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ORIGINAL RESEARCH

MYC2 regulates *ARR16*, a component of cytokinin signaling pathways, in Arabidopsis seedling development

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Abstract

MYC2 is a basic helix-loop-helix transcription factor that acts as a repressor of blue light-mediated photomorphogenic growth; however, it promotes lateral root formation. MYC2 also regulates different phytohormone-signaling pathways in crucial manner. Arabidopsis response regulator 16 (*ARR16*) is a negative regulator of cytokinin signaling pathways. Here, we show that MYC2 directly binds to the E-box of *ARR16* minimal promoter and negatively regulates its expression in a cytokinin-dependent manner. While ARR16 and MYC2 influence jasmonic acid and cytokinin signaling, the expression of *ARR16* is regulated by cry1, GBF1, and HYH, the components of light signaling pathways. The transgenic studies show that the expression of *ARR16* is regulated by MYC2 at various stages of development. The mutational studies reveal that ARR16 positively regulates the hypocotyl growth in blue light, and phenotypic analysis of *atmyc2 arr16* double mutant further reveals that *arr16* can suppress the short hypocotyl phenotype of *atmyc2*. Altogether, this work highlights MYC2-mediated transcriptional repression of *ARR16* in Arabidopsis seedling development.

KEYWORDS
Arabidopsis, ARR16, light signalling, MYC2

1 | INTRODUCTION

Plants are photosynthetic and non-motile, and thus they need to be especially plastic in response to their light environment. The multiple responses of plants to light require complicated sensing of its intensity, direction, duration, and wavelength (Chen & Chory, 2011; Fankhauser & Chory, 1997; Josse & Halliday, 2008; Pfeiffer et al., 2016; Wang et al., 2014). Plants possess distinct types of photoreceptors such as phytochromes (phyA to phyE) for far-red (FR) and red light (RL), cryptochromes (cry1, cry2, and cry3), phototropins (phot 1 and phot 2), and ZEITLUPE (ZTL) families for blue (BL) and UV-A light; and UVR8 for UV-B light perception (Ahmad & Cashmore, 1993; Furuya, 1993; Jiao, Lau, & Deng, 2007; Kendrick & Kronenberg, 1994; Li et al., 2011; Lin, 2002; Neff, Frankhauser, & Chory, 2000; Quail, 2002; Rizzini et al., 2011). Arabidopsis seedlings are genetically capable of following two distinct developmental pathways: skotomorphogenesis in the dark is characterized by elongated hypocotyl and closed cotyledons with apical hook; while photomorphogenesis in the light is characterized by short hypocotyl with open and expanded cotyledons (von Arnim & Deng, 1996; Chen & Chory, 2011; Josse & Halliday, 2008; Pfeiffer et al., 2016; Wang et al., 2014).

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Transcriptional regulatory networks have a key role in mediating light signaling through the coordinated activation and repression of many downstream regulatory genes. Therefore, there is considerable interest in elucidating the hierarchy of networks that are formed by transcription factors, and in identifying the key regulatory elements in different light-responsive developmental processes (Jiao et al., 2007). Moreover, the cross talks of this signaling pathway with other signaling cascades are largely unknown.

MYC2 is a basic helix-loop-helix (bHLH) transcription factor. The analysis of *atmyc2/zbf1* mutants has demonstrated that the short hypocotyl phenotype of *atmyc2* seedlings is restricted to BL and low intensity of white light (WL) (Gangappa, Prasad, & Chattopadhyay, 2010; Yadav, Mallappa, Gangappa, Bhatia, & Chattopadhyay, 2005). Although *MYC2* is expressed in the dark and in various light-grown seedlings, it functions as a negative regulator of BL-specific photomorphogenic growth mediated by cryptochromes (Gangappa et al., 2010; Yadav et al., 2005). MYC2 has been shown to regulate the expression of *SPA1*, an associated factor of COP1 ubiquitin ligase, in BL-mediated photomorphogenic growth (Gangappa et al., 2010). Recent studies have shown that MYC2 works in a module of MKK3-MPK6-MYC2 to regulate BL-mediated photomorphogenic growth- and light-regulated gene expression (Sethi, Raghuram, Sinha, & Chattopadhyay, 2014). MYC2 also plays important roles in abscisic acid (ABA), gibberellic acid (GA), jasmonic acid (JA), and JA-ethylene signaling pathways (Abe, Urao, Seki, Shinozaki, & Yamaguchi-Shinozaki, 2003; Anderson et al., 2004; Boter, Ruiz-Rivero, Abdeen, & Prat, 2004; Chini, Gimenez-Ibanez, Goossens, & Solano, 2016; Hong, Xue, Mao, Wang, & Chen, 2012; Kazan & Manners, 2013; Liu et al., 2019; Lorenzo, Chico, Sanchez-Serrano, & Solano, 2004; Yadav et al., 2005). In JA signaling pathway, MYC2 acts as a master regulator by monitoring the transcriptional regulation of different JA-responsive genes. In presence of the bioactive JA-Ile (JA ligand jasmonyl-isoleucine), JAZ (JASMONATE ZIM DOMAIN) repressor proteins forms a coreceptor complex with COI1 (CORONATINE-INSENSITIVE1) which is the F-box subunit of the SCF (Skp-Cullin-F-box) complex leading to the proteasomal degradation of JAZ repressor via SCF^{COI} protein complex. This, in turn releases the JAZ-mediated transcriptional repression of MYC2 which causes the recruitment of other transcriptional activating proteins and chromatin-modifying enzymes resulting in the transcriptional expression of JA-responsive genes (An et al., 2017; Chen et al., 2012; Chini et al., 2007, 2016; Fonseca et al., 2009; Goossens, Mertens, & Goossens, 2017; Kazan & Manners, 2013; Liu et al., 2019; Sheard et al., 2010; Thines et al., 2007; Yan et al., 2007).

Arabidopsis Response Regulator 16 (*ARR16*) is a type-A *ARR* gene containing a receiver domain at the N terminal region along with short variable C terminal extension that contains less than 30 amino acids beyond the receiver domain. Expression of mRNA transcript level of *ARR16* gets induced by the application of exogenous cytokinin. However, *ARR16* itself acts as a negative regulator of cytokinin signaling (D'Agostino & Kieber 2000; Efroni et al., 2013; Ren et al., 2009). *Arabidopsis* histidine kinase protein also known as AHK4/CRE1 (CYTOKININ RESPONSE1)/WOL1 (WOODEN LEG1) acts as cytokinin receptor. In the roots of *cre1-1* mutant, which is a loss of function mutant of *AHK4*, the expression of *ARR16* gets significantly reduced indicating a link between *ARR16* and *AHK4*-mediated signal transduction (Inoue et al., 2001; Kiba, Yamada, & Mizuno, 2002; Yamada et al., 2001). Very recently, it has been shown that transcription factor CIN-TCP4 and SWI/SNF chromatin remodeling ATPase *BRAHMA* (*BRM*) bind to the *ARR16* promoter resulting in the induction of *ARR16* expression (Efroni et al., 2013; Xiao, Jin, & Wagner, 2017). The microarray studies carried out in our laboratory have shown that one of the key regulatory genes that is up-regulated in *atmyc2* mutant background is *ARR16* in BL (Gene Expression Omnibus database under the series accession number GSE8955).

In this work, we have characterized the function of *ARR16*, a component of cytokinin signaling pathways, in light signaling pathways, and have shown how MYC2 is functionally connected to *ARR16* during seedling development. This study further demonstrates that ARR16 and MYC2 work in light, jasmonic acid, and cytokinin signaling pathways.

