SCIENCE CHINA
Life Sciences

SPECIAL TOPIC: Plant biology: chromatin, small RNA and signaling
• RESEARCH PAPER •

March 2015 Vol.58 No.3: 270–275
doi: 10.1007/s11427-015-4807-6

Site-1 protease cleavage site is important for the ER stress-induced activation of membrane-associated transcription factor bZIP28 in *Arabidopsis*

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Received August 11, 2014; accepted December 26, 2014; published online January 27, 2015

Many sources of stress cause accumulation of unfolded or misfolded proteins in endoplasmic reticulum (ER), which elicits the unfolded protein response (UPR) to either promote cell survival or programmed cell death depending on different developmental context or stress severity. The *Arabidopsis* membrane-associated transcription factor, bZIP28, is the functional equivalent of mammalian ATF6, which relocates from the ER to the Golgi where it is proteolytically processed and released from the membrane to the nucleus to mediate the UPR. Although the canonical site-1 protease (S1P) cleavage site on the ER lumen-facing domain is well conserved between bZIP28 and ATF6, the importance of S1P cleavage on bZIP28 has not been experimentally demonstrated. Here we provide genetic evidence that the $RRIL^{573}$ site, but not the $RVLM^{373}$ site, on the lumen-facing domain of bZIP28 is critical for the biological function of bZIP28 under ER stress condition. Further biochemistry and cell biology studies demonstrated that the $RRIL^{573}$ site, but not the $RVLM^{373}$ site, is required for proteolytic processing and nuclear relocation of bZIP28 in response to ER stress. Our results reveal that S1P cleavage site plays a pivotal role in activation and function of bZIP28 during UPR in plants.

membrane-associated transcription factor, ER stress, unfolded protein response, bZIP28, S1P, S2P

Citation: Sun L, Zhang SS, Lu SJ, Liu JX. Site-1 protease cleavage site is important for the ER stress-induced activation of membrane-associated transcription factor bZIP28 in *Arabidopsis*. Sci China Life Sci, 2015, 58: 270−275, doi: 10.1007/s11427-015-4807-6

In eukaryotic cells, all the secreted proteins and most of the membrane proteins including membrane receptors enter the endoplasmic reticulum (ER) for protein folding and post-translational modifications [1]. When unfolded or misfolded proteins accumulate in ER, a well-conserved response called the unfolded protein response (UPR) operates to mitigate the ER stress by coordinating the protein folding demand with protein folding capacity [2–5]. UPR is fundamentally important not only for development but also for environmental stress adaptation [3,6,7].

In mammals, the UPR encompasses three pathways, the IRE1, PERK and ATF6 pathways [1]. The IRE1 pathway is the most conserved branch among yeast, plants and human. The ER-localized type I membrane protein IRE1 (for inositol requiring kinase 1) has an N-terminal ER stress sensing domain in the ER-lumen and C-terminal protein kinase and RNase domain facing the cytosol [8,9]. Oligomerization of IRE1 under ER stress conditions activates its cytosolic kinase and endo-ribonuclease activity [10], leading to non-conventional splicing of "intron" sequences presented on the mRNAs of yeast *HAC1*, mammalian *XBP1* or plant *bZIP60* [11–18]. Mammalian cells also have the PERK (for dsRNA-activated protein kinase-like ER kinase) pathway, which has not been found in yeast and plants [19]. ER stress induces activation of the ER-localized PERK to attenuate bulk protein translation by phosphorylating the eukaryotic

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translation initiation factor 2A subunit (eIF2A) [20].

