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Gene Note

Transcriptional regulation of an *Arabidopsis* gene encoding a CCT domain-containing protein during endoplasmic reticulum stress

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Abstract In eukaryotic cells, accumulation of unfolded proteins in the endoplasmic reticulum (ER) triggers the transcriptional induction of ER-resident molecular chaperones and folding enzymes to maintain cellular homeostasis, termed the ER stress response or the unfolded protein response (UPR). In this study we focused on a putative transcriptional regulator of *Arabidopsis thaliana* whose transcripts accumulate in response to the ER stress inducer tunicamycin. This gene, designated as *TCR1* (tunicamycin-induced CONSTANS-like-related 1), encodes a predicted protein of 195 amino acids with a plant-specific CCT (CONSTANS, CONSTANS-like, TOC1) domain considered to function in transcriptional regulation. In a dual-luciferase assay using *Arabidopsis* protoplasts, we showed that induction of the *TCR1* gene by tunicamycin is independent of a nucleotide sequence similar to the cis-element ER stress response element on its promoter. Our result further indicates that this induction could be mediated by the transcription factor AtbZIP60. This study is a first step to understand the role of TCR1 in the *Arabidopsis* ER stress response.

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

The endoplasmic reticulum (ER) plays a central role in synthesis and modification of secretory and membrane proteins in all eukaryotic cells. These proteins are correctly folded and assembled in the ER, but when the cellular environment is perturbed, they accumulate as unfolded or misfolded forms in the ER. Eukatyotic cells induce adaptive responses to relieve such ER stress, termed the ER stress response or the unfolded protein response (UPR), which upregulates the transcription of genes encoding ER-resident molecular chaperones such as *BiP* (Ron and Walter 2007). Recently in mammals, importance of the ER stress response has been recognized in a wide variety of cellular processes. In plants, the ER stress response has been implicated in plant-specific cellular processes such as pathogen response and seed development (Tajima et al. 2008; Vitale and Ceriotti 2004).

In the mammalian cells, ATF6, a basic leucine zipper (bZIP) transcription factor with a transmembrane domain, plays an important role in the ER stress response. ATF6 resides in the ER membrane and is subjected to proteolytic cleavage in response to ER stress (Haze et al. 1999). This cleavage localizes the cytoplasmic fragment containing a bZIP domain to the nucleus and the truncated protein activates downstream genes through the cis-elements ER stress-response element (ERSE, consensus sequence CCAAT-N9-CCACG) and ERSE-II (consensus sequence ATTGG-N-CCACG) (Yamamoto et al. 2004; Yoshida et al. 2000; Yoshida et al. 2001).

Although observation of the plant ER stress response itself has been documented (Iwata and Koizumi 2005b; Koizumi et al. 1999; Martinez and Chrispeels 2003; Wrobel et al. 1997), the molecular mechanism has not yet been fully clarified. Analyzing *Arabidopsis BiP* promoters identified the cis-elements plant-UPR element (P-UPRE) and ERSE (Noh et al. 2003; Oh et al. 2003). Even though a simple homology search could not find any transcription factors homologous to ATF6, we have isolated the ER stress-inducible *AtbZIP60* gene from *Arabidopsis* and proposed a model that AtbZIP60 is synthesized as a precursor protein anchored in the ER membrane and is activated by proteolytic cleavage in response to ER stress. This allows translocation of its cytoplasmic domain containing a bZIP domain into the nucleus and activates ER chaperone genes through P-UPRE and ERSE (Iwata and Koizumi 2005a). In order to investigate genome-wide transcriptional profile in the

Abbreviations: bZIP, basic leucine zipper; CCT, CONSTANS, CONSTANS-like, TOC1; ER, endoplasmic reticulum; ERSE, ER stress response element; GUS, β-glucuronidase; TCR1, tunicamycin-induced CONSTANS-like-related 1; UPR, unfolded protein response; P-UPRE, plant-UPR element.

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Arabidopsis ER stress response, we carried out microarray analysis using tunicamycin, an inhibitor of asparagine-linked glycosylation inducing ER stress. We used an oligonucleotide array (Agilent Arabidopsis 2 Oligo Microarray), which contains 60-mer oligonucleotides for each 21,500 gene derived from ATH1 ver. 3 database of The Institute for Genomic Research. For this experiment, *Arabidopsis thaliana* Col-0 seedlings were grown in one-half strength MS medium supplemented with 1% (w/v) sucrose in a 16 h light and 8 h dark cycle. RNA was isolated from 10-day-old seedlings before or after 5 μg ml^{-1} tunicamycin treatment for 5 h using RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions, and subjected to microarray analysis. The report of the detailed results of the microarray analysis is being preparation for publication (Iwata et al. in preparation).

