The Plant Cell, Vol. 26: 4200–4213, October 2014, www.plantcell.org

UV-B-Responsive Association of the *Arabidopsis* bZIP Transcription Factor ELONGATED HYPOCOTYL5 with Target Genes, Including Its Own Promoter[W][OPEN]

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In plants subjected to UV-B radiation, responses are activated that minimize damage caused by UV-B. The bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) acts downstream of the UV-B photoreceptor UV RESISTANCE LOCUS8 (UVR8) and promotes UV-B-induced photomorphogenesis and acclimation. Expression of *HY5* is induced by UV-B; however, the transcription factor(s) that regulate *HY5* transcription in response to UV-B and the impact of UV-B on the association of HY5 with its target promoters are currently unclear. Here, we show that HY5 binding to the promoters of UV-B-responsive genes is enhanced by UV-B in a UVR8-dependent manner in *Arabidopsis thaliana*. In agreement, overexpression of REPRESSOR OF UV-B PHOTOMORPHOGENESIS2, a negative regulator of UVR8 function, blocks UV-B-responsive HY5 enrichment at target promoters. Moreover, we have identified a T/G-box in the *HY5* promoter that is required for its UV-B responsiveness. We show that HY5 and its homolog HYH bind to the T/G^{HY5}-box *cis*-acting element and that they act redundantly in the induction of *HY5* expression upon UV-B exposure. Therefore, HY5 is enriched at target promoters in response to UV-B in a UVR8 photoreceptor-dependent manner, and HY5 and HYH interact directly with a T/G-box *cis*-acting element of the *HY5* promoter, mediating the transcriptional activation of *HY5* in response to UV-B.

INTRODUCTION

UV-B radiation (UV-B; 280 to 315 nm) is a critical regulatory signal that induces photomorphogenic responses in plants (Heijde and Ulm, 2012; Li et al., 2013; Jenkins, 2014). These UV-B-induced responses are mediated by the photoreceptor UV RESISTANCE LOCUS8 (UVR8) in *Arabidopsis thaliana* (Rizzini et al., 2011) and include hypocotyl growth inhibition (Ballare et al., 1995; Kim et al., 1998; Favory et al., 2009), altered leaf morphogenesis (Hectors et al., 2007; Wargent et al., 2009), stomatal closure (Tossi et al., 2014), and the biosynthesis of UV light-absorbing "sunscreen" compounds (Beggs and Wellmann, 1994; Kliebenstein et al., 2002; Stracke et al., 2010). Thus, UVR8 regulates the expression of a broad panel of genes that underlie UV-B-dependent photomorphogenic responses and acclimation (Brown et al., 2005; Favory et al., 2009). The acclimation response helps to prevent or repair UV-B damage, and *uvr8* mutants are hypersensitive to chronic levels of UV-B (Kliebenstein et al., 2002; Brown et al., 2005; Favory et al., 2009). Such *uvr8* mutants are specifically impaired in UV-B acclimation and not in the response to acute UV-B stress (González Besteiro et al., 2011).

UVR8 exists as a homodimer in plants and rapidly monomerizes in response to UV-B (Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012). Photoactivated UVR8 then interacts with the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) (Favory et al., 2009; Rizzini et al., 2011; Cloix et al., 2012; Huang et al., 2014), which is a well-known repressor of photomorphogenesis (Lau and Deng, 2012) and also plays an important role in UV-B signaling (Oravecz et al., 2006). As part of the UVR8 photocycle, regeneration of reactive UVR8 occurs by rapid reversion from the monomer to the dimer (Heijde and Ulm, 2013; Heilmann and Jenkins, 2013). The UVR8-interacting and negative feedback regulators REPRESSOR OF UV-B PHOTOMORPHOGENESIS1 (RUP1) and RUP2 (Gruber et al., 2010) facilitate UVR8 redimerization in planta that consequently disrupts the UVR8-COP1 interaction and halts signaling (Heijde and Ulm, 2013).

An important, largely unresolved issue is how UV-B photoreception by UVR8 leads to transcriptional changes. It has been shown that UVR8 itself binds to chromatin in the vicinity of putative target genes via an interaction with histone H2B (Brown et al., 2005; Cloix and Jenkins, 2008). It was suggested subsequently that UVR8 may mediate the recruitment or activation of transcription factors and/or chromatin remodelers. However, the molecular events and the identity of the components mediating the transcriptional regulation of target genes by UVR8 remained

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www.plantcell.org/cgi/doi/10.1105/tpc.114.130716

elusive. It is known that the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) mediates UV-B-induced gene expression changes downstream of UVR8, in partial redundancy with its homolog HYH (Ulm et al., 2004; Brown et al., 2005; Oravecz et al., 2006; Brown and Jenkins, 2008; Stracke et al., 2010; Fehér et al., 2011; Huang et al., 2012). Indeed, HY5 and HYH are thought to govern the majority of the UVR8-mediated UV-B transcriptional responses (Tilbrook et al., 2013; Jenkins, 2014). HY5 is implicated in a positive feedback loop promoting *COP1* expression by binding to a specific UV-B-responsive ACGT-containing element within the *COP1* promoter (Huang et al., 2012). *HY5* itself, as well as *HYH*, is one of the UVR8- and COP1-regulated genes (Brown et al., 2005; Oravecz et al., 2006; Favory et al., 2009), but the transcription factor(s) and *cis*-regulatory element(s) mediating its transcription are not known. HY5 is also known to be posttranslationally stabilized by UV-B (Favory et al., 2009; Huang et al., 2013).

HY5 is a target of the ubiquitin ligase activity of COP1 (Osterlund et al., 2000; Lau and Deng, 2012); thus, its UV-B-sensitive stabilization is likely a consequence of the UVR8-COP1 interaction (Favory et al., 2009). Supporting an important role for HY5 in the UV-B acclimation response, *hy5* mutants are UV-B stress hypersensitive (Brown et al., 2005; Oravecz et al., 2006; Huang et al., 2012). HY5 is known to be a critical positive regulator of light responses, and chromatin immunoprecipitation (ChIP) combined with microarray analysis has demonstrated its association with the promoter region of over 9000 potential target genes (Zhang et al., 2011). However, HY5 is abundant mainly in young seedlings and declines during later developmental stages, in agreement with its main activity at early stages of photomorphogenesis in visible light (Hardtke et al., 2000). Thus, UV-B responses in older seedlings and adult plants depend upon the reengagement of HY5 through UV-B-induced *HY5* expression and protein stabilization (Ulm et al., 2004; Oravecz et al., 2006). Accordingly, ChIP experiments have shown that HY5-yellow fluorescent protein associates with HY5-dependent UV-B-induced genes (Stracke et al., 2010). However, the dynamics of HY5 chromatin association in response to environmental cues, including exposure to UV-B, have been defined to a much lesser extent (Lee et al., 2007; Stracke et al., 2010; Zhang et al., 2011).

To further our understanding of UV-B-induced gene expression changes, we have investigated the UV-B-responsive HY5 association with chromatin. We report here (1) that HY5 chromatin association is enhanced by UV-B in a UVR8 photoreceptor-dependent manner and (2) that this process is impaired in plants overexpressing *RUP2*, a negative feedback regulator of UVR8. We also identify two *cis*-regulatory elements, designated T/G- and E-boxes, which mediate the transcriptional activation of *HY5* in response to UV-B. Furthermore, we show that HY5 and HYH bind to the T/G^{HY5}-box and that they are both required for UV-B-activated *HY5* gene expression.

