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

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Running head: *Arabidopsis thaliana* RFNR isoforms

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Abstract

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

Keywords

Arabidopsis, ferredoxin-NADP$^+$ oxidoreductase, gene expression, low temperature, ozone, plastid, root, stress response

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Introduction

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

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

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

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

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

Materials and methods

Plant material

Arabidopsis thaliana Col-0 (WT), *rfnr1-1* (SALK_085009), *rfnr2-1* (SAIL_527G10) and *rfnr2-2* (SALK_133654) (in Col-0 background) as well as L*er*-0 and *rfnr1-2* (GT20582) (in L*er*-0 background) were grown under the light rhythm of 8 h light/16 h darkness at PPFD of 100 μmol 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 ligh/ 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 Scientifics) 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 Clonase™ 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 Clonase™ II
155 enzyme mix (Invitrogen). The binary vectors were transformed into Agrobacterium and used for
156 floral inoculation of Arabidopsis.

GUS staining and histology

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

Fluorescence microscopy

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

Protein extraction

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

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

Root plastid isolation

Roots of four week old plants were ground in homogenization buffer (50 mM Tris-HCl, pH 7.5, 330 mM sorbitol, 1 mM EDTA, 1 mM MgCl2, 0,1% BSA, Pierce^{TM} Protease inhibitor, 1 tablet/10 mL), filtered through one layer of Miracloth and centrifuged (4000 x g, 3 min, 4 ºC). The plastid enriched pellet was resuspended in homogenization buffer, loaded on 10% Percoll and centrifuged (4000 x g 5 min, 4 ºC). The plastid fraction was collected, washed and treated with shock buffer (5 mM sucrose, 10 mM Hepes- NaOH, 5 mM $MgCl_2$) as previously described (Bowsher et al., 1989).

SDS-PAGE and protein gel blot analysis

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

Determination of chlorophyll content

Chlorophyll content in leaf discs was calculated according to Inskeep and Bloom (1985). The 0.5 cm^2 discs were cut from the leaves of 4-week old plants and incubated overnight in 1 mL of dimethylformamide in darkness and room temperature. The absorbance was read at 646.6 nm, 663.6 nm and 750 nm.

Glucosinolate analysis

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

Statistical analysis

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

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

Stress treatments

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

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

Pathogen infections. For induced resistance experiments, one leaf per 18-day-old plant was infiltrated with an avirulent DC3000 *Pseudomonas syringae pathovar tomato* (Pst) bearing transgenically-expressed avrPpt2 (10^8 cell/mL suspension (OD_{600}= 0.2) in 10 mM $MgCl_2$), or mock inoculated with 10 mM $MgCl_2$, using a needleless syringe. The treated leaves exhibited visible hypersensitive cell death within 24 h of treatment. At three weeks of age plants were subsequently spray infected with virulent DC3000 (with an empty vector; $5x10^7$ cells/mL suspension (OD_{600}= 0.1) in 10 mM $MgCl_2$ containing 0.04 % Silwet). Plants were photographed 72 h later to document visible symptoms.

Botrytis infections were performed using four-week-old plants by dropping 3 µl of *Botrytis cinerea* suspension ($1x10^6$ spores/mL) in half-strength potato dextrose broth (1/2 PDB). For mock treatment ½ PDB was used. Plants were photographed 48 h post infection and lesions were measured in ImageJ (version 1.47v; Schneider, Rasband, & Eliceiri, 2012).

