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Jasmonic acid promotes degreening via MYC2/3/4- and ANAC019/055/072-mediated regulation of major chlorophyll catabolic genes

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SUMMARY

Degreening caused by rapid chlorophyll (Chl) degradation is a characteristic event during green organ senescence or maturation. Pheophorbide *a* oxygenase gene (*PAO*) encodes a key enzyme of Chl degradation, yet its transcriptional regulation remains largely unknown. Using yeast one-hybrid screening, coupled with *in vitro* and *in vivo* assays, we revealed that Arabidopsis MYC2/3/4 basic helix-loop-helix proteins directly bind to *PAO* promoter. Overexpression of the *MYC*s significantly enhanced the transcriptional activity of *PAO* promoter in Arabidopsis protoplasts, and methyl jasmonate (MeJA) treatment greatly induced *PAO* expression in wild-type Arabidopsis plants, but the induction was abolished in *myc2 myc3 myc4*. In addition, MYC2/3/4 proteins could promote the expression of another Chl catabolic enzyme gene, *NYC1*, as well as a key regulatory gene of Chl degradation, *NYE1/SGR1*, by directly binding to their promoters. More importantly, the *myc2 myc3 myc4* triple mutant showed a severe stay-green phenotype, whereas the lines overexpressing the *MYC*s showed accelerated leaf yellowing upon MeJA treatment. These results suggest that MYC2/3/4 proteins may mediate jasmonic acid (JA)-induced Chl degradation by directly activating these Chl catabolic genes (*CCG*s). Three NAC family proteins, ANAC019/055/072, downstream from MYC2/3/4 proteins, could also directly promote the expression of a similar set of *CCG*s (*NYE1*/*SGR1*, *NYE2*/*SGR2* and *NYC1*) during Chl degradation. In particular, *anac019 anac055 anac072* triple mutant displayed a severe stay-green phenotype after MeJA treatment. Finally, we revealed that MYC2 and ANAC019 may interact with each other and synergistically enhance *NYE1* expression. Together, our study reveals a hierarchical and coordinated regulatory network of JA-induced Chl degradation.

Keywords: Arabidopsis, chlorophyll degradation, jasmonic acid signaling, MYC2/3/4, ANAC019/055/072.

INTRODUCTION

Rapid chlorophyll (Chl) degradation is one of the earliest events initiated in the chloroplast during green organ senescence (Thomson and Plat-Aloia, 1987; Noodén, 1988), and the resulting degreening phenotype is often considered the visual marker of senescence and maturation. Over the last decade or so, the biochemical pathway of Chl degradation has been largely revealed in Arabidopsis by the identification of Chl catabolic genes (*CCG*s), and has more or less been validated in other species (Christ and Hörtensteiner, 2014; Liang *et al.*, 2014). According to our current understanding, Chl *a* is the degradable form of Chl; Chl *b*, before being degraded, needs to be reduced to Chl *a* via a two-step reduction, catalyzed by Chl *b* reductase (CBR, encoded by *NYC1* and *NOL*) and hydroxymethyl Chl *a* reductase (HCAR) (Kusaba *et al.*, 2007; Horie *et al.*, 2009; Sato *et al.*, 2009; Meguro *et al.*, 2011). The removal of chelated magnesium is deduced as the initial step of Chl *a* breakdown, whereas cleavage of the phytol group was validated as the subsequent step via a hydrolytic reaction catalyzed by pheophytin pheophorbide hydrolase (pheophytinase, PPH) (Morita *et al.*, 2009; Schelbert *et al.*, 2009; Ren *et al.*, 2010). The porphyrin macrocycle of the resulting pheophorbide *a* (Pheide *a*) is then oxygenolytically opened by pheophorbide *a* oxygenase (PAO) to

generate a red Chl catabolite (RCC) (Pružinská *et al.*, 2003, 2005; Yang *et al.*, 2004). The RCC, catalyzed by red Chl catabolite reductase (RCCR), is subsequently reduced to a primary fluorescent Chl catabolite (*p*FCC) (Wüthrich *et al.*, 2000; Mach *et al.*, 2001; Pružinská *et al.*, 2007). The macrocycle-opening event leads to the loss of the green color of the Chl catabolite. Non-yellowing 1 (NYE1, also known as SGR1) is a general regulator of Chl degradation and is essential for recruiting Chl catabolic enzymes (CCEs) into a possible multiprotein complex in senescing chloroplasts (Sakuraba *et al.*, 2012). Although the major *CCG*s (*NYC1*, *NOL*, *HCAR*, *PPH*, *PAO*, *RCCR* and *NYE*s/*SGR*s) have been identified in Arabidopsis, their transcriptional regulation has not been thoroughly explored.

A number of phytohormones can initiate and/or promote degreening and induce senescence in general (Lim *et al.*, 2007). Nevertheless, the underlying mechanism has remained untouched until recently. ABSCISIC ACID INSENSITIVE 3 (ABI3), one of the major abscisic acid (ABA) signaling components, was reported to directly regulate the expression of both *NYE1* and *NYE2* during seed maturation (Delmas *et al.*, 2013); ABI5 and ENHANCED EM LEVEL (EEL), two other components of ABA signaling, could directly bind to the promoters of *NYE1* and *NYC1*, and regulate their expression downstream of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF5 (Sakuraba *et al.*, 2014). OsNAP, a newly identified ABA-inducible transcription factor (TF) in rice (*Oryza sativa*), links ABA signaling and Chl degradation by directly regulating the expression of some *CCG*s (*NYE1*, *NYC1*, *NYC3* and *RCCR1*) (Liang *et al.*, 2014).

Jasmonic acid (JA) induces senescence in various plant species (Ueda and Kato, 1980; Parthier, 1990; He *et al.*, 2002; Reinbothe *et al.*, 2009; Shan *et al.*, 2011; Yan *et al.*, 2012; Lee *et al.*, 2015), and was therefore considered an inducer of senescence. During leaf senescence in Arabidopsis, JA levels were four-fold higher in senescing than in non-senescing leaves, and JA biosynthesis-related genes were differentially activated (He *et al.*, 2002). Schommer *et al.* (2008) found that miR319-controlled TCP4 positively regulates leaf senescence by directly activating the JA biosynthetic gene *LIPOXYGENASE 2* (*LOX2*). The exogenous application of JA promotes senescence (He *et al.*, 2002). Shan *et al.* (2011) showed that Rubisco activase (RCA) plays an important role in JA-induced leaf senescence. Various senescence-associated genes (*SAG*s) were induced by JA (Buchanan-Wollaston *et al.*, 2005; Jung *et al.*, 2007). For example, the expression of *SENESCENCE 4* (*SEN4*) and *SAG12* was increased by JA treatment in wild-type plants, but was severely reduced in the *coi1-2* mutant (Xiao *et al.*, 2004). Some WRKY TFs were found to be involved in JA-induced senescence. WRKY70 and WRKY53 function as the node of JA- and SA-mediated signals, and act as regulators of senescence (Li *et al.*, 2004; Miao and Zentgraf, 2007; Ülker *et al.*, 2007; Xie *et al.*, 2014). Jiang *et al.* (2014) confirmed that WRKY57, a repressor of *SEN4* and *SAG12*, functions as a node of convergence for JA- and auxin-mediated signaling pathways during JA-induced leaf senescence. Although JA was found to promote leaf degreening nearly three decades ago (Ueda and Kato, 1980), how JA signaling regulates Chl degradation at the molecular level has yet to be revealed.

In this study, we initially identified MYC2/3/4 proteins, the key JA signaling components, as direct transcriptional regulators of *PAO*. Then we found that MYC2/3/4 proteins can also directly regulate the expression of *NYE1* and *NYC1* by binding to their promoters. Three newly identified components of JA signaling downstream of the MYCs, ANAC019/055/072 (Bu *et al.*, 2008; Zheng *et al.*, 2012), could directly regulate the expression of a similar set of *CCG*s. We further revealed an interaction between MYC2 and ANAC019, and their synergistic effect on *NYE1* expression. Collectively, our study reveals a hierarchical and coordinated regulatory network of JA-induced Chl degradation during leaf senescence.

