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Note

Overexpression of the endoplasmic reticulum stress-inducible gene *TIN1* causes abnormal pollen surface morphology in Arabidopsis

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Abstract The unfolded protein response (UPR) or the endoplasmic reticulum (ER) stress response occurs when folding and maturation of secretory and membrane proteins are impaired in the ER. The UPR induces a number of genes that encode ER-localized molecular chaperones and folding enzymes to increase folding capacity in the ER. We have identified *Tunicamycin Induced 1* (*TIN1*), an Arabidopsis gene that is highly induced during the UPR. We have shown that TIN1 protein is localized in the ER but its physiological function remains to be elucidated. In the present study we generated and analyzed transgenic Arabidopsis plants expressing *TIN1* under CaMV35S promoter to obtain insights into the physiological role of TIN1. We found that although *TIN1*-overexpressing plants grew as did wild-type plants under ambient laboratory conditions, their pollen grains exhibited abnormal surface morphology. The result suggests a specific role of TIN1 in secretion of proteins and/or lipids during pollen development.

Key words: Arabidopsis, endoplasmic reticulum, pollen, unfolded protein response.

Secretory and membrane proteins are synthesized on the endoplasmic reticulum (ER) in which folding and maturation of proteins occur before delivered to their final destinations via vesicle trafficking. When protein folding and maturation in the ER are perturbed or the amount of proteins loaded into the ER exceeds the capacity of the folding machinery in the ER, unfolded proteins accumulate in the ER. Under these so-called ER stress conditions, the unfolded protein response (UPR) or the ER stress response is initiated to induce transcriptional activation of genes encoding ER-resident molecular chaperones and folding enzymes to increase the protein folding capacity in the ER (Ron and Walter 2007).

In Arabidopsis, two transcription factors, bZIP60 and bZIP28, are activated during the UPR to induce transcription of genes encoding ER-resident molecular chaperones and folding enzymes (Howell 2013; Iwata and Koizumi 2012). bZIP60 is activated by cytoplasmic splicing, in which the removal of the 23-nt intron of *bZIP60* mRNA causes a frameshift and results in the production of an active, nuclear form of bZIP60 (Nagashima et al. 2011). bZIP28 is a membrane-bound transcription factor activated by proteolytic processing within its transmembrane domain, which liberates the cytoplasmic portion of bZIP28 from the membrane (Iwata et al. 2017). Activation of bZIP60 and bZIP28 and transcriptional induction of genes encoding ER-resident molecular chaperones and folding enzymes have been observed during male gametophyte development (Iwata et al. 2008, 2010b) as well as in response to heat shock and pathogen attack (Gao et al. 2008; Nagashima et al. 2014; Wang et al. 2005), implicating a role of the UPR in these phenomena.

Polypeptides translated by ribosomes attached on the ER membrane enter the ER lumen and undergo folding and maturation (Ellgaard and Helenius 2003). BiP is an ER-resident heat shock protein 70 cognate and binds to nascent polypeptides to assist folding. Calreticulin and calnexin are molecular chaperones for proteins with *N*-glycan chains. Genes encoding those molecular chaperones are induced during the UPR to increase the folding capacity in the ER under stress conditions (Iwata et al. 2010b; Martínez and Chrispeels 2003). Although their functions have been well known, there are also a number of ER stress-inducible genes whose functions remain to be characterized (Iwata et al. 2010b). Our transcriptomic analysis using tunicamycin, an inhibitor of *N*-linked glycosylation that causes ER stress, identified

Abbreviations: ER, endoplasmic reticulum; SEM, scanning electron microscope; TIN1, Tunicamycin Induced 1; UPR, unfolded protein response.
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Tunicamycin Induced 1 (*TIN1*, AGI code At5g64510) (Iwata et al. 2010a, 2010b). We showed that *TIN1* is induced in bZIP60-dependent manner during the UPR and that *TIN1* encodes an ER-localized protein (Iwata et al. 2010a). We also showed that *TIN1* is highly expressed in pollen grains and that the *tin1* mutant exhibits altered pollen surface structure, suggesting its role in pollen development and function (Iwata et al. 2012). In the present study, we generated and analyzed transgenic plants expressing *TIN1* under CaMV35S promoter (hereafter referred to as *TIN1-OX* plants) to obtain deeper insights into the function of TIN1.

