The suspensor: not just suspending the embryo

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The suspensor is a terminally differentiated embryonic region that connects the embryo to surrounding tissues during early seed development. Most seed-bearing plant embryos contain suspensor regions, which occur in a wide variety of sizes and shapes, and suspensor-like structures are present in the embryos of some lower land plants. Recent technological advances, including novel genomics approaches, have provided insights into the function of the suspensor and the DNA sequences that control suspensor-specific gene expression. The molecular mechanisms controlling embryo basal-cell lineage specification and suspensor differentiation events are also beginning to be illuminated. Here, we summarize the role of the suspensor in plant embryogenesis and discuss future directions of suspensor biology, including the dissection of suspensor gene regulatory networks.

Suspensors are ancient and morphologically diverse embryo structures

The suspensor (Figure 1a) was first discovered almost 170 years ago, but was originally thought to be a part of the pollen tube [1,2]. Further investigation of embryo development in several plant species showed that the suspensor is a unique embryonic region, connects the embryo to the seed coat, and is morphologically diverse throughout the plant kingdom, in contrast with the embryo proper (Figure 1b; Box 1) [3–8]. For example, the scarlet runner bean (*Phaseolus coccineus*) suspensor, which has been a classic model system for investigating suspensor function [9–12], has at least 200 cells and is massive compared with the much smaller *Arabidopsis* (*Arabidopsis thaliana*) suspensor that is comprised of a simple file of seven cells (Figure 1). Both, however, contain a uniform set of cells with a large basal cell at their micropylar ends (Figure 1). On the other hand, the orchid suspensor consists of only one, or a few, large, highly vacuolated cells (Figure 1b).

Regardless of shape or size, the suspensor is derived from the basal cell of the two-cell proembryo and is fully differentiated prior to the beginning of embryo proper maturation and the onset of dormancy (e.g., scarlet runner bean suspensor development, Figure 1a) [13,14]. During seed maturation, the suspensor undergoes programmed cell death and does not contribute to the development of next plant generation following seed germination (Box 1) [6]. The mechanisms responsible for shifting the suspensor from a differentiation program to a cell death program are not understood. Because the suspensor is a terminally differentiated and highly-specialized part of the embryo that has no role in subsequent plant development, it has been able to evolve independently of the embryo proper with respect to both form and function throughout the plant kingdom.

With few exceptions (e.g., swamp wattle, Figure 1b), the embryos of most seed-bearing plants have suspensors, suggesting that the suspensor is evolutionarily conserved and that it plays an important role in plant embryogenesis. It is remarkable that well-preserved suspensors are observed in gymnosperm seed fossils uncovered in Permian rocks found in Antarctica, indicating that highly differentiated suspensors were a prominent part of higher plant embryos over 300 million years ago (mya) [15,16]. Some lower plant embryos, including those of mosses and other bryophytes, also possess a suspensor, or an embryonic region that might perform similar functions (e.g., foot) (Figure 1c) [5]. It remains unclear whether the suspensor and foot evolved from a common ancestral cell type. Nevertheless, the suspensor is an ancient embryonic structure, and the processes by which suspensor cell fate is determined in higher plants may have already existed in lower plants, which diverged from seed-bearing plants ∼450 mya [17].

The suspensor plays an important role in embryo development

The position and structure of the suspensor within the embryo provide clues for its possible role(s) during embryogenesis. For example, the suspensor pushes the embryo proper into the endosperm cavity and connects the embryo proper to surrounding maternal and endosperm tissues - serving as a conduit for nutrients and growth regulators required for embryonic development (Figure 1a). Cellular structures that enhance the ability to transfer molecules from cell to cell are present in many suspensors, including (i) cell-wall ingrowths, (ii) haustorial outgrowths, and (iii) numerous plasmodesmata [6,8,18]. In addition, the fact that the suspensor does not have a waxy cuticle layer facilitates direct communication with adjacent seed tissues (e.g., seed coat, endosperm), in contrast with the embryo proper that is surrounded by a cuticle that covers the protoderm surface [19,20].

