Original Paper

THESEUS1 is involved in tunicamycin-induced root growth inhibition, ectopic lignin deposition, and cell wall damage-induced unfolded protein response

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Abstract Endoplasmic reticulum (ER) stress activates unfolded protein responses (UPRs), such as promoting protein folding under the control of specific gene expression. Our previous study showed that ER stress induced by ER stress inducers such as tunicamycin (Tm), an inhibitor of N-linked glycan synthesis, causes ectopic lignin deposition in Arabidopsis roots, but the relationship between UPR and ectopic lignin deposition remains unclear. The receptor-like kinase THESEUS1 (THE1) has been shown to sense cell wall damage (CWD) induced in Arabidopsis by cellulose synthase inhibitors such as isoxaben (ISO) and to activate ectopic lignin deposition. In this study, we assessed the involvement of THE1 in ectopic lignin deposition caused by the ER stress inducer Tm. The loss-of-function mutation of *THE1*, *the1-3*, suppressed Tm-induced root growth inhibition and ectopic lignin deposition, revealing that THE1 is involved in root growth defects and ectopic lignin deposition caused by ER stress. Similarly, ISO treatment induced ectopic lignin deposition as well as the expression of the UPR marker genes *binding protein 3* (*BiP3*) and *ER-localized DnaJ 3b* (*ERdj3b*). Conversely, in the *the1-3* mutant, ISO-induced ectopic lignin deposition and the expression of *BiP3* and *ERdj3b* were suppressed. These results showed that THE1 is involved in not only root growth inhibition and ectopic lignin deposition caused by ER stress but also CWD-induced UPR.

Key words: *Arabidopsis thaliana*, cell wall damage, ectopic lignin deposition, THESEUS1, UPR.

Introduction

Protein secretion is an important pathway in cell wall architecture (Liu and Howell 2016) through which nascent proteins are loaded into the endoplasmic reticulum (ER), folded properly, and subsequently transported to the Golgi apparatus. However, adverse environmental conditions can cause accumulation of unfolded or misfolded proteins in the ER, leading to cellular damage. To prevent this, ER stress activates the cellular unfolded protein response (UPR), whereby genes involved in protein folding in the ER, including *binding protein 3* (*BiP3*) and *ER-localized DnaJ 3b* (*ERdj3b*), or

in the degradation of unfolded or misfolded proteins, are induced (Strasser 2018). In plants, these UPR-related genes are regulated by two signaling pathways, namely, the inositol-requiring enzyme 1 (IRE1)–basic leucine zipper 60 (bZIP60) and bZIP17/28 pathways (Fanata et al. 2013; Howell 2013; Iwata and Koizumi 2012). Additionally, UPR is involved in plant resistance to pathogen attack and heat stress (Bao and Howell 2017; Moreno and Orellana 2011).

We previously isolated the *lignescens* (*lig*) mutant of *Arabidopsis thaliana* (Nozaki et al. 2012), which shows a G68S point mutation in glucosamine-6-phosphate N-acetyltransferase (*GNA1*). The mutant was found to be

Abbreviations: BiP, binding protein; bZIP, basic leucine zipper; CCR, cinnamoyl CoA reductase; CESA, cellulose synthase; Col, Columbia; CrRLK1L, *Catharanthus roseus* RLK 1-like; CWD, cell wall damage; CWI, cell wall integrity; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; ERdj, ER-localized DnaJ; GM, germination medium; GNA, glucosamine-6-phosphate N-acetyltransferase; IRE1, inositol requiring enzyme 1; ISO, isoxaben; *lig*, *lignescens*; PME, pectin methylesterase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RBOH, respiratory burst oxidase homolog; RLK, receptor-like kinase; ROS, reactive oxygen species; SA, salicylic acid; SE, standard error; SUB, STRUBBELIG; THE1, THESEUS1; Tm, tunicamycin; UPR, unfolded protein response; WS, Wassilewskija.

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temperature-sensitive because of the low thermal stability of GNA1^{G68S} and was deficient in uridine diphosphate-*N*-acetylglucosamine, which is required for the formation of *N*-glycans, under restrictive temperatures. Therefore, *N*-glycosylation of proteins in the ER was inhibited in the *lig* mutant, which caused ER stress, root growth inhibition, and ectopic lignin deposition. The phenotype of the *lig* mutant resembled those caused by the ER stress inducer tunicamycin (Tm), an inhibitor of *N*-linked glycan synthesis, and by dithiothreitol, a reducing agent that disrupts disulfide bonds in the wild type. These observations suggested that ER stress may be involved in root growth inhibition and ectopic lignin deposition.

