Article

EFR-mediated innate immune response in ER α–glucosidase II *rsw3* and UGGT *uggt1-1* *Arabidopsis thaliana* mutants.

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Abstract: Plants offer a simpler and cheaper alternative to mammalian animal models for the study of Endoplasmic Reticulum glycoprotein folding Quality Control (ERQC). In particular, the *Arabidopsis thaliana* (*At*) innate immune response to bacterial peptides provides an easy means of assaying ERQC function *in vivo.* A number of mutants that are useful to study ERQC *in planta* have been described in the literature, but only for a subset of these mutants the innate immune response to bacterial elicitors has been measured beyond monitoring plant weight and some physio-pathological parameters related to the plant immune response. In order to probe deeper into the role of ERQC in the plant immune response, we monitored expression levels of the *PHI-1* and *RET-OX* genes in the *At* ER α-Glu II *rsw3* and the *At* UGGT *uggt1-1* mutant plants, in response to bacterial peptides elf18 and flg22. The elf18 response was impaired in the *rsw3* but not completely abrogated in the *uggt1-1* mutant plants, raising the possibility that the latter enzyme is partly dispensable for ERF signalling. In the *rsw3* mutant, seedling growth was impaired only by concomitant application of the *At* ER α-Glu II *N*B-DNJ inhibitor at concentrations above 500 nM, suggesting residual activity in this mutant. The study highlights the need for extending plant innate immune response studies to assays sampling EFR signalling at the molecular level.

Keywords: *Arabidopsis thaliana; ER α–glucosidase II; glycoprotein folding quality control; plant immune response; UGGT; elf18; flg22; EFR; FLS2; rsw3; uggt1-1; NB-DNJ.*

1. Introduction

Glycoproteins traversing the secretory pathway of eukaryotic cells reach their cellular or extracellular destinations after folding in the Endoplasmic Reticulum (ER) *[1]*. Glycoprotein folding in the ER is aided by the ER glycoprotein folding Quality Control (ERQC) machinery, also known as "calnexin cycle" *[2]*. A number of chaperones and foldases associate with the ER lectins calnexin (CNX) and/or calreticulin (CRT) , which bind glycoproteins carrying an *N*-linked mono-glucosylated glycan, $Glc_1Man_9GlcNAc_2$. Thanks

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to ER α–glucosidase II (Glu II), the ERQC usher, all glycoproteins have at least one chance to associate with CNX/CRT and profit from chaperone/foldase assisted folding. Glu II mediates entry of a glycoprotein into the calnexin cycle by cleaving the outer glucose from a glycoprotein's $Glc_2Man_9GlcNAc_2$ *N*-linked glycan, yielding a mono-glucosylated *N*-linked glycan *[3]*.

Remarkably, the same ERQC usher allowing glycoprotein entry into the calnexin cycle, enables escape from it: after having mediated the exposure of a mono-glucosylated glycan, Glu II catalyses a second Glc cleavage, removing the inner glucose from the glycan, thus making it no longer a substrate for ER lectins. As every *N*-glycosylated glycoprotein originally carries one or more *N*-linked glycans, all glycoproteins can profit from one or more rounds of CNX/CRT association via the Glc residues present on the glycans upon entry into the ER. Indeed, simply thanks to the mono-glucosylated *N*-linked glycans generated by Glu II, easy/quick-to-fold glycoproteins bind ERQC lectins and chaperones/foldases long enough to form successfully, and then progress towards the Golgi down the secretory pathway.

Glycoproteins that are slower/trickier to fold need further rounds of interaction with ERQC lectins and chaperones/foldases. The ERQC checkpoint, UDP-glucose glycoprotein glucosyltransferase (UGGT), evolved to take care of these glycoproteins, preventing their premature exit from the ER. The enzyme recognises a mis-folded glycoprotein and adds back a glucose residue to a $Man_9GlcNAc_2$ *N*-linked glycan. At this end of the cycle, many glycoproteins undergo multiple re-glucosylation and CRT/CNX binding events. UGGT deletion results in premature release of these glycoproteins from the cycle. For other proteins, UGGT deletion substantially delays release from calnexin *[4]*, perhaps because UGGT itself associates with chaperones required for structural maturation export from the ER *[5]*.

Confirming the importance of Glu II and UGGT in development, no multicellular organism is known to survive Glu II KO to adulthood *[6]*, and homozygous UGGT deletion is embryonically lethal in mice *[7]*. Mutations to either the Glu II or UGGT genes can impair glycoprotein folding and cause ER retention and/or degradation, with the accompanying loss of function *[8-11]*. Yet, the centrality of ERQC to viral glycoprotein folding and secretion makes Glu II and UGGT appealing targets for host-acting antivirals, with clinical relevance for broad-spectrum antiviral therapy. Indeed, iminosugars (a class of glycomimetics) are antiviral ER α-Glu II inhibitors that are well tolerated in mammals *[12]*, with ER α-Glu iminosugar inhibitors having reached clinical trials against dengue fever (https://clinicaltrials.gov/ct2/show/NCT01619969) and HIV (in combination with AZT, *[13]*). UGGT too has been advocated as a pharmacological target, UGGT modulation having therapeutic potential for the rescue of the secretion of misfolded but functional glycoprotein mutants in congenital protein folding diseases *[14-16]*. No UGGT inhibitors are known (apart from its product, UDP *[17]*); heterozygous $UGGT1^{+/-}$ knockout mice have been reported to express approximately half of the wild-type (WT) amount of $UGGT1$ but they undergo normal development and have no obvious aberrant phenotype *[18]*.

