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ORIGINAL RESEARCH

Glutaredoxin *AtGRXS8* represses transcriptional and developmental responses to nitrate in *Arabidopsis thaliana* roots

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Abstract

Glutaredoxins (GRXs) are small oxidoreductase enzymes that can reduce disulfide bonds in target proteins. The class III GRX gene family is unique to land plants, and *Arabidopsis thaliana* has 21 class III GRXs, which remain largely uncharacterized. About 80% of *A. thaliana* class III GRXs are transcriptionally regulated by nitrate, and several recent studies have suggested roles for these GRXs in nitrogen signaling. Our objective was to functionally characterize two nitrate-induced GRX genes, *AtGRXS5* and *AtGRXS8*, defining their roles in signaling and development in the *A. thaliana* root. We demonstrated that *AtGRXS5* and *AtGRXS8* are primarily expressed in root and shoot vasculature (phloem), and that the corresponding GRX proteins display nucleocytosolic subcellular localization. Ectopic expression of *AtGRXS8* in transgenic plants caused major alterations in root system architecture: Normal primary root development, but a near absence of lateral roots. RNA sequencing demonstrated that the roots of *AtGRXS8*-overexpressing plants show strongly reduced transcript abundance for many primary nitrate response genes, including the major high-affinity nitrate transporters. Correspondingly, high-affinity nitrate uptake and the transport of nitrate from roots to shoots are compromised in *AtGRXS8*-overexpressing plants. Finally, we demonstrated that the AtGRXS8 protein can physically interact with the TGA1 and TGA4 transcription factors, which are central regulators of early transcriptional responses to nitrate in *A. thaliana* roots. Overall, these results suggest that *AtGRXS8* acts to quench both transcriptional and developmental aspects of primary nitrate response, potentially by interfering with the activity of the TGA1 and TGA4 transcription factors.

KEYWORDS

Arabidopsis thaliana, glutaredoxin, nitrate, root development, signaling

Ahmad Ehrary, Miguel Rosas contributed equally to this work.

1 | INTRODUCTION

Glutaredoxins (GRXs) are small disulfide oxidoreductase enzymes found in almost all living organisms (Alves, Vilaprinyo, Sorribas, & Herrero, 2009). There are three major classes of GRXs, as defined by the sequence of amino acids in their active site motifs: Class I (CGFS-type), Class II (CPYC-type), and Class III (CC-type). Class I and class II GRXs have been relatively well studied in plants and are primarily involved in oxidative stress response, maintenance of redox homeostasis, and iron-sulfur cluster assembly (Rouhier, 2010; Ströher & Millar, 2012; Yu et al., 2017). Class III GRXs are exclusively found in land plants, and in contrast with other GRXs, it is not clear whether they are enzymatically active in thiol-disulfide exchange reactions (Couturier, Didierjean, Jacquot, & Rouhier, 2010). The class III GRX gene family has expanded substantially in higher plants—for example, *Arabidopsis thaliana* has 21 class III GRXs, six class II GRXs, and five class I GRXs (Gutsche, Thurow, Zachgo, & Gatz, 2015; Ziemann, Bhave, & Zachgo, 2009). Functional characterization of most class III GRXs is still in its infancy.

The class III GRX gene family has been best studied in *A. thaliana*, with several functional links established to plant development and stress responses. For example, *AtGRXC7* (*ROXY1*) is required for petal development (Xing, Rosso, & Zachgo, 2005), while *AtGRXC7* and *AtGRXC8* (*ROXY2*) play redundant roles in another development (Xing & Zachgo, 2008). In terms of biotic stress, *AtGRXS13* (*ROXY18*) and *AtGRXC9* (*ROXY19*) are linked to susceptibility to the fungal pathogen *Botrytis cinerea* and are also involved in jasmonic acid and salicylic acid signaling (La Camera et al., 2011; Lai et al., 2014; Ndamukong et al., 2007). *AtGRXS13* limits oxidative damage caused by high light stress (Laporte et al., 2012). In most of the studies described above, class III GRXs were suggested to mediate their biological effects through protein:protein interactions with one or more members of the TGA transcription factor family (Gutsche et al., 2015). Many GRX:TGA protein interactions appear to be ubiquitous and may be controlled primarily by partner availability through overlapping expression domains (Zander, Chen, Imkampe, Thurow, & Gatz, 2012).

Several recent studies have provided direct or indirect evidence linking class III GRXs to nitrogen response. Strikingly, 80% of the class III GRXs are transcriptionally regulated by nitrate availability, with 11 members of the gene family upregulated by nitrate and six members of the gene family downregulated by nitrate (Table S1) (Jung, Ahn, & Schachtman, 2018; Patterson et al., 2016). Three of the nitrate-repressed GRX genes, *AtGRXS11* (*ROXY6/CEPD1*), *AtGRXC13* (*ROXY9/CEPD2*), and *AtGRXC14* (*ROXY8/CEPDL2*) encode proteins that act as mobile signals of nitrogen starvation, moving from the shoots to the roots of nitrogen starved plants, and activating high-affinity nitrate transport (Ohkubo, Tanaka, Tabata, Ogawa-Ohnishi, & Matsubayashi, 2017; Ota, Ohkubo, Yamashita, Ogawa-Ohnishi, & Matsubayashi, 2020). For the nitrate-induced GRXs, it has been shown that the tandemly arranged glutaredoxin gene cluster *AtGRXS3/4/5/7/8* (*ROXY11-15*) acts to negatively regulate primary root growth in response to nitrate availability (Patterson et al., 2016). The objective of the current study was to further characterize the functional roles of class III GRXs in nitrate response, with a specific focus on *AtGRXS5* and *AtGRXS8*. We found that *AtGRXS8* acts as a negative regulator of the primary transcriptional response to nitrate and suppresses lateral root outgrowth, thus acting to limit or quench primary molecular and developmental responses to nitrate availability.

