low temperature, some of TPases may escape from host-specific regulation and transfer into nuclei, for the expression of Tam3 TPase is not changed by the temperature, we deduce that this leakage is caused by the down-regulation of the expression of host factor(s) at low temperature (Uchiyama et al., 2008) (Fig. 1.3).

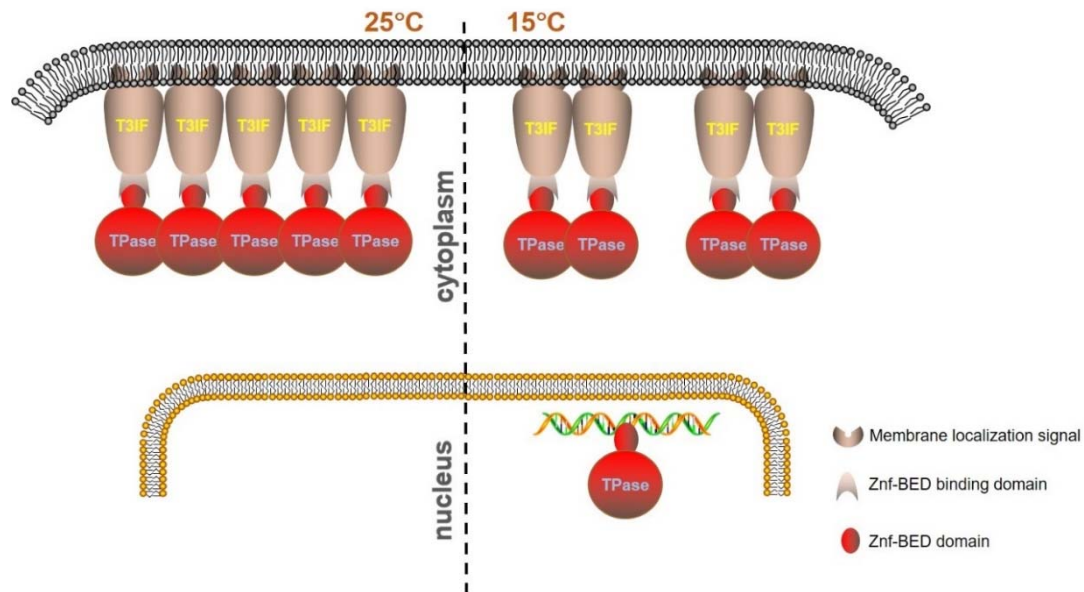


Figure 1.3 A model for low-temperature dependent transposition of Tam3.

At high temperature, the transposition activity of Tam3 is completely silenced by the host factor(s) (Tam3 TPase interacting factors, T3IF) by detaining Tam3 TPase at the cell membrane. While, at low temperature, the expression of host factor(s) are down-regulated. Hence, some Tam3 TPase can escape from the control of host factor(s) and enter into the nucleus to initiate the transposition of Tam3.

At the same time, I try to make clear the mechanisms underlying low temperature dependent transposition (LTDT). To make clear LTDT, I need to know what the host factor(s) are first. I used yeast-two-hybrid cDNA library and will try in vitro pull-down experiment to isolate the host factor(s). And now, I have already got some Tam3 TPase interacting proteins.

Chapter 2 Materials and Methods

2.1 Plant materials

The Tam3-active *Antirrhinum majus* line HAM22, originated from John Innes Centre (Norwich, UK) stock JI: 1 line (*pallida*^{recurrens}: *pal*^{rec}), was used in this study. This line was initially grown for 1.5 months at 25°C in a green house, and subsequently transferred into 15°C or 25°C growth chambers. Before use, materials were grown in the growth chamber for at least 2 weeks. Onions were gifted from the Sapporo Experimental Station of Sumika Agrotech, Japan.

2.2 Plasmid construction

The 2409bp Tam3 *TPase* coding sequence was amplified from HAM22 genomic DNA by PCR using Tks Gflex DNA polymerase (Takara). The amplified segment was induced into a pMD-20T vector using a ligation mix following manufacturer's instructions (Takara). Following verification by sequencing, the target segment was inserted into XhoI and SpeI sites of the pA7-GFP vector, at the N-terminus of the GFP gene. Truncated Tam3 *TPase* constructs (*TPase*Δ55, *TPase*Δ170, *TPase*Δ179, *TPase*Δ200, *TPase*Δ231 and *TPase*Δ244) were produced by PCR, and inserted into the same position of the pA7-GFP vector as the construct *TPase*.

To confirm whether BED-zinc finger domain could guide anonymous proteins to the plasma membrane, *AmCSBL* and *AmDnaJh1* coding sequences, without stop codons, were amplified using HAM22 cDNA products as template. The fragments were also cloned into XhoI and SpeI sites of the vector pA7-GFP, generating the constructs pA7-*AmCSBL* and pA7-*DnaJh1*. Overlapping PCR was adopted to add the sequence of the BED-zinc domain of Tam3 *TPase* to the N-terminus of these two genes (Ge and Rudolph, 1997). Take the construction of pA7-(Znf-*AmCSBL*) for example, during the first round PCR reaction, product 1 was amplified by a pair of primers T3*TPase*Δ170 (XhoI)-F (CCGCTCGAGCGGATGGCCTCTACATCAAGACC) and Znf_BED&*AmCSBL*-R (GATGAAGCACTACCCATTGTACCGTCTGGTTGTC)

using plasmids of construct TPase Δ 170 as template, product 2 was amplified by a pair of primers Znf_BED&AmCSBL-F (GACAACCAGACGGTACAATGGGTAGTGCTTCATC) and AmCSBL (SpeI)-R (GGACTAGTCCGTCAATGGGGACTTCCATC) using plasmids of construct pA7-AmCSBL as template. In the second round PCR reaction, product 3 was amplified by a pair of primers T3TPase Δ 170 (XhoI)-F and AmCSBL (SpeI)-R using the mixture of product1 and 2 as template. The product 3 was inserted into XhoI and SpeI sites of the pA7-GFP vector to generate the construct pA7-(Znf-AmCSBL). The construct pA7-(Znf-DnaJh1) was produced in a similar way. Primers used are listed in Table S1.

2.3 Point mutation

Point mutation constructs were created using overlapping PCR (Ge and Rudolph, 1997). Primers were designed to include the desired changes. The presence of a desired mutation(s) was confirmed for all constructs by sequencing. Primers used are listed in Table S1.

2.4 Transient expression assay

Transient expression assays of GFP in *Antirrhinum* petal and onion epidermal cells were performed using the Helium Biolistic gene transformation system, IDERA GIE-III (TANAKA). Tissues were placed on the surface of 1/2 MS medium. Approximately 2.0 μ g plasmid DNA was precipitated onto 1.0 μ m gold microcarriers (BIO-RAD). Bombardment delivery into prepared tissue was performed according to the methods described by Uchiyama et al. (Uchiyama et al., 2008).

2.5 Protoplast transformation

Protoplasts were prepared from young *Antirrhinum* leaves and fresh tobacco BY2 cells. PEG-calcium transfections were performed in accordance to Yoo et al. (Yoo et al., 2007). For each construct, 20 μ g of plasmid DNA was used. Following transformation, all samples were cultivated under 25°C conditions for 20 h.

2.6 Microscopic observation

Fluorescence signals were observed using a UV-fluorescence microscope (Olympus) equipped with a U-MNIBA filter set (470–490 nm excitation filter, 505 nm dichroic mirror, 515–550 nm barrier filter) for GFP, and a U-MWIG filter set (520–550 nm excitation filter, 560 nm dichroic mirror, 580 nm barrier filter) for chloroplast auto-fluorescence.

2.7 Active protein extraction and native-PAGE western blot

After determining fluorescence, protoplasts were harvested by centrifugation at $100 \times g$ for 2 min. Active protein was extracted with 50 μ l protein extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich)) for 1 mL protoplasts (approximately 1×10^6 cells). The extracts were gently shaken for 30 min at 0°C, centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants were collected for native-PAGE western blot analysis. Proteins were prepared in a non-reducing and non-denaturing sample buffer (50 mM Tris-HCl (pH 6.5), 10% glycerol, 12.5 mM EDTA, and 0.02% bromophenol blue), then separated on 6% PAGE gel without sodium dodecyl sulfate (SDS) on ice. The running buffer was also SDS-free. Gel electrophoresis was performed at 20 mA, then the proteins were transferred to a polyvinylidene fluoride membrane. Independently, the same samples were electrophoresed in adjacent lanes to check the running profile after CBB staining. The membrane was treated for blocking with 5% (w/v) ECL prime™ blocking agent (GE), and incubated overnight at 4°C with monoclonal mouse anti-GFP antibody diluted 1000-fold (anti-GFP, Monoclonal Antibody (mFX75), Wako Pure Chemical Industry, Ltd.). After washing three times with Tris-buffered saline, 0.1% Tween 20, the membrane was incubated for 2 h with secondary anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase (GE), diluted 3000-fold and then stained with ECL Western Blotting Detection Reagent

(GE). Signals were detected using the chemical luminescent detection system (Ez-Capture MG, ATTO).

2.8 Protein alignments

TPase coding sequences of the *hAT* family were acquired from GenBank and EMBL databases. Sequence alignments were performed by ClustalX Multiple Sequence Alignment (Thompson et al., 1994; Thompson et al., 1997). Jalview software was employed to perform alignment editing for visualization of final results (Waterhouse et al., 2009).

