

Chapter 5

The Development of the Maize Root System: Role of Auxin and Ethylene

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5.1 Introduction

Maize (*Zea mays*) is an herbaceous monocotyledon with an annual cycle. When the seed germinates, a primary or seminal root arises from the embryonic radicle. This root elongates rapidly and, as it grows, forms very many new lateral roots (LRs). The primary root may persist throughout the plant's life, although frequently it ceases growing and branching (Feldman 1994). Consequently, it has relatively little functional importance after the first stages of seedling development (Hochholdinger et al. 2004). The mature plant has an extensive fibrous root system consisting of several whorls of adventitious roots, some of which become widely spread, horizontally growing roots, while others become deeply penetrating vertical roots. Because they originate from shoot nodal regions, these adventitious roots are often categorized as nodal roots. Post-embryonic nodal maize roots have been subdivided into crown and brace roots according to whether they initiated from underground or aboveground nodes, respectively (Hochholdinger et al. 2004).

Despite its relatively poor functional importance, the primary root is a wonderful system in which to analyse basic processes of development such as (i) organ polarity establishment, (ii) cell proliferation regulation, (iii) cell fate decisions,

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(iv) cell signalling, (v) cell growth coordination, etc. Together, these processes occur in a beautiful and highly ordered histological context and are affected by multiple physicochemical factors, some of which have long been well known, while others are the object of current research.

In order to draw rigorous conclusions, the study of the physiology and molecular biology which regulates root development requires an adequate analysis of the temporal evolution of the histological structure of the root. In this sense, the present study is an attempt to contribute to understanding how the primary root system proceeds in maize and what is the presumptive role played in this process by two specific phytohormones: auxin and ethylene.

The development of the maize root system has two main aspects which occur simultaneously although they are conceptually different. Firstly, it is necessary to understand how each individual root develops by a continuous contribution of new cells at the root apex and the subsequent growth of these cells. Secondly, one must consider how new root meristems destined to originate lateral and adventitious roots are formed endogenously in mature root or shoot tissues.

The present work is mainly concerned with the description of the development and branching of the primary roots. Adventitious roots follow paths of development which have been appropriately described in maize and other related species elsewhere (Erdelska and Vidovencova 1993; Hetz et al. 1996; Mergemann and Sauter 2000).

5.2 Morphology and Structure of the Primary Maize Root

5.2.1 *General Morphology and Growth Zones*

The maize primary root is a cylindrical structure, finished at the tip of its distal end by the presence of the root apex (Fig. 5.1a). It is generally considered to consist of a series of consecutive root zones. The root apex comprises approximately the distal-most 2 mm of the root extension (Fig. 5.1a). It contains the root apical meristem, consisting of undifferentiated meristematic cells that are in continuous cell division. Its function is to produce the cells destined to constitute the various tissues of the root.

Just above the root apex is located the elongation zone that extends approximately from 2 to 10 mm behind the tip (Fig. 5.1a). In this region, the cells stop dividing and begin to grow intensely and predominantly in a direction parallel to the longitudinal root axis in a process known as cell elongation. Initially, in the apical-most part of this zone, cell growth is both longitudinal and transverse, whereas subsequently, in the rapid elongation zone, it is only longitudinal (Ivanov 1997).

In the root zones located at more than 10 mm from the tip, one may consider the cells to have reached their definitive length, and cell differentiation begins. This region is defined as a maturation zone. In it, the vascular tissues progressively

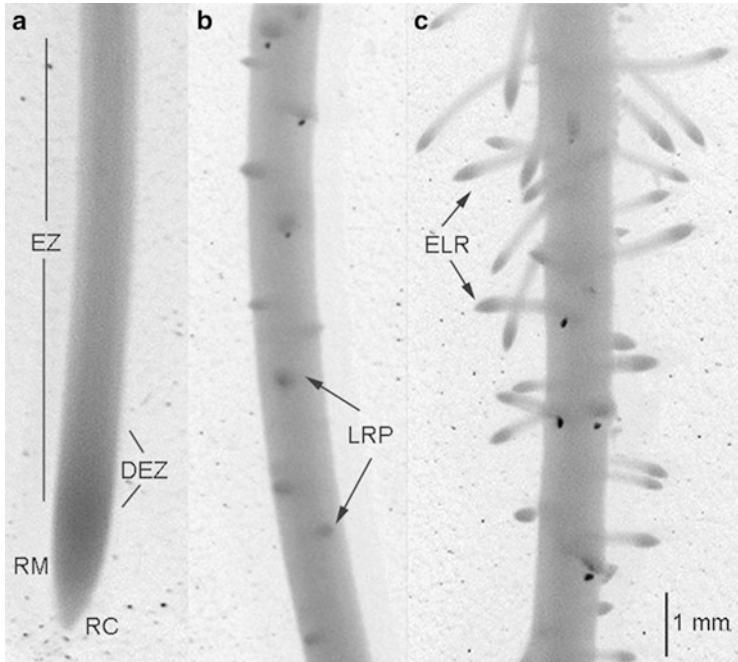


Fig. 5.1 Segments of the primary root of *Zea mays* showing the morphology of root apex and the development of lateral roots. (a) Root apex and elongation zone. (b) Maturation zone (distal). (c) Maturation zone (proximal). *RC* root cap, *RM* root meristem, *DEZ* distal elongation zone, *EZ* elongation zone, *LRP* lateral root primordia, *ELR* emerged lateral roots. Bar: 1 mm

acquire maturity. The root hairs are formed and begin their development at this level of the root, and the LRs continue their growth (Fig. 5.1b, c).

5.2.2 Histology of the Primary Root

The arrangement of tissues that form the body of the primary root can be studied by means of transverse and longitudinal sections. In the root, as in the rest of the plant organs, three main systems of tissues can be distinguished: the epidermis, the cortex, and the vascular cylinder.

The outermost layer of cells of the root is the epidermis (Fig. 5.2a). The epidermis is uniseriate in maize, and the cell walls are asymmetrically thickened. Epidermal cells are elongated parallel to the long axis of the main root, and their internal content is very clear because almost all of the cell volume is occupied by a large vacuole. The cell nucleus is usually located away from the centre in a position adjacent to the cell wall (Fig. 5.2a).

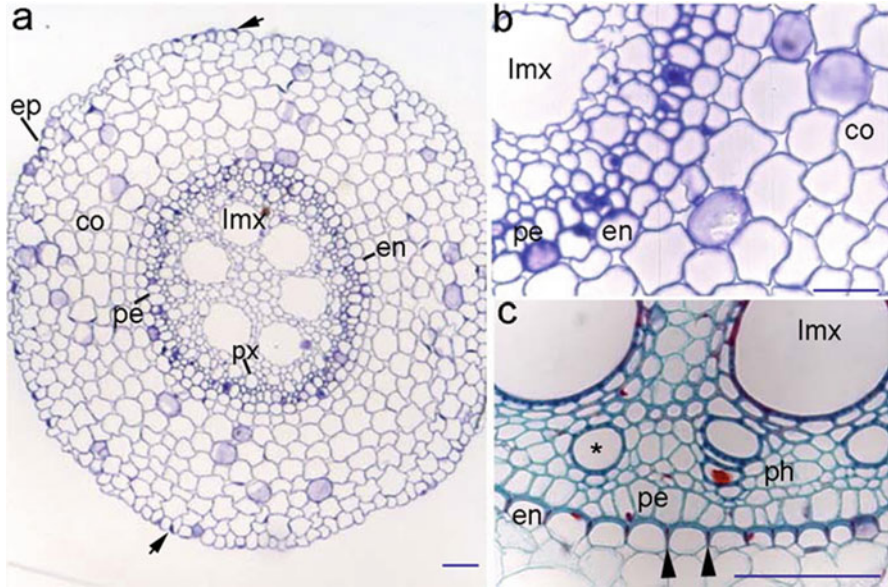


Fig. 5.2 Cross section of maize roots. **(a)** General view at the end of the elongation zone (toluidine blue staining, TBS). **(b)** Detail of the vascular cylinder and inner cortex at the end of the elongation zone (TBS). **(c)** Detail of the vascular cylinder and inner cortex at the proximal part of the maturation zone (safranin-fast green staining procedure). Note in **(a)** that the nucleus of the epidermal cells are displaced to the periphery of the cell (*arrows*) and in **(c)**, the U-shaped wall of endodermal cells (*arrowheads*) and the early metaxylem elements (*asterisk*). *ep* epidermis, *co* cortex, *en* endodermis, *pe* pericycle, *px* protoxylem pole, *lmx* late metaxylem, *ph* phloem. Bars: 50 μm