RESULTS

Identification of MYC2/3/4 proteins as putative trans-regulators of *PAO*

Pheophorbide *a* oxygenase gene (*PAO*) encodes one of the key enzymes for catalyzing degreening. To explore its transcriptional regulation, we aimed to identify its putative transcriptional regulators by yeast one-hybrid (Y1H) screening. To obtain a suitable bait fragment, we dissected the 1.2-kb fragment upstream of its start codon and fused four 5′-truncated fragments to the *GUS* gene. After obtaining transgenic plants harboring the fused constructs, we observed that one fragment, between the start codon of *PAO* gene and the stop codon of the upstream gene, as short as 252 bp, responded to dark treatment, a conventional way of inducing synchronized senescence of leaves (Figure S1a). We then amplified a 2.6-kb *PAO* genomic fragment, harboring the 252-bp promoter region, and transferred it into the *acd1-20* mutant, which is impaired in PAO function and displays stay-green and photosensitive phenotypes during leaf senescence (Greenberg and Ausubel, 1993; Yang *et al.*, 2004). The phenotype of *acd1-20* was largely restored in homozygous transgenic plants, as indicated by enhanced yellowing (Figures 1a and S1b,c). Consistently, we found no Pheide *a* accumulation in the senescing leaves of completely complemented transgenic plants, as compared with wild-type (WT) plants, in contrast to a relatively high level of Pheide *a* in the leaves of *acd1-20* plants (Figure S1d). These results suggest that the 252-bp fragment is the functional *PAO* promoter.

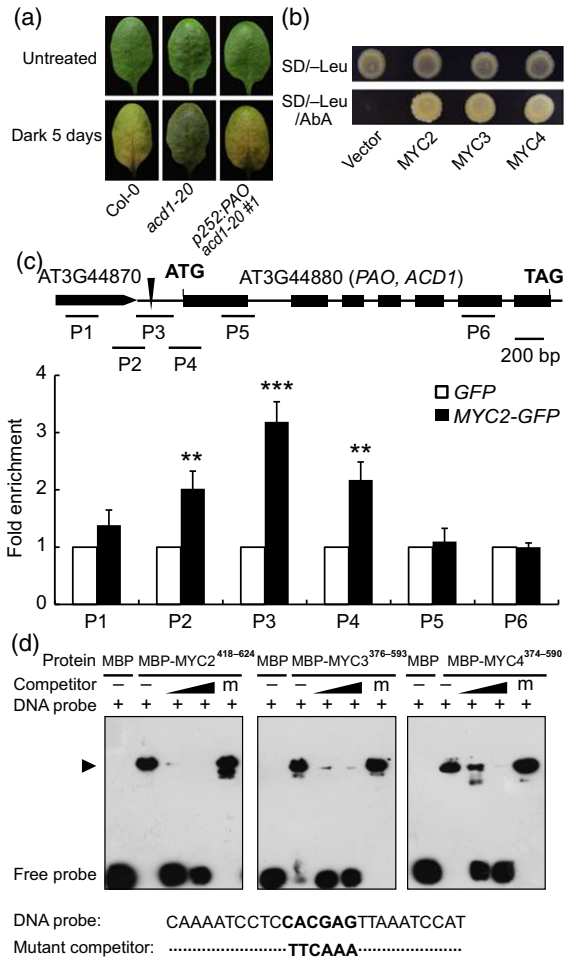

Figure 1. MYC2/3/4 proteins bind to the pheophorbide *a* oxygenase gene (*PAO*) promoter *in vitro* and *in vivo*.

(a) Rescue of the stay-green phenotype of *acd1-20* by the expression of *p252:PAO*. The fifth and sixth detached leaves of 4-week-old plants were incubated in darkness for 5 days. *p252*, 252-bp *PAO* promoter fragment.

(b) Interaction of MYC2/3/4 proteins with the *PAO* promoter in the yeast one-hybrid (Y1H) assay. The transformed yeast cells containing both bait (*p252:AUR-1C*) and prey (*MYC2/3/4-AD*) constructs were plated on selective medium (SD/−Leu/AbA). Vector, empty vector control; AbA, Aureobasidin A.

(c) Association of MYC2 protein with the *PAO* promoter *in planta*. Upper panel, schematic diagram of the *PAO* gene showing the positions of the G-box element (black triangle) and the six ChIP amplicons (P1–P6). Lower panel, chromatins isolated from *35S:MYC2-GFP* and *35S:GFP* transgenic plants were used for the qPCR assay. Data are means ± SDs of two independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-tests).

(d) Binding of MYC2/3/4 proteins to the *PAO* promoter on EMSA. MBP-MYCs proteins were used in EMSA. A biotin-labeled PAO promoter fragment containing the wild-type G-box was used as the probe. The non-labeled wild-type (WT) fragment (200- or 400-fold excess) or the fragment with a mutated G-box (400-fold excess) were used as competitors; −, absence; +, presence. The arrow points to DNA–protein complexes. The 26-bp probe sequence is shown below EMSA images, with WT and mutant G-boxes in bold letters.

We used the 252-bp fragment as a bait to screen for putative TFs of *PAO* against a cDNA library generated from the senescent leaves of Arabidopsis plants; 15 of 73 positive clones encoded MYC2, a basic helix-loop-helix (bHLH)-type stress-related TF involved in JA signaling. To confirm this result, MYC2 and its two close homologs, MYC3 and MYC4, were re-tested by Y1H assay. The three MYCs interacted strongly with the *PAO* promoter (Figure 1b).

MYC2/3/4 proteins specifically bind to the G-box in the *PAO* promoter

A previous study showed that MYC2/3/4 proteins could bind to a DNA sequence with a core 6-mer motif, named G-box, upon activation of JA signaling (Fernández-Calvo *et al.*, 2011). A G-box motif was identified in the 252-bp *PAO* promoter (Figure 1c). To verify whether the MYC proteins interact with the *PAO* promoter *in planta*, we performed a chromatin immunoprecipitation (ChIP)-qPCR assay with a methyl jasmonate (MeJA)-treated transgenic line harboring *35S:MYC2-GFP* in the WT Columbia-0 (Col-0) background. Immunoprecipitated DNA fragments of *35S:MYC2-GFP* transgenic plants and *35S:GFP* control plants were used as templates in quantitative PCR (qPCR) to quantify the fold enrichment of particular regions of the *PAO* promoter. Up to three-fold enrichment was observed in the region between −284 and −78 bp upstream of the start codon of *PAO* gene, with no enrichment for a *PAO* coding region (Figure 1c). The result suggests an association of MYC2 protein with the *PAO* promoter *in vivo*.

To test whether MYC2/3/4 proteins directly bind to the *PAO* promoter, we used an electrophoretic mobility shift assay (EMSA). A 26-bp DNA fragment harboring the G-box was labeled and used as the probe, with non-labeled DNA fragments with or without mutations in the G-box as competitors. All three MYC proteins were able to bind to the labeled WT probe, and an excess quantity of the non-labeled fragment with the WT G-box effectively abolished the binding; however, the non-labeled fragment with mutations in the G-box could not compete with the labeled WT probe, indicating that the binding was specific (Figure 1d). These results suggest that MYC2/3/4 proteins could specifically bind to the G-box of the *PAO* promoter.

MYC2/3/4 proteins promote JA-induced Chl degradation in part via the activation of *PAO* expression

Jasmonic acid (JA) accelerates leaf yellowing in adult Arabidopsis plants (He *et al.*, 2002). CORONATINE INSENSITIVE1 (COI1) is the receptor of JA signaling (Fonseca *et al.*, 2009; Yan *et al.*, 2009; Sheard *et al.*, 2010), and plays a crucial role in JA-induced leaf senescence (He *et al.*, 2002; Robson *et al.*, 2010; Shan *et al.*, 2011; Qi *et al.*, 2015). In the JA signaling pathway, MYC2/3/4 proteins were reported to regulate several JA-dependent processes downstream of COI1 (Cheng *et al.*, 2011; Fernández-Calvo *et al.*, 2011). This finding prompted us to examine whether the MYCs are involved in JA-induced Chl degradation. The

252-bp *PAO* promoter was responsive to JA treatment because the stay-green phenotype of *acd1-20* was largely rescued by *p252:PAO* transformation under MeJA treatment (Figure S2). We then determined whether mutations of the *MYCs* could cause a phenotype in Chl degradation upon JA treatment. Both detached and attached leaves were treated with or without MeJA. Considering that similar phenotypes were observed in both the attached and detached leaves (Figures 2a,b and S3), detached leaves were used for all the following analyses for their convenience of preparation. Although detached leaves of *myc2* turned yellow, as those of Col-0, after 4 days of MeJA treatment, the relative Chl level was significantly higher in *myc2* than in Col-0 leaves (35 vs. 22%; Figure 2a,b). As expected, the double mutants *myc2 myc3*, *myc2 myc4* and *myc3 myc4* showed clearly greener phenotypes, with significantly higher Chl contents (Figure S4). Similar to the phenotype of *coi1-1*, leaves of *myc2 myc3 myc4* stayed green, even until the end of the treatment (Figure 2a,b). This result is consistent with those in a recent publication (Qi *et al.*, 2015). Therefore, the MYCs play a crucial role in JA-induced Chl degradation.

