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Posted Date: 17 June 2024

doi: 10.20944/preprints202406.1076.v1

Keywords: Arabidopsis thaliana; MYC2; MYC3; ethylene; hypocotyl; ERF1 doi:10.20944/preprints202406.1076.v1

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Arabidopsis thaliana MYC2 and MYC3 Are Involved in Ethylene-Regulated Hypocotyl Growth as Negative Regulators

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Abstract: The ethylene-regulated hypocotyl elongation of *Arabidopsis thaliana*'s involves many transcription factors. The specific role of MYC transcription factors in ethylene signal transduction is not completely understood. The results here revealed that two MYCs, MYC2 and MYC3, act as negative regulators in ethylene-suppressed hypocotyl elongation. Etiolated seedlings of the lost-of-function mutant of *MYC2* or *MYC3* were longer than wild type seedlings. Single- or double-null mutants of *MYC2* and *MYC3* displayed an enhanced response to ACC(1-aminocyclopropane-1-carboxylate), the ethylene precursor, compared to wild type seedlings. MYC2 and MYC3 directly bind to the promoter zone of *ERF1*, suppressing its expression. Additionally, EIN3, a key component in ethylene signaling, interacts with MYC2 or MYC3 and suppresses their binding to *ERF1*'s promoter. MYC2 and MYC3 play crucial roles in the ethylene-regulated expression of functional genes. The results revealed these transcription factors' novel role and functional mechanism in ethylene signal transduction.

Keywords: *Arabidopsis thaliana*; MYC2; MYC3; ethylene; hypocotyl; ERF1

1. Introduction

During seed germination, the elongation of the hypocotyl pushes the primary root out of the seed coat and allows it to absorb water and minerals from the soil. Hypocotyl elongation also helps in uncovering the cotyledons, which is essential for photomorphogenesis and the development of autotrophic seedlings [1,2]. The hypocotyl is sensitive to various internal and external signals [3], making it an ideal model for studying the regulation of plant growth and development [4,5].

When elongating hypocotyls encounter obstacles, ethylene levels increase to suppress the elongation rate and boost the jacking force, which is necessary for pushing seedlings out of the soil [6]. In ethylene-regulated growth and development, five ethylene receptors in the endoplasmic reticulum (ER) membrane, including ETR1, ERS1, ETR2, ERS2, and EIN4, are involved in responding to ethylene and triggering downstream signaling events [7–9]. When ethylene is absent, these receptors bind to CTR1 to form a complex. As a serine/threonine protein kinase, CTR1 phosphorylates EIN2. Since phosphorylated EIN2 is unable to trigger EIN3/EIL1, which act as transcription factors, the signaling cascade is blocked. When ethylene content increases, it binds and disassembles the receptor complex, releasing the inhibitory effect of CTR1 on EIN2. Activated EIN2 migrates from the ER membrane to the nucleus and stimulates EIN3/EIL1, which ultimately alters functional gene expression related to growth and development [9–11].

The growth of Arabidopsis hypocotyl is influenced by ethylene in relation to light exposure. Ethylene suppresses elongation of etiolated hypocotyls in the dark but promotes elongation in the light [3,12]. In darkness, hypocotyls of the ethylene-overproducing mutant *eto1-1* and the null mutant of CTR1 (*ctr1-1*) are shorter than the wild type, whereas hypocotyls of the ethylene-insensitive mutant *ein2-5* and *ein3eil1* are longer than the wild type [13,14]. In the light, hypocotyls of the EIN3-overexpression line and *ctr1-1* are longer than the wild type, whereas hypocotyls of *ein2-5* and *ein3eil1* are shorter than the wild type [12]. The expression of Ethylene Response Factor 1 (ERF1) is regulated by ethylene, and overexpression of *ERF1* significantly inhibits elongation of etiolated hypocotyls, thereby positively regulating ethylene-inhibited elongation [15,16].

MYC transcription factors belong to the plant bHLH transcription factor family. The first plant MYC gene was discovered in maize, and it is involved in regulating anthocyanin biosynthesis [17]. In Arabidopsis, AtMYC2 and its three homologues, AtMYC3, AtMYC4, and AtMYC5, have been extensively studied [18,19]. MYCs are involved in four physiological processes: (1) Plant growth and development. MYC2, MYC3, MYC4, and MYC5 inhibit cell proliferation of integument, thus negatively regulating seed enlargement [20]. MYC3 interacts with the DELLA protein, antagonizes the regulation of CO (CONSTANS) on *FT* (FLOWERING LOCUS T) gene expression, and participates in the flowering process which is regulated by photoperiod and gibberellin(GA) [21]. MYC2, MYC3, and MYC4 negatively regulate Jasmonic Acid(JA)-inhibited root and hypocotyl growth, by regulating the expression of *HY5* [22,23]. MYC2 promotes the expression of a senescence-related gene, *Dof2.1*, thus enhancing JA-induced leaf senescence [24]. (2) Plants' response to biotic stresses. In *Solanum lycopersicum*, magnesium oxide triggers the expression of resistance genes by stimulating JA signaling and activating MYC2, thus inducing the immune response to *Fusarium* wilt [25]. In rice, overexpression of *OsMYC2* induces up-regulation of pathogen resistance genes and enhances resistance to bacterial blight [26]. In Arabidopsis, AtPIFs and AtMYC2 form a homodimer that binds the promoter of the *TPS* (terpene synthase) gene. An increase in terpene biosynthesis enhances the defensive ability of whitefly (*Bemisia tabaci*) [27]. (3) Plants' response to abiotic stresses. MYC1 inhibits the binding of FIT (FER-like Iron Deficiency-induced transcription factor) with bHLH38/39 heterodimer, suppressing the expression of its target genes (*FRO2* and *IRT1*), thus inhibiting iron absorption and tolerance to iron deficiency [28]. MYC2 enhances Arabidopsis' tolerance to salinity by regulating proline biosynthesis [29]. The MYC2/MYC3/MYC4-dependent transcription factor network regulates water spray-responsive gene expression, as well as JA accumulation, responding to water spray stress caused by simulated rain [30]. (4) Regulating the synthesis of secondary metabolites. Some MYCs regulate gene expression of components in the biosynthesis of tryptophan, alkaloids, or flavonoids, promoting the accumulation of these secondary metabolites [26,31,32]. The accumulation of flavonoids inhibits the growth of insects and enhances the recovery ability of plant cells, increasing resistance to insect attacks [33].

The growth and development of plants are regulated by phytohormones, which also induce stress response by stimulating or suppressing the expression of functional genes using multiple transcription factors. In ethylene signaling, several agents can bind and regulate the activity of some bHLH transcription factor members. Phytochrome-interacting factors (PIFs), a group of well-characterized bHLH transcription factors, are involved in the cross-talk of ethylene- and photo-signaling [34]. Overexpression of *PIF5* increases ethylene levels in etiolated seedlings by stimulating the expression of ACC synthase [35,36]. Ethylene promotes hypocotyl elongation of green seedlings by increasing *PIF3* expression [12]. PIF1 and EIN3/EIL1 inhibit photobleaching by inhibiting the expression of protochlorophyllide biosynthetic genes and activating the expression of *POR* genes [37]. EIN3 and EIL1 participate in skotomorphogenesis by regulating the activity of PIF3 [16]. PIFs and EIN3/EIL1 are involved in apical hooks development, which is being regulated by phytohormones (e.g., ethylene, gibberellin, JA, salicylic acid, cytokinin) and light, by regulating the expression of *HLS140* [38–40]. MYCs are involved in antagonism of JA- and ethylene-signaling in Arabidopsis. MYC2/3/4 repress the transcriptional activity of EIN3/EIL1, thus suppressing ethylene-promoted resistance to necrotic fungi and apical hook formation [41], vice versa, EIN3/EIL1 inhibits the transcriptional activity of MYC2/3/4 to inhibit the expression of wound response genes (*TAT3*, *VSP1*, and *VSP2*) and herbivore-induced genes (*CYP79B3*, *BCAT4*, and *BAT5*) induced by JA [42].

Several MYCs have been studied for their physiological functions and functional mechanisms, but the specific role of MYC2 and MYC3 in ethylene signaling, is not fully understood. This study confirms the involvement of MYC2 and MYC3 in regulating hypocotyl growth in response to ethylene and explores their relationship with various ethylene signaling agents. The findings offer new insights into the role of MYC2 and MYC3 in the ethylene signal transduction pathway and shed light on the mechanism behind ethylene-regulated seedling growth.

