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HFR1 encodes an atypical bHLH protein that acts in phytochrome A signal transduction

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Phytochromes are informational photoreceptors through which plants adapt their growth and development to prevailing light conditions. These adaptations are effected primarily through phytochrome regulation of gene expression by mechanisms that remain unclear. We describe a new mutant, hfr1 (long hypocotyl in far-red), that exhibits a reduction in seedling responsiveness specifically to continuous far-red light (FRc), thereby suggesting a locus likely to be involved in phytochrome A (phyA) signal transduction. Using an insertionally tagged allele, we cloned the HFR1 gene and subsequently confirmed its identity with additional alleles derived from a directed genetic screen. HFR1 encodes a nuclear protein with strong similarity to the bHLH family of DNA-binding proteins but with an atypical basic region. In contrast to PIF3, a related bHLH protein previously shown to bind phyB, HFR1 did not bind either phyA or B. However, HFR1 did bind PIF3, suggesting heterodimerization, and both the HFR1/PIF3 complex and PIF3 homodimer bound preferentially to the Pfr form of both phytochromes. Thus, HFR1 may function to modulate phyA signaling via heterodimerization with PIF3. HFR1 mRNA is 30-fold more abundant in FRc than in continuous red light, suggesting a potential mechanistic basis for the specificity of HFR1 to phyA signaling.

[Key Words: gravitropism; photomorphogenesis; transcription factor; high irradiance response; T-DNA tagging; light-regulated gene]

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responsive mutants, phyA signaling pathways, respectively. Also, two hyper-gests loci important to early events in separate phyB and

class to the nucleus. tochrome with sequence-specific DNA-binding proteins
gene expression is the direct interaction of activated phy-
troxin-Garcia et al. 2000). Together, these results suggest

bound PIF3 on conversion to the active Pfr form (Mar-

cate to the nucleus by photoconversion to Pfr (Sakamoto

culated in various genetic screens (Deng and Quail 1999;

spond to any other mutant with a FRc-specific long-hy-

frame to white light (Barnes et al. 1996). We have de-
volved a novel, directed genetic screen based on the

large-scale fertilization of a mutagenized male-sterile

population with hfr1-1 pollen. Using an insertionally
tagged allele of hfr1, we have cloned the HFR1 gene and

find that it encodes a bHLH protein with strong similarity
to PIF3. We explore the light-regulation of the HFR1
gene, the subcellular localization of the HFR1 protein,

and the propensity of HFR1 to interact with PIF3, phyA,

and phyB. Our results suggest that HFR1 may act in the
direct regulation of gene expression hypothesized for phyA.

Results

Isolation of hfr1 mutants

Using a FRc fluence rate below saturation for the de-
etiolation response, we screened variously mutagenized

populations of Arabidopsis for a long-hypocotyl phen-
type and selected seedlings displaying a partial response
to the FRc. The progeny of these candidates were tested
by germination and growth in darkness and in RC, as well
as in FRc. Of those judged to have a FRc-specific long-
hypocotyl phenotype, 13 were assessed for allelism to

known FRc long-hypocotyl mutants. Eleven proved to be

allelic to phyA, and one to phyB. One mutant resulting

from T-DNA mutagenesis, which we have named hfr1

(long hypocotyl in far-red), shows incomplete linkage to

phyA at the top of chromosome I and does not corre-

spond to any other mutant with a FRc-specific long-hy-

pocotyl phenotype (phy1, phy3, fin2, far1, pat1).

To obtain additional alleles of hfr1 beyond the one

initially isolated, hfr1-1, we employed a directed genetic

screen [see Materials and Methods]. In this approach, the

population screened is F1 seed that results from cros-

sing a mutant to a second line, where one parent has

been mutagenized. To obtain loss-of-function alleles

from a dominant, gain-of-function mutation, the mutant

parent is further mutagenized (Timpte et al. 1994); to

obtain additional alleles of a recessive, loss-of-function

mutation like hfr1-1, the wild-type parent is muta-

genized. The method we devised overcomes the primary

obstacle to directed screening, namely, the tedious na-

ture of standard techniques for the cross-pollination of

Arabidopsis. For our screen, ethylmethanesulfonate-mu-
tagenized, male-sterile plants were fertilized in bulk

with pollen from hfr1-1, and 12 F1 progeny with long

hypocotyls in FRc were selected for F2 analysis. Two

of the twelve mutants lacked wild-type segregants in

their progeny, indicating the presence of new hfr1 alle-
des, designated hfr1-2 and hfr1-3. One, hfr1-2, was iso-
lated in its homozygous state and grown for two gene-
rations with selection against deleterious mutations in