To elucidate a possible role of the MYCs in the regulation of *PAO* expression, we quantified the relative abundance of *PAO* transcripts after MeJA treatment. *PAO* transcription was induced by five-fold in Col-0 leaves treated with 100 μM MeJA for 4 days; as expected, the induction was abolished in *myc2 myc3 myc4* (Figure 2c). Conversely, upon treatment with MeJA, the transcript level of *PAO* was increased more dynamically, and the Chl content was further reduced, in *MYC2/3/4* overexpression lines, compared that in Col-0 or *35S:GFP* transgenic plants (Figure S5). The accelerated Chl degradation phenotype was similar to that shown by Qi *et al.* (2015). Thus, MYC2/3/4 proteins positively regulate *PAO* expression in JA-induced Chl degradation.

The regulation of *PAO* by MYC2/3/4 proteins was further examined by a dual luciferase assay. The reporter construct *pPAO:LUC* (Figure 2d) was transferred into the protoplasts of Col-0 and *myc2 myc3 myc4*, and the effect of MeJA treatment on the activation of the *PAO* promoter was determined by measuring relative luciferase activity. The *PAO* promoter was *trans*-activated by MeJA treatment in Col-0, but not in *myc2 myc3 myc4* (Figure 2e), suggesting that the MYCs are necessary for JA-mediated *PAO* expression. Consistently, when the effector constructs *35S:MYC2/3/4* individually co-expressed with the reporter construct, the activity of *PAO* promoter was enhanced by by two- to ten-fold (Figure 2d, f). These observations indicate that MYC2/3/4 proteins can *trans*-activate *PAO* expression.

After revealing that the MYCs are positive regulators of *PAO* expression in JA-induced Chl degradation, we wondered whether the stay-green phenotype of *myc2*

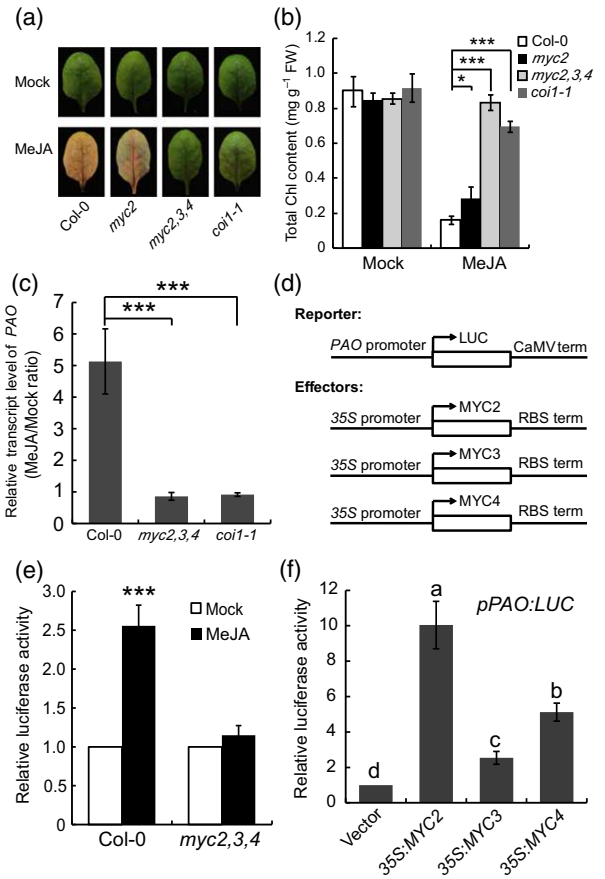

Figure 2. MYC2/3/4 proteins upregulate the expression of the pheophorbide *a* oxygenase gene (*PAO*) by binding to its promoter.
(a) Mutations of the MYC2/3/4 proteins resulted in stay-green phenotypes. The fifth and sixth detached leaves of 4-week-old plants were treated with or without 100 μM methyl jasmonate (MeJA) for 4 days.
(b) Chlorophyll (Chl) contents in the detached leaves shown in (a).
(c) Transcript levels of *PAO* in detached leaves shown in (a). β-*ACTIN2* was an internal control.
(d) Schematic diagram of the reporter and effector constructs used in the dual luciferase assay. The firefly luciferase (*LUC*) reporter was driven by the *PAO* promoter, and *MYC2/3/4* was driven by a CaMV *35S* promoter in each of the effector constructs.
(e) Luciferase assay of the inhibition of MeJA-induced *PAO* expression by the mutation of MYC2/3/4. Protoplasts of Col-0 and *myc2 myc3 myc4* (*myc2, 3, 4*) were transformed with the *pPAO:LUC* reporter construct with or without 100 μM MeJA.
(f) Luciferase assay of enhancement of *PAO* promoter activity by the overexpression of *MYC2/3/4* in protoplasts. Col-0 protoplasts were co-transformed with the *pPAO:LUC* reporter and respective *35S:MYC2/3/4* effector constructs. Vector, empty vector control. Different letters indicate significant differences at $P < 0.001$ (one-way ANOVA). Data are means ± SDs of three biological replicates; $*P < 0.05$, $***P < 0.001$ (Student's *t*-tests).

myc3 myc4 upon MeJA treatment was due to the decreased *PAO* expression. To test this, we ectopically overexpressed *PAO* in *myc2 myc3 myc4* and Col-0 by using the dexamethasone (DEX)-inducible system (Aoyama and Chua, 1997). Elevated levels of *PAO* transcripts were confirmed by RT-qPCR in homozygous transgenic lines (Figure S6). Two representative lines, *iPAO-*

OE #6 and *iPAO-OE*
myc2,3,4 #2, were chosen for evaluating the effect of its inducible expression. The application of DEX accelerated leaf yellowing in the *iPAO-OE #6* line upon MeJA treatment. As expected, leaf yellowing was also accelerated by the application of DEX in the *iPAO-OE myc2,3,4 #2* line, although to a lesser extent, after MeJA treatment (Figure 3a). Consistently, Chl contents in *iPAO-OE #6* and *iPAO-OE myc2,3,4 #2* transgenic lines were further reduced compared with those in their respective non-transgenic counterparts (Figure 3b). These phenotypes were presumably resulted from the enhanced *PAO* expression (Figure 3c). Thus, the MYCs modulate JA-induced Chl degradation at least in part by activating *PAO* expression.

MYC2/3/4 proteins activate the expression of other *CCG*s

Besides PAO, Chl degradation is also regulated by a group of other *CCG*s. To determine whether MYC2/3/4 proteins also regulate the expression of other *CCG*s, we examined the change in their transcript levels in Col-0, *myc2 myc3 myc4* and *coi1-1* plants after MeJA treatment. The transcript levels of *NYE1*, *NYE2*, *NYC1* and *PPH*, as well as *PAO*, were significantly induced by MeJA treatment in Col-0, but the induction was abolished in *myc2 myc3 myc4* and *coi1-1* (Figure 4a). This finding suggests that the MYCs may also positively regulate the expression of *NYE1*, *NYE2*, *NYC1* and *PPH*. To validate this result, we performed a dual-luciferase assay and found that the overexpression of MYC2/3/4 proteins significantly enhanced the activity of *NYE1*, *NYC1* and *PPH* promoters, but not the *NYE2* promoter, by two- to six-fold (Figure 4b). To test whether the MYCs regulate the expression of *NYE1*, *NYC1* and *PPH* by binding to their promoters, we analyzed the promoter regions of *NYE1*, *NYC1* (Sakuraba *et al.*, 2014) and *PPH*, and found that *NYE1* and *NYC1* promoters contained a canonical G-box (Figure 4c). We then examined a possible association of MYC2/3/4 proteins with the promoter regions of *NYE1* and *NYC1.* ChIP assays clearly demonstrated a significant enrichment of the promoter fragments containing a G-box by *MYC2-GFP* overexpression (Figure 4c). Direct binding of MYC2 to the *NYE1* and *NYC1* promoter fragments was further confirmed by EMSA (Figure 4d). These findings, along with the observation of a stay-green leaf phenotype in *nye1-1* plants upon treatment with MeJA (Figure 4e, f), suggest that MYC2/3/4 proteins are involved in JA-induced Chl degradation not solely by activating the expression of *PAO* but simultaneously by activating the expression of *NYE1* and *NYC1*. Mutating *NYC1* did not confer a stay-green phenotype, however, presumably because of functional redundancy between *NYC1* and its homologous gene *NOL* (*NYC1-like*). We indeed observed a clearly greener phenotype in the double mutant *nol nyc1* upon treatment with MeJA (Figure S7).

