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Temperature controls nuclear import of Tam3 transposase in *Antirrhinum*.

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Summary (204 words)

It has been proposed that environmental stimuli can activate transposable elements (TEs), while few substantial mechanisms have been shown so far. The class II element *Tam3* from *Antirrhinum majus* exhibits a unique property of low-temperature-dependent transposition (LTDT). LTDT has proved invaluable in developing the gene isolation technologies that have underpinned much of modern plant developmental biology. Here, we reveal that LTDT involves differential sub-cellular localization of the *Tam3* transposase (TPase) in cells grown at low (15°C) and high (25°C) temperatures. The mechanism is associated with the nuclear import of *Tam3* TPase in *Antirrhinum* cells. At high temperature, the nuclear import of *Tam3* TPase is severely restricted in *Antirrhinum* cells, while at low temperature, the nuclear localization of *Tam3* TPase is observed in about 20% of the cells. However, in tobacco BY-2 and onion cells, *Tam3* TPase is transported into most nuclei. In addition to three nuclear localization signals (NLSs), the *Tam3* TPase is equipped with a nuclear localization inhibitory domain (NLID), which functions to abolish nuclear import of the TPase at high temperature in *Antirrhinum*. NLID in *Tam3* TPase is considered to interact with *Antirrhinum* specific factor(s). The host-specific regulation of the nuclear localization of transposase represents a new repertoire controlling class II TEs.

Introduction (502 words)

With unfavorable environmental changes, plants immediately respond in physiological way to avoid fatal consequences, but also plants can alter genome *per se* to produce potential progenies to adapt to the given environmental changes. Mechanisms by which the physiological responses in plants follow the environmental changes have been intensively studied in various aspects. It is known that the structural changes of the plant genomes are occasionally induced by transposable elements (TEs) activated by environmental stresses (Grandbastien 1998, Kalendar *et al.* 2000), while the mechanisms regarding the activation processes of TEs are limited in our knowledge. To maintain genome integrity, plants may adopt diverse strategies to regulate the activities of transposable elements.

Tam3 from *Antirrhinum majus* is a class II transposon belonging to the *hAT* superfamily (Calvi *et al.* 1991). *Tam3* excision and, by implication, transposition, exhibit the unusual feature of activation at low growth temperatures (15°C) and inhibition at high temperatures above 25°C (Carpenter *et al.* 1987, Harrison and Fincham 1964) (Figure 1A). Activation of*Tam3* can occur as a rapid and reversible response to a temperature change during the lifetime of a single plant (Hashida *et al.* 2003). Such low-temperature-dependent transposition (LTDT) of *Tam3* represents a response of transposon to environmental stimuli. Because the activity of *Tam3* can be controlled by temperature, this transposon system has been used for the generation of mutations and a gene tagging system that have made remarkable contributions to the study of

developmental and metabolic systems in plants (Coen *et al.* 1989, Schwarz-Sommer *et al.* 2003).

Unlike known mechanisms in the stress responses of other TEs, LTDT is not regulated at the level of transcription of the *TPase* gene (Hashida *et al.* 2003). Although a reversible change of DNA methylation state in *Tam3* that occurred in somatic tissues was positively correlated with growth temperature (Hashida *et al.* 2003), methylation was not a cause of LTDT of *Tam3* (Hashida *et al.* 2005). Instead, nuclear extracts from plants grown at 15°C were capable of binding to the *Tam3* sequence, but the extract from plants grown at 25°C did not bind, suggested that the methylation change in *Tam3* is affected by the binding ability of its transposase (TPase) to *Tam3*, which is dependent on the growth temperature (Hashida *et al.* 2006). Previous results led to a conclusion that *Tam3* TPase *in vivo* exhibits differential binding abilities to *Tam3* between 15°C and 25°C that might be related to LTDT.

Here, it is reported that the nuclear localization of *Tam3* TPase in *Antirrhinum* is arrested at temperatures at which *Tam3* transposition is inhibited. The *Tam3* TPase reporter constructs fused with green fluorescence protein (GFP) and the luciferase (LUC) assay demonstrated that the nuclear localization of *Tam3* TPase in *Antirrhinum* cells is severely restricted at high temperature and is permitted in about 20% of the cells at low temperature. The results are provided to demonstrate that LTDT of *Tam3* is controlled by the nuclear localization of TPase, and evidence is shown for specific

interactions between *Tam3* TPase and host factor(s) to establish LTDT.

Results (2057 words)

Presence of *Tam3* TPase in *Antirrhinum* cells

LTDT of *Tam3* is not regulated by transcription of the *Tam3 TPase* gene or the DNA methylation state of *Tam3* (Hashida *et al.* 2005, Hashida *et al.* 2003). To test whether LTDT is correlated with the amount of the *Tam3* TPase in *Antirrhinum* cells, immunoblot analysis was performed using an anti-peptide antibody, pN1 (Uchiyama *et al.* 2008) (see Figure S1). pN1 detected a polypeptide of about 100 kD corresponding to *Tam3* TPase in young leaf proteins extracted from *Antirrhinum* plants grown at 15°C and 25°C (Figure 1B). This result demonstrated that the processes prior to translation of TPase are not responsible for LTDT.

Because TPases should be transported into the nucleus in order to catalyze the transposition reaction (Heinlein *et al.* 1994, Ono *et al.* 2002), it was considered that LTDT might reflect different accessibility of the TPase to the *Tam3* DNA sequences at the two temperatures: the low temperature could enhance the TPase accessibility to the *Tam3* sequences, while the high temperature could abolish the accessibility. In the previous results, proteins in a nuclear extract from 15°C- but not 25°C-grown plants bind to the *Tam3* sequence (Hashida *et al.* 2006). Differential nuclear localization of the TPase at high and low temperatures is thus a possible mechanism to explain LTDT.

Nuclear localization of *Tam3* TPase

To confirm the above assumption, transient expression analyses were conducted using a plasmid, pTam3TPase-GFP, containing the *Tam3 TPase* gene fused to the GFP gene. The plasmid was introduced into young petal epidermal cells of *Antirrhinum* plants grown at 15°C or 25°C using a particle bombardment method, and the bombarded petals were further incubated for 24 hours at 15°C or 25°C (Figure 2). The continuous low temperature (Ll) or low temperature after bombardment (Hl) conditions did not allow the plasmid to express TPase-GFP in the *Antirrhinum* petals, because the transient expression of the plasmid required relatively high temperatures. Continuous high temperature (Hh) conditions allowed the cells to express GFP, and most expressed GFP in the cytoplasm, while only 1% of the cells expressed GFP in their nuclei. In high temperature after bombardment (Lh) conditions, GFP was detected in the nuclei in about 18% of the cells with green fluorescence (Figure 2). These results showed that the TPase-GFP was not directed to the nucleus in most cells of the *Antirrhinum* plants grown at high temperature, while about 20% of the cells localized TPase-GFP to the nuclei at low temperature. When pTam3TPase-GFP was delivered into tobacco BY-2 cells and onion epidermal cells, GFP signals were observed in the nuclei of nearly 100% of the cells (see Figure 5 and Figure S2), suggesting that LTDT of *Tam3* is an *Antirrhinum*-specific phenomenon.

Previously, *Antirrhinum* callus showed LTDT of Tam3 similar to the plants (Hashida *et*

al. 2005). Then, we examined transgenic calli of *Antirrhinum* leaf that produced a chimera protein of Tam3 transposase and GFP. The plasmid pGWB5-Tam3 that contains *Tam3* TPase gene in the pGWB5 construct consisting of 35S promoter and *GFP* gene (Figure 3A) was introduced into the *Antirrhinum* genome by *Agrobacterium*-mediated transformation. In contrast to the transient expression of GFP, the transgenic calli stably expressed the fluorescence even at 15°C of continuous low temperature (Figure 3B). The nucleus-to-cytoplasm GFP (NCG) ratio was calculated based on the GFP fluorescent intensities of the nucleus and cytoplasm in the cell. The *Antirrhinum* calli transformed with the plasmid pGWB5 encoding merely GFP did not exhibit any difference in distributions of the NCG ratio between cells grown under low and high temperatures (Figure 3C). In the calli expressing GFP-fused TPase, however, the NCG ratios of the cells grown under low temperatures were clearly distributed in a higher ratio compared with that of cells grown under high temperatures, which was similar to the findings observed in calli transformed with pGWB5 (Figure 3C). Therefore, the transgenic *Antirrhinum* calli verified that the low temperature induces the GFP-fused TPase to accumulate in the nuclei.