This microarray analysis identified a tunicamycin-inducible gene encoding a plant-specific CCT (CONSTANS, CONSTANS-like, TOC1) domain-containing protein, here designated as *TCR1* (for tunicamycin-induced CONSTANS-like-related 1). The CCT domain-containing proteins are found in a wide range of flowering plants, but have not been described in mammals or yeast. The CCT domain-containing proteins have been considered to function as transcriptional regulators, implicated in diverse processes such as photoperiodic flowering (Putterill et al. 1995), regulation of circadian rhythms (Strayer et al. 2000), light signaling (Kaczorowski and Quail 2003) and gene expression in response to sugars (Masaki et al. 2005). In *Arabidopsis*, a CCT domain has been identified in CO and 16 COL proteins (Putterill et al. 1995; Robson et al. 2001), TOC1 and four other structurally related pseudo-response regulators, APRRs (Matsushika et al. 2000; Strayer et al. 2000) (Figure 1A). In addition, the GATA-type zinc-finger protein expressed in inflorescence meristem, ZIM, and two homologous proteins, ZML1 and ZML2, contain a CCT domain (Shikata et al. 2004). ASML2 family proteins also contain a CCT domain, without any of the domains that COL, APRR and ZIM family proteins have (Masaki et al. 2005). The *TCR1* gene (AGI code; At1g07050) is predicted to contain two introns (Figure 1B) and encode a putative protein of 195 amino acids with high homology to a CCT domain at its C-terminus (Figure 1C). TCR1 and three other predicted proteins do not belong to any of other families (Figure 1A), thus representing a novel class of CCT domain-containing proteins. In the current study, we focused on the TCR1 gene and investigated the transcriptional regulation of the *TCR1* gene during ER stress as a first step of elucidating the role of TCR1 in the *Arabidopsis* ER stress response.

To confirm the microarray analysis, we performed semi-quantitative RT-PCR analysis. Total RNA was extracted from 10-day-old seedlings treated with 5 μg ml^{-1} tunicamycin using RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. Each 100 ng of RNA was reverse transcribed using PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer's instructions with oligo(dT) primers. For semi-quantitative RT-PCR, *TCR1* cDNAs were amplified using the primers TCR1sq1 (5′-GTCGACATGGATACACAAAGGTTGCCGAAG-3′) and TCR1sq2 (5′-CCATGGAGTCTCTCTTTTCACGA-ACCGACC-3′) in 30 cycles. PCR products were separated by electrophoresis and visualized by ethidium bromide staining. Real-time quantitative RT-PCR measurements were performed using LightCycler 480 (Roche). Each transcript for *TCR1* and *Act8* was amplified using SYBR Premix Ex Taq (TaKaRa) with the primers TCR1q1 (5′-CAACTCTGGACAATTCCGTTT-3′) and TCR1q2 (5′-TGCTTCTCTTCGCATGTTTC-3′) for *TCR1* and Act8q1 (5′-TCAGCACTTTCCAGCA-GATG-3′) and Act8q2 (5′-ATGCCTGGACCTGCTT-CAT-3′) for *Act8*. Expression values of *TCR1* were normalized to those of *Act8*.

As shown in Figure 2A, accumulation of *TCR1* transcripts was observed by tunicamycin treatment. The rate of this induction was quantified by quantitative RT-PCR (Figure 2B), showing approximately 8 fold induction in response to tunicamycin treatment in 5 h. Transcriptional induction of *TCR1* was also observed in response to dithiothreitol, another ER stress inducer inhibiting disulfide bond formation, albeit to a lesser extent (data not shown). These results showed that the *TCR1* gene is transcriptionally induced by ER stress.

