A new insight to explore the regulation between S-nitrosylation and N-glycosylation

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Abstract
Nitric oxide (NO) is a signal molecule in plants and animals. *Arabidopsis* GSNO reductase1 (AtGSNOR1) catalyzes metabolism of S-nitrosoglutathione (GSNO) which is a major biologically active NO species. The GSNOR1 loss-of-function mutant gsnor1-3 overaccumulates GSNO with inherent high S-nitrosylation level and resistance to the oxidative stress inducer paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride). Here, we report the characterization of dgl1-3 as a genetic suppressor of gsnor1-3. DGL1 encodes a subunit of the oligosaccharyltransferase (OST) complex which catalyzes the formation of N-glycosidic bonds in N-glycosylation. The fact that dgl1-3 repressed the paraquat resistance of gsnor1-3 meanwhile gsnor1-3 rescued the embryo-lethal and post-embryonic development defect of dgl1-3 reminded us the possibility that S-nitrosylation and N-glycosylation crosstalk with each other through co-substrates. By enriching glycoproteins in gsnor1-3 and mass spectrometry analysis, TGG2 (thioglucohydrolase2) was identified as one of co-substrates with high degradation rate and elevated N-glycosylation level in gsnor1-3 ost3/6. The S-nitrosylation and N-glycosylation profiles were also modified in dgl1-3 and gsnor1-3. Thereby, we propose a linkage between S-nitrosylation and N-glycosylation through co-substrates.

**KEYWORDS**
*Arabidopsis*, DGL1, gsnor1-3, N-glycosylation, S-nitrosylation, TGG2

1 | INTRODUCTION

S-nitrosylation is a crucial mechanism for the exertion of nitric oxide (NO) biological functions in animal and plants. During S-nitrosylation modification, NO molecules are covalently added to consensus cysteine residues flanked by acidic and basic amino acid residues of peptides forming endogenous S-nitrosothiols and modifying protein structures and functions (Greco et al., 2006; Seth & Stamler, 2011; Stamler, Toone, Lipton, & Sucher, 1997). In animals, three major isoforms of NO synthases (NOSs: iNOS, eNOS, nNOS) have been identified since 1989 (Alderton, Cooper, & Knowles, 2001) however not any NOS similar to that in animal models has been identified in plants, although the S-nitrosoglutathione reductase (GSNOR) has been characterized. GSNOR irreversibly catalyzes the metabolism of GSNO which is a major biological active form of NO to GSSG and NH3 as main products thus indirectly controls the S-nitrosylation level (Malik, Hussain, Yun, Spoel, & Loake, 2011). In *Arabidopsis*, mutations in the singled-copied GSNOR1 gene cause defects in development (Feechan et al., 2005; Kwon et al., 2012). The gsnor1-3 is a loss-of-function mutant in GSNOR1 gene with increased SNO level, accompanied by severe developmental defects such as semi-dwarf, bushy, and reduced fertility (Feechan et al., 2005; Kwon et al., 2012; Lee, Wie, Fernandez, Feeisch, & Vierling, 2008). In our previous study (Chen et al., 2009), we performed a genetic screen for the paraquat resistant mutant and characterized the *par2-1* mutant which was identical to *gsnor1-3/hot5*. Recent researches have shown
gsnor1-3 plays critical roles in studying NO function and GSNO1-regulated S-nitrosylation (Feng et al., 2013; Hu et al., 2015, 2017; Tada et al., 2008; Yang et al., 2015; Zhan et al., 2018). In order to further analyze the role of GSNO1 in Arabidopsis development, we performed another genetic screen for the suppressor of gsnor1-3 based on its paraquat resistance. Map-based clone revealed that the suppressor was a mutation in one subunit of the oligosaccharyltransferase (OST) complex which catalyzes the transfer of oligosaccharide onto a nascent protein in N-glycosylation.