The high conservation of sequence and function of ERQC components across eukaryotes makes it possible to use fungi and plants for basic studies of ERQC *in cellula [19]* and *in vivo [20]*, respectively, without the ethical and economical complications of mammalian animal

models. Any phenotype mediated by glycoproteins whose folding is controlled by ERQC enables the study of ERQC function *in vivo*. In plants, such phenotypes are provided by the response to microbe-associated molecular patterns (MAMPs), measured in terms of MAMP-induced events, *e.g.* reduced plant weight or detectable reactive oxygen species (ROS) generation *[9]*.

Amongst the best studied MAMPs are bacterial proteins such as EF-Tu (elongation factor thermo unstable), recognised by the EF-Tu receptor (EFR), a pattern-recognition receptor in *Arabidopsis thaliana [21]*. Plant cells recognise and bind EF-Tu, preventing genetic transformation and protein synthesis in pathogens such as *Agrobacterium tumefaciens*. *At* Glu II point mutations and deletions impair the plant response to bacterial peptide elf18, derived from EF-Tu, likely because the elf18 receptor (EFR) or another protein in the elf18 signalling pathway requires calnexin cycle assisted folding *[21]*. Two more genetic studies showed that UGGT - like Glu II - is also important for the correct function of the plant innate immune response to elf18 *[8,9]*. The implication of those studies is that EFR and/or a glycoprotein involved in elf18 EFR-mediated signalling depend on repeated cycles of association with the ER lectins and the associated chaperones and foldases - explaining the need for both ER α-Glu II and UGGT.

Somewhat surprisingly, the plant immune response to a different bacterial peptide, flg22, mediated by the flagellin sensing 2 receptor (FLS2), which belongs to the same fold family as the EFR receptor (subfamily XII of the leucine-rich repeat receptor kinases *aka* LRR-RKs), is not affected by Glu II impairment *[22]* nor by one of the UGGT mutants that impair elf18 response *[9]*. Loss of *At* UGGT also shows no discernable defects in the plant response to the brassinosteroid hormone, mediated by the BRI1 receptor, another member of the LRR-RK family.

A number of mutants that are useful to study ERQC *in planta* have been described in the literature, but only for a subset of these mutants the innate immune response to bacterial elicitors has been measured beyond monitoring plant weight and some physio-pathological parameters related to plant immune response. In order to probe deeper into the role of ERQC in the plant immune response, we monitored expression levels of the *PHI-1* and *RET-OX* genes in the *At* ER α-Glu II *rsw3* and the *At* UGGT *uggt1-1* mutant plants, in response to bacterial peptides elf18 and flg22. The elf18 response was impaired in the *rsw3* but not completely in the *uggt1-1* mutant plants. Moreover, in the *rsw3* mutant, seedling growth was impaired only by concomitant application of the *At* ER α-Glu II *N*B-DNJ inhibitor at concentrations above 500 nM, suggesting residual activity of the *At* ER α-Glu II *rsw3* mutant and/or extra toxic effects due to *N*B-DNJ glycolipid metabolism inhibition *[23]*. The study highlights the need for extending plant innate immune response studies assays to assays sampling ERQC function at the molecular level.

2. Materials and Methods

2.1 Plant material, growth conditions and treatments.

2.1.1 Testing the effect of elf18/flg22 elicitors and NB-DNJ on plant weight.

The surface of the seeds was sterilized, the seeds were vernalized for 4 days prior being sown in 12-wells plates containing one-half strength Murashige and Skoog (MS) medium

[24]. About 10 seeds were placed per each well containing 2 mL of liquid medium supplemented with 0.5% (w/v) sucrose. 70 µM *N*B-DNJ treatment was carried out on 3 days-old seedlings germinated and grown in one-half-strength MS medium, together with 100 nM elf18 (ac-SKEKFERTKPHVNVGTIG) or flg22 (QRLSTGSRINSAKDDAAGLQIA) peptide elicitors. The effect of treatment on seedling growth was analysed 13 days post sowing by photography and recording the fresh weight of plants. To test the toxicity of *N*B-DNJ on *rsw3* plants *N*B-DNJ was used in different concentrations (10 µM, 5 µM, 2 µM, 1 µM, 500 nM, 300 nM, 100 nM, 50nM) on 3 days-old seedlings. Seedlings were grown at 22°C and 70% relative humidity under a 16-h-light/8-h-dark cycle (approximately 120 µmol m^{-2} s^{-1}). Statistical analysis of the weights was performed using the software GraphPad (La Jolla, USA), test ANOVA with Bonferroni's test.

2.1.2 Growth condition for gene expression analysis experiment.

For gene expression analysis the surface of the seeds was sterilized, they were vernalized for 4 days and sown in multiwell plates (approximately 10 seeds per well) containing one-half strength MS medium with 0.5% sucrose (w/v) (2 mL per well). After 5 days 70 µM of *N*B-DNJ was added to the medium in the wells indicated in Fig. 2A and 4A. After 9 days, the medium was replaced with fresh medium (plus *N*B-DNJ as indicated) and treatments with elicitors elf18 and flg22 (100 nM) were performed after 24 h (the 10^{th} day) for 30 minutes before freezing the tissues in liquid nitrogen.

