

## Introduction

A plant's ability to assess light quantity and quality is fundamental to maintaining healthy growth. One way that plants sense changing light conditions is via the perception of red (R~660 nm) and far red (FR~730 nm) wavelengths by a group of light receptors called the phytochromes (PHYs). PHY mediated responses include de-etiolation (which includes reduced hypocotyl elongation and increased cotyledon expansion) and shade avoidance responses (which include stem and petiole elongation) (Mathews, 2006).

We have found that two highly similar genes *LIGHT RESPONSE BTB1* (*LRB1*) and *LIGHT RESPONSE BTB2* (*LRB2*) act as negative regulators of phy-mediated red light-responses in *Arabidopsis* (Christians *et al.*, 2012). Plants with disruptions of these two genes are red light hypersensitive and thus display enhanced de-etiolation in response to red light and significant shade tolerance (Figure 1). *LRB1* and *LRB2* code proteins with a BTB (Bric-a-Brac, Tramtrack, Broad Complex) domain. One well characterized function of these domains is to link BTB proteins to Cullin 3 (CUL3) proteins in BTB/CUL3 E3 ubiquitin (Ub)-ligase complexes. In these complexes the BTB protein acts as the target adapter, recruiting the Protein(s) to be ubiquitinated by the E3 (Figure 2). We hypothesize that *LRB1* and *LRB2* regulate, via ubiquitination, some component in the red light signaling pathway

In order to identify the component(s) of the red light signaling pathway that the *LRBs* are regulating (or other actors in the pathway) we have conducted a genetic suppressor/enhancer screen, identifying mutations which relieve or increase red light inhibition of hypocotyl elongation in the *lrb1/lrb2* red hypersensitive double mutants. Here we describe this screen and characterization of these mutants.



Figure 1. Four day old seedlings grown for three days in continuous 10 μmol/m<sup>2</sup>/sec. LED-generated red light. The *lrb1/lrb2* double mutant has significantly shorter hypocotyls and larger cotyledons, indicating increased red light sensitivity.