N-glycosylation is a ubiquitous protein modification in eukaryotes, almost 70% of eukaryotic proteins are glycosylated (Mononen & Karjalainen, 1984). In secretory pathway, glycosylation is essential for proteins to be secreted or integrated in membranes. Nascent polypeptides in the endoplasmic reticulum (ER) lumen which are covalently attached with oligosaccharide to asparagine (Asn) side-chains will be folded into the native structures and delivered to the Golgi apparatus for further folding and conformational maturation, while underglycosylation will cause proteins misfolding and trigger ER stress, unfolded proteins response (UPR), and ER-associated degradation (ERAD) (Aebi, 2013; Lanno & Van Damme, 2015; Moremen, Tiemer, & Naïr, 2012). The ERAD system translocates misfolded proteins across the ER membrane into the cytosol where ubiquitin-conjugated enzymes target these misfolded proteins for degradation.

OST is a multi-subunit complex which catalyzes oligosaccharides attached to conserved asparagine residues in Asn-X-Ser/Thr (N-X-S/T) motif of polypeptides in N-glycosylation. At least seven subunits of OST have been reported in Arabidopsis such as STT3a, STT3b, OST3/6, DGL1, DAD1, DAD2, and HAP6 (Aebi, 2013; Farid et al., 2013; Mohorko, Glockshuber, & Aebi, 2011). These subunits are believed to exert distinct functions, for example DAD1 and DAD2 such as STT3a, STT3b, OST3/6, DGL1, DAD1, DAD2, and HAP6 (Aebi, 2013; Farid et al., 2013; Mohorko, Glockshuber, & Aebi, 2011). These subunits are believed to exert distinct functions, for example DAD1 and DAD2.

2 MATERIALS AND METHODS

2.1 Plant materials, growth conditions, and genetic screen for gsnor1-3 dgl1-3

Plants were grown under a 16 hr light/8 hr dark cycle or continuous white light (120–130 μmol m⁻² s⁻¹) at 22°C in soil or on a 1/2 MS medium containing 3% sucrose and 0.8% agar.

To screen gsnor1-3 dgl1-3 mutant, EMS-mutagenized M2 seeds based on gsnor1-3 in the Col-0 background were germinated and grown on 1/2 MS agar plates for 7 days then transferred to 1/2 MS with or without 0.1 μM paraquat for additional 5 days. In the first 7 days, the seedlings were grown on the vertically placed plates for elongation of roots, after transferring the seedlings were placed in the inverted orientation for bending roots. By comparing the relative bending roots lengths with and without paraquat treatment, paraquat hypersensitive mutant was identified. Genetic analyses were performed by pair-wise crossing of individual mutants followed by assessing segregation patterns in F1 and F2 generations.

2.2 Genetic mapping of dgl1-3

F2 seeds derived from crosses between gsnor1-3 -/ dgl1-3 -/ (Col-0) and gsnor1-3 -/ (Ler) were germinated on 1/2 MS medium for 7 days and seedlings with dark cotyledons were selected for genetic mapping. An F2 population of about 300 mutant seedlings was analyzed to define the dgl1-3 mutation between K1F13-2 and MSN2 (see Supporting information for sequences of primers). The sequences of dCAPS marker used to identify homozygous and heterozygous dgl1-3 were as follow (5’ to 3’): F: TTATAGTCATCTACTCAATGCAAGAA; R: TTTAGTTTACAGCGTCCCGTGGGCTTC.

2.3 Genetic complementation

A DGL1 (At5g66680) genomic DNA fragment of 4226-bp containing 3’-UTR was obtained by PCR from wild-type (Col-0) plants, verified
by sequencing and then cloned into the XhoI and SpeI sites of a binary vector pER8 to yield pER8-DGL1. The Flag or GFP sequence was linked to the C or N terminal of DGL1 in a head-to-tail configuration. The constructed vector was transformed into both dgl1-3+/− and gsnor1-3−/+ plants by floral dipping and the plants with the homogeneous dgl1-3 mutation background in T3 generation were isolated by sequencing the PCR products amplified by primers anchored between the mutated site and 5′-UTR.

