Molecules and Cells

Effect of an Endoplasmic Reticulum Retention Signal Tagged to Human Anti-Rabies mAb SO57 on Its Expression in *Arabidopsis* and Plant Growth

Ilchan Song[1,11], Young Koung Lee[2,11], Jin Wook Kim[3,11], Seung-Won Lee[1], Se Ra Park[1], Hae Kyung Lee[4], Soyeon Oh[1], Kinarm Ko[5], Mi Kyung Kim[6], Soon Ju Park[7], Dae Heon Kim[8], Moon-Soo Kim[9], Do Sun Kim[10], and Kisung Ko[1,*]

[1]Department of Medicine, College of Medicine, Chung-Ang University, Seoul 06974, Korea, [2]Plasma Technology Research Center, National Fusion Research Institute, Gunsan 54004, Korea, [3]Department of Urology, College of Medicine, Chung-Ang University, Seoul 06974, Korea, [4]Division of Zoonotic and Vector Borne Diseases Research, Korea National Institute of Health, Osong 28159, Korea, [5]Department of Stem Cell Biology, Konkuk University School of Medicine, Seoul 05029, Korea, [6]Department of Pathology, College of Medicine, Chung-Ang University, Seoul 06974, Korea, [7]Division of Biological Sciences, Wonkwang University, Iksan 54538, Korea, [8]Department of Biology, Sunchon National University, Sunchon 57922, Korea, [9]Department of Chemistry, Western Kentucky University, Bowling Green, KY 42101, USA, [10]Vegetable Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration (RDA), Wanju 55365, Korea, [11]These authors contributed equally to this work.
*Correspondence: ksko@cau.ac.kr
https://doi.org/10.14348/molcells.2021.2002
www.molcells.org

Transgenic *Arabidopsis thaliana* expressing an anti-rabies monoclonal antibody (mAb), SO57, was obtained using *Agrobacterium*-mediated floral dip transformation. The endoplasmic reticulum (ER) retention signal Lys-Asp-Glu-Leu (KDEL) was tagged to the C-terminus of the anti-rabies mAb heavy chain to localize the mAb to the ER and enhance its accumulation. When the inaccurately folded proteins accumulated in the ER exceed its storage capacity, it results in stress that can affect plant development and growth. We generated T_1 transformants and obtained homozygous T_3 seeds from transgenic *Arabidopsis* to investigate the effect of KDEL on plant growth. The germination rate did not significantly differ between plants expressing mAb SO57 without KDEL (SO plant) and mAb SO57 with KDEL (SOK plant). The primary roots of SOK agar media grown plants were slightly shorter than those of SO plants. Transcriptomic analysis showed that expression of all 11 ER stress-related genes were not significantly changed in SOK plants relative to SO plants. SOK plants showed approximately three-fold higher mAb expression levels than those of SO plants. Consequently, the purified mAb amount per unit of SOK plant biomass was approximately three times higher than that of SO plants. A neutralization assay revealed that both plants exhibited efficient rapid fluorescent focus inhibition test values against the rabies virus relative to commercially available human rabies immunoglobulins. KDEL did not upregulate ER stress-related genes; therefore, the enhanced production of the mAb did not affect plant growth. Thus, KDEL fusion is recommended for enhancing mAb production in plant systems.

Keywords: antibody, glycosylation, plant stress, rabies virus, transgenic

Received 27 December, 2020; revised 20 August, 2021; accepted 27 August, 2021; published online 29 October, 2021

eISSN: 0219-1032

INTRODUCTION

Rabies is one of the most frequently diagnosed viral diseases worldwide, particularly in developing countries in Asia and Africa (Cleaveland and Hampson, 2017; Taylor and Nel, 2015). Recombinant therapeutic anti-viral antibodies and preventive vaccines have been produced in plants (Kang et al., 2016; Kim et al., 2018; Shin et al., 2019; Yao et al., 2015). *Arabidopsis* plants have a short life span, easily manageable growth conditions, and high total soluble proteins (TSPs). Thus, it is often selected as a host for the production of anti-rabies monoclonal antibodies (mAbs) (Koornneef and Meinke, 2010; Song et al., 2015; 2018b). In plants, the endoplasmic reticulum (ER) retention signal KDEL has been fused to recombinant proteins in the ER to increase their stability and production level (De Meyer and Depicker, 2014; Kim et al., 2021; Song et al., 2018b; 2019; 2020). The glycoprotein fused with KDEL is retained in the ER and consequently has a glycan structure without the plant-specific glycans α(1,3)-fucose and β(1,2)-xylose, which can cause an immune response (Song et al., 2018a; Sriraman et al., 2004).

However, high accumulation of recombinant proteins in the ER can result in ER stress via the ER-mediated protein quality control (ERQC) function (Deng et al., 2013; Qian et al., 2015). ERQC activates the unfolded protein response (UPR) signaling pathways. The main UPR pathway is controlled by ER-membrane-associated activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase RNA-like ER kinase (PERK) (Lee and Ozcan, 2014; Sanderson et al., 2015). Three basic leucine zipper transcription factor family proteins (bZIP17, bZIP28, and bZIP60) (Nawkar et al., 2018), two binding immunoglobulin proteins (BiP1 and BiP3) (Nawkar et al., 2018), two plant-specific NAC (NAM, no apical meristem; ATAF, Arabidopsis transcription activation factor; CUC, cup-shaped cotyledon) transcription factors (NAC103 and NAC089) (Nawkar et al., 2018), regulators of ER stress-induced programmed cell death (BAX inhibitor 1), B-cell lymphoma 2 (Bcl-2)-associated athanogene 7 (BAG7), and ER oxidoreductin 1 (ERO1) have all been investigated in terms of ER stress regulation (Lisbona et al., 2009; Nawkar et al., 2018; Tajima et al., 2008). In our previous study, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed responses of ER stress-related genes by high ER accumulation of anti-colorectal cancer mAbs in transgenic *Arabidopsis* plants (Song et al., 2018a).

In this study, to confirm the effects of ER retention of the human anti-rabies recombinant mAb in transgenic *Arabidopsis* on its expression and ER stress responses, we tagged the KDEL ER retention signal to the heavy chain (HC) of a human anti-rabies mAb (mAb SO) expressed in transgenic *Arabidopsis*. Homozygous seeds were obtained from both transgenic plants expressing non-KDEL-tagged mAb SO (SO plants) and transgenic plants expressing KDEL-tagged mAb SO (mAb SOK) (SOK plants).

Germination rates, primary root lengths, mAb protein expression levels, and plant-derived mAb (mAb^P) virus-neutralizing functions were observed in SO and SOK plants.

Furthermore, the expression of ER stress-related genes in both transgenic plants (SO and SOK) was verified by qRT-PCR and transcriptomic analyses to determine the effect of the ER retention signal.

