RESEARCH PAPER

The physiological importance of photosynthetic ferredoxin NADP\(^+\) oxidoreductase (FNR) isoforms in wheat

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Abstract

Ferredoxin NADP\(^+\) oxidoreductase (FNR) enzymes catalyse electron transfer between ferredoxin and NADPH. In plants, a photosynthetic FNR (pFNR) transfers electrons from reduced ferredoxin to NADPH for the final step of linear electron flow, providing reductant for carbon fixation. pFNR is also thought to play important roles in two different mechanisms of cyclic electron flow around photosystem I; and photosynthetic reductant is itself partitioned between competing linear, cyclic, and alternative electron flow pathways. Four pFNR protein isoforms in wheat that display distinct reaction kinetics with leaf-type ferredoxin have previously been identified. It has been suggested that these isoforms may be crucial to the regulation of reductant partition between carbon fixation and other metabolic pathways. Here the 12 cm primary wheat leaf has been used to show that the alternative N-terminal pFNRI and pFNRII protein isoforms have statistically significant differences in response to the physiological parameters of chloroplast maturity, nitrogen regime, and oxidative stress. More specifically, the results obtained suggest that the alternative N-terminal forms of pFNRI have distinct roles in the partitioning of photosynthetic reductant. The role of alternative N-terminal processing of pFNRI is also discussed in terms of its importance for thylakoid targeting. The results suggest that the four pFNR protein isoforms are each present in the chloroplast in phosphorylated and non-phosphorylated states. pFNR isoforms vary in putative phosphorylation responses to physiological parameters, but the physiological significance requires further investigation.

Key words: Chloroplast maturity, ferredoxin NADP\(^+\) oxidoreductase (FNR), nitrogen, oxidative stress, photosynthetic ferredoxin NADP\(^+\) oxidoreductase (pFNR), Triticum aestivum.

Introduction

Ferredoxin NADP\(^+\) oxidoreductase (FNR) enzymes transfer electrons between the one-electron carrier ferredoxin and the two-electron carrier NADPH (Carrillo and Ceccarelli, 2003). Leaf metabolism is dominated by photosynthesis. At the end of the photosynthetic electron transport chain, energized electrons are transferred from the photosystem I protein complex (PSI) to ferredoxin (Haehnel, 1984; Chhitnis, 2001). In linear electron flow (LEF), which is the dominant electron pathway, electrons are transferred from ferredoxin to NADPH by a photosynthetic FNR (pFNR) enzyme (Chhitnis, 2001). Most of the reductant (in the form of NADPH) and ATP generated by photosynthesis is used for carbon fixation in the Calvin cycle (Oja et al., 2007). However, a number of additional electron sinks, grouped under the collective term ‘alternative electron flow’ (AEF; Bukhov and Carpentier, 2004), require reduced ferredoxin generated at PSI as their direct reductant supply. The ferredoxin:NADPH ratio demanded downstream of photosynthesis varies according to the relative activities (dependent on the physiological state of the chloroplast) of carbon fixation and these AEF sinks. Electron partition between NADPH supply (LEF) and reduced ferredoxin supply (AEF) depends, in part, on the catalytic properties of the pFNR enzyme that connects the two reductant pools.

Abbreviations: AEF, alternative electron flow; CEF, cyclic electron flow; DTT, dithiothreitol; 2DE, two-dimensional gel electrophoresis; FNR, ferredoxin NADP\(^+\) oxidoreductase; FQR, ferredoxin plastoquinone reductase; IEF, isoelectric focusing; IPG, immobilized pH gradient; LEF, linear electron flow; MS, mass spectrometry; MV, methyl viologen; NDH, NADPH dehydrogenase; PSI, photosystem I; pFNR, photosynthetic ferredoxin NADP\(^+\) oxidoreductase.

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Two pathways of cyclic electron flow (CEF), which recycle electrons under conditions of oxidative stress to minimize the production of damaging reactive oxygen species (Endo et al., 1999; Munekage et al., 2004), are thought to require pFNR. CEF also pumps protons into the thylakoid lumen, powering the generation of ATP. Under normal physiological conditions CEF might have a role in adjusting the stoichiometry of ATP:NADPH generated by photosynthesis (Munekage et al., 2002, 2004). pFNR has been identified as a component of the cytochrome_6 complex (Clark et al., 1984) and implicated in ferredoxin plastoquinone reductase (FQR)-dependent CEF by measurements of FNR-dependent quinine reduction (Cleland and Bendall, 1992). In this context, pFNR would bind ferredoxin molecules to allow the subsequent transfer of electrons to plastoquinone (by an unknown mechanism), but would not generate NADPH. pFNR is also bound to the NADPH dehydrogenase (NDH; Guedeney et al., 1996) complex, and NDH-dependent CEF can be inhibited using an FNR antibody (Mills et al., 1979). It seems probable that pFNR generates a locally enriched pool of NADPH at the NDH complex that is then dehydrogenated in situ to release electrons back into the electron transport chain (Gummadova et al., 2007).

