

Should I stay or should I go? Trafficking of plant extra-nuclear transcription factors

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Abstract

At the heart of all biological processes lies the control of nuclear gene expression, which is primarily achieved through the action of transcription factors (TFs) that generally contain a nuclear localization signal (NLS) to facilitate their transport into the nucleus. However, some TFs reside in the cytoplasm in a transcriptionally inactive state and only enter the nucleus in response to specific signals, which in plants include biotic or abiotic stresses. These extra-nuclear TFs can be found in the cytosol or associated with various membrane systems, including the endoplasmic reticulum and plasma membrane. They may be integral proteins with transmembrane domains or associate peripherally with the lipid bilayer via acylation or membrane-binding domains. Although over 30 plant TFs, most of them involved in stress responses, have been experimentally shown to reside outside the nucleus, computational predictions suggest that this number is much larger. Understanding how extra-nuclear TFs are trafficked into the nucleus is essential for reconstructing transcriptional regulatory networks that govern major cellular pathways in response to biotic and abiotic signals. Here, we provide a perspective on what is known on plant extranuclear-nuclear TF retention, nuclear trafficking, and the post-translational modifications that ultimately enable them to regulate gene expression upon entering the nucleus.

Introduction

The response of plant cells to developmental cues as well as abiotic and biotic inputs is carefully orchestrated through intricate signal recognition and relay pathways, which ultimately trigger differential gene expression. This dynamic process of gene expression regulation is tightly controlled by transcription factors (TFs), proteins that bind DNA in a sequence-specific manner and either promote or repress transcription of specific genes. TFs are usually classified into 50 to 70 different families according to the structure of their DNA-binding domains, with some TF families being unique to the plant kingdom. TFs represent 5% to 7% of all the plant protein-coding genes, with *Arabidopsis thaliana* predicted to contain about 2,000 TFs (Riechmann et al. 2000; Davuluri et al. 2003; Riaño-Pachón et al. 2007; Yilmaz et al. 2010). However, additional TFs are likely yet to be identified from the large number of genes for which functions

remain to be determined. Just as a reference, even in the best described plant genome, *Arabidopsis*, there are still more than 5,000 genes with unknown functions (Shang et al. 2023).

Although TFs regulate gene transcription inside the nucleus, a fraction of them reside in the cytoplasm (extra-nuclear TFs) as transcriptionally inactive forms. Upon activation, they are transported into the nucleus. Some TFs are retained in the cytoplasm because they either form complexes with cytoplasmic proteins or are unable to interact with proteins required for their nuclear translocation. In other instances, TFs associate with particular membrane compartments, and only upon activation they are released for nuclear import (Liu et al. 2018). This phenomenon has been well characterized for a relatively small number of animal and plant TFs, yet computational studies suggest that the number of TFs that are regulated in this way is much larger (Zupicich et al. 2001).

How does cytoplasmic TF retention affect the kinetics of the regulatory cascade in which it is involved? Simple intuition suggests that having a TF preformed in the cytoplasm might allow for a faster response than having to synthesize it *de novo* (involving transcription, splicing, translation). Consistent with this idea, most extranuclear TFs are activated and translocated into the nucleus upon stress conditions that require rapid transcriptional responses. In mammalian cells, transcription elongation rates vary between 30 to 100 nts/s (Fuchs et al. 2014), RNA splicing takes in average 5 to 10 min (Singh and Padgett 2009), and translation elongation proceeds at 6 amino acid/s (Ingolia et al. 2011). Newly synthesized TFs may need to undergo post-translational modifications before they are ready to be translocated to the nucleus. Therefore, we can estimate that synthesizing TFs *de novo* can require between a few minutes and a few hours. How long is required to release and activate extranuclear TF from the cytoplasm? Unfortunately, the information on extranuclear TF kinetics is rather limited but based on the few cases that have been studied in plants, it may require even longer than *de novo* protein synthesis. For example, the release of ANAC013 from membranes has been estimated to occur 1.5 h after the onset of low-oxygen conditions (hypoxia) (Eysholdt-Derzso et al. 2023). However, ANAC017, another extranuclear TF that also responds to hypoxia, activates the transcription of ANAC013 within minutes of mitochondrial dysfunction onset (He et al. 2022), suggesting that activation of preformed TFs and *de novo* synthesis of the same TFs may represent regulatory circuits to balance the timing and amplitude of complex TF kinetics.

Here, we build on a number of studies that have discussed the extranuclear retention of TFs to provide a perspective of what is known in plants, with particular emphasis given to membrane retention and mechanisms associated with TF activation.

General mechanisms of TF nuclear import and export

Most TFs are synthesized in the cytosol and translocated into the nucleus through the recognition of a nuclear localization signal (NLS), which consists of 1 or more short sequences with basic amino acids (Lu et al. 2021). Whereas proteins smaller than ~40 kDa can enter and exit the nucleus by diffusion, larger proteins require an NLS or a nuclear export signal (NES) to move through nuclear pores. Certain amino acid features are common to many NLSs, but they are not characterized by strict consensus sequences. The general translocation mechanism into the nucleus typically begins with interaction between the NLS sequence in the client protein (e.g. TF) and 2 nuclear import proteins, importin α and importin β , in the cytosol. The complex is then channeled through the nuclear pore complex. Within the nucleus, the GTP-bound Ran (Ras-related nuclear) G protein dissociates the importin-TF complex and releases the TF that can then bind DNA and regulate gene expression (Lu et al. 2021). Proteins that shuttle between the nucleus and cytoplasm also contain an NES. Inside the nucleus, the NES in the client

protein is recognized by the GTP-bound Ran-exportin complex, which mediates exit back to the cytoplasm (Merkle 2011) (Fig. 1). As the complex moves through the nuclear pore and into the cytoplasm, the Ran hydrolyzes GTP, causing the disassembly of the complex and the release of the client protein. Occasionally, the NLS and NES sequences can be found in a partner protein of the TF and not in the TF itself. In such cases, partner proteins are essential to control the movement of TFs in and/or out of the nucleus. A good example in plants is provided by the JAZ9 transcriptional repressor, which requires MYC2 to translocate to the nucleus (Withers et al. 2012).

There are several mechanisms by which extra-nuclear TFs are retained outside the nucleus; some of these TFs are soluble in the cytosol (Figs. 2 and 3), and others are associated with membranes. Extra-nuclear TFs have been identified in numerous TF families, including the plant-specific TFs NAC (NO APICAL MERISTEM, ATAF1/2, CUP-SHAPED COTYLEDON 2), SBP (SQUAMOSA-PROMOTER BINDING PROTEIN), WRKY, ARF (AUXIN RESPONSE FACTOR) families, and TF families shared with other eukaryotes, such as bHLH (basic HELIX-LOOP-HELIX), bZIP (basic LEUCINE ZIPPER), MADS, MYB, and C2H2. In addition, some TF families, such as the bZIP and bHLH TFs, have evolved multiple mechanisms of retention outside the nucleus.

Over 30 extranuclear TFs have been experimentally identified in *Arabidopsis*, including 14 with transmembrane domains. However, it is difficult to estimate how many more there really are due to the limitation in predicting and even detecting extranuclear TFs. In this review, we discuss the main mechanisms that mediate retention of TFs outside the nucleus as an important regulatory component of gene expression and comment on new experimental techniques and computational methods to address challenges in identifying new extra-nuclear TFs. We focus primarily on *Arabidopsis* extra-nuclear TFs because they have been best studied but refer to examples from other plant species when contributing new knowledge.

Cytoplasmic retention of TFs through protein-protein interactions

Eukaryotes use diverse mechanisms to retain TFs in the cytosol (cTFs) as a mechanism to control their transcriptional activity. These mechanisms usually involve interactions with cytosolic proteins and may require specific post-translational modifications such as phosphorylation. Nucleo-cytoplasmic trafficking of cTFs in plants is commonly mediated by hormone signaling and environmental stresses. In *Arabidopsis*, approximately 16 cTFs across 8 TF families have been experimentally characterized (Fig. 2).