2.9 Accession numbers

Sequence data from this article can be found in the GenBank/EMBL database libraries under accession numbers CAA38906 (Tam3), CAA29005 (Ac), CAB68118 (*Daysleeper*), AM087608 (*b-gary*), AM087609 (*w-gary1*), AAC37217 (*Hermes*), U22467 (*hermit*), AAS21248 (*Herves*), 85002 (*Hobo*), AAD03082 (*Homer*), AAL93203 (*Hopper*), CAA93759 (*Restless*), AF051562 (*Tag1*), ABF20545 (*TcBuster*), AAP59878 (*THELMA13*), BAA87039 (*Tol2*), CAA76545 (*tramp*), KX016023 (*AmCSBL*), and KX016024 (*AmDnaJh1*).

2.10 Total RNA isolation and purification of poly(A) RNA

Total RNA was extracted from leaves and flowers of *Antirrhinum* line HAM22 growing at 25°C by using TRIzol[®] Plus RNA Purification Kit (Invitrogen). The quality of total RNA was checked by nanodrop 2000 (Thermo) and running gel.

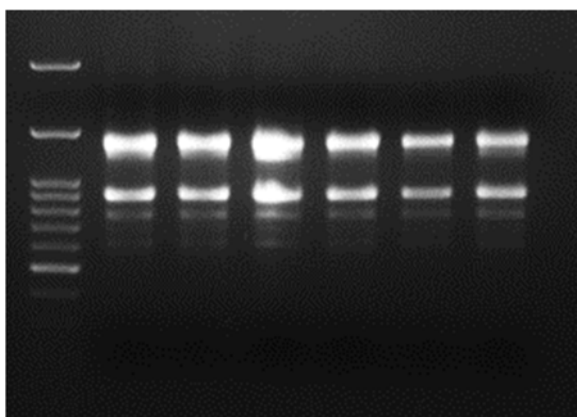


Figure 2.1 Isolation of total RNA.

The total RNA used for constructing the two-hybrid library is checked by electrophoresis.

About 250µg total RNA was used for purification of poly(A) RNA by using NucleoTRap® mRNA kit (mini) (Machery-nagel). The concentration of the isolated poly(A) RNA was measured by nanodrop 2000 (Thermo).

2.11 cDNA library construction

About 1µg high-quality poly(A) RNA was used for the cDNA library construction. Each step was operated following the instructions in Make Your Own “Mate & Plate™” Library System User Manual (PT4085-1, Clontech). First-strand cDNA was synthesized by using oligo-dT primer, and ds-cDNAs were amplified using long-distance PCR (LD-PCR) with 20 thermal cycles and further purified with CHROMA SPIN™ TE-400 columns. The purified ds-cDNA was checked by electrophoresis. 2-5 µg should be harvested.

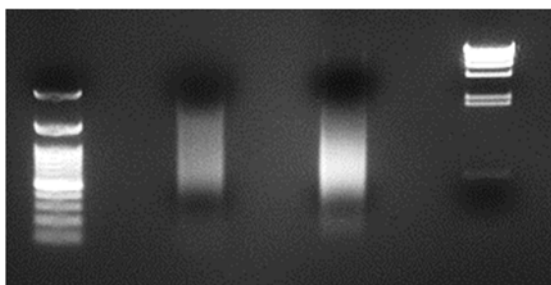


Figure 2.2 Ds-cDNA generated using SMART cDNA synthesis.

The Ds-cDNA used for constructing the two-hybrid library is checked by electrophoresis.

2.12 Construction of a Two-Hybrid Library

Library-scale yeast transformation following the Yeastmaker Yeast Transformation System 2 User Manual (PT1172-1, Clontech), in which 20 µl of ds-cDNA (5 µg) and 6 µl of Sma I-linearized pGADT7-Rec (3 µg) were co-transformed into competent Y187 yeast cells. At the last step, the transformed yeast cells were resuspended in 15 ml of 0.9% (w/v) NaCl and spread on 90 mm SD/–Leu agar plates containing 50 µg/ml kanamycin sulfate (100 µl per plate on ~150 plates). After incubation at 30°C for 4 days, all transformants were collected in 500 ml of freezing medium (YPDA medium and 25% glycerol) and prepared in 1 ml aliquots for short-term use and 50 ml aliquots for long-term storage at -80°C. The cell density should be over 2×10^7 per ml.

2.13 Bait construction

The cDNA encoding the full-length *Antirrhinum* Tam3 TPase (1-803 aa) or fragments (55-803 aa) was also amplified by PCR using HAM22 gDNA as template. The amplified segments were induced into the pMD-20T vector using a ligation mix following manufacturer's instructions (Takara). Following verification by sequencing, the target segments were inserted into EcoRI and BamHI sites of the pGBKT7 vector.

2.14 Screening of the *Antirrhinum* two-hybrid library by yeast mating and the yeast two-hybrid assay

A 1-ml aliquot of this cDNA library was used for screening following the Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1, Clontech). Firstly, the pGBKT7 plasmids containing truncated Tam3 TPase (55-803 aa) were introduced into the Y2H gold yeast strain using the LiAc method following Yeast Protocols Handbook (PT3024-1, Clontech), and the transformants were selected on SD/-Trp plates. Mating between the selected Y2H gold and Y187 yeast cells was performed in the 2 L flask. After 20-24 h incubation till the “Mickey Mouse” face appeared, yeast cells were collected and spread on agar plates SD/–Ade/–His/–Leu/–Trp containing 5 mM 3AT. Colonies grew at 30°C for 5-7 days. Those colonies were then grown on agar plates SD/–Ade/–His/–Leu/–Trp/X-α-Gal/AbA containing 5 mM

3AT, and blue colonies were furtherly selected on agar plates SD/–Ade/–His/–Leu/–Trp/X- α -Gal/AbA containing 5 mM 3AT again, finally the remaining blue colonies were selected as the positive clones for further cDNA insert analysis. using Matchmaker™ Insert Check PCR Mix (PT4102-2, Clontech). The cDNA inserts were amplified by PCR using MATCHMAKER 3' AD LD-Insert Screening Amplimers. After analyzed with agrose gel electrophoresis, the PCR products were recycled using the NucleoSpin® Gel and PCR Clean-up Kit (Machery-nagel) and then sequenced using pGADT7 sequencing primers: T7 Sequencing Primer, 5'-TAATACGACTCACTATAGGGC-3'; 3' AD Sequencing Primer, 5'-AGATGGTGCACGATGCACAG-3'(Clontech). Each sequence was finally blasted on Genebank.

The yeast plasmids were extracted using Easy Yeast Plasmids Isolation Kit (Clontech). To further test the interactions, the prey plasmids from the positive yeast colonies were rescued and then co-transformed with the bait plasmid into Y2H gold yeast cells. The interactions were checked by using agar plates SD/–Ade/–His/–Leu/–Trp/X- α -Gal/AbA containing 5 mM 3AT.

2.15 5' RACE

The total RNA used for 5' RACE should be fresh. 5' RACE was processed using 5'-Full RACE Core Set kit (D6122, TAKARA).

2.16 Protein expression in E.coli cells

The cDNA encoding the full-length TPase (1-803 aa) or fragments (55-803 aa) or mutants (m10) were amplified by PCR and then inserted into vector pVP68K by the aid of the recombination system of E.coli DH5 α . E. coli BL21 (DE3) transformed with sequence-verified plasmids encoding the constructs are grown in 2 mL of non-inducing medium (MDAG media) for 12 h at room temperature. For scale-up, 2 mL aliquots are transferred into 200 mL of non-inducing medium and grown for 12 h at room temperature. The 25 mL culture is then transferred into 1 L of auto-induction medium

(TB+G) containing kanamycin (50 µg/mL) and chloramphenicol (34 µg/mL), and incubate for 25 h at 25°C. Cells will be harvested by centrifugation at 5,000×g for 15 min.

Chapter 3 Results

3.1 Trapping nuclear import of Tam3 TPase for Tam3 inactivation

Antirrhinum line HAM22 carries *pallida*^{recurrens::Tam3} (*pal*^{rec}), which contains a 3.6 kb Tam3 insertion in the promoter of the Pallida locus encoding dihydroflavonol-4-reductase that is required for anthocyanin synthesis (Uchiyama et al., 2009) (Fig. 3.1A). The insertion of Tam3 suppressed expression of *pal*^{rec}, resulting in an ivory petal color (Almeida et al., 1989) (Fig. 3.1A). This state was maintained unless the transposition of Tam3 occurred at low temperatures of around 15°C, after which variegation with red spots appeared in the petal (Fig. 3.2A). The Tam3 TPase protein was restricted to the PM at high temperatures around 25°C (Fig. 3.1B), while a portion of the Tam3 TPase was able to enter the nucleus at permissive temperatures of around 15°C (Fig. 3.2B) (Fujino et al., 2011). Only nuclear extracts from plants grown at 15°C are capable of binding the Tam3 sequence, with nuclear extracts from plants grown at 25°C unable to do so (Hashida et al., 2006). Because initiation of the transposition reaction is dependent on the nuclear transport of TPase (Heinlem et al., 1994; Ono et al., 2002), inhibiting the nuclear import of Tam3 TPase is the likely cause of Tam3 stabilization at high temperatures. I determined the localization of Tam3 TPase in onion epidermis cells and tobacco BY2 cells; in these two plants, most Tam3 TPase localized to both the PM and the nucleus and was not influenced by temperature changes (Fig. 3.3).