other loci.
hfr1 mutants are defective in a subset of seedling responses to FRc

The seedling phenotype of hfr1 mutants is shown in Figure 1. Seedlings of wild-type and mutant phyA and hfr1 seedlings all exhibit a normal etiolated phenotype when grown in complete darkness [Fig. 1A]. FRc suppresses hypocotyl elongation in both the wild-type and the hfr1 mutants, but this response is significantly impaired in the hfr1 mutants in moderate and strong FRc [Fig. 1B,C,E,F]. This contrasts with the complete blindness to FRc of the phyA mutant [Fig. 1B,C]. This effect is FRc specific, as the suppression of hypocotyl elongation in Rc is not altered in the hfr1 mutants [Fig. 1C,G,H]. A quantitative examination of hypocotyl elongation responses over a range of FRc and Rc fluence rates corroborates this FRc specificity and shows that hfr1-2 is slightly more impaired in this response than is hfr1-1 [Fig. 2A]. The reason for the slightly longer hypocotyls of wild-type and phyA mutants than of the hfr1 mutants in darkness has not been determined. However, this difference was not consistently observed in other experiments.

A second response to FRc is also strongly affected in the hfr1 mutants: the suppression of hypocotyl negative gravitropism [Poppe et al. 1996; Robson and Smith 1996; Hangarter 1997]. Seedling hypocotyls extend vertically, against gravity [negative gravitropism], when grown in darkness [Fig. 1A]. Moderate FRc greatly suppresses the hypocotyl negative gravitropism of the wild type but not of the hfr1 or phyA mutants [Fig. 1B]. This deficiency, like the reduced suppression of hypocotyl elongation, is FRc specific: the suppression of hypocotyl negative gravitropism by Rc is largely unaffected in hfr1 mutants.

Quantitation of the suppression of hypocotyl negative gravitropism over a range of FRc fluence rates reveals a stronger defect for hfr1-2 than for hfr1-1 [Fig. 2B], as was seen for the suppression of hypocotyl elongation. The hfr1 mutants, unlike phyA, do show some suppression of hypocotyl negative gravitropism in higher fluence rates of FRc. The FRc specificity of the gravitropic phenotype is less absolute, in that there is a somewhat reduced amplitude of Rc response in hfr1 mutants as well as phyA [Fig. 2B]. Clearly, the suppression of wild-type hypocotyl negative gravitropism by FRc does not have a monotonic relationship with FRc fluence rate [Fig. 2B], which may explain why the suppression of hypocotyl negative gravitropism by phyA has been considered exclusively a "very low fluence response" [Poppe et al. 1996; Robson and Smith 1996]. The data shown here implicate a phyA-mediated "high-irradiance response" in the suppression of hypocotyl negative gravitropism, which is diminished in hfr1 mutants.

We have examined other seedling responses to FRc that are absent in phyA null mutants and found them to be unaltered in hfr1-1 over a range of fluence rates similar to those used in Figure 2B [data not shown]. These include apical hook opening and cotyledon separation [Liscum and Hangarter 1993], anthocyanin production [Kunkel et al. 1996], and lack of greening in FRc-grown seedlings on transfer to white light [Barnes et al. 1996].

The loss of responsiveness to FRc observed in the hfr1 mutants could, in principle, result from a reduction in phyA protein level or spectral activity [the ability to interconvert between Pr and Pfr forms on absorption of light]. No difference in phyA protein levels between wild-type and hfr1-1 was observed in darkness, and a similar pattern of decline in levels for wild-type and mutant was observed in Rc and FRc [data not shown]. As the decline in protein level of phyA depends on its spectral activity, we can conclude that HFR1 does not act primarily through the regulation of phyA level or spectral activity.

Molecular cloning of the HFR1 locus

We were able to clone the HFR1 locus by virtue of an inserted T-DNA "tag" in hfr1-1. Though T-DNA-born kanamycin resistance did not cosegregate with the mutation, a set of T-DNA right-border [RB] insertions, detectable by Southern blotting, did cosegregate. The Arabidopsis genomic sequence flanking one of these RB insertions was cloned and found to be physically linked to the nearest genetic marker known to be linked to hfr1 (cer1; Fig. 3A). Complete cosegregation of a codominant marker for the T-DNA junction and hfr1 was observed, indicating a map distance between the T-DNA insertion and hfr1 of <0.07 cM.

We determined 6 kb of DNA sequence around the T-DNA insertion site [Fig. 3B]. This sequence is identical to that recently deposited by the Arabidopsis Genome Project for the BAC T6A9. A candidate HFR1 gene was obtained by analysis of transcribed regions near the T-DNA insertion of hfr1-1. The insertion point was not within a predicted gene, and a probe spanning the insertion point did not allow detection of a transcript in a Northern blot of wild-type RNA. Two transcribed sequences flanking the insertion point, each beginning about 1 kb away, were delineated by a search of the set of Arabidopsis ESTs in GenBank with the 6-kb genomic sequence [Fig. 3B]. The centromere-proximal transcript contains regions with strong homology to plant protochlorophyll oxidases but lacks an ORF of significant length and thus is unlikely to be the template for a functional enzyme. The centromere-distal transcript has a substantial ORF that includes homology to the bHLH family of DNA-binding proteins. Northern analysis indicated a greatly reduced level of this transcript in hfr1-1 relative to wild type [Fig. 4], making it an excellent candidate to be HFR1. We sequenced the corresponding genomic region in the two hfr1 alleles derived from the directed screen, hfr1-2 and hfr1-3. Both had point mutations in the transcribed region of the putative HFR1 gene, confirming its identity as HFR1. In addition, the 6-kb genomic region depicted in Figure 3B fully complements the hfr1-1 and hfr1-2 mutations when present as a transgene (C.D. Fairchild, M.S. Schumaker, and P.H. Quail, unpubl.).