RESULTS

HY5 Association with Target Genes Is Regulated by UV-B

HY5 has been shown previously to associate with the promoters of its UV-B-responsive target genes *CHALCONE SYNTHASE*

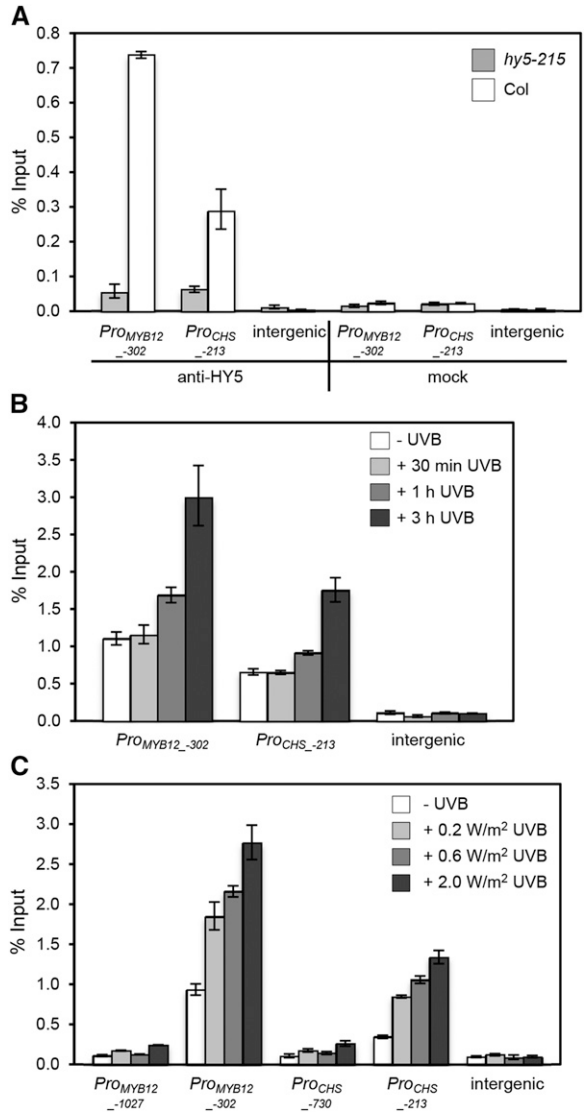

Figure 1. UV-B Enhances HY5 Binding to the Promoters of Its Target Genes *MYB12* and *CHS*.

(A) ChIP of DNA associated with HY5. ChIP-qPCR was performed for the *MYB12* and *CHS* promoters and an intergenic region between the *At4g26900* and *At4g26910* genes using 10-d-old wild-type plants (Col) or *hy5-215* null mutants grown in white light (no UV-B). ChIP was performed with an anti-HY5 antibody or without addition of the antibody (mock). Data shown are representative of five independent experiments.

(B) HY5 ChIP-qPCR using 7-d-old wild-type seedlings grown in weak light and treated with narrowband UV-B for 0.5, 1, and 3 h compared with a −UV-B control.

(C) HY5 ChIP-qPCR using 7-d-old wild-type seedlings grown in weak light and treated for 2 h with different intensities of narrowband UV-B compared with an untreated control (−UV-B).

The numbers of the analyzed DNA fragments indicate the positions of the 5′ base pair of the amplicon relative to the translation start site (referred to as position +1). ChIP efficiency of DNA associated with HY5 is presented as the percentage recovered from the total input DNA (% Input). Error bars represent SD of three technical replicates.

(*CHS*) and *MYB12* (Stracke et al., 2010; Zhang et al., 2011); however, the dynamics of this process in response to UV-B are unclear. In order to test whether UV-B specifically affects the association of endogenous HY5 with its target genes, we performed ChIP experiments using anti-HY5 antibodies. First, we analyzed the chromatin immunoprecipitated DNA for specific enrichment of previously established HY5 target genes, such as *CHS* and *MYB12*. Indeed, endogenous HY5 specifically associated with *CHS* and *MYB12* promoter fragments in wild-type seedlings (Figure 1A). The specificity of the ChIP data was demonstrated by the negative controls provided by the *hy5* mutant and a mock immunoprecipitation without primary antibody as well as by an intergenic region not bound by HY5 (Figure 1A). Together, the data confirm that HY5 ChIP is specific for DNA immunoprecipitated with endogenous HY5 protein and that HY5 associates specifically with its target genes.

We further tested whether UV-B affects the association of HY5 with target promoters. Our data show that HY5 binding to *CHS* and *MYB12* target promoters increased in response to UV-B, detectable as early as 1 h after UV-B exposure (Figure 1B), and that its accumulation at target promoters was dose dependent (Figure 1C). As expected, HY5 did not associate with the intergenic region under any condition tested (Figures 1A to 1C). The specificity of binding was further supported by the absence of a signal for regions ~0.5 kb upstream (*ProMYB12*$_{-1027}$ and *ProCHS*$_{-730}$) of the apparent binding site regions (*ProMYB12*$_{-302}$ and *ProCHS*$_{-213}$) (Figure 1C). Altogether, we conclude that increased promoter occupancy of HY5 at its target genes is an integral part of the UV-B response.

UV-B-Mediated Enhancement of HY5 Chromatin Association Requires UVR8 and Is Negatively Regulated by RUP2

To test whether the UV-B-enhanced association of HY5 with target promoters is regulated by UVR8, we performed HY5 ChIP using the *uvr8-7* null mutant in comparison with the wild type. Whereas the association with HY5 target promoters was similar to that in the wild type in white light, UV-B-mediated enhancement was absent in *uvr8-7* mutants (Figure 2A).

The WD40-repeat protein RUP2 acts as repressor of the UV-B response by facilitating UVR8 redimerization and thus ground state reversion (Gruber et al., 2010; Heijde and Ulm, 2013). In agreement with this activity, *RUP2* overexpression resulted in a reduced UV-B-specific response (Gruber et al., 2010), and this was associated with the absence of UV-B-induced enhancement of HY5 chromatin association at target genes (Figure 2B). Together, the absence of enhanced HY5 association with its target promoters in the *uvr8-7* mutant and *RUP2* overexpression lines underlines the importance of UVR8-mediated UV-B perception and signaling impinging on HY5 activity.

HY5 Binds to Genes Encoding Proteins Involved in UV-B Signaling but Not to *UVR8*

We tested the capacity of HY5 to bind the genomic regions of key UV-B pathway factors, namely *RUP1*, *RUP2*, the B-box family gene *BBX24*/*STO*, *UVR8*, and *COP1* (Tilbrook et al., 2013; Jenkins, 2014). Except for *UVR8*, which is constitutively expressed, these genes are known to be transcriptionally activated by UV-B irradiation (Ulm et al., 2004; Gruber et al., 2010; Huang et al., 2012). In accordance with the UV-B responsiveness, the HY5 association with *RUP1*, *RUP2*, *COP1*, and *BBX24* genomic regions was enhanced in response to UV-B in the wild type (Columbia [Col]) but not in *uvr8-6* null mutants (Figure 3). In contrast with UV-B-regulated genes, no clear association of HY5 with the constitutively expressed *UVR8* gene was detectable (Figure 3). The chromatin association of HY5 with *RUP1*, *RUP2*, *COP1*, and *BBX24* is in agreement with the HY5 dependence of

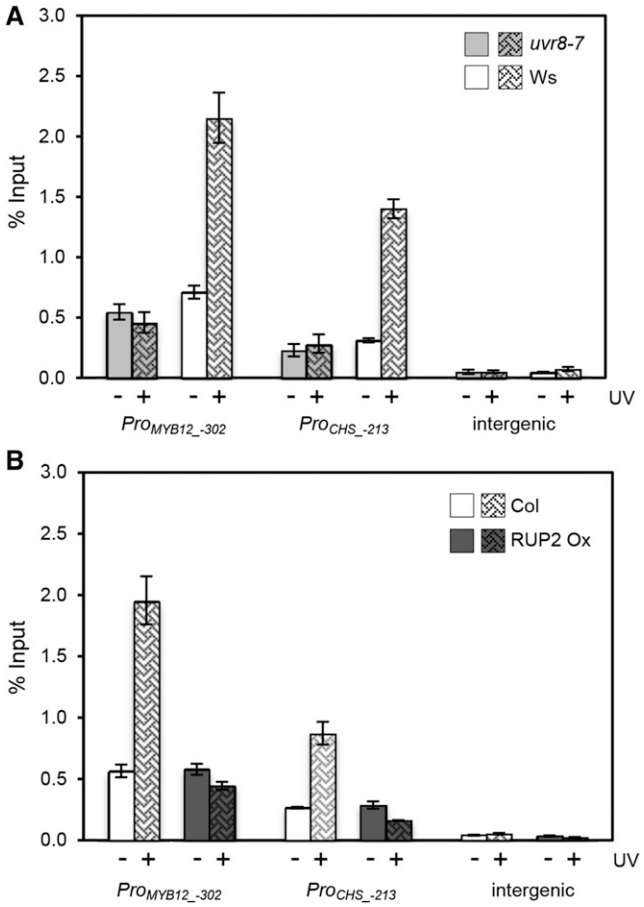

Figure 2. HY5 Chromatin Association Is Regulated by the UVR8 Photoreceptor Pathway.