Figure 3.1 Phenotype of temporarily inactive Tam3 *Antirrhinum majus* line HAM22

A, Structure of the *pallida*^{recurrens::Tam3} allele (*pal*^{rec}) from HAM22, and its flower phenotype at 25°C. Tam3 was embedded in the promoter of *pal*^{rec}, resulting in the silencing of *pal*. The flower showed an ivory petal color owing to abortion of anthocyanin synthesis. B, Subcellular localization of Tam3 TPase at 25°C in the protoplast of HAM22.

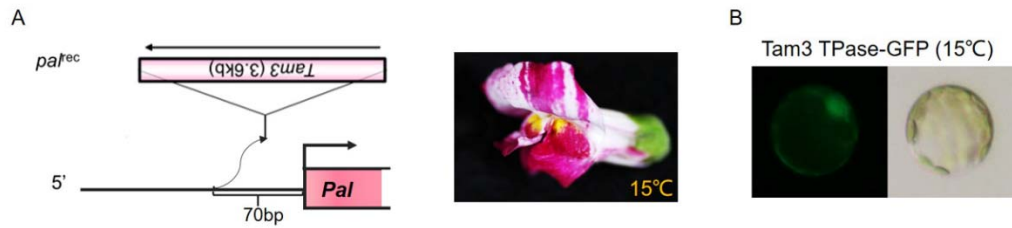


Figure 3.2 Phenotype of Tam3 temporarily inactive *Antirrhinum* line at 15°C.

A, Structure of the *pal* allele from *Antirrhinum majus* line HAM22 and its flower phenotype at 15°C. The transposition of Tam3 happened under low temperature in some *Antirrhinum* petal cells. In this case, Tam3 was removed from the promoter of *pal*. The expression of *pal* was recovered. Hence, the flower showed red spots in the petal. B, The subcellular localization of Tam3 TPase at 15°C in the protoplast of HAM22. Some of Tam3 TPase can go into the nuclei under low temperatures.

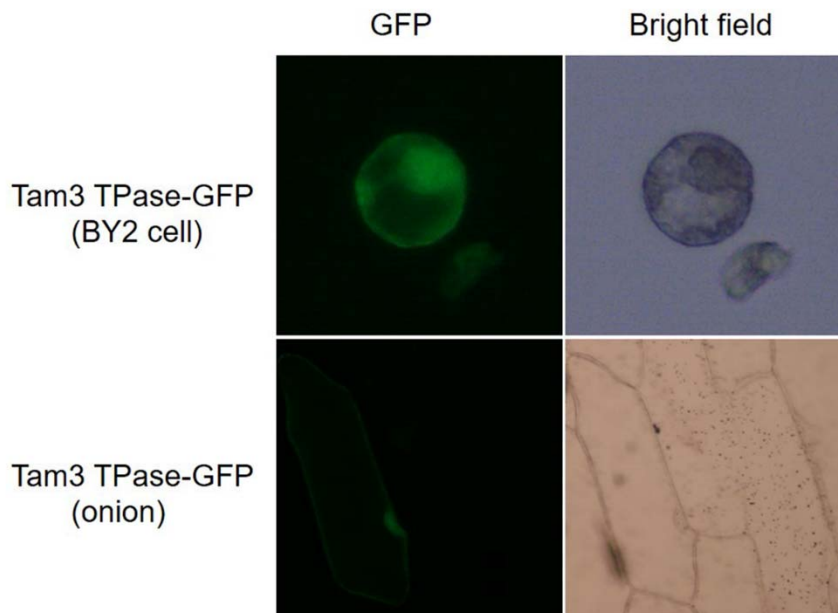


Figure 3.3 Subcellular localization of Tam3 TPase in tobacco BY2 cells and onion epidermis cells.

Plasmid DNA was introduced into tobacco BY2 cells and onion epidermal tissues by the PEG-mediated way or the bombardment method, respectively. Then, the tissues were incubated at 25°C for 20 h. In these two plants, most of cells showed GFP signal at both cell membrane and nuclei.

3.2 The Znf-BED domain is responsible for membrane localization of Tam3 TPase

Nuclear localization signals (NLSs) direct the nuclear transport of nuclear proteins following translation in the cytoplasm (Fujino et al., 2011). Tam3 TPase contains at least three experimentally confirmed NLSs (Fujino et al., 2011) (Fig. 3.4A). The nuclear transport of Tam3 TPase was found to be strictly controlled under a high temperature (Figs. 3.1B and 3.4B), indicating that other domain(s) in Tam3 TPase

function to suppress the nuclear transport of Tam3 TPase. Fujino et al. (2011) previously identified an N-terminal region, located within the 55–231 amino acid sequence of Tam3 TPase, which contains a possible nuclear inhibitory domain for arresting the nuclear translocation of itself (Fujino et al., 2011).

I performed further transient assays to identify the potential functional domain responsible for inhibiting the nuclear import of Tam3 TPase. We constructed a series of green fluorescent protein (GFP)-fused Tam3 TPase deletion constructs, including one intact and six truncated sequences (Fig. 3.4B). These plasmids were transformed into the protoplasts and petal cells of *Antirrhinum* line HAM22 grown at 25°C. The GFP signal from the series of truncated constructs TPase, TPase Δ 55, TPase Δ 170, and TPase Δ 179 only appeared on the PM (Fig. 3.4B; Table 3.1). However, constructs with a shorter TPase than TPase Δ 200 produced green fluorescence in the nucleus of most cells (Fig. 3.4B; Table 3.1). Interestingly, the region between TPase Δ 179 and TPase Δ 244 corresponded to an integral Znf-BED motif (Hashida et al., 2006). These results suggest that the Znf-BED motif served as a functional domain to confine the nuclear import of Tam3 TPase.

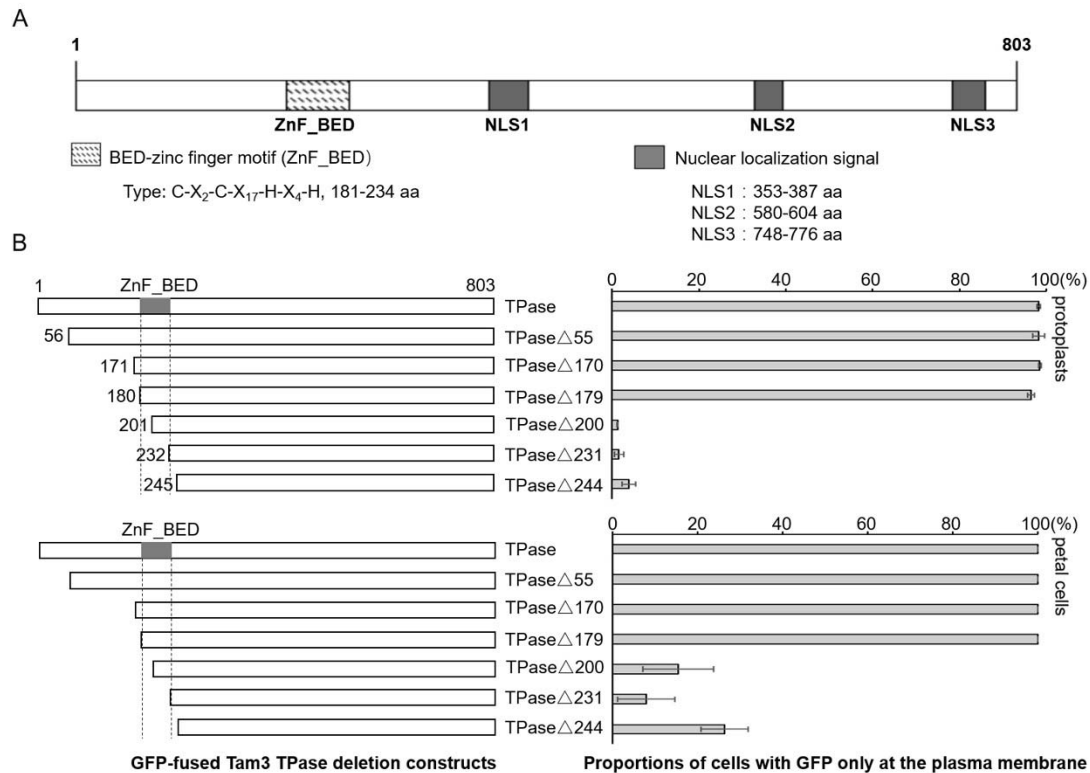


Figure 3.4 The Znf-BED motif of Tam3 TPase functions to direct Tam3 TPase to the PM

A, Protein domains of Tam3 TPase. The TPase contains a conserved Znf-BED motif and three experimentally confirmed nuclear localization signals (NLSs). B, Identification of the potential functional domain of Tam3 TPase which is responsible for directing Tam3 TPase to the PM. Left panel shows schematic of the fusion proteins used. Full-length or truncated TPase was fused to the N-terminal region of GFP. Each of these was transformed into protoplasts (upper part) and petal cells (lower part) of HAM22 to analyze subcellular localization. Numbers in the constructs represent the amino acid position in the Tam3 TPase sequence. The right panel consists of two histograms showing the proportions of cells with GFP signal in the PM compared with the total number of cells with green fluorescence (Table 3.1). Data represent means $\pm$ s.d.

