Supporting Information

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SI Materials and Methods

Semiquantitative RT-PCR. Total RNA was extracted from *Arabidopsis* leaf material ground in liquid N_2. RNA was extracted with TRIzol (Invitrogen). RNA was reverse transcribed in cDNA using the M-MLV Reverse Transciptase kit (Promega). glutathione reductase (GR)1 cDNA was PCR amplified with the primers 5′-CATACCACCACTAGCTGTAGTG-3′ and 5′-TGCATGATCTCAGCTGCATCAG-3′. The reference cDNA of the constitutively expressed actin 7 was amplified with primers 5′-CAACCGGTATTGTGCTCGATTC-3′ and 5′-GAGTGAGTCTGTGAGATCCCG-3′. PCR was performed with Taq polymerase (New England Biolabs) in a standard PCR program.

Isolated spores from male gametophyte were obtained by modification of the protocol of Honys and Twell (1); 500 mg of *Arabidopsis* inflorescences were collected and gently ground using a mortar and pestle in 5 mL of 0.3 M mannitol. The slurry was filtered through 100 and 60 μM nylon mesh. Mixed spores were concentrated by centrifugation (15 mL Falcon tubes, 450 × *g*, 3 min, 4 °C). Concentrated spores were washed in 1 mL of 0.3 M mannitol, and spores were concentrated again by centrifugation (Eppendorf tubes, 2,000 × *g*, 1 min, 4 °C) and stored at −80 °C. The purity of isolated fractions was determined by light microscopy and DAPI staining. Viability was assessed by fluorescein 3′,6′-diacetate (FDA) treatment. RNA isolation and RT-PCR experiments were performed as described above; cDNA were amplified with the following primers: *GR1* forward primer, 5′-CTAATATACCTAGCATATGGGCTGTAGGAG-3′, and reverse primer, 5′-TCATAGATTTGTCTTAGGTTTGGG-3′. *GR2* forward primer, 5′-GGATTTCGTTGGAGAGCAGATGTCTTTAAGA-3′, and reverse primer, 5′-CTACACCCCAGCAGCTGTTTTAG-3′. *NTRA* forward primer, 5′-GCAAAATGTGTTGGATCTCAATGAG-3′, and reverse primer, 5′-CATGGATCCTTCTCCTACAGCTTC-3′. *NTRB* forward primer, 5′-CGAAAGCTTTGCACGGCTTGGTGGTG-3′, and reverse primer, 5′-GATCAATCAACAATAACTCAATGACCT-3′. *SPIK* forward primer, 5′-GTATAGCAGTGAGCCTACAATG-3′, and reverse primer, 5′-TCATTACTCAAAATCGAAAGAG-3′. *EF1α* forward primer, 5′-CTAAGGATGGTCAGACCCG-3′, and reverse primer, 5′-CTTCAGGTATGAAGACACC-3′.

Molecular Cloning and Plasmid Constructs. For expression of recombinant protein, *GR1* was amplified by PCR with Phusion polymerase (Finnzymes) using a cDNA template. The AseI and BamHI restriction sites (underlined in primer sequence) were added to forward (5′-ATTAATGAGAATCTTTATTTTCAGGGCATGGCGAGGAAGATGCTTG-3′) and reverse (5′-GGATCCTCATAGATTTGTCTTAGGTTTGG-3′) primers, respectively. PCR product was purified and blunt ligated into the MluNI site of pCAP (Roche). Accuracy of the construct and the cloned sequence was confirmed by sequencing (Seqlab). Subsequently, the *GR1* sequence was cut out with AseI and BamHI and cloned into pET28a (Novagen) behind an N-terminal His_6 tag.

NTRA was PCR amplified using the primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGAAACTCACAAAACCA-3′, and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATCACTCTTACCCTCCT-3′. TRXh3 was amplified by PCR with the primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGCCGCAGAAGGAGAAG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGG*GTCTCAAGC*AGCAGCAACAACTG-3′. Both PCR products were cloned into pETG10 (Novagen) to generate an N-terminal His_6 tag using the Gateway technology (Invitrogen).