UV-B-responsive HY5 chromatin association in wild-type plants (Ws and Col) was compared with that in the *uvr8-7* mutant **(A)** and a *RUP2* overexpression line (RUP2 Ox) **(B)**. Seedlings were grown for 7 d in a weak light field and exposed for 2 h to narrowband UV-B. ChIP-qPCR was performed for the *MYB12* and *CHS* promoters and an intergenic region between the *At4g26900* and *At4g26910* genes. The numbers of the analyzed DNA fragments indicate the positions of the 5′ base pair of the amplicon relative to the translation start site (referred to as position +1). ChIP of DNA associated with HY5 is presented as the percentage recovered from the total input DNA (% Input). Data shown are representative of three **(A)** and two **(B)** independent biological replicates. Error bars represent SD of three technical replicates.

their UV-B-induced expression (Gruber et al., 2010; Huang et al., 2012; Jiang et al., 2012) and further underlines the regulatory activity of HY5 chromatin association in UV-B signaling.

HY5 and HYH Bind to the Promoter of *HY5* and Act Redundantly in UV-B-Induced *HY5* Gene Activation

We performed two lines of experiments to test our hypothesis that HY5 and/or its homolog HYH regulates *HY5* gene induction. First, we tested whether HY5 binds to the promoter region of *HY5*. ChIP assays demonstrated that HY5 is indeed associated with its own promoter in white light-grown seedlings and UV-B increases binding in a dose-dependent manner (Figure 4A). Binding of HY5 was specific for the promoter region, as no binding to the *HY5* coding region was detected (Figure 4B). Furthermore, as with the promoters of other HY5 target genes (Figures 2A and 3), the association of HY5 with its own promoter did not depend on UVR8 in the absence of UV-B (Figure 4B).

Sequence conservation, the ability to form heterodimers, and partial functional redundancy between HY5 and HYH predicted a possible involvement of HYH in regulating the activity of the *HY5* promoter (Holm et al., 2002; Brown and Jenkins, 2008; Stracke et al., 2010). Accordingly, ChIP data clearly demonstrated a specific association of endogenous HYH with chromatin in the *HY5* promoter region in wild-type seedlings (Figure 4C). In the ChIP assays, the strongest signal for HY5 and HYH binding to the *HY5* promoter was obtained with the primer pair amplifying the region −414 to −251 bp of the *HY5* promoter (with the first base pair in the cDNA defined as +1) ($ProHY5_{-414}$ in Figures 4B and 4C). Thus, we concluded that both HY5 and HYH bind to the *HY5* promoter in vivo.

To address the dependency of UV-B-mediated *HY5* induction on HY5 and/or HYH proteins, we used transgenic plants expressing a chimeric *ProHY5:LUCIFERASE* (*LUC*) reporter (Ulm et al., 2004) in *hy5*, *hyh*, and *hy5 hyh* mutant backgrounds. After applying supplemental UV-B for 30 min to light-grown seedlings, luciferase activity was monitored over several hours. Induction of luminescence peaked around 4 h after UV-B irradiation to a similar extent in *hy5*, *hyh*, and wild-type background seedlings (Figures 4D and 4E; note that the slight reduction of *hyh* in Figure 4D is due to a slightly elevated basal level of luciferase activity; see Figure 4E). By contrast, *ProHY5:LUC* induction was almost absent in the *hy5 hyh* double mutant background and completely absent in *uvr8* knockout mutants (Figures 4D and 4E). These data demonstrate that HY5 and HYH act redundantly in regulating the UV-B-induced transcription of *HY5*.

Three *cis*-Regulatory Elements Mediate the Transcription of *HY5*, Including a T/G-Box Required for Its UV-B Responsiveness

The rapid and transient induction of *HY5* upon UV-B exposure requires the binding of HY5 and HYH to the promoter of *HY5* (Figures 4A to 4D). To identify the underlying UV-B-specific *cis*-regulatory sequences, we created stable transgenic *Arabidopsis* lines containing chimeric gene constructs of different *HY5* promoter fragments fused to the *LUC* reporter gene. The extent of LUC induction upon UV-B treatment in plants harboring the *HY5* promoter at −157 to −1 bp ($ProHY5_{[-157/-1]}$:*LUC*) (the last base pair before the 5′ untranslated region being defined as −1) was similar to that of plants containing the chimeric construct of the complete *HY5* promoter ($ProHY5_{[-565/+192]}$:*LUC*) (defined as the

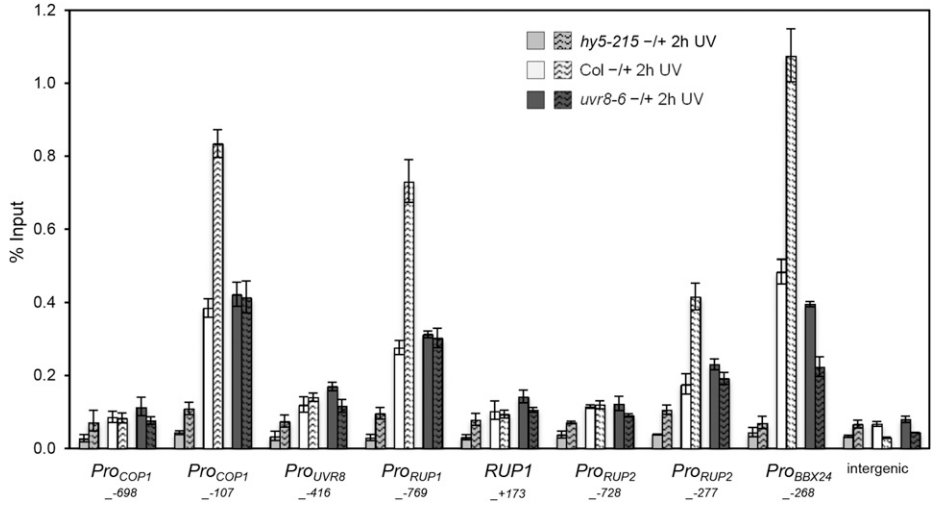

Figure 3. HY5 Associates with *RUP1*, *RUP2*, *COP1*, and *BBX24* but Not with the *UVR8* Promoter.

hy5-215, *uvr8-6*, and wild-type (Col) seedlings were grown for 7 d in a standard growth chamber followed by weak light acclimation for 12 h and narrowband UV-B irradiation for 2 h. ChIP was performed with an anti-HY5 antibody, and copurified DNA was analyzed by qPCR for different primer pairs amplifying parts of the *COP1*, *UVR8*, *RUP1*, *RUP2*, and *BBX24* genomic regions and an intergenic region between genes *At4g26900* and *At4g26910*. The numbers of the analyzed DNA fragments indicate the positions of the 5′ base pair of the amplicon relative to the translation start site (referred to as position +1). ChIP of DNA associated with HY5 is presented as the percentage recovered from the total input DNA (% Input). Data shown are representative of two independent experiments. Error bars represent SD of three technical replicates.

