Letter to the Editor

Endoplasmic reticulum stress-induced accumulation of VAMP721/722 requires CALRETICULIN 1 and CALRETICULIN 2 in Arabidopsis

Running Title: ER stress-induced VAMP721/722 accumulation

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Excessive demand for translation and protein folding in the endoplasmic reticulum (ER) can cause ER stress in plants. Here, we show that CALRETICULIN 1 (CRT1) and CRT2 are critical components in the accumulation of VESICLE-ASSOCIATED MEMBRANE PROTEIN 721 (VAMP721) and VAMP722 during ER stress responses. We show that CRT2 interacts with VAMP722 and that CRT1/2 post-translationally maintain elevated VAMP721/722 levels under ER stress. The greater growth inhibition in VAMP721/722-deficient plants, induced by tunicamycin, suggests that plants under ER stress maintain physiological homeostasis, at least in part, by regulating VAMP721/722 levels, as VAMP721/722 are known to participate in various biological processes.

The VESICLE-ASSOCIATED MEMBRANE PROTEIN 721 (VAMP721) and VAMP722 are among the core soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) factors driving exocytosis in plants (Kwon et al. 2008). VAMP721/722 influence a range of biological processes, including cell division, growth, development and environmental stress responses (Kwon et al. 2008; El Kasami et al. 2013; Yi et al. 2013; Yun et al. 2013; Ichikawa et al. 2014). Although the mechanism remains poorly understood, plants are thought to control the distinct cellular activities of VAMP721/722 through formation of different SNARE complexes with various plasma membrane (PM) syntaxins and/or by regulating their expression levels (Yun and Kwon 2017).

ER stress, which is caused by accumulation of misfolded proteins in the ER, can also be induced by environmental stresses, leading to a higher burden on secretory proteins in the ER in plants (Bao and Howell 2017; Nawkar et al. 2018). This ER stress often results in growth retardation in Arabidopsis (Christensen et al. 2008; Christensen et al. 2010). As VAMP721/722 are required for various stress responses, and as they are also required for sustained growth during stress responses (Kwon et al. 2008; Yi et al. 2013; Yun et al. 2013), we investigated their function during ER stress response in Arabidopsis. Expression of VAMP721 and VAMP722 were examined upon induction of ER stress by treating 10-day-old Arabidopsis seedlings with tunicamycin (TM), which interferes with N-glycosylation, resulting in unfolded proteins, thereby driving ER stress. Immunoblot assays using anti-VAMP721/722 antibody showed that 5 μg/mL TM caused a slight increase in VAMP721/722 at 1 day after initiation of treatment (DAT), and reached near maximum levels by 2 DAT (Figure S1). Using quantitative RT-PCR, we determined that transcription of both VAMP721 and VAMP722 was also elevated by TM at 2 DAT (Figure 1A), accompanying the increased VAMP721/722 expression.
protein levels (Figure 1B). Similar result was observed with both the ER stress inducers L-azetidine-2-carboxylic acid (AZC) (Figure 1C), which is analogous to proline, and dithiothreitol (DTT) (Figure S2), which induces ER stress by blocking disulfide bond formation in newly synthesized proteins, in that they enhanced VAMP721/722 protein levels. Taken together, these results indicate that VAMP721/722 expression is induced by ER stress, resulting in correspondingly higher protein accumulation. The higher level of transcription by VAMP722 over VAMP721 (Figure 1A) implies that the increased VAMP721/722 protein levels are primarily contributed to by VAMP722.

CALRETICULIN (CRT) is a key protein responsible for maintaining intracellular Ca²⁺ homeostasis and directing proper folding of newly synthesized proteins in the ER (Michalak et al. 2009). In Arabidopsis, CRT1 and CRT2 function redundantly as folding-related chaperones, whereas CRT3 plays a specific role in the maturation of BRI1 and EFR PM receptors (Liu and Li 2014). Since CRT1/2 are required for sustained growth under ER stress (Christensen et al. 2008; Christensen et al. 2010), and VAMP721/722 are important for steady growth during abiotic and biotic stress responses (Yi et al. 2013; Yun et al. 2013), we tested whether CRT1/2 are involved in the ER stress-induced VAMP721/722 accumulation.