Quantitative measurements of nuclear localization of *Tam3* TPase

For quantitative measurement of the amount of *Tam3* TPase in nuclei, the experiments using the GAL4 system were adopted (Aoyama *et al.* 1995, Igarashi *et al.* 2001). This system utilizes the effector protein containing GAL4 DNA-binding domain (GAL4 BD) and VP16 activator domain (VP16), both of which are required for transcriptional

activation of the reporter gene carrying GAL4 binding sites in its promoter sequence. We constructed two effector plasmids, pGATam3 and pGAGUS, where the *Tam3* TPase or GUS proteins were fused to GAL4 BD and VP16 activator domains (Figure 4A). The reporter plasmid, pGBL, contained two operons for *Renilla* luciferase and firefly luciferase genes as shown in Figure 4A: the *Renilla* luciferase gene driven by the 35S promoter is a reference to standardize differences in efficiency of the bombardment delivery, and the transcription of the firefly luciferase gene can be induced by binding of the effector protein to the GAL4 binding sites (six tandem copies). The firefly luciferase is produced by nuclear import of the effector proteins containing GAL4 BD and VP16. The constructs were introduced into young petal epidermal cells of *Antirrhinum* plants grown at 25°C by bombardment method, and the bombarded petals were incubated for 16 h at 15°C or 25°C, and then the firefly and *Renilla* luciferase activities were measured. The outcome for the level of nuclear import of *Tam3* TPase or GUS protein was indicated by ratio of the firefly luciferase activity to the *Renilla* luciferase activity (LUC ratio). When pGATam3 was bombarded along with pGBL, the incubation at 15°C of the petal cells showed as much as five time higher LUC ratio relative to the incubation at 25°C (Figure 4B). The delivery of pGAGUS and pGBL into the petal cells resulted in the higher LUC ratio at 15°C relative to that at 25°C, but it was not statistically significant (Figure 4B). In the case where pGBL alone was introduced, the LUC ratios were considerably low in both the temperature conditions (Figure 4B). It is noteworthy that GAL4 BD in the effector protein contains an NLS, and so is the effector protein directed to nucleus. Accordingly, the GUS-fusion protein

from the pGAGUS construct should be fully imported into nuclei, as pGAGUS induced highly LUC ratios. LUC ratio in the pGATam3-introduced cells incubated at 15°C showed about one fifth of that of pGAGUS-introduced cells incubated at 15°C (Figure 4B). This was the same proportion with the one estimated with pTam3TPase-GFP: about 20% of the cells with *Tam3* TPase-GFP had the signal in nuclei at low temperature (Figure 2). Therefore, although low temperature facilitated nuclear localization of *Tam3* TPase in *Antirrhinum*, about 80% of *Tam3* TPase might not be permitted to be present in *Antirrhinum* nuclei. At high temperature, nuclear localization of *Tam3* TPase was even more strictly restricted. The quantitative measurements using the luciferase gene constructs verified the results in the analyses using the GFP fusion proteins.

Arrest of nuclear import of *Tam3* TPase at high temperature

As described in the previous sections, two mechanisms for the differential nuclear localization of the TPase dependent on temperature were considered; 1) TPase is imported into the nucleus only at low temperature, 2) TPase is exported from the nucleus into the cytoplasm at high temperature. To test these possibilities, leptomycin B (LMB), which inhibits the export of nuclear proteins into the cytoplasm by targeting exportin 1, was applied to protoplasts prepared continuously at 25°C. The effect of LMB was verified using the plasmid pJ4-RSG-GFP, which encodes a GFP-fusion protein containing a nuclear localization signal (NLS) along with a nuclear export signal (NES) (Igarashi *et al.* 2001). RSG-GFP fusion protein was predominantly localized to

the cytoplasm of both *Antirrhinum* and BY-2 protoplasts (Figure 5C, G and J). In protoplasts treated with 20 ng/ml LMB, the ratios of cells that accumulated RSG-GFP protein in the nucleus to the number of total cells expressing GFP increased in both *Antirrhinum* (28% to 88%) and BY-2 (15% to 53%) protoplasts (Figure 5D, H and J). In the same protoplasts, LMB failed to alter the ratios for the nuclear localization of GFP from pTam3TPase-EGFP, which was mostly observed in the cytoplasm of *Antirrhinum* and in the nuclei of BY-2 cells (Figure 5F and J). Therefore, the differential nuclear localization of TPase dependent on temperature must be because of inhibition of the import of TPase into the nucleus in cells grown at 25°C and not inhibition of export. The control of nuclear import of TPase underlies the LTDT of *Tam3* in *Antirrhinum*. Even under low temperature conditions, the nuclear translocation of *Tam3* TPase-fusion protein was limited to about 20% of the total cells expressing GFP or LUC in *Antirrhinum* (Figures. 2 and 3). This may be a mechanism to suppress transposon activity and maintain genome stability that has evolved in *Antirrhinum*.

Nuclear localization signals in *Tam3* TPase

NLSs are intrinsic for nuclear proteins to enter the nucleus after translation in the cytoplasm. Based on the amino acid sequence of the *Tam3* TPase, three short stretches (~35 amino acids) were predicted as NLSs, which contains 6 or 7 basic amino acid residues (K/R) (Figure 6 and Figure S1). Each of the three regions was fused to a GFP plus GUS protein (to prevent GFP from autonomously entering the nucleus).

Bombardment with every construct resulted in green fluorescence that was mostly detected in the nuclei in both *Antirrhinum* petals and onion epidermal cells incubated at 25°C (Figure 6). The results revealed that *Tam3* TPase possesses at least three NLSs, which permitted entry into the nucleus. These findings were supported by other data obtained using onion cells bombarded with deletion mutants of the *Tam3* TPase (Figure S2). These results suggest that the *Tam3* TPase is equipped with a domain for the arrest of nuclear translocation of the TPase at high temperatures.

Nuclear localization inhibitory domain in *Tam3* TPase

To test whether *Tam3* TPase contains a domain required for the arrest of nuclear translocation at high temperatures, further transient assays were carried out. One intact and four truncated sequences of the *Tam3 TPase* gene fused to the *GFP* gene were introduced into the young *Antirrhinum* petals of plants grown at 15°C or 25°C through the bombardment method (Figure 7 left). The bombarded tissues were incubated in Hh or Lh conditions as described in Figure 2. The truncated constructs contained at least one NLS, so that the GFP fusion proteins potentially entered the nuclei as depicted in Figure 7. When the two plasmids pTP231-577E and pTP578-803E were introduced into the petals of plants grown at 15°C and 25°C, most of all the cells (87.5~100% of cells with GFP signal) expressed green fluorescence in the nuclei in either temperature conditions (Figure 7 right). The GFP fusion protein from pTam3TPase-GFP was delivered into the nuclei in 1% and 18% of the cells observed in Hh and Lh conditions, respectively (Figure 2 and 5). Similarly, low frequencies of nuclear localization of

GFP signal were observed in the assays with pTP1-577E and pTPΔBE (Figure 7). These three constructs, pTam3TPase-GFP, pTP1-577E, and pTPΔBE, all encoded the amino acid sequence 55 to 244 of the *Tam3* TPase (Figure 7). This 55-244 amino acid sequence should be associated with the arrest of nuclear translocation of *Tam3* TPase itself. Therefore, this region was designated as the nuclear localization inhibitory domain (NLID), which is potentially capable of suppressing the translocation of *Tam3* TPase to the nuclei in *Antirrhinum* cells. Compared with pTP231-577E and pTP578-803E, these three constructs had even lower frequencies of nuclear localization of the GFP signal in both the low and high temperature conditions. The NLID of *Tam3* may not only function at high temperature to strictly prevent nuclear translocation of the TPase but also function at low temperature to confine nuclear translocation to around 20% of the total TPase yield.

