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Endoplasmic reticulum quality control and metal tolerance: reduced activity of glucosidase II enzyme affects positively cadmium tolerance in *Arabidopsis thaliana*

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ABSTRACT

The Endoplasmic Reticulum Quality Control (ERQC) machinery is highly conserved among eukaryotes and assists the newly synthetized proteins in the folding process. Previous works have reported the involvement of ERQC in plant immunity and biotic stress response. However, the interaction between ERQC pathway and heavy metals exposure has been poorly investigated in plants. In the present study, we showed that the *Arabidopsis thaliana rsw3* mutant, characterised by a reduced activity of the ER Glucosidase II enzyme, exhibits an increased tolerance to cadmium (Cd) stress. Under standard conditions, *rsw3* seedlings exhibit shorter primary roots compared to Wild-type (Wt) plantlets, because of a constitutive ER stress and a consequent upregulation of both ERQC and Unfolded Protein Response (UPR) stress markers in root or shoot tissues. Interestingly, differently from Wt seedlings, these markers remain unchanged in *rsw3* under Cd stress. Biochemical data here provided linked the enhanced Cd tolerance of *rsw3* to the brassinosteroid receptor 1, BRI1, as the partial impairment of GII activity positively affects the accumulation of the active form of BRI1 receptor on the plasma membrane under Cd stress.

1. Introduction

Pollution by heavy metals is a global problem due to their wide distribution and mobilization in the environment and their toxic effects on human health (Angon et al., 2024). Among them, cadmium (Cd) has been classified by the World Health Organization as group-1 human carcinogens (Moulis and Thévenod, 2010) and it is in the top ten list of hazardous substances (ATSDR, 2007). One of the main sources of human exposure to heavy metals is represented by plant-based food. Research on plants Cd response aims, on one hand, to reduce metal uptake in edible crops to ensure food safety, and on the other, to enhance the same process in plants used for phytoremediation strategies to decontaminate soil.

Many papers have investigated the response of plants to heavy metal stress by characterizing their uptake, translocation and mobilization at cellular and molecular levels (Tang et al., 2023). However, only a few studies have focused on heavy metal-induced ER stress (Xi et al., 2016; De Benedictis et al., 2023; Demircan et al., 2024). Exposure to heavy metals can lead to the accumulation of misfolded proteins in the ER lumen. To restore protein homeostasis, the cell activates the Unfolded Protein Response (UPR) pathway, which in turn induces the expression of genes encoding ER resident chaperones (e.g. BiP), heat shock proteins (such as HSP70) and foldases (PDIs) with the aim of increasing ER folding capacity (Karagöz et al., 2019). In our recent paper, we demonstrated that defects in the UPR pathway increase Cd tolerance in *bzip28/60 Arabidopsis thaliana* mutant (De Benedictis et al., 2023). The UPR pathway is strictly connected with the Endoplasmic Reticulum Quality Control (ERQC) machinery, which assists newly synthetized proteins in the folding process allowing that only correctly folded proteins can move along the secretion pathway while terminally misfolded proteins are targeted for degradation (Liu and Howell, 2010). In detail, the newly synthetized proteins are glycosylated by en bloc transfer of a

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preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) precursor to an asparagine residue within the sequence motif Asn-X-Ser/Thr (where X can be any amino acid except proline) by the oligosaccharyltransferase (OST) protein complex. Subsequently, the transmembrane protein glucosidase I (GI) and the luminal enzyme glucosidase II (GII) sequentially remove the two outermost glucose residues, generating the monoglucosylated *N*-glycan ($Glc_1Man_9GlcNAc_2$), which by interacting with the lectins calnexin (CNX) and calreticulin (CRT), enters the glycan-dependent folding cycle. At this point, GII in its second interaction with glycoprotein, removes the final glucose residue, releasing it from its association with the lectins. At this stage, the glycoprotein undergoes a quality control process by UDP-Glc glycoprotein glucosyltransferase (UGGT), which determines its fate. If the protein is correctly folded, it can leave the ER for its final cellular localization. Instead, if UGGT detects folding defects, it reglucosylates *N*-glycans and the protein can attempt another CNX/CRT folding cycle. Proteins that are finally misfolded are sent to the ER-Associated Degradation pathway (Strasser, 2018).

Severe loss of the catalytic subunit of the OST complex, as well as of the GI and GII enzymes, causes embryonic lethality, highlighting the importance of protein N-glycosylation in eukaryotes (Koiwa et al., 2003; Boisson et al., 2001; Burn et al., 2002). The role of ERQC machinery in plant response to metal stress has so far been less investigated.

We took advantage of the availability of the *A. thaliana rsw3* mutant line, characterized by a reduced GII activity due to a single nucleotide substitution resulting in an aminoacidic change (Ser599 to Phe) within the GII catalytic domain (Burn et al., 2002), to better investigate the ERQC involvement in heavy metal response. In the present work, we present new data about *A. thaliana* response to this environmental stress at phenotypic, biochemical and molecular levels in Wild type (Wt) and *rsw3* genetic background.

2. Materials and methods

2.1. Plant lines and growth conditions

In this study, seeds of *A. thaliana* Wild-type (Wt) ecotype Col-0, the *rsw3* mutant (Burn et al., 2002), the *bri1-9* mutant (Vert et al., 2005) and the BRI1-GFP overexpressing line (Friedrichsen et al., 2000) were sterilized and grown in ½ MS medium supplemented with vitamins, in the growth room at 22 °C, 60 % relative humidity, 16/8 h (light/dark) photoperiod, with or without the indicated concentrations of $CdCl_2$ or NBDNJ.

Primary root length and fresh weight of aerial parts were measured on plants grown vertically on agar medium 14 days after sowing, and chlorophyll content was quantified according to Hiscox and Tsraelstam (1979).

2.2. ICP-AES analysis

Roots and shoots of 14-day-old Wt and *rsw3* seedlings grown under control conditions or in the presence of 50 μM $CdCl_2$ were processed according to Zhai et al. (2014) for the quantification of Cd content using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), as described by Scortichini et al. (2018). Dried samples were weighed and mixed with 4 mL of H_2O_2 and 6 mL of superpure HNO_3 69 %, then treated at 180 °C for 10 min using a microwave digestion system (Milestone START D). The samples were then cooled, diluted with superpure water to a final volume of 20 mL, filtered through syringe filters (pore size 0.45 μm), and then measured for Cd content using an ICP-AES (Thermo Scientific, iCap 7000 Series) spectrometer. The spectrometer was previously calibrated for quantitative analysis with five standard solutions containing known Cd concentrations: 0.001, 0.01, 0.1, 1.0, and 10.0 mg/L. The calibration line showed a correlation coefficient greater than 0.99. The results were expressed as the average of three different measurements, and Cd concentrations were expressed as ppm (mg/kg of sample weight).

2.3. Protein extraction and Western blot

Total protein extract was obtained from 0.2 g of 14-day-old roots or shoots, or from whole seedlings grown under control conditions, or with 50 μM $CdCl_2$, 40 μM NBDNJ, or in the presence of both treatments following the protocol reported in De Benedictis et al. (2023). Plant extracts were separated on 10 % polyacrylamide gels (Bio-Rad), either untreated or after digestion with Endoglycosidase H (Endo H; Sigma-Aldrich, A0810), according to the manufacturer's instructions. After transfer onto PVDF stain free membrane (Bio- Rad), the proteins were blotted with 1 μg/mL Concanavalin A (Sigma-Aldrich, L6397), or with anti-PDI5 (PhytoAB, PHY3848S), anti-BiP (Agrisera, AS09481), anti-BRI1 (Agrisera, AS121859) antibodies following the company's recommendations. The relative amount of PDI5, BiP and BRI1 protein bands was calculated using Image Lab Software (Bio-Rad).

2.4. Gene expression level (qRT-PCR)

Total RNA was isolated from ground tissues of *A. thaliana* lines following the protocol reported by RNeasy Plant Mini Kit (Qiagen), and treated with RNase-Free DNase I (Qiagen). One microgram of RNA was used for cDNA synthesis with SuperScript III First-Strand Synthesis System kit (Invitrogen). qRT-PCR analysis was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with the SYBR Green Supermix (Bio-Rad).

