Jasmonic acid-dependent MYC transcription factors bind to a tandem G-box motif in the YUCCA8 and YUCCA9 promoters to regulate biotic stress responses

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Abstract: The indole-3-pyruvic acid pathway is the major route for auxin biosynthesis in higher plants. Tryptophan aminotransferases (TAA1/TAR) and members of the YUCCA family of flavin-containing monooxygenases catalyze the conversion of L-tryptophan via indole-3-pyruvic acid into indole-3-acetic acid (IAA). It has been described that locally produced jasmonic acid (JA) in response to mechanical wounding, triggers de novo-formation of IAA through the induction of two YUCCA genes, YUC8 and YUC9. Here, we report the direct involvement of a small number of basic helix-loop-helix transcription factors of the MYC family in this process. We show that the JA-mediated regulation of YUC8 and YUC9 gene expression depends on the abundance of MYC2, MYC3, and MYC4. In support of this observation, seedlings of myc knockout mutants displayed a strongly reduced response to JA-mediated IAA formation. In addition, transactivation assays provided experimental evidence for the binding of the MYC transcription factors to a particular tandem G-box motif abundant in the promoter regions of YUC8 and YUC9, but not in those of the other YUCCA genes. Moreover, we clearly demonstrate that YUC8ox and YUC9ox overexpressing plants show less damage after spider mite infestation, thereby underlining a role of auxin in plant responses toward biotic stress cues.

Keywords: Arabidopsis thaliana, indole-3-acetic acid, jasmonic acid, plant hormone crosstalk, transcriptional regulation, wound response, biotic stress, growth-defense trade-off

1. Introduction

Since its discovery in the thirties of the last century [1-3], many genetic and biochemical studies clearly demonstrated that auxins govern virtually every aspect of plant life, related
with growth and development, i.e. cell division, cell elongation, cell differentiation, tropisms, apical dominance, emergence of new primordia, initiation of lateral and adventitious root growth, senescence, leaf abscission, and flowering [4-6]. However, despite the tremendous importance of auxins for plant development, the role of auxin in plant defense is still not fully understood. Several lines of evidence indicate that indole-3-acetic acid (IAA), the major auxin in plants, exerts a negative role in plant stress resistance, and that investing in plant growth imposes a penalty on plant defense, and vice versa [7-10]; a phenomenon referred to as the growth-defense trade-off [11]. In line with these affirmations, it has been described that activation of IAA mediated stem elongation in response to light, increases the susceptibility of Arabidopsis thaliana and Chenopodium album to different pathogens, such as the bacteria Pseudomonas syringae or the herbivore Spodoptera exigua [12,13]. Likewise, Mutka and co-workers (2013) showed that elevation of endogenous IAA levels in Arabidopsis decreases plant tolerance against P. syringae. Conversely, another series of studies suggests that auxin can positively influence plant tolerance. For example, it has been demonstrated that the auxin signaling component AUXIN RESPONSE FACTOR3 (ARF3) controls the formation of leaf trichomes, considered a direct defense mechanism against predators [15,16]. In addition, analysis carried out using the Arabidopsis auxin reporter line DR5::GUS showed that mechanical wounding stimulates IAA signaling in neighboring unwounded plants [17], which suggests an indirect defense mechanism that allows plants to get prepared for a possible imminent attack. Taking all together, we intend not only to shed light on the implication of auxin in plant biotic stress responses, but also to provide new molecular targets that mediate the trade-off between plant growth and defense.

In this context, jasmonates play a prominent role. Jasmonates are a group of lipid-derived hormones comprising jasmonic acid (JA) and several JA derivatives [18]. These molecules have an essential role in counteracting abiotic and biotic stress responses, such as pathogen and herbivore attack [19-21]. Upon stress recognition, the production of the bioactive JA, jasmonoyl-L-isoleucine (JA-Ile) is stimulated and perceived by the protein CORONATINE INSENSITIVE 1 (COI1). This enables the Skp-cullin 1-F-box (SCF) E3 ligase complex to bind and ubiquitinate specific repressor proteins called JASMONATE ZYM DOMAIN (JAZ). Degradation of JAZs by the 26S proteosome releases the MYC family of basic helix-loop-helix (bHLH) transcription factors from repression, which subsequently triggers the expression of different subsets of JA-responsive genes [11,22,23].

Over the past few years, a wide array of links between JA and auxin signaling pathways has been reported. For instance, JA has been demonstrated to negatively affect primary root growth in A. thaliana through the transcriptional repression of PLETHORA genes expression, namely of PLT1 and PLT2, which are known as essential transcription factors controlling auxin-regulated root meristem specification and maintenance [24]. A more recent work described a crosstalk model in which wound-inducible amidohydrolases contribute to the cellular regulation of JA and auxin levels to coordinate stress responses by controlling JA- and IAA-amino acid conjugate contents [16]. In contrast, a series of
publications emphasized that JA exerts a direct stimulating effect on various phases of auxin biosynthesis. Dombrecht et al. (2007) reported that the JA-associated transcription factor MYC2 controls the formation of auxin biosynthesis precursors as well as auxin-related defense compounds, such as indole glucosinolates. Furthermore, JA has been shown to promote auxin \textit{de novo}-biosynthesis through the transcriptional activation of two anthranilate synthase genes, \textit{ASA1} and \textit{ASB1}, resulting in elevated L-tryptophan levels and, thus, increased precursor availability for auxin biosynthesis [26]. A more direct impact on auxin biosynthesis has been reported by Hentrich et al. (20013) . The authors provided evidence for the JA-dependent transcriptional activation of two Arabidopsis \textit{YUCCA} genes, \textit{YUC8} and \textit{YUC9}. Intriguingly, \textit{YUCCA} enzymes are considered key players in general IAA biosynthesis, along with tryptophan aminotransferases [28-31]. Importantly, the gene expression studies presented by Hentrich et al. (2013a) demonstrated that the \textit{YUC8} and \textit{YUC9} transcriptional response towards the treatment with different oxylipins, such methyl jasmonate (MeJA) and its precursor 12-oxo-phytodienoic acid (OPDA), is almost entirely impaired in the \textit{coi1} mutant background. Based on this experiment, it can be speculated that the transcriptional regulation of \textit{YUC8} and \textit{YUC9} depends on the COI-JAZ-MYC signaling module.