In order to characterize the transcriptional regulation of *TCR1* during ER stress in more detail, we carried out a dual-luciferase reporter assay by using transiently transformed protoplasts from *Arabidopsis* suspension cells. Plasmids BiP1pro-Luc, 35Spro-RLuc and 35Spro-AtbZIP60ΔC were generated as previously described (Iwata and Koizumi 2005a). A plasmid carrying the β-glucuronidase (*GUS*) gene driven by the CaMV35S promoter, pBI221 (Clontech), was used as 35Spro-GUS. For TCR1pro-Luc, the 1,500 bp promoter fragment of the *TCR1* gene was PCR amplified using the primers TCR1p1 (5′-GTCGACCAAACTTTGCTGCTTCATGT-TCATTGAAAAACAGG-3′) and TCR1p2 (5′-GGATC-CATCCATATATCTCTTATCTTTTTCTCTGTTTTGTT TTCCCTTCA-3′), and the PCR fragment with SalI and BamHI was cloned into pGEM-T Easy (Promega). The promoter fragment was then substituted with the CaMV35S promoter of pBI221-Luc digested with XhoI and BamHI. For TCR1mpro-Luc, two mutated PCR fragments were amplified using the primers TCR1p1 and TCR1p3 (5′-CCTAGCGTCTCATCGGCTAGGAAAG-AGCTA-3′) as well as TCR1p4 (5′-GACGCTAG-GGACATGCTATTAGTATAAAGA-3′) and TCR1p2. Subsequent PCR was performed using the primers

