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Hypocotyl Growth During Seed Germination  
In Lupinus angustifolius

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ABSTRACT

Hypocotyl growth during seed germination in Lupinus angustifolius cv. New Zealand Bitter Blue was studied.

There was an initial period of rapid hypocotyl elongation over the whole length of the hypocotyl from 0.5 to 1.5 days. Cell division in the cortex occurred between 1 and 3 days, with a peak at 1.5 days. Total hypocotyl length increased rapidly from 2 to 4 days. The distribution of regions of elongation within the hypocotyl changed during this expansion phase, with a wave of cell elongation proceeding acropetally from the base.

The embryo hypocotyl in the seed contained 50 % of the maximum amount of RNA found in the germinating hypocotyl. The peak rate of RNA synthesis for the hypocotyl as a whole, occurred at 12 hours. RNA content of whole hypocotyls doubled between 0.5 and 1.5 days. Nucleoli reached their maximum size within the first day of germination. The loss of vacuoles and granular zones accounted for the subsequent decrease in nucleolar size. At the time of maximum nucleolar size, the nucleus was often convoluted and the nuclear membrane had a high frequency of pores. Cytoplasm was densely packed with polyribosomes and rough e.r. until the cells started their rapid elongation phase at approximately 2 days. RNA synthesis declined rapidly after this time.

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CHAPTER ONEINTRODUCTION

In the preface to the publication of the Proceedings of the Sixth International Conference on Plant Growth Substances, Wightman and Setterfield (1968) state that for detailed understanding of developmental processes, complex systems involving integration of events at the molecular, cell, tissue, organ and organism levels will have to be invoked.

In this project an integrative approach to the study of development of the hypocotyl of lupin (Lupinus angustifolius) is used. The hypocotyl is described at the levels of its morphology, cellular events, subcellular organelles, and molecular content (specifically RNA). The characteristics of the hypocotyl are monitored from the time of seed germination until the seedling is five days old.

In recent years the electron microscope has been used extensively to study ultrastructure of cells at various stages of development. However, such studies have been concentrated on either meristematic cells or mature cells which are engaged in a specialized function. Relatively few workers have made a comparative electron microscope study of dividing, elongating and maturing cells, particularly in non-specialized tissue such as cortex parenchyma. Even a recently published comprehensive book on plant ultrastructure (Robards, 1974), which covers a wide range of organelles and specialized cell types, does not contain a chapter on general formation and development of parenchyma cells.

Although cellular growth from division to maturation is often divided into stages, it is actually a dynamic continuum of events. A sigmoid curve describes the increase in length of an expanding cell with time. The cell elongation stage may be divided into the early exponential

phase and the later linear phase. In the early phase the relative growth rate of a cell is high but the actual increase in length is low. Conversely, in the second phase, the actual increase in length is high but the relative growth rate is lower than in the early phase.

Elongating cells, especially in the second phase of elongation, have not been extensively studied under the electron microscope. This may be the result of the technical difficulties of satisfactorily fixing the highly vacuolated cells that are typical of this stage of development. And also, the thin layer of cytoplasm initially looks to be of little interest compared with the contents of meristematic or specialized mature cells.

Several comparative electron microscope studies of developing plant tissue have been made. The tissues studied include root apices of Plantago ovata (Hyde 1967), Allium cepa (Chounard 1975) and Pisum sativum (Chaly and Setterfield 1975), the wheat coleoptile (Rose 1974) and reactivating storage tissue of Jerusalem artichoke (Fowke and Setterfield 1968, Jordan and Chapman 1971). These electron microscope studies complement analytical and light microscope studies of such tissues and, together with the many other approaches to the study of cell development, will help in forming an understanding of the general pattern of growth in plants.

Information on the developmental process has resulted from several different approaches to its study. In this particular laboratory, the role of auxin in elongation of the hypocotyl of light-grown lupin seedlings has been studied. Penny (1969) found that there is a lag phase of about 15 minutes before auxin stimulated elongation occurs, which suggests that cell elongation is not the primary action of auxin. An understanding of the physiological processes in expanding cells may be aided by an electron microscope study of the region. Penny (1971) did an electron

microscope study of the rapidly elongating zone of a 4 day old lupin hypocotyl and described a typical expanding non-vascular cell as being highly vacuolated and containing only a few golgi and free ribosomes but frequent rough endoplasmic reticuli. Paramural bodies were also seen commonly. These are membranous structures associated with the cell wall and are postulated to be involved in cell wall synthesis (Marchant and Robards 1968).

