Multisite Phosphorylation of *Arabidopsis* HFR1 by Casein Kinase II and a Plausible Role in Regulating Its Degradation Rate*

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*Arabidopsis* Long Hypocotyl in Far-Red Light 1 (HFR1), a bHLH transcription factor, plays a critical role in promoting seedling photomorphogenesis and in balancing the shade-avoidance response under canopy shade conditions. Previous studies have established that HFR1 protein is degraded in darkness and is stabilized under light conditions to promote light signaling. How light regulates HFR1 stability is not well understood. In this study, we show that *Arabidopsis* HFR1 can be phosphorylated by recombinant casein kinase II (CKII) and plant extract *in vitro* and that phosphorylation of HFR1 can be effectively reduced by treatments with two CKII-specific inhibitors, 5,6-dichloro-1-β-o-ribofuranosyl-benzimidazole (DRB) and heparin. We demonstrate that HFR1 physically interacts with the CKB1 and CKB2 regulatory subunits of CKII. Mutagenesis studies indicate that HFR1 is phosphorylated at multiple serine (Ser) residues in the N-terminal regulatory domain of HFR1. We also show that phosphorylation of HFR1 is promoted by light and that a predicted CKII site, Ser122, represents a major phosphorylation site of HFR1 under both dark and light conditions. Comparison of wild-type, phosphorylation-deficient, and phosphorylation-mimic mutant proteins suggests that phosphorylation acts to reduce the degradation rate of HFR1. Together, our results suggest that CKII-mediated phosphorylation represents an important post-translational modification influencing the stability and signaling activity of *Arabidopsis* HFR1.

As sessile organisms, higher plants have evolved sophisticated photosensory mechanisms to sense changes in the ambient light environment and undergo adaptive growth and development. Plants can detect almost all facets of light such as direction, duration, quantity, and wavelength using three major classes of photoreceptors: the red (R)/far-red (FR) light (600–750 nm) sensing phytochromes (phy), and the blue (B)/UV-A (320–500 nm) absorbing cryptochromes (cry) and phototropins (1, 2). It has been established that, in *Arabidopsis*, phyB to phyE predominantly regulate light responses under continuous R and white light in a conditionally redundant manner, whereas phyA is the primary photoreceptor responsible for mediating the high irradiance response under continuous FR light (3).

Phytochromes have been well characterized for their roles in regulating seedling de-etiolation and mediating shade-avoidance response. In response to light, *Arabidopsis* seedlings develop short hypocotyls, with open and expanded cotyledons, and differentiated chloroplasts (4). During vegetative growth, plants use phytochromes to measure the R/FR ratios in their surroundings. Under a canopy, light is depleted of red but not far-red wavelengths, thus lowering the R/FR ratio. By sensing this change in light quality, plants initiate the shade-avoidance response by increasing the elongation growth of petioles and stems, the length-to-width ratio of leaves, and accelerating flowering (5).

Genetic studies revealed that *Arabidopsis* HFR1 acts as a positive regulator of phyA-mediated FR and cry1-mediated B light signaling in regulating seedling de-etiolation, and as a general negative regulator of shade-avoidance response (6–9). HFR1 encodes an atypical bHLH transcription factor of 292 amino acids and is closely related to PIF3, the first identified bHLH protein that can interact with the biologically active Pfr form of both phyA and phyB (10–12). Previous studies have shown that HFR1 protein is targeted for degradation in darkness by the COP1-SPA1 E3 ubiquitin ligase complex through the ubiquitin-26 S proteasome pathway and is stabilized under light conditions to promote light signaling (13–17). However, the molecular mechanisms by which light regulates HFR1 stability and functionality remain largely unknown. Although the abundance of nuclear COP1 is diminished through a light-mediated nuclear exclusion mechanism, the kinetics of COP1 nuclear depletion is very slow (taking up to 24 h) (18). In addition, nuclear accumulation of SPA1 protein increases significantly upon light exposure (19). Thus, the observed rapid accumulation of HFR1 protein under light conditions (detected in less than 30 min of light exposure; Ref. 13) suggests that there must be additional mechanisms regulating HFR1 stability.

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3 The abbreviations used are: R, red; FR, far-red; CKII, casein kinase II; GST, glutathione S-transferase; λ-PPase, λ-phosphatase; GFP, green fluorescent protein; HA, hemagglutinin; phy, phytochrome; DRB, 5,6-dichloro-1-β-o-ribofuranosyl-benzimidazole; bHLH, basic helix loop helix; YFP, yellow fluorescent protein; PKA, cAMP-dependent kinase.
In this study, we show that Arabidopsis HFR1 is phosphorylated in vitro and in vivo. We present multiple lines of evidence to support the notion that CKII is a cognate kinase responsible for HFR1 phosphorylation. We demonstrate that HFR1 is phosphorylated at multiple serine (Ser) residues in the N-terminal regulatory domain of HFR1 and that a predicted CKII site, Ser122, is a major phosphorylation site of HFR1 under both dark and light conditions. We also show that phosphorylation of HFR1 is promoted by light. Using an in vitro degradation assay, we gathered evidence to support the proposition that phospho-