Transfer of nutrients and growth factors to the embryo proper

What direct experimental evidence is there for the participation of the suspensor in transferring nutrients and growth factors to the embryo proper? Experiments
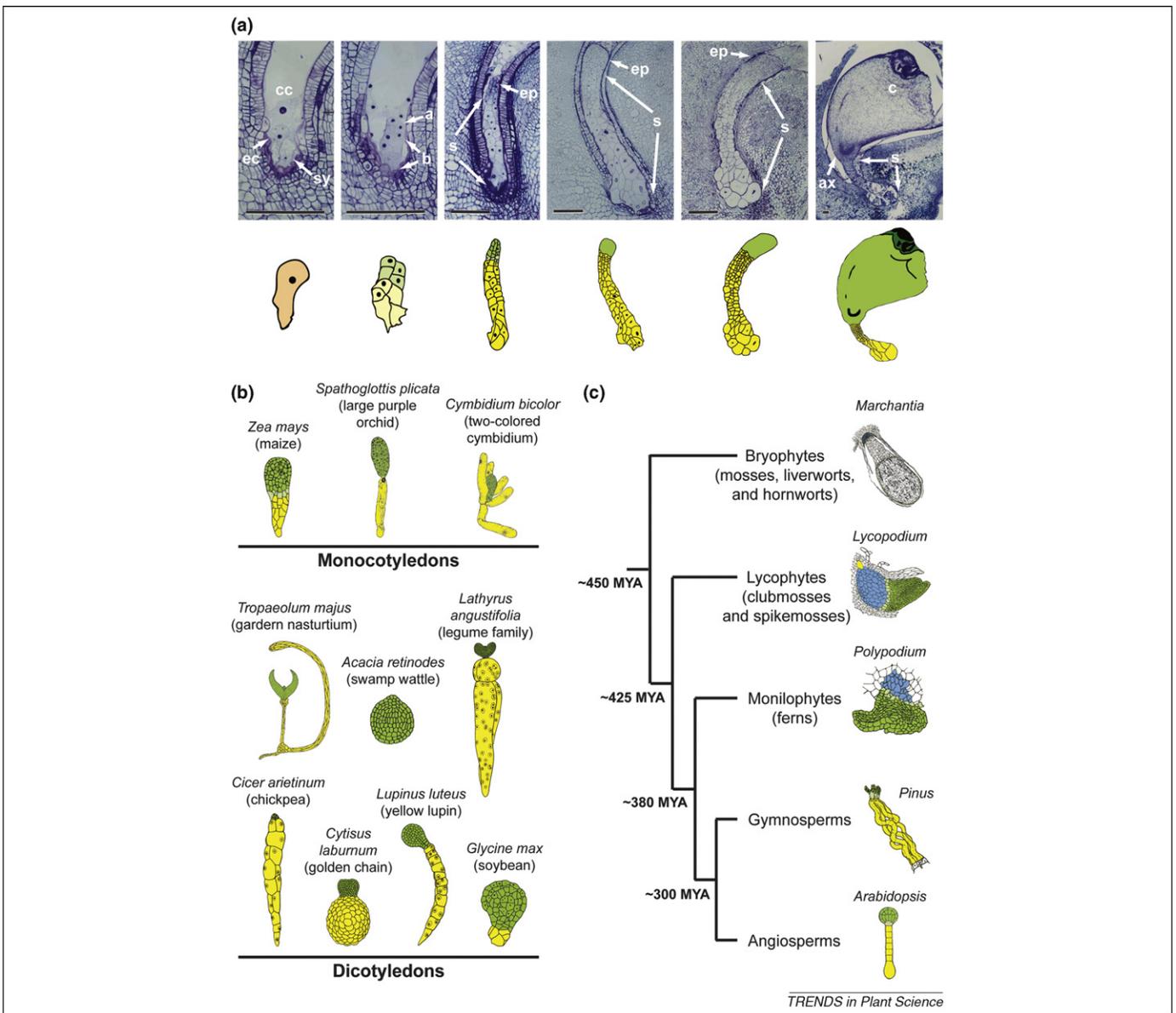
Figure 1. Development, diversity, and evolution of the suspensor. **(a)** Scarlet runner bean embryo development. Schematic images (lower row) were drawn from the bright-field photographs (upper row) that were taken from Weterings *et al.* [14]. **(b)** Schematic images of embryos and suspensors from different flowering plant species. Green and yellow colors indicate the embryo proper and suspensor, respectively. *A.r.* does not have an obvious suspensor structure. *C.b.* and *T.m.* suspensors possess haustorial structures. *L.a.* and *L.l.* suspensor cells are multinucleated. Images were taken and adapted from Guignard in [8] for *A.r.*; Guignard in [5] for *C.a., C.l., L.a.,* and *L.l.*; Chamberlin *et al.* [71] for *G.m.*; Raddolph in [5] for *Z.m.*; Swamy in [8] for *C.b.* and *S.p.*; Nagl and Kuhner in [8] for *T.m.* **(c)** Suspensor and suspensor-like structures in the plant kingdom. Phylogenetic tree was adapted from Bowe *et al.* [72] and Langdale [17]. Green, yellow, and blue colors indicate the embryo proper, suspensor, and foot, respectively. Images were taken and adapted from Goldberg *et al.* [13] for *Arabidopsis*, and Raven *et al.* [73] for *Lycopodium, Marchantia, Polypodium,* and *Pinus*. a, apical region of proembryo; ax, axis; b, basal region of proembryo; c, cotyledon; cc, central cell; ec, egg cell; ep, embryo proper; s, suspensor; sy, synergid. Bars = 100 μm.

