Rapid responses of chloroplast metabolism and adjustments to photosynthetic machinery are of utmost importance for plants’ survival in a fluctuating environment. These changes may be achieved through posttranslational modifications of proteins, which are known to affect the activity, interactions, and localization of proteins. Recent studies have accumulated evidence about the crucial role of a multitude of modifications, including acetylation, methylation, and glycosylation, in the regulation of chloroplast proteins. Both of the Arabidopsis (Arabidopsis thaliana) leaf-type FERREDOXIN-NADP⁺ OXIDOREDUCTASE (FNR) isoforms, the key enzymes linking the light reactions of photosynthesis to carbon assimilation, exist as two distinct forms with different isoelectric points. We show that both AtFNR isoforms contain multiple alternative amino termini and undergo light-responsive addition of an acetyl group to the α-amino group of the amino-terminal amino acid of proteins, which causes the change in isoelectric point. Both isoforms were also found to contain acetylation of a conserved lysine residue near the active site, while no evidence for in vivo phosphorylation or glycosylation was detected. The dynamic, multilayer regulation of AtFNR exemplifies the complex regulatory network systems controlling chloroplast proteins by a range of posttranslational modifications, which continues to emerge as a novel area within photosynthesis research.

Plants adjust their metabolism according to environmental stimuli by inducing adaptation mechanisms, which ultimately lead to changes in intracellular protein levels and activities. In addition to modulating gene expression, which results in relatively long-term changes, rapid responses are required in order to maintain the functionality and fitness of plants upon sudden shifts in ambient conditions. These responses are achieved through posttranslational modifications (PTMs) of proteins (Prabakaran et al., 2012). In general, PTMs of proteins are known to affect enzyme activity, localization, and turnover as well as interaction with other proteins, RNA, DNA, and lipids, thus providing mechanisms for the rapid adjustment of cellular metabolism (Mann and Jensen, 2003). PTMs include proteolytic cleavage and covalent addition of modifying groups to amino acids, such as phosphorylation, acetylation, methylation, and glycosylation.

In chloroplasts, phosphorylation is the best-characterized PTM, which regulates photosynthetic performance and other responses to environmental stimuli. For example, under high light intensity, PSII subunits D1, D2, CHLOROPHYLL BINDING 43-kD PROTEIN (CP43), and PSII REACTION CENTER PROTEIN H are strongly phosphorylated with the simultaneous suppression of light-harvesting complex (LHC) phosphorylation, while a decrease in light intensity results in the opposite phosphorylation pattern (Tikkanen and Aro, 2012). The reversible phosphorylation of PSII and LHCII is a prerequisite for the efficient repair of photodamaged PSII, and it also guarantees balanced excitation of the photosystems, thus preventing oxidative damage of the photosynthetic machinery (Aro et al., 1993; Tikkanen and Aro, 2012).

Recently, acetylation of nonhistone plant proteins has drawn more and more attention (Xing and Poirier, 2012). N⁺-acetylation refers to the addition of an acetyl group to the α-amino group of the N-terminal amino acid of proteins, which are known to affect the activity, interactions, and localization of proteins. Recent studies have accumulated evidence about the crucial role of a multitude of modifications, including acetylation, methylation, and glycosylation, in the regulation of chloroplast proteins. Both of the Arabidopsis (Arabidopsis thaliana) leaf-type FERREDOXIN-NADP⁺ OXIDOREDUCTASE (FNR) isoforms, the key enzymes linking the light reactions of photosynthesis to carbon assimilation, exist as two distinct forms with different isoelectric points. We show that both AtFNR isoforms contain multiple alternative amino termini and undergo light-responsive addition of an acetyl group to the α-amino group of the amino-terminal amino acid of proteins, which causes the change in isoelectric point. Both isoforms were also found to contain acetylation of a conserved lysine residue near the active site, while no evidence for in vivo phosphorylation or glycosylation was detected. The dynamic, multilayer regulation of AtFNR exemplifies the complex regulatory network systems controlling chloroplast proteins by a range of posttranslational modifications, which continues to emerge as a novel area within photosynthesis research.
acid of proteins. Cotranslationally occurring N\textsuperscript{\alpha}-acetylation of preprotein transit peptides has been shown to be crucial for efficient translocation into the plastids (Pesaresi et al., 2003; Bischof et al., 2011). In addition to the preproteins, many mature nucleus-encoded chloroplast proteins are targets of posttranslational N\textsuperscript{\alpha}-acetylation inside the chloroplast (Zybailov et al., 2008; Bienvenut et al., 2011, 2012), and a number of chloroplast-encoded proteins, such as D1, D2, CP43, Rubisco Large Subunit, and ATP Synthase, have also been shown to be N\textsuperscript{\alpha}-acetylated (Michel et al., 1988; Mulligan et al., 1988; Zybailov et al., 2008; Hoshiyasu et al., 2013). N\textsuperscript{\alpha}-acetylation was shown to protect AtpE from degradation upon drought stress (Hoshiyasu et al., 2013), while no specific role has been defined for the N\textsuperscript{\alpha}-acetylation of the other mature chloroplast proteins. N\textsuperscript{\alpha}-acetylation of the side chain amino group of Lys residues (Lys acetylation), in turn, is a well-known modification of histone proteins, which regulates the chromatin structure and, thus, affects transcriptional activity (Jenuwein and Allis, 2001). Lys acetylation has only recently been described in nonhistone proteins (Choudhary et al., 2009), including several proteins from the chloroplasts of higher plants (Finkemeier et al., 2011; Wu et al., 2011). Intriguingly, the activity of some central chloroplast proteins, such as Rubisco, phosphoglycerate kinase, and NAD\textsuperscript{+}-dependent malate dehydrogenase, has been shown to change upon Lys acetylation (Finkemeier et al., 2011).

Ferredoxin-NAD\textsuperscript{+} Oxidoreductase (FNR) links the light reactions of photosynthesis to stromal metabolism by accepting electrons from two reduced Ferredoxin (FD) molecules and using them for the reduction of NADPH to NADPH, in turn, is a well-known modification of histone proteins, which regulates the chromatin structure and, thus, affects transcriptional activity (Jenuwein and Allis, 2001). Lys acetylation has only recently been described in nonhistone proteins (Choudhary et al., 2009), including several proteins from the chloroplasts of higher plants (Finkemeier et al., 2011; Wu et al., 2011). Intriguingly, the activity of some central chloroplast proteins, such as Rubisco, phosphoglycerate kinase, and NAD\textsuperscript{+}-dependent malate dehydrogenase, has been shown to change upon Lys acetylation (Finkemeier et al., 2011).

Ferredoxin-NAD\textsuperscript{+} Oxidoreductase (FNR) links the light reactions of photosynthesis to stromal metabolism by accepting electrons from two reduced Ferredoxin (FD) molecules and using them for the reduction of NADPH to NADPH. NADPH, in turn, is mainly used for carbon assimilation. In Arabidopsis (Arabidopsis thaliana), chloroplast FNR is encoded by two distinct nuclear genes, At5g66190 and At1g20020, encoding AtFNR1 and AtFNR2 (Hanke et al., 2005). The mature isoforms share 82% amino acid sequence identity (Fig. 1), and their function is partly redundant. Either AtFNR1 or AtFNR2 alone is able to support the autotrophic growth of plants, although the growth and development of the fur1 and fnr2 single mutant plants are compromised compared with wild-type plants (Lintala et al., 2007, 2009; Hanke et al., 2008). Interruption of both leaf-type FNR genes simultaneously results in pale, yellowish plants capable of only heterotrophic growth on Suc medium (Lintala et al., 2012). Based on structural studies, FNR is predicted to be a soluble protein, but it is nonetheless divided into membrane-bound and soluble pools (Hanke et al., 2005). It has been shown that the AtTIC62 (for the 62-kD subunit of the translocon of inner chloroplast envelope; Benz et al., 2009, 2010) and AtTROL (for thylakoid rhodanese-like protein; Jurić et al., 2009) proteins serve as the membrane anchors for AtFNR (Lintala et al., 2014). Despite the partly redundant function and high similarity of the isoforms, AtFNR1 and AtFNR2 show distinct molecular properties, as AtFNR1 is required for thylakoid membrane binding of AtFNR2 in planta (Lintala et al., 2007) and upon growth under low temperature only fnr2 plants show changes in oxidative stress responses. Hence, the AtFNR2 isoform is proposed to have a specific role in directing the reducing power from PSI to stromal reactions (Lintala et al., 2009).

