The Roles of Photoreceptor Systems and the COP1-Targeted Destabilization of HY5 in Light Control of Arabidopsis Seedling Development[1]

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Arabidopsis seedlings display contrasting developmental patterns depending on the light environment, e.g. photomorphogenesis in the light and skotomorphogenesis (etiolation) in darkness. Recent studies have implicated protein degradation as a means of regulating this developmental switch (Osterlund et al., 2000). For example, HY5, a positive photomorphogenic regulator that acts to promote the light developmental pattern (Koornneef et al., 1980; Oyama et al., 1997), is subject to the control of negative photomorphogenic regulators such as COP1 and COP9 signalosome at the level of protein stability (Osterlund et al., 1999, 2000). Light controls this process by modulating the subcellular localization of COP1. COP1 is predominantly cytoplasmic in the light, but accumulates in the nuclei in the darkness where it directly interacts with HY5 (von Arnim and Deng, 1994; Ang et al., 1998). A nuclear COP1-HY5 interaction is necessary for the degradation of HY5 (Osterlund et al., 2000).

HY5 is a basic Leu zipper-type transcription factor and is localized in the nucleus constitutively (Oyama et al., 1997; Ang et al., 1998). It has been shown that HY5 binds to the G-box motif of multiple light-inducible promoters and is necessary for optimal expression of the corresponding genes (Ang et al., 1998; Chattopadhyay et al., 1998a, 1998b). The dark-dependent degradation of HY5 provides a reasonable means by which the activity of HY5 and ultimately HY5-mediated gene expression could be regulated in responsive to light. We set out to examine HY5-mediated gene expression as a measure of its activity in correlation with HY5 protein levels.

THE ACTIVITY OF HY5 CORRELATES TO ITS CELLULAR ABUNDANCE

Previous reports have shown that the abundance of HY5 directly corresponds with an etiolated seedling development (Osterlund et al., 2000). It is important to verify that the activity of HY5 correlates to protein levels. To determine the activity of HY5, we used a previously established promoter-reporter transgene (*RbcS-GUS*) that expresses the β-glucuronidase reporter gene (GUS) from a Rubisco small subunit gene (Rbcs) promoter. It has been shown that light-regulated optimal expression of GUS in the *RbcS-GUS* transgenic seedlings is dependent on HY5 (Chattopadhyay et al., 1998a, 1998b). Consistent with the recent reports (Osterlund et al., 2000), Figure 1A (bottom) shows that the abundance of HY5 protein decreases with decreasing fluence of the light. It is important to note that in transgenic seedlings grown under various intensities of continuous white light, the RbcS-GUS expression decreases proportionally with the level of HY5 and light fluence (Fig. 1A, top). This finding suggests that the activity of the RbcS promoter correlates with the cellular level of HY5, and that modulation of HY5 abundance by COP1 could directly influence HY5-mediated gene expression.

