Short title: Development and analysis of tobacco HY5 mutant

Corresponding author:
Prabodh Kumar Trivedi, CSIR-National Botanical Research Institute (CSIR-NBRI), Rana Pratap Marg, Lucknow-226001, INDIA; **Telephone number: +91 522 2297958; e-mail address:** prabodht@hotmail.com; prabodht@nbri.res.in

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Tobacco HY5, NtHY5, positively regulates flavonoid biosynthesis and enhances salt stress tolerance

Deeksha Singh[1,2], Hiteshwari Sinha[1,2], Nivedita Singh[1,$], Shambhavi Dwivedi[3] & Prabodh Kumar Trivedi[1,2,3]

[1]CSIR-National Botanical Research Institute, Council of Scientific and Industrial Research (CSIR-NBRI), Rana Pratap Marg, Lucknow-226001, INDIA
[2]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad- 201002, India

[3]Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India.

[&]Present address:
Department of Botany, University of Lucknow, Lucknow-226007, India.

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One sentence summary: Analysis of *NtHY5* overexpressing and mutant plants suggests role of NtHY5 in flavonoid biosynthesis and salt stress tolerance.

Responsible author for distribution of materials: Prabodh K. Trivedi

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ABSTRACT

Plants have evolved complex signaling networks to regulate their growth and development. Some of these signaling components also play a crucial role in secondary metabolite biosynthesis. Among the signaling components identified to date, ELONGATED HYPOCOTYL 5 (HY5), a bZIP family transcription factor is the most investigated and known as the center of transcriptional network hub. However, HY5 has not been characterized from plants known to synthesize important secondary metabolites. In this study, based on homology search and phylogenetic analysis, HY5 has been identified from *Nicotiana tobaccum,* and characterized for its role in secondary plant product biosynthesis and stress response through developing overexpressing lines and CRISPR/Cas9-based knockout mutant plants. NtHY5 was able to complement the *Arabidopsis thaliana hy5* mutant at molecular, morphological and biochemical levels. Overexpression of *NtHY5* in tobacco led to the up-regulation of the phenylpropanoid pathway genes and enhanced the flavonoid content, whereas mutant plants had the opposite effect. Electrophoretic Mobility Shift Assay (EMSA) suggested that NtHY5 interacts with the promoter of *NtMYB12,* a transcription factor known to regulate flavonoid biosynthesis. In addition, *NtHY5* enhanced the abiotic stress tolerance as evident by the salt tolerance ability of HY5 overexpressing lines by diminishing the ROS accumulation after salt treatment. These data provide credible evidence about the potential role of NtHY5 in light-mediated flavonoid biosynthesis, plant growth and abiotic stress tolerance in tobacco. The photomorphogenic mutant, *Nthy5*, developed in this study, will help in elucidating the role of the HY5 in different biological processes in tobacco.

Keywords: *Arabidopsis thaliana,* flavonoid synthesis, HY5, light-dependent regulation, MYB transcription factor, *Nicotiana tobaccum,* salt stress

INTRODUCTION

Major plant developmental processes such as flowering, seed development, organ development, seed germination and seedling de-etiolation are governed by light (Chory et al., 2010; Vanhaelewyn et al., 2016; Gangappa et al., 2016; Bhatnagar et al., 2020). Many transcription factors of various families, including bHLH, bZIP, MYB, GATA and Zinc-finger get activated after light perception by photoreceptors (Oyama et al., 1997; Chory et al., 2010; Yogev et al., 2020). Constitutive nuclear-localized ELONGATED HYPOCOTYL 5 (HY5), a bZIP family transcription factor, acts downstream to the photoreceptors and plays a central role in seedling development via integrating light and phytohormone signaling pathways (Koornneef et al., 1980; Osterlund et al., 2000; Ulm et al., 2012; Li et al., 2013). As established by ChIP-chip experiments, HY5 regulates the expression of one-third of Arabidopsis genes and binds to promoters of more than 3000 genes directly (Zhang et al., 2011a). Thus, it acts as a master regulator by binding to *cis*-regulatory elements.

HY5 promotes photomorphogenesis in red, far-red, blue and UV-B light and acts downstream of cryptochromes, phytochromes and UVR8 (Jiang et al., 2012; Zheng et al., 2013; Yadav et al., 2020). The *hy5* mutant shows a long-hypocotyl phenotype in light (Koornneef et al., 1980) with an increased number of lateral roots, modified gravitropic and touch responses in roots (Oyama et al., 1997). The content of flavonoids, anthocyanin and chlorophyll was also reduced substantially in *hy5* seedlings compared to wild type in spite of the fact that seedlings of *hy5* mutant had larger cotyledons compared to wild type (Oyama et al., 1997; Sibout et al., 2006; Bhatia et al., 2018; Bhatia et al., 2021). In addition to plant growth and development, there are reports suggesting an involvement of HY5 in the regulation of terpenoid biosynthesis (Michael et al., 2020) and the expression and accumulation of miRNA-encoded peptide, miPEP858a (Sharma et al., 2021).

Flavonoids are low molecular weight polyphenolic secondary metabolites with a C_6-C_3-C_6 backbone. Flavonoids with more than 9000 distinct structures are synthesized via the phenylpropanoid/flavonoid pathway (Falcon Ferreyra et al., 2012). These molecules are divided into several classes, including chalcones, flavanones, dihydroflavonols, flavonols, flavones, isoflavonoids and anthocyanins on the basis of structural diversity (Ververidis et al., 2007; Saito et al., 2013). Sequential action of enzymes like chalcone isomerase (CHI), flavanone 3-

hydroxylase (F3H), flavonoid 3′ hydroxylase (F3′H) and flavonol synthase (FLS) leads to the formation of a aglycone flavonoid backbone. Furthermore, various modifications like glycosylation, methylation and acylation lead to huge chemical diversity in flavonoids (Ferrer et al., 2008). Flavonoids have received tremendous attention in the past few years because of their ubiquitous presence, diversity, and diverse roles in plants as well as in the pharmaceutical industry (Cai et al., 2010; Schulz et al., 2015). Flavonoids are known to have diverse roles in plants ranging from tissue and flower pigmentation to provide protection against various kinds of abiotic and biotic stresses (Winkel-Shirley 2002; Misra et al., 2010; Schulz et al., 2016). Overproduction of rutin was found to be responsible for insecticidal activity of AtMYB12 overexpressing transgenic tobacco plants and callus (Misra et al., 2010; Pandey et al., 2012).

Past studies in *Arabidopsis* demonstrated that R2R3 MYBs, bHLHs and WD40 regulatory proteins form the MBW ternary complex that coordinately regulates the flavonoid biosynthesis at the transcriptional level (Hichri et al., 2011). The early biosynthetic genes of the flavonoid biosynthetic pathway are transcriptionally regulated by a set of three closely related R2R3 MYBs; AtMYB12/PFG1, AtMYB11/ PFG2 and AtMYB111/ PFG3 and they belong to "PRODUCTION OF FLAVONOL GLYCOSIDES" family (Stracke et al., 2007; Stracke et al., 2010a). *AtMYB12, AtMYB11*, *AtMYB111* and *NtMYB12* are reported to regulate the flavonoid biosynthesis by activating the phenylpropanoid pathway genes (Misra et al., 2010; Pandey et al., 2015; Fu et al., 2016). Studies also suggest that the expression of these MYB transcription factors is regulated by miR858 (Sharma et al., 2016) and miRNA-encoded peptide miPEP858a (Sharma et al., 2020). The light regulatory mechanism of the flavonoid pathway in tobacco is not that much explored, but there are few studies suggesting that light plays an important role in flavonoid biosynthesis in tobacco (Fu et al., 2016; Pandey et al., 2015).

Tobacco is one of the important cash crops worldwide and its leaves contain various secondary plant products, including flavonoids. Therefore, tobacco is a suitable model plant to explore regulatory mechanisms related to various secondary plant products. The extensive characterization of HY5 and flavonoid biosynthesis has been done in Arabidopsis, Rice, *Pisum sativum*, *Physcomitrella patens* and *Lotus japonicus* (Burman et al., 2018; Yamawaki et al., 2011, Nishimura et al., 2002; Weller et al., 2009, Li et al., 2015; Zhai et al., 2016; Zhai et al 2019) but, there are no reports of characterization of *Nicotiana tobaccum* HY5 and its role in light-regulated photomorphogenesis as well as flavonoid biosynthesis pathway. In this study, we

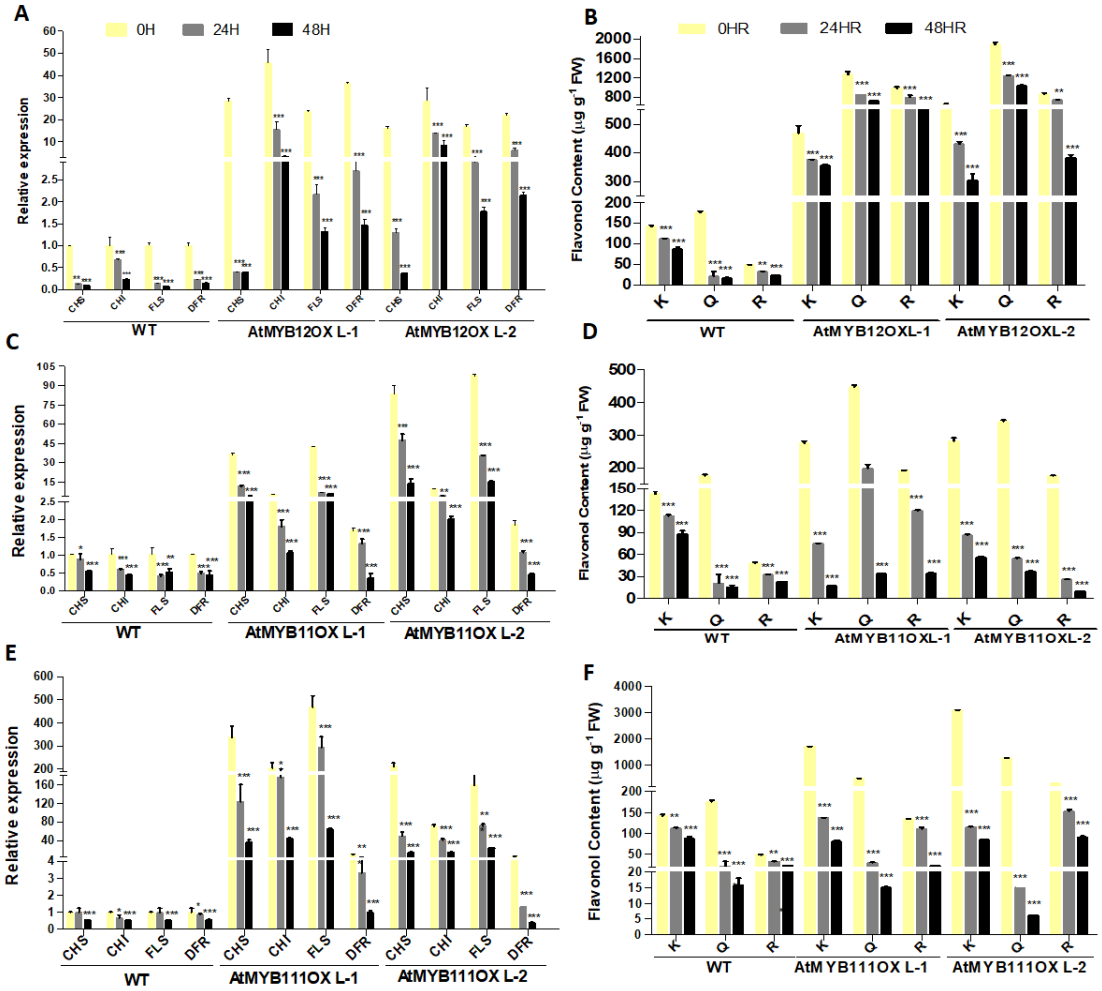
demonstrated that NtHY5, an important light signaling component, regulates light-mediated flavonoid biosynthesis in tobacco. To decipher the function of NtHY5 in detail, we functionally complemented it in the corresponding Arabidopsis mutants. Additionally, the binding of NtHY5 to the promoter of *NtMYB12* was validated, which suggests that HY5 activates the expression of NtMYB12 in response to light. We also developed the CRISPR/Cas9-based mutant of HY5 in tobacco, which will be a resource to study the involvement of HY5 in various developmental processes, secondary plant product biosynthesis and stress responses. Using NtHY5 overexpressing tobacco lines and developed mutant, we have demonstrated the role of NtHY5 in providing salt stress tolerance in tobacco.

RESULTS

Light modulates the flavonol accumulation in tobacco

In tobacco, the role of light in the regulation of flavonoid biosynthesis has not been studied in detail. To broaden our understanding of the flavonol regulation through light, we analyzed the expression of genes associated with the phenylpropanoid pathway and accumulation of the major flavonol aglycones and glycosides in *Nicotiana* overexpression lines of AtMYB12 (AtMYB12OX), AtMYB11 (AtMYB11OX), and AtMYB111 (AtMYB111OX). An increased expression of pathway genes and flavonoid accumulation was observed in 10-day light-grown transgenic seedlings (AtMYB12OX, AtMYB11OX and AtMYB111OX) compared to WT **(Fig. 1A, B, C, D, E and F)**. Additionally, an increase in antioxidant activity in these transgenic lines compared to WT was observed **(Supplemental Fig. S1)**. All these data suggested that similar to Arabidopsis, flavonoid biosynthesis in tobacco is also under the transcriptional control of R2R3-MYBs PRODUCTION OF FLAVONOL GLYCOSIDE1 (PFG1／MYB12, PFG2／MYB11 and PFG3／MYB111).

