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**Original Paper**

# Transcriptomic response of *Arabidopsis thaliana* to tunicamycin-induced endoplasmic reticulum stress

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**Abstract** Secretory and membrane proteins of eukaryotic cells must be properly folded and assembled in the endoplasmic reticulum (ER) before translocation to their final destination where they function. Perturbation of this process results in accumulation of unfolded proteins in the ER, so-called ER stress. The cells initiate a protective response to maintain cellular homeostasis, which is termed the ER stress response or the unfolded protein response (UPR). In the present study, we performed time-series transcriptome analysis of the ER stress response in Arabidopsis (*Arabidopsis thaliana*) with the N-linked glycosylation inhibitor tunicamycin, which causes misfolding of proteins in the ER, and therefore, triggers ER stress. A total of 259 genes were identified as tunicamycin-responsive genes, 175 of which were upregulated and 84 were downregulated. Hierarchical clustering and bioinformatic analysis demonstrated that 259 tunicamycin-responsive genes can be assigned to one of the six distinct expression classes and identified a potential novel cis-element, as well as known cis-elements, i.e., ER stress response element and UPR element. We also observed that a considerable number of tunicamycin-inducible genes, including those encoding the ER chaperone BiP and the membrane-bound transcription factor AtbZIP60, are coordinately upregulated at a late pollen development stage in Arabidopsis. This observation suggests that the ER stress response plays an important role in the development and function of pollens.

**Key words:** *Arabidopsis thaliana*, endoplasmic reticulum stress, pollen, tunicamycin, unfolded protein response.

In eukaryotic cells, secretory and membrane proteins first enter the endoplasmic reticulum (ER) as unfolded polypeptide chains. Before being transported to their final destination, these proteins need to undergo proper folding and modification, including N-linked glycosylation and disulfide bond formation in the ER, to ensure their functionality. Perturbation of this process results in accumulation of unfolded proteins in the ER, a condition called "ER stress," and cells respond in different manners to obviate such a situation. These responses are collectively referred to as the ER stress response or the unfolded protein response (UPR) (Kaufman et al. 2002; Mori 2000; Ron and Walter 2007). In mammals, the importance of the ER stress response has been recently recognized in different cellular processes (Schroder 2008; Todd et al. 2008; Yoshida 2007).

The ER stress response was first characterized as the process responsible for transcriptional induction of genes coding for the ER chaperone and folding enzymes that included BiP, an ER cognate of Hsp70. Tunicamycin, an N-linked glycosylation inhibitor, has been commonly used to induce ER stress, and many studies have been conducted to clarify the molecular mechanism of *BiP* induction in yeast and mammalian cells using tunicamycin. In yeast, the ER stress sensor protein IRE1 plays a vital role in transducing ER stress signaling. Upon activation, IRE1 catalyzes spliceosome-independent cytosolic splicing of *HAC1* mRNA, coding for a basic leucine zipper (bZIP) transcription factor (Cox et al. 1993; Mori et al. 1993; Mori et al. 2000). HAC1 protein is synthesized from spliced *HAC1* mRNA and in turn activates *BiP*/*Kar2* transcription (Mori et al. 1996; Shamu and Walter 1996). Transcriptome analyses have shown that many aspects of secretory pathway functions are upregulated in response to ER stress. These not only include the genes for ER-resident molecular chaperones but also those for ER–Golgi transport, ER-associated degradation (ERAD) components, and lipid/inositol

Abbreviations: BCP, bicellular pollen; bZIP, basic leucine zipper; CNX, calnexin; CR, common reference; CRT, calreticulin; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated protein degradation; ERSE, endoplasmic reticulum stress response element; MPG, mature pollen grains; MS, Murashige and Skoog; PDI, protein disulfide isomerase; qRT-PCR, quantitative RT-PCR; RSAT, Regulatory Sequence Analysis Tools; TAIR, The Arabidopsis Information Resource; TCP, immature tricellular pollen; TIGR, The Institute for Genomic Research; UNM, uninucleate microspores; UPR, unfolded protein response; UPRE, unfolded protein response element

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metabolism, comprising ~6% of the yeast genome (Travers et al. 2000).

In contrast to yeast cells, where ER stress signal transduction is solely dependent on the IRE1–HAC1 pathway, mammalian cells have three ER stress transducers: IRE1, ATF6, and PERK. IRE1 regulates cytosolic splicing of mRNA coding for the bZIP transcription factor XBP1 in a manner similar to that for yeast IRE1 (Shen et al. 2001; Yoshida et al. 2001). XBP1 in turn activates the expression of genes coding for ER chaperones and ERAD components including *BiP*. This induction has been shown to depend on the following ER stress-responsive cis-elements: UPR element (UPRE; consensus sequence TGACGT-GR), ER stress-response element (ERSE; consensus sequence CCAAT-N9-CCACG), and ERSE-II (consensus sequence ATTGG-N-CCACG) (Kokame et al. 2001; Wang et al. 2000; Yamamoto et al. 2004; Yoshida et al. 1998). The ER membrane-bound bZIP transcription factor ATF6 is cleaved in response to ER stress, and its cytosolic fragment containing the bZIP domain translocates into the nucleus, where it acts as a transcription factor through the cis-elements ERSE and ERSE-II (Haze et al. 1999; Yamamoto et al. 2004; Ye et al. 2000; Yoshida et al. 2000). In addition to the transcriptional response mediated by IRE1–XBP1 and ATF6 pathways, the transmembrane protein kinase PERK phosphorylates the eukaryotic translation initiation factor eIF2$\alpha$, which leads to the inhibition of mRNA translation, and thus, reduces the load of newly synthesized ER proteins in the stressed ER (Harding et al. 2001; Harding et al. 1999). Transcriptome analysis has revealed that genes upregulated during ER stress have rather been found to be restricted to ER-resident chaperones and ERAD components (Okada et al. 2002), perhaps reflecting a mechanistic difference between yeast and mammalian cells in the ability to respond to ER stress. That is, the mammalian ER stress response comprises both transcriptional and translational control, whereas only the former is present in its yeast counterpart; hence, a more strong transcriptional response would be required to cope with ER stress.