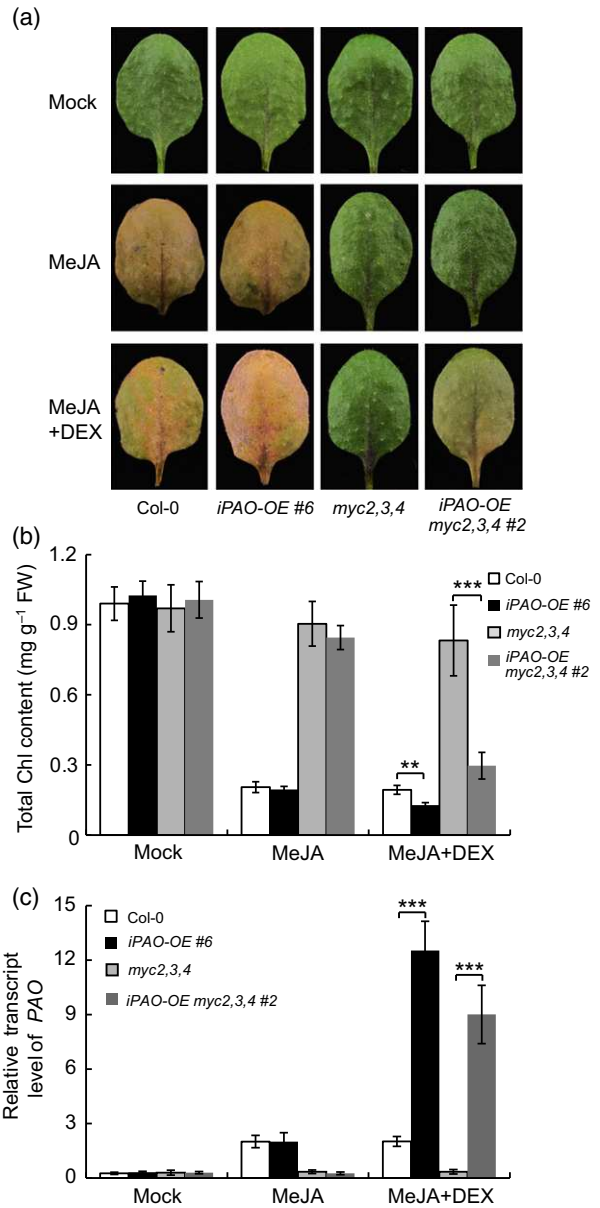

Figure 3. MYC2/3/4 proteins promote chlorophyll (Chl) degradation by inducing pheophorbide *a* oxygenase gene (*PAO*) expression.
(a) The inducible expression of *PAO* partially rescued the stay-green phenotype of *myc2 myc3 myc4* upon methyl jasmonate (MeJA) treatment. The fifth and sixth detached leaves were soaked in water (mock), 100 μM MeJA or 100 μM MeJA + 30 μM dexamethasone (DEX), and then incubated for 4 days.
(b) Chl contents in the leaf samples shown in (a).
(c) qRT-PCR analysis of *PAO* transcript levels. RNA was isolated from the leaves shown in (a), and β-*ACTIN2* was used as an internal control. Data are means ± SDs of three biological replicates; $*P < 0.05$, $***P < 0.001$ (Student's *t*-tests).

Involvement of ANAC019/055/072 in the regulation of JA-induced Chl degradation

The MYC proteins promote JA-induced Chl degradation by regulating major *CCG*s via directly binding to their

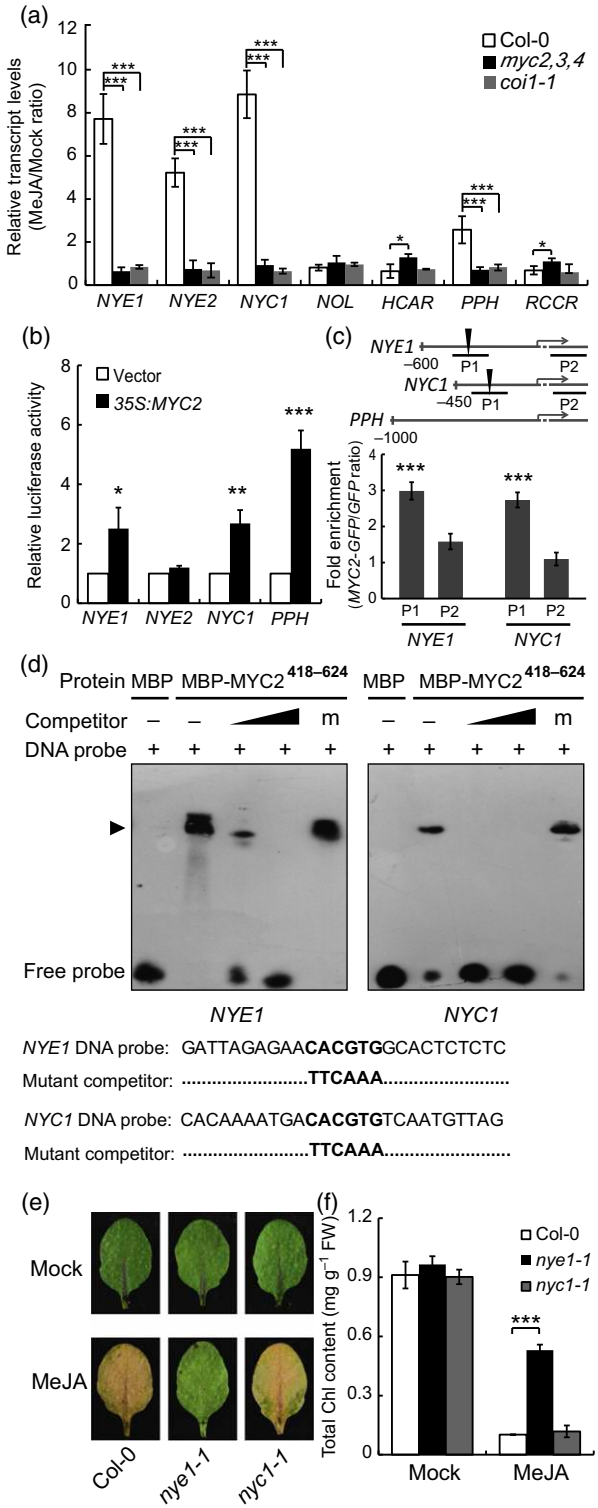

Figure 4. MYC2/3/4 proteins promote the transcription of *NYE1* and *NYC1*.
(a) Transcript levels of other major *CCG*s in the detached leaves shown in Figure 2a. β-*ACTIN2* was used as an internal control.
(b) Overexpression of *MYC2* enhanced the promoter activities of *NYE1*, *NYC1* and *PPH* in the dual luciferase assay. Effectors, *35S:MYC2* or the empty vector (control); reporters, a *LUC* expression cassette driven by *NYE1*, *NYE2*, *NYC1* or *PPH* promoters, respectively.
(c) Association of MYC2 protein with *NYE1* and *NYC1* promoters *in planta*. Upper panel, schematic diagrams of *NYE1*, *NYC1* and *PPH* genes, showing the positions of the G-box element (black triangles) and ChIP amplicons (P1 and P2). Lower panel, chromatin isolated from *35S:MYC2-GFP* and *35S:GFP* transgenic plants was used for the ChIP-qPCR assay.
(d) Binding of MYC2 to *NYE1* and *NYC1* promoters in EMSA. Details of the EMSA assay were presented in Figure 1(d). Key: –, absence; +, presence; competitor, non-labeled wild-type (WT) or mutant (m) fragment; DNA probe, biotin-labeled *NYE1* or *NYC1* promoter fragment; MBP, recombinant MBP protein; MBP-MYC2, recombinant MBP-MYC2 protein. The arrowhead points to DNA–protein complexes.
(e) Phenotypes of the detached leaves of *nye1-1*, *nyc1-1* as well as Col-0, with or without MeJA treatment.
(f) Chlorophyll (Chl) content in the detached leaves shown in (e). In (a), (b) and (f), data are means ± SDs of three biological replicates. In (c), data are means ± SDs of two biological replicates. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-tests).

