

<https://helda.helsinki.fi>

Root-type ferredoxin-NADP(+) oxidoreductase isoforms in Arabidopsis thaliana : Expression patterns, location and stress responses

Grabsztunowicz, Magda

2021-02

Grabsztunowicz , M , Rantala , M , Ivanauskaite , A , Blomster , T , Koskela , M M , Vuorinen , K , Tyystjarvi , E , Burow , M , Overmyer , K , Mähönen , A P & Mulo , P 2021 , ' Root-type ferredoxin-NADP(+) oxidoreductase isoforms in Arabidopsis thaliana : Expression patterns, location and stress responses ' , Plant, Cell and Environment , vol. 44 , no. 2 , pp. 548-558 . <https://doi.org/10.1111/p>

<http://hdl.handle.net/10138/338073>

<https://doi.org/10.1111/pce.13932>

unspecified

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.



This is a self-archived – parallel published version of an original article. This version may differ from the original in pagination and typographic details. When using please cite the original.

Wiley:

This is the peer reviewed version of the following article:

CITATION: Grabsztunowicz, M, Rantala, M, Ivanauskaite, A, et al. Root-type ferredoxin-NADP⁺ oxidoreductase isoforms in *Arabidopsis thaliana*: Expression patterns, location and stress responses. *Plant Cell Environ.* 2021; 44: 548– 558.

which has been published in final form at

DOI <https://doi.org/10.1111/pce.13932>

This article may be used for non-commercial purposes in accordance with [Wiley Terms and Conditions for Use of Self-Archived Versions](#).

1 **Root-type ferredoxin-NADP⁺ oxidoreductase isoforms in *Arabidopsis thaliana*: expression**
2 **patterns, location and stress responses**

3 **Authors:**

4 Magda Grabsztunowicz^{a,f}, Marjaana Rantala^a, Aiste Ivanauskaitė^a, Tiina Blomster^{b,c}, Minna M.
5 Koskela^{a,g}, Katariina Vuorinen^b, Esa Tyystjärvi^a, Meike Burow^{d, e}, Kirk Overmyer^b, Ari Pekka
6 Mähönen^{b, c} and Paula Mulo^a

7
8 **Contact Information:**

9 ^aDepartment of Biochemistry, Molecular Plant Biology, University of Turku, Biocity A, Turku,
10 Finland; ^bOrganismal and Evolutionary Biology Research Program, Faculty of Biological and
11 Environmental Sciences, and Viikki Plant Science Centre, University of Helsinki, P.O. Box 65
12 (Viikinkaari 1), FI-00014 Helsinki, Finland; ^cInstitute of Biotechnology, HiLIFE, University of
13 Helsinki, P.O. Box 65 (Viikinkaari 1), FI-00014 Helsinki, Finland; ^dDynaMo Center, Department of
14 Plant and Environmental Sciences, University of Copenhagen, Frederiksberg C, Denmark;
15 ^eCopenhagen Plant Science Centre, Department of Plant and Environmental Sciences, University of
16 Copenhagen, Frederiksberg C, Denmark.

17
18 **Present address:**

19 Magda Grabsztunowicz^g Department of Plant Physiology, Adam Mickiewicz University, ul.
20 Uniwersytetu Poznańskiego 89, 61-614 Poznań, Poland

21
22 Minna Koskela^f Institute of Microbiology, Czech Academy of Sciences, Centre Algatech,
23 Novohradská 237 - Opatovický mlýn, 379 81 Třeboň, Czech Republic.

24
25 **Corresponding author:** Paula Mulo, Department of Biochemistry, Molecular Plant Biology,
26 University of Turku, Biocity A, Turku, Finland. E-mail: pmulo@utu.fi

27
28 **Running head:** *Arabidopsis thaliana* RFNR isoforms

29
30 **Funding:** This study was financially supported by Academy of Finland (307335) for M.G., A.I.,
31 M.R., T.B., M.M.K, E.T., A.P.M., K.V., K.O. and P.M., (321616) for A.I., M.R. and P.M., Doctoral
32 Programme in Molecular Life Sciences at the University of Turku (A.I., M.M.K), University of
33 Helsinki Doctoral Program in Plant Science (K.V.), Turku University Foundation (M.R.) and The
34 Danish National Research Foundation, DNRf grant 99 (M.B.).

35 **Abstract**

36 In *Arabidopsis* two leaf-type ferredoxin-NADP⁺ oxidoreductase (LFNR) isoforms function in
37 photosynthetic electron flow in reduction of NADP⁺, while two root-type FNR (RFNR) isoforms
38 catalyze reduction of ferredoxin in non-photosynthetic plastids. As the key to understanding the
39 function of RFNRs might lie in their spatial and temporal distribution in different plant tissues and
40 cell types, we examined expression of *RFNR1* and *RFNR2* genes using β -glucuronidase (GUS)
41 reporter lines and investigated accumulation of distinct RFNR isoforms using a GFP approach and
42 Western blotting upon various stress conditions. We show that while *RFNR1* promoter is active in
43 leaf veins, root tips and in the stele of roots, *RFNR2* promoter activity is present in leaf tips and root
44 stele, epidermis and cortex. RFNR1 protein accumulates as a soluble protein within the plastids of
45 root stele cells, while RFNR2 is mainly present in the outer root layers. Ozone treatment of plants
46 enhanced accumulation of RFNR1, whereas low temperature treatment affected RFNR2
47 accumulation. We further discuss the physiological roles of RFNR1 and RFNR2 based on
48 characterization of *rfnr1* and *rfnr2* knock-out plants and show that even if the function of these
49 proteins is partly redundant, the RFNR proteins are essential for plant development and survival.

50 **Keywords**

51 *Arabidopsis*, ferredoxin-NADP⁺ oxidoreductase, gene expression, low temperature, ozone, plastid,
52 root, stress response

53 **Acknowledgements**

54 Dr. Guido Durian is thanked for help with root protein extraction, Viivi Lindholm for assisting with
55 the root and leaf cross sections, Kai Wang for assistance with ozone and *Pseudomonas* experiments
56 and Tuomas Puukko, Airi Lamminmäki, Leena Grönholm, Henriette Kehlet Jepsen and Laura
57 Laihonen for excellent technical support. Prof. Toshiharu Hase is warmly thanked for sharing the
58 RFNR antibody. GFP imaging was performed at the Cell Imaging Core, Turku Bioscience, Turku,
59 Finland, and glucosinolate analysis at the DynaMo MS-Analytics Facility, Department of Plant and
60 Environmental Sciences, University of Copenhagen, Denmark. Biocenter Finland is acknowledged
61 for the imaging/flow cytometry instrumentation, and Jouko Sandholm for help in confocal
62 microscopy. The authors declare no conflicts of interest.

63 **Introduction**

64 In the plastids of higher plants, ferredoxin-NADP⁺ oxidoreductase (FNR, EC 1.18.1.2) mediates
65 electron transfer between ferredoxin (Fd) and NADP⁺. Leaf-type FNRs (LFNRs) function in the last
66 step of linear photosynthetic electron flow by mediating electron transfer from Fd for the reduction
67 of NADP⁺ to NADPH, while root-type FNRs (RFNRs) catalyze the opposite reaction (reduction of
68 Fd) in non-photosynthetic plastids (Bowsher, Dunbar, & Emes, 1993; Green et al., 1991; Morigasaki,
69 Takata, Suzuki, & Wada, 1993; Morigasaki et al., 1990; Suzuki, Oaks, Jacquot, Vidal, & Gadal,
70 1985). Even if the functional features of non-photosynthetic plastids have remained relatively poorly
71 characterized, it is known that NADPH in these plastids originates from oxidative pentose-phosphate
72 pathway. NADPH is used to reduce Fd, which in turn provides reducing power for the function of
73 enzymes involved in assimilation of nitrogen and sulfur (NiR and SiR, respectively), desaturation of
74 fatty acids as well as redox regulation (FTR) and biosynthesis of glutamate (GOGAT) (Bowsher,
75 Boulton, Rose, Nayagam, & Emes, 1992; Bowsher, Hucklesby, & Emes, 1989; Hanke and Mulo,
76 2013; Oji, Watanabe, Wakiuchi, & Okamoto, 1985).

