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The jasmonic acid signaling pathway is linked to auxin homeostasis through the modulation of *YUCCA8* and *YUCCA9* gene expression

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SUMMARY

Interactions between phytohormones play important roles in the regulation of plant growth and development, but knowledge of the networks controlling hormonal relationships, such as between oxylipins and auxins, is just emerging. Here, we report the transcriptional regulation of two Arabidopsis *YUCCA* genes, *YUC8* and *YUC9,* by oxylipins. Similarly to previously characterized *YUCCA* family members, we show that both *YUC8* and *YUC9* are involved in auxin biosynthesis, as demonstrated by the increased auxin contents and auxin-dependent phenotypes displayed by gain-of-function mutants as well as the significantly decreased IAA levels in *yuc8* and *yuc8/9* knockout lines. Gene expression data obtained by qPCR analysis and microscopic examination of promoter-reporter lines reveal an oxylipin-mediated regulation of *YUC9* expression that is dependent on the COI1 signal transduction pathway. In support of these findings, the roots of the analyzed *yuc* knockout mutants displayed a reduced response to methyl jasmonate (MeJA). The similar response of the *yuc8* and *yuc9* mutants to MeJA in cotyledons and hypocotyls suggests functional overlap of YUC8 and YUC9 in aerial tissues, while their function in roots show some specificity, likely in part related to different spatio-temporal expression patterns of the two genes. These results provide evidence for an intimate functional relationship between oxylipin signaling and auxin homeostasis.

Keywords

YUCCA genes; auxin; jasmonic acid; hormone signaling; plant hormone interaction; root development; Arabidopsis

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INTRODUCTION

Oxylipins, such as jasmonates and their precursor 12-oxo-phytodienoic acid (OPDA), are pleiotropic plant growth regulators that are widely represented throughout the plant kingdom (Böttcher and Pollmann, 2009). These bioactive signaling molecules regulate various plant developmental processes including anther dehiscence, pollen maturation, and root growth. In addition, they are well-known mediators of abiotic and biotic stress responses, e.g. to pathogen attack and herbivory (Howe and Jander, 2008; Wasternack, 2007).

There is mounting evidence of extensive interaction between phytohormones in the regulation on plant growth and development or their adaptation to external stimuli (Wolters and Jürgens, 2009). These interactions are in some cases governed by shared components of signal transduction pathways, but in other cases reflect the modulation that a given hormone exerts on the synthesis or action of another one (Hoffmann *et al.*, 2011). Jasmonates make no exception and numerous interactions of jasmonates with other hormones have been reported (Kazan and Manners, 2008). Among those, the interaction between jasmonic acid (JA) and auxin is still poorly understood despite growing evidence for significant interactions between these two seemingly antagonistic signaling molecules, in particular in regulating adventitious root initiation (Gutierrez *et al.*, 2012), and coleoptile photo- and gravitropism (Gutjahr *et al.*, 2005; Riemann *et al.*, 2003). In addition, there seems to be an intimate interplay between JA and IAA in the control of lateral root formation (Sun *et al.*, 2009).

Besides shared components in ubiquitin-dependent protein degradation pathways such as AXR1 and AXR6 (Ren *et al.*, 2005; Tiryaki and Staswick, 2002), and shared co-repressors like TPL (Pauwels *et al.*, 2010; Szemenyei *et al.*, 2008), auxin and JA pathways are linked through the action of auxin response factors (ARFs) which regulate the transcription of auxin-dependent genes. In addition to an impaired auxin response, *arf6/arf8* mutants exhibit aberrant flower development, and JA deficiency as well as reduced transcription of JA biosynthetic genes in their flowers (Nagpal *et al.*, 2005). The essential role of JA in the regulation of flower development has been previously documented (Ishiguro *et al.*, 2001). Moreover, auxin is capable of inducing the expression of JA biosynthetic genes (Tiryaki and Staswick, 2002), while JA increases the metabolic flux into the L-tryptophan (Trp) pathway by inducing gene expression of *ANTHRANILATE SYNTHASE α1* (*ASA1*) and *β1* (*ASB1*). Elevated *ASA1* and *ASB1* expression leads to increased production of Trp, a precursor for the synthesis of active auxin, namely indole-3-acetic acid (IAA), and indole glucosinolate (Dombrecht *et al.*, 2007; Sun *et al.*, 2009). Recently, jasmonates have also been reported to be involved in the regulation of auxin transport through effects on the distribution of the auxin exporter PIN-FORMED 2 (PIN2) (Sun *et al.*, 2011).

Although the intricacies of IAA biosynthesis still need to be fully elucidated, both genetic and biochemical studies suggest that YUCCA enzymes play a role in the production of IAA (Dai *et al.*, 2013; Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011). In Arabidopsis, the *YUCCA* gene family consists of eleven members encoding flavin-containing monooxygenase-like proteins. Several YUCCA enzymes, i.e. YUC1-6 and YUC10-11, have been implicated in the local production of auxin (Cheng *et al.*, 2006, 2007; Kim *et al.*, 2011; Kim *et al.*, 2007; Woodward *et al.*, 2005; Zhao *et al.*, 2001) and the transcription of the *YUCCA* genes appears to be strictly spatio-temporally regulated (Zhao, 2008). Knowledge of the transcriptional networks by which the expression of the *YUCCA* genes is regulated, however, just emerges.

Recent studies attracted our attention by providing circumstantial evidence for the induction of two *YUCCA* genes, *YUC8* and *YUC9*, by wounding. For example, induction of *YUC9*

expression was described in response to OPDA treatment both in the *tga2/5/6* triple-mutant and the wild type background (Mueller *et al.*, 2008). Another dataset from an Arabidopsis cell culture experiment gave evidence for a 4-fold rapid and transient increase of *YUC8* gene expression in response to MeJA application (Pauwels *et al.*, 2008). These results prompted us to hypothesize that *YUC8* and *YUC*9 may play an important role in linking oxylipin signaling and IAA metabolism.

Here, we analyze these two as yet uncharacterized members of the Arabidopsis *YUCCA* gene family. We demonstrate a role of the two genes in auxin homeostasis and root development as well as in hypocotyl and leaf phenotypes typically associated with changes in IAA levels. We show that both genes participate in the induction of auxin synthesis by MeJA and other oxylipins. Our data provide evidence for partial redundancy between the two genes, but also reveal specific functions with respect to their spatio-temporal expression patterns as well as their involvement in the interplay of auxin and oxylipins.