Further, 10-day-old light-grown AtMYB12, AtMYB11 and AtMYB111 overexpression transgenic seedlings were incubated in darkness for 24h and 48h. Analysis suggested that dark incubation led to significant down-regulation in the expression of genes (*NtCHS, NtCHI, NtFLS* and *NtDFR*) involved in the flavonol biosynthesis **(Fig. 1A, C and E)**. This is accompanied by a



decrease in the accumulation of flavonol aglycones like kaempferol, quercetin and glycosides rutin in overexpressing MYB lines as compared to the WT **(Fig. 1 B, D and F; Supplemental Fig. S2)**. These results indicated that light positively regulates flavonoid biosynthesis, and there could be a possible involvement of other light-associated signaling components, in addition to AtMYB12, AtMYB11, and AtMYB111.

Figure 1. Light affect flavonoid biosynthesis in transgenic tobacco. (A),(C),(E) Relative transcript abundance of *AtCHS*, *AtCHI*, *AtFLS* and *AtDFR* that are involved in phenylpropanoid pathway in AtMYB12, AtMYB11 and AtMYB111 overexpressing transgenic lines respectively

by real time PCR using RNA from the 10-d-old light grown seedlings transferred to dark for 24hr and 48hr. Tubulin was used as endogenous control to normalize the relative expression levels. **(B), (D), (F)** Effect of light over flavonol biosynthesis in different transgenic lines expressing AtMYB12, AtMYB11 and AtMYB111 respectively. Compounds (K, Kamepferol; Q, Quercitin; R; Rutin) were quantified using methanolic extracts of 10-d-old old seedlings of different transgenic lines through HPLC. Data are derived from the mean of three technical replicates. Error bars represent SE of means (n=3). The statistical analysis was performed using two-tailed Student's t-tests. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; ***P< 0.001.

NtHY5 and light-dependent flavonoid accumulation

The participation of light through associated transcription factors in the regulation of flavonoid biosynthesis has been demonstrated in plants. Studies in *Arabidopsis, Oryza sativa, Zea mays* and many other species have shown that HY5 is an important light signaling component (Nawkar et al., 2017; Burman et al., 2018); thus, we studied the role of tobacco HY5 in flavonol accumulation. The BLAST search using AtHY5 in the NCBI database identified a homolog in tobacco with more than 90% sequence similarity (XM_009590053). This was named NtHY5 as it showed significant sequence similarity with sequences from other plant species like tomato, capsicum, etc. NtHY5 gene contains 4 exons and 3 introns similar to Arabidopsis **(Supplemental Fig. S3A)**. Phylogenetic and pairwise distance analysis suggests thatNtHY5 is closer to CaHY5 and SlHY5 compared to AtHY5 (**Fig. 2A)**. The NtHY5 showed high homology with Arabidopsis and tomato amino acid sequences **(Fig. 2B**) and contains a VPE motif (COP1-interaction motif) and leucine-rich zinc finger motif (as described by Holm et al., 2002). Analysis through the conserved domain database suggested NtHY5 belongs to the bZIP superfamily, like HY5 proteins of other plant species **(Supplemental Fig. S3B)**.

To study the involvement of NtHY5 in flavonol biosynthesis through controlling the expression of genes associated with the phenylpropanoid pathway genes expression, tobacco (WT) seedlings were grown in the light and continuous dark for 10 days. Seedlings grown in dark showed longer hypocotyl length compared to light-grown seedlings **(Fig. 2C)**. The phytochemical analysis and expression of *NtHY5* and phenylpropanoid pathway genes revealed a significantly higher expression and enhanced flavonoid accumulation and expression of genes in 10-day-old light-grown compared to dark-grown seedlings **(Fig. 2D, E and F)**. To study the responsiveness of flavonols towards light, 5-day-old dark-grown seedlings were transferred to light conditions for 3 h. This dark-to-light transition of seedlings showed significant enhancement in the flavonol

content **(Supplemental Fig. S4)**, suggesting light-responsiveness of flavonol biosynthesis. To investigate the spatio-temporal expression of endogenous *NtHY5*, a comparative analysis was carried out at different stages of tobacco development and the result showed 4- to 5-fold higher expression in the mature leaf and young leaf, compared with seedling, root, stem, and flower of the young plant **(Fig. 2G)**.

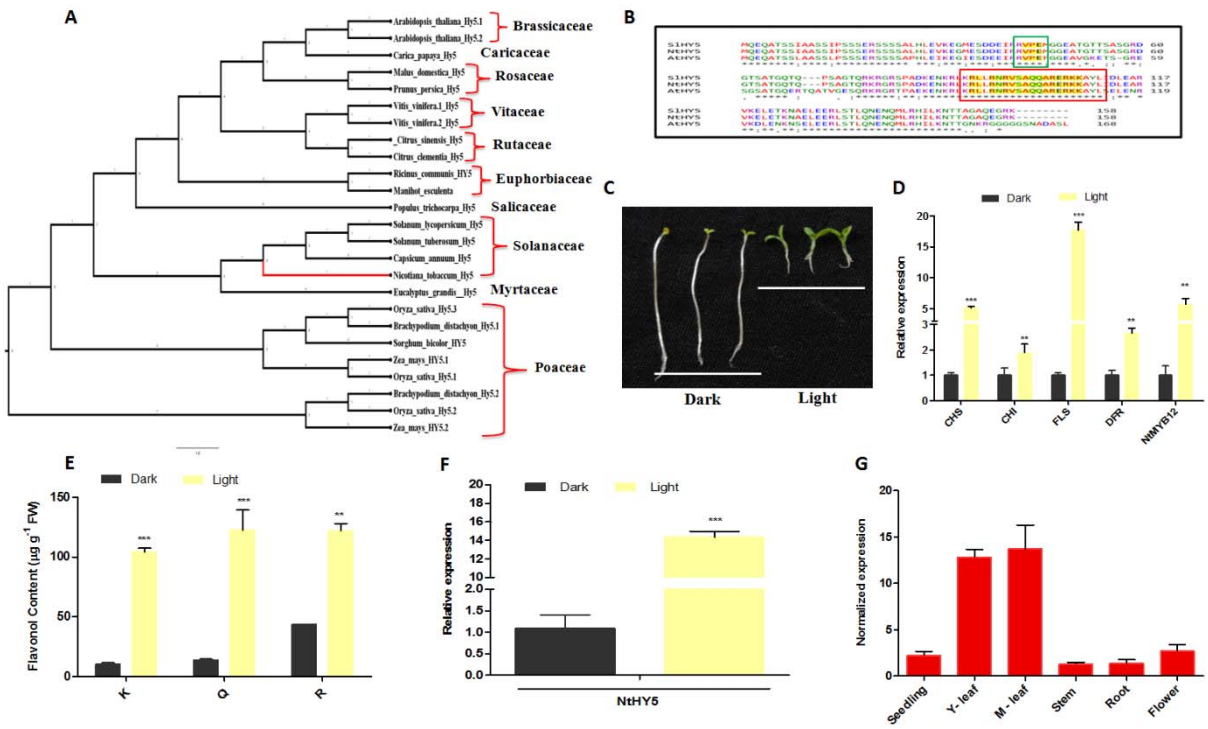


Figure 2. NtHY5, the light signaling component, alters flavonoid content in tobacco. **(A)** Phylogenetic tree of HY5 homologous proteins (sequence obtained from NCBI database) from across species of different families. The numbering at the nodes represents the number of trees that have the same topology as the consensus tree. NtHY5 is denoted through the red branch, and the scale bar indicates the branch length. **(B)** Amino acids alignment of NtHY5 and its homologs in Tomato and Arabidopsis using multallignment tool. The VPE motif and zinc finger domain is boxed in green and red color respectively. **(C)** Phenotype of 10-d-olddark and light grown WT seedlings. **(D)** Expression analysis of phenylpropanoid pathway genes *NtCHS*, *NtCHI*, *NtFLS* and *NtDFR* in WT seedlings grown in light and dark for 10 days. **(E)** Quantitative estimation of flavonoids (K, Q and R) in 10-d-light and dark grown seedlings through HPLC. **(F)** Relative expression of NtHY5 in 10 light and dark grown seedlings. **(G)**Normalized expression of NtHY5 in different tissue [seedling, young (Y) leaf, mature (M) leaf, stem, root and flower]. Data are derived from the mean of three technical replicates. Tubulin was used as endogenous control to normalize the relative expression levels. Error bars represent SE of means (n=3). The statistical analysis was performed using two-tailed Student's t-tests. The asterisks indicate significant difference, **P< 0.01; ***P< 0.001.

NtHY5 Binds to the promoter of NtMYB12

As the expression of flavonol biosynthetic genes enhanced in response to light, it was of interest to determine whether the Nicotiana transcription factor, NtMYB12, is controlled by the light signaling component, NtHY5. The *in silico* analysis of the promoters of NtMYB12 revealed the presence of putative LREs (GATA, BOX-4, ACE and LTR) upstream of the transcription start

site **(Supplemental Table S1)**. Analysis revealed the presence of two G-boxes with core motif (ACGT) within the 1.5-kb region upstream of the transcription start site of NtMYB12 **(Fig. 3A)**. Electrophoretic Mobility Shift Assay (EMSA) using NtHY5 suggested that NtHY5 binds to G-box at -220 bp (G-box I) from the transcription start site and to the other G-box at -1249 bp (G-box II) elements **(Fig. 3B)**. Results also suggested that NtHY5 binds to the labeled probe containing G-box but not the mutated version (G-mut I and G-mutII) where the G-box had been disrupted **(Fig. 3C and D)**. These results indicate that NtHY5 binds directly to the promoter of NtMYB12 and might regulate its expression.

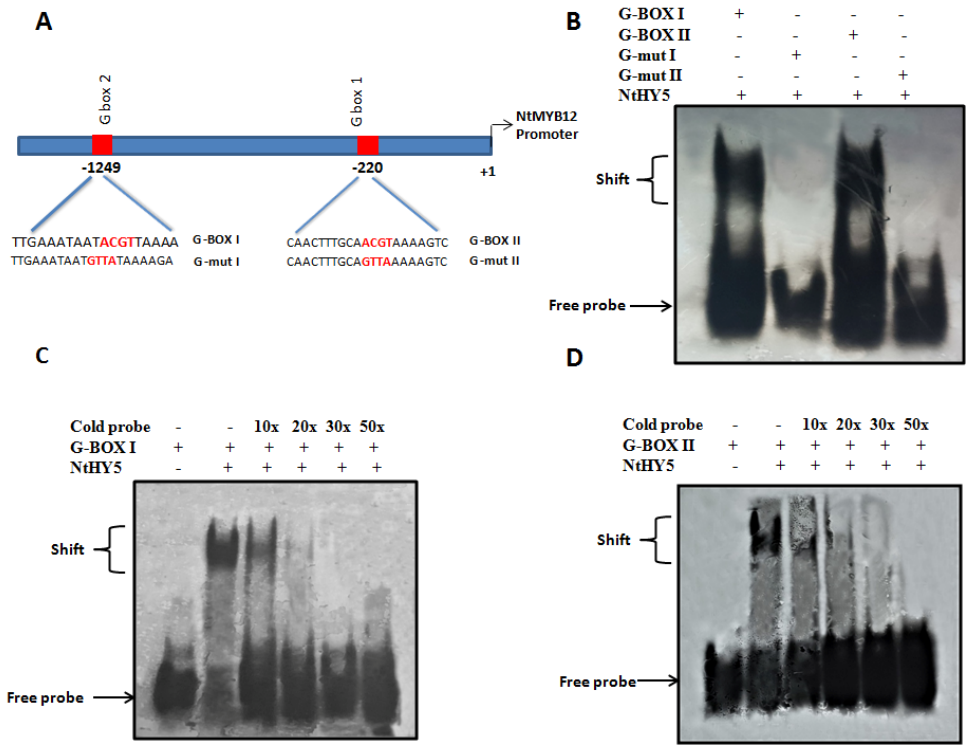


Figure 3. NtHY5 interacts with NtMYB12 promoter. (A) Representation of the probes (G-BOXI, G-mut I, G-BOXII and G-mut II) designed upstream of the transcription start site of NtMYB12 promoter. G-BOX is altered by multiple base substitutions. **(B)** EMSA (Electrophoretic mobility shift assay) for the binding of 6X-His-HY5 (NtHY5) with core ACE-DIG element (HY5 binding site that is G-BOXI and G-BOXII) present in the LRE motifs of the NtMYB12 promoter. Upper and lower arrows indicate shift and free probe respectively. **(C), (D)** EMSA with competition between digoxigen in labelled G-BOXI, unlabeled G-BOX I (cold), G-BOXII and unlabeled G-BOX II (cold) probe to bind to 6X-His-HY5 respectively. Superscript values 10X, 20X, 30X and 50X represent increasing amount of cold probe. Upper and lower arrows indicate shift and free probe respectively.