Plasmid Construction—To generate various GFP-HFR1 fusion protein constructs, the plasmid pRTL2-GFP-HFR1 (15) was used as a template for mutagenesis using a QuickChange Multi Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instruction. The primers used were: S28A and S33A: 5′-gtagagttgacgatgtgaagcggaggagtttacaagag-3′; S84A: 5′-gtcctgtagaattcatgtcgaataatcaagctttc-3′; S121A, S122A, and S126A: 5′-cgtactcagctctagctgcatg-3′; S171A: 5′-caacagcagaacttgctggtctctgcaagac-3′; S28E and S33E: 5′-gattcgaggaattcatgtcgaataatcaagctttc-3′; S84E: 5′-gtctctgtagaattcatgtcgaataatcaagctttc-3′; S121E and S126E: 5′-cgtactcagctctagctgcatg-3′; S121E and S126E: 5′-cgtactcagctctagctgcatg-3′; S122E: 5′-gatgaaagcggaggagtttacaagag-3′; S171E: 5′-caacagcagaacttgctggtctctgcaagac-3′.

To construct the GST-NT131 and various mutant protein expression plasmids, NT131 fragments of HFR1 (containing N-terminal 131 amino acids, wild-type and mutant forms) were amplified by PCR using the pRTL2-GFP-HFR1 wild-type and mutant plasmids as templates. The primers used are: HFR1-1F (5′-ggctctagactcgagtcatcttgtaaactcctccgattc-3′) and HFR1-1R (5′-agttaagtgaagatgatgaatcggagg-3′). The purified PCR products were inserted into the pGEM-T Easy vector (Promega, Madison, WI) to generate pGEM-NT131 (wild-type and mutant forms). The inserts were then released by BglII and SalI and cloned into the DNA binding domain containing yeast two-hybrid vector pBD-GAL4 Cam (Stratagene) at EcoRI and SalI sites, producing BD-HFR1. Full-length cDNAs of CKA1 (At5g67380), CKA2 (At3g50000), CKB1 (At5g47080), and CKB2 (At4g17640) were amplified by reverse transcriptase (RT)-PCR from wild-type Arabidopsis (ecotype Columbia), and cloned into the pGEM-T easy vector to generate pGEM-CKA1, pGEM-CKA2, pGEM-CKB1, and pGEM-CKB2. The CKA and CKB fragments were then released from their respective pGEM plasmids by digestion with BamHI and Xhol, and the released fragments were cloned into the activation domain containing yeast two-hybrid vector pAD-GAL4–2.1 (Stratagene) at BamHI and Xhol sites, giving rise to GAD-CKA1, GAD-CKA2, GAD-CKB1, and GAD-CKB2.

To construct the various plasmids for the subcellular co-localization assay, HFR1 was released from yy64-HFR1 by digestion with BglII and SalI and then subcloned into the pSAT6-EYFP-C1 vector (21) at BglII and SalI sites to generate pSAT6-CFP-HFR1. The BamHI-Xhol fragment of CKB1 and CKB2 was released from pGEM-CKB1 and pGEM-CKB2 and cloned into the pSAT6-EYFP-C1 vector (21) at BglII and SalI sites to generate pSAT6-YFP-CKB1 and pSAT6-YFP-CKB2, respectively.

To generate 3HA-tagged full-length HFR1 wild type, A5, and E5 mutant constructs, HFR1, A5 and E5 were amplified by PCR using pRTL2-HFR1, pRTL2-A5, or pRTL2-E5 as the templates respectively. The primers used were HFR1–1F (5′-gagagatctgaattcgtgcatgactgcatgttcatgacct-c-3′) and HFR1–292R (5′-ggctctactgcatgtatccttcgattcgtgcatgacct-c-3′). The PCR products were digested with EcoRI and Xhol, and then subcloned into the pSAT6–3HA vector4 at EcoRI and SalI sites to generate pSAT6-HFR1–3HA, pSAT6-A5–3HA, and pSAT6-E5–3HA, respectively. All PCR products generated in this study were confirmed by DNA sequencing.

Preparation of GST-NT131 Wild-type and Mutant Fusion Proteins—The pGEX-NT131 plasmids (wild-type and mutant forms) were transformed into the bacterial strain BL21 (DE3). To prepare bacterial extract, the transformed cells were inoculated in LB and grown overnight. The culture was diluted (1:100) in fresh medium and cultured at 37 °C until the cell A600 is 0.8. The cells were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 25 °C and harvested by centrifugation. The cell pellet was resuspended in 4 ml of 1× PBS, pH 7.4, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and lysed with a French Press. The cell lysate was applied on the glutathione-Sepharose 4B column (Amersham Biosciences). The column was washed with 1× phosphate-buffered saline (1× PBS, 0.1% Triton X-100), and bound proteins were eluted with an elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM glutathione).