with labeled metabolites, such as sucrose and polyamine, in developing seeds demonstrated the direct movement of molecules from the suspensor to the embryo proper in an energy dependent manner [21,22]. More recent studies using transgenic plants have shown that the *Arabidopsis* globular-stage suspensor is connected symplastically to the embryo proper by visualizing the movement of green fluorescent protein (GFP) from the suspensor to the embryo proper [23]. In addition, genes involved in transferring molecules, such as the *Arabidopsis AtSUC3 sucrose transporter* and the loblolly pine (*Pinus taeda*) *PtNIP1;1 aquaglyceroporin*, are up-regulated in the suspensor [23,24]. Finally, auxin is transported from the suspensor to the embryo proper in *Arabidopsis* embryos by the suspensor-specific PIN7 auxin efflux carrier protein, and

PIN7-mediated auxin transport is essential for embryo development [25]. Taken together, these data show that the suspensor transports molecules involved in both nutrition and growth regulation to the embryo proper to support embryo development. The cellular processes by which the suspensor transfers substances to the embryo proper remain to be determined.

Biosynthesis of plant hormones
The suspensor has an abundance of organelles such as mitochondria, endoplasmic reticulum, and specialized plastids [8,18]. In addition, the suspensor cells of many plant species have undergone extensive endopolyploidization, possess polytene chromosomes - most notably in their basal cells (e.g., scarlet runner bean; Figure 1a), and are

often multinucleated forming a syncytium (e.g., *Lathyrus angustifolia*; Figure 1b) (Box 1) [8,12,18]. These features generally reflect high levels of cellular biosynthetic activity, and are markers for terminally differentiated regions, such as the suspensor, that produce specialized products over a short period of time. In fact, classical experiments carried out with the giant scarlet runner bean suspensor (Figure 1a) over a generation ago, showed that it has higher levels of RNA and protein synthesis in comparison with the embryo proper during the early stages of development [9–11].

What is known about specific molecules that are synthesized in the suspensor as a consequence of its elevated metabolic activity? Several plant hormones, including gibberellic acid (GA), auxin, cytokinin, and abscisic acid, are found in the suspensor of several species [6]. For example, the giant scarlet runner bean suspensor (Figure 1a) has been used as a system to investigate suspensor hormone activity because it can be hand-dissected from embryos at the globular stage and separated from the embryo proper [4,9,26–28]. The scarlet runner bean suspensor has the highest level of free indole-3-acidic acid (IAA) in the early embryo, suggesting that it is the site of auxin synthesis [29]. In addition, scarlet runner bean suspensor extracts are able to synthesize GAs [30], and mRNAs encoding all enzymes in the GA biosynthetic pathway are highly prevalent in the scarlet runner bean suspensor compared with the embryo proper [4,31]. Experimental manipulation of scarlet runner bean embryonic regions in culture demonstrated that (i) the suspensor is required for the embryo

proper development during the early stages of embryogenesis and (ii) the presence of the suspensor can be substituted by the application of exogenous GAs. These results confirm the importance of GA production in the suspensor for embryo development, at least for the scarlet runner bean [32–35].

Do the suspensors of all plant embryos synthesize GAs? Several studies have identified the presence of bioactive GAs in suspensors of several different species in addition to the scarlet runner bean [36,37]. What about the small *Arabidopsis* suspensor (Figure 1c)? Recent experiments have shown that *GA3-ox4*, a gene encoding the last enzyme required for GA biosynthesis, GA3-oxidase, is active in the endosperm, but not in the suspensor during early *Arabidopsis* seed development [38]. Related *GA3* genes (*GA3-ox1, GA3-ox2, and GA3-ox3*) are also inactive in the suspensor [38]. This suggests that GA biosynthesis is not a common feature of all suspensors, and that there are differences in suspensor function among plant species. What functions are shared by the suspensors of all plant embryos, and what species-specific suspensor activities are coupled with specialized suspensor morphologies (Figure 1b) remain to be investigated.

