Signaling and Communication in Plants

Soumya Mukherjee
František Baluška *Editors*

Rhizobiology: Molecular Physiology of Plant Roots

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František Baluška, IZMB, Department of Plant Cell Biology, University of Bonn, Bonn, Nordrhein-Westfalen, Germany

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Soumya Mukherjee · František Baluška
Editors

Rhizobiology: Molecular Physiology of Plant Roots

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Editors
Soumya Mukherjee
Department of Botany
University of Kalyani, Jangipur College
West Bengal, India

František Baluška
Institute of Cellular and Molecular Botany
University of Bonn
Bonn, Nordrhein-Westfalen, Germany

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Preface

Roots are the primary organs which support plant adaptive responses to various environmental conditions via cognitive processes and behavioral adaptations. Rhizobiology is a dynamic subdiscipline of plant science which collates investigations from various aspects of plant physiology, biochemistry, genetic analysis and plant-microbe interactions. In this context, it is worth mentioning that the physiology and molecular mechanisms of root development have undergone significant advancements in the last couple of decades. Apart from the already known conventional phytohormones (IAA, GA, cytokinin, ethylene and ABA), certain novel biomolecules have been considered as potential growth regulators for plant growth and development. Nitric oxide, carbon monoxide and hydrogen sulfide have emerged as three potential gaseous signaling molecules in plants. Serotonin, melatonin, dopamine, GABA and acetylcholine are some of the important neurotransmitters capable of modulating root development and signaling. Jasmonates, brassinosteroids, polyamines and strigolactones have also been reported to possess a plethora of effects in regulating the physiology of root growth. Root phenotyping and plasticity analysis with respect to the specific functional mutants of each biomolecule shall provide substantial information on the molecular pathways of root signaling. The present volume shall discuss the recent advancements in the role of various biomolecules in regulating root architecture, growth and development. In this context, special emphasis shall provide insights into the sensing, tolerance and modulatory mechanisms of root physiology in response to environmental stresses. Thus, the collation of recent developments shall summarize our current understandings of the molecular mechanisms of root physiology. Future investigations on the characterization of specific receptors for new biomolecules and deciphering complex signaling pathways and crosstalk mechanisms shall remain as a priority.

West Bengal, India — Soumya Mukherjee
Bonn, Germany — František Baluška

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Root Apex Cognition: From Neuronal Molecules to Root-Fungal Networks

František Baluška, Felipe Yamashita, and Stefano Mancuso

What we see is the blossom, which passes. The rhizome remains.
Jung (1963)

Abstract Plant roots are generally hidden from our sight, growing and living underground in alliances with symbiotic fungi. In order to find enough water and critical mineral nutrients, they explore large areas of soil with their root apices acting as plant cognition-based brain-like organs allowing them to use kin recognition, self/non-self recognition as well as swarm intelligence. Importantly, fungal hyphae integrate root systems into huge root-wide webs which allow not only the sharing of water and mineral nutrients, but also support long-distance chemical and electric signals. Roots use neuronal molecules such as glutamate and GABA supported by their specific receptors, as well as actin-based synapses and the plant-specific action potentials, to perform all their social activities and cognitive navigation for soil exploration.

1 Introduction

Plants conquered land in a tight co-evolution with symbiotic fungi, especially with the soil-borne members of the phylum Glomeromycota: arbuscular mycorrhiza (AM) fungi which teamed up with plant roots some 400 million years ago (Selosse and

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F. Baluška (✉) · F. Yamashita
IZMB, University of Bonn, Kirschallee 1, 53115 Bonn, Germany
e-mail: baluska@uni-bonn.de

S. Mancuso
Department of Agrifood Production and Environmental Sciences, University of Florence, Florence, Italy

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Le Tacon 1998; Redecker 2000; Selosse et al. 2015; Remy et al. 1994; Field et al. 2015; Hoysted et al. 2018). These so-called endomycorrhizal fungi were followed in evolutionary history by ectomycorrhizal (ECM) fungi, which grow as saprotrophs in soil and enter into mutualistic symbiosis with many trees by enveloping their root tips with mycelial mantles (Bonfante and Genre 2010; Genre et al. 2020). Whereas hyphae of the AM fungi enter root cells and form intracellular arbuscules, hyphae of the ECM fungi remain outside of root apex cells, forming Hartig nets and mantles surrounding the root apices (see Fig. 1 in Bonfante and Genre 2010; Genre et al. 2020). A unique feature of AM fungi is that the hyphae of their extraradical mycelium typically interconnect several root apices not only of the same plant, but also different plants of different species, forming 'common mycorrhizal networks' also known as the 'wood-wide-web' (Simard et al. 1997; Read 1997; Giovannetti et al. 2006; Beiler et al. 2010; Rog et al. 2020; Gorzelak et al. 2020). Besides plants specialized for either AM or ECM symbiosis, there are also so-called dual-symbiosis plants capable of associating their root apices with both the AM and ECM fungi (Brundrett and Tedersoo 2018; Teste et al. 2020).

2 Root Apex Transition Zone: Oscillatory Brain-Like Cognitive Organ in Soil Exploration

Evolution of roots in land plants was accomplished via root-fungal co-evolution when the first ancient plants succeeded in overcoming the difficult transition from sea to land (Taylor et al. 1995; Redecker 2000). This is obvious not only from paleontological records but also from the root-fungal symbiosis found in the earliest plant lineages of evolutionary ancient plants including Lycophytes, Liverworts and Hornworts (Rimington et al. 2020). Although it is generally accepted that the roots of vascular plants evolved later than their shoots (Raven and Edwards 2001), the lower capacity of roots to fossilize make this scenario less stringent. Furthermore, several extant plants lacking roots lost them secondarily, making it difficult to properly evaluate fossil plants lacking roots as this may also be the derived condition (Raven and Edwards 2001). Regardless, it is clear that the evolution of roots was accomplished in a stepwise manner with numerous progressive changes culminating in the generation of complex root systems found among contemporary flowering plants (Kenrick and Strullu-Derrien 2014; Hetherington and Dolan 2017, 2018; Hetherington et al. 2016; Fujinami et al. 2020).

In 1880, Charles Darwin suggested that the root apex acts as a brain-like organ, '*…brain being seated within the anterior end of the body, receiving impressions from the sense-organs, and directing the several movements*' (Darwin 1880; Baluška et al. 2006a, 2009a; Barlow 2006). This surprising claim received severe criticism from Julius Sachs, an influential contemporary botanist who accused Charles Darwin and

his son Francis of performing flawed experiments in their country house (Heslop-Harrison 1980; de Chadarevian 1996; Ayres 2008). This dispute was a crucial crossroads in plant science, which was won by Julius Sachs not with scientific arguments but rather using his scientific political influence as leading figure in the field of plant physiology at that time. He asked his technical assistant Emil Detlefsen to repeat the experiments involving the surgical removal of maize root caps (originally reported by Ciesielski 1872) but he was not able to repeat this rather simple experiment properly (Detlefsen 1881), even though he was a skilled assistant of Sachs. However, strong support in favour of Sachs also came from Julius Wiesner, professor of plant anatomy and physiology at the University of Vienna (Wiesner 1881, 1884a, b). Now we can only speculate what would have been the outcome for plant science if Julius Sachs and Julius Wiesner would have accepted that even experiments performed in a country house can produce good results. Later, Francis Darwin and Wilhelm Pfeffer published data confirming that maize roots, with the caps cleanly removed, are well-suited for experiments and that the allegedly flawed Down House root experiments outcompeted the laboratory experiments of Sachs and Detlefsen (Krabbe 1883; Heslop-Harrison 1980; de Chadarevian 1996; Ayres 2008; Kutschera and Briggs 2009). Currently, the removal of maize root caps is accepted methodology and removed root caps regenerate completely within 30–40 h (Juniper et al. 1966; Barlow 1974; Barlow and Sargent 1978; Barlow and Hines 1982; Bennet et al. 1985; Iijima et al. 2003; Feldman 1976). The roots of dicot plants such as pea and arabidopsis are also capable of root cap regeneration (Barlow and Hines 1982; Sena et al. 2009; Efroni et al. 2016). For example, when plant regeneration is accomplished using callus tissue then it occurs via root development pathways (Sugimoto et al. 2010, 2011).

In 1997, we succeeded at immunofluorescence labelling of F-actin cytoskeletons in the intact root apices of maize (Baluška et al. 1997a), the same model structure which caused the severe dispute between Sachs and Darwins in 1880. This was the first time the actin cytoskeleton was visualized not in protoplasts or isolated plant cells, but in cells organized intact within tissues of the root apex. Abundant F-actin meshworks were found to be associated with the non-growing end-poles/cross walls of the transition zone cells (Baluška et al. 1997a, 2000, 2003a). In 2003, we outlined the plant synapse concept for the first time (Baluška et al. 2003b, 2005). Our data showed that this F-actin-based recycling of vesicles, including cell wall components, especially pectins, allows for effective cell–cell communication in the root apex (Baluška et al. 2002, 2003a, b, 2005, 2009b). Later studies revealed that this endocytic vesicle recycling is linked with the polar auxin transport accomplished via PIN-based export of auxin out of cells in root apices (Šamaj et al. 2004; Mancuso et al. 2005; Baluška et al. 2009b, McLamore et al. 2010). The same situation was found also for the transition zone in *Arabidopsis thaliana* roots (Verbelen et al. 2006; Schlicht et al. 2006; Mancuso et al. 2007; Dhonukshe et al. 2009; Mettbach et al. 2017). Later it emerged that this is part of the actin-auxin oscillator that drives polar trans-cellular transport of auxin through plant tissues (Holweg 2007; Nick 2007; Nick et al. 2009; Baluška and Mancuso 2013a, b, c).

There are several critical features suggesting that the root apex transition zone represents the root *brain* as proposed by Charles and Francis Darwin in 1880 (Darwin 1880; Baluška et al. 2006a, 2009a). First of all, cells in this developmentally unique zone are not distracted by any obvious tasks. They are neither dividing nor rapidly elongating, which allows them to focus on sensory integration tasks. They are located in very close proximity to phloem unloading sites which means that they are flooded with abundant levels of sucrose (Complainville et al. 2003; Ross-Elliott et al. 2017). This is associated with high activities of cell wall invertase, an enzyme which cleaves sucrose to hexoses (Hellebust and Forward 1962; Giaquinta et al. 1983; Roitsch and Gonzales 2004). Moreover, a high level of apoplastic sucrose induces osmotic stress which is relieved via induction of the fluid-phase endocytosis in cells close to phloem unloading sites (Baluška et al. 2004d). Another way to relieve this stress due to high sucrose levels is to synthesize large starch grains within the amyloplasts of the root apex transition zone cells (Fig. 6 in Baluška et al. 1993a and Fig. 2 in Baluška et al. 1993b).

This exceptional status of the transition zone cells allows them to focus mainly on cognitive tasks, resembling the situation of neurons of the central nervous system (CNS) seated within animal brains. Moreover, similar to CNS neurons, cells in the root apex transition zone also require greater levels of nutrient resources and oxygen (Baluška and Mancuso 2013a, b, c) in order to produce the ATP molecules necessary to drive the energetically demanding endocytic vesicle recycling and to support abundant and synchronized electrical spiking activities (Masi et al. 2009, 2015). This view is supported by a study reporting high cytosolic phosphate (Pi) concentrations in the transition zone for both epidermal and cortical cells of *Arabidopsis thaliana* root apices (Sahu et al. 2020). Pi is critical for ATP synthesis in mitochondria and for the synthesis of membrane phospholipids. In roots facing low levels of Pi in their environment, root caps act as the sensing organ which promptly stops root growth under Pi deficiency (Svistoonoff et al. 2007; Kanno et al. 2016). In this sensory circuit, the STOP1 transcription factor and ALMT1 anion/GABA (Ramesh et al. 2015, 2017, 2018; Žárský 2015; Kamran et al. 2020) act together to stop root growth (Abel 2017; Balzergue et al. 2017; Godon et al. 2019). ALMT1 also acts as a GABA receptor when, as in animal and human neurons, GABA lowers excitability of the plasma membrane (Žárský 2015).

There are intriguing similarities between animal brains and plant root apex *brains*: both enjoy uniquely protected as well as privileged locations within animal and plant bodies. Animal brains are protected mechanically within the skull, provided preferentially with nutrition and oxygen. Animal brains are free to perform only activities relevant to the control of cognitive behaviour of animals. Similarly, the Darwinian root-apex *brains* are positioned between the dividing cells of the root apical meristem and rapidly elongating cells pushing the whole root apex forward. In both maize and arabidopsis root apices, the size of the transition zone is similar to the size of the apical meristem, and unloading phloem elements define the basal border of the transition zone (Baluška et al. 1990, 1996a, 2001a, b; Verbelen et al. 2006). Finally, the brain is the only animal organ which is not in direct contact with blood. In fact, blood is toxic to neurons, and the blood–brain-barrier (BBB)

effectively prevents direct contact of brain neurons with blood (Hagan and Ben-Zvi 2015; Righy et al. 2016; Abdullahi et al. 2018; Madangarli et al. 2019; Nian et al. 2020; Segara et al. 2021). Intriguingly, the etymological origin of the term neuron comes from the ancient Greek, meaning 'vegetal fibre' (Brenner et al. 2006; Mehta et al. 2020). More importantly, the allegedly unique features of neurons, formulated and popularized as the 'Neuron Doctrine' by Wilhelm Waldeyer in 1891 (Shepherd 1991; Jones 1994), are no longer considered to be so unique (Gold and Stoljar 1999; Guillery 2007).

Rather surprisingly, many so-called neuronal features are present in plant cells, especially in the transition zone of root apices (Baluška 2010). Recent advances in plant cell biology have revealed that plant cells, especially those located in the root apex transition zone, show almost all of the features which were defined, according the 'Neuron Doctrine', to be neuron-specific (Baluška 2010; Baluška et al. 2005, 2009a, b; Masi et al. 2009). As noted by Rainer Stahlberg, nerves in animals and vascular bundles in plants share analogous functions of conducting rapid electric signals (Stahlberg 2006a, b). Similar analogies to the cellular basis of plants and animals resulted in the acceptance of the Cell Theory. Therefore, it is puzzling that plant electrophysiology is considered to be esoteric (Alpi et al. 2007; Taiz et al. 2019). The most significant differences between plant and animal cells are associated with their different extracellular matrices, and their interactions with the plasma membrane and elements of cytoskeletal polymers (Reuzeau and Pont-Lezica 1995; Baluška et al. 2003b, Seymour et al. 2004; Halbleib and Nelson 2006; Campbell and Humphries 2011). For example, sodium is the major ion driving action potentials in animals but it is toxic for plants with pectinic cell walls (Feng et al. 2018; Verger and Hamant 2018), which rely instead on calcium fluxes (Hope 1961; Beilby and Coster 1979; Beilby and Al Khazaaly 2016; Hedrich and Neher 2018; Iosip et al. 2020). While plant cell walls pose additional problems for the excitability of plant cells and tissues, they also provide them with additional layers of signalling complexity (Baluška et al. 2003b; Ringli 2010; Wolf et al. 2012; Wolf 2017). Our discovery that cell wall molecules, such as calcium, boron cross-linked pectins and xyloglucans, are actively recycled from cell walls via endosomal vesicles (Baluška et al. 2002, 2009a, b; Dhonukshe et al. 2009) is crucial for our conceptual advancement of plant-specific synapses in the root apex transition zone.

3 Neuronal Molecules Relevant for Root Apex Cognitive Navigation and Soil Exploration

Plant root apices are supported via numerous molecules which were originally characterized as neuronal molecules. Among these, we will briefly discuss glutamate and GABA with their receptors, which control the electrical properties of the plasma membrane. Importantly, in both neurons as well as in plant cells, glutamate stimulates and GABA inhibits excitability of the plant plasma membrane. Although there are

some differences in their receptors, especially with respect to GABA (Ramesh et al. 2015, 2017; Žárský 2015), the electrophysiological impacts on plasma membrane potentials and excitability are very similar. The same is true for another neurotransmitter, glutamate, in that the glutamate receptors of plants are very similar to those of animal brains (Weiland et al. 2016; Wudick et al. 2018; Qiu et al. 2020).

Evolutionary analysis even suggests that plant glutamate receptors might predate the animal glutamate receptors of the NMDA class which have a central role in the control of the brain's synaptic plasticity (Stroebel and Paoletti 2020). Importantly, both glutamate and GABA shape action potentials (APs) in plants, partially through their control of voltage-gated potassium channels (Cuin et al. 2018; Adem et al. 2020; Koselski et al. 2020). Similar to the neuronal APs in humans and animals, plant-specific APs are also blocked by diverse anesthetics and this prevents the movements of plant organs (Yokawa et al. 2018, 2019; Pavlovič et al. 2020; Baluška and Yokawa 2021).

4 Synaptic Principles Relevant for Root Apex Cognitive Navigation

Root apex cells located in the transition zone are unique with respect to their cytoarchitecture, endocytic vesicle trafficking, arrangement of actin cytoskeleton elements, polar transport of auxin, and bioelectric activities of their plasma membranes. In 1987, we discovered that the actin cytoskeleton is organized via unique bundles of F-actin anchored at the cellular end poles (cross-walls) which are densely populated with plasmodesmata (Baluška et al. 2000, 2003a, b; Baluška and Hlavacka 2005). Later, the plant-specific myosin VIII was discovered in plants and was also localized abundantly to these cross-walls (Reichelt et al. 1999). It emerged that myosin VIII supports plasmodesmata structure and function, anchoring the F-actin cables at the cross-walls, and driving endocytosis and endocytic vesicle recycling (Baluška et al. 2000; Volkmann et al. 2003; Baluška and Hlavacka 2005; Golomb et al. 2008; Sattarzadeh et al. 2008; Haraguchi et al. 2014). Importantly, myosin VIII-based end-poles of cells in the transition zone assemble cell–cell adhesion domains which fulfil several synaptic criteria and support the brain-like status of the root apex transition zone (Baluška et al. 2005, 2009a, b; Baluška and Mancuso 2013a, b, c). Auxin emerges as acting not only as a plant hormone but also as a plant-specific neutrotransmitter-like molecule which is integrating sensory inputs into the context of root tropism outputs (Baluška et al. 2005, 2008, 2009a, b; Baluška and Mancuso 2013a, b, c; Schlicht et al. 2006; Baluška et al. 2008). Interestingly, the root apex transition zone acts as the specific target of aluminium toxicity (Sivaguru and Horst 1998; Kollmeier et al. 2000; Sivaguru et al. 1999, 2000, 2003a; Illés et al. 2006; Yang et al. 2014; Li et al. 2018). The central role of aluminium toxicity in the transition zone is especially relevant for the basipetal (shootward) flow of auxin driven via the PIN2 auxin efflux transporter (Kollmeier et al. 2000; Shen et al. 2008; Yang

et al. 2014; Wu et al. 2014, 2015), and is mediated by the activity of plant glutamate receptors (Sivaguru et al. 2003b).

5 Transition Zone Energides in the Driver's Seat to Control Root Apex Navigation

One of the most prominent features of cells in the root apex transition zone is the fact that the nucleus is centralized and suspended in dynamic cytoplasmic strands organized by cytoskeletal polymers (Baluška et al. 1990, 1997a, 2000, 2001a, b, 2003a, 2006b, 2010). Whereas the F-actin bundles are organized conically between cellular end-poles and are the most prominent structure, the dense F-actin baskets that suspend the centrally positioned nuclei and perinuclear radiating microtubules are also important for the integral roles of these cells in sensory signal perception and integration, resulting in adaptive root tropisms (Baluška et al. 2004a, 2006a, b, 2009a, b, 2010; Baluška and Mancuso 2013a, b, c). The current version of the Cell Theory is facing skepticism due to the existence of multinuclear coenocytic (cell division not followed by cytokinesis) and syncytia (fusion of cells) cellular assemblies. In fact, almost all plant cells have free cytoplasmic channels known as plasmodesmata. We have extended and fully developed the Cell Body concept which was originally proposed by Daniel Mazia in 1993, and correlates well with the Energide concept of Julius Sachs from 1891 (Baluška and Barlow 1993; Baluška et al. 1997b, 1998, 2001b, 2004b, c, 2006a, b). The Energide-Cell Body is the smallest unit of cellular life originating from still unknown ancient and centrin-based archaea with microtubular flagella (Baluška and Lyons 2018, 2021). It is hypothesized that the cytoplasmic strands, supported by vibrating and oscillating F-actin cables and microtubules (Tuszyński et al. 2004; Cifra et al. 2010; Kučera and Havelka 2012), are transmitting sensory signals received at the plasma membrane to the central nuclei (Matzke et al. 2019). Similar neuronal synapse—nucleus communication is involved in the formation and maintenance of neuronal circuits (Saha and Dudek 2008; Cohen and Greenberg 2008). Action potentials seem to have originated from the repair of damaged plasma membranes of ancient cells and contributed to preservation and homeostasis of plasma membrane and cellular integrity (Goldsworthy 1983; Steinhardt et al. 1994; Brunet and Arendt 2016; Baluška and Mancuso 2019).

6 Changing Metaphor for Transition Zone Energide: From 'Bug in Cage' to 'Spider in Web'

In 2004, we proposed the metaphor *Bug in Cage* for the Cell Body/Energide enclosed by the plasma membrane and cytoplasm (Baluška et al. 2004b). The idea behind this metaphor was that the symbiotic evolutionary origin of the Cell Body/Energide

implies its semi-autonomous nature and biological agency behind its organization and behaviour (Baluška et al. 1997b, 1998; Baluška and Lyons 2018, 2021). The Cell Body/Energides in the root apex transition zone cells are acting as navigators of root apices (Fig. 1, Baluška and Mancuso 2018) in their search for water and critical mineral nutrients and avoidance of toxic soil patches. They can act as kind of sensitive radar for both acoustic and chemical cues (Falik et al. 2005; Schenk 2006; Gagliano et al. 2012a, b; Yokawa et al. 2014; Rodrigo-Moreno et al. 2017).

Our proposal here is that the Nuclei/Energides suspended within the cytoskeleton-supported cytoplasmic strands (Fig. 1a, b) of the root apex transition zone are perfectly suited to control the root apex navigation *akin* to navigators seated in the driver's seat (Fig. 1c). As the F-actin cables enclosing the nuclei are anchored at the root synapses (Baluška et al. 1997a, 2000, 2005, 2009b; Baluška and Hlavacka

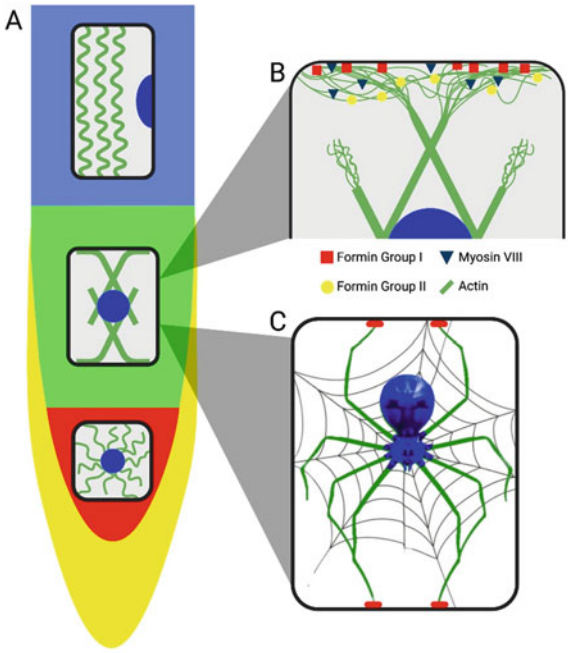

Fig. 1 Schematic Overview of the Root Apex Zones Relevant for Root Apex Navigation. **a** The root cap (yellow) encloses the apical meristem (red) and the transition zone (green). The zone of rapid cell elongation (blue) follows, which pushes all the other more apical zones forward. The nucleus (in blue) is enclosed by F-actin elements (in green) in the form of a meshwork (cells in meristem) or conical bundles anchored at the synaptic end poles (cells in transition zone). In cells of the rapid cell elongation zone, the nucleus is pushed to the cell periphery by the large central vacuole and relaxed F-actin bundles are organized longitudinally. **b** Detail of the two conical F-actin bundles organized at the synaptic cell periphery by actin-binding formins and myosin VIII. **c** Hypothetic scenario of root apex navigation via the transition zone Cell Bodies/Energides, depicted metaphorically in the form of a spider-in-web. For more details, see Baluška and Hlavacka 2005; Baluška and Mancuso 2013a, 2013b, 2013c, 2018; Baluška and Lyons 2018, 2021)

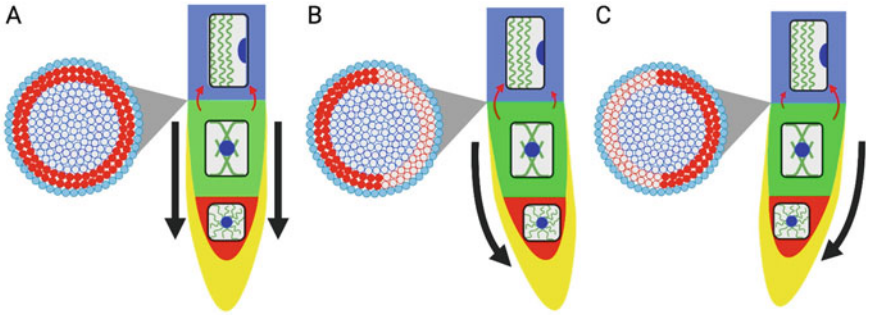

Fig. 2 Smart Border at the Basal Limit of the Transition Zone. The transition zone Cell Bodies/Energides control root apex navigation through their contacts at the synaptic end-poles of cells at the basal limit of the transition zone. This translates sensory perceptions into motoric root apex tropisms at this smart border between the basal limit of the transition zone. **a** If there is no relevant cue registered by the Cell Body/Energide, then all the transition zone cells are released into the rapid cell elongation zone in a coordinated fashion. **b, c** Differential release of cells from the transition zone into the rapid cell elongation zone allows root tropisms which are finely-tuned by relevant cues. The most critical cells for root apex tropisms are PIN2 expressing cells (shown as red circles) at the root periphery. **b** Repelling cues slow-down (small red arrow) the release of PIN2 cells (unfilled red circles in the root cross-section view) from the transition zone (green) into the region of rapid cell elongation (blue) at the opposite side of the root apex periphery. Attracting cues speed-up (large red arrow in the root cross-section view) the release of PIN2 cells (filled red circles) from the transition zone (green) into the region of rapid cell elongation (blue) at the opposite side of the root periphery

2005), the Nuclei/Energide are optimally placed to navigate root apex trajectories. The most effective means to control root tropisms is to manipulate the onset of rapid cell elongation in a coordinated fashion across the root epidermis and cortex (Fig. 2). In the maize root apex, there are hundreds of cells located at the basal limit of the transition zone that are primed for rapid cell elongation. Their Energides give their 'yes' for the burst-like onset of the rapid cell elongation (Fig. 2) which is under the control of auxin, calcium, ethylene and actin-myosin forces (Baluška et al. 1993a, b, 1996a, 1997a, 2000, 2001a, b). On the other hand, microtubules are not involved in this developmental switch as maize root tropisms are completed with all microtubules depolymerized (Baluška et al. 1996b). In some way, the active Energides of the transition zone cells resemble spiders sitting within their webs (Fig. 1c), feeling web vibrations to inform them of the presence of prey, as well as of other relevant cues from their environment (Mortimer 2019; Mortimer et al. 2019). This sensitive cytoarchitecture would explain the surprising ability of growing roots to respond to specific acoustic signals via positive root phonotropism (Rodrigo-Moreno et al. 2017) or to recognize barriers from distance (Falik et al. 2005; Schenk 2006).

How could the Energide sense relevant sensory signals and integrate this information to control root cell elongation? Here the 'Plasma Membrane Control Centers' (Pickard and Ding 1993; Pickard 1994, 2013; Gens et al. 2000) and the 'Hechtian Growth Oscillator' (Lamport et al. 2014, 2018, 2020) concepts are relevant. For the

root apex, important cues are water and critical minerals which, when perceived, are associated with changes in tension and vibrations of the cytoplasmic strands (Fig. 1). The contact of F-actin and myosin VIII with the critical plasma membrane domains can control the ion fluxes across the plasma membrane. Interestingly, the conical bundles of F-actin that enclose nuclei are straight and thick, as if under tension, in the transition zone; in contrast, they instantly appear thin and wrinkled as root cells initiate their rapid cell elongation (Baluška et al. 1997a, 2000; Baluška and Hlavacka 2005).

Such sensitive and vibrating networks could allow effective perceptions from the root apex rhizosphere, including possible sound waves bouncing back from soil portions ahead of the growing root apices. For example, maize root apex generates sound waves in regular frequencies (Gagliano et al. 2012a, b). Analysis of growing roots of arabidopsis revealed that they are attracted by sound waves of 200 Hz which are close to the sound waves generated by streams of water (Rodrigo-Moreno et al. 2017). This root phonotropism can be expected to be useful for roots in their search for water (Rodrigo-Moreno et al. 2017; Fromm 2019). Acoustic root navigation, resembling bat echolocation, would also allow recognition of physical barriers in advance (Falik et al. 2005; Schenk 2006).

7 Evolution of the Root Apex Brain: From Ancient Roots Towards Complex Root Systems

In early root evolution, some 400 million years ago, ancient roots teamed-up with symbiotic AM fungi and have tightly co-evolved ever since (Pirozynski and Malloch 1975; Selosse and Le Tacon 1998; Selosse et al. 2015). Moreover, roots also attract specific bacteria which help roots to cope with diverse stresses. In order to control their rhizosphere, roots release large amounts of exudates and diverse infochemicals (Baluška and Mancuso 2020, 2021). These substances help them not only to develop the surrounding soil as their living niche but also to enjoy complex social lives with the roots of neighbouring plants (Baluška and Mancuso 2020, 2021). Roots are territorial (Schenk 2006; Novoplansky 2019). They discriminate self—non/self roots and apply the kin recognition (Bais 2018; Novoplansky 2019) in their behaviour (Baluška and Mancuso 2021). The root apex transition zone plays a central role in this social aspect of root life. Auxin transport via neurotransmitter-like modes based on synaptic-like vesicle recycling is critical aspect of root behaviour. In the evolution of roots, the auxin-transporting synapses (Baluška et al. 2005, 2008, 2009b) have been proposed to evolve from the ancient symbiotic synapses (Baluška et al. 2005; Kwon et al. 2008; Lima et al. 2009; Baluška and Mancuso 2013c).

Plants compete for light, water and mineral nutrients (Craine and Dybzinski 2013). In shoots, the shade avoidance syndrome is behind the light competition between neighbour plants (Smith and Whitelam 2007, Keuskamp et al. 2010; Martínez-García et al. 2010, 2014). In plant roots, fierce competition for water and critical minerals

shapes root behaviour (Gersani et al. 2001; Schenk 2006; McNickle et al. 2009; Farrior 2019). Root apices apply their plant-specific perception, cognition and intelligence in order to succeed in their difficult task of finding sufficient water and mineral nutrients (Hodge 2009; Barlow 2010a, b; Gruntman et al. 2017; Baluška and Mancuso 2018; Fromm 2019; Novoplansky 2019; Parise et al. 2020). In plant evolution, roots evolved from structurally and cognitively simple rhizoids up to the complex root systems of contemporary flowering plants which enjoy complex foraging behaviour. Plants use their root systems for plant-plant communication of sensory and stress cues (Falik et al. 2012; Elhakeem et al. 2018; Novoplansky 2019; Volkov and Shtessel 2020; Yamashita et al. 2021).

8 Root-Fungal Networks Control Underground Supracellular Life

Plant root evolution started with the earliest colonization of barren land with help from symbiotic AF fungi some 400 billions of years ago (Pirozynski and Malloch 1975; Remy et al. 1994; Heckman et al. 2001; Schüßler and Walker 2011; Feijen et al. 2018). Roots are hidden underground in the soil, leading to the prevailing view of plants as simply green organisms which flower when mature. As an example, the value of the largest living organism on Earth, the giant sequoia tree, is generally based on its shoot parts, while its root parts are ignored. However, the true nature of plants and trees is based on the fact that their roots are structurally and functionally connected through fungal hyphae networks. In some sense, these networks are analogous to our human invention of the internet because the latest advances suggest that they serve not only for exchange of nutrients and water, but also for chemical and electrical long-distance signaling (Simard et al. 1997; Song et al. 2010; Barto et al. 2012; Gorzelak et al. 2015, 2020; Sasse et al. 2018; Simard 2018; Volkov et al. 2019; Volkov and Shtessel 2020). Obviously, the true nature of plants is hidden underground, which would explain why plants are generally considered to be devoid of agency, cognition, and intelligence. The aboveground parts of plants, visible to us, are just support organs specialized for photosynthesis and sexual reproduction (Baluška and Mancuso 2021). Roots demonstrate kin recognition, self/non-self recognition and swarm intelligence (Baluška et al. 2010; Ciszak et al. 2012; Baluška and Mancuso 2018, 2020, 2021). They invest their carbon-based photosynthetic substances to control the rhizosphere microbiota communities and soil as a life-friendly biotop (Barlow 2010a, b; Barlow and Fisahn 2013; Novoplansky 2019; Baluška and Mancuso 2020, 2021). Future experimental studies will focus on the ecological, cognitive and electrophysiological aspects of the root-wide-web (Simard et al. 1997; Lee et al. 2013; Simard 2018; Giovannetti et al. 2006; Fukasawa et al. 2020; Volkov et al. 2019; Volkov and Shtessel 2020; Kokkoris et al. 2021) spanning large areas of the Earth surface. Unfortunately, these intact forest areas are shrinking and this has serious consequences for the life-friendly climate (Baluška and Mancuso 2020).

Circadian clocks have emerged as critical players in decoding sensory information obtained from the environment (Hearn and Webb 2020; Koronowski and Sassone-Corsi 2021), which is crucial for cognitive aspects of all organisms. With respect to plants, which live both above-ground (shoots) and below-ground (roots), the situation is unique (Baluška and Mancuso 2018, 2021; Lee et al. 2019). Although the shoot clock was proposed to be the primary plant clock and the root clock is viewed as a simplified slave-like version of the shoot clock (James et al. 2008), recent studies revealed that the root clock coupling strength is extraordinary especially in the root apex (Gould et al. 2018; Maric and Mas 2020). Light can reach the root apices via internal tissues down to under-ground roots (Mandoli and Briggs 1984; Lee et al. 2016). This then allows them direct light-mediated entrainment of the root clock (Nimmo 2018; McClung 2018). As the AM fungi have their own circadian clocks (Lee et al. 2018, 2019), it can be expected that the huge symbiotic root—AM fungi networks are integrated via their supra-organismal circadial clocks (Lee et al. 2019). Similar trans-kingdom clocks are found in animals and humans (Thaiss et al. 2014; Page 2019). We can look forward to future studies in this newly emerging field of supra-organismal chronobiology.

9 Conclusions and Gaian Outlook

Land plants are decisive organisms with respect to the Earth's climate ever since they evolved from rather simple and small predecessors living in seas. The first terrestrial plants cooled the Ordovician Earth (Lenton et al. 2012). Their roots, in co-operation with symbiotic AM fungi, generated soil as a central habitat for terrestrial ecosystems (Rillig and Mummey 2006; van der Heijden et al. 2008). Ever since then, land plants have been integral in establishing and maintaining the climate of the Earth (Beerling 2019). Tree root systems are integrated and networked with the symbiotic fungal hyphae into huge super-organismal phenomenon known as wood-wide-web (Simard et al. 1997; Helgason et al. 1998; Giovannetti et al. 2006; Simard 2021). This wood-wide-web participates in homeostatic processes (Power et al. 2015) also known as the Gaia hypothesis proposed by James Lovelock in 1972 (Lovelock 1972, 1979, 2019: Lenton and van Oijeb 2002; Lenton and Latour 2018, Lenton et al. 2018). In this respect, although this seems to be counter-intuitive, plants are socially and cognitively active mostly underground as only roots, but not shoots, can enter into the long-lasting symbiotic interactions (Baluška and Mancuso 2018, 2020). There are examples of plants and even trees (Henschel and Seely 2000; Maurin et al. 2014) that live underground, and numerous myco-heterotrophic plants that are not green at all, obtaining all their food from fungal partners (Bidartondo 2005; Merckx et al. 2009). It is possible that future studies will reveal even more surprising connections between roots, fungal hyphae and microbial populations which control the terrestrial ecosystems and the Earth's climate. If we would like to solve the current climatic crisis and better understand the Earth's ecosystems, we should focus more on the

underground life which is dominated by plant roots and their AM fungal partners. Here is where the key to our future life on the planet Earth is hidden.

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Root Architectural Plasticity in Changing Nutrient Availability

Prakhar Awasthi and Ashverya Laxmi

Abstract Plant roots have been an intriguing subject to plant biologists for a long time. Roots serve a wide range of functions in plants, including anchorage and absorption of water and nutrients. Roots are highly plastic in their development and directional growth to facilitate better absorption and assimilation of water and nutrients under different environmental conditions. Plasticity of root system architecture (RSA) confers different architecture in response to immediate soil-based and endogenous signals. Nutrient heterogeneity in the soil is a major factor determining RSA. Plants sense local nutrient availability and facilitates directional root development to enhance nutrient acquisition. Among the mineral nutrients, Nitrogen (N) and Phosphate (Pi) are major macronutrients important for plant development. N dissolves in water and accumulates in deep soil, thus compelling plants for producing deeper roots. Pi retains in topsoil and during pi deficiency, root system grows more laterally to enhance pi uptake. Thus, RSA is highly dynamic in nature which varies according to soil condition and nutrient availability and enhancing the plasticity of RSA will improve the nutrient uptake and use efficiency of crops. In this chapter, we are discussing how N and pi availability remodel different aspects of RSA in model plants and crops.

Keywords Ammonium · Lateral root differentiation · Lateral root primordia · Nitrate · Nutrient acquisition · Nutrient crosstalk · Nutrient use efficiency · Phosphate · Primary root growth · Root system architecture · Root plasticity · Root hairs · Root meristem · Soil heterogeneity · Topsoil foraging

Abbreviations

RSA	Root system architecture
N	Nitrogen

P. Awasthi · A. Laxmi (✉)
National Institute for Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India
e-mail: ashverya_laxmi@nipgr.ac.in

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Pi	Phosphate
NUE	Nutrient use efficiency
LRP	Lateral root primordia
PHR1	Phosphate starvation response
PSR	Phosphate starvation responses
LR	Lateral roots
PR	Primary root
P1BS	PHR1-binding sites
ARFs	Auxin-response factors
GWAS	Genome-wide association study
QTLs	Quantitative trait locus

1 Introduction

Roots played a crucial role in the evolutionary success of land plants. They search for minerals and water in the soil and supply it to the above-ground parts of the plant. Plants need mineral nutrients for optimal growth. Roots forage the soil to maximize nutrient acquisition. Plants require a range of essential nutrients, among them, nitrogen (N), phosphate (Pi), potassium (K) are required in large quantities. The inorganic forms of phosphate (HPO_4^-, Pi) and nitrate (NO_3^-) or ammonium (NH_4^+) are taken up from the soil (Bouain et al. 2019). The heterogeneous distribution of these nutrients in the soil limits their acquisition. Moreover the assimilation of pi in plant is also poor as inorganic phosphate reacts with iron (Fe_3^+), aluminum (Al_3^+), and calcium (Ca_2^+) ions present in soil to form an insoluble complex. Limitation of phosphate and nitrogen in the plant severely affects plant growth and productivity and nitrogen and phosphate are externally supplied to the crop in form of fertilizers to enhance productivity. Excessive input of fertilizers causes water and air pollution. Ongoing soil degradation, water pollution, and climatic changes negatively affect sustainable agriculture (Li et al. 2016). An increasing population and limited agricultural space aggravate this problem. One effort in mitigating the situation would be to improve nutrient use efficiency (NUE) of crop plants. Global agriculture demands nutrient efficient crops with improved nutrient uptake even under nutrient-deficient conditions. Plants employ multi-level pathways for nutrient uptake, such as modification of root system, enhancing the activity of nutrient transporters, releasing organic acids in soil, and developing symbiosis with fungi or bacteria (Chen and Liao 2017).

The intricate three-dimensional web of roots in the soil is known as root system architecture (RSA) (Satbhai et al. 2015). Enhancing the resilience and plasticity of RSA is one of the ways to improve the nutrient uptake of crops. Root length, branching, angle, and surface area, are major tenets of RSA. The RSA of a plant is the cumulative product of genetic and environmental interaction (Lynch 2019). Plants sharing the same genotype might develop different root systems depending on the immediate requirement. This interaction of genome and environment provides

plasticity to the plant roots system. Root plasticity is more advanced in higher plants to support much complex physiology (Satbhai et al. 2015). Phenotypic plasticity of roots shapes root system in response to local root environment which includes, soil texture and compactness, water and nutrient availability, soil fauna, and gravity (Satbhai et al. 2015). As nitrogen and phosphate both are involved in RSA remodeling in response to their availability in soil, but the phenotypical changes in roots are contrasting to each other in terms of primary root growth and lateral root formation. Plants faces deficiencies of multiple nutrients in soil at a time and in such combinatorial stress, a crosstalk between the nutrients signaling is required (Bouain et al. 2019). In this chapter, we will briefly discuss the idea of RSA in model and crop plant then we will move forward to study the role of nitrogen in mediating RSA. We will zoom into the molecular machinery of plant RSA in response to phosphate, and progress in utilizing the underlying machinery to improve the nutrient efficiency of crops.

2 RSA and Nitrogen Mediated Root Remodeling

Nutrient efficiency has been an important trait in the evolution of roots in land plants (Lynch 2019). Plants utilize the root system for the acquisition of mineral nutrients such as nitrogen and phosphate from the soil. Plants need to forage the soil in search of nutrients which varies from place, time, and depth of soil. Exploration of soil is mediated by roots in terms of root growth, branching, root hair formation, and microbial symbiosis. Dicots plants such as Arabidopsis have tap root system, which consists of the main primary root and several branched lateral roots along with root hairs (Satbhai et al. 2015). Monocots such as maize and rice develop fibrous roots primarily consisting of adventitious roots. Adventitious roots rising from any non-root tissue, such as junction roots, crown roots, etc. (Del Bianco and Kepinski 2018). Root system architecture (RSA) has been considered essential for soil exploration and NUE. Modifying the RSA for improving the nutrient acquisition of the plants is the most basic yet complicated trait for crop improvement.

In general, the distribution of nutrients in soil depends on the amount of nutrients, soil composition, nutrient solubility, etc. Nitrogen (N) is an essential structural and functional component of primary and secondary organic compounds. Limitation of nitrogen in plants constraints plant growth and development with subsequent reductions of plant productivity (Luo et al. 2020). Owing to Haber's process of artificial nitrogen fixation, the production of ammonium has increased (Jenkinson 2001). However, the high solubility of nitrate and ammonium in water causes nitrogen to leach out with the groundwater, causing poor nitrogen acquisition and environmental pollution. The most logical and accessible option to reduce wastage and pollution is to improve nitrogen utilization efficiency (Xu and Takahashi 2020). In dicots, such as model plant Arabidopsis, four methods of root remodeling have been studied in the presence of nitrogen, (i) presence of nitrate promotes localized elongation of lateral root growth, (ii) high tissue nitrate systemically inhibits the lateral root meristems, (iii) exogenously supplied L-glutamate inhibits primary root growth and promotes

root branching, (iv) high carbon to nitrogen ratio inhibits the lateral root initiation (Zhang et al. 2007).

The effect of normal nitrate on primary root growth was either insensitive or modestly promoting the primary root growth (Forde 2014). Although moderately higher concentration of KNO_3 inhibited the primary root growth and this was mediated via the miR393/AFB3 regulatory module (Vidal et al. 2010). microRNA393 is induced by nitrate and it specifically cleaves transcripts of AFB3, an auxin receptor which has a role in auxin dependent root growth in response to nitrate (Vidal et al. 2010). L-glutamate, an amino acid when exogenously provided specifically inhibited the primary root growth (Forde 2014). Nitrate was seen to monitor the stem cell dynamics by regulating the cell division genes and differentiation of distal stem cells (Guan et al. 2017; Wang et al. 2017). TCP20, a component of nitrate signaling interacts with NIN-like proteins, NLP6 and NLP7, and upregulates the expression of nitrate-dependent genes and downregulates the G2/M cell cycle gene *CYCB1;1* in the root meristem (Guan et al. 2017).

Local patches of nitrate promote preferential lateral root growth (Mounier et al. 2014). Nitrate being the major source of nitrogen has an intrinsic role as a nutrient and signaling molecule perceived by plant nitrate transporter 1.1 (*NRT1.1*) (Maghiaoui et al. 2020b). This nitrate-dependent lateral root growth is the outcome of nitrate transporter/sensor NRT1.1 (also known as *NPF6.3* or *CHL1*) and auxin interaction (Krouk et al. 2010). NRT1.1 regulates lateral root growth by orchestrating the basipetal transport of auxin out of lateral root primordia (LRPs) (Krouk et al. 2010). In a recent study, it was found that NRT1.1 also regulates the auxin biosynthesis (Maghiaoui et al. 2020a), NRT1.1 negatively regulated the expression of auxin biosynthetic gene, TAR2 and auxin influx carrier, LAX3 to reduce acropetal transport of auxin in LRPs (Maghiaoui et al. 2020a). Interestingly, the supply of NH_4^{+a} a preferential source of nitrogen for crops, stimulate lateral root branching (Lima et al. 2010; Jia and von Wirén 2020). In Arabidopsis, it was found that local NH_4^+ promoted branching with the help of NH_4^+ transporter AMT1;3. This branching was found absent in quadruple mutant of *AMMONIUM TRANSPORTER* (*amt1;1, amt1;2, amt1;3, amt2;1*), reconstituting the expression of *AMT1;3* in quadruple mutant restored the branching (Lima et al. 2010). Ammonium uptake releases protons in the apoplast, this acidification of apoplast modifies the auxin mobility which in turn increased the lateral root density (Meier et al. 2020; Pélissier et al. 2021). A detailed overview of nitrogen-dependent lateral root formation is published recently (Pélissier et al. 2021).

Along with local signaling, systemic signaling also regulates root architecture in foraging soil nitrogen. Systemic signals of nitrogen deficiency shape the root architecture by modulating the phytohormones such as auxin, cytokinin, and Brassinosteroids (Kiba et al. 2011). The *steep, cheap, and deep* roots in crops attain root architecture exploring deeper sections of soil in search of mobile nitrate (Lynch 2019). This architecture includes *steep* lateral growth angle, fewer branching of root or *cheaper* in carbon units, and *deeper* roots in foraging nitrogen. Several reports in maize and rice support *steep, cheap, and deep* root ideotype of crops in low nitrogen condition (Lynch 2013; Trachsel et al. 2013; Ju et al. 2015; Chen and Liao 2017; Lynch 2019). Recent studies have suggested an integration of phosphate and nitrate signaling in

plant root development (Medici et al. 2015, 2019a; Bouain et al. 2019). GARP type transcription factors, HRS1 and NIGT1.2 both have been shown to involved in nitrate and phosphate signaling (Medici et al. 2015; Wang et al. 2020). A crosstalk between Nitrate receptor NRT1.1 and PHOSPHATE STARVATION RESPONSE1 (PHR1) was observed (Medici et al. 2019a). PHOSPHATE2 (PHO2) integrated the N availability into the phosphate starvation responses (PSR). Nitrogen mediated PSR were significantly affected in *pho2* mutants (Medici et al. 2019a).

3 Root System Architecture in Response to Phosphate (Pi)

Phosphate (HPO_4^-, Pi) is another essential nutrient required for plant growth and development. Being a component of DNA, protein, and cell membrane, it is requisite along with carbon and nitrogen for optimal growth. The acquisition of phosphate in plants is also inefficient, which is supported by the fact that only 10–25% of applied phosphate is taken up by the plant (Crombez et al. 2019). Limitation of pi in soil affects plant growth and reduces plant productivity. Unlike nitrogen, the sources of phosphorus are non-renewable and has been predicted to deplete in the next few decades (Lynch 2013, 2019). The predominant form of phosphorus in soil is orthophosphate (HPO_4^-), which reacts with cations such as Mg^{2+} and Ca^{2+} (Chen and Liao 2017). Low mobility of orthophosphate or pi in the soil makes the topsoil rich in phosphate. To increase the uptake of pi, plants forage the topsoil (Lynch 2019). Topsoil foraging in crops generally reorganizes the root system architecture with more production of axial roots, shallower axial root angle, greater lateral root density, and longer and denser root hairs (Lynch 2019). In Arabidopsis shallow root system is optimized with reduced primary root length, increasing lateral root length and density, and producing denser root hairs (Fig. 1) (Huang and Zhang 2020).

3.1 Primary Root Growth Under Pi Deficiency

Inhibition of primary root growth in low phosphate is orchestrated in a coordinated fashion, by arresting cell division, inhibiting the cell elongation, and stimulating the cell differentiation (Péret et al. 2014). Numerous genetic screenings identified the role of several mutants in regulating the root meristem under pi deficiency. In Arabidopsis, LOW PHOSPHATE ROOT 1 (LPR1) and PHOSPHATE DEFICIENCY RESPONSE 2 (PDR2) appeared in regulating the meristem activity (Ticconi et al. 2009; Müller et al. 2015). *PDR2* encodes a single P5-type ATPase which supports the expression of Scarecrow (*SCR*) in maintaining root patterning (Ticconi et al. 2009). *LPR1* (Ferroxidase) and *PDR2* both works together under pi deficiency to arrest root apical meristem (RAM). LPR1-PDR2 module promotes iron deposition specifically in the cell wall of RAM. Accumulation of Fe stimulates callose deposition which interfere the cell to cell communication and inhibiting transport of SHORTROOT

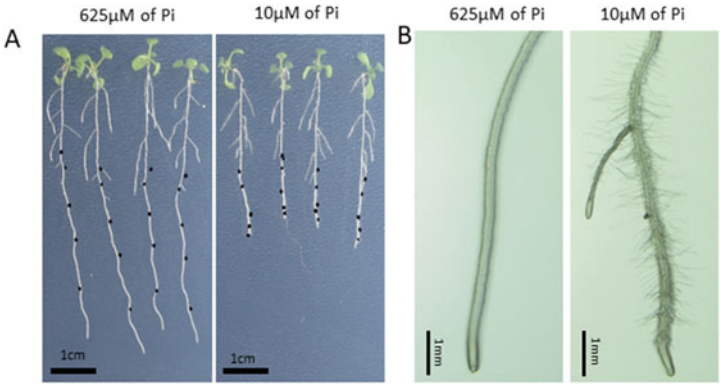

Fig. 1 RSA of Arabidopsis wildtype in phosphate deficient (10 μM) and sufficient medium (625 μM). 7 DAG old Col-0 subjected to pi deficiency for five days showed inhibition of primary root growth, an increase in lateral root density (panel **a**), and root hair (panel **b**). Dots represent the primary root growth kinetics for a period of five days

(SHR) from stele into the quiescent center (Müller et al. 2015). Inhibition of primary root growth in pi deficiency was completely abolished in *lpr1lpr2* double mutant. In contrast, the primary root of *pdr2* mutant was hypersensitive to pi deficiency (Müller et al. 2015). Phosphate-dependent callose deposition in RAM was due to enhanced reactive oxygen species (ROS) production in stem cells, which increased in pdr2 mutant (Müller et al. 2015). Reduced cell elongation and stimulation of cell differentiation are two other very important characteristics of pi-mediated inhibition of PR growth. STOP (SENSITIVE TO PROTON TOXICITY1) and ALMT1 (ALUMINUM-ACTIVATED MALATE TRANSPORTER1) module coordinate the cell expansion in Arabidopsis (Balzergue et al. 2017). STOP is a transcription factor that controls the cell elongation by modulating the expression of its direct target gene, *ALMT* which codes for malate channel. Together they mediate cell wall stiffening with the help of iron and peroxidase (Balzergue et al. 2017). SIZ1 a SUMO E3 ligase regulates PSR, *siz1* mutant exhibited reduced primary root and extensive lateral root and root hair development in pi deficient conditions (Miura et al. 2005).

In crops, the effect of primary root growth inhibition in pi deficiency is less pronounced (Péret et al. 2014). Different crops and cultivars showed a different response to primary root growth under pi deficiency (Péret et al. 2014). Crop plants favor proliferation of adventitious roots in pi deficiency. Maize genotypes with more crown roots and lateral root branching have higher topsoil foraging, growth, and a better yield under low pi conditions (Jia et al. 2018; Lynch 2019). In addition to that, different plant families such as Proteaceae and Leguminosae develop denser lateral roots in cluster are known as proteoid roots in response to low pi (Li et al. 2016). Pi deficiency in plants stepwise coordinates the reduction in primary root growth to mobilize the carbon allocation from the primary root to lateral root density and root hair formation.

3.2 *Lateral Root Growth in Pi Deficiency*

Lateral roots in plants have an important role in the acquisition of pi from the topsoil by increasing the total surface area (Waidmann et al. 2020). In pi deficiency, plants have to minimize the resources allocation as well as it has to explore the soil. As a result, the primary root growth needs to be reduced along with the elongation of lateral roots and the formation of higher-order LRs (Waidmann et al. 2020). A repertoire of genes gets differentially activated in response to lateral root formation. These genes are majorly involved in auxin biosynthesis, transport, and signaling (Banda et al. 2019; Crombez et al. 2019). Systemic responses of phosphate sensing and signaling are largely managed by the transcription factor PHR1 (Rubio et al. 2001; Gutiérrez-Alanís et al. 2018). PHR1 binds on PHR1-binding sites (P1BS) and regulates the expression of phosphate starvation responses (PSR) genes (Fig. 2) (Rubio et al. 2001; Castrillo et al. 2017). Auxin signaling interacts with phosphate signaling in response to lateral root formation and elongation.

Lateral root initiation begins from the cells present in the pericycle known as lateral root founder cells (LRFCs). Auxin is known to mark the pre-branch site for future lateral roots (Banda et al. 2019). Pi starvation increases auxin level and sensitivity in root tip and lateral root primordia of Arabidopsis. Auxin receptor *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) expression increases under pi deficiency, causing the degradation of AUX/IAA proteins, which overall increases the auxin sensitivity in pi deficiency (Pérez-Torres et al. 2008). *TIR1* expression in low phosphate was found to be directly regulated by PHR1 (Castrillo et al. 2017). Auxin-response factors (ARFs) are downstream to the auxin signaling controlling various aspects of auxin signaling. ARF7 and ARF19 play important role in lateral root priming, lateral root

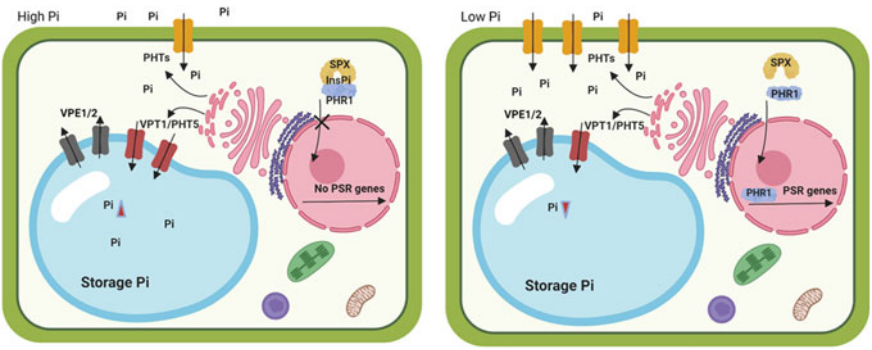

Fig. 2 Phosphate signaling in plant cell. Phosphate (pi) is taken up by phosphate transporters present on the cell membrane (PHTs). Vacuolar efflux transporters situated on tonoplast controls cytosolic pi concentration by transporting the pi in and out of the vacuole. PHOSPHATE STARVATION RESPONSE (PHR) is sequestered by SPX domain harboring proteins in the presence of Inositol Phosphate (InsPs). In the deficiency of phosphate SPX allows PHR to bind to cis-regulatory elements P1BS to regulate the expression of phosphate starvation-response (PSR) genes. Image created in BioRender.com

initiation, patterning, and initiation (Banda et al. 2019; Crombez et al. 2019). Interestingly, expression of PHR1 was also positively regulated by ARF7/19, and the impairment of lateral root growth in *arf7arf19* double mutant was also rescued by overexpressing the PHR1 (Huang et al. 2018). PIN-FORMED (PIN) transporters of auxin and several ARFs are also possible targets of PHR1 (Castrillo et al. 2017). Along with auxin several other mutants such as *lpr1, pnp, plt1;1plt1;4, alf3, siz1,* and *wrky75* showed differential regulation of lateral roots in low pi (Niu et al. 2013). (Huang and Zhang 2020) recently reviewed the role of different phytohormone in pi deficiency.

The response to low pi conforms to differential root growth in crops. Crop plants such as maize and rice have a fibrous root system that is generally deeper and shallower than the taproot system of Arabidopsis, and bean. Several genes have been identified in maize, rice, tomato, and bean that showed robust remodeling of root in varying pi conditions (Yang et al. 2007; Zhou et al. 2008; Dai et al. 2012; Postma et al. 2014; Zhou et al. 2014; Jia et al. 2018; Gonçalves et al. 2020). Simulation modeling of *Zea mays* roots using SimRoot suggested densely spaced but shorter lateral roots were more optimal to phosphorus acquisition (Postma et al. 2014). Maize with greater lateral root branching outperformed others in terms of phosphate acquisition and crop productivity. Higher uptake of phosphate led to 14% greater grain yield than control maize plants (Jia et al. 2018). Considering the above-mentioned studies of lateral root growth in pi deficiency, lateral roots show a promising role in battling pi deficiency. Branching of lateral roots overall improves the phosphate uptake and thus the growth of the plant.

3.3 Role of Root Hairs in Pi Deficiency

Root hairs are specialized tubular epidermal cells of plant roots that play a significant role in nutrient and water absorption (Salazar-Henao et al. 2016). The presence of root hairs near the root tip is considered a hotspot for pi assimilation as root hairs can be responsible for up to 90% of phosphate uptake by the plants (Brown et al. 2013). Restricted cell elongation in the primary root is the prerequisite for increasing root hair frequency (Salazar-Henao et al. 2016). Pi deficiency-induced callose deposition inhibits the cell to cell signaling in epidermal cells leading to shorter epidermal cells and increased expression of ENHANCER OF TRY AND CPC 1 (ETC), which results in a higher frequency of hairs per unit root length (Savage et al. 2013; Salazar-Henao et al. 2016b). Auxin has a significant role in root hair initiation and elongation (Knox et al. 2003; Salazar-Henao et al. 2016a; Bhosale et al. 2018; Giri et al. 2018). Pi deficiency-induced root hair growth is determined by bHLH transcription factor ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4). RSL4 protein synthesis increases in low pi and initiates the hair elongation and it gets gradually degraded by 26S proteasomal pathway. The amount of RSL4 synthesized directly determines the final size of differentiated root hair cells (Datta et al. 2015). Rise in auxin level ultimately promotes downstream ARFs. ARF19 induction in root apex shown to induce RSL4

in the root differentiation zone (Bhosale et al. 2018). Notably, AXR3/IAA17 and SHY2/IAA3 module are also involved in root hair initiation and elongation (Knox et al. 2003).

Improving RH density is an important agronomic trait (Brown et al. 2013). In rice and *Brachypodium*, overexpression of ROOT HAIR DEFECTIVE SIX-LIKE (RSL) class I bHLH transcription factor improved the root hair length (Kim et al. 2017; Zhang et al. 2018). Genome-wide association study (GWAS) of desi chickpea, maize, common bean identified several genetic loci associated with RH length (Yan et al. 2004; Zhu et al. 2005; Kohli et al. 2020). These QTLs analysis will help in modifying the surface area of root system architecture to improve crop productivity.

4 Conclusion and Future Perspective

Root system architecture is the underground three-dimensional arrangement of roots. RSA of a plant is the cumulative output of genomic and the immediate environmental conditions. Root plasticity reorganizes the RSA in response to water, mineral, microorganisms present in the soil. Nutrients are present in a heterogeneous manner in the soil. The limitation of nutrients in plants drastically affects the yield of the plant. Plants adapt to local patches of nutrients in the soil by proliferating the root growth for greater nutrient acquisition. Nitrogen and phosphorus are two essential nutrients that employ differential root systems for respective nutrient absorption. Deeper RSA with longer primary root and sparsely spaced lateral roots favor nitrogen uptake, while shorter primary root with higher lateral root branching with denser root hairs promotes phosphate acquisition. Molecular integration of nitrate and phosphate signaling also affected the RSA. Expression of nitrate transporter *NRT1.5* was strongly induced by Pi starvation, while its mutants observed an significant increase in primary root and reduced lateral roots in pi deficiency (Cui et al. 2019). Inhibition of cell division in phosphate deficiency is largely determined by the Fe-stimulated callose deposition in the root meristem. Promotion of lateral root increases the root surface that assists in topsoil foraging. Also, while increasing the foraged soil volume, the higher number of lateral roots also leads to a greater number of root tips. Root tips along with the root hairs are hotspots for pi uptake (Brown et al. 2013; Crombez et al. 2019). Integration of various nutrient signaling the in response to combinatorial stress project to develop the smart and sustainable agriculture.

Most studies done in Arabidopsis have been carried out under the artificial system by adding and reducing exogenous nutrient to "mimic" natural environmental conditions. These conditions are much more informative than the studies done in natural conditions. However, such studies propose the question of translation efficiency of nature mimicked studies to the natural conditions (Shahzad and Amtmann 2017). Buffered delivery of phosphate to Arabidopsis roots showed a non-canonical phenotype of pi deficiency. Phosphate buffered with Al_2O_3 particles supplied realistic low phosphate to plants. This buffered pi delivery resulted in smaller plants with reduced

lateral root branching density, longer root hair, and differential expression of canonical phosphate starvation genes (Hanlon et al. 2018). This inefficient translation of the lab generated information to the field calls for much robust and updated experimental design.

An interconnected hub underlies RSA remodeling, wherein different phytohormone, signal peptides, and nutrient signals integrate to regulate primary root, lateral root, and root hair formation and elongation. Several reports have identified genes and QTLs to develop a smart root system. Different nutrients interact with each other in the remodeling of the root system (Bouain et al. 2019; Medici et al. 2019a, b). In future scientists need to consider a much practical soil conditions with multiple nutrient deficiency. Our challenge would be to develop a smarter network root system to coordinate mineral nutrient homeostasis and root growth.

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Molecular Physiology of Nitrate Sensing by Roots

L. Ravazzolo, S. Trevisan, and S. Quaggiotti

Abstract Nitrogen (N) is needed by plants in great quantities. Besides being a nutrient, it also acts as a signal, regulating many downstream processes. Understanding the physiological and molecular processes regulating nitrogen use efficiency (NUE), particularly the below-ground traits related to root architecture, is crucial to reducing N loss and improving the efficacy of N fertilisation. Nitrate is the predominant source of nitrogen in aerobic agricultural soils and many studies have investigated the molecular mechanisms underlying the root response to nitrate, especially in Arabidopsis, one of the best studied model plants in plant biology. Maize is a very important crop, and its root apparatus is quite different from and more complex than that of Arabidopsis. Elucidating the molecular events underlying nitrate regulation of the root architecture in both these species is a crucial step towards improving technology transfer in the field. Auxin has been shown to play a prominent role in the transduction process leading to root architecture adjustments in response to nitrate availability in both Arabidopsis and maize, but the two plants differ in many other specific molecular components of this response.

Keywords Arabidopsis · Auxin · Maize · Nitrate · Nitrogen use efficiency · Root · Strigolactones · Transition zone

L. Ravazzolo · S. Trevisan · S. Quaggiotti (✉)
Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padua, Viale dell'Università 16, 35020 Legnaro, Italy
e-mail: silvia.quaggiotti@unipd.it

L. Ravazzolo
e-mail: laura.ravazzolo@unipd.it

S. Trevisan
e-mail: sara.3visan@libero.it

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1 NUE and Roots for a Second Green Revolution

In the twentieth century, novel technological innovations introduced during the Green Revolution led to massive changes in crop productivity worldwide. For the most part, success was achieved by a combination of high rates of investment in crop research (e.g., genetic improvement of crops to obtain high-yielding varieties) and in public services, market development and appropriate policy assistance (Pingali 2012). A central role was also played by the wide use of chemical fertilisers, in particular artificial nitrogenous fertilisers, made possible by the development of the Haber–Bosch process: almost 50% of people still rely on this process today (Godfray et al. 2010).

Nitrogen (N) is the essential macronutrient most needed by plants. It is a building block for nucleic acids, proteins, enzymes, and some metabolic components, such as chlorophyll, ATP and phytohormones (Andrews et al. 2013; Gojon 2017). Since it comprises almost 2% of plant dry matter and nearly 16% of total plant protein (Frink et al. 1999), unavailability limits plant development, crop yield and global primary production (Gutiérrez 2012). Besides its importance as a crucial plant nutrient, N also acts as a signalling molecule by regulating many plant processes, such as resistance to biotic and abiotic stresses, root development, dormancy, flowering time, leaf expansion, seed germination and hormone signalling (Bouguyon et al. 2012; O'Brien et al. 2016; Guan 2017; Izmailov and Nikitin 2020). Terrestrial plants can absorb N from the soil in two forms: inorganic compounds, such as nitrate (NO_3^-) and ammonium (NH_4^+), or organic compounds, such as amino acids, peptides, urea and proteins (Miller et al. 2007). Of these, nitrate (NO_3^-) is the preferred source in aerobic agricultural soils, while ammonium (NH_4^+) is critical in acidic and anaerobic environments.

Under most cropping situations, reduced N availability in the soil limits plant productivity (Dechorgnat et al. 2018). Furthermore, when the soil is subjected to massive synthetic N fertilisation, only half of the N fertiliser applied is absorbed by plants, while the remainder can have negative consequences on both the environment and human health (Gruber and Galloway 2008). As a result, understanding the physiological and molecular processes regulating nitrogen use efficiency (NUE) in plants is now crucial.

NUE is an important but complex concept that can be defined in terms of the total biomass or yield (e.g., of grain) produced per unit of N fertiliser applied to the soil (Xu et al. 2012). At each step of N metabolism, NUE is controlled by multiple interacting genetic and environmental factors, such as the nature of the N source, its interactions with microorganisms, soil type and management, and climate (Moll et al. 1982), and also by the efficiency of N uptake, remobilisation and assimilation (Hirel et al. 2007; Chen et al. 2020). In order to increase crop yield while decreasing N fertilisation, more sustainable agricultural practices along with controlled genetic manipulation and breeding strategies to improve crop NUE should be the new targets of a second green revolution (Han et al. 2015; Hirel and Lea 2018). For instance, a crop plant optimised for NUE should not just have a high rate of N uptake from

the soil and N incorporation into organic forms, but also be highly efficient in N use, recycling and remobilisation into grains (Omara et al. 2019). In this scenario, below-ground traits related to root architecture, N uptake and N fixation are crucial elements in defining NUE, and improving them is a key step towards a second green revolution (Den Herder et al. 2010). For example, the increased growth rate and biomass accumulation in maize, which correlated with the increase in yield in the USA Corn Belt, was very much dependent on changes in the root system architecture (RSA) (Hammer et al. 2009).

Root plastic development is pivotal in determining soil exploration and nutrient acquisition. In particular, the lateral spread and depth of root foraging are primary traits for the acquisition of soluble nutrients such as nitrate (Lynch 2007). Ground anchorage, water seeking and uptake, and establishing an advantageous relationship with symbiotic organisms are also highly dependent on the root system. Improving NUE in crops must, therefore, be based on an understanding of the physiological, molecular and signalling mechanisms directing root development in response to N fluctuations.

2 Root Morphology: Maize Root Versus Arabidopsis Root

Arabidopsis is one of the best studied model plants in plant biology, particularly the root system because of its transparent, simple organisation and its invariant cell lineage that can be traced back to a few founder cells (Benfey and Scheres 2000). The Arabidopsis root system comprises a single primary root (PR) and several lateral roots (LR) that remain active during the whole plant life cycle (Benfey and Schiefelbein 1994). It has no shoot-borne root system, and the number of epidermal cells that will form root hairs is quite predictable (Hochholdinger and Zimmermann 2008). Moreover, a single tiny layer of 8 cells forms the cortex, and just four cells form the quiescent centre (QC), while maize has as many as 800–1200 cells in the QC and 10–15 cortical cell layers (Smith and De Smet 2012) (Fig. 1).

Maize (*Zea mays* L.) is one of the most important cereal crops for both human and animal consumption. In 2019 global production reached 1124 million tons of grain over a cultivation area of 197 million ha (FAOSTAT 2019). In the developed world, maize is mainly used to feed livestock and produce biofuel, while in many developing countries it is primarily grown and consumed directly as food. Maize is particularly important in the diets of the people of sub-Saharan Africa and Latin America (Shiferaw et al. 2011). A simultaneous rise in demand and decline in productivity of the crop has been predicted, which will incur an annual cost of US$30 billion by 2050 (Rosegrant et al. 2009).

The maize root system is organised into an embryonic and a post-embryonic system. The embryonic root system derives directly from the seed to produce a primary root (PR) and seminal roots (SR). The PR elongates rapidly and forms many lateral roots (LR), although these usually do not persist throughout the maize plant's life (Feldman 1994). The post-embryonic root system, also called the shoot-borne

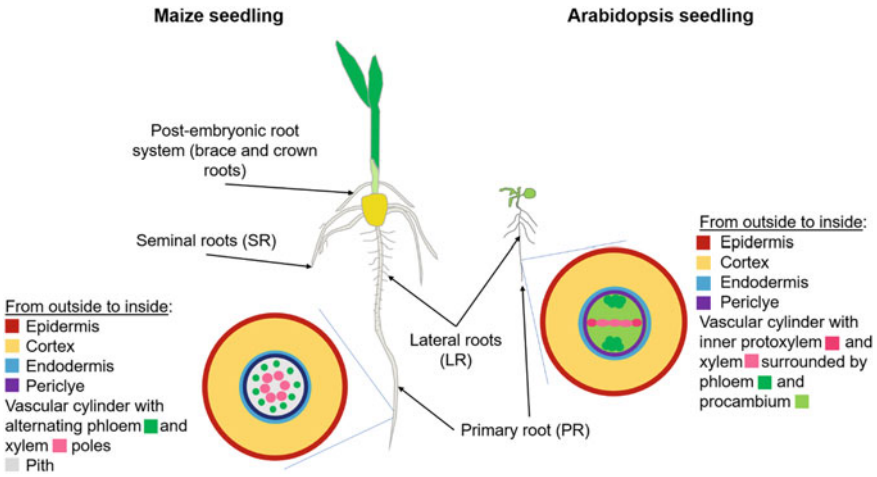

Fig. 1 Comparison of maize and Arabidopsis root systems with diagrams of the transverse sections of their primary roots. While the maize seedling has both an embryonic (PR: primary root; SR: seminal root) and a post-embryonic root system (brace and crown roots), Arabidopsis forms only a primary root (PR) with some lateral roots (LR) throughout its development

system, derives from the shoot and produces crown roots (CR) below the soil, and brace roots (BR) above the soil. Both CR and BR have many LR. Although LR were formerly considered part of the post-embryonic system, they may represent the link between the embryonic and post-embryonic systems, growing on PR, SR, CR and BR (Yu et al. 2015).

Maize PR and LR have a cylindrical structure with a distal extremity called the root apex (Alarcón et al. 2014). From outside to inside, the maize root consists of the epidermis, the cortex and the vascular cylinder (Alarcón et al. 2014). While the epidermis is uniseriate, the cortex comprises 6–10 layers of parenchymatous tissue, the innermost being the endodermis, the outermost the exodermis, both of which have highly specialised cells. The vascular cylinder consists of an outer layer, the pericycle, within which is the typical alternating organisation of xylem and phloem poles. In maize, the LR originate from pericycle and endodermis cells located opposite the phloem poles, while in eudicots, such as Arabidopsis, they originate from pericycle cells opposite the protoxylem poles (Hochholdinger et al. 2004; Casimiro et al. 2003; Jansen et al. 2012). Moreover, while Arabidopsis has only two protoxylem poles, maize can have ten or more phloem poles, resulting in a highly radial root branching phenotype (Smith and De Smet 2012).

Finally, roots can also be divided longitudinally into four consecutive zones (Baluška et al. 2010), namely the meristem (M, the first 2 mm up from the root tip), the transition zone (TZ, the next 2 mm above the M), the elongation zone (EZ, the next 4 mm above the TZ) and the maturation zone (MZ, from the EZ up to the seed). The TZ is a crucial root zone that integrates external and internal stimuli

into adaptative responses, and will therefore be described in some detail in the last paragraph.

3 Main Molecular Actors for Nitrate Sensing in Arabidopsis Root

Exogenous signals directly control plant growth and development by activating a large number of regulatory networks. Endogenous cellular sensors are able to quantitatively measure environmental fluctuations, which can arise in a very short time (milliseconds) and can last for several hours. This sensing/signalling pathway optimises the plants' adaptation to changing environments and variations in nutrient availabilities. Plants are able to sense NO_3^- in their environment and can rapidly respond to fluctuations in its availability. Nitrate sensors are thus elements that perceive alterations in the environment through ion binding, and transduce those alterations to an output.

Nitrate sensing systems have been partly identified in Arabidopsis (Fig. 2). They consist of membrane transporters that have already been shown to be involved in the uptake of NO_3^- into root cells. This dual ability gave rise to the term 'transceptor' (transporter/receptor). Transceptors have been described in yeast and animals. Interestingly, their function as sensors is independent of their function as transporters in plants (Ho et al. 2009).

Among the nitrate transporters, NITRATE TRANSPORTER 1 (NRT1.1)/PEPTIDE TRANSPORTER FAMILY 6.3 (NPF6.3) is representative of this class of transceptors (Bouguyon et al. 2015), acting also as a nitrate sensor (Muños et al. 2004).

NRT1.1/NPF6.3 participates in nitrate-dependent regulation of gene expression that leads to root development (Muños et al. 2004; Bouguyon et al. 2015). The activity of NRT1.1/NPF6.3 in N sensing is governed by Thr-101 (T101) phosphorylation. NRT1.1 acts as a dual-affinity transporter and nitrate sensor and responds to changes in soil nitrate concentrations by switching between phosphorylated and dephosphorylated forms (Liu et al. 1999; Liu and Tsay 2003; Ho et al. 2009; Léran et al. 2014).

Nitrate uptake and the related transceptor NRT1.1 activation induces phospholipase C (PLC) activity, which in turn increases cytoplasmic calcium (Riveras et al. 2015). CBL-INTERACTING SERINE/THREONINE-PROTEIN KINASE 23 (CIPK23), CALCINEURIN B-LIKE PROTEINS 1 AND 9 (CBL1 and CBL9), and the protein phosphatase ABSISSIC ACID INSENSITIVE 2 (ABI2) are then involved in decoding the calcium signal and triggering a phosphorylative cascade.

Under low nitrate conditions, phosphorylation of T101 promotes recruitment of NRT1.1 into functional membrane microdomains at the plasma membrane. This facilitates NRT1.1-dependent auxin flux and reduces auxin concentrations in the

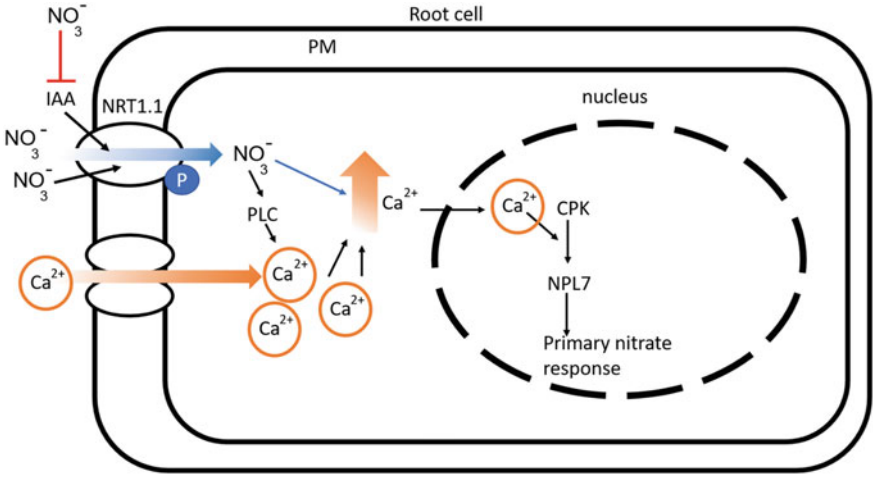

Fig. 2 Nitrate sensing in the Arabidopsis root cell. Nitrate is absorbed into the root cell by the plasma membrane-localised nitrate transporter family. In low nitrate conditions, the phosphorylation mechanism switches NRT1.1 into the high-affinity system. This sensing of NO_3^- alters the phosphorylation state of NRT1.1 causing calcium (Ca^{2+}) efflux through the activation of phospholipase C (PLC), which increases the cytosolic IP3 levels. The increased IP3 concentration in the cytosol induces the opening of Ca^{2+} channels and the accumulation of cytosolic Ca^{2+}. This calcium-dependent signalling results in variation in the expression of nitrate transporter genes. This activation stimulates LR growth, induces NRT1.1-auxin transport activity at the PM, and stimulates Ca^{2+}-ANR1 signalling from the endosomes. The signals resulting from the increased Ca^{2+} can be sensed by CPK10/30/32, which then phosphorylates NLP7. The phosphorylated NLP7 is retained in the nucleus to activate primary nitrate responsive genes. NRT1.1 also transports auxin, and nitrate-dependent auxin transport is an integral part of nitrate sensing, allowing root growth to adjust locally to variations in the nitrate supply in the soil

lateral root primordia (LRP) inhibiting their outgrowth. If nitrate increases, the non-phosphorylated NRT1.1 oligomerises and lateral mobility at the plasma membrane decreases, resulting in faster, inducible endocytosis. These processes could promote LR development by suppressing NRT1.1-auxin transport activity on the plasma membrane and initiating Ca_2^+-ANR1 (Ca_2^+-ARABIDOPSIS NITRATE REGULATED 1) signalling from the endosome (Zhang et al. 2019). ANR1 is the nuclear target of the nitrate-Ca^{2+} signalling cascade.

Once nitrate is sensed by NRT1.1, this signal needs to be sent to the nucleus to transduce its action on gene expression. NLP7 plays a prominent role in transmitting the nitrate signal from the membrane to the nucleus (Liu et al. 2017).

The nitrate-dependent calcium signal is necessary for changes in gene expression for the primary response which involves the regulation of several genes, such as *NRT2.1* and *TGA1*. This nitrate-dependent calcium influx activates the protein kinases CPK10/30/32, which in turn promotes phosphorylation of the transcription factor NLP7 (NIN-LIKE PROTEIN 7). The calcium signalling cascade can then influence other transcription factors (TFs) to control and coordinate additional responses,

which could ultimately regulate nitrate uptake by the roots and/or modulate the root architecture.

Nevertheless, NRT1.1 is also able to transport auxin, thereby generating nitrate-dependent auxin transport. The resulting auxin reallocation allows root growth to locally adjust to variations in the nitrate supply in the soil (Krouk et al. 2010b). When nitrate availability to the plant is low, CHL1/NRT1.1 functions as an auxin transporter to remove auxin from the LR primordia, thereby blocking their development (Krouk et al. 2010b; Mounier et al. 2014).

Nitrate perception triggers rapid transcriptional reprogramming (<5 min), suggesting that several TFs are involved in this regulation (Coneva et al. 2014; Obertello et al. 2015; Alvarez et al. 2020). For example, the aforementioned ANR1 would induce cell proliferation in LR tips and thus LR growth.

Several gene regulatory networks have been identified in which different TFs play a major role, including NLP7 (reviewed in Wang et al. 2018).

NLP7 directly regulates the nitrate-dependent transcriptional response (Alvarez et al. 2020; Marchive et al. 2013) and nitrate-assimilation processes (Marchive et al. 2013; Castaings et al. 2009; Konishi and Yanagisawa 2013). NLPs belongs to the family of RWP-RK transcription factors (Schauser et al. 2005; Chardin et al. 2014). *NLPs* genes are closely related to the leguminous *NIN* (nodule inception) genes. When the transceptor NRT1.1 perceives nitrate at the plasma membrane, the consequent calcium influx triggers nuclear retention of NLP7, which then binds with and regulates the transcription of its downstream target genes (Liu et al. 2017; Alvarez et al. 2020). The resulting transcription cascade enables the plant to respond to nitrate within minutes without requiring de novo protein synthesis (Wang et al. 2018; Marchive et al. 2013).

NRT2 proteins are members of the major facilitator superfamily (MFS) of transporter and transporter-like proteins (Pao et al. 1998) and their expression has been shown to be related to repression of LR initiation, suggesting their role either as nitrate sensors or signal transducers to coordinate the development of the root system using nutritional cues (Little et al. 2005).

Sensors need to be capable of responding to and reporting spatially-delimited signalling processes that might be restricted to specific organs, tissues, cells, organelles, or even to a subregion of the cytosol. Spatially-delimited sensing can be achieved by cell type expression of endogenous sensors and subcellular targeting of proteins. Endogenous sensors are often restricted to specific regions of the cytosol by tethering to membranes or other signalling components.

With regard to spatial localisation of nitrate sensing, it is tempting to suggest that root tips are central in N perception: they would rapidly sense N signals and send a signal to the shoots via the stele. A systemic signal would then come back to the roots in a root-shoot–root interplay, as described by Ruffel et al. (2011).

4 Regulation of Root Development by Nitrate Availability: Maize Versus Arabidopsis

As mentioned above, the effects of the N source on the root system are complex and depend on several factors, such as its concentration and form in the soil, the N endogenous status of the plant and the responsiveness of the different genotypes. Nitrate (NO_3^-) is the main N source for crops in aerobic soils (Vidal et al. 2020) and it became evident in the late 1990s that it was involved in root response (Zhang et al. 1999). Studies on root system architecture (RSA) and morphology are critical, given that RSA determines the ability of plants to explore the soil for water and nutrients, including nitrate (Asim et al. 2020).

The primary root (PR) is the first root to emerge in both dicots and monocots (Smith and De Smet 2012). The effect of nitrate on PR growth is controversial, but appears to vary greatly according to NO_3^- concentration, and temporal and spatial factors (Andrews et al. 2013; Vidal et al. 2015; Ruffel and Gojon 2017). PR growth in Arabidopsis is typically found to be relatively insensitive to or even induced by moderate nitrate availability, but it can also be inhibited by high nitrate supply (Vidal et al. 2013; Tian et al. 2014). For example, many *A. thaliana* accessions were found to have a nitrate stimulatory effect on PR growth after nine days of NO_3^- exposure at concentrations ranging from 0.05 to 5 mM (Walch-Liu and Forde 2008). A similar effect was obtained by Gifford et al. (2013) after twelve days of exposure to NO_3^- at concentrations ranging from 0 to 20 mM. In contrast, Zhang and Forde (1998) observed no changes in PR length with nitrate concentrations ranging from 0.01 to 100 mM (fourteen days of exposure), nor were any effects found by Signora et al. (2001) after seven days of exposure to NO_3^- in concentrations ranging from 0.1 to 10 mM. These last authors, however, reported an inhibitory effect at concentrations higher than 50 mM. Moreover, long-term exposure (17/18 days) to NO_3^- at a low concentration (0.01/1 mM) significantly inhibited PR elongation (Linkohr et al. 2002), while Naulin et al. (2020) recently reported that provision of 5 mM nitrate for 3–14 days stimulated PR growth, further suggesting increased meristem activity due to the involvement of cytokinin (CK) in nitrate signalling. Overall, these results indicate that the regulation of PR growth in response to nitrate in the model species *Arabidopsis thaliana* is highly complex and not entirely clear.

In maize, a consistent inhibitory effect on PR length was observed by Tian and co-authors (2005) after twelve days of growth at a nitrate concentration of 20 mM. These authors subsequently showed that after twelve days of treatment, nitrate concentrations lower than 0.5 mM had no effect on the elongation of primary, seminal and crown roots, while concentrations above 5 mM affected root elongation more significantly (Tian et al. 2008). A similar inhibitory effect was also observed after growing maize seedlings under two different NO_3^- concentrations (0.1 and 10 mM) for seven days, then exposing them, respectively, to 0.1 and 1 mM NO_3^- for 48 h (Zhao et al. 2007). More recently, maize PR growth was monitored over 48 h of NO_3^- provision (1 mM) to seedlings previously starved of N, revealing a dual effect: PR growth was stimulated after 2 h of nitrate provision and inhibited after 24–48 h (Manoli et al.

2016; Ravazzolo et al. 2019). The maize primary root would therefore seem able to adapt its pattern of growth according to variations in nitrate concentrations and length of exposure.

Unlike nitrate regulation of PR growth, which is in many ways still unclear, more is known about the molecular and morphological mechanisms governing lateral root (LR) formation and development in response to nitrate. LR create an extraordinary, extensive underground branching network (Atkinson et al. 2014) and perform fundamental functions of soil exploration, water and nutrient uptake, and establishment of beneficial symbioses. LR are generally more sensitive to variations in the nitrogen source than PR (Tian et al. 2014; Hachiya and Sakakibara 2017), but the role played by nitrate is complex and is bound by both genetics and the environment (Sun et al. 2017). LR development is usually still stimulated under mild nitrate deficiency, but is inhibited under severe N shortage (Krouk et al. 2010a). High nitrate supply, on the other hand, always exerts an inhibitory effect on LR growth.

According to Malamy and Benfey (1997), LR development generally unfolds in four stages: LR initiation, LR primordia (LRP) formation, LR outgrowth and emergence, LR elongation.