It has been found also, that RNA synthesis is not necessary, at least initially, for the action of auxin on elongation (Penny P. et al. 1972). Kinetic studies were made on the effect of Actinomycin D, an RNA synthesis inhibitor, on auxin induced growth. Auxin induced growth continued the same in tissue pretreated for up to 2 hours in Actinomycin D and remaining in Actinomycin D during treatment with indole acetic acid (IAA), as it did in control tissue treated with IAA only. The growth rate in inhibitor-treated tissue dropped below that of the control about 2 hours after addition of IAA. These results suggest that RNA synthesis is not necessary for the initial action of auxin on elongation.

There is a lot of evidence to suggest that RNA synthesis in its various forms has a central role to play in developmental processes in plants. It is important in the chain of physiological events between the hormonal stimulus and the cellular response. Several approaches to the study of the role of RNA in developmental processes have been used and, because of their relevance to this project, some of these results are reported here.

One approach has been to determine the absolute amount of RNA present in tissue at different times or at a range of distances from a meristematic apex, and also to determine the actual time when RNA synthesis is occurring in relation to the stage of development of the tissue.

Heyes (1960) studied the root tip of Pisum sativum and found that

RNA content per cell increased from  $4 \times 10^{-5}$   $\mu\text{g}$  per cell at 1mm from the tip to  $16 \times 10^{-5}$   $\mu\text{g}$  per cell in mature cells 8-10 mm from the tip.

In the coleoptile of germinating wheat, Wright (1961) found that the period of rapid cell division is from 24 to 48 hours and the most rapid cell expansion from 48 to 84 hours. On a per coleoptile basis, the increase in RNA from 24 to 48 hours was 275% and from 48 to 72 hours was 31%. This indicates that the rate of RNA synthesis is much higher during the cell division than cell expansion stage.

Giles and Myers (1964) studied growth of the hypocotyl of Lupinus albus. They report that, from germination to 14 days, growth is purely by cell expansion, all cell division having occurred during formation of the seed. (Buis, 1967, reports the presence of some cell division up to 5 or 6 days in hypocotyls of the same species.) A period of rapid increase in RNA content of the hypocotyl precedes the period of rapid increase in hypocotyl length. In the dark the hypocotyl expands to about 20 cm in 14 days compared with about 5cm in continuous light. The maximum RNA content of the dark grown hypocotyl is twice as great as for the light grown one. Van Oostveldt et al. (1976) found, similarly, that light strongly inhibits growth of the hypocotyl in Lupinus angustifolius and also causes a reduction in synthesis of RNA.

Chaly and Setterfield (1975) used autoradiographic methods to determine the time of RNA synthesis relative to the stage of development of the tissue. They found that incorporation of  $^3\text{H}$ -cytidine into RNA is very high in cells from 0 to 1mm from the root tip of Pisum sativum, but decreases exponentially from 2mm basipetally. There is still a small amount of incorporation up to 7 mm from the tip.

Rose (1974) studied incorporation of  $^3\text{H}$ -uridine into the nucleoli of germinating wheat coleoptile cells. The nucleoli in tissue excised

at 15 hours show the highest incorporation, while the nucleoli from tissue excised at 73 hours show very little incorporation of the labelled uridine. The time of highest incorporation slightly precedes the time of initiation of cell division.

These studies indicate that RNA synthesis is most rapid either during or slightly preceding the cell division phase in the tissue and declines very rapidly in elongating and maturing tissue.

A second and very informative approach to the investigation of the role of RNA in developmental processes is electron microscope study of the nucleolus. A lot has been learned from studies of nucleoli of cells at various stages of development.

The nucleolus of cells in tissue prepared for electron microscopy exhibits three zones. These are described by Jordan and Chapman (1971). The granular zone is made up of 15 nm densely staining granules. The fibrillar zone is composed of densely packed fibrils measuring about 10 nm in diameter. The third region appears under the electron microscope as lightly staining vacuoles within the nucleolus. This region is considered by Jordan and Chapman (1971) to be the nucleolar-organizing region of the nucleolar chromosome. That is, a part of the nucleolar chromosome which has expanded so that it can function as the template on which ribosomal RNA is synthesized. Jordan and Chapman regard the change in distribution of the vacuolar region as fundamental and the other nucleolar changes as being consequent upon it.

