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Molecular interactions of BBX24 and BBX25 with HYH, HY5 HOMOLOG, to modulate *Arabidopsis* seedling development

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BBX24 and BBX25 are two important transcriptional regulators, which regulate seedling photomorphogenesis in *Arabidopsis*. Very recently, we have shown that BBX24 and BBX25 negatively regulate the expression of BBX22, reducing the function of HY5, by physically interacting with its bZIP domain.[1] Furthermore, HY5 HOMOLOG, HYH, has been reported to heterodimerize with HY5 and enhances its photomorphogenic function in seedling de-etiolation by serving as coactivator.[8] Here, we further report that BBX24 and BBX25 physically interact with HYH. The physical interactions of BBX24 and BBX25 with HYH could lead to depletion of HYH molecules from the active pool and, thus indirectly, reduce the function of HY5 in promoting photomorphogenesis. Hence, our results suggest another mode of regulation by which BBX24 and BBX25 exert their negative effects on HY5 indirectly through HYH for the fine-tuning of seedling photomorphogenesis.

Light is one of the most important abiotic factors, which determines the fate of plant growth and development. To cope with constant diurnal and seasonal variations in quality, quantity, duration and direction of light, plants have evolved a complex signaling network that involves photoreceptors, transcriptional regulators and downstream targets. The pathway begins with the perception of light signals by photoreceptors and ultimately leads to the modulation of the transcriptome through the action of transcription factors to switch on specific signaling cascades. Different photoreceptors perceive different wavelengths of light: red/far-red light is perceived by phytochromes (phyA-phyE), blue/UV-A light by cryptochromes (cry1 and cry2) and phototropins (phot1 and phot2) and UV-B by UVR8 photoreceptor.[2,3]

Light is known to regulate nearly 30% of *Arabidopsis* transcriptome.[4] Different laboratories around the world have identified several transcriptional regulators, which integrate signals from photoreceptors.[14] Some of them integrate different light signals and function downstream to different photoreceptors such as HY5, CAM7, BBX21, BBX22, BBX24, BBX25[1,6,7] and other are involved in specific light signaling such as HYH, MYC2, GBF1, BIT1 that integrate signals from blue light[8-11] or LAF1, HFR1, FHY1 and FHL from far-red light.[12-14] In the light, the photoreceptors activate transcription factors such as HY5, HYH, CAM7, LAF1 and HFR1 by attenuating the repressive action of negative regulators such as COP/DET/FUS. HY5, a major photomorphogenic transcription factor, regulates physiological processes such as inhibition of hypocotyl elongation, anthocyanin and chlorophyll synthesis and lateral root formation.[15,16] HY5 preferentially binds to G-, Z- and ACE-box cis-acting elements present in the promoters of genes related to light, hormonal and metabolic pathways.[17] Although HY5 binding at the promoter is required for the regulation of photomorphogenic genes, for the correct transcriptional regulation, cooperation of other cofactors is necessary.[17] In fact, HY5 has been reported to function co-operatively with HYH, CAM7, BBX21 and BBX22.[5,7,8] HY5 physically interacts with HYH at the G-box element in the promoters of target genes[8] regulating photomorphogenesis in blue, far-red and low UV-B.[8,18,19] Furthermore, BBX proteins can physically interact with HY5 enhancing (BBX21 and BBX22) or suppressing (BBX24 and BBX25) its function.[1,6,7] Although the physical interactions of BBX24 and BBX25 proteins with HY5 and their physiological significance is known to some extent, it is not clear whether these proteins can also interact with its close homolog, HYH. Here, we report that BBX24 and BBX25 physically interact with HYH and probably modulate indirectly the HY5 activity by the inactivation of HYH leading to the fine-tuning of photomorphogenesis.