2 | METHODS

2.1 | Plant materials, growth conditions, and generation of transgenic lines

The wild-type *Arabidopsis thaliana* and *arr16* T-DNA mutant used in this study are in Col-0 background. *arr16* mutant line (SALK_142105C) was confirmed for its homozygousity by genomic PCR analysis and seeds were bulked for further experiments to determine its photomorphogenic phenotype. To know the exact location of T-DNA insertion in *ARR16* promoter sequence, we amplified the PCR product using T-DNA-LBP and gene-specific reverse primer and sequenced, which showed T-DNA is inserted in 5'-UTR at 33 bases upstream of the ATG start codon. Complementation test of *arr16* mutant line was performed by agro-infiltration of construct containing *ARR16* along with 1.2 kb upstream promoter fragment cloned in pBI101.2 vector. The *arr16/ARR16* complemented transgenic lines were screened on kanamycin containing Murashige and Skoog medium. The *ProARR16*-GUS transgenic lines in Col-0 and *atmyc2* background were generated as described by Abbas, Maurya, Senapati, Gangappa, and Chattopadhyay (2014); and cMyc-ARR16OE lines were generated as described by Kushwaha, Singh, and Chattopadhyay (2008). Seeds were surface sterilized and plated on Murashige and Skoog agar medium and 1% sucrose. The plates were then kept for stratification (cold and dark condition) for 3 days and subsequently transferred to light chambers maintained at 22°C with the required wavelength at particular light intensity (Kushwaha et al., 2008).

For generation of *ARR16* promoter-GUS transgenic lines, the 1.2 Kb DNA fragment upstream of the start codon was PCR amplified and cloned into the *Bam*HI and *Xba*I restriction sites of pBI101.2 promoter-less cloning vector. The *ARR16* promoter-fused *GUS* transgene was agro-infiltrated (using Agrobacterium GV3101 strain) into the wild type (Col-0) by floral dip method and transformants carrying the targeted transgene were screened on MS medium containing

kanamycin (20 μg/ml). The homozygous transgenic lines were generated as described by Hettiarachchi, Yadav, Reddy, Chattopadhyay, and Sopory (2003). The *ProARR16-GUS* transgene was then transferred to *atmyc2-3* mutant (Yadav et al., 2005) background by genetic crossing with the wild-type homozygous transgenic lines as described by Yadav et al. (2002). The homozygous mutant transgenic lines were obtained in the T4 generation for further studies.

For generation of ARR16OE transgenic lines, the full-length CDS of ARR16 was cloned in pBI121 vector using *Xba*I and *Bam*HI restriction sites using overlapping primers to add cMyc tag at the N terminal of the full-length ARR16. To study the possible genetic interaction between *MYC2* and *ARR16*, *atmyc2 arr16* double mutant lines were generated by taking *atmyc2* mutant background and crossing it with the pollens of *arr16* flower and obtained the homozygous line in the F4 generation. Homozygous *atmyc2 arr16* transgenic lines were confirmed by genomic PCR and RT-PCR analyses for further study.

2.2 | Gel-shift assay and yeast one-hybrid assay

To determine the interaction between MYC2 and *ARR16* promoter, 137-bp DNA fragment from −229 to −93 bp containing E-Box (CACATG) was cloned into *Eco*RI and *Xho*I restriction sites in the pBluescript SK+ vector. The double digested, PAGE-purified DNA fragment was radiolabelled at the 3′ end with [α-^{32}P] dATP as mentioned in Chattopadhyay, Ang, Puente, Deng, and Wei (1998) and used as probe. The same DNA fragment without radiolabelling was used for competition assay. Recombinant GST-MYC2 was purified from *Escherichia coli BL21* (DE3 strain) transformed with pGEX-4T-2-MYC2 construct, and the gel-shift experiment was carried out as described by Yadav et al. (2005). The incubated reaction was loaded on 7.5% NATIVE-PAGE gel and after running the gel, it was kept for drying and then autoradiographed.

For yeast one-hybrid assay, same region of the promoter fragment was used as in gel-shift assay. We have cloned the 137-bp promoter fragment in pLacZi2μ vector using *Eco*RI and *Xho*I restriction sites. The DNA fragment containing the mutated version of the E-Box was constructed by primer-based site-directed mutagenesis using the same restriction sites as mentioned above in the same vector. The full-length MYC2 was cloned in AD-vector using *Nde*I and *Cla*I. Both the constructs were co-transformed into EGY48 yeast strain following Clontech LiAc protocol. Transformed colonies were selected on double dropout (2D) plate devoid of leucine and uracil. Then, those transformed colonies were restreaked on plate devoid of leucine and uracil but supplemented with X-gal substrate to confirm the interaction between the questioned protein and promoter fragment.

2.3 | Chromatin immunoprecipitation (ChIP) assay

The ChIP assays were performed according to the protocol described by Gangappa et al. (2010) with some modifications. Wild-type (Col-0) and transgenic MYC2OE were used for the experiment. Both wild-type and MYC2OE seedlings were grown under WL (15 μmol m^{-2} s^{-1}) for 6 days followed by 12 hr of 10 μM zeatin or mock solution treatment. The anti-c-Myc antibody (Sigma-Aldrich) was used for immunoprecipitation. Real-time PCR analysis was performed for monitoring the enrichment of *ARR16* promoter fragment in immunoprecipitated products using *ARR16* promoter-specific primers as well as Non-box primers. We have used Power SYBR® Green PCR Master Mix (Applied Biosystems) for real-time PCR analysis.

2.4 | RT-PCR analysis

For RT-PCR experiment, RNA was isolated from the 6-d-old seedlings grown under desired light condition using RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA was converted into cDNA by using Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit followed by RT-PCR using Power SYBR® Green PCR Master Mix (Applied Biosystems) with gene-specific and *ACTIN* primers. *ACTIN* of WT-Col was kept as endogenous control.

2.5 | Root growth

Seeds were placed on 0.5× MS medium (1% sucrose and 1% agar) on vertical square plates and stratified (cold and dark condition) for 3 days before placing it under desired light condition. The seedlings were grown under 100 μmol m^{-2} s^{-1} light intensity for 16 days. In case of methyl jasmonate treatment, we have used the same light intensity in presence of 20 μM methyl jasmonate. While in case of cytokinin treatment, we have used 1 μM of trans-zeatin. Here also, we grew the seedlings for 16 days under the same light condition as control (only MS medium without hormone) and MeJa treatment.

2.6 | Primers used

The primers used in this study are listed below:

RT-ARR16-FP: 5′-TGTTGCAAAGTGACAACAGCAGAG-3′
RT-ARR16-RP: 5′-GATGACTCCTGCTTCACTTTCTTG-3′
Y1H-ARR16-FP:5′-CGGAATTCCCATCACCAGAGATTCACATG-3′
Y1H-ARR16-FP-MUT: 5′CGGAATTCCCATCACCAGAGATTTTA CAAATTCATGCATACACACTTC-3′
Y1H-ARR16-RP:5′-CCGCTCGAGCAGACATTGCTTTTAGTTCTC-3′
ACTIN2-FP: 5′-AAAGGCTTAAAAAGCTGGGG-3′
ACTIN2-RP: 5′-GGGACTAAAACGCAAAACGA-3′
ChIP-ARR16-FP: 5′-CATCATAAATACCATCACCAG- 3′
ChIP-ARR16-RP: 5′-CAGACATTGCTTTTAGTTCTC- 3′
Non-box -ARR16-FP: 5′ CAAACATCATTGTTTCAATTTCC 3′
Non-box -ARR16-RP: 5′ TGGACTTGCACAGCTTGAAC 3′

3 | RESULTS

3.1 | MYC2 directly interacts with the E-box of *ARR16* promoter

MYC2 works as a negative regulator of blue light (BL)-mediated inhibition of hypocotyl elongation and gene expression (Gangappa et al.,

2010; Sethi et al., 2014; Yadav et al., 2005). The microarray studies have shown that *ARR16* is up-regulated in *atmyc2* mutant seedlings in BL (Gene Expression Omnibus database under the series accession number GSE8955). To further investigate the observation, we carried out quantitative reverse transcription (RT)-PCR analysis. The transcripts of *ARR16* indeed showed ~2-fold higher accumulation in *atmyc2* mutant than wild-type seedlings in BL (Figure 1A). The transcript level of *ARR16* was also found to be significantly higher in *atmyc2* mutant (SALK_017005) as compared to wild type in dark and white light (WL) (Figure 1A). These results indicate that MYC2 negatively regulates the expression of *ARR16*, either directly or indirectly.