ATF6 (for activating transcription factor 6) is a type II membrane-associated bZIP transcription factor. In response to ER stress, ATF6 relocates from the ER to the Golgi where it is processed by two Golgi-resident site-1 protease (S1P) and site-2 protease (S2P) [21]. Although ortholog of ATF6 is not presented in the *Arabidopsis* genome, the ER membrane-associated bZIP transcription factor bZIP28 is found to be the functional equivalent of mammalian ATF6, which is activated in a similar way to ATF6 [22–24]. The type I membrane protein site-1 protease (S1P), also known as the subtilisin kexin isozyme-1 (SKI-1), is a subtilisin-like serine protease (subtilase) that is conserved between mammals and plants [25,26]. Cleavage of ATF6 by S1P reduces the size of the lumen-facing domain of ATF6, which enables more efficient sequential cleavage of ATF6 within the Golgi membrane by site-2 protease (S2P) [27]. Although bZIP28 has little sequence similarity to ATF6, the canonical S1P cleavage site is well conserved between bZIP28 and ATF6 [22]. However, the importance of the S1P cleavage site in the lumen-facing domain of bZIP28 has not been experimentally demonstrated.

In the current study, we have examined function of the S1P cleavage site presented in bZIP28 in *Arabidopsis*. Genetic complementation analysis showed that the canonical S1P site $RRIL^{573}$ of bZIP28 is critical for its biological function while the $RVLM^{373}$ site of bZIP28 is less important. Subsequently protein immuno-blotting and sub-cellular localization studies provided evidence that S1P cleavage at $RRIL^{573}$ site is required for proteolytic processing and nuclear relocation of bZIP28 under ER stress condition in Arabidopsis.

1 Materials and methods

All plant materials were grown under standard conditions as reported previously [28]. For phenotypic analysis of ER stress sensitivity, seeds were germinated on agar plates containing half-strength Murashige and Skoog (MS) salts, 1.2% sucrose, 0.05% MES, plus or minus tunicamycin (TM, 0.05 μg mL^{-1}), pH 5.7. Seedlings were photographed and root length was measured 10 days after germination. For gene expression analysis and Western blotting, 10-day-old seedlings grown on 1/2 MS plates were transferred to liquid 1/2 MS medium plus or minus TM (5 μg mL^{-1}) or DTT (2 mmol L^{-1}) for 4 h, and whole seedlings were harvested for RNA or total protein extraction, respectively. First-strand cDNA was synthesized using Moloney Murine Leukemia Virus (M-MLV, Invitrogen, USA) reverse transcriptase according to the manufacturer's instructions. Primers for RT-PCR were the same as previously reported [28]. For complementation analysis, the full-length of native or mutated form of bZIP28 was expressed driven by the bZIP28 native promoter [28]. For protein sub-cellular localization and processing, N-terminus of bZIP28 (aa1-300) was replaced by GFP tag and 4× MYC tag and over-expressed using the 35S promoter [28]. The mutated forms of bZIP28 were generated using a Quick-Change Site-directed Mutagenesis Kit (Stratagene, USA). For stable transformation, various constructs were introduced into the wild-type (wt) or the double mutant (*zip28zip60*) of *bZIP28* and *bZIP60* by floral dip [29]. For confocal analysis, various constructs were co-transformed with the ER marker [30], Golgi marker [30] or the nucleus marker [31] in tobacco (*Nicotiana benthamiana*). After agro-infiltration for 2–3 d, tobacco epidermal leaves were treated with or without 2 mmol L^{-1} DTT for 4 h and observed under confocal microscope (Zeiss LSM A710). All the phenotypic data in the paper were subjected to Student's *t*-test.