These five plasmids used in the above analysis were delivered into onion epidermal cells by the bombardment method, and most of the cells with GFP clearly exhibited green fluorescence in nuclei (Figure S2). The NLID may interact directly with *Antirrhinum* specific factor(s) to prevent nuclear translocation of the TPase itself. The NLID is considered as a *Tam3*-unique region; no homology was detected in the N terminal 170 amino acids of *Tam3* TPase, which partly comprises the NLID, although the rest of the TPase amino acid sequence was homologous to rice putative TPases (Dart) (AB206820) (Fujino *et al.* 2005, Tsugane *et al.* 2006).

Discussion (618 words)

This study elucidated the differential nuclear localization of *Tam3* TPase dependent on temperature; low temperature allowed the TPase to enter the nuclei, but high temperature did not allow. Control of the nuclear translocation of the TPase thus underlies LTDT of *Tam3* in *Antirrhinum*. The regulatory systems of transposon activities identified so far are known to have specific limitations; the germ line-specific transposition of *P* element in *Drosophila* due to splicing being limited to the germ cells (Laski *et al.* 1986); the inactivation of *Tc1* elements in the germ line of *Caenorhabditis elegans* attributable to RNA interference (Ketting *et al.* 1999); and the developmentally regulated transposition of maize transposons (Levy and Walbot 1990). Repressor genes have also been identified against *P* (Black *et al.* 1987), *Spm* (Cuypers *et al.* 1988), and *Mu* (Slotkin *et al.* 2005) transpositions, which result from aberrant transposase genes or the rearrangements of their derivative elements. In addition, non-specific regulation of transposon activity has been observed due to DNA methylation and/or RNA silencing (Buchon and Vaury 2006, Colot and Rossignol 1999, Martienssen 1998, McDonald *et al.* 2005). LTDT of*Tam3* occurring at a post-translational stage is considered as a unique regulatory mechanism relative to the above regulation mechanisms of transposon activity.

For *Tam3* TPase, the differential subcellular localization regulated by temperature is unlikely to be accomplished by itself, because the same phenomenon did not arise when

pTam3TPase-GFP was introduced into tobacco BY-2 and onion cells where green fluorescence was preferentially detected in the nuclei at high temperature. The control of nuclear localization of *Tam3* TPase should be established by cooperation with *Antirrhinum* host factor(s). Martin et al. (1989) and Harring et al. (1991) also showed no enhancement of *Tam3* transposition at low temperature in transgenic tobacco plants. These previous results support the prediction that LTDT requires *Antirrhinum* specific factor(s).

In general, TPases are transported from the cytoplasm into the nucleus after translation, because of the presence of NLS-related domains, to allow TPases to bind to nuclear DNA for the cut-and-paste processes. Thus, NLSs seem to be essential for TPases. The two transposons, *Ac* (Boehm *et al.* 1995) and *Mu* (Ono *et al.* 2002), in maize have at least one major transcript in their autonomous elements encoding proteins containing NLS signals, which are capable of directing the proteins to the nuclei in petunia protoplasts and onion epidermal cells, respectively. *Tag1* in *Arabidopsis* was also inferred to possess a NLS from the amino acid sequence (Liu and Crawford 1998). At least three monopartite NLSs were identified in *Tam3* TPase. When GFP fusion proteins carrying each of the NLSs were introduced into *Antirrhinum* or onion cells, the green fluorescence was localized to the nuclei at both temperatures. In addition to these monopartite NLSs, *Tam3* TPase harbors an NLID that was identified as a region to inhibit nuclear localization of the TPase. The NLID consisted of an amino acid sequence unique to *Tam3* TPase and is a possible region to regulate LTDT of *Tam3* and

to interact with *Antirrhinum* specific factor(s). The NLID might also play a role to limit nuclear translocation up to approximately 20~30% of total *Tam3* TPase production even at low temperature. These results seem to reflect the complexity of the host mechanism involved in LTDT.

Tam3 is classified into the *hAT* superfamily (Calvi *et al.* 1991), of which related elements have been widely found in multicellular organisms, implying that the *hAT* superfamily probably arose before the separation of plants, animals and fungi. These elements are considered to have co-evolved vertically with individual host organisms, because there is no evidence for their horizontal transfer (Rubin *et al.* 2001). The distinct behavior of *Tam3* in *Antirrhinum* might reflect coordinate evolution between host specific factor(s) and the transposon.

Experimental procedures (1574 words)

Plant materials

We used the *Antirrhinum majus* line HAM5 (*nivea*$^{\text{recurrens: }Tam3}$), which gives rise to variegated petals at low temperatures of around 15˚C because of *Tam3* transposition. This line was initially grown for 2 months at 25˚C and subsequently transferred to a 15˚C growth chamber or grown continuously at 25˚C. The tobacco BY-2 cell line was maintained in the liquid MS medium and subcultured every week. Onion was used within one week after purchase.

Antibody production and purification

Antibody production was performed by Molecular Biology Laboratory (Nagoya, Japan). An oligopeptide, GCGTGTLTRHLTAKHKNRD, which corresponds to the amino acid residues 212-230 of *Tam3* TPase (see Figure S1) was synthesized and used to immunize a rabbit. To purify the antibody, the serum obtained was incubated with the resin-immobilized oligopeptide (FMP-activated Cellulofine; Seikagaku Kogyo, Tokyo, Japan) overnight at room temperature. The resin was washed extensively with wash buffer containing 20 mM Tris-HCl (pH 7.5), 1 M NaCl, and 1% Triton X-100 and then with Tris-buffered saline (TBS). Absorbed antibody molecules were eluted with 0.1 M glycine-HCl (pH 2.5) and neutralized immediately with 1/20 volume of 1 M Tris at 4˚C. The purified antibody was designated as pN1.

Immunoblot analysis

Total protein was extracted from the young leaves or flower petals of *Antirrhinum* plants. About 0.5 g of tissue was homogenized with 1 ml of protein sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5 μg of aprotinin, and 35 μg of PMSF, and then supplemented with 50 μl of 2-mercaptoethanol. The homogenate was centrifuged for 5 min at 15,000 rpm. The resulting supernatant was separated from the cell debris. The protein concentrations of the extracts were measured using a Protein Assay kit (BioRad, Hercules, CA, USA). About 20 μg of protein was separated by 10% (w/v) SDS-PAGE and transferred to a poly-vinylidenedifluoride membrane.

After blocking with 4% (w/v) skim milk, the membrane was incubated overnight at 4˚C with 2,500-fold-diluted primary antibody. The membrane was incubated for 1 h with 5,000-fold-diluted secondary antibody (anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase) and then stained with ECL Western Blotting Detection Reagent (RPN2106; Amersham Bioscience, UK) according to the manufacturer's recommendations. Signals were detected using Fuji X-ray films.