When we treated crt1/2 double mutant plants (Figure S3) with TM, we observed that VAMP721/722 protein levels were not elevated (Figure 1D). These results indicated that CRT1/2 are necessary for VAMP721/722 accumulation under ER stress. However, the induction of VAMP721/722 transcription by TM was maintained in these crt1/2 plants (Figure 1A). This differential effect on transcript vs. protein accumulation indicated that the ER stress-induced VAMP721/722 elevation occurs via regulation at the post-transcriptional level. Such regulation might involve CRT1/2-directed monitoring of VAMP721/722 stability, but does not appear to occur via a CRT1/2-stabilized transcription factor, as the VAMP721/722 transcriptional response was not disrupted in crt1/2 double mutant plants.

The comparable VAMP721/722 levels observed under TM treatment in the wild-type (WT) and crt1 and crt2 single mutant plants (Figure 1D) additionally suggested that CRT1/2 redundantly control the stability of ER stress-induced VAMP721/722. As misfolded proteins are detrimental to growth and development, they are cleared in stressed cells via the 26S proteasome pathway (Liu and Li 2014). To test whether ER stress-induced VAMP721/722 proteins are degraded via the 26S proteasome pathway in CRT1/2-depleted plants, we pre-treated plants with the 26S proteasome inhibitor MG132, followed by TM treatment. As previously reported (Yi et al. 2013), MG132 slightly increased VAMP721/722 abundance in all tested genotypes (Figure S4), suggestive of constitutive degradation of a part of VAMP721/722 proteins in plants. In crt1/2 plants pre-treated with MG132, we observed that
TM-induced VAMP721/722 protein levels were indistinguishable from those in the other genotypes (Figure 1D). This finding strongly suggested that during ER stress, VAMP721/722 are under the control of CRT1/2, in the absence of which excessive VAMP721/722 proteins are removed by the 26S proteasome pathway.

We next examined CRT1/2–VAMP721/722 interactions. CRT1 and CRT2 are very similar, having 86% identity and 92% similarity, and VAMP721 and VAMP722 are different only in seven amino acids (Kwon et al. 2008). Thus, recombinant hemagglutinin-tagged CRT2 (HA-CRT2) and glutathione-S-transferase-fused VAMP722 proteins lacking the transmembrane motif (GST-VAMP722ΔTM) were expressed in and purified from Escherichia coli. Immunoblot analysis of Glutathione Sepharose 4B-precipitates with anti-HA antibody demonstrated that VAMP722 directly interacted with CRT2 (Figure 2A). The lower molecular weight of recombinant HA-CRT2, compared to the endogenous CRT2, indicated that N-glycosylation of CRT2 is taking place in plants (Figures 2A, S3) (Christensen et al. 2010). Co-immunoprecipitation with protein extracts from GFP-VAMP722-expressing transgenic plants (Kwon et al. 2008) enabled detection of GFP-VAMP722 in CRT1/2 immunoprecipitates (Figure 2B). In the reciprocal co-immunoprecipitation assay, we also detected CRT1/2 in the GFP-VAMP722 immunoprecipitates (Figure 2C). These results indicated that CRT1/2 interact with VAMP721/722 in planta. The enhanced interactions between CRT1/2 and GFP-VAMP722 after TM treatment (Figure 2B, C) additionally suggested that CRT1/2 have a VAMP721/722-stabilizing function under ER stress.

Although CRTs are thought to mainly reside in the ER lumen, they were reported to be translocated to the cytoplasm (Afshar et al. 2005; Jia et al. 2009), where the majority of VAMP721/722 is exposed. In addition, a portion of CRT was also reported to be retranslocated from the ER to the cytosol (Brandizzi et al. 2003). As CRT1/2 interact with GFP-VAMP722 and their interaction was increased by TM (Figure 2B, C), we therefore examined CRT1/2 localization by comparing cytosolic and microsomal fractions. Interestingly, we detected CRT1/2 in the cytosolic fraction even in the absence of TM (Figure S5). TM treatment greatly elevated the cytosolic CRT1/2 levels (Figure S5), further supporting a protective function of CRT1/2 in stabilizing VAMP721/722 during ER stress responses. However, it is also possible that an additional mediator may stabilize TM-induced VAMP721/722 levels, because the elevation of VAMP721/722 abundance in response to TM is rather late (Figure S1).

