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Original Paper

Role of the plant-specific endoplasmic reticulum stress-inducible gene *TIN1* in the formation of pollen surface structure in *Arabidopsis thaliana*

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Abstract Accumulation of unfolded proteins in the endoplasmic reticulum (ER) of eukaryotic cells triggers the transcriptional activation of ER-resident molecular chaperones and folding enzymes to maintain cellular homeostasis. This process is known as the ER stress response or the unfolded protein response. We have identified *tunicamycin induced 1* (*TIN1*), a plant-specific ER stress-inducible *Arabidopsis thaliana* gene. The TIN1 protein is localized in the ER; however, its molecular function has yet to be clarified. In this study, we performed functional analysis of *TIN1 in planta*. RT-PCR analysis showed that *TIN1* is highly expressed in pollen. Analysis using the *β*-glucuronidase reporter gene demonstrated that the *TIN1* promoter is active throughout pollen development, peaking at the time of flowering and in an ovule of an open flower. Although a T-DNA insertion mutant of *TIN1* grows normally under ambient laboratory conditions, abnormal pollen surface morphology was observed under a scanning electron microscope. Based on the current and previous observations, a possible physiological function of *TIN1* during pollen development is discussed.

Key words: *Arabidopsis thaliana*, endoplasmic reticulum, molecular chaperone, pollen, unfolded protein response.

Secretory and membrane proteins synthesized on the rough endoplasmic reticulum (ER) of eukaryotic cells enter the ER as unfolded polypeptide chains. These proteins must undergo proper folding and modifications, such as N-linked glycosylation and disulfide bond formation, in the ER before transportation to subcellular compartments where they function. This process is assisted by molecular chaperones and folding enzymes located in the ER (Dudek et al. 2009; Michalak et al. 2009; Tu and Weissman 2004). BiP is an ER-localized HSP70 family protein that assists in protein folding by binding to a hydrophobic peptide region exposed on the protein surface. BiP activity is regulated by ER-localized J domain-containing protein (J protein), an HSP40 family protein that functions as a co-chaperone with HSP70. Protein disulfide isomerase catalyzes disulfide bond formation, allowing proteins to efficiently find the correct arrangement of disulfide bonds, thereby facilitating protein folding. Calreticulin and its membrane-bound isoform, calnexin, are lectin chaperones that recognize an N-linked glycan chain. Unfolded or misfolded proteins that fail to fold are retrotranslocated to the cytosol from ER and degraded by the ubiquitin–proteasome system, a process called ER-associated protein degradation (ERAD) (Vembar and Brodsky 2008). Proteins involved in these processes constitute an ER protein quality control (ERQC) system that ensures full functionality of secretory and membrane proteins.

Perturbation of the ERQC system causes accumulation of unfolded proteins in the ER, a condition called ER stress. To cope with ER stress, eukaryotic cells activate a number of ERQC component genes. This protective response is termed the ER stress response or the unfolded protein response (UPR) (Ron and Walter 2007). In Arabidopsis (*Arabidopsis thaliana*), three basic leucine zipper (bZIP) transcription factors, bZIP17, bZIP28,

Abbreviations: bZIP, basic leucine zipper; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; ERQC, endoplasmic reticulum quality control; GUS, *β*-glucuronidase; IRE1, inositol-requiring enzyme 1; J protein, J domain-containing protein; SEM, scanning electron microscopy; TAIR, The Arabidopsis Information Resource; *TIN1*, *tunicamycin induced 1*; UPR, unfolded protein response.

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and bZIP60, have been shown to mediate the ER stress response (Iwata and Koizumi 2005; Iwata et al. 2009b; Liu et al. 2007; Tajima et al. 2008). Inositol-requiring enzyme 1 (IRE1), an ER-localized transmembrane kinase/RNase conserved in yeast, animals, and plants, regulates bZIP60 by unconventional mRNA splicing (Deng et al. 2011; Nagashima et al. 2011). bZIP17 and bZIP28 are membrane-bound transcription factors activated by regulated intramembrane proteolysis, liberating their bZIP domain from the ER membrane (Che et al. 2010; Liu et al. 2007; Tajima et al. 2008). The active form of the bZIP60 protein is present in anthers and *bZIP60* is highly expressed in pollen and tapetal cells, indicating the importance of the ER stress response in these tissues (Iwata et al. 2008; Iwata et al. 2009a).