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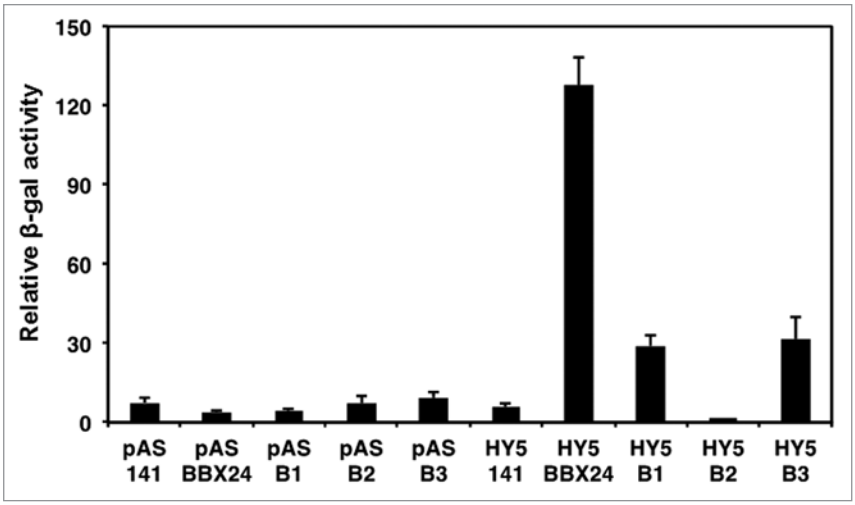

Figure 1. BBX24 physically interacts with HY5 through its B-Boxes. B1, B2, B3 represent Asp to Ala substitutions in the B-boxes at positions 20 (B-box1), 72 and 81 (B-box2) residues, respectively, in the BBX24 protein. Yeast two-hybrid interactions of BBX24 and its mutated versions with HY5 as measured by β-Galactosidase enzymatic activity. Error bars indicate SD (n-6). The experiment is the representative of the one of the three independent experiments. In the figure, 141 and pAS represent empty vectors pYX141 and pAS2-1, respectively.

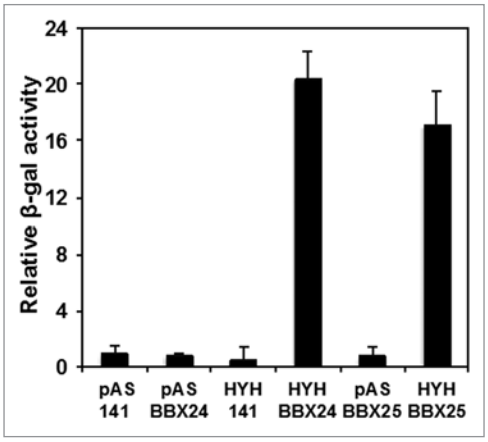

Figure 2. BBX24 and BBX25 physically interact with HYH. Yeast two-hybrid interactions of BBX24 and BBX25 with HYH as measured by β-Galactosidase enzymatic activity. Error bars indicate SD (n = 6). The experiment is the representative of one of the three independent experiments.

BBX24 Physically Interacts with HY5 through B-box Domain

The B-boxes in BBX21, BBX22 and BBX25 were found necessary for the interaction with HY5.[1,6,7] To see whether also the B-boxes in BBX24 were required for the interaction with HY5, we individually substituted three conserved Asp residues in the B-boxes to Ala and cloned in pYX141 vector. The substituted proteins in BBX24 were named B1 (D20A), B2 (D72A) and B3 (D81A). Two of these residues (D20 and D72) correspond to Zn^{2+} ligating residues in the B-box protein MIDLINE,[20] and substitution of these is likely to disrupt the structure of the B-box. All the three substitutions resulted in dramatic reduction of β-Gal activity compared with wild-type levels indicating that both the B-boxes are required for interaction with HY5 (**Fig. 1**).

BBX24 and BBX25 Physically Interact with HYH in Yeast Cells

Very recently, we have shown that BBX24-BBX25 co-operatively function to downregulate the expression of *BBX22* by targeting HY5.[1] Because HY5 and HYH are closely homologous (share 69% homology at the amino acid level) and both function positively in seedling de-etiolation, we reasoned that BBX24 and BBX25 might interact with HYH. To this end, we cloned full-length HYH in Gal4-DBD vector and co-transformed with either BBX24 or BBX25 into Y190 strain in yeast to measure β-Gal activity. When we expressed BBX24 and BBX25 along with Gal4-DBD empty vector, they did not activate transcription. However, when they are expressed with Gal4-DBD-HYH, β-Gal activity resulted in a ~25- and 20-fold increase over the vector control (**Fig. 2**). These results clearly demonstrate that BBX24 and BBX25 physically interact with HYH in yeast cells. Collectively, the results showed here along with the previous data reported[1] indicate that both BBX24 and BBX25 interact with HY5 and HYH in yeast and both B-boxes encoded by BBX24 and BBX25 are important for the interaction with HY5. It is evident that HYH is an important functional cofactor partner of HY5 and together they regulate a variety of developmental processes such as hypocotyl growth, pigment accumulation, gene regulation and lateral root development.[8,18] Hence, our results suggest that targeting HYH by both BBX24 and BBX25 could be a mechanism through which BBX24 and BBX25 regulate the HY5 function via HYH. This proposed mechanism perfectly complements with our previous evidences demonstrating that BBX24 and BBX25 regulate HY5 activity.[1] In fact, we have shown that BBX24 and BBX25 repress *BBX22* expression by interfering with HY5 transcriptional activity. Since HY5 is a central positive hub for photomorphogenesis, we suggest that the action of different mechanisms to keep on its function is crucial for the fine-tuning of seedling development.