The most common mechanism of cytosolic retention of cTFs is binding to 14-3-3 proteins in the cytosol upon phosphorylation. The highly conserved 14-3-3 proteins can interact with phosphorylated forms of their client proteins (Huang et al. 2022). Twelve 14-3-3 genes are expressed in

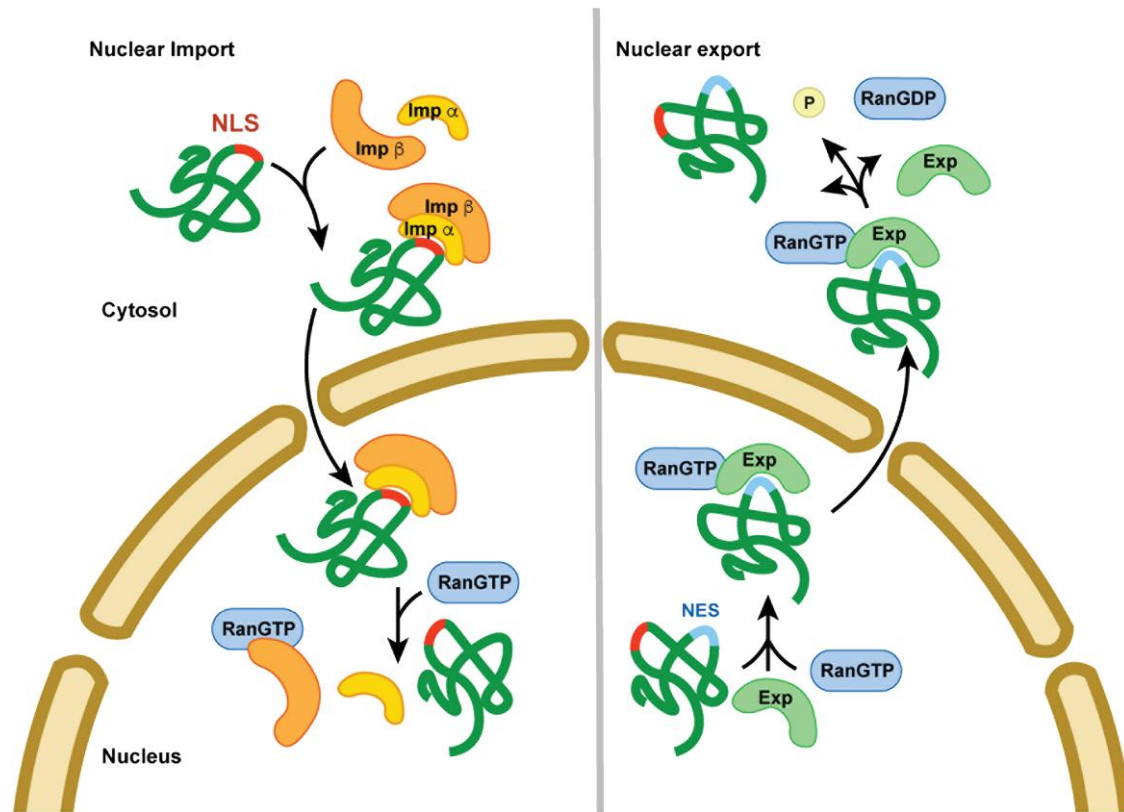


Figure 1. General mechanisms of nuclear protein import and export. NLS sequences are recognized by importins (importins a and b, indicated as Imp a and Imp b, respectively) in the cytosol and mediate the translocation into the nucleus. RanGTP mediates the dissociation of the importin complex and the NLS, facilitating the release of the client protein inside the nucleus. Exportins binds the NES in client proteins and, together with RanGTP, form a complex that moves through the nuclear pore. At the cytosol, GTP hydrolysis in the Ran GTPase facilitates the disassembly of the complex and the release of the client protein.

Arabidopsis, with several involved in gene transcription regulation (Sehnke et al. 2002), including those retaining cTFs outside the nucleus (see Fig. 2).

For example, in *Arabidopsis*, BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (bri1-EMS-SUPPRESSOR 1)/BRZ2 are 2 important brassinosteroid (BR)-responsive cTFs that directly or indirectly control the expression of thousands of genes during plant development and in response to environmental signals (Sun et al. 2010; Nolan et al. 2020). When BR levels are low, BZR1 and BES1 are phosphorylated by the GSK3-like kinase BIN2 (BRASSINOSTEROID INSENSITIVE 2) (Li and Nam 2002; Zhao et al. 2002), which increases their affinity for several 14-3-3 proteins in the cytosol (Gampala et al. 2007) and promotes their degradation, by either the 26S proteasome or autophagy (He et al. 2002; Nolan et al. 2017; Kim et al. 2019). BR perception by the cell surface receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) and its co-receptor BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) leads to the inhibition and degradation of BIN2 (Li and Nam 2002; Yin et al. 2002) and the enhanced dephosphorylation of BES1 and BRZ1 by PP2A-type phosphatases (Tang et al. 2011). Dephosphorylated BES1 and BRZ1 dissociate from 14-3-3 proteins and are translocated into the nucleus (Ryu et al. 2007) to drive expression of BR-responsive genes.

Interestingly, BIN2 itself partitions between the nucleus and cytosol and can phosphorylate BES1 and BZR1 in both locations (Ryu et al. 2010a). The nuclear phosphorylation of BZR1 triggers their exportin-dependent nuclear export back to the cytosol (Ryu et al. 2007, 2010b) (Fig. 3). Regulation of BZR1's nuclear translocation can also be cell specific. For example, in differentiating phloem cells, the plasma membrane-associated protein OCTOPUS binds and inhibits BIN2 at the cell surface (Anne et al. 2015), promoting the dephosphorylation of BES1 and BZR1 and their nuclear import.

At least 5 isoforms of 14-3-3 proteins were shown to interact with BZR1; however, the analysis of single and double mutants for 14-3-3 genes has failed to confirm the role of 14-3-3 proteins in BR signaling (Gampala et al. 2007). This could be due to functional redundancy within the 14-3-3 protein family or the occurrence of additional mechanism of cytosolic retention. In fact, several studies support the idea that larger molecular scaffolds hold phosphorylated BZR1 in the cytosol. For example, BZR1, together with other BR-signaling proteins, interacts with TTL3 (TETRATRICOPEPTIDE THIOREDOXIN-LIKE 3) in the cytosol. *Arabidopsis* TTL proteins contain 6 tetratricopeptide repeat commonly involved in protein-protein interactions and a C-terminal domain similar to thioredoxins

TF Family	TF	cytosolic form				other features	Signal for nuclear translocation	References
		phosphorylated	bound to 14-3-3 proteins	bound to other proteins	in condensates			
BES1/BZR	BES1	✓	✓				BR	Ryu et al., 2010
	BZR1	✓	✓	✓ BSS1 TLL3		sumoylation causes nuclear accumulation	BR	Ryu et al., 2007; Srivastava et al., 2020
bZIP	bZIP51	✓	✓				mechanical or hypo-osmotic stress	Takeo and Ito, 2017
	bZIP52	✓	✓				heat stress	Wiese et al., 2021
	bZIP18	✓	✓					
bHLH	PIF7	✓	✓				shade	Huang et al., 2018
	bHLH039					Phosphoryl. triggers nuclear localization	low iron	Gratz et al., 2019; Trofimov et al., 2019
	FIT							
MYB	PHR1	✓		✓ SPX4			low PI	Puga et al., 2014; Osorio et al., 2019
	RVE4; RVE8						cold and heat stress	Kidokoro et al., 2021
HSF	HSFA8			✓ HSP		Cys disulfide bridges needed for cytosolic retention	oxidative stress	Giesguth et al., 2015
NAC	VND6			✓ XND1			xylem differentiation	Zhong et al., 2020
ARF	ARF7 ARF19				✓ ✓		auxin	Powers et al., 2019; Jing et al., 2022
E2F	E2Fa					Moves to nucleus upon binding DPa	cell differentiation	Kosugi and Ohashi, 2002

Figure 2. Table of *Arabidopsis* cTFs retained in the cytosol in their transcriptionally inactive forms and known mechanisms of cytosolic retention and activation. BSS1 (BRZ-SENSITIVE-SHORT HYPOCOTYL1); HSF, heat stress factor; HSP, heat shock proteins; SPX4, SYG1/Pho81/XPR1 (SPX) domain-containing protein; RSG, REPRESSION OF SHOOT GROWTH); TLL3 (TETRATRICOPEPTIDE THIOREDOXIN-LIKE 3); XND1 (XYLEM NAC DOMAIN 1).