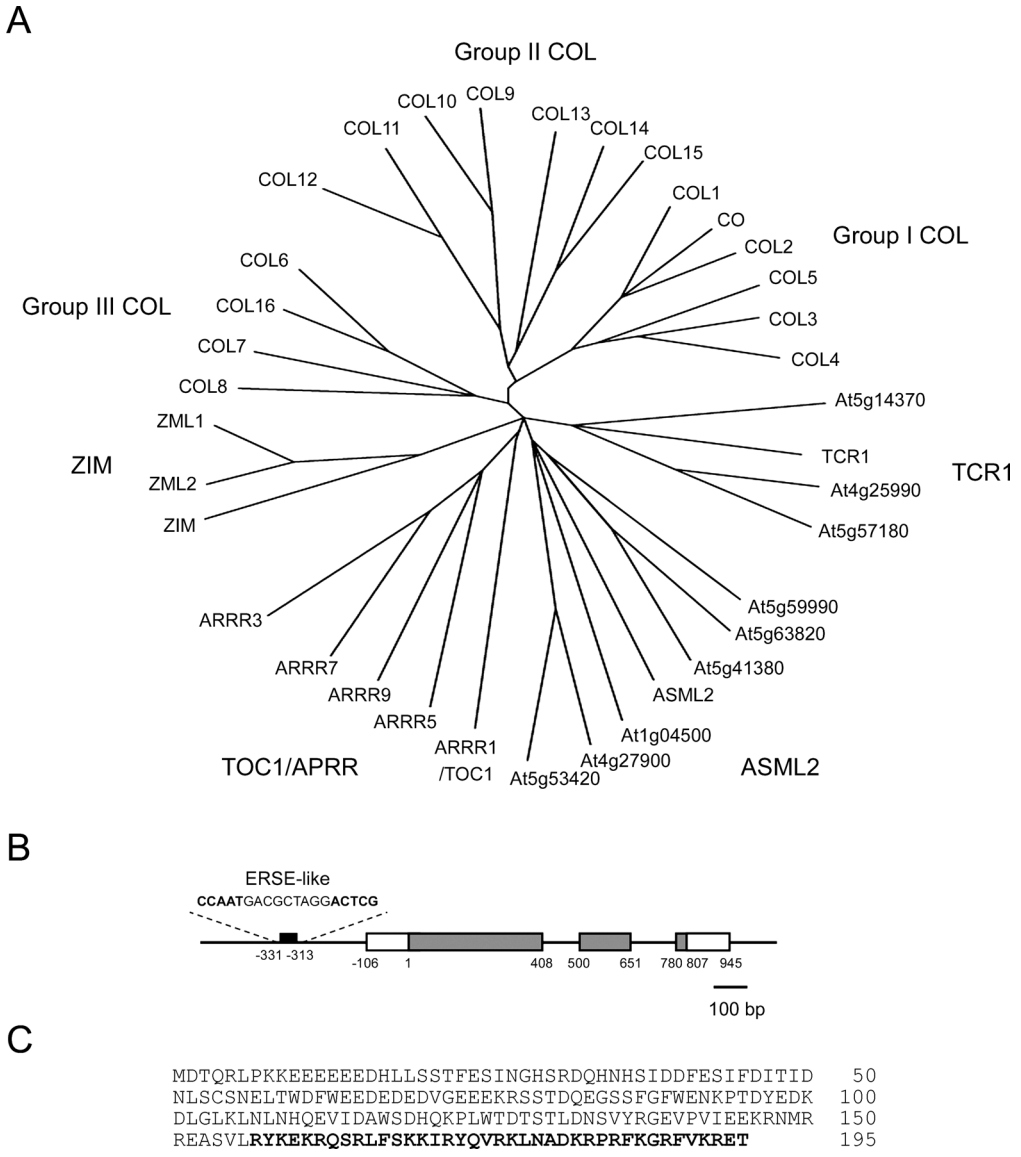

Figure 1 CCT domain-containing proteins. (A) Phylogenetic tree of CCT domain-containing proteins in *Arabidopsis*. A phylogenetic tree was generated using amino acid sequences corresponding to CCT domains of *Arabidopsis* CCT domain-containing proteins. Groups I, II and III of the COL family, TOC1/APRR family, ZIM family, ASML2 family and TCR1 family proteins are indicated. (B) Gene structure of *TCR1*. Protein coding regions and untranslated regions are indicated by gray and white boxes, respectively. An ERSE-like element on the promoter (CCAAT-N9-ACTCG) is indicated by a black box. (C) Deduced amino acid sequence of TCR1. The CCT domain is indicated in bold.

TCR1p1 and TCR1p2 to obtain a full-length mutated promoter, which was substituted for the CaMV35S promoter of pBI221-Luc in the same way as TCR1pro-Luc. *Arabidopsis* suspension cell MM2d in the Landsberg *erecta* ecotype (Menges and Murray 2002) was cultured in MS medium supplemented with 3% (w/v) sucrose, 200 mg l^{-1} KH_2PO_4, 1 mg l^{-1} thiamine hydrochloride, 100 mg l^{-1} myoinositol and 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid at 25°C. A proportion of cells were transferred to new medium once a week. Protoplasts were isolated from *Arabidopsis* suspension cells and transiently transformed by using polyethylene glycol according to Ueda et al. (Ueda et al. 2001). Transformed protoplasts were incubated at 23°C for 16 h in the dark, and luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

The 1,500 bp promoter of the *TCR1* gene fused to the firefly luciferase gene (TCR1pro-Luc) was introduced into protoplasts. When cells were treated with tunicamycin, higher luciferase activity was observed (Figure 3A). The *BiP1* promoter, which was previously shown to respond to tunicamycin in this assay system, also responded to tunicamycin (Figure 3A).

Because the *TCR1* gene has one element similar to the cis-element ERSE in its promoter region (Figure 1B), we

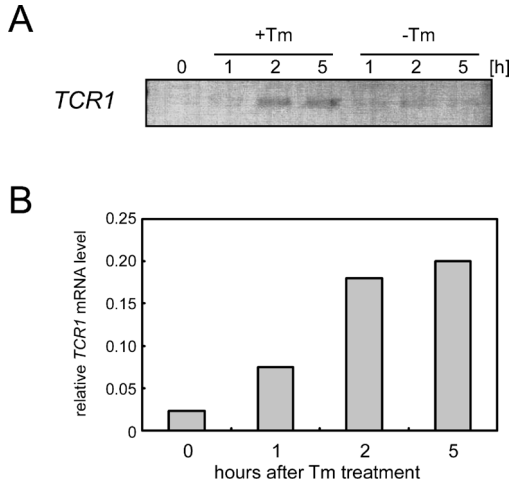

Figure 2 Expression profile of *TCR1*. (A) Induction of time course after tunicamycin treatment analyzed by semi-quantitative RT-PCR. *Arabidopsis* seedlings were treated with DMSO (−Tm) or 5 μg ml^{-1} tunicamycin (+Tm). Total RNA was extracted at the indicated time points and analyzed by RT-PCR. (B) Induction of time course after tunicamycin treatment analyzed by quantitative RT-PCR. Total RNA was extracted as in A, and analyzed by quantitative RT-PCR. The transcript level of *TCR* was normalized by that of *Act8*.