Using genomics to uncover suspensor function
One way to answer the question of what are common and specialized suspensor functions among different species is to isolate suspensor mRNAs from diverse plants and compare their transcriptional profiles. This, of course, is challenging because of the size, accessibility, and limited temporal presence of the suspensor during the plant life cycle. Newly emerging genomics and laser-capture microdissection (LCM) technologies, however, have changed this and opened up a new era in understanding suspensor biology [4].

Recently, our laboratory sequenced ~300,000 ESTs obtained from hand-dissected globular-stage scarlet runner bean giant suspensors (Figure 2) (GenBank Accession Numbers GD372310-GD660862 and CA899870-CA916678) [4]. In collaboration with John Harada's laboratory at UC Davis, we also used LCM and Affymetrix GeneChip technologies to profile (i) soybean (*Glycine max* (L.) Merr.) suspensor mRNAs at three developmental stages [(globular -GEO Series GSM147936-GSM14938); (heart - GEO Series GSM182032-GSM182034); and (cotyledon - GEO Series GSM191077-GSM191078)] [4], and (ii) *Arabidopsis* suspensor mRNA at the globular stage (GEO Series GSM284386-GSM284387) (Figure 2). These are the first comprehensive sets of genomics data to show (i) the number of mRNAs required for suspensor development, (ii) the diverse functions carried out by the suspensors of different plant species, and (iii) the types of transcription factors represented in the suspensor. Soybean and *Arabidopsis* suspensors contain at least 10,000 and 15,000 diverse mRNAs during early embryo development, respectively (Figure 2a) - a significant proportion (~20%) of which are in the "unclassified" category (Figure 2b). This indicates that a large number of genes are required for suspensor development and that many novel, yet undiscovered, biological processes take place in the suspensor. Taken together, comparative genomics from these, and

(a)

Method	Species	Stage	# of mRNAs	# of TF mRNAs	Reference
LCM & GeneChip	*Glycine max*	Globular	10,439	642	http://seedgenenetwork.net/
		Heart	14,930	953	
		Cotyledon	14,729	952	
	Arabidopsis thaliana	Globular	10,692	777	

Method	Species	Stage	# of ESTs	# of TF ESTs	Reference
EST-Sequencing	*Phaseolus coccineus*	Globular	305,363	3,984	http://estdb.biology.ucla.edu/PcEST/

(b)
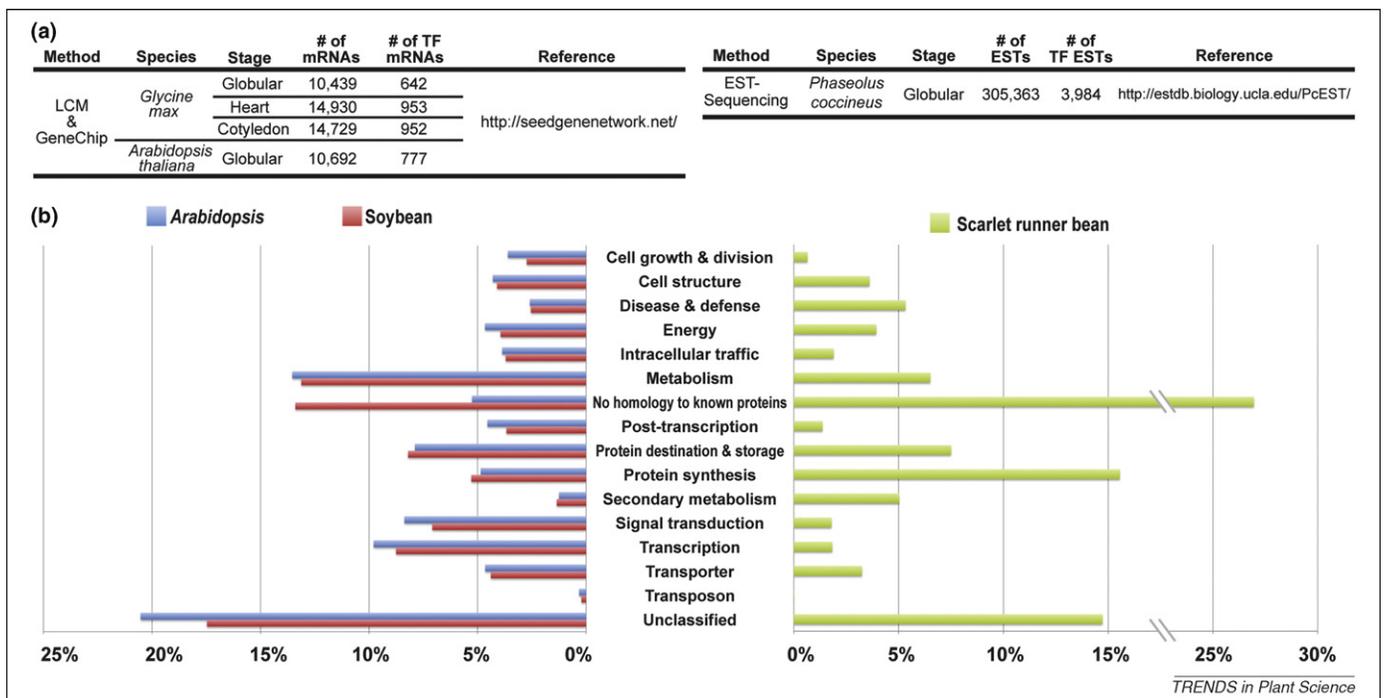
Figure 2. Soybean, scarlet runner bean, and *Arabidopsis* suspensor mRNA profiles. **(a)** Numbers of mRNAs and ESTs detected by GeneChip and 454 high-throughput DNA sequencing studies, respectively. **(b)** Functional classes of proteins encoded by mRNAs present in globular-stage *Arabidopsis*, soybean, and scarlet runner bean suspensors. TF, transcription factor.