2 | MATERIALS AND METHODS

2.1 | Plant growth and root growth assays

A. thaliana ecotype Columbia-0 was used as the wild type in all described studies. Plants were grown in a controlled environment growth chamber with a 16 hr light period at 22°C and ~100 µmol/m^2 s^{-1} illumination and an 8 hr dark period at 18°C. Seed stratification was performed for 2–3 days at 4°C. For plant growth on soil, Sungro Sunshine Mix #1 was used. For root growth assays, seeds were initially germinated on plates containing half-strength Murashige and Skoog medium, 1% sucrose, pH 6.0. After 5 days, seedlings were transferred to vertically oriented plates containing Somerville and Ogren medium (5 mM KNO_3, 2.5 mM KH_2PO_4 [pH5.6], 2 mM $MgSO_4$, 2 mM $Ca[NO_3]_2$, 50 µM Fe-EDTA, and the reported micronutrient mix) (Somerville & Ogren, 1982) with no exogenous carbon source. To analyze root system architecture, plates were scanned using an Epson V600 flatbed scanner after 6–7 days of vertical growth (11- to 12-day-old plants), and images were analyzed using the EZ-Root-VIS software pipeline (Shahzad et al., 2018).

2.2 | DNA constructs and plant transformation

The coding sequences of *AtGRXS5* and *AtGRXS8* (309 bp each) and the promoters of *AtGRXS5* and *AtGRXS8* (2000 bp directly upstream of the GRX gene start codon) were PCR amplified and cloned into the pENTR/D-TOPO vector (Invitrogen). PCR primers used for amplification are listed in Table S4. After sequence verification by Sanger sequencing, Gateway cloning was used to insert the gene coding sequences into the pEarleygate100 vector (35Spro::GRX), the pEarleygate104 vector (35Spro::YFP-GRX), and the pGWB433 vector (GRXpro::GUS) (Earley et al., 2006; Nakagawa et al., 2007). Sequence-verified binary vectors were electroporated into *Agrobacterium tumefaciens* strain EHA101 or EHA105, and *A. thaliana* plants were transformed by floral dip (Clough & Bent, 1998). Transgenic plants were selected on half-strength Murashige and Skoog medium containing 50 mg/L kanamycin (pGWB433-derived vectors) or on soil by spraying seedlings with a 300 µM glufosinate ammonium solution (pEarleygate-derived vectors). For subcellular protein localization (Figure 2), the *Nicotiana benthamiana* leaves were agroinfiltrated with *A. tumefaciens* EHA105 pEarleygate104-*AtGRXS5* (35S::*YFP-AtGRXS5*) or *A. tumefaciens* EHA105 pEarleygate104-*AtGRXS8* (35S::*YFP-AtGRXS8*), using previously described

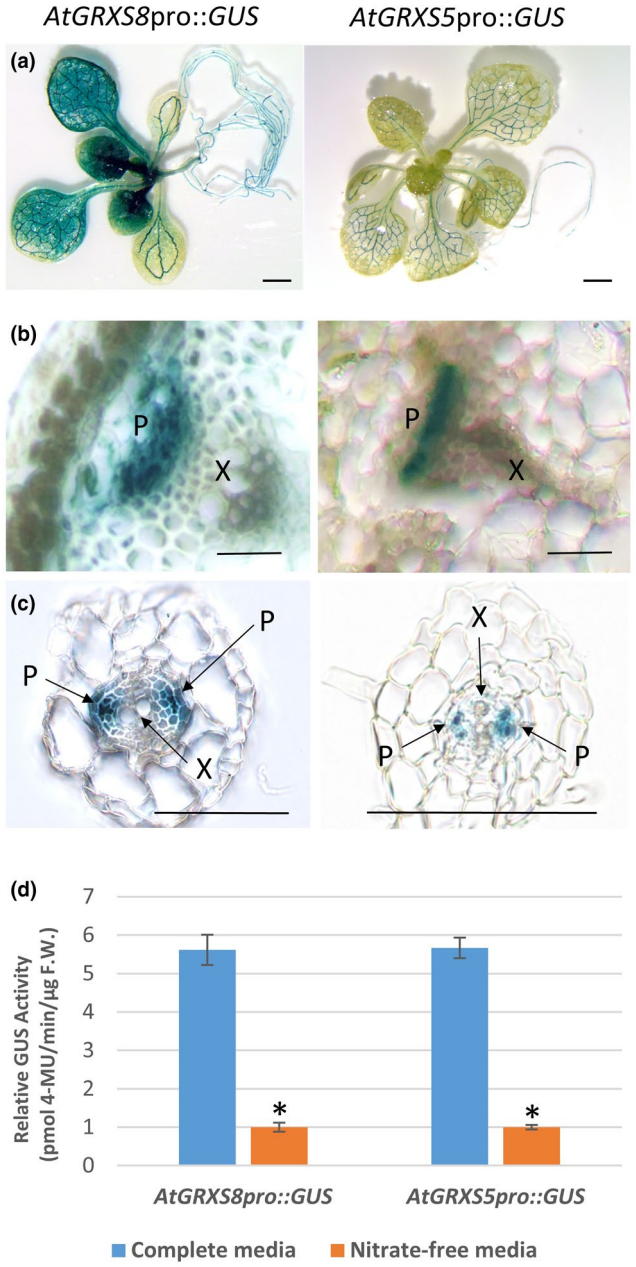

FIGURE 1 Expression domains of *AtGRXS5* and *AtGRXS8*. (a) Histochemical GUS staining of 14-day-old seedlings expressing *AtGRXS8*promoter::GUS (left) and *AtGRXS5*promoter::GUS (right). Scale bars = 1 mm. (b) GUS-stained cross-sections of stem (bolt) vascular bundles from soil-grown plants expressing *AtGRXS8*promoter::GUS (left) and *AtGRXS5*promoter::GUS (right). Scale bars = 40 µm. X = xylem, P = phloem. (c) GUS-stained cross-sections of primary roots from plants expressing *AtGRXS8*promoter::GUS (left) and *AtGRXS5*promoter::GUS (right). Scale bars = 100 µm. X = xylem, P = phloem. (d) Fluorometric GUS assays performed on total protein extracts from 5-day-old seedlings expressing *AtGRXS8*promoter::GUS and *AtGRXS5*promoter::GUS. Seedlings were grown on either nitrate-replete complete media or nitrate-free media. Values were normalized to set GUS activity on nitrate-free media equal to 1.0. Averages ± *SEM* are shown, $N \geq 3$. An asterisk indicates a significant difference ($p \leq .05$) in GUS activity, as determined by Student's *t* test

methods (Zhao, Tan, Wen, & Wang, 2017). Two days after agroinfiltration, leaves were imaged with a Leica SP5 confocal microscope using a 20X water immersion objective. A 514 nm argon laser was used for excitation, and YFP emission data were collected from 520 to 550 nm.