The AtFNR1 and AtFNR2 isoforms can be separated into two forms with different pls by isoelectric focusing (Lintala et al., 2007), which indicates that both proteins are posttranslationally modified. Therefore, we have now focused on clarifying the type(s) of PTMs of these proteins and their impact on FNR function. In this study, we show that neither of the FNR isoforms is likely to be phosphorylated or glycosylated, whereas Lys acetylation and N-terminal trimming were detected in all four forms of AtFNR. Both AtFNR isoforms were also found to contain partial N\textsuperscript{\alpha}-acetylation, which was attributed to the shift in pI. The generated
three-dimensional (3D) model for the AtFNR1-AtFNR2 heterodimer in complex with the ATTIC62 peptide indicates that the acetylated Lys-321 in AtFNR1 and Lys-330 in FNR2 are located in close proximity to the active site, but none of the detected modifications interfere with ATTIC62 binding. Moreover, the degree of N-terminal acetylation of AtFNR was shown to change in response to light, implying a dynamic control of FNR function through PTMs.

RESULTS

AtFNR1 and AtFNR2 Are N-Terminally Trimmed and N\(^\text{\textsuperscript{\alpha}}\) Acetylated

The AtFNR1 and AtFNR2 isoforms are localized in the chloroplasts, where they are found as soluble proteins as well as bound to the thylakoid and inner chloroplast envelope membranes (Hanke et al., 2005; Lintala et al., 2007; Benz et al., 2009; Fig. 2). After separation of the membrane-bound proteins by isoelectric focusing and two-dimensional (2D) SDS-PAGE, both AtFNR isoforms form two clear spots with different pIs (the acidic [a] and basic [b] forms) but with similar molecular masses (Fig. 2, A and B; Lintala et al., 2007). As the same pattern was identified from the soluble fraction of chloroplast proteins (Fig. 2, C and D), the PTMs do not appear to be the sole determinants of AtFNR localization. Moreover, the additional faint spots in the proximity of AtFNR1 (1 and 2 in Fig. 2A) and AtFNR2 (3 in Fig. 2A) were identified as AtFNR1 and AtFNR2, respectively.

In wheat (Triticum aestivum), three amino acid residues in the N terminus of photosynthetic FNR1 (pFNRI) and two amino acid residues in pFNRII have been shown to be trimmed, which results in changes in their enzyme activity (Gummadova et al., 2007). Therefore, the N termini of the membrane-bound and soluble pools of AtFNR were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after excision of the spots from 2D gels, and several different N-terminal peptides were identified for both AtFNR1 and AtFNR2. The spots representing AtFNR1 (both acidic and basic) contained peptides starting with AQVTDDIT, QVTTDIT, and VTTDIT, and the spots representing AtFNR2 (both acidic and basic) contained N-terminal peptides starting with AQVITTEDT, QITTEDT, and ITTETD (Table I). More peptides starting with Gln and Ile or Val were identified, as compared with those starting with Ala, in both the soluble and membrane-bound FNR pools (Supplemental Table S1). Results from Edman degradation corroborated the LC-MS/MS findings: analysis of the basic AtFNR1 spot revealed two alternative sequences for the N terminus of AtFNR1 (QVTDD and VTTDIT) and one for the N terminus of the basic AtFNR2 spot (QITTED; Table II). Sequencing of the acidic spots remained unsuccessful, indicating possible N-terminal blocking of the proteins (Table II), which prompted us to search for possible N-terminal modifications of the proteins.

Indeed, several N\(^\text{\textsuperscript{\alpha}}\)-acetylated peptides were detected in the acidic AtFNR1 and AtFNR2 spots in both the membrane-bound and soluble pools, while none (AtFNR1b) or only one (AtFNR2b) was found in the basic spots with high confidence (Table III; Supplemental Table S1). In addition to acetylation, Gln cyclization to pyro-Glu was detected in all FNR forms. Detection of some unmodified peptides in the acidic spots, and of a few modified peptides in the basic spots, might result from incomplete isoelectric focusing during 2D SDS-PAGE. As pyroglutamylated peptides, identified from the Q-starting peptides in all spots, are common artifacts in mass spectrometry (MS) analysis, we conclude that N\(^\text{\textsuperscript{\alpha}}\)-acytlation of FNR is the modification resulting in the appearance and N-terminal blocking of the acidic forms of both AtFNR isoforms in planta.

AtFNR1 and AtFNR2 Are Lys Acetylated

Acetylation and methylation of chloroplast proteins have been identified recently (Houtz et al., 1992; Finkemeier et al., 2011; Wu et al., 2011; Mininno et al., 2012; Alban et al., 2014). Therefore, the acetylation and methylation states of the four membrane-bound AtFNR forms were analyzed. Acetylation of Lys residues results in a mass gain of 42.0105 D due to the substitution of a hydrogen atom with an acetyl group (\(\text{CH}_3\text{CO}_2\)), while trimethylation increases the mass by 42.0471 D. It is important to note that the small 0.03-D mass difference between Lys-acetylated and Lys-trimethylated peptides can be distinguished in the spectra of the LTQ Orbitrap mass spectrometer. All membrane-bound AtFNR forms possessed acetylated Lys residues, while no methylation could be reliably detected (Table IV; Fig. 1). In the acidic AtFNR1 spot, Lys-175 and Lys-321 were found to be acetylated, while in the basic AtFNR1, only Lys-321 acetylation could be detected. Both AtFNR2 spots, in turn, possessed Lys acetylation on Lys-96 and Lys-330 (Table IV; Fig. 1). Lys acetylation could not be assessed reliably from the soluble FNR pool due to the low protein abundance in the soluble fraction.

AtFNR Is Neither Phosphorylated Nor Glycosylated

Both of the AtFNR isoforms contain putative phosphorylation sites (Lintala et al., 2007) and a conserved N-glycosylation site (Asn-Ala-Thr; Fig. 1), similar to other leaf-type FNRs (Supplemental Fig. S1). Nevertheless, immunoblot analysis using anti-phospho-Thr, anti-phospho-Ser, or anti-phospho-Tyr antibody, as well as staining the 2D gels with Pro-Q Diamond Phosphoprotein Gel Stain, provided negative results, and no shift in the electrophoretic mobility of AtFNR was detected after phosphatase treatment of immunoprecipitated AtFNR using Phos-tag acrylamide (Supplemental Fig. S2). Moreover, when the isolated membranes were treated either with calf
alkaline phosphatase and Lambda protein phosphatase or with the protein phosphatase inhibitors, the pattern of FNRs on the 2D gels remained unchanged (Supplemental Fig. S2). Staining of the 2D gel with the glycoprotein stain ProQ Emerald Glycoprotein or immunoblotting using a Fuc antibody resulted in a weak signal from all AtFNR spots (Supplemental Fig. S3). As a similar response was also recorded by using a complex-glycan-deficient mutant of Arabidopsis, which lacks complex glycans (Strasser et al., 2005), the signal appears to be unspecific. Moreover, LC-MS/MS analysis of putative glycans revealed numerous peptides from the conserved N-glycosylation site, but without the expected +3-D mass shift at the Asn (Supplemental Table S2). Although several glycoproteins have been identified in Arabidopsis chloroplasts (Villarejo et al., 2005; Nanjo et al., 2006), our results suggest that AtFNR is not one of those.

