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Oscillating Gene Expression Determines Competence for Periodic *Arabidopsis* Root Branching

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Abstract

Plants and animals produce modular developmental units in a periodic fashion. In plants, lateral roots form as repeating units along the root primary axis; however, the developmental mechanism regulating this process is unknown. We found that cyclic expression pulses of a reporter gene mark the position of future lateral roots by establishing prebranch sites and that prebranch site production and root bending are periodic. Microarray and promoter-luciferase studies revealed two sets of genes oscillating in opposite phases at the root tip. Genetic studies show that some oscillating transcriptional regulators are required for periodicity in one or both developmental processes. This molecular mechanism has characteristics that resemble molecular clock–driven activities in animal species.

Formation of periodic modular structures is a common developmental feature in both animals and plants (1,2). The body axis of many metazoans—including species of arthropods, annelids, and vertebrates—is organized into segments. This organization is established during embryogenesis by successive addition of segments to an elongating posterior body region (3). In plants, branching of shoots and roots during postembryonic development produces repeating units (phytomers and lateral roots, respectively) along the growing longitudinal axis. Leaves are generated in a regular phyllotactic pattern, and lateral roots (LRs) are continuously produced from the primary root (Fig. 1A) as it grows, following the gravity vector (4).

In vertebrates, segmentation is coordinated by a molecular clock that converts temporal information into a periodic spatial pattern, which precisely positions somites along the anterior-posterior axis (3,5). In the plant shoot apical meristem, there is evidence that

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differential hormone distribution may be involved in positioning leaf primordia (6). In the root, the developmental mechanism by which newly formed organs are positioned in time and space along the primary root remains largely uncharacterized. LR formation begins when selected pericycle cells become LR founder cells. However, the process underlying the selection of these cells is unknown. After specification, LR founder cells undergo a stereotypic patterning to form a LR primordium (7). Eventually, the newly formed primordia develop into LRs that emerge through the primary root tissues. Emergence is highly dependent on environmental conditions (8). An auxin-based oscillatory mechanism has been hypothesized to be operating to initiate LR primordia in roots (9). Our evidence suggests that LR positioning is instead mediated by oscillating genes that establish the temporal and spatial distribution of LRs along the primary root axis.

Periodic branching and bending require an endogenous mechanism

Enhanced transcriptional response to auxin in the basal meristem, as reported by the DR5:GUS synthetic promoter construct, has been proposed to be involved in formation of LR primordia (10). To investigate this hypothesis in vivo and in real time, we fused the DR5 promoter to the Luciferase coding region to conduct nondestructive reporter assays (11). We observed that DR5:Luciferase expression rhythmically pulsed with a period of ~6 hours in the basal meristem and the elongation zone (Fig. 1, B and C, and movies S1 and S2). We designated this whole region as the oscillation zone (OZ). Examination of gene expression relative to the root's developmental zones is relevant because, in the *Arabidopsis* root, cells at a fixed spatial position change their status over time. In addition, we observed that the pulses of DR5 expression preceded the establishment of static points of DR5:Luciferase expression, which we termed prebranch sites. Prebranch sites can subsequently develop into new LRs (Fig. 1D and fig. S1). Prebranch sites from which LRs did not emerge were examined microscopically, and early-stage LR primordia (7) were found in all cases. Thus, prebranch site formation predicts the spatial position of LRs in the primary root, and we hypothesize that the oscillation reported by DR5 results in competence to form LRs at the prebranch sites.

It has been previously described that distance or cell number between consecutive LRs or LR primordia was variable (12); we therefore investigated whether LR positioning might follow a temporal distribution. We measured the time between adjacent prebranch sites and tested the distribution of the cumulative function for normality. We found that the frequency of prebranch site production followed a normal distribution (P-value = 1.8e-06 in an Anderson-Darling normality test) with a mean period of ~6 hours (Fig. 1E). In addition, because previous reports had shown a correlation between the bends formed by the primary root while it grows and LR formation, we also measured the time between consecutive bends (Fig. 1F). Remarkably, we found that the frequency of bending also followed a normal distribution (P = 0.017 in an Anderson-Darling normality test) with a mean period similar to that of prebranch site initiation. We observed that the DR5 oscillation seems to occur prior to changes in growth direction of the primary root (Fig. 1C). This suggests that bending may also be a consequence of a developmental mechanism reported by DR5, although additional regulation by external cues, such as gravity, cannot be excluded.