77 The unique primary structure of RFNR isoforms as compared to LFNR (Hanke, Kurisu, Kusunoki,
78 & Hase, 2004; Onda et al., 2000; Shinohara et al., 2017) results in marked differences in their
79 biochemical properties. For instance, when diaphorase activity of the maize LFNR and RFNR was
80 measured by using 2,6-dichlorophenol indophenol (DCPIP) as an electron acceptor, RFNR showed a
81 higher ($71 \pm 1 \mu\text{M}^{-1}\text{s}^{-1}$) $k_{\text{cat}}/K_{\text{m}}$ value for NADPH than LFNR ($9.9 \pm 0.1 \mu\text{M}^{-1}\text{s}^{-1}$) (Onda et al., 2000).
82 Moreover, distribution of distinct FNR isoforms in photosynthetic and non-photosynthetic parts of
83 plants (Hanke *et al.*, 2005) is accompanied by the presence of specific forms of Fd. Two leaf-type
84 Fds (Fd1 and Fd2), characterized by relatively low redox potentials (-425 and -433 mV, respectively),
85 are found exclusively in leaves, while the gene encoding root-type Fd (Fd3, -337 mV) is expressed
86 in all examined tissues except flowers (Hanke et al., 2004, 2005). The catalytic efficiency and affinity
87 of RFNR was found to be much higher with Fd3 as compared to the leaf-type Fd (Onda et al., 2000),
88 and also *in vitro* sulfite reduction was more efficient when RFNR interacted with a Fd3 as compared
89 to Fd1 (Yonekura-Sakakibara et al., 2000). These properties indicate that electron transfer from
90 NADPH to Fd3 through RFNR is thermodynamically more favorable than to Fd1 (Akashi,
91 Matsumura, Taniguchi, & Hase 1997), and reflects the metabolic needs of non-photosynthetic
92 plastids.

93 Both LFNR and RFNR exist as multiple isoforms in *Arabidopsis thaliana* (hereafter *Arabidopsis*;
94 Hanke et al., 2005) and in maize (Okutani et al., 2005; Onda et al., 2000). The LFNR isoforms have

95 been studied extensively (Hanke, Endo, Satoh, Hase, & Hanke, 2008; Lintala et al., 2007; 2009; 2012;
96 Mulo, 2011; Mulo and Medina, 2017), but currently the two RFNR isoforms are poorly characterized
97 and only limited information is available on their specific functions (Figure 1). In Arabidopsis, two
98 LFNR proteins LFNR1 (At5g66190) and LFNR2 (At1g20020) accumulate exclusively in green
99 tissues, while the genes encoding the two root-type isoforms RFNR1 (At4g05390) and RFNR2
100 (At1g30510) are expressed in both shoots and roots (Hachiya et al., 2016; Hanke et al., 2005).
101 However, accumulation of *RFNR1* and *RFNR2* mRNA is much more prominent in roots, transcripts
102 of *RFNR2* being more abundant than those of *RFNR1* (Hachiya et al., 2016; Hanke et al., 2005). In
103 line with the gene expression analysis, crude shoot and root protein extracts contained both RFNR1
104 and RFNR2 isoforms, and RFNR2 was the prominent isoform under all various conditions studied
105 (Grabsztunowicz, Rokka, Farooq, Aro, & Mulo, 2020; Hachiya et al., 2016; Hanke et al., 2005).
106 Previous studies have shown that nitrate induces the expression of both *RFNR* genes as compared to
107 ammonium (Hanke et al., 2005; Wang, Guegler, LaBrie, & Crawford, 2000; Wang, Okamoto, Xing,
108 & Crawford, 2003), and that that growth of plants in the presence of nitrite specifically induces gene
109 expression of *RFNR2* (Hachiya et al., 2016). As the primary root growth was aberrant upon
110 cultivation of *rfnr2* knock-out mutants on nitrite medium, probably due to increased accumulation of
111 toxic nitrite in the roots, it has been suggested that RFNR2 protein plays an important role in the
112 detoxification of nitrite (Hachiya et al., 2016).

113 Because the spatial distribution of RFNR isoforms in the plant tissues both under standard and stress
114 conditions might provide a key to understand their function, we have examined expression of *RFNR1*
115 and *RFNR2* genes in different tissues of Arabidopsis using β -glucuronidase (GUS) reporter lines and
116 investigated accumulation of distinct RFNR isoforms using a GFP approach and Western blotting.
117 We further studied the impact of ozone exposure and low temperature treatment of the plants on
118 protein accumulation, as these conditions are known to specifically induce expression of the *RFNR*
119 genes. We also characterized knock-out plants lacking one of the RFNR isoforms and discuss the
120 functional properties of RFNR proteins.

121 **Materials and methods**

122 ***Plant material***

123 *Arabidopsis thaliana* Col-0 (WT), *rfnr1-1* (SALK_085009), *rfnr2-1* (SAIL_527G10) and *rfnr2-2*
124 (SALK_133654) (in Col-0 background) as well as *Ler-0* and *rfnr1-2* (GT20582) (in *Ler-0*
125 background) were grown under the light rhythm of 8 h light/16 h darkness at PPFD of 100 $\mu\text{mol m}^{-2}$

126 s⁻¹ (Osram Powerstar HQI-BT 400W/D daylight), 50 % humidity, and 23 °C for five weeks on 50%
127 soil/ 50% vermiculite.

128 Root material for protein analyses and GFP studies was obtained from Arabidopsis plants grown on
129 half-strength Murashige and Skoog (1/2 MS) medium (Murashige and Skoog 1962) in 50 mM MES
130 buffer pH 5.7 containing 0.8 % Agar. After surface sterilization with 70 % ethanol and 0.5 % Triton
131 X- 100 Arabidopsis seeds were sown on the uppermost surface of square Petri dishes and kept at 4
132 °C in the dark for three days. Plants were grown in a vertical position at PPFD of 100 μmol m⁻² s⁻¹,
133 and 23 °C in a long a day conditions (16 h light/ 8 h darkness) for two to five weeks, as indicated.
134 Growth of GUS reporter lines is described below.

135 For ozone treatment and pathogen infections, plants were grown on a 1:1 mixture of peat and
136 vermiculite with 12 h light/ 12 h dark at 23 °C/18 °C with 65 % / 75 % relative humidity. Three-week-
137 old plants were used for the experiments.

138 *Construction of mutant lines*

139 ***pRFNR1::GUS and pRFNR2::GUS***. Putative promoter regions of *RFNR1* and *RFNR2* (-702 bp and
140 -879 bp from the first ATG codon, respectively) were amplified from Arabidopsis genomic DNA
141 using Phusion hot start II DNA polymerase (ThermoFisher Scientific) and specific primers (Table
142 S1). PCR products were cloned into pGreen II 0029-GUS plasmid using T4 DNA ligase
143 (ThermoFisher Scientific). The binary vector was transformed into *Agrobacterium tumefaciens* strain
144 GV3101:pMP90:pSoup and used for floral inoculation of Arabidopsis Col-0 plants according to
145 Narusaka, Shiraishi, Iwabuchi, & Narusaka (2010). The transgenic seedlings were selected from 1/2
146 MS plates containing 50 μg/mL kanamycin, transferred to soil and allowed to self- pollinate. T3
147 generation of plants (2-3 lines per construct) were used for the localization analyzes.

148 ***pRFNR1::RFNR1-GFP and pRFNR2::RFNR2-GFP***. The putative promoter regions of *RFNR1*
149 and *RFNR2* were amplified from Arabidopsis genomic DNA as described above. PCR products were
150 cloned into pK7FWG2 plasmid without the 35S promoter (Karimi, Inzé, & Depicker, 2010) in SacI-
151 SpeI restriction sites. The coding regions of *RFNR1* and *RFNR2* were amplified from Arabidopsis
152 cDNA (Table S1) and cloned into pDONR221 vector using BP ClonaseTM II enzyme mix (Invitrogen)
153 according to manufacturer's instruction. Coding regions of *RFNR* or *RFNR2* were subcloned into
154 pK7FWG2 vector containing respective promoter sequences by LR reaction using LR ClonaseTM II
155 enzyme mix (Invitrogen). The binary vectors were transformed into *Agrobacterium* and used for
156 floral inoculation of Arabidopsis.

157 ***GUS staining and histology***

158 Seeds of GUS-reporter lines were surface-sterilized within an exicator containing chlorine gas and
159 stratified in 4 °C in 0.1 % sterile agarose until plating on in vitro growth media containing 1/2 MS
160 with vitamins (Duchefa), 0.8 % Plant Agar (Duchefa), 1 % sucrose (Duchefa) and pH adjusted to 5.7-
161 5.8 with MES buffer (Duchefa). Plants grown vertically in 16h/ 8h (day /night) photoperiod and 23°C
162 temperature were fixed with cold 90 % (v/v) acetone on ice for 30 min. Samples were washed twice
163 on ice with cold 0.05 M sodium phosphate buffer (pH 7.4) for 10 minutes and vacuum-infiltrated in
164 GUS staining solution (0.05 M sodium phosphate buffer, pH 7.4; 1.5 mM ferrocyanide, 1.5 mM
165 ferricyanide, 1 mM X-glucuronic acid, 0.1 % Triton X-100) for 20 min and incubated further in 37
166 °C in darkness for a total staining time of 2 h (5- and 7-day samples) or 3 h (14-day samples). After
167 washing twice with sodium phosphate buffer (pH 7.4), the staining was imaged under
168 stereomicroscope (Leica MZ10F with Leica DFC490 digital camera) and/or the samples were fixed
169 for cross sectioning according to Idänheimo et al. (2014). No further staining was used prior imaging
170 with Leica 2500 microscope (10x, 20x and 40x objectives). The experiment was repeated twice.