To examine whether MYC2 directly binds to the *ARR16* promoter to regulate its expression, we carried out gel-shift assay (Electrophoretic Mobility Shift Assay). In silico analysis of the upstream promoter region of *ARR16* showed the presence of an E-box (CACATG) about 10 bp upstream to the transcriptional start site of *ARR16* promoter (Figure 1B). We used a 137-bp *ARR16* promoter DNA fragment containing the E-box as probe and a purified glutathione S-transferase (GST)-MYC2 fusion protein in gel-shift assays (Figure 1C). GST-MYC2 was able to bind to the *ARR16* promoter fragment, forming a lower mobility DNA–protein complex (Figure 1C, lane 3). However, GST alone did not show any protein–DNA complex formed (Figure 1C, lane 2). Moreover, excess unlabeled DNA fragment containing the E-box competed for the binding activity of GST-MYC2 (Figure 1C, lanes 4 and 5). These results indicate that MYC2 is able to bind to the E-box of *ARR16* promoter.

We then performed yeast one-hybrid assays to reexamine the protein–DNA interaction between MYC2 protein and *ARR16* promoter fragment. Yeast colonies co-transformed with the constructs containing the *ARR16* promoter and MYC2 coding sequence were grown on defined double dropout (2D) medium, which is devoid of leucine and uracil but supplemented with X-gal substrate. The GAL4 transcriptional activation domain-fused MYC2 (AD-MYC2) binds to the E-Box of the *ARR16* promoter fragment resulting in the induction of the *lacz* reporter gene expression that causes the blue coloration of the transformed yeast colonies (Figure 1D). The specificity of this interaction was determined by using a mutated version of the E-Box (from CACATG to TTACAA) that results in the disruption of the interaction causing no induction of the reporter gene expression, and hence no blue coloration of the yeast cells (Figure 1D). Taken together, these results suggest that MYC2 directly binds to the E-Box of *ARR16* promoter.

To examine the in vivo interaction of MYC2 with *ARR16* promoter, we performed chromatin immunoprecipitation (ChIP) assays. *ARR16* belongs to type-A ARR family, and it is reported that *ARR16* expression is induced by cytokinin treatment (Bhargava et al., 2013; Rashotte, Carson, To, & Kieber, 2003; Ren et al., 2009; Taniguchi, Sasaki, Tsuge, Aoyama, & Oka, 2007). We used wild-type (Col-0) and MYC2OE (MYC2 overexpressor) transgenic seedlings containing three copies of c-Myc epitopes fused to MYC2 (Maurya, Sethi, Gangappa, Gupta, & Chattopadhyay, 2015) grown in WL (15 μmol m^{-2} s^{-1}) for 6 days followed by 12 hr of 10 μM zeatin and mock solution treatment (Figure 1E). The c-Myc antibodies were used for immunoprecipitation. Detailed diagrammatic representation in Figure 1E showed the position of the E-box and Non-box (used as control; contains no light-responsive elements (LREs)) in *ARR16* promoter. The qPCR analysis of the co-immunoprecipitated genomic DNA fragment showed the enrichment of *ARR16* promoter fragment containing the E-box in zeatin-treated MYC2OE seedlings by about ~ 7-fold with respect to zeatin-treated wild-type seedlings (Figure 1E). No differential enrichment of the *ARR16* promoter was observed in zeatin-untreated samples. These results suggest that in vivo binding of MYC2 to the promoter of *ARR16* requires cytokinin.

3.2 | MYC2 negatively regulates the activity of *ARR16* promoter

Light-induced transcriptional regulation is conferred by promoter containing LREs of the target genes (Abbas et al., 2014; Chattopadhyay et al., 1998; Terzaghi & Cashmore, 1995; Tobin & Kehoe, 1994). To determine whether the interaction between MYC2 and *ARR16* promoter has a functional relevance in vivo, we examined the activity of *ARR16* promoter in *atmyc2* mutant. Stable transgenic lines were generated by introducing *ProARR16-GUS* construct into wild type (Col-0), and several independent homozygous transgenic lines were selected for further experiment (Figure S1). One representative homozygous transgenic line containing *ProARR16-GUS* transgene (line no. 12; L-12) was used for transferring the transgene into the *atmyc2-3* null mutant background (Yadav et al., 2005) through genetic crosses. The homozygous transgenic lines containing *ProARR16-GUS* transgene in *atmyc2* mutant background were then generated for further studies (Figure S1). The activity of *ARR16* promoter was determined by GUS reporter enzymatic activity measurements.

The activity of *ARR16* promoter was found to be restricted to the cotyledons in wild type and *atmyc2* mutant backgrounds in dark (Figure 2A). Under WL condition, the *ARR16* promoter activity was observed in cotyledons and hypocotyl of 6-day-old wild-type seedlings. Similar pattern of expression was also found in *atmyc2* mutant background; however, the level of expression appeared to be increased in *atmyc2* as compared to wild type. Although no GUS stain was detected in the roots of wild type seedlings, *ARR16* promoter activity was detected in roots of *atmyc2* (Figure 2A). The *ARR16* promoter activity was observed in cotyledons and hypocotyl of both wild type and *atmyc2* mutant seedlings in BL condition (Figure 2A). The quantitative GUS activity measurements revealed that the *ARR16* promoter activity was 1.5- to 4-fold higher in *atmyc2* mutant background than the wild type under constant dark or light conditions (Figure 2B). These results suggest that MYC2 represses the promoter activity of *ARR16*.

To study the light-mediated induction kinetics of the promoter of *ARR16* in *atmyc2* background in comparison to wild type in BL, we transferred 4-day-old dark-grown seedlings to BL for various time points and measured GUS activity; whereas *ARR16* promoter was induced to about ~2.5-fold in *atmyc2* mutant, the level of induction