2.3 | RNA analyses

For routine screening of target gene expression in transgenic lines (e.g. Figure 3), ~100 seedlings were grown in shaking liquid cultures for 9 days prior to RNA isolation, as previously described (Escobar,

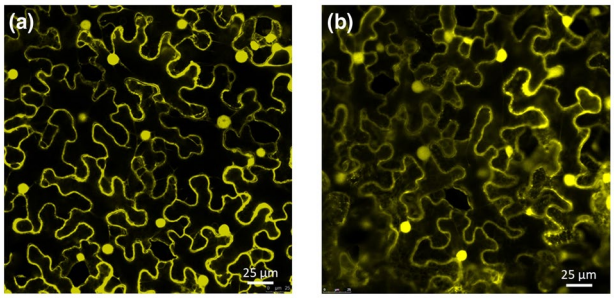

FIGURE 2 Subcellular localization of the AtGRXS5 and AtGRXS8 proteins. (a) Confocal micrograph of epidermal cells from *Nicotiana benthamiana* leaves Agroinfiltrated with a 35Spro::*YFP-AtGRXS5* construct. (b) Confocal micrograph of epidermal cells from *N. benthamiana* leaves Agroinfiltrated with a 35Spro::*YFP-AtGRXS8* construct. Scale bars = 25 µm

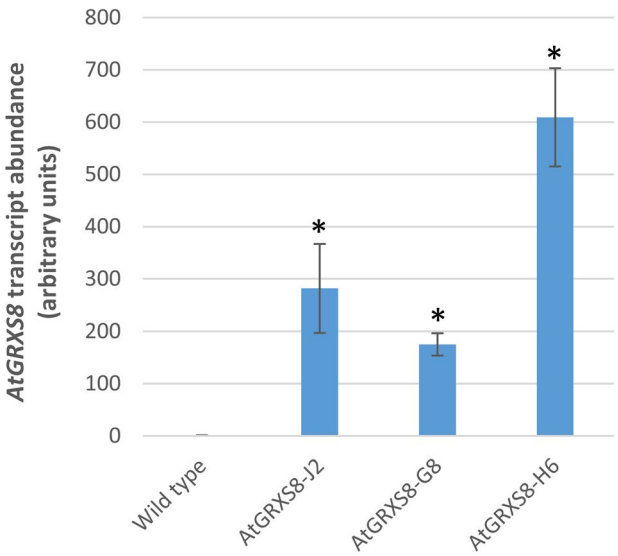

FIGURE 3 Overexpression of *AtGRXS8*. Transcript levels of *AtGRXS8* were quantified via real-time RT-PCR in 9 day old seedlings grown in liquid culture. Values were normalized to set *AtGRXS8* transcript abundance in wild type (Columbia-0) equal to 1.0. Averages ± *SEM* are shown, $N \geq 3$. An asterisk indicates a significant difference ($p \leq .05$) in *AtGRXS8* transcript abundance compared to wild-type, as determined by Student's *t* test

Geisler, & Rasmusson, 2006). Alternatively, plants were grown on vertically oriented Somerville and Ogren media (exactly as described for root growth assays, above), and RNA was isolated from the roots of 11-day old plants (RNA-seq analysis) or 6-day-old plants (Figure 5).

For RNA-seq analysis, total RNA was isolated from the seedling roots using an RNeasy Plant Kit (Qiagen). There were four independent biological replicates for wild-type plants and four independent biological replicates for transgenic line H6. RNA quality was assessed using an RNA 6000 Nano Assay on a Bioanalyzer 2100 (Agilent). All RIN values were above 9.0. RNA-seq libraries were created using the NEBNext Ultra II Directional Library Prep Kit (New England Biolabs), and library quality was assessed via Bioanalyzer analysis (High Sensitivity DNA Kit, Agilent). A 1 × 75 nt DNA sequencing reaction was carried out using an Illumina NextSeq500, generating ~50 million reads per sample. FASTQ files were trimmed and aligned using the systemPipeR RNA-seq pipeline (Backman & Girke, 2016), and edgeR (Robinson, McCarthy, & Smyth, 2010) was used to identify differentially expressed genes. Differential expression was defined by a false discovery rate ≤0.01 and a fold change ≥2.0. GO term enrichment (Table 1) was performed using Panther

TABLE 1 Ectopic expression of *AtGRXS8* alters specific functional categories in the root transcriptome

GO category (biological process)[a]	Fold enrichment	False discovery rate
Decreased in *AtGRXS8* overexpression line		
Regulation of amino acid export	32.5	3.84E-03
Leucine biosynthetic process	19	3.22E-03
Glucosinolate biosynthetic process	13.5	1.78E-05
Response to nitrate	11.4	1.35E-03
Nitrate transport	10	6.18E-03
Ion homeostasis	3.2	7.43E-03
Response to light stimulus	2.4	3.96E-03
Response to hormone	1.9	4.32E-03
Increased in *AtGRXS8* overexpression line		
Indole glucosinolate metabolic process	11	8.36E−03
Drug transmembrane transport	6.9	8.23E−03
Response to wounding	4.4	2.27E−03
Defense response to bacterium	3.5	6.05E−04
Oxidation-reduction process	2.3	3.32E−05
Defense response	2.2	7.03E−04
Response to hormone	2	2.45E−03
Response to stress	1.8	2.55E−05

[a]Only top-level categories with false discovery rate ≤ 0.01 are shown.