The cortex consists of six to ten layers of parenchymatous tissue with small intercellular spaces at the angles of the cells (Fig. 5.2a). The outermost and innermost cortex layers have special characteristics and are called the exodermis and endodermis, respectively. The cells of these two layers are highly specialized, and in most higher plants, their cell wall development passes through three stages (Clarkson and Robards 1975). In Stage I, they deposit Casparian strips on their radial and transverse cell walls. Later in ontogeny (Stage II), a suberin lamella is deposited between the primary wall and the plasmalemma. Thanks to the Casparian strips and the suberin lamellae, the system of cortical extracellular spaces remains sealed, preventing the access of ions to the vascular cylinder via the apoplast (Enstone et al. 2002). In some species, these cells progress to a third stage of development (Stage III) with the deposition of a tertiary cellulose wall. This can be observed very clearly in the maize endodermis which, near the apex, presents a slightly thickened cell wall (Fig. 5.2b), while further away it presents the characteristic U-shaped tertiary cell wall with clearly discernible asymmetric thickening (Fig. 5.2c). The transition from Stage II to Stage III is not always sharply demarcated (Clarkson and Robards 1975).

The first layers of the vascular cylinder form the pericycle. The maize root pericycle is initially single layered, consisting of thin-walled parenchymatous cells (Fig. 5.2a–c) which may become thick-walled later in ontogeny (Sutherland and McCully 1976). Pericycle cells are capable of recovering their meristematic activity and of initiating the process of LR production.

The vascular cylinder has an alternating organization of xylem and phloem poles, which is typical of most roots. The number of protoxylem poles is variable in maize roots, ranging from 20 to 40. There are commonly two or three protoxylem strands to each large metaxylem vessel (Fig. 5.2c). The primary phloem poles alternate with the protoxylem poles and are in contact with pericycle cells with a particularly large cross-sectional area (Fig. 5.2c). Often, parenchymatous cells that are located between the xylem and phloem poles eventually present thickened and lignified cell walls. The centre of the root is occupied by a parenchymatous tissue called pith, which extends between the vascular tissues until it reaches the pericycle (Fig. 5.2a).

The LRs originate as endogenous anlagen named lateral root primordia (LRP). The LRP primarily consist of an arc of pericycle which more or less extends over three phloem and two intervening xylem poles (Fig. 5.3a). Although most of the cells that constitute the LRP derive from the pericycle, there is an endodermal cover which is clearly incorporated into the primordium (Fig. 5.3a). Longitudinal sections show that the early LRP consist of two short pericycle cells lying end to end in the same column, flanked above and below by two longer pericycle derivatives (Fig. 5.3b). More developed LRP gradually make their way through the cortex of the parent root (Fig. 5.3c, d) until they reach the level of the epidermis and break through it to emerge as young LRs (Fig. 5.1c).

5.3 Root Development

In general terms, biological development can be defined as a series of gradual changes in size, structure, and function affecting individuals, organs, or cells. The development of a root basically consists of the processes of growth and differentiation that represent, respectively, quantitative and qualitative changes (Segura 2000). In the study of the development of the root system of any plant, it is necessary to consider two specific aspects: the growth and differentiation of each individual root and how new LRs are formed.

The classical concept of the development of an individual root considers successive zones of division, elongation, and maturation. The source of cells for the body of the growing root is the root apex. Cells produced in the root apex are usually regarded as members of a larger unit, undergoing more or less abrupt transitions between different developmental stages at the same time as they transit specific root zones.

In most angiosperms, root branching occurs far from the apical meristem when specific founder cells initiate a sequence of events that leads them to create a new

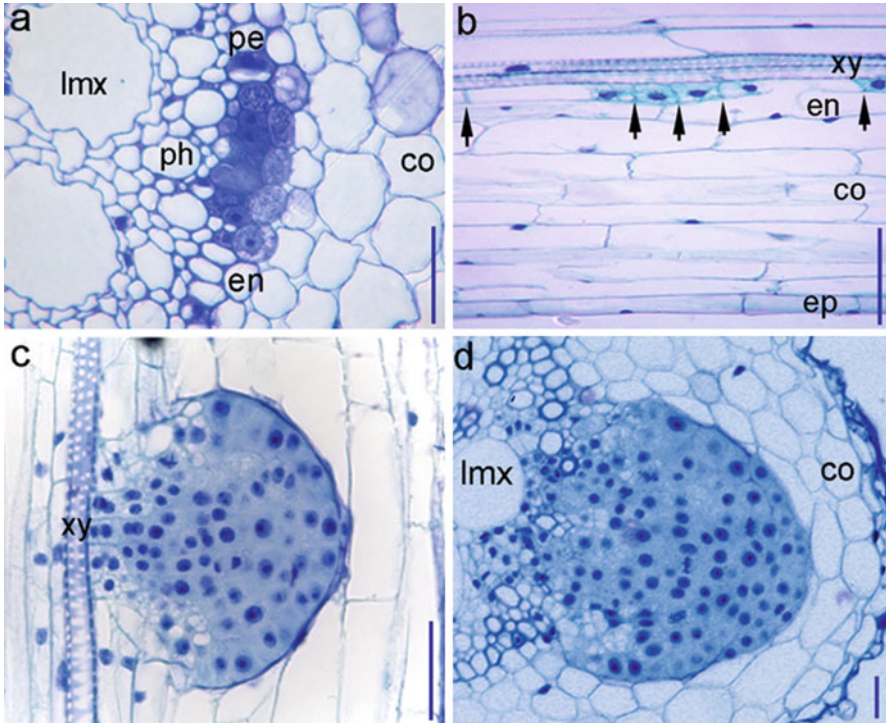
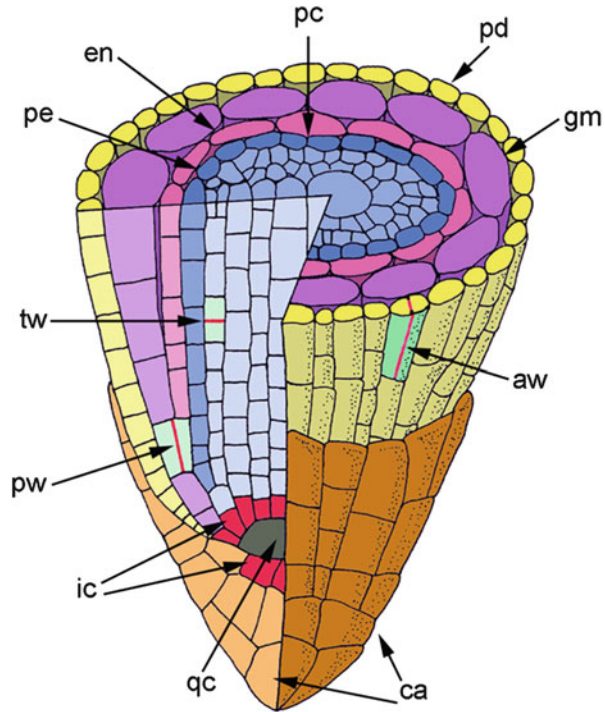