MATERIALS AND METHODS

Plant expression vector and transformation

The plant binary expression vectors pBI SO57 (pBI SO), harboring the HC and light chain (LC) of anti-rabies virus mAb SO57, and pBI SO57K (pBI SOK), carrying HC fused to the KDEL signal peptide and LC, were introduced into *Agrobacterium tumefaciens* GV3101::pMP90. Prior to the transformation experiment, five wild-type *Arabidopsis thaliana* seeds (Col-0 ecotype) were sown in each pot (Song et al., 2019). The plants were incubated in a plant growth chamber (23°C, 16-h light/8-h dark). The primary bolts of *Arabidopsis* were removed when the bolts were 4-6 cm long to induce the growth of many secondary bolts. *A. tumefaciens* carrying mAb SO or mAb SO fused to KDEL (mAb SOK) expression cassettes cultured at 28°C in Luria–Bertani (LB) medium containing kanamycin was pelleted at 4,000× *g* for 10 min. The pelleted inoculum was suspended with infiltration media to adjust it to an optical density (OD) value of 0.9. A surfactant (Silwet L-77) was added to the inoculum solution to adjust the concentration to 0.02% (vol/vol). The flower buds were dipped in the inoculum medium for 6 min. The plants were covered with black plastic bags to ensure high humidity and darkness for 24 h. The next day, the plastic bags were removed. When the transformed seeds were ripe, they were sown on Murashige and Skoog (MS) agar medium (Song et al., 2018a). All surviving seedlings were transferred to pots filled with soil in a growth chamber under standard conditions (23°C, 16-h light/8-h dark).

PCR analysis

One hundred milligrams of 4-week-old fresh rosette leaves (non-transgenic [NT], SO, and SOK) selected from T_1 plants were used for PCR. Plant genomic DNA was extracted from the leaves using a DNA extraction kit (RBC Bioscience, Korea). Primer pairs were designed to determine the presence of the HC (281 bp) and LC (227 bp) genes of mAb SO: HC forward primer, 5′-CAG ACT CAC CAT TAC CGC-3′; HC reverse primer, 5′-AGT AGT CCT TGA CCA GGC-3′; LC forward primer, 5′-CAC TGG AAC CAG CAG TGA-3′; and LC reverse primer, 5′-TGT AGT CGC CTG CAT ATG A-3′. Leaves from the NT plant and pBI SO vector were used as positive and negative controls, respectively. PCR analysis of all samples was performed in at least three technical replicates.

Western blot analysis

Before bolt formation, we investigated the protein expression of the rosette leaves of T_1 plants previously confirmed to have mAb SO and mAb SOK HC and LC DNA insertion by PCR analysis. One hundred milligrams of 4-week-old fresh leaf samples were ground in liquid nitrogen (LN). The ground samples were treated with 100 μl of sample buffer (Song et al., 2018a), boiled for 5 min, and placed on ice before being transferred to a fresh tube. TSPs were separated by 12.5% SDS-PAGE and then transferred to a nitrocellulose membrane. Membranes were incubated in 1× PBST (1× phosphate

buffered saline plus 0.5% [v/v] Tween 20) with 5% skim milk for 16 h at 4°C and incubated at room temperature for 2 h with goat anti-human IgG Fcγ (1:5,000) and anti-human IgG F(ab)′$_2$ (1:5,000), which recognize the HC and LC, respectively. The membranes were washed three times with 1× PBST for 10 min at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA) was used to treat the membranes, and an X-ray film (Agfa, Belgium) was used to detect the targeted protein band. Fresh leaflets of NT *Arabidopsis* plants were used as negative controls.

Assessment of *Arabidopsis* germination and growth traits

To obtain homozygous lines of SO and SOK, we repeated the antibiotic seed selection process for the T_3 generation. For agar media–mediated plant growth, one hundred and fifty seeds of SO, SOK, and NT were surface-sterilized with 70% ethanol and distilled water and planted in plates with MS agar containing kanamycin (50 mg·L^{-1}) (Song et al., 2018a). The seedling plates were stored at 4°C for 3 days under dark conditions. The plates were then vertically incubated at 23°C under 16 h light/8 h dark conditions. The number of germinated seeds was counted to calculate the germination rate (number of germinated seeds/number of total seeds × 100). The germinated shoots were observed 4 days after incubation, and primary root lengths were measured using a ruler on days 3, 5, 11, and 13. For soil media-mediated plant growth, subsequently, 32 randomly selected shoots of SO, SOK, and NT plants were transplanted into pots with soil under natural environments. Rosette leaf lengths were measured every 5 weeks. Photographs (Digital Gross System; Humintec, Korea) were taken 13 days after germination. All experiments were performed with three technical and biological replicates.

Reverse transcription PCR analysis

Total RNA was isolated from fresh leaflets of the NT, SO, and SOK plants. Four-week-old fresh rosette leaves were ground in LN. Total RNA was isolated from plant leaf tissue using TRIzol reagent (Invitrogen, USA). The cDNA was used as a template for RT-PCR analysis to confirm the transcription of HC and LC genes using the Maxime PCR Premix Kit (iNtRON Biotechnology, Korea). Actin 8 was used as a reference gene for normalization, and the following primer sets were used to amplify the gene: actin 8 forward primer, 5′-CAA CTA TGT TCT CAG GTA TTG CAG A-3′ and actin 8 reverse primer, 5′-GTC ATG GAA ACG ATG TCT CTT TAG T-3′. The analyses were independently conducted three times with an NT plant as the negative control, and the amplified products were analyzed by 1.0% agarose gel electrophoresis.

Quantitative RT-PCR analysis

The root tissues of *Arabidopsis* seedlings grown vertically for 13 days after agar media grown seeding were collected for qRT-PCR. Two micrograms of total RNA extracted from the root tissue was used for cDNA production using the Superscript III First Strand Synthesis Kit (Invitrogen). Primer sets for conducting qRT-PCR were used to amplify a ~200 bp region of the 11 target transcripts. AtRTPrimer was used to design the primers (Han and Kim, 2006), and the primers previously described by Song et al. (2018) were also used for qRT-PCR conducted using a C1000 Thermal Cycler (Bio-Rad, USA). Primer quality was determined in a previous study (Song et al., 2018a). The analyses were performed using three technical replicates. *UBI10* was used as the control.

RNA sequencing

To obtain total RNA, 13-day-old agar media grown fresh shoots of SO and SOK were collected, ground in LN, and applied using a tri-reagent procedure. The experiments were performed in two biological replicates. To construct cDNA libraries, 1 μg of mRNA was used with the TruSeq RNA Sample Prep Kit v2 (Illumina, USA). The prepared libraries were quantified using qPCR according to the Illumina qPCR Quantification Protocol Guide. To verify the size of PCR-enriched fragments, we confirmed the template size distribution using an Agilent Technologies 2100 Bioanalyzer with a DNA 1000 chip. cDNA library sequencing was performed using the NovaSeq 6000 System (Illumina). mRNA sequencing (mRNA-seq) reads were aligned to *the A. thaliana* reference genome (TAIR10) using HISAT2 (Kim et al., 2015; 2019). The transcript read counts were calculated, and the relative transcript abundances were measured in fragments per kilobase of exon per million fragments mapped (FPKM) from StringTie. For quality control, we excluded 11,557 of 33,602 genes with at least one zero count, leaving 22,045 genes to be analyzed. These raw data were visualized with a smear plot, volcano plot, and heat map.

Statistical analysis of gene expression level

The relative abundances of genes were measured in read counts using StringTie. Statistical analysis was performed to identify differentially expressed genes (DEGs) using the estimates of abundances for each gene in the samples. Genes with more than one zero read count value in the samples were excluded. The filtered data were $\log_2$-transformed and subjected to relative log expression normalization. The statistical significance of the differential expression data was determined using the DESeq2 nbinomWaldTest (Love et al., 2014) and fold change. The null hypothesis was that no difference exists among groups. A P value < 0.05 was considered statistically significant.