Photosynthesis thus appears to require varying catalytic properties from the pFNR enzyme, in order to adjust the ratio of ferredoxin:NADPH reductant supplied and to function effectively in LEF and the two CEF pathways. These differing requirements for pFNR catalytic properties could be met by different isoforms, whether arising from different genes or from alternative translated protein products of the same gene. Plants possess two evolutionarily conserved classes of pfnr genes that encode more basic (pFNRI) and more acidic (pFNRII) forms of the protein. The two genes respond differently at the mRNA level to an altered supply of nitrate—high levels induce pfnrI (but not pfnrII) expression in both wheat (Gummadova et al., 2007) and Arabidopsis (Hanke et al., 2005). Precursor pFNR protein is translated in the cytosol from the nuclear pfnr genes and then imported into the chloroplast, with the removal of an N-terminal transit peptide domain. Multiple forms of pFNR varying at the N-terminus have been identified in the past (Karplus et al., 1984) and suggested to arise as a result of N-terminal proteolytic degradation (Shin et al., 1990). However, it has recently been demonstrated that the presence of mature wheat pFNR proteins with alternative N-terminal start points, differing by a three amino acid truncation in pFNRI and a two amino acid truncation in pFNRII, is not the result of N-terminal proteolytic degradation (Gummadova et al., 2007). Recombinant versions of these four pFNR protein isoforms (pFNRIKVS, pFNRIKKQ, pFNRIIKSK, and pFNRIIKKD) were overexpressed in Escherichia coli. Each of the four recombinant pFNR proteins resolves to a unique isoelectric point (pI) with two-dimensional gel electrophoresis (Gummadova et al., 2007). The purified enzymes have distinct reaction kinetics with leaf-type ferredoxin, as determined using an NADPH-dependent cytochrome_6 reduction assay (Gummadova et al., 2007). The pFNRI isoforms have half the maximum activity (V_max) of the pFNRII isoforms but higher affinity for ferredoxin, as indicated by a lower Michaelis–Menten constant (K_m). Further differences were seen between alternative N-terminal truncations. pFNRIKQ, pFNRIKKQ, pFNRIKSV, and pFNRIKKQ, while pFNRIKKQ has 3-fold higher catalytic efficiency and ferredoxin affinity in comparison with pFNRIKKQ (Gummadova et al., 2007).

The 12 cm primary wheat leaf is a well-characterized developmental model (Leech et al., 1986) that allows exploration of changes in pFNR expression with chloroplast maturity and other physiological parameters (Gummadova et al., 2007). In the current study, the developing wheat leaf model system is used to test the hypothesis that the presence of multiple forms of pFNR, with varied expression and post-translational modification, provides the plant with flexibility to respond to changes in reductant demand. More specifically, it is demonstrated that the alternative N-terminal pFNRI protein isoforms in wheat have statistically significant differences in response to the physiological parameters of chloroplast maturity, nitrogen regime, and oxidative stress. Alternative N-terminal processing of pFNRI is also identified as being important for thylakoid targeting. In addition to the four pFNR spots in the chloroplast proteome that match the isoelectric positions demonstrated for the recombinant forms (Gummadova et al., 2007), a further four pFNR spots match the isoelectric positions predicted to arise with protein phosphorylation. The significance of these findings for understanding the regulation of photosynthetic electron flow is discussed in the context of specific metabolic roles for pFNR isoforms.

**Materials and methods**

Unless otherwise stated, all chemicals were purchased from Sigma.

**Plant material and growth conditions**

Wheat (Triticum aestivum cv. Paragon; RAGT Seeds) was grown in a controlled-environment cabinet (Sanyo) with a 240 W photon light flux cycle of 16 h light and 8 h dark at 20 °C for 6–7 d until the primary wheat leaf attained 12 cm length. Plants for the controlled (0 mM or 10 mM) nitrate experiment were carried out 3 h into the light period, with the primary leaf dissected as described previously (Gummadova et al., 2007). For dark adaptation experiments, wheat was first grown on compost in a cycle of 16 h light and 8 h dark for 6 d. Plants were then moved to a dark room before the end of the dark period on the sixth day and harvested in the dark after a total of 11 h darkness.

**Chloroplast and subchloroplast protein samples**

Intact wheat chloroplasts were isolated using a protoplast digestion and density gradient purification method adapted from Tobin and Bowsher (2004). Fractionation of stroma and thylakoid proteins from intact chloroplasts used the method of Okutani et al.
Each biological sample from whole leaves used 15 g of 12 cm primary wheat leaf tissue prepared from ~250 plants. Biological samples of 15 g of leaf tissue prepared from middle (4–6 cm from the base) or tip sections (10–12 cm from the base) required a concomitant increase in plant number to ~1500.

Oxidative stress induction by methyl viologen treatment

Intact chloroplasts were isolated in 500 mM sorbitol, 50 mM tricine, pH 8.4, 10 mM EDTA from wheat grown on potting compost. Methyl viologen (MV) at a final concentration of 100 μM was added to intact chloroplasts (at a concentration of ~200 μg protein ml⁻¹) and subjected to constant illumination (90 μmol m⁻² s⁻¹ photon light flux) for 1 h prior to separation into stroma and thylakoid protein fractions. Control chloroplasts were treated identically, but in the absence of MV.

2D gel electrophoresis

Two-dimensional electrophoresis (2DE) used isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension. All protein samples for 2DE were precipitated overnight at –20 °C with 80% (v/v) acetone and then resuspended in 7 M urea, 2 M thiourea, 40 mM dithiothreitol (DTT), 2% (w/v) CHAPS, 0.005% (w/v) bromophenol blue, with pH 3–10 ampholytes (Bio-Rad) and used to rehydrate a pH 4.0–7.0 IPG strip (GE Healthcare) over 24 h. Rehydrated IPG strips were washed with D₂O before isoelectric focusing in a Protean IEF Cell (Bio-Rad) according to the manufacturer’s instructions. Focused strips were equilibrated for 30 min with 0.375 M TRIS-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT, and then equilibrated for a further 30 min with 0.375 M TRIS-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide. The second dimension was run on a 20 cm 15% acrylamide–bisacrylamide, 500 mM TRIS-HCl, pH 8.8, 0.1% (w/v) SDS resolving gel using the Protean XL gel system (Bio-Rad). For total protein visualization, gels were stained for 8.8, 0.1% (w/v) SDS resolving gel using the Protean XL gel system (Bio-Rad). For total protein visualization, gels were stained for TRIS-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT, and then equilibrated for a further 30 min with 0.375 M TRIS-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide. The second dimension was run on a 20 cm 15% acrylamide–bisacrylamide, 500 mM TRIS-HCl, pH 8.8, 0.1% (w/v) SDS resolving gel using the Protean XL gel system (Bio-Rad). For total protein visualization, gels were stained for 24 h with Coomassie G-250 following the modified ‘blue-silver’ recipe of Candiano et al. (2004).

