

The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in *Arabidopsis*

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Tight control of cellular redox homeostasis is essential for protection against oxidative damage and for maintenance of normal metabolism as well as redox signaling events. Under oxidative stress conditions, the tripeptide glutathione can switch from its reduced form (GSH) to oxidized glutathione disulfide (GSSG), and thus, forms an important cellular redox buffer. GSSG is normally reduced to GSH by 2 glutathione reductase (GR) isoforms encoded in the *Arabidopsis* genome, cytosolic GR1 and GR2 dual-targeted to chloroplasts and mitochondria. Measurements of total GR activity in leaf extracts of wild-type and 2 *gr1* deletion mutants revealed that ≈65% of the total GR activity is attributed to GR1, whereas ≈35% is contributed by GR2. Despite the lack of a large share in total GR activity, *gr1* mutants do not show any informative phenotype, even under stress conditions, and thus, the physiological impact of GR1 remains obscure. To elucidate its role in plants, glutathione-specific redox-sensitive GFP was used to dynamically measure the glutathione redox potential (E_{GSH}) in the cytosol. Using this tool, it is shown that E_{GSH} in *gr1* mutants is significantly shifted toward more oxidizing conditions. Surprisingly, dynamic reduction of GSSG formed during induced oxidative stress in *gr1* mutants is still possible, although significantly delayed compared with wild-type plants. We infer that there is functional redundancy in this critical pathway. Integrated biochemical and genetic assays identify the NADPH-dependent thioredoxin system as a backup system for GR1. Deletion of both, NADPH-dependent thioredoxin reductase A and GR1, prevents survival due to a pollen lethal phenotype.

redox homeostasis | redox imaging | redox-sensitive GFP | thioredoxin reductase

Thio-redox biochemistry is a common feature of life and is involved in a broad range of physiological and pathological processes. Thus, tight control of cellular redox homeostasis is essential for maintenance of normal metabolism and redox-dependent signaling. In general, several metabolic reactions and, to a larger extent, stress-induced processes lead to the formation of reactive oxygen species (ROS). The concomitant oxidation is buffered through a tight network of antioxidant enzymes and low-molecular weight antioxidants (1). The most prevalent non-protein thiol-based redox buffer is the tripeptide glutathione, which is present in low millimolar concentrations in most eukaryotic cells. Under nonstress conditions, cytosolic glutathione is present mainly in the reduced form (GSH) with only nanomolar concentrations of the oxidized form, glutathione disulfide (GSSG) (2, 3). In plants, ROS are at least partially detoxified through the glutathione-ascorbate-cycle at the expense of electrons from the GSH pool (4), causing a transient oxidation of the GSH pool. This transient change in glutathione redox potential (E_{GSH}) has been suggested to be part of signaling cascades leading to changes in gene expression during stress responses and developmental processes (5, 6). The

transmission of such signals is likely to be facilitated by glutaredoxins (GRXs) (6), which equilibrate E_{GSH} with the redox potential of target protein thiols. Indeed, GRXs have been shown to be involved in disease resistance and developmental processes, suggesting that downstream events in these processes are modified by E_{GSH} (7, 8).

NADPH-dependent glutathione reductase (GR), a member of the FAD-binding disulfide reductase superfamily, is the major enzyme responsible for reduction of GSSG to GSH in most organisms, with only few GR-deficient exceptions (9, 10). The *Arabidopsis* genome contains 2 GR genes. One gene codes for an organellar isoform (GR2), which is dual-targeted to chloroplasts and mitochondria (11). GR2 is essential for plant development, which is evident from lethality of deletion mutants in early embryo development (12). The second gene codes for the cytosolic GR1. Expression analysis by scrutiny of the Genevestigator microarray database (13) shows *GR1* to be responsive to several stress factors. Although GR activity is one of the most frequently monitored enzyme activities after stress application, there is still very little information on the biochemical properties of *Arabidopsis* GR1. Because *gr1* deletion mutants have not been reported in plants, we questioned whether other oxidoreductases may provide a functional backup system for GR1 and, thus, conceal function on gene disruption (14).