2 Results and discussion

2.1 The S1P cleavage site $RRIL^{573}$ on the lumen-facing domain of bZIP28 is important for its biological function

S1P translates as a preprodomain-containing protein with a transmembrane domain near its C-terminus, which undergoes autocatalytic maturation in the ER through proteolytic cleavages [25,26]. Other substrates of S1P include the sterol-regulatory element binding protein (SREBP), ATF6, Luman/CREB3 and old astrocyte specifically induced substance (OASIS) [21,32–34]. Extensive biochemical studies conclude that S1P processes precursors on the consensus motif (R/K)X(hydrophobic)Z where Z is any amino acid, preferentially Leu or Thr, but excluding Val, Pro, Glu, Asp, or Cys [25,35]. We compared the putative S1P cleavage sites of *Arabidopsis* bZIP28 and its paralogs bZIP17 and bZIP49, as well as the *Arabidopsis* S1P maturation sites with that of human SREBP, ATF6 and S1P prosegments. It was found that Arg at P4 site and I/L at P2 site are well conserved (Table 1). In order to know whether site A and site B of bZIP28 are indeed processing sites, genetic complementation analysis was performed. Previous studies have successfully demonstrated the important roles of bZIP28 and bZIP60 in plant UPR [22,31,36,37]. Knock-outs both the *bZIP28* and *bZIP60* confer high ER stress sensitivity, and restoring the expression of either *bZIP28* or *bZIP60* with the respective native promoter rescues the ER stress sensitive phenotype of the double mutant *zip28zip60* [28]. Mutated forms of bZIP28 in which $RVLM^{373}$ and $RRIL^{573}$ were replaced with $GVSM^{373}$ and $GGIL^{573}$, respectively (Figure 1A), were expressed in the *zip28zip60* mutant plants under the bZIP28 native promoter. In each genetic complementation experiment, at least five transgenic lines were examined. It was found that the $GGIL^{573}$ and $GVSM^{373}$ expressing plants grew as well as the wild-type (wt) plants and the *zip28zip60* (*dm*) mutant plants under normal growth condition (Figure 1B and C). When the ER stress inducer tunicamycin (TM) was added into the growth medium, the

Table 1 S1P cleavage sites in human and *Arabidopsis*[a)]

Precursor protein	S1P cleavage site sequence								
	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′
Human pro-S1P Site B	V	F	**R**	S	**L**	K↓	**Y**	A	E
Human pro-S1P Site B′	P	Q	**R**	K	**V**	F↓	R	S	L
Human pro-S1P Site C	S	S	**R**	R	**L**	L↓	R	A	I
Human SREBP-2	S	G	**R**	S	**V**	L↓	S	F	E
Human SREBP-1a	P	G	**R**	N	**V**	L↓	G	T	E
Human ATF6	Q	R	**R**	H	**L**	L↓	G	F	S
Arabidopsis pro-S1P Site A	Y	Q	**R**	V	**L**	L↓	G	G	S
Arabidopsis pro-S1P Site B	W	S	**R**	H	**L**	L↓	A	Q	K
Arabidopsis bZIP17 Site A	R	D	**R**	V	**L**	D	T	S	R
Arabidopsis bZIP17 Site B	Q	N	**R**	R	**I**	L	R	G	L
Arabidopsis bZIP28 Site A	K	G	**R**	V	**L**	M	V	G	D
Arabidopsis bZIP28 Site B	K	N	**R**	R	**I**	L	E	G	L
Arabidopsis bZIP49 Site A	R	G	**R**	V	**L**	V	V	D	S
Arabidopsis bZIP49 Site B	K	N	**R**	R	**I**	L	S	G	G
Arabidopsis RALF23	I	N	**R**	R	**I**	L	A	T	R

a) The S1P cleavage sites in human proteins were experimentally demonstrated which are denoted with arrows. The conserved amino acids at P2 and P4 positions are highlighted in bold. S1P, Site-1 Protease; SREBP, Sterol-Regulatory Element Binding Protein; ATF6, Activating Transcription Factor 6; RALF23, Rapid Alkalinization Factor 23.