GRX1-roGFP2 (2) was PCR amplified with Phusion polymerase (Finnzymes) using the primers 5′-GATCCATGGCTCAAGAGTTTGTGA-3′ and 5′-GTCGACTTACTTGTACAGCTCGTCC-3′ to add BamHI and SalI restriction sites. The PCR product was cloned blunt-end into the vector pCAP (Roche). The GRX1-roGFP2 was then subcloned into the expression vector pBinAR with BamHI and SalI. The construct was transformed into the *Agrobacterium* strain AGL-1 and used for *Arabidopsis* transformation.

Protein Purification. Recombinant roGFP protein was cloned, expressed, and purified as previously described (3). For all other proteins the pET constructs were transformed into *Escherichia coli* HMS 174 cells. Cells were grown at 37 °C to an OD_{600} of 0.8 in selective media containing 50 μg/mL kanamycin. Protein expression was induced by adding IPTG to a final concentration of 1 mM and cells were harvested after 4 h. Cells were pelleted by centrifugation and resuspended in 50 mM Tris·HCl, pH 8/250 mM NaCl buffer supplemented with 0.5 mM PMSF. After sonication the cell debris was pelleted by centrifugation and filtered through a 45 μM sterile filter. Soluble proteins were loaded on a Ni^{2+}-NTA column (Amersham) for 30 min with a flow rate of 1 mL min^{-1}. The loaded column was washed with 10 mL wash buffer (50 mM Tris·HCl, pH 8/250 mM NaCl/80 mM imidazole). Last, the protein was eluted with elution buffer (wash buffer with 250 mM imidazole). The protein concentration was determined by Bradford.

Identification of T-DNA Insertion Lines. Both mutant alleles *gr1-1* (SALK_105794) and *gr1-2* (SALK_060425) were obtained form the SALK collection. Homozygous knock-out plants were identified by PCR using gene-specific primers and primers binding to the left border of the T-DNA. The following primers were used for *gr1-1*: GR1-1_f 5′-TCGTCTATGGAGCTACTTACGGTGG-3′ GR1-1_r 5′-CGCAAAAATATCCAATCTACTGAGCAC-3′, *gr1-2:* GR1-2_f 5′-CTCCAGCTGTCTAAGGATGTATC-3′ GR1-2_r 5′-CTACTGCTTCTTCTTCGCTGAGAC-3′ T-DNA left border was 5′-GACCGCTTGCTGCAACTCTCTCAGG-3′. PCR performed with DNA from homozygous knockout plant and gene specific primers yielded no PCR product. T-DNA insertions were confirmed by PCR with the following combinations: GR1-1: GR1-1_r and T-DNA left border primer, GR1-2_f and T-DNA left border primer. Alleles of *ntra* and *ntrb* were identified as described (4).

Antibody Production and Protein Gel Blot Analysis. Antiserum against *At*GR1 and *At*GR2 was generated by injection of purified recombinant protein in rabbit. For protein gel blot analysis, 15 μg of extracted proteins were separated by discontinuous SDS/PAGE in MiniProtean II cells according to Laemmli (Bio-Rad). Proteins were transferred to nitrocellulose using the Mini Trans-Blot system (BioRad). To confirm a successful transfer and equal loading, the nitrocellulose membrane was stained with Ponceau [0.1% Ponceau S (wt/vol) in 5% acetic acid]. Subsequently, the membrane was blocked over night at room temperature with 5% Milk powder in TBS (20 mM Tris/137 mM NaCl, pH 7.6) supplemented with 0.1% (vol/vol) Tween 20. The membrane was washed 3 times with 0.5% Milk powder in TBS and 0.01% Tween and incubated with primary antiserum at

dilution of 1:5,000 for 2 h. After washing 3 times for 5 min with 0.5% Milk powder in TBS, the membrane was incubated with secondary antibody [Anti-rabbit IgG-Alkaline Phosphatase (AP) Conjugate, 1:10,000] for 1 h and washed twice with AP buffer (100 mM Tris·HCl, pH 9.5/100 mM NaCl/50 mM $MgCl_2$). The AP reaction was done by adding the substrate nitroblue tetrazolium/5-bromo-5-chloro-3-indolyl phosphate (Roche).

1. Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. *Genome Biol* 5:R85.
2. Gutscher M, et al. (2008) Real-time imaging of the intracellular glutathione redox potential. *Nat Methods* 5:553–559.
3. Meyer AJ, et al. (2007) Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. *Plant J* 52:973–986.
4. Reichheld J-P, et al. (2007) Inactivation of thioredoxin reductases reveals a complex interplay between thioredoxin and glutathione pathways in Arabidopsis development. *Plant Cell* 19:1851–1865.

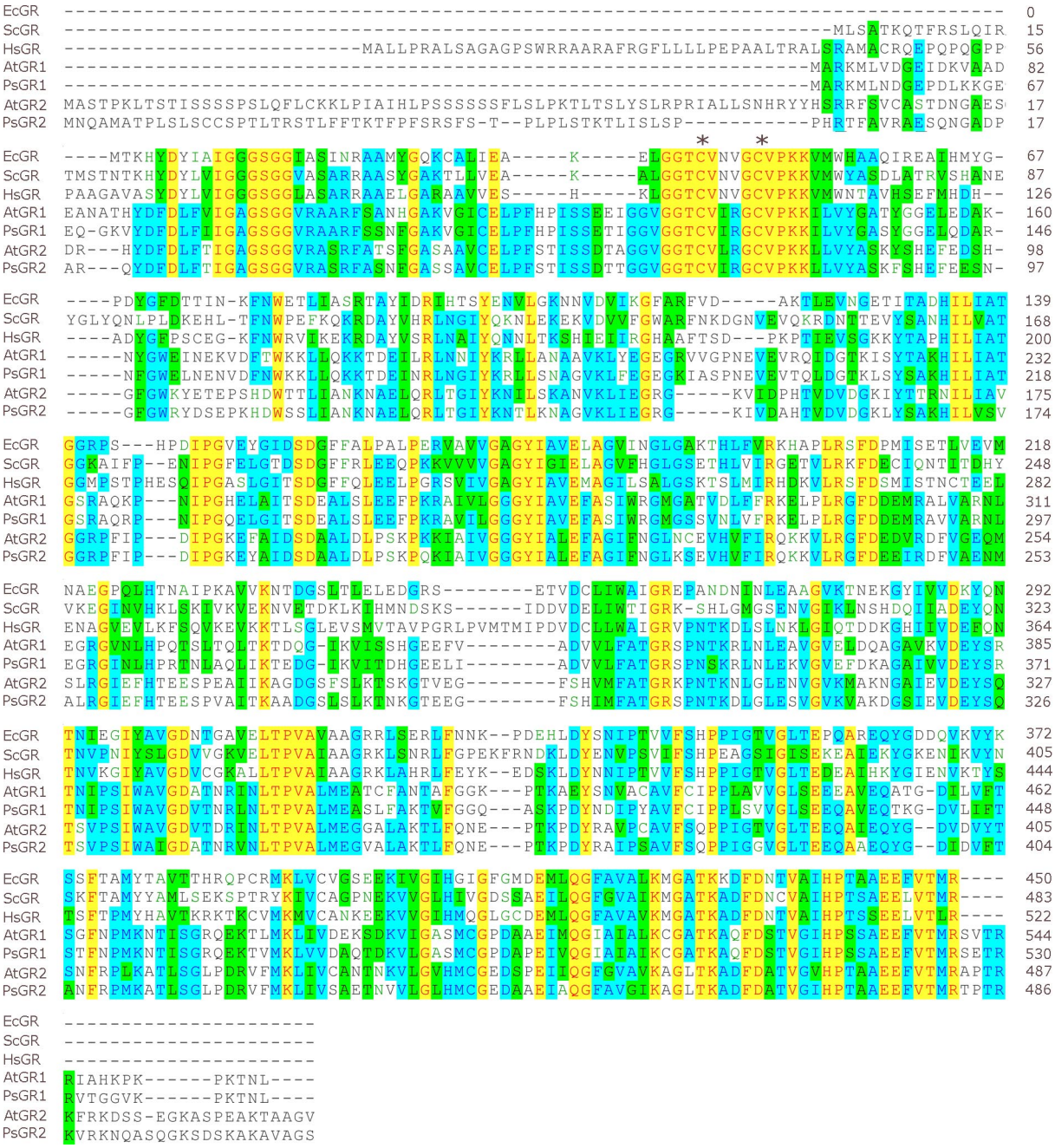

Fig. S1. Protein alignment of *Arabidopsis* GRs with 5 homologues from other species. Identical residues are highlighted in yellow, conservative residues are highlighted in cyan, and blocks of similar residues in green. Green letters on white background represent residues with weak similarity. *Ec*GR: *Escherichia coli* (gi|121674); *Sc*GR: *Saccharomyces cerevisiae* (gi|1708060); *Hs*GR: *Homo sapiens* (gi|14916998); *At*GR2: *Arabidopsis thaliana* (At3g54660; gi|1170040); *Ps*GR2: *Pisum sativum* (gi|121676). *At*GR1: *A. thaliana* (At3g24170; gi|1346194); *Ps*GR1: *P. sativum* (gi|2500116). Cysteines within the redox active center are marked (*). The alignment was performed with ClustalX.