In this work, we show that the Arabidopsis transcription factor MYC2, and its closest homologs MYC3 and MYC4 [32], play a direct role in the regulation of auxin biosynthesis through the control of \textit{YUC8} and \textit{YUC9} gene expression. We demonstrate that different \textit{myc} knockout mutants display a significant reduction not only in JA-mediated IAA production, but also in \textit{YUC8} and \textit{YUC9} transcript accumulation. Moreover, our transient transactivation analyses in \textit{Nicotiana benthamiana} and Arabidopsis protoplasts clearly demonstrate that MYC2, MYC3 and MYC4 bind to a specific tandem G-box motif abundant in the promoter regions of \textit{YUC8} and \textit{YUC9}, which is not abundant in those of the other \textit{YUCCA} genes. Finally, \textit{YUC8} and \textit{YUC9} mutants have been exposed to the two-spotted spider mite \textit{Tetranychus urticae}, which provided compelling evidence that the overexpression of the two genes rendered the mutant plants more resistant toward herbivore predators. Taken together, these results provide evidence for a signal transduction mechanism that employs the COI-JAZ-MYC module to fine-tune the expression of auxin biosynthesis-related genes in response to wounding and resistance to phytophagous mites.

2. Results

The jasmonate signaling components MYC2, MYC3 and MYC4 are required for auxin formation after JA treatment

Firstly, we investigated whether MYC transcription factors are directly involved in \textit{de novo} IAA synthesis. To this end, we quantified the endogenous IAA levels in \textit{A. thaliana} wild-type seedlings (Col-0), as well as different \textit{myc} knockout mutants, 4 h after the treatment with 50 \textmu M MeJA by gas chromatography-tandem mass spectrometry (GC-MS/MS) (Figure 1). Confirming previous observations [27], application of MeJA to WT seedlings
resulted in more than 5-fold enhanced IAA formation relative to mock-treated control seedlings (0.5% MeOH (v/v)). In contrast, the IAA production in response to MeJA-treatment appeared to be clearly impaired in all myc single mutants, as well as in the myc2/myc3 and myc2/myc4 double and myc2/myc3/myc4 triple mutants. The strongest effect was visible in the triple mutant, displaying a 0.5-fold reduction of IAA levels in comparison to the respective mock-treated control. The additive effect observed in the triple mutant indicates that MYC2, MYC3 and MYC4 transcription factors collaborate in the control of JA-mediated IAA accumulation. Remarkably, although all single myc mutants displayed a general tendency of impaired MeJA-mediated IAA formation (no significant differences to the mock-treated controls), myc3/myc4 seedlings exhibited a remaining significant increase in IAA contents in response to the MeJA application. However, with an increase of approximately 3.5-fold over the corresponding mock-treated myc3/myc4 seedlings, the exhibited response was still weaker than the one detected for wild-type seedlings.

Figure 1. JA-mediated IAA biosynthesis is impaired in several Arabidopsis myc knockout mutants. IAA levels were measured by GC-MS/MS in 10 days-old seedlings treated with 50 µM MeJA or mock solution (0.5% MeOH (v/v)) for 4h. The IAA accumulation was calculated as a ratio of the endogenous IAA to the stable isotope-labelled [2H2]-MeIAA. Then, the final relative IAA content is given by the difference of auxin levels in each MeJA treated sample and the respective control and expressed as pmol IAA per gram fresh weight. The levels of samples without MeJA treatment (0.5% MeOH (v/v)) were arbitrarily set to 1. Values are mean ± SE (n = 3). Statistical analysis was performed using Student’s t-test comparing the mock treated seedlings with the respective MeJA treated WT or myc loss-of-function mutant. Significant differences between means are indicated with asterisk (*P < 0.05, **P < 0.01).

In summary, the presented results support the hypothesis of an intimate crosstalk between JA signaling and IAA biosynthesis. At the same time, the obtained data suggest a leading role of MYC2 in this process.

**YUC8 and YUC9 expression is suppressed in myc loss-of-function mutants**

To evaluate the role of MYC transcription factors in the transcriptional activation of YUC8 and YUC9, we conducted quantitative reverse transcriptase PCR (qRT-PCR) analyses after
seedlings were treated with exogenous MeJA. In line with the results presented above, we observed that the abundance of YUC8 and YUC9 transcripts increased 1.3-fold and 8.7-fold in WT seedlings respectively (Figure 2). Moreover, we found that the gene expression of both YUC genes was unaffected by MeJA treatment in practically all single, double, and triple myc knockout mutants, relative to the respective mock treated seedlings. Surprisingly, in both myc3 and myc2myc3 the YUC8 transcription was slightly induced after 4 h of MeJA treatment (0.67-fold induction and 1.4-fold induction, respectively) (Figure 2A). Likewise, in the myc4 background YUC9 expression was highly induced after 2h of MeJA application (7.8-fold induction) (Figure 2B). In summary, these data highlight that YUC8 transcription is driven by MYC2 and MYC4. While MYC2 and MYC3 act as major regulators of YUC9 gene expression in response to oxylipins. It is noteworthy that, when studying the myc2myc3myc4 triple mutant, we did not observe a complete suppression of the YUC8 and YUC9 transcription (Figure 2A,B). This observation may be interpreted as an indication for a compensatory mechanism to attenuate the absence of JA response in plants.

