N-Terminal Structure of Maize Ferredoxin:NADP⁺ Reductase Determines Recruitment into Different Thylakoid Membrane Complexes

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INTRODUCTION

The ATP and NADPH that support photautotrophic growth are provided by photoexcitation and electron transport through protein complexes at the thylakoid membrane. Under natural growth conditions, light intensity does not remain constant, and it is a challenge to maximize energy conversion under low light conditions while minimizing damage due to overexcitation at high light. Photosynthetic organisms can respond to these environmental changes by altering photosynthetic electron transport (PET) at the thylakoid membrane. In the main pathway of linear electron transport (LET), water is split to O₂ and H⁺, and NADP⁺ is the final electron acceptor (Arnon et al., 1963; Shin and Amon, 1965). The final transfer of electrons to NADP⁺ is catalyzed by the enzyme ferredoxin:NADP⁺ reductase (FNR), which is both bound to the thylakoid as a peripheral protein and present as a soluble protein in the chloroplast stroma. In the alternative pathway of cyclic electron transport (CET), electrons are passed back to the plastoquinone pool from either ferredoxin or NADPH, which generates a proton gradient resulting in ATP synthesis, with minimal water splitting and no net reduction of NADP⁺ (Arnon et al., 1963; Hosler and Yocum, 1985; Munekage et al., 2004). A role for FNR in CET has been proposed due to reported interactions between FNR and characterized components of the CET apparatus in higher plants and algae (Zhang et al., 2001; DalCorso et al., 2008; Iwai et al., 2010), perturbation of CET in cyanobacteria that lack membrane-bound FNR (van Thor et al., 2000), electron donation from FNR to quinones (Bojko et al., 2003), and the disruption of CET in spinach (Spinacia oleracea) thylakoids by FNR inhibitors (Shahak et al., 1981). However, the precise function of FNR in the CET pathway remains an open question.

These roles in different electron transport pathways have led to intense research interest in the mechanism and location of FNR binding to the thylakoid membrane (Andersen et al., 1992; Jose Quiles and Cuello, 1998; Zhang et al., 2001; Peng et al., 2008; Benz et al., 2009; Jurić et al., 2009; Alte et al., 2010; Baniulis et al., 2011). Two thylakoid FNR binding proteins named TROL and Tic62 have been identified in Arabidopsis thaliana. TROL mutants are impaired in photosynthesis (Jurić et al., 2009), and Tic62 has no direct photosynthetic role but seems rather to stabilize FNR (Benz et al., 2009). An FNR binding domain containing a Ser/Pro-rich motif has been identified in both TROL and Tic62, and its interaction with FNR has recently been thoroughly characterized (Alte et al., 2010). In the crystal structure, FNR dimerizes around this Ser/Pro-rich motif. This interaction increases the surface area available for FNR:FNR binding from 380 to 2070 Å², and the strength of this interaction is pH dependent. In addition to these specific binding proteins,
FNRI copurifies with the cytochrome b₅f complex (Cyt b₅f) of several higher plants and cyanobacteria (Zhang et al., 2001; Okutani et al., 2005; Baniulis et al., 2011), although the area on the complex to which FNRI binds is not yet clear. Isolation from Chlamydomonas reinhardtii of a supercomplex capable of CET, and composed of only FNRI, photosystem I (PSI), Cyt b₅f, and PgrL1 (Iwai et al., 2010), supports the involvement of FNRI bound to Cyt b₅f in CET.

In all higher plants with extensive sequence availability, two different FNRI isoproteins are present. The best studied example is Arabidopsis, where both proteins (At-FNRI and At-FNRII) were identified in membrane-bound and unbound states and were found to have similar electron transfer properties (Hanke et al., 2005). Reciprocal knockouts are quite similar in phenotype but have revealed that At-FNRI is dependent on At-FNRI for membrane association (Lintala et al., 2007; Hanke et al., 2008), and At-FNRII may have a role in stress signaling (Lintala et al., 2009). By contrast, maize (Zea mays) chloroplasts contain three separate FNRI isoproteins (Zm-FNRI, Zm-FNRII, and Zm-FNRIII) (Okutani et al., 2005).

These three FNRIIs have distinct membrane association patterns (Okutani et al., 2005). Zm-FNRI is restricted to the thylakoid, Zm-FNRII is only recovered from chloroplasts as a soluble protein, and Zm-FNRIII is found in both membrane-bound and unbound states. The physiological role of the uniquely soluble FNRIII in maize chloroplasts is not clear, although it has a slightly higher efficiency than maize FNRII or FNRII in nonphotosynthetic electron transfer, from NADPH to ferredoxin, and transcripts are unbound:free ratio of FNRI is completely different between these cell types. In determining membrane association by solving the crystal structures of all three maize FNRIIs, truncated FNRII sequences, and a chimeric FNRI construct with exchanged N termini established that the N-terminal contribution to membrane binding could be a universal mechanism in higher plants and that N-terminal structure also contributes to relative recruitment into Tic62 or TROL coordinated thylakoid membrane complexes.

RESULTS

Variable FNRI Composition of Maize Bundle Sheath and Mesophyll Cells

Evidence from maize thylakoid proteomic studies suggests that FNRII is enriched in the mesophyll, and FNRI is equally abundant in mesophyll and bundle sheath cells (Majeran et al., 2008). No information is available for FNRIII. To clarify the contribution of FNRI isoproteins to the different electron transfer processes that occur in these cell types, we compared their abundance in separated mesophyll and bundle sheath cells by immunoblotting. Figure 1A shows that mesophyll cells contain a higher abundance of FNRI than the bundle sheath, and Figure 1B demonstrates the different FNRI profiles of these cell types. Membrane-bound forms were detected in both the bundle sheath and mesophyll cells, with the ratio of Zm-FNRI to Zm-FNRII higher in the bundle sheath than the mesophyll. By comparison, soluble proteins were only detected in the mesophyll cells, where FNRIII is the most abundant isoprotein. The higher proportion of FNRI at the membrane in the bundle sheath therefore correlates with a higher demand for CET, while the greater abundance of soluble FNRI occurs in cell types with a greater demand for LET. It should be noted that, in our experiments, soluble proteins were extracted from leaves under growth light conditions in 100 mM NaCl ionic strength buffer; thus, transiently or weakly membrane-bound FNRI might be recovered in the soluble fraction.