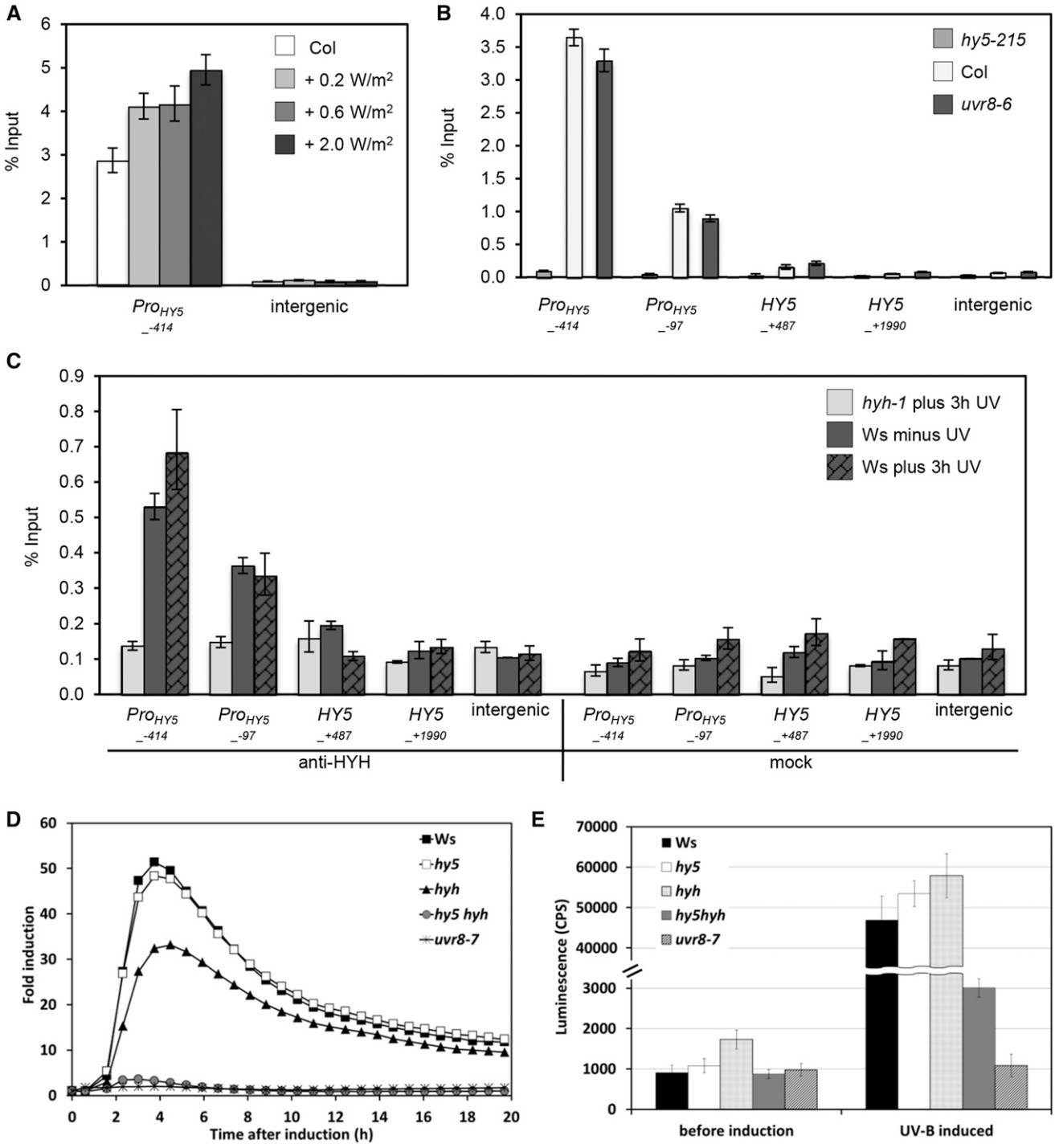

Figure 4. HY5 and HYH Act Redundantly in Inducing *HY5* Gene Expression and Associate with Common Target Genes, Including the *HY5* Promoter.

(A) and **(B)** HY5 associates with its own promoter. Seven-day-old wild-type (Col) seedlings grown in weak light were treated for 2 h with different intensities of narrowband UV-B **(A)**, and *hy5-215*, *uvr8-6*, and wild-type (Col) seedlings were grown for 7 d in a standard growth chamber **(B)**. ChIP was performed with an anti-HY5 antibody, and copurified DNA was analyzed by qPCR for different primer pairs covering the *HY5* genomic locus and an intergenic region between genes *At4g26900* and *At4g26910*. The numbers of the analyzed DNA fragments indicate the positions of the 5′ base pair of the amplicon relative to the translation start site (referred to as position +1). ChIP of DNA associated with HY5 is presented as the percentage recovered from the total input DNA (% Input). Error bars represent SD of three technical replicates.

sequence between the stop TGA of the upstream gene *At5g11270* and the start ATG of *HY5*/*At5g11260*), whereas LUC induction was markedly lower in plants containing *ProHY5*$_{[-65/+192]}$*:LUC* (Figure 5A, compare constructs 1 and 5 with construct 4). Therefore, we performed a linker scan analysis examining the UV-B-responsive region between −157 and −74 within the *ProHY5*$_{[-565/+192]}$*:LUC* construct (Figure 5B). To characterize how the mutations modulate *HY5* activity with or without UV-B irradiation, we measured the basal activity (the luminescence measured in white light-grown seedlings; Figures 5C and 5D) as well as the UV-B-modulated activity of the promoter (the maximal luminescence measured after UV-B treatment; Figures 5C and 5E) and calculated the fold UV-B induction displayed by the various mutants (Figure 5F). Using this approach, we identified three *cis*-regulatory domains designated as a previously undescribed ACG-box (−108-TAACGGC-102), a T/G-box (−90-CACGTT-85), and an E-box (−77-CAATTG-72) that appear to be involved in modulating the activity and UV-B induction of *HY5* (Figure 5). We found that mutations located in the 5′ proximal region of the *HY5* promoter (−157 to −109) did not affect UV-B responsiveness. Mutation of sequences perturbing the ACG-box located between −108 and −102 (MUT8) drastically elevated the basal activity of the mutated promoter in white light (Figure 5D) and marginally increased maximal UV-B-induced fluorescence (Figure 5E). It follows that this mutation significantly lowered the relative UV-B induction of the *HY5* promoter compared with the wild type (6-fold versus 40-fold; Figure 5F). Mutation of sequences between −101 and −95 (MUT9) did not modify either the basal or the maximal UV-B-induced activity of the *HY5* promoter. Mutations perturbing the T/G-box, including MUT10 (−94 to −88) and MUT11 (−87 to −81), uniformly but only moderately elevated the basal activity of the *HY5* promoter in white light (Figure 5D) and reduced maximal UV-B-induced fluorescence (Figure 5E). Accordingly, mutations of the T/G-box strongly reduced the fold UV-B induction of the *HY5* promoter (Figure 5F). The activity of the *HY5* promoter containing a disrupted E-box (MUT12) but intact ACG- and T/G-boxes was again slightly higher than that of the wild type in white light (Figure 5D), and it produced a maximal UV-B-induced fluorescence similar to that in the wild type (Figure 5E), an ~20-fold UV-B induction (Figure 5F).

Interestingly, the *HY5* promoter containing both mutant ACG- and T/G-boxes (MUT8-10) behaved similarly to that of the single T/G-box mutants (MUT10 and MUT11) (Figures 5D to 5F; Supplemental Figure 1A). This suggests (1) that the T/G-box is essential for the full activity of *HY5* in response to UV-B, (2) that the unleashed activity of MUT8 (mutated repressive ACG-box) in visible light also requires a functional T/G-box, and (3) that the ACG element itself is likely to be superfluous for UV-B induction. It is important to note that luminescence levels in the various transgenic seedlings kept in light for 48 h after the UV-B treatment were almost identical to the levels before treatment (Supplemental Figure 1B). Thus, the results above also indicate that perturbation of the ACG-box (1) elevates the activity of the *HY5* promoter in seedlings grown in visible light but (2) does not interfere with deactivation after UV-B irradiation. To test whether the ACG-box is required in the regulation of *HY5* promoter activity in the dark, we determined *LUC* mRNA abundance in wild-type and MUT8 *ProHY5:LUC* plants grown in darkness and in light. Supplemental Figure 1C shows that MUT8 did not significantly affect the activity of the *HY5* promoter in etiolated material but drastically elevated *LUC* mRNA levels, as expected, in light-grown material compared with wild-type *HY5*. We note that Abbas et al. (2014) recently reported the involvement of T/G- and E-boxes in regulating the activity of the *HY5* promoter in visible light. Thus, we also analyzed the induction of the corresponding mutated promoter versions (T/G-box mutated MUT10 and MUT11 as well as E-box mutated MUT12; Figure 5B) and *hy5*, *hyh*, and *hy5 hyh* mutants after illuminating etiolated seedlings with continuous white light for 72 h. Our data illustrate that the induction of *HY5* is transient, it reaches a maximum level 1 to 4 h after the onset of light, and it declines to low levels afterward (Supplemental Figures 2A to 2D). This figure also demonstrates that (1) mutations of the T/G-box eliminate the induction of *HY5* transcription, (2) mutation of the E-box has a much less pronounced effect, and (3) activation of *HY5* transcription by white light is below the detection level in the *hy5 hyh* double mutant. Taken together, we conclude that the T/G-box positively regulates *HY5* activity in visible light, whereas the T/G-box and to a minor extent the E-box, but not the ACG-box, are essential for full induction by UV-B light.

HY5 and HYH Bind to the *cis*-Regulatory T/G-Box in Vitro

We showed above that the activity of the *HY5* promoter in transgenic plants is regulated by three *cis*-regulatory domains, the ACG-, T/G-, and E-boxes, located between −108 and −72. To test which of these binds HY5 and/or HYH, we performed electrophoretic mobility shift assays (EMSAs) using recombinant HY5 and HYH proteins. The target sequence spanned the promoter region of *HY5* between −113 and −62 and thus contained all identified *cis*-regulatory domains. The design of the mutations

Figure 4. (continued).