Plasmid construction

To construct plasmids able to express GFP, modifications were made to the basic construct pJB1414, which contains the Cauliflower mosic virus (CaMV) 35S promoter, EGFP coding sequence, and the terminal sequence of nopaline synthase (TNOS) in a cistron, and the ampicillin resistance gene. For the TPase gene-fusion plasmid, pTam3TPase-GFP, the full-length *Tam3* TPase coding sequence (2409 bp in AB038406) was inserted between the 35S promoter and EGFP sequences in pJB1414 (Uchiyama *et al.* 2008). This insertion process was mediated by the Gateway reaction (Invitrogen, Carlsbad, CA, USA) and the primers used in GATEWAY cloning are listed in Table S1. Similarly, the plasmid, pGWB5-Tam3, for *Agrobacterium*-mediated transformation were constructed based on pGWB5 (Nakagawa *et al.* 2007) by the GATEWAY cloning system. The plasmid, pGWB5-Tam3, locates the Tam3 TPase gene upstream of the synthetic green fluorescent protein with the S65T mutation (sGFP gene), which is present between the 35S promoter and TNOS in pGWB5 (Figure 3A). Deletions of parts of the *Tam3* TPase coding sequence for pTP1-577E, pTP231-577E, pTP578-803E,

and pTP1-351E were produced by PCR, and the deletion of pTPΔBE was performed by self-ligation after BspT1071 digestion with the PCR segment of the *Tam3 TPase* gene (the sequence corresponding to *Tam3* TPase amino acid positions 55-803), which can take away the DNA sequence between amino acid positions 245 and 588 of *Tam3* TPase. These truncated sequences were also introduced between the 35S promoter and EGFP coding sequence in pJB1414 through the GATEWAY method as described above. To survey the NLS sequences, each of the candidate NLSs (Table S2) with a basic amino acid cluster was inserted between the 35S promoter and EGFP sequences in pJB1414. These plasmids also contained the GUS gene, which was inserted between the EGFP coding and TNOS sequences of pJB1414, to prevent autonomous translocation to the nucleus (the EGFP molecule by itself tends to enter the nucleus).

Transient expression assay

Transient expression of GFP in *Antirrhinum* petal and onion epidermal cells was carried out using the Helium Biolistic gene transformation system (BioRad, Hercules, CA, USA). The epidermis of each tissue was peeled and placed on 0.5 × MS medium. Plasmid DNA (~2 μg) was precipitated onto gold particles (1.6 μm) as described previously (Hashida *et al.* 2003). The bombardment delivery into intact tissue was performed according to the method of Uchiyama *et al.*(2008). The subcellular localization of GFP fusion proteins was observed using a UV-fluorescence microscope (Olympus, Tokyo, Japan) equipped with a UV-A fluorescence filter set (330-380 nm excitation filter, 400 nm dichroic mirror, 420 nm barrier filter). Bombardments were

repeated three or more times for each plot.

***Agrobacterium*-mediated transformation and GFP microscopic observation**

Leaves from *in vitro*-grown plants were used for callus induction in MS medium containing 5mgl^{-1} Picloram (Sigma, Missouri, USA). Two weeks after callus induction, 5-mm-square pieces of leaf laminae were inoculated with freshly grown *Agrobacterium* tumefaciens strain LBA4404, transformed with pGWB5-Tam3 or pGWB5 (Figure 3A). Inoculated leaf segments were cocultivated on 0.2% Gellan gum (Wako Pure Chemical Industries LTD, Osaka Japan) solidified MS medium containing 30 gl^{-1} sucrose. A total of 150 leaf explants were treated with *Agrobacterium* and cultured in plastic dishes. After five days of cocultivation, the leaf segments were transferred to the same medium with the addition of 300 mgl^{-1} cefotaxime (Hoechst AG, Frankfurt-Höchst, Germany) to eliminate *Agrobacterium* and the additions of 25mgl^{-1} Hygromycine and 50 mgl^{-1} Kanamycine were added for the selection of transgenic cells. The induced calli were subcultured every two weeks on the same medium. The proliferated calli grown for one week after the second subculture at 25˚C or 15˚C were excised and examined for GFP observation using a UV-fluorescence microscope (Nikon, Tokyo, Japan) equipped with B-2A (450-490 nm excitation filter) for EGFP and UV-1A (360 nm excitation filter) for DAPI. In order to estimate the nucleus-to-cytoplasm GFP (NCG) ratio, GFP fluorescence intensities were measured by scanning of the microscopic images with ImageJ 1.43 (http://rsb.info.nih.gov./ij/). An average of GFP fluorescence intensities at points with an equal distance within the nucleus or cytoplasm was used to estimate the

NCG ratio. In each plot, 50 cells collected from at least three independent transgenic lines were randomly selected from the microscopic images and were analyzed to determine the NCG ratio.

Quantitative transient assay using GAL4 system

The plasmid pGBL has two operons as shown in Figure 4A: one consists of the firefly luciferase gene (pGL4.12, Promega, Madison, USA) with TNOS sequences driven by the six tandem copies of the GAL4 binding site upstream of the TATA box, and the other, which is a reference to standardize differences in efficiency of the bombardment delivery, carries the 35S promoter + the *Renilla* luciferase gene (pRL-null, TOYO B-Net CO., LTD) with TNOS sequences. The segment containing the GAL4 binding sites and TATA-box was adopted from pTA7001 (Aoyama *et al.* 1995) as a PCR segment with 5'-GAC GTT GTA AAA CGA CGG CCA C-3' and 5'-AGG CCA GCG TGT CCT CTC C-3'. pTA7001 was provided from Dr. N. H. Chua. In the two effector plasmids, pGATam3 and pGAGUS, GAL4 DNA-binding domain (amino acid positions 1 to 74) and Activator of VP16 (75 to 154) were inserted following a viral translation enhancer (Ω) present at the 3' end of the 35S promoter sequence. The segment of GAL4 BD and Activator were also amplified from pTA7001 with 5'-ATG AAG CTA CTG TCT TCT ATC GAA C-3' and 5'-CCC ACC GTA CTC GTC AAT TCC-3'. As shown in Figure 4, each of pGATam3 and pGAGUS contains *Tam3 TPase* gene or *GUS* gene, respectively, followed by TNOS sequence.

The introduction of the plasmids into the flower petals (HAM5 line) was performed by the bombardment method. A mixture of pGBL (~2.0 μg) and pGATam3 or pGAGUS (~0.8 μg) was precipitated onto 1.2 mg of gold particles (1.6 μm) with ethanol, and 0.12 mg of the plasmid DNA-coated gold particles was delivered into intact tissue as described above. The bombarded buds were incubated for 16 h at 25°C or 15°C. As the luciferase detection system is very sensitive, the pre-incubation of the bombarded buds at 25˚C was not necessary. Firefly luciferase and *Renilla* luciferase activities were assayed using a Dual-luciferase Assay Kit (Promega) in accordance with the manufacturer's instructions. Chemical luminescence was measured using a luminometer (Mithras, LB 940, Berthold Technology, Bad Wildbad, Germany).

PEG transformation and microscopy observation

Protoplasts were isolated from young leaves of *Antirrhinum* plants (line HAM5) and tobacco BY-2 cells. Incubation of the protoplasts in isolation buffer (1.5% Cellulase Onozuka R10, 0.05% Pectolyase Y-23, 0.55 M mannitol) was performed for 3 h at a temperature at 15˚C or 25˚C. For polyethylene glycol (PEG) transformation, purified protoplasts were washed in 0.55 M mannitol, 0.5% MES, pH 5.6, and carrier salmon sperm DNA and plasmid DNA were added to 100 μl of protoplast suspension per tube. Subsequently, the same volume of 40% PEG 4000 was added. These protoplasts were washed once and incubated in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 5 mM glucose, pH 5.8) 24 h at 25˚C with 20 nM LMB until the observation. The protoplasts expressing GFP fusion proteins were observed using a UV-fluorescence

microscope (Olympus, Tokyo, Japan) equipped with the U-MNIBA filter set (470-490 nm excitation filter, 505 nm dichroic mirror, 515-550 nm barrier filter) for EGFP.

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Short legends for supporting information

Table S1

Primer combinations used for GATEWAY cloning of Tam3-transposase sequences.

Table S2

Primer combinations for the amplification of NLSs from the *Tam3 TPase* gene.

Figure S1

Amino acid sequence of *Tam3* TPase, and positions of oligopeptide used as the antigen for pN1 and NLSs.

Figure S2

Subcellular localization of the intact- and truncated- *Tam3* TPase in onion epidermal cells.

