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Cellular and molecular events in a newly organizing lateral root meristem

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SUMMARY

Spontaneous or auxin-induced lateral root formation in radish and *Arabidopsis* provides an efficient system in which to examine molecular and cellular events associated with the initiation of a new meristem. Subtracted cDNA libraries made at different times in lateral root initiation were used as a source of genes that are expressed differentially during this developmental process, and expression studies on a small gene family of ribosomal protein genes were conducted. From analysis of cell division patterns in pericycle cells the number of founder cells for lateral roots was established. By the use of *in vitro* growth assays lateral root formation was determined to be a two-stage process. First a primordium is formed, and subsequently a subset of primordial cells begins to function as the lateral root apical meristem. This mode of root development has implications for pattern formation in newly organizing organs.

1. INTRODUCTION

The shoot and root apical meristems that function to produce postembryonic plant organs are initiated in the embryo. The time at which meristems first become identifiable during embryogeny differs in different species, and may also differ because different histological, genetic, and molecular criteria have been used to define them (Steeves & Sussex 1989).

As an alternative approach to the analysis of meristem initiation we have studied the origin and early development of lateral root meristems in radish and *Arabidopsis*. In these two species lateral roots originate in the pericycle, the boundary cell layer of the root stele. Radial positioning of lateral roots is quite predictable. Lateral roots are initiated only from pericycle cells that lie on or close to the xylem radius of the stele. Longitudinal positioning is variable, resulting in irregular spacing between lateral roots in each of the two files. Longitudinal spacing is also responsive to exogenously applied auxin which results in increased numbers of roots per file (Blakely *et al.* 1988). Furthermore, in response to auxin application, differentiated pericycle cells lying on the xylem radius of the stele along the whole length of a seedling primary root are competent to initiate lateral roots.

We have used this system of auxin-induced lateral root initiation to analyse how differentiated pericycle cells are reactivated into the cell cycle, leading to the formation of lateral roots that grow by the activity of an apical meristem.

2. PATTERNS OF GENE EXPRESSION IN NEWLY ORGANIZING LATERAL ROOTS

Two subtracted cDNA libraries enriched for genes that are expressed at specific times and stages in lateral root development in radish were generated (Kerk 1990). One was a 4 h library, from mRNA collected from roots 4 h after auxin application, by subtraction against mRNA from untreated, differentiated root tissue. This library was expected to be enriched in genes that are expressed before the first division of pericycle founder cells. The second was a 24 h library, from mRNA collected from roots 24 h after auxin application, by subtraction against mRNA from roots auxin treated for 4 h, and untreated, differentiated root tissue. This library was expected to be enriched in genes expressed just before meristem initiation.

From these two libraries we have cloned and sequenced 61 cDNAs that represent 51 different genes. The most abundant class of cDNAs represents ribosomal protein (RP) genes. We have identified 14 different RP genes. The protein products of ten of these are components of the large ribosomal subunit, and four are components of the small subunit. Other cDNAs that we have identified code for proteins required for functions associated with cell growth (ribosomal RNA synthesis, mRNA translation, and tRNA synthesis), as well as metabolic enzymes. Ten of the cDNA sequences have no significant similarity to sequences in any data bank that we have searched.

mRNA from roots exposed to auxin for 0–96 h was slot-blotted and probed with each of the cDNAs. Expression was low in the 0 h roots, increased to

