HCF164 Receives Reducing Equivalents from Stromal Thioredoxin across the Thylakoid Membrane and Mediates Reduction of Target Proteins in the Thylakoid Lumen*5

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HCF164 is a membrane-anchored thioredoxin-like protein known to be indispensable for assembly of cytochrome b_{6}f in the thylakoid membranes. In this study, we report the finding that chloroplast stroma m-type thioredoxin is the source of reducing equivalents for reduction of HCF164 in the thylakoid lumen, providing strong evidence that higher plant chloroplasts possess a trans-membrane reducing equivalent transfer system similar to that found in bacteria. To probe the function of HCF164 in the lumen, a screen to identify the reducing equivalent acceptor proteins of HCF164 was carried out by using a resin-immobilized HCF164 single cysteine mutant, leading to the isolation of putative target thylakoid proteins. Among the newly identified target proteins, the reduction of the PSI-N subunit of photosystem I by HCF164 was confirmed both in vitro and in isolated thylakoids. Two components of the cytochrome b_{6}f complex, the cytochrome f and Rieske FeS proteins, were also identified as novel potential target proteins. The data presented here suggest that HCF164 serves as an important transducer of reducing equivalents to proteins in the thylakoid lumen.

The redox state of higher plant chloroplasts undergoes significant fluctuations in both light and dark conditions. In the light, photosynthetic electron transport via ferredoxin and ferredoxin-NADP^{+} reductase (FNR)2 results in the synthesis of NADPH, which is used as a source of reducing equivalents for carbon fixation. However, a portion of the electrons produced are transferred to PSI-N subunit of photosystem I by HCF164 and in isolated thylakoids. A proteomic based screen then allowed us to identify putative thylakoid-localized HCF164 target proteins, one of which was confirmed to undergo HCF164-mediated insulin reducing activity in vitro (22) leads to the possibility that, in a similar way to Trx, the HCF164 protein may act as a transducer of reducing equivalents in the thylakoid lumen.

To understand the role of HCF164 in the thylakoid lumen, we have carried out a thorough investigation of the biochemical properties of the HCF164 protein. We found that m-type Trx is able to effectively transfer electrons to luminal HCF164 across the thylakoid membranes. A proteomic based screen then allowed us to identify putative thylakoid-localized HCF164 target proteins, one of which was confirmed to undergo HCF164-dependent reduction. The results presented here underline the physiological significance of HCF164 as a transducer of reducing equivalent within the thylakoid lumen.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of HCF164 and PSI-N—The genes for HCF164 and the N subunit of photosystem I (PSI-N) were obtained by PCR amplification from an Arabidop-
s cDNA library, using the following oligonucleotides: for the soluble domain of HCF164 (HCF164sol, amino acid residues 116–261 (supplemental Fig. S1)) (At4g37200), 5′-aactgccatgattttgggatttctttgaa-3′ (Ndel) and 5′-cggatctcatcatggtccgtgataggat-3′ (EcoRI); for the mature form of PSI-N (amino acid residues 87–171) (At5g64040), 5′-aactgcagacgaatacct-3′ (EcoRI) and 5′-cggatccatttttccagaaaca-3′ (EcoRI). The restriction sites for the enzyme shown in parentheses are underline. The amplified DNA fragments were cloned into the Ndel and EcoRI sites of pET23c (Novagen), and these are underlined. The amplified DNA fragments were expressed in Escherichia coli BL21 (DE3) cells and purified as follows. E. coli cells were suspended in 25 mM Tris-HCl (pH 8.1) and disrupted by French press (5501-M, Ohtake Works, Tokyo, Japan) at 4 °C. The disrupted cells were centrifuged at 100,000 g for 1 h. The supernatant was diluted 50–100-fold with 25 mM Tris-HCl (pH 8.1) and 2 mM EDTA and disrupted by French press (5501-M, Ohtake Works, Tokyo, Japan) at 4 °C. The disrupted cells were centrifuged at 100,000 g for 1 h. The supernatant was applied to a QAE-Toyopearl 650M column (Tosoh) and eluted with a 0–250 mM linear gradient of NaCl in 25 mM PIPES-NaOH (pH 8.1). The protein solution was applied to a SP-Toyopearl 650M column (Tosoh) and eluted with a 0–500 mM gradient of NaCl in 25 mM Tricine-KOH (pH 7.1). After incubation, both thylakoids samples were washed twice with the same solution without TCEP, precipitated with trichloroacetic acid (final 5%), washed with ice-cold acetone, and finally dissolved in 50 mM Tris-HCl (pH 8.6), 1% SDS, and 10 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) (12, 17). The protein samples were then analyzed by 13% nonreducing SDS-PAGE and Western blotting using anti-HCF164 serum.