171 ***Fluorescence microscopy***

172 The GFP fusion proteins from 2-week-old Arabidopsis roots were imaged with confocal microscope
173 Zeiss LSM780. Single-plane images were acquired using Plan-Apochromat 20x/0.8 objective. For
174 3D image reconstruction of Z-stacks, sequential confocal images were collected (21 slices, 20 µm
175 and 49 slices 48 µm for plants expressing *pRFNR1::RFNR1-GFP* and *pRFNR1::RFNR1-GFP*,
176 respectively GFP was excited with 488 laser and detected at 507-560 nm. Localization of chloroplast
177 marker protein pt-ck has been described in Nelson, Cai, & Nebenführ (2007). The CFP was excited
178 with 458 nm laser and the emission detected at 460-506 nm. Zeiss Zen imaging software (version
179 2.3) was used for creating the images. To improve the RFNR2-GFP signal 8 days old seedlings were
180 treated in +5 °C for five days.

181 ***Protein extraction***

182 Arabidopsis root protein were extracted as described by Raorane, Narciso, & Kohli (2016) with some
183 modifications. The root material was ground with mortar and pestle in liquid nitrogen in the presence
184 of homogenization buffer (50 mM Tris-HCl, pH 7.5, 2% SDS, PierceTM Protease inhibitor, 1 tab/10
185 mL) in proportion 1:2 (roots: buffer w/v). The homogenate was filtered through one layer of
186 Miracloth, then heated at 56 °C for 20 min and cooled on ice. After centrifugation (118 500 x g, 5
187 min, at room temperature), the supernatant was collected and used for further experiments.

188 Proteins from Arabidopsis rosettes were isolated by grounding the plant material in liquid nitrogen
189 with the extraction buffer (100 mM Tris- HCl, pH 8, 100 mM NaCl, 10 mM dithiothreitol, 0.5% (v/v)
190 Triton X-100, Pierce™ Protease inhibitor, 1 tablet/10 mL). After centrifugation (18500 x g, 20 min,
191 4 °C) the supernatant was collected, divided into small aliquots, frozen and stored at -80 °C until
192 further use.

193 ***Root plastid isolation***

194 Roots of four week old plants were ground in homogenization buffer (50 mM Tris-HCl, pH 7.5, 330
195 mM sorbitol, 1 mM EDTA, 1 mM MgCl₂, 0,1% BSA, Pierce™ Protease inhibitor, 1 tablet/10 mL),
196 filtered through one layer of Miracloth and centrifuged (4000 x g, 3 min, 4 °C). The plastid enriched
197 pellet was resuspended in homogenization buffer, loaded on 10% Percoll and centrifuged (4000 x g
198 5 min, 4 °C). The plastid fraction was collected, washed and treated with shock buffer (5 mM sucrose,
199 10 mM Hepes- NaOH, 5 mM MgCl₂) as previously described (Bowsher et al., 1989).

200 ***SDS-PAGE and protein gel blot analysis***

201 Root and leaf extracts as well as root plastid proteins were solubilized with 4 x Laemmli buffer
202 (Laemmli, 1970) and run on 12 % - 15 % SDS-PAGE gels. The gels were blotted to PVDF membrane
203 (Merck), membranes were blocked with 5 % milk and the proteins immunodetected using the ECL
204 system (GE Healthcare). The RFNR antibody was a generous gift from T. Hase (Hanke et al., 2005).

205 ***Determination of chlorophyll content***

206 Chlorophyll content in leaf discs was calculated according to Inskeep and Bloom (1985). The 0.5 cm²
207 discs were cut from the leaves of 4-week old plants and incubated overnight in 1 mL of
208 dimethylformamide in darkness and room temperature. The absorbance was read at 646.6 nm, 663.6
209 nm and 750 nm.

210 ***Glucosinolate analysis***

211 Glucosinolate contents were measured for root and shoot of each plant individually (n=4-5).
212 Glucosinolates were extracted as desulfo-glucosinolates as described before (Crocoll, Mirza,
213 Reichelt, Gershenzon, & Halkier, 2016). Desulfo-glucosinolates were then quantified by
214 UHPLC/TQ-MS on an Advance™-UHPLC/EVOQ™Elite-TQ-MS instrument (Bruker, Bremen,
215 Germany) equipped with a C-18 reversed phase column (Kinetex 1.7 u XB-C18, 10 cm × 2.1 mm,
216 1.7 µm particle size, Phenomenex, Torrance, CA) as described previously (Crocoll et al., 2016,
217 Alternate Protocol 2).

218 ***Statistical analysis***

219 *Botrytis* lesion size data was analyzed with a linear mixed effect model and Tukey-Post hoc test;
220 analysis was performed in R (2017, version 3.4.0) using nlme (Pinheiro, Bates, DebRoy, Sarkar, R
221 Core Team, 2017) and multcomp (Hothorn, Bretz, & Westfall, 2008) packages.

222 Statistical analysis for glucosinolate analysis was performed with R version 3.3.2 (R Core Team
223 2016). Differences between glucosinolate levels in extracts from *Arabidopsis* wt and mutant lines
224 were tested using One-Way Anova (aov function) followed by pairwise t-test function with Holm-
225 adjustment for multiple testing.

226 ***Stress treatments***

227 **Low temperature.** 4-week old plants (grown either on soil or on plates, as indicated) were exposed
228 to +5 °C for 0, 2 and 6 days under standard growth light conditions (see above). The cold growth was
229 performed at 5 °C in 16 h light/ 8 h darkness. Rosettes of soil-grown plants and roots from plates
230 were sampled after 18 weeks and 11 weeks of growth, respectively.

231 **Ozone.** Three-week old soil-grown plants were exposed to 300 nl L⁻¹ ozone for 6 h and then recovered
232 at ambient ozone concentrations (< 20 nl L⁻¹) for 18 h.

233 **Pathogen infections.** For induced resistance experiments, one leaf per 18-day-old plant was
234 infiltrated with an avirulent DC3000 *Pseudomonas syringae pathovar tomato* (Pst) bearing
235 transgenically-expressed avrPpt2 (10⁸ cell/mL suspension (OD₆₀₀= 0.2) in 10 mM MgCl₂), or mock
236 inoculated with 10 mM MgCl₂, using a needleless syringe. The treated leaves exhibited visible
237 hypersensitive cell death within 24 h of treatment. At three weeks of age plants were subsequently
238 spray infected with virulent DC3000 (with an empty vector; 5x10⁷ cells/mL suspension (OD₆₀₀= 0.1)
239 in 10 mM MgCl₂ containing 0.04 % Silwet). Plants were photographed 72 h later to document visible
240 symptoms.

241 *Botrytis* infections were performed using four-week-old plants by dropping 3 µl of *Botrytis cinerea*
242 suspension (1x10⁶ spores/mL) in half-strength potato dextrose broth (1/2 PDB). For mock treatment
243 ½ PDB was used. Plants were photographed 48 h post infection and lesions were measured in ImageJ
244 (version 1.47v; Schneider, Rasband, & Eliceiri, 2012).

245

246 **Results and discussion**

247 ***RFNR1* and *RFNR2* show distinct expression patterns in roots and shoots**

248 *RFNR* genes have been shown to be expressed both in the green tissues and roots (Hanke *et al.*, 2004),
249 but detailed analyses of the *RFNR1* and *RFNR2* gene expression patterns and localization of the
250 *RFNR* isoforms have not been performed. As the key to understanding the function of *RFNRs* might
251 lie in their spatial and temporal distribution in different plant tissues and cell types, we produced and
252 analyzed plants expressing GUS (*pRFNR1::GUS* and *pRFNR2::GUS*) or GFP-tagged *RFNR1* and
253 *RFNR2* (*pRFNR1::RFNR1-GFP* and *pRFNR2::RFNR2-GFP*) under the native promoters of *RFNR1*
254 or *RFNR2* to assess the specific location of the promoter activity and protein accumulation of the
255 distinct *RFNR* isoforms.

