Subcellular Distribution of NTL Transcription Factors in *Arabidopsis thaliana*

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Abbreviations: NTL, NAC with transmembrane motif1-like; ND, N-terminal NAC domain; RR, middle regulation region; TM, C-terminal transmembrane domain.

Brief Synosis

NTLs are a class of NAC transcription factors containing a transmembrane domain at their C-terminus. Using a transient transformation approach in *Arabidopsis* mesophyll protoplasts we showed that twelve NTLs, with the exception of NTL11 and NTL5, are anchored to the ER membrane. Furthermore, the N-terminal NAC domain and the regulation region of NTLs were proved to be responsible for NTLs entering the nucleus. These results indicated that proteolytic activation of NTLs is a regulatory scheme active in plants during various stress situations.

Summary

NTL transcription factors, containing three regions: the N-terminal NAC domain (ND), the middle regulation region (RR), and the C-terminal transmembrane domain (TM), belong to the tail-anchored proteins. Although these NTLs play numerous essential roles in plants, their subcellular distribution and the mechanism of translocation into the nucleus remain unclear. In this study, we found that most of the full-length NTLs were localised in the endoplasmic reticulum (ER), with the exception of NTL11 and NTL5, which were restricted to the nucleus. Furthermore, we found that NTL11 contains a TM, whereas NTL5 does not. The ND of all of the NTLs was responsible for nuclear localisation in plants. After truncation of the TM, NTL8_NR, NTL10_NR and NTL13_NR localised in the cytoplasm and nucleus, and other NTL_NRs were only in the nucleus, suggesting that the RR of NTL8, NTL10 and NTL13 contains some inhibitory region to mask the NLS sequence in the ND domain and permit their diffusion between in cytoplasm and nucleus. Furthermore, the N-terminus of NTL11 was translocated to the nucleus, but the C-terminal was degraded in *Arabidopsis* mesophyll protoplasts. The chimeric construct of NTL11_ND with NTL10_RR and TM (11ND-10RT) was localised exclusively in the ER, and not in the nucleus. However, 10ND-11RT was found mainly in the nucleus. Our results indicated that the TM is essential for NTL targeting the ER and the N-terminal fragment, including ND and RR, is translocated into the nucleus after activation through proteolytic cleavage events upon stimulation by internal and external environmental signals.

Introduction

Tail-anchored (TA) proteins, a class of polypeptides integrated into the membrane by a single transmembrane domain (TM) at their C-terminus, are present in all three domains of life (1). They are found on all cellular membranes with the N-terminal functional domain facing the cytosol and participate in remarkably diverse cellular processes, such as vesicle trafficking, redox reactions, protein translocation, signal transduction, regulation of transcription, and apoptosis (2-4). The C-terminal TM, which is located at approximately 30 residues from the C-terminus, constitutes the only membrane-targeting sequence and emerges from the ribosome tunnel only after termination of translation. The TA proteins are inserted into all target membranes: endoplasmic reticulum (ER), outer mitochondrial, outer chloroplast, peroxisomal, and prokaryotic cytoplasmic membranes, through post-translational pathways (5, 6). Membranes delimiting other compartments of the secretory pathway, including the Golgi complex, plasma membrane, vacuoles, endosomes, and lysosomes can obtain TA proteins from the ER by vesicular transport (7-10).

Based on the structural definition of the C-terminal TM, over 500 TA proteins have been identified in the *Arabidopsis* genome via web-based TM prediction programs such as TMHMM and THMMPRED (1, 3, 5). Notably, an interesting group of putative TA proteins are considered to be transcription factors. Membrane-bound transcription factors (MTFs) have been found in prokaryotes, yeast, animals, and plants (11-13). Upon stimulation by developmental and external signals, dormant MTFs are activated either by regulated intramembrane proteolysis (RIP) or regulated ubiquitin/proteasome-dependent processing (RUP), and the transcriptional active MTF forms are translocated into the nucleus to regulate the expression of target genes (14). A previous survey identified 85 MTFs, which generally possess either one or three TMs and are released from the membrane by regulated proteolysis to permit their relocation to the nucleus in *Arabidopsis* (15). AtbZIP60 was the first plant MTF to be functionally studied (16). ER stress triggers activation of the ER membrane-associated AtbZIP60 protein, which is mediated by proteolysis (17). Therefore, signal transduction across intracellular membrane systems, such as the ER membrane, appears to require less intracellular mediators in plants than animals because of the high availability of TFs, which are directly bound to the membrane (1).

A group of MTFs showing structures similar to TA proteins contains about 30 members, many of which fall into the NAC group (NAM, ATAF and CUC) and are named NAC with transmembrane motif1-like (NTL) proteins in plants. About 13 NTLs were predicted using the ARAMEMNON protein database in *Arabidopsis* (18). Typically, NTL proteins share a conserved N-terminal NAC domain (ND), the diversified middle transcription regulatory region (RR), and the C-terminal TM domain (TM). Previous studies showed that NTL12/NTM1 resides on the ER membrane (19). When the tail anchor of NTL12 is removed by proteolysis, the cytosolic portion, containing the NAC domain, enters the nucleus and activates genes involved in cellular division (19). Ng *et al* reported that activation of NTL7/ANAC017 occurs via proteolytic activation by a rhomboid protease, allowing the N-terminus of NTL7 to migrate from the ER membrane to the nucleus. The C-terminal TM remained in the ER upon antimycin A treatment (20). Similar to NTL7, NTL1/ANAC013 is also targeted to the ER and characterised as a regulator of mitochondrial retrograde regulation in response to stress in *Arabidopsis* (21). ANAC089 was also reported to be a NTL protein and named as NTL14, which

was released from ER membrane upon treatment with reducing agents or ER stress conditions and targeted to the nucleus (22-24). In addition, the membrane-associated NTL4, NTL6, NTL8, NTL9, and NTL13/NTM2 were characterised to link external signals, such as salt, cold, and osmotic stress, to plant growth and development (15, 25-28). Although the functions of most NTLs have been elucidated, our current understanding of their subcellular distribution and translocation into the nucleus in plants is limited.

Here, we employed a transient transformation approach to characterise the subcellular localisation of 14 NTLs in *Arabidopsis* mesophyll protoplasts. Twelve NTLs, with the exception of NTL11 and NTL5, were anchored to the ER membrane. Furthermore, we showed that the N-terminal NAC domain and the regulation region of NTLs are responsible for NTLs entering the nucleus. These results indicated that proteolytic activation of NTLs is a regulatory scheme active in plants during various stress situations.

Results

Full-length NTLs are localised in the ER membrane, but not in the plasma membrane

NTLs are TA proteins that do not contain any signal peptides, but contain a single transmembrane domain (TM) at the C-terminus (Figure S1). To investigate the overall subcellular membrane distribution of the 14 NTLs, the different truncated forms of NTLs fused with yellow fluorescent protein (eYFP) were generated (Figure 1) and transiently co-transformed with mCherry-labelled marker constructs in the *Arabidopsis* mesophyll protoplasts. The full-size NTLs were dispersed in the cytoplasm and mainly co-localised with the ER marker. Exceptions were NTL5 and NTL11, which were found exclusively in the nucleus (Figure 2). Comparing the cDNA sequence of cloned NTL5 with the predicted sequence in The *Arabidopsis* Information Resource (http://www.arabidopsis.org/), we found that NTL5 lacks the C-terminal TM domain and is not a TA protein, as its 3' splice site of the third intron in pre-mRNA is at the -20 bp of the predicted position which result in early termination. This result is same as previous reports from two different groups (29, 30). We further analysed the subcellular localisation of NTL11 in more detail in two ways: eYFP-NTL11 was co-localised with the mCherry-labelled nucleus marker (NU) and the C terminal of ~60 aa containing the TM domain fused with a eYFP was overlapped with the ER marker, but not with other organelle markers including the cytoplasm (CT), plasma membrane (PM), Golgi (G), mitochondria (MT), peroxisome (PX) and plastids (PT) (Figure 3). These results suggest that NTL11 is indeed a TA protein, and the C-terminal tail anchor of NTL11 is possibly removed by proteolysis during protoplast culture, releasing the N-terminal portion to migrate into the nucleus. In order to eliminate the cross color from mCherry to eYFP when co-transformed eYFP-NTLs_FL and mCherry-labelled ER marker in the *Arabidopsis* mesophyll protoplasts, we transformed only the eYFP-NTLs_FL construct without any marker and found that the localization of the full-size NTLs is same as co-transformation experiment (Figure S2).