**Trx-dependent Reduction Assay of HCF164 and PSI-N in Thylakoids**—Intact thylakoids or sonicated thylakoids from *Arabidopsis* (cholorophyll concentration, 200 μg/ml) were incubated with 5 mM tris-(2-carboxyethyl) phosphine (TCEP), 0.1 μM sorbitol, 5 μM MgCl₂, 10 mM NaCl, 20 mM KCl, and 30 mM HEPES-KOH (pH 7.1) for 30 min at 25 °C. After incubation, both thylakoid samples were washed twice with the same solution without TCEP, precipitated with trichloroacetic acid (final 5%), washed with ice-cold acetone, and finally dissolved in 50 mM Tris-HCl (pH 6.8), 1% SDS, and 10 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) (12, 17). The protein samples were then analyzed by 13% nonreducing SDS-PAGE and Western blotting using anti-HCF164 serum.

**Orientation of Active Cysteines of HCF164 on Thylakoid Membranes**—Intact thylakoids or sonicated thylakoids from *Arabidopsis* (cholorophyll concentration, 200 μg/ml) were incubated with 5 mM tris-(2-carboxyethyl) phosphine (TCEP), 0.1 μM sorbitol, 5 μM MgCl₂, 10 mM NaCl, 20 mM KCl, and 30 mM HEPES-KOH (pH 7.1) for 30 min at 25 °C. After incubation, both thylakoid samples were washed twice with the same solution without TCEP, precipitated with trichloroacetic acid (final 5%), washed with ice-cold acetone, and finally dissolved in 50 mM Tris-HCl (pH 6.8), 1% SDS, and 10 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) (12, 17). The protein samples were then analyzed by 13% nonreducing SDS-PAGE and Western blotting using anti-HCF164 serum.

**Insulin Reduction Assay**—To check the disulfide reduction activity of the recombinant soluble domain of HCF164, we compared with that of stromal Trx proteins, we measured the change in turbidity of the insulin solution because of precipitation of free insulin B chain by reduction (14, 26). The assay mixture contained 100 mM potassium phosphate (pH 7.0), 2 mM EDTA, and 130 μM bovine insulin. The reaction was initiated by adding 330 μM DTT into the assay mixture, and the change in turbidity was monitored at 650 nm at 25 °C.

**Determination of the Redox Potential of HCF164sol Protein**—Wild type HCF164sol (1 μM) was incubated at 25 °C for 3 or 16 h in 50 mM potassium phosphate buffer (pH 7.0), 100 mM oxidized DTT, and various concentrations of reduced DTT (0.5 μM to 2 mM), under nitrogen atmosphere. To minimize oxidation by air, buffer solutions were thoroughly degassed. After incubation, samples were treated with trichloroacetic acid (final 5%). The protein precipitates were washed with ice-cold...
acetone and then dissolved in buffer containing 50 mM Tris-HCl (pH 6.8), 1% SDS, and 2 mM AMS. Reduced (AMS derivative) and oxidized (nonderivative) forms of HCF164sol were separated by 15% nonreducing SDS-PAGE, stained with Coomassie Brilliant Blue R-250, and quantified by using LAS-3000mini CCD-imaging (Fujifilm, Tokyo, Japan) (27). The equilibrium constant and the standard redox potential were calculated as described (28), using a value of -330 mV for the standard redox potential of DTT as a reference (29). When Trx-f (1 μM) and Trx-m (1 μM) were incubated in the redox equilibrium buffer, the reduced DTT concentrations were varied from 20 μM to 100 mM against 100 mM oxidized DTT.