Antibody production

Wheat pFNR proteins [pFNRIKKVS, pFNRI[HKKK-] and pFNRIIN+22AQVS] overexpressed in E. coli and purified using a ferredoxin affinity column (Gummadova et al., 2007) were used to produce pFNR antiserum. A 1 mg aliquot of each protein was divided into three aliquots and each aliquot mixed with an equal volume of Freund’s adjuvant prior to injecting into a sheep at monthly intervals (Diagnostics Scotland, UK). Three individual aliquots were washed with dH2O before isoelectric focusing in a Protean IEF Cell (Bio-Rad) and used to rehydrate a pH 4.0–7.0 IPG strip (GE Healthcare) over 24 h. Rehydrated IPG strips were washed with D₂O before isoelectric focusing in a Protean IEF Cell (Bio-Rad) according to the manufacturer’s instructions. Focused strips were equilibrated for 30 min with 0.375 M TRIS-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT, and then equilibrated for a further 30 min with 0.375 M TRIS-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide. The second dimension was run on a 20 cm 15% acrylamide–bisacrylamide, 500 mM TRIS-HCl, pH 8.8, 0.1% (w/v) SDS resolving gel using the Protean XL gel system (Bio-Rad). For total protein visualization, gels were stained for 24 h with Coomassie G-250 following the modified ‘blue-silver’ recipe of Candiano et al. (2004).

Immunoblotting

Immunoblotting of gels onto nitrocellulose membranes (Millipore) and detection of proteins was performed as described previously (Gummadova et al., 2007). The three pFNR antibodies generated were used in preliminary experiments to determine their ability to detect and distinguish distinct forms of pFNR. As they all gave similar results, the antibody raised against pFNRIKKVS was used (at a dilution of 1:1000) for all subsequent experiments.

Gel and immunoblot analysis

All gels and immunoblots were scanned using QuantityOne software (Bio-Rad) with a GS-710 scanning densitometer (Bio-Rad) and imported into PDQuest software (Bio-Rad) for spot analysis. Eight pFNR spots were identified using 2DE, with pairs of spots identified as representing a phosphorylated and non-phosphorylated version of each pFNR isoform (see Results for further information). Individual protein spot intensity was read as the peak optical density. The total protein level for each individual pFNR isoform on a blue-silver-stained 2D gel was determined by combining the sum intensity of the putative phosphorylated and non-phosphorylated protein spots normalized to the intensity of the 23 kDa oxygen-evolving complex subunit spot = 1.0. Since the three intense fructose bisphosphate aldolase spots (predominantly localized to the stromal fraction) obscured the pFNRII spots, stromal pFNR levels were estimated by subtracting thylakoid pFNR isoform levels from chloroplast pFNR isoform levels. To distinguish between putative phosphorylated and non-phosphorylated pFNR isoforms, the proportion of each isoform present in the putative phosphorylated state was calculated as a percentage of the combined intensity of the total specific isoform (phosphorylated and non-phosphorylated spots). Changes in pFNR isoform levels and phosphorylation state over different physiological treatments were all quantified from blue-silver-stained 2D gels of the thylakoid proteome.

Seven landmark protein spots were used as reference points for protein size/pI, stroma/thylakoid markers, and to match immunoblots (landmark positions marked with ink after temporary staining with Ponceau Red) to 2D gels of the chloroplast and thylakoid proteinomes stained with blue-silver. The large and small subunit proteins of Rubisco (RBCL and RBCS, respectively) provided stromal markers, and the 23 kDa and 32 kDa protein subunits of the PSII oxygen-evolving complex provided thylakoid markers. Three isoforms of fructose bisphosphate aldolase were adjacent to the pFNRII protein spots and found in both stroma and thylakoid fractions. In all cases the identity of each protein spot was confirmed by mass spectrometry (MS).

MS analysis

Tryptsin protease digest and MS analysis of pFNR and landmark spots from 2D gels used the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) method of Shevchenko et al. (1996, 2006). Automated analysis of mass peak data used Mascot software (Perkins et al., 1999) and the Swiss-Prot/Trembl UniProt protein database (Apweiler et al., 2004; Uniprot Consortium, 2008).

PeptideMass software (Wilkins et al., 1997) was used to generate theoretical cleavage patterns of pFNR proteins with a trypsin digest. Theoretical peptides were used as a checklist against which pFNR peaks identified by Mascot software were matched to pFNRI and/or pFNRII. Peptides shared by both pFNRI and pFNRII correspond to lengths of sequence between protease cleavage points that have 100% identity; pFNRI or pFNRII unique peptides correspond to lengths of the sequence between cleavage points that have one or more non-identical positions and are therefore isoform specific.