Previous studies have shown that several NTLs, including NTL4 (23), NTL6 (25), NTL8 (26), NTL9 (27), and NTL13 (28), are located at the PM. We performed additional experiments to confirm our results. First, these NTL constructs fused with eYFP were transiently co-transformed with mCherry-labelled PM marker in the *Arabidopsis* mesophyll protoplasts. We found that these five NTLs were not co-localised with the PM marker (Figure 4A). Next, we analysed the subcellular localisation of the C terminus of ~60 aa containing the TM domain of these five NTLs, because Ng *et al* demonstrated that the TM region is critical for localisation of NTL7 into the membrane of the ER (20). The results showed that the TM regions of these NTLs perfectly co-localised with the ER marker (Figure 4B). Taken together, these results proved that all NTLs are post-translationally localised at the ER membrane by the TM regions.

The TM domain is critical for NTLs to be localised in the ER membrane

To further characterise the role of TM in the subcellular localisation of NTLs, we analysed the subcellular distribution pattern of the truncated NTL forms containing the N-terminal ND and the middle RR in the *Arabidopsis* mesophyll protoplast. The eYFP fluorescence of NTL_NRs was only detected in the nucleus, with the exception of NTL8_NR, NTL10_NR, and NTL13_NR, which were located in the cytoplasm and nucleus together, overlapping with the CT marker, but not with the ER marker (Figure 5, Figure S3). These results suggest that the TM is critical for NTL transcription

factors targeted to the ER membrane, and if lost, the NTLs cannot be inserted into the ER membrane. Next, we constructed the eYFP-NTLs_RR vectors containing only the middle RR of NTL8, NTL10 and NTL13 to investigate the subcellular localisation of NTLs_RR. We found NTL8_RR, NTL10_RR and NTL13_RR to be localised in the cytoplasm and nucleus and perfectly merged with the CT marker, but not the ER marker (Figure S4), which is similar to the localization of NTL8_NR, NTL10_NR and NTL13_NR. These results indicated that the RR of NTL8, NTL10 and NTL13 does not contain any NLS signal, but has some inhibitory region to mask the NLS sequence in the ND of NTL8, NTL10 and NTL13 and permit their diffusion between in cytoplasm and nucleus.

N-terminal NAC domain of NTLs is responsible for localisation in the nucleus

Previous studies have indicated that the N-terminal ND of NTLs is the DNA-binding domain, which contains the nuclear localisation signal (NLS) and is responsible for NTL localisation in the nucleus (31). We used the NLStradamus program (32) to predict the NLS sequence in NTLs and found that all NTLs have the NLS sequence in the ND domain. In addition, NTL1 and NTL9 possess another NLS sequence in the RR region close to TM (Figure S1). Furthermore, we investigated the subcellular distribution of the truncated NTLs containing only the ND domain (NTLs_ND) in the *Arabidopsis* mesophyll protoplast . As anticipated, when the RR and TM regions of NTLs were removed, all NTLs_ND were completely localised in the nucleus (Figure 6).

The TM of NTL11 is removed by proteolysis in the *Arabidopsis* protoplast

In this study, we found that full-length NTL11 is localised in the nucleus when transiently expressed in the *Arabidopsis* mesophyll protoplast (Figure 2). However, NTL11 is a TA protein and has a potential TM motif, which is responsible for NTL11 localisation on the ER membrane (Figure 3). Therefore, we hypothesised that NTL11 was expressed as a membrane-associated, dormant form, then triggered by a proteolysis event in protoplast culture, and was rapidly released from the membranes and localised in the nucleus. To support this hypothesis, the new constructs which fused the eYFP to the N-terminal and the mCherry to the C-terminal of NTL11_FL and NTL11_NR was designed and expressed in the *Arabidopsis* mesophyll protoplast. And the same constructs of NTL10_FL and NTL10_NR were as a control. We observed eYFP fluorescence of NTL11_FL localised in the nucleus, but no mCherry was detected. In contrast, the eYFP and mCherry of NTL10_FL were co-localised in the ER membrane (Figure 7A). After the TM of NTL11 and NTL10 were deleted, the eYFP and mCherry of NTL11_NR were co-localised in the nucleus, and NTL10_NR in the cytoplasm and nucleus (Figure 7B). We also generated the transgenic *Arabidopsis* plant overexpressing eYFP-NTL11_FL-mCherry drived by CaMV 35S promoter. Through observing the subcellular localisation of NTL11_FL in the leaves of transgenic plants, we found that the N-terminal and C-terminal fluorescence both localised on the ER membrane (Figure 7C). These results suggested that the RR region of NTL11 has some sites recognised and cleaved by proteolysis under protoplast culture conditions, after which the N-terminus was released from ER membrane and translocated into the nucleus and the C-terminus was degraded through another undiscovered mechanism.

To further confirm this hypothesis, the chimeric constructs 11ND-10RT and 10ND-11RT were obtained by exchanging the middle RR and C-terminal TM of NTL10 and NTL11 to each other's N-terminal NAC domain and transiently transformed the *Arabidopsis* mesophyll protoplast (Figure

8A). It was interesting to find that 10ND-11RT was detected mainly within the nucleus, similar to the localisation pattern of full-size NTL11 (Figure 8B). However, 11ND-10RT was densely localised to the ER membrane, similar to NTL10 (Figure 8C). Therefore, it was verified that NTL11, similar to other NTLs, is associated with the ER membranes, from which the C-terminal TM is removed and the N-terminus is translocated in the nucleus to play the role of a transcription factor depending on some elusive signal related to the environment and plant development.

Discussion

NTL transcription factors are one subgroup of NAC family characterized with the C-terminal TM domain. Previous studies showed that the transgenic plants over-expressing partial-size NTL constructs devoid of the TMs, or similar constitutively expressing T-DNA mutants, but not those over-expressing full-size constructs, showed distinct dominant-negative phenotypes. For example, dysfunction of NTL7 resulted in significantly reduced drought/moderate light stress tolerance (20), and 35S:NTL4-ΔC transgenic plants over-expressing an active form of NTL4 showed the phenotype of accelerated leaf senescence under drought conditions (33). Li *et al* (34) found that the truncated NTL14 lacking a membrane association domain will be an active form of transcription factor to be responsible for the physiological function in flowering time control. So, there will be a valuable topic to disect the localization and translocation mechanism of NTLs in *Arabidopsis*. We have presented evidence that NTLs are a group of posttranslationally activated transcription factors localised in the ER membrane and possibly translocated to the nucleus after proteolysis in response to internal and environmental signals in *Arabidopsis*. The localisation of different truncated NLT forms expressed in the *Arabidopsis* mesophyll protoplast showed that the C-terminal TM is essential for targeting of 13 NTLs to the ER, the N-terminal ND is sufficient for localisation to the nucleus, and the middle RR may contain some modification sites related to proteolytic cleavage and migration to the nucleus (Table S8). Our results increase our understanding of the subcellular distribution and biological function of NTLs in *Arabidopsis*.