An important study was made by Fowke and Setterfield (1968). They used electron microscope techniques to compare the ultrastructure of cells from dormant Jerusalem artichoke tuber with cells from tuber tissue which has been "aged" by being cut in thin slices and placed in aerated water

at 25°C. Observations relevant to the nucleolus are mentioned here. In cells fixed directly from the intact tuber, nucleoli were dense, homogeneous and fibrillar. There were no vacuolar or granular zones. Nucleoli in "aged" tissue showed distinct granular and fibrillar zones, and vacuoles within the nucleolus were common. Fowke and Setterfield showed that nucleolar size increases during aging of discs of Jerusalem artichoke. Ultrastructural changes can be related to the changes in nucleolar size. Nucleoli of dormant tuber cells appeared homogeneous and compact and were small. In cells "aged" for 24 hours the nucleoli were enlarged and showed distinct fibrillar and granular zones. Vacuoles were found within both granular and fibrillar zones. Chapman and Jordan (1971) have suggested that nucleolar size is directly related to nucleolar activity. This generalization seems to be true in many cases but Chaly and Setterfield (1975) warn that this size-activity correlation may be misleading. In Pisum sativum roots cut into ten 1 mm segments basipetally, nucleoli 1 mm and 3 mm from the apex were very similar in size, as were nucleoli 5, 7 and 9 mm from the apex, but according to autoradiographic data, segments 1 and 3 incorporated <sup>3</sup>H-cytidine at very different rates, as did nucleoli of segments 5 and 9. Also the nucleoli differed in ultrastructure within each group.

These studies show that marked changes in the ultrastructure of the nucleolus accompany changes in the level of RNA synthetic activity.

The third approach to studying the role of RNA in developing plant tissue has been to determine the effect of 5-fluorouracil (5-FU) on RNA metabolism. 5-FU is a pyrimidine analogue which selectively disrupts synthesis of ribosomal and transfer RNA without significantly influencing synthesis of messenger RNA (Key and Ingle 1964).

Masuda, Setterfield and Bayley (1966) found that 5-FU failed to inhibit the rapid expansion growth of either intact coleoptiles or isolated coleoptile segments. Setterfield (1963) found that 5-FU given during the "aging" period inhibited subsequent auxin-induced elongation in Jerusalem artichoke tuber slices and Fowke and Setterfield (1968) have shown by electron microscope studies that ribosomes are synthesized in this aging period. Rose and Setterfield (1971) found that RNA precursors were incorporated into the nucleolus during 5-FU treatment but that label did not appear in the cytoplasm. Such nucleoli were found to lack a distinct granular zone and appeared as homogeneous fibrillar structures under the electron microscope. King and Chapman (1972) found that when discs are aged in 5-FU, the nucleolar volume increases 50 to 70 % more than control tissue, which suggests that 5-FU causes accumulation of ribosomal-RNA precursors within the nucleolus.

These results suggest that the rapid elongation phase of growth depends on the formation of ribosomes at an earlier stage. The effect of 5-FU is probably to disrupt the actual assembly of ribosomes or their transport from the nucleolus, since RNA precursors are still incorporated and appear to be accumulated in the nucleolus and no granular zone forms.

The fourth approach has been to determine the level of RNA polymerases in tissue at various stages of development. At least one enzyme (RNA polymerase I) is compartmentalized within the nucleolus and is presumably involved in synthesis of ribosomal RNA in vivo. There is also at least one polymerase (RNA polymerase II) in the non-nucleolar nucleoplasm. It is involved in the formation of messenger RNA from the DNA template.

Mature hypocotyl of soybean, when treated with 2,4-D, becomes

swollen and proliferates abnormally. In such tissue Guilfoyle *et al.* (1975) found a 5 to 8 fold increase in the amount of Polymerase I. The level of Polymerase II remained similar to that in the untreated control.

Rizzo and Cherry (1975) measured the relative levels of RNA polymerases in soybean hypocotyls during various stages of development. The meristematic region has more total polymerase activity per gram fresh weight and a greater proportion of Polymerase I to Polymerase II than the differentiated regions. The fully elongated tissue contains mainly Polymerase II.

The level of RNA polymerase I in the nucleolus gives an indication of the amount of ribosomal-RNA synthesis occurring. There is more to be learned from studies like that of Rizzo and Cherry in which the level of RNA polymerase is correlated with either the stage of development of the tissue or with the ultrastructure of the nucleolus.