2.2. Gene expression analysis. After treatment with elicitors, 10 days-old seedlings were frozen and ground with pestle and mortar in liquid nitrogen. Total RNA was extracted from three independent replicates, each composed of 20 seedlings, using the NORGEN-Total RNA Purification Kit (Biotek Corp., Thorold, Canada) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase I (ThermoFisher scientific, Waltham, USA) to eliminate any potential DNA contamination. First-strand cDNA was synthesized from 1 µg of total RNA by using Maxima H Minus cDNA Synthesis Master Mix (ThermoFisher scientific, Waltham, USA) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (qRT-PCR) was used to analyse the expression level of the *PHI1* and *RET-OX* genes; the constitutively expressed *UBIQUITIN5* gene served as an internal reference to normalize gene expression. Analysis was performed on a StepOne™ Real-Time PCR System (Applied Biosystem, Warrington, United Kingdom) in a reaction volume of 20 µL containing 2X iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and 0.5 µM of each primer. The following amplification conditions were used: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Specificity of the PCR amplification was confirmed by dissociation curve analyses. The relative quantification of gene expression was established using the comparative $2^{-\Delta\Delta CT}$ method. The PCR efficiency of each oligonucleotide pair was calculated from linear regressions of the standard curves. Real-time PCR derived data were relatively quantified taking into account the divergent efficiencies. Three replicates were performed for each

experiment in addition to a no-template control included for each primer pair. Statistical analysis was performed using Student's t-test.

Primers sequences are shown in Supplementary Table 1.

2.3 Homology modelling. Homology modelling of *A. thaliana* UGGT (Uniprot database entry UGGG_ARATH) was carried out using the HHPred server *[25]* to align the sequence of *At* UGGT with the one of the *Chaetomium thermophilum* orthologue and the homology model created with MODELLER *[26].* The figure was made with PyMol *[27].*

3. Results

3.2 Development of A. thaliana ER α-Glu II rsw3 mutant seedlings is sensitive to NB-DNJ iminosugar dose higher than or equal to 500 nM.

Two missense mutants have been reported in Arabidopsis for the ER α–Glu II α catalytic subunit: the *At* Glu II S517F aka psl5-1 mutant and the *At* ER α–Glu II S599F aka rsw3 mutant (Supplementary Table 2). Both these mutants grow normally under standard conditions but show impaired response to elf18 treatment [28]. Both psl5-1 and rsw3 are missense mutations, raising the question as to any residual ER α–Glu II activity. In previous work, we showed that germinated embryos of *At* psl5-1 were unable to growth in the presence of 70 μM NB-DNJ iminosugar *[20]*, suggesting that *At* psl5-1 may possess residual activity, the latter only completely abrogated by the iminosugar treatment. Alternatively, the extra toxic effects due to NB-DNJ glycolipid metabolism inhibition may explain the toxicity *[23]*. As the site of the *At* Glu II rsw3 S599F amino acid substitution is located further away from the catalytic residues (*At* Glu II Asp512 and Asp588) than the site of the S517F mutations in psl5-1 *[28]*, it is expected that the rsw3 mutant may also carry residual activity, as suspected also by Soussillane et al. *[29].*

To check if this is the case, we repeated the *N*B-DNJ iminosugar treatment on the *At* ER α–Glu II *rsw3* mutant. We then treated *rsw3* embryos with elf18 or flg22 elicitors. Concentrations of *N*B-DNJ lower 500 nM were not toxic to *rsw3* mutants: embryos growth was not significantly impaired by 300 nM *N*B-DNJ (Figure 1). At *N*B-DNJ iminosugar dose higher than or equal to 500 nM, the growth of *rsw3* embryos was completely abolished (Figure 1). Similar results were obtained treating the *psl5-1* mutant with the same concentrations of *N*B-DNJ (not shown).

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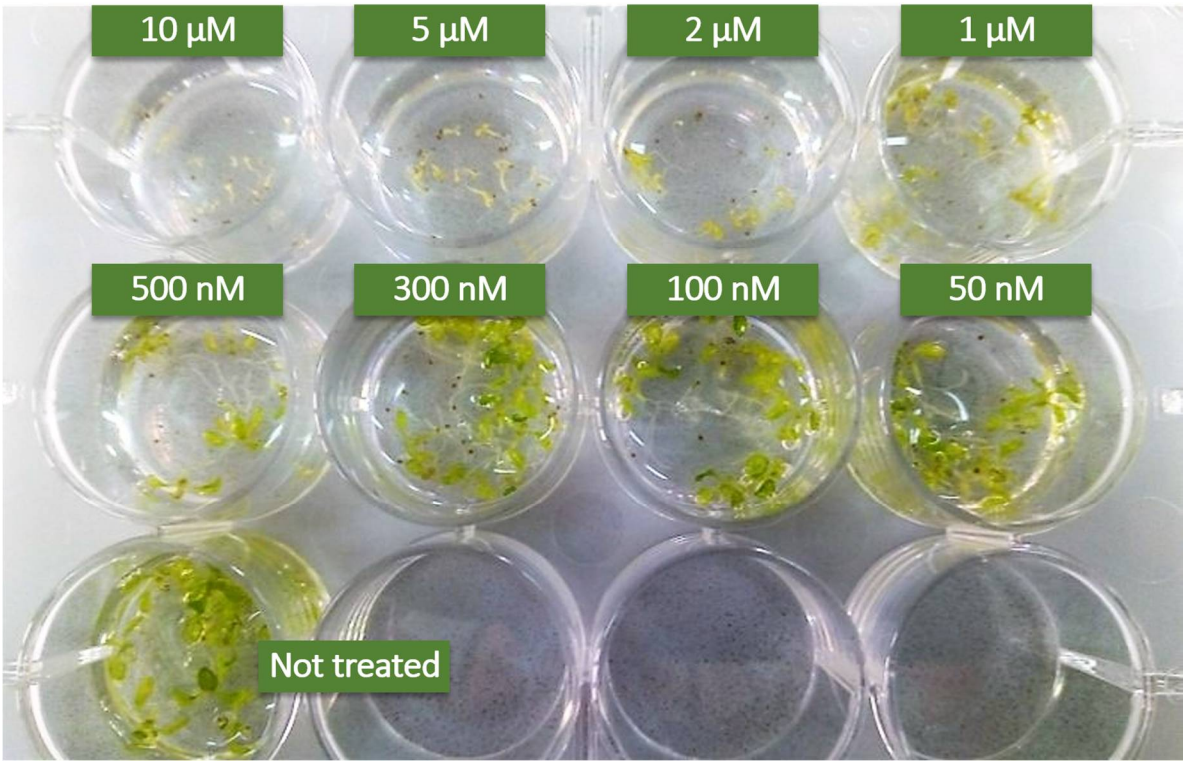

