Advances in experimental methods for root system architecture and root development

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Abstract Plant roots play important roles in acquisition of water and nutrients, storage, anchoring, transport, and symbiosis with soil microorganisms, thus quantitative researches on root developmental processes are essential to understand root functions and root turnover in ecosystems, and at the same time such researches are the most difficult because roots are hidden underground. Therefore, how to investigate efficiently root functions and root dynamics is the core aspect in underground ecology. In this article, we reviewed some experimental methods used in root researches on root development and root system architecture, and summarized the advantages and shortages of these methods. Based on the analyses, we proposed three new ways to more understand root processes: (1) new experimental materials for root development; (2) a new observatory system comprised of multiple components, including many observatory windows installed in field, analysis software,

and automatic data transport devices; (3) new techniques used to analyze quantitatively functional roots.

Keywords Root system analysis · Fractal geometry · Novel materials

Introduction

There are great differences in development between above- and below-ground parts of plants. The development of above-ground plant parts is less affected by environmental factors, thus branching patterns of above-ground parts are similar among individuals of the same species. In contrast, the development of root systems is greatly affected by the soil environmental factors, such as soil nutrients (especially the availability of nitrogen and phosphate) (Zhang and Forde 1998; Williamson et al. 2001; Linkohr et al. 2002; Wu et al. 2005; Hill et al. 2006; Jain et al. 2007; Vidal et al. 2010; Peret et al. 2011; Gan et al. 2012; Gruber et al. 2013; White et al. 2013), soil water (Berntson and Woodward 1992; Manschadi et al. 2006, 2008; Henry et al. 2011; Bengough et al. 2011), soil temperature (Mackay and Barber 1984; Kaspar and Bland 1992; Clausnitzer and Hopmans 1994), soil pH (Walter et al. 2000), soil compaction (Bushamuka and Zobel 1998; Busscher et al. 2000; Tracy et al. 2010a), and slope gradients (Di Iorio et al. 2005; Pierret et al. 2007; Sun et al. 2008), thus the morphology and physiology of roots show great plasticity. Because of the relationships between above- and below-ground parts of plants, it is necessary to maximize root/rhizosphere efficiency to improve plant productivity and nutrient efficiency (Shen et al. 2013). Root traits can be used to create phosphate efficient crop varieties in response to limited global phosphate resources (Gahoonia and

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Nielsen 2004). Therefore, root development, root system architecture, and root dynamics have been the focuses in botany and ecology. An obstacle to the study of root system architecture is to measure and quantify the three-dimensional configuration of root systems in soil. At the same, root dynamics is not monitored efficiently because of hidden roots in dark medium. Researchers have attempted to overcome the two obstacles in order to understand root functions and root responses to soil environmental factors. In this paper, we review some advantages and shortages in experimental methods for study of root development, root system architecture, and root dynamics, and propose some new ways to enhance root research.

The theoretic bases for analysis of root system

Root system architecture, as defined by Lynch (1995), refers to the spatial configuration of the root system, i.e. the explicit geometric deployment of the root axes. The root system architecture of plant seedlings is relatively simple but adult plants, especially adult trees, such as *Sequoia sempervirens*, have complex root system architecture (Nadezhkina and Čermák 2003; Di Iorio et al. 2005) that is difficult to study.

Although plant root systems show plasticity and complexity, within a given species root systems are similar because of shared genetic characteristics. The similarity provides the basis for their analysis.

Mandelbrot (1975) coined the term “fractal”. A fractal is generally a rough or fragmented geometric shape that can be split into parts, each of which is (at least approximately) a reduced-size copy of the whole shape, i.e. self-similarity. A fractal often has traits including: (1) self-similarity; (2) fine or detailed structure at arbitrarily small scales; (3) simple and recursive definition; (4) irregularity which is difficult to describe by Euclidean geometry; (5) Hausdorff dimension which is greater than its topological dimension. Based these traits of fractals, smaller parts of roots are fractal representations of larger parts of roots, thus fractal analysis is an appropriate tool for study of root systems. Therefore, fractal analysis was used to describe root systems of many plant species (Table 1). Fractal analysis has also been used to compare the responses of root system architecture to nutrient deficiency (Eghball et al. 1993; Nielsen et al. 1999), drought stress (Wang et al. 2009), and efficiency of soil exploration by roots (Walk et al. 2004).