Figure legends (1268 words)

Figure 1.

Phenotypic changes in *Antirrhinum* flowers due to variegation in plants grown at 25°C and 15°C and detection of *Tam3* TPase.

(A) The different variegated flower phenotypes at 25°C and 15°C resulted from LTDT of *Tam3* in the *pallida*$^{recurrens::Tam3}$ allele of *Antirrhinum*. (B) Immunoblot analysis to detect the *Tam3* TPase using an anti-peptide antibody, pN1, against amino acid positions 212-230 of *Tam3* TPase (see Figure S1). Total proteins were extracted from the leaves of *Antirrhinum* plants grown at 25°C or 15°C. The antibody pN1 specifically recognizes a peptide of 100 kDa, which corresponds to the 803 aa peptide of Tam3 TPase (Uchiyama *et al.* 2008).

Figure 2

Subcellular localization of *Tam3* TPase at different temperatures.

Plasmids were delivered into *Antirrhinum* petal cells by the bombardment method. Before introduction of the plasmid, the plants were grown at 25°C (H) or 15°C (L) for at least 3 weeks. The young petals bombarded with the plasmid were incubated at 25°C (h) or 15°C (l) for 24 hours, and the subcellular localization of GFP was observed by fluorescence microscopy. Petal cells bombarded with a plasmid carrying only the GFP gene showed preferential nuclear localization of the GFP protein, while in the case of pTam3TPase-GFP, two GFP localization patterns were observed: one in which GFP was

present in the nucleus and the other in which it was absent from the nucleus. The histogram shows the frequencies of cells with nuclear localization among those expressing GFP. N.D.: GFP was not detected. Scale bars in the GFP images indicate 10 μm in length.

Figure 3

Differential localization of the *Tam3* TPase in transgenic *Antirrhinum* calli at different temperatures.

(A) The construction of the plasmid pGWB5-Tam3 was accomplished by replacement of the *ccdB* gene in pGWB5 with Tam3 TPase ORF using GATEWAY Technology (Invitrogen). P35S; CaMV 35S promoter, B1 and B2; *att*B1 and *att*B2 as the sites for BP recombinase, R1 and R2; *att*R1 and *att*R2 as the sites for LR recombinase, TNO; the terminater sequence of Nopalin syntase gene (TNOS). (B) The two constructs (pGWB5-Tam3 and pGWB5) were introduced into the *Antirrhinum* calli, which were grown at 15°C or 25°C. The GFP fluorescence was detected in the cells grown at both temperatures, but strong localization at the nuclei was observed in the calli carrying pGWB5-Tam3 grown at 15°C. Nuclear localization was confirmed by DAPI. Each scale bar indicates 10 μm in length. (C) The nucleus-to-cytoplasm GFP (NCG) ratios for GFP fluorescence intensities were estimated in 50 cells randomly selected in each plot, and the frequencies (number of cells) were depicted in two histograms for the calli containing pGWB5-Tam3 and pGWB5. The blue bar indicates cells grown at 15°C, while the red bar indicates cells grown at 25°C.

Figure 4

Quantitative analysis of nuclear localization of *Tam3* TPase using GAL4 system. (A) Two effector and one reporter plasmids were constructed as described in Experimental procedures. Both the effector plasmids, pGATam3 and pGAGUS, commonly contain Cauliflower mosaic virus 35S promoter sequence (CaMV 35S) and omega motif (Ω) for the promoter sequences, and the terminater sequence of Nopalin syntase gene (TNOS). The coding sequences in pGATam3 and pGAGUS also shared GAL4 DNA-binding domain (GAL4 BD) and VP16 activator domain (VP16), which were fused to *Tam3* TPase or GUS polypeptides, respectively. The reporter construct, pGBL, consists of the firefly LUC and *Renilla* LUC genes. The firefly LUC gene is induced by binding of GAL4 BD plus VP16 domain to six tandem motifs of GAL4 binding site, while *Renilla* LUC gene is constitutively transcribed with CaMV 35S promoter sequence. (B) Measurements of the luciferase activities were carried out with *Antirrhinum* petal cells, which were incubated for 16 h at 25°C or 15°C after bombardment of the plasmids. LUC activity is the ratio of firefly LUC activity to *Renilla* LUC activity (× 1000) and indicates a level of nuclear localization of *Tam3* TPase. Single star mark shows that LUC ratios between 25°C and 15°C were significantly different at 1% ($t = 0.0003$), while double star marks show that LUC ratios between 25°C and 15°C were not significantly different at 5% level ($t = 0.116$).

Figure 5

Effect of LMB on nuclear localization of *Tam3* TPase.

Each of the plasmids, pTam3TPase-GFP or pJ4-RSG-GFP (RSG), was introduced into protoplasts prepared from *Antirrhinum* leaves and tobacco BY-2 cells grown at 25°C, and the protoplasts were incubated with 20 ng/ml of LMB (+) or without LMB (-) for 24 h at 25°C (J). For the control, pTam3TPase-GFP was delivered into leaf protoplasts from *Antirrhinum* leaves grown at 15°C. Representative images of the subcellular localizations of GFP fusion proteins in *Antirrhinum* leaf protoplasts (A-D) and tobacco BY-2 protoplasts (E-H). (A) The leaf protoplasts from *Antirrhinum* plants grown at 15°C show GFP signals derived from pTam3TPase-GFP in the nucleus. (B) Leaf protoplasts from *Antirrhinum* plants grown at 25°C show GFP signal derived from pTam3TPase-GFP in the cytoplasm. (C) Leaf protoplasts from *Antirrhinum* plants grown at 25°C show GFP signals derived from pJ4-RSG-GFP in the cytoplasm. (D) Leaf protoplasts from *Antirrhinum* plants grown at 25°C, which were treated with leptomycin B (LMB), show GFP signals derived from pJ4-RSG-GFP in the nucleus. (E) Protoplasts from BY-2 cells cultured at 25°C show GFP signals derived from pTam3TPase-GFP in nucleus. (F) Protoplasts from BY-2 cells cultured at 25°C, which were treated with leptomycin B (LMB), show GFP signals derived from pTam3TPase-GFP in the nucleus. (G) Protoplasts from BY-2 cells cultured at 25°C show GFP signals derived from pJ4-RSG-GFP in the cytoplasm. (H) Protoplasts from BY-2 cells cultured at 25°C, which were treated with leptomycin B (LMB), show GFP signals derived from pJ4-RSG-GFP in the nucleus. (I) Microscopic field image indicating multiple *Antirrhinum* protoplasts that show GFP signals derived from

pJ4-RSG-GFP. Each scale bar of A – I indicates 10 μm in length. (J) The histogram indicates the percentage of the cells expressing GFP in the nucleus among the cells with GFP signals. Standard deviations were obtained from three repetitions.

Figure 6

Identification of three NLSs in *Tam3* TPase.

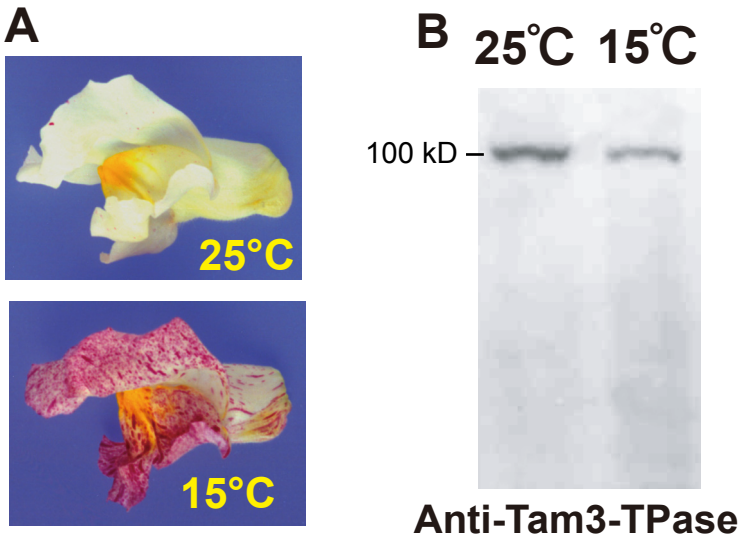
Three acidic domains of *Tam3* TPase were tested as NLS candidates. Each of the candidate domains was fused to the GFP-GUS gene construct, and the resultant plasmids were introduced into onion epidermal cells or *Antirrhinum* petal cells at 25°C (Hh). The GFP-GUS fusion protein could not be transported to the nucleus, but each NLS could direct the GFP-GUS protein to the nucleus in most of the cells. The positions and amino acid sequences of the three NLSs are indicated below images and Figure S1. Scale bars indicate 30 μm or 10 μm in the images of onion cell and *Antirrhinum* cell, respectively.