Given that VAMP721/722 are required for sustained growth during stress responses (Yi et al. 2013; Yun et al. 2013), we next tested whether plant growth under ER stress is dependent upon VAMP721/722 abundance. To rule out stress from tissue damage during
genotyping, we included only vamp721 and vamp722 plants, in which functional
gene-dependent differences in VAMP721/722 levels were clearly observed (Figure 2E)
(Kwon et al. 2008). In this experiment, we treated 10-day-old seedlings with 0.1 μg/mL TM, as
the concentration used in expression analyses was too high for plant growth measurement
(Christensen et al. 2008). After growth for an additional 7 d in the presence of TM, we
observed overall growth inhibition but not any other defects in all genotypes, as previously
reported (Christensen et al. 2008; Christensen et al. 2010). Interestingly, we detected greater
inhibition of growth in vamp721 and vamp722 mutants than in WT plants (Figure 2D). The
reduced levels of VAMP721/722 in vamp721 and vamp722 plants compared to WT at 7 DAT
(Figure 2E) strongly suggested that VAMP721/722 are responsible for steady growth during
ER stress responses. Notably, we determined that VAMP721/722 levels at 7 DAT were
decreased in all genotypes when compared to the untreated controls, although, as previously
reported, CRT1/2 levels were elevated (Figure 2E) (Christensen et al. 2008). Based on these
findings, we monitored VAMP721/722 levels in WT plants treated with 0.1 μg/mL TM over the
7-day period. Both CRT1/2 and VAMP721/722 levels increased gradually and reached a
maximum at 3 DAT (Figure S6). While CRT1/2 levels were maintained until 7 DAT,
VAMP721/722 levels gradually diminished until 7 DAT, compared to untreated plants (Figures
2E, S6). As VAMP721/722 are required for plant growth, these results suggested that plant
growth inhibition by TM is, at least in part, due to the down-regulated VAMP721/722 levels
(Kwon et al. 2008).

In summary, we show that CRT1/2 are required for VAMP721/722 accumulation during
ER stress. Our findings that VAMP721/722 transcript levels in crt1/2 plants were
indistinguishable from WT, but that TM treatment did not elevate VAMP721/722 protein levels
in crt1/2, indicate that CRT1/2 post-transcriptionally regulate VAMP721/722 levels under ER
stress. As CRT2 and VAMP722 directly interact, and MG132 treatment elevates
VAMP721/722 levels in crt1/2 plants in a manner similar to that in WT, it may be concluded
that CRT1/2 could monitor the stability of ER stress-induced VAMP721/722, which is
otherwise degraded via the 26S proteasome.

As key components of a default secretory pathway, VAMP721/722 are engaged in
diverse physiological processes, including division, growth and stress responses (Kwon et al.
2008; El Kasami et al. 2013; Yi et al. 2013; Yun et al. 2013 Ichikawa et al. 2014). Therefore,
plants likely maintain cellular homeostasis, at least in part, by elevating VAMP721/722 under
ER stress. As such, it is not surprising that CRT1/2-deficient plants, which fail to increase
VAMP721/722 levels, are hypersensitive both to TM (Christensen et al. 2008; Christensen et
al. 2010) and abiotic stresses (Kim et al. 2013). Intriguingly, TM-triggered ER stress elevated
the cytosolic levels of CRT1/2 as well as BiP (Figure S5). The same size of VAMP721/722-interacted CRT1/2 as non-interacted glycosylated forms (Figure 2C) implies that CRT1/2 might be translocated from the ER lumen to the cytosol, which can be promoted by ER stress.