(Amorim-Silva et al. 2019). When BR signaling is activated, TTL3 relocates and brings several BR signaling proteins to the plasma membrane, where it promotes the dephosphorylation of BZR1 and its nuclear translocation (Fig. 3). BZR1 also binds to the BROAD COMPLEX/TRAMTRACK/BRIC-A-BRAC family protein BSS1 (BRZ-SENSITIVE-SHORT HYPOCOTYL1). BSS1 localizes to both the cytosol and nucleus and forms oligomers that bind BZR1 under low BR levels. These BSS1 oligomers bind BRZ1 and trap it in the cytosol. Increased BR concentration leads to the disassembly of BSS1 oligomers, the release of BRZ1, and its translocation into the nucleus (Shimada et al. 2015). How the 14-3-3 proteins, TLL3, and BSS1 cooperate to retain BZR1 and BES1 in the cytosol is unclear, but they could provide a multilayered mechanism of BR signaling through the dynamic control of BES1 and BZR1 localization (Fig. 3).

BES1 and BZR1 can also be subjected to SUMOylation, that is, the reversible conjugation of SUMO (small ubiquitin-like modifier) through biochemical reactions similar to ubiquitination. However, the effects of SUMOylation in the 2 TFs are very different. SUMOylated BES1 is rapidly degraded (Zhang

et al. 2019), whereas SUMOylation of BZR1 prevents its interaction with the BIN2 kinase and causes its accumulation within the nucleus (Srivastava et al. 2020) (Fig. 3). Salt stress stimulates the deSUMOylation of BZR1, which results in the phosphorylation of BZR1 by BIN2 and its nuclear export and degradation (Srivastava et al. 2020).

For other phosphorylated cTFs retained in the cytosol by 14-3-3 proteins, the signal that triggers their dephosphorylation and nuclear translocation is environmental (Fig. 2). For example, activation of photomorphogenesis in response to light depends on the repression of several PIFs (PHYTOCHROME INTERACTING FACTORS) (Pham et al. 2018). These are bHLH TFs that physically interact with phytochromes. Light induces phosphorylation of PIFs, promoting their ubiquitylation and degradation by the 26S proteasome (Al-Sady et al. 2006). However, phosphorylated *Arabidopsis* PIF7 is not degraded, and instead interacts with 14-3-3 proteins in the cytosol. When plants are under shade conditions, PIF7 is dephosphorylated and unable to bind 14-3-3 proteins, resulting in the translocation of PIF7 into the nucleus, where it activates expression of shade-induced genes (Huang et al.

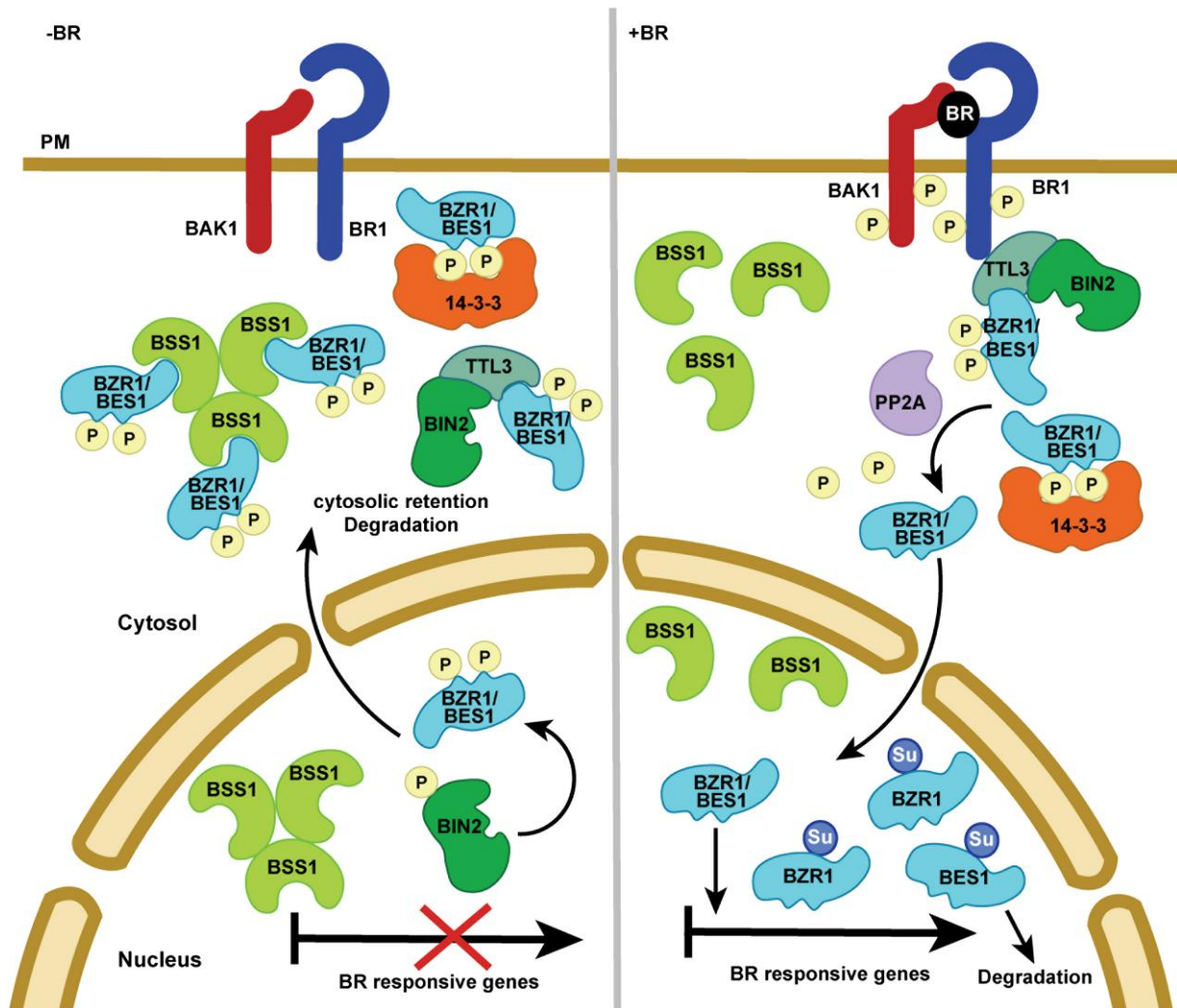


Figure 3. Main cytosolic retention mechanisms for the regulation of BR-responsive TFs BRZ1 and BES1. BZR1/BES1 are retained in the cytosol by various mechanisms involving interaction with 14-3-3 proteins, BSS1, and TTL3. How these 3 mechanisms are interconnected is currently unclear. Some components of the BR signaling pathways are omitted in this figure for clarity. P, phosphorylation; Su, SUMOylation.

2018). Interestingly, PIF7 can bind BES1 and BZR1 to regulate crosstalk between BR and light signaling (Martínez et al. 2018). PIF7 was also recently identified as being important in the integration of growth responses to high temperatures in the shade (Burko et al. 2022).

Some bZIP TFs (e.g. *Arabidopsis* bZIP18, bZIP51/VIP1, bZIP52, and *Nicotiana tabacum* RSG) are also retained in the cytosol in their transcriptionally inactive, phosphorylated form through interactions with 14-3-3 proteins (Igarashi et al. 2001; Nakata et al. 2009; Takeo and Ito 2017; Wiese et al. 2021) (Fig. 1). VIP1/bZIP51 (VIRE2-INTERACTING PROTEIN 1/BASIC-LEUCINE ZIPPER 51) is dephosphorylated and translocated into the nucleus upon mechanical or hypo-osmotic stress (Takeo and Ito 2017; Tsugama et al. 2019), whereas dephosphorylation and nuclear translocation of bZIP18 and bZIP52 are triggered by heat stress (Wiese et al. 2021).