Generally, a plant cell has a very large central vacuole and many chloroplasts, and sometimes the ER is clustered with the cytomembrane at some regions of the cell or huddled at the perphery region of nucleus. In fact, the ER membrane could form some linkage with the plasma membrane so that the ER could directly connect with the outside of the cell (35). Thus, it is hard to distinguish the ER and plasma membrane in the periphery region of cell if there is no co-transformed marker to display the localisation of membrane protein. Previous studies showed that full-size NTL8 and NTL9 are membrane-bound transcription factors when transiently expressed in onion epidermal cells (26, 27), and NTL4, NTL6 and NTL13 are present in the plasma membrane of *Arabidopsis* protoplasts (23, 25, 28). A common issue in these research works is no any marker to delimit the plasma membrane. In our study, we used mCherry-labelled markers to distinguish between the ER and PM membrane, and confirmed that all 13 NTLs localised to the ER membrane through the C-terminal TM after transiently expressed in the *Arabidopsis* mesophyll protoplast. We further proved NTL11 is localized in the ER membrane when stably expressed in transgenic *Arabidopsis* plants. However, we must not exculde the possibility that one or several NTLs reach the plasma membrane or other compartments along the secretory pathway under some kinds of physiological conditions.

Previous study proved that smaller non-nuclear proteins, such as eYFP and mCherry without the localisation signals, are able to enter the nucleus and equilibrate between the nucleus and cytoplasm, while molecules larger than a certain size (approximately 60 KDa) are excluded, which indicated that the nuclear envelope is not a barrier to small proteins but restricts access of large proteins (36). In this study, we found NTL8_ND, NTL10_ND and NTL13_ND completely localised in the nucleus. However, NTL8_NR, NTL10_NR and NTL13_NR were localised in the cytoplasm and

nucleus and perfectly merged with the mCherry-labelled CT marker, which is similar to the localization of NTL8_RR, NTL10_RR and NTL13_RR. These results indicated that the RR of NTL8, NTL10 and NTL13 contain no NLS signal, but the inhibitory region to mask the NLS sequence in the ND of NTL8, NTL10 and NTL13 and permit their free diffusion between the cytoplasm and nucleus, which resembles the mechanism of the autoinhibitory region of calcium-dependent protein kinases to keep the whole enzyme in an inactive mode by restricting the substrate access to the kinase catalytic center (37).

NTL11 was previously be shown to up-regulate the expression of genes related to flavonoid biosynthesis, leading to the accumulation of anthocyanins in response to high light stress (38). In this paper, we confirmed that NTL11 containing the ER-targeted C-terminal TM domain was localised to the nucleus in *Arabidopsis* mesophyll protoplasts, which suggested that NTL11_FL first targets the ER after translation and the transcriptionally active N-terminus NTL11-NR is translocated into the nucleus after cleavage of the C-terminus TM by either RIP or RUP pathway, due to the culture condition during the transformation of *Arabidopsis* mesophyll protoplast cells. This also indicated that the middle RR of NTL11 contained some unique sites, which could be recognised, modified, and cleaved to release the N-terminal ND for migration into the nucleus. We also presumed that the ND of NTLs is responsible for transportation to the nucleus and is independent of any modification of the middle RR. Localisation of the double fluorescence construct of NTL11 and the chimeric constructs 11ND-10RT and 10ND-11RT after transient expression in *Arabidopsis* mesophyll protoplast supported this hypothesis.

The NTL transcription factors are known to possess two distinct localisation signal sequences. One is a nuclear localisation signal related to their transactivation function, while the other is a transmembrane domain responsible for targeting the organelle membrane. To date, most studies have explored the molecular mechanism underlying translocation of the N-terminal portion to the nucleus after cleavage of the C-terminal TM by proteolysis and the biological function of NTLs. However, the mechanism of NTLs targeting the ER membrane remains unclear. Previous studies described the insertion of TA proteins into the ER membrane by two chaperone-mediated pathways: one is mediated by a novel ATPase identified as TRC40/Asna1 or the Get3 complex in mammals and yeast, respectively (39-41). The second is mediated by the cytosolic chaperone complex Hsp40/Hsc70 (5, 6, 9, 42). Thus, identifying the chaperone and receptor responsible for NTL targeting of the ER membrane, characterising proteases related to NTL processing, and determining the effects of internal and external signals is important for future studies.

Materials and Methods

Plant materials and growth conditions

All plant materials were grown in growth chambers at 22□ with a relative humidity of 55% under 12 h 120 μmol m^{-2} s^{-1} light /12 h dark. Seeds were germinated on agar plates containing half-strength Murashige and Skoog (MS) salts, 1% sucrose, 0.75% (w/v) agar, pH 5.7, after stratification at 4□ for at least 2 days before being transferred to growth chambers. The eYFP-NTL11-mCherry overexpressing plants drived by CaMV 35S promoter were transformed into *Arabidopsis* Col-0 by Agrobacterium tumefaciens–mediated floral dipping methods (43).

RNA extraction from *Arabidopsis* leaves

Total RNA from *Arabidopsis* was extracted from *Arabidopsis* leaves using TRIzol Reagent according to the manufacturer's (Invitrogen, USA) instructions and quantified using a Nanodrop ND-1000 spectrophotometer (LabTech, USA). Total RNA (5 μg) was used for cDNA synthesis with the Revert-Aid First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions.

Cloning of NTLs and DNA construction

The CDS of *NTLs* was PCR amplified, separately digested with *BamH*I and *Not*I (for *NTL1, 2, 3, 5, 8, 9, 10* and *14*) or with *Xma*I and *Asc*I (for *NTL4, 6, 7, 11, 12* and *13*) and cloned into pE6n (addgene, USA) or pE6nXA (reformed by pE6n, the *BamH*I and *Not*I sites were replaced by *Xma*I and *Asc*I sites). The primers used to clone the *NTLs* are listed in Table S1.

To generate the various truncated NTL constructs, such as NTL_TMs (containing only TM), NTL_NRs (containing ND and RR), NTL_NDs (containing only ND), and NTL_RRs (containing only RR), the corresponding NTL fragments were amplified with the primers listed in Table S2-S5 and ligated into pE6n or pE6nXA. Construct maps containing different parts of NTLs are shown in Figure 1.

To produce the double fluorescence tagged vectors, NTL10_FL, NTL11_FL, NTL10_NR, and NTL11_NR were subcloned into the pE6n-mC with the primers listed in Table S6. The pE6n-mC was reformed by the pE6nXA by fusing the mCherry coding sequence (from ER marker) into the downstream of MCS.

To construct the cross-over vectors between NTL10 and NTL11, the ND of NTL10 was fused to the NTL11 C-terminal region of RT (containing RR and TM) and the ND of NTL11 with RT of NTL10 using two-step PCR methods with the primers listed in Table S7. The fragments of 10ND-11RT and 11ND-10RT were cloned into pE6nXA (Figure 8A).

The pE6n, pE6nXA, or pE6nXA-mC constructs containing different NTL fragments were used in combination with the destination vector pMDC32 (http://www.arabidopsis.org/) with the Gateway LR II kit (Invitrogen, USA) to generate all of the plant expression vectors used in *Arabidopsis* mesophyll protoplast transfection. All vectors were confirmed by sequencing.