Ectopic lignin deposition is associated with cell wall damage (CWD) caused by the pharmacological or genetic inhibition of cellulose synthesis (Bischoff et al. 2009; Caño-Delgado et al. 2000, 2003). A likely candidate for mediating ectopic lignin deposition by CWD is THESEUS1 (THE1), which is a receptor-like kinase (RLK) that belongs to the *Catharanthus roseus* RLK 1-like (CrRLK1L) protein kinase subfamily (Hématy et al. 2007; Nissen et al. 2016). Hypocotyl growth inhibition and ectopic lignin deposition in *pcr1-1*, which is defective in the cellulose synthesis-related subunit CESA6, were partially attenuated by a loss-of-function mutation in THE1 without influencing cellulose synthesis. Therefore, THE1 presumably mediates the response to cellulose synthesis perturbation and acts as a CWD sensor (Hématy et al. 2007).

N-glycosylation and processing of *N*-glycans in the secretory pathway are thought to be related to the synthesis of cellulose, which is the main load-bearing component of plant cell walls (Strasser 2014). A point mutation in α -1,2-mannosyltransferase, whose function is to transfer mannose residues to dolichol-linked core oligosaccharides during the synthesis of *N*-glycans precursors, results in defective cellulose synthesis and xylem collapse (Zhang et al. 2009). The defects in glucosidase I or glucosidase II, which process *N*-glycans in the ER, lead to reduced cellulose content (Burn et al. 2002; Gillmor et al. 2002). KORRIGAN I, a membrane-anchored endo- β -1,4-glucanase, necessary for normal cellulose biosynthesis (Nagashima et al. 2018), contains eight potential *N*-glycosylation sites in the extracellular domain, and utilization of several *N*-glycosylation sites, not the structure of the attached *N*-glycan of, is important for KORRIGAN I activity (Liebminger et al. 2013). Based on this evidence, we speculated that ER stress due to a defect in *N*-glycans causes impaired cell walls.

Therefore, in this study, we hypothesized that ER stress and subsequent cell wall perturbation lead to root growth inhibition and ectopic lignin deposition via THE1 signaling. Root growth inhibition and ectopic lignin deposition by Tm treatment were reduced by *THE1* mutation, confirming our hypothesis that THE1 accounts

for these phenotypes induced by ER stress. Surprisingly, the *the1-3* mutant exhibited low expression of *BiP3* and *ERdj3b*, known UPR marker genes, under Tm treatment conditions. Moreover, we found that cellulose synthesis inhibition caused by the cellulose synthesis inhibitor isoxaben (ISO) partially induced the expression of *BiP3* and *ERdj3b* through THE1 signaling.

Overall, our study revealed that ER stress caused by defects in *N*-glycans activated THE1, leading to root growth inhibition and ectopic lignin deposition. Furthermore, we showed that THE1 signaling promoted UPR activation.

Materials and methods

Plant materials

In this study, we used several *Arabidopsis thaliana* mutants and strains, as described below. The double mutant *bzip60,28* was obtained by crossing *bzip60* (SALK_050203C) and *bzip28* (SALK_132285C) in Columbia (Col)-0 background; the *rbohdf* (N68522) in Col-0 background was obtained from the Nottingham Arabidopsis Stock Center; and the *the1-3* mutant (FLAG_201C06) in the Wassilewskija (WS) background was obtained from the Versailles Arabidopsis Stock Center.

Plant culture conditions

Sterilized seeds were treated at 4°C in the dark for 3 days and sown on germination medium (GM) solidified with 1.5% agar. The GM was Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 10 g l⁻¹ sucrose and buffered to pH 5.7 with 0.5 g l⁻¹ 2-morpholinoethanesulfonic acid. Seedlings were vertically grown under continuous light (25–37 μ mol m⁻² s⁻¹) at 22°C. In liquid culture conditions, 30 to 40 seeds were sown in 1/10 $\times$ liquid GM (10 ml) in a 50 ml flask and incubated on a rotary shaker at 110 rpm under continuous light (25–37 μ mol m⁻² s⁻¹) at 22°C.