We used *Arabidopsis thaliana* ecotype Col-0 in this study. Plants were grown on soil in a growth chamber at 23°C under a 16-h-light/8-h-dark cycle. For tunicamycin treatment, 10-day-old seedlings grown in half strength Murashige and Skoog medium supplemented with 1% sucrose were treated with 5 μg ml^{-1} tunicamycin or 0.1% dimethyl sulfoxide as a solvent control for 5 h. To generate transgenic plants expressing *TIN1* gene under CaMV35S promoter, *TIN1* cDNA was PCR amplified using the primers TIN1-F-XbaI (5′-GTC TAG AAT GGG TCA CAG AGT ATT GGT TT-3′) and TIN1-R-PacI (5′-GTT AAT TAA TTA CAA GGT AAA AGG GCT TGG-3′), cloned into the pGEM-T Easy vector (Promega), and subcloned into the modified pBI121 vector, which harbors *Xba*I and *Pac*I sites between CaMV35S promoter and Nos terminator. Transformation was carried out by the floral dip method (Clough and Bent 1998).

For quantitative RT-PCR (qRT-PCR), RNA was extracted from leaves of 4-week-old wild-type and *TIN1-OX* plants grown on soil and 10-day-old seedlings grown on half strength Murashige and Skoog medium using RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using ExScript RT Reagent Kit (TaKaRa). Quantitative real-time PCR was carried out using SYBR Premix Ex Taq (TaKaRa) with LightCycler 480 Instrument (Roche). *TIN1* and *Act8* fragments were amplified using the following primers; TIN1-F-qPCR (5′-AAT ATG CGC CTT TCT TTA CCA T-3′) and TIN1-R-qPCR (5′-GCA CCA TCT GCT AAT ATC ACT TTC-3′) for *TIN1*, Act8-F-qPCR (5′-TCA GCA CTT TCC AGC AGA TG-3′) and Act8-R-qPCR (5′-ATG CCT GGA CCT GCT TCA T-3′) for *Act8*. The expression level of *TIN1* was normalized to that of *Act8*. The experiment was carried out with three biological replicates.

Mature pollen grains from wild-type and *TIN1-OX* plants were observed under a scanning electron microscope (SEM) as previously described (Iwata et al. 2012). In brief, mature pollen grains were placed on double-sided sticky carbon tape to the aluminum stub, which was then mounted on the cooling stage at −20°C. The specimen was observed with a SEM (S-3200N, Hitachi) at a chamber pressure of 30 Pa and an accelerating voltage of 15 kV.

