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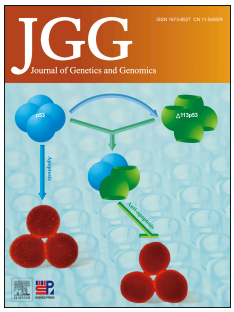

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Control of *de novo* root regeneration efficiency by developmental status of *Arabidopsis* leaf explants

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ABSTRACT

De novo root regeneration (DNRR) has wide applications in agriculture such as those related to cutting technology. Detached *Arabidopsis thaliana* leaf explants can regenerate adventitious roots without added hormones. The regenerative ability is highly dependent on the developmental status of the leaf. An immature leaf has a higher regenerative ability, while a mature leaf is difficult to regenerate. Using RNA-Seq analysis, we showed that the expression levels of many genes, including those in the auxin network, changed during leaf maturation. Particularly, the expression levels of many *YUCCA* (*YUC*) genes in the auxin biosynthesis pathway are responsive to leaf maturation. Overexpression of *YUC1* in the *yuc-1D* dominant mutant rescued the rooting defect caused by leaf maturation. In addition, *YUC4* expression levels were also affected by circadian rhythms. The regenerative ability was reduced in both immature and mature mutant leaf explants from the new *wuschel-related homeobox 11-3* (*wox11-3*) and *wox12-3* mutant alleles created by the CRISPR/Cas9 method. Overall, the transcriptome and genetic data, together with the auxin concentration analysis, indicate that the ability to upregulate auxin levels upon detachment may be reduced during leaf maturation. Thus, multiple developmental and environmental signals may converge to control auxin accumulation, which affects the efficiency of the *WOX11*/*12*-mediated DNRR from leaf explants.

Keywords: *De novo* root regeneration; *WOX11*; *Arabidopsis*; Developmental status; Circadian rhythms; *YUCCA*

1. Introduction

De novo root regeneration (DNRR) gives rise to adventitious roots from injured plant tissues and has been widely applied in many agricultural technologies, such as using cuttings in the vegetative propagation of plants (De Klerk et al., 1999; De Klerk, 2002; Falasca and Altamura, 2003; da Costa et al., 2013; Atkinson et al., 2014; Bellini et al., 2014; Verstraeten et al., 2014; Xu and Huang, 2014; Birnbaum, 2016; Steffens and Rasmussen, 2016; Xu, 2018). Successful applications of DNRR are dependent on the developmental status of the explant, which impacts the regenerative ability to form adventitious roots (Woo et al., 1994; Sanchez et al., 1995; Swamy et al., 2002; Abarca et al., 2014; Abu-Abied et al., 2014; Leakey, 2014; de Almeida et al., 2015; Aumond et al., 2017). Usually, mature or old organs have a lower regenerative ability than immature organs. However, the mechanism behind the development-dependent control of the regenerative ability is largely unclear.

Adventitious rooting from *Arabidopsis thaliana* leaf explants is a simple system for the study of DNRR (Chen et al., 2014). A preliminary framework of the DNRR process has been established based on this system (Xu, 2018). Many early signals, including those from wounds, the environment and the developmental status of the explant, can be sensed by converter cells (i.e., mesophyll cells, leaf margin cells and some vascular cells) in the leaf explant. Guided by these early signals, the converter cells produce auxin, which is then transported from converter cells to regeneration-competent cells (i.e., procambium and some vascular parenchyma cells) to transition into roots. In the regeneration-competent cells, the expression levels of *WUSCHEL-RELATED HOMEOBOX 11* (*WOX11*) and *WOX12*, which encode two homeodomain transcription factors, are upregulated by auxin. *WOX11/12* can promote the transition of the regeneration-competent cells to root founder cells, initiating the organogenesis of adventitious roots (Liu et al., 2014; Hu and Xu, 2016; Sheng et al., 2017).

Auxin is the core hormone in DNRR (Thimann and Went, 1934; Zimmerman and Wilcoxon, 1935; Hitchcock and Zimmerman, 1936). The level of auxin produced in converter cells is rigorously controlled by the combination of early signals. For example, the developmental status of the leaf explants has an impact on the regenerative ability. Among *Arabidopsis* rosette leaves, the immature leaves have a great ability to regenerate adventitious roots, while fully mature leaves have difficulty forming adventitious roots (Chen et al., 2014). Auxin can partially rescue the rooting defect caused by leaf maturation, suggesting that the reduced auxin accumulation might be

responsible for the reduced regenerative ability in fully mature leaves (Chen et al., 2014). Currently, it is not clear how auxin accumulation is affected by changes in the developmental status of leaf explants.

In this study, we used the DNRR system of *Arabidopsis* leaf explants to analyze the effect of leaf maturation on the regenerative ability. We found that the expression levels of many genes changed during leaf maturation. In particular, the expression levels of many *YUCCA* (*YUC*) genes, which encode flavin-containing monooxygenases in the auxin biosynthesis pathway (Zhao et al., 2001) and are critically involved in auxin production in converter cells during DNRR (Chen et al., 2016), respond to leaf maturation. In addition, *YUC* expression has multiple upstream regulators, including wounding and circadian rhythms. The effects of those early signals may eventually converge to guide auxin production and *WOX11*/*12*-mediated rooting.

2. Results

2.1. The developmental status of leaf explants affects gene expression during DNRR

To analyze the molecular mechanism behind the relationship between *Arabidopsis* leaf maturation and DNRR, we first carried out an RNA-Seq analysis using detached first-pair rosette leaves before culturing (time 0) and 1 day after culturing (DAC) from 9-, 12- and 15-day-old wild-type Columbia-0 (Col-0) seedlings, respectively (Fig. 1A–C). The leaves from 9-day-old seedlings were in the immature stage, with short petioles and small blades (Fig. 1A); the leaves from 12-day-old seedlings were at the partially mature stage (Fig. 1B); the leaves from 15-day-old seedlings were at the fully mature stage, with fully elongated petioles and expanded blades (Fig. 1C). The seedlings were grown under a constant 24-h light condition to avoid the effects of light/dark transitions on gene expression.

