Role of HFR1 in Shade Avoidance and Phytochrome A Signaling

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ABSTRACT

Phytochromes are the photoreceptors mainly responsible for the detection of red and far-red (FR) light and the following responses. HFR1 is a basic helix-loop-helix type putative transcription factor involved in Phytochrome A signaling pathway. First we look at the early phenotype of mutant seedlings lacking a functional *HFR1* gene and we show that auxin is involved in the increased hypocotyl phenotype of these seedlings. Northern blots and RT-PCRs showed that *ATHB-2*, a gene involved in shade avoidance is regulated by HFR1 under FR light. Microarray experiments were performed to find the genes that are early targets of regulation by HFR1.

INTRODUCTION

DETECTION OF LIGHT IN PLANTS

Organisms have to adapt to their environment in order to increase the survival chance of themselves and their progeny. This depends on getting information from the environment and responding to the environment. Plants do not posses the sensory organs the animals have. Therefore they have to rely on other mechanisms to detect environmental stimuli. The way plants detect the properties of light depends on the molecules called photoreceptors. Photoreceptors are classified into three groups: Cryptochromes (Cashmore et al. 1999), phototropins (Briggs et al. 2001), and phytochromes (Neff et al. 2000). Cryptochromes and phototropins are responsible for the detection of blue/ultraviolet light. Phytochromes are mainly responsible for the detection of red and far-red light.

PHYTOCHROMES

Phytochromes are chromoproteins; they are composed of two parts: the chromophore part is able to absorb specific wavelengths of light while the polypeptide part causes the downstream reactions (Butler et al. 1959, 1964, Quail 1997). Phytochromes exist as homodimers containing two polypeptides with a molecular weight around 125 kDa. The chromophore of the phytochromes is phytochromobilin. Phytochromobilin binds to a cysteine residue present in a conserved domain in the amino terminal of phytochromes. The amino terminal domain is necessary and sufficient for chromophore binding and normal spectral properties while the carboxy terminal domain mediates dimerization and is essential for signaling (Fankhauser 2000, Quail 1997). Light can cause isomerization between rings C and D of phytochromobilin giving phytochromes an ability to be present in two forms. The role of

phytochromes to act as photosensors depends on their ability to cycle in these two photoconvertible forms. Phytochromes are synthesized in their inactive Pr form (λmax: 665 nm). This form has the ability to be converted to the active Pfr form (λmax: 730 nm) upon exposure to red light. Pfr form can be converted to Pr form by far-red light. Absorption spectra of the two forms of phytochromes are shown in figure 1 (Smith 2000).

There are 5 phytochromes in Arabidopsis, phytochrome A (phyA) to phytochrome E (phyE) (Sharrock and Quail. 1989, Clack et al. 1994). PhyA is the most abundant phytochrome in the dark grown tissues, while its level may decrease 100 fold upon light exposure (Clough et al. 1999). PhyB is the most abundant phytochrome in light grown tissues, phyC to phyE are less abundant light-stable phytochromes (Clack et al. 1994, Hirschfield et al. 1998).

To better understand the functions of the phytochromes it is necessary to know a few stages of development in the Arabidopsis life cycle. When seeds are germinated in dark, they develop long hypocotyls, closed apical hook and light yellow colored appressed cotyledons. This phenomenon is called etiolation or skotomorphogenesis. Light-grown seedlings have shorter, thicker hypocotyls, no apical hook and open, green, expanded cotyledons. The growth of seedlings under light is called de-etiolation or photomorphogenesis.

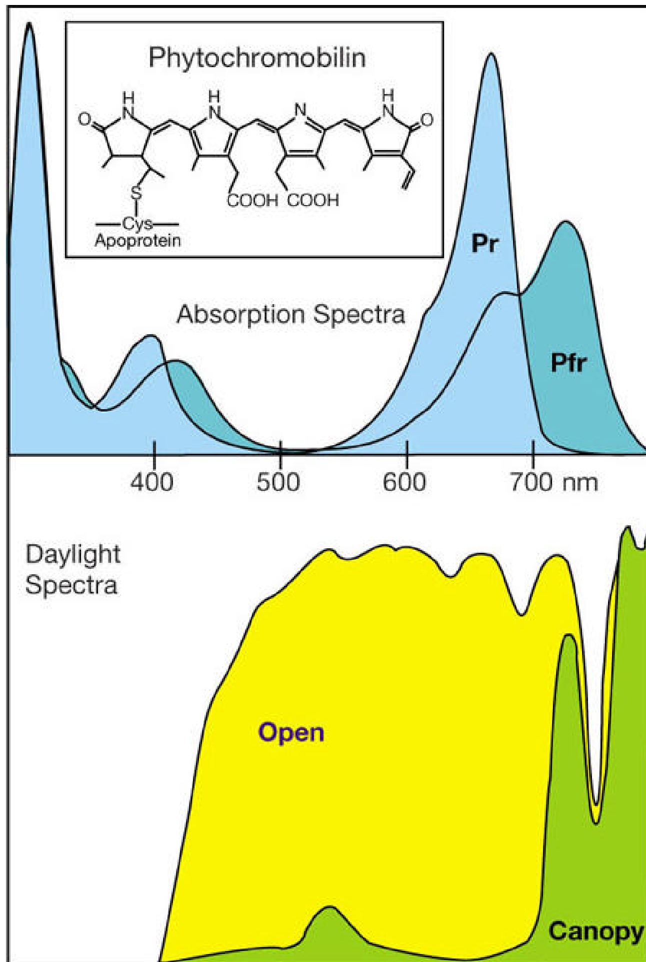


Figure 1: The absorption spectra of the two forms of phytochromes and the daylight spectra (Smith 2000).

There are three physiologically distinct modes of phytochrome action that have been found to date. These modes can be classified according to the amount of light required. In very-low-fluence responses (VLFR), plants respond to between 0.1 and 1 µmole.m^{-2} of light, low fluence responses (LFR) occur between 1 and 1000 µmole.m^{-2} of light. In high-irradiance responses (HIR), plants respond to > 1000 µmole.m^{-2} of light (Neff et al. 2000, Mancinelli 1994).

PhyA is the phytochrome that is mainly responsible for the detection of far-red light and de-etiolation by far-red light while phyB is mainly responsible for the detection of red light (Whitelam and Devlin 1997). PhyA is also involved in the very-low-fluence responses of blue and red light (Neff et al. 2000). Overexpression studies that were done indicate a role for phyC in primary leaf expansion (Halliday et al. 1997, Qin et al. 1997).

Originally it was thought that phytochromes are cytoplasmic molecules that effect the transcription of other genes through other molecules, but it was recently shown that although phyA and phyB are cytoplasmic in the dark, they are transported to the nucleus after exposure to light (Kircher et al. 1999; Yamaguchi et al. 1999). These studies were done using jellyfish green fluorescent protein (GFP) fused to phytochrome molecules, which were able to rescue the null mutants, showing that they were functional. An important observation in these studies was the formation of speckles in the nucleus upon the transportation of the GFP fused phytochromes to the nucleus (Kircher et al. 1999, Yamaguchi et al. 1999). Speckled structures were previously found in animal cells and they contained molecules involved in RNA transcription and processing, like snRNPs (Lamond and Earnshaw, 1998).

PHYTOCHROME SIGNALING

Among the efforts to solve the phytochrome-signaling network, phytochrome interacting molecules were found using yeast two-hybrid screens. Also mutagenesis studies enabled the finding of light signaling mutants, which were analyzed to find the components that are defective. The possible roles of these components were then investigated.

The components that were identified using the yeast two-hybrid screen were PKS1 (phytochrome kinase substrate) (Fankhauser et al. 1999), NDPK2 (nucleoside diphosphate

kinase 2) (Choi et al. 1999) and PIF3 (phytochrome interacting factor 3) (Ni et al. 1998). PKS1 was identified in attempts to find the proteins that interact with the carboxyl-terminus of phyA. In addition to its interaction with carboxyl-terminus of phyA PKS1 also interacts with the carboxyl-terminus of phyB. Phosphorylation of PKS1 increases under red light vs. dark but not under far-red light. This evidence together with the finding that Arabidopsis seedlings overexpressing PKS1 have a reduced sensitivity to red light suggests that PKS1 is a negative regulator of phyB signaling (Fankhauser et al. 1999).

NDPK2 was also identified in a screen for phyA carboxyl-terminus interacting proteins. NDPK2 interacts with both Pr and Pfr form of phyA but more with the Pfr form. The γ-phosphate-exchange activity of NDPK2 is greater when it interacts with the Pfr form of phyA than when it interacts with the Pr form. A loss-of-function allele of NDPK2 was shown to have reduction in cotyledon greening and apical hook opening under both red and far-red light. According to these results NDPK2 seems to be involved in phyA and phyB signaling as a positive regulator (Choi et al. 1999).

PIF3 was identified in a yeast two-hybrid screen where carboxyl-terminus of phyB was used as bait. PIF3 also interacts with the carboxyl-terminus of phyA. Analysis of PIF3 showed that it was a bHLH (basic helix-loop-helix) protein containing a bipartite nuclear localization signal (NLS), and has a region with some similarity to a PAS (Per-Arnt-Sim-like) domain. Overexpressing sense *PIF3* resulted in decreased responsiveness to continuous red and far-red light leading to longer hypocotyls and smaller cotyledons under red light and reduced cotyledon opening under both red and far-red light. The *pif3* mutant had the opposite phenotypes. The amount of anthocyanin increased while the amount of chlorophyll decreased in the sense *PIF3* overexpressers. The *pif3* mutant had lower chlorophyll content (Kim et al.,

2003). PIF3: GUS fusion protein was used to study the nuclear localization of PIF3 in onion epidermal cells and the fusion protein was localized to the nucleus (Ni et al. 1998). Further experiments showed that PIF3 was only bound to the Pfr form of phyB and this binding could be reversed converting phyB to the Pr form by far-red light. It was also shown that both carboxyl and amino-terminus of phyB were involved in PIF3 binding (Ni et al. 1999).

The experiments done to compare the binding of PIF3 to phyA and phyB showed that, as for phyB, PIF3 bound to the Pfr form of phyA but not the Pr form. PIF3 had more affinity for phyB than for phyA. It was shown that PIF3 bound to phyB stoichiometrically at an equimolar ratio (Zhu et al. 2000). The experiments that were done to find the domains of PIF3 and phyB that were required for binding showed that a 37-aa part at the amino-terminus of phyB was required for the high affinity binding of phyB to PIF3. This 37-aa part is not present in phyA, which may explain the higher affinity of phyB. For PIF3, the PAS domain was identified as the main domain involved in the interaction (Zhu et al. 2000).

Two classes of genes functioning downstream of phytochrome signaling have been found. Mutants of one class includes have altered photomorphogenetic responses under light conditions, like long hypocotyls. *Eid1*, *far1*, *fhy1*, *fhy3*, *fin2*, *fin219*, *hfr1*, *laf1*, *pat1*, *spa1* and *vlf* are such mutants that have altered phyA dependent responses (Whitelam et al., 1993; Soh et al., 1998; Hoecker et al., 1999; Hudson et al., 1999; Bolle et al., 2000; Büche et al., 2000; Fairchild et al., 2000; Fankhauser and Chory, 2000; Hsieh et al., 2000). The *hy5* mutant has long hypocotyl under blue, R and FR light, which suggests that HY5, a bZIP transcription factor, acts as a positive regulator downstream of the photoreceptors phyA, phyB, and cry1 (Koorneef et al., 1980; Oyama et al., 1997).

Mutants of the other class have photomorphogenic development in the absence of light, like shorter hypocotyls and expanded cotyledons. These mutants are collectively named *cop/det/fus* mutants (Chory et al., 1989; Deng et al., 1991; Kwok et al., 1996). COP1 is a RING-finger protein that has WD40 repeats and its nuclear localization is negatively regulated by light (Deng et al., 1992; von Arnim and Deng, 1994). It was shown that COP1 targets HY5 (a positive regulator) to proteasome-mediated destabilization, which suggests a mechanism for COP1 for repressing photomorphogenesis (Ang et al., 1998; Hrdtke et al., 2000; Osterlund et al., 2000).

SHADE AVOIDANCE

When plants grow in close proximity, they appear to attempt to outgrow one another in competition for light. Plants sense neighbors by analyzing the red to far-red light ratio. Under normal light conditions this ratio is around 1.2, while under canopy, or even with nearby green plants, it decreases (Smith 2000). When a plant senses a low red to far-red light ratio the first strategy is to grow longer than the other plants. If this strategy fails, the plant flowers early and produces seeds early to increase the chance of survival of the progeny (Smith, 2000, Smith and Whitelam, 1997). This is called shade avoidance. PhyB, phyD and phyE are responsible for the shade avoidance syndrome in Arabidopsis (Aukerman et al. 1997, Devlin et al. 1998, 1999).

ATHB-2 and *ATHB-4* were the first genes that were found to be specifically and reversibly regulated by R: FR ratio in green plants (Carabelli et al. 1993 and 1996); *ATHB-2* has been more extensively studied than *ATHB-4*. *ATHB-2* is present at high levels in etiolated seedlings and upon exposure of the seedlings to R or FR light the levels decline. When young seedlings and mature plants are grown under light with high R: FR ratio *ATHB-2* levels are low

but they increase rapidly when the ratio decreases (higher FR in the light). The process is reversible, *ATHB-2* levels decrease rapidly when R: FR ratio increases (Carabelli et al. 1993).

It has been shown that when wild type seedlings grown under low R:FR ratio are treated with auxin polar transport inhibitor naphthylphthalamic acid (NPA) they grow like wild type seedlings grown under high R:FR ratio without NPA. When *ATHB-2* overexpressing seedlings were grown under high R:FR ratio they behaved very much like wild type seedlings grown under low R:FR ratio, with or without NPA (Steindler et al. 1999).These results indicate an involvement of auxin and *ATHB-2* in shade avoidance.

HFR1

This work involves a gene that is involved in phyA signaling network. Fairchild et al. performed screening experiments for mutants using a continuous far-red light (FRc) fluence rate below saturation for the de-etiolation, to find the mutants less responsive to FRc. The idea of using a FRc fluence rate below saturation was to identify the genes whose loss-of-function mutants give weak phenotypes that are unidentifiable under saturating FRc. Among the mutants that had a FRc specific long hypocotyl phenotype, one had an incomplete linkage to phyA and did not correspond to any of the other mutants that had a FRc specific long hypocotyl phenotype. This mutant was named *hfr1* (long hypocotyl in far-red). Wild type and mutant *phyA* and hfr1 seedlings have a normal etiolated phenotype when grown in dark. FRc suppresses hypocotyl elongation in wild type and *hfr1* mutants, but *hfr1* mutants have longer hypocotyls in moderate or strong FRc. The decreased response of *hfr1* mutants is FRc specific since hypocotyl lengths of *hfr1* mutants are quite similar to wild type ones in Rc (Fairchild et al., 2000).

HFR1 was identified at the same time by two other groups and was given the names *REP1*(reduced phytochrome signaling) and *RSF1*(reduced sensitivity to FR light) (Soh et al., 2000; Fankhauser and Chory, 2000).

Another response to FRc that is affected in *hfr1* mutants is the suppression of hypocotyl negative gravitropism. When seedlings are grown in darkness, their hypocotyls extend vertically against gravity. Although this response is highly suppressed in wild type seedlings in moderate FRc, *hfr1* mutants, like *phyA* mutants have hypocotyls showing negative gravitropism under moderate FRc. This deficiency is FRc specific; Rc causes suppression of hypocotyl negative gravitropism in *hfr1* mutants (Fairchild et al., 2000).

After the cloning of *HFR1* locus, the alleles *hfr1-2* and *hfr1-3* were sequenced and it was found that both of them had point mutations in the transcribed region of *HFR1*. The *hfr1-2* allele had two base changes in the transcribed region of *HFR1*. One, at residue 159, results in a nonsense codon that causes the truncation of the predicted *HFR1* protein in the loop between the helices of the bHLH domain. This truncation causes the size of the protein to become less than half of the normal one and inactivation of the bHLH domain. The only base change in the transcribed region of *hfr1-3* is in the 5' untranslated region. Northern blots have shown that size and amount of *hfr1-3* mRNA is similar to that of wild type in dark-grown seedlings. This result suggests that *hfr1-3* mutation may be causing a decrease in HFR1 protein levels and thereby leading to its phenotype (Fairchild et al., 2000).

The search of GenBank for proteins similar to HFR1 showed that the two closest homologs were two *Arabidopsis* bHLH proteins, PIF3 (Ni et al., 1998) and AAD2380, a protein predicted from genomic sequence. The HLH region of these proteins has the highest homology. HFR1 does not have the PAS domain of PIF3. Amino-terminal part of the HLH in

HFR1 has a basic character. This part includes some residues that are often basic in DNA binding bHLH proteins but not in the ones that do not bind DNA (Fairchild et al., 2000).

Following the finding that the predicted HFR1 protein had two potential monopartite nuclear localization signals and its similarity to DNA-binding proteins, it was hypothesized that HFR1 might be functioning in the nucleus. To test this possibility the coding region of HFR1 was fused to the reporter ß-glucuronidase (GUS) in a plant expression construct and particle bombardment was used to deliver the construct into peels of onion epidermis. While the GUS control localized to the cytoplasm, GUS-HFR1 was mostly present in nuclei, whether the peels were kept in darkness or in FRc (Fairchild et al., 2000).

Fairchild et al. performed northern blots to find the *HFR1* mRNA levels in seedlings grown in darkness, FRc, or Rc for 3 days. These experiments showed that *HFR1* mRNA level was two times more in wild type seedlings grown in FRc and 14 times less in Rc grown seedlings when compared to those grown in darkness. This means that there is approximately 30 times more *HFR1* mRNA in seedlings when grown in FRc than in Rc (Fairchild et al., 2000). *ATHB-2* and *ATHB-4* are the first genes that were shown to be regulated in a similar fashion (Carabelli et al. 1993, 1996).

INTERACTION OF HFR1 WITH OTHER PROTEINS

The possibility of binding of HFR1 to phyA, which could explain the FRc specific action of HFR1, was tested using yeast two-hybrid assay and coimmunoprecipitation assays. No direct interaction between HFR1 and phyA or phyB was found after these assays. Since HLH proteins can form homodimers and heterodimers with other HLH proteins, and PIF3 is the closest homolog of HFR1 and acts in phytochrome signaling by binding to phyA, it was

thought that HFR1 can interact with PIF3 and in that way act in phyA signaling. Yeast two-hybrid assays done to check for this possibility showed an interaction between HFR1 and PIF3. This result led to another experiment to test for the binding of an HFR1/PIF3 complex to phyA or phyB. Coimmunoprecipitation experiments showed that like the PIF3 homodimer, the HFR1/PIF3 complex binds preferentially to the Pfr forms of phyA and phyB (Fairchild et al., 2000).

To investigate the role of HFR1 in light signaling Kim et al. made double and triple mutants of *HFR1*, *HY5* and *COP1* using the mutants *hfr1-201*(previously rep1-1), *hy5-1* and *cop1-6*. Using these mutants they were able to show additive interactions between *hfr1-201* and *hy5-1* in the inhibition of hypocotyl elongation under FR light. This supports the hypothesis of HFR1 and HY5 functioning in different branches of phyA signaling. The analysis of the *cop1-6hfr1-201* double mutant showed that HFR1 is partially required for the shortened hypocotyl and expanded cotyledon phenotypes of *cop1-6* in darkness and FR light. It was also shown using the same double mutant that HFR1 is required for randomized hypocotyl growth of *cop1-6* mutants in darkness. *HFR1* transcript levels were found to be increased in the *cop1-6* mutant, compared to wild type in seedlings grown in the dark. These results suggest that HFR1 acts downstream of COP1 to mediate photomorphogenic development in darkness and that HFR1 is negatively regulated by COP1 (Kim et al., 2002).

HFR1 IN CRYPTOCHROME SIGNALLING

It was shown that *hfr1-101*(previously *rsf1*) mutants had a reduced sensitivity to blue light in addition to FR light (Fankhauser and Chory, 2000). When the other *hfr1* mutants (*hfr1-2*, *hfr1-201*) were investigated, a similar reduction in sensitivity to blue light was observed.

Analysis of *phyAhfr1-101* double mutant showed that HFR1 had some phyA independent functions under blue light but HFR1 and phyA acted in together in some pathways under certain fluence rates of blue light. Analysis of other double mutants of *hfr1* suggested that HFR1 acted mainly in the cry1 pathway (Duek and Fankhauser, 2003).

In this work we use the *hfr1-2* mutant that was obtained by ethylmethanesulfonate (EMS) mutagenesis (This mutant was three times backcrossed to eliminate other mutations).

We have done northern blots, RT-PCRs and microarray experiments using *hfr1-2* mutants and the corresponding wild type plants to search for early HFR1-regulated genes. Also, we made unsuccessful attempts to detect endogenous HFR1 protein using anti-HFR1 polyclonal antibodies.

MATERIALS AND METHODS

PLANT MATERIAL

SEEDLING GROWTH

Seeds were surface sterilized for 10 min in 1% commercial bleach with 0.1% SDS, rinsed at least five times with sterile water, and sown onto Murashige and Skoog (MS) (Murashige and Skoog, 1962) plates with 0.9% agar. 1% sucrose was included in the medium for NPA (Chem Service, West Chester, PA) experiments. NPA stock solution (10mM) was made with 100% ethanol and added to the tempered autoclaved medium to get the appropriate concentrations. Ethanol was added to the negative control plates. Plates were wrapped with aluminum foil to prevent the exposure of seeds to light. Plates were kept at 4^oC for 4 days, transferred to white light for 6 hr at 23^oC, transferred to dark for 1 day for hypocotyl length measurement and NPA experiments or 2 days for RNA isolation experiments, and transferred to FR light (3.4 $\mu mol.m^{-2}s^{-1}$). If the seeds did not germinate well after 1 day in the dark, another protocol was applied for those. In this protocol after the seeds were placed on the trays the seeds were exposed to 15 min of high FR light and then transferred to 4^oC. The plates were transferred to dark for two days after 4^oC and then to white light for 6 hr. The rest of the protocol is the same.

GROWING PLANTS ON SOIL

To grow the plants on soil first the trays with wet soil were prepared. The trays (25.5cm wide, 52cm long) can accommodate 8 square pots (12.5cm wide, 12.5cm long and 6cm height). Pots were placed in the trays. Soil (Premier Pro-Mix BX) and water were mixed in a 4 1

container until soil was completely wet. The wet soil was placed in each pot and pressed to make it tight. The seeds to be sown were placed in a microfuge tube and water was added into tubes. Using a Pasteur pipette 2-3 seeds or more were placed on specific spots of 5 or 9 on each pot. After all the seeds were sown the tray was covered with plastic wrap and put in the cold room (4^oC) for four days. After that they were transferred to the growth chamber. The growth chamber was adjusted to 23^oC for a 18 hours day period and to 21^oC for a 6 hours night period. The plants were watered once in a week. Fertilizer (Miracle-Gro®) was included in the water after two weeks of growth in the growth chamber at a concentration of 0.35g/l. Watering was stopped when most of the plants were dry. After the plants were completely dry, seeds were collected from them. The seeds were put in microfuge tubes and the tubes were left in the dessicator for two weeks to further dry the seeds.

PHYSIOLOGICAL ANALYSIS

For hypocotyl length measurement experiments seeds were sown in a grid pattern, 1 or 2 seeds per spot, 40 seeds for wild type and *hfr1-2* on each plate. Plates were grown vertically; photographs were taken under FR light using the night mode of a digital camera and the seedling hypocotyl lengths were measured using the Image J 1.27 program (NIH). For NPA experiments seeds were sown the same way, plates were kept under FR light ($1\ \mu mol.m^{-2}.s^{-1}$) for 10 days and photographs were taken under white light.

DNA EXTRACTION FROM A.thaliana LEAVES

DNA extraction from the *A.thaliana* leaves was done as described in Edwards et al. A round piece of leaf was collected from each plant by capping the lid of a sterile microfuge tube.

The collected tissues were grind using blue pelet pestle from Nalge Nunc in the tube until no ungrinded tissue was visible. 400μl extraction buffer (200mM Tris HCl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5 % SDS) was added onto the grinded samples and they were vortexed for 5 minutes. Samples in the buffer were centrifuged in a microfuge at 13000 rpm for 1 minute and 300 μl of the supernatant was transferred to a new microfuge tube. 300 μl isopropanol was mixed with each supernatant and the samples were left at room temperature for 2 minutes. Samples were centrifuged at 13000 rpm for 5 minutes and the supernatant was emptied into a waste bottle. The microfuge tubes were inverted on a tissue paper to dry the pellets. After no liquid was visible to the eye, the pellets were dissolved in 100 μl 1X TE.

POLYMERASE CHAIN REACTION (PCR)

For PCRs the user manuals for the DNA polymerases were followed. The polymerase buffer (usually 10X) was diluted to give a final concentration of 1X. Primers (10μM) were diluted to give a concentration of 0.4μM. dNTP mix (10mM) was diluted to give a concentration of 0.4mM. 1.25 unit of DNA polymerase was included to each reaction. A master mix that contained water, the polymerase buffer, the primers, dNTP mix and the polymerase for all the PCRs was prepared in a single tube and aliquoted into the tubes where the reactions were going to be performed. To amplify the certain fragments from the DNA isolates from the leaves, 2.5μl out of 100μl DNA solution was used for each 50μl reaction volume. No DNA was put into the negative control reaction. The PCR machine was programmed according to the user manuals of the polymerases and the melting temperatures (T_m) of the primers. Primers CO10 and CO20 were used for *hfr1-2* and CO12 and CO14 were used for *hfr1-3* PCRs.

Name of Primer	Sequence	T_M (^oC)
CO10	5'-ATCGATGATCTGATCTCTCTGAAAAATC-3'	63.9
CO12	5'-TGATCTTTCACAGCAAGTGATCCGA-3'	67.2
CO14	5'-TGTTCATGTGTCAAAGTGGTAGTGAATC-3'	64.2
CO20	5'-CGGACAAAGCTGAAATCCCTAAAGA-3'	65.6
eIF2a-F	5'-ATTGCTTGCTGAGCACATG-3'	62.7
eIF2a-R	5'-CTACTAACCATGTTTGGGGTCA-3'	62.4
ATHB-2-L	5'-GCCCCCAGCTAGTCACATAC-3'	61.4
ATHB-2-R	5'-TTGAGCCTTGTGGATCTGTG-3'	57.3

Table 1: Primers used for PCRs.

RESTRICTION ENZYME DIGESTION

To cut the DNA fragments with a restriction enzyme a 2X concentrated master mix of enzyme was prepared. The master mix contained 2X enzyme buffer (usually 1:5 diluted 10X buffer), 0.2µg/µl acetylated BSA and the necessary amount of the restriction enzyme as written in the product sheet supplied with the enzyme. For each DNA fragment to be digested 10µl of master mix was prepared and mixed with 10µl of the solution containing the DNA fragment. The reaction mix was incubated at the specific temperature for the specific time in a water bath. At the end of the reaction the enzyme was heat inactivated, if necessary. For the cases when the digestion was incomplete the amount of the restriction enzyme was increased and/or the length of the incubation was increased.

RNA METHODS

RNA ISOLATIONS AND NORTHERN BLOTS

For RNA isolation, seeds were sown on sterile Whatman filter paper placed on the medium in the plates. After seedlings were collected from the plates they were placed on a standard testing sieve (# 30) and the ungerminated seeds were washed away with sterile water. Seedling samples were dried, weighed and ~ 1 g from each sample was wrapped with aluminum foil and frozen in liquid nitrogen and kept at -80°C. These steps were done in dark with a green LED light. RNeasy Midiprep kit (Qiagen) was used for RNA isolation. 1:9 volume of Plant RNA Isolation Aid (Ambion) was included in the extraction buffer. Tissue Tearor (Biospec Products, Inc.) was used for homogenization. After homogenization samples were filtered through Miracloth (Calbiochem, La Jolla, CA) placed in a 50 ml polypropylene conical tube, by centrifuging at 3000 g for 4 min. The supernatant was put in 50 ml Oak Ridge tubes and centrifuged at 20000 g for 10 min. The rest of the protocol was done as described in the protocol supplied with the RNeasy Midiprep Kit. Northern blots were done as described in Molecular Cloning (2001). 20 µg total RNA from each sample was used. 522 bp long 5' end of Athb-2 cDNA (between 2nd and 523rd base pairs) was randomly labeled with a-^{32}P-dATP using Strip-EZ DNA Kit (Ambion) and used as the probe.

RT-PCRs

For RT-PCRs Superscript III (Invitrogen) was used and the supplied protocol was followed, volumes of RTs and PCRs were 25 µl, 5 µg total RNA, 1 µg oligo dT primer and 70 units of Superscript III was included in the RT together with 0.05 µl a-^{32}P dATP (0.25 mCi, 3000 Ci/mmol) per reaction. RTs were done at 50°C for 3 hr and 5 µl of samples were run on a

4% denaturing polyacrylamide gel for 2 hr at 80 volts as described [18], gel was dried and exposed to imaging plate for 15 hr and scanned with a phosphoimager (Fujix Bas 1000). The program MacBas v1.01 was used to analyze and quantify the bands on the gels. cDNA amounts were normalized for all samples and control PCR was done using primers for eIF2a with 0.5 µl a-^{32}P dATP and 1.25 units of Hotmaster Taq DNA polymerase (Eppendorf) per reaction for 25 cycles. PCR samples were run on the same type of gel and exposure was done in the same way. cDNA amounts were normalized again using the values for the bands in the control PCR gel. PCR was done using primers for Athb-2 with 0.5 µl a-^{32}P dATP per reaction for 25 cycles. Gel running and exposure were the same as before. Primers used for eIf2a were eIf2a-F and eIf2a-R. Primers used for ATHB-2 were ATHB-2-L and ATHB-2-R.

DETECTING HFR1

IGY ISOLATION FROM EGG YOLKS

Isolation of IgY from egg yolks was done using 5 protocols. For the first protocol a funnel was placed over a 50 ml conical screw cap polypropylene tube. Eggs were washed with warm water and the yolk was separated from the white by keeping the yolk in the shell while getting rid of the white. The yolk was put in the funnel and passed into the tube. The volume of the yolk was written down. An equal volume of dilution buffer (10mM $NaPO_4$, 100mM NaCl, pH 7.5) was added in the tube and the tube was vortexed. The mixture was centrifuged at 3000g for 30 minutes at room temperature and the supernatant containing the IgY was recovered.

The second and the third protocols were done as described in Polson (1990) with some modifications. For the second protocol the steps until recovering the first supernatant are the

same as protocol 1. The supernatant was then mixed with an equal volume of chloroform and centrifuged at 10000g for 30 minutes at room temperature. The upper phase was transferred to another tube and solid PEG (Polyethylene Glycol) 8000 was added to reach a 12% (w/v) concentration. The mixture was vortexed, left at 4^oC for 30 minutes and centrifuged at 14000g for 10 minutes. The supernatant was removed and the pellet was washed with water and resuspended in 0.5 volume of dilution buffer.

For the third protocol supernatant was prepared as in protocol 1 and PEG 8000 was added to reach a 3.5% (w/v) concentration. The mixture was vortexed, left at 4^oC for 30 minutes and centrifuged at 14000g for 10 minutes. The middle clear supernatant was recovered and PEG 8000 was added to reach a 12% (w/v) concentration. The mixture was vortexed, left at 4^oC for 30 minutes and centrifuged at 14000g for 10 minutes. The supernatant was removed and the pellet was washed with water and resuspended in 0.5 volume of dilution buffer.

The fourth protocol was done according to Polson et al. (1980), modified by Sturmer et al. (1992). The isolated egg yolk was mixed with 2 volumes of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and PEG 8000 was added to reach a 3.5% (w/v) concentration. The mixture was vortexed, left at 4^oC for 30 minutes and centrifuged at 14000g for 10 minutes. All further centrifugations were done in the same conditions. The supernatant was filtered with a gauze plug and PEG 8000 was added to reach a 12% (w/v) concentration. After the samples were centrifuged at the pellet was dissolved in ½ volume of yolk and centrifuged again. 2 M ammonium sulfate was added to the recovered supernatant (1:2 v/v) and the solution was stirred at 4^oC for 30 min and centrifuged. The pellet was dissolved in sterile water and dialyzed against PBS with 0.01% sodium azide and stored at -80^oC.

The fifth protocol was done according to Sun et al. (2001). The isolated egg yolk was diluted with four volumes of sterile water and kept at 4°C for 6 hr and centrifuged at 3500 g for 25 min. Sodium sulfate was added to the recovered supernatant to reach a 19% concentration and the samples were centrifuged at 3500 g for 10 min and the precipitate was dissolved in sterile water. Sodium sulfate was added to reach a 14% concentration and after another centrifugation at 3500 g for 10 min the precipitate was dissolved in sterile water.

After the concentration of proteins in the IgY samples were calculated, equal amounts from each sample (highest volume from the lowest concentrated sample being 22.5 µl) was mixed with an equal volume of 2X SDS loading buffer (50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% bromophenol, 10% (v/v) glycerol). The rest of the protocol is done as described in Molecular Cloning. Immunoblotting was done as described in Molecular Cloning, using Protran nitrocellulose membrane (Schleicher & Schuell) the transfer apparatus from Idea Scientific. 5% nonfat dry milk was used in the blocking buffer. Goat anti-chicken IgG coupled to horseradish peroxidase was used to detect IgY antibodies on the membrane. PBS was used to wash the membrane after incubating the membrane with the buffer containing the antibody. Detection of secondary antibodies was done using 1-Step TMB-Blotting reagent (Pierce).

For dot-blot IgY samples were diluted 1/10 and 1/100 and equal amounts in 10 µl volume were spotted on Protran nitrocellulose membrane (Schleicher & Schuell). The blocking of the membrane the detection was done as previously described.

BRADFORD ASSAY

Bradford Assay was used for protein concentration determination. Bradford's Reagent (Bradford, 1976) was made by dissolving 100mg of Coomassie Brilliant Blue G-250 in 50ml 95% ethanol, adding 100ml 85% Phosphoric acid and adjusting the volume to 1 liter with distilled water. The final concentrations of constituents are: 0.01% w/v Coomassie Brilliant Blue G-250, 4.7% ethanol, and 8.5% Phosphoric acid. The solution was filtered through Whatman paper after a day passed. 0.5mg/ml BSA stock was made. The BSA stock was diluted 1:5, 1:6.25, 1:8.33 (100/12), 1:10, 1:12.5, 1:20 fold with water to a final volume of 100µl. Protein samples were diluted 1:5 or 1:10 as default with water to a final volume of 100µl. 1ml Bradford's Reagent was added to all of the standard solutions and the samples. After 5 minutes the absorption of all the solutions at 595nm were measured using the spectrophotometer. Using the absorptions of the standard samples a plot of amount of protein vs. the absorption was made using the Excel® program. The best fitting line passing through 0 was drawn and the slope was calculated. Using the slope, the amount of protein in the samples and the concentrations of the samples were calculated.

TOTAL PROTEIN ISOLATION FROM SEEDLINGS

For total protein isolation from the seedlings, the seedlings were scraped from the plate and dried on paper towels. The mass of the seedlings was determined. The seedlings were enclosed with aluminum foil and frozen in liquid nitrogen until the next step. All these steps were done in the dark using green LED light. Next steps were performed in normal laboratory conditions. The frozen seedlings were placed in a mortar and ground for 20-30 seconds with the pestle. Liquid nitrogen was added into the mortar and the grinding step was repeated two

more times. After a fine powder was isolated after grinding, liquid nitrogen was added into the mortar and the powder was transferred with liquid nitrogen to a polypropylene conical tube with an appropriate volume. After liquid nitrogen evaporated ten volumes of ice-cold extraction buffer (8M Urea, 20mM MOPS (3-(N-Morpholino) Propane Sulfonic Acid), 20mM 2-Mercaptoethanol, 2mM EDTA (Ethylenediaminetetraacetic acid), 2mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), and 1% SDS (Sodium dodecyl sulfate)) was added into the tube and the powder was resuspended. The solution was aliquoted, frozen in liquid nitrogen and kept at –20°C.

NUCLEAR PROTEIN ISOLATION FROM SEEDLINGS

We thought that using nuclear protein extracts could help us detect HFR1 easier since HFR1 is localized in the nucleus. For nuclear protein extraction the protocol from Busk and Pagès (1997) was used with some adjustments. The beginning of the protocol is the same as the one done for total protein isolation until the transfer of the powdered seedlings with liquid nitrogen to the 50 ml polypropylene conical tube. After liquid nitrogen evaporated ten volumes of ice-cold extraction buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 10 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 2 mM AEBSF, 1:300 diluted plant protease inhibitor complex (Sigma), 250 mM sucrose, 0.5% Triton X-100) was added into the tube. The sample was vortexed and filtered through Miracloth placed in a 50 ml polypropylene conical tube, by centrifuging at 3000 g for 15 min. The pellet was resuspended in four volumes of extraction buffer and centrifuged at 2000 g for 10 min. The pellet was resuspended in 0.1 volume of low salt buffer (20 mM HEPES (pH 7.8), 20 mM KCl, 0.2 mM EDTA, 2 mM AEBSF, 1:300 diluted plant protease inhibitor complex, 1.5 mM $MgCl_2$, 0.5 mM DTT, 25% glycerol) and stored at -80°C.

DETECTING HFR1

After the concentration of proteins in the nuclear protein extracts were calculated, equal amounts from each sample (highest volume from the lowest concentrated sample being 22.5 µl) was mixed with an equal volume of 2X SDS loading buffer (50 mM Tris-Cl (ph 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% bromophenol, 10% (v/v) glycerol). The rest of the protocol is done as described in Molecular Cloning. Immunoblotting was done as described in Molecular Cloning, using Protran nitrocellulose membrane (Schleicher & Schuell) the transfer apparatus from Idea Scientific. 5% nonfat dry milk was used in the blocking buffer. The primary antibody that was used was either rabbit affinity purified anti-HFR1 (Kasia Galecka, unpublished) or chicken anti-HFR1. The secondary antibody was either goat anti-rabbit IgG (Sigma) or goat anti-chicken IgG, both coupled with horseradish peroxidase. PBS was used to wash the membrane after incubating the membrane with the buffer containing the antibody. Detection of secondary antibodies was done using SuperSignal West Substrates (Pierce). The exposure time of the membranes was adjusted according to the signal received after a short exposure (15 sec or 1 min).

MICROARRAY EXPERIMENTS

POST-PROCESSING OF MICROARRAY SLIDES

cDNA microarray slides were supplied from Keck Foundation Biotechnology Resource Laboratory, Molecular Biotechnology Services, Yale University, New Haven, Connecticut. Oligonucleotide arrays were supplied from Dr. David Galbraith, Department of Plant Sciences, University of Arizona, Tucson, Arizona. Rehydration and fixing of the spots of oligonucleotide arrays was done as in the supplied protocol. Boundaries of the arrays were

marked with a diamond scriber. Slides were kept over 50°C water bath face down for 10 seconds and snap-dried on 65°C heating block for 5 seconds. This was repeated 4 more times for each slide. Slides were placed on the floor of the chamber of Fisher Scientific UV Crosslinker FB-UVXL-1000. DNA on the slides was crosslinked to the slide by delivering 150 mJ of UV light from the crosslinker. Slides were washed with 1% SDS for 5 minutes, SDS was removed by keeping the slides in 100% ethanol for 30 seconds with gentle shaking and the slides were placed in 50 ml falcon tubes and dried by spinning them in a centrifuge for 2 min at 1000 rpm. For blocking oligonucleotide slides 6g succinic anhydride was dissolved in 350 ml 1-methyl-2-pyrrolidinone, 15 ml sodium borate was added into the solution and the solution was poured into an empty slide chamber. Slide rack with the slides was plunged into the solution and after a few seconds of vigorous shaking the chamber was shaken on an orbital shaker for 20 min. Slides were washed with distilled water for 2 min and with 95% ethanol briefly and spun in a centrifuge for 2 minutes at 1000 rpm.

MICROARRAY EXPERIMENTS

The protocol supplied by the Keck Foundation Biotechnology Resource Laboratory was used for all of the experiments. For each labeling reaction 5-6μg mRNA with 30μl volume was mixed with 8μg oligo dT and 10 μg random hexamers. The volume was adjusted to 40 μl and the samples were kept at 65° for 5 minutes and transferred to ice for at least 2 minutes. 16 μl 5X first strand buffer, 8 μl 0.1M DTT, 1.2 μl 33.3 mM dACG (33.3 mM of each dATP, dCTP, dGTP), 0.8 μl 20 mM dTTP, 8 μl 2 mM aa-dUTP, 1 μl SUPERase.In RNase inhibitor (Ambion) and 6 μl Superscript III (Invitrogen) was added to the reaction mix and the samples were kept at 50°C for 3 hr. 10 μl 0.5 M EDTA and 20 μl 1 M NaOH was added to each

reaction to degrade RNA and the tubes were incubated at 65°C for 20 min. 5 µl 1 M HCl and 10 µl Tris (pH 7.5) were added to neutralize the reaction mix. 1 volume phenol and 1 volume chloroform: isoamylalcohol (25:1) were added, samples were vortexed, spun at maximum speed for 2 min and the upper layer in each sample was recovered. To remove unincorporated nucleotides sample volumes were adjusted to 400 µl; samples were added to Microcon YM-30 columns (Millipore) and spun for 10 minutes at 14000g. This was repeated for 3 times and after the last centrifugation remaining sample volume on the membrane was adjusted to less than 18 µl. Samples were recovered by spinning the columns upside down in a clean microfuge tube for 2 minutes at 8000 rpm. Sample volumes were adjusted to 18 µl, 2 µl 1 M sodium bicarbonate (pH 8.0) was added to each sample and 10 µl of each sample was added into a tube of Cy3 mono NHS ester dye and remaining 10 µl was added to a tube of Cy5 mono NHS ester dye (Amersham Biosciences) and samples were kept at room temperature (RT) for an hour in the dark.

To prehybridize the microarray slides thick lifter cover slips (25X60I-M-5439) (Erie Scientific) were placed on the slides and 65 µl prehybridization buffer (48% formamide, 3.2X SSPE, 0.4% SDS, 2X Denhardt's Reagent, 0.177 mg/ml single strand salmon sperm DNA) was added under each cover slip. Slides were put into hybridization chamber containing 55 µl hybridization chamber buffer (3.2X SSC, 23% formamide). The chamber was kept in 50°C water bath for an hour. Slides were than washed with distilled water, 70% and 100% ethanol respectively for 2 min each and air dried. Dye coupling reaction was stopped by adding 1 µl 2 M ethanolamine to each reaction and incubating the samples at RT for 5 minutes. For removal of unincorporated dyes Microcon YM-30 columns were used as previously described, the centrifugation step was repeated until no color was observed in the flow throughs (usually 3

times). Alternatively, CyScribe GFX Purification Kit (Amersham Biosciences) was used as described by the supplier to remove the unincorporated dyes. Microcon YM-30 columns were used afterwards to remove the contents of the elution buffer and to concentrate the samples (2 centrifugations). Wild type Cy3 labeled cDNA was mixed with mutant Cy5 labeled cDNA and vice versa in the columns. The end volumes of the samples were adjusted to 12.5 µl. 2.5 µl 20X SSPE, 15 µl poly-A and 32.5 µl hybridization buffer (62.8% formamide, 0.8% SDS, 4X Denhardt's Reagent, 5X SSPE) was added to the samples, samples were heated to 90°C for 2 min and kept in 42°C water bath until placed on the array. Thick cover slips were placed on the slides and the samples at 42°C water bath were added under the cover slips and the slides were put into hybridization chamber containing 55 µl hybridization chamber buffer and incubated in 42°C water bath for 18-20 hours. Slides were washed with 250 ml of 2X SSC, 0.1% SDS for few minutes and 10 min in the same solution, 2 times with 250 ml of 0.2XSSC, 0.1% SDS for 10 min and lastly 2 times with 250 ml of 0.2X SSC for 10 min and spun to dryness for 2 min at 1000 rpm. Slides were vacuum packaged, wrapped with aluminum foil and sent to Keck Foundation Biotechnology Resource Laboratory for scanning and the images were analyzed using Scanalyze Program.

RESULTS

DETECTING HFR1

IGY ISOLATION FROM EGG YOLKS

Five procedures were tested for purifying IgY from egg yolk. Normal commercially available eggs were used for this purpose. The total amount of protein detected using Bradford's reagent in the IgY isolations was compared for the five procedures (Table 2). Coomassie staining of the SDS-PAGE of the samples and the western did not reveal a clear result (Figure 2 and 3).

	Total Protein Amount (mg)	Volume (ml)
Protocol 1	365.4	14.5
Protocol 2	80.16	12
Protocol 3	64.92	12
Protocol 4	7.776	9
Protocol 5	13.412	56

Table 2: The total protein amount and volume of IgY isolations for the protocols.

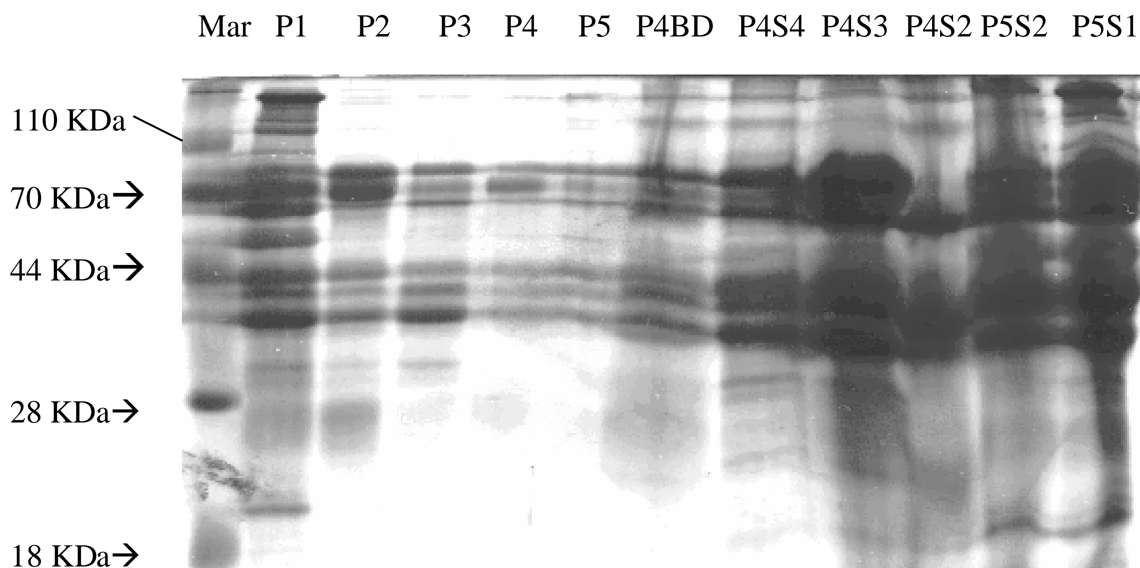


Figure 2: Coomassie staining of the SDS PAGE done for the IgY isolation protocols. Mar: Protein Marker (BRL, MD, USA), P: Protocol, BD: Before Dialysis, S: Supernatant. Heavy chain of IgY is 65 Kda.

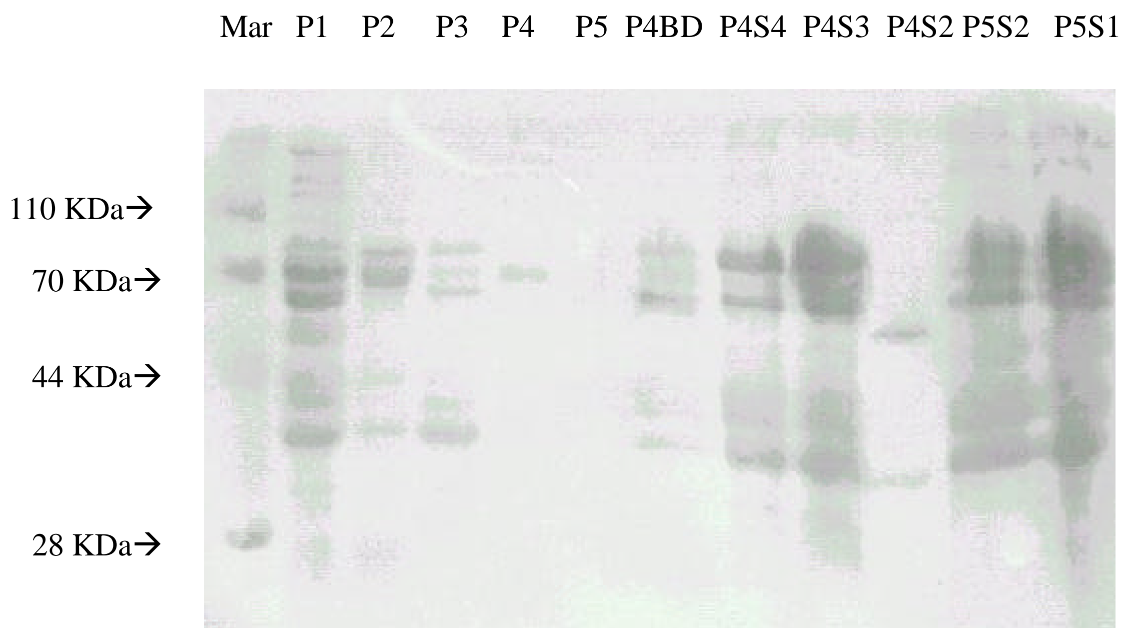


Figure 3: The western blot of the IgY isolations done with the five different procedures. Goat anti-chicken IgG was used to detect IgY.

A dot blot was performed to investigate the highest dilution of the IgY (from each of the five procedures) that can be detected with the secondary antibody. Among the 1/10 IgY dilutions, the detection of IgY from protocol 2 and 4 were quite similar but 1/100 dilution from protocol 2 was better. This may be a result of saturation of spots due to lack of TMB reagent or high amount of IgY on the spots of 1/10 diluted IgY of 2 and 4 (Figure 4).

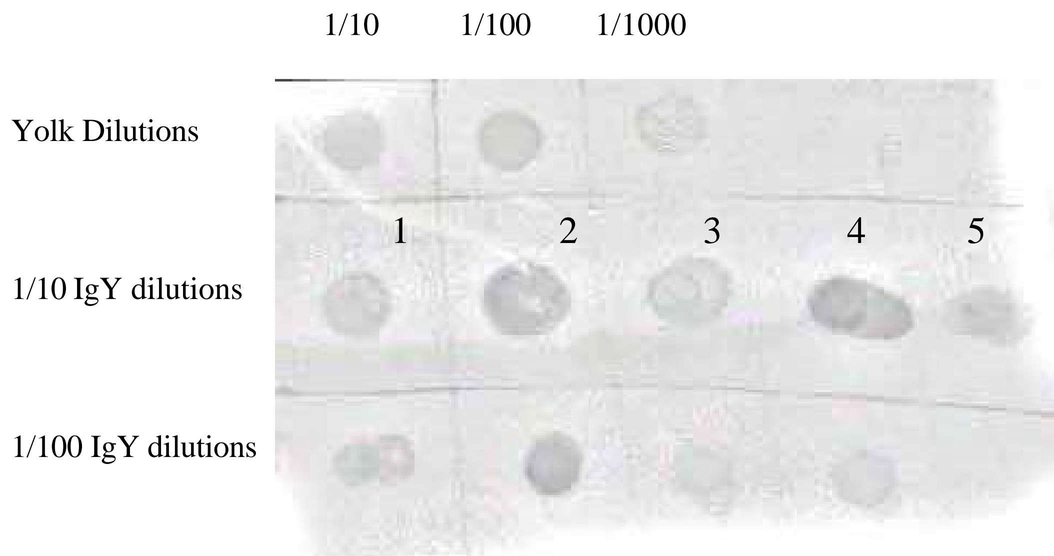
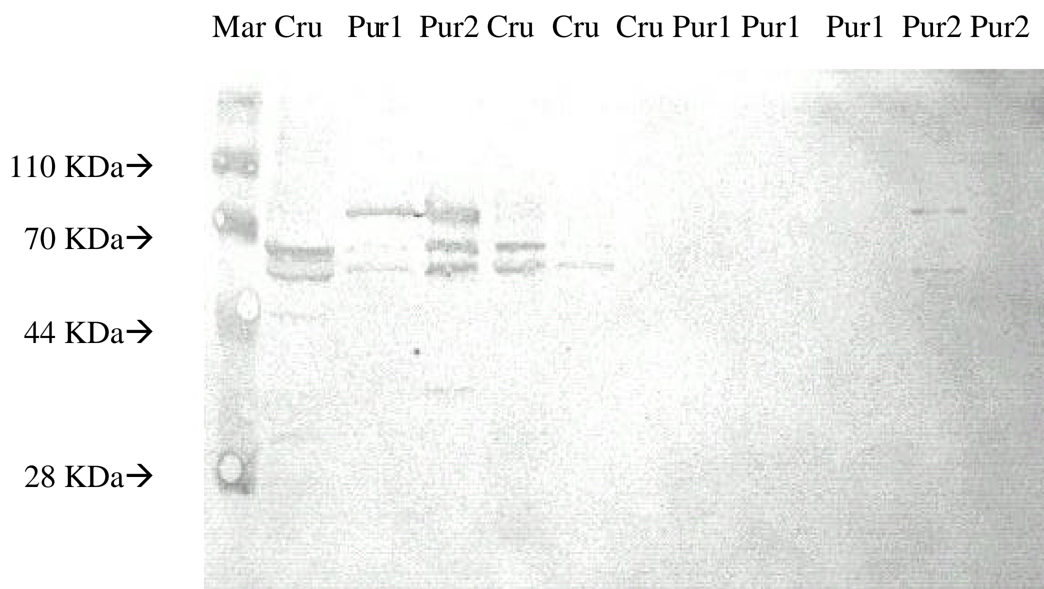


Figure 4: Dot-blot of diluted egg yolk and IgY samples. Numbers on the image are protocol numbers.

The next step was to isolate IgY from an egg that contains anti-HFR1 IgY and find if the isolated IgY was capable of detecting crude and purified MBP-HFR1. For this purpose a western blot was performed using undiluted and diluted crude and purified MBP-HFR1 samples (Figure 5). The result showed that 1/1000 diluted purified IgY (isolated using protocol 2) could detect 1/10 times diluted purified MBP-HFR1. After IgY was isolated from all of the 12 eggs using protocol we had ~ 73 ml of IgY.



Dilutions: 1/10 1/50 1/100 1/10 1/50 1/100 1/10 1/50

Figure 5: Detection of crude and purified MBP-HFR1 using purified IgY diluted 1/1000. Mar: Protein Marker, Cru: Crude, Pur: Purified. Detection was done using 1-Step TMB-Blotting reagent.

TESTING PRIMARY RABBIT ANTIBODIES

Several elutions produced during an affinity purification of primary rabbit antibodies developed against HFR1 (Kasia Galecka, unpublished) were tested to find the ones that have the highest amount of antibody. The detection of the primary antibodies on the dot blot with the anti-rabbit antibody (from goat) showed that the highest amount of antibody was present in tubes labeled # 20 and 21 (Figure 6, table 3).

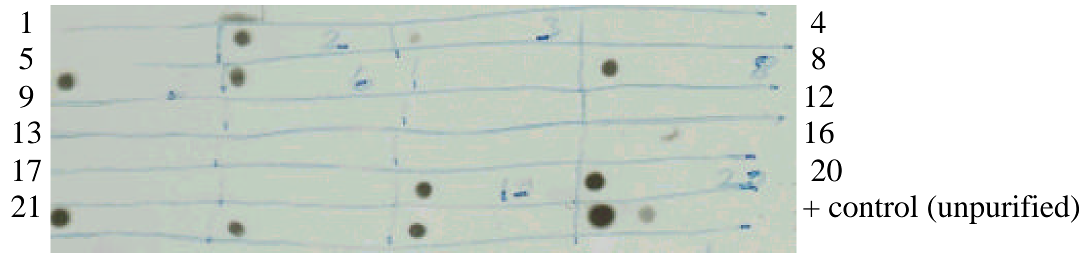


Figure 6: Film after detection of rabbit primary antibodies using goat anti-rabbit antibody. Purified anti-HFR1 rabbit antibodies were spotted on small strips of nitrocellulose membrane. Detection was done as previously described. Each box is a strip with four spots, From left to right they are 1/10, 1/100, 1/500 and 1/1000 diluted antibody samples.

Amount	**Highest**	**High**	**Low**	**Lower**
Elution number	20 21	23 19 8	6, 5 2, 22	3

Table 3: The amount of anti-HFR1 rabbit antibodies in different elutions.

HFR1 DETECTION

Unfortunately all the HFR1 detection efforts failed. We were not able to observe a band that was present only in the protein isolations from wild type but not from mutant *hfr1* seedlings. An example is shown in figure 7, sample # 21 of rabbit anti-HFR1 antibody was used as the primary antibody. IgY sample isolated from egg yolks was also used in other experiments but those failed too.

Mar W1 M1 T + W2 M2

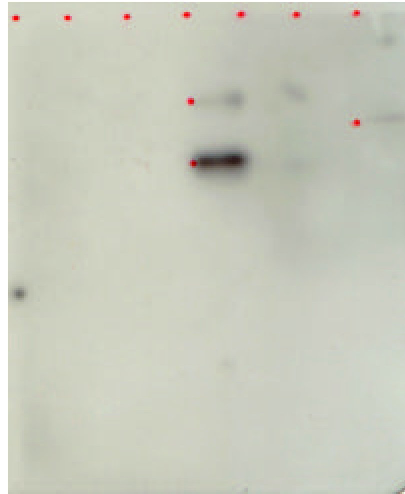


Figure 7: Film after exposure to the membrane. Mar: Protein Marker, W1: wild type 24 hr nuclear protein sample, M1: *hfr1-2* mutant 24 hr nuclear protein sample, T: In vitro translated HFR1 (using TNT/T7 Quick Coupled Transcription Translation System (Promega)), W2: wild type 6 hr nuclear protein sample, M2: *hfr1-2* mutant 6 hr nuclear protein sample.

ROLE OF HFR1 IN SHADE AVOIDANCE

NORTHERN BLOTS

After we made the observation that the wild-type and *hfr1-2* seedlings showed a hypocotyl length difference as early as 24 hours, we decided to perform the northern blot experiments using total RNA isolated after the seedlings were grown under far-red light for 24 hours.

The first candidate to check the mRNA level for was *ATHB-2*. *ATHB-2* gene encodes a homeodomain-leucine zipper protein. The mRNA level of *ATHB-2* increases upon exposure of plants to far-red light or after a decrease of red to far-red light ratio (Carabelli et al. 1993).
Since ATHB-2 is proposed to be involved in shade avoidance during which plants increase their length, we thought ATHB-2 might be involved in the long hypocotyl phenotype of *hfr1-2*

seedlings (Steindler et al. 1999). The northern blot showed that mRNA level of *ATHB-2* increased 9 times in *hfr1-2* seedlings compared to wild-type seedlings when they were grown under far-red light for 24 hours (Figure 8).

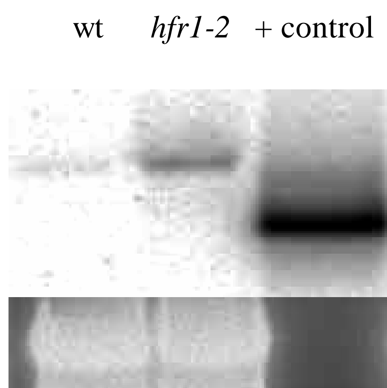


Figure 8: The northern blot done to detect the amount of *ATHB-2* mRNA. wt and *hfr1-2* lanes contain 20 µg total RNA. + control is 10 ng of *ATHB-2* cDNA fragment that was used to prepare the probe. The bottom part shows the EtBr staining of the total RNA loaded on the gel.

Another northern was done to check the *ATHB-2* mRNA levels at 0 hour far-red time point. This experiment showed that the *ATHB-2* mRNA level was 3 times downregulated in the *hfr1-2* seedlings (Figure 9).

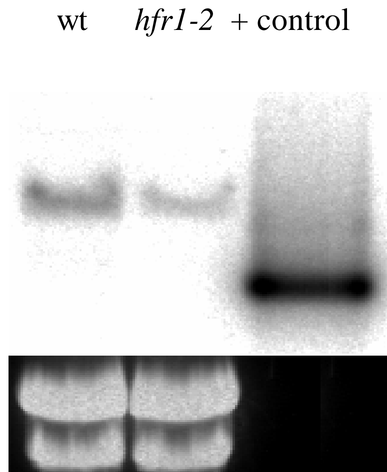


Figure 9: The northern blot showing the *ATHB-2* mRNA levels in wild-type and *hfr1-2* seedlings at 0 hour far-red time point. wt and *hfr1-2* lanes contain 20 µg total RNA. + control is 1 ng of *ATHB-2* cDNA fragment that was used to prepare the probe. The lower part shows the EtBr staining of the RNA loaded on the gel.

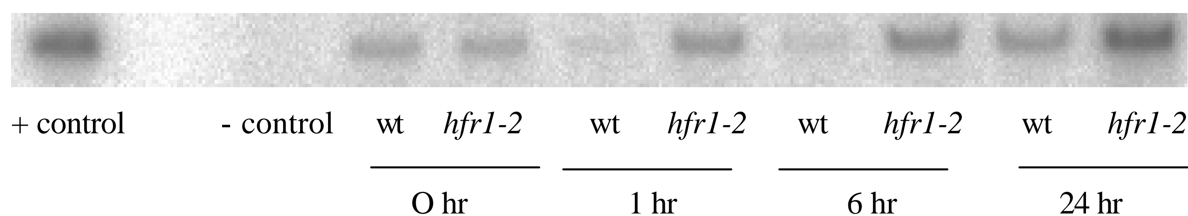
Next step was to look at another gene *ATHB-4*, which also gets upregulated by far-red rich light like *ATHB-2* (Carabelli et al. 1993).Unfortunately until this time we were not successful to get a result from this experiment. The blots showed two bands in both lanes for wild-type and *hfr1-2*. These bands probably correspond to ribosomal RNA. The appropriate way to pursue this northern would be probably by using mRNA instead of total RNA, which should eliminate the cross-hybridization.

RT-PCRS

To confirm the northern blot results for *ATHB-2* and look for the expression levels of *ATHB-4*, we performed RT-PCR experiments using RNA isolated from wild-type and *hfr1-2* seedlings. The PCR result for *ATHB-2* confirmed its upregulation in *hfr1-2* seedlings. A 5-fold difference in *ATHB-2* mRNA amount is observed between wild type and *hfr1-2* at 24 hr time point, 6 and 7-fold differences at 1 hr and 6 hr time points respectively, while little difference

was found at 0 hr (Figure 10). These and northern results show that HFR1 is required for downregulation of *ATHB-2* and thus that *ATHB-2* may be to some extent responsible for the long hypocotyl phenotype of *hfr1-2* mutants. *ATHB-4* also turned out to be upregulated in *hfr1-2* seedlings but by a similar ratio (Figure 11).

A.



B.

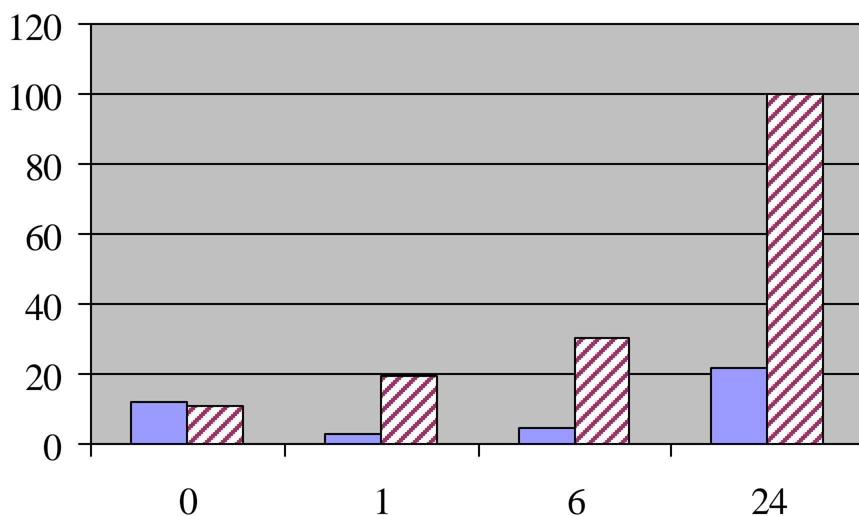
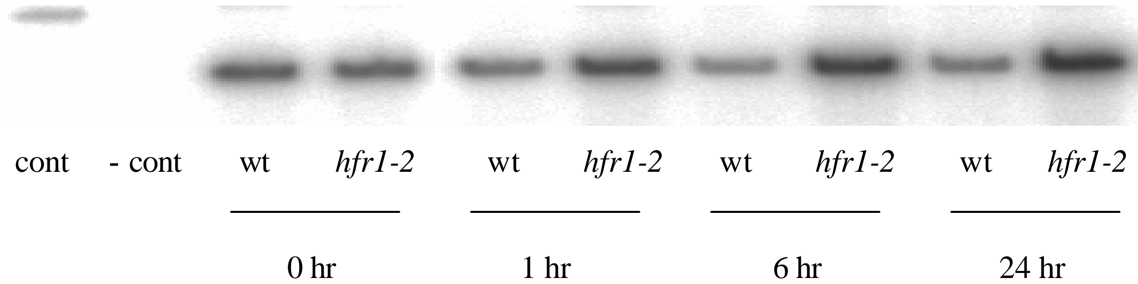


Figure 10: The result of RT-PCR done for *ATHB-2*. A) The bands corresponding to the amplified part of cDNA. + control is the PCR product from a previous experiment where the size of the band (520 bp) was confirmed with a radioactively labeled DNA ladder that was run together with the sample. Genotypes and time points are indicated. B) Graph showing relative amounts of *ATHB-2* mRNA. Solid bars are wild type, dashed bars are *hfr1-2*. *ATHB-2* mRNA amount in *hfr1-2* seedlings at 24 hr time point is taken as 100.

A.



B.

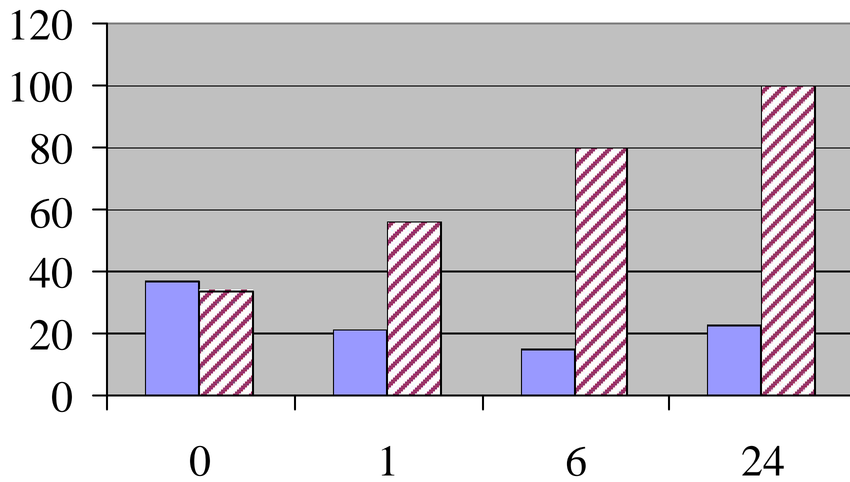


Figure 11: The result of RT-PCR done for *ATHB-4*. A) The bands corresponding to the amplified part of cDNA. + control is the PCR product *ATHB-2* from a previous experiment where the size of the band (520 bp) was confirmed with a radioactively labeled DNA ladder that was run together with the sample. Genotypes and time points are indicated. B) Graph showing relative amounts of *ATHB-4* mRNA. Solid bars are wild type, dashed bars are *hfr1-2*. *ATHB-4* mRNA amount in *hfr1-2* seedlings at 24 hr time point is taken as 100.

INVOLVEMENT OF AUXIN IN HFR1 PHENOTYPE

NPA experiments were done to find out if auxin was involved in the hypocotyl phenotype of *hfr1-2* mutants, since it was shown that NPA could decrease hypocotyl lengths of *ATHB-2* overexpressers by blocking the polar transport of auxin, and we have seen that *hfr1-2*

mutants have higher levels of *ATHB-2* than wild type seedlings (Steindler et al., 1999). The first experiment done using NPA gave the result as in table 4.

	Average hypocotyl length (mm)	**Standard error**	**Number of seedlings**
hfr1-2 **- NPA**	10.432	0.242	51
wt - NPA	4.309	0.220	31
hfr1-2 **1 mM NPA**	6.715	0.182	57
wt 1 mM NPA	2.459	0.112	31

Table 4 : The result of the first NPA experiment. Far-red light intensity was 0.6 μmoles.m^{-2}.s^{-1}.

The result of this experiment was interpreted in two ways. Since the average hypocotyl length of *hfr1*-2 seedlings is still not close to that of wild-type (It is 2.51 times more, compare with 2.42 without NPA) in the presence of 1 μM NPA, either auxin is not involved in *hfr1-2* phenotype or 1 μM NPA was not enough to block the effect of auxin. To test the latter idea we performed another NPA experiment by increasing the concentration of NPA to 2 and 20 μM. The results are shown in table 5 and figure 12. The effect of NPA is evident when its concentration is 2 μM but there is still a large difference between the hypocotyl lengths of wild type and *hfr1-2* seedlings. However at 20 μM concentration of NPA the difference is largely eliminated. These results suggest that auxin is involved in the long hypocotyl phenotype of *hfr1-2* mutants, together with *ATHB-2* and probably because of high levels of ATHB-2 protein.

	Average hypocotyl length (mm)	Standard Error	Number of seedlings
wt - NPA	4.223	0.226	18
***hfr1-2* - NPA**	7.991	0.246	8
wt 2µM NPA	2.061	0.07	22
***hfr1-2* 2µM NPA**	3.715	0.257	8
wt 20µM NPA	1.425	0.06	21
***hfr1-2* 20µM NPA**	1.807	0.144	6

Table 5: The result of the second NPA experiment. The intensity of far-red light was 1 µmol.$m^{-2}.s^{-1}$.

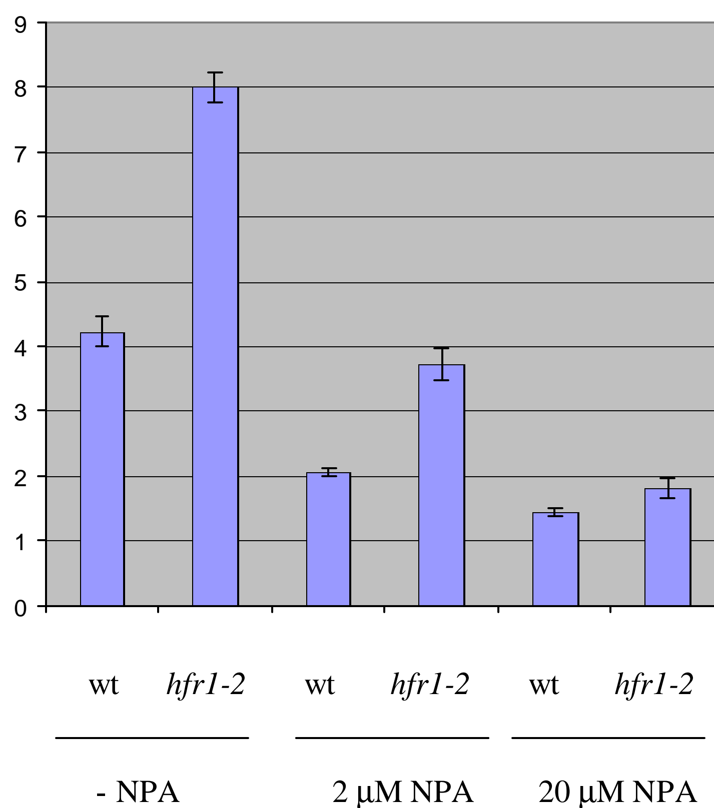


Figure 12: The result of the second NPA experiment in figure form.

FINDING HFR1 REGULATED GENES

Our aim was to find the genes regulated by HFR1 since HFR1 is a transcription factor. We also want to find the early genes targeted by HFR1 rather than the late ones. Since the *hfr1-2* mutant we are using is obtained by EMS mutagenesis it also contains other mutations in other genes that can effect the expression of those and other genes. That's why first we wanted to have two populations, one wild type and other *hfr1-2* containing the same extra mutations so that the differences would not come up as HFR1 regulated in experiments like microarray experiments. To do this we had to genotype plants grown from the seeds from a plant heterozygote for *HFR1* as described in the following results. After that we went on to find out at which earliest time-point we could see a hypocotyl length difference between wild type and *hfr1-2* seedlings. After finding that out we planned our experiments accordingly.

GENOTYPING ARABIDOPSIS ADULT PLANTS FOR HFR1-2

209 plants were grown from seeds isolated from a two times backcrossed plant heterozygote for *HFR1* with a genotype of *HFR1* x *hfr1-2* and with a Col-5 background. PCRs were done to amplify the part of the hfr1 gene, which included the mutation in the *hfr1-2* plants. The PCRs worked for all of the plants (Figure 13). After the restriction enzyme digestions were performed using the enzyme BsmAI (Figure 14), we found that there were 53 *hfr1-2* homozygous plants and an equal number of segregated wild-type plants. Each of these groups was present as 25.4 % of the segregating population. This result was very close to the perfect expected percentage of 25.

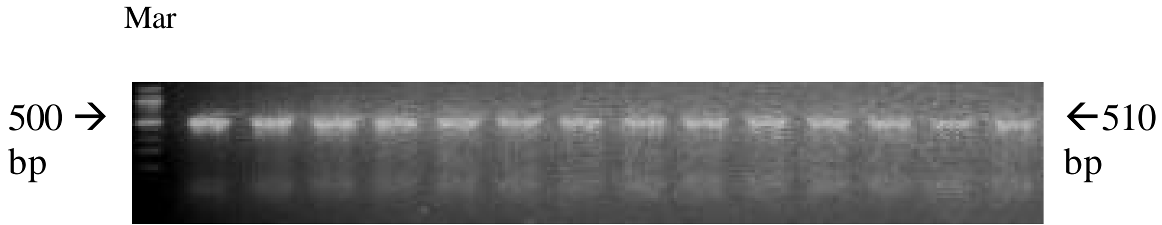


Figure 13: A part of the gel run to check the PCRs done to amplify the part of hfr1, which had a mutation in *hfr1-2* plants. Mar: 100 bp DNA marker.

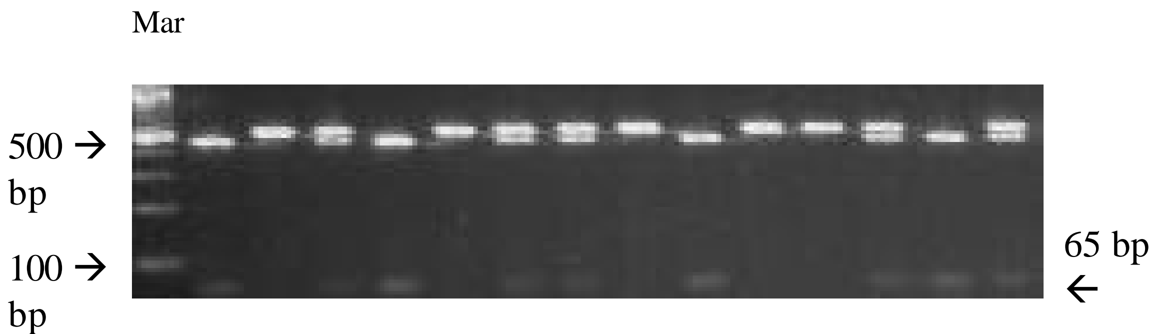


Figure 14: A part of the gel run to see the restriction enzyme digestion pattern of the part of hfr1 amplified with PCR. Of the three bands that can be present in a lane the uppermost band is 510 bp (No enzyme cutting, observed either in *hfr1-2* homozygous or heterozygous plants.), the lower band is 435 bp (The larger fragment formed after enzyme cutting, observed either in *hfr1-2* heterozygous or wild-type plants.), the lowest band is 65 bp and is present together with 435 bp fragment.

ANALYZING THE PHENOTYPE OF HFR1-2 SEEDLINGS

After the hypocotyl phenotypes of wild-type and *hfr1-2* seedlings were investigated, it was seen that the hypocotyl lengths of wild-type and *hfr1-2* seedlings were different after 24 hours under far-red light following growth in dark for two days (Table 6, figure 15).

Hours	wt	*hfr1-2*	wt St Error	*hfr1-2* St Error
24	1.005	1.229	0.088	0.099
30	1.132	1.629	0.110	0.117
43	1.524	3.007	0.044	0.240
50	1.638	3.202	0.146	0.159
56	1.722	3.529	0.107	0.132
70	1.997	3.833	0.109	0.146
96	1.876	4.108	0.061	0.218

Table 6: The result of the analysis of the phenotypes of the wild-type Col-5 and *hfr1-2* seedlings grown under far-red light.

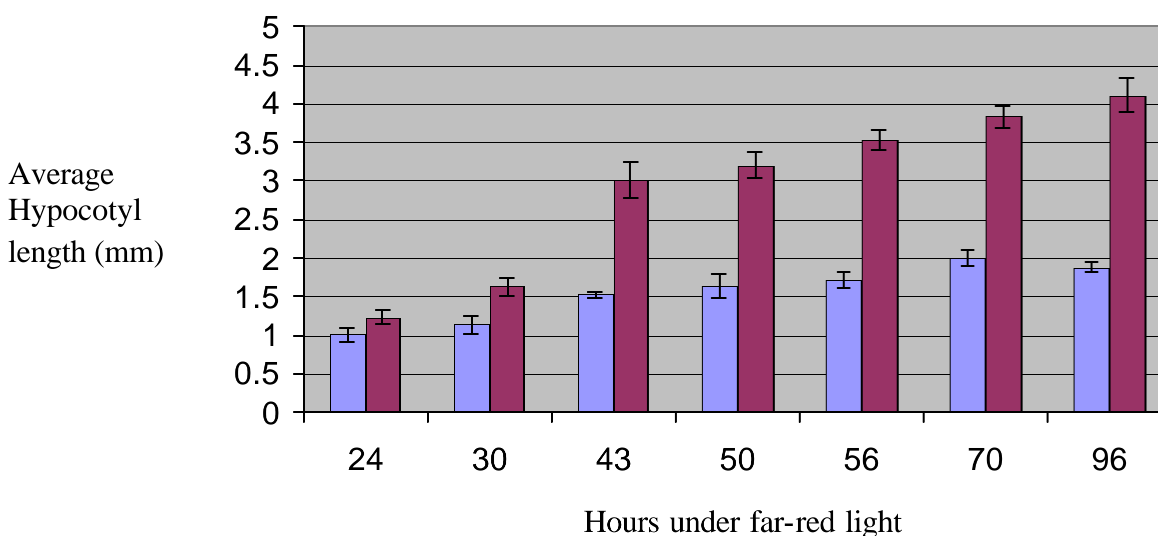


Figure 15: Average hypocotyl lengths of wild type (blue bars) and *hfr1-2* (red bars) seedlings grown under far-red light. Error bars show the standard error.

MICROARRAY EXPERIMENTS

The first microarray experiment was done using a spotted cDNA microarray, which contained 9216 spots representing more than 6000 genes. The slide had 16 blocks arranged 4 by 4, and each block normally has 24 rows and 24 columns. The image of a block (23 rows and 24 columns in our array) is shown in figure 16.

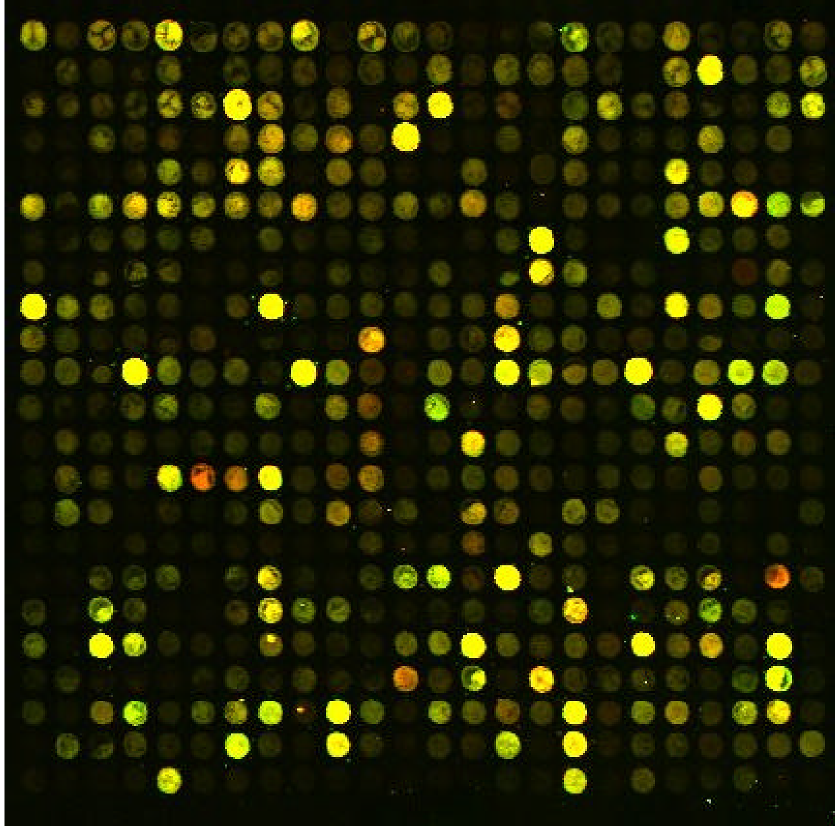


Figure 16: A block of the cDNA microarray hybridized with Cy3 and Cy5 labeled cDNA samples. Green spots are the genes down regulated in *hfr1-2* seedlings while the red spots are the upregulated ones. Yellow spots show the cDNAs that have the same level in both of the samples.

A publicly available program, ScanAlyze 2.50, developed by Michael Eisen's lab was used to analyze the images (http://rana.lbl.gov/). The analysis program gave us the ratios of colors of each spot representing the ratios of transcripts of genes in *hfr1-2* mutants versus the wild type. After the analysis 77 genes were found to have more than 1.5 fold differential expression in both of the experiments (the two replicates). Among these 77 genes 61 were downregulated in *hfr1-2* seedlings while the remaining 16 were upregulated. Some examples are given in table 7.

GenBank Name	Expression Ratio		Function
	5 days FR*	24 hr FR	
N65145	0.382	0.623	Serine/threonine protein kinase
W43222	2.162	2.467	Putative CHP-rich zinc finger protein
N65533	1.510	1.584	Chloroplast ribosomal protein

Table 7: Comparison of expression ratios of some of the genes, which were found by Wang et al. and me. The mutant allele used by Wang et al. was Rep1. * Wang H, Ma L, Habashi J, Li J, Zhao H, Deng XW. (2002). Analysis of far-red light-regulated genome expression profiles of phytochrome A pathway mutants in Arabidopsis. Plant J.Dec;32(5):723-33

After the cDNA microarray experiment we wanted to do the next experiments using the newly available 70mer oligonucleotide microarrays representing 26090 genes. These microarrays would let us look at the expression of more than 4 times more genes than cDNA microarrays. 0 hr and 1 hr time point experiments were done to find the earliest genes that were regulated by HFR1. GenePix Pro 5.0 was used to analyze the images. The results are shown in table 8.

	Upregulated in *hfr1-2* mutants	Downregulated in *hfr1-2* mutants
0 hr FR light	92	66
1 hr FR light	21	191

Table 8: The number of genes upregulated or downregulated in *hfr1-2* mutants after indicated hours of FR light.

We then looked at the ratios of transcripts for ATHB-2. The results are shown in table 7.

	0 hr	0 hr	1 hr	1 hr
ATHB-2	0.394	0.454	2.691	2.638

Table 7: Gene transcript ratios for ATHB-2 shown for duplicate experiments for 0 and 1 hr time points. Ratios are *hfr1-2*/wild type.

GENOTYPING ARABIDOPSIS LINES FOR HFR1-3

PCR was performed followed by restriction digestion to identify the genotype of 2 Arabidopsis lines for *hfr1-3*. Figure 17 shows the PCR result, figure 18 shows the restriction enzyme reaction result. Line number 4 was identified as *hfr1-3* homozygous while number 12 was heterozygous.

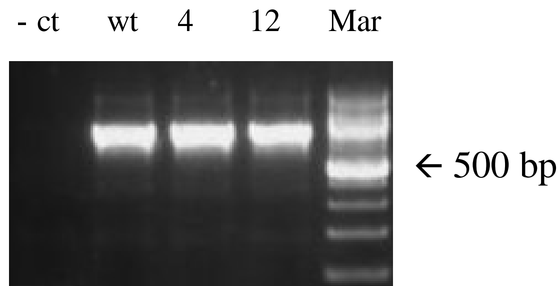


Figure 17: Result of the PCR done to amplify the part of hfr1 containing the mutation for *hfr1-3*. – ct: negative control, wt: wild-type, Mar: 100 bp DNA marker.

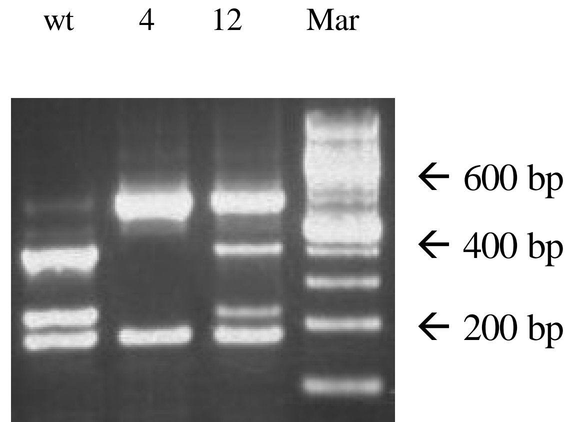


Figure 18: The restriction enzyme reaction of the PCR results. Line 4 is missing the RsaI cutting site in both copies of the hfr1 gene while line 12 is missing in one copy.

DISCUSSION

IgY samples were successfully isolated from egg yolks but we were not able to detect HFR1 using these antibodies or by using affinity purified rabbit anti-HFR1 antibodies. Purification of nuclear proteins from transgenic plants overexpressing *HFR1*, which presumably have higher HFR1 levels may lead to the detection of HFR1.

We were able to show that hypocotyl length difference between wild type and *hfr1-2* seedlings could be observed after 24 hr of FR but we believe that with the use of more sensitive imaging and measuring techniques this difference can be observed at earlier time points.

The 9-fold *ATHB-2* mRNA difference between wild type and *hfr1-2* seedlings observed at 24 hr in northern blot shows that *ATHB-2* is regulated by *HFR1*. We did the RNA isolations and RT-PCRs for time points earlier than 24 hr because we thought that the physiological difference observed at this point is a result of earlier changes in gene and protein levels, since *HFR1* encodes a transcription factor. We wanted to see if it was possible to observe a difference in *ATHB-2* mRNA levels at an earlier time point which would indicate that it is an early target of HFR1 protein. As seen in figure 10 the difference in *ATHB-2* mRNA levels between wild type and *hfr1-2* at 1 and 6 hr time points indicates that *ATHB-2* is an early target of HFR1. The decrease of *ATHB-2* mRNA during early hours of FR treatment has not been reported before and northern blots have to be done to confirm the results obtained by RT-PCRs for the amounts of *ATHB-2* in wild type and *hfr1-2* seedlings at 1 and 6 hr time points.

Although the result of the northern for *ATHB-2* for 0 hr time point conflicts with the RT-PCR result the oligonucleotide microarray result is in accordance with the northern. Additional northern experiments for 0 hr time point would probably resolve this conflict.

NPA experiments showed the involvement of auxin in the increased hypocotyl length of *hfr1-2* seedlings. As for the other factors involved there are two current possible explanations: Either increased *ATHB-2* level is causing this shade avoidance-like phenotype through a path other than auxin or the lack of HFR1 is leading to changes in the levels of other proteins that are causing the phenotype.

The results of our microarrays experiments are not very conclusive at the moment but we believe that by doing more microarray experiments for the time points we did using a salk line, salk_037727 which has a T-DNA insertion in *HFR1* that causes a *hfr1* phenotype, we will be able to get more conclusive results and find the genes regulated by HFR1.

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