In maize, LR initiate from a few pericycle cells at the phloem poles, called founder cells, which undergo de-differentiation and proliferation to produce the LRP. Founder cell priming involves asymmetric cell division by cell cycle reactivation and auxin accumulation at the quiescent centre (Jung and McCouch 2013). Arabidopsis LRs, on the other hand, originate exclusively from pericycle founder cells positioned at opposite xylem poles (Dolan et al. 1993) and their initiation is positively regulated by auxin, gibberellins (GA) and jasmonate (JA), but negatively regulated by CK, abscisic acid (ABA) and high concentrations of ethylene (ET) (Péret et al., 2009; Guan et al. 2017; Vega et al. 2019). For instance, it has been hypothesised that ABA and CK reverse the auxin effect by reducing its polar transport (Shkolnik-Inbar and Bar-Zvi 2010), while JA promote both LR initiation and emergence (Raya-González et al. 2012). In addition, LR development and emergence are stimulated by treatments that raise ethylene production in the root (Ivanchenko et al. 2008). In maize, auxin modulates LR initiation, thereby determining pericycle cell length (Alarcón et al. 2019) as well as LR development (Ravazzolo et al. 2021). Furthermore, strigolactones (SLs) negatively regulate LR development in Arabidopsis (Ruyter-Spira et al. 2011) and rice (Sun et al. 2014, 2019), while inhibition of SL in maize seems to reactivate auxin signalling, so it is, at least in part, responsible for stimulating LR development by nitrate (Ravazzolo et al. 2021). Finally, a negative effect of ET and CK on maize LR initiation has also been hypothesised (Alarcón et al. 2014).

Two proteins involved in high affinity nitrate transport, NRT2.1 and NAR2.1, have been shown to act as positive regulators of the stimulatory effect of LR initiation by low nitrate (0.5 mM) in Arabidopsis (Remans et al. 2006b; Orsel et al. 2007). The aforementioned ANR1 (ARABIDOPSIS NITRATE-REGULATED1), a MADS-box transcription factor specifically expressed in LRP, and the NO_3^- "transceptor" NRT1.1/NPF6.3 have been identified as furthers key actors in LR development in response to nitrate in this model plant (Remans et al. 2006a). Transgenic Arabidopsis lines, in which *ANR1* expression was down-regulated or even

suppressed, gave rise to an Arabidopsis phenotype that was less responsive to localised NO_3^-. Consequently, it has been suggested that *ANR1* acts downstream of *NRT1.1/NPF6.3* in the signalling pathway stimulating LR initiation in response to low nitrate (Remans et al. 2006a). The Arabidopsis auxin biosynthetic gene *TAR2* has been reported to play a role in the stimulatory effect of low nitrate on LR development (Ma et al. 2014). *TAR2* encodes a tryptophan aminotransferase-related protein 2 and its expression in the pericycle and vasculature of developed roots close to the root tip is enhanced under low nitrogen conditions, but *tar2* null mutants do not display N-stimulated auxin accumulation in the root tip. Recently, it has also been shown that NRT1.1 phosphorylation in Ser 101 plays a key role in this response by facilitating auxin flow under low-nitrate conditions (Zhang et al. 2019). As previously reported, the nitrate "transceptor" NRT1.1/NPF6.3 transports both auxin and nitrate (Krouk et al. 2010b) and negatively regulates LR emergence at low nitrate concentrations by promoting auxin lateral basipetal transport out of the LR. High nitrate levels, on the other hand, inhibit NRT1.1/NPF6.3-dependent basipetal auxin transport leading to auxin accumulation in LR tips and stimulating their growth.

It has also been suggested that miR167 and its target AUXIN RESPONSE FACTOR 8 (ARF8) (Gifford et al. 2008) and miR393 and the auxin receptor AFB3 (AUXIN SIGNALING F-BOX PROTEIN 3) (Vidal et al. 2010) are additional important players in the regulation of LR initiation and LR outgrowth in Arabidopsis. MiR167/ARF8 is a module that regulates the ratio between LR initiation and development (Gifford et al. 2008), while the miR393/AFB3 regulatory module has been studied for its modulatory effect on both LR and PR growth in response to nitrate by integrating nitrate and auxin signalling (Vidal et al. 2010, 2013). In Arabidopsis, miR393 is encoded by the two loci MIR393a and MIR393b, and post-transcriptionally regulates mRNAs for the ubiquitin protein ligase SCFTIR1/AFB, auxin receptors TIR1 (Transport Inhibitor Response Protein 1), AFB1 (Auxin Signalling F-box Protein 1), AFB2 and AFB3 (Parry et al. 2009). The TIR1/AFBs constitute a small subset of F-box-containing auxin receptors and mediates proteasomal degradation of Aux/IAA transcriptional repressors to release the activities of auxin response factors (ARFs), thus promoting the transcription of auxin-responsive genes (dos Santos et al. 2009). In particular, AFB3 was found to be the only auxin receptor transcriptionally induced by nitrate and subsequently post-transcriptionally repressed by miR393. These studies suggest that, besides modulating auxin gradients in roots through NRT1.1/NPF6.3 activity (Krouk et al. 2010b), nitrate can also increase root auxin sensitivity by affecting *AFB3* expression (Bouguyon et al. 2016).

In cereals, such as maize, the molecular regulation of LR development in response to nitrate is complex (Bray and Topp 2018, and references therein) and, indeed, only a few lateral root mutants have been described in these crops, generally those related to auxin pathways (Hochholdinger and Tuberosa 2009; Atkinson et al. 2014; Yu et al. 2019). One example is the maize *rum1*, which encodes an Auxin/indole-3-acetic acid (Aux/IAA) protein called RUM1 (ROOTLESS WITH UNDETECTABLE MERISTEM 1) (von Behrens et al. 2011). Aux/IAA protein degradation leads to the release of ARFs (Auxin Response Factors), which can then bind to the promoters of downstream auxin-responsive genes involved in lateral and seminal root formation

(reviewed in Taylor-Teeples et al. 2016). *rul1* (*rum1-like1*) is the homolog of *rum1*, originating from an ancient maize genome duplication (Zhang et al. 2016). Both RUM1 and RUL1 have the canonical four-domain structure of Aux/IAA proteins and nuclear localisation, and they interact in vivo with ZmARF25 and ZmARF34 (Zhang et al. 2016), probably blocking LR formation in non-precursor pericycle cells (von Behrens et al. 2011). Moreover, RUM1 can directly bind to the promoter of *lrp1* (*lateral root primordia 1*) which encodes an auxin-inducible transcriptional activator (Zhang et al. 2015). In Arabidopsis, *AtLRP1* encodes a member of the SRS (short internodes-related sequence) family with a zinc finger motif and is involved in early lateral root formation (Smith and Fedoroff 1995). Maize *lrp1* expression is localised in the root meristem and emerging lateral root primordia (LRP), and is repressed by binding with RUM1, suggesting the involvement of LRP1 in maize auxin signal transduction downstream of *rum1* (Zhang et al. 2015). RUM1 can specifically interact also with RAP1 (RUM1 ASSOCIATED PROTEIN 1) (Zhang et al. 2016), while no interaction was observed between RUL1 and RAP1. The RAP1 family includes six other members, called RAP1-like: RAL1, RAL2, RAL3, RAL4, RAL5, RAL6 (Zhang et al. 2016). RAP1 is the homolog protein of AtSPR1 (SPIRAL 1), a nitrilase-associated microtubule-localised protein in Arabidopsis involved in the directional control of rapidly expanding cells (Nakajima et al. 2004).

Besides LR development, regulation of the shoot-borne roots of maize is also crucial (Taramino et al. 2007). The paralogous LOB-domain maize proteins RTCS (ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS) and RTCL (RTCS-Like) are important for development of the crown root (Xu et al. 2015). In particular, the LBD (LATERAL ORGAN BOUNDARIES Domain) protein family plays a role in defining organ borders and in many other plant developmental processes (Majer and Hochholdinger 2011). As mentioned above, maize LBD-dependent signalling in root development includes *RTCS* and *RTCL* expression, both of which have auxin-responsive elements and are preferentially expressed in roots. Their expression is activated through binding with auxin-induced *ZmARF34*. Consequently, RTCL and RTCS bind to the promoters of the genes operating downstream by acting as transcription factors (Xu et al. 2015). In maize, RTCS is the closest homolog of AtLBD29, and *rtcs* acts upstream of *rtcl*. Although *AtLBD19*, *AtLBD16* and *AtLBD29* have redundant functions in LR emergence in Arabidopsis (Okushima et al. 2007), the maize *rtcs/rtcl* double mutants did not reduce LR density. Hence, LBD proteins in maize seem to be involved only in shoot-born root formation (Xu et al. 2015).

As with Arabidopsis, the establishment of auxin response maxima in LR initiation is also crucial in maize (Atkinson et al. 2014; Ötvös et al. 2021). Auxin transport is fundamental to the generation of these local auxin maxima, and PIN transporters determine polar auxin transport (PAT). It has been shown that where LR develop, the monocot-specific *PIN9* can modulate auxin efflux to pericycle cells at the phloem poles, thereby activating the cell cycle (Yu et al. 2015).

5 Maize Transition Zone and Nitrate Sensing

The transition zone (TZ) is the part of the root between the meristem and the elongation zone. It is a distinctive zone that translates several endogenous and exogenous signals into adaptative differential growth (Baluška and Mancuso 2013; Trevisan et al. 2014). It has been known for a long time that TZ cells undergo a series of crucial changes in their cytoarchitecture and are characterised by a complex system of polar auxin transport circuits that make them highly responsive to auxin (Baluška et al. 2010).

These characteristics make the TZ a dynamic sensor that can reorganize root growth in response to various stimuli, such as gravity (Masi et al. 2015), touch and extracellular calcium (Ishikawa and Evans 1992; Baluška et al. 1996), osmotic stress (Baluška and Mancuso 2013), hypoxia (Pucciariello and Perata 2017; Manrique-Gil et al. 2021), oxidative stress and auxin (Mugnai et al. 2014) and heavy metal stress (Sivaguru et al. 2013; Yang et al. 2014; Sahay and Gupta 2017; Kong et al. 2018; Wei et al. 2020). In addition, the TZ seems to be the designated nitrate responsive region in maize (Manoli et al. 2014; Trevisan et al. 2014).

Coordinated spatio-temporal regulation of the expression of genes encoding a nitrate reductase (NR) and a non-symbiotic haemoglobin (nsHb) soon after nitrate provision to N-depleted roots has been observed in the root epidermal TZ cells of maize (Trevisan et al. 2011). Nitrate perception triggers a sudden rise in NO production by NR and concomitant activation of nsHbs to rapidly detoxify high intracellular NO concentrations, thus protecting the cell from oxidative stress. This process was explored further by Manoli et al. (2014), who demonstrated in vivo NR-dependent NO production soon after nitrate supply using confocal microscopy. Remarkably, the TZ appeared to be the elected zone for NO production, making it one of the maize root regions most responsive to nitrate. Together, these studies present a new perspective on the contribution of nitric oxide (NO) to the root response to nitrate (Trevisan et al. 2011, 2015; Manoli et al. 2014). NO is a general bioactive plant signalling molecule involved in many physiological and developmental processes, and regulates both biotic and abiotic stress responses and hormonal crosstalk (Arora et al. 2016; Kolbert et al. 2019; Sánchez-Vicente et al. 2019). It has been reported to be required for RSA development (Prakash et al. 2020), particularly in PR growth (Fernández-Marcos et al. 2012; Manoli et al. 2014), adventitious root formation (Pagnussat et al. 2003), LR formation (Wang et al. 2010) and root hair formation (Lombardo and Lamattina 2018). It has been suggested that auxin and NO act together in the process of LR development (Correa-Aragunde et al. 2015) and regulation of the stem-cell niche (Sanz et al. 2014). It has also been demonstrated that the NO produced after nitrate provision triggers auxin and PIN1 re-localisation in TZ cells, which favours cell expansion over cell division and guides root apex elongation (Manoli et al. 2016). Detailed RNA-sequencing has subsequently revealed the transcriptional signature of the TZ cells in response to nitrate supply, and many crucial transcripts have been identified (Trevisan et al. 2015). Strigolactone (SL) biosynthesis and signalling has

emerged from this study as a further pivotal element regulating the response to N availability in TZ cells.

SLs are carotenoid-derived phytohormones that regulate plant development in response to various environmental stimuli and in concert with many other regulators through their action as both endogenous and exogenous signalling molecules (Machin et al. 2020). Since the interplay of NO and auxin is important in regulating multiple aspects of root biology (Sami et al. 2018) and a connection between SLs and NO has been shown (Kolbert et al. 2019; Oláh et al. 2020, 2021), the role of SLs in the pathway where NO acts as a coordinator of nitrate and auxin signalling to regulate the overall maize root response is interesting. A recent survey (Ravazzolo et al. unpublished data) suggests that the observed shutdown of SL production in response to nitrate might occur independently of NO production, leading us to suppose that there are two distinct signalling pathways controlling NO production and SL inhibition in response to nitrate provision.

Many studies have shown that soil nutrient deficiencies, particularly phosphate starvation, induce increased SL biosynthesis, which in turn influences the root architecture (Kohlen et al. 2011; Koltai, 2015; Ito et al. 2016; Marzec and Melzer 2018). Recently, it has been shown that a lack of nitrogen could be more effectual than phosphorous deficiency in stimulating SL exudation in maize root, while nitrate availability rapidly switched off SL exudation (Ravazzolo et al. 2019). In the same study, it was also suggested that the shutdown of SL production by nitrate could play a role in the complex LR developmental pathway. The negative regulation of LR by SLs has already been documented in Arabidopsis (Ruyter-Spira et al. 2011) and rice (Sun et al. 2014, 2019). Indeed, a lower number of lateral root primordia (LRP) were found in both these species when plants were treated with a racemic mixture of an SL analogue (*rac*-GR24). Moreover, the impact of SLs on root development in response to nutrient deprivation appeared to be dependent on auxin levels (Omoarelojie et al. 2019), and some studies on rice (Zhang et al. 2010), pea (Ligerot et al. 2017) and Arabidopsis (de Jong et al. 2014) have focused more specifically on the interaction between SLs and auxin (Rameau et al. 2019). With regard to maize root, a more recent systemic molecular study based on RNA-sequencing highlighted some genes whose transcription is regulated in response to nitrate and is dependent on auxin, SLs, or both (Ravazzolo et al. 2021). Four independent clusters of transcripts regulated by nitrate and dependent on either auxin or SLs, or on both, or independent of both of them have been described in maize root. Each cluster holds several putative molecular candidates potentially attributable to these alternative transduction pathways. They represent a useful starting point for broadening current knowledge of the entire process.

A transcriptomic study on TZ cells (Trevisan et al. 2015) has also enabled identification of further previously unknown players contributing to nitrate perception by this root region. Particular attention should be paid to the coordinated opposite regulation of the transcription of *ZmUPB1* and *ZmPRX112* that occurs in TZ cells and that seems to affect the overall balance between H_2O_2 and $O_2^{\bullet -}$ in the first mm of the primary root and the equilibrium between cell division and elongation that in turn drives PR growth in response to nitrate in maize (Trevisan et al. 2019). It is known

that differences in superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) accumulation in the root tip significantly affect PR growth and differentiation (Dunand et al. 2007) and the transition of cells from a zone of cell division to a zone of cell elongation and differentiation (Tsukagoshi et al. 2010). *ZmUPB1* orthologue expression in the TZ is highly repressed by nitrate supply but induced by N deficiency (Trevisan et al. 2015).

From the above, it appears that the perception of nitrate is very high in the TZ of maize, and that this region is a promising reservoir of useful information for studying and modelling the process leading to the adaptative response of roots to nitrate availability. A sophisticated interplay of highly specific events, including NO and ROS homeostasis regulation, and hormonal (auxin and SLs) accumulation and signalling seems to characterise the early stages of nitrate perception in these dedicated root cells, which in turn activate a transduction pathway to the whole root (Fig. 3).

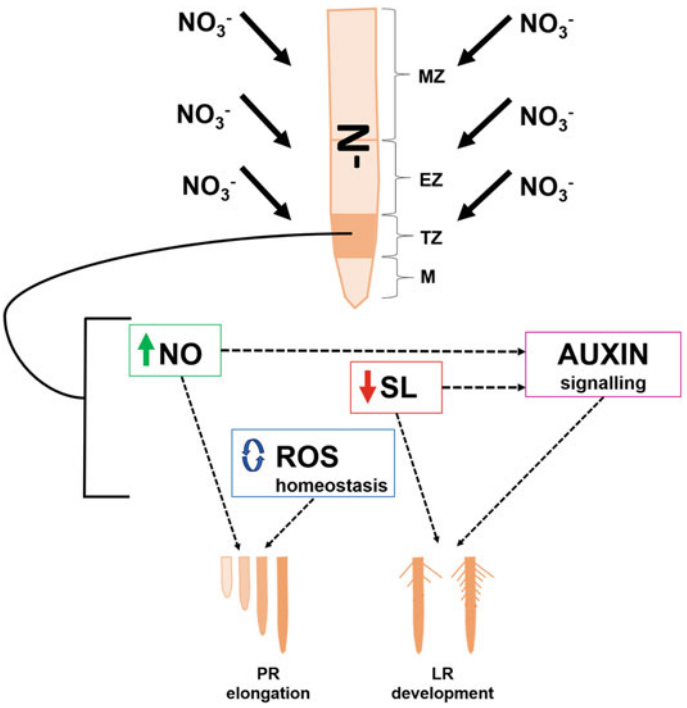

Fig. 3 Proposed model of nitrate response in the maize root transition zone (TZ). After experiencing nitrate deficiency (-N), the maize root perceives the nitrate (NO_3^-) supply and many transduction pathways from the transition zone (TZ) are activated leading to an adaptative response. Some key signals are represented by nitric oxide (NO) and reactive oxygen species (ROS), which are implicated in primary root (PR) elongation, while strigolactones (SL) and auxin are involved in lateral root (LR) development. *Abbreviations*: MZ, maturation zone; EZ, elongation zone; TZ, transition zone; M, meristem zone

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Role of Arbuscular Mycorrhizal Fungi in Root Development with a New Dimension in the Root Web Network

Palak Nagpal, Rachna Kapila, and Shantanu Mandal

Abstract A number of interactions take place in the soil. These interactions are important for the physiology, growth and development of the plant. It is one of the important interactions is the symbiotic relationship between the arbuscular mycorrhizal fungi and the roots of the plant. It tends to affect the growth patterns of plant indirectly by forming colonization in roots through hyphae. Many benefits like more nutrient uptake, more water absorption and an increase in biomass are provided to plants through the roots. The hyphal colonization also helps the plant to survive in harsh environmental conditions and from biotic and abiotic stress. Many studies and researches have proved that the inoculation of arbuscular mycorrhiza to the roots of the plant acts as a benefit for them. Findings also say that arbuscular mycorrhiza resists weeds by decreasing the amount of nutrition for them. In the presented chapter, the studies, researches and Findings, about the relationship between roots and Arbuscular mycorrhizal fungi and their importance have been covered.

Keywords Arbuscular mycorrhizal fungi · Root colonization · Nutrient uptake · Biotic and abiotic stress

1 Introduction

Arbuscular mycorrhizal (AM) fungi, is one of the most well-known group of obligate symbiotic fungi that colonizes approximately 70–80% of the land plant species and belongs to a monophyletic group, the Glomeromycota (Parniske 2008; Harrison 2005). Arbuscular mycorrhizae are present in the soil in forms like spores. During germination, the hyphal germ tube grows in search of the host root. As the symbiotic relationship is formed, the fungus forms appressorium in the root surface through which it enters the root. However, the growth depends upon the species and type of plant (Cavagnaro et al. 2001; Harrison 2005).

P. Nagpal · R. Kapila · S. Mandal (✉)
Department of Biology, Institute of Home Economics, University of Delhi, New Delhi, India
e-mail: shantanu.mandal@ihe.du.ac.in

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These spores can even germinate in water without the signal of plants (Bécard and Piché 1989; Harrison 2005). It uses triglyceride and glycogen reserves for the growth and hyphal germ tube extension. The symbiotic development of Arbuscular Mycorrhiza results in the formation of Arbascules. These are tree-shaped subcellular structures along with plant cells. It acts as the main door for nutrition exchange between the plant and symbiotic fungal partners (Parniske 2008; Harrison 2005). The association connects the plant and fungi with hyphae that can be 100 m long or hyphae per cubic centimetre of soil. These hyphae proceed with the intake of water and nutrients, phosphate predominantly. In return for providing nutrients, it gets carbohydrates from plants (Parniske 2008; Harrison 2005).

Mycorrhizal colonisation can also increase root longevity through a number of mechanisms, including improved resistance to drying soil and increased protection against root pathogens (Eissenstat et al. 2000). According to the reports, arbuscular mycorrhiza leads to an increase in root branching of plants and increasing plant biomass (Fig. 1) (Hodge et al. 2001; Sukumar et al. 2013; Gutjahr and Paszkowski 2013) but another report by Hetrick (1991), Gutjahr and Paszkowski (2013) says that it reduces root branching and length of roots. Depending on the environmental conditions and genetic variations, arbuscular mycorrhizae induces root growth in maize and soybeans cultivars (Zhu et al. 2005; Gutjahr and Paszkowski 2013).

The inoculation of arbuscular mycorrhizae to only one side of the roots of peanut and pigeon pea resulted in a higher number of lateral roots as compared to non-inoculated ones (Volpe et al. 2013; Gutjahr and Paszkowski 2013). Arbuscular mycorrhiza can rescue reduced lateral root growth and restore wild type root system morphology (Gutjahr and Paszkowski 2013). Another example can be seen in maize mutants. The maize mutant which lacked embryonic lateral roots when inoculated with fungi developed bushy lateral roots even at elevated phosphate levels (Fig. 1) Paszkowski and Boller 2002; Gutjahr and Paszkowski 2013). Indicating, the importance of AM fungi in plant roots production/development.

However, AM fungi not only improves the plant roots growth and its physiology but also improves the overall physiological and ecological aspects of the plants by providing the plants with a horde of beneficial effects and this chapter is a summarization of such effects that the AM fungi provide to the host plants as a result of root colonization.

2 Improved Nutrient Uptake

Arbuscular mycorrhizae increase the uptake of nitrogen in mycorrhizal plants (Araim et al. 2009; Rasouli-Sadaghiani et al. 2010; Miransari 2011) by activating the ammonium transporter of plants (Guether et al. 2009). Nitrogen in plants is responsible for the synthesis of amino acids, nucleic acids, proteins and chlorophylls (Marschner 1995). Thus improves the overall physiology of the plants (Fig. 1).

The hyphal network of arbuscular mycorrhizae tends to reach the depth of soil and absorb more nutrients. Phosphorus is one of the nutrients found in depths of

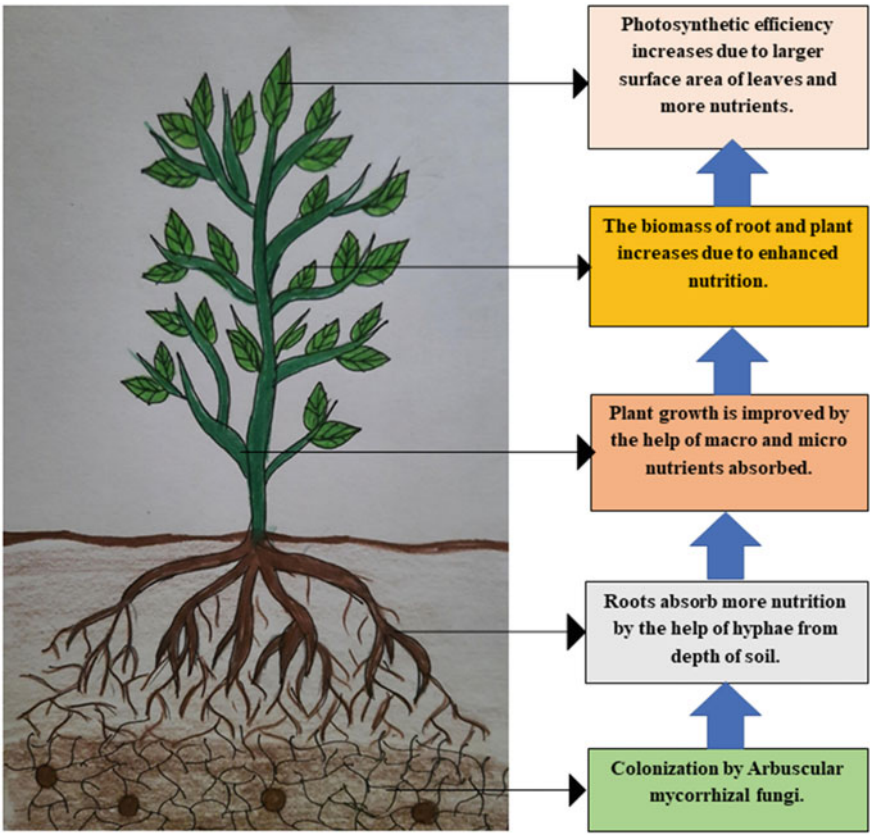

Fig. 1 Enhancement in morphological and physiological processes occurring in plants through Arbuscular mycorrhizal colonisation. The figure above shows Arbuscular mycorrhizal colonization in plants through the fungal hyphae. These hyphae help the roots of the plants to absorb nutrients from the soil that are found in the depth of soil like phosphorous. More nutrition absorbing efficiency leads to appropriate plant growth and development. Leaves with good surface area help the plant to perform photosynthesis efficiently

soil (Ruiz-Lozano et al., 2012). The hyphae have more tendencies to hold Phosphate ions even at low concentration for absorption than the plants roots (Fig. 1). This factor facilitates the continuous supply of phosphorus to the plants (Bolan 1991), an important essential macro-nutrient that is required by the plants in large amounts.

Along with nitrogen and phosphorus, the plants are also boosted with micro-nutrients as a result of AM colonization. Micro-nutrients are those that are required in small amounts but if deficient, can cause damage to the plant in various aspects (Bacha et al. 1997). They help in the photosynthesis and synthesis of proteins in plants (Marschner 1995). Inoculation of arbuscular mycorrhiza to the plants enhances the uptake of Fe, Mn, Cu and Zn (Smith and Read 2008; Mathur et al. 2006; Araim

et al. 2009). As a result, mycorrhizal colonisation increases the host plant's overall physiological aspects by supplying balanced nutrients (Fig. 1).

3 Promotes Growth

Arbuscular mycorrhizal symbiosis is an ancient association formed between plant and fungi. The hyphae system of mycorrhizae helps the plant root to absorb more and more nutrients which are delivered to the plant in return for photosynthetically assimilated carbon. Thus, it is to be expected that a host plant will benefit directly from the AM symbiosis through increased nutrient uptake, and, consequentially, increased growth (Fig. 1) (Smith and Read 2008).

4 Improves Photosynthetic Efficiency

The plant is said to be more photosynthetic when it converts more light energy into chemical energy through which it carries out the process (Zhu et al. 2010c). The inoculation of Arbuscular mycorrhiza to the plants leads to more well-developed leaves with greater surface areas (Harris and Paul 1987) leading to more synthesis of Chlorophyll (Giri and Mukerji 2004), and more is the chlorophyll more it will result in a quality of photosynthesis positively (Fig. 1) (Sheng et al. 2009).

5 Alters the Level of Phytohormone

Inoculation of arbuscular mycorrhiza leads to change in phytohormone level in the plants like jasmonic acid, gibberellic acid and cytokinins (Allen et al. 1980, 1982; Hause et al. 2002; Shaul-Keinan et al. 2002). According to (Perazza et al. 1998; Traw and Bergelson 2003; Li et al. 2004; Maes et al. 2008; Maes and Goossens 2010), these hormones improves the secondary metabolite production by modulating epidermal differentiation programmes, which results in increased trichome densities, ectopic trichome formation or aberrant trichome morphologies. Therefore, opens a whole new dimension in plant-pathogen interaction mediated by mycorrhizal fungi.

6 Provides Resistance from Abiotic Stress

6.1 Salinity

Salinity or more salt in plants can decrease its growth rate and can affect the leaf expansion rate and net assimilation capacity of the plant (Hasanuzzaman et al. 2009, 2013) Due to the AM colonization, the plant absorbs more water from the soil through hyphae of the fungi (Porcel et al. 2012; Hameed et al. 2014). For plants that survive at saline conditions, inoculation of the arbuscular mycorrhiza can enhance its hydraulic conductivity (Kapoor et al. 2008) and can increase its stomatal conductance (Sheng et al. 2008, 2011). It also reduces stress by decreasing the membrane lipid peroxidation in salinity exposed plants. The plants accumulate a high amount of soluble sugars in host plants when inoculated (Fig. 2) (Abdel Latef and Chaoxing 2014; Talaat and Shawky 2014; Yang et al. 2014).

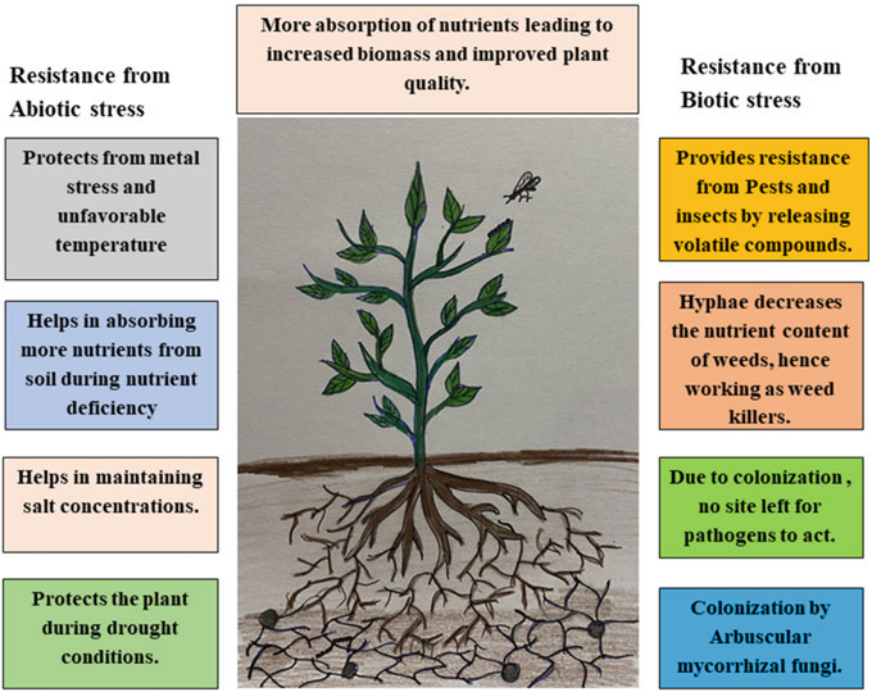

Fig. 2 Resistance from biotic and abiotic stress provided by Arbuscular mycorrhizal fungi colonization. The plant shows the colonization by the hyphae of Arbuscular mycorrhiza. With the help of these, the plant gets the power to resist biotic and abiotic stress. Hyphae absorb more water from the soil helping the plants survive in drought and hence decrease salt concentration. More macro and micronutrients are absorbed through hyphae. AM colonisation also helps in resisting pests by releasing volatile compounds, and works as weed killers by decreasing their nutrients amount. This all contributes to the healthy growth and development of the plant

7 Drought Stress

Water deficiency in plants affects their growth and development (Moussa and Abdel-Aziz 2008; Hasanuzzaman et al. 2013). Arbuscular Mycorrhizal colonization improves the root length, leaf area and nutrient uptake of the host plants (Al-Karaki et al. 2004; Gholamhoseini et al. 2013; Kapoor et al. 2013). The mycorrhizae secrete glomalin which enhances water absorption and nutrient uptake (Miransari 2010; Gholamhoseini et al. 2013; Gong et al. 2013; Pagano 2014). It is directly involved in some of the important plant processes like direct uptake and transfer of water, increased osmotic adjustment and better protection against damage (Rapparini and Peñuelas 2014). The colonization results in more leaf water potential, increased stomatal conductance and transpiration and photosynthesis during drought (Fig. 2) (Lee et al. 2012; Gholamhoseini et al. 2013).

8 Metals

Metals in plants cause damage to plants as well as humans by reducing the productivity of plants (Garg and Singla 2012). Toxic metals in plants leads to harmful effects such as inhibition of growth, seed germination, root elongation and decreased photosynthesis rate (Drzewiecka et al. 2012). The inoculation of mycorrhizal fungi restricts the metals by the secreting compounds and by changing its pH to regulate plant processes (Malekzadeh et al. 2011). Glomulin secreted seizes metals and reduces their toxicity (Fig. 2).

9 Temperature Stress

Temperature is another factor that affects plant growth. At low temperature, the cellular metabolism of plants is affected (Thakur and Nayyar 2013). Cold temperatures suppress plant development and symbiotic efficiency (Wu and Zou 2010; Gavito and Azón-Aguilar 2012). Arbuscular mycorrhizal Fungus improves plants tolerance to cold temperatures (Fig. 2). Reports have proved that the AM inoculated plants grow better than the non-arbuscular mycorrhizal plants in terms of growth and development (Zhu et al. 2010a, b; Abdel Latef and Chaoxing 2011).

10 Provides Resistance from Biotic Stress

Resistance to biotic stress as a result of AM colonisation is a novel feature of the AM symbiosis (Whipps 2004; Pozo et al. 2009). Although, this bio-protection has been

widely observed in a variety of plant systems, the basic mechanisms remain relatively unknown. Plant defence responses are adjusted during mycorrhiza establishment to achieve a stable symbiosis. This modulation will lead to a mild but successful activation of plant immune responses, both locally and systemically. The plant enters a primed state as a result of this stimulation, allowing for more successful activation of defence (Pozo et al. 2009). The relationship typically decreases harm inflicted by soil-borne pathogens, but the effects on shoot-targeting species are strongly dependent on the attacker's lifestyle (Fig. 2) (Pozo et al. 2009).

Pozo et al. (2009) stated that aboveground mycorrhiza-induced resistance seems to be successful against necrotrophic pathogens and generalist chewing insects, but not against biotrophs. The authors also suggested that the output spectrum of mycorrhizal induced resistance correlates with jasmonic acid-dependent plant defence potentiation. They also speculated that this form of mediated resistance may be one of the reasons why root associations with AM fungi have continued throughout plant evolution (Pozo et al. 2009).

An increased spectrum of secondary metabolites (terpenoids) by mycorrhizal symbiosis is also an effective way to boost both direct and indirect plant defence against herbivorous insects. This expanded terpenoids compound repertoire is associated with AM-mediated nutrient absorption, overall plant growth and physiology, and increased transcription levels of specific genes involved in the terpenoids biosynthesis pathway (Fig. 2).

Terpenoidal compound primed defences responses in mycorrhizal plants may also be transmitted to subsequent plants through a common below-ground network. As a result, this provides an additional arsenal in plant defence policy strategy and pest controls in agricultural environments (Sharma et al. 2017).

11 Arbuscular Mycorrhizal Fungi (AMF) as (Agro) Ecosystem Engineers

Changes in the structure and operation of host plant species are found to be facilitated by symbiotic relationships. Parasitic plants, for example, can influence plant diversity by suppressing competitive population dominants (Smith and Read 2008; Irving and Cameron 2009; Cameron 2010). Arbuscular Mycorrhizal fungi show alteration in host populations by increasing the nutrient uptake and development and suppressing non-mycorrhizal organisms. The reduction in the nutritional content of weeds is related to the allopathic effect of mycorrhizal fungi reducing numbers of root hairs and hence surface area for nutrient uptake of non-mycorrhizal plant species (Francis and Read 1994; Cameron 2010). Arbuscular Mycorrhizal fungi can thus serve as ecosystem engineers, fostering changes in host plant communities by physiologically suppressing non-contributing or non-mycorrhizal plant species (Cameron 2010). This contrast in plant reaction to AM fungi has been proposed as a method for weed suppression (many of which are non-mycorrhizal) in agroecosystems where

mycorrhizal crop species are grown (Cameron 2010). Rinaudo et al. (2010) have extensively recorded this occurrence (Fig. 2). They demonstrated the suppression of the weed species *Chenopodium album* while benefiting the crop plant *Helianthus annuus* in their report. These beneficial properties of Am fungi may be exploited as potential herbicides, lowering the cost and environmental impact of chemical herbicides (Fig. 2) (Cameron 2010). However, this mechanism is not suitable for those weeds plants that do not form AM symbiosis.

12 Relationship Between Strigolactone and AM Fungi

The relationship between Strigolactones (SLs) and AMF has been discovered recently. The resting spores or chlamydospores of AMF germinates under appropriate condition and shows a hyphal extension to a limited extent (Bécard and Piché 1989; Mosse 1988; Xie et al. 2010). During this time of forage, if the growing hyphae do not encounters the plant roots, the hyphae stop growing and becomes quiescent. The hyphae of AMF differentiates into complex morphological systems characterised by extensive branching in the presence of host roots (Xie et al. 2010; Giovannetti et al. 1993; Buee et al. 2000). This is visible without any obvious contact between the host roots and the fungus, and host root exudates alone are sufficient to induce hyphal branching (Xie et al. 2010; Giovannetti et al. 1996). Branching influences are cues for hyphal branching that are produced from host plant roots (Xie et al. 2010; Giovannetti et al. 1996; Nagahashi and Douds 2000). Strigolactones (5-deoxystrigol) were later identified as one of many secondary metabolites extracted from root exudates by Akiyama et al. (2005). Strigolactones are a carotenoid analogue that has attracted a lot of recognition since their discovery as a novel category of plant hormone (Mitra et al. 2021; Kumar et al., 2015). Some experiments have also shown how pathogenic and saprotrophic fungi responds to SL's (Mitra et al. 2021; Akiyama et al. 2010; Carvalhais et al. 2019; Choi et al. 2020; Lanfranco et al. 2017). The discovery that SLs allow parasites to germinate and attract AM fungi nutrients suggested that parasites can be controlled by regulating AMF colonisation (Mitra et al. 2021; Akiyama et al. 2005). According to some studies, there is a decrease in seed germination of parasitic weeds in reaction to AMF inoculation, which may be attributed to the down-regulation of the SL's. This is likely to be an autoregulation strategy for the host plant to prevent unnecessary colonisation, which may be metabolically costly (Mitra et al. 2021; Staehelin et al. 2011).

13 Conclusions

In the last decade, there has been a substantial advancement in plant mycorrhizal symbiosis owing to its enormous importance to plant communities. This symbiosis not only improves the physiological and mechanical aspects of plants but also opens

whole new dimensions of opportunities and research for the plant biologist. The present chapter depicts a few of its avenues. Therefore a holistic approach should be taken to understand its importance for Mother Nature.

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Ally or Foe: Role of Soil Microbiota in Shaping Root Architecture

Srayan Ghosh and Shraboni Ghosh

Abstract The rhizosphere comprises of a plethora of microorganisms that either partage or exploits nutrients from the host plants. Together, these microorganisms constitute the root microbiota that can govern the root shape and architecture of plants. Certain microorganisms secrete volatiles that promote lateral root branching and foster the growth of the aerial shoots whereas many cause root necrosis culminating into death of the entire plant. In the current scenario wherein, there is an increased demand in crop production to feed the growing human population it is important to devise ways or strategies that promote root biomass and associated plant yield. A healthy root biomass along with efficient nutrient mobilization from the soil plays a crucial role in increasing the yield of the plant. In this chapter, we discuss how the root microbiota modulates the plant root architecture. We also highlight through manipulation of microbiome; we can not only increase the root biomass but also promote the overall plant growth. We have also discussed how the soil microbiota governs the gene expression pattern in the roots which governs the overall developmental and physiological status of the host plant.

Keywords Beneficial microbes · Symbiosis · Pathogen · Lateral root · Microbiome

1 Introduction

Plants behave as the 'ecosystem engineers' in our biome. The plant roots play an central role in recycling and conversion of minerals/organic compounds across different spheres of our ecosystem (Freschet et al. 2021). The rhizosphere is a reservoir to millions of microorganisms which survive in close association with plants growing on a particular soil profile. The plant roots are exposed to such microorganisms ranges from bacteria, fungi, nematodes, oomycetes, viruses and archaebacteria etc. The roots often secrete certain compounds such as nutrients, mucilage, exudates

S. Ghosh (✉) · S. Ghosh
National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India

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and border cells in the rhizosphere which attracts the growth of microorganisms (Philippot et al. 2013). Such organisms have a profound impact on the growth and development of plants growing in an ecosystem. However, the interaction of plants with such microorganisms can be either beneficial or detrimental for the growth of the plant. The association of plants with such microorganisms can be of several types such as mutualism, commensalism, and parasitism. Mutualism refers to the interaction wherein both the host plant and the microorganism sustain and promote the growth of each other, for example N_2 fixing bacteria, *Rhizobium*. However, when one interactor benefits from the association at the cost of the other such interaction is known as parasitism.

The root traits are a part of a complex range of strategies for nutrient uptake adopted by plants. Fast growing plants possess comparatively thinner roots follow a 'do-it-yourself strategy' wherein the roots itself have been developed for efficient soil exploration strategy. On the other hand, slow growing plants possess a 'outsourcing' nutrient acquisition strategy wherein they form thicker and denser root system to harbor arbuscular mycorrhizal (AM) fungal partner and in trade the plant produced carbon with that of nutrients absorbed by fungi from the soil (Kong et al. 2014; Brundrett and Tedersoo 2018). Root architecture study of 1810 plant species has revealed that host plants that form association with AM fungi predominantly develops root with higher diameter and larger cortex to house its symbiont partner. However, plants that survive independently forms a higher root length for efficient nutrient uptake (Bergmann et al. 2020). Each root phenotype enables the plant to survive under specific environmental conditions. A symbiotic carbon allocation within AM enables an efficient Carbon utilization strategy in resourceful environment with high intraspecific competition whereas plants with thinner roots have a rapid root growth which allows them to survive under stressful environmental conditions (cold or drought) (Ma et al. 2018).

The plant roots secrete a wide array of chemical compounds known as rhizodeposits that attract and promote the growth of microorganisms. Such compounds can be low-molecular mass compounds (for example organic and amino acids) mucilage (polymerized sugar), root border cells, dead root cap cells and even secondary metabolites (for example flavonoids, nematocidal and antimicrobial compounds) (Bais et al. 2006; Jones et al. 2009).

It is important to study the root development associated with its microbiome as its not only acts as a system that supports the plant growth but also play an important role in crop productivity. There are several parameters of root system architecture study however lateral root formation is a principle component that governs the overall root growth (Dubrovsky and Fordeb 2012). The lateral root morphology not only plays a crucial role in maintaining effective water-use efficiency but also absorption of macro/micro-nutrients from the soil (Casimiro et al. 2003). Hence lateral root development study is indeed an important parameter to study the plant root architecture.

2 Different Microorganisms that Modulate Root Architecture

Plants are exposed to a diverse array of microorganisms in the soil may either be beneficial or pathogenic. Plants possess an enigmatic way of co-habiting with such organisms while maintaining its growth and development simultaneously. Unlike beneficial interactions which aids overall plant growth, negative interactions often have a detrimental effect of the overall physiology of plant growth. Plants in order to avoid or endure such pathogenic interactions modulate their morphology in such a manner so as it enable them to overcome the physiological stresses encountered during such interactions (Ney et al. 2013). We have further elaborated how plants modulate their morphology with special emphasis on root architecture during its interactions with different microorganisms (Table 1).

2.1 Bacteria as Modulators

Bacteria associated with rhizosphere can influence plant growth and development. Such bacteria often known as plant growth promoting bacteria (PGPRs). *Pseudomonas putida* not only enhance effective Mg, K and Ca uptake from the soil but also provides tolerance against salinity stress in the soil. *Bacillus amyloliquefaciens* collected from wild plants when inoculated onto cotton seeds exhibit better gemination rate, root biomass and tolerance against salinity stress (Irizarry and White 2017). It has been reported that plants through endocytosis degrade microbes present within root cells and uptake its nutrients, a phenomenon also known as microbivory (Paungfoo-Lonhienne et al. 2010; White et al. 2019). Interestingly, it has been observed that plants degrade associated bacterial population (endophytes or free soil dwelling) to obtain nutrients from it also known as the rhizophagy cycle. *Pseudomonas* sp. found associated with the rhizosphere of wild grass *Phragmites australis* penetrate into the root cell periplasm through the root meristem. The host plants obtain its nutrients from the bacterium though oxidation/degradation and allows the escape of surviving bacterium from the root hair tips (White et al. 2018).

Bacteria belonging to Actinomycete phylum such as *Frankia* sp. forms actinorhizal symbiosis with host tree plants belonging to Butulaceae, Casuarinaceae and Myricaceae (Péret et al. 2018). The plant hosts the bacteria in specialized root structure called nodules. The bacteria help in fixation of atmospheric nitrogen and increases the overall growth of the plant however, they do not cause any significant changes its overall root architecture.

Table 1 Interactions and impact of different microorganisms on host plant roots

Organism	Microbe associated	Host plant	Outcome of the interaction	Reference
Bacteria	*Pseudomonas putida* Rs-198	*Gossypium hirsutum*	Imparts tolerance during salt stress	Yao et al. (2010)
	Klebsiella oxytoca	*Gossypium hirsutum*	Promote seedling growth and protection against salinity	Yue et al. (2007)
	Bacillus sp.	*Zea mays*	Solubilization of phosphate compounds Production of IAA-like molecules, and promote root growth	de Sousa et al. (2020)
	Bacillus sp	*Arabidopsis thalliana*	Volatiles produced trigger overall plant growth	Ryu et al. (2003)
Fungi	Arbuscular mycorrhizal fungi	*Nicotiana rustica*	Promotes plant nutrient uptake and attracts parasitic wasps to feed upon silver leaf whitefly (*Bemisia argentifolii*)	Wooley and Paine (2011)
	Aphanomyces euteiches	*Pisum sativum*	A root system with larger and longer roots can impart good levels of resistance against the pathogen	Desgroux et al. (2018)
	Gaeumannomyces graminis	*Triticum aestivum*	Increase in uptake of Nitrogen per unit root	Schoeny et al. (2003)
	Fusarium solani	*Phaseolus vulgaris*	Increased adventitious root formation and tolerance to pathogen	Snapp et al. (2003)
	Trichoderma viride	*Arabidopsis thalliana*	Volatile organic compounds produced that increased lateral root formation	Hung et al. (2013)
Nematode	*Acrobeloides* sp.	*Oryza sativa*	Increased P uptake along with higher branching of lateral roots	Ranoarisoa et al. (2018)

(continued)

Table 1 (continued)

Organism	Microbe associated	Host plant	Outcome of the interaction	Reference
	Heterorhabditis megidis	*Zea mays*	Nematode feeds on insect pest *Galleria mellonella*	Demarta et al. (2014)
	Steinernema carpocapsae	*Pinus* sp.	Effective in killing pine weevil, *Hylobius abietis*	Ennis et al. (2010)
	Rhabditis sp.	*Pinus pinaster*	Presence of bacterivorous (*Bacillus subtilis*) nematode increases N and P availability to host plants	Irshad et al. (2011)
Parasite	*Nuytsia floribunda*	Woody plants	Obtain water and solutes from the xylem from host plant root	Calladine and Pate (2000)
	Dactylanthus taylorii	Woody plants	Form fine root structure to adhere to host and uptake nutrients	Holzapfel et al. (2016)
	Balanophora sp.	Woody plants	Tuber attaches to host plant root and visible only when develops inflorescence	Eberwein et al. (2009)
Insects	Vine weevil (*Otiorhynchus sulcatus*)	*Plantago lanceolata*	Defense priming by production of defense metabolites and increased AM fungal colonization	Bennett et al. (2013)
	Otiorhynchus sulcatus	*Fragaria x ananassa*	Roots of plants colonized with AM fungus (*Glomus* sp.) shows enhanced tolerance to pest	Gange (2001)
	Tipula paludosa larvae	*Agrostis capillaris*	Change in the composition of root exudates and increased colonization of AM fungi	Currie et al. (2006)
Virus	Wheat streak mosaic virus	*Triticum aestivum*	Decrease in root biomass and water use efficiency in susceptible cultivars upon viral infection	Price et al. (2010)

(continued)

Table 1 (continued)

Organism	Microbe associated	Host plant	Outcome of the interaction	Reference
	Cucumber mosaic virus	*Arabidopsis thalliana*	Increased branching of lateral roots in infected plants	Vitti et al. (2013)

2.2 Fungi as Modulators

The root phenotype can play a crucial role in modulating the composition of fungal community associated with the rhizosphere (Schroeder et al. 2019). Traits such as root diameter, nitrogen content, total length have been important in shaping the plant-fungal community in the rhizosphere of grassland ecosystem (Sweeney et al. 2021).

Approximately 200 different species of fungal species belonging to Glomeromycota (also known as arbuscular mycorrhizae) have been found to infect more than 80% of land plants (James et al. 2006). Such interactions between plant roots and fungi increase plant nutrient and water uptake. The fungal hyphae of mycorrhizae penetrate and colonizes the root cortex. Further the interactions results in increase in lateral roots, root diameter and alterations in root topology (Péret et al. 2018).

As a part of tolerance strategy, the root phenotype of a host plant also gets altered depending on the population of the fungal pathogen causing infection. Certain wheat cultivars stimulate overall root growth upon low pathogen density however, with rise in inoculum density the overall root growth is eventually retarded (Bailey et al. 2006). This suggests that epidemiological disease modelling can be a useful tool in management of fungal diseases in plants. Similarly, in another study, it has been shown that *Phaseolus vulgaris* having a higher lateral root density and adventitious root formation demonstrates enhanced tolerance to root rot pathogen *Fusarium solani* (Snapp et al. 2003).

Several fungal pathogens have been reported to secrete volatile organic compounds (VOCs) that modulate the growth pattern of plants. Isolates of *Fusarium* have been found to increase the primary root length of host plants (Schenkel et al. 2018). In another report strains of *Rhizoctonia solani* have been found to induce certain VOCs that increases root biomass and lateral root formation to favour pathogen colonization process in Arabidopsis plants. However, increase in VOCs can result in resistance against insect herbivory by *Mamestra brassicae* (Cordovez et al. 2017). The study of chemical composition of the VOCs and their mechanism in modulating the underground plant growth turns out to be an exciting area of future research.

2.3 Nematodes as Modulators

Nematodes are motile microorganisms ubiquitously present in nature. Several nematodes are found to inhabit the soil wherein they can either be beneficial or pathogenic to the host plant. Plant-parasitic nematodes also known as entomopathogenic nematodes can prey on several harmful pests of crop plants (Demarta et al. 2014). These nematodes can utilize rhizosphere cues to attract and use roots as paths to feed on soil dwelling herbivorous insects (Hui and Webster 2000; Ennis et al. 2010). Nematode infected roots produce rhizodeposits in the form of secreted mucilage, degraded root cells and other root exudates. The rhizodeposits not only play an important role in altering the root system but also governs the microbial community structure in the rhizosphere (Haase et al. 2007; Dennis et al. 2010).

The while clover (*Trifolium repens*) is a host to clover cyst nematode (*Heterodera trifolii*) and develops specialized feeding structure called syncytium in the root cortex. The nematode infected plants exhibited drastic modifications in the root architecture including decrease in lateral root length and increase in number of lateral secondary roots branching off from the primary root (Treonis et al. 2007). A pathogenic nematode *Melidogyne incognita* causes disease in diverse plant species. It induces gall formation in roots associated with damaged root epidermis, cortex and xylem vascular bundles thereby disrupting the plant-water continuum (Koenning et al. 2004). Although infection in cotton by root knot nematode causes severe growth deformation, however the overall root length increases (Ma et al. 2013). The roots of maize plants secrete certain volatile organic compounds which diffuse through the roots and attracts nematode *Heterorhabditis megidis* towards its prey *Galleria mellonella* (Demarta et al. 2014). Plants with a reduced root angle attracted increased number of nematode as it guided specifically towards its prey *G. mellonella* than plants with a wider root angle.

2.4 Insects as Modulators

Herbivorous insects feed not only on aerial plant parts but also on underground parts and results in major alterations. Unlike shoot herbivores, root herbivores have a lower diversity, a longer life span and are more adaptive to changes in the external environment (Johnson et al. 2016). As a part of control strategy, certain insect pests are utilized as beneficial herbivores to control the population of weeds. One such example is that of *Agapeta zoegana* which is used as a biocontrol agent for weed *Centaurea maculosa.* Interestingly, it has been observed *C. maculosa* infected roots show enhanced nitrogen uptake. The pest infested roots of *C. maculosa* transports nitrogen from the site of infection to aerial plant parts in response to herbivory (Newingham et al. 2007).

In a study featuring root herbivory in cotton plants by *Agriotes lineatus* causes an increase in accumulation of secondary metabolites such as terpenoids in roots which

gets transported to the aerial plant parts. This results in deterrence of *Spodoptera exigua* to feed on the aerial plant plants and result in resistance against the foliage feeding insect (Bezemer et al. 2003).

2.5 Parasitic Plants as Modulators

Plant-parasite interactions serves a vital role in modulating plant phenotype. *Striga hermonthica* is a major crop parasite that is found to infected several crop species worldwide. It gets attracted to the host plants root exudates preferentially strigolactones and attaches to the host roots to obtain its nutrition (Bouwmeester et al. 2003). Interestingly, it has been observed that certain cultivars of sorghum exhibit resistance against *S. hermonthica*. In-depth analysis has revealed such cultivars to harbour a *LOW GERMINATION STIMULANT1 (LGS1)* mutant that is responsible for production of altered root exudate strigolactone called orobanchol which is a weak stimulant for *S. hermonthica* thereby preventing its growth (Gobena et al. 2017). However, increased farming of *lgs1* cultivar has caused an emergence of *S. hermonthica* strains that show increased susceptibility (Bellis et al. 2020). This reveals the evolutionary forces that coexist between both the plant host as well as root parasite.

Similarly, *Orobanche* is a widely predominant root parasite that gets attracted to host root exudates and establishes itself to obtains nutrition from its host. However, certain resistant host cultivars have been found to secrete excess of root phenolic compounds and peroxidases that deter *Orobanche* from colonizing its host root (Pérez-De-Luque et al. 2005).

2.6 Viruses as Modulators

Viruses also infect several of plant species and modulate their host root architecture. These viruses are carried and spread to their host by means of propagating vectors such as insects. Apple Stem Grooving Virus (ASGV) infection in pear plants results in modulation of cytokinin, auxin and abscisic acid levels causing reduced formation of roots, and overall reduction in root length (Chen et al. 2017). In another study, Sweet Potato Chlorotic Stunt Virus (SPCSV) infected sweet potato exhibited lower lateral root length, density and number. Interestingly, the decrease in the lateral root formation simultaneously decreases the competency of adventitious roots (potato tubers) to undergo storage root initiation culminating in total yield loss (Villordon et al. 2014).

3 Different Levels of Modulation of Root Architecture

3.1 Anatomical and Structural Changes

The root architecture is determined by the fate of the root apical meristems which govern the rate of root elongation or emergence of lateral roots or development of root nodules (Fig. 1). Legume plants growing on Nitrogen-deprived soil form root nodules to harbour symbiotic nitrogen-fixing bacteria. Under low N_2 conditions, *CEP* (C-terminally Encoded Peptide) gets induced which gets transduced in the shoots through systemic signalling via *CRA2* kinase (Gautrat et al. 2020). The *CRA2*

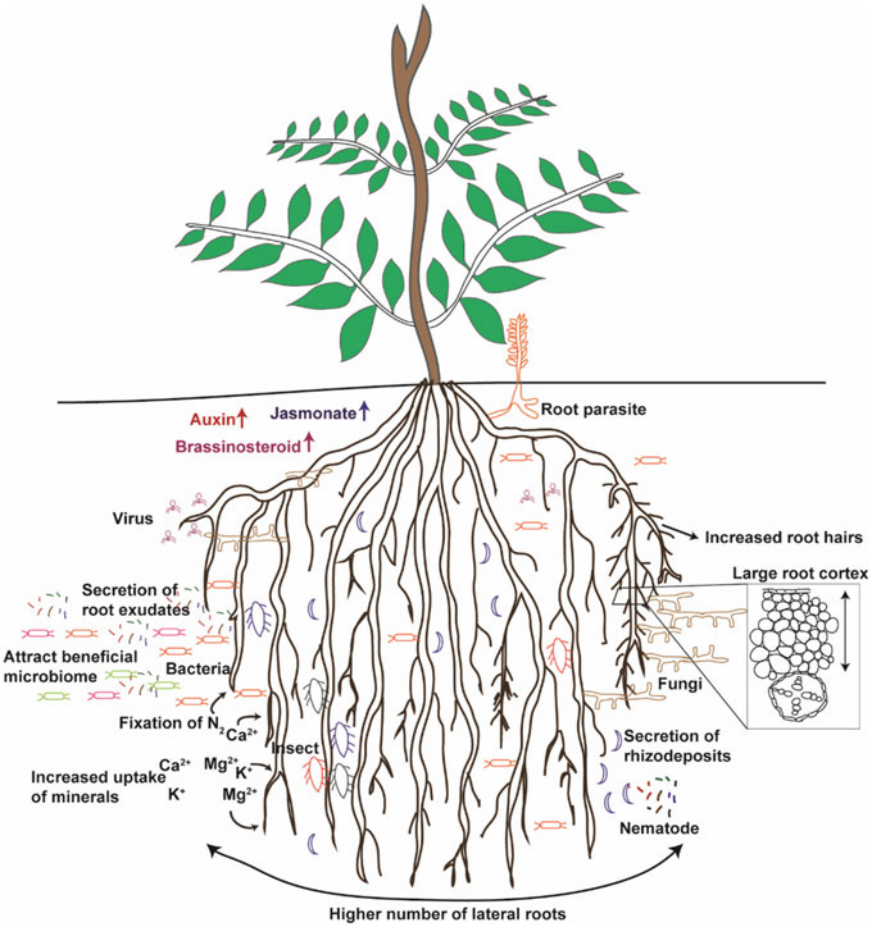

Fig. 1 Impact of microbiota in modulating different aspects of root system architecture of the host plant

gene encoding a Leucine-Rich Repeat Receptor-Like Kinase negatively regulates lateral root formation locally but promotes formation of symbiotic N_2 fixing nodule systematically from the shoots (Huault et al. 2014). Also, nodule development can be promoted by other systemic signals from the shoots, such as SUNN (Super Numeric Nodules) that induce higher nodule formation in roots.

Similarly, AM symbiosis facilitates enhanced uptake of P, Zn and other essential micronutrients from the soil through formation of arbuscles inside the root cortical tissue of the host plant. In this context, it is interesting to note that depending on the host genotype, there are two types of root architecture that gets promoted during AM colonization (Li et al. 2016). Under Type I category, colonization by AMF results in elongation and a greater number of lateral root formation along with root hair formation (Yao et al. 2009; WU et al. 2011). However, type II form of root architecture is represented as constricted root growth along with decreased root length and surface area. This type of interaction occurs when the host is inhabited by both N_2 fixing bacteria as well as AM fungi, wherein the host root morphology gets reduced to lower the carbon cost for maintaining both the organisms (Wang et al. 2011). Moreover, an analysis of root architecture of diverse plant types revealed that plants having increased root length were more favourable for AM symbiosis rather than roots with increased lateral root formation (Yang et al. 2015).

3.2 Physiological Changes

In response to root herbivory, plants exhibit relocation of photo-assimilates and variation in its growth pattern (Johnson et al. 2016). Some of the examples highlighting the changes in root physiology have been discussed (Fig. 1). Root herbivory can cause depletion of stored photo assimilates underground, as observed in *C. maculosa* infected roots show enhanced nitrogen uptake and transport to the aerial plant parts in response to herbivory by *Agapeta zoegana* (Newingham et al. 2007).

Upon herbivory there is an increased allocation of photo-assimilates to promote root repair and defense (Johnson et al. 2013). In maize, it has been reported that feeding by specialist herbivore *Diabrotica virgifera* causes increased accumulation of primary as well as secondary metabolites including insecticides and phenolic compounds in the crown roots. In turn, the increased accumulation of these toxic metabolites deter feeding from generalist herbivores such as *Spodoptera littoralis* and *Diabrotica balteata* (Robert et al. 2012). Accumulation of certain secondary metabolites can also cause alteration in root morphology. Fungal sesquiterpene produced from ectomycorrhizal fungi *Laccaria bicolor* can increase lateral root formation even in the absence of fungi (Ditengou et al. 2015).

3.3 Hormonal Changes

Rhizosphere microflora also induce several developmental changes in the host plant through modulation in the hormonal levels through either direct or indirect manner. Brassinosteroid signalling transcription factor *BZR1* regulates the expression of several genes involved in cell growth and elongation along with cell cycle regulation (Sun et al. 2010). The *bzr1* mutant not only exhibit abnormal root architecture but also low susceptibility to RKN, *M. incognita*. The RKN modulates the expression of *BZR1* thereby regulating cell cycle and promoting the cell wall plasticity to form giant cells (Warmerdam et al. 2018).

Nutrient deficiency can often have severe impact on root morphology. For example, phosphorous deficiency causes elongation in primary root length and increases the lateral root formation along with root hair formation (Chiou and Lin 2011; Zhang et al. 2014). However, phosphate limitation has also been found to be associated with increase in Jasmonic acid (JA) biosynthetic and signaling pathways in the plant. The elevated JA levels in-turn provides resistance against herbivory by generalist feeding insect *Spodoptera littoralis* (Khan et al. 2016). During root herbivory different phytohormonal levels get altered when compared to shoot herbivory. Roots exposed to herbivores show more accumulation of JA levels, whereas SA, ABA and ethylene levels majorly seem to be unaffected (Lu et al. 2015; Johnson et al. 2016).

One of the major functions of plant hormone auxin is development of root system architecture. Also, studies have shown that auxin functions as a signaling molecule in a bacteria community as well as between bacteria and host plants (Spaepen and Vanderleyden 2011). Indole derived compounds secreted by roots stimulate the growth of plant growth promoting bacteria (Kamilova et al. 2006). Also, it has been observed that viral infected roots have higher accumulation of auxin. It has been observed that upon CMV (Cucumber mosaic virus) infection IAA (Indole acetic acid) levels in the root tissues along with upregulation of *AtNIT* (nitrilase, IAA biosynthesis). Moreover, CMV infected roots demonstrate a higher lateral root count along with increased root hair formation (Vitti et al. 2013). Beneficial microbes have been reported to modulate auxin biosynthesis, transport and signaling to promote root growth. Different microorganisms target a common or specific component of the auxin pathway to induce a particular type of root architecture (SUKUMAR et al. 2013). Similarly, aerobic Nitric Oxide (NO) produced by *Azospirillum brasilense* promote auxin signaling (IAA) to induce lateral and adventitious root formation in host tomato plants (Molina-Favero et al. 2007).

3.4 Molecular Changes

It is evident that soil microorganisms have the ability to promote root growth and architecture. Several of the effects on modulation of root growth occurs on the development of roots at the post-embryonic level. Interestingly, the effect of root growth promotion is most prominent when the microbes are found in high density and in close proximity to the host roots (Verbon and Liberman 2016). Bacterial species such as *Pseudomonas simiae* WCS417 and *Bacillus megaterium* UMC1 has the ability to promote the transition from cell division to cell elongation in the root meristem of Arabidopsis plants. They also increase the number of lateral root founder cells in the pericycle region that induce lateral root formation (López-bucio et al. 2007; Zamioudis et al. 2013).

Further, high density of bacterial population have been found associated with Casparian strips in the root endodermis. In this context, it is speculated that accumulation of bacteria these breakpoints in the root endodermis in the Casparian strips may account for the emergence of lateral roots from these zones (Verbon and Liberman 2016).

4 Evolutionary Pressure between the Plant and Rhizobiome

Since millions of years of coexistence, plants and microbes have co-evolved to either benefit from each other presence (such as symbiotic interaction) or survive at the cost of the other (such as pathogenic interaction). Incidentally, the transition of plants from aquatic to terrestrial phase has been largely mediated via symbiotic interactions between plants and microbes. However, even before the evolution of multicellular organisms, the cell–cell interaction between the prokaryotic organisms had given rise to endosymbiosis. The higher evolutionary advantage of the endosymbiotic organism propelled the evolution of multicellularity and tissue formation. The gene families associated with symbiosis arose during evolution of early land plants and have been diversified in several plant lineages. These genes were manipulated by pathogens during pathogenesis which in turn were recognized by the host plants through elaborate defense signaling mechanisms (Delaux and Schornack 2021).

There has been a constant molecular crosstalk between plant and microbes which had given rise to several forms of interactions ranging from pathogenic to mutualism (Lagunas et al. 2015). There is a tight regulation that occurs between pathogenic and mutualistic association so as both interactions can activate the host defense responses. However, in case of mutualistic interactions the defense response gets suppressed which allows co-existence of host plant and microbe (Zamioudis and Pieterse 2011). One such theory suggest that the molecular evolution of plant LysM receptor has allowed difference in detection system between pathogenic and mutualistic association (Nakagawa et al. 2011). The LysM receptor genes have evolved into NFP

(for nodulation) during symbiosis and LYK related 1 (LYK1) during mycorrhizal interaction in *Medicago trunculata* (Young et al. 2011).

Lateral root organogenesis into lateral root or nodules are often regulated by common environmental signal, i.e., nitrogen levels in the soil. Moreover, nodulation and lateral root development share a common regulatory pathway. For example, lateral root organ defective (*latd*) mutant forms abnormal root nodules and lateral root formation (Bright et al. 2005). In *Lotus japonicus*, Hypernodulation Aberrant Root formation (*LjHAR1*) is involved in lateral root and nodule formation. The *har1* mutant either develops higher number of lateral roots or nodules in the absence and presence of rhizobia respectively (Wopereis et al. 2000). Several plant species rely on symbiosis for acquisition of resources and defense. Mycorrhizal association of plants and fungi are amongst the most primitive symbiotic association known so far (Hoeksema et al. 2018).

However, other than nutrient availability, the mycorrhizal symbiosis have been largely governed by community ecology and evolutionary biology. Ectomycorrhizal symbiosis with fungi has evolved multiple times in different plant phylogenies, whereas endo-mycorrhizal association (arbuscular mycorrhiza; AM) have evolved from different nutrient sharing strategies that affect the growth of their host plants (Hoeksema et al. 2018).

5 Strategies to Improve Plant Health by Manipulating Microbiome

Roots of plant produce a plethora of secondary metabolites that shape the microbial communities that survive in the rhizosphere (Das et al. 2021). Some of the metabolites can act as a source of nutrients and promote the growth of microbes whereas some metabolites are toxic and suppress the growth of microbes (Berendsen et al. 2012). Maize plants produce certain indole derived compounds also known as benzoxazinoids (BXs) that act as a gatekeeper of microbes that are found in the rhizosphere and play a dual role in attracting as well as deterring microbes (Kudjordjie et al. 2019). BXs are antagonistic to herbivores insects (aphids, corn borers), fungal pathogen *Fusarium* (Bacon et al. 2007; Betsiashvili et al. 2015). However, BXs promote the growth of bacteria *Pseudomonas putida* (Neal et al. 2012). The aerial roots of maize growing in N_2 poor soil secretes nutrient rich mucilage which attracts and promotes the growth of diazotrophic bacteria. In turn the resident bacteria helps in fixing of atmospheric N_2 and enable the maize to sustain under N_2 poor soil (Van Deynze et al. 2018).

Amidst the increasing usage of chemical fertilizers and pesticides to promote plant growth and yield, it is imperative to devise strategies to promote agriculture in an environment friendly way. A novel way could be harnessing the potential of rhizosphere microbiome to promote the nutrient availability for the plant as well as prevent infection from pathogenic microorganisms (Lareen et al. 2016). In this

strategy, utilizing plant breeding approach a plant trait is selected which promote the growth of certain microbiome in the rhizosphere (Ryan et al. 2009). For example, plant growing in disease suppressive soil secrete certain antimicrobial and signalling compounds that not only prevents the growth of harmful microorganisms but also aid in the degradation of toxic metabolites present in the soil (Yergeau et al. 2014; Pagé et al. 2015). Also, genetic engineering of plants to produce metabolites (such as rhizopines) for the growth and promotion of nitrogen fixing rhizobia can be another interesting avenue to promote the growth of microorganisms (Geddes et al. 2019). The manipulation of microbiome by synthetic or natural microbial communities to promote the root system associated with plant growth is an emerging to be an exciting avenue for research.

6 Conclusion

It is evident that the rhizosphere is a habitat of millions of microorganisms that under continuous interaction with the host plant. These microorganisms have co-evolved to survive under close association of the host plants. Many such microorganisms enhance the root architecture and enhance nutrient uptake by the host plant. It is important to devise and adopt strategies to promote the growth of beneficial microorganisms in the soil so as to promote sustainable means of agriculture and protect our natural ecosystem.

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miRNA Mediated Signaling Involved in *Arabidopsis thaliana* Root Development

Vibhav Gautam, Priyamvada Gupta, Prafull Salvi, Akanksha Sharma, Deepak Kumar, and Anuj Dwivedi

Abstract Root is an important component of the plant body which is crucial for providing anchorage to the aerial part, nutrient uptake and storage of the reserve foods. Development of root system consists of embryonic and post-embryonic stages where embryonic roots include primary root and seminal root, whereas post-embryonic roots include lateral, crown and brace roots. Root Apical Meristem undergoes division, patterning and differentiation to give rise to entire plant root. Root system architecture of the higher plants is regulated by various factors such as gene regulatory networks, plant hormones, signalling peptides and non-coding RNAs. Different transcription factors or genes are targeted by miRNAs (20-24 nucleotides) and thereby plays a significant role in root growth and spatiotemporal patterning. Interaction of miRNAs and their targets may evolve as a more intricate network in order to coordinate exogenous environmental cues and endogenous developmental regulation. In the recent past, miRNAs have also emerged as a key controller in shaping root growth and development. miRNAs are involved in regulating most of the developmental processes by negatively regulating the expression of their target genes . In this chapter, we have summarized the role of miRNAs in the root specification and development in model plant *Arabidopsis thaliana.*

V. Gautam (✉) · P. Gupta
Centre of Experimental Medicine and Surgery, Institute of Medical Sciences, Banaras Hindu University-Varanasi, Varanasi 221005, India
e-mail: vibhav.gautam4@bhu.ac.in

P. Salvi
Agriculture Biotechnology Department, National Agri-Food Biotechnology Institute, Sahibzada Ajit Singh Nagar, Sector 81, Mohali, Punjab 140306, India

A. Sharma
Department of Botany, Central University of Jammu, Rahya-Suchani (Bagla), Samba, J&K 181143, India

D. Kumar
Department of Botany, Institute of Science, Banaras Hindu University-Varanasi, Varanasi 221005, India

A. Dwivedi
Division of Biochemistry, Indian Agriculture Research Institute, New Delhi, India

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1 Introduction

Root is an important organ of the plant body which is responsible for water and nutrient uptake and provides a robust anchorage to the plant body (Gautam et al. 2017; Atkinson et al. 2014; Chapman et al. 2012; Chen et al. 2012). A very rapid response is shown by plant root towards the environmental signals, so by identifying the factors that are involved in controlling the architecture of root system, root system architecture can be modulated (Atkinson et al. 2014; Bellini et al. 2014; Coudert et al. 2010; Hochholdinger and Tuberosa 2009; Hochholdinger and Zimmermann 2008; Marcon et al. 2013). In higher plants root system architecture is determined by distinct root types which include embryonic and post embryonic root types (Atkinson et al. 2014; Hochholdinger et al. 2004; Hochholdinger and Zimmermann 2008; Muthreich et al. 2013). Roots are involved in the vital processes like absorption and water and nutrient and provides anchorage to the plant body (Bellini et al. 2014; Chapman et al. 2012; de Dorlodot et al. 2007; Rich and Watt 2013). Roots of different plant species can have significantly distinct cellular organization and root system architecture (Gautam et al. 2021; Bellini et al. 2014; Hochholdinger and Zimmermann 2008). Due to simple and transparent organization, *Arabidopsis* root is one of the best studied models and its invariant cell lineage can be used to trace back to a few founder cells (Hochholdinger and Feix 1998; Hochholdinger and Hoecker 2007; Hochholdinger et al. 2001; Hochholdinger and Tuberosa 2009; Hochholdinger and Zimmermann 2008; Marcon et al. 2013). High throughput reverse genetics-based approach focuses at identification of diverse root specific genes which will be helpful to unravel the complex network involved in root development (Dolan and Roberts 1995; Garay-Arroyo et al. 2012; Pace et al. 2015; Ubeda-Tomas et al. 2012).

Root comprises of a root cap, root apical meristem, the distal elongation zone and maturation zone in its longitudinal arrangement (Hahn et al. 2008; Kumpf and Nowack 2015; Overvoorde et al. 2010). For the deep penetration of growing roots into the soil, root cap serves as an important structure for secretion of mucilage (Hahn et al. 2008; Kumpf and Nowack 2015; Overvoorde et al. 2010). Root cap, at its proximal end, consists of root apex that is present sub terminally and includes about 800–1200 cells which are mitotically inactive and termed as quiescent centre (QC) (Aichinger et al. 2012; Busch et al. 2010; Sablowski 2011; Sena 2014; Yadav et al. 2013). The proximal and distal meristem surrounds QC. The distal elongation zone is present next to the proximal meristem which consists of newly generated cells. It is quite difficult to demarcate cells on the basis of their mitotic activity in this region, therefore, the region of distal elongation zone is considered as the transition zone between elongation zone and meristematic zone (Rogers and Benfey 2015). The elongation zone is responsible for controlling the distal elongation zone where cells do not undergo division but elongate at their greatest extent. Maturation zone is present proximal to the elongation zone which is marked by the presence of root

hairs (Aichinger et al. 2012; Busch et al. 2010; Sablowski 2011; Sarkar et al. 2007; Sena 2014; Yadav et al. 2013).

Model plants such as *Arabidopsis* display a single primary root and consist of the lateral roots which functions throughout life cycle. *Arabidopsis* embryonic root includes only primary root (Atkinson et al. 2014; Dolan et al. 1993; Sena 2014; Sozzani and Iyer-Pascuzzi 2014; Wang and Li 2008). Shoot borne root system is missing in *Arabidopsis* (Hochholdinger and Tuberosa 2009). In *Arabidopsis*, the site of lateral root formation is at xylem poles, the number of cortical cell layer is only one in *Arabidopsis*, QC cells are only 4 in *Arabidopsis* (Hochholdinger et al. 2004; Hochholdinger and Zimmermann 2008). In dicots such as *Arabidopsis,* initiation of longitudinal root growth takes place from root tip. The central part of the root harbours the mitotically inactive region referred to as QC. QC is surrounded by stem cells and generate several types of cell files including epidermis, cortex, endodermis, stele and columella and the lateral root cap that comprises the root. The division in each stem cell occurs asymmetrically, thus, giving rise to one daughter cell and that persist as a stem cell by remaining in contact with the QC. Several rounds of cell divisions occur in a cell that is located one cell far from the QC and it further undergoes differentiation. Columella stem cells (CSCs) lie at the distal side of QC. CSCs divide to produce daughter cells and further differentiates into starch containing columella cells. Several genes are involved in the maintenance of stem cell niche such as *WUSHEL RELATED HOMEOBOX5 (WOX5)* which expresses in the QC and is responsible for promoting stem cell fate (Sarkar et al. 2007; Atkinson et al. 2014; Breakfield et al. 2012; Dolan et al. 1993; Drisch and Stahl 2015; Garay-Arroyo et al. 2012; Hochholdinger and Zimmermann 2008; Meyerowitz 1994; Peret et al. 2009; Yruela 2015; Zhang and Yu 2014).

Besides protein coding genes and phytohormones, various non coding RNAs are also known to regulate the plant growth and development (Gautam et al. 2017; Chitwood and Timmermans 2010; Kidner and Timmermans 2007). There exists a intricate network between small RNAs and gene/hormones which in turn regulates the entire developmental cycle of the plant (Chen 2009; Ghildiyal and Zamore 2009). The size range for regulatory small RNAs is from 20 to 24 nucleotides (Axtell et al. 2006; Axtell 2013; Chen 2009). The production of sRNAs has been shown to be served by four different pathways in plants which is mediated by different DICER LIKE proteins (DCLs) (Gasciolli et al. 2005; Chapman and Carrington 2007). The production of 24 nucleotide siRNA, as well as the regulation of post transcriptional gene silencing, is mediated by *DICER LIKE3* (*DCL3*). *DICER LIKE4* (*DCL4*) along with *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*), *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*), and DOUBLE STRANDED RNA BINDING PROTEIN 4 (DRB4) aids in the formation of 21 nucleotide trans-acting (ta-siRNAs) small interference RNAs, derived from miRNA-mediated TAS RNA precursors (Peragine et al. 2004; Montgomery et al. 2008; Felippes and Weigel 2009; Allen et al. 2005; Yoshikawa et al. 2005).

In plants, the developmental course is divided into two major phases i.e., embryonic and post-embryonic development (Meinke 1991; Scheres 2007). The formation of root apical meristem (RAM) occurs during embryogenesis so it serves as a primary

source to attain sustainable growth and development of the root (Willmann et al. 2011; Nodine and Bartel 2010; Petricka et al. 2012; Sabatini et al. 2003). The stem cell identity in the nearby cells of RAM is maintained by QC that plays a very crucial role in differentiated tissue production (Möller and Weijers 2009; Peris et al. 2010). Root development is tightly controlled and well-organized phenomenon which is extensively studied in model plant *Arabidopsis thaliana* (Gautam et al. 2017). The mechanism of root development varies from monocot to dicot plants due to the difference in the root system architecture. Genetic analysis has shown a set of genes with overlapping functions inspite of having developmental and morphological diversity.

In cereals crops, endodermis cells are designated to produce the new root cap and subsequently cortical cells divide to produce the lateral root primordium. However, in dicot model plant system such as *Arabidopsis*, exclusively, pericycle cells situated at opposite poles of protoxylem produce lateral root primordia (Lavenus et al. 2013). Multiple genes like *AUXIN RESPONSE FACTORS 10 (ARF10)*, *ARF16* and *ARF17* in dicot are targeted by miR160 (Mallory et al. 2005; Wang et al. 2005). Overexpression of miR160 or loss of function mutant of both *ARF10* and *ARF16* leads to root elongation. Remarkably, it has been observed that phenotypes of miR160 overexpression are suppressed in miR resistant lines of *ARF10* or *ARF16*, while, in *ARF17*, it was not found (Wang et al. 2005). Thus, it suggested that *ARF17* is not involved in root cap formation reliant on miR160 (Mallory et al. 2005; Wang et al. 2005). miR160 is considered as a primary regulator of root growth and gravitropism and it is a negative regulator of the three genes namely, *ARF10*, *ARF16* and *ARF17*.

In plants, the conserved targets of miR396 family are *GROWTH-REGULATING FACTORS* (GRFs). The conserved targets of miR396 in plants are *GRFs* (Debernardi et al. 2012) where expression occurs in transit-amplifying cells (TACs) at the root meristem and thereby suppressing *PLETHORA* (*PLT*) transcript expression (Debernardi et al. 2014; Rodriguez et al. 2015). Studies in *Arabidopsis thaliana* and *Medicago truncatula* have shown that overexpression of miR396 decreases root elongation with other deleterious effects on root meristem size (Bazin et al. 2013; Rodriguez et al. 2015). Furthermore, Rodriguez et al. decoded a regulatory module of miR396/*GRFs-PLT,* to establish equilibrium between the division activities in the TACs and stem cell niche (SCN) (Rodriguez et al. 2015). A conclusion has been drawn based on the designed model, in SCN miR396 was activated by *PLT* which, subsequently, suppresses *GRF* in this region. In SCN, the stable expressions of *GRFs* have deleterious effect on QC and columella cells (Galinha et al. 2007). In *Arabidopsis* genome, four copies of *HAIRY MERISTEM (HAM 1–4)* are present, of these, *HAM 1–3* are targeted by the miR171 family. Four copies of *HAIRY MERISTEM (HAM 1–4)* are present in *Arabidopsis* genome, where miR171 family target *HAM 1–3* (Engstrom et al. 2011; Llave et al. 2002). They are associated with various developmental responses (Engstrom et al. 2011). Remarkably, miR171c overexpression reduces the primary root length, as is the case with simultaneous mutation in the *HAM* genes. Notably, the primary root growth is reduced by miR171c overexpression, like in the case of simultaneous mutation in the *HAM* genes (Engstrom et al. 2011; Zhou et al. 2015).

2 Role of miRNAs in Primary Root Development

Plants are continuously confronted with variable environmental cues that alter their growth and development; however, plants possess a remarkable root system that enables them to sense, respond, adapt, and grow accordingly (Kellermeier et al. 2014; Rellán-Álvarez et al. 2016). The root system comprises of primary and secondary (lateral) roots which apparently determine the overall root architecture. The foremost organ to develop from germinating seed is the primary root, whereas the LR emerges from the outermost layer of vasculature i.e., pericycle (founder cell) (Bellini et al. 2014; Waidmann et al. 2020). Robust root system architecture is a steppingstone for plant growth, as it facilitates water and nutrient acquisition (de Dorlodot et al. 2007). The overall root architecture largely relies on the growth and development of primary root which is a dynamic yet highly regulated process. This complex regulation is attributed to multiple factors including, phytohormonal regulation, transcriptional syndicate, reactive oxygen signaling, miRNA mediated regulation, etc. (Dietz et al. 2016; Khan et al. 2011; Lavenus et al. 2016). miRNA (21–22 nucleotide), the small non-coding regulatory RNA, are encoded by primary transcript with a significant contribution in developmental as well as a stress responses in plants. miRNAs transcribed via RNA-Pol-II, similarly like protein-coding genes, harbors TATA-box in their *cis*-regulatory region (Megraw et al. 2006; Voinnet 2009). The miRNAs are the negative regulator of gene expression, as they implicate translational repression via transcript breakdown. Moreover, the transcription factors (TFs) are considered as a master regulator of gene expression, as in the promoter region of a gene it binds to the cis-regulatory elements and regulates its transcript level. These TFs are regulated by the key regulator, miRNAs and miRNA-mediated regulation of TFs facilitates the strategy of "regulating the regulators" to orchestrate the multiple gene expression at a time (Ng et al. 2018; Samad et al. 2017; Song et al. 2019).

miRNAs are involved in a wide array of biological processes; however, here in this section we have taken into account the role of miRNAs in primary root development of *Arabidopsis thaliana.* The miRNA-driven regulation of root initiation, elongation, and development is coordinated by the interplay of phytohormones. Among different phytohormones, the root growth and development is majorly controlled by auxin and its molecular crosstalk with other hormones (Curaba et al. 2014; Saini et al. 2013).

Auxin response involves the *ARFs* (auxin response factors), Aux/IAA (auxin/indole acetic acid) protein, and the protein degradation machinery. *ARFs* belong to a specific group of transcription factors which is a key determinant for root growth i.e., auxin response factors (*ARFs*) that binds to the promoter region at the cis-regulatory element of auxin-responsive genes. However, *ARF* activity remains restricted by its interaction with the Aux/IAA and other repressor components like *TOPLESS* (*TPL*). The auxin signaling instigates the ubiquitin-mediated proteolysis of Aux/IAAs which is a cognate repressor of *ARF* and the proteolysis of Aux/IAAs relieves the ARF and allow it to regulate the transcription of the downstream gene associated with root development (Chandler 2016; Choi et al. 2018; dos Santos et al. 2009).

ARF6 and *ARF8* transcripts are targeted by miR167 which are known to contribute towards the developmental module. The cleavage of *ARF* transcript has been indicated in diverse plant species including soyabean, tomato, rice to name a few (Jain and Khurana 2009; Pang et al. 2009; Van Ha et al. 2013; Wu et al. 2011). The miRNA level affects the *ARFs* cellular pool; thereby altering the root morphology especially under stressful environments. For instance, the miR393/*ARF3* negatively regulates the primary root elongation and triggers the secondary root emergence during nutrient acquisition. It has been found that nitrate can regulate the auxin response via miRNA, transcriptional, and post-transcriptional mechanisms which affect the root system architecture. The miR393 regulates the transcript of gene encoding auxin receptors TIR and ABFs. Expression of Xyloglucan endotransglucosylases (XTHs) is regulated by auxin in response to nitrogen signals, which are known to regulate growth of cell wall and root primordia. Auxin signaling F-box protein 3 (AFB3) perceive nitrate, instigate the proteolysis of IAA and desuppress *ARF7/19* and regulates primary and lateral root growth (Kulcheski et al. 2015; Vidal et al. 2010; Xu and Cai 2019).

Under phosphate starvation condition, the miR399 shows remarkable induction, cleave the transcript that encodes for a ubiquitin-conjugating enzyme (UBC), and regulate the primary root growth to confront the low-Pi condition. The constitutive overexpression lines of miR399 in *Arabidopsis* displayed comparatively higher accumulation of Pi when compared to the wild-type. The miR399 targets 5-UTR of gene encoding UBC which resulted in the reduction of UBC mRNA accumulation even under phosphate abundance. Besides, overexpression of UBC coding gene without 5'UTR region was less responsive to low-Pi and displayed reduced primary root growth repression than wildtype plant under Pi-starvation (Fujii et al. 2005). In *T. chinensis*, miRNA regulates the rooting process by controlling the target genes involved in phytohormone and nutrient response during rooting (Fei et al. 2019). During nitrogen inadequacy, miR171c has been found to increase up to 3-folds which cleave the *SCARECROW-LIKE*-6 (*SCL-6*) and suppress the primary root growth during nitrogen starvation condition (Kulcheski et al. 2015). Similarly, miR167 and its target *ARF8* have been found to associate with the regulation of root growth during nitrogen and its metabolite response. The auxin response factors *ARF10* and *ARF16* coordinate the cell differentiation of the root cap which is targeted by miR160. Further, overexpression lines with increased miR160 exhibited compromised cell differentiation and showed root tip defect. Interestingly the double mutants of *ARF10* and *ARF16* i.e., *arf10-2 arf16-2* were phenotypically similar to that of the overexpression of miR160 (Wang et al. 2005). Thus, during N-deficiency, miR160 mediated regulation of *ARFs* serves as an important regulator of root cap formation to control the root architecture (Wang et al. 2005). miR165/166 is also shown to regulate the expression of *HD-ZIP IIIs* which promote meristematic activity and play a crucial role in primary root growth in *Arabidopsis thaliana* (Singh et al. 2014).