Although observations regarding the ER stress response have been documented in plants (Boston et al. 1991; Iwata and Koizumi 2005b; Jelitto-Van Dooren et al. 1999; Koizumi et al. 1999; Leborgne-Castel et al. 1999), the molecular mechanism has not yet been completely elucidated. Analysis of *BiP* promoters in Arabidopsis (*Arabidopsis thaliana*) has shown conservation of cis-elements between plant and mammalian cells (Noh et al. 2003; Oh et al. 2003). Although proteins homologous to yeast and mammalian IRE1 have been reported in Arabidopsis and rice (*Oryza sativa*) (Koizumi et al. 2001; Noh et al. 2002; Okushima et al. 2002), their involvement in the ER stress signaling pathway has not yet been determined; neither has any mRNA that is spliced been identified. Arabidopsis AtbZIP60 is the best characterized transcription factor involved in the ER stress response in plants (Iwata et al. 2008; Iwata and Koizumi 2005a; Iwata et al. 2009b). *AtbZIP60* was first identified as a gene transcriptionally induced in response to ER stress (Iwata and Koizumi 2005a). The AtbZIP60 protein resides in the ER membrane with its transmembrane domain under unstressed conditions and is activated by proteolysis in response to ER stress. This allows the cytosolic fragment containing the bZIP domain to translocate into the nucleus, where it acts as a transcription factor (Iwata et al. 2008). AtbZIP60 can also amplify ER stress signaling by activating its own transcription through the ERSE-like sequence on the *AtbZIP60* promoter (Iwata and Koizumi 2005a). The AtbZIP60 protein is activated in anthers and the *AtbZIP60* gene is highly expressed in pollen and tapetal cells within an anther without an exogenous supply of stress reagents, thus suggesting the importance of the ER stress response in the development and function of pollen and tapetal cells (Iwata et al. 2008). Silencing of *NbbZIP60*, an *AtbZIP60* homolog in *Nicotiana benthamiana*, has been reported to compromise host defense against the non-host pathogen *Pseudomonas cichorii*, suggesting its involvement in innate immunity in plants (Tateda et al. 2008). In addition to AtbZIP60, AtbZIP28 has also been shown to act as a transcription factor activating *BiP* genes during the Arabidopsis ER stress response (Liu et al. 2007; Tajima et al. 2008).

Transcriptome analysis has also been reported in Arabidopsis using Affymetrix GeneChip covering ~8000 protein-coding genes (Martinez and Chrispeels 2003; Noh et al. 2003) and a fluid microarray (Kamauchi et al. 2005), and genes related to protein folding and degradation have been shown to be upregulated by ER stress. In this study, time-series transcriptome analysis using Agilent Arabidopsis 2 Oligo Microarrays, covering 21,500 Arabidopsis genes, was conducted. The current transcriptome data was compared with the previously published Arabidopsis transcriptome data on pollen in which AtbZIP60 activation had been observed (Iwata et al. 2008).

## Materials and methods

### Plant materials

*Arabidopsis thaliana* (Col-0) seeds were surface sterilized, germinated in half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2% (w/v) sucrose, and cultured under a 16 h light/8 h dark cycle at 23°C with gentle shaking for 10 days.

### RNA blot

Total RNA was extracted from 10-day-old seedlings treated with 5 $\mu$g ml$^{-1}$ tunicamycin (Wako Pure Chemical) at the indicated time points using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. RNA was loaded (5 $\mu$g per lane) and fractionated on a 1.2% agarose gel containing 2% formaldehyde. It was capillary blotted onto a nylon membrane (Hybond N; Amersham Bioscience) in 20×SSC. An approximately 200-bp fragment of the 5′ region of *BiP3* cDNA (Iwata et al. 2008) was labeled with [$\alpha$-$^{32}$P]dCTP using a DNA labeling kit (BcaBEST labeling kit; Takara). The membrane was hybridized with labeled probes in 1 mM EDTA, 0.5% SDS, 50 mM Tris-HCl, pH 7.5, 1×Denhardt's solution, 3×SSC, 50% formamide, and 10% dextran sulfate at 42°C overnight. The membrane was washed 3 times with 0.2×SSC and 0.1% SDS at 55°C and then analyzed using a BAS-2500 bioimaging analyzer (Fuji Photo Film).