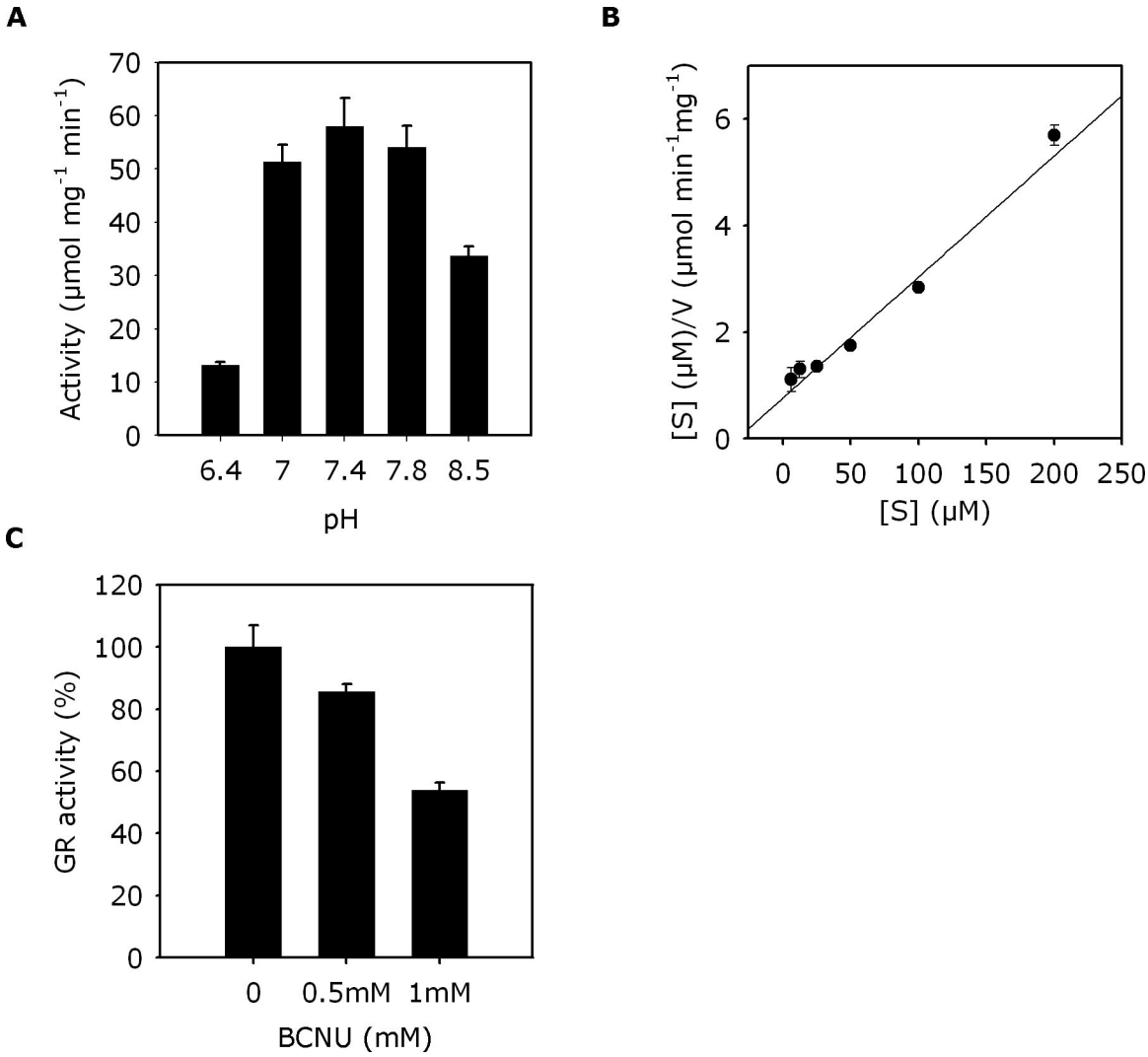

Fig. S2. Biochemical characterization of cytosolic glutathione reductase GR1. (*A*) pH dependence of GR1. Enzymatic assays were performed in sodium phosphate buffers at pH 6.4–8.5, highest activity was reached at pH 7.4. (*B*) Hanes plot for kinetic analysis of GR1 regarding the substrate NADPH. The enzyme assay was performed with 1 mM GSSG and 6.25–200 μM NADPH. All values are means ± SD ($n = 3$). $R^2 = 0.97$. (*C*) Inhibition of GR1 by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BCNU was added to the assay to the indicated concentrations and the decrease in the initial velocity of GR1 was compared with the control. Means ± SD ($n = 3$).

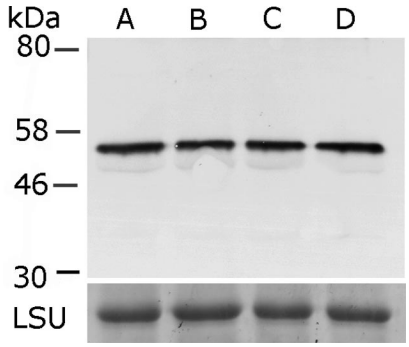

Fig. S3. Gel blot analysis of GR2 protein in different mutant backgrounds. Protein gel blot analysis with antiserum raised against GR2; 15 μg of total protein of Col-0 (*A*), *gr1-1* (*B*), *gr1-2* (*C*), and *ntra ntrb* double mutant (*D*) were separated on a 10% SDS/PAGE and electro blotted to nitrocellulose. Equal loading in is confirmed by staining of the large subunit (LSU) of 1,5-bisphosphate carboxylase/oxygenase (Rubisco).