Relative expression levels were calculated for *BiP1/2* (At5g28540/At5g42020), *BiP3* (At1g09080), *PDI5* (At1g21750), *bZIP60* (At1g42990) genes. *Actin2* (At3g18780) and *Ubiquitin5* (At3g62250) were used as multiple reference genes for normalization. Data represent the mean of three biological replicates. Primer sequences are listed in Table S1.

2.5. Confocal laser scanning microscopy

Stably transformed BRI1-GFP overexpressing plants (Friedrichsen et al., 2000) were grown under the following conditions: (i) control; (ii) 50 μM $CdCl_2$; (iii) 40 μM NBDNJ; (iv) 50 μM $CdCl_2$ combined with 40 μM NBDNJ.

Seedlings were examined 7 days after germination using a confocal laser scanning microscope LSM 710 Zeiss (ZEN Software, GmbH, Germany). After excitation at 488 nm, GFP fluorescence was detected in the 505–530 nm emission range, corresponding to the green fluorescent signal. Grayscale images of roots were obtained using transmitted light from the same roots used for fluorescent frame. The laser power was set to a minimum, and appropriate controls were included to ensure no bleed-through from different channels. Images were assembled into a single figure panel using Adobe Photoshop 7.0 software (Mountain View, CA, USA). Quantitative analysis was performed with ImageJ-Win64 (Schindelin et al., 2012).

3. Results

3.1. rsw3 mutant displays increased tolerance to Cd stress

We first investigated the phenotypic traits of *rsw3* seedlings grown under control conditions or Cd stress. Under control conditions, *rsw3* mutant showed a significantly reduced length of the primary root compared to Wt seedlings, while no differences were observed in the aerial tissues, either in terms of shoot fresh weight or chlorophyll content (Fig. 1).

When Wt plantlets were challenged with Cd stress, the primary root length was significantly reduced in a dose-dependent manner. The *rsw3* mutant showed a slight but significant increase in root length in the presence of 25 μM Cd and remained unchanged at 50 μM Cd, so that under this latest stress condition the primary root length of Wt and

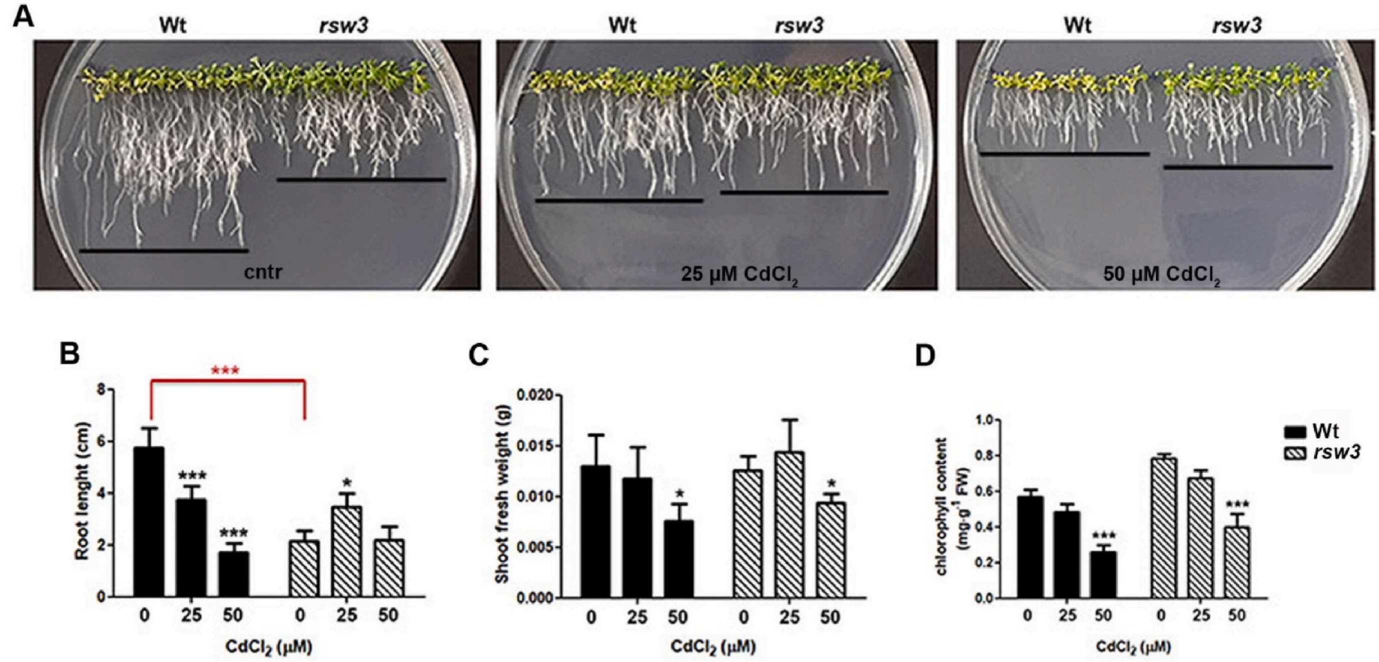

Fig. 1. *rsw3* is more tolerant to Cd stress than Wt seedlings. Plants were grown with or without (cntr) $CdCl_2$ for 14 days (A). Root length (B), shoot fresh weight (C) and chlorophyll content (D) were measured in both plant lines in all conditions tested. Data represent the mean of at least three biological replicates ($n > 20$). Error bars indicate standard deviation. Black asterisks show significant differences between control and $CdCl_2$ treated plants of the same genotype; red asterisks are indicative of the significant differences between the two genotypes under the same growing conditions (one-way ANOVA followed by Bonferroni's tests, $^*p \leq 0.05$; $^{***}p \leq 0.001$).

mutant was similar (Fig. 1A and B). Concerning the aerial tissues, a significant reduction of shoot fresh weight and the chlorophyll content was observed in both Wt and *rsw3* plantlets under 50 μM Cd stress (Fig. 1C and D).

To confirm the role of GII, Wt plants were treated with NBDNJ, a well-known inhibitor of this enzyme (Caputo et al., 2016; Marti et al., 2018). In a previous work, we demonstrated that NBDNJ was not toxic to *A. thaliana* plants at a concentration lower than 50 μM (Marti et al., 2018), even though it causes some reduction in primary root length (Fig. 2 A).

When Wt plants were grown in the concomitant presence of 40 μM NBDNJ and 25 or 50 μM Cd, they exhibited a phenotype similar to that of *rsw3* mutant, with a significant increase in primary root length (Fig. 2A and B) and no significant change in terms of shoot fresh weight (Fig. 2C).

We also monitored whether the *rsw3* mutant had any defects in Cd uptake or translocation by ICP-AES analyses. Therefore, both plant lines were grown for 14 days in the presence of 50 μM Cd, then root and shoot samples were analysed to evaluate Cd content. Results shown in Fig. S1 clearly indicated similar Cd levels in either root or shoot tissues, suggesting that the enhanced tolerance shown by the *rsw3* mutant to Cd stress was not due to a lower metal intake.

3.2. *rsw3 mutant shows defects in N-glycosylation*

The GII enzyme plays a central role in ERQC pathway since it catalyses the removal of the second glucose from the *N*-glycan of newly synthesised glycoproteins, regulating the interaction with the molecular chaperones (*i.e.* CNX, CRT, BiP) assisting glycoproteins to reach a proper folding. Genetic impairments in the ERQC enzymes often result in a reduced glycoprotein folding capacity. To verify this hypothesis in the *rsw3* mutant in the presence or not of a Cd stress, total protein extracts from Wt and *rsw3* seedlings were separated by gel electrophoresis (Fig. 3 A) and incubated with Concanavalin A (ConA), a lectin which selectively binds high mannose ER retained glycoproteins (Fig. 3 B). As previously reported (Soussillane et al., 2009), under control conditions, an increase in ConA reacting glycoproteins was observed in *rsw3* in comparison with Wt seedlings, with no further increase recorded after Cd treatment (Fig. 3). By converse, a slight increase in the ConA bound glycoproteins was observed in Wt plantlets when challenged by Cd stress. To further verify the ER localization of these glycoproteins, samples were also digested with endoglycosidase H (Endo H), which cleaves high mannose residues from glycoproteins. As shown in Fig. 3 EndoH digestion resulted in a reduction of the ConA reacting glycoproteins, thus indicating that the mutant constitutively accumulates higher levels of unfolded glycoproteins (Fig. 3).