Table 3.1 Measurements of the rate of nuclei-located GFP signal of intact- and truncated- Tam3 TPase in protoplasts and petal cells of HAM22.

Subcellular localization of Tam3 TPases using protoplasts of HAM22 (25°C)			
(numerator: number of cells showing nuclear GFP localization; denominator: total number of cells with GFP)			
	repeat 1	repeat 2	repeat 3
T3TPase	4/200	2/99	1/78
T3TPase Δ 54	0/56	1/48	2/59
T3TPase Δ 169	1/78	1/67	2/101
T3TPase Δ 179	3/66	2/56	2/78
T3TPase Δ 200	70/71	88/89	80/81
T3TPase Δ 230	58/58	95/97	74/76
T3TPase Δ 243	45/46	56/58	47/50
Subcellular localization of Tam3 TPases using petal cells of HAM22 (25°C)			

(numerator: number of cells showing nuclear GFP localization; denominator: total number of cells with GFP)			
	repeat 1	repeat 2	repeat 3
T3TPase	0/89	0/52	0/105
T3TPase Δ 54	0/62	0/58	0/69
T3TPase Δ 169	0/51	0/54	0/68
T3TPase Δ 179	0/39	0/38	0/42
T3TPase Δ 200	33/43	49/51	21/26
T3TPase Δ 230	41/49	36/36	36/39
T3TPase Δ 243	61/76	32/48	34/46

To establish whether the Znf-BED motif could function to confine the nuclear import, I fused the Znf-BED motif into the 5' terminal of two *Antirrhinum majus* genes: calcineurin subunit B-like (*AmCSBL*; KX016023) and dnaJ homolog 1 (*AmDnaJh1*; KX016024); the proteins encoded by these genes are originally located in the nucleus and mitochondria, respectively (Fig. 3.5A and B). When fused with the Znf-BED domain, these two fusion proteins were restricted to the PM (Fig. 3.5C and D; Table 3.2), indicating that the Znf-BED motif acted as a strong PM localization signal in *Antirrhinum*. We initially called this functional segment the nuclear localization inhibitory domain, but hereafter I refer to it as the PM localization signal.

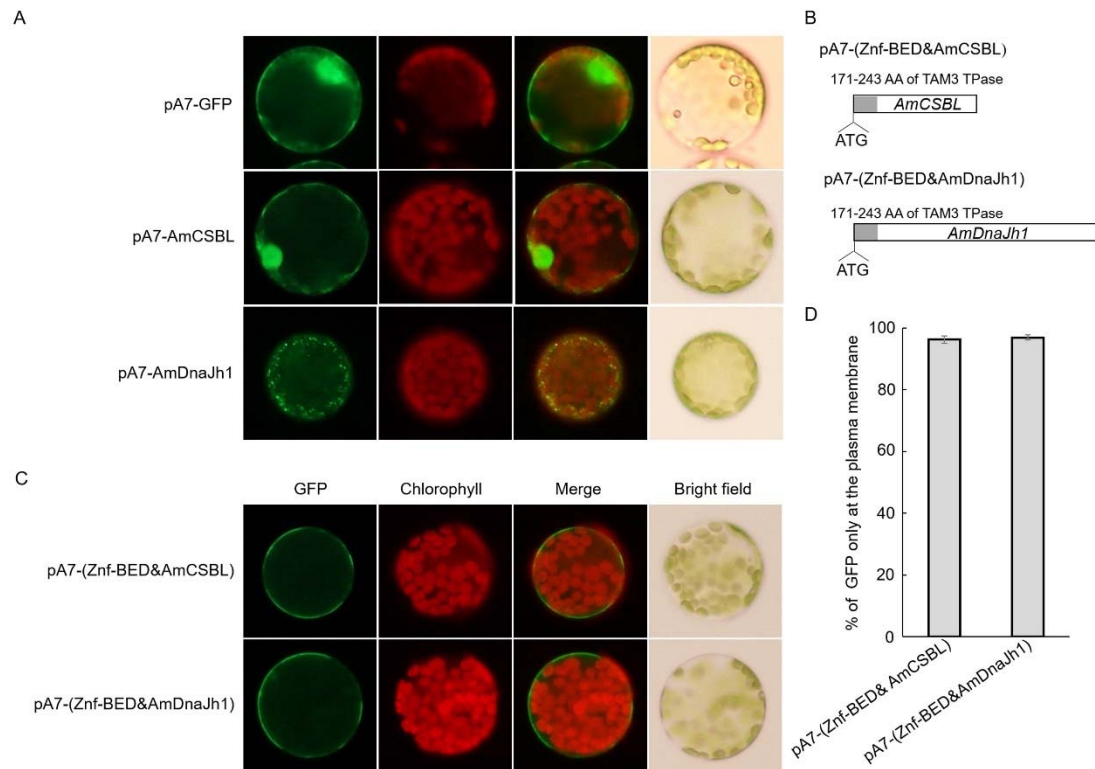


Figure 3.5 The Znf-BED motif in Tam3 TPase is a strong PM localization signal

A, Subcellular localization of *AmCSBL* and *AmDnaJh1*; pA7-GFP was used as a control vector. B, Constructs with Znf-BED motif inserted into the N-terminals of *AmCSBL* and *AmDnaJh1*. C, Subcellular localization of Znf-BED motif fused to *AmCSBL* and *AmDnaJh1*. D, The proportions of cells with a GFP signal focused on the PM compared with the total number of cells with green fluorescence (Table 3.2). Data represent means $\pm$ s.d.

Table 3.2 Measurements of the rate of plasma membrane-located GFP signal of pA7-(Znf-AmCSBL) and pA7-(Znf-DnaJh1) in protoplasts of HAM22.

Subcellular localization of pA7-(Znf-AmCSBL) and pA7-(Znf-DnaJh1) using protoplasts of HAM22 (25°C) (numerator: number of cells showing only plasma membrane GFP localization; denominator: total number of cells with GFP)			
	repeat 1	repeat 2	repeat 3
pA7-(Znf_BED&AmCSBL)	92/97	120/123	79/82
pA7-(Znf_BED&AmDnaJh1)	48/49	49/51	31/32

3.3 The Znf-BED domain is prevalent in the transposases of *hAT* DNA transposons

Zinc finger proteins were first discovered in 1985 and have since been recognized as one of the most common regulatory factors in plants, animals, and fungi (Miller et al., 1985). They vary largely in both structure and function. One subclass of the zinc fingers

is the Znf-BED, named after the two *Drosophila* proteins in which it is found: BEAF and DREF (Aravind, 2000). The Znf-BED is a protein domain of about 50–60 amino acid residues in length, with two highly conserved regions: aromatic amino acids (tryptophan and phenylalanine) at the N terminus and a shared pattern of cysteines and histidines predicted to form a zinc finger, characterized by the signature $Cx_2Cx_nHx_3$ - $s[H/C]$ (where x_n is a variable spacer) (Aravind, 2000; Saghizadeh et al., 2009). The Znf-BED is found in one or more copies in cellular regulatory factors and TPases (Aravind, 2000). Here, I found the Znf-BED to be widespread in the *hAT* superfamily TPase of different species (Fig. 3.6). These alignments of the Znf-BED domains featured aromatic amino acids at the N terminus as well as the cysteine and histidine signature, the CCH[H/C] motif (Fig. 3.6). Little is known about the N-terminal motif in Tam3 TPase, while the CCH[H/C] motif is predicted to form a zinc finger. In the N terminal region, the secondary structure predicted using the PHD program indicated that the T184V185W186K187W188F189 amino acid residues formed a β strand (Aravind, 2000). Because it has a well-known DNA binding ability, the BED-zinc domain may have a conserved function in regulating the transposition of *hAT* superfamily transposons.

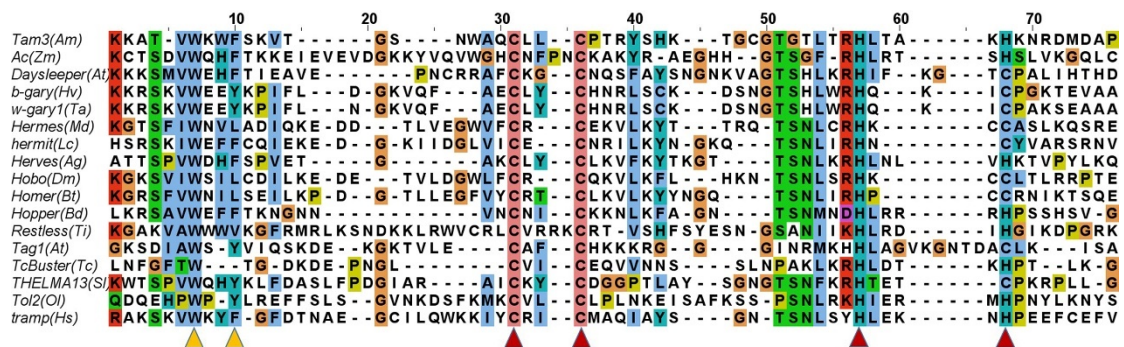


Figure 3.6 Multiple alignment of the Znf-BED domain in transposases of the *hAT* superfamily

Residues are colored according to the background coloring program of ClustalX Multiple Sequence Alignment. Yellow triangles represent the two conserved aromatic positions at the N terminal region; red triangles indicate conserved cysteines and histidines predicted to form a zinc finger. Each protein is labeled using its name, followed by species abbreviation in parentheses. Abbreviations: Am, *Antirrhinum majus*; Zm, *Zea mays*; At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*; Ta, *Triticum aestivum*; Md, *Musca domestica*; Lc, *Lucilia cuprina*; Ag, *Anopheles gambiae*; Dm, *Drosophila melanogaster*; Bt, *Bactrocera*

tryoni; Bd, *Bactrocera dorsalis*; Ti, *Tolypocladium inflatum*; Tc, *Tribolium castaneum*; Sl, *Silene latifolia*; Ol, *Oryzias latipes*; Hs, *Homo sapiens*.

