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The segmentation clock: inherited trait or universal design principle?

David L Richmond and Andrew C Oates

Metamerism is a widespread feature of multicellular body plans; however, our understanding of the underlying mechanisms that generate these patterns is currently based on only a few model organisms. In particular, vertebrate embryos use a segmentation clock to rhythmically and sequentially add segments in concert with posterior elongation of their body. Recent evidence of a segmentation clock acting in arthropods indicates that this mechanism may be a widely used strategy for generating serial anatomy in animals. Whether this is due to homology or convergence is not yet known, but the recent discovery of an oscillatory process associated with the production of sequential root primordia in plants suggests that a segmentation clock is a fundamental patterning principle in growing tissues, independent of ancestry. In this review, we consider the principles of the segmentation clock that may be conserved across the animal and plant kingdoms, and discuss opportunities for cross-fertilization between these active fields of research.

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Introduction

Segmentation of the body axis into a series of repeating units is a canonical strategy in morphogenesis and evidence of this can be seen from the skeletal system of vertebrates to the regular architecture of the plant root system. The process of segmentation in many of these disparate systems is surprisingly similar, and is characterized by the rhythmic and sequential addition of segments to an elongating body axis. In vertebrates, this is regulated by an oscillating mechanism termed the segmentation clock [1], and the overt similarity of this process in different organisms across the tree of life raises the question of whether they follow a common underlying principle (Figure 1).

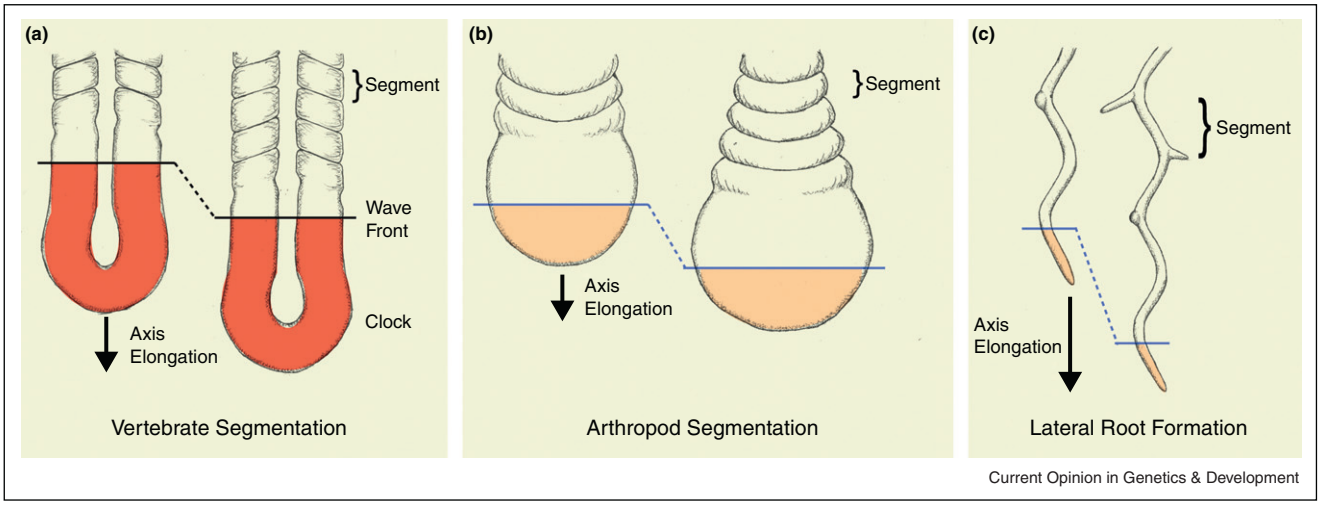
One argument for the similarity of animal segmentation is heredity. Examples of segmented body plans can be found in animals from the three major clades of bilaterians, Annelids (Lophotrochozoans), Arthropods (Ecdysozoans) and Chordates (Deuterostomes), hinting at a common segmented ancestor [2]. Recent evidence that these different animals use similar molecular pathways to control segmentation seems also to corroborate this story [3–5]; however, these findings have been contested based on the pleiotropy of the involved signaling pathways [6•,7]. An alternative hypothesis proposes that Urbilateria, the common ancestor of all bilaterally symmetric animals, exhibited a mechanism for posterior growth that was later independently co-opted by multiple lineages to generate the common process of sequential segmentation [8,9]. This argument accounts for molecular similarities without assuming common ancestry of segmentation itself, and explains why each bilaterian clade also contains numerous examples of unsegmented animals. Thus, the question of whether a segmented body plan was 'discovered' multiple times by evolution, or whether it was present in Urbilateria and subsequently lost by multiple lineages is still an open question [10–13]. By contrast, since the last common ancestor of plants and animals was unicellular, any similarities in body segmentation that span this divide cannot be owing to common ancestry and may therefore reflect the existence of general patterning principles.

