Functional interconnections of HY1 with MYC2 and HY5 in Arabidopsis seedling development

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Abstract

Arabidopsis seedling development is controlled by many regulatory genes involved in multiple signaling pathways. The functional relationships of these genes working in multiple signaling cascades have started to be unraveled. Arabidopsis HY1/HO1 is a rate-limiting enzyme involved in biosynthesis of phytochrome chromophore. HY5 (a bZIP protein) promotes photomorphogenesis, however ZBF1/MYC2 (a bHLH protein) works as a negative regulator of photomorphogenic growth and light regulated gene expression. Further, MYC2 and HY1 have been shown to be important roles in jasmonic acid (JA) signaling pathways. Here, we show the genetic interactions of HY1 with two key transcription factor genes of light signaling, HY5 and MYC2, in Arabidopsis seedling development. Our studies reveal that although HY1 acts in an additive manner with HY5, it is epistatic to MYC2 in light-mediated seedling growth and gene expression. This study further demonstrates that HY1 additively or synergistically functions with HY5, however it works upstream to MYC2 in JA signaling pathways. Taken together, this study demonstrates the functional interrelations of HY1, MYC2 and HY5 in light and JA signaling pathways.

Background

Light is one of the most important environmental factors for plant growth and development throughout its life cycle [1,2]. Plants have evolved with multiple photoreceptor systems to monitor the surrounding light quality, quantity, and direction. In Arabidopsis, these photoreceptors include the blue/UV-A light absorbing cryptochromes (CRY1 to CRY3) and phototropins (PHOT1 and PHOT2); the red/far-red light absorbing phytochromes (phy: phyA to phyE) [3-7]. Arabidopsis phytochromes form homo and hetero dimers with each other [8-10]. Formation of such heteromeric photoreceptors increases the potential complexity of R/FR light sensing and signaling mechanism in plants. Similarly, light induced activation of cryptochromes leads to possible autophosphorylation and dimerization [11]. Moreover, phytochromes and cryptochromes work together either by interaction with each other in a light-dependent and interdependent manner [12,13].

Arabidopsis seedlings exhibit two distinct developmental patterns, photomorphogenesis or skotomorphogenesis depending on the presence or absence of light, respectively [13-15]. Skotomorphogenesis is the strategy followed under dark conditions where Arabidopsis seedlings exhibit elongated hypocotyl, closed cotyledons with apical hooks; whereas in presence of light, photomorphogenesis is initiated, characterized by short hypocotyl with fully developed cotyledons. This developmental change from skotomorphogenesis to photomorphogenesis is carried out by different classes of photoreceptors, and characterised by a change in the expression of about one-third of genes in the Arabidopsis genome [16-18].

Genetic screen of Arabidopsis seedlings for developmental defects under light conditions have led to the identification of several transcription factors that either act as a positive or negative regulator downstream to specific photoreceptor or a set of photoreceptors [19-27]. Recently, a DNA-ligand binding screen has led to the identification of three Z-box binding factors, ZBF1/MYC2, ZBF2/GBF1 and ZBF3/CAM7 [28-33]. MYC2 is a bHLH transcription factor that acts downstream to cry1 and cry2 photoreceptors, and negatively regulates blue light-mediated photomorphogenic growth and blue and far red-light regulated gene expression [29]. MYC2 also functions as a transcriptional regulator for ABA and JA signaling pathways [29,34-37].

HY5 is one of the first known and most extensively studied bZIP transcription factor involved in promoting photomorphogenesis. Arabidopsis seedlings mutant for
HY5 exhibit elongated hypocotyl under various wavelengths of light, suggesting that functionally HY5 is downstream to multiple photoreceptors [19,38-40]. Further, hy5 mutant seedlings exhibit defects in root growth and reduction in chlorophyll and anthocyanin accumulation [19,41]. In addition, studies have shown the involvement of HY5 in both auxin and cytokinin signaling pathways [42-44], suggesting that HY5 might be a common intermediate in light and hormone signaling pathways. The chromatin immunoprecipitation (CHIP) assays have revealed that HY5 preferentially binds to more than 3000 chromosomal sites that were distributed in all the five chromosomes [45].