Large Scale Preparation of Arabidopsis Thylakoids for Screening of HCF164 Target Proteins—Arabidopsis (ecotype Columbia) rosette leaves from 4- to 5-week-old plants were harvested and then rapidly homogenized in a Waring blender in ice-cold 330 mM sorbitol, 5 mM sodium ascorbate, 0.05% BSA, 2 mM and then rapidly homogenized in a Waring blender in ice-cold EDTA, 1 mM MgCl2, 1 mM MnCl2, and 50 mM HEPES-NaOH (pH 7.6), filtered through Miracloth (Calbiochem), and centrifuged at 2,000 g for 5 min. The precipitated chlo-roplasts were resuspended in 330 mM sorbitol, 5 mM sodium ascorbate, 1 mM MgCl2, and 50 mM HEPES-NaOH (pH 7.6) and centrifuged at 1,300 × g for 5 min. They were then resuspended with 330 mM sorbitol, 2 mM sodium ascorbate, 1 mM MgCl2, 1 mM MnCl2, 2 mM EDTA, 2 mM NaNO3, 5 mM NaHCO3, 0.5 mM K2HPO4, 5 mM sodium dihydrogen pyrophosphate, and 50 mM HEPES-NaOH (pH 7.6) and centrifuged at 1,300 × g for 5 min. The precipitated chlo-roplasts were resuspended with 50 mM Tricine-KOH (pH 8.0), 2 mM EDTA, 1% protease inhibitor mixture for plant cell extracts (Sigma) at a final concentration of 0.5 mg/ml chlorophyll. The broken chloroplast suspension was then centrifuged at 7,500 × g for 10 min at 4°C and washed twice with 10 mM sodium dihydrogen pyrophosphate-NaOH (pH 7.8) to remove peripheral proteins (30). Arabidopsis thylakoids were obtained as a precipitate of this suspension following centrifugation for 10 min at 7,500 × g, for screening of HCF164 target proteins.

Screening of HCF164 Target Proteins in Arabidopsis Thylakoids—HCF164sol (CS)-mutant (149WCEVs153 to 149WCEVS153) was immobilized on CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. Arabidopsis thylakoids were solubilized in 50 mM Tricine-KOH (pH 8.0), 2 mM EDTA, 1% protease inhibitor mixture for plant cell extracts (Sigma) and 1% n-octyl-β-D-glucoside for 60 min at 4°C with gentle mixing and centrifuged at 140,000 × g for 30 min. The solubilized thylakoid protein fraction was obtained as a supernatant. The detergent-solubilized fraction was then incubated with the HCF164sol (CS)-mutant immobilized resin for 60 min at room temperature. The resin was washed with 50 mM Tricine-KOH (pH 8.0), 2 mM EDTA, and 1% n-octyl-β-D-glucoside (Buffer A) to remove any non-specifically bound proteins. The resin was then washed with Buffer A plus 500 mM NaCl and with Buffer A containing 500 mM NaCl and 0.1% SDS. Each washing step was repeated until the absorbance of the washed solution at 280 nm approached zero. The resin was finally suspended in Buffer A plus 0.1% SDS and 20 mM DTT for 60 min, and the eluted proteins were analyzed by SDS-PAGE (15% (w/v)) and stained with Coomassie Brilliant Blue R-250. Stained protein bands were identified by N-terminal amino acid analysis using a peptide sequencer and by peptide mass fingerprint (PMF) analysis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (12, 14, 31).

HCF164-dependent Reduction of the Recombinant PSI-N in Vitro—PSI-N (8 μM) in 25 mM Tris-HCl (pH 7.5) was incubated for 60 min at 25°C with 50 μM CuCl2 or various concentrations of DTT in the presence or absence of HCF164sol (5 mM). The redox states of PSI-N were assessed by the AMS labeling as described (12, 17). PSI-N bands were visualized by Western blotting using anti-PSI-N antibody because the mobility of reduced form of PSI-N was almost same as that of HCF164sol.