RESULTS

YUC8 and YUC9 contribute to auxin production *in vivo*

As a first step to investigate *YUC8* and *YUC9* function, we generated stable *35S::YUC8* and *35S::YUC9* overexpression lines (YUC8ox, YUC9ox). Several independent homozygous YUC8ox and YUC9ox lines were recovered. All displayed a phenotype similar to other auxin overproducers, e.g. *yucca,* CYP79B2ox*,* or *rooty* (Zhao *et al.*, 2002), with elongated hypocotyls, epinastic cotyledons, reduced primary root lengths, and narrow elongated leaf blades and petioles (Figure 1a). Hypocotyl length in YUC8ox and YUC9ox was 1.6- and 2-fold greater, respectively, than in wild type, similar to hypocotyl elongation earlier observed in *sur1/rooty* or *sur2* mutants, showing a 2- to 3-fold increase (Boerjan *et al.*, 1995; King *et al.*, 1995). It is noteworthy, however, that free IAA levels in both YUC8ox and YUC9ox (Figure 1b), as well as in other previously characterized YUCCA overexpressing lines (Zhao *et al.*, 2001) are only 1.5- to 2-fold higher than those in wild type. This contrasts with the 5- to 20-fold increase of IAA contents in *sur1/rooty* and *sur2* (King *et al.*, 1995; Sugawara *et al.*, 2009). This may emphasize the importance of the spatio-temporal distribution of IAA-overproduction, constitutive and unspecific in the YUCox lines due to the use of a 35S promoter to drive overexpression, but most probably tissue/cell specific in the *superroot* knockout mutants.

Like *35S::YUC6* transgenic plants (Kim *et al.*, 2011), adult YUC8ox and YUC9ox plants showed considerable longevity, with some lines reaching heights of more than one meter (Figure 1c). To test the hypothesis that elevated IAA levels were the cause of the aberrant phenotypes associated with *YUC8/9* overexpression, auxin levels were measured in a number of independent transgenic lines by gas chromatography-mass spectrometry. In agreement with previous reports on auxin contents in other *YUCCA*-overexpressing lines (Zhao *et al.*, 2001), endogenous auxin levels in our lines were increased by 1.5- to 2-fold when compared to wild type levels (Figure 1b). Consistent with the assumption of a role of *YUCCA* genes in Trp-dependent auxin synthesis (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011; Zhao *et al.*, 2001), the examined lines showed an increased resistance towards toxic 5-methyl-tryptophan (5-mT) (Figure S1). Even though 5-mT is ultimately lethal to wild type and YUCCA overexpressing plants by inhibiting Trp formation and blocking the function of proteins in which this amino acid is incorporated (Hull *et al.*, 2000), it seems as if part of the 5-mT is shunted into non-toxic 5-methyl IAA thereby increasing the resistance of YUC8ox and YUC9ox.

We also used the 35S::YUC8 and 35S::YUC9 overexpression vectors to perform transient assays on *Nicotiana benthamiana* leaves. Within 18 hrs post-infiltration, leaves infiltrated

with either construct showed strong curling compared to control leaves infiltrated with *Agrobacterium tumefaciens* harboring an empty vector (Figure S2a–b). Inspection of leaf epidermis revealed that expression of both *YUC8* and *YUC9* caused a 2–2.5-fold increase in pavement cell size (Figure S2c-e). These observations are in agreement with previous studies on auxin overproducers showing similar leaf curling (Zhang *et al.*, 2007; Zhao *et al.*, 2001). To confirm the results obtained in our *YUC8* and *YUC9* overexpression experiments, we analyzed IAA levels in seven-day-old *yuc8* and *yuc9* single- and *yuc8/yuc9* double-knockout mutants. Relative to the wild type, two out of three mutants showed significantly reduced IAA contents (Figure 2a). The most severe reduction was detected in *yuc8/yuc9* double-knockout seedlings with an IAA level approximately 50% of that in wt compared to 70% for the *yuc8* single mutant. The impact of YUC9 loss-of-function on the endogenous IAA pool was not statistically significant with a decrease of only 5–10% compared to wild-type levels. All mutants showed slightly reduced hypocotyl (75%) and petiole (83%) lengths (Figure 2b–c), in line with observations in other mutants with low auxin contents such as the *ilr1/iar3* double-and the *ilr1/iar3/ill2* triple-knockout mutants or the *sav3-2* mutant (Rampey *et al.*, 2004; Tao *et al.*, 2008). Taken together, these results indicate that YUC8 and YUC9 both contribute to auxin synthesis in Arabidopsis. In addition, the additive effects on the IAA levels detected in the mutant seedlings under normal conditions (Figure 2a) reveal that YUC8 and YUC9 seemingly act in parallel to maintain auxin homeostasis.

YUC8 and YUC9 have distinct expression patterns

To examine the degree of redundancy between the two genes, we analyzed tissue-specific expression levels by qRT-PCR (Figure 3). *YUC8* expression was highest in very young 3 and 7d-old seedlings, thereafter declining. In adult 6–8 week-old plants, *YUC8* transcript levels were highest in young sink leaves, lower in mature source leaves, roots, and flower buds, and hardly detectable in stems (Figure 3a). *YUC9* gene expression was generally one order of magnitude lower than *YUC8* expression (Figure 3b). It was also developmentally regulated in young seedlings but with a peak 7 days after germination. In adult plants, *YUC9* was predominantly expressed in roots and at a much lower level in sink leaves, while being undetectable in floral tissue. These patterns are consistent with microarray data deposited in public databases, e.g. the Genevestigator database (Hruz *et al.*, 2008), and indicate marked differences in the spatio-temporal expression patterns of the two genes.

We next generated *pYUC8::GUS* and *pYUC9::GUS* reporter lines allowing for finer resolution in the examination of cellular and tissue specificity in *YUC8* and *YUC9* expression. Within two days of germination, expression of both *YUC8* and *YUC9* was detectable in primary root tips, and within the next two days became visible in the root vasculature (Figure S3). In older plants, GUS reporter activity was absent from leaves (*YUC8*) or confined to the very margin of sink leaves (*YUC9*, Figure S4h). The two genes were expressed in reproductive organs but with quite distinct patterns. *YUC8* showed high specific expression in flower buds (Figure S4c). Closer examination of flowers revealed *YUC8* promoter activity in the young anthers of immature flowers (Figure S4i) and in the vascular tissues of sepals and petals of mature flowers as well as in anther filaments (Figure S4i–m) and the funiculus and integuments of young ovules (Figure S5b–d). These results extend the previously described pattern of *YUC8* promoter activity (Rawat *et al.*, 2009) and suggest a role for *YUC8* in reproductive development. By contrast, there was no detectable *YUC9* promoter activity in floral tissues, but transient expression in the suspensor of the embryo, at the 8- and 16-cell stage (Figure S5a,e–g). As suspensor cells are known to synthesize IAA (Kawashima and Goldberg, 2010), the expression pattern of *YUC9* suggests a role of YUC9 in IAA supply during early embryo development. Overall and consistent with our qRT-PCR data, these results confirm tissue specificity in *YUC8* and *YUC9* gene expression. In order to compare the subcellular localization of the encoded proteins, both

genes were fused to the green fluorescent protein (*GFP*) reporter gene. The resulting constructs were then used to transiently transfect Arabidopsis pavement cells by ballistic gene transfer. Confocal laser scanning microscopy revealed an exclusive cytoplasmic localization of the fusion proteins (Figure S6).