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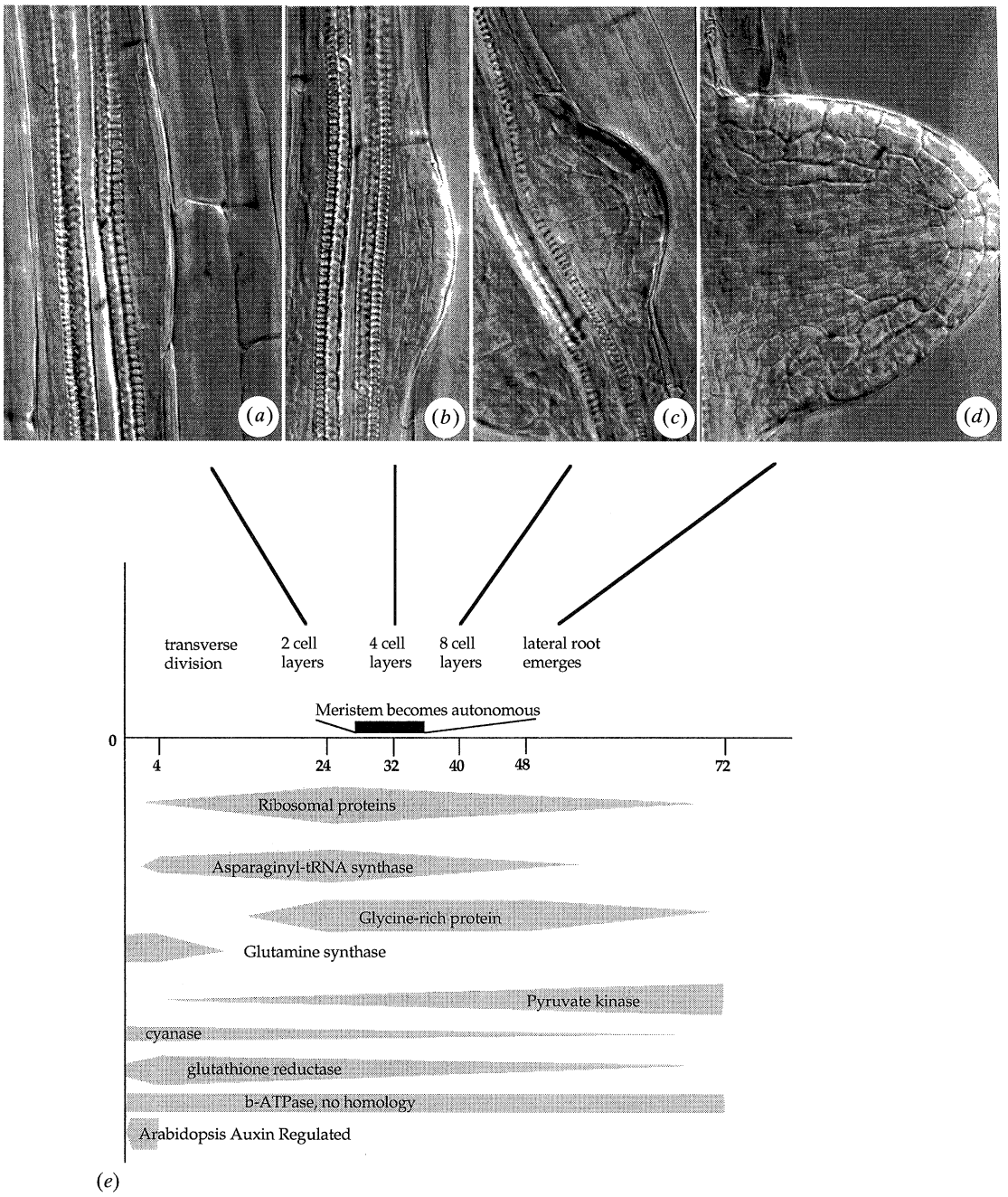

Figure 1. Cellular and molecular events in the formation of lateral roots in *Arabidopsis thaliana*. (*a*)–(*d*) Early cellular changes (magnification ×450): (*a*) the first periclinal division in pericycle cells to form a two-layered primordium; (*b*) a four-layered primordium; (*c*) an eight-layered primordium with periclinal division in the surface cells to establish the root cap; (*d*) a lateral root with an organized apical meristem; (*e*) time line in hours showing when an autonomous apical meristem is established in a primordium, and expression patterns of representative genes.

maxima at 24 or 48 h, and generally declined after that time (see figure 1). There were two exceptions to this general expression pattern. Glutamine synthase was expressed at high levels in the 0 h roots and was not expressed after 8 h, and the expression of pyruvate kinase increased continuously (see figure 1).

Using differential display (Liang & Pardee 1992) we identified a gene in *Arabidopsis* roots that is upregulated by 10 min after auxin treatment and reaches a peak at 30 min (*Arabidopsis* Auxin Upregulated). This has sequence similarity with *WrbA* of *E. coli*, a gene that codes for a tryptophan repressor binding protein (Yang *et al.* 1993).