Figure 7

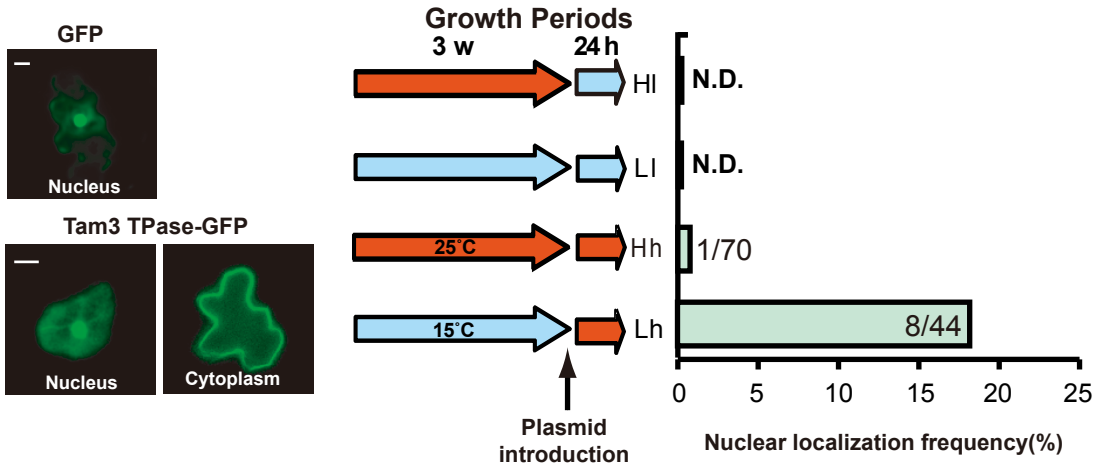
Identification of the nuclear localization inhibitory domain (NLID) of *Tam3* transposase.

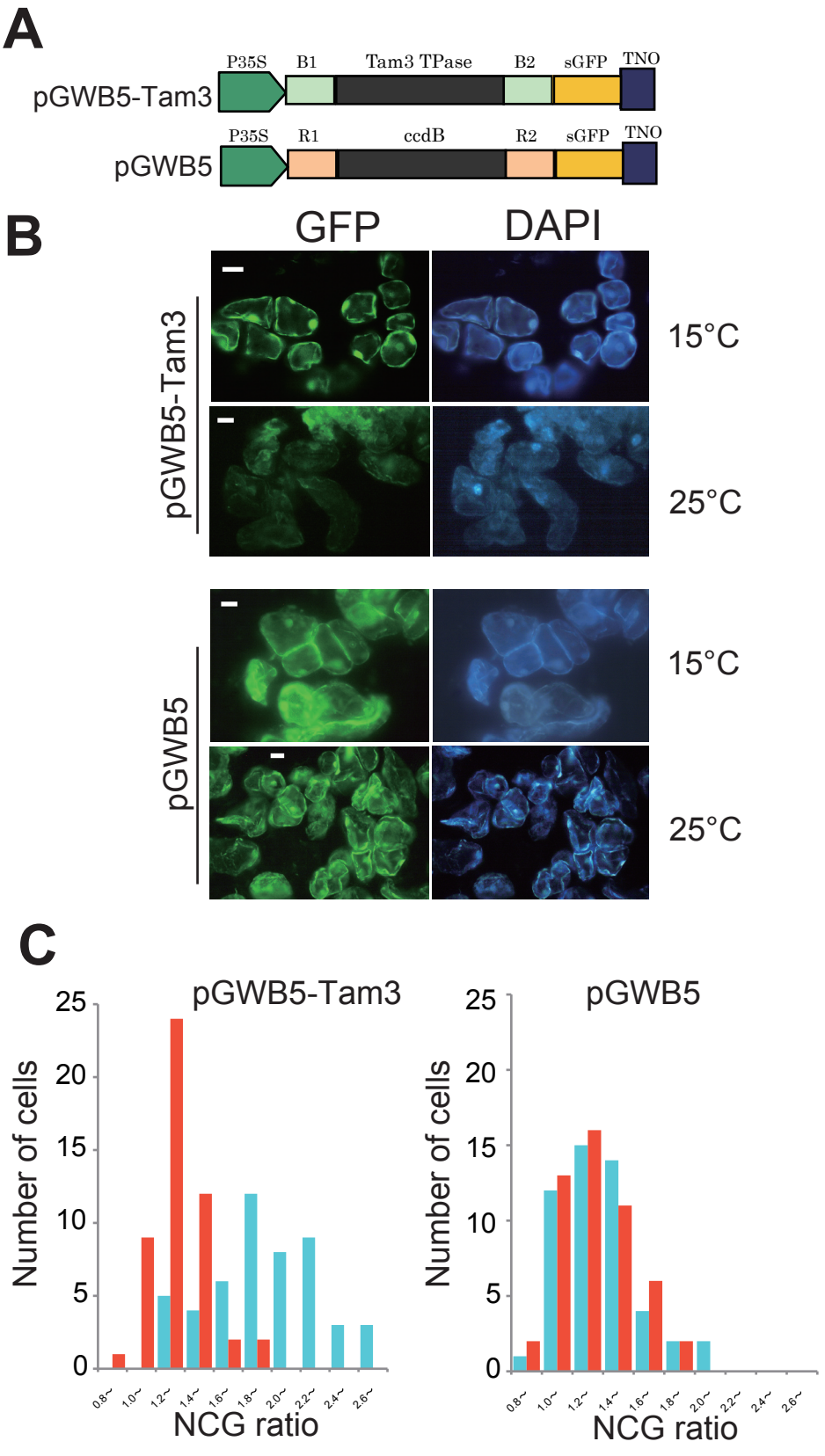
The left panel shows the intact and truncated constructs derived from the *Tam3 TPase* gene. Each construct fused with the GFP gene was introduced into young petals of *Antirrhinum* by the bombardment method. Numbers in the constructs show the amino acid positions in *Tam3* TPase. Black strips indicate NLS positions. pTPΔBE has two deletions of the *Tam3* TPase at amino acid positions 1-54 and 245-588. The right