Molecular Interactions of FNR

AtFNR is present in several high-molecular-mass (approximately 500 and 190 kD) thylakoid protein complexes together with the AtTIC62 (Benz et al., 2009) and AtTROL (Jurić et al., 2009) proteins, which contain highly similar Pro-rich FNR-binding domains. In principle, each domain is able to bind two FNR molecules. To get insights into the possible role of PTMs in complex formation, the 3D structure of the AtFNR1-AtFNR2 heterodimer in complex with the AtTIC62 peptide (Fig. 3) was modeled. The model is based on the crystallized pea (Pisum sativum) FNR (PsFNR)-PsTIC62 complex (Protein Data Bank [PDB] code 3MHP; Alte et al., 2010), where Tyr-308 was rotated into a conformation that allows NADP⁺ binding. Analysis of the amino acids interacting with PsTIC62 in both chains of the PsFNR dimer and comparison with those of AtFNR1 and AtFNR2 show that the residues in AtFNR1 have a similar conservation rate with both the A and B chains, while AtFNR2 residues are more conserved with the A chain. Hence, to create the 3D model of the heterodimeric AtFNR1-AtFNR2 complex with AtTIC62, we modeled AtFNR2 in the position of the A chain and AtFNR1 in the position of the B chain of the PsFNR homodimer. The model evaluations show that the 3D fold can be considered reliable and that the model is of good quality, with 93.6% of the residues in most favored regions in the Ramachandran plot produced by PROCHECK (Laskowski et al., 1993). The high sequence identity between the PsFNR and AtFNRs further increases the reliability of the 3D models.
In the modeled complex, the AtTIC62 peptide holds the AtFNR1-AtFNR2 heterodimer together in a back-to-back conformation (Fig. 3), which is further stabilized by interactions between Arg-87, Leu-89, Leu-90, Thr-109, and Pro-187 in AtFNR1 and Leu-98, Leu-99, Leu-160, Asn-162, Glu-164, Pro-196, and Gly-197 in AtFNR2. Importantly, none of the acetylated Lys residues coincide with the binding sites of AtTIC62; thus, the Lys acetylations are not likely to regulate the membrane binding of FNR, at least directly. This result is in line with the fact that all four FNR spots are present in both the membrane-bound and soluble pools (Fig. 2). Moreover, we separated the thylakoid protein complexes by blue native (BN)-PAGE and analyzed the FNR-containing protein complexes using LC-MS/MS (Fig. 4). Even if the complex of approximately 500 kD is known to be composed of FNR and TIC62 (Benz et al., 2009) and the complex of 190 kD is known to be composed of FNR and TROL (Jurić et al., 2009), all the studied complexes contained both AtFNR isoforms as well as TIC62 and TROL, which indicates an incomplete separation capacity of BN-PAGE (Fig. 4). All complexes also contained alternatively trimmed N termini of AtFNR, but the other types of PTMs were not reliably detected, possibly due to the high number of other proteins present in the complexes. As the long and flexible N terminus of FNR does not allow reliable structural modeling (Fig. 3), the question about the effect of N-terminal trimming on the interaction of AtFNR with either TIC62 or TROL still remains open.

Next, we wanted to clarify whether the PTMs could have an effect on the interaction of FNR with FD. AtFD1 and AtFD2, representing approximately 5% to 10% and 90% of the total chloroplast FD pool, respectively (Hanke et al., 2004), share 87.2% sequence identity with each other. Interaction of FD with FNR, studied in numerous species, depends on the charges, hydrophobic forces, and conformational changes of both proteins (Jelesarov and Bosshard, 1994; Martínez-Júlvez et al., 2009; Lee et al., 2011; Hanke and Mulo, 2013), and both AtFD isoforms are known to interact with AtFNR in a very similar way (Hanke et al., 2004). There are two available crystal structures of FNR in complex with FD: maize (Zea mays) FNR (ZmFNR; PDB code 1GAQ; Kurisu et al., 2001) and Anabaena sp. PCC7119 FNR (PDB code 1EWY; Morales et al., 2000). As the sequence identity of AtFNR1 (86.5%) and AtFNR2 (84.1%) to ZmFNR is higher than those for Anabaena sp. PCC7119 FNR (51.5% and 51.8%, respectively), only the ZmFNR-ZmFD complex was used for studying the AtFNR-AtFD interaction. Comparison of the FD-binding amino acids in ZmFNR with those in PsFNR, AtFNR1, and AtFNR2 shows that these amino acids or their chemical properties are strictly conserved (Supplemental Fig. S4). Additionally, the amino acids on ZmFD interacting with ZmFNR (Kurisu et al., 2001) are conserved in both AtFD isoforms (Supplemental Fig. S5), indicating that both AtFNR isoforms interact with both AtFDs through identical salt bridges. Of the conserved residues,

| Isoform | Peptide Sequence | Mass Measured | Change | Expectation Value | Ion Score |
|---------|-----------------|---------------|--------|------------------|-----------|
| FNR1    | AQVTTDTTEAPPVK  | 1,457.749     | 0.21   | 1.60E-05          | 78        |
|         | QVTTDTTEAPPVK   | 1,386.711     | 0.14   | 1.80E-04          | 67        |
|         | VTTDTTEAPPVK    | 1,258.652     | -0.25  | 2.40E-04          | 66        |
| FNR2    | AQITTETDTPPAK   | 1,473.7387    | -3.05  | 3.30E-04          | 66        |
|         | QITTETDTPPAK    | 1,402.70586   | -0.15  | 5.90E-05          | 73        |
|         | ITTETDTPPAK     | 1,274.646     | -1.14  | 2.80E-06          | 86        |

Table II. N-terminal sequences of the acidic and basic membrane-bound AtFNR1 and AtFNR2 proteins determined by Edman degradation

The membrane proteins of Arabidopsis plants were separated by 2D gel electrophoresis and electroblotted, and the membrane was stained with Coomassie Brilliant Blue. The FNR spots were excised, and N-terminal sequencing was performed using the Procise 494A sequencer. The previously identified N-terminal sequences are also indicated. a denotes acidic form, and b denotes basic form. –, Sequence not detected.
Table III. Summary of the N-terminal modifications in the acidic and basic membrane-bound AtFNR1 and AtFNR2 proteins

The membrane proteins of Arabidopsis plants were separated by 2D gel electrophoresis. The four spots representing AtFNR1 and AtFNR2 were excised from the gel, digested with trypsin, and subjected to LC-MS/MS using the LTQ Orbitrap Velos Pro mass spectrometer. a denotes acidic form, and b denotes basic form.

| Isoform | Peptide Sequence | Modification | Mass Measured | Change | Ion Score | Expectation Value |
|---------|------------------|--------------|---------------|--------|-----------|-------------------|
| FNR1a   | QVTTDTTEAPVK     | N terminus (Gln → pyro-Glu) | 1,369.6845 | -0.09 | 67 | 1.60E-04 |
|         | VTTDTEAPVK       | N terminus (acetyl) | 1,428.7203 | -1.02 | 62 | 5.00E-04 |
| FNR1b   | QVTTDTTEAPVK     | N terminus (Gln → pyro-Glu) | 1,369.6850 | 0 | 55 | 2.40E-03 |
| FNR2a   | AQITTETDTTPA     | N terminus (acetyl) | 1,515.7551 | 0.86 | 60 | 9.70E-04 |
| FNR2b   | QITTETDTTPA      | N terminus (Gln → pyro-Glu) | 1,385.6802 | 0.51 | 61 | 5.90E-04 |
|         | QITTETDTTPA      | N terminus (acetyl) | 1,444.7177 | 0.74 | 81 | 8.30E-06 |
|         | ITTETDTTPA       | N terminus (acetyl) | 1,316.6583 | 0.15 | 78 | 1.60E-05 |
|         | QITTETDTTPA      | N terminus (Gln → pyro-Glu) | 1,385.6789 | -0.46 | 70 | 8.30E-05 |
|         | QITTETDTTPA      | N terminus (acetyl) | 1,444.7170 | 0.23 | 65 | 3.70E-04 |

Table IV. Summary of Lys-acetylated peptides of acidic and basic membrane-bound AtFNR1 and AtFNR2 proteins

The membrane-bound proteins of Arabidopsis plants were separated by 2D gel electrophoresis. The four spots representing AtFNR1 and AtFNR2 were excised from the gel, digested with trypsin, and subjected to LC-MS/MS using the LTQ Orbitrap Velos Pro mass spectrometer. a denotes acidic form, and b denotes basic form.