Using oligonucleotide arrays covering 21,500–24,000 Arabidopsis genes, we performed transcriptome analysis of Arabidopsis using tunicamycin, an N-linked glycosylation inhibitor that causes ER stress by triggering accumulation of unglycosylated proteins that do not fold correctly (Koizumi et al. 1999), and identified a number of tunicamycin-responsive genes (Iwata et al. 2008; Iwata et al. 2010b; Nagashima et al. 2011). In addition to genes involved in ERQC, such as ER-resident molecular chaperones and ERAD components, we identified some tunicamycin-inducible genes that do not show homology to any gene with known function. To better understand the ERQC components in plants, we analyzed one such gene, *tunicamycin induced 1* (*TIN1*, AGI code At5g64510) (Iwata et al. 2010a). *TIN1* orthologs are found in several plant species but not in some organisms, including yeast and animals. *TIN1* is highly induced by ER stress and the TIN1 protein is localized in the ER (Iwata et al. 2010a); however, its physiological function remains unclear. In the present study, we conducted further analysis to gain insight into the physiological functions of *TIN1* and have presented evidence that suggests the involvement of *TIN1* in certain aspects of pollen development and function.

Materials and methods

Plant materials and stress treatment

We used *Arabidopsis thaliana* (Col-0) as the wild-type. The T-DNA insertion mutant *tin1-1* (line ID: GABI_123G05) was obtained from GABI-Kat (http://www.gabi-kat.de/) (Rosso et al. 2003). Plants were grown at 23°C under a 16-h-light/8-h-dark cycle. For tunicamycin treatment, *Arabidopsis* seedlings were grown in half strength Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 1% sucrose for 10 days and treated with 5 μg ml^{-1} tunicamycin or 0.1% dimethylsulfoxide (DMSO) as a solvent control for indicated time periods.

RT-PCR

To examine the expression level of *TIN1*, roots, stems, rosette leaves, cauline leaves, buds, open flowers, pollen grains, and siliques of 8-week-old plants and 2-week-old seedlings were used. Pollen grains were collected using vacuum as previously described (Johnson-Brousseau and McCormick 2004). Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen) and reverse transcribed with an oligo(dT) primer using the ExScript RT reagent Kit (Takara). *TIN1* and *Act8* transcripts were PCR amplified using Primer_TIN1-F (5′-ATGGGTCAC AGAGTATTGGTTTATG-3′) and Primer_TIN1-R (5′-TGG TAAAGAAAGGCGCATATTATTAGC-3′) for *TIN1* and Primer_Act8-F (5′-GTCGCTGTCGACTACGAGCAAG-3′) and Primer_Act8-R (5′-CTGTGGACAATGCCTGGA CCTGC-3′) for *Act8*. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Generation of TIN1 Promoter::β-glucuronidase (GUS) transgenic plants

The genomic sequence data of *TIN1* were downloaded from The Arabidopsis Information Resource (TAIR) web site (Swarbreck et al. 2008). An approximately 1-kb region of the *TIN1* promoter was amplified by PCR using Primer_TIN1pro-F (5′-GGGTCGACAAAAAATAACTTAATAGTCCATTACGAC ATG-3′) and Primer_TIN1pro-R (5′-GGGGATCCACCCATC TTGGATTGACCAAAAAAAACC-3′) and cloned into the pGEM-T Easy vector (Promega). The *TIN1* promoter fragment was then subcloned into *Bam*HI and *Sal*I sites of the pBI101 vector (Clontech), resulting in the binary vector pBI101-TIN1pro-GUS. Transformation was performed by the floral-dip method (Clough and Bent 1998).

GUS assay

For quantitative GUS activity measurement, 10-day-old TIN1pro-GUS seedlings were treated with 5 μg ml^{-1} tunicamycin or 0.1% DMSO for 24 h and subjected to an assay using a substrate, 4-methylumbelliferyl-β-D-glucuronide, as previously described (Iwata and Koizumi 2005).

For histochemical GUS staining, each tissue of 8-week-old TIN1pro-GUS plants was immersed in the GUS staining solution {0.1 M sodium phosphate buffer, 10 mM EDTA (pH 8.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 1 mM X-glucuronide, 0.1% Triton X-100}, applied with vacuum for 15 min, and then incubated at 37°C. GUS-stained tissues were dehydrated with ethanol and embedded in Spurr's resin (Spurr Low-Viscosity Embedding Media, Polysciences, Inc.). The tissues were then sectioned to a 2–3-μm thickness and observed using a light microscope.