RESULTS

A Disulfide Bond in the Thioredoxin Domain of HCF164 Faces the Thylakoid Lumen—To probe the redox-related function of HCF164, we initially sought to determine the source of the reducing equivalents supplied to the luminal side of the thylakoid membranes. To achieve this, we first prepared Arabidopsis thaliana (Arabidopsis) thylakoid membranes and determined their degree of intactness; incubation of isolated thylakoids with the protease thermolysin resulted in the rapid degradation of FNR, which is located on the stroma-exposed side of the thylakoid membranes. To this end, we first prepared Arabidopsis thaliana (Arabidopsis) thylakoid membranes and determined their degree of intactness; incubation of isolated thylakoids with the protease thermolysin resulted in the rapid degradation of FNR, which is located on the stroma-exposed side of the thylakoid membranes. To achieve this, we first prepared Arabidopsis thaliana (Arabidopsis) thylakoid membranes and determined their degree of intactness; incubation of isolated thylakoids with the protease thermolysin resulted in the rapid degradation of FNR, which is located on the stroma-exposed side of the thylakoid membranes.
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protected for at least 30 min. In contrast, the largest component of the HCF164 protein was immediately degraded in sonicated thylakoid membranes by thermolysin treatment. Moreover, we determined the orientation of the active cysteines of the thioredoxin domain of HCF164 using the membrane-impermeable reducing reagent, TCEP (34–36). In intact thylakoids, HCF164 was found to be present in the oxidized form and not reduced by TCEP (Fig. 1B, Intact). In contrast, HCF164 was easily reduced when thylakoids were disrupted by sonication (Fig. 1B, Sonicated). These results substantiate existing evidence that the large hydrophilic domain of HCF164, which includes the active site Cys motif, is located in the thylakoid lumen, as suggested previously by Lennartz et al. (22).

HCF164 Receives Electrons from Trx Located on the Stromal Side—Having characterized the orientation of HCF164 within the membrane, we proceeded to determine the source of the reducing equivalents for reduction of the luminal portion of the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the HCF164 protein.

To determine the source of reducing equivalents, which could reduce the active cysteines of HCF164 on the luminal side, we monitored the redox state of HCF164 in intact thylakoids treated with an exogenous supply of reductant. The freshly prepared intact HCF164 protein was found to occur in the oxidized form in thylakoid membranes by thermolysin treatment. Moreover, we determined the orientation of the active cysteines of the thioredoxin domain of HCF164 using the membrane-impermeable reducing reagent, TCEP (34–36). In intact thylakoids, HCF164 was found to be present in the oxidized form and not reduced by TCEP (Fig. 1B, Intact). In contrast, HCF164 was easily reduced when thylakoids were disrupted by sonication (Fig. 1B, Sonicated). These results substantiate existing evidence that the large hydrophilic domain of HCF164, which includes the active site Cys motif, is located in the thylakoid lumen, as suggested previously by Lennartz et al. (22).

HCF164 Receives Electrons from Trx Located on the Stromal Side—Having characterized the orientation of HCF164 within the membrane, we proceeded to determine the source of the reducing equivalents for reduction of the luminal portion of the HCF164 protein. In chloroplasts, electrons produced by photosynthetic electron transport ultimately accumulate within the stroma, the site of the chloroplast Trxs. In prokaryotic bacteria, the polytopic membrane protein cytochrome c defective A (CcdA) is known to act as a transducer of reducing equivalents, bridging the transfer of electrons from the cytoplasmic reduced form Trx to substrates within the periplasm across the membrane (37); an ortholog of the bacterial CcdA protein is also present in Arabidopsis (38). We therefore sought to investigate the possibility of a related pathway in thylakoid membranes.

To determine the source of reducing equivalents, which could reduce the active cysteines of HCF164 on the luminal side, we monitored the redox state of HCF164 in intact thylakoids treated with an exogenous supply of reductant. The freshly prepared intact Arabidopsis leaf thylakoids (Fig. 2A, −Trx without DTT). This oxidized form of HCF164 could be partially reduced by the reduced form of Trx-m (Fig. 2A, Trx-m with DTT) but not by oxidized Trx-m (Fig. 2A, Trx-m without DTT). The reduced forms of Trx-f or 10 μM DTT without Trx were ineffective in reducing HCF164 (Fig. 2A, Trx-f with DTT and −Trx with DTT). Reduction of HCF164 was accomplished by Trx-m even in the presence of low concentrations of DTT but not by Trx-f (Fig. 2B). The reduction rate of HCF164 by Trx-m was fast (t1/2 <10 min) reaching a maximum within 30 min (Fig. 2C). These results clearly indicate that the stromal reduced form Trx-m is able to transfer reducing equivalents across the membrane to the luminal HCF164 protein.

Disulfide Reduction Activity and Redox Potential Determination of the HCF164 Protein—To further characterize the biochemical properties of HCF164, we constructed an expression system consisting of the soluble C-terminal domain of the HCF164 protein containing the Trx motif alone (hereafter designated as HCF164sol), lacking both the N-terminal chloroplast signal sequence and the membrane spanning domain (supplemental Fig. S1) (22). The insulin reduction activity displayed by purified HCF164sol was found to be lower than that measured for the well characterized stromal Trxs Trx-f and Trx-m (Fig. 3).