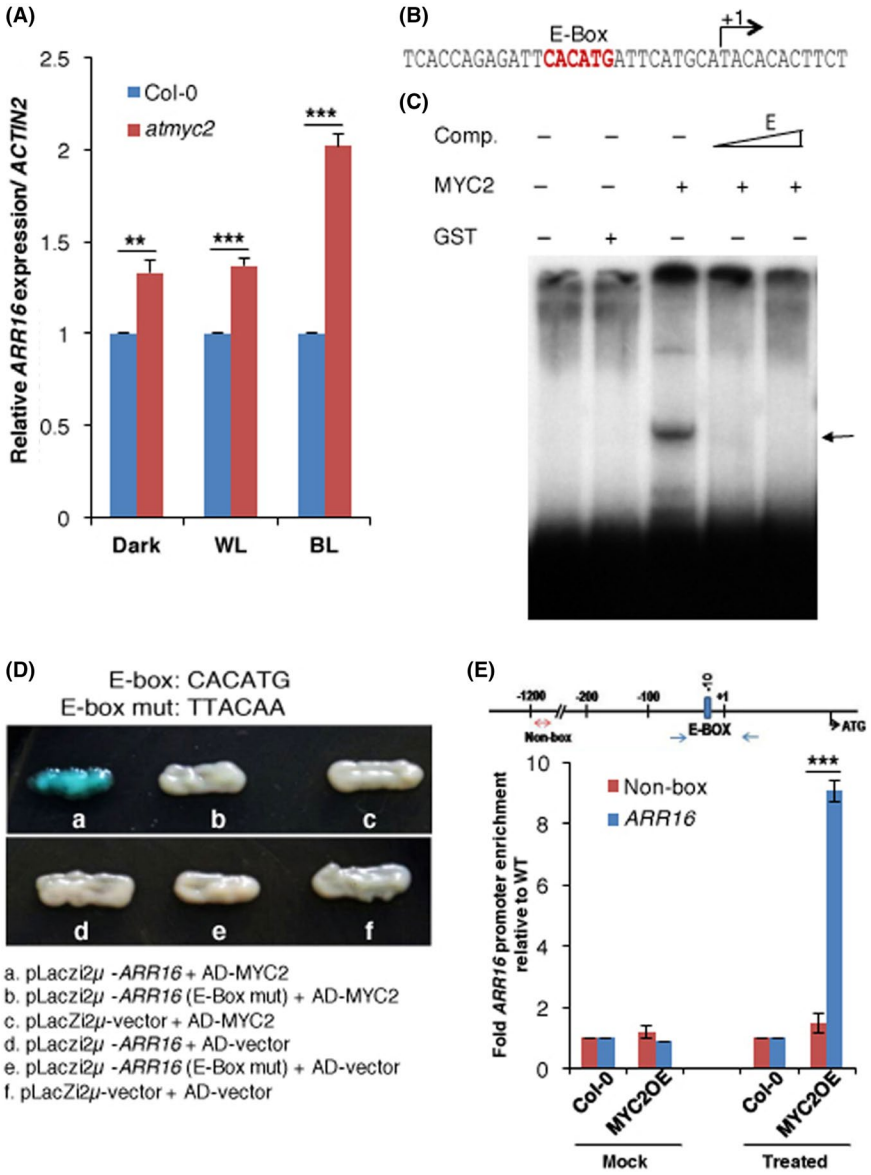

FIGURE 1 MYC2 Directly Interacts with *ARR16* Promoter and negatively regulates its activity. (A) Real-time PCR analysis of *ARR16* transcripts in 6-day-old wild-type (Col-0) and *atmyc2* (SALK_017005) seedlings grown in constant dark, blue light (15 μmol m^{-2} s^{-1}) and white light (30 μmol m^{-2} s^{-1}) conditions. Error bars represent ± *SD* of the mean of four biological replicates. Asterisks represent statistically significant differences (**p < .01 and ***p < .001) as determined by Student's t test. (B) Diagrammatic representation of *ARR16* minimal promoter region showing cis-acting element (E-Box). The transcriptional start site is designated as position + 1. (C) Electrophoretic mobility shift assay by using GST-MYC2 recombinant protein and *ARR16* promoter fragment containing E-box. Approximately 500 ng of recombinant protein GST-MYC2 was added (lane 3, 4 and 5) to the radioactively labeled E-Box containing *ARR16* promoter which is used as probe. No protein was added in lane 1 and 300 ng of GST protein was added in lane 2. The DNA–protein complexes were resolved on 7.5% native polyacrylamide gel. The triangle indicates the increasing concentration of unlabeled E-Box containing *ARR16* promoter fragment used as competitors (Comp.); the plus and the minus signs indicate the presence and absence, respectively. The arrow shows the DNA–protein complex formed. One representative result has been shown out of five independent experiments. (D) Yeast one-hybrid interaction between *ARR16* promoter fragment and MYC2 by co-transforming Yeast EGY48 strain and plating on double dropout media (2D) devoid of leucine and uracil however supplemented with X-gal. Result of one representative experiment out of three has been shown. (E) ChIP assay of *ARR16* promoter from wild type (Col) and MYC2 overexpressor (MYC2OE) transgenic seedlings grown in constant WL (15 μmol m^{-2} s^{-1}) for 6 days followed by 12 hr of 10 μM zeatin or mock solution treatment. Diagrammatic representation of the *ARR16* promoter showed E-box element. The position (+1) indicates the transcriptional start site. The arrows indicate the position of the primers used for the ChIP assay, whereas the double-headed arrow indicates the position of DNA fragment without any LRE (termed as "non-box") used as negative control in ChIP assay. c-Myc antibodies were used for immunoprecipitation. qPCR analyses of the E-box containing *ARR16* promoter fragment in mock solution (left panel) or zeatin (right panel)-treated seedlings grown in WL. The ChIP values were normalized first by their respective input values, and then fold enrichment relative to the wild type was calculated. Error bars indicate ± *SD* of three biological replicates. Asterisks represent statistically significant differences (***p < .001) based on two-way ANOVA factorial analysis followed by Tukey's HSD test indicating the genotype that differs significantly in *ARR16* promoter enrichment in comparison to its respective wild type (Col-0)

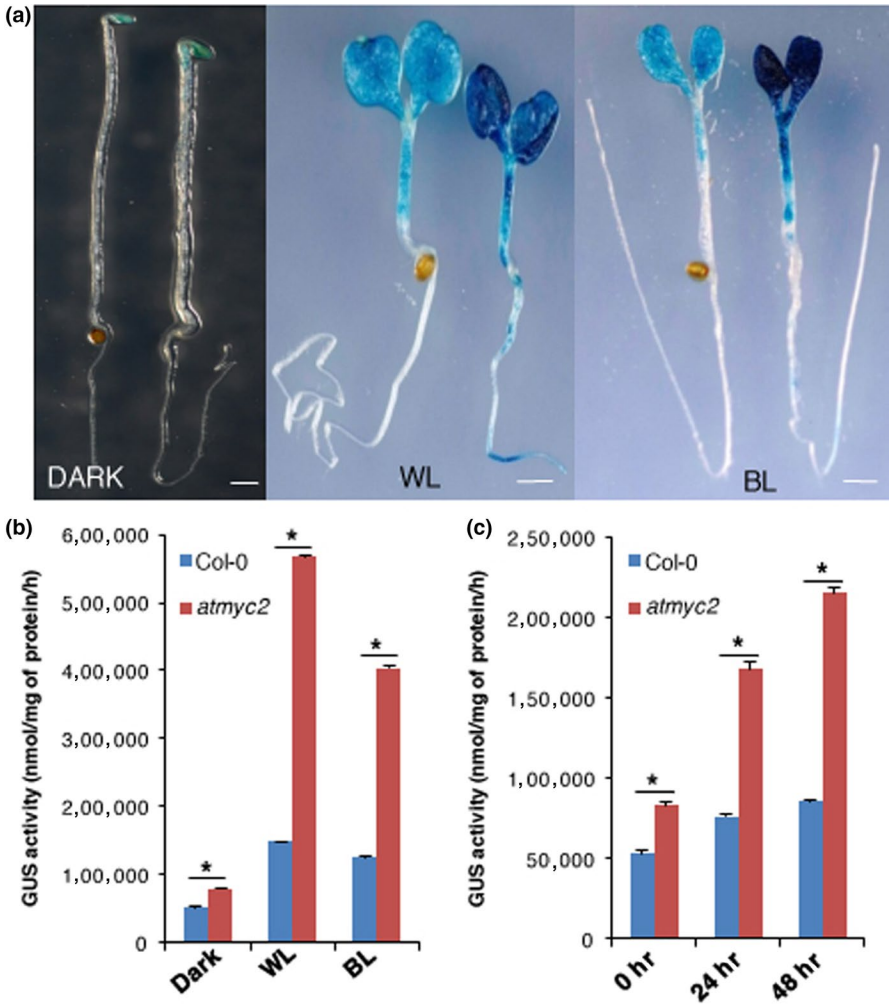

FIGURE 2 MYC2 negatively regulates *ARR16* promoter activity. (a) In each panel, wild type (Col-0) seedling is shown on the left and *atmyc2* mutant seedling is shown on the right. Six-day-old seedlings carrying the *ProARR16-GUS* transgene were grown under constant dark, WL (30 µmol m^{-2} s^{-1}), and BL (15 µmol m^{-2} s^{-1}) conditions. The promoter activity in wild-type and *atmyc2* mutant backgrounds was determined by GUS staining for the same length of time. Bar = 1 mm. (b) Quantitative GUS activities of 6-day-old constant dark, WL (30 µmol m^{-2} s^{-1}) and BL (15 µmol m^{-2} s^{-1}) grown seedlings. Error bars represent ± *SD* of the mean of three biological replicates. Asterisks represent statistically significant differences (*p < .05) as determined by Student's *t* test. (c) GUS activity of 4-day-old dark-grown seedlings transferred to BL (15 µmol m^{-2} s^{-1}) for 24 hr and 48 hr. Error bars represent ± *SD* of the mean of three biological replicates. Asterisks represent statistically significant differences (*p < .05) as determined by Student's *t* test