These applications of fractal analysis are deficient because they were only involved in two-dimension structures of root systems, and spatial configurations of root systems are not taken into consideration. In natural ecosystems, most soil nutrients are in the soil layer of 10–20 cm deep, thus root spatial configurations are related to nutrition uptake.

Therefore, some parameters involved in description for root spatial configuration, such as the angles between primary root and the first lateral roots and the angles between root links in a root system, should be analyzed in fractal analysis. Data obtained from a complex root system are great, thus new software will be developed for analyzing them.

Root topology is another term used for delineation of root systems. As defined by Lynch (1995), the term refers to how individual root axes are connected to each other through branching. Unlike branching of above-ground parts, root branching shows great spatiotemporal changes, for example, first-order fine roots can be connected with second-order and the third-order roots (Pregitzer et al. 2002), and even with fourth-order roots (Wang et al. 2006), leading to the complexity of root branching. Regardless of its complexity, root branching links can be classified into three types, i.e. exterior–exterior (EE), exterior–interior (EI), and interior–interior (II) (Fig. 1, Fitter and Stickland 1991).

Three topological parameters can be calculated to describe the relationships between links:

Magnitude (α): number of exterior links. The total number of root tips categorized as EI and EE (Fig. 1a) or total numbers of root tips categorized a EE (Fig. 1b).

Altitude (μ): number of links in the longest unique path from the base link to an exterior link. In Fig. 1a, from an exterior link labeled EE to the base link, the number of root links is 10, i.e. $\mu = 10$. In Fig. 1b, from any exterior link labeled EE to the base link, the number of root links is 4, i.e. $\mu = 4$.

Total exterior pathlength (p_e): sum of numbers of links in all possible unique paths from the base link to all exterior links. In Fig. 1a, $p_e = 64$; in Fig. 1b, $p_e = 32$.

Two topological indices can be used

Altitude-slope: the slope of the regression of $\log_{10}\alpha$ on $\log_{10}\mu$ for a given root system. In the plot with $\log_{10}\mu$ as the x axis and $\log_{10}\alpha$ as the y axis, the slope of the regression is the altitude-slope for a set of root system.

Pathlength-slope: the slope of the regression of $\log_{10}p_e$ on $\log_{10}\mu$ for a given root system. In the plot with $\log_{10}\mu$ as the x axis and $\log_{10}p_e$ as the y axis, the slope of the regression is the pathlength-slope for a given root system.

In addition, some parameters are used to describe the morphology of root systems, such as root hair density, root diameter, the occurrence pattern of lateral roots, the distance from the tip to the lattermost lateral root. Anatomical traits such as the sizes of epidermal cells in the elongation zone, the cell density of the meristem zone, and the size of meristem zone are not usually involved in the description of root systems.

Similar to fractal analysis, root topology does not take root spatial configuration into consideration.

Table 1 The plant species which were analyzed using fractal analysis

Plant species	References
<i>Phaseolus vulgaris</i>	Nielsen et al. (1997, 1999); Walk et al. (2004)
<i>Betula populifolia</i>	Berntson (1994)
<i>Betula alleghaniensis</i>	Berntson (1994)
<i>Zea mays</i>	Costa et al. (2003); Tatsumi et al. (1989); Eghball et al. (1993)
<i>Arachis hypogaea</i>	Tatsumi et al. (1989)
<i>Panicum miliaceum</i>	Tatsumi et al. (1989)
<i>Pisum sativum</i>	Tatsumi et al. (1989)
<i>Secale cereale</i>	Tatsumi et al. (1989)
<i>Sorghum bicolor</i>	Tatsumi et al. (1989)
<i>Triticum aestivum</i>	Tatsumi et al. (1989)
<i>Trifolium pratense</i>	Fitter and Stickland (1992)
<i>Trifolium repens</i>	Fitter and Stickland (1992)
<i>Plantago lanceolata</i>	Fitter and Stickland (1992)
<i>Festuca voina</i>	Fitter and Stickland (1992)
<i>Festuca nigrescens</i>	Fitter and Stickland (1992)
<i>Oryza sativa</i>	Wang et al. (2009)
<i>Strychnos cocculoides</i>	Oppelt et al. (2000)
<i>Strychnos spinosa</i>	Oppelt et al. (2000)
<i>Vangueria infausta</i>	Oppelt et al. (2000)
<i>Grewia flava</i>	Oppelt et al. (2000)
<i>Reaumuria soongorica</i>	Shan et al. (2013)
<i>Nitraria tangutorum</i>	Shan et al. (2013)