RSG (REPRESSION OF SHOOT GROWTH), a *Nicotiana tabacum* bZIP TF closely related to *Arabidopsis* VIP1/bZIP1,

represents a special case within plant TFs retained in the cytosol by 14-3-3 proteins. Different from other plant TFs statically retained in the cytosol until activation, RSG undergoes continuous nucleo-cytoplasmic shuttling mediated by importins and exportins (Igarashi et al. 2001; Nakata et al. 2009; Ito et al. 2014). How exactly the dynamic shuttling of RSG is controlled or what functional advantage it provides is unclear. Whereas RSG seems to be the only identified plant cTF that shows continuous nucleo-cytoplasmic shuttling in plants, several examples are well known for animal cTFs. For example, under unstimulated conditions, the animal TF NF- κ B (nuclear factor κ B) binds I κ B α , which promotes the nuclear translocation of both proteins, mediated by the NLS of NF- κ B. Because I κ B α contains an NES, the NF- κ B-I κ B α complex quickly exits the nucleus (Huang et al. 2000; Tam et al. 2000). Thus, NF- κ B-I κ B α complex mostly resides in the cytosol because the effect of the NES in I κ B α dominates that of the NLS in NF- κ B. However, upon

activation by perception of pathogens or cytokines, I κ B α is phosphorylated and degraded, which enables the rapid localization of NF- κ B to the nucleus for transcriptional regulation. The biological relevance of continuous nucleo-cytoplasmic shuttling of TFs is not always evident (Ghosh and Karin 2002) but could allow for very fast responses by keeping readily available pools of TFs ready for nuclear accumulation.

For other plant cTFs retained in the cytosol, such as PHR1 (PHOSPHATE STARVATION RESPONSE1), HSF8, and VND6 (Puga et al. 2014; Giesguth et al. 2015; Zhong et al. 2020), no 14-3-3 binding partners have been identified. However, these 3 cTFs interact with other types of cytosolic proteins that mediate their cytosolic retention (Fig. 2). For example, PHR1, which together with other MYB TFs control gene expression under low inorganic phosphate (Pi), binds SPX (SYG1/PHO81/XPR1) domain-containing proteins. In both *Arabidopsis* and rice (*Oryza sativa*), in the presence of Pi, SPX4 binds PHR1 and retains it in the cytosol (Puga et al. 2014; Wang et al. 2014), whereas under low Pi, SPX4 is degraded, allowing free PHR1 to enter the nucleus and activate Pi-starvation response gene expression (Lv et al. 2014; Osorio et al. 2019).

Another mechanism of cytosolic retention discovered recently for some ARF (AUXIN RESPONSE FACTOR) cTFs is protein condensation. In root cells with reduced auxin responses, ARF7 and ARF19 form condensates in the cytosol that prevent them from entering the nucleus (Powers et al. 2019; Jing et al. 2022). In *Arabidopsis* root tips, ARF19 is predominantly inside the nucleus, but in mature root tissues it forms cytoplasmic condensates through its Phox and Bem1p (PB1) domains and its intrinsically disordered middle region. ARF7 and ARF19 interact with AFF1 (AUXIN RESPONSE FACTOR F-BOX1), an E3 ligase that mediates ubiquitylation of target proteins. In mutants lacking AFF1 function, ARF proteins showed elevated condensation. Although the mechanism by which AFF1 alters ARF dynamics is not entirely clear, it has been postulated that ubiquitination of ARF7 and ARF19 blocks their NLS, affecting their nucleo-cytoplasmic partition (Jing et al. 2022).

The *Arabidopsis* MYB TFs RVE4 (REVEILLE4) and RVE8 move quickly and reversibly from the cytoplasm into the nucleus in response to either low or high temperatures to activate the expression of *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 1* (Kidokoro et al. 2021). What molecular mechanism promotes the nuclear import of RVE4 and RVE8 under unfavorable temperature conditions is currently unknown.

Although not considered a bona fide TF because it does not appear to bind DNA directly, the salicylic acid receptor NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) shows 1 of the most complex mechanisms of dynamic cytosolic-nuclear localization that affects directly gene expression. Inside the nucleus and in the presence of salicylic acid, NPR1 forms a complex with either transcriptional activators (e.g. TGAs within the bZIP TF family

(Zhang et al. 1999) for the expression of PR (PATHOGENESIS-RELATED) genes (Cao et al. 1997) or transcriptional repressors (e.g. WRKY TFs), reprogramming gene expression in response to pathogens and other stresses (Zhou et al. 2023). NPR1 is approximately 66 kDa in size and contains an NLS sequence at its C-terminal domain (Kinkema et al. 2000). In its uninduced state, NPR1 is an oligomer formed by intermolecular disulfide bridges between cysteine (Cys) residues (Mou et al. 2003) (Fig. 4). Oligomerization of NPR1 in the cytosol is further promoted by S-nitrosylation of Cys156 from S-nitrosoglutathione, a natural nitric oxide donor that accumulates during innate immune responses (Tada et al. 2008). Salicylic acid accumulation causes a change in cellular redox potential, leading to the reduction of Cys thiol groups in NPR1 mediated by thioredoxins and the disassembly of NPR1 oligomers (Tada et al. 2008) (Fig. 4). Reduced NPR1 can then enter the nucleus, associates with TFs, and orchestrates plant immune responses. Consistent with the critical role of Cys residues in the retention of NPR1 in the cytosol, mutations of Cys82 or Cys216 result in constitutive nuclear localization and upregulated expression of defense genes (Mou et al. 2003; Tada et al. 2008). Nuclear import of monomeric NPR1 is also promoted by phosphorylation through the action of SnRK2.8 (SNF1-RELATED PROTEIN KINASE 2.8) in the cytosol (Fig. 4). SnrK2.8 phosphorylates Thr 373 and Ser 589, the latter located in the NLS region of NPR1. Phosphorylation of either residue is required for NPR1 nuclear import in distal leaves (systemic signaling) but not in the infected leaves (local immunity). Inside the nucleus, activated NPR1 can be SUMOylated, which switches NPR1 interactions from transcription repressors to activators and promotes its ubiquitination and degradation (Saleh et al. 2015) (Fig. 4).

Interestingly, under high levels of salicylic acid, NPR1 condensates both inside the nucleus and in the cytosol, forming salicylic acid-induced NPR1 condensates that contain stress response proteins. In the nucleus, within these condensates, NPR1 is a major target of ubiquitylation and degradation, whereas, in the cytoplasm, NPR1 condensates associate with CULLIN 3 to assemble a E3 ligase complex that mediates ubiquitination of other proteins, such as the lipase-like protein EDS1 involved in effector-triggered immunity (Zavaliev et al. 2020).

Cytosolic retention of TFs through membrane associations

Membrane-associated TFs can be fully integrated into membranes through transmembrane domains or peripherally associated with membranes through various means (lipidation/acylation or hydrophobic membrane-binding domains).