Chemical treatments

Seedlings vertically grown on plates for 5 days were transferred to plates containing Tm and incubated under the same conditions as the pre-culture. In liquid culture, seedlings grown in liquid medium for 4 days were directly supplied with a 2,000 $\times$ concentration stock solution of Tm, ISO in dimethyl sulfoxide (DMSO), or DMSO as a mock treatment and incubated under the same conditions as used for the pre-culture.

Quantitative reverse transcription (qRT)-PCR analysis

Seedlings grown in liquid medium for 4 days were treated with either 100 ng ml⁻¹ Tm for 24 h or 600 nM ISO for 12 h, after which, entire seedlings were harvested for total RNA extraction. Total RNA was isolated from the seedlings as described in Chang et al. (1993), treated with DNase I (Takara Bio, Shiga, Japan), and reverse transcribed with ProtoScript II Reverse

Transcriptase (New England Biolabs Japan, Tokyo, Japan). First-strand cDNA was used as a template for PCR amplification of cDNA fragments. *BiP3* and *ERdj3b* were quantified based on qRT-PCR analysis performed using the Fast Start Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland) with ABI PRISM 7000 (Applied Biosystems, MA, USA) under the following conditions: an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and elongation at 60°C for 1 min. For quantification of *cellulose synthase 5* (*CESA5*), *CESA8*, *pectin methylesterase 20* (*PME20*), and *cinnamoyl CoA reductase 2* (*CCR2*), qRT-PCR was performed using the Luna Universal qPCR Master Mix (NEB, MA, USA) with MyGo Pro (IT-IS Life Science Ltd., Ireland) under the following conditions: an initial denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing, and elongation at 60°C for 30 s. The gene-specific primers used in this study were as follows: *ACT2* (5'-TCAGGAAGGATCTGTACGGTAAC-3' and 5'-TTCAGGTGGTGCAACGAC-3'), *BiP3* (5'-ACCCAGCAAAGGACTAAC-3' and 5'-AATACCGAGACTCAAAGGCG-3'), *ERdj3b* (5'-GAGGAGGCGGCATGAATAT-3' and 5'-CGTCATCCCCCTTGACA-3'), *CESA5* (5'-GAGGGCACCAAGGCACTAA-3' and 5'-CATTCCTAGCAAGCCACCA-3'), *CESA8* (5'-GCAAATTTATCATCCCAACGCTATC-3' and 5'-ATACTGACTCCGCTCCATCG-3'), *PME20* (5'-GCCCTTTCTCCAAAGGTTG-3' and 5'-TGCCCTTCCGTCATCTTA-3'), and *CCR2* (5'-CCTCAGGCCGTTACATCCTC-3' and 5'-TTCTCGTCCGAACACTTGGTG-3'). The results from three technical replicates are shown. The experiments were repeated at least twice, with similar results being obtained.

Phloroglucinol-HCl staining and image analysis

Lignin staining was performed as described by Nakano and Meshitsuka (1992), with minor modifications. Seedlings grown in liquid medium for 4 days were treated with 100 ng ml⁻¹ Tm for 72 h or 600 nM ISO for 24 h, fixed with 70% ethanol overnight at 4°C, and then stored at 4°C. The fixed seedlings were washed with deionized water, and the roots and cotyledons were cut into sections. These sections were stained with 1% (w/v) phloroglucinol in 20% (w/v) HCl. The stained samples were then observed under an optical microscope (Labophot-2, Nikon, Tokyo, Japan). Lignification was compared using Lignin (Nakamura et al. 2020). The experiments were repeated at least twice, with similar results being obtained.

Measurement of root length

Root length was measured from seedling pictures with NeuronJ plugin (Meijering et al. 2004) using ImageJ software (Rasband 1997–2021). The experiments were repeated at least twice, with similar results being obtained.

Statistical analysis

All treatment differences were tested for significance using R version 3.6.3 (R Core Team 2021) using R commander 2.6-2 (Fox 2005). A Shapiro–Wilk test was used to determine

whether the data groups obtained in this study were normally distributed. In cases in which either or both groups showed a non-normal distribution, the significance of any difference between the two data groups was tested using the nonparametric two-sided Wilcoxon rank-sum test. In cases in which both data groups showed a normal distribution, the *F* test was used to determine whether the variances of the data from the two groups were equal. Significant differences between the two groups with homoscedasticity were assessed using Student's *t*-test, while Welch's *t*-test was used to assess significant differences between the two groups with heteroscedasticity.