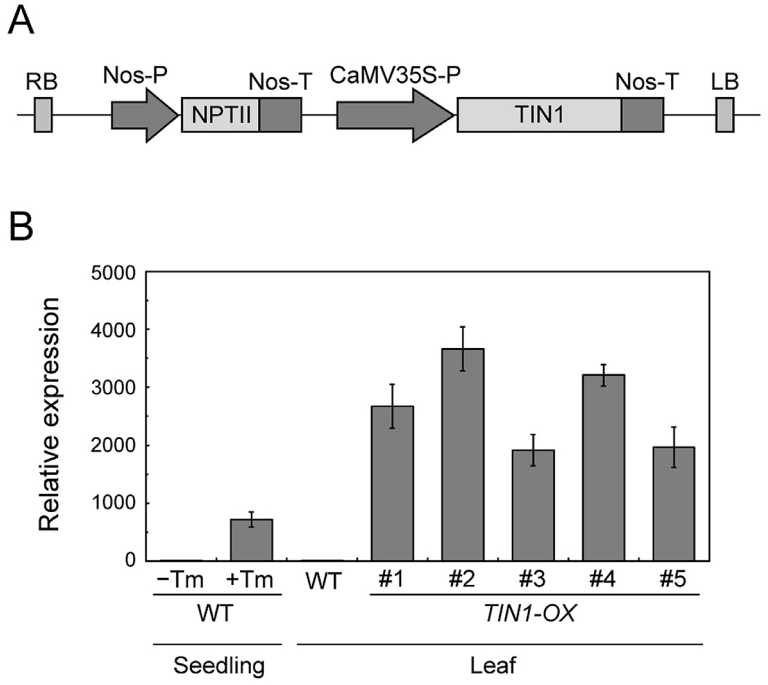

Figure 1. Generation of *TIN1-OX* plants. (A) A schematic diagram of the construct used to generate *TIN1-OX* plants. CaMV35S-P, CaMV35S promoter; Nos-P, Nos promoter; Nos-T, Nos terminator; NTPII, neomycin phosphotransferase II; RB, right border; LB, left border; (B) Expression level of *TIN1* in wild-type and *TIN1-OX* plants. RNA was extracted from leaves of 4-week-old wild-type and *TIN1-OX* plants and subjected to quantitative RT-PCR analysis. Ten-day-old wild-type seedlings treated with 5 μg ml^{-1} tunicamycin (+Tm) or 0.1% dimethyl sulfoxide (−Tm) were also analyzed for comparison. The expression level of *TIN1* was normalized to that of *Act8*.

To generate *TIN1-OX* plants, we constructed a binary vector that expresses *TIN1* gene under CaMV35S promoter (Figure 1A) and introduced it into Arabidopsis wild-type plants. We obtained five T3 lines that exhibit high *TIN1* expression even without the ER stress inducer tunicamycin (Figure 1B). The expression level of *TIN1* in *TIN1-OX* was comparable to that of tunicamycin-treated wild-type plants (Figure 1B). When we grew wild-type and *TIN1-OX* plants side-by-side and compared the growth phenotype, *TIN1-OX* plants were indistinguishable from wild-type plants in growth and development, flower morphology, silique length, and seed size (Figure 2).

We have shown that *TIN1* gene is highly expressed in pollen and that the *tin1-1* mutant exhibits pollen grains with altered surface morphology (Iwata et al. 2012). We therefore observed pollen grains from wild-type and *TIN1-OX* plants under SEM and examined whether *TIN1-OX* plants have any defects in pollen grains. We found altered pollen grain morphology of *TIN1-OX* plants (Figure 3). We observed adherence of a substance on the pollen surface of *TIN1-OX* plants, which was not seen in that of wild-type plants.

We have shown that *tin1-1* mutant plants also harbor pollen grains with abnormal surface structure but did not exhibit any defects in growth and development in ambient laboratory growth conditions (Iwata et al. 2012). The present study also demonstrated that the effect of *TIN1* overexpression was confined to pollen grains. A possible explanation for this pollen-specific phenotypic alteration is that the role of TIN1 is to assist folding and maturation of pollen-specific secretory or membrane proteins, and altered levels of *TIN1* affects the secretion of proteins and lipids, culminating in altered pollen surface morphology. A role of the UPR during male gametophyte development has been suggested by the previous observations that an Arabidopsis UPR-related mutant, *ire1a ire1b*, exhibit reduced male fertility (Deng et al. 2013, 2016). It is tempting to speculate that reduced *TIN1* expression in the *ire1a ire1b* mutant contributes to some extent to the reduced male fertility.