We first analyzed the gene expression levels in the leaves before detachment (at time 0) from the three developmental states. Changes in gene expression could be grouped into six clusters (Fig. 1D–F and Table S1). Many genes were upregulated (clusters 1 and 2; Fig. 1D) or downregulated (clusters 3 and 4; Fig. 1E) during leaf maturation. Notably, the expression levels of many of the genes involved in the auxin network were affected during leaf maturation (Fig. 1D–F). For example, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*), *YUC2* and *YUC6*, which are involved in the auxin biosynthesis pathway (Cheng et al., 2006; Stepanova et al., 2008; Tao et al., 2008) and have been shown to be required in DNRR from leaf explants (Chen et al., 2016; Sun et

al., 2016; our unpublished data), were downregulated during leaf maturation (Fig. 1E). qRT-PCR analysis confirmed the downregulation of *TAA1*, *YUC2* and *YUC6* during leaf maturation (Fig. 2).

Next, we analyzed the gene expression levels in the leaf explants from 9- and 15-day-old seedlings at 1 DAC and compared them with the gene expression levels at time 0. By comparing up- or downregulated genes (1 DAC *vs* time 0) between leaves from 9- and 15-day-old seedlings, we found that many of the genes were up- or downregulated only in immature leaves or only in mature leaves at 1 DAC (clusters a to l; Fig. 1G–J and Table S1). Many of the auxin network genes were also included in these clusters. For example, *YUC5*, which functions in response to darkness and wounding in DNRR from leaf explants (Chen et al., 2016), was upregulated during leaf maturation (Fig. 1D); however, its expression levels were downregulated at 1 DAC compared with time 0 in mature leaf explants but not in immature leaf explants (Fig. 1G and I). In addition, the expression levels of *YUC8* and *YUC9*, which also act in response to darkness and wounding during DNRR (Chen et al., 2016), were downregulated at 1 DAC compared with time 0 in mature leaf explants but not in immature leaf explants (Fig. 1G and I). These data suggest that immature and mature leaf explants may have different responsive abilities to wounding.

Overall, gene expression patterns were not only affected by leaf maturation before detachment, but also by their different responses to wounding. Therefore, the reduced ability of rooting in mature leaves may result from the complex and combined changes in gene expression levels. Further analysis of the genes involved in leaf maturation will improve our understanding of the effect of maturation on regeneration.

2.2. *YUC1/4* expression levels are responsive to leaf maturation

Auxin biosynthesis is critically required for DNRR after leaf explant detachment, and the expression levels of auxin biosynthesis genes are usually affected by multiple signals (Chen et al., 2016; Sun et al., 2016). Auxin concentration analysis (Sun et al., 2017; Sun et al., 2018) showed that immature leaf explants were able to upregulate the auxin level at 1 DAC, while fully mature leaf explants barely had this competence for upregulation of the auxin level at 1 DAC (Fig. S1), indicating that the auxin production ability after detachment might be reduced during leaf maturation.

The auxin biosynthesis genes *YUC1/4* are involved in auxin production in converter cells at the early stage of DNRR from leaf explants (Chen et al., 2016). Because *YUC1/4* expression levels are

relatively low in the RNA-Seq data, we carried out a qRT-PCR analysis. We analyzed the expression levels of *YUC1*/*4* in regeneration using the first-pair leaves from 9-, 12- and 15-day-old seedlings grown under 24-h light conditions to avoid the influence of circadian rhythms on gene expression (see below). Time-0 and 1-DAC leaf explants cultured on B5 medium in 24-h light conditions were used for the qRT-PCR analysis (Fig. 3A and B). *YUC1* had very low expression levels in time-0 immature and partially mature leaves from 9- and 12-day-old seedlings, respectively, while its expression levels were not detected in time-0 fully mature leaves from 15-day-old seedlings (Fig. 3A). Although *YUC1* expression levels were upregulated after 1 day of culturing on B5 medium in all leaf explants at different developmental states, the upregulated levels were more evident in mature leaf explants than in immature leaf explants (Fig. 3A). *YUC4* expression levels were progressively downregulated in time-0 leaves during maturation (Fig. 3B). *YUC4* expression levels in immature leaf explants from 9-day-old seedlings did not show upregulation after 1 day of culturing on B5 medium, while its expression levels in the 1-DAC partially and fully mature leaf explants from 12- and 15-day-old seedlings, respectively, were upregulated compared with the corresponding time-0 leaf explants (Fig. 3B).

Next, we analyzed the adventitious rooting phenotype in the wild type (Col-0) and the *yuc-1D* dominant mutant which has a higher *YUC1* expression and a higher auxin biosynthesis level (Zhao et al., 2001) (Fig. 3C–E). Using the first-pair leaves from 9-, 12- and 15-day-old seedlings, we found that the wild-type leaf explants had a reduced rooting ratio during leaf maturation. At 14 DAC, almost all (~99%) immature wild-type leaf explants from 9-day-old seedlings produced roots; many (~87%) of the leaf explants from 12-day-old wild-type seedlings regenerated roots; only a few (~24%) mature leaf explants from 15-day-old wild-type seedlings had a rooting ability. The *yuc-1D* leaf explants from 9- and 12-day-old seedlings had similar rooting ratios to the wild-type leaf explants at 14 DAC. However, about 79% of the mature *yuc-1D* leaf explants from 15-day-old seedlings produced roots at 14 DAC, showing a significant higher rooting ratio than the wild-type mature leaf explants (Fig. 3 C–E), suggesting that enhanced *YUC1* expression could partially rescue the rooting defect caused by leaf maturation. In addition, it is possible that some mechanisms besides the *YUC1*-mediated auxin biosynthesis pathway may function in DNRR in response to leaf maturation, because enhanced *YUC1* expression in *yuc-1D* could not promote the rooting ratio in partially mature leaves from 12-day-old seedlings (Fig. 3E).

Thus, *YUC1/4* expression levels are controlled by both leaf maturation (before culturing) and wounding (after culturing). Before culturing (i.e., at time 0), *YUC1/4* expression levels are reduced during leaf maturation, probably contributing to the reduced competence for auxin upregulation upon detachment of mature leaves. *yuc-1D* had a higher auxin level in mature leaves, resulting in a relatively higher regenerative ability compared with the wild-type seedlings. Thus, the reduced regenerative ability in mature leaves could be, at least partially, due to the reduction of the potential auxin biosynthesis ability upon detachment during leaf maturation. *YUC1/4* expression levels could be increased in partially and fully mature leaf explants in response to wounding after culturing on B5 medium (e.g., at 1 DAC). However, the upregulation of *YUC1* did not appear to fully rescue the rooting defects caused by leaf maturation, because the wild-type mature leaf explants still had severe defects in root regeneration (Fig. 3C and E), although *YUC1/4* were upregulated to levels even higher than those in immature leaf explants at 1 DAC (Fig. 3A and B). One explanation is that the expression levels of many other auxin-related genes are still low in mature leaf explants (Fig. 1).