Double B-Box Proteins Function as Transcriptional Co-Regulators

A group of zinc-finger proteins, which contain either one or two B-boxes in their N-terminal region, are called B-box proteins. The functions of B-box protein family has been implicated in the light mediated plant growth and developmental processes, such as photomorphogenesis, flowering, shade avoidance and circadian rhythms.[1,6,7,21,26] Recently, involvement of

these proteins in abscisic acid and brassinosteroid signaling has been reported.[22,23] The fourth sub-family of B-box proteins contain two tandem B-boxes in their N-terminal region, are called Double B-Box (DBB) proteins. There are eight DBB proteins in *Arabidopsis*: BBX18-BB25.[24] Interestingly, out of eight, five have been reported to physically interact with COP1 (BBX20-BBX22, BBX24-BBX25) and four (BBX21, BBX22, BBX24 and BBX25) have been reported to physically interact with HY5.[1,6,7,25] BBX21 and BBX22 function independently and co-operatively promoting photomorphogenic growth and pigment accumulation and inhibiting hypocotyl length in shade.[6,7,26,27] Genetic analysis of double mutants between BBX21 and BBX22 with COP1 and HY5 suggested that, they suppress COP1 functions, whereas enhance HY5 functions. The other two proteins such as BBX24 and BBX25 function additively as negative regulators of photomorphogenesis. Epistatic analysis with COP1 and HY5 suggest that, BBX24 and BBX25 enhance the COP1 function, whereas they supress HY5 function. Surprisingly, BBX21-BBX22 and BBX24-BBX25 pairs share ~70% homology at the amino acid level. Furthermore, BBX21, BBX22, BBX24 and BBX25 physically interact with HY5 through its bZIP domain.[1,6,7,25] Whereas BBX24 and BBX25 suppress the HY5 function,[1] it is not exactly known how BBX21 and BBX22 enhance the function of HY5.[7] BBX32 has been also shown to negatively regulate the HY5 function by physically interacting with BBX21 (one of the probable HY5 co-activator) and forming inactive heterodimers.[28] Considering these evidences, we propose a model for the action of BBX24 and BBX25 together with COP1, HY5 and HYH (**Fig. 3**). The fine-tuning of developmental processes such as photomorphogenesis, skotomorphogenesis and gene regulation and pigment accumulation involve the balance between positive and negative regulators like as BBX proteins for the plant adjustment to the changing light environments.

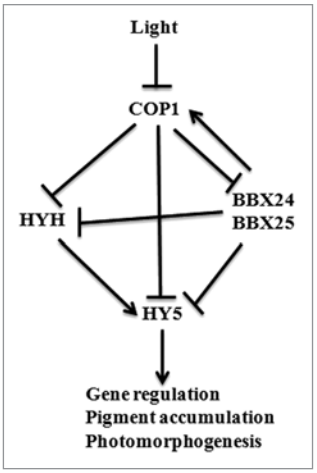

Figure 3. Working model of BBX25 and BBX24 interaction with HYH for the fine-tuning of Seedling De-etiolation. COP1 negatively regulates HYH and HY5 by degrading them. HYH enhances the function of HY5 specifically in a blue light dependent manner, as it acts as co-activator of HY5. BBX24 and BBX25 could target HYH to put a check on HY5 function via HYH by forming inactive heterodimers. BBX24 and BBX25 enhance the function of COP1, but in a feedback regulatory loop COP1 attenuates the function of BBX25 and BBX24 by degrading them in light.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work is dedicated to the memory of Dr Magnus Holm.

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