To evaluate the feasibility of a putative electron cascade from stromal Trx to HCF164 in the thylakoid lumen, the redox potential values of HCF164sol and Trxs were determined using a DTTred/DTTox redox buffer. Following equilibration of HCF164 and Trxs with increasing ratios of DTTred/DTTox at 25 °C, the proteins were acid-denatured and treated with the thiol modifier AMS (27, 39). The above samples were then separated by nonreducing SDS-PAGE to resolve the reduced (AMS-derivatized) and oxidized (nondervatized) forms of the proteins (Fig. 4A). Incubation for 3 and 16 h gave identical results, indicating that the redox states of these proteins had reached equilibrium under each of the DTTred/DTTox conditions. To calculate their equilibrium constants, the fractions of the reduced form of the proteins were plotted against the DTTred/DTTox ratios (Fig. 4B); using the reported redox potential of DTT (−330 mV) as a reference (29), we determined the standard redox potential value of HCF164sol to be −224 mV.

![FIGURE 2. Reduction of HCF164 in thylakoid lumen. A, reduction of thylakoid lumen HCF164 by stromal Trx. Intact thylakoids were treated with Trxs as described under “Experimental Procedures.” After incubation, quenched samples were incubated with AMS (10 mM) to specifically modify free sulfhydryl groups. The proteins were visualized by using an anti-HCF164-specific antibody. B, DTT concentration dependence of the reduction of HCF164. Intact thylakoids were incubated at various concentration of DTT with or without Trxs (5 μM) for 60 min at 25 °C, and the redox states of HCF164 were determined using AMS modification as described. C, reduction rate of HCF164 in intact thylakoids by Trx-m. Intact thylakoids were incubated at various times with Trx-m (5 μM) and DTT (10 μM) at 25 °C, and the redox states of HCF164 were determined using AMS modification. red, reduced; ox, oxidized.](image-url)
The same reference was used to determine the redox potential values of the stromal Trx-f and Trx-m proteins, which were found to be $-291$ and $-282$ mV respectively (Fig. 4C). Because the redox potential value for the active site cysteines of HCF164 was found to be significantly less negative than those of the stromal Trx-f and Trx-m proteins, we propose that HCF164 may act as an electron acceptor from stromal Trx-m, most likely through an additional thylakoid membrane-bound component to transfer the reducing equivalents across the membrane.

Screening of HCF164 Target Proteins in the Thylakoid Lumen—
To identify the potential substrates of the HCF164-derived reducing equivalents, we prepared a single cysteine mutant at the active site Cys$^{153}$ (HCF164$_{sol}$ (CS)) and immobilized this mutant protein on the resin surface (supplemental Fig. S1) (12–14, 40, 41). The resin was then used to screen HCF164 target proteins obtained from solubilized thylakoid fractions. The Arabidopsis thylakoids were first solubilized with 1% n-octyl-$\beta$-D-glucoside, and the resulting solubilized fraction was incubated with HCF164$_{sol}$ (CS)-mutant immobilized resin. Following thorough washing procedures, the captured proteins bound to HCF164 via an intermolecular disulfide bond were eluted from the resin with DTT. Analysis of the eluted proteins by SDS-PAGE and Coomassie Brilliant Blue R-250 staining revealed the presence of at least 15 putative target proteins. The Coomassie Brilliant Blue R-250-stained protein bands were identified using a combination of N-terminal peptide sequencing and PMF analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Using these techniques, we were able to identify nine proteins from the Arabidopsis genome data base (42). All the identified proteins were found to be thylakoid membrane proteins as follows: cytochrome f, Rieske FeS protein, PSI-N, LHCBI5, FTSH2, and FTSH8 (Fig. 5 and Table 1). Although facing the stromal side of the thylakoid membrane, three CF$_1$ subunits were also identified; these are likely not to be the target of HCF164 in vivo because the HCF164 active cysteine pair faces the thylakoid lumen (Fig. 1B).