2.3. *YUC4* expression is affected by circadian rhythms

The expression levels of many *YUC* genes are sensitive to environmental signals, such as dark and light conditions (Tao et al., 2008; Hornitschek et al., 2012; Chen et al., 2016). Previously, the growth conditions of our seedlings included a 16-h light and 8-h dark period (Chen et al., 2014; Liu et al., 2014; Chen et al., 2016). We tested whether this circadian condition affects *YUC4* expression by qRT-PCR using the first-pair leaves from 12-day-old seedlings grown in a 16-h light and 8-h dark period. *YUC4* is indeed regulated by circadian rhythms, showing relatively higher expression levels in light-on conditions and relatively lower expression levels during the night (light-off conditions) (Fig. 4). Therefore, *YUC4* appears to be regulated by multiple upstream signals, including wounding, circadian rhythms, leaf developmental status and probably many other signals. The upregulation of *YUC4* after leaf explant detachment under 16-h light and 8-h dark conditions (Chen et al., 2016) may be the combined result of multiple upstream inducers, including wounding, circadian rhythms and probably other signals. However, it is still unclear how the upstream signals regulate *YUC* expression and whether *YUC1/4* are direct or indirect targets of wounding.

2.4. *wox11* and *wox12* mutant alleles generated by CRISPR/Cas9

The auxin produced in the leaf explants is transported into regeneration-competent cells for their fate transition (Liu et al., 2014). Auxin promotes the first fate transition step from

regeneration-competent cells to root founder cells through the direct activation of *WOX11* expression and probably also the expression of its partially redundant homologue *WOX12*. The T-DNA insertion-derived single-mutant alleles *wox11-2* and *wox12-1*, and their double mutant, showed relatively mild rooting defects. Because the T-DNA insertion sites in the two alleles both caused disruptions in the C-terminal regions of the proteins and did not affect the homeodomains, it is likely that the *wox11-2* and *wox12-1* mutant alleles are weak alleles (Fig. 5A and B) (Liu et al., 2014).

To further analyze the roles of *WOX11* and *WOX12* in DNRR, we designed new mutant alleles using the CRISPR/Cas9 method (Figs. 5A, 5B and S2) (Yan et al., 2015). The *wox11-3* and *wox12-3* mutant alleles have an 11-bp deletion and 1-bp insertion in the homeodomains, respectively (Figs. 5A, 5B and S2). These alleles caused frameshift mutations in the homeodomains of *WOX11* and *WOX12* and probably abolished the functions of the two proteins.

Next, we analyzed the adventitious rooting phenotype of the mutant leaf explants form the 9-, 12- and 15-day-old *wox11-3* and *wox12-3* single-mutant and *wox11-3 wox12-3* double-mutant seedlings grown under 24-h light conditions. The detached leaf explants were cultured under 24-h light conditions on B5 medium. The *wox11-3* single mutant and the *wox11-3 wox12-3* double mutant were defective in rooting from all leaf explants compared with the wild-type seedlings (Fig. 5C). The data suggest that the *WOX11*/*12* pathway is involved in rooting from leaf explants with different developmental states. However, we could still find the effect of the leaf maturation on the rooting ability of the *wox11-3 wox12-3* double mutant, indicating that other pathways may have partial redundant roles with *WOX11*/*12* in DNRR.

3. Discussion

In this study, we showed that leaf maturation might cause a reduced auxin accumulation during DNRR from leaf explants. This may explain why mature explants have more difficulty regenerating roots than immature explants (Woo et al., 1994; Sanchez et al., 1995; Swamy et al., 2002; Abarca et al., 2014; Abu-Abied et al., 2014; Leakey, 2014; de Almeida et al., 2015; Aumond et al., 2017). Multiple early signals, including wounding, circadian rhythms and leaf maturation, may converge to regulate auxin biosynthesis. For example, the expression levels of *YUC1*/*2*/*4*/*6* and some other *YUC* genes and auxin-related genes are associated with the developmental stages of leaf explants; the *YUC1*/*4*/*5*/*8*/*9* genes could act in response to detachment (Chen et al., 2016); the expression of

YUC4 and probably other *YUC* genes could be affected by circadian; the *YUC5*/*8*/*9* expression levels are also upregulated by darkness (Chen et al., 2016). All of these early signals may affect the level of auxin accumulation and influence the *WOX11*/*12*-mediated cell transition ability of regeneration-competent cells (see a model in Fig. 6).

4. Materials and methods

4.1. Plant materials and culture conditions

Arabidopsis Col-0 was used as the wild type. The *yuc-1D* mutant was previously described (Zhao et al., 2001). *Arabidopsis* seeds were sterilized with 75% alcohol and kept at 4 °C for 2 days. The seeds were then germinated on 1/2 MS medium (half-strength of MS basal medium with 1% sucrose, 1% agar and 0.5 g/L MES, pH 5.7) (Murashige and Skoog, 1962) at 22 °C under 24-h constant light conditions, except during the circadian rhythm analysis in which seedlings were grown under 16-h light and 8-h dark conditions. The first-pair rosette leaves were used for regeneration in this study. Detached leaf explants were cultured on B5 medium without sucrose (Gamborg B5 basal medium with 0.5 g/L MES and 0.8% agar, pH 5.7) (Gamborg et al., 1968) at 22 °C under 24-h light conditions.