Arabidopsis HY1 encodes heme oxygenase (HO) that catalyses the committed step in the conversion of heme to biliveridin IXa (BV), which is further converted to phochromobilin through sequential steps and exported to cytoplasm where it binds to the newly synthesized apo-phys by an autocatalytic process to form functional holo-phytochrome. The HOs are encoded by a small gene family that includes HY1, HO2, HO3 and HO4. Among all the four members of the HO family HY1 is highly expressed in almost all the tissues and plays a major role in synthesis of holo-phytochrome [46]. Seedlings mutant for HY1 exhibits elongated hypocotyl in red and far red light, and display defects in root development. Further, the light inducible genes such as CAB, RBCS and CHS are under-expressed in hy5 mutant background [47-49]. Recently, it has been reported that seedlings mutant for HY1 show elevated levels of JA and expression of JA-inducible defense genes [50].

In this study, in order to identify genes that might be working parallel to HY5, a genetic screen was set up using hy5-ks50 mutant lines through EMS mutagenesis. Gene cloning and genetic complementation analysis revealed that one of these mutants (enhancer of HY5: ehy5) contains a mutation in the HY1 gene. We have investigated the interrelations of HY1 with two transcription factors, HY5 and MYC2, with respect to light-controlled Arabidopsis seedling development and JA responsiveness.

Results
Mutations in EHY5 modulate HY5-controlled hypocotyl elongation
HY5 is a key transcription factor in light signaling pathways that promote photomorphogenesis under a broad spectrum of light [32,40]. Although the hy5 mutant seedlings display elongated hypocotyl in light, the seedlings are not completely etiolated similar to dark grown seedlings. Therefore, there might be additional factors present that are involved in the promotion of photomorphogenesis under various wavelengths of light [39]. Recent studies have shown that CAM7/ZBF3 works in various wavelengths of light to promote photomorphogenic growth and light regulated gene expression. Further, HY5 and CAM7 work synergistically or additively in the promotion of photomorphogenesis [32].

In order to find additional factors that promote photomorphogenesis in concert with HY5, an extragenic enhancer screen was set up using hy5 mutant lines (hy5-ks50 mutant; 19) through EMS mutagenesis. Several double mutant lines that showed enhanced hypocotyl growth as compared to that of hy5 mutants were identified. One such mutant line, hy5 ehy5 (enhancer of HY5) double mutant, was selected for further study. The segregated ehy5 line (obtained in F2 population from a back cross with wild type (Ws) was repeatedly backcrossed with wild type (Ws) to purify the mutation from any other background mutations.

The examination of seedling morphology revealed that neither ehy5 alone nor ehy5 hy5 double mutants exhibited any altered morphology in the dark (Figure 1A (a) and 1B). The characteristic long hypocotyl phenotype of hy5 in WL (white light) irradiation was further enhanced in ehy5 hy5 double mutants, exhibiting a super tall phenotype under various fluences of WL (Figure 1A (b) and 1C). To determine whether this reduced sensitivity of ehy5 hy5 phenotype is specific to a particular wavelength of light, the growth of 6-day-old ehy5 hy5 double mutant seedlings was tested in various wavelengths of light. As shown in Figure 1A (c) and 1D, ehy5 hy5 double mutants displayed further reduced sensitivity to far-red light (FR) as compared to ehy5 and hy5 single mutants, suggesting that EHY5 and HY5 additively control the hypocotyl growth in FR. On the other hand, hypocotyl length of ehy5 hy5 double mutants was found to be closer to either of the single mutants in red light (RL), suggesting that EHY5 and HY5 are likely to work in the same branched pathways in controlling the hypocotyl length in RL (Figure 1A (d) and 1E). The ehy5 mutants exhibited similar hypocotyl length to that of wild type in blue light (BL), and the hypocotyl length of ehy5 hy5 double mutants was similar to that of hy5 single mutants, suggesting that additional mutation in EHY5 does not affect the hy5 phenotype in BL (Figure 1A (e) and 1F).

Map based cloning reveals that EHY5 encodes HY1
To determine the genetic basis of EHY5 mutation, we followed map-based cloning strategy. The ehy5 mutants (WS) were genetically crossed to wild type (Col), and the resulting F1 progeny showed wild type phenotype. F1 plants were self-pollinated and since the ehy5 long hypocotyl phenotype is easy to score at the seedling stage, the EHY5 locus has served as a useful landmark for classical mapping. For fine mapping, the segregating F2 populations with the ehy5 phenotype were used for mapping with Simple Sequence Length Polymorphism (SSLP) and Cleaved Amplified Polymorphic Sequence (CAPS) markers
that we developed during this study and also that are available in the database at the Arabidopsis Information Resource (TAIR). Initially, the target locus was mapped between the markers ER and T20P8 on Chromosome 2 (Figure 2A). Further fine mapping with seven genetic markers delimited the target gene to a 20-Kb region on the F18A8 BAC clone. To further identify the exact position of the EMS mutation, we have sequenced the genomic DNA fragment of the 20-Kb region from the ehy5 background and compared with that of wild type (WS) genomic DNA sequence, which revealed that a single C to T nucleotide substitution in the first exon of the HY1