3.4 The N-terminal region containing the two highly conserved aromatic amino acid regions of the Znf-BED domain is directly targeted by the host for membrane-associated localization of Tam3 TPase

To test the functions of these two motifs, point mutations were introduced into the conserved positions (Fig. 3.7A). First, C200, C203, H221, and H226 were individually mutated to create m1, m2, m3, and m4, respectively (Fig. 3.7A). These mutations introduced changes to the subcellular localization of Tam3 TPase (Fig. 3.7B; Table 3.3). A side-by-side mutation at H221 and L222 contained in m6 also showed a similar result to m3 (Fig. 3.7A and B; Table 3.3), suggesting that these amino acids are not the direct action site of the host factor(s). It is feasible that the presence of H209 or C213 compensates for any defect caused by these mutations to the zinc-finger structure. However, in m5, where cysteines C200 and C203 were simultaneously changed to R (Fig. 3.7A), irreparable damage to the zinc finger structure occurred, with most *Antirrhinum* cells displaying GFP in their nuclei (Fig. 3.7B; Table 3.3). This indicates that maintenance of the zinc finger structure is indispensable for controlling the PM localization of Tam3 TPase. In N terminus of the Znf-BED domain, m7 and m8 carrying mutations at the two conserved aromatic amino acids, W186 or F189, dramatically reduced the number of the cells with GFP at the PM (Fig. 3.7A and B; Table 3.3). In addition to these two positions, m9 revealed that V185 was also functionally necessary for the PM localization of Tam3 TPase (Fig. 3.7A and B; Table 3.3). These data suggested that the β strand was important for the protein binding ability of the Znf-BED domain and was directly targeted by the host to detain Tam3 TPase in the PM. Hence, the m10 construct, which contained mutations in both the β strand and the CCH[H/C] motif, expressed GFP in the nuclei of most cells, but not at the PMs (Fig. 3.7A and B; Table 3.3). These results indicated that protein modifications such as phosphorylation within the Znf-BED domain were not responsible for altering the

localization of Tam3 TPase, because not all mutated amino acids were associated with phosphorylation.

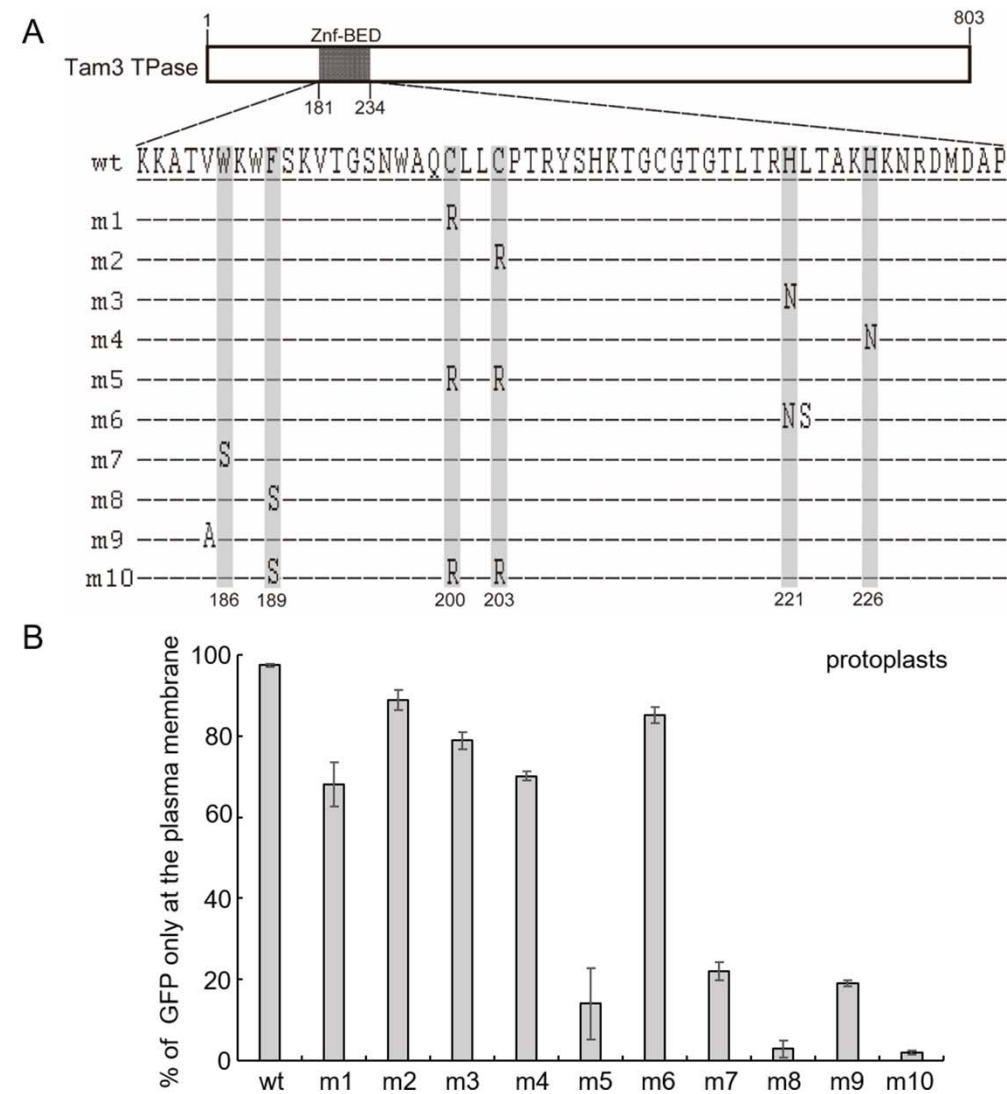


Figure 3.7 Identification of binding sites for Tam3 TPase interacting factors for detaining Tam3 TPase at the PM.

A, Amino acid sequences of ZnF-BED domains in the 10 constructs (m1 to m10) carrying point mutations and wild-type (wt) are depicted. Each construct was fused with the GFP gene at C termini. The grey background represents conserved sites; amino acids positions 186 and 189 for the N terminal aromatic amino acids; 200, 203, 221 and 226 for the zinc finger. Dashes represent the same amino acid as wild-type. B, Subcellular localization of point mutation constructs. Ten constructs were transformed into protoplasts of *Antirrhinum*. Proportion of the cells with GFP in the PM to the total number of cells with GFP were counted under a fluorescence microscope (Table 3.3). Data represent means $\pm$ s.d.

Table 3.3 Measurements of the rate of nuclei-located GFP signal of intact- and mutated- Tam3

TPase in protoplasts of HAM22.

Subcellular localization of Tam3 TPase and its mutant constructs using protoplasts of HAM22 (25°C) (numerator: number of cells showing nuclear GFP localization; denominator: total number of cells with GFP)			
	repeat 1	repeat 2	repeat 3
TPase(wt)	3/100	1/49	1/39
TPase(m1)	12/48	14/43	13/34
TPase(m2)	5/34	6/62	3/33
TPase(m3)	10/54	17/72	13/61
TPase(m4)	35/119	37/128	34/108
TPase(m5)	56/60	50/55	59/80
TPase(m6)	6/42	5/39	7/40
TPase(m7)	77/96	84/112	30/38
TPase(m8)	58/58	62/65	72/75
TPase(m9)	79/98	85/106	86/105
TPase(m10)	70/71	80/82	90/90

3.5 Detainment of Tam3 TPase at the PM through the protein binding ability of its Znf-BED domain

Two possibilities exist for the regulation of Tam3 TPase localization. The first is that membrane protein(s) interact with Tam3 TPase and confine it to the PM. The Znf-BED domain might interact with the NLS and prevent the nuclear localization of Tam3 TPase. However, it seems unlikely that only one Znf-BED domain prevents the function of all three NLSs simultaneously, and Tam3 TPase would be more likely to localize to the cytosol, not the PM. The second possibility involves an alteration of the Tam3 TPase configuration following a temperature shift. However, this would be expected to occur in all plant species, yet PM localization of Tam3 TPase has only been observed in *Antirrhinum* cells, not in onion or tobacco. To clarify this issue, I conducted native polyacrylamide gel electrophoresis (PAGE) western blot analysis to detect the non-denatured protein structure of the two Tam3 TPase-GFP constructs, wt and m10 (Fig. 3.7). wt and m10 with different Znf-BED domains were transformed into the protoplasts of *Antirrhinum* (Fig. 3.8). After incubation for 20 h, I confirmed that the protoplasts with the wt construct expressed GFP at the PM, while GFP was expressed in the nuclei of m10 protoplasts (Fig. 3.8A). The non-transformed protoplast was also prepared as a control (Fig. 3.8). Active proteins were extracted from these protoplast

samples without denaturation and were loaded on a native PAGE gel. Following electrophoresis, western blot analysis using a monoclonal mouse anti-GFP antibody revealed three specific bands (Fig. 3.8C). The first band (grey arrowhead) was specific to wt samples, the second band (white arrowhead) was shared by wt and m10 samples, and the third band was common to all samples (Fig. 3.8C). The wt-specific band reflected the interaction with the host protein(s) through the Znf-BED domain of the Tam3 TPase. The second band indicated the GFP-related protein(s) in both wt and m10 samples. The common band appeared to be an artifact of GFP-independent binding, because it also appeared in the Coomassie Brilliant Blue (CBB)-stained gel as a major fraction in all samples including non-transformed protoplasts (Fig. 3.8B).