Fig. 1 Diagram showing the distinction between extreme branching patterns (a: herringbone; b: dichotomous). Exterior–exterior (EE); exterior–interior (EI); interior–interior (II) [e.g. a link between the two dashed lines in (b) cited from (Fitter and Stickland 1991)]

Experimental methods for measurement of root systems

The complexity of root system architecture raises obstacles to data acquisition that are addressed by advances in technology. Digital images help to quickly acquire data about root system architecture. Meanwhile, related software is helpful for processing these data.

Root system analysis technologies based on scanners were developed, including WinRhizo and RootSnap. Specific root analysis software was also developed, including Multi-ADAPT (Ishikawa and Evans 1997), AMAPmod (Danjon et al. 1999a, b, 2008), Delta-T Scan and WinRHIZO (Boumma et al. 2000), REGR (Walter et al. 2002), RootFlow (van der Weele et al. 2003, KineRoot (Basu et al. 2007), SmartRoot (International Atomic Energy Agency 2006; Draye 2008), RootLM (Qi et al. 2007), Phytomorph (Miller et al. 2007; Spalding, 2009), RootNav (Pound et al. 2013), RootTrace (French et al. 2009), and Growth Tracer (Iwamoto et al. 2013). RootFlow and REGR made use of optic flow-based techniques (Barron et al. 1994; Barron and Liptay 1994). All of these applications have their strengths and weaknesses. The combination of scanner and software was used to measure some root parameters using three-dimension techniques that provided more configuration information about root system architecture (Danjon and Reubens 2008). For entire root systems, some morphological traits can be analyzed, including total root length, root average diameter, total root surface, project surface, root tips, root volume. Some parameters related to root links can be measured, such as total number of links in a root system, their average length and diameter, volume, and branching angles. Topological parameters can also be measured, such as altitude, magnitude, and exterior path length.

Some root analysis systems have their shortcomings, one of which is the fact that roots must be excavated and washed from soil cores before they can be scanned (Gregory 2006; Smit et al. 2000). Excavation and washing must break some parts of the root system, leading to the underestimation of fine roots through breakage and loss of information on the spatial distribution and root system architecture. More seriously, data obtained later are lack of the information about root spatial configuration.

More advanced devices were developed. More than ten years ago, X-ray computed tomography (CT) was first used to analyze root systems (Heeraman et al. 1997; Pierret et al. 1999). Because the scanning process of X-ray CT is non-destructive, the technique can track root growth and changes in root system architecture. Based on these advantages, the technique has been developed and widely used (Gregory et al. 2003; Lontoc-Roy et al. 2005; Perret et al. 2007; Tracy et al. 2010a, b; 2012a, b; Mooney et al. 2012; Mairhofer et al. 2012, 2013).

X-ray CT can visualize the internal structure of opaque objects. X-ray CT scanners acquire a series of projections from different facets of a vessel that contains plants, measuring the attenuation of ionizing radiation passing through the vessel. These projections are combined to reconstruct a three-dimensional data set. Data values recorded at each voxel reflect the density of the imaged material and are usually mapped to greyscale intensity values for visualization purposes (Mooney 2002). But variations in the x-ray attenuation values of root material and the overlap in attenuation values between roots and soil caused by water and organic materials (especially non-decomposed litter) represent major challenges to data recovery. Software (such as RooTrack) has been developed to separate root systems from their soil environment in images obtained by X-ray CT and recover root system architecture traits (Mairhofer et al. 2012, 2013).