Differences in Maize FNRI Recruitment into Arabidopsis Thylakoid Complexes

The lack of soluble FNRI in bundle sheath chloroplasts, in comparison to the abundant soluble FNRI in mesophyll chloroplasts, could either be due to variation between the chloroplast environment in these cell types or FNRI composition. Variable membrane association of Zm-FNRI, Zm-FNRII, and Zm-FNRIII suggests that their intrinsic properties might determine the ratio between soluble and membrane-associated FNRI. We therefore further tested their membrane association by introducing the maize (Zm) FNRI, FNRII, and FNRIII coding sequences into Arabidopsis and examining the location of the proteins. Figure 2A shows higher FNRI activity than the wild type in two independent lines expressing each Zm-FNRI gene. The phenotype of the transgensics varied with the Zm-FNRII gene introduced. Figure 2B shows that lines overexpressing Zm-FNRII and Zm-FNRIII were larger than the wild type. By contrast, lines overexpressing Zm-FNRII were smaller, and increasing FNRI activity in the Zm-FNRII lines correlated with decreasing size. The maize FNRIIs have similar molecular mass (~35 kD) and can only be clearly separated from native Arabidopsis FNRI isoproteins by native-PAGE followed by immunoblotting, as shown in Figure 2C. On introduction to Arabidopsis, the maize FNRIIs appear to associate with the membrane in a comparable pattern to that seen in maize chloroplasts. In Arabidopsis, transgenically introduced Zm-FNRI was found principally at the membrane, Zm-FNRIII was equally distributed between membrane and soluble fractions, while Zm-FNRIII was mostly recovered in the soluble fraction, indicating that differences between the proteins contribute to their membrane association. Apparent differences in Zm-FNRI abundance in the transgenic lines are probably due to variable antigenicity of the isoproteins to the antisera, which was raised against maize FNRIII.

Two proteins have recently been reported to bind FNRI at the thylakoid membrane in Arabidopsis: TROL (Jurić et al., 2009) and...
transgenic Zm-FNR plants. Figure 2D shows the distribution of FNR in thylakoids from representative lines expressing Zm-FNR1, Zm-FNR2, or Zm-FNR3. In comparison to the wild type, Zm-FNR1–expressing plants showed enrichment in FNR bound to the 140- to 190-kD complexes coordinated by TROL. In comparison, thylakoids from Zm-FNR2–expressing plants were specifically enriched in the higher molecular mass complexes coordinated by Tic62. Membranes from Zm-FNR3 lines were not noticeably enriched, and relative staining of TROL and Tic62 complexes was similar to that seen in untransformed Arabidopsis. The kinetics of FNR binding to a motif conserved between TROL and Tic62 are well described (Alte et al., 2010), and the data in Figure 2D now indicate there may be differences between the mechanism of FNR interaction with TROL and Tic62.

N-Terminal Structure Varies between Maize FNR Proteins

The recruitment of maize FNR1 and FNR2 into different membrane complexes and the weakened membrane association of maize FNR3 offer an opportunity to identify the structural determinants of FNR membrane recruitment. To investigate this, we crystallized the purified, mature maize FNR proteins and solved their structures. Crystal data and refinement statistics are given in Supplemental Table 1 online. The maize FNR isoforms closely resemble each other (Figure 3A), with the only major difference between them in the orientation of the N terminus. In the Zm-FNR1 structure, resolution of N-terminal amino acids was excellent, showing a rigid structure, perpendicular to the surface of the molecule. In Zm-FNR2, the N-terminal was poorly resolved, presumably due to a high degree of flexibility. The N-terminal region in the Zm-FNR3 structure was resolved clearly enough to identify its orientation, lying flat across the molecule.

All three maize FNRs were crystallized as homodimers (Figure 3B). The orientation of Zm-FNR1 and Zm-FNR2 subunits in the homodimer is very similar to that in the cocystal of pea (Pisum sativum) FNR with a 27–amino acid fragment of Tic62 (Alte et al., 2010). By contrast, the structure of the Zm-FNR3 homodimer is much more open, with the potential Tic62 binding surfaces further apart. This is consistent with the poor membrane association behavior of Zm-FNR3. Interestingly, in the Zm-FNR1 homodimer, the N termini of two adjacent Zm-FNR1 molecules are inserted at the interface of the homodimer (Figure 3C; coordinates taken from Alte et al., 2010). In addition to N-terminal differences, there is variation between the charge distribution at the homodimer-forming surface of FNR proteins (Figures 3C and 3D). Maize Zm-FNR1 is similar to pea FNR (Figure 3E), with a basically charged area to one side of the mostly acidic region to which the Tic62 peptide binds. By contrast, Zm-FNR2 has a much more acidic surface in this region (Figure 3D). Zm-FNR3 shows almost identical surface charge to Zm-FNR2 and so is not shown. Although the FNR binding domains on Tic62 and TROL are very similar, the increased TROL binding in Arabidopsis transformed with Zm-FNR1 indicates that these differences in N-terminal and homodimer interface may determine preferential recruitment of Zm-FNR1 into TROL and Zm-FNR2 into Tic62-mediated complexes.
The Maize FNR1 N Terminus Confers Membrane Binding Activity on Maize FNR3