Reduction of the Mature Form PSI-N in Vitro and in Intact Thylakoids—Of the proteins captured by the HCF164$_{sol}$ (CS)-mutant immobilized resin, the PSI-N subunit of photosystem I, which is known to be located on the luminal side of the thylakoid membrane (32, 33), was found to be of particular interest. We therefore proceeded to carry out a functional verification of the HCF164-PSI-N interaction by determining whether HCF164 could reduce the PSI-N disulfide bond in vitro. The mature form of PSI-N contains four cysteine residues, which can potentially form two disulfide bonds. Indeed, purified PSI-N was shown to gradually convert from an oxidized to a reduced form with increasing concentrations of DTT by a two-step reduction (Fig. 6A, upper panel). More than 250 $\mu$M DTT was required to completely reduce PSI-N. Upon addition of 5 $\mu$M HCF164$_{sol}$ to DTT, PSI-N was efficiently reduced at significantly lower DTT concentrations (about 50 $\mu$M) (Fig. 6A, lower panel), suggesting that HCF164 can enhance the reduction of the disulfide bonds of PSI-N.
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this protein. To investigate the nature of this interaction in vivo, we attempted the reduction of PSI-N by addition of the reduced form Trx to intact thylakoids. When the reduced form of Trx-m was added to intact thylakoids, PSI-N was partially reduced (Fig. 6B, Trx-m + DTT). In contrast, the reduced form Trx-f or 10 μM DTT alone had no effect on the redox state of PSI-N. These results were similar to those obtained for HCF164 reduction in the thylakoid lumen by Trxs (Fig. 2A).

DISCUSSION

Characterization of a Novel Transport System of the Reducing Equivalents across the Thylakoid Membrane in Chloroplasts—This study aimed to determine the orientation of HCF164 in the thylakoid membrane and to characterize the potential electron donor(s) of this intriguing protein. In previous work (22), protease susceptibility experiments led to the conclusion that HCF164 is anchored to the thylakoid membrane by the N-terminal transmembrane domain, with the large C-terminal hydrophilic domain exposed in the thylakoid lumen. However, in these experiments, the orientation of the hydrophilic domain of HCF164 in the thylakoid membranes could not be conclusively determined; the protease protection assay revealed that HCF164 protein in intact thylakoids was not completely protected, as degradation only showed a delay compared with sonicated thylakoids (22). Because the precise location of the large hydrophilic domain of HCF164 is critical in determining its electron source, we carried out a thorough investigation of its localization by using highly intact thylakoid membranes. The protease protection assay and TCEP-based HCF164 reduction assay carried out here clearly demonstrate that the protease-insensitive 19-kDa fragment containing the active cysteine residues in the Trx-like domain of HCF164 is oriented toward the thylakoid lumen (Fig. 1, A, left upper panel, and B).

This conclusion gave rise to the question as to the potential source(s) of the HCF164 reducing equivalents. Reducing equivalents present in chloroplasts are produced by the photosynthetic electron transport system and are known to predominantly accumulate within the stroma. However, the mode of translocation of reducing equivalents, which are used for reduction of the disulfide bond, from the stromal to the luminal side of the thylakoid membrane has not yet been determined. The possibility that photosynthetic electron transport may act as an electron donor for HCF164 is highly unlikely given the

![FIGURE 5. Screening and identification of HCF164 target proteins in Arabidopsis thylakoids. Proteins captured by HCF164 sol (CS)-mutant immobilized resin were separated by SDS-PAGE (15%) and visualized by Coomassie Brilliant Blue R-250 staining. Stained protein bands were identified by N-terminal amino acid analysis, PMF analysis, or tandem mass spectrometry.](https://example.com/figure5.png)