2.4 | Analysis of trypan blue and DAB staining

For trypan blue staining, the seedlings were stained with lactophenol-trypan blue (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 10 mg of trypan blue, dissolved in 10 ml of distilled water) (Keogh, Deverall, & McLeod, 1980). The whole seedlings were boiled for approximately 1 min in the stain solution and then decolorized in chloral hydrate (2.5 g of chloral hydrate dissolved in 1 ml of distilled water) for at least 30 min. The seedlings were viewed and photographed under a stereoscope.

DAB(Beyotime, Cat#: ST003) was dissolved into a 50 mM Tris-HCl, pH 3.8 solution with a concentration of 1 mg/ml. The seedlings were submersed into the DAB solution in dark for 5–6 hr then dehydrated in 95% ethanol (Yokawa, Kagenishi, Kawano, Mancuso, & Baluška, 2011).

2.4.1 | Glycoprotein enrichment

Plant material was homogenized in liquid nitrogen and extracted in extraction buffer (20 mM Tris-HCl, pH 7.0 and 20 mM β-mercaptoethanol). Homogenate was filtered through Miracloth and centrifuged at 10,000 rpm for 10 min. Proteins in cleared extracts were precipitated by 80% saturation of ammonium sulfate and centrifuged in column buffer (20 mM Tris-HCl, pH 7.0, and 500 mM NaCl), centrifuged, and loaded onto Con A affinity columns (Chen et al., 2009).

2.4.2 | Mass spectrometry analysis

The enriched glycoproteins in gels were digested by trypsin (0.01 mg/ml; Promega, Cat #: V5111) (Hu et al., 2015). The digested peptides were sent to National Center for Protein Sciences (Beijing) (http://www.phoenix-center.cn) for analyzing by mass spectrometer (Thermo Q Exactive, USA).

Raw data were used for a search against Arabidopsis protein database (www.ncbi.nlm.nih.gov/guide/proteins/; Version, Aug 8, 2018) with the taxonomy restriction to "Arabidopsis thaliana". The BioWorks TurboSequest software was used for the database searching using the following parameters: the mass tolerances for peptides and fragment ions were set to 0.5 Da; (FDR) < 0.05; PSM FDR, Protein FDR, and Site FDR were set under 0.01; minima peptide length was 6; top MS/MS peaks per 100 Da (TOF) was 10.

2.4.3 | Biotin-switch and Western blot assay

S-nitrosylated proteins were analyzed by the biotin-switch assay as described previously (Feng et al., 2013; Yang et al., 2015).

For Western blot analysis, 7-day-old seedlings were homogenized in a denaturing buffer [20 mM Tris-HCl, pH 6.8, 0.3% β-mercaptoethanol, 5% (v/v) glycerol, and 1% (w/v) SDS]. After boiling for 5 min, the proteins were separated by SDS-PAGE in 8% or 15% polyacrylamide gels. Polypeptides were then transferred onto a PVDF membrane. For immunodetection, PVDF membranes were probed with HRP antibody (GenScript, Cat#: A00619) raised against β(1,2)-xylose and α(1,3)-fucosyl N-glycan residues (Lerouxel et al., 2005).

3 | RESULTS

3.1 | Genetic screen for the suppressor of gsnor1-3

Paraquat (PQ) is a kind of nonselective herbicide which has an efficient inducer of cell death in animal and plant cells (Suntres, 2002). In our previous research, we got a par2-1 mutant with strong paraquat resistance which is allelic to gsnor1-3/hot5 (Chen et al., 2009). The gsnor1-3/hot5/par2-1 mutant showed anti-cell death, reduced fertility and heat acclimation phenotypes. We hypothesized that mutations render gsnor1-3 sensitive to paraquat may represent important genetic loci that are involved in regulation of cell death or NO metabolism. Therefore, we carried out a genetic screen for paraquat sensitive mutants by surveying ethylmethane sulfonate (EMS) generated library based on gsnor1-3. As the mutants after paraquat treatment should be feeble and require to recover, a low concentration of paraquat and delicate method were used for screening the mutants (Figure S1, Supporting Information). About 8,000 lines of seeds in the library were germinated on 1/2 MS medium vertically for 7 days then transferred to 1/2 MS medium with or without 0.1 μM paraquat and invertedly placed and cultured for additional 5 days. The bent roots lengths of seedlings grown on mediums were measured and the relative root lengths were calculated with roots grown on 1/2 MS medium without PQ as controls. After screening, we got a double mutant named gsnor1-3 dgl1-3 based on the gene cloned in the mutant (Figure 1a,b). Indeed, gsnor1-3 dgl1-3 was so tender that high humidity or pests in greenhouse caused plant death easily. Therefore we crossed gsnor1-3−/− dgl1-3−/− background plants (developed normally) selected from original M2 population with gsnor1-3−/− and Col-0 separately to obtain gsnor1-3−/− dgl1-3−/− and dgl1-3−/− mutants in F2 populations.