Like GRs, NADPH-dependent thioredoxin reductases (NTRs) also belong to the FAD-binding disulfide reductase superfamily (15). In *Arabidopsis*, NADPH-dependent thioredoxin reductase A (NTRA) and NADPH-dependent thioredoxin reductase B (NTRB) result from recent gene duplication and constitute the major route to reduce mitochondrial and cytosolic thioredoxins (TRXs) (16, 17). Nevertheless, the double knockout *ntra ntrb* was found to be viable, although it was hypersensitive to depletion of the GSH pool (18). A different, although less efficient, reduction system consisting of GRX and TRX was apparently able to complement the NTR function (18). To be maintained in the reduced form, GRX requires GSH, GR, and NADPH as the primary electron donor. Partial redundancies between the NTR/TRX system and the GSH redox system have also been observed in *Escherichia coli* and *Saccharomyces cerevisiae* (19, 20). However, in fission yeast, GR is indispensable for growth under aerobic conditions (21).

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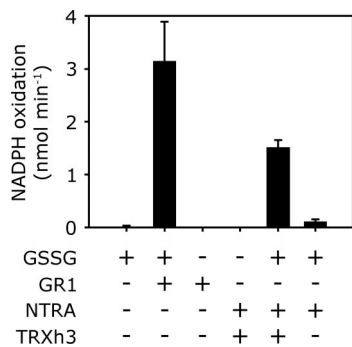


Fig. 5. Biochemical activity of GSSG reduction by NTRA and TRXh3. TRXh3 is first reduced for 30 min with NTRA and NADPH. Activity is monitored as NADPH oxidation. Note that the amount of disulfide reductase protein varied between the assays. Enzymes and substrates were used at the following concentrations: NADPH, 200 μ M; GSSG, 400 μ M; GR1, 0.01 μ M; NTRA, 1 μ M; TRXh3, 10 μ M. Means \pm SD ($n = 3$).

smaller rosettes (Fig. S5). To test for functional redundancy, we examined whether the TRX system is substituting the lack of GR1 in the *gr1* mutants, we generated triple mutants lacking NTRA, NTRB, and GR1. Both mutant lines, *gr1-1* and *gr1-2*, were crossed with an *ntra ntrb* double knockout. From the F2 generation, mutants homozygous for *ntra* and *ntrb*, but heterozygous for *gr1* were selected and selfed. These mutants segregated a ratio of 1 (*GR1/gr1*):1 (*gr1/gr1*) with no homozygous triple mutant for either *gr1* allele (Table S2), suggesting that triple mutants are lethal. Lack of aborted seeds (Fig. S7) indicated a pollen lethal effect while female transmission of *gr1* was not impaired.

To further test whether the triple mutation affects male fertility, pollen of a *GR1/gr1-1 ntra ntrb* was transferred to wild-type stigma. Progeny testing failed to identify plants heterozygous for *gr1-1* (Table 1), suggesting that no *gr1-1 ntra ntrb* pollen had successfully fertilized the wild-type ovules. Alexander staining (25) revealed no cytoplasmic or pollen wall abnormalities. The reciprocal cross did result in 50% *GR1/gr1* plants, which again confirmed that female transmission of the defective *gr1* allele in the *ntra ntrb* background was normal.

The genetic evidence suggests that at least one of the cytosolic disulfide Rs, GR1, NTRA, or NTRB, is required for development of the male gametophyte. To test this hypothesis, the presence of the 3 transcripts was measured in mature pollen. Semiquantitative RT-PCR indicated that GR1 and NTRA were

Table 1. Reciprocal cross between *GR1/gr1-1 ntra/ntra ntrb/ntrb* and Col-0

A:	Female parent	×	Male parent	Progeny genotype	
	<i>GR1/gr1-1</i>		Col-0	<i>GR1/gr1-1</i>	<i>GR1/GR1</i>
	<i>ntra/ntra</i>	×		<i>NTRA/ntra</i>	<i>NTRA/ntra</i>
	<i>ntrb/ntrb</i>			<i>NTRB/ntrb</i>	<i>NTRB/ntrb</i>
	Frequency*			13	16
B:	Female parent	×	Male parent	Progeny genotype	
	Col-0		<i>GR1/gr1-1</i>	<i>GR1/gr1-1</i>	<i>GR1/GR1</i>
		×	<i>ntra/ntra</i>	<i>NTRA/ntra</i>	<i>NTRA/ntra</i>
			<i>ntrb/ntrb</i>	<i>NTRB/ntrb</i>	<i>NTRB/ntrb</i>
	Frequency			0	38

*Twenty-nine progeny were genotyped by PCR. $\chi^2 = 0.416$ for 1:1 segregation, $P = 0.52$.