**Figure 2.** MY2, MYC3 and MYC4 control YUC8 and YUC9 gene expression in response to MeJA. (A) Quantitative RT-PCR analysis of YUC8 after 4 h of MeJA induction treatment. (B) YUC9 expression levels after 2 h of MeJA treatment. Both YUC8 and YUC9 transcript levels are given relative to the reference genes APT1 and UBI10 and normalized using the mock treated seedlings (0.5% MeOH (v/v)). Data shown are mean ± SE (n = 3). A two-fold change between the control mock treated seedlings and the respective myc ko mutant was considered as differentially regulated (* fold-change ≥ 2).

**In silico** analysis of YUCCA promoter sequences indicates a conserved MYC2, MYC3 and MYC4 binding sites in the promoter of YUC8 and YUC9

To further confirm the role of MYC2, MYC3 and MYC4 in the transcriptional regulation of YUC8 and YUC9, the 3000 bp sequence upstream of the transcription start was isolated for each A. thaliana YUCCA gene and used to predict MYC binding motifs, i.e., G-box and sixteen described G-box variants [32]. As shown in Figure 3, we observed that the jasmonate responsive elements, G-box and G-box variants, are highly enriched in all YUCCA promoter sequences. However, we detected that only pYUC8 and pYUC9 contain a specific “tandem” DNA binding motif configuration. This “tandem” includes two
canonical G-boxes (5'-CACGTG-3') and one G-box variant 9 (5'-CACGTC-3') at the nucleotide positions -535, -555 and -571 in case of pYUC8, and -1240, -1247 and -1272 in pYUC9. In addition, we found that this configuration is followed by the G-box variant 3 (5'-CATGTG-3') in positions -140 and -207 of pYUC8 and pYUC9, respectively. Remarkably, the G-box variant 3 was relatively close to a 5'-TATAAA-3' sequence, in positions -153 (pYUC8) and -267 (pYUC9). This sequence was identified as the consensus TATA-box, which is commonly recognized as a transcription enhancer [33]. In conclusion, the prediction of putative cis-elements prompted us to hypothesize that this G-box configuration may be crucial for the transcriptional regulation of both YUC8 and YUC9.
Figure 3. The promoter region of YUC8 and YUC9 presents a specific G-boxes binding motif configuration. Schematic representation of the distribution of cis-regulatory elements (G-box and G-box variants) in the promoter of Arabidopsis YUCCA genes. The conserved promoter region (from -3000 to -1) upstream to the transcriptional start point (ATG) of the eleven YUCCA family members is shown. All putative G-boxes are color-coded (square) and assigned with a specific sequence. Numbers indicate the corresponding G-box sequence, with number 1 being the canonical G-box. Different color indicates different experimental MYC2 binding affinities [32].
Myc2 regulates YUC8 and YUC9 expression through the interaction with G-box elements

We next studied whether the observed DNA binding sites are indeed involved in the transcriptional regulation of YUC8 and YUC9. To this end we performed a transient transactivation effector-reporter experiment in N. benthamiana leaves. To prepare the effector plasmid, the independent MYC2, MYC3 or MYC4 open reading frames (ORFs) were isolated and fused to the Cauliflower mosaic virus (CaMV) 35S promoter to generate three constructs, i.e. 35S::MYC2, 35S::MYC3, and 35S::MYC4 (Figure 4A). As reporter, a series of truncated promoter fragments from YUC8 or YUC9, containing the tandem DNA motifs (191) or the final cis-acting element (#3), as well as a promoter segment containing none of these regulatory sequences (Ø), were amplified by PCR and cloned into a vector carrying the β-glucuronidase (GUS) reporter gene. This resulted in the generation of three promoter constructs for pYUC8, i.e. 191::GUS, 3::GUS and Ø::GUS, and three promoter constructs for pYUC9, i.e. 191::GUS, 3::GUS and Ø::GUS (Figure 4A). Thereafter, we investigated GUS expression levels after Agrobacterium tumefaciens-mediated plant transformation (Figure 4B). Remarkably, the blue coloration demonstrated that the GUS expression, driven by the 191::GUS and 3::GUS constructs of both YUC8 and YUC9 promoters, was strongly activated in presence of either of the transcription factors MYC2, MYC3 or MYC4 relative to the fusion constructs Ø::GUS. Likewise, the detected weak blue staining perceived for the Ø::GUS constructs indicated a very weak background activity.

In a second experiment, another group of agroinfiltrated leaf discs were utilized to quantify the transactivation of GUS reporter gene expression employing a fluorometric technique (Supplementary Figure A1). In agreement with the histochemical results we observed that the GUS activity of 191::GUS and 3::GUS samples increased 2-fold and 0.8-fold in pYUC8, and 2.3-fold and 1.3-fold in pYUC9, respectively in presence of MYC2. However, when the N. benthamiana leaves were co-infiltrated with different pYUC8 constructs and 35::MYC3, the enzymatic analysis indicated that only the 191::GUS construct was significantly activated, with almost 1.5-fold increase of the GUS activity compared to the negative control (empty vector). Interestingly, the absence of GUS activity in 3::GUS may indicate that MYC3 does not effectively bind to the 5’-CATGTG-3’ element #3. Nevertheless, the analysis of the YUC9 promoter called this interpretation into question, since the fluorometric assay showed a strong activation of the GUS activity in 191::GUS and 3::GUS relative to the empty vector control (2.5-fold and 11.5-fold increased GUS activity, respectively). Finally, we detected that the transcription factor MYC4 significantly activated the 3::GUS construct in the pYUC8, whereas in the pYUC9, both 191::GUS and 3::GUS showed 4.5-fold and 4-fold increased GUS activity levels, respectively, when compared to the negative control (Supplementary Figure A1). In contrast to MYC2 and MYC3, the presence of MYC4 only resulted in a moderate GUS activity for the Ø::GUS construct of pYUC8. This promoter fragment contains the G-box variant #11 (5’-CAAATG-3’) in its sequence, suggesting that this cis-acting element could be important in the transcriptional regulation of YUC8 exerted by MYC4. Overall, these analyses highlight MYC2 as the powerful positive regulator of both auxin biosynthetic genes, most probably through the interaction with the promoter G-box elements included in the “tandem” 1-9-1 (5’-CACGTG - CACGTC - CACGTG-3’), and/or the final cis-
regulatory G-box #3 (5’-CATGTG-3’). The results also suggest that MYC3 and MYC4 could act as possible co-operators in the transcriptional regulation of YUC8 and YUC9.