Figure 1. Effects of *N*B-DNJ-treatment on the *At rsw3* mutant. 15 days-old *rsw3* plants grown with different concentration of *N*B-DNJ. Each well contains about 10 seeds. Above 500 nM, *N*B-DNJ impairs plant embryo growth.

To check the ability of the *rsw3* plant to respond to bacterial peptides, we preliminary evaluated the response of 3 days old WT plants (Col-0 ecotype) to *elf18* or *flg22* elicitation (for 10 days). As expected both treatments reduced significatively plant growth (Fig. S1). In our hands, the *rsw3* mutant was insensitive to elf18 and highly sensitive to flg22 (Fig. 2 B and C), in keeping with what is reported in the literature *[28].*

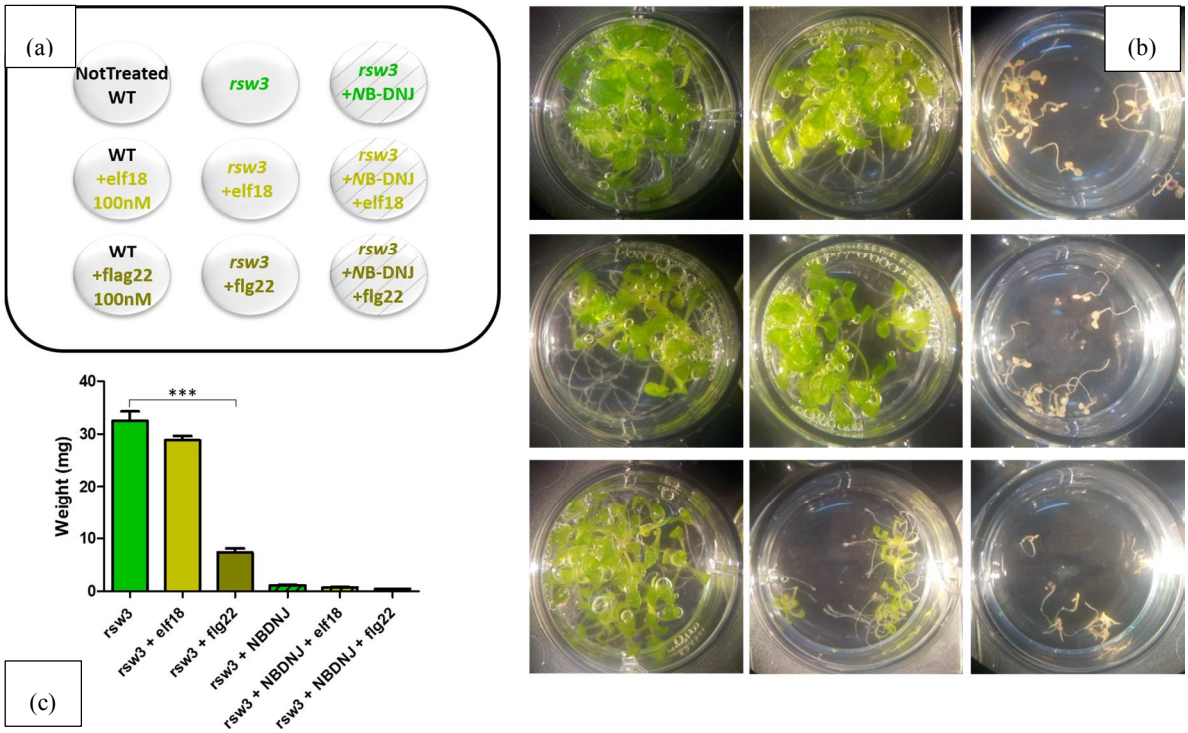

Figure 2. Effects of elicitors-treatment on *rsw3* mutant. 13-days-old seedlings control or treated with 100 nM elf18 or flg22 elicitors with and without *70 μM N*B-DNJ. A: scheme of the treatments distribution on the plate. B: images of the plants after the treatments. Each well contains about 10 seedlings. C: mean of the plants fresh-weight (expressed in mg) of at least three independent experiments (± s.e. n=10), statistical analysis determined by ANOVA with Bonferroni's test (*=P<0.5, **=P<0.01, ***=P<0.001).