NtHY5 is a functional ortholog of AtHY5

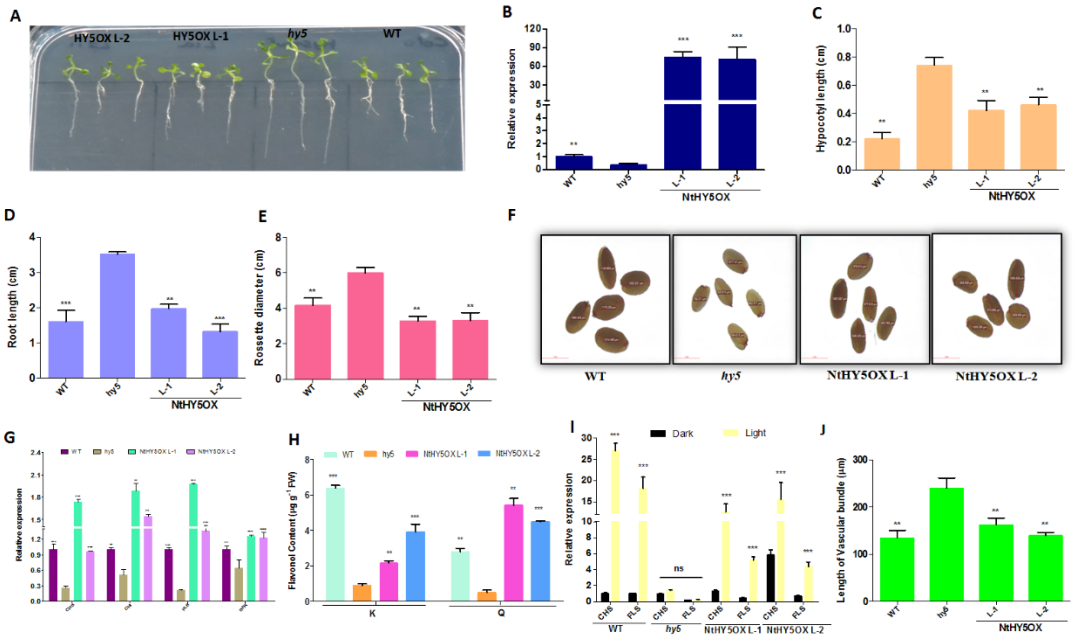
To investigate the biological function of NtHY5, it was used to complement Arabidopsis *hy5* mutant **(Fig. 4A; Supplemental Fig. 5A)**. The homozygous transgenic lines in the *hy5* background, NtHY5OXL-1 and NtHY5OXL-2, were used for detailed analyses. Higher expression of *NtHY5* was observed in complemented lines compared to WT as well as *hy5* mutant **(Fig. 4B)**. Since the Arabidopsis, *hy5* mutant is reported to have an elongated hypocotyl length in light (Koornneef et al., 1980; Jing et al., 2013), thus 7-d-old white light-grown homozygous complemented transgenic lines were analyzed for hypocotyl length. The average hypocotyl lengths of 7-d-old wild-type (WT), *hy5* mutant, and NtHY5;*hy5* seedlings were approximately 0.2, 0.7, and 0.4 mm, respectively. The decrease in hypocotyl length in complemented lines indicated that NtHY5 could compensate for the loss of function of HY5 in the *Athy5* mutant **(Fig. 4A and C)**.

As it is previously reported that AtHY5 plays a major role in light-regulated root growth in Arabidopsis (Zhang et al., 2017), we analyzed the root length of 7-d-old light-grown WT and *hy5* mutant *Arabidopsis* seedlings and found root length to be longer in *hy5* mutant compared with WT. The complementation of the *hy5* with NtHY5 regained the root length as of WT **(Fig. 4D)**. The average length of *Athy5* mutant rosette diameter was approximately 6 cm whereas, the length of WT and complemented lines were 4 and 3 cm, respectively. Similarly, seeds of *hy5* mutant also regained the size similar to WT after complementation **(Fig. 4E and F; Supplemental Fig. 5B and C)**. These results suggested that NtHY5 could complement the phenotypic function of the *Athy5* mutant.

NtHY5 modulates the expression of flavonol biosynthetic genes in Arabidopsis

To understand the function of *NtHY5*, the expression of flavonol biosynthetic pathway genes was studied in WT, *hy5* mutant and NtHY5;*hy5* complemented seedlings. The analysis suggested that overexpression of *NtHY5* in the *hy5* mutant background restored the expression of genes associated with the phenylpropanoid pathway (*AtCHS, AtCHI, AtFLS and AtDFR)* **(Fig. 4G)**. Moreover, the levels of flavonols, such as kaempferol and quercetin, were also restored in NtHY5; *hy5* overexpressed lines compared with *hy5* mutant **(Fig. 4H)**.

To investigate the importance of NtHY5 in the regulation of genes associated with flavonol biosynthesis, expression of genes was studied in 5-days-old dark-grown seedlings exposed to light for 3 h. The transcript level of structural genes increased on exposure to light for 3h. By contrast, *hy5* mutant showed inhibited light-dependent induction of genes expression **(Fig. 4I)**. The expression analysis of MYB family transcription factors revealed a higher expression of these transcription factors in NtHY5 complemented lines after 3h of light induction compared with *hy5* mutant seedlings. These results suggested that *NtHY5,* through regulation of MYB family transcription factors (*AtMYB12*, *AtMYB11* and *AtMYB111*), affects the flavonoid accumulation in Arabidopsis **(Supplemental Fig. S6)**.



These results also suggest that NtHY5 has the potential to complement *Athy5* at the phenotypic as well as the molecular level.

Figure 4. Complementation of Arabidopsis *hy5* mutant by NtHY5. (A) Phenotype of 7-d-old light-grown WT (wild type; Col-0), *hy5* and NtHY5OX; *hy5* seedlings (L1 and L2 are two independent transgenic lines overexpressing NtHY5 in *hy5* mutant background). **(B)** Relative expression of NtHY5 in 7-d-old light grown WT, *hy5*, L1 and L2. **(C)** Hypocotyl length of 7-d-old white light grown WT, *hy5*, L1 and L2 seedlings. **(D)** Root length of 7-d-old white light grown WT, hy5, L1 and L2. **(E)**Rosette diameter of 30-day-old WT, *hy5*, L1 and L2 plants. **(F)** Representative photographs of the seed size of WT, *hy5* and transgenic lines (L1 and L2). Bars = 1 mm. **(G)** Relative expression of pheylpropanoid pathway genes (*AtCHS, AtCHI, AtFLS and AtDFR*) in 7-d-light grown WT, *hy5*, L1 and L2 seedlings. **(H)** Quantitative estimation of flavonol (Kaempferol and Quercitin) content in 7-d-old light grown WT, *hy5*, L1 and L2 seedlings through HPLC. **(I)** Expression analysis of *AtCHS, AtCHI, AtFLS* and *AtDFR* in WT, *hy5*, L1 and L2 seedlings grown for 5 days in dark followed by an exposure to light for 3hr. (J) Length of vascular bundle of 30-day-oldstem of WT, *hy5*, L1 and L2 plants. Statistical analysis

was performed using two-tailed Student's t-test. Error bars represent SE of means (n=3) For hypocotyl and root length (n=10-12). Tubulin was used as endogenous control to normalize the relative expression levels. Error bars represent standard deviation. Asterisks indicate a significant difference, *P < 0.1, **P < 0.01, ***P < 0.001. ns represent non-significant.

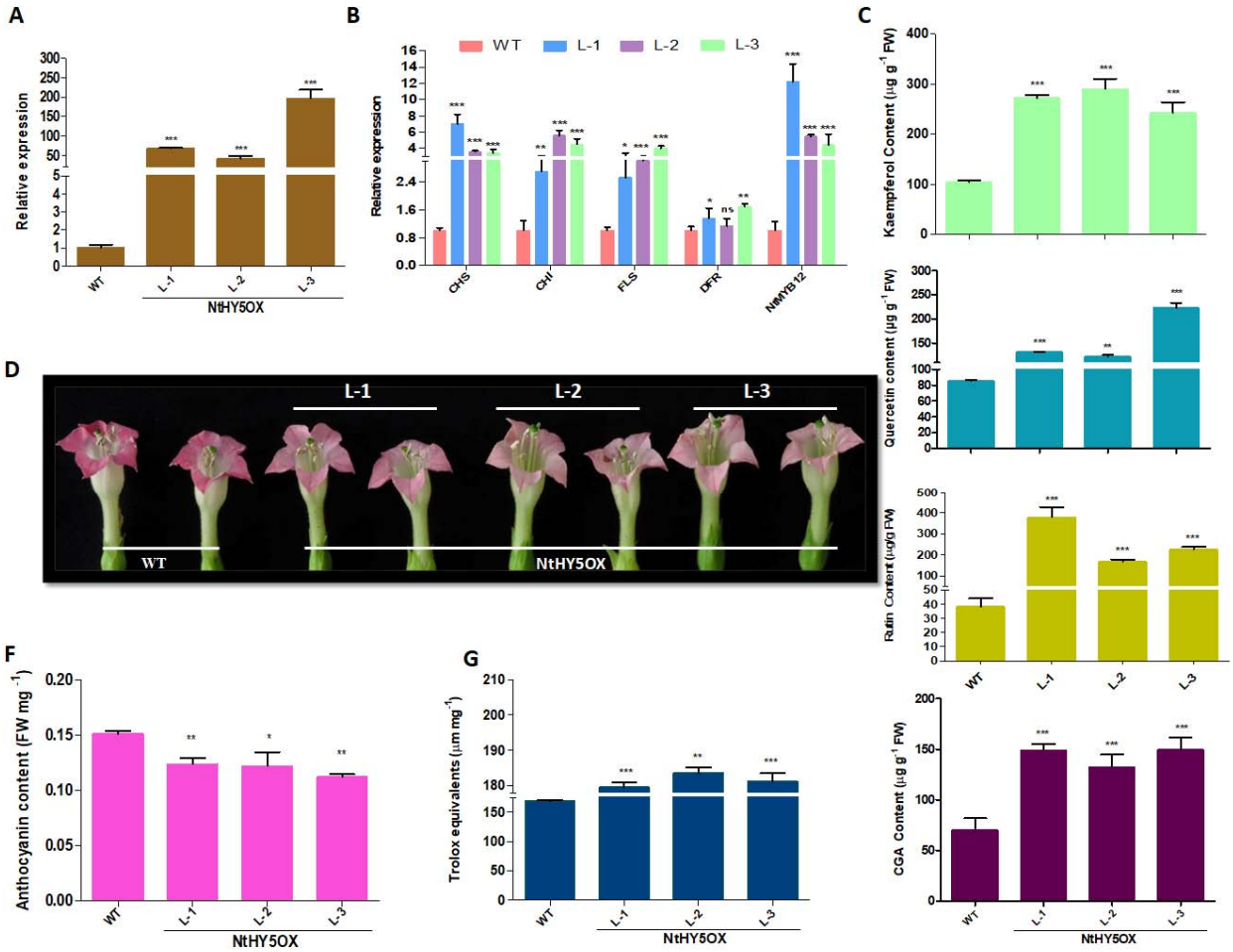
NtHY5 modulates the levels of lignin, anthocyanin and chlorophyll in Arabidopsis

The phenylpropanoid pathway synthesizes various secondary metabolites, including flavonols, flavones, flavonols, isoflavones, flavan-3-ol, proanthocyanidins (PAs), anthocyanin and lignin. Phenylalanine is reported to be a common precursor for all of them (Vogt et al., 2010). Thus, we also studied the effect of NtHY5 on lignin and anthocyanin production in Arabidopsis. The stem cross-sections of WT, *hy5* and NtHY5;*hy5* overexpression lines (NtHY5OXL-1 and NtHY5OXL-2) were used for lignin staining. The results showed a decreased lignification in the interfascicular and vascular tissues. In addition, the length of the vascular bundle decreased in NtHY5;*hy5* compared with WT and *hy5* mutant plants **(Fig. 4J; Supplemental Fig. S5D)**. As AtHY5 is known to be involved in anthocyanin and chlorophyll biosynthesis (Oyama et al., 1997; Chattopadhyay et al., 1998), we estimated anthocyanin and chlorophyll contents of WT, *hy5*, and NtHY5;*hy5* seedlings. In 7-d-old light-grown seedlings, the total chlorophyll content of NtHY5;*hy5* was higher compared with WT and *hy5* **(Supplemental Fig. S7A)**. The anthocyanin content in NtHY5;*hy5* was higher than *hy5* seedlings but found to be comparatively lesser than WT **(Supplemental Fig. S7B)**. Overall, these results suggest that NtHY5 can compensate for the loss in *Athy5* mutant at the transcript level and at the metabolite level.