Figure 4. Transactivation of the pYUC8::GUS and pYUC9::GUS by MYC2, MYC3 and MYC4 proteins using agroinfiltrated N. benthamiana leaves. (A) Schematic representation of the effector and reporter constructs used in the transient expression experiment. The effector constructs contain the CaMV 35S promoter fused to the MYC2, MYC3 and MYC4 coding sequences. The reporter constructs contain different combinations of the G-box binding sites found in the YUC8/9 promoters, i.e., the tandem 1-9-1 (5’-CACGTG - CACGTC - CACGTG-3’); the final cis-regulatory G-box #3 (5’-CATGTG-3’). Moreover, Ø refers to the promoter fragment lacking any of the mentioned DNA binding sites. All reporter constructs were fused to the GUS reporter gene, followed by the Nos terminator cassette. (B) Histochemical GUS staining of N. benthamiana leaf discs agroinfiltrated with the 191::GUS and 3::GUS constructs contain in the pYUC8/9 promoters in presence of the constitutively expressed MYC2, MYC3 and MYC4 transcription factors. The Ø::GUS construct was used as negative control.

To independently validate the interaction between MYC2 and YUC8/9 promoters by an alternative in planta method, we performed a third experiment in mesophyll protoplast of A. thaliana (Figure 5). Here, constructs analogous to those used for transient expression in N. benthamiana leaves were employed. They only differ in the plasmid backbone used for cloning. In addition, an empty pBT-10 vector was co-transfected with the 35S::MYC2 effector constructs as negative control (Figure 5A). Confirming our previous findings, the relative values of GUS activity revealed that, in comparison to the negative control, the transcription factor MYC2 significantly activates the GUS reporter gene of the 191::GUS and 3::GUS constructs in both pYUC8 and pYUC9 (Figure 5B). Remarkably, the detected enzymatic GUS activity for the construct pYUC9-Ø::GUS, showed an increment of approximately 1.5-fold in comparison to the empty vector control. Like in the transactivation assay in N. benthamiana, this could be explained by the presence of the G-
box variants 5 (5'-CACGCG-3') and 10 (5'-TACGTG-3') in the promoter sequence used for these particular constructs. However, this conclusion is mainly based on the observation that no significant differences were found when compared to the negative control, which might be due to the high variability registered for the Ø::GUS samples. Most importantly, our results further support the notion that MYC2 directly binds to the G-box elements to promote YUC8 and YUC9 gene expression.

**Figure 5.** Transcriptional activity assay in *A. thaliana* mesophyll protoplasts. (A) Schematic representation of the effector constructs 35S::MYC2 and the 191::GUS, 3::GUS and Ø::GUS reporter constructs used. (B) Fluorometric GUS activity quantification. The empty vector was used as negative control. The GUS activation levels were relativized to the NAN reporter gene activity and normalized to the empty vector. Final GUS activity levels are expressed as pmol 4-methylumbelliferone (MU)/min. Values are mean ± SE. Three aliquots per protoplast suspension were analyzed. Similar results were obtained in two independent experiments. Asterisks indicate Student’s *t*-test significant differences (*P < 0.05, **P < 0.01).
YUC9 plays a role in biotic stress responses

The bHLH transcription factor MYC2 plays a key role in JA-mediated defense response against herbivores and necrotrophic pathogens [23],[34-36]. This prompted us to investigate the activation of YUC8 and YUC9 expression by a phytophagous pest. To this end, 3 to 4 weeks-old wild-type plants and the reporter lines pYUC8::GUS, pYUC9::GUS, and pAOS::GUS were exposed to the two-spotted spider mite, Tetranychus urticae. Subsequent GUS staining clearly revealed a strong reporter activity for the positive control, the AOS (At5g42650) promoter line, and the YUC9 promoter driven construct (Figure 6A). On the contrary, the absence of visible GUS activity in pYUC8::GUS leaves exposed to T. urticae suggests that YUC8 is possibly not involve in the defense against pests or that the response of YUC8 is slower than the response of YUC9, which has previously been suggested for oxylipin treatments (Hentrich et al., 2013a). In view of this result, we intended to shed light on the biological meaning of auxin synthesis driven by MYC2. Thus, WT, the overexpression line YUC9ox, and the yuc9 knockout line (yuc9ko), were used to analyze as to whether alterations in YUC9 expression has an influence on the susceptibility of plants toward herbivorous predators. To this end, ten adult female spider mites were placed on single leaves of ten plants for each genotype and allowed to feed for 4 days. The leaf damage quantification highlighted a preference of adult mites to feed on WT rather than YUC9ox plants. This is displayed by approximately 40% lower leaf damage area of YUC9ox compared to WT (Figure 6B). Interestingly, the yuc9ko mutants exhibited a decreased leaf damage area in comparison to WT, but the difference is statistically insignificant. Consistent with these observations, the DAB staining and the trypan blue exclusion test determined a visibly higher accumulation of reactive oxygen species (ROS) and cell death in WT and yuc9ko plants in comparison to YUC9ox (Supplementary Figure A2, Supplementary methods A2 and A3).