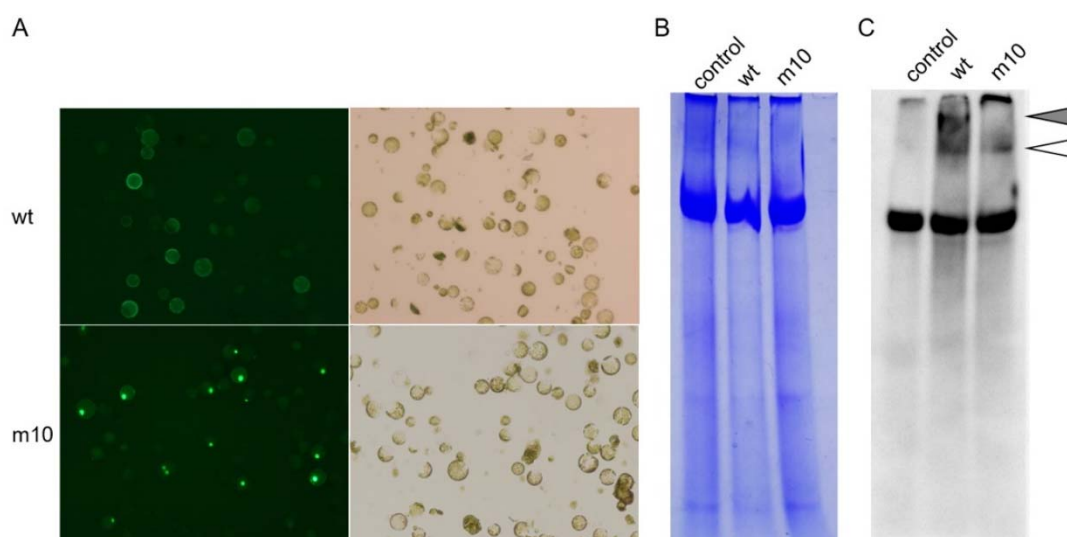


Figure 3.8 Protein binding profile of the Znf-BED domain of Tam3 TPase.

A, Mass protoplasts prepared from young *Antirrhinum* leaves transformed with Tam3 TPase constructs wt and m10 (see Fig. 3.7). The protoplasts with wt and m10 showed GFP expression in the PM and nucleus, respectively. Transformation efficiencies for wt and m10 constructs were 40%–50%. Protein preparations were made using these *Antirrhinum* protoplasts. B, CBB staining of the native PAGE gel for non-denatured proteins extracted from the three protoplast samples, non-transformant protoplasts (control), transiently transformed protoplasts with wt construct (wt) and m10 construct (m10). Active proteins were extracted using native protein extraction buffer containing 0.20% Triton X-100. C, GFP-Tam3 TPase complex detected by western blotting using an anti-GFP antibody. The duplicate of the gel blot of panel B was made by loading adjacent lanes with the same samples.

3.6 Selection of the conditions for two-hybrid library screening

Before performing a two-hybrid screening, test the bait for self-activation is necessary. The aim of this test is to measure the background reporter activity (here: *His3*) of bait proteins. This measurement is used for deciding the selection conditions of two-hybrid library screening. The full-length Tam3 TPase had very strong self-activation ability, which even could not be eliminated by 30 mM 3AT (Figure 3.9). So The full-length Tam3 TPase was not used for the two-hybrid screening. While self-activation ability of the truncated Tam3 TPase can be inhibited by using 5 mM 3AT. Therefore, the selection condition of two-hybrid library screening was SD/–Ade/–His/–Leu/–Trp/X- α -Gal/AbA containing 5mM 3AT.

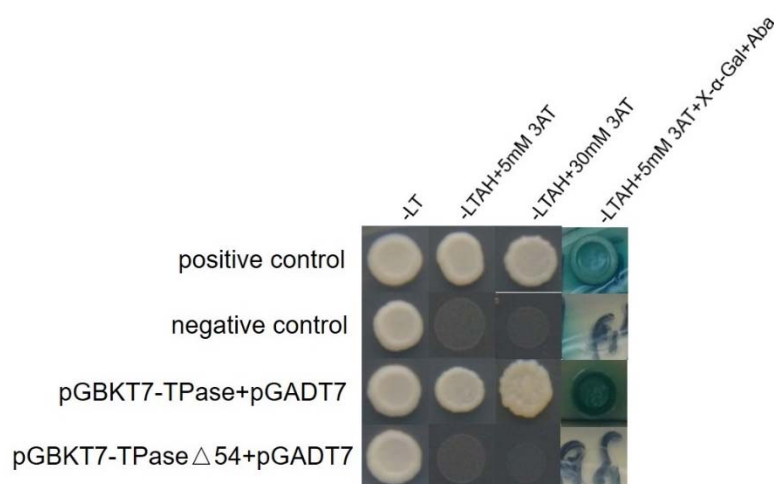


Figure 3.9 Assessing self-activation of bait proteins.

3.7 Isolating of Tam3 TPase interacting proteins

After three times two-hybrid screening, dozens of positive colonies were isolated. Through sequencing, the sequences of these prey proteins were uncovered. But nearly all of these prey proteins are truncated. So 5' RACE experiment was carried on to obtain the full cDNA sequence of some of those genes, which were thought to be membrane-located. Next, the interaction between Tam3 TPase and those genes should be checked again by using the full cDNA sequence of them. Lastly, several Tam3 TPase interacting proteins were isolated (Figure 3.10). Further experiments will be carried on to identify the host factors.

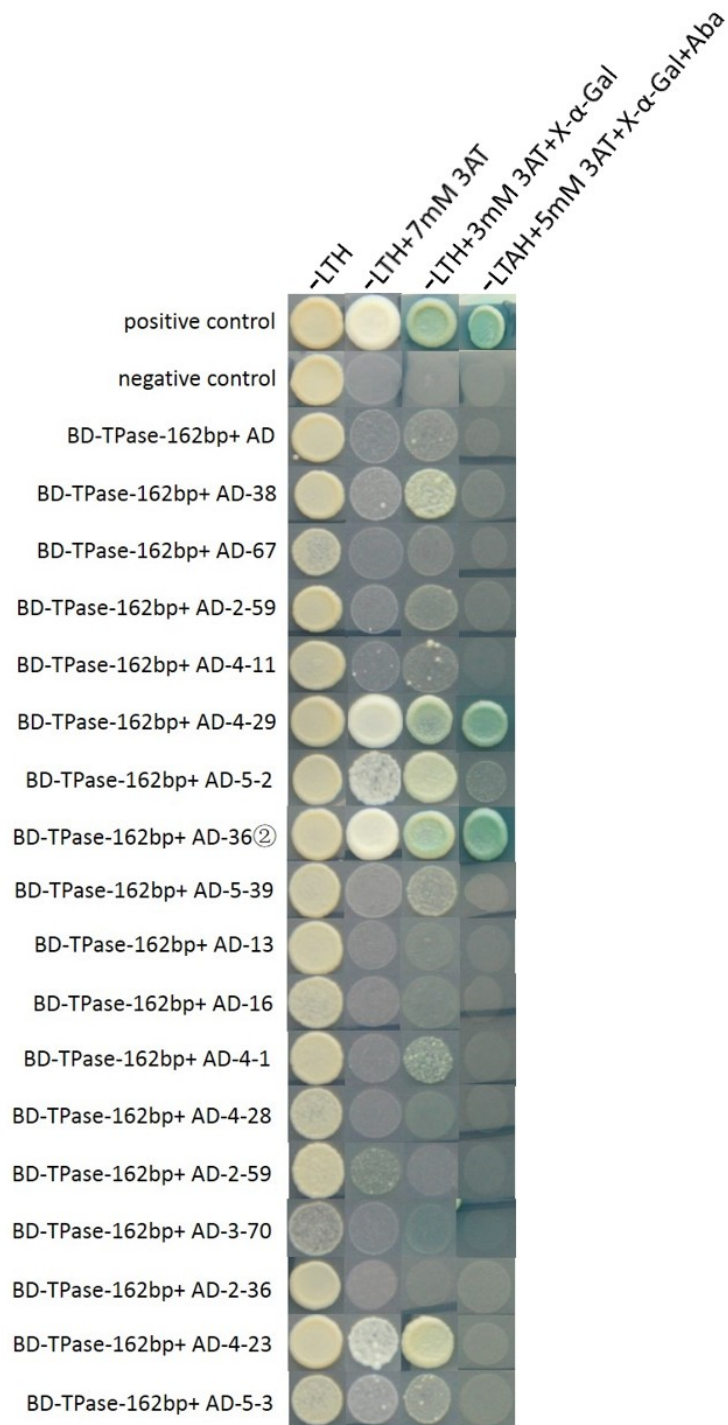


Figure 3.10 Isolating of Tam3 TPase interacting proteins.