Transient Gene Expression in Onion Epidermal Cells—The procedure for transient expression in living onion epidermal cells using particle bombardment was described previously (15). For the co-localization assay, 15 μg of CFP-HFR1 plasmid DNA and the same amount of YFP-CKB1 or YFP-CKB2 were co-coated on tungsten particles and bombarded into onion epidermal cells, and incubated overnight before observation.

Fluorescence and Confocal Microscopy—For observing co-localization of CFP-HFR1 with YFP-CKB1 and YFP-CKB2 in onion epidermal cells, transformed onion epidermal peels were

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4 M. Dai and H. Wang, unpublished results.

5 H. Park and H. Wang, unpublished results.
examined and recorded using a LEICA TCS SP5 Confocal Laser Scanning Microscope (458-nm argon laser for ECFP and 514-nm argon laser for YFPP). Overlay of CFP and YFPP images were generated using Image J software.

**Yeast Two-hybrid Assay—BD-HFR1 (or the empty vector control pBD-GAL4 Cam) was co-transformed into the yeast strain Pj69-4A with GAD-CKA1, GAD-CKA2, GAD-CKB1, and GAD-CKB2, or the empty AD vector pAD-GAL4-2.1, respectively. Yeast cells were selected on Leu− Trp− drop-off media and then patched onto His− plus 0.05 mg/liter Ade medium. The yeast cells were allowed to grow for 4–8 days.**

**In Vitro Phosphorylation Assay—For the in vitro phosphorylation assay using plant extracts as the kinase source, Arabidopsis seedlings were grown in the desired light conditions for 4–5 days and then harvested and frozen in liquid N2. Then the seedlings (∼1 g) were homogenized in 1 ml of CKII assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 25 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor mixture (Roche Applied Science)). The extracted protein solution was spun down at 12,000 rpm for 10 min at 4 °C, the supernatant was transferred into a new tube, and protein concentrations of the extracts were measured using the Bradford assay (Bio-Rad). For 40 μl of reaction mixture, we combined 10–30 μg of plant extracts, 3–5 μg of substrate proteins, 20 μM ATP and 5 μCi of [γ-32P]ATP (PerkinElmer Life Science) in the CKII assay buffer. The reaction was incubated at 30 °C for 30 min, then 10 μl of 5× SDS-sample buffer (1×: 0.0625 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% SDS, 0.001% (w/v) bromphenol blue) was added, and the sample boiled for 3 min. Then the samples were separated on 11% SDS-PAGE and examined by autoradiography. Phosphorylation signals were quantified with a Storm Scanner (Molecular Dynamics, Sunnyvale, CA). For the in vitro phosphorylation assay using recombinant kinases, the human glioblastoma recombinant CKII (αβ2 tetrameric holoenzyme), CKI (rat testis), and protein kinase A (PKA, murine PKA catalytic subunit) were purchased from NEB (New England Biolabs, Ipswich, MA). 25 units of CKI or CKII, or 150 units of PKA were used for the phosphorylation assay of GST-NT131.

**Protoplast Transient Expression and Dephosphorylation Assay—Isolation of Arabidopsis protoplast and polyethylene glycol-mediated transfection were performed as described (22). After transformation, the protoplasts were incubated under darkness or weak light conditions for 12–15 h. Then the protoplasts were spun down at 100–200 × g for 1 min, and the supernatant was removed. Next, an equal volume of 2× protoplast extraction buffer (2× λ-PPase buffer from NEB, 4 mM MnCl2, 2% Triton X-100, 2× complete protease inhibitor mixture) was added to the protoplast pellets, and the protoplasts were resuspended by vortexing for several seconds. The protoplast solution was incubated on ice for 5 min, then aliquoted into three separate tubes (25–30 μl each). 1 μl of λ-PPase was added to one of the tubes. 1 μl of λ-PPase plus 10 μl of a broad-band phosphatase inhibitor Na2VO3 were added to another tube. The reaction mixtures were incubated at 30 °C for 30 min. Then the samples were mixed with 8 μl of 5× SDS-sample buffer, boiled for 3 min, and separated by NuPAGE gel (Invitrogen). As previously indicated, NuPAGE gel provides better resolution for discriminating phosphorylated versus unphosphorylated forms of proteins, compared to a homemade SDS-PAGE gel (23). Then the GFP-HFR1 protein was detected with immunoblot using anti-GFP antibodies (Invitrogen).