was found to be ~1.6-fold in wild type background after 48 hr of exposure to BL (Figure 2C). These results indicate that the induction of the *ARR16* promoter was significantly enhanced in *atmyc2* background.

3.3 | *ARR16* expression is regulated by MYC2 in various tissue types of adult plants

Although it is evident from the above results that the activity of *ARR16* promoter is suppressed by MYC2 at the seedling stage, it also raised the question of its role in young adult and flowering plants since MYC2 plays important roles at various stages of plant growth (Yadav et al., 2005). To elucidate the effect of *MYC2* mutation on the *ARR16* promoter activity in different organs, GUS staining as well as quantitative GUS assays of 15-day-old young adult plants and 30-day-old flowering plants grown under 16-hr-light/8-hr-dark cycles were carried out.

The activity of *ARR16* promoter was detected in root and stem in *atmyc2* mutant, however not in the wild-type background in 15-d-old BL grown plants (Figure 3A,B). Moreover, increased GUS staining was detected in the leaves of *atmyc2* mutants as compared to wild type (Figure 3C). Quantitative GUS activity measurements showed that *ProARR16-GUS* transgene was expressed ~2-fold higher in various tissue types of *atmyc2* mutants as compared to wild-type background (Figure 3H).

The GUS staining of 30-day-old plants showed that the expression of the transgene was detectable in root and flower in wild-type background, while in *atmyc2* mutant background, the expression could be observed in root, stem, leaf, and flower (Figure 3D–G). One interesting observation was that though *ARR16* promoter activity was more pronounced in almost all the organs in *atmyc2* mutant background, GUS staining was reduced in flowers of *atmyc2* background. Quantitative GUS activity measurements revealed that *ARR16* promoter activity was ~1.5-fold enhanced in *atmyc2* mutants as compared to wild-type background in root, stem, and leaf (Figure 3I). However, *ARR16* promoter activity was significantly reduced in *atmyc2* mutants as compared to wild type in flowers (Figure 3I).

3.4 | ARR16 promotes photomorphogenic growth in blue light

MYC2 is a well-established blue light-specific negative regulator of photomorphogenesis (Gangappa et al., 2010; Maurya et al.,

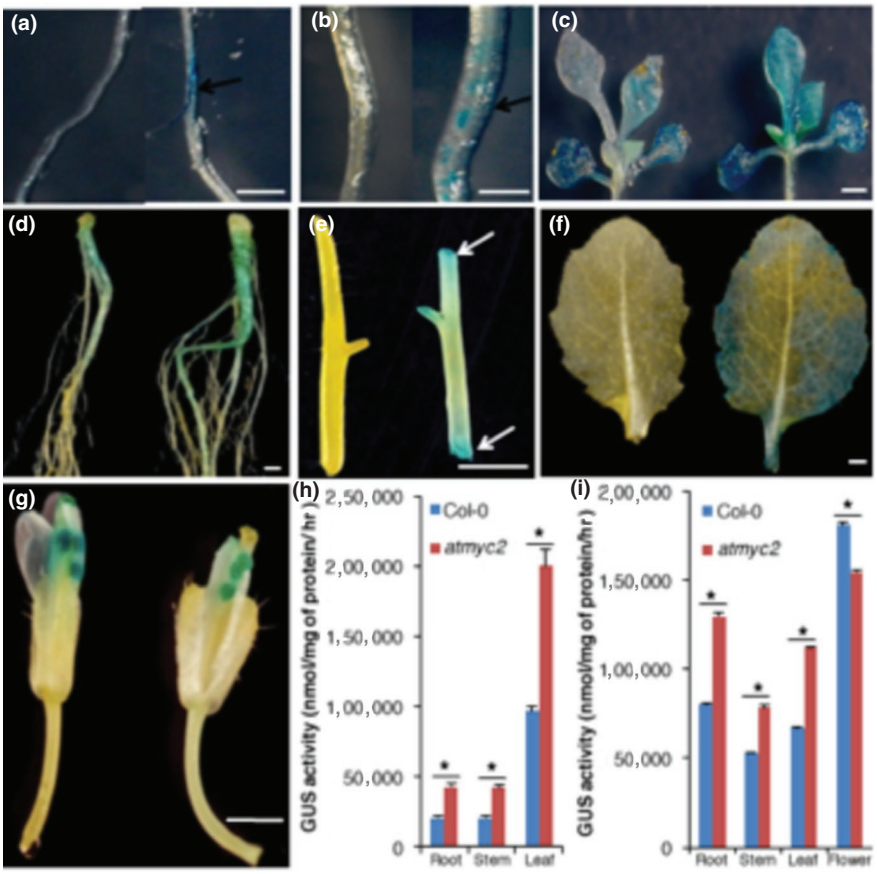

FIGURE 3 Effect of *atmyc2* Mutation on Tissue-Specific Expression of *ProARR16-GUS* Transgene. In each panel, wild-type (Col-0) plants are shown on left and *atmyc2* mutant plants are shown on right. (a–c) Root (a), stem (b), and leaf (c) of 15-day-old constant BL (15 µmol m^{-2} s^{-1}) grown plants carrying the *ProARR16-GUS* transgene, respectively. (d–g) Root (d), Stem (e), Leaf (f) and Flower (g) of 30-day-old WL (16 hr light/8 hr dark) grown plants, respectively. Tissue-specific expression of *ARR16Pro-GUS* transgene (a–g) in wild type (Col-0) and *atmyc2* mutant backgrounds was observed after GUS staining for same length of time. Bar = 1 mm. (h) Quantification of GUS activity in root, stem, and leaf of 15-day-old constant BL (15 µmol m^{-2} s^{-1}) grown plants. Promoter activities were monitored by measuring the GUS activities of WT (Col-0) and *atmyc2* mutant plants carrying the *ProARR16-GUS* transgene. Error bars indicate ± *SD* of the mean of four biological replicates. Asterisks represent statistically significant differences (*$p < .05$) as determined by Student's *t* test. (i) Quantification of GUS activities of different parts of the 30-day-old WL (16 hr light/8 hr dark) grown adult plant. Error bars indicate ± *SD* of the mean of three biological replicates. Asterisks represent statistically significant differences (*$p < .05$) as determined by Student's *t* test