promoters. Recently, ANAC019, ANAC055 and ANAC072, three functionally overlapping members of the NAC TF family, were found to be direct targets of MYC2 in pathogenesis (Bu *et al.*, 2008; Zheng *et al.*, 2012). In addition, some members of the NAC family have been found to regulate leaf senescence (Balazadeh *et al.*, 2010; Liang *et al.*, 2014). Therefore, the MYCs may also promote JA-mediated Chl degradation during leaf senescence by upregulating the expression of the *ANAC*s. Consistent with this speculation, our data showed that MeJA induced the transcription of *ANAC019*, *ANAC055* and *ANAC072* in Col-0 leaves, whereas their induction was abolished in *myc2 myc3 myc4* (Figure S8a). ChIP assay revealed that *ANAC019* was the direct target of MYC2 (Figure S8b). We then analyzed the involvement of the ANACs in the regulation of JA-induced Chl degradation. Detached leaves from the *anac019 anac055 anac072* triple mutant and Col-0 were treated with or without 100 μM MeJA for 4 days. Loss-of-function mutations of the *ANAC*s resulted in a stay-green phenotype after treatment with MeJA (Figure 5a). Chl content measurement confirmed the phenotypic observations (Figure 5b). These results suggest that ANAC019, ANAC055 and ANAC072 also positively regulate JA-induced Chl degradation.

The ANACs promote Chl degradation, probably by a parallel pathway or simply by sharing the same targets of the MYCs. To clarify the possibilities, we explored the downstream targets of ANAC019/055/072 in Chl degradation. We found that MeJA induced the transcription of *NYE1*, *NYE2*, *NYC1*, *PPH* and *PAO*, and that their induction was abolished in *anac019 anac055 anac072* (Figure 5c). Thus, these five genes might also be targets of ANAC019/055/072. A previous study identified a complete DNA-binding sequence that contains both the NAC recognition sequence (NACRS) 'CATGTG' and the core-binding site 'CACG', and can be recognized by ANAC019/055/072 (Tran, 2004). We

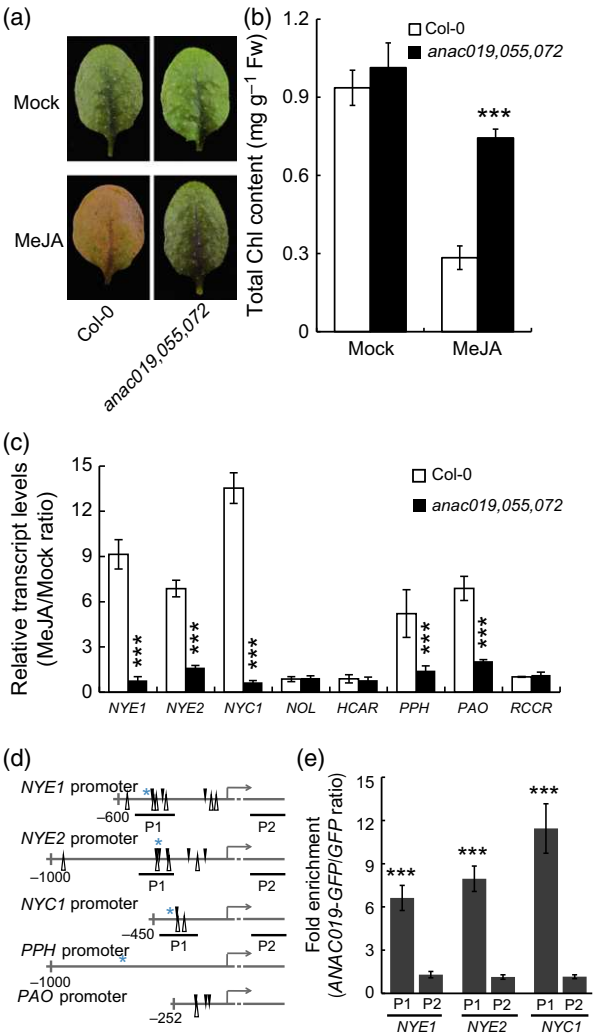

Figure 5. ANACs regulate the expression of major chlorophyll (Chl) catabolic genes (*CCG*s).

(a) Phenotypes of the detached leaves of *anac019 anac055 anac072* (*anac019, 055, 072*) and Col-0 plants after methyl jasmonate (MeJA) treatment.

(b) Chl contents in the leaf samples shown in (a).

(c) qRT-PCR analysis of the mRNA levels of major *CCG*s in the detached leaves shown in (a). β-*ACTIN2* was used as an internal control.

(d) Putative ANAC protein binding sites in the promoter regions of the *CCG*s. Asterisks indicate the recognition sequence (NACRS) 'CATGTG' and the core-binding site 'CACG', marked by black triangles on the sense strand and white triangles on the antisense strand. Positions of the examined regions in ChIP-qPCR are indicated (P1, P2).

(e) Association of ANAC019-GFP fusion protein with *NYE1*, *NYE2* and *NYC1* promoters *in planta*. Chromatins isolated from *35S:ANAC019-GFP* and *35S:GFP* transgenic plants were used for the chromatin immunoprecipitation (ChIP) assay. In (b), data are means ± SDs of three independent experiments. In (c) and (e), data are means ± SDs of two independent experiments. ***$P < 0.001$ (Student's *t*-tests).

scanned the promoter regions of the above *CCG*s and identified the complete DNA-binding sequence in the promoters of *NYE1*, *NYE2* and *NYC1* (Figure 5d). Importantly, ChIP-qPCR analyses demonstrated that the ANAC019-GFP fusion protein was associated with the regions of *NYE1*, *NYE2* and *NYC1* promoters containing the complete DNA-binding sequence, with enrichments ranging from six- to 12-fold; no associations with their coding regions were detected (Figure 5e). Therefore, MYC2/3/4 proteins promote JA-induced Chl degradation either by directly regulating the expression of major *CCG*s (*NYE1*, *NYC1* and *PAO*), or by hierarchically regulating the expression of a similar set of *CCG*s (*NYE1*, *NYE2* and *NYC1*) via the mediation of intermediate regulators ANAC019, ANAC055 and ANAC072.

MYC2 interacts with ANAC019 to co-regulate the expression of *NYE1*

MYC2 contains a bHLH domain with two amphipathic alpha-helices connected by a loop structure, and can form homo- or heterodimers accordingly with other TFs, including MYC3 and MYC4 (Fernández-Calvo *et al.*, 2011). In addition, the C-terminal of MYC2 contains a leucine zipper domain possibly functioning as a dimerization domain that affects the specificity of interactions with other TFs (Amoutzias *et al.*, 2008). We identified binding sites for both MYCs and ANACs in the adjacent regions of *NYE1* and *NYC1* promoters (Figure 4c, 5d). Our ChIP-qPCR results indicated that both MYCs and ANACs were associated approximately with the same regions in *NYE1* and *NYC1* promoters (Figure 4c, 5e). Therefore, a direct interaction might exist between MYC2 and ANAC019. A bimolecular fluorescence complementation (BiFC) assay was initially performed in tobacco *(Nicotiana benthamiana)* mesophyll cells. No YFP fluorescence was observed when only one of the two proteins was fused to an unfolded YFP fragment; however, when both MYC2 and ANAC019 were present in tobacco mesophyll cells, a strong YFP signal was detected (Figure 6a). The interaction was confirmed by *in vitro* pull-down assay, in which the purified MBP-MYC2 fusion protein successfully pulled down the ANAC019-3×FLAG fusion protein expressed in tobacco leaves (Figure 6b). Moreover, GST-ANAC019 and MBP-MYC2 could mutually pull down each other when expressed in *Escherichia coli* (Figure S9). In addition, we performed a co-immunoprecipitation (co-IP) assay to examine the interaction between ANAC019 and MYC2 *in planta*. ANAC019-3×FLAG was co-expressed with GFP or MYC2-GFP in tobacco leaves. Total proteins were extracted and used for co-IP. ANAC019-3×FLAG was indeed co-immunoprecipitated with MYC2-GFP (Figure 6c). These results collectively indicate that MYC2 interacts with ANAC019 in living cells.