2. Results

2.1. MYC2 and MYC3 Negatively Regulate the Ethylene-Inhibited Elongation of Etiolated Hypocotyls

To analyze the role of MYC3 and MYC3 in ethylene-inhibited etiolated hypocotyl elongation, the response of seedlings, including Col-0 and two MYC2 single null mutants (*myc2-1* and *myc2-2*) and a MYC3 null mutant (*myc3*), to ethylene precursor ACC was detected. The hypocotyl length of *myc2-1*, *myc2-2*, and *myc3* seedlings were 13.86±0.74, 14.00±1.07, and 13.84±1.09 mm, respectively, significantly longer than hypocotyl of Col-0 seedlings (11.83±0.74 mm) (p<0.05) (Figure 1A), indicating that MYC2 and MYC3 may play negative roles in elongation of etiolated hypocotyls. The etiolated hypocotyl of the double null mutant (*myc2myc3*) seedlings was longer than *myc2* and *myc3* seedlings (p<0.01), indicating that MYC2 and MYC3 may regulate hypocotyl elongation synergistically (Figure 1A,B).

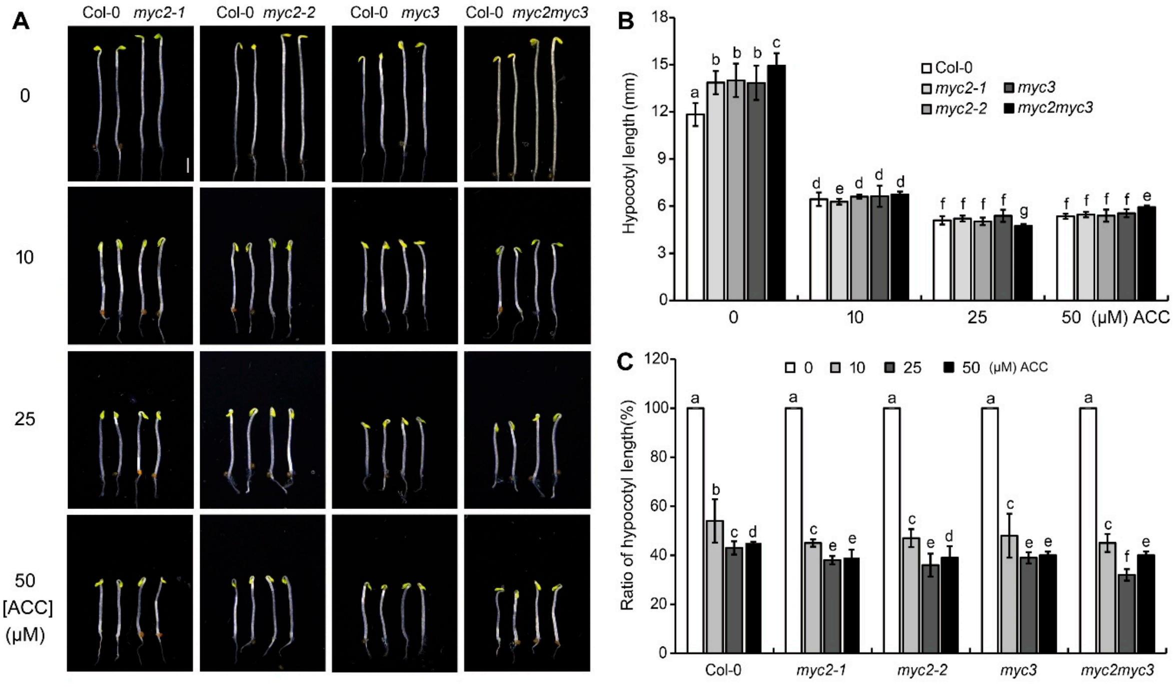

Figure 1. MYC2 and MYC3 negatively regulates ethylene-inhibited elongation of etiolated hypocotyls. (**A**). Photograph of etiolated seedlings which were grown on 1/2 MS medium containing serial concentration of ACC for 4 days. Scale bar =1 mm. (**B-C**)**.** Mean±SE value of hypocotyl length (**B**) and the ratio ((ACC treatment/control)*100%) of hypocotyl length (**C**). In each experiment, at least 40 seedlings were measured. Data from 3 replicates were calculated and statistically analyzed using one-way ANOVA.

On a medium containing ACC, the growth rate of etiolated hypocotyls significantly decreased. The effect of 25 μM ACC was stronger than that of 10 μM ACC, and the effect of 50 μM ACC was similar to that of 25 μM ACC. The length of etiolated hypocotyls of *myc2-1*, *myc2-2*, *myc3*, and *myc2myc3* seedlings was similar to that of Col-0 seedlings after treatment with 10, 25, or 50 μM ACC (*p*>0.05) (Figure 1A–C). To clarify the role of MYC2 or MYC3 in ethylene-inhibited etiolated hypocotyl elongation, the ratio of etiolated hypocotyl length under ACC to control was calculated. Results showed that the ratio of any null mutant was higher than that of Col-0, indicating that mutants respond to ACC more sensitively than the wild type (Figures 1 and S1).

To elucidate the role of MYCs in etiolated seedlings' responses to ethylene, growth parameters of seedlings that were cultured on ACC-containing medium were detected. 0.1 μM ACC significantly inhibited elongation of etiolated hypocotyls of *myc2-2*, *myc3*, and *myc2myc3*, while did not affect etiolated seedlings of *myc2-1* and Col-0. On 0.25 μM ACC containing medium, all *myc* mutants' seedlings were notably suppressed, while Col-0 seedlings were only slightly suppressed. Furthermore, on a medium containing 0.5 μM ACC, the elongation of hypocotyls in *myc2myc3* displayed increased sensitivity compared to Col-0 and the three single null mutants. Col-0 and all mutants' seedlings responded to 0.75 or 1 μM ACC in a similar manner and to a similar extent. The results indicated that the effectiveness of MYC2 and MYC3 in etiolated hypocotyls' response to ethylene depends on ethylene dosage (Figure S1).

To further verify the role of MYC2 and MYC3 in ethylene-regulated seedling growth, the response of etiolated seedlings of *MYC2* or *MYC3* complemention lines to ACC was detected. Results showed that seedlings of two *MYC2* complemention lines (*MYC2#COM1*, *MYC2#COM2*) and two *MYC3* complemention lines (*MYC3#COM1*, *MYC3#COM2*) responded to ACC in similar sensitivity with Col-0 seedlings (Figure S2).

It was reported that while ethylene suppresses the elongation of etiolated hypocotyls, it promotes the elongation of hypocotyls in green seedlings [3,12]. To further investigate the role of MYC2 and MYC3 in ethylene-regulated seedling growth, seedlings were cultured under light on ACC-containing medium. The results showed that the seedling growth rate was similar in Col-0 and *MYC2* or *MYC3* null mutants. In other words, the hypocotyls of Col-0, *myc2-1*, *myc2-2*, *myc3*, and *myc2myc3* seedlings were all significantly promoted by ACC (Figure S3), suggesting that MYC2 and MYC3 may not be involved in the ethylene-promoted elongation of hypocotyls in green seedlings.

To further investigate the role of MYC2 and MYC3 in ethylene-suppressed etiolated hypocotyl elongation, scanning electron microscopy (SEM) was used to detect etiolated hypocotyl cells. The epidermal cell count showed that the number of hypocotyl cells in both Col-0 and all mutant seedlings is similar, with each row containing 20-22 epidermal cells. Etiolated seedlings grown on ACC-free medium showed that the hypocotyl cells' length of *myc2-1*, *myc2-2*, and *myc3* were significantly longer than Col-0, but shorter than *myc2myc3*. On ACC-containing medium, the hypocotyl cells' length decreased significantly, and the cell length of *myc2-1*, *myc2-2*, and *myc3* was not significantly different from Col-0 (Figure 2). This suggests that mutants were more sensitive to ACC than the wild type, indicating that MYC2 and MYC3 may play a negative role in ethylene-regulated cell elongation in etiolated hypocotyls.

2.2. MYC2 and MYC3 Inhibit Expression of ERF1 by Binding Its Promoter

To verify the possible mechanism of MYC2 and MYC3-regulated hypocotyl growth, expression of *ERF1* in *myc2* or *myc3* was detected. In 4-days-old etiolated seedlings, expression of *ERF1* in *myc2*, *myc3* was markedly higher than in Col-0 (*p*<0.01), the expression level in *myc2-2* was the highest among the three mutants, which was 7-8 times of Col-0 (Figure 3A). The results indicate that *ERF1* expression is negatively regulated by MYC2 or MYC3.