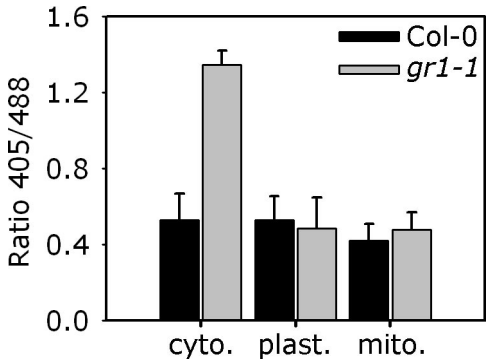

Fig. S4. Compartment-specific analysis of E_{GSH} in wild-type Col-0 and *gr1* plants. For measurement of cytosolic E_{GSH}, plants were transformed with GRX1-roGFP2. Measurements in plastid stroma and the mitochondrial matrix were done with free roGFP2. Images of seedling leaves were taken by CLSM with excitation at 405 and 488 nm, respectively. Data show means ± SD ($n = 5$).

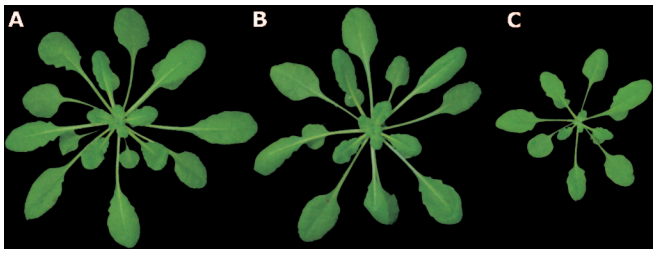

Fig. S5. Phenotypes of *gr1* and *ntra ntrb* deletion mutants. (*A*) Col-0, (*B*) *gr1-1*, and (*C*) *ntra ntrb* double mutant. All plants were 6 weeks old.