3.3. *Reduced GII activity induces a constitutive ER stress and limits further response to Cd treatment in mutant seedlings*

To monitor the ER stress levels in *rsw3* mutant under standard growth condition or in the presence of Cd stress, we monitored the level of PDI5 (Lu and Christopher, 2008; Yuen et al., 2013; Sun et al., 2022) and BiP (Srivastava et al., 2013; Iwata et al., 2018), both well-known ER stress markers. Total protein extracts from root and shoot tissues of Wt and *rsw3* seedlings were separated by SDS-PAGE (Fig. S2) and probed with anti-PDI5 or BiP (which recognises all three BiP isoforms) primary antibodies. Our results indicated that the *rsw3* mutant showed constitutively higher levels of both markers in comparison with Wt in root and shoot samples. Furthermore, under stress conditions, both markers showed a general increase in Wt plantlets, with a more sustained increase of PDI in shoots and BiP in roots. A similar increase was observed only for PDI5 levels in the aerial tissues of the *rsw3* mutant (Fig. 4).

The expression levels of the genes encoding these markers (*BiP1/2*, *BiP3* and *PDI5*) were also verified by qRT-PCR analysis on RNA samples extracted from the same tissues (Fig. 5). All the tested genes showed significantly higher expression levels in the *rsw3* mutant compared to Wt in root tissue under standard growth conditions and their expression level did not change significantly under Cd stress. Conversely, either the *BiP3* or *PDI5* transcript was upregulated in stressed roots of Wt plants.

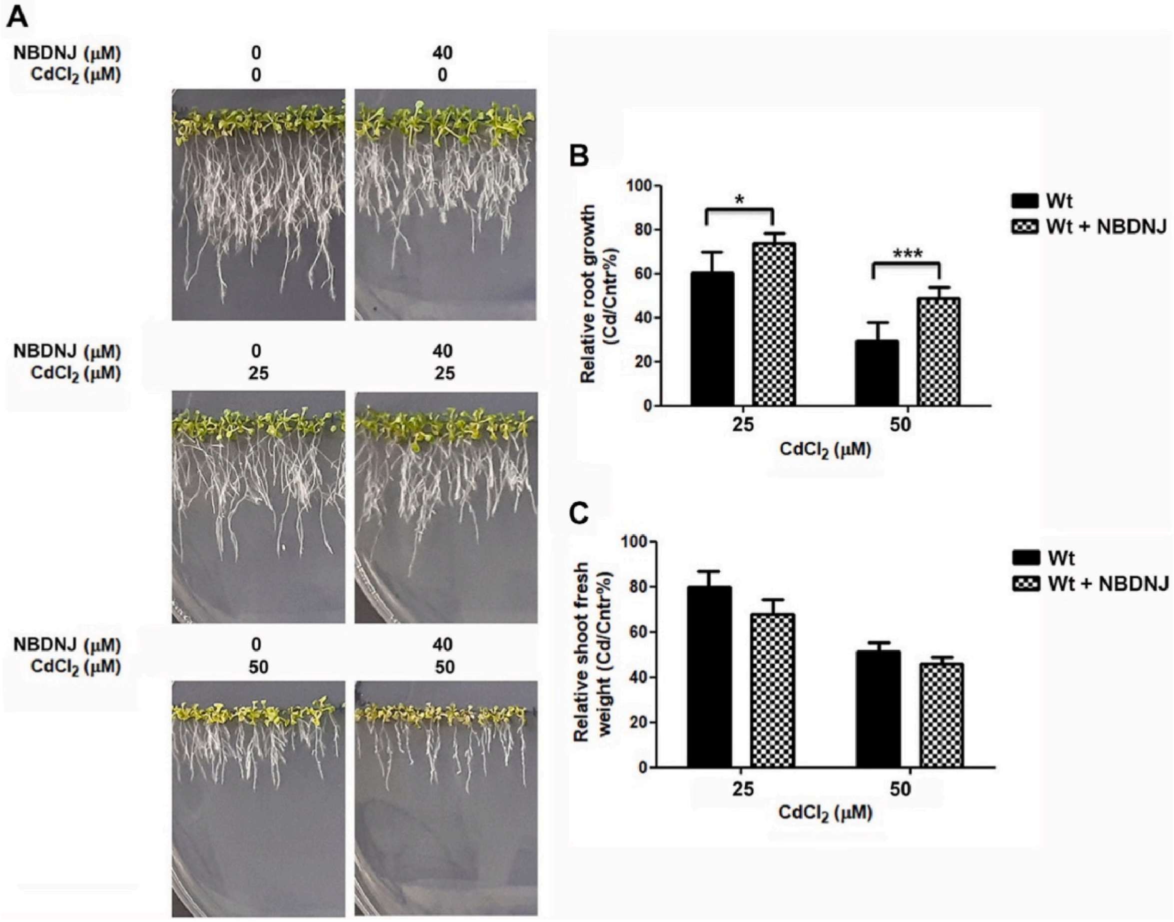

Fig. 2. NBDNJ plant treatment increases Cd tolerance in *A. thaliana.* (A) Wt plants were grown with 0 or 40 μM NBDNJ and in the presence of $CdCl_2$ concentrations for 14 days. (B) Relative root length and (C) relative shoot fresh weight of Cd-treated Wt plants in comparison with their respective controls. Error bars indicate standard deviation. The values represent the average of at least three biological replicates (one-way ANOVA followed by Bonferroni's tests, $^*p \leq 0.05$; $^{***}p \leq 0.001$).

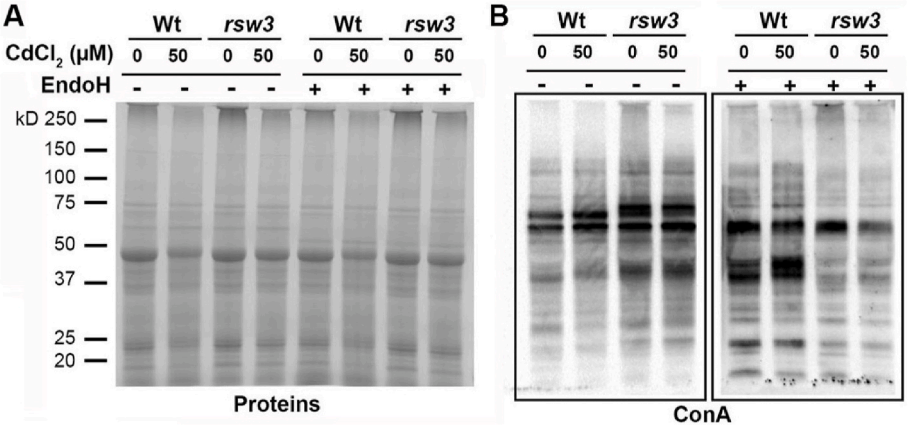

Fig. 3. (Glyco)proteins accumulated in *rsw3* seedlings in the absence/presence of $CdCl_2$. Stain free gel of total proteins extracted from Wt and *rsw3* mutant plants grown in control condition (0) or in the presence of 50 μM $CdCl_2$ for 14 days and treated without (−) or with (+) Endo H treatment (A). Samples were stained using Concanavalin A (ConA) for detection of high mannose bearing glycoproteins (B).

Moving to the aerial tissues, our results indicated no significant differences between the two genotypes, under control or stress conditions. Taken together biochemical and molecular results demonstrate the constitutive activation of ER stress-related marker in *rsw3* mutant. By contrast, both *BiP3* and *PDI5* transcripts were significantly upregulated in Wt roots under Cd stress.