In addition, as a complementary tool to other predictive techniques, gel growth systems with superior optical clarity have been introduced to facilitate noninvasive two-dimensional (2D; Iyer-Pascuzzi et al. 2010) and three-dimensional (3D; Fang et al. 2009; Clark et al. 2010) imaging. These methods are only used for root systems of seedlings, thus its applications are limited.

At present, there are not available techniques used in field for in situ dynamic observation. Ground penetrating radar (GPR) should be useful to investigate root systems of forest trees on forest ecosystem level (Nadezhkina and Čermák 2003; Butnor et al. 2003; Barton and Montagu 2004; al Hagrey 2007). GPRs have not only the lowest wave length and resolution to detect small targets but also the highest wave attenuation that limits resolution and penetration in wet conducting media, thus they have been used to estimate single root segments or biomass with a

relatively low precision (Butnor et al. 2003; Barton and Montagu 2004; al Hagrey 2007). But, at present, the accuracy and resolution of GPRs are not enough for fine roots (root diameter <1 mm), and more advanced GPRs are needed. Software applications have also been developed to analyze information collected by GPR, providing detailed investigation on root systems at the ecosystem level.

Based on the analyses mentioned above, two new techniques will be developed: one is the combination of continuous three-dimensional imaging and fractal analysis/topology used in laboratories; another is in situ continuous multiple-site observatory root systems with automatic data transport used in ecosystem level.

Novel plant materials used in root development

Root development includes three main aspects, i.e. elongation of roots, branching (including proteoid roots), and differentiation of root epidermal cells (i.e. root hair formation, including dauciform roots; Playsted et al. 2006; Shane et al. 2006). Lateral root formation is the base of development of the whole root system in which root elongation enlarges the volume of soil explored by roots, and root hairs greatly increase the absorptive surface area for water and nutrients in soil. Of the three aspects, lateral root formation seems more important, thus investigation on lateral root formation has been the focus of the root development research. While various types of advanced microscopes are used in studies of root development, new plant materials are also used. Many mutants related to specific genes that affect root development and many transgenic *Arabidopsis* lines with GFP or GUS in root tissues showed novel traits, i.e. specific occurrence in a tissue in roots. Thus these transgenic plants are often used in studies of root development. Lateral root formation starts with a series of anticlinal and periclinal divisions of pericycle cells, a layer of root tissue surrounding the vasculature (Malamy and Benfey 1997; Kurup et al. 2005). Thus, detection of the activity of pericycle cells near the xylem poles is important for lateral root formation. Quiescent centers (QC) in root tips maintain the activities and sizes of the meristems in roots, so detection of QC is also important.

In the MRC Laboratory of Molecular Biology, led by Jim Haseloff (<http://data.plantsci.cam.ac.uk/Haseloff/>), GAL4-GFP lines were produced by Agrobacterium-mediated transformation of the C24 *Arabidopsis* line using the binary vector pBIN m-gfp5-ER. The *m-gfp5-ER* gene is GAL4 responsive and is linked to a GAL4 gene, a foreign transcription activator derived from the yeast GAL4 gene in the GAL4-GFP lines. Because of the traits of GFP, the patterns of GAL4 expression in the GAL4-GFP lines can be immediately and directly detected. Some lines in the GAL4-

GFP set show specific patterns of GAL4 expression in roots, thus they are widely used in studies on root development.

Of all the GAL4-GFP lines, J0121 seems to be the most useful for studies on root development. In J0121, GAL4::GFP expression is specific to 2–3 root pericycle cell files adjacent to the xylem poles (Fig. 2; Laplace et al. 2005), thus the line can be used for detection of the first cell division of cells at the initial stage of lateral root formation. In J0192, GAL4::GFP is expressed in young lateral root primordia (Fig. 2; Laplace et al. 2005), thus it is very useful for detecting the development of young lateral root primordial. After these two lines were produced, they have often been used in studies of root systems (Table 2).

Most recently, a GAL4-based targeted activation tagging system in *Arabidopsis* has been established (Waki et al. 2013). Host plants that expressed a synthetic transcription activator GAL4:VP16 (GV) in an organ- or tissue-specific manner were transformed with a T-DNA harboring tandem copies of UAS, a GAL4-binding sequence. Thus the activation tagging system is a powerful tool for discovering novel genes that affect root development.