4.2. *wox11* and *wox12* mutants generated by CRISPR/Cas9

To generate *wox11-3* and *wox12-3* mutants, a *WOX11*-specific target (5′-CAGAACCGGTTCGGTCCCGA-3′) and a *WOX12*-specific target (5′-CCGAACCAGTCCGGGCACGT-3′) were selected as the targets for Cas9 to mutate *WOX11* and *WOX12*, respectively. Vector construction was performed as previously described (Yan et al., 2015). Briefly, the target sequences were first cloned into the pBluescript-AtU6-26-sgRNA vector. Then, the AtU6-26-WOX11-sgRNA or AtU6-26-WOX12-sgRNA fragment was digested and inserted into the pCAMBIA1300-pYAO:hSpCas9 vector to generate the pCAMBIA1300-pYAO:hSpCas9-WOX11-sgRNA or pCAMBIA1300-pYAO:hSpCas9-WOX12-sgRNA plasmid. The plasmids were introduced into the wild-type *Arabidopsis* by *Agrobacterium*-mediated floral dip transformation. The genomic fragments covering the mutation sites were amplified from the T_1 transgenic plants by PCR and then sequenced.

4.3. Determination of auxin concentrations

Thirty leaf explants from each sample were harvested and ground by liquid nitrogen. The powder

was dissolved by 200 μL PBS buffer for 10 min on ice and then centrifuged for 2 min at 12000 r/min at 4 ºC. We used 10 μL supernatant for each technical repeat of electrochemical detection of auxin as previously described (Sun et al., 2017; Sun et al., 2018).

4.4. qRT-PCR and RNA-Seq

RNA extraction, reverse transcription and qRT-PCR were carried out as described previously (He et al., 2012). The qRT-PCR results represented the relative expression levels, which were normalized against those produced by the *ACTIN* primers that had an arbitrarily fixed value of 1.0. Primers for qRT-PCR are listed in Table S2.

For RNA-Seq analysis, RNA was extracted using TRIzol (Invitrogen, USA). Library construction and deep sequencing were carried out using the Illumina HiSeq 3000 platform following the manufacturer's instructions by Genergy Biotechnology (Shanghai, China). Raw RNA-Seq reads were trimmed based on quality using Trimmomatic (Bolger et al., 2014), and paired reads were mapped to the *Arabidopsis* genome (TAIR10) using STAR 2.5.3.a (Dobin et al., 2013) with default settings. The returned alignments were stringently filtered to remove ambiguously mapped reads and read pairs with conflicting alignments. For the RNA-Seq data analysis, RSEM v1.3.0 (Li and Dewey, 2011) was used to quantitate transcript abundance and expression values of individual genes, which are shown as the average of transcripts per million (TPM) in three biological replicates. Differentially expressed genes were detected by EBSeq (Leng et al., 2013) based on the combined criteria: $|\log_2(\text{fold change})| > 1$ and false discovery rates < 0.05. To compare expression dynamics between different genes, genes were filtered by combined criteria: the average of TPM > 1 (in three developmental states) and coefficient of variation (CV) > the median CV of all expression genes. Finally, TPM values of 8775 genes were z-score normalized and clustered into six groups using the k-means clustering in R version 3.5.1 (https://www.R-project.org/). The RNA-Seq data were deposited in Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE108253. The analyzed data are shown in Table S1.

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Figure legends

Fig. 1. Analysis of transcriptome during leaf maturation. **A**–**C**: Wild-type 9- (**A**), 12- (**B**) and 15-day-old (**C**) seedlings grown on 1/2 MS medium. Arrows indicate the first pair of rosette leaves. c, cotyledon. Scale bar, 1 mm. **D**–**F**: RNA-Seq analysis of the first-pair leaves from 9-, 12- and 15-day-old seedlings. Changes of gene expression patterns are grouped into six clusters (clusters 1 to 6), showing upregulation (**D**), downregulation (**E**) or other changes (**F**) of gene expression levels. Auxin-related genes are listed in each cluster. Also see Table S1 for the gene lists of each cluster. **G**–**J**: RNA-Seq analysis of changes in gene expression levels (1 DAC *vs* time 0) of leaf explants from 9- and 15-day-old seedlings. Twelve clusters of genes (clusters a to l) are shown. Auxin-related genes are listed in each cluster. Also see Table S1 for the gene lists of each cluster. All plants and leaf explants were grown and cultured under 24-h light conditions.

Fig. 2. Expression of *TAA1*, *YUC2* and *YUC6* in response to leaf maturation. **A**–**C**: qRT-PCR analysis of the expression levels of *TAA1* (**A**), *YUC2* (**B**) and *YUC6* (**C**) in time-0 leaf explants from 9-, 12- and 15-day-old seedlings. Bars show SEM of at least three biological replicates. Each biological replicate was performed with three technical replicates. * $P < 0.05$ and ** $P < 0.01$ in two-sample *t*-test compared with 9-day-old seedlings.