Heat map analysis

Heat map analysis was also performed using complete linkage and Euclidean distance as a measure of similarity to display the expression patterns of differentially expressed transcripts that were satisfied with |fold change| ≥ 2 and raw $P < 0.05$. All data analysis and visualization of DEGs were conducted using R 3.6.3 (https://www.r-project.org/).

Human anti-rabies mAb purification from plant biomass

One hundred fifty grams of leaves obtained from transgenic *Arabidopsis* plants were ground in 900 ml homogenizing buffer, as described in a previous study (Song et al., 2018a). The purification processes, including chloroplast removal and TSP isolation, have been described in detail in previous studies (Song et al., 2018a; 2019). Both mAb SO and mAb SOK pro-

teins were eluted using protein A affinity chromatography. The eluted mAbs were visualized by SDS-PAGE, as described by Song et al. (2019).

In vitro anti-rabies assay for plant-derived mAb SO and mAb SOK

The rapid fluorescent focus inhibition test (RFFIT) described by Song et al. (2019) was conducted to determine the *in vitro* antiviral activity of mAb SO, mAb SOK, and human rabies immunoglobulin (HRIG; National Institute for Biological Standards and Control [NIBSC], UK). The initial concentrations of the mAbs were 25 $\mu g \cdot ml^{-1}$, and the mAbs were diluted to ratios of 1:2.5, 1:12.5, 1:62.5, and 1:312.5 in dilution media, as described in a previous study (Song et al., 2018b; 2019). Virus neutralization tests with the challenge virus standard (CVS-11) were performed as described by Song et al. (2018b; 2019).

RESULTS

Generation of transgenic plants

The genes encoding the HC and LC of mAb SO and mAb SOK in plant expression vectors (pBI SO and pBI SOK, respectively) were transferred to *Arabidopsis* using *Agrobacterium*-mediated floral dip transformation (Fig. 1A). After transformation, 3,000 randomly chosen T_1 seeds were placed on *in vitro* germination media to select transformants. Most of the seeds developed yellow shoots without true leaves (Supplementary Fig. S1). Twenty-four to thirty seedlings with true leaves that were transformants for mAb SO and mAb SOK (SO and SOK, respectively) were observed in the kanamycin selection medium and grown in soil. PCR analyses showed the amplified HC (281 bp) and LC (227 bp) genes in the rosette leaves (Fig. 1B, upper). The PCR product was not detected in NT *Arabidopsis*. After confirming the presence of the HC and LC genes, mAb SO and SOK protein expression was confirmed by immunoblotting (Fig. 1B, lower). Among the plants transferred to pots, SO and SOK plants exhibited mAb SO and SOK protein bands, respectively. One more antibiotics selection process was performed until T_3 homozygous seeds were obtained from T_1 screening (Supplementary Fig. S2).

Effect of tagging ER retention signal peptide KDEL to HC on plant growth

Among the 150 seeds plated on germination media, most seeds of NT (98.3%), SO (96.1%), and SOK (95.8%) plants germinated in the kanamycin-containing medium within 2-3 days, with high seedling survival percentages (Fig. 2A). Thirteen-day-old SO and SOK plants showed mean root length growth of 11.6 mm and 8.3 mm, respectively (Fig. 2B). In SOK plants, the mean root length was 1.4 times shorter than that of SO plants (Fig. 2C). Within 4-5 days, all NT seedlings

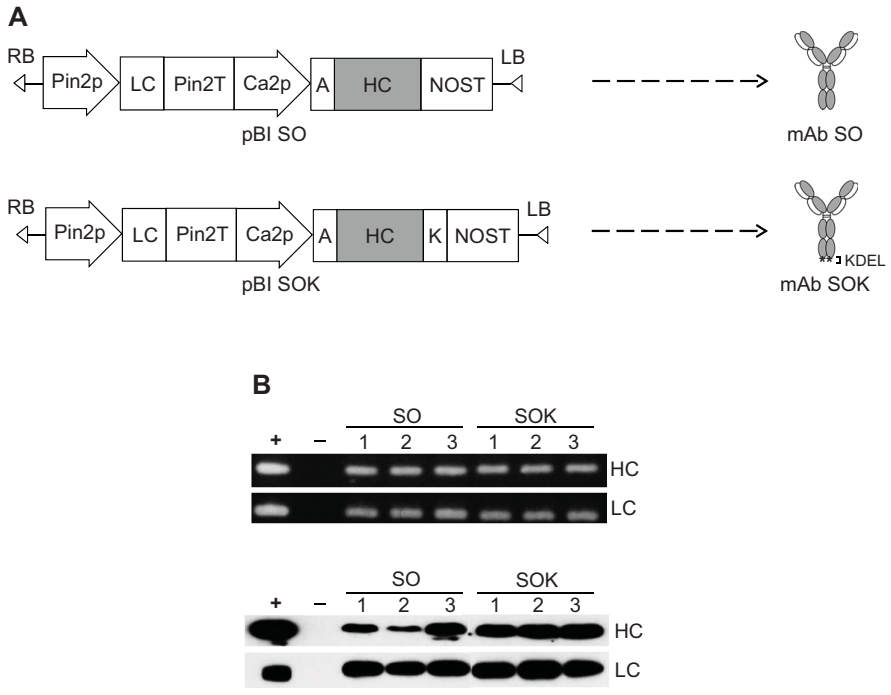

Fig. 1. Schematic diagram of the transformation vectors and selection of transformants. (A) Gene expression cassettes for mAb SO57 without KDEL (SO) and mAb SO57 with KDEL (SOK) in plant binary vectors pBI mAb SO and pBI mAb SOK. Pin2p, promoter of proteinase inhibitor II (Pin2) gene from potato; Ca2p, cauliflower mosaic virus 35s promoter with duplicated upstream B domain; A, an alfalfa mosaic virus untranslated leader sequence of RNA4; Pin2T, terminator of Pin2 gene; NOST, terminator of nopaline synthase (NOS) gene; K, ER retention signal peptide. (B) Selection of positive T_1 transformants using PCR and western blot analyses. Rosette leaves (mAb SO and mAb SOK) were sampled to confirm the gene existence of the HC and LC. The kanamycin selection process was repeated until T_3 homozygous lines were identified (Supplementary Fig. S2).