To determine the contribution of the different N-terminal structures to membrane association, we investigated whether exchange of N-terminal regions between FNR proteins could change their membrane association (Figure 4). Chimeric constructs were generated, encoding the transit peptide and mature N-terminal of Zm-FNR1 and the remaining part of the Zm-FNR3 molecule (Figure 4A). Transformation of Arabidopsis with these Zm-FNR1-3 constructs yielded several independent lines. The introduced Zm-FNR chimeras could not be distinguished from indigenous Arabidopsis FNRs by SDS-PAGE (data not shown), so native PAGE was used to determine whether the chimeras were membrane bound or not. The typical distribution of chimeric Zm-FNR1-3 between membrane and soluble fractions is shown in Figure 4B, and a single additional band migrating faster than the endogenous At-FNRs is visible. In addition, these plants appear enriched in native At-FNR1, presumably due to some pleiotropic effect, as described in previous studies of FNR overexpression (Higuchi-Takeuchi et al., 2011). In comparison to transgenic Arabidopsis plants expressing the parent molecules, in which Zm-FNR3 was predominantly recovered in the soluble fraction, the additional Zm-FNR1-3 band is more abundant at the membrane. When thylakoids from Zm-FNR1-3 lines were separated by blue native-PAGE and immunoblotted, enrichment of TROL coordinated FNR complexes was clearly seen (Figure 4C). This result demonstrates that introduction of the maize FNR1 N-terminal to maize FNR3 increases membrane association and specifically enhances interaction with TROL.

Figure 2. Transformation of Arabidopsis with Maize FNR Isoproteins.

(A) Arabidopsis transformed with maize FNR proteins. Genes encoding maize FNR1 (Zm-FNR1), FNR2 (Zm-FNR2), and FNR3 (Zm-FNR3) were cloned and used to stably transform wild-type (wt) Arabidopsis under control of the Arabidopsis FNR1 promoter. Crude protein extracts were made from leaves of the transformants and assayed for FNR activity, measured as ferredoxin-dependent cytochrome c reduction in crude protein extracts from leaves of the wild type and two independent Arabidopsis lines overexpressing Zm-FNR1, Zm-FNR2, and Zm-FNR3. Values are means ± SE of three independent measurements.

(B) Phenotype of Zm-FNR transgenics. Typical representative plants of two independent lines expressing either Zm-FNR1, Zm-FNR2, or Zm-FNR3 are shown following 10 weeks of growth.

(C) Subcellular location of Zm-FNR proteins in Arabidopsis. Crude extracts from the wild type and representative Zm-FNR1, Zm-FNR2, and Zm-FNR3–expressing lines were separated into soluble (S) and membrane (M) protein fractions. For each sample, 20 µg of total protein was divided into S and M fractions with equal volume, before native-PAGE, and immunoblotting with an antibody raised against Zm-FNR2. Position of native Arabidopsis FNR1 (At-FNR1) and FNR2 (At-FNR2) proteins is indicated to the left of the blot. Positions of introduced Zm-FNR proteins are indicated to the right of the blot.

(D) Recruitment of maize FNR proteins into Arabidopsis thylakoid membrane complexes. Chloroplasts were isolated from the wild type and representative Arabidopsis lines expressing the maize FNR genes. Thylakoid membranes were extracted, digested, and subjected to blue native-PAGE (BNP) before immunoblotting and detection of FNR-containing complexes. Samples were loaded on an equal chlorophyll basis, with 4 µg per lane. Left panel shows the blue native-PAGE gel following the run, with major native complexes indicated to the left of the gel. Right panel shows an immunoblot of the gel, detected with an antibody raised against maize FNR2, with FNR-containing complexes indicated to the right of the blot. Position of molecular mass markers is indicated between the gel and the blot in kilodaltons.
Figure 3. Structural Variation at the N-Terminal and Homodimer Interface of Maize FNR Proteins.

Maize FNR1, FNR2, and FNR3 were expressed recombinantly as mature proteins, purified, and crystallized. (A) Crystal structures of single FNR molecules. Structures are shown as ribbon with α-helices in red and β-sheets in yellow. The FAD moiety is shown in blue. Lower resolution of structure is indicated by the green and red dotted lines representing parts of the Zm-FNR2 and Zm-FNR3 N termini. (B) Arrangement of Zm-FNR homodimers. Structures are shown as a ribbon with α-helices in red and β-sheets in yellow. In the Zm-FNR1 structure, N termini of two adjacent Zm-FNR1 molecules are shown in either white or blue balls and sticks.

(C) to (E) Charge distribution at the interface of Zm-FNR homodimers (red, area of acidic charge; blue, area of basic charge). Charge distribution on space-filled homodimers of Zm-FNR1 (C), Zm-FNR2 (D), and pea FNR (E) (taken from Alte et al., 2010). The dimer structure is shown in the center, composed of two monomers [A] and [B]. To the left and right of each dimer, the constituent FNRA and FNRB monomers of each dimer are shown rotated through 90° to display the surface area of interaction. In the structure of Zm-FNR1, the N termini of other Zm-FNR1 molecules adjacent to the dimer in the crystal matrix insert into the dimer interface. These adjacent FNR molecules are labeled FNR1A' and FNR1B' and are shown as ball and stick models in white (FNR1 A') and blue (FNR1 B'). The location of the 27-amino acid fragment of Tic62 in the structure of the pea FNR structure is shown in a yellow ball and stick model.
Truncated Maize FNR1 Retains Some TROL Binding Ability