Generation of organelle marker lines

The ER marker (ER-rk, *CD3-959*), Golgi marker (G-rk, *CD3-967*), mitochondria marker (MT-rk, *CD3-991*), peroxisomes marker (px-rk, *CD3-983*) and plastids marker (pt-rk, *CD3-999*) were purchased from the Arabidopsis stock centre (http://www.arabidopsis.org). The nucleus marker (NU) was constructed by fusing the N-terminal 158 amino acids of AtBZR2 (*ARABIDOPSIS*

BRASSINAZOLE-RESISTANT 2) (44) to the N-terminus of mCherry under control of double 35S promoter in a pMDC32 vector. The plasma membrane (PM) marker was constructed by fusing the N-terminal 120 amino acids of AtCAN2 (*ARABIDOPSIS* CALCIUM DEPENDENT NUCLEASE 2) (45) to the N-terminus of mCherry under control of a double 35S promoter in a pMDC32 vector. The mCherry under control of a double 35S promoter in pMDC32 vector was the the cytoplasm marker (CT). All vectors were confirmed by sequencing.

Subcellular localisation of the NTL proteins in *Arabidopsis* mesophyll protoplasts

The vectors containing eYFP-NTLs and a marker gene were co-transformed and transiently expressed in *Arabidopsis* mesophyll protoplasts, as described previously (46, 47). After incubation for 16 h at 22□ in the dark, fluorescence was visualised using a Zeiss LSM 700 confocal microscope. Observations were made using a ×63 objective under oil immersion. eYFP fluorescence was excited at 488 nm and collected at SP 550 IR. The chloroplast autofluorescence was also excited at 488 nm but collected at LP 640 IR. The mCherry fluorescence was excited at 555 nm and collected at SP 630 IR. The pinholes was about 1.0 unit and the thickness of optical section was about 0.5 μm. The different organelles were identified using mCherry-labelled markers as a control.

Multiple sequence alignment of NTLs

The protein sequence of 13 NTLs were analysed using the DNAMAN software and modified manually. The NAC domain was characterized by NCBI server, the NLS sequence was predicted with the NLStradamus program (32), and the TM domains were annotated according to TMHMM Server v. 2.0 prediction (http://www.cbs.dtu.dk/services/TMHMM/).

Acknowledgments

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Figure Legends

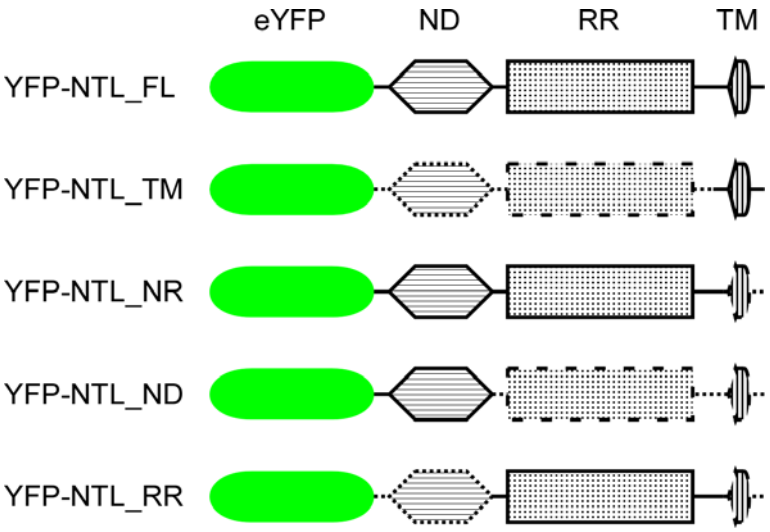

Figure 1. Schematic of different eYFP-NTL constructs. Fusion proteins were constructed, consisting of full-length and different truncated NTL forms with eYFP (rectangles) fused to the N-terminal region. Highly conserved NAC domains (ND) are present in their N-terminal regions (hexagon boxes), the regulation regions (RR) are localised in the middle (ellipse boxes), and the α-helical transmembrane domains (TM) are located in their distal C-terminal regions (wavy boxes). Dashed line around the box indicates that this box was removed in the fusion construct.

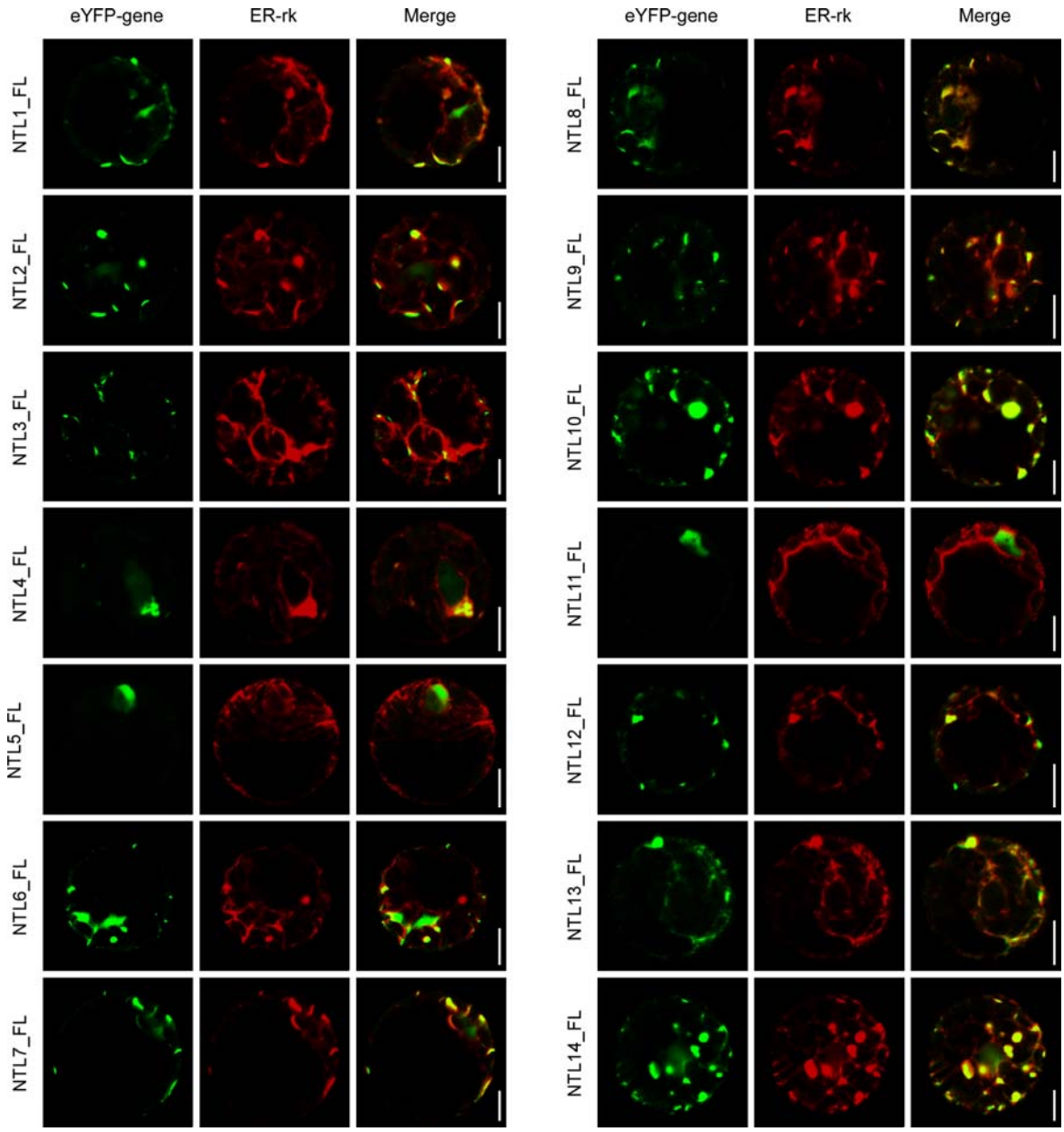

Figure 2. Subcellular localisation of full-length NTLs. Localisation of eYFP-NTLs_FL and mCherry-labelled ER marker after transient co-transformation in *Arabidopsis* mesophyll cells. All images in this figure were obtained from one optic section. Scale bars are equivalent to 10 µm.