MYC2 and MYC3 can bind G-box, a cis-acting element in the promoter of target genes, to regulate gene expression [23]. In the promoter of *ERF1*, there are 4 potential MYC2-binding sites, located at -1233, -1279, -1830, and -1921 upstream of the transcription start point; and 2 potential MYC3-binding sites, located at -1789 and -1921 upstream of transcription start point, respectively (Figure 3B). MYC2 or MYC3 may binds these elements and regulates *ERF1* expression.

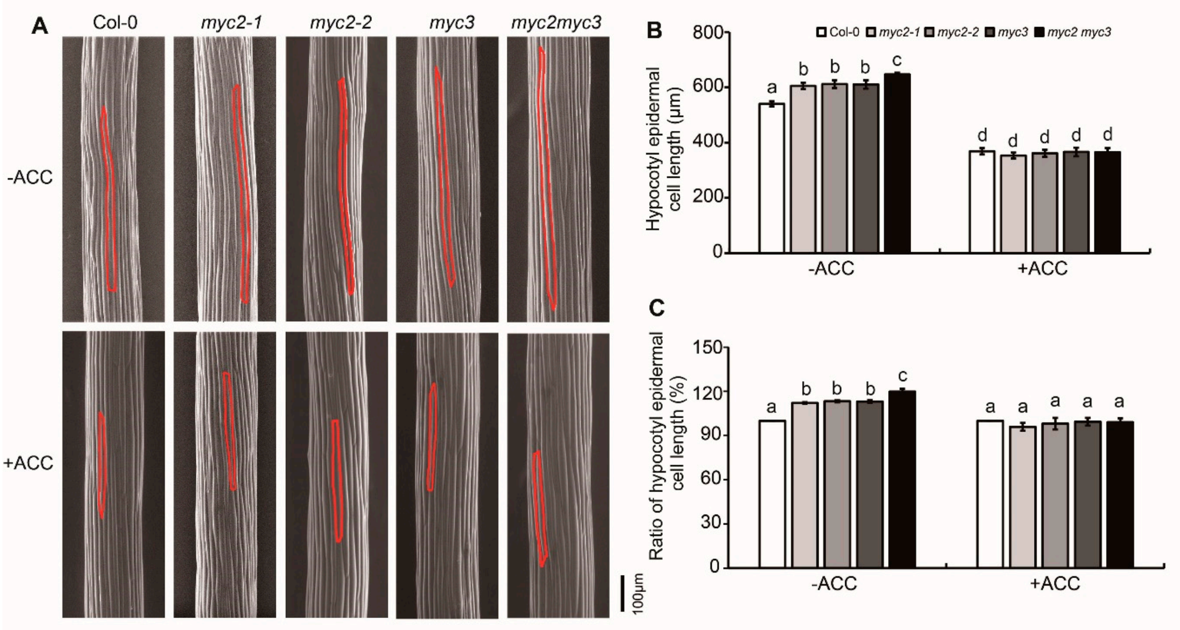

Figure 2. MYC2 and MYC3 negatively regulated ethylene-inhibited elongation of etiolated hypocotyl epidermal cell. (**A**). SEM photograph of epidermal cells of etiolated seedlings which were grown on MS medium containing 25 μM ACC for 4 days. (**B-C**). Mean±SE of hypocotyl epidermal cell length (**B**) and the ratio of hypocotyl epidermal cell length after/before ACC treatment (**C**). In each experiment, at least 10 seedlings were investigated. Data from 3 replicates were calculated and statistically analyzed using one-way ANOVA.

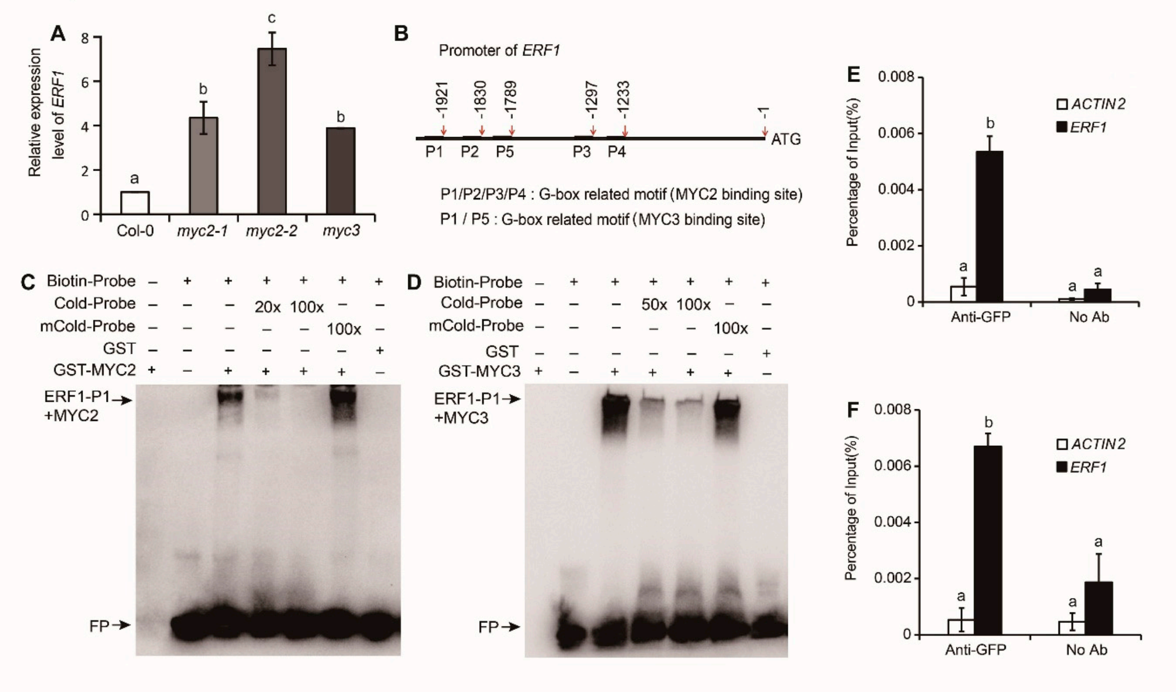

Figure 3. *ERF1* is a target of MYC2 and MYC3. (**A**). *ERF1* expression level in etiolated wild type and mutants' seedlings. (**B**). Five predicted MYCs-binding sites in the promoter region of *ERF1*. (**C-D**). EMSA results of the binding of MYC2 (**C**) or MYC3(**D**) with *ERF1* promoter. (**E-F**). ChIP-qPCR assay of MYC2 and MYC3 binding to *ERF1 in vivo*. In figure **A**, **E** and **F**, mean±SE of data from three replicates were calculated and statistically analyzed using one-way ANOVA.

To verify the binding of MYC2 or MYC3 with the *ERF1* promoter, GST-MYC2 and GST-MYC3 proteins were purified. Probe primers were designed based on the potential binding sequence in the *ERF1* promoter, and EMSA (electrophoretic mobility shift assay) was performed. The results showed that GST-MYC2 or GST-MYC3 bound to P1 (Figure 3C,D), while they did not bind to P2, P3, P4, or P5 of the *ERF1* promoter (Figure S4). The binding was abolished by adding an unlabeled P1 probe, but it was not affected by mutated probes.

To confirm the binding of MYC2 or MYC3 with the *ERF1* promoter, *MYC2* or *MYC3* was cloned to *pSuper::pCAMBIA1300GFP* binary vector and transformed into Col-0, DNA was extracted and purified from transformant seedlings for chromatin immunoprecipitation assay, which was performed by using GFP (green fluorescent protein) antibody. Results showed that *ERF1* promoter sequences in genomic DNA fragments were co-immunoprecipitated by MYC2 or MYC3 (Figure 3E,F), proving the *in vivo* binding of MYC2 or MYC3 with *ERF1* promoter.

To evaluate the effect of MYC2 or MYC3 on the expression of *ERF1*, the *ERF1* promoter-drived GUS(β-glucuronidase) reporter was transiently expressed. *pSuper::MYC2* and *pSuper::MYC3* were used as effectors. GUS vectors driven by *ERF1* promoter, including G-box or mutated G-box, were used as reporters (Figure 4A). Genes were transiently expressed in *Nicotiana benthamiana* leaves. Results showed that MYC2 or MYC3 inhibited *ERF1* expression through the G-box region in the *ERF1* promoter, since the inhibitory effect was not detected when G-box was mutated (Figure 4B,C).