Fig. 5.3 Development of lateral root primordia (LRP) in *Zea mays*. (a) Transverse section of an arc of basophilic cells from the pericycle and the endodermis that are initiating an LRP (toluidine blue staining technique, TBS). (b) Longitudinal section of a column of pericycle where two founder cells have undergone the first transverse divisions during LRP initiation (Feulgen staining technique). Note that four cells, two short central and two long peripheral, can be distinguished. Arrows indicate the transverse walls of each cell. (c) Longitudinal section of a maize root segment along a xylem pole (TBS). Note that the LRP of the image has a perfectly organized apical meristem and has crossed more than half of its way towards the external surface of the parent root. (d) Transverse section of maize root showing an LRP in a more advanced stage of development (just emerging) which extends covering an arc of pericycle of approximately three phloem poles and two intervening xylem poles (TBS). *co* cortex, *en* endodermis, *pe* pericycle, *xy* xylem strand, *lmx* late metaxylem, *ph* phloem. Bars: 50 μ m

apical meristem inside the parent root. Because founder cells belong to the outer cell layer of the vascular cylinder, i.e., the pericycle, LRP are initially endogenous structures (Lloret and Casero 2002). Nevertheless, they quickly grow through the parent root cortex (Fig. 5.3c, d) and break the epidermis to emerge as young LRs (Fig. 5.1c).

In the following, we shall discuss some basic aspects of the development of the root system of plants, with special emphasis on the situation in maize.

Fig. 5.4 Schematic representation of the maize root apex including an explanation of the different orientation planes of cell divisions in meristematic cells. *ca* root cap, *qc* quiescent centre, *ic* initial cells, *pw* periclinal wall, *aw* anticlinal wall, *tw* transverse wall, *pe* pericycle, *en* endodermis, *pc* procambium, *pr* protodermis, *gm* ground meristem



5.3.1 The Root Apex

The structure and developmental dynamics of the apex of both the main root and the LRs are similar. There is always a terminal root cap and a sub-terminal root meristem (Figs. 5.4 and 5.5).

The root cap forms a cover at the tip of the apex and consists of cells that differentiate quickly, secrete mucilaginous compounds, and slough off as they reach the organ's surface. In addition to its protective function, the root cap also serves to sense the direction of gravity and other environmental signals (Sievers et al. 2002).

Interposed between the root cap and the meristem, there is a special region which is very important for both the organization and the physiology of roots: the quiescent centre (QC). The QC is discoid in shape (Fig. 5.4) and consists of cells which, on average, divide relatively infrequently (Fig. 5.5c). Clowes (1958) showed that, in the root apex, the cells undergoing DNA synthesis and mitoses are located around the QC. This means that the functional initials of root tissues are really two groups of cells located above and below the QC. The initial cells located below the QC will, by repeated cell divisions, form the root cap, whereas those located above the QC will form the rest of the root tissues. Often this latter cell population is considered to constitute the promeristem, from which originate the true primary

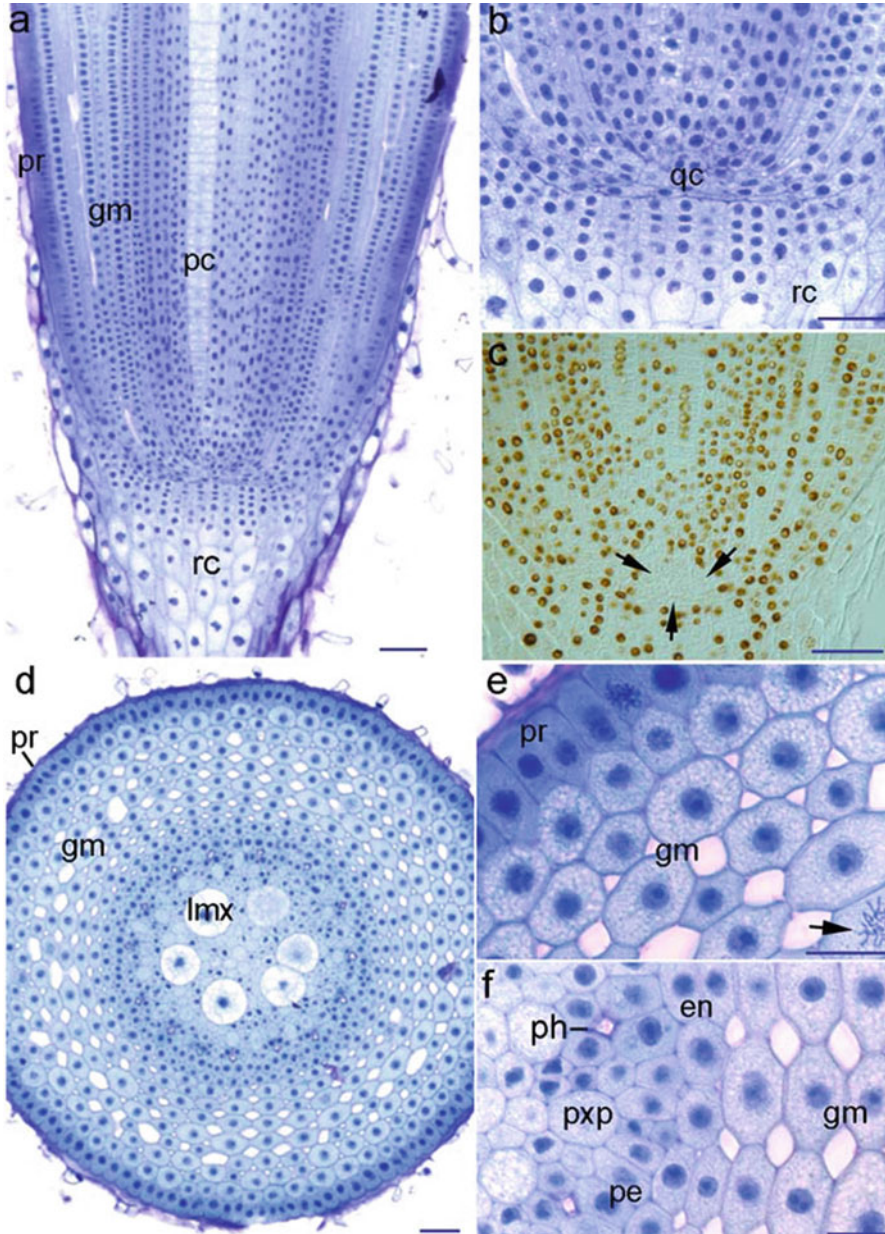


Fig. 5.5 (a) Longitudinal section of a maize root apex showing the overall organization (toluidine blue staining technique, TBS). (b) Detail of the promeristem and the quiescent centre as well as the base of the root cap. The cell columns of the ground meristem can be followed through the quiescent centre, leaving the initial cells of the root cap at a lower typical position of closed meristems (TBS). (c) The application of an immunocytochemical technique to detect proliferative cells (BrdU labelling technique) in the promeristem region of the corn root apex delimits clearly

meristematic tissues of the root apex: protodermis, ground meristem, and procambium (Fig. 5.4). To prevent depletion of the initials, after every division of each initial cell, the distal daughter retains its status of initial cell. Therefore, the initial cells are in theory capable of self-perpetuating indefinitely.