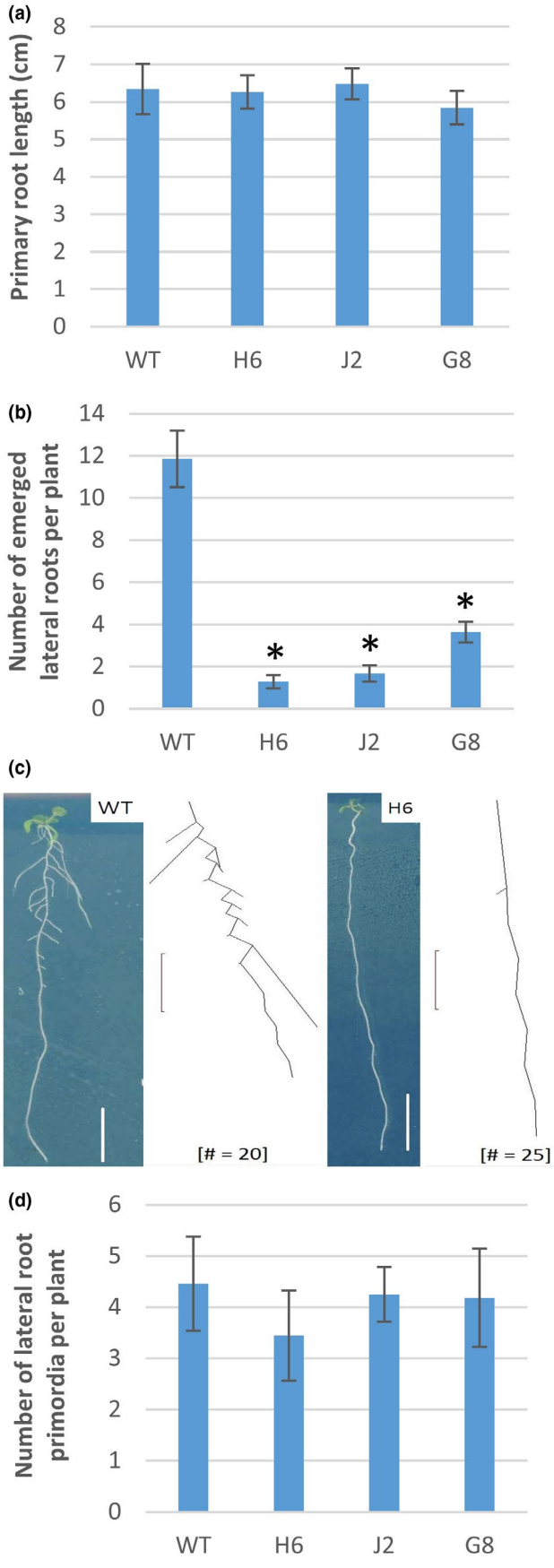

FIGURE 4 Root system phenotypes in transgenic plant lines overexpressing *AtGRXS8* (lines H6, J2, G8). (a) Primary root lengths of 12-day-old seedlings grown on vertically oriented media. Averages ± *SEM* are shown, $N \geq 20$. (b) Number of lateral roots in 12-day-old seedlings grown on vertically oriented media. Averages ± *SEM* are shown, $N \geq 20$. An asterisk indicates a significant difference ($p \leq .05$) compared with wild-type, as determined by Student's *t* test. (c) Representative images of 12-day-old wild-type (WT) and transgenic line H6 seedlings. Corresponding line drawings represent binned average root system architectures from 20 to 25 seedlings (Shahzad et al., 2018). Scale bars = 1 cm. (d) Number of lateral root primordia (stages IV–VIII; Péret et al., 2009) in 12-day-old seedlings grown on vertically oriented media. Averages ± *SEM* are shown, $N \geq 9$

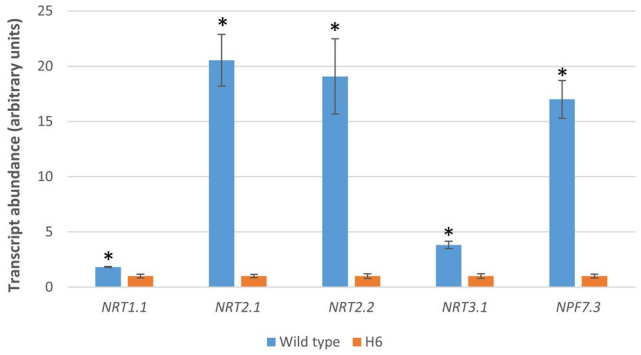

FIGURE 5 *AtGRXS8* represses nitrate transporter gene expression. Transcript abundance of the noted nitrate transporter genes was measured by real-time RT-PCR in wild-type and transgenic line H6 (which ectopically expresses *AtGRXS8*) seedlings. Total RNA was isolated from the roots of the 6-day-old seedlings, prior to lateral root emergence. All values were individually normalized to set transcript abundance in line H6 equal to 1.0. Averages ± *SEM* are shown, $N \geq 3$. An asterisk indicates a significant difference in transcript abundance compared with wild type ($p \leq .05$), as determined by Student's *t* test

(Mi et al., 2017), and gene list comparisons (Figure S4) were performed using Genesect (Katari et al., 2010; Krouk, Mirowski, LeCun, Shasha, & Coruzzi, 2010).

For real-time RT-PCR analysis of target transcripts, total RNA was isolated from the plant tissue using an RNeasy Plant Kit (Qiagen), and isolated RNA was DNAse treated using the Turbo DNA-free Kit (Invitrogen). RNA integrity was examined by formaldehyde agarose gel electrophoresis and RNA concentration was quantified spectrophotometrically (Patterson et al., 2010). First strand cDNA synthesis was performed using a Verso cDNA Synthesis Kit (Thermo Scientific) using 500 ng of total RNA and oligo dT primers. Real-time PCR was carried out using an Absolute Blue qPCR SYBR Green Master Mix (Thermo Scientific) and a RotorGene 6000 Real-Time Cycler (Corbett Research), as previously described (Patterson et al., 2010). Real-time data were normalized using the constitutively expressed internal standard gene *UBC* (At5g25760) (Czechowski, Stitt, Altmann, Udvardi, & Scheible, 2005). Primers used for real-time PCR are included in Table S4.

2.4 | Nitrate analyses

To quantify steady-state tissue nitrate levels (Figure 6b), plants were grown as described above for root growth assays (11-day-old plants on vertically oriented media containing 9 mM NO_3^-). Extraction and quantification of nitrate in the roots and shoots of these seedlings were carried out as previously described (Hachiya & Okamoto, 2017). For high-affinity nitrate uptake experiments (Figure 6a), plants were grown hydroponically on nitrate-free ammonium succinate medium for 15 days (Patterson et al., 2016; Vidal et al., 2010). 0.1 mM KNO_3 was then added to the media, with roots harvested 45 min later. Nitrate was extracted and quantified from the root tissue using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical), as previously described (Canales, Contreras-López, Álvarez, & Gutiérrez, 2017).