In this review, we discuss the possibility that a similar set of principles underlie the generation of metameric anatomy in vertebrates, arthropods and even plants. We begin by reviewing the vertebrate segmentation clock, with a focus on its organizing principles and its surprising evolutionary plasticity. We then discuss recent evidence that a clock-based mechanism underlies segmentation in the red flour beetle, *Tribolium castaneum* [14••]. In the third part of this review, we examine the intriguing possibility that the serially segmented architecture of the elongating root system of the flowering plant *Arabidopsis thaliana* is likewise regulated by a segmentation clock [15••]. In closing, we consider the implications of finding common principles underlying segmentation in such distantly related organisms.

The vertebrate segmentation clock

In vertebrates, segmentation occurs by the rhythmic and sequential formation of segments along the anterior–posterior axis of the growing embryo, with a species specific period: 30 min in zebrafish, 90 min in chick

Figure 1



Metameric body plans arise in vertebrates, arthropods and plant roots through a process of growth and iterative segmentation. Vertebrate body segmentation during embryogenesis **(a)** is organized by a segmentation clock: a population of genetic oscillators in the posterior tip of the elongating embryo (Clock, orange) that are sequentially arrested by the passage of a wavefront (Wavefront, black line), which converts the temporal rhythm of the clock into a permanent periodic pattern of cellular differentiation. The segmentation of arthropod bodies **(b)** and plant roots **(c)** during development appears similarly organized, with oscillatory gene expression (yellow) and a putative wavefront of differentiation (blue line).

and *Xenopus*, 2 hours in mouse, and 6 hours in humans [1]. The striking temporal periodicity of this process led Cooke and Zeeman to propose their 'Clock and Wavefront' model in 1976 [16]. This model was the first to predict that segmentation resulted from the interaction of a population of cellular oscillators (the clock) with a posteriorly sweeping front that induced a rapid change in cell properties to define the segment (the wavefront). Subsequent theoretical studies examined how oscillating activator–inhibitor systems could segment a body axis in concert with a moving gradient [17]. In each case, temporal information is converted into a permanent periodic spatial pattern.

The presence of an oscillator underlying vertebrate segmentation was confirmed in 1997 [18]. *c-hairy1*, a homolog of the *Drosophila hairy* pair-rule gene was observed to have a highly variable striped expression pattern in the presomitic mesoderm (PSM) of similarly staged chick embryos. To confirm that these patterns were dynamic, the authors bisected embryos along the midline and fixed one side immediately, allowing the other side to grow in culture. The expression patterns of the incubated and unincubated tissue always differed, unless the incubation time was an integer multiple of the somitogenesis period. By tracking the position of small clusters of labeled cells over 30 min, they showed that the dynamic expression pattern was not owing to cell motion, thereby ruling out a cell lineage-based explanation and confirming that they resulted from oscillating or cyclic expression of the *c-hairy1* gene. Masamizu *et al.* have since directly observed cyclic expression of the orthologous transcriptional repressor gene *Hes1* in individual cells from dissociated mouse PSM using a bioluminescent reporter [19]. Importantly, this suggests that the observed genetic oscillations are cell autonomous, and favors a model for the cellular oscillator based on delayed transcriptional repression [20].

The molecular details of a wavefront that slows and stops the cellular oscillators and thereby defines the segmental pattern are similarly beginning to be revealed. Transient perturbation of Wnt, Fibroblast Growth Factor (FGF) or Retinoic Acid (RA) signaling pathways leads to a distortion in the length of forming somites, consistent with a transient shift in the positioning of the wavefront, although concomitant changes to oscillator period and axial elongation have not been ruled out [21–24]. These results suggest a molecular model for the wavefront where Wnt and/or FGF signaling, which are high in the posterior PSM, promote the transcriptional oscillations and are opposed by RA signaling which is produced in newly formed somites at the anterior end of the PSM [25–27]. It is proposed that cells transiting the PSM use the positional information from these signaling gradients to gradually arrest their oscillations, although how this occurs is also not yet clear. Interestingly, Wnt also influences elongation of the body axis and may have provided the mechanism of posterior growth in Urbilateria [9]. Thus, the wavefront may have preceded the cellular oscillator in evolution.