It has been found that there is a very high rate of auxin release in the QC by the mechanism of polar auxin transport (Kerk et al. 2000). Also, high auxin concentrations in the QC have been correlated with high ascorbic acid oxidase activity, which in turn results in low ascorbic acid concentrations and the establishment of the characteristic quiescence of this region (Kerk et al. 2000). Functionally, there is growing evidence that the QC is an organizing structure, and it has been shown to maintain the stem cell's niche necessary for continuous root growth via the expression of the homeobox gene *WOX5* (Sarkar et al. 2007).

Most cell divisions in the root meristem occur with the new cell wall in a plane perpendicular to the root's long axis (transversal divisions), and the cells are arranged in long columns (Fig. 5.4). The outer columns of the meristem constitute the protodermis which, when it matures, forms the epidermal covering of the root. Further inwards is the ground meristem which gives rise to the cortex in the mature root, and in the centre is the procambium which gives rise to the vascular cylinder (Fig. 5.4).

The development of the primary root includes the processes of cell division, elongation, and differentiation. In principle, cell divisions are restricted to the promeristem and the meristem in the proximal part of the root apex and to the initial cells of the root cap and their immediate derivatives in the distal face of the QC. All the cells which will eventually form the root body are generated through the proliferation of the procambium, ground meristem, and protodermis. In maize roots, longitudinal growth occurs within the region extending 12 mm from the tip (Silk 2002). Although the maximum growth rate is attained at a distance of 4–6 mm from the apex (Silk 2002), cells in the meristematic region also elongate along the apical-basal axis at a slower growth rate. Consequently, one may assume that the processes of cell division and elongation overlap at the root apex.

Within the apical meristem, two types of cell division take place: formative and proliferative (Barlow 1984). The formative divisions follow an anticlinal (i.e., perpendicular to the root surface) or periclinal (parallel to the root surface) plane and have the function of producing new columns of cells (Fig. 5.4). New tissues can be established by means of this kind of cell division. For example, in most angiosperms the epidermis and cortex share a common ontogenetic origin in the root apex initial cell zone (Clowes 1994). The proliferative divisions have the equatorial plane oriented transversely with respect to the root's long axis, thereby



Fig. 5.5 (continued) the location of the quiescent centre (*arrows*). (**d–f**) Transversal sections of maize root apex taken at the middle of the meristematic region (TBS). Note in (**e**) a mitotic figure in a cell of the ground meristem (*arrow*). *qc* quiescent centre, *rc* root cap, *pe* pericycle, *en* endodermis, *lmx* late metaxylem, *ph* phloem, *pc* procambium, *pr* protodermis, *gm* ground meristem, *pxp* protoxylem pole. Bars: 50 μ m

giving rise to daughter cells that belong to the same column. Consequently, it becomes possible for the number of cells constituting a given column to increase while the total number of columns remains unaltered (Fig. 5.4).

Anatomically, the apical meristem of the maize root is considered to be “closed” because it is possible to continue along all cell columns up to the groups of initial cells located around the QC (Fig. 5.5b). Closed root apices are also characterized by having a clear separation between the root cap and the rest of the root body. In other species, the meristem is considered as “open” because there is no such clear continuity of columns to the QC region, and the boundaries between cortex, epidermis, and cap are unstable (Clowes 1994).

The meristematic cells of the maize root apex are very small isodiametric cells, with there being scarcely any intercellular spaces observable between them (Fig. 5.5a). They present a very thin primary cell wall and middle lamella. Their cytoplasm is dense, with many small vacuoles, and the nucleus is located centrally (Fig. 5.5d–f).

The maize root apex reaches a distance of about 2,000 μm from the distal end of the QC (root cap junction, RCJ). Most of the root apex is occupied by the meristematic region where cell divisions occur (Barlow 1987). The behaviour of cells in this region is affected by environmental changes monitored in the aerial part of the plant (Muller et al. 1998). Cell division by itself does not increase the length of the root. However, the cell division rate, the fraction of proliferating cells, and how long these cells remain mitotically active determine the rate at which cells are incorporated from the apex into the elongation zone, and therefore the root elongation rate (Beemster and Baskin 1998, 2000; Baskin 2000).

Beemster et al. (2003) divide the root apical meristem into two halves—the apical and the basal. In the apical half, cell divisions and root growth are correlated in such a way that the average cell length remains constant. Along the basal meristem, cells divide roughly at the same rate as in the apical meristem, but the elongation rate increases progressively. As a consequence, cell size also increases. The basal meristem cells share some common features with cells that are located at the beginning of the elongation zone (De Smet and Jürgens 2007). For example, it is assumed that these two areas are particularly important for integrating environmental and/or hormonal stimuli and that they modulate root growth (Ishikawa and Evans 1995; Beemster et al. 2003).

5.3.2 *The Elongation Zone*

In maize roots, the elongation zone (EZ) reaches to a distance of about 10–12 mm from the RCJ. It is characterized basically by the cessation of cell division, with the cells continuing their elongation at a variable rate that depends on their position. Ishikawa and Evans (1993) subdivide the EZ into five segments depending on the growth rate of the cells at each point. In the distal-most segment, the elongation growth rate is fairly slow (less than 0.3 times the maximum rate), and the cells seem

to be particularly sensitive to auxin and to environmental changes. This root segment has been termed the transition zone (Baluška et al. 1996) or the distal elongation zone (Ishikawa and Evans 1993) and is located between the apical meristem and the region where rapid elongation takes place (Ivanov 1997). Some authors argue that the distal EZ, the transition zone, and the basal meristem are terms referring to the same region of the root (Beemster et al. 2003). The key to choosing which term to use would be whether or not cell division has ceased at this level of the root.

In the segment termed the rapid elongation zone, which lies at between 3,000 and 6,000 μm from the RCJ, major growth takes place. The beginning of this segment coincides with the cessation of transverse growth (García-Sánchez et al. 1991). Further away from the RCJ, the elongation gradually diminishes, until the expansion of the wall completely ceases at about 10 mm from the RCJ (Ishikawa and Evans 1993).

5.3.3 *The Maturation Zone*

In their transition through the three zones, as a result of polarized growth, the morphology of the root cells changes, and they gradually acquire traits of maturity through cell differentiation. From 10 mm from the RCJ onwards, the cell length remains constant, and those cells that acquire specific features of cellular differentiation gradually mature. This is particularly noticeable in cells such as those that constitute the hypodermis and endodermis that acquire Casparian strips and suberin lamellae (Zeier et al. 1999). In this zone, the conductive elements of the xylem, and even pericycle and stellar parenchyma cells, increase the thickness of their walls and deposit lignin (Sutherland and McCully 1976). The root hairs are also formed and eventually wither (Wen and Schnable 1994). Another remarkable phenomenon that occurs in this region is the beginning of the formation of the LRP. This is initiated by a series of cell divisions in some cells of the parent root pericycle. The first divisions are transversal and significantly reduce the length of the LRP's founder cells (Fig. 5.3b) (Dubrovsky et al. 2000; Casimiro et al. 2003).