Isolation of tin1-1

A T-DNA insertion mutant of *TIN1* (line ID: GABI_123G05) was obtained from GABI-Kat (Rosso et al. 2003) and designated as *tin1-1*. The presence of T-DNA in *tin1-1* was verified by genomic PCR using Primer_TIN1-F, Primer_

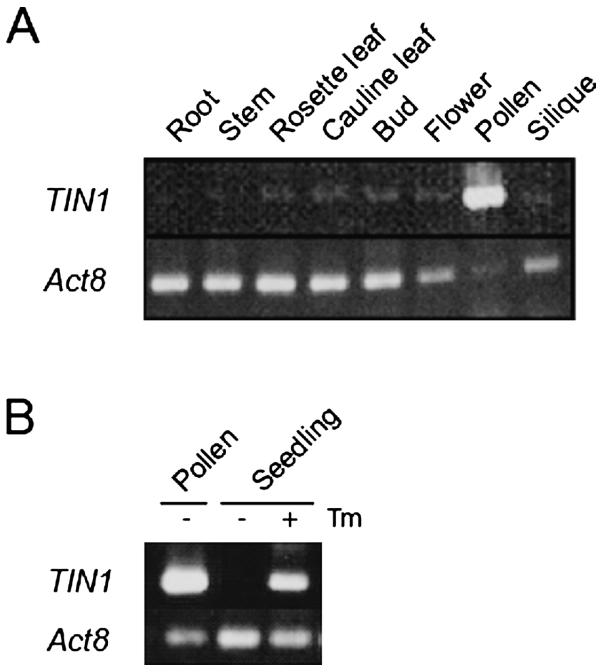

Figure 1. Tissue-specific expression of *TIN1*. (A) Expression of *TIN1* in various tissues. RT-PCR analysis was performed using RNA extracted from indicated tissues. *Act8* was used as a control. (B) Comparison of the expression level of *TIN1* in pollen with that in tunicamycin-treated seedlings. Ten-day-old seedlings were treated with 5 μg ml^{-1} tunicamycin or 0.1% DMSO as a solvent control and subjected to RT-PCR analysis.

TIN1-R and Primer_T-DNA (5′-CCCATTTGGACGTGAATG TAGACAC-3′).

Scanning electron microscopy (SEM)

Mature pollen grains from wild-type and *tin1-1* were observed as previously described (Iwano et al. 1999). In brief, mature pollen grains were attached with double-sided sticky carbon tape to the aluminum stub, which was then mounted on the cooling stage at −20°C. The specimen was observed and analyzed with a SEM (Hitachi S-3200N) at a chamber pressure of 30 Pa and an accelerating voltage of 15 kV.

Results

Tissue-specific expression of TIN1

We previously showed that Arabidopsis *TIN1* is strongly induced in seedlings and suspension cells in response to treatment with ER stress inducers (Iwata et al. 2010a). To gain insight into the function of *TIN1* throughout plant development, we first investigated the expression profile of *TIN1* by RT-PCR using RNA extracted from various tissues. As shown in Figure 1A, a high level of *TIN1* transcripts was detected in mature pollen grains. Consistent with this result, eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), a web-based tool for exploring publicly available microarray data, shows high expression of *TIN1* in pollen (Winter et al. 2007).