(C) HYH binds to the *HY5* promoter in vivo. Wild-type plants (Ws) and null mutant *hyh-1* were grown for 7 d in a weak light field and exposed for 3 h to narrowband UV-B. ChIP was performed with an anti-HYH antibody (left) or without the addition of antibody (mock; right). ChIP-qPCR was performed for different primer pairs covering the *HY5* genomic locus and an intergenic region between genes *At4g26900* and *At4g26910*. The numbers of the analyzed DNA fragments indicate the positions of the 5′ base pair of the amplicon relative to the translation start site (referred to as position +1). ChIP of DNA associated with HYH is presented as the percentage recovered from the total input DNA (% Input). Data shown are representative of two independent experiments. Error bars represent SD of three technical replicates.

(D) and **(E)** HY5 and HYH play redundant but essential roles in mediating the responsiveness of the *HY5* promoter to UV-B. Luciferase activity is shown for transgenic wild-type *Arabidopsis* (Ws), *hy5*, *hyh*, *hy5 hyh*, and *uvr8-7* plants carrying the same copy of the full-length *HY5* promoter fused to the *LUC* reporter gene. Thirty-six individual seedlings were assayed for each genotype. Error bars represent SE.

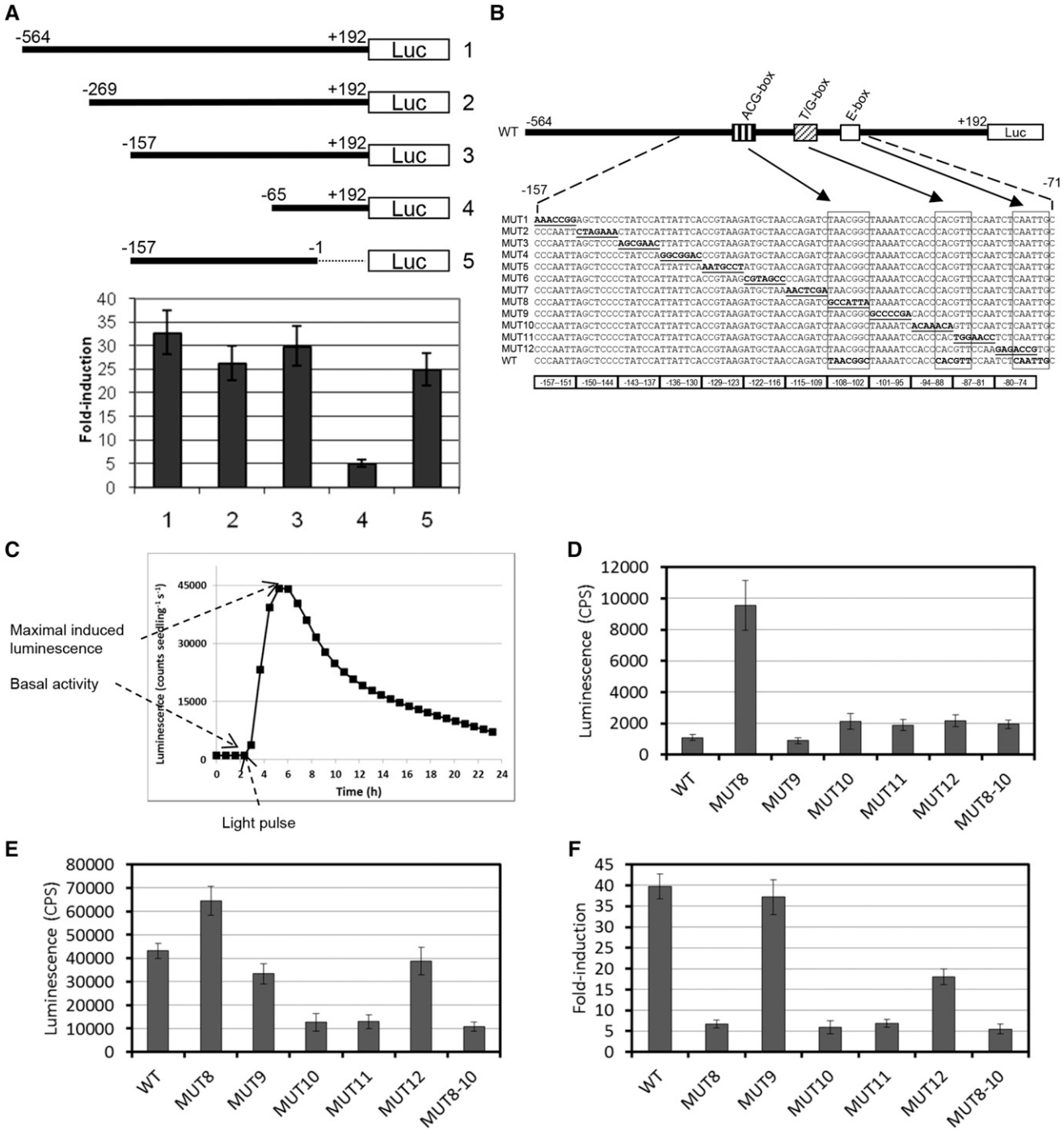

Figure 5. Identification of Functional *cis*-Regulatory Elements of the *HY5* Promoter.

(A) Deletion analysis of the *HY5* promoter. A schematic representation of the 5′ truncated derivatives of the *HY5* promoter fused to the *LUC* reporter gene is shown. Constructs 1 to 4 carry the full 5′ untranslated region of *HY5*, whereas the 5′ untranslated region was replaced by a 35S minimal promoter in construct 5. Nucleotide positions relative to the first base pair of the 5′ untranslated region (transcriptional start site defined as position +1) are shown. Wild-type *Arabidopsis* (Ws) seedlings carrying the reporter constructs were grown in a standard growth chamber for 1 week. Plants were transferred to continuous white light at 10 μmol m^{-2} s^{-1} fluence rate for 48 h. Luminescence measurements were started 3 to 4 h before application of the light pulse. Plants were irradiated with narrowband UV-B for 30 min and then returned to white light, where luminescence measurement was resumed. Twenty-four to 36 individual seedlings were assayed for each line, and two independent transgenic lines were tested for each construct. Error bars represent SE.

facilitated the identification of regulatory elements that can bind HY5 and HYH independently of the other two. The EMSA results clearly demonstrated (1) that the wild-type T/G-box efficiently binds HY5 (Figure 6A) as well as HYH (Figure 6B), even when the ACG- and E-box sequences (MUT8-12) are perturbed, and (2) that mutation of the T/G-box (MUT10) eliminates the binding of HY5 and HYH to the probe containing intact ACG- and E-box elements (Figures 6A and 6B). The results in Figures 6C and 6D demonstrate that binding of HY5 and HYH to the T/G-box cannot be competed off with ACG- and E-box sequences but only with an excess amount of cold probe including an intact T/G-box and, therefore, is sequence-specific. Taken together, we conclude that the T/G-box binds HY5 and HYH and thus plays a critical role in regulating the induction of *HY5* by UV-B irradiation.

DISCUSSION

UV-B-activated UVR8 photoreceptor signaling alters gene expression, which also underpins induced UV-B acclimation and subsequently elevated UV-B tolerance. However, despite their physiological importance, the processes involved in UV-B-mediated transcriptional regulation are poorly understood. HY5 is the major transcription factor required downstream of UVR8. In addition, the *HY5* gene is an excellent and widely used marker for UVR8-mediated gene expression changes (Ulm et al., 2004; Brown et al., 2005, 2009; Oravecz et al., 2006; Gruber et al., 2010; Huang et al., 2012; Jiang et al., 2012). Thus, it is thought that HY5 is required for UV-B-responsive gene activation and that its own transcriptional induction as well as posttranslational stabilization further enforce the UV-B response (Li et al., 2013; Tilbrook et al., 2013; Jenkins, 2014). However, the impact of UV-B on HY5 chromatin association as well as the identity of the *cis*-regulatory elements and transcriptional regulators required for UV-B-mediated *HY5* gene activation were unclear. Our analyses reveal that the HY5 association with target gene promoters is enhanced by UV-B in a UVR8-dependent manner. Furthermore, HY5 itself, together with HYH, is required for transcriptional activation of its own gene by binding the T/G^{HY5}-box, a *cis*-regulatory element required for *HY5* gene activation by UV-B. Thus, our study provides increased understanding of the transcriptional response to UV-B. In particular, it reveals HY5 as a transcriptional regulator of its own gene expression in a positive feedback loop and identifies a UV-B-responsive *cis*-element targeted by HY5.