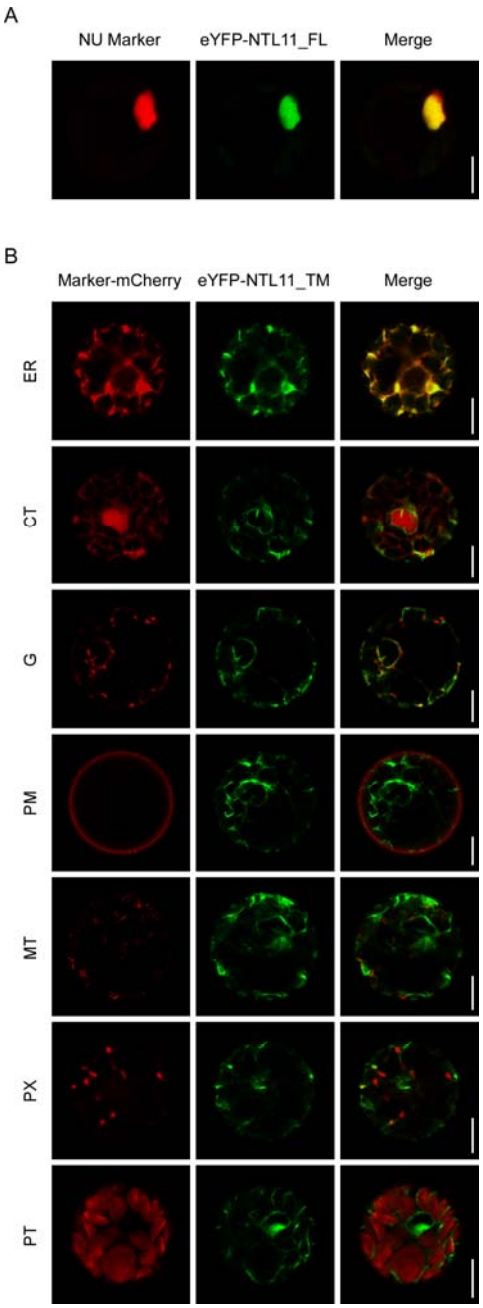

Figure 3. Co-localisation of NTL11_FL with the mCherry-labelled NU marker in *Arabidopsis* mesophyll cells. (A) Localisation of eYFP-NTL11_FL and mCherry-labelled NU marker after transient co-transformation in *Arabidopsis* mesophyll cells. (B) Localisation of NTL11_TM and several mCherry-labelled organelle markers in *Arabidopsis* mesophyll cells, respectively. All images in this figure were obtained from one optic section. Scale bars are equivalent to 10 μm.

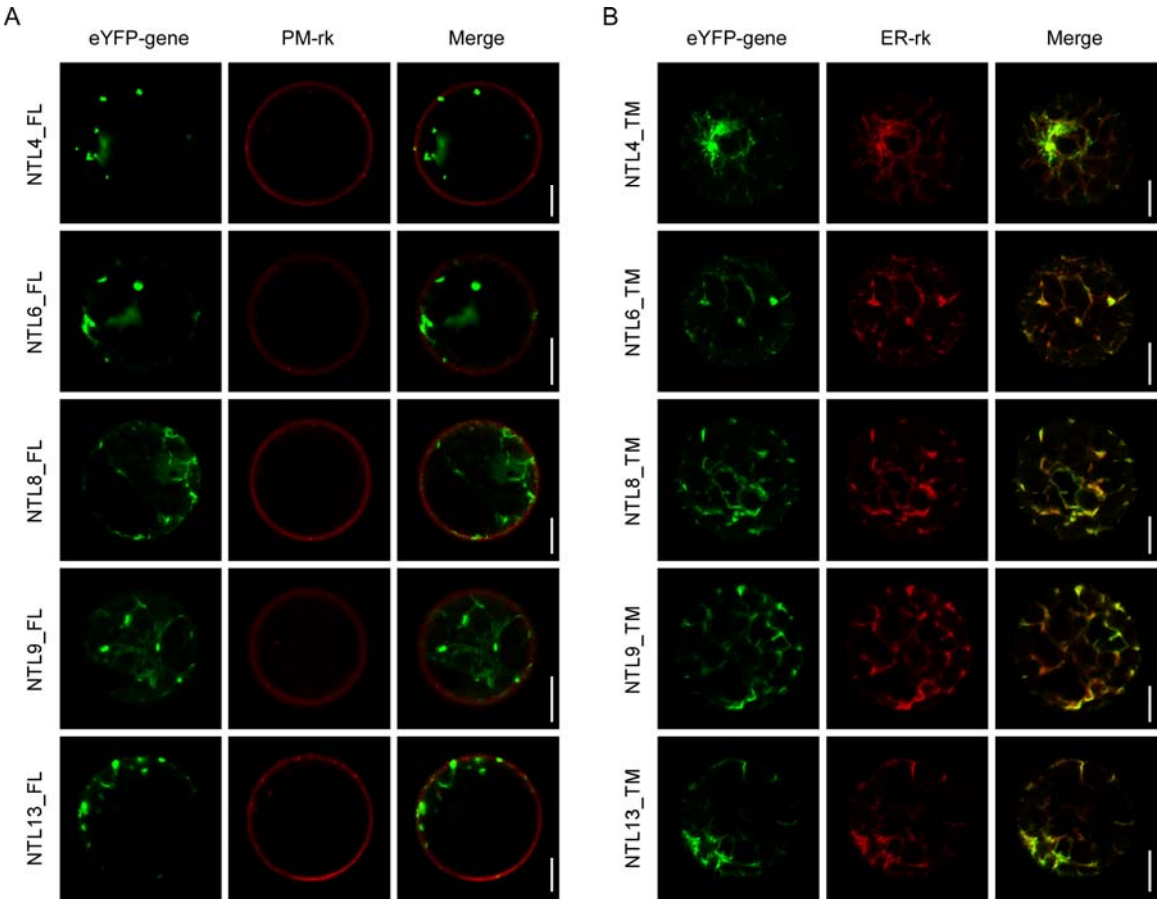

Figure 4. Subcellular localisation of five NTLs with PM marker and their TM region with ER marker. (A) Localisation of five eYFP-NTLs_FL and mCherry-labelled PM marker or (B) eYFP-NTLs_TM and mCherry-labelled ER marker in *Arabidopsis* mesophyll cells. All images in this figure were obtained from one optic section. Scale bars are equivalent to 10 μm.