3.2 | The phenotypes of dgl1-3 and gsnor1-3 dgl1-3 mutants

Besides the sensitivity to paraquat, the cotyledons of both dgl1-3 and gsnor1-3 dgl1-3 were dark, suggesting accumulation of
anthocyanin (Figure 1c). Trypan blue staining showed that the cell death level was much higher in dgl1-3 than in gsnor1-3 dgl1-3 (Figure 1d). The hydrogen peroxide level of dgl1-3 was also depressed in gsnor1-3 dgl1-3 detected by 3,3-diaminobenzidine (DAB) staining (Figure 1e). After approximately 12 days of culture on 1/2 MS medium dgl1-3 was dead indicating a post-embryonic development cease phenotype as reported by Lerouxel et al. (2005), while gsnor1-3 dgl1-3 survived and bloomed, and few seeds were obtained (Figure 1f). The phenotype of gsnor1-3 dgl1-3 double mutant still looks like gsnor1-3 with dwarf and fertility defective but fewer branches (Figure 1g).

Among the F2 population of self-pollinated gsnor1-3 dgl1-3/gnor1-3 dgl1-3−/− seedlings, the dark cotyledons phenotype was segregated in a 1:3 ratio (dark: green = 34: 125, $\chi^2 = 1.1$), indicating that the mutation is recessive in a single nuclear gene. But the F2 of self-pollinated dgl1-3−/− segregated dark cotyledons seedlings less than 25% of total, fluctuated from 8% to 15%. In fact, when we used gsnor1-3−/− dgl1-3−/− (Col-0 background) to cross gsnor1-3−/− (Landsberg erecta background) to generate F2 population for mapping, we noticed that the segregation ratio was 1:3 in gsnor1-3−/− background population but in other background populations the number of dark cotyledons seedlings was less than that in theory. Thus the dgl1-3 mutant was embryo lethal as reported (Lerouxel et al., 2005) while gsnor1-3 rescued the defect. The phenotypes of dgl1-3−/− and gsnor1-3−/− indicated an interactive genetic regulation between S-nitrosylation and N-glycosylation.
3.3 Molecular cloning of DGL1

Using the gsnor1-3−/− background seedlings with dark cotyledons in the F2 population obtained from hybridization of gsnor1-3−/− dgl1-3−/− (Columbia) × gsnor1-3−/− (Landsberg erecta), we mapped the mutation on chromosomes V. By monitoring genetic recombination in a population of about 300 mutant seedlings, we located dgl1-3 in a ~70 kb region containing 20 open reading frames. DNA sequencing analysis of all 20 genes revealed a mutation in At5g66680, characterized as a G-to-A transition in exon 4, which converts a glycine (G) into an arginine acid (R) at residue 186 (Figure 2a,b). This glycine residue is highly conserved in related proteins across plants and animals (Figure 2b). According to the nucleic acid transition of G to A, we designed a dCAPS marker to distinguish homozygous and heterozygous dgl1-3 mutant (Figure 2c).