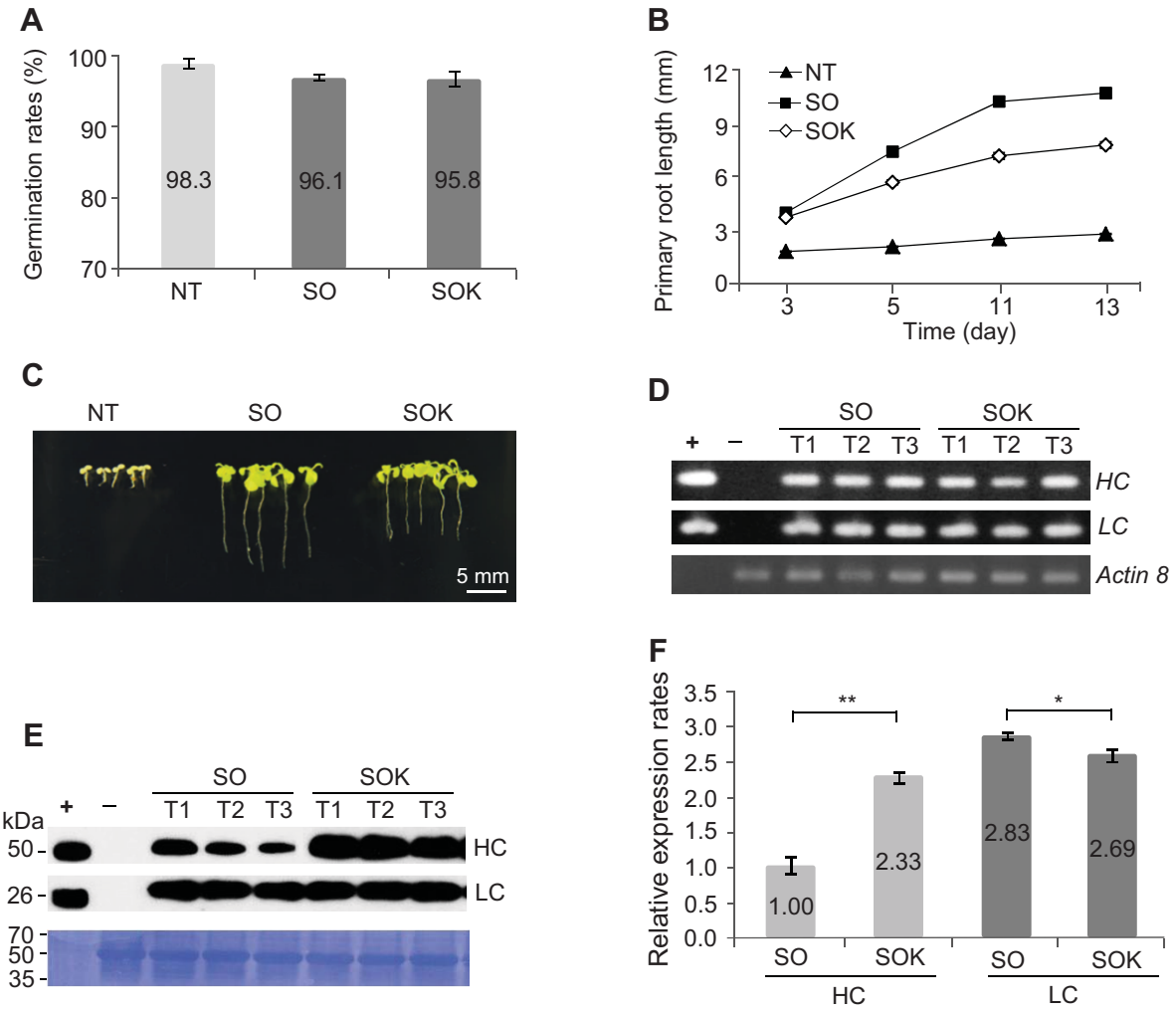

Fig. 2. Effect of ER retention signal on plant growth and transgene expression of transgenic *Arabidopsis* for mAb SO57 without KDEL (SO) and mAb SO57 with KDEL (SOK) under kanamycin-containing agar medium. (A) The germination rate was calculated 4 days after sowing of T_3 homozygous seeds on kanamycin-containing medium. The values in the graph represent the mean value of each group. Error bars indicate the mean ± SD of three replicates. (B) After sowing seeds on a square dish, the primary root lengths of non-transgenic (NT), SO, and SOK seedlings were measured at 3, 5, 11, and 13 days. (C) A photograph was taken 13 days after sowing each seed group on Murashige and Skoog (MS) media. Scale bar = 5 mm. The mRNA and protein expression levels of HC and LC were determined by RT-PCR (D) and western blot (E) analyses. Lane 1, human rabies immunoglobulin (HRIG); Lane 2, NT; Lanes 3-5, mAb SO-expressing plants; and Lanes 6-8, mAb SOK-expressing plants. The nitrocellulose (NC) membrane was stained with Coomassie Brilliant Blue R-250 containing buffer (bottom). (F) Western blot analysis to determine relative HC and LC protein levels. The band density was statistically analyzed by the ImageJ program. Values indicated in the graph represent the mean for each group, and the error bars indicate the SD. Asterisks represent statistical significance ($*P < 0.05$; $**P < 0.01$).

showed etiolation under kanamycin selection conditions.

Effect of ER retention signal on transcription and translation of HC and LC of mAb SO

The transcription and translation levels in the rosette leaves of the SO and SOK T_3 plants were observed using RT-PCR and western blot analyses, respectively (Figs. 2D and 2E). The HC and LC transcription levels of SO plants did not differ from those of SOK plants (Fig. 2D). These results are consistent with those of previous studies showing that transcription is not directly correlated with translation (Gygi et al., 1999). The HC band density of SOK plants was more than twice that of SO plants (Figs. 2E and 2F).

Effect of ER retention signal on ER stress related genes in plant

To determine the effect of tagging KDEL to mAb SO on the stress response in transgenic *Arabidopsis*, we visualized the normalized DEGs between SO and SOK by volcano plot (Figs. 3A and 3B). ER stress-related genes (bZIP17, bZIP28, bZIP60, BiP1, BiP3, IRE1a, NAC103, NAC089, BAG7, BAX inhibitor 1 [BI1], and ERO1), are labeled as red points. The volcano plot

showed the differential expression of genes between SO and SOK plants in agar media or soil. In the plot, the ER stress-related genes were not distributed in the gray region (Fig. 3A [agar media] and Fig. 3B [soil]). Furthermore, heat map analysis was conducted to determine how KDEL affects ER stress-related gene expression (Fig. 3C). To this end, mRNA was isolated from SO57 and SO57K plants grown in both agar media and soil and was used for RNA-seq. In general, the expression trends were dissimilar between SO57 and SO57K under both agar media and soil growth conditions. However, the gene expression pattern was significantly different between the agar media and soil growth conditions. The analysis was grouped by one-way hierarchical clustering (Euclidean distance and complete linkage) using Z-scores for normalized values (1,479 genes) (Fig. 3C).

In addition, qRT-PCR was conducted to investigate the transcription levels of 11 ER stress-related genes (*bZIP17*, *bZIP28*, *bZIP60*, *BiP1*, *BiP3*, *IRE1a*, *NAC103*, *NAC089*, *BAG7*, *BAX inhibitor 1*, and *ERO1*) in both mAb SO and mAb SOK plants under agar media conditions (Fig. 4A). Among the 11 tested genes, *BiP1* and *BAX inhibitor* 1 showed the highest relative mRNA expressions (~0.5). *bZIP17, bZIP28, biZP60,*