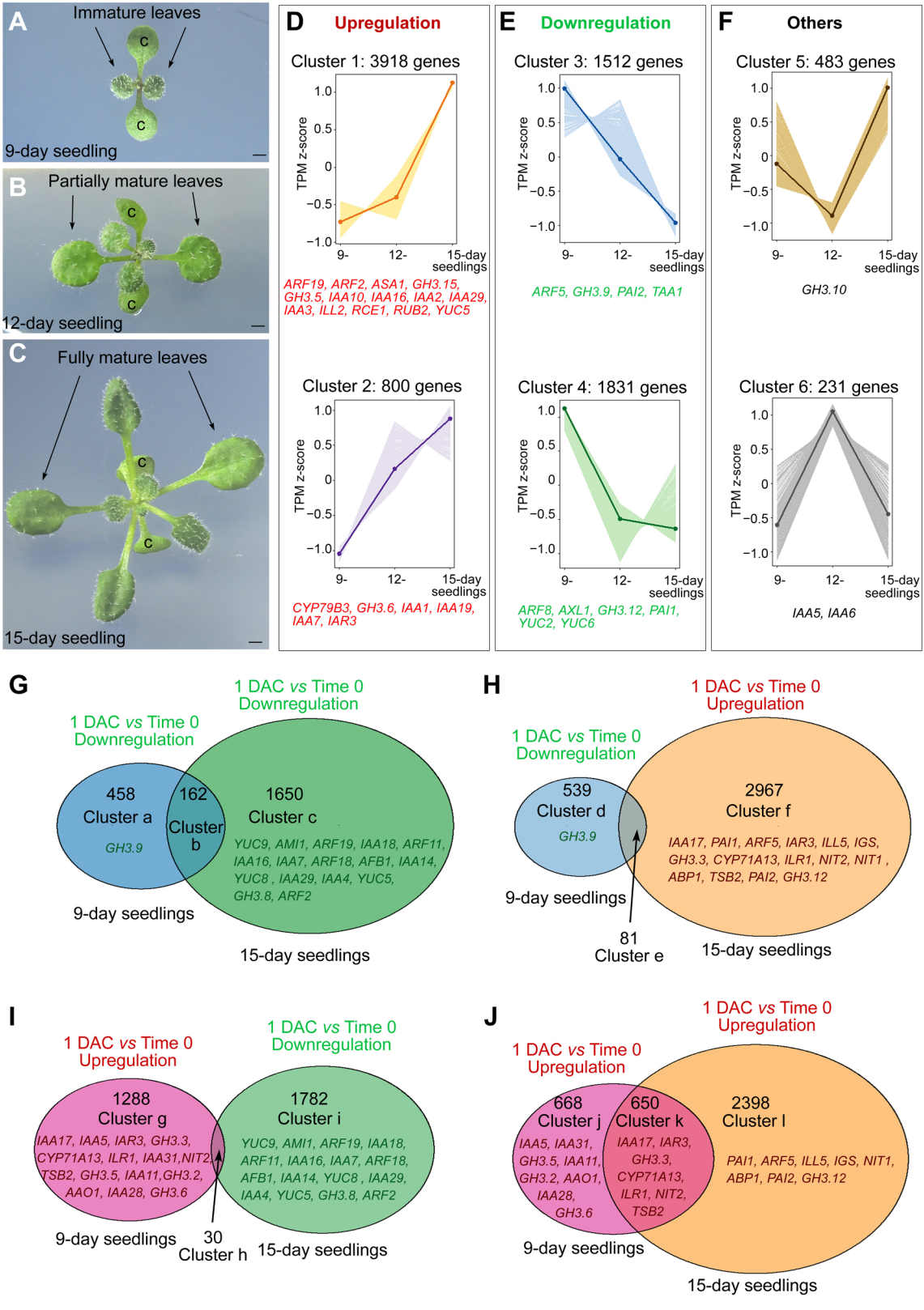
Fig. 3. *YUC1/4* are expressed in response to leaf maturation and wounding. **A** and **B**: qRT-PCR analysis of the expression levels of *YUC1* (**A**) and *YUC4* (**B**) in time-0 and 1-DAC leaf explants from 9-, 12- and 15-day-old seedlings. Bars show SEM of three biological replicates. Each biological replicate was performed with three technical replicates. * $P < 0.05$ and ** $P < 0.01$ in two-sample *t*-test. **C** and **D**: Leaf explants from 15-day-old wild-type (**C**) and *yuc-1D* (**D**) seedlings were cultured on B5 medium at 14 DAC. Scale bar, 5 mm. **E**: Percentage of leaf explants with regenerated adventitious roots at 14 DAC. Leaf explants from 9-, 12- and 15-day-old wild-type (Col-0) and *yuc-1D* seedlings were cultured on B5 medium. Bars show SD of three biological repeats ($n = 30$ per repeat). ** $P < 0.01$ in two-sample *t*-test compared with Col-0 control. In **A**–**E**, all plants and leaf explants were grown and cultured under 24-h light conditions.

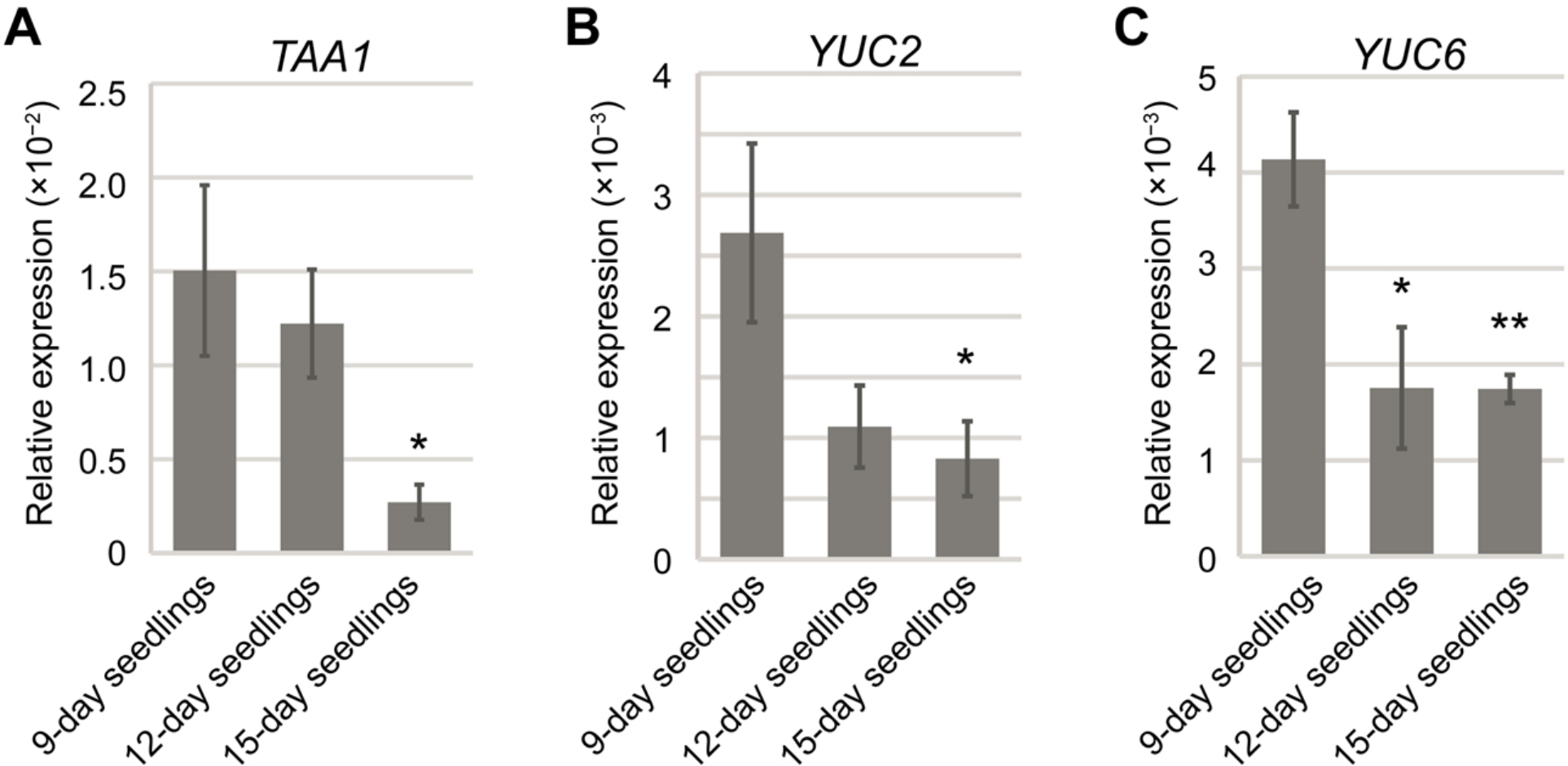
Fig. 4. Circadian rhythms regulate *YUC4* expression. qRT-PCR analysis of the expression levels of *YUC4* in the first-pair rosette leaves from 12-day-old seedlings in a 24-h circadian period (16-h light and 8-h dark). The light was turned on at 9:00 in the morning and turned off at 1:00 at night.