Figure 1 The ehy5 mutants display elongated hypocotyl. A. Phenotype of segregated wild-type (WS), ehy5, hy5, and ehy5 hy5 double mutants in dark and different light conditions. Six-day old constant dark, WL (90 μmol m⁻² s⁻¹), FR (90 μmol m⁻² s⁻¹), RL (90 μmol m⁻² s⁻¹) and BL (45 μmol m⁻² s⁻¹) grown seedlings (a to e). B-C. Quantification of hypocotyl length of 6-day-old seedlings grown in constant dark and various fluences of WL, respectively. D-F. Quantification of hypocotyl length of 6-day-old constant FR (90 μmol m⁻² s⁻¹), RL (90 μmol m⁻² s⁻¹) and BL (45 μmol m⁻² s⁻¹) grown seedlings, respectively. The error bar indicates standard deviation (SD). The experiment was repeated more than twice and similar results were obtained each time. A representative result is presented. For measuring hypocotyl length, ~30 seedlings were used in each genotype.
AT2G26670) DNA leads to the conversion of Glutamine (CAA) to stop codon (TAA), resulting in the premature termination of the protein translation (Figure 2A). This EMS induced substitution in HY1 first exon introduces a DdeI recognition site adjacent to the mutation region. We developed a dCAPS marker to confirm the mutation in ehy5 (Figure 2B).

As a final step to establish that the EHY5 locus encodes HY1 transcript, we tested whether a wild type genomic fragment containing the entire HY1 gene could complement ehy5. Fragment containing entire HY1 coding region with its native promoter was introduced into ehy5-mutant background. As shown in Figure 2C, ehy5 seedlings transformed with full length HY1 genomic DNA
fragment exhibited wild-type phenotype. The positive transformants were confirmed by RT-PCR (Figure 2D). These results indicate that the ehy5 mutant is an allele of hy1 mutant, and henceforth we refer to ehy5 as hy1.

HY1 and HY5 additively regulate the expression of light regulated genes and accumulation of chlorophyll and anthocyanin during early seedling development

The loss-of-function mutants of HY5 display partial photomorphogenic growth at various wavelengths of light with reduced expression of light-regulated genes such as CAB1 and RBCS-1A. Similarly, hy1 also shows reduced accumulation of CAB and RBCS transcripts [51]. To examine how HY5 and HY1 genetically interact to regulate the expression of light inducible genes, we monitored the expression of CAB1 and RBCS-1A by real-time PCR. As shown in Figure 3A-B, the expression of CAB1 and RBCS-1A was reduced in both hy1 and hy5 single mutants as compared to wild-type, and the accumulation of transcript was further reduced in hy1 hy5 double mutants compared to either of the single mutants. These results indicate that HY1 and HY5 act in an additive manner to regulate the expression of CAB1 and RBCS-1A genes.

Earlier studies have shown that hy5 and hy1 mutant seedlings display reduction in the accumulation of chlorophyll and anthocyanin. To determine the genetic interaction of HY1 and HY5 for chlorophyll and anthocyanin

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Figure 3 HY1 and HY5 additively regulate the light-induced gene expression. A-B, Relative expression of CAB1 and RBCS-1A in 6-day-old seedlings grown in WL (90 μmol m⁻² s⁻¹). C, Accumulation of chlorophyll in 6-day-old wild-type and mutant seedlings grown in WL (90 μmol m⁻² s⁻¹). D, Accumulation of anthocyanin in 6-day-old wild-type and mutant seedlings grown in WL (90 μmol m⁻² s⁻¹). The error bars indicate SD. *** indicates significant difference from hy5 (p > 0.001 student’s t-test, n = 30, number of seedlings used for hypocotyl measurement). Real-time PCR was repeated more than thrice and in each biological experiment, three technical replicates were used. Similar results were obtained in all the experiments. A representative figure is shown here. For chlorophyll and anthocyanin estimation, 50 seedlings was used in each genotype and the experiment was repeated thrice and in each biological experiment, four technical replicates were used. Similar results were obtained in all the experiments. A representative figure is presented.
accumulation, chlorophyll and anthocyanin contents were estimated from six-day-old WL grown seedlings. As shown in Figure 3C and 3D, the hy1 hy5 double mutants showed less accumulation of chlorophyll and anthocyanin as compared to that of hy1 and hy5 single mutants, suggesting that HY1 and HY5 act in an additive manner to control the accumulation of chlorophyll and anthocyanin in WL.