5.3.4 *Lateral Root Ontogeny*

The initiation of LRs is a fascinating developmental process because it involves the production of an entire organ from a small number of differentiated cells in response to intrinsic and environmental cues (Malamy 2005). In *Zea mays*, LR initiation occurs endogenously and far from the root apex, in particular, at a distance of approximately 12–15 mm behind the tip (Casero et al. 1995) when a few pericycle cells are stimulated to dedifferentiate and proliferate to form an LR primordium. The first sign of this process that is visible under light microscopy is

that, by means of coordinated asymmetrical transverse divisions, one column of pericycle cells produces two very short cells (about 25 μm long, Fig. 5.3b) lying end to end and flanked above and below by two longer cells (Fig. 5.3b). The initiation of LRs by this procedure has been described in many species, and it can be assumed that this type of LR initiation is the commonest case among angiosperms (Lloret and Casero 2002).

In the circumferential plane, the columns which start the development of LR primordia are always located just in front of the protophloem poles of the parent root (Fig. 5.3a). Later, successive rounds of transversal root divisions extend longitudinally along the same column and transversally to adjacent columns, and a small plate of short cells is formed. This plate has been termed the rhizogenous plate (Van Tieghem and Douliot 1888) and extends transversally to an arc of approximately 12 columns of pericycle cells which cover three phloem poles and the two intervening xylem poles (Fig. 5.3a). In the longitudinal plane, the long founder cells of the LR primordium undergo a series of transverse divisions which give rise to many new short cells [see Fig. 5.6 in Lloret and Casero (2002)].

The pairs of cells of adjacent columns that give rise to the primordial rhizogenous plate are known as founder cells (Laskowski et al. 1995). Once these founder cells have undergone the first asymmetric transverse divisions, the process of LR development continues quickly. The following events are a radial enlargement of cells located at the centre of the rhizogenous plate, a reduction in the size of vacuoles, and, finally, periclinal divisions. In maize LR primordia, these cell divisions are asymmetrical, giving rise to an inner cell larger than the outer cell (Casero et al. 1995). The ulterior development of LRs in *Raphanus sativus* and *Arabidopsis thaliana* has been subdivided into seven and six developmental stages, respectively (Blakely et al. 1982; Malamy and Benfey 1997).

As the LRP pass through successive stages of development, they become ever more complex. During this process, the number of cells and the size of the LRP increase (Malamy and Benfey 1997), and the LRP acquire the capacity for autonomous growth, i.e., their growth becomes self-sufficient, no longer requiring growth regulators from the parent root. This phenomenon apparently occurs when the primordium organizes its own root apex (Laskowski et al. 1995).

The LRP must break through the parent root cortex and epidermis to emerge as young LRs. As the primordia cross these parental tissues, the primordial cells of pericyclic origin are covered by a cap of cells deriving from the endodermis termed the endodermal covering (Seago 1973). From the beginning of LR development, the endodermal covering cells present morphological features like those of pericyclic-derived cells, including increased protoplast stainability and transverse divisions which produce very short cells, etc. (Bell and McCully 1970; Seago 1973). Periclinal divisions are scarce, however, so that this layer does not constitute a permanent covering of the primordia, but must instead be regarded as a temporary structure to be replaced by a true root cap formed from cells of pericyclic origin (Seago 1973; Clowes 1978).

The outgrowth of the LRP through parent root tissues requires the “dissolution” of cells located in the way of the young developing primordium. In the case of the

parent root cortex, it has been proposed that cells are pushed out by a combination of pressure (Pond 1908) and the secretion of hydrolases by the young primordium (Bonnett and Torrey 1966; Sutcliffe and Sexton 1968; Keller and Lamb 1989; Peretto et al. 1992). Also, it has been demonstrated recently that auxin promotes LR emergence through the regulation of the spatial and temporal distribution of aquaporin channels (Péret et al. 2012).

The final stage of LR development is emergence (Fig. 5.1c). In order to be fully functional, the vascular systems of the young LR and the parent root must be connected. This vascular connection matures either at about the time of LR emergence or later in freshly emerged LRs. Phloem and xylem connector elements are formed from the pericycle derivatives and vascular parenchyma cells of the parent root vascular cylinder (Peterson and Peterson 1986). It is generally accepted that maturation of the vascular connectors proceeds acropetally into the LRs (Peterson and Peterson 1986). These maturing vascular elements connect with newly formed vascular elements that have originated from the root apex of the young LR which is already active at this time.

5.4 Role of Auxin, Ethylene, and Other Phytohormones in the Development of Root Systems

An important challenge for plant developmental biologists is to understand the mechanisms that control the patterned development of more or less complex systems. The focus of the present section will be on the hormonal mechanisms that shape the growth, differentiation, and architecture of the maize root system. In particular, we shall analyse the effects on maize's primary root development of treatments with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and with the synthetic auxin 1-naphthaleneacetic acid (NAA), alone or in combination. Experiments have also been carried out using an ethylene antagonist and inhibitors.

Plant development sciences have evolved from being mostly descriptive and comparative to a field dominated by genetic and molecular approaches. This change has been favoured by the systematization of experimental procedures and the emergence of ever more sophisticated research tools. Nevertheless, classical studies of root development using such simple techniques as the exogenous application of growth regulators are still useful in this context, while recent physiological studies using new tools such as auxin transport inhibitors, ethylene biosynthesis inhibitors, or ethylene action inhibitors, for example, continue to improve our knowledge of the process (Casimiro et al. 2001; Alarcón et al. 2009; Strader et al. 2009; Steinitz et al. 2010).

Usually, the exogenous application of auxin promotes three main alterations in root system development: inhibition of elongation, increased transversal expansion, and enhanced lateral root formation. Physiologically, these changes occur in

association with increased ethylene production due to increased transcription of ACC synthase genes (Abel et al. 1995). This leads to the accumulation of the ethylene precursor ACC and its product, ethylene, in tissues as a result of the auxin treatment.

The morphological changes observed in dark-grown seedlings treated with ethylene or its metabolic precursor ACC have been termed the triple response syndrome: exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and shortening of the hypocotyl and root (Ecker 1995).

Since the effects of auxin and ethylene on root development share some common features, it is difficult to assign the causality of these effects to one or the other of these growth regulators or to the interaction between the two. As auxin stimulates ethylene production by increasing the biosynthesis rate of ACC synthase, it has been posited that ethylene is the mediator of, and directly responsible for, the effects of auxin on root growth (Eliasson and Bollmark 1988; Jackson 1991). Hansen and Grossmann (2000) report that auxin-induced ethylene triggers the production of abscisic acid (ABA) and inhibits root growth through this growth regulator. The fact that there is some crosstalk between auxin- and ethylene-resistant mutations suggests at least that these two phytohormones interact in the regulation of root development (Stepanova et al. 2007). Consequently, there is a need for a precise dissection of the particular effects of each growth regulator on different aspects of root development.

5.4.1 *Phytohormones and Root Elongation*

The most obvious generalization about the effect of exogenous auxin on root elongation is that its inhibition of growth is dose dependent (Blakely et al. 1982; Lloret and Pulgarín 1992). Indeed, when it is applied at very low doses, it can even stimulate root growth (Díez et al. 1971). Such stimulation by exogenous auxin at very low concentrations has also been observed in maize roots pretreated with ethylene biosynthesis inhibitor (Mulkey et al. 1982). Moreover a negative correlation has been found between root growth and endogenous indole-3-acetic acid (IAA) content (Pilet and Barlow 1987). These results support the idea that the endogenous IAA level should be optimal or supraoptimal for elongation, and consequently any increment in the auxin level would result in root growth inhibition (Evans et al. 1994).