| Arabidopsis Genome Initiative Code | Protein | Peptide | Side Chain Site and Type (A, Acetylation Site) | Mass Measured | Change | Expectation Value | Ion Score |
|-----------------------------------|---------|---------|-----------------------------------------------|---------------|--------|-------------------|----------|
| At5g66190 FNR1a                    | LVYTNDDGEIVKGCVSNFLCDKLPGDEAK | K175A | 3,254.57077 | -0.05 | 3.8E-005 | 52 |
|                                   | DNTFVYMCGLKGMEK | K321A | 1,834.81792 | 0.32 | 2.0E-004 | 42 |
| At5g66190 FNR1b                    | DNTFVYMCGLKGMEK | K321A | 1,834.81804 | 0.39 | 1.1E-004 | 45 |
| At1g20020 FNR2a                    | EPYTGKCLLNTK | K96A | 1,465.73467 | -0.63 | 9.8E-004 | 40 |
|                                   | DNTFVYMCGLKGMEK | K330A | 1,834.81816 | -0.45 | 3.0E-003 | 30 |
| At1g20020 FNR2b                    | EPYTGKCLLNTK | K96A | 1,465.73552 | -0.05 | 2.4E-003 | 36 |
|                                   | DNTFVYMCGLKGMEK | K330A | 1,834.81792 | 0.32 | 9.4E-003 | 25 |

Glu-312 in spinach (Spinacia oleracea) FNR is essential for electron transfer between FNR and FD (Aliverti et al., 1998), which is also reflected by its strict conservation in ZmFNR, PsFNR, AtFNR1, and AtFNR2. Furthermore, Phe-65 in Anabaena sp. PCC7119 FD has been proposed to be important for the rapid electron transfer to FNR (Hurley et al., 1993). However, the corresponding residue, Tyr-63 in ZmFD, does not seem to be important for the electron transfer (Kurisu et al., 2001), although the aromatic property of the residue is conserved in the aligned proteins (Supplemental Fig. S4). Importantly, even if Lys-321 in AtFNR1 and Lys-330 in AtFNR2 are located in close proximity to the active site, Lys acetylation does not appear to sterically hinder the binding of either AtFD isoform.

Although structural modeling could not resolve the impact of N termini on the molecular interactions (see above), we studied the effect of N-terminal acetylation on FD binding experimentally. To this aim, mature AtFD1 and AtFD2 were expressed in Escherichia coli, purified, and covalently bound to a Sepharose resin. Thereafter, crude protein extract from Arabidopsis leaves was loaded on the FD columns, and the proteins bound to AtFD1 or AtFD2 were analyzed by 2D SDS-PAGE. Figure 5 shows that although both AtFD isoforms are able to bind all four forms of AtFNR, more acidic (N-terminal acetylated) AtFNR forms as compared with basic (nonacetylated) AtFNRs were bound by both AtFDs in vitro.

Although one of the acetylated Lys residues, Lys-321 (AtFNR1)/Lys-330 (AtFNR2), is situated close to the NADP(H)-binding site (Fig. 3), 3D modeling did not provide proof that the acetylation would limit the access of FAD or NADP* to the cofactor-binding sites. However, this Lys is conserved (Supplemental Fig. S4) and occupies the same position in the 3D model (Fig. 3) when AtFNR1 and AtFNR2 are compared, which may indicate a role of Lys-321/Lys-330 acetylation in the regulation of enzyme activity or other molecular interactions. The other acetylation sites, Lys-175 in AtFNR1 and Lys-96 in AtFNR2, are located very differently: Lys-175 in AtFNR1 points toward the solvent, while Lys-96 in AtFNR2 is at the interface between the two monomers in the heterodimeric complex (Fig. 3).

The N-terminal Acetylation Status of FNR Is Regulated by Light

Protein import (Hirohashi et al., 2001) and the accumulation of AtFNR-containing protein complexes at the thylakoids (Benz et al., 2009) depend on the chloroplast redox state, which is strictly regulated by light. We investigated the distribution of the different
AtFNR forms in the membrane-bound and soluble pools upon various light conditions in order to see whether some form(s) dominate during the photosynthetically inactive period of the day and whether some of these are needed upon the activation of light reactions or when the photosynthetic machinery is challenged with high irradiation. To this end, leaf samples were collected at the end of the dark period, at growth light (100 \( \text{mol photons m}^{-2} \text{s}^{-1} \)), and after a 2-h moderate high light treatment (550 \( \text{mol photons m}^{-2} \text{s}^{-1} \)). Thereafter, the membrane-bound and soluble proteins were isolated, separated by 2D SDS-PAGE, and detected by immunolabeling with FNR antibody. Blots were quantified, and the relative amounts of different forms were determined by comparing the intensity of the spots with the total AtFNR amount on the blot (Fig. 6). Distribution of the soluble AtFNR forms in the dark differed drastically from those in the light. In darkness, AtFNR1 was clearly more abundant than AtFNR2, but upon the shift to light, the relative amount of soluble AtFNR2 increased drastically (Fig. 6, A and B). In contrast, no such significant differences were observed in the distribution of the thylakoid-bound AtFNR forms between the different treatments (Fig. 6, C and D). In addition, the proportion of non-N\(^{-}\)-acetylated AtFNR1 (FNR1b) increased with increasing illumination both at membranes and in the soluble fraction compared with the nonacetylated form. A similar trend, but less pronounced, was also observed for the AtFNR2 acidic and basic forms.

**DISCUSSION**

Recent studies have shown that the processing of organelle-targeting peptides is not as precise as earlier considered, and many chloroplast-imported proteins may contain up to five different transit peptide cleavage positions in their sequence, distributed along a string of 10 amino acid residues (Bienvenut et al., 2012). It is noteworthy that the proteins with multiple cleavage sites contain significantly more frequently Ala at positions -1, 2, 3, 4, and 5 than the proteins with only a single cleavage site (Bienvenut et al., 2012), which also holds true for both AtFNR1 and AtFNR2 (Fig. 1). Nevertheless, it is not yet known whether the Ala residues as such are required for differential cleavage or whether the absence of hydroxylated or acidic amino acid residues interferes with the cleavage of the transit peptide (Bienvenut et al., 2012). In spite of the fact that many chloroplast proteins undergo N-terminal trimming (Zybailov et al., 2008; Plöscher et al., 2009; Bienvenut et al., 2012), this concept seems to be overlooked, perhaps because of its unknown physiological relevance. Some examples are Cys synthase, whose N terminus may start with either the sequence AVSIKPEA or VSIKPEA (Zybailov et al., 2008), and NADPH-protochlorophyllide oxidoreductase A, starting with either AAAVSAP or AAVSAP (Plöscher et al., 2009). To date, there are no data available allowing conclusions about whether the alternative
trimming might result from a nonprecise transit peptide cleavage by stromal processing peptidase or from the action of chloroplast aminopeptidases (Zybailov et al., 2008). In fact, several aminopeptidases are known to be located in chloroplasts, and after the cleavage of the transit peptide, they may cleave N-terminal residue(s) and reveal penultimate amino acids (Walleng, 2006). This, in turn, may directly affect the longevity of the proteins, thus influencing metabolic reactions (Walleng, 2006).

Our results show alternative N-terminal trimming of AtFNR isoforms in both the membrane-bound and soluble AtFNR pools (Tables I and II; Supplemental Table S1) as well as in all AtFNR-TIC62/TROL protein complexes at the thylakoid membrane (Fig. 4). This indicates that the trimming of the AtFNR N terminus would not determine recruitment of the protein into different protein complexes at the thylakoid membrane. Dimerization and membrane association of the three ZmFNR isoforms have been shown to depend on structural variation in the N termini (Twachtman et al., 2012), but the impact of N-terminal trimming has not yet been studied. Two alternative N termini have also been reported for the wheat pFNRI and pFNRII isoforms: KKVSKKQE and SKKQE for pFNRI and ISKKQD and KKQD for pFNRII (Gummadava et al., 2007). The longer form of pFNRII was able to discriminate between the ZmFD isoforms, but the truncation of two amino acid residues eliminated this ability (Gummadava et al., 2007; Bowsher et al., 2011). It should be noted that the N-terminal start point of the mature wheat pFNRI and pFNRII is 19 to 23 amino acids after the end of the predicted transit peptide, while the N termini of AtFNR1 and AtFNR2 match with the predicted cleavage site (Moolna and Bowsher, 2010), which may indicate differences in the functional properties of the FNR N termini between the species. Nevertheless, as the flexible N terminus of AtFNR does not allow structural modeling (Fig. 3), the ultimate role of N-terminal trimming of AtFNR requires further biochemical analyses.