To determine whether MYC2 and ANAC019 co-regulate major *CCG*s, we performed a transient co-expression assay with the dual luciferase system. Overexpression of MYC2 or ANAC019 induced the expression of *pNYE1-LUC* by

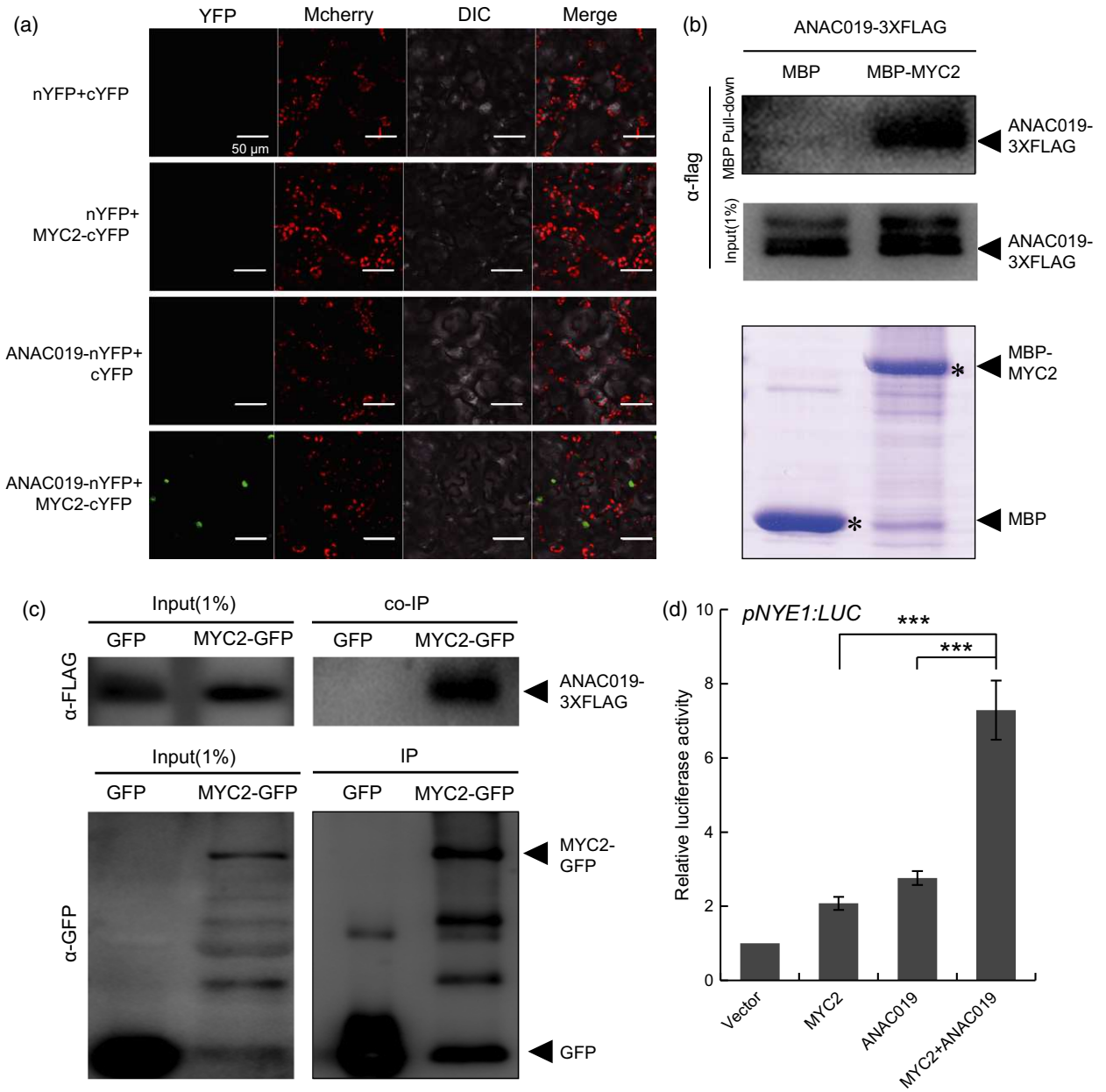

Figure 6. An interaction between MYC2 and ANAC019.
(a) An *in vivo* bimolecular fluorescence complementation (BiFC) assay of interaction between MYC2 and ANAC019. MYC2 was fused to the C terminus of YFP (cYFP), and ANAC019 was fused to the N terminus of YFP (nYFP). Combinations of nYFP + cYFP, nYFP + MYC2-cYFP and ANAC019-nYFP + cYFP were included as negative controls. From left to right: YFP, mCherry, DIC and merged. Scale bar: 50 μm.
(b) An *in vitro* pull-down assay of interaction between MYC2 and ANAC019. Purified MBP and MBP-MYC2 proteins from *Escherichia coli* were used to pull down FLAG-tagged ANAC019 from plant extracts. ANAC019-3×FLAG was transiently transformed into *Nicotiana benthamiana*. Purified MBP and MBP-MYC2 proteins were stained with Coomassie blue. The input and pull-down proteins were detected with FLAG antibody.
(c) A co-immunoprecipitation (Co-IP) assay of interaction between MYC2 and ANAC019 *in planta*. The ANAC019-3×FLAG was coexpressed with GFP (control) or MYC2-GFP in *N. benthamiana* leaves. Immunoprecipitation involved GFP-Trap beads, and co-immunoprecipitated proteins were detected with anti-FLAG antibody.
(d) MYC2 and ANAC019 synergistically enhanced the expression of *NYE1* in Arabidopsis protoplasts. Col-0 protoplasts were co-transformed with the *pNYE1:LUC* reporter construct and different combinations of effector constructs (*35S:MYC2* and *35S:ANAC019*). Vector, empty vector control. Data are means ± SDs from three biological replicates; $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-tests).

approximately two-fold, and the induction was increased to as high as seven-fold when *MYC2* and *ANAC019* were co-expressed (Figure 6d); however, *pPAO-LUC* or *pNYE2-LUC* could only be regulated by MYC2 or ANAC019, respectively (Figure S10). These results suggest that, by interacting with each other, MYC2 and ANAC019 may

coordinately amplify the JA signal during Chl degradation by synergistically regulating the expression of *NYE1*.

DISCUSSION

Our data reveal the molecular basis of MYC2/3/4 and ANAC019/055/072 in regulating JA-induced Chl degradation in Arabidopsis. MYC2/3/4 proteins promote this process by activating the expression of major *CCG*s (*PAO*, *NYE1* and *NYC1*) via directly binding to their promoters. ANAC019/055/072, functioning downstream of MYC2/3/4 in JA signaling in the defense response (Bu *et al.*, 2008; Zheng *et al.*, 2012), induce the expression of *NYE1*, *NYE2* and *NYC1* in a similar manner. Moreover, MYC2 and ANAC019 interact with each other at the protein level, so they may form a complex to efficiently promote JA-induced Chl degradation by synergistically regulating the expression of *NYE1*.

PAO encodes a key enzyme responsible for opening the porphyrin macrocycle of Pheide *a*, a photosensitive intermediate of Chl catabolism, which consequently yet prominently leads to the eventual loss of the green color of the Chl catabolite (Pružinská *et al.*, 2003). By Y1H screening and further *in vitro* and *in vivo* assays, we initially identified MYC2/3/4 proteins as the direct regulators of *PAO* expression. This finding was confirmed by phenotypic and physiological analyses of their triple mutant *myc2 myc3 myc4* (Figure 2a, b). Molecularly, the MeJA-induced expression of *PAO* was nearly blocked in *myc2 myc3 myc4* (Figure 2c), and the activity of *PAO* promoter could be activated by the three MYCs in the protoplasts of Col-0, with MYC2 being the most effective (Figure 2f). Overexpression of *PAO* in *myc2 myc3 myc4* only partially rescued the stay-green phenotype (Figure 3), suggesting that the MYCs may not solely upregulate *PAO* expression in promoting Chl degradation. Our further analysis revealed that other major *CCG*s, including *NYE1* and *NYC1*, are also the targets of the MYCs. NYE1 is known to be an essential CCE recruiter during Chl degradation, whereas *NYC1* encodes a major CCE responsible for catalyzing an upstream reaction of Chl degradation. The direct involvement of the MYCs in regulating multiple *CCG*s implies that they likely function as orchestrators of the expression of major *CCG*s, which presumably guarantees the highest efficiency of JA-induced Chl degradation.