Overexpression of NtHY5 in tobacco

In addition to the functional complementation of the *hy5* mutant of Arabidopsis by NtHY5, we generated transgenic tobacco lines to study if NtHY5 performs additional or unique functions in tobacco. The expression of *NtHY5* and pathway genes (*NtCHS*, *NtCHI*, *NtFLS* and *NtDFR*), as well as *Nicotiana* MYB family transcription factor (*NtMYB12*), enhanced in 10-d-old light-grown transgenic compared to WT tobacco seedlings **(Fig. 5A, B)**. The analysis of metabolites in the NtHY5OXL-1, NtHY5OXL-2 and NtHY5OXL-3 lines suggested a significantly higher accumulation of flavonols (kaempferol, quercetin and rutin) and the CGA levels **(Fig. 5C)**. The flower of NtHY5OXL-1, NtHY5OXL-2 and NtHY5OXL-3 lines showed reduced pigmentation compared to WT **(Fig. 5D)**. Analysis suggested reduced anthocyanin content in overexpression lines compared to the WT **(Fig. 5F)**. As it is known that flavonol content leads to the antioxidant

activity of plants (Pandey et al., 2015; Song et al., 2020), total antioxidant activity was measured in extracts from NtHY5OXL-1, NtHY5OXL-2 and NtHY5OXL-3 tobacco seedlings. Analysis



suggested enhanced antioxidant activity in transgenic lines compared to the WT **(Fig. 5G)**. Altogether, these results suggest that overexpression of NtHY5 modulates metabolic content such as flavonol and anthocyanin along with the antioxidant activity of tobacco.

Figure 5. Overexpression of NtHY5 modulates gene expression and metabolite level in tobacco. (A) Relative expression of NtHY5 in 10-d-old light grown WT and NtHY5OX lines of tobacco (L1, L2 and L3). **(B)** Expression analysis of pheylpropanoid pathway genes (*NtCHS, NtCHI, NtFLS and NtDFR*) in 10-d-old light grown WT and NtHY5OX lines. **(C)**Quantification of flavonoids (K, Q and R) and polyphenolic compound (CGA) in 10-d-old light grown WT and NtHY5OX lines of tobacco using HPLC. **(D)** Representative photograph of flower color alterations in petals of NtHY5OX transgenic lines in compared with WT. **(E)** Total anthocyanin content in petals of WT and NtHY5OX transgenic lines. **(F)** Estimation of antioxidant activity by

360 trolox equivalent in WT and NtHY5OX lines. TUBULIN was used as the endogenous control to
361 normalize the relative expression levels. The statistical analysis was performed using two-tailed
362 Student's t-tests. The data are plotted as means ± SD (n= 3). The error bars represent standard
363 deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; ***P< 0.001.

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Development and analysis of NtHY5 mutants

To gain a deeper insight into NtHY5 function, we developed NtHY5 mutant plants using the CRISPR–Cas9 approach. To mutate NtHY5, we used guide RNAs (gRNAs) targeting the coding region **(Supplemental Fig. S8)**. The nucleotide sequence analysis of the mutants revealed deletions and insertions in the coding regions of NtHY5 **(Fig. 6A)**. The *NtHy5*^{CR} lines showed frame-shift mutations leading to truncated peptides **(Supplemental Fig. S8)**. The expression analysis of NtHY5 in mutants revealed a significant reduction in NtHY5 expression **(Fig. 6B)**. Expression of *NtMYB12* and other phenylpropanoid pathway genes, like *NtCHS, NtCHI, NtFLS* and *NtDFR,* was also found to be significantly reduced **(Fig. 6C)**. Phytochemical analysis suggested a significantly decreased level of flavonols (kaempferol, quercetin and rutin), CGA and anthocyanin content in *NtHy5*^{CR} lines compared to WT **(Fig. 6D, E, F, G and H)**. The decrease in the expression of phenylpropanoid pathway genes and NtMYB12 and reduced flavonoid content suggested the pivotal role of NtHY5 in flavonoid biosynthesis in tobacco.

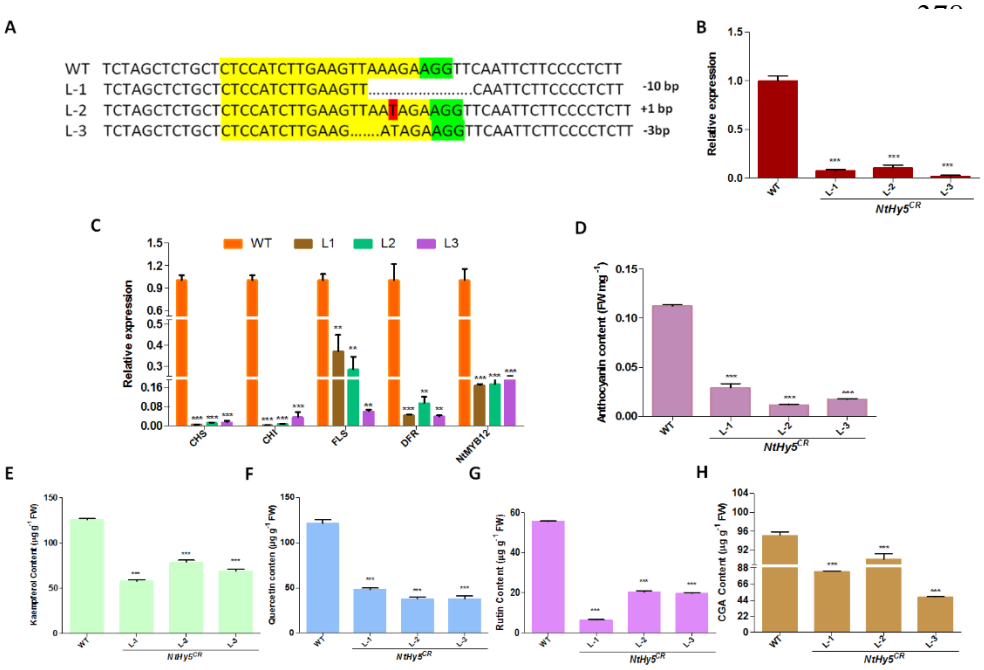


Figure 6. CRISPR-Cas9-mediated knockout of NtHY5 alters gene expression and accumulation of metabolite content. (A) Representation of mutations in target region in three independent transgenic lines of tobacco. Dots indicate deleted nucleotides. Yellow highlight denotes gRNA region used for targeting the sequence. Red highlights denote indels in nucleotides. **(B)** Relative expression of NtHY5 in 10-d-old light grown WT and *NtHY5*^{CR} lines of tobacco (L1, L2 and L3). **(C)** Relative transcript abundance of pheylpropanoid pathway genes (*NtCHS, NtCHI, NtFLS and NtDFR*) in 10-d-old light grown WT and *NtHY5*^{CR} lines. **(D)** Quantification of flavonoids (K, Q and R) and polyphenolic compound (CGA) in 10-d-old light grown WT and *NtHY5*^{CR} lines of tobacco. **(E)** Quantification of Anthocyanin content in 10-d-old light grown WT and *NtHY5*^{CR}lines. **(F)** Quantification of antioxidant activity by trolox equivalent in 10-d-old light grown WT and *NtHY5*^{CR} lines. Tubulin was used as the endogenous control to normalize the relative expression levels. The statistical analysis was performed using two-tailed Student's t-tests. The data are plotted as means ± SD (n=3). The error bars represent standard deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; ***P< 0.001.

NtHY5 regulates growth and development of tobacco

Several previous studies have demonstrated that AtHY5 regulates the hypocotyl length by repressing the expression of hypocotyl cell elongation-related genes (Liu et al., 2013). To study the difference in hypocotyl length in *NtHY5* overexpression and *NtHy5*^{CR} lines, we measured the hypocotyl length of 10-day-old light- and dark-grown tobacco seedlings. In light-grown seedlings, the hypocotyl length of *NtHy5*^{CR} mutant seedlings was significantly higher compared to overexpression lines **(Fig. 7A, B)**. At the same time, hypocotyl length of the dark-grown overexpressing lines was significantly higher compared to *NtHy5*^{CR} mutant plants **(Fig. 7C; Supplemental Fig. S9A)**.

It is reported that the asymmetric accumulation of flavonoids in root tips in Arabidopsis affects the direction of root growth (Wan et al., 2018). Thus, we determined the effect of NtHY5 on root length and root agravitropic response. For this, the root bending angle of 10-d-old light- and dark-grown, NtHY5 overexpression and *NtHy5*^{CR} seedlings was measured. A significant increase in root length of *NtHy5*^{CR} seedlings compared to overexpression lines was observed **(Fig. 7D)**. Furthermore, observations suggested that the root-bending angle of NtHY5OXL-1 and NtHY5OXL-2 seedlings was significantly higher compared to *NtHy5*^{CR}L-1, *NtHy5*^{CR}L-2 and WT plants **(Fig. 7E)**. Our analysis suggested that the *NtHy5*^{CR} has a smaller seed size compared to the overexpression plants **(Fig. 7F; Supplemental Fig. S9B)**. We also counted the number of seeds per unit (5 mg) weight and *NtHy5*^{CR} had a significantly larger number of seeds compared to overexpression lines **(Fig. 7G)**. In addition to these factors, the plant height of 40-days-old *NtHy5*^{CR} plants was significantly higher than *NtHY5* overexpression and WT plants **(Fig. 7H and I)**. The dark-to-light transition was able to enhance the expression of genes to several folds in *NtHY5* overexpressed lines. However, such an increase was not observed in *NtHy5*^{CR} plants **(Fig. 7J and K)**. These results suggested that NtHY5 is crucial for plant growth and light-responsive expression of genes associated with the phenylpropanoid pathway.

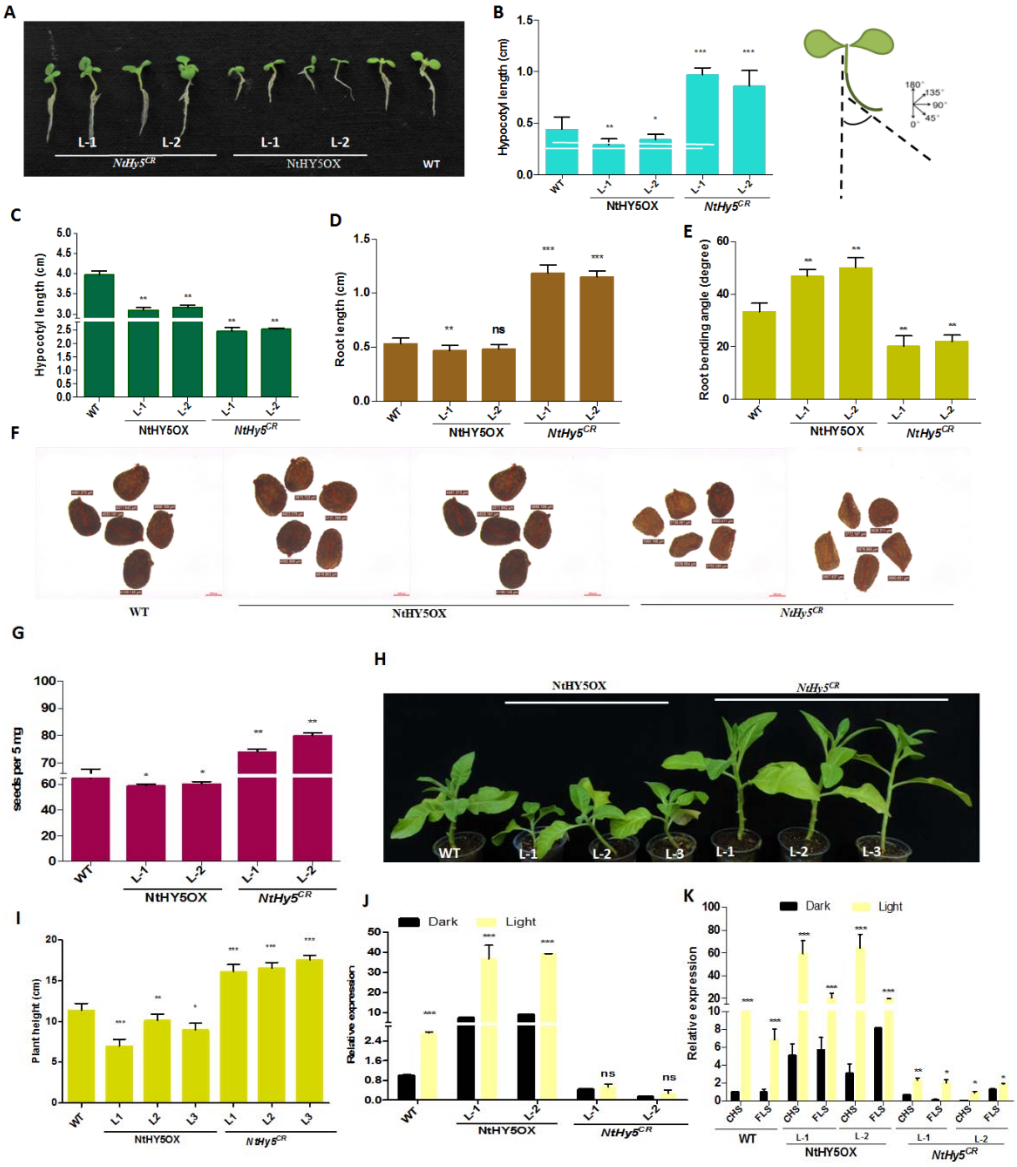


Figure 7. NtHY5OX and CRISPR–Cas9-derived knockout mutants show altered phenotypes and gene expression. **(A)** Representative image of 10-d-old light grown WT, NtHY5OX and *NtHY5*^{CR}seedlings grown on half-strength MS medium. Scale bars, 1 cm. **(B), (C)** Hypocotyl length of 10-d-old light and dark grown WT, NtHY5OX and *NtHY5*^{CR} seedlings. **(D)** Root length of 10-d-old light grown WT, NtHY5OX and *NtHY5*^{CR} seedlings. **(F)** Measurement of root bending angle of 10-d-old light grown WT, NtHY5OX and *NtHY5*^{CR} seedlings. **(F)** Quantitative analysis of seed size of WT, NtHY5OX and NtHY5^{CR} plants. **(G)** Quantitative estimation of Number of seeds per 5 mg of WT, NtHY5OX and *NtHY5*^{CR} plants. **(H)** Representative image of 40-day-old WT, NtHY5OX and *NtHY5*^{CR} plants showing plant heights. **(I)** Data presenting difference in height of 40-day-old WT, NtHY5OX and *NtHY5*^{CR} plants. **(J), (K)** Relative expression of NtHY5 and phenylpropanoid pathway genes (*NtCHS, NtCHI, NtFLS* and *NtDFR*) in 10-d-old light grown seedlings transferred to dark for 3h. Tubulin was used as the endogenous control to normalize the relative expression levels. The statistical analysis was performed using

two tailed Student's t-tests. The data are plotted as means ± SD (n= 3). For measurement of root bending angle, root length, hypocotyls length and seed size (n=10-12). The error bar represents standard deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; 0.001.