3.8 Protein expression in E.coli cells

To isolate the host factor(s), except two-hybrid screening, I also want to use in vitro pull-down experiment to get some Tam3 TPase interacting proteins. So the first step is to acquire some tags fused Tam3 TPase proteins. But, unfortunately, nearly all the

target proteins were expressed in the inclusion bodies by using the E.coli system. Nextly, I prepare to express protein using yeast system.

Chapter 4 Discussion

4.1 Post-translational regulation of Tam3 in *Antirrhinum*

Plants have evolved a set of strategies to control the activity of TEs. Among these, DNA methylation is a stable mark used by hosts to fix transposons at the “off” position. Accumulated evidence previously revealed a negative relationship between DNA methylation and the active state of TEs (Slotkin and Martienssen, 2007). An increased level of DNA methylation in the promoter regions of TEs, such as Ac, Spm, and MuDR, tends to suppress the expression of TPase transcripts, eventually silencing these autonomous elements (Hashida et al., 2005). In plants, the hypermethylation state in these regions can be inherited over several generations (Habu et al., 2001). Although higher temperatures result in an increased level of DNA methylation in both terminal regions of Tam3, DNA methylation is not the cause of Tam3 silencing (Hashida et al., 2003). Moreover, because both transcripts and translational products of Tam3 TPase can be detected in *Antirrhinum* (Uchiyama et al., 2008; Fujino et al., 2011), mRNA cleavage and translation inhibition do not determine the inactivation of Tam3. Hence, regulation of the Tam3 transposition in *Antirrhinum* occurs in both epigenetic independent and post-translational manners.

4.2 Mechanisms involved in regulating Tam3 activity depend on the Znf-BED domain

Initiation of the transposition of a DNA transposon depends on the nuclear localization of its TPase. The mRNA of Tam3 TPase is translated to protein in the cytoplasm, and then translocated into the nucleus where it recognizes and binds specific sites for cut-and-paste processes. In the ovarian somatic cells of *Drosophila*, Piwi-interacting (pi)RNA-loaded Piwi proteins are selectively transported into the nucleus, where they exert TE silencing (Saito et al., 2009; Ishizu et al., 2011). N-terminally truncated Piwi loaded with piRNAs cannot translocate into the nucleus, and thus cannot silence TEs (Saito et al., 2009; Saito et al., 2010). Although exhibiting the opposite

function to TPase, this finding enabled us to speculate that the inhibition of TPase nuclear transport can silence TEs.

In the present study, I detected a positive correlation between Tam3 TPase PM localization and Tam3 suppression. When Tam3 was in an inactive state, the localization of Tam3 TPase was confined to the PM (Fig. 3.1). During cell division, plant cells undergo rupture and restructuring of the nuclear envelope. In the process of nuclear envelope restructuring, it is possible that some cytoplasmic contents, such as soluble proteins and organelle fragments, become incorporated into the nucleus. However, the PM structure is maintained during cell division. Hence, the PM would be suitable for preventing Tam3 TPase from entering the nucleus to strongly control Tam3 activity in *Antirrhinum*. This is supported by our finding that little variegation caused by Tam3 transposition occurred in the continuous growth of *Antirrhinum* petals under high temperatures. As long as the Znf-BED domain remained intact, Tam3 TPase was unable to enter the nuclei. However, if the domain was partially or completely destroyed, the majority of *Antirrhinum* cells expressed GFP in the nucleus (Fig. 3.4). This suggests that the cis-element was indispensable for regulating the membrane-associated localization of Tam3 TPase, and that this domain is essential for the epigenetic-independent regulation of Tam3. Mediating the localization of TPase may be a means of arresting TE transposition. Relative to the epigenetic mechanisms of TGS and PTGS, this mechanism is considered an optional function specifically established between the host and TE. It is conceivable that Tam3 TPase plays additional biological roles for the host given that many TPase-derived genes have important functions in plant development (Bundock and Hooykaas, 2005; Lin et al., 2007; Roccaro et al., 2007). Indeed, the DNA binding ability of Tam3 TPase was indicated by the fact that the Znf-BED domain binds to Tam3 to enable its transposition. Although the main function of TPase is to mediate the transposition of TEs, under certain conditions such as low temperature for Tam3 they may also function as gene activators or suppressors to enable the host to adapt to stress.

4.3 Control of Tam3 transposition through the Znf-BED domain is an outcome of coordinated evolution between the host and transposon

The BED-zinc domain is widely present in *hAT* superfamily transposons of different species (Rubin et al., 2001). The Znf-BED domain is thought to play a conserved role in starting the transposition of TEs belonging to the *hAT* family (Mack and Crawford, 2001; Hashida et al., 2006; Hickman et al., 2014). To date, no Znf-BED domains in TEs have been shown to interact with host proteins, except for Tam3, which may be involved in bipartite abilities to bind DNA and protein. Tam3 is under strict regulation from the host at the protein level. Unlike in *Antirrhinum*, I found that the localization of Tam3 TPase in onion epidermis cells and tobacco BY2 cells was not limited to the PM, but was also observed in the nucleus with no temperature regulation (Fig. 3.3). This indicates that an *Antirrhinum*-specific factor(s) or regulatory pathway(s) is involved in the regulation of Tam3. Although *hAT* superfamily transposons are widely found in multicellular organisms, no reports of horizontal transfer of these elements have been made (Rubin et al., 2001). Therefore, elements of the *hAT* superfamily are considered to have co-evolved vertically with individual host organisms. The distinct behavior of Tam3 in *Antirrhinum* might be an outcome of coordinate evolution between host and transposon. While the host needs to silence Tam3 to ensure its own safety under normal conditions, Tam3 TPase activity might be beneficial to the host under stress.

4.4 Mechanisms involved in the detainment of Tam3 TPase at the PM

Zinc finger proteins are one of the most common regulatory factors found in organisms. While the Znf-BED domain is mainly thought to be involved in DNA binding, accumulating evidence suggests that it can also interact with proteins (Diaz-Meco et al., 1996; Becker and Kunze, 1997; Chen et al., 2009). However, the mechanism involved in this protein binding ability is poorly understood. The Znf-BED domain constitutes two parts: an N-terminal region of two highly conserved aromatic amino acids, and a C-terminal CCH[H/C] patch. In TPases of TAG1 and DAYSLEEPER, site-directed mutagenesis of each cysteine or histidine predicted to

form a zinc finger led to loss of the DNA binding capacity of the Znf-BED domain (Mack and Crawford, 2001; Bundock and Hooykaas, 2005). Our present study also indicated that unrecoverable damage to the CCH[H/C] patch of the Znf-BED domain abolishes the protein binding ability of the Znf-BED domain of Tam3 TPase. Although the zinc finger structure is essential for protein binding activity, our results implied that the N-terminal motif was the direct target site for the host factor(s).

The trapping of protein nuclear translocation can be mediated by the nuclear pore complex or by protein modification (Greber and Gerace, 1992; Zhou et al., 2010). However, retention of Tam3 TPase at the PM seems to be regulated differently. In humans, the endoplasmic reticulum (ER) membrane protein NSIG-1 binds the NH₂-terminal membrane domain of the SREBP cleavage-activating protein (SCAP), and facilitates retention of the SCAP/SREBP complex in the ER (Yang et al., 2002). NSIG-1-mediated ER retention of SCAP is sterol-dependent. Binding with sterols causes a conformational change of SCAP, which increases the affinity of SCAP for INSIG-1 (Yang et al., 2002). The SREBP/SCAP/INSIG-1 complex becomes trapped in the ER by an as yet unknown mechanism (Yang et al., 2002). In the absence of sterols, the SCAP/SREBP complex is free to exit the ER and reach the Golgi complex (DeBose-Boyd et al., 1999; Nohturfft et al., 2000). The mutant SCAP(TM1-6) contains a Y298C substitution and fails to be retained in the ER upon sterol addition (Yang et al., 2002). I have demonstrated that *Antirrhinum* stores Tam3 TPase in the PM under non-stressed conditions. This study revealed that the Znf-BED domain in Tam3 TPase acts as a PM localization signal to interact with host factor(s). In this process, the host factor(s) binds mature Tam3 TPase proteins in the Golgi complex, and the protein complex is then transported to the PM via a transport vesicle. It appears that N-terminal aromatic amino acids in the Znf-BED domain have protein binding ability, while the zinc finger structure is necessary for controlling the PM localization of Tam3 TPase.