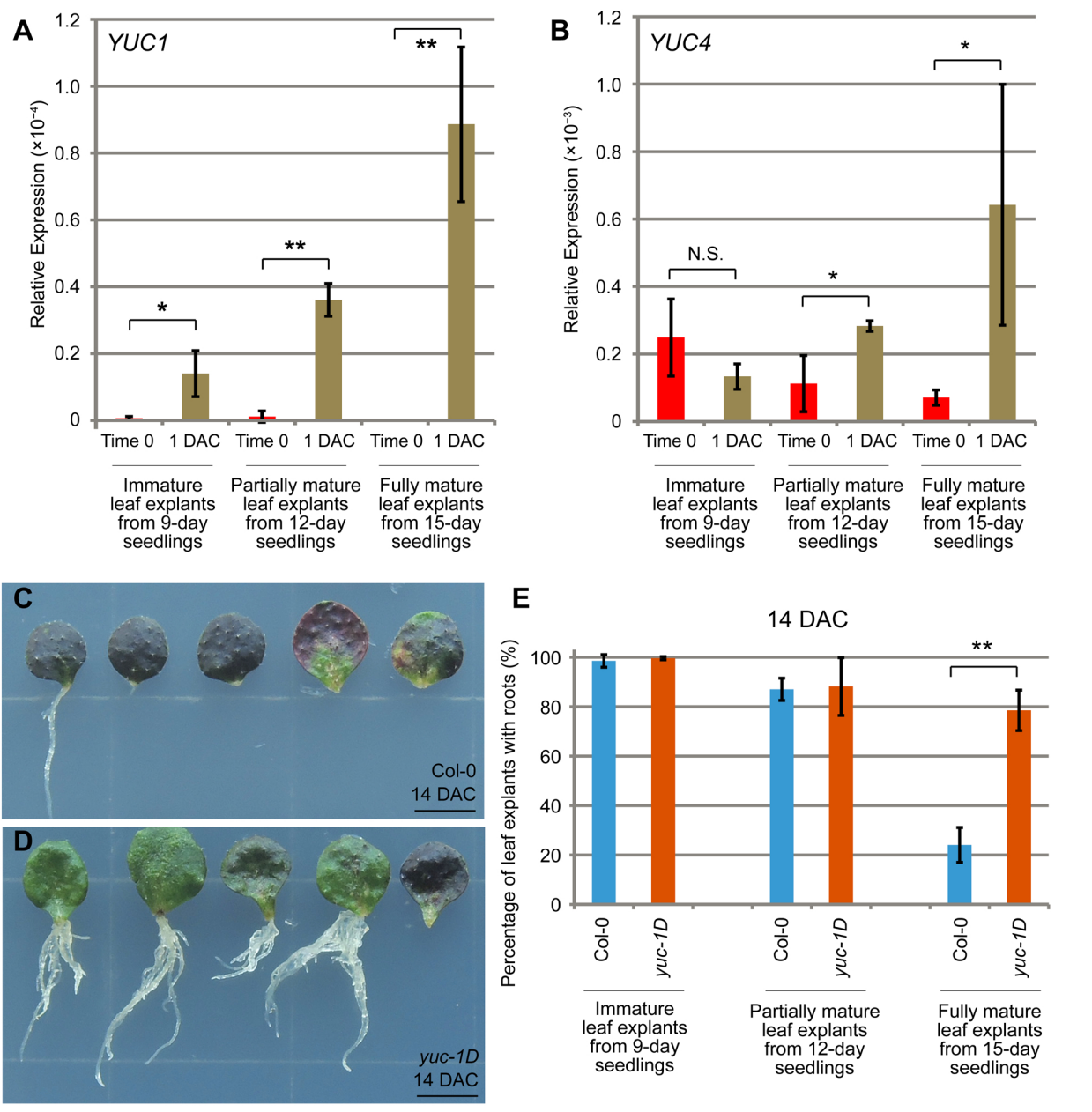
Bars show SEM of three biological replicates. Each biological replicate was performed with three technical replicates.