Next, we monitored the expression levels of both isoforms of the *bZIP60* transcription factor, the main arm of the UPR pathway, whose involvement in the response to Cd stress was recently reported (De Benedictis et al., 2023) (Fig. 5). RT-PCR analysis carried out in root and shoot samples of both genotypes, showed that both the unspliced (*bZIP60u*) and spliced (*bZIP60s*) transcripts are significantly upregulated in shoots but not in roots of *rsw3* already under standard growth conditions. Cd stress induced a significant induction of both the transcripts

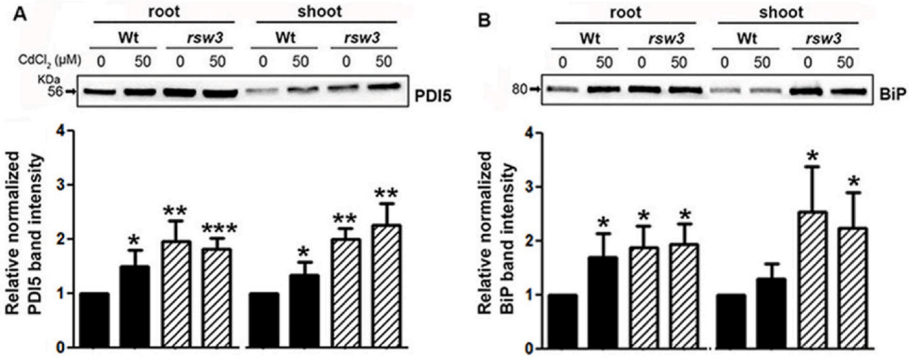

Fig. 4. Effects of Cd treatment on marker proteins of ER stress. Total proteins extracted from root and shoot tissues of Wt and *rsw3* seedlings grown for 14 days with or without 50 µM $CdCl_2$ were probed with anti-PDI5 (A) and anti-BiP (B) antibodies. Histograms reported the abundance of PDI5 and BiP marker proteins compared to the protein of Wt samples grown under control conditions after normalization with total proteins transferred onto the PVDF membrane, using the Image Lab 6.1 software. Data represent the mean and the standard deviation of three biological replicates. Asterisks show significant differences, *t*-tests, *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001).

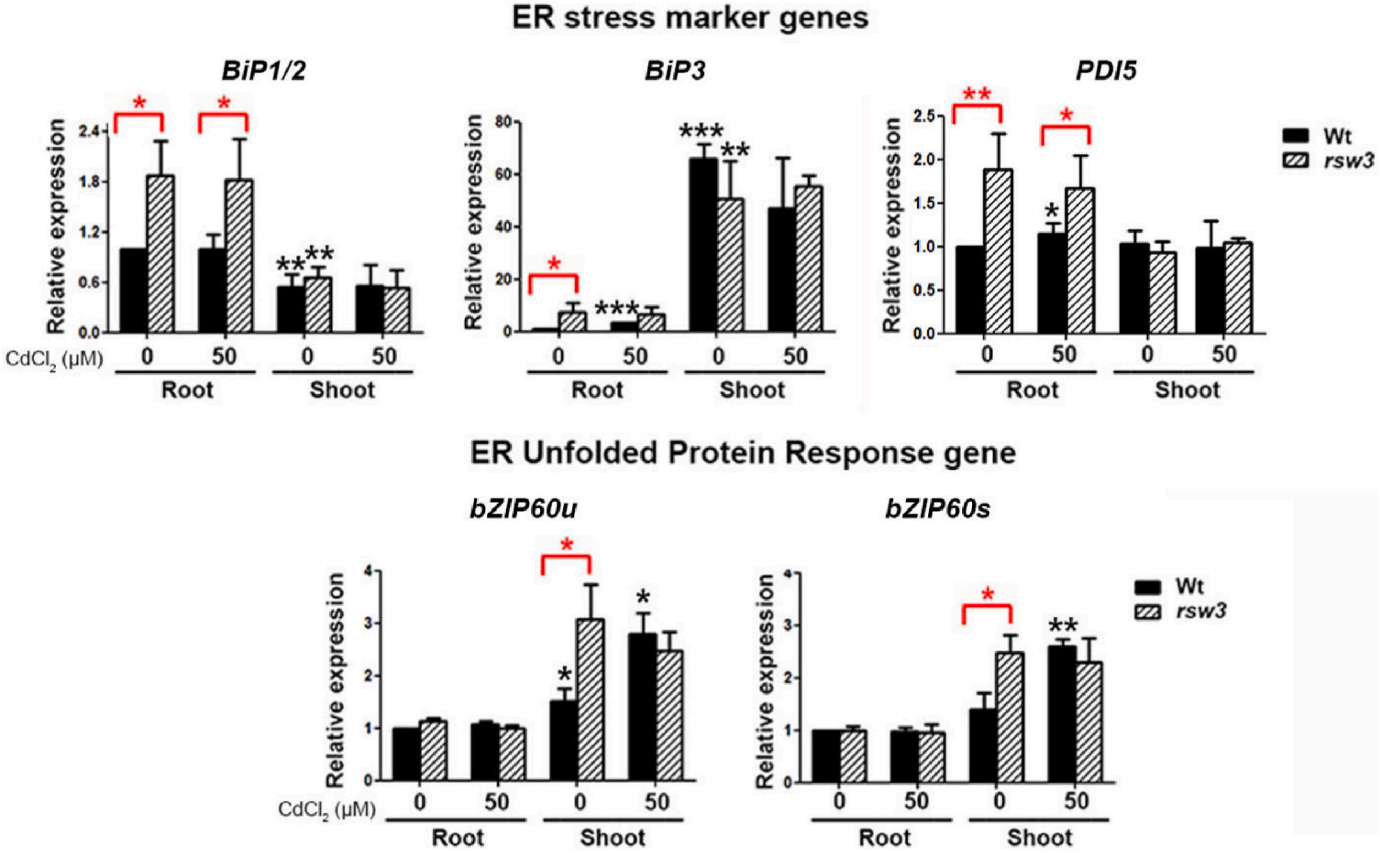

Fig. 5. Impact of the *rsw3* mutation on the expression level of ER stress and UPR marker genes. Expression of *BiP1/2*, *BiP3*, *PDI5 and bZIP60* unspliced (u) and spliced (s) was analysed by qRT-PCR in roots and shoots of Wt and *rsw3* mutant seedlings grown without $CdCl_2$ or treated with 50 µM $CdCl_2$ for 14 days. Data are shown relative to the expression level in Wt root tissues under control conditions. Black asterisks indicate significant difference between control and Cd-treated samples of the same genotype; red asterisks indicate significant differences between the two genotypes under the same conditions of growth (Student's *t*-tests, *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001).

in shoot samples of Wt plants but not in the *rsw3* mutant (Fig. 5). These data confirm that a reduced GII activity, as observed in *rsw3* mutant, is sufficient for inducing a constitutive ER stress condition and Cd treatment do not result in any further response.

3.4. *Cd stress differently impacted on BRI1 receptor protein levels in Wt and rsw3 mutant*

Brassinosteroid receptor kinase 1 (BRI1) is a glycoprotein bearing 14 potential N-glycosylation sites (Li and Chory, 1997) and, once it reaches the correct folding into the ER, assisted by the ERQC machinery, is sorted to the plasma membrane (PM) through the secretory pathway. During its sorting, it binds brassinosteroid hormones (BRs), which play a crucial role in plant growth, modulating cell expansion and elongation (Planas-Riverola et al., 2019).

To verify the possible involvement of BRI1 receptor in the reduced root growth and increased tolerance to Cd stress displayed by the *rsw3* mutant, we monitored BRI1 accumulation levels. With this aim, Wt and *rsw3* total proteins were extracted, separated by gel electrophoresis (Fig. S3A) and blotted before immunostaining with a specific BRI1 antibody. The results shown in Fig. 6A–B indicated that Cd stress resulted in a general reduction of BRI1 protein in Wt plants and, as expected, Endo H treatment slightly impacts on protein electrophoretic mobility, thus indicating that the protein is not retained in the ER.