Results and discussion

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

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

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

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

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

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

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

Low temperature and ozone affect accumulation of RFNR isoforms

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

Ozone and pathogen responses

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

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

To pinpoint the function of the RFNR isoforms further, we characterized *rfnr1* and *rfnr2* knock-out mutants under standard conditions and upon exposure to various stresses. For RFNR1, we studied two independent knock-out lines SALK_085009 (*rfnr1-1*) in the Columbia-0 (Col-0) and Gt20582 (*rfnr1-2*) in Landsberg *erecta* accession, while for RFNR2, SAIL_527G10 (*rfnr2-1*) and SALK_133654.52 (*rfnr2-2*), both in Col-0 background, were used. Figure 4 shows that accumulation of either RFNR1 or RFNR2 protein in the *rfnr1* and *rfnr2* knock-out lines was under detection limit of the antibody, even if it has been reported that SAIL_527G10 is actually a knock-down line, which accumulates 18-25 % of the *RFNR2* mRNA as compared to WT (Hachiya et al., 2016). Under standard growth conditions, the phenotype of *rfnr1* and *rfnr2* plants did not differ from that of WT (Figure S2A; Figure 4). Moreover, Figure S2 shows that the rosette growth, root growth, chlorophyll content, and chlorophyll a/b ratio of the leaves was not affected by the loss of either RFNR isoform. Because the *rfnr1* and *rfnr2* knock-out mutants did not show any major morphological phenotypes, we crossed the single mutants in order to produce double knock-out plants, which might reveal the processes dependent on RFNR proteins. When the F2 progeny from the *rfnr1 rfnr2* crossing was self-pollinated, no double-knockout seedlings were identified, but the T3 generation was composed of 50% heterozygotes (*rfnr1* RFNR2*rfnr2* and RFNR1*rnfr1 rfnr2)* and 50 % WT plants (Table 1). The double knock out appears to be lethal rather during the gamete development than upon embryo development, since no aborted seeds were detected in the siliques of the T3 plants (Haruta et al., 2010). Taken together, RFNR isoforms appear to be redundant but essential components in the growth and development of Arabidopsis plants.

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

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

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

As scavenging of ROS within the chloroplast requires a complex network of metabolites and enzymes (ascorbate-glutahione or Foyer-Halliwell-Asada pathway), including Fd and NADPH, the FNR proteins lie in the very center of cellular redox metabolism. Several studies have indeed implicated FNR in the responses to oxidative stress, even if its exact role has not been resolved yet (Palatnik, Valle, & Carrillo, 1997). For instance, decreased accumulation of LFNR has been shown to result in increased production of $^1O_2$ in LFNR antisense tobacco plants (Palatnik et al. 2003), while LFNR overexpression led to enhanced protection from oxidative stress (Rodriguez et al. 2007). Additionally, imbalance in the FNR/Fd ratio affected both ROS scavenging and ROS production (Kozuleva et al. 2016). Interestingly, it has been shown that the enucleate sieve elements, which are rich in non-photosynthetic plastids (Cayla et al., 2015) and presumably contain RFNR proteins (Figure 3) possess a functional antioxidant system with various ROS scavenging enzymes (Walz, Juenger, Schad, & Kehr, 2002). Our results demonstrating increased accumulation of RFNR1 upon ozone exposure (Figure 3G) as well as sensitivity of *rfnr2-1* plants to ozone treatment (Figure 4B) further support the view that FNR proteins might play a role in ROS metabolism, ROS responses and/or ROS signaling in Arabidopsis. It will be intriguing to elucidate the detailed functions and specificities of the RFNR isoforms in ROS signaling network, and to pinpoint the sequence of stress response events both in roots and in shoots.

Low temperature

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

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

Conclusions

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

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

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Tables

Table 1. Genotypes of T3 generation plants.

Parents genotype	Progeny genotype	Ob.	Ex. 1	Ex.2	Ex.3
rfnr1 RFNR2 rfnr2	*rfnr1 RFNR2*	20	9.75	13	19.5
	rfnr1 RFNR2 rfnr2	19	19.5	26	19.5
	rfnr1 rfnr2	0	9.75	0	0
RFNR1rfnr1 rfnr2	*RFNR1 rfnr2*	17	9.75	13	19.5
	RFNR1rfnr1 rfnr2	22	19.5	26	19.5
	rfnr1 rfnr2	0	9.75	0	0

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

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 (O_3 6h) for 6 h. Thereafter, control plants (ctrl 24h) and O_3
665 treated plants (O_3 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

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

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

Supportive Information

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

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

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

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

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