### Microarray hybridization and data analysis

Total RNA was extracted from 10-day-old seedlings treated with tunicamycin (5 $\mu$g ml$^{-1}$) at the indicated time points using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. Total RNA samples were processed to cDNA, labeled, and hybridized to Arabidopsis 2 Oligo Microarrays (Agilent Technologies), which were then scanned using an Agilent Technologies Microarray Scanner and processed using Feature Extraction 7.5.1 (Agilent Technologies). Each RNA sample, labeled with Cy5, was hybridized competitively with common reference (CR) labeled with Cy3; an equal mixture of each total RNA was used for CR. Two independent batches of seedlings were used as the source of RNA for each condition.

Genes showing at least a 2.5-fold change during tunicamycin treatment were defined as tunicamycin-responsive genes, and 259 genes with reliably altered levels were identified by one-way ANOVA (*P* value cutoff, 0.05) using GeneSpring GX software (Silicon Genetics). Hierarchical clustering was also performed using the same software. The averaged values from two independent batches of seedlings per condition are shown in Supplemental Table 1.

To predict the hydrophobic regions that may constitute a transmembrane domain or a signal peptide, a known or predicted amino acid sequence of each gene in clusters V and VI was obtained from The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org/) (Swarbreck et al. 2008) and analyzed by the DAS-TMfilter website (http://mendel.imp.ac.at/sat/DAS/DAS.html) (Cserzo et al. 2002).

### Quantitative RT-PCR

Ten-day-old Arabidopsis seedlings were treated with tunicamycin (5 $\mu$g ml$^{-1}$) or 0.1% DMSO as a solvent control for the indicated time periods. Three independent batches of seedlings for each condition were used as the source of RNA. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. Each 100 ng of RNA was reverse transcribed with oligo(dT) primers using the PrimeScript RT reagent kit (Takara) according to the manufacturer's protocol. Real-time PCR measurements were performed using a 7300 Real-Time PCR System (Applied Biosystems) and SYBR Premix *Ex Taq* (Takara). Primers used were as follows: 5′-TCGTGATGTTGGAAAATTTGA-3′ and 5′-TCTTCATCTTGCTCTGTAGCTGA-3′ for *SRO2* (AGI code; At1g23550), 5′-CGATGATGCTGTGGCTAAAA-3′ and 5′-TCTCAAGCATTCTCTTTCGAGAT-3′ for *AtbZIP60* (At1g42990), 5′-GCTGATGAAGATGTGAGGATGA-3′ and 5′-CACAGGCCTTGGCAAAAA-3′ for *Hap5b* (AGI code; At1g56170), 5′-TCGTCCCATTCATGTCAAAG-3′ and 5′-TTTGTAGTGTTTCCAGGAGTGC-3′ for *GLP1* (AGI code; At1g72610), 5′-CCAGCACCAACTGCAACA-3′ and 5′-TCGAAACATCCGCTAGTAGCTT-3′ for *LTP2* (AGI code; At2g38530), and 5′-TCAGCACTTTCCAGCAGATG-3′ and 5′-ATGCCTGGACCTGCTTCAT-3′ for *Act8* (AGI code; At1g49240). Transcript abundance was normalized to that of *Act8*.

### Motif analysis

To detect the presence of ERSE, ERSE-II, and UPRE in promoters of genes in clusters I and II, we used Patmatch (Yan et al. 2005), which is available at the TAIR website (http://www.arabidopsis.org/) (Swarbreck et al. 2008). Since the first cytosine residue of CCACG in ERSE (CCAAT-N9-CCACG) and ERSE-II (ATTGG-N-CCACG) was reported to have a minor effect, consensus sequences used were as follows: ERSE, CCAAT-N10-CACG; ERSE-II, ATTGG-N2-CACG. For UPRE, the consensus sequence (TGACGT-GR) was used without modification. A 500-bp upstream sequence from the known or predicted transcription start site was used as a promoter.

To identify potential cis-elements, we retrieved 500 bp of promoter sequences upstream from the known or predicted transcription start site for each gene in cluster I from the TAIR website (http://www.arabidopsis.org/) (Swarbreck et al. 2008) and analyzed them using Regulatory Sequence Analysis Tools (RSAT) oligo-analysis (http://rsat.ulb.ac.be/rsat/) (Thomas-Chollier et al. 2008) with oligomer length 8. Having identified two 8 nucleotide sequences with 7 nucleotides overlapping each other, we surveyed for the combined 9 nucleotide sequence AAAATATCT using Patmatch to study the presence of this element on promoters of tunicamycin-responsive genes.

To determine if these cis-elements are over-represented among the promoters of tunicamycin-responsive genes, the frequency of each motif in 500 bp of promoter sequences in all Arabidopsis genes was first determined using Patmatch. The probability of finding promoter regions having one or more elements in the set of promoter regions of tunicamycin-responsive genes in clusters I and II was then calculated.

### Expression of tunicamycin-inducible genes during pollen development

We first obtained genes that were upregulated more than or equal to 4-fold at all time points in tunicamycin-treated seedlings. Expression values for these genes were retrieved from the pollen transcriptome data described by Honys and Twell (2004) and showed them in a graph format. Among 30 genes that show more than or equal to 4-fold at all time points in tunicamycin-treated seedlings, 3 genes (At3g51980, At4g05010 and At5g23575) were missing in the pollen transcriptome data. We therefore used the remaining 27 genes in this analysis.