256 Analysis of the GUS signals from the transgenic homozygous *pRFNR1::GUS* and *pRFNR2::GUS* T3
257 plants showed that although both *RFNR* genes were expressed in the roots, they showed spatially
258 divergent expression patterns dependent on the developmental stage of the root (Figure 2; Summary
259 of the results presented in Table S1). The *RFNR1* gene was expressed in the root tip, especially
260 pericycle (Figure 2A,C), and weakly in the stele of young roots (Figure 2 B). The *RFNR2* gene
261 showed high expression level in root cortex (Figure 2I, N), and weak expression in epidermis and
262 stele (Figure 2H-I). The strong *pRFNR2::GUS* signal from the mature cortex implies that the detected
263 signals are not simply dependent on the plastid density, which is much higher in the root tip than in
264 mature root tissue (Bramham and Pyke 2017). Both *RFNR* genes were expressed in the xylem
265 parenchyma and more variably in outer tissues such as periderm in later developmental stages (Figure
266 2F-G, M-N). It should be noted that the *RFNR2* gene was expressed in the tips of developing lateral
267 roots (Figure 2O-Q), while no signal was detected from the primary root tip (Figure 2H,J).

268 As the regulation of gene expression may not be reflected at the protein level, we focused on the
269 accumulation of the *RFNR1* and *RFNR2* proteins using two distinct approaches. First, localizations
270 of the GFP-tagged *RFNR1* and *RFNR2* proteins were observed by confocal microscopy (Figure 3,
271 Table S1) using the plastid indicator Arabidopsis line pt-ck CS16265 as a control. In roots, the
272 speckled CFP signal representing root plastids was dispersed evenly throughout the root (Figure 3A),
273 and both *RFNR* isoforms appeared to accumulate within the plastids (Figure 3). Distribution of
274 *RFNR1* and *RFNR2* in the roots, however, showed marked differences. GFP signal representing
275 *RFNR1* was restricted to the inner layers of the root (Figure 3B, E), which is in line with the data
276 obtained from the transcriptional reporter lines (Figure 2). In addition to inner root layers, plastids
277 containing *RFNR2* were also detected in the cortex that did not show any *RFNR1-GFP* signal (Figure
278 3C-E). The strict regulation of *RFNR* gene expression and differential accumulation of the *RFNR*
279 isoforms within the root suggests that *RFNR1* and *RFNR2* may have distinct physiological roles in
280 the root metabolism.

281 Secondly, we extracted proteins from the leaves and the roots of the plants, separated them using
282 SDS-PAGE and performed protein immunoblot analysis using RFNR antibody that also recognizes
283 the LFNR isoforms. Notably, as the predicted molecular weight of RFNR1 and RFNR2 (42.4 kDa
284 and 42.9 kDa, respectively) as well as pI (8.84 and 8.85, respectively) is very close to each other,
285 separation of the RFNR isoforms from each other is somewhat problematic. In line with previous
286 findings (Grabsztunowicz et al., 2020; Hachiya et al., 2016; Hanke et al., 2005) as well as our GFP
287 studies (Figure 3B, C), both RFNR isoforms were detected in the roots, RFNR2 giving stronger signal
288 than RFNR1 (Figure 3F). LFNR was not detectable in the root tissue (Figure 3F). Moreover, to study
289 the localization of RFNR isoforms in more detail, root plastids were isolated and further fractionated
290 into soluble and membrane pools. In contrast to chloroplast-targeted LFNR, which is distributed
291 between the membrane-bound and soluble fractions (Benz et al., 2009; Lintala et al., 2007; Matthijs,
292 Coughlan, & Hind, 1986), both RFNR isoforms existed exclusively as soluble proteins (Figure 3F).
293 It should be noted that neither RFNR1 nor RFNR2 are subjected to such a strong N-terminal
294 acetylation as the LFNR proteins (Grabsztunowicz et al. 2020; Lehtimäki et al. 2014), but we can not
295 exclude the possibility that other, so far unidentified modifications may have an impact on the
296 localization of RFNR isoforms.

297 As for the leaves, expression patterns of *RFNR1* and *RFNR2* genes differed markedly from each other.
298 *RFNR1* was strongly expressed in leaf veins (Fig. 1A, D-E), while expression of the *RFNR2* gene
299 was detectable only in leaf tips (hydathodes) of young seedlings (Figure 2H, K-L). Despite clear
300 expression of the *RFNR1* and *RFNR2* genes in the vasculature, no GFP signal was detected from the
301 leaves. A weak GFP signal, presumably resulting from a low level of RFNR protein in the leaves,
302 could be masked by high chlorophyll autofluorescence. Nevertheless, both RFNR isoforms gave a
303 signal when studied using immunoblotting with an FNR antibody (Figure 3F). The antibody also
304 detected LFNRs (LFNR1 and LFNR2 migrate as a single band designated as 'LFNR'), which showed
305 high accumulation in leaves and complicates detection of the RFNR signal (Figure 3). It is intriguing
306 that both RFNR isoforms were clearly immunodetected in the leaf veins (Figure 3F), even if no
307 *RFNR2* promoter activity was observed in the leaf vascular tissue (Figure 2). Within veins, all types
308 of phloem cells, i.e. phloem parenchyma cells, companion cells and sieve elements, contain plastids.
309 Phloem parenchyma cells contain chloroplasts organized at the cell periphery, while in companion
310 cells plastids form a thin parietal layer occupying a large portion of cell volume (Cayla et al., 2015).
311 The enucleate sieve elements, in turn, contain numerous small, non-photosynthetic plastids (Cayla et
312 al., 2015), which might accommodate RFNR proteins. Although our current results do not allow us
313 to conclude the exact distribution of RFNR isoforms between these cell and plastid types, it appears

314 likely that RFNRs are mainly present in the non-photosynthetic plastids. As the functional role(s) of
315 non-photosynthetic plastids in leaves and in leaf veins is still poorly understood, more research is
316 required to elucidate the impact of RFNRs in this compartment.

317 **Low temperature and ozone affect accumulation of RFNR isoforms**

318 Even if the RFNR1 and RFNR2 proteins share high sequence identity (89%) (Hanke et al., 2005;
319 Figure 1), differences in expression patterns and location (Figure 2, 3) imply that the isoforms may
320 play unique functional roles. In root plastids RFNR utilizes NADPH originating from oxidative
321 pentose phosphate pathway for the reduction of Fd, which is required for the function of NiR and thus
322 reduction of toxic nitrite to ammonium. Hachiya et al. (2016) have suggested that in the absence of
323 RFNR2 the function of NiR is restricted, which leads to high accumulation of nitrite. This, in turn,
324 appears to disturb primary root growth when nitrite is supplied as the sole source of nitrogen. In
325 addition to nitrogen source, also other environmental factors regulate expression of the *RFNR1* and
326 *RFNR2* genes, as revealed by analysis of the publically available databases (Figure S1). Expression
327 of *RFNR1* was upregulated upon different kinds of biotic stresses that resulted in cell death (including
328 infection with *Botrytis cinerea* and avirulent strains of *Pseudomonas syringae*) as well as upon
329 exposure of plants to ozone, while treatment of plants under low or high temperature had a profound
330 effect on the expression of *RFNR2* (Figure S1). Therefore, we focused on studying the accumulation
331 of RFNR1 and RFNR2 under conditions resulting in differences in *RFNR1* and *RFNR2* gene
332 expression, i.e. upon ozone treatment and low temperature. Moreover, we applied *rfnr1* and *rfnr2*
333 mutants to study the physiological roles of RFNR isoforms in stress responses as well as in
334 accumulation of secondary metabolites, i.e. glucosinolates.

335 ***Ozone and pathogen responses***

336 Exposure of the plants to ozone was performed using a similar experimental set up as earlier described
337 (intact soil-grown Arabidopsis plants treated with ozone), which results in clear visual damage of the
338 leaves in ozone sensitive Arabidopsis lines (e.g. Blomster et al., 2011). This approach allowed us to
339 compare ozone sensitivity of the *rfnr* knock-out mutants to previous studies (see below), and to study
340 the impact of ozone on the accumulation of RFNR proteins in leaves. Root protein extraction of the
341 plants grown on soil, however, was not successful and prevented analyses of the RFNR isoform
342 accumulation in the roots. Figure 3G shows that induction of gene expression was indeed
343 accompanied by an increased accumulation of the specific RFNR isoform. Accumulation of the
344 RFNR1 isoform was markedly increased in the leaves of soil-grown plants that were exposed to 300
345 nl L^{-1} ozone for 6 h. The level of RFNR1 remained high during 18 h of recovery under ambient ozone

346 concentration ($< 20 \text{ nl L}^{-1}$), while no such increase in the accumulation of RFNR2 could be detected
347 (Figure 3G).