**In Vitro Degradation Assay—In vitro degradation assay was performed essentially as previously described (24, 25) with minor modifications. For degradation of the GST-NT131, GST-A5, and GST-E5 recombinant proteins, plant extracts were prepared from 5-day-old dark-grown seedlings, grounded in liquid nitrogen, and resuspended in a cell-free degradation assay buffer (25 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 5 mM dithiothreitol, 10 mM NaCl, and 10 mM ATP). Cell debris was removed by centrifugation before adding to the recombinant proteins. Then equal amounts (∼300 μg) of plant extracts were added to the recombinant protein samples (5 μg) (total reaction volume 110 μl). The reaction mixtures were incubated in darkness, and 20 μl of reaction mixture was taken at different time points: 0, 20, 40, 60, or 120 min, into new tubes containing 5 μl of 5× SDS sample buffer to stop the degradation process. Then the samples were boiled for 3 min and separated on 11% SDS-PAGE gel and subjected to immunoblot analysis using anti-HFR1 polyclonal antibodies (15).

For the cell-free degradation assay with full-length HFR1–3HA, A5-3HA, and E5-3HA fusion proteins, the pSAt6-HFR1–3HA, pSAt6-A5-3HA, and pSAt6-E5-3HA plasmids were individually transfected into Arabidopsis protoplasts to transiently express the HFR1–3HA, A5-3HA, and E5-3HA fusion proteins. After incubation in darkness for 12 h, the expressed proteins were extracted with 2× protoplast cell-free degradation assay buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 10 mM dithiothreitol, 20 mM NaCl, and 20 mM ATP, 2% Triton X-100). The extracted proteins were used for degradation assay by adding equal amounts of plant extracts prepared from 5-day-old dark-grown seedlings as described above. The degradation assay was performed as described above for the recombinant proteins, and the fusion proteins were detected via immunoblot using anti-HA-specific antibodies (Roche Applied Science).

**RESULTS**

**HFR1 Can Be Phosphorylated by CKII and Plant Extracts in Vitro—**A recent study reported that HFR1 is a phosphoprotein in vivo (13); however, the phosphorylation site(s) is unknown, and the responsible kinase(s) remains to be identified. Sequence analysis revealed that HFR1 contains five predicted CKII phosphorylation sites (see below). Thus, we first tested whether recombinant CKII can phosphorylate HFR1 in vitro. An N-terminal 131-amino acid fragment of HFR1 (NT131) was selected for this assay as this domain was previously shown to be responsible for interacting with COP1 and is the regulatory domain of HFR1 stability (13–15). Second, this domain contains four of five predicted CKII phosphorylation sites. NT131 of HFR1 was expressed in Escherichia coli and purified as a GST fusion protein (GST-NT131). This fusion protein was used as the substrate for an in vitro phosphorylation assay in the CKII buffer containing [γ-32P]ATP. GST-NT131 has no autophosphorylation activity, but can be effectively phosphorylated by recombinant CKII and slightly phosphorylated by casein kinase.
Phosphorylation of Arabidopsis HFR1

I (CKI). However, PKA is not effective in phosphorylating HFR1. As previously reported (26), GST-HY5 was also phosphorylated by CKII, whereas GST itself was not phosphorylated by CKII (Fig. 1A).

Next, we tested whether the GST-NT131 recombinant protein can be phosphorylated by plant extracts in vitro. GST-NT131 can be effectively phosphorylated by plant extracts from light-grown seedlings using [γ-32P]GTP as the phosphate donor. Boiling of the plant extracts before adding to the phosphorylation assay completely abolished HFR1 phosphorylation. Moreover, addition of a CKII-specific inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), effectively inhibited HFR1 phosphorylation in a dosage-dependent manner (Fig. 1B). Further, heparin, another potent and CKII-specific inhibitor, also effectively blocked HFR1 phosphorylation by plant extracts (Fig. 1C). The capacity to use both [γ-32P]ATP and [γ-32P]GTP as the phosphate donors, which is a characteristic of CKII kinase, and the inhibition of HFR1 phosphorylation by DRB and heparin, strongly support that CKII could be a bona fide kinase responsible for phosphorylation of HFR1 in vivo.

CKII Subunits Physically Interact with HFR1—The CKII holoenzyme that has been purified from many different eukaryotes is generally a tetrameric complex formed by two catalytic subunits (α) and two regulatory subunits (β), with a molecular mass of ~130 kDa (27). In Arabidopsis, there are four genes (CKA1-CKA4) encoding the α subunits of CKII and four genes (CKB1-CKB4) encoding the β subunits of CKII. It is believed that the β subunits of CKII are inactive by themselves, but they can stimulate CKIIα catalytic activity, confer stability to the enzyme, and provide specificity for the interactions with substrates and inhibitors (28). To investigate whether HFR1 physically interacts with CKII, we have cloned two α subunits (CKA1 and CKA2) and two β subunits (CKB1 and CKB2) as fusions with the GAL4 activation domain (GAD-CKII) and tested their interaction with HFR1 that is fused with the GAL4 DNA binding domain (DB-HFR1), using a yeast two-hybrid assay. We selected these CKII subunits for this assay as they are localized in the nucleus as HFR1 (15, 29). Both the CKB1 and CKB2 subunits interact with HFR1, judging from the yeast growth on a selection plate that lacks histidine (a selective marker for the HIS3 reporter gene expression) (Fig. 2A). No physical interaction was detected between HFR1 with the CKA1 and CKA2 subunits of CKII (data not shown). To confirm the observed physical interaction between HFR1 with CKB1 and CKB2 in yeast, we performed a subcellular co-localization assay in planta. The CKB1 and CKB2 subunits were tagged with yellow fluorescence protein (YFP-CKB1/2) and co-expressed with a cyan fluorescence protein (CFP)-tagged HFR1 (CFP-HFR1) in onion epidermal cells. Both CFP-HFR1 and CFP-CKB1/2 are localized into the nucleus, and above a uniform nucleoplasm distribution, these fusion proteins also form distinct speckle-like nuclear bodies that overlay with each other (Fig. 2B), indicating that CFP-HFR1 and YFP-CKB1/2 co-localize in the nucleus. This observation provides strong supporting evidence for the in vivo interaction between HFR1 and CKB1/2.