MULTIPLE WAVELENGTHS OF LIGHT REGULATE HY5 ACTIVITY

It has been shown that HY5 acts as a positive regulator of photomorphogenesis in all light conditions, confirmed by the long hypocotyl phenotype of *hy5* mutants in white, red, blue, and far-red light (Koornneef et al., 1980; Ang and Deng, 1994). As a transcription factor, HY5 must act downstream of multiple wavelength-specific signal transduction pathways to regulate the expression of its target genes (Ang et al., 1998; Chattopadhyay et al., 1998). We recently showed that HY5 abundance is regulated by multiple photoreceptor systems (Osterlund et al., 2000). To extend this study, we examined the effect of an *hy5*-null mutation on the downstream target gene expression under the light wavelengths that selectively activate individual photoreceptors. The *Rbcs-GUS* reporter transgene was introduced into the null *hy5–1* mutant and its expression under various wavelengths of light was examined (Fig. 1B). It is clear that the activity of the Rbcs promoter is reduced in an *hy5* mutant background relative to the
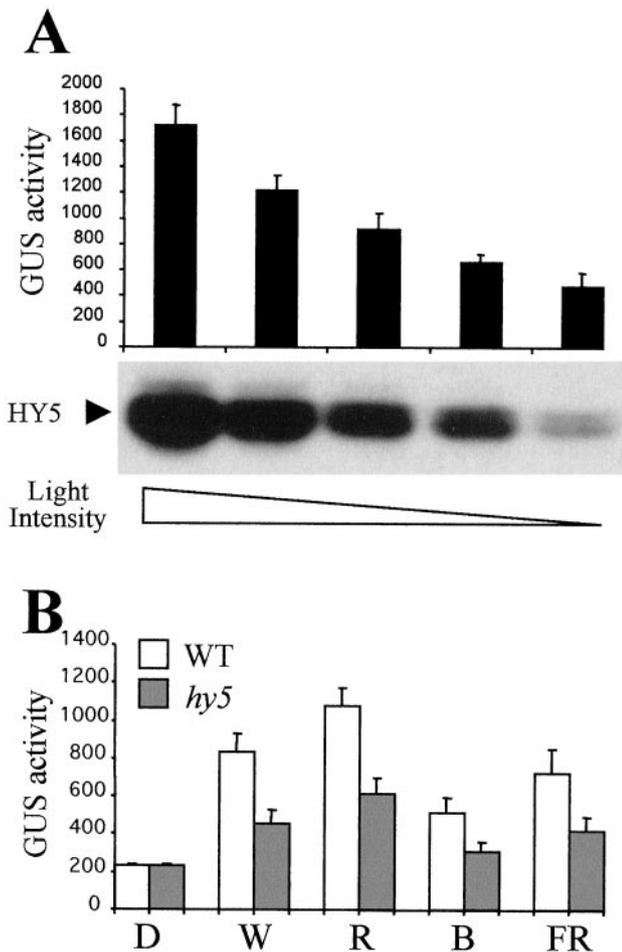
Figure 1. Analysis of HY5 in mediating light activation of its target promoter. A, Rbcs promoter activity as estimated via GUS from *RbcS-GUS* transgenic seedlings grown in continuous white light for 5 d (top). Seedlings were grown in decreasing light intensities from left to right. The fluence rates were 127.5, 19.0, 8.7, 2.8, and 0.6 μmol s^{-1} m^{-2}, respectively (Osterlund et al., 2000). GUS activity is measured in arbitrary units. αHY5 western on the seedlings used for the GUS assay is shown at the bottom. B, The RbcS-GUS activity from wild type and the *hy5–1* seedlings grown in continuous darkness (D), white light (W), red light (R), blue light (B), and far-red light (FR). The reporter GUS activity is measured in arbitrary units. All error bars indicate SD between four samples.

wild type in all light conditions. This result supports the notion that the binding of HY5 to the RbcS promoter is essential for optimal expression under all light conditions. This result also implied that signaling pathways, initiated by different photoreceptors, converge to regulate HY5.

Although HY5 is required for optimal light activation of the RbcS promoter, we noted that in no light environment has the RbcS promoter activity in the *hy5–1* mutant reduced to that of dark-grown seedlings (Fig. 1B). This finding suggests that additional positive regulators other than HY5 must contribute to the expression of the light-inducible gene. This finding is consistent with the partially etiolated phe-

notype of the *hy5*-null mutant (Oyama et al., 1997) and a previous finding that overexpression of HY5 alone has no obvious phenotypic effect in darkness (Ang et al., 1998).

ISOLATION OF NEW *hy1* AND *hy2* ALLELES AS ENHANCER MUTATIONS OF AN *hy5*-NULL MUTANT

In an effort to identify additional factors that act together with HY5 to promote photomorphogenesis, we employed genetic screening for extragenic mutations that enhance the partially etiolated phenotype of a null *hy5* mutant (*hy5-ks50*; Oyama et al., 1997). Using ethyl methanesulfonate as the mutagen, 31 double mutants exhibited elongated hypocotyls (compared with the *hy5-ks50* parent) with either open or closed cotyledons (Fig. 2A). Seven mutants that exhibited consistent and the most dramatic long hypocotyl phenotypes under white, red, blue, and far-red lights were selected for further analysis. Complementation tests revealed that these seven recessive mutants belong to two complementation groups. The first group was represented by a single allele, mutant *88.1* (Fig. 2A). In addition to the seedlings' long hypocotyl phenotype, the *88.1* mutant displayed a severe adult phenotype with small flowers and dramatically reduced fertility (data not shown). The remaining six mutations are all allelic. *134.1* displayed the most dramatic seedling phenotype (Fig. 2A).