other, plant species (Figure 1) should provide novel insights into previously unknown roles of the suspensor in embryo development and enable suspensor functions to be investigated across the plant kingdom (Figure 1c).

Differentiation of the suspensor from the basal cell lineage

Suspensor development precedes that of the embryo proper (Figure 1a), and the suspensors of many plants become fully differentiated a few divisions after basal cell formation (e.g., tobacco and *Arabidopsis*) [39,40]. Furthermore, suspensor cells are direct clonal descendants of the basal cell. Thus, it is possible that the molecular mechanisms that control suspensor-specific gene activity are directly linked to the processes that establish basal cell fate.

How does the basal-cell lineage become specified? Both autonomous and position-dependent signaling hypotheses have been proposed for how apical and basal cells of the two-cell plant embryo might become specified to follow different developmental pathways [14,41,42]. One hypothesis proposes that there is an asymmetrical distribution of morphogenetic factors (e.g. transcription factors) within the egg cell and/or the zygote, and after zygotic division the apical and basal cells obtain different sets of factors that are able to program distinct developmental fates [14,41]. By contrast with this cell-autonomous process, cell–cell signaling events have been proposed in which signals derived from adjacent seed tissues (e.g., seed coat and/or endosperm) interact with the basal region of the zygote and/or the basal cell, and these signals trigger a cascade of events leading to the differentiation of the basal cell into the suspensor [42]. Although it is still not clear how the basal cell is specified, recent findings suggest that both cell-autonomous and position-dependent processes may be essential for the establishment of the basal cell lineage and suspensor development [41–43].

Asymmetrical distribution of transcripts from the zygote to the basal cell

Are there mRNAs that are distributed asymmetrically within the egg and/or zygote? Experiments with apical and basal cells isolated from maize eggs fertilized *in vitro* demonstrated the presence of basal-cell specific mRNAs. One class of basal cell-specific mRNAs is present in the egg cell, whereas another class accumulates in the zygote following fertilization [44]. These results suggest that mRNAs present in the egg and zygote can asymmetrically accumulate within the basal cell following zygotic division. Whether this is a result of (i) an asymmetric distribution of pre-existing transcripts to the basal cell, (ii) *de novo* synthesis of basal-cell-specific mRNAs, and/or (iii) selective turnover of basal-cell-specific mRNAs in the apical cell is not known [44].

The *Arabidopsis* WUSCHEL-RELATED HOMOEOBOX TRANSCRIPTION FACTOR8 (WOX8)/STIMPY-LIKE (STIPL) mRNA is also distributed asymmetrically to the basal cell from the zygote and is highly prevalent in the suspensor at the globular stage [41,45]. WOX9/STIP mRNA, a close WOX8/STIPL mRNA relative, is also up-regulated in the basal cell, although, in comparison with WOX8/STIPL mRNA, it is less prevalent in the suspensor [41,45]. A *wox8/9 (stip/stipl)* double mutant results in embryo lethality, including the failure to establish an auxin gradient from the basal cell lineage to the embryo proper (see online Supplementary Material, Table S1 for a list of *Arabidopsis* genes that affect suspensor development when mutated) [41,45,46]. Genes related to *WOX8* and *WOX9* in maize (e.g., *ZmWOX9A, ZmWOX9B, ZmWOX9C*) [47] and the scarlet runner bean (e.g., *PcWOX9-like*) [4] have suspensor-specific transcriptional patterns, and *WOX-related* genes are found in the moss, *Physcomitrella patens,* that has a suspensor-like foot (Figure 1c) [22,48], suggesting a conserved role for

WOX8 and *WOX9* genes in suspensor development across the plant kingdom. Taken together, these results show that the basal cell can accumulate specific mRNAs, including those encoding transcription factors, and that *WOX8* and *WOX9* genes play an important role in basal cell specification and subsequent suspensor differentiation.