N-acetylation of proteins is a major PTM taking place in eukaryotes (Ferro et al., 2003; Meinnel et al., 2005; Kleffmann et al., 2007; Bienvenut et al., 2012). Although it is not a common phenomenon among prokaryotes, it has been described in both nucleus- and chloroplast-encoded plastid proteins (Bienvenut et al., 2011, 2012). In Arabidopsis, about 30% of the N-acetylation is related to chloroplast proteins translated via the translocon of outer/inner chloroplast envelope system (Bienvenut et al., 2012), and our results also show that AtFNR belongs to this group (Table III). It has been shown that N-acetylation occurs preferentially at Ala, Val, and Ser in Arabidopsis (Zybailov et al., 2008) or Ala, Val, Ile, Ser, and Thr in Chlamydomonas reinhardtii (Bienvenut et al., 2011). Indeed, the N-terminal peptides representing the N-acetylated AtFNR1 (FNRIa) started with Val or Glu and the N-terminal peptides representing the N-acetylated AtFNR2 (FNRIIa) started with Ala, Ile, or Glu (Table III; Supplemental Table S1). However, similar N-terminal peptides were also identified in nonacylated forms, mainly in the basic spots (FNRI/2b), indicating that AtFNRs undergo partial N-acetylation independent of the N-terminal trimming. In a recent study, it was shown that partial N-terminal acetylation of the ε-subunit of ATP synthase in Citrullus lanatus resulted in a practically identical migration pattern on the 2D gels as compared with that of AtFNR (Hoshiyasu et al., 2013). In this case, N-acetylation was shown to protect the protein from degradation under drought stress conditions (Hoshiyasu et al., 2013).

It has been suggested that the phosphorylation of FNR might provide another level of regulation by determining whether cyclic or linear electron transfer flow is supplied with electrons (Hodges et al., 1990). Indeed, in vitro phosphorylation of pea FNR has been detected (Hodges et al., 1990), and a large-scale Arabidopsis chloroplast phosphoproteome profiling study reported a phosphopeptide, 168LVpTNDGGEIVK, for AtFNR1 (Fig. 1; Yang et al., 2013). Additionally, the four distinct wheat FNR spots observed after 2D SDS-PAGE matched the theoretical pIs predicted for phosphorylated FNRs (Moolna and Bowsher, 2010). In contrast, Sugiymama et al. (2008) did not report AtFNR as a phosphoprotein. In this study, we performed a number of experiments trying to gain evidence for AtFNR phosphorylation (Supplemental Fig. S2). Although our results do not completely exclude the possibility that AtFNR isoforms could be phosphorylated, at least under some environmental conditions, we could not find any evidence that AtFNRs would be phosphorylated in planta.

Lys acetylation of several membrane-bound and soluble proteins, such as the ATP/ADP translocator...
AAC1, ATP synthase, Rubisco, and various LHC proteins, was recently detected in the chloroplast proteome (Finkemeier et al., 2011; Wu et al., 2011). Acetylation of Lys may affect enzyme activity and/or protein-protein interactions by hindering the formation of hydrogen bonds, depending on the position of the Lys residue within the protein. Indeed, the activities of Rubisco and phosphoglycerate kinase increased upon in vitro deacetylation by HUMAN SIRTUIN-3, a broad substrate specificity Lys deacetylase, whereas the activity of malate dehydrogenase decreased (Finkemeier et al., 2011). Intriguingly, it has been shown that the peripheral pool of LHCB1 and LHCB2 possesses a high level of Lys acetylation, whereas the LHC proteins present in the PSI supercomplexes are less acetylated (Wu et al., 2011). Although acetylation of Lys was not found to be light responsive (Wu et al., 2011), these results indicate a regulatory impact of Lys acetylation on photosynthetic reactions and yield. Nevertheless, the location of the acetylated Lys residues on the surface of AtFNR1 and AtFNR2 suggests that these modifications do not affect the interaction with the AtTIC62 and AtTROL proteins (Fig. 3). However, as AtFNR1 (Lys-321) and AtFNR2 (Lys-330) acetylation occurs on a conserved Lys residue in the same 3D position near the active site (Fig. 3; Supplemental Fig. S4), it is plausible that acetylation of Lys-321/330 affects the enzyme activity, even if the 3D models could not provide direct evidence for restricted binding of AtFD, FAD, or NADP+ to the acetylated AtFNR isoforms. Interestingly, a conserved Lys-175 in AtFNR1 was acetylated while the corresponding residue in AtFNR2 was not (Table IV), which could confer distinguishing features between the isoforms. It is also tempting to speculate that the acetylation of surface Lys residues in AtFNR might mediate weaker interactions with other (photosynthetic) protein complexes.

A shift from darkness to light affected the ratio of N\textsuperscript{\textalpha}-acetylated to nonacetylated forms of AtFNR (Fig. 6), suggesting that N\textsuperscript{\textalpha}-acetylation plays a role in the adaptation of the plants to ambient environmental conditions. The relative ratio of all membrane-bound AtFNR forms remained quite stable in all light conditions studied, while the soluble pool showed marked fluctuations (Fig. 6). Upon the transition from darkness to light, the relative amount of both AtFNR2 forms increased drastically compared with AtFNR1. This result suggests a central role for AtFNR2 in light-dependent metabolism, which is in line with our previous results showing a decreased rate of carbon assimilation in the fnr2 mutant plants as compared with the wild type and fnr1 (Lintala et al., 2009).

Taken together, our results show that AtFNR is under a complex, multilayer regulation dynamically responding to ambient environmental conditions. In addition to regulation at the levels of gene expression (Hanke et al., 2005; Gummadova et al., 2007), protein import (Hirohashi et al., 2001), and subchloroplastic location (Benz et al., 2009), AtFNR is subjected to N-terminal trimming, N\textsuperscript{\textalpha}-acetylation, and acetylation...
of Lys residues. The development of novel techniques enabling the identification of various PTMs is likely to reveal other chloroplast proteins that may be regulated by a number of PTMs. The functional significance of these modifications on chloroplast proteins in general, and specifically on AtFNR, obviously requires further research.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia plants were grown in a phytotron under standard conditions (100 μmol photons m⁻² s⁻¹, 8-h-light/16-h-dark cycle, and +23°C). Four- to 5-week-old plants were used in all experiments unless mentioned otherwise. High-light treatment was performed under 500 to 600 μmol photons m⁻² s⁻¹ for 2 h. Temperature measured under the high-light conditions was +28°C to 30°C. Dark samples were taken at the end of the 16-h-dark period prior to the light period.

Isolation of Chloroplasts

Isolation of chloroplasts was performed as described (Zhang et al., 1999) with slight modifications. Leaves of 6-week-old plants were harvested and briefly homogenized in buffer (50 mM HEPES-KOH, pH 7.6, 330 mM sorbitol, 5 mM EDTA, 1 mM MgCl₂, 2.5 mM ascorbate, and 0.025% [v/v] bovine serum albumin, filtered through Miracloth (Calbiochem, Merck), and centrifuged (950g; 1.5 min, and 4°C). Buffer A (50 mM HEPES-KOH, pH 8, 330 mM sorbitol, and 2 mM EDTA) was used to resuspend the chloroplast-containing pellets before loading them on top of Percoll (Amersham Pharmacia Biotech) step gradients (40% [v/v] and 70% [v/v] in buffer A). After centrifugation (4,000g, 5 min, and 4°C), intact chloroplasts were collected from the border of the gradients, washed twice gently with buffer A (centrifuged at 1,250g; 1.5 min, and 4°C and at 8,000g; 1 min, and 4°C), and finally resuspended in buffer A.

Isolation of Proteins

For the extraction of leaf proteins, leaf material was frozen in liquid nitrogen. For the isolation of membrane proteins with intact protein complexes, leaves were homogenized in grinding buffer (330 mM sorbitol, 50 mM HEPES-NaOH, pH 7.5, 2 mM EDTA, and 1 mM MgCl₂), filtered through Miracloth (Calbiochem, Merck), and centrifuged (4,000g, 5 min, and 4°C). The pellet was resuspended in shock buffer (10 mM HEPES-KOH, pH 7.6, 5 mM Suc, and 5 mM MgCl₂) and centrifuged (4,000g, 5 min, and 4°C), and the resulting pellet was resuspended in storage buffer (10 mM HEPES-KOH, pH 7.5, 100 mM Suc, 5 mM NaCl, and 10 mM MgCl₂).