To further test the in vivo functional relationship between CKII and HFR1, we examined the phosphorylation efficiencies of HFR1 by plant extract from CKB1 and CKB4 overexpression transgenic plants. The degrees of GST-NT131 phosphorylation were significantly higher when incubated with plant extracts from light-grown CKB1 and CKB4 overexpression transgenic plants, compared with wild-type plant extract (Fig. 2C). This result further supports that CKII acts as the cognate kinase for phosphorylating HFR1 in vivo.

HFR1 Is Phosphorylated at Multiple Serine Residues in Vivo—To identify the phosphorylation sites of HFR1, we transiently overexpressed GFP-HFR1 protein (driven by the constitutive 35S mosaic virus promoter) in Arabidopsis protoplasts using a PEG-mediated transfection method (22). After incubating under dark or weak light conditions for 12 h, the protein was
extracted and treated with λ-PPase in the presence or absence of a phosphatase inhibitor sodium orthovanadate (Na₃VO₄). After separation using NuPAGE gel, the proteins were transferred onto a polyvinylidene fluoride membrane and probed with anti-GFP antibodies. As shown in Fig. 3, treatment with λ-PPase caused the GFP-HFR1 protein to shift into a faster migrating band, and this migration shift was inhibited by Na₃VO₄. This result suggests that GFP-HFR1 is indeed phosphorylated in vivo. We concluded that the faster migrating form represents dephosphorylated HFR1, whereas the slower migrating band represents the phosphorylated HFR1. Interestingly, migration of GFP-CT161 was not affected by λ-PPase treatment (Fig. 3). This observation suggests that the phosphorylation site(s) responsible for HFR1 migration shift resides in the N-terminal 131 amino acids (NT131), a regulatory domain responsible for interacting with COP1 and the targeted degradation of HFR1 (13–15).