348 To pinpoint the function of the RFNR isoforms further, we characterized *rfnr1* and *rfnr2* knock-out
349 mutants under standard conditions and upon exposure to various stresses. For RFNR1, we studied
350 two independent knock-out lines SALK_085009 (*rfnr1-1*) in the Columbia-0 (Col-0) and Gt20582
351 (*rfnr1-2*) in Landsberg *erecta* accession, while for RFNR2, SAIL_527G10 (*rfnr2-1*) and
352 SALK_133654.52 (*rfnr2-2*), both in Col-0 background, were used. Figure 4 shows that accumulation
353 of either RFNR1 or RFNR2 protein in the *rfnr1* and *rfnr2* knock-out lines was under detection limit
354 of the antibody, even if it has been reported that SAIL_527G10 is actually a knock-down line, which
355 accumulates 18-25 % of the *RFNR2* mRNA as compared to WT (Hachiya et al., 2016). Under
356 standard growth conditions, the phenotype of *rfnr1* and *rfnr2* plants did not differ from that of WT
357 (Figure S2A; Figure 4). Moreover, Figure S2 shows that the rosette growth, root growth, chlorophyll
358 content, and chlorophyll a/b ratio of the leaves was not affected by the loss of either RFNR isoform.
359 Because the *rfnr1* and *rfnr2* knock-out mutants did not show any major morphological phenotypes,
360 we crossed the single mutants in order to produce double knock-out plants, which might reveal the
361 processes dependent on RFNR proteins. When the F2 progeny from the *rfnr1 rfnr2* crossing was self-
362 pollinated, no double-knockout seedlings were identified, but the T3 generation was composed of
363 50% heterozygotes (*rfnr1 RFNR2rfnr2* and *RFNR1rfnr1 rfnr2*) and 50 % WT plants (Table 1). The
364 double knock out appears to be lethal rather during the gamete development than upon embryo
365 development, since no aborted seeds were detected in the siliques of the T3 plants (Haruta et al.,
366 2010). Taken together, RFNR isoforms appear to be redundant but essential components in the growth
367 and development of Arabidopsis plants.

368 Because of differential accumulation of RFNR1 and RFNR2 upon exposure of WT Arabidopsis plants
369 to ozone (Figure 4), the *rfnr1* and *rfnr2* mutant plants were subjected to ozone treatment. The
370 appearance of cell death lesions following exposure of the plants to 300 nl L^{-1} ozone for 6 h
371 demonstrated that the *rfnr2-1* plants were more sensitive to ozone than the *rfnr1* mutants or WT
372 (Figure 4B). This is intriguing, since ozone specifically induced expression of RFNR1 at mRNA and
373 protein level, indicating an important role for RFNR1 in ozone responses (Figure 4, Figure S1). It
374 should be noted, however, that the *rfnr2-2* plants showed WT-like ozone tolerance (Figure 4B), and
375 that we do not currently know the ultimate reason behind this difference. Acute ozone exposure of
376 plants leads to formation of secondary reactive oxygen species (ROS) in the apoplast, which induces
377 endogeneous, enzymatic production of ROS. This 'oxidative burst' is similar to that induced by
378 pathogen attack, and ozone has been widely used as a non-invasive tool to study signaling pathways

379 leading to programmed cell death and hypersensitive response (Vainonen and Kangasjärvi 2015).
380 Therefore, we also tested the defense responses of *rfnr1* and *rfnr2* plants against *Botrytis cinerea* and
381 *Pseudomonas syringae*, and measured the accumulation of Met-derived aliphatic and Trp-derived
382 indolic glucosinolates in the WT and *rfnr* mutant plants.

383 Glucosinolates, important compounds rich in sulfur and nitrogen, are involved in defense reactions
384 against herbivores and microbial pathogens and they are known to accumulate both in Arabidopsis
385 shoots and roots (Andersen et al., 2013; Burow and Halkier, 2017; Clay, Adio, Denous, Jander, &
386 Ausubel, 2009). Our results support the earlier findings showing that total glucosinolate content of
387 roots is higher than that of shoots and that short chain aliphatic glucosinolates (derived from
388 methionine elongated by 1-3 methylene groups) dominate in shoots, while indole glucosinolates
389 accumulate predominantly in roots (Figure 4C, Andersen et al., 2013; Brown, Tokuhisa, Reichelt, &
390 Gershenzon, 2003; Petersen, Chen, Hansen, Olsen, & Halkier, 2002). No significant differences,
391 however, could be detected between the glucosinolate profiles of the *rfnr1*, *rfnr2* and WT plants
392 (Figure 4C), which is in line with the finding that the defense responses of *rfnr1* and *rfnr2* plants
393 against *Botrytis cinerea* and *Pseudomonas syringae* were similar as in the WT (Figure S3).

394 As scavenging of ROS within the chloroplast requires a complex network of metabolites and enzymes
395 (ascorbate-glutathione or Foyer-Halliwell-Asada pathway), including Fd and NADPH, the FNR
396 proteins lie in the very center of cellular redox metabolism. Several studies have indeed implicated
397 FNR in the responses to oxidative stress, even if its exact role has not been resolved yet (Palatnik,
398 Valle, & Carrillo, 1997). For instance, decreased accumulation of LFNR has been shown to result in
399 increased production of $^1\text{O}_2$ in LFNR antisense tobacco plants (Palatnik et al. 2003), while LFNR
400 overexpression led to enhanced protection from oxidative stress (Rodriguez et al. 2007). Additionally,
401 imbalance in the FNR/Fd ratio affected both ROS scavenging and ROS production (Kozuleva et al.
402 2016). Interestingly, it has been shown that the enucleate sieve elements, which are rich in non-
403 photosynthetic plastids (Cayla et al., 2015) and presumably contain RFNR proteins (Figure 3) possess
404 a functional antioxidant system with various ROS scavenging enzymes (Walz, Juenger, Schad, &
405 Kehr, 2002). Our results demonstrating increased accumulation of RFNR1 upon ozone exposure
406 (Figure 3G) as well as sensitivity of *rfnr2-1* plants to ozone treatment (Figure 4B) further support the
407 view that FNR proteins might play a role in ROS metabolism, ROS responses and/or ROS signaling
408 in Arabidopsis. It will be intriguing to elucidate the detailed functions and specificities of the RFNR
409 isoforms in ROS signaling network, and to pinpoint the sequence of stress response events both in
410 roots and in shoots.

411 ***Low temperature***

412 Next, we studied the effect of low temperature on RFNR isoform accumulation both in leaves and in
413 roots. First, *Arabidopsis* plants were grown under standard conditions on plates for four weeks, and
414 treated under low temperature (+5 °C) for six days followed by sampling and protein extraction at
415 day 0 (ctrl), day 2 and day 6. Exposure of plants to low temperature for two days resulted in decreased
416 accumulation of RFNR2 in the roots, but after six days the RFNR2 content increased and exceeded
417 the control level (Figure 3H). This result is consistent with the fact that treatment of the
418 *pRFNR2::RFNR2-GFP* plants at low temperature (8-days old seedlings were transferred to +5 °C for
419 five days) resulted in markedly increased GFP signal from the root plastids (Figure 3D). In the leaves,
420 however, low temperature treatment resulted in decreased accumulation of both RFNR isoforms
421 (Figure 3I).

422 A possible explanation for the induction of the *RFNR2* gene and accumulation of RFNR2 protein in
423 response to low temperature may be related to the fact that RFNR proteins produce reducing power
424 in the form of Fd, which is needed for desaturation of fatty acids in plastids. Desaturation of fatty
425 acids is related to temperature-induced changes in membrane fluidity, and polyunsaturated lipids have
426 a crucial role in maintenance of cellular functions and plant viability upon exposure to low
427 temperature (Miquel, James, Dooner, & Browse, 1993). The location of ω -3 fatty acid desaturase 7
428 (FAD7) in the plastids of cells surrounding the vasculature (Soria-Garcia et al., 2019), coinciding
429 with that of RFNR2 (Figure 3), supports the view that RFNR2 might serve as a link between primary
430 metabolism and cold acclimation. Surprisingly, however, when the *rfnr1* and *rfnr2* mutant plants
431 were exposed to low temperature no visual differences could be detected between the WT and mutant
432 lines (Figure 5A, B). It is plausible that FAD3, which is located in the endoplasmic reticulum and
433 accepts reducing power from cytochrome b_5 instead of Fd (Shanklin and Cahoon 1998), may be
434 capable of maintaining appropriate root lipid desaturation level (Soria-Garcia et al., 2019) even if low
435 temperature induced lipid desaturation in plastids would be impaired due to the loss of RFNR2.

436 **Conclusions**

437 In the present study, we show that (i) the *Arabidopsis thaliana* RFNR isoforms show distinct gene
438 expression patterns in the roots and in the leaves, and that (ii) both isoforms accumulate mainly in
439 root plastids (iii) as a soluble protein. (iv) Even if only the RFNR1 gene is expressed in leaf veins,
440 both isoforms can be detected in leaf vasculature. (v) Ozone exposure induces accumulation of
441 RFNR1, while (vi) low temperature affects accumulation of RFNR2. (vi) The defect of one RFNR

442 isoform does not impair growth and development of the plants, while double knock-out appears to be
443 lethal, implying that the RFNRs have redundant, but essential roles in Arabidopsis metabolism.