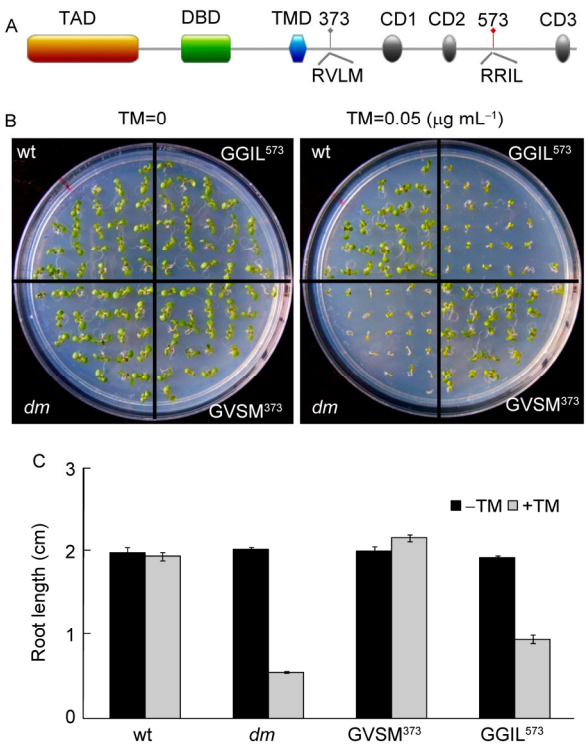

Figure 1 The canonical S1P site of bZIP28 is important for its biological function. A, Diagram of bZIP28 domains. The transcriptional activation domain (TAD), DNA binding domain (DBD), transmembrane domain (TMD) and three sequence conserved domains (CD1-CD3) are illustrated. Two putative S1P cutting sites (RVLM and RRIL) with their respective amino acid positions are also included. B and C, Phenotypic analysis of transgenic plants transformed with mutated forms of bZIP28 in the *zip28zip60* (*dm*) background driven by the bZIP28 native promoter. Plants were photographed (B), roots were pulled out from the growth medium and root length was measured (C) 10 days after germination. Bars depict SE (n=3).

GVSM^{373} expressing plants grew similarly to the wt (Figure 1B and C), indicating that mutations at RVLM^{373} does not affect the function of bZIP28. On the contrary, the GGIL^{573} expressing plants remained as sensitive to TM as the *dm* plants (Figure 1B and C), suggesting that the Args at RRIL^{573} site are critical for the function of bZIP28 under ER stress condition. Following 4 h of TM treatment, the UPR marker genes *BiP3* and *CNX1* were highly up-regulated in the wt while not in the *dm* plants (Figure 2A and B). When the mutated form GVSM^{373} of bZIP28 was expressed in the *dm* plants, both *BiP3* and *CNX1* were up-

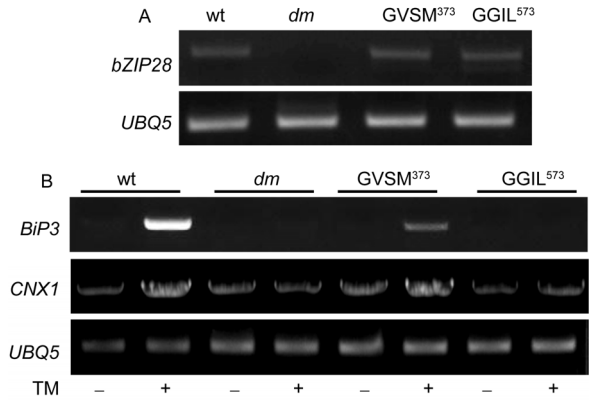

Figure 2 The canonical S1P site of bZIP28 is important for transcriptional regulation of UPR downstream genes. A, Validation of *bZIP28* expression in the transgenic plants under normal growth condition. Primer was designed to amplify either the native gene or the transgene. B, Up-regulation of UPR genes by ER stress in the wild-type, *zip28zip60* double mutant (*dm*), and transgenic plants transformed with mutated form of bZIP28 in the *dm* background. Semi-quantitative RT-PCR was performed and *UBQ5* was used as a control.

regulated by TM treatment (Figure 2A and B). Partial restoration of UPR downstream gene expression could be explained because *bZIP60* was still not functional in the transgenic plants. However, when the mutated bZIP28 form GGIL^{573} was expressed in the *dm* plants, neither *BiP3* nor *CNX1* was up-regulated by TM treatment (Figure 2A and B), which is consistent with the ER stress sensitive phenotype of GGIL^{573} expressing plants (Figure 1B and C). Thus, RRIL^{573} site is crucial for the biological function of bZIP28 during UPR.