Disruption of Delta-Notch signaling, which doesn't appear to be part of the core oscillator or the wavefront in zebrafish [28•,29], leads to segmental defects and

points to an additional level of control in the vertebrate segmentation clock. Jiang et al. proposed that Delta-Notch signaling is required to maintain the synchronization of neighboring PSM cells [30]. This is consistent with observations in dissociated PSM cells that single cell oscillations are highly variable and unstable [19]. Thus, the 'coupling' that is observed between neighboring cells in the vertebrate segmentation clock may be an essential feature of any clock constructed from noisy cell autonomous oscillations. From these findings, a three-tiered model emerges with (i) cellular oscillators at the ground level, (ii) local synchronization of oscillators at the next level, and finally (iii) a tissue level signaling gradient that sets the position of the advancing arrest front [1] (Figure 2). Importantly, these basic organizing principles do not rely on the particular molecules involved, allowing the genetic program to differ across species.

Consistent with this notion, comparison of the segmentation clock in three model vertebrate organisms – mouse, chick and zebrafish – reveals a high degree of variability in its genetic makeup [31••,32]. Krol *et al.* identified the set of cyclically expressed genes in the PSM of these animals by bisecting the PSM and amplifying the mRNA in the posterior half of one side, using the other side to stage the embryo. This process was repeated for ~20 time points covering one cycle, and periodic traces were identified using statistical methods. This study identified 40–100 cyclic genes belonging to the Notch, FGF and Wnt signaling pathways, and surprisingly the sets of the oscillatory genes showed little overlap in the three species. In fact, the only conserved genes were orthologs of the Her/Hes transcriptional repressors, Hes1 and Hes5, which are also downstream effectors of Delta-Notch signaling in many contexts. This study supports the idea that it is the organizing principles of the segmentation clock – *cellular oscillations*, *local synchronization*, *and global control of arrest* – that are the key to segmental patterning, and that the molecular details are free to vary.

A segmentation clock in arthropods

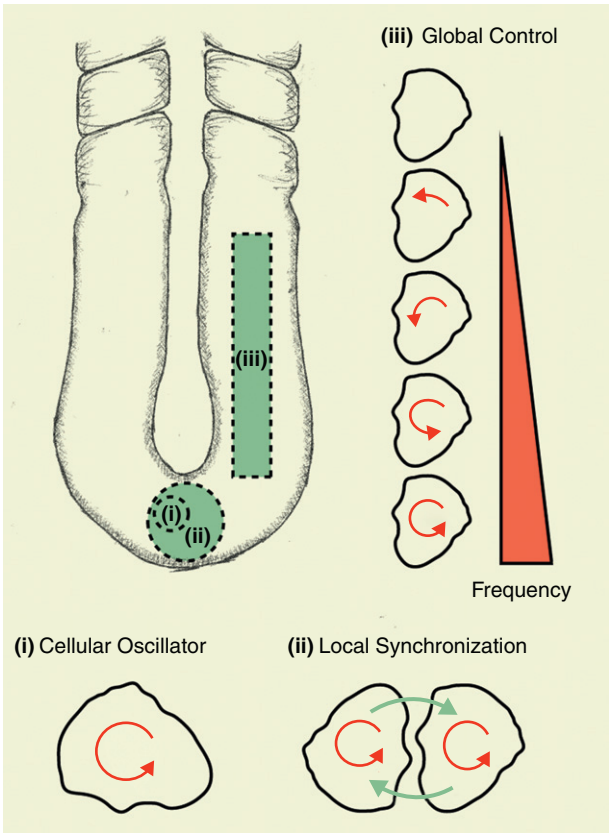
Most arthropods accomplish segmentation in a sequential manner, similar to vertebrates, adding one segment at a time from an elongating posterior zone. One notable exception is *Drosophila*, which exhibits a hierarchical segmentation cascade in a syncytial environment [33]. With its body length set by the size of the oocyte, it forms 14 segments almost simultaneously along its axis. This hierarchical mode of segmentation appears to be highly derived, and most other arthropods exhibit both modes of segmentation, hierarchical segmentation of the anterior tissue including the head, and sequential segmentation of the posterior tissue [34]. However, sequential segmentation does not in itself guarantee the presence of a clock-based mechanism analogous to vertebrate segmentation.