Figure 6. YUC9 activation and plant damage assay after 4 days of spider mite (Tetranychus urticae) herbivory. (A) Histochemical GUS staining of leaves from wild-type Arabidopsis, pYUC8::GUS, pYUC9::GUS and pAOS::GUS plants, using 20 females from T. urticae per plant (n = 5). Scale bar = 50 μm. (B) Quantification of the total plant damage area (expressed in mm²) in WT, yuc9ko and YUC9ox mutant lines. Represented are means ± SE (n = 5). Student’s t-test: **P < 0.01.

3. Discussion

The first indication of a positive JA-IAA crosstalk arouses from the observation that both
hormones share a conserved signal transduction mechanism that utilizes the 26S-proteosome system [37-39]. In line with this, during a mutant screening it became evident that a newly identified MeJA insensitive mutant was allelic to *axr1* defective mutant, which is impaired in auxin signaling [40], suggesting that AXR1 contributes to both, IAA and JA, perception. Likewise, it has been demonstrated that a point mutation in one subunit forming the SCF-E3 ligase complex of Arabidopsis resulted not only in reduced auxin response, but also in diminished expression of several JA-related genes [41]. In addition, it has been observed that JA and IAA cooperate spatiotemporally to regulate flower development and fertility through the action of ARF6 and ARF8 [42]. In rice, the IAA-gradient generated during gravitropism is accompanied by a reciprocally oriented JA-gradient to co-regulate asymmetrical growth [43]. On the other hand, interconnectivity between JA signaling and IAA biosynthesis pathways have also been reported. Thus, two independent transcriptomic analysis revealed that among the group of up-regulated genes two *YUCCA* genes, *YUC8* and *YUC9*, were differentially induced by OPDA and MeJA [44, 45]. Meanwhile, Hentrich et al., (2013a) clearly demonstrated that wound-induced formation of MeJA in Col-0 Arabidopsis leaves is sufficient to trigger *YUC9* expression. Nevertheless, the molecular mechanism that controls *YUC8/9* expression remained uncertain.

In our effort to address whether these two auxin biosynthetic genes are direct downstream targets of the JA signaling pathway, we uncovered that MYC2, MYC3 and MYC4 are involved in *YUC8* and *YUC9* transcriptional regulation. Our GC-MS/MS and qRT-PCR experiments highlighted that JA-dependent IAA production driven by *YUC8* and *YUC9* is considerably dependent on the master JA regulator MYC2 (Figures 1-2). Likewise, we observed that MYC3 and MYC4 additionally contributed to auxin homeostasis, thereby confirming that these two transcription factors are phylogenetically closely related to MYC2 (Figure 1). We furthermore confirmed that MYC3 and MYC4 might also have a role in the transcriptional regulation of *YUC8* and *YUC9*, respectively. However, since we detected an induction of both genes in some loss-of-function mutants after MeJA treatment, the possibility that alternative regulatory elements also contribute to the translational control of *YUC8* and *YUC9* cannot be excluded (Figure 2A,B). Further extending this hypothesis, it is known that PIF4, which triggers hypocotyl elongation under high temperatures, can effectively bind to the G-motif located in the *YUC8* promoter [46]. Furthermore, the jasmonate inducible ETHYLENE RESPONSE FACTOR 109 (ERF109) has been reported to physically interact with the DNA-binding site 5'-GCCGCC-3' to control ASA1 and YUC2 transcript accumulation [47]. Nevertheless, we did not identify the mentioned GCC-box motif in the promoter region of neither *YUC8* nor *YUC9*.

Here we demonstrated that all three described MYC proteins bind with similar, although not identical affinities to the core 5'-CACGTC-3' motif, called G-box, and its variants [32], [48]. We analyzed the presence of these JA-responsive elements in the promoter sequence of the eleven Arabidopsis YUCCA members. Our results clearly identified a specific G-box motif configuration composed by the “tandem” 1-9-1 (5'-CACGTG-CACGTC-CACGTG-3') followed by the G-box 3 (5'-CATGTG-3') in the non-coding region of *YUC8* and *YUC9* (Figure 3). We provided multiple lines of supporting evidence, including
effector-reported assay in *N. benthamiana* and *A. thaliana* leaf protoplasts, to demonstrate that all three MYC transcription factors bind to the YUC8 and YUC9 promoters *in vivo*, when the 1-9-1 G-box-tandem or the 3 G-box variants are present (Figures 4, 5 and Supplementary Figure A1). Intriguingly, our experiments employing leaf protoplast further corroborated that MYC2 functions as a direct regulator of YUC9 (Figure 5B).

Recently, Santamaria *et al.*, (2017) demonstrated that *T. urticae* infestation of Arabidopsis plants activates the MYC2 defense pathway. Taking advantage of this finding, we investigated the biological role of JA-induced IAA biosynthesis by performing a *T. urticae* feeding experiment. As shown in Figure 6A, the pest not only activated the AOS promoter, which is known to respond to wounding [50], but also the YUC9 promoter. Recently, Zhurov *et al.*, (2014) reported the significant induction of JA production by *T. urticae* feeding in Arabidopsis Col-0 plants. Moreover, a more recent publication performed in *Nicotiana attenuata* showed the accumulation of auxin at the site of herbivory by *Manduca sexta* [52]. Intriguingly, this auxin accumulation was accompanied by a rapid activation of two YUCCA-like proteins. Thus, our results highlight the importance of the intimate crosstalk between JA and IAA through the modulation of YUC9 expression in plant defense responses. Moreover, the *T. urticae* infestation experiments showed that the auxin overproducer line, YUC9ox, exhibited reduced plant-damage, H2O2 accumulation and cell death in comparison to similarly treated wild-type plants (Figure 6B and Supplementary Figure A2). It has been demonstrated that feeding of *T. urticae* on plant leaves proceeds via the insertion of their stylet between the pavement cells or through the open stoma [53]. Thus, it can be speculated that epidermal cell expansion is the cause of this enhanced tolerance by limiting spider mite feeding, rather than IAA-mediated immune activation. Congruent with this hypothesis, it has been observed that the transient overexpression of YUC9 in *N. benthamiana* leaves resulted in significantly expanded pavement cells [27]. Alternatively, it is known that IAA and the biotic-stress related hormone ethylene, can interact at multiple levels [54]. For instance, earlier studies indicated that IAA stimulates ethylene production thought the action of specific ACC-synthase genes (ACS), which encode enzymes involved in a rate-limiting step of ethylene biosynthesis [55-59]. Likewise, Hentrich *et al.*, (2013b) reported up-regulation of several ethylene biosynthesis and signaling related genes in YUC8ox and YUC9ox lines. Therefore, we suggest that the JA-IAA-ET induced lignification contributes to complicate mite feeding or reduce palatability by increasing leaf rigidity and possibly reducing leaf nutritional values. The latter aspect is particularly supported by the observation that the spider mites actively left YUC9ox leaves, indicating that the spider mites detest feeding on those leaves.