CKII phosphorylates serine (Ser) and threonine (Thr) residues immersed in acidic sequences within proteins and peptides. The minimum requirements for phosphorylation by CKII are depicted by the sequence (S/T)XX(D/E) (X stand for any nonbasic amino acid) (27, 30). HFR1 contains five predicted CKII phosphorylation sites (predicted with NetPhos 2.0 Server), including Ser³³ (possibility score 0.998), Ser⁸⁴ (possibility score 0.990), Ser¹²¹ and Ser¹²² (possibility scores are 0.988 and 0.996, respectively), and Ser¹⁷¹ (possibility score 0.986). In addition, Ser²⁸ and Ser¹²⁶ are predicted with a possible phosphorylation score of 0.890 and 0.996, respectively (Fig. 4A). Of note, among these predicted CKII sites, Ser¹⁷¹ is located in the second helix region of the bHLH domain of HFR1, and this serine residue is conserved in all members (includes PIF1, PIF3, PIF4, and PIF5) of the subfamily15 bHLH proteins, suggesting a functional significance of this residue (31). To test whether Ser¹⁷¹ is phosphorylated, we generated a short peptide (Asn-Cys-His-Lys-Thr-Asp-Lys-Val-Ser¹⁷¹-Val-Leu-Asp-Lys-Thr-Ile-Glu-Tyr-Met) containing Ser¹⁷¹ and expressed it as a GST fusion protein. This peptide was not phosphorylated by recombinant CKII or Arabidopsis plant extracts (data not shown). Thus the functional significance of this residue was not pursued further in this study. It is also worth noting that Ser¹²¹-Ser¹²² (in the context of Gln-Val-Leu-Ser¹²¹-Ser¹²²-Asp-Asp-Glu-Ser-Glu-Glu) are followed by a stretch of acidic amino acids and thus they are particularly suitable for contacting the basic residues in the active site of CKII and being phosphorylated by CKII. To determine which of the serine residues are phosphorylated, we generated a series of mutant forms of HFR1 using site-directed mutagenesis (Fig. 4B). The serine residues were changed to either alanine (A) or glutamic acid (E). These mutant forms of HFR1 were fused with GFP, and their fusion proteins were transiently expressed in Arabidopsis protoplasts, separated on NuPAGE gel and analyzed by immunoblot analysis. As shown in Fig. 4B, mutating six Ser residues to Ala (construct GFP-A5) completely abolished the migration shift by λ-PPase treatment, whereas several other mutants (GFP-A1, GFP-A3, GFP-A4) in which three of the Ser residues were mutated into Ala still display migration shift upon λ-PPase treatment.
Ser⁹⁶, Ser⁹⁹ and Ser¹²²) by CKII or other kinases. Notably, plant extracts from light-grown seedlings phosphorylated HFR1 more effectively, compared with plant extracts from dark-grown seedlings (Fig. 5E, compare lanes 2), suggesting that HFR1 phosphorylation might be light-regulated. Phosphorylation of HFR1 Is Promoted by Light—To further test whether phosphorylation of HFR1 is regulated by light, we compared the phosphorylation efficiency of GST-NT131 by plants extracts of light- and dark-grown Arabidopsis seedlings. GST-NT131 was less phosphorylated by the plants extracts of seedlings initially grown in white light conditions but transferred into darkness for 2 h, compared with the plant extracts from seedlings that have been kept in white light conditions. Similarly, the plant extracts from seedlings that were initially grown in darkness but exposed to white light for 2 h displayed an increased phosphorylation efficiency toward GST-NT131, when compared with the plant extracts from seedlings kept in darkness (Fig. 6, A and B). In addition, plant extracts from dark-grown seedlings irradiated with red or far-red light for 30 min exhibited significantly increased phosphorylation efficiencies toward GST-NT131, compared with the plant extracts of seedlings kept in darkness (Fig. 6C-D). Together, these results support the notion that phosphorylation of HFR1 is promoted by light treatments. However, it should be noted that this increased phosphorylation by light-grown plant extracts could be due to either increased kinase activity or decreased phosphatase activity, or a combination of both.

We next performed experiments to determine which phosphorylation site(s) might be regulated by light. Wild-type GST-NT131 and various mutant forms were incubated with either light- and dark-grown plant extracts in the presence of [γ²³³²P]ATP. As shown in Fig. 7, phosphorylation of wild-type GST-NT131, GST-S121A, and GST-A3 (S28A, S33A, and S84A) was similarly reduced by dark-grown plant extracts, compared with light-grown plants extracts, suggesting that Ser⁷⁸, Ser⁹³, Ser⁹⁹, and Ser¹²¹ are not the major light-regulated phosphoresidues. In contrast, phosphorylation of the GST-S122A mutant protein and other mutant forms containing the S122A mutation (GST-A1 and GST-A5) was much reduced by either dark- or light-grown plant extracts. This result suggests that Ser¹²² is the major phosphorylation site under both darkness and light conditions.

Phosphorylation Reduces the Degradation Rate of HFR1 in Vitro—The NT131 domain of HFR1 was previously shown to be responsible for interacting with COP1 and the targeted degradation of HFR1 by the 26 S proteasome (13–15). We next tested whether phosphorylation of NT131 correlates with its...
stability. To this end, we first performed a cell-free degradation assay with GST-tagged HFR1-NT131 recombinant proteins. Equal amounts (1 μg) of recombinant GST-NT131, GST-A5 (NT131), and GST-E5 (NT131) proteins were incubated with plant extracts from dark-grown seedlings (supplemented with 10 mM ATP) under darkness for an extended time period. As shown in Fig. 8A, GST-NT131 is notably more stable than GST-A5 (a phosphorylation-deficient form), whereas GST-E5 (a hyperphosphorylated form) is clearly more stable than both the GST-NT131 and the GST-A5 mutant protein. This result suggests that phosphorylation likely acts to enhance the stability of HFR1 protein.

To provide further support for the notion that phosphorylation may act to enhance HFR1 stability, we examined the effects of phosphorylation with full-length HFR1 protein. Full-length wild-type HFR1–3HA (tagged with 3 copies of HA epitope at the C terminus of HFR1), A5-3HA, and E5-3HA were expressed in Arabidopsis protoplasts and then incubated with plant extracts derived from 5-day-old dark-grown seedlings. The reactions were incubated under darkness, and samples were taken at different time points to stop the degradation process. Immunoblot analysis using anti-HA antibodies showed that E5-3HA fusion protein is clearly more stable than both wild-type HFR1–3HA and A5-3HA, whereas the wild-type HFR1–3HA is visibly more stable than A5-3HA (Fig. 8B). This result further supports the notion that phosphorylation acts to reduce the degradation rate of HFR1, at least under our assay conditions.