To further characterize the enhancer mutations, we first outcrossed mutant *134.1* to wild-type plants. In the F$_2$ generation, we noted that seedlings carrying the homozygous *134.1* mutation exhibited a long hypocotyl phenotype on its own (Fig. 2B), and segregated as a single recessive trait. The white light-grown seedlings containing the *134.1* mutation in a wild-type background resembled hy5 mutants with slightly closed cotyledons (Fig. 2B). To confirm the genotypes of the segregated populations (wild type, *hy5*, *134.1*, and *hy5/134.1*), F$_2$ seedlings were allowed to mature and their offspring were used for an HY5 western blot. Figure 2B (bottom) shows that HY5 is present in the *134.1* mutant but not in the *hy5* or *hy5/134.1* mutants. Therefore, *hy5* and *134.1* have additive effects that result in a highly etiolated seedling phenotype in the white light.

To determine if *134.1* is allelic to any known long hypocotyl mutants (Koornneef et al., 1980), the location of the mutation was mapped using PCR-based markers and located close to marker nga126, on the upper arm of chromosome three (Fig. 2C). This position was confirmed by the mapping positions of four additional allelic mutants isolated from this screen (*174.2*, *92.1*, *208.2*, and *1.1*). Of the known long hypocotyl mutants, *hy2* is located within 5 centimorgans of nga126. A complementation test revealed that *134.1* is allelic to *hy2*, indicating that the *1.1*, *1.3*, *92.1*, *134.1*, *174.2*, and *208.2* mutations defined six new alleles of the *HY2* locus (data not shown).
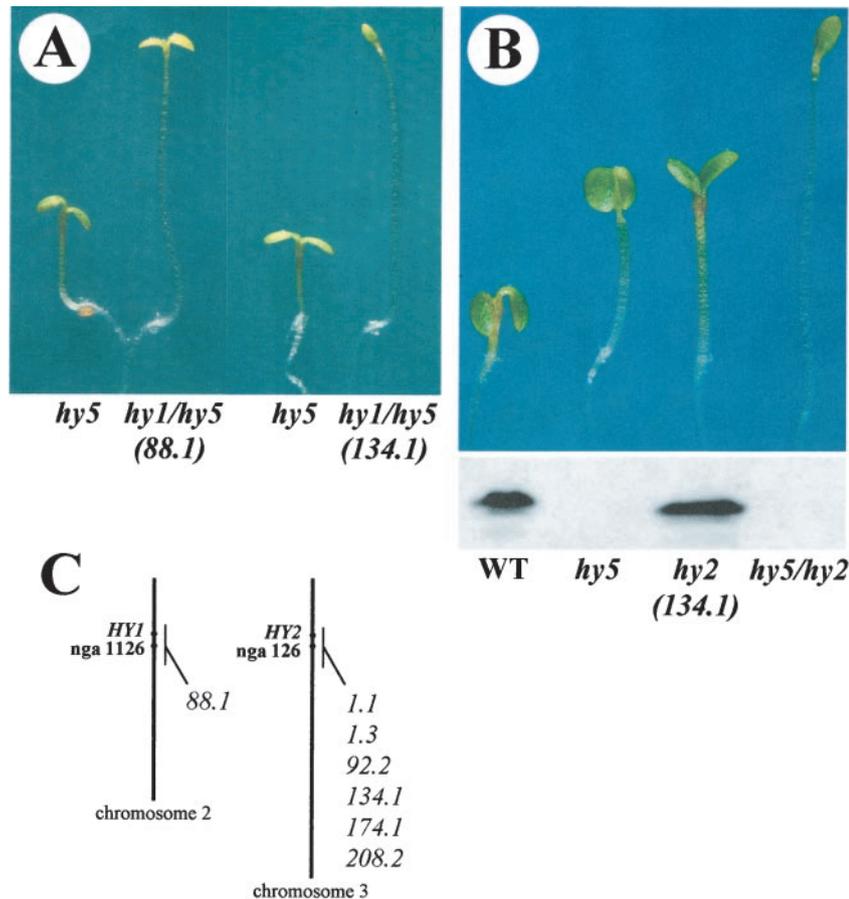
Figure 2. Characterization of the *hy5* enhancer mutants. A, Enhancer mutants are shown in comparison with the parental *hy5-ks50* mutant. Seedlings were grown in continuous white light for 5 d. Left, The *hy5* seedling beside a *hy1/hy5* (*88.1*) double mutant. Right, The *hy5* seedling beside a *hy2/hy5* (*134.1*) double mutant. B, The representative F₂ segregating seedlings from the *hy2/hy5* double mutant crossed to a wild-type plant. The αHY5 western corresponding to the segregating populations is shown in the bottom. The western verifies the wild-type (WT), *hy5*, and *hy2* genotypes in the four types of segregants. C, Genomic map positions of the seven *hy5* enhancer mutations as determined by PCR-based mapping.