3. ISOLATION AND EXPRESSION OF RIBOSOMAL PROTEIN L16 GENES FROM *ARABIDOPSIS*

A radish ribosomal protein cDNA that showed a high level of sequence similarity to the genes encoding the ribosomal protein L16 of yeast (Leer *et al.* 1984) was used to isolate corresponding cDNAs from an

Arabidopsis library, and one of these was used to isolate corresponding genomic sequences. Two members of a small gene family were characterized. Each contained three introns whose positions were precisely conserved. The predicted protein sequences of the two genes differed by only three amino acids. These two *Arabidopsis* RP genes showed considerable sequence similarity to comparable RP genes of *Dictyostelium*, and the radish and *Arabidopsis* sequences were highly conserved (Williams & Sussex 1995).

When *RPL16* cDNA (which hybridizes to mRNAs derived from all RPL16 genes) was used as a probe, Northern analysis showed increased levels of *RPL16* mRNA in *Arabidopsis* roots, but not in shoots, in response to auxin treatment. This suggests that the levels of the *RPL16* transcripts are not generally auxin responsive, but may be correlated with a root-specific, auxin-induced developmental event. To test this, *RPL16* mRNA was localized by *in situ* procedures in auxin-treated radish roots. *RPL16* mRNA was first detected in the pericycle in founder cells of lateral root primordia, *RPL16* mRNA levels remained high in all cells of the developing primordium. Subsequently, as the lateral root meristem became functional, *RPL16* mRNA expression was restricted to dividing cells. Expression was absent from cells of the quiescent centre of the lateral root apical meristem and from non-dividing cells at the base of the lateral root.

To characterize the expression patterns of *RPL16* in more detail fusions were made between the promoters of *RPL16A* and *RPL16B* and the *E. coli* gene *uidA* which codes for β-glucuronidase (GUS) (Jefferson *et al.* 1987). Using *Agrobacterium*-mediated transformation, eight independent transgenic plants of Wassilewskija or Landsberg *erecta* ecotypes were obtained in F2 seedlings, expression of the *RPL16A/GUS* construct was observed in internal cell layers of the root between 0.1 and 0.3 mm behind the tip. There was no X-Gluc staining in the root cap, the apical meristem, or in mature root tissues. In auxin-treated seedlings X-Gluc staining occurred in dividing pericycle cells that lay in the xylem radius of the primary root. During development of the lateral root primordium all cells, and those of adjacent parts of the primary root stele showed X-Gluc staining. As the lateral root elongated staining became progressively localized to the stelar region.

In addition to *RPL16A* expression in lateral roots, the *RPL16A/GUS* construct was also expressed in developing pollen and anthers, first appearing in the tapetum at stage eight (Smyth *et al.* 1990) then extending to the pollen.

Expression patterns of the *RPL16B/GUS* fusion construct were broader than those of *RPL16A*. In F2 seedlings expression was observed in cotyledons, shoot apex, vascular tissue and root tip. In older plants X-Gluc staining occurred also in leaf primordia and in all organs of developing flowers.

4. INITIATION AND EARLY DEVELOPMENT OF LATERAL ROOTS

In radish and *Arabidopsis* lateral roots are initiated from pericycle cells located adjacent to a protoxylem pole. Primary roots of both species are diarch with two protoxylem poles. This results in the formation of lateral roots in two files, on opposite sides of the primary root. Pericycle cells on the protoxylem radius of radish are approximately one-third the length of those on the protophloem radius, and in *Arabidopsis* protoxylem radius pericycle cells are one-half the length of those on the protophloem radius (Laskowski *et al.* 1995). The shorter length of xylem radius pericycle cells results from additional division in these cells. In addition, xylem radius pericycle cells of *Arabidopsis* have a greater cross sectional area than do those on the phloem radius of the root. Thus the functional distinction between pericycle cells in the xylem radius files and those in phloem radius files is correlated with anatomical differences.

The formation of a lateral root in both species begins with transverse divisions in xylem radius pericycle cells, each cell becoming subdivided into isodiametric cells which expand radially and undergo a succession of periclinal divisions. Although the first pericycle cells to respond to the root-inducing stimulus are located on or near the xylem radius of the primary root, laterally adjacent pericycle cells are also activated into division and contribute to the formation of the lateral root. In *Arabidopsis* the arc of pericycle cells that is activated for lateral root formation is one-half the circumference of the pericycle and consists of an average of six cells. In radish an arc approximately one-third of the pericycle circumference and consisting of an average of 9.8 cells is activated. The number of pericycle cells in the longitudinal dimension that is activated for lateral-root formation in radish is approximately 4, and in *Arabidopsis* 2.4 (Laskowski *et al.* 1995). From these numbers we calculate that the number of pericycle founder cells (Poethig, 1984) for each lateral root is approximately 11 in *Arabidopsis*, and 40 in radish.