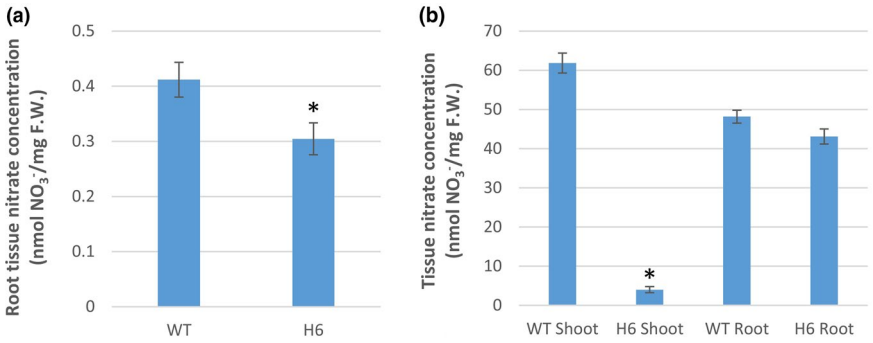

FIGURE 6 Plant nitrate uptake and distribution is altered by ectopic expression of *AtGRXS8*. (a) High-affinity nitrate uptake, as measured by quantifying root tissue nitrate content of 15-day-old plants grown on ammonium succinate media and then supplied with 0.1 mM KNO_3 for 45 min (Canales et al., 2017). Averages ± *SEM* are shown, $N \geq 20$. (b) Tissue nitrate content of 11-day-old seedlings grown on vertically oriented plates of growth media containing 9 mM nitrate. Averages ± *SEM* are shown, $N \geq 9$. Asterisks indicate significant differences ($p \leq .05$) in tissue nitrate concentration compared with wild-type (WT) tissue, as determined by Student's *t* test

2.5 | GUS staining and imaging

GUS staining was performed as previously described (Notaguchi, Wolf, & Lucas, 2012), and sections of stained root and stem tissue were hand sectioned using a razor blade. Root whole mounts were used for examination of lateral root primordia (Péret et al., 2009). Light microscopy of GUS-stained tissue was carried out using a Keyence BZ-X700 inverted microscope. Quantitative GUS activity assays (Figure 1d) were carried out on five-day-old seedlings grown on either standard Somerville and Ogren medium (9 mM NO_3^-) or a nitrogen-free version of the same medium (KCl replaces KNO_3 and $CaCl_2$ replaces $Ca(NO_3)_2$) (Patterson et al., 2010). Total protein extraction and the 4-methylumbelliferyl β-D-glucuronide (MUG) assay were performed as previously described (Jefferson, 1987).

2.6 | Yeast two-hybrid assays

Yeast two-hybrid assays were performed by Hybrigenics Services. The coding sequence of *AtGRXS8* was PCR-amplified and cloned in frame with the Gal4 DNA binding domain into plasmid pB35 as a C-terminal fusion to Gal4 (Gal4-bait fusion). pB35 was constructed by inserting the Gal4 DNA-binding domain from pAS2ΔΔ (Fromont-Racine, Rain, & Legrain, 1997) into the pFL39 backbone (Bonneaud et al., 1991) under the control of MET25 promoter (Mumberg, Müller, & Funk, 1994). The DNA fragment corresponding to amino acids 6–368 of TGA1 and the DNA fragment corresponding to amino acids 131–364 of TGA4 were extracted from the ULTImate Y2H *A. thaliana* seedling library (Hybrigenics Services). These prey fragments are fused out-of-frame (OOF) with the Gal4 activation domain (AD) in plasmid pP6, derived from the original pGADGH (Bartel & Fields, 1995). *Saccharomyces cerevisiae* are able to use ribosomal frameshift to generate a small percentage of the correct AD fusion protein with OOF1 and OOF2 prey fragments (Fromont-Racine et al., 1997; Vidal & Legrain, 1999). As a consequence, out-of-frame clones can be identified as interaction partners in Y2H experiments.

Bait and prey constructs were transformed in the yeast haploid cells CG1945 (mata) and YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, matα), respectively. The diploid yeast cells were obtained using a mating protocol with both yeast strains (Fromont-Racine et al., 1997). These assays are based on the HIS3 reporter gene (growth assay without histidine). As negative controls, the bait plasmid was tested in the presence of empty prey vector (pP6) and all prey plasmids were tested with the corresponding empty bait vector (pB35). The known interaction between the human SMAD and SMURF proteins was used as a positive control (Colland et al., 2004). Controls and interactions were tested in the form of streaks of three independent yeast clones for each control and interaction on DO-2 and DO-3 selective media. The DO-2 selective medium lacking tryptophan and leucine was used as a growth control and to verify the presence of the bait and prey plasmids. The DO-3 selective medium without tryptophan, leucine, and histidine selects for the interaction between bait and prey.

3 | RESULTS

The class III glutaredoxins *AtGRXS3*, *AtGRXS4*, *AtGRXS5*, *AtGRXS7*, and *AtGRXS8* are arranged in a tandem array on *A. thaliana* chromosome 4, and show very high sequence identity (Walters & Escobar, 2016). *AtGRXS3/4/5/7/8* are strongly and specifically upregulated by nitrate in nitrogen-limited plants (Patterson et al., 2016). This study focuses on *AtGRXS5* and *AtGRXS8*, the two most "divergent" members of this gene cluster (though they encode proteins with 91% amino acid sequence identity). First, we set out to define the expression domains of *AtGRXS5* and *AtGRXS8*, as well as the subcellular localization of the corresponding proteins.

Transgenic plants expressing *AtGRXS5*promoter::*GUS* and *AtGRXS8*promoter::*GUS* fusions showed expression in the vasculature of all root and shoot tissues in 14-day-old seedlings (Figure 1a). While *AtGRXS5* was exclusively vascular localized, *AtGRXS8* also displayed some expression in the mesophyll of true leaves. Imaging of stem and root cross-sections demonstrated that *AtGRXS5* and *AtGRXS8* are specifically expressed in the phloem (Figure 1b,c). As expected, GUS activity was nitrate-dependent in these transgenic lines (Figure 1d). To define the subcellular localization of the GRX proteins, the *AtGRXS5* and *AtGRXS8* coding sequences were fused to the *YFP* gene, and these constructs (35S::*YFP-AtGRXS5* and 35S::*YFP-AtGRXS8*) were used in Agroinfiltration of *N. benthamiana* leaves (Zhao et al., 2017). Confocal microscopy of agroinfiltrated leaves demonstrated that the AtGRXS5 and AtGRXS8 proteins are localized to both nuclei and the cytosol (Figure 2). While this subcellular localization pattern is similar to GFP alone (Figure S1), it is also consistent with the nucleo-cytosolic localization of the previously characterized class III GRX proteins AtGRXC7 (Li, Gutsche, & Zachgo, 2011), AtGRXS17 (Wu et al., 2012), and AtGRXS11 (Ohkubo et al., 2017). Collectively, these findings suggest that AtGRXS5 and AtGRXS8 accumulate in the nuclei and cytosol of phloem cells in response to nitrate availability.