NtHY5 enhanced tobacco tolerance to salt stress

Flavonoids are known to positively regulate the abiotic stress tolerance ability of plants (Wan et al., 2018; Chen et al., 2019; Bhatia et al., 2018). To study the response of NtHY5 under salt stress, leaf disc assay was performed to analyze the comparative effect of NaCl (200 mM) on leaves of NtHY5OX lines and*NtHy5*^{CR}and WT plants. After 3 days of salt stress, higher chlorosis was observed in the leaves of *NtHy5*^{CR} compared with leaves of NtHY5OX and WT plants **(Fig. 8A and B)**. Chlorosis affects the survival of leaves by decreasing the chlorophyll content (Pandey et al., 2014). Thus, the survival of leaves was measured by analyzing the total chlorophyll content as well as chlorophyll a and b. Higher chlorophyll content was observed in NtHY5OX leaves compared with *NtHy5*^{CR} and WT leaves after 3 days of salt stress **(Fig. 8C)**. It is well known that salt stress leads to excessive accumulation of ROS, leading to cell death (Chen et al., 2019; Ahanger et al., 2017). Higher NBT and DAB staining were observed in *NtHy5*^{CR} compared with NtHY5OX and WT, suggesting that NtHY5 might play a crucial role in salt stress tolerance in tobacco by inhibiting ROS accumulation **(Fig. 8D; Supplemental Fig S10)**. A higher ROS accumulation suggests the lower antioxidant activity in *NtHy5*^{CR} lines compared with NtHY5OX and WT.

487 **Discussion**

488 **HY5 participates in light-induced flavonoid biosynthesis in *Nicotiana tobaccum***

489 Studies on flavonol biosynthesis in different plant species suggest a tight regulation by multiple
490 exogenous and endogenous factors, involving many transcription factors (Stracke et al., 2007;
491 Stracke et al., 2010a; Pandey et al., 2014; Sharma et al., 2016). In tobacco, regulation of
492 flavonoids is not studied extensively; however, few reports suggest the importance of
493 transcription factors in the regulation of flavonoid biosynthesis. According to previous studies,
494 up-regulation of three functionally redundant transcription factors, namely AtMYB12,
495 AtMYB11 and AtMYB111, enhance the flavonoid biosynthesis in tobacco by activating a
496 branch of the phenylpropanoid pathway (Mishra et al., 2010; Pandey et al., 2012; Pandey et al.,
497 2014; Pandey et al., 2015). Our result suggests that dark exposure inhibits flavonol accumulation
498 by decreasing the expression of pathway genes in spite of overexpression of AtMYB12,
499 AtMYB11 and AtMYB111 in tobacco **(Fig. 1)**. Similar to our result, a decrease in flavonol
500 content after dark exposure in AtMYB12 and AtMYB111 overexpressing lines in Arabidopsis
501 and mature tobacco plants, respectively, was reported (Bhatia et al., 2021; Pandey et al., 2015).
502 Our data also suggest that the up-regulation of genes involved in the flavonoid biosynthesis
503 pathway by AtMYB111 is significantly higher compared to AtMYB12 and AtMYB11, although
504 they are functionally redundant. This might be due to the differential influence of MYB11,
505 MYB12 and MYB111 on the spatial accumulation of specific flavonol derivatives, as reported
506 by Stracke et al., (2010a). All these observations gave rise to a fundamental question, i.e.,

whether there is an additional factor other than these transcription factors involved in the regulation of flavonol accumulation in tobacco.

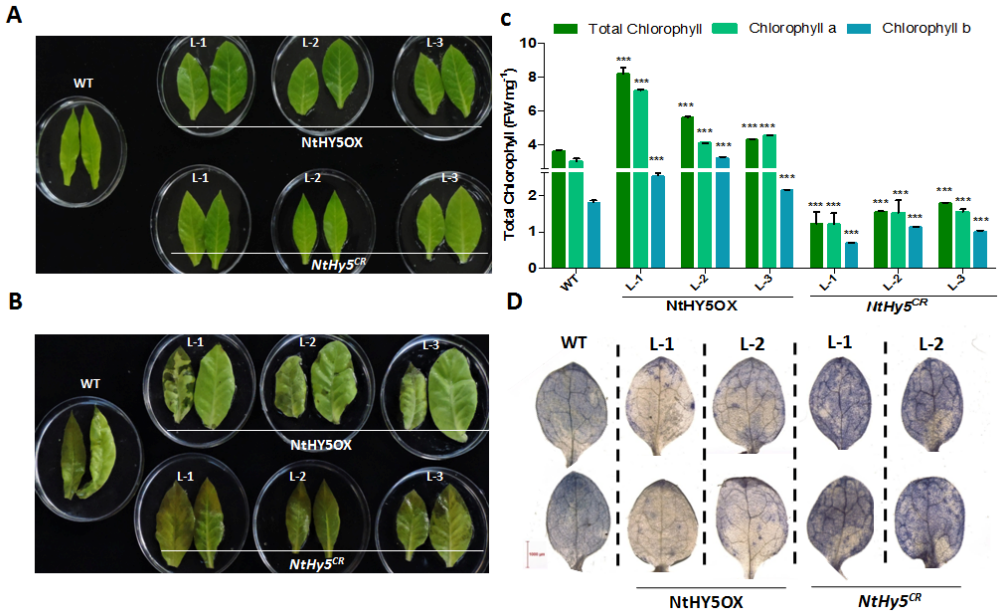


Figure 8. Effect of salt stress on different transgenic. Fully expanded leaves of 30-d-old plants to floated on 6 ml of 200 mM NaCl solution (for salt stress) for 3 days. **(A), (B)** The phenotypic (chlorosis) changes due to salt treatment on leaf discs were assessed on 0 and 3 days respectively. **(C)** Quantification of chlorophyll content of WT, NtHY5OX and *NtHY5*^{CR} leaves after 3day of salt stress. **(D)** Detection of Superoxide (O^{2-}) by NBT staining in 10-d-old light grown WT, NtHY5OX and *NtHY5*^{CR} tobacco seedlings subjected to 200 mM salt stress for 3 hours. The statistical analysis was performed using two-tailed Student's t-tests. The data are plotted as means ± SD (n=3). The error bars represent standard deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; ***P< 0.001.

Previous studies in Arabidopsis and other plant species suggest HY5 as a regulator of the photomorphogenesis and a key signaling component of light (Ulm et al., 2004; Lee et al., 2007; Nawkar et al., 2017). HY5 is known to interact with LREs present in the minimal promoters of several genes, including *CHS, CHI, FLS*, and *MYB12* (Chattopadhyay et al., 1998; Shin et al., 2013; Zhou et al., 2017). We hypothesized that HY5 might be one of the potential factors responsible for light-mediated flavonol accumulation in tobacco. Our results suggest that *NtHY5* was light-responsive; therefore, it is possible that NtHY5 might be a key regulatory factor in flavonoid biosynthesis in tobacco. Expression and metabolite analyses suggest that NtHY5 together with NtMYB12 regulate flavonol biosynthesis. The expression profile also suggested the highest expression of NtHY5 in young and mature leaf, indicating that NtHY5 is developmentally regulated.

NtHY5 interacts with NtMYB12 promoter to promote flavonoid biosynthesis

HY5 is reported to have a strong binding affinity to G-box (CACGTG), T/G-box (CACGTT), E-box (CAATTG), GATA-box (GATGATA), ACE-box (ACGT), CT-Box (GACGTT), Z-box (ATACGGT), Cbox (GTCANN), A-box (TACGTA), hybrid C/G-(G) and C/A-boxes present in promoters of many genes and transcription factors (Song et al., 2008). Previous studies confirmed that AtHY5 regulates flavonoid biosynthesis by binding to promoters of specific members of the MYB family transcription factors to promote their expression (Stracke et al., 2010, Shin et al., 2013, Nguyen et al., 2015). Further, MYB12 and MYB111 regulate the expression of early flavonoids biosynthetic genes by binding to their promoters, whereas MYB75 binds to the late flavonoids biosynthetic genes (Stracke et al., 2010a, Shin et al., 2013). We analyzed the promoter sequence of NtMYB12 and identified several light-responsive elements such as G-box I (at -220 bp) and G-box II (at -1249 bp) **(Supplemental Table S1)**, which suggested that NtHY5 might bind to the promoter of NtMYB12 to regulate the flavonol content in tobacco. Our EMSA results suggested the binding of NtHY5 to the G-BOX element of the NtMYB12 promoter. This result suggests that NtHY5 binds directly to the conserved light-responsive element that is ACE (ACGT) and promotes *NtMYB12* expression, which in turn up-regulates the expression of genes associated with the phenylpropanoid pathway.

564 **NtHY5 rescue the *Athy5* mutant at phenotypic and metabolite levels**

565 Several HY5 orthologs from different plant species have been identified and characterized
566 (Cheong et al., 1998; Nishimura et al., 2002; Burman et al., 2018). OsZIP48 is reported as a
567 functional ortholog of AtHY5 in rice as it was able to rescue the phenotype of Arabidopsis *hy5*
568 mutant plants after complementing with OsZIP48. Similarly, overexpression of STF1, the
569 HY5ortholog in soybean, can fully complement *hy5* phenotype for hypocotyl length, chlorophyll,
570 anthocyanin and root gravitropic response (Song et al., 2008). In our study, we demonstrated that
571 NtHY5 could complement Arabidopsis *hy5* mutant for phenotypes such as hypocotyl length, root
572 length, seed size and rosette diameter **(Figure 4 A, C, D, E and F; Supplemental Fig. B, C and**
573 **D)**. Moreover, the RT-qPCR analysis showed that flavonoid biosynthetic structural genes,
574 including *AtCHS*, *AtCHI*, *AtFLS*, *AtDFR* and MYB transcription factors, were significantly up-
575 regulated in *NtHY5;hy5*. The content of metabolites, such as anthocyanin and chlorophyll, was
576 also restored in complemented lines. Cross-sections of stems in Arabidopsis *hy5* mutant showed
577 more lignin-specific staining **(Supplemental Fig. S5D)** compared with *NtHY5*;*hy5*, indicating
578 the higher accumulation of flavonoids in complement lines due to the diversion of the substrate
579 flux towards flavonoid synthesis at the cost of lignin production. These findings suggest that
580 NtHY5 had a conserved function across different species, and it regulates flavonoid biosynthesis
581 in complemented lines of Arabidopsis.

582 **Modulated expression of genes and metabolite content in NtHY5OX and *NtHy5^{CR}* plants**

583 There are plenty of reports, which suggest that the expression of MYBs in various plant species
584 regulates flavonoid content (Misra et al., 2010; Bhatia et al., 2018). In our study, overexpression
585 of NtHY5 in tobacco led to the enhanced expression of genes involved in the phenylpropanoid
586 pathway. Quantitative estimation of flavonol content suggested increased accumulation of
587 kaempferol, quercitin, rutin and CGA in overexpression lines compared with WT. On the
588 contrary, mutant lines showed a decrease in flavonoid content due to the reduced expression of
589 pathway genes as well as *NtMYB12*. Altogether, these results suggest that NtHY5 is functionally
590 similar to HY5 of other plant species in the regulation of flavonoid biosynthesis and NtHY5 up-
591 regulates the flavonol accumulation via increasing the expression of NtMYB12 and other
592 phenylpropanoid pathway genes. Additionally, anthocyanin content reduced in *NtHy5^{CR}*,

593 indicating the decreased accumulation of precursor substrate common for both flavonoid and
594 anthocyanin biosynthesis.