Prediction of phosphorylation sites and 3D modelling of pFNR protein structure

pFNR protein structures were predicted using 3D-Jigsaw modelling software (Bates and Sternberg, 1999; Bates et al., 2001; Contreras-Moreira and Bates, 2002) and images generated using Polyview-3D software (Porollo et al., 2004; Porollo and Meller, 2007). pFNR protein sequences were analysed for potential phosphorylation sites using NetPhos 2.0 software (Blom et al., 1999). Positions with a prediction score above the 0.500 threshold are potential phosphorylation sites; however, scores of <0.900 are considered low and phosphorylation highly unlikely (Blom et al., 1999; Nikitina and Tishchenko, 2005). Possible phosphorylation sites not conserved across wheat, rice, and Arabidopsis protein sequences or that are inside the globular protein (and hence not accessible to phosphorylating protein kinases) were not considered.
Statistical analysis and protein property prediction

Sixteen biological replicates of blue-silver gels and immunoblots were initially performed to map the chloroplast proteome and locate pFNR protein spots. Four biological replicates were subsequently performed for every physiological treatment. Statistical analysis was performed using a two-tailed Student’s t-test with Microsoft Office Excel software (Microsoft) and graphs were drawn with standard deviation error bars using GraphPad Prism 4 software (GraphPad Software). The pI and molecular weight were predicted for proteins from their amino acid sequences using Compute pI/mw software (Bjellqvist et al., 1993). Phosphorylation effects on pFNR protein pI were predicted using Scansite software (Obenauer et al., 2003). pFNR N-terminal transit peptide sequences were analysed using ChloroP software (Emanuelsson et al., 1999, 2007) and the precise end of the transit peptide defined as the first position within the approximate end with a cleavage site score >1.000.

Gene and protein accession numbers

Throughout this paper pFNRI is used to refer to the more basic form of the enzyme and pFNRII to refer to the more acidic form. All protein sequences are listed in Table 2 and referenced to their accession number at the US National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

Results

pFNR proteins in the 2D wheat chloroplast proteome

To confirm the presence of multiple pFNR forms in vivo, wheat was grown for 7 d, chloroplasts isolated from whole primary leaves, and total protein separated by 2DE. The chloroplast proteome was first mapped and landmark protein spots identified using MS (Fig. 1). Immunoblotting of the 2D chloroplast proteome with a pFNR-specific antibody was then used to identify pFNR protein spots (Fig. 2A). MS confirmed that four protein spots (A–D) at pH 5.30–5.51 and four protein spots (W–Z) at pH 5.90–6.62 represented clusters of pFNRII and pFNRI, respectively (Table 1; Fig. 2A). The four protein spots C, D, X, and Z have previously been identified as pFNRIIISK, pFNRIIKKD, pFNRIISKKQ, and pFNRIKKVS, respectively (Fig. 2B; Gummadova et al., 2007). To investigate the identity of the additional protein spots, the effect of protein phosphorylation on the pI of the four pFNR proteins during 2DE was predicted using Scansite software. A predicted shift in an acidic direction (Fig. 2B) was comparable with the pattern of the four additional spots observed in vivo (Fig. 2A). These data suggest that protein spots A, B, W, and Y represent the
phosphorylated forms of pFNRI_{SSKK}, pFNRII_{KKQ}, pFNRII_{KKKD}, and pFNRI_{KKSYS}, respectively. As each of the eight pFNRI protein spots was clearly identifiable on blue-silver-stained 2D gels of chloroplast or thylakoid proteins (Fig. 2C), subsequent quantification of protein expression from the spot intensity could be made.

Viable protein phosphorylation sites for the wheat pFNRI proteins were analysed in silico with NetPhos 2.0 software. This identified a single putative serine phosphorylation site for wheat pFNRI at position 75, which is the fourth residue at the N-terminus of the pFNRI_{KKSYS} isoform and the first residue of the pFNRI_{SSKK} isoform (Fig. 3A). This putative phosphorylated serine position is located on the surface of the 3D protein (Fig. 4A). Furthermore, it has a high phosphorylation score and is also conserved in Arabidopsis and rice (Fig. 3B). In wheat pFNRII, however, Ser75 (S75) is not predicted to be a phosphorylation site (Fig. 3B) and instead two putative threonine phosphorylation sites located on the surface of the 3D model were predicted at positions 104 and 293 (Fig. 4B, C). Both of these sites, neither of which was predicted for phosphorylation in pFNRI, are also conserved in Arabidopsis and rice (Fig. 3B). In wheat pFNRII the T104 position, at the closure of the hinge between the FAD-binding and NADP(H)-binding domains (Fig. 4B), and with its higher phosphorylation score (0.911, Fig. 3B), is considered most likely to represent a genuine phosphorylation site. The use of commercially available phosphoserine and phosphothreonine antisera failed to detect pFNRI phosphorylation on immunoblots (data not shown). Similarly, MS analysis of the putative phosphorylated spots failed to identify any phosphopeptides (data not shown).