QC maintains the activity of root meristem and specific lines were produced to observe QC status (Table 2). GFP only occurs in QC25, thus QC25 is used in root development, especially used in monitoring the activity of QC and root meristem of plants treated with phytohormones and environmental stresses.

In other GAL4-GFP lines, GFP specifically occurs in columella (Q1630), root epidermis (J2301, J0631, and J2921), lateral root cap (J0951), and stele (J2501) (Table 2).

Lateral roots originate from the pericycle cells opposite to xylem poles. These pericycle cells are distant from the root meristem zone and stop division. Therefore, the first step of lateral root formation is the recovery of the cell cycle of some pericycle cells. CYCB1;1 is a mitotic cyclin in *Arabidopsis*, and is expressed only around the G2/M transition in the cell cycle (Doerner et al. 1996; Shaul et al. 1996), therefore, *CycB1;1::uidA* reporter construct (Fig. 3) is often used as the marker to detect the process of the cell cycle during lateral root formation (Table 2; Casimiro et al. 2001; Aida et al. 2004; Hutchison et al. 2006; Ioio et al. 2007).

Fig. 2 GFP expression in the enhancer trap lines J0121 (a–c) and J0192 (d–g). (a, b, d, e, f, g) Longitudinal confocal sections of roots and (c) projected confocal view of the top part of a hypocotyl of living plants showing GFP fluorescence (green) and counterstained with propidium iodide (red). a GFP expression starts in the pericycle cells of the elongation zone. No GFP fluorescence is visible in the root

meristem. b Expression in pericycle cells adjacent to a xylem strand. c Expression in the hypocotyl epidermis. d Expression in a stage I primordium. e Expression in a stage II primordium. f Expression in a stage III primordium. g Expression in a stage V primordium. Cited from Laplace et al. 2005

Table 2 Experimental materials often used in studies on root development

Material names	Expression patterns	Application
J0121	Xylem pole pericycle	Dubrovsky et al. (2006), Casimiro et al. (2001), Ohashi-Ito and Bergmann (2007), Laplace et al. (2007), Sugimoto et al. (2010)
J0192	Young lateral root primordia	Sabatini et al. (2003), Laplace et al. (2007)
J0571	Cortex	Sabatini et al. (1999), Casimiro et al. (2001), Sabatini et al. (2003), Ioio et al. (2007)
J2301	Root epidermis	Sabatini et al. (1999)
J2341	QC and columella initials	Sabatini et al. (2003), Willemsen et al. (2003), Ding and Friml (2010)
J1892	Columella	Ioio et al. (2007)
J0631	Epidermis	Ioio et al. (2007)
J2921	Epidermis	Ioio et al. (2007)
J3411	Root cap	Birnbaum et al. (2003), Petersson et al. (2009)
J0951	Lateral root cap	Swarup et al. (2005), Petersson et al. (2009)
J2812	Epidermis and cortex	Petersson et al. (2009)
J2501	Stele	Petersson et al. (2009)
Q1630	Columella	Sabatini et al. (1999, 2003); Willemsen et al. (2003), Ohashi-Ito and Bergmann (2007), Ioio et al. (2007), Ding and Friml (2010)
QC25	QC	Sabatini et al. (2003), Ohashi-Ito and Bergmann (2007)
QC46	Endodermis	Sabatini et al. (1999, 2003); Willemsen et al. (2003)
QC148	QC	Sabatini et al. (2003)
Q0990	QC and stele initials	Petersson et al. (2009)
M0028	Root cap, columella, initials, QC	Swarup et al. (2005), Petersson et al. (2009)
DR5::GFP/GUS	QC, columella	Ottenschläger et al. (2003), Willemsen et al. (2003), Laplace et al. (2007), Ohashi-Ito et al. (2007), Petersson et al. (2009)
Pscr::GFP/GUS	Endodermis	Birnbaum et al. (2003), Sabatini et al. (2003), Petersson et al. (2009), Sugimoto et al. (2010)
CYCB1;1::GFP/GUS	Xylem pole pericycle	Casimiro et al. (2001), Aida et al. (2004), Hutchison et al. (2006), Ioio et al. (2007)

Root development is affected by environmental factors, thus lateral root initiation is a probabilistic event, but its frequency is set by fluctuating levels of auxin response (Laskowski 2013). In *Arabidopsis*, the process of lateral root formation consists of two major stages: cell cycle reactivation in the xylem pericycle and establishment of a new meristem (Himanen et al. 2002). Pericycle reactivation depends on local concentration of auxin in the roots, whereas the outgrowth of lateral roots is regulated by shoot-derived auxin (Bhalerao et al. 2002). Therefore, detection of local concentration of auxin in roots is very important for lateral root formation and root system development.