The frequency of lateral root initiation is increased in response to indole acetic acid (IAA) supplied exogenously in the culture medium (Blakely *et al.* 1988), and this facilitates examination of early events in root initiation. The early developmental stages of *Arabidopsis* lateral root meristems were examined in detail in auxin-treated roots cleared in lactic acid and examined under Nomarski optics. Twenty-four hours after IAA application the majority of lateral root primordia consisted of 2–4 periclinal layers of isodiametric cells extending in parallel files out from the stele of the primary root (see figure 1*a*, *b*). By 32 h the number of cell layers had increased to 5–8, and by 48 h the number of cell layers was 8–10. At this time the lateral root emerged through the primary root surface. A change in the pattern of cell files occurred when the primordium consisted of about eight cell layers. Periclinal divisions occurred in the outermost cells located at the tip of the primordium (see figure 1*c*), followed by longitudinal divisions in the central body of the primordium that produced a procambium-like

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tissue (see figure 1*d*). These histological changes indicate that a region within the primordium becomes organized into a cellular pattern characteristic of a functioning root apical meristem between 32 and 48 h after IAA treatment, when there are about 8–10 cell layers.

5. LATERAL ROOT FORMATION IS A TWO-STAGE PROCESS

We observed that when radish roots were supplied with 90 μM IAA all of the xylem radius pericycle cells underwent division, resulting in the formation of two continuous columns of pericycle-derived tissue. Over the course of several days these columns gave rise to a large number of closely packed, separate lateral roots (Laskowski *et al.* 1995). This suggested that the formation of a lateral root occurs in two distinct stages. First a primordium is formed, and subsequently a subset of cells within the primordium begins to function as the apical meristem of the lateral root. We define an apical meristem as a group of cells that is capable of forming an organ directly (Ball 1980).

To identify the stage at which a root apical meristem begins to function, 0.5 mm segments of primary root that had not been treated with auxin and that contained lateral root initials varying from the first transverse division stage in the pericycle to more than six cell layers were cultured on a Murashige and Skoog medium containing sucrose but no growth regulators. Segments that had not initiated a lateral root before excision failed to do so in separate culture. Those that contained transversely divided pericycle cells or two layered primordia when excised failed to form lateral roots. Primordia of six or more layers at excision always gave rise to morphologically normal roots. Those of 3–5 layers gave variable results, some developing as roots, others forming short deformed outgrowths. We conclude that a primordium is first formed and initiates an autonomous meristem capable of forming a root when it consists of 3–5 cell layers (see figure 1). This is slightly before the pattern of cell divisions indicative of a functioning root apical meristem is detectable.

6. DISCUSSION

We have identified molecular and cellular events that are associated with the initiation of lateral roots from differentiated pericycle cells in radish and *Arabidopsis*. Of the 51 different gene sequences that we have isolated from subtracted cDNA libraries made at stages of lateral root formation, all of the identified sequences represent genes coding for ribosomal proteins, cell growth activities, or metabolic enzymes. Thus, these appear to be genes that are required for the reactivation of differentiated pericycle cells for renewed protein synthesis and growth. In the absence of complete sequences for genes that correspond to the ten cDNAs that did not have significant homology to identified genes, we do not know whether any of these are likely to have regulatory functions for lateral root initiation or development.

By examining the pattern of division activity in newly forming lateral roots we have established two important points. First, a large number of pericycle cells contribute to each lateral root as founder cells, and second, there are two distinct stages in lateral root formation. An initial primordium is formed, and subsequently a meristem is initiated in the primordium and generates the distal parts of the root.

The fact that lateral roots are formed from a large number of founder cells, approximately 11 for *Arabidopsis* and 40 for radish, is consistent with the large number of founder cells for leaf formation in tobacco and maize (Poethig 1984). Thus angiosperm lateral organs in both roots and shoots appear to be polyclonal in origin.