Table 1. MS identification of pFNRI and pFNRII spots

| Spot          | A | B | C | D | W | X | Y | Z |
|---------------|---|---|---|---|---|---|---|---|
| Score         | 488 | 372 | 529 | 377 | 178 | 297 | 269 | 268 |
| pFNRI-unique peaks | 0 | 0 | 0 | 0 | 1 | 6 | 3 | 3 |
| Shared peaks  | 4 | 2 | 4 | 3 | 3 | 4 | 4 | 4 |
| pFNRII-unique peaks | 9 | 7 | 9 | 8 | 0 | 0 | 0 | 0 |
| pFNRI gene identity | II | II | II | II | I | I | I | I |
| pFNRII gene identity | II | II | II | II | I | I | I | I |

**Analysis of a putative secondary N-terminal processing motif**

The N-terminal differences between the four wheat pFNRI isoforms were explored by comparative analysis of transit peptide sequences and mature N-terminal sequences. Full-length precursor pFNRI protein sequences from a number of plant species were aligned and their transit peptide lengths predicted using ChloroP software (Fig. 3A). The transit peptide ends with a conserved R/K-T-A-A/Q motif at positions 51–53 inclusive. However, the mature N-terminal start point of wheat pFNRI proteins (Gummadova et al., 2007) is 19–23 positions after the end of the transit peptide at K72 (pFNRI_{KKSYS}), I74 (pFNRII_{ISKK}), S75 (pFNRII_{SKKQ}), or K76 (pFNRII_{KKQD}). Similarly, the N-terminal start point for the mature spinach FNR, at position V71, is 18 positions after the end of the transit peptide (Hasumi et al., 1983). In wheat and spinach, the region between the transit peptide and the highly conserved lysine position K70 (approximately corresponding to the N-termini of the mature pFNRI protein) has an alanine/proline content of 56%. In contrast, the corresponding region in pFNRI and pFNRII of Arabidopsis, which is retained in the mature proteins, has an alanine/proline content of only 23%. The N-terminal start point of Arabidopsis pFNRI and pFNRII mature protein sequences (Hanke et al., 2005) aligns with the end of the predicted transit peptide.

**Subchloroplast localization of pFNRI isoforms**

Using PDQuest software, it was possible to quantify the total protein level of each pFNRI isoform on a blue-silver-stained 2D gel as the sum intensity of its two spots normalized to the intensity of the 23 kDa oxygen-evolving complex subunit protein spot. To examine the subchloroplast localization of pFNRI isoforms between stroma and thylakoid fractions, a comparison of pFNRI protein levels in the chloroplast and thylakoid proteomes was made. Based on the information collated above, spots A and C, spots B and D, spots W and X, and spots Y and Z were quantified.
and combined in pairs to represent the total protein levels of pFNRIISKK, pFNRIIKKQD, pFNRIKQ, and pFNRIKKVS, respectively (Fig. 5). The total pFNR protein was distributed equally between the stroma (52%) and thylakoid (48%) pools (Fig. 5). Furthermore, the two alternative N-terminal forms of pFNRII were both distributed equally between the subchloroplast pools. In contrast, the alternative N-terminal forms of pFNRI showed differential distribution, with 76% of pFNRIKQ and only 33% of pFNRIKKVS being found in the thylakoid pool.

**pFNR isoform levels change with different physiological conditions**

The abundance of the pFNR spots in the thylakoid proteome in response to different metabolic demands was examined to provide an insight into the dynamics of pFNR levels in vivo. There was no significant difference in the levels of total pFNR protein in the thylakoids of chloroplasts isolated from either the middle of the leaf or the more photosynthetically active leaf tip (Fig. 6). However, when examining the response of individual pFNR isoforms, the level of pFNRIKQ was significantly higher in the leaf tip (P < 0.05; Fig. 7A). Comparison of pFNR levels between light- or dark-adapted plants showed no significant differences in either total pFNR levels or individual isoform levels (Figs 6, 7D). The total pFNR protein levels did increase significantly (P < 0.05) when plants were grown in the presence of 10 mM nitrate compared with when plants were grown in the absence of nitrate. Furthermore, pFNRIKKVS levels showed a significant increase in response to nitrate supply (P < 0.05; Fig. 7B). Total pFNR protein level also increased significantly when chloroplasts were treated with 100 μM MV to induce oxidative stress (Fig. 6).

**Fig. 3.** N-terminal processing region of the mature pFNR protein. (A) Alignment of wheat (Ta), rice (Os), Arabidopsis (At), and spinach (So) FNR sequences (taken from the NCBI database) starting from the processing region of the pFNR transit peptide and extending into the catalytic domain. Positions are given relative to the conserved lysine residue at position 70 (K70) that corresponds to the approximate start of the FNR catalytic domain (highlighted in lowercase and underlined). pFNR N-termini experimentally determined for wheat (Gummadova et al., 2007), Arabidopsis (Hanke et al., 2005), and spinach (Hasumi et al., 1983) are highlighted in black with white writing. Transit peptide sequences (shaded grey) were predicted using ChloroP software (Emanuelsson et al., 1999, 2007) as described in the Materials and methods. Serine and threonine positions matching known phosphorylation motifs were identified using NetPhos 2.0 software (Blom et al., 1999); those located on the surface of the wheat pFNR protein (Fig. 4) and, within pFNRI or pFNRII, also conserved in rice and Arabidopsis are shown (highlighted in bold and underlined). (B) NetPhos 2.0 scores for these candidate phosphorylation sites in wheat, Arabidopsis, and rice pFNR sequences, with scores >0.900 highlighted in bold.

**Differences between isoforms in putative phosphorylation patterns**

To investigate the impact of putative phosphorylation status, the intensity of the putative phosphorylated spot for each isoform was expressed as a percentage of the combined intensity of the two spots. The putative phosphorylation level in the thylakoid fraction compared with total chloroplast protein extract was significantly higher for pFNRIISKK (P < 0.05), but significantly lower for pFNRIKQ (P < 0.05; Fig. 8). Further analysis examined whether different reductant demands led to altered phosphorylation status of the pFNR isoforms in the thylakoid proteome. In all experimental conditions examined there were no significant differences identified in the phosphorylation status of any of the pFNR...
isoforms (data not shown). However, over all the physiological conditions examined in this study (Fig. 7) the mean level of phosphorylation of pFNRI\textsubscript{IKKQ} was significantly lower ($P < 0.05$) than that of pFNRI\textsubscript{KVS}. There was no significant difference in the mean phosphorylation level of the pFNRII isoforms.