MYC2 is involved in the transcriptional regulation of JA-responsive gene expression (Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). Accumulating data indicate that MYC2 acts as a master regulator in the JA signaling pathway, mediating the JA-dependent regulation of diverse aspects of plant growth and development, as well as responses to pest and pathogen attacks or wounding and synthesis of secondary metabolites (Hong *et al.*, 2012; Wasternack and Hause, 2013). During the review of our article, Qi *et al.* (2015) reported that MYC2 plays a positive role in JA-mediated leaf senescence, and that MYC2/3/4 proteins function redundantly in activating the promoter of *SAG29*. Here, we reveal the molecular basis of MYC2's involvement in the regulation of JA-induced Chl degradation. Previously, MYC3 and MYC4, two closely related bHLH TFs, were shown to regulate both overlapping and distinct functions with MYC2 in Arabidopsis (Fernández-Calvo *et al.*, 2011). Consistently, we found that MYC3 and MYC4 redundantly promote JA-induced Chl degradation with MYC2, as evidenced by a more severe stay-green phenotype of *myc2 myc3 myc4* than that of *myc2* or any of their double mutants (Figures 2a, b and S4). Compared with *myc3 myc4*, both *myc2 myc3* and *myc2 myc4* showed a more severe stay-green phenotype (Figure S4), indicating that MYC2 may play a major role in mediating JA-induced Chl degradation.

Recently, three ANAC TFs, ANAC019, ANAC055 and ANAC072, were found to act downstream of MYC2 as mediators of a JA-triggered defense response by regulating the expression of *VSP1*, *LOX2*, *ICS1* and *BSMT1* (Bu *et al.*, 2008; Zheng *et al.*, 2012). In this study, we found that the ANACs also mediate JA-induced Chl degradation by directly regulating the expression of *NYC1*, *NYE1* and *NYE2* (Figure 5a, b). Importantly, both the MYCs and ANACs directly target *NYE1* and *NYC1*, suggesting that they may exert their regulatory roles by promoting the early and key steps of Chl degradation. In addition, the ANACs target an additional *CCG*, *NYE2*, which may allow them to strengthen and broaden the signal from the MYCs.

Our ChIP analysis demonstrated that the MYCs and ANACs were associated with the same regions of *NYE1* and *NYC1* promoters (Figures 4c and 5d, e). BiFC, pull-down and co-IP analyses revealed a direct interaction between MYC2 and ANAC019 (Figure 6a–c), which is supported by the observation that MYC2 and ANAC019 synergistically activated the expression of *pNYE1:LUC* in Arabidopsis protoplasts (Figure 6d). The MYCs and ANACs may coordinately regulate the transcription of some of their target genes by forming some kind of protein complex(es) on their binding sites.

In summary, we reveal that MYC2/3/4 proteins act as a key regulatory node of JA-induced Chl degradation, which directly or indirectly, via the intermediate mediators of ANAC019/055/072, activate the expression of major *CCG*s. The MYCs and ANACs may form some kind of complex(es) to coordinately mediate the regulation of their target genes and constitute a feed-forward regulatory module to magnify the JA signal, along with major *CCG*s, to efficiently regulate Chl degradation in particular scenarios (Figure 7). Our discovery of the mode of direct involvement of MYCs and ANACs in the regulation of *CCG*s helps to elucidate the molecular mechanism of Chl degradation regulation, and extends our understanding of the hierarchical network of JA signaling in regulating functional genes in general.

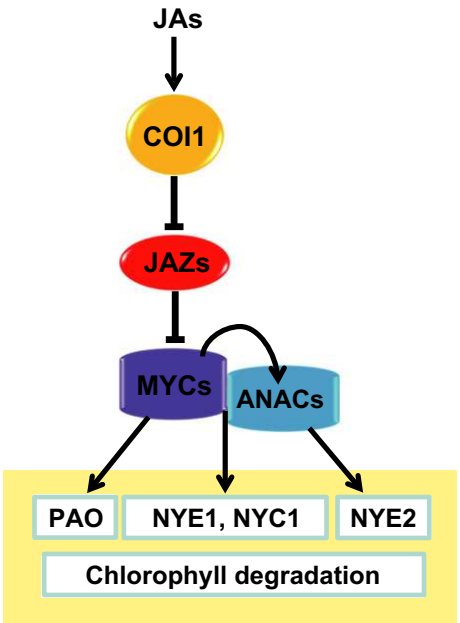

Figure 7. A proposed model for the molecular regulation of jasmonic acid (JA)-induced chlorophyll (Chl) degradation. MYC2/3/4 proteins positively regulate JA-induced Chl degradation, downstream of COI1 and JAZs in the JA signaling pathway, by binding to the promoters of major Chl catabolic genes (*NYE1*, *NYC1* and *PAO*). Meanwhile, the MYCs also regulate the Chl degradation indirectly by mediating its downstream transcription factors, *ANAC019/055/072*, which can activate the expression of the same (*NYE1* and *NYC1*) or distinctive (*NYE2*) Chl catabolic genes (*CCG*s). Furthermore, MYC2 and ANAC019 may form a complex to promote the expression of some *CCG*s. This model illustrates a hierarchical and coordinated regulation of JA-induced Chl degradation by MYC and ANAC proteins.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All mutants and transgenic lines used in this study were derived from Arabidopsis ecotype Col-0. The *myc2* (SALK_017005) and *acd1-20* (CS3733) mutants were obtained from the ABRC Stock Center (http://abrc.osu.edu), and their homozygous lines were identified by PCR-based genotyping (the primers are in listed in Table S1). *nye1-1* (Ren *et al.*, 2007), *nyc1-1* (Horie *et al.*, 2009), *myc2 myc3 myc4* (Fernández-Calvo *et al.*, 2011), *coi1-1* (Xie *et al.*, 1998), *anac019 anac055 anac072*, *35S:MYC2-GFP*, *35S:ANAC019-GFP* (Zheng *et al.*, 2012), and *35S:GFP* (Wang *et al.*, 2015) were described previously.

Plants were grown at 22–24°C under long-day conditions (16-h light/8-h dark) in a growth room equipped with cool-white fluorescent lights (100 μmol m^{-2} s^{-1}). Transgenic lines were generated by the floral-dip method (Clough and Bent, 1998). For selection, 50 mg L^{-1} kanamycin or 25 mg L^{-1} hygromycin was included in the MS medium.

INDUCTION TREATMENTS

For dark treatment, the fifth and sixth rosette leaves of 4-week-old plants were detached and incubated as described by Ren *et al.* (2007). For MeJA treatment, whole plants were sprayed with 1 mM MeJA water solution (Sigma-Aldrich, http://www.sigmaaldrich.com) containing 0.01% (v/v) Silwet L-77; fifth and sixth rosette leaves of 4-week-old plants were soaked in 100 μM MeJA. For DEX treatment, 30 μM DEX water solution was added to 100 μM MeJA.

Plasmid constructs

A 252-bp promoter fragment (*p252*), upstream of the *PAO* start codon, was cloned into the pCHF3 vector to generate *p252:PAO*. For Y1H, *MYC2/3/4* coding sequence (CDS) was cloned into the pGADT7 vector (Clontech, http://www.clontech.com). For the dual luciferase assay, the *PAO* promoter (*p252*) was cloned into the pGreenII 0800-LUC vector (Hellens *et al.*, 2005). To produce their proteins in *E. coli* Rosetta, the DNA binding region of the MYC2/3/4 proteins (Li *et al.*, 2006; Kazan and Manners, 2013) was cloned into the pMAL-C5G vector (New England Biolabs, http://www.neb.com). All primers used for plasmid construction are listed in Table S1.

Chl content measurement

The fifth and sixth rosette leaves of 4-week-old plants were detached for measuring Chl content. Chl extraction and quantification were performed as described by Benedetti and Arruda (2002).

Yeast-one-hybrid screening

The Y1H screening involved the Matchmaker Gold Y1H Library Screening system (Clontech). The bait sequence (252-bp fragment upstream of the *PAO* start codon) was cloned into the pAbAi vector that harbors the *AUR-1C* gene, conferring resistance to Aureobasidin A (AbA, a cyclic depsipeptide antibiotic used as a yeast selection marker). The resulting pAbAi-Bait plasmid was then integrated into the yeast genome (strain Y1HGold) to make a Bait-reporter strain. The Bait-reporter strain was used to screen a cDNA library generated from senescing Arabidopsis Col-0 leaves, according to the manufacturer's instructions. Approximately 5×10^5 transformants were initially screened on selective medium containing 50 ng ml^{-1} AbA (SD/–Leu/AbA). The prey fragments of the positive colonies were identified by DNA sequencing.