For total protein extraction and fractionation into soluble and membrane fractions, leaves were homogenized directly in shock buffer and filtered through Miracloth. Total protein samples were collected, and the rest of the homogenate was centrifuged (18,600g; 5 min, and 4°C). Supernatant was stored as the soluble fraction, and the pellet was resuspended into storage buffer to obtain the membrane fraction. Isolation was performed in the presence of Complete Protease Inhibitor Cocktail Tablets in the shock buffer (Roche Diagnostics).

To further separate membrane and stroma fractions from isolated chloroplasts, intact chloroplasts were centrifuged (9,500g; 2 min, and 4°C), and the pellets were resuspended in shock buffer, incubated 10 min on ice, frozen with liquid nitrogen, melted, and centrifuged (18,600g; 10 min, and 4°C). Supernatants were stored as stroma fractions, and pellets that were resuspended in storage buffer were stored as membrane fractions.

Immunoprecipitation of AtFNR

Immunoprecipitation of AtFNR was performed by lysing chloroplasts (4 μg of chlorophyll) isolated either in the absence or presence of 10 mM NaF in 100 μL of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) with 1.5% (w/v) deoxycholate either in the presence or absence of 10 mM NaF. Chloroplast lysate was centrifuged at 9,500g for 10 min at 4°C. The resulting supernatant was incubated with 0.4 μL of FNR antibody and 40 μL of Protein A (Millenyi Biotech) for 50 min on ice. The mixture was loaded into rebonded μMACS columns (Millenyi Biotech), and the columns were washed four times in 200 μL of immunoprecipitation buffer containing 0.15% (w/v) deoxycholate and one time in 100 μL of 1× Thermo Scientific buffer.

Phosphatase Treatments

The immunoprecipitated AtFNR proteins were subjected to phosphatase treatment (immunoprecipitated AtFNR equal to 4 μg of chlorophyll and 20 μL of calf intestinal alkaline phosphatase (CIP) in Thermo Scientific buffer). Reaction was carried out at +30°C for 30 min. Columns were rinsed with 100 μL of 1× NEB Lambda protein phosphatase buffer (New England Biolabs) and subjected to Lambda phosphatase (New England Biolabs) treatment (10 μL of Lambda phosphatase, NEB protein metallophosphatase buffer, and +37°C for 30 min). Control reactions contained only corresponding buffers.

In vitro protein phosphatase treatment of membrane proteins was performed using Lambda protein phosphatase (New England Biolabs) and CIP alkaline phosphatase (New England Biolabs). Membrane extract (25 μg of chlorophyll) was first treated with CIP alkaline phosphatase (500 units) for 30 min at +37°C and centrifuged (4,000g, 5 min, and +4°C). The pellet was resuspended in 1× NEB Lambda protein phosphatase buffer in the presence of Lambda protein phosphatase (16,000 units) and incubated for 30 min at +30°C. Alternatively, the membrane proteins were isolated in the presence of phosphatase inhibitors (PhosSTOP; Roche Diagnostics) and 10 mM NaF. The treated proteins were separated by 2D SDS-PAGE and stained with either silver stain or Pro-Q Diamond Phosphoprotein Gel Stain.

Detection of Glycosylation

Proteins were isolated and separated by 2D SDS-PAGE using standard methods. The gels were either stained with Pro-Q Emerald Glycoprotein Gel Stain (Molecular Probes) or electroblotted and the membrane immunolabeled with a Fuc antibody purchased from Agrisera.

For MS analysis, protein spots were excised from Coomassie Brilliant Blue-stained 2D gels and subsequently destained, reduced, and alkylated as described (Shevchenko et al., 2006). After complete dehydroyation with two washes with MS-grade acetonitrile, gel pieces were incubated with 500 units (1 μL) of peptide-N-glycosidase F (NEB P0705S) in 100 μL of 100 mM ammonium bicarbonate (pH 8) prepared in 18O-labeled water (97 atom % 18O; Sigma). The enzymatic reaction was performed overnight at 37°C. The supernatant containing released glycans was removed, and samples were subjected to i-metal gel tryptic digestion (Shevchenko et al., 2006). LC-MS/MS analyses were carried out on an Ultimate 3000 Nano HPLC system (Dionex) coupled via a nanoospray interface to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). A concatenated target-decoy database consisting of Arabidopsis representative gene models (The Arabidopsis Information Resource 10; January 3, 2011) supplemented with a list of common contaminants (common Repository protein lists) was used for database searching (http://www.thepm.org/asta/). The Posttranslational Modifications of FNR protein data was analyzed using X!Tandem (version 2013.09.01; Craig and Beavis, 2004).

Decoy sequences were generated by amino acid shuffling of tryptic peptide sequences. For details regarding instrument settings, peptide identification, and data analysis, refer to Mathieu-Rivet et al. (2013).

Determination of Protein and Chlorophyll Content

Measurements of protein concentration were done using the Bio-Rad Protein Assay. Chlorophyll content was determined according to Porra et al. (1989).

Preparation of AtFD1- and AtFD2-Bound Protein Samples

Mature sequences of AtFD1 and AtFD2 proteins were expressed in Escherichia coli and purified as described (Hanke et al., 2004), after which the proteins were covalently bound to Sepharose resin (Hanke et al., 2011). Crude protein fractions were prepared from Arabidopsis leaves by grinding at 4°C in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% (v/v) Triton X-100, with Complete Protease Inhibitor Cocktail, in the presence of preswollen polyvinylpolypyrrolidone. Particulate matter was removed by centrifugation at 4°C and 33,000 g, and the resulting solution was desalted into 50 mM Tris-HCl, 100 mM NaCl, and 0.1% (v/v) Triton X-100.

Plant Physiol. Vol. 166, 2014 1773

Posttranslational Modifications of FNR
ph 7.5, over Sephadex G-25 before being used to challenge a column made of the AfTfD-bound resin. Following extensive washing, bound proteins were eluted with 50 mM Tris-HCl, pH 7.5, and 500 mM NaCl and concentrated for further analysis with Amicon spin concentrators with a 30-kDa cutoff (Millipore).

One-Dimensional SDS-PAGE

Proteins were separated in the linear range for each primary antibody using SDS-PAGE (12%–15% [w/v] acrylamide). The phosphorylation status of AfFNR was studied using Phos-tag acrylamide gels (Wako Pure Chemical Industries). 150 μL Phos-tag ligand, and 2.3 μmol of MnCl₂. After gel electrophoresis, the Phos-tag gels were incubated in transfer buffer containing 1 mmol of EDTA prior to western blotting.

2D Electrophoresis

Immobiline DryStrip gels (IPG strips; pH 3–11 NL, 18 cm; GE Healthcare Life Sciences) were rehydrated in rehydration buffer (8 M urea, 2 M thiourea, 4% [w/v] CHAPS, 100 mM dithiothreitol, and 0.5% [v/v] IPG buffer, pH 3–11 NL) overnight. A total of 200 μg of protein (membrane, soluble, or stroma) extract was solubilized with rehydration buffer in a final volume of 150 μL by vortexing for 2 to 3 h at room temperature and loaded into rehydrated IPG strips via cup loading. The first-dimension separation was performed using the Ettan IPGphor 3 isoelectric focusing system. The proteins were focused at 150 V for 3 h and at 300 V for 3 h, then the voltage was raised to 1,000 V during 6 h and to 10,000 V during 2 h, and then kept at 10,000 V for 3 h. After isoelectric focusing, the IPG strips were treated for 15 min with equilibration solution (0.375 mM Tris-HCl, pH 8.8, 6 M urea, 30% [w/v] glycerol, and 2% [w/v] SDS) containing 130 mM dithiothreitol and for 15 min with equilibration solution containing 153 mM iodoacetamide. The second dimension separation was carried out by linear SDS-PAGE (14% [w/v] acrylamide), and the gels were silver stained using standard methods.

BN-PAGE

Thylakoid protein complexes were separated using BN-PAGE as described (Lintala et al., 2009).

Immunoblotting and Quantification

After gel electrophoresis, proteins were electroblotted to a polyvinylidene fluoride membrane (Immobilon-P; Millipore). Immunolabeling was carried out using protein-specific primary antibodies. The FNR antibody was a kind gift from Henrik V. Scheller (Andersen et al., 1992). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare) with ECL Western Blotting Detection Reagents (GE Healthcare) were used for the detection of proteins on x-ray films (FujiFilm SuperRX).