Collectively, micro RNAs have been proposed to regulate diverse biological processes including environmental stress, nutrient signaling, as well as the developmental aspect. Owing to their remarkable association with root growth and architecture, it is important to expand our understanding of miRNA profile and their

regulatory role in the root system. The evolving data available for miRNA can be exploited to decipher the key node for root development and their underlying molecular and genetic components that can apparently be utilized for crop improvement. Various physiological processes are regulated by the crucial role played by majority of miRNAs (Osmont et al. 2007; Péret et al. 2009; Petricka and Benfey 2008). The important processes comprise of the modulation of nutrient absorption, sensing, embryogenesis, transportation and acculturation to an adaptive response via the regulation of organ and floral development, root architecture and abiotic stress tolerance (Huang et al. 2012; Kim 2005; Luo et al. 2013; Mallory et al. 2004b; Sunkar et al. 2012). Additionally, some micro-RNA families also play important part in regulating the development of both lateral and adventitious roots by pessimistically modulating the associated protein coding genes. Adventitious and lateral roots vary primarily on the account of their origin, the latter originating from the root tissue while the former originating from non-root tissues (Atkinson et al. 2014; Bellini et al. 2014). While monocots characteristically possess adventitious roots, dicots are known to have primary tap root with numerous associated lateral roots (LR). Adventitious roots (AR) give rise to brace and crown roots subsequently, each respectively originating from underground or below ground shoot nodes. On the contrary, the primary root system comprises primary tap root and the lateral branches which emerge from the embryonic radicle. Pericycle cells function as the progenitor of lateral roots. These are situated adjacent to the xylem which in turn is located near the apex of primary root (Péret et al. 2009). The lateral roots originate from a dome carved primordium which is formed by anticlinal divisions followed by periclinal divisions in the pericycle progenitor cells (Malamy and Benfey 1997). Recently, the focus has shifted towards the study of growth and development of both lateral and adventitious roots and is an emerging field of research because it encompasses the role of already differentiated cells to a post-embryonic specification mechanism. Pictorial representation of the role of miRNAs in regulating different root types of *Arabidopsis* is shown in Fig. 1.

3 miRNA Mediated Regulation of Lateral Root Development

Increasing body have evidences have indicated the role of miRNAs in the homeostasis of Phosphorus (P), Nitrogen (N) and Potassium (K) (Kulcheski et al. 2015; Nguyen et al. 2015). The formation of lateral roots i.e., from events of preinitiation to the formation of meristem is governed by various factors (Overvoorde et al. 2010; Péret et al. 2009; Petricka et al. 2012). Amid these factors, phytohormone auxin involves an prominent role in transport, signalling and homeostasis (Fukaki et al. 2007; Lavenus et al. 2013). Sequential regulation of the *AUXIN REPONSE FACTOR* (*ARF7–ARF19*)—*SOLITARY ROOT* (*SLR/IAA14*) along with *LATERAL ORGAN BOUNDARIES DOMAINS* (*LBD/ASL*) by auxin regulates the cell divisions

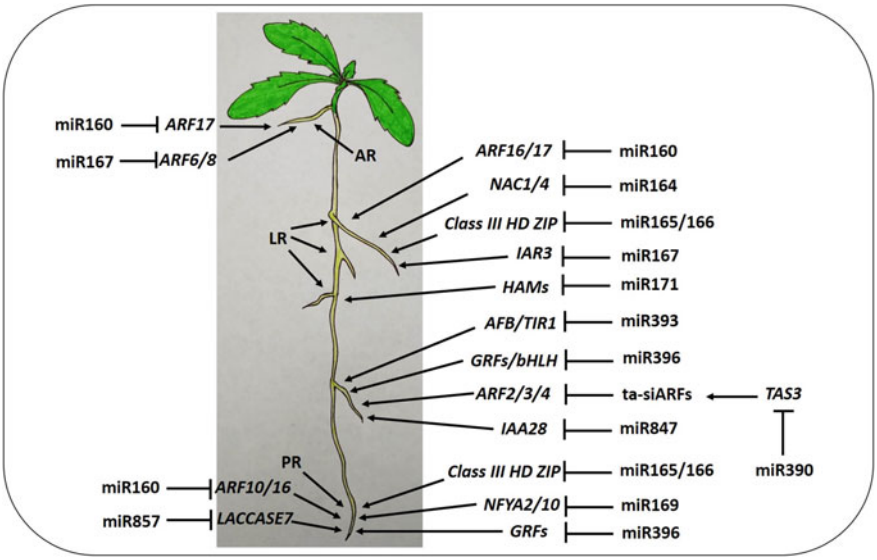

Fig. 1 miRNA dependent regulation of root branching and development in *Arabidopsis*. Activity of various miRNAs in AR, PR and LR growth and development is demonstrated, along with their respective targets in the root tissue of *Arabidopsis*. AR—adventitious roots, PR—primary root, LR—lateral root

in early primordium. Similarly, *BODENLOS* (*BDL/IAA12*)—*ARF5* also controls the LR organogenesis (Fukaki et al. 2007). *ARF16* and *ARF17* are downregulated by miR160 and therefore, employ a pragmatic effect on the lateral root development in *Arabidopsis*. Astonishingly, none among the miR-resistant *arf10* and *arf10/arf16* single loss-of-function mutants show an obvious phenotype (Liu et al. 2007; Wang et al. 2005). *ARF2, ARF3* and *ARF4* are few other members of *ARF* gene family, exhibiting an important role in auxin homeostasis which is targeted by ta-siRNAs (Allen et al. 2005; Gautam et al. 2021; Williams et al. 2005). miRNA regulates breakdown of non-coding transcripts of *TAS* gene. A very important role is played by an *RNA-DEPENDENT RNA POLYMERASE (RDR6)* in the development of the cleavage product, i.e., the double stranded RNA (dsRNA) (Howell et al. 2007). *DICER-LIKE 4 (DCL4)* then slices the long dsRNA which later on converts the dsRNA into gradual small 21 base pairs in size dsRNAs. These small fragments of dsRNAs are known as ta-siRNAs which ultimately trans-regulate the target genes. This can be explained by an example, where miR390 cleaves *TAS3*. The resulting ta-siRNAs antagonistically synchronises *ARF2*, *ARF3* and *ARF4* which further play a significant part in the establishment of leaf polarity along with the developmental timings (Fahlgren et al. 2006; Garcia et al. 2006; Hunter et al. 2006). A crucial role of *TAS3*/mi390/*ARF* modules has been recognized in the formation of lateral roots (Marin et al. 2010; Yoon et al. 2010). Expression of miR390 is induced by auxin along with *ARF2, ARF3* and *ARF4* (Marin et al. 2010; Yoon et al. 2010). miR390 expresses in pericycle cells

towards the ends of xylem poles during the initiation of lateral root and eventually produces ta-siARF. It further acts antagonistically to the function of *ARF2*, *ARF3* and *ARF4* present in the lateral root primordia (Marin et al. 2010). The multiplex autoregulatory loop between miR390, ta-siARFs, *ARF2, ARF3 and ARF4* and auxin have been well shown in one of the report (Martin et al. 2010).

The five transcription factors *ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR (ATAF)*, *NO APICAL MERISTEM (NAM)*, *CUP SHAPED COTYLEDON (CUC*) are targeted by miR164 (Guo et al. 2005; Olsen et al. 2005). Along with the role played in development of aerial plant parts, *NAC1* is also observed to be associated in the LR development. It relays signals downstream of an F-box auxin receptor, TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Blein et al. 2010; Guo et al. 2005; Laufs et al. 2004; Mallory et al. 2004a; Ruegger et al. 1998; Xie et al. 2000). The NAC1 transcript is dissected into fragments by auxin regulated miR164 (Guo et al. 2005). It is present in pericycle cells and plays crucial role in LR formation (Xie et al. 2000). Similar results were observed in *Medicago* plants, where it was observed that *ARF10/16/17*-miR160, *HAM1/2/3*-miR171 and *ARF6/8*-miR167 also affected the LR development under nitrogen starved conditions (Liang et al. 2012). Thus, miR164, *NAC1* and auxin together function as a conserved unit in regulating root structure and development in nutrient starved conditions. Also, the role of auxin in root development in association with nitrate signals is also reported (Forde 2002; Walch-Liu et al. 2006).

4 Role of miRNA in Adventitious Root Development

Some common mechanisms and genes that account for regulating LR and AR development have been identified previously (Hochholdinger and Zimmermann 2008). Several *ARFs* along with auxin and miRNAs are reported in *Arabidopsis thaliana* to modulate the development of AR (Gutierrez et al. 2009). miR160 governs the formation of adventitious roots homologous to the lateral root development, but this regulation is most likely associated with *ARF17* and not to the suppression of *ARF16*. On the contrary, miR167 pessimistically affects AR development by regulating *ARF6* and *ARF8*. Although, *Arabidopsis thaliana* harbours both *ARF8* and *ARF17* but these act as rivals in maintaining auxin homeostasis (Sorin et al. 2005; Tian et al. 2004). *ARF8* and *ARF17* however, regulate the functioning of certain auxin-amino acid-conjugating enzymes coded by *GH3*-like genes. Gutierrez et al. carried out experiments to highlight the partial dependency of miR160, miR167 and their associated targets on light (Gutierrez et al. 2009).

ARF10 and *ARF16* in conjunction with miR160 promotes growth of the root tip along with their ability to sense gravity (Wang et al. 2005). miR164 along with the transcription factor *NAM/ATAF/CUC1* (*NAC1*) synchronises the branching and LR initiation (Guo et al. 2005). miR167 regulates the expression of *IAA-Ala Resistant3* (*IAR3*) to control the LR and PR development under high osmotic stress (Kinoshita et al. 2012). Another report has shown that miR393 regulates the expression of

TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and AUXIN SIGNALLING F-BOX 2 (AFB2) which in turn affects LR growth and development (Meng et al. 2011 and Chen 2009), Table 1 highlights the role of selected miRNA along with their targets, involved in *Arabidopsis* root development.

In a previous report an insensitive auxin-resistant rice mutant has been observed (Meng et al. 2009). This rice mutant exhibited diminished root cap, lateral root and adventitious root development (Meng et al. 2009). Among the multitude of miRNAs involved in auxin signalling or root development, some of the major auxin related miRNAs have shown significant impaired expression pattern in *osaxr* (Meng et al 2009). A suitable example of this case has been observed in osa-miR164abf that showed higher expression level whereas osa-miR167d-j and osa-miR390 were repressed in *osaxr*. In contrast, in case of *Arabidopsis thaliana*, miR167 was observed as an enhancer for the development of adventitious roots in rice (Meng et al 2009). Thus, we can conclude that these auxins related miRNA/ta-siRNA regulatory pathways in case of evolutionarily distinct species cannot be fully conserved.

5 Conclusion and Future Perspectives

Plant developmental and physiological processes are influenced by exogenous and endogenous factors. Recent reports have shown the regulatory networks of miRNAs in various biological processes including epigenetic silencing of transposable elements, pattern formation, response to environmental stress, and defence against invading pathogens (Singh et al. 2018). The potential role of miRNAs in meristem development, vegetative and reproductive organ growth and establishment of lateral organ polarity and boundaries, etc. suggest the significant effect of miRNAs on plant development (Singh et al. 2018). Moreover, miRNA are known to play a crucial role in the plant development by regulating protein–protein and protein DNA interactions, as many target genes are responsible for encoding transcription factors. The transcription factors are the major player controlling cell division/differentiation/expansion, phase transition, nutrition homoeostasis and specification of organ identities thus serve as, effectors of mRNA in plant growth (Bartel and Bartel 2003; Jones-Rhoades et al. 2006). miRNA associated with plant development can be highly conserved in plant kingdom or may be unique in particular plant species (Axtell and Bowman 2008; Cuperus et al. 2011; Djami-Tchatchou et al. 2017; Zhang et al. 2007).

Mutation in key genes involved in miRNA biogenesis mainly, *DICER LIKE1* (Schauer et al. 2002), *ARGONAUTE1* (Lynn et al. 1999; Vaucheret et al. 2004), *HEN1* (Boutet et al. 2003), *HYPONASTIC LEAVES1* (Han et al. 2004; Lu and Fedoroff 2000) and *ZIPPY* (Hunter et al. 2003) leads to range of developmental defects in plants, thereby illustrating the significant role of miRNA in plant growth and development. In *Arabidopsis*, miRNA miR165/166 have been reported to target at least five members of the CLASS *III HOMEODOMAIN-LEUCINE*

Table 1 Role of miRNAs in root development of *Arabidopsis thaliana* (primary root, lateral root and adventitious root)

miRNA	Target	Function (root development)	References
miR156	*SPL3, 9, 10*	Involved in lateral root development	(Gautam et al. 2017; Yu et al. 2015)
miR160	*ARF10,16,17*	Involved in root cap formation, lateral root and primary root development	(Mallory et al. 2005; Wang et al. 2005)
miR164	*NAC1*	Involved in lateral root development	(Guo et al. 2005; Meng et al. 2011)
miR165/166	*HD-ZIP IIIs*	Involved in primary root development	(Meng et al. 2011; Singh et al. 2014)
miR167	*ARF6, 8* and *IAR3*	Involved in lateral root and adventitious root development	(Gutierrez et al. 2009; Meng et al. 2011)
miR169	*NF-YA2, 10*	Involved in primary root development	(Meng et al. 2011; Sorin et al. 2014)
miR171	*HAIRY MERISTEM 1 (HAM1), HAM2* and *HAM3*	Involved in primary root development, maintenance of QC and columella stem cells	(Llave et al. 2002; Wang et al. 2010)
miR390	*TAS3*	Involved in lateral root development	(Marin et al. 2010; Meng et al. 2011)
miR393	*TIR1-NAC1*	Involved in lateral root development	(Meng et al. 2011)
miR394	*LCR*	Involved in ABA-(abscisic acid) dependent seed germination and root growth	(Song et al. 2013)
miR395	*SULTR2, APS1, APS4*	Unknown role in root development	(Meng et al. 2011)
miR396	*GRF1, GRF3*	Involved in reprogramming of root cells	(Hewezi et al. 2012; Meng et al. 2011)
miR398	*CSD1, CSD2*	Unknown role in root development	(Meng et al. 2011)
miR399	*PHO2*	Involved in phosphate signalling and essential for total root development	(Kim et al. 2011; Meng et al. 2011)
miR847	*IAA28*	Involved in lateral root initiation	(Wang and Guo 2015)

(continued)

Table 1 (continued)

miRNA	Target	Function (root development)	References
miR857	*LACCASE7*	Involved in secondary xylem differentiation	(Zhao et al. 2015)
miR858	*MYB11, MYB12, and MYB111*	Involved in primary root development	(Mehrtens et al. 2005; Stracke et al. 2007)
ta-siRNA	*ARF2/3/4*	Involved in primary root and lateral root development	(Gautam et al. 2021; Yoon et al. 2010)

ZIPPER (*HD-ZIPIII*) family genes including *PHAVOLUTA* (*PHV*)/*ATHB9*, *INTERFASCICULAR FIBERLESS/REVOLUTA* (*IFL/REV*) *PHABULOSA* (*PHB*)/*ATHB14*/*INCURVATA*/*CORONA*/*ATHB15* and *ATHB8* and thereby control meristem functions (Byrne 2006; Barton 2010; Emery et al. 2003; Prigge et al. 2005). In *Arabidopsis, ARGONAUTE10* (*AGO10*) is mainly responsible for sequestering miR165/166, dowregulation of *AGO10* results in an increased miR165/166 loading, leading to the decreased expression of *HD-ZIPIII* genes which eventually is responsible for the differentiation of SAM (Zhu et al. 2011). miR396 regulates the expression of *GROWTH RESPONSE FACTORS* (*GRFs*) and regulates the specification of root cell identity by *AP2/EREBP* family gene *PLETHORA* (*PLT*). miRNA also takes part in the regulation of root architecture by modulating the auxin signalling by targeting *ARF* family genes (Curaba et al. 2014; Rodriguez et al. 2015). The regulation of *ARF* family genes, namely *ARF10*, *ARF16* and *ARF17* is controlled by miR160 while its overexpression led to reduction in root length but an increase in the number of lateral roots (Wang et al. 2005).

The most important miRNA in the floral transition, responsible for controlling phase transition is miR156 which targets *SQUAMOSA-PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes (Cho et al. 2012). Extended juvenile phase and delayed flowering has been reported in *Arabidopsis* due to the presence of constitutively overexpressing miR15 (Huijser and Schmid 2011). Another miRNA, miR172 upon direct activation by *SPL* family members promotes flowering and it is accelerated through overexpression of miR172 while a group of *AP2* domain transcription factors is downregulated, comprising *TARGET OF EAT1 (TOE1), TOE2, TOE3, SCHNARCHZAPFEN (SNZ) SCHLAFMUTZE (SMZ),* and *AP2* that together with *AP1*, *SOC1* and *FT* regulates floral transition (Huo et al. 2016; Jung et al. 2007; Mathieu et al. 2009). Flowering is found to be mediated by miR159 that along its downstream *GAMYB-like* genes repress the floral transition and is a conserved pathway in *Arabidopsis,* ornamental plant gloxinia (*Sinningia speciosa*) and rice and to some extent depends on GA-mediated signalling and floral identity gene *LFY* (Achard et al. 2004; Li et al. 2013; Schwab et al. 2005; Tsuji et al. 2006).

Recent advances and thorough research in the field considering non-coding RNAs have discovered several types of functional non-coding RNAs that possess great potential to regulate major agronomic characteristics (Raven and Edwards 2001).

This potential can be taken into use as a new breeding tool to develop genetically improved plants. However, many of the miRNAs have remained unexplored in terms of their biogenesis, regulatory mechanism and target. The species-specific miRNAs due to their weaker expression and lack of target genes as compared to conserved miRNAs are considered as "inert" and are thus responsible for functionality of miRNAs. To explore more about non-conserved miRNAs there is a need to examine more carefully the fine changes caused by miRNAs. In large gene families, for the conserved miRNAs, redundant functions are played by different members that account for either tissue-specific or organ-specific roles. The spatial and temporal-specific expression along with the function of different members is therefore worth investigating. RNA sequences have been elucidated through the use of high throughput technologies, like next generation sequencing. This technology on combining with appropriate tools and algorithm can help to predict the target miRNAs, their experimental verification and functional analysis.

The mechanism behind the developmental role of miRNAs needs to be elucidated. The functional analysis and control mechanism of other non-coding RNAs and siRNA on plant development needed to be elucidated and their interactions with miRNAs along with the resulting impact on plant development can be of great significance.

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Rooting the Right Way: Role of Glucose Signaling in Regulating Root Development in Plants

Harshita B. Saksena and Ashverya Laxmi

Abstract Root growth and development is highly plastic and adapts to the fluctuating environmental conditions via modulating the root system architecture (RSA). RSA principally comprises of primary root (PR), lateral roots (LR), adventitious root (AR) and root hairs. Optimum root architecture is crucial for plant growth as it aids in providing mechanical support to the aerial organs of the plant, proper anchorage to the soil, uptake of essential nutrients and minerals and interaction with symbiotic organisms. Glucose regulates numerous aspects of root development through different signalling pathways including Hexokinase1 (HXK1) dependent signalling, RGS1 mediated heterotrimeric G-protein (HXK1-independent) signalling or via energy mediated signalling through Target of Rapamycin (TOR) kinase and SUCROSE-NON-FERMENTATION KINASE1 (SnRK1). Additionally, crosstalk of glucose with various phytohormones like auxin, cytokinin (CK), brassinosteroid (BR), ethylene, jasmonic acid (JA), abscisic acid (ABA), gibberellic acid (GA) is also essential to achieve optimal RSA. Thus, this book chapter will provide an insight on the significance of glucose in modulating the root growth and development in plants.

1 Introduction

Roots are an integral organ of the plant that not only aid in proper anchorage to the soil but also helps in uptake of water and nutrients for the plant growth and development. The root system comprises of primary root (PR), lateral root (LR), adventitious root (AR) and root hairs (RH). The PR develops from root apical meristem during embryogenesis and later gives rise to secondary roots which further produce tertiary roots. These secondary and tertiary roots are together known as the LRs (Malamy 2005). AR includes roots which develop from non-root tissue like junction roots, brace roots, crown roots, stem roots and hypocotyl roots (Steffens and Rasmussen 2016; Bianco and Kepinski 2018). Parameters including number, length and growth

H. B. Saksena · A. Laxmi (✉)
National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India
e-mail: ashverya_laxmi@nipgr.ac.in

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angle of PR, LR, AR and RH form the root system architecture (RSA) (Bianco and Kepinski 2018). Roots system possesses a high developmental plasticity (Malamy 2005) and can modulate its architecture in response to various internal (eg. phytohormones) and external (eg. touch, gravity, nutrient starvation, environmental stress) factors (Kushwah et al. 2011a). Sugars are not only a source of energy but also function as signalling molecules. Complex crosstalk between sugar signalling, phytohormones and environmental cues govern plant growth and development. Among the diverse sugar molecules that play role in plant development, glucose has been identified the most evolutionary conserved signal which controls several aspects of plant growth and development including cell cycle progression, primary and secondary metabolism, expression of numerous genes and proteins etc. (Sheen 2014). Perception and transduction of glucose signal occurs via direct or indirect sensing mechanisms. Direct glucose sensing mechanisms include Hexokinase (HXK1) dependent and HXK1 independent pathways and indirect glucose sensing occurs via energy and metabolite sensors (Sheen 2014; Li and Sheen 2016). HXK1 was discovered as the first intracellular glucose sensor in plants. HXK1 functions both in glucose metabolism and signalling in Arabidopsis. The role of HXK1 as a glucose sensor was determined through a S177A mutation which disrupts its catabolic activity but retains its function as a glucose sensor (Moore et al. 2003; Sheen 2014; Li and Sheen 2016). The functions of HXK1 are also evolutionary conserved across the plant kingdom (Li and Sheen 2016). Extracellular glucose can also be perceived via HXK1 independent pathway which involves regulator of G-protein signaling (RGS1) as a glucose sensor. In the presence of glucose, RGS1, seven transmembrane protein, forms a complex with G-PROTEIN ALPHA-SUBUNIT1 (GPA1) and frees $G\beta\gamma$ which recruits WNK8 (WITH NO LYSINE8) to phosphorylate RGS1 followed by RGS1 endocytosis and uncoupling of GPA1 to activate downstream G-protein mediated signalling (Urano et al. 2012). GPA1 further regulates glucose response through THYLAKOID FORMATION1 (THF1) (Huang et al. 2006). Apart from direct sensing, indirect sensing of glucose also occurs via energy sensors like the TARGET OF RAPAMYCIN (TOR) protein kinase to coordinate energy status of plant during growth and survival (Xiong et al. 2013; Li and Sheen. 2016). TOR is a serine/threonine kinase and is composed of two structurally and functionally different complexes known as TOR complex I (TORC1) and TOR complex II (TORC2) (Xiong and Sheen 2014). Some components of mammalian TORC1 have been identified in plants including Arabidopsis such as RAPTOR1/2 (REGULATORY ASSOCIATE PROTEIN OF TOR), LST8-1/2 (LETHAL WITH SEC-13 PROTEIN8), S6K1/2 (RIBOSOMAL PROTEIN S6 KINASE), RPS6a/b (RIBOSOME PROTEIN SMALL SUBUNIT6) and TAP46 (TYPE 2A-PHOSPHATASEASSOCIATED PROTEIN 46 KD) (Anderson et al. 2005; Deprost et al. 2005; Mahfouz et al. 2006; Ahn et al. 2011; Moreau et al. 2012; Ren et al. 2012; Xiong and Sheen 2014; Sheen 2014) Under energy deprivation conditions, SUCROSE-NON-FERMENTATION KINASE1 (SnRK1) is activated to regulate stress signalling and plant development (Crozet et al. 2014; Tomé et al. 2014; Li and Sheen 2016). KIN10/11 kinase form the catalytic part of the SnRK1 complex in Arabidopsis and repress the expression of genes responsible for growth and development (Baena-González et al. 2007; Li

and Sheen 2016). TOR kinase and SnRK1 are the major players in glucose mediated energy signalling and show antagonistic regulation of glucose responsive genes (Xiong et al. 2013; Baena-González et al. 2007; Li and Sheen 2016). Considering the important role of glucose signalling in regulating plant growth and development, this book chapter will discuss regarding the regulation of root development and architecture via different glucose signalling mechanism in concert with diverse phytohormones.

2 Role of HXK1 Dependent Pathway in Regulating Root Development

Glucose led to increase in root length, number of LRs and RH and regulated gravitropic response of PR in a dose dependent manner. The glucose mediated root responses were HXK1 pathway dependent as the *glucose insensitive 2 (gin2)* (HXK1) showed decreased PR growth, LR number and a constitutive phenotype for root deviation (Fig. 1) Thus, suggesting a role of glucose mediated HXK1 signalling for optimal RSA (Mishra et al. 2009). In a study by Kushwah et al. (2011a), CK induced asymmetrical root growth caused by differential cell elongation. Two component system of CK signalling involving CK receptor, ARABIDOPSIS HISTIDINE KINASE4 (AHK4) and Type-A and Type-B ARRs were required for CK induced asymmetrical root growth. Further, downstream to CK signalling, ethylene signalling and auxin

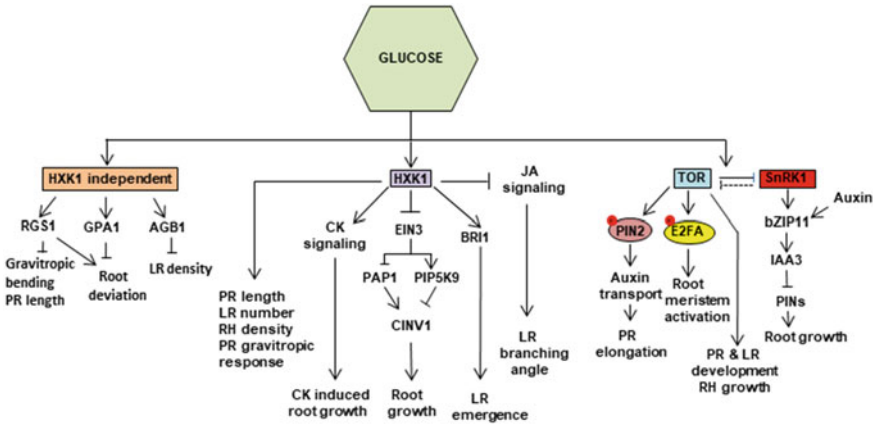

Fig. 1 Role of diverse glucose signalling pathways in regulating root development in plants. Glucose is perceived via direct sensing including HXK1 sensor and RGS1 mediated G-protein signalling (HXK1 independent) or via indirect sensing through TOR and SnRK1 mediated energy signaling. Glucose controls several aspects of root development through these signaling pathways. The HXK1 mediated glucose signalling regulates root development by interacting with different phytohormone signalling pathways. Similarly, TOR-SnRK1 signaling translates the energy status of the plant to control root growth via presenting an interplay with auxin

signalling and transport were essential to transduce the signal for CK dependent root growth. Also, glucose augmented this response in HXK1 dependent manner as *gin2* mutant displayed a decreased CK induced root growth (Kushwah et al. 2011a) (Fig. 1). Another study by Kushwah and Laxmi (2017) determined that glucose and CK function antagonistically at lower glucose concentration while agonistically at higher glucose concentration to control PR length in a HXK1 dependent manner as *gin2* was resistant to all glucose concentrations tested towards CK for PR length inhibition (Kushwah and Laxmi 2017). Glucose also affected the root gravitropism kinetics of gravistimulated seedlings and led to slow down in gravitropic bending of roots with increasing concentrations. This gravitropic root curvature response was significantly reduced in *gin2* mutant roots in the presence of glucose suggesting HXK1 mediated glucose signalling is involved in regulating the root bending in response to gravistimulation (Singh et al. 2014a) (Fig. 1) High light fluxes led to photosynthetically generated sugar signals which mimic exogenously supplementation of glucose (Singh et al. 2014a). A study by Singh et al. (2014a) revealed that *gin2* mutant displayed perturbation towards high light flux induced root deviation from the vertical. Glucose induced LR formation and density was significantly reduced in the *gin2* mutant, however, the HXK1 overexpression seedlings exhibited enhanced LR production and density (Mishra et al. 2009; Gupta et al. 2015). Similar to the impaired root deviation response in high light fluxes, *gin2* mutant also exhibited reduced LR emergence. In addition, *gin2* mutant showed an impaired response to exogenous BR for LR production and diminished expression of BR-related genes in the presence of glucose. Application of glucose and BR to *bri1-6* mutant and *gin2-1bri1-6* double mutant further abolished the emergence of LR indicating that BRASSINOSTEROID INSENSITIVE 1 (BRI1) works downstream to HXK1 signalling to regulate glucose-BR mediated LR production (Gupta et al. 2015) (Fig. 1). Recently, Meng et al. (2020) determined that endogenous or exogenous sucrose led to increase in the CYTOSOLIC INVERTASE1 (CINV) activity and produced endogenous glucose which further promoted root growth. Further, the authors observed that *ethylene-insensitive3-1 (ein3-1)* mutants showed increased root length due to accumulation of high endogenous glucose levels as a result of enhanced CINV1 activity suggesting EIN3 functions as a negative regulator of glucose mediated root growth. The HXK1 mutant *gin2* exhibited reduced root growth and endogenous glucose levels. However, *ein3/gin2-1* double mutant exhibited enhanced root growth suggesting EIN3 functions downstream to HXK1-dependent glucose signalling to negatively regulate glucose mediated root growth. Moreover, EIN3 showed binding to the promoters of *PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1)* and *PHOSPHATIDYLINOSITOL MONOPHOSPHATE 5-KINASE 9 (PIP5K9)* and further suppressed and enhanced their transcription respectively. Meanwhile, PAP1 was further bound to *CINV1* promoter to enhance its expression and PIP5K9 showed negative regulation of CINV1. The *gin2* mutant exhibited enhanced *EIN3* and *PIP5K9* gene expression; while reduced expression of *PAP1* and *CINV1* was observed thus suggesting glucose-HXK1-EIN3-PAP1/PIP5K9-CINV1-glucose loop governs root growth by HXK1-mediated glucose signalling (Meng et al. 2020) (Fig. 1). Arabidopsis seedlings treated with exogenous methyl jasmonate (MeJA) showed decrease in LR branching angle

which was further enhanced on application of glucose thus suggesting an antagonistic interaction between glucose and MeJA. Glucose mediated regulation of LR branching angle was HXK1 dependent as *gin2* mutant showed perturbed response to MeJA as well as to the combined treatment of MeJA and glucose (Sharma et al. 2020) (Fig. 1). Collectively, HXK1 dependent glucose signalling contributes to various aspects of RSA independently or in concert with other factors including phytohormone signalling.

3 Role of RGS1 Mediated Heterotrimeric G-protein Signalling (HXK1-Independent) in Regulating Root Development

Glucose signal transduction also occurs through HXK1-independent pathway via heterotrimeric G-protein coupled receptor signalling wherein RGS1 acts as glucose sensor (Chen and Jones 2004). Very few reports document the role of glucose-mediated heterotrimeric G-protein coupled receptor signalling in modulating RSA. Booker et al. (2010) determined that auxin-induced, LR formation becomes bimodal with respect to auxin concentration in the presence of glucose in a G protein-complex dependent manner. Auxin induced the production of lateral root primordial (LRP) at both low and high concentrations. However, glucose only displayed its effect in the presence of high dosage of auxin by decreasing the LRP number. In contrast, glucose was required for auxin induced LR emergence at both modes but particularly more at high dose of auxin. Abolishment of either AtGPA1 or AtRGS1, but not AGB1, eliminated the glucose response suggesting heterotrimeric G-protein complex mediated signalling is required for glucose attenuation of auxin bimodality for LR development (Booker et al. 2010). Glucose mediated root deviation responses in *rgs1-1, rgs1-2,* and *thf1-1* were reduced while *gpa1-1, gpa1-2,* and *gpa1-3* showed increased degree of glucose induced root deviation from the vertical suggesting that HXK1-independent pathway plays a role in root deviation response (Fig. 1).Gravitropic bending was increased in *rgs1-1* mutant after 90° gravistimulation corresponding to involvement of HXK1-independent pathway in modulating the gravitropic bending responses (Singh et al. 2014a) (Fig. 1). Mudgil and coworkers also found that *agb1* mutant showed increased LR density as it had higher level of photosynthetically derived glucose and other sugars like fructose and sucrose in the roots. In addition, AGB1 was critical to achieve glucose- induced auxin patterning in the primary root apical meristem and LR primordia and also attenuated glucose mediated PIN2 membrane localization. Also, *rgs1* mutants exhibited increased PR length while LR density was slightly compromised (Fig. 1).Therefore, it concludes that G-protein signalling extends the glucose effect on RSA (Mudgil et al. 2016).

4 Role of Glucose Mediated TOR-SnRK1 Energy Signalling in Regulating Root Development

Glucose is indirectly sensed in the form of high energy or low energy status by the energy sensors TOR kinase and SnRK1 respectively (Xiong et al. 2013; Crozet et al. 2014; Tomé et al. 2014). TOR kinase and SnRK1 function antagonistically to each other to modulate glucose mediated energy signalling (Xiong et al. 2013; Baena-González et al. 2007; Li and Sheen 2016). Arabidopsis seedlings treated with rapamycin, a TOR inhibitor, showed retardation of PR and LR growth. Also, estradiol-inducible *tor* mutants (*tor-es*) exhibited a similar response in the presence of glucose thus suggesting the involvement of glucose-TOR signalling in PR and LR development (Xiong and Sheen 2012) (Fig. 1). Rapamycin exerts its inhibitory effect by formation of a complex with the FK506-BINDING PROTEIN 12 (FKP12) and the FKBP12-RAPAMYCIN BINDING (FRB) domain of TOR kinase (Wullschleger et al. 2006). Xiong and Sheen (2012) also showed the inhibitory effect of rapamycin on the root hair growth of Arabidopsis seedlings supplemented with glucose. However, the *fkp12* mutants were resistant to rapamycin mediated inhibition of root growth. The estradiol inducible *tor* mutant also displayed diminished root hair growth (Xiong and Sheen 2012). Active-site TOR inhibitors (asTORis) caused inhibition of Arabidopsis root growth by affecting the length and division area in the meristematic zone, epidermal cells in elongation zone and the root hair cells in a dose dependent manner (Montane and Menand 2013). Xiong et al. (2013) revealed an important observation that endogenous glucose derived from photosynthesis, activated TOR signalling through glycolysis and mitochondria mediated energy relay to control root meristem activation. Application of glycolysis and mitochondrial electron transport inhibitors abolished root growth and reactivation of quiescent root meristem. Similarly, in oestradiol inducible *tor* mutants, the reactivation of root meristem was blocked and root growth was inhibited. TOR kinase phosphorylates downstream E2F transcription factors to regulate cell proliferation. The *e2fa* null mutant displayed compromised root growth and root meristem expansion in the presence of glucose signifying glucose mediated TOR-E2F module function in regulating root development (Xiong et al. 2013) (Fig. 1). In line with this, Li et al. (2017) revealed that glucose promoted PR growth and root meristem reactivation independent of light. Application of yucasin, an auxin biosynthesis inhibitor led to reduction in auxin accumulation and *pCYCB1;1::GUS* expression in the root meristem in the presence of glucose which was rescued by auxin treatment. Both auxin and glucose were required to activate root meristem in the dark (Li et al. 2017). A recent study by Yuan et al. (2020) demonstrated that *PIN- FORMED 2 (PIN2)* mutants were less sensitive to inhibition by Torin2 (TOR inhibitor) on glucose induced primary root growth. This Torin2 resistant phenotype in *eir1-1*, a *PIN2* mutant was a result of changes in cell elongation rather than cell division. Therefore, glucose-TOR signaling mediated regulation of *PIN2* was required to maintain low auxin concentrations in the root to stimulate elongation (Yuan et al. 2020) (Fig. 1). An interaction of sulfur signalling and glucose-TOR signalling in controlling root meristem activity was

discovered by Dong et al. (2017) wherein roots of *SULFITE REDUCTASE (SiR)* mutant had reduced meristematic activity due to inhibition of TOR activity in *sir1-1*. Glucose treatment led to restoration of root meristematic activity in *sir1-1* and was blocked in the presence of TOR inhibitors (Dong et al. 2017; Wu et al. 2019). Apart from scientific studies directing the role of high energy, glucose mediated TOR in root development, a report by Weiste and coworkers demonstrated the involvement of low energy induced SnRK1-C/S1 basic leucine zipper (bZIP) signalling in regulating root development. bZIP transcription factors modulated the expression of *IAA3* under starvation conditions and thus interfered with PR growth. Arabidopsis seedlings showed inhibition of PR growth under energy deprivation conditions. However, supplementation with glucose led to a rescued phenotype. Both bZIP knockdown lines and *iaa3* mutants exhibited less repression of root growth under starvation conditions, thus implying bZIP function via IAA3 to modulate root growth depending on energy conditions. bZIP11 also led to reduction in accumulation of PIN1 and PIN3 in the roots thereby affecting the polar auxin transport and causing auxin depletion at the root apex. Thus, auxin depletion at the root apical meristem led to cell differentiation and inhibition of root growth (Fig. 1). Therefore, interplay of bZIP and SnRK1 signalling via auxin signalling and transport coordinate energy mediated PR growth (Weiste et al. 2017).

5 Crosstalk Between Glucose and Phytohormones in Regulating Root Development

Root development is highly complex and involves an intricate network of different cellular and molecular pathways to achieve optimum structure and function. This particular segment of the chapter discusses how exogenous glucose modulates RSA by regulating various phytohormone signalling. A study by Mishra et al. (2009) reported that glucose interacts with auxin at the global transcriptomic level and modulates the expression of auxin affected genes. The authors revealed that increasing concentration of glucose could alter the root length and LR formation in auxin perception *transport inhibitor response1 (tir1)* and auxin signalling *solitary root/ indole-3-acetic acid inducible 14 (slr1/iaa14), auxin resistant 3/ indole-3-acetic acid inducible 17(axr3/iaa17)* and *auxin resistant 2/ indole-3-acetic acid 7(axr2/iaa7)* mutants. Also, in *slr1/iaa14* and *axr2/iaa7* mutants, roots are agravitropic and show randomized growth in presence of increasing concentrations of glucose. Thus, glucose can heighten the growth defects in the mutants having perturbed auxin signalling by further interfering with auxin signalling and transport. Auxin signalling mutants such as *slr1/iaa14*, *axr2/iaa7* and *axr3/iaa17* are devoid of root hair formation. However, high glucose concentrations could rescue root hairless phenotype of these mutants (Fig. 2).The study proposes that glucose may induce the root hair initiation in auxin signalling mutants via destabilizing mutated AUX/IAA or by inducing a transcription factor which can release AUXIN RESPONSE FACTORS (ARFs) from the inhibitory

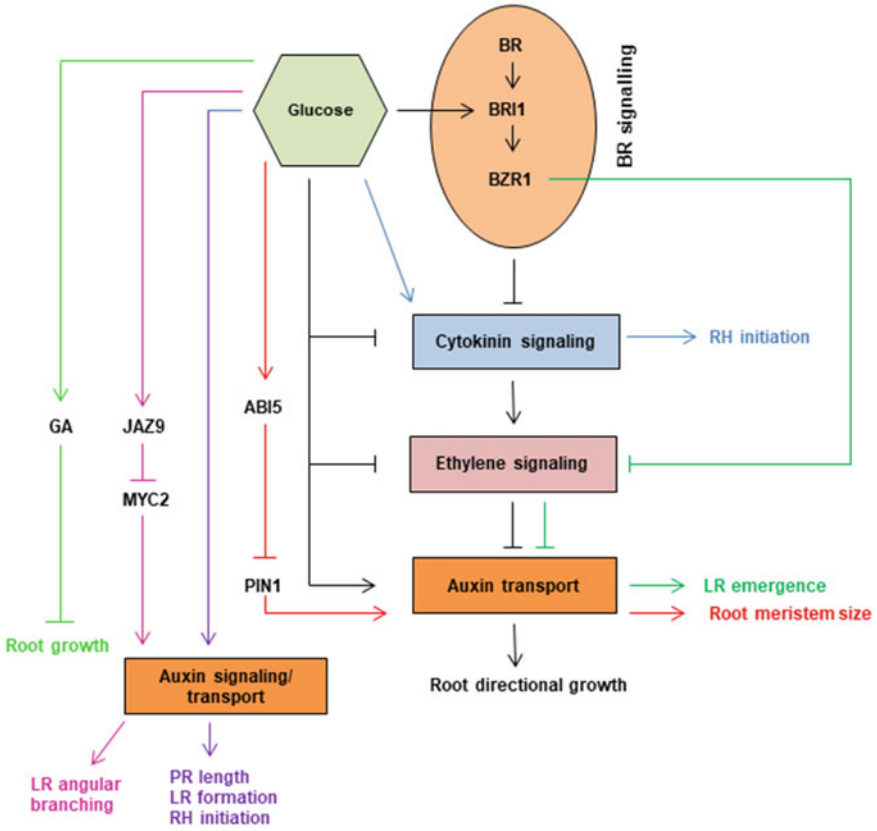

Fig. 2 Crosstalk between glucose and various phytohormones regulates root development in plants. Exogenous glucose modulates various aspects of root system development via regulating the components of different phytohormone signaling pathways

effect of AUX/IAAs and thus promote transcription of auxin regulated genes (Mishra et al. 2009). Exogenous application of indole-3-acetic acid (IAA) and/or glucose led to increase in total root length, root surface area, and root volume in *Malus baccata* (L.) Borkh. seedlings (Lang et al. 2019). Also, glucose and IAA regulated the expression of *SHY2, SHR, ALF4, and LBD11* to promote RSA. Treatment of seedlings with 2,3,5-triiodobenzoic acid (TIBA), an auxin polar transport inhibitor caused reduction in root topological traits, however supplementation of glucose to TIBA treated seedlings reduced the adverse effects on RSA by increasing expression of auxin biosynthesis genes such as *YUCCA8, TAR2, TAA1, and CYP79B3* and polar transport genes including *PIN1, AUX1, and LAX2* thereby increasing the endogenous auxin in roots (Lang et al. 2019). Singh et al. (2014b) demonstrated an interplay of glucose and phytohormones in modulating the root directional growth in Arabidopsis. The CK receptor mutants, *ahk2* and *ahk4* and type- B ARR mutants exhibited an

increased root directional response while mutants of type -A ARR showed an attenuated response. In line with this, the ethylene signalling mutants *ethylene resistant1–1 (etr1–1)* and *ethylene insensitive2 (ein2–1)* displayed an enhanced root directional response in the presence of glucose and application of ethylene biosynthesis inhibitor also promoted glucose induced root directional response thus suggesting negative regulation of glucose induced root directional response by CK signalling and ethylene signalling. The authors also observed that 6-benzylaminopurine (BAP) and 1-aminocyclopropane-carboxylic acid hydrochloride (ACC) were able to diminish the synergistic effect of glucose and BR in regulating root directional response. Thus, CK and ethylene signalling functions downstream to glucose-BR signalling and antagonize this response. Finally, auxin transport lies downstream to CK and ethylene signalling to exhibit the glucose induced root deviation response (Singh et al. 2014b). Ethylene signalling exerts a negative effect on the glucose-BR regulated LR emergence (Singh et al. 2015). (Fig. 2). Application of ACC to *brassinazole resistant1-1D (bzr1-1D)* mutant in the presence of glucose led to reduction in LR emergence. Further, ethylene signaling mutants *ein2-1* and *ein3-1* displayed enhanced LR emergence upon glucose treatment thus implying ethylene signalling works downstream and is antagonistic to glucose and BR to regulate LR emergence (Fig. 2). Auxin functioned downstream to ethylene signalling as LR emergence defect in *ethylene overproducer2 (eto2)* mutant was rescued on exposure to IAA in the presence of glucose (Singh et al. 2015). Glucose along with BR synergistically promoted root growth deviation from the vertical by enhancing BRI1 internalization. Treatment with protein phosphatase inhibitors like okadaic acid and cantharidin caused increase in BRI1 endocytosis and glucose induced root deviation. Mutant of *ROOTS CURL IN NAPHTHYL PHTHALAMIC ACID1 (RCN1)* having reduced PP2A activity showed amplified root growth deviation response in the presence of glucose which was restored by brassinazole (BRZ) treatment. In addition, inhibition of auxin transport by NPA in *bri1-6* in the presence of glucose led to root deviation response which was not observed in independent glucose treatment. Further, auxin transport mutants *ethylene insensitive root 1 (eir1-1)*, *auxin resistant 1(aux1-7)*, and *multidrug resistance 1 (mdr1-1)* exhibited a magnified glucose-induced root deviation response thus suggesting auxin functions downstream to glucose-BR signalling to control root directional responses (Singh et al. 2014a). In the roots of 35S::GFP-ABD2-GFP transgenic lines, presence of glucose resulted in vertical orientation of actin filaments. However, application of CK caused loss of this vertical arrangement of actin filaments. Similar effect was observed for auxin transport inhibitor, NPA thus actin filament organization is required for differential cell elongation and changes in root directional growth (Kushwah et al. 2011b). Kushwah and Laxmi (2017) also observed that *ahk4* and type B ARR triple mutant *arr1,10,11* showed increased PR length even in the absence of glucose as opposed to inhibition of root length in wild type in absence of glucose. Both increasing concentrations of glucose and CK led to compromised root length in auxin signalling mutants suggesting auxin might function to glucose-CK signalling to control PR length. Increasing concentration of glucose (upto 3%) led to disruption in RH initiation in CK signalling mutants and less number of RH were seen at 5%glucose suggesting a crosstalk between

glucose and CK signalling to mediate root hair initiation (Kushwah and Laxmi 2017) (Fig. 2).Recently, Sharma et al. (2020) proposed MeJA and glucose function antagonistically to regulate LR branching angle. Glucose stabilized JASMONATE-ZIM-DOMAIN 9 (JAZ9), a negative regulator of JA signalling. Reduced levels of JAZ9 were observed in the presence of glucose and MeJA thus suggesting the increase in MeJA induced LR branch angle by glucose. Auxin transport and signalling was required downstream to MeJA for LR angular branching as the auxin transport and signalling mutants had wider branch angle of LRs than wild type in the presence of glucose and MeJA (Sharma et al. 2020) (Fig. 2). High concentrations of glucose caused reduction in the size of primary root meristem in a dose dependent manner. Accumulation of PIN1 was diminished in the roots in presence of high concentrations of glucose thus affecting auxin levels in roots (Yuan et al. 2014). ABA INSENSITIVE 5 (ABI5), a bZIP transcription factor is involved in ABA signalling (Finkelstein and Lynch 2000; Lopez-Molina et al. 2001; Yuan et al. 2014). *ABI5* expression was induced by high concentrations of glucose in the root meristem and *ABI5* overexpression lines had shorter root meristem. PIN1 accumulation was regulated by ABI5 as *abi5-1* showed a marginal reduction in PIN1 levels in the presence of high concentrations of glucose (Yuan et al. 2014) (Fig. 2). Gabryszewska 2010 studied the effects of glucose and phytohormones including kinetin, indole-3-butyric acid (IBA), gibberellic acid (GA_3) on various aspects of plant development including root growth in *Paeonia lactiflora*' Jadwiga. Increasing concentrations of glucose without the supplementation of exogenous phytohormones promoted root growth in explants having leaves. However, application of kinetin or GA_3 in glucose media reduced root growth (Fig. 2). Addition of IBA along with glucose to the media promoted root.

6 Conclusions

Optimal root system development is necessary for plant growth and survival. Comprehensive understanding of root development is essential to improve agronomic productivity as these organs are majorly responsible for acquisition of water and nutrients from soil. Since, roots adapt to rapidly changing environmental conditions, they are an excellent system to explore developmental plasticity. Root system development is not only governed by external cues but also responds to the internal factors like plant growth regulators. Glucose, apart from being an important energy source, also functions as a signalling molecule and regulates various aspects of plant development. In this book chapter, we have highlighted the role of glucose in coordinating numerous aspects of root development via different signalling pathways. HXK1 mediated signalling contributed to PR growth, LR number and angle, RH growth and gravitropic bending response either independently or by interplay with different hormone signalling pathways. Similarly, HXK1 independent pathway also regulated PR and LR development and root deviation responses in plants. Several reports pointed towards the role of energy sensing via TOR-SnRK1 signalling in controlling root meristematic activity. In addition, crosstalk between exogenous glucose and

hormone signalling is also involved to control glucose- induced root development phenotypes. Taken together, glucose is an indispensible signal to balance different aspects of RSA.

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Plant Hormonal Crosstalk: A Nexus of Root Development

Shivani Saini, Isha Sharma, Priya, Aparna Maitra Pati, and Pratap Kumar Pati

Abstract The plant root is an essential organ that participates in several biological functions such as water and nutrient uptake as well as anchorage. Besides, roots also play a critical role in conferring stress tolerance in plants. In recent years, a lot of emphasis has been given to understand the key mechanisms involved in root morphogenesis and development. Among various factors controlling root growth and development, the role of plant hormones has been studied extensively. The modern experimental tools and resources have uncovered the critical role of plant hormones such as auxin, gibberellins, brassinosteroids, and strigolactones in regulating the signaling mechanisms involved in plant root development. Plant hormones are perceived by their respective receptors, which subsequently activate various signal transduction pathways to regulate root development. These plant hormones also integrate their signals to interact either synergistically or antagonistically in a coordinated manner to control various aspects associated with root morphogenesis and development. The present book chapter highlights some critical conceptual developments in the participation of plant hormones and their crosstalk in regulating various signaling components involved in root development.

Keywords Root · Phytohormones · Auxin · Brassinosteroids · Gibberellins · Strigolactones

S. Saini
Department of Botany, Goswami Ganesh Dutta SD (GGDSD) College, Sector-32C, Chandigarh 160030, India

I. Sharma
Agribiotech Foundation, PJTS Agricultural University Campus, Rajendranagar, Hyderabad 500030, Telangana, India

Priya · P. K. Pati (✉)
Department of Biotechnology, Guru Nanak Dev University, Amritsar 143005, Punjab, India

A. M. Pati
Chief Scientist, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

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1 Introduction

The root is crucial for the acquisition of water and nutrients, translocation of nutrients, anchorage of plants, symbiotic interactions, photoassimilates accumulation, storage of nutrients, synthesizing secondary metabolites and phytohormones (Meng et al. 2019; Vissenberg et al. 2020). Besides, the root also perceives various environmental signals to trigger defense related mechanisms for acclimatization (Saini et al. 2018). Owing to its multi-dimensional roles, efforts are being employed to explore the hidden part of the plant i.e. root, as it is underutilized for improving crop productivity (Den Herder et al. 2010; Comas et al. 2013). Moreover, targeting a particular organ rather than a whole plant has become an area of interest among plant biologists for improving growth and yield (Seo et al. 2020). As plant productivity significantly depends upon root development, hence, root modulation and optimization are the key mechanisms for targeting the next green revolution (Seo et al. 2020). The great potential of root in improving yield and plant performance through optimization of root traits such as root length, root number, lateral roots (LR), root angle etc. has been realized (Lynch 2007; Comas et al. 2013). In current years, it has been elucidated that the root traits are controlled by various intrinsic as well as extrinsic factors. These root traits regulating factors involve plant hormones, nutrients, mycorrhizal associations, microbes, sugars, genes and transcription factors (Montiel et al. 2004; Saini et al. 2013; Verbon and Liberman 2016; Egamberdieva et al. 2017; Shahzad and Amtmann 2017; Hennion et al. 2019; Campo et al. 2020; Xu and Watahiki 2020).

Among various factors known phytohormones are critically involved in mediating interactions and different physiological processes participating in root growth and development (Saini et al. 2013; Chaiwanon et al. 2015). Phytohormones such as auxin, gibberellins (GA), brassinosteroids (BR), and strigolactones (SL) control various aspects of root development. There are several evidence which point towards the critical role of plant hormonal interactions in regulating signal transduction pathway for directing root growth (Schwechheimer 2012; Saini et al. 2013; Chaiwanon et al. 2015; Kumar et al. 2015). Among various phytohormones known auxin is considered as a master regulator which controls every aspect of root development (Saini et al. 2013). Recent findings link the processes of auxin biosynthesis, transport and its signaling with root formation (Morffy and Strader 2018). In current years, the role of another phytohormone BR in mediating root development related processes has also been elucidated. Various BR deficient and signaling mutants exhibited significantly shortened root phenotypes marking them as potential regulators of root growth (Müssig et al. 2003; Wei and Li 2016; Retzer et al. 2019). BR participate in LR formation, maintenance of meristem size, root hair initiation, gravitropic response, mycorrhizal association, and formation of nodules in leguminous plants (Müssig et al. 2003; Wei and Li 2016; Retzer et al. 2019). In current years, the role of GA in the regulation of root development has also been unveiled. Using GA biosynthesis inhibitors and GA-deficient mutants it is now evident that GA controls root cell elongation and size of the meristem at a low concentration range (Tanimoto 2012). SL has recently been discovered as a novel class of phytohormones which are

mainly synthesized in root and are largely associated with root formation process (Sun et al. 2016). They orchestrate root architecture by regulating the length of root hair, root hair density, prevent LR formation and increase the cell number of primary root meristem (Kumar et al. 2015; Sun et al. 2016). Hence, in the present chapter, the role of auxin, GA, BR and SL in root development has been emphasized. Furthermore, the crosstalk of these phytohormones in the regulation of signaling components associated with root development has also been highlighted.

2 Auxin and Its Crosstalk in Root Development

Auxin is one among several essential plant hormones which play a pivotal role in plant development and also mediate responses under environmental constraints (Korver et al. 2018). Auxin regulates plant responses by modulating auxin biosynthesis, transport, signaling, conjugation and degradation process (Potters et al. 2007). The biosynthesis of auxin has been extensively investigated suggesting the key role of *tryptophan aminotransferase of arabidopsis* (*TAA*) and *YUCCA* (*YUC*) genes in the production of auxin in young growing parts of root and shoot (Zhao 2008, 2014). Once synthesized, auxin is transported to different parts of the plant through passive diffusion and active transport mechanism (Casimiro et al. 2001; Blakeslee et al. 2007). The passive diffusion is mediated by phloem while the active transport is regulated by auxin influx (auxin1/like-aux1; Aux1/LAX family) and auxin efflux (pin-formed; PIN and ATP-binding cassette b4/p-glycoprotein; ABCB/PGP family) proteins (Bennet et al. 1996; Geisler and Murphy 2006). Once auxin enters the cell, it is perceived by the receptors of proteasome independent and proteasome dependent auxin signaling pathway. Proteasome independent auxin signaling pathway is regulated by auxin binding protein1 (ABP1) (Ljung 2013). While, proteasome dependent auxin signaling is mediated by the perception of auxin by transport inhibitor response1/auxin signaling f-box1 (TIR1/AFB1) family of auxin receptors (Kepinski and Leyser 2005; Leyser 2018). At high auxin levels, the formation of (TIR1/AFB1)-IAA-auxin/indole-3-acetic acid (AUX/IAA) co-receptor complex promotes the proteolytic degradation of AUX/IAA transcriptional repressors and the subsequent activation of auxin response factor (ARF) proteins which triggers auxin-related genes involved in plant growth and development process (Leyser 2018). Hence, AUX/IAA and ARF proteins regulate the expression of auxin-related genes (Leyser 2018). The storage of excess IAA in the form of their conjugates with glucose, or protein/peptide is performed by Gretchen Hagen3 (GH3) to maintain auxin homeostasis (Okrent and Wildermuth 2011; Fu et al. 2011).

Auxin is considered a key regulator of primary root elongation, gravitropism, LR formation, tuber and storage root development (Muday and Haworth 1994; Alarcón et al. 2019; Kondhare et al. 2021). Auxin interacts with various plant hormones to control the root development process. Cytokinin antagonizes auxin response of LR formation by degrading auxin efflux PIN proteins and interfering with polar auxin transport, suggesting their antagonistic interaction (Marhavý et al. 2011, 2014; Jing

and Strader 2019). Further, the auxin-CK crosstalk signals are relayed in the root by signaling component Arabidopsis histidine kinase3 (AHK3, a receptor of cytokinin) and a downstream transcription factor Arabidopsis response regulator1 (ARR1) (Fig. 1A). These regulate the transcript levels of IAA3/short hypocotyl2 (SHY2) which represses auxin signaling by attenuating the expression of PIN genes and inhibiting their intracellular trafficking thus hindering positioning and LR organogenesis in Arabidopsis (Fig. 1A) (Ioio et al. 2008; Yoshida et al. 2011; Marhavý et al. 2011; Bielach et al. 2012; Pacifici et al. 2015). Besides, SHY2 also play a critical role in regulating the size of the root meristem and its development. In Arabidopsis it was noticed that loss-of-function of *shy2-31* mutant alters balance between cell division and cell differentiation thereby, enlarging size of root meristem due to perturbed auxin distribution and its transport (Ioio et al. 2008; Moubayidin et al. 2010; Peng et al. 2013). Root growth is also known to be regulated by ARR12, which activates ARF19 (a member of ARF family), in conjunction with SHY2, thus triggering cell differentiation of meristematic cells at the transition zone (Perilli et al. 2013). Hence, crosstalk between auxin and ABA controls polar auxin transport for regulating root development. Abscisic acid insensitive4 (ABI4), which is induced

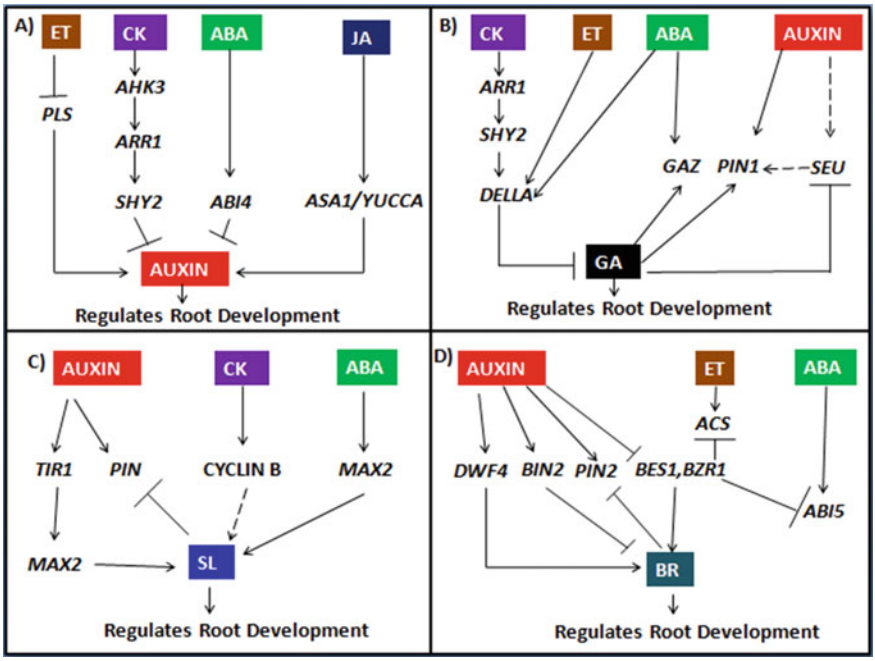

Fig. 1 Regulation of root development through interaction of (**A**) Auxin with ethylene (ET), cytokinin (CK), abscisic acid (ABA) and jasmonic acid (JA) (**B**) Gibberellins (GA) with CK, ABA, ET and auxin (**C**) Strigolactones (SL) with auxin, CK and ABA (**D**) Brassinosteroids (BR) with auxin, ET and ABA. Arrows show activation processes, bar represents repressed events, while dashed arrows show unidentified interactions

by ABA and CK, suppresses the expression of an auxin transporter PIN1 (Table1) (Shkolnik-Inbar and Bar-Zvi 2010). In Arabidopsis, *abi4* mutants develop more LR, while ABI4-overexpressing plants possess fewer LR suggesting ABA-mediated LR inhibition (Shkolnik-Inbar and Bar-Zvi 2010; Munguía-Rodríguez et al. 2020). Further, it has also been explored that ABI5 a basic leucine zipper (bZIP) transcription factor also suppresses polar auxin efflux by inhibiting PIN1 thereby regulating root growth and development (Fig. 1A) (Yuan et al. 2014; Pacifici et al. 2015). A strong hypersensitivity towards ABA, longer primary root and higher number of LR was observed in 35S::YUC4 seedlings in Arabidopsis (Munguía-Rodríguez et al. 2020). The study also suggests that higher content of the endogenous auxin acts synergistically with ABA for inhibiting root growth (Thole et al. 2014; Rowe et al. 2016; Munguía-Rodríguez et al. 2020). Further, augmenting ABA concentration has also been shown to delay primary root growth in a dose-dependent manner suggesting the negative impact of ABA in root development (Munguía-Rodríguez et al. 2020). In YUC4 overexpressing roots, PIN1 exhibited down-regulation, while PIN2, PIN3 and PIN7 showed up-regulation with respect to the wild-type (Munguía-Rodríguez et al. 2020). The study suggested that YUC4 overexpression regulates the process of auxin homeostasis and auxin-ABA crosstalk mediates primary root development. Auxin and ABA play integral roles in the regulation of root elongation which depends upon cell division and cell elongation process (Emenecker and Strader 2020). It was suggested that alleles of abscisic acid (ABA) signaling (ABI2), ethylene signaling (EIN2, ETR1), auxin signaling (IAA16, AXR4) and auxin transport (AUX1, PIN2) could mediate ABA resistance in primary root elongation assay. Further, ABA signaling mutants such as *abi1-1, abi2-1,* and *abi3-1* exhibited wild-type responses to 2,4-dichlorophenoxy acetic acid (a synthetic auxin) (Emenecker and Strader 2020). Hence, the study indicates that auxin, ABA, and ethylene crosstalk in the regulation of the root development process. In Arabidopsis, it has been observed that the POLARIS (PLS) gene transcribes mRNA which is repressed by ethylene while induced by auxin (Fig. 1A) (Casson et al. 2002; Chilley et al. 2006; Liu et al. 2014). The roots of *pls* mutants are short, exhibit less cell elongation, reduced auxin content, while elevated ethylene and cytokinin concentration. These mutants are also hyper-responsive to external application of cytokinins, as they show higher transcript levels of cytokinin-inducible *ARR5/induced by cytokinin6* (*IBC6*) gene (Table 1) (Casson et al. 2002; Chilley et al. 2006; Liu et al. 2010). In PLS overexpressing transgenics, enhanced auxin content was observed, while ethylene concentration remained unchanged. Further, in *pls* mutants, auxin efflux PIN1 and PIN2 proteins were also decreased (Casson et al. 2002; Chilley et al. 2006; Liu et al. 2010). Therefore, the study suggested potential crosstalk between auxin, ethylene and cytokinin in controlling root growth and development (Liu et al. 2014). The effect of exogenous application of jasmonic acid on the alteration of roots, endogenous content of IAA and its distribution has also been investigated in rice. The endogenous JA induces the expression of the auxin synthase gene (*ASA1*) and YUCCA, leading to the enhancement of auxin content (Fig. 1A) (Ronzan et al. 2019). Besides regulating the expression of auxin biosynthesis genes, JA also controls the expression of JAZ1 and root growth. Furthermore, alteration of the DR5::GUS auxin-localization signal

Table 1 Summary of the plant hormones crosstalk in root development

Plant hormones crosstalk	Genes involved	Root development process	References
Auxin crosstalks			
Cytokinin	*PIN1, ARR1, AHK3, SHY2*	Regulates LR organogenesis	Marhavý et al. (2014)
	ARR12, ARF19, SHY2	Regulates cell differentiation of meristematic cells	Perilli et al. (2013)
Abscisic acid and cytokinin	*PIN1, ABI4*	Regulates LR formation	Shkolnik-Inbar and Bar-Zvi (2010), Yuan et al. (2014),
Cytokinin and ethylene	*PLS, ARR5, IBC6, PIN1, PIN2*	Regulates cell elongation in roots	Casson et al. (2002), Chilley et al. (2006)
Cytokinin	*ASA1, YUCCA*	Regulates LR and adventitious root formation	Ronzan et al. (2019)
Gibberellin crosstalks			
Cytokinin	*SHY2, ARR1, PIN1, DELLA*	Controls root meristem size	Ioio et al. (2008), Moubayidin et al. (2010), Weiss and Ori (2007), Fu and Harberd (2003)
Abscisic acid	*GAZ*	Regulates formation of middle cortex of root	Choi and Lim (2016)
Auxin	*SEU, PIN, SHR, SCR, SCL3*	Regulates middle cortex formation of root	Gong et al. (2016), Lee et al. (2014)
Strigolactone crosstalks			
Auxin	*PIN*	Regulates LR emergence	Ruyter-Spira et al. (2011), Faizan et al. (2020)
	TIR1, MAX2	Regulates root hair density	Mayzlish-Gati et al. (2012)
Cytokinin	*MAX2, CYCLIN B1*	Regulation of adventitious root primordial	Rasmussen et al. (2012)
Abscisic acid	*MAX2*	Regulates AMF colonization in root	López–Ráez et al. (2010), Ren et al. (2018)
Brassinosteroid crosstalks			
Auxin	*DWF4, BIN2*	Regulates LR elongation	Yoshimitsu et al. (2011), Maharjan et al. (2011)
	PIN2	Regulates root gravitropism	Retzer et al. (2019)

(continued)

Table 1 (continued)

Plant hormones crosstalk	Genes involved	Root development process	References
	BZR1	Regulates root cell elongation	Chaiwanon and Wang (2015)
Ethylene	*BRI1, ACS, BES1, BZR1*	Regulates root cell elongation and primary root growth	Fridman et al. (2014), Lv et al. (2018)
Abscisic acid	*BIN2, SnRK*	Regulates primary root growth	Cai et al. (2014)
	ABI5, BZR1	Regulates primary root	Yang et al. (2016)

in the roots was also found to be caused by exogenous jasmonic acid, suggesting auxin and jasmonic acid crosstalk in root development (Yang et al. 2019; Ronzan et al. 2019).

Several studies indicate that microbial interaction in roots influences auxin biosynthesis and its action thereby influencing root growth and development. With the increase in scientific data, the role of auxin is becoming evident in the establishment of successful plant-microbial interaction particularly in relation to arbuscular mycorrrhizal fungi (AMF). Lipo-chitooligosaccharides (LCOs) produced by AMF modulates auxin homeostasis and promote the formation of LR (Buendia et al. 2019). Under mild drought conditions, mycorrhizal inoculation in *Poncirus trifoliate* root resulted in significant enhancement of ABA, IAA, methyl jasmonate, and BR levels in the root, ultimately positively impacting the root hair trait (Zhang et al. 2019). AMF *Funneliformis mosseae* inoculation significantly augmented root hair density in *Poncirus trifoliate* through up-regulation of auxin biosynthesis *PtYUC2* and auxin transporter *PtLAX3* genes. Further, the transcript levels of *Poncirus trifoliate expansins PtEXP* (*PtEXPB2, PtEXPA2,* and *PtEXPA4*) were also found to be up-regulated in the root. However, the expression levels of root auxin transporter *PtPIN2* and *PtPIN8* were significantly down-regulated in the root (Liu et al. 2018). In Arabidopsis (non-mycorrhizal plant), *Laccaria bicolor,* an ectomycorrhizal fungus promotes LR formation by increasing the expression of *AtPIN2* that stimulates ba

sipetal auxin transport from root apex towards the elongation zone (Felten et al. 2009). It was observed that *Pseudomonas putida* and *Pseudomonas fluorescens* enhanced LR formation, growth of root hair and biomass of plant (Ortiz-Castro et al. 2020). The bacteria released bioactive cyclodipeptides which triggered auxin-related genes and auxin signaling, thus enhacing root development process (Ortiz-Castro et al. 2020). There are clear evidence that illustrates that nod factors promote LR development and enhance mycorrhizal colonization. Interestingly, a diffusible component from AMF was also found to promote LR formation (Olah et al. 2005). Several genes were found to be up-regulated during interaction of Eucalyptus and ectomycorrhiza wherein EgHypar exhibited a great degree of homology with auxin-induced glutathione-S-transferases (Tagu et al. 2003). *Bacillus altitudinis* played a critical role in regulating endogenous IAA contents in rice roots by

controlling the auxin-responsive AUX/IAA genes, thereby causing alterations in root architecture (Ambreetha et al. 2018).

3 Gibberellins and Their Crosstalk in Root Development

Bioactive GA regulates several major aspects of plant development processes, such as stem elongation, flower formation, fruit development, seed germination and flowering time (Kaneko et al. 2003; Schwechheimer 2012). The biosynthesis of GA firstly involves the genesis of ent-kaurene from geranyl geranyl diphosphate (GGDP). The ent-kaurene is then converted into GA12 via cytochrome P450 monooxygenases. In the third step, C20- and C19 GAs are formed in the cytoplasm and finally, GA 3-oxidase (GA3ox) converts GA intermediates into bioactive GAs (Sun 2008). Once synthesized, GA binds to the gibberellin insensitive dwarf1 (GID1) receptor, which stimulates the synthesis of the GA-GID1-DELLA complex. The formation of this complex stimulates the degradation of the DELLA protein repressors thus, activating GA responsive genes involved in the regulation of root growth. However, in the absence of GA, DELLA accumulates and represses GA-mediated responses (Sun 2008; Davière and Achard 2013).

Crosstalk between GA, auxin, and cytokinin is known to be regulated by SHY2, PIN and ARR1 proteins for controlling root meristem size (Fig. 1B) (Pacifici et al. 2015). During the growth phase of the root meristem, an elevated GA content mediates the repression of ARR1, consequently degrading DELLA protein (Moubayidin et al. 2010) which decreases the levels of SHY2. Lower levels of SHY2 activates auxin signaling which promotes auxin efflux transporters such as PINs, thus enhancing the distribution of auxin and cell division in the root (Ioio et al. 2008; Moubayidin et al. 2010). In Arabidopsis, GA and ABA crosstalk are involved in the formation of the middle cortex during the post-embryonic root development process (Pacifici et al. 2015; Lee et al. 2016; Shu et al. 2018). Transcriptomic studies have revealed a previously uncharacterized C2H2-type zinc finger gene *GAZ* (*GA- and ABA-responsive zinc finger*) whose expression is regulated both by ABA and GA (Choi and Lim 2016) (Fig. 1B). Transgenic seedlings overexpressing GAZ (GAZ-OX) exhibited sensitivity to ABA and GA during the formation of the middle cortex, while, RNAi-GAZ seedlings showed opposite phenotypes. Further in Arabidopsis roots, the formation of ground tissue is mediated by GAZ which participates in maintaining the homeostasis of ABA and GA (Lee et al. 2016). In Arabidopsis, SEUSS (SEU) gene also participates in the formation of the middle cortex of the root (Gong et al. 2016; Shu et al. 2018). The *seu* mutants showed significantly reduced transcript levels of SHORT-ROOT (SHR), SCARECROW (SCR), and SCARECROW-LIKE3 (SCL3). The study indicates the positive role of SEU in regulating these genes through binding physically with their upstream regulatory sequences in controlling middle cortex formation of the root. SEU was found to be repressed by GA while induced by the paclobutrazol (GA biosynthesis inhibitor) (Gong et al. 2016). This indicates that SEU work downstream of GA signaling to control middle cortex formation. In addition, it was

suggested that SEU also regulates auxin distribution through altering PIN1 expression indicating a nexus between GA and auxin in root development which requires detailed investigations (Fig. 1B) (Lee et al. 2014; Gong et al. 2016; Shu et al. 2018). Another crosstalk between GA and ABA in mediating root growth has been revealed in Arabidopsis (Achard et al. 2006; Weiss and Ori 2007). It was observed that DELLA proteins regulate GA-mediated promotion while, ABA-mediated suppression of root growth. The exogenous application of ABA hinders GA-induced degradation of DELLA and increases its stability. Furthermore, the quadruple DELLA mutant (*GAI*, *RGA*, *RGL1* and *RGL2*) exhibits resistance against the inhibitory effects of ABA on growth. This indicates the positive role of GA in controlling root development (Weiss and Ori 2007). GA and auxin crosstalks in regulating root elongation process, as removal of the shoot apex, (a major source of auxin) inhibits root elongation. Furthermore, 1-N-naphthylphthalamic acid (NPA) application (auxin transport inhibitor) or mutation of auxin efflux transporter AtPIN1 prevented GA-mediated root elongation. In auxin signaling mutant *axr1*, GA-mediated degradation of DELLA protein RGA was suppressed. Hence, auxin and GA synergistically promote root elongation by degrading DELLA protein (Fu and Harberd 2003; Weiss and Ori 2007). Crosstalk between GA and ethylene in promoting root elongation in Arabidopsis through DELLA proteins has been suggested (Fig. 1B). In double mutants *gai rga*, GA promoted root elongation as ethylene inhibited the degradation of RGA (Achard et al. 2003; Guo and Ecker 2004; Weiss and Ori 2007). Hence, the phytohormones interact either synergistically or antagonistically, to regulate various aspects associated with root development. It has been reported that several members of *GRAS* transcription factor family-like *SCR/SHR/SCL3* were induced in mycorrhizal roots of *Solanum lycopersicum* and controls GA signaling and root development. The *SCR* and *SHR* transcription factors control radial root organization while *SCL3* mediates GA-promoted cell elongation during symbiosis (Ho-Plagaro et al. 2019).

4 Strigolactones and Their Crosstalk in Root Development

Strigolactones (SL) are the derivatives of carotenoids which are naturally present in a wide variety of dicot as well as monocot plants (Waters et al. 2017; Faizan et al. 2020). SL participates in symbiotic interactions and controls branching of the shoot as well as root development (Xie et al. 2010; Faizan et al. 2020). SL have been classified as a novel class of phytohormones that were first isolated in 1966 from the cotton plant's root (Zwanenburg et al. 2016). The precursor for the biosynthesis of SL is carlactone, a derivative of all-trans-β-carotene. In subsequent steps carlactone oxidation, ring closures and functionalizations leads to the synthesis of SL and SL like compounds (Jia et al. 2019). In Arabidopsis, *MORE AXILLARY GROWTH 1 (MAX1)* transforms carlactone into carlactonoic acid which synthesize SL-like compounds. Once synthesized, SL are transported through xylem or via. the pleiotropic drug resistance 1 (PhPDR1), a member of the ABC family transporter (Mashiguchi et al. 2021). The receptor of SL, D14 acts both as an enzyme as well as a receptor. Upon

binding to SL, D14 undergoes significant conformational changes to form a new surface. This permits D14 to interact with various other signaling components for triggering SL induced signal transduction pathways. In the presence of SL, MAX2 participates as a substrate for recruiting SKP1–CULLIN–F-BOX (SCF) ubiquitin ligase protein complex for interacting with D14. This promotes the degradation of D53 (which acts as a repressor of SL mediating signaling) by the SCF^{MAX2} complex, thus activating SL mediated responses in plants (Jia et al. 2019; Mashiguchi et al. 2021).

SL crosstalks with auxin in controlling polar auxin transport from shoot to root and it also mediates auxin efflux in the roots to regulate primary root, LR and root hair development (Saini et al. 2013; Sun et al. 2016). Recent reports have suggested the key role of SL in augmenting primary root length and root hairs via. *MAX2* dependent signaling pathway (Kapulnik et al. 2011; Mayzlish-Gati et al. 2012; Faizan et al. 2020). However, SL prevents LR emergence by affecting *PIN* proteins in root primordia which are involved in regulating the origin, positioning and length of LR (Fig. 1C) (Faizan et al. 2020; Ruyter-Spira et al. 2011). However, SL signaling mutants possess enhanced LR formation demonstrating the role of SL in inhibiting LR formation (Faizan et al. 2020; Kapulnik et al. 2011; Ruyter-Spira et al. 2011). SL might modulate the levels of auxin mandatory for root growth and development (Mayzlish-Gati et al. 2012). Depending upon the analysis of SL and auxin signaling mutants, it was observed that auxin components act downstream of SL signaling for regulating the formation of the LR, seminal root, adventitious root and root hair. SL and auxin also regulate patterns of root morphology along with inorganic phosphate (Pi) (Mayzlish-Gati et al. 2012). In Arabidopsis, the SL signaling (*max2-1*) or biosynthesis (*max4-1*) mutants exhibited decreased response towards limited Pi conditions with respect to the wild type plant (Mayzlish-Gati et al. 2012). However, in *max4-1* mutants, the decreased response to low Pi was compensated upon GR24 (a synthetic SL) treatment. While auxin application compensated for the decreased *max2-1* response to low Pi conditions. Furthermore, the enhanced transcript level of *TIR1* gene was observed upon low Pi conditions in wild type plants, compared to the *max2-1* mutants. Further, auxin signaling mutant *tir1-1*, exhibited reduced root hair density, under low Pi condition, with respect to the wild type (Mayzlish-Gati et al. 2012). Hence, the study suggested crosstalk between SL and auxin is mediated by MAX2 component of SL signaling and TIR1 auxin receptor for root development (Fig. 1C). Another interaction between auxin and SL in regulating adventitious root formation has been investigated in rice (Sun et al. 2015, 2016). The SL biosynthesis (*d10, d17* and *d27*) as well as signaling (*d3, d14* and *d53*) mutants of rice showed reduced adventitious root production with respect to the wild type plants. The exogenous application of GR24 enhanced adventitious root number per tiller in d10 mutants indicating a positive role of SL in adventitious root production. Furthermore, elevated IAA content, higher DR5::GUS expression and enhanced activity of IAA was found in the d mutants. The exogenous GR24 treatment reduced DR5::GUS expression suggesting the role of SL in inhibiting polar auxin transport for modulating adventitious root formation. Hence, the study provided an insight to understand the

key mechanism involved in SL-auxin crosstalk in regulating adventitious root formation (Sun et al. 2015, 2016). In Arabidopsis and Pea, SL mediated repression of the process of adventitious rooting was observed. On the contrary, enhanced adventitious root formation was found in SL-deficient and response mutants (Rasmussen et al. 2012; Sun et al. 2016). In a SL response mutant, *more axillary growth2* (*max2*), an elevation in the expression of CYCLIN B1 (regulated by CK also), which initiates adventitious root primordia was observed (Fig. 1C) (Rasmussen et al. 2012; Sun et al. 2016). The study suggested that SL repressed the initial divisions of the founder cells (pericycle cells adjacent to the xylem poles). Cytokinin treatment to the SL mutants such as *max1, max2, max3,* and *max4* decreased adventitious rooting. Similarly, SL application to cytokinin biosynthesis (*ipt1 ipt5 ipt7*) and cytokinin perception (*ahk3 ahk4*) mutants lead to a significant reduction of adventitious roots indicating that SL and cytokinins act independently in suppressing the adventitious root formation (Rasmussen et al. 2012; Sun et al. 2016). The role of SL and auxin crosstalk in adventitious root formation has also been suggested. As SL can partially regress the adventitious root formation mediated by auxin, while in *max* mutants, auxin can promote the number of adventitious roots (Rasmussen et al. 2012; Sun et al. 2016). The study suggested that adventitious root formation is regulated by SL and auxin content. In roots, the biosynthesis of SL and *MAX2* expression decreased significantly in ABA deficient mutants of tomato, reducing AMF colonization (Fig. 1C). The study suggested that appropriate levels of ABA and SL production are the key factors regulating the establishment of AMF (López–Ráez et al. 2010; Ren et al. 2018).

5 Brassinosteroids and Their Crosstalk in Root Development

BR are polyhydroxylated steroidal plant hormones that play key roles in their normal growth and development (Clouse and Sasse 1998). BR binds with plasma-membrane situated leucine-rich repeat receptor-like kinase, brassinosteroid insensitive 1 (BRI1) (Li and Chory 1997) which stimulates the formation of heterodimer complex, BRI1/brassinosteroid insensitive 1-associated receptor kinase 1 (BRI1/BAK1), to trigger a series of intracellular phosphorylation cascade (Russinova et al. 2004). The intracellular signaling cascade subsequently promotes the activity and stability of brassinazole resistant 1 (BZR1) and BRI1-EMS-suppressor 1 (BES1) transcription factors (Planas-Riverola et al. 2019) to regulate the transcription of BR-responsive genes and mediating several growth and development related processes of plants.

BRs signaling is essential for primary root growth as mutants defective in BR biosynthesis and signaling display short root phenotype. Mutant of BR receptor, *bri1*, displays reduced root meristem size and all other mutants (signaling and biosynthetic) display reduced root cell elongation (Gonza´lez-Garcı´a et al. 2011; Hacham et al. 2011; Chaiwanon and Wang 2015). A wide variety of BR have been found in the roots

of plant species (Yokota et al. 2001; Shimada et al. 2003). While application of small doses of BR can promote root growth, large doses of bioactive BR are deleterious to normal root development (Clouse et al. 1996; Mussig et al. 2003). Therefore, the fine tuning of BR led growth response is essential and is often mediated by a range of complex interactions between BR and other plant hormones.

For the past few decades, BR crosstalk with auxin has been known to regulate various aspects of root growth and development. In contrast to the synergism in shoot development, BR-auxin interacts antagonistically in roots for optimum root growth. An opposite regulation of BR biosynthetic gene *DWF4* by BR and auxin was observed during LR elongation (Fig. 1D) (He et al. 2005; Yoshimitsu et al. 2011). Brassinosteroid-insensitive 2 (BIN2), a key negative regulator of BR signaling serves as another node for opposite regulation of LR development by BR and auxin (Fig. 1D), as auxin-mediated increase in LR development is further elevated in a gain-of-function mutant, *bin2* (Maharjan et al. 2011). Further, BR control the auxin availability in the cellular milieu by modifying the polar auxin transport and distribution in roots (Bao et al. 2004; Li et al. 2005). Recently, an interesting study has shown that brassinolide (BL) acts as an antagonist of PIN2 endocytosis and alter degradation patterns of PIN2, particularly in gravity-responding roots to mediate root gravitropism (Fig. 1D). BL stabilized the PIN2 reporter, PIN2:ubq:VEN, with a ubiquitin-tag to signal protein internalization and degradation. BL showed a dose-dependent and de novo protein synthesis-independent effect on PIN degradation, suggesting a BR led non-genomic mode of regulation of PIN sorting in roots. Further, upon application of BL, constitutively endocytosed PIN2:ubq:VEN showed inhibition in PIN2 internalization from plasma membrane while the effect was less pronounced in wild type PIN2 reporters representing that PIN2 aimed for degradation, as a favored target for such hormonal regulation (Retzer et al. 2019). Furthermore, BR and auxin develop opposite gradient patterns along the root developmental zones that result in a spatial segregation of BR led-root growth control. Endogenous BR induce BZR1 more strongly at the transition from meristem to elongation zone and at low levels in the stem cell niche, in a manner opposite to the auxin levels in root tips. This spatial distribution instigates antagonistic responses of auxin on BZR1 nuclear localization and cell elongation partly due to auxin gradient mediated BR catabolism and BR mediated auxin transport (Fig. 1D). The analysis of BR-responsive, auxin-responsive, and developmental zone-specific transcriptomes suggests that BR and auxin induce opposite patterns of gene stimulation and repression along the various developmental zones which are in correlation to their endogenous distribution. Interestingly, several genes that show opposite coregulation by BR and auxin were the direct targets of BZR1, predominantly in the quiescent center and transition elongation zones (Chaiwanon and Wang 2015). Moreover, BR mainly induces target genes in the epidermis while it frequently represses genes in the stele, indicating tissue-specific responses in gene elicitation by BR. Epidermal BR perception results in activation of auxin genes, which are required for the initiation of cell division in the meristem zone. Authors proposed that BR signaling triggered auxin biosynthesis and transport in the epidermis led to its translocation to initiate stem cell divisions and delay the onset of cellular differentiation (Vragovic et al. 2015).

Interaction between BR and ethylene has been observed during root cell elongation. Root sensitivity to BR is established by two types of epidermal root cells, hair and nonhair cells. Targeted expression of BRI1 in hair cells promotes cell elongation in all tissues while its expression in non-hair cells is inhibitory as BRI1 activation in non-hair cells enhances the expression of 1-aminocyclopropane-1-carboxylate synthases (ACS) genes leading to increased cellular ethylene signaling that inhibits unidirectional cell expansion and consequently whole root growth (Fridman et al. 2014). A novel BR biosynthetic mutant allele in Arabidopsis, *Det2-9* displays a short-root phenotype and hyper-accumulation of ethylene. The level of ethylene content increases with the increasing concentration of exogenously applied BR and the short-root phenotype of *det2-9* is partially rescued by the chemical inhibition of ethylene. A chromatin immunoprecipitation (ChIP) and yeast one-hybrid assays confirmed that promoters of ethylene biosynthetic genes (*ACS*s) were directly regulated by the BR responsive transcription factors, BES1 and BZR1 (Fig. 1D). Thus, BR regulate primary root growth by controlling ethylene biosynthesis, as low cellular level of BR repress the expression of ACS through BES1 and BZR1 activation, resulting in inhibition of ethylene biosynthesis (Lv et al. 2018).

BRs signaling and biosynthetic mutants display hypersensitivity response to ABA in primary root inhibition assays (Clouse et al. 1996; Rodrigues et al. 2009). BIN2 is an important mediator of cross-talk between BRs and ABA for modulating root development. ABA induced repression of DWF4 expression and LR development was reduced in *bin2* background (Maharjan et al. 2011). Moreover, *bin2* displayed enhanced sensitivity to ABA in primary root inhibition was abolished by *Snf1-related kinase 2 s (SnRK2)*-RNAi, and showing that BIN2 modulates ABA signaling through the phosphorylation of SnRK module (Table 1) (Cai et al. 2014). BZR1 is another important mediator for BR-ABA crosstalk for root development. Dominant mutant allele *bzr1-1D* with elevated BR signaling display reduced sensitivity to ABA-inhibited primary root growth. BZR1 binds directly to cis-elements in the promoter of a major ABA signaling component ABA insesnitive5 (*ABI5*) to suppress the expression of ABI5 and resulting in plants with less sensitive to ABA (Fig. 1D) (Yang et al. 2016).