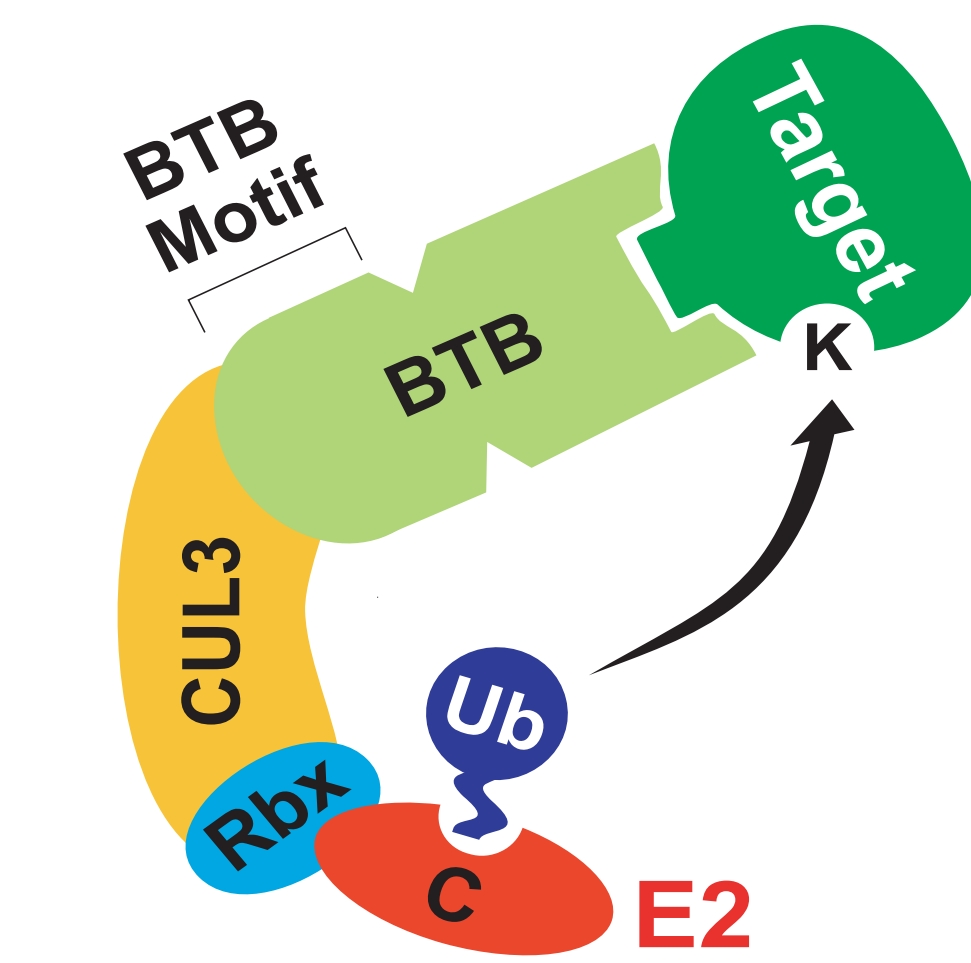


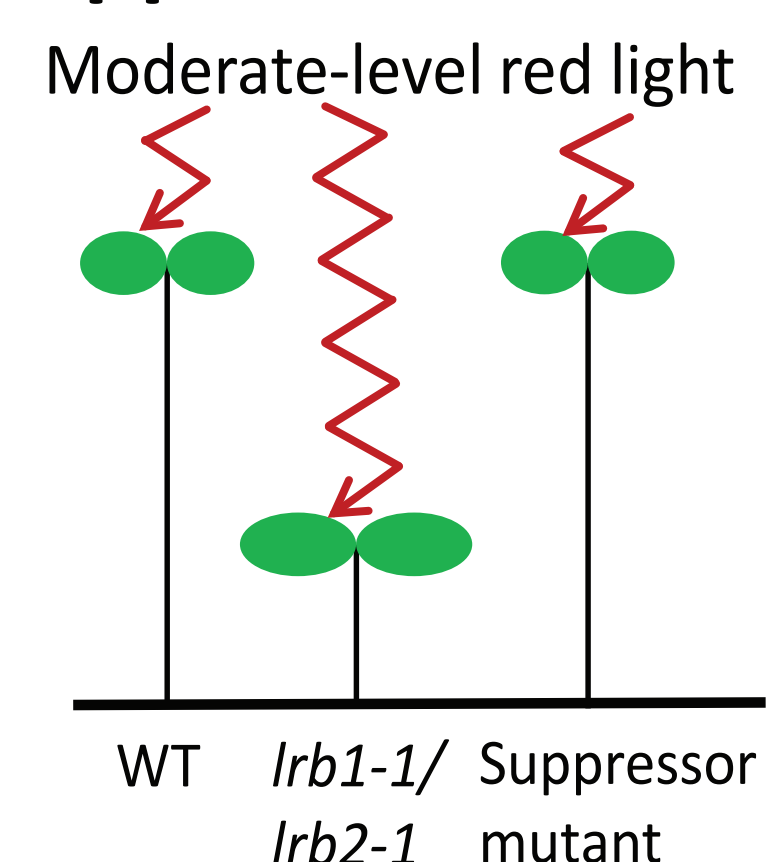
Figure 2. Predicted structure of BTB/CUL3 E3 ubiquitin ligase complexes. The E3 binds the target and ubiquitinates it. This