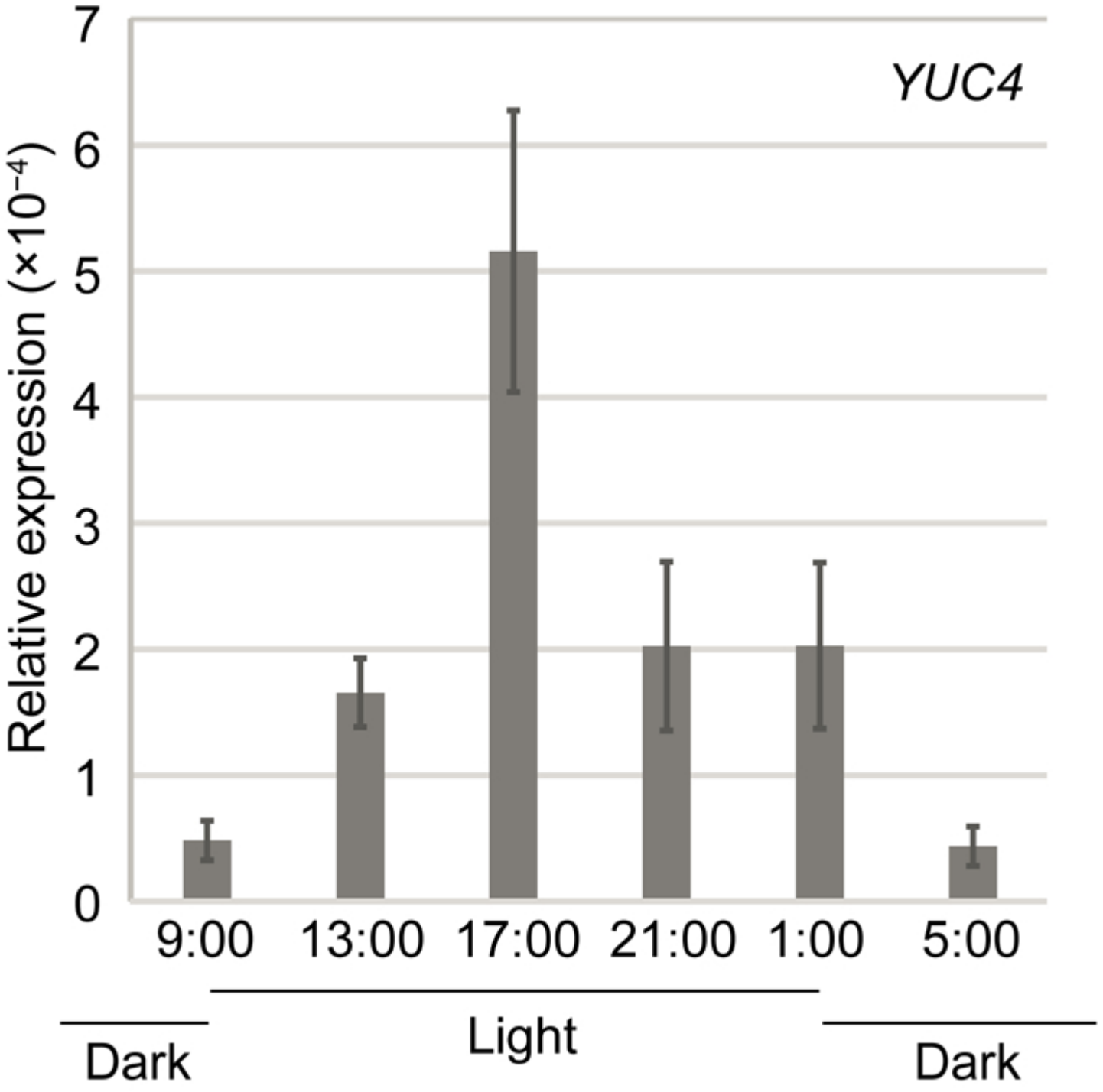
Fig. 5. Analysis of adventitious rooting ability in *wox11-3* and *wox12-3* mutants. **A** and **B**: Structural diagrams of the *WOX11* (**A**) and *WOX12* (**B**) genes, showing the T-DNA insertion alleles *wox11-2* and *wox12-1* and the CRISPR/Cas9 alleles *wox11-3* and *wox12-3*. The sequencing results of the CRISPR/Cas9 alleles are listed in the boxed regions. HD, homeodomain. **C**: Percentage of leaf explants with regenerated adventitious roots at 14 DAC. Leaf explants from 9-, 12- and 15-day-old Col-0, the *wox11-3* and *wox12-3* single mutants and the *wox11-3 wox12-3* double mutant were cultured on B5 medium for 14 days. Bars show SD of three biological replicates (n = 30 per replicate). ** $P < 0.01$ in two-sample t-test compared with each Col-0 control. All plants and leaf explants were grown and cultured under 24-h light conditions.

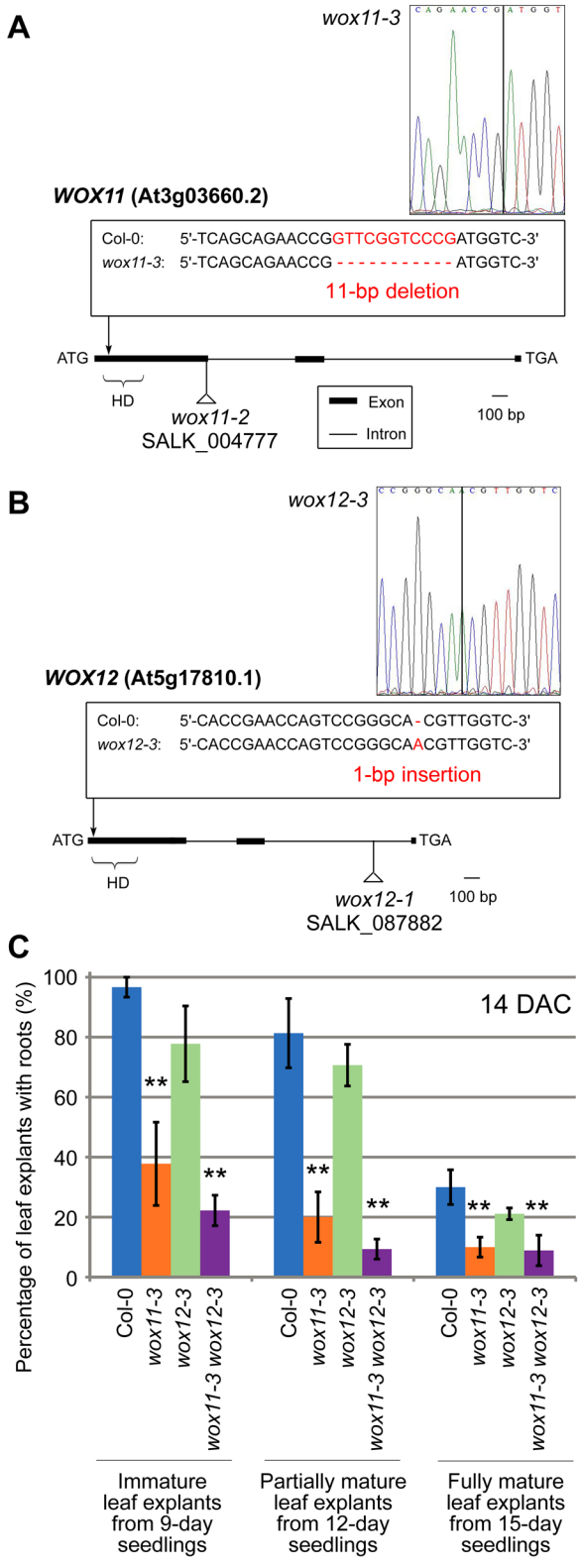
Fig. 6. Model of early signals in regulation of DNRR from leaf explants. Multiple early signals may regulate auxin production in converter cells (i.e., mesophyll cells, leaf margin cells and some vascular cells), therefore influencing the efficiency of *WOX11*/*12*-mediated cell fate transition of regeneration-competent cells. Currently it is not clear whether circadian may have an effect on DNRR.