A general reduction in BRI1 protein level was observed in *rsw3* grown under control conditions. Noteworthy, Cd stress did not impact on BRI1 levels in *rsw3* which were similar than those observed in Wt grown under the same conditions. Furthermore, Endo H digestion of *rsw3* protein sample, resulted in a double BRI1 protein band. The lowest Endo H sensitive molecular weight band was already reported for several *bri1* mutants (Chen et al., 2020), and it is also visible in the Western blot shown in Fig. S4, in which all the *bri1-9* mutant protein is Endo H sensitive, thus most likely indicating an ER-retained BRI1 protein.

Of note is that Cd stress resulted in an increase in the level of only the high molecular mass, Endo H resistant BRI1 but not in the lower molecular weight fraction of BRI1 (Fig. 6A and B).

To confirm these results, Wt plants were treated with NBDNJ, total proteins separated by gel electrophoresis (Fig. S3B) and probed with the anti-BRI1 antibody. Also in this case, Cd stress and partial GII inhibition resulted in a slight increased levels of BRI1 protein (Fig. 6C and D).

We finally verified the BRI1 receptor pattern *in vivo* using Wt plants stably over-expressing the BRI1-GFP chimeric protein (Friedrichsen et al., 2000) under standard growth conditions or challenged with Cd stress in the presence or absence of NBDNJ. Fig. 7 shows that BRI1-GFP was distributed at the plasma membrane (PM) and Early Endosomes (EE), when seedlings were grown under standard conditions, as already reported in previous works (Russinova et al., 2004; Geldner et al., 2007; Irani et al., 2012). Cd stress drastically decreased the fluorescence intensity, probably due to the general reduction in biosynthesis levels and/or the increased rate of BRI1 endocytosis or degradation. NBDNJ treatment resulted in a reduced fluorescence level even though a similar number of GFP-labelled EE was recorded. The concomitant application of Cd and NBDNJ gave a fluorescence pattern very similar to that observed after NBDNJ treatment, with an increase in general fluorescence than that observed after Cd stress (Fig. 7 A, B, C). Finally, we measured the primary root length of BRI1:GFP overexpressing line. Our results (Fig. 7D and E) indicated that the fluorescence pattern paralleled well with the phenotypic analysis, with a positive correlation of levels of BRI1 fluorescence at the PM and primary root growth.

4. Discussion

4.1. GII and ERQC are involved in *A. thaliana* primary root development and plant response to Cd stress

Previous works have reported the involvement of ER GII in plant immunity, pathogen response (Lu et al., 2009; von Numers et al., 2010) and cell wall cellulose biosynthesis (Burn et al., 2002). However, the interaction between ERQC pathway and heavy metals exposure has been poorly investigated in plants. In the present work, we provided new evidence regarding the concomitant effects of the reduced enzymatic activity of GII and Cd stress. With this aim, we took advantage of the availability of the *A. thaliana rsw3* mutant, which is characterised by a point mutation in the GII catalytic domain (Burn et al., 2002) or NBDNJ, a well-known inhibitor of this enzyme (Caputo et al., 2016).

Firstly, we showed that, similar to other mutants of the ERQC pathway, such as those deficient in *uggt* (Blanco-Herrera et al., 2015), the *rsw3* mutant exhibits reduced primary root length compared to Wt plants grown under standard conditions (Fig. 1). This suggests that different components of the ERQC pathway influence the root phenotype, most likely through the reduced folding rate of some glycoproteins involved in this physiological process.

As previously reported (Hu et al., 2013; Bruno et al., 2017), Cd stress had a strong impact on primary root length in Wt plants, which showed about 37 % and 71 % root length reduction when plants were challenged with 25 or 50 μM Cd, respectively (Fig. 1). In contrast, the primary root length of *rsw3* was not significantly affected by Cd application. Indeed, under Cd stress, *rsw3* exhibited a comparable or even slightly longer primary root than Wt, despite both genotypes accumulating the same amount of Cd in root and aerial tissues (Fig. S1).

To confirm these results, we treated Wt plants with NBDNJ to partially inhibit GII enzymatic activity. Also in this case, we observed a significant increase in primary root lenght when plants were concomitantly treated with NBDNJ and Cd, compared to plants exposed only to

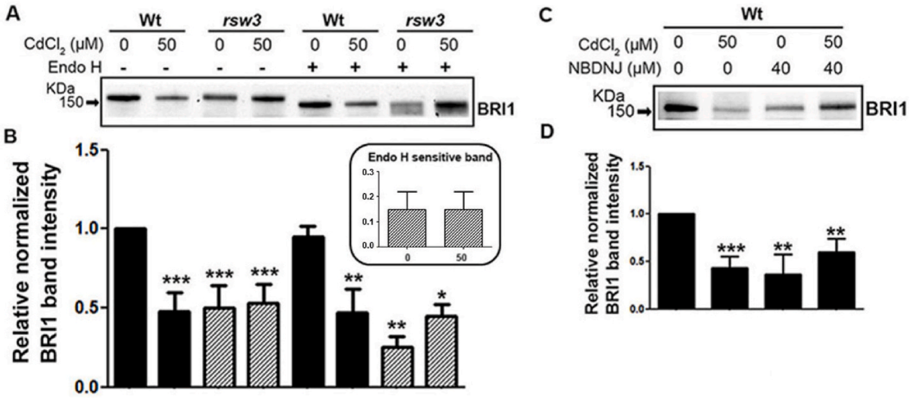

Fig. 6. Cd stress differently impacted on BRI1 protein levels in Wt and *rsw3* seedlings. Total protein extracts obtained from Wt and *rsw3* seedlings grown under control conditions or in the presence of 50 μM $CdCl_2$ for 14 days were treated with (+) or without (−) Endo H and analysed by immunoblotting using an anti-BRI1 antibody (A).

The BRI1 protein bands were normalized to total proteins, and the relative band intensity was calculated after comparison with the signal detected in Wt seedlings grown under control conditions (scored as 1; B). The inset reports the normalization of lower molecular mass BRI1 band.

(C): Total protein extracts obtained from Wt seedlings grown with 40 μM NBDNJ alone or in combination with $CdCl_2$ for 14 days and analysed by immunoblotting using an anti-BRI1 antibody. (D): The BRI1 protein bands were normalized to total proteins as in Fig. 6 B.

Data represent the mean of three biological replicates and error bars show the standard deviation. Asterisks show significant differences compared to Wt seedlings grown under control conditions (Student's *t*-tests, $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$).