**Fig. 4.** Predicted phosphorylation sites on 3D models of pFNRI and pFNRII. Wheat pFNRI and pFNRII 3D models were drawn with Polyview-3D software (Porollo et al., 2004; Porollo and Meller, 2007) from tertiary structures predicted using 3D-Jigsaw software (Bates and Sternberg, 1999; Bates et al., 2001; Contreras-Moreira and Bates, 2002). (A) A putative phosphorylation site at Ser75 (S75) for pFNRI was identified. (B) Putative threonine phosphorylation sites for pFNRII at position 104 (T104); and (C) position 293 (T293) were identified.

**Fig. 5.** Subchloroplast localization of pFNRI protein isoforms. Stacked abundance of pFNRI isoforms in stroma and thylakoid pools. pFNRI\textsubscript{IkKQ} is shown in black, pFNRI\textsubscript{KVS} is shown in dark grey, pFNRI\textsubscript{IKKD} is shown in light grey, and pFNRI\textsubscript{IkK} is shown in white. The thylakoid pFNRI component was compared with total chloroplast pFNRI levels (thylakoid plus stroma) to deduce the stromal component. Data were from four replicate gels for each of the chloroplast proteome and the thylakoid proteome.

**Fig. 6.** Total pFNRI protein levels change with various physiological parameters. Total pFNRI protein levels in the thylakoid proteome were examined in experimental conditions altering various physiological parameters: leaf developmental position (middle/tip of leaf); nitrate induction (0/10 mM nitrate growth conditions); effects of oxidative stress [0/100 \mu M methyl viologen (MV) treatments]; and leaves harvested 3 h into the light period were compared with dark-adapted leaves (light/dark treatment). Total pFNRI protein abundance was measured from blue-silver total protein staining relative to the 23 kDa protein of the oxygen-evolving complex (=1.0) in four replicate gels for each treatment. Within each experiment, the mean total pFNRI protein levels in the two different treatments were compared using a two-tailed Student’s $t$-test. A horizontal line and star above the bars indicates a significant difference ($P < 0.05$) between the samples; error bars show the standard deviation.
Discussion

pFNR protein isoforms and phosphorylation

The recent identification in wheat of alternative N-terminal versions of \( pFNR_1 \) and \( pFNR_2 \) proteins with distinct catalytic properties suggested isoform-specific metabolic roles (Gummadova et al., 2007). The developmental model of the primary wheat leaf was therefore used in this study to investigate whether specific \( pFNR \) isoforms were induced by changes in physiological parameters connected to photosynthetic electron flow. 2DE used the differences in pI previously identified (Gummadova et al., 2007) to distinguish between the four wheat \( pFNR \) protein isoforms. Four \( pFNR \) spots were identified at approximately equivalent IEF positions to those previously demonstrated for recombinant versions of the isoforms. The identification from IEF positions of spots C and Z as the longer N-terminal forms of \( pFNR_2 \) and \( pFNR_1 \), respectively, was fully confirmed by their molecular weights (indicated by SDS–PAGE migration distances), which are \( \sim 300 \) Da larger than their proposed corresponding short N-terminal forms at spots D and X.

(D) leaves harvested 3 h into the light period were compared with dark-adapted leaves (light/dark treatment). \( pFNR \) isoform abundance was measured from blue-silver total protein staining relative to the 23 kDa protein of the oxygen-evolving complex (=1.0) in four replicate gels for each treatment; mean values were then calculated for each isoform in each treatment. Pairwise comparison of treatments used a two-tailed Student’s \( t \)-test. A horizontal line and star above the bars indicates a significant difference \( (P < 0.05) \) between the samples; error bars show the standard deviation.
An additional four protein spots were identified by Scansite software to be at equivalent isoelectric positions to the predicted shift arising from protein phosphorylation (Obenauer et al., 2003). This interpretation was further supported by the equivalent molecular weights of the putative phosphorylated proteins to those of their non-phosphorylated counterparts. In silico identification of putative phosphorylation sites at serine (S75) and threonine (T104 or T293) positions for wheat pFNRI and pFNRII, respectively, supported previous suggestions from work on purified spinach pFNR proteins (Hodges et al., 1990; Hodges and Migniac-Maslow, 1993). Similarly, the two pFNR proteins in Arabidopsis, representing single N-terminal versions of pFNRI and pFNRII (Hanke et al., 2005), also have additional spots in the 2D thylakoid proteome that were identified as potential phosphorylated forms (Lintala et al., 2007).

The disruption in pFNRIKKVS of the predicted pFNR phosphorylation motif (with the N-terminal residue being the predicted S75 phosphorylation site) may explain why its phosphorylation is consistently lower than that of pFNRIKKVS (35% compared with 46% across all experiments). The fact that pFNRIKKVS maintains some level of phosphorylation, despite the disrupted motif, suggests that this is a kinase-independent autophosphorylation. Serine autophosphorylation was previously demonstrated to occur both in the dark and in the absence of kinase enzymes for spinach pFNR (Hodges et al., 1990). For pFNRII, the significantly higher levels of pFNRIISKK phosphorylation seen at the thylakoid membrane support the previous proposal that threonine phosphorylation of spinach pFNR in vivo requires a membrane-bound kinase (Hodges et al., 1990). Although no associations of pFNRII phosphorylation with specific physiological conditions were discernible, phosphorylation levels for the two alternative N-terminal forms did vary between 45% and 65% across the different experiments.