ubiquitination typically leads to degradation of the target. *LRB1* and *LRB2* are predicted to encode BTB proteins.

## Suppressor/Enhancer Screen Methods

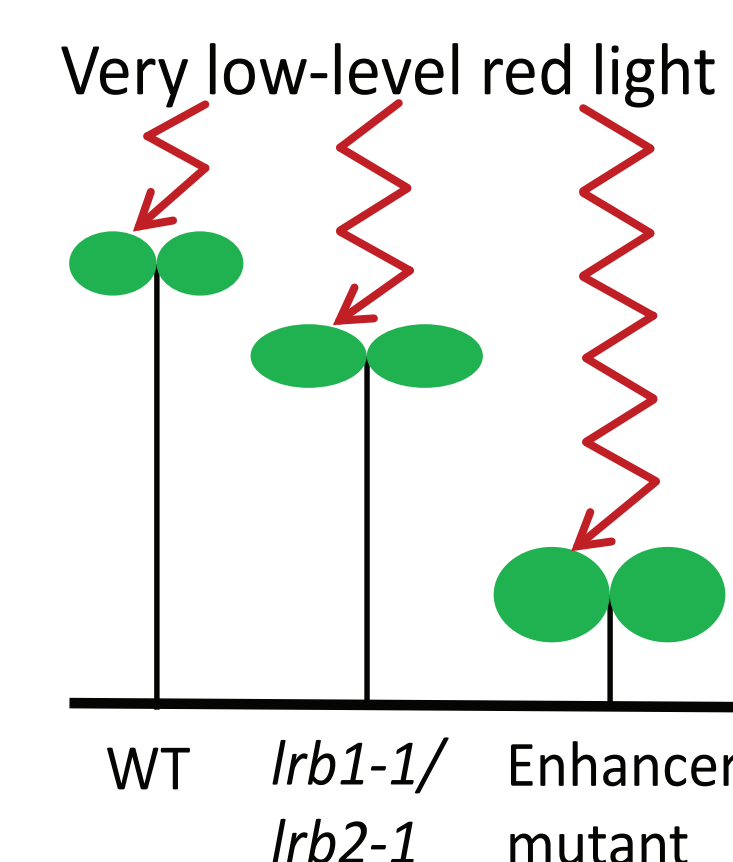
### Strategy:

- Mutagenize population of *lrb1-1 lrb2-1* seeds with ethylmethanesulfonate (EMS).
- Germinate seeds and grow plants (10 plants/pot), 2000 individuals total.
- Collect seed from these individuals.
- Germinate and grow this next generation (M2) under red filtered light; identify individuals that have reduced or increased red light sensitivity compared to the *lrb1-1 lrb2-1* double mutants.

### Suppressor Screen



### Enhancer Screen



- Approximately 30,000 M2 generation plants were screened for both suppressor and enhancer phenotypes.
- 100+ individuals with putative suppressor phenotypes and 400+ enhancer phenotypes were identified. 3-4 individuals with similar phenotypes were typically identified per M2 pool.
- M3 generation seedlings are being tested for various red, far red, and blue light responses.

## Characterization of the Suppressor Mutant *lbs1*

Suppressor mutant line *lbs1* hypocotyl length is less than WT and the *lrb1/lrb2* parental line in the dark (Figure 3). It is less sensitive to R inhibition of hypocotyl elongation than WT (Figure 3), but has WT-sensitivity to FR and B light (Figure 4).

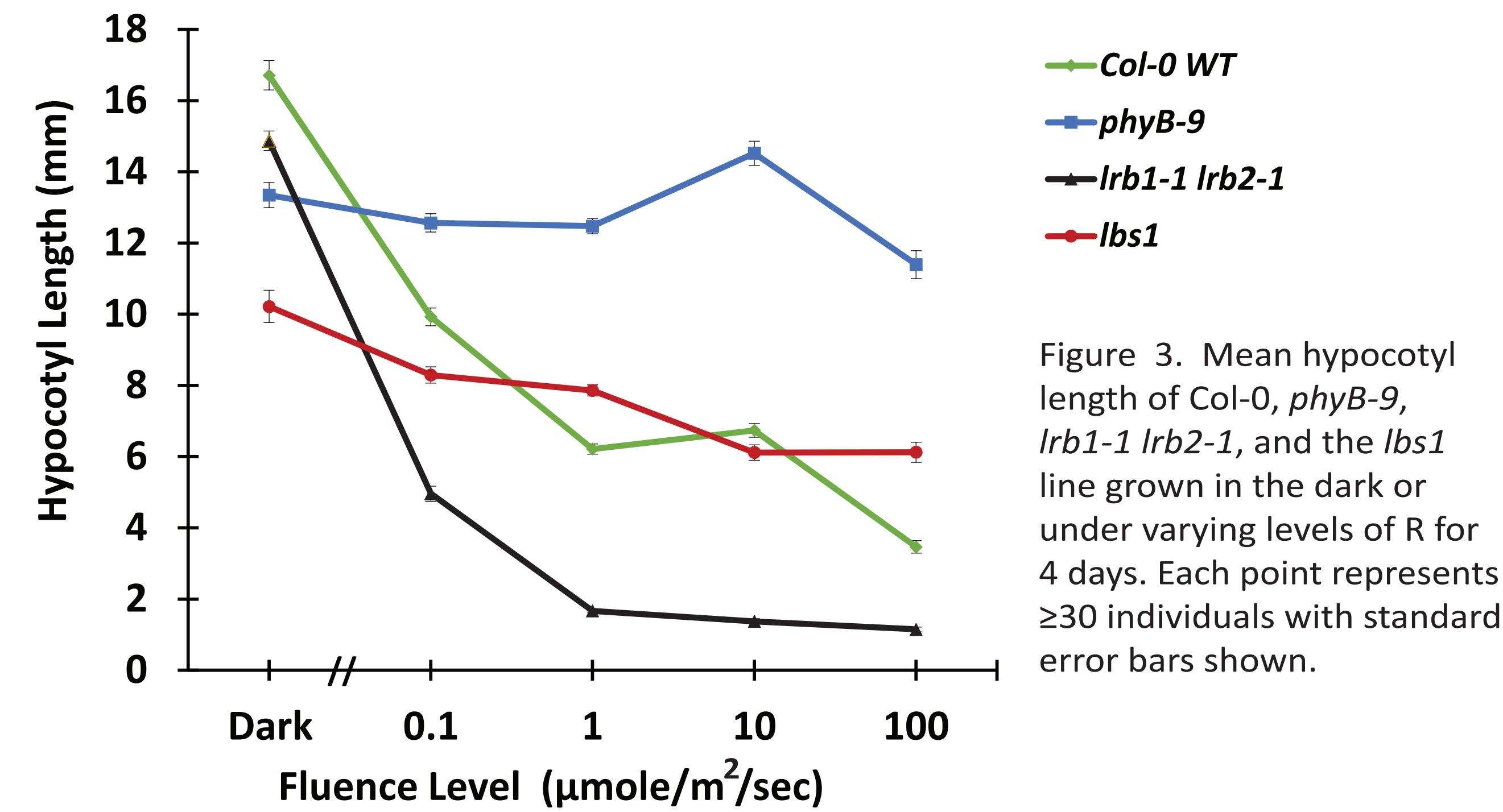


Figure 3. Mean hypocotyl length of Col-0, *phyB-9*, *lrb1-1 lrb2-1*, and the *lbs1* line grown in the dark or under varying levels of R for 4 days. Each point represents ≥30 individuals with standard error bars shown.