Because the addition of the Zm-FNR1 N-terminal enhanced Zm-FNR3 binding to the membrane in general, and to TROL mediated complexes in particular, we tested whether truncation of this domain would affect the properties of Zm-FNR1. We constructed a truncated Zm-FNR1 coding sequence (Zm-FNR1t), with the transit peptide still intact (Figure 5). The truncated construct lacked the N-terminal 14 amino acids of the mature protein that correspond to those raised from the globular structure of Zm-FNR1 in Figure 3A. To confirm correct processing of the transit peptide of Zm-FNR1t and investigate membrane association, we used SDS-PAGE, rather than native-PAGE, because the smaller, truncated protein can easily be distinguished from the native At-FNR isoproteins. The smaller size of the additional protein confirms that the transit peptide was correctly processed (Figure 5B). The relative proportion of Zm-FNR1t bound to the membrane decreased in comparison to Zm-FNR1, but some membrane binding capacity was retained. Blue native-PAGE analysis demonstrated that Zm-FNR1t-expressing plants were still enriched in TROL binding FNR relative to the wild type, although this was reduced in comparison to Zm-FNR1-expressing plants. This suggests that there are additional factors that contribute toward specific recruitment of maize FNR1 to TROL. The most likely of these is the Zm-FNR1 homodimer interface surface, which is more basic than that seen in Zm-FNR2 or Zm-FNR3 (Figure 3), and this would also provide some explanation for the enriched Tic62 binding in one line expressing the Zm-FNR1-3 chimera (Figure 4C), as Zm-FNR3 is also more acidic in this region.

FNR Location Influences Electron Transport around the Photosystems

Since the discovery that two different proteins, Tic62 and TROL, are capable of mediating FNR association with the thylakoid exchange the transit peptides and amino acids perpendicular to the surface of Zm-FNR1 in the structure shown in Figure 3. The first amino acids of the mature proteins are indicated as white lettering on black. (B) Detection of chimeric Zm-FNR1-3 proteins expressed in transgenic Arabidopsis. Plants were stably transformed with the chimeric Zm-FNR1-3 construct under control of the At-FNR1 promoter. Protein extracts were made from Arabidopsis wild-type (wt), Zm-FNR1, and Zm-FNR3-expressing plants, and a representative line expressing Zm-FNR1-3 (Figure 4), and this would also provide some explanation for the enriched Tic62 binding in one line expressing the Zm-FNR1-3 chimera (Figure 4C), as Zm-FNR3 is also more acidic in this region.

Figure 4. Impact of Chimeric N-Terminal Exchange between Maize FNR Proteins.

(A) Construction of chimeric Zm-FNR1-3. Amino acid sequence alignment, comparing Zm-FNR1 (black on white) and Zm-FNR3 (black on gray) with chimeric Zm-FNR1-3, in which the Zm-FNR1 N-terminal sequence was exchanged for that of Zm-FNR3. Chimeras were created to

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(Benz et al., 2009; Jurić et al., 2009), there has been much speculation that FNR bound to different protein complexes, and in the soluble phase, might coordinate different electron transfer processes (Benz et al., 2010; Joliot and Johnson, 2011). As we found Arabidopsis plants expressing maize FNR1, FNR2, and FNR3 are respectively enriched in FNR bound to TROL, Tic62, and in soluble FNR, we compared their PET capacity, to gain further insight into this question. There are early reports that FNR is the likely point of light regulation at the reducing side of PSI on light induction (Satoh and Katoh, 1980; Satoh, 1982), and more recent articles have provided further evidence that FNR may regulate PET (Ilík et al., 2006; Joliot and Johnson, 2011) and that this might be dependent on the NADP+/NADPH ratio (Rajagopal et al., 2003; Hald et al., 2008). In addition, FNR membrane association is known to change during dark-to-light transitions: FNR bound to Tic62 is released at basic pH values that could be generated in PET (Alte et al., 2010), and interaction with thylakoid Tic62- and TROL-mediated complexes is reported to be disrupted in the light (Benz et al., 2009). We therefore examined electron transport processes at PSII and PSI over the transition from dark to moderate light. Figure 6 shows parameters calculated from chlorophyll fluorescence at PSII and P700 absorption at PSI. As expected, differences from the wild type were mainly seen during the initial stages of light induction. Electron transport rates at PSI and PSII in the maize FNR-expressing plants show relatively small deviations from the wild type, but there is a more rapid increase in $\Phi_{I}/\Phi_{II}$ for Zm-FNR1-expressing plants when the light is switched on, reflecting higher activity at PSI relative to PSII. This rise is mirrored in a more rapid increase in nonphotochemical quenching (NPQ). By contrast, Zm-FNR2– and Zm-FNR3–expressing plants show slower increases in both these parameters, and this is most dramatic for Zm-FNR3–expressing plants. The differences of Zm-FNR2– and Zm-FNR3–expressing plants disappear over the light induction, but Zm-FNR1–expressing plants retain a higher $\Phi_{I}/\Phi_{II}$ for the duration of the measurement.

Figure 5. Impact of N-Terminal Truncation on Maize FNR1 Assembly into Thylakoid Membrane Complexes.

(A) Truncation of maize FNR1 sequence. Alignment comparing the N-terminal portion of the native (Zm-FNR1) and truncated (Zm-FNR1t) nucleotide and amino acid sequences. The Zm-FNR1 sequence was truncated to retain the transit peptide, while shortening the mature protein sequence by 14 amino acids that are perpendicular to the surface of the protein in the structure shown in Figure 3. The first amino acid of the mature Zm-FNR1 protein is indicated as white lettering on black.