444 **References**

445 Akashi, T., Matsumura, T., Taniguchi, I., & Hase, T. (1997). Mutational analysis of the redox
446 properties of the [2Fe-2S] cluster in plant ferredoxins. *Journal of Inorganic Biochemistry* 67, 255.

447 Andersen, T. G., Nour-Eldin, H. H., Fuller, V. L., Olsen, C. E., Burow, M., & Halkier, B. A.
448 (2013). Integration of biosynthesis and long-distance transport establish organ-specific
449 glucosinolate profiles in vegetative arabidopsis. *Plant Cell*, 25, 3133–3145.
450 <https://doi.org/10.1105/tpc.113.110890>

451 Benz, J. P., Stengel, A., Lintala, M., Lee, Y. H., Weber, A., Philippar, K., ... Bölder, B. (2009).
452 Arabidopsis Tic62 and ferredoxin-NADP(H) oxidoreductase form light-regulated complexes that
453 are integrated into the chloroplast redox poise. *Plant Cell*, 21, 3965–3983.
454 <https://doi.org/10.1105/tpc.109.069815>

455 Blomster, T., Salojärvi, J., Sipari, N., Brosché, M., Ahlfors, R., Keinänen, M., ... Kangasjärvi, J.
456 (2011). Apoplastic reactive oxygen species transiently decrease auxin signaling and cause stress-
457 induced morphogenic response in Arabidopsis. *Plant Physiology*, 157, 1866–1883.
458 <https://doi.org/10.1104/pp.111.181883>

459 Bowsher, C.G., Dunbar, B., & Emes, M. G. (1993). The purification and properties of ferredoxin-
460 NADP⁺ oxidoreductase from roots of *Pisum sativum* L. *Protein Expression and Purification* 4, 512-
461 518.

462 Bowsher, C. G., Boulton, E. L., Rose, J., Nayagam, S., & Emes, M. J. (1992). Reductant for
463 glutamate synthase in generated by the oxidative pentose phosphate pathway in non-photosynthetic
464 root plastids. *The Plant Journal*, 2, 893–898. <https://doi.org/10.1111/j.1365-313X.1992.00893.x>

465 Bowsher, C. G., Hucklesby, D. P., & Emes, M. J. (1989). Nitrite reduction and carbohydrate
466 metabolism in plastids purified from roots of *Pisum sativum* L. *Planta*, 177(3), 359–366.
467 <https://doi.org/10.1007/BF00403594>

468 Bramham, L., & Pyke, K. (2017). Changing plastid dynamics within early root and shoot apical
469 meristem-derived tissue of *A. thaliana*. *Bioscience Horizons*, 10, 1–10.
470 <https://doi.org/10.1093/biohorizons/hzx001>

471 Brown, P. D., Tokuhsa, J. G., Reichelt, M., & Gershenzon, J. (2003). Variation of glucosinolate
472 accumulation among different organs and developmental stages of *Arabidopsis thaliana*.
473 *Phytochemistry*, 62, 471–481. [https://doi.org/10.1016/S0031-9422\(02\)00549-6](https://doi.org/10.1016/S0031-9422(02)00549-6)

474 Burow, M., & Halkier, B. A. (2017). How does a plant orchestrate defense in time and space? Using
475 glucosinolates in *Arabidopsis* as case study. *Current Opinion in Plant Biology*, 38, 142–147.
476 <https://doi.org/10.1016/j.pbi.2017.04.009>

477 Cayla, T., Batailler, B., Le Hir, R., Revers, F., Anstead, J. A., Thompson, G. A., ..., Dinant, S. (2015).
478 Live imaging of companion cells and sieve elements in *Arabidopsis* leaves. *PLoS ONE*, 10(2), 1–22.
479 <https://doi.org/10.1371/journal.pone.0118122>

480 Clay, N. K., Adio, A. M., Denoux, C., Jander, G., & Ausubel, F. M. (2006). Innate immune response.
481 *Encyclopedic Reference of Immunotoxicology*, 323, 347–348. [https://doi.org/10.1007/3-540-27806-](https://doi.org/10.1007/3-540-27806-0_812)
482 [0_812](https://doi.org/10.1007/3-540-27806-0_812)

483 Crocoll, C., Mirza, N., Reichelt, M., Gershenzon, J., & Halkier, B. A. (2016). Optimization of
484 engineered production of the glucoraphanin precursor dihomomethionine in *Nicotiana benthamiana*.
485 *Frontiers in Bioengineering and Biotechnology*, 4, 1–9. <https://doi.org/10.3389/fbioe.2016.00014>

486 Grabsztunowicz, M., Rokka, A., Farooq, I., Aro, E-M., & Mulo, P. (2020). Gel-based proteomic
487 map of *Arabidopsis thaliana* root plastids and mitochondria. *BMC Plant Biol*, 20, 413.
488 <https://doi.org/10.1186/s12870-020-02635-6>

489 Green, L. S., Yee, B. C., Buchanan, B. B., Kamide, K., Sanada, Y., & Wada, K. (1991). Ferredoxin
490 and ferredoxin-NADP reductase from photosynthetic and nonphotosynthetic tissues of tomato. *Plant*
491 *Physiology*, 96(4), 1207–1213. <https://doi.org/10.1104/pp.96.4.1207>

492 Hachiya, T., Ueda, N., Kitagawa, M., Hanke, G., Suzuki, A., Hase, T., & Sakakibara, H. (2016).
493 *Arabidopsis* root-type ferredoxin: NADP(H) oxidoreductase 2 is involved in detoxification of nitrite
494 in roots. *Plant and Cell Physiology*, 57(11), 2440–2450. <https://doi.org/10.1093/pcp/pcw158>

495 Hanke, G. T., Endo, T., Satoh, F., & Hase, T. (2008). Altered photosynthetic electron channelling
496 into cyclic electron flow and nitrite assimilation in a mutant of ferredoxin:NADP(H) reductase. *Plant,*
497 *Cell and Environment*, 31(7), 1017–1028. <https://doi.org/10.1111/j.1365-3040.2008.01814.x>

498 Hanke, G. T., Kurisu, G., Kusunoki, M., & Hase, T. (2004). Fd: FNR electron transfer complexes:
499 Evolutionary refinement of structural interactions. *Photosynthesis Research*, 81(3), 317–327.

500 <https://doi.org/10.1023/B:PRES.0000036885.01534.b8>

501 Hanke, G. T., & Mulo, P. (2013). Plant type ferredoxins and ferredoxin-dependent metabolism. *Plant,*
502 *Cell and Environment*, 36(6), 1071–1084. <https://doi.org/10.1111/pce.12046>

503 Hanke, G. T., Okutani, S., Satomi, Y., Takao, T., Suzuki, A., & Hase, T. (2005). Multiple iso-proteins
504 of FNR in Arabidopsis: Evidence for different contributions to chloroplast function and nitrogen
505 assimilation. *Plant, Cell and Environment*, 28(9), 1146–1157. <https://doi.org/10.1111/j.1365-3040.2005.01352.x>

507 Haruta, M., Burch, H.M., Nelson, R.B., Barrett-Wilt, G., Kline K.G., Mohsin, S.B., ... Sussman,
508 M.R. (2010). Molecular characterization of mutant Arabidopsis plants with reduced plasma
509 membrane proton pump activity. *The Journal of Biological Chemistry*, 285, 17918 –17929.
510 <https://doi.org/10.1074/jbc.M110.101733>

511 Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models.
512 *Biometrical Journal*, 50(3), 346–363. <https://doi.org/10.1002/bimj.200810425>

513 Idänheimo, N., Gauthier, A., Salojärvi, J., Siligato, R., Brosché, M., Kollist, H., ... Wrzaczek, M.
514 (2014). The *Arabidopsis thaliana* cysteine-rich receptor-like kinases CRK6 and CRK7 protect against
515 apoplastic oxidative stress. *Biochemical and Biophysical Research Communications*, 445(2), 457–
516 462. <https://doi.org/10.1016/j.bbrc.2014.02.013>

517 Inskeep, W. P., & Bloom, P. R. (1985). Extinction coefficients of chlorophyll *a* and *b* in N,N -
518 dimethylformamide and 80% acetone. *Plant Physiology*, 77, 483–485.
519 <https://doi.org/10.1104/pp.77.2.483>

520 Karimi, M., Inzé, D., & Depicker, A. (2002). GATEWAY™ vectors for *Agrobacterium*-mediated
521 plant transformation. *Trends in Plant Science* 7, 193–195. [https://doi.org/10.1016/S1360-1385\(02\)02251-3](https://doi.org/10.1016/S1360-1385(02)02251-3)