**HY1 and HY5 work in an additive or synergistic manner to control JA responsiveness**

In the presence of jasmonic acid (JA), hy1 mutants have stunted root growth and expression of JA-inducible defence genes [50]. We asked whether mutation in HY5 can modulate the JA sensitiveness of hy1 mutants. To examine that, we grew the seedlings in the presence or absence of JA and examined the root growth. Although very little difference, if any, was observed between the wild type and hy5 single mutants, a significant difference in root length was observed among hy1 hy5 double mutants in the absence of JA (Figure 4A), whereas there was very little expression of VSP2 in the presence of JA, the expression of VSP2 was increased in the presence of JA in wild-type and hy1 mutant plants. Further, the hy1 mutants showed significantly higher level of accumulation of VSP2 transcript as compared to wild-type background. However, the expression of VSP2 was less in atmyc2 plants, as expected from its less sensitiveness to JA, and was found to be similar to hy1 atmyc2 double mutants. These results suggest that MYC2 works downstream to HY1 in JA-induced expression of VSP2 gene.

**Overlapping functions of HY1 and MYC2 in Arabidopsis seedling development**

The atmyc2 mutants display hypersensitive response to BL, and are epistatic to cry1 and cry2 [29]. In order to determine how these two light signaling components, HY1 and MYC2, genetically interact to control early seedling development, we measured the hypocotyl length of hy1 atmyc2 double mutants in various light conditions. Similar to hy1 or atmyc2 single mutants, hy1 atmyc2 double mutants did not show any altered growth in the dark. However, under WL conditions, hy1 atmyc2 double mutants displayed hypocotyl length similar to hy1 single mutants (Figure 6A and 6B). Furthermore, as shown in Figure 6A and 6C to 6E, hy1 atmyc2 double mutants showed less accumulation of chlorophyll and anthocyanin by real time PCR in various mutant backgrounds. As shown in Figure 5C, whereas there was very little expression of VSP2 in the absence of JA, the expression of VSP2 was increased in the presence of JA in wild-type and hy1 mutant plants. Further, the hy1 mutants showed significantly higher level of accumulation of VSP2 transcript as compared to wild-type background. However, the expression of VSP2 was less in atmyc2 plants, as expected from its less sensitiveness to JA, and was found to be similar to hy1 atmyc2 double mutants. These results suggest that MYC2 works downstream to HY1 in JA-induced expression of VSP2 gene.

**Discussion**

Although many components of light signaling pathways are known, the interconnections of these components in Arabidopsis seedling development is unclear. Moreover, very little information is available on cross talks of various components of light signaling with other signaling cascades and vice versa. In this study, we have demonstrated the genetic interactions of HY1 with two other light-signaling components, HY5 and MYC2, which
belong to two important families of transcription factors, bZIP and bHLH, respectively, in Arabidopsis seedling development. Furthermore, this study reveals that HY1, HY5 and MYC2 are functionally connected in JA signaling pathways.

An attempt to identify new genes that might enhance hy5 phenotype, similar to CAM7/ZBF3 led to the identification of EHY5 [32]. Map based cloning and genetic complementation of ehy5 mutants reveal that EHY5 codes for HY1 (HO1), a rate-limiting enzyme that catalyzes the conversion of heme to biliverdin IXα (BV) in the chromophore biosynthesis pathway [54]. Phenotypic analyses under various light conditions have revealed that HY1 and HY5 function in an additive manner.