Regulation of the HY5 Association with Chromatin

Even though initial data suggested that binding of HY5 in vivo is not affected by light (Lee et al., 2007), more recent results suggest that the HY5 association with target genes is enhanced in response to white light (Zhang et al., 2011). However, the photoreceptor(s) mediating this enhanced binding in response to visible light have remained unclear. Here, we provide evidence for UV-B-responsive enrichment of promoter-associated HY5 at target genes, dependent on the UVR8 UV-B photoreceptor. In agreement, the UV-B-responsive increased binding is also affected by overexpression of the negative regulatory *RUP2* gene. In *RUP2* overexpression lines, UVR8 remains in its inactive homodimeric form, which blocks signaling (Gruber et al., 2010; Heijde and Ulm, 2013). Thus, enhanced chromatin association of HY5 is a response closely associated with UVR8-mediated gene expression changes. However, it is still unclear whether the detected UV-B-enhanced binding is due to stronger binding per individual target promoter (e.g., multiple *cis*-elements) or to binding of previously unoccupied promoters in the same or additional cells. This is further complicated by the fact that HYH binds to the same *cis*-element in the *HY5* promoter; thus, the relative abundance of the competing transcription factors can further distort the actual data. These dilemmas are not specific to the results for HY5 but represent general uncertainties when interpreting ChIP data. In addition, it is yet uncertain whether the UV-B-induced HY5 association with chromatin of target genes primarily reflects the stabilization of the HY5 protein (Favory et al., 2009; Huang et al., 2013) or the activity of the HY5 protein is affected otherwise by UV-B. It was previously reported that phosphorylation of HY5 within its COP1 binding domain may regulate HY5 stability and activity (Hardtke et al., 2000). However, any potential impact of UV-B on modifying the phosphorylation of HY5 remains to be demonstrated.

There are several genes among the direct targets of HY5 that encode components of the UV-B signaling pathway. For example, HY5 binds and regulates *COP1* as well as *RUP1* and *RUP2*. This transcriptional modulation appears to increase the abundance of active UVR8-COP1-HY5 core pathway components but, in parallel, also to induce the negative feedback regulators RUP1 and RUP2 (Oravecz et al., 2006; Favory et al., 2009; Gruber et al., 2010; Heijde and Ulm, 2013). The induction of *COP1* by UV-B was shown to be modulated by combinatorial regulation of FHY3 and HY5 (Huang et al., 2012). Interestingly, HY5 and FHY3 physically

Figure 5. (continued).

(B) to **(F)** Linker scan mutant derivatives of the *HY5* promoter.

(B) Putative *cis*-elements (ACG-box, T/G-box, and E-box) are indicated as boxes, and corresponding nucleotides are framed in the sequence panel and shown in boldface in the wild-type sequence. Mutations are underlined and printed in boldface and are shown in the corresponding mutant sequence line. All mutations were generated in the −565 to +192 fragment of the *HY5* promoter, but only sequences from −157 to −71 are shown. Nucleotide positions relative to the first base pair of the 5′ untranslated region (transcriptional start site defined as position +1) are shown.

(C) Induction profile of *ProHY5*$_{[-565/+192]}$*:LUC* in response to UV-B pulses (plants were treated as described in **[A]**). Basal activity was calculated from average counts at the three time points just before the light pulse. The highest luminescence value detected after the light pulse is taken as the maximal induced luminescence. Fold induction is the ratio of maximal induced luminescence to basal activity. Data represent averages of 36 individual seedlings for each condition.

(D) to **(F)** Wild-type *Arabidopsis* (Ws) seedlings carrying the indicated mutant derivatives of the *HY5* promoter fused to the *LUC* reporter gene were grown and assayed as in **(C)**. Twenty-four to 36 individual seedlings were assayed for each line, and two independent transgenic lines were tested for each construct. Basal activity **(D)**, maximal induced luminescence **(E)**, and fold induction **(F)** were calculated according to **(C)**. Error bars represent SE.

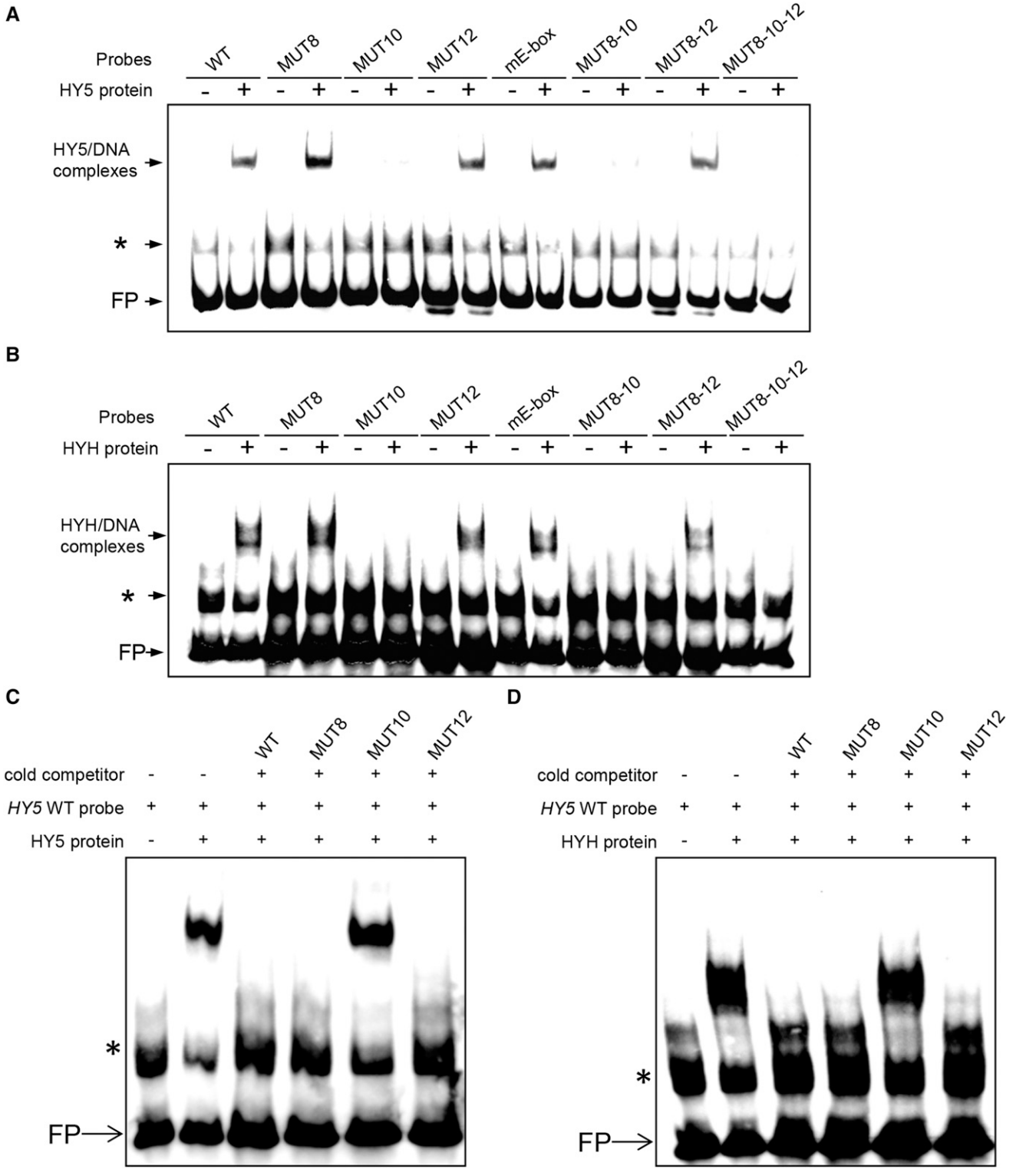

Figure 6. HY5 and HYH Bind to the *cis*-Regulatory Element T/G^{HY5}-Box.