Cell-cell signaling plays a role in suspensor differentiation

Evidence for the importance of cell–cell signaling events in basal cell specification and suspensor differentiation has been obtained from studies with the *Arabidopsis YODA* (*YDA*) and *SHORT SUSPENSOR* (*SSP*) genes that encode mitogen-activated protein kinase kinase (MPKK) kinase [42] and interleukin-1 receptor-associated kinase/Pelle-like kinase [43] proteins, respectively. Mutant *yda* and *ssp* zygotes do not elongate and produce a small basal cell following zygotic division that fail to develop into a suspensor [42,43] (see online Supplementary Material, Table S1). In both cases, suspensor-specific marker genes are not expressed [42,43].

SSP acts upstream of *YDA* and appears to activate the YDA MPKK kinase pathway in the zygote and basal cell through a unique paternal parent-of-origin effect [43]. Surprisingly, SSP mRNA is transferred from the sperm cell to the egg, and then translated into SSP proteins in the zygote following fertilization [43]. Together, these results indicate that the SSP-YDA signaling pathway plays a major role in suspensor differentiation events, and that basal cell specification is, in part, paternally regulated. Mitogen-activated protein kinases, MPK3 and MPK6, that are downstream in the YDA pathway operating in stomatal cells have been shown to phosphorylate the SPEECHLESS transcription factor *in vitro*, and *mpk3/mpk6* double mutants fail to develop suspensor (see online Supplementary Material, Table S1) [49,50]. How the SSP-YDA pathway intersects with transcription factors that are important for suspensor differentiation and the identity of signals within the seed that trigger this pathway during early embryo development remain to be determined.

cis-regulatory sequences that activate suspensor-specific transcription

How are specific sets of genes activated in the suspensor once basal cell fate becomes established? We utilized the *G564* gene of the scarlet runner bean to identify suspensor *cis*-regulatory sequences [14,51]. G564 mRNA encodes a protein with unknown function, accumulates asymmetrically within the basal cells of a three-cell proembryo, and is highly prevalent in the suspensor at the globular stage (Figure 1a) [14]. The *G564* upstream region is able to activate suspensor-specific transcription of the *E. coli β-glucuronidase* (*GUS*) reporter gene in transgenic tobacco and *Arabidopsis* embryos shortly after fertilization, indicating that both the temporal and spatial transcriptional machinery responsible for *G564* transcription in the suspensor is conserved in flowering plants (Figure 3a) [14,51].

Functional dissection of the *G564* upstream region in transgenic tobacco plants uncovered a 54-bp positive regulatory module located within a 150-bp tandem repeat that is both necessary and sufficient to activate transcription in the suspensor (Figure 3c) [51]. In addition, mutagenesis experiments showed that this regulatory module contains at least three short sequence motifs that are required for suspensor transcription during early embryo development (Figure 3a and 3c) [51]. These motifs consist of two similar 10-bp flanking sequences, designated as 10-bp (5'-GAAAAGCGAA-3') and 10-bp-like (5'-GAAAAACGAA-3'), and a central 6-bp sequence referred to as Region 2 (5'-TTGGT-3') (Figure 3c) [51]. Knocking out each of these motifs either eliminated or reduced significantly transcription in the suspensor [51]. What transcription factors interact with these suspensor *cis*-regulatory elements and the connection, if any, between the *G564* suspensor transcriptional machinery and the SSP-YDA signaling cascade remain to be determined (Figure 3c).

There are many thousands of genes that are active in the suspensor (Figure 2a), only a small fraction of which encode suspensor-specific mRNAs [4]. Whether *G564* suspensor *cis*-regulatory elements are used by other suspensor-specific genes is not yet known. For example, it is possible that there are several suspensor gene regulatory networks that operate in parallel within the basal cell lineage, and only a subset employ *G564* regulatory motifs. Sequences related to the 10-bp motif that are present in other suspensor-specific upstream regions are able to substitute for the 10-bp motif to program transcription in the suspensor [51]. Whether these 10-bp-like motifs operate in the context of their own promoters remains to be determined.