Blots were imaged using the Geliance 1000 imager and GeneSnap imaging software (PerkinElmer). 2D blots were quantified using the ProFINDER 2D Gel Analysis software (PerkinElmer).

MS Analysis and N-Terminal Sequencing

For MS analysis of PTMs, protein spots were excised from silver-stained 2D gels and in-gel digested with trypsin according to Shevchenko et al. (2006). LC-MS/MS analyses were performed on a nanoflow HPLC system (Easy-Nano; nanoHPLC System; Ettan TP10000; Ettan TP10000 System). Peptides were first loaded on a trapping column and subsequently separated on line on an in-house-made 15-cm C18 column (75 μm × 15 cm, Magic 5-μm, 200-Å C18, Michrom Bio-Resources). The mobile phase consisted of a mixture of water:acetonitrile (98:2, v/v) with 0.2% (v/v) formic acid (solvent A) and acetonitrile:water (95:5, v/v) with 0.2% (v/v) formic acid (solvent B). A linear 20-min gradient from 5% to 35% (v/v) B was used to elute peptides. Mass spectra were acquired in a data-dependent manner with an automatic switch between MS and tandem MS scans using a top-10 method. Peptide fragmentation was performed with collision-induced dissociation.

The data files were searched for protein identification and modifications using Proteome Discoverer (version 1.4) connected to in-house Mascot (version 2.4) software. To detect Lys acetylation and methylation, data were searched against the SwissProt database (release 2011_08) by using Arabidopsis as a taxonomy filter. To observe the N-terminal peptides, data were searched against an in-house-made database consisting of reviewed canonical Arabidopsis proteins (SwissProt; release 2014_02) and common laboratory contaminants. Database search settings included precursor mass tolerance of 5 ppm, fragment mass tolerance of 0.5 D, semimyptic peptides, one missed trypsin cleavage, fixed modification of carbamidomethylation of Cys, variable modifications of Met oxidation, acetylation of Lys or peptide N-terminus, and methylation of Lys or Arg. A significance threshold of P < 0.05 was used. To confirm acetylation sites, a manual verification of the matched spectrum was performed.

For N-terminal sequencing, 2D-separated proteins were electroblotted to a polyvinylidene fluoride membrane (Immobilon-P; Millipore) followed by Coomassie Brilliant Blue staining. The AfTfD spots were excised, and N-terminal sequencing was performed using the Procie 494A sequencer (PerkinElmer).

Structural Modeling

For homology modeling of the complex between AfFNR1, AfFNR2, and AtTIC62, a modified version of the crystal structure of PsFNR in complex with PsTIC62 (PDB code 3MHP; Alte et al., 2010) was used as a template. NADP⁺ was included in the model by superimposing the structure of PsFNR with NADP⁺ (PDB code 1QFY; Deng et al., 1999) on top of the PsFNR-TIC62 complex structure and saving the NADP⁺ molecule into this structure. Additionally, rotamers of Tyr-308 in the PsFNR-TIC62 complex structure were checked in the BODIL modeling environment (Lehtonen et al., 2004), and the only rotamer allowing access to the NADP⁺-binding site was selected. The resulting structure was a PsFNR-TIC62 complex with bound FAD and NADP⁺, which was then used as a template for modeling the AfFNR1-FNR2 heterodimer in complex with AtTIC62, FAD, and NADP⁺. The AfFNR1, AfFNR2, and AtTIC62 sequences were downloaded from UniProtKB (accession codes Q9FKW6, Q8W493, and Q9H0U5, respectively) and aligned to PsFNR and PsTIC62 with MALCIGN (Johnson et al., 1996) in the BODIL modeling environment (Lehtonen et al., 2004). The sequence identity between PsFNR and AFNFR1 is 89.5%, while the identity for AfFNR2 is 84.8%. The INTIC62 peptide shares 69.2% identity with the corresponding region in AtTIC62. Based on the template crystal structure, amino acids Ser-66 to Thr-360 from AfFNR1, Ser-75 to Thr-369 from AfFNR2, and Lys-440 to Ser-465 from AtTIC62 could be modeled. A set of 10 models was created with MODELLER (Sali and Blundell, 1993), and the model with the lowest value of the MODELLER objective function was chosen as the best model. Model evaluations were performed with PROCHECK (Laskowski et al., 1993) and QMEAN (Benkert et al., 2009). Alignment images were created with ESPript (version 3.0; Cout et al., 2003) and model images with PYMOL (version 1.6; Schrödinger).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or UniProtKB databases under the following accession numbers: AfFNR1 (At5g66190/Q9FKW6), AfFNR2 (At1g20020/Q8W493), AfFHD1 (At1g10960), AfFHD2 (At1g60950), AtTIC62 (At3g18890/Q9H0U5), AtTRIO (At4g01050), and ZmFD (P27776).

Supplemental Data

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

Supplemental Figure S1. The conserved N-glycosylation site in the plant-type FNRs.

Supplemental Figure S2. Phosphorylation of AtFNR isoforms.

Supplemental Figure S3. Glycosylation of AfFNR.

Supplemental Figure S4. Sequence alignment of ZmFNR, PsFNR, AfFNR1, and AfFNR2.

Supplemental Figure S5. Sequence alignment of ZmFD, AtFD1, and AfFD2.

Supplemental Table S1. MS analysis of AtFNR1 and AfFNR2 N-terminal peptides from the chloroplast membrane and stromal fractions.

Supplemental Table S2. Detection of glycosylated peptides in the acidic and basic membrane-bound AtFNR1 and AfFNR2 proteins.
ACKNOWLEDGMENTS

We thank Dr. Andrea Trotta for fruitful discussions, Ilaf Bilal and Markus Palonen for skillful technical assistance, Dr. Taina Tyyystajärvi for critical reading of the article, and Mark S. Johnson and Biocenter Finland (bioinformatics, structural biology, and translational activities) for the excellent computational infrastructure at the Structural Bioinformatics Laboratory, Åbo Akademi University.

Received August 26, 2014; accepted October 7, 2014; published October 9, 2014.

LITERATURE CITED

Alban C, Tardif M, Mininno M, Brugière S, Gilgen A, Ma S, Mazzoleni M, Gigarel O, Martin-Laffon J, Ferro M, et al. (2014) Uncovering the protein lysine and arginine methylation network in Arabidopsis chloroplasts. PLoS ONE 9: e85512

Aliverti A, Deng Z, Ravasi D, Piubelli L, Karplus PA, Zanetti G (1998) Probing the function of the invariant glutamyl residue 312 in spinach ferredoxin-NADP+ reductase. J Biol Chem 273: 3408–34015

Alle F, Stengel A, Renz JP, Petersen E, Soll J, Groll M, Böller B (2010) Ferredoxin:NADPH oxidoreductase is recruited to thylakoids by binding to a polyproline type II helix in a pH-dependent manner. Proc Natl Acad Sci USA 107: 19260–19265

Andersen B, Scheller HV, Moller BL (1992) The PSI-E subunit of photosystem I binds ferredoxin:NADPH oxidoreductase. FEBS Lett 311: 169–173

Arn-C, Virgin I, Andersson B (1993) Photoinhibition of photosystem II: inactivation, protein damage and turnover. Biochim Biophys Acta 1143: 113–134

Benkert P, Künzli M, Schwede T (2009) QMEAN server for protein model quality estimation. Nucleic Acids Res 37: W510–W514

Benz JP, Lintala M, Soll J, Mulo P, Böller B (2010) A new concept for ferredoxin-NADP(H) oxidoreductase binding to plant thylakoids. Trends Plant Sci 15: 608–612

Benz JP, Stengel A, Lintala M, Lee YH, Weber A, Philipp K, Gügel IL, Kaieda S, Ikekami T, Mulo P, et al. (2009) Arabidopsis Tic62 and ferredoxin-NADP(H) oxidoreductase form light-regulated complexes that are integrated into the chloroplast redox poise. Plant Cell 21: 3965–3983

Bienvenut WV, Espagne C, Martínez A, Majeran W, Valot B, Zivy M, Vallon O, Adam Z, Meinnel T, Giglione C (2011) Dynamics of post-translational modifications and protein stability in the stroma of Chlorymodoxas reinhardii chloroplasts. Proteomics 11: 1734–1750

