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; Funkhauser & 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, Funkhauser, & 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 and expanded cotyledons (von Arnim & Deng, 1996; Chen & Chory, 2011; Josse & Halliday, 2008; Pfeiffer et al., 2016; Wang et al., 2014).
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 atmyc2/2b/1 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 corepressor complex with COI1 (CORONATINE-SENSITIVE1) which is the F-box subunit of the SCF (Skp-Cullin-F-box) complex leading to the proteasomal degradation of JAZ repressor via SCFCOI 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 homozygosity by genomic PCR analysis and seeds were bulked for further experiments to determine its photomorphogenetic 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 pB101.2 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 BamHI and XbaI 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

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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 XbaI and BamHI 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 EcoRI and XhoI restriction sites in the pBluescript SK+ vector. The double digested, PAGE-purified DNA fragment was radiolabelled at the 3' end with [α-32P]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 pLaCZ2µ vector using EcoRI and XhoI 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 Ndel and Clal. 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 RNasy 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’-GTGGCAAGGCAAACGCAGAG-3’
- **RT-ARR16-RP**: 5’-GATGACTCTCTGGTTCACTTCTTG-3’
- **Y1H-ARR16-FP**: 5’-CGGAATTCATCCACAGATTACATG-3’
- **Y1H-ARR16-RP**: 5’-CAGACATTGCTTTAGTTCTTC-3’
- **RT-ARR16-FP-MUT**: 5’-CGGAATTCATCCACAGAGAGTTA-3’
- **RT-ARR16-RP**: 5’-CATTCACTACTACAGAGATAGA-3’
- **ACTIN2-FP**: 5’-AAAGGGTAAAAAGCTTGGGG-3’
- **ACTIN2-RP**: 5’-GGGAAAGAAGCAGAAACCA-3’
- **ChIP-ARR16-FP**: 5’-CATCATATAATCCACAGAGATAGA-3’
- **ChIP-ARR16-RP**: 5’-CAGACATTGCTTTAGTTCTC-3’
- **Non-box-ARR16-FP**: 5’-CAACACATCTGTTCACTTCC-3’
- **Non-box-ARR16-RP**: 5’-TGGACATTGCAACAGTTGAAC-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).
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, and leaf (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.,
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).
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...
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 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
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 hy5 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; 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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