Past studies has revealed that the HY2 protein defines a biochemical step in the biosynthesis of the phytochrome chromophore, phytochromobilin from heme (Parks and Quail, 1991; Goto et al., 1993). Thus the *hy2* mutants are expected to have reduced activity of all phytochromes. Therefore, it is possible that the other nonallelic *88.1* mutant might be related to *HY1*, the only other known gene in addition to *HY2* that is involved in phytochrome chromophore biosynthesis (Parks and Quail, 1991; Davis et al., 1999). Fine mapping of the *88.1* mutation located it close to the *HY1* locus. To confirm an allelic relationship between *88.1* and *hy1*, *88.1/hy5* double mutants were crossed to *hy1* plants. Although the F₁ seedlings were shorter than the *88.1/hy5* parent, they are all quite similar to *hy1* mutants (data not shown). Thus mutant *88.1* is allelic to *hy1*. Therefore, all strong enhancers of *hy5* that were selected for the study disrupt chromophore biosynthesis and phytochrome activity.

GUS-COP1 IS A CONSTITUTIVE NUCLEAR LOCALIZED IN AN *hy1* MUTANT

The identification of new *hy1* and *hy2* mutations as enhancers of *hy5* indicates that the phytochrome sig-naling pathways promote seedling photomorphogenic development by both HY5-dependent and -independent mechanisms. This finding is consistent with the speculation that other transcription factors in addition to HY5 are involved in promoting photomorphogenesis (Osterlund et al., 1999). In contrast, null *cop1* mutants completely lose the ability to undergo etiolation and affect expression of a wide range of genes (Deng et al., 1991). It has been proposed that multiple photoreceptors regulate COP1 activity, whereas COP1 in turn regulates multiple transcription factors, including HY5, that promote photomorphogenesis (Hardtke et al., 2000; Osterlund et al., 2000). Because COP1 targets proteins for degradation (Osterlund et al., 2000), mutations that increase the nuclear abundance of COP1 could also decrease the activities of these transcription factors, causing etiolated phenotypes. Therefore, we investigated whether the enhancement of the *hy5* phenotype by the *hy1* and *hy2* mutants might be attributable to increased nuclear accumulation of COP1. To test this, the subcellular localization of COP1 fusion protein was analyzed in an *hy1* mutant background. A translation fusion between the GUS and COP1 (*GUS-COP1*) has been shown to represent the subcellular distribution of COP1 (von Arnim and Deng, 1994).

After crossing the *GUS-COP1* transgene with the *hy1–1* (20.84N) mutant (Koornneef et al., 1980), we analyzed the subcellular localization of COP1.