595 **Role of NtHY5 in plant growth and development**

596 HY5 and specific MYBs are known to be involved in plant growth and development (Zhong et
597 al., 2008; McCarthy et al., 2009; Gangappa et al., 2016; Misra et al., 2010). OsbZIP48 ortholog
598 is reported to cause a reduction in cell size, thus reduces the plant height of OsbZIP48
599 overexpression transgenic plants (Burman et al., 2018). HY5 negatively regulates hypocotyl
600 elongation by suppressing auxin signaling (Cluis et al., 2004, Sibout et al., 2006). Similar to the
601 above results, we also observed variation in phenotypic characters of the developed NtHY5
602 overexpression and mutant plants of tobacco. The results suggested that modulation in the
603 NtHY5 expression changes in various morphological characteristics, with contrasting phenotypes
604 between NtHY5OX and *NtHy5*^{CR}plants. Hypocotyls length of mutants was significantly longer
605 than overexpressed and WT seedlings grown in light for 10 days. Interestingly, shorter hypocotyl
606 length was observed in mutants in comparison to overexpressed and WT in dark-grown seedlings
607 **(Fig 7 A, B)**. This suggests that mutation in NtHY5 eradicates the light-dependent repression of
608 cell elongation and does not promote cell elongation independent of the light stimulus. Similar to
609 our result, Oyama et al., (1997) also reported the loss of light-dependent cell elongation in *Athy5*
610 mutant plants in the dark.

611 Previous reports suggest that HY5 negatively regulate the auxin signaling pathway by promoting
612 the expression of auxin signaling inhibitors such as INDOLE ACETIC ACID 14 (SLR/IAA14)
613 and INDOLE ACETIC ACID 7 (AXR2/IAA7), whereas mutation in *hy5* promotes up-regulation
614 of auxin signaling genes (Sibout et al., 2006). Flavonoids negatively regulate plant growth and
615 root length by down-regulating the auxin signaling genes (Brown et al., 2001; Peer et al., 2007;
616 Yin et al., 2014; Sharma et al., 2020). In our study, we found that up-regulated flavonol
617 accumulation in NtHY5OX lines resulted in a decrease in root length, plant height as well as
618 high flavonol content. In addition, increased root bending angle in was observed in NtHY5OX
619 lines compared with tobacco *hy5* mutant and WT **(Fig. 7B)**. Additionally, the seed size of the
620 NtHY5 mutant was significantly smaller in comparison to mutant and WT plants **(Fig. 7K)**. This
621 significant change in seed size might be due to modulation in hormone signaling mediated by
622 flavonoids. Rapid changes in auxin distribution by ZmPIN1 in the endosperm of maize affect the

seed size (Lur et al., 1993). There are some other reports, which suggest that flavonol accumulation regulates the seed development in Arabidopsis (Doughty et al., 2014; Sharma et al., 2018). Thus, the relation between hormone signaling and NtHY5 could be the subject for further study.

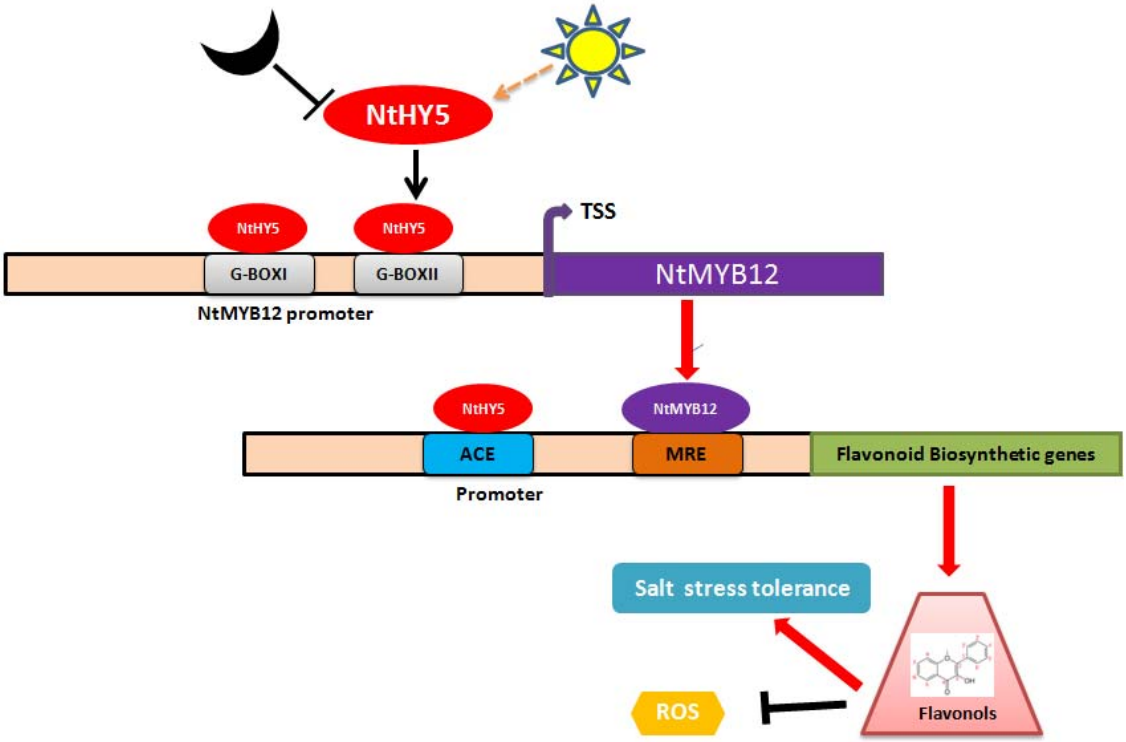
NtHY5 enhances the salt stress tolerance in tobacco

It is well explored that flavonoids have antioxidant properties and have an important role in abiotic stress tolerance. Previous studies showed that AtHY5 plays a role in abiotic stress tolerance by enhancing the flavonol level in plants (Agati et al., 2012; Emiliani et al., 2013 and Muhlemann et al., 2018). In tobacco, several studies reported that high flavonoid content causes high antioxidant properties, which may result in abiotic stress tolerance (Docheva et al., 2014; Pandey et al., 2015). Recent studies indicate that over-expression lines of HDA15 were found to be resistant to salt stress and also displayed increased accumulation of abscisic acid. It was also shown that HY5 directly interacts with HDA15 promoting salt tolerance (Truong et al., 2021). RNAi of NtMYB4 causes up-regulation of NtCHS, leading to enhanced levels of flavonol content and higher antioxidant property and salinity tolerance in tobacco (Chen et al., 2019). Similarly, overexpression of NtMYB12 causes more flavonol accumulation and higher ROS scavenging property thus enhancing tolerance to low Pi stress in tobacco (Song et al., 2020). In our study, the antioxidant activity was higher in overexpression lines in comparison to WT and *NtHY5*^{CR} plants. This leads to lesser chlorosis in salt-treated young leaves of NtHY5OX lines compared with *NtHy5*^{CR}, suggesting that high flavonoid content leads to higher ROS scavenging ability of overexpression lines. As tobacco *NtHy5*^{CR} mutants were salt-sensitive, thus more ROS is accumulated in salt-treated mutant leaves **(Fig. 8D)**. These results suggest that NtHY5 promotes salt stress tolerance at least partially through the antioxidant activity of flavonoids.

Conclusion

In conclusion, the present study reveals that NtHY5 is responsible for light-mediated flavonoid biosynthesis as well as for the enhanced antioxidant property of tobacco plants. The CRISPR-edited tobacco *NtHy5*^{CR} plants showed a reduced expression of regulatory and structural genes of the flavonoid biosynthesis pathway, which is accompanied by a lower accumulation of flavonoid and antioxidant properties. The high flavonol content and antioxidant property caused less chlorosis in *NtHY5* overexpressed plants and enhanced ROS scavenging property, which leads to

653 an increase in salt-tolerance property in NtHY5 overexpressing tobacco leaves. This response
654 likely occurs through NtHY5 binding to the ACE and G-BOXES (cis-elements) present within
655 the promoters of flavonoid biosynthetic genes and NtMYB12, respectively. Thus, NtHY5 and
656 NtMYB12 share common target genes, which are involved in flavonol production and exhibit



657 combinatorial regulation of the flavonoid biosynthesis genes. Therefore, we propose that these
658 regulatory factors act in concert for light-mediated flavonoid production **(Fig. 9)**. The *NtHy5*
659 mutant developed in this study will be used as an essential tool in elucidating the involvement of
660 NtHY5 in various biological processes in tobacco.

661

662
663 **Figure 9.** Proposed working model for the role of NtHY5 and NtMYB12 in regulating the
664 expression of flavonoid pathway genes during light exposure. We propose that NtHY5 positively
665 regulates the expression of NtMYB12 by directly binding to promoter of NtMYB12 through G-
666 BOX I and G-BOX II in turn NtMYB12 directly binds to the MRE (MYB recognition element)
667 in the promoters of flavonoid pathway genes and enhances flavonol accumulation whereas
668 NtHY5 also binds independently to ACE element present on the promoter of flavonoid pathway
669 genes and regulate their expression. Thus, together NtHY5 and NtMYB12 lead to flavonol
670 production. Flavonol ultimately leads to salt tolerance by inhibiting ROS production.
671 Arrowheads and tees indicate positive and negative regulation respectively.

672

673

674

675 **MATERIALS AND METHODS**

676 **Isolation and sequence analysis of NtHY5.** To identify the ortholog of AtHY5 in tobacco,
677 BLASTn searches were performed on National Center for Biotechnology Information
678 (http://www.ncbi.nlm.nih.gov/BLAST). The sequence showing 90% similarity with AtHY5 and
679 SlHY5 was retrieved and amino acid sequence was analyzed for the leucine rich bZIP Zinc
680 finger domain and VPE domain using multallignment tool. Conserved domain database (CCD)
681 was also employed to ensure the bZIP family of the identified NtHY5 protein. Promoter
682 sequences (~1.5 kb upstream of translation start site) of NtMYB12 gene was retrieved from the
683 respective databases AGRIS (Palaniswamy et al., 2006) and PlantCare (Lescot et al., 2002),
684 which was utilized for the scanning of light-responsive cis-elements (LREs).

685 **Phylogenetic analysis.** Homologs of AtHY5 that belongs to different families were identified by
686 using the National Center for Biotechnology Information BLASTn tool. The genes retrieved
687 were checked for the presence of the requisite domains using InterProScan (http://
688 www.ebi.ac.uk/Tools/pfa/iprscan/; Quevillon et al., 2005; Jones et al., 2014; Mitchell et al.,
689 2015). A phylogenetic tree was constructed using the Neighbor-Joining (NJ) algorithm in
690 MEGA6.0 software (Kumar et al., 2008).

691 **Plant material, growth conditions and treatments.** Young leaves of *Nicotiana tabacum* cv.
692 *Petit Havana* have been used for raising the transgenic plants. Tobacco plants were grown in
693 glass house at 25°C±2°C and 16 h/8 h light-dark photoperiods for harvesting of tissues belonging
694 to different developmental stage. For dark treatment, seeds were surface sterilized using 70%
695 EtOH for 1 min and then dipped in 50% bleach for 10 min after that washed repeatedly with
696 autoclaved Milli-Q water, and grown for 10 days in culture room with 16 h/8 h light-dark
697 photoperiods, 24°C temperature on one-half-strength Murashige and Skoog (MS) medium
698 (Sigma) plates and then transferred in dark chamber with similar growth conditions as in culture
699 room. AtMYB12, AtMYB11 and AtMYB111-expressing seedlings were harvested after 24 and

700 48 h of dark treatment for gene expression as well as phytochemical analysis. Samples were
701 frozen in the liquid nitrogen and kept in -80°C deep freezer until further use. The experiment was
702 performed by using three independent replicates. Arabidopsis (ecotype Col-0) was used as the
703 wild-type plant in complementation study. Seeds were surface sterilized and placed on one-half-
704 strength MS medium containing 1% Sucrose. After stratification for 2 to 3 d at 4°C in the dark,
705 seeds were transferred to a growth chamber under controlled conditions of 16-h-light/8-h-dark
706 photoperiod cycle, 22°C temperature, 150 to 180 µmol $m^{-2}s^{-1}$ light intensity, and 60% relative
707 humidity for 10 d unless mentioned otherwise.

708 **Plasmid construction and generation of Arabidopsis and Tobacco transgenic lines.** To
709 overexpress NtHY5, the NtHY5 was amplified from the single-stranded complementary DNA
710 (cDNA) library prepared from 10-d-old tobacco seedlings through PCR using specific primers.
711 The full-length open reading frame of the NtHY5 cDNA, under the control of cauliflower mosaic
712 virus 35S promoter in the binary vector pBI121 (Clontech, USA) was transferred into
713 *Agrobacterium tumefaciens* strain GV3101 and used to transform Arabidopsis (Col0) by floral
714 dip method (Clough and Bent, 1998) and tobacco plants by leaf disk method (Horsch et al.,
715 1985). Several transgenic tobacco lines constitutively expressing NtHY5 cDNA were selected on
716 the basis of RT-PCR. Seeds were harvested, sterilized and plated on solid half strength MS
717 medium supplemented with 100 mg/l kanamycin. Antibiotic resistant plants were shifted to the
718 glass house and grown until maturity.