For the re-transformation assay, the full-length CDSs of candidate genes were amplified with gene-specific primers (listed in Table S1). The PCR products were cloned into the pGADT7 (Clontech) prey vector and the resulting constructs were then transferred into the aforementioned bait-reporter yeast strain. The cells were grown on SD/–Leu plates at 30°C for 3 days, collected, resuspended and diluted to 10^7 cells ml^{-1} (OD_{600} = 0.5) with sterile water. A 2-μl volume of each of the cell suspensions was plated onto SD/–Leu/AbA medium (50 ng ml^{-1} AbA). The empty vector pGADT7 was used as a negative control.

Protoplast transformation and dual-luciferase assay

Arabidopsis protoplasts were isolated from 4-week-old Col-0 rosette leaves by using an enzymatic hydrolysate [0.15% (w/v) pectolyase Y-23 (Yakult, http://www.yakult.co.jp), 0.35% (w/v) cellulose RS (Yakult), 0.4 M mannitol, 20 mM 2- (*N*-morpholine)-ethanesulphonic acid (MES), 20 mM KCl and 10 mM $CaCl_2$]. Plasmids were transiently transferred into protoplasts by a polyethylene glycol (PEG)-mediated method. Sixteen hours after incubation in the dark at 20°C, protoplasts were harvested and lysed with cell lysis buffer. Firefly and *Renilla* luciferase activities were quantified using a dual-luciferase assay kit (Promega, http://www.promega.com) and detected by using the Synergy 2 Multi-Mode Microplate Reader (Bio-Tek, http://www.biotek.com), as described previously (Sun *et al.*, 2013).

Chromatin immunoprecipitation (ChIP) assay

Rosette leaves from 6-week-old Arabidopsis transgenic plants of *35S:MYC2-GFP* and *35S:ANAC019-GFP* were used for the ChIP-qPCR assay, with those from *35S:GFP* plants used as a control. Rosette leaves were harvested 24 h after being sprayed with 1 mM MeJA water solution containing 0.01% (v/v) Silwet L-77. ChIP assays were performed as described by Zhang *et al.* (2011), with the following modifications: nuclei were isolated and chromatin was sonicated into 0.3- to 1-kb DNA fragments. After reverse cross-linking, the DNA fragments were purified using the ChIP DNA Clean & Concentrator kit (Zymo Research, http://www.zymoresearch.com). The enriched DNA fragments were quantified by qPCR with the primers in Table S1. *ACTIN2* was used to normalize the qPCR result in each ChIP sample. Fold enrichment of each region in the *35S:MYC2-GFP* and *35S:ANAC019-GFP* transgenic lines was calculated in comparison with the control *35S:GFP* line.

Electrophoretic mobility shift assay (EMSA)

MBP-MYC plasmids and pMAL-c5G empty vector were transferred into the *E. coli* strain Rosetta (DE3; Merck, http://www.merck.com). The expression of MBP-MYCs or MBP was induced by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 16°C for 10 h. The recombinant proteins were purified with amylose resin (New England Biolabs) according to the manufacturer's instructions. EMSA involved the Light Shift Chemiluminescent EMSA Kit (ThermoFisher Scientific, http://www.thermofisher.com). The 26-bp biotin-labeled probe used for EMSA is listed in Table S1. Unlabeled competitors were added in 200- or 400-fold molar excess.

Bimolecular fluorescence complementation assay (BiFC)

The CDS of *MYC2* without the stop codon was inserted into the pXY104 (cYFP) vector. The CDS of *ANAC019* without the stop codon was amplified and cloned into the pXY103 (nYFP) vector. The resulting constructs were introduced into *Agrobacterium* strain GV3101. For the BiFC assay, the transformed *Agrobacteria* were grown in the YEB medium containing 50 mg L^{-1} spectinomycin, 30 mg L^{-1} rifampicin, 100 mg L^{-1} streptomycin and 20 μM acetosyringone to an OD_{600} value of 1.2. Cells were collected and resuspended in infiltration medium (10 mM $MgCl_2$; 10 mM MES, pH 5.6; and 200 μM acetosyringone). After incubation at 20°C for 2 h, paired fusions with an OD_{600} of 0.8 were co-infiltrated into the leaves of 4-week-old *N. benthamiana*. The plants were left at 22–24°C for 36 h before the infiltrated leaves were imaged by confocal laser scanning microscopy.

Pull-down assay

GST-ANAC019, MBP-MYC2 and MBP proteins were expressed in *E. coli* strain BL21. Pull-down assays were performed as described by Wang *et al.* (2015). The recombinant proteins were separated by 10% SDS-PAGE and detected by western blot analysis with anti-GST or anti-MBP antibodies (TransGen, http://www.transgenbiotech.com).

Agrobacterium harboring *35S:ANAC019-3×FLAG* was infiltrated into *N. benthamiana* leaves. After 48 h, 2 g of *N. benthamiana* leaves transiently expressing ANAC019-3×FLAG were harvested and total proteins were extracted in 5 ml of extraction buffer [150 mM NaCl; 50 mM Tris-HCl, pH 7.5; Triton X-100; 1% (v/v) glycerol; 1 mM phenylmethylsulfonyl fluoride (PMSF); complete protease inhibitor cocktail (Sigma-Aldrich); and 5 mM 2-mercaptoethanol]. The extracted total proteins were centrifuged twice at 16 000 ***g*** at 4°C. About 50 μg of purified MBP or MBP-MYC2 were incubated with 120 μl of amylose beads for 2 h at 4°C. These amylose beads were then washed five times with 1 ml of extraction buffer and incubated with 200 μl of concentrated total proteins containing ANAC019-3×FLAG for 2 h at 4°C. After five washes with 1 ml of extraction buffer, the mixture was resuspended in 1×SDS loading buffer, boiled for 5 min, separated on 10% SDS-PAGE and immunoblotted with anti-flag antibody (Sigma-Aldrich).

Co-immunoprecipitation of MYC2 and ANAC019

Agrobacterium cells harboring *35S:MYC2-GFP* and *35S:ANAC019-3×FLAG* were resuspended in infiltration media, mixed to make a final OD_{600} of approximately 0.6 for each strain and infiltrated into 4-week-old *N. benthamiana* leaves; 48 h after the transformation, transiently transformed *N. benthamiana* leaves were harvested for the co-IP assay. A 2-g sample of leaves was ground into fine powder in liquid nitrogen and total proteins were extracted as described in the pull-down experiment. The plant extracts were centrifuged twice at 20 000 ***g*** at 4°C.

The supernatant was incubated with GFP-Trap (ChromoTek, http://www.chromotek.com) for 4 h at 4°C, with rotation. After three washes with 1 ml of extraction buffer, the GFP-Trap beads underwent immunoblot analysis with anti-FLAG (Sigma-Aldrich) or anti-GFP (Covance, http://www.covance.com) antibodies.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Information Resource (TAIR) or GenBank/EMBL databases with the following accession numbers: *SGR1*/*NYE1* (AT4G22920), *SGR2*/*NYE2* (AT4G11910), *NYC1* (AT4G13250), *NOL*(AT5G04900), *HCAR*(AT1G04620), *PPH*(AT5G13800), *PAO* (AT3G44880), *RCCR*(AT4G37000), *MYC2*(AT1G32640), *MYC3* (AT5G46760), *MYC4*(AT4G17880), *ANAC019*(AT1G52890), *ANAC055*(AT3G15500), and *ANAC072*(AT4G27410).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The 252-bp DNA fragment upstream from the start codon of *PAO* is a functional promoter.

Figure S2. The *PAO* promoter is responsive to MeJA treatment.

Figure S3. Attached leaves of Col-0 and *myc2 myc3 myc4* show a phenotype concordant with those of their detached counterparts after MeJA treatment.

Figure S4. Phenotypes of *myc* double mutants after MeJA treatment.

Figure S5. Overexpression of *MYC2/3/4* accelerates Chl degradation upon MeJA treatment.

Figure S6. Transcript levels of *PAO* in the inducible transgenic lines in WT and the *myc2 myc3 myc4* triple mutant backgrounds.

Figure S7. The *nol nyc1* double mutant showed a stay-green phenotype.

Figure S8. *ANAC019*, *ANAC055* and *ANAC072* are the targets of MYC2.

Figure S9. *In vitro* pull-down assay of MYC2 and ANAC019.

Figure S10. MYC2 and ANAC019, respectively, regulate the expression of *PAO* and *NYE2*.

Table S1. Primers used in this study.

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