2.2 The S1P cleavage site RRIL^{573} on the lumen-facing domain of bZIP28 is important for its proteolytic processing

The bZIP28 precursor is a type II membrane protein with the C-terminus facing the ER lumen. It is processed to a shorter form upon ER stress [22,28]. We were interested in knowing how the RRIL^{573} site contributes to the proteolytic processing. To increase protein stability, green fluorescent protein (GFP) and 4x MYC were fused to the bZIP28 C-terminus (aa 301–675) (M-WT, Figure 3A). Mutated forms of bZIP28 were also generated (Figure 3A). These bZIP28 derivates were over-expressed in *Arabidopsis* plants driven by the 35S promoter. Western blotting analysis indicated that M-WT was processed after DTT treatment (Figure 3B). Although the M-GVSM^{373} precursor was less stable, DTT treatment accelerated the proteolytic processing (Figure 3B). DTT treatment did not induce the processing of

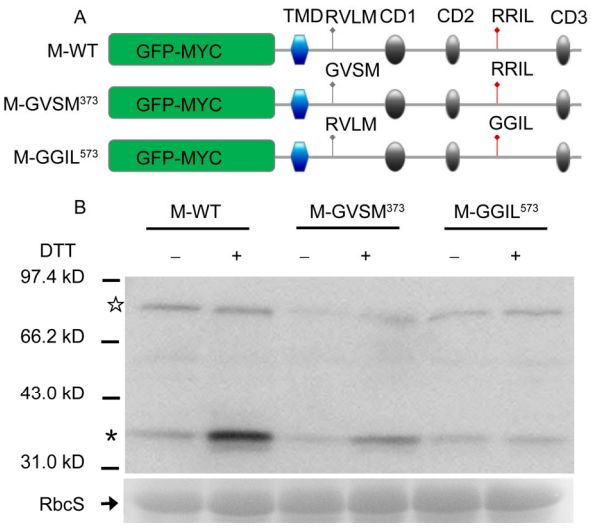

Figure 3 The canonical S1P site of bZIP28 is important for proteolytic processing of bZIP28. A, Diagram showing different forms of bZIP28 derivates. N-terminus of bZIP28 (aa 1–300) is replaced with GFP-MYC in M-WT. Abbreviations of TMD and CD1 to CD3 are the same as in Figure 1. The putative S1P cutting sites and the mutated sites are listed above the illustrations, respectively. B, Western blotting analysis of protein processing of fusion proteins in response to DTT treatment. The star and asterisk denote position of the precursor and the activated form, respectively. Fusion proteins were detected with *anti*-MYC antibody. Coomassie blue staining of RbcS serves as a loading control.

M-GGIL^{573} (Figure 3B). The consensus motif (R/K)X (hydrophobic)Z where Z is any amino acid, preferentially Leu or Thr, but excluding Val, Pro, Glu, Asp, or Cys, was identified for S1P processing in mamalian cells [25,35]. Based on our results, Met at Z site blocks S1P processing in *Arabidopsis* plant. Previously, the peptide growth factor rapid alkalinization factor 23 (RALF23) was identified as one of the S1P substrates in *Arabidopsis*, and the two Args at RRIL^{85} site on RALF23 were important for its proteolysis [38]. Our results further demonstrate that the RRIL^{573} site on the lumen-facing domain of bZIP28 is required for its proteolytic processing.