Auxin is mostly synthesized in the shoot, whence it is transported to the root. Inhibition of this transport from shoot to root reduces root elongation (Rashotte et al. 2003). In the root, IAA moves acropetally towards the root apex through the central cylinder and basipetally from the root apex towards the base through the epidermal and cortical cells (Ruzicka et al. 2009), controlling root elongation (Rashotte et al. 2000). In *Arabidopsis*, basipetal auxin transport is sufficient to control root elongation, and inhibition of this transport by *N*-1-naphthylphthalamic acid (NPA) reduces root elongation (Casimiro et al. 2001).

Auxin can regulate the elongation of root cells by modifying different aspects of their physiology. Elongation requires both a driving force and a modification of the cell wall elasticity by loosening cross-links between cell wall components. In coleoptiles, auxin has been demonstrated to promote a rapid increase in cell wall elasticity (Cleland 1992; Taiz 1994; Cosgrove 2000). The acid growth hypothesis (Rayle and Cleland 1970) is that hydrogen ions are the agents responsible for cell wall loosening during *Avena* coleoptile growth, and auxin-induced stimulation of growth in coleoptiles is driven by proton extrusion into the apoplastic space. The pH falls to below 5.5, and this acidification enhances cell wall extensibility and increases the activity of enzymes involved in cell growth. In roots, exogenous auxin usually inhibits proton extrusion and hence cell elongation (Evans et al. 1980).

However, as was noted above, root elongation is stimulated by very low auxin concentrations if ethylene biosynthesis has been inhibited (Mulkey et al. 1981). In maize, exogenous NAA at concentrations greater than 0.001 μM inhibit root elongation, and complete inhibition is observed at 1 μM , with the inhibition correlating with the logarithm of the NAA concentration (Alarcón et al. 2012). This inhibitory effect is probably due to the greater sensitivity of roots than of stem tissues such as coleoptiles, in which these concentrations are stimulatory (Thimann 1936).

Similarly, ethylene has long been recognized as a growth inhibitor, but there has recently been reported evidence of its effects in promoting plant growth. In particular, the inhibitory effect of ethylene on root growth was described in 1901 by Neljubov (Dugardeyn and Van Der Straeten 2008), but today it is understood that while high concentrations inhibit root elongation and increase swelling, very low concentrations may promote root elongation (Chang et al. 2004; De Cnodder et al. 2005; Dugardeyn and Van Der Straeten 2008).

ACC reduces cell length in the rapid elongation zone, and ethylene also controls cell elongation (De Cnodder et al. 2005). However, a certain threshold of ethylene is also known to be necessary to maintain root growth in rice (Yin et al. 2011), and other evidence for ethylene's stimulation of growth has been reported (Pierik et al. 2006). The stimulatory effects occur mostly at very low concentrations and are more pronounced in situations of stress. In P-sufficient white clover, low ACC concentrations stimulate root growth, while higher concentrations have no such effect (Dinh et al. 2012). In P-deprived seedlings, however, not only is root elongation enhanced, but ACC at higher concentrations super-stimulates growth, suggestive of an increased ethylene sensitivity in response to the low availability of P (Dinh et al. 2012). *Arabidopsis* root growth is promoted by ethylene under phosphorus stress (Ma et al. 2003). Also, ethylene is required for root development under non-stressful growth conditions. In particular, in *Oryza sativa*, the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) reduces primary root elongation (Yin et al. 2011). In coherence with this result, AVG or cobaltous ions also cause a strong reduction in ethylene levels and inhibited root elongation in maize (Alarcón et al. 2009).

Pierik et al. (2006) propose a biphasic ethylene response model which integrates growth inhibition and stimulation, with low ethylene levels promoting, and high

levels inhibiting, growth. The levels required to stimulate or inhibit growth depend on the species, plant organ, environmental conditions, and endogenous hormone levels. Four types of plant have been distinguished according to their ethylene dose–response relationship. In terrestrial plants, aerial organ growth can be stimulated by ethylene at low concentrations, but ethylene always has an inhibitory effect at high levels. In semi-aquatic plants, ethylene stimulates petiole elongation at high concentrations.

Ethylene is needed for the development of the root cap, the organ which facilitates the penetration of the roots into the growth medium (Zacarias and Reid 1992). In particular, high impedance triggers an increase in ethylene biosynthesis which then serves to facilitate the penetration of the root into the soil (Sarquis et al. 1992). Recently, it has been reported that a coaction between auxin and ethylene is required for root penetration during tomato seed germination (Santisree et al. 2011).

The inhibitory effect of aluminium on root elongation is well known, and it has recently been shown that ethylene mediates this effect in *Arabidopsis* (Sun et al. 2010). The inhibitory effects of aluminium are stronger in the wild type than in ethylene signalling-defective (*etr1-3* and *ein2-1*) and auxin polar transport-defective (*aux1-7* and *pin2*) mutants. Inhibitors of ethylene and auxin polar transport partially prevent the aluminium-induced inhibition. Aluminium and ACC increase the transcripts of *aux-1* and *pin-2*, but this increase is not observed in the presence of ethylene inhibitors. It has therefore been suggested that aluminium-induced ethylene alters the auxin distribution by disrupting AUX1- and PIN2-mediated auxin polar transport, inhibiting root growth elongation (Sun et al. 2010).

Root development is not only regulated by auxin and ethylene. Gibberellins, cytokinins, and other phytohormones play a significant role (Hansen and Grossmann 2000). Also, ethylene biosynthesis is stimulated by other growth regulators: ACC synthase is up-regulated by cytokinin (Rodrigues-Pousada et al. 1999), abscisic acid (Wang et al. 2011), and brassinosteroids (Joo et al. 2006).

5.4.2 Root Transversal Expansion

Generally, the inhibition of root elongation produced by auxin is associated with an increased rate of transverse expansion, altering the predominant direction of root growth from longitudinal to transversal (Burström and Svensson 1974).

This change in growth polarity may be related to changes in the orientation of the cortical microtubules, which pass from an essentially transversal orientation to one which is predominantly longitudinal. Such a relationship is proposed by the alignment hypothesis that there is a causal link between the orientation of cortical microtubules and the orientation of nascent cell wall microfibrils (Baskin 2001), which would in turn regulate cell expansion (Green 1984; Baskin and Williamson 1992). With respect to this hypothesis, it has been demonstrated that treatment with auxin affects the direction of cell expansion and the orientation of the cortical

microtubules in the epidermal cells of maize coleoptiles (Bergfeld et al. 1988). It seems, however, that auxin treatment does not affect the orientation of microtubules in the cells of the root vascular cylinder in the same way (Blancaflor and Hasenstein 1997), indicating that the response to auxin treatment could be tissue dependent.

As with auxin, ethylene also increases the root's radial expansion (Smalle and Van der Straeten 1997; Buer et al. 2003), which change in the growth pattern has also been related to a change in the disposition of the cortical microtubules (Baskin and Williamson 1992).

Treatments of maize roots with the synthetic auxin NAA or with the ethylene precursor ACC inhibit root elongation and increase radial growth, although the responses to the two treatments differ in degree (Alarcón et al. 2012). In particular, NAA enhances radial expansion by nearly 200 %, whereas ACC only increases it by about 40 %, so that NAA seems to be more effective than ACC as a root swelling promoter (Alarcón et al. 2012).