There are some auxin-responsive elements (AuxRE) in the promoters of auxin-responsive genes, such as *IAA5* and *IAA19* genes (Nakamura et al. 2003), and AuxRE contains the TGTCTC sequence. ARF proteins can bind to AuxRE after Aux/IAA proteins are degraded and ARF proteins are released from Aux/IAA proteins, promoting auxin responses. A highly active synthetic AuxRE, referred to as DR5, was created by performing site-directed mutations in a natural composite AuxRE found in the soybean GH3

Fig. 3 Schematic of the *CycB1;1::uidA* reporter construct (pCDG). The upper panel highlights functional motifs of cyclin *CycB1;1*. The lower panel shows the 1.8 kb genomic fragment, including approximately 1.2 kb of promoter sequence and the first three exons of *CycB1;1* coding sequence up to amino acid 116, translationally fused to the *E. coli uidA* gene. Thick lines represent promoter sequences or introns, boxes represent exons. Cited from Colón-Carmona et al. 1999

promoter (Ulmasov et al. 1997). Because DR5 showed greater auxin responsiveness than a natural composite AuxRE (as shown in Fig. 4), it became a useful reporter gene (acting as DR5::GFP or DR5::GUS) for studying auxin responses in wild-type plants and various mutants (Sabatini et al. 1999; Friml et al. 2002a, b; Ottenschläger

Fig. 4 Histochemical staining for GUS activity in *Arabidopsis* seedlings transformed with DR5($\times 7$)-GUS. Seedlings were treated for 24 h with 50 μ M 1-NAA (right) or H₂O (left) and then stained for GUS activity for ~8 h (top) or 16 h (bottom). Cited from Ulmasov et al. (1997)

et al. 2003; Willemsen et al. 2003; Laplace et al. 2007; Ohashi-Ito and Bergmann, 2007; Petersson et al. 2009).

SCARECROW (SCR) gene encodes a putative transcription factor (Di Laurenzio et al. 1996) that is first expressed in QC precursor cells during embryogenesis, after which it extends to the initial cells for the ground tissue (cortex and endodermis) (Wysocka-Diller et al. 2000). Because *SCR* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* roots (Di Laurenzio et al. 1996) and is involved in positioning the stem cell niche in the *Arabidopsis* root meristem (Sabatini et al. 2003), its fusion constructs with GFP or GUS are widely used to study root development (Table 2).

All the novel plant experimental materials provide us with information about root developmental processes, especially initiation of lateral roots and QC activity. Since root brain hypothesis have been accepted gradually (Baluška et al. 2009, 2010), some of the plant experimental materials will be useful for investigation on the functions of root tips. In view of complexity of root brain, new plant experimental materials will be developed, especially those involved in neurotransmitters.

Additionally, since root development is affected by other phytohormones, such as ethylene (Rahman et al. 2001; Stepanova et al. 2007), cytokinin (Aloni et al. 2006;

Laplace et al. 2007), and nitric oxide (Correa-Aragunde et al. 2004, 2006; Stöhr and Stremlau 2006), novel transgenic plants, like the above-mentioned transgenic lines with DR::GFP and DR5::GUS, are needed for investigation on the effects of ethylene, cytokinin and nitric oxide on root development, especially their effects on lateral root formation. New techniques and experimental materials are needed to promote study of root development.

Concluding remarks

In view of multiple functions of roots, we have to face crypticity and high plasticity of root systems. Development of new techniques for data acquisition and analysis and experimental materials will help us overcome difficulties and understand more details about root functions and root dynamics.

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