523 Kozuleva, M., Goss, T., Twachtmann, M., Rudi, K., Trapka, J., Selinski, J., ... Hanke, G.T. (2016).
524 Ferredoxin: NADP(H) oxidoreductase abundance and location influences redox poise and stress
525 tolerance. *Plant Physiology* 172, 1480–1493. <https://doi.org/10.1104/pp.16.01084>

526 Laemmli, U. K. (1970). Glycine-SDS-PAGE for separation of proteins. *Nature* 227, 680–685.

527 Lehtimäki, N., Koskela, M., Dahström, K., Pakula, E., Lintala, M., Scholz, M., ... Mulo P. (2014)
528 Post-translational modifications of ferredoxin-NADP⁺ oxidoreductase in *Arabidopsis thaliana*

529 chloroplasts. *Plant Physiology*, 166, 1764-76. doi: 10.1104/pp.114.249094

530 Lintala, M., Allahverdiyeva, Y., Kangasjärvi, S., Lehtimäki, N., Keränen, M., Rintamäki, E., ...
531 Mulo, P. (2009). Comparative analysis of leaf-type ferredoxin-NADP⁺ oxidoreductase isoforms in
532 *Arabidopsis thaliana*. *Plant Journal*, 57, 1103–1115. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-313X.2008.03753.x)
533 313X.2008.03753.x

534 Lintala, M., Allahverdiyeva, Y., Kidron, H., Piippo, M., Battchikova, N., Suorsa, M., ... Mulo, P.
535 (2007). Structural and functional characterization of ferredoxin-NADP⁺-oxidoreductase using knock-
536 out mutants of *Arabidopsis*. *Plant Journal*, 49(6), 1041–1052. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-313X.2006.03014.x)
537 313X.2006.03014.x

538 Lintala, M., Lehtimäki, N., Benz, J. P., Jungfer, A., Soll, J., Aro, E. M., ... Mulo, P. (2012). Depletion
539 of leaf-type ferredoxin-NADP⁺ oxidoreductase results in the permanent induction of photoprotective
540 mechanisms in *Arabidopsis* chloroplasts. *Plant Journal*, 70, 809–817. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-313X.2012.04930.x)
541 313X.2012.04930.x

542 Matthijs, H. C. P., Coughlan, S. J., & Hind, G. (1986). Removal of ferredoxin:NADPH⁺
543 oxidoreductase from thylakoid membranes, rebinding to depleted membranes, and identification of
544 the binding site. *Journal of Biological Chemistry*, 261, 12154–12158.

545 Miquel, M., James, D., Dooner, H., & Browse, J. (1993). *Arabidopsis* requires polyunsaturated lipids
546 for low-temperature survival. *Proceedings of the National Academy of Sciences of the United States*
547 *of America*, 90, 6208–6212. <https://doi.org/10.1073/pnas.90.13.6208>

548 Morigasaki, S., Jin, T., & Wada, K. (1993). Comparative studies on ferredoxin-NADP⁺
549 oxidoreductase isoenzymes derived from different organs by antibodies specific for the radish root-
550 and leaf-enzymes. *Plant Physiology*, 103, 435–440. <https://doi.org/10.1104/pp.103.2.435>

551 Morigasaki, S., Takata, K., Suzuki, T., & Wada, K. (1990). Purification and characterization of a
552 ferredoxin-NADP⁺ oxidoreductase-like enzyme from radish root tissues. *Plant Physiology*, 93(3),
553 896–901. <https://doi.org/10.1104/pp.93.3.896>

554 Mulo, P. (2011). Chloroplast -targeted ferredoxin-NADP⁺ oxidoreductase (FNR): Structure,
555 function and location. *Biochimica et Biophysica Acta* 1807, 927-934.
556 doi.org/10.1016/j.bbabi.2010.10.001

557 Mulo, P., & Medina, M. (2017). Interaction and electron transfer between ferredoxin–NADP⁺

558 oxidoreductase and its partners: structural, functional, and physiological implications. *Photosynthesis*
559 *Research*, 134, 265–280. <https://doi.org/10.1007/s11120-017-0372-0>

560 Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with
561 Tobacco Tissue Cultures. *Physiologia Plantarum*, 15, 473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>

563 Narusaka, M., Shiraishi, T., Iwabuchi, M., & Narusaka, Y. (2010). The floral inoculating protocol: A
564 simplified *Arabidopsis thaliana* transformation method modified from floral dipping. *Plant*
565 *Biotechnology*, 27, 349–351. <https://doi.org/10.5511/plantbiotechnology.27.349>

566 Nelson, B. K., Cai, X., & Nebenführ, A. (2007). A multicolored set of in vivo organelle markers for
567 co-localization studies in *Arabidopsis* and other plants. *Plant Journal*, 51, 1126–1136.
568 <https://doi.org/10.1111/j.1365-313X.2007.03212.x>

569 Oji, Y., Watanabe, M., Wakiuchi, N., & Okamoto, S. (1985). Nitrite reduction in barley-root plastids:
570 Dependence on NADPH coupled with glucose-6-phosphate and 6-phosphogluconate
571 dehydrogenases, and possible involvement of an electron carrier and a diaphorase. *Planta*, 165, 85–
572 90. <https://doi.org/10.1007/BF00392215>

573 Okutani, S., Hanke, G. T., Satomi, Y., Takao, T., Kurisu, G., Suzuki, A., & Hase, T. (2005). Three
574 maize leaf ferredoxin:NADPH oxidoreductases vary in subchloroplast location, expression, and
575 interaction with ferredoxin. *Plant Physiology*, 139, 1451–1459.
576 <https://doi.org/10.1104/pp.105.070813>

577 Onda, Y., Matsumura, T., Kimata-Arigo, Y., Sakakibara, H., Sugiyama, T., & Hase, T. (2000).
578 Differential interaction of maize root ferredoxin:NADP⁺ oxidoreductase with photosynthetic and
579 non-photosynthetic ferredoxin isoproteins. *Plant Physiology*, 123, 1037–1045.

580 Palatnik, J. F., Tognetti, V. B., Poli, H. O., Rodríguez, R. E., Blanco, N., Gattuso, M., ... Carrillo, N.
581 (2003). Transgenic tobacco plants expressing antisense ferredoxin-NADP(H) reductase transcripts
582 display increased susceptibility to photo-oxidative damage. *Plant Journal*, 35, 332–341.
583 <https://doi.org/10.1046/j.1365-313X.2003.01809.x>

584 Palatnik, J. F., Valle, E. M., & Carrillo, N. (1997). Oxidative stress causes ferredoxin-NADP⁺
585 reductase solubilization from the thylakoid membranes in methyl viologen-treated plants. *Plant*
586 *Physiology*, 115, 1721–1727. <https://doi.org/10.1104/pp.115.4.1721>

587 Petersen, B. L., Chen, S., Hansen, C. H., Olsen, C. E., & Halkier, B. A. (2002). Composition and
588 content of glucosinolates in developing *Arabidopsis thaliana*. *Planta*, *214*, 562–571.
589 <https://doi.org/10.1007/s004250100659>

590 Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team. (2017). nlme: Linear and nonlinear
591 mixed effects models. *R package version 3.1-131*.

592 Raorane, M. L., Narciso, J. O., & Kohli, A. (2016). Total soluble protein extraction for improved
593 proteomic analysis of transgenic rice plant roots. *Methods in Molecular Biology*, *1385*, 139–147.
594 https://doi.org/10.1007/978-1-4939-3289-4_10

595 R Core Team. (2016). R: A Language and Environment for Statistical Computing. *R Foundation for*
596 *Statistical Computing*, Vienna, Austria.

597 Rodriguez, R. E., Lodeyro, A., Poli, H. O., Zurbriggen, M., Peisker, M., Palatnik, J. F., ... Carrillo,
598 N. (2007). Transgenic tobacco plants overexpressing chloroplastic ferredoxin-NADP(H) reductase
599 display normal rates of photosynthesis and increased tolerance to oxidative stress. *Plant Physiology*,
600 *143*, 639–649. <https://doi.org/10.1104/pp.106.090449>

601 Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image
602 analysis. *Nature Methods*, *9*, 671–675. <https://doi.org/10.1038/nmeth.2089>

603 Shanklin, J., & Cahoon, E. B. (1998). Desaturation and related modifications of fatty acids. *Annual*
604 *Review of Plant Biology*, *49*, 611–641. <https://doi.org/10.1146/annurev.arplant.49.1.611>

605 Shinohara, F., Kurisu, G., Hanke, G. T., Bowsher, C., Hase, T., & Kimata-Arigo, Y. (2017). Structural
606 basis for the isotype-specific interactions of ferredoxin and ferredoxin: NADP⁺ oxidoreductase: an
607 evolutionary switch between photosynthetic and heterotrophic assimilation. *Photosynthesis*
608 *Research*, *134*(3), 281–289. <https://doi.org/10.1007/s11120-016-0331-1>

609 Soria-Garcia, A., Rubio, M.C., Lagunas, B., Lopez-Gomollon, S., de los Angeles Lujan, M., Diaz-
610 Guerra, R., ... Alfonso, M. (2019). Tissue distribution and specific contribution of Arabidopsis FAD7
611 and FAD8 plastid desaturases to the JA- and ABA-mediated cold stress or defense responses. *Plant*
612 *and Cell Physiology* *60*, 1025-1040 doi.org/10.1093/pcp/pcz017

613 Suzuki, A., Oaks, A. N. N., Jacquot, J., Vidal, J., & Gadal, P. (1985). An electron transport system in
614 maize roots for reactions of glutamate synthase and nitrite reductase: physiological and
615 immunochemical properties of the electron carrier and pyridine nucleotide reductase. *Plant*

616 *Physiology* 78, 374-78.