The fifth approach to the study of the central role of RNA in development is to follow the changes in density of ribosomes either per cell or per volume of cytoplasm, as tissue passes through the developmental stages.

Chaly and Setterfield (1975) covered this in their study of pea roots cut into 1 mm segments basipetally. The cytoplasm of segment one was packed with free ribosomes, and only isolated elements of rough endoplasmic reticuli (e.r.) were present. By segment 3, extensive rough e.r. had formed and this persisted through to segment 9. Segments 3 to 7 had the highest density of ribosomes on e.r. In the non-growing segments, 7 and 9, the free ribosome population appeared greatly reduced. The density of membrane-bound ribosomes and the amount of e.r. per wall length also decreased.

Hsiao (1970) estimated the number of ribosomes per cell in 3 regions of the root tip of Zea mays. In the apical region, 0 to 3 mm, there were  $17 \times 10^6$  ribosomes per cell. The regions 6 to 12 mm and 12 to 40 mm both had about  $25 \times 10^6$  ribosomes per cell.

Jordan and Chapman (1971) report an increase in ribosomes and rough e.r. in the cytoplasm after 24 hours aging of carrot discs.

Masuda et al. (1966) found the concentration of both free and bound ribosomes does not increase in growing oat coleoptiles and may even decline. Free ribosomes decline markedly in fully grown coleoptiles, while the proportion of bound ribosomes increases. They conclude that young coleoptiles contain a full complement of ribosomes necessary for subsequent growth.

Tata (1971) suggests that the association of ribosomes with e.r. may function to segregate different populations of ribosomes synthesizing different classes of proteins.

In summary, these five approaches lead to the general conclusion that RNA synthesis is greatest in meristematic and early elongating tissue and that during this time the number of ribosomes formed is sufficient to sustain subsequent expansion growth. It is the aim of this project to study this generalization in lupin hypocotyl tissue and find how it relates to other processes at the molecular, subcellular, cellular and tissue levels.

Lupin, Lupinus angustifolius cv New Zealand Bitter Blue, was chosen as the study species because it is used in physiological experiments, particularly in this laboratory. Therefore a description of the growth of the plant at cellular and subcellular levels may be useful for

hypothesizing a mechanism to explain a physiological response in an experimental situation.

Other advantages of lupin, which were found after the study had begun, were that cells of the hypocotyl show two phases of elongation. Presumably, certain cytological events are common to the process at both stages. Other commonly studied systems contain a meristematic apex from which cells arise and show one phase of elongation before maturing. In the hypocotyl of lupin the initial phase of expansion is by cells which have been in an inactive state for a variable time period in the seed, whereas, in say a root, it is active meristematic cells which elongate.

The epidermal cells of lupin were found not to divide after germination, so the cytological events which occur in these cells at an early stage are required for elongation. Early events in cortical cells may be required for cell division as well as elongation.

In this study the growth of the hypocotyl is defined by its increase in total length over the growth period. Then growth is explained in terms of the contributing factors at the cellular level; cell division and cell elongation. By knowing the distribution of these cellular events in the hypocotyl, tissue can later be taken from a region whose cellular activities are known, and used for analyses of cellular contents. This will enable correlations to be made between cellular activity and cell contents. This type of correlation has often been made for cells with a specialized function, but not so often for unspecialized cells such as cortex parenchyma.

At the sub-cellular level this study concentrates on the formation of ribosomal RNA in the nucleolus and its presence in the cytoplasm. This is considered to give an indication of protein synthetic activity in the cell.

Cells at a known stage of development are studied at the subcellular level. The first approach is a light microscope study of the nucleolus, measuring its size and noting the extent of nucleolar vacuoles. Secondly, electron microscope techniques are learned and used to study the ultrastructure of the nucleolus at various stages, and also the density and distribution of ribosomes in the cytoplasm. The third approach is to chemically isolate ribosomal RNA and determine the relative amounts in cells at different stages of development. Similar methods are used to determine the stage at which RNA synthesis is most rapidly occurring.

As well as the RNA-associated organelles, description of other organelles is included in the hope that they will help in forming a fuller, more general description of plant growth, in conjunction with other more experimental approaches.

By using several approaches to the study of the lupin hypocotyl, a wide range of experimental techniques will be learnt and used.