719 For generation of mutant plants of NtHY5, CRISPR-Cas9 technology was used. A 20-bp gRNA
720 was selected from the identified coding region. NtHY5 gRNAs was cloned into the binary vector
721 pHSE401 using the BsaI restriction site (Xing et al., 2014). This vector contains Cas9
722 endonuclease-encoding gene under dual CaMV35S promoter as well as genes encoding
723 neomycin phosphotransferase and hygromycin phosphotransferase as selection markers. All the
724 constructs were sequenced from both the orientations using plasmid-specific forward and vector
725 reverse primers after that transferred to GV3101 and then transformed to tobacco.

726 **Gene expression analysis.** Total RNA was isolated using a Spectrum Plant Total RNA kit
727 (Sigma Aldrich) following the manufacturer's instruction. For qRT-PCR analysis, 2 µg of
728 DNAfree RNA was reverse transcribed using a RevertAid H minus first-strand cDNA synthesis
729 Kit (Fermentas) according to the manufacturer's instructions. qRT-PCR was performed using

730 Fast Syber Green mix (Applied Biosystems) in a Fast 7500 Thermal Cycler instrument (Applied
731 Biosystems). Expression was normalized using Tubulin and analyzed through the comparative
732 CT method (Livak and Schmittgen 2001). The oligonucleotide primers used to study expression
733 of different genes were designed using the Primer Express 3.0.1 tool (Applied Biosystems) and
734 information is provided in Supplementary Table S1.

735 **Extraction and quantitative estimation of flavonols.** Flavonols were determined either as
736 aglycones or as flavonol glycosides by preparing acid-hydrolyzed or non-hydrolyzed extracts
737 respectively. Extraction of flavonols was carried out by grinding the plant material (300mg) into
738 the fine powder in liquid N_2 and then extracted with 80% methanol overnight at room
739 temperature with brief agitation. The extract was hydrolyzed with an equal amount of 6 N HCl at
740 70°C for 40 min followed by addition of an equal amount of methanol to prevent the
741 precipitation of the aglycones (Jiang et al. 2015). Extracts were filtered through 0.2 mm filter
742 (Millipore, USA) before HPLC. For non-hydrolyzed extracts, samples were extracted as
743 described previously (Pandey et al., 2014). Breeze 2 software (Waters) was used for the
744 quantification of various metabolites through HPLC. The mobile phase was a gradient prepared
745 from 0.05% (v/v) ortho-phosphoric acid in HPLC-grade water (component A) and methanol
746 (component B). Before use, the components were filtered through 0.45-mm nylon filters and de-
747 aerated in an ultrasonic bath. The gradient from 25 to 50% B in 0–3 min, 50 to 80% B in 3–18
748 min, 80 to 25% B in 25 min and 25% B in 30 min was used for conditioning of the column with
749 a flow rate of 1 ml/min. All the samples were analysed by HPLC–PDA with a Waters 1525
750 Binary HPLC Pump system comprising PDA detector following the method developed by
751 (Niranjan et al., 2011).

752 **Extraction of total anthocyanin, chlorophyll and lignin staining.** Anthocyanin was measured
753 according to the method described by Li et al., (2016). Briefly, 300mg tissue were boiled for 3
754 min in extraction solution (propanol:HCl:H2O, 18:1:81), and incubated at room temperature in
755 the dark for at least 2 h. Samples were centrifuged, and the absorbance of the supernatants was
756 measured at 535 and 650 nm. Anthocyanin content was calculated as

757 $$\text{Anthocyanin content} = (A_{535} - 2.2 * A_{650}) / \text{g FW}.$$

758 Chlorophyll content was measured by using the 300mg tissue of 10-d-old WT and transgenic
759 seedlings. Sample was crushed in 2 ml (80 %, v/v) chilled acetone (Arnon 1949). Homogenized
760 tissues were centrifuged at 7,800g for 10 min. Supernatant was collected and absorbance was
761 recorded at 663, 645, 510 and 480 nm. Total chlorophyll content was calculated in mg g−1 fresh
762 wt using formula of Machlachalan and Zalik (1963).

763 Total chlorophyll= [34.5(A652)*V]/d *1000*W

764 Where, V=volume of the final extract (ml), W= weight of the plant sample (g) and d=width of
765 the cuvette(1cm).

766 To visualize lignified cells in stems, hand-cut sections were stained using phloroglucinol (Sigma-
767 Aldrich) for 1min and visualized on a Leica DM2500 microscope (Sharma et al., 2020).

768 **Determination of total antioxidant activity.** A method by Wong et al., (2006) with slight
769 modification was used to determine the antioxidant activity. DPPH (1, 1-diphenyl-2-
770 picrylhydrazyl free radical) assay was used in determining the free radical scavenging activity. In
771 brief, 0.1 mM solution of DPPH was prepared in methanol. The initial absorbance of DPPH in
772 methanol was measured at 515 nm and did not change throughout the period of assay. An aliquot
773 of the samples (50–100 ml) was used to make up volume to 1.5 ml with DPPH solution. The
774 sample was incubated at room temperature for 30 min. The absorbance of the samples was
775 recorded at 515 nm using Ultraspec 3000 UV/Vis spectrophotometer (Pharmacia Biotech Ltd.,
776 Cambridge, CB4, 4FJ, UK). Trolox was used to make the calibration curve using different
777 dilutions. The antioxidant capacity based on the DPPH free radical scavenging ability of the
778 extract was expressed as mM Trolox equivalents per gm of plant material on fresh weight basis.

779 **Expression and purification of NtHY5.** The complete open reading frame of NtHY5 was
780 amplified and cloned into pET-28b(+) vector (Novagen, Germany). The construct was
781 transformed into *E.coli* BL21 (DE3) pLysS (Invitrogen, USA) for prokaryotic expression,
782 induced by IPTG. Recombinant 6X-Histagged HY5 was purified using a Ni-NTA column
783 (Nucleopore, India) and the eluted and was quantified through Bradford assay.

784 **Electrophoretic mobility shift assay.** The 2nd generation DIG Gel Shift EMSA kit (Roche,
785 USA) was used to label the probes with digoxigenin as per manufacturer's instructions. Labelled

786 probes were incubated at 21 C for 30 min in binding buffer [100 mM HEPES (pH 7.6), 5 mM
787 EDTA, 50 mM $(NH4)_2SO_4$, 5 mM DTT, Tween 20, 1% (w/v), 150 mM KCl] with or without
788 recombinant protein. Unlabeled probes were added to the reaction solution in increasing
789 concentrations to test specific binding. The binding reaction was resolved on a 6%
790 polyacrylamide gel in 0.5X TBE (pH 8.0) buffer and was semi-dry blotted (Transblot, BIO-
791 RAD, USA) onto a positively charged nylon membrane (BrightStar, Invitrogen, USA) followed
792 by UV cross-linking. The membrane was finally incubated with CSPD chemiluminescent
793 solution and exposed to X-ray blue film (Retina, India).

794 **Salt stress treatment.** Salt stress analysis was performed by using the young leaves from 2nd
795 and 3rd nodes from 1-month-old *NtHY5OX*, *NtHy5*CR and WT plants to determine the
796 comparative response during the leaf discs' salt assay. These leaves were transferred into salt
797 solution of 200 mM. The treatment was carried out at 25 °C for 3 days. In order to determine the
798 effect of salinity stress, survival of leaves and chlorophyll content of NaCl treated leaves were
799 analysed. All the data were recorded from 0 to 3 day of NaCl treatment. The experiment was
800 repeated three times.

801 **Nitro blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining.** Superoxide (O_2-)
802 staining was performed by infiltration by the nitro blue tetrazolium (NBT) method (Liu et al.,
803 2018; Song et al., 2019) with some modification. WT as well as transgenic seedlings were grown
804 for 10 days on half strength MS media then transferred to 200mM salt solution for 3hours. After
805 3 hours of salt stress seedlings were dipped in 0.5mg/ml NBT solution for 2h followed by
806 removal of chlorophyll by heating at 70ºC temperature until chlorophyll is removed. Leaves
807 were directly visualized for NBT staining.

808 Hydrogen peroxide (H_2O_2) staining of 10-day light grown tobacco seedlings was performed by
809 staining with 3,3′-diaminobenzidine (DAB) using an adaptation of previous methods (Zhao et al
810 2016). DAB is oxidized by hydrogen peroxide in the presence of some haem-containing proteins,
811 such as peroxidases, to generate a dark brown precipitate. WT as well as transgenic seedlings
812 were grown for 10 days on half strength MS media then transferred to 200mM salt solution for
813 3hours. After 3 hours of salt stress seedlings were dipped in 1mg/ml DAB solution for 5 hours.
814 Following the incubation, DAB staining solution was replaced with bleaching solution (ethanol:

815 acetic acid: glycerol = 3:1:1). After that chlorophyll was removed by heating at 90-95ºC
816 temperature. Leaves were directly visualized for DAB staining.

817 **Statistical analysis.** Data are plotted as means ±SE (n=3) in the figures. Data of flavonol
818 accumulation were evaluated for statistical significance using the Two-tailed Student's t-test
819 using Graphpad prism 5.01 software. Asterisks indicate significance levels with (* $P < 0.1$; ** P
820 < 0.01; *** $P < 0.001$) as confidence intervals.

821

822 **Supplemental Data**

823 **Figure S1.** Antioxidant activity by trolox equivalent. Antioxidant activity was analysed in 10-
824 day-old AtMYB12, AtMYB11 and AtMYB111 overexpressing tobacco transgenic lines grown
825 in light. Statistical analysis was performed using two-tailed Student's t-test. Data are plotted as
826 means ±SD (n=3). Error bars represent standard deviation. Asterisks indicate a significant
827 difference***$P < 0.001$.

828 **Figure S2.** Metabolite analysis of MYB12OX lines of tobacco through HPLC. (A)
829 Chromatograms showing peak of standard (Kaempferol, Quercitin and Rutin). (B)
830 Chromatograms showing peak of Kaempferol and Quercitin at 0 and 48 h of dark treatment
831 different in AtMYB12OX line of tobacco. (C) Chromatograms showing peak of Rutin at 0 and
832 48 h of dark treatment different in AtMYB12OX line of tobacco.

833 **Figure S3.** Estimation of flavonol content in WT tobacco. Flavonol aglycones (kaempferol and
834 quercetin) were measured in 10-d-old dark-grown WT tobacco seedlings and WT seedlings
835 transferred to light for 3 h after growing 10 days in darkness. Statistical analysis was performed
836 using two-tailed Student's t-test. Data are plotted as means ±SD (n=3). Error bars represent
837 standard deviation. Asterisks indicate a significant difference, **$P < 0.01$, ***$P < 0.001$.

838 **Figure S4.** Sequence analysis of NtHY5 gene. (A) Gene structure of NtHY5 in comparison to
839 AtHY5. The black boxes represent exons, lines represent introns and numbers represent the
840 length of the exons and introns (bp). (B) Representation of NtHY5 conserved domain that is
841 basic leucine zipper (bZIP) domain through conserved domain database.

Figure S5. Phenotypic analysis of different transgenic lines. (A) A schematic representation of NtHY5 construct under the control of CaMV35S promoter. (B) Representative photographs of rosette diameter of transgenic WT, *hy5* and *NtHY5;hy5* plants. (C) Measurement of seed size of *NtHY5;hy5* lines of Arabidopsis in comparison to WT and *hy5* plants. (D) Transverse section of stem to show lignin content in *NtHY5; hy5* in comparison to *hy5* and WT. Statistical analysis was performed using two-tailed Student's t-test. Data are plotted as means ±SD (n=10-12) for rosette diameter. Seed size, and lignin staining experiments. Error bars represent standard deviation. *hy5* was taken as control for significance. Asterisks indicate a significant difference, *P < 0.1, **P < 0.01.

Figure S6. Relative expression of MYB transcription factors associated with phenylpropanoid pathway. Expression analysis of *AtMYB12*, *AtMYB11* and *AtMYB111*through real time PCR in*NtHY5;hy5*, *hy5* and WT after growing for 7-d-dark condition and then transferred to light for 3 hours. Statistical analysis was performed using two-tailed Student's t-test. Tubulin was used as endogenous control to normalize the relative expression levels. Data are plotted as means ±SD (n=3). Dark grown seedlings were used as control for significance. Error bars represent standard deviation. Asterisks indicate a significant difference, **P < 0.01, ***P < 0.001.

Figure S7. Estimation of total chlorophyll and anthocyanin content. (A) Chlorophyll content in 30-d-old rosette leaves of WT, *hy5* and *NtHY5; hy5* plants. (B) Total Anthocyanin content in WT, *hy5* and *NtHY5OX;hy5* in 7-d-old light-grown seedlings. Statistical analysis was performed using two-tailed Student's t-test. Data are plotted as means ±SD (n=3). Error bars represent standard deviation. Asterisks indicate a significant difference, **P < 0.01, ***P < 0.001.