3.2 The A. thaliana ER α-Glu II rsw3 is insensitive to EFR signalling.

Next, we investigated the expression level of two genes typically induced by elf18 and flg22 elicitation. Phosphate-induced 1 (*PHI-1*) and reticulin-oxidase homologue (*RET-OX*) are well known early elicitor-induced genes *[30]*. 10-days old Col-0 seedlings and *rsw3* seedlings were treated with elf18 (or flg22 used as a control) for 30 minutes. Low expression levels of both *PHI-1* and *RET-OX* were recorded in the *rsw3* mutant seedlings in response to elf18 but not flg22, while Col0 seedlings showed a normal defense response characterized by higher expression levels of defence genes with both elicitors (Figure 3).

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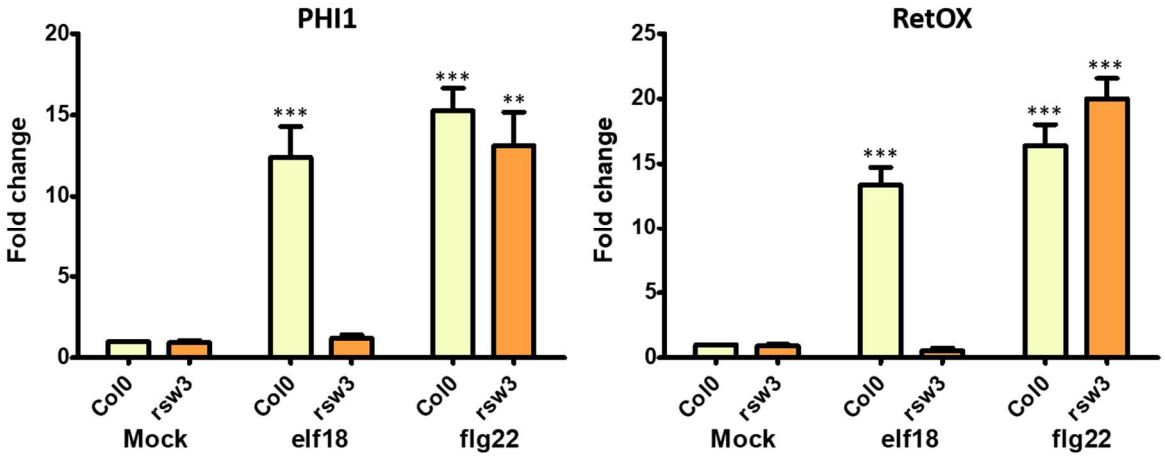

Figure 3. Expression of defence genes in the *At rsw3* plant. 10-days-old seedlings were treated with water or elf18 and flg22 elicitors. Expression of *PHI1* and *RET-OX* genes were determined by qRT-PCR 30 minutes after treatment. Transcript levels are shown as the mean of at least three independent experiments (± s.e. n=20 in each experiment) normalized to *UBQ5* (ubiquitin 5) used as a reference. In both panels asterisks indicate statistically significant differences among mock and treatments within the same genotype (same colour) according to Student's t-test (*=P<0.5, **=P<0.01, ***=P<0.001).

3.1.2 *The A. thaliana UGGT uggt1-1 mutant retains some EFR signalling*

At the calnexin cycle checkpoint end, both insertional UGGT mutants and point mutants were previously reported (Supplementary Table 3). In the first category fall *uggt1-1* and *uggt1-2*, (carrying T-DNA insertions in the 11th and 16th exons, respectively *[10]*) and the *uggt-4* mutant, characterised by a T-DNA insertion in the 24th intron *[8]*. With regard to point mutants that showed impaired immune response to EFR, Saijo et al. *[9]* reported three point mutants (*psl2-1, aka At*UGGT D1497N, *psl2-2, aka At*UGGT E306K and *psl2-4, aka At*UGGT W1443STOP), together with a double mutant (*psl2-3, aka At*UGGT R1409K/W1443STOP) and a large deletion mutant (*psl2-5, aka At*UGGT Δ304-1613). Furthermore, Li et al. *[8]* reported another point mutant of UGGT, which was named *uggt-3, aka At*UGGT W1523STOP.

We mapped these mutants on a homology model of *At*UGGT (Figure 4).

Concerning the insertional mutants *uggt1-1* and *uggt1-2*, Blanco-Herrera et al. *[10]* reported some residual re-glucosylation activity for *uggt1-1* in comparison with *uggt1-2*, used in the same study. Both these mutants were reported to be more susceptible to pathogen attack, but their response to bacterial elicitors has not been investigated so far.