One of the challenges of studying the biological function of the *AtGRXS3/4/5/7/8* gene cluster is the high likelihood of functional redundancy. We previously used RNA interference to silence *AtGRXS3/4/5/7/8* in transgenic *A. thaliana* plants; however, suppression of GRX expression in these lines was uneven, with *AtGRXS5* retaining ~50% of wild type mRNA levels and *AtGRXS8* retaining ~25% of wild-type mRNA levels (Patterson et al., 2016). Thus, we decided to ectopically express *AtGRXS5* and *AtGRXS8* in order to gain a better understanding of their functions in plant nitrate response and root development.

Multiple transgenic lines strongly overexpressing *AtGRXS8* (35S::*AtGRXS8*) were characterized (Figure 3). In contrast, repeated attempts to overexpress *AtGRXS5* (35S::*ATGRXS5*) were unsuccessful, as T1 generation transformants that were recovered consistently displayed near wild-type *AtGRXS5* transcript levels. This suggests that high-level ectopic expression of *AtGRXS5* may produce a lethal phenotype or interfere with fertility.

The *AtGRXS8*-overexpressing transgenic lines H6, G8, and J2 displayed a clear dwarf shoot phenotype, with major reductions in

rosette area (Figure S2). Surprisingly, the tiny shoot systems of these plants supported the development of a primary root that was essentially indistinguishable from wild type (Figure 4a). However, lateral roots were almost entirely absent in the *AtGRXS8*-overexpressing plants (Figure 4b,c). Comparable numbers of lateral root primordia developed in the primary root of transgenic and wild type lines (Figure 4d), demonstrating that *AtGRXS8* primarily acts by suppressing lateral root extension, not the early development of lateral root primordia. It is noteworthy that the media used to grow the plants lacks an exogenous carbon source, so the limited photosynthate generated from the dwarf shoot systems of *AtGRXS8*-overexpressing plants appears to be disproportionately invested in the growth of the primary root. Accordingly, the shoot:root fresh weight biomass ratio of 12-day-old-seedlings from the transgenic line H6 was 0.8:1.0, compared to a 2.2:1.0 shoot:root ratio in wild-type seedlings.

Auxin plays a critical role in lateral root outgrowth and emergence, and many auxin biosynthesis, transport, or signaling mutants are deficient in lateral root development (Fukaki, Tameda, Masuda, & Tasaka, 2002; Goh, Kasahara, Mimura, Kamiya, & Fukaki, 2012; Hobbie & Estelle, 1995; Lee, Kim, Lee, & Kim, 2009; Ruegger et al., 1997). To determine whether *AtGRXS8*-overexpressing plants have altered auxin signaling within the root system, we crossed the *AtGRXS8*-overexpressing line H6 to the synthetic DR5promoter::*GUS* auxin reporter line (Ulmasov, Murfett, Hagen, & Guilfoyle, 1997). F1 crosses (heterozygous) did not display any obvious changes in typical DR5::GUS staining patterns (i.e. weak staining of lateral root primordia and strong staining of root tips), suggesting that ectopic expression of *AtGRXS8* does not cause major alterations in auxin signaling in the root (Figure S3). However, this result obviously does not preclude subtle changes in auxin concentration or distribution in the *AtGRXS8*-overexpressing plants.

To better understand how ectopic expression of *AtGRXS8* affects the root at the molecular level, we isolated RNA from roots of wild-type plants and transgenic line H6 plants, and then characterized their transcriptomes via RNA sequencing. 426 genes displayed significantly increased expression in line H6 compared with wild type (Table S2), and 488 genes displayed significantly decreased expression in line H6 compared with wild type (Table S3). The lists of differentially regulated genes were analyzed via Gene Ontology category enrichment (Mi et al., 2017). As shown in Table 1, ectopic expression of *AtGRXS8* specifically upregulates GO Biological Processes associated with stress and defense responses. In contrast, nitrogen response processes (Nitrate transport, Response to nitrate, Regulation of amino acid export) are clearly repressed in the roots of plants ectopically expressing *AtGRXS8*. Given that *AtGRXS8* is strongly induced by nitrate (Figure 1d and Patterson et al., 2016), we were surprised that *AtGRXS8* appears to have a negative effect on nitrate signaling in roots. Thus, we focused follow-up studies on the specific effects of *AtGRXS8* on nitrate signaling.

A recent meta-analysis identified a core group of 50 genes that are consistently upregulated in response to nitrate in *A. thaliana* roots across multiple studies (Canales, Moyano, Villarroel, & Gutiérrez, 2014). We compared this list of core nitrate induced genes to the list of genes that were significantly downregulated by ectopic expression of *AtGRXS8*. There was a highly significant overlap, with 22% of the core nitrate induced genes being suppressed in the roots of the H6 transgenic line ($p < 6.4E\text{-}10$) (Figure S4). Particularly noteworthy is the effect of *AtGRXS8* in suppressing the expression of the genes encoding the major high-affinity nitrate uptake transporters in the root: *NRT1.1* (three-fold decrease), *NRT2.1* (13-fold decrease), *NRT2.2* (14-fold decrease), and *NRT3.1* (five-fold decrease). In addition, the gene encoding the nitrate transporter NPF7.3, which is involved in xylem loading of nitrate and corresponding root-to-shoot nitrate transport (Lin et al., 2008), was also strongly transcriptionally downregulated in line H6 (49-fold decrease).

One potential confounding factor in our RNA-seq analyses is the fact that wild-type plants have expanded lateral roots, while transgenic lines ectopically expressing *AtGRXS8* do not (Figure 4). Thus, it is possible that the observed patterns of differential gene expression could be attributed to genes that are exclusively or preferentially expressed in lateral roots. We tested this hypothesis on a targeted set of nitrate response genes by isolating RNA from the roots of young (6-day old) plants, prior to lateral root emergence in the wild type. Real-time RT-PCR was then used to quantify the levels of target transcripts. As shown in Figure 5, patterns of nitrate transporter gene expression were consistent with the RNA-seq data: *NRT1.1*, *NRT2.1*, *NRT2.2*, *NRT3.1*, and *NPF7.3* all showed significantly reduced transcript abundance in transgenic line H6. The fact that *AtGRXS8* can act to suppress the expression of an array of nitrate transporter genes suggests that nitrate uptake and transport *in planta* might also be compromised by ectopic expression of *AtGRXS8*.