In a recent paper, Sarrazin *et al.* reported the first conclusive evidence of a clock underlying segmentation of the emerging model organism, the red flour beetle *Tribolium castaneum* [14••]. The authors adapted a similar slew of experiments that initially led to the discovery of the vertebrate segmentation clock to investigate expression of the pair-rule gene *odd skipped* (*Tc-odd*). Their initial observation was that *Tc-odd* expression is highly variable in the posterior growth zone of *T. castaneum*, even among similarly staged embryos. By arranging these embryos into a putative sequence, based on a combination of morphological features and the anterior *Tc-odd* expression pattern, they demonstrated that *Tc-odd* levels in the posterior growth zone appear to cyclically rise and fall. To confirm that *Tc-odd* expression is dynamic, they bisected embryos, immediately fixed one side and maintained the other half in culture. They observed that the expression levels changed within the bilateral sections of individual embryos, repeating with a periodicity of 95 min at 30 °C and corresponding to the formation of two consecutive segment boundaries. Finally, to dispel the possibility that the dynamic expression levels resulted from cell movements, they developed live imaging to track individual cells over a 60 min window. They observed that cells remain in the posterior growth zone for multiple clock cycles and therefore must exhibit cyclic expression of *Tc-odd*, consistent with the notion of a segmentation clock in *T. castaneum*.

A network of transcriptional interactions underlying segmentation in *T. castaneum* has previously been proposed [35,36]. From these findings, we assemble a minimal cell-autonomous genetic network consisting of a feedback loop of the three pair-rule genes *even*, *run* and *odd* (Figure 3). A striking feature of this proposed oscillator is the role of transcriptional repression, similar to the vertebrate oscillator. Simulated evolution of transcriptional networks *in silico* shows that oscillatory gene expression arising from transcriptional repression appears to be a strongly favored mechanism to generate segments under the condition of a traveling morphogen gradient [37]. Both direct auto-repression, as appears in the vertebrate segmentation clock, and indirect auto-repression, as appears in this model for the *T. castaneum* clock, commonly arise in these simulations. These networks recapitulate the basic property of the Clock and Wavefront mechanism, that segment size is the direct result of the wavefront velocity and the oscillatory period, and points to a fundamental physical principle favoring a Clock and Wavefront mechanism in growing systems.

Unifying vertebrate and arthropod segmentation mechanisms under a common principle offers numerous advantages. If transcriptional oscillations in the arthropod segmentation clock are found to be cell autonomous, then theoretical frameworks developed for the vertebrate clock can be directly applied [38]. For this

Figure 2



A conceptual framework for the organization of a segmentation clock. Using a three-tier model of the vertebrate segmentation clock as a template, we propose that all segmentation clocks may share the following organizational principles: **(i)** Cellular oscillators, capable of autonomous oscillation. **(ii)** An active local synchronization of cellular oscillators, so that genetic and other noise sources are suppressed, allowing a coherent rhythm to be generated. **(iii)** A global control of how the frequency of oscillators is slowed and stopped, such that a precise position of arrest is coordinated with elongation and growth of the body axis.

reason, observing transcriptional oscillations in cultured dissociated *T. castaneum* cells is an important step forward in understanding arthropod segmentation. Notably, this will require development of transgenic techniques for visualizing single cell oscillations, such as the bioluminescent reporter developed in mouse [19]. It also appears that the 2nd organizing principle of the vertebrate segmentation clock – local coupling of autonomous oscillators – may be at play in arthropods. A role for constituents of the Delta-Notch signaling pathway has now been reported in posterior segmentation of spiders [3,39], cockroaches [4] and centipedes [40]. Although Delta-Notch signaling has additional roles in germ layer specification in arthropods [6,7,41•], it will be interesting to see if it plays a similar role of synchronizing neighboring cell oscillations as it does in vertebrates [28•,29,30,42]. There is currently no direct evidence from arthropods concerning the 3rd organizing principle, a tissue-level control of the arrest of the oscillations, but if this were forthcoming, it would further develop the analogy between vertebrate and arthropod segmentation clocks, and reaffirm our notion that animal segmentation clocks follow a common design principle.