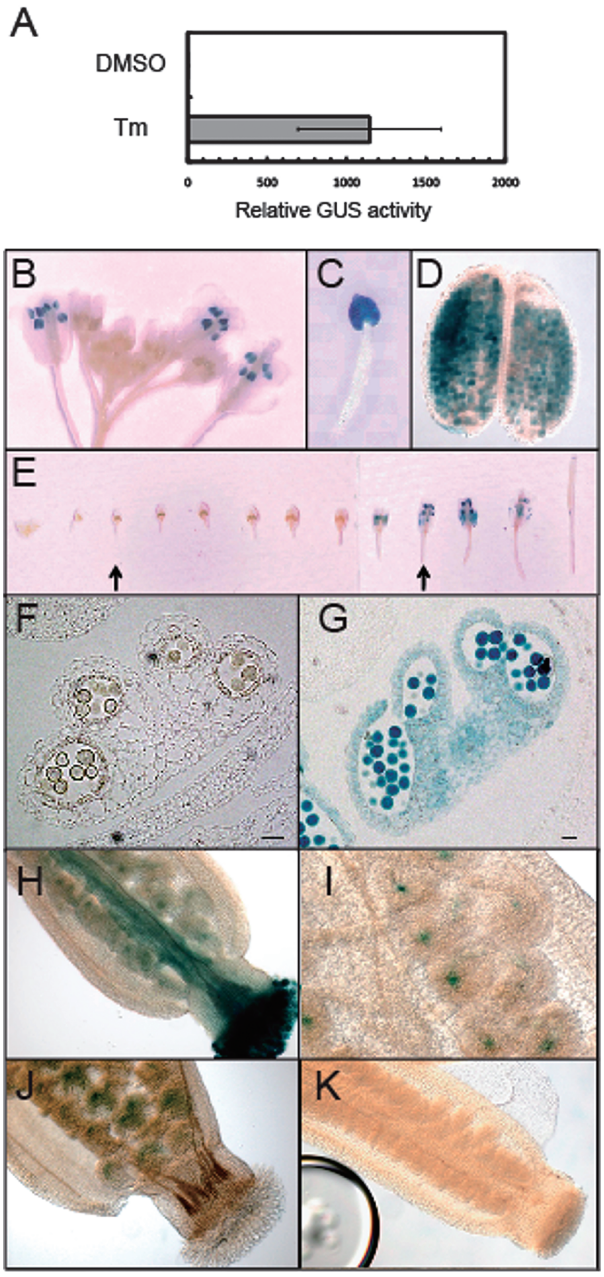

Figure 2. Analysis of TIN1pro::β-glucuronidase (GUS) transgenic plants. (A) Quantitative measurement of GUS activity in TIN1pro::GUS plants with or without tunicamycin treatment. Protein extracts were prepared from 10-day-old TIN1pro::GUS seedlings treated with 5 μg ml^{-1} tunicamycin or 0.1% DMSO as a solvent control for 24 h. Quantification of GUS activity was performed using 4-methylumbelliferyl-β-D-glucuronide as a substrate. (B–K) GUS staining of TIN1pro::GUS plants without stress treatment. (B) Inflorescence. (C) Stamen. (D) Anther. (E) Flowers with various developmental stages. (F, G) Sections of anthers. The anthers of a bud (F) and an open flower (G) used for sectioning are indicated by arrows in (E). The second flower from the right was used for staining two days after flowering. Bar=20 μm. (H, I) Pollinated pistil of an open flower. (J) Unpollinated pistil of an open flower. (K) Pistil of a bud.

The levels of *TIN1* transcripts in pollen were comparable with those in tunicamycin-treated seedlings (Figure 1B).