617 Vainonen, J. P., & Kangasjärvi, J. (2015). Plant signalling in acute ozone exposure. *Plant, Cell and*
618 *Environment*, 38, 240–252. <https://doi.org/10.1111/pce.12273>

619 Walz, C., Juenger, M., Schad, M., & Kehr, J. (2002). Evidence for the presence and activity of a
620 complete antioxidant defence system in mature sieve tubes. *Plant Journal*, 31, 189–197.
621 <https://doi.org/10.1046/j.1365-313X.2002.01348.x>

622 Wang, R., Guegler, K., LaBrie, S. T., & Crawford, N. M. (2000). Genomic analysis of a nutrient
623 response in arabidopsis reveals diverse expression patterns and novel metabolic and potential
624 regulatory genes induced by nitrate. *Plant Cell*, 12, 1491–1509. <https://doi.org/10.1105/tpc.12.8.1491>

625 Wang, R., Okamoto, M., Xing, X., & Crawford, N. M. (2003). Microarray analysis of the nitrate
626 response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new
627 linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. *Plant Physiology*, 132, 556–
628 567. <https://doi.org/10.1104/pp.103.021253>

629 Yonekura-Sakakibara, K., Onda, Y., Ashikari, T., Tanaka, Y., Kusumi, T., & Hase, T. (2000).
630 Analysis of reductant supply systems for ferredoxin-dependent sulfite reductase in photosynthetic
631 and nonphotosynthetic organs of maize. *Plant Physiology*, 122, 887–894.
632 <https://doi.org/10.1104/pp.122.3.887>

633

634

635 **Tables**636 **Table 1. Genotypes of T3 generation plants.**

| Parents genotype | Progeny genotype | Ob. | Ex. 1 | Ex.2 | Ex.3 |
|--------------------------|--------------------------|------------|--------------|-------------|-------------|
| <i>rfnr1 RFNR2 rfnr2</i> | <i>rfnr1 RFNR2</i> | 20 | 9.75 | 13 | 19.5 |
| | <i>rfnr1 RFNR2 rfnr2</i> | 19 | 19.5 | 26 | 19.5 |
| | <i>rfnr1 rfnr2</i> | 0 | 9.75 | 0 | 0 |
| <i>RFNR1rfnr1 rfnr2</i> | <i>RFNR1 rfnr2</i> | 17 | 9.75 | 13 | 19.5 |
| | <i>RFNR1rfnr1 rfnr2</i> | 22 | 19.5 | 26 | 19.5 |
| | <i>rfnr1 rfnr2</i> | 0 | 9.75 | 0 | 0 |

637 The genotypes of T3 progeny were determined by PCR. (Ob.) denotes the
638 number of individuals observed, (Ex.1) the expected number based on
639 Mendelian inheritance, (Ex.2) the expected number when the homozygous
640 double mutants are lethal during embryogenesis, and (Ex.3) the expected number
641 when de double mutant plants are not viable due to the lethality during
642 gametogenesis. Primers are shown in Table S1.

643

644 **Figure legends**

645 **Figure 1. Amino acid sequence comparison of RFNR1 and RFNR2.** Full-length amino acid
646 sequences were aligned by Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>). Residues
647 common to both sequences are highlighted in yellow, dash lines indicate a gap.

648 **Figure 2. Tissue-specific activity of GUS in the *pRFNR1::GUS* (A-G) and *pRFNR2::GUS* (H-Q)**
649 **seedlings grown on ½ MS-plates.** A, H, GUS activity in the 5-day-old seedlings; B, I, cross-section
650 of the roots of 7-day-old plants. C, J, cross-section of the root tips of 7-day-old plants. D, K, GUS
651 activity in the shoots of 14-day-old plants. E, L, leaf cross-section of the 14-day-old plants. F, M,
652 GUS activity in the roots of 14-day-old plants. G, N, cross-section of the roots of 14-day-old plants.
653 O, P, Q, GUS activity in the lateral roots of 7-day-old *pRFNR2::GUS* plants. The scale bar denotes 2
654 mm in A, D, F, H, K, M, 50 µm in B, C, E, I, J, L, 100 µm in G, N and 200 µm in O, P and Q.

655 **Figure 3. Localization and accumulation of RFNR1 and RFNR2 proteins in Arabidopsis.**
656 Confocal microscopy image of Arabidopsis roots expressing (A) plastid marker pt-ck CS16265
657 (*pSSRubisco::CFP*), (B) *pRFNR1::RFNR1-GFP* fusion protein, or C, *pRFNR2::RFNR2-GFP* fusion
658 protein. D, *pRFNR2::RFNR2-GFP* fusion protein in plants treated in +5 °C for 5 days. Left panel
659 shows the brightfield image, middle panel GFP signal, right panel a merged image of the two. E,
660 Reconstructed 3D model from the z-stacks of the roots expressing RFNR1/2-GFP fusion proteins.
661 Bar = 50 µm. F, Immunodetection of RFNR proteins from Arabidopsis leaf and root extract, enriched
662 leaf veins as well as soluble and membrane fraction of root plastids. G, Representative immunoblot
663 of RFNR proteins isolated from the leaves of intact, soil-grown Col-0 Arabidopsis plants exposed to
664 ambient air (ctrl 6h) or 300 nl L⁻¹ ozone (O₃ 6h) for 6 h. Thereafter, control plants (ctrl 24h) and O₃
665 treated plants (O₃ 24h) were shifted to standard conditions for 18 h. n=4. H, Representative
666 immunoblot of RFNR proteins isolated from the roots of plate-grown Col-0 plants exposed to low
667 temperature (+5 °C) for two and six days. n=5. I, Representative immunoblot of RFNR proteins
668 isolated from the leaves of intact Col-0 Arabidopsis plants grown on soil and treated under low
669 temperature (+5 °C) for two and six days. n=6. Proteins were separated on 12 % acrylamide gel and
670 immunodetection performed using RFNR antibody. Coomassie stained membranes show equal
671 loading of the gels (G-I).

672 **Figure 4. Characteristics of the *rfnr1* and *rfnr2* mutant plants upon ozone exposure.** A, RFNR
673 content in the roots of WT, *rfnr1* and *rfnr2* plants. Proteins were separated on 12 % acrylamide gel
674 and immunodetection performed using RFNR antibody. Ten micrograms of protein was loaded per
675 sample. B, Phenotype of ozone exposed plants. The plants were treated with 300 nl L⁻¹ ozone for 6 h

676 and then recovered at ambient ozone concentrations ($< 20 \text{ nl L}^{-1}$) for 18 h. C, Accumulation of indole,
677 short chain and long chain glucosinolates in the roots and leaves of Col-0, *rfnr1-1*, *rfnr2-1*, and *rfnr2-*
678 2, plants grown under standard growth conditions for five weeks. SC, short chain aliphatic
679 glucosinolates; LC, long chain aliphatic glucosinolates; IG, indole glucosinolates.

680 **Figure 5. Characteristics of the *rfnr1* and *rfnr2* mutant plants upon low temperature.** A,
681 Phenotype of plants grown at +5 °C for 11 weeks. B, Root phenotype of plants grown at +5 °C for 11
682 weeks.

683

684 **Supportive Information**

685 **Figure S1.** *RFNR1* (A) and *RFNR2* (B) gene expression data from GENEVESTIGATOR upon
686 selected abiotic and biotic stresses.

687 **Figure S2.** Characteristics of the *rfnr1* and *rfnr2* mutant plants.

688 **Figure S3.** Arabidopsis WT (Col-0 and Ler-0), *rfnr1* and *rfnr2* mutant plants after infection with
689 *Botrytis cinerea* or *Pseudomonas syringae*.

690 **Table S1.** Summary of the *pRFNR* promoter activity and accumulation of the RFNR proteins.

691 **Table S2.** Sequences of PCR primers used in the work.

692