To test this hypothesis, we directly studied the ability of wild type and *AtGRXS8*-overexpressing plants to take up nitrate in a hydroponic system. As previously described, plants were grown in ammonium succinate-based hydroponic media for 15 days, and then provided with 0.1 mM KNO_3 for 45 min to measure high-affinity nitrate uptake (Canales et al., 2017; Patterson et al., 2016). Roots were then thoroughly rinsed and the nitrate content of the root tissue was quantified (Canales et al., 2017). As shown in Figure 6a, the roots of transgenic line H6 take up significantly less nitrate (~25%) than the roots of wild-type plants, indicating a reduction in high-affinity nitrate transport. In a follow-up experiment, plants were grown for 11 days on media containing relatively high nitrate levels (9 mM KNO_3), and the nitrate content of roots and shoots were quantified separately. While the root nitrate content in transgenic line H6 was not significantly different than wild type in this system, shoot nitrate content in line H6 was reduced by >90% compared with wild type (Figure 6b). These findings suggest that ectopic expression of *AtGRXS8* reduces both nitrate uptake by the root (mediated primarily by NRT2.1, NRT2.2, NRT3.1 and NRT1.1) and the transport of nitrate from the root to the shoot (mediated primarily by NPF7.3) (Fan et al., 2017).

As mentioned previously, the biological activities of many class III GRXs have been linked to their ability to physically interact with

TGA transcription factors. The partially redundant clade I TGA transcription factors TGA1 and TGA4 have previously been identified as early activators of nitrate transcriptional response and are also involved in lateral root growth (Alvarez et al., 2014; Brooks et al., 2019). TGA1 and TGA4 are nuclear localized, and they are expressed in root and shoot vasculature- TGA1 in xylem, and TGA4 in both xylem and phloem (Bae, Cho, Choi, & Park, 2003; Pontier et al., 2002; Wang et al., 2019). Given the compatibility of their expression domains, we wondered whether AtGRXS8 could physically interact with TGA1 and TGA4. Indeed, yeast two-hybrid assays demonstrated protein:protein interactions between AtGRXS8 and both TGA1 and TGA4 (Figure 7). Unfortunately, multiple attempts to independently verify the AtGRXS8:TGA1/4 interaction *in planta* via biomolecular fluorescence complementation (BiFC) were inconclusive due to high background fluorescence in multiple tested BiFC vectors that contained the *TGA1* or *TGA4* coding sequences. Overall, it appears that AtGRXS8 could affect primary transcriptional and developmental responses to nitrate by binding to and altering the activity of the TGA1 and/or TGA4 transcription factors. However, further study is needed to fully test this hypothesis and explore possible indirect effects of AtGRXS8 on nitrate signaling.

4 | DISCUSSION

Both our findings and the recently published work of others highlight the fundamental importance of class III GRXs in plant nitrate response. Seventeen of the 21 class III GRXs in the *A. thaliana* genome are transcriptionally regulated by nitrate availability (Table S1). Of these nitrate-regulated GRXs, two groups have been studied: The nitrate-induced *AtGRXS3/4/5/7/8* gene cluster and nitrate-repressed *CEPD1/AtGRXS11*, *CEPD2/AtGRXC13*, and *CEPDL2/AtGRXC14*. *CEPD1*, *CEPD2*, and *CEPDL2* are components of the C-terminally encoded peptide (CEP), CEP receptor (CEPR), and CEP downstream (CEPD/CEPDL) system that mediates systemic nitrogen starvation signaling in *A. thaliana* (Ohkubo et al., 2017; Ota et al., 2020; Tabata et al., 2014). In response to nitrogen starvation, CEPD1, CEPD2, and CEPDL2 are produced in shoots and are then translocated via the phloem to the roots, activating high-affinity nitrate uptake and root-to-shoot nitrate translocation (Ohkubo et al., 2017; Ota et al., 2020). There are striking parallels between the *CEPD1-CEPD2-CEPDL2* system, which *activates* nitrate uptake and translocation in response to nitrogen limitation, and *AtGRXS8*, which appears to *repress* high-affinity nitrate uptake and translocation in response to nitrogen sufficiency (Figures 5 and 6). Indeed, a very recent study

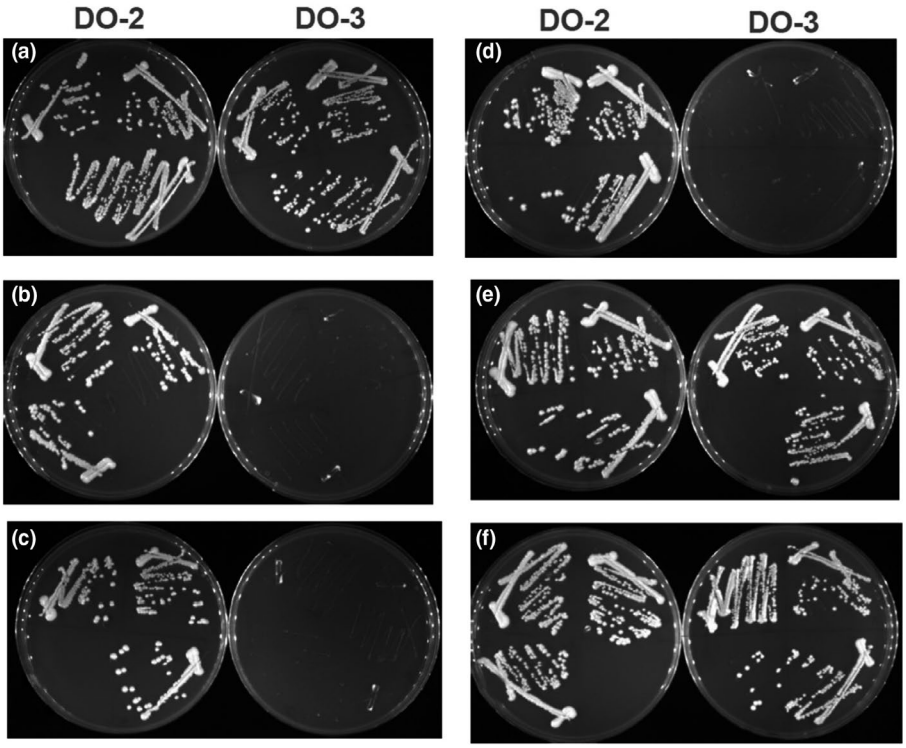

FIGURE 7 Characterizing protein–protein interactions between TGA transcription factors and AtGRXS8 via yeast two-hybrid assay. DO-2 indicates media without tryptophan and leucine, which selects for yeast containing both bait and prey vectors. DO-3 indicates media without tryptophan, leucine, and histidine, which selects for yeast containing both bait and prey vectors, and a protein–protein interaction between the bait and prey proteins. (a) Positive control (bait-SMAD, prey-SMURF- Colland et al., 2004). (b) Negative control (bait-empty vector; prey-TGA1). (c) Negative control (bait-empty vector; prey-TGA4). (d) Negative control (bait-AtGRXS8; prey-empty vector). (e) AtGRXS8-TGA1 protein–protein interaction (bait-AtGRXS8; prey-TGA1). (f) AtGRXS8-TGA4 protein–protein interaction (bait-AtGRXS8; prey-TGA4)