To verify the identity of the candidate DGL1 gene, we performed a genetic complementation experiment. A 3 × Flag or GFP tag sequence was connected with a 4226-bp wild-type genomic DNA fragment which contains the putative promoter region, 5'-untranslated region (UTR), coding sequence of At5g66680, and was cloned into a binary vector. The resulting construct was transformed into dgl1-3−/− and gsnor1-3−/− dgl1-3−/− background plants.
by floral dipping. Among the analyzed transgenic lines in T3 generation with homogenous dgl1-3 mutation, all seedlings showed a phenotype similar to that of gsnor1-3 or Col-0 (Figure 2d). But the Flag or GFP tag in these transgenic lines could not be detected by Western blot, neither of the tags were linked to the C or N terminal while the integration of DGL1 into the genome was confirmed by conventional PCR analyses (Figure S2, Supporting Information).

Previous researches have reported that human OST48 is allelic to DGL1 and consists of 456 residues with the first 42 residues in N terminal including a signal sequence meanwhile the nine residues in the C terminal constitutes a cytosolic segment which interacts with DAD1 (Fu, Ren, & Kreibich, 1997; Mohorko et al., 2011). Pig OST48 contains a double lysine motif at the very C terminus which was suggested to confer ER residency (Hardt, Aparicio, & Bause, 2000; Hardt, Aparicio, Breuer, & Bause, 2001; Mohorko et al., 2011). Because of the homolog in function and structure between DGL1 and OST48, we believed some special structure or functions might be possessed by both terminals of DGL1 and the tags in our transgenic lines possibly be sheared before protein maturation.

3.4 | The gsnor1-3 mutation endows OST mutants with tunicamycin resistance

DGL1 encodes a critical subunit of the OST complex which catalyzes transfer of oligosaccharide onto nascent peptides in N-glycosylation. The mutation of DGL1 causes the underglycosylation of proteins. Immunoblot analysis using a Horseradish Peroxidase (HRP) antibody specific for α(1,3)-fucose and β(1,2)-xylose N-linked glycan epitopes showed different profiles in dgl1-3 and gsnor1-3 dgl1-3 (Figure 3a left, arrowheads). Actually, the bands which represented glycoproteins were hardly to be detected in dgl1-3 by HRP antibody indicating a mess of glycosylation in dgl1-3 which was recovered in gsnor1-3 dgl1-3.
The OST complex consists of at least eight subunits in Arabidopsis. We crossed stt3b (SALK_134449C) and ost3/6 (SALK_067271C) with gsnor1-3. Unfortunately the seeds of dgl1 SALK lines obtained from donors were inactive or without mutation. All those related mutations and Col-0 were also detected by immunoblot analysis with the HRP antibody. The immunoblot showed that the profiles of glycoproteins in gsnor1-3 were differently expressed to that in Col-0; meanwhile the depression of glycosylation in stt3b and ost3/6 were highly elevated in double mutants (Figure 3a right, arrowheads).

Tunicamycin (TM) is a Streptomyces-derived inhibitor of eukaryotic protein N-glycosylation and also an inducer of endoplasmic reticulum (ER) stress. Plants treated with TM will accumulate unglycosylated, misfolded proteins in ER. We tested if gsnor1-3 endowed the stt3b and ost mutants resistance to TM. An experiment similar to screening for the gsnor1-3 suppressor was carried out but TM inhibition was so strong that the bending roots were ceased to elongate, thus we measured the fresh weight of per plant (Figure 3b). The relative fresh weight of gsnor1-3 after TM treatment was higher than that of Col-0 indicating a strong resistance to TM. Similarly all the gsnor1-3 ost double mutants showed higher resistance than relative ost single mutants (Figure 3c). From these experiments we have reasons to believe that the elevated S-nitrosylation of proteins promoted N-glycosylation.

3.5 The S-nitrosylation pattern was strongly changed in gsnor1-3 dgl1-3

Using biotin-switch analysis, we checked the S-nitrosylation patterns in relative mutants. In line with previous research, gsnor1-3 had much more bands representing nitrosylated proteins, but here we cannot tell the level difference of S-nitrosylation between gsnor1-3 and gsnor1-3 dgl1-3 as some bands in gsnor1-3 dgl1-3 were missing while some bands were aggravated. However, the profiles of S-nitrosylation were apparently different between gsnor1-3 and gsnor1-3 dgl1-3 (Figure 4a).