A characteristic of endogenous mechanisms that track time in living organisms is their capacity to compensate for changes in temperature, which buffers the periodic processes against variations due to external conditions (13,14). To determine if this is the case for prebranch site formation, we grew plants at different temperatures and observed the number of prebranch sites formed during 6 days of growth. We found that the number of prebranch sites remained largely unchanged in plants grown between 18° and 24°C. The number also remained constant when plants were grown in altered nutrient conditions (no sucrose) or in

continuous light (Fig. 1G). All of these conditions affect the growth rate of the primary root as evidenced by root length that is considerably reduced by growth at lower temperatures, on media without sucrose, and in continuous light (Fig. 1G). In addition, we examined bending under altered nutrient conditions (no sucrose), and we found that the number of bends after 72 hours of growth was similar to those in the standard conditions (altered: 14.5 ± 2.1, n = 25, and standard: 13.6 ± 1.6, n = 25). Taken together, our results indicate that both prebranch site formation and root bending are periodic responses that may be regulated by an endogenous clock mechanism.

Auxin is not sufficient to specify prebranch sites

Given the known role of auxin in several stages of LR formation (9,15,16) and the general use of DR5 as an auxin-signaling reporter (17), we investigated whether prebranch site formation was related to changes in levels of this hormone. We generated different auxin-signaling reporters by fusing the promoters of INDOLE-3-ACETIC ACID INDUCIBLE 7 (IAA7), IAA14, and IAA19 to the luciferase coding region and tested them for response to exogenous application of auxin in the OZ. pIAA19 was expressed in the OZ and responded to exogenous auxin in a fashion similar to that of DR5:Luciferase (Fig. 2, A and B, and movies S3 and S4), whereas pIAA7 and pIAA14 responded to auxin at later times. When we evaluated the expression of pIAA19 in the OZ without any hormone treatment, we did not detect an oscillatory behavior in its expression over time (Fig. 2C and movie S5). Although additional transcriptional regulation of pIAA19 cannot be excluded, our data suggest that fluctuation in auxin is unlikely to be sufficient to drive the periodic pulses of DR5 expression in the OZ.

Induction of auxin biosynthesis in pericycle cells has been linked to acquisition of LR founder-cell identity (18). Thus, we tested whether auxin could generate prebranch sites independently of the oscillation reported by DR5. We performed localized auxin treatments in the OZ, both when natural peaks of DR5 expression were detected and when they were not detected. Exogenous auxin treatment or auxin plus 1-*N*-naphthylphthalamic acid (NPA), which should prevent auxin movement, did not lead to production of a prebranch site when the treatment was not concurrent with a DR5 oscillation (Fig. 2, D and E). When the DR5 oscillation was concurrent with application of auxin or auxin plus NPA, the prebranch site appeared to be shifted from the predicted position of a natural prebranch site in ~60% of the cases (Fig. 2E). However, auxin did not seem to specify a new prebranch site, because two prebranch sites were never observed in close proximity after the auxin treatment (fig. S2). As DR5 expression also marks LR founder cells (18), this indicates that functional founder-cell specification normally requires the oscillation reported by DR5. However, we cannot rule out the possibility that at very high, sustained doses of auxin there may be de novo production of LRs through a mechanism that does not depend on prebranch site formation.

Additionally, it has been proposed that root bending could promote auxin accumulation preferentially at the outside of the curve, which, in turn, could induce LR formation (19,20). This was particularly intriguing because we had observed that primary root bending occurred with a frequency similar to that of prebranch site production (Fig. 1, E and F), and prebranch sites tend to be located in the middle of the bends. Thus, we examined whether ectopic prebranch sites were induced by manual bending (generating J-hooks) near the root tip (19). When J-hooks were formed ~5 mm from the root tip, the J-hook occurred at a position where prebranch sites were already specified (fig. S3). We found that the first prebranch site was located at an average of 3 mm from the root tip, and we did not detect ectopic formation of prebranch sites at J-hooks (fig. S3). Lateral roots always emerged from preexisting prebranch sites. In addition, we examined gravity-induced bends. We altered the root's position relative to the gravity vector by rotation of either 90° or 180° in the vertical