## Results and discussion

### *Identification of tunicamycin-responsive genes*

We used Agilent Arabidopsis 2 Oligo Microarrays, which contain 60-mer oligonucleotides for each of the 21,500 genes derived from ATH1 ver. 3 database of The Institute for Genomic Research (TIGR). RNA for microarray analysis was isolated from 10-day-old wild-type seedlings before tunicamycin treatment and from those treated with tunicamycin for 2, 5, and 10 h. These time points were determined on the basis of RNA blot analysis, which showed that the mRNA level of *BiP3*, a typical marker gene for the Arabidopsis ER stress response (Iwata et al. 2008; Noh et al. 2003), increases at 2 h, peaks at 5 h, and decreases at 10 h after tunicamycin administration (Figure 1).

Of the 21,500 genes represented on the microarray, 19,583 showed significant signal intensity. Genes showing at least 2.5-fold change at one or more time points during tunicamycin treatment were defined as tunicamycin-responsive genes, and it was observed that 259 genes were tunicamycin responsive. Among them, 175 genes were upregulated and 84 were downregulated at different times in the experiment. These changes in gene expression were statistically significant at $P<0.05$ (see Materials and methods). On comparing with previous transcriptome studies that used an oligonucleotide array comprising ~8000 genes (Martinez and Chrispeels 2003; Noh et al. 2003) and a fluid microarray (Kamauchi et al. 2005), which detected ~70 tunicamycin-inducible genes, our time-series transcriptome study covering 21,500 genes was able to identify a larger number of tunicamycin-responsive genes.

### *Tunicamycin-responsive genes can be assigned to one of six expression clusters*

The 259 tunicamycin-responsive genes were subjected to hierarchical clustering based on their relative transcript levels in plants after 2, 5, and 10 h of tunicamycin treatment. Each gene was assigned to one of six expression clusters (Figure 2). Expression clusters I, II, and IV comprised upregulated transcripts and clusters V and VI comprised downregulated transcripts. Clusters II and IV comprised genes that were upregulated at 2 h,

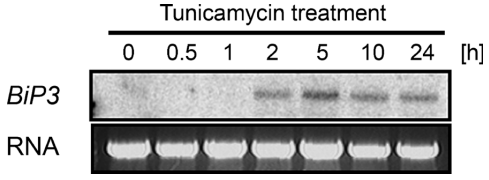


Figure 1. RNA blot analysis of *BiP3* transcripts. Total RNA was extracted from Arabidopsis seedlings treated with 5 $\mu$g ml$^{-1}$ tunicamycin for the indicated times. Total RNA (5 $\mu$g per lane) was fractionated on an agarose gel, transferred to a nylon membrane, and probed with *BiP3*-specific cDNA fragments. rRNA was visualized by ethidium bromide staining as a loading control.

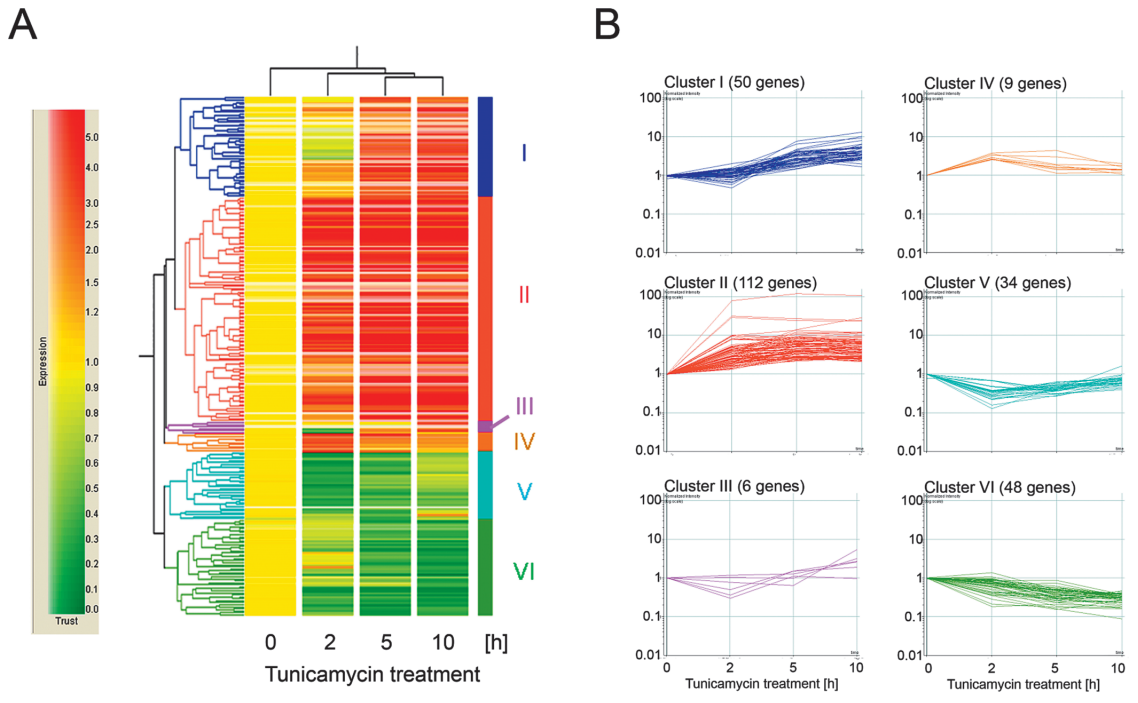


Figure 2. Hierarchical cluster and expression profiles of tunicamycin-responsive genes. (A) Hierarchical cluster analysis of the 259 tunicamycin-responsive genes. RNA was extracted from Arabidopsis seedlings treated with 5 $\mu$g ml$^{-1}$ tunicamycin for the indicated times and used in microarray analyses. Each horizontal bar represents a single gene. Green indicates a relatively low expression level of a given mRNA, and red indicates a relatively high expression level. (B) Expression profiles for genes in each cluster are presented in a graph format. The graphs plot log of ratio values over time.

whereas genes in cluster I started to increase at a later phase (i.e., 5 and 10 h). The major difference between clusters V and VI was that the transcript levels in cluster VI continued to decrease between 5 and 10 h, whereas those in cluster V showed some recovery over this time interval. Cluster III comprised both upregulated and downregulated transcripts. A complete list of these genes is given in Supplemental Table 1.