6 Conclusion

In current years, a lot of effort has been done to understand various mechanisms regulating root growth and development with the aim to optimize root architecture under various environmental constraints. The advent of modern molecular tools has enormously enhanced our understanding of how phytohormones such as auxin, GA, SL and BR regulate root development. Furthermore, it is also evident that hormonal pathways are interconnected and hence, much complex hormonal crosstalk exists in determining root patterning. Although, past research has shed considerable light on understanding the molecular mechanisms underlying root development. However, interdisciplinary research, mathematical modelling and computer-aided phenotyping

can greatly improve our knowledge in the field of root biology. Adopting such modern techniques will also enable plant biologists to engineer roots under different environmental conditions in the future.

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Dynamic Pool of Nitric Oxide (NO) in Rhizosphere Modulates Root Architecture, Nutrient Acquisition and Stress Tolerance in Plants

Piyush Mathur and Soumya Mukherjee

Abstract Among various abiotic factors influencing the biology of rhizosphere, soil organic matter (SOM) and humus formation play a major role in regulating the nutrient acquisition capability of roots. Nitric oxide present in the rhizosphere is a widely distributed gaseous biomolecule which plays a pivotal role in regulating plant growth and metabolism. There exists a possible functional link associated with the soil organic matter and NO generation in the rhizosphere. It is important to understand the various biotic and abiotic sources of rhizospheric NO being accumulated by the activity of microbes and in-vivo production of NO by plant roots. Rhizosphere microclimate affects NO generation both from soil and plant roots, however, excessive accumulation of NO may turn toxic for microbial and plant growth. Plants synthesize NO both in the apoplast and symplast region of root tissues. Thus, plant derived-NO contributes to the total available NO in the rhizosphere which in turn affects root functioning. Soils harbour different types of microbes which involve nitrifying-denitryifying bacteria, photoautotrophs, chemotrophs or facultative/obligate symbionts. Nitric oxide levels in the rhizosphere largely depends upon the nature of biotic community present in the soil which in turn affects soil C:N ratio resulting from root exchange. Abiotic stress factors like heavy metal stress, drought stress or hypoxia stress are alleviated by rhizospheric NO. Furthermore, rhizospheric NO has been associated with management of mineral deficiency in plants. NO stands to be an important molecule in nitrate-sensing process of roots. NO acts differently at low and high levels of N present in the soil. Humus mediated-NO formation also results in NO-IAA crosstalk which acts upstream to PM-H^+ATPase expression. Among various physiological effects exhibited by NO, protein modification at cysteine residues, tyrosine nitration and mobilization of secondary messengers have been reported to be active in response

P. Mathur (✉)
Department of Botany, University of North Bengal, Darjeeling, Raja Rammohunpur 734013 West Bengal,, India

S. Mukherjee
Department of Botany, Jangipur College, University of Kalyani, Kalyani 742213 West Bengal,, India

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to abiotic stress. The rhizosphere-plant-atmosphere continuum of NO functioning is therefore associated with plant-environment interactions.

Keywords Abiotic stress · Nitric oxide · Nitrate · Rhizosphere · Microbes

1 Introduction

Rhizosphere is a unique facet of plant-environment interaction affected by various biotic and abiotic factors. In this context, it is important to assess the microbial flora of rhizosphere which largely regulates plant growth and nutrient exchange across the roots. Among various abiotic factors influencing the biology of rhizosphere, soil organic matter (SOM) and humus formation play a major role in regulating the nutrient acquisition capability of roots. A diverse group of biomolecules like sugars, amino acids, peptides, organic acids, minerals and other secondary metabolites are exchanged in the soil-root interface. Nitric oxide present in the rhizosphere is a widely distributed gaseous biomolecule which plays a pivotal role in regulating plant growth and metabolism. It is important to understand the various biotic and abiotic sources of rhizospheric NO being accumulated by the activity of microbes and *in-vivo* production of NO by plant roots. Nitric oxide is chemically amphipathic in nature and capable of free diffusion across plasma membranes. Plants synthesize NO both in the apoplast and symplast of root tissues. Thus, plant derived-NO contributes to the total available nitric oxide in the rhizosphere which in turn affects root functioning. Soils harbour different types of microbes which involve nitrifying-denitryifying bacteria, photoautotrophs, chemotrophs or facultative/obligate symbionts. Therefore, nitrification and denitrification activities play a major role in the formation of NH_3, NO_2, NO_3 and NO in rhizosphere (Ġodde and Conrad 2000). Mineralization of soil components by heterotrophic microbes yield NH_4 compounds which are further converted to NO_2, NO_3 and NO. Furthermore, bio-fertilization results in agricultural soils being rich in nitrification activities which may also result in higher accumulation of NO. Nitric oxide levels in the rhizosphere largely depend upon the nature of biotic community present in the soil which in turn affects soil C:N ratio resulting from root exchange. Thus, NO levels in the rhizosphere affect the rate of N cycling between the plant and soil. A part of the NO produced also reacts with other organic components and is responsible for ozone formation in the troposphere. Soil-generated NO in the rhizosphere has chemical abilities to form various nitrogenous forms like N_2O, N_2O_3 or peroxynitrites (Stöhr and Ullrich 2002). This in turn affects various metabolic pathways within the plant roots. Additionally, different plant species have been reported to exhibit varied levels of NO emission resulting due to nitrification reactions (Wildt et al. 1997). Rhizospheric soils have been reported to exhibit higher nitrification abilities in comparison with non-rhizospheric soils (Chowdhury et al. 2016). Thus, microbial activity is likely to decrease with increase in soil distance from plant roots. Root exudations and mycorrhizal associations contribute to the levels of humus formation in the rhizosphere. This in turn affects the microbial flora present

in the rhizosphere. Rhizospheric NO has been reported to regulate various morphological and physiological responses in plants. Regulation of root architecture and morphology is partially controlled by available free NO present in the rhizosphere. Abiotic stress factors like heavy metal stress, drought stress or hypoxia stress are alleviated by rhizospheric NO (Arasimowicz-Jelonek et al. 2011b; Molina-Favero et al. 2007). Furthermore, rhizospheric NO has been associated with management of mineral deficiency in plants (Zhang et al. 2012). NO uptake has also been reported to be regulated by NO concentrations in the rhizosphere (Simon et al. 2013). NO plays a major role in communication between rhizospheric microbes and roots (Pande et al. 2021). The current chapter thus summarizes the aspects of plant growth and stress tolerance mechanisms mediated by rhizospheric nitric oxide.

2 Sources of NO Generation and Its Distribution in the Rhizosphere

Nitric oxide formation in the rhizosphere results from both microbial activity and plant based NO biosynthesis in the roots. Bacterial nitrification and denitrification are major microbial pathways responsible for NO accumulation in the rhizosphere. NO in soil is likely to be produced by autotrophic or heterotrophic nitrification or denitrification reactions (Robertson and Groffman 2005). Soil based variation in the levels of NO largely depend upon the types of ecosystem. Tropical moist forest lands have been reported to exhibit high turnover of NO prevalent in the rhizosphere (Butterbach-Bahl et al. 2001). NO and N_2O are the major nitrogen oxides produced in the rhizosphere among which N_2O flux usually appears higher than NO. Various parameters of soil texture, temperature, fertilization and microbial activity affect NO levels in the rhizosphere (Stange et al. 2000; Parton et al. 2001; Butterbach-Bahl et al. 2001). Rhizosphere microclimate affects NO generation both from soil and plant roots, however, excessive accumulation of NO may turn toxic for microbial and plant growth (Zumft 1997). Simon et al. (2009) has reported the evidences of rhizospehric NO being absorbed by plant roots. This has been attributed to NO-induced regulation of pedospheric nitrogen allocation among various components of the rhizosphere. NO level in the rhizosphere is indicative of the relative levels of microbial and plant metabolism prevalent in the zone. Microbial metabolism involves requirement of various amino acids and ammonium compounds in the soil. Thus, low levels of NO in the rhizosphere signify poor N-turnover generated by microbial metabolism (Simon et al. 2013). Various nitrifying bacteria like Rhizobium, Azotobacter or Azospirullum influence the rate of NO flux from the rhizosphere. Chemolithotrophic bacteria present in the rhizosphere may also alter nitrate use efficiency and influence the rate of NO generation (Laanbroek and Woldendorp 1995). Presence of arbuscular-vescicular mycorhizal fungi (*Glomus* sp.) associated with the rhizospher regulate NO generation through nitrification and denitrification activities (Zhang et al. 2013). Evidences suggest the crucial role of soil fungi in regulating

N_2O and NO emission from the rhizosphere (Ma et al. 2008). Fungal respiration pathway thus involves the conversion of N_2O to NO. However, unlike bacterial denitrification process fungal metabolism involves aerobic conditions in the rhizosphere. Thus, oxygen levels in the rhizosphere may regulate the rate of fungal and bacterial metabolism contributing to NO generation (Ma et al. 2008). Hypoxic condition in the rhizosphere alters the rate of heterotrophic nitrification thus causing changes in the NO flux. The conversion of rhizospheric NO to N_2O catalyzed by the activity of nitric oxide reductase has been reported in some fungal members (Zhang et al. 2001; Zhou et al. 2002; Watsuji et al. 2003).

Nitric oxide produced in the rhizosphere is transient and freely diffusible. Plant-derived nitric oxide also contributes to rhizospheric NO. NO by the virtue of its unpaired electron has been suggested to possess high reactivity with O^2 or O^{2-}. In this regard it is worth mentioning that certain amount of NO formed in the rhizosphere gets converted to nitrite by oxidation (Stohr and Ulrich 2002). Additionally autooxidation of NO in the rhizosphere also yields peroxynitrite species ($ONOO^-$) (Huie and Padmaja 1993). Subsequently NO toxicity affects plant metabolism which is attributable to the formation of oxidizing species of peroxynitrite. Furthermore during hypoxic conditions NO tends to react with thiols and secondary amines. Alkaline soil in the rhizosphere supports the formation of N_2O. Thus formation and distribution of NO in the rhizosphere is precisely regulated by the edaphic factors associated with the nature of microflora.

3 Rhizosphere Composition Regulates Apoplastic and Symplastic NO Production in Roots

Nitric oxide in plants is biosynthesized both by enzymatic and non-enzymatic pathways. In animal systems NO is mainly synthesized by the enzyme nitric oxide synthase (NOS). Although putative NOS like activity has also been detected in plants (Durner et al. 1998; Foissner et al. 2000) major part of NO in cytosol is produced by the enzyme nitrate reductase (cNR). Nitrite has been reported to be an important precursor of NO in plant cells (Delledonne et al. 1998). Non-enzymatic pathway of NO biosynthesis involves protonation of nitrite to form nitrous acid which subsequently yields NO and NO_2^-. Bethke et al. (2004) suggested that such mechanism of NO generation is likely to be prevalent in the apoplast of plant roots. Various factors like low pH, nitrite permeable transporters and nitrite present in the apoplast support apoplastic pathway of NO production in plants. However, root tissues may vary in their apoplastic nitrite content which partially depends upon the N turnover rate of rhizosphere. Interestingly Bethke et al. (2004) have reported the presence of phenolics to promote NO formation in the apoplastic regions. Rhizospheric region has been reported to contain higher amount of NO_2^- compare to the soil solutions away from the vicinity of plan roots (Binnerup and Sorensen 1992). Intriguing facts remain to be deciphered as to whether plant roots involve more of enzymatic or non-enzymatic

pathway leading to NO generation. There are possibilities of rapid changes in root apoplastic pH mediated by various signaling events like auxin efflux, gravitropic response or changes in ion flux (Fasano et al. 2001; Pagnussat et al. 2002). Furthermore, root plasma membranes are known to possess NR activity which subsequently draws the possibility of apoplastic NO generation both through enzymatic and non-enzymatic pathways (Stohr and Ullrich 2002). According to Wildt et al. (1997) different plant species have been reported to possess variable NO emission limits in their rhizosphere. Thus, both apoplastic and symplastic NO produced by plant roots is likely to diffuse into the rhizosphere. Tobacco root cell plasma membrane has been reported to possess a nitrite: NO reductase enzyme capable of NO generation from nitrite (Stöhr et al. 2001). Earlier investigations suggested underground NO formation only under the control of microbial sources (Stöhr and Ullrich 2002). However, investigation across the last decade has put forward some intriguing facts about the rhizospheric regulation of NO generation in plant roots. The absorptive zone of roots contains abundant root hairs. This zone is active for nutrient exchange from soil solution. Furthermore, the morphology and architecture of root is under the precise control of rhizosphere nutrient levels (Forde and Lorenzo 2001; Forde 2002). Nitrate-induced lateral root formation thus coincides with cellular NO generation manifested as an effect of soil nitrate levels (Zhang and Forde 2000). Cytoslic nitrate reductase activity is induced by soil and apoplastic nitrite levels. Anoxic condition in the rhizosphere is likely to exert compartmentalisation of nitrite in the apoplast thus leading to NO generation in the apoplast (Botrel et al. 1996; Stoimenova et al. 2003). Nitrite mediated NO formation in the apoplast is upregulated by ascorbic acid and phenolic substances (Bethke et al. 2004). According to Stöhr and Stremlau (2006) it is difficult to quantify plant liberated NO present in the rhizosphere as bacterial nitrification–denitrification process remains active in the vicinity of roots. Soil liberated NO lies in the range of 1 mg N m^{-2} h^{-1} which, however, is a function of rhizosphere pH, soil temperature, moisture and fertilization (Stöhr and Ulrich 2002). Anoxic conditions result in high amount of apoplastic NO generation in plant roots. This amount of NO generation inadvertently protects cellular biomolecules from NO toxicity.

4 Rhizospheric Organic Matter Elevates NO Biosynthesis and Subsequent Upregulation of Plant Growth Hormones

Rhizospheric nitric oxide can possibly modulate the activity of various plant growth regulators like auxin (IAA), cytokinin (Cyt), abscisic acid (ABA) and ethylene (Et). Crop productivity is largely regulated by the content of soil organic matter in the field (MacCarthy et al. 1990; Magdoff and Weil 2004). Root-generated NO and humus (SOM) affect microbial metabolism in the rhizosphere. Humus originates primarily from fresh organic matter of plant and animal debris. Microbial and fungal activity in the rhizosphere causes degradation of complex organic substances. This regulates the nutrient availability of the soil viz. rhizosphere (Tipping et al. 2002; Chen

et al. 2004). Investigations have suggested that a certain amount of humus with low molecular weight can penetrate root apoplast thus affecting nutrient exchange capability of the roots (Vaughan et al. 1985; Vaughan 1986; Nardi et al. 2002, 2009). Humification has been reported to directly regulate NO biosynthesis in roots which is also associated with increase in plasma membrane bound H^+ ATPase activity and hormone biosynthesis (IAA, ABA, Et) (Mora et al. 2014). Furthermore, humus mediated effects can be manifested through variable concentration of growth regulators prevalent in root and shoot of plants (Mora et al. 2014). Since NO is a biologically active signaling molecule, therefore it is worthwhile to state that humus mediated regulation of PGRs is likely mediated by root NO levels. Rhizospheric humus has been suggested to elevate auxin activity (Mora et al. 2014). Interestingly, humus mediated upregulation of root PM-H^+ ATPase activity enhances nitrate uptake by plants (Mora et al. 2014). In this context, Jannin et al. (2012) reported that rhizospheric humus preferably up regulates the genes associated with nitrate transport in roots. Humus-induced nitric oxide surge acts as a rapid response which triggers the expression of PM H^+ATPase, IAA and Et in the roots (Zandonadi et al. 2010; Mora et al.2014). These physiological changes later manifest in the form of better root growth, root hair proliferation and increased root dry weight. Humus mediated-NO formation also results in NO-IAA crosstalk which acts upstream to PM-H^+ATPase expression (Xu et al. 2010; Terrile et al. 2012; Freschi 2013). According to Terrile et al. (2012) NO can preferably S-nitrosylate the TIR-1 region of auxin receptor and down regulate IAA-oxidase activity. Humus mediated enhancement in nitrate uptake therefore promotes cytosolic NO biosynthesis in plant roots. Thus, in the context of rhizopsheric NO humus acts as a positive modulator of plant growth. However, further investigations are required to decipher the analysis of NO contributed by the plant root and microflora in the rhizosphere separately. Humus associated rhizosphere acidification is likely to regulate the microbial metabolism thus affecting NO flux from rhizosphere.

5 Rhizospheric NO Regulates Nitrate Assimilation and Root Architecture in Plants

Various investigations have deciphered the role of endogenous nitric oxide in growth promoting effects on various plant organs. However, rhizospheric NO also exerts unique regulation on root architecture and its proliferation. Autotrophic or heterotrophic nitrification promotes rhizospheric NO in soils (Fig. 1). *Azospirillum brasilense* is a soil dwelling plant—growth-promoting bacteria which liberates NO through its metabolic pathways. Aerobic denitrification process mediated by *Azospirillum brasilense* has been considered as a major source of NO flux from rhizosphere. Tomato (*Solanum lycopersicum* Mill.) plants investigated for *Azospirillum* mediated growth promoting effects were observed to exhibit better root proliferation (lateral and adventitious root) in presence of bacterial inoculation. The malleability

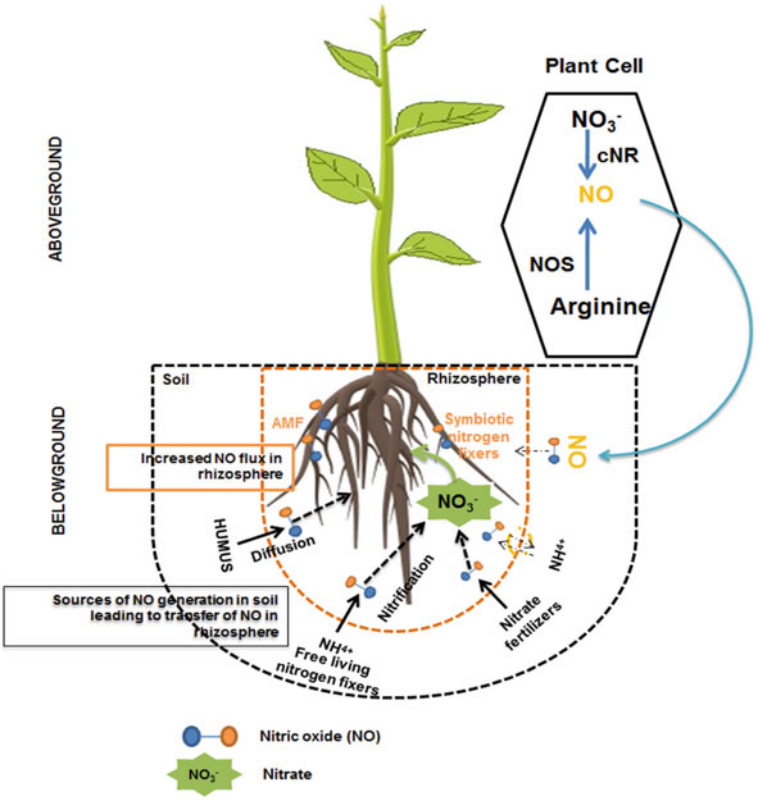

Fig. 1 Sources of rhizospheric NO. In the aboveground parts of plants NO are being synthesized inside plant cell and is translocated to below ground. In the belowground rhizospheric region has substantial NO that is uptaken up by plant roots. Region outside rhizosphere acts as supplier of NO through soil organic matter (humus) that transport NO through diffusion into rhizosphere followed by roots. A number of free living nitrogen fixing bacteria converts NH^{4+} into NO which is then transported to rhizosphere where it gets converted into NO^{3-} (nitrate) that is readily taken up by plant roots. Additionally, number of nitate fertlizers also acts as a source of NO subsequently into nitrate. Furthermore, ammonia itself in solution gets translocated to rhizospheric region in the form of NO. Inside the rhizospheric region, a number of symbiotic nitrogen fixing bacteria and arbuscular mycorrhizal fungi (AMF) are found associated with plant roots that also acts NO source in plant roots

of NO-induced root growth was confirmed by treatments of NO scavengers. *Azospirillum brasilense* is unique among various plant-growth-promoting bacteria (PGPB) and regulates root architecture through root hair proliferation and LR formation in wheat and tomato (Kolb and Martin 1985; Okon and Kapulnik 1986; Fallik et al. 1994; Dobbelaere et al. 1999; Creus et al. 2005). According to Hartmann and Zimmer (1994) dissimilatory nitrate reduction pathway of *Azospirillum* produces nitrite in addition to nitric oxide and nitrous acid. NO prodiction by *Azospirillum*

has been suggested to be accomplished by various pathways during aerobic conditions (Molina-Favero et al. 2007). Aerobic denitrification has been suggested to be accomplished by periplasmic nitrate reductase activity (Jetten et al. 1997). Other metabolic pathways likely to be prevalent are heterotrophic denitrification of ammonium compounds which liberate hydroxylamine and NO as intermediates (Wrage et al. 2001). Different strains of *Azospirillum* have been used for inoculation with plants. Nutrient status of the rhizosphere regulates the nature of metabolism exhibited by *Azospirillum* and energised by NH_4^+, NO_3^-, or arginine availability. NO production, however, can also result from stressful situations. Rhizospheric NO is a volatile membrane permeable gaseous growth modulator likely to diffuse into plant roots. Nitrite uptake by roots can promote apoplastic NO formation in plants. NO-induced root proliferation is mediated by downstream auxin response (Pagnussat et al. 2002). Molina-Favero et al. (2008) suggest the possibility of NO-induced activation of cyclin D3 proteins necessary for cell cycle regulation, which in turn regulates cell division leading to root proliferation. Exogenous humus application has also been reported to increase PM H^+-ATPase activity in maize seedlings (Zandonadi et al. 2010). Interesting observations have been obtained for the rate of reductive denitrification activity prevalent in the rhizosphere of barley crops. NO has been reported to be associated with nitrate assimilation at varying levels of nitrogen in the rhizosphere. The process of nitrate uptake through roots is followed by long distance from roots to shoots. NO stands to be an important molecule in nitrate-sensing process of roots (Sanz-luque et al. 2013, Sun et al. 2015a). NO has been reported to regulate the expression of transcripts associated with nitrate assimilation pathway. However, NO-induced modulation of NR activity showed varietal differences in its expression levels. Interestingly, NO liberated by NR activity counter-regulates the activity of nitrate transporters thus increasing N uptake. This is further manifested by increased lateral root growth (Zhang et al. 2007; Ruffel et al. 2011; Mounier et al. 2013; Sun et al. 2015b). NO acts differently at low and high levels of N present in the soil. Low N content leads to NO-induced activation of nitrate transporters to increase internal N levels in the plant roots. However, during high N-levels in soil NO inhibits the phosphorylation of 14–3–3 and causes S-nitrosylation of the protein (Frungillo et al. 2014). Non-mycorhizal beech roots (*Fagus sylvatica*) were reported to be associated with increased uptake of ammonium and glutamate sources induced by NO treatment (Simon et al. 2009). These observations thus imply that Rhizospheric NO-mediated N partitioning possibly occurs between plant roots and soil microbes in natural soil. Variable concentrations of NO in presence of different N levels in soil rhizosphere exhibit differences in the intensity of N-uptake in Scots pine (*Pinus sylvestris* L.) seedlings (Simon et al. 2013). Higher concentrations of NO supported preferential uptake of nitrate and arginine. Amonium uptake was, however, independent of NO concentration. This substantiates the fact that high concentration of NO preferentially increases nitrate and arginine uptake through roots. Thus, rate of N assimilation by plants mostly appears to be a function of rhizospheric NO concentrations. To summarize, N-sources potentially available to pine seedlings were mostly ammonium, nitrates, glutamine and arginine. The authors also stated that NO-mediated response of N-uptake varies in different plant species. Dong et al. (2015) suggests the

synergistic effects of rhizospheric NO and CO_2 to affect N-uptake in *Fagus sylvatica* seedlings. Rhizospheric CO_2 is another important regulatory molecule released by microbial or plant respiration. CO_2 functioning associated with such soil-root interface also regulates N-uptake (Cramer et al. 1996; Van der Merwe and Cramer 2000; Viktor and Cramer 2005). The effect of NO on N-uptake was more pronounced at ambient CO_2 concentrations. High and low CO_2 concentrations prioritised the uptake of organic or inorganic N-uptake in presence of NO.

6 Nitric Oxide Mediated Abiotic Stress Tolerance in Plants is Partially Regulated by Rhizospheric Interactions

Unfavourable growth environment for plants are mostly associated with high nutrient depletion, heavy metal infusion, poor cation exchange capability of soils, sodicity or temperature adversities. Furthermore, anoxic and hypoxic conditions also result in physiological imbalances occurring in various plant organs. Changes in the pH, temperature and oxygen content of the rhizosphere primarily affect root metabolism and membrane functioning. Nitric oxide synthesis and its distribution in plants is regulated at the root-soil interface and accompanied by various factors like composition of microflora, available soil NO and N availability. Natural soils mostly involve NO flux obtained due to microbial metabolism. Agricultural fields, however, are subject to variable NO flux regulated by fertilization, irrigation and type of cultivation practiced. Alkaline and acidic soils show differences in microbial communities and available sources of NO. NO mediated imbalance in the reactive nitrogen species (RNS) levels triggers various nitrosative responses in plants (Corpas et al. 2011). Environmental adversities led to formation of reactive radical species thus causing harm to various cellular and metabolic processes. In this context, roots are likely to produce high amounts of apoplastic NO which prevents cytoplasmic NO toxicity. Thus beneficial effect of NO is largely mediated by signaling response associated with stress stimuli. Roots are subjected to higher rates of environmental variations in comparison with shoot or aerial organs. Thus root-mediated abiotic stress signals are transduced to aerial organs through long distance signal transduction. Among various physiological effects exhibited by NO, protein modification at cysteine residues, tyrosine nitration and mobilization of secondary messengers have been reported to be active in response to cadmium toxicity in soils (Gill et al. 2013). Persistence of cadmium in soils is mostly due to its prolonged biological half-life and common in areas contaminated with industrial effluents or phosphate fertilization (Gill et al. 2013). This heavy metal has been reported to be readily uptaken by roots. Conversely, NO has been attributed to facilitate cadmium uptake from rhizosphere which contradicts to its role in alleviating cadmium toxicity (Arasimowicz-Jelonek et al. 2011a). However, NO mediated amelioration depends upon rhizosphere composition, N turnover and NO flux from root-soil interface. Upregulation of endogenous NO biosynthesis has been reported to be regulated by cadmium stress. Hypoxia is

a major soil-mediated abiotic stress signal inducing morpho-anatomical and physiological changes in plants. Water logging in soils cause oxygen deficiency which affects plants at the early vegetative stages. Liu et al. (2015) have reported that hypoxia-induced NO formation in *Populus* is primarily regulated by nitrate levels available in the nutrient solution. Thus nitric oxide production in roots induced by oxygen deficiency is likely to be a function of rhizospheric nitrate concentration and subsequent nitrite formation catalyzed by cNR activity in roots. Similar investigations by Wany et al. (2017) revealed that ethylene-induced aerenchyma formation in wheat roots was possibly regulated by NO activity during hypoxic conditions. Hypoxia-induced NO formation was catalyzed by NR activity prevalent in roots. The authors also reported that NO-signal during hypoxia was possibly transduced by xylem mediated long distance transport of NO-precursor or NO derivative from root to aerial shoots of *Populus*. Soil-dwelling chemolithotrophs are responsive to nitrification process induced by stressful conditions (Laanbroek and Woldendorp 1995). Thus, abiotic stress-induced microbial nitrification in the rhizosphere is one of the major regulators of NO production and its downstream action in plants. Mora et al. (2014) have reviewed the beneficial role of rhizospheric humus in promoting abiotic stress tolerance mediated by NO. Sodic stress-induced NO signaling primarily operates through NO-IAA crosstalk in cucumber plant roots (Gong et al. 2015).

Interestingly, plant based non-symbiotic haemoglobins have been reported to act as important endogenous regulator of NO in roots (Dordas et al. 2003). Plants subjected to hypoxia often exhibit a surge in cytosolic NO content. Hypoxia-induced haemoglobin synthesis has been reported in maize roots (Taylor et al. 1994). Rhizospheric changes associated with O_2 defeciency and water logged conditions promote haemoglobin accumulation in roots. This family of protein in its oxyhaemoglobin form ligates with NO to form nitrosylhaemoglobin. Thus, haemoglobin-NO interaction triggered by hypoxia stress is involved with plant tolerance to O_2 defeciency, adventitious rooting and prevention of nitrosative stress (Dordas et al. 2003). In this context, it is important to understand that exogenous nitrate levels also regulate haemoglobin mediated NO interaction in roots (Dordas et al. 2003). NO has been reported to increase Fe availability in *Arachis hypogaea* Linn. hsuji grown in iron deficient calcareous soils (Zhang et al. 2012). NO-induced increased Fe uptake has been suggested to be accomplished by increased Fe^{III} reductase activity in the roots followed by increased levels of available Fe form in the rhizosphere (Zhang et al. 2012). Thus rhizospheric NO level is important in regulating the pH, available Fe content and Fe reducing ability in the soil-root interface. NO-mediated alleviation of Fe deficiency thus manifests in better plant growth and increased biomass. Zn-stress tolerance in *Solanum nigrum* plants have been reported to be operative through NO production and subsequent modulation of antioxidative homoeostasis. Zn stress-induced NO formation has been suggested to cause programmed cell death at the root apex followed by regulation of root architecture. Salinity stress and plant community assemblage have been reported to regulate microbial denitrification in natural wet land vegetations of coastal regions (Bañeras et al. 2012). Al-induced NO production, however, exerts negative impact on Al-tolerance of plants (Sun et al. 2015a, 2015b). NO produced by Al toxicity further decreases pectin methylation in root cell wall.

This results in increased Al uptake from rhizosphere. Thus rhizosphere composition affected by nutrient availability and concentration of essential and non-essential minerals regulate NO-mediated responses during abiotic stress.

7 Rhizobacteria Mediated NO Formation in the Rhizosphere Regulates Abiotic Stress Tolerance in Plants

Root-colonizing rhizobacteria are important regulators of abiotic stress tolerance in plants. Plant-bacteria interaction in the rhizosphere triggers a series of physiological events associated with stress tolerance, free radical detoxification and amelioration of the effects of soil toxicity. The rhizosphere is suitable for root colonizing bacteria due to the fact that around 85% of soil organic carbon is obtained from roots and underground plant tissues (Barber and Martin 1976). Rhizobacteria respond to the root exudates and colonize by the mechanism of chemotaxis. A complex networking has been suggested to develop between the bacterial proteins and plant root exudates obtained in the rhizosphere (Gomez-Gomez and Boller 2002; Navarro et al. 2006). Among various biomolecules produced by rhizobacteria, NO is liberated in the rhizosphere. NO further induces resistance to soil pathogens and increases bioavailability of soil associated essential minerals (Dimkpa et al. 2009). Rhizobacteria mediated NO liberation regulates hormone biosynthesis pathway in the roots and aerial organ of the plants. Auxin, gibberllin and ethylene are the major target biomolecules modulated by NO. However, stress response induces elevation of ABA in the roots exposed to stress factors in the rhizosphere. Rhizobacteria can colonize both in the external and internal region of root cortex and hypodermis. The various soil dwelling rhizobacteria include *Bacillus, Pseudomonas, Klebsiella*, and *Streptomyces* which can also grow as endophytes in the roots (Hallmann et al. 1997; Long et al. 2008). NO mediated regulation of root architecture has been reported in *Azospirullum*-plant interaction (Creus et al. 2005; Molina-Favero et al. 2008). NO-mediated ethylene biosynthesis can, however, be reduced by the action of bacterial ACC deaminase activity which reduced ACC and ethylene levels in the roots. Thus, rhizobacteria colonization imparts abiotic stress tolerance mediated by reduced ethylene levels in plants. NO and IAA produced by the bacterial metabolism promotes lateral root development in plants. Furthermore, the beneficial effect of NO is manifested by increased accumulation of compatible solutes and antioxidants. Belimov et al. (2009) has suggested growth improvements and increased water use efficiency in pea plants supplemented with ACC deaminase producing bacterial strains. Plant growth promoting bacteria can possibly facilitate NO-induced salt stress amelioration. The process of NO-mediated modulation of enzyme activity (lipoxygenase, peroxidase, phenylalanine ammonialyase, catalase, superoxide dismutase) and subsequent proline accumulation has been reported to be enhanced by bacterial inoculation in salt-stressed soybean plants (Vaishnav et al. 2013). Furthermore, bacterial growth in association

to exogenous nitric oxide effectively modulates ion transport mechanisms manifested by altered sodium and potassium levels. Evidences therefore imply that microbial composition at the rhizosphere region possibly regulate NO-induced processes of ion transport and metabolism in roots. Rhizobacteria-mediated NO production has been reported in response to drought, salinity and heavy metal stress (Dimkpa et al. 2009). *Rhizobium* sp. has been reported to trigger IAA induced-NO production in *Pisum*, *Medicago* and sugar beet plants (Ramachandran et al. 2011; Molina-Favero et al. 2007). Thus application of rhizobacteria in the form of biofertilizer promotes nutrient availability and NO production in the rhizosphere.

8 Future Perspectives: Rhizospheric NO Regulates Symbiotic Associations with Plant Roots

Nitric oxide has been reported to play a pivotal role in the aspects of plant-fungi and plant-bacterial symbiosis. Soil nitrate levels accompanied by N-uptake and rhizospheric NO production are some of the primary factors associated with the establishment of symbiotic associations. NO primarily regulates process of nodule formation and its senescence during legume-rhizobium symbiosis (Puppo et al. 2013). In this context both plant and bacterial metabolism associated with NO liberation provides important insights to the signaling process. Nitrate reductase, putative NO synthase and nitrate levels play a major role in the intensity of NO liberation (Meilhoc et al. 2011). However, excess NO causes inactivation of nitrogenase in the rhizobium colonies of root nodules. Thus bacterial enzymatic systems include haemoglobin, nitric oxide reductase and flavoredoxins which convert NO into nitrates, nitrous oxide or amonia (Cabrera et al. 2011). Bethke et al. (2004) considers both plant and bacteria as potential sources contributing to rhizospheric NO involved in symbiotic process. Different metabolic pathways are likely to be trigger NO generation in aerobic and anaerobic conditions. (Gupta et al. 2011; Mur et al. 2013). Thus O_2 environment is an important determinant of NO-mediated root-bacterial signaling. According to Leach et al. (2010) NO production during soybean-*Bradyrhizobium japonicum* interaction is likely to be NOS dependent. Contradictory observations by Boscari et al. (2013), however, do not state the possibilities of NOS mediated NO generation during the early phase of NO-mediated symbiosis. Rhizospheric NO levels can effectively upregulate leg haemoglobin genes (*Lj*HB1) in plants (Shimoda et al. 2005; Nagata et al. 2008). Interestingly NO burst occurring at the early stage of root-bacterial symbiosis induces haemoglobin synthesis which down regulates further NO production thus facilitating nodulation process in the roots. NO in general has been reported to exert both positive and negative regulation in the nodulation process in various plant systems (Pii et al. 2007; Leach et al. 2010; Shimoda et al. 2009). Hypoxic condition in the rhizosphere regulates NO-mediated symbiotic interaction associated with nitrate levels and subsequent NR activity (Meakin et al. 2007; Sanchez et al. 2010; Horchani et al. 2011). Nitrate mediated NO generation is likely to be

accomplished by mitochondrial and bacterial electron transfer chain (Horchani et al. 2011). NO mediated regulation of nitrogenase levels has been reported to be regulated by S-nitrosylation activity (Xue et al. 2010; Puppo et al. 2013). Mycorhizal inoculation by *Gigaspora margarita* has also been reported to induce NO formation in Medicago trunculata (Calcagno et al. 2012). NO mediated mycorhizal symbiosis has been reported to be assisted by downregulation of defence resposne thus facilitating mycorhizal associations in the root (Boscari et al. 2013; Espinosa et al. 2014). Plasma membrane associated NR activity, NO reducatse and rhizospheric nitrate levels are important determinants of mycorhizal symbiosis (Moche et al. 2010). NO, ROS and phytohormone crosstalk has been reported to be crucial in establishing both mycorhizal and lichen symbiosis (Hichri et al. 2015). Rhizospheric NO has been reported to exhibit differential effects on N-uptake rates in mycorhizal and non-mycorhizal roots (Simon et al. 2009). Thus, further investigations are necessary to decipher complex reguations of rhizosphere NO in regulation of mycorhizal and lichen associations. The rhizosphere-plant-atmosphere continuum of NO functioning is therefore associated with plant-environment interactions.

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Role of Nitric Oxide as a Double Edged Sword in Root Growth and Development

Suchismita Roy

Abstract Nitric oxide is a gaseous molecule which has been given the status of signalling hormone in animals as well as plants. It has expansive continuum of regulatory functions involving all aspect of plant's growth and development, and also under several environmental cues. As NO is a part of a redox network, it's intermediate forms each of which form the signalling molecules. In prospect of this book chapter we discuss the role of this gaseous molecule and its impact in roots in plants, highlighting its interplay with other well featured hormones, in terms of its detection and how it acts as a double edged sword.

1 Introduction

In recent times nitric oxide (NO) have evolved as a potent endogenous signalling molecule in both animals as well as plant system. In animals, NO took a place as a signal transducer that has function in vivid tissues and has the potential to interact with multiple target compounds. Initially, it was in the form of knowledge, that hormones may influence smooth muscles cells via NO (Beligni and Lamattina 2002; Murad et al. 1978). In later years NO was identified as a potential gaseous candidate for signalling as characterized by its role in maintaining blood pressure in cardiovascular system (Murad et al. 1978), stimulating host defences in immune system, regulating neural transmission in the brain, regulating gene expression, sexual functions of males, cytotoxicity and cellular protection in animal system. In fact, in the last few decades, there has been a flare-up in the amount of research done on NO. In 1998, Nobel Prize in Physiology and Medicine was awarded for the discovery of NO as a biological mediator produced in mammalian cells, as joint venture of three persons between Furchgott, Murad and Ignarro (Nicholls 2019). Much on later, Beligni and Lamattina (Beligni and Lamattina 2002) categorised NO as a non-traditional regulator of plant growth. Research provoked the fact that plants not only respond to atmospheric

S. Roy (✉)
Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, La Jolla, San Diego, USA

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level of NO, but also hold the capability to produce NO using its own enzymes. Rather it seemed like NO might work in plants as a signalling molecule via pathways extraordinarily similar to those existing in mammals. These investigations also rule as an evidence highlighting many signal transduction pathways that are similar in both plants and animals.

2 Chemical Nature of Nitric Oxide

NO is a free, radical lipophilic diatomic gas under atmospheric conditions. Its small radius and being neutrally charged allow it for rapid diffusion through the membrane. NO is a very reactive gas with a half-life less than that of air (Beckman and Koppenol 1996). NO reacts very quickly with oxygen and thus has the potential to generate a variety of nitrogen based oxides. It is soluble in water as well as lipid. It has the potential to exist in 3 interchangeable form (NO.), nitrosonium cation (NO^+) and nitroxl anion (NO^-) which forms part of the intermediates of its redox network. Its stability & decaying nature depends on its redox status of the system in which it is existing. Neutral NO has a single electron in its 2p-pπ anti bonding orbital (Beckman and Koppenol 1996). Of its most important characteristics is its existence as an unpaired electron, which allows it to be highly reactive with oxygen and also superoxide, nitrogen derivatives and transition metals (Beligni and Lamattina 2002).

3 Different Routes of NO Synthesis

In animals, synthesis of NO is undertaken by three different isoforms of nitric oxide synthases (NOS) (Alderton et al. 2001). The three isoforms of NOS are iNOS (inducible NOS), eNOS (endothelial NOS), and nNOS (neuronal NOS). The overall reaction for these enzymes is the same i.e., nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-dependent oxidation of L-arginine to N-hydroxy arginine and then to NO and citrulline (Alderton et al. 2001).

In an experiment, treatment of isolated macrophages with an immunogenic elicitor as bacterial lipopolysaccharides (LPS), induced nitrate and nitrite synthesis (Stuehr and Marletta 1985). This experiment was the key to the research of NO, as it provided a tissue culture system to study nitrate and nitrite synthesis and was used to show that L-arginine is essentially needed by macrophages to produce inorganic nitrogen (Hibbs et al. 1987) and that nitrate and nitrite arise from oxidation reaction of NO (Hibbs et al. 1987). These experiments also established arginine as a substrate for NO synthesis. NO is generated mainly by nitric oxide synthase (NOS), which initiates the NADPH dependent oxidation of L-Arginine to L-citrulline and NO.

NO is a very unstable molecule that is produced at very meagre concentrations in various compartments of the cells in plants. In 1979, Klepper provided the first evidence of NO being produced due to nitrate reductase activity (Klepper 1979). Later

on NO production was studied in plants such as *Saccharum officinale, Helianthus annus, Nicotiana tabacum* as a consequence of varied stress responses (Yu et al. 2014). Basically, there are two well proposed existing pathways for production of NO. First is the pathway involving enzymes as nitric oxide synthesis (NOS), nitrate reductase (NR) (Yamasaki and Sakihama 2000), Xanthine oxidoreductase (XOR), peroxidise and cytochrome 450. The second route is the non-enzymatic pathway in which NO is synthesized by reduction reaction of nitrite by the carotenoids at a lower pH or in the presence of light (Cooney et al. 1994). Plants are more susceptible to the toxic effects of NO_2 when exposure takes place under dark conditions. Beta-carotene and other common carotenoids react with NO_2 in dark to yield intermediate nitrosating agents and forms nitrate esters. Simultaneous exposure of carotenoids to NO_2 and light significantly reduced formation of nitrosating intermediates and resulted in the release of NO into the gaseous phase. Light facilitated NO_2 to NO conversion by reduction by carotenoids may be an important mechanism for preventing damage in plants exposed to NO_2 (Cooney et al. 1994). NO_2 is also absorbed by grass and plants like Ginkgo in which they were found to adsorb NO_2 and release as NO (Nishimura et al. 1986). Processes as denitrification and nitrification are also effective for biological nitrogen fixation. NO is produced non enzymatically by the NO donors as sodium nitroprusside (SNP) & S-nitroso-N-acetylpenicillamine (SNAP) as an exogenous supply. NO scavengers are (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (cPTIO) (Akaike and Maeda 1996). These compounds have been routinely studied in most of the NO related studies.

NO production depends on the accumulation, rate of NO formation and also enzymatic activities of enzymes such as, nitrate reductase (NR) activity. Nitrite is produced in the cytosol and then it is translocated to the chloroplast and reduced to a rather simplified form of $NH4^+$ by the enzyme nitrite reductase (NR). Nitrite generally gets accrued when photosynthetic activity is repressed or absent or there is a prevailing cellular anaerobic condition. NO^{2-} on the other hand, gets accumulated in the cytosol together with the over generation of reactive oxygen species (ROS) (Planchet and Kaiser 2006). NR would carry out conversion of NO^{2-} to NO. NO would further get along the stroma of chloroplast. Role of the enzyme NR in NO production has been further established with *Arabidopsis* nia mutants and with R-free plants made under such growth conditions. Arabidopsis has two known NR genes, NIA1 and NIA2 (Hao et al. 2010). In the mutant set up experiments, ammonium was given as the sole source of nitrogen and tungstate supplied was given to be a non-functional state of the enzyme NR. Comparative studies of individual and double mutants, nia1/nia2, showed a significant drop in NO synthesis and different level of contribution to the synthesis of NO in different tissues (Bright et al. 2006; Planchet et al. 2006). Gusts of NO being produced was noticed by hormones such as auxins, abscisic acid (ABA) or also hydrogen peroxide (Kolbert et al. 2008; Wang et al. 2010; Yamamoto-Katou et al. 2006) and all were dependent on NR activities. As a response, NR activity causes stomatal closure (Bright et al. 2006; Desikan et al. 2002), works in response to various biotic stressors (Mur et al. 2013, 2005; Prats et al. 2005), respond to abiotic stress (Asgher et al. 2017) and during developmental

processes such as flowering (Seligman et al. 2008) or lateral root induction (Wang et al. 2013).

Nitrite-dependent NO production has been observed in plants as *Glycine max* (soybean) (Delledonne et al. 2001) and *Helianthus annuus* (Rockel et al. 2002), algae as *Chlamydomonas reinhardtii* (Astier et al. 2020; Sakihama et al. 2002). In some of the cases, NO is likely to be produced by the regular NR activity. From several other evidences it was also detected that biosynthesis of NO in plants can also be from arginine dependent NOS-like activities. Plants possess another enzyme called nitric oxide synthase (NOS), that is completely independent of nitrite and converts the amino acid L-arginine into L-citrulline and NO using a NADPH dependent pathway involving O_2 and also involving Ca^{2+} and calcium binding protein such as calmodulin, as second messengers (Ma et al. 2008). In plants, NOS had not been noted, but from antibody mediated reaction it was detected that NOS shares certain domain similarity with cytochrome P-450 reductase. NOS is a known bioenzymes with a carboxy-terminal domain with high sequence similarity to cytochrome P450 reductases (Bredt et al. 1991), and this makes many anti-NOS cross-react with many oxido reductases. Cyt 450-mediated NO formation from organic nitrates in vascular vessels has been reported (Minamiyama et al. 2007), showing an additional pathway of NO liberation in living organisms. Cytochrome P450 proteins have been demonstrated to catalyze the oxidation of NOHA by NADPH and O_2 releasing NO in plants (Boucher et al. 1992; Mansuy and Boucher 2002). Arabidopsis encodes a protein previously named AtNOS1 and this sequence was much similar to a protein from snail *Helix pomatia.* AtNOS1 is involved in NO synthesis. By loss of AtNOS1 gene, in T-DNA insertional mutant Atnos1 resulted in improper NO production in roots and reduced NOS activity in leaf extracts (Guo et al. 2003). Overexpression of AtNOS1 convened higher levels of NOS activity in leaf extracts. Unfortunately, AtNOS1 recombinant protein did not show up any NOS activity in-vitro suggesting (Guo et al. 2003) the involvement of AtNOS1 in NO biosynthesis may be an indirect reaction (Crawford 2006). Later AtNOS1 was renamed as AtNOA1 (NO-associated 1) (Crawford 2006). A direct correlation between arginine and NO would provide an additional proof of the existence of a plant NOS. NO measurement from arginine incubation with plant extracts provided more credit to the search for a plant NOS-like enzyme (Chaki et al. 2009; Corpas et al. 2009).

Xanthine oxidoreductase (XOR) was shown to produce NO (Harrison 2002). XOR occurs as a interconvertible form one of which is a superoxide producing XO and the other as xanthine dehydrogenase (XDH) (Palma et al. 2002). XOR's high expression was traced in pea leaf peroxisomes due to presence of XO gene (Corpas et al. 1997; Sandalio et al. 1988). XOR in its catalytic breakdown can generate free radicals in animals (Harrison 2002). The important property of producing O_2 and NO radicals establishes XOR a key role in and as a source of signal molecules in plant cells (Corpas et al. 2001). Recent reports provided a new possibility for oxidative NO formation from polyamines (Tun et al. 2006; Wimalasekera et al. 2011) or hydroxylamines (Rümer et al. 2009) in plants, although the molecular and enzymatic components are not yet clear under physiological conditions.

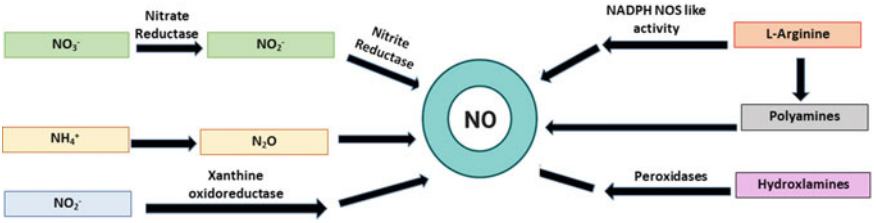

Fig. 1 A simplistic diagram showing the different routes of NO formation in plants involving different enzymes and substrates

As there is the relevancy of two different enzymatic pathways for NO production in plants a comparative study was undertaken to measure the efficacy of these two pathways (Wendehenne et al. 2001). It was traced out that the enzymatic mechanism for NO production by NOS is complex process and the reaction works with many cofactors (Wendehenne et al. 2001). In relation, the mechanism of NO production from nitrite is simpler. NO production via the nitrite pathway happens to be in the acidic compartments of the cells or in tissues under healthy conditions. The fundamental differences between the nitrite and arginine pathways are firstly, the nitrite pathway can proceed even in the absence of an enzyme and secondly the presence of O_2 is important for arginine pathway, whereas the nitrite way does not necessitate the presence of O_2, thus stating that this route is even active in anoxic or hypoxic conditions. In a recent study where NO production in crude extracts from Sorghum embryonic axes by NR and by NOS was quantified by EPR, the estimated NO production capacity of NOS was almost 10% higher than NR. Thus, these two pathways operating gives a system a sumptuous amount of NO, vital for functioning in the plants regulatory functions. A simplified schematic of the existing NO biogenesis pathways in plants is given in Fig. 1 adapted from Mukherjee and Corpas (2020).

4 Ways and Means to Study NO in Plants

Though within the last few decades there has been tremendous progress in research related to NO, but there lacks a true method to detect NO concentration which earlier narrowed down the rate of discovery of NO and its related downstream cascades. Nonetheless, various methods have been modified and redefined to study these processes. Initially NO detection was carried out using in In planta assays involving measurement of oxyhaemoglobin using a spectrophotometric approach which basically was a diazotization reaction in presence of a azo dye (Grisham et al. 1996). Assays made for studying NOS activity by analysing the conversion rate from arginine to citrulline was another important addendum to NO research. Several fluorescent binding dyes with NO such as 4,5-diaminofluorescein diacetate

(DAF-2DA) and 4-amino-5-methylamino-2,7-difluorofluorescein (DAF-FM) have also gained prominence in research work (Namin et al. 2013). DAF dyes were first described by Kojima et al. (1998) where they were shown to react with N_2O_3 as a by-product of oxidation reaction of NO, with an increase in the detection level of fluorescence. DAF-2DA is readily taken up by live cells. The reaction works by removing the diacetate group by the esterases which are already present in the cells, making the membrane to be impermeable to the DAF-2, which is a form available for nitration by N_2O_3 to generate the highly fluorescent triazole (DAF-2 T) (Kojima et al. 1998). DAF-2 is used in flow cytometry (Strijdom et al. 2004) but have mostly been used to image patterns of cellular NO production using fluorescence microscopy. DAF-FM is cell permeable making it a better sensor for NO. It is also more photostable, majorly cytosolic and more sensitive to DAF-2 with wide range of sensitivity from approx 5 nM and approx 3 nM respectively. DAF-FM is also effective in basic conditions with pH above 5.

The discovery of NO donors as SNP which has the capacity to work in a dose responsive manner for many physiological experiments (Filippou et al. 2013) has facilitated NO research. SNP was demonstrated to regulate the production of endogenous proline and polyamine metabolites in time, dose based and development-dependent manners (Filippou et al. 2013) thus giving a possible chance of NO estimation in the species *Medicago truncatula.* To study the effect of NO and also NO donors and scavengers and their relative quantification some method has been conceptualised to be good for detection, one such example is cyclic guanosine monophosphate (cGMP) estimation using radio labelling assay using 125I and liquid chromatography followed by tandem mass spectrometry (Newton and Smith 2004). Here cGMP acts an indirect marker for NO release (Bansinath et al. 1994). S-nitrosoglutathione (GSNO) is another NO donor accepted in various studies (Prince et al. 2010). EPR (Electronic Paramagnetic resonance) spectroscopy or EPR spin technique (Kleschyov et al. 2007) has been used as a technique in plants to report NO production from pollen (Bright et al. 2009), sorghum embryonic axis (Jasid et al. 2006) and also Arabidopsis infected with bacterial pathogens (Modolo et al. 2005). This EPR technique was further enhanced by the usage of 14 and 15 N labels, in such a way that both NO and enzymatic resource can be estimated (Maia and Moura 2016).

Another approach of indirect identification of NO components is by analysing the posttranslational protein modification of S-nitrosylation, a reduction oxidation based reaction with modification in amino acid residue cysteine and thiol group by NO (Kovacs and Lindermayr 2013). Kato et al. 2013) could identify proteins modified by S-nitrosylation in potato tissues. A biotin switch assay (BST) and nano-liquid chromatography combined with mass spectrometry (MS) based approach was applied here. BST, which notably is the first developed assay to study S-nitrosylated (SNO) proteins from cells and tissues (Han and Chen 2008). BST mainly follows three steps involving conversion of the cysteine residues of SNO into cysteine residues which are biotinylated. These biotinylated residues can be detected using streptavidin or a specific antibody (Jaffrey and Snyder 2001). Firstly, the protein which are reduced to thiols are blocked under denaturing conditions with S-thiomethylating agents, such

as monomethyl thiosulfonate (MMTS), N-ethylmaleimide, or iodo-acetic acid. This is followed by a blocking method in which the SNO-bond is specifically reduced to a free thiol in presence of ascorbate. The final step involves the conversion of free thiols with a thiol-specific reversible biotinylating agent, such as biotin-HPDP. Biotinylated proteins are then visualized directly using an avidin antibody. Alternatively, biotinylated proteins are then precipitated with immobilized avidin or streptavidin and routine Western blotting is used for fishing out protein-of-interest or by MS (Han and Chen 2008). After the introduction of BST based studies, increasing numbers of modifications have been reported due to some critical steps in the assay, which may result in false-positive detection of SNO sites too. Though the BST method had problem with detection with false positives and efficiency and specificity for the use of ascorbate in the second reduction step (Huang and Chen 2006). BST methodology can even cause reduction of protein disulfides as in the case of microtubule proteins (Landino et al. 2006). To date, around 20 different S-nitrosylated proteins have been characterized in details in plants and most of them have been reviewed recently with regard to their functional significance in NO signalling (Astier et al. 2012). Interestingly, there exists another unique prototype of NOS inhibitor such as nanoshutter (NS1). NSI hits the NADPH site of NOS and produces specific fluorescence enhancement upon biding to constitutive NOS (Li et al. 2012). This is a good non-invasive imaging method of NOS worthy of assessing NO in living cells and tissues. Problems have arisen for a number of reasons, mostly from the physical properties of NO itself. In the presence of O_2 it has a half-life of 29 s only and can be rapidly scavenged by haem containing proteins, and thiols such as glutathione (Wink et al. 1995). NO production may also be restricted to very few cells, such as guard cells (Bright et al. 2006). Thus, modes of measurements must be very sensitive to be able to detect NO production from plants. In addition, significant doubts have been expressed as to the specificity of the detection methods, for example the use of DAF dyes which are used by large numbers of NO researchers routinely. So, these detection methods all have pros and cons but at least methods on NO detection has gained pace which have worked as a timely help for understanding NO research.

5 Where is NO Produced in a Plant Cell

NO is liberated in the cytosol by the reaction mediated NR which is already present in the cytosol. NOS activity in the cytosol has also been reported by Zhang (Zhang et al. 2003) and this was tested by blocking the pathway using arginine substrate analogue of NO. NO also has the potential to be produced in the apoplast of the cell by local NR reactions by the plasma membrane bound nitrate reductase (Stohr et al. 2001). Apoplastic NO are generally liberated by enzymatic breakdown of plasma membrane lipids during the heightened response and during wounding (Stohr et al. 2001).

NO affects mitochondrial functionality in plant cells and reduces total cell respiration rate in course of stronger inhibition of the cytochrome pathway. NO development

can be both oxygen dependent and independent in the mitochondria, where arginine or nitrate are the substrates. Assemblage of NO from L arginine by NOS present in the mitochondria was reported in leaves and roots (Gupta et al. 2010). Plant mitochondria also yield NO from its nitrite sources (Planchet et al. 2005). Though, anaerobic conditions are still needed in vivo and in vitro for appreciable amount of NO synthesis. Inhibitors of mitochondria electron transport chain (ETC) retard synthesis of NO. This study highlighted, that electrons from ETC promotes reduction of nitrite (Planchet et al. 2005) generating most of the NO in the mitochondrial compartment itself. This liberation of NO in those specific organelle depends on the ratio of nitrite to nitrate, which is present in the in the cytosol. NR mutants of the green algae *Chlorella* sp showed up the ability to release copious amounts of NO when provided nitrite under anoxic conditions (Tischner et al. 2004), which was studied in comparison to control and two mutants as nitrate reductase (NR)- and nitrite-reductase (NiR)-deficient cells of Chlorella. Suspension cells of the tobacco nia-double mutant (or ammonium-grown WT cells) gave no chemiluminescence signal and hardly any increase in DAF-fluorescence, neither in air nor in nitrogen. With a supplementation of nitrite in range of $\leq$0.5 mM under nitrogen, the NR-deficient cells could produce as much NO as NR expressing WT cells. The elevated amount of anoxic NO production was completely turned off by inhibitors of respiratory ETC, raising facts for similar nitrite: NO reductase activity in the plant mitochondria (Planchet et al. 2005). This pathway was confirmed with assay set up with purified mitochondria from various plant which produced NO under nitrogen when supplied with NADH and nitrite. Peroxisomes also shows NOS activity with L-arginine, oxygen and NADPH as substrates (Corpas and Barroso 2014). It has been demonstrated that NO is an endogenous metabolite of peroxisomes, where it is produced from L-arginine by a protein as iNOS, and the NO. NO has also been reported from the guard cells of the chloroplasts, but its production has been attributed to the enzyme NR (Corpas et al. 2004; Gayatri et al. 2013). NO thus has different localization contributing integral role in different signalling pathways. This supports the involvement of NO everywhere in the plant system.

6 Role of NO in Root Growth and Development

Finally, in context to this chapter, we will be discussing the most pertinent role of NO in growth and development. In plants, increasing evidence indicates NO as a key component of signalling network, controlling numerous physiological and metabolic processes such as seed germination (Albertos et al. 2015), flowering (He et al. 2004), root growth (Fernandez-Marcos et al. 2011), respiration and stomatal conductance (Wang et al. 2015), and adaptive retorts to stresses (Shan et al. 2015; Wang et al. 2015). The envelopment of NO in promoting root growth has been observed vividly. The much studied role of NO is as a reagent of cell elongation, which is much similar to that of the plant growth hormone auxin, i.e. the root proliferation hormone. A transient increase in NO concentrations was shown to be involved in

adventitious root development by exogenous application of IAA (Pagnussat et al. 2002). We have earlier discussed how NO is routinely generated in several cellular compartments. The role of NO is as a double edged sword, as a causative agent for root proliferation as well as an inhibitor of root development, which most likely depends on the concentration or dose of NO prevailing then and there in the system. It has also been studied that NO employs in a powerful role in regulating plant NR activity at posttranslational levels, probably via a direct interaction and mechanism, nitrogen assimilation mechanisms being the process for it. NO notably regulates distribution of nitrogen and uptake in several plant species. In a rice cultivars, NR based release of NO, played a pivotal role in improving N uptake capacity by increasing root growth and improvising inorganic N_2 uptake, representing a potential strategy for rice adaption to a changeable nitrate resource (Sun et al. 2017). NO from soils is liberated in range from a few mg to $Nm^{-1}\ h^{-1}$ (Stöhr and Stremlau 2005), however, the variation in liberation rate of NO are directly dependent on various factors such as temperature, availability of oxygen, alkalinity or acidity of the soil, the rate at which nitrogen fertilizers are used in the soil Plant systems are more open to the environment than are those of animals. Consequently, plant systems may be closely linked to the activities of soil bacteria through changes in NO levels that can vary in response to nitrogen and oxygen availability (Stöhr and Ullrich 2002). Naturally, processes as nitrification and denitrification of bacteria, are responsible for the liberation of the nitrogen content in the soil as well. Under the scope of this chapter we will be discussing involvement of NO in terms of providing advantages/disadvantages to root growth and proliferation only. A summary of what NO does to the entire root system is given in Table 1.

7 Role of NO in Adventitious Rooting

The formation of adventitious roots is a fundamental process of root biology, as these roots are post embryonic roots which arise from the nonpericycler tissues in older roots. The development of adventitious roots is a complex process regulated by environmental cues as well as plant growth hormones. Root cells have the capacity to respond to NO, which enhanced the elongation of maize root segments as noted (Zhao et al. 2007). It was proposed (Pagnussat et al. 2002) that IAA and NO shares mutual pathway as both of them show similar kind of responsiveness to root growth in plants. Seedlings of Arabidopsis were treated with NO donors and they had enhanced root initiation at certain high concentrations of NO. On the otherhand, root elongation was inhibited in higher dose of these donors, again was a double sided effect of NO. It was hypothesized that there exists a connection between NO and cGMP in Arabidopsis (Pagnussat et al. 2003)**.** In another report in maize, the hormone brassinosteroid (BR) enhanced water stress tolerance, was studied to have BR-induced NO production and NO triggered ABA biosynthesis (Zhang et al. 2011). 10 mM of NO donors such as SNP or SNAP prompted adventitious root development in cucumber explants, where the primary roots were already separated from 10 days old germinated hypocotyls

Table 1 List of changes in the root system brought by NO. Enlisted in the table is the process, NO mediated effect and other effectors involved and in which plants

Process	NO mediated effect	Other hormone/messenger/external factor involved	Studied in plants
Adventitious root development	Enhanced root initiation	Auxin and cGMP, Brassinosteroid, Strigolactone	*Arabidopsis* (Pagnussat et al. 2002) Cucumber (Li et al. 2020) *Helianthus* (Bharti and Bhatla 2015)
Adventitious root development	Facilitate nitrate signaling is at the root environment edge	H_2O_2	*Tagetes erecta* (Liao et al. 2009)
Adventitious root development under submergence state	Increased adventitious root formation	Flooding	*Suaeda salsa* (Chen et al. 2015)
Gravitotropic bending of roots	Facilitate responses to gravistimulation in primary roots	cGMP	Soybean (Hu et al. 2005)
Lateral root formation	Lateral root formation	Auxin	*Arabidopsis* (Pagnussat et al. 2004), *Oryza sativa* (Sun et al. 2018)
Lateral root formation and inhibiting of elongation of the adventitious roots under Cd stress	Prevent oxidative damage	Cd	Pea (Rodríguez-Serrano et al. 2006)
Lateral root formation	Appearance of lateral roots	Ethylene, Se	(Feigl et al. 2019)
Lateral root development under stress	Lateral root formation	CO_2	Tomato (Wang et al. 2013)
Root hair formation under Mg deficient condition	Induction of root hairs	Auxin, Ethylene	Arabidopsis (Liu et al. 2018)
Root hair formation	Differentiation of rhizodermal cells to form root hairs	Auxin	Lettuce (Lombardo et al. 2006)

(continued)

Table 1 (continued)

Root hair development	Regulation of cytoskeleton for modifying root hair growth as well as propagation of new root hairs	ABA	Arabidopsis (Lombardo and Lamattina 2018)

(Pagnussat et al. 2002). The effect of these NO donor on the induction of adventitious root formation was noticed to be dose dependent here as well. This group also noted NO operating downstream of IAA signalling cascade, promoting adventitious root development through the GC-catalysed synthesis of cGMP (Pagnussat et al. 2003a). In the proposed study they featured NO as a second messenger which get liberated in due course of the IAA mediated pathway (Pagnussat et al. 2003a). It was seen that the activation of defence related genes by NO was also induced by cGMP which in turn regulated Ca^{2+} based signalling leading to various other signalling pathways and even initiating mitotic processes and differentiation of cells to initiate root development. Hu et al. reported the influence of NO in gravitropic bending in soybean roots (Hu et al. 2005). NO and cGMP facilitate responses to gravistimulation in primary roots of soybean. Horizontal orientation of these soybean roots caused gathering of both NO and cGMP, and due to uneven spread out of NO for gravity based simulation (Hu et al. 2005).

NO and H_2O_2 both played crucial roles and had synergistic effect on adventitious root development in *Tagetes erecta* (Liao et al. 2009). A possible role of NO in mediating root development, in response to nitrate and other signaling is at the environment and root edge (Liao et al. 2009). This was inferred by the coordinated doings of the root specific plasma membrane bound enzymes, NR i.e. PM-NR and nitrite:NO reductase NI-NOR (Stöhr and Stremlau 2005). NO can act in a cGMP independent pathway, activating phosphatises and protein kinases including MAPKs (Pagnussat et al. 2004). Exogenous application of BR around 1 mM could significantly increase the rate of adventitious rooting, while higher concentrations of this same in the range of 2–8 Mm upturned its effect and inhibited the rate of adventitious rooting (Li et al. 2020). SNAP promoted the occurrence of adventitious roots. BR and SNAP when applied at one go could promote adventitious rooting and the combined effect was drastic in comparison to the application of BR or SNAP as a standalone. Moreover, NO scavenger c-PTIO and inhibitors L-NAME and tungstate inhibited the positive effects of BR on adventitious root. On further assessing it was noticed that endogenous levels of NO, NO synthase and NR activities also increased, while the application of BR specific inhibitor BRz lessened these effects. In addition, the relative expression level of NR was up-regulated by BR and SNAP, whereas BRz showed a potential down-regulation. The application of NO inhibitor tungstate in BR also inhibited the up-regulation of NR in cucumber (Li et al. 2020).

Adventitious root development is one of the most important physiological effects which a species encompasses when submergence stress prevails (Chen et al. 2015).

SNP and cPTIO were applied to the euhalophyte *Suaeda salsa* seedlings to examine the effects of NO on flooding or submergence tolerance (Tianshu et al. 2016). SNP alleviated growth inhibition and increased adventitious root formation. This featured that endogenous NO has a role in adventitious root cell integrity in S. salsa under waterlogged conditions. NO donor mediated effects were prevented or reversed by the application of the scavenger cPTIO. On determining the pathway, SNP treatment decreased NR activity but increased NOS activity suggesting these euhalophytes partakes in waterlogging tolerance by enhancing adventitious root formation and NO generation with the NOS mediated pathway (Tianshu et al. 2016). NO induced modulations in adventitious root growth, lignin content and lignin synthesizing enzymes in the hypocotyls of *Vigna radiate* was also noted (Sharma et al. 2019). Niu et al. studied the effect of NO and calcium on the process of adventitious rooting in cucumber plants under osmotic stress. They found outcomes which showed the both NO and Ca^{2+} on exogenous application under osmotic stress potentiated the growth of adventitious roots in cucumber in a dosage dependent manner. A maximum of reaction was recorded at 10 μM NO donor SNP or 200 μM Ca^{2+}. Application of Ca^{2+} chelators or channel inhibitors and the secondary messenger calmodulin (CaM) antagonists drastically overturned NO-induced adventitious rooting, inferring that endogenous Ca^{2+}/CaM is responsible for NO-induced adventitious rooting under osmotic stress (Niu et al. 2017). All these studies, highlights apposite role of NO in stress management in plants, influencing the adventitious root system.

8 Role of NO in Lateral Root Formation

NO by now is known to act downstream of auxin in regulating lateral root formation (Pagnussat et al. 2004) and affect patterning of root elongation and channelizing polar auxin transport. SNP when applied exogenously to germinating tomato seeds could proliferate lateral root formation in the same rate, as similar to the exogenous application of the rooting hormone auxin (Correa-Aragunde et al. 2004). NO has been studied to have similar tenacity to alter the expression of regulatory genes involving cell cycle regulation (Correa-Aragunde et al. 2004) as seen in case of tomato plants. On the other hand, a kind of stress involving induced level of carbon dioxide also enhanced growth of lateral root development as a phenotypic change to alleviate the nutrient loss (Wang et al. 2013). On further assessing the cause of such phenotypes, scientists detected endogenous NO level in roots using the dyes DAF-FM DA. Elevated CO_2 had an influential effect on the enzyme NOS in roots, but not over NR activity. Thus, increase in NOS promoted production of NO, which in term involved propagation of lateral roots in tomato plants under CO_2 stress (Wang et al. 2013).

In a study, lateral root formation and the length of seminal roots of *Oryza sativa* were measured along with auxin concentrations. Their results highlighted that NO influences rice root growth by regulating auxin transport in response to NO_3^-. Manoli et al. (2016) emphasized the role of NO mediated root apex responses to NO_3^- are

also regulated by auxins in maize plants. Their findings suggested a subtle interaction between auxin and NO in regulating root growth. In *Oryza sativa*, as Sun et al. (2018) studied, NO functions downstream of auxin pathway in regulating lateral root generation but prevents elongation of root by reducing the levels of the hormone auxin at root tips under iron deficient conditions. The work of Sun et al. (2018) focused that NO is involved in NO_3^- regulated auxin transport in roots system. However, upon treatment with IAA it did not affect the NO content in roots under the supply of NH_4^+. When the expression of auxin related genes such as PIN1b and PIN1d in roots were screened, they had higher expression under high SNP treatment in occurrence of NH_4^+ and developing more number of lateral roots. In case of the pin1b mutants, the phenotype reversed showing less root formation. Thus, interfaces between NO and auxin signalling in facilitating root growth are may be a closer call for each other (Fernandez-Marcos et al. 2011; Sun et al. 2018).

Lateral root formation is dependent on lateral root primordia initiation under NO_3^- supply (Sun et al. 2018). The activity of meristematic cells in the roots affects root elongation (Blilou et al. 2005). NO_3^- source escalated the root meristem activity by regulating the expression of CYCB1;1 gene in *Arabidopsis* (Liu et al. 2013). NO_3^- supply increased pCYCB1;1::GUS construct and CYCB1;1 expression levels in root but did not disturb the measurement of existing mature cells (Sun et al. 2018). These findings suggest that root elongation is regulated by accumulative cell division in the root meristematic zone under the influence of NO_3^- concentration relative to the supply of NH_4^+ (Sun et al. 2018). There is report of existing crosstalk between strigolactone, auxin and NO in lateral root development as studied in sunflower seedlings (Bharti and Bhatla 2015). NO has a role in in H_2 induced lateral root development (Cao et al. 2017). H_2^- and NAA promoted lateral root formation, but it was conceded in the presence of the scavenger cPTIO. Progressive accumulation of NO was traced back on 48 h of treatment of both NAA- and H2, compared with control. Co-treatment with cPTIO reduced the above NO content. (Cao et al. 2017). In another study using two cultivars of tobacco plant, elongation of lateral roots was relatable to the concentration of the NO. Furthermore, on studying the expression pattern of the cell division marker CYCB1;1, based on the application of SNP vs control plants they could notice a relational association with SNP and expression of CYCB1;1 signifying a role for NO in the regulation of stem cell decisions (Song et al. 2018). They also conferred that NO might be involved in auxin-regulated lateral root elongation in retort to potassium ion deficient conditions as well. They highlighted K^+ ion deficiency induced NO was inversely related with elongation of lateral roots (Song et al. 2018).

9 Role of NO in Root Hair Development

Further along the study of root growth and development, root hairs are very important to increase the surface area for absorption of nutrients. Root hairs are formed from root epidermis. In lettuce plants grown hydroponically, supplemented with 10 μM

SNP it had an substantial effect, in which all the rhizodermal cells could differentiate and form root hairs (Lombardo et al. 2006). Magnesium ion (Mg) deficient condition elevates the levels of auxin, ethylene and NO in roots; each facilitates the accumulation of the other two by stimulating the activities of enzymes such as 1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO) and ACC synthase (ACS) for ethylene; NR and NOS-L for NO or the expression of transporters such as AUX1, PIN1 and PIN2 for auxin, thus forming a regulatory positive feedback network of ethylene-NO-auxin. Auxin acts downstream of ethylene and NO, leading to the induction of root hairs under Mg^{2+} deficiency existing in soil (Liu et al. 2018). Experimental evidence from Mg-deficient plants treated with ethylene inhibitor silver thiosulphate (STS) or NO scavenger cPTIO, and Mg sufficiently plant treated with ethylene precursor ACC or NO donor SNP showed that the elevation of ethylene and NO levels is associated with an increase in the root auxin level under Mg^{2+} deficiency (Liu et al. 2018).

10 Role of NO During Different Stages of the Legume Rhizobium Interaction

NO has sufficient investigatory role in legume-rhizobia interaction which have been elaborately studied as different phases in legume rhizobia interaction such as early stage of recognition, establishment stages such as infection and nodule development, and senescence of the nodule at the final stage (Signorelli et al. 2020). As they studied different concentrations of NO being present at different stages of nitrogen fixation happening biologically, based on interaction between *Lotus japonicas and Mesorhizobium loti,* and *Medicago sativa and Ensifer meliloti*, they traced NO at recognition as a signaling molecule rather than as a stress element (Nagata et al. 2008). Nodule organogenesis and lateral root formation has some similarities. Both organs require auxin at junctures as expansion of the primordia and also when there is further differentiation of the nodule vasculature. NO is produced in root nodules of *M. truncatula* and *M. sativa* and NO is also more in IAA-overproducing nodules. They noted that aerobically grown stationary phase produce larger amount of IAA and *S. meliloti* yield NO and possess NOS like activity as well. Therefore, these NO level in nodules could be because of both plant and bacterial association. However, no difference in NO production was observed in free living wildtype and IAA strains. Their study stated NO synthesis in nodule is enhanced to a larger scale in plants with larger nodulation capacity and that NO scavenger has the potential to reduce the rate of nodule formation. This was one of the first experiments which showed that nodule formation is regulated by NO and auxin signaling (Pii et al. 2007). Higher NO levels were detected in indeterminate nodules bearing plants as *Medicago*, shaped by the IAA overproducing rhizobia. cPTIO could markedly reduce the nodulation pattern which was induced by wild type and IAA-overproducing strains (Baudouin et al. 2006).

11 Role of NO in Protecting Plant Roots from Stress

Several researches have highlighted the involvement of NO in the regulation of plant response to toxic elements. Kopyra et al. investigated the role of NO in cell suspension treated with cadmium (Cd). SNP reduced the negative impact of Cd^{2+} on cell growth (Kopyra et al. 2006). This was one of the premiere studies where the role of NO as a quencher of ROS was established. NO provided aid by preventing cellular damage by reducing oxidative damage. This study was further strengthened from a recent report which stated that Cd exposure to pea roots resulted in oxidative damage and reduced in vivo NO level (Rodríguez-Serrano et al. 2006). Results featured higher NO levels improved the roots under toxic conditions by lateral root formation and inhibiting elongation of the adventitious roots, with enhanced lignin deposition in the endodermis of the cell-walls (Rodríguez-Serrano et al. 2006). Their study was significant in a way, as it revealed that different toxic elements have different effect on NO modulating the root system (Piacentini et al. 2020). NO is involved highly with physiological as well as metabolic processes, mainly due to their capability to alter numerous proteins, through transcriptional and translational processes. For post-translational processes, such as S-nitrosylation, nitration and nitrosylation were effected. Transcriptional regulation was by affecting the transcription of genes that encode proteins involved in stress responses. Another element treatment, i.e. Molybdenum (Mo) supply upregulated the expression of genes as nitrate transports 1 (NRT1.1) in the presence of sources as NH_4^+. Mo supply elevated expressions of NRT1.1, NRT2.1, and NAR2.1 transporter under both NO_3^- and NH_4NO_3 treatment (Imran et al. 2020). This concurrently not only increased the contents of nitrogen but alongside changed morphological traits of the root system. This study featured that Mo induced improvement in root growth and development that might have occurred through changes in nitrogen acquisition via alterations of NRT gene expressions (Imran et al. 2020).

12 Crosstalk Between NO and Other Plant Hormones in Terms of Root Growth and Development

NO forms a close orchestrated network with other hormones. Here we are going to focus on the role of NO and its interplay with other plant hormones and its relation with developing the root architecture. ABA and NO has put up vivid mechanistic approaches in relation with ABA induced stomatal closure (Lombardo and Lamattina 2018) and also in reactions to UV-B-induced stress (Tossi et al. 2009) and its relation in terms of root formation is remarkable. Microtubulins (Mt) and actin cytoskeleton were studied to be the known targets of ABA and NO related signaling processes, changing the pattern of root hair growth and their formation ectopically. The cross-talk between ABA and NO was demonstrated in several plant responses to abiotic stresses. NO is a non-traditional plant regulator modulating root hair growth,

including the promotion of root hair differentiation in plants like lettuce (Lombardo et al. 2006). The inhibitory effect on root hair growth by the hormone ABA, was along with a significant increase level of NO level. On the other hand, the depletion of NO with the scavenger cPTIO also resulted in a severe inhibition of root hair growth in this study, which highlights the role of NO as precision specific and works within a certain range of its concentration i.e. having a double sword effect (Lombardo et al. 2006). Addition to ABA, the role of NO came in role play as a dose response dependent one. NO dosage generates different root phenotype as well, lower concentration influences the orientation of the microtubule without any casual effect on the root hairs but when higher dosage was given it grew root hairs in a different angle. In short NO has a pertinent role with cytoskeleton remodeling based on the environmental cues (Lombardo and Lamattina 2012). Posttranslational modifications as tyrosine nitration of α-tubulin is one of a direct phenomenon induced by NO. They control microtubule rearrangement in the epidermal cells and guard cells as noticed in *Arabidopsis* (Yemets et al. 2011; Zhang et al. 2008). NO affects cytosolic streaming especially at the growing tips as observed in pollen tubes and root hairs (Wang et al. 2008). In the NO deficient Arabidopsis G′4,3 mutant plants there was no involvement of ABA, but its actin reorganization was mediated by NO. Such reorganization of actin filaments were specific to cell type, cell development and subcellular specificities as noticed in roots of *Zea mays* (Kasprowicz et al. 2009). For better adaptability to drought conditions it's better to have shorter roots with more number of roots hairs as a phenotype. Lombardo & Lamattina highlighted the interplay between ABA and NO in regulation cytoskeleton for modifying root hair growth as well as propagation of new root hairs to bring in better endurance to dry (Lombardo and Lamattina 2018).

Root hair morphogenesis is delimited by variation in the current endogenous factors induced by the source of nutrient elements obtainable by the plants (Chandrika et al. 2013). In a study under Magnesium (Mg) ion shortage the level of the gaseous hormone ethylene and along with gaseous element NO was heightened especially in roots and root hairs. Ethylene and NO both has role in increasing the radial anatomy of root hairs (Giehl and Wirén 2014; Leitner et al. 2010). Their experiment was further validated by adding extra dosage of ethylene or NO in roots and also by generating mutation of related genes which had effect on root hair development. (Liu et al. 2017). Along with NO, deficiency of many other nutrients, such as phosphorus (P), potassium (K) and iron (Fe), was also found to elevate the production of ethylene or NO (Jin et al. 2012). GSNO (a no donor) and cPTIO (a NO scavenger) has an inhibitory and inducing effect, respectively on levels of ethylene (Niu et al. 2017). This study forecasted for the first time the antagonistic interplay between ET and NO which orders the appearance of lateral roots in *Arabidopsis* under selenium (Se) stress. (Feigl et al. 2019) Interestingly, in the etr1-1 mutant which is insensitive to NO in the presence of Se, GSNO showed an increase in lateral roots and cPTIO had an insignificant inhibitory outcome (Feigl et al. 2019).

A relationship of jasmonic acid (JA) mediated NO production and its associated phenotype of higher number of lateral root formation was due to a positive role of NO, stimulating cells in the pericycler region of the roots or via auxin signaling

in lateral root primordia (Schlicht et al. 2013). NO donors prompt enhance expression of auxin-dependent genes and its absence hinders Aux/IAA protein degradation (Terrile et al. 2012). TIR1 S-nitrosylation enhances protein protein interaction between TIR1-Aux/IAA, enabling degradation of Aux/IAA. NO causes overexpression of Jasmonate zim domain (JAZ1) gene, which is regulated by JA (Grunewald et al. 2009) at the highly differentiating region of the roots mainly the vascular cylinder and cortex and lateral root primordia and the protoxylem of primary root tips. NO appeared as a mediator in JA signaling with high chances of its involvement in lateral root formation and elongation. Ethylene and JA share targets for their regulatory mechanism. WT primary root growth when compared to the ethylene insensitive mutants of ein2-1 and ein2-1/jar1-1 mutants in response to either JA or NO donor SNP showed that the ein2 mutant makes plants resilient to JA and SNP for primary root growth ethylene mutants of ein2-1 and ein2-1/ jar1-1 mutants. This propagated the idea that EIN2 could be part of a NO sensing pathway and also synchronizes reactions on degradation of ERFs (Gibbs et al. 2014).

NO propagated elongation of maize root segments is dependent on the concentration of NO supplemented. IAA and NO might share some steps in common, in the signalling cascade as both of them prompt the same kind of reactions in plants (Pagnussat et al. 2004). Auxin and NO interplay for adventitious root development was not only demonstrated in cucumber explants but also in some wood species (Pagnussat et al. 2004). The NO in the cucumber explants stimulated cGMP as also validated using a GC inhibitor which reduced adventitious root formation in both IAA and NO treated ones (Pagnussat et al. 2003a). NO and auxin can also regulate nodulation. NO regulate the expression of PIN AUX efflux carriers genes in *Arabidopsis* and rice (Berger et al. 2020). NO does this by controlling AUX transport, by suppressing the level of expression of PIN proteins which in turn cause accumulation of AUX proteins and affecting cell division in the nodule (Signorelli et al. 2020). In legumes, phytoglobins (Phytogbs) control NO in early phase of the nitrogen-fixing and aids in buffering oxygen level in nodules (Singh et al. 2020). A potential mechanism can be through the NO induced cytokinin (CK) signalling mentioned via CRE 1. This occurs as a mechanism for symbiosis as seen in *Medicago truncatula* and *E. meliloti* association. CRE1 gene is required for the CK receptor CRE1 and induces nodulation (Gonzalez-Rizzo et al. 2006), Nodulation is also affected by NO (Ferrarini et al. 2008). Downstream CK signaling, the transcription factors as nodule inception (NIN) and nodule signalling pathway 2 (NSP2) promote nodule development (Suzaki et al. 2012). Thus, the induction of CRE1 by NO illustrates a potential mechanism by which NO produced soon after the infection could promote the establishment of symbiosis at early stages. NO facilitates strigolactone signalling in auxin and is ethylene-sensitive thus initiating lateral root formation as observed in case of sunflowers (Bharti and Bhatla 2015). Strigolactones aid in lateral root and adventitious root formation and development by regulating the auxin flux and polar transport. NO in the tissue system inhibits carotenoid cleavage dioxygenase (CCD activity), reducing the rate of biosynthesis of strigolactone. Bharti et al. proposed a mechanistic approach of CCD being negatively controlled by the endogenous level of NO, which is turn affecting strigolactone, auxin flux and the root formation in

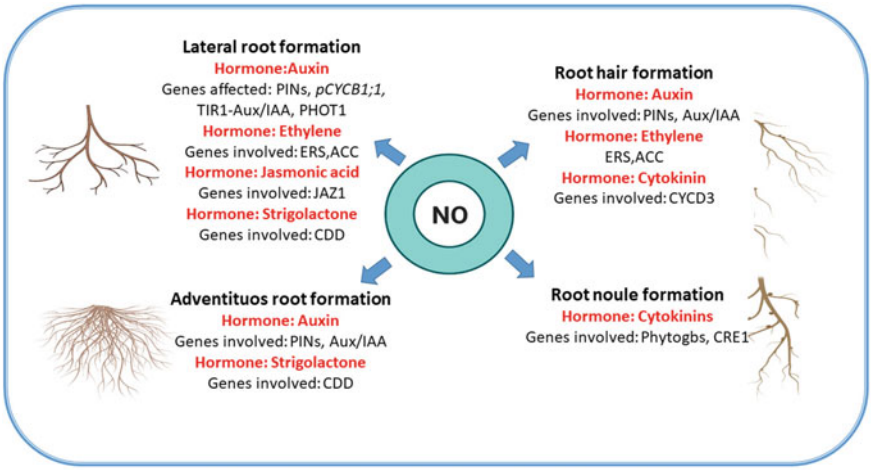

Fig. 2 A schematic showing NO associates with different hormones to bring in changes in the root architecture in plants

sunflower seedlings (Bharti and Bhatla 2015). In another study, role of blue light induced photoreceptor kinase phototropin 1 (Phot1) expression was analysed, which ultimately inhibits lateral root formation via decreasing the effects of auxin. Auxin binds to the promoter region of Phot1 and causes transcription of various targets which are responsible for lateral root formation. These evidences suggest that auxin supported lateral root formation may be regulated by interact between Phot1 and NO. NO also promotes TIR1-Aux/IAA interaction as evidenced by pull-down and two-hybrid assays. NO-mediated modulation of auxin signalling by S-nitrosylation of cysteine 140 of TIR1 auxin receptor. These findings underline the importance of NO in phytohormone signaling pathways (Terrile et al. 2012). All in all, NO and other hormones have a kin association right from processes rooted to establishment of the root systems. A schematic showing NO associates with different hormones to bring in changes in the root architecture in plants in Fig. 2.

13 NO a Double Edged Secondary Sword

NO exerts both beneficial and harmful effects on plants, depending on its concentrations in cells. One of the most intriguing issues in NO biology is its dual function as a potent oxidant and effective antioxidant. This dual role of NO might depends on its concentration as well as on the status of the environment. High concentrations of NO are sometimes phytotoxic. The observed effects of elevated NO levels are caused by its high reactivity as a radical with oxygen, radical oxygen species (ROS), and metals. High concentrations of NO can either be beneficial (e.g. it activates defence responses or, together with ROS, it directly kills the pathogen) or detrimental for

the plant cell in itself (Beligni and Lamattina 2000). Among the deleterious effects are lipid peroxidation, oxidation of tyrosine, as well as S-nitrosylation. The interaction of NO with iron containing proteins can lead to the inhibition of enzymes such as the cytochrome C oxidase of mitochondrial ETC (Yamasaki et al. 2001). It was reported that overproduction of NO strongly inhibits respiratory ATP synthesis in the mitochondria and also inhibits photosynthetic activity. These inhibitory actions are due to the inhibition of redox enzymes as a direct effect of NO. In the presence of ROS, irreversible damage may occur through ONOO– formation. ONOO–, a reaction product between NO and, is the most toxic RNS that potentially causes cellular dysfunctions via DNA damage and protein nitration or nitrosative stress. NO concentrations above 10–6 M inhibits expansion of leaf lamina, increased the viscosity of stimulated thylakoid lipid monolayers. Thus, living organisms must strictly control NO level to tune a fine balance between the beneficial (signaling) and harmful (deleterious effect) actions of NO (Hosseini et al. 2014). NO in phytohormone signalling pathways highlight its role as a second messenger during the configuration of the root system architecture.