plane or by turning the plants 90° from the vertical to horizontal plane (fig. S4). We observed that gravity-induced bends occurred in an asynchronous manner. Roots rotated 180° showed gravity-induced bending after an average of 3 hours; however, many roots took longer to bend, as seen in the skewed distribution in Fig. 2F. Interestingly, the time between the last periodic bend prior to the 180° rotation and the subsequent gravity-induced bend followed a distribution similar to that detected for roots under normal conditions (Fig. 2F and Fig. 1F). This suggests that the root's natural periodic bends are completed prior to gravity-induced bends. It has been reported that gravistimulation at 6-hour intervals is optimal for increasing LR density (21). As LR development requires previous formation of a prebranch site, it is possible that the observed increase in LR density is due to the gravistimulus-promoted development of each prebranch site into a LR primordium. In addition, after rotation of the roots, we detected peaks of DR5 expression approximately every 3 hours; this is more frequent than expected for the periodic DR5 oscillation or the response to gravity alone (Fig. 2G). It has been previously reported that auxin may accumulate following gravity-induced bending (19). To examine this further, we observed pIAA19 expression after gravistimulation. We found that pIAA19 peaks of expression were less frequent than peaks of DR5 expression after gravistimulation (Fig. 2H). Because pIAA19 does not oscillate, but responds to auxin, these pIAA19 peaks may report increases in auxin signaling or content due to gravity response. Under gravity response conditions, pulses of DR5 expression generated static points of DR5 expression; however, not all of these points persisted and only a few formed a prebranch site that eventually developed a LR (fig. S4). In addition, we tested whether prebranch site formation was affected by conditions that reduced root bending by growing roots through the growth medium. We detected approximately the same number of prebranch sites for roots that grew through the agar as for roots grown along its surface (standard conditions) (Fig. 2I). This indicates that prebranch site specification occurs in the absence of visible root bends.

Taken together, our results indicate that competence to form a LR through establishment of prebranch sites is determined by internal cues reported by peaks in DR5 expression. Auxin may contribute to this endogenous mechanism, because application of auxin appears to shift the location of a prebranch site. In addition, the root gravitropic response, which likely involves altered endogenous auxin concentrations, appears to be able to change the period of the endogenous mechanism.

Genes oscillate in opposite phases before LR initiation

From our previous results, we hypothesized that the periodic behavior of the DR5 reporter in the OZ might represent a more general oscillatory transcriptional mechanism. To determine what genes, if any, were expressed in a periodic pattern similar to that of DR5, we dissected the OZ of 40 individual roots and divided it into an upper (OZ2) and lower (OZ1) half (fig. S5). Because we were unable to synchronize the roots, we analyzed DR5:GUS expression by real-time reverse transcription polymerase chain reaction (RT-PCR) in the two halves of the OZ to infer an approximate chronological order of the different roots along one cycle of the DR5 oscillation (supporting online text). On the basis of the inferred order, we selected 20 OZ1 and 19 OZ2 representative samples and performed microarray experiments on each one (fig. S6). The microarray data from these samples were concatenated to generate an inferred time series. To identify gene expression profiles that exhibited periodic behavior, we used two different pattern-detection methods. One was a modification of the Lomb-Scargle periodogram (3), which is related to Fourier transform analysis, and the other was the address-reduction method, which is data-driven and, unlike Lomb-Scargle, does not assume periodicity of the patterns of interest (22). Although both methods have been shown to identify known periodic genes within diverse microarray time series (23), we considered significant only those genes identified by both approaches and that met other filtering

criteria based on fold-change and expression levels (Fig. 3A and table S1). In addition, randomization of the samples showed that substantial numbers of periodic gene expression patterns were only associated with the order given by the analysis of the DR5:GUS expression (fig. S8). Two distinct sets of genes were identified as oscillating in the OZ. One set comprised 2084 genes oscillating in phase with DR5, and the second set was made up of 1409 genes, which oscillated in antiphase to DR5 (Fig. 3B). The predicted oscillating expression pattern of several of these genes in the OZ was validated by constructing fusions of their promoters to the luciferase gene (movies S6 to S10). Quantification of the luciferase activity in the OZ showed that expression of pZAT11 and pARF7 rhythmically pulsed (Fig. 3, C to F) with the same period as DR5 (Fig. 1C).