(B) Detection of the mature, truncated Zm-FNR1 protein expressed in transgenic Arabidopsis. Plants were stably transformed with the truncated (Benz et al., 2009; Jurić et al., 2009), there has been much speculation that FNR bound to different protein complexes, and in the soluble phase, might coordinate different electron transfer processes (Benz et al., 2010; Joliot and Johnson, 2011). As we found Arabidopsis plants expressing maize FNR1, FNR2, and FNR3 are respectively enriched in FNR bound to TROL, Tic62, and in soluble FNR, we compared their PET capacity, to gain further insight into this question. There are early reports that FNR is the likely point of light regulation at the reducing side of PSI on light induction (Satoh and Katoh, 1980; Satoh, 1982), and more recent articles have provided further evidence that FNR may regulate PET (Ilík et al., 2006; Joliot and Johnson, 2011) and that this might be dependent on the NADP+/NADPH ratio (Rajagopal et al., 2003; Hald et al., 2008). In addition, FNR membrane association is known to change during dark-to-light transitions: FNR bound to Tic62 is released at basic pH values that could be generated in PET (Alte et al., 2010), and interaction with thylakoid Tic62- and TROL-mediated complexes is reported to be disrupted in the light (Benz et al., 2009). We therefore examined electron transport processes at PSII and PSI over the transition from dark to moderate light. Figure 6 shows parameters calculated from chlorophyll fluorescence at PSII and P700 absorption at PSI. As expected, differences from the wild type were mainly seen during the initial stages of light induction. Electron transport rates at PSI and PSII in the maize FNR-expressing plants show relatively small deviations from the wild type, but there is a more rapid increase in $\Phi_{I}/\Phi_{II}$ for Zm-FNR1–expressing plants when the light is switched on, reflecting higher activity at PSI relative to PSII. This rise is mirrored in a more rapid increase in nonphotochemical quenching (NPQ). By contrast, Zm-FNR2– and Zm-FNR3–expressing plants show slower increases in both these parameters, and this is most dramatic for Zm-FNR3–expressing plants. The differences of Zm-FNR2– and Zm-FNR3–expressing plants disappear over the light induction, but Zm-FNR1–expressing plants retain a higher $\Phi_{I}/\Phi_{II}$ for the duration of the measurement.

Zm-FNR1t construct under control of the At-FNR1 promoter. Protein extracts were made from Arabidopsis wild type (wt), one representative line expressing Zm-FNR1, and one representative line expressing Zm-FNR1t (Zm-FNR1t-7). For each sample, 20 µg of total protein was divided into soluble (S) and membrane-bound (M) fractions, before SDS-PAGE, and immunoblotting with an antibody raised against maize FNR2. Position of native Arabidopsis FNR proteins (At-FNR1 + At-FNR2) is indicated to the left of the blots, and positions of introduced Zm-FNR1 and Zm-FNR1t are indicated to the right of the blots.

(C) Assembly of Zm-FNR1 into thylakoid membrane complexes. Thylakoid membrane protein complexes from Arabidopsis wild type, transgenics expressing Zm-FNR1, and two independent lines overexpressing Zm-FNR1t were solubilized and subjected to blue native-PAGE (BNP) before immunoblotting and detection of FNR-containing complexes. Samples loaded contained 4 µg chlorophyll per lane. Approximate molecular masses are given in kilodaltons to the left of the blot, and FNR-containing complexes coordinated by Tic62 and TROL are indicated to the right of the blot.
DISCUSSION

N-Terminal Structure Promotes FNR Dimerization, Enhancing Recruitment to the Membrane

The three FNR proteins in maize chloroplasts are all unique in their association with the membrane. The aim of our study was to identify structural variation between these proteins that could determine membrane interaction. In Zm-FNR1 (membrane bound), the N terminus sticks out from the surface of the protein, while in Zm-FNR2 (dual location), this region is not well resolved, and in Zm-FNR3 (soluble), it is folded back across the surface of the protein. N termini from adjacent Zm-FNR1 molecules fill the space between the dimerized Zm-FNR1 molecules in the crystal. By contrast, the relative orientation of the crystallized Zm-FNR3 in the homodimer is markedly different, with a very open structure. Assuming the orientation of the Zm-FNR1 N terminus in the crystal structure is not an artifact, this suggests that the Zm-FNR1 N terminus may promote dimer formation and that dimerization is crucial for recruitment to the thylakoid. Excluding the N terminus, maize FNR2 and FNR3 monomer structures are very similar (Figure 3), and amino acid sequences are 93% identical (see Supplemental Figure 1 online). We therefore speculate that the more efficient dimerization of Zm-FNR2 is also due to the N terminus. Although it is not resolved in the crystal structure, it may adopt an orientation favorable to dimer formation, such as that shown by Zm-FNR1. Because Zm-FNR2 is the only maize FNR capable of both membrane and soluble location, structural rearrangement of the flexible Zm-FNR2 N terminus could thus regulate its recruitment and release from the thylakoid by influencing dimer formation. Interestingly, regulation of wheat (*Triticum aestivum*) FNR association with the thylakoid is also influenced by N-terminal structure, with truncation of the FNR1 N terminus by only three amino acids, resulting in an increase from 33 to 76% of the enzyme found associated with the membrane (Moolna and Bowsher, 2010).

In contrast with their native environment in the chloroplast, the membrane and soluble localization of maize FNR1 and FNR3, respectively, is not as exclusive when the genes are expressed in *Arabidopsis*; a small amount of Zm-FNR3 is bound to the membrane, and a small amount of Zm-FNR1 is soluble (Figure 2C). We speculate that this is due to evolutionary refinement of FNR–binding protein interactions in maize and that the evolutionary distance from *Arabidopsis* means relative affinities are not completely conserved. FNR binding to complexes mediated

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**Figure 6.** PET in *Arabidopsis* with FNR Enriched in Soluble, TROL-Bound, or Tic62-Bound FNR.