5.4.3 Auxin–Ethylene Interaction

Since auxin specifically stimulates ethylene biosynthesis, it is often unclear whether the effects observed are due to auxin alone, to ethylene alone, or to an interaction between the two. In maize roots, both hormones inhibit elongation and promote swelling in the root tips (Alarcón et al. 2012). Two hypotheses have been proposed to explain auxin's regulation of root growth. One is that auxin's action is mediated by ethylene, as indeed has been demonstrated in the case of light-promoted inhibition of root growth (Eliasson and Bollmark 1988; Jackson 1991). The other is that auxin directly affects root growth, as seems to be the case in *Pisum sativum* for which it has been demonstrated that auxin inhibits elongation without the mediation of ethylene (Eliasson et al. 1989). Recent studies in *Z. mays* and *Oryza sativa* are consistent with this concept of auxin exerting its effect directly (Yin et al. 2011; Alarcón et al. 2012). Nevertheless, the fact that auxin has a direct effect on root elongation and transversal growth does not imply that it cannot interact with other growth regulators (including ethylene). Indeed, our results show that auxin and ethylene can inhibit root elongation and increase radial growth cooperatively. The effect of combined treatments is synergistic when NAA or ACC are applied at very low concentrations and additive at slightly higher concentrations. Cooperation between the two hormones is absent, however, at high concentrations of either (Alarcón et al. unpublished results).

The synergy between auxin and ethylene has been described in processes affecting seedling development (Muday et al. 2012), root hair growth and differentiation (Pitts et al. 1998), root gravitropism (Buer et al. 2006), and root growth (Swarup et al. 2007). Particularly interesting for the present context is the work of Santisree et al. (2011) on the coactions between auxin and ethylene required for roots to penetrate into the soil during tomato seed germination.

Another possible mechanism of interaction could be related to the role of ethylene in the regulation of auxin transport and/or biosynthesis. It is known that ethylene stimulates the biosynthesis of auxin and its basipetal transport to the elongation zone (Ruzicka et al. 2007). This would increase the concentration of auxin in this zone and hence decrease the root elongation rate (Lee et al. 1990).

Molecular studies have demonstrated an interaction between auxin and ethylene at the genetic level. Auxin response factors ARF19 and ARF7 participate in auxin signalling and play a critical role in ethylene responses in the *Arabidopsis* root, indicating that ARFs serve as a point of crosstalk between the two hormones (Li et al. 2006a).

5.4.4 Lateral Roots

The generation of LR has a decisive influence on the root system's capacity to anchor the plant to the soil and to acquire water and nutrients. It is unsurprising therefore that the regulation of the formation of LR is under redundant control mechanisms performed through both endogenous and exogenous agents (Lloret and Casero 2002).

It is generally accepted that, among the endogenous factors that regulate LR formation, the most important is the phytohormone auxin (Casimiro et al. 2003). Many lines of experimental evidence relate auxin to diverse aspects of LR development. In particular, it appears to be involved in LR initiation, in the organization of the apical meristem, and in the emergence and ulterior growth of LR.

Increased production of LR has been demonstrated in most species after treatment with exogenous auxins (Torrey 1962; Blakely et al. 1972, 1982; Webster and Radin 1972; Wightman et al. 1980; Zeadan and MacLeod 1984; Hinchee and Rost 1986; MacIsaac et al. 1989; Lloret and Pulgarín 1992; Baum et al. 1998; Vuylsteker et al. 1998; Zhang and Hasenstein 1999). Nevertheless, the experimental procedures involved in these exogenous auxin treatments have been subject to criticism (Lloret and Casero 2002). The results of more recent studies with mutants and transgenic plants strongly relate LR formation to the biosynthesis or metabolism of auxin (Casimiro et al. 2003). For example, the *sur1* mutants of *A. thaliana* that overproduce auxins also show increased LR formation (Boerjan et al. 1995). Similarly, transgenic plants that overexpress bacterial *iaa* genes, and consequently have high auxin levels, also tend to form many LR (Klee et al. 1987). On the contrary, the *diageotropica* (*dgt*) tomato mutant and the combination of two auxin resistance mutations (*axr4* and *axr1*) both reduce the number of LR (Hobbie and Estelle 1995; Muday et al. 1995). The common feature of these two kinds of mutation is their reduced sensitivity to auxins.

Auxin overproducers and auxin-resistant mutants show striking physiological alterations in addition to changes in LR production. For example, there are specific effects on LR formation in *alf1-1*, *alf3-1*, and *alf4-1* mutants of *A. thaliana* (Celenza et al. 1995). The *alf1-1* mutation promotes the formation of LR, *alf4-1* inhibits

their initiation, and *alf3-1* is defective in their maturation. The *alf1-1* mutants are likely to have a defect in auxin catabolism that favours the accumulation of the hormone in the root, resulting in an increased number of LRs. The *alf4-1* mutant seems to be affected in the perception or the response to auxin and does not produce LRs, and *alf3-1* initiates the LRP but soon aborts its development. The treatment with auxin reverts *alf-3* but not *alf4-1* (Celenza et al. 1995). These findings suggest a model for LR formation in which IAA is required for at least two stages in LR development: (i) to initiate cell division in the pericycle and (ii) to promote cell division and maintain cell viability in the developing LR.

The regulation of the development of LRs is not just a matter of the concentration of auxin but is also related to the transport of this hormone. Auxin transport is a directional process. Applied to the stem, auxin is transported to the root (McDavid et al. 1972), where it moves acropetally through the vascular cylinder (Kerk and Feldman 1995) to accumulate in the LRP and in the apex of the main root (Rowntree and Morris 1979; Sabatini et al. 1999). When the hormone reaches the root apex, its transport is inverse, i.e., basipetally from the apex to the elongation zone (Muday and DeLong 2001). This transport seems to occur through the root epidermis or the external layers of the cortex (Tsurumi and Ohwaki 1978; Yang et al. 1990). The acropetal movement of auxin has been associated with the regulation of LRP growth (Reed et al. 1998), while the basipetal movement would be associated with the initiation of the LRs (Casimiro et al. 2001).

There are essentially three lines of experimental evidence concerning the transport of auxin in regulating the LR formation process. First, elimination of the plant's aerial organs (the source of auxin) reduces the LR frequency, and then application of exogenous auxin to the aerial part of the mutilated plant reverses this effect (Hinchee and Rost 1986). Second, mutants with defects in the auxin transport system (*tir3*) present fewer LRs (Ruegger et al. 1997). And third, NPA, an inhibitor of auxin transport, suppresses the formation of LRs in tomato (Muday and Haworth 1994) and *Arabidopsis* (Reed et al. 1998; Casimiro et al. 1999, 2001). Together, these three lines of evidence clearly reflect a fundamental role for auxin transport in the formation of LRs.

Unlike auxin, ethylene is a negative regulator of LR formation in both *A. thaliana* and *Solanum lycopersicum* (Ivanchenko et al. 2008; Negi et al. 2008, 2010), with enhanced ethylene synthesis resulting in reduced LR initiation independently of whether the enhancement is due to ACC treatment or to *eto1* or *ctr1* mutations. Similarly, Prasad et al. (2010) report that the protein XBAT32 negatively regulates ethylene biosynthesis by modulating the abundance of the enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS), and, as expected, *xbat32* mutants produce an excess of ethylene and have a limited number of LRs.

Since auxins promote ethylene biosynthesis, it is difficult to evaluate any possible direct influence of ethylene on LR formation. The enzyme ACS is involved in the ethylene biosynthesis pathway. It is well known that, during the initiation of LRs, ACS activity increases in response to auxin (Rodrigues-Pousada et al. 1993). This is a perfect example of how these two hormones work together in regulating a biological process.

Another form of interaction between auxin and ethylene could be by the effect of ethylene on the regulation of auxin transport. It has been suggested that the increase in the concentration of ethylene in the root affects the distribution of proteins involved in auxin transport, causing two effects: accumulation of auxin at the root apex and depletion in the zone where LR_s are initiating (Muday et al. 2012).