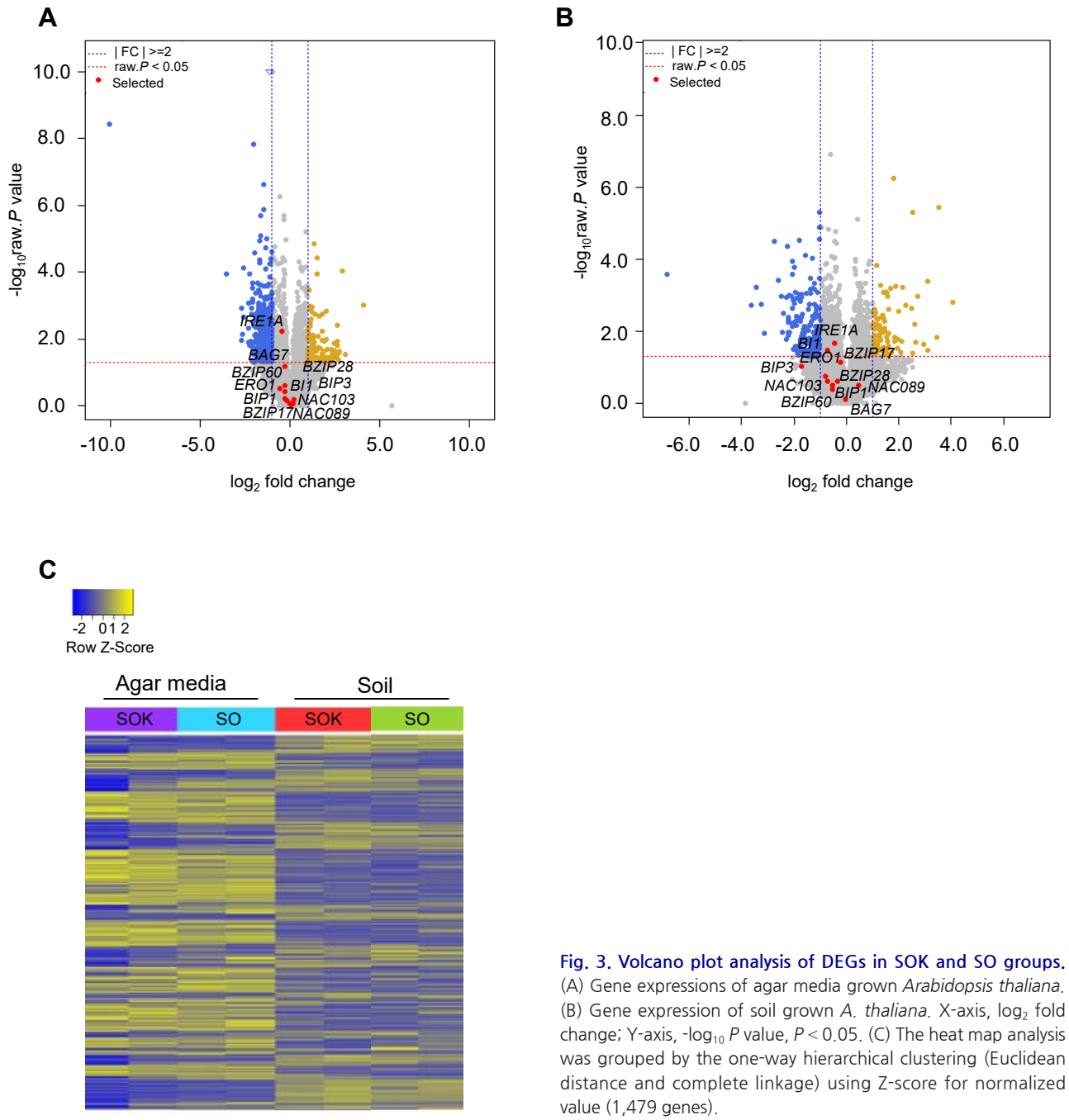

Fig. 3. Volcano plot analysis of DEGs in SOK and SO groups. (A) Gene expressions of agar media grown *Arabidopsis thaliana*. (B) Gene expression of soil grown *A. thaliana*. X-axis, $\log_2$ fold change; Y-axis, $-\log_{10} P$ value, $P < 0.05$. (C) The heat map analysis was grouped by the one-way hierarchical clustering (Euclidean distance and complete linkage) using Z-score for normalized value (1,479 genes).

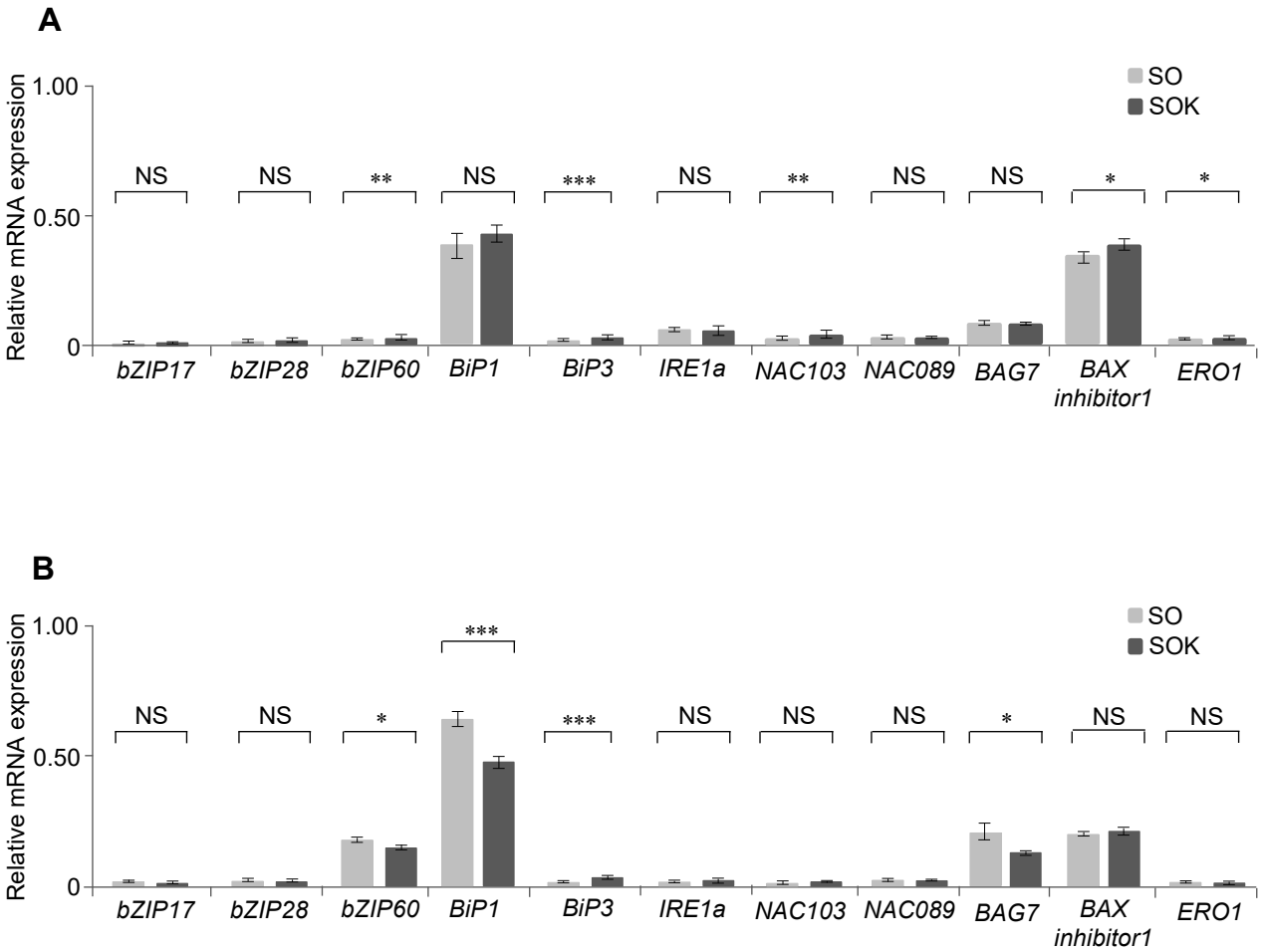

Fig. 4. Transcription levels of ER stress-related genes (*bZIP17*, *bZIP28*, *bZIP60*, *BiP1*, *BiP3*, *IRE1a*, *NAC103*, *NAC089*, *BAG7*, *BAX inhibitor 1*, and *ERO1*) in transgenic SO57 without KDEL (SO) and SO57 with KDEL (SOK) *Arabidopsis* grown under agar media and soil conditions. The transcription levels were measured using qRT-PCR analysis. (A) Total RNA was isolated from the roots of plants (SO and mAb SOK) grown on kanamycin-containing plant media for 13 days (B) and isolated from 4-week-old leaves of plants (mAb SO and mAb SOK) grown in greenhouse under soil conditions. The ubiquitin 10 (*UBI10*) gene was applied as a reference gene for normalization. Error bars indicate the mean ± SD of three technical replicates with three biological replicates. Asterisks represent statistical significance (NS, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). ER stress-related genes are listed on the X-axis. The relative mRNA expression levels of each transgenic shoot (SO and SOK) are expressed on the Y-axis.