4. Material and Methods

Plant Material

In our study the *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as wild type (WT). All different Arabidopsis myc mutants, *i.e.* single, double and triple T-DNA insertion lines myc2, myc3, myc4, myc2myc3, myc2myc4, myc3myc4 and myc2myc3myc4, and the overexpression lines YUC9ox, as well as the T-DNA insertion mutant yuc9ko and the
reporter lines pAOS::GUS, pYUC8::GUS and pYUC9::GUS have been previously described elsewhere [27], [32], [50], [60]. Seeds were surface sterilized and sown on solidified ½-strength MS medium supplemented with 1% sucrose and stratified at 4 ºC for 48 h in darkness. Plant growth was performed under controlled conditions (22 ºC, 16 h light/8 h dark and 100 μmol/m² s light intensity). For plant defense experiments, we transferred 10-days old Arabidopsis plants to a mixture of peat and vermiculite (3:1) and we grew them under the same condition described above. The transactivation assay was carried out in 14-days old N. benthamiana seedlings transplanted to independent 13 cm diameter pots containing peat-based soil. Plants were grown under controlled greenhouse conditions (25 ºC and 40-65 % relative humidity) and a long-day photoperiod (16 h light/8 h dark) for 2 to 3 weeks.

qRT-PCR analysis

Here, we incubated 10-days old Arabidopsis seedlings with either 50 µM of MeJA or a control solution (0.5% methanol, v/v) over either 2 or 4 h, respectively [27]. Afterwards, total RNA was isolated using the phenol:chloroform method, coupled to lithium chloride precipitation, according to Box et al., (2011). Extracted RNA was reverse-transcribed into complementary DNA (cDNA) using the RNA-dependent DNA polymerase from Moloney-Murine Leukemia Virus (M-MLV) (Promega) following the manufactured instructions. Quantitative RT-PCRs were performed using a LightCycler® 480 (Roche) thermocycler following the manufacturer’s instructions. For data accuracy, three independent biological replicates were tested in triplicates. The relative gene expression levels were calculated according the $2^{\Delta \Delta Ct}$ method [62,63]. Primers used for analyzing mRNA levels are listed in Supplemental Table A1. APT1 and UBI10 were selected as the reference genes for data normalization [64].

Auxin quantification

Extraction of IAA was carried out according to Carrasco-Loba & Pollmann (2017). To this end, we harvested approximately 100 mg of 10 days-old seedlings in microcentrifuge tubes containing 1 ml of methanol and 50 pmol of the internal standard [2H2]-IAA (OlChemIm Ltd). After hormone extraction, IAA concentrations was examined by gas chromatography-mass spectrometry (GC-MS/MS). In brief, dried samples were resuspended in 20 µl of the freshly prepared derivatization solution [88 % acetone:methanol (9:1, v/v), 11.8 % diethyl ether, 1.2 % Trimethylsilyl diazomethane, 2 M in diethyl ether]. After incubation for 30 min at RT, 1 µl of the derivatized sample was injected splitless using a CLC CombiPal autosampler into a BRUKER Daltonics 451 gas chromatograph equipped with a stationary phase ZB-35 (30 m x 0.25 mm, 0.25 µm film) fused silica capillary column (Phenomenex). Helium at a flow rate of 1 ml min⁻¹ was used as the mobile phase for separation. The injector temperature was set to 250 ºC and the column was held at 50 ºC for 1.2 min, then temperature was increased by 30 ºC min⁻¹ to
120 °C, and finally to 325 °C by 10 °C min⁻¹ and held there for 4 min more. The column effluent was introduced into the ion source of a Scion-TQ triple quadrupole mass spectrometer (BRUKER Daltonics). The transfer line and the ion source temperatures were maintained at 250 °C and 200 °C, respectively. Ions were generated by a 70 eV electron beam at an ionization current of 80 µA, and 30 spectra s⁻¹ were recorded in the mass range of 50 to 600 m/z. Under the given conditions the retention time for the endogenous methylated-IAA hormone was 13.6 min. For quantification, we selected the following precursor ions and corresponding diagnostic product ions; MeIAA (189/130) and [²H₂]-MeIAA (191/132).

**In silico analysis of YUCCA promoter sequences**

Firstly, the full-length promoter sequences from the *A. thaliana* YUC8 and YUC9 genes were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/gene/) and aligned using the online EMBOSS Matcher tool for pairwise sequence alignment. Following the determination of similarities in the promoter sequences, the predicted conserved regions obtained for the *A. thaliana* YUC8 and YUC9 promoter were used to identify and isolate the equivalent promoter sequences for the rest of the *A. thaliana* YUCCA family members. All different promoter sequences used in this work were retrieved from the NCBI gene database using the corresponding gene accession numbers: At4g32450 (YUC1), At4g13260 (YUC2), At1g04610 (YUC3), At5g11320 (YUC4), At5g43890 (YUC5), At5g25620 (YUC6), At2g32320 (YUC7), At4g28720 (YUC8), At1g04180 (YUC9), At1g48910 (YUC10), At1g21430 (YUC11). MYC2 binding motifs in the YUC promoter sequences were predicted by running target sequences against known cis-regulatory elements in the AtPan collection (http://atpan.itps.ncku.edu.tw/)[66] and PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [67] databases. To ensure the incorporation of all the possible G-box variants described by Fernández-Calvo et al. (2011) the promoter sequences were also manually inspected.