2015; Sethi et al., 2014; Yadav et al., 2005). Since this study reveals that MYC2 regulates *ARR16* expression, we were curious to determine the possible role of ARR16 in light-mediated seedling development. We searched for the T-DNA insertion mutant line (Alonso et al., 2003). A mutant line with T-DNA insertion at the 5′ end (in 5′-UTR at 33 bases upstream of the ATG start codon) of *ARR16* (SALK_142105C) was identified and referred to as *arr16* (Figure S2a,b). RT-PCR analyses showed that the mutant line was a knockdown mutant of *ARR16* (Figure S2c). We have also generated cMyc-tagged ARR16 homozygous overexpressor transgenic lines (ARR16OE) for this study (Figure S2d–f). To examine the photomorphogenic growth of *arr16*, we grew the *arr16* and ARR16OE seedlings in dark, WL, and BL conditions. Although the *arr16* seedlings displayed significantly shorter hypocotyl in dark (Figure 4A and d), the hypocotyl length of *arr16* seedlings was found to be significantly higher than that of wild type in WL and BL. On the other hand, ARR16OE transgenic lines displayed drastically shorter hypocotyl than wild type in WL and BL with no effect in the darkness (Figure 4B,C and E,F). Further, when we complemented *arr16* mutant with *ARR16* functional gene, we found that the hypocotyl length of *arr16/ARR16* was similar to that of wild type (Col-0) under BL condition. These results suggest that the phenotypic defects of *arr16* mutant are specific to mutation in *ARR16* (Figure S3). Taken together, these results suggest that ARR16 works as a positive regulator of photomorphogenic growth in BL and WL, however likely to play, if any, a negative regulatory role in the darkness.

To determine the genetic interaction between *MYC2* and *ARR16*, we constructed *atmyc2 arr16* double mutant plants by genetic crosses between *atmyc2* and *arr16*, and homozygous double mutant lines were generated. Examination of the hypocotyl length of *atmyc2 arr16* revealed that *arr16* mutation suppressed the short hypocotyl phenotype of *atmyc2* in BL, suggesting that *arr16* works downstream to *atmyc2* (Figure 4G and h, and Figure S4).

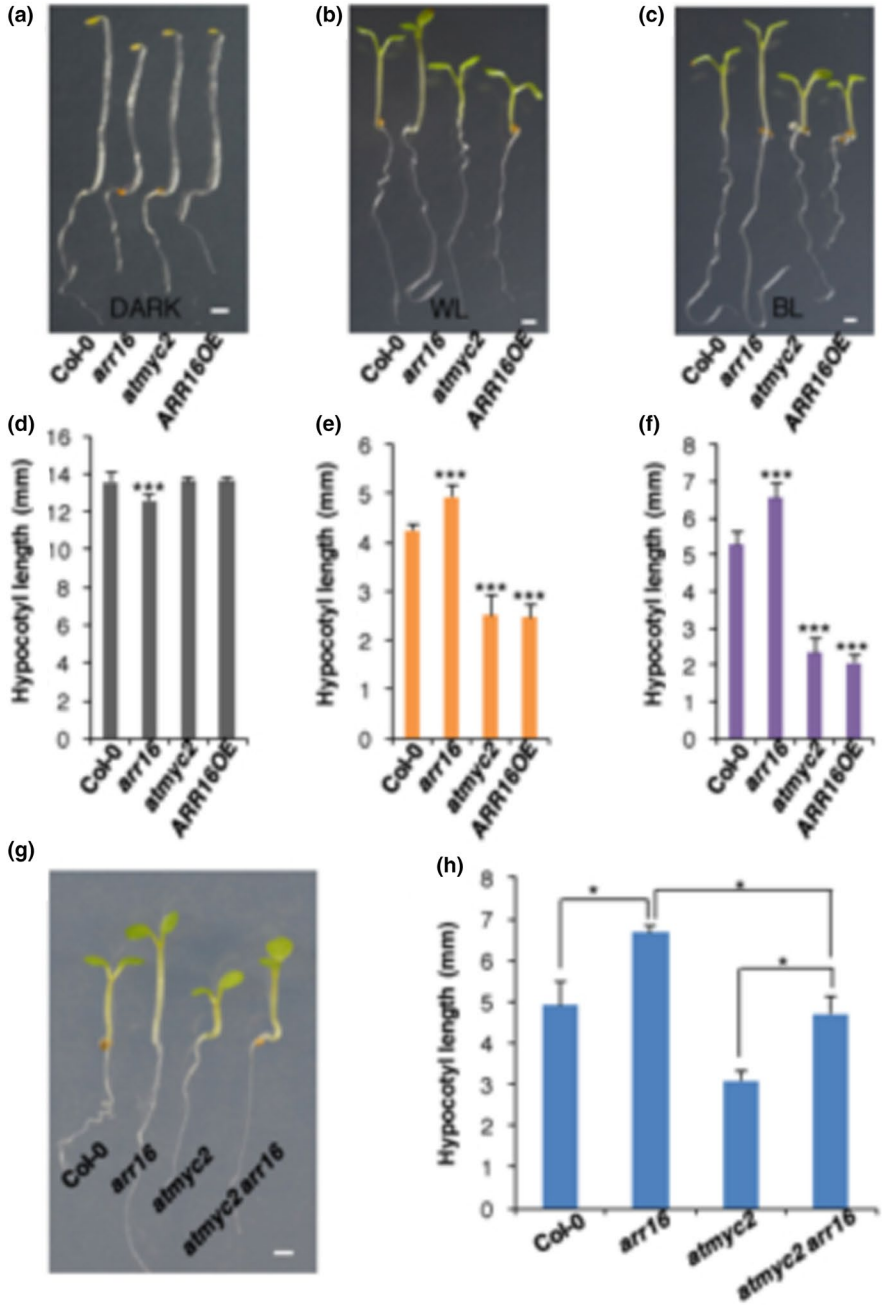

FIGURE 4 Phenotypic Characterization of *arr16* and *atmyc2 arr16*. (a–c) Phenotype of 6-day-old wild type (Col-0), *arr16*, *atmyc2*, and ARR16OE seedlings grown under constant dark, WL (15 µmol m^{-2} s^{-1}), and BL (15 µmol m^{-2} s^{-1}) conditions. Bar = 1 mm. (d–f) Quantification of hypocotyl length of seedlings grown in dark, WL and BL, respectively. Error bars represent ± *SD* (*n* ≥ 30); Student's *t* test, ***$p < .001$. (g) Phenotype of 6-day-old Col-0, *arr16*, *atmyc2*, and *atmyc2 arr16* seedlings grown under BL (15 µmol m^{-2} s^{-1}). Bar = 1mm. (h) Quantification of the hypocotyl length of 6-d-old Col-0, *arr16*, *atmyc2*, and *atmyc2 arr16* seedlings grown under BL (15 µmol m^{-2} s^{-1}). Error bars represent *SD* (*n* ≥ 30); Student's *t* test, *$p \leq .05$

3.5 | ARR16 and MYC2 influence jasmonic acid and cytokinin signaling

Since *ARR16* is a negative regulator of cytokinin signaling pathways (Ren et al., 2009), we examined the root growth response of *arr16* in the absence and presence of cytokinin. In the absence of cytokinin, the primary root length of *arr16* was significantly shorter than that of wild type (Col-0), while ARR16OE transgenic lines showed the opposite root length phenotype (Figure 5A and 5). On the other hand, in the presence of cytokinin, both *arr16* and ARR16OE were less sensitive to cytokinin response in comparison with that of wild type (Col-0). Reduction in the primary root length upon cytokinin treatment was about 6-fold in wild type (Col-0), while in case of *arr16* and ARR16OE the reduction was found to be about 4.5- and 4.8-fold, respectively, indicating the saturation of the response of *ARR16* to cytokinin responsiveness. Interestingly, *atmyc2* mutant showed only about 2.6-fold reduction in the primary root length, which is significantly less in comparison to that of the ~6-fold in case of the wild type (Col-0). Therefore, mutation in *MYC2* resulted in less sensitive response to cytokinin treatment, suggesting the role of MYC2 in cytokinin signaling in addition to that of light and jasmonic acid signaling pathways (Figure 5B and 5).