Identification of two stages in lateral root formation, initial primordium formation and subsequent meristem initiation, raises issues of pattern formation in lateral roots. According to our analysis, cells of the primordium are not derived from the meristem of the lateral root. Instead, the meristem originates from a subset of primordium cells. Yet the radial and longitudinal patterning of the basal portion of the lateral root that derives from the primordium is similar to that of the more distal parts of the root that derive from the apical meristem. This raises the question of whether the lateral root apical meristem imposes radial and longitudinal pattern on the basal primordial region by processes comparable to induction in animal embryos, or whether the basal region is patterned independently of the meristem. Recent studies on the origin of patterning in the *Arabidopsis* embryo have suggested that the basal region of the primary root closest to the hypocotyl is not derived from the root apical meristem and acquires its radial and longitudinal patterns independently of root apical meristem activity (Dolan *et al.* 1993; Scheres *et al.* 1994). It is not clear yet whether different mechanisms operate to establish patterning at the base of primary and lateral roots.

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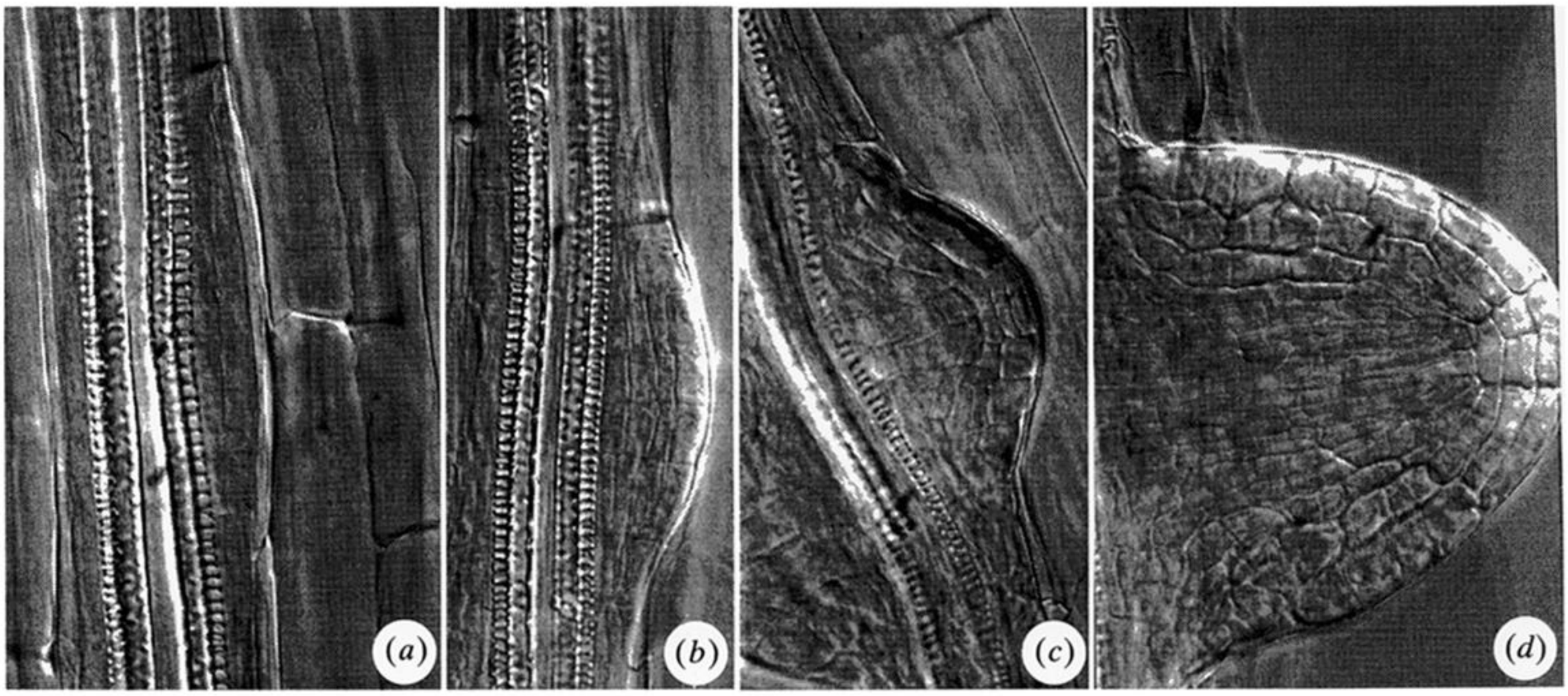