TFs with transmembrane domains (tmTFs)

Although a relatively small number of tmTFs have been experimentally characterized in plants and algae, there are

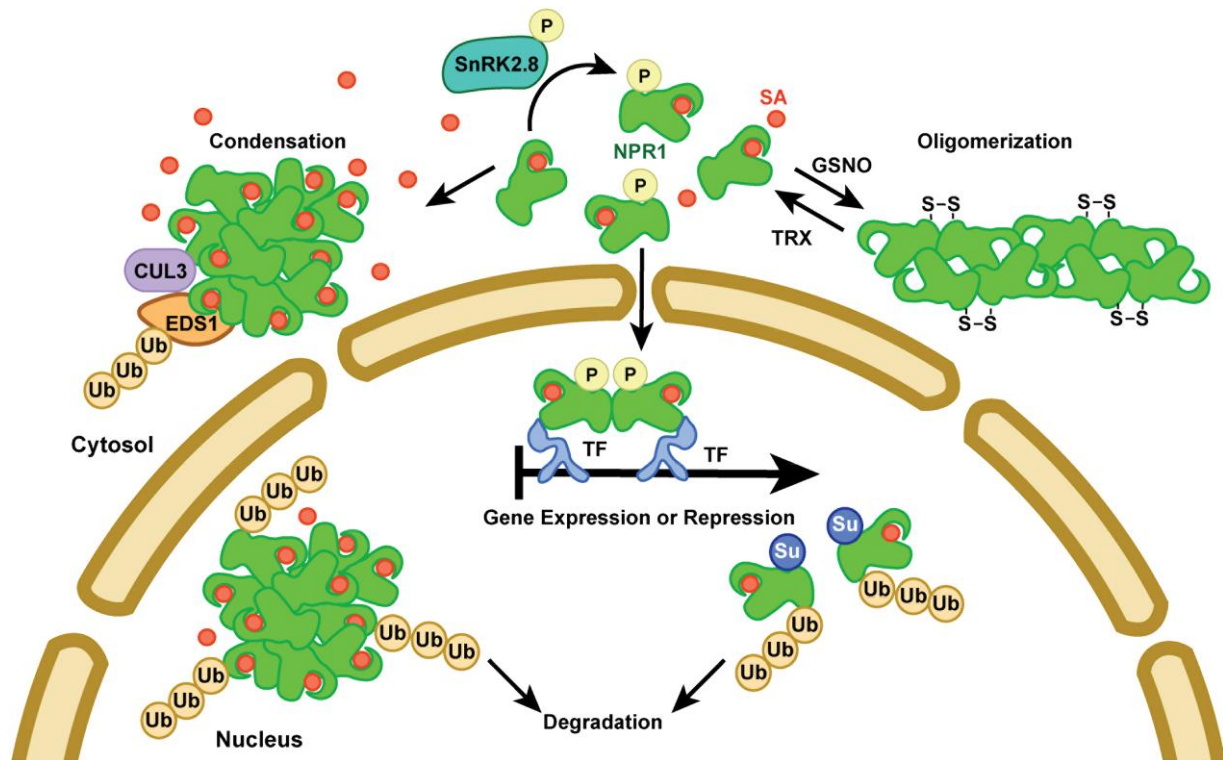


Figure 4. Trafficking of NPR1. Depending on the concentration of salicylic acid (SA) and redox state, NPR1 can be found as an oligomer, monomer, or condensate. At low SA concentration, the oligomeric form is stabilized by S-nitrosylation mediated by GSNO (S-nitrosoglutathione) and disulfide bridges (S-S) between Cys residues. Increased concentration of SA and action of thioredoxins (TRX) facilitate the disassembly of the oligomers and release of monomeric NPR1, which can be phosphorylated by the SNRK2.8 kinase and translocated into the nucleus to mediate either gene activation or repression. SUMOylation or condensation within the nucleus promote ubiquitination and degradation. NPR1 condensates in the cytosol mediate the ubiquitination and degradation of stress-related proteins such as the lipase-like protein EDS1. Su, SUMO; Ub, ubiquitin.

reasons to believe they represent an important fraction of all TFs. In silico studies of 14 species spanning from algae to flowering plants predicted that 16% of the 230 TFs in *Chlamydomonas reinhardtii* and 5% to 10% of the TFs in flowering plants contain 1 to 13 transmembrane domains (Yao et al. 2017) and that from the approximately 2,000 *Arabidopsis* TFs, 64 to 85 TFs corresponding to 24 to 27 families are tmTFs (Kim et al. 2010; Yao et al. 2017) (Fig. 5, A and B). Their representation within these families is variable, being as much as 75% of the SiF2-like (3 of 4), 50% of the NF-X1 (2 of 4), 25% of the YABBY (2 of 8), 20% of the CAMTA (2 of 10), 16% of the SBP (5 of 30), 12% of the NAC (17 of 138), and 5% of the bZIP (5 of 127) TF families. Based on their general topology, tmTFs are classified as type I and type II, both with a single transmembrane domain, and type III, with more than 1 transmembrane domain (Fig. 5C). Type I and II integral proteins differ in their orientations; the domain exposed to the cytosol is the C terminus for type I proteins and the N terminus for type II proteins (Fig. 5C), and this distinction is important because they involve different insertion mechanisms. There is a broad variation in the distribution of transmembrane domains among TF families (Kim et al. 2010; Yao et al. 2017). Most

Arabidopsis tmTFs have 1 or 2 (a few with 3 or 4) transmembrane domains at either the C- or the N-terminal regions (Fig. 5A). The bZIP and NAC families have the largest number of identified tmTFs (Fig. 5B; Fig. 6). All the NAC tmTFs are type II proteins, whereas other families contain both type I and type II proteins (Kim et al. 2010).

Localization of tmTFs

Similar to other integral proteins, tmTFs are synthesized in the endoplasmic reticulum (ER)-associated polysomes, from where they can be trafficked to other membranes, such as the plasma membrane or be retained in the ER as an inactive form. The topology and orientation of membrane proteins is primarily determined by the direction through which the first transmembrane helix is translocated across ER membranes. However, 50% to 70% (Kim et al. 2010; Yao et al. 2017) of the *Arabidopsis* tmTFs, including many NAC and bZIP tmTFs, are predicted to be inserted post-translationally into membranes as type II proteins. These tmTFs shared features with the so-called tail-anchored proteins, which contain N-terminal cytosolic domains with diverse functions (e.g. membrane trafficking, redox reactions, signal transduction) (Kutay et al. 1993) and a single