14 Conclusion

Plant are more exposed to the environmental vagaries than animals due to their sessile nature. Thus, plants need more adaptative phenomenon to follow up for their survival. Many. have highlighted the importance of gaseous molecules as NO in signalling and interactions in plants. NO is a extensive intracellular and intercellular messenger with a broad spectrum of regulatory functions in many physiological processes in plants. The group of scientist involving Suemastu and coworkers have emphasized on a separate field of research for ‘gas biology’. This field should highlight mainly on the reactive species of oxygen, nitrogen and sulphur (Kajimura et al. 2012). To cut an entire picture of the existing NO process, it becomes essential to integrate all fields of biology. Studies ranging from single molecule, to cellular, tissue, organ, whole plant-plant, microbe-plant interactions to global issues eventually is required.

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Interaction of Cytokinin and Ethylene in the Regulation of Primary Root Growth and Development

Samina N. Shakeel, Swadhin Swain, Sitwat Aman, and G. Eric Schaller

Abstract Cytokinin and ethylene inhibit growth of the primary root through their effects on cell proliferation and cell expansion, doing so independently as well as cooperatively. Here we provide an overview of the cytokinin and ethylene signal transduction pathways. We discuss points of crosstalk between the pathways based on the ability of cytokinin to induce ethylene biosynthesis, the ability of ethylene to signal through the multi-step phosphorelay that mediates cytokinin signal transduction, and the ability of both hormones to cross-regulate gene expression for key elements in each other's biosynthesis and signaling pathways. The ability of these two hormones to regulate auxin activity plays a major role by which they inhibit primary root growth. To this end, mechanisms by which these two hormones regulate rootward and shootward auxin transport to control cell proliferation in the root meristem and cell expansion within the elongation zone, respectively, are discussed. Auxin-independent mechanisms to regulate root growth by these hormones are also considered. A model is proposed that provides a framework for the interaction of these hormones in the regulation of primary root growth.

1 Introduction

The root system of plants is essential for survival, and plays an indispensable role in absorption of water and nutrients and their translocation, as well as in anchoring the plant to the soil (Gowda et al. 2011; Lynch et al. 2014; Jung and McCouch 2013; Smith and De Smet 2012; Peret et al. 2014; Giehl and von Wiren 2014; Jones and Ljung 2012). In a simple taproot system, as found in eudicots such as Arabidopsis, there

Samina N. Shakeel and Swadhin Swain contributed equally to this chapter.

S. N. Shakeel · S. Swain · S. Aman · G. E. Schaller (✉)
Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA
e-mail: george.e.schaller@dartmouth.edu

S. N. Shakeel
Department of Biochemistry, Quaid-I-Azam University, Islamabad 45320, Pakistan

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are two major types of roots: primary and secondary roots. Primary roots develop embryonically and serve to initially anchor the plant to the soil. Secondary roots, such as lateral roots, develop post-embryonically and are major determinants of the overall root system architecture. This root system architecture contributes to many agronomic traits, including nutrient uptake and drought tolerance (Gowda et al. 2011; Lynch et al. 2014; Jung and McCouch 2013; Smith and De Smet 2012; Peret et al. 2014; Giehl and von Wiren 2014; Jones and Ljung 2012), and so its regulation is of broad agricultural interest. The root is a dynamic system that adapts its architecture in response to the environment and nutrient availability (Lopez-Bucio et al. 2003; Niu et al. 2013).

A variety of phytohormones regulate the growth and development of roots (Overvoorde et al. 2010; Pacifici et al. 2015; Vanstraelen and Benková 2012). Hormonal responses help mediate the plasticity of the root system architecture throughout the life cycle of the plant, doing so by controlling the rate of root growth, whether such growth involves changes in cell proliferation or cell expansion, the initiation and angle of lateral root growth, and the production and length of root hairs. A prominent role for auxin in regulating cell proliferation and cell expansion of the primary root is well established, principally through studies in Arabidopsis (Overvoorde et al. 2010; Pacifici et al. 2015; Vanstraelen and Benková 2012; Perrot-Rechenmann 2010; Takatsuka and Umeda 2014). However, additional phytohormones including cytokinin (CK), ethylene, abscisic acid (ABA), gibberellin (GA), jasmonic acid (JA), strigolactone (SL), and brassinosteroid (BR) also modulate root growth and development, this occurring through both auxin-dependent and auxin-independent mechanisms (Artner and Benkova 2019; Moubayidin et al. 2009; Qin et al. 2019; Xu et al. 2020; Liu et al. 2017). Extensive research making use of various molecular and genetic tools in Arabidopsis has revealed roles for both cytokinin and ethylene as inhibitors of primary root growth; for example cytokinin serves to signal the availability of nutrients such as nitrogen and phosphorus (Gu et al. 2018; Jia and von Wiren 2020; Sakakibara et al. 2006; Peret et al. 2011, 2014; Niu et al. 2013; Wu et al. 2013), and ethylene as a stress hormone serves to signal mechanical soil impediments and compaction (Okamoto et al. 2008; Hussain et al. 1999; Potocka and Szymanowska-Pulka 2018; Pandey et al. 2021). Initial models suggested a dichotomy of action with cytokinin negatively regulating cell proliferation and ethylene negatively regulating cell expansion in the root (Růžička et al. 2007, 2009; Swarup et al. 2007). More recent studies point to greater complexity of action, with cytokinin and ethylene each capable of regulating both cell proliferation and cell expansion, doing so at least partially independently but also achieving an additive response due to an ability to cooperatively influence signaling by each other (Street et al. 2015, 2016).

Here we focus on the roles of cytokinin and ethylene in the regulation of growth and development of the post-embryonic primary root, discussing these two phytohormones within the context of auxin signaling, the activity and distribution of which is instrumental in controlling both root cell proliferation and cell expansion. The mechanisms we discuss are primarily based on the model established in Arabidopsis through extensive genetic and molecular studies. Although the basic features of this model are also likely to pertain to other plant systems, novel regulatory mechanisms

will undoubtedly be uncovered based on the diversity found in the plant kingdom. We do not discuss lateral root initiation in this chapter, but the tips of lateral roots share a similar organization to that of the primary root tip, and thus much of the model established from studies of the Arabidopsis primary root is pertinent to considering the regulation of lateral root growth through hormonal effects on cell proliferation and expansion.

To address the hormonal regulation of primary root growth and development, we discuss the following. First, we lay a foundation for our understanding for the hormonal regulation of primary root growth by discussing the three phytohormones cytokinin, ethylene, and auxin, and their mechanisms for signal transduction. Second, we describe some of the mechanisms for hormonal crosstalk that occur with cytokinin and ethylene, such crosstalk being of significance in how they regulate both cell proliferation and cell expansion in the root. Third, we discuss the organizational structure of the primary root and the role that auxin plays in maintaining and regulating cell proliferation and expansion at the root tip. We then turn to how cytokinin and ethylene function through auxin-dependent and independent mechanisms to regulate cell proliferation and expansion, and thereby control root growth. We also address the roles cytokinin and ethylene play in regulating cell division in the root quiescent center. Finally, we conclude by highlighting some of the key elements related to how cytokinin and ethylene regulate primary root growth and development.

2 Signal Transduction by Cytokinin, Ethylene, and Auxin

In this section we discuss cytokinin, ethylene, and auxin and their mechanisms for signal transduction as a foundation for understanding how these three phytohormones interact to control primary root growth. Many of the key players involved in the biosynthesis and signal transduction by these hormones were uncovered through genetic studies in Arabidopsis, and the mutants uncovered in these studies now serve as critical tools in elucidating the roles of these hormones in the regulation of root growth as well as in other physiological studies.

2.1 Cytokinin Signaling

Cytokinins are adenine derivatives with N6 substitutions (Sakakibara 2006; Kieber and Schaller 2014; Hwang et al. 2012). Cytokinin profoundly influences various aspects of plant growth development such as maintenance of shoot and root apical meristems, branching, inflorescence development, phyllotaxis, nodule formation, and stress responses (Sakakibara 2006; Kieber and Schaller 2018, 2014; Hwang et al. 2012). The predominant naturally-occurring cytokinins are trans-zeatin (tZ), isopentyladenine (iP), dihydrozeatin, and benzyladenine. These are produced in both shoot and root tissues, have local effects, and are also transported rootward and

shootward, thereby providing a mechanism for root-shoot communication (Hirose et al. 2008; Kudo et al. 2010; Kieber and Schaller 2018).

Cytokinin levels are controlled by biosynthesis, conjugation, and degradation (Kudo et al. 2010). Cytokinin biosynthesis is initiated by the addition of a prenyl group to the N6 position of ADP or ATP by the enzyme isopentenyl transferase (IPT) to form an iP ribotide (Kakimoto 2001 #578; Takei et al. 2001; Sakakibara 2006). Formation of *t*Z-type cytokinins from the iP ribotide is catalyzed by the cytochrome P450 enzymes CYP735A1 and CYP735A2. At this point the iP and tZ cytokinin-ribotide derivatives are inactive. Activation requires their conversion to their free-base form by the LONELY GUY (LOG) family of cytokinin nucleoside 5′-monophosphate phosphoribohydrolases (Kurakawa et al. 2007; Kuroha et al. 2009). The level of cytokinin activity can be further modulated by conjugation of the cytokinins to sugars such as glucose or degradation by cytokinin oxidases (CKXs) (Werner et al. 2006).

Cytokinin signal transduction occurs through a multistep phosphorelay system evolutionarily related to bacterial two-component signaling systems (Fig. 1) (Kieber and Schaller 2014, 2018; Hwang et al. 2012). In such systems, the signal is propagated by sequential phosphorylation of conserved histidine to aspartate residues within the signaling elements. In Arabidopsis cytokinin signal transduction, this involves initial binding of cytokinin to the ARABIDOPSIS HISTIDINE KINASE (AHK) receptor, initiating autophosphorylation on a conserved histidine residue within a histidine kinase domain and subsequent intramolecular transfer to a conserved aspartate residue within the receptor receiver domain to begin the phosphorelay. The phosphate is then transferred to the histidine residue of an ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEIN (AHP). From there, the phosphate is transferred to the aspartate residue on a response regulator, of which there are two families chiefly implicated in regulating the cytokinin response: the type-B ARABIDOPSIS RESPONSE REGULATORS (ARRs), which mediate the transcriptional response to cytokinin, and the type-A ARRs, which are rapidly transcriptionally induced by the type-B ARRs and negatively regulate cytokinin responses. Mutations of the *AHK*s, *AHP*s, and type-B *ARR*s reduce the plant sensitivity to cytokinin, whereas mutations in the type-A *ARR*s enhance the plant cytokinin response.

In Arabidopsis, as in most plant species, each of these signaling elements is encoded by a multigene family, typically of overlapping function (Kieber and Schaller 2014, 2018; Hwang et al. 2012). For example, Arabidopsis has three cytokinin receptors: AHK2, AHK3, and AHK4. Single loss-of-function mutants are hyposensitive in some respects for cytokinin perception, but are phenotypically similar to the wild type under normal growth conditions. In contrast, the triple mutant *ahk2/3/4* is strongly insensitive to cytokinin and exhibits severe growth defects (Cheng et al. 2013a; Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006). The AHK receptors are primarily localized in the ER membranes, with the cytokinin binding CYCLASE/HISTIDINE KINASES ASSOCIATED SENSOR EXTRACELLULAR (CHASE) domain on the luminal side and the histidine kinase output domain on the cytosolic side of the ER membrane (Fig. 1) (Caesar et al. 2011; Lomin et al. 2011; Wulfetange et al. 2011; Romanov et al. 2018). A percentage of the AHK receptors are also detected at the plasma membrane, suggesting that they could

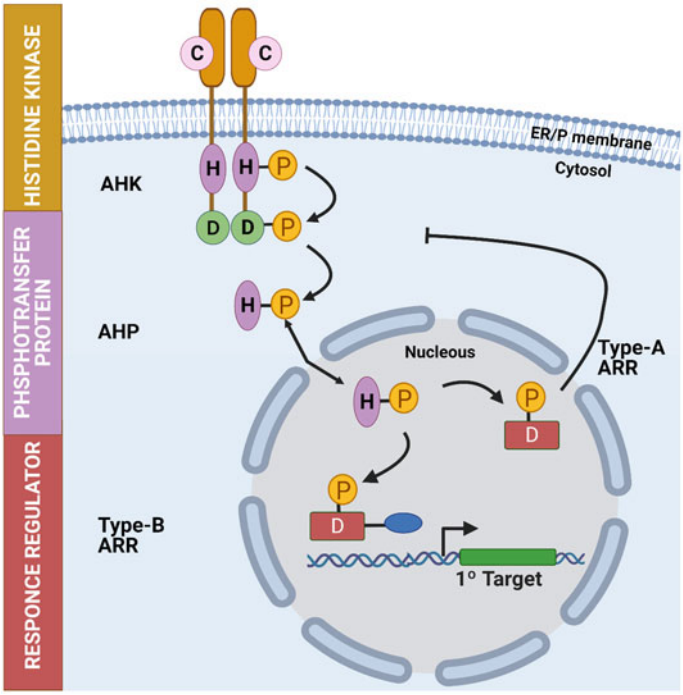

Fig. 1 The cytokinin signaling pathway. Cytokinin (c) signals through a multistep phosphorelay that involves hybrid histidine kinases with histidine kinase and receiver domains (AHKs), histidine-containing phosphotransfer proteins (AHPs), and response regulators (ARRs). The AHK receptors localize to the ER membrane and, to a lesser extent, to the plasma membrane (ER/P membrane). The response regulators are of two main types, the type-B ARRs, which mediate the transcriptional response, and the type-A ARRs which are encoded by genes that are primary targets of the type-B ARRs. The type-A ARRs negatively regulate cytokinin signal transduction

play a role in detecting apoplastic cytokinin (Zurcher et al. 2016; Antoniadi et al. 2020; Kubiasova et al. 2020). The receptors vary in their affinities for the different cytokinin varieties (Heyl et al. 2012). AHK3 has a high affinity for trans-zeatin (tZ) and is primarily localized in the shoot where it can perceive tZ that moves shootward from the root through the xylem (Hirose et al. 2008; Kudo et al. 2010). In contrast, both AHK2 and AHK4 have a high affinity for isopentyladenine (iP) and are primarily located in root where they can perceive iP that moves rootward from the shoot through the phloem. In this manner, hormonal communication can occur between root and shoot to coordinate their growth with respect to each other.

The AHP proteins shuttle the phosphate signal from the membrane-localized receptors to the nucleus (Fig. 1) (Kieber and Schaller 2014, 2018; Hwang et al. 2012). They are capable of moving in and out of the nucleus and their localization is independent of the cytokinin concentration (Punwani et al. 2010). Arabidopsis has five AHP proteins (AHP1 to AHP5) and one pseudo-AHP (APHP1/AHP6), which

lacks the conserved histidine residue needed for phosphorylation. AHP1, AHP2, AHP3, and AHP5 act redundantly and positively in cytokinin signaling, with higher order mutations in their genes resulting in cytokinin insensitivity (Hutchison et al. 2006). APHP1/AHP6 is a negative regulator of the pathway, potentially interacting with the receiver domains of the AHKs to interfere with the phosphorelay, and genetic analysis supports roles in protoxylem differentiation, leaf phyllotaxy, and lateral root initiation (Mähönen et al. 2006; Besnard et al. 2014; Bishopp et al. 2011; Moreira et al. 2013).

The final step of the multistep phosphorelay is the transfer of phosphoryl groups from the AHPs to the downstream ARR response regulators (Fig. 1) (Kieber and Schaller 2014, 2018; Hwang et al. 2012). The type-B ARRs contain a receiver domain with the conserved aspartate residue for phosphorylation as well as long C-terminal extensions that contain a Myb-like DNA binding domain (Argyros et al. 2008; Ishida et al. 2008). Upon phosphorylation, the type-B ARRs bind to the promoters of cytokinin primary response genes to initiate the transcriptional response to cytokinin (Xie et al. 2018; Zubo et al. 2017). Among the primary response genes are the type-A *ARR*s, which exhibit some of the most rapid inductions and highest levels of expression of the cytokinin targets in response to the cytokinin signal, their strong induction often being used as a molecular assay for the cytokinin response (Brandstatter and Kieber 1998; D'Agostino et al. 2000). The type-A ARRs have a receiver domain for phosphorylation, but lack a DNA binding domain. The type-A ARRs can dampen the cytokinin response by competing with type-B ARRs for phosphorylation by the AHPs and thus act as negative regulators in a feedback loop (To et al. 2004). Arabidopsis has 11 type-B and 10 type-A *ARR* genes, higher order mutant combinations in these resulting in cytokinin hyposensitivity or hypersensitivity, respectively (Argyros et al. 2008; Ishida et al. 2008; To et al. 2004).

2.2 Ethylene Signaling

Ethylene (C_2H_4) is a simple gaseous olefin and, having been identified as an endogenous plant growth regulator in 1934 (Gane 1934), was the first gaseous hormone identified (Bakshi et al. 2015). Although most widely known for its role in stimulating fruit ripening, ethylene performs regulatory functions throughout the life cycle of the plant including the regulation of seed germination, root and shoot development, senescence, and abscission, as well as modulating biotic and abiotic responses (Abeles et al. 1992; Mattoo and Suttle 1991; Schaller and Kieber 2002; McManus 2012). Plants are extremely sensitive to ethylene, being able to sense and respond to ethylene concentrations as low as 0.2 nL L^{-1}, but also able to recognize and respond to changes in ethylene concentration as high as 1000 μL L^{-1}, a range in sensitivity of over six orders in magnitude (Binder et al. 2004a; Chen and Bleecker 1995).

Ethylene biosynthesis, initiated from methionine in plants, occurs in three enzymatically catalyzed steps. Methionine is first converted to S-adenosyl methionine (SAM) by SAM synthetase, then in the second step SAM is converted to

1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and finally in the third step ACC is oxidized to release ethylene by ACC oxidase (ACO) (Booker and DeLong 2015; Abeles et al. 1992; Mattoo and Suttle 1991). Under most conditions, ACS catalyzes the rate-limiting step in this pathway and has been subject to the most molecular and genetic characterization in Arabidopsis. Both ACS and ACO enzymes are encoded by multi-gene families, and both recessive and dominant mutations have been identified in the ACS family, the dominant ACS mutations overproducing ethylene and resulting in a constitutive ethylene response (Chae et al. 2003; Vogel et al. 1998). Chemical inhibitors of the ACS and ACO enzymes have often been used as means to circumvent the genetic redundancy of these gene families to block ethylene biosynthesis, and are used both commercially and in laboratory experiments (Schaller and Binder 2017). For example, aminoethoxyvinylglycine (AVG) is an effective inhibitor of ACC synthase although, as with most chemical inhibitors there can be off-target effects, in this case inhibition of the tryptophan aminotransferase involved in auxin biosynthesis, and so caution is needed when interpreting such experiments.

Once produced, ethylene can diffuse from its site of production and bind to ethylene receptors to initiate signaling and induce an ethylene response (Abeles et al. 1992; Mattoo and Suttle 1991; Schaller and Kieber 2002; McManus 2012). The ethylene signal transduction pathway is comprised of elements of diverse evolutionary origin (Fig. 2) (Binder 2020; Azhar et al. 2019; Shakeel et al. 2013). The ethylene receptors, like those involved in cytokinin signaling, are related to bacterial histidine kinases (Chang et al. 1993; Schaller and Bleecker 1995). However, genetic analysis revealed that the downstream signaling elements that comprise the major ethylene signal transduction pathway are not related to the two-component signaling systems of bacteria. Instead, the signal is transmitted from the receptors to the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) protein kinase, then to the multi-pass transmembrane protein ETHYLENE INSENSITIVE2 (EIN2), and from there to the EIN3/(EIN3-LIKE) EIL family of transcription factors (Kieber et al. 1993; Alonso et al. 1999; Chao et al. 1997). This pathway involves both positive and negative regulators, such that in the absence of ethylene, the receptors activate CTR1 which then suppresses the downstream ethylene response (Ju and Chang 2015; Merchante et al. 2013; Shakeel et al. 2013; Binder 2020). In the presence of ethylene, CTR1 is inactivated and the positive signaling elements downstream of it then induce the ethylene response.

Arabidopsis has a five-member family of ethylene receptors comprised of ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and EIN4 (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). As with most plant species, the receptors fall into two subfamilies based on their phylogeny as well as some of their structural features (Fig. 2b). ETR1 and ERS1 compose subfamily 1 and ETR2, ERS2, and EIN4 compose subfamily 2, subfamily 1 receptors typically playing the more predominant role in ethylene signaling. The receptors localize to the ER membrane with the ethylene binding site being found within the transmembrane domain, a site compatible with the diffusability of ethylene (Chen et al. 2002; Dong et al. 2008). The histidine-kinase-like output domains are cytosolic.

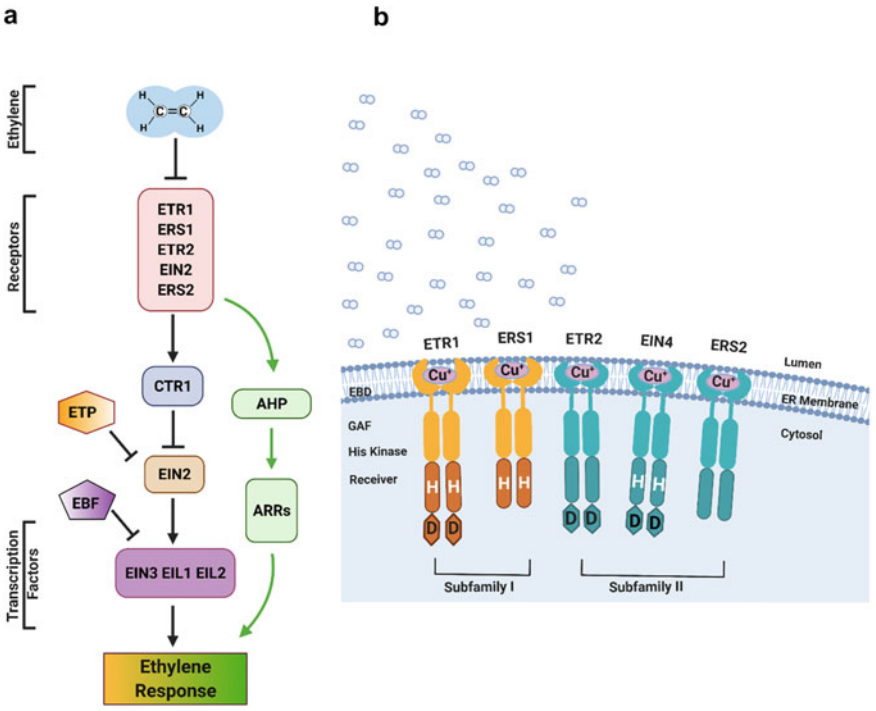

Fig. 2 The ethylene signaling pathway. **a** Elements of the ethylene signal transduction pathway of Arabidopsis. The primary signaling pathway contains positive and negative signaling elements. Ethylene binds to members of the ethylene receptor family (ETR1, ERS1, ETR2, EIN4 and ERS2), and the signal is transduced through the protein kinase CTR1, the transmembrane protein EIN2, and the EIN3/EIL transcription factor family. As part of a secondary pathway, the ethylene receptors may also signal through the AHP and ARR two-component signaling elements that play a role in cytokinin signal transduction. See text for details. **b** The Arabidopsis ethylene-receptor family. The ethylene receptors are dimers localized to the ER membrane, with the ethylene binding site residing within the transmembrane domain of the receptors and requiring a copper cofactor. The cytosolic portion of the receptors contains a GAF domain, a histidine kinase-like domain, and in some cases a receiver domain. The receptors group into two subfamilies based on phylogeny and some structural features

The subfamily-1 receptors have all the conserved features necessary for histidine-kinase activity, with such activity having been demonstrated for ETR1 and ERS1; the subfamily-2 receptors have diverged histidine-kinase domain, evidence indicating that they as well as ERS1 have serine/threonine kinase activity (Gamble et al. 1998; Moussatche and Klee 2004). Neither histidine kinase nor serine/threonine kinase activities play a major role in receptor signal transduction. Both gain-of-function and loss-of-function mutations have been isolated in the receptors. Gain-of-function mutations are ethylene insensitive, and typically arise due to mutations in the ethylene binding domain such that the receptors are unable to bind ethylene and/or to transduce the signal (Bleecker et al. 1988; Yen et al. 1995; Wilkinson et al. 1995; Hua et al.

1995; Sakai et al. 1998; Chang et al. 1993; Schaller et al. 1995; Wang et al. 2006). The dominant gain-of-function mutant *etr1-1* has proved a particularly useful molecular tool for generating ethylene insensitivity in transgenic experiments. Loss-of-function mutations result in ethylene hypersensitivity, higher order loss-of-function mutations involving multiple family members resulting in a constitutive ethylene response (Hua and Meyerowitz 1998; Wang et al. 2003; Qu and Schaller 2004; Cancel and Larsen 2002; Hall and Bleecker 2003; Tieman et al. 2000). Ethylene insensitivity can also be conferred on plants by treatment with the 1-methylcyclopropene (1-MCP), a high affinity competitive inhibitor for ethylene binding (Schaller and Binder 2017).

CTR1 is a serine/threonine protein kinase that negatively regulates the ethylene response (Fig. 2a) (Huang et al. 2003; Kieber et al. 1993). CTR1 physically associates with the ethylene receptors (Cancel and Larsen 2002; Clark et al. 1998; Gao et al. 2003) and, in the absence of ethylene, phosphorylates the transmembrane protein EIN2 (Alonso et al. 1999), a positive regulator of the ethylene response, inactivating it and also potentially targeting it for degradation (Ju et al. 2012; Qiao et al. 2012; Chen et al. 2011). Under this condition, the downstream EIN3/EIL family of transcription factors (Guo and Ecker 2004; Chao et al. 1997; Alonso et al. 2003), also positive regulators of the ethylene response, are ubiquitinated and degraded. Degradation of EIN2 and EIN3/EIL is mediated through the action of the *ETP* and *EBF* genes, respectively, which encode the F-box proteins that participate in SKP1-CULLIN-F-BOX PROTEIN (SCF) E3 ubiquitin ligase complexes (Qiao et al. 2009; Guo and Ecker 2003; Potuschak et al. 2003). Ethylene binding to the receptors results in an inhibition of CTR1 kinase activity, and a proteolytic cleavage of EIN2 to release its cytosolic C-terminal portion (EIN2-C) (Ju et al. 2012; Qiao et al. 2012; Chen et al. 2011). EIN2-C apparently has two functions, one is to inhibit the mechanism by which the EIN3/EIL transcription factors are turned over (Li et al. 2015; Merchante et al. 2015), the presence of ethylene resulting in a dramatic increase in protein levels of the transcription factors; the second to regulate EIN3/EIL dependent transcription in the nucleus (Zhang et al. 2017). Stabilization of EIN3/EIL proteins elevate ethylene dependent transcription, among the targets being genes encoding the ERF family of transcription factors, which also play a substantial role in regulating ethylene responses (Chao et al. 1997; Solano et al. 1998). Loss-of-function mutations in *CTR1* result in a constitutive ethylene-response phenotype (Huang et al. 2003; Kieber et al. 1993), whereas such mutations in *EIN2* or the *EIN3/EIL* family result in ethylene insensitivity (Alonso et al. 1999; Chao et al. 1997).

2.3 Auxin Signaling

The auxin class of plant hormones, of which the tryptophan derivative indole-3-acetic acid (IAA) is the main naturally occurring form, regulates practically all aspects of plant growth and development (Lavy and Estelle 2016; Ljung 2013). Auxins play key roles in embryogenesis, root and shoot development, tropic responses, and

plant defense. Of particular significance, and contributing to the exquisite regulation possible through auxin signaling, is its carefully controlled directional transport between cells (Sauer and Kleine-Vehn 2019; Adamowski and Friml 2015; Michniewicz et al. 2007; Petrasek and Friml 2009; Zwiewka et al. 2019). This polar auxin transport is a critical mechanism in plant embryogenesis and organogenesis. Furthermore, regulation of polar auxin transport is a major control point by which other hormones and regulators can alter plant growth and development.

Auxin levels are controlled by biosynthesis, conjugation, and degradation (Zhao 2014; Ljung 2013; Ludwig-Muller 2011). There are several routes by which auxins are synthesized, with evidence supporting both tryptophan-dependent and tryptophan-independent routes for biosynthesis (Zhao 2014). IAA, the predominant auxin in plants, is primarily synthesized by a two-step pathway starting from tryptophan (Zhao 2014). In the first step, indole-3-pyruvate is generated by the removal of the amino group from tryptophan, this step being catalyzed by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) (Tao et al. 2008; Stepanova et al. 2008; Yamada et al. 2009). In the second step, the indole-3-pyruvate undergoes oxidative decarboxylation to produce IAA, this step being catalyzed by the YUCCA (YUC) family of flavin monooxygenases (Zhao et al. 2001; Cheng et al. 2006). The free form of IAA is the active form but the level of auxin activity can be further modulated by reversible conjugation or degradation (Ludwig-Muller 2011). The GRETCHEN HAGEN3 (GH3) family of amido synthases catalyze the formation of IAA-amino acid conjugates to inactivate IAA, amidohydrolases then serving to release the active free IAA. In this manner a ready pool of mobilizable auxin can be maintained, transitioning between active and inactive forms, without the necessity of new biosynthesis.

Auxin perception and signal transduction involves three major signaling elements: an SCF E3 ubiquitin-ligase complex ($SCF^{TIR1/AFB}$) containing TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) as the F-box component, the Aux/IAA family of transcriptional repressors, and the AUXIN RESPONSE FACTOR (ARF) family of transcription factors (Fig. 3) (Lavy and Estelle 2016; Salehin et al. 2015). The ARFs are positive regulators for the auxin response and bind to specific sequences in the promoters of target genes. However, in the absence of auxin, the ARFs are inactive due to dimerization with the Aux/IAA repressors. $SCF^{TIR1/AFB}$ and the Aux/IAA proteins function as a co-receptor complex for auxin, such that when auxin is present it serves as a 'molecular glue' in a shared binding pocket between $SCF^{TIR1/AFB}$ and Aux/IAA. The Aux/IAA protein is then ubiquitinated and targeted for degradation through the proteasome, the decrease in Aux/IAA levels resulting in derepression of the ARF transcription factors and the initiation of the auxin transcriptional response.

As previously mentioned, the auxin transport mechanisms between cells for auxin are particularly fine-tuned, with such polar auxin transport serving to establish the local auxin minima and maxima that play critical roles in embryogenesis and organogenesis (Sauer and Kleine-Vehn 2019; Adamowski and Friml 2015; Petrasek and Friml 2009; Zwiewka et al. 2019). As we will subsequently see when considering roots in more detail, the establishment of such auxin maxima within the quiescent

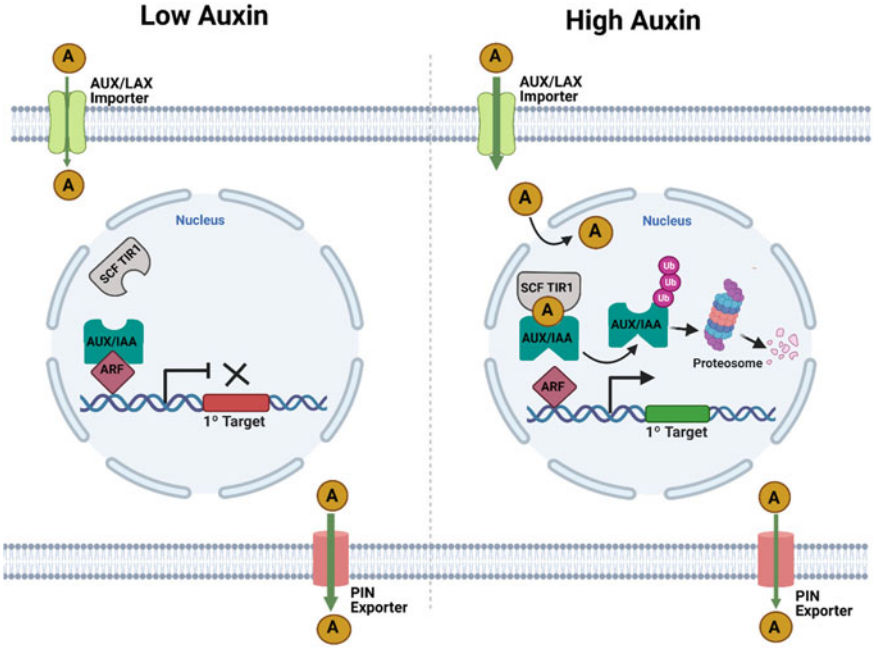

Fig. 3 The auxin signaling pathway. Auxin levels in the plant cell are regulated by the AUX/LAX family of auxin importers and the PIN family of auxin exporters. The ARF family of transcription factors are inhibited by members of the Aux/IAA family. Auxin binds to a shared binding pocket between its $SCF^{TIR1/AFB}$ receptor and Aux/IAA. The Aux/IAA protein is then ubiquitinated and targeted for degradation through the proteasome, allowing for transcription to be initiated by the ARFs

center is a key element in establishing the meristematic structure and, furthermore, the rootward and shootward transport of auxin is of particular importance in regulating cell proliferation and cell expansion in the root. Auxin transport depends on influx carriers and efflux carriers (Sauer and Kleine-Vehn 2019; Adamowski and Friml 2015; Petrasek and Friml 2009; Zwiewka et al. 2019). Auxin influx across the plasma membrane is mediated by the AUXIN RESISTANT1 (AUX1)/LIKE AUX1 (LAX) family of transmembrane proteins, the family member AUX1 serving the major role in shootward transport of auxin in the root (Bennett et al. 1996; Swarup et al. 2008; Peret et al. 2012). Auxin efflux across membranes is mediated by the PIN-FORMED (PIN) and ATP-BINDING CASSETTE SUBFAMILY B (ABCB) families of carriers, the PIN family being the most thoroughly characterized (Petrasek et al. 2006; Petrasek and Friml 2009; Krecek et al. 2009; Adamowski and Friml 2015; Noh et al. 2001). The PIN family plays the most significant role among the auxin influxers/effluxers in establishing directional cell-to-cell transport by their dynamic and asymmetric distribution on the plasma membrane, this being accomplished due to cycles of endocytotic degradation and recycling (Mravec et al. 2008; Petrasek and Friml 2009; Krecek et al. 2009; Adamowski and Friml 2015). Although many

members of the PIN family function at the plasma membrane, some like PIN5 and the related PIN-LIKES (PILS) family members localize to the ER membrane, potentially serving as an alternative means by which to regulate intracellular auxin levels (Mravec et al. 2009; Sauer and Kleine-Vehn 2019).

3 Mechanisms for Crosstalk Between Cytokinin and Ethylene

Cytokinin and ethylene are best known for their antagonistic roles in the shoot, where cytokinin promotes cell proliferation, stimulates chloroplast development, and delays senescence, and ethylene counteracts these same processes (Abeles et al. 1992; McManus 2012; Hwang et al. 2012; Kieber and Schaller 2014; Mok and Mok 2001; Rai et al. 2015). However, they are also capable of acting in a synergistic manner, cytokinin in fact inducing ethylene biosynthesis under some growth conditions (Cary et al. 1995; Chae et al. 2003; Vogel et al. 1998). Of relevance to this chapter, cytokinin and ethylene both inhibit root growth by inhibiting cell expansion and cell proliferation (Werner et al. 2003; Werner et al. 2010; Růžička et al. 2007; Růžička et al. 2009; Street et al. 2015; Street et al. 2016; Dello Ioio et al. 2007; Dello Ioio et al. 2008; Moore et al. 2015), pointing to the likelihood of cooperativity and overlap in the mechanisms employed. Here we discuss some of the mechanisms for crosstalk by which components in the cytokinin and ethylene pathways can affect each other, including transcriptional cross-talk, the induction of ethylene biosynthesis by cytokinin, and evidence that ethylene can signal through the cytokinin phosphorelay.

3.1 Transcriptional Cross-Talk

Plant growth and development involves the integration of multiple hormonal signals, some antagonistic and some cooperative, and so it is not surprising that one hormone will regulate the expression of genes controlling the activity of another. What has become much clearer with the advent of genome-wide chromatin-immunoprecipitation (ChIP) studies are the breadth of this regulation, the specific players involved, and how much of this regulation represents a primary transcriptional target for hormones. Recent ChiP-Seq studies with EIN3 and various type-B ARRs, the transcription factors that regulate the primary response to ethylene and cytokinin, respectively, identify hormone-related targets as an enriched category (Chang et al. 2013; Xie et al. 2018; Zubo et al. 2017; Zubo and Schaller 2020). These hormone-related targets cover the breadth of possibilities and include genes involved in biosynthesis, degradation and inactivation, and signal transduction.

One of the first such studies to be performed was with EIN3, in which Chip-Seq and RNA-Seq analyses were performed with three-day old dark-grown seedlings, across a six-point time course ranging from 15 min to 24 h (Chang et al. 2013). ChIP-Seq was performed with an antibody that recognizes the native EIN3 protein. From this study 375 EIN3 targets were identified for which there was also expression information, the majority of these targets being induced in response to ethylene. Over 900 additional candidate targets were identified for which there was no evidence of a change in ethylene-dependent expression, potentially due to other co-factors being necessary for changes in expression. Although robust cross-talk with many hormone related targets was identified, the level of EIN3-dependent crosstalk with cytokinin signaling was less than that found for most other hormone signaling pathways, representing only 1% of the total possible targets. By comparison hormone related targets for ethylene comprised 42% of the total, ABA 9%, GA 6%, and auxin 5%. The cytokinin targets of EIN3 included the positive regulator *AHP1* and the negative regulator *ARR3*, both of which were transcriptionally induced in response to ethylene, and AHK4 cytokinin receptor gene as a candidate target.

Far greater numbers of candidate and target genes were identified in two individual studies making use of type-B ARRs to examine the cytokinin response (Xie et al. 2018; Zubo et al. 2017; Zubo and Schaller 2020). The greater number of such genes is owed at least in part due to studies having been performed more recently than that involving EIN3, improved technologies allowing for greater depth of genomic coverage. From both studies, approximately 800 genes were identified as high-confidence targets for which there was confirming evidence for cytokinin regulation of the genes; 1000s of additional candidate targets were also identified. For ethylene-related genes, type-B ARR binding sites were found associated with genes involved in biosynthesis and signal transduction. Related to biosynthesis, two *ACS* genes (*ACS2* and *ACS6*) were identified, expression of the *ACS2* gene being cytokinin-induced, a finding consistent with the known role for cytokinin in up-regulating ethylene biosynthesis (Qin et al. 2019; Zdarska et al. 2015). Although the gene for the ethylene receptor *ETR2* was identified as a type-B ARR target whose expression is down-regulated by cytokinin, most of candidate genes related to ethylene signal transduction were modulators of the process rather than integral parts of the signaling circuit (Xie et al. 2018; Zubo et al. 2017; Zubo and Schaller 2020). These include members of the *ARGOS* gene family, negative regulators that function at the level of the receptors, the positive regulator *TRP1* that also acts at the level of the receptors, and the *ETP* and *EBF* genes, negative regulators that encode the F-box proteins involved in degradation of EIN2 and EIN3, respectively.

3.2 Induction of Ethylene Biosynthesis by Cytokinin

Cytokinin as well as other phytohormones induce ethylene biosynthesis, an effect that can be elegantly demonstrated by hormonal treatment of dark-grown Arabidopsis seedlings with cytokinin (Cary et al. 1995; Hansen et al. 2009; Woeste et al. 1999).

Such treatment induces a diagnostic morphological response to ethylene characterized by inhibition of hypocotyl and root growth, and the formation of a pronounced apical hook, and is largely blocked by inhibitors of ethylene biosynthesis as well as by ethylene-insensitive mutations. The finding that type-B ARRs bind to promoters for the ethylene biosynthetic genes *ACS2* and *ACS6*, as described above, might suggest that transcriptional regulation is the major mechanism by which cytokinin induces ethylene biosynthesis, however post-translational mechanisms appear to play a greater role.

The ACC synthase (ACS) family of Arabidopsis can be divided into three subtypes based in large part on the C-terminal extensions that lie outside the catalytic core of the enzyme (Booker and DeLong 2015; Chae and Kieber 2005; Pattyn et al. 2021). These C-terminal extensions turn out to particularly important in the regulation of ACS protein stability. The significance of the C-terminal extensions was initially suggested based on the discovery that the dominant ethylene-overproducing mutants *eto2* and *eto3* resulted from alterations in the C-termini of ACS5 and ACS9, respectively, and that ACS5 containing the eto2 mutation had a slower turnover rate than the wild-type version (Chae et al. 2003; Vogel et al. 1998). Subsequent analysis and cloning of *eto1*, a recessive mutant that overproduced ethylene, provided a clear indication that proteasome-dependent turnover played a significant role in ACS stability (Wang et al. 2004). First, ETO1 is a type of protein that links the CUL3-based ubiquitin ligase to substrate proteins; second, ETO1 physically interacts with ACS5; and third, ACS5 protein levels are substantially higher in the *eto1* mutant background. Since these initial experiments, a variety of protein kinases, phosphatases, and additional regulators have been identified that mediate ACS stability, the majority functioning through modification of these C-terminal ACS extensions (Booker and DeLong 2015; Chae and Kieber 2005; Pattyn et al. 2021).

ACS mutants have facilitated the analysis of how ethylene biosynthesis mediates the cytokinin response, and indicate that the type-2 ACS family, which includes ACS5 and ACS9, plays the predominant role in this regard. For this purpose, the *ACS5* loss-of-function *cin*5 (*cytokinin insensitive5*) mutants were particularly informative (Vogel et al. 1998). These were isolated based on insensitivity to cytokinin in a dark-grown seedling assay, but were still sensitive to exogenous ethylene treatment, indicating that the genetic lesion did not fall in the ethylene signaling pathway. The *cin5* mutants all mapped to ACS5 and were predicted to result in a loss of function. The *cin5* mutants of *ACS5* produced substantially less ethylene than the wild type when treated with cytokinin. Furthermore, treatment of wild-type seedlings with cytokinin did not result in appreciable changes in *ACS5* transcript levels, consistent with cytokinin playing a post-transcriptional role in the regulation of ACS5. This hypothesis was confirmed by use of epitope-tagged versions of ACS5, where it was demonstrated that cytokinin treatment stabilized the ACS5 protein (Chae et al. 2003). The closely related ACS9 was similarly demonstrated to have its protein stability increased in the presence of cytokinin (Hansen et al. 2009).

More recently, additional enzymes of the ethylene biosynthetic pathway have been demonstrated to be up-regulated in the presence of cytokinin by taking a proteomic approach with roots and shoots (Zdarska et al. 2013). Green seedlings were treated

for either 30 or 120 min so as to focus on the short-term response to cytokinin. A SAM synthetase (MAT3) and an ACC oxidase (ACO2) were up-regulated in the root at the 30-min timepoint, and the SAM synthetase MAT4 was downregulated in the shoot at the 120 min timepoint. This study identifies additional players in the ethylene biosynthetic pathway that are regulated by cytokinin and, furthermore, emphasizes that differences in regulation can occur between the root and the shoot. How much transcriptional or post-transcriptional mechanisms play in the cytokinin-dependent regulation of these enzymes is still unclear, but none are in the high-confidence list of type-B ARR targets based on ChIP-seq analysis (Zubo and Schaller 2020).

3.3 Signaling by Ethylene Through the Multi-step Phosphorelay

As previously discussed, ethylene receptors are histidine kinase-like proteins, the histidine-kinase activity of ETR1 and ERS1 of subfamily 1 having been biochemically confirmed (Gamble et al. 1998; Moussatche and Klee 2004). However, the major pathway (CTR1 → EIN2 → EIN3/EIL) operating downstream of the ethylene receptors is not a two-component phosphorelay. Thus, the ethylene-signaling pathway illustrates how two-component elements have been evolutionarily adapted to the needs of eukaryotes, with a histidine-kinase ancestor evolving toward new biochemical function, and incorporating novel downstream elements into the signal transduction pathway (Schaller et al. 2011). The histidine kinase activity of the receptors appears to play a role in modulating the ethylene response based on standard assays (Hall et al. 2012), but this alone is not sufficient to demonstrate action through a two-component based phosphorelay; for example, such autophosphorylation could affect interactions with CTR1 as part of a receptor complex. Nevertheless, there is evidence that ethylene receptors such as ETR1 mediate a subset of the ethylene responses through action of the two-component signaling pathway (Binder et al. 2012; Shakeel et al. 2013; Qu and Schaller 2004; Binder et al. 2004b; Hall et al. 2012; Scharein et al. 2008; Urao et al. 2000; Hass et al. 2004; Binder et al. 2018; Street et al. 2015).

The major role of the AHP and ARR two-component signaling elements is in mediating the cytokinin signal, but they have also been implicated as playing a minor role in the ethylene signaling pathway, potentially operating downstream of the ethylene receptors (Hass et al. 2004; Binder et al. 2018; Street et al. 2015). Consistent with potential participation in a phosphorelay, ETR1 is capable of interacting with multiple AHP proteins based on a variety of assays (Zdarska et al. 2019; Scharein et al. 2008). Analysis of the interaction with AHP1 indicates that the interactions occur with higher affinity when one protein is in its phosphorylated state and the other not, lower affinity when both proteins are in their phosphorylated or nonphosphorylated states (Scharein and Groth 2011). The clearest role for the two-component phosphorelay functioning in ethylene receptor signaling comes from kinetic analysis

to examine the ability of ethylene-treated seedlings to recover normal growth after removal of ethylene (Binder et al. 2004b; Binder et al. 2018). Treatment of wild-type etiolated seedlings with ethylene inhibits their growth rate, but upon removal of ethylene, the seedlings return to their normal growth rate within two hours. A loss-of-function mutant in ETR1 (*etr1-7*) exhibits a significantly slower recovery than wild-type, and this recovery was shown to be dependent on the receptor's histidine-kinase activity (Binder et al. 2004b). Further analysis identified *ahp* and type-B *arr* mutant combinations that exhibited a similar slow growth recovery phenotype (Binder et al. 2018). These mutant effects are specific for ethylene signaling, because this mutant phenotype is not observed in cytokinin receptor mutants such as an *ahk2/3* double mutant (Argueso et al. 2012; Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006; Binder et al. 2018).

4 The Arabidopsis Root System

The Arabidopsis root system consists of one primary root and multiple lateral roots. The roots absorb nutrients and water from the soil and provide anchorage (Giehl and von Wiren 2014; Petricka et al. 2012; Scheres et al. 2002). The primary root originates from the embryo and the lateral roots emerge from the primary root during postembryonic development (Petricka et al. 2012). The root tip has 3 distinct developmental regions—the meristematic zone, the transition zone, and the elongation/differentiation zone—all of which are critical for normal root growth and development (Fig. 4). Cell proliferation occurs within the meristematic zone and cell expansion within the elongation/differentiation zone, with the phytohormone auxin playing a key role in the meristematic zone as a positive regulator of cell proliferation, and in the elongation zone as a negative regulator of cell expansion (Overvoorde et al. 2010; Petrasek and Friml 2009).

The meristematic zone has 4–8 undifferentiated and rarely dividing cells referred to as the quiescent center (QC) surrounded by slowly dividing meristem initials, collectively referred to as the stem cell niche (Petricka et al. 2012). The QC suppresses the differentiation of meristem initials and maintains their identity. The meristem initials divide in an anticlinal fashion to produce meristem daughter cells which then continue to divide until reaching a developmental state where cell division ceases and elongation starts. The stem cell niche along with the meristematic cells constitute the root apical meristem (RAM). The RAM drives postembryonic growth and development of the root by forming root cell layer-specific meristem initials, by maintaining meristem cell identity, and by proliferation of meristem daughter cells (Petricka et al. 2012).

Moving shootward from the root tip, the meristematic zone is followed by the elongation/differentiation zone (Petricka et al. 2012). The meristematic and elongation/differentiation zones are separated by a transition zone, this being the region at which cell proliferation ceases and cell expansion initiates (Petricka et al. 2012). The proximal meristematic cells continually lose their meristematic identity and enter

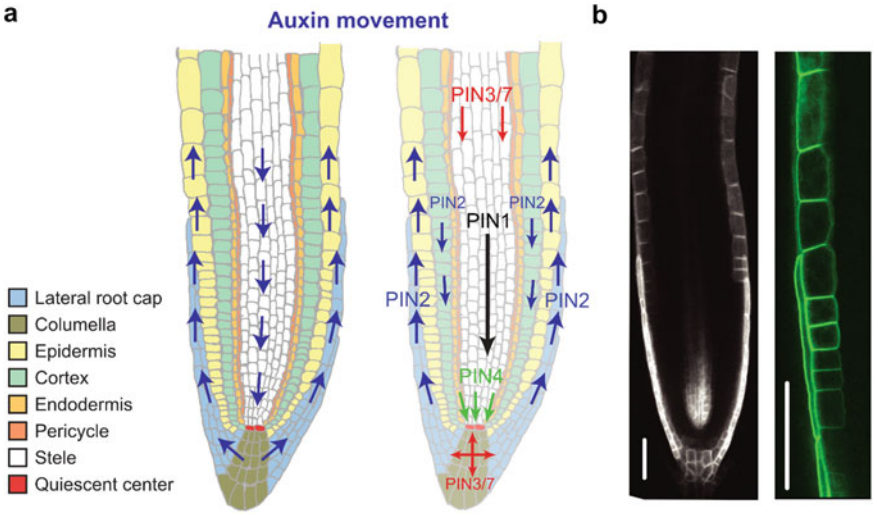

Fig. 4 Cellular organization and factors controlling auxin movement in the primary root tip of Arabidopsis. **a** Auxin movement. The diagram on the left illustrates the various tissue types of the primary root tip and "inverted fountain" of auxin movement. The diagram on right illustrates the role of PINs in establishing auxin movement, with arrows indicating the direction of auxin transport by PIN1, PIN2, PIN3, PIN4, and PIN7. **b** Localization of the AUX1 auxin importer based on fluorescence of a *pAUX1:AUX1:YFP* reporter (from Street et al. 2016, permission obtained from author (copyright holder)). Closeup shows localization to the lateral root cap and the epidermal layer of the root just above the root cap. Scale bar = 50 μm

into the transition zone, shifting from cell cycle to endoreduplication as they expand and elongate (Schaller et al. 2014; Petricka et al. 2012). This equilibrium between cell proliferation and differentiation is vital to maintain normal root growth.

As previously discussed, polar auxin transport plays a major role in the regulation of plant growth and development, with this being of particular significance in the regulation of root growth and development (Blilou et al. 2005; Grieneisen et al. 2007; Sabatini et al. 1999). Polar auxin transport in the root relies upon the polar subcellular distribution of the plasma-membrane associated PIN efflux carriers, as well as the overall tissue expression pattern of the PINs and the AUX/LAX influx carriers. Auxin transport in the primary root is often likened to an inverted fountain, based on its pattern of rootward (acropetal) and shootward (basipetal) transport (Fig. 4) (Overvoorde et al. 2010; Petrasek and Friml 2009). For rootward transport, auxin is transported through the vasculature toward the root tip, the directionality being primarily dependent on the action of PIN1 and PIN7, where it forms an auxin maxima at the QC (Michniewicz et al. 2007; Petrasek and Friml 2009). For shootward transport, auxin is transported through the columella of the central root cap, to the lateral root cap, and then to the epidermal cell layer, the shootward transport being dependent on AUX1 and PIN2 (Wisniewska et al. 2006; Swarup et al. 2001, 2005). Contributions to root auxin can come from long-distance rootward transport from the

shoot through the phloem, as well as from locally produced auxin in the root (Brumos et al. 2018). In addition, recycling of auxin during transport can occur, for once the shootward transported auxin reaches the transition zone, the auxin can move toward the interior vascular tissue. The auxin gradient formed due to polar auxin transport defines the developmental fate of the cells based on location and concentration. The auxin maxima in the QC ensures its quiescence nature, an intermediate auxin level in the meristematic zone is associated with cell proliferation, and an auxin minimum near the transition zone is associated with cell differentiation (Petricka et al. 2012; Overvoorde et al. 2010; Petrasek and Friml 2009; Perrot-Rechenmann 2010; Takatsuka and Umeda 2014).

Auxin regulates proliferation in the meristematic region in part through the *PLETHORA* (*PLT*) family of genes (Aida et al. 2004; Galinha et al. 2007). *PLT*s encode AP2/EREBP-like transcription factors that play roles in both embryonic organ patterning and the postembryonic growth of the root. High auxin concentrations in the region of the stem cell niche induce the expression of *PLT* genes (Aida et al. 2004; Mahonen et al. 2014), which positively regulate the expression of *PIN1*, *PIN3*, and *PIN4* (Galinha et al. 2007). Analysis of PLT protein accumulation indicates that they form a concentration gradient in the meristem similarly to what is observed for auxin (i.e. highest at the stem cell niche and decreasing in concentration toward the transition zone). However, the PLT gradient is not actually a direct readout of the auxin gradient, but arises due to PLT dilution following each cell division as well as due to intercellular movement of the *PLT* mRNA. The high level of PLT protein is necessary to maintain the stem cell identity of the QC and meristem initials, whereas the intermediate level induces cell division in the meristem zone, and the low levels coincide with cell differentiation and elongation (Galinha et al. 2007; Mahonen et al. 2014).

5 Auxin-Dependent Mechanisms by Which Cytokinin and Ethylene Regulate Cell Proliferation in the Primary Root

Both cytokinin and ethylene inhibit root growth in part by suppressing cell proliferation within the meristematic region of the primary root, evidence indicating that both phytohormones accomplish this activity through auxin-dependent mechanisms. There is considerably more information about the mechanisms by which cytokinin inhibits root cell proliferation and so, in this section, we initially focus on the roles played by cytokinin and then discuss more recent data that also support a role for ethylene in suppressing cell proliferation.

5.1 Auxin-Dependent Mechanisms by Which Cytokinin Regulates Cell Proliferation in the Primary Root

Early physiological studies on the role of cytokinins in plant growth relied upon exogenous application of the hormone. Key evidence on the role of endogenous cytokinins in the regulation of plant growth came from the transgenic overexpression of Arabidopsis cytokinin-oxidase (*CKX*) genes in tobacco and Arabidopsis, the cytokinin oxidases serving to degrade and thus lower the levels of endogenous cytokinins (Werner et al. 2003, 2001). Transgenic plants overexpressing the *CKX* genes exhibited reduced shoot growth and had smaller shoot apical meristems with fewer cells compared to the wild-type plants, a finding consistent with the concept that cytokinin is a positive regulator of cell proliferation in plants. In contrast, the overall root growth was enhanced in the transgenic *CKX* lines, due to more rapid growth of primary roots and increased formation of lateral roots. Examination of the root apical meristem in the *CKX* lines demonstrated an increase in its size coupled to an increase in the number of dividing cells based on use of cyclin-GUS reporter. Increases in cell expansion were also noted. These studies established that cytokinin has opposite effects on shoot and root growth and that, in the root, cytokinin negatively regulates cell proliferation and expansion (Werner et. al. 2001 PNAS, 2003 Plant Cell).

The identification of mutants in the cytokinin signaling pathway provided additional genetic tools by which to examine the role of cytokinin in root growth and development. Primary root growth is inhibited by exogenous cytokinin, and the higher-order *ahk*, *ahp*, and type-B *arr* mutants all exhibit insensitivity to cytokinin based on this root growth assay (Hutchison et al. 2006; Ishida et al. 2008; Kieber and Schaller 2014; Mason et al. 2005; Yokoyama et al. 2007; Cheng et al. 2013a; Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006). A careful examination of type-B *ARR* mutants, coupled to a more exact measurement of meristem size based on the number of cortical cell between the QC and the transition zone, revealed that loss of individual members of the family resulted in an enlarged root meristem, with the five most abundantly expressed type-B ARRs in the root all affecting meristem size (Dello Ioio et al. 2007; Hill et al. 2013). Some differences in developmental timing for the effects of the *arr* mutants on meristem size is observed following germination, however functional redundancy for the effects on meristem size is supported by the finding that double mutants such as *arr1 arr12* have larger root meristems than the single mutants (Dello Ioio et al. 2007).

Since type-B ARRs mediate the transcriptional response to cytokinin, the question then becomes what are the transcriptional targets by which they and cytokinin regulate root cell proliferation (Fig. 5). One such type-B ARR target turns out to be *SHORT HYPOCOTYL2* (*SHY2/IAA3*), a member of the Aux/IAA family, which is a cytokinin primary response gene (Dello Ioio et al. 2008). As previously described, members of the Aux/IAA family such as SHY2, negatively regulate auxin signaling by physical interaction with the ARF transcription factors, this leading to the repression of auxin response genes, some of which encode PIN efflux carriers. Indeed, application of

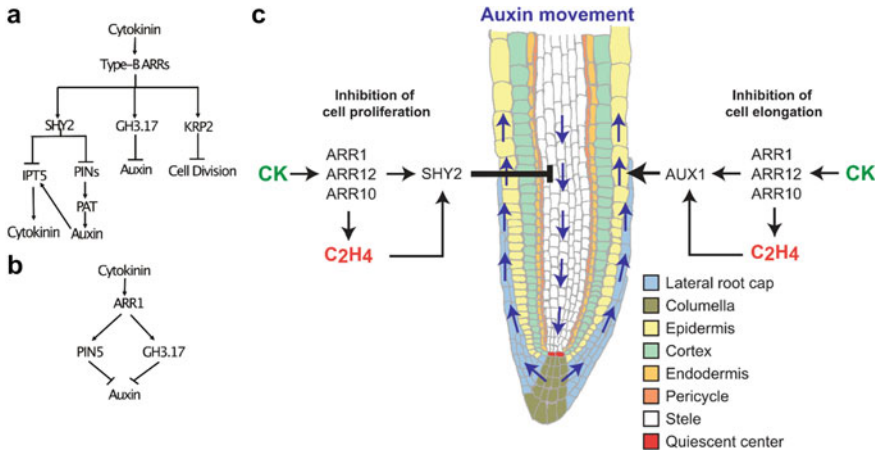

Fig. 5 Cytokinin and ethylene dependent mechanisms for regulation of cell proliferation and elongation at the Arabidopsis root tip. **a** Genetic circuitry involved in the mechanisms by which cytokinin regulates cell proliferation in the RAM and the positioning of the transition zone through auxin-dependent and independent mechanisms. PAT stands for polar auxin transport. **b** Additional proposed circuitry by which cytokinin regulates cell proliferation in the RAM by effects at the lateral root cap. **c** Crosstalk between cytokinin and ethylene in the regulation of root cell proliferation and elongation. Positive and negative genetically defined interactions are shown. For the inhibition of cell proliferation, the ethylene and cytokinin signaling pathways converge on *SHY2*, a member of the Aux/IAA family to inhibit the rootward transport of auxin. For the inhibition of cell elongation, the ethylene and cytokinin signaling pathways converge on *AUX1* to regulate the shootward transport of auxin. Cytokinin induces ethylene biosynthesis, which contributes to the effects of cytokinin on cell proliferation and elongation

cytokinin represses expression of *PIN1*, *PIN2*, and *PIN3*, and activates *PIN7* (Růžička et al. 2009). *ARR1* and *ARR12* are most highly expressed in the transition zone, and so their activation will dampen the rootward auxin transport to the meristematic zone, and thereby reduce cell proliferation. In contrast, high auxin levels in the meristematic zone degrade SHY2 through proteasomal degradation and de-repress auxin response genes, which facilitates cell proliferation (Dello Ioio et al. 2008; Maraschin Fdos et al. 2009). The activation of *SHY2* by cytokinin, besides interfering with auxin activity, also functions in a negative feedback loop to reduce cytokinin levels by inhibiting the expression of the cytokinin biosynthesis gene *IPT5*, both positive and negative regulatory loops being a key to maintaining the balance between cell division and cell differentiation (Dello Ioio et al. 2008; Salvi et al. 2020a).

However, the type-B ARR induction of *SHY2* expression is not sufficient to inhibit the rootward transport of auxin in seedlings in response to cytokinin. The likelihood for additional targets was suggested by the fact that a *shy2* loss-of-function mutant does not completely eliminate the cytokinin effect, being most effective when evaluated for its role following a short-term exogenous cytokinin treatment (Dello Ioio et al. 2008). Subsequent studies have identified some of these additional factors (Fig. 5a). ARR1 and ARR12 activate *GH3.17* expression in the uppermost meristem

cells. As previously described, GH3 family members play a role in amino acid conjugation of auxin (Di Mambro et al. 2017). Thus, cytokinin can reduce auxin levels in addition to reducing the rootward transport of auxin. ARR12 also, in coordination with the cell cycle protein RETINOBLASTOMA RELATED (RBR), activates the expression of ARF19, which promotes cell differentiation (Perilli et al. 2013). Both ARR1 and ARR12 binds to the promoter of SHY2 and GH3.17. SHY2 activation, auxin degradation, and ARF19 induction are necessary to stabilize the TZ by creating an auxin minimum and suppress cell division in the uppermost meristem cells.

The PLT transcription factors are an additional auxin-related signaling element that functions as part of the regulatory network with the type-B ARRs (Salvi et al. 2020b). Type-B ARRs and the PLTs antagonize the expression of each other, thereby regulating the size of the meristem and the position of the transition zone. ARR12 represses the expression of both *PLT1* and *PLT2*, with *PLT2* demonstrated to be a cytokinin primary response gene, the high level of type-B ARR activity at the transition zone effectively restricting *PLT* expression to the meristematic zone. Conversely, it is also the dilution of PLT proteins through cell division as one moves shootward from the QC that allows for the higher level of type-B ARR expression at the transition zone.

Cytokinin also appears to regulate meristematic auxin levels in part through effects at the lateral root cap (Fig. 5b) (Di Mambro et al. 2019). This possibility was suggested because expression of a *CKX1* gene in the lateral root cap, so as to locally reduce cytokinin levels, resulted in an enlarged meristem. Conversely, expression of an activated type-B *ARR1* construct in the lateral root cap resulted in a reduced meristem. Further analysis indicated that this effect could be due to an ability for *ARR1* to induce the expression of *GH3.17* as well as *PIN5*. GH3.17 serves to conjugate and inactivate the auxin and the PIN5 efflux carrier transports auxin from the cytosol to the ER lumen. Together, these would reduce the level of available auxin in this region, and loss-of-function mutants of *GH3.17* and *PIN5* both exhibit larger meristems.

5.2 *Auxin-Dependent Mechanisms by Which Ethylene Regulates Cell Proliferation in the Primary Root*

The role of ethylene as an inhibitor of cell expansion has been extensively documented in Arabidopsis, but ethylene can also inhibit cell proliferation. Ethylene inhibits cell proliferation, along with cell expansion, in Arabidopsis leaves based on treatment with the ethylene precursor ACC and the analysis of ethylene pathway mutants (Rai et al. 2015; Skirycz et al. 2011). Recent studies demonstrate that ethylene also inhibits cell proliferation in the primary root meristem. Treatment of seedlings with ethylene or its biosynthetic precursor ACC results in a reduced RAM size based on the analysis of cortical cell number in the RAM (Street et al. 2015; Zdarska et al. 2019). In addition, RAM cortical cells in ethylene-treated roots

switch over from the mitotic cycle to endoreduplication at an earlier point than does the wild type, based on the analysis of nuclear size (Street et al. 2015).

Ethylene inhibits cell proliferation in the RAM through the canonical EIN2/CTR1/EIN3 signaling pathway and, to a lesser extent, through the AHP/type-B ARR phosphorelay (Zdarska et al. 2013, 2019; Street et al. 2015). The canonical pathway is implicated because ethylene-insensitive mutants, such as *etr1-1*, *ein2-50*, and *ein3/eil1*, are insensitive to ethylene for the RAM response (Street et al. 2015; Zdarska et al. 2019). Furthermore, constitutive ethylene-response mutants, such as *ctr1-2* or higher-order receptor mutants like *etr2/ein4/ers2* or *etr1-9/ers1-3*, have a reduced RAM size in the absence of ethylene (Street et al. 2015). The multistep phosphorelay is implicated because transgenic plant lines carrying a histidine-kinase-deficient version of ETR1 are less effective than those carrying wild-type ETR1 for the RAM response (Street et al. 2015; Zdarska et al. 2019). Analysis of type-B *ARR* mutants implicated *ARR1*, but not *ARR10* or *ARR12* in mediating the RAM response (Street et al. 2015). In addition, the ethylene precursor ACC is able to induce a reporter construct (*pTCSn*:*GFP*), which contains a concatemerized type-B ARR promoter binding site; this ethylene-dependent induction occurs at the transition zone, but is substantially less than the induction possible for *pTCSn*:*GFP* by treatment with cytokinin (Zdarska et al. 2019).

The earlier finding that cytokinin inhibited RAM cell proliferation through action of the auxin inhibitor SHY2 suggested this might also be the case for ethylene (Fig. 5c). This hypothesis was confirmed because the RAM of a loss-of-function *shy2* mutant is resistant to a 12-h ethylene treatment when compared to the wild type (Street et al. 2015). In contrast, a gain-of-function *shy2* mutant exhibits a substantial reduction in RAM size compared to the wild type, and ethylene treatment of this mutant has no additional effect on RAM size. In addition, ethylene treatment induces expression of *SHY2* and the constitutive ethylene-response mutant *ctr1*, which has reduced RAM size, exhibits an elevated basal level of *SHY2* (Street et al. 2015).

The role for ethylene in the inhibition of RAM cell proliferation facilitates the ability of cytokinin to inhibit RAM cell proliferation (Fig. 5c). As previously discussed, one effect of cytokinin in many tissues is the induction of ethylene biosynthesis. ACO2, an ACC oxidase involved in ethylene biosynthesis, increases in response to cytokinin based on proteomic studies in the root, and the *aco2* mutant RAM is less responsive to cytokinin than the wild type (Zdarska et al. 2013). Additionally, ethylene-insensitive mutants such as *etr1-1* and *ein2-1* exhibit reduced sensitivity to cytokinin in the RAM inhibition assay (Street et al. 2015; Zdarska et al. 2019). Treatment of plants with MCP, an inhibitor of ethylene binding to its receptors, also reduces RAM sensitivity to cytokinin (Street et al. 2015). These results are consistent with ethylene biosynthesis and signal transduction playing a role in the RAM cytokinin response. Based on the analysis of the ethylene-insensitive mutants, ethylene facilitates the cytokinin response but is not absolutely required, because the mutants responded like the wild type at higher cytokinin concentrations (Street et al. 2015). Cross-talk between ethylene and the cytokinin signaling pathway is likely to occur due to the shared use of the AHP/type-B ARR phosphorelay in signaling and the convergence on *SHY2* as a means to regulate auxin activity.

6 Regulation of the Cell Cycle by Cytokinin and Ethylene

In the previous section, we discussed key auxin-dependent mechanisms by which cytokinin and ethylene inhibit cell proliferation. A key aspect to such regulation was the inhibition of rootward auxin transport, this occurring as a result of auxin degradation coupled to the inhibition of polar auxin transport itself. The reduction in auxin levels within the meristematic zone and transition zone are predicted to result in reduced cell proliferation based on the roles that auxin can play in stimulating the cell cycle and inhibiting entry into the endocycle, associated with cell division and cell differentiation, respectively. More specifically, auxin is implicated in inducing the expression of genes such as cyclin dependent kinase A (*CDKA;1*) the facilitates the G_1/S and G_2/M transitions and the cyclin CYCD3;1 that is rate-limiting for the G_1/S transition, as well as inhibiting expression of *KRP1* and *KRP2* that negatively regulate the cell cycle (Perrot-Rechenmann 2010; Takatsuka and Umeda 2014), thus facilitating cell division. Auxin can also inhibit transition from the mitotic cycle to the endocycle associated with cell differentiation, one target being the cyclin *CYCA2;3*, expression of which inhibits entry into the endo cycle (Perrot-Rechenmann 2010; Takatsuka and Umeda 2014; Ishida et al. 2010). Here, we consider how cytokinin and ethylene may regulate cell proliferation in the RAM through more direct control of the cell cycle, with such mechanisms not necessarily being mediated through auxin signal transduction. Substantially more is known about the regulation of the cell cycle by cytokinin, but there is also evidence beginning to accumulate that ethylene can inhibit the cell cycle and so this possibility is also addressed.

6.1 Regulation of the Cell Cycle by Cytokinin

The inhibitory effects of cytokinin on cell proliferation in the RAM can potentially arise due to effects on the rate of cell division in the meristem and/or the transition from a mitotic cell cycle to an endocycle at the transition zone. An influential model proposed to explain the inhibitory effects of cytokinin on the RAM was that cytokinin solely regulated the transition to elongation/differentiation, suggesting an effect on the commitment to an endocycle rather than on the rate of the meristematic cell cycle (Dello Ioio et al. 2007). However, prior analysis using a kinematic approach to quantify the rate and extent of meristematic cell division in the root, found that cytokinin reduced the average cell division rate by over 30% (Beemster and Baskin 2000). More recently, an analysis of cell cycle duration in the root found that exogenous cytokinin prolonged the mitotic cycle in the RAM, thereby decreasing the rate of cell proliferation, and that this effect could substantially contribute to the effect of cytokinin on RAM size (Ivanov and Filin 2018). Consistent with a role for cytokinin in regulation of the RAM cell cycle, an *ipt3 ipt5 ipt7* cytokinin biosynthesis mutant, predicted to lower the endogenous level of root cytokinins, exhibited an increase in the rate of cell proliferation (Ivanov and Filin 2018).

Taking into account what we now know about the critical role of rootward auxin transport on cell proliferation, the initial data used to support a role for cytokinin in the transition to elongation/differentiation can readily be reconciled with a model in which cytokinin regulates the cell cycle at both the transition zone (endocycle) and the meristematic zone (rate of cell division) (Dello Ioio et al. 2007). The original model was largely based on the use of tissue-specific promoters to drive the expression of a *CKX* gene to degrade and reduce cytokinin levels, and then evaluating how the reduced level of cytokinin affected meristem size (Dello Ioio et al. 2007). The authors reported that the expression of *CKX* in the transition zone results in a larger meristem, but that expression of *CKX* in the meristem itself has no effect on meristem size. However, the meristem-specific promoter (*pRCH1*) used by the authors does not express throughout the meristem, notably lacking expression in the provascular tissue above the QC that serves to mediate the rootward transport of auxin toward the QC (Dello Ioio et al. 2007). On the other hand, a vascular tissue promoter (*pQ0990*) exhibits heightened expression in the provascular tissue above the QC, with minimal expression in the transition zone, and results in an enlarged meristem when used to drive *CKX* expression. Thus promoters used to drive *CKX* expression to reduce cytokinin levels exhibit effects on meristem size in both the transition zone and the provascular tissue of the meristem zone, supporting roles for cytokinin in regulation of the endocycle and the mitotic cycle to regulate meristem size. Below we discuss some of the modes by which cytokinin may regulate the cell cycle, not all of which are auxin-dependent.

Many effects of cytokinin are mediated through its ability to regulate progression through the cell cycle (Schaller et al. 2014). Interestingly, cytokinin can promote mitosis and cell division, as it does at the shoot apical meristem, or inhibit these same processes and promote entry into the endocycle as it does in the root apical meristem. Entry into the endocycle bypasses the G2 and M phases of the cycle resulting in endoreduplication such that the nuclear content is increased by DNA replication in the absence of mitosis or cytokinesis. Cytokinin can regulate both the G_1/S and G_2/M transitions of the cell cycle. Effects of cytokinin on the G_1/S transition are mediated through D-type cyclins (CYCDs), *CYCD3* being induced by cytokinin in Arabidopsis in a type-B *ARR* dependent manner (Argyros et al. 2008; Riou-Khamlichi et al. 1999; Scofield et al. 2013). CYCD3s are not required for cell cycle progression but their activity favors the mitotic cell cycle over the endocycle (Dewitte et al. 2007). Cytokinins also play a major role in regulating the G_2/M transition of the cell cycle (Lipavska et al. 2011; Francis 2011). Much of this evidence comes from the study of cell division in cell culture. However, the cytokinin receptor triple mutant *ahk2 ahk3 ahk4* has smaller shoot and root apical meristems; fluorescence-activated cell sorting of root cells suggests that defects in cell division correlate with a delay in the G_2/M transition (Higuchi et al. 2004). The role of cytokinin in controlling the G_2/M transition is the critical point for the commitment to mitosis or endoreduplication.

Cytokinin may inhibit cell division and promote endoreduplication in the root through auxin-independent mechanisms affecting both the G_1/S and G_2/M transitions of the cell cycle. The type-B ARR (ARR1) induces expression of

CYCLIN-DEPENDENT KINASE INHIBITOR2 (*KRP2*) at the root tip (Salvi et al. 2020b) (Fig. 5a). KRP2 inhibits the kinase activity of CYCD2-1/CDKA-1 complex that mediates the G_1/S transition, thus slowing down the rate of cell division. Significantly, members of the KRP family are likely to be direct targets of the type-B ARRs based on analysis of ChIP-Seq data which indicates a strong peak of binding near the transcriptional start site of *KRP1*, and weaker peaks near the transcriptional start sites of *KRP2* and *KRP6* ((Zubo et al. 2017) Another type-B ARR (ARR2) induces the expression of *CELL CYCLE SWITCH 52A1* (*CCS52A1*) (Takahashi et al. 2013). *CCS52A1* encodes an activator of the E3 ubiquitin ligase, anaphase-promoting complex/cyclosome (APC/C) that facilitates endoreduplication by the degradation of mitotic cyclins (Larson-Rabin et al. 2009).

The balance between root cell proliferation and differentiation is also regulated by the HD-ZIP III transcription factors PHABULOSA (PHB) and PHAVOLUTA (PHV) (Dello Ioio et al. 2012). The gain- and loss-of-function mutants have smaller and larger RAMs, respectively. Application of cytokinin phenotypically rescues the loss-of-function double mutant *phb phv* (Dello Ioio et al. 2012). The effects of *PHB* and *PHV* operate at the level of cytokinin biosynthesis as they are able to induce expression of *IPT7*, which will increase the level of cytokinins. However, the type-B ARR1 represses expression of *PHB* as well as its repressor microRNA165, potentially creating an incoherent regulatory loop that may help maintain the balance between cell division and differentiation.

There are additional data that suggest an indirect relationship between cytokinin and the ubiquitin receptor DA1-RELATED PROTEIN1 (DAR1), DAR2, TCP14, and TCP15 in the root. DAR1 and DAR2 positively and TCP14 and TCP15 negatively influence endoreduplication, DAR1 and DAR2 physically interacting with TCP14 and TCP15 for their inactivation in in the leaf (Peng et al. 2015). *DAR2*, *TCP14*, and *TCP15* are expressed in the root transition zone (Peng et al. 2013b; Resentini et al. 2015), the same region where several key type-B *ARR*s are maximally expressed. The mutant and overexpression lines are defective in root growth, specifically in the meristem and transition zone. DAR2 functions downstream of SHY2 and upstream of PLT2. These data suggest that SHY2, which suppresses the expression of *PIN2* may also suppress DAR2, creating an incoherent loop for proliferation and differentiation (Peng et al. 2013a, b). These pathways also act on *CCA52A1*, which facilitates endoreduplication (Larson-Rabin et al. 2009). Given that *SHY2* and *CCA52A1* are points by which cytokinin regulates cell proliferation, it will be interesting to gain a better understanding of how cytokinin interacts with the *DAR* and *TCP* families of genes, and whether auxin-dependent and/or auxin-independent mechanisms are involved.

6.2 Regulation of the Cell Cycle by Ethylene

Less in known about ethylene and its effect on the cell cycle in the primary root, but several lines of evidence indicate that ethylene may also regulate RAM size

through direct effects on the cell cycle. In shoots, ethylene was found to inhibit the cell cycle through a post-transcriptional reduction in CDKA activity, CDKA serving to promote both the G_1/S and G_2/M transitions (Skirycz et al. 2011). In the root, as previously described, ethylene induces an earlier developmental switch from the mitotic to the endoreduplication cycle (Street et al. 2015). In addition, analysis of the RAM using a cyclin-GUS reporter, indicates an ethylene-dependent reduction in the mitotic index within the zone of cell proliferation. Of particular interest, similarly to what has been found with cytokinin, ethylene induces the expression of the cyclin-dependent kinase inhibitor *KRP1*/*ICK1* (Street et al. 2015), a regulator of cell cycle progression in roots and shoots (Cheng et al. 2013b; Beemster et al. 2005). Furthermore, ethylene-insensitive mutants exhibit reduced levels of *KRP1* expression, whereas the constitutive ethylene-response mutant *ctr1* exhibited substantially heightened levels of *KRP1* expression (Street et al. 2015).

7 Auxin-Dependent Mechanisms by Which Cytokinin and Ethylene Regulate Cell Expansion in the Primary Root

Changes in root growth arise due to changes in cell proliferation and cell expansion, cell proliferation varying in the meristematic zone and cell expansion varying in the elongation zone. Both ethylene and cytokinin are each capable of regulating cell expansion, doing so at least partially independently but also achieving an additive response due to an ability to cooperatively influence signaling by each other. In particular, the ability of cytokinin to induce ethylene biosynthesis plays a substantial role in cytokinin's ability to inhibit cell elongation, although cytokinin can also inhibit cell expansion through an ethylene-independent mechanism. As with cell proliferation, cell expansion in the primary root is regulating by auxin movement, but in contrast to what is observed for cell proliferation, inhibition of cell expansion is primarily driven by shootward auxin movement rather than rootward auxin movement.

7.1 Auxin-Dependent Mechanisms by Which Ethylene Regulates Cell Expansion in the Primary Root

Ethylene strongly inhibits root growth due to its ability to inhibit cell expansion in the elongation zone (Le et al. 2001; Růžička et al. 2007; Swarup et al. 2007). This inhibitory effect of ethylene on cell elongation is blocked in ethylene-insensitive mutant lines such as *ein2-1* and *etr1-3*, whereas the constitutive ethylene-response mutant *ctr1-1* exhibits short cell lengths similar to those observed in the presence of ethylene (Le et al. 2001; Růžička et al. 2007). A kinematic analysis of different cell types reveals that the ethylene precursor ACC inhibits cell elongation of multiple cell

types, including cortical cells, the epidermal trichoblast cells that produce root hairs, and the epidermal atrichoblast cells that lack root hairs (Swarup et al. 2007). ACC has no significant effect on cell expansion rates in the meristematic zone but sharply reduces the expansion rates when cells leave the meristematic zone and reach the elongation zone.

Two types of auxin transporters are implicated in mediating the inhibitory effect of ethylene on cell expansion—the AUX1 auxin importer and the PIN2 auxin exporter—both of which are expressed in the lateral root cap and epidermis and mediate shootward auxin transport (Fig. 4). An *aux1* mutant sharply curtails the ability of ACC to inhibit cell elongation (Swarup et al. 2007; Růžička et al. 2007). However, the *aux1* mutant defect can be rescued by expressing *AUX1* in the mutant lateral root cap and epidermal tissues using a GAL4-based transactivation system, confirming that these tissues of the root can mediate the ethylene response through AUX1-dependent auxin transport (Swarup et al. 2007). Multiple PINs provide directionality to auxin movements in the root (Fig. 4), however only a loss-of-function *pin2* mutant is insensitive to ACC for root growth; mutants for *pin1*, *pin4*, and *pin7,* which are primarily implicated in rootward auxin transport, have no effect (Růžička et al. 2007).

The effects of the *aux1* and *pin2* mutants point to a role for shootward auxin transport in mediating the effects of ethylene on cell expansion. Consistent with this hypothesis, use of auxin reporters such as *IAA2:GUS*, *DR5:GUS*, and *DR5:GFP* reveal an ethylene-dependent induction of auxin activity in the outer layers of the lateral root cap, and the epidermis of more mature meristematic cells and newly expanded cells, a region coincident with the transition zone (Swarup et al. 2007; Růžička et al. 2007). Ethylene-dependent induction of the *DR5:GUS* reporter is blocked in ethylene-insensitive mutant backgrounds (Růžička et al. 2007). Consistent with the increase in auxin activity, measurements of auxin transport indicate that ethylene stimulates shootward auxin transport and this is effect is largely abolished in an *aux1* mutant (Negi et al. 2010). These results are all consistent with a model in which the shootward auxin transport initiated by ethylene results in an increase in auxin activity in the elongation zone to inhibit cell expansion.

Two complementary approaches, both employing cell-type specific expression, have been taken to resolve the tissues that initiate and respond to the ethylene-dependent auxin signal (Vaseva et al. 2018; Swarup et al. 2007). To resolve which tissues are required to initiate the ethylene signal the EBF F-box proteins were employed (Fig. 2) (Vaseva et al. 2018); these target the EIN3/EIL transcription factors for degradation and so will generate ethylene hyposensitivity or insensitivity in cell types required for mediation of the ethylene signal. Based on expression of *EBF*s in different tissues using GAL4 driver lines as well as from specific root promoters, only the lateral root cap and epidermis are required for ethylene-induced root growth inhibition, the strongest effect being found with a *pLRC1:EBF2* construct. Furthermore, a *pLRC1:EBF2* construct partially complements the constitutive ethylene response phenotype of *ctr1-1*, consistent with the significance of the lateral root cap and epidermis for mediating ethylene signal transduction in the root. To determine which elongation zone tissues are required to respond to the ethylene-dependent auxin signal, the mutant auxin repressor protein axr3-1 was expressed in

various root tissues using GAL4 driver lines (Swarup et al. 2007). Strong ethylene resistance is obtained when axr3-1 is expressed throughout tissues of the elongation zone, but only partial ethylene resistance is obtained when axr3-1 is expressed within a subset of tissues in the elongation zone (e.g. root epidermis, epidermis and cortex, or cortex and endodermis) (Swarup et al. 2007). Taken together these results support a model in which ethylene is only required in the lateral root cap and epidermis to initiate the AUX1-dependent auxin movement, but that the effects of auxin in the inhibition of cell elongation require activity throughout the tissues of the elongation zone.

7.2 *Auxin-Dependent Mechanisms by Which Cytokinin Regulates Cell Expansion in the Primary Root*

Cytokinin, like ethylene, inhibits cell expansion in the root and the features for inhibition share many of the same features noted for ethylene in the previous section. Not surprisingly, the effects of cytokinin on root cell expansion are mediated in part through its ability to induce ethylene biosynthesis. The inhibitory effects of cytokinin on root growth are blocked by the ethylene biosynthesis inhibitor AVG (Růžička et al. 2009), although interpretation of this result is complicated due to AVG also being an inhibitor of auxin activity (Schaller and Binder 2017). More significantly, root growth of an ethylene-insensitive *ein2* mutant is almost completely insensitive to treatment with 0.1 μm of the cytokinin benzyladenine (BA) (Růžička et al. 2009). However, an *ein2* mutant is only partially insensitive to treatment with 1 μM BA, based on measurement of root length as well of cell length in the elongation zone (Street et al. 2016). These data indicate that ethylene biosynthesis mediates the majority of the root growth response at lower cytokinin levels but that ethylene-independent mechanisms come into play at higher cytokinin levels.

As with ethylene, the ability of cytokinin to inhibit cell expansion is completely dependent on AUX1, implicating the shootward transport of auxin in mediating the cytokinin response (Street et al. 2016). An *aux1* mutant blocks the inhibitory effect of cytokinin on elongation zone cell length, but has no effect on the ability of cytokinin to inhibit cell proliferation in the RAM. In contrast, the type-B ARR mutant *arr1 arr12* curtails the inhibitory effect of cytokinin on cell expansion as well as on cell proliferation in the RAM. The *aux1* mutant defect can be rescued by expressing *AUX1* in the mutant lateral root cap and epidermal tissues using the GAL4-based transactivation system, consistent with a role in shootward transport of auxin (Street et al. 2016). These data indicate that cytokinin can inhibit cell expansion independently from cell proliferation, and suggest differing roles for rootward and shootward auxin movements in mediating the effects of cytokinin.

As found with ethylene treatment, use of a *DR5:GFP* reporter for auxin activity indicates that, in response to cytokinin treatment, auxin activity increases in the outer cell layer of the lateral root cap (Street et al. 2016). Cytokinin induction of *DR5:GFP*

activity in the lateral root cap is eliminated in the *aux1* and cytokinin insensitive *arr1 arr12* mutants. The region of cytokinin-enhanced auxin activity in the lateral root cap of the wild type extends from a point parallel to the QC up to the transition zone, consistent with a model that increased in auxin activity in this region inhibits cell elongation. Interestingly, cytokinin inhibits the expression of *AUX1* when examined at both the message and protein level (Street et al. 2016); furthermore, chromatin-immunoprecipitation analyses indicates that *AUX1* is a primary target of the type-B ARRs and contains a cytokinin-dependent type-B ARR binding site in intron 8 (Zubo et al. 2017; Zubo and Schaller 2020). The identification of *AUX1* as a primary target for the type-B *ARR*s is consistent with cytokinin having an ethylene-independent effect on cell expansion. Taken together these data suggest a model in which AUX1 is necessary for the shootward transport of auxin but that, due to the reduction in AUX1 levels, a bottleneck for auxin transport is generated at the transition zone. This bottleneck results in a local build-up of auxin concentration and the resulting inhibition of cell elongation.

8 Ethylene and Cytokinin Regulate Cell Division in the QC

Our focus throughout this chapter has been on the mechanisms by which the phytohormones cytokinin and ethylene regulate the cell proliferation and expansion to control the plasticity of root growth. However, these same hormones also play a role in controlling activity of the QC, which serves as the source for all the cells in the root. The auxin maxima in the QC inhibits cell division in the QC to maintain its quiescent state (Petricka et al. 2012), but increased levels of ethylene or cytokinin can interfere with the suppressive activity of auxin and lead to additional cell divisions within the QC. This hormonal effect was first discovered for ethylene (Ortega-Martinez et al. 2007). Application of the ACC biosynthetic precursor for ethylene induces cell division in the QC. Similarly, the ethylene-overproducing mutants *eto1* and *eto2* as well as the constitutive ethylene-response mutant *ctr1* enhance cell division in the QC. The additional QC cells induced by ethylene retain QC cell identity based on the use of molecular markers; they also retain QC cell function as they induce the production of an additional cellular layer of the columella (Ortega-Martinez et al. 2007). Conversely, inhibition of ethylene responses by use of the biosynthetic inhibitor AVG or the ethylene-insensitive mutant *ein2* results in the loss of a cellular layer of the columella. As such, ethylene appears to antagonize the role of auxin so as to induce additional cell divisions in the QC.