All the available in vitro evidence presented here supports the suggestion that the four additional pFNR spots identified by 2DE represent phosphorylated pFNR proteins. To date it has proved more difficult to confirm this in vivo, with phosphopeptides not detected in the putative phosphorylated pFNRI and pFNRII spots by either immunoblots with phosphoserine and phosphothreonine antisera or MS analysis (data not shown). Although the attachment of a phosphate group adds the small mass of 80 Da to a peptide (Goshe, 2006), the phosphate groups are prone to detach from peptides during MS sample processing and this can lead to problems in detection. Similarly, the location towards the N-terminus of the predicted pFNR phosphorylation at S75 and the most likely pFNRII phosphorylation at T104 will result in peptides rich in small and hydrophobic amino acids for which it is particularly difficult to obtain mass peaks (T Nühse, personal communication). This, together with the incomplete phosphorylation of pFNR (Hodges and Migniac-Maslow, 1993), suggests that the putative phosphopeptides in pFNR may not be detectable with current methodology.

Subchloroplast localization

Total wheat pFNR protein, as in previous work for pea (Newman and Gray, 1988) and Arabidopsis (Hanke et al., 2005), was evenly distributed between stroma and thylakoid pools. However, whilst both alternative N-terminal forms of wheat pFNRII are equally distributed between stroma and thylakoid pools, the net distribution of wheat pFNRII proteins was more stromal. Interestingly, of the alternative N-terminal forms of wheat pFNRI, the more basic pFNRIKKS was mostly stromal, whilst the less basic pFNRIKSKQ was mostly thylakoid bound. Since the basic pFNR protein in Arabidopsis was also more stromal in distribution, whilst the acidic pFNR protein was more thylakoid localized (Hanke et al., 2005, Lintala et al., 2009), this suggests that stroma-thylakoid distribution may in part be related to isoform acidity. However, a greater acidity of pFNR protein does not correlate directly to greater thylakoid localization, as the mostly thylakoid-bound pFNRIKSKQ isoform is actually less acidic than the two alternative N-terminal forms of pFNRII that are equally distributed between the stroma and thylakoid pools. Clearly other differences between pFNRI and pFNRII isoforms contribute to the strength of thylakoid binding. A partial link between pFNR protein acidity and subchloroplast localization could be due to pFNR being loosely bound to the thylakoid membrane by electrostatic forces (Carrillo and Vallejos, 1982). Based on a study of an Arabidopsis mutant line lacking the acidic pFNR form, the basic pFNR was only found in the stroma, suggesting that heterodimer formation may also play an essential role in membrane attachment of pFNR (Lintala et al., 2007).

Alternative mature N-termini

pFNRIKSKQ was the only isoform that changed significantly in abundance along the developmental and photosynthetic gradient between the leaf middle and tip, whilst pFNRIKKS was the only isoform induced significantly by the MV treatment or by nitrate. Although pFNRIISKK was consistently more abundant than pFNRIKSKQD across all eight treatments (P < 0.001), neither pFNRII isoform was significantly induced by any of the treatments. The two forms of pFNRI also showed contrasting distribution patterns between stroma and thylakoid pools. The alternative forms of protein from each pfnr gene differ by just a short truncation (2/3 amino acids) at the N-terminus. Analysis of the full-length precursor protein sequences suggests that wheat pFNRs undergo a secondary processing event after the initial removal of the transit peptide sequence. This secondary removal of an amino acid length between the cleavage site of the pFNR transit peptide and its core catalytic domain would then leave the alternative mature N-terminal versions of wheat pFNR.

In pFNR, this alanine- and proline-rich spacer region has previously been demonstrated to be important for rates of protein import to the chloroplast (Rial et al., 2002). If secondary processing of pFNR occurs during chloroplast
import, then this spacer region may also be involved in generating the alternative N-terminal versions. Interestingly, Arabidopsis pFNRI and pFNRII each appear to have only a single mature N-terminal version corresponding to the transit peptide cleavage site predicted here (Hanke et al., 2005). This may be because the lower alanine/proline content of the putative spacer region in Arabidopsis (a mean of 23% for Arabidopsis pFNRI/II compared with a mean of 56% for wheat pFNRI/II) precludes this process.

How pFNR binds to chloroplast envelope translocons and to photosynthetic electron transport chain complexes still needs to be clarified. pFNR binds to the chloroplast side of the protein import machinery at the Tic complex (Küchler et al., 2002) and has been proposed to connect to a redox signalling cascade through the Tic62 translocon (Stengel et al., 2007). A thylakoid-localized pool of Tic62 forms light- and pH-regulated complexes with pFNR and is proposed to be important for stabilizing pFNR membrane binding in Arabidopsis (Benz et al., 2009). Furthermore, a thylakoid rhodanese-like (TROL) protein has recently been identified in Arabidopsis and suggested to play a role in anchoring pFNR to the thylakoid membrane (Juric et al., 2009). The metabolic importance of this process has been confirmed by analysis of Arabidopsis mutant lines lacking TROL which showed severely decreased levels of LEF (Juric et al., 2009).

Physiological state and pFNR isoform protein levels

Comparisons of the location, expression pattern, and ferredoxin interaction of native isoforms, together with the analysis of recombinant enzymes and mutant lines, indicate that multiple forms of wheat (Gummadova et al., 2007), maize (Okutani et al., 2005), and Arabidopsis (Hanke et al., 2008; Lintala et al., 2009) pFNR provide plants with the flexibility to respond to changing reductant demands. In studies of Arabidopsis mutant lines both pFNR forms are necessary for optimal growth, with the absence of either form leading to low chlorophyll content, low accumulation of photosynthetic thylakoid proteins, and reduced carbon fixation rate (Lintala et al., 2009). In the results reported herein, different experimental conditions were used to provide an insight into potential roles of specific wheat pFNR isoforms.