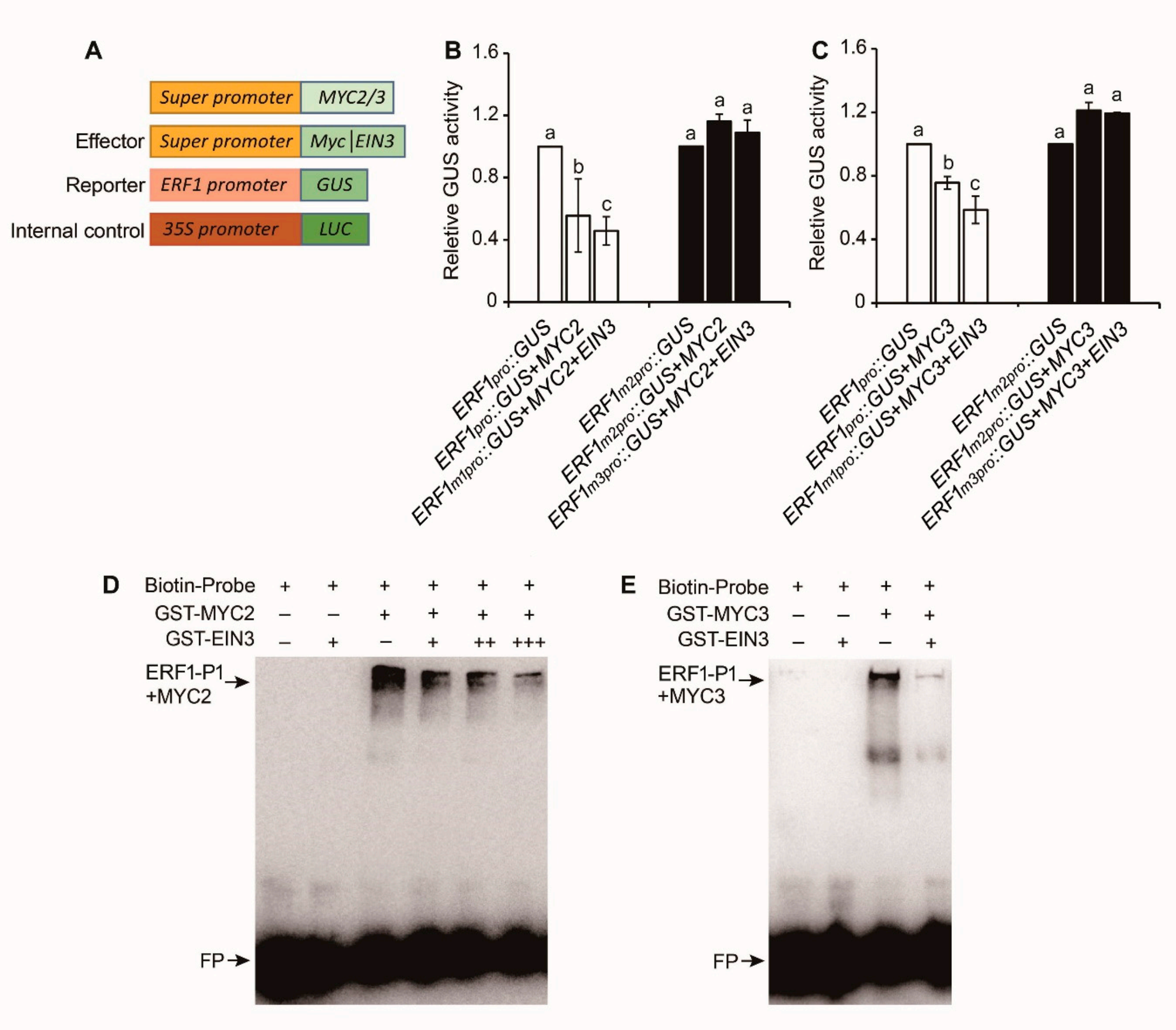

Figure 4. EIN3 suppressed the transcriptional activity of MYC2 or MYC3. (**A**). Schematic image of the reporter and effector used in transient transactivation assays. (**B-C**). Transient expression of *MYC2*, *MYC3*, *EIN3* and *ERF1*$_{pro}$*::GUS* or *ERF1*$_{mpro}$*::GUS* (*ERF1*$_{m1pro}$: EIN3 binding site was mutated; *ERF1*$_{m2pro}$: P1 was mutated; *ERF1*$_{m3pro}$: EIN3 binding site and P1 were both mutated) in *N. benthamiana* leaves.

ERF1$_{pro}$*::GUS* or *ERF1*$_{mpro}$*::GUS* expression level was set to 1. Mean±SE of data from three replicates were calculated and statistically analyzed using one-way ANOVA. (**D-E**). EIN3 blocked the binding of MYC2 or MYC3 with *ERF1* promoter. The binding of MYC2 (**D**) or MYC3 (**E**) with P1 was detected using EMSA.

2.3. EIN3 Suppresses the Binding of MYC2 or MYC3 with ERF1's Promoter

Interaction between some MYCs (e.g. MYC2, MYC3, MYC4) and EIN3 has been reported [41,42]. Yeast two hybridization result primarily showed that MYC2 and MYC3 did not interact with EIN2 (Figure S5). To investigate whether EIN3 can affect the binding and inhibitory effect of MYC2 or MYC3 on *ERF1* expression, fusion genes including *EIN3-MYC, MYC2-GFP*, *MYC3-GFP, ERF1pro::GUS,* and *ERF1mpro::GUS* were separately combined and co-expressed in *N. benthamiana* leaves. Results showed that the inhibitory effect of MYC2 or MYC3 on *ERF1* expression was further enhanced by EIN3 (Figure 4B,C).

It was reported that EIN3 can bind *ERF1*'s promoter [15]. To further explore the impact of EIN3 on the association between MYC2, MYC3, and *ERF1*'s promoter, the EIN3 binding site, MYC2 and MYC3 binding site (P1) in *ERF1*'s promoter were mutated for following EMSA. Results showed that when the EIN3 binding site was mutated, the expression of *ERF1* decreased, when the EIN3 binding site and P1 were both mutated, the expression of *ERF1* was not altered (Figure 4B,C), indicating that EIN3 inhibits the transcriptional activity of MYC2 or MYC3. EMSA results showed that, EIN3 inhibited the binding of MYC2 or MYC3 with *ERF1*'s promoter, along with the increasing of EIN3 dosage, the binding of MYC2 with P1 was gradually weakened (Figure 4D,E).

2.4. MYC2 and MYC3 Genetically Act Upstream of ERF1

To verify the role of ERF1 in ethylene-suppressed etiolated hypocotyl elongation, seedings' response of 4 *ERF1* null mutants (*erf1-1* and *erf1-2* were generated using CRISPR/Cas9, as showed in Figure 5A; *erf1-3* and *erf1-4* were generated using RNAi) and two *ERF1* overexpression lines (*ERF1-OE1* and *ERF1-OE2*) to ACC was detected. On ACC-free medium, hypocotyl length of the *ERF1* null mutants was similar to wild type, while hypocotyls of the *ERF1 OE* lines were significantly shorter than Col-0. On ACC-containing medium, the hypocotyl length of the null mutants' seedlings was similar to Col-0, while hypocotyls of the *ERF1 OE* lines were significantly shorter than Col-0 (Figure 5B,C). The ratio of hypocotyl length for seedlings grown on ACC/ACC-free medium showed that the ratio of *ERF1 OE* lines were significantly higher than Col-0, indicating overexpression of *ERF1* increased seedlings' sensitivity to ethylene (Figure 5D).