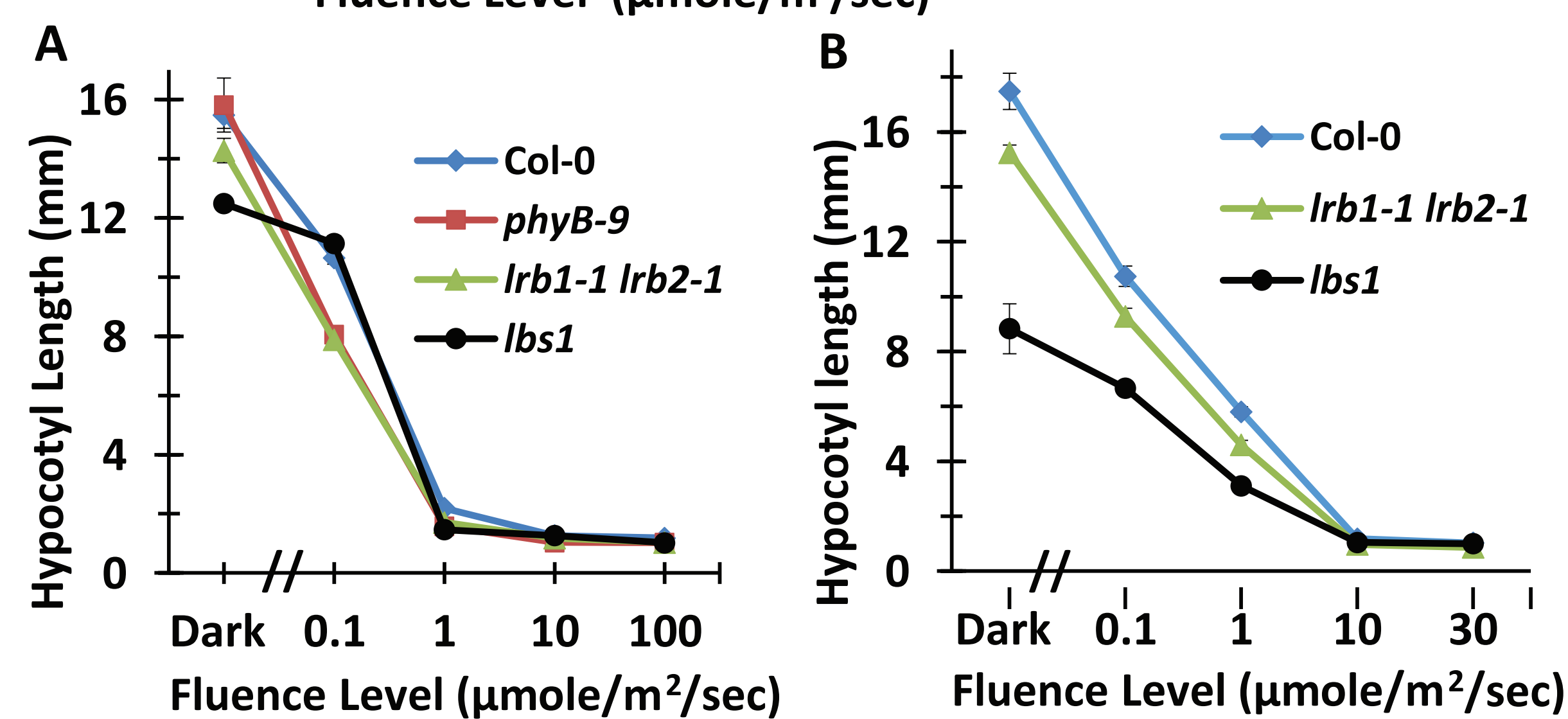


Figure 4. Mean hypocotyl length of Col-0, *phyB-9*, *lrb1-1 lrb2-1*, and *lbs1* grown in the dark or under varying levels of FR (A) or B (B) for 4 days. Each point represents ≥30 individuals with standard error bars shown.

## The *lbs1* Line Has a Mutation in the *PHYB* Gene

The location of the mutation in *lbs1* was determined through mapping using genetic markers. These markers narrowed the region of the mutation to be in a small section of the genome that contained amongst others the *PHYB* gene. We sequenced the *PHYB* gene in the *lbs1* mutant and discovered the mutation in the last nucleotide of the first intron (Figure 5). We discovered that the mutation was preventing proper *PHYB* splicing of intron 1 (Figure 6B), and resulted in no *PHYB* protein being detected in extracts from seedlings with the *lbs1* mutation (Figure 6A). This suggests *lbs1* is a *PHYB* null mutant.

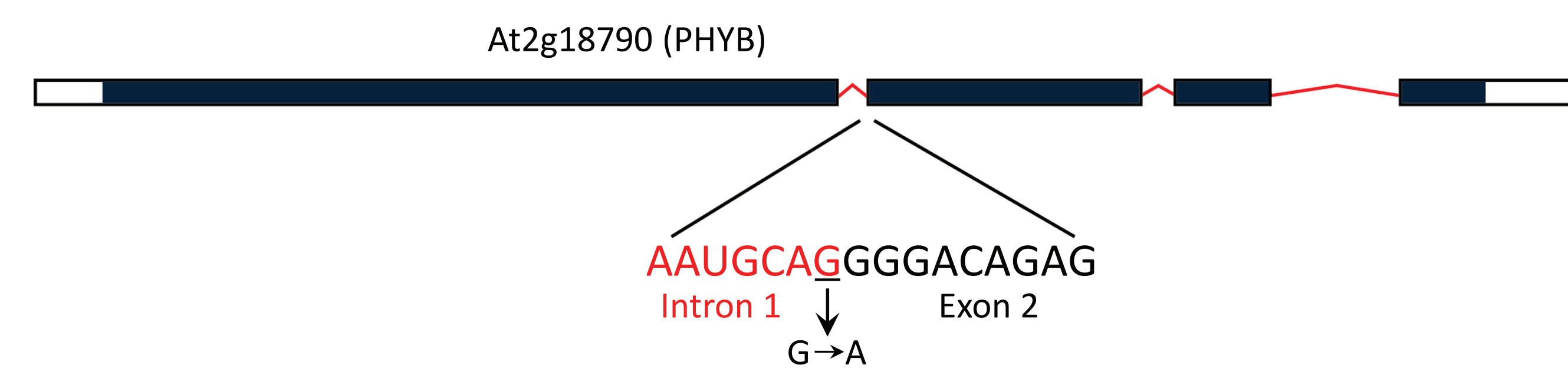


Figure 5. The *lbs1* line has a single mutation in the *PHYB* gene, changing the final nucleotide of the first intron from a G to an A. This mutation prevents splicing of intron 1 (figure 6B).