The root tip oscillator

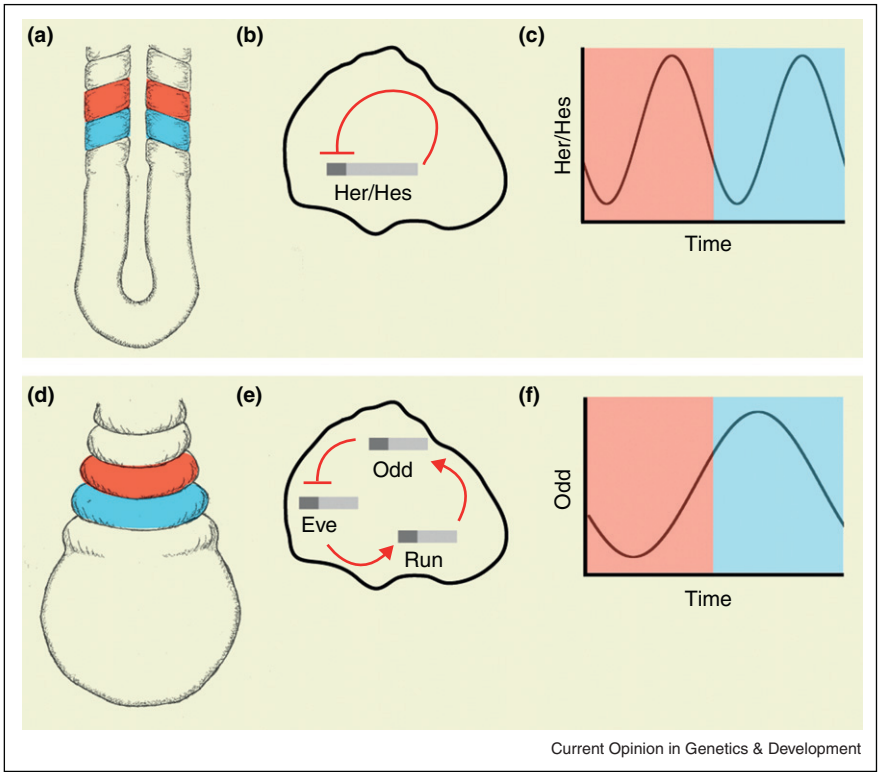
We have discussed evidence that a segmentation clock is a common principle for generating metameric structures in growing systems as disparate as vertebrates and arthropods. If this is true, we might expect to find examples of this strategy in other multicellular organisms that evolved independently. While the search for extraterrestrial life continues, we can for the moment consider an example that is closer to home – plants.

The architecture of the root system in plants is modular and highly regular. As a general principle, plants extend a primary root (PR) into the earth along the direction of gravity, forming lateral branches at regular spatial intervals. These lateral branch sites generate lateral roots (LR) that explore the soil for nutrients and improve anchorage. The formation of LR primordia has been linked to an enhanced transcriptional response to auxin, as reported by the *DR5* promoter in the mouse-ear cress *Arabidopsis thaliana* [43]; however the mechanism for generating periodic spacing between LRs was previously unknown. Recently, Moreno-Risueno et al., demonstrated that the formation of pre-branch sites in *A. thaliana* is both spatially and temporally periodic, with a temporal periodicity of approximately 6 hours, and reported the first evidence for a clock underlying this process [15••].

The authors generated a Luciferase-based live reporter of auxin signaling from the *DR5* promoter and observed oscillations in the tissue just proximal to the tip meristem, which they called the oscillation zone (OZ), and subsequent maintenance of *DR5* expression at a position marking the future site of LR branch formation. The period of oscillations corresponded to the period of pre-branch site formation, and interestingly this timing was invariant under different growth conditions. Thus, the speed of primary root growth corresponded to differing anatomy of the root network, with slower PR growth (e.g. at higher temperature) resulting in closer spacing of LRs. This represents a fundamental difference between metamerism in plants, which regulate their anatomy in direct response to changing environment, and animals, which strictly conserve body proportions over a wide range of environmental conditions.