In contrast to LR initiation, the development of LR_s is stimulated by treatments that raise ethylene production in the root (Ivanchenko et al. 2008). In the maturation and abscission processes, ethylene increases the hydrolytic activity of the enzymes that degrade the cell wall (Roberts et al. 2000). It has therefore been proposed that ethylene also favours the degradation of the cortical cells located just in front of the LRP during its development. Obviously, this would facilitate the emergence of the LRP (Bonfante and Peretto 1993).

LR development is regulated antagonistically by the plant hormones auxin and cytokinin. Indeed, cytokinins are considered to be good inhibitors of the formation of LR_s because their exogenous application frequently reduces this formation (MacIsaac et al. 1989). However, very low concentrations of these hormones can stimulate auxin-promoted LR formation (Torrey 1962). Furthermore, the application of cytokinins not only inhibits the initiation of LR_s but also their emergence (Van Staden and Ntingane 1996). The root apex is the main site in the plant at which cytokinins are synthesized (Van Staden and Davey 1979). From there, they are transported by the xylem to the rest of the root, inhibiting elongation and LR formation (MacIsaac et al. 1989).

Cytokinins inhibit LR formation both in wild *A. thaliana* plants and in mutants defective in auxin transport or response mechanisms (Li et al. 2006b). The application of exogenous auxin to these mutants does not reverse this inhibition. It is therefore posited that auxin and cytokinin act in this process via different signalling pathways, with the cytokinins exerting their inhibitory effect by blocking pericycle founder cells from progress in the cell cycle from G₂ to mitosis (Li et al. 2006b). This does not mean, however, that there is no crosstalk between auxin and cytokinin in regulating the formation of LR_s. Xylem-pole pericycle cells have been described as being sensitive to cytokinins, whereas young LRP are not (Laplaze et al. 2007). It appears that cytokinins perturb the expression of PIN genes in LR founder cells and inhibit the accumulation of auxin in them, which in turn promotes LR initiation and regulates cell spacing (Laplaze et al. 2007).

ABA is also considered to be an LR inhibitor. Recent studies by Guo et al. (2009) in *Arachis hypogaea* report that exogenous ABA treatments decrease root branching in a dose-dependent manner. In *Arabidopsis*, mutation in the Abscisic Acid Insensitive 4 (ABI4) transcription factor promotes an increase in the number of LR_s. It has been demonstrated that the expression of ABI4 is enhanced by ABA and cytokinin but repressed by auxin. The production of LR_s in *abi4* mutants is not affected by cytokinin or ABA (Shkolnik-Inbar and Bar-Zvi 2010). As the expression of the auxin efflux carrier protein PIN1 is reduced in ABI4 over expressors and enhanced in *abi4* mutants, it has been suggested that ABA and cytokinin counteract the effect of auxin in the regulation of LR formation by reducing polar auxin transport (Shkolnik-Inbar and Bar-Zvi 2010).

Gibberellins are reported to interact with auxin in the regulation of LR development in *Populus*. GA-deficient and GA-insensitive transgenic plants show increased LR proliferation and elongation which is reversed by exogenous GA. Microarray analyses suggest a crosstalk of GA with auxin and that GA modulates LR development by modifying polar auxin transport (Gou et al. 2010).

In sunflowers, the lipid-derived hormone jasmonic acid and its derivatives (collectively named jasmonates), applied exogenously, inhibit primary root elongation and reduce LR growth and number. Treatment with ibuprofen, an inhibitor of jasmonate synthesis, enhances primary root and LR lengths, but auxin elicits its typical response even in the presence of ibuprofen. Jasmonates, therefore, may induce primary root and LR growth inhibition via an auxin-independent pathway (Corti-Monzón et al. 2012).

In contrast, recent studies on *A. thaliana* indicate that jasmonates are promoters of both the initiation and emergence of LRs (Raya-González et al. 2012). In addition, regulation of the development of the root system seems to operate through mechanisms of two types, some dependent on auxin and others independent of this hormone. These results indicate that it is still unclear how jasmonates contribute to many aspects of root development and that there may be differences between species.

In sum, while auxins seem to constitute the main regulator of LR formation, other growth regulators appear to collaborate with it in fine-tuning the process and accommodating it to environmental influences.

5.4.5 Root Hairs

Auxin and ethylene are required for the normal development of root hairs (Pitts et al. 1998; Rahman et al. 2002). Ethylene increases their growth in various species including pea, fava bean, and lupin (Abeles et al. 1992). Analysis of *Arabidopsis* mutants has shown that root hair elongation in plants with enhanced ethylene production (*eto1-1*) was greater than in wild-type plants, and the elongation was negatively affected in the ethylene-insensitive mutant *etr1* (Pitts et al. 1998).

The constitutive ethylene mutant of *Arabidopsis ctr1* has more root hairs than the wild-type phenotype, and the application of ethylene to the wild type produces root hairs with a morphology similar to that of the mutant. In addition, application of an inhibitor of ethylene biosynthesis (AVG) or action (silver thiosulfate, STS) to the wild-type phenotype reduces the production of root hairs (Tanimoto et al. 1995).

The root hair in *Arabidopsis* mutants insensitive to ethylene (*etr1*) or auxin (*aux1*) exhibits normal morphology, whereas double mutants show reductions in root hair formation (Smalle and Van der Straeten 1997). These reductions can be reverted by the application of IAA but not ACC, suggesting that ethylene may act via more than one route (Masucci et al. 1996).

5.5 Conclusions

The organs of higher plants are complex multicellular organizations which grow by means of coordinated activity from their constituent cell types. It has long been recognized that hormones play a crucial role in this coordination. The root is no exception in this respect. It has only been recently that researchers have begun to understand how different growth regulators interact to properly regulate the development of the root system and adapt it to changing environmental conditions. It is now clear that the root cells exchange signals and that the signalling network they form is far more intricate than was previously assumed. Over the past few decades, particular attention has been paid to analysing how individual tissues may contribute to the regulation of overall root growth, and there is a need to continue on this line of inquiry in even greater depth.

In genetic studies of higher plants, the material of choice has been *A. thaliana*. While such a concentration of research effort on this plant model has yielded impressive results, it may now be advisable to expand our vision somewhat to look at other species. In this sense, it would be very interesting to compare the situation in monocots and dicots. Even if it were only for being one of humanity's largest volume crops, the maize plant merits particular attention in the immediate future.

The maize primary root is a cylindrical structure formed by consecutive zones: (a) the root apex contains the apical meristem, where cell divisions occur; (b) the elongation zone in which cells stop dividing and start to elongate; and (c) the maturation zone, where cells reach their definitive lengths, cell differentiation begins, and lateral roots initiate. In the root, three main tissue systems can be distinguished: the epidermis, the cortex, and the vascular cylinder. The first layer of the vascular cylinder is the pericycle. Cell cycle activation in pericycle cells is clearly connected with lateral root initiation.

Root grows basically by the elongation of its cells and branches through proliferation of pericycle founder cells. Auxin is the main hormone in regulating these both processes. Exogenous auxin inhibits root growth, increases transversal expansion, and enhances lateral root formation. As auxin also enhances ethylene production, it is difficult to know whether certain auxin effects are mediated by ethylene or not. Based on own results and on the specialized literature, we discussed on regulation by auxin and ethylene of the development of the maize root system. The emerging model is that auxin and ethylene regulate root elongation depending on concentration and that both regulators interact to regulate root growth. The role of auxin in regulating lateral root formation is clearly established. However, ethylene does not seem to have such a direct role in this process.

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