### TABLE 1

| Band no. | Identity | Accession no. | Method | Determinants |
|----------|----------|---------------|--------|--------------|
| 1.       | VAR2(FTSH2) | AT2G30950 | PMF    | 698.33, 728.35, 762.33, 1198.59, 1396.76, 1452.77, 1471.81, 1615.84, 1650.86, 1674.86, 1707.99, 1798.89, 1814.98, 1903.93, 1947.05, 2043.04, 2013.09, 2011.33, 2188.08, 2327.28, 2359.26, 2466.34, 2700.44, 2861.46, 3252.59 |
| 2.       | FTSH8 | AT1G06430 | PMF    | 698.33, 728.35, 762.33, 1071.51, 1140.53, 1396.76, 1452.77, 1471.81, 1547.69, 1615.84, 1707.99, 1798.89, 1814.98, 1903.93, 2013.09, 2171.19, 2188.08, 2327.28, 2359.26, 2466.34, 2700.44, 2861.46, 3252.59 |
| 3.       | CF-α | ATCG00120 | PMF    | 736.45, 775.55, 815.57, 860.60, 876.58, 903.67, 1030.68, 1101.66, 1107.67, 1209.75, 1252.80, 1416.87, 1474.87, 1553.81, 1574.86, 1604.91, 1630.93, 1942.10, 2273.20, 2292.14, 2429.28, 2629.23, 2804.30 |
| 4.       | CF-β | ATCG00470 | PMF    | 997.49, 1007.55, 1144.55, 1526.70, 1706.85, 1834.93, 2652.12 |
| 5.       | Cytochrome f(PetA) | ATCG00540 | PMF    | 793.41, 833.43, 949.50, 970.50, 1158.71, 1247.72, 1314.79, 1418.81, 1498.71, 1696.89, 1801.94, 2125.10, 2370.23, 2507.23, 2551.21, 2590.10 |
| 6.       | Chlorophyll a/b-binding protein CP26 (LHCB5) | AT4G03280 | PMF    | 734.31, 833.43, 949.50, 970.50, 1158.71, 1247.72, 1314.79, 1418.81, 1498.71, 1696.89, 1801.94, 2125.10, 2370.23, 2507.23, 2551.21, 2590.10 |
| 7.       | Rieske FeS (PetC) | AT4G03280 | PMF    | 734.31, 833.43, 949.50, 970.50, 1158.71, 1247.72, 1314.79, 1418.81, 1498.71, 1696.89, 1801.94, 2125.10, 2370.23, 2507.23, 2551.21, 2590.10 |
| 8.       | CF-ε | ATCG00470 | PMF    | 1016.62, 1128.71, 1284.76, 1284.77, 1300.60, 1426.82, 2355.16, 3978.85 |
| 9.       | PSI-N | AT5G64040 | PMF    | 997.49, 1007.55, 1144.55, 1526.70, 1706.85, 1834.93, 2652.12 |

*a Accession numbers are from The Arabidopsis Information Resource.

*b PMF, peptide mass fingerprint analysis; ES, Edman sequencing; MS/MS, tandem mass spectrometry.

The queried mass peak data, which coincided with the value of the database, were shown for PMF.
discrepancy in their redox potentials; those of plastoquinone and plastocyanin were +80 to +110 mV (43, 44) and +360 mV (45), respectively.

In E. coli, cytoplasmic Trx is known to act as an electron donor for the membrane domain of DsbD protein, a three-domain inner membrane protein component of the Dsb (disulfide bond formation) protein reduction system, which catalyzes the transfer of electrons from the cytoplasm to periplasmic substrate proteins across the membrane (46, 47). A protein called CcdA, which has been shown to be the functional homolog of the membrane domain of DsbD in some prokaryotes (37), is also encoded by the Arabidopsis genome (38). The CcdA-deficient Arabidopsis T-DNA insertion mutant lines (48) show a defect in the accumulation of cytochrome b_{6f} complex similar to that observed in the HCF164-deficient mutant (hcf164). This has lead to the suggestion that a functional link may exist between CcdA and the HCF164-dependent reduction pathway in the assembly of the cytochrome b_{6f} complex, and to the hypothesis that the CcdA protein may function as a component of the HCF164-dependent reduction pathway (48).

In this study, we examined the effect of spinach Trx-f and Trx-m on this pathway, because these two Trxs are well known to be functionally localized in the chloroplast stroma (5, 7). Four different m-type Trxs have been reported recently for Arabidopsis (49). Based on amino acid sequence homology, spinach Trx-m is similar to Trx-m2 in Arabidopsis, although the differences in physiological roles among the two m, m2, and m4 isoforms are unknown (9). Comparison of the reducing efficiency between Trx-f and Trx-m suggests that Trx-m, the prokaryotic homolog of Trx in chloroplasts, may be the preferred candidate for the stromal source of reducing equivalents used for reduction of luminal HCF164 target proteins. Indeed, addition of the reduced form of exogenous Trx-m to intact thylakoids resulted in the partial reduction of both HCF164 and the HCF164 target candidate PSI-N in the lumen (Fig. 2 and Fig. 6B). These results also demonstrate the existence of a transmembrane reducing equivalent transfer system in higher plant chloroplasts resembling the system found in bacteria (50, 51). The suggested flow of the reducing equivalents from the stroma to the luminal proteins via HCF164 in this study is shown in Fig. 7. Our results suggest that the membrane component(s) that transfers the reducing equivalents from Trx in the stroma to HCF164 in the thylakoid lumen shows a preference for Trx-m. The Trx-x and Trx-y, which are predicted to be imported into chloroplasts, have been identified recently in the Arabidopsis genome (6–8). Although Trx-y was targeted to chloroplasts in a Trx-fused GFP experiment (10), the accurate localization of these Trx isoforms in chloroplasts (stroma side or lumen side) has not been determined. Whether Trx-x and Trx-y can assist in the reducing equivalent transfer to HCF164 should be confirmed together with their accurate localization in the chloroplasts.