bZIP60, *biP3*, *NAC103*, *NAC089*, and *ERO1* showed low relative mRNA expression (<0.1). The relative mRNA expression levels of *IRE1a* and *BAG7* were approximately 0.1. In the highest expression groups (*BiP1* and *BAX inhibitor 1*), the transcription levels were not significantly different in SOK plants relative to SO plants. Furthermore, the other low expression groups did not show dramatic changes. Only the *BiP3* transcription level was higher in SOK plants than that in SO plants. However, under soil growth conditions, only the *BiP3* gene tended to be upregulated in SOK plants (Fig. 4B).

Growth of SO and SOK *Arabidopsis* plants in soil

Of the seeds cultured on MS agar media containing kanamycin, we transplanted healthy SO (40), SOK (40), and NT (16) seedlings with true leaves to soil in plastic pots. After transplantation, the plastic pots were covered with a wrap and maintained under standard plant growth conditions. After one week, the average lengths of rosette leaves were measured in each experimental group. At the first measurement, the average lengths for SO, SOK, and NT were 1.15, 1.07, and 1.18 cm, respectively (Supplementary Fig. S3). After two weeks, the average lengths for each group were 1.93, 1.79, and 1.78 cm, respectively. Three weeks after transplantation, these values were 2.73, 2.82, and 2.84 cm, respectively. After four weeks, those of each group were 3.32, 3.28, and 3.34 cm, respectively. Finally, after five weeks, these values were 3.43, 3.40, and 3.44 cm, respectively (Supplementary Fig. S3).

Isolation of SO and SOK mAbs from transgenic plants

Both SO and SOK homozygous seedlings expressing human anti-rabies mAbs were planted in pots and cultivated in a

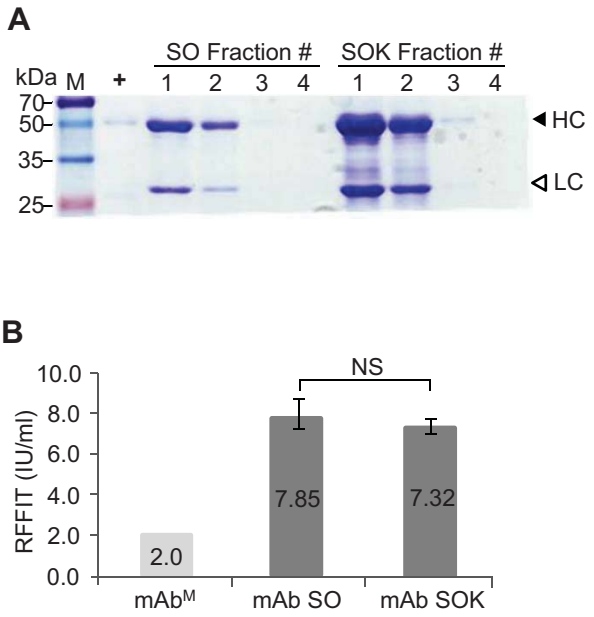

Fig. 5. mAb purification and *in vitro* function of the purified mAb SO57 without KDEL (SO) and mAb SO57 with KDEL (SOK) as compared to mAb^{M} against target rabies viruses (CVS-11). (A) Confirmation of eluted fractions 1-4 of mAbs obtained from transgenic SO and SOK *Arabidopsis* plants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, protein marker; Lane 2, human rabies immunoglobulin (HRIG); Lanes 3-6, SO; Lanes 7-10, SOK. To investigate antibody-neutralizing activities, antibodies (mAb^{M}, mAb SO, and mAb SOK) were mixed with rabies virus standard strain CVS-11, respectively, and treated on the murine neuroblastoma N2a cell line. (B) Rapid fluorescent focus inhibition test (RFFIT) values (IU·ml^{-1}) are mean values of each group, performed in duplicate. Error bars indicate the SD of three biological replicates. NS, not significant.

greenhouse (Supplementary Fig. S3A). Forty grams of transgenic *Arabidopsis* leaves were obtained from a single tray. To purify anti-rabies mAbs SO and SOK, 150 g of biomass was used. We confirmed the presence of purified mAb SO and SOK HC (50 kDa) and LC (25 kDa) by SDS-PAGE. Both mAb SO and SOK were mainly eluted in fractions 1 and 2 (Fig. 5A). The eluted samples (fractions 1-4) were quantified using NanoDrop analysis. The total amount of mAb SOK (1,500 μg) obtained was three-fold higher than that of mAb SO (500 μg).

Neutralization of plant-derived mAbs SO and SOK

Both mAb SO and mAb SOK were dialyzed with 1× PBS, and their concentration (20 μg·ml^{-1}) was adjusted for virus-neutralizing analysis. Ten microliters of samples (commercially available HRIG, mAb SO, and mAb SOK) were used for each analysis. The rabies virus-neutralizing activities of mAb SO and mAb SOK purified from transgenic *Arabidopsis* were compared with that of HRIG. The mean RFFIT values of each group were as follows: HRIG (2.00 IU·ml^{-1}), mAb SO (7.85 IU·ml^{-1}), and mAb SOK (7.32 IU·ml^{-1}) (Fig. 5B). Both mAbs (mAb SO and mAb SOK) purified from transgenic *Arabidopsis* showed approximately four-fold higher RFFIT activity against the rabies virus strain CVS-11 than that of HRIG (Fig. 5B; Supplementary Fig. S4).

DISCUSSION

The present study revealed that the KDEL ER retention motif affects human mAb production and plant growth in *Arabidopsis*. In transgenic tobacco, KDEL tagging has been shown to enhance mAb protein accumulation in the ER, eventually increasing the production of recombinant therapeutic human anti-rabies mAbs (Lee et al., 2013; Song et al., 2018b). Here, *Arabidopsis* was used for the expression of anti-rabies mAb SO and mAb SOK, and its growth phenotypes were observed to investigate the effect of KDEL-tagging on human anti-rabies mAb SO57 (Song et al., 2018b).

We aimed to confirm how tagging an ER retention signal to mAb SO57 affects its expression in *Arabidopsis*, and whether this affects plant growth differently to the origins and disease targets presented in Song et al. (2018a). In Song et al. (2018a), the mAbs produced in plants were murine antibodies targeting colorectal cancer. Our study focuses on the gene expression of human-originated anti-rabies mAbs. Plant growth physiology and transgene expression may vary depending on different transgenes (Han et al., 2016; Hay and Tsiantis, 2010). Thus, our study provides a meaningful reconfirmation of how ER retention tagging affects transgenic *Arabidopsis*.