has demonstrated that ectopic expression of *CEPDL2* causes increased expression of *NRT2.1*, *NRT2.2*, *NRT3.1*, and *NPF7.3/NRT1.5* (Ota et al., 2020), precisely the same set of nitrate transporter genes that are strongly repressed by ectopic expression of *AtGRXS8*. Both AtGRXS8 (Figure 7) and CEPD2 (Li et al., 2019) can physically interact with the TGA1 and TGA4 transcription factors, which are direct transcriptional regulators of *NRT2.1*, *NRT2.2*, and many other nitrate response genes (Alvarez et al., 2014; Brooks et al., 2019). There is also extensive overlap in the expression domains of these GRX and TGA proteins: *CEPD1*, *CEPD2*, *and CEPDL2* are expressed in shoot phloem (and then transported to root phloem); *AtGRXS5* and *AtGRXS8* are expressed in both root and shoot phloem (Figure 1); and TGA1 and TGA4 are expressed in root xylem (TGA1) or root xylem and phloem (TGA4) (Ohkubo et al., 2017; Wang et al., 2019). In addition, all of these proteins show nuclear or nucleo-cytosolic localization (Figure 2) (Li et al., 2011; Ohkubo et al., 2017; Ota et al., 2020). Thus, it appears that nitrate availability may control the relative balance/abundance of nitrate-induced GRXs (e.g. *AtGRXS8*) and nitrate-repressed GRXs (e.g. *CEPD1*, *CEPD2* and *CEPDL2*) in the phloem, and that these GRXs may act to "fine tune" nitrate uptake and transport, likely via interactions with the TGA1 and/or TGA4 transcription factors.

As discussed previously, the *AtGRXS3/4/5/7/8* genes are strongly and specifically upregulated by nitrate (Patterson et al., 2016), so it was initially surprising to find that the ectopic expression of *AtGRXS8* causes the suppression of typical transcriptional responses to nitrate (Table 1). However, previous studies of the *NIGT1/HHO* family of nitrate-associated transcriptional repressors show several similarities with our findings (Kiba et al., 2018; Maeda et al., 2018). The *NIGT1* gene family (*NIGT1.1–1.4*) is transcriptionally activated by nitrate, and the corresponding NIGT1 proteins act to suppress the expression of many primary nitrate response genes. Specifically, 20% of all nitrate-induced genes in *A. thaliana* roots were found to be downregulated in transgenic plants ectopically expressing NIGT1.2 (Maeda et al., 2018). Similarly, 22% of the core nitrate-induced genes identified by Canales et al. (2014) are repressed in transgenic plants ectopically expressing *AtGRXS8* (Figure S4). Many primary nitrate response genes, including *NRT2.1* and *NRT3.1*, display transient induction: A rapid increase in transcript abundance immediately after nitrate exposure followed by a decrease to basal transcript levels despite the continued presence of nitrate (Bi, Wang, Zhu, & Rothstein, 2007; Maeda et al., 2018; Sawaki et al., 2013). Notably, *AtGRXS8* is not itself a primary nitrate response gene. Instead, *AtGRXS8* is indirectly regulated by increased cytokinin levels (and the cytokinin-associated transcription factors *ARR1/10/12*), which are triggered by nitrate availability (Patterson et al., 2016; Sakakibara, Takei, & Hirose, 2006). Accordingly, a recent study of the temporal dynamics of nitrogen signaling demonstrated that many primary nitrate response genes such as *NRT1.1*, *NRT2.1*, *NRT2.2*, and *NRT3.1* are induced 5–15 min after nitrogen provision in *A. thaliana*, while *AtGRXS8*, which acts to represses these primary response genes, is induced 90 min after nitrogen provision (Varala, Marshall-Colón, & Cirrone, 2018). Thus, negative regulators such as *NIGT1.2* and *AtGRXS8* appear to provide a delayed "time limit" to many primary transcriptional responses to nitrate.

Beyond its effects on nitrate signaling, *AtGRXS8* also negatively regulates lateral root development, which is typically stimulated by nitrate availability (Zhang & Forde, 1998). Transgenic plants ectopically expressing *AtGRXS8* almost entirely lack lateral roots, with a specific inhibition of the extension/emergence of lateral root primordia (Figure 4). We previously demonstrated that RNA silencing of the *AtGRXS3/4/5/7/8* gene cluster resulted in increased primary root length, suggesting that these GRXs act as negative regulators of primary root growth (Patterson et al., 2016). Thus, it was somewhat unexpected that ectopic expression of *AtGRXS8* specifically suppressed lateral root growth, but had no obvious effect on primary root growth. Still, it is clear that these nitrate-induced GRXs negatively regulate root growth and can dramatically affect overall root system architecture (e.g. the relative growth of primary vs. lateral roots). Given that AtGRXS8 can interact with TGA1 and TGA4, it is noteworthy that the *tga1/tga4* mutant of *A. thaliana* displays deficiencies in both primary root growth and lateral root growth (Alvarez et al., 2014). During the course of our studies, Jung et al. (2018) published an analysis of transgenic *A. thaliana* plants overexpressing several GRXs, including *AtGRXS8*. Although no effects on primary or lateral root development were reported, they did demonstrate that overexpression of *AtGRXS8* resulted in a significant decrease in root hair length (Jung et al., 2018). This phenotype was also observed in our *AtGRXS8* overexpression lines, when they were grown under experimental conditions identical to those described by Jung et al. (2018) (Figure S5). Given that root hair length is also influenced by nitrate availability in *A. thaliana* (Vatter, Neuhäuser, Stetter, & Ludewig, 2015), this finding further supports the import role of the nitrate-regulated GRXs in root system developmental responses to nitrate.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS

M.A.R., A.E., and S.C. helped to design the research and performed the research. O.D., C.C., and F.F. performed the research. M.A.E designed the research and wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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