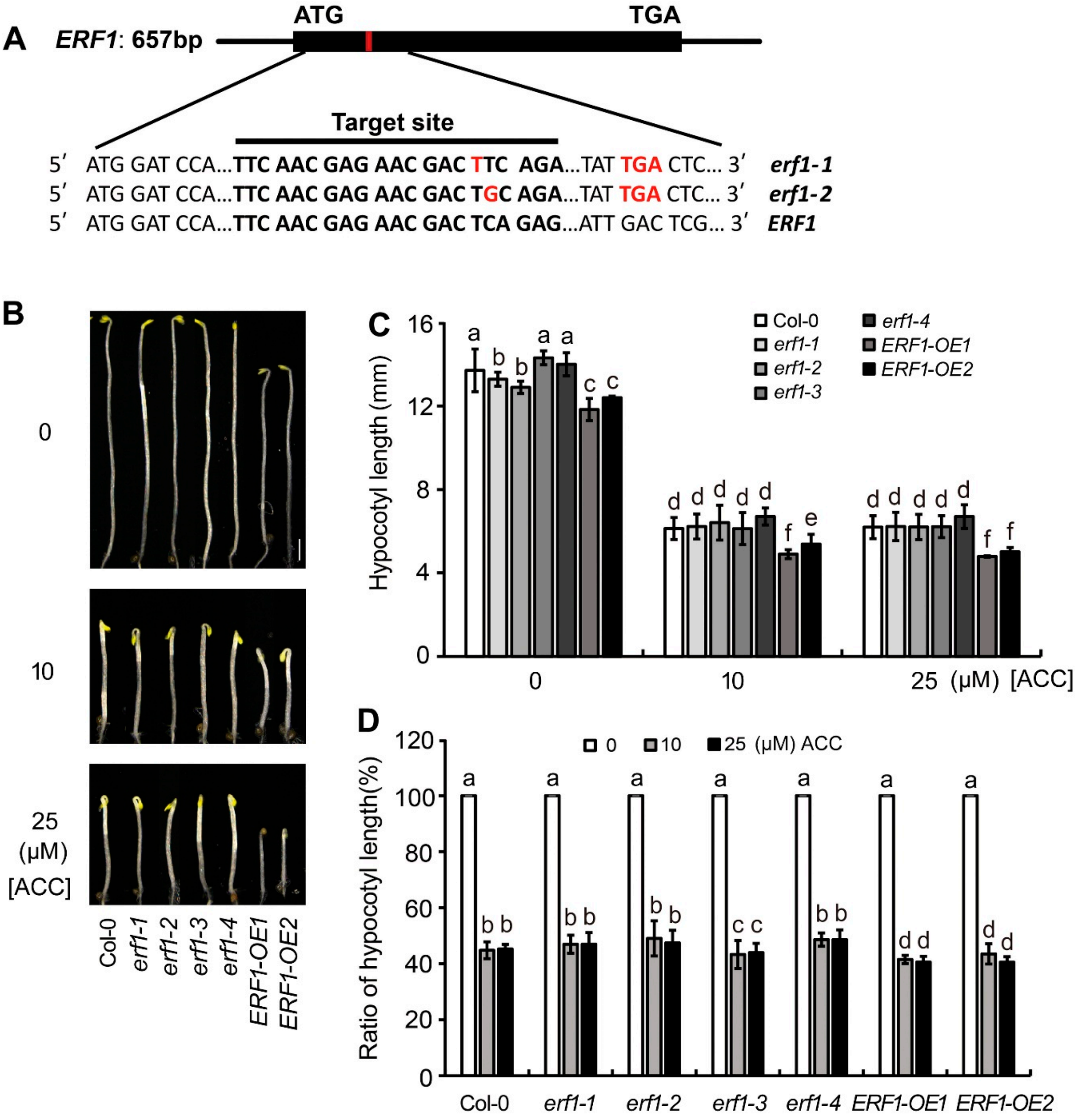

Figure 5. ERF1 is involved in ethylene regulated elongation of etiolated hypocotyl. (**A**). Schematic diagram of *ERF1* gene structure. The black boxes note the exons. Red frame note the point of a T base insertion. Sequence of gRNA target site of *ERF1* was noted below. (**B**). Overexpression of *ERF1* suppressed elongation of etiolated hypocotyls. Seedlings were grown on 1/2 MS medium in darkness for 4 days. (**C-D**). The effect of ACC treatment on elongation of etiolated hypocotyl of *ERF1* null mutants or OE lines. Hypocotyl length (**C**) and ratio of hypocotyl length (**D**) were showed, respectively. In each experiment, at least 40 seedlings were measured. Data from 3 replicates were calculated and statistically analyzed using one-way ANOVA.

To further investigate the genetic link of MYC2, MYC3, and ERF1, *erf1-3* and *ERF1-OE1* were hybridized with null mutants of *MYC2* or *MYC3*, materials with different genetic backgrounds were identified from their offsprings. The response of etiolated seedlings to ACC showed that the sensitivity of three double null mutants (*myc2-1erf1-3*, *myc2-2erf1-3* and *myc3erf1-3*) was similar to *erf1-3*. However, seedlings of *myc2-1ERF1-OE1*, *myc2-2ERF1-OE1*, and *myc3ERF1-OE1* showed sensitivity to ACC similar to *ERF1-OE1* (Figure 6). These results further demonstrate that MYC2 and MYC3 act upstream of ERF1.

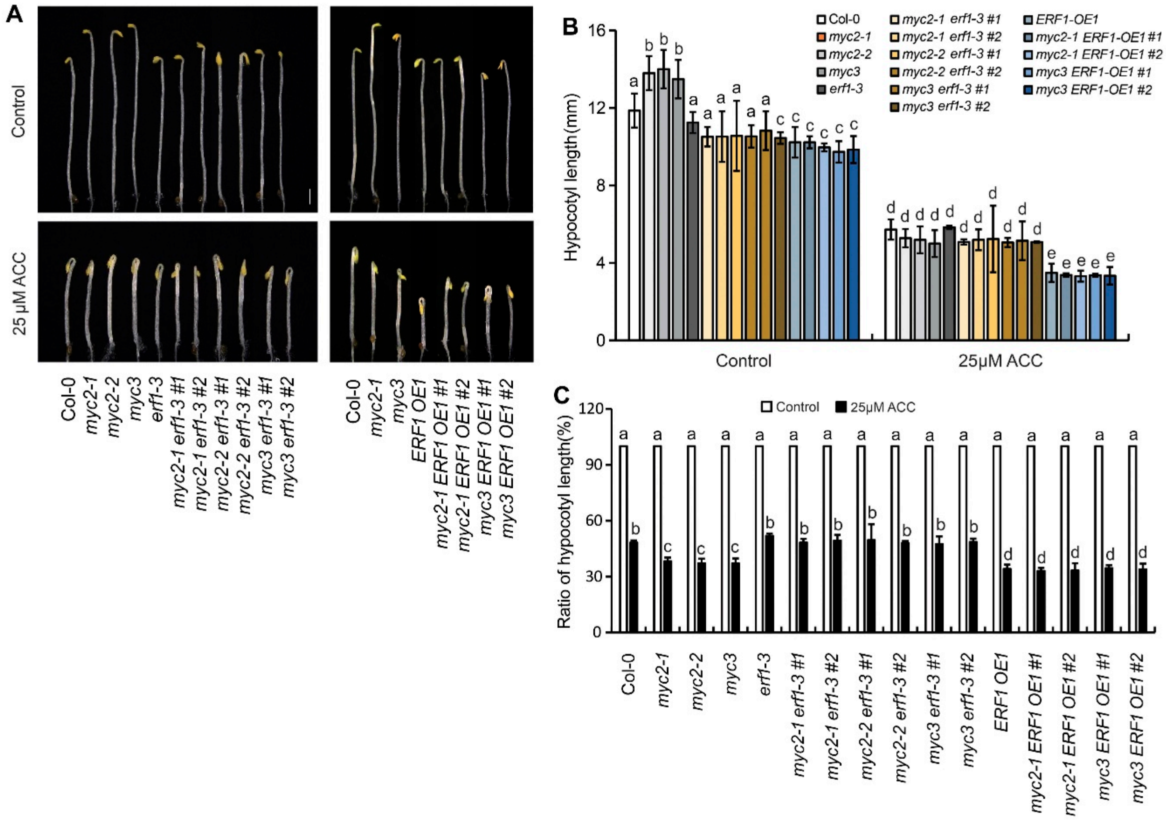

Figure 6. MYC2 and MYC3 function at upstream of *ERF1*. Null mutants of *MYC2* or *MYC3* were hybridized with *erf1-3* or *ERF1-OE1*. Effect of ACC on seedlings of hybrid progenies was detected. (**A**). Photograph of etiolated seedlings which were grown on ACC-containing medium for 4 days. In figure **B** and **C**, hypocotyl length (**B**) and ratio of hypocotyl length (**C**) after/before ACC treatment were showed, respectively. In each experiment, at least 40 seedlings were measured. Data from 3 replicates were calculated and statistically analyzed using one-way ANOVA.

2.5. MYC2 and MYC3 Are Involved in Ethylene-Regulated Gene Expression

To better understand the role of MYC2 and MYC3 in ethylene-regulated hypocotyl growth, high-throughput RNA-sequencing was used to analyze changes in gene expression pattern after ACC treatment in both Col-0 and *myc2myc3*. Following 25 μM ACC treatment, 1651 differentially expressed genes (DEGs) (fold change > 2) were identified in Col-0 seedlings, with 853 genes up-regulated and 798 genes down-regulated. In *myc2myc3* seedlings, 1559 DEGs were identified, with 636 genes up-regulated and 923 genes down-regulated (Figure 7A). Of these genes, 724 were differentially expressed both in Col-0 and *myc2myc3* seedlings (Figure 7B). Gene ontology (GO) analysis revealed that in Col-0 seedlings, DEGs were enriched in response to stress, plant hormone signaling, cell wall organization, and cellular metabolism; while in *myc2myc3* seedlings, DEGs were enriched in response to ethylene, water or salt stress, plant hormone signaling, and secondary metabolism (Figures 7C and S6).