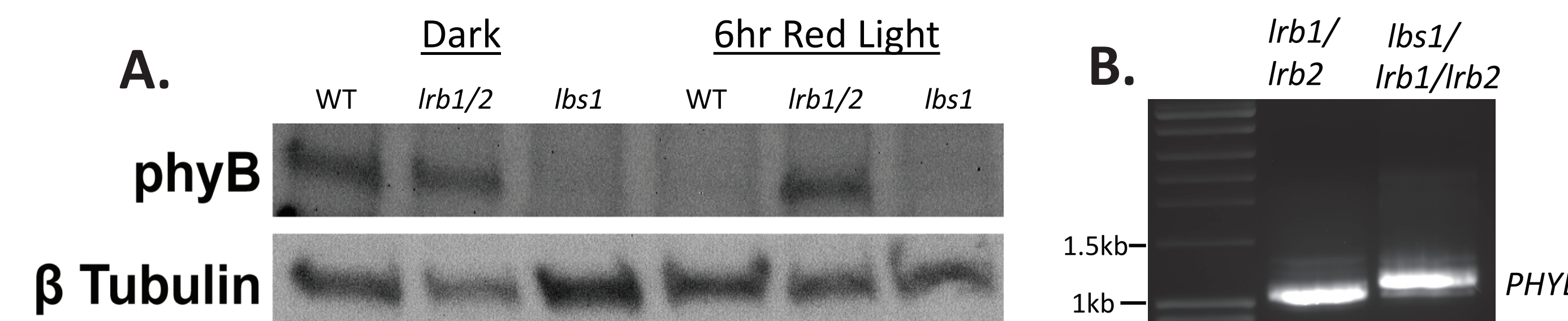


Figure 6. A. Immunoblot analysis of phyB protein levels in crude extracts from wild-type (WT), *lrb1/lrb2*, and *lbs1/lrb1/lrb2* seedlings. Extracts were taken from seedlings grown for 5 days in the dark ("Dark") and seedlings that received an additional 6 hour-long 20 μmol/m<sup>2</sup>/sec R treatment after the 5-day dark treatment ("R"). An anti-β Tubulin antibody was used to confirm equal protein loading. B. RT-PCR analysis of *PHYB* in *lrb1/lrb2*, and *lbs1/lrb1/lrb2*. This result suggests improper splicing of intron 1 as the RT-PCR product produced from plants with the *lbs1* mutation is longer. Sequencing of the RT-PCR products confirmed that intron 1 is not spliced out in plants with the *lbs1* mutation. *PAE2* was used as a control.

## Other *lrb1/lrb2* Suppressor/Enhancer Mutant Lines

Suppressor mutant lines *lbs3*, *lbs6*, *lbs9*, and *lbs10* display suppression of the *lrb1/lrb2* parental line with reduced sensitivity to R inhibition of hypocotyl elongation (Figure 7). Immunoblot analysis shows *PHYB* levels vary between mutants with some having high levels and others having no detectable *PHYB* present (Figure 8). Enhancer mutant *lbe1* displays an unusual phenotype with hypersensitivity to R and B (Figure 9), but WT-like sensitivity to FR (not shown).