The hfr1-2 allele was found to have two base changes
Figure 1. Visible defects in hfr1 seedling photomorphogenesis. Col-5 wild-type (wt) and mutant (phyA-211 [Reed et al. 1994], hfr1-1 and hfr1-2) seedlings were grown for 4 d in complete darkness or in various light conditions on vertically oriented agar surfaces. They were then photographed without rearrangement. Seedlings grown in (A) darkness, (B) moderate FRc, (C) strong FRc, (D) Rc. (E–H) Representative wt (E,G) and hfr1-2 (F,H) seedlings grown in strong FRc (E,F) or in Rc (G,H). In (E–H), the root/hypocotyl junction is roughly marked by the empty seed coats.
in the transcribed region of HFR1 (Fig. 3C). One, at residue 159, results in a nonsense codon that truncates the predicted HFR1 protein in the loop between helices of the bHLH domain. With this truncation, more than half of the predicted protein would be absent and the bHLH domain inactivated. Thus, based on molecular data, the hfr1-2 mutant would be predicted to have a more complete loss of HFR1 function than has hfr1-1, which produces a lower level of unaltered mRNA, and this prediction is in accord with the physiological data from the two mutants.

The only base change in the transcribed region of hfr1-3 is in the 5′ untranslated region, 6 nucleotides from the longest 5′ end of the HFR1 transcript as determined by RACE (Fig. 3C). Preliminary evidence from Northern blot analysis indicates that the size and abundance of the hfr1-3 mRNA is similar to that of wild type in dark-grown seedlings, suggesting that the hfr1-3 mutation
may reduce translation. We have not directly tested this possibility.

HFR1 encodes a bHLH protein with strong similarity to the phy-interactor PIF3

A search of GenBank with the HFR1 sequence revealed that the closest homologs are two Arabidopsis bHLH proteins, PIF3 [Ni et al. 1998] and a protein predicted from genomic sequence, AAD24380. The homology to both of these proteins is highest in the HLH region (>50% identity) but extends in both directions beyond the bHLH domains (Fig. 5A). There is no significant homology between HFR1 and these proteins beyond the region shown. HFR1 lacks the PAS domain of PIF3. A notable difference between them is in the basic region of the bHLH domains, where there appears to be a deletion in HFR1 relative to the others [discussed below]. A comparison of HFR1 and representative members of the broader HLH protein family (Fig. 5B) reveals extensive conservation in HFR1 of residues that define the HLH domain [Atchley et al. 1999].

The region amino-terminal of the HLH in HFR1 has a basic character, including residues 23–25 in the alignment [Fig. 5B] that are often basic in DNA-binding bHLH proteins but not in HLH proteins that do not bind DNA, such as ID1 [Atchley et al. 1999]. The more amino-terminal part of the basic domain corresponds to the apparent deletion relative to PIF3 and AAD24380. In Figure 5B, these proteins are aligned to ungapped HFR1 to show the lack of colinear similarity. This region of HFR1 is not similar to the corresponding region of most bHLH proteins but does show strong similarity to the basic region of the Achaete/Scute subfamily of animal bHLH proteins [Fig. 5C].

Two amino acid residues of HFR1 that are notably different from most, or perhaps all, bHLH proteins are indicated with arrows in Figure 5B,C. The indicated ar-
Figure 5. Sequence comparison of HFR1 to other bHLH proteins. Identical amino acid residues are shaded black; similar residues are shaded grey. (A) Alignment of HFR1 (amino acid residues 105–248) and its closest known homologs, the Arabidopsis protein PIF3 (GenBank accession no. AF100166) and predicted protein AAD24380, over the region of significant sequence similarity. Solid bars indicate prospective monopartite nuclear localization signals in HFR1. (B) Alignment of a more restricted region of HFR1 (amino acid residues 122–194) to a broader set of representative members of the bHLH protein family from various organisms. Arrows indicate key positions where HFR1 is dissimilar to most (perhaps all) known bHLH proteins. GenBank accession nos.: AhR, P30561; Sim, P05709; R-Lc, P13526; Achaete, P10083; Hairy, P14003; MyoD, CAA40000; Arnt, P14739; PHO4, P07270; ID1, P20067. (C) Similarity of the basic region of the HFR1 bHLH domain (amino acid residues 132–153) to the basic regions of the Achaete/Scute bHLH subfamily. In this alignment, only amino acid residues with similarity to HFR1 are shaded. GenBank accession nos.: L-sc, P09774; Scute, P10084; MASH2, P19360; MASH1, P19359; Asense, P09775.
Thus, there is 30-fold more seedlings relative to those grown in darkness (Fig. 4C). Seedlings grown in FRc, but a 14-fold decrease in Rc-grown shows more than twofold induction in wild-type seedlings and in HFR1 protein, compared to darkness (Fig. 4B). The mRNA analysis of seedlings grown in darkness, FRc, or Rc. The HFR1 mRNA levels were assayed by Northern blot analysis of the HD-Zip-encoding genes ATHB-2 and ATHB-4 have been shown to exhibit a similar induction by FR and suppression by R (Carabelli et al. 1993, 1996). The FRC induction of HFR1 is absent in the phyA mutant, suggesting that this induction is a result of phyA signaling. By contrast, the Rc suppression of HFR1 mRNA levels is like wild type in the phyA mutant, implicating a phytochrome other than phyA in this regulation.