A
Immature leaves
C
C
9-day seedling
B
Partially mature leaves
C
C
12-day seedling
C
Fully mature leaves
C
C
15-day seedling
D
Upregulation
Cluster 1: 3918 genes
TPM z-score
1.0
0.5
0
−0.5
−1.0
9-
12-
15-day seedlings
ARF19, ARF2, ASA1, GH3.15, GH3.5, IAA10, IAA16, IAA2, IAA29, IAA3, ILL2, RCE1, RUB2, YUC5
Cluster 2: 800 genes
CYP79B3, GH3.6, IAA1, IAA19, IAA7, IAR3
E
Downregulation
Cluster 3: 1512 genes
ARF5, GH3.9, PAI2, TAA1
Cluster 4: 1831 genes
ARF8, AXL1, GH3.12, PAI1, YUC2, YUC6
F
Others
Cluster 5: 483 genes
GH3.10
Cluster 6: 231 genes
IAA5, IAA6
G
1 DAC vs Time 0
Downregulation
1 DAC vs Time 0
Downregulation
458
Cluster a
GH3.9
162
Cluster b
1650
Cluster c
YUC9, AMI1, ARF19, IAA18, ARF11, IAA16, IAA7, ARF18, AFB1, IAA14, YUC8 , IAA29, IAA4, YUC5, GH3.8, ARF2
9-day seedlings
15-day seedlings
H
1 DAC vs Time 0
Upregulation
1 DAC vs Time 0
Downregulation
539
Cluster d
GH3.9
2967
Cluster f
IAA17, PAI1, ARF5, IAR3, ILL5, IGS, GH3.3, CYP71A13, ILR1, NIT2, NIT1 , ABP1, TSB2, PAI2, GH3.12
9-day seedlings
81
Cluster e
15-day seedlings
I
1 DAC vs Time 0
Upregulation
1 DAC vs Time 0
Downregulation
1288
Cluster g
IAA17, IAA5, IAR3, GH3.3, CYP71A13, ILR1, IAA31,NIT2, TSB2, GH3.5, IAA11,GH3.2, AAO1, IAA28, GH3.6
1782
Cluster i
YUC9, AMI1, ARF19, IAA18, ARF11, IAA16, IAA7, ARF18, AFB1, IAA14, YUC8 , IAA29, IAA4, YUC5, GH3.8, ARF2
9-day seedlings
30
Cluster h
15-day seedlings
J
1 DAC vs Time 0
Upregulation
1 DAC vs Time 0
Upregulation
668
Cluster j
IAA5, IAA31, GH3.5, IAA11, GH3.2, AAO1, IAA28, GH3.6
650
Cluster k
IAA17, IAR3, GH3.3, CYP71A13, ILR1, NIT2, TSB2
2398
Cluster l
PAI1, ARF5, ILL5, IGS, NIT1, ABP1, PAI2, GH3.12
9-day seedlings
15-day seedlings


A
TAA1
Relative expression (×10⁻²)
2.5
2.0
1.5
1.0
0.5
0
*
9-day seedlings
12-day seedlings
15-day seedlings
B
YUC2
Relative expression (×10⁻³)
4
3
2
1
0
*
9-day seedlings
12-day seedlings
15-day seedlings
C
YUC6
Relative expression (×10⁻³)
5
4
3
2
1
0
*
**
9-day seedlings
12-day seedlings
15-day seedlings


A
YUC1
Relative Expression (×10⁻⁴)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
**
Time 0
1 DAC
Time 0
1 DAC
Time 0
1 DAC
Immature leaf explants from 9-day seedlings
Partially mature leaf explants from 12-day seedlings
Fully mature leaf explants from 15-day seedlings
B
YUC4
Relative Expression (×10⁻³)
1.2
1.0
0.8
0.6
0.4
0.2
0
N.S.
*
*
Time 0
1 DAC
Time 0
1 DAC
Time 0
1 DAC
Immature leaf explants from 9-day seedlings
Partially mature leaf explants from 12-day seedlings
Fully mature leaf explants from 15-day seedlings
C
Col-0
14 DAC
D
yuc-1D
14 DAC
E
14 DAC
Percentage of leaf explants with roots (%)
100
80
60
40
20
0
**
Col-0
yuc-1D
Col-0
yuc-1D
Col-0
yuc-1D
Immature leaf explants from 9-day seedlings
Partially mature leaf explants from 12-day seedlings
Fully mature leaf explants from 15-day seedlings


YUC4
Relative expression (×10⁻⁴)
7
6
5
4
3
2
1
0
9:00
13:00
17:00
21:00
1:00
5:00
Dark
Light
Dark


A
wox11-3
WOX11 (At3g03660.2)
Col-0: 5'-TCAGCAGAACCGGTTCGGTCCCGATGGTC-3'
wox11-3: 5'-TCAGCAGAACCG - - - - - - - - - - ATGGTC-3'
11-bp deletion
ATG
TGA
HD
wox11-2
SALK_004777
Exon
Intron
100 bp
B
wox12-3
WOX12 (At5g17810.1)
Col-0: 5'-CACCGAACCAGTCCGGGCA - CGTTGGTC-3'
wox12-3: 5'-CACCGAACCAGTCCGGGCAACGTTGGTC-3'
1-bp insertion
ATG
TGA
HD
wox12-1
SALK_087882
100 bp
C
14 DAC
Percentage of leaf explants with roots (%)
100
80
60
40
20
0
**
Col-0
wox11-3
wox12-3
wox11-3 wox12-3
Immature leaf explants from 9-day seedlings
Partially mature leaf explants from 12-day seedlings
Fully mature leaf explants from 15-day seedlings