Figure 4 JA responsiveness of hy1 hy5 double mutants. A, Quantification of root length of 16-day-old wild-type and hy1 mutant plants grown in constant WL (90 μmol m\(^{-2}\) s\(^{-1}\)) without hormone (JA). B, Quantification of root length of A. C, Relative induction of VSP2 expression by JA in wild-type and mutant plants. Six-day-old wild-type and mutant seedlings were treated with MS (Mock) or with JA (50 μM) for 5-hours and total RNA was isolated from 100 mg of tissue and used for quantitative real-time PCR analysis. ACTIN2 was used as internal control. Approximately 25 to 30 seedlings were used for the root growth measurement. The error bars indicate standard error (SE) of three biological replicates.
resulting in a super tall phenotype in WL. Similar additive function of HY5 and HY1 was also observed in the regulation of hypocotyl growth in FR. Genetic interaction studies between HY1 and HY5 reveal that they are likely to work in the same branched pathways of light signaling. On the other hand, mutations in HY1 does not affect the hy5 phenotype in BL. However, the additional mutation in HY1 is able to suppress the atmyc2 phenotype in BL. These results strongly suggest the wavelength specific interdependent functions of HY1, HY5 and MYC2 in the regulation of hypocotyl growth in Arabidopsis seedling development.

The expression of light regulated genes is down-regulated in hy1 mutant background. HY5 directly binds to the G-box present in the promoters of light regulated genes and promote their expression [39]. MYC2/ZBF1 also interacts with the Z-/G-box LRE present in the light-inducible promoters such as CAB1 and RBCS1A, however down-regulates their expression [29,33,45]. Analysis of light-regulated gene expression in hy1 hy5 double mutants reveal that HY1 and HY5 function in an additive manner and elevate the expression of light regulated genes. These two proteins also function in an additive manner to regulate the accumulation of chlorophyll and anthocyanin. On the other hand, the expression of CAB1 and RBCS-1A in hy1 atmyc2 double mutant seedlings was similar to that of hy1 single mutants, and thus suggesting that HY1 works downstream to MYC2 in the regulation of CAB1 and RBCS-1A expression. It has been shown earlier that although atmyc2 works downstream
to cry1 and cry2 photoreceptors, phyA is epistatic to atmyc2 in BL [29].

Plant growth and development is a complex phenomenon, which is likely to be regulated through interactions between light and phytohormone signaling pathways. Recent studies have shown that signals from light and multiple hormonal signaling pathways cross talk through common downstream regulatory proteins such as MYC2 and HY5 [29,34-36,42-44,52,55]. For example, seedlings mutant for HY5 show altered balance of auxin and cytokinin signaling and also has decreased expression of two negative regulators of auxin signaling pathways such as AXR2/IAA7 and SLR/IAA14. The functional overlap of light and JA signaling in defence, wound and shade response has been reported [56,57]. MYC2 regulates JA responses via differential regulation of an intermediate spectrum of transcription factors with activating or repressing roles. Furthermore, a JA activated MKK3-MPK6 pathway negatively regulates the expression of MYC2 [53]. It has been shown that phytochrome deficient hy1 mutant seedlings overproduce JA and also display constant expression of JA inducible defense related genes such VSP1. The possible reason may be that there is reduction in the total photoactive phytochrome pool in Figure 6 Light-mediated seedling development of hy1 atmyc2. A. Phenotype of wild-type and various mutant seedlings in different light conditions. Six-day-old constant WL (90 μmol m⁻² s⁻¹), RL (90 μmol m⁻² s⁻¹), FR (90 μmol m⁻² s⁻¹) and BL (45 μmol m⁻² s⁻¹) grown seedlings. B-E. Quantification of hypocotyl length of 6-day-old constant WL (90 μmol m⁻² s⁻¹), RL (90 μmol m⁻² s⁻¹), FR (90 μmol m⁻² s⁻¹) and BL (45 μmol m⁻² s⁻¹) grown seedlings, respectively. F-G. The relative expression of CAB1 and RBCS-1A in 6-day-old seedlings grown in WL (90 μmol m⁻² s⁻¹). The error bars indicate SD. Approximately 25 to 30 seedlings were used for hypocotyl length measurement. For gene expression studies, total RNA was isolated from 100 mg of tissue was used for cDNA preparation. The real-time PCR experiments were repeated more than twice and three technical replicates were used for each genotype. Similar results were obtained in all the experiments. A representative graph is shown. H. Working model shows the role of HY1, HY5 and MYC2 in photomorphogenesis and JA responsiveness. HY1 and HY5 act additively in response to JA and light signaling pathways. MYC2 acts downstream to HY1 in JA responsiveness, and HY1 acts negatively to MYC2-mediated BL specific photomorphogenic growth.
the hy1 mutant background and thereby resulting an altered light sensitivity. This may lead to photo-oxidative stress resulting in upregulation of JA synthesis in hy1 mutants [50]. The cross talks among multiple signaling pathways occur at the level of intermediate components of the signaling pathways rather than at the receptor level. For example, cross talks between light and JA signaling is mediated by the transcription factor (intermediate component) MYC2/ZBF1. MYC2/ZBF1 works in cryptochrome mediated blue light signaling pathways [29], however cry1/cry2 mutants do not have altered JA responses.