YUC8 and YUC9 contribute to MeJA-induced auxin synthesis at the organ level

Previous studies reported that treatment with either MeJA or coronatine promotes auxin production (Dombrecht *et al.*, 2007; Sun *et al.*, 2009; Uppalapati *et al.*, 2005). Hence, we next analyzed auxin levels and responsiveness to MeJA application in wild type and *yuc8* and *yuc9* single- and double-knockout mutants. Seven-day-old seedlings were treated for 2 hrs with either 50 μM MeJA or a control solution (0.5% methanol (v/v)) and then dissected into cotyledons, hypocotyls, and roots for separate analysis of IAA contents. Endogenous IAA levels in control mock treated seedlings were affected by *YUC* loss-of-function to varying degrees depending on the organ considered, with no effect in cotyledons, a decrease in hypocotyls in *yuc9* and more so in *yuc8*, and for roots a decrease in the *yuc8* mutant only (Figure S7). Combining the two mutations had no effect in cotyledons, but data for the double mutant suggested some additive effects of the *yuc8* and *yuc9* mutations in hypocotyls while in roots IAA levels in the double mutant mirrored those measured in *yuc8*. MeJA application consistently caused an elevation of IAA contents (Figure 4a), but the extent of this effect was organ- and line-dependent. While the double mutant showed reduced responsiveness to MeJA in all organs, single mutations affected responsiveness not significantly. These results indicate a role of both YUC8 and YUC9 in overall auxin homeostasis and MeJA-induced IAA production.

The root response to MeJA treatment is impaired in *yuc8* and *yuc9*

Wild-type seedlings respond to jasmonate treatment by a reduction of primary root growth (Figure 4b), consistent with earlier reports (Staswick *et al.*, 1992). This MeJA-induced inhibition of root elongation was partially suppressed in all mutant lines (Figure 4b), most significantly so in the *yuc8/yuc9* double mutant. Primary roots of MeJA-treated seedlings of this mutant were 53% shorter than corresponding untreated *yuc8/yuc9* mutants, while treated *yuc8* and *yuc9* mutants showed 62% shorter primary roots. Under these same conditions primary roots in MeJA-treated wild-type seedlings were 82% shorter relative to the untreated wild-type plants. These gradual effects are in agreement with the differential reductions in MeJA-induced IAA synthesis in these mutants (Figure 4a).

MeJA treatment also caused a considerable reduction in the number of lateral roots, in all lines (Figure 4c). In wild type, this reduction was of relatively smaller magnitude than in the *yuc8* and *yuc9* single and double mutants. While in MeJA-treated wild-type seedlings the lateral root number is decreased to 48% compared to the untreated control, the reduction was more pronounced in *yuc9* and *yuc8/yuc9* mutants, with a decrease to 27% and 30%, respectively, relative to the corresponding untreated samples. However, when combining the observation of primary root growth inhibition with those on lateral root numbers, considering lateral root density as the index figure, we were able to detect that MeJA-treatment did not result in a diminished lateral root density, which is likely due to the pronounced inhibition of primary root growth (Figure 4d). While wild-type seedlings responded to MeJA application with a nearly 2-fold increase in lateral root density, this promoting effect was partly suppressed by the *yuc8* mutation and completely abolished in by the *yuc9* mutation. In both the *yuc9* and *yuc8/yuc9* double mutants, MeJA no more enhanced lateral root formation: lateral root density was similar on treated and untreated roots. These results show that *YUC9,* and possibly *YUC8* are involved in mediating the promoting effect of MeJA on lateral root formation.

The different responses to MeJA of the *yuc8* and *yuc9* mutants prompted us to more closely examine the expression patterns of the two genes in roots using our GUS reporter lines. Both lines were consistently expressed in root tips (Figure 5a–d), *YUC8* promoter activity was visible early during lateral root formation at the sites of primordia initiation and was very high in the vasculature of the young emerged lateral roots (Figure 5e). *YUC9* expression was detectable even earlier, but was more diffuse in young lateral roots and also expressed in the vasculature of subtending roots (Figure 5f). These not totally overlapping expression pattern may suggest distinct roles of *YUC8* and *YUC9* in the local auxin supply through which the growth of lateral roots may be supported. Overall, these data suggest that YUC8 and YUC9 promote lateral root formation, possibly in a synergistic manner.

Induction of *YUC9* expression by oxylipins is COI1 dependent

Our findings here of an increase in IAA levels after MeJA treatment as well as published gene expression datasets (Mueller *et al.*, 2008; Pauwels *et al.*, 2008) indicated oxylipin-dependent changes of *YUC8* and *YUC9* gene expression and/or activity. We therefore investigated the hypothesis that the reduced responsiveness of the *yuc8* and *yuc9* mutants to MeJA may reflect a role of oxylipins on the transcriptional regulation of *YUC8* and *YUC9*. To address this, we measured *YUC8* and *YUC9* transcript abundance by qRT-PCR (Figure 6) following application of MeJA, OPDA or coronatine, a bacteriotoxin that mimics the bioactive jasmonate JA-Ile (Fonseca *et al.*, 2009). In wild-type seedlings, all treatments resulted in a statistically significant reduction of *YUC8* transcript levels within 30', followed by a progressive recovery within the next 3 hrs (Figure 6a). This pattern is in line with published expression data on transcriptional responses mediated by jasmonates (Mandaokar *et al.*, 2006), from which one can extract a similar kinetic of *YUC8* transcripts. The *YUC8* induction by MeJA previously observed in cell culture (Pauwels *et al.*, 2008) may result from a transient and more general stress response and not from a specific oxylipin induction as suggested by the recent report that protoplasting of root tissue leads to a 10-fold increase of *YUC8* expression (Dinneny *et al.*, 2008). In contrast to *YUC8*, *YUC9* transcription was not repressed but rather induced by oxylipins (Figure 6a), with a 7-fold increase in transcript levels after 120 min of OPDA treatment while MeJA and coronatine caused an even stronger induction (11- and 12-fold increase in transcript abundance 240 min post-treatment, respectively). *YUC9* induction by MeJA is consistent with the increases in IAA levels and alterations of root development shown in Figure 4. The transient down-regulation of *YUC8* was, however, unexpected given the apparent synergy of the two genes in increasing responsiveness to oxylipins. To further examine the interaction between *YUC8* and *YUC9*, we asked whether a loss of either gene function might be compensated for by an up-regulation of the other one; therefore we analyzed *YUC8/9* expression in the corresponding knockout background (Figure 6b). Relative to untreated seedlings *YUC8* and *YUC9* transcript levels were significantly more abundant after short-term MeJA treatments, which may also explain why *yuc8* mutants show oxylipin-related phenotypic defects. These experiments indicate that the two genes can, at least partially, compensate for a loss of function of the other and that both genes are functionally linked. This highlights that in addition to the already reported *YUC8/9* induction by high temperatures (Stavang *et al.*, 2009), the transcriptional regulation of both genes can be considerably affected by MeJA.