The UPR is conserved among diverse eukaryotic cells,

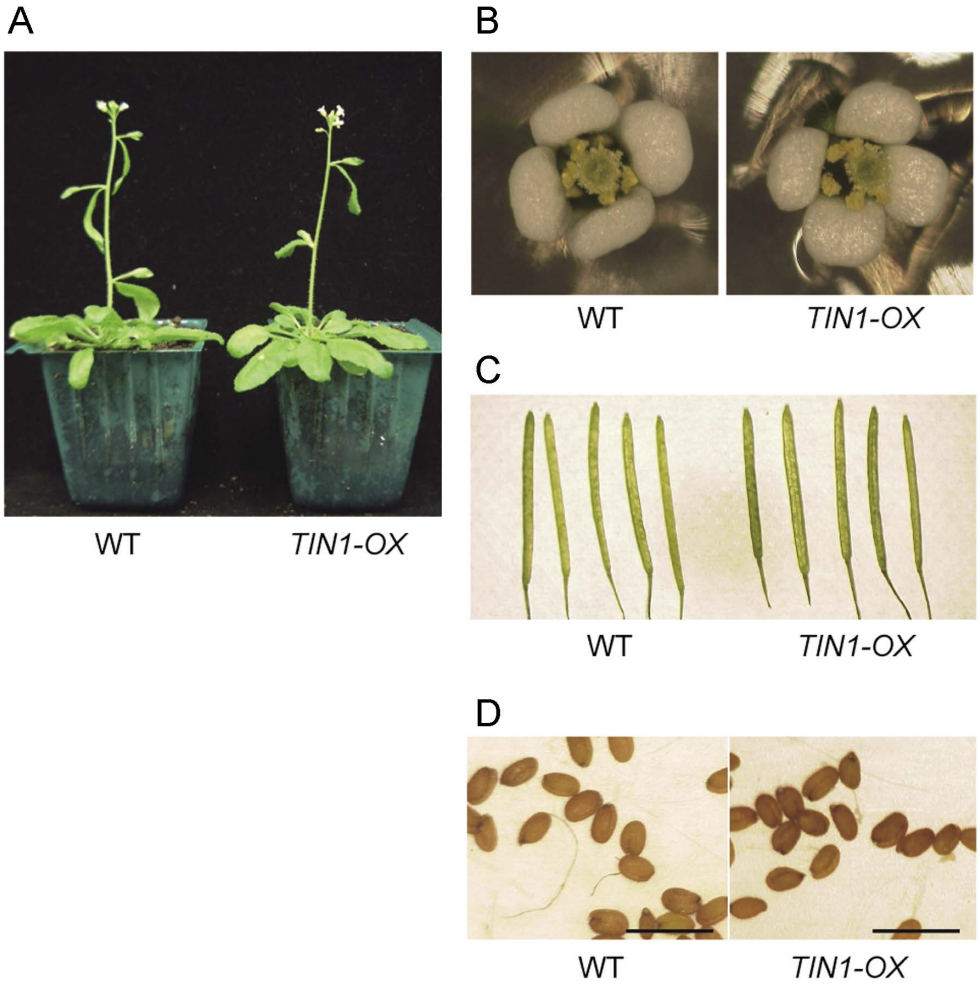

Figure 2. Observation of *TIN1-OX* plants. (A–D) Pictures of soil-grown wild-type and *TIN1-OX* (line #2) plants. (A) Five-week-old plants. (B) Eight-week-old flowers. (C) Eight-week-old siliques. (D) Ten-week-old seeds. Bar = 1 mm.

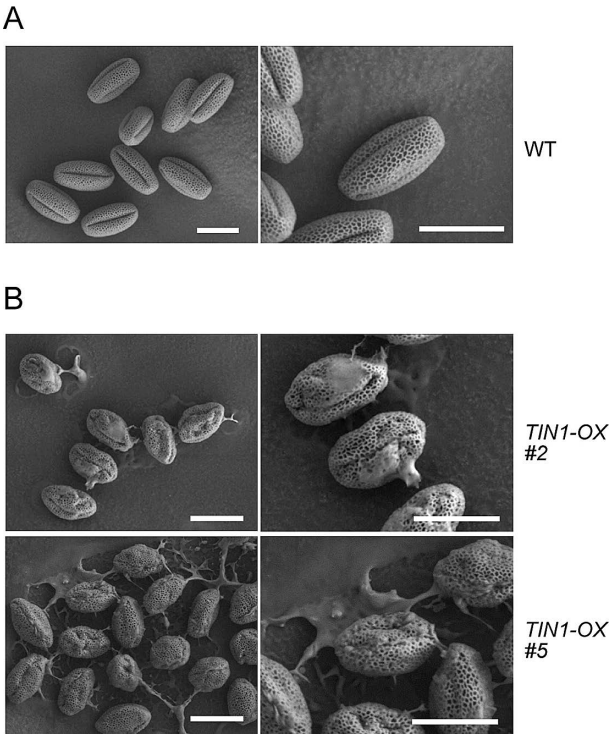

Figure 3. SEM observation of pollen grains of *TIN1-OX* plants. (A) SEM images of pollen grains of wild-type plants. (B) SEM images of pollen grains of *TIN1-OX* plants. Images from two independent *TIN1-OX* lines (#2 and #5) were shown. Bar = 20 μm.

including yeast, animals, and plants (Howell 2013; Iwata and Koizumi 2012; Ron and Walter 2007). Although the molecular mechanism of transcription factor activation differ to some extent among different species (Iwata and Koizumi 2012), the UPR induces a similar set of genes, which encode ER molecular chaperones such as BiP and calnexin, to increase the protein folding capacity in the ER. However, although *TIN1* is an ER stress-inducible gene that encodes an ER-localized protein (Iwata et al. 2010a), *TIN1* homologs are found in sequenced genomes of plant species but not in those of other species. Elucidation of the function of TIN1 during pollen development would provide interesting insights into the diversity of the UPR and the ER folding machinery.

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