Bienvenut WV, Sumpton D, Martínez A, Lilla S, Espagne C, Meinnel T, Giglione C (2012) Comparative large-scale characterization of plant versus mammal proteins reveals similar and idiosyncratic N-terminal regions. Mol Cell Proteomics 11: 5074–5091

Bischof S, Baerenfaller K, Wildhaber T, Troesch R, Vidi PA, Roschitzki B, Hirsch-Hoffmann M, Hennig L, Kessler F, Gruissem W, et al. (2011) Plastid proteome assembly without Toc159: photosynthetic protein import and plastid differentiation in rice. Plant Physiol 158: 3917–3930

Bowsher CG, Eyres LM, Gummadoja JO, Hothi P, McLean KJ, Munro AW, Scrutton NS, Hanke GT, Sakakibara Y, Hase T (2011) Identification of N-terminal regions of wheat leaf ferrodoxin NADP+ oxidoreductase important for interactions with ferredoxin. Biochemistry 50: 1778–1787

Choudhary C, Kumar C, Gnäd F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325: 834–840

Craig R, Beavis RC (2004) TANDEM: matching proteins with tandem mass spectra. Bioinformatics 20: 1466–1467

Deng Z, Aliverti A, Zanetti G, Arakaki AK, Oottado J, Hase T, et al. (2009) Plant type ferredoxins and ferredoxin-dependent metabolism. Plant Cell Environ 32: 1071–1084

Hanke GT, Endo T, Satoh F, Hase T (2008) Altered photosynthetic electron channelling into cyclic electron flow and nitrite assimilation in a mutant of ferredoxin:NADP(H) reductase. Plant Cell Environ 31: 1017–1028

Hanke GT, Kimata-Ariga Y, Taniguchi I, Hase T (2004) A post genomic characterization of Arabidopsis ferredoxins. Plant Physiol 134: 255–264

Hanke GT, Oktiani S, Satomi Y, Takao T, Suzuki A, Hase T (2005) Multiple iso-proteins of FNR in Arabidopsis: evidence for different contributions to chloroplast function and nitrogen assimilation. Plant Cell Environ 28: 1146–1157

Hanke GT, Satomi Y, Shinmura K, Takao T, Hase T (2011) A screen for potential ferredoxin electron transfer partners uncovers new, redox dependent interactions. Biochim Biophys Acta 1814: 366–374

Hirashashi T, Hase T, Nakai M (2001) Maize non-photosynthetic ferredoxin precursor is mislocalized to the chloroplast envelope space of chloroplasts in the presence of light. Plant Physiol 125: 2154–2163

Hodges M, Migniac-Maslow M, Le Marechal P, Rémy R (1990) The ATP-dependent post translational modification of ferredoxin:NADP+ oxidoreductase. Biochim Biophys Acta 1052: 446–452

Hoshiyasu S, Kohzuma K, Yoshida K, Fujimura M, Fukao Y, Yokota A, Arai Y (2013) Photosynthesis and heterologous expression of N-terminal acetylation in the quantitative regulation of the e subunit of chloroplast ATP synthase under drought stress. Biosci Biotechnol Biochem 77: 998–1007

Houtz RL, Poneleit L, Jones SB, Royer M, Stults JT (1992) Posttranslational modifications in the amino-terminal region of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from several plant species. Plant Physiol 98: 1170–1174

Hurley JK, Salamon Z, Meyer TE, Fitch JC, Cusanovich MA, Markley JL, Cheng H, Xia B, Chae YK, Medina M, et al. (1993) Amino acid residues in Anabahua ferredoxin crucial to interaction with ferredoxin-NADP+ oxidoreductase: site-directed mutagenesis and laser flash photolysis. Biochemistry 93: 9346–9354

Jelezarov I, Bosshard HR (1994) Thermodynamics of ferredoxin binding to ferredoxin:NADP+ reductase and the role of water at the complex interface. Biochemistry 33: 13321–13328

Jenuwein T, Allis CD (2001) Translating the histone code. Science 293: 1074–1080

Johnson MS, May AC, Rodionov MA, Overington JP (1996) Discrimination of protein complexes: application to protein sequence to-structure comparisons. Methods Enzymol 266: 575–598

Jurš S, Hazer-Plepic K, Tomasić A, Lepusid H, Jelicić B, Pulihaiveets S, Brownlee T, Vočja L, Altwegg R, et al. (2009) Tethering of ferredoxin-NADP+ oxidoreductase to thylakoid membranes is mediated by novel plastid complex protein TRL. Plant J 60: 783–794

Kleffmann T, von Zychlinski A, Russenberger D, Hirsch-Hoffmann M, Gehrig P, Gruißem W, Baginsky S (2007) Proteome dynamics during plastid differentiation in rice. Plant Physiol 143: 912–923

Kurisu G, Kusunoki M, Katoh E, Yamazaki T, Teshima K, Onda Y, Kimata-Ariga Y, Hase T (2001) Structure of the electron transfer complex between ferredoxin and ferredoxin-NADP+ reductase. Nat Struct Biol 8: 117–121

Laskowski RA, Moss DS, Thornton JM (1993) Main-chain bond lengths and bond angles in protein structures. J Mol Biol 231: 1049–1067

Lee YH, Ikekami T, Standley DM, Sakurai K, Hase T, Goto Y (2011) Binding energetics of ferredoxin-NADP+ reductase with ferredoxin and its relation to function. ChemBioChem 12: 2062–2070

Lehtonen JV, Stil DJ, Rantanen VV, Ekholm J, Björklund D, Itikhar Z, Huhtala M, Repo S, Jussila A, Jaakkola J, et al. (2004) BODIL: a molecular modeling environment for structure-function analysis and drug design. J Comput Aided Mol Des 18: 401–419

Lintala M, Allahverdiyeva Y, Kangasjärvi S, Lehtimäki N, Keränen M, Rintamäki E, Aro EM, Mulo P (2009) Comparative analysis of leaf-type ferredoxin-NADP+ oxidoreductase isoforms in Arabidopsis thaliana. Plant J 57: 1103–1115

Lintala M, Allahverdiyeva Y, Kidron H, Piippo M, Batchikova N, Suorsa M, Rintamäki E, Salminen TA, Aro EM, Mulo P (2007) Structural and...
functional characterization of ferredoxin-NADP⁺-oxidoreductase using knock-out mutants of Arabidopsis. Plant J 49: 1041–1052.

Lintala M, Lehtimäki N, Benz JP, Jungfer A, Soll J, Aro EM, Böler B, Mulo P (2012) Depletion of leaf-type ferredoxin-NADP⁺ oxidoreductase results in the permanent induction of photoprotective mechanisms in Arabidopsis chloroplasts. Plant J 76: 809–817.

Lintala M, Schuck N, Thommähn I, Jungfer A, Weber KL, Weber AP, Geigenberger P, Soll J, Böler B, Mulo P (2014) Arabidopsis tic62 trol mutant lacking thylakoid-bound ferredoxin-NADP⁺ oxidoreductase shows distinct metabolic phenotype. Mol Plant 7: 45–57.

Mann M, Jensen ON (2003) Proteomic analysis of post-translational modification reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiol 130: 889–903.

Sali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 234: 779–815.

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and protein cleomes. Nat Protoc 1: 2856–2860.

Strasser R, Stadlmann J, Svoboda B, Altman F, Glössl J, Mach L (2005) Molecular basis of N-acetylglucosaminyltransferase I deficiency in Arabidopsis thaliana plants lacking complex N-glycans. Biochem J 387: 385–391.

Sugiyama N, Nakagami H, Mochida K, Daudi A, Tomita M, Shirasu K, Ishihama Y (2008) Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in Arabidopsis. Mol Syst Biol 4: 193.

Tikkannen M, Aro EM (2012) Thylakoid protein phosphorylation in dynamic regulation of photosystem II in higher plants. Biochim Biophys Acta 1817: 232–238.

Twachtmann M, Altman B, Muraki N, Voss I, Okutani S, Kurisu G, Hase T, Hanke GT (2012) N-terminal structure of maize ferredoxin-NADP⁺ reductase determines recruitment into different thylakoid membrane complexes. Plant Cell 24: 2979–2991.