DISCUSSION

Phosphorylation and dephosphorylation often serve as an on-and-off switch for rapid regulation of transcription factor activity through modulating their DNA binding activity, cellular localization, stability, and interaction with other proteins (32). Arabidopsis HFR1 encodes a bHLH-type transcription factor that plays a key role in regulating light signaling. A previous study reported that HFR1 is a phosphoprotein in vivo (13); however, its sites of phosphorylation, the responsible kinase(s), and the potential biological role of this post-translational modification remain unknown. In this study, we demonstrated that HFR1 can be phosphorylated both in vitro and in vivo. We present multiple lines of evidence to support the notion that CKII is a kinase for phosphorylating HFR1. First, we show that HFR1 can be phosphorylated by recombinant CKII and plant extracts in vitro. Second, we show that both [γ^{32}-P]ATP and [γ^{32}-P]GTP can be used as the phosphate donors for HFR1 phosphorylation, which is a characteristic hallmark of CKII activity (29, 33). Third, we show that two CKII specific inhibitors, DRB and heparin, can effectively reduce HFR1 phosphorylation by plant extracts in vitro. Fourth, we demonstrate that HFR1 physically interacts with the CKB1 and CKB2 regulatory subunits of CKII using a yeast two-hybrid assay and a subcellular co-localization assay in planta. Moreover, we show that HFR1 is phosphorylated at multiple Ser residues in vivo (including four predicted CKII sites, Ser^{33}, Ser^{84}, Ser^{121}, and Ser^{122}) and that Ser^{122}, a predicted CKII site, is a major phosphorylation site of HFR1 under both dark and light conditions. Further,
transgenic plants overexpressing two different subunits of CKII (CKB1 and CKB4) display higher activities toward phosphorylating HFR1, compared with wild-type plants. Together, these data provide strong support for the proposition that CKII serves as a cognate kinase responsible for phosphorylating HFR1.

CKII is a ubiquitous and highly conserved Ser/Thr protein kinase in all higher eukaryotes. Studies from several organisms have identified numerous substrates of CKII and demonstrated that CKII participates in the regulation of a variety of cellular processes, such as cell cycle progression and cell growth, circadian clock, signal transduction, or transcriptional control (27). Studies with transgenic plants expressing reduced levels of CKII catalytic subunits have demonstrated a role of CKII in regulating both general plant growth and light-responsive gene expression (34). Interestingly, several light signaling- or clock-related transcription factors have been shown to be phosphorylated by CKII in vitro or in vivo. For example, the DNA binding activity of GBF1 was shown to be modulated through phosphorylation by a CKII activity (35). CKII-dependent phosphorylation also regulates DNA binding, stability, and activity of the bZIP transcription factor HY5 (26). It has also been shown that CCA1 phosphorylation by CKII is important for the normal functioning of the clock (33, 36, 37). Interestingly, the binding of CCA1 and HY5 to the Lhcb*1 promoter was reported to be increased by incubation with CKII regulatory β-subunits, which, alone, cannot phosphorylate CCA1 or HY5 (38). Further, overexpression of CKB3 or CKB4 in Arabidopsis alters multiple outputs of the clock function, including clock-regulated gene expression, hypocotyl elongation, and flowering time (36, 39). CKII has also been implicated as an important component in the circadian clock of Drosophila and Neurospora (40, 41). Interestingly, our data suggest that HFR1 can be phosphorylated by plant extracts of both dark- and light-grown seedlings, consistent with the notion that CKII is constitutively active, being unresponsive to known second-messenger molecules (27, 28). The observed higher phosphorylation activity of light-grown seedling extracts toward HFR1, compared with plant extracts from dark-grown seedlings, is likely to reflect the balance between CKII and a light-regulated phosphatase activity. Regardless, our results indicating that Arabidopsis HFR1 is a substrate of CKII highlight the importance of CKII as an evolutionarily conserved kinase in regulating light signaling and circadian clock function in higher plants and other eukaryotes.

Recent studies showed that targeted proteolysis via the ubiquitin/26 S proteasome plays a critical role in modulating the signal transduction processes of light and various phytohormones, such as ethylene, auxin, gibberellin, cytokinin, jasmonate acid, abscisic acid, and brassinosteroid (42, 43). For example, the FR light photoreceptor phyA and the B light photoreceptor cry2, as well as several key light signaling molecules (including HY5, PIF1, PIF3, PIF4, PIF5, and LAF1) have been shown to be subject to 26 S proteasome-mediated degradation in a light-dependent fashion (24, 44–47). Phosphorylation is the most common type of substrate modification and could serve as a prerequisite for substrate interaction with the F-box subunit of an SCF E3 ubiquitin ligase complex (48). Consistent with this, phosphorylation has been shown to correlate with, and possibly trigger the degradation of, phyA (49), cry2 (23), and the phytochrome-interacting bHLH transcription factors PIF3, PIF4, and PIF5 (46, 50, 51). In this study, we show that recombinant HFR1 protein can be phosphorylated by both dark- and light-grown plant extracts, but the levels of HFR1 phosphorylation are clearly higher by light-grown plant extracts toward HFR1, compared with plants extracts from dark-grown seedlings (13). We conclude that HFR1 is phosphorylated under both dark and light conditions and that light acts to promote HFR1 phosphorylation. This notion is consistent with the previous observations that light induces accumulation of both phosphorylated and unphosphorylated HFR1 isoforms and that phosphorylated HFR1 form can be stabilized in the presence of proteasome inhibitors in dark-grown seedlings (13). Duek et al. (13) speculated that phosphorylation of HFR1 rendered it particularly unstable in the dark based on the observation that upon transfer into darkness, the slow-migrating form (presumably the phosphorylated form) disappeared faster than...
the fast-migrating form (13). However, it is worth pointing out that phosphorylation and dephosphorylation is a reversible and dynamic process. Degradation of one form will most likely break the equilibrium and cause conversion of one form into the other. Thus, such data should be interpreted with caution.