JA signal transduction proceeds via the COI1 signal transduction pathway (Browse, 2009; Chini *et al.*, 2009; Feys *et al.*, 1994). The root phenotype of *yuc8* and *yuc9* mutants grown on MeJA-containing media is intermediate between that of wild type and the jasmonate-insensitive *coi1* mutant (Figure 4b). The *yuc8* and *yuc9* single and double mutants, however, retain a residual sensitivity to MeJA, as shown by our phenotypic analyses and quantification of IAA levels, even though their responsiveness is significantly reduced (Figure 4b). To investigate whether oxylipin-dependent *YUCCA* expression is also

governed by COI1, we treated the *coi1* mutant with the various oxylipins and examined changes in *YUC8* and *YUC9* expression (Figure 6c). When compared to responses obtained 2 hrs after induction of wild-type plants, transcriptional responses to oxylipin treatment appeared almost entirely suppressed in the *coi1* background. This suggests that the tested oxylipins most likely regulate *YUC8* and *YUC9* transcription through a COI1-dependent pathway (Figure 6c).

In agreement with the qRT-PCR results, the *pYUC9::GUS* reporter showed substantial induction in primary roots transiently treated with 50 μM MeJA, while no significant change in *YUC8* promoter activity could be detected (Figure 7b,c). When plants were grown continuously on plates containing MeJA, activation of the *pYUC8::GUS* reporter was detectable but at an obviously lower level than that of the *pYUC9::GUS* reporter (Figure S8). Local application of MeJA on leaves also activated the *YUC9* promoter (Figure 7a). We next examined whether the oxylipin levels produced in response to wounding (Glauser *et al.*, 2009) were sufficient to trigger *YUC8* and *YUC9* promoter activation. *YUC9* expression responded strongly, similar to the wound-inducible *ALLENE OXIDE SYNTHASE* promoter (Kubigsteltig *et al.*, 1999), while there was no detectable reporter activity in the *pYUC8::GUS* lines (Figure 7d). Overall, these data indicate that *YUC9*- but not *YUC8* expression is up-regulated by jasmonates through a COI1-dependent pathway.

DISCUSSION

Auxin is an essential plant growth hormone ubiquitous throughout the plant kingdom and well known to contribute to a wide array of physiological processes, acting either alone or in interaction with other phytohormones (Davies, 2010). However, the intricacies of the biosynthesis of the primary plant auxin, IAA, are yet to be fully uncovered. Currently, IAA formation is proposed to mainly proceed through four Trp-dependent pathways (Lehmann *et al.*, 2010; Novák *et al.*, 2012). In this context, YUCCA enzymes have received much attention since their discovery. The molecular function of YUCCA monooxygenases has been disclosed recently (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011). They are thought to play a key-role in auxin production, catalyzing the conversion of indole-3-pyruvate to IAA. YUCCA monooxygenases have been extensively studied, not only in Arabidopsis but also in tomato, maize, petunia, and rice (Expósito-Rodríguez *et al.*, 2011; LeClere *et al.*, 2010; Tobeña-Santamaria *et al.*, 2002; Yamamoto *et al.*, 2007), but despite intensive work their transcriptional regulation is still not entirely clear. Thus far, from the eleven *YUCCA* homologues in Arabidopsis eight have been studied in more detail (Cheng *et al.*, 2006, 2007; Kim *et al.*, 2011; Kim *et al.*, 2007; Woodward *et al.*, 2005; Zhao *et al.*, 2001). At present, only the functions of *YUC7-9* have not been settled. The results described here give evidence that, as other YUCCAs, both YUC8 and YUC9 also participate in auxin production in Arabidopsis. In addition, detailed analysis of *YUC8* and *YUC9* gene expression during the course of plant development and in response to oxylipins demonstrates partial functional overlap between the two genes but also specificity based on their different spatio-temporal expression patterns.

Phenotypically, *yuc8* and *yuc9* single and double mutants grown on MS media do not substantially differ from the wild type, except for slightly shorter hypocotyls and petioles as well as a slightly reduced number of lateral roots (Figure 2, 4). The root phenotype of the *yuc8* and *yuc9* mutants and the localized GUS staining observed in our reporter lines (Figure 5) imply an involvement of *YUC8* and *YUC9* in lateral root development.

Our data also give evidence for a direct link between oxylipin signaling and auxin biosynthesis. The examined oxylipins seem to transiently repress the expression of *YUC8*, although *YUC8* expression in the *yuc9* background appears to be increased after MeJA

treatment (Figure 6a,b). Also, the *YUC8* promoter gets activated when seedlings are raised on plates containing MeJA (Figure S8). Similarly to the increased *YUC8* response in *yuc9* mutants, induction of *YUC9* expression is more pronounced in *yuc8* seedlings, suggesting that the two genes are functionally linked and that they can, to a certain extent, compensate for each other (Figure 6a,b). The regulatory effect of oxylipins both on *YUC8* and *YUC9* expression is, however, abrogated in the *coi1* background, pointing to an involvement of the COI1-dependent signaling pathway in the regulation of the two genes (Figure 6a,c).

A limited number of previous studies have already provided evidence for a tight connection between JA and auxin homeostasis as well as for an involvement of the jasmonate signaling machinery in the regulation of auxin biosynthesis (for review see, Hoffmann *et al.*, 2011). Those studies revealed a moderate impact of MeJA on *YUC2* expression (Sun *et al.*, 2009), mainly focusing on JA-mediated processes upstream of the auxin biosynthetic pathways (i.e. Trp production), auxin transport or auxin responsiveness (Dombrecht *et al.*, 2007; Gutjahr *et al.*, 2005; Riemann *et al.*, 2003; Sun *et al.*, 2011). The present results on the regulation of *YUC8* and *YUC9* provide novel insight into the intimate relationship between jasmonate signaling and auxin biosynthesis, pointing out that JA not only triggers substrate production for subsequent auxin synthesis (Sun *et al.*, 2009), but also induces the formation of IAA by the direct induction of IAA synthesis-related genes.

The *asa1-1* mutation appears to interfere with the enhancing effect of MeJA on lateral root formation, which is completely abolished upon treatment with more than 20 μM MeJA (Sun *et al.*, 2009). Under these same conditions (20 μM MeJA), the number of lateral roots in *yuc8* and *yuc9* mutants is also substantially reduced. This effect is most significant in the *yuc8/yuc9* double mutation (Figure 4d), but unlike in *asa1-1* where lateral root development is totally suppressed, *yuc8/yuc9* mutants still exhibit lateral roots (70% of the number of emerged lateral roots compared to untreated *yuc8/yuc9* seedlings). This indicates that *YUC8* and *YUC9* are likely not the only components of auxin formation downstream of *ASA1*, linking jasmonate signaling to auxin biosynthesis.