Analysis of the underlying molecular mechanisms of auxin-mediated LR initiation has been examined by microarray analyses under different experimental conditions (24,25). To address whether the molecular oscillation in the OZ contained components similar to those previously described for LR initiation, we compared all these data sets (fig. S9). We found little overlap (<15%) between the oscillating genes and the various LR initiation data sets (fig. S9), which suggested that prebranch site production and LR initiation use distinct molecular mechanisms, as would be expected for distinct developmental processes. Among the common genes, we found LOB-DOMAIN 16 (LBD16) and LBD33, which have roles in LR initiation (26). However, we did not find IAA14-SOLITARY ROOT (SLR), which is required for LR initiation (27) but not for establishment of LR primordia (10,27). One of the genes found only in the OZ data set is AUXIN RESPONSIVE FACTOR 7 (ARF7), which is upstream of LBD16 (26); and unexpectedly, OZ-localized auxin treatment did not result in increased expression of pARF7:Luciferase. Thus, it is plausible that the pathways leading to auxin-mediated LR initiation and prebranch site formation are largely independent, although specific molecular connections might ensure coordination of both processes.

Oscillating genes change expression along the primary root

Because we performed the microarray experiments with tissue only from the OZ, we were also interested in examining the behavior of the oscillating genes over developmental time. Recently, a spatiotemporal transcriptional map of the *Arabidopsis* root (the RootMap) identified dominant gene expression patterns that fluctuate over developmental time and from root to root (28). We examined the expression of the oscillating genes in the RootMap longitudinal data set (28), which separately profiles 12 sections from two independent roots. We performed *K*-means clustering of the phase and antiphase gene expression patterns for one of the roots. The oscillating genes were grouped into five phase gene clusters and four antiphase gene clusters. Gene expression patterns for every cluster coordinately changed when examined in the second root (Fig. 3G,fig. S10, and table S1). Expression analyses of the oscillating genes using promoter fusions to the luciferase gene showed that expression of pARF7, pATAF1, pLBD16, pZAT11, and pWRK65 expression changed both across developmental zones and over time in the *Arabidopsis* root (Fig. 3H,fig. S11, and movies S6 to S10). Gene expression also seems to move spatially, propagating along the root's longitudinal axis (Fig. 3H; note that pARF7 and pATAF1 expression moves across the dotted lines). This movement might involve some cell-to-cell communication or signaling process. It is possible that different waves of gene expression might link prebranch site formation with other developmental processes. For instance, ATAF1, in wave A4, has been described as negatively regulating the expression of stress-responsive genes (29), and VND6 in wave P2 has been shown to be involved in metaxylem specification (30).

To investigate the possible biological functions of these waves of gene expression, we analyzed Gene Ontology (GO) term enrichment (28). We found that genes oscillating in opposing phases show significant enrichment in different GO categories and that different

waves tend to be enriched in GO categories associated with specific biological processes (Fig. 3I and table S2). Particularly intriguing was the P3 wave (Fig. 3J), which was enriched in GO categories associated with cell cycle progression, mitosis-specific expressed genes, and genes putatively involved in asymmetric division (24). Formation of LR primordia requires that LR founder cells undergo a series of asymmetric divisions (7), and although progression through the cell cycle has been shown to be involved in LR initiation (31), it is not sufficient to specify new LR primordia (10,25,27). Thus, genes within the P3 wave may be required before LR initiation. In accordance with this, we found that most of the genes in common between the OZ data set and the LR initiation data set are in waves P3 and A1 (Fig. 3J and table S3).