Recently, we observed that the suspensor-specific pine *PtNIP1;1 aquaglyceroporin* gene upstream region [52] can activate transcription of the *GUS* reporter gene in tobacco globular-stage embryos (Figure 3b) (T. Kawashima, PhD thesis, University of California, Los Angeles, 2009). This observation suggests that suspensor gene regulatory networks are conserved in both angiosperms and gymnosperms, which diverged from a common ancestor over 300 mya (Figure 3a and 3b). The precise nature of suspensor gene regulatory networks, how they are activated in the basal cell lineage after zygotic division, and how they originated during plant evolution remain major unanswered questions.

Suspensor identity is controlled in part by the embryo proper

Is communication between the suspensor and embryo proper unidirectional, or does the embryo proper transfer signals to the suspensor that are important for its development and/or function? Classical embryological studies in several species showed that destruction and/or alteration of the embryo proper results in abnormal suspensor enlargement, and, in the case of *Eranthis* (winter aconite), a new embryo proper regenerates from the suspensor [6,53,54]. Similarly, mutations in genes that cause defects in *Arabidopsis* embryo proper development can result in the suspensor acquiring an embryo proper-like state (e.g., *raspberry1, sus1*) and, in the extreme, generate a new embryo proper (e.g., *twn1*) (see online Supplementary Material, Table S2) [7,55].

These observations indicate that the suspensor has the potential to follow an embryo proper-like pathway, and
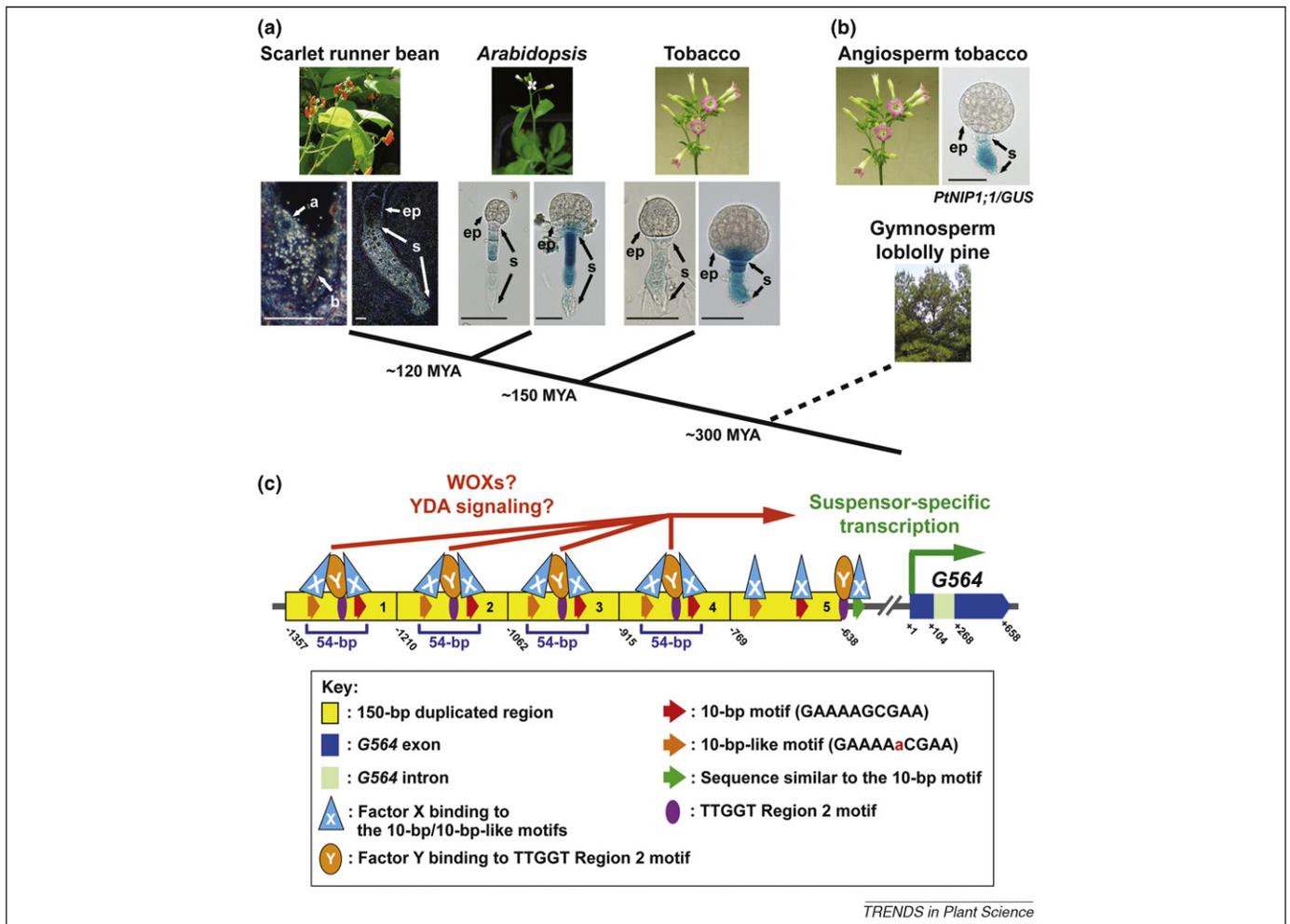
Figure 3. Conservation of suspensor gene regulatory networks among seed-bearing plants. **(a)** Accumulation of G564 mRNA in scarlet runner bean embryos, and suspensor GUS activity in *Arabidopsis* and tobacco embryos carrying a *G564/GUS* transgene. Phylogenetic tree was adapted from Paterson *et al.* [74] and Bowe *et al.* [72]. Scarlet runner bean G564 mRNA *in situ* images and *G564/GUS* tobacco and *Arabidopsis* embryo photos were taken from Weterings *et al.* [14] and Kawashima *et al.* [51], respectively. **(b)** GUS activity in transgenic tobacco embryos carrying a *PtNIP1;1/GUS* gene. Loblolly Pine image was taken from http://bioimages.vanderbilt.edu/, photographed by Steve Baskauf. **(c)** A schematic model of the scarlet runner bean *G564* upstream region taken from Kawashima *et al.* [51]. Suspensor *cis*-regulatory sequences were identified by functional *G564* promoter/GUS gene fusion and mutagenesis experiments in transgenic tobacco plants [51]. Unknown transcription factors X and Y bind the 10-bp/10-bp-like motifs and 5′-TTGGT-3′ Region 2 motif, respectively. Transcription factors X and Y are hypothesized to form a complex (X-Y-X) that recruits the basal transcriptional machinery to activate *G564* transcription in the suspensor. The identity of putative X and Y transcription factors is not yet known. It is also possible that there is an additional short sequence required for suspensor-specific transcription within the 54-bp suspensor regulatory module. a, apical region of proembryo; b, basal region of proembryo; ep, embryo proper; s, suspensor. Bars = 50 μm.