Electron transport parameters of *Arabidopsis* wild type (closed triangles) and representative lines expressing either Zm-FNR1 (Zm-FNR1-5, open boxes), Zm-FNR2 (Zm-FNR2-2, open triangles), or Zm-FNR3 (Zm-FNR3-4, open circles) over light induction. Plants were dark adapted before exposure to growth light intensity actinic light. Saturation pulses were given at the indicated times for calculation of the indicated parameters. Values are means ± SE of measurements on mature leaves from five to six different individuals, and the experiment was repeated three times with basically the same result. The experiment was also repeated with other lines expressing the same genes to confirm tendencies.
by TROL is necessary for optimal photosynthetic electron flow (Jurić et al., 2009), while binding to Tic62 is not (Benz et al., 2009). It is therefore of interest to speculate on how maize FNR1 and FNR2 are differentially recruited to these two different complexes (Figure 2). Addition of the Zm-FNR1 N terminus results in recruitment of the normally soluble Zm-FNR3 to TROL, so the N terminus has a clear role in this specific association. Tic62:FNR association is weaker than TROL:FNR association (Jurić et al., 2009), and because the N terminus of Zm-FNR1 appears to sterically hinder Tic62-type binding (Figure 3), it may be that this prevents binding to Tic62. The stronger affinity of TROL for FNR could allow it to displace the N terminus, enabling FNR dimerization around TROL. However, truncation of the Zm-FNR1 N terminus could not completely eliminate recruitment into TROL complexes; therefore, other structural factors must contribute. We propose a model (Figure 7) in which a combination of N-terminal structure and charge distribution on the FNR: FNR dimer interface determines membrane association. Here, the N termini of adjacent Zm-FNR1 molecules promote the preformation of an FNR dimer, by occupying the space normally filled by an FNR binding domain on Tic62 or TROL (Alte et al., 2010). This preformed dimer has a greater tendency to dimerize in turn around the TROL binding domain, an association that is enhanced by the basic charges on the Zm-FNR1 dimer interface.

**Differential Roles of Membrane-Bound and Soluble FNR in CET**

There is strong evidence that membrane-bound FNR is necessary for optimal electron transport. In higher plants, it has been shown that membrane-bound FNR has a much higher activity in NADP⁺ photoreduction than that of soluble FNR (Forti and Bracale, 1984) and even addition of 5 μM soluble FNR could not restore wild-type NADP⁺ photoreduction rates to mutant thylakoids lacking native FNR (Hanke et al., 2008). In addition, mutants lacking TROL were perturbed in LET at high light intensities (Jurić et al., 2009). Finally, work on cyanobacteria (Korn et al., 2009) showed that membrane-bound FNR is predominantly involved in NADP⁺ photoreduction, while soluble FNR reduces ferredoxin using NADPH under heterotrophic conditions. Interestingly, not all membrane-bound FNR is directly involved in electron transport, as FNR recruited to Tic62-mediated complexes was stabilized against degradation rather than involved in PET (Benz et al., 2009).

The differential distribution of membrane-bound and soluble FNR isoproteins in maize bundle sheath and mesophyll cells is therefore enlightening. Homologous Tic62 and TROL cDNA sequences have been identified in maize (see Supplemental Figure 2 online). The maize FNR protein contains two of the FNR binding domains defined previously (Stengel et al., 2008), but maize Tic62 cDNAs lack any sequence encoding an FNR binding motif. The FNR binding motif is also missing from Tic62 sequences of sorghum (Sorghum bicolor), indicating evolutionary loss in some C4 type grasses. Proteomic work has shown that maize TROL is equally abundant in both mesophyll and bundle sheath cells (Majeran et al., 2008), so membrane-bound FNR is presumably recruited to TROL or Cyt b₆f in both cell types in maize. When genes were expressed in Arabidopsis, we found that maize FNR1 and FNR2 were bound to the recently identified FNR binding proteins TROL and Tic62, respectively, but that maize FNR3 was not (Figure 2D). Both maize FNR1 and FNR2 were also copurified from maize leaves with Cyt b₆f, but maize FNR3 was not (Okutani et al., 2005), indicating that FNR association with the Cyt b₆f might be either similar to, or dependent on, Tic62 or TROL.

Bundle sheath cells perform predominantly CET, with little need for regulatory adjustment of PET. The fixed membrane localization of FNR in bundle sheath chloroplasts therefore points to its permanent localization at specific complexes involved in CET, such as that reported by Iwai et al. (2010). By contrast, mesophyll cells must have the capacity to adjust electron flow between LET and CET, depending on environmental conditions (Munekage et al., 2004). There is evidence for FNR recruitment to several different thylakoid complexes (Andersen et al., 1992; Jose Quiles and Cuello, 1998; Zhang et al., 2001; Peng et al., 2008; Benz et al., 2009; Jurić et al., 2010).
2009; Baniulis et al., 2011), and the greater abundance of soluble FNR in maize mesophyll cells may provide capacity for dynamic release and rapid recruitment of FNR to different complexes, as has recently been suggested (Joliot and Johnson, 2011).

Because of the differences in FNR binding protein composition and PET, between the chloroplasts of Arabidopsis (a C3 plant) and the bundle sheath and mesophyll cells of maize, we can only tentatively interpret the physiological roles of FNR isoproteins using the Arabidopsis maize FNR transgenics. However, the data in Figure 6 support a model where tight binding to TROL enhances CET, as the Zm-FNR1-expressing plants showed an increased ratio of PSI to PSII activity, particularly during light induction. This is reflected in the stimulation of NPQ, which is dependent on CET enhancement of ΔpH formation. Such activity is consistent with the higher abundance of Zm-FNR1 in bundle sheath cells (Figure 1), where CET predominantly occurs. Arabidopsis expressing maize FNR2 showed almost no differences from the wild type in the PET parameters measured. As these plants are enriched in FNR bound to Tic62, this is consistent with reports that Tic62-associated FNR is not involved in PET (Benz et al., 2009).

In contrast with the Zm-FNR1 plants, Arabidopsis expressing maize FNR3 shows a slightly slower rise in ΔpH and a slower induction of NPQ on light induction (Figure 6). As Zm-FNR3 is predominantly soluble when expressed in Arabidopsis (Figure 2B), one interpretation of these results is that CET is enhanced by FNR recruitment to a specific complex, as previously proposed (Joliot and Johnson, 2011) and that this is facilitated if FNR is already localized at the membrane. The greater abundance of soluble FNR in the Zm-FNR3 plants may therefore compete with CET-recruited FNR for electrons carried by ferredoxin, slowing the onset of ΔpH and NPQ. The physiological role of the FNR3 in maize remains unclear, although it appears to be restricted to chloroplasts capable of performing LET (Figure 1), and one possibility is that it channels electrons from NADPH to support ferredoxin dependent reactions, such as those involved in N and S assimilation, when the PET chain is not operating, in a system analogous to that seen in cyanobacteria (Korn et al., 2009).