Oscillating transcription factors regulate root periodic responses

The identification of two main sets of genes oscillating in opposite phases in the OZ suggested that an oscillatory network might be involved in establishing the pace of periodic branching and bending in the *Arabidopsis* root. To further investigate this hypothesis we focused on determining the role of transcription factors (TFs) in regulating root periodic responses. Wave P5 was enriched in genes with an unknown biological function and in transcription factor activity, which suggested that oscillating TFs in wave P5 could be involved in periodic prebranch site formation and bending in the *Arabidopsis* root. Particularly intriguing was the overrepresentation of TFs from the MADS-box protein family (Fig. 3I and table S3) as they have not been described to have a role in root development. Among these TFs were SHATTERPROOF1 (SHP1), SHATTERPROOF2 (SHP2), and SEEDSTICK (STK), which are involved in carpel and ovule development and seed dispersal (32), and AGAMOUS-LIKE20 (AGL20), which regulates the transition to the reproductive stage (33). Notably, *shp1shp2* and *shp1shp2stk* mutant plants showed striking root phenotypes when compared with the wild type (Fig. 4A). When we examined LR and prebranch site production in *agl20-2*, *shp1shp2*, and *shp1shp2stk*, we observed a substantial decrease in both the total number of LRs and prebranch sites as reported by DR5 (Fig. 4B). These defects were not related to delayed seed germination in the mutants. Furthermore, analysis of the time between consecutive bends showed that periodic bending was also defective in *shp1shp2* and *shp1shp2stk* (Fig. 4C). Using a transcriptional reporter, we found that expression conferred by the SHP1 promoter fluctuated over time in the OZ (Fig. 4, D and E) and that it appeared transiently in static points of expression resembling prebranch sites (Fig. 4E). Examination of these points microscopically after 1 day revealed early stages of LR primordia. Our results indicate that MADS-box TFs are involved in periodic processes in the *Arabidopsis* root.

Additionally, we screened T-DNA insertion lines for 55 oscillating transcription factors identified in our data set. Several TFs belonging to the ARF (ARF7 and ARF2) and NAC [VND2, FEZ, and SOMBRERO (SMB)] families showed defects in prebranch site initiation and reduced numbers of LRs (Fig. 4B). In addition, the double and triple mutants *arf7shp1* and *arf7shp1shp2* showed some reduction and increased variability in the number of prebranch sites compared with *arf7* and *shp1shp2*, respectively. These findings indicate that there is some genetic interaction between ARF7, SHP1, and SHP2, which suggests that these TFs may function in the same pathway to periodically produce prebranch sites. In *arf7* plants, we observed that DR5 expression in the OZ was more persistent compared with wild-type plants and lacked periodicity (Fig. 4F), resulting in irregular locations of prebranch sites along the primary root (Fig. 4G). This suggests a role for ARF7 in regulation of periodic oscillating gene expression leading to prebranch site formation.

We observed that *fez-3* mutants were impaired in prebranch site formation (Fig. 4B), but quantification of the time between bends showed a distribution similar to that in wild-type

plants (Fig. 4H). This indicates that prebranch site formation and bending can be genetically separated and that these two periodic processes may be regulated by different sets of genes. Taken together, our results strongly suggest that an oscillatory network located in the OZ is the endogenous developmental mechanism triggering periodic branching and bending in the *Arabidopsis* root.

Discussion

How plants position newly formed organs during postembryonic development is a major unanswered question. In the root, this requires that subsets of cells be specified to generate LR primordia. We present evidence that in the *Arabidopsis* primary root, a process involving oscillating gene expression, which has characteristics of a biological clock, is the first step in positioning new LRs. This temporal prepatterning mechanism results in establishment of prebranch sites, which determines the position from which LRs will develop. Auxin may contribute to this process, but it does not appear to be sufficient to initiate a prebranch site. In addition, subsequent effects of auxin on modulating LR emergence likely participate in the final LR spatial distribution pattern. Remarkably, the root's response to gravity seems to affect the period of the endogenous mechanism producing prebranch sites. The integration of external cues with an endogenous developmental mechanism may represent an advantage for plants growing in soil where roots are forced to bend in response to various obstacles. In this case, branching in precise locations would maximize the root system's ability to explore heterogeneous territory for resources to support overall plant growth.

Current models for shoot phyllotactic patterning indicate that auxin accumulation controls the spatial patterning of aerial organs (34). In contrast, oscillating gene expression in the OZ of the *Arabidopsis* root seems to be responsible for establishing the spatial-temporal distribution of LR primordia. Remarkably, this shares similarities with the segmentation clock in vertebrates, where a large network of genes with oscillating expression in the presomitic mesoderm has been described (5). Somite length and number is temperature-compensated (35), whereas there is temperature compensation of the period of prebranch site production. In animals where development results in a fixed body plan, maintenance of somite size and number would be critical; however, in plants where the final body axis is flexible, a fixed production rate might optimize the root system's exploration of the soil. Thus, it appears that independently evolved multicellular organisms have converged upon analogous mechanisms to position modular units along specific growing axes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

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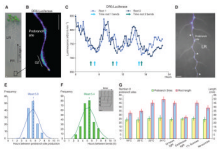

Fig. 1.