The germination rates of SOK seeds were not significantly different from those of SO seeds. This result was consistent with that presented in Song et al. (2018a). However, SOK plants had shorter primary roots than those of SO plants. This result suggests that KDEL tagging of the HC affected root growth. Indeed, in this study, the ER stress-related genes were turned on in SOK plants with mAb ER accumulation, affecting plant growth in agar media.

Eleven representative ER stress-related genes, controlling the ATF6 and IRE1 pathways, were investigated as these genes have been extensively studied in plants under ER stress conditions (Chen and Brandizzi, 2012; Hayashi et al., 2012).

It has been reported that the UPR pathways are activated to maintain ER homeostasis by ER stress resulting from accumulation of proteins in the ER (Deng et al., 2013; Liu et al., 2011; Yamamoto et al., 2003). Among the ER stress-related pathways (Hollien, 2013; Sanderson et al., 2015), we analyzed genes associated with the ATF6 and IRE1 pathways. In the volcano plot analysis, all the tested ER stress-related genes of agar media and soil grown plants (SO and SOK) were distributed in the gray region, indicating that the expression of ER stress-related genes is not affected by accumulation of anti-rabies virus mAb in the ER. Unlike the volcano plot analysis, in qRT-PCR analysis, *bZIP60, BiP3, NAC103, BAX inhibitor 1,* and *ER01* exhibited differing expression levels between SO and SOK plants grown under agar media conditions. However, with the exception of BAX inhibitors, the relative mRNA expression levels *of bZIP60, BiP3, NAC103,* and *ER01* were approximately 0.1, suggesting that differences in ER stress-related gene expression would be insignificant. The *bZIP60, BiP3, NAC103*, *BAX inhibitor 1*, and *ERO1 UPR* path-

way genes assist protein folding and suppress ER stress-induced cell death (Ruberti et al., 2018; Wan and Jiang, 2016; Williams et al., 2014). Under soil conditions, the relative mRNA expression of *bZIP60, BiP1,* and *BAG7* were reduced in SOK plants compared to SO plants. There were no consistent trends in transcriptional changes of stress-related genes in plants soil grown compared to agar media grown plants. In addition, SO and SOK plants that survived in the antibiotic selection were moved into soil pots to confirm plant growth and mAb expression patterns. In contrast to the agar media plants, there were no differences in growth among SO, SOK, and NT plants grown under soil conditions. These results are similar to those reported by Song et al. (2018a). Taken together, it can be speculated that soil growth conditions such as natural light, soil, nutrients, and water overcame ER stress (Rickes et al., 2019; Tang et al., 2017).

The HC and LC gene transcription levels of SOK plants was similar to those of SO plants, whereas the mAb production level in SOK plants was three-fold higher than that in SO plants. It is speculated that KDEL, the ER signal peptide, stably retains anti-rabies antibodies in the ER, resulting in high production levels (Lee et al., 2013; Song et al., 2018b).

To utilize transgenic *Arabidopsis* for therapeutic protein production, it is crucial to screen homozygous plant lines with high expression levels under both agar media and soil conditions. Approximately 2,000 seeds from SO and SOK plants were planted in pots under greenhouse conditions, and mature plants were harvested for anti-rabies mAb purification. Phenotypic differences in SO and SOK plants were not observed under greenhouse conditions. SDS-PAGE results showed that SOK plants had a higher purified mAb quantity per unit mass than SO plants did following purification by affinity chromatography.

Both mAb SO and mAb SOK purified from transgenic *Arabidopsis* showed higher neutralizing activities toward rabies virus strain CVS-11 than that exhibited by HRIG. These results were also similar to those of Song et al. (2018b), in which anti-rabies mAbs were expressed in transgenic tobacco plants. In the present study, we demonstrated that *Arabidopsis* is another potential alternative to produce human anti-rabies antibodies comparable to HRIG, a counterpart antibody. Tagging KDEL to HC increases the mass production of mAbs in the plant without provoking a significant stress response under the soil conditions. Thus, in plant molecular biofarming, ER protein accumulation is recommended for the production of therapeutic antibodies.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This research was supported by grants from Cooperative Research Program for Agriculture Science and Technology Development (project No. PJ016266), and Chung-Ang University Research Scholarship Grants in 2018.

AUTHOR CONTRIBUTIONS

I.S., S.W.L., and S.O. performed the experiments. Y.K.L., S.R.P., D.H.K., and J.W.K. gave technical support and analyzed the data. K.K.(Kisung Ko), S.J.P., and M.S.K. conceived and supervised the study. H.K.L. and M.K.K. provided expertise and feedback. D.S.K., J.W.K., and K.K.(Kinarm Ko) wrote and edited the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Ilchan Song https://orcid.org/0000-0002-9432-7009
Young Koung Lee https://orcid.org/0000-0001-5822-3387
Jin Wook Kim https://orcid.org/0000-0003-4157-9365
Seung-Won Lee https://orcid.org/0000-0001-8893-8332
Se Ra Park https://orcid.org/0000-0002-1733-2453
Hae Kyung Lee https://orcid.org/0000-0002-9560-862X
Soyeon Oh https://orcid.org/0000-0002-2779-4045
Kinarm Ko https://orcid.org/0000-0002-8495-9790
Mi Kyung Kim https://orcid.org/0000-0003-1610-4536
Soon Ju Park https://orcid.org/0000-0002-8525-5891
Dae Heon Kim https://orcid.org/0000-0001-9481-1557
Moon-Soo Kim https://orcid.org/0000-0002-6778-9881
Do Sun Kim https://orcid.org/0000-0003-4306-607X
Kisung Ko https://orcid.org/0000-0003-4964-0845

REFERENCES

Chen, Y. and Brandizzi, F. (2012). AtIRE1A/AtIRE1B and AGB1 independently control two essential unfolded protein response pathways in Arabidopsis. Plant J. *69*, 266-277.

Cleaveland, S. and Hampson, K. (2017). Rabies elimination research: juxtaposing optimism, pragmatism and realism. Proc. Biol. Sci. *284*, 20171880.

De Meyer, T. and Depicker, A. (2014). Trafficking of endoplasmic reticulum-retained recombinant proteins is unpredictable in Arabidopsis thaliana. Front. Plant Sci. *5*, 473.

Deng, Y., Srivastava, R., and Howell, S.H. (2013). Endoplasmic reticulum (ER) stress response and its physiological roles in plants. Int. J. Mol. Sci. *14*, 8188-8212.

Gygi, S., Rochon, Y., Franza, B., and Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. Mol. Cell. Biol. *19*, 1720-1730.

Han, S. and Kim, D. (2006). AtRTPrimer: database for Arabidopsis genome-wide homogeneous and specific RT-PCR primer-pairs. BMC Bioinformatics *7*, 179.

Han, Z., Yu, H., Zhao, Z., Hunter, D., Luo, X., Duan, J., and Tian, L. (2016). AtHD2D gene plays a role in plant growth, development, and response to abiotic stresses in Arabidopsis thaliana. Front. Plant Sci. *7*, 310.

Hay, A. and Tsiantis, M. (2010). KNOX genes: versatile regulators of plant development and diversity. Development *137*, 3153-3165.

Hayashi, S., Wakasa, Y., Takahashi, H., Kawakatsu, T., and Takaiwa, F. (2012). Signal transduction by IRE1-mediated splicing of bZIP50 and other stress sensors in the endoplasmic reticulum stress response of rice. Plant J. *69*, 946-956.