**HFR1 is constitutively nuclear localized when transiently expressed in onion epidermal cells**

The predicted HFR1 protein contains two potential monopartite nuclear localization signals (Fig. 5A). These signals and the similarity of HFR1 to DNA-binding proteins suggest that HFR1 might function in the nucleus. To test the subcellular localization of HFR1, and the possibility that this localization might be light-regulated, we fused the coding region of HFR1 to the reporter β-glucuronidase (GUS) in a plant expression construct. This construct was transfected into peels of onion epidermis by particle bombardment. Whether the peels were then incubated in darkness or in FRC, the GUS-HFR1 protein was found predominantly in nuclei, in contrast to the cytoplasmic localization of the GUS control (Fig. 6).

**HFR1 expression is light regulated**

HFR1 mRNA levels were assayed by Northern blot analysis of seedlings grown in darkness, FRc, or Rc. The HFR1 mRNA is about 1.3 kb in size, as predicted from 5′-RACE and the poly-A ends of the cDNA clones, and is detectable in all light conditions (Fig. 4A). The mRNA shows more than twofold in wild-type seedlings grown in FRc, but a 14-fold decrease in Rc-grown seedlings relative to those grown in darkness (Fig. 4C). Thus, there is 30-fold more HFR1 mRNA in FRc than in Rc. Many genes in seedlings are either induced or repressed by both FRc and Rc compared to darkness (Terzaghi and Cashmore 1995), but to our knowledge, only the HD-Zip-encoding genes ATHB-2 and ATHB-4 have been shown to exhibit a similar induction by FR and suppression by R (Carabelli et al. 1993, 1996). The FRC induction of HFR1 is absent in the phyA mutant, suggesting that this induction is a result of phyA signaling. By contrast, the Rc suppression of HFR1 mRNA levels is like wild type in the phyA mutant, implicating a phytochrome other than phyA in this regulation.

**HFR1 lacks affinity for phyA or phyB**

A simple mechanism by which HFR1 might provide FRC specificity in its action is by binding preferentially to phyA itself. We explored the possibility of an HFR1/phyA interaction by two methods used previously to demonstrate interactions between PIF3 and phytochromes: the yeast two-hybrid assay and coimmunoprecipitation. PIF3 has been shown to bind the C-terminal halves of both phyA and phyB in two-hybrid assays [Ni et al. 1998]. These interactions are used here as positive controls (Fig. 7A). However, in the analogous experiment with HFR1, there is no indication of an interaction with either phytochrome fragment (Fig. 7A).

We also looked for evidence of phyA/HFR1 binding in vitro. PIF3 has also been shown to bind full-length phyB in vitro with a dramatic preference for the Pfr form [Ni et al. 1999]. Ni et al. used the GAL4 activation domain (GAD) as an epitope tag fused to PIF3 [GAD-PIF3] as “bait,” unfused GAD as a negative control bait, and phyB as prey. They expressed phyB in vitro and combined it with chromophore to make spectrally active holoprotein; bait proteins were produced in Escherichia coli. In the experiment described here, all proteins were expressed in vitro [Fig. 8A]. We were able to establish conditions that allow not only the preferential precipitation of phyB Pfr by GAD-PIF3 [Fig. 8B, lanes 15,16; Fig. 8C, right, GP] but also the preferential precipitation of phyA Pfr [Fig. 8B, lanes 7,8; Fig. 8C, left, GP]. Under these conditions, even with a greater amount of GAD-HFR1 bait than GAD-PIF3, there is no sign of phyA or phyB binding to GAD-HFR1 (Fig. 8B, lanes 3,4,11,12, Fig. 8C). Thus, both by two-hybrid and immunoprecipitation assays, we have no evidence for a direct interaction between HFR1 and phyA or phyB.
HFR1 can bind PIF3

In many cases, HLH proteins can form both homodimers and heterodimers with other HLH proteins. Frequently, the heterodimers involve related HLH domains. PIF3 is the closest known relative to HFR1 and appears to act in phytochrome signaling through a direct interaction with phytochrome. Thus, another possible mechanism by which HFR1 might act in phyA signaling is through interaction with PIF3.