Like ethylene, cytokinin has also been found to stimulate cell division in the QC (Zhang et al. 2013). Growth of seedlings in the presence of exogenous cytokinin stimulates QC cell divisions as does the elevation of endogenous cytokinin in *ckx* mutants. Additional QC cell divisions are also observed in the cytokinin-hypersensitive type-A *ARR* mutants (Zhang et al. 2013, 2011). Cytokinin-insensitive mutants involving the receptors (*ahk2 ahk3*) are type-B ARRs (*arr1 arr12*) block the effect of exogenous cytokinin, indicating a role of the cytokinin signaling pathway in the QC cell

divisions (Zhang et al. 2013). The effects of cytokinin on QC cell division are independent of the ability of cytokinin to induce ethylene biosynthesis as cytokinin can still induce QC cell divisions in an *ein2* ethylene-insensitive mutant. An examination of molecular markers for QC cell identity indicates that these are still present in cytokinin-treated seedlings, similarly to what is observed with ethylene, but that their expression is reduced, suggesting a partial loss of QC cell function.

Significantly, the effect of cytokinin on QC cell divisions correlates with effects of cytokinin on the auxin transport system, supporting a model in which cytokinin inhibits auxin transport into the QC cells so that the suppressive role of auxin on cell division is ameliorated (Zhang et al. 2013, 2011). Of particular significance to this model is the suppressive effect cytokinin has on *LAX2* expression, which encodes a member of the AUX/LAX family of auxin importers (Zhang et al. 2013). Cytokinin suppresses the expression of *LAX2* within 8 h, this effect on the *LAX2* message and protein being particularly noticeable in the provascular cells above the QC, which would likely decrease the ability of rootward auxin transport to reach the QC cells. Consistent with down-regulation of *LAX2* resulting in increased cell divisions of the QC, a *lax2* loss-of-function mutant also exhibits increased QC cell division. Cytokinin may also regulate auxin activity in the QC independently of its role in regulating auxin transport, as treatment of the *lax2* mutant with cytokinin further enhances the level of QC cell division. In addition, cytokinin inhibits the expression of *SCARECROW* (*SCR*) which in turn attenuates the auxin response in the QC (Dello Ioio et al. 2008; Zhang et al. 2011; Pernisova et al. 2009), but the expression of *SCR* is not affected in the *lax2* mutant (Zhang et al. 2013). Together these effects result in decreased auxin activity in the QC, as confirmed by a decrease in expression of the auxin response reporter *DR5:GFP*, and a reduction in levels of such key regulatory genes as *SCR* and *WUSCHEL RELATED HOMEOBOX5* (*WOX5*) which play auxin-dependent roles in the prevention of stem cell differentiation (Sarkar et al. 2007).

9 Conclusion

Great progress has been made in elucidating mechanisms by which cytokinin and ethylene inhibit primary root growth and development in Arabidopsis, along with the key role that polar auxin transport plays in this process. These results support a general model in which the rootward transport of auxin principally serves to regulate cell proliferation in the meristematic zone and the shootward transport of auxin principally serves to regulate cell expansion in the elongation zone (Fig. 5c). Cell proliferation and cell expansion both contribute to the overall growth of the root and are targets by which environmental factors operating through phytohormones can control the root system architecture. For example, cytokinin inhibits primary root growth and the production of lateral roots under replete nitrogen or phosphorus conditions (Gu et al. 2018; Jia and von Wiren 2020; Sakakibara et al. 2006; Peret et al. 2011, 2014; Niu et al. 2013; Wu et al. 2013). Ethylene, as a stress hormone,

inhibits root growth in the presence of mechanical impediments or compacted soil (Okamoto et al. 2008; Hussain et al. 1999; Potocka and Szymanowska-Pulka 2018; Pandey et al. 2021; Růžička et al. 2009), and also mediates effects of cytokinin, principally on cell expansion.

Several points deserve emphasis when considering the mechanisms by which cytokinin and ethylene inhibit root cell proliferation and cell expansion (Fig. 5c). First, historically, there has been a tendency to over-simplify the effects of these hormones on root growth and development, resulting in models by which cytokinin directly regulates cell proliferation but not cell expansion, ethylene directly regulates cell expansion but not cell proliferation, and that cytokinin regulates cell proliferation by effects at the transition zone but not the meristematic zone. But, as discussed in the previous sections of this chapter, accumulating evidence indicates that both cytokinin and ethylene can regulate root cell proliferation and cell expansion independently of each other, and that cytokinin likely inhibits cell proliferation through effects at the transition zone (entry into the endocycle) as well as at the meristematic zone (rate of cell cycle). Second, the regulation of cell proliferation and cell expansion are not obligately coupled one to the other. This point is based on the finding that *aux1* mutants specifically block the inhibitory effects of cytokinin and ethylene on cell expansion, but not on cell proliferation, indicating a specific role for AUX1-dependent shootward transport of auxin in the regulation of cell expansion. Third, cytokinin and ethylene signal transduction both converge on a similar set of gene regulators to control cell proliferation and cell expansion. These include *AUX1* for the regulation of cell expansion and *SHY2* for the regulation of cell proliferation, both serving to modulate polar auxin transport in the root. It also appears likely that both cytokinin and ethylene will regulate cell proliferation in part through an alternative auxin-independent mechanism by targeting *KRP* inhibitors to prolong the cell cycle. Fourth, there are many potential avenues for crosstalk between cytokinin and ethylene in regulating root cell proliferation and expansion, whether from the ability of cytokinin to induce ethylene biosynthesis or from the ability of ethylene to signal through the AHP/ARR phosphorelay.

As mentioned at the beginning of this chapter, much of the data supporting the regulatory circuits and mechanisms involved in control of primary root growth and development has come from studies in Arabidopsis. Although the basic features of this model are also likely to pertain to other plant systems, novel regulatory mechanisms will undoubtedly be uncovered based on the diversity found in the plant kingdom. Similarly, study of other plant systems is likely to also reveal fundamental aspects of regulation that could not be readily uncovered in Arabidopsis. For instance, genetic studies of cytokinin signaling in rice are starting to revise the model for cytokinin as functioning solely as an inhibitor of cell proliferation in the RAM, a strong cytokinin-receptor mutant of rice exhibiting a severe reduction in root growth and RAM size (Burr et al. 2020). These results may indicate that a basal level of cytokinin signal transduction is needed in the root to maintain the cell cycle, with higher levels of cytokinin activity then becoming inhibitory. Such a possibility is consistent with some studies in cell culture where both auxin and cytokinin were required for entry into the cell cycle (Trehin et al. 1998).

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Role of Brassinosteroids in Root Growth and Development

Suman Sharma and Madhumita Banerjee

Abstract Phytohormones are naturally occurring organic compounds in plants which act as chemical messenger at micro molar to pico molar concentration. They play significant role in regulation of growth and development processes as well as response to biotic and abiotic stress factors so that plants can better adapt to environmental changes. Root system in plant plays significant role in providing anchorage to soil and absorption of water and nutrients. The root is highly dynamic part of plant having indeterminate growth of root tissue. Phytohormones play important role at molecular level in fine tuning the root development process. The discovery of new class of phytohormones, Brassinosteroids (BRs) almost four decades ago opened new areas in the field of plant growth and development and also provided deep understanding of their potential role in regulating root development. Recent studies based on mutants have revealed that BRs are not only involved in root cell elongation but also in maintenance of meristem size, root hair formation, gravitropic response, lateral root initiation, mycorrhiza formation, and nodulation in legume species. Studies based on mutant have also led to the conclusion that different concentrations of BR are required in different regions of root for its normal growth and function. Moreover BRs interplay with other phytohormones to regulate root growth and development. The present chapter focuses on role of BRs in root development, its genetic background and crosstalk with other phytohormones in regulating this phenomenon.

S. Sharma (✉) · M. Banerjee
Department of Botany, Ramjas College, University of Delhi, Delhi 110007, India
e-mail: sumansharma@ramjas.du.ac.in

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Abbreviations

BRs	Brassinosteroids
BL	Brassinolide
BRI1	Brassinosteroid insensitive 1
BAK 1	BRI1- associated receptor kinase
BIN 2	Brassinosteroid-insensitive 2
QC	Quiescent centre
RALF	Rapid alkalization factor
VSP	Vegetative storage protein
CSC	Columella stem cell
BSK	BR-Signaling Kinase 1
CDG1	Constitutive Differential Growth 1
BSU1	BRI1 Suppressor 1

1 Introduction

Plants synthesize a diverse class of organic compounds, termed as plant hormones or plant growth regulators. These compounds are biologically active within plants and at a very low concentration influence physiological processes such as growth, development, differentiation as well as response to biotic, abiotic stress helping plants to maintain balance and also in adapting to the changing environment. The effects of few plant hormones, such as auxin, gibberellins, cytokinins, abscisic acid (ABA), and ethylene, have been described and characterized for almost last 50 years. The discovery of a new class of phytohormones, the Brassinosteroids (BRs) and Jasmonates (JA), almost 40 years ago further unravelled new avenues in the studies of plant growth and development and future perspectives in the regulation of agronomic traits through their use in agriculture.

2 Brassinosteroids

Brassinosteroids (BRs) belong to category of polyhydroxy steroids. The first Brassinosteroid was isolated from rape (*Brassica napus* L.) pollen in 1979. It was characterized as Brassinolide, (Grove et al. 1979). Since then more than 60 BRs have been isolated and identified, out of which 31 have been fully characterized, including

two conjugates (Sakurai and Fujioka 1993). BRs are ubiquitously present in plants, including dicots, monocots, gymnosperms, green alga and fern (Sakurai and Fujioka 1993). BRs have been isolated from almost all parts of a plant like seeds, fruits, shoots, leaves and flower buds, however their concentration is relatively higher in pollen in comparison to other parts of plants. All the known variants of BRs differ from each other due to different substitutions in the NB rings and side chains of the basic structure occurred during oxidation and reduction reaction in the biosynthesis pathway. Out of all known BRs the most abundant are brassinolide and its precursors.

3 Biosynthesis of Brassinolide

Biosynthesis of brassinolide begins with campesterol a derivative of sterol biosynthesis to form campestanol by reduction of 5,6 double bond in campesterol. The pathway is further followed by an early oxidation of campestanol to form cathasterone at carbon 6 or a late oxidation at carbon 6. Both pathways give rise to an intermediate compound castasterone which is ultimately converted into brassinolide.

4 Brassinosteroid Signaling Pathway

Signalling pathway of Brassinosteroid involves two membrane spanning kinase proteins, BRI1 (BRASSINOSTEROID INSENSITIVE 1) and BAK1 (BRI1—ASSOCIATED RECEPTOR KINASE 1). BRI1 is a leucine–rich repeat receptor kinase which functions with its co-receptor BAK1. In presence of Brassinosteroid both will bind together and stimulate phosphorylation of BKI1 an inhibitor of BRI1 leading to its dissociation from the plasma membrane and gets further associated with some protoplasmic proteins which are involved in interaction with two more proteins in the cascade and their retention in cytoplasm (Fig. 1). These two proteins are BZR1 and BES1. Activated BRI1 is also involved in phosphorylation of the BSKs (BR-SIGNALING KINASE 1) and CDG1 (CONSTITUTIVE DIFFERENTIAL GROWTH 1), which both subsequently activate BSU1 phosphatase (BRI1 SUPPRESSOR 1) (Fig. 1). BSU1 is responsible for dephosphorylating BIN2 (BRASSINOSTEROID-INSENSITIVE 2), an important repressor of the BR signaling pathway (Ryu et al. 2010). On dephosphorylating BIN2 become inactivated as a result of this BZR1 and BES1 are rapidly dephosphorylated by PP2A (PHOSPHATASE 2A) and subsequently gets dissociated from 14-3-3 cytoplasmic proteins, causing them to accumulate into the nucleus, resulting in the regulation of many BR-responsive genes (Wang et al. 2002). In the absence of BR, BKI1 binds to the intracellular domain of BRI1, preventing its association with its co receptor

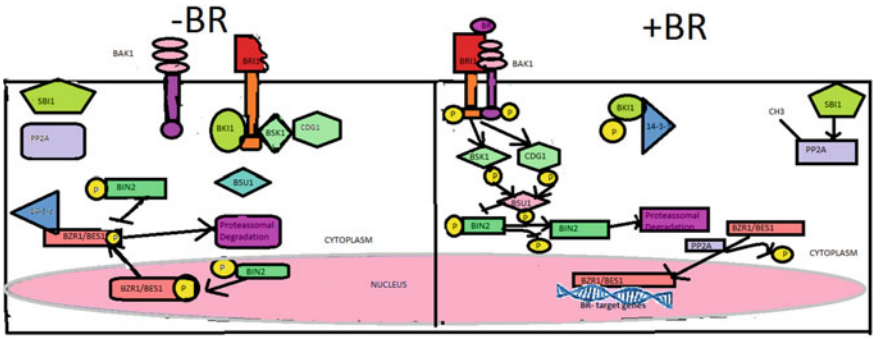

Fig. 1 Signalling pathway of Brassinosteroids in presence or absence of BRs (with permission from Ana Laura G. L. Peres et al. 2019)

BAK1 (Wang and Chory 2006). As a result of this, BIN2 is activated, and 14–3-3 proteins remain associated with BZR1 and BES1, keeping them dephosphorylated and blocking their capability of shuttling to the nucleus for the regulating BR responsive genes (Jaillais et al. 2011).

5 Physiological Roles of Brassinosteroid

Brassinosteroid regulate several growth and development processes in plants like cell division, cell elongation, responses to biotic and abiotic stress, morphogenesis, differentiation, reproduction and senesence (Clouse and Sasse 1998; Divi and Krishna 2009). In addition to their growth-promoting activities, exogenous BRs have been reported to inhibit root growth, enhance gravitropism, retard leaf abscission, enhance resistance to stress, and promote xylem differentiation (Sakurai and Fujioka 1993).

Growth and development of a plant root system needs coordinated regulation of endogenous cues as well as environmental signals (Wei and Li 2016). Previous studies demonstrated that plant root growth and development are intricately linked with phytohormones (Pacifici et al. 2015). Several types of Brassinosteroids have been reported in roots of plants like maize and *Arabidopsis* (Yokota et al. 2001; Shimada et al. 2003; Kim et al. 2005). Mutants impaired in BR biosynthesis or signal transduction, display a short-root phenotype (Li et al. 1996; Müssig et al. 2003). Physiological studies have demonstrated that application of BRs promote root growth at low concentration whereas at high concentration they become inhibitory for root growth (Roddick et al. 1993; Clouse et al. 1996; Müssig et al. 2003). In this chapter molecular physiology of BR signal transduction, homeostasis, roles of BRs and their interplays with other signalling pathways in regulating root growth and development are summarised briefly.

5.1 Maintenance of Meristem Size in Roots

Root meristem size in plants is regulated by BRs at molecular level. Many transcription factors like PLETHORA (PLT), GRAS, SHORT ROOT (SHR) and SCARECROW (SCR) are identified as important regulators for maintaining root stem cells and quiescent centre (QC) (Di Laurenzio et al. 1996; Helariutta et al. 2000; Sabatini et al. 2003; Levesque et al. 2006; Cui et al. 2007). One homeodomain transcription factor WOXS is also found to be involved in maintenance of stem cell identity. This factor is strictly expressed in QC and its expression is controlled by two Auxin Response Factors ARF 10 and 16. Cell division and cell elongation in root meristem is dependent on BRs concentration. Low concentration of BL promotes root growth, whereas high concentration is inhibitory for root growth (González-García et al. 2011). Further in a study done on BR deficient mutant dwf4, exogenous application of BL in low concentration helped in restoring cell organisation and cell length (Chaiwanon and Wang 2015). Maintenance of root meristem size by BRs depends on their site of action, since in one of the BR signalling mutant bri1-116 meristem size can be restored by targeted expression of BRI1 expression in epidermis but restoration of meristem size failed if BRI1 expressed in QC or stele, morever even exogenous application of BL also failed to restore meristem size. Expression of bzr1-1D an active, hypo phosphorylated form of BZR1 in epidermis of bri1-116 enhanced growth of root meristem whereas its expression in endodermis or QC has no effect on meristem size of bri1-16 (Wang et al. 2002; Chaiwanon and Wang 2015). This emphasizes that epidermal BR signalling is sufficient enough to maintain root meristem. More studies done on this also indicates that BR signalling in different cell type has differential effect on root meristem size for example BRI1 activity in epidermis enhances cell proliferation, whereas in stele it promotes cell differentiation. Tissue specific protein profiling have clearly shown that BR inducible genes are mainly expressed in epidermal basal meristem, whereas BR repressible genes are predominantly present in apical meristem of stele (Vragovic et al. 2015) (Fig. 2). Studies have shown that Brassinosteroids plays significant role in maintaining meristem size by promoting cells to progress through cell cycle. In a research done on one of the BR insensitive mutant bri1-116, for analysing the expression pattern of cell division markersCYCB1, ICK2/KRP2 and KNOLLE, have shown that meristem size was highly reduced in this mutant due to decreased mitotic activity and this can be overcome by overexpression of a gene CYCD3. Further it was also observed that the activity of Quiescent centre (QC) was also low in this mutant. Plants when treated with brassinolide-an active BR or in *besI-D* (an enhanced BR signalling mutant) premature exit from cell cycle is observed thus causing early differentiation of meristems. This also reduces the meristem size and growth of root. BRs also play critical role in differentiation and maintenance of distal Columella stem cells (CSCs) which is dependent on its concentration and BZR1/BES1 availability (Fig. 2). Low concentration of BRs inhibits while higher concentration promotes stem cell differentiation. Overall it may be concluded that BRs affect root meristem size by three different mechanisms. First by maintain normal frequency of cells and their expansion in root

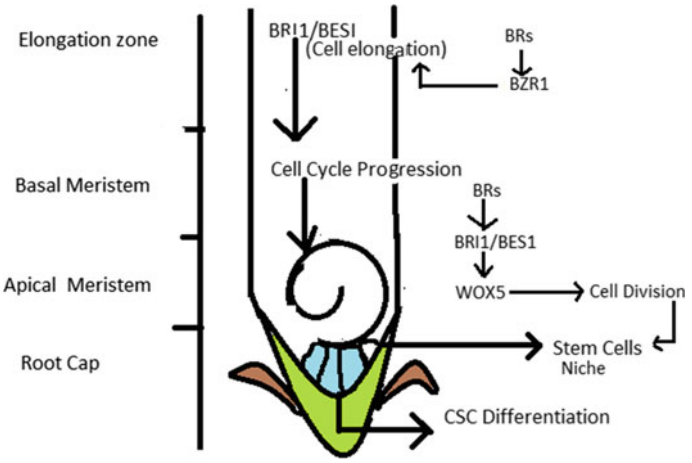

Fig. 2 Crosstalk of Brassinosteroids with other signalling molecules for lateral root development. Arrows and bar end denote activation and inhibitory effects respectively (Wei and Li 2016)

meristem. Second by maintain QC identity. Third, by both enhancing and retarding differentiation of columella stem cells by BES-1 and BZR1, respectively. In addition, the promoting and inhibiting effects of BRs on root meristem size depend on hormonal concentration and their site of action.

5.2 Growth of Root by Cell Elongation

Root growth by cell elongation depends on physical property of cellulose which is the major component of cell wall. Conversion of crystalline cellulose to amorphous form promotes unidirectional cell expansion. BRs regulate cell elongation in a spatial manner. Expression of BRI1 in hair cell promotes root cell elongation whereas its expression in non hair cells is inhibitory. Along with BRs, auxins are also involved in root cell elongation via BZR1. Increased concentration of BZR1 in the epidermal nuclei of elongation zone activates certain genes which are involved in cell wall organization and biogenesis. Physiological and genetic assays have shown that BR and auxin show opposite effects on the expression of these genes to antagonistically control root elongation (Chaiwanon and Wang 2015). In *Arabidopsis* a peptide hormone RALF activates a cell surface receptor FERONIA which suppresses cell elongation in primary root (Pearce et al. 2001; Covey et al. 2010; Mingossi et al. 2010; Haruta et al. 2014). A RALF overexpressing mutant AtRALF1 showed reduced size of root cells as well as reduced sensitivity to BL. When BL and RALF were applied simultaneously it resulted in low expression of RALF inducible genes required for

cell wall rearrangement and BR biosynthesis (Bergonci et al. 2014). These observations suggest that RALF and BR work antagonistically in *Arabidopsis* roots and both interact together for cell expansion.

5.3 Root Hair Formation

Root hair is a tubular structure originating from epidermal cells present in differentiation zone. Presence of root hairs increases surface area of roots thereby enhancing uptake of water and nutrients from soil. Differentiation of a cell into root hair cell (H) or non-hair cell (N) depends purely on their position relative to the underlying cortical cells (Wei and Li 2016). Several studies done in past have shown that differentiation of root hair is genetically controlled. A complex of TF like WEREWOLF (WER), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA GLABRA 1 (TTG1) in N cell upregulate expression of two genes GLABRA2 (GL2) and CAPRICE (CPC) (Galway et al. 1994; Wada et al. 1997; Lee and Schiefelbein 1999; Bernhardt et al. 2003; Zhang et al. 2003; Ryu et al. 2005; Shen et al. 2006; Song et al. 2011). Out of this transcription factor complex, GL3 and EGL3 are bHLH type of TF which are formed initially in H cells and then they move to adjacent N cell nuclei. In N cells activation of GL2 and its expression carried out by WER-GL3/EGL-3-TTG1 decide fate of these cells (Shen et al. 2006; Schiefelbein et al. 2009). On the other hand CPC travel from N cells to H cells and there it competes with WER to form a complex with GL3/EGL-3- TTG1 which is unable to induce expression of GL2 (Wada et al. 1997; Ryu et al. 2005; Song et al. 2011). Recent studies have shown that BR signalling plays significant role in supressing H-cell differentiation and promotes N-cell fate in both N and H position cells (Cheng et al. 2014). Studies conducted on some mutants revealed that number of root hair is high in BR deficient mutants but lower in BR signalling enhanced plants compared to their wild counterparts (Cheng et al. 2014). In presence of BR, WER gets activated whereas activity of BIN 2 kinase is inhibited resulting in formation of WER-GL3- TTG1 and WER-EGL3-TTG1 in H and N cells respectively. Both of these complexes are activated to promote GL2 expression and N-cell fate. BR signalling in N-position cells leads to accumulation of CPC. The CPC then diffuse in surrounding H-position cells inhibiting thereby expression of WER and GL2 along with accumulation of SCM (a LLR-RLK). SCM further decreases activity of WER and inhibits GL2 expression in H-position cells.

5.4 Initiation of Lateral Roots

Lateral roots have significant physiological role to play in plants. Their presence not only increase efficiency of water uptake but also provide mechanical support to aerial parts of the plant. Lateral roots arise from pericycle founder cell, after

several rounds of division followed by cell expansion (Wei and Li 2016). Unlike primary root, lateral roots are formed throughout the life cycle of plant. Auxin play significant role in lateral root initiation and development by maintaining high auxin gradient in root tip through its acropetal movement and BRs interplay with auxin in regulating this response in plants. Higher concentration of BL supresses lateral root formation by activating several AUX/IAAs genes which act as repressor of auxin- responsive gene (Fig. 3). High concentration of BRs induce expression of IAAs which inhibit auxin signalling and hence auxin gradient in root tip (Bao et al. 2004). Besides auxin, glucose is also involved in regulating initiation of lateral roots. Low concentration of glucose promotes emergence of lateral roots by increasing auxin transport while higher concentration of glucose is inhibitory (Mishra et al. 2009; Gupta et al. 2015). Genetic studies conducted have shown that BRs interplay with Glucose in mediating this response by increasing auxin transport (Gupta et al. 2015). Studies have also revealed that cytokinin also inhibit initiation and growth of lateral roots by altering cell division and auxin gradient in founder cell of pericycle (Fig. 3). Rapid alkalyzation factor (RALF) is involved in lateral root initiation and elongation since silencing the AtRALF1 gene in Arabidopsis increased the lateral root number, while AtRALF1 overexpression showed the opposite effect. Based on results obtained from various studies conclusion can be drawn that BRs significantly

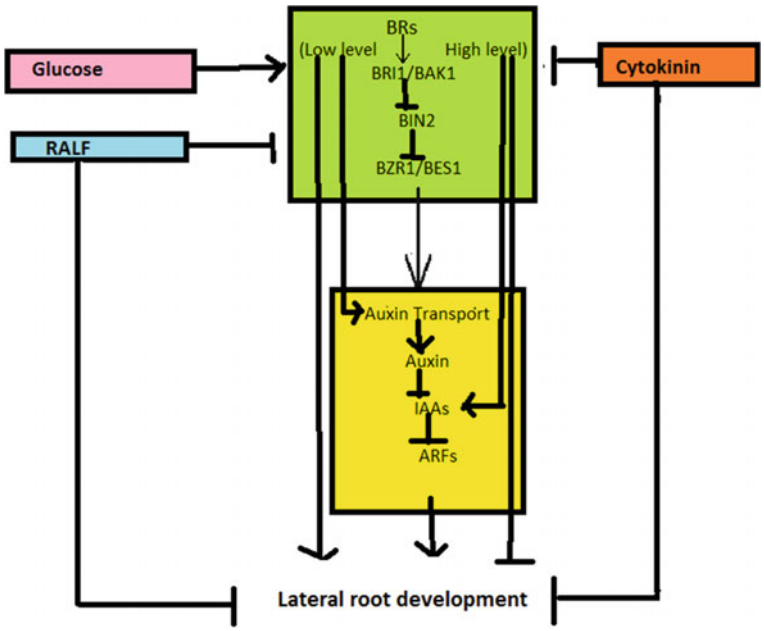

Fig. 3 Model depicting role of Brassinosteroids in maintaining meristem size and root elongation. BR promotes progression of cell through cell cycle (at root apex) and maintains balance between stem cell renewal, differentiation (middle portion) and their elongation (bottom portion)

regulate initiation of lateral root primordia without affecting the later development stage of lateral roots.

5.5 Gravitotropic Responses Shown by Roots

Root development process is regulated by several environmental factors like drought, gravity, temperature and nutrients. Gravitotropic stimulus is perceived by root cap cells. In some plant species like maize and *Arabidopsis,* exogenous BL application increases the gravitotropic curvature (Kim et al. 2000, 2007; Li et al. 2005). BR signalling mutant in *Arabidopsis* show reduced gravitotropic curvature whereas transgenic raised for overexpressing BRI1 showed increase in gravitotropic curvature compared to wild type of plants (Kim et al. 2007). Effect of BL on gravitotropic responses can be explained in three ways (1) low level of auxin IAA promotes whereas increase level of IAA inhibits effect of BL on gravitotropic responses in roots (Kim et al. 2000, 2007; Li et al. 2005) (2) Exogenus application of BL increase gravitotropic responses by activating ROP2 GTPase which alter Auxin distribution in roots by polar accumulation of PIN 2 root meristem (Li et al. 2005). (3) BR modulates root gravitotropic responses by inducing actin cytoskeleton configuration (Lanza et al. 2012). Presence of glucose works in additive manner along with BRs in deviating root growth direction (Singh et al. 2014).

5.6 Nodulation and Mycorrhiza Formation

Roots of leguminous plants show symbiotic association with nitrogen fixing bacteria present in soil. These bacteria invades roots of legumes and result in nodule formation in cortex. Bacteria gets shelter and food through this symbiotic association and in return help the plant in fixing atmospheric nitrogen into ammonia (Mylona et al. 1995). Development of nodules is an energy dependent process and number of nodules per plant is regulated by AON (autoregulation of nodulation) pathway. Four enzymes, LK, LKB, 5alpha reductase and C-24 reductase identified in BR biosynthetic pathways in Pea out of which mutation in two genes LK and LKB, resulted in low nodulation thereby indicating role of BRs in regulating nodulation, however in soyabean BRs inhibit nodulation. Further studies are required to clearly understand role of BR in nodulation.

Arbascular mycorrhiza is a symbiotic association between fungi and land plants (Strack et al. 2003) in which plants are benefited from their fungal partner by getting better uptake of soil nutrients like phosphorous especially in nutrient deficient environment at the same time fungus receives nutrients like carbohydrates from plants. Role of BRs in mycorrhizal association is studied in a BR biosynthetic mutant D^X in tomato. This mutant has suboptimal amount of sugars which are required for mycorrhizal colonization and infection (Bitterlich et al. 2014).

6 Crosstalk of BRs with Other Phytohormones Operating During Root Development

BRs interact with other phytohormone synergistically as well as antagonistically to regulate root growth and development (Kim et al. 2000, 2007; Bao et al. 2004; Li et al. 2005; Chaiwanon and Wang 2015). Expression of gene DWF4 which is involved in lateral root elongation is induced by Auxin whereas BRs suppresses its expression. Studies have shown that auxin promotes lateral root development by activating BIN 2 but at the same time BR signalling pathway inhibits BIN 2 activity (He et al. 2002; Wang et al. 2002; Yin et al. 2002). Further expression of some AUX/IAA gene which are involved in root development are activated by exogenous application of BL. It is evident from studies that BRs control root growth by altering polar auxin transport and distribution in roots (Bao et al. 2004; Li et al. 2005) ABA mediated inhibition of lateral root development is regulated by the activity of BIN2. Biochemical and genetical analysis have demonstrated that BIN 2 interact with a kinase SnRK2 which can positively regulate ABA signalling to inhibit root elongation (Cai et al. 2014). However in some case BRs may also promote ABA signalling and activates certain transcription factors acting downstream of BIN2 (Rodrigues et al. 2009). BRs interact with ethylene for regulating root cell elongation. Expression of BRI1 in root hair cells promotes their elongation but in non hair cells elongation is inhibited by BRI1. The negative effect of BRI1 in non hair cells is actually due to overexpression of ACS gene resulting in accumulation of ethylene biosynthesis precursor there which enhances ethylene signalling and inhibits unidirectional expansion of cell (Fridman et al. 2014). BRs also interplay with JA in controlling root growth. JA inhibits root growth in *Arabidopsis* (Browse 2005) and this effect of JA can be reversed by exogenous application of BL.

7 Conclusions

Roots are important part of plants as they help in nutrient uptake, provide anchorage and mechanical support to plants. They are also involved in storage and synthesis of some essential compounds required for growth and development. In order to facilitate all these functions, root system must be studied in depth and should be optimized for better utilization of available nutrient resources. Studies done in recent past have enhanced our understanding in role of BRs on root growth and development. At low concentration BRs regulate root meristem size and lateral root development but at higher concentration the effects are inhibitory. BRs generally function in a cell specific manner for controlling root meristem size (González-García et al. 2011; Hacham et al. 2011; Chaiwanon and Wang 2015; Gupta et al. 2015; Lee et al. 2015). They also regulate root cell growth in root hair as well as non hair cells (Cheng et al. 2014; Fridman et al. 2014). Further BRs also regulate symbiotic association of bacteria and fungus in root through nodulation and mychorrhiza (Terakado et al.

2005; Bitterlich et al. 2014; Foo et al. 2014). BRs interplay with other phytohormones for root growth and development is a synergistic as well as antagonistically manner.

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Precise Role of Strigolactones and Its Crosstalk Mechanisms in Root Development

Kaiser Iqbal Wani, Sadaf Chaudhary, Andleeb Zehra, M. Naeem, and Tariq Aftab

Abstract Strigolactones (SLs) constitute a group of carotenoid-derived phytohormones with butenolide moieties. These hormones are involved in various functions, including regulation of secondary growth, shoot branching and hypocotyl elongation, and stimulation of seed germination. Root and shoot extracts of various plant species, including *Arabidopsis*, have indicated the presence of various SLs. A single plant species can produce various types, combinations and levels of SL molecules. To regulate shoot branching, root-derived SLs are transported to shoots through the xylem. In addition, SLs initiate AM fungal hyphal branching even before host root infection. The inhibition of plant shoot branching is associated with both endogenous SL production and exogenous SL application in SL hyper branching mutants. Accumulating evidence indicates that SLs participate in root growth in many plant species. SLs promote the elongation of seminal/primary roots and root hair (RH) formation, and they repress lateral root (LR) formation. SLs are generally considered as positive regulators of AR formation, however it appears that the positive or negative regulation of AR formation by SLs is dependent on experimental conditions and the type of plant species. The Levels of SLs, which are produced mainly by plant roots, increase under low nitrogen and phosphate levels to regulate plant responses. Various functions of SLs are regulated by their interaction with other hormones; due to this hormonal interplay, plants can suitably respond to changing environmental factors, such as nutrient availability, shading and temperature. In this article, we review our current mechanistic understanding of strigolactones and its crosstalk mechanisms in root development. We also highlight recent advances regarding the interaction of SLs with other hormones during developmental processes and stress conditions.

Keywords Carotenoid-derived phytohormone · Phytohormone crosstalk · Strigolactone biosynthesis · Strigolactone receptors · Strigolactone signalling

K. I. Wani · S. Chaudhary · A. Zehra · M. Naeem · T. Aftab (✉)
Department of Botany, Aligarh Muslim University, Aligarh 202002, India

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1 Introduction

Strigolactones (SLs) are a class of signaling molecules found in plants which play important roles in several aspects of plant growth and development. These signaling molecules were called SLs due to their role in stimulating germination of Striga seeds and due to their lactone ring containing chemical structure (Butler 1995; Wani et al. 2020). These signaling molecules were identified as plant hormones by two research groups, due to their role in repression of lateral bud outgrowth, thus regulating shoot development (Gomez-Roldan et al. 2008; Umehara et al. 2008). The plants which are deficient in SLs had a hyper-branching phenotype, which could not be correlated with any other known hormone (Koltai 2014) and the exogenous application of SLs (GR24) resulted in the inhibition of this hyper-branching phenotype (Umehara et al. 2008).

Initially, SLs were identified as potent seed germination inducers in some parasitic plant species (Cook et al. 1966). For a long time these SLs were thought to be associated with these parasitic weeds only. However later on they were found as stimulants of arbuscular mycorrhizal (AM) fungal branching (Akiyama et al. 2005), and after that they were recognized as phytohormones as discussed above. They were found to be involved in a number of plant developmental processes and regulation of plant architecture. They are involved in regulating secondary growth in plants via cambium stimulation (Agusti et al. 2011). Furthermore, they are also involved in inhibition of hypocotyl elongation which is dependent on phytochrome and cryptochrome signaling, and light (Jia et al. 2014). Due to their role in enhancing germination of parasitic plant species, they were initially considered to be harmful, but later on they were considered beneficial due to their role in inducing AM fungal colonization (Akiyama et al. 2005; Besserer et al. 2006). They are involved in mediating defence responses against pathogens (Torres-Vera et al. 2014) and regulation of plant symbiotic relations like nodulation in legumes due to their interaction with rhizobia (Foo et al. 2014).

Strigolactones have been found to be involved in regulating plant adaptation to various abiotic stresses, such as drought and salinity stress (Van Ha et al. 2014; Ma et al. 2017; Lu et al. 2019), with SL-deficient plants showing hypersensitive response under such conditions. The application of SL analogue GR24 on such mutant plants is able to rescue this sensitive phenotype (Van Ha et al. 2014). These phytohormones also interact with many other hormones to enable plants to adapt to changing environmental conditions, such as temperature and nutrient availability (Cheng et al. 2013; Wani et al. 2020). They are involved in regulating root development and may act either synergistically or antagonistically by their crosstalk with other hormones. They promote root hair (RH) elongation (Kapulnik et al. 2011b), repress Lateral Root (LR) formation (Kapulnik et al. 2011a), and adventitious root (AR) (Rasmussen et al. 2012a) formation from the stem and promote primary root (PR) and seminal root (SR) growth (Ruyter-Spira et al. 2011).

2 Structure, Diversity and Biosynthesis of Strigolactones

Natural SLs have a conserved tricyclic lactone structure in which three rings called ABC rings are connected with a fourth ring (butenolide group) called D ring via an enol ether bridge. The A ring contains one or two methyl groups, whereas A/B ring moiety has acetyloxyl or hydroxyl groups attached to it (Xie et al. 2010; Wani et al. 2020). Natural SLs have a common C-D ring moiety. Strigol is the first naturally occurring SL to be identified, produced by *Gossypium hirsutum* as a germination stimulant of Striga seeds (Cook et al. 1966). Interestingly, *Gossypium hirsutum* is not a true host plant of Striga and strigol was isolated from the roots of true Striga host plants, namely proso millet and maize. Since then, a large number of different SLs have been identified in many different host and non-host species of plants, acting as germination stimulants of different parasitic root parasitic plants (Siame et al. 1993). Sorgolactone is another naturally occurring SL which was first isolated from the roots of *Sorghum bicolor*, a genuine host of *Striga hermonthica* and *Striga asiatica* (Hauck et al. 1992). After sorgolactone, orobanchol was another SL to be isolated from the roots of *Trifolium pretense* (red clover), which is a host of parasitic plant *Orobanche minor* (Yokota et al. 1998). The root and shoot extracts of numerous plants have shown the presence of various types, levels, and combinations of SLs, with root derived SLs transported through xylem in order to regulate shoot braching (Kapulnik and Koltai 2014; Wani et al. 2020).

Naturally occurring SLs can be classified into two main groups, namely canonical and non-canonical SLs, which are structurally different from each other. Canonical SLs, such as strigol and orobanchol possess the ABCD ring formation, whereas non-canonical SLs (both natural and synthetic) such as avenaol, methyl carlactonoate (MeCLA), and Yoshimulactone Green lack A, B, and/or C ring; however they contain D ring (Butler 1995; Alder et al. 2012; Wani et al. 2020). Depending on the presence of α or β oriented C-ring, the canonical SLs can be of orobanchol or strigol type, respectively (Xie et al. 2013). The structural diversity of canonical SLs arises due to various modifications in the AB ring system, involving many SL biosynthetic genes (Bhattacharya et al. 2009; Wani et al. 2020). Although. Non-canonical SLs have mostly been characterized in the last decade only, most of the recently identified SLs have been reported to be non-canonical. Based on this, it is expected that non-canonical SLs will surpass canonical SLs as the former allow more diversity and require the presence of enol-ether-D ring moiety, proposed to be an indispensable requirement for SL activity (Zwanenburg et al. 2009; Yoneyama 2020). Based on certain reports, it has also been suggested that only D ring is essential for SL activity, as synthetic SL agonists containing D ring but lacking enol-ether bridge have been developed (Fukui et al. 2017). Besides their role in plants, certain synthetic SL analogues have shown anticancer properties in hepatocellular carcinoma (Hasan et al. 2018), which shows their potential as chemotherapy agents in future.

5-deoxystrigol (5-DS), isolated from *Lotus japonicas* in 2005 is a simplest SL without acetyloxyl, hydroxyl, or other oxygen atoms containing substituents on A and B rings (Akiyama et al. 2005; Xie et al. 2010). It has been reported in the

root exudates of a large number of species throughout the plant kingdom, and is thought to be a common precursor of all these SLs (Zwanenburg et al. 2009). Strigol or orobanchol are produced by allylic hydroxylation of 5-DS (Rani et al. 2008), whereas sorgomal, the third monohydroxy-SL (Xie et al. 2008), is produced by hydroxylation on homoallylic position. Sorgolactone is produced by the oxidation of the hydroxymethyl group of sorgomal, followed by decarboxylation. In plants orobanchol is the most widely distributed hydroxyl-SL among three hydroxyl SLs namely strigol, orobanchol and sorgomal.

Strigolactone biosynthetic pathway starts in chloroplasts with all-trans-β-carotene, which is acted upon by β-carotene isomerase (DWARF27/D27) to form a 40 carbon compound 9-cis-β-carotene (Alder et al. 2012). The involvement of carotenoids in SL biosynthesis was established by the fact that the plants treated with carotenoid biosynthesis inhibitors, such as fluridone had reduced SL levels. This was further confirmed by the lack of SLs in carotenoid deficient mutants (Matusova et al. 2005). After D27, two carotenoid cleavage dioxygenases (CCDs) work sequentially and produce carlactone. First 9-cis-β-carotene is acted upon by CCD7 (encoded by *MAX3*) to produce 9-cis-β-apo-10-carotenal and β ionone, having 27 and 13 carbon skeleton respectively (Waters et al. 2012). After this carlactone is produced by the action of CCD8 (encoded by *MAX4*) via intra molecular rearrangement of 9-cis-β-apo-10- carotenal, and it acts as a mobile intermediate, which is transported into cytoplasm for further processing (Alder et al. 2012; Al-Babili and Bouwmeester 2015).

Carlactone has a SL like carbon skeleton, contains A and D rings along with the enol ether bridge and has properties similar to that of SLs (Alder et al. 2012; Wani et al. 2020). Carlactone, with two incomplete middle rings, has SL like properties and acts as a putative intermediate during the biosynthesis of other SLs, but its further processing takes place in the cytoplasm (Al-Babili and Bouwmeester 2015). Carlactone is converted into carlactonoic acid by the enzyme cytochrome P450 monooxygenase (MAX1) (Zhang et al. 2014) and the role of MAX1 in this conversion has been confirmed by Booker et al. (2005) by reciprocal grafting experiments. This observation was also confirmed by the formation of carlactonoic acid from carlactone in vitro in yeast microsomes, using recombinant MAX1 proteins (Abe et al. 2014). Carlactonoic acid is further converted into 5-DS and 4-deoxyorobanchol (4-DO), which are then converted into other types of SLs like strigol, orobanchol, sorgomal, which may be modified further as well (Rani et al. 2008; Xie et al. 2010).

Carlactonoic acid can also be converted into its methyl ester called methyl carlactonoate (SL-LIKE1) by a methyl transferase enzyme (Seto and Yamaguchi 2014). Recently, it has been reported that another enzyme Lateral Branching Oxidoreductase (LBO) acts on methyl carlactonoate and converts it into hydroxymethyl carlactonoate (Yoneyama et al. 2020).

3 Spatial Expression Analysis of SL Biosynthesis Genes in Roots

Strigolactone biosynthesis takes place predominantly in the roots, from where they are transported to shoots or secreted into the rhizosphere (Xie et al. 2010; Wani et al. 2020). In comparison to relatively low levels of SLs in the stem, leaves, and hypocotyl, plant roots have relatively high content of SLs (Yoneyama et al. 2007). Furthermore, there are also certain cell types in the shoot with localized high concentration of SLs. In roots, the localization of SL biosynthetic gene expression gives an idea about the region wherein they are produced. Two CCDs i.e. *CCD7* and *CCD8* involved in SL biosynthesis as described above, are expressed throughout the vascular parenchyma cells of roots (Zou et al. 2006; Arite et al. 2007). *MAX1* shows predominant expression in root vasculature, whereas *MAX4* (*CCD8*) shows predominant expression in root cap columella of primary and lateral roots (Sorefan et al. 2003; Bainbridge et al. 2005). Furthermore; *CCD8* is up-regulated in the (pro) vasculature of the primary root and the cortical tissue of the root apex and elongation zone upon treatment by synthetic auxin 1-naphthaleneacetic acid (NAA) (Sorefan et al. 2003; Bainbridge et al. 2005). Moreover, the relatively high expression of SL signaling components namely *D14* and *MAX2* in the root elongation zone suggests a possible co-localization of SL biosynthesis and signaling in this region of the root (Brady et al. 2007).

4 Strigolactones and Root Development

Root growth is the result of three main phases i.e. cell division, cell elongation, and cell maturation. The fates of these cells are determined by local auxin gradients, maintained by a complex polar auxin transport circuit and its biosynthesis (Benkova et al. 2003; Ljung et al. 2005); with elongation zone crucial in the dynamics of this circuit. In the elongation zone of roots, lateral transport of auxin takes place from epidermis into the PAT (Polar auxin transport) stream (Benkova et al. 2003), and the initiation of LRs also occurs in this zone (De Smet et al. 2007). Based on accumulating evidences, SLs have been reported to be involved in regulating root growth in a number of plant species. As reported by Crawford et al. (2010), SLs have been found to play important role in regulating the transport of auxins in the main stem, so the regulation of PR growth and LR initiation by SLs could probably be due to regulation of lateral auxin reflux in the elongation zone of roots (Ruyter-Spira et al. 2013). They promote the elongation of seminal/PRs and ARs, but repress LR formation (Ruyter-Spira et al. 2011; Sun et al. 2015). In SL-deficient rice, crown roots are shorter than those of corresponding wild type plants (Ruyter-Spira et al. 2011), and in case of tomato the inhibitory effect of exogenous auxin application on root elongation was reversed by SLs (Koltai et al. 2010). The PR length in SL-deficient Arabidopsis mutants has been found to be shorter than the wild type, due to reduction in the number of cells in meristem, and this effect was reversed upon the

application of synthetic SL analogue GR24 (Ruyter-Spira et al. 2011; Koltai 2011). This increase in PR length correlates with an increase in meristem and elongation zones upon GR24 treatment. The decreased lateral auxin transport from epidermis into PAT stream results in increased meristem and elongation zone (Grienesein et al. 2007). These observations suggest that SLs are involved in the regulation of lateral auxin reflux. The elongation zone of the root is important in adjusting the growth of roots as per the developmental stages and environmental conditions due to the action of other hormones as well, and it thus acts as a regulatory hotspot (Ruyter-Spira et al. 2011).

SLs have been found to regulate root development under nitrogen and phosphate deficiencies. Under such conditions, plants show increased SL content in the roots, which induce AM fungal hyphal branching and inhibit outgrowth of buds as an adaptation to nitrogen and phosphate deficiencies (Umehara et al. 2008; Czarnecki et al. 2013; De Jong et al. 2014; Xu et al. 2015). SL biosynthesis and signaling mutants in Arabidopsis show reduced response to phosphate limiting conditions as compared to wild type plants (Mayzlish-Gati et al. 2012). This reduced response was compensated by the application of GR24 in case SL-synthesis mutants, but not in case signaling mutants. SLs have been suggested to regulate the plant architecture, mainly under phosphate deficient conditions.

4.1 Strigolactones and Primary Root Development

The growth of the PR is determined by the activity of root apical meristem, which is a region present at the root tip consisting of actively dividing cells and from which all the tissues of PR are derived. The root apical meristem consists of a stem cell niche, a distal meristem and a proximal meristem. Cell division, elongation and differentiation during root development in this region is controlled by phytohormones, among which auxin is the main player. Auxin is imported from the shoot but local auxin biosynthesis also takes place in roots (Chen and Xiong 2009; Petersson et al. 2009). The root growth is determined by the auxin concentration gradient along the longitudinal axis of root meristem. This gradient is established by the action of auxin transporters including efflux carriers such as PINs and ABC transporters and auxin influx carriers such as AUX1 and LIKE-AUX1 (Kleine-Vehn et al. 2006; Grieneisen et al. 2007; Zazimalova et al. 2010). In the PR, PIN1, PIN3, and PIN7 are basally localized in the stele and help in the acropetal transport of auxins towards the root tip (Petrasek and Friml 2009). In root columella, PIN3 and PIN7 redirect the auxin flow towards epidermis and then PIN2 helps in the transport of auxins upwards into the elongation zone (Petrasek and Friml 2009). PIN2 in cortex regulates both rootward and shootward auxin flux, maintaining a high auxin maxima at the root tip (Rahman et al. 2010).

The reports regarding the role of SLs in regulating PR length are a bit inconsistent, with its effects modified by a number of environmental factors and sometimes its effects seem to be species dependent (Marzec and Melzer 2018). Under normal sugar

and phosphate conditions, the application of *rac*-GR24 (a racemic mixture of two enantiomers) increases the PR length in SL-biosynthesis mutants (*max1*, *max3* and *max4*), but not in case of SL-signaling mutants (*max2*) and this response occurs in a MAX2 dependent manner (Jain et al. 2007; Ruyter-Spira et al. 2011). The increased PR length upon GR24 treatment correlates with increase in the number of cortical cells in primary root meristem and transition zone size (Ruyter-Spira et al. 2011; Koren et al. 2013). This rescue effect of GR24 in increasing the PR length in SL-biosynthesis mutants was observed at 1.25 μM concentration, and an inhibition in the length of PRs was observed beyond 2.5 μM concentration, most probably due to its non-physiological concentration having a toxicity affect. This inhibitory effect of GR24 at high concentration was MAX2 independent (Ruyter-Spira et al. 2011; Shinohara et al. 2013). They further reported that the stimulatory effect of SLs (GR24) in increasing the length of primary roots was also seen under carbohydrate limiting conditions. Furthermore, they found that this response is seen in case of SL-biosynthesis mutants only, and not in *max2* mutants, confirming the fact that the role of GR24 is MAX2 dependent. Under limited carbon availability, the plants have reduced length of PRs. Under such conditions, both SL biosynthesis and signaling Arabidopsis mutants have shorter PRs than wild type plants (Claassens and Hills 2018). The SL biosynthetic and signaling mutants in rice possess short seminal roots under limited phosphate and nitrate; however upon GR24 treatment, the root lengths were restored in case of biosynthetic mutants, but not in case of signaling mutants (Sun et al. 2014). However, in case of wild type plants GR24 application had no effect on length of seminal roots. Similarly, in case of *Medicago truncatula* and tomato, the application of *rac*-GR24 causes an increase in the length of PRs in SL-biosynthetic mutants, but it remained ineffective in increasing PR length in wild type plants (Koltai et al. 2010; De Cuyper et al. 2015). Contrastingly, in *Lotus japonicus* the silencing of *MAX3* ortholog results in the development of a longer PR than a shorter one (Liu et al. 2015). Based on the results, it is clear that it is difficult to make a general conclusion on the impact of SLs on PRs. However, we may say that SLs act as positive regulators of PR elongation, however this response varies between species and is also dependent on the growth conditions (Fig. 1).

The changes in root meristem mediated by GR24 are indicative of altered local auxin gradients (Mayzlish-Gati et al. 2012). The SL-auxin interplay during root development is also clear due to regulatory role played by SLs during PAT from roots to shoots (Sun et al. 2014), and also during auxin flux within root tissues (Sun et al. 2014; Kumar et al. 2015; Omoarelojie et al. 2019). As high auxin concentration promotes cell division, and low auxin concentration promotes cell elongation, it may be concluded that low SL content causes an increase in auxin content in PR meristem, whereas high SL content decreases its concentration (Ruyter-Spira et al. 2011). This shows that the activity of SLs in regulating PR growth are mediated by auxins. The local auxin concentrations in primary root tip are maintained by the action of auxin efflux carriers belonging to the PIN protein family as discussed above (Blilou et al. 2005). Thus along with SLs, auxin transport acts as a major determinant of root meristem patterning (Friml et al. 2003). The SLs regulate the allocation of PINs to plasma membrane by controlling their cycling between the endomembrane system

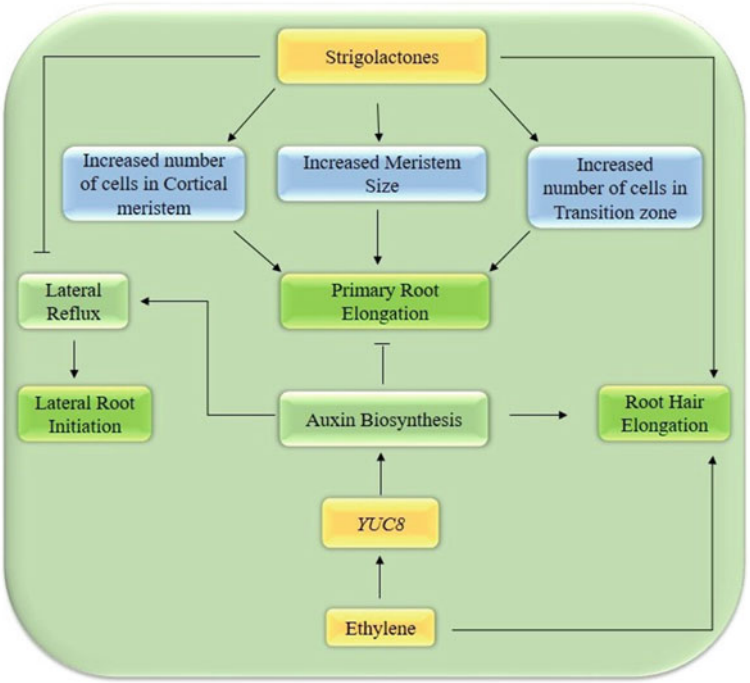

Fig. 1 Proposed interplay of Strigolactones, auxin and ethylene hormones in regulating PR, LR and RH development. SLs promote PR elongation due to increased cell number in cortical meristem and transition zone, whereas auxin inhibits PR elongation. Auxin acts as a positive regulator of LR development, whereas SLs act as negative regulators of LR development, and they mediate their responses via inhibition of lateral reflux. Ethylene positively regulates auxin biosynthesis via induction of *YUC8* expression. SLs, ethylene and auxin act as positive regulators of RH elongation

and plasma membrane (Prusinkiewicz et al. 2009). The reduction in PIN protein cycling due to GR24 application results in reduced auxin transport in root vascular tissues, which also act as the main spots of *MAX2* expression (Stirnberg et al. 2007). The reduction in basipetal auxin transport due to endogenous SLs and upon GR24 treatment has also been demonstrated by Crawford et al. (2010). The accumulation of auxins within meristem cells of the primary root due to modulation of PIN activities by SLs results in increased cell division, and expansion of meristem and transition zones, which ultimately results in increased PR length (Ruyter-Spira et al. 2011). SL–auxin interaction controls root development by adjusting or regulating intercellular auxin flow, auxin sensitivity, and shoot-to root transport (Mayzlish-Gati et al. 2012; Omoarelojie et al. 2019).

Recently, Oláh et al. (2020) conducted a study involving *Arabidopsis thaliana* to determine any possible interaction between SLs and nitric oxide in root development. They found that SL biosynthetic and signaling mutants have elevated levels

of nitric oxide and S-nitrosothiol due to decreased abundance and activity of S-nitrosoglutathione reductase. These observations indicate that there is a possible signal interaction between S-nitrosoglutathione reductase regulated levels of nitric oxide and SLs, which was further supported by the down-regulation of SL-biosynthetic genes in S-nitrosoglutathione reductase deficient *gsnor1-3* having high nitric oxide content (Oláh et al. 2020). They speculated that S-nitrosoglutathione reductase is required to control nitric oxide levels during SL induced PR elongation. However, it needs to be seen if there is any involvement of karrikins or auxins as other partners in this interplay, and this needs to be clarified in the future studies.

4.2 Strigolactones and Lateral Root Development

Lateral root formation is initiated by a series of divisions controlled by auxins in the primed founder cell. This process is regulated by the auxin reflux in the transition zone (Cheng et al. 2013; Marhavy et al. 2013). The LR initiation from founder cell requires an auxin gradient, which is regulated by transient PIN3 expression in the endodermis (Marhavy et al. 2013). After LR initiation LR primordia is formed and finally LR emergence takes place (De Smet 2012; Cheng et al. 2013). The LR emergence from LR primordia requires the establishment of a suitable auxin maximum by auxin efflux carriers at the tip of primordia (Petrasek and Friml 2009). This establishment of auxin maximum at the tips serves as a signal to remodel adjacent cells due to expression of LAX3 (an auxin influx carrier) in epidermal and cortical cells, which then leads to separation of cells in the LR primordial overlying tissue, which finally results in LR emergence as explained by Swarup et al. (2008). After LR initiation, the subsequent development of the LR is dependent on shoot derived auxins transported into LR primordia via PAT stream (Wu et al. 2007; Cheng et al. 2013).

SLs negatively regulate the initiation and development of LRs. The SL-biosynthetic mutants (*max3* and *max4*) and SL-signaling mutants (*max2*) show increased LR density as compared to the wild type (Kapulnik et al. 2011a). The treatment of Arabidopsis seedlings with GR24 decreased the LR density (Ruyter-Spira et al. 2011; Kapulnik et al. 2011a) in wild type plants, and *max3* and *max4* mutants; however this response was not seen in case of *max2* mutants. From these observations it is evident that the repression of LR development by SLs is MAX2 dependent (Kapulnik et al. 2011a). Ruyter-Spira et al. (2011) reported that the application of GR24 at 2.5 μM concentration results in decreased LR density, however the initiation of LRs is negatively regulated only upon application of 5 μM GR24. Therefore they concluded that the reduced LR density at 2.5 μM GR24 is the result of delayed LR development. This delayed LR development occurs due to reduced auxin levels in LR primordia as they displayed reduced levels of PIN1-GFP intensities (Ruyter-Spira et al. 2011). They also observed that the inhibitory effect of GR24 upon LR development was not seen upon the application of sufficient exogenous auxin. They observed that there was no reduction in PIN1-GFP intensity in

LR primordia upon simultaneous application of auxin and SLs These results indicate that the SL mediated regulation of LRs is mediated through the modulation of local auxin concentration. These local auxin gradients are maintained due to asymmetrical localization of PINs (Marhavy et al. 2013). Moreover, Sun et al. (2019) reported that in addition to primary LRs, rice SL biosynthesis and signaling mutants display increased production of secondary LRs as well. The exogenous application of GR24 on SL biosynthetic mutants decreased the number of secondary LRs in rice. Low nitrate and phosphate concentrations have been found to enhance SL exudation and LR elongation in rice but decrease the LR density (Sun et al. 2014). The enhanced SL production under nitrate and phosphate deficiency reduces the transport of auxins from shoots to roots which reduces the density of LRs (Sun et al. 2014). So SLs regulate the LR development by regulating the auxin transport from shoots to roots.

Furthermore, the SL signaling in endodermis, mediated by MAX2 signaling component has been found to be sufficient in restoring the root responses and involves the participation of SHY2 in SL signaling to regulate LR formation (Kohlen et al. 2012). SHY2 is an important component involved in auxin-cytokinin interplay, controls meristem size by regulating PIN auxin transporters, and is involved in the development of LRs (Marhavy et al. 2013). As auxin reflux between endodermis and pericycle mediated by PIN3 is found to be crucial in LR initiation, and MAX2 mediated SL signaling in endodermis is enough in restoring responses to LR formation indicates that SL signaling modulates auxin flux in the elongation zone to regulate LR development (Koren et al. 2013).

4.3 Strigolactones and Adventitious Root Formation

Adventitious roots (AR) are post embryonic plant roots that arise from non-root tissues—usually a stem. The application of IAA (Indole-3-acetic acid) in tomato has a positive impact on AR formation, and this response is dose dependent (Negi et al. 2010). The positive impact of auxin on AR formation is further elucidated by the fact that the over-expression of *YUCCA1* gene (an auxin biosynthetic gene) in *Oryza sativa* results in increased formation of Crown roots (ARs) (Yamamoto et al. 2007). Moreover, the formation of ARs in Oryza sativa PIN1 RNAi lines is significantly suppressed (Xu et al. 2005), suggesting the involvement of PIN1 dependent PAT during AR formation. As SLs result in PIN1 depletion from the xylem parenchyma cells in stem (Shinohara et al. 2013), it is quite reasonable to predict their inhibitory effect on AR development via PAT inhibition.

Based on the studies conducted in *Pisum sativum* and *Arabidopsis thaliana*, it has been revealed that SL biosynthetic and signaling mutants possess increased number of ARs as compared to wild type plants (Rasmussen et al. 2012a, b), which indicates that SLs negatively regulate AR formation. It has been suggested that SL mediated suppression of AR formation occurs by inhibition of early divisions of founder cell (Rasmussen et al. 2012b). The expression of *MAX2* in *max2* mutants under a xylem

specific promoter results in formation of ARs similar to that of wild type. This is in agreement with the fact that *MAX2* is expressed throughout the plant vasculature. The SL application of Arabidopsis biosynthetic mutants and wild type plants, results in reduced number of ARs even under elevated auxin levels (Rasmussen et al. 2012b; Cheng et al. 2013). Furthermore, both auxin response mutant *axr1* and double mutant *axr1max1-4* hardly form ARs, and auxin application increases AR number in *max* mutants (Rasmussen et al. 2012b). These observations suggest that auxins may promote AR formation independently of SLs. The negative role of SLs in AR formation is also confirmed by the presence of increased number of ARs in tomato plants with reduced *CCD8* expression, and thus less SL production (Kohlen et al. 2012).

Like SLs, cytokinins have also been reported to be negative regulators of AR formation. However, cytokinins and SLs work independently during the suppression of AR rooting (Rasmussen et al. 2012b). This is clarified by the fact that SL mutants are cytokinins responsive and cytokinins mutants are SL responsive (Fig. 2).

Due to availability of certain inconsistent data, the role of SLs in AR formation becomes a bit doubtful. The rice biosynthetic and signaling mutant (*d10* and *d3*) plants possess reduced number of ARs as compared to wild type plants (Sun et al. 2015). The application of racGR24 resulted in increased number of ARs

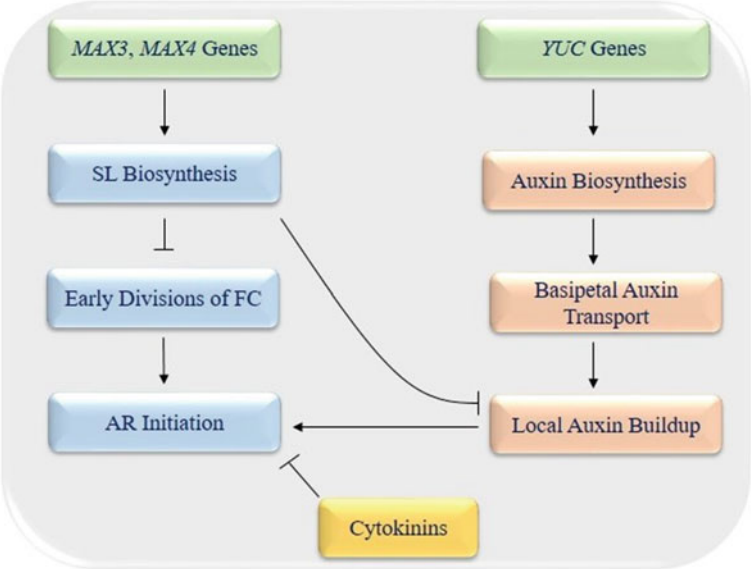

Fig. 2 Regulatory role of SLs, auxin and cytokinins in AR development. SLs act as negative regulators of AR initiation, as they inhibit the early divisions of FC. SLs also prevent the local auxin build-up needed for AR initiation. Cytokinins act as inhibitors of AR initiation, however cytokinins mediated inhibition does not involve any crosstalk with SLs (SL: Strigolactones; FC: Founder cell; AR: Adventitious root)

in SL-biosynthetic mutant *d10*, but not in case of SL-response mutant (Sun et al. 2015). They further suggested that the negative effect of *N*-1-naphthylphalamic acid (polar auxin transport inhibitor) and positive effect of α-naphthylacetic acid (synthetic auxin) application indicates the significance of auxin in AR formation. These observations indicate that AR formation in rice is positively regulated by SLs, but the SL-auxin interplay during this seems very complex.

4.4 Strigolactones and Root Hair Elongation

Root hairs (RHs) are delicate, unicellular (except in aerial ARs of *Kalanchoe* where they are multicellular), and unbranched tube like extensions from the root epidermis that help in the uptake of water and nutrients from the soil. They are formed in the differentiation zone of the roots from special epidermal cells called trichoblasts. The differentiation of epidermal cells into trichoblasts depends upon the activity of *CAPRICE* (*CPC*) gene, which is a positive regulator of trichoblasts differentiation (Thomas 2016). RH formation has been proposed to be mediated by optimal auxin content and signaling, whereas ethylene acts in it through regulation of intracellular auxin levels (Muday et al. 2012). The ideal auxin concentration required for RH elongation is regulated by auxin influx and efflux carriers. AUX1 (an auxin influx carrier) dependent auxin transport through non-hair cells can maintain auxin supply for developing trichoblasts and ensure RH outgrowth (Jones et al. 2009). Ethylene also acts a positive regulator of RH elongation, as Arabidopsis *ein2* mutant and ethylene-resistant mutant *aux1* possess short RHs (Rahman et al. 2002). Moreover, they also observed that the recovery of RH length of *ein2* mutants required the application of very high amount of 1-naphthaleneacetic acid (100 nM), suggesting that roots become less sensitive to auxins upon the loss of ethylene signaling.

The SLs interact with ethylene and auxin in regulating RH elongation. The SLs increase RH elongation, as their exogenous application results in increased RH length in SL deficient and wild type plants, but not in signaling mutants, suggesting that their effect is MAX2 dependent (Kapulnik et al. 2011a; Kapulnik and Koltai 2014). Liu et al. (2018) reported that the overexpression of PDR1 involved in SL transport results in enhanced root biomass, LR growth and RH elongation. The regulation of RH elongation by SLs requires auxin signaling and ethylene signaling as well (Kapulnik et al. 2011b). Ethylene has been found to be positive regulator of auxin biosynthesis due to up-regulation of *YUC8* expression, which in turn promotes RH elongation (Stepanova and Alonso 2009). The application of IAA in *max2* mutant results in RH elongation similar to that of wild type plants, indicating that auxin response is independent of SL-signaling (Kapulnik et al. 2011b). However, they reported that SL response requires auxin perception in regulating RH elongation. The ethylene signaling mutants show reduced SL sensitivity and the application of ethylene biosynthetic inhibitor aminoethoxyvinylglycine results in reduced impact of SLs on RHs (Kapulnik et al. 2011b). This shows that during RH elongation ethylene is epistatic to SLs. These observations suggest that SLs regulate RH elongation at least

partially through their interplay with ethylene. We may therefore conclude that there is a complex interplay between SLs, auxin and ethylene in regulating RH elongation.

5 Conclusion

To summarize, SLs play an important regulatory role in root initiation and development. Based on the above discussion it is quite obvious that root development is not regulated by a single hormones, but it involves a complex interplay between SLs, auxin, ethylene, and cytokinins, in which auxin modulation by SLs plays an important part.

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Crosstalk of Jasmonates with Phytohormones Accompanying Root Growth, Development and Microbe-Interaction

Suman Sharma and Madhumita Banerjee

Abstract Jasmonates (JAs) are well known new class of lipid based phytohormones which are produced endogenously in plants growing under stress. They play significant role in regulating plant adaptation to several biotic and abiotic stresses like wounding, predator attack, salt stress and UV radiation etc. Studies have shown that besides playing role of stress hormone JAs are also involved in many growth and development activities in vegetative as well as reproductive parts of plants including roots. Since the time of its discovery many detailed studies have been done in understanding its biosynthetic and signalling pathway, its crosstalk with other phytohormones. Many genes and transcription factors have been identified which are involved in positive and negative regulation of these pathways and other root growth related activities such as inhibition of primary root growth, growth of lateral and adventitious roots, gravitotropic response, root—microbe interactions. In this chapter we have given an overview on mechanism of JA action and its effects on various aspects of root growth and development.

Keywords Jasmonates · Biotic stress · Abiotic stress · Root-microbe interaction · Gravitotropic response

Abbreviations

α-LeA	α-Linolenic acid
LOX	Lipoxygenase
COI 1	Coronatine insensitive 1
MeJA	Methyl jasmonates
AOS	Allene oxide synthase
AOC	Allene oxide cyclase
OPDA	(9S,13S)-12-oxo-phytodienoic acid

S. Sharma (✉) · M. Banerjee
Department of Botany, Ramjas College, University of Delhi, Delhi 110007, India
e-mail: sumansharma@ramjas.du.ac.in

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JAZ	JASMONATE ZIM DOMAIN
AUT	Autoregulation of nodulation
PLT	Plethora
JA-Ile	Jasmonate isoleucine
bHLH	Basic loop helix
HR	Hairy root
ASA1	Anthranicate sysnthase A1
TF	Transcription factor

1 Introduction

Jasmonates (JAs) are phospholipids derived cyclopentanones that included Jasmonic acid (JA) and its derivatives methyl Jasmonates (MetJA). In year 1962 (Met JA) was first isolated from essential oil of *Jasminium grandilorum* flower (Demole et al. 1962) and the free acid was isolated later from the culture filtrate of fungus *Botryodiplodia theohormae* (Aldrige et al. 1971), *Cucurbita pepo* (Fukui et al. 1977), *Vicia faba* (Dathe et al. 1981). In 1980, JA and its derivatives were synthesised chemically and their biological activity was tested on growing rice seedling (Yamane et al. 1980). JA are basically stress hormone as they slow down normal growth and development processes in plants which are sensitive to environmental stress and promotes several stress related responses in plants. Since the MeJA is a volatile compound it can easily escape from a plant under stress and raises an alarm in neighbouring plants to the prevailing biotic and abiotic stresses. Therefore this hormone has several ecological and physiological implications. In last few years almost all genes, proteins and transcription factors involved in biosynthetic and signalling pathways of JA have been identified, isolated and characterized. In this chapter we have outlined the mechanism of JAs biosynthesis, signal transduction, crosstalk with other phytohormones and molecular basis of the effects shown by them in regulating various growth and development related activities in roots.

2 Jasmonates

Jasmonates include Jasmonic acid (JA) its methyl ester MeJA and isoleucine conjugates of JA. This is a class of phytohormones which are involved in plant defence against biotic and abiotic stress (Du et al. 2013). Along with plant defence these are also involved in plant growth and development, reproduction (Wasternack 2007; Browse 2009), floral development, trichome formation, vegetative storage protein (VSP) formation, fruit ripening, tendril formation, mycorrhizal association, male fertility and development of roots in plant. Chemically JA is 3-oxo-2'-2'-cis pentenyl-cyclopentane 1-actic acid.

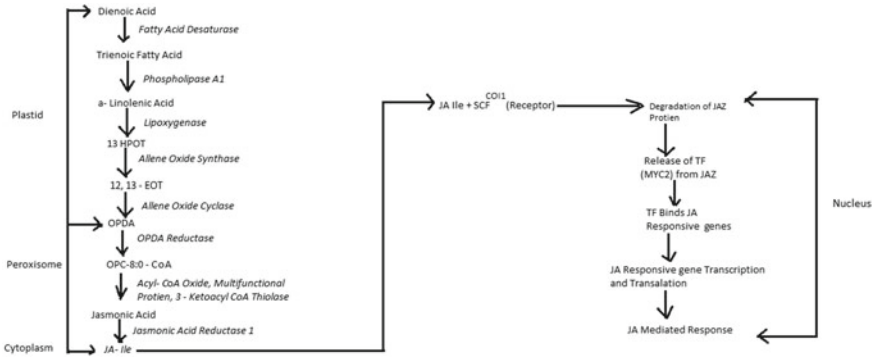

Fig. 1 Jasmonates (JAs) biosynthesis and signaling pathway

3 Biosynthesis of Jasmonates

α-linolenic acid (α-LeA) serves as a precursor for biosynthesis of JA (Browse 2005; Wasternack and Hause, 2013) (Fig. 1). Biosynthesis process begins in plastids, where α-linolenic acid is produced by joint action of two enzymes fatty acid desaturase (FAD) and phospholipase A1 (PLA). It is then converted to (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT), 12,13(S)-epoxyoctadecatrienoic acid (12,13-EOT), and (9S,13S)-12-oxo-phytodienoic acid (OPDA) in a stepwise manner through the action of enzymes 13-lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), respectively (Fig. 1). OPDA thus formed is transported to peroxisomes, where it is reduced to 3-oxo-2-(*cis*-2′-pentenyl)-cyclopentane-1-octanoic acid by OPDA reductase (OPR). OPC is subsequently shortened to jasmonic acid by three rounds of β-oxidation catalyzed by three different enzymes: acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT). Jasmonic acid is finally exported to the cytoplasm, where it is conjugated with isoleucine to form bioactive (+)-7-*iso*-JA-Ile (Wasternack and Strnad 2016).

4 Biosignalling of Jasmonates

A JA response mutant *coronatine insensitive 1* (coi1) in *Arabidopsis thaliana* enlighten our understanding in JA signalling pathway (Feys et al. 1994). COI1 encodes for a F-box protein which is a putative JA receptor and it function in E3—ubiquitin ligase mediated degradation of target proteins (Thines et al. 2007; Chini et al. 2007; Xu et al. 2002) JASMONATE ZIM DOMAIN (JAZ) protein (Fig. 1). Further, identification of JA responsive MYC transcription factors, revealed pathways for JA perception and JA dependent gene regulation. At low level of JA, expression of JA response and JA responsive genes are not active and MYC2 transcription factors

are also inactive by interacting with JAZ protein, the JA signalling repressor. JAZ protein contain two domains, ZIM and JAS. The ZIM domain regulate its dimerization and interaction with NINJA further connecting it to another protein TOPLESS, a transcription suppressor in JA signalling pathway (Huang et al. 2017). The JAS domain regulate interaction of JAZ with COI1. In response to endogenous or environmental signals JA biosynthesis pathway gets activated by binding of JA to COI1 receptor. This interaction results in degradation of JAZ protein followed by release of MYC2 from JAZ (Fig. 1). On its release MYC2 activates transcription of JA responsive genes. Both JAZ and MYC2 play significant role in plant growth and development as positive and negative regulator by regulating JA dependent inhibition of growth under various biotic and abiotic stresses.

5 Role of Jasmonates in Root Growth and Development

5.1 Gravitotropism Response

According to Cholodny and Went, shift in auxin transport from basipetal to lateral results in development of lateral auxin gradient and hence asymmetric growth thereby showing gravitotropism. In a study done on rice coleoptiles (Gutjahr et al. 2005) it is clearly evident that gravitropism is not caused only by auxin gradient but it also involve role of JA. The total JA content rises significantly in the gravitropically stimulated rice coleoptiles during the time course of stimulation and also JA is distributed in a gradient reciprocally oriented to the IAA-gradient. In *Arabidopsis* (Moseyko et al. 2002) wheat seedling (Kramer et al. 2003) expression of enzyme lipoxygenase the first enzyme of JA biosynthesis pathway is upregulated during gravitropic stimulation (León and Sánchez-Serrano 1999) further in rice coleoptile split in two halves, the transcript level of the JA-responsive gene GER1 increases in both halves. Since JA concentration generally increases in both flanks during initial stages of gravitropic stimulation but later a gradient is developed due to more synthesis in the upper flank. In order to find out whether the JA-gradient has any significant role in gravitropism two test were done in rice coleoptile (1) stimulated coleoptiles were flooded the with exogenous methyl-jasmonate (Me-JA) (2) JA-deficient rice mutant hebiba was compared with the wild type for time course of bending (Gutjahr et al. 2005). Results obtained showed that flooding with jasmonate delays the onset of gravitropic bending moreover a jasmonate-deficient rice mutant bends more slowly and much late in comparison to the wild type. This clearly indicates that that JA is not absolutely necessary for gravitropic bending but mainly seems to accelerate the bending process.

Investigations were also carried using 5 uM concentration of NPA to determine whether the JA-gradient is induced independently or it's a downstream effect of the IAA-gradient. Results obtained showed that 5uM NPA efficiently suppressed the

establishment of an IAA-gradient but has no effect on the JA-gradient (Gutjahr et al. 2005).

In conclusion JA is not absolutely necessary for gravitropic bending but at the same time it accelerates the gravitropic response.

5.2 Inhibition of Primary Root Growth

Exogenous application of JA inhibits growth of primary root. In *Arabidopsis thaliana* COI1 together with JAZ and inositol pentakisposphate (InsP5) form a coreceptor for JA–Ile (Sheard et al. 2010; Huang et al. 2017) to inhibit root growth. Mutation in COI1 makes the coreceptor complex insensitive to this inhibitory response. Several JAZ proteins have been identified in *Arabidopsis* of which few can directly recruit the corepressor TPL and related proteins to suppress JA response whereas many perform this function by interacting with NINJA and uses its EAR domain to recruit these co-repressors (Chini et al. 2007, 2016; Pauwels et al. 2010; Shyu et al. 2012; Thines et al. 2007; Thireault et al. 2015; Yan et al. 2007). The inhibitory effect of JAs on primary root growth can be suppressed by overexpression of some NINJA or JAZ protein mutants (e.g. JAZ1Δ3A, JAZ3ΔC, JAZ10.3/JAS1, JAZ10.4, JAZ8, and JAZ13). In response to JA-Ile the E3-ligase SCF^{COI1} targets JAZ for degradation via 26S proteasome pathway. Several TFs in *Arbidopsis* including MYC2, 3 and 4 which are present in primary root apex function to promote inhibitory action of JA on growth of primary root (Fig. 2). MYC 2 interact with a mediator complex (MED 25) and repress expression of two genes PLT1 and PLT2 (PLETHORA genes), which results in restricted activity of root meristem and hence inhibit growth of primary root. MYC3 also interact with MED 25 and regulate the effect. Ubiquitination and phosphorylation of MYC2 by PLANT U-Box protein (PUB-10) and MAPK decreases inhibitory effect of JA on primary root growth (Fig. 2). Basic loop helix (bHLH) like TF also interact with JAZ. They compete with transcription activators MYC2 for common promoter sequences of target genes, inactivate them subsequently by binding to them and hence negatively regulate inhibition of primary root growth by JA.

An ethylene signalling TF EIN3-LIKE1 (EIL1) also interact with JAZ protein and positively regulate JA induced primary root growth inhibition and JA dependent root hair formation (Zhu et al. 2011) (Fig. 2). Effect of high salt condition on JA mediated inhibition of root growth was analysed in some rice mutants and it was observed that the inhibitory effect of JA on root growth decreases in JA biosynthesis mutants whereas in loss of function mutants the root growth was severely affected under high salt conditions (Hazman et al. 2015).

Lateral root formation is promoted by JA in *Arabidopsis*. This response is mediated by overexpression of ERF 109 which binds and activate promotor of an auxin biosynthetic gene ANTHRANICATE SYSNTHASE A1 (ASA1) and YUCCA (Cai et al. 2014b; Sun et al. 2009). At the same time JA negatively regulate adventitious

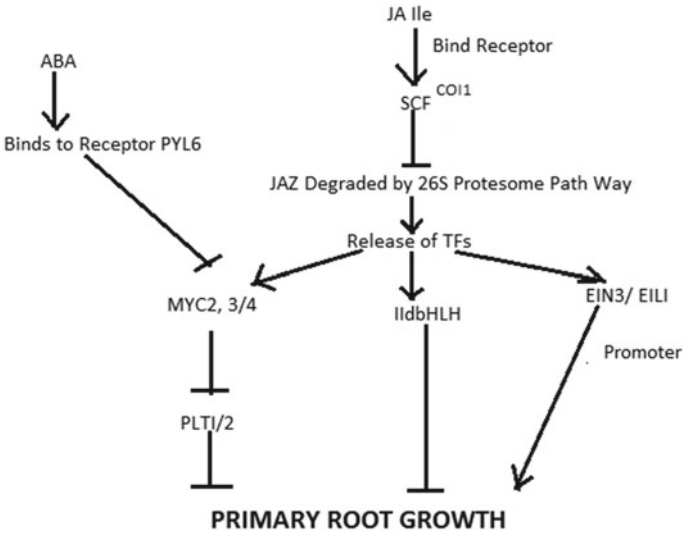

Fig. 2 Regulation mechanism for JA induced inhibitory effect on primary root growth. JAZ on being associated with NINJA and TPL attenuates the inhibitory effect but in presence of JA-Ile, JAZ gets degraded and the inhibitory effect is removed

root formation in *Arabidopsis*. This effect is controlled by auxin induced overexpression of GH3 enzymes. These enzymes inactivate JA by conjugating it to amino acids aspartic acid, methionine and tryptophan and hence promotes adventitious root formation (Gutierrez et al. 2012). In *Petunia* plant however JA enhances adventitious root formation (Lischweski et al. 2015) emphasizing differential effect of JA on adventitious root formation in different plant species.

5.3 Effect on Nodulation

In order to maintain balance in symbiotic relation, leguminous plants have a systemic regulation system called autoregulation of nodulation (AUT). Mechanism of AUT is similar to systemic acquired resistance (SAR). Exogenous application of methyl jasmonates (MeJA) in *Lotus japonicas* wild type and one of its mutant har-1 resulted in suppression of nodulation in wild type and also suppression of hypernodulation in the har1-4 (Nakagawa and Kawaguchi 2006). Higher concentration of MeJA showed similar response in both wild type as well as mutant whereas at lower concentration suppression effect is more pronounced in mutant. Since JA are known to inhibit plant growth and degradation of photosynthetic pigment therefore suppression of nodule formation may be a secondary effect of growth inhibition. When MeJA applied at conc 10^{-4} M it result in significant reduction of root hair curling, infection threads and nodule primordia formation moreover higher concentration of the rate 10^{-3}

completely block root hair deformation and curling. These results indicate that shoot applied with MeJA inhibits early stages of bacterial infection and nodule initiation. A gene NIN which is already nodulin gene induced in response to nod factor, coded for a putative transcription regulator which is required for formation of infection thread and inception of nodule primordia. Expression of NIN gene is significantly reduced in legumes shoot treated with MeJA. These finding suggests that inhibitory effect of MeJA on infection and nodulation in legumes occur upstream of the induction of NIN transcript.

5.4 Jasmonate Mediated Root Curling

A gene identified as Oryza sativa root meander curling (OSRMC) is expressed largely in roots of rice plant. It results in production of a putative receptor protein OSRLK, AAL87185. Expression of this gene is induced by application of JA. RNAi based knockdown of this gene in transgenic rice plants results in altered root development and coiling pattern. The primary root in RNAi transgenic rice plant meanded and curled more efficiently than wild type plant roots when treated with JA. In these transgenic plants primary roots were shorter, number of lateral was low whereas adventitious roots increased in number. Transgenic rice also showed increased expression of one of the JA signalling pathway gene RSO_sPR10. It is very evident from the results obtained that OSRMC a DUF26 subfamily gene is directly involved in JA signalling mediated root development process and negatively regulates root curling in rice (Jiang et al. 2007).

5.5 Disruption of Root Mitochondria

Application of MeJA results in reduction in accumulation of protein related to energy metabolism. Treating the hairy roots (HR) with MeJA increases in accumulation of H_2O_2 in the initial 48 h and gradually the concentration decreases thereafter due to disruption of root tissues and also the mitochondrial membrane in the roots. The disintegration of mitochondrial membrane, reduction in ATP synthesis and increased accumulation of H_2O_2 suggest that mitochondria in hairy root (HR) might be the target organelle for MeJA signalling. Activity of enzymes like POX and CAT al decreases in HR treated with MeJA which are responsible for accumulation of H_2O_2. In overall H_2O_2 outburst due to MeJA could be a initiating response for disruption of root mitochondria (Loyola-Vargas et al. 2012).

5.6 Regulation of Beneficial Microbe—Root Interaction

JA promotes interaction between plant roots and beneficial bacteria or fungi. Generally JA signalling at moderate rate promotes symbiotic association while at the same time high rate of JA signalling inhibits this response. Besides JA signalling rate the mutualism is also dependent on compatibility between microbe—host and environmental factors. A recent study has shown that when arbascular mycorrhiza colonizes barley roots it results in elevation of endogenous JA level, expression of JA responsive genes and JA biosynthetic genes in cells containing arbascular (Hause et al. 2002). Further, studies have also shown that treatment with JA stimulates mycorrhizal development in endo and ectomycorrhizal associations (Regvar et al. 1996, 1997) and expression of symbiotic nod genes in Rhizobium (Rosas et al. 1998).

6 Crosstalk of Jasmonates with Other Phytohormones During Root Development

Auxin: Wild plants of *Arabidopsis* when treated with JA showed shorter roots due to decreased apical growth of roots while JA signalling mutant showed normal size roots even on treatment with JA (Jang et al. 2017). In contrast auxin deficient or auxin signalling mutants like (trp2-12) and (arx 3-1) from very short roots compared to wild type plants (Ursache et al. 2014; Zhang et al. 2019). This clearly indicates that JA induced inhibition of root growth might be regulated by its interaction with auxin (Chen et al. 2011). Inhibition of root growth is actually a result of reduced meristem activity. Application of JA on plants suppress expression of auxin responsive transcription factor PLETHORAs (PLTs) which maintain stem cells and their proliferation in meristem (Mähönen et al. 2014). However in JA signalling mutant like coi I and myc2, PLTs expression is not suppressed indicating thereby that COI1—dependent JA signalling mediates JA induced root phenotype and transcription factor MYC2 suppresses expression of PLTs. Therefore JA and auxin acts antagonistically for regulating apical growth of roots. Formation of lateral and adventitious roots in plants is also due to interplay between JA and auxin biosynthetic genes.

Cytokinin: Water and minerals transporting xylem elements develop form procambium cells in their roots. Cytokinin transcription mutant of type B, ARRSs and transgenics overexpressing AHP6—a negative regulation of cytokinin signalling form extra xylem (Yokoyama et al. 2007; Jang et al. 2017). JA deficient OPDA reductase 3 (opr3) when treated with JA showed an extra xylem phenotype, whereas JA signalling mutant coiI, jasmonate resistant I (jar I), failed to do so (Jang et al. 2017). From these studies conclusion can be drawn that stress hormone JA antagonistically interact with cytokinin in xylem development in plant roots. Molecular studies done further validates that JA reduces expression of cytokinin responsive gene PINFORMED (PIN 7) which controls xylem development. Further myc2 mutant fail

to form extra xylem on exogenous application of JA and it also lacks expression of AHP6 a cytokinin signalling inhibitor.

Ethylene: JA and ethylene coordinate together to regulate many plant stress responses via JAZs—MYC2 and EIN3/EIL1. In plants EIL 1 an essential TF in ethylene signalling, interact with JAZ protein and positively regulates both JA—dependent primary root growth inhibition and JA induced root hair formation (Zhu et al. 2011).