In this context, it appears noteworthy that we did not observe an increase in lateral root number upon MeJA-treatment as point out previously (Sun *et al.*, 2009). Rather, we detected a substantial decrease (52%) in the number of lateral roots when comparing untreated and 20 μM MeJA-treated wild-type seedlings. This discrepancy may be explained by the fact that Sun and colleagues (2009) included lateral root primordia in their assessment, while we focused on already emerged lateral roots only. On the other hand, differences in growth conditions, e.g. sugar contents in the medium, could be responsible for this inconsistency. While the *asa1-1* mutation does not considerably impact on the MeJA-triggered suppression of primary root elongation, *yuc8* and *yuc9* mutations attenuate the inhibitory effect of MeJA on primary root elongation. This finding extends the previous observations made by Sun and colleagues (2009). Collectively, these results highlight that application of MeJA triggers an increase in IAA levels by inducing both the production of the major auxin precursor, L-Trp, and auxin synthesis.

YUC9 expression also responds to wounding and MeJA treatment in a COI1-dependent manner (Figure 6a,7). Moreover, the *yuc8* and *yuc9* knockouts show a root phenotype that is intermediate between the wild type and the JA-insensitive *coi1* mutant, with both *YUCCA* gene products seemingly forming a functional unit acting synergistically on root development and root growth responses. These observations suggest that reduced primary root elongation in response to MeJA treatment is not solely due to primary JA effects, e.g. through inhibition of mitosis (Zhang and Turner, 2008). Rather, MeJA-mediated primary root growth inhibition also involves the induction of auxin synthesis and the subsequent action of this additionally formed plant hormone.

As pointed out before, the *asa1-1* mutation does not result in altered primary root elongation responses to MeJA when compared to wild type plants. Thus, the inhibitory effect of MeJA on root elongation does not seem to depend on *ASA1*. By contrast, the examined combined *yuc8* and *yuc9* mutations do significantly suppress the inhibitory effect of MeJA on primary root elongation (Primary roots of MeJA-treated *yuc8/yuc9* mutants appeared 2.9-fold longer than the roots of equally treated wild-type plants, *P*-value = 0.0077) (Figure 4b). Both *ASA1* and the *YUCCA* genes impact on IAA levels, but *ASA1* might play a more general role and act on the formation of L-Trp, a primary building block in protein synthesis. Data deposited in public databases (Genevestigator) show a medium to strong *ASA1* expression in nearly all tissues and organs. In contrast, all *YUCCA* genes examined so far are characterized by a very tight spatio-temporal regulation of their expression. This more specific expression pattern may be the reason why *yuc8* and *yuc9* mutations do alter the inhibitory effect of MeJA on primary root elongation.

In summary, we propose a modified model in which the observed root growth responses are collectively mediated by JA and IAA action. Besides its primary functions, we demonstrate that JA induces the overexpression of *YUC9*. Together with the action of other induced enzymes, the increased YUC levels cause transient IAA overproduction. In fact, this is a good example of an indirect mode of interaction (Hoffmann *et al.*, 2011). In turn, the increased IAA levels result in altered lateral root development and local YUC action seems to also contribute to the inhibition of primary root elongation by MeJA-induced IAA production. As phytohormones act in a dose-dependent manner, with elevated auxin concentrations exerting inhibitory effects on growth (Thimann, 1938), the increased IAA content may reinforce the jasmonate growth inhibiting effect. A transient, local increase in IAA levels may lead to local growth inhibition and also override positional auxin signals, for example, auxin gradients or local auxin maxima. In this context, it is interesting that jasmonates have been reported to interfere with auxin polar transport in a concentration dependent manner (Sun *et al.*, 2011; Sun *et al.*, 2009).

The relationships between JA signaling and IAA production described here might provide a further regulatory mechanism for the control of the plant responses to biotrophic or necrotrophic pathogens. The defense against these two types of pathogens is generally governed by an antagonistic relationship between salicylic acid (SA, induced by biotrophs) and jasmonic acid/ethylene (JA/ET, induced by necrotrophs and herbivores). Several studies have, however, already pointed to an interconnection between auxin and SA or JA/ET dependent pathogen responses (Chen *et al.*, 2007; Truman *et al.*, 2010). As the complex networks of phytohormone interaction are slowly deciphered, further insights on the sophisticated cellular processes that are affected by YUCCA enzymes will be revealed.

METHODS

Plant material and growth conditions

In this study, we analyzed the *Arabidopsis thaliana* ecotype Col-0, the mutants *coi1* (Feys *et al.*, 1994), *yuc8* and *yuc9* (Tao *et al.*, 2008), and the promoter fusions *35S::GUS* (Biesgen and Weiler, 1999) as well as *pAOS::GUS* (Kubigsteltig *et al.*, 1999). Seeds were germinated on solidified ½-strength MS medium supplemented with 1% sucrose in a growth chamber under controlled conditions (110 $\mu E \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation, supplied by standard white fluorescent tubes. 8 h light at 24 °C, 16 h darkness at 20 °C).

Oxylipin treatment

Oxylipin treatment prior to the quantification of free IAA levels and RNA extraction was carried out as follows: If not stated otherwise, seedlings were grown on ½-MS medium (1%

sucrose) for seven days. Next, they were transferred to either 50 μM MeJA solution (in liquid ½ MS media containing 0.5% MeOH (v/v)) or a control solution (liquid ½ MS media, 0.5% MeOH (v/v)) and incubated for the indicated period of time. In case of long-term treatments, i.e. seven days, seedlings were grown on ½-MS media (1% sucrose) containing 20 μM MeJA and directly taken from the plates. Control plants were grown on similar plates lacking MeJA. For the GUS staining of roots on plates (Figure 7b–c), seedlings of the reporter lines were grown on vertical ½-strength MS media plates supplemented with 1% sucrose. To trigger responses, 5 ml of either a mock or a 50 μM MeJA solution (vide supra) was administered to the plates. After the indicated period of time the plants were washed with ½ MS solution before they were stained for β-glucuronidase activity as explained below.

RT-PCR

Reverse-transcription was performed on total RNA isolated from wild-type plants as well as *yuc8*, *yuc9*, and *coi1* mutant lines. RNA was prepared from 100 mg plant tissue by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The mRNA was further purified using an Oligotex mRNA kit (Qiagen). First strand synthesis was performed according to the supplier's instructions, using M-MLV-reverse transcriptase and oligo$(dT)_{15}$ primer (Promega).