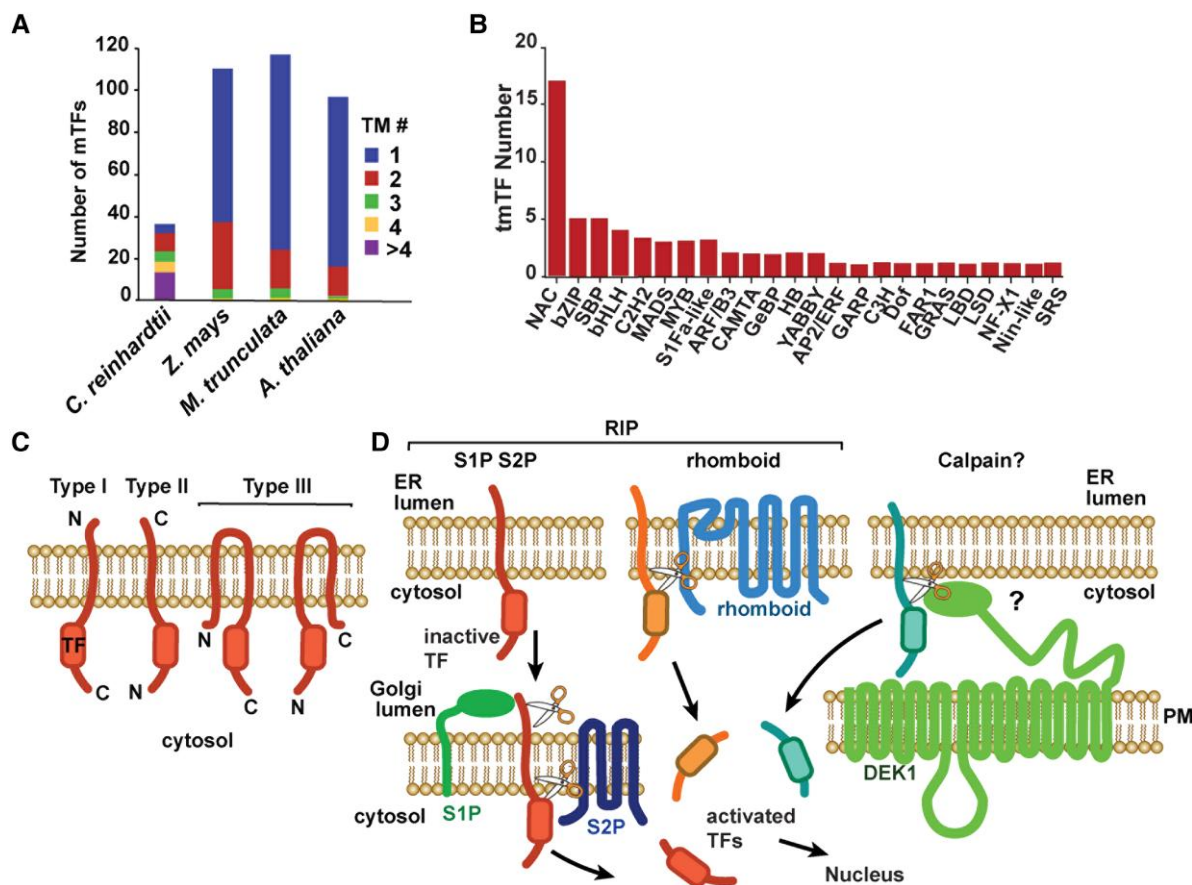


Figure 5. TFs with transmembrane domains (tmTFs). **A**) Number of predicted tmTFs in different plant and algal species, modified from Yao et al. (2017). **B**) Number of predicted tmTFs across different *Arabidopsis* TF families according to Yao et al. (Yao et al. 2017). **C**) Most predicted tmTFs in *Arabidopsis* contain either 1 (type I and type II) or 2 (type III) transmembrane domains at either the N or C terminus. **D**) Mechanism of tmTF activation by proteolytic cleavage and release from membranes. RIP, regulated intramembrane proteolysis; S1P, Site-1-Protease.

C-terminal transmembrane domain that is inserted post-translationally into many different types of membranes (e.g. ER, outer chloroplast and mitochondrial envelopes, peroxisomal membranes) (Chio et al. 2017). Because of this mechanism of membrane insertion, it is very hard to predict the subcellular localization of tail-anchored tmTFs.

Seventeen of the approximately 20 *Arabidopsis* tmTFs whose localization has been tested by expression of fluorescently tagged proteins seem to localize to the ER (Fig. 2). Four of the 75 *Arabidopsis* bZIP tmTFs (bZIP17, bZIP28, bZIP49, and bZIP60) are predicted to contain a single transmembrane domain, and 3 of them (bZIP17, bZIP28, and bZIP60) have been experimentally shown to localize to the ER in their inactive forms (Fig. 2). NAC tmTFs also show preference for ER localization. Twelve *Arabidopsis* NAC (ANAC) TFs with predicted single transmembrane domains (also called NAC with TMmotif1-like or NTL) (Liang et al. 2015) and 15 NAC TFs from soybean expressed as GFP fusions in *Arabidopsis* protoplasts localize to the ER (Ng et al. 2013; Li et al. 2016). In addition, maMYB, a member of the plant-specific R2R3-MYB TF family that controls root hair elongation (Slabaugh et al. 2011), also localizes to the ER, whereas SPL1 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 1),

involved in thermotolerance, localizes to the cell periphery (ER or plasma membrane) (Chao et al. 2017). As an example of tmTFs that localize to other organelles besides ER, *Arabidopsis* PTM (PHD type TF with transmembrane domains) localizes to the chloroplast envelope and mediates chloroplast retrograde signaling to the nucleus (Sun et al. 2011), whereas NACMTF3, involved in responses to stress in tomato (*Solanum lycopersicum*) (Bhattacharjee et al. 2017), localizes to the plasma membrane when transiently expressed as a GFP fusion.

Other predicted *Arabidopsis* tmTFs, such as CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 5, AGAMOUS-LIKE 69, SPL16, among others have been detected outside the nucleus in proteomic studies of isolated subcellular compartments such as plasma membrane, Golgi, mitochondria, and chloroplasts (De Backer et al. 2022), but their extra-nuclear localization has not been confirmed through other methods.

Once the transmembrane domains are removed from the tmTFs, the rest of the protein is free to move throughout the cytosol. According to in silico predictions, 73% of the *Arabidopsis* tmTFs contain NLSs, but it is likely that NLS motifs in other tmTFs have gone undetected (De Backer et al. 2022).

TF Family	TF	Localization	Alternative splicing	Proteolytic processing by	Signal for activation	References
bZIP	bZIP17	ER		S1P and likely S2P	ER stress, salt stress	Liu et al 2007
	bZIP28	ER		S1P and ?	ER stress, heat, BR, SA	Iwata et al 2017
	bZIP60	ER	✓		ER stress, heat, SA	Nagashima et al., 2011
NAC	ANAC013	ER		RBL	hypoxia mitoch. retrograde signaling	Eysholdt-Derzsó et al., 2023 De Clercq et al (2013)
	ANAC016 ANAC017	ER		RBL?	hypoxia hypoxia; ER stress	Eysholdt-Derzsó et al., 2023 Meng et al., 2019
	ANAC040	ER			Salt stress	Kim et al 2007 and 2008
	ANAC053	ER			heat stress anther maturation	Lee et al., 2014 Shih et al., 2014
	ANAC060	no TM in Col-0	✓		ABA signaling	Li et al., 2014
	ANAC062	ER			cold	Seo et al., 2010a Seo et al., 2010b
	ANAC068	ER		Calpain?	cell division	Kim et al (2006)
	ANAC069	ER			Salt stress	Park et al 2011
	ANAC078	ER			flavonoid synthesis	Moshita et al (2009) Yabuta et al (2010)
	ANAC089	ER			ER stress	Klein et al., 2012
	NTL9	ER			oxidative stress	Hye-Kyung et al., 2008
PTM	PHD	Chloropl. envelope			stress retrograde signaling	Sun et al., 2011
maMYB	R2R3-MYB	ER			root hair elongation?	Slabaugh et al., 2011
SBP	SPL1	ER or PM			heat?	Chao et al., 2017

Figure 6. Table of *Arabidopsis* tmTFs with subcellular localization experimentally tested. The table also includes the tmTF-activating protease, when known, and the activation signal.

There are many gaps in our knowledge about tmTFs to fully appreciate their cellular distribution and dynamics. First, in most studies, fluorescently tagged tmTFs are only characterized at low resolution; for tmTFs showing a peripheral signal in vacuolated epidermal cells or protoplasts, it is difficult to distinguish clearly between plasma membrane and ER localization. Second, most localization studies of tmTFs have been restricted to transient overexpression experiments, many times in heterologous systems. It is possible that the final and functional localization of many tmTFs requires additional binding partners expressed in specific cell types and certain developmental stages. More systematic efforts to characterize the subcellular distribution and dynamics of tmTFs will be needed to fully understand the range of cellular localization of plant tmTFs.

Activation of tmTFs

The active form of a tmTF lacks the transmembrane domains and is free to move into the nucleus through recognition of their NLSs and regulate gene expression. This is typically

achieved through the proteolytic cleavage of the tmTF protein to release the cytosolic domain. Activation of tmTFs by proteolytic processing is triggered by specific signals and involves either regulated intramembrane proteolysis (Fig. 5D) or regulated ubiquitin/proteasome-dependent processing (Seo et al. 2008). The handful of tmTFs studied in plants are activated by regulated intramembrane proteolysis (no examples of regulated ubiquitin/proteasome-dependent processing-mediated activation have been yet reported in plants), but in most cases, the specific proteases required for their release are unknown. The proteases known to process tmTFs in eukaryotes include Site-1-Proteases (S1P), S2P, rhomboid proteases, and presenilins, all of which are present in plants.

In animals, the bZIP tmTF ATF6 localizes to the ER and activates gene expression as part of the Unfolded Protein Response (UPR) during ER stress. For activation, ATF6 moves from the ER to the Golgi, where it is sequentially processed by S1P and S2P (Ye et al. 2000). S1P is located in the lumen of the *cis* and medial Golgi cisternae and cleaves the luminal

domains of the tmTFs. This first cleavage by S1P facilitates the second cleavage by the intramembrane protease S2P, which releases the cytosolic domain of the tmTFs in the cytosol to enter the nucleus (Ye et al. 2000; Shen and Prywes 2004). All the tmTFs that share this proteolytic activation mechanism, including ATF6, have in common the presence of a cytosolic domain able to act as a TF, a S1P recognition motif RXX(R/L) in the lumen, and a helical destabilization motif, such as an NP, NXXP, or PXXP sequence in the transmembrane helix for S2P cleavage (Ye 2020).