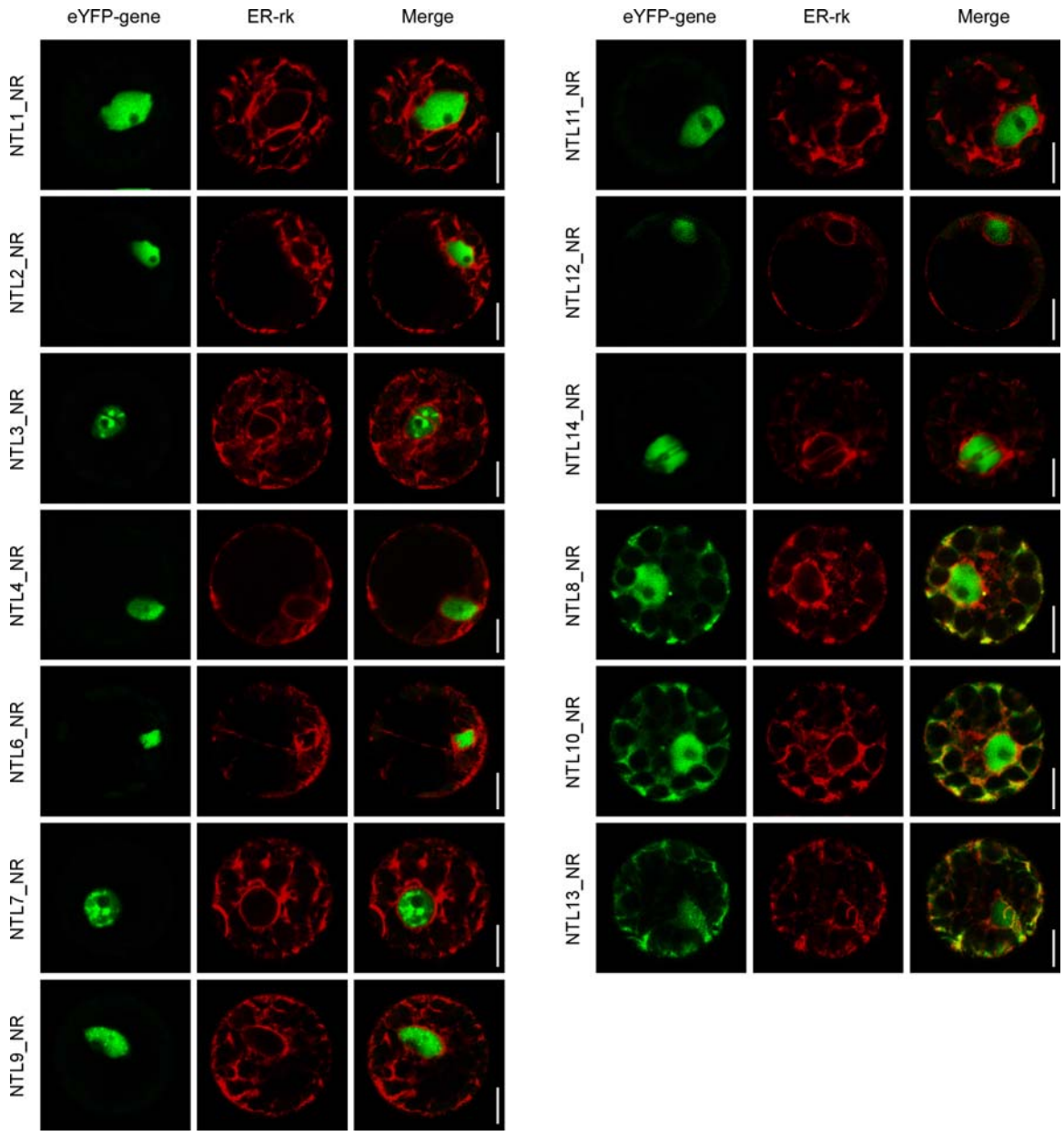

Figure 5. Subcellular localisation of NTLs_NR. Localisation of truncated eYFP-NTL_NRs lacking the C-terminal TM and mCherry-labelled ER marker in *Arabidopsis* mesophyll cells. All images in this figure were composed of one optic section. Scale bars are equivalent to 10 µm.

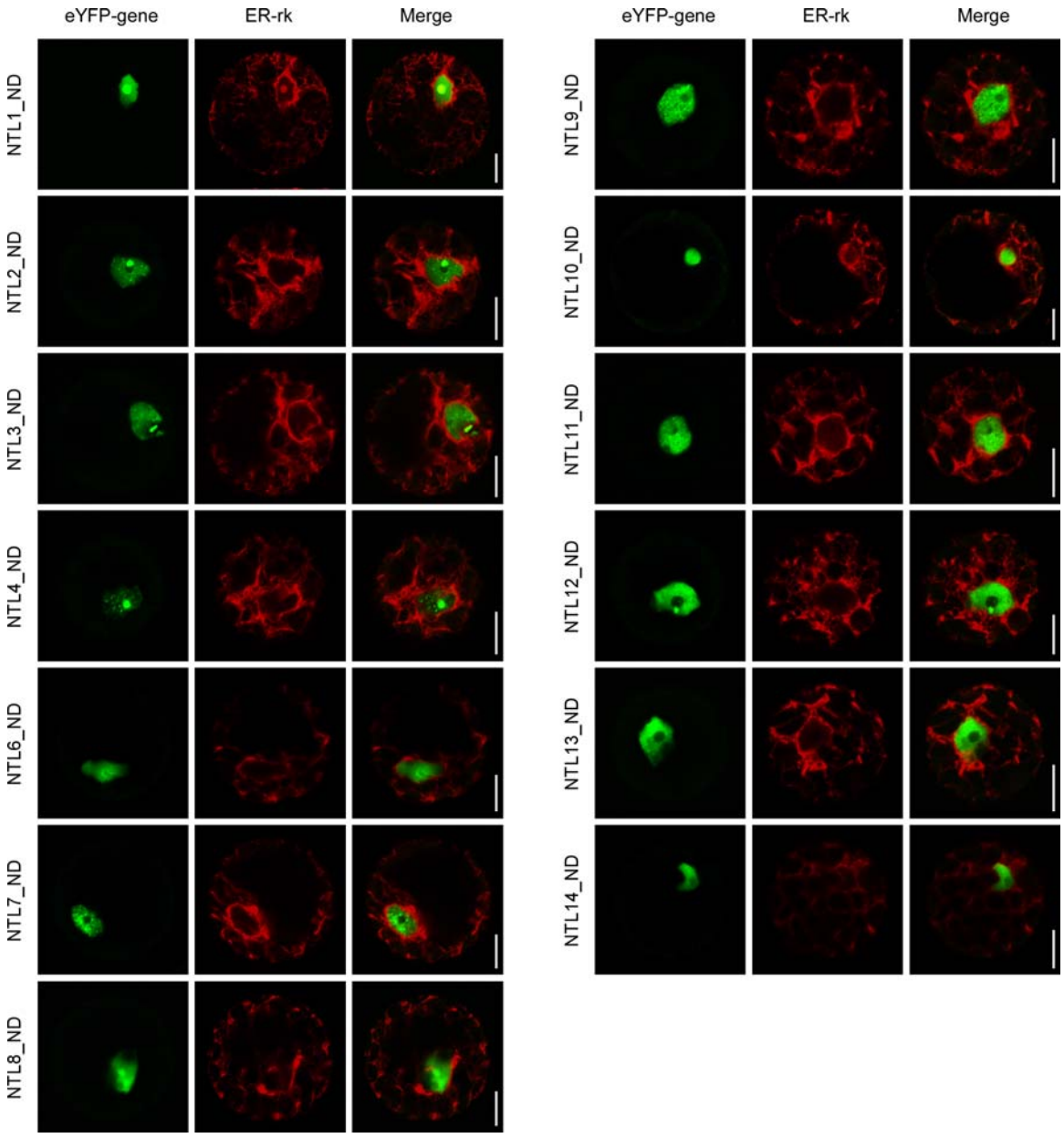

Figure 6. Subcellular localisation of NTLs_ND. Localisation of the isolated ND of 12 eYFP-NTLs_ND and ER-mCherry in *Arabidopsis* mesophyll cells. All of the images in this figure were obtained from one optic section. Scale bars are equivalent to 10 μm.