To confirm the microarray results, selected genes were subjected to quantitative RT-PCR (qRT-PCR) analysis. The results showed consistency with the characteristics of each cluster mentioned above (Figure 3). That is, for genes upregulated by tunicamycin treatment, transcripts of the *SRO2* gene (AGI code; At1g23550) from cluster I showed slower induction in response to tunicamycin than those of *AtbZIP60* (AGI code; At1g42990) and *Hap5b* (AGI code; At1g56170), genes assigned to clusters II and IV, respectively. For genes downregulated by tunicamycin treatment, transcript levels for the cluster V gene *GLP1* (AGI code; At1g72610) dropped rapidly, followed by some recovery over this time interval, whereas *LTP2* (AGI code; At2g38530), a gene assigned to cluster VI, remained downregulated.

### *Genes upregulated in response to tunicamycin treatment*

Most genes that were upregulated by tunicamycin treatment were found in clusters I and II. There was a marked difference between these two clusters. Cluster II comprised genes that were upregulated after 2 h of tunicamycin treatment, whereas genes that started to increase at a later phase (i.e., 5 and 10 h) were assigned to cluster I. Transcriptional induction of genes that encode molecular chaperones and folding enzymes functioning in the ER is a hallmark of the ER stress response, and most of them were found in cluster II. These include three BiPs, five protein disulfide isomerases (PDIs) (Houston et al. 2005; Lu and Christopher 2008), ER oxidoreductin ERO1 (Dixon et al. 2003), two calnexins (CNX1 and CNX2) (Boyce et al. 1994; Huang et al. 1993), calreticulin CRT2 (Christensen et al. 2008), three ER-localized J domain-containing proteins (ERdj2B, ERdj3A, and ERdj3B) (Yamamoto et al. 2008), and GRP94 (Klein et al. 2006). Also found in cluster II were genes coding for proteins involved in protein import into the ER (two Sec61$\alpha$, two Sec61$\beta$, two Sec61$\gamma$, and signal peptidase), vesicular transport (Sar1, three Arfs, and three Emp24-like proteins), ERAD (Der1 and Sel1) (Kirst et al. 2005), and glycosylation (AtUTR1 and AtUTR3) (Reyes et al. 2010; Reyes et al. 2006).

In contrast, only 4 genes coding for such proteins were identified in cluster I: peptidyl-prolyl cis-trans isomerase Roc7 (He et al. 2004; Romano et al. 2004), CRT1, PDI1, and a protein homologous to yeast Emp24 (Kuehn et al. 1998; Schimmoller et al. 1995). Given that genes assigned to cluster I are upregulated later than those in cluster II, cluster I would be expected to include genes encoding proteins necessary for recovery from cellular damage caused by ER stress. In this respect, genes coding for thioredoxin and glutathione S-transferase (AtGSTU22) in cluster I are interesting. One glutathione S-transferase gene (AtGSTU11), assigned to cluster III, was also found to be upregulated in a later phase. Because ER-stressed cells were considered to be exposed

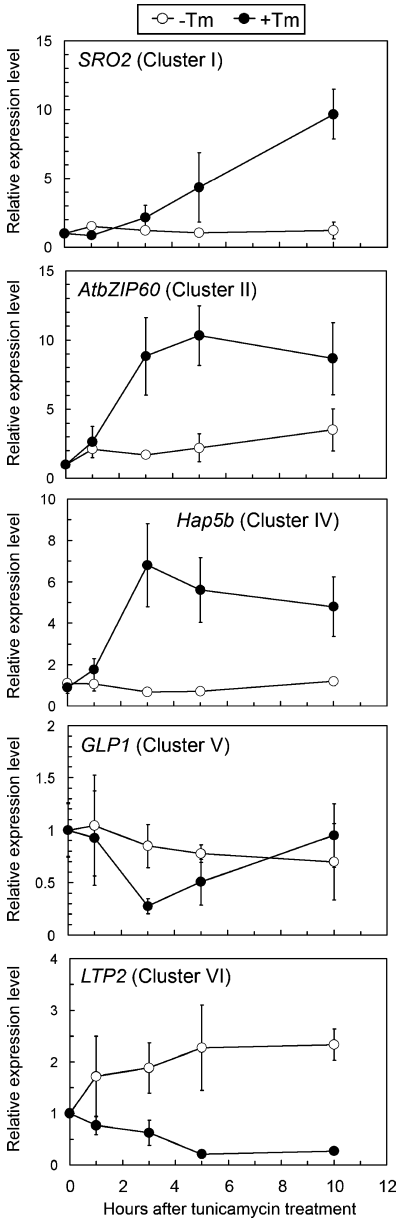


Figure 3. qRT-PCR analysis of selected tunicamycin-responsive genes. Arabidopsis seedlings were treated with 5 $\mu$g ml$^{-1}$ tunicamycin (+Tm, closed circles) or 0.1% DMSO as a solvent control (−Tm, open circles). RNA was extracted at each time point, reverse transcribed, and subjected to real-time PCR analysis. Abundance of transcripts for *SRO2* (cluster I), *AtbZIP60* (cluster II), *Hap5b* (cluster IV), *GLP1* (cluster V), and *LTP2* (cluster VI) was normalized to that of *Act8* transcripts. Data represent means with SE of three independent experiments.