4.5 Characteristics of host factor(s)

DNA transposon Tam3 is famous for its unusual and remarkable feature of LTDT: activation at low growth temperatures (15°C) and inhibition at high temperatures above 25°C (Harrison and Fincham, 1964; Carpenter et al., 1987). Although Tam3 has been studied for dozens of years, the mechanism involved in LTDT is still a mystery. However, based on the existing data, a model for LTDT has been raised (Fig. 1.3). It was proposed that certain *Antirrhinum*-specific factor(s) were involved in this process (Fig. 3.8). These proteins can interact with the Znf-BED domain of Tam3 TPase, and fully trap the TPase at PM under the high temperature, thereby abolishes nuclear import of the TPase. Therefore, to make clear LTDT, isolation of the host factor(s) is really a key process. The characteristics of the host factor(s) should include: 1) PM located; 2) interaction with Tam3 TPase through binding to the Znf-BED domain; 3) low temperature sensitive: its expression should be down-regulated by low temperature.

4.6 Difficulties in isolation of host factor(s)

Up to now, the genome sequence of *Antirrhinum* has not been published yet. So the two-hybrid screening seems to be the best way to isolate Tam3 TPase interacting proteins. I used the matchmaker® gold yeast two-hybrid system to process the two-hybrid screening. Considering that the host factor(s) are cell membrane proteins, there are some difficulties to isolate the host factor(s) through this system, which is more suitable for isolating nuclear proteins. Although the prey vector contains a NLS which can guide the downstream fused proteins entering into the nucleus, it's still possible that the interaction between Tam3 TPase and host factor(s) cannot be detected, for the formation of the right conformation of host factor(s) is probably membrane binding dependent. Another problem with yeast two-hybrid system is the high false positive rate. Therefore, in vitro pull-down experiment will be carried on at the same time. As long as I cannot acquire transgenic *Antirrhinum* plants currently, I will express tags fused Tam3 TPase in yeast cells. The key point for in vitro pull-down experiment is to get

purified Tam3 TPase proteins. At last, I hope I can acquire some candidates by comparing the results of these two experiments.

Chapter 5 Concluding remarks

My data imply that the function of the Znf-BED domain of Tam3 TPase undergoes strong selection in *Antirrhinum*. Besides the DNA binding ability, the Znf-BED domain, which also serves as a PM localization signal, is targeted by host factor(s) to detain Tam3 TPase at the PM. This is closely related to Tam3 activity. Further experiments identified amino acids in the Znf-BED domain that are involved in the interaction between the host and Tam3 TPase. The N-terminal region comprises two highly conserved aromatic amino acids that appear to be the direct target site, while the zinc finger conformation seems to be indispensable for TPase detainment. This work provides insights into the mechanism and function of a Znf-BED domain involved in protein binding and TE regulation.

Chapter 6 Summary

Transposable elements (TEs) are widespread in the plant genomes. As characterized as mobile elements, TEs can move and insert into new positions within a genome, resulting in gene disruption, chromosome breakage, illegitimate recombination, and genome rearrangement. To maintain the stability and integrity of plant genomes, TE activities are under rigorous control of the hosts. Although hosts adopt a series of mechanisms in eliminating TE activity, a variety of conditions can trigger transposon activation, which are necessary for both TEs and their hosts. TEs need to maintain a certain level of activity to ensure their propagation and survival, while sometimes hosts need to activate TEs to serve for their growth and development, especially under stress. Because TEs are able to benefit the host organisms through their functions in genome organization, gene expression regulation and new gene creation. In this study, we performed detailed analyses on how cold stress affect the activities of TEs in plants and the mechanisms involved in regulating the TEs activation.

In *Antirrhinum*, DNA transposon Tam3 exhibits an unusual and remarkable feature of LTDT: activation at low growth temperatures (15°C) and inhibition at high temperatures above 25°C. Most especially, we found that LTDT of Tam3 is epigenetic-independent. LTDT of Tam3 was associated with temperature-dependent nuclear transfer of Tam3 TPase. The low temperature allows the TPase to transfer into nuclei resulting in the transposition of Tam3, but high temperature inhibits this nuclear import, causing the silencing of Tam3. Our previous study found that Tam3 TPase was equipped with a functional domain in the N-terminal region in confining nuclear transport of Tam3 TPase. In this study, this functional domain was identified to be comparable to Znf-BED domain that detained Tam3 TPase on the plasma membrane. This Znf-BED domain could also direct the other proteins with the different subcellular localizations to the PM in *Antirrhinum* cells. Some particular point mutations in the Znf-BED domain abolished detainment of the TPase at the PM. Functional amino acids in the Znf-BED domain were also determined by these mutation analysis. Znf-BED

domains in transposable elements have been generally known to be capable of binding ability to their own element DNAs to initiate the transposotion. Our study reveals that the Znf-BED domain of Tam3 TPase has bidirectional functions in regulating the activity of Tam3, which depends on the subcellular localization of TPase. Besides DNA binding ability in targeting transposon sequences in the nucleus, the Znf-BED domain in Tam3 facilitates to interact with certain host factor(s) causing inhibition of TPase nuclear transport, resulting in inactivation of Tam3. In addition, this post-translational control, in an epigenetic-independent manner, is a first finding regarding silencing of transposon that the Znf-BED domain targeted by host factor(s) resulted in detainment of transposase at PM.

Supplementary Tables

Table S1 Primers list of plasmid constructions.

primers for subcellular localization of Tam3 TPases	
T3TPase(XhoI)-F	CCGCTCGAGCGGATGGCAAACGAAGAAAACCTCAAATC
T3TPase Δ 55(XhoI)-F	CCGCTCGAGCGGATGGACACGAGCAATATTCA
T3TPase Δ 170(XhoI)-F	CCGCTCGAGCGGATGGCCTCTACATCAAGACC
T3TPase Δ 179(XhoI)-F	CCGCTCGAGCGGATGACGAAGAAAGCGACGGTA
T3TPase Δ 200(XhoI)-F	CCGCTCGAGCGGATGTTACTTTGTCCTACAAG
T3TPase Δ 231(XhoI)-F	CCGCTCGAGCGGATGGACGCTCCGGATATGCA
T3TPase Δ 244(XhoI)-F	CCGCTCGAGCGGATGGCACCCTGGAGGTATGACCAAAAT
T3TPase(SpeI)-R	GGACTAGTCCGTGGATGTTTGTAATCATATGGC
primers for subcellular localization of AmCSBL and AmDnaJh1	
AmDnaJh1(XhoI)-F	CCGCTCGAGCGGATGGCCATCATTCCTTGTTGA
AmDnaJh1(SpeI)-R	GGACTAGTCCCCTACTACTGGGAGCCTTC
AmCSBL(XhoI)-F	CCGCTCGAGCGGATGGGTAGTGCTTCATCAATG
AmCSBL(SpeI)-R	GGACTAGTCCGTCAATGGGGACTTCCATC
primers for inserting BED-zinc finger motif into N-terminals of AmCSBL and AmDnaJh1	
T3TPase Δ 170(XhoI)-F	CCGCTCGAGCGGATGGCCTCTACATCAAGACC
Znf_BED&AmDnaJh1-F	GACAACCAGACGGTACAATGGCCATCATTCCTTG
Znf_BED&AmDnaJh1-R	CAAGGAATGATGGCCATTGTACCGTCTGGTTGTC
Znf_BED&AmCSBL-F	GACAACCAGACGGTACAATGGGTAGTGCTTCATC
Znf_BED&AmCSBL-R	GATGAAGCACTACCCATTGTACCGTCTGGTTGTC
primers for point mutations	
T3TPase(XhoI)-F	CCGCTCGAGCGGATGGCAAACGAAGAAAACCTCAAATC
T3TPase(SpeI)-R	GGACTAGTCCGTGGATGTTTGTAATCATATGGC
T3TPase(m1)-F	CTGGGCTCAGCGTTTACTTTGTCCTACAAG
T3TPase(m1)-R	CTTGTAGGACAAAGTAAACGCTGAGCCAG
T3TPase(m2)-F	CTGGGCTCAGTGTTTACTTCGTCCTACAAG
T3TPase(m2)-R	CTTGTAGGACGAAGTAAACACTGAGCCAG
T3TPase(m3)-F	GAACACTTACAAGAAATTTGACGGCAAAG
T3TPase(m3)-R	CTTGCCGTCAAATTTCTTGTAAGTGTC
T3TPase(m4)-F	CATTTGACGGCAAAGAATAAGAATCGCGAC
T3TPase(m4)-R	GTCGCGATTCTTATTCTTTGCCGTCAAATG
T3TPase(m5)-F	CTGGGCTCAGCGTTTACTTCGTCCTACAAG
T3TPase(m5)-R	CTTGTAGGACGAAGTAAACGCTGAGCCAG
T3TPase(m6)-F	GAACACTTACAAGAAATTCGACGGCAAAG
T3TPase(m6)-R	CTTGCCGTCAAATTTCTTGTAAGTGTC
T3TPase(m7)-F	GAAAGCGACGGTATCGAAATGGTTTTTC
T3TPase(m7)-R	GAAAACCATTTGATACCGTCGCTTTC
T3TPase(m8)-F	TATGGAAATGGTCTTCAAAGGTGAC
T3TPase(m8)-R	GTCACCTTTGAAGACCATTTCATA

T3TPase(m9)-F	TGAAAGCGACGGCATGGAAATGG
T3TPase(m9)-R	CCATTTCCATGCCGTCGCTTTCA

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