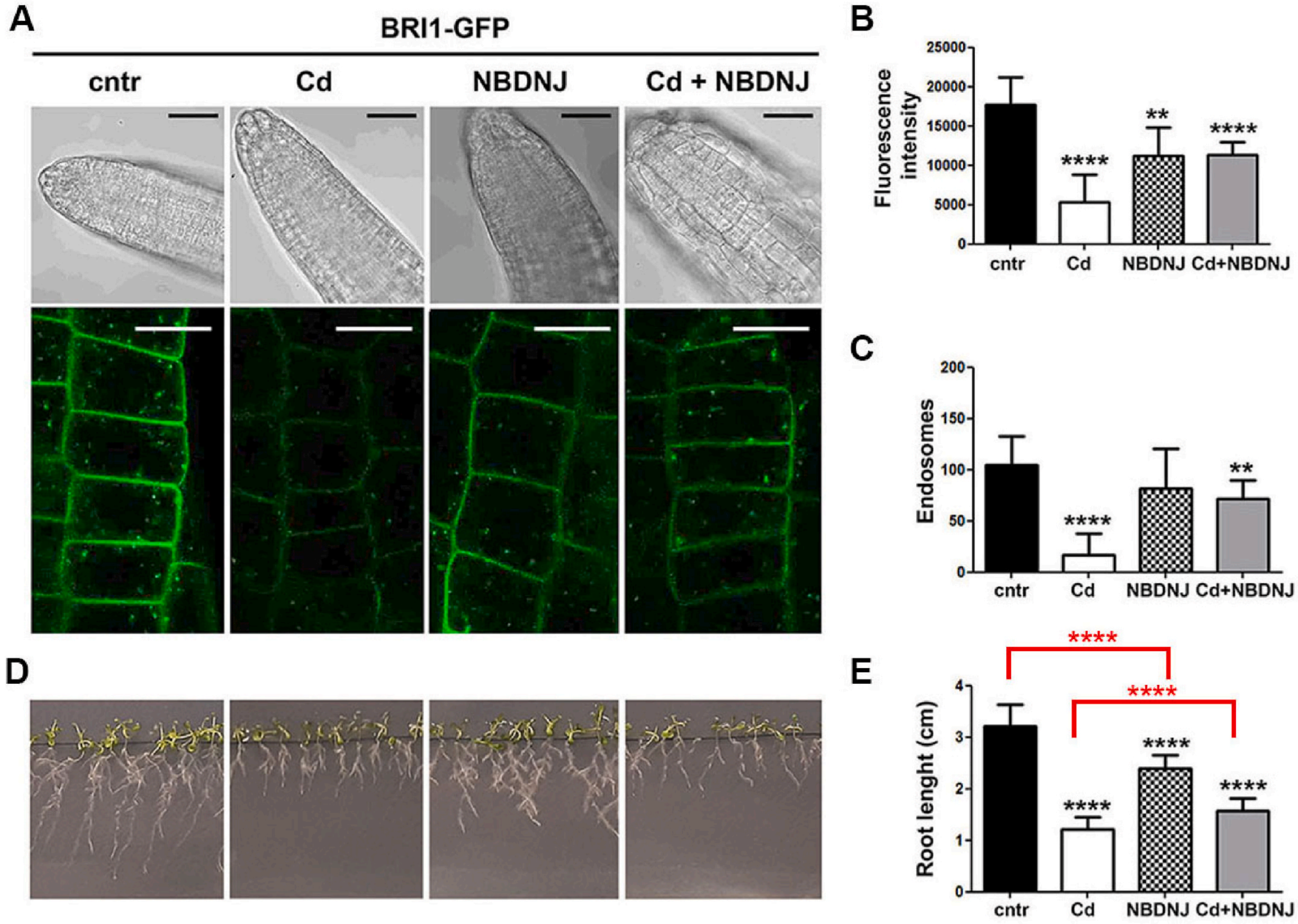

Fig. 7. BRI-GFP localization is affected by Cd or NBDNJ treatments. (A) CLSM of root cells of BRI1-GFP overexpressing line grown for 7 days under control conditions (cntr) or in the presence of 50 μM $CdCl_2$, or 40 μM NBDNJ or with both treatments combined. Bars, 20 μm. (B) Quantification of GFP signal intensity. (C) Number of endosomes. (D) Phenotype and (E) root length of the BRI1-GFP overexpressing line grown under the same conditions. Error bars indicate standard deviation ($n \geq 10$). Asterisks show a significant difference (Student's *t*-test, $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$).

Cd stress (Fig. 2). Taken together, these results support the role of ERQC components, and specifically GII, in root growth and in the plant response to chronic Cd stress.

4.2. *The rsw3 mutant shows a constitutive ER stress condition*

Result shown in Fig. 3 indicated that a reduced activity of GII enzyme is sufficient to cause an over-accumulation of ER retained misfolded glycoproteins (see also Soussillane et al., 2009), and a constitutive ER stress condition as clearly indicated by increased levels of protein (in root and aerial tissues) and mRNA levels (at root level) for BiP and PDI5 (at shoot level; Figs. 4 and 5). *BiP* is one of the key players in alleviating ER stress (Leborgne-Castel et al., 1999; Reyes-Impellizzeri and Moreno, 2021; Wilson et al., 2025) and its over-expression has already been reported in other mutants of the ERQC pathway, such as the *lew3 (leaf wilting 3)* mutant, which has a defect in an α-1,2-mannosyltransferase responsible for the final transfer of two mannose residues to the dolichol-linked core oligosaccharide (Zhang et al., 2009); the *alg3* mutant, which has a defect in an α-1,3-mannosyltransferase (Henquet et al., 2008), and the *stt3a* mutant, which is defective in one of the nine subunits of the OST complex (Koiwa et al., 2003). At the same time, transgenic plant species (e.g. tobacco, soybean and *A. thaliana*) over-expressing *BiP3* gene showed an increased tolerance to drought stress (Alvim et al., 2001; Valente et al., 2009; Wang et al., 2017), Cd stress (Guan et al., 2015), heat, salt and osmotic stresses (Wang et al., 2017). These findings suggest that genetic manipulation impacting on ERQC and glycoprotein folding capacity share the common feature of a constitutive ER stress and support the prediction that high levels of molecular chaperones, as in the case of BiP isoforms, may help to mitigate this stress condition and/or counteract further new stressing conditions. Indeed, upon Cd application, only the Wt plantlets, but not the *rsw3* mutant, over-reacted, inducing the expression of all the ER and UPR stress markers here considered (BiP3, PDI5 at root level and bZIP60 at shoot level). These results, together with the phenotypic analyses of Fig. 1 showing no detrimental effects on the main growth parameters at both the root and aerial tissues, indicate a greater tolerance of *rsw3* mutant to Cd stress.

Interestingly, the molecular analyses carried out on different organs (below or upward) indicated that changes in the BiP levels occurred mainly at root level (Figs. 4 and 5), while the opposite trend was recorded for the UPR marker *bZIP60*, which over-reacted only in the shoots (Fig. 5). This trend has been already reported in Wt plants grown under phosphate deficiency conditions (Montpetit et al., 2023). Taken together, these results indicate that the ER stress response and UPR pathway induced by Cd treatment trigger a tissue specific response.

4.3. Cd stress and brassinosteroid perception mediated by BRI1 receptor

Brassinosteroid receptor 1 (BRI1) with its 14 N-glycosylation sites is folded under the strict supervision of the ERQC machinery (Jin et al., 2007). Starting from this evidence, we wondered whether genetic impairment in GII activity and the concomitant Cd stress condition may have an impact on folding/routing of this important growth regulator. Under standard growth conditions, the nascent BRI1 receptor is correctly folded in the ER (Fig. 6) and it is delivered to its final destinations, the PM or the EE (Fig. 7). In agreement with literature, Cd stress induces a general reduction in BRI1 protein level in Wt seedlings (Figs. 6 and 7; see also Spielmann et al., 2022; De Benedictis et al., 2023), suggesting a general decrease in glycoproteins biosynthesis or an activation of protein degradation mechanisms, including ERAD or autophagy, due to the ER stress caused by heavy metal application. Accordingly, it has been reported that increased autophagic activity is associated with enhanced Cd tolerance in *A. thaliana* plants (Tang et al., 2025). Because of the reduced amount of active, plasma membrane localised BRI1, the primary roots of Wt seedlings showed a significant reduction in primary root length in comparison with the seedlings grown under standard growth conditions (Figs. 1 and 7).

The reduction in GII activity in the *rsw3* mutant likely leads to a reduction in the folding rate and finally the accumulation of misfolded BRI1 receptor (Fig. 6), which may be retained in the ER and subsequently targeted for degradation, as observed in the *bri1-9* mutant (Fig. S4). Alternatively, the presence of unprocessed N-glycans on misfolded BRI1 may hinder its recognition by ER chaperones (Petrescu et al., 2004) or may alter its local hydrophobicity (Tan et al., 2014), thereby impairing interaction with the ERQC machinery and allowing a partial escape from the ER (Chen et al., 2020). In any case, the final status of BRI1 glycans and trafficking dynamics of this important receptor under Cd stress and/or reduced GII activity need further work and a better elucidation.