It is worth mentioning here that it has earlier been reported that hy1 mutants display shorter roots than wild type plants [50]. However, this study does not find such difference in the absence of JA. The apparent discrepancy may be attributed to the developmental stages the observations were made. Whereas Zhai et al., 2007 found the difference at the early seedling stage, this study demonstrates the results of 16-day-old young adult plants, where the altered hypocotyl length was fairly maintained. In this study, our results demonstrate that HY5 and HY1 act additively or synergistically to regulate the JA-induced root-growth-inhibition and expression of JA-responsive genes. Although hy5 mutants do not show altered root growth in JA, the JA inducible gene VSP2 was upregulated in hy5 mutants in the presence of JA. These results indicate a negative regulatory role of HY5 in JA-mediated regulation of VSP2. On the other hand, MYC2 which acts as a negative regulator of light signaling, acts as a positive regulator of JA-mediated VSP2 expression Figure 5; [35]. Thus, both these transcription factors work in an opposite manner in light and JA signaling pathways.

Conclusions
This study demonstrates an overlapping function of HY1 with two important transcription factors of light signaling, HY5 and MYC2, in light and JA signaling pathways. The findings in this work will help to better understand the light signaling in Arabidopsis, and the cross talk of light and JA signaling pathways.

Methods
Plant materials, growth conditions and generation of double mutants
Arabidopsis (Arabidopsis thaliana) seeds were surface sterilized and sown on Murashige and Skoog plates, then kept at 4°C in darkness for 3 to 5 days, and transferred to specific light conditions at 22°C. The intensities of WL and various colour lights (in the light-emitting diode chamber, Q-Beam 3200-A; (Quantum Devices)) used were described in Yadav et al. (2002). For the generation of double mutants such as hy1 atmcy2, homozygous hy1 (WS) mutant plants were genetically crossed with atmcy2-1 (Col-0) homozygous mutant lines. In the F2 generation, seedlings were grown in WL (90 μmol m-2 s-1) for the identification of hy1 homozygous lines, and long hypocotyl hy1 mutants were selected and transferred to soil. To determine the genotype of AtMYC2 locus, about 40 seedlings from each line were tested by genomic PCR. F3 progenies that were homozygous for atmcy2 mutant plants were further examined by RT-PCR and considered as hy1 atmcy2 double mutants. For measurement of hypocotyl length, ∼30 seedlings were used in each genotype. The hypocotyl length measurement was repeated more than twice with similar results.

Mutant screen and map-based cloning
Ethyl methanesulphonate (EMS)-mutagenized, hy5KS50 M2 seeds of Arabidopsis thaliana ecotype Wassilewskija (Ws) were grown on MS media under WL conditions and hypocotyl length was compared with that of wild type and hy5KS50 mutant lines. Seedlings that showed enhanced and elongated hypocotyl length (as compared to hy5KS50) under all light conditions tested were selected and used for further studies. To identify the genetic basis of the EMS mutation in eh5, we isolated the new eh5 mutant, from the hy5KS50 eh5 double mutant background. The double mutant plants were back-crossed successively to wild-type (WS) and the segregated eh5 mutant seedlings in the F2 generation was selected and used for further back-crosses with wild type (WS) for four generations (to purify the background mutations in the EMS treated hy5-ks50 mutant plants) before physiological and genetic analysis. The eh5 mutant was out-crossed with Wild-type (Col) ecotype, and the mapping population was selected from F2 generation. A total of 823 individual F2 plants showing the eh5 phenotype were selected for genetic mapping. Genomic DNA was prepared using the protocol described by Edwards et al. (1991). Cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers between Col and Ws were used for mapping EHY5. For genetic complementation analysis, a genomic fragment containing the entire HY1/At2g26670 coding sequence along with its promoter was amplified from the wild-type (Ws) by PCR and the PCR product was digested with HindIII and SmaI and inserted into same sites of modified pBI121 binary vector. The construct obtained was then introduced into eh5 (hy1) mutant plants using A. tumefa-
ciens-mediated transformation. Transformants were selected based on their resistance to kanamycin.