†Thirty-eight progeny were genotyped by PCR. $\chi^2 = 29$ for 1:1 segregation, $P = 0$.

both expressed in pollen, whereas no signal could be detected for NTRB (Fig. S8). In flowers, GR1 and both NTR isoforms were detected. Selfing of a mutant homozygous for *ntra* and heterozygous for *gr1* resulted in a 1:1 segregation of the progeny ($15:15$, $\chi^2 = 0$, $P = 1$) expected if the double knockout is lethal. In combination with the *ntrb* knockout, *gr1* segregated with a 1:2:1 ratio ($7:17:6$; $\chi^2 = 0.6$, $P > 0.5$).

Discussion

Catalytic Activity of *Arabidopsis* GR1. *Arabidopsis* GR1 is present as a FAD-bound homodimer (Fig. 1) similar to homologous flavoprotein disulfide reductases from other organisms (15). The K_m value of 77 μ M for the substrate GSSG is similar to K_m values reported for GRs from *E. coli* (61 μ M), yeast (55 μ M), and human (72 μ M) (26–28). GRX1-roGFP2 reports the actual E_{GSH} (3), and due to its midpoint potential of -280 mV (24), allows the determination of residual amounts of GSSG in the presence of GR and the second substrate NADPH. Both, in vitro (Fig. 2) and in vivo (Fig. 4) GR1 maintain an E_{GSH} of ≈ -310 mV, which is consistent with earlier in vivo measurements in *Arabidopsis* and HeLa cells (2, 3). Due to irreversibility of the reaction, GR1 can, thus, maintain nanomolar GSSG concentrations in the cytosol.

GR1 Is Not Essential for Plant Development. In mammals and bacteria, the inhibitor BCNU has been used (22, 23). However, it does not inhibit plant GR1. Thus, to confirm the importance of GR1, T-DNA knockout mutants were selected and characterized. In the absence of detectable GR1 transcript and GR1 protein in both isolated mutants and no elevation of organellar GR2 protein, the remaining GR activity of total protein extract of $\approx 35\%$ in *gr1* mutants compared with wild-type plants can be attributed to GR2 activity (29) and possibly other cytosolic reduction systems. The 65% share of the overall GR activity in leaf extracts for cytosolic GR1 is much higher than 20% activity associated with the cytosol in pea leaves (30).

ATP-binding cassette (ABC) transporters with micromolar K_m values have been proposed as backup systems for insufficient cytosolic GR activity (31). Although roGFP at this stage is not capable of detecting increased accumulation of GSSG in the ER or the vacuole, the absence of continuous accumulation of GSSG and the less reducing cytosolic E_{GSH} of *gr1* mutants rather suggest an alternative cytosolic reduction system with lower efficiency than GR1.

TRX and NTR Can Replace GR In Vitro. An E_{GSH} of -270 mV in the cytosol is still sufficiently negative to maintain metabolic functions under normal conditions, which is consistent with reports of GR deletion mutants in *S. cerevisiae* (32). The use of a ratiometric probe in this work allowed dynamic in vivo measurements of E_{GSH} under induced oxidative stress. These measurements, showed (i) that the buffer capacity of *gr1* against ROS is significantly diminished, and (ii) that another less efficient reduction system is in place (Fig. 4G). In mammalian cells, GR and NTR share many common features, including similar primary and tertiary structures and high similarities of active site residues (33). However, lack of electrostatic attraction in the potential GSSG binding pocket prevents efficient GSSG reduction by human NTR (34). However, this observation cannot be directly transferred to plants, because GR1 and NTRA in this case are not related to each other (35). Also, our results for *Arabidopsis* clearly disproved the hypothesis of direct GSSG reduction for NTRA (Fig. 5). Only together with TRX and NADPH as electron donor, NTRA is capable of reducing GSSG, suggesting that the TRX system as a whole may constitute a backup system for GR1. When measured in vitro, the activity of NTRA/TRXh3 toward GSSG was 200-fold lower than the activity of GR1 (Fig. 5). This large difference in activity high-