**Transient expression analysis in Nicotiana benthamiana**

The YUC8 and YUC9 promoter sequences, as well as the coding sequences from MYC2, MYC3 and MYC4 were amplified using PCR specific primers (Supplementary Table A1) and introduced into the entry vector pSP-Entry1 (Stephan Pollmann, not published). Subsequently, pYUC8/9::GUS and 35S::MYC2/3/4 constructs were obtained by transferring the target DNA fragments into the destination vectors pMDC-163 [68] or p35S-HA-GW [69,70] by LR reactions (Invitrogen). Then, we performed the *A. tumefaciens*-mediated transient expression as described in Ma et al., (2012). Briefly, Agrobacterium strain C58C1 carrying the desired construct and the Agrobacterium strain P19 carrying the suppressor of gene silencing from tomato bushy stunt virus (TBSV) were infiltrated into 3 to 4 weeks-old *N. benthamiana* plants. 3 days post inoculation, the infiltrated leaves were collected and the β-glucuronidase (GUS) activity was evaluated by histochemical analysis as detailed by Jefferson et al. (1987).
We also quantified GUS expression levels using a fluorometric analysis [73]. For this purpose, two leaf discs were frozen in liquid nitrogen (N\textsubscript{2}), ground and re-suspended in 150 µl of GUS extraction solution [50 mM sodium phosphate buffer Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4} pH 7.5, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauroylsarcosinate (Sigma-Aldrich), 0.05% (v/v) β-MeEtOH]. An aliquot of 10 µl was utilized for total protein content determination [74] using bovine-γ-globulin as the protein standard (Bio-Rad). Whereas an aliquot of 100 µl of the suspension was transferred to a 96-well clear flat bottom microtiter plate (Falcon) and mixed with 100 µl GUS extraction solution containing 4 mM of 4-methylumbeliferyl-β-D-glucuronide (4-MUG) (Duchefa). Samples were then incubated at 37 °C in the dark for 10 min (T\textsubscript{0}). After the incubation, 100 µl the 4-MUG solution were transferred to a new 96-well plate and the reaction was stopped by the addition of 100 µl of 200 mM Na\textsubscript{2}CO\textsubscript{3}. The remaining sample (100 µl) was further incubated at 37 °C in darkness for 1 h (T\textsubscript{60}). Subsequently, fluorescence was measured at 360 nm excitation and 460 nm emission (56 gain, 10 flashes, 50 % mirror) using a TECAN Genios Pro fluorescence spectrometer (MTX Lab Systems). The GUS activity was calculated using the following equations:

\[
\text{GUS Activity} = \frac{GUS - Activity}{\text{mg of total protein}}
\]

\[
GUS - Activity \ [\text{pmol/min}] = \frac{\Delta F / 10}{t}
\]

Where ∆F is the difference in fluorescence intensity T\textsubscript{60}-T\textsubscript{0}, 10 are the fluorescence units corresponding to 1 pmol of hydrolysed 4-MUG and t is the time of incubation. Two independent experiments were carried out and GUS activity was quantified in triplicates to ensure accuracy.

**Arabidopsis protoplast-based transient expression**

To generate the reporter plasmids pYUC8::GUS and pYUC9::GUS, we amplified the promoter sequences of YUC8 and YUC9 containing different MYC2/3/4 binding sites, using PCR specific primers (Supplementary Table A1) and ligated into the pGEM\textsuperscript{®}-T vector (Promega). Thereafter, DNA fragments were digested by restriction endonucleases and cloned into the PBT-10 plasmid [75]. On the other hand, the effector plasmids 35S:MYC2/3/4 were made as described above. In this case, however, pEarlyGate-210 [76] was used as destination vector. After construct generation, mesophyll protoplast isolation and PEG-calcium mediated DNA transfection were performed according to Mathur & Koncz (1998), Yoo et al. (2007), and Alonso et al. (2009). In this work, 9 µg of each reporter plasmid and 14 µg of the effector plasmid were utilized. Moreover, to normalize the transfection efficiency, 3 µg of the 35S::neuroaminidase (NAN) plasmid [73] were used. Then, GUS transactivation was quantified by fluorometric analysis as already described. Furthermore, NAN activity was determined according to Kirby & Kavanagh (2002). To do this, from the 150 µl resuspended protoplasts in GUS extraction solution a 10 µl aliquot
was mixed with 10 µl NAN extraction solution [50 mM NaHPO₄/NH₄PO₄ pH 7.0, 10 mM EDTA, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) sodium lauroylsarcosinate] containing freshly added 0.05 % (v/v) β-MeEtOH and 1 mM 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUN) (Duchefa). The protoplasts were then incubated at 37 ºC in the dark for 10 min (T₀). After the incubation, 3.3 µl of the protoplast suspension was transferred to a 200 µl of NAN stop solution [330 mM Na₂CO₃]. The remaining protoplast/4-MUN solution was incubated at 37 ºC in darkness for 1 h (T₆₀). Afterwards, the fluorescence was measured as described before. NAN activity was calculated as [80]:

\[ \text{NAN Activity [pmol/min]} = \frac{\Delta F/10}{t} \]  

(3)

Where ΔF is the difference in fluorescence T₆₀-T₀, 10 are the fluorescence units corresponding to 1 pmol of hydrolysed 4-MUN and t is the time of incubation. Normalization of the GUS-activity was performed by calculating the ratio of GUS and NAN activities, represented as relative GUS/NAN units, following the equation:

\[ \frac{\text{GUS Activity}}{\text{NAN Activity}} = \frac{\text{GUS Activity}}{\text{NAN Activity}} \]  

(4)

To ensure data accuracy GUS and NAN activities were measured in triplicates and each experiment was repeated at least twice.