Based on previous observation that light acts to promote stabilization of HFR1 (13–17) and our observation that light acts to promote HFR1 phosphorylation, we propose that light-regulated differential phosphorylation of HFR1 may serve as a fine-tuning mechanism to help stabilize Arabidopsis HFR1, and consequently the degree of light signaling and photomorphogenesis (Fig. 9). This notion is further supported by our in vitro degradation results. We showed that two differential phosphorylation-deficient mutant forms of HFR1 (GST-A5 and A5-3HA) degraded faster than the wild-type GST-NT131 and HFR1–3HA, respectively. Further, two phosphorylation-mimic mutant forms of HFR1 (GST-E5 and E5-3HA) show significantly enhanced stability compared with wild-type HFR1 and the phosphorylation-deficient mutant forms of HFR1. Similarly, phosphorylation has been shown to stabilize several important cellular proteins, such as the antiapoptotic protein Bcl-2, the mammalian transcription factors c-Jun, and the plant transcription factors HY5 and RGL2 (26, 52–54). Together, these studies support the role of phosphorylation as a versatile molecule tag for regulating protein stability in various cellular signaling and physiological processes. Identification of the phosphorylation sites now set up the stage for future investigation into the effects of phosphorylation on HFR1 stability.

FIGURE 7. Ser122 is a major phosphorylation site under both darkness and light conditions. A, 4-day-old dark (D)-grown seedlings were kept in darkness or transferred to white light conditions for 2 h. The plant extracts were used to phosphorylate GST-NT131 and its mutant variants (GST-A1 (S121A, S122A, S126A), GST-A3 (S28A, S33A, S84A), GST-S121A, GST-S122A, and GST-A5 (S28A, S33A, S84A, S121A, S122A, S126A)). Arrowhead indicates the position of GST-NT131. B, quantification of the phosphorylation efficiencies of GST-NT131 and its mutant variants by light- and dark-grown plant extracts shown in A.

FIGURE 8. Phosphorylation promotes stabilization of HFR1 in vitro. A, immunoblot analysis showing that GST-E5 (GST-NT131 (S28E, S33E, S84E, S121E, S126E)) recombinant protein is more stable than GST-NT131 wild-type HFR1 protein and the phosphorylation-deficient mutant protein GST-A5 (GST-NT131 (S28A, S33A, S84A, S121A, S122A, S126A)) in an in vitro degradation assay. 1 μg of recombinant protein is subjected to a cell-free degradation assay over selected time points indicated at the top. B, immunoblot analysis showing that E5-3HA (HFR1 (S28E, S33E, S84E, S121E, S126E)-3HA) fusion protein expressed in protoplasts is more stable than wild-type HFR1–3HA and A5-3HA (HFR1 (S28A, S33A, S84A, S121A, S122A, S126A)-3HA) fusion proteins in the in vitro degradation assay over selected time points indicated at the top.

FIGURE 9. A molecular model depicting regulation of Arabidopsis HFR1 stability and activity by reversible phosphorylation. In darkness, HFR1 physically interacts with COP1 and SPA1 and is targeted for degradation by the COP1-SPA1 E3 ubiquitin ligase complex. Exposure to light enhances HFR1 phosphorylation, which may lead to altered interaction with COP1 and SPA1, and subsequent stabilization of HFR1 to promote light signaling. In addition, light triggers depletion of nuclear COP1 by regulating its nucleocytoplasmic partitioning. The phosphatase (s) (PPase) responsible for dephosphorylating HFR1 is currently unknown (denoted by ?).
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vivo and to assess the effects of phosphorylation on its interaction with the COP1-SPA1 E3 ubiquitin ligase complex and ubiquitination, its interaction with DNA, and its transcriptional regulatory activity. It is also important to identify the phosphatase(s) responsible for dephosphorylating HFR1, which is needed to understand how the reversible and dynamic phosphorylation/dephosphorylation cycles of HFR1 contribute to the regulation of HFR1 stability and functionality in response to light signals.

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