The levels of pFNRIKKVS doubled between the greening middle section of the leaf (4–6 cm from the base) and the leaf tip. Since photosynthetic rates at the leaf tip are one-third higher than at the leaf middle (Tobin et al., 1988), then the most obvious function for pFNRIKKVS would be to fulfil the proposed requirement for CEF to generate additional ATP at high rates of photosynthesis and carbon fixation (Cruz et al., 2005). However, such provision of ATP for the normal functioning of photosynthesis is linked to the FQR-dependent pathway (Munekage et al., 2004), requiring a pFNR with a high enzyme $K_m$ to bind ferredoxin for FQR without generating NADPH. This does not fit the low $K_m$ of the pFNRIKKQ isoform (Gummadova et al., 2007). Instead, with half the catalytic efficiency of pFNRIKKVS (Gummadova et al., 2007), pFNRIKKQ has catalytic properties more suitable for decreasing the tight coupling of PSI to LEF generation of NADPH. By increasing the amount of reduced ferredoxin that can escape to the stroma and enter CEF (Fig. 9A), this fits the redox

![Fig. 9. Speculative roles of the pFNRI isoforms. Results here suggest that the two alternative N-terminal forms of pFNRI play distinct roles in the partition of photosynthetic reductant. (A) The less efficient isoform, pFNRIKKQ, is specifically induced at the leaf tip, where it is proposed to allow reduced ferredoxin to diffuse to the stroma to enter FQR-dependent CEF around the cytochrome$_{b6}$f complex. (B) pFNRIKKVS, in contrast, is the most efficient isoform and its induction by nitrate is proposed to be linked to higher demands for LEF to supply NADPH. (C) pFNRIKKVS induction by methyl viologen treatment is proposed to provide NADPH for NDH-dependent CEF in response to oxidative stress when the electron transport chain (ETC) is over-reduced.](image-url)
poise paradigm for electron flux partition expounded by Breyton and co-workers (2006).

pFNR protein levels are influenced by the nitrogen supply in the growth medium (Hanke et al., 2005, 2008; Okutani et al., 2005; Gummadova et al., 2007; Lintala et al., 2009). Under low nitrogen conditions, Arabidopsis mutants lacking individual pFNR isoproteins accumulated greater biomass than wild-type plants under low nitrogen conditions, indicating marked physiological effects as a result of altered chloroplast electron channelling (Hanke et al., 2008). Plants grown in the presence of nitrate have higher rates of photosynthesis (Dale, 1972), and the generation of the carbon-fixing Rubisco protein is one of the largest requirements for nitrogen in plant metabolism (Feller et al., 2008). Increased nitrate availability is therefore likely to increase carbon fixation demands for NADPH supply by LEF. Total pFNR protein levels were significantly higher (P < 0.05) when wheat plants were grown on 10 mM nitrate than when grown in the absence of nitrate, due specifically to the doubling (P < 0.05) of levels of the pFNR1KKVS isoform. Of the four pFNR isoforms, pFNR1KKVS has the highest catalytic efficiency (Gummadova et al., 2007) and so would favour the high rates of LEF likely to be induced by nitrate (Fig. 9B).

The MV experiment was based on previously published approaches for stimulating oxidative stress (Bowler et al., 1991; van Camp et al., 1996; Palatnik et al., 1997). The 100 μM MV treatment also significantly increased levels of pFNR1KKVS (P < 0.05), suggesting a role for this specific isoform in one or both of the two CEF pathways (FQR dependent and NDH dependent) required for tolerance to oxidative stress conditions (Endo et al., 1999; Munekage et al., 2004). pFNR has previously been linked to both FQR-dependent (Clark et al., 1984; Cleland and Bendall, 1992) and NDH-dependent (Mills et al., 1979) CEF pathways. FQR-dependent CEF would require a pFNR enzyme with low catalytic efficiency, which does not fit the catalytic properties of pFNR1KKVS. For pFNR to act in NDH-dependent CEF, as originally suggested by Mills et al. (1979), would similarly require low catalytic efficiency. As such the high catalytic efficiency of pFNR1KKVS and its specific induction by MV suggests that it most probably generates a locally enriched pool of NADPH at the NDH complex to feed CEF (Fig. 9C; Gummadova et al., 2007). Recently distinct differences in functions of pFNR have become more evident when Arabidopsis mutants lacking pFNR isoproteins were grown at low temperature (Lintala et al., 2009). More specifically, Arabidopsis plants were more tolerant to oxidative stress when only the acidic pFNR protein was present (Lintala et al., 2009). It has been suggested that, under such conditions that impact the photosynthetic capacity of the plant, the acidic pFNR protein has an important role in the redistribution of electrons to different reducing pathways (Lintala et al., 2009). The mature pFNR protein is essentially globular with a small, flexible N-terminal ‘flap’ protruding from the surface (Gadda et al., 1990; Maeda et al., 2002). Because the ferredoxin affinity of pFNR changes with different N-terminal start points (Maeda et al., 2005; Gummadova et al., 2007), it seems that the N-terminal flap (and its length) plays an important role in holding the ferredoxin substrate. Since enzymes are ‘dynamic molecular machines’ and intrinsic motions are important for mechanisms of interaction (Huang and Montelione, 2005), then the ability of the enzyme to move and change shape might be important in this regard. Ongoing research investigating the effects of artificial N-terminal truncations of wheat pFNR1 and pFNR2 on catalytic properties will form the topic of a future publication.

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