Expression analysis by qRT-PCR

Seedlings were grown, RNA isolated and qRT-PCR carried out as previously described (Lehmann and Pollmann, 2009). Samples were run in triplicate. Relative quantification of expression was calculated after data analysis using the Opticon monitor software (ver. 2.02.24, MJ Research) by the comparative $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001) with *APT1* and *UBQ10* as reference genes (Czechowski *et al.*, 2005). For validation of reference genes and determination of amplification efficiencies the geNORM and LinRegPCR algorithms, respectively, were used (Ramakers *et al.*, 2003; Vandesompele *et al.*, 2002) Primer sets were as follows: *APT1* (At1g27450): Pri15-Pri16; *UBQ10* (At4g05320): Pri17-Pri18; *YUC8*: Pri19-Pri20; *YUC9*: Pri21-Pri22 (primer sequences are given in supporting Table S1).

GUS staining

All analyzed seedlings were grown on plates as described above. For analysis of the older plants and generative organs, plantlets were transferred into liquid MS media (1% sucrose) two weeks after germination. Plant material was pre-cleared with 90 % acetone for 0.5 hour and then stained in 50 mM citrate buffer (pH 7) containing 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0,1 % Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt for 14 h at RT. Thereafter, plant material was dehydrated with an ethanol series (20%, 35%, 50%, 70%, 80%, 90% (v/v)). Prior to microscopy, GUS-stained tissues were incubated in Hoyers solution overnight (Liu and Meinke, 1998).

Microscopy

Arabidopsis leaves were transformed by particle bombardment and the microscopic analysis of the distribution of GFP-tagged fusion proteins was carried out as previously described (Pollmann *et al.*, 2006). After incubation for 16–18 h, the YUC-GFP expressing cells were analyzed by confocal laser scanning microscopy.

GUS-stained tissues were examined with a Zeiss Axioskop 100. Images were captured on a Zeiss Axiocam MRc camera. Whole seedlings were analyzed with a Leica MZ12 microscope, and digital images were captured with a Nikon D200 camera.

Auxin quantification

Whole or dissected seedlings were immediately frozen in liquid N_2. Approx. 100 mg tissue was pooled per sample and at least 3 biological replicates were harvested for each independent experiment. IAA was quantified on a Varian Saturn 2000 GC-MS/MS system as previously described (Pollmann *et al.*, 2009).

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's-B post-hoc test to allow for comparisons among all means or with Student's *t*-test when two means were compared. Statistical analyses were conducted using Prism version 5.03 (GraphPad Software, La Jolla, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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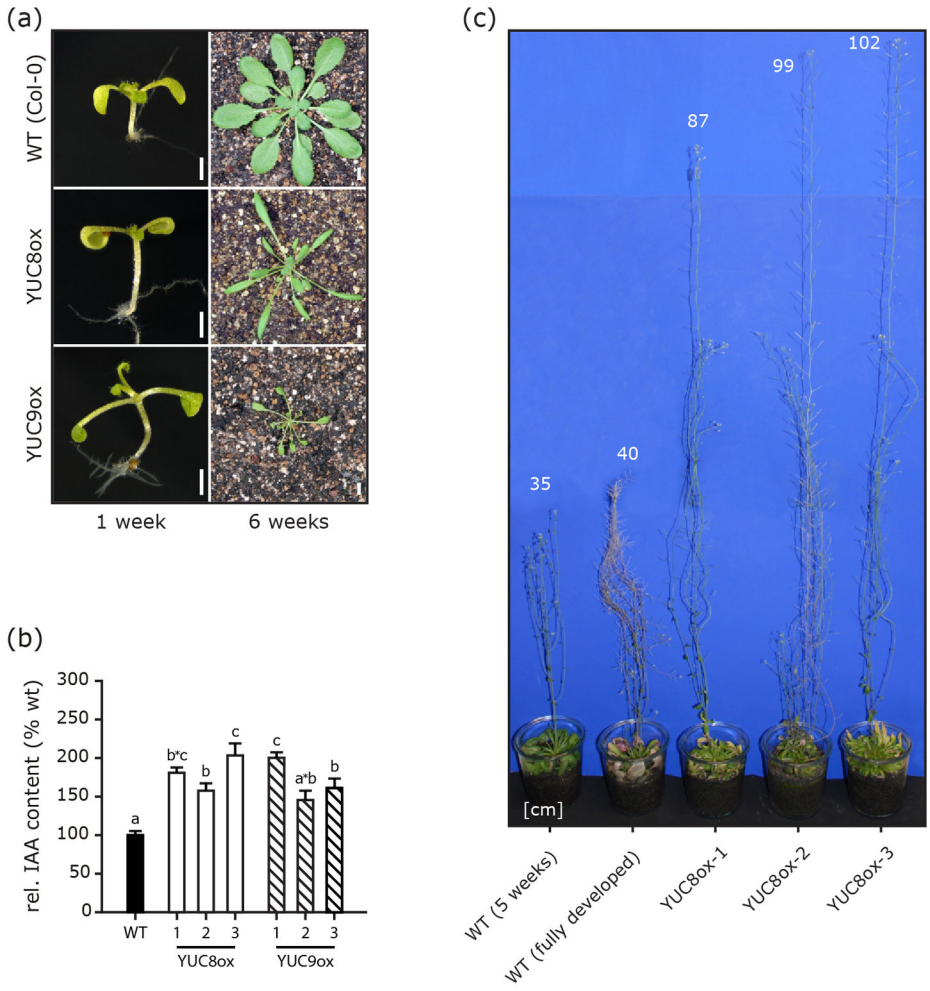

Figure 1.

Phenotype of *YUC8* and *YUC9* overexpression lines. One-week-old and six-week-old plants of wt as well as *YUC8ox* and *YUC9ox* lines are shown (a). [Scale bars, 1 mm on the left, 5 mm on the right]. (b) Elevated levels of IAA in *YUC8*- and *YUC9*-overexpressing lines. IAA levels were determined by GC-MS analysis. For each construct, three independent lines were analyzed and compared to wt. IAA levels are normalized to 100% in wt. Means are given with their SE ($n = 6$). Similar results were obtained in two independent experiments. Different letters indicate significant differences between means [$P < 0.05$]. (c) *Arabidopsis* overexpressing *YUC8* exhibit strongly enhanced shoot growth and stem elongation. Numbers in the figure indicate plant heights in cm. Similar results were obtained for *35S::YUC9* lines, showing maximum heights of 120 cm (data not shown).

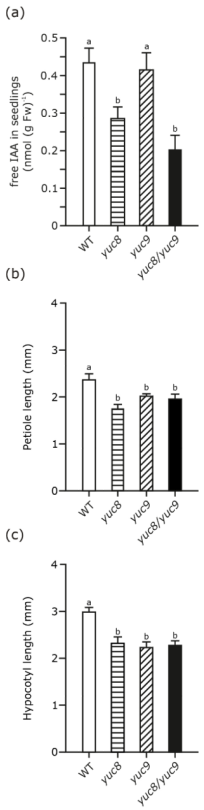

Figure 2.