lights the difficulties of direct extrapolation from in vitro data to the in vivo situation where the *gr1* mutant still has a considerable reduction capacity for GSSG (Fig. 4). Within the pool of 11 cytosolic type h TRX (36), some isoforms may be more efficient than the tested TRXh3. Also, the in vitro assay does not consider the relative abundance of the respective oxidoreductases. The abundance of documented ESTs indicates that NTR (60 ESTs) and TRXh (>100 ESTs) may be far more abundant than GR1 (30 ESTs), at least at mRNA level. The situation in the *Arabidopsis gr1* mutant to some extent resembles the naturally occurring situation in *Drosophila melanogaster*, where the TRX system substitutes for the lacking GR (9). It can also not be excluded that some other alternative GSSG reduction systems may act in *Arabidopsis* and contribute to maintenance of GSH redox homeostasis in *gr1* mutants. A multifunctional TRX GR has been found in *Schistosoma mansoni*, which has an N-terminal GRX-like domain for TRX-independent GSSG reduction (37). In *Synechocystis*, which also lacks a genuine GR, NTR-reducible GRXs may act as intermediate reducers of GSSG (38). Due to the high number of isoforms, further dissection of individual contributions to GSSG is a difficult challenge. However, the genetic evidence provided here (see below) clearly shows the need of either GR1 or NTR in plants. The NTRs are highly similar across the plant kingdom, and wheat NTR is a highly efficient reducer of *Arabidopsis* TRXh. Thus, it can be assumed that the backup of cytosolic GR1 by the NTR/TRX system is universal across plant species. In contrast to *S. cerevisiae*, where mutual functional backup between NTR/TRX and GR has been shown (20), redundancy between GR and the NTR/TRX system in plants seems to be restricted to the cytosol, because mutants lacking organellar GR2 are embryo lethal (12). Deletion of GR1 may be tolerable, because oxidative stress in the cytosol is relatively low compared with plastids and mitochondria (39). A drop in cytosolic E_{GSH} is likely to gradually affect downstream signaling and developmental processes. Antisense lines for *Arabidopsis* GSH1 indicated that 5% of wild-type GSH, which would result in an E_{GSH} of ≈ -240 mV, already limits the growth rate and renders plants more sensitive to environmental stress (40). Progressive growth inhibition and increasing stress sensitivity is also apparent for the even more severely affected *rml1* mutant, which has <5% of wild-type GSH, and embryo lethal *gsh1* deletion mutants (41, 42). Deletion of GR and a concomitant 21-fold increase in GSSG have been reported to cause oxidation of GRXs in *S. cerevisiae* (20). Even though this oxidation does not have an immediate effect on plant development, it can be assumed that excessive oxidation of GRXs can affect downstream processes under conditions of severe stress.

Either GR1 or NTRA Is Required for Pollen Viability. Genetic evidence for the functional overlap between GR1 and the TRX system was gained from the combination of the *gr1* mutation with a *ntra ntrb* double knockout. In *Arabidopsis*, the *ntra* and *ntrb* deletion mutants on their own do not show any phenotype (16). A growth phenotype of the *ntra ntrb* double knockout indicated functional redundancy between the 2 NTRs in the diploid phase (18). Interestingly, our present results also show functional overlap between cytosolic GR1 and NTRA during the haploid phase. Cross-pollination experiments showed that the lethal phenotype can be attributed to the male gametophyte, whereas oocytes with the triple mutation in GR1, NTRA, and NTRB genes are viable, as shown by the segregations after pollination with the wild-type (Table 1, Fig. S7). The difference between male and female gametophytes remains elusive, but it may be that pollen depends on a more robust redox buffer system. Pollen tube growth is associated with increased ROS production (43), which may be deleterious in the triple mutant. Oocytes, in contrast, are resting and also much better protected by maternal tissues. Transmission of defective *gr1* alleles in pollen can only occur in the presence of functional NTRA, suggesting that

GR1 and NTRA proteins have a major role in pollen fertility. Indeed, detection of GR1 and NTRA transcripts in pollen are also supported by proteomic analysis (44). However, NTRB mRNAs were hardly detected in pollen, and this protein has never been found in the pollen proteome. The fact that no NTRB transcript was found in pollen and that *gr1* in a homozygous *ntrb* background segregated 1:2:1 unambiguously shows that NTRB is not important for *gr1* transmission. However, this does not exclude the possibility that NTRB might contribute to cytosolic GSH redox homeostasis in mature plants, especially under conditions of severe oxidative stress. Because *gr1 ntra* pollen are not viable, homozygous mutants required to test for the function of GR1 and NTRs in embryonic tissue and mature plants can only be generated from conditional knockouts activated after fertilization. RNAi and inducible expression constructs will be helpful to investigate this question in more detail.