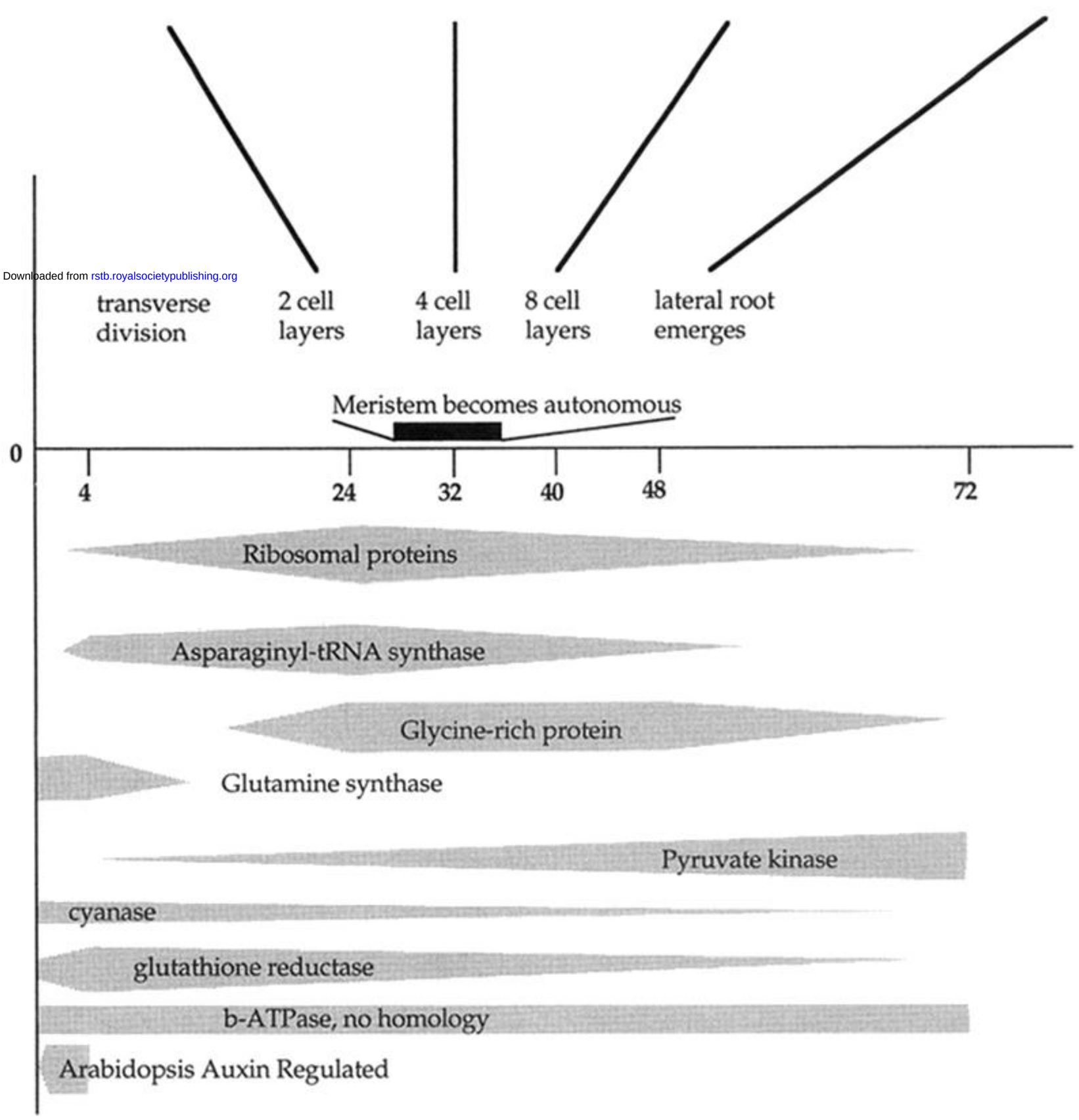

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