We checked the parquat sensitivity of gsnor1-3 ost double mutants by measuring the relative bending root lengths of 7-day-old seedlings transferred to 1/2 MS mediums containing 0.1 μM paraquat. The gsnor1-3 ost3/6 double mutant showed a hypersensitivity to paraquat while gsnor1-3 stt3b still had a strong resistance...
cued the embryo lethal phenotype of N-sylation and some co-Sgsnor1-3 glycoprotein list of Arabidopsis were analyzed by mass spectrometry and cross referenced to the Col-0 (Con A) to enrich distinct glycoproteins in DGL1 substrates. To identify the co-substrates, the concanavalin A (Con A)-conjugated agarose beads were used in immunoprecipitation (IP) to enrich distinct glycoproteins in dgl1-3 and gsnor1-3 mutants that had the same molecular weight as that in Col-0 (Figure 5a, arrowheads). The proteins between 40 and 55 kDa potential target of DGL1 functions in binding lipid substrates. To identify the co-substrates, the concanavalin A (Con A)-conjugated agarose beads were used in immunoprecipitation (IP) to enrich distinct glycoproteins in dgl1-3 and gsnor1-3. On the basis of these phenomena we suspected that S-nitrosylation and N-glycosylation crosstalks with each other through some co-substrates. To identify the co-substrates, the concanavalin A (Con A)-conjugated agarose beads were used in immunoprecipitation (IP) to enrich distinct glycoproteins in gsnor1-3 compared to that in Col-0 (Figure 5a, arrowheads). The proteins between 40 and 55 kDa were analyzed by mass spectrometry and cross referenced to the glycoprotein list of Arabidopsis reported in the N-glycoproteomes research (Zielinska Dorota, Gnad, Schropp, Wiśniewski Jacek, & Mann, 2012). A total of 116 glycoproteins were identified and 22 proteins were also found in the list of S-nitrosylated proteins reported in the Arabidopsis nitrosoproteomic analysis (Hu et al., 2015). Interestingly, nine out of 116 proteins related to unfolded protein reaction (UPR) such as CRT1, CRT3, PDILs were also identified (Table S1, Supporting Information). We picked TGG2 protein as a target for forward research because it had been proved to be a glycoprotein and the glycosylated and unglycosylated TGG2 proteins were easy to distinguish on a denaturating polyacrylamide gel by Western blot (Liebming et al., 2012; Ueda et al., 2006). Using chemically synthesized antigen (sequence: AHALDPSPPEKLT) (Ueda et al., 2006), we prepared the antibody of TGG2.

As glycoproteins in underglycosylated mutants were fast degraded for misfolding through ERAD pathway, TGG2 protein volume in ost3/6 was lower with higher electrophoretic mobility compared to that in Col-0. However, no difference of TGG2 was observed between stt3b and Col-0 suggesting that stt3b was a weak underglycosylation mutant which was coincident with the paraquat resistant phenotype (Figure 4b,c). TGG2 in dgl1-3 was suspected to be degraded to low molecular weight fragments (arrowheads) which were completely degraded in gsnor1-3 dgl1-3. In addition, TGG2 is a potential target of S-nitrosylation which degrades faster in gsnor1-3, it is possible that S-nitrosylation promoted TGG2 degradation. As one N-glycan side chain was removed from a glycoprotein, the protein molecular weight would minus 1 kDa, when comparing the electrophoretic mobility of TGG2 in ost3/6, gsnor1-3 stt3b, and gsnor1-3 ost3/6, TGG2 was obviously glycosylated in gsnor1-3 ost3/6 which had the same molecular weight as that in gsnor1-3 stt3b intimating that S-nitrosylation promotes N-glycosylation of TGG2 and a crosstalk between S-nitrosylation and N-glycosylation involving co-substrates.