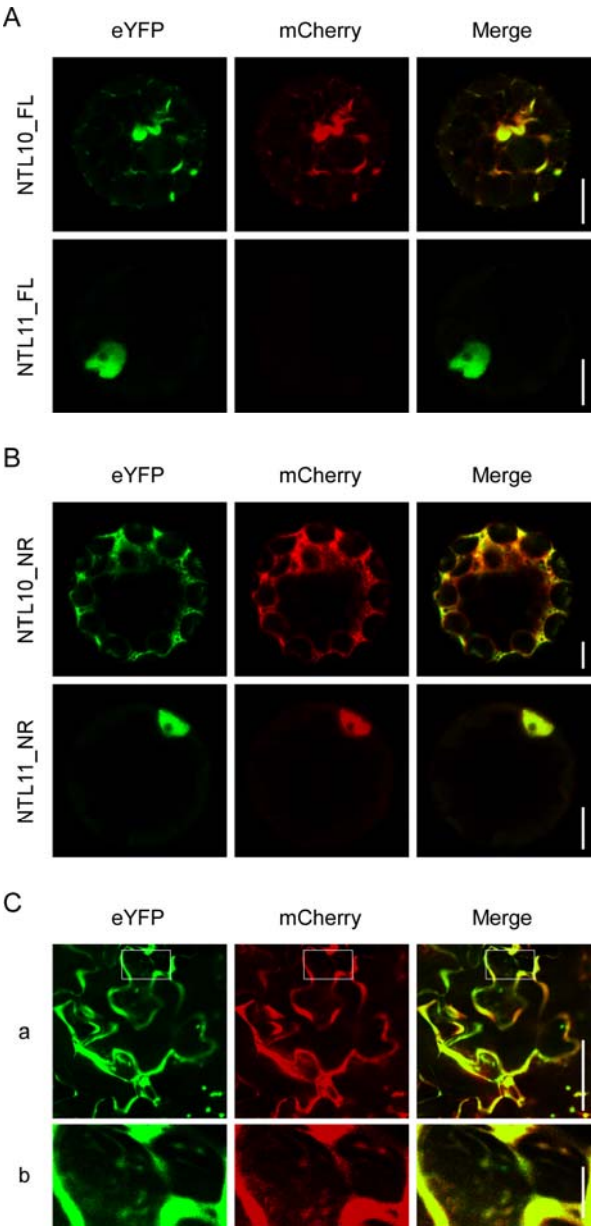

Figure 7. Subcellular localizations of NTL10 and NTL11 labelled with different fluorescence proteins at the N and C terminals. (A) Localisations of eYFP-NTL10_FL-mCherry and eYFP-NTL11_FL-mCherry in *Arabidopsis* mesophyll cells. Scale bars are equivalent to 10 μm. (B) Localisations of eYFP-NTL10_NR-mCherry and eYFP-NTL11_NR-mCherry in *Arabidopsis* mesophyll cells. Scale bars are equivalent to 10 μm. (C) Localisation of the eYFP-NTL11_FL-mCherry (a) in transgenic *Arabidopsis* leaves. Image b is the magnification of the selected areas in image a. Scale bars are equivalent to 50 μm in image a and 10 μm in b. All images in this figure were obtained from one optic section.

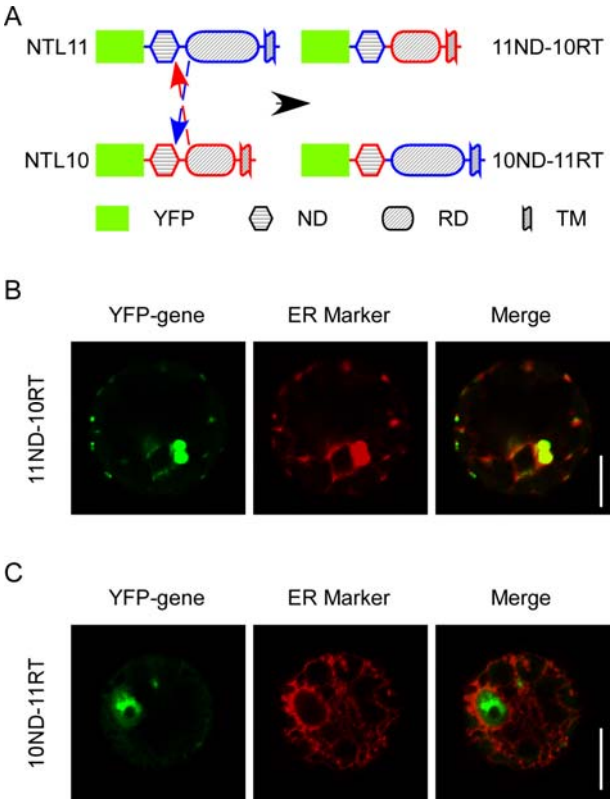

Figure 8. Subcellular localisation of chimeric constructs of NTL10 and NTL11. (A) Schematic presentation of chimeric eYFP-NTL constructs. The middle RR and c-terminal TM fragments of NTL10 were fused to the N-terminal ND of NTL11, and vice versa. (B) Localisation of eYFP-11ND-10RT and ER-mCherry in *Arabidopsis* mesophyll cells. (C) Localisation of eYFP-10ND-11RT and ER-mCherry in *Arabidopsis* mesophyll cells. All images in this figure were obtained from one optic section. Scale bars are equivalent to 10 µm.

References

1. Pedrazzini E. Tail-Anchored Proteins in Plants. Journal of Plant Biology 2009;52(2):88-101.

2. Kutay U, Hartmann E, Rapoport TA. A class of membrane proteins with a C-terminal anchor. Trends in Cell Biology 1993;3(3):72-75.

3. Kriechbaumer V, Shaw R, Mukherjee J, Bowsher CG, Harrison AM, Abell BM. Subcellular distribution of tail-anchored proteins in *Arabidopsis*. Traffic 2009;10(12):1753-1764.

4. Chartron JW, Clemons WM, Jr., Suloway CJ. The complex process of GETting tail-anchored membrane proteins to the ER. Current opinion in structural biology 2012;22(2):217-224.

5. Abell BM, Mullen RT. Tail-anchored membrane proteins: exploring the complex diversity of tail-anchored-protein targeting in plant cells. Plant cell reports 2011;30(2):137-151.

6. Borgese N, Fasana E. Targeting pathways of C-tail-anchored proteins. Biochimica et biophysica acta 2011;1808(3):937-946.

7. Borgese N, Brambillasca S, Colombo S. How tails guide tail-anchored proteins to their destinations. Current opinion in cell biology 2007;19(4):368-375.

8. Borgese N, Colombo S, Pedrazzini E. The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. Journal of Cell Biology 2003;161(6):1013-1019.

9. Rabu C, Schmid V, Schwappach B, High S. Biogenesis of tail-anchored proteins: the beginning for the end? Journal of cell science 2009;122(Pt 20):3605-3612.

10. Wattenberg B, Lithgow T. Targeting of C-terminal (tail)-anchored proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. Traffic 2001;2(1):66-71.

11. Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated Intramembrane Proteolysis: A Control Mechanism Conserved from Bacteria to Humans. Cell 2000;100(4):391-398.

12. Hoppe T, Matuschewski K, Rape M, Schlenker S, Ulrich HD, Jentsch S. Activation of a Membrane-Bound Transcription Factor by Regulated Ubiquitin/Proteasome-Dependent Processing. Cell 2000;102(5):577-586.

13. Wang X, Sato R, Brown MS, Hua X, Goldstein JL. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell 1994;77(1):53-62.

14. Hoppe T, Rape M, Jentsch S. Membrane-bound transcription factors: regulated release by RIP or RUP. Current opinion in cell biology 2001;13(3):344-348.

15. Kim SG, Lee S, Ryu J, Park CM. Probing protein structural requirements for activation of membrane-bound NAC transcription factors in *Arabidopsis* and rice. Plant Science 2010;178(3):239-244.