Root growth measurement
Seeds were on MS media in vertical square plates and stratified at 4°C in dark conditions for 4 days to induce uniform germination. The plates were placed vertically
in racks, and the seedlings were grown under constant white light conditions (90 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) for 16 days. The root length of wild type, single and double mutants was measured. Approximately 25 to 30 seedlings were used for the root length measurement. The experiments were repeated for three times with similar results.

**Root growth response to methyl jasmonate**

Seeds of wild type and mutant plants were plated on MS with 15 \( \mu \text{M} \) of methyl jasmonate (Sigma) in square plates, after four days of stratification in cold (4\(^{\circ}\)C), plates were placed vertically in racks, and the seedlings were grown under constant white light conditions (90\(\mu\text{mol m}^{-2} \text{s}^{-1}\)) for 16 days. The root length of wild type, single and double mutants was measured. For determining the VSP2 expression, six-day-old white-light grown seedlings were mock (only MS solution) or JA treated (50 \( \mu \text{M} \) JA in MS solution) for 5 hour. After the time period, seedlings were washed with sterile milliQ, excess water was removed with the tissue paper and the tissue was harvested and snap freeze in liquid nitrogen and total RNA was extracted from 100 mg of tissue, using the RNeasy plant mini kit (Qiagen), and cDNA were synthesized from total RNA using titan one-tube RT-PCR system (Roche Applied Science) following the manufacturer’s instructions. Real-time PCR analysis of gene expression was carried out using LightCycler-FastStart DNA Master-PLUS SYBR Green (Roche Applied Science) and was performed using Step-one Real-Time PCR system (ABI). \( C_T \) values of VSP2 were normalized, relative to that of \( ACTIN2 \) (Internal control).

The following primers were used for the experiment:

- **VSP2-RP**: 5\' GTAGTAGTGGATTTGGGAGC 3\'
- **CABI-3'-RP**: 5\' GGACTAAACGCAAAACGA 3\'
- **HY1-RP**: 5\' TCTGAATCTAGGTCAGGG 3\'
- **CABI-1'-RP**: 5\' GTTAACAAACACGTAGGG 3\'
- **RBCS1A-3'-RP**: 5\' GTAGTAGAGTGGATTTGGGAGC 3\'
- **RBCS1A-1'-RP**: 5\' AACGGCTTAAAAAGCTGGG 3\'
- **ACTIN2-RP**: 5\' GGACTAAACGCAAAACGA 3\'

**Real-time PCR analysis**

Total RNA was extracted from 100 mg of tissue, using the RNeasy plant mini kit (Qiagen), according to manufacturer’s protocol. RT-AMV reverse transcriptase (Roche Applied Science) was used for both semi-quantitative RT-PCR and cDNA synthesis. Real-time PCR analysis of gene expression was carried out using LightCycler-FastStart DNA Master-PLUS SYBR Green (Roche Applied Science) and was performed using Step-one Real-Time PCR system (ABI). \( C_T \) values of \( CABI \) and \( RBCS1A \) were normalized, relative to that of \( ACTIN2 \) (Internal control). Real-time PCR was repeated more than thrice and in each biological experiment three technical replicates were used.

The following primers were used for the experiment:

- **VSP2-3'-FP**: 5\' GTAGTAGTGGATTTGGGAGC 3\'
- **ACTIN2-3'-FP**: 5\' TCCTCTCAGTCCAAGG 3\'
- **CABI-3'-RP**: 5\' GTTAACAAACACGTAGGG 3\'
- **RBCS1A-3'-RP**: 5\' GTAGTAGAGTGGATTTGGGAGC 3\'
- **HY1-RP**: 5\' TCTGAATCTAGGTCAGGG 3\'
- **CABI-1'-RP**: 5\' GTTAACAAACACGTAGGG 3\'
- **RBCS1A-1'-RP**: 5\' AACGGCTTAAAAAGCTGGG 3\'
- **ACTIN2-RP**: 5\' GGACTAAACGCAAAACGA 3\'

**Chlorophyll and anthocyanin measurements**

Chlorophyll and anthocyanin contents were measured following essentially the same protocols as described in [41]. For chlorophyll and anthocyanin estimation, 50 seedlings were used in each genotype and the experiment was repeated thrice; and in each biological experiment, four technical replicates were used.

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