In plants, the UPR signaling response is largely coordinated by ER-localized tmTFs of bZIP family and consists of 2 pathways, one mediated by bZIP17 and bZIP28 and the other by bZIP60. Similar to animal ATF6, *Arabidopsis* bZIP17 and bZIP18 are released from the ER by proteolytic cleavage. The first cleavage step of bZIP17 is mediated by S1P (Liu et al. 2007), and although the identity of the protease that releases bZIP17 from the membrane has not been experimentally determined, bZIP17 contains a S2P cleavage motif close to its transmembrane segment (De Backer et al. 2022). For bZIP28, instead, the first cleavage of the luminal domain is not mediated by S1P but by an unknown protease, whereas S2P cleaves the cytosolic domain and releases the active form for nuclear translocation (Iwata et al. 2017). Just like ATF6, the C-terminal domain of bZIP28 binds the ER chaperon BiP (Binding Protein), preventing its relocalization to the Golgi under unstressed condition (Shen et al. 2002). In response to ER stress, bZIP28 dissociates from BiP, facilitating its transport out of the ER (Srivastava et al. 2013). A conserved mechanism of bZIP tmTF activation in UPR has been reported for maize (*Zea mays*) (Yang et al. 2013) and other vascular plant species (Howell 2021).

As mentioned above, many NAC tmTFs also localize to the ER. *Arabidopsis* ANAC013 interacts with the RHOMBOID-LIKE 2 (RBL2) intramembrane protease at the ER. Under low-oxygen conditions (hypoxia), dysfunctional mitochondria initiate a retrograde signaling that leads to the cleavage of the cytosolic domain of ANAC013 by RBLs and its translocation into the nucleus (Eysholdt-Derzso et al. 2023). ANAC017, a closely related NAC tmTF, also interacts with RBL2 (Eysholdt-Derzso et al. 2023) and has been predicted to be cleaved by rhomboid-type proteases (Ng et al. 2013).

Besides the intramembrane S2P and rhomboid proteases, there is evidence that other proteases may mediate the release of tmTFs in plants. For example, based on pharmacological studies using specific protease inhibitors, it was proposed that the release from ER membranes of *Arabidopsis* NAC068, a tmTF that mediates cytokinin signaling during cell division, is catalyzed by a calpain protease (Kim et al. 2006). Interestingly, the only functional calpain in plants is DEK1 (DEFECTIVE KERNEL 1), with 21 transmembrane domains and localized to the plasma membrane (Liang et al. 2013) (Fig. 5D). However, whether DEK1 directly cleaves ANAC068 has not been experimentally proven. For some tmTFs, phosphorylation after proteolytic cleavage is important for nuclear translocation. For example,

ANAC062, which upon cold induction is cleaved from the ER membrane and regulates pathogen-resistance responses, needs to be phosphorylated by SnRK2.8 to enter the nucleus (Kim et al. 2012).

Although most NAC tmTFs are released from the ER in response to stress, there seems to be some specificity for the activation of at least some of them. For example, ANAC013 and ANAC017 are released during hypoxic conditions and ER stress (Meng et al. 2019; Eysholdt-Derzso et al. 2023), ANAC053 is released in response to heat stress (Lee et al. 2014), ANAC062 in response to low temperatures (Seo et al. 2010a, 2010b) and ER stress (Yang et al. 2014), NTL9 in response to osmotic stress (Hye-Kyung et al. 2008), ANAC089 in response to ER stress (Klein et al. 2012), and ANAC068 to promote cell division (Kim et al. 2006). Considering that only a few proteases are known to cleave tmTFs, it is unclear what provides the specificity to release only the pertinent TF from the ER membrane. Recent evidence suggests that the timing of mTF release from the ER varies among tmTFs. For example, although ANAC013, ANAC016, and ANAC017 interact with the same RBL2 rhomboid protease at the ER membrane, ANAC013 seems to be cleaved by RBL2 before the other 2 TFs, at the onset of hypoxia (Eysholdt-Derzso et al. 2023). *Arabidopsis* contains 13 RBLs (RBL1-7 and RBL10-15) with different predicted subcellular localizations (ER, mitochondria, chloroplasts) (Kmiec-Wisniewska et al. 2008), which could also provide additional specificity to tmTF activation.

Adding to the complexity of the regulation of these ER-localized TFs, many of them are part of the same gene regulatory networks. For example, ANAC017 binds the promoters and mediates the expression of both ANAC013 and bZIP60 during mitochondrial retrograde signaling (He et al. 2022).

PTM, the only tmTF known to localize to the chloroplast envelope, contains a DNA-binding domain and an NLS at its N-terminal cytosolic domain and 4 transmembrane domains at its C terminus. Upon chloroplast stress induction by factors such as high light, PTM is cleaved by an unknown protease, released in the cytosol, and translocated into the nucleus to activate *ABI4* transcription and mediate retrograde signaling from plastids (Sun et al. 2011).

As mentioned above, bZIP60 is also an ER-localized tmTF mediating UPR such as bZIP28. However, different from bZIP28, bZIP60 is activated by alternative splicing and not by proteolytic cleavage. Upon perceiving ER stress, the ER-localized ribonuclease IRE1 (INOSITOL REQUIRING ENZYME1) cleaves the unspliced *bZIP60* mRNA in the cytosol, which is different from conventional spliceosome-dependent intranuclear RNA splicing. The spliced *bZIP60* transcript encodes a protein without transmembrane domain that moves freely into the nucleus (Nagashima et al. 2011). This signaling mechanism is conserved across eukaryotes with IRE1 mediating the unconventional splicing of bZIP60 in plants, *HOMOLOGOUS TO ATF/CREB 1* in yeast, and *X-BOX BINDING PROTEIN 1* in metazoans.

The other 18 predicted tmTFs, including LSD ONE LIKE 1, a zinc finger TF regulating oxidative stress-induced cell death,

have annotated alternative transcripts that would result in TF variants without a transmembrane domain (Yao et al. 2017; De Backer et al. 2022). However, the lack of detection of alternatively spliced tmTF transcripts should be taken with caution because they may be produced only under very specific conditions, such as ER stress for the splicing of *bZIP60*. Interestingly, whereas in most *Arabidopsis* accessions ANAC060 is predicted to have a transmembrane domain as many other members of the NAC family, a single-nucleotide polymorphism in the ecotype Columbia alters the splicing pattern of the ANAC060 transcript, resulting in a shorter protein without a transmembrane domain that constitutively localizes to the nucleus and confers sugar insensitivity (Li et al. 2014). Although in principle alternative splicing and proteolytic cleavage are not mutually exclusive, there are no known examples of plant tmTFs activated by both mechanisms.

TFs peripherally associated with membranes

Acylated TFs (acTFs)

Protein acylation is the covalent attachment of often short fatty acid chains to glycine (Gly), serine (Ser), lysine (Lys), or Cys residues (Shang et al. 2022). The most important protein acylations are myristoylation, prenylation, and, the best studied, palmitoylation (Fig. 7A). S-palmitoylation is a reversible cysteine modification tightly regulated by palmitoyltransferases and acyl protein thioesterases (Spinelli et al. 2018). Very few acTFs have been identified in eukaryotes. The only known plant acTF is MfNACsa from *Medicago falcata* (Duan et al. 2017), which regulates tolerance to drought stress. Under normal conditions, MfNACsa is palmitoylated and associates with the plasma membrane. Under drought stress, MfNACsa is depalmitoylated by MtAPT1 and translocated to the nucleus. MtAPT1 (Duan et al. 2017) is the only APT characterized in plants (Smokvarska et al. 2021). Interestingly, protein palmitoylation levels are affected by the nutritional stress and metabolic status in mammalian cells (Spinelli et al. 2018), opening the possibility that acTFs could be responsive to metabolic stress (Shang et al. 2022).