Periodic branching and bending are marked by repeated pulses of DR5 reporter expression. (**A**) LRs develop from the primary root (PR), and younger LRs are located closer to the PR tip. (**B**) DR5 fused to the luciferase reporter gene is expressed in the oscillation zone (OZ) and prebranch site(s). (**C**) Quantification of DR5 expression in the OZ over time. Arrows mark the time when the primary root bends. (**D**) Overlay of luciferase and bright-field images of a DR5-expressing root. Bright-field image was taken 5 days after the luciferase picture. Asterisks indicate LR primordia that did not emerge. (**E** and **F**) Graph of the distribution of prebranch site production and bending over time (n = 15 and 30, respectively). (Inset) Root bends with time interval (t_i) between bends. (**G**) Prebranch site production and root length under different growth conditions (n = 40). Scale bars, 1 mm.

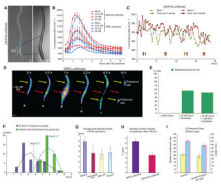

Fig. 2.

Manipulation of auxin levels or signaling is not sufficient to induce prebranch site formation or ectopic branching. (**A**) Overlay (O) of bright-field and luciferase images, and detailed bright-field image (BF), showing expression of pIAA19 fused to the luciferase reporter. (**B**) Quantification of pIAA19 and DR5 expression in the OZ after localized auxin treatments (*n* = 10). (**C**) Quantification of pIAA19 expression in the OZ over time. (**D**) Time series of a DR5:Luciferase-expressing root after OZ-localized auxin treatment. Yellow arrows indicate natural DR5 oscillations. Red arrow shows the location of the auxin treatment. (**E**) Percentage of plants showing misplaced prebranch sites in response to OZ-localized auxin and NPA treatments. (**F**) Distribution of the time to bend in response to gravity after a 180° turn (*n* = 39). (**G**) Time between consecutive pulses of DR5 expression over a 20-hour period under normal conditions and after changing the relative position of roots to gravity by turning. (**H**) Number of peaks of DR5 or pIAA19 expression detected over a 20-hour period after a 180° turn (*n* = 10). (**I**) Prebranch site production and root length for roots grown on the surface (control) or through the growth medium (*n* = 15 for each). Scale bars, 250 μm. Error bars represent SD.

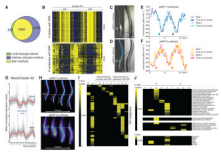

Fig. 3.

Identification of genes with periodic expression behavior in the oscillation zone. (**A**) Venn diagram showing the number of genes exhibiting periodic behavior as identified by two different methods. (**B**) Heat maps of the two sets of genes found to oscillate in the OZ. Yellow indicates higher and blue indicates lower relative expression. (**C** and **D**) Overlay (O) of bright-field and luciferase images showing expression of the promoter of (C) ZAT11 in phase with DR5, and (D) ARF7, in antiphase, fused to the luciferase gene. Bright-field images (BF) detailing the OZ are shown to the right (C and D). Scale bars, 250 μm. (**E**) Quantification of pZAT11:Luciferase and (**F**) pARF7:Luciferase expression in the OZ over time. (**G**) Expression profiles for oscillating genes in cluster A3 as expressed in the RootMap. Red line represents average gene expression. (**H**) Time series of pARF7:Luciferase (top) and pATAF1:Luciferase (bottom) expression (cluster A3). Dotted lines mark a fixed spatial position. (**I**) Gene Ontology (GO) term enrichment in the phase (P) and antiphase (A) gene clusters and (**J**) their enrichment in data sets profiling LR initiation and in transcription factor families.