Endogenous auxin contents and phenotype of *yuc8* and *yuc9* single- and double-knockout mutants. (a) IAA contents in seven-day-old seedlings. Auxin contents were quantified by GC-MS analysis. Means are given with their SE ($n = 6$). Similar results were obtained in three independent experiments. Different letters indicate significant differences between means [$P < 0.05$]. Seedlings grown for seven days on vertical MS plates were analyzed for their (b) petiole and (c) hypocotyl length. Both petioles and hypocotyls are significantly shorter in the mutants. Given are means with their SE ($n = 8–10$ seedlings). Different letters indicate significant differences between means [$P < 0.05$].

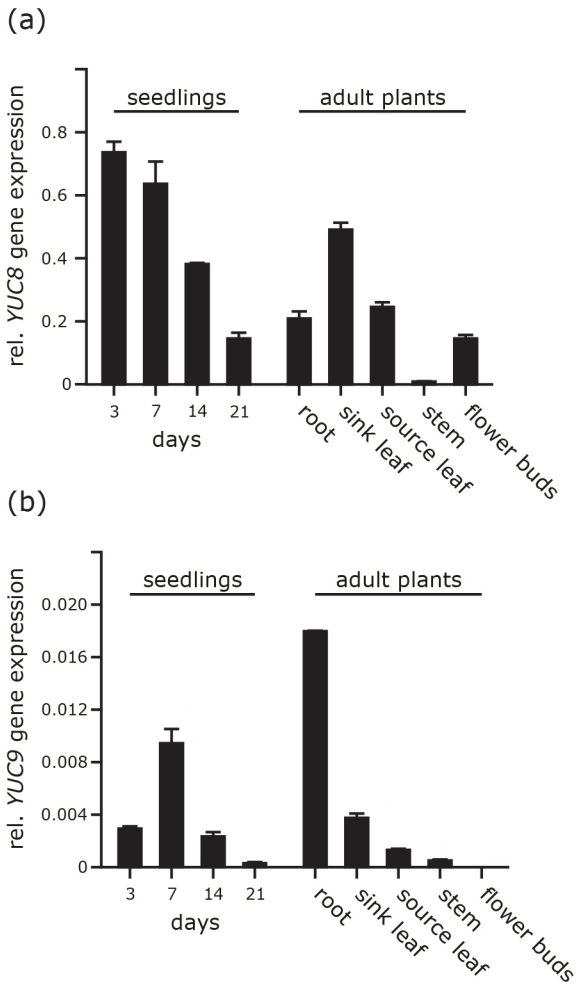

Figure 3.

Tissue specific expression of *YUC8* (a) and *YUC9* (b) in wild type. Transcript abundance values are given relative to the geometric means of *APT1* and *UBQ10* transcripts. Error bars are given ± SE.

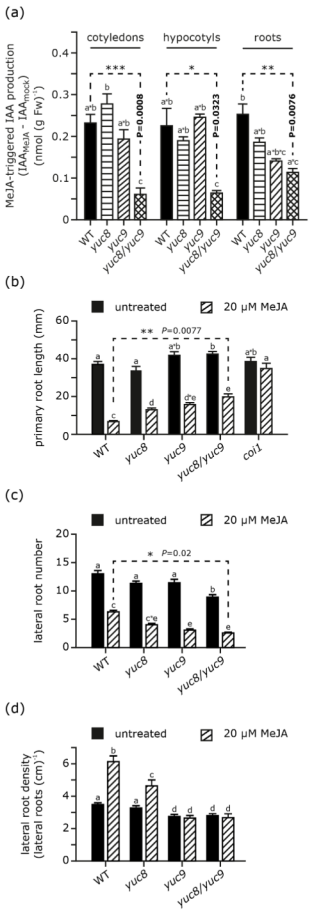

Figure 4.

Comparison of MeJA-induced auxin formation as well as root phenotypes in wild type and both *yuc8* and *yuc9* single and double mutants. (a) Auxin accumulation after MeJA treatment is given by the difference of IAA levels in mock treated samples and those in MeJA treated ones. The corresponding absolute amounts are given in Figure S7. After the treatment seedlings were dissected to separate cotyledons, hypocotyls, and roots. All organs were immediately frozen in liquid nitrogen. Means are given ± SE (n = 6). Similar results were obtained in three independent experiments. (b) Analysis of wild type, *coi1*, *yuc8*, *yuc9* and *yuc8*/*yuc9* mutant primary root lengths. Without supplementation the primary root length of the compared lines does not significantly differ. When grown on media containing 20 µM MeJA, the examined *yuc* mutant roots are significantly longer than the wt ones; however, they are considerably shorter than *coi1* primary roots. Means are given with their SE (n = 8–10 seedlings) (c) Relative to wild type roots, the roots of *yuc8* and *yuc9* single- and double-mutants show a significantly reduced number or lateral roots. Moreover, lateral root density given in number of lateral roots calculated per cm primary root is lower (d). For comparison, root systems of seedlings grown on ½ MS media or media supplemented with MeJA were analyzed. Illustrated are means ± SE (n = 15–20). Similar results were obtained in three independent experiments. Mean values within a graph are significantly different [P < 0.05] where superscript letters differ.