MYC2 plays both positive and negative regulatory roles in jasmonic acid (JA) signaling pathways (Lorenzo et al., 2004; Yadav et al., 2005; Dombrecht et al., 2007; Robson et al., 2010; Kazan & Manners, 2013; Zhai et al., 2013; Zhang et al., 2014). Since this study shows that ARR16 is involved in BL-mediated photomorphogenic

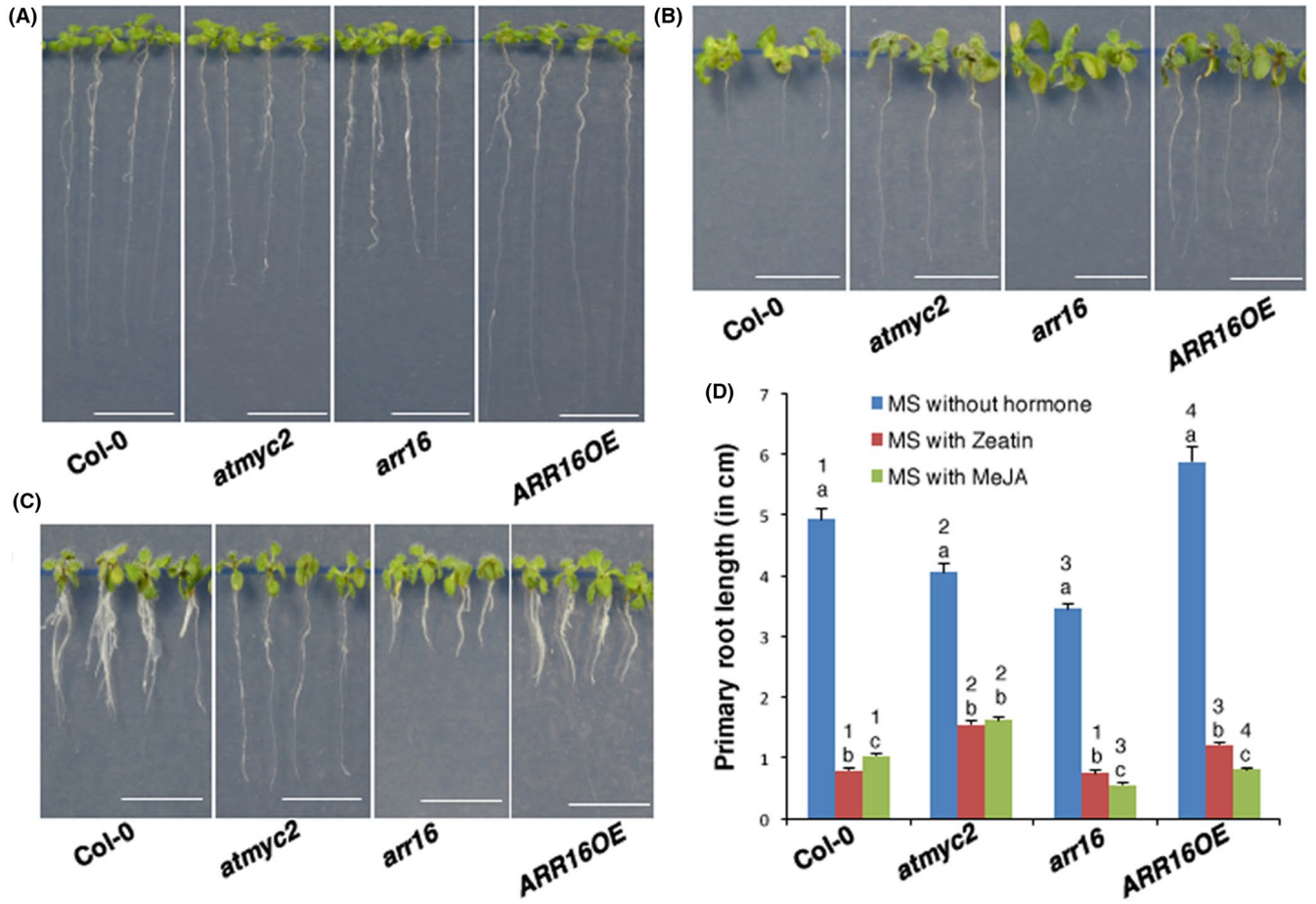

FIGURE 5 ARR16 and MYC2 modulate Jasmonic Acid and Cytokinin Signaling pathways, Respectively. (A) Root phenotype of 16-d-old WL (100 µmol m^{-2} s^{-1}) grown wild type (Col-0), *atmyc2*, *arr16*, and ARR16OE. Bar = 1 cm. (B) Root phenotype of 16-day-old WT (Col-0), *atmyc2*, *arr16*, and *ARR16OE* grown under WL (100 µmol m^{-2} s^{-1}) for cytokinin treatment with 1 µM zeatin. Bar = 1 cm. (C) Root growth of 16-day-old WL (100 µmol m^{-2} s^{-1}) grown wild type (Col), *atmyc2*, *arr16*, and ARR16OE in presence of 20 µM methyl jasmonate. Bar = 1 cm. (D) Diagrammatic presentation of the mean (±*SD*; *n* = 15) values of the primary root length in different genotypes (wild-type (Col-0), *atmyc2*, *arr16*, and ARR16OE) under different hormonal (zeatin and methyl jasmonate) treatments and in control (without hormone) conditions. Different small alphabetical letters on the error bars indicate significant (*p*-value < .05) differences in the root length between different hormonal treatment condition within a particular genotype; while different numerals on the error bar indicate significant (*p*-value < .05) differences in the primary root length between different genotype within a particular treatment condition (MS without hormone, MS with 1 µM Zeatin, MS with 20 µM methyl jasmonate). The data were compared by using two-way ANOVA factorial analysis followed by Tukey's HSD test

growth, and its expression is negatively regulated by MYC2, we ask whether ARR16 is also involved in JA signaling pathway. As shown in Figure 5C and 5, upon JA treatment *atmyc2* mutants were less sensitive (2.5-fold reduction in primary root length in comparison with that of wild-type Col-0 seedlings that showed 4.8-fold reduction) to JA as observed earlier (Dombrecht et al., 2007; Kazan & Manners, 2008; Lorenzo et al., 2004; Yadav et al., 2005), while mutation as well as overexpression of *ARR16* resulted in the reduction of the primary root length by 6.1- and 7.2-fold, respectively. Therefore, it is evident that both *arr16* and ARR16OE seedlings are hypersensitive to JA responses. Taken together, these results indicate that in addition to cytokinin and light, ARR16 also plays an important role in JA-mediated root growth. Furthermore, it also emerges that MYC2 plays important role in cytokinin signaling in addition to JA and light signaling pathways.