ABA: JAZ—MYCs are involved in crosstalk between JA and ABA signalling pathways, affecting various aspects of plant growth (Chen et al. 2011). A ABA receptor PYRABACTIN RESISTANCE 1 Like protein (PYLS) forms a complex with JA2, which interacts with MYC2 and activates its transcriptional activity. The activated MYC2 inhibits the expression of PLT 1 and PLT2 (PLETHORA 1 and 2) and hence the primary root growth.

7 Conclusions

Jasmonic acid and its derivatives are involved in regulating various developmental and growth related processes in plants. It is produced in response to various biotic and abiotic stresses. JA controls various aspect of root growth and development like, inhibition of primary growth of roots, promotes lateral roots, inhibits formation of adventitious roots, colonization of roots with beneficial microbes, gravitropism, root curling behaviour, nodulation etc. Most of the effect on roots are negatively controlled by JA signalling in plants. JA is involved in crosstalk with several other hormone like auxin, cytokinin, ethylene and ABA for mediating root growth and development in plants. All effects regulated by JA and its interaction with other phytohormone in genetically controlled and well elucidated by several studies carried out in recent past.

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Jasmonates: A Thorough Insight into the Mechanism of Biosynthesis, Signaling and Action in Root Growth and Development

Manvi Sharma and Ashverya Laxmi

Abstract The roots are a highly plastic system of plants that function both as an anchor and site of interaction with microorganisms and nutrient uptake. Studies in the past two decades revealed that Root System Architecture (RSA) is highly dynamic and its development is shaped by several external factors such as light, gravity, water, nutrients, etc. Endogenously, the phytohormone pathways were found to be crucial sensing and signalling component determining the root growth and tropic responses by these environmental factors. The importance of hormone such as jasmonates (JAs) is still emerging in the context of RSA. JAs are lipid-derived phytohormones that regulate diverse range of processes ranging from pant growth and development, secondary metabolism, defense against insect attack, pathogen infection as well as tolerance to abiotic stresses. The history of JAs is very old and dates back to 1960s when MeJA was first identified from jasmine flower. Since then the physiological functions, biosynthesis, distribution, perception, signalling and crosstalk of JA have been elucidated. A balance between production, distribution and abolishment contributes to the fine-tuning of JA responses. The chapter addresses JA biosynthetic enzymes, their activity, regulation, metabolic pathways and COI1-JAZ-based perception and signalling, co-existence of signalling activators and repressors as well as complex nature of JA in root growth and development.

1 Introduction

Sufficient perception, union and transmission of signals are mandatory for appropriate growth of an organism. Plants sense the changes around them and transmit signals as part of normal development. Plant hormones are structurally varied signal molecules that control all cellular processes such as cell division, elongation and differentiation, thus ensuring a valuable developmental plan and optimal use of resources. In the past, hormones have been identified by conservative chemical

M. Sharma · A. Laxmi (✉)
National Institute of Plant Genome Research, New Delhi, India
e-mail: ashverya_laxmi@nipgr.ac.in

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isolation techniques along with plant phenotypic assays. However, combined biosynthesis/genetic analysis approach has enabled the identification of previously unknown plant hormones, such as SL (Gomez-Roldan et al. 2008; Umehara et al. 2008).

Apart from the five classical hormones, the last decade has witnessed the emergence of many new plant growth regulators such as oxylipins, phenolics, polyamines, BRs and SL. Oxylipins are highly diverse group of oxidized compounds that carry out various physiological functions. One of their well characterized examples are JAs that regulate several important roles in plant development. JAs are involved in vegetative growth, stamen and trichome development, senescence, cell cycle regulation, anthocyanin biosynthesis regulation, fruit ripening, cell cycle regulation (Parthier 1991; Sembdner and Parthier 1993; Creelman and Mullet 1995, 1997; Engelberth et al. 2004; Browse 2005; Wasternack 2007; Tani et al. 2008; Howe and Jander 2008; Pauwels et al. 2008; Yoshida et al. 2009; Reinbothe et al. 2009; Pauwels and Goossens 2011; Yan et al. 2012). Additionally, JAs participate in plant defense responses to pathogen attack, insect-driven wounding, biological response to injury and environmental stresses (Creelman and Mullet 1997; Wasternack 2007; Howe and Jander 2008; Browse 2009a; Pauwels and Goossens 2011). JAs also have an indispensable role in sex determination, reproductive bud initiation, leaf senescence and defense responses in monocots (Wasternack and Hause 2013).

2 Initial Isolation and Identification

Hydroxyl-jasmone esters of chrysanthemic acid were the first JAs obtained from *Chrysanthemum cinerariifolium* (Crombie and Elliott 1961). In 1962, Demole and co-workers isolated a volatile compound with sweet fragrance and called it methyl jasmonate (MeJA) from *Jasminium grandiflorum* (Demole et al. 1962) (Fig. 1). Later on, many of these compounds were chemically synthesized followed by elucidation of their structures. Aldridge and co-workers isolated the growth inhibitor JA from the fungal culture filtrate of *Lasiodiplodia theobromae* (Aldridge et al. 1971) (−)-JA was first time detected in faba bean (Dathe and Sembdner 1981). (+)-7-iso-JA was first isolated from fungal culture filtrate of *Botryodiplodia theobromae* (Miersch et al. 1987). Table 1 provides a snapshot of establishment of JA during years.

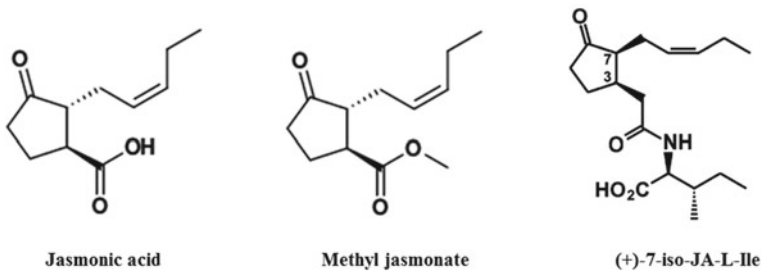

Fig. 1 General structure of JA and its derivatives

Table 1 Establishment of Jasmonic acid (JA) during years

Year	Advancements with respect to Jasmonates
1962	MeJA in flowers of *Jasminium grandiflorum*
1971	JA structure, growth inhibition
1980	Promotion of senescence
1983	1st pathway of JA biosynthesis
1987	Isolation of (+)-7-iso-JA from *Botryodiplodia theobromae*
1992	Isolation of first JA-insensitive mutant *jar1*
1995	Involvement of chloroplast LOX in wound-induced JA
1997	Stereo-specific action of JA-Ile isomers
1997	Identification of JA pathway from dnOPDA (16:3)
1998	COI1 is an F-box protein
2002	JA is linked to to the SCF complex by COI1
2004	JIN1 encodes TF MYC2
2007	First identification of JAZ genes
2007/2008	JA-Ile (isomer mixture) promotes COI1-JAZ interaction
2009	most active JA compound is (+)-7-iso-JA-L-Ile
2010	Crystallization of SCF^{COI1}-JAZ-co-receptor
2010	TOPLESS and NINJA associates to SCFCOI1-JAZ
2012	Involvement of MED25 in JA signalling
2014	Identification of JAR1 inhibitor, jarin-1

3 Biosynthesis of Jasmonates

Vick and Zimmermann first elucidated the biosynthesis of JA from the substrate α-linolenic acid (18:3) (α-LeA) by enzyme-catalyzed reactions in plastid, peroxisome and cytoplasm, followed by reductions and successive β-oxidations (Vick and Zimmerman 1983) (Fig. 2). In flowers, α—LeA is released from the galactolipids stored in the chloroplast of membranes by phospholipase A1 (PLA_1). *DEFECTIVE IN ANTHER DEHISCENCE 1*(*DAD1*), a flower-specific PLA_1 is essential for JA formation and filament elongation (Ishiguro et al. 2001). DAD-like lipases of leaves are still unidentified. In *Nicotiana attenuata*, galactolipase A1 (GLA1) forms JA in roots and leaves except during *Phytophothora parasitica infection* (Bonaventure et al. 2011). Thus, suggesting the formation of JA is catalyzed by stimuli-specific lipases.

The formation of oxylipins is initiated by dioxygenases called lipoxygeneases (LOXs) that catalyzes the insertion of oxygen at C-13/C-9 position leading to

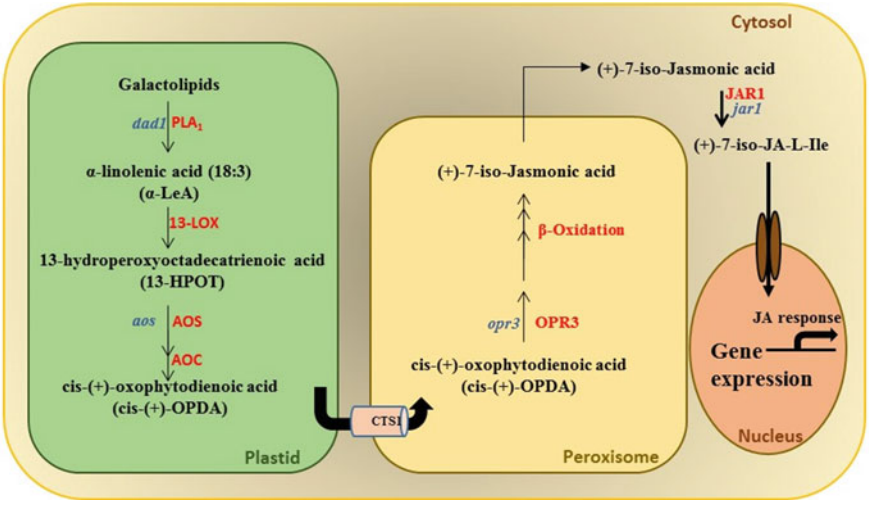

Fig. 2 Scheme of JA biosynthesis pathway from α-linolenic acid generated from galactolipids in *Arabidopsis thaliana*. Steps impaired in mutants of Arabidopsis are indicated in blue. AOC, allene oxide α-LeA, α-linolenic acid, 13-LOX, 13-lipoxygenase; OPR3-OPDA reductase; PLA_1, phospholipase A_1

the formation of (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) or (9S)-hydroperoxyoctadecatrienoic acid (9-HPOT) respectively which represent branch points within the LOX pathway. The Arabidopsis *LOX* family consists of six members including four encoding *13-LOX* (*AtLOX2*, *AtLOX3*, *AtLOX4* and *AtLOX6*) and are involved in JA formation that show specificity in wound responses and lipid peroxidation, fertility and flower development and natural and dark-induced senescence. The *lox3lox4* double mutant is male sterile, has defective dehiscence, non-viable pollen and abnormal anther maturation. 13-LOXs are also implicated in abiotic and biotic stress responses. *AtLOX3* is involved in salinity stress response (Ding et al. 2016). *AtLOX3* and *AtLOX4* are involved in defense against cyst nematode and root knot nematode infections (Ozalvo et al. 2014). There are six 13-LOXs in tomato (TomLoxA-F), out of which TomLoxC and TomLoxD are chloroplast located. TomLoxD is essential for all defense against herbivores (Yan et al. 2013). Whereas, TomloxC is vital for the formation of C5 flavor volatiles without any important role in defense (Shen et al. 2014). The first committed step of JA biosynthesis is carried out by 13-ALLENE OXIDE SYNTHASE (13-AOS) leading to the formation of an unstable allene oxide. AOS belongs to the CYP74A enzyme family. Beside the single copy gene of Arabidopsis (Park et al. 2002), AOS are present as gene family in other plant species. Chloroplast localized ALLENE OXIDE CYCLASE (AOC) coverts unstable allene oxide into cis (+)-2-oxophytodienoic acid (OPDA), the final product formed in the plastid. Arabidopsis consist of 4 differentially expressed AOC as reported by promoter:: GUS activity (Stenzel et al. 2012). All 4 AOCs show high activity in the meristematic tissues, the elongation zone, leaf tissues as well as the LR

primordial of roots. The AOCs form hetero or homodimers indicating an additional level of regulation (Stenzel et al. 2012). Plastid localized JA biosynthesis enzymes together catalyze hexadecatrienonic acid (C16:3) to form dinor-OPDA (dnOPDA).

The subsequent step, i.e. the reduction of the cyclopentanone rings of OPDA and dnOPDA by peroxisomal OPDA reductase (OPR) (Strassner et al. 2002). OPDA and dnOPDA formed in the plastid are transported to the peroxisomal membrane possibly by COMATOSE (Theodoulou et al. 2005) and are converted to oxophytoenic acid (OPC-8) and OPC-6, respectively. Due to defects in stamen development and in JA-induced root growth inhibition, *opr3-1* is male sterile. Important role of OPR was recently demonstrated in maize wherein *opr7opr8 showed* strong defects in development and low JA levels in all organs (Yan et al. 2012). The *opr7opr8* plants are also involved in herbivory and are susceptible to root-rotting oomycetes (Pythium spp.). Interestingly, a novel function of AtOPR3 has also been implicated under Pi deficiency (Zheng et al. 2016). Experiments with labeled OPDA revealed the formation of OPC-6 and OPC-4 derivatives and finally JA by the enzymes of ß oxidation machinery (Miersch and Wasternack 2000) such as acyl-CoA oxidase (ACX) (Li et al. 2005; Schilmiller et al. 2007), multifunctional protein (MFP) (Richmond and Bleecker 1999), L-3-ketoacyl CoA thiolase (KAT) (Castillo et al. 2004) and 4-coumarate: CoA ligase-like enzymes (Schneider et al. 2005). This is followed by cleavage of Jasmonoyl-CoA by THIOESTERASE (TE) to form *cis*-7-iso-jasmonic acid [(+)-7-iso-JA].

4 Regulation of JA Biosynthesis

There is always a balance between production, distribution and abolishment in living system, without which a proper life cycle is not possible. Similar is the case of plant hormones, they are synthesized; distributed and their levels are regulated either by degradation or by feedback regulation. All these events take place so as to regulate optimal growth at a cost of minimal energy. There are many elements that modify JA levels, regulate JA biosynthesis and metabolism. Substrate availability, tissue specific expression patterns and a positive feedback loop affecting the expression of JA biosynthesis genes are some of the endogenous factors that determine JA levels (Wasternack 2007; Browse 2009a, b). It is already known that external stimulus causes the release of α-LeA which act as substrate in JA biosynthesis. In nature, the fully developed leaves of Arabidopsis carry LOX, AOS and AOC proteins abundantly. However, JA formation does not take place without any stimuli from the outside. Additionally, this stimuli-induced rise in JA is temporary and appears only before *LOX, AOS* and *AOC* expression. Thus, suggesting that JA biosynthesis is regulated by substrate availability (Wang et al. 1999; Laudert et al. 2000; Park et al. 2002; Stenzel et al. 2003). Additionally, JA biosynthesis is regulated by a positive feedback loop as all genes encoding JA biosynthesis enzymes are JA inducible. Upon the activation of JA-Ile, JAZ undergo proteasomal degradation that liberates MYC2 to further activate JA biosynthesis genes. However, *MYC* and *JAZ* genes are also JA-Ile

responsive, thus permanently renewing negative and positive regulators that lead to a balance in JA biosynthesis and signalling (Hoo et al. 2008). Furthermore, tissue specificity of JA biosynthetic genes contributes to the outcome in JA biosynthesis. For example, tomato *AOC* is confined to vascular bundles and sieve elements (Hause et al. 2000). Post-translational regulation of JA biosynthetic genes has also been indicated. For example, the OPR3 activity seems to result from its dimerization and phosphorylation (Breithaupt et al. 2006). Moreover, interaction studies using BiFC detected dimerization of all the four AOCs that partially led to altered enzyme activity (Stenzel et al. 2012).

5 Jasmonic Acid Metabolism

There are plethora of JAs compounds that are formed when JA undergoes several biochemical modifications. Numerous JAs compounds have been identified and isolated in algae, fungi, mosses, gymnosperms and angiosperms. Homeostasis among various JAs metabolites is a common mechanism in plants to sustain active forms of JAs in plants. JA can be converted into active, partially active and inactive compounds by various metabolic reactions such as conjugation with amino acids, hydroxylation, carboxylation, decarboxylation, methylation, esterification, sulfation, O-glycosylation, and lactone formation of 12-OH-JA derivatives (Fig. 3). Some reac-

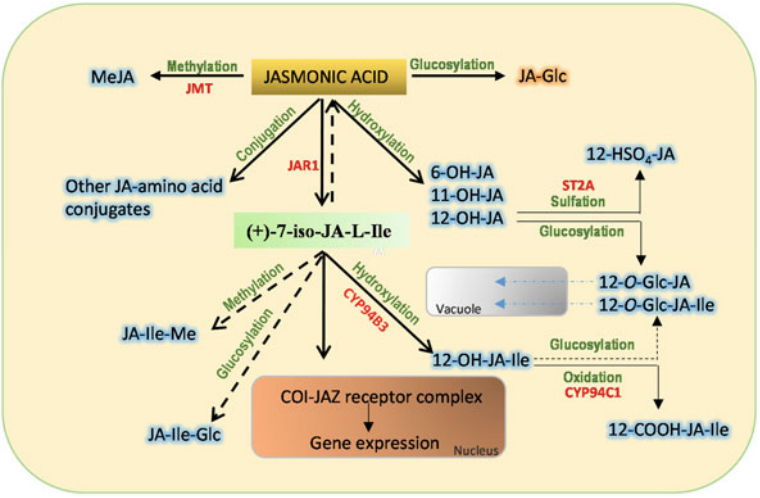

Fig. 3 Scheme of JA metabolic pathway in *Arabidopsis thaliana*. JAR1, jasmonoyl isoleucine synthetase; JA-Ile-12-hydroxylase; CYP94B3; 12-OH-JA-Ile carboxylase, CYP94C1; amidohydrolases; JMT, JA methyl transferase; ST2A, 12-OH-JA sulfotransferase. Solid lines indicate confirmed pathway whereas dotted lines indicate pathways for which there is little or no evidence

tions lead to the formation of compounds that are specific to certain stress responses and developmental processes.

6 Jasmonic Acid Signalling

JA signal transduction research has now grown by leaps and bounds from the initial discovery of the JA receptor to a complete definition of molecular components required to relay the JA signal. In the last decade, a vast amount of data has been accumulated by transcriptomic, lipidic, proteomic and metabolomic studies and many key players involved inperception of external signals, signal integration and finally in responses to development and stress have been identified.

In an attempt to identify the JA receptor, Turner and co-workers performed JA-insensitive mutant screens after EMS mutagenesis to identify individuals showing reduced or abolished JA regulated root growth inhibition on Murashige and Skoog medium containing COR, a phytotoxin produced *P. syringae* that mimics JA-Ile (Feys et al. 1994; Xie et al. 1998). The *coi1-1* mutant identified showed perturbations of JA response such as male sterility, anthocyanin accumulation, MeJA inhibited root growth, susceptibility to necrotrophic pathogens and insects; thus, suggesting a central role in JA signal transduction. Successive map based cloning strategy revealed that COI1 encodes a F-box motif and 16 imperfect leucine-rich repeats (LRRs) Further research indicated that COIlis related to human SkpZ and yeast Grrl and Arabidopsis TIRl with 34% identity (Xie et al. 1998). These two lines of evidences suggested COI1 as the receptor. However, various pull down experiments with COI1 failed to prove its putative receptor function.

In 2007, three independent research groups simultaneously identified JAZ proteins (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). JAZ proteins belong to a family of TIFY proteins that is named for the presence of a highly conserved TIF[F/Y]XG motif that is present within the ZIM (initially named for a zinc-finger protein expressed in the inflorescence meristem) domain of all family members (Vanholme et al. 2007; Bai et al. 2011) (Fig. 4b). The ZIM domain is responsible for JAZ dimerization and interaction with Novel interactor of JAZ (NINJA) (Fig. 4c). The basic difference between JAZ and other TIFY proteins is the presence of an approximately 27-amino-acid, multifunctional Jas motif located near the C terminus that facilitates interaction with various transcription factors (TFs) and COI1 and is defined by the $SLX_2FX_2KRX_2RX_5PY$ (Melotto et al. 2008; Chung et al. 2010). It is also known to repress JAZ proteins. The Arabidopsis genome encodes 13 JAZ proteins (JAZ1–JAZ13) that are classified into 5 phylogenetic groups (Thireault et al. 2015). These five groups are present in all angiosperms, with significant expansion of group I JAZ proteins in monocots. The repressive function of JAZ in JA-signalling came in light by studying the mutant of *JAZ3/JAI3* gene.*jai3-1* is deficient in the C-terminal region which disturbs its binding and degradation via SCFCOI1 complex, thereby leading to the accumulation of truncated JAI3/JAZ3 proteins in the mutant. This further blocks the JA-induced degradation of other JAZ proteins, thus, showing

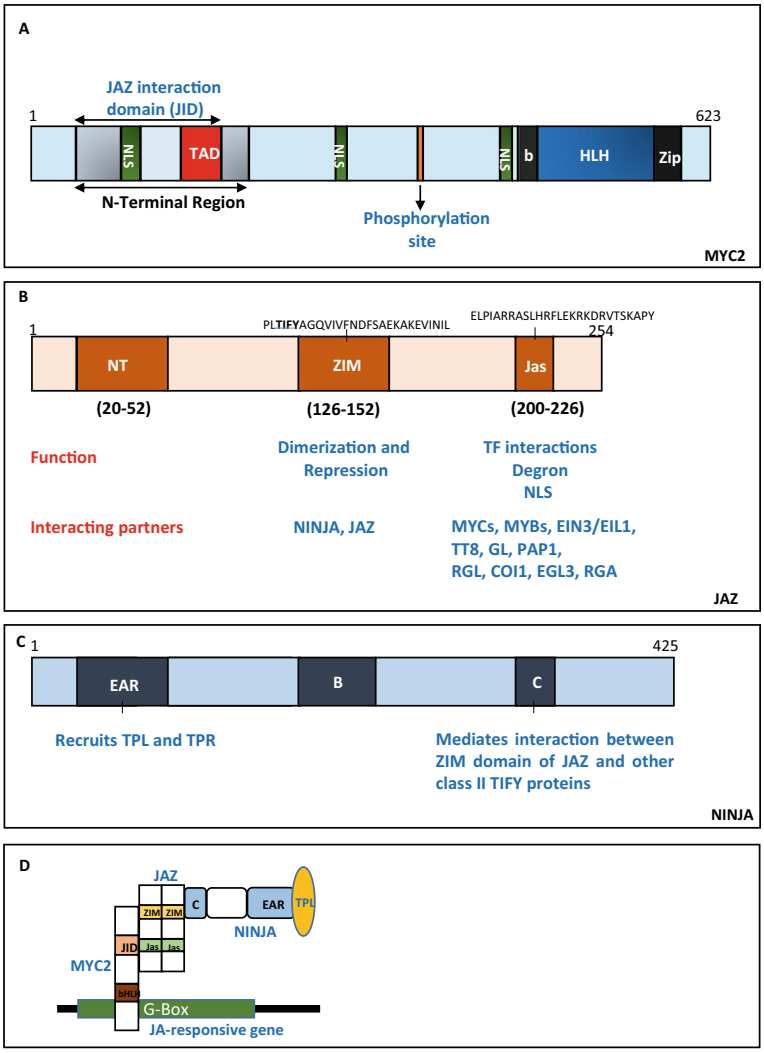

Fig. 4 Domain structure of MYC2, JAZ, NINJA and a hypothetical arrangement of interaction between MYC2-JAZ-NINJA. bHLH, basic helix-loop-helix; COI1, CORONATINE INSENSITIVE1; DBD, DNA-binding domain; EAR, ETHYLENE RESPONSE FACTOR (ERF)-associated amphiphilic repression; InsP5, inositol pentakisphosphatephosphate; JA-Ile, Jasmonate-isoleucine; JID, JAZ interaction domain; ZIM, Zinc-finger protein expressed in Inflorescence Meristems

dominant JA-insensitivity (Chini et al. 2007). Alternative splicing of JAZ genes leads to the formation of dominant JAZ variants. For example JAZ10 has naturally occurring splice variants that lack a part of the Jas motif (JAZ10.3) or complete Jas domain (JAZ10.4) (Yan et al. 2007; Chung et al. 2009, 2010). Recently it has been found that JAZ10 possess a cryptic MYC-interaction domain (CMID) near

the N terminus (Moreno et al. 2013) through which it retains the ability to repress JAs responses. X-ray crystallography studies comparing the structure of theMYC3–JAZ10CMID andMYC3–JAZ10Jas complexes showed that whereas the Jas motif binds MYC3 as a single continuous α-helix, the CMID adopts a bipartite structure in which one α-helix occupies the Jas-binding groove of MYC and a second helix makes contact with the backside of this groove (Zhang et al. 2017). This clamp-like action of the CMID engages MYC3 with higher affinity than the Jas helix does and also effectively masks the MED25 binding site of this MYC transcription factor. Functional CMIDs have been identified in other Arabidopsis JAZ proteins (e.g., JAZ1) and likely exist in other plants as well.

Furthermore, reports revealed the identification of the degron sequence (ELPI-ARRA) of some JAZ proteins (Sheard et al. 2010). The presence of the degron motif varies among different JAZ proteins, thus defining the distinct affinities in different JAZ-COI1 combinations and the physiological output of the JA pathway. For example JAZ8 possess PKASMK motif that shows limited affinity with COI1 (Shyu et al. 2012), thus suggesting distinctJA sensing properties. Besides this, JAZ8 mediated repression was shown to be dependent on the presence of EAR (ERF associated amphiphilic repression) motif at the N terminus (Shyu et al. 2012) that can directly bind the co-repressor TOPLESS (TPL) and TPL-related proteins (TPRs). These co-repressors mediate repression by recruiting histone deacetylases (HDAs) and demethylases (JUMONJI8) that cause chromatin modifications leading to suppression of gene expression (Long et al. 2006). JAZ1, JAZ3, and JAZ9 can physically interact with HDA6 (Zhu et al. 2011). Other JAZ proteins lack this EAR motif execute repression by recruiting an adapter protein called NINJA that further interacts with co-repressors TPL/TPR. The EAR motif containing A domain of NINJA is required for recruiting TPL and TPL-related proteins, whereas the C domain of NINJA facilitates the interaction with the ZIM domain of the JAZ and other class II TIFY proteins (Pauwels et al. 2010).

Earlier COI1 was assumed to be the JA receptor on the basis of its analogy to auxin receptor TIR1. However, 10 years after cloning of COI1, its function as the JA receptor is now established (Xie et al. 1998). COI1 interacts with the Jas domain of JAZ proteins in the presence of the bioactive ligand, JA-Ile and forms a loop that traps JA-Ile in the ligand binding pocket, thus forming a stable COI1–JA-Ile–JAZ ternary complex. The interaction occurs via N terminal $SLX_2FX_2KRX_2RX_5PY$ conserved pattern present in Jas domain and is strongly increased by the presence of inositol pentakisphosphate (IP_5) (Sheard et al. 2010; Mosblech et al. 2011).

JAZ repressors show strong affinity for basic-helix-loop-helix (bHLH) TFs such as MYC2, MYC3, and MYC4 as well as other TFs such as EGL1 (ENHANCER OF GLABRA3 1), TT8 (TRANSPARENT TESTA8), GL3 (GLABRA3) among a few (Cheng et al. 2011a; Fernández-Calvo et al. 2011b; Niu et al. 2011a; Qi et al. 2011). MYC TF possess a JAZ interaction domain (JID) at the N terminus (Chini et al. 2007; Fernández-Calvo et al. 2011a) (Fig. 4a). The JID domain is also present in other TFs such as GL3, TT8 etc. In its N-terminal, there is a transcriptional activation domain (TAD) required for interaction with the mediator complex and transactivation. TAD specifically interacts MED25 subunit of the plant Mediator complex (Çevik et al.

2012; Chen et al. 2012). MED25 recruits HAC1 to catalyze histone 3 Lys 9 (H3K9) acetylation near the transcriptional start sites of MYC2 target genes (An et al. 2017). A conserved bHLH domain at C terminal is required for homo and hetero-dimerization of MYCs (Fernández-Calvo et al. 2011a). The C terminal leucine zipper domain possibly functions as an additional dimerization domain that affects the specificity of interaction with other TFs (Amoutzias et al. 2008). The basic domain present next to bHLH domain is required for binding to the G-box (5′-CACGTG-3′) present in MYC2 target promoters (Carretero-Paulet et al. 2010). A phosphorylation site consisting of serine (S) residues is also present (Sugiyama et al. 2008). However, the consequence of this phosphorylation is still unknown.

Stable transformation in Arabidopsis and transient expression in tobacco cells led to the identification of nuclear localization of MYC (Lorenzo et al. 2004) (Monte et al. 2014). Later on MYC3 and MYC4 were also found to be nuclear localized (Lorenzo et al. 2004). MYC2 shows strong affinity for the 5′-CACNTG-3′ sequence (E-box), G-box and its variants such as 5′-AACGTG-3′ and 5′-CATGTG-3′, 5′-CACGAG-3′, 5′-CACGCG-3′ (Boter et al. 2004; Chini et al. 2007; Dombrecht et al. 2007). Of all the JAZ targets, MYC2 is called the master regulator of JA signalling since it acts as both activator and repressor of distinct JA-responsive gene expressions in Arabidopsis (Lorenzo et al. 2004). At one hand it activates JA-induced root growth inhibition, anthocyanin biosynthesis and oxidative stress tolerance whereas, on the other hand MYC2 inhibits tryptophan biosynthesis, indole glucosinolates and in mediating resistance to necrotrophic pathogens (Dombrecht et al. 2007). PDF1.2, CHIB, PR4 are some of the important genes that are negatively regulated by MYC2 (Lorenzo et al. 2004).

There are other factors that function downstream to MYC2. Genetic and biochemical approaches have identified ANAC019 and ANAC055 that positively regulate JA induced *LOX2* and *VSP1* expression downstream of COI1 and MYC2 (Bu et al. 2008). MYC2 interacts with a variety of factors such as key components of circadian clock TIME FOR COFFEE (TIC) which negatively regulates JA signalling. TIC is known to inhibit MYC2 accumulation, and thus repress COI1 expression (Shin et al. 2012). Yeast-two-hybrid (Y2H) screening using JAZ as a bait helped in identifying MYC3 and MY4 (Cheng et al. 2011b; Fernández-Calvo et al. 2011b; Niu et al. 2011b). They do not have much role in root growth inhibition as compared to MYC2, but show strong involvement in the expression of wound responsive genes; thus indicating redundancy in their family. Y2H screening also identified MYB21 and MYB24 using JAZ1 and JAZ8 as targets (Song et al. 2011). MYCs also associate with MYB21 and MYB24, which themselves interact with JAZ to control stamen and pollen development in a tripartite JAZ–bHLH–MYB complex (Song et al. 2011; Figueroa and Browse 2015; Qi et al. 2015; Goossens et al. 2017). The homologous JAZ–MYC–MYB complexes regulate anthocyanin biosynthesis in the epidermis of apple fruit (An et al. 2016) and fiber initiation in cotton (Hu et al. 2016) indicates that these JAZ–transcription factor modules are widespread in the plant kingdom.

7 From JA-Ile Perception to Transcriptional Activation-Mechanism of JA-Induced Gene Expression

In the absence of a stimulus (Fig. 5a), the cellular levels of JA-Ile are below a threshold concentration. During this, Jas motif of JAZ repressors adopts an extended α-helix conformation that binds to the JID near the amino terminus of MYCs (Zhang et al. 2015). Structural analysis of JAZ9-MYC3 complexes have shown that JID and TAD of MYC2 functionally overlap to form a continuous groove that encompasses the Jas helix (Zhang et al. 2015). Jas helix competitively inhibits MYC binding to the ACID of MED25 of the mediator complex. As a result, JAZ binding to JID-TAD region obstructs MYC-MED25 coupling. JAZ proteins also stop MYC activity by recruiting TPL that interacts with HDA6 and HDA19 to induce gene silencing (Pauwels et al. 2010; Ke et al. 2015; Zhang et al. 2015). An and co-workers have showed that MED25 and JAZ1 can simultaneously bind to MYC2 in the repressed state and MED25-MYC2 interactions are enhanced only upon JA-Ile elicitation (An et al. 2017). Upon stimulation (Fig. 5b), there is a hormone dependent formation of a co-receptor complex consisting of JA-Ile, JAZ and COI1. The conserved degron sequence forms a loop that traps JA-Ile in the ligand binding pocket, thus forming a stable COI1–JA-Ile–JAZ ternary complex (Sheard et al. 2010). The C terminal region of the Jas α-helix appears to dock the Jas peptide to the surface of COI1. JAZ proteins that are recruited to SCFCOI1 in this hormone-dependent manner are tagged with polyubiquitin chains and subsequently degraded by the 26S proteasome This leads to the disassociation of co-repressor modules (NINJA-TPL) from the promoter of MYC TF and to permit binding of MED25 and engagement of RNA polymerase II via the Mediator complex, thus establishing an activated transcriptional state (Fig. 4d) (Browse 2009a; Fonseca et al. 2009a; Koo and Howe 2009; Pauwels and Goossens 2011)).

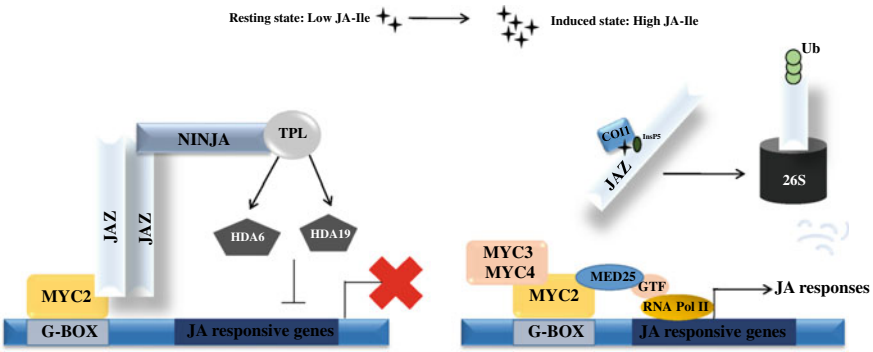

Fig. 5 Diagrammatic representation of JA perception and signal transduction pathway

8 Role of Jasmonates in Modulating Root System Architecture (RSA)

The inhibitory role of JA was first elucidated in late 1900's when it was observed that JA promotes senescence and growth inhibition (Ueda and Kato 1980; Dathe and Sembdner 1981). The first mutant known to be insensitive to JA application was *jar1-11* which was later cloned and characterised as JA-Ile synthase (Staswick et al. 1992). Apart from this, there are other components of JA biosynthesis and signal transduction that show inhibition of root growth and development. For example, mutations in COI1 leads to insensitivity to JA mediated root growth inhibition (Xie et al. 1998; Yan et al. 2009). The co-receptor complex formed by COII1-$InsP_5$-JAZ also show reduced root growth. Reports suggests that $InsP_5$ not only increases the interaction between COI1-JAZ but also the inhibition of root growth by JA (Mosblech et al. 2011). COI1 has been shown to be at interface of JA and ethylene signalling in mediating root growth inhibition. Previous reports show that *coi1-16* shows unresponsiveness to ethylene induced root growth inhibition in light but not in dark. Also this response did not require any other components of JA biosynthesis and signalling such as *jar1-1, jin1, aos* and *opr3*. Thus, the inhibition of Arabidopsis root growth to ACC is light, *COI1* dependent but JA independent and occurs due to inhibition in cell elongation (Ellis and Turner 2002; Adams and Turner 2010). The interaction between auxin and JA in regulating root growth has been very well demonstrated in the past. Root growth has been shown to be under the regulation of *PLETHORA* gene family, required to maintain stem cell niche and cell proliferation (Mähönen et al. 2014). Previous reports suggest that MYC2 directly causes transcriptional repression of *PLT1* and *PLT2* leading to root growth inhibition (Fig. 6) (Chen et al. 2011). Moreover, JA may increase auxin levels by inducing the expression of auxin biosynthetic gene *ANTHRANILATE SYNTHASE 1* (*ASA1*), thereby leading to root growth inhibition (Fig. 6) (Sun et al. 2009). Previous reports claim that JA might also work via an auxin independent mechanism to inhibit PR and growth in the seedlings of *Helianthus annuus* (Corti Monzón et al. 2012). Monzon and co-workers demonstrated that both endogenous and exogenous JA are required to inhibit PR length. Addition of ibuprofen, a potent JA biosynthesis inhibitor caused increase in PR and LR length. Also, when PAT inhibitor NPA was applied, JA was still able to exert its repressive effect. Besides, the auxin produced its phenotype even when ibuprofen was applied. Hence, contrary to general notion, JA also works via an auxin independent pathway (Corti Monzón et al. 2012). A recent report highlights the synergistic role of JA and auxin in stem cell activation and root regeneration. Wound induced JA causes ERF109 activation which further stimulates *CYCD6;1* and *ERF115*. They then modulate RBR-SCR module to allow root tissue regeneration. Upon wounding, auxin accumulation also takes place that then activates several regeneration regulators of this pathway (Zhou et al. 2019). Crosstalk between JA and cytokinin (CK) remain largely unknown. A recent report has shed light on CK-JA crosstalk in xylem development in Arabidopsis roots. CK acts as a negative regulator of xylem differentiation. Studies using the *wooden leg (wol)* mutants with defects in cytokinin signalling show

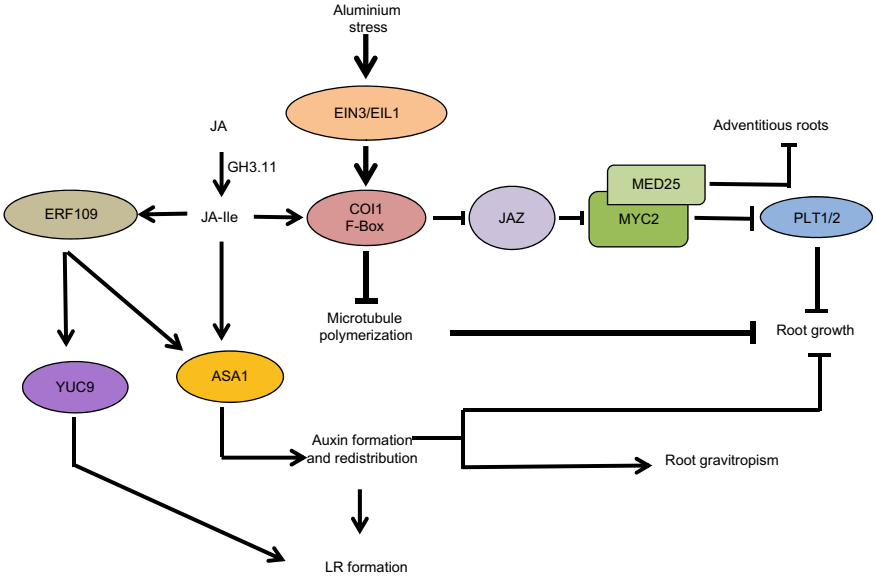

Fig. 6 Role of JA in modulating various parameters of RSA. JA inhibits root growth via multiple ways. JA activates MYC2 which represses PLT1 and PLT2 expression resulting in root growth inhibition. JA also impedes root growth by modulating auxin levels through ASA1 induction. Under Al stress condition, JA causes microtubule depolymerization and thereby inhibition of root growth through COI1. JA positively regulates lateral root formation by modulating auxin biosynthesis and homeostatsis. JA induces the expression of ASA1 both through COI1 and ERF109. ERF109 also induce the expression of another auxin biosynthetic gene YUC9. In Arabidopsis, JA signalling inhibits adventitious root formation

an all xylem phenotype. Additionally, *arr1*, *arr10* and *arr12* mutants or transgenic plants overexpressing *AHP6*, a negative regulator of cytokinin signalling have been shown to form extra xylem (Jang et al. 2017). Similarly, *opr3* mutants showed extra xylem, however, *coi1* and *jar1-11* did not (Jang et al. 2017). Hence, suggesting that JA suppresses CK response and that the effect of JA on extra xylem formation is nullified by cytokinin, thus, there is an antagonistic interaction occurring between JA and CK in xylem formation and differentiation in Arabidopsis roots (Jang et al. 2017).

Jasmonic acid also contributes to inhibition of root growth under stress conditions. Earlier reports by Kang et al. (2005) have shown that the exogenous application of JA on rice recovered salt-induced damages in terms of root dry weight in salt sensitive cultivar as compared to the tolerant cultivar by decreasing salt induced damaged caused by Na and increasing the level of Mg, K and Ca levels (Kang et al. 2005). Another report highlights the involvement of *COI1* dependent root growth inhibition under aluminium stress (Fig. 6) (Yang et al. 2017). The expression of COI1 and MYC2 was up-regulated in response to Al stress in the root tips. This process together with COI1-mediated Al-induced root growth inhibition under Al stress was

controlled by ethylene but not auxin. The differential responsive of microtubule organization-related genes between the wild-type and *coi1-2* mutant is consistent with the changed depolymerization of cortical microtubules in *coi1* under Al stress. In addition, Aluminium activate malate transporter (ALMT)-mediated malate exudation and thus Al exclusion from roots in response to Al stress was also regulated by COI1-mediated JA signalling and not auxin signalling. Thus, suggesting that auxin and JA act independently in regulating Al stress (Yang et al. 2017). A very recent report highlights the activation of JA signalling under hypoxia that negatively affect PLT2, PLT3 and WOX5 activity and that the oxygen-sensing transcription factor RAP2.12 can directly induce JAZs to establish feedback inhibition (Shukla et al. 2020).

Like root growth, LR formation and development is a complex process that is governed by hormone and environmental crosstalk. Analogous signalling mechanisms of JA and auxin point out on the role of JA in regulating LR formation development. For example, a previous report demonstrated the presence of LR primordia in the promoters of all four *AOC* genes (Stenzel et al. 2012). Additionally, data from transcriptional studies have shown that JA promotes LR initiation and growth in a COI dependent manner. Sun and co-workers have shown that MeJA induces the expression of *ASA1* that ultimately lead to increased local auxin accumulation in the root basal meristem (Fig. 6). They have also showed that JA-induced LRP initiation was repressed in *asa1-1* (Sun et al. 2009). COI1 is also involved in JA-induced pericycle cell activation and LR formation, positioning and emergence on bends and requires a canonical auxin signalling pathway (Raya-González et al. 2012). LR development by JA is also triggered by the expression of *ETHYLENE RESPONSE FACTOR 109 (ERF 109)* which then binds to the promoters of *ASA1* and *YUCCA 9 (YUC9)* (Fig. 6) (Cai et al. 2014). Apart from having a positive role in LR formation, a very recent report highlights the negative role of JA in regulating auxin induced LR formation, that was shown to be independent of COI receptor (Ishimaru et al. 2018). Higher concentrations of (−)JA and (+)JA were shown to counter the promotory effect of auxin by stabilizing DII-VENUS and suppressing the expression of *PUCHI* and *LATERAL ORGAN BOUNDARIES-DOMAIN 29 (LBD29)*, involved in LR formation (Ishimaru et al. 2018).

Lateral and adventitious roots mostly share common regulatory networks except a few regulatory mechanisms which distinguish lateral and AR pathways (Wilmoth et al. 2005; Liu et al. 2014). JA has shown to differentially regulate AR formation in different species. In Arabidopsis, JA has been shown to inhibit AR development and works downstream of auxin pathway (Gutierrez et al. 2012). In Arabidopsis hypocotyls, JAR1 has shown suppression of adventitious rooting. Enhancement of adventitious rooting was observed in the leafy cuttings of *Petunia hybrid* (Lischweski et al. 2015). They found that in the *PhAOC* RNAi construct, there was less accumulation of wound induced JA that resulted in the formation of lower numbers of ARs. Very recently, Druege and co-workers proposed that early wound-induced induction of JA stimulates AR induction possibly via enhanced IAA accumulation (Druege et al. 2019). Recently Lakehal and co-workers proposed that TIR1 and AUXIN SIGNALLING F-BOX 2 (AFB2) interact with IAA6, IAA9 and IAA17 to control JA homeostasis and AR initiation in Arabidopsis (Lakehal et al. 2019a). They also

proposed a feedback circuit between IAA and JA where they have demonstrated that DIOXYGENASE FOR AUXIN OXIDATION (DAO1) converts free IAA to oxindole-3-acetic acid (oxIAA), hence leading to reduced free IAA level and AR initiation in Arabidopsis. They also showed that the expression of DAO1 is induced by JA signalling (Lakehal et al. 2019b).

JA is also found to be a crucial regulator of root gravitropism. Studies on rice coleoptiles revealed an increased amount of JA upon gravistimulation. They also observed the formation of a JA gradient opposite to the internal auxin gradient across the stimulated organ during gravitropic response that worked in an IAA manner. A JA deficient rice mutant *hebiba* was identified that bended slowly upon gravitropic stimulus, thus, suggesting that JA might accelerate the bending response (Gutjahr et al. 2005). Moreover, JA-Trp, an IAA antagonist has been reported to cause agravitropism. The response has been shown to be TIR1 dependent but COI1 independent (Staswick 2009), suggesting that another branch of JA signalling is also involved in regulating gravitropic responses. JA also influences gravitropism via ASA1 which further leads to changes in auxin homeostasis (Fig. 6). Apart from the main root, other organs also show gravitropic behaviour. Recent study by Sharma and co-workers have shown that apart from auxin that causes vertically oriented LRs, JA also influences this behaviour and work upstream of auxin transport and signalling module (Sharma et al. 2020). They also showed that this JA-auxin regulation of LR angle occurs via MYC2 that directly activates the transcription of *CYP79B2*, an auxin biosynthetic gene and *LAZY2/4*. In addition, JA treatment also affects PIN2 localization in the stage II LRs, leading to a downward LR orientation (Sharma et al. 2020).

9 Conclusions and Perspective

In the recent years, there have been significant advances in understanding the mechanism of JA biosynthesis and signalling. An increasing number of new partners involved in JA biosynthesis, metabolism and signalling have been identified and many new players are expected to be identified with the omics studies. For example, recently, an alternative JA biosynthesis pathway has been identified which is found to be OPR3 independent (Wasternack and Hause 2018). Also, fine-tuning of JA responses is dependent on the homeostasis between active and inactive JA compounds. Newer and sensitive methods have enabled the identification of new active and inactive JA compounds. However, extensive work is required to understand how these derivatives are involved in systemic responses. Moreover, how these derivatives regulate the downstream signalling cascade is another challenge that will enhance our knowledge in understanding JA signalling networks.

The action of JA is not only restricted to mitigate biotic and abiotic stressors or understanding plant-plant/plant-herbivore/plant–microbe interactions. JA is widely known to regulate various physiological parameters of the root system. However, the action of JAs largely depends on specific hormone combinations. This chapter has focussed on the regulatory role of JA in modulating RSA, citing various examples of

crosstalk of JA with auxin and other plant hormones. Additionally, several reports in this chapter have made clear that the action of JAs require the modulation of auxin homeostasis (Sun et al. 2009; Ishimaru et al. 2018). Moreover, these interactions do not occur via simple linear mechanisms, rather, a number of downstream effectors and numerous feedback loops are involved in driving a single developmental process. However, there are still lots of gaps in understanding the underlying mechanisms of root development and there is a need to identify newer components involved in regulating root growth and development. The advancement of newer technologies and system biology aided approach will enable us to integrate information related to the overall organization of how roots sense various endogenous and environmental signals, and how they turn them into cellular responses.

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Serotonin and Melatonin: Role in Rhizogenesis, Root Development and Signaling

Madhumita Banerjee and Suman Sharma

Abstract The indoleamines serotonin (5-hydroxyl-tryptamine) and melatonin (*N* acetyl 5 methoxy-tryptamine) are naturally occurring signaling molecules first discovered in animals but now known to be present in plants from diverse angiosperm families. They perform multiple functions and impact diverse aspects of a plants life. Their role in rhizogenesis is well documented. Melatonin and serotonin induce root elongation, formation and growth of lateral and adventitious roots and root hair development thereby altering the root architecture. Their mode of action in rooting appears to be diverse—they may act in concert with auxins or through independent signaling pathways. As mediators of biotic and abiotic stress, there is considerable interaction of these molecules with ROS and NO species and crosstalk with other plant growth regulators. Analysis of transcript and gene expression profiles has provided insights into the mechanism and pathway of melatonin/serotonin induced promotion of root induction and growth. As of today no receptor has been identified for serotonin. A receptor for melatonin was reported in *Arabidopsis thaliana,* but a subsequent report identified the protein as being located in the cytoplasm.

Abbreviations

AR	Adventitious root
ASMT	*N* Acetyl serotonin *O*-methyl transferase
Col1	Essential gene in JA signaling
CAMT	Caffeic acid *O*-methyl transferase
DEG	Differentially expressed genes
Et	Ethylene
GSNOR	*S* Nitrosoglutathione reductase
HSP	Heat shock protein
JA	Jasmonic acid
JAR1	Essential gene in JA signaling

M. Banerjee (✉) · S. Sharma
Department of Botany, Ramjas College, University of Delhi, Delhi 110007, India

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LR	Lateral root
LRP	Lateral root primordia
MAPK	Mitogen activated protein kinase cascade
NO	Nitric acid
NR	Nitrate reductase
PAT	Polar auxin transport
RAM	Root apical meristem
RCD 1	Radical-induced cell death 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNAT	Serotonin *N* acetyl transferase
TF	Transcription Factor
T5H	Tryptophan 5 hydroxylase
TDC	Tryptophan decarboxylse
TIBA	Tri-iodo benzoic acid
WT	Wild type

1 Introduction

The indole amines melatonin (*N* acetyl 5 methoxy tryptamine) and its precursor serotonin (5-hydroxy tryptamine) are naturally occurring signaling molecules that mediate a range of physiological activities in humans, animals and plants (Arnao 2014; Erland et al. 2015).

In plants serotonin was discovered in the medicinal herb *Mucuna pruriens* L. (Bowden et al. 1954) in 1954. Melatonin was isolated from bovine pineal gland in 1958 (Lerner 1958) and was considered to be a molecule unique to the animal kingdom until it was identified in the unicellular dinoflagellate *Lingulodinium polyedrum* (syn. *Gonyaulux polyedra*) (Balzer and Hardeland 1991). In higher plants melatonin was detected in the ivy morning glory (*Pharbitis nil* L. syn. *Ipomoea nil* L.) and fruits of *Solanum lycopersicum* (van Tassel and O'Nielle 1993; van Tassel et al. 1995) in *Chenopodium rubrum* (Kolar et al. 2003) and in *Nicotiana tabacum* and many edible plants (Dubbels et al.1995; Hattori et al.1995).

Melatonin is present in more than 300 plant species representing almost all angiosperm families (Paredes et al. 2009; Simlat et al. 2018; Yan et al. 2020), while serotonin is reported from a fewer but significant number of plants (Gonzalez-Gomez et al. 2009; Huang and Mazza 2011), including many edible, medicinal and horticultural plants (Chen et al. 2003; Reiter et al. 2007; Jemima et al. 2017; Arnao and Hernadez-Ruiz 2018; Yan et al. 2020) in endogenous concentrations ranging from pico to micrograms per g of dry tissue (Jemima et al. 2017; Mir et al. 2020).

Melatonin occurs in almost all plant tissues—root, shoots, leaves, flowers, fruits, seeds and bulbs (Nawaz et al. 2016). Melatonin levels are usually high in seeds and low in fruits and show a gradient from high to low in seeds, leaves, roots, flowers

and fruits in many plants (Arnao 2014). Endogenous concentration of melatonin can vary depending on the genotype, external factors like temperature, photoperiod and developmental stage (Zhao et al. 2012). Endogenous levels of melatonin are enhanced by biotic and abiotic stress (Reiter et al. 2015). A concentration gradient of melatonin, from apical to basal parts of the hypocotyl and root, similar to the gradient exhibited by auxins, was seen in *Lupinus albus* L (Arnao and Hernandez–Ruiz 2006).

Serotonin has been found in roots, leaves, fruits and seeds of several plant species (Erland et al. 2018). The endogenous content of serotonin is also known to vary in response to developmental and seasonal changes, under light and dark conditions, and is upregulated in response to biotic stress (Ramakrishna et al. 2011; Ishihara et al. 2008). In Rice, two TDC-like enzymes involved in the biosynthesis of melatonin and serotonin from tryptophan, encoded by LOC_Os08g04540-*TDC1* and LOC_Os08g04560-*TDC3*, are highly induced by both abiotic and biotic stresses. Both *TDC1* and *TDC* 3 are induced by a broad spectrum of pathogens (Dharmawardhana et al. 2013). Melatonin is a good bio-stimulator, improving not only seed germination, seedling/plant growth but also crop production especially under stress conditions.

Endogenous concentrations of melatonin and serotonin are reported to increase at specific stages of flower development in *Datura metel* L. (Murch et al. 2010) and synchronously with circadian rhythms in *Chara australis* (Beilby et al. 2015).

Melatonin and serotonin influence almost all aspects of a plants growth and survival by regulating germination and seedling development (Simlat et al. 2018), morphogenesis (Ramakrishna et al. 2009), parthenocarpy (Liu et al. 2018) root induction, growth and architecture and tropic responses (Pelagio-Flores et al. 2011, 2012; Arnao and Hernandez-Ruiz 2014), delaying senescence (Kang et al. 2009) flowering (Kolar et al. 2003), tolerance to biotic (Ishihara et al. 2008) and abiotic stress (Posmyk et al. 2008; Lei et al. 2004; Bajwa et al. 2014) improving iron deficiency tolerance by inducing Fe-responsive gene expression (Wan et al. 2018) Serotonin is present in the xylem and phloem parenchyma cells suggesting its involvement in maintaining the cellular integrity for facilitating efficient nutrient recycling (Ramakrishna et al. 2009).

Melatonin is an evolutionarily ancient, conserved molecule. As a strong antioxidant (Tan et al. 2015) and a scavenger of reactive oxygen and nitrogen species, melatonin protects plants from oxidative stress and stress due to salinity, heavy metals, drought, extreme temperature, herbicides, pathogens and senescence (Mukherjee et al 2014; Bajwa et al. 2014); Lei et al. 2004; Hardeland 2009; Arnao 2014; Shi et al. 2015b; Pelagio-Flores et al. 2016). Melatonin accumulation in response to these factors acts as a signal to activate transcription factors and antioxidant genes that mitigate these diverse biotic and abiotic stresses. The growth promoting role of melatonin was demonstrated in *Lupinus* albus (Hernandez-Ruiz et al. 2004). In the rooting processes of primary, secondary and adventitious roots, melatonin regulates the expression of many factors, such as PIN auxin transporters and AUX1, and others (Arnao and Hernadez-Ruiz 2018; Wen et al. 2016; Wang et al. 2016).

2 Biosynthetic Pathway

In plants, the indoleamines melatonin and serotonin are synthesized primarily in the root through a common biosynthetic pathway operative in chloroplasts and mitochondria (Tan et al. 2013; Tan and Reiter 2019; Wei et al. 2021). Tryptophan, biosynthesized through the shikimic acid pathway, is the common precursor for the synthesis of serotonin, melatonin, auxin as well as indole alkaloids. TDC is a rate limiting step in melatonin biosynthesis (Zhao et al. 2018).

The biosynthetic pathway for serotonin and melatonin was first described by Murch et al. (2000) in *Hypericum perforatum* L. through the use of C14 labeled tryptophan. The pathway (classical pathway) is almost identical in animals and plants. An alternate pathway for melatonin biosynthesis was proposed (Tan et al. 2016; Geun-Hee et al. 2017) Melatonin is synthesized from tryptophan through four reaction steps involving several enzymes (Fig. 1).

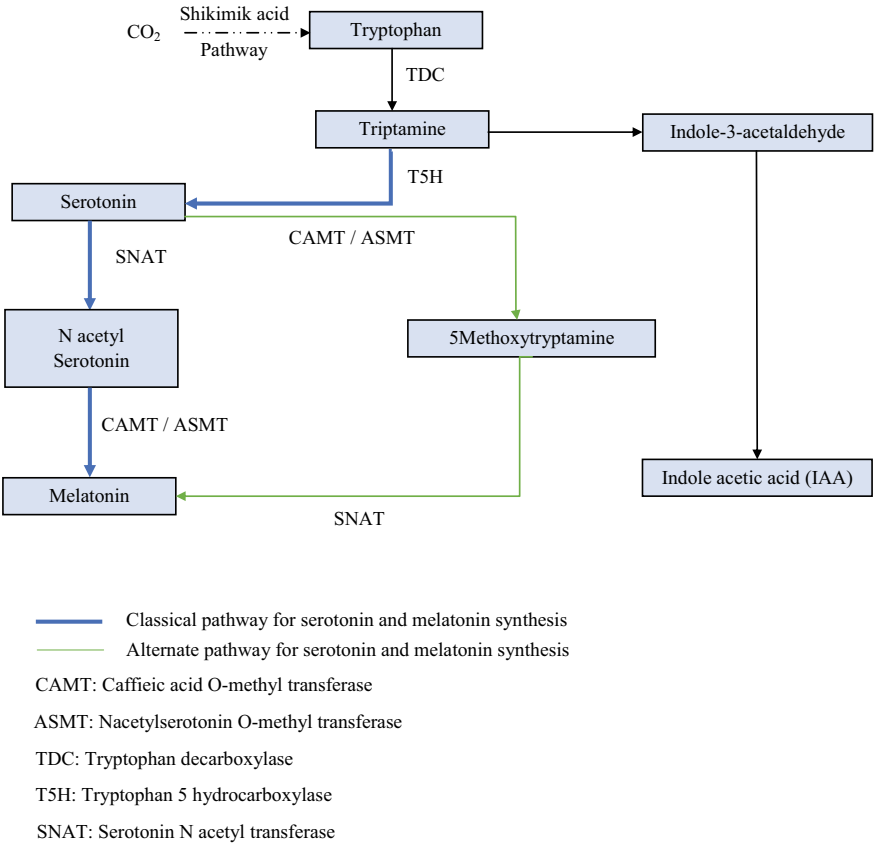

Fig. 1 Biosynthetic pathways of serotonin, melatonin and IAA in plants

Genes of the biosynthetic pathway have been identified and cloned in rice, apple, cassava (Kang et al. 2012; Lee et al. 2014; Lei et al. 2013; Wei et al. 2021). Melatonin can regulate its own biosynthesis by upregulating the gene expression SNAT, ASMT, and CAMT. Over-expression of TDC increases the content of 5-hydroxytryptamine and also confers stress resistance (Kang et al. 2007; Yu et al. 2019; Moustafa-Farag et al. 2020).

Upstream TF s of some melatonin biosynthesis enzymes have been identified. These include heat shock factor 20 (Me Hsf20), Me WrKY79 which bind to the promotor region of MeASMT 1. Transcripotome analysis has revealed the network between melatonin biosynthetic enzymes and signaling pathways for stress resistance (Wei et al. 2021). Melatonin biosynthetic enzymes also interact with ascorbate peroxidase resulting in improved oxidative stress resistance (Bai et al. 2020). Abiotic stress induces an increase in endogenous melatonin through the upregulation of melatonin biosynthetic genes. This is a useful strategy, given the strong antioxidant property of melatonin.

Melatonin can be metabolized to hydroxyl melatonin, β hydroxyl melatonin or converted to a cyclic form. Several studies have reported that the melatonin metabolite, cyclic-3-hydroxymelatonin, is more potent than melatonin to scavenge hydroxyl radical and other ROS (Tan et al. 2015).

3 Role of Melatonin and Serotonin in Rooting

Root initiation, development and architecture are critical for the growth and survival of plants. Besides anchorage, roots source water and nutrients from the soil and are exposed to a multitude of biotic and abiotic stress. Roots are the primary interface between the plant and the soil, and they sense and respond to unfavorable soil environments, enabling plants to overcome these stress related challenges. The primary root (PR) is embryogenic in origin, being formed from the radical and developed by the activity of the root apical meristem. Lateral roots (LR) are initiated from primordia formed on the pericycle of the primary root (Pelagio-Flores et al. 2011). The lateral roots (LR) in turn produce many more LRs which together form a robust root system. Adventitous roots (AR) are formed by redifferentiation of meristematic tissue at the base of the stem, after removal of the primary root system. Auxins establish a new meristem at the base of the stem (Pagnussat et al. 2004). Root hairs arise from specialized epidermal cells called trichoblasts, and vastly increase the root surface area (Slovak et al. 2016).

The redirection of plant growth is initiated by changes in the relative ratio of plant growth regulators, auxin and cytokinin (Skoog and Miller 1957). Cytokinin signaling leads to the formation of embryonic root but cytokinin induced disruption of the auxin gradient leads to the formation of lateral roots (Werner et al. 2009). The control of post-embryonic root growth and LR formation is tightly regulated by auxin (IAA). IAA moves throughout the plant in the phloem or by a more controlled polar transport system (polar auxin transport (PAT) (Baluska et al. 2010). PAT is

regulated by AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX) uptake proteins, PIN-FORMED (PIN) efflux carriers and P-GLYCOPROTEIN (MDR/PGP/ABCB) efflux/conditional transporters (Swarup et al. 2008; Mravec et al. 2009; Slovac et al. 2016).

Differentiation is now recognized as a complex process regulated by multiple endogenous factors like hormonal interactions and signals, nutrient status and external factors like biotic and abiotic stress and light and temperature regimes (Casimiro et al. 2003; Lopez-Bucio et al. 2003; Peret et al. 2009).

The roles of melatonin and serotonin in plant growth and differentiation is well documented (Hardeland 2015; Erland et al. 2015, 2018; Arnao and Hernandez-Ruiz 2018). Exogenous application of melatonin and serotonin promotes/inhibits elongation of the primary root and initiation and growth of lateral and adventitious roots in many plant species often resulting in an altered root architecture.

Murch et al. (2001) published one of the earliest reports on the effect of melatonin and serotonin on root organogenesis in the medicinal plant St. John's wort (*Hypericum perforatuum* L.). Low concentrations of IAA and melatonin decreased de novo root formation, while increased levels of melatonin led to a corresponding increase in root initiation. Increased serotonin levels increased shoot formation, indicating that a balance between endogenous levels of melatonin and serotonin regulates morphogenesis in plants.

In *Lupinus alba* (Hernandez–Ruiz et al. 2004) melatonin induced LR and AR formation from pericycle and stem cuttings at a similar concentration as IAA. The growth response was concentration dependent and exogenous melatonin could replace the auxin stimuli when the apical meristem was excised. This was the first experimental data that clearly demonstrated the auxinic role of melatonin.

IAA and melatonin induced rhizogenesis in hypocotyl cultures of *Lupinus albus* through the initiation of root primordia on the pericycle., producing an increased the number of newly formed lateral roots. The number and length of adventitious roots were also enhanced which impacted the root structure (Arnao and Hernández-Ruiz 2007). Since then a number of reports have confirmed the role of MT as well as 5HT in the induction of lateral and adventitious roots in a number of diverse species including *Arabidopsis thaliana*,*Brassica juncea*,*Vigna radiata*,*Oryza sativa*, *Prunus* and *Malus* species (Table 1).

4 Melatonin and Serotonin—Auxin like Function in Root Induction?

IAA and MT are structurally related, both are biosynthesized from tryptophan and elicit similar responses (Murch et al. 2000; Tan et al. 2016). In many plants exogenous MT up regulates endogenous IAA levels (Chen et al. 2009; Wang et al. 2016). However, the IAA content decreased in transgenics over expressing MT biosynthesis genes (Zuo et al. 2014). IAA improves root growth in a dose dependant manner, with

Table 1 Effect of melatonin and serotonin on root induction and growth

	Plant	Treatment	Observation	Reference
1	*Arabidopsis thaliana* transgenic line	Ten-day-old seedlings treated with melatonin/IAA/NAA	Enhanced number of lateral roots	Koyama et al. (2013)
2	*Arabidopsis* Ecotype Col-o	In vitro grown seedlings cultured on 10–300 μM serotonin	LR development at low concentrations (10–160 mM) higher concentrations inhibited PR growth and LR development	Pelagio-Flores et al. (2011)
3	*Arabidopsis thaliana* Col0, Ws and Ler ecotypes)	Etiolated hypocotyl from in vitro grown seedlings	melatonin did not inhibit primary root growth even at 200 μm, increased lateral root number and density in all three ecotypes	Pelagio-Flores et al. (2012)
4	*Arabidopsis* ecotype Columbia (Col-0), mutant and wildtype lines	In vitro raised seedlings	serotonin-induced root growth inhibition due to a ROS imbalance at the root tip in a process mediated by RCD1 and Et–JA crosstalk	Pelagio-Flores et al. (2016)
5	*Arabidopsis* Ecotype Col-o	3-day-old in vitro grown seedlings	Melatonin suppressed Pr growth in a dose dependent manner. Inhibited polar auxin transport	Wang et al. (2016)
6	Ecotype Col-o *Arabidopsis*	Melatonin and serotonin applied exogenously	PR growth unaffected LR formation increased at higher dose	Wan et al. (2018)
7	*Arabidopsis thaliana*	In vitro seedlings treated with ten pM to 500 micro-M Melatonin	Primary growth inhibited, lateral root growth enhanced in a dose dependent manner. Melatonin altered expression of 16 auxin related genes	Ren et al. (2019)

(continued)

Table 1 (continued)

	Plant	Treatment	Observation	Reference
8	*Brassica juncea* L	2 and 4 days old seedlings	0.1 microM melatonin stimulated root growth, 100 microM inhibitory for 2d old seedlings	Chen et al. (2009)
9	*Cucumis sativus* L	Seedlings treated with PEG and melatonin	Increase in number of lateral roots compared to control	Zhang et al. (2012)
10	*Cucumis sativus* L	Seeds primed in melatonin germinated in DW/PEG/Melatonin	n Melatonin improved root volume, diameter, improved water stress tolerance	Zhang et al. (2013)
11	Cucumis sativus L. cv. Jingyu-1	Seeds subjected to Nacl stress, treated with melatonin	Radical emergence 0.1–100 microM Melatonin reduced the inhibitory effects of high salinity on germination	Zhang et al. (2014)
12	St. John's wort (*Hypericum* perforatum L.)	In vitro raised shoot explants treated with IAA, melatonin, serotonin	Rooting increase in the endogenous concentration of melatonin increased root formation increased serotonin levels increased shoot formation	Murch et al. (2001)
13	*Hypericum perforatum L.* germplasm lines created by mutation and haploid breeding	medium supplemented with melatonin, serotonin, trptophan, intermediates of the biosynthetic pathway	Partially recovered growth and regenerative recalcitrance of the germplasm	Erland et al. (2018)
14	*Hypericum perforatum* L	Root explants from WT/anther culture (line112/mutant line 4 on modified MS medium with 10 μM tryptophan, or IAA	Shoot formation increased in line 112 but inhibited in line 4. Differential effect of short pulse and prolonged exposure	Erland and Saxena (2019)
15	*Helianthus annuus*	2.4.6.day old seedlings treated with melatonin, serotonin and Na Cl	Elongation of primary root	Mukherjee et al. (2014)

(continued)

Table 1 (continued)

	Plant	Treatment	Observation	Reference
16	*Hordeum vulgare*	Seeds germinated for 72 h on filter paper soaked with serotonin	Serotonin increased root length but had no effect on root weight and mitotic index	Csaba and Pal (1982)
17	*Lupinus albus L.* KBSH 44	6-day-old de-rooted lupin hypocotyls treated with melatonin and IAA	Formation of root primordia from pericycle cells, development of lateral and adventitious roots	Arnao and Hernández-Ruiz (2007)
18	*Malus prunifolia*	Nodal explants treated with melatonin. IBA	AR formation through increased function of *MdWOX11*	Mao et al. (2020a)
19	*Oryza sativa* cv. Dongjin (WT and transgenics expressing sheep SNAT)	3, 10 day old seedlings of WT and T3 transgenic lines	In transgenic lines, seminal root length was 75% longer, and root biomass increased 44% compared to WT In WT melatonin stimulated root growth at lower concentrations, inhibitory at higher concentrations	Park and Back (2012)
20	*Oryza sativa* ssp. Japonica	Seedlings in hydroponic cultures with melatonin (0, 10, 20, and 50 μmol/L)	embryonic and crown root elongation inhibited, number and length of LR increased in 10 and 20 μmol/L melatonin	Liang et al. (2017)
21	cherry rootstock PHL-C (*Prunus avium* L. x *Prunus cerasus*	Shoot explants (from in vitro regenerated plants) treated with melatonin	Melatonin increased root induction and root length Inhibitory at higher concentrations	Sarropoulou et al. (2012a)

(continued)

Table 1 (continued)

	Plant	Treatment	Observation	Reference
22	in vitro cultures of (*Prunus cerasus* L.), Gisela 6 (*P. cerasus, P. canescens*), and M, M 60 (*P. avium, P. mahaleb*	Shoot explants from in vitro regenerated plantlets	Melatonin increased the number roots number, length and percentage of rooting in three commercial cherry rootstocks at low concentrations. inhibited root growth, at higher concentrations	Sarropoulou et al. (2012b)
23	*Punica granatun* cv Wonderful	Shoot cuttings from four year old plant	Melatonin increased number and length of roots, improved rooting percentage	Sarrou et al. (2014)
24	*Solanum lycopersicum*	10 day old de- rooted seedlings treated with melatonin, NO donours and scavenger	Exogenous melatonin induced ARF, NO accumulation	Wen et al. (2016)
25	*Juglans nigra* x *Juglans regia* clone A35	Shoot explants from in vitro raised plants	Endogenous serotonin increased in the shoot top of shoots that rooted in an auxin containing medium. The level of serotonin was unchanged at the bases of these shoots	Gatineau et al. (1997)
26	Leaves of Aspen (*Populus tremuloides* x P. *trémula)*	Aspen leaves treated with crude extract of serotonin from embryos of *Juglans ailanthifolia* var. ailanthifoliaCarr	Serotonin influenced rhizogenesis in Aspen leaves cultivated in *vitro* to the same extent as indole-3-acetic acid	Regula et al. (1989)
27	*Vigna radiata*	Melatonin pretreated 3d old seedlings, chilled at 5 °C for 2 days and transferred to 25 °C	Root growth after transfer to 25 °C. 20% increase in root length in MT treated seeds over control	Szafrańska et al. (2012)
28	*Vitis vinifera* L Rootstock 5BB and cv. Cabernet Sauvignon	2-bud cuttings with basal ends dipped indifferent concentration of melatonin	Root induction genotype dependant. MT decreased rooting compared to IAA	Gokbayrak et al. (2020)

an inhibitory effect at higher concentrations. The root promoting effect of melatonin is also expressed in the formation of lateral and adventitious roots as a dose dependant response, but the stimulatory and inhibitory concentration may vary from plant to plant-eg. melatonin is inhibitory to cherry root stock at5mM whereas it is inhibitory at 100 mM in *Brassica juncea* roots (Sarropoulou et al. 2012a, b; Chen et al. 2009).

There are fewer reports of a stimulatory effect of melatonin and serotonin in the growth of primary roots. The growth-promoting effect of melatonin is high when a stress condition affects plant development, as in the case of salinity induced stress in *Helianthus* (Mukherjee et al. 2014) *Zea mays* (Ren et al. 2020; Su et al. 2021) and *Cynodon* (Oxidative stress) (Shi et al. 2015a, b, c), *Arabidopsis* (cold stress) (Bajwa et al. 2014). Arnao and Hernandez-Ruiz (2006) proposed that melatonin may have auxin like functions in the regulation of plant growth and development.

In *Arabidopsis thaliana* (Pelagio-Flores et al. 2012) primary root growth was unaffected at high concentrations (600-lm) of melatonin, but the number of lateral roots increased three -fold over the control at lower concentrations (150–600-lm) of exogenously supplied melatonin. The CycB1:uidA marker which is active only in mitotic cells, was not expressed in primary root tips. The marker was expressed in lateral root primordia (LRP) at the development stage 1 (as defined by Malamy and Benfey 1997). The lateral roots were produced by the maturation of preformed LRPs. Melatonin also failed to activate the auxin mediated degradation of the Aux/IAA protein indicating that melatonin action was through an auxin independent signaling pathway (Pelagio-Flores et al. 2012). In this study melatonin modulated root system architecture by stimulating lateral and adventitious root formation but minimally affected primary root growth and root hair development.

Pelagio-Flores et al. (2011) also investigated the role of serotonin in rhizogenesis and root growth in WT and transgenic lines *of Arabidopsis thaliana*. Serotonin treatment stimulated LRP formation by decreasing auxin responses during LRP development. Exogenous application of serotonin inhibited root developmental processes which are under auxin control, such as primary root growth, LR formation and root hair development. Serotonin blocked auxin-responsive DR5:uidA and BA3:uidA gene expression and auxin-regulated LR formation. Mutant analyses indicated that serotonin inhibited primary root growth and promoted adventitious root formation independently of the auxin-related loci axr2-1, axr4-1 and aux1-7 but required AXR1 and AXR2. This indicated that serotonin regulates root development probably by acting as a natural auxin inhibitor (Pelagio-Flores et al. 2011).

NaCl stress blocked IAA biosynthesis/transport resulting in accumulation of serotonin and melatonin in the roots and cotyledons of *Helianthus annuus* indicating that NaCl-induced endogenous serotonin accumulation possibly regulates root growth, independent of auxin action (Mukherjee et al.2014).

In *Arabidopis* moderate concentrations of melatonin and serotonin did not affect primary root (PR) growth but induced lateral root (LR) formation through the expression of cell-wall-remodeling genes *LBD16* and *XTR6* (Wan et al. 2018). The authors concluded that melatonin and serotonin do not have auxin-like activity.

5 Insights from Gene Expression Patterns

Techniques like RT PCR and in silico analysis pinned the role of melatonin and serotonin in mediating morphogenesis through altered transcription and gene expression patterns. Wang et al. (2016) found high concentration (600 μM) of melatonin inhibited root growth in *Arabidopsis thaliana* by reducing root meristem and down regulating auxin biosynthesis, the expression of PINFORMED (PIN-PIN1/3/7) proteins as well as the auxin response. The PIN formed proteins are secondary transporters involved in the efflux of auxin from cells. Transcription and protein expression levels of Pin 1,3 and7 were down regulated. Melatonin mediated decrease of the root apical meristem (RAM) was not altered by the auxin transport inhibitor TIBA suggesting that melatonin altered RAM by influencing polar auxin transport (PAT). Expression of key auxin biosynthesis genes (YUC1, YUC2, YUC5, YUC6 and *TAR2*) was also down regulated. This combined effect of melatonin- decreased auxin biosynthesis and altered PAT, resulted in a reduced root apical meristem and inhibited root growth. The authors proposed that melatonin regulates root growth in *Arabidopsis*, through auxin biosynthesis and polar transport, which cause optimal auxin accumulation and distribution in the root apex during the developmental process (Wang et al. 2016) In another study in *Arabidopsis* (Wan et al. 2018) abundance of the auxin carrier AUX1 and PIN 1,2,4,7 was unaltered in response to moderate concentrations of melatonin and serotonin. In *Malus domestica* increased IAA levels and overexpression of MdWOX11 led to increased AR formation in transgenic lines (Mao et al. 2020a, b).

Transcriptome analysis showed that melatonin regulates root development in a partially auxin-dependent manner in rice (Liang et al. 2017). Genome wide expression profiling by RNA-sequencing revealed that a total of 120transcription factors (TF), were up- or down-regulated in the melatonin treated samples compared with the control. The expression of roughly 25 auxin-induced TFs were upregulated whereas the expression of several auxin-inhibited TFs were down–regulated. Among 44 co-up- or co-down- regulated TFs, 21 genes, were specifically or primarily expressed in roots identifying these TFs as key regulators of melatonin signaling pathway (Liang et al. 2015, 2017).

Melatonin induced lateral roots had improved osmic tolerance (Zhang et al. 2013) Analysis of RNA seq profiles in melatonin treated seedlings of *Cucumis sativus* (Zhang et al.2014) revealed differential expression of transcription factors. Ethylene-responsive transcription factors and NAC domain containing proteins were down-regulated by melatonin. In WRKY (a stress related TF) over-expressed lines, lateral root formation was upregulated. Genes that participate in cell wall biogenesis were up-regulated. Melatonin also up regulated peroxidase which controls cell elongation in roots through its auxin oxidase activity. The authors concluded that melatonin affected LR formation in an auxin independent manner (Zhang et al. 2014).

Genome wide expression profiling by RNA sequencing in rice showed that root architecture is modulated by melatonin through auxin signaling pathways (Liang et al. 2017). Melatonin activated the expression of many auxin induced TFs amongst

which 21 genes showed a root specific expression. Many of the DEGs were involved in auxin stimulus response and the auxin mediated signaling pathway. Melatonin induced modification of root architecture through increased length of root hairs and an increase in the number of roots, is a well characterized auxin response (Overvoorde et al. 2010; Liang et al. 2017).

However, according to Pelagio-Flores et al. (2012), melatonin does not regulate AXR3/IAA17 nor activate auxin-inducible gene expression in root development in *Arabidopsis*. The differences in the observation of the two groups may be due to the differential expression profiles of auxin related genes or due to species specific responses (Liang et al. 2017).

According to Yan et al. (2020) melatonin-induced root development is independent of IAA in the signal transduction pathway but melatonin can promote root development through IAA synthesis, polar transport and hormone perception.

Transcriptome analysis revealed that auxin metabolism-related genes exhibited minimal changes in melatonin-treated *Arabidopsis* plants with respect to untreated plants. Only one IAA-amino synthase was upregulated, with no change in the expression of auxin biosynthesis genes (Weeda et al. 2014). Several auxin influx carrier proteins (AUX1/LAX) were down regulated in response to melatonin. AUX 1 regulates lateral root development, root hair development and the gravitropic response (Swarup and Péret 2012) Several efflux genes (PIN 1,2 and3) and auxin signaling transduction genes (IAA19 andIAA24) were upregulated. (Weeda et al. 2014; Arnao and Hernández-Ruiz 2015a, b, c). The same auxin PIN proteins (Pin 1/2/3) were down-regulated in *Lycopersicon* (Wang et al. 2016). In the roots of lupin and some monocots a melatonin gradient similar to IAA gradient is operative (Hernandez-Ruiz and Arnao 2008).

Melatonin and auxin share structural similarity and a common precursor, but there is lack of consensus on the signaling pathway of the two molecules (Wang et al. 2016; Wen et al. 2016; Pelagio-Flores et al. 2012; Ren et al. 2019).

In *Arabidopsis thaliana* (Ren et al. 2019) identified 16 auxin related genes whose expression was altered on exogenous application of melatonin. Many genes coding for auxin transport (PIN5, TT4, TT5)and LAX2 and the auxin /IAA proteins (IAA3 and IAA 17) were down regulated indicating that melatonin modulates lateral root development by regulating the intracellular distribution of auxins. In the *Arabidopsis* ecotype Col-0, auxin and melatonin acted synergistically to promote lateral root envelopment but in the ecotype Ler-O, the two indoleamines had an additive effect which was not expressed in the knock out mutants. The authors proposed a model (Fig. 2) to explain auxin, melatonin reactions in lateral root development. Auxin transporters present in the plasma membrane allow inflow of exogenous auxin into the cell. Exogenous auxin and melatonin inactivates the PIN 5 influx carrier located in the endoplasmic reticulum directly or indirectly through the protein kinase WAG1, thereby inhibiting auxin transport from the endoplasmic reticulum to the nucleus. Exogenous melatonin also down-regulates flavonoid biosynthesis by reducing TT4and TT5 transcripts. Down regulation of flavonoids activates auxin transporters in the plasma membrane. This dual action of melatonin—inactivating PIN 5 and activating auxin transporters in the plasma membrane through the TT4

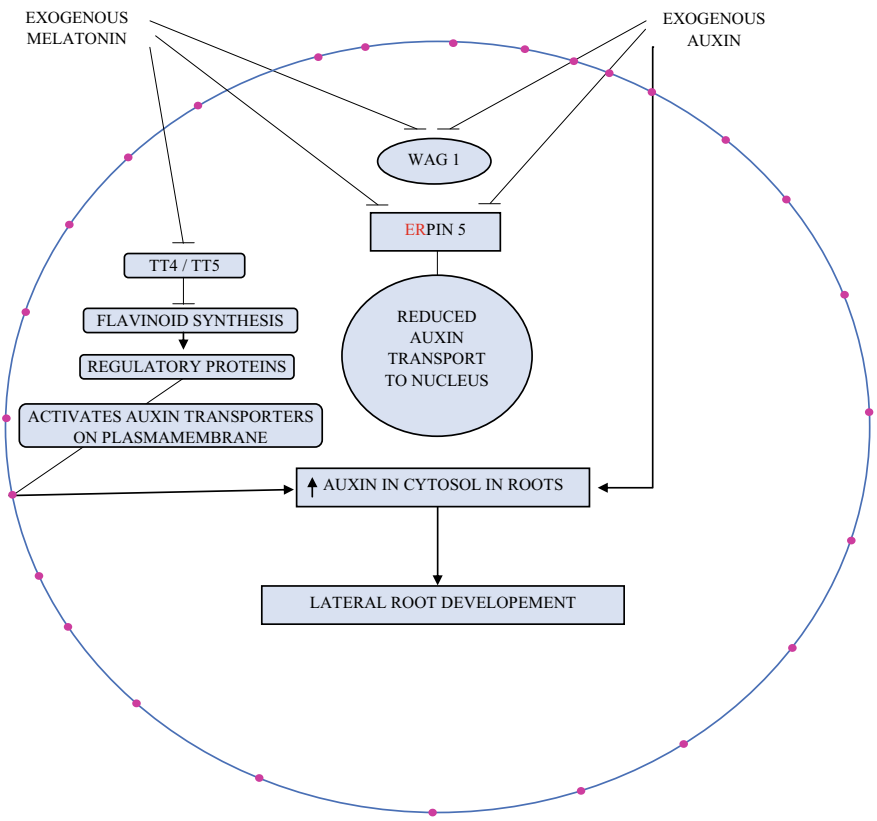

Fig. 2 Proposed mechanism of auxin/melatonin interaction in lateral root development (Ren et al. 2019)

and TT5 transcripts, results in a high level of auxin in the cytosol, leading to calcium signaling and increased lateral roots. Cross talk between melatonin and the flavanoid pathway regulates lateral root development (Ren et al. 2019). (with permission provided under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.)

6 Gravitropic Response—An Auxin Like Response to Melatonin

Application of either IAA or melatonin enriched agar blocks elicited a gravitropic response in roots of *Lupinus*. Disruption of the endogenous level of auxin or melatonin through the agar blocs resulted in a gradient that induced the gravitropic response (Arnao and Hernandez-Ruiz 2017). Roots of rice seedlings also showed a gravitropic curvature when treated with exogenous melatonin. The difference in tip angles between the treatments and the control, showed that melatonin modulates root growth through effects on the auxin signaling pathway (Liang et al. 2017).

7 Nitric Oxide, Auxin and Melatonin Signaling Pathways in Root Induction

NO (nitric oxide) and auxin signaling pathways are interconnected in regulating several plant responses. NO and auxins interact to regulate growth, development, and morphology of plant roots (Sanz et al. 2015). In the auxin -regulated formation of adventitious roots in cucumber hypocotyl cuttings, NO interaction with auxins involve the regulation of Ca^{2+}—dependent protein kinase (CDPK) activity. Ca^{2+} and CDPK act as downstream messengers in the signaling pathway triggered by auxins and NO to promote AR development (Pagnussat et al. 2002). NO activates at least two different pathways during the induction of AR (cGMP-dependent and cGMP independent pathways) that involves a MAPK signaling cascade. The MAPK signaling cascade involved in ARF in cucumber explants is cGMP-independent (Pagnussat et al. 2004).

NO donours can mimic the effect of auxin suggesting an important role of NO in auxin induced processes (Chen and Kao 2012). Melatonin increases the nitric oxide (NO) level through the upregulation of nitrate reductase (Arnao and Harnedez-Ruiz 2018). NO as a down stream signal is involved in melatonin induced AR formation in *Solanum lycopersicum* (Wen et al. 2016). In S*olanum lycopersicum* melatonin triggered NO production by upregulating nitrate reductase and down regulating the expression of GSNOR. NO induced auxin accumulation led to ARF induction. Enhanced endogenous concentrations of IAA and IBA, expression of several genes

of the auxin signaling pathway–auxin carriers (PIN1,3,7) and auxin signaling transduction genes (IAA19, IAA24) in response to melatonin, indicated that melatonin influenced auxin transport, accumulation and signal transduction through the NO signaling pathway (Wen et al. 2016; Arnao and Harnedez-Ruiz 2018). NO is required for growth and development of roots; Sanz et al. 2015). NO can modify hormonal action by chemical modification of transcription factors/chemical modification of proteins (enzymes) or by directly interacting with plant hormones, leading to changes in hormone levels and signaling patterns (Asgher et al. 2017).

8 Tryptophan as an Inductive Signal

Tryptophan is the precursor of three classes of growth modulators—auxins as well as serotonin and melatonin. Erland and Saxena (2019) proposed that tryptophan also functions as an inductive signal and triggers diverse morphogenetic pathways. Exogenous application of tryptophan resulted in root formation while IAA treatment resulted in de novo shoot formation. Trptophan appeared to have altered the melatonin and serotonin balance and serotonin acted as a transient signal modulating diverse morphogenetic pathways (Erland and Saxena 2019).

9 Stress and Rhizobiology: Role of Melatonin and Serotonin

Roots being the interface between the soil and the plant is subject to diverse biotic and abiotic stress. Roots perceive the stress and respond, often through enhanced endogenous melatonin production, in an effort to alleviate the stress induced harmful effects. Melatonin is a powerful antioxidant and has a cascade effect i.e. melatonin through its secondary and tertiary derivatives is able to neutralize many toxic oxygen derivatives (Tan et al. 2015). One melatonin molecule can scavenge up to ten reactive oxygen species (ROS).Melatonin produced as a response to external stress improves the survival of plants under such conditions. The growth-promoting effect of melatonin is high when a stress condition affects plant development, as in the case of root growth in *Helianthus annuus* (Mukherjee et al. 2014).

Growth inhibition of roots can occur due to deficiency in auxin concentrations caused by disruption of the acropetal gradient of PIN proteins (Sun et al. 2008) In *Arabidopsis thaliana* high salt stress enhanced the proliferation of LR due to accumulation of auxin in the developing primordia in response to salt stress (Zolla et al. 2010). ABA synthesis and ethylene signaling network was also involved in the response.