to more oxidized conditions in yeast and mammalian cells (Haynes et al. 2004; Marciniak et al. 2004), upregulation of these genes might be a protective response to reduce reactive oxygen species generated in such situations. In fact, the IRE1–HAC1 pathway in yeast has been reported to possess the ability to activate antioxidative stress genes (Kimata et al. 2006). Another interesting gene found in cluster I is *ATG8D*, an essential gene for autophagosome formation (Yoshimoto et al. 2004). Because activation of autophagy in response to ER stress has also been reported to play an important role in survival of mammalian cells (Ogata et al. 2006), the observed upregulation of *ATG8D* implies a similar mechanism in plants.

Many genes encoding a transcription factor were found to be upregulated. The tunicamycin-inducible membrane-bound transcription factor *AtbZIP60* was assigned to cluster II. We previously observed that although the induction of ER stress-responsive genes is much less pronounced in the *atbzip60* mutant, some genes are still inducible (Iwata et al. 2008). This indicates that additional transcription factors play an important role in the Arabidopsis ER stress response. In fact, AtbZIP28 was recently shown to be involved in the induction of *BiP* genes in response to ER stress (Liu et al. 2007; Tajima et al. 2008). As identified by our microarray analysis, highly upregulated genes encoding a transcription factor are other possible candidate transcription factors activating ER stress-responsive genes. Analysis of these candidate genes awaits future study.

### *Known and potential cis-elements*

The number, order, and type of transcription factor-binding cis-elements present in promoters are major determinants of the differences in gene expression patterns. We first searched promoter sequences of ER stress-responsive genes for ERSE, ERSE-II, and UPRE that are known cis-elements responsible for transcriptional induction in response to ER stress (see Materials and methods for details). As shown in Table 1, both ERSE and UPRE were enriched in promoter sequences of genes in clusters I and II, with higher enrichment in cluster II. ERSE was found to be highly enriched in cluster II, as much as 26.8-fold. ERSE-II was present only in the promoters of *BiP1* and *BiP2* genes, which were classified in cluster II.

Although ERSE and UPRE were significantly over-represented in both clusters I and II, their enrichment was less pronounced in cluster I, which comprises genes that were upregulated later than those in cluster II. This prompted us to speculate that other potential cis-acting regulatory elements might also be present in cluster I. Therefore, we then took advantage of a bioinformatic approach to identify such elements involved in coordinate gene regulation. In this regard, oligo-analysis in Regulatory Sequence Analysis Tools (RSAT; http://rsat.ulb.ac.be/rsat/) (Thomas-Chollier et al. 2008) was used to search for 8-bp sequences that were significantly over-represented in the promoter regions of genes in this cluster (see Materials and methods for details). The results showed that two motifs, AAAATATC and AAATATCT, were over-represented. Since 7 bases overlap between these two motifs, promoters of genes in cluster I were searched for the combined motif AAAATATCT. This element was 4.9-fold enriched in cluster I compared with random occurrence in promoters of the whole Arabidopsis genome (Table 1). Enrichment of this element was also observed in cluster II, albeit to a lesser extent (2.5-fold).

Given that this potential novel cis-element was more clearly enriched in cluster I than in cluster II, it will be interesting to test whether this element is actually responsive to ER stress at a later stage. If this can be verified, this cis-element could then be used to identify additional transcription factors involved in shaping the transcriptome in response to ER stress.

### *Genes downregulated in response to tunicamycin treatment*

Most of the genes that were downregulated in response to tunicamycin treatment were assigned to clusters V and VI. Prediction of hydrophobic regions revealed that more than half of the genes (46 out of 82 genes in clusters V and VI) have potential hydrophobic regions that could be a transmembrane domain or a signal peptide in their protein products (data not shown). However, none of them seems to be involved in maintaining homeostasis of the ER and subsequent secretory pathway. Thus, a considerable number of downregulated genes were likely to encode proteins entering ER and being secreted or vacuole targeted through the secretory pathway.