Results

Response of the *the1-3* mutant to Tm treatment

To investigate the relationship between ER stress and THE1, the effects of treatment with Tm on root growth were examined in the *the1-3* mutant. Root growth of *the1-3* and wild-type WS was measured 3 days after Tm treatment on agar medium (Figure 1). Root growth of *the1-3* was less sensitive to Tm at 80 and 100 ng ml⁻¹ compared with that of WS. Therefore, we used Tm to induce ER stress by inhibiting protein *N*-glycosylation. Thus, it was shown that THE1 may be involved in the inhibition of root growth caused by Tm-induced ER stress.

We then examined ectopic lignin deposition in the *the1-3* mutant under Tm-induced ER stress. Three-day lignin deposition in *the1-3* and WS under treatment with 100 ng ml⁻¹ Tm in liquid culture was analyzed by image analysis after phloroglucinol-HCl staining (Figure 2A, B). Significantly higher ectopic lignin deposition was detected in the roots and cotyledons of WS than in those of *the1-3* upon treatment with Tm. In addition, the expression of two UPR-related genes, *BiP3*, induced through the bZIP60 pathway, and *ERdj3b*, induced through the bZIP17/28 pathway (Ruberti et al. 2018), was analyzed using qRT-PCR (Figure 2C). The expression of *BiP3* and *ERdj3b* upon Tm treatment was significantly

Figure 1. Effect of the ER stress inducer Tm on root growth in the *the1-3* mutant. Seedlings of WS and *the1-3* mutant grown on vertical agar plates for 5 days were transferred to vertical agar plates containing the indicated concentrations of Tm, and root growth was measured after 3 days. Vertical bars indicate standard error (SE) for 20 seedlings. Asterisks indicate significant differences between WS and *the1-3* mutants ("80 ng ml⁻¹ Tm", Student's *t*-test; "100 ng ml⁻¹ Tm", Welch's *t*-test; **p* < 0.05, ***p* < 0.01).

lower in *the1-3* than in WS.

These results indicate that THE1 mediates not only the signaling pathway from ER stress caused by *N*-glycosylation inhibition to the arrest of root growth and ectopic lignin deposition in roots and cotyledons, but also the acceleration of UPR.

Suppressive involvement of UPR in Tm-induced root growth inhibition and ectopic lignin deposition

We assessed how UPR is involved in root growth

Figure 2. Effect of Tm treatment on ectopic lignin deposition and UPR in the *the1-3* mutant. Seedlings grown in liquid medium for 4 days were treated with 100 ng ml^{-1} Tm for the indicated periods. (A) and (B) show ectopic lignin deposition in the roots and cotyledons of WS and *the1-3* mutants treated with Tm for 72h. (A) Analysis of lignified area. Mean values are shown as $\times$; root: $n=5$ (0h), $n=8$ (72h); cotyledon: $n=10$ (0h), $n=25-29$ (72h). Asterisks indicate significant differences between data groups in each graph ("Root", Student's *t*-test; "Cotyledon", two-sided Wilcoxon rank-sum test; * $p<0.05$, *** $p<0.001$). (B) Representative pictures of roots and cotyledons stained with phloroglucinol-HCl to detect lignin. Bar=100 μm . (C) qRT-PCR analysis of the expression of UPR-related genes, *BiP3* and *ERdj3b*, in WS and *the1-3* mutants treated with Tm for 24h. *ACT2* was used as the standard. Asterisks indicate significant differences between WS and *the1-3* mutants ($n=3$; bar: SE; Student's *t*-test; * $p<0.05$, ** $p<0.01$).

inhibition and ectopic lignin deposition caused by the inhibition of *N*-glycosylation upon treatment with Tm. Most UPR target genes in plants are regulated by the IRE1-bZIP60 and bZIP17/28 signaling pathways (Fanata et al. 2013; Howell 2013; Iwata and Koizumi 2012). Root growth of *bzip60,28*, a knockout mutant of *bZIP60* and *bZIP28*, which is defective in major UPR signaling components, was significantly inhibited in the presence of 50 and 100 ng ml^{-1} Tm, compared to that of Col (Figure 3). Subsequently, ectopic lignin deposition and expression of UPR-related genes were analyzed under 100 ng ml^{-1} Tm treatment in liquid culture (Figure 4). Ectopic lignin deposition in Tm-treated roots and cotyledons was lower in *bzip60,28* than in Col (Figure 4A, B). In addition, the expression of *BiP3* and *ERdj3b* induced by Tm treatment was not induced in the *bzip60,28* mutant (Figure 4C). These results suggest that UPR alleviates Tm-induced root growth inhibition and preserves ectopic lignin deposition ability by increasing Tm tolerance.