4 | DISCUSSION

In this study, by the isolation of paraquat sensitive mutant under gsnor1-3 background, we characterized a new mutation on DGL1 nominated as dgl1-3. Genetic and biochemical experiments showed that dgl1-3 suppressed the bushy phenotype and changed the S-nitrosylation pattern of gsnor1-3. As one important subunit of OST complex, it is believed that DGL1 functions in binding lipid-linked oligosaccharide donor substrates (Pathak et al., 1995). The
dysfunction of DGL1 in Arabidopsis (dgl1-1and 2) causes severe development defects, such as embryo lethality, reduced cell elongation, and post-embryonic development cease (Lerouxel et al., 2005), while rice Osdgl1 mutant exhibited shorter root length, smaller root meristem, and cell death in the root (Qin et al., 2013). Here, we also observed that dgl1-3 was embryo lethal like dgl1-2 and ceased post-embryonic development as dgl1-1. Furthermore, the cell death level in the cotyledons of dgl1-3 was elevated. Both dgl1-1 and 2 are T-DNA insertions in the promoter region while Osdgl1 is a point mutation resulting in premature termination of protein synthesis. Using total cDNA of Col-0 and dgl1-3 as PCR templates and primers for the full length of DGL1 (1,314 bp), we amplified the same length of bands, and by qRT-PCR the expression of DGL1 in Col-0 and dgl1-3 were at the same level (data not shown). Therefore, the development defects of dgl1-3 must be caused by dysfunction of DGL1 protein and the Gly186 is of great importance.

By segregation ratio assay of F2 population from different backgrounds, we further observed that gsnor1-3 rescued the embryo lethality phenotype of dgl1-3. Furthermore, gsnor1-3 dgl1-3 double mutant was reproductive meaning gsnor1-3 rescued the post-embryo cease phenotype of dgl1-3. DGL1 has two cysteine residues, by assuming that these cysteine residues were S-nitrosylated and transforming cysteine to serine mutated DGL1 to dgl1-3 background, the transformed plant developed normally (data not shown). Therefore, it is impossible for S-nitrosylation rescuing the dysfunction of DGL1, not to mention that DGL1 is just a subunit of OST complex and its S-nitrosylation is hardly to influence the enzyme activity of OST during the N-glycosylation reaction in gsnor1-3 dgl1-3.

Both S-nitrosylation and N-glycosylation are post-translational modifications with enormous amount of substrates. As the S-nitrosylation and N-glycosylation patterns were changed in dgl1-3 and gsnor1-3, the gsnor1-3 dgl1-3 also showed the intermediate phenotypes, we hypothesized that S-nitrosylation and N-glycosylation might crosstalk with each other through some common structures. As the S-nitrosylation happen even before the nascent glycopeptide is translated by membrane-anchored OST complex. Dose S-nitrosylation happen even before N-glycosylation? It is mysterious to answer this question.

In our previous work, we assumed that PAR2/GSNOR1/HOT5 functions downstream of superoxide to regulate cell death (Chen et al., 2009). Here, because H2O2 as a superoxide in dgl1-3 was depressed in gsnor1-3 dgl1-3, it seems our assumption was reasonable.

5 | CONCLUSION

By characterizing the suppressor of gsnor1-3, we identified a new point mutation on the DGL1 gene which genetically repressed the paraquat resistance of gsnor1-3. We also presented genetic and biochemical evidences to shed light on the crosstalk between S-nitrosylation and N-glycosylation based on co-substrates. As TGG2 was one of the co-substrates of S-nitrosylation and N-glycosylation, the elevated S-nitrosylation level rescued its underglycosylation in gsnor1-3 ost3/6 double mutant. We hypothesized that the transformation
between S-nitrosothiols and disulfides bracketing N-glycosylation sites regulated the crosstalk between S-nitrosylation and N-glycosylation, but further investigation is needed.

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AUTHOR CONTRIBUTIONS

J.Z. designed the study; H.D. performed the experiments, data analysis, and wrote the article; L.C. constructed the EMS-mutant library; N.Z., J.M., and B.R. provided necessary assistance in the study.

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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