suggest that the embryo proper sends an 'inhibitory' signal to the suspensor that is required to maintain its differentiated state [6,7,56]. Alternatively, it is also possible that there is a critical balance of growth regulators in the entire embryo that maintains the developmental state of both, the suspensor and the embryo proper [57]. Disruption of these signals by defects in the embryo proper might cause the suspensor to take on an embryo proper-like fate similar to the induction of somatic embryos from differentiated cells *in vitro* [55]. The precise mechanism and the nature of putative signals by which the embryo proper influences suspensor cell fate remain a mystery.

Future Perspectives
Rapidly evolving genomic technologies have opened up new opportunities to investigate the role and evolution of the suspensor in the plant kingdom. It is now possible to investigate gene activity in the suspensor of almost any plant species and uncover novel genes and pathways that play important roles in establishing suspensor form and function. The development of *in vitro* culture systems that generate somatic embryos with suspensors in both angiosperms (e.g., *Brassica napus*) [58] and gymnosperms (e.g., Japanese larch - *Larix leptolepis*) [59–61] should permit the dissection of suspensor molecular processes, including regulatory networks, in a high-throughput manner. There are many questions yet to be answered in suspensor biology. For example, what are the precise molecular events that enable the basal cell to differentiate into a suspensor and what are the gene regulatory networks that control this process? We are now in a new era of possibility to answer these questions and explore this unique embryo region. One thing is clear, however: the suspensor does a lot more than simply 'suspend' the embryo.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2009.11.002.

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Plant Science Conferences in 2010

The 5th EPSO Conference: Plants for Life
18–22 April, 2010
Olos Polar Center (Lapland), Finland
http://www.epsoweb.org/catalog/conf2010.htm

21st International Conference on Arabidopsis Research
6–10 June 2010
Yokohama, Japan
http://arabidopsis2010.psc.riken.jp/

BANFF Conference on Plant Metabolism
24–28 June, 2010
Banff, Canada
http://www.ucalgary.ca/plantmetabolism/

XVII Congress of the Federation of European Societies of Plant Biology (FESPB)
4–9 July, 2010
Valencia, Spain
http://www.geyseco.es/fespb/principal.php?seccion=welcome