Previous transformation of tobacco (Nicotiana tabacum) with pea FNR did not yield a phenotypic difference (Rodriguez et al., 2007), indicating that factors other than FNR limit photosynthesis in tobacco. It was recently reported that expression of rice (Oryza sativa) FNR2 in Arabidopsis perturbed both LET and CET, while rice FNR1 disturbed N assimilation (Higuchi-Takeuchi et al., 2011). No information about association with different FNR binding proteins is available for this study, and interpretation of the phenotypes is complicated by the large decreases in native Arabidopsis FNRs, which were not seen in our study, where the native At-FNR1 promoter was used to drive Zm-FNR expression. The phenotypes of transgenics with variable FNR localization indicate that FNR microcompartmentation to different locations in the chloroplast has a big impact on the physiology of Arabidopsis. The more abundant FNR at the TROL complex in Zm-FNR1 plants seems to have a negative impact, whereas enrichment in Tic62 or soluble FNR results in plants larger than the wild type under these conditions (Figure 2B). Taken together with the analysis of PET in these plants, these results could indicate that under the growth conditions used in this work, FNR has a high control coefficient for PET but that dynamic association and dissociation from different membrane complexes is necessary for optimal efficiency. When association to thylakoid membrane complexes (specifically TROL) is enhanced, as in the maize FNR1-expressing Arabidopsis, dynamic dissociation is retarded and the balance of excitation between the photosystems disturbed, with deleterious consequences for PET and photosynthesis.

In summary, it has long been proposed that some structural change in FNR may regulate PET at the acceptor side of PSI (Satoh and Katoh, 1980; Satoh, 1982). The data presented in this article demonstrate that this regulation is probably related to the N-terminal structure of FNR, which determines the recruitment of the enzyme to thylakoid membrane complexes in maize, in a mechanism that can also operate in Arabidopsis. Analysis of PSI and PSII activity in Arabidopsis with FNR enriched either at TROL, Tic62, or in the soluble phase shows that PET activity over light induction is influenced by FNR location. Therefore, changes in N-terminal structure may be involved in controlling relative recruitment into different membrane complexes, allowing for crucial regulation of electron flow around the thylakoid membrane through changes in FNR location.

**METHODS**

**Plant Material and Sample Preparation**

Maize plants (Zeae mays cv Golden Cross Bantam T51) were grown on soil in a glasshouse, with supplementary light from Son-T Agro lamps (400 W; Philips). Arabidopsis thaliana plants were all Columbia ecotype and grown under long-day conditions, basically as described previously (Voss et al., 2011). Membrane and soluble protein fractions were prepared from whole plants and isolated chloroplasts and subjected to native-PAGE or SDS-PAGE and immunoblotting as described previously (Hanke et al., 2008). Mesophyll protoplasts and bundle sheath strands were isolated from mature maize leaves by physical disruption, basically as described by Sheen (1995). Antibodies used in immunoblotting detection were raised against maize FNR2 (1:50,000), spinach (Spinacia oleracea) NADP-MDH (1:10,000), and pea (Pisum sativum) ribulose-1,5-bisphosphate carboxylase/oxygenase (1:20,000).

**Cloning, Plasmid Construction, and Plant Transformation**

Mature maize FNR coding sequences and the Arabidopsis FNR1 promoter (At-FNR1 pro) were amplified from maize cDNA and Arabidopsis genomic DNA, respectively, with the primers listed in Supplemental Table 2 online as described previously (Okutani et al., 2005), and cloned using the Mighty TA-cloning kit (Takara). The At-FNR1 pro sequence was subcloned into pSMAB704 plant transformation vector (kind gift of Hiroaki Ichikawa) using a native HindIII site and an XbaI site introduced during cloning to give pSAFNFRI pro-GUS (for β-glucuronidase). Zm-FNR sequences were then separately subcloned into pSAFNFRI pro-GUS, using NcoI (native including the start codon following At-FNR1 pro and the start codon of Zm-FNR2 and introduced during cloning at the start codon for Zm-FNR1 and Zm-FNR3) and Smal sites (Zm-FNR sequences) or SacI digestion followed by blunting with T4 polymerase (pSAF-FNR1 pro-GUS), yielding pSAF-FNR1 pro-Zm-FNR1, pSAF-FNR1 pro-Zm-FNR2, and pSAF-FNR1 pro-Zm-FNR3. Plasmids were introduced into Arabidopsis via Agrobacterium tumefaciens using the floral dip method as described previously (Hanke and Hase, 2008) and transformants selected on 100 μg mL⁻¹ glyphosate.
FNR Activity Assay

FNR proteins were extracted from Arabidopsis leaves in the presence of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, and 0.1 mg mL\(^{-1}\) polyvinyl-polypyrrolidone. Extracts were desalted and detergent removed over Sephadex-G50. Ferredoxin-dependent electron transfer from NADPH to cytochrome c was measured in 100 μg total protein, basically as described previously (Hanke et al., 2004), in 50 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 200 μM NADPH, and 200 μM cytochrome c with 20 μM Arabidopsis ferredoxin 2.

Blue Native-PAGE

All steps were performed at 4°C. Washed chloroplasts were resuspended in 20% (v/v) glycerol, 25 mM Bis-Tris HCl, pH 7, and 250 mM NaCl, and solubilized with 2% Sodium deoxycholate and 0.1% hyamine acetate, and 0.1 M sodium acetate buffer, pH 5.5, as precipitant in a solution containing 30% (w/v) polyethylene glycol 4000, 0.1 M ammonium acetate, and 0.1 M sodium acetate buffer, pH 5.5, as precipitant at 293K. The crystals of Zm-FNR3 were obtained using a solution containing 18% (v/v) polyethylene glycol 8000, 0.2 M sodium chloride, and 0.1 M sodium acetate buffer, pH 5.5, as precipitant at 293K.