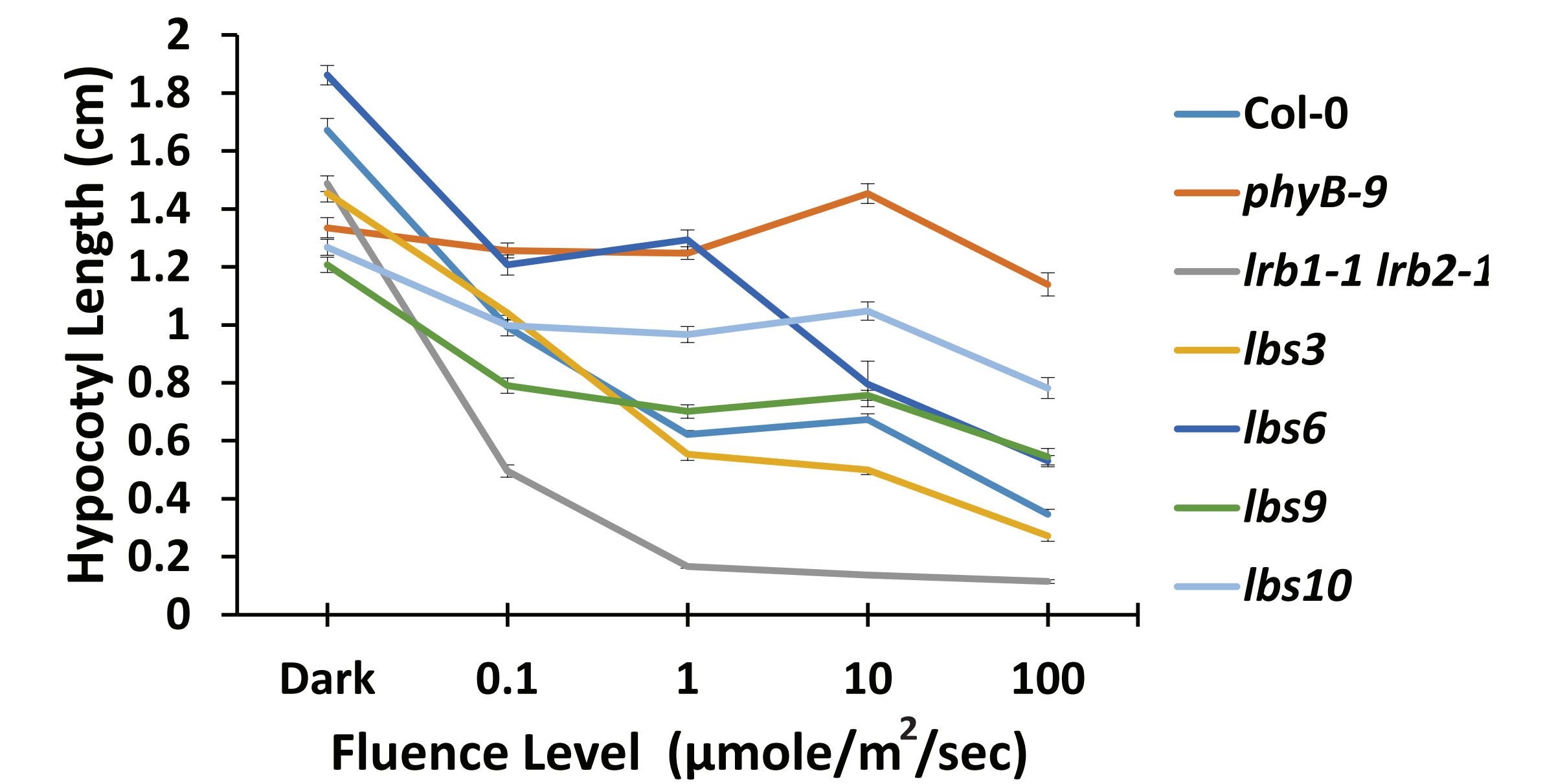


Figure 7. Mean hypocotyl length of Col-0, *phyB-9*, *lrb1-1 lrb2-1*, *lbs3*, *lbs6*, *lbs9*, and *lbs10* lines grown in the dark or under varying levels of R for 4 days. Each point represents ≥30 individuals with standard error bars shown.

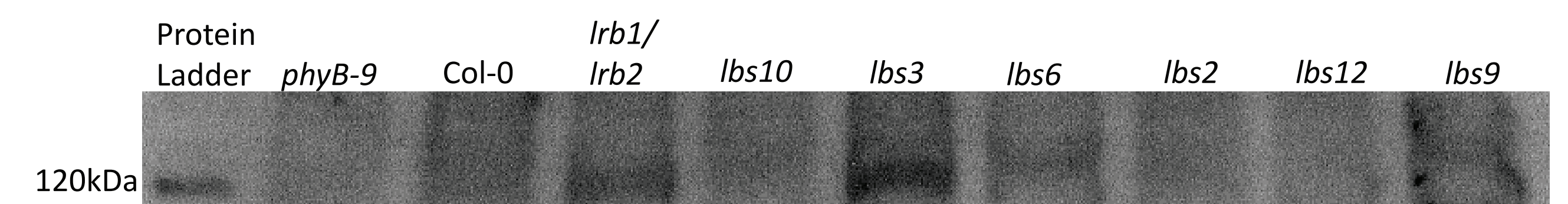


Figure 8. Immunoblot analysis of *PHYB* protein levels in crude extracts from *phyB-9* mutants, wild-type (WT), *lrb1/lrb2*, *lbs10*, *lbs3*, *lbs6*, *lbs2*, *lbs12*, and *lbs9* seedlings. Extracts were taken from seedlings grown for 5 days in the dark ("Dark"). *lbs10* and *lbs6* appear to have low levels of *PHYB* and *lbs2* and *lbs12* appear to have none.