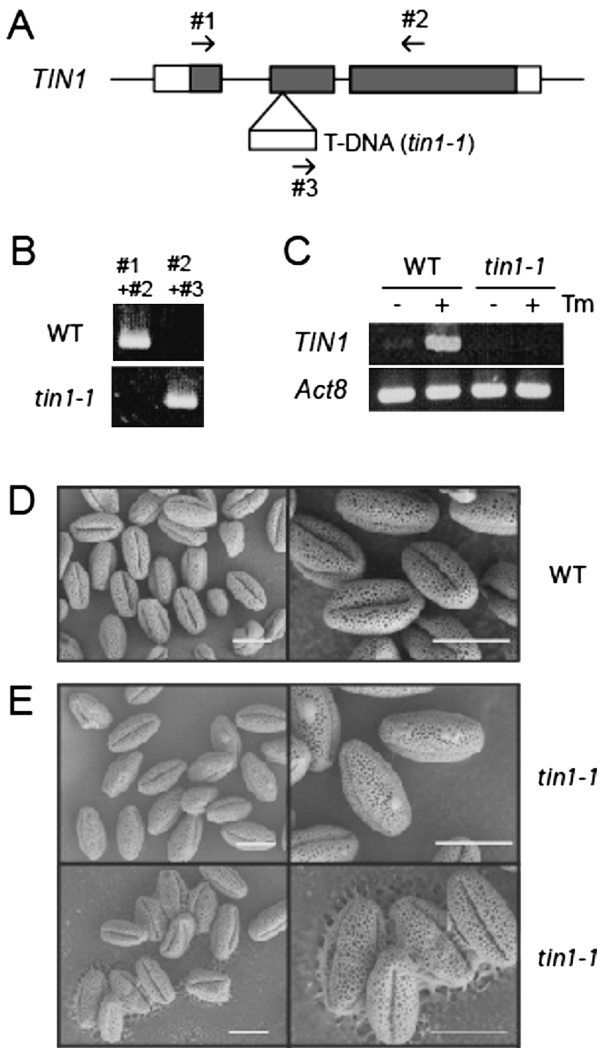

Figure 3. Isolation and analysis of *tin1-1*. (A) Schematic representation of *TIN1* and the location of T-DNA insertion in *tin1-1*. The gray and white boxes represent a coding and untranslated sequence, respectively. Primers used in the following experiments are indicated. (B) Isolation of homozygous *tin1-1*. Genomic PCR was performed using primers mentioned in (A) to confirm the presence of T-DNA and the disruption of the gene in the *tin1-1* homozygous mutant. (C) Expression of *TIN1* in wild-type and *tin1-1* in response to tunicamycin treatment. Two-week-old Arabidopsis seedlings were treated with 5 μg ml^{-1} tunicamycin or 0.1% DMSO as a solvent control. RT-PCR analysis was carried out using primers 1 and 2 mentioned in (A) to detect *TIN1* transcripts. *Act8* was used as a control. (D, E) SEM observation of pollen grains from wild-type and *tin1-1*. (D) Wild-type pollen. (E) *tin1-1* pollen. Bar = 20 μm.

TIN1 promoter activity

To investigate the expression profile of *TIN1* in more detail, we utilized the *GUS* reporter gene. We first defined the 937-bp genomic DNA fragment upstream from the start codon of *TIN1* as the *TIN1* promoter. We then introduced the construct harboring the *TIN1* promoter fused to the *GUS* reporter gene into the wild-type Arabidopsis plants. The resulting TIN1pro::GUS transgenic plants showed strong GUS activity when treated with tunicamycin (Figure 2A), indicating that the *TIN1* promoter used here responds to tunicamycin treatment in the same manner as the endogenous *TIN1*. Under ambient laboratory growth conditions, marked GUS staining was observed in anthers (Figure 2B, C, D).

To investigate the temporal activity of the *TIN1* promoter throughout anther development, histochemical GUS staining was performed using flowers at various developmental stages. The signal intensity of GUS staining in anthers increased over the course of bud growth, peaking at the day of anthesis (Figure 2E). A decrease in signal intensity was observed two days after anthesis (Figure 2E). Strong GUS staining of anthers at the day of anthesis was mostly attributed to pollen, as evidenced by analysis of a section of stained buds, with a faint GUS signal also evident in the epidermis (Figure 2F, G).

GUS staining was also observed in a pistil of an open flower (Figure 2H, I). The signal in an ovary originated from a part of the ovules, most likely from the charazal region. To determine whether GUS activity in the pistil was attributable to the pistil itself or the penetrating pollen tubes, we observed the pistil of an open flower whose stamens were removed before flowering to prevent pollination. As shown in Figure 2J, the GUS staining pattern of an unpollinated pistil was indistinguishable from that of a pollinated pistil. Furthermore, GUS activity was not detectable in the pistil of a bud (Figure 2K). These results indicate that the expression of *TIN1* in an ovule is temporally controlled, regardless of whether the pistil is pollinated.

Isolation of tin1-1

To gain insight into the physiological function of *TIN1*, we isolated a T-DNA insertion mutant of *TIN1*, *tin1-1*, from GABI-Kat collection (Rosso et al. 2003). This mutant contains a T-DNA in the second exon of *TIN1* (Figure 3A). The homozygous mutant was isolated and confirmed by genomic PCR (Figure 3B). RT-PCR analysis showed the absence of *TIN1* transcripts in *tin1-1* seedlings even when treated with tunicamycin (Figure 3C). When grown on soil or plates in ambient laboratory conditions, *tin1-1* did not show any detectable growth defect compared with the growth of the wild-type plant.