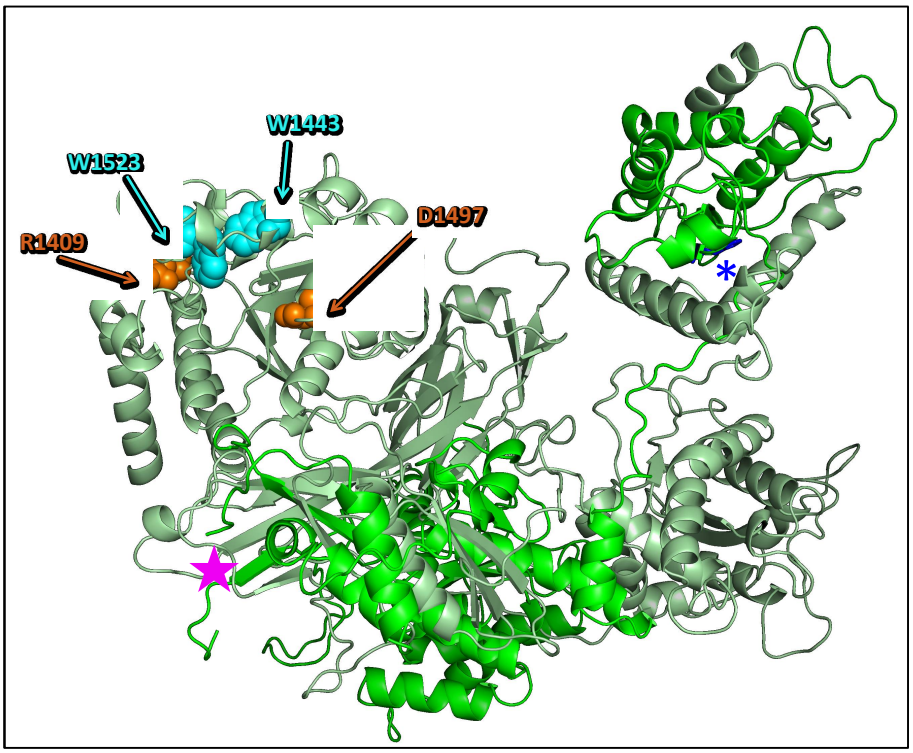

Figure 4. Mapping of *At* UGGT mutants on a homology model. The portion of *At* UGGT preceding the site of the *psl2-5* Δ604-1613 deletion mutant is in bright green; in grey/green the portion of At UGGT that is missing in the same *psl2-5* mutant. In cyan spheres, the sites of the premature stop mutations (*uggt3 aka* W1523stop and *psl2-4 aka* W1443stop); in orange spheres, the missense mutants (*psl2-3 aka* R1409K and *psl2-1 aka* D1497N. The purple star marks the missing loop containing the site of the *psl2-2* E306K mutation. The blue asterisk marks the site of the T-DNA insertion in the *uggt1-1* mutant whose response to elicitors was characterised in this study.

However, the *uggt1-1* mutant is characterised by a T-DNA insertion in the 11th exon, at a position corresponding to amino acid *At* UGGT H499, and thus much earlier than the *uggt 1-2* mutant (where T-DNA was mapped on the 24th intron) and the *uggt4* mutant (where T-DNA was mapped on the 16th exon) for which efl18 insensitivity was reported *[8]*. We chose to investigate the *A. thaliana* T-DNA UGGT insertion mutant (*uggt1-1*) using EFR signalling. To confirm the dependency of the elf18 response on *At ER α–Glu II* we decided to investigate the effects of *N*B-DNJ treatment on the *uggt1-1* mutant response to elf18 elicitation, using the plant response to flg22 as a positive control.

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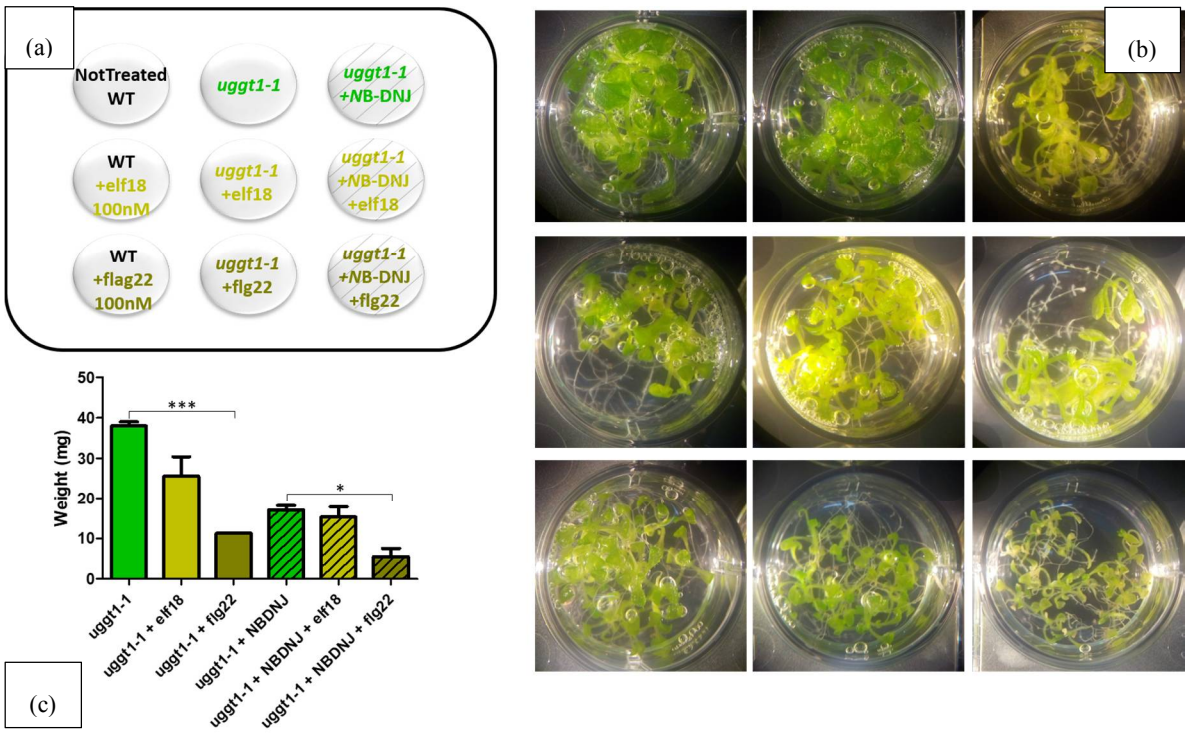

Figure 5. Effects of elicitor treatments on the *At uggt1-1* mutant. 13-days-old seedlings control or treated with 100 nM elf18 or flg22 elicitors with and without 70 µM *N*B-DNJ. A: scheme of the treatments distribution on the plate. B: images of the plants after the treatments. Each well contains about 10 seedlings. C: mean of the plants fresh-weight (expressed in mg) of at least three independent experiments (± s.e. n=10), statistical analysis determined by ANOVA with Bonferroni's test (*=P<0.5, **=P<0.01, ***=P<0.001).