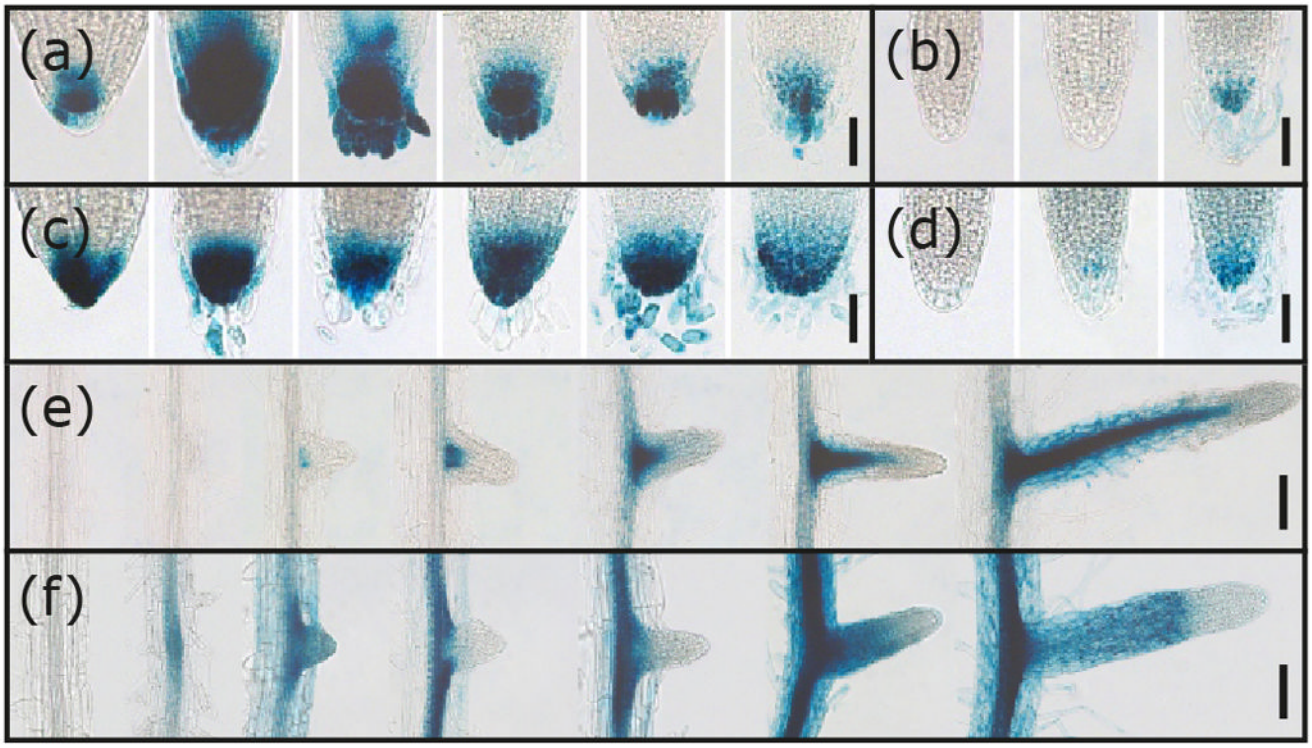

Figure 5.

Histochemical staining of root tips and emerging lateral roots in transgenic promoter reporter lines. Depicted are GUS-stained primary root tips of *pYUC8::GUS* (a) and *pYUC9::GUS* (c) at 1, 2, 4, 7, 10, and 14 days after germination. *YUC8* (b) and *YUC9* (d) promoter activity in the tips of lateral roots at 8, 12, and 14 days after emergence from the epidermis. [Scale bars, 50 μm (a–d)]. β-glucuronidase activity in *pYUC8::GUS* (e) and *pYUC9::GUS* (f) reporter lines during lateral root formation. Plants were grown sterilely on plates and then stained for reporter activity. [Scale bars: 100 μm (e–f)].

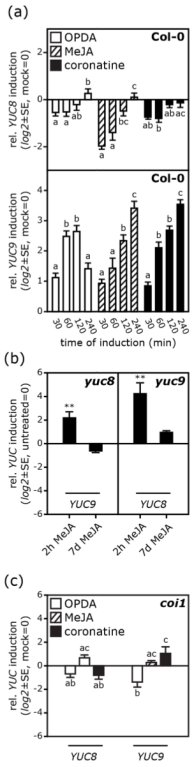

Figure 6.

Oxylipin-mediated induction of *YUCCA* gene expression. Quantitative real time reverse transcriptase PCR for *YUC8* and *YUC9* gene expression in wild type (a), and both *yuc8* and *yuc9* (b), as well as *coi1* (c) mutant seedlings after treatment under variable conditions. Seedlings were either treated with a 50 μM MeJA, 50 μM OPDA, 10 μM coronatine or control solution (½ MS media, 0.5% MeOH (v/v)). The duration of induction varied between 30 to 240 min (a) and 2 h (b,c). Additionally, a long-term treatment with 20 μM MeJA in plates was conducted (b). Then, seedlings were harvested, total RNA extracted, and transcript levels examined by qRT-PCR. Relative expression levels of the genes are normalized to levels in mock controls or untreated control samples and represent the average of three biological replicates, with error bars denoting the standard error. [Student's *t* test; ** $P < 0.01$]. Mean values within a graph are significantly different [$P < 0.05$] where superscript letters differ.

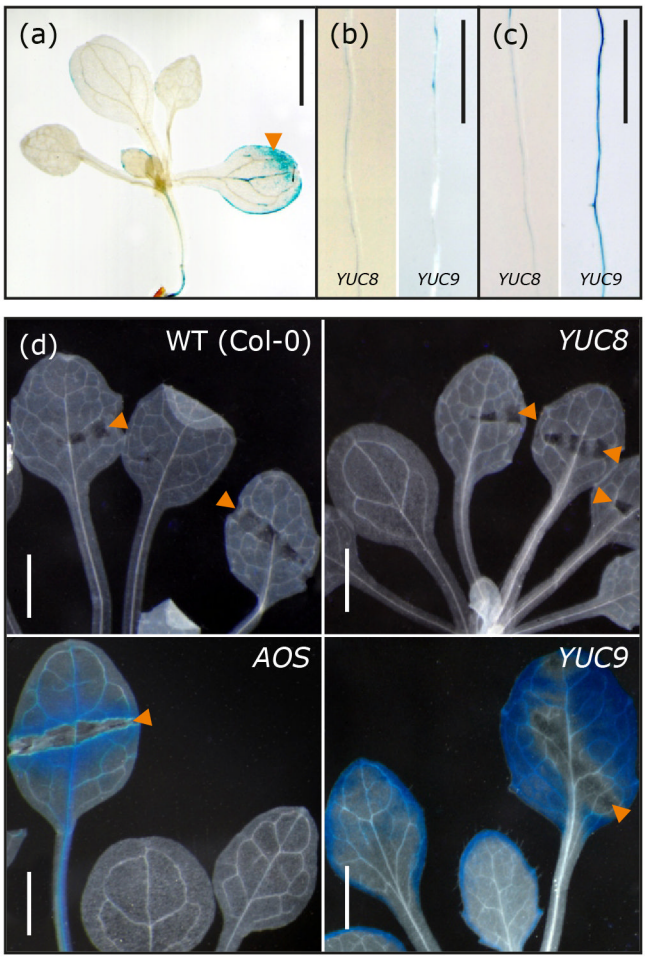

Figure 7.

Induction of *YUC* gene expression. (a–c) GUS staining of *pYUC8::GUS* and *pYUC9::GUS* promoter reporter lines after treatment with MeJA. (a) *YUC9* promoter activation 2 hours after application of 10 μL 1 mM MeJA on a single leaf (arrow). Promoter activity in primary root segments of seven-day-old *pYUC8::GUS* and *pYUC9::GUS* lines 2 hours after treatment with either a mock control (b) or with a solution containing 50 μM MeJA (c). [Scale bars: 1 cm (a–c)]. (d) Pattern of GUS activity in four-week-old sterilely grown wild type, *AOS*-promoter GUS line 4-1 (*AOS*) (Kubigsteltig *et al.*, 1999), *pYUC8::GUS* (*YUC8*) and *pYUC9::GUS* (*YUC9*) lines 12 hours after wounding with sterile forceps (arrows). [Scale bars: 20 μm (d)].