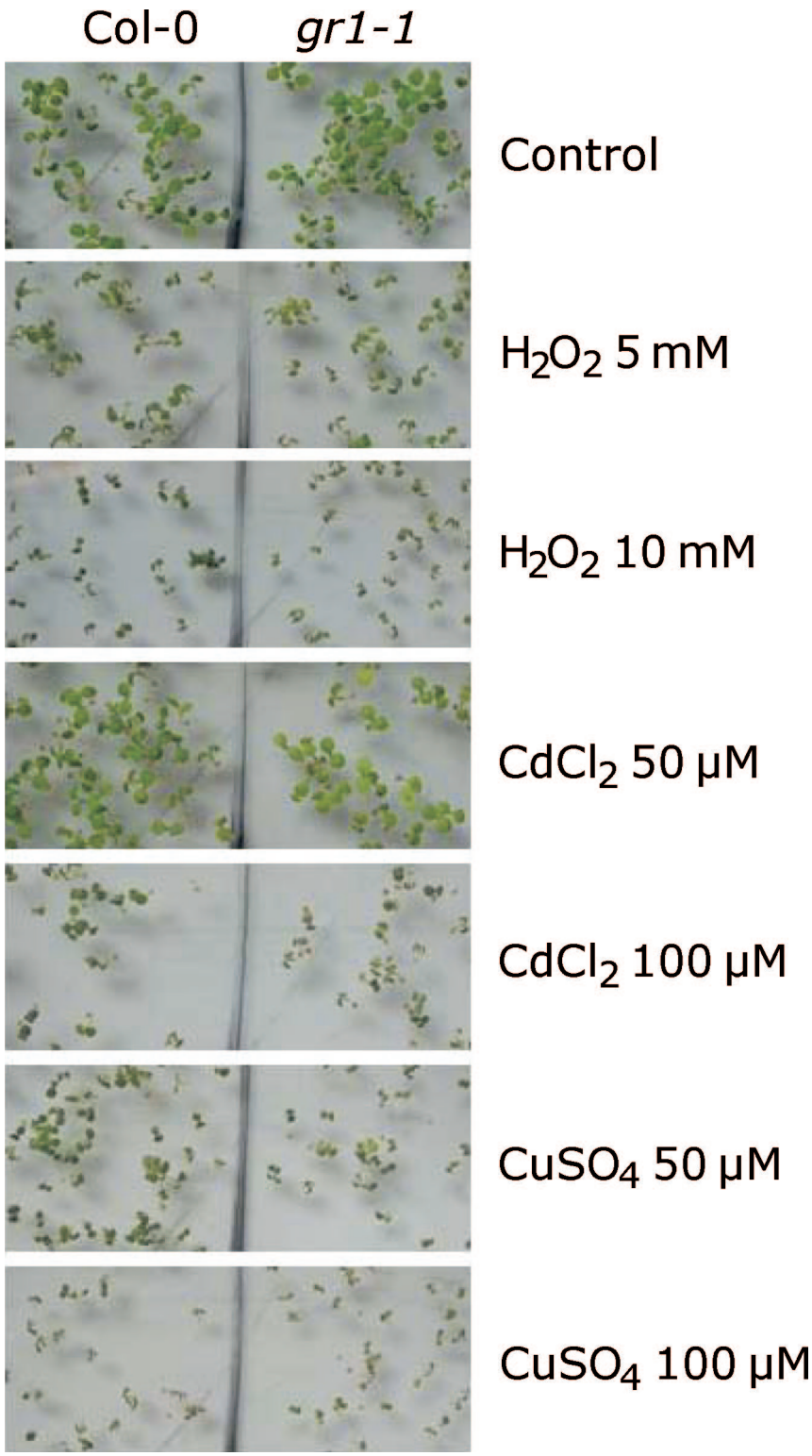

Fig. S6. Growth of wild-type (Col-0), and *gr1-1* mutant under stress conditions. Seeds were soaked on 0.5× MS agar plates supplemented or not (C) with different concentrations of H_2O_2, $CdCl_2$, and $CuSO_4$. Plants were grown under a 16-h light/8-h dark regime for 5 d.

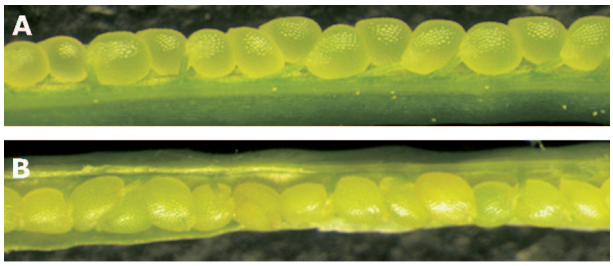

Fig. S7. Self-fertilization of *GR1/gr1-1 ntra ntrb* plants does not cause premature ovule abortion. (*A*) Col-0; (*B*) *GR1/gr1-1 ntra ntrb*. All seeds in siliques developing on a *GR1/gr1-1 ntra ntrb* show a wild-type phenotype. Lack