2.3 The S1P cleavage site RRIL^{573} on the lumen-facing domain of bZIP28 is important for its nuclear relocation

The hallmark of bZIP28 pathway is the relocation of bZIP28 from the ER to the nucleus via the Golgi [22,28]. The failure of protein processing with M-GGIL^{573} prompted us to investigate the sub-cellular localizations of each bZIP28 derivate. The GFP tagged bZIP28 with native C-terminus (M-WT, Figure 3A) was shown to be translocated from the ER to the nucleus in response to DTT treatment in tobacco (*Nicotiana benthamiana*) epidermal cells after agro-infiltration (Figure 4). Prior to DTT treatment, M-GVSM^{373} and M-GGIL^{573} were co-localized with the ER marker CD3-959 [30] (Figure 4). Upon DTT treatment, M-GVSM^{373} protein was found in the nucleus (Figure 4), overlaying with a nuclear marker [31]. However, M-GGIL^{573} protein was co-localized with a Golgi body marker CD3-967 [30] after DTT treatment (Figure 4). These results strongly suggest that GGIL^{573} mutation does not affect ER-to-Golgi movement but rather affects proteolytic processing and further nuclear relocation. Previously we have shown that the bZIP28 derivate MLD2 in which the downstream sequences of RRIL^{573} site were deleted, was sufficient for S2P cleavage and ER-to-Golgi-to-nucleus movement [28], indicating that S1P cleavage is to generate a shorter ER lumen-facing C-terminus of bZIP28 for further S2P processing.

3 Conclusion

Regulated nuclear relocation of transcription factors from organelle membrane represents a regulatory control on gene expression in eukaryotic cells. Proteolytic cleavage can be mediated by regulated intramembrane proteolysis. S1P plays important roles in activation of membrane-associated transcription factors, in which pre-cleavage of the lumen-facing sequences by S1P enables the substrate more accessible to further cleavage by S2P [39]. Previously we have demonstrated the important role of S1P in activation of bZIP17 and RALF23 in Arabidopsis [26,38]. In the current paper we have shown that S1P cleavage is also important

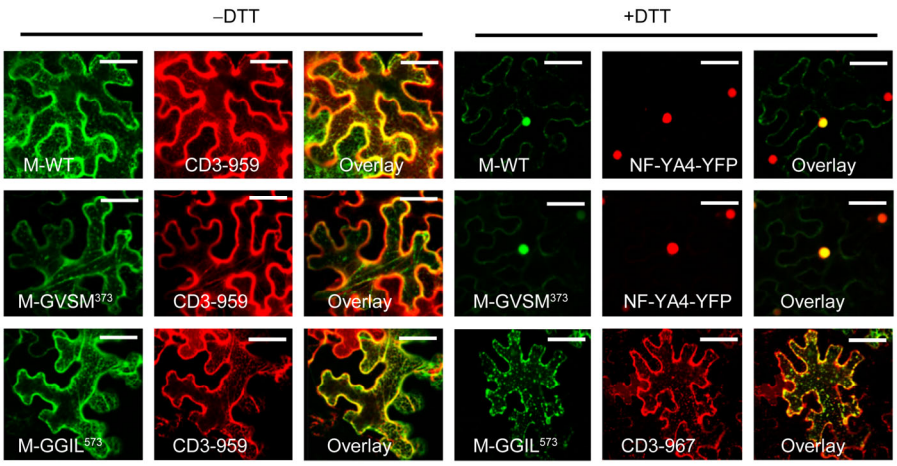

Figure 4 The canonical S1P site of bZIP28 is important for nuclear relocation of bZIP28. Confocal images of various fusion proteins (details in Figure 3A) in tobacco epidermal cells. The ER marker (CD3-959), Golgi marker (CD3-967) and nucleus marker (NF-YA4-YFP) were co-transformed with bZIP28 derivate, respectively. Scale bar, 50 μm.

for the biological function of the membrane-associated transcription factor bZIP28. The RRIL^{573} site, but not RVLM^{373} site, on the ER lumen-facing domain of bZIP28 is required for the proteolytic processing and nuclear relocation of bZIP28 during plant UPR.

This work was supported by grants from the National Basic Research Program of China (973 Program, 2012CB910500), the National Natural Science Foundation of China (31171157, 31222008), and the Specialized Research Fund for the Doctoral Program of Higher Education (20130071110011).

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