By the yeast two-hybrid assay, positive signals for an interaction in yeast between HFR1, PIF3, phyA C-terminal half, or phyB C-terminal half, along with negative controls nuclear lamin and unfused GAL4 activation domain (GAD). GBD refers to the GAL4 DNA-binding domain. Asterisks mark assay results that are also shown in an expanded view in the inset. Bars represent the standard error of the mean. By the yeast two-hybrid assay, positive signals for an HFR1/PIF3 interaction were obtained in both arrangements of the chimeras (Fig. 7A). Positive signals were also obtained from the combinations PIF3/PIF3 and HFR1/HFR1. The HFR1 homodimerization signal is relatively weak but is significantly above the HFR1/phyA background (Fig. 7A, inset). The relative intensity of these signals may not have a simple relationship to the relative affinities of the proteins, as immunoblots with monoclonal antibodies to the GAL4 domains indicate a lower level of expression in yeast for the HFR1 chimeras than for the PIF3 chimeras. The positive results for HFR1 interaction with PIF3 verify the efficacy of the HFR1 two-hybrid chimeras and lend credence to the lack of interaction observed between HFR1 and phyA or phyB.

We were able to confirm the propensity of HFR1 and PIF3 to form a complex by coimmunoprecipitation of the two proteins coexpressed in vitro. GAD was again used as an epitope tag in bait constructs in fusion with PIF3 (GAD-PIF3) and HFR1 (GAD-HFR1). Prey HFR1 (HFR1) and PIF3 (PIF3) were expressed without the epitope tag. Bait proteins were immunoprecipitated using antibody to GAD immobilized on beads. Input lanes were loaded with half the fraction of each binding reaction that was loaded for washed precipitate lanes (Ppt.). (Lanes 1–3) precipitation of HFR1 by GAD control (1), GAD-HFR1 (2), and GAD-PIF3 (3). [Lanes 4,5] precipitation of PIF3 by GAD control (4) and GAD-HFR1 (5).

Like PIF3, an HFR1/PIF3 complex preferentially binds the Pfr form of phyA and phyB

As HFR1 can bind PIF3 and PIF3 can bind phyA and phyB, it was of interest to test for the ternary complexes HFR1-PIF3-[phyA or phyB]. In the same conditions under...
which we fail to see precipitation of phytochrome by GAD-HFR1 (Fig. 8B, lanes 3, 4, 11, 12; Fig. 8C, GH), the GAD-HFR1/PIF3 complex does preferentially precipitate phyA and phyB Pfr forms (Fig. 8B, lanes 5, 6, 13, 14; Fig. 8C, GH + P), with an efficiency similar to that of GAD-PIF3 alone (Fig. 8B, lanes 7, 8, 15, 16; Fig. 8C, GP). Almost all of the PIF3 in the GAD-HFR1/PIF3 bait is present by virtue of its association with HFR1 because the GAD-HFR1/PIF3 complex bait was prepared under conditions that remove most of the PIF3 from beads incubated with coexpressed GAD and PIF3 (see Fig. 8B, lanes 1, 2, 9, 10). The negative control bait, GAD with residual PIF3, has scant preferential affinity for the phytochrome Pfr forms (Fig. 8B, lanes 1, 2, 9, 10; Fig. 8C, G + P). Thus, like the PIF3 homodimer, the HFR1/PIF3 complex binds preferentially to the Pfr forms of phyA and phyB.

**Discussion**

Considerable progress has been made in recent years in efforts to define phytochrome signal transduction pathways [Wei and Deng 1999; Nagy and Schafer 2000; Neff et al. 2000]. Genetic and molecular approaches have identified a significant number of components that potentially function as signaling intermediates and have provided evidence of both shared and separate pathway branches for individual phytochrome family members (Soh et al. 1998; Bolle et al. 2000; Bueche et al. 2000; Neff et al. 2000; Osterlund et al. 2000). However, lacking until recently was evidence of a contiguous transduction pathway, consisting of identified molecular intermediates, that leads from phytochrome photoconversion to changes in gene expression. The recent discoveries that phytochromes translocate to the nucleus in response to light (Sakamoto and Nagatani 1996; Kircher et al. 1999; Yamaguchi et al. 1999) and that phyB can interact directly with the bHLH protein, PIF3, bound to a DNA target site [Martinez-Garcia et al. 2000] have suggested that one mode of phytochrome signal transduction is the direct transcriptional regulation of target genes. PIF3 was initially isolated as a phytochrome-interacting factor in a yeast two-hybrid screen. Here we have genetically identified a second member of the bHLH family, HFR1, in a screen for components specific to the phyA signaling pathway and have shown that HFR1 can heterodimerize with PIF3. These data support and extend the hypothesis that phytochromes can regulate target genes directly and open up the possibility that they may do so via multiple heterodimerizing members of the bHLH family of transcription factors, which might regulate gene expression in combinatorial fashion.