Protein domains that mediate membrane association

Some protein domains can mediate peripheral association with membranes (Fig. 7B). *Arabidopsis* XVP/ANAC003 has been experimentally characterized as a plant membrane-bound TF (mbTF). XVP is involved in vasculature differentiation, localizes to the plasma membrane, and does not contain any transmembrane domain (Yang et al. 2020). Instead, a positively charged domain at the C terminus enriched in Lys and arginine (Arg) is required for its plasma membrane localization (Lee et al. 2021a). In this case, the release of ANAC003 from the plasma membrane does not depend on proteolytic processing but on the phosphorylation status of a threonine (Thr) residue located close to Lys-Arg-rich domain (Lee et al. 2021a). In silico prediction of mbTFs is challenging and requires a more in-depth analysis of putative membrane-binding domains among plant TFs.

Unknown mechanisms

Rice OsMADS18 has been localized to the cell periphery/plasma membrane and can be translocated to the nucleus in response to abscisic acid signaling (Yin et al. 2019). However, OsMADS18 does not seem to have any predicted transmembrane domain, which suggests that it is retained close to the plasma membrane by membrane tethering (either by acylation or a membrane binding domain) or by interacting with a membrane-associated protein.

Computational identification of plant extra-nuclear TFs—not that easy

There are many challenges associated with the computational prediction of TF subcellular localization, including (1) limitations in determining if a protein is a TF, particularly when no conserved domains are present; (2) limitations in the prediction of NLSs (De Backer et al. 2022); and (3) limitations associated with establishing whether a TF associates with cytoplasmic proteins that prevents translocation into the nucleus. The prediction of tmTFs is probably the most reliable (>80% accuracy) as a number of algorithms are available to detect the presence of trans-membrane domains in proteins (Tsirigos et al. 2018). Predicting whether a TF will be acylated remains challenging, although computational tools are becoming available for at least some of these post-translational modifications [e.g. GPS-Lipid; (Xie et al. 2016)]. The prediction of mbTFs remains very difficult because whether a protein domain is able to bind a membrane is highly dependent on the protein conformation and cellular environment. Thus, we are a long way to accurately predicting whether a particular TF will have extra-nuclear residence; experimental determination, primarily through subcellular localization, biochemistry, and proteomics (Walley et al. 2016; Lee et al. 2021b), remains essential.

Future challenges and perspectives

The retention of TFs outside the nucleus, either by interaction with other proteins or through attachment to membranes, provides one of several mechanisms by which the regulatory activity of a TF is controlled. It is unclear how many TFs are regulated in this fashion, but given what is currently known in plants and other organisms, as many as 10% to 20% of all TFs could be controlled by such mechanisms. This is a significant number, but as discussed here, it is remarkable how much remains to be discovered regarding the mechanisms of the cytoplasmic retention of TFs and their biological implications.

There are several reasons why it is particularly challenging to determine if a TF is regulated through cytoplasmic retention using standard high-throughput methods such as GFP-tagging and ectopic overexpression as well as mass spectrometry detection. For example, low stability outside the nucleus makes it difficult to detect by mass spectrometry and establish TF extranuclear residency. It is also possible that cytoplasmic

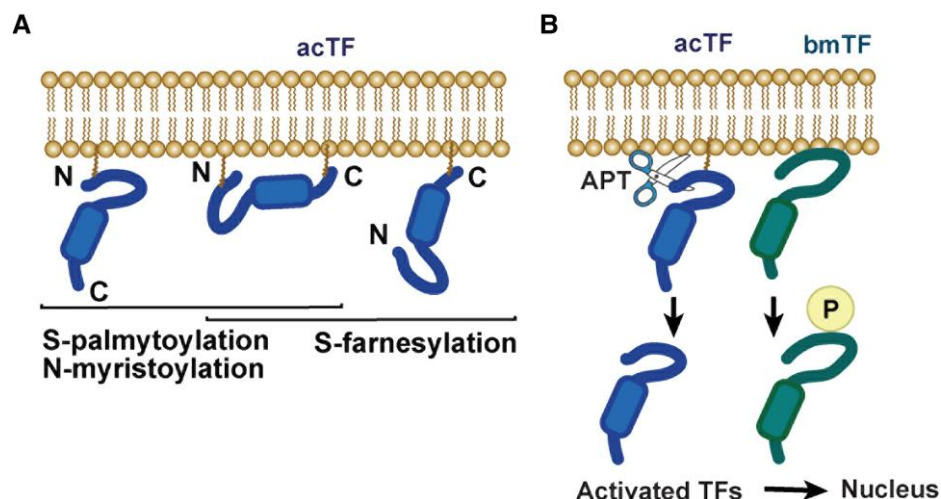


Figure 7. TFs peripherally associated with membranes. **A)** Types of possible acylation in TFs: S-palmitoylation, N-myristoylation, S-farnesylation. **B)** Mechanisms of acTF and mbTF release from membranes. De-acylation of a S-palmitoylated protein and phosphorylation of a mbTF. APT, acyl protein thioesterase.

retention is deployed only in some cell types or under specific developmental stages and therefore is not detectable under ectopic overexpression of fluorescently tagged TFs.

Orthology is often used to infer similar regulatory mechanisms across species, but this might be more challenging for predicting cytoplasmic retention, as shown for *Arabidopsis* ANAC060, which even within the same species has alleles that include or lack the transmembrane domain due to a polymorphism that alters splicing patterns (Li et al. 2014). Indeed, alternative splicing of exons harboring transmembrane domains can result in the formation of isoforms with nuclear and non-nuclear localizations, as described for bZIP60 (Nagashima et al. 2011) but are also potentially found in a number of other TFs (De Backer et al. 2022). While not formally an instance of cytoplasmic retention, as this mechanism involves a different translation product being directed to the nucleus, experimentally it can certainly be confused with nuclear shuttling if experiments are not conducted carefully. Alternate transcription start site selection can potentially also result in distinct protein isoforms, one, for example, with a N-terminal transmembrane domain, and the other without, as has been found for other plant protein domains (Mejía-Guerra et al. 2015).

The cytoplasmic retention of tmTFs offers another opportunity to control TF activity comparable to the use of glucocorticoid receptor fusions (Yamaguchi et al. 2015). TFs could be fused to a transmembrane domain containing a proteolytic site corresponding to a protease whose expression can be tightly controlled.

The impact of TF cytoplasmic retention on the kinetics of the regulatory cascade controlled by the TF is unclear and will likely need to be studied on a case-by-case basis, as the extra time involved in de novo TF synthesis could easily be comparable or even shorter than the activation of a signal transduction cascade necessary to activate and release the TF from the cytoplasm. Moreover, it cannot be ruled out

that the inactive extranuclear TF plays other yet to be determined functions that would provide a better reason than just simple kinetic considerations to the question of why so many TFs are controlled in this fashion.

Accession numbers

Gene identifications of TF listed in Figs. 1 and 2: BES1 (AT1G19350), BZR1 (AT1G75080), bZIP51 (AT1G43700), bZIP52 (AT1G06850), bZIP18 (AT2G40620), PIF7 (AT5G61270), bHLH039 (AT3G56980), FIT (AT2G28160), PHR1 (AT4G28610), RVE4 (AT5G02840), RVE8 (AT3G09600), HSFA8 (AT1G67970), VND6 (AT5G62380), ARF7 (AT5G20730), ARF19 (AT1G19220), E2Fa (AT2G36010), bZIP17 (AT2G40950), bZIP28 (AT3G10800), bZIP60 (AT1G42990), ANAC053 (AT3G10500), ANAC062 (AT3G49530), ANAC013 (AT1G69850), ANAC017 (AT1G34190), ANAC016 (AT1G34180), ANAC078 (AT5G04410), ANAC089 (AT5G22290), ANAC040 (AT2G27300), (AT4G35580), ANAC069 (AT4G01550), ANAC068 (AT4G01540), ANAC060 (AT3G44290), PHD (AT5G35210), R2R3-MYB (AT5G45420), SPL1(AT2G47070).

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