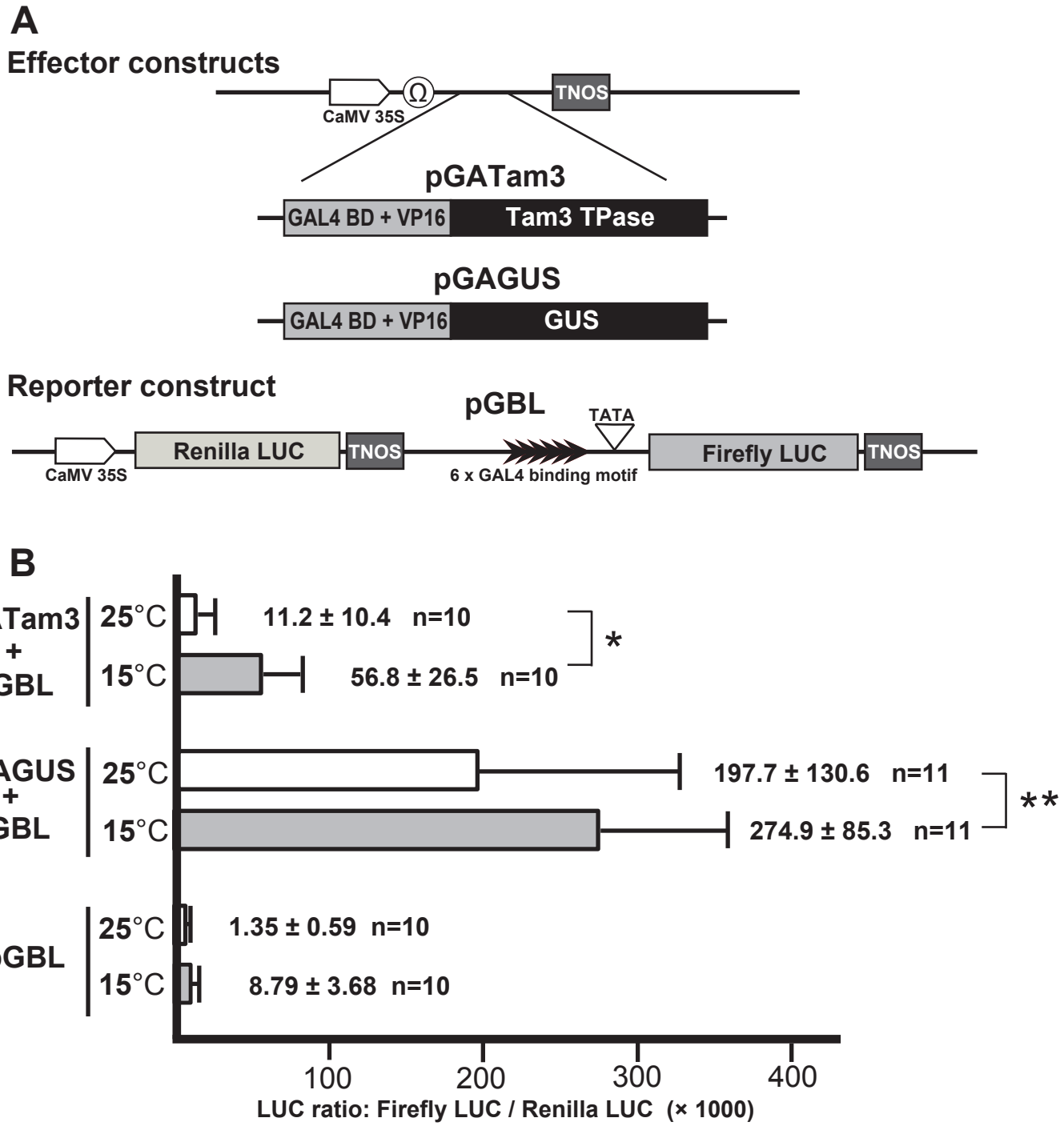
panel shows histograms to indicate the proportions of cells with nuclear localization of GFP compared with the total number of cells with green fluorescence and also by fractions in the columns (numerator: number of the cells showing nuclear GFP localization, and denominator: total number of cells observed). Each of the constructs was introduced to the young petals of the plants grown at 15°C (L) or 25°C (H) for at least 3 weeks, and the bombarded petals were incubated at 25°C (h) for 24 hours; white column (Lh) and gray column (Hh).