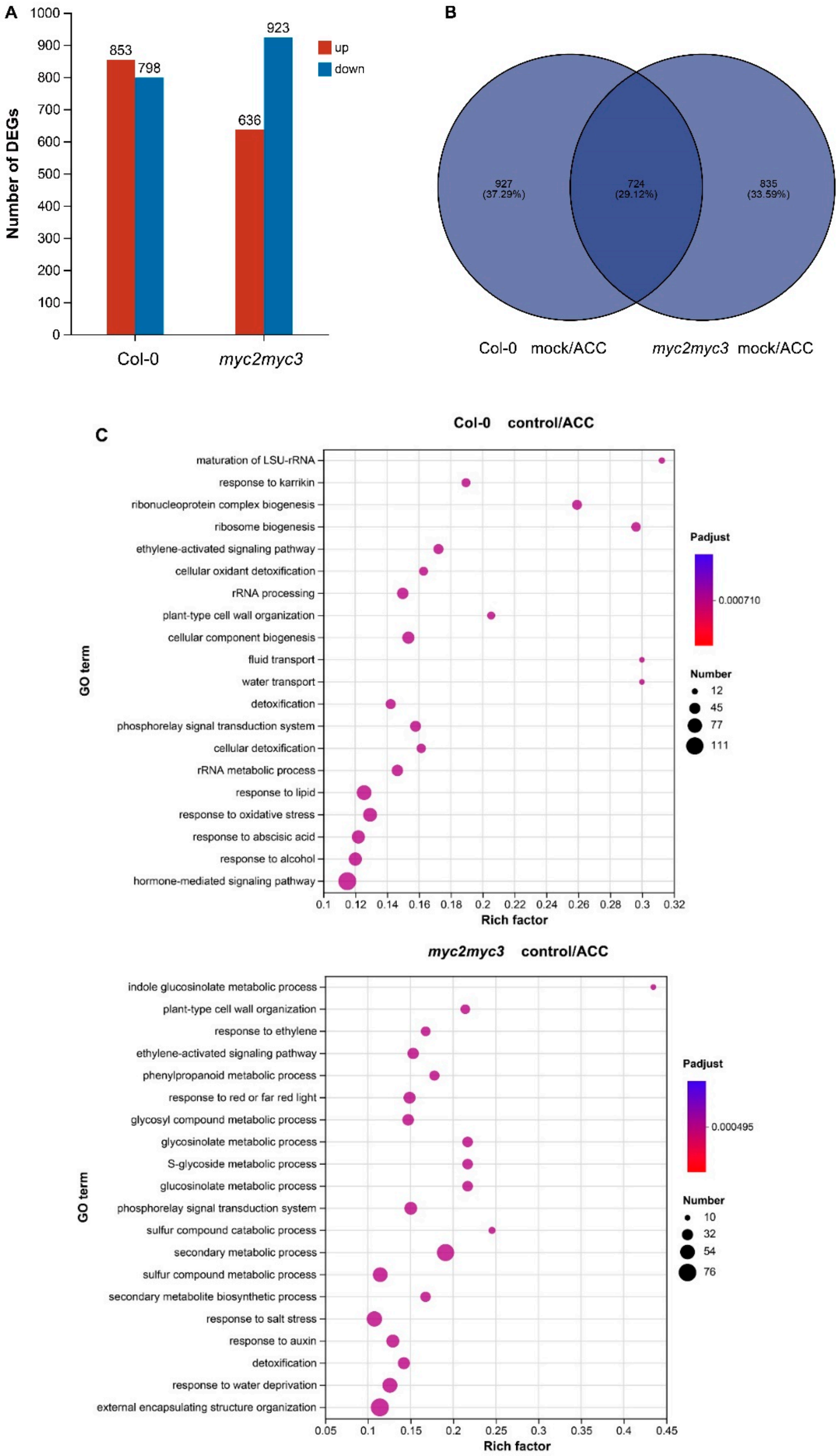

Figure 7. MYC2 and MYC3 are involved in ethylene-regulated expression of functional genes. Etiolated seedlings were grown on a medium containing 25 μM ACC for 4 days. High-throughput RNA-seq was used to analyze the expression pattern of functional genes. (**A**). Number of ethylene-regulated DEGs in Col-0 and *myc2myc3*. (**B**). Venn analysis of DEGs. (**C**). GO (gene ontology) analysis of DEGs.

To further verify the role of MYC2 and MYC3 in ethylene-regulated gene expression, DEGs with a fold change >5 in both Col-0 and *myc2myc3* were analyzed in detail. DEGs that were up-regulated in Col-0 while not up-regulated in *myc2myc3* are involved in cell wall organization, oxidation &

reduction, stress response, and gene expression regulation, etc. (Table 1) This suggests that MYC2 and MYC3 may have a positive role in the ethylene-regulated expression of these genes. DEGs that were down-regulated in Col-0 while not down-regulated in *myc2myc3* are involved in cellular metabolism, growth & development, signal transduction, oxidation & reduction, and gene expression regulation, etc. (Table 2) This suggests that MYC2 and MYC3 may play a negative role in the ethylene-regulated expression of these genes. Further analysis revealed that DEGs that were up-regulated in *myc2myc3* while not up-regulated in Col-0 are involved in cell wall organization, cellular metabolism, signal transduction, and gene expression regulation, etc. (Table S1) DEGs that were down-regulated in *myc2myc3* while not down-regulated in Col-0 are involved in cellular metabolism, hormone signaling, oxidation & reduction, stress response, and detoxification, etc. (Table S2)

Table 1. DEGs which were up-regulated in Col-0 while not up-regulated in *myc2myc3* after ACC treatment.

Functional category	Gene_id	Gene name	Gene description
cell wall organization	AT1G54970	PRP1	involved in cell wall structure formation, inducible by ethylene or auxin
	AT1G26250		proline-rich extensin-like protein
	AT1G02460		pectin lyase-like superfamily protein
	AT1G62980	EXPA18	Expansin-A18, involved in cell wall loosening
growth & development	AT5G49870	JAL48	involved in plant growth and development
	AT1G18630	RBG6	involved in regulation of growth & development
hormone metabolism or signaling	AT1G04180	YUC9	involved in auxin synthesis
	AT4G00880		SAUR-like auxin-responsive protein
	AT5G25350	EBF2	EIN3-binding F-box protein 2, involved in ethylene signaling
oxidation reduction	AT3G49960	PER35	Peroxidase 35
	AT1G69880	TRX8	Thioredoxin H8, involved in cell redox homeostasis
	AT5G25180	CYP71B14	Cytochrome P450 family protein
	AT4G20240	CYP71A27	Cytochrome P450 family protein
	AT4G26010		a peroxidase
signal transduction	AT4G04220	AtRLP46	Receptor-like protein 46
	AT3G23120	AtRLP38	Receptor-like protein 38
	AT1G03010		a NPH3 family protein, involved in response to light stimulus
stress response	AT2G05230		DNA-J heat shock protein
	AT1G53130	GRI	involved in extracellular ROS-induced cell death
	AT4G09435		involved in disease resistance
	AT4G38410		dehydrin family protein, involved in response to water stress
	AT5G14200	IMDH3	3-isopropylmalate dehydrogenase, involved in glucosinolate synthesis
transcription factor	AT5G57520	ZFP2	Zinc finger protein 2
	AT1G03790	SOM	Zinc finger CCCH domain-containing protein 2
	AT3G62090	PIF6	Myc-related bHLH transcription factor
	AT5G17430	BBM	AP2-domain containing protein
	AT3G60490	ERF035	Ethylene-responsive transcription factor 035

Table 2. DEGs which were down-regulated in Col-0 while not down-regulated in *myc2myc3* after ACC treatment.