of ovule abortion indicates that haploid ovules carrying mutant alleles for all 3 genes are still viable.

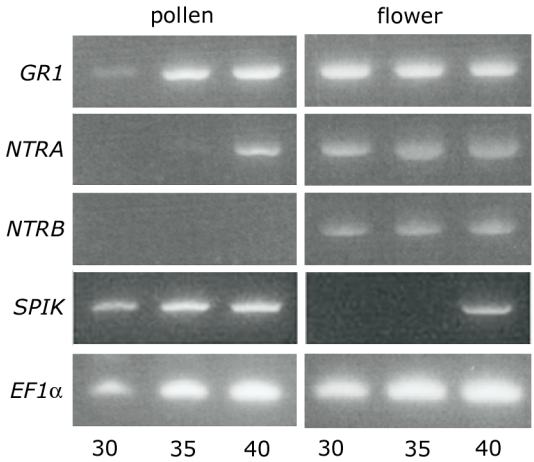

Fig. S8. Semi-quantitative RT-PCR was done on RNA extracted from mature pollen and mature flowers for 30, 35, and 40 cycles. *SPIK* was used as a pollen specific control gene. Note that the level of SPIK cDNA is almost saturating after 30 cycles in pollen. However, 40 cycles are required to detect SPIK cDNAs in flowers, because of the low ratio of pollen RNAs in flower RNAs.

Other Supporting Information Files

Table S1 (PDF)
Table S2 (PDF)