Promotion of UPR activation by CWD through THE1 signaling

To investigate the relationship between CWD and UPR and the involvement of THE1 signaling, we used ISO, a cellulose synthesis inhibitor that activates THE1 signaling (Merz et al. 2017). We first analyzed ectopic lignin deposition induced by treatment with 600 nM ISO for 24h in WS and *the1-3* mutant (Figure 5A, B). ISO-induced ectopic lignin deposition occurred in the roots of both WS and *the1-3* and was significantly lower in *the1-3* than in WS. The results of ectopic lignin deposition in the roots of ISO-treated *the1-3* were similar to those previously reported (Engelsdorf et al. 2018; Merz et al. 2017; van der Does et al. 2017). In contrast, in the cotyledons, ectopic lignin deposition occurred in WS but rarely in *the1-3*. Then, the expression of *BiP3* and *ERdj3b* in WS and *the1-3* was analyzed by qRT-PCR (Figure 5C)

Figure 3. Effect of Tm treatment on root growth of the *bzip60,28* mutant. Seedlings of Col and *bzip60,28* mutants grown on vertical agar plates for 5 days were transferred to vertical agar plates containing the indicated concentrations of Tm, and root growth was measured after 3 days. Vertical bars indicate SE for 20 seedlings. Asterisks indicate significant differences between Col and *bzip60,28* mutants ("50 ng ml^{-1} Tm", two-sided Wilcoxon rank-sum test; "100 ng ml^{-1} Tm", Welch's *t*-test; *** $p<0.001$).

Figure 4. Effect of Tm treatment on ectopic lignin deposition and UPR in the *bzip60,28* mutant. Seedlings grown in liquid medium for 4 days were treated with 100 ng ml⁻¹ Tm for the indicated periods. (A) and (B) show ectopic lignin deposition in the roots and cotyledons of Col and *bzip60,28* mutants, respectively, treated with Tm for 72 h. (A) Analysis of lignified area. Mean values are indicated by ×: root: *n*=5 (0h), *n*=9–11 (72h); cotyledon: *n*=8 (0h), *n*=10–12 (72h). Asterisks indicate significant differences between data groups in each graph (two-sided Wilcoxon rank-sum test; ****p*<0.001). (B) Representative pictures of roots and cotyledons stained with phloroglucinol-HCl to detect lignin. Bar=100 μm. (C) qRT-PCR analysis of the expression of UPR-related genes, *BiP3* and *ERdj3b*, in Col and *bzip60,28* mutants treated with Tm for 24 h. *ACT2* was used as the standard. Asterisks indicate significant differences between Col and *bzip60,28* mutants (*n*=3; bar: SE; “*BiP3*”, Welch’s *t*-test; “*ERdj3b*”, Student’s *t*-test; ****p*<0.001).

at 12 h after ISO treatment. The expression of *BiP3* and *ERdj3b* increased at 12 h after ISO treatment; however, it was lower in *the1-3* than in WS. These results indicate that CWD induces UPR and that THE1 signaling contributes to promoting UPR activation.

To further investigate the relationship between CWD and UPR in plants, we analyzed ectopic lignin deposition induced by 600 nM ISO treatment for 24 h in Col and *bzip60,28* mutants. ISO treatment induced