16. Iwata Y, Koizumi N. An *Arabidopsis* transcription factor, AtbZIP60, regulates the endoplasmic reticulum stress response in a manner unique to plants. Proc Natl Acad Sci USA 2005;102(14):5280-5285.

17. Iwata Y, Fedoroff NV, Koizumi N. *Arabidopsis* bZIP60 Is a Proteolysis-Activated Transcription Factor Involved in the Endoplasmic Reticulum Stress Response. The Plant cell 2008;20(11):3107-3121.

18. Kim SY, Kim SG, Kim YS, Seo PJ, Bae M, Yoon HK, Park CM. Exploring membrane-associated NAC transcription factors in *Arabidopsis*: implications for membrane biology in genome regulation. Nucleic acids research 2007;35(1):203-213.

19. Kim YS, Kim SG, Park JE, Park HY, Lim MH, Chua NH, Park CM. A membrane-bound NAC transcription factor regulates cell division in *Arabidopsis*. The Plant cell 2006;18(11):3132-3144.

20. Ng S, Ivanova A, Duncan O, Law SR, Van Aken O, De Clercq I, Wang Y, Carrie C, Xu L, Kmiec B, Walker H, Van Breusegem F, Whelan J, Giraud E. A membrane-bound NAC transcription factor, ANAC017, mediates mitochondrial retrograde signaling in *Arabidopsis*. The Plant cell 2013;25(9):3450-3471.

21. De Clercq I, Vermeirssen V, Van Aken O, Vandepoele K, Murcha MW, Law SR, Inze A, Ng S, Ivanova A, Rombaut D, van de Cotte B, Jaspers P, Van de Peer Y, Kangasjarvi J, Whelan J*, et al.* The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in *Arabidopsis*. The Plant cell 2013;25(9):3472-3490.

22. Li P, Wind JJ, Shi X, Zhang H, Hanson J, Smeekens SC, Teng S. Fructose sensitivity is suppressed in *Arabidopsis* by the transcription factor ANAC089 lacking the membrane-bound domain. Proc Natl Acad Sci USA 2011;108(8):3436-3441.

23. Klein P, Seidel T, Stocker B, Dietz KJ. The membrane-tethered transcription factor ANAC089 serves as redox-dependent suppressor of stromal ascorbate peroxidase gene expression. Frontiers in plant science 2012;3:247.

24. Yang ZT, Wang MJ, Sun L, Lu SJ, Bi DL, Sun L, Song ZT, Zhang SS, Zhou SF, Liu JX. The membrane-associated transcription factor NAC089 controls ER-stress-induced programmed cell death in plants. PLoS genetics 2014;10(3):e1004243.

25. Seo PJ, Kim MJ, Park JY, Kim SY, Jeon J, Lee YH, Kim J, Park CM. Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in *Arabidopsis*. Plant Journal 2010;61(4):661-671.

26. Kim SG, Kim SY, Park CM. A membrane-associated NAC transcription factor regulates salt-responsive flowering via FLOWERING LOCUS T in *Arabidopsis*. Planta 2007;226(3):647-654.

27. Yoon HK, Kim SG, Kim SY, Park CM. Regulation of leaf senescence by NTL9-mediated osmotic stress signaling in *Arabidopsis*. Moleculars and Cells 2008;25(3):438-445.

28. Park J, Kim YS, Kim SG, Jung JH, Woo JC, Park CM. Integration of auxin and salt signals by the NAC transcription factor NTM2 during seed germination in *Arabidopsis*. Plant physiology 2011;156(2):537-549.

29. Castelli V, Aury JM, Jaillon O, Wincker P, Clepet C, Menard M, Cruaud C, Quetier F, Scarpelli C, Schachter V, Temple G, Caboche M, Weissenbach J, Salanoubat M. Whole genome sequence comparisons and "full-length" cDNA sequences: a combined approach to evaluate and improve Arabidopsis genome annotation. Genome research 2004;14(3):406-413.

30. Li P, Zhou H, Shi X, Yu B, Zhou Y, Chen S, Wang Y, Peng Y, Meyer RC, Smeekens SC, Teng S. The ABI4-induced Arabidopsis ANAC060 transcription factor attenuates ABA signaling and renders seedlings sugar insensitive when present in the nucleus. PLoS genetics 2014;10(3):e1004213.

31. Olsen AN, Ernst HA, Leggio LL, Skriver K. NAC transcription factors: structurally distinct, functionally diverse. Trends in Plant Science 2005;10(2):79-87.

32. Nguyen Ba AN, Pogoutse A, Provart N, Moses AM. NLStradamus: a simple Hidden Markov Model for nuclear localization signal prediction. BMC bioinformatics 2009;10:202.

33. Lee S, Seo PJ, Lee HJ, Park CM. A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in *Arabidopsis*. Plant Journal 2012;70(5):831-844.

34. Li J, Zhang J, Wang X, Chen J. A membrane-tethered transcription factor ANAC089 negatively regulates floral initiation in *Arabidopsis thaliana*. Science China Life sciences 2010;53(11):1299-1306.

35. Wang P, Hawkins TJ, Richardson C, Cummins I, Deeks MJ, Sparkes I, Hawes C, Hussey PJ. The plant cytoskeleton, NET3C, and VAP27 mediate the link between the plasma membrane and endoplasmic reticulum. Current Biology 2014;24(12):1397-1405.

36. Dingwall C, Laskey RA. Protein import into the cell nucleus. Annual review of cell biology 1986;2:367-390.

37. Hamel L-P, Sheen J, Séguin A. Ancient signals: comparative genomics of green plant CDPKs. Trends in Plant Science 2014;19(2):79-89.

38. Morishita T, Kojima Y, Maruta T, Nishizawa-Yokoi A, Yabuta Y, Shigeoka S. *Arabidopsis* NAC transcription factor, ANAC078, regulates flavonoid biosynthesis under high-light. Plant & cell physiology 2009;50(12):2210-2222.

39. Ast T, Cohen G, Schuldiner M. A network of cytosolic factors targets SRP-independent proteins to the endoplasmic reticulum. Cell 2013;152(5):1134-1145.

40. Stefanovic S, Hegde RS. Identification of a targeting factor for posttranslational membrane protein insertion into the ER. Cell 2007;128(6):1147-1159.

41. Mariappan M, Li X, Stefanovic S, Sharma A, Mateja A, Keenan RJ, Hegde RS. A ribosome-associating factor chaperones tail-anchored membrane proteins. Nature 2010;466(7310):1120-1124.

42. Rabu C, Wipf P, Brodsky JL, High S. A precursor-specific role for Hsp40/Hsc70 during tail-anchored protein integration at the endoplasmic reticulum. Journal of Biological Chemistry 2008;283(41):27504-27513.

43. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. The Plant journal : for cell and molecular biology 1998;16(6):735-743.

44. Wang ZY, Nakano T, Gendron J, He J, Chen M, Vafeados D, Yang Y, Fujioka S, Yoshida S, Asami T, Chory J. Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Developmental Cell 2002;2(4):505-513.

45. Leśniewicz K, Poręba E, Smolarkiewicz M, Wolff N, Stanisławski S, Wojtaszek P. Plant plasma membrane-bound staphylococcal-like DNases as a novel class of eukaryotic nucleases. BMC Plant Biology 2012;12(1):195.

46. Yoo SD, Cho YH, Sheen J. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature protocols 2007;2(7):1565-1572.

47. Wu FH, Shen SC, Lee LY, Lee SH, Chan MT, Lin CS. Tape-*Arabidopsis* Sandwich - a simpler *Arabidopsis* protoplast isolation method. Plant methods 2009;5:16.