Biotin-labeled double-stranded probes (40 fmol) were incubated with (+) or without (−) expressed and purified HY5 (**[A]** and **[C]**) or HYH (**[B]** and **[D]**) protein (400 ng). Binding reactions were resolved on 6% native polyacrylamide gels. WT corresponds to the −117 to −62 fragment of the *HY5* promoter, whereas MUT8, MUT10, MUT12, MUT8-10, and MUT8-10-12 carry the corresponding single, double, and triple linker scan mutations in this

interact with each other, and this is influenced by UV-B. FHY3 binds to an FHY3 binding site and HY5 to an ACGT-containing element in the *COP1* promoter, both of which seem to be required for the UV-B responsiveness of *COP1* (Huang et al., 2012). Combinatorial regulation exerted by the interaction of other transcription factors with HY5/HYH is widely present, with negative or positive regulatory effects (Shin et al., 2007; Andronis et al., 2008; Holtan et al., 2011; Huang et al., 2012; Jiang et al., 2012; Singh et al., 2012; Gangappa et al., 2013a, 2013b; Jing et al., 2013; Abbas et al., 2014). Moreover, HY5 interacts with the chromatin-remodeling factor PICKLE (PKL), recruiting PKL to promoters of target genes repressing the H3K27me3-repressive histone mark (Jing et al., 2013). Thus, an exact understanding of HY5/HYH function in response to UV-B will also require systematic analysis of known HY5-interacting transcriptional regulators and an unbiased approach to identify those that are specifically involved.

Regulation of the *HY5* Promoter

Our ChIP experiments clearly support the binding of HY5 to its own promoter, suggesting that HY5 is one of the missing transcription factors for *HY5* gene activation by UV-B. However, our previous results showed that HY5 is superfluous for transcriptional activation of itself (Ulm et al., 2004). Here, we also demonstrate that HY5 or its homolog HYH can individually activate *HY5* transcription in the absence of the other. This redundancy is clearly demonstrated by the similar UV-B-mediated activation of *ProHY5:LUC* in the wild type and the *hy5* and *hyh* single mutants but the very strongly reduced activation in *hy5 hyh* double mutants. Notably, whereas *ProHY5:LUC* activation was not found in *uvr8*, an approximately 3-fold induction was still apparent in *hy5 hyh* (compared with >50-fold in the wild type). This indicates the possible involvement of additional transcriptional regulators of likely minor importance. Next to transcriptional regulation, the expression of *HY5* was recently shown to involve light-regulated posttranscriptional regulation by the small regulatory microRNA miR157d (Tsai et al., 2014); however, whether UV-B affects miR157d expression remains unknown. This notwithstanding, HY5 and HYH both associate with the *HY5* promoter and are required in vivo for mediation of the early event of *HY5* gene induction, which strengthens the concept of their central regulatory role in rapid UV-B responses downstream of UVR8.

Regulatory *cis*-Elements Contributing to *HY5* Gene Expression

In a linker scan analysis, we identified three *cis*-regulatory elements that mediate the transcriptional activity of *HY5*, namely an ACG-box, a T/G-box, and an E-box. Mutation of the ACG-box resulted in elevated basal expression of the luciferase reporter after extended white light treatment (24 to 72 h) but not in darkness, indicating that this box functions as a light-induced *HY5* repression element. In contrast, mutation of the T/G or E element did not significantly affect the expression of *ProHY5:LUC* after 24 to 72 h of illumination with white light. It was reported recently that HY5 and CALMODULIN7 (CAM7) bind to T/G and E in vitro and interact in vivo; the expression of a *ProHY5:GUS* reporter is downregulated in *hy5* and *cam7* mutants after irradiation with visible light (Abbas et al., 2014). We found that the activity of the *HY5* promoter is not strongly responsive to extended irradiation with visible light. It is notable, however, that after transfer from darkness to white light, the induction of luminescence (*ProHY5:LUC*) peaked around 4 h to a similar extent in *hy5*, *hyh*, and wild-type seedlings, and mutation of the E-box did not significantly affect this response (Supplemental Figures 2C and 2D). By contrast, this acute induction by white light was eliminated by the mutation of the T/G-box as well as in the *hy5 hyh* double mutant background (Supplemental Figures 2C and 2D). These data indicate that other transcription regulators, including CAM7, may play a minor role in the absence of HY5 and HYH for acute white light induction of *HY5* expression during the dark-to-light transition. Similarly, mutation of the T/G-box or the lack of

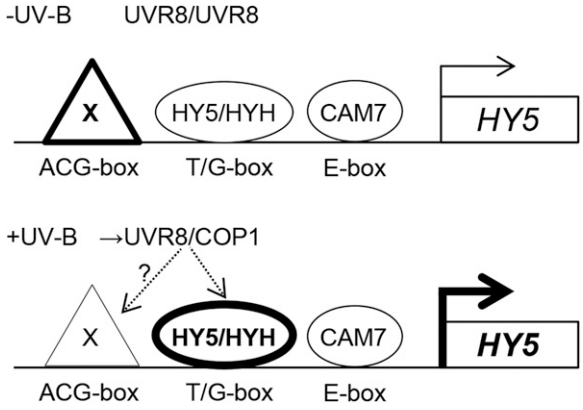

Figure 7. Working Model of the Transcriptional Regulation of *HY5*.

HY5 and HYH binding to the T/G-box mediates UV-B responsiveness of the *HY5* promoter. An ACG-box is postulated to be bound by an unknown repressor protein X that is potentially removed from the *HY5* promoter or overruled by the UV-B responsiveness of the T/G-box in response to UV-B (note that the ACG-box requires the T/G-box for its repressive function in –UV-B). The E-box was previously found to be bound by CAM7 (Abbas et al., 2014) and seems to make only a very minor contribution to UV-B responsiveness. The accumulated HY5 protein (combination of new synthesis and stabilization) then binds to and activates multiple downstream target genes, including genes encoding UV-B signaling components (*COP1*, *BBX24*, *RUP1*, and *RUP2*).

Figure 6. (continued).

context. The mE-box probe has mutations identical to those described by Abbas et al. (2014). In **(C)**, reactions were performed as in **(A)** using *HY5* wild-type probe and HY5 protein, but unlabeled *HY5* MUT8 or *HY5* MUT10 fragments were used as cold competitors at a 200-fold molar excess. In **(D)**, reactions were performed as in **(B)** using *HY5* wild-type probe and HYH protein, but unlabeled *HY5* MUT8 or *HY5* MUT10 fragments were used as cold competitors at a 200-fold molar excess. Sequences of all probes are provided in Supplemental Table 2. FP indicates free (nonbound) probe. Asterisks mark a nonspecific band that appears independent of the presence of HY5 or HYH protein.

HY5/HYH in *hy5 hyh* double mutants dramatically reduced UV-B-induced transcription of the *ProHY5:LUC* reporter (Figures 4D and 4E). HY5 and HYH bind specifically to the T/G-box, and mutations disrupting this binding in vitro also block UV-B-mediated activation of the *HY5* promoter in planta, even in the presence of functional HY5/HYH. Together, these data suggest that HY5 and HYH are major and critical factors for regulating the UV-B and visible light responsiveness of the *HY5* promoter via binding to the T/G^{HY5} motif. Mutation of the E-box also reduced UV-B-induced *HY5* transcription in planta, albeit much less effectively than T/G-box mutation. HY5 and HYH do not bind to the E-box, and its mutation does not affect the binding of HY5/HYH to the T/G^{HY5}-box (Figure 6). By contrast, Abbas et al. (2014) found that binding of HY5 and CAM7 to the *HY5* promoter required the presence of intact T/G and E elements. The apparent contradiction between our data and those of Abbas et al. (2014) may be due to the use of differentially tagged HY5 proteins (HY5-6xHis versus HY5-GST) in the in vitro binding assays.

Independent of this, our data clearly demonstrate (1) that full induction of the *HY5* promoter by UV-B also requires an intact E-box (Figure 5F) but (2) that the functional significance of this *cis*-element is low in the absence of HYH/HY5 (Figure 4D) or an intact T/G^{HY5}-box (Figures 6E and 6F). Mutation of the ACG-box identified in this study also appears to compromise UV-B-induced activity of the *HY5* promoter in planta, but the molecular mechanism underlying this phenomenon is not understood. The drastically (50-fold) elevated expression of the *HY5* promoter carrying a mutant ACG-box in white light clearly demonstrates that this *cis*-regulatory element mediates the binding of an as yet unknown transcription factor, downregulating the activity of HY5 in continuous visible light. Upon exposure to UV-B, this repressor may be degraded or competed off by an activating transcription factor with a higher affinity for the ACG motif. Accordingly, mutation of the ACG-box might not only interfere with binding of the repressor but also of an activator. The high activity of the mutant *HY5* promoter in white light, however, could deplete one or more components and, thus, limit further induction by UV-B.