Although little is known in plants, it was reported that the amino-terminal arginylation of ER-residing proteins, including CRT and BiP, stimulates their cytosolic relocalization, which is induced by ER stress in HeLa cells (Cha-Molstad et al. 2015). This post-translational modification is regarded to be conserved in plants, to regulate various aspects of plant growth and stress responses (Tasaki et al. 2012; Gibbs et al. 2016; Dissmeyer et al. 2018). Therefore, a possible scenario is that under ER stress, cytosolically retranslocated CRT1/2 interact with and protect VAMP721/722, which are synthesized in the cytosol due to the lack of a signal peptide. It was recently reported that a set of SNARE complex proteins, including VAMP721/722, are delivered in an inactive form to a target site (Karnahl et al. 2017). Hence, it will be of great interest in future work to understand whether VAMP721/722 accumulation is controlled in either the monomeric form or in a SNARE complex by CRT1/2 when under ER stress.

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AUTHOR CONTRIBUTIONS
S.K., C.K., and H.S.Y. designed the experiments. S.K. performed the experiments. C.K., and H.S.Y. wrote the manuscript. All authors read and approved the manuscript.
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Figure legends

Figure 1. CRT1/2-dependent elevation of VAMP721/722 levels by ER stress
(A) Transcription of VAMP721 and VAMP722 was increased by tunicamycin (TM). Plants grown in liquid medium for 10 d were treated without (blue) or with (green) 5 µg/mL TM for 2 d, and RNA extracts were subject to quantitative RT-PCR analysis. Relative transcript amounts of VAMP721 and VAMP722 were normalized against those of ACTIN2. Data are mean ± SD (n = 6 biological replicates). (B, C) VAMP721/722 protein levels were elevated by ER stress. Plants grown in liquid medium for 10 d were treated with the indicated amounts of TM (B) or AZC (C) for 2 d. Protein extracts were then subject to immunoblot with anti-VAMP721/722 antibody. Equal loading was visualized by Rubisco staining with Ponceau S. (D) TM-induced VAMP721/722 protein degradation, via the 26S proteasome pathway, in crt1/2 plants.
indicated genotypes were grown in liquid medium and treated with 5 \( \mu \text{g/mL} \) TM for 2 d. To test the involvement of the 26S proteasome, plants were pretreated with 10 nM MG132 4 h before TM treatment. Protein extracts were then subject to immunoblot with anti-VAMP721/722 antibody. Equal loading was visualized by Rubisco staining with Ponceau S.

**Figure 2. Sustained growth under ER stress requires VAMP721/722**

(A) CRT2 directly interacts with VAMP722. Recombinant HA-CRT2 was mixed with recombinant GST or GST-VAMP722ΔTM, all of which were purified from *E. coli*. Glutathione Sepharose 4B-bound precipitates were then subjected to immunoblotting with anti-HA or anti-GST antibody. (B, C) *In planta* interaction between CRT1/2 and VAMP722. Protein extracts from GFP-VAMP722-expressing plants treated with or without 5 \( \mu \text{g/mL} \) tunicamycin (TM) for 2 d were immunoprecipitated (IP) with anti-CRT1/2 (B) or anti-GFP antibody (C). The immunoprecipitates were then subjected to immunoblotting (IB) with anti-GFP (B) or anti-CRT1/2 antibody (C). A portion of the protein extracts (30 \( \mu \text{g}, 3\% \)) was analyzed by immunoblotting with anti-CRT1/2 or anti-GFP antibody (Input). Equal loading was visualized by Rubisco staining with Ponceau S. To show a change in molecular weights of CRT1/2, immunoprecipitated CRT1/2 with anti-GFP antibody were blotted next to non-precipitated CRT1/2 (input) (C). short, short exposure; long, long exposure. (D, E) TM-induced exaggerated growth inhibition in VAMP721/722-deficient plants. Plants of the indicated genotypes, grown in liquid medium, were treated with or without 0.1 \( \mu \text{g/mL} \) TM, and grown for an additional 7 d. Weight of TM-treated plants was then divided by that of untreated control plants (D). Data are mean \( \pm SD \) \((n = 7 \) biological replicates\) *, <0.05; **, <0.01 in comparison to WT (Student t-test). Protein extracts from TM-treated and untreated plants were subject to immunoblot with anti-VAMP721/722 or anti-CRT1/2 antibody. (E). Equal loading was visualized by Rubisco staining with Ponceau S.