To determine the abundance of GUS-COP1 in the hypocotyl nuclei, a semiquantitative analysis of nuclear GUS staining (Osterlund and Deng, 1998) was used. In this analysis, hypocotyl cells showing strong nuclear staining were given a value of three plus signs (+++), those with weak but obvious nuclear staining were given a value of two plus signs (++), and those with undetectable nuclear staining were given a value of one plus sign (+). This analysis was conducted with the *hy1* seedlings that were grown in various wavelengths of light for 5 d. As shown in Figure 3, GUS-COP1 accumulates to high levels in the hypocotyl nuclei of *hy1* seedlings grown in all light conditions tested. This result reinforces the notion that COP1 activity is under the control of upstream photoreceptors (Osterlund and Deng, 1998) and further indicates that the phytochrome system is required not only for the red and far-red light control of COP1 nuclear localization, but it is also essential for the blue-light control of COP1 nuclear abundance. Therefore, it represents another example of functional interplay between phytochrome and cryptochrome systems (Ahmad and Cashmore, 1997; Chattopadhyay et al., 1998a, 1998b; Neff and Chory, 1998).
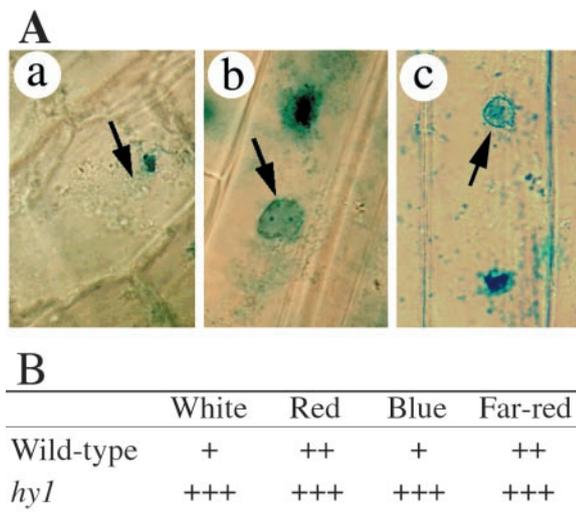
Figure 3. Effect of *hy1* mutation on GUS-COP1 nuclear localization. A, Subcellular localization of GUS-COP1 in representative wild-type and *hy1* seedlings. a, Typical cytoplasmically localized GUS-COP1 in a hypocotyl cell from wild-type seedlings grown in continuous white light. b, Typical nuclear-localized GUS-COP1 in a hypocotyl cell from wild-type seedlings grown in continuous darkness. c, Nuclear-localized GUS-COP1 in a hypocotyl cell from *hy1* seedlings grown in continuous white light. Arrows indicate the locations of the nuclei. B, The subcellular localization of GUS-COP1 in wild-type and *hy1* seedlings grown in white light, red light, blue light, and far-red light. Seedlings showing strong nuclear staining of GUS-COP1 received a value of +++, seedlings with slight but obvious nuclear staining received a value of ++, and seedlings displaying no observable nuclear staining received a value of +.

Taken together, the enhancement of the *hy5* phenotype by *hy1* and *hy2* might be a result of the increased nuclear localization of COP1, which might down-regulate HY5 and, more importantly, other photomorphogenesis-promoting factors.

CONCLUDING REMARKS

In an extension of our recent report (Osterlund et al., 2000), we verified that the abundance of HY5 does correspond with expected HY5 activity. There is a strong correlation between the activity of HY5-dependent RbcS promoter and the level of HY5 protein under changing light intensity. Further, optimal activation of the RbcS promoter under different light regions also requires HY5, correlating well with the reported HY5 abundance under those light conditions (Osterlund et al., 2000). These data together support the notion that COP1-targeted degradation of HY5 can in fact act as a means to regulate HY5-mediated gene expression. In addition, the likely influence of COP1 on multiple transcription factors, along with the light-regulated subcellular localization of COP1, provides an attractive model to explain the molecular switch between photomorphogenic and etiolated development.

The isolation of new alleles of *hy1* and *hy2* as enhancers of the *hy5-ks50* mutation revealed additional insights about the role of photoreceptors, COP1, and HY5 in mediating light control of Arabidopsis development. The *hy1* and *hy2* mutations, which are defective in all phytochromes, likely disrupt the normal activation of parallel photomorphogenic-promoting factors that act in coordination with HY5 to promote photomorphogenic development. As COP1 is constitutively localized in the nuclei in the *hy1*-null mutants, it seems possible that the increased nuclear accumulation of COP1 negatively regulates activities of multiple photomorphogenesis-promoting transcription factors, resulting in the enhanced etiolation of the *hy1/hy5* or *hy2/hy5* double mutants.

It is tempting to speculate why all the dramatic enhancer mutations of *hy5* in our screen are new *hy1* or *hy2* alleles. Because the functions of individual photoreceptors are partially overlapping, mutations in individual phytochromes or cryptochromes might only cause weak or subtle enhancement of the *hy5* mutant phenotype, whereas the chromophore deficiency would result in a defect in all phytochromes and lead to dramatic enhancement of etiolation. We cannot rule out that the limited nature of our screen might simply miss the identification of parallel transcription factors that act together with HY5 to promote photomorphogenesis. However, an alternative explanation is that mutations in any individual transcription factor beside HY5 would cause weak or subtle effect on the seedling development and would not be included in this dramatic enhancer collection.

In the latter case, the future characterization of the remaining weak *hy5* enhancer mutations from our initial screen could prove quite valuable.

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