Figure S8. Position of gRNA in NtHY5 sequences. (A) Coding sequence of NtHY5 showing location of gRNA in red color, PAM sequences (NGG) are green in color. (B) Amino acid sequence of WT and $NtHY5^{CR}$edited plants.

Figure S9. Comparative analysis of HY5OX vs. $NtHy5^{CR}$. (A) Phenotype of WT, $NtHYR^{CR}$ and NtHY5OX 10-d-dark grown seedling. (B) Measurement of seed size of WT, NtHY5OX and $NtHY5^{CR}$ plants of tobacco. (C) Quantitative analysis of chlorophyll content of 1-month-old WT, NtHY5OX and $NtHY5^{CR}$ plants of tobacco. Statistical analysis was performed using two-tailed

870 Student's t-test. Data are plotted as means ±SD (n=3). Error bars represent standard deviation.
871 Asterisks indicate a significant difference, ***P < 0.001.

872 **Figure S10.** Detection of hydrogen peroxide by DAB staining in 10-d-old light grown WT,
873 NtHY5OX and *NtHY5*^{CR}tobaccoseedlings subjected to 200 mM salt stress for 3 hours. The
874 statistical analysis was performed using two-tailed Student's t-tests. The data are plotted as
875 means ± SD (n=3). The error bars represent standard deviations. The asterisks indicate significant
876 difference, *P< 0.1; **P< 0.01; ***P< 0.001.

877 **Table S1**. Putative *cis*-acting elements in NtMYB12 promoter

878 **Table S2.** List of Primers used in the study

879 **Figure legends**

880 **Figure 1. Light affect flavonoid biosynthesis in transgenic tobacco. (A),(C),(E)** Relative
881 transcript abundance of *AtCHS*, *AtCHI*, *AtFLS* and *AtDFR* that are involved in phenylpropanoid
882 pathway in AtMYB12, AtMYB11 and AtMYB111 overexpressing transgenic lines respectively
883 by real time PCR using RNA from the 10-d-old light grown seedlings transferred to dark for
884 24hr and 48hr. Tubulin was used as endogenous control to normalize the relative expression
885 levels. **(B), (D), (F)** Effect of light over flavonol biosynthesis in different transgenic lines
886 expressing AtMYB12, AtMYB11 and AtMYB111 respectively. Compounds (K, Kamepferol; Q,
887 Quercitin; R; Rutin) were quantified using methanolic extracts of 10-d-old old seedlings of
888 different transgenic lines through HPLC. Data are derived from the mean of three technical
889 replicates. Error bars represent SE of means (n=3). The statistical analysis was performed using
890 two-tailed Student's t-tests. The asterisks indicate significant difference, *P< 0.1; **P< 0.01;
891 ***P< 0.001.

892 **Figure 2. NtHY5, the light signaling component, alters flavonoid content in tobacco**. **(A)**
893 Phylogenetic tree of HY5 homologous proteins (sequence obtained from NCBI database) from
894 across species of different families. The numbering at the nodes represents the number of trees
895 that have the same topology as the consensus tree. NtHY5 is denoted through the red branch, and
896 the scale bar indicates the branch length. **(B)** Amino acids alignment of NtHY5 and its homologs
897 in Tomato and Arabidopsis using multallignment tool. The VPE motif and zinc finger domain is

898 boxed in green and red color respectively. **(C)** Phenotype of 10-d-olddark and light grown WT
899 seedlings. **(D)** Expression analysis of phenylpropanoid pathway genes *NtCHS*, *NtCHI*, *NtFLS*
900 and *NtDFR* in WT seedlings grown in light and dark for 10 days. **(E)** Quantitative estimation of
901 flavonoids (K, Q and R) in 10-d-light and dark grown seedlings through HPLC. **(F)** Relative
902 expression of NtHY5 in 10 light and dark grown seedlings. **(G)**Normalized expression of NtHY5
903 in different tissue [seedling, young (Y) leaf, mature (M) leaf, stem, root and flower]. Data are
904 derived from the mean of three technical replicates. Tubulin was used as endogenous control to
905 normalize the relative expression levels. Error bars represent SE of means (n=3). The statistical
906 analysis was performed using two-tailed Student's t-tests. The asterisks indicate significant
907 difference, **P< 0.01; ***P< 0.001.

908

909 **Figure 3. NtHY5 interacts with NtMYB12 promoter. (A)** Representation of the probes (G-
910 BOXI, G-mut I, G-BOXII and G-mut II) designed upstream of the transcription start site of
911 NtMYB12 promoter. G-BOX is altered by multiple base substitutions. **(B)** EMSA
912 (Electrophoretic mobility shift assay) for the binding of 6X-His-HY5 (NtHY5) with core ACE-
913 DIG element (HY5 binding site that is G-BOXI and G-BOXII) present in the LRE motifs of the
914 NtMYB12 promoter. Upper and lower arrows indicate shift and free probe respectively. **(C), (D)**
915 EMSA with competition between digoxigen in labelled G-BOXI, unlabeled G-BOX I (cold), G-
916 BOXII and unlabeled G-BOX II (cold) probe to bind to 6X-His-HY5 respectively. Superscript
917 values 10X, 20X, 30X and 50X represent increasing amount of cold probe. Upper and lower
918 arrows indicate shift and free probe respectively.

919 **Figure 4. Complementation of Arabidopsis *hy5* mutant by NtHY5. (A)** Phenotype of 7-d-old
920 light-grown WT (wild type; Col-0), *hy5* and NtHY5OX; *hy5* seedlings (L1 and L2 are two
921 independent transgenic lines overexpressing NtHY5 in *hy5* mutant background). **(B)** Relative
922 expression of NtHY5 in 7-d-old light grown WT, *hy5*, L1 and L2. **(C)** Hypocotyl length of 7-d-
923 old white light grown WT, *hy5*, L1 and L2 seedlings. **(D)** Root length of 7-d-old white light
924 grown WT, hy5, L1 and L2. **(E)**Rosette diameter of 30-day-old WT, *hy5*, L1 and L2 plants. **(F)**
925 Representative photographs of the seed size of WT, *hy5* and transgenic lines (L1 and L2). Bars =
926 1 mm. **(G)** Relative expression of pheylpropanoid pathway genes (*AtCHS, AtCHI, AtFLS and*
927 *AtDFR*) in 7-d-light grown WT, *hy5*, L1 and L2 seedlings. **(H)** Quantitative estimation of

flavonol (Kaempferol and Quercitin) content in 7-d-old light grown WT, *hy5*, L1 and L2 seedlings through HPLC. **(I)** Expression analysis of *AtCHS, AtCHI, AtFLS* and *AtDFR* in WT, *hy5*, L1 and L2 seedlings grown for 5 days in dark followed by an exposure to light for 3hr. (J) Length of vascular bundle of 30-day-oldstem of WT, *hy5*, L1 and L2 plants. Statistical analysis was performed using two-tailed Student's t-test. Error bars represent SE of means (n=3) For hypocotyl and root length (n=10-12). Tubulin was used as endogenous control to normalize the relative expression levels. Error bars represent standard deviation. Asterisks indicate a significant difference, *P < 0.1, **P < 0.01, ***P < 0.001. ns represent non-significant.

Figure 5. Overexpression of NtHY5 modulates gene expression and metabolite level in tobacco. (A) Relative expression of NtHY5 in 10-d-old light grown WT and NtHY5OX lines of tobacco (L1, L2 and L3). **(B)** Expression analysis of pheylpropanoid pathway genes (*NtCHS, NtCHI, NtFLS and NtDFR*) in 10-d-old light grown WT and NtHY5OX lines. **(C)**Quantification of flavonoids (K, Q and R) and polyphenolic compound (CGA) in 10-d-old light grown WT and NtHY5OX lines of tobacco using HPLC. **(D)** Representative photograph of flower color alterations in petals of NtHY5OX transgenic lines in compared with WT. **(E)** Total anthocyanin content in petals of WT and NtHY5OX transgenic lines. **(F)** Estimation of antioxidant activity by trolox equivalent in WT and NtHY5OX lines. TUBULIN was used as the endogenous control to normalize the relative expression levels. The statistical analysis was performed using two-tailed Student's t-tests. The data are plotted as means ± SD (n= 3). The error bars represent standard deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; ***P< 0.001.

Figure 6. CRISPR-Cas9-mediated knockout of NtHY5 alters gene expression and accumulation of metabolite content. (A) Representation of mutations in target region in three independent transgenic lines of tobacco. Dots indicate deleted nucleotides. Yellow highlight denotes gRNA region used for targeting the sequence. Red highlights denote indels in nucleotides. **(B)** Relative expression of NtHY5 in 10-d-old light grown WT and *NtHY5*^{CR} lines of tobacco (L1, L2 and L3). **(C)** Relative transcript abundance of pheylpropanoid pathway genes (*NtCHS, NtCHI, NtFLS and NtDFR*) in 10-d-old light grown WT and *NtHY5*^{CR} lines. **(D)** Quantification of flavonoids (K, Q and R) and polyphenolic compound (CGA) in 10-d-old light grown WT and *NtHY5*^{CR} lines of tobacco. **(E)** Quantification of Anthocyanin content in 10-d-old light grown WT and *NtHY5*^{CR}lines. **(F)** Quantification of antioxidant activity by trolox equivalent in 10-d-old light grown WT and *NtHY5*^{CR} lines. Tubulin was used as the endogenous

959 control to normalize the relative expression levels. The statistical analysis was performed using
960 two-tailed Student's t-tests. The data are plotted as means ± SD (n=3). The error bars represent
961 standard deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01;
962 ***P< 0.001.

963 **Figure 7.** NtHY5OX and CRISPR–Cas9-derived knockout mutants show altered phenotypes and
964 gene expression. **(A)** Representative image of 10-d-old light grown WT, NtHY5OX and
965 *NtHY5*^{CR}seedlings grown on half-strength MS medium. Scale bars, 1 cm. **(B)** Measurement of
966 root bending angle of 10-d-old light grown WT, NtHY5OX and *NtHY5*^{CR}seedlings. **(C), (D)**
967 Hypocotyl length of 10-d-old light and dark grown WT, NtHY5OX and *NtHY5*^{CR} seedlings. **(E)**
968 Root length of 10-d-old light grown WT, NtHY5OX and *NtHY5*^{CR} seedlings. **(F)**Quantitative
969 analysis of seed size of WT, NtHY5OX and NtHY5^{CR} plants. **(G)** Quantitative estimation of
970 Number of seeds per 5 mg of WT, NtHY5OX and *NtHY5*^{CR}plants. **(H), (I)**Relative expression of
971 NtHY5 and phenylpropanoid pathway genes (*NtCHS, NtCHI, NtFLS* and *NtDFR*) in 10-d-old
972 light grown seedlings transferred to dark for 3h. **(J)** Representative image of 40-day-old WT,
973 NtHY5OX and *NtHY5*^{CR} plants showing plant heights. **(K)** Data presenting difference in height
974 of 40-day-old WT, NtHY5OX and *NtHY5*^{CR} plants. Tubulin was used as the endogenous control
975 to normalize the relative expression levels. The statistical analysis was performed using two
976 tailed Student's t-tests. The data are plotted as means ± SD (n= 3). For measurement of root
977 bending angle, root length, hypocotyls length and seed size (n=10-12). The error bar represents
978 standard deviations. The asterisks indicate significant difference, *P< 0.1; **P< 0.01; 0.001.

979 **Figure 8.** Effect of salt stress on different transgenic. Fully expanded leaves of 30-d-old plants to
980 floated on 6 ml of 200 mM NaCl solution (for salt stress) for 3 days. **(A), (B)** The phenotypic
981 (chlorosis) changes due to salt treatment on leaf discs were assessed on 0 and 3 days
982 respectively. **(C)** Quantification of chlorophyll content of WT, NtHY5OX and *NtHY5*^{CR} leaves
983 after 3day of salt stress. **(D)** Detection of Superoxide (O^{2-}) by NBT staining in 10-d-old light
984 grown WT, NtHY5OX and *NtHY5*^{CR} tobacco seedlings subjected to 200 mM salt stress for 3
985 hours. The statistical analysis was performed using two-tailed Student's t-tests. The data are
986 plotted as means ± SD (n=3). The error bars represent standard deviations. The asterisks indicate
987 significant difference, *P< 0.1; **P< 0.01; ***P< 0.001.

988 **Figure 9.** Proposed working model for the role of NtHY5 and NtMYB12 in regulating the
989 expression of flavonoid pathway genes during light exposure. We propose that NtHY5 positively
990 regulates the expression of NtMYB12 by directly binding to promoter of NtMYB12 through G-
991 BOX I and G-BOX II in turn NtMYB12 directly binds to the MRE (MYB recognition element)
992 in the promoters of flavonoid pathway genes and enhances flavonol accumulation whereas
993 NtHY5 also binds independently to ACE element present on the promoter of flavonoid pathway
994 genes and regulate their expression. Thus, together NtHY5 and NtMYB12 lead to flavonol
995 production. Flavonol ultimately leads to salt tolerance by inhibiting ROS production.
996 Arrowheads and tees indicate positive and negative regulation respectively.

997

998

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