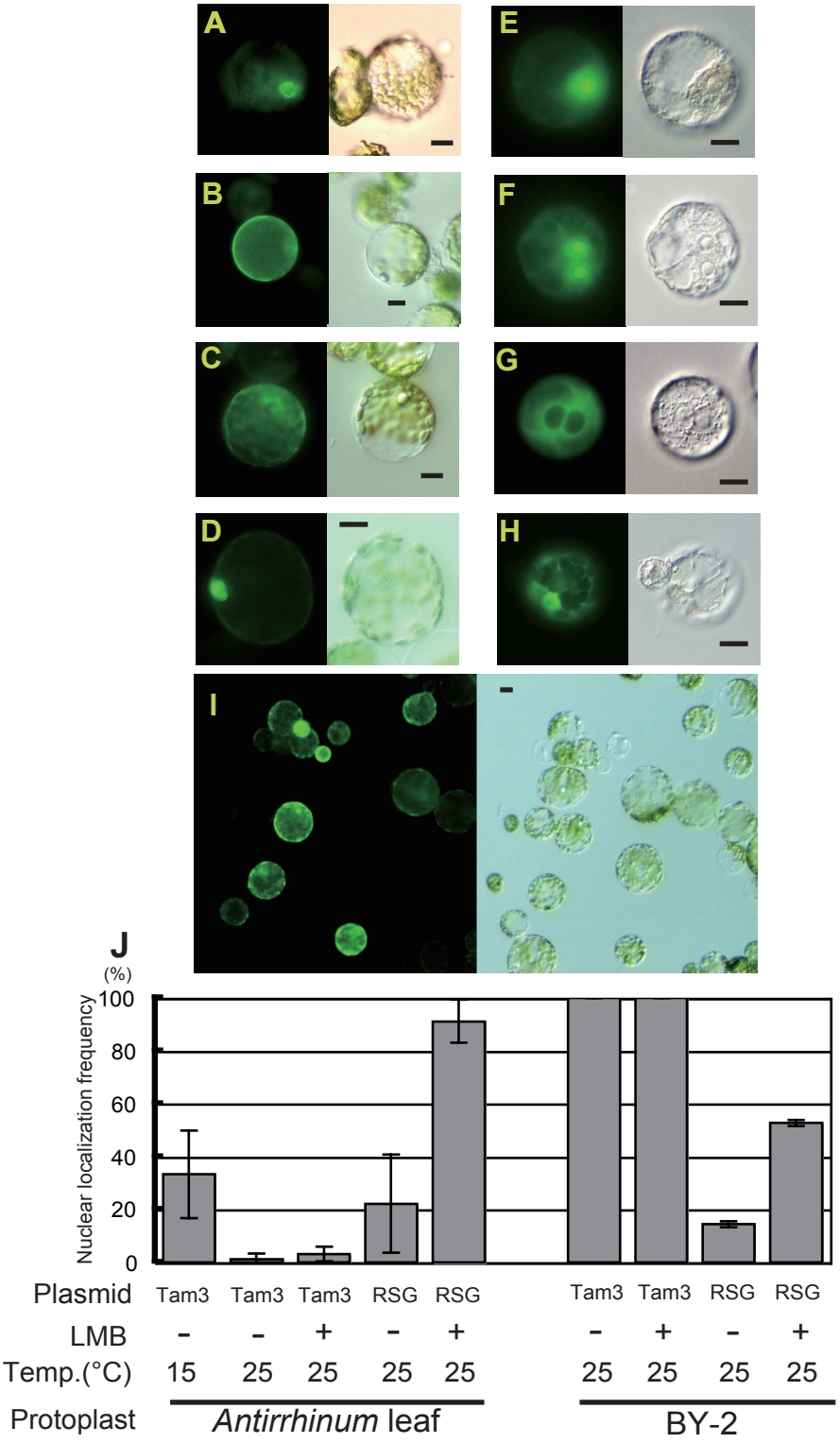

A
25°C
15°C
B
25℃ 15℃
100 kD –
Anti-Tam3-TPase

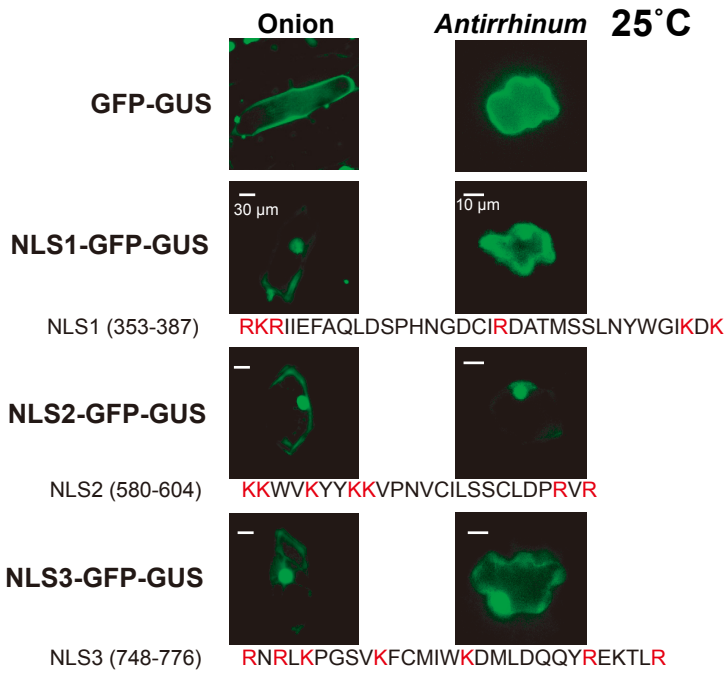
Fujino Fig. 2

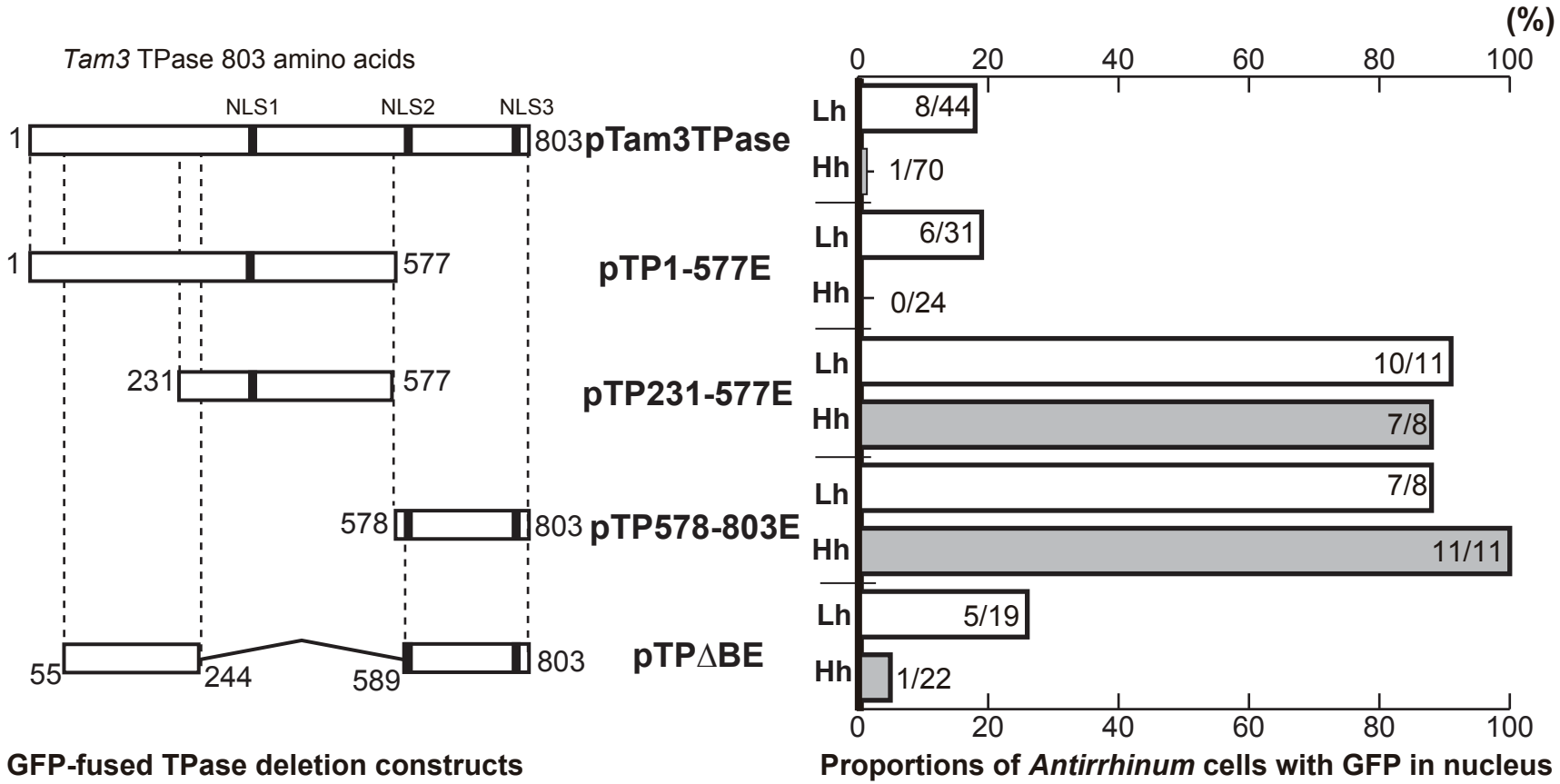

GFP
Nucleus
Tam3 TPase-GFP
Nucleus
Cytoplasm
Growth Periods
3 w
24 h
25˚C
15˚C
Hl
Ll
Hh
Lh
N.D.
N.D.
1/70
8/44
0
5
10
15
20
25
Plasmid introduction
Nuclear localization frequency(%)


A
P35S
B1
Tam3 TPase
B2
sGFP
TNO
pGWB5-Tam3
P35S
R1
ccdB
R2
sGFP
TNO
pGWB5
B
GFP
DAPI
pGWB5-Tam3
15°C
25°C
pGWB5
15°C
25°C
C
pGWB5-Tam3
pGWB5
Number of cells
NCG ratio
0.8~
1.0~
1.2~
1.4~
1.6~
1.8~
2.0~
2.2~
2.4~
2.6~


A
Effector constructs
CaMV 35S
Ω
TNOS
pGATam3
GAL4 BD + VP16
Tam3 TPase
pGAGUS
GAL4 BD + VP16
GUS
Reporter construct
pGBL
TATA
CaMV 35S
Renilla LUC
TNOS
6 x GAL4 binding motif
Firefly LUC
TNOS
B
pGATam3 + pGBL
25°C
11.2 ± 10.4 n=10
15°C
56.8 ± 26.5 n=10
*
pGAGUS + pGBL
25°C
197.7 ± 130.6 n=11
15°C
274.9 ± 85.3 n=11
**
pGBL
25°C
1.35 ± 0.59 n=10
15°C
8.79 ± 3.68 n=10
100
200
300
400
LUC ratio: Firefly LUC / Renilla LUC (× 1000)


A
B
C
D
E
F
G
H
I
J
(%)
Nuclear localization frequency
100
80
60
40
20
0
Plasmid
Tam3 Tam3 Tam3 RSG RSG Tam3 Tam3 RSG RSG
LMB
- - + - + - + - +
Temp.(°C)
15 25 25 25 25 25 25 25 25
Protoplast
Antirrhinum leaf
BY-2


Onion
Antirrhinum
25˚C
GFP-GUS
30 µm
10 µm
NLS1-GFP-GUS
NLS1 (353-387) RKRIIEFAQLDSPHNGDCIRDATMSSLNYWGIKDK
NLS2-GFP-GUS
NLS2 (580-604) KKWVKYYKKVPNVCILSSCLDPRVR
NLS3-GFP-GUS
NLS3 (748-776) RNRLKPGSVKFCMIWKDMLDQQYREKTLR


Tam3 TPase 803 amino acids
NLS1
NLS2
NLS3
1
803
pTam3TPase
1
577
pTP1-577E
231
577
pTP231-577E
578
803
pTP578-803E
55
244
589
803
pTPΔBE
(%)
0
20
40
60
80
100
Lh
Hh
8/44
1/70
6/31
0/24
10/11
7/8
7/8
11/11
5/19
1/22
GFP-fused TPase deletion constructs
Proportions of *Antirrhinum* cells with GFP in nucleus