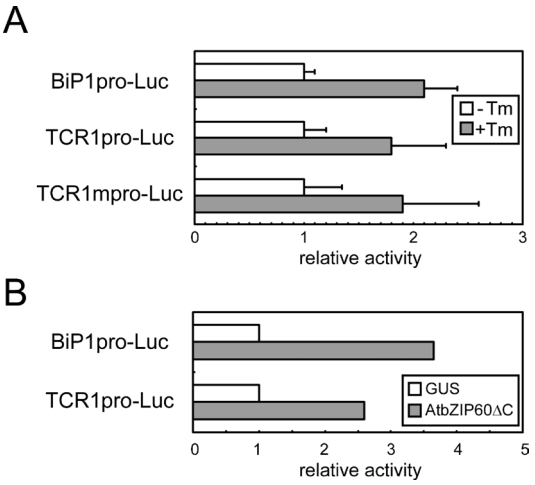

Figure 3 Regulation of the *TCR1* promoter. (A) Activation of *TCR1* promoters with tunicamycin treatment. Protoplasts were transiently transformed with reporter plasmids carrying the firefly luciferase gene driven by the authentic or mutated *TCR1* promoter (TCR1pro-Luc or TCR1mpro-Luc) or by the *BiP1* promoter (BiP1pro-Luc). A reference plasmid carrying *Renilla* luciferase gene driven by the CaMV35S promoter (35Spro-RLuc) was also introduced. After transformation, protoplasts were incubated with or without 5 μg ml^{-1} tunicamycin for 16 h. Luciferase activities were normalized by *Renilla* luciferase activities. Relative activity represents the activities relative to that obtained from a plasmid with the *BiP1* promoter without tunicamycin treatment. (B) Effect of expression of AtbZIP60ΔC on the *TCR1* promoter. Transient assay was performed as in A, except for co-transfection of an effector plasmid carrying *AtbZIP60ΔC* or *GUS* driven by the CaMV35S promoter (35Spro-AtbZIP60ΔC or 35Spro-GUS). Relative activity represents the activities relative to that obtained from plasmids with the *BiP1* promoter and *GUS*.

suspected that induction of *TCR1* is dependent on this element. To test this hypothesis, a mutation was introduced into this ERSE-like element on the *TCR1* promoter to obtain TCR1mpro-Luc (ACTCG of the ERSE-like sequence to GACAT). Unexpectedly, the mutated promoter still responded to tunicamycin (Figure 3A). These results suggest that induction of the *TCR1* gene in response to tunicamycin is independent of the ERSE-like sequence on its promoter. The *TCR1* promoter might contain an unknown cis-element responsible for the induction during ER stress.

Previously we showed that co-expression of AtbZIP60ΔC, an active form of AtbZIP60 without its C-terminal region including a transmembrane domain, activated promoters of ER stress-responsive genes including *BiP* (Iwata and Koizumi 2005a). Therefore we examined the effect of AtbZIP60ΔC on induction of the *TCR1* promoter. Co-expression of AtbZIP60ΔC activated the *TCR1* promoter as well as the *BiP1* promoter when compared to that of GUS, which was used as a control (Figure 3B). This result suggests that transcriptional induction of *TCR1* can be mediated by AtbZIP60. Recently, another bZIP transcription factor with a transmembrane domain, AtbZIP28, was reported to be involved in the signal transduction of the *Arabidopsis* ER stress response (Liu et al. 2007). Thus, in addition to AtbZIP60, AtbZIP28 may also play roles in the regulation of *TCR1*. The functional analysis of AtbZIP28 in the regulation of *TCR1* will be an interesting research subject.

In the present study we analyzed the transcriptional regulation of the *TCR1* gene during ER stress. A next question is whether the TCR1 protein is involved in the transcriptional induction of ER stress-inducible genes. As described above, CCT domain-containing proteins have been considered to function as transcriptional regulators. A CCT domain contains a nuclear localization signal, but lacks DNA-binding activity (Robson et al. 2001). Therefore it is suggested that CCT domain-containing proteins function with other transcription factors through CCT domains. In the mammalian cells, activation through ERSE or ERSE-II by ATF6 requires the NF-Y transcription factor complex (Yamamoto et al. 2004; Yoshida et al. 2000; Yoshida et al. 2001). Under unstressed conditions, CCAAT of ERSE or ATTGG (complementary to CCAAT) of ERSE-II is occupied by the NF-Y complex, which consists of NF-YA, NF-YB and NF-YC. ATF6 activated in response to ER stress binds CCACG, the other part of ERSE or ERSE-II, via direct interaction with NF-YC of the NF-Y complex, resulting in activation of downstream genes. Namely, binding of ATF6 to ERSE or ERSE-II is achieved only when the NF-Y complex is present. Recent studies suggested that CCT domain-containing proteins function in cooperation with the NF-Y complex.

For instance, a tomato homolog of CO, one of the founding members of the CCT protein family, interacts with a tomato HAP5/NF-YC protein (Ben-Naim et al. 2006). Another report also suggested that *Arabidopsis* CO and related protein COL15 interact with HAP5/NF-YC and regulate flowering time (Wenkel et al. 2006). With the importance of the NF-Y complex in the function of ERSE in the mammalian ER stress response described above, it is conceivable that TCR1 mediates ERSE-dependent induction of ER stress-responsive genes in cooperation with the NF-Y complex. Functional characterization of the TCR1 protein will be required to address these questions.

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