The observation that loss-of-function mutations in HFR1 result in a FR-specific phenotype indicates that HFR1 functions positively in the phyA signaling pathway. Furthermore, the normal, or even increased, level of phyA protein and its normal photoconversion activity in hfr1 mutants imply that HFR1 is an authentic signal transducer, rather than a protein involved in phyA synthesis or assembly. In these respects, hfr1 mutants are similar to other FR-specific, loss-of-response mutants that have been identified previously, far1 [Hudson et al. 1999], fhy1 and fhy3 [Whitelam et al. 1993], fin2 [Soh et al. 1998], and pat1 [Bolle et al. 2000]. However, the subset of developmental responses to FRc that are affected in hfr1 mutants differs from the subset affected in other phyA-signaling mutants. The
most consistent hfr1 phenotype is limited to a clear, partial loss of FRc-suppression of hypocotyl elongation and negative gravitropism. The hypocotyl elongation and gravitropism defects of hfr1-1 strictly cosegregate, and this correlation is maintained in the hfr1-2 homozygotes that lack the hfr1-1 T-DNA insertion, thereby indicating that both defects result from mutation of HFR1. Except for fin2, which appears to show a loss of FRc suppression of hypocotyl gravitropism similar to hfr1 {Soh et al. 1998}, the effects on hypocotyl gravitropism of mutations in other phyA-signaling loci have not been established.

In phyA-signaling mutants other than hfr1, a wider range of responses to FRc is affected, including the stimulation of anthocyanin production and the loss of greening on transfer to white light {Barnes et al. 1996; Soh et al. 1998; Hudson et al. 1999; Bolle et al. 2000}. These FRc responses are consistently unaffected in hfr1 mutants. The specificity of the hfr1 phenotype for particular FRc responses suggests that HFR1 may direct phyA signals primarily to the subset of phyA-regulated genes that drive these responses.

Although three other genes specific for phyA signaling have been molecularly cloned—SPA1 {Hoeccker et al. 1999}, FAR1 {Hudson et al. 1999} and PAT1 {Bolle et al. 2000}—HFR1 is the only one of these whose sequence offers an obvious prediction of its biochemical role. The sequence homology of HFR1 to members of the bHLH family of transcription factors, along with potential nuclear localization signals, suggests that it might be a transcription factor. The demonstrated constitutive nuclear localization of transiently expressed HFR1 in onion epidermal cells and the binding affinity of HFR1 for its closest homolog, PIF3, are consistent with this suggestion. PIF3 has similarly been shown to be constitutively nuclear {Ni et al. 1998}, and there is more substantial evidence that PIF3 may act as a transcription factor. PIF3 alone can bind DNA, with a strong preference for the G-box core motif and some specificity for one flanking base on either side of it {cCACGTGg}, and appears to be involved in the induction of the genes CCA1 and LHY within 1 h of a light signal {Martinez-Garcia et al. 2000}. In addition, the PIF3 two-hybrid chimera with the GAL4 DNA-binding domain has transcriptional activation activity in yeast {Ni et al. 1998}. However, we have not yet attempted to determine whether HFR1 binds directly to DNA, and in contrast to PIF3, the analogous HFR1 two-hybrid chimera exhibits no detectable intrinsic transcriptional activation activity in yeast.

Does the unusual basic region of HFR1 predict a lack of affinity for DNA or DNA binding with a sequence specificity distinct from that of other bHLH proteins? In all reported cases, other bHLH proteins that have unusual basic regions ultimately have been found to bind DNA with an altered sequence specificity {Littlewood and Evan 1998; Atchley et al. 1999}. The two key differences between HFR1 and a consensus basic region {Fig. 4B, arrows} are partly reflected in other bHLH proteins. The highly conserved glutamate residue [arginine at position 22 in Fig. 5B], which is integral to E-box {CACGTG} sequence recognition {Atchley et al. 1999}, is not re-
function, given that PIF3 binds both phyA and phyB. It is tempting to speculate that in place of, or in addition to PIF3, a phyA-specific binding protein might bridge HFR1 and phyA. Obvious candidates for such a phyA-specific binding protein are the apparently phyA-specific signal transducers that have been identified genetically, but no evidence has yet been presented for a phyA-specific interacting protein.

The 30-fold greater abundance of HFR1 mRNA in FRC relative to Rc that we have observed might be sufficient to explain the FRC specificity of HFR1 action, if this difference in mRNA level translates to a similar difference in HFR1 protein activity in the two light conditions. The role of HFR1 could be to confer a different DNA sequence specificity on the PIF3/phyA complex and, thus, to adjust the gene-regulatory output of phyA (Fig. 9). The apparent FRC-specificity of HFR1 activity might then be determined by a complex, reciprocal regulation of HFR1 abundance by Rc and FRC (Fig. 9).

Materials and methods

Isolation of mutants

As part of a comprehensive screen of available mutagenized populations for long-hypocotyl mutants in FRC, we used T2 seed from 2000 T-DNA transformed parents, which were a gift from Robert Fischer (University of California, Berkeley). The Fischer lines had been transformed with T-DNA from a vector that was derived from pBI121 (Jefferson et al. 1987) by deletion of the GUS gene. Seedlings were grown on FRC (2–3 µmole m−2 sec−1) for a total of 4–5 d before mutant selection.

In the directed screen for additional alleles of hfr1, F2 seed from a cross of Ler wild type to the male-sterile mutant ms1 [Ler background] was mutagenized in 0.3% or 0.45% [v/v] ethyl-methanesulfonate for 13 h and sown onto mesh-covered soil at a rate of 400 per 4-inch pot. All self-sterile plants (three-quarters of the population) were weeded out as they became evident by the presence of elongating siliques, leaving 10–40 male-sterile plants per pot and a mutagenized male-sterile population total (∼5000). Concurrently, several flats of hfr1-1 plants were grown as a source of pollen. We found that dragging the flowering, male-sterile plants through a dense stand of young, flowering hfr1-1 plants was an effective mass cross-pollination method. We found that dragging the flowering, male-sterile plants through a dense stand of young, flowering hfr1-1 plants was an effective mass cross-pollination method.

Several rounds of pollination were performed on each pot of male-sterile plants over the course of one week. The result was cross-pollination at a rate of several seeds per inflorescence branch. Seed was collected in pools of two pots each and screened directly for long-hypocotyl mutants in FRC. Leaf tissue samples were taken from the young rosettes of selected putative mutants for small-scale DNA preparation.

New, noncomplementing alleles of hfr1 were confirmed by testing F2 progeny in FRC for the presence of wild-type seedlings (for hfr1-2, >2000 F2 individuals; for hfr1-3, 200).

Genetic mapping and complementation tests

An F2 population resulting from a cross of hfr1-1 to Ler wildtype was used for mapping. DNA for PCR was prepared from leaf samples (Edwards et al. 1991). With the assessment of 16 PCR markers (CAPS and SSLP; Konieczny and Ausubel 1993; Bell and Ecker 1994) in a population of 17 hfr1-1 homozygotes, along with the visible markers er and gl1 in a larger population, hfr1 was mapped to the top of chromosome I. This map position excluded the possibility of allelism to some long-hypocotyl mutants. Others that were linked (phyA) or unmapped (fhy1 and fhy3) failed to complement hfr1-1 in the F1 and were confirmed as nonallelic by the segregation of wild types in the F2 generation.

The expanded mapping population (758 individuals) consisted largely of hfr1 homozygotes and a few homozygous wild type. As hfr1-1 exhibits partial dominance and a subtle phenotype, a larger population of potential hfr1 homozygotes was picked, and then heterozygotes for flanking markers cer1 and nF21B7 were retested as probable hfr1 heterozygotes. For F2 individuals with an apparent recombination between these markers, the hfr1/ hfr1 genotype was confirmed by a lack of wild-type segregants in the F3 population.

Seedling growth and measurements

For seedling growth, seeds were surface sterilized, sown on agar-solidified medium [lacking sucrose] in petri dishes, and germinated in darkness or under defined light conditions as previously described (Hudson et al. 1999). For mutant screening, seed was suspended in sterile 0.15% agar [aq.] and sown densely in horizontal rows. For hypocotyl length and gravitropism measurements, seeds in 0.15% agar were spotted one per 0.5 cm2 in a staggered grid pattern. For RNA and protein extractions, seed was sown on filter paper laid over agar-solidified medium.

For most purposes, seedlings were germinated and grown in vertically oriented petri dishes, such that both hypocotyls and radicals grew along the agar surface, with horizontal R or FR illumination. Seedlings for RNA and protein extractions were
grown in petri dishes in the normal, horizontal orientation with illumination from above. For measurement of hypocotyl length and angle-from-vertical, measurements (~40 per genotype at each fluence rate) were made from digital images of unarranged seedlings using the program NIH Image.

For recovery of seedlings germinated in FRC for further growth in white light, seedlings were aseptically transferred under dim green light (in some cases after taking digital images in green light) to growth medium containing 1% or 2% sucrose. These seedlings were then kept in darkness for 3 d, exposed to filtered room light for 3 h, and then transferred to full white light. After 1 wk, during which the seedlings partially greened and initiated normal leaves, they were transferred to soil.

Yeast two-hybrid binding assays

HFR1 yeast two-hybrid vectors were constructed from the plasmids pGAD424 and pGBT9. The other two-hybrid constructs, based on the same plasmids, were described previously [Ni et al. 1998]. The yeast strain Y187 was transformed with combinations of vectors, and LacZ activity was assayed with ONPG as a substrate according to the Clontech Yeast Protocols Handbook [Clontech]. The LacZ activities are the mean of six values from two independent cultures of each vector combination assayed in triplicate.