**SUPPORTING INFORMATION**

**Figure S1. Time-dependent induction of VAMP721/722 by tunicamycin**
WT seedlings grown in liquid MS medium were treated with the indicated amounts of tunicamycin (TM) for the indicated times. Protein extracts were separated on a 10% acrylamide gel and blotted with anti-VAMP721/722 antibody. Equal loading was visualized by staining Rubisco with Ponceau S.

**Figure S2. Dithiothreitol-induced VAMP721/722 elevation**

WT seedlings grown in liquid MS medium were treated with the indicated amounts of dithiothreitol (DTT) for 24 h. Protein extracts were separated on a 10% acrylamide gel and blotted with anti-VAMP721/722 antibody. Equal loading was visualized by staining Rubisco with Ponceau S. As DTT is toxic, plants could not be treated for longer than 1 d.

**Figure S3. Specificity of anti-CRT1/2 antibody**

(A) Protein extracts from the indicated plant genotypes were separated on a 10% acrylamide gel and blotted with anti-CRT1/2 antibody, generated in a rabbit using full-length recombinant CRT2. Note that either or both bands, corresponding to CRT1 or CRT2, disappeared in the indicated single or double mutant plants. Equal loading was visualized by staining Rubisco with Ponceau S. (B) Protein extracts from GFP-VAMP722-expressing plants treated with 5 μg/mL tunicamycin for 2 d were immunoprecipitated with rabbit-raised anti-CRT1/2, or mouse-raised anti-GFP antibody (IP). Immunoprecipitates were then subjected to immunoblotting with anti-GFP or anti-CRT1/2 antibody (IB). As a negative control, rabbit IgG or mouse IgG was used for immunoprecipitation.

**Figure S4. Effect of MG132 on VAMP721/722 levels**

The indicated plant genotypes were grown in liquid medium and treated with either 10 nM MG132 or 5 μg/mL tunicamycin (TM) for 2 d. Protein extracts were then subject to immunoblot with anti-VAMP721/722 antibody. Equal loading was visualized by Rubisco staining with Ponceau S.

**Figure S5. Cytosolic localization of CRT1/2**

WT seedlings grown in liquid MS medium were treated for 2 d, with or without the indicated amounts of tunicamycin (TM). Seedlings were homogenized and separated into cytosolic (Cy) and microsomal (M) fractions. Both fractions were then subject to immunoblot with anti-CRT1/2 antibody. To detect BiP, PEN1 or CCOAOMT1 (caffeoyl-CoA o-methyltransferase 1), as microsomal or cytosolic markers, respectively, both fractions were also blotted with anti-BiP, anti-PEN1 or anti-CCOAOMT1 antibody.
Figure S6. Accumulation pattern of VAMP721/722 in response to ER stress
WT seedlings grown in liquid MS medium were treated with tunicamycin (TM) for the indicated time. Protein extracts were separated on a 10% acrylamide gel and blotted with anti-VAMP721/722 or anti-CRT1/2 antibody. Equal loading was visualized by staining Rubisco with Ponceau S.

Supplementary Methods

Plant growth
All plants used for experiments were grown in liquid MS medium containing 1% sucrose at 22°C with a 10 h photoperiod. To analyze the expression of VAMP721 and VAMP722, 10-d-old plants were treated with the indicated amounts of TM or AZC for the indicated time. To test an involvement of the 26S proteasome in VAMP721/722 degradation, the indicated plant genotypes including crt1/2 mutants were pretreated with 10 nM MG132, a 26S proteasome inhibitor, at 4 h before TM treatment. To analyzed the TM-induced growth inhibition, WT, vamp721 and vamp722 plants grown for 10 d were treated ± 0.1 μg/mL TM, and further grown for 7 d. Due to small seedling size, 3 plants were weighed and averaged. The degree of growth inhibition was assessed by dividing the weight of TM-treated plants by that of untreated controls. To monitor VAMP721/722 levels over the period of TM treatment, 10-d-old WT plants were treated with 0.1 μg/mL TM for the indicated time.