For data collection under cryogenic conditions, both crystals were briefly soaked in reservoir solution containing 15% (v/v) glycerol. Crystals were mounted in a nylon loop and flash-cooled in a stream of gaseous nitrogen at 100K. Diffraction data from crystals of Zm-FNR2 and Zm-FNR3 were collected with oscillation method (Δψ = 1.0°) using synchrotron radiation at beamline NW-12 of Photon Factory. Diffraction images were collected at 100K using a Quantum4R charge-coupled device detector (ADSC) and GN2 cryosystem (Rigaku). The data were processed and scaled using the HKL2000 program package.

Initial phase angles of Zm-FNR2 and Zm-FNR3 were both calculated by the molecular replacement method, using MOLREP in the CCP4 suite. The crystal structure of Zm-FNR1 (PDB ID: 1GAW) was used as the search model in both cases. Iterative manual model buildings were performed using the program COOT. Throughout the crystallographic refinement, the programs CNS and Refmac5 in the CCP4 suite were used. The final models of Zm-FNR2 and Zm-FNR3 were refined at 2.00 and 1.39 Å, respectively. The coordinates and structure factors for Zm-FNR2 and Zm-FNR3 have been deposited at the Protein Data Bank under accession codes 3V01 and 3V02, respectively.

The crystallographic data and the statistics of the refinement are summarized in Supplemental Table 1 online.

Construction of Chimeric and Truncated Maize FNR Constructs

Plasmids for expression of chimeric and truncated maize FNR sequences were constructed by extracting At-FNR1pro·Zm-FNR1 and At-FNR1pro·Zm-FNR3 constructs from plant expression vectors by HindIII and XbaI digestion, followed by subcloning into pASK IBA3 (Cayman Chemical Company) to give pIBA3-At-FNR1pro·Zm-FNR1 and pIBA3-At-FNR1pro·Zm-FNR3. Restriction sites for BsiII were inserted by mutagenizing the common sequence coding for amino acids Ser61-Lys62 (Zm-FNR1 nomenclature) in both Zm-FNR1 and Zm-FNR3 (primers in Supplemental Table 2 online) using the QuikChange site-directed mutagenesis kit (Stratagene) to give pIBA3-At-FNR1pro·Zm-FNR1B and pIBA3-At-FNR1pro·Zm-FNR3B. Mutagenized plasmids were digested with BsiII and HindIII, and the excised At-FNR1pro·5'-FNR regions ligated into the appropriate vectors containing 3'-FNR regions, to create chimeras. The truncated Zm-FNR1 sequence (Zm-FNR1t) was created by mutagenizing the codons for amino acids Arg43-Ala44 (primers in Supplemental Table 2 online) in the pIBA3-At-FNR1pro·Zm-FNR1B vector to introduce a restriction site for BsiII, giving the plasmid pIBA3-At-FNR1pro·Zm-FNR1BB. This plasmid was digested with BsiII and BsaHI to excise the region coding for the N terminus in the mature protein. The oligonucleotides 5'-CGCGCAGGCAGGT-3' and 5'-GTCCGCAAGCGC-3' were then annealed and ligated into the linearized pIBA3-At-FNR1pro·Zm-FNR1BB, yielding a Zm-FNR1 sequence with an intact transit peptide, but truncated mature sequence. Chimeric and truncated At-FNR1pro·Zm-FNR constructs were subcloned into pSMAB704 following digestion with Xbal (At-FNR1pro·Zm-FNR constructs) or ScaI (pSMAB704) followed by blunting with T4 polymerase and then digestion with HindIII.

Measurement of Chlorophyll Fluorescence and P700

All measurements were performed on 8-week-old plants between 1 and 6 h into the light period. Plants were dark incubated for 20 min before following the light induction response of a single mature leaf using a DUAL-PAM-100 (Walz). For the measurement, chlorophyll fluorescence and P700 maxima were established followed by a further 2-min dark adaptation and then light induction at 126 μE red actinic light and 29 μE blue actinic light. Chlorophyll fluorescence parameters were calculated basically according to Genty et al. (1989) from dark-adapted maxima and measurements taken at saturating pulses, except that qP = (Fm - Fo) / (Fm - F), where Fo was estimated according to Oxborough and Baker (1997), and NPO was calculated according to Kramer et al. (2004). ΦF was calculated basically according to Klughammer and Schriebe (1994). ETRI and ETRII were calculated as μmol electrons(m2·s) from the photosynthetically active radiation and either ΦI or ΦII, respectively, based on the assumptions that PSI and PSII absorbed equal quanta in all plants and that leaves of all plants absorb 0.84 of quanta available.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: maize FNR1, BAA88236; maize FNR2, BAA88237; maize FNR3, ACF85815; Arabidopsis FNR1, AT5G66190; Arabidopsis FNR2, AT1G20020; maize Tic62, ACG28394.1; Arabidopsis Tic62, AT3G18890; maize TROL, ACF79627.1; Arabidopsis TROL, AT4G30150.1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of FNR Isoproteins from Arabidopsis and Maize.

Supplemental Figure 2. Comparison of FNR Binding Proteins from Arabidopsis and Maize.

Supplemental Table 1. Data Collection and Refinement Statistics for Maize FNR2 and FNR3.

Supplemental Table 2. Primers Used in Preparing Experimental Material.
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AUTHOR CONTRIBUTIONS

M.T. performed research and analyzed data. B.A. performed research. N.M., I.V., and S.O. performed research. G.K. analyzed data. T.H. designed and performed research. G.T.H. designed and performed research, analyzed data, and wrote the article.

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N-Terminal Structure of Maize Ferredoxin:NADP+ Reductase Determines Recruitment into Different Thylakoid Membrane Complexes
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