Figure 5. Effect of ISO treatment on ectopic lignin deposition and UPR in the *the1-3* mutant. Seedlings grown in liquid medium for 4 days were treated with 600 nM ISO for the indicated periods. (A) and (B) show ectopic lignin deposition in the roots and cotyledons of WS and *the1-3* mutants, respectively, treated with ISO for 24 h. (A) Analysis of lignified area. Mean values are indicated by ×: root: *n*=8–9 (0h), 15–18 (24h), cotyledon: *n*=15–18 (0h), 19–23 (24h). Asterisks indicate significant differences between WS and *the1-3* mutants (two-sided Wilcoxon rank-sum test; **p*<0.05, ****p*<0.001). (B) Representative pictures of roots and cotyledons stained with phloroglucinol-HCl to detect lignin. Bar=100 μm. (C) qRT-PCR analysis of the expression of UPR-related genes, *BiP3* and *ERdj3b*, in WS and *the1-3* mutants treated with ISO for 12 h. *ACT2* was used as the standard. Asterisks indicate significant differences between WS and *the1-3* mutants (*n*=3; bar: SE; “*BiP3*”, Student’s *t*-test; “*ERdj3b*”, Welch’s *t*-test; **p*<0.05, ****p*<0.001).

ectopic lignin deposition in the roots and cotyledons of Col and *bzip60,28* mutants, and no significant difference was observed between Col and *bzip60,28* (Figure 6A, B). Then, the expression of *BiP3* and *ERdj3b* in Col and *bzip60,28* treated with ISO was analyzed by qRT-PCR (Figure 6C). In the *bzip60,28* mutant, *BiP3* expression was completely repressed, while *ERdj3b* expression was partially downregulated. These results suggest that ISO-induced UPR did not have a significant effect on ectopic lignin deposition.

Figure 6. Effect of ISO treatment on ectopic lignin deposition and UPR in the *bzip60,28* mutant. Seedlings grown in liquid medium for 4 days were treated with 600 nM ISO for the indicated periods. (A) and (B) show ectopic lignin deposition in the roots and cotyledons of Col and *bzip60,28* mutants, respectively, treated with ISO for 24 h. (A) Analysis of lignified area. Mean values are indicated by ×; $n=8-20$. (B) Representative pictures of roots and cotyledons stained with phloroglucinol-HCl to detect lignin. Bar = 100 μm. (C) qRT-PCR analysis of the expression of *BiP3* and *ERdj3b* in Col and *bzip60,28* mutants treated with 600 nM ISO for 12 h. Asterisks indicate significant differences between Col and *bzip60,28* mutants ($n=3$; bar: SE; “*BiP3*”, Welch’s *t*-test; “*ERdj3b*”, Student’s *t*-test; * $p<0.05$, ** $p<0.01$). *ACT2* was used as the standard.

Comparison of cell wall synthesis-related gene expression in response to Tm and ISO treatment

ER stress due to *N*-glycosylation inhibition by Tm is presumed to affect cell wall synthesis and induce CWD. It has been reported that CWD induced by ISO treatment alters the expression of cell wall synthesis-related genes (Bischoff et al. 2009). Therefore, we compared the expression of *CESA5* as a primary cell wall synthesis-related gene, *CESA8* as a secondary cell wall synthesis-related gene, *PME20* as a pectin synthesis-related gene, and *CCR2* as a stress-responsive lignin synthesis-related gene in response to treatment with Tm and ISO

(Figure 7). The expression of *CESA5* by ISO treatment was increased two-fold in all strains, while that by Tm treatment increased only in the *bzip60,28* mutant. The increased expression in the *bzip60,28* mutant suggests that UPR suppresses upregulation of *CESA5* by Tm treatment. The expression of *CESA8* by ISO treatment was increased in all strains, while that by Tm treatment increased only in WS and the *the1-3* mutant. The expression of *PME20* by ISO treatment was markedly increased in all strains, and that by Tm treatment also increased in WS, the *the1-3* mutant, and Col. These results are consistent with those of a previous study in which *PME20* expression was upregulated by CWD induced by ISO treatment (Bischoff et al. 2009). *CCR2* expression was markedly increased in the ISO and Tm groups. The overall pattern of expression by ISO and Tm in wild-type WS and Col was similar, suggesting that CWD also occurs by Tm treatment. In addition, the expression of *CCR2* was suppressed by Tm and ISO treatments in the *the1-3* mutant, consistent with the results presented in Figures 2 and 5, showing the suppression of lignin deposition in the *the1-3* mutant.