As a result of the reduced amount of the active BRI1 receptor delivered to PM and EE, the primary root of the *rsw3* mutant showed about 61 % reduction in comparison with Wt seedlings grown under the same standard conditions (Fig. 1A). Surprisingly, the concomitant reduction of GII (caused by genetic impairment or NBDNJ treatment) and Cd stress application, resulted in a less severe phenotype (Figs. 1 and 2) in terms of primary root length. These results can be due, at least in part, to the increased level of BRI1 observed at biochemical (Fig. 6) and cellular levels (Fig. 7). Indeed, the biochemical data reported in Fig. 6 clearly indicated that the concomitant application of Cd and GII impairment resulted in an increase of only the active, high molecular mass BRI1 receptor but not of the endoH sensitive protein, thus indicating that the receptor protein delivered to the PM and evidenced in Fig. 7 is functionally active and can efficiently alleviate the effects of Cd stress. We can therefore hypothesize that the increased tolerance of *rsw3* mutant or NBDNJ treated Wt plants to Cd stress might be partially explained by the higher levels of active BRI1 localised at PM or EE level and, therefore, a better BRs brassinosteroids signalling.

Overall, the results here presented confirm the protective role of BRs against Cd toxicity, adding new hints to those already reported by others (Villiers et al., 2012).

Further investigations are needed to better elucidate the molecular mechanism underlying BRI1 folding and release from the ER under Cd stress and a reduced activity of GII enzyme.

5. Conclusions

Understanding the cellular and molecular bases of tolerance to abiotic stresses is mandatory to develop future crops more adapted to harsh environmental conditions or anthropogenic pollutants.

In this framework, this research work is aimed to better decipher the molecular mechanisms of plant response to Cd stress. The results presented here highlighted a new role for GII and BRI1 protein receptor in the tolerance response of *A. thaliana* seedlings to this stress. Further work is needed to verify the same mechanism on other glycoproteins trafficking from the ER to their final destination. Data here presented indicated that GII partial impairment positively impacted on the rate of functional BRI1 delivered to the PM and EE under Cd stress condition. As a consequence, seedlings showed a less severe phenotype in terms of primary root length and a greater tolerance to this stress. Taken together results here presented and previous works (Villiers et al., 2012) highlighted the role of BR signaling and BRI1 in particular, in the modulation of plant response to Cd stress. The molecular mechanism by which the partial impairment of ERQC components, as in the case of GII, resulted in increase rate of glycoproteins dismissal from the ER and its general physiological significance on relieving other abiotic stress conditions still need further experimental work on other glycoproteins and other (a)biotic stresses.

CRediT authorship contribution statement

Maria De Benedictis: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Antonia Gallo:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Danilo Migoni:** Writing – review & editing, Formal analysis. **Paride Papadia:** Writing – review & editing, Formal analysis. **Francesco Paolo Fanizzi:** Writing – review & editing, Formal analysis. **Gian Pietro Di Sansebastiano:** Writing – review & editing, Formal analysis. **Pietro Roversi:** Writing – review & editing, Formal analysis. **Angelo Santino:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix. ASupplementary data

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Data availability

No data was used for the research described in the article.

References

Alvim, F.C., Carolino, S.M., Cascardo, J.C., Nunes, C.C., Martinez, C.A., Otoni, W.C., Fontes, E.P., 2001. Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. Plant Physiol. 126, 1042–1054.

Angon, P.B., Islam, MdS., Kc, S., Das, A., Anjum, N., Poudel, A., Suchi, S.A., 2024. Sources, effects and present perspectives of heavy metals contamination: soil, plants and human food chain. Heliyon 10, e28357.

ATSDR, 2007. Agency for Toxic Substance and Disease Registry, U.S. Toxicological Profile for Cadmium. Department of Health and Humans Services, Public Health Service, Centers for Disease Control, Atlanta.

Blanco-Herrera, F., Moreno, A.A., Tapia, R., Reyes, F., Araya, M., D'Alessio, C., Parodi, A., Orellana, A., 2015. The UDP-glucose: Glycoprotein glucosyltransferase (UGGT), a key enzyme in ER quality control, plays a significant role in plant growth as well as biotic and abiotic stress in Arabidopsis thaliana. BMC Plant Biol. 15, 127.

Boisson, M., Gomord, V., Audran, C., Berger, N., Dubreucq, B., Granier, F., Lerouge, P., Faye, L., Caboche, M., Lepiniec, L., 2001. Arabidopsis glucosidase I mutants reveal a critical role of N-glycan trimming in seed development. EMBO J. 20, 1010–1019.

Bruno, L., Pacenza, M., Forgione, I., Lamerton, L.R., Greco, M., Chiappetta, A., Bitonti, M. B., 2017. In Arabidopsis thaliana cadmium impact on the growth of primary root by altering SCR expression and auxin-cytokinin cross-talk. Front. Plant Sci. 8, 1323.

Burn, J.E., Hurley, U.A., Birch, R.J., Arioli, T., Cork, A., Williamson, R.E., 2002. The cellulose-deficient arabidopsis mutant rsw3 is defective in a gene encoding a putative glucosidase II, an enzyme processing n-glycans during ER quality control. Plant J. 32, 949–960.

Caputo, A.T., Alonzi, D.S., Marti, L., Reca, I.B., Kiappes, J.L., et al., 2016. Structures of mammalian ER α-glucosidase II capture the binding modes of broad-spectrum iminosugar antivirals. Proc. Natl. Acad. Sci. U.S.A 113, E4630–E4638.

Chen, T., Zhang, H., Niu, G., Zhang, S., Hong, Z., 2020. Multiple N-glycans cooperate in balancing misfolded BRI1 secretion and ER retention. Plant Mol. Biol. 103, 581–596.

De Benedictis, M., Gallo, A., Migoni, D., Papadia, P., Roversi, P., Santino, A., 2023. Cadmium treatment induces endoplasmic reticulum stress and unfolded protein response in Arabidopsis thaliana. Plant Physiol. Biochem. 196, 281–290.

Demircan, N., Ozgur, R., Turkan, I., Uzilday, B., 2024. Heavy metal toxicity leads to accumulation of insoluble proteins and induces endoplasmic reticulum stress–specific unfolded protein response in Arabidopsis thaliana. Environ. Sci. Pollut. Res. 31, 53206–53218.

Friedrichsen, D.M., Joazeiro, C.A., Li, J., Hunter, T., Chory, J., 2000. Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. Plant Physiol. 123, 1247–1256.

Geldner, N., Hyman, D.L., Wang, X., Schumacher, K., Chory, J., 2007. Endosomal signaling of plant steroid receptor kinase BRI1. Genes Dev. 21, 1598–1602.

Guan, C., Jin, C., Ji, J., Wang, G., Li, X., 2015. LcBiP, a endoplasmic reticulum chaperone binding protein gene from Lycium chinense, confers cadmium tolerance in transgenic tobacco. Biotechnol. Prog. 31, 358–368.

Henquet, M., Lehle, L., Schreuder, M., Rouwendal, G., Molthoff, J., Helsper, J., van der Krol, S., Bosch, D., 2008. Identification of the gene encoding the α1,3-Mannosyltransferase (ALG3) in arabidopsis and characterization of downstream N-Glycan processing. Plant Cell 20, 1652–1664.

Hiscox, J.D., Tsraelstam, G.F., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57, 1332–1334.

Hu, Y.F., Zhou, G., Na, X.F., Yang, L., Nan, W.B., Liu, X., et al., 2013. Cadmium interferes with maintenance of auxin homeostasis in Arabidopsis seedlings. J. Plant Physiol. 170, 965–975.

Irani, N.G., Di Rubbo, S., Mylle, E., Van den Begin, J., Schneider-Pizon, J., et al., 2012. Fluorescent castasterone reveals BRI1 signaling from the plasma membrane. Nat. Chem. Biol. 8, 583–589.

Iwata, Y., Iida, T., Matsunami, T., Yamada, Y., Mishiba, K.I., Ogawa, T., Kurata, T., Koizumi, N., 2018. Constitutive BiP protein accumulation in Arabidopsis mutants defective in a gene encoding chloroplast-resident stearoyl-acyl carrier protein desaturase. Genes Cells 23, 456–465.

Jin, H., Yan, Z., Nam, K.H., Li, J., 2007. Allele-specific suppression of a defective brassinosteroid receptor reveals a physiological role of UGGT in ER quality control. Mol. Cell 26, 821–830.