**Plant-arthropod interactions**

Adult female *T. urticae* spider mites, London strain, isolated from infested bean plants, were placed on leaves from 3 to 4-weeks-old *A. thaliana* plants according to Santamaria *et al.* (2017). The mites feed for four days in growth chambers (25 ºC, 70% relative humidity and with a 16/8 h (light/dark) regime). Histochemical analyses of GUS activity were performed as described by Jefferson *et al.* (1987). For leaf damage quantification whole rosette of infested and control plants were scanned using a resolution of 1200 dpi. Plant damage was assessed as total area of chlorotic spots based on scanned leaves overlaid with a grid of 0.25 mm x 0.25 mm using Adobe Photoshop CS5 software (Adobe Systems, San Jose, CA) according to the following equation:

\[ \text{Area damage [mm}^2]\text{ = number of dots x 0.25/0.25} \]

Herein, the number of dots was calculated from the total number of pixels (derived from the histogram tool) divided by the number of pixels per dot (52 pixel/dot) [81]. We assessed plant damage was in five infested independent samples from each genotype.

**Statistics**
The data were analyzed with Student’s t-test when two means were compared. Statistical analyses were realized employing the STATGRAPHICS® Centurion XVI (Statpoint Technologies, INC.).

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure A1. Fluorometric quantification of GUS activity in N. benthamiana leaves agroinfiltrated with different YUC8 and YUC9 promoter fragments. Figure A2. Histochemical analysis after spider mite herbivory. Methods A1. Trypan Blue staining. Methods A2. DAB staining. Table A1. List of primers used for cloning and qRT-PCR analysis.

**Author Contributions:** S.P. and M.-M.P.-A. conceived and designed the research; M.-M.P.-A., B.S.-P., P.O.-G., M.E.S., I.D. and S.P. performed the research and analysed the data; S.P. was responsible for funding acquisition and wrote and edited the manuscript together with M.-M.P.-A.. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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**Conflict of interest:** The authors declare no conflict of interests.

**Appendix A**

**Methods A1.** Trypan Blue staining. Three to four weeks old infested A. thaliana leaves were harvested after 4 days of T. urticae feeding and stained with 5 ml lactophenol-trypan blue solution (10 ml lactic acid, 10 ml phenol, 10 ml glycerol, 10 ml tryptan blue (Sigma), dissolved in 10 ml of distilled H2O) [1]. Before its use, the TB solution was diluted 1:2 with 100 % ethanol. The solution including two leaves was then boiled for 1 min and destained for 30 min at room temperature in 2 ml chloral hydrate solution (5 g of chloral hydrate dissolved in 2 ml distilled water). After overnight destaining, the chloral hydrate solution was removed and then 2 ml 50 % glycerol were added. Leaves were then placed on a microscope slide, covered with a cover slip and analyzed under bright-field lighting using a light stereomicroscope Leica MZ10F (Leica Microsystems) at a magnification of x8 and x40, respectively. Images were captured using a Leica DFC 400C camera (Leica Microsystems). Trypan blue staining was performed for five spider mite infested plants from each genotype and two control plants.

**Methods A2.** DAB staining. We examined H2O2 accumulation in three to four A. thaliana leaves exposed to 4 days of T. urticae feeding using the DAB staining method [2]. For this purpose, two leaves were placed in a 15 ml Falcon tubes, covered with 5 ml DAB solution (0.1 % (w/v) of 3,3-diaminobenzidine-HCl (pH 3.8)), vacuum infiltrated for 5 min and incubated overnight. After incubation, the DAB solution was supplemented with 10 mM ascorbic acid. Then, three subsequent washing steps with 5 ml ethanol/acetic acid/glycerol (v/v, 3:1:1) of 2 h each were performed to clear leaf tissues. Then 2 ml of 50% glycerol were added. Microscopy and imaging were carried out using a light stereomicroscope Leica MZ10F (Leica Microsystems), at a magnification of x8 and x40, and a Leica DFC 400C camera (Leica Microsystems). For this experiment were used, from each genotype, five spider mite infested plants and two control plants.
Appendix A References


Appendix B

A1. Fluorometric quantification of GUS activity in *N. benthamiana* leaves agroinfiltrated with different YUC8 and YUC9 promoter fragments. (A) Relative GUS activity of the 191::GUS, 3::GUS and ø::GUS reporter constructs co-transformed with the effector construct 35S::MYC2, (B) 35S::MYC3, and (C) 35S::MYC4. In this case the empty pMDC163 vector was generated by the deletion of ccdB operon [1] and used as negative control. GUS activity was then normalized according the empty vector and expressed as pmol 4-methylumbelliferone (MU)/mg protein/min. Protein amount was determined using Bradford method [2]. Data are mean ± SE. Three different leaves per plant were agroinfiltrated. The quantification of GUS activity for each promoter construct was measured in triplicates. Statistical analysis was performed using Student’s t-test comparing between the negative control and each promoter construct. Significant differences between means are indicated with asterisks (*P < 0.05, **P < 0.01).

A2. Histochemical analysis after spider mite herbivory. (A) DAB staining, (B) Trypan blue staining. Arrows indicate H2O2 accumulation and cell death, respectively. Images represent leaf details of control plants (top row) and plants exposed to 4 days spider mite feeding (bottom row). Scale bar = 1 mm.
**Table S1.** List of primers used for cloning and qRT-PCR analysis ([www.quantprimer.de](http://www.quantprimer.de)) [3]

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YUC9-qPCR For
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Appendix B references


References