Taken together, our data support a model in which HY5 and HYH act as major regulators of the UV-B-enhanced transcription of *HY5* and other target genes (Figure 7). In broader terms, by demonstrating the impact of UV-B via UVR8 on the dynamics of chromatin occupancy by HY5 and HYH and revealing *cis*-elements and transcription factors regulating *HY5* transcription in response to UV-B, these results provide a conceptual framework for further experiments towards a better understanding of UV-B-induced transcriptional responses.

METHODS

Plant Material

hy5-215 (Oyama et al., 1997), *uvr8-6* (Favory et al., 2009), and *rup2-1*/*Pro35S:RUP2* (Gruber et al., 2010) are in the *Arabidopsis thaliana* Col background, while *hy5-ks50* (Oyama et al., 1997), *hyh-1* (Holm et al., 2002), *hy5-ks50 hyh-1* (Holm et al., 2002), *ProHY5:LUC* (Ulm et al., 2004), and *uvr8-7* (Favory et al., 2009) in the Wassilewskija (Ws) background.

ProHY5:LUC was introduced into *hy5-ks50*, *hyh-1*, and *hy5-ks50 hyh-1* by genetic crossing. The parental line has a stable single-copy insertion of the *ProHY5:LUC* transgene and was used previously for a genetic screen identifying *cop1* and *uvr8* mutants (Ulm et al., 2004; Oravecz et al., 2006; Favory et al., 2009).

Gene Constructs and Transgenic Plants

Linker scan mutagenesis on the *HY5* promoter region was performed essentially as described (Gustin and Burk, 1993). Twelve mutant derivatives of *ProHY5*$_{[-565/+192]}$*:LUC* were produced spanning nucleotides −157 to −74 relative to the *HY5* transcription initiation site.

Constructs were transformed by the floral dip method into the *Arabidopsis* Ws accession (Clough and Bent, 1998), and transformants were selected on Murashige and Skoog (MS) medium supplemented with 15 μg/mL hygromycin. Ten to 15 independent transformants for each construct were self-fertilized, and individuals of the T2 or the homozygous T3 progeny were used for luminescence assays.

Growth Conditions and Light Treatments

Arabidopsis seeds were surface-sterilized with sodium hypochlorite and plated on half-strength MS medium (Duchefa) containing 1% sucrose and 0.8% agar. Seeds were stratified for at least 2 d at 4°C and germinated aseptically at 25°C in a standard growth chamber (MLR-350; Sanyo) at 80 μmol m^{-2} s^{-1} with a 12-h/12-h light/dark cycle (moderate white light) or under continuous irradiation in a white light field with Osram L18W/30 tubes (3.6 μmol m^{-2} s^{-1}; measured with an LI-250 Light Meter) (weak white light). UV-B irradiation was performed for the indicated times in a weak white light field supplemented by Philips TL 20W/01 RS narrowband UV-B tubes (0.6 W/m^2 or as indicated; measured with a VLX-3W UV Light Meter equipped with a CX-312 sensor; Vilber Lourmat). The UV-B range was modulated using 3-mm transmission cutoff filters of the WG series with half-maximal transmission at the indicated wavelength (WG305 and WG345; Schott Glaswerke). For ChIP assays, seedlings were grown for the indicated times under weak white light conditions or in moderate white light. Seedlings grown in moderate white light were acclimated under weak white light for 12 or 24 h prior to UV-B treatment.

ChIP

ChIP was performed as described previously (Stracke et al., 2010). The chromatin was immunoprecipitated with antibodies against HY5 (Oravecz et al., 2006) and HYH (using a 1:120 dilution of sera). Polyclonal HYH antibodies were raised against recombinant His_6-tagged HYH protein (Eurogentec). Quantitative real-time PCR ChIP data were obtained using the ABsolute SYBR Green Rox Mix Kit according to the manufacturer's instructions (Thermo Scientific). The samples were amplified using a 7900HT real-time PCR system (Applied Biosystems) with the primer pairs listed in Supplemental Table 1. The intergenic region between the *At4g26900* and *At4g26910* genes was described before (Lee et al., 2007; Stracke et al., 2010). Quantitative PCR (qPCR) data were analyzed according to the percentage of input method (Haring et al., 2007). Technical error bars were calculated according to the Applied Biosystems user manual.

Luminescence Measurements

Surface-sterilized seeds were grown in a 12-h-white-light (50 μmol m^{-2} s^{-1})/12-h-dark cycle at 22°C (MLR-350; Sanyo) for 7 d. Plants were grown on MS medium supplemented with 3% sucrose. All experiments were performed at 22°C. Continuous white light was produced by Philips TL-D 18W/33-640 tubes (10 μmol m^{-2} s^{-1}). UV-B was produced by Philips TL 20W/01 RS tubes and filtered through LP305 cutoff filters (Rapp Optoelectronic), providing a final fluence rate of 1.5 μmol m^{-2} s^{-1}. Plants were transferred to continuous white light 48 h prior to UV-B induction.

Luminescence was monitored with the TopCount NXT luminometer (Perkin-Elmer) at 0.5- to 1.5-h intervals depending on the number of samples, as described previously (Kevei et al., 2006).

EMSAs

Double-stranded probes and nonlabeled fragments (competitors) were produced by annealing complementary oligonucleotides (IDT) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 50 mM NaCl (sequences are shown in Supplemental Table 2). Equal amounts of complementary oligonucleotides were mixed at a final concentration of 40 μM, heated to 95°C for 5 min in a block heater, and cooled to room temperature overnight. For probes, the 5′ end of the forward oligonucleotide was labeled by biotin (IDT). *HY5* and *HYH* cDNA molecules were PCR-amplified from a size-selected cDNA library (CD4-13; TAIR) and cloned in pET28a vectors (Novagen). Proteins with an N-terminal His_6 tag were expressed in *Escherichia coli* BL21 cells and purified using Ni-NTA agarose matrix (Qiagen). Protein expression and purification were performed according to the manufacturer's instructions (Qiagen; QIAexpressionist). Binding reactions contained 10 mM Tris-HCl, pH 7.5, 85 mM KCl, 5% (v/v) glycerol, 0.1 μg/μL poly(dI•dC), 40 fmol of probe, and 100 ng of purified HY5 or HYH protein in a 20-μL volume. Reactions were incubated at room temperature for 20 min and loaded onto native 4% polyacrylamide gels made with 0.5× TBE buffer. Gels were run in 0.5× TBE for 70 min and electroblotted to Hybond N+ (Amersham) nylon membranes in 0.5× TBE for 60 min. Biotin-labeled fragments were detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to the manufacturer's protocols. Chemiluminescent signals were captured by a deep-cooled Orca II CCD camera (Hamamatsu).

Accession Numbers

Sequence data from this work can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT1G06040 (*BBX24*), AT5G13930 (*CHS*), AT2G32950 (*COP1*), AT5G11260 (*HY5*), AT3G17609 (*HYH*), AT2G47460 (*MYB12*), AT5G52250 (*RUP1*), AT5G23730 (*RUP2*), AT5G63860 (*UVR8*), and AT4G26900/AT4G26910 (intergenic region).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Effect of the ACG^{HY5}-Box Mutation on Basal Promoter Activity.

Supplemental Figure 2. T/G-Box Mutation Impairs White Light Induction of *HY5* Expression.

Supplemental Table 1. Primer Pairs Used in ChIP-qPCR.

Supplemental Table 2. List of Oligonucleotides Used to Produce Probes for EMSA Experiments.

ACKNOWLEDGMENTS

We thank Xing-Wang Deng for providing *hyh*, *hy5*, and *hyh hy5* mutant seeds and Alexander Baumann for generating anti-HYH antibodies. Research performed in Szeged, Hungary was supported by grants from the Hungarian Scientific Research Fund (OTKA-106361 to L.K.-B. and OTKA-108559 to F.N.) and the New Hungary Development Plan (TÁMOP-4.2.2A-11/1/KONV-2012-0035 to F.N.). Research performed in Edinburgh, UK was supported by a Biotechnology and Biological Science Research Council grant (BB/K006975/1) to F.N. and that in Geneva, Switzerland by the State of Geneva as well as grants from the Swiss National Science Foundation (Grant 31003A-132902) and the European Research Council (ERC-StG 310539) to R.U.

AUTHOR CONTRIBUTIONS

The research was designed by M.B., L.K.-B., F.N., and R.U. and performed by M.B., L.K.-B., and K.T. L.D.V. provided new experimental tools. M.B., L.K.-B., F.N., and R.U. analyzed data and wrote the article.

Received August 4, 2014; revised September 18, 2014; accepted October 13, 2014; published October 28, 2014.

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