Non-involvement of RBOH D- and F-dependent ROS and ectopic lignin deposition in UPR activation

NADPH oxidase produces reactive oxygen species (ROS) downstream of CrRLK1L signaling (Cheung and Wu 2011; Galindo-Trigo et al. 2016). Arabidopsis has 10 genes encoding NADPH oxidases, known as respiratory burst oxidase homologs (RBOHs) (Torres and Dangel 2005). Among them, RBOH D and F act downstream of THE1 signaling and are seemingly involved in ectopic lignin deposition (Denness et al. 2011). Moreover, low concentrations of ROS induce ER stress-related genes (Ozgur et al. 2015).

To investigate whether RBOH D and F are involved in promoting ectopic lignin deposition and UPR activation, seedlings of *rbohdf* and Col grown in liquid medium for 4 days were treated with 100 ng ml⁻¹ Tm or 600 nM ISO, and lignin deposition and the expression of *BiP3* and *ERdj3b* were analyzed. The *rbohdf* mutant did not accumulate ectopic lignin in the roots or cotyledons after Tm or ISO treatment (Figure 8A). These results support those of a previous study, which showed that ROS produced by RBOH D and F are required for lignin deposition in response to CWD caused by ISO (Denness et al. 2011). In contrast, the expression of *BiP3* and *ERdj3b* induced by Tm or ISO treatment did not differ much between *rbohdf* and Col (Figure 8B, C). This result is consistent with that of Angelos and Brandizzi (2018) who reported that Tm treatment also induced *BiP3* expression in the *rbohdf* mutant. Taken together, these results suggest that UPR activation is not caused by ROS produced by RBOH D and F and the associated ectopic

Figure 7. Effects of ISO and Tm treatments on the expression of the cell wall synthesis-related genes *CESA5*, *CESA8*, *PME20*, and *CCR2* in WS, *the1-3* mutant, Col, and *bz1p60,28* seedlings. Seedlings grown in liquid medium for 4 days were treated with 600 nM ISO or DMSO (mock) for 12 h or 100 ng l⁻¹ Tm or DMSO for 24 h. *ACT2* was used as a standard ($n=3$; bar: SE).

lignin deposition but by other factors downstream of THE1.

Discussion

A model of THE1 function under ER stress

Inhibition of *N*-glycosylation by Tm treatment induces root growth inhibition, ectopic lignin deposition, and UPR (Nozaki et al. 2012). However, the factors that mediate this effect remain unclear. In this study, we found that THE1 mediated root growth inhibition and ectopic lignin deposition and promoted UPR activation caused by Tm-induced inhibition of *N*-glycosylation. THE1 is known to be a receptor kinase required for the response to inhibition of cellulose synthesis, and its activation is suggested to be induced by CWD (Galindo-Trigo et al. 2016; Lindner et al. 2012; van der Does et al. 2017). Additionally, cellulose content decreases when *N*-glycosylation is compromised (Strasser 2014). Based on these findings, together with the results of the present study, we propose a model to account for the function of THE1 under ER stress due to impaired *N*-glycosylation (Figure 9). ER stress due to Tm-induced impairment of *N*-glycosylation seemingly leads to CWD, which in turn inhibits root growth and promotes ectopic lignin deposition via THE1. Conversely, Tm-induced UPR alleviates ER stress by promoting protein folding in the ER, thereby mitigating the inhibitory effects of ER stress on ectopic lignification (Figure 4). Moreover, by alleviating ER stress, Tm-induced UPR also mitigates the inhibition of root growth. RBOH D and F were shown to be involved in ectopic lignin deposition but

not in UPR activation by Tm or ISO. As the promotion of UPR activation by CWD also occurs in part in *the1-3*, it would seem that THE1 is not the sole factor involved. Thus, for example, MIK2 has been suggested to function downstream of THE1 (Engelsdorf et al. 2018; Gigli-Bisceglia et al. 2020; van der Does et al. 2017), and recently, STRUBBELIG (SUB) has been shown to be involved in cell wall integrity (CWI) independent of THE1 (Chaudhary et al. 2020), suggesting that other factors, such as SUB, may also be involved in the promotion of UPR activation by CWD.