3.6 | The expression of *ARR16* is regulated by multiple regulatory proteins of light signaling pathways

It has been shown earlier that MYC2 works downstream to cry1 photoreceptor (Yadav et al., 2005). Moreover, the MKK3-MPK6-MYC2 module works specifically under BL in Arabidopsis seedling development (Sethi et al., 2014). Although MYC2-mediated regulation of *ARR16* is observed in different light conditions, MYC2 is a negative regulator of photomorphogenic growth in BL. Therefore, we wanted to examine whether *ARR16* expression is regulated by cry1 photoreceptor and other regulatory proteins such as HYH and GBF1 that work specifically in BL (Holm, Ma, Qu, & Deng, 2002; Mallappa, Yadav, Negi, & Chattopadhyay, 2006; Singh, Ram, Abbas, & Chattopadhyay, 2012); and HY5 and CAM7 that work at various wavelengths of light

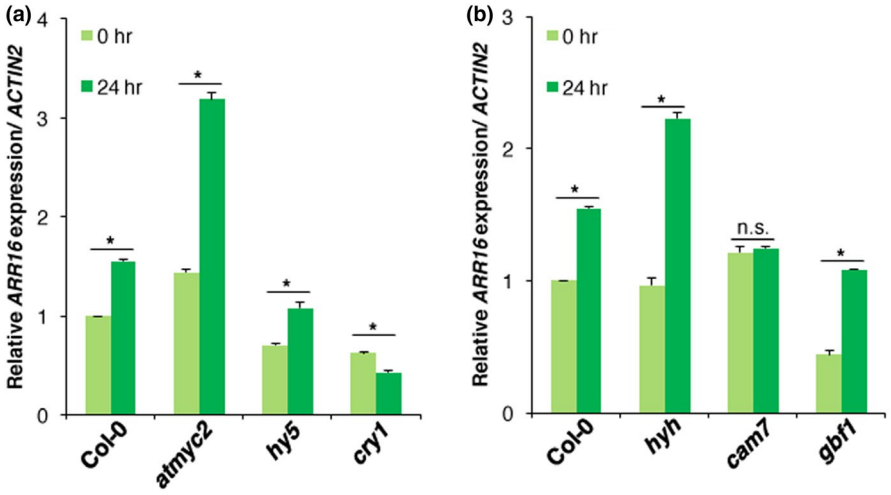

FIGURE 6 Regulation of *ARR16* Expression by Various Components of Light Signaling Pathways. (a) Real-time PCR of *ARR16* transcripts in 5-day-old wild-type (Col-0), *atmyc2*, *hy5*, and *cry1* etiolated seedlings transferred to BL (15 µmol m^{-2} s^{-1}) for 24 h. (b) Real-time PCR of *ARR16* transcripts in 5-day-old wild-type (Col-0), *hyh*, *cam7*, and *gbf1* etiolated seedlings transferred to BL (15 µmol m^{-2} s^{-1}) for 24 hr. In each of (a) and (b), error bars represent ± *SD* of the mean of four biological replicates. Asterisks indicate genotypes that differ significantly (ANOVA followed by Tukey's HSD, *$p \leq .05$) in *ARR16* expression level after 24 hr of BL induction in comparison to 0 hr. n.s. indicates no significant difference in the *ARR16* expression level after 24 hr of BL exposure

including BL (Abbas et al., 2014; Ang et al., 1998; Kushwaha et al., 2008). We tested the expression of *ARR16* in wild type versus various mutant backgrounds after transferring the 4-day-old dark-grown seedlings to BL for 24 hr. As shown in Figure 6A, the induction of *ARR16* was found to be significantly elevated (~3-fold) in *atmyc2* mutant as compared to wild-type background (~1.5-fold) (Figure 6A). Also, there was *ARR16* induction in *hy5* mutant background; however, the expression of *ARR16* was significantly decreased in *cry1* mutant (Figure 6A). The induction of *ARR16* was increased to more than 2-fold in *hyh* and *gbf1* mutant backgrounds, although there was no induction of *ARR16* in *cam7* mutant background upon BL exposure (Figure 6B). Taken together, these results suggest that *ARR16* expression is negatively regulated by HYH and GBF1 bZIP transcription factors that work specifically in BL. On the other hand, cry1 is required for the optimum expression of *ARR16* in BL.

4 | DISCUSSION

Recent studies have started unraveling that at least some of the light signaling components work as point of cross talk with other signaling pathways. MYC2 bHLH transcription factor has been shown to be working in light, abscisic acid, and jasmonic acid signaling pathways (Abe et al., 1997; Aleman et al., 2016; Anderson et al., 2004; Boter et al., 2004; Gangappa et al., 2010; Yadav et al., 2005). In this study, it has been shown that MYC2 regulates a bona fide cytokinin signaling pathway component, ARR16, in BL and WL. Working downstream to MYC2, ARR16 promotes BL-mediated seedling development. MYC2 directly binds to the promoter of *ARR16* to negatively regulate its expression.

Transcriptional regulation of the target genes of MYC2 by binding to the cis-acting elements such as G-box (5′-CACGTG-3′), Z-box (5′-ATACGTGT-3′), and G-box variants like 5′-CACATG-3′ which is also known as E-box (CACNTG), located in the promoter region of these targeted genes were already well reported (Abe et al., 2003; Boter et al., 2004; Dombrecht et al., 2007; Gangappa et al., 2010; Maurya et al., 2015; de Pater, Pham, Memelink, & Kijne, 1997; Sethi et al., 2014; Yadav et al., 2005). Here, in this study, molecular data show that MYC2 binds to the E-box of *ARR16* promoter in order to regulate its expression. The GUS activity measurements of the transgenic seedlings and adult plants demonstrate that the transcriptional activity of *ARR16* promoter is increased in *atmyc2* mutant background from seedling stage to flowering plants. These results are in line with the molecular data establishing an important role of MYC2 in transcriptional regulation of *ARR16*. Genetic analyses of *atmyc2 arr16* double mutants demonstrate that ARR16 works downstream to MYC2 in BL-mediated photomorphogenic growth (Figure 4). The additional mutation of *ARR16* in *atmyc2* mutant background resulted in the loss of *atmyc2* short hypocotyl phenotype in BL. Therefore, functional ARR16 is required to exhibit the photomorphogenic response of *atmyc2* mutant in BL.

ARR16 acts as a negative regulator of cytokinin signaling (Ren et al., 2009). Interestingly, the root growth studies in presence of cytokinin supports that ARR16 is functionally redundant in cytokinin signaling, which is in contrast to its specific role in light signaling pathways. Also, involvement of MYC2 in the control of primary root length in response to cytokinin treatment hinted a new role of MYC2 in cytokinin signaling pathways, setting the stage for further elaborated studies. This study further shows the role of ARR16 in jasmonic acid signaling pathways.

MYC2 works as a negative regulator of photomorphogenesis in BL and negatively regulates the expression of several regulatory genes such as *SPA1* and *MPK6* in BL (Gangappa et al., 2010; Sethi et al., 2014). The expression of *ARR16*, as revealed in this study, is also

negatively regulated by MYC2 in BL. The binding of a transcription factor to the promoter of downstream target genes, or upstream regulatory genes in a signaling pathway is not unprecedented. HY5 bZIP transcription factor has been shown to bind to *RBCS-1A* and *CHS* promoters as well as several upstream regulatory genes including its own promoter in light signaling pathways (Abbas et al., 2014; Lee et al., 2007). In this study, gel-shift and yeast one-hybrid results indicate the transcriptional regulation of *ARR16* by the direct binding of MYC2 to the E-box of *ARR16* promoter. However, in vivo interaction of MYC2 with the promoter of *ARR16* in ChIP assays indicates the involvement of cytokinin signaling pathways in the process. Also, based on ChIP data, it appears that in the absence of cytokinin some other regulatory component, that might work downstream of MYC2, is involved in the regulation of *ARR16*. Similar observation has also been made by Jang et al., 2017.

The negative effect of MYC2 on *ARR16* expression is partial, since residual expression is observed in various tissue types. Therefore, although MYC2 represses the expression of *ARR16* under BL and WL conditions, it does not seem to be the only regulatory protein involved in the process. This study reveals that BL-specific regulators of light signaling pathways such as HYH and GBF1 regulate the expression of *ARR16*, however mutation in *CAM7*, which works at multiple wavelengths of light including BL failed to cause the induction of *ARR16* expression upon BL exposure. ARR16 works in cytokinin signaling pathways, and this study further reveals that ARR16 also works in light and jasmonic acid responsive pathways. Therefore, the regulation of *ARR16* is likely to be complex as controlled by multiple signaling pathways.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTIONS

A.S., S.D. and S.C. designed the research. A.S. and S.D. carried out the experiments. A.S. S.D. and S.C. analyzed the data; and A.S and S.C. wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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