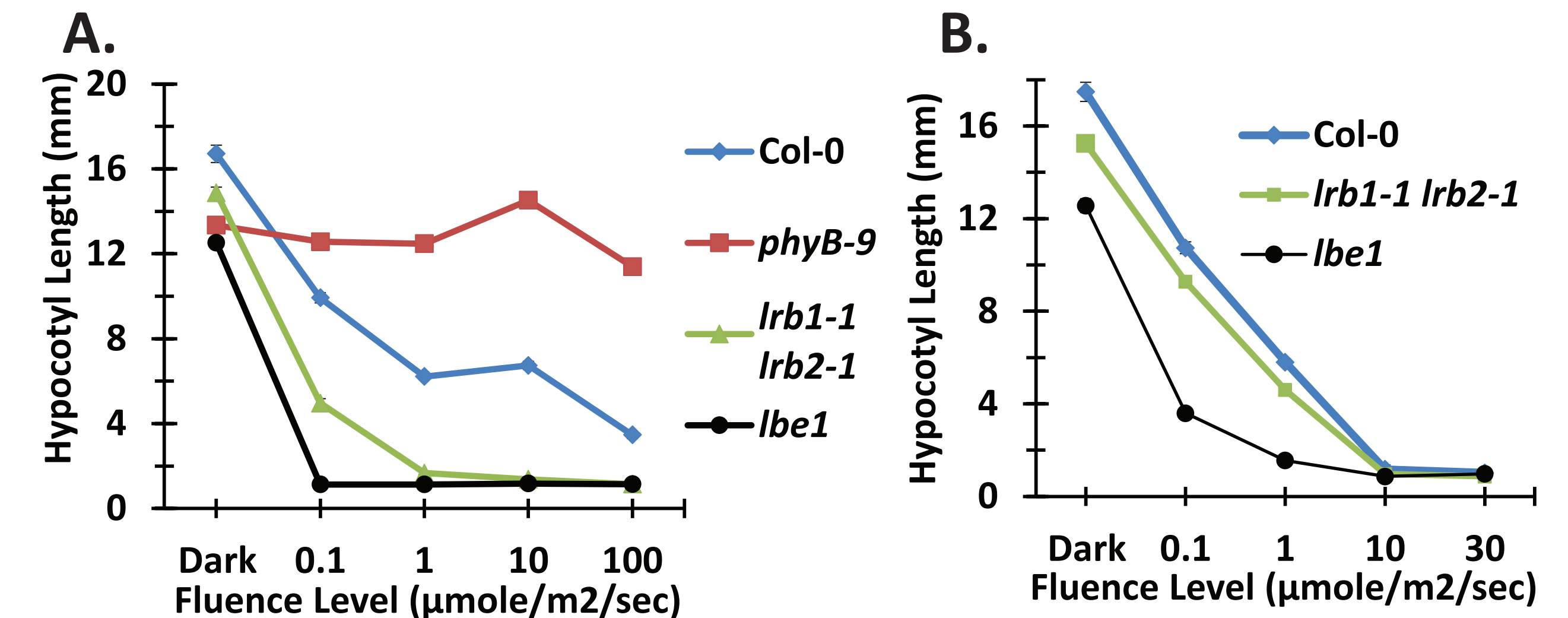


Figure 9. Mean hypocotyl length of Col-0, *phyB-9*, *lrb1-1 lrb2-1*, and *lbe1* grown in the dark or under varying levels of R (A) or B (B) for 4 days. Each point represents ≥30 individuals with standard error bars shown.

## Conclusions and Future Work

- Our suppressor and enhancer screen successfully identified mutants which reduce or enhance the phenotype of a red light hypersensitive mutant in *Arabidopsis thaliana*.
- We have identified one suppressor mutant that has a single mutation in the *PHYB* gene preventing expression of *PHYB*.
- We are currently investigating more mutants, including some that have reduced or are lacking *PHYB* expression.
- We are beginning to identify more mutations present in our mutants using full genome sequencing.

## References

- Christians, Matthew J., Gingerich, Derek J., Hua, Zhihua, Lauer, Timothy D., Vierstra, Richard D. (2012). The Light-Response BTB 1 and 2 Proteins Assemble Nuclear Ubiquitin Ligases That Modify Phytochrome B and D signaling in *Arabidopsis*. *Plant Physiology*, published online June 25th, 2012. doi:10.1104/pp.112.199109
- Mathews, Sarah. (2006). Phytochrome-mediated Development in Land Plants: Red Light Sensing Evolves to Meet the Challenges of Changing Light Environments. *Molecular Ecology*, 15, 3483-3503.