Functional category	Gene_id	Gene name	Gene description
cell wall organization	AT3G50220	IRX15	involved in xylan synthesis and deposition
	AT3G55090	ABCG16	required for synthesis of cell wall

cellular metabolism	AT1G05675UGT74E1	UDP-Glycosyltransferase superfamily protein
	AT4G25835	AAA-ATPase, P-loop containing NTP hydrolases
	AT4G25150	Acid phosphatase-like protein
	AT1G06520GPAT1	Glycerol-3-phosphate acyltransferase 1
	AT1G66040ORTH4	E3 ubiquitin-protein ligase
	AT1G06030	fructokinase-2
	AT5G05270CHI3	chalcone--flavonone isomerase 3, involved in response to karrikin
	AT1G650604CL3	4-coumarate-CoA ligase 3, involved in phenylpropanoid synthesis
	AT1G72520LOX4	Lipoxygenase 4, involved in growth and defense response
	AT2G28210ATACA2	alpha carbonic anhydrase 2
growth & development	AT2G29950EFL1	ELF4-LIKE 1, involved in circadian and flowering
	AT3G18217MIR157C	MIR157C, involved in regulating vegetative phase
	AT5G51720NEET	involved in plant development
	AT1G43790TED6	involved in tracheary element differentiation
	AT2G46830CCA1	involved in regulating circadian rhythms
	AT1G65620AS2	involved in formation of a symmetric flat leaf lamina
	AT1G52690LEA7	Late embryogenesis abundant protein 7
hormone metabolism or signaling	AT2G14960GH3.1	IAA-amido synthetase
	AT2G16660	involved in response to karrikin
	AT5G66260	SAUR-like auxin-responsive protein
oxidation reduction	AT3G49110PER33	Peroxidase 33
	AT1G32780	Alcohol dehydrogenase-like 3
	AT3G44970	Cytochrome P450 family protein
	AT2G30540GRXS9	Monothiol glutaredoxin-S9
	AT5G25130CYP71B12	Cytochrome P450 family protein
signal transduction	AT3G15050IQD10	a calmodulin binding protein
	AT5G15430	a calmodulin-binding protein-like protein
	AT3G01830CML40	Calmodulin-like protein 40
	AT1G21550CML44	Calmodulin-like protein 44
	AT2G24850TAT3	a tyrosine aminotransferase that is responsive to jasmonic acid
	AT5G43300GDPD3	PLC-like phosphodiesterase
	AT3G45430LECRK15	L-type lectin-domain containing receptor kinase I.5
	AT3G45390LECRK12	L-type lectin-domain containing receptor kinase I.2
	AT5G55830LECRKS7	L-type lectin-domain containing receptor kinase S.7
stress response	AT1G12280SUMM2	a disease resistance protein, involved in defense response
	AT3G59930	Defensin-like protein 206
	AT2G03020	Heat shock protein HSP20
transcription factor	AT5G61620	myb-like transcription factor family protein
	AT1G73870COL7	zinc finger protein CONSTANS-LIKE 7
	AT1G62700NAC026	NAC (No apical meristem) domain transcriptional regulator
	AT5G64750ABR1	ethylene-responsive transcription factor
	AT1G22810ERF019	ethylene-responsive transcription factor

3. Discussion

MYCs are important transcription factors involved in JA, ABA, and GA signaling, as well as photoreceptor-mediated light signaling [43–45]. In 2020, Yi *et al.* reported that light induces JA synthesis, and the binding of JA to its receptor COI1 activates MYC2, MYC3, and MYC4. These transcription factors bind to the promoter of *HY5*, leading to suppressed hypocotyl elongation [23]. The role of MYCs in skotomorphogenesis has been rarely reported. We investigated the growth of etiolated seedlings of MYCs null mutants (including *myc2*, *myc3*, *myc4*, *myc5*) and found that the etiolated hypocotyls of *myc2* and *myc3* were significantly longer than those of wild type, indicating their novel role in regulating etiolated hypocotyl elongation.

As a typical ethylene-induced response, the "triple response" had been used to verify the involvement of ethylene in physiological processes. As a precursor of ethylene synthesis, ACC has been used in studies about the physiological function and mechanism of ethylene [11,46,47]. In this work, etiolated hypocotyls of *myc2* or *myc3* seedlings showed increased sensitivity to ACC, illustrating that MYC2 and MYC3 play negative roles in ethylene-inhibited etiolated hypocotyl elongation. In un-stimulated seedlings, endogenous growth substrates, including ethylene, finely modulate seedling growth, ensuring the steady growth of seedlings in darkness [3,4,48]. MYC2 and MYC3 may be downstream targets of ethylene signaling, acting as growth suppressors, and playing synergistic roles.

The signal transduction process of ethylene-induced triple response has been illustrated. Ethylene binds its receptors, releases their inhibitory effect on CTR1 and EIN2, and then regulates cell elongation through transcription factors EIN3/EIL1 and ERFs, which ultimately inhibits hypocotyl elongation [6]. Results here showed that ethylene inhibited cell elongation rather than proliferation since cell number was not altered after ACC treatment, consistent with reported data [49]. Following the illustration of the framework of ethylene signal transduction, more detailed mechanisms were revealed, e.g. EIN3 induced the transcription of *PIF3*, PIF3 regulated expression of a microtubule-related protein, MDP60, led microtubule re-organization, and enhanced hypocotyl growth [50]. Cross-talk between ethylene-CTR1 and Glc-TOR signaling pathway regulated the etiolated hypocotyl elongation by affecting multiple phosphorylation sites in EIN2 [51]. Moreover, ethylene regulates hypocotyl elongation by crosstalk with light signaling components, e.g. PhyB, PIF3, COP1, and HY5. Ethylene stimulates PIF3 to promote hypocotyl elongation under light, whereas photoactivated phyB may attenuate the promoting effect of ethylene. In darkness, ethylene inhibited hypocotyl elongation by stimulating ERF1 and WDL5. When seedlings grow up to the soil surface, increased light inhibit ethylene synthesis and eliminate the inhibitory effect of EIN3 on hypocotyl elongation [6]. Dozens of transcription factors are involved in ethylene signal transduction, e.g. PIFs, MYCs, HSFs, and CBFs, some of which are bHLH family members (PIF1/3/4/5, MYC2/3/4, RLS2/4, RHD6, etc.) [52]. Nevertheless, so far, the position and role of MYC2 or MYC3 in the ethylene signaling pathway have not been elucidated. Results here are of good help for understanding the role of the two transcription factors in ethylene signaling, as well as their role in the regulation of growth and development.

To verify the role of the two MYCs in ethylene signaling, the relationship of MYC2 or MYC3 with ERF1 and EIN3 was intensively investigated. The interaction between EIN3 and MYC2, MYC3, and MYC4, have been reported; as well as EIN3 binds and regulates transcription of *ERF1* [15,53]. However, the relation between MYC2 or MYC3 and ERF1 was not revealed so far. As a crucial component in ethylene signaling, EIN3 can be stimulated by EIN2, and then stimulates multiple downstream signaling agents, including the apical curvature regulator *HLS1* [38], the root hair elongation factor *RSL4* [54], the senescence-related genes *ORE1*, *SAG29* [55], *ESE1* [56], cold stress-related genes *CBF1/2/3* [57], and pathogenicity-related genes *ERF1* and *ORA59* [42], which in turn regulate growth of vegetative organs (e.g. apical hook, root hair, and leaves), as well as response to (a)biotic stresses [58]. The results in this work suggest that MYC2 and MYC3 are closely associated with EIN3, and involved in the ethylene-regulated hypocotyl growth, providing new evidence to clarify the mechanism of EIN3-involved regulation of plant growth.

AP2/ERF are essential transcription factors, which are involved in multiple physiological functions’ regulation [59–61]. ERF1 is involved in plant growth, development, and stress responses, which were modulated by various phytohormones, e.g. ethylene, JA, auxin, and ABA(Abscisic Acid) [42,62–65]. Expression of *ERF1* is regulated by EIN3/EIL1, ABI3, or NIGT1.4, at the transcriptional level [65–67]. After translation, UBC18, MPK3/MPK6 may regulate ERF1 activity by protein modification, e.g. ubiquitination and phosphorylation. The abundance and activity of ERF1 directly affect cell metabolism, proliferation, and differentiation [68,69]. The regulatory mechanism of *ERF1* expression was also intensively explored [64,65,70]. In this work, *ERF1* expression was significantly increased in *MYC2* and *MYC3* null mutants, indicating that MYC2 and MYC3 may be involved in regulating *ERF1* expression. Subsequently, EMSA and ChIP assays showed that MYC2 and MYC3 directly bind the promoter of *ERF1* and inhibit its expression. The data indicated that MYCs may act at upstream of ERF1, providing novel clues for understanding the regulation mechanism of *ERF1* expression. An assay of the relationship among EIN3, MYCs, and ERF1 showed that EIN3 suppressed the effect of MYC2 or MYC3 on *ERF1* expression. MYC2 and MYC3 have been revealed to antagonize the effect of EIN3 on the expression of *HLS1* [41]. Here, it is verfied that EIN3 inhibits the binding of MYC2 or MYC3 with *ERF1* promoter, clarifying the inhibitory mechanism of EIN3 on MYCs-regulated *ERF1* expression.