Results shown in Fig. 5 indicated that, unlike what was observed with the Glu II mutants *psl5-1* and *rsw3*, *N*B-DNJ is not lethal in the background of the *uggt1-1* mutant: treatment with the iminosugar caused a growth reduction similar to that observed in Col0 WT plants.

Elicitation experiments showed that this mutant was highly susceptible to flg22 (about 70% weight reduction). Elf18 elicitation impacted on *uggt1-1* plant growth much less than flg22, causing a lower plant weight reduction (about 35%), which was not statistically significant when compared to untreated plants. Only co-treatment of *At uggt1-1* with 70 µM *N*B-DNJ caused complete insensitivity to elf18, likely because of Glu II inhibition (Fig. 5 B and C).

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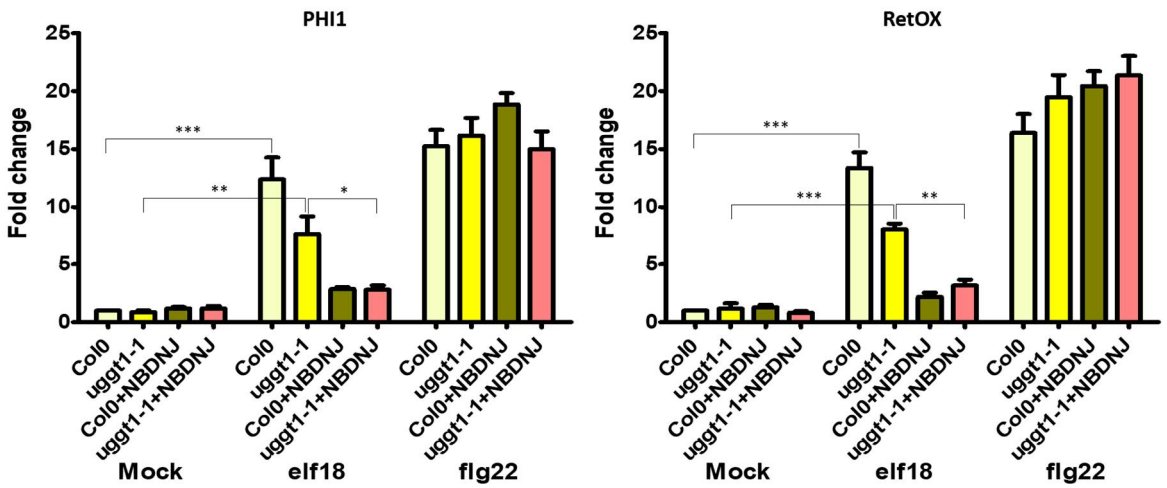

Figure 6. Expression of defence genes in the *At uggt1-1* plant. 10-days-old seedlings were treated with water or elf18 and flg22 elicitors, with and without 70 µM *N*B-DNJ. Expression of *PHI1* and *RET-OX* genes were determined by qRT-PCR 30 minutes after treatment. Transcript levels are shown as the mean of at least three independent experiments (± s.e. n=20 in each experiment) normalized to *UBQ5* (ubiquitin 5) used as a reference. In both panels asterisks indicate statistically significant differences among mock and treatments within the same genotype (same colour) according to Student's t-test (*=P<0.5, **=P<0.01, ***=P<0.001).

Finally, we verified the expression of innate immune defence genes in the *uggt1-1* mutant upon elf18 or flg22 elicitation. Under our experimental conditions, we found that both elicitors triggered a significant increase in the expression levels of both *PHI-1* and *RET-OX* and only the concomitant application of *N*B-DNJ blocked the EFR signalling pathway (Fig. 6).

4. Conclusions

High sequence and functional conservation of the two main components of the ERQC, ER α–Glu II and UGGT, enables the use of plant ER α–Glu II and UGGT mutants for *in vivo* ERQC studies. In a previous piece of work *[16]* we were able to recomplement the insertional UGGT mutant in the genetic background *ebs1-3/bri1-9* using a functional UGGT from the thermophilic filamentous fungus *Chaetomium thermophilum*. In a separate study, we showed that the iminosugar *N*B-DNJ, which reached phase I clinical trials as an antiviral drug, was able to inhibit *A. thaliana* ER α–Glu II *[20]*. Taken together these results confirmed the potential of *A. thaliana* as a low-cost, easy-to-use organism to study ERQC function. In this context, leucine-rich repeat receptor kinases (LRR_RKs) are very useful ERQC client glycoproteins. Even though EFR, FLS2 and the brassinosteroid receptor BRI1 belong to the same sub-family (LRR-RK, sub-family XII), EFR-mediated signalling depend on ERQC, while the BRI1- and FLS2-mediated responses do not *[8]*.