Hollien, J. (2013). Evolution of the unfolded protein response. Biochim. Biophys. Acta *1833*, 2458-2463.

Kang, Y., Shin, Y.K., Park, S.W., and Ko, K. (2016). Effect of nitrogen deficiency on recombinant protein production and dimerization and growth in transgenic plants. Hortic. Environ. Biotechnol. *57*, 299-307.

Kim, D., Langmead, B., and Salzberg, S.L. (2015). HISAT: a fast spliced aligner with low memory requirements. Nat. Methods *12*, 357-360.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat. Biotechnol. *37*, 907-915.

Kim, D.S., Song, I., and Ko, K. (2018). Low risk of pollen-mediated gene flow in transgenic plants under greenhouse conditions. Hortic. Environ. Biotechnol. *59*, 723-728.

Kim, K., Kang, Y.J., Park, S.R., Kim, D., Lee, S., Ko, K., Ponndorf, D., and Ko, K. (2021). Effect of leaf position and days post-infiltration on transient expression of colorectal cancer vaccine candidate proteins GA733-Fc and GA733-FcK in Nicotiana benthamiana plant. PeerJ *9*, e10851.

Koornneef, M. and Meinke, D. (2010). The development of Arabidopsis as a model plant. Plant J. *61*, 909-921.

Lee, J. and Ozcan, U. (2014). Unfolded protein response signaling and metabolic diseases. J. Biol. Chem. *289*, 1203-1211.

Lee, J.H., Park, D.Y., Lee, K.J., Kim, Y.K., So, Y.K., Ryu, J.S., Oh, S.H., Han, Y.S., Ko, K., Choo, Y.K., et al. (2013). Intracellular reprogramming of expression, glycosylation, and function of a plant-derived antiviral therapeutic monoclonal antibody. PLoS One *8*, e68772.

Lisbona, F., Rojas-Rivera, D., Thielen, P., Zamorano, S., Todd, D., Martinon, F., Glavic, A., Kress, C., Lin, J.H., Walter, P., et al. (2009). BAX inhibitor-1 is a negative regulator of the ER stress sensor IRE1alpha. Mol. Cell *33*, 679-691.

Liu, L., Cui, F., Li, Q., Yin, B., Zhang, H., Lin, B., Wu, Y., Xia, R., Tang, S., and Xie, Q. (2011). The endoplasmic reticulum-associated degradation is necessary for plant salt tolerance. Cell Res. *21*, 957-969.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Nawkar, G.M., Lee, E.S., Shelake, R.M., Park, J.H., Ryu, S.W., Kang, C.H., and Lee, S.Y. (2018). Activation of the transducers of unfolded protein response in plants. Front. Plant Sci. *9*, 214.

Pertea, M., Kim, D., Pertea, G.M., Leek, J.T., and Salzberg, S.L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat. Protoc. *11*, 1650-1667.

Qian, D., Tian, L., and Qu, L. (2015). Proteomic analysis of endoplasmic reticulum stress responses in rice seeds. Sci. Rep. *5*, 14255.

Rickes, L.N., Klumb, E.K., Benitez, L.C., Braga, E.J.B., and Bianchi, V.J. (2019). Differential expression of the genes involved in responses to water-deficit stress in peach trees cv. Chimarrita grafted onto two different rootstocks. Bragantia *78*, 60-70.

Ruberti, C., Lai, Y., and Brandizzi, F. (2018). Recovery from temporary endoplasmic reticulum stress in plants relies on the tissue-specific and largely independent roles of bZIP28 and bZIP60, as well as an antagonizing function of BAX-Inhibitor 1 upon the pro-adaptive signaling mediated by bZIP28. Plant J. *93*, 155-165.

Sanderson, T.H., Gallaway, M., and Kumar, R. (2015). Unfolding the unfolded protein response: unique insights into brain ischemia. Int. J. Mol. Sci. *16*, 7133-7142.

Shin, C., Kang, Y., Kim, H.S., Shin, Y.K., and Ko, K. (2019). Immune response of heterologous recombinant antigenic protein of viral hemorrhagic septicemia virus (VHSV) in mice. Anim. Cells Syst. (Seoul) *23*, 97-105.

Song, I., Kang, Y., Lee, Y.K., Myung, S.C., and Ko, K. (2018a). Endoplasmic reticulum retention motif fused to recombinant anti-cancer monoclonal antibody (mAb) CO17-1A affects mAb expression and plant stress response. PLoS One *13*, e0198978.

Song, I., Kang, Y.J., Choi, S.L., Han, D., Kim, D.S., Lee, H.K., Lee, J.C., Park, J., Kim, D.S., and Ko, K. (2019). Purification of plant-derived anti-virus mAb through optimized pH conditions for coupling between protein A and epoxy-activated beads. PeerJ *7*, e6828.

Song, I., Kang, Y.J., Kim, D.H., Kim, M.K., and Ko, K. (2020). Expression and in vitro function of anti-cancer mAbs in transgenic Arabidopsis thaliana. BMB Rep. *53*, 229-233.

Song, I., Kim, D.S., Kim, M.K., Jamal, A., Hwang, K.A., and Ko, K. (2015). Comparison of total soluble protein in various horticultural crops and evaluation of its quantification methods. Hortic. Environ. Biotechnol. *56*, 123-129.

Song, I., Park, S.A., Han, D., Lee, H.K., An, H.J., and Ko, K. (2018b). Expression, glycosylation, and function of an anti-rabies virus monoclonal antibody in tobacco and Arabidopsis plants. Hortic. Environ. Biotechnol. *59*, 285-292.

Sriraman, R., Bardor, M., Sack, M., Vaquero, C., Faye, L., Fischer, R., Finnern, R., and Lerouge, P. (2004). Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-alpha(1,3)-fucose residues. Plant Biotechnol. J. *2*, 279-287.

Tajima, H., Iwata, Y., Iwano, M., Takayama, S., and Koizumi, N. (2008). Identification of an Arabidopsis transmembrane bZIP transcription factor involved in the endoplasmic reticulum stress response. Biochem. Biophys. Res. Commun. *374*, 242-247.

Tang, Y., Liu, K., Zhang, J., Li, X., Xu, K., Zhang, Y., Qi, J., Yu, D., Wang, J., and Li, C. (2017). JcDREB*2*, a physic nut AP2/ERF gene, alters plant growth and salinity stress responses in transgenic rice. Front. Plant Sci. *8*, 306.

Taylor, L.H. and Nel, L.H. (2015). Global epidemiology of canine rabies: past, present, and future prospects. Vet. Med. (Auckl.) *6*, 361-371.

Wan, S. and Jiang, L. (2016). Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) in plants. Protoplasma *253*, 753-764.

Williams, B., Verchot, J., and Dickman, M.B. (2014). When supply does not meet demand-ER stress and plant programmed cell death. Front. Plant Sci. *5*, 211.

Yamamoto, K., Hamada, H., Shinkai, H., Kohno, Y., Koseki, H., and Aoe, T. (2003). The KDEL receptor modulates the endoplasmic reticulum stress response through mitogen-activated protein kinase signaling cascades. J. Biol. Chem. *278*, 34525-34532.

Yao, J., Weng, Y., Dickey, A., and Wang, K.Y. (2015). Plants as factories for human pharmaceuticals: applications and challenges. Int. J. Mol. Sci. *16*, 28549-28565.