Promotion of UPR activation via THE1 signaling in ISO-induced CWD, and involvement of UPR in ectopic lignin deposition

Analysis of wild type and *the1-3* mutant suggested that ISO-induced CWD promoted UPR activation through THE1 signaling (Figure 5C). In yeast, cell wall stress activates UPR (Krysan 2009; Scrimale et al. 2009). An interrelationship between CWI and UPR has also been reported in the rice blast fungus, *Magnaporthe oryzae* (Yin et al. 2016). In the present study, we showed, to our knowledge for the first time in plants, that CWD activates UPR and that THE1 signaling is involved in the process. However, it should also be considered that the induction level of UPR-related genes was much lower by ISO than by Tm, a direct ER stress inducer. UPR was not as strongly induced by ISO as that by Tm treatment, suggesting no large difference in ectopic lignin deposition between Col and *bz1p60,28* mutants (Figure 6A, B). Overall, this study revealed the interrelationship of CWD, CWI, and UPR in plants.

Figure 8. Effect of Tm and ISO treatments on ectopic lignin deposition and UPR in the *rbohdf* mutant. Seedlings grown in liquid medium for 4 days were treated with 100 ng ml⁻¹ Tm or 600 nM ISO for the indicated periods. (A) Representative pictures of the roots and cotyledons of Col and *rbohdf* mutants stained with phloroglucinol-HCl for detection of lignin. Bar=100 μm. (B, C) qRT-PCR analysis of the expression of *BiP3* and *ERdj3b* in Col and *rbohdf* mutants treated with 100 ng ml⁻¹ Tm for 24 h (B) or 600 nM ISO for 12 h (C). Asterisk indicates significant difference between Col and *rbohdf* mutants ($n=3$; bar: SE; Student's *t*-test; ** $p<0.01$). ACT2 was used as the standard.

Possible factors mediating the promotion of UPR activation downstream of THE1 signaling

We considered ROS produced by RBOH D and F as putative factors triggering UPR downstream of THE1 signaling. Our data showed that *rbohdf* did not cause ectopic lignin deposition under Tm or ISO treatments because of the lack of ROS required for monolignol polymerization (Figure 8A). However, the expression of *BiP3* and *ERdj3b* was not affected by *rbohdf* mutation (Figure 8B, C). Consistently, Angelos and Brandizzi (2018) reported that Tm treatment induced the expression of *BiP3* in the wild type as well as in *rbohdf*. Altogether, these results suggest that ROS do not contribute to the promotion of UPR by Tm or

Figure 9. Proposed model showing the role of THE1 in ectopic lignin deposition in Tm- and ISO-treated seedlings. The model shows the involvement of THE1 and UPR in root growth inhibition and ectopic lignin deposition under ER stress caused by Tm-induced inhibition of N-glycosylation. The model also shows the involvement of THE1 and UPR in CWD caused by ISO-induced inhibition of cellulose synthesis.

ISO treatment. In contrast, salicylic acid (SA), a plant hormone, induces UPR gene expression (Moreno et al. 2012; Nagashima et al. 2014). Similarly, ISO treatment increases SA accumulation in the wild type but reduces SA accumulation in *THE1* loss-of-function mutants and increases SA accumulation in *THE1* gain-of-function mutants (Engelsdorf et al. 2018; Gigli-Bisceglia et al. 2018; Hamann et al. 2009). These results suggest that SA may be a candidate factor that functions downstream of THE1 signaling to promote UPR activation under Tm and ISO treatments. SUB has also been shown to act as a CWD sensor (Chaudhary et al. 2020), and it is possible that SUB signaling is also involved in promoting UPR, in addition to THE1 signaling.

In this study, we showed that THE1 is involved in root growth inhibition and ectopic lignin deposition induced by ER stress through the inhibition of N-glycosylation. Furthermore, we showed that UPR is promoted in response to CWD and that THE1 is also involved in this process. Thus, this study expands our understanding of the role of THE1 in ER stress and CWD. Several components upstream and downstream of THE1 signaling have been identified recently (Engelsdorf et al. 2018; Gigli-Bisceglia et al. 2018; Gonneau et al. 2018). Future studies will surely reveal additional details of the mechanisms underlying the interrelationship between UPR and CWI.

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Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *THESEUS1*, AT5G54380; *bZIP60*, AT1G42990; *bZIP28*, AT3G10800; *BiP3*, AT1G09080; *ERdj3b*, AT3G62600; *CESA5*, AT5G09870; *CESA8*, AT4G18780; *PME20*, AT2G47550; *CCR2*, AT1G80820; *RBOHD*, AT5G47910; *RBOHF*, AT1G64060; and *ACT2*, AT3G18780.

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