Exogenous stress disturbs the ion and redox homoeostasis in plants, resulting in the accumulation of ROS (reactive oxygen species) and RNS (reactive nitrogen

species). ROS and RNS play a dual role—they are harmful at higher concentrations, damaging membranes, organelles and even nucleic acids but at lower concentrations they act as signaling molecules that re-establish homoeostasis (Arnao and Hernandez-Ruiz 2019a, b). ROS production is needed by plants as it functions as a secondary messenger in signal transduction (Baxter et al. 2014). Melatonin biosynthesis is upregulated by external stress and increased levels of endogenous melatonin mitigate the effect of the stress by directly scavenging ROS/RNS or indirectly by upregulating the expression of genes coding for antioxidant enzymes—catalase/peroxidase/superoxide dismutase etc. (Arnao and Hernandez-Ruiz 2020a, b). Weeda et al. (2014) showed that plants vary in their sensitivity to melatonin and some genes are regulated by low concentrations of melatonin while others are regulated by higher concentrations.

Melatonin production, induced by abiotic stress also increases the level of NO by upregulation of nitrate reductase. Melatonin and NO induce changes in hormonal levels and also alter the expression of Tfs and hormone signaling elements that lead to easing the stress (Arnao and Hernandez-Ruiz 2018).

In *Zea mays* seedlings exogenous melatonin enhanced salt tolerance through osmotic adjustment, ion balance, and alleviation of salt-induced oxidative stress (Ren et al. 2020). Melatonin alleviated high salinity and proline induced water stress and promoted seed germination in *Cucumis sativus* by upregulating the activity of ROS scavenging enzymes (Zhang et al. 2012, 2013). Melatonin promoted the expression of stress tolerant proteins as well as proteins involved in ATP production and promoted the degradation of storage proteins to produce energy for germination of *Cucumis sativus* seeds. Melatonin also regulated heat shock proteins to protect seed germination under salt stress (Zhang et al. 2017).

In mutant and transgenic lines of *Arabidopsis thaliana*, stress due to incubation with Hydrogen—rich water stimulated the expression of the hydrogenase gene CrHYD1. A hydrogen signaling cascade upregulated melatonin biosynthesis. Melatonin re-established ion and redox homoeostasis in part through Na^+/H^+ antiport across the plasma membrane leading to improved tolerance to salinity stress (Su et al. 2021). The authors concluded that melatonin acts downstream to the hydrogen signaling cascade.

Melatonin is known to re-establish ion/redox homoeostasis. In *Helianthus annuus* seedlings melatonin eliminated the harmful effect of ROS and RNS by the modulation of two superoxide dismutases (SOD) Cu/Zn SOD and Mn SOD (Arora and Bhatla 2017). Melatonin also affects the catabolism of hormones like GA and ABA and increases the expression of genes down regulated by salt stress (Kaur et al. 2015; Reiter et al. 2015).

In response to biotic stress melatonin through its crosstalk with plant hormones activates pathogen related gene expression (Arnao and Hernandez-Ruiz 2018) NO is also produced by melatonin in response to stress (Shi et al. 2015a).

In *Arabidopsis thaliana* melatonin up-regulated the expression of C-repeat-binding factors, Drought Response Element Binding factors, a cold-responsive gene, *COR15a*, a transcription factor involved in freezing and drought-stress tolerance *CAMTA1* and transcription activators of reactive oxygen species (ROS)-related

antioxidant genes, *ZAT10* and *ZAT12*, in a response to mitigate cold stress (Bajwa et al. 2014). In *Citrullus lanatus* melatonin treatment given to the roots improved cold tolerance of the leaves. Melatonin was detected in the xylem sap indicating that it is transported from roots to leaves via the xylem. Exogenous melatonin promoted cold-induced up-regulation of genes involved in signal transduction and transcriptional regulation in leaves, but not in roots suggesting that melatonin is involved in the sensing the cold signal and subsequent signal transduction (Li et al. 2017a, b). In *Arabidopsis* high temperature tolerance was achieved through endogenous melatonin production which led to the expression of heat shock factors and heat shock proteins (HSP90, HSP10; Shi et al. 2015b).

Abiotic stress leads to the production of ROS which acts as a second messenger in signal transduction (Baxter et al. 2014).

Melatonin is an antioxidant and can scavenge ROS by triggering the production of endogenous antioxidants or through activating redox—sensitive regulating pathways Melatonin is active in ROS scavenging which results in a good redox balance essential for the development of a robust root system (Yan et al. 2020; Shi et al. 2015c).

In plants, ROS signaling occurs through complex mechanisms and hormone response crosstalk via salicylic acid, jasmonic acid (JA) and ethylene (Et) genetic components (Mittler et al. 2011).

Serotonin is known to regulate developmental processes via ROS scavenging (Ramakrishna et al. 2011). Serotonin induced redistribution of ROS in the root tip mediated through the RCD1 locus and the JA—Et signaling pathway, is responsible for inhibition of primary root growth in *Arabidopsis thaliana.* (Pelagio–Flores et al. 2016). The inhibition of root growth is due to serotonin and not due to the conversion of serotonin to melatonin (Pelagio-Flores et al. 2016).

10 Is Melatonin a Phytohormone?

Melatonin regulates diverse plant processes. Its mode of action is similar to auxin in many aspects it influences root initiation and growth in a dose dependent manner through the establishment of a gradient. Like auxins melatonin can induce the gravitropic response. As a scavenger of ROS and RNS melatonin can mitigate stress through its interaction with downstream signaling molecules and crosstalk with hormones. Melatonin is now viewed as a master regulator or a new plant hormone (Arnao and Hernandez Ruiz 2018, 2020a, b).

The signaling pathway of melatonin and serotonin is not clear as receptors for the two indoleamines have not been discovered in plants. Based on the similarity of serotonin and IAA activity, it is proposed that serotonin may function through auxin receptors on the cell membrane in shared/similar signaling pathways (Mukherjee 2020). Wei et al. (2018), for the first time reported a phytomelatonin receptor (CAND2/PMTR1) in *Arabidopsis thaliana.* The membrane bound receptor mediates the phytomelatonin induced stomatal closure through a H_2 O_2 and Ca^{2+} signaling transduction cascade. Ca^{++} permeable ion channels exist in plants (Zimmermann et al.

1999). The role of calcium channels and calcium ionophore (A23187) in the perception of melatonin and serotonin induced responses has been reported (Ramakrishna et al. 2009).

However, a receptor for melatonin has not been detected in other plants. Lee and Back (2020) claimed that CAND 2 protein is located in the cytoplasm rather than the plasma membrane, is not involved in melatonin induced MAPK activation or in melatonin-mediated defense signaling pathway via G protein components and is therefore not a melatonin receptor. The authors concluded that it may be a melatonin binding protein that can decrease the free melatonin level in cells. So while the role of melatonin and serotonin on plant growth and viability is well established it is still not considered to be a plant hormone in the absence of a receptor.

The ability of melatonin and serotonin to induce root induction and its positive impact on root growth can be used for clonal propagation of improved varieties of plants. Seen in the perspective of their role in promoting growth, protecting photosystem II, delaying senescence and combating stress, melatonin and serotonin have the potential to improve yield and reduce dependence on synthetic chemicals to counter biotic and abiotic stress.

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Suberin in Monocotyledonous Crop Plants: Structure and Function in Response to Abiotic Stresses

Paul Grünhofer, Lukas Schreiber, and Tino Kreszies

Abstract Apoplastic barriers, formed by Casparian bands and suberin lamellae, represent important means of plant roots to adapt water and nutrient homeostasis to changing environmental conditions. To understand and evaluate the precise physiological role of suberin lamellae in water and nutrient transport characteristics, it is important to understand root anatomy, including main deposition sites and microstructure of suberin. Here we review suberin localization, chemistry, biosynthesis, and differential implementation in dependence of different abiotic stimuli in roots of monocotyledonous crop plants. Furthermore, we add results on the formation of suberized barriers in barley roots under nitrogen and phosphate deficiency, as well as ABA treatments. We conclude that the degree of suberin accumulation is essentially independent of absolute root length, while endodermal plasticity strongly and differentially responds to external environmental stimuli and thus affects plant physiology.

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P. Grünhofer · L. Schreiber (✉)
Department of Ecophysiology, Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, 53115 Bonn, Germany
e-mail: lukas.schreiber@uni-bonn.de

P. Grünhofer
e-mail: p.gruenhofer@uni-bonn.de

T. Kreszies (✉)
Plant Nutrition and Crop Physiology, Department of Crop Sciences, University of Göttingen, Carl-Sprengel-Weg 1, 37075 Göttingen, Germany
e-mail: tino.kreszies@uni-goettingen.de

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1 Introduction

Suberin and cutin are chemically closely related biopolymers forming lipophilic barrier structures for water, solutes, gases, and pathogens in specialized plant-environment interfaces (Schreiber 2010). Suberin is found in specialized tissue layers including periderms of shoots and the rhizo-, hypo-, and endodermis of roots, whereas cutin is restricted to the epidermis of above-ground organs (Ranathunge et al. 2011b). Since deposition sites of suberin and its chemical composition in plants have been very well characterized (Bernards 2002; Brundrett et al. 1991; Graça and Santos 2007; Kolattukudy and Espelie 1989; Schreiber et al. 1999), research of the past decade has focused on elucidating its three-dimensional structure, biosynthesis, genetic regulation, and functional implications. Oligomeric building blocks (Graça et al. 2015; Graça 2015), as well as many key enzymes and reaction steps (Vishwanath et al. 2015) orchestrated by a multitude of different transcription factors (Capote et al. 2018; Cohen et al. 2020; Kosma et al. 2014; Krishnamurthy et al. 2021), are known to date. Also, the general effect of suberization processes on transport physiology has been very well described (Barberon 2017; Kim et al. 2018; Ranathunge et al. 2017; Ranathunge and Schreiber 2011). All of this allowed for a slow and still ongoing transition and transfer from the suberin model species cork oak (*Quercus suber* L.), potato (*Solanum tuberosum* L.), and *Arabidopsis thaliana* to further important crop species employed in agriculture (Kreszies et al. 2018) and agroforestry (Rains et al. 2017).

Here we summarize and discuss studies on root suberization of monocotyledonous crop species in response to abiotic stresses (excess and deficiency) and possible effects on root transport physiology. We conclude that apoplastic barriers, formed by the Casparian bands and suberin lamellae, are important means for a plant root to adapt to changing environmental conditions. To understand and evaluate the precise physiological role of suberin lamellae development, it is important to consider root anatomy, including main deposition sites and microstructure of suberin, biosynthesis, genetic regulation, and transport physiology.

2 Suberin

2.1 Localization

Suberin is a plant-unique biopolymer deposited in various organs and tissues throughout the organism, displaying a multitude of physiological functions. It is

probably best known as an integral component of cork oak (*Quercus suber* L.) periderm (Graça and Pereira 2000b), even providing this plant with its specific epithet. But it can also be found in tubers (Kolattukudy et al. 1975), seeds (Compagnon et al. 2009), wounding tissue (Yang and Bernards 2006), trichomes and glands (Kolattukudy 2001), and roots (Schreiber 2010). A noteworthy difference between suberin and cutin, its counterpart which is only to be found in above-ground tissues, is that suberin may be deposited into, whereas cutin is deposited onto cell walls (Andersen et al. 2015). Suberin is typically found incrusted in primary cell walls or deposited in lamellate structures between the plasma membrane and carbohydrate cell walls (Peterson and Cholewa 1998). In roots, suberin is frequently observed in endodermal and hypo-/exodermal cell layers, where it serves as a bidirectional apoplastic transport barrier for water and solutes as well as against pathogen invasion (Enstone et al. 2003). If a hypodermis exhibits Casparian bands, it is by definition referred to as exodermis (Perumalla and Peterson 1986). Cells of the endodermis may species-dependent originate from periclinal divisions of cortex-endodermis or epidermis-endodermis initial cells (Dolan et al. 1993; Pauluzzi et al. 2012) in the root apical meristem under influence of the SHORT-ROOT and SCARECROW transcription factors (Gallagher et al. 2004; Koizumi et al. 2012a, b). After cell divisions and maturations, endodermal development is characterized by three distinct stages, firstly described by Krömer (1903). Stage I: deposition of Casparian bands in transverse and radial cell walls. Stage II: accumulation of protoplast-enclosing suberin lamellae, and stage III: tertiary thickening of cell walls. Stage I differentiation is reported to be under the main control of the MYB36 transcription factor (Kamiya et al. 2015), whereas the master regulator of stage II yet remains elusive (Andersen et al. 2015). The third stage, however, may only be observed in certain species and is not ubiquitously found (Zeier and Schreiber 1998). These three stages are typically associated with the maturity of a given root segment and may develop earlier (i.e. closer to the root apex) or even later under stress conditions (Kreszies et al. 2019; Melino et al. 2021; Ranathunge et al. 2015; Stoláriková et al. 2012). Single cells in the endodermis lacking suberin lamellae are called passage cells, which have also been identified in the exodermis, and are hypothesized to aid in retaining transport abilities (Andersen et al. 2018; Peterson and Enstone 1996). In contrast to the endodermis, such a precise chronological order of differentiation has not been reported for the exodermis (Tylová et al. 2017). Furthermore, an exodermis may form constitutively as it has been observed in many rice (*Oryza sativa* L.) cultivars (Pedersen et al. 2020), optionally only due to stress as in maize (*Zea mays* L.) (Zimmermann et al. 2000), or mature even before the endodermis as in some wetland species (Soukup et al. 2002). Some other plant roots appear to be entirely devoid of exodermis formation (Nawrath et al. 2013). In many modern and some wild barley (*Hordeum vulgare* L.) cultivars no exodermis could be induced even under severe environmental stresses (Armand et al. 2019; Coffey et al. 2018; Kreszies et al. 2020a; Ranathunge et al. 2017), but not necessarily the whole species is incapable of forming this protective feature (Kreszies et al. 2020a; Reissinger et al. 2003). Nonetheless, an endodermis and hypo-/exodermis do share the most important common features such as developmental plasticity in response to biotic and abiotic stress, function as apoplastic

barriers, and band plasmolysis due to Casparian bands (Enstone et al. 2003; Enstone and Peterson 1997; Hose et al. 2001; Karahara et al. 2004).

2.2 Composition and Structure

Patterns of suberin deposition are investigated by light microscopy using different types of (fluorescent) dyes, as Sudan red or fluorol yellow (Brundrett et al. 1991). In contrast, qualitative investigation of polymer building blocks and true quantification may best be achieved by precise analytical methods such as mass spectrometry and gas chromatography (Zeier and Schreiber 1997). Hints on the chemical nature of suberin already originate from the nineteenth century, where the renowned French chemist Michel Eugène Chevreul firstly used the term "subérine" to describe non-soluble compounds characteristic of cork (Chevreul 1815). This non-solubility, even in modern analyses, is used to specifically isolate suberized tissues for subsequent monomer extraction. Today, suberin is known as a biopolyester made of a polyaliphatic and a polyaromatic domain with glycerol as its backbone (Bernards 2002; Franke and Schreiber 2007; Graça 2015; Graça and Pereira 2000a; Rains et al. 2017).

The polyaliphatic fraction (aliphatics) consists of saturated or unsaturated long-chain mono- or bi-functional fatty acids, ω-hydroxy acids, α,ω-dicarboxylic acids, mid-chain oxygenated fatty acids, 2-hydroxy acids, and alcohols of species-dependent chain lengths, most commonly between C_{16} and C_{26} and in varying relative amounts (Holloway 1983; Kolattukudy 2002). In some species, such as sweet potato, even chain lengths of up to C_{32} in the alcohol compound class have been reported (Bernards 2002; Kolattukudy et al. 1975). Chain length distribution as well as the abundance of individual monomers are not only species-specific but may also depend on the tissue of origin. It is documented, that bark suberin of a given species not necessarily has to share the same composition as suberin being deposited in roots (Matzke and Riederer 1991). ω-hydroxy acids and α,ω-dicarboxylic acids represent the most important aliphatic monomers (Graça 2015) and have therefore been termed as "suberin diagnostic".

The polyaromatic domain (aromatics) is composed of monolignols and/or hydroxycinnamic acids, yet still subject to frequent debates (Bernards and Razem 2001; Rains et al. 2017). Due to solubilized monolignols, the aromatic fraction of suberin has historically been attributed to the lignin polymer (Kolattukudy 1980). However, modern approaches consider it to be distinctly different from lignin due to relatively higher amounts of hydroxycinnamic acids (Bernards 2002). The latter functional group is typically composed of ferulic and coumaric acid (Ranathunge et al. 2015), of which ferulic acid frequently represents <1% of the fractionalized suberin polymer (Graça 2010). Coumaric acid may also lack entirely, as in bark suberin of poplar (Rains et al. 2017) or roots of *Arabidopsis* (Molina et al. 2009). In many recent studies, released monolignols appear to be considered as co-solubilized, and only hydroxycinnamic acids are reported as core aromatic domain (Graça 2015; Kreszies et al. 2019; Ranathunge et al. 2017; Shiono et al. 2014b). Nonetheless, when interpreting

aromatic compounds, great care needs to be taken due to several reasons. On the one hand, typically employed chemical reactions aimed at investigating suberin are best in depolymerizing the aliphatic fraction (Graça and Pereira 2000b; Ranathunge et al. 2011b; Zeier and Schreiber 1999), resulting in non-representative amounts of aromatics (Graça 2015) in some species. On the other hand, especially *Poaceae* species such as barley, maize, and rice are known to have high amounts of aromatic molecules bound to all cell walls (Carpita 1996; Chabbert et al. 1994), which may lead to a strong overestimation of truly suberin-specific aromatic compounds. For example, if comparing these monocotyledonous species to the dicot model plant *Arabidopsis*, their aromatic suberin fraction appears to be disproportionally large (Fleck et al. 2015; Franke et al. 2005; Ranathunge and Schreiber 2011; Schreiber et al. 2005b). The puzzle of how exactly aliphatics, aromatics, and glycerol are interlinked and three-dimensionally organized is still not solved and restricted to models (Graça 2015; Graça and Santos 2007; Ranathunge et al. 2011b) since the observation of the suberin polymer in its unaltered native form has not yet been achieved.

2.3 Biosynthesis

To obtain a fully functional suberin polymer several crucial reaction steps are needed: (i) biosynthesis of glycerol, hydroxycinnamic acids, and long-chain fatty acid precursors; (ii) elongation of precursors to very-long-chain fatty acids; (iii) α,ω-bifunctionalization by the introduction of additional hydroxy- and carboxy-groups and mid-chain oxygenation of fatty acids; (iv) reduction of fatty acids to alcohols; (v) conjugation of acyl chains to glycerol and ferulic acid; (vi) export of monomers and oligomers out of the cell into the apoplast; and (vii) polymerization to a three-dimensional structure. To achieve this, an array of highly orchestrated enzymes has to be recruited. In the following, we will focus on the most commonly described genes. Suberin C_{16} and C_{18} fatty acid precursors of the polyaliphatic domain are synthesized by the fatty acid synthase (FAS) complex in the plastids and subsequently transported to the endoplasmic reticulum (Li-Beisson et al. 2016). In the endoplasmic reticulum, most importantly fatty acid elongation (FAE) and various functionalization steps are taking place (Franke and Schreiber 2007). Elongation with C_2 units is achieved by β-ketoacyl-CoA synthases (KCS) (Franke et al. 2009; Lee et al. 2009; Serra et al. 2009b) and may, at various intermediate chain lengths, be succeeded by oxidation reactions to introduce additional hydroxy- and carboxy groups, resulting in the most characteristic suberin monomers ω-hydroxy acids and α,ω-dicarboxylic acids. Oxidations are carried out, depending on the chain length of their substrate, by members of the cytochrome P450 (CYP) monooxygenase/ω-hydroxylase enzyme family, two members of which are most renowned as HORST (CYP86A1) and RALPH (CYP86B1) in *Arabidopsis* suberin biosynthesis (Compagnon et al. 2009; Höfer et al. 2008). Alternatively, fatty acid precursors of any given chain length may be reduced by fatty acid reductases (FAR) to yield primary alcohols (Vishwanath et al. 2013) or just remain unmodified. Entirely independent of this, hydroxycinnamic acids of the polyaromatic fraction are synthesized in the phenylpropanoid

pathway (Bernards 2002; Bernards and Razem 2001; Tsai et al. 2006). First esterification reactions even within the cell at the endoplasmic reticulum are carried out by glycerol 3-phosphate acyltransferases (GPAT) (Beisson et al. 2007) and aliphatic suberin feruloyl transferases (ASFT) (Molina et al. 2009) to conjugate acyl chains with glycerol or ferulic acid, respectively, before secretion of the suberin building blocks into the apoplast. Very little is known about the export process from the endoplasmic reticulum through the plasma membrane and even less about polymerization outside of the protoplast. ABC transporters have been characterized in suberin monomer secretion (Panikashvili et al. 2010; Shiono et al. 2014b; Yadav et al. 2014). Also, lipid transfer proteins (LTP) (Plett et al. 2016; Shiono et al. 2014a), as well as Golgi-mediated vesicle trafficking (Vishwanath et al. 2015) are speculated to participate in suberin transportation. Once the building blocks reached their destination, assembly to a functional polymer by esterification has to be executed. However, no candidate genes have yet been characterized by true experimental confirmation to be involved in this very crucial step of suberin biosynthesis (Ranathunge et al. 2011b).

2.4 Regulation of Suberin Biosynthesis

So far only limited knowledge is available about the phytohormonal and genetic regulation of the spatiotemporal tightly controlled suberization process. As discussed in the section environmental stimuli (Sect. 4), a multitude of external stimuli has to be sensed and translated to adapt the root physiology to the changing environmental conditions. Early studies investigating supplementation effects of abscisic acid (ABA) on suberization in potato tubers were able to show that fairly high concentrations of 100 μM ABA significantly increased suberin amounts and diffusion resistance (Cottle and Kolattukudy 1982; Soliday et al. 1978). Enhanced suberization was later also confirmed with roots of maize subjected to 10 μM ABA (Zeier 1998). In *Arabidopsis*, it could be demonstrated that ABA significantly enhances suberization processes, whereas ethylene appears to be involved in the delay of suberin lamellae development, even under non-stress conditions (Barberon et al. 2016). Delayed suberization, which simultaneously goes along with an increased number of passage cells (Ogden et al. 2018), was shown to also be mediated by auxin-influenced cytokinin signaling as its suppression in the root apical meristem resulted in increased numbers of passage cells (Andersen et al. 2018). This effect was surprisingly not to be antagonized by additional ABA treatment, suggesting that cytokinin determines the responsiveness of endodermal cells to ABA (Andersen et al. 2018). Based on co-expression studies, members of the MYB, NAC, and WRKY transcription factor gene families have been suggested to take part in orchestrating suberization processes (Ranathunge et al. 2011b), and as of today, at least nine independent genes of these families have been described in greater detail (Capote et al. 2018; Cohen et al. 2020; Kosma et al. 2014; Krishnamurthy et al. 2021; Lashbrooke et al. 2016; Legay et al. 2016; Mahmood et al. 2019; Verdaguer et al. 2016). Aside from this fast progress in elucidating suberin biosynthesis and regulation, still, no suberin mutants of barley and maize and only one for rice (Shiono et al. 2014b) have

been reported, whereas most studies were done with *Arabidopsis* or potato (Bernards 2002; Franke et al. 2005; Lulai and Corsini 1998; Schreiber et al. 2005c; Serra et al. 2009a; Vogt et al. 1983; Yang and Bernards 2006). Future studies are needed to transfer the acquired knowledge on suberin biosynthesis and regulation from model to crop plants. In contrast, environmental stimuli (Sect. 4) have been widely described for both, model and crop plants. Therefore, we will focus on findings based on the monocotyledonous crop species barley, maize, and rice.

2.5 Function of Suberin

Suberin displays an array of important functions, which may depend on the site of deposition. It acts as a sealing agent in wounding tissue after physical injuries (Yang and Bernards 2006), protects barks against fire (Dantas and Pausas 2013), serves as a diffusion barrier for atmospheric gases in bundle sheaths (Mertz and Brutnell 2014), leads to abscission of specific plant organs (van Doorn and Stead 1997) and may even be employed by humans for insulation and several further industrial applications (Gandini et al. 2006; Graça 2015). In plant roots, it is well established for many species that suberization of the endodermis and/or exodermis continuously increases over the length of the root (i.e. its maturity) (Andersen et al. 2015; Kotula et al. 2009, 2017; Kreszies et al. 2019; Ranathunge et al. 2017; Schreiber et al. 1999). Differential development, being induced or delayed suberization, has been observed as a reaction to certain environmental stimuli, as will be thoroughly discussed later (Sect. 4, Tables 1, 2, 3, and 4). Due to these findings, it was concluded that suberin lamellae must play an important role in plant water and nutrient homeostasis. It does act as an apoplastic transport barrier that influences water conductivity and nutrient uptake dynamics, which has repeatedly been proven by the employment of suited measurement techniques such as the root pressure probe or ion bypass measurements (Krishnamurthy et al. 2011; Ranathunge et al. 2005; Zimmermann et al. 2000).

The question of whether the endodermis or the exodermis, if present, greater contributes to the apoplastic barrier properties, was investigated extensively with studies on rice roots. It could be shown that an increase of exodermal suberization did not significantly decrease water transport conductivities whereas the endodermis was the rate-limiting factor for water flow (Ranathunge et al. 2003; Ranathunge et al. 2011a). The endodermis, which is always present, fulfills a bi-directional function. Besides controlling the uptake of solutes, it must as well prevent leakage of solutes from the stele into the cortex (Barberon 2017; Enstone et al. 2003). The exodermis, if developed, will have the same function as the endodermis. In addition, due to its deposition in the hypodermis, suberin can also serve as a barrier against pathogens (Reissinger et al. 2003) and has been proven to represent a barrier against radial oxygen loss of many wetland species (De Simone et al. 2003; Kotula et al. 2009, 2017; Soukup et al. 2007). When rice plants are flooded, the exodermis forms a strong barrier against radial oxygen loss around the developing aerenchyma, which in combination greatly facilitate the diffusion of oxygen from the shoot to the growing root tip (Pedersen et al. 2020).

Even though one might conclude, as it has also been indicated by some studies (Kotula et al. 2009; Ranathunge et al. 2017; Zimmermann et al. 2000), that more suberin always yields a stronger transport barrier, this is not always the case. Not only the amount of suberin but probably also its microstructure needs to be considered (Kreszies et al. 2018). The findings of Ranathunge et al. (2003) indicated that despite a strong suberization of the rice exodermis, its water conductivity was still comparably high. This could be due to a porous structure allowing small water molecules to pass, whereas diffusion of larger oxygen molecules and NaCl ions are hindered (Schreiber et al. 2005b). This was later verified by sealing potential wall pores with different precipitates and particles which in turn significantly decreased the conductivity of the outer part of the root (Ranathunge et al. 2004, 2005). Comparable conclusions were drawn after investigating mutant lines of *Arabidopsis*. Even though *enhanced suberin1 (esb1)* mutants (Baxter et al. 2009) exhibit two-fold more suberin than their wildtypes, this ectopic suberin accumulation failed to significantly reduce water and NaCl permeabilities (Ranathunge and Schreiber 2011).

It is well accepted that especially the hydrophobic aliphatic domain conveys water and gas repellency in roots (Graça and Pereira 2000b; Hose et al. 2001; Ranathunge et al. 2011a; Shiono et al. 2014b; Zimmermann et al. 2000). Impregnation with associated waxes, especially in above-ground organs, can greatly enhance barrier properties, as it was shown for aerial roots of *Monstera deliciosa* and air-exposed potato tubers (Schreiber et al. 2005c; Vogt et al. 1983; Zeier and Schreiber 1998). However, little to no wax has yet been reported for isolated endodermal and exodermal cell walls from soil-grown roots (Schreiber et al. 1999). In turn, there is still speculation about the precise role of aromatic suberin compounds. It has been suggested that the aromatic monomers link the "core suberin" (i.e. being aliphatics) to the primary cell wall and lignified cell walls (Graça 2015). In addition, the aromatic fraction might contribute to mechanical stability and act as a barrier especially for pathogens (Bernards and Razem 2001; Lulai and Corsini 1998).

3 The Effect of Suberized Barriers on Water and Solute Transport

Three pathways for radial solute and water transport, summarized as composite transport model (CTM), have been described (Steudle et al. 1993; Steudle and Brinckmann 1989) and this was further expanded and refined in the following years (Kim et al. 2018; Ranathunge et al. 2017; Steudle 2000a, b; Steudle and Peterson 1998). The model suggests three theoretical pathways: (i) the apoplastic (cell wall) pathway, (ii) the symplastic (cellular) pathway, and (iii) the transcellular pathway, which represents a combination of the former two pathways (apo- and symplastic). Experimentally, the symplastic and the transcellular pathway cannot be measured separately to date (Steudle and Peterson 1998). Purely symplastic and purely apoplastic pathways would represent the two extremes of water and solute uptake. If water and solute transport would exclusively take place symplastically, molecules would enter

the protoplast across the plasma membrane of the rhizodermis and travel through plasmodesmata from cell to cell, without ever directly crossing further membranes, before being exported into the xylem vessels (Maurel and Chrispeels 2001; Steudle 2000a). With a purely apoplastic pathway, water and solutes would diffuse across the porous cell wall continuum without ever crossing any cell membrane. However, latest at the endodermis further radial transport is controlled by the symplast via membrane-bound transporters and channels regulating which solutes are taken up into the central cylinder (Ranathunge et al. 2017). This sealing of the apoplast may only be interrupted at sites where lateral roots emerge, thus breaking the continuum, or at the very root tip where no functional Casparian bands and suberin lamellae have yet been developed (Kreszies et al. 2018; Steudle and Peterson 1998). In contrast, the transcellular pathway is characterized by constant vectorial influx and efflux processes (Geldner 2013).

Axial (i.e. longitudinal) movement may be neglected at this point since the resistance of dead xylem vessels is low (Steudle and Peterson 1998) and radial uptake has proven to be the rate-limiting step (Frensch and Steudle 1989). This is different for immature root tips where functional xylem has not yet formed (Ranathunge et al. 2017).

Sophisticated experimental approaches, for example, root pressure probes, exudation experiments, or pressure chambers, have to be applied for measuring and quantifying radial transport across roots (Miyamoto et al. 2001; Steudle et al. 1987; Suku et al. 2014). Thus, hydraulic conductivities (Lp_r, in $m^3\ m^{-2}\ s^{-1}\ MPa^{-1}$ or simplified $m\ s^{-1}\ MPa^{-1}$), which are a measurement of water flow per unit surface area perpendicular to flow path and per unit biophysical driving force (Knipfer et al. 2020; Kreszies et al. 2018), as well as membrane permeabilities (P_{sr}, in $m\ s^{-1}$) and reflection coefficients (σ_{sr}, no dimension) of given solutes are obtained (Ranathunge et al. 2017). The latter two are inversely correlated (Steudle and Peterson 1998), which means that, for example, a high P_{sr} indicates a low σ_{sr}. The reflection coefficient is by definition a dimensionless value between zero and unity, where zero resembles non-selectivity for a solute and one (unity) indicates perfect semi-permeability of a given barrier, as would be the case in an ideal osmometer (Steudle and Brinckmann 1989). In roots, σ_{sr} of the apoplast is virtually zero whereas the semi-permeable cell-to-cell path may theoretically reach values close to unity (Kim et al. 2018; Steudle and Peterson 1998). The root hydraulic conductivity (Lp_r) may be dissected into hydrostatic Lp_r(HY) and osmotic Lp_r(OS) by considering the two driving forces hydrostatic (i.e. transpirational tension) and osmotic (i.e. osmolyte gradients) pressure, which are influencing the Lp_r (Steudle and Peterson 1998). Hydrostatic hydraulic conductivity reflects composite water flow along all three pathways in parallel (Steudle 2000b). In turn, osmotic pressure gradients may not impact the non-selective apoplastic route, which is why Lp_r(OS) solely resembles the combined cell-to-cell pathways (Kreszies et al. 2018). The ratio of both (Lp_r(HY):Lp_r(OS)) yields information of relative contributions. If it is close to one, as for *Arabidopsis* (Ranathunge and Schreiber 2011), a dominant cell-to-cell pathway is indicated, whereas a value substantially greater than one depicts a higher contribution of the apoplastic pathway (Steudle and Peterson 1998). In the case of rice, for example, ratios may easily reach values of up

to 20 resembling a strong apoplastic contribution (Miyamoto et al. 2001). All of the above-described water and solute uptake processes are highly dependent on several variables, for example, plant species (Kreszies et al. 2020a; Schreiber et al. 2005b), plant age, developmental state (Ranathunge et al. 2017), formation of an exodermis (Zimmermann et al. 2000), or specific growth conditions (Kreszies et al. 2019).

A controversially discussed topic is the detailed composition and exact physiological role specifying Casparian bands and suberin lamellae as apoplastic barriers in water and nutrient relations of a plant, which has already been mentioned shortly after their discovery (Krömer 1903). Undeniably, primary cell walls exhibit pores with diameters being too big to selectively regulate water and low molecular weight solutes (Marschner 1995; Ranathunge et al. 2005, 2011b). Secondary modifications in the form of lignin or suberin, however, might be sufficient to shrink pore sizes to a degree that allows higher semi-permeability of solutes and potentially affect the small water molecules to a lower extent (Kreszies et al. 2019; Schreiber et al. 2005b). The contribution of Casparian bands in effectively sealing the apoplastic pathway at the endodermis is indisputable (Caspary 1865; Clarkson 1993; Peterson 1987). However, for Casparian bands, it was only shown experimentally that the apoplastic movement of large fluorescent dyes is blocked (Kamiya et al. 2015; Naseer et al. 2012; Peterson 1975). To our best knowledge, experiments proving that small water molecules are completely blocked by Casparian bands are still missing. In contrast for suberin lamellae, which are chemically hydrophobic, reductions of radial water transport under osmotic stress or radial oxygen loss under anaerobic conditions have been shown (Kotula et al. 2009, 2017; Kreszies et al. 2019). A further compelling example is the potato periderm, which is composed of suberin and completely lacks Casparian bands. Potato periderm has barrier properties comparable to a leaf cuticle. Thus all cell walls, even the primary cell walls, must obviously be suberized and encrusted with waxes. Otherwise, such efficient barrier properties could not be obtained (Schreiber et al. 2005c).

Moreover, the precise chemical composition of Casparian bands is still a matter of debate. A broad consensus is achieved in their structure being mainly made of lignin, but the exact contribution of suberin to their chemical properties remains yet elusive and might even be species-dependent (Soukup and Tylová 2018; Tylová et al. 2017). On one hand, analytical methods, as well as Raman scattering microscopy, showed that suberin aside from lignin was identified in endodermal Casparian bands of many species, even if no developmental stage II had been observed (Man et al. 2018; Schreiber et al. 1994; Schreiber 1996; Thomas et al. 2007; Zeier et al. 1999; Zeier and Schreiber 1998). On the other hand for *Arabidopsis*, reporter gene systems and expression studies indicated no involvement of suberin in endodermal Casparian band biosynthesis (Kamiya et al. 2015; Naseer et al. 2012). Yet additional endodermis-specific transcriptomic and chemical analyses are still missing to provide further reliable data in this controversy (Kreszies et al. 2019). Recently, direct analysis of Casparian bands via high-resolution Raman spectroscopy indicated the simultaneous deposition of both, lignin as well as suberin, in Casparian bands of maize (Man et al. 2018) and Chinese fir (Song et al. 2019). It can be summarized here, that neither Casparian bands nor suberin lamellae establish perfect barriers but rather convey

increased resistances against diffusion of water as well as solutes (Barberon et al. 2016; Ranathunge et al. 2003, 2005; Schreiber et al. 2005b; Schreiber 2010; Steudle 2000a; Vogt et al. 1983), which in turn is highly dependent on the environmental conditions the plant is confronted with.

4 Environmental Stimuli

For suberin lamellae development, it has repeatedly been shown that biotic (Borg-Olivier and Monties 1993; Lulai and Corsini 1998; Ranathunge et al. 2008; Reissinger et al. 2003; Salas-González et al. 2021; Thomas et al. 2007) and abiotic (Tables 1, 2, 3, and 4; Enstone et al. 2003; Stoláriková et al. 2012) environmental stimuli are capable of differentially affecting suberization. Some may induce suberization, whereas others delay the development of suberin lamellae. Inoculation of *Arabidopsis* roots with microbiota was recently shown to convey enhanced performance under abiotic stress treatments (Salas-González et al. 2021). This greatly emphasizes the need for future investigations on potentially beneficial microbiome-root interactions and how they influence suberization processes of crop species in field trials. In root research, plants are typically grown under axenic gel-based or hydroponic conditions, as it allows precise control over treatments and provides easy access to the roots at harvest. Especially for agar plates, which are frequently employed to cultivate only several-day-old seedlings of *Arabidopsis*, illumination of roots should generally be avoided, but at the very least be considered as a potential additional stimulus (Baluška et al. 2009). Unconscious secondary outcomes might also be produced by the employment of multi-faceted stresses such as salinity, which not only exerts ion toxicity upon roots but also considerable osmotic effects. Studies comparing hydroponics with additional methods of cultivation (Table 1), such as growth in aeroponics or most naturally on soil, have proven that cultivation alone yields a stimulus strong enough to differentially affect suberization and root morphology (Krishnamurthy et al. 2009; Miyamoto et al. 2001; Ranathunge et al. 2015; Redjala et al. 2011; Zimmermann et al. 2000). Hydroponic growth generally seems to induce less suberin development than the other two methods of cultivation (Table 1). When comparing results, this should always be taken into account. Also, just unilateral subjection to stimuli was shown to have specific effects, as gel-grown maize roots developed asymmetrical suberization after treatment with cadmium and air exposure (Líška et al. 2016). It must be emphasized that also under control conditions even different parts of the same root along its developmental axis may exhibit distinct features and properties (Ranathunge et al. 2017). Lastly, genotypic differences between cultivars or wild types of the very same species might lead to different reactions (Kreszies et al. 2020a). To better compare specific developmental conditions, the percentage distance of roots measured from the apex should be chosen, as this ensures root segments to have the same age, irrespective of potentially varied lengths under environmental stress conditions (Ranathunge et al. 2015). Due to all of the above, a "detailed map" (see Fig. 7) over the length of the root should be created for the most

reliable and conclusive interpretation of experimental results (Kreszies et al. 2018). Besides, such a map would also allow feasible and reliable comparisons to other species or cultivars/genotypes within the same species.

The sensing of external conditions by plants is closely integrated into phytohormone signaling pathways as it has been mainly investigated in the dicotyledonous model plant *Arabidopsis* (Sect. 2.4). The phytohormone signaling network of *Arabidopsis* is highly reactive to nutrient availability (Table 1): manganese (Mn), iron (Fe), zinc (Zn) and phosphorus (P) deficiency lead to decreased suberization by affecting ethylene and cytokinin activity, whereas potassium (K) and sulfur (S) starvation, as well as sodium chloride (NaCl) addition, stimulated suberization by triggering ABA signaling in *Arabidopsis* (Andersen et al. 2018; Barberon et al. 2016). The exact sensing mechanism of nutrient availability appears to be still unknown (Barberon et al. 2016). However, partly due to the nature of thin and fragile *Arabidopsis* roots, adequate chemical quantification after nutrient stress exposure and analysis of resulting transport properties have not or rarely been performed. Data on various crop species may be taken as assistance to further elucidate the effects of specific environmental stimuli.

The following sections are focused on the developmental processes of rice, maize, and especially barley roots. Until now, most studies investigated seminal and/or adventitious main roots but explicitly not their laterals. However, studies by Tylová et al. (2017) and Knipfer et al. (2020) have emphasized that lateral roots must get detailed consideration in the future, and more research is needed to draw further conclusions on this often-overlooked part of the plant root system.

Table 1 Summary of studies reporting about effects of different cultivation methods and various treatments on root morphology, suberization reactions, and hydraulic properties of monocotyledonous crop species and *Arabidopsis*

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
Cultivation conditions	*Zea mays*	Hydroponics and aeroponics (solution specified in this paper)	6-8d (3-4d G + 2-5d T)	Hydroponics versus aeroponics with the same nutrient solution	Microscopy: investigation at apical 20/40/60/80/100/150/200/250 mm GC (to DW): zonation into 0-50/50-100%, separation into ECW/RHCW RPP and CPP	-	Comparing aeroponics to hydroponics: = Aliphatics and aromatics, in both zones in EN ↑ Aliphatics, most remarkably in 0-50% = Aromatics in (newly formed) EX	Comparing aeroponics to hydroponics: ↓ Lp_r(HY) = Lp_r(OS) P_{sr} not determined	-	Zimmermann et al. 2000
Cultivation conditions	*Oryza sativa* 2 cultivars	Hydroponics and aeroponics (solution specified in this paper)	31-40d (5-6d G + 25-35d T)	Hydroponics versus aeroponics with the same nutrient solution	Microscopy: investigation at apical 10/20/40/60/80/100/150/200/250 mm RPP and pressure chamber	Comparing aeroponics to hydroponics: ↑ RL	Comparing aeroponics to hydroponics: =	Comparing aeroponics to hydroponics: = Lp_r(HY) and Lp_r(OS) (↓Lp_r(OS) in one cultivar)	-	Miyamoto et al. 2001
Cultivation conditions	*Oryza sativa* 3 cultivars	Soil (Red laterite /alfisols) Hydroponics (½ Hoagland solution)	22-35d (3d G + 2w3d-3w4d C + 2/7d T)	Soil (watered with same nutrient solution) versus Hydroponics	GC (to DW): zonation into 0-50/50-100%, separation into CC/OPR	-	Comparing soil to hydroponics: ↑ Aliphatics, most remarkably in 50-100% = (trend to ↑) Aromatics in EN and EX	-	-	Krishnamurthy et al. 2009
Cultivation conditions	*Zea mays*	Hydroponics and aeroponics (solution specified in this paper) and soil (Loamy)	15-16d (3d G + 12d C + 21h T)	Hydroponics versus aeroponics versus Soil with same nutrient solution	Microscopy: investigation at 0-50%	Comparing aeroponics to soil: = RL Comparing above with hydroponics: ↓ RL	Comparing aeroponics to soil: = in EN and EX Comparing hydroponics to the above: ↓ in EN and EX	-	-	Redjala et al. 2011
Cultivation conditions	*Zea mays*	Agar plates (MS) Air	13d (3d G + 10d T)	Unilateral air exposure	Microscopy: investigation over whole roots	-	↑ unilaterally to air-exposed side in EN and EX	-	-	Líška et al. 2016

(continued)

Table 1 (continued)

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
Cultivation conditions	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	16-20d (6d G + 10-14d C)	Basal versus apical root zone	Microscopy: investigation at apical 10/20/30/40/50/60/100 mm GC: zonation roughly into 0-50/50-100%, no separation RPP and CPP	-	Comparing 50-100% to 0-50%: ↑ Aliphatics and aromatics in EN	Comparing whole roots to apical part: ↓ Lp_r(HY) $P_{s/}$ of NaCl is similar to that of KCl, higher than that of Mannitol and lower than that of Ethanol	-	Ranathunge et al. 2017
Various	*Arabidopsis thaliana*	Agar plates (½ MS)	5d	-Fe, -Mn, -Zn, -S, -K, +100 mM NaCl (-0.5 MPa), +1 µM ABA, +1/2/5 µM ACC (ethylene)	Microscopy: investigation mostly with longitudinal cuts GC (to DW) for ABA and ACC treatment: no zonation, no separation	(for NaCl treatment only) ↓ RL	↓, ↓, ↓, ↑, ↑, ↑, ↑, ↓ in EN	-	-	Barberon et al. 2016
Various	*Arabidopsis thaliana*	Agar plates (½ MS)	5d	-Zn, -Fe, -P	Microscopy: investigation mostly with longitudinal cuts	-	↓, ↓, ↓ in EN	-	-	Andersen et al. 2018
Various	*Zea mays*	Hydroponics (¼ Hoagland solution) Solid media (Soil/1:1quartz:perlite)	18d (4d G + 14d T)	Hydro: oxygen saturation <20% (+organic acid toxicity), +100 mM NaCl, +5/50 µM Cd; Solid: flooded, mild drought stress	Microscopy: investigation at the most differentiated root base segments with a high additional focus on LR	↓ RL	= in EN ↑ in EX	-	-	Tylová et al. 2017

[a] G, germination; C, cultivation under control conditions; T, treatment

[b] Only the most relevant methods for this summary are given. Microscopy, fluorescence or confocal; LR, lateral root; GC, gas chromatography; DW, dry weight; RL, root length; ROL, radial oxygen loss; zonation, axial division of roots into zones; no zonation, analysis performed with whole roots; separation, isolation of specific tissue layers; CC, central cylinder; OPR = outer part of the root; ECW, endodermal cell walls; RHCW, rhizo-/hypodermal cell walls; CP, cortical parenchyma; qPCR, quantitative PCR; RPP, root pressure probe; CPP, cell pressure probe

[c,d,e,f] =, no observable trend; ↓ and ↑, observed trends (histochemistry) or significant changes (gas chromatography)

[c] SA, surface area

[d] EN, endodermis; HY, hypodermis; EX, exodermis; ROL, radial oxygen loss; WT, wildtype

[e] Lp_r, root hydraulic conductivity; Lp_r(HY), hydrostatic hydraulic conductivity (apoplastic, symplastic, transcellular combined); Lp_r(OS), osmotic hydraulic conductivity (symplastic, transcellular combined); $P_{s/}$, solute permeability; $\sigma_{s/}$, reflection coefficient; $J_{v/}$, water flow

4.1 Water Deficiency and Osmotic Stress

Precisely controlled drought stress (i.e. water deficit) in hydroponic systems (Table 2), as opposed to water withdrawal on soil, is most often mimicked by different types of polyethylene glycol (PEG) addition to the medium (Michel 1983). This non-toxic and inert polymeric agent introduces osmotic stress by allowing the precise adjustment of the nutrient solution's water potential (Ψ, in MPa). Investigating effects of a water potential of −1.07 MPa on maize roots found decreased root lengths but simultaneously increased amounts of suberin in the endo- and exodermis (Zeier 1998). For barley roots, this general setup was refined with PEG8000 by not only dissecting roots into three zones according to their developmental stage but also investigating stepwise decreased water potentials of −0.4, −0.8, and −1.2 MPa (Kreszies et al. 2019). Due to the fine nature of barley roots, separation into the central cylinder (endodermis) and outer part of the root (hypo-/exodermis) was not feasible. Nonetheless, since in the modern cultivar Scarlett no exodermis was ever observed, changes of suberin amounts could solely be attributed to specific effects in the endodermis. It was shown that root lengths decreased with decreasing water potential of the medium and aliphatic suberin amounts conversely increased, most remarkably in the root zones of 25–50 and 50–100%. These findings were supported by a significant upregulation of suberin biosynthesis genes. The aromatic fraction, in contrast, was found to be non-responsive to the imposed stresses. Interestingly, the onset of suberization remained at a similar distance of 25%, not resulting in a shift of suberization towards the root tip. Water and nutrient transport analyses of the −0.8 MPa treatment revealed a significant reduction in Lp_r(HY) with no effects on Lp_r(OS) and P_{sr} of NaCl if compared to control conditions (Kreszies et al. 2019). The addition of silicon (Si) at a water potential of −0.8 MPa was not able to show additional effects on root lengths or the degree of suberization in cultivar Scarlett (Kreszies et al. 2020b). These investigations were subsequently expanded to further barley cultivars and wild barley accessions (Kreszies et al. 2020a). Most findings for the cultivars were highly similar to that of the cultivar Scarlett, but especially the wild barley accessions reacted differently. In wild barley accessions, suberization was more restricted to the most basal root zone (50–100%) and also aromatic compounds did show significant increases. Furthermore, one of the wild accessions from Jordan was observed to exhibit a properly developed exodermis. Still, when comparing wild accessions with modern cultivars, the induction of core suberin genes was less pronounced, potentially reflecting the overall slightly lower aliphatic suberin amounts of wild accessions (Kreszies et al. 2020a).

Table 2 Summary of studies reporting about effects of osmotic stress, salinity, and ABA supplementation on root morphology, suberization reactions, and hydraulic properties of monocotyledonous crop species

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
Osmotic	*Zea mays*	Hydroponics (¼ Hoagland solution)	10d (4d G + 6d T)	300 g/kg PEG6000 (-1.07 MPa)	Microscopy: investigation at apical 20 mm, 50%, and 10 mm below the root-shoot junction GC (to DW and RL): no zonation, separation of ECW/RHCW	↓ RL	↑ Aliphatics and aromatics combined in EN and HY/EX (best observable in EN if related to RL)	-	-	Zeier 1998
Osmotic	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	12d (3d G + 3d C + 6d T)	17.5/25.4/31.6% (w/w) PEG8000 (-0.4, -0.8, -1.2 MPa)	Microscopy: investigation at 12.5/25/37.5/50% GC: zonation into 0-25/25-50/50-100%, no separation RNA-Seq. RPP	↓ RL with increasing osmotic stress	↑ Aliphatics, with increasing osmotic stress, most remarkably in 25-50% and 50-100% = Aromatics in EN	In -0.8 MPa: ↓ Lp_r(HY) = Lp_r(OS) = P_{sr} of NaCl (trend to ↑)	↑ most remarkably in 25-50%	Kreszies et al. 2019
Osmotic	*Hordeum vulgare* 3 wild and 3 cultivated varieties	Hydroponics (½ Hoagland solution)	12d (3d G + 3d C + 6d T)	25.4 (w/w) PEG8000 (-0.8 MPa)	Microscopy: investigation over whole roots GC: zonation into 0-25/25-50/50-100%, no separation RNA-Seq. RPP	↓ RL for all varieties	= Aliphatics in 0-25% of all varieties ↑ Aliphatics in 25-50% of modern cultivars ↑ Aliphatics in 50-100% of modern and wild (except one) varieties ↑ Aromatics in 50-100% of wild (except one) varieties in EN	In -0.8 MPa: One cultivated variety: ↓ Lp_r(HY) = Lp_r(OS) = P_{sr} of NaCl One wild variety: = Lp_r(HY) = Lp_r(OS) (trend to ↑) = P_{sr} of NaCl (trend to ↑)	Comparing wild varieties to modern cultivars under the same stress conditions: ↓ for key suberin genes (some others were differentially expressed)	Kreszies et al. 2020a
Osmotic Silicon	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	12d (3d G + 3d C + 6d T)	1 mM $NaSiO_3$, also in combination with osmotic stress by 25.5% (w/w) PEG8000 (-0.8 MPa)	Microscopy: investigation at 25/50/90% GC: zonation into 0-25/25-50/50-100%, no separation	↑ RL for Si, ↓ RL for PEG, and no additional effect for PEG+Si	= Aliphatics for Si ↑ Aliphatics for PEG, mostly in 25-50% and 50-100% No significant additional effect for PEG+Si in EN	-	-	Kreszies et al. 2020b

(continued)

Table 2 (continued)

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
NaCl Osmotic	*Zea mays*	Hydroponics (¼ Hoagland solution)	10d (4d G + 6d T)	100 mM NaCl (-0.5 MPa)	Microscopy: investigation at apical 20 mm, 50%, and 10 mm below the root-shoot junction GC (to DW and RL): no zonation, separation of ECW/RHCW	↓ RL	↑ Aliphatics and aromatics combined in EN and HY/EX (best observable in EN if related to RL)	-	-	Zeier 1998
NaCl Osmotic	*Oryza sativa* 3 cultivars	Hydroponics (½ Hoagland solution)	33/38d (3d G + 4w C + 2/7d T)	50/100/200 mM NaCl (-0.25/-0.5/ -1 MPa) supplemented with various concentrations of $CaCl_2$	Microscopy: investigation at apical 10/20/30/50/100/200 mm GC (to DW): zonation into 0-50/50-100%, separation of CC/OPR qPCR	-	↑ Aliphatics and aromatics with increasing salt (50 to 100 mM) concentration, most remarkably in EN of 0-50% and EX of 50-100% in EN and EX 2d of 200 mM NaCl treatment did not increase suberin amount	-	↑ after 0.5 h = after 4 h	Krishnamurthy et al. 2009
NaCl Osmotic	*Oryza sativa* 2 cultivars	Hydroponics (½ Hoagland solution)	33/38/40 d (3d G + 4w C + 2/7/2+7 [pre-conditioning] T)	100/200 mM NaCl (-0.5/-1 MPa) in various combinations (pre-conditioning or not)	Microscopy (for Casparian bands only): investigation at apical 10/20 mm Exudation experiments combined with pressure chamber Measurement of Na+ bypass flow	-	↑ (referred to Krishnamurthy et al. 2009)	↓ Lp_r(HY), with their method expected to mainly reflect apoplastic water movement Lp_r(OS) after stress not to be determined ↓ P_{sr} of NaCl (estimated with Na+ bypass flow measurement)	-	Krishnamurthy et al. 2011
NaCl Osmotic	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	15-17d (7d G + 3d C + 5-7d T)	100 mM NaCl (-0.5 MPa)	Microscopy: investigation at apical 9-14 mm and 25/50% with an additional focus on LR Exudation experiments, Transpiration-Lp, RPP and CPP, Modelling of Lp_r based on CPP	↓ RL	↑, only at the root apex in EN	↓ Lp_r(HY) ↓ Lp_r(OS) this decrease is attributed to a reduction in aquaporin expression and activity rather than to the effect of suberin No P_{sr} to be measured, as indicated by σ_{sr} of 1	-	Knipfer et al. 2020

(continued)

Table 2 (continued)

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
ABA	*Zea mays*	Hydroponics (¼ Hoagland solution)	10d (4d G + 6d T)	10 µM ABA	Microscopy: investigation at apical 20 mm, 50%, and 10 mm below the root-shoot junction GC (to DW and RL): no zonation, separation of ECW/RHCW	↓ RL	↑ Aliphatics and aromatics combined in EN and HY/EX (best observable in EN if related to RL)	-	-	Zeier 1998
ABA	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	12d (3d G + 3d C + 6d T)	10/50 µM ABA	GC: zonation into 0-25/25-50/50-100%, no separation	↓ RL for 50 µM ABA	↑ Aliphatics and aromatics, most remarkably in 0-25% and 25-50% in EN	-	-	This study

[a] G, germination; C, cultivation under control conditions; T, treatment

[b] Only the most relevant methods for this summary are given. Microscopy, fluorescence or confocal; LR, lateral root; GC, gas chromatography; DW, dry weight; RL, root length; ROL, radial oxygen loss; zonation, axial division of roots into zones; no zonation, analysis performed with whole roots; separation, isolation of specific tissue layers; CC, central cylinder; OPR = outer part of the root; ECW, endodermal cell walls; RHCW, rhizo-/hypodermal cell walls; CP, cortical parenchyma; qPCR, quantitative PCR; RPP, root pressure probe; CPP, cell pressure probe

[c,d,e,f] =, no observable trend; ↓ and ↑, observed trends (histochemistry) or significant changes (gas chromatography)

[c] SA, surface area

[d] EN, endodermis; HY, hypodermis; EX, exodermis; ROL, radial oxygen loss; WT, wildtype

[e] Lp_r, root hydraulic conductivity; Lp_r(HY), hydrostatic hydraulic conductivity (apoplastic, symplastic, transcellular combined); Lp_r(OS), osmotic hydraulic conductivity (symplastic, transcellular combined); P_w, solute permeability; σ_w, reflection coefficient; J_w, water flow

4.2 Salt Stress

Salinity, which frequently goes along with drought conditions as salts (e.g. NaCl) accumulate in the soil (Table 2), is not only capable of exerting osmotic stress similar to PEG but may also impose the additional stress of severe ion toxicity on root organs, especially in the case of NaCl. Quantitative investigations on maize roots indicated that suberin amounts increased upon 100 mM NaCl treatment (Zeier 1998). Further and more elaborate studies were carried out on roots of rice with concentrations of 50, 100, and 200 mM NaCl (Krishnamurthy et al. 2009, 2011). These concentrations exhibit theoretical water potentials of −0.25, −0.5, and −1.0 MPa, respectively. Taken together, salt treatments were shown to increase the expression of suberin genes within only 30 min, but control levels were reached again after 4 h of exposure. This upregulated gene expression coincided with significantly enhanced suberization after 7 d of treatment, where aliphatics as well as aromatics accumulated in both the endo- and exodermis. Interestingly, the most pronounced changes of the endodermis were observed in the apical half (0–50%), whereas the exodermis reacted more severely in the basal half of the root (50–100%). It was further observed that a just two-day-long treatment with the highest 200 mM concentration was not sufficiently long to yield increased suberin amounts (Krishnamurthy et al. 2011). This shows that the formation of enhanced suberin lamellae needs to be considered as a long-term adaptation to abiotic stress. Pre-conditioning with lower concentrations of sodium chloride as well as supplementation of calcium (Ca) greatly improved the survival rate of rice plants. Subsequent investigations of water and solute transport after salt exposure showed a significant reduction in Lp_r(HY) and P_{sr} of NaCl, whereas Lp_r(OS) could due to methodological limitations not be determined (Krishnamurthy et al. 2011). This data may be complemented with findings of barley, where 100 mM of NaCl significantly reduced root lengths and, in histochemistry, comparably increased suberization especially in the endodermis of the root tip (Knipfer et al. 2020). Here, in addition to a decreased Lp_r(HY), the Lp_r(OS) was found to be negatively affected as well. Furthermore, the σ_{sr} of NaCl was close to 1.00 (Knipfer et al. 2020). This is in strong contrast to our studies on barley where we reported reflection coefficients for sodium chloride of 0.29 up to 0.69 (Kreszies et al. 2019, 2020a; Ranathunge et al. 2017). These differences may be explained by different experimental procedures, calculations, and points of view of whether roots behave as perfect osmometers or not (for detailed reviews see Kim et al. 2018; Kreszies et al. 2018).

4.3 Exogenous Abscisic Acid Treatment

Most of the environmental stress conditions presented here will most probably influence ABA homeostasis, as ABA is commonly described as the most important phytohormone regulating abiotic stress reactions (Aasamaa et al. 2002; Barberon 2017; Bauer et al. 2013; Chen et al. 2014; Kosma et al. 2009; Macková et al. 2013). As

endogenous ABA was shown to be involved in wound-induced suberization of potato tubers (Lulai et al. 2008; Lulai and Suttle 2009), one might consider investigating its effects after artificial exogenous application. However, surprisingly very little is known for monocot crop species (Table 2). In maize, external application of 10 μM ABA resulted in significantly reduced root lengths and an increased suberin accumulation in endo- and hypo-/exodermis (Zeier 1998), but no subsequent effects on transport physiology were investigated. This was performed by Schraut et al. (2005), where a physiological dosage of 0.1 μM ABA significantly enhanced water flow (J_v) within minutes even after various long-term nutrient starvation treatments in maize. These short-term responses of root hydraulic conductivity might substantially be regulated by aquaporin activity (Kaneko et al. 2015) rather than suberization effects occurring after several days.

A proof of concept analysis of ABA application to roots of barley has been performed in our laboratory (see Fig. 1 and Supplementary for experimental details) to confirm results previously reported for *Arabidopsis* (Barberon et al. 2016) and maize roots (Zeier 1998). The average root length of 12 d old plants under control conditions in the ABA experiment was 19.3 ± 3.7 cm (Fig. 2). An addition of 10 μM ABA did not affect the root length (19.8 ± 3.5 cm) compared to its control. In contrast, subjection to 50 μM ABA for 6 d significantly reduced the average root length (14.2 ± 2.9 cm). Suberin lamellae development has been affected differentially in between the treatments (Fig. 3). Under control conditions, no suberization was visible until 25% of the root length (zone A), whereas patchy suberization was observed in zone B (25–50%) and developed into full suberization in zone C (50–100%) (Fig. 3a, e, i, m). Suberization of zone C did not seem to vary considerably in either treatment, since microscopy indicated consistent full suberization (Fig. 3a, b). However, if roots were subjected to ABA, staining with fluorol yellow revealed an increased suberization in zone A and B, because full suberization was reached already very close behind the root apex (Fig. 3j, n). Microscopic observations have

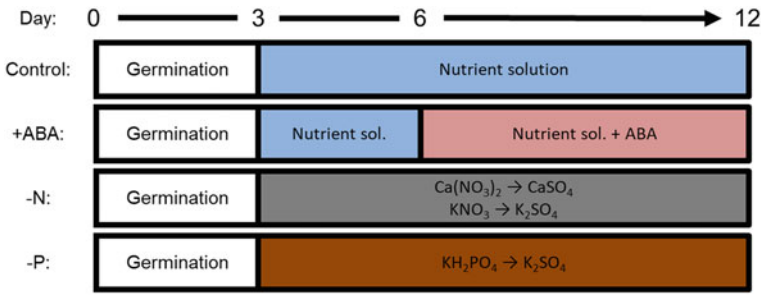

Fig. 1 General hydroponic cultivation procedure. After 3 d of germination, seedlings were transferred into (modified) ½ Hoagland nutrient solution. In control and deficiency (-nitrogen, -phosphorus) treatments, seedlings were left to grow for further 9 d. In excess ABA treatments, ABA was added to the nutrient solution at day 6, and plants were grown for another 6 d to ensure comparability to previous osmotic stress studies (Kreszies et al. 2019, 2020a, b). All plants were harvested at 12 d of age

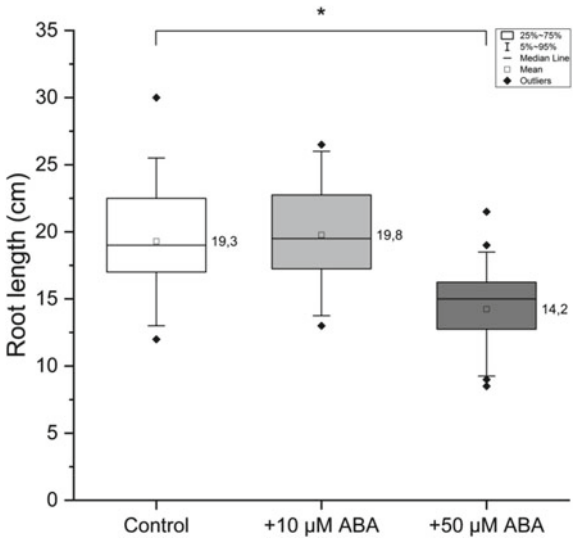

Fig. 2 Seminal root lengths of 12 d old barley plants grown in control or ABA supplemented (+10/50 µM ABA) conditions. The number beside each box (n = 35–45 individual roots) represents the mean. Significant differences at $p \leq 0.05$ based on t-tests are indicated by asterisks

only been carried out for the 50 µM ABA concentration, which severely affected root length (Fig. 3b, f, j, n). Under influence of ABA, no exodermis formation could be observed in barley seminal roots. Aliphatic and aromatic suberin monomer distribution of barley roots were exactly as previously described elsewhere (Kreszies et al. 2019; Ranathunge et al. 2017). The aliphatic fraction consisted of ω-hydroxy acids, α,ω-dicarboxylic acids, fatty acids, and alcohols of chain-lengths between C_{16} and C_{26}. Aromatic suberin was composed of ferulic and coumaric acid. Furthermore, relative amounts of individual monomers, as well as substance class composition did not differ significantly in between growth conditions. Due to these abovementioned reasons, only sums of aliphatic (Fig. 4a) and aromatic (Fig. 4b) suberin are shown. It is evident, that with increasing maturity of the root (i.e. from tip to base), the suberin content of both fractions steadily increased, which is perfectly in line with the histochemical observations (Fig. 3). The addition of both 10 and 50 µM ABA considerably increased the total aliphatic suberin amount, most remarkably in zone A of 50 µM ABA treatment and zone B of both ABA applications. Zone C was not affected much, with only a slight increase by the addition of 50 µM ABA. It seems, that between 5 to 8 µg cm^{-2} a plateau of aliphatic suberin accumulation has been reached. The aromatic fraction behaved accordingly, but with less steep increases of amounts if compared to aliphatics, especially in zone A and B.

ABA treatment did not exert osmotic stress on roots, which means that observable effects are solely due to the excess conditions employed, without having to consider secondary effects. Since no exodermis formation could be observed, quantitative changes in suberin amounts can be attributed exclusively to differences in endodermal development. Root lengths measured and suberin lamellae deposition observed in control conditions fit very well to that reported previously for the very same growth conditions (same plant age and climate chamber) for barley cultivar Scarlett (Kreszies

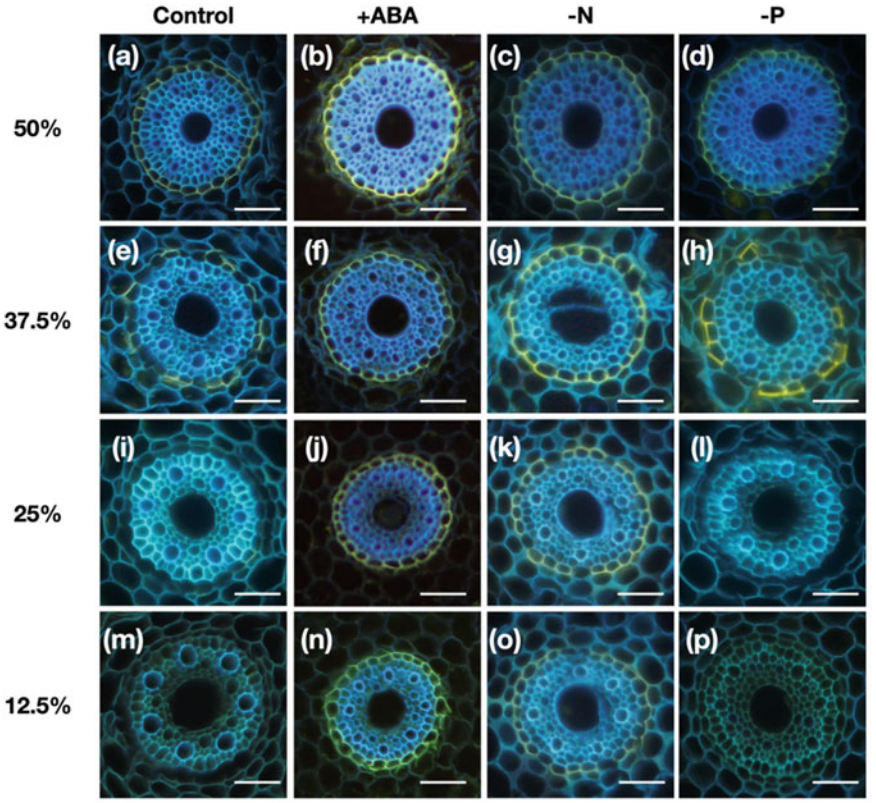

Fig. 3 Development of endodermal suberin lamellae in barley seminal roots. Suberin lamellae are indicated by yellow fluorescence through staining with fluorol yellow 088. Apical zone A (0–25%) and intermediate zone B (25–50%) are depicted, as suberization in distances of >50% (basal zone C) was always fully developed. Under control conditions, suberization started at approx. 25%, developed in a patchy manner and became fully suberized at 50% (**a**, **e**, **i**, **m**). 50 μM ABA (+ABA) and nitrogen deficiency (−N) treatment showed full suberization already at 12.5% distance (**n**, **o**), whereas phosphorus starvation (−P) led to similar suberin lamellae development as under control conditions. Size bars = 50 μm

et al. 2019, 2020b). However, the newly introduced environmental stimulus employed in this study provoked significantly different reactions regarding root morphology and suberin deposition. Barley seminal root lengths were found to be significantly decreased only at concentrations higher than 10 μM ABA (Fig. 2). Differently in maize, this dose of 10 μM was sufficient to severely affect the average lengths of primary roots (Zeier 1998). The suberization was strongly enhanced (Fig. 3f, j, n). Already at the very root tip, full suberization had been reached, which continuously persisted to the root base. Fluorol yellow signals for ectopic suberin could not be observed in cortex cells of any distance, as it had previously been reported for roots of *Arabidopsis* (Barberon et al. 2016). Roots also had no exodermis, which indicates

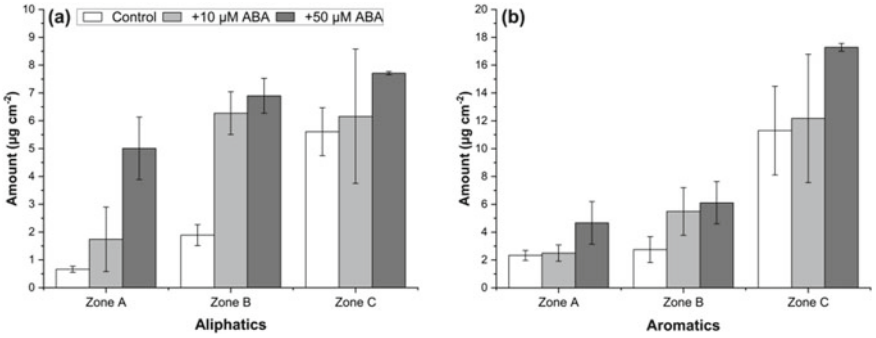

Fig. 4 Total amounts of aliphatic (**a**) and aromatic (**b**) suberin components under control ABA supplemented (+10/50 µM ABA) conditions. Seminal roots were divided into three zones: apical zone A (0–25%), intermediate zone B (25–50%), and basal zone C (50–100%). Bars represent means ± standard deviation of n = 2 replicates. Due to the lack of a third biological replication, no statistical tests were performed

that the barley cultivar Scarlett might just not be able to additionally suberize the hypodermal layer, even in the most severe environmental stress conditions (Kreszies et al. 2019, 2020b). Chemical analysis confirmed the strong suberization induced by ABA (Fig. 4), as it has already been reported before in different species (Barberon et al. 2016; Zeier 1998). It is noteworthy that root zones A and B were most enhanced with suberin deposition and amounts were similar to root zone C (Fig. 4). This was the strongest reaction of this cultivar ever observed yet (Kreszies et al. 2019, 2020b). Total aliphatic suberin amounts seem to approach a threshold value, which is also found in zone C of control roots. This was not found for the aromatic fraction. Different from nitrogen and phosphorus deficiency (Sect. 4.4), synthesis of the aliphatic fraction seemed to be induced more intensely than aromatic compounds. The effect of ABA-induced suberization will probably decrease root hydraulic properties as shown earlier in various species in response to abiotic stress (Armand et al. 2019; Kreszies et al. 2019; Krishnamurthy et al. 2011; Ranathunge et al. 2015; Zimmermann et al. 2000).

4.4 Nitrogen, Phosphorus, and Potassium Excess and Deficiency

Comparatively, extensive research has been performed investigating the effects of nitrogen (N) excess and deficiency (Table 3). Nitrogen is typically available to the plant in the form of nitrate (NO_3^-) or, less frequently, ammonium (NH_4^+) (Bang et al. 2021). Phenotypic reactions induced by excess or deficiency conditions may not only depend on the nitrogen source but also the species exposed (Armand et al. 2019; Melino et al. 2021; Ranathunge et al. 2015). Non-optimal dosages of NH_4^+

differentially affected root morphology and water and solute permeability of rice. Low concentrations and deficiencies resulted in increased root lengths and decreased amounts of aliphatics and aromatics, whereas excess concentrations led to shorter roots and enhanced accumulation of suberin in both endo- and exodermis, at the root tip as well as in the basal part (Ranathunge et al. 2015). These findings were perfectly reflected in qPCR analyses: suberin gene expression was down-regulated under low ammonium conditions but increased in high concentrations. Effects on root water and solute transport appeared well correlated in this case. Accordingly, reduced amounts of suberin resulted in increased Lp_r (both osmotic and hydrostatic) and P_{sr} of NaCl. Increased suberin concentration was able to decrease P_{sr}, even though the Lp_r remained unchanged (Ranathunge et al. 2015). In strong contrast to these findings on ammonium are the effects of low concentrations or absence of nitrate with maize and barley, which independently and repeatedly led to opposite findings (Armand et al. 2019; Melino et al. 2021; Plett et al. 2016; Schraut et al. 2005). First indications for this might be extrapolated from measurements of radial water flows (J_v) that were found to be significantly reduced after treating maize plants with nitrate deficiency (Schraut et al. 2005), indicating a potentially increased deposition of suberin. Later, maize transcriptomic data revealed co-expression of lipid metabolism genes that were attributed to a suberization response to nitrogen supply and demand (Plett et al. 2016). Two studies on nitrate deficiency that investigated several barley cultivars observed that roots were also longer under starvation conditions compared to control (Armand et al. 2019; Melino et al. 2021). However, compared to rice, the opposite behavior regarding suberization and transport properties was found. In barley, based on microscopy and exudation experiments, suberization of the endodermis was increased at 25 and 50% distance from the tip, and Lp_r(OS) was significantly reduced (Armand et al. 2019). The increased suberization due to nitrogen limitation was also quantitatively confirmed recently (Melino et al. 2021). Most significant increases of both the aliphatic and aromatic fraction of suberin were found in the endodermis at 25–50 or 50–75%, depending on the cultivar investigated. Additionally, the expression of suberin genes was significantly upregulated in all cultivars (Melino et al. 2021).

Surprisingly, less is known about the effect of phosphorus (P) and potassium (K) deficiency on suberization and subsequent effects on transport processes of monocot crop plants, even though they are two macronutrients in plant mineral nutrition (Table 3). In *Arabidopsis*, potassium deficiency resulted in enhanced suberization (Barberon et al. 2016). However, phosphorus starvation has not been investigated in more detail. Maize grown in phosphorus- or potassium-limited conditions were found to have an increased radial water flow (J_v) (Schraut et al. 2005), which might point towards reduced suberin amounts. Conversely, for barley roots, an increased degree of suberization at 25 and 50% distance after phosphorus deficiency treatment was histochemically observed. This coincided with significantly decreased rates of Lp_r(OS) (Armand et al. 2019). Also, barley roots were found to be decreased in length, but due to thicker roots under phosphorus depletion, the combined root surface area remained stable. The root:shoot surface area ratio had increased significantly (Armand et al. 2019).

Table 3 Summary of studies reporting about effects of plant mineral nutrients on root morphology, suberization reactions, and hydraulic properties of monocotyledonous crop species

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
Nitrogen	*Zea mays*	Hydroponics (solution specified in this paper)	11d (4d G + 7d T)	Replacement of KNO_3 by KCl	Water flow determination	-		↓ J_v	-	Schraut et al. 2005
Nitrogen	*Oryza sativa*	Hydroponics (solution specified in Miyamoto et al. 2001)	34d (6d G + 4w T)	Low (30/100), optimum (300) and high (1000/3000 µM) NH_4^+ with $(NH_4)_2SO_4$ as the only nitrogen source	Microscopy: investigation at 30/50/70% GC: zonation into 0-33/33-100%, separation of CC/OPR RPP qPCR	↑ RL for low and ↓ RL for high concentrations if compared to optimum	Comparing to optimum: ↓ Aliphatics and aromatics for low concentrations ↑ Aliphatics and aromatics for high concentrations in EN and EX in both zones	Comparing to optimum: Low concentrations: ↑ Lp_r(HY) and Lp_r(OS) ↑ P_w of NaCl High concentrations: = Lp_r(HY) and Lp_r(OS) ↓ P_w of NaCl	Comparing to optimum: ↓ for low concentrations ↑ for high concentrations	Ranathunge et al. 2015
Nitrogen	*Zea mays*	Hydroponics (Johnson's solution)	15-40d (4d G + 11-36d T)	Non-growth-limiting supply of 0.5 or 2.5 mM NO_3^-	RNA-Seq.	-	-	-	Some lipid metabolism genes are co-expressed with those encoding NRT2 transporters, indicating suberization response to N supply and demand	Plett et al. 2016
Nitrogen	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	13-17d (6d G + 7-11d T)	3.33% of control N (0.017 mM NH_4^+ and 0-0.2 mM NO_3^-)	Microscopy: investigation at apical 9-11mm and 25/50% Exudation experiments and CPP	↑ RL	↑, most remarkably at 25% and 50% in EN	Lp_r(HY) not determined ↓ Lp_r(OS) P_w not determined	-	Armand et al. 2019

(continued)

Table 3 (continued)

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
Nitrogen	*Hordeum vulgare* 3 cultivars	Hydroponics (Johnson's solution)	24d (3d G + 21d T)	1.5 mM NO_3^- in control conditions and switch to 0 mM NO_3^- after 14d for 7d in the treatment group	Microscopy: investigation at 0-25/25-50/50-75% GC: zonation into 0-25/25-50/50-75%, no separation qPCR	↑ RL	↑ Aliphatics and aromatics, most remarkably in 25-50% and 50-75% depending on cultivar in EN	-	↑ highest in 0-25% of all cultivars	Melino et al. 2021
Nitrogen	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	12d (3d G + 9d T)	Replacement of $Ca(NO_3)_2$ by $CaSO_4$ and KNO_3 by K_2SO_4	Microscopy: investigation at 25/50/90% GC: zonation into 0-25/25-50/50-100%, no separation	↑ RL	↑ Aliphatics and aromatics, most remarkably in 0-25% and 25-50% in EN	-	-	This study
Phosphorus	*Zea mays*	Hydroponics (solution specified in this paper)	11d (4d G + 7d T)	Replacement of KH_2PO_4 by KCl	Water flow determination	-	-	= J_v (trend to ↑)	-	Schraut et al. 2005
Phosphorus	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	13-17d (6d G + 7-11d T)	2.5% of control P (0-0.0125 mM PO_4^{3-})	Microscopy: investigation at apical 9-11mm and 25/50% Exudation experiments and CPP	↓ RL = SA ↑ root:shoot SA ratio	↑, most remarkably at 25% and 50% in EN	Lp_r(HY) not determined ↓ Lp_r(OS) P_{sr} not determined	-	Armand et al. 2019
Phosphorus	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	12d (3d G + 9d T)	Replacement of KH_2PO_4 by K_2SO_4	Microscopy: investigation at 25/50/90% GC: zonation into 0-25/25-50/50-100%, no separation	↑ RL	↓ Aliphatics and aromatics, most remarkably in 0-25% and 25-50% in EN	-	-	This study
Potassium	*Zea mays*	Hydroponics (solution specified in this paper)	11d (4d G + 7d T)	Replacement of K^+ by Na^+	Water flow determination	-		↑ J_v	-	Schraut et al. 2005
Potassium	*Hordeum vulgare*	Hydroponics (½ Hoagland solution)	15-18d (6d G + 9-12d T)	2.5% of control K (0.05 mM K^+)	Microscopy: investigation at apical 9-11mm and 65-70mm Exudation experiments and CPP	↑ RL	=, in both zones in EN	Lp_r(HY) not determined ↓ Lp_r(OS) P_{sr} not determined	-	Coffey et al. 2018

(continued)

Table 3 (continued)

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
Heavy metal	*Zea mays*	Hydroponics (¼ Hoagland solution)	10d (4d G + 6d T)	100 µM $CdCl_2$	Microscopy: investigation at apical 20 mm, 50%, and 10 mm below the root-shoot junction GC (to DW and RL): no zonation, separation of ECW/RHCW	↓ RL	↑ Aliphatics and aromatics combined in EN and HY/EX (best observable in EN if related to RL)	-	-	Zeier 1998
Heavy metal	*Zea mays*	Hydroponics and aeroponics (solution specified in this paper)	15-16d (3d G + 12d C + 21h T)	1 µM radio-labelled $CdCl_2$	Microscopy: investigation at 0-50% Cd uptake and content determination	Comparing hydroponics to aeroponics: ↓ RL	Comparing hydroponics to aeroponics ↓ in EN and EX	Comparing hydroponics to aeroponics: ↑ P_{sr} of Cd (estimated by Cd content measurement)	-	Redjala et al. 2011
Heavy metal	*Zea mays*	Agar plates (MS)	8d (3d G + 5d T)	50 µM Cd applied locally and unilaterally	Microscopy: investigation over whole roots	↓ RL	↑ unilaterally to Cd exposed side in EN and EX	-	-	Líška et al. 2016

[a] G, germination; C, cultivation under control conditions; T, treatment

[b] Only the most relevant methods for this summary are given. Microscopy, fluorescence or confocal; LR, lateral root; GC, gas chromatography; DW, dry weight; RL, root length; ROL, radial oxygen loss; zonation, axial division of roots into zones; no zonation, analysis performed with whole roots; separation, isolation of specific tissue layers; CC, central cylinder; OPR = outer part of the root; ECW, endodermal cell walls; RHCW, rhizo-/hypodermal cell walls; CP, cortical parenchyma; qPCR, quantitative PCR; RPP, root pressure probe; CPP, cell pressure probe

[c,d,e,f] =, no observable trend; ↓ and ↑, observed trends (histochemistry) or significant changes (gas chromatography)

[c] SA, surface area

[d] EN, endodermis; HY, hypodermis; EX, exodermis; ROL, radial oxygen loss; WT, wildtype

[e] Lp_r, root hydraulic conductivity; Lp_r(HY), hydrostatic hydraulic conductivity (apoplastic, symplastic, transcellular combined); Lp_r(OS), osmotic hydraulic conductivity (symplastic, transcellular combined); P_{sr}, solute permeability; σ_{sr}, reflection coefficient; J_v, water flow

A comparative study but with potassium starvation yielded entirely different findings (Coffey et al. 2018). Root lengths had significantly increased, but no changes in suberization could be observed via microscopy. Nonetheless, the $Lp_r(OS)$ was found to be significantly reduced, which was argued to be due to aquaporin activity to counterbalance the increased surface area of roots (Coffey et al. 2018). Unfortunately, we cannot conclude the final quantitative suberin amounts from the studies mentioned about phosphorous and potassium deficiency at the moment. Thus, we add and discuss new data about phosphorus deficiency. In general, more suberin observed by microscopy such as with staining via fluorol yellow can be supported by findings of enhanced suberin amounts via analytical methods (Kreszies et al. 2019). However, all microscopy staining methods have the disadvantage that there is a certain threshold needed to bind and show a signal and no fine differences, for example, for a reduced suberin amount besides more passage cells can be detected (Kreszies et al. 2020b).

To further complement the set of intensively studied abiotic stresses in barley roots (Tables 1, 2, 3, and 4), additional histochemical, as well as chemical analyses (see Fig. 1 and Supplementary for experimental details), have been carried out on nitrogen and phosphorus deficiency in our laboratory. Control roots of 12 d old plants in the deficiency experiment (Fig. 5) were similar in length (21.4 ± 4.5 cm) to the control roots of the ABA treatment (19.3 ± 3.7 cm). In contrast, root lengths of both deficiency treatments were significantly longer than their control. Roots subjected to nitrogen deficiency (35.7 ± 10.6 cm) were additionally considerably longer than those of phosphorus deficiency (30.0 ± 6.2 cm). Microscopic investigations of Casparian bands did not show any differences between control and treatments (data not shown). Starting in the middle of zone A, all roots displayed continuous Casparian bands in the radial endodermal cell walls and no Casparian bands or suberin

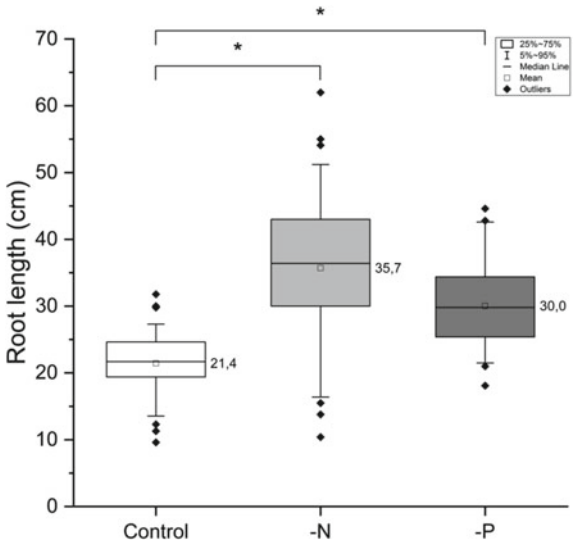

Fig. 5 Seminal root lengths of 12 d old barley plants grown in control or nutrient deficiency (-nitrogen, -phosphorus) conditions. The number beside each box (n = 40–66 individual roots) represents the mean. Significant differences at $p \leq 0.05$ based on t-tests are indicated by asterisks

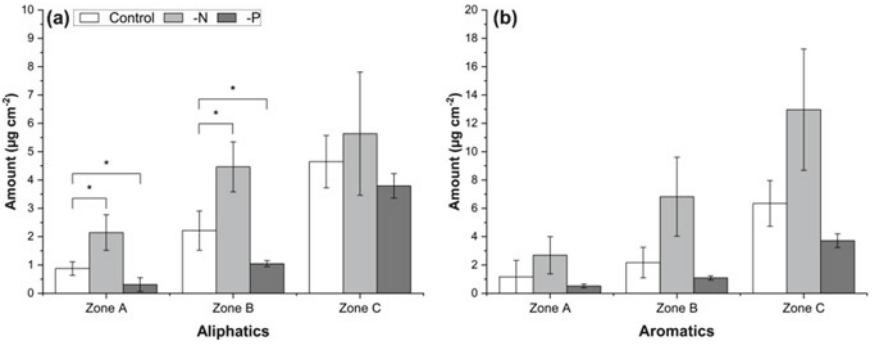

Fig. 6 Total amounts of aliphatic (**a**) and aromatic (**b**) suberin components under control or nutrient deficiency (-nitrogen, -phosphorus) conditions. Seminal roots were divided into three zones: apical zone A (0–25%), intermediate zone B (25–50%), and basal zone C (50–100%). Bars represent means ± standard deviation of $n = 3$ biological replicates. Significant differences at $p \leq 0.05$ based on t-tests are indicated by asterisks

lamellae have been observed in the hypodermis. Thus, no exodermis has been formed. Full suberization of zone C was consistent throughout all treatments (Fig. 3a, c, d). It is evident, that nitrogen deficiency (Fig. 3c, g, k, o) induced suberization patterns very similar to that of the ABA treatment (Fig. 3b, f, j, n). Treatment with phosphorus deficