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Plants can adapt and respond to their environment in a variety of ways. They have evolved sophisticated mechanisms, specifically, to respond to light. The LRBs (Light Response BTB) are E3 ligases, which are proteins involved in the degradation of other proteins in light. The LRBs are also involved in the degradation of Frigida (FRI), a protein that regulates flowering time. LRB is able to degrade FRI and allow the plant to flower. The mechanism that LRB employs to degrade FRI remains unknown. There is evidence that LRB attaches a protein tag to FRI and this may play a role in its degradation. To investigate this notion, we attempted to purify GFP-tagged LRB's from plants grown in different light conditions to detect the FRI interaction. We may have been able to successfully purify GFP-LRB. We were unable to determine if FRI was attached to the purified LRB's since we could not get the FRI antibody to work. This work may aid in our understanding of light and flowering in plants.

INTRODUCTION

Light plays a significant role in many key developmental processes in plants including shade avoidance, flowering, plant defense, and germination. Therefore, the mechanisms by which plants are able to detect and respond to light are of crucial importance. There are many variations in light, with the photoreceptors, known as Phytochromes (phys), constituting the system that plants employ to detect red light (1, 2). There are several sub-types of phys in plants; specifically, phyB is primarily responsible for Red (R) light responses, while phyA is involved in Far-Red (FR) responses (1, 3). Upon activation by R light, the phys localize to the nucleus as the Pfr conformer, or far-red light-absorbing conformer (1, 3, 4). During inactivation, the phys are located in the cytoplasm as the Pr conformer, or red light-absorbing conformer (1, 2, 3). Previous studies have demonstrated that phyB is degraded in R light by a BTB E3 ligase known as LRB1 and 2 (Light Response BTB1/2), which requires an interaction with the scaffold protein Cullin3 (Cul3) in order to execute degradation (3, 5). The LRBs interact with Cul3 strongly in R light, but exhibit little to no interaction with Cul3 in Dark (D) conditions (3). Furthermore, plants containing mutations in which *LRB1* and *2* are absent are hypersensitive to R light, providing evidence of a role for the LRBs in the R light response pathway (3, 5).

Arabidopsis thaliana is capable of surviving throughout many different climates, due to variations in flowering time between accessions (5, 6, 7, 8). The wild type accessions Columbia (Col-0) and Landsburg *erecta* (L*er*) exhibit early flowering (5, 8). These early-flowering accessions, unlike many of the other wild type accessions, do not require a period of cold (vernalization) in order to flower (5). This is the result of a mutation in the gene known as Frigida (*FRI*), one of the principal genes involved in the regulation of flowering time (5, 8). Col-0 contains a deletion in the *FRI* allele, while L*er* contains a deletion in the promoter region of *FRI*, and both mutations render the protein either truncated or expressed at low levels, respectively (8). In late-flowering accessions, such as H51 and Sf-2, FRI upregulates Flowering Locus C (*FLC*), which suppresses the activity of specific flowering genes including *LEAFY* and Flowering Locus T (*FT*) (5, 6, 8). Previous studies have demonstrated that FRI degrades subsequent to a period of vernalization, therefore allowing the plant to flower (5). In the early-flowering accessions, mutated FRI is unable to upregulate *FLC*, so these flowering genes, among others, are expressed, allowing the plant to flower (5).

Recently, FRI's C-terminal region has been found to interact with the N-terminal region of Cul3, and FRI's N-terminal region interacts with the N-terminal region of the LRB's (5). The interaction between LRB and Cul3, at least, appears to be light-dependent because the two proteins interact in R light but not in the dark (3). Thus far, no studies have been conducted to determine whether the interaction between LRB and FRI is light-dependent. A post-translational modification similar to that of ubiquitylation, known as neddylation, changes the conformation of various proteins via the protein Neural Precursor Cell-Expressed Developmentally Down-Regulated Protein8 (NEDD8), which is attached to target proteins in a similar manner as ubiquitin (9). This modification could explain some of the interactions that have been observed. In this study, the interaction between LRB and FRI is examined in order to determine whether it is light-dependent as well as whether FRI becomes neddylated when exposed to particular light conditions.

METHODS AND MATERIALS

PLANT GROWTH CONDITIONS

Arabidopsis thaliana seeds were sterilized by first washing in 70% ethanol and then washing for 5 minutes in 25% bleach. The seeds were then left on MS media with 2% sucrose at 4°C for 3 days in the dark. The seeds were transferred to white light in long day conditions (16-hr-light/8-hr-dark) and were then placed in red (R) light for 4 days. Far-red (FR) light-treated tissue was exposed for an additional 24 hours in FR light following the R light treatment. Treated tissue was collected by freezing in liquid nitrogen and stored at -80°C.

SELECTION OF TRANSGENIC PLANTS

Selection was based on the paper by Harrison et al, 2006 (9). At least 20 lines of seeds from the F2 generation of FLAG-LRB1 (Basta resistant) X HA-Strep-Nedd8 (Hygromycin resistant) transgenic plants that were already selected for homozygous growth on Hygromycin were plated on MS+2% sucrose with either Hygromycin (15 μg/ml) or Basta (50 μM) supplanted in the media. For Basta selection, plates were placed in white light with one sheet of paper covering the plate, to allow some light to pass through. The seeds were allowed to germinate and grow for 5 days, after which, resistance was determined by resistant seeds germinating and greening up. For Hygromycin resistance, plates were wrapped in 2 layers of foil for 4 days. After 4 days, resistant seeds had longer hypocotyls. Some plates were hard to call, so these were left in the white light an additional 3 days, after which resistant seeds greened up.

IN-VIVO GFP PULL-DOWN

Frozen, 10-day old seedlings were ground in liquid nitrogen with a mortar and pestle and combined with protein extraction buffer (100 mM MOPS, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM Phenylmethylsulfuryl fluoride, 0.1% Nonidet P-40, 20 mM Iodoacetamide, 2 μg/L Aprotinin, 5 μg/L Leupeptin, 1 μg/L Pepstatin, and 2x Complete Protease Inhibitor Cocktail). The buffer and tissue mixture was filtered through double-layered cheese cloth and spun down at 12,000 rcf for 10 minutes at 4°C. Protein G Sepharose beads were washed 3x in protein extraction buffer with spins at 1,000 rpm for 2 minutes between washes. To pre-clear the lysate, prewashed beads were added to the samples and incubated at 4°C for 30 minutes with gentle rotation. Those beads were then spun down at 1,000 rpm for 2 minutes and the supernatant was used for the immunoprecipitation. Polyclonal rabbit anti-GFP antibody (1:500 Santa Cruz Biotechnology) was added to the samples, which were incubated at 4°C for 1 hour with gentle rotation. Prewashed Protein G Sepharose beads were added to the antigen/antibody mixture and incubated at 4°C for 1 hour with gentle rotation. The beads were then spun down at 1,000 rpm for 2 minutes. The beads were washed 3x in protein extraction buffer with spins at 1,000 rpm for 2 minutes

between washes. Finally, 2% SDS Sample Buffer was added to the beads which were then stored at -80°C.

PROTEIN EXTRACTION

Protein extraction buffer (100 mM MOPS, pH 7.6, 50 mM NaMetabisulfate, 2% SDS, 20% Glycerol, 4 mM EDTA) was added to frozen, 10-day old seedlings. The seedlings were then ground with a small pestle. The samples were then boiled for 10 minutes and spun down for 10 minutes at 12,000 rcf. 0.5 volumes of SDS Sample buffer was added to the supernatant after boiling.

IMMUNOBLOTTING

Protein samples were separated on a 10% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. For detecting GFP-LRBs, the membrane was probed with polyclonal rabbit anti-GFP antibody (1:500 Santa Cruz Biotechnology) and then goat anti-rabbit antibody (1:4000 Li-Cor CW 800). For detecting Frigida (FRI), the membrane was probed with polyclonal rabbit anti-FRI (N) antibody (1:2000 Abiotech) and then with goat anti-rabbit antibody (1:4000 Li-Cor CW 800). The membrane was visualized using the Odyssey Fc imager (Li-Cor).

RESULTS

IMMUNOPRECIPITATION

The E3 ubiquitin ligase, LRB, interacts with FRI, a regulator of flowering time, in order to facilitate its degradation. While the mechanism by which this occurs is still unclear, there is reason to suspect that FRI may be neddylated in a light-dependent manner. Using 35S::GFP-LRB2 (GFP-LRB2) transgenic plants, an *in vivo* pull-down assay was performed with both the red (R) light-treated GFP-LRB2 and the far-red (FR) light-treated plants. GFP-LRB2 was recognized by anti-GFP antibodies (AbBio). Once the antibodies recognized the GFP-LRB2 proteins, they were captured using Pierce Thermo Scientific protein A/G magnetic beads. The proteins were eluted using SDS Sample Buffer and separated on a polyacrylamide gel. They were then immunoblotted using polyclonal rabbit anti-GFP antibody and then washed with goat anti-rabbit antibody as the secondary antibody.

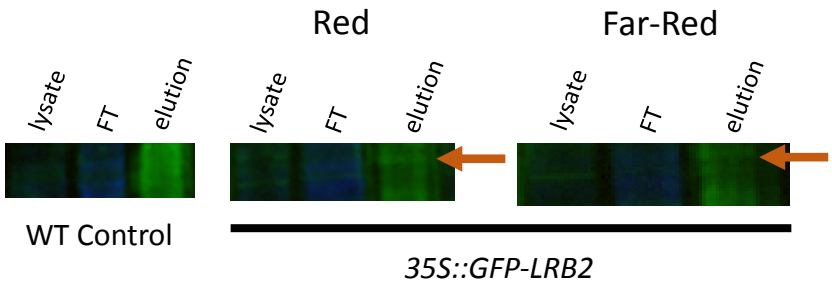

Figure 1. Purification results from GFP-LRB2.

Our first attempt at the purification of GFP-LRB2. The 96kd GFP-LRB2 protein seems to be visible in the elution.
Lysate is the crude, unpurified tissue. FT is the flow through, and Elution is the beads after they were washed.
The Columbia wild ecotype of *Arabidopsis thaliana* was used as the control in the experiment. GFP-LRB2 was inserted into the Columbia background under the control of the 35S promoter. The plants were grown for 1 day in long day conditions (16-hr-light/8-hr-dark) followed by 4 days in red (R) light. The far-red (FR) treated plants were then grown for 1 more day in FR conditions.

In Figure 1, a band at about 90kd, the size at which GFP-LRB2 separates, can be seen in the elutions of the GFP-LRB2 samples grown in R and FR. The wild-type Columbia control that does not contain the GFP-tagged LRB should not show any band. There is no band in the wild type in the lysate, which seems to confirm that the band seen in the GFP-LRB2 lines is, indeed, GFP-LRB2.

However, there is quite a high signal-to-noise ratio on these blots, causing the bands to be difficult to see due to the brightness, particularly in the elution lanes. This may be due to insufficient blocking of the membrane prior to probing with the antibodies or a high amount of non-specific banding.

There is a possibility that there is a band in the WT elution lane. That could be due Col-0 seeds being contaminated with GFP-LRB2 seeds or the presence of a protein that is naturally present in high amounts and auto-fluoresces at the same size as GFP-LRB2. Yet another explanation is that the polyclonal rabbit anti-GFP antibody that was used for the *in vivo* pull-down was not fully reduced. When the antibody is not fully reduced, it can cause a band around 100kd, right around the size at which GFP-LRB2 runs. If there were a band in the wild type lane, it would indicate that GFP-LRB2 was not successfully purified as we had hoped.

In order to eliminate the possibility that the band we see in all three samples (WT control, Red and FR, Figure 1) is simply non-reduced antibody or a non-specific band, a second *in vivo* pull-down of GFP was performed with a slightly altered immunoblot protocol. The immunoblot protocol was altered by blocking the membrane twice. It was blocked before adding the primary antibodies and before adding the secondary antibodies. This alteration was made to the protocol in order to decrease the signal-to-noise ratio in the elution lanes.

In Figure 2, the samples from the second immunoprecipitation are much less dirty when run through a SDS PAGE and blocked prior to adding the secondary antibodies. A band around 96kd, the correct size of GFP-LRB2, can clearly be seen in the R and FR elution lanes. However, that same band is also present in the wild type control lane.

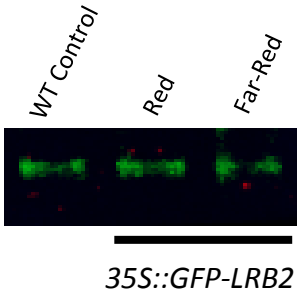

Figure 2. Purification results from GFP-LRB2 using altered protocol.
Plants were grown in 4 days of red light (Red-treated samples) or 4 days of red light followed by 24 h of FR (FR-treated).

There are a number of possible explanations as to why the band around 90kd in the wild type control is present. It is possible that the wild type control seeds that were used were contaminated with GFP-LRB2 seeds. These seeds were tested for contamination on Hygromycin plates because GFP-LRB2-containing plants are Hygromycin resistant. 100% of the wild type Col-0 seeds (from a population of at least 50) failed to grow on the Hygromycin plates, which indicates that this batch of Col-0 was most likely not contaminated with GFP-LRB2 seeds (data not shown).

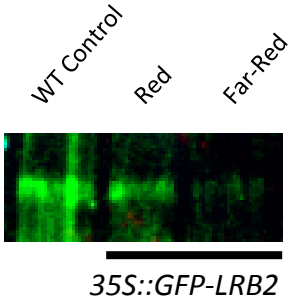

Figure 3. Purification results from GFP-LRB2 using non-magnetic beads.
Plants were grown in 4 days of red light (Red-treated samples) or 4 days of red light followed by 24 h of FR (FR-treated).

Another possible explanation is that the antibody that is being used is not being fully reduced by the elution conditions. As such, the samples were eluted once more using 200 mM Diothiothreitol (DTT) in addition to the 10% concentration of β-mercaptoethanol (BME) already present in the elution buffer. Unfortunately, that band was still present in the WT control lane (Figure 3).

The source of the band around 90 kDa in the wild type control lane was not able to be determined from this experiment. An *in vivo* pull-

down with the wild type control with and without anti-GFP antibodies could be performed in order to determine if the band we see in the elution samples is due to non-reduced antibody. To reduce the possibility of the rabbit anti-GFP antibody used in the pull down being recognized by the secondary antibody we use in the immunoblots possibly causing the band seen in the wild type lane, a mouse anti-GFP antibody could be used for the immunoblot.

CROSS BETWEEN HSN AND FLAG-LRB1

Since no known *Arabidopsis* Nedd8 antibody has been developed, we wanted to identify the neddylation status of the LRBs and FRI directly. To do this, we crossed a *35S::*FLAG-LRB1 line with transgenic plant conditionally expressing the HA-Strep-Nedd8 (HSN) protein (9) with the purpose of purifying the LRBs or FRI and identify if the HSN is covalently attached. The F2 plants were selected on Basta (FLAG-LRB1) and Hygromycin (HSN) and several plants were identified that were resistant to both. These were planted in soil and the seeds from these plants were screened on Basta and Hygromycin to identify several homozygous lines for both FLAG-LRB1 (Basta) and HSN (Hygromycin) (Table 1).

Table 1: These lines are F3 lines that were selected for Basta resistance (homozygous). Blue: Lines that show complete resistance to Basta, but Hygromycin resistance was unknown. Pink: Lines that show almost complete resistance to Basta, and complete resistance to Hygromycin. Yellow: Lines that show complete resistance (homozygous) for both Basta and Hygromycin.

	Basta Resistance		
Line:	Resistant	Non-Resistant	No Germination
Col-0	0	35	4
FLAG-LRB1	26	3	3
Ha-Nedd8	0	35	2
1	35	0	1
2	29	1	0
3	0	31	7
4	26	6	0
5	27	10	1
6	22	8	0
7	0	32	3
8	24	7	0
9	1	40	1
10	0	29	0
11	0	38	1
12	25	0	3
13	35	0	1
14	0	31	0
15			
16			
17	16	14	1
18	29	0	0
19	25	7	0
20	28	0	0

Table 2: These lines are F3 lines that were selected for Hygromycin resistance (homozygous). Blue: Lines that show complete resistance to Basta, but Hygromycin resistance was unknown. Pink: Lines that show almost complete resistance to Basta, and complete resistance to Hygromycin. Yellow: Lines that show complete resistance (homozygous) for both Basta and Hygromycin.

	Hygromycin Resistance		
Line:	Resistant	Non-Resistant	No Germination
Col-0	0	29	1
FLAG-LRB1	0	35	2
Ha-Nedd8			
1			
2	29	0	0
3	25	1	3
4	22	2	4
5	36	0	1
6	29	0	0
7	27	1	2
8	29	0	0
9	36	2	1
10	31	0	0
11	30	1	3
12	29	0	2
13	41	0	1
14	39	0	0
15			
16			
17	37	1	0
18	29	0	0
19	39	1	0
20	31	0	0

Lines 12, 13, 18, and 20 are all double homozygous for FLAG-LRB1 and Ha-Nedd8. Tissue from line 20 will be used to perform an in vivo pull-down with anti-Ha antibody. An immunoblot using anti-FRI antibody will then be performed in order to determine its neddylation status. This tissue will also be used to immunoblot with FLAG in order to determine the neddylation status of LRB.

DISCUSSION

The LRB proteins have an important role in the R light-signaling pathway because they are responsible for the degradation of both the phys and the PIFs. The LRBs are also important in the regulation of flowering due to their role in the degradation of FRI, which controls flowering time. We hypothesized that FRI and the LRBs may interact in a light-dependent manner. We tested this idea by extracting protein from tissue grown in different light conditions and probing for FRI. We also hypothesized that FRI is neddylated. To test this hypothesis, we wanted to purify FRI from *Arabidopsis thaliana* and probed with Nedd8 antibody. The research into a potential light-dependent relationship between LRB and FRI was not

conclusive. The neddylation status of FRI also remains unclear.

The first in-vivo pull-down that was performed seemed promising. However, after a second pull-down, a band corresponding to the same size as GFP-LRB2 showed up in the wild type sample. Prior to the results in this experiment, we were unable to visualize GFP-LRB2 even in the lysate. We thought that it was because the protein was being broken down by proteases in the lysate. However, we are now able to show that the protein is not being broken down during this purification because the lysate shows the GFP-LRB2 proteins in only the transgenic GFP-LRB2 plants, and not in wild type Columbia. In Figure 1, it seems that GFP-LRB2 is present in only the experimental treatment elution lanes, however, after further experiments, a band around the size of GFP-LRB2 in the wild type lane in figure 2 was present, suggesting that the band is not GFP-LRB2, but some artifact of the immunoprecipitation process. Therefore, there may be non-reduced antibody present or the bands we see in the R and FR lanes are not GFP-LRB2.

Another purification of LRB is required in order for the relationship between it and FRI to be examined. The unusual result in Figure 2 will require further investigation. In the case that the antibody is not separating fully, a mouse anti-GFP antibody may be used to visualize the purified GFP-LRB2 in order to prevent cross-reactivity with the rabbit antibody used in the purification. Once completed, a co-purification of LRB and FRI may be performed and the light conditions under which the two proteins interact may be observed. We suspect that either the LRBs or FRI may be neddylated when they interact. In order to investigate this notion, a transgenic, double homozygous line containing both FLAG-tagged LRB1 and *HS*-Nedd8 proteins, has been created. A purification of FLAG-LRB1 from the transgenic line will be performed to determine the neddylation status of the LRBs and FRI.

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