Given the sequence similarity of Trx-m to prokaryotic Trx (52), the functional similarity observed between the bacterial and chloroplast transmembrane-reducing equivalent transfer system is of significant interest, particularly with regard to the evolution of the Trx system.

To assess the viability of the redox system suggested in this study, we determined the redox potential value for HCF164 by AMS labeling and compared it to those obtained for Trx-f and Trx-m (Fig. 4). The observed values for Trx-f and Trx-m were not dissimilar to those obtained in previous work using fluorescence quantification of monobromobimane, which is covalently linked to thiol groups (53). The redox potential value obtained for HCF164 was found to be less negative than stromal Trxs and was consistent with the idea that reducing equivalents may be transferred from stromal Trx-m to HCF164 via a component(s) located in the thylakoid membranes. Although glutaredoxin and glutathione are also potential electron donors within the chloroplast, they are unlikely to act as such for HCF164 because the redox
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potentials of the glutaredoxin family are around −190 to −230 mV (54, 55) and that of glutathione are −240 mV (56, 57), and both are almost equivalent to that of HCF164. This alternative possibility should, however, be subjected to further investigation because glutathione is known to be an abundant reductant in chloroplasts (58, 59).

HCF164 Mediates the Transfer of Reducing Equivalents to Target Proteins in the Thylakoid Lumen—By using the thylakoid proteome for our screen of HCF164 target proteins, we were able to capture a number of target protein candidates, although several peripheral proteins such as the CF$_1$-α, -β, and -ε subunits, which face the stromal side, were also captured because we used the detergent-solubilized protein fractions of whole thylakoids for our screening (Fig. 5 and Table 1). The α and β subunits of CF$_1$ have been captured previously by Trx affinity chromatography (15, 60, 61); although, because of the luminal location of the active disulfide bond of HCF164, they cannot interact with HCF164 in vivo, their high abundance and surface Cys residues are likely to make them prone to be captured by the chromatography method used here. Among the potential target proteins of HCF164, we identified the luminal PSI-N (32, 33) and proceeded to characterize its redox properties. PSI-N was reduced by HCF164 in vitro and was also reduced in intact thylakoids under the same conditions as HCF164 (Fig. 6). These results strongly suggest that PSI-N is an authentic target of HCF164 in thylakoid membranes. However, a number of questions regarding the reduction process of this protein remain unanswered. Although PSI-N has four cysteine residues, both of which can be reduced to form two disulfide bridge-linked cysteine pairs in vitro (Fig. 6A, red2), only one of these disulfide bonds could be reduced in intact thylakoids (Fig. 6B, red1). This suggests that only one PSI-N disulfide bond may be susceptible to reduction by HCF164, whereas reduction of the other pair may be hindered by conformational restrictions of PSI-N occurring within the photosystem I complex. In view of the fact that the majority of PSI-N on the thylakoid membrane remained oxidized, another possibility is that the reducing equivalents supplied by the exogenously added reduced form Trx-m may have been insufficient to reduce both disulfide bonds. This possibility cannot be excluded in our current study because PSI-N could be completely reduced by high concentrations of DTT alone (Fig. 6A, upper panel).

HCF164 was originally identified as an indispensable factor for assembly of the cytochrome $b_6f$ complex in chloroplasts (22). This study indicates that HCF164 may directly interact with two components of the cytochrome $b_6f$ complex, the cytochrome f and Rieske FeS proteins, through formation of an intermolecular disulfide bond with the HCF164 active site cysteine (Fig. 5). These results strongly suggest that the interaction between HCF164 and both the cytochrome f and Rieske FeS proteins may be important prerequisites for correct assembly of the cytochrome $b_6f$ complex. The significance of HCF164 as a transducer of reducing equivalents in the thylakoid lumen requires further investigation by corroboration of the luminal target proteins identified herein.

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