Real-time RT-PCR analysis
Total RNA was extracted from plants with easy-spin™ IIp Plant RNA Extraction Kit (Intron Biotechnology) according to the manufacturer’s instruction. Reverse transcribed cDNAs were then subject to PCR with cycles of 10 s at 95°C, 10 s at 55°C, and 15s at 72°C with a LightCycler® Nano System (Roche). Used primers are; 5’-ATCCCTTGCGAACCAGTC and 5’-AACCAGCACACATCAGC for VAMP721, 5’-GATTCCTATGGCTTTCTT and 5’-CTTGCTATCATCAGG for VAMP722, and 5’-ATGGAAATCTGCTTGGAAATCCAC and 5’-TTTGCTCAGTACGCGT for Actin2. Relative amounts of VAMP721 and VAMP722 transcripts were normalized against Actin2, and calculated by LightCycler® Nano Software (Roche).

Immunoblot analysis
To extract total proteins, plants were ground in liquid nitrogen and suspended in 1 x PBS (pH 8.0) containing 1% Triton X-100. After centrifugation, the supernatant was used for
immunoblotting. Proteins were separated on a 1% acrylamide gel and blotted with anti-CRT1/2 or anti-VAMP721/722 antibody. To show equal loading, Rubisco was stained with Ponceau S. To separate cytosolic and microsomal fractions, liquid-grown WT seedlings treated \( \pm 5 \mu g/mL \) TM for 2 d were homogenized and subject to cytosolic/microsomal fractionation with Minute\(^\text{TM}\) Plant Microsomal Membrane Extraction Kit (Invent Biotechnologies), according to the manufacturer’s instructions. The localization of CRT1/2 was analyzed by immunoblot with anti-CRT1/2 antibody. BiP, PEN1 or CCOAOMT1 (caffeoyl-CoA o-methyltransferase 1) was immuno-detected as a marker of microsomal or cytosolic fraction, respectively.

**Protein-protein interaction assay**

To bacterially express recombinant proteins, DNA sequences corresponding to CRT2 full-length and TM-lacking VAMP722 (VAMP722\(\Delta\)TM) were amplified by PCR and separately cloned into the pGEX-6p-1 plasmid vector (GE Healthcare Life Sciences). After introducing the plasmid DNA constructs into *E. coli* BL21-CodonPlus (Agilent Technologies), expression of recombinant proteins was induced by 1 mM IPTG. By passing bacterial lysates through glutathione Sepharose 4B (GE Healthcare Life Sciences), GST-fused recombinant proteins were purified. To remove the GST moiety, bead-bound GST fusion proteins were digested with Prescision protease (GE Healthcare Life Sciences), and the eluted proteins were collected. Purified CRT2 was used for generating anti-CRT1/2 antibody in a rabbit. To N-terminally tag CRT2 with an HA, DNA sequence corresponding to HA was added in a forward primer when amplified.

To test the *in vitro* interaction between GST-VAMP722\(\Delta\)TM and HA-CRT2, equimolar purified proteins were mixed in a binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, and 5% glycerol). Interacted proteins were then obtained by precipitating GST-VAMP722\(\Delta\)TM with glutathione Sepharose 4B. Precipitates were subject to immunoblot with anti-HA or anti-GST antibody. For testing the *in planta* interaction between CRT1/2 and VAMP721/722, proteins were extracted from GFP-VAMP722-expressing transgenic plants treated \( \pm 5 \mu g/mL \) TM for 2 d. Protein extracts were first pre-cleared with Protein A/G-agarose beads (Santa Cruz Biotech) and incubated with either anti-CRT1/2 or anti-GFP antibody. The immunoprecipitates with Protein A/G-agarose beads were then analyzed by immunoblot with anti-GFP or anti-CRT1/2 antibody. As negative control, mouse IgG or rabbit IgG was used for immunoprecipitation.
Figure 1
Figure 2