A possible explanation of this different behaviour is that EFR (or one of its downstream effectors) evolved more recently and depends on the ERQC machinery more than FLS2 and BRI1 *[8]*. Alternatively, macroscopic phenotypes such as loss of plant weight or decreased plant size are not subtle enough to probe the complex roles that ERQC components play in the folding of the many glycoproteins likely involved in the plant's responses to elicitors. Differences in protein structure, and/or numbers of *N*-linked glycans and/or the relative

positions of the *N*-glycosylation site(s) with respect to the sites of misfold can all affect the degree to which either the EFR/FLS2/BRI1 receptors, or other glycoproteins involved in their signalling cascades, depend on Glu II and UGGT.

Our results confirm those from Lu et al. *[28]*, who showed for the first time that EFR-dependent ligand binding activity was impaired in *psl5-1* and *rsw3* mutants. While a T-DNA insertion mutant was reported for the β-subunit of ER α–Glu II *[28]*, homozygous insertional mutants disrupting the fold of the α catalytic subunit of ER α–Glu II cause embryonic death *[6]*, pointing to a likely essential role of the enzyme for seedling development. Interestingly, treatment of both ER α–Glu II *rsw3* and *psl5-1* mutants with *N*B-DNJ at concentrations higher than 500 nM (see Fig. 1) was lethal to *Arabidopsis thaliana* embryos, likely because of complete inhibition of *At* Glu II, and/or extra toxic effects due to *N*B-DNJ glycolipid metabolism inhibition *[23]*. Taken together our results confirm that ER α–Glu II is essential for seedling development and EFR signalling, and that *At* Glu II *rsw3* may retain some residual activity.

In the *At uggt1-1* mutant, EFR signalling as measured by plant size is not significantly impaired, as reported in previous works for the *psl2-1*, *uggt-3* and *uggt-4* UGGT mutants *[8, 9]*. Yet, under our experimental conditions, both elicitors triggered a significant increase in the expression levels of both *PHI-1* and *RET-OX*, and only the concomitant application of *N*B-DNJ blocked completely the EFR signalling pathway (Fig. 6). These results indicate that EFR-mediated *PHI-1* and *RET-OX* upregulation depends on glycoproteins that can fold successfully thanks to mono-glucosylated glycans afforded by ER α–Glu II alone and thus, UGGT activity is not needed for some of the EFR response.

Finally, the observation that insertional mutants have been reported for *At* UGGT but not for ER α–Glu II indicate that UGGT activity modulation might have a less drastic impact on the glycoprotein folding machinery and ER homeostasis than ER α–Glu II deletion, boding well for future studies of UGGT modulation for the rescue of the secretion of misfolded but functional glycoprotein mutants in congenital protein folding diseases.

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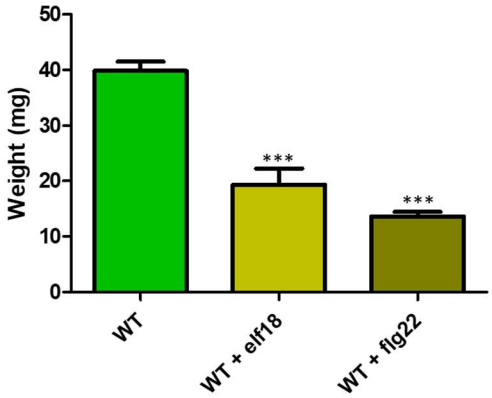
Supplementary information



Supplementary Figure 1. Effects of elicitors treatment on *At* Col0. 13-days-old seedlings control or treated with 100 nM elf18 or flg22 elicitors. Values represent the mean of the plants fresh-weight (expressed in mg) of at least three independent experiments (± s.e. n=10). Statistical analysis was determined by ANOVA with Bonferroni's test (*=P<0.5, **=P<0.01, ***=P<0.001).

452

GENE	AG CODE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
UBQ5	AT3G62250	GGAATCGACGCTTCATCTCG	ATGAAAGTCCCAGCTCCACA
PHI1	AT1G35140	TTGGTTTAGACGGGATGGTG	ACTCCAGTACAAGCCGATCC
RET-OX	AT1G26380	AGGTTCTCGAACCCTAACAACA	GCACAGACGACACGTAAGAAAG

453 **Supplementary Table 1.** Primers used in the qRT-PCR analysis showed in this work.

454

Mutant name	**Kind of mutation**	**Was it tested for elf18 response?**	**References**
psl5-1	S517F	Yes	*[28]*
rsw3	S599F	Yes	
psl4-1	T-DNA insertion	Yes	
psl4-2	W360stop	Yes	

455 **Supplementary Table 2.** Known *At* ER α–Glu II mutants.

456

457

Name of mutant	Kind of mutation	Was it tested for elf18 response?	References
uggt1	Aberrant mRNA splicing variant	No	*[8]*
uggt2	Aberrant mRNA splicing variant	No	
uggt3	W1523stop	Yes	
uggt4	T-DNA insertion in 16th exon	Yes	
uggt1-1	T-DNA insertion in 11th exon	No	*[10]*
uggt1-2	T-DNA insertion in 24th intron	No	
psl2-1	D1497N	Yes	*[9]*
psl2-2	E306K	No	
psl2-3	R1409K, W1443stop	No	
psl2-4	W1443stop	No	
psl2-5	Δ604-1613	No	

458 **Supplementary Table 3.** Known *At* UGGT mutants.