Table 1. Known and potential cis-elements found in promoters of tunicamycin-inducible genes

| | Cluster I (50 genes) | | | Cluster II (112 genes) | | |
|---|---|---|---|---|---|---|
| | Motif occurrence Observed (Expected) | Fold-Enrichment | *P* value | Motif occurrence Observed (Expected) | Fold-Enrichment | *P* value |
| ERSE | 3 (0.55) | 5.5 | 0.0173 | 34 (1.27) | 26.8 | <0.0001 |
| ERSE-II | 0 (0.10) | 0 | | 2 (0.24) | 8.3 | 0.0242 |
| UPRE | 6 (1.44) | 4.2 | 0.0031 | 22 (3.34) | 6.6 | <0.0001 |
| AAAATATCT | 10 (2.06) | 4.9 | <0.0001 | 12 (4.79) | 2.5 | 0.0032 |

This downregulation may be a protective response that alleviates the excess load of newly synthesized proteins in the ER. This inference is based on observations in other organisms. In mammalian cells, the ER stress sensor PERK initiates inhibition of translation by phosphorylating eIF2$\alpha$ (Harding et al. 2001; Harding et al. 1999). In fact, enhanced translation in cells experiencing ER stress exacerbated ER stress-induced cell death (Marciniak et al. 2004). However, an extensive homology search did not find any PERK homologs in any plant species, including Arabidopsis. Consistent with this fact, a previous study showed that the regulation of translation did not seem to be altered under ER stress in Arabidopsis (Kamauchi et al. 2005). These observations imply that in response to ER stress, Arabidopsis decreases mRNA levels rather than attenuates translation to achieve the same purpose. At present it is unclear whether the decrease in mRNA abundance in response to tunicamycin treatment observed here is due to active degradation of existing mRNA or cessation of transcription. Because we could not detect any over-represented sequences in the promoters of downregulated genes using RSAT oligo-analysis (data not shown), it could be that the downregulation of these genes might not be mediated by a transcriptional repressor binding to promoters in a sequence-dependent manner. Metazoan IRE1 was recently found to mediate the rapid degradation of a specific mRNAs subset coding for secreted proteins in response to ER stress (Hollien et al. 2009; Hollien and Weissman 2006). Of particular interest is whether Arabidopsis IRE1 homologs, whose target mRNA is yet to be identified, are involved in this process.

### *Regulation of tunicamycin-inducible genes during pollen development*

We have reported that the AtbZIP60 protein is activated in anthers, and the *AtbZIP60* gene is highly expressed in pollen and tapetal cells within an anther without an exogenous supply of stress agents such as tunicamycin and dithiothreitol, thus suggesting the importance of the ER stress response regulated by AtbZIP60 in these tissues (Iwata et al. 2008; Iwata et al. 2009a). This observation prompted us to compare the present microarray results with publicly available transcriptome data using these tissues.

Honys and Twell (2004) conducted transcriptome profiling throughout microgametogenesis at the following four different stages of pollen development: uninucleate microspores (UNM), bicellular pollen (BCP), immature tricellular pollen (TCP), and mature pollen grains (MPG). Hierarchical clustering analysis resulted in 39 coregulated clusters covering all phases of male gametophyte development. We therefore asked whether genes upregulated by ER stress are coordinately regulated during pollen development.

To this end, we focused on genes that were highly upregulated in the course of tunicamycin treatment. We obtained genes that were upregulated more than or equal to 4-fold at all time points in tunicamycin-treated seedlings. Having identified 27 genes (see Materials and methods for details), all of which were assigned to cluster II, we retrieved transcript levels of these genes from the pollen transcriptome data. We found that a considerable number of these genes were upregulated during pollen development (Figure 4). More specifically, almost half of these genes (13 out of 27 genes; 48%), including *BiP1*, *BiP2*, *BiP3*, *AtbZIP60*, *ERdj3A*, *Sec61*$\alpha$, *Arf1Ad*, *AtUTR1*, *AtUTR3*, and *ERO1*, were found in clusters 1 and 3, which comprise genes whose transcripts continue to increase through TCP and MPG stages and cover only 5.3% of genes on the Affymetrix gene chip (1190 out of 22591 genes) (Honys and Twell 2004) (Figure 4A). Five genes were assigned to clusters 5, 6, and 31, which comprise 309, 1229, and 153 genes whose transcripts show a relatively steady state level, and 6 genes were assigned to clusters 25, 27, and 29, which comprise 2064, 702, and 4464 genes whose transcripts decrease after the TCP stage (Honys and Twell 2004) (Figure 4B). Three genes showed no detectable transcript levels at any pollen development stage (Honys and Twell 2004) (Figure 4B). Thus, a considerable number of genes that are highly upregulated by tunicamycin treatment were coordinately upregulated during the late pollen development stages.

It has been reported that pollen cells have a high secretory capacity to support pollen tube growth after pollination (Hepler et al. 2001). Consistent with pollen's secretory functions, extensive ER development has been documented in pollens of many plant species (Ciampolini et al. 1988; Polowick and Sawhney 1993; Rodriguez-Barcia and Fernandez 1990; Weber 1989). These observations imply that ER expansion is required to support pollen's proper function. There is accumulating evidence that the mammalian ER stress response is required for the development of specialized secretory cells having highly developed ER. For instance, the IRE1–XBP1 pathway is indispensable for the differentiation of B cells to plasma cells in which a large amount of immunoglobulin is synthesized and assembled in the ER (Iwakoshi et al. 2003; Reimold et al. 2001). It was also reported that a deficient ER stress response results in poor ER development in pancreatic and salivary gland acinar cells, accompanied by decreased expression of ER chaperone genes (Lee et al. 2005). These observations support the notion that the ER stress response modulates the capacity of the ER and subsequent secretory pathway in response to cellular demands (Yoshida 2009).