At a conceptual level, oscillating gene expression associated with the regular branching of lateral roots is tantalizingly similar to segmentation observed in animals. However, one intriguing difference is that cells appear

Figure 3



Transcriptional oscillators underlying the vertebrate and arthropod segmentation clocks. A comparison of the segmenting body axis of vertebrate **(a)** and arthropod embryos **(d)** where red and blue mark successive segments. Models for the corresponding genetic circuit driving the cellular oscillators contrasting a single gene negative feedback loop in vertebrates **(b)** and a three-gene negative feedback loop in arthropods **(e)**. The phase relationship between a segment and a cycle of the oscillator is shown for vertebrates (**c**, 1:1) and for arthropods (**f**, 2:1) where the blue and red colors correspond to the colored segments in **a** and **d**.

to oscillate only once as they pass through the OZ of the root tip. This could represent a qualitative difference in the underlying mechanism or a simply a quantitative difference in the relative rates of the constituent processes. For example, PSM cells in vertebrates typically oscillate multiple times before they arrest in a forming somite. However, if the period of the oscillator were significantly slower, or the passage of the wavefront much faster, this could result in the typical PSM cell oscillating only once during its transit, resembling the passage of a root cell through the OZ.

A remaining key question is whether the temporal periodicity of this root oscillator is causally related or simply correlated with the observed metameric structure. The authors rule out the possibility that oscillations in the levels of auxin signaling molecules underlie the observed oscillations in *DR5* expression by designing live reporters of additional auxin-sensitive promoters. They observe that other auxin sensitive reporters exhibit constant expression, suggesting that auxin levels are constant in the oscillating zone throughout this process. Additionally, pulses of exogenous auxin did not generate additional pre-branch sites. This appears to rule out an auxin based oscillator upstream of OZ.

The authors hypothesized that a genetic network may underlie the root oscillator. Using a similar phase ordered microarray strategy as described above [31••,32], the authors identified the set of oscillating genes in the root tip using RT-PCR to stage the roots along one cycle of *DR5* expression. This returned almost 3500 candidate genes, with ~2100 oscillating in phase with DR5 and ~1400 genes oscillating in anti-phase. A number of these candidates were validated by generating live reporters, and several mutants exhibited strong phenotypes in LR formation, with reduced numbers of pre-branch sites and LRs. Of particular interest is the *arf7* phenotype, which appears to disrupt rhythmic *DR5* expression and produces irregular branching, consistent with an important role for *arf* 7 in regulating the periodic behavior of the root.

An alternative hypothesis for the dynamic gene expression observed in the OZ is that it encompasses the region of the root tip where cells begin a differentiation program

accompanied by transient pulses of gene expression. This would be akin to the behavior of a live reporter for *Mesp2*, a marker of the rostral half of presumptive somites which turns on and then off downstream of the segmentation clock in each forming somite [44]. In this case, perhaps the OZ is operating downstream of a clock in the root tip meristem. To distinguish between these possibilities, it will be necessary to test whether cells of the OZ have the potential to oscillate multiple times. This could be accomplished by delaying the progression of a differentiation wavefront, or even by sustaining oscillations in dissociated root tip cells in culture. Additionally, the potential autonomy of the OZ could be tested by ablating the root tip meristem and observing whether oscillations persist, analogous to the posterior transection experiments of Palmeirim [18].

Another curious feature of the root tip oscillator is the number of genes involved. This might signify a fundamental difference between plant and animal segmentation clocks, or that these are effector genes that are regulated by an upstream clock, akin to the many hundreds of genes that oscillate in peripheral tissues in accordance with the rhythm of the circadian clock [45–48]. Given the scale of transcription in the root oscillator, searching naively for a potential core transcriptional circuit or mechanism may be difficult, and subsequent experiments should first attempt to establish the relevant underlying segmentation principles.

Conclusions and outlook

A number of questions remain regarding the origins and molecular basis for the metameric body plans observed across the plant and animal kingdoms. However, with the recent report of a segmentation clock in the red flour beetle *T. castaneum* and oscillatory expression in the root tip of *A. thaliana*, it appears that a unifying principle is emerging. Each system exhibits signatures of a segmentation clock, first identified in vertebrates over a decade ago. Confirmation of a segmentation clock in plants will play a particularly important role in this debate, as it would conclusively demonstrate the independent adoption of this design principle. This result may also favor the argument for convergence when considering more closely related species, such as vertebrates and arthropods.

Cross-fertilization between study in these distinct systems offers numerous potential rewards. For example, the conceptual framework developed for understanding the vertebrate segmentation clock offers an opportunity to focus inquiry in these new systems, and could greatly increase the pace of discovery. We anticipate that this approach will shed new light on the segmentation clock as a general developmental principle for generating metamerism in growing systems.

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