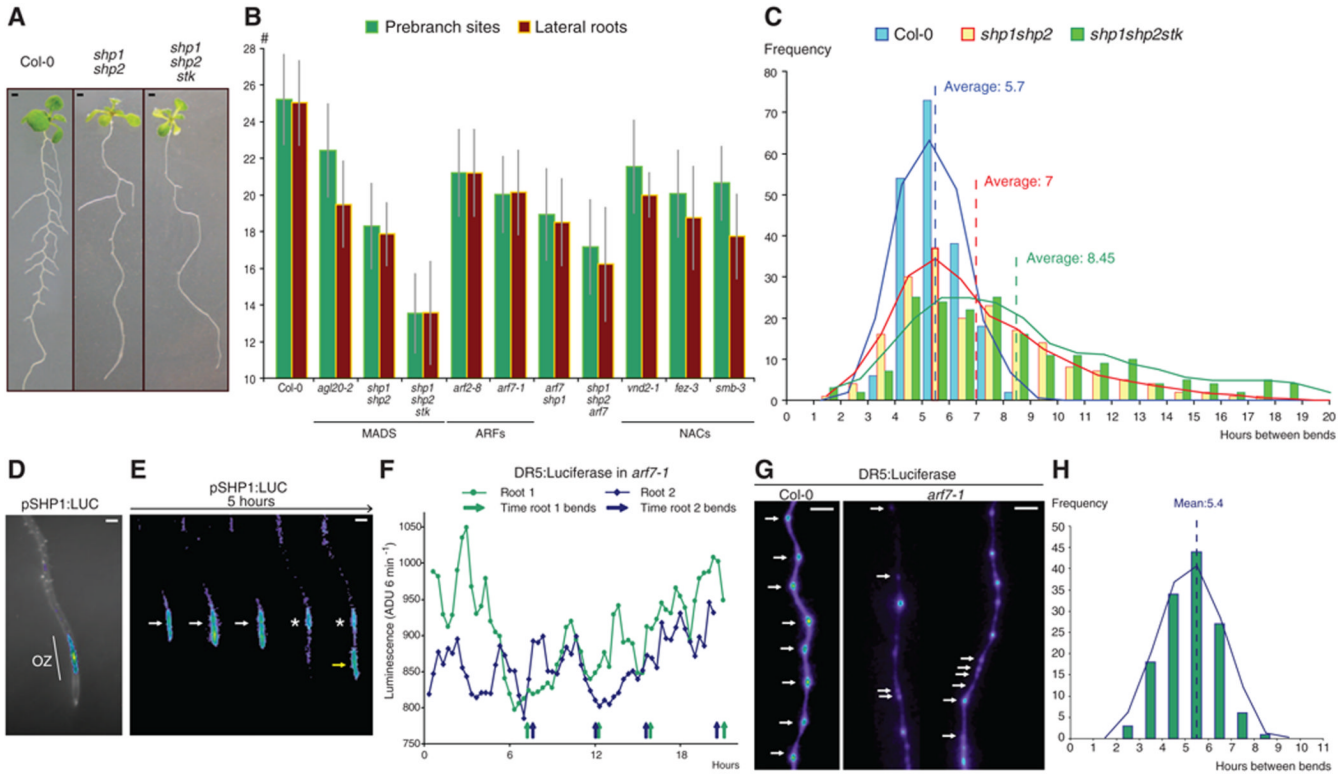

Fig. 4.

Oscillating genes encoding transcription factors are necessary for regular prebranch site formation and root bending. (**A**) Bright-field images of seedlings showing root bending and emerged LRs of wild type (Col-0) and mutants. (**B**) Quantification of the number of prebranch sites and LRs for a variety of genotypes (n = 25). Error bars, SD. (**C**) Distribution of bending over time in Col-0 compared with mutants (n = 25). Note the increased distribution of time between bends in the mutants. (**D** and **E**) Expression of pSHP1:Luciferase in the OZ. (D) Bright-field and Luciferase images overlaid. (E) Time series showing SHP1 expression pulses over time. White and yellow arrows mark first and second expression pulses, respectively; asterisk marks position of a future LR primordium. (**F**) Quantification of DR5:Luciferase expression in the OZ in *arf7-1* mutants. Arrows indicate the times when primary root bending occurred. (**G**) Luciferase images of Col-0 (wt) and *arf7-1* expressing the DR5:Luciferase reporter. White arrows mark prebranch sites. (**H**) Quantification of the frequency of bending in *fez-3* mutants (n = 15). Bending of *fez-3* and wild type is similar [compare with (C)]. Scale bars: (A and G), 1 mm; (D and E), 250 μm.