Corroborating this notion, XBP1 has been shown to possess the ability to drive ER biogenesis. In particular,

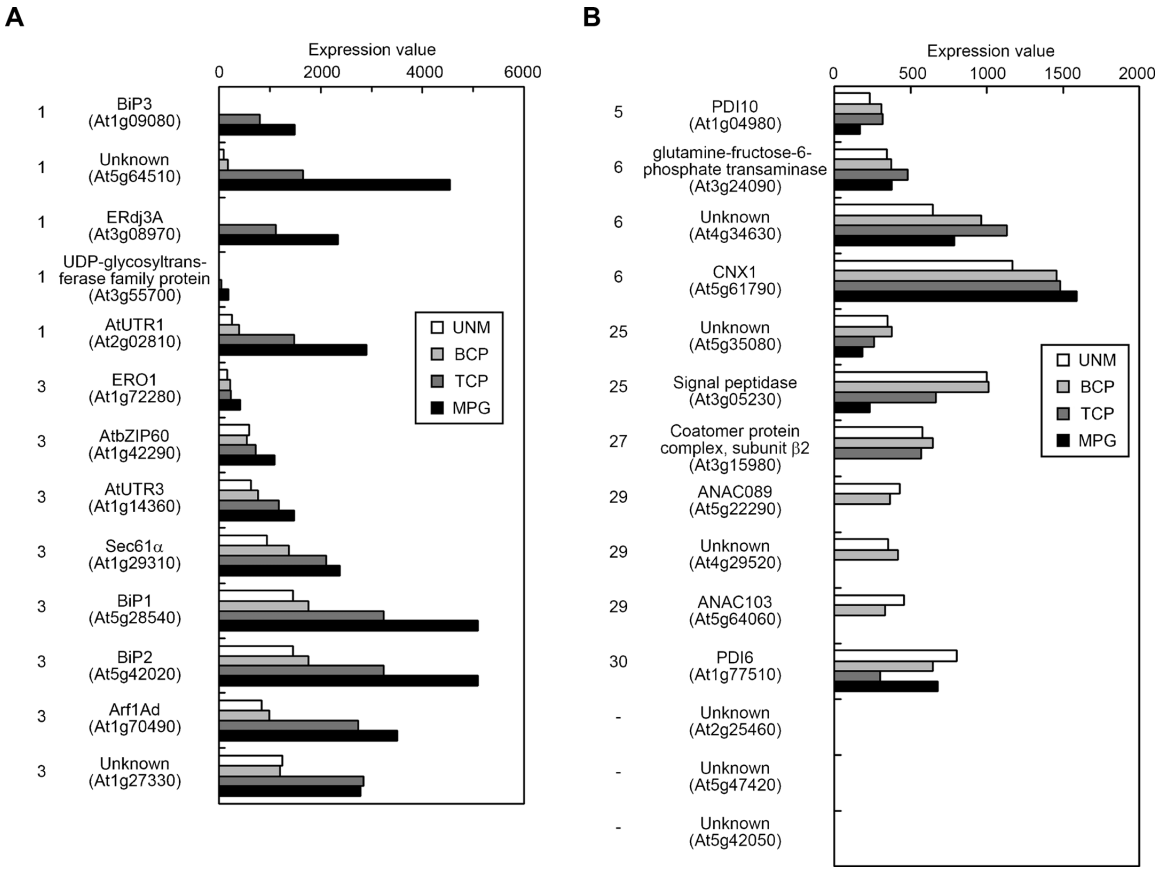


Figure 4. Expression of genes highly upregulated by tunicamycin treatment during pollen development. Expression values at each stage of pollen development were retrieved from the pollen transcriptome data reported by Honys and Twell (2004) and presented in a graph format. Shown are 13 genes assigned to cluster 1 or 3 (A) and 11 genes assigned to other clusters (B). Also included in (B) are 3 genes at the bottom of the graph showing no detectable transcript levels at any pollen development stage. On the leftmost of each graph is the cluster number assigned by Honys and Twell (2004) for each gene. For details of those clusters, see the main text. The gene name and AGI code of each gene are indicated to the left of each graph. Note that the maximum scale value in (A) is three times as large as that in (B). UNM, uninucleate microspores; BCP, bicellular pollen; TCP, immature tricellular pollen; MPG, mature pollen grains.

expression of the active form of XBP1 suffices for triggering the expansion of the rough ER, which is correlated with increased phospholipid biosynthesis and expression of many genes that function in the secretory pathway (Shaffer et al. 2004; Sriburi et al. 2007; Sriburi et al. 2004). These observations are in accordance with the essential role of XBP1 as professional secretory cells. The exact mechanism by which XBP1 activates ER biogenesis is unclear, since the enzymatic activities of phospholipid biosynthesis are enhanced by overexpression of the active form of XBP1, whereas the expression levels of the corresponding genes remain unaffected (Sriburi et al. 2004). The same may hold true for plants, since a link between the ER stress response and lipid biosynthesis has also been implicated in plants. A number of lipid biosynthetic activities are increased in the endosperm of maize (*Zea mays*) mutant *floury-2* (Shank et al. 2001), which experiences ER stress caused by the accumulation of an aberrant 24-kDa $\alpha$-zein storage protein in ER and ER-derived protein bodies due to defective signal peptide processing (Boston et al. 1991; Coleman et al. 1995).

The role of the ER stress response in ER biogenesis, together with our previous and current observations, support the idea that certain aspects of the ER stress response that are regulated by AtbZIP60 and possibly by AtbZIP28 are important for the development and function of pollens. Analysis of mutant plants that are defective in genes involved in the ER stress response is necessary to elucidate how the ER stress response supports the development and function of pollens.

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