In vitro binding assays

Immunoprecipitations were performed as previously described [Ni et al. 1999] with the following variations. All proteins for immunoprecipitations were expressed from T7 promoters in the TnT in vitro transcription/translation system [Promega] in the presence of 35S-methionine. The GAD-HFR1 vector was constructed by replacement of PIF3 by HFR1 in the GAD-PIF3 vectors. The HFR1 prey construct consisted of the HFR1 coding region from pBluescript [Stratagene]. The PIF3 prey was inserted into the NcoI/BamHI sites of the vector pET21 [Novagen] with the addition of a six-His tag at its N terminus. PhyA was expressed from the new plasmid T7-A.BS, which consists of phyA in-frame with the insert from the cDNA clone EST 209K19T7. By 5’-RACE, the HFR1 transcript was found to extend beyond the 5’ end of the insert from the cDNA clone EST 209K19T7. For the determination of subcellular localization of a GUS-HFR1 chimeric protein, the HFR1 was fused to GUS in the pET3 in a pBluescript backbone.

Preclcled phyA or phyB in the Pr or Pfr form was mixed with washed bait beads. After 6 h at 4°C, the beads were collected and washed once with 1.4 mL Tris binding buffer containing 0.5% Tergitol NP-40.

Samples for SDS-PAGE were mixed with SDS sample buffer and either boiled or heated to 65°C for 5 min [paramagnetic beads]. Gels were fixed and then dried for autoradiography by phosphor screen [Phosphorlmager Storm 860, Molecular Dynamics]. For relative quantification of precipitated phytochrome, in-lane backgrounds of mock precipitation controls were subtracted and the amount precipitated expressed as a percent of the preclelced input phytochrome.

Subcellular localization

For the determination of subcellular localization of a GUS-HFR1 chimeric protein, the HFR1 was fused to GUS in the vector TEx2. TEx2, onion epidermal cell transient transfection, and staining were as described previously [Ni et al. 1998].

Molecular cloning of HFR1

Northern blot analysis of mRNA levels in seedlings was as described previously [Hoecker et al. 1999]. The HFR1 probe was the insert from the cDNA clone EST 209K19T7. By 5’-RACE, the HFR1 transcript was found to extend beyond the 5’-end indicated by this cDNA clone and to, thus, include the hfr1-3 mutation site.

For the determination of subcellular localization of a GUS-HFR1 chimeric protein, the HFR1 was fused to GUS in the pET3 in a pBluescript backbone. Holo-phytochrome was formed by a 1 : 4 dilution of TnT protein in vitro transcription/translation system [Promega] in the presence of 35S-methionine. The GAD-HFR1 vector was constructed by replacement of PIF3 by HFR1 in the GAD-PIF3 vectors. The HFR1 prey construct consisted of the HFR1 coding region from pBluescript [Stratagene]. The PIF3 prey was inserted into the Ncol/BamHI sites of the vector pET21 [Novagen] with the addition of a six-His tag at its N terminus. PhyA was expressed from the new plasmid T7-A.BS, which consists of phyA in-frame with the insert from the cDNA clone EST 209K19T7. By 5’-RACE, the HFR1 transcript was found to extend beyond the 5’ end of the insert from the cDNA clone EST 209K19T7.

For coimmunoprecipitations with phyA or phyB as prey, the PBS binding buffer [pH 7.2] contained 0.1% [w/v] Tergitol NP-40, 1 mM EDTA, 0.1% BSA, and Complete protease inhibitors. PIF3 and GAD or GAD-HFR1 were coexpressed. Mixtures of expressed proteins were precleared by incubation for 1 h at 4°C with Protein A-agarose in binding buffer, and the supernatants were added to pelleted anti-GAD/Protein A beads. After 4 h at 4°C, beads were pelleted and washed three times with 1 mL of binding buffer and once with 1 mL binding buffer without BSA or protease inhibitors.

Communoprecipitations with HFR1 as prey were performed similarly, with the exceptions that the binding buffer contained 50 mM Tris-HCl [pH 7.5 at 25°C] in place of PBS [Tris binding buffer], paramagnetic Protein A beads were used [DynaBeads Protein A, Dynal] in place of Protein A-agarose, and both final washes contained 0.5% Tergitol NP-40.

For communoprecipitations with phyA or phyB as prey, bait beads were prepared and mixed with separately expressed phytochrome. For bait beads, bait proteins expressed individually or in combinations were bound to anti-GAD/Protein A magnetic beads in PBS binding buffer. The beads were then washed three times with 1.4 mL PBS binding buffer with 0.5% Tergitol NP-40 and once with Tris binding buffer with 150 mM NaCl. Holo-phytochrome was formed by a 1 : 4 dilution of TnT solutio into Tris binding buffer containing 30 µM phycocyanobilin.

Holo-phytochrome was formed by a 1 : 4 dilution of TnT solutio into Tris binding buffer containing 30 µM phycocyanobilin.
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(HFR1, EST 209K19T7 [Arabidopsis Biological Resource Center]. The HFR1 coding region for various constructs was derived from EST 209K19T7.

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