In summary, our study shows that NTRA together with TRXh3 exhibit functional redundancy with cytosolic GR1. Biochemical and genetic evidence shows that GR1 constitutes the main GR activity in *Arabidopsis*. Despite the presence of an efficient backup system, deletion of GR1 significantly lowers the buffering capacity of the cytosolic GSH pool against ROS. The delayed reduction of GSSG after an exogenously triggered oxidation in the *gr1* mutant indicates that *gr* mutants expressing GRX1-roGFP2 may constitute a suitable system for detection of stress-induced ROS signals.

Materials and Methods

Plant Material and Growth Conditions and Treatments. Plants were soil grown in 8.5:15.5-h day/light cycle at a temperature between 18 and 22 °C, 100 μ E $m^{-2} s^{-1}$ and 50% humidity. For Confocal laser scanning fluorescence microscopy (CLSM) analysis, transgenic seeds were surface sterilized and grown on 0.5 \times Murashige and Skoog (MS) medium (pH 5.7, 1% sucrose, 0.7% agar). Rosette leaves of 19- to 22-d old T₂-seedlings were used for imaging.

For stress treatments of in vitro seedlings, seeds were surface sterilized and plated on 0.5 \times MS medium including Gamborg B5 vitamins [pH 5.7, 1% (wt/vol) sucrose, 0.8% (wt/vol) plant agar] (Duchefa). To supplement the growth medium with H₂O₂, CdCl₂, and CuSO₄ at various concentrations, appropriate amounts of the respective filter-sterilized stock solutions were added to 0.5 \times MS medium before gelling.

Isolation of Total RNA and Semiquantitative RT-PCR. Total RNA was extracted from *Arabidopsis* leaves, pollen, or inflorescences, and reverse transcribed in cDNA; cDNA was subsequently used for semiquantitative RT-PCR. For details, see *SI Materials and Methods*.

Molecular Cloning and Plasmid Constructs. Cloning and expression of proteins is described in *SI Materials and Methods*.

Protein Purification. Recombinant roGFP protein was cloned, expressed, and purified as previously described (2). For all other proteins, details are described in *SI Materials and Methods*.

Identification of T-DNA Insertion Lines. Two T-DNA insertion lines, *gr1-1* and *gr1-2*, were obtained from the SALK collection, and characterized as described in *SI Materials and Methods*. Alleles of *ntra* and *ntrb* were identified as described (18).

Mutant Crosses and Pollen Competition. For generation of *ntra ntrb gr1* triple mutant, the *ntra ntrb* double mutant was crossed with either *gr1-1* or *gr1-2*. The F₁ plants were selfed, and plants with the desired genotype were selected in the F₂ generation. Pollen competition experiments were performed as described (18).

Protein Extraction from *Arabidopsis* Leaves. Protein was extracted from 250-mg ground leaf material using extraction buffer (50 mM Hepes, pH 7.4/10 mM KCl/1 mM EDTA/1 mM EGTA/10% vol/vol glycerol) supplemented with 1 mM PMSF. The extract were desalted with NAP5 columns (GE Healthcare), and resuspended in 1 mM K₂HPO₄/KH₂PO₄, pH 7.4/1 mM EDTA. Protein concentrations were determined by Bradford.

GR Activity. Twenty micrograms of proteins were assayed in 100 mM K_2HPO_4/KH_2PO_4 , pH 7.4/1 mM EDTA/750 μ M DTNB/200 μ M NADPH/400 μ M GSSG. Absorbance was measured at 412 nm. NADPH and GSSG were always freshly prepared. For inhibition experiments, BCNU was dissolved in ethanol and added to the concentrations indicated.

Antibody Production and Protein Gel Blot Analysis. Experimental details are provided in *SI Materials and Methods*.

HPLC Analysis of Glutathione. GSH and GSSG were analyzed from 7-week-old, soil grown *Arabidopsis* plants as described (2). The amount of GSH measured after extraction with NEM refers to the amount of GSSG present in

the sample. After subtraction from the total GSH pool measured after extraction with DTT, the difference corresponds to the amount of GSH present in the sample.

Redox-Sensitive GFP Imaging and GR Assay Using GRX1-roGFP. Ratiometric imaging of roGFP, perfusion experiments, and image analysis were done as described previously (45).

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