The analysis of DEGs after ACC treatment indicated that MYC2 and MYC3 may be essential components in ethylene-regulated gene expression. The complicated data showed that MYC2 and MYC3 may play positive roles in some genes' expression, while playing negative roles in other genes' expression. The data suggests that MYC2 and MYC3 affect the expression of multiple sets of functional genes that are involved in cellular metabolism, cell wall organization, growth and development, and stress responses. These effects may ultimately impact the elongation of hypocotyls. In the double-null mutant, impaired regulation of gene expression by MYC2 and MYC3 resulted in altered gene expression pattern and the enhanced sensitivity to ethylene. The mechanism needs to be intensively investigated in the future.

The data in the work suggest that EIN3 may be located upstream of the two MYCs and inhibit their transcription activity. MYC2 and MYC3 inhibit *ERF1* expression. When *MYC2* and *MYC3* were mutated, *ERF1* gene expression may be enhanced and the hypocotyls' ethylene responsiveness is markedly increased (Figure 8).

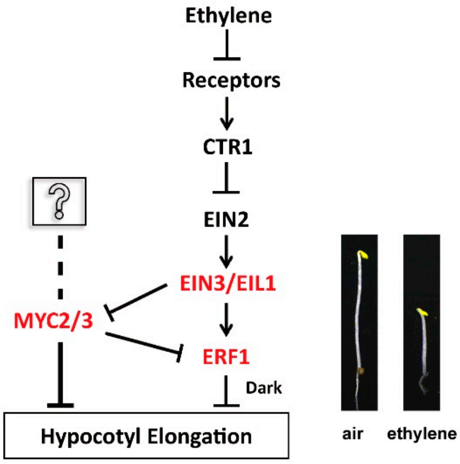

Figure 8. Model of the function of MYC2 or MYC3 in ethylene-inhibited elongation of etiolated hypocotyls. Arrows and bars indicate positive and negative regulation, respectively.

4. Materials and Methods

4.1. Plant Materials

All Arabidopsis materials were derived from ecotype Col-0 background. The mutants, including *myc2-1* (SALK_017005), *myc2-2* (SALK_061267), and *myc3* (SALK_01 2763), were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). The double null mutant *myc2myc3* was obtained by crossing *myc2-1* and *myc3*. The *ERF1* knock-out mutants, *erf1-1 and erf1-2*, were obtained by CRISPR/Cas9 mutation in Col-0. Two *ERF1* knock-out mutants, *erf1-3* and *erf1-4*, were obtained by *RNAi.*

doi:10.20944/preprints202406.1076.v1

Seeds were surface sterilized with 70% ethanol, after being washed with sterilized water 3 times, seeds were sown on a solid medium consisting of 1/2 MS salt, 1% (w/v) agar, and 3% (w/v) sucrose, and then cultured under light (22°C, 16/8h light/dark, 130 μmol·m^{-2}·s^{-1}). For etiolated hypocotyl growth measurement, culture dishes were stored at 22°C under light for 6 h, and then coated with foil and cultured at 22 °C for 4 days.

After being cultured for some time, seedlings were photographed using a scanner (Perfection V39II, Epson), images were analyzed by using Image J software to measure hypocotyl length. Statistical analysis of the data was performed using IBM SPSS Statistics 22.0 software (IBM, New York, NY, USA).

4.2. Gene Transformation

The *pSuper::MYC2* and *pSuper::MYC3* plasmids were introduced into *Agrobacterium tumefaciens* (strain GV3101) using the freeze-thaw method. The fused genes were then transformed into wild type (Col-0) plants through floral dipping. Transgenic plants were screened using 1/2 MS medium containing 100 mg·mL^{-1} kanamycin.

4.3. PCR Analysis

Total RNA was isolated from seedlings for quantitative real-time PCR analysis, which were performed using CFX96™ real-time PCR system (Bio-Rad, Hercules, CA, USA). Primers used for subsequent detection of *ERF1* expression were 5′-ACACGTGCGGGATATCAAATA-3′ and 5′-ACACGTGCGGGATATCAAATA-3′. *ACTIN2* was used as a reference (5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′). Three biological replicates and three technical replicates were used each time. Mean±SE was calculated and statistically analyzed.

4.4. Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed according to the reported method [49]. The recombinant GST-MYC2 and GST-MYC3 protein was expressed in *E. coli* and then extracted and purified. EMSA was conducted using the Light Shift Chemiluminescent EMSA Kit (Thermo Scientific, Rockford, IL, USA) with biotin-labeled and cold probes.

Nucleotide sequences of the double-stranded oligonucleotides were as follows:***ERF1* P1**: 5′-AAAACTTTGAACACGTGCGGGATATCAA-3′ and 5′-TTGATATCCCGCACGTGTTCAAAGTTTT-3′. ***ERF1* P2**: 5′-GAAAAAATGGCACATGAAGTATCTTT-3′ and 5′-AAAGATACTTCATGTGCCATTTTTTC-3′. ***ERF1* P3**: 5′-ACTCAGGATGCATGTGATGATGTGTA-3′ and 5′-TACACATCATCACATGCATCCTGAGT-3′. ***ERF1* P4**: 5′-TTATCTTCTAAACATGAGATGGCTCG-3′ and 5′-CGAGCCATCTCATGTTTAGAAGATAA-3′. ***ERF1* P5**: 5′-TTAGTTGCGTATCCGTTCGAATAATT-3′ and 5′-AATTATTCGAACGGATACGCAACTAA-3′.

4.5. Chromatin Immunoprecipitation (ChIP)

10-days-old seedlings of *pSuper::MYC2-GFP* or *pSuper::MYC3-GFP* transformant were fixed in 1% formaldehyde, and then were homogenized and sonicated. The sheared chromatins were immunoprecipitated, washed, reverse cross-linked, and amplified. The monoclonal anti-GFP antibody was used for immunoprecipitation. Approximately 10% of sonicated but non-immunoprecipitated chromatin was reverse cross-linked and used as an input DNA control. The immunoprecipitated DNA and input DNA were analyzed by using real-time q-PCR. Primer used to detect the MYC2 and MYC3 target *ERF1* promoter was 5′-ACACGTGCGGGATATCAAATA-3′ and 5′-CATGCATCCTGAGTCC AGTAG-3′, *ACTIN2* as a control (5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′).

4.6. Gene Transient Expression in *N. benthamiana*

ERF1$_{pro}$*::GUS* or *ERF1*$_{mpro}$*::GUS* with pCAMBIA1391, *pSuper::MYC2*, *pSuper::MYC3* or *pSuper::EIN3* with pCAMBIA1300 constructs were transformed into *A. tumefaciens* (strain GV3101). Cultured *A. tumefaciens* cells were harvested by centrifugation and suspended and diluted with a solution consisting of 10 mM MES, 10 mM $MgCl_2$, and 200 mM acetosyringone, pH 5.6, to an optical density (600 nm, OD=0.7). Cells were injected into *N. benthamiana* leaves by using a needle-free syringe. GUS activity of the infiltrated leaves was quantitatively assessed.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1. The effect of serial concentration of ACC on elongation of etiolated hypocotyls of wild type and null mutants of *MYC2* or *MYC3*. Figure S2. The effect of serial concentration of ACC on elongation of etiolated hypocotyls of wild type and the complementation lines of *MYC2* or *MYC3*. Figure S3. MYC2 or MYC3 is not involved in ethylene-promoted hypocotyl elongation of seedlings grown under light. Figure S4. MYC2 or MYC3 did not bind P2-P5 of *ERF1* promoter. Figure S5. MYC2 or MYC3 did not interact with EIN2. Figure S6. KEGG analysis of ethylene-regulated DEGs in Col-0 and *myc2myc3*. Table S1. DEGs that were up-regulated in *myc2myc3* while not up-regulated in Col-0 after ACC treatment. Table S2. DEGs that were down-regulated in *myc2myc3* while not down-regulated in Col-0 after ACC treatment.

Author Contributions: E.K. and Z.S designed the experiment and finished writing the article. Y.L.(Yuke Li), Y.C., F.W., Y.L.(Yingxiao Liu), and R.Z. performed research. C.X., P.Z., and J.Z. provided partial material.

Funding: This work was supported by the National Natural Science Foundation of China (Grant No. 31800233, 31871409), the Science Research Project of Hebei Education Department (BJK2023093), the Advanced Talents Foundation of Hebei Education Department (Grant No. GCC2014037), the Graduate Innovation Research Project of Hebei Normal University (Grant No. XCXZZBS202309), the Provincial Fundamental Research Funds for Universities (JYZ202007), the Outstanding Youth Fund of Hebei University of Traditional Chinese Medicine (YQ2020002).

Data Availability Statement: Data available on request from the authors.

Acknowledgments: We thank all the colleagues who helped with the development of the different parts of this manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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