Karagöz, G.E., Acosta-Alvear, D., Walter, P., 2019. The unfolded protein response: detecting and responding to fluctuations in the protein-folding capacity of the endoplasmic reticulum. Cold Spring Harbor Perspect. Biol. 11, a033886.

Koiwa, H., Li, F., McCully, M., Mendoza, I., Koizumi, N., et al., 2003. The STT3a subunit isoform of the Arabidopsis oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. Plant Cell 15, 2273–2284.

Leborgne-Castel, N., Jelitto-Van Dooren, E.P.W.M., Crofts, A.J., Denecke, J., 1999. Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress. Plant Cell 11, 459–469.

Li, J., Chory, J., 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90, 929–938.

Liu, J.X., Howell, S.H., 2010. Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. Plant Cell 22, 2930–2942.

Lu, D.P., Christopher, D.A., 2008. Endoplasmic reticulum stress activates the expression of a sub-group of protein disulfide isomerase genes and AtbZIP60 modulates the response in Arabidopsis thaliana. Mol. Genet. Genom. 280, 199–210.

Lu, X., Tintor, N., Mentzel, T., Kombrink, E., Boller, T., et al., 2009. Uncoupling of sustained MAMP receptor signaling from early outputs in an Arabidopsis endoplasmic reticulum glucosidase II allele. Proc. Natl. Acad. Sci. U.S.A 106, 22522–22527.

Marti, L., Lia, A., Reca, I.B., Roversi, P., Santino, A., Zitzmann, N., 2018. In planta preliminary screening of ER glycoprotein folding quality control (ERQC) modulators. Int. J. Mol. Sci. 19, 2135.

Montpetit, J., Clúa, J., Hsieh, Y.F., Vogiatzaki, E., Müller, J., Abel, S., Strasser, R., Poirier, Y., 2023. Endoplasmic reticulum calnexins participate in the primary root growth response to phosphate deficiency. Plant Physiol. 191, 1719–1733.

Moulis, J.M., Thévenod, F., 2010. New perspectives in cadmium toxicity: an introduction. Biometals 23, 763–768.

Petrescu, A.-J., Milac, A.-L., Petrescu, S.M., Dwek, R.A., Wormald, M.R., 2004. Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding. Glycobiology 14, 103–114.

Planas-Riverola, A., Gupta, A., Betegón-Putze, I., Bosch, N., Ibañes, M., Caño-Delgado, A. I., 2019. Brassinosteroid signaling in plant development and adaptation to stress. Development 146, dev151894.

Reyes-Impellizzeri, S., Moreno, A.A., 2021. The endoplasmic reticulum role in the plant response to abiotic stress. Front. Plant Sci. 12, 755447.

Russinova, E., Borst, J.-W., Kwaaitaal, M., Cano-Delgado, A., Yin, Y., Chory, J., de Vries, S.C., 2004. Heterodimerization and endocytosis of arabidopsis brassinosteroid receptors BRI1 and AtSERK3 (BAK1). Plant Cell 16, 3216–3229.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., et al., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

Scortichini, M., Chen, J., De Caroli, M., Dalessandro, G., Pucci, N., et al., 2018. A zinc, copper and Citric acid biocomplex shows promise for control of Xylella fastidiosa Subsp. pauca in olive trees in Apulia region (Southern Italy). Phytopathol. Mediterr. 57, 48–72.

Soussillane, P., D'Alessio, C., Paccalet, T., Fitchette, A.C., Parodi, A.J., et al., 2009. N-glycan trimming by glucosidase II is essential for Arabidopsis development. Glycoconj. J. 26, 597–607.

Spielmann, J., Cointry, V., Devime, F., Ravanel, S., Neveu, J., Vert, G., 2022. Differential metal sensing and metal-dependent degradation of the broad spectrum root metal transporter IRT1. Plant J. 112, 1252–1265.

Srivastava, R., Deng, Y., Shah, S., Rao, A.G., Howell, S.H., 2013. BINDING PROTEIN is a master regulator of the endoplasmic reticulum stress sensor/transducer bZIP28 in Arabidopsis. Plant Cell 25, 1416–1429.

Strasser, R., 2018. Protein quality control in the endoplasmic reticulum of plants. Annu. Rev. Plant Biol. 69, 147–172.

Sun, X., Guo, C., Ali, K., Zheng, Q., Wei, Q., et al., 2022. A non-redundant function of MNS5: a class I α-1, 2 mannosidase, in the regulation of endoplasmic reticulum-associated degradation of misfolded glycoproteins. Front. Plant Sci. 13, 873688.

Tan, N.Y., Bailey, U.-M., Jamaluddin, M.F., Mahmud, S.H.B., Raman, S.C., Schulz, B.L., 2014. Sequence-based protein stabilization in the absence of glycosylation. Nat. Commun. 5, 3099.

Tang, Z., Wang, H.Q., Chen, J., Chang, J.D., Zhao, F.J., 2023. Molecular mechanisms underlying the toxicity and detoxification of trace metals and metalloids in plants. J. Integr. Plant Biol. 65, 570–593.

Tang, H., Zang, S., Hong, X., Jiang, R., Huang, H., et al., 2025. Autophagy and glycolysis synergistically mediate Cd^{2+} transport to enhance Cd tolerance in Arabidopsis. J. Hazard. Mater. 497, 139639.

Valente, M.A., Faria, J.A., Soares-Ramos, J.R., Reis, P.A., Pinheiro, G.L., et al., 2009. The ER luminal binding protein (BiP) mediates an increase in drought tolerance in soybean and delays drought-induced leaf senescence in soybean and tobacco. J. Exp. Bot. 60, 533–546.

Vert, G., Nemhauser, J.L., Geldner, N., Hong, F., Chory, J., 2005. Molecular mechanisms of steroid hormone signaling in plants. Annu. Rev. Cell Dev. Biol. 21, 177–201.

Villiers, F., Jourdain, A., Bastien, O., Leonhardt, N., Fujioka, S., et al., 2012. Evidence for functional interaction between brassinosteroids and cadmium response in Arabidopsis thaliana. J. Exp. Bot. 63, 1185–1200.

Von Numers, N., Survila, M., Aalto, M., Batoux, M., Heino, P., Palva, E.T., Li, J., 2010. Requirement of a homolog of glucosidase II β-subunit for EFR-mediated defense signaling in Arabidopsis thaliana. Mol. Plant 3, 740–750.

Wang, H., Niu, H., Zhai, Y., Lu, M., 2017. Characterization of BiP genes from pepper (Capsicum annuum L.) and the role of CaBiP1 in response to endoplasmic reticulum and multiple abiotic stresses. Front. Plant Sci. 8, 1122.

Wilson, C.A., Alfaro-Valdés, H.M., Kaplan, M., D'Alessio, C., 2025. Mechanical effect of protein glycosylation on BiP-mediated post-translational translocation and folding in the endoplasmic reticulum. Biophys. Rev. 17, 435–447.

Xi, H., Xu, H., Xu, W., He, Z., Xu, W., Ma, M., 2016. A SAL1 loss-of-function arabidopsis mutant exhibits enhanced cadmium tolerance in association with alleviation of endoplasmic reticulum stress. Plant Cell Physiol. 57, 1210–1219.

Yuen, C.Y.L., Matsumoto, K.O., Christopher, D.A., 2013. Variation in the subcellular localization and protein folding activity among Arabidopsis thaliana homologs of protein disulfide isomerase. Biomolecules 3, 848–869.

Zhai, Z., Gayomba, S.R., Jung, H.I., Vimalakumari, N.K., Pineros, M., et al., 2014. OPT3 is a phloem-specific iron transporter that is essential for systemic iron signaling and redistribution of iron and cadmium in arabidopsis. Plant Cell 26, 2249–2264.

Zhang, M., Henquet, M., Chen, Z., Zhang, H., Zhang, Y., et al., 2009. LEW3, encoding a putative α-1, 2-mannosyltransferase (ALG11) in N-linked glycoprotein, plays vital roles in cell-wall biosynthesis and the abiotic stress response in Arabidopsis thaliana. Plant J. 60, 983–999.