Observation of tin1-1 pollen using SEM

Our observation that *TIN1* is strongly expressed in pollen grains prompted us to ask whether a mutation in *TIN1* affects some aspects of pollen development and function. Therefore, we observed the pollen of wild-type and *tin1-1* by SEM and found abnormal morphology in *tin1-1* pollen (Figure 3D, E). We detected altered morphology in pollen surface structure with protrusion and/or adherence of a substance on the pollen surface (Figure 3E). We also observed pollen adhering to each other through the substance on their surfaces (Figure

3E). Such abnormalities were observed in 35% of *tin1-1* pollen (n=112), but only in 2.4% of wild-type pollen (n=124).

Discussion

Arabidopsis *TIN1* was originally identified as a gene highly induced by tunicamycin, an N-linked glycosylation inhibitor causing ER stress (Iwata et al. 2010a), and initial studies of *TIN1* were carried out in the context of the ER stress response. Our previous study showed that the induction of *TIN1* by ER stress is partly dependent on bZIP60, a transcription factor involved in the Arabidopsis ER stress response (Iwata et al. 2010a). We have also shown that *bZIP60* is strongly expressed in pollen and the active form of the bZIP60 protein is present in anthers (Iwata et al. 2008). Taking into account our current observation that *TIN1* is highly expressed in pollen, it is plausible that pollen cells suffer from ER stress during microgametogenesis and that high expression of *TIN1* in pollen is at least partly dependent on the ER stress-activated IRE1–bZIP60 pathway. However, because we do not have direct evidence supporting this notion, an equally viable possibility is that the activation of *TIN1* in pollen is independent of ER stress or bZIP60 function.

TIN1 homologs are present in plants but not in other species, including yeast and animals, suggesting that the TIN1 protein functions in plant-specific cellular and developmental processes. In the present study, we found that *TIN1* is highly expressed in pollen and *tin1-1* exhibits abnormal morphology of pollen grain surface. However, the nature of the substance found on *tin1-1* pollen is unknown. In addition, it remains to be determined whether *tin1-1* pollen is functionally impaired (e.g., dysfunctional pollen tube germination and elongation). Although it remains to be analyzed whether a wild-type *TIN1* gene construct can complement *tin1-1* phenotype or whether other knockout or knockdown alleles of *TIN1* gene show similar defect, the abnormal pollen surface morphology observed in *tin1-1* suggests the involvement of *TIN1* in aspects of pollen development and function.

We previously showed that the TIN1 protein fused with a fluorescent protein localizes to the ER (Iwata et al. 2010a). The fact that many ER stress-inducible genes encode an ER-localized protein involved in ERQC, such as ER-resident molecular chaperones and ERAD components (Iwata et al. 2010b; Martinez and Chrispeels 2003), indicates the involvement of the TIN1 protein in ERQC. Indeed, *SDF2*, a tunicamycin-inducible gene whose molecular function had been unclear, was recently shown to be involved in ERQC through interaction with certain ER-resident molecular chaperones (Nekrasov et al. 2009; Schott et al. 2010). Furthermore, the co-expression database ATTED-II (Obayashi et al. 2007) identifies a gene encoding an ERQC component, *ERdj3A*, as the gene that shows the strongest correlation in an expression profile with *TIN1*. *ERdj3A* encodes an ER-resident J protein that functions as a co-chaperone with BiP. Similar to *TIN1*, *ERdj3A* is transcriptionally induced by tunicamycin treatment (Yamamoto et al. 2008) and its promoter is highly active in pollen (Yang et al. 2009). Interestingly, the *ERdj3a* mutant has functionally deficient pollen characterized by a marked decrease in pollen tube growth at high temperatures (Yang et al. 2009). Most notably, the *bzip34* mutant pollen has been reported to exhibit an adherent pollen surface phenotype, reminiscent of the *tin1-1* mutant pollen, with less developed ER and altered transcript level of some of the ER-related genes (Gibalova et al. 2009).

It should also be noted that the prevailing view is that the pollen surface components are derived from tapetal cells, which surround the developing pollen (Edlund et al. 2004). Nevertheless, some pollen surface components may be derived from pollen cells, because PCP-A1 (pollen coat protein, class A, 1), a defensin-like pollen coat protein, has been reported to be the product of a gametophytically expressed gene (Doughty et al. 1998). Therefore, it is conceivable that defective ERQC during pollen development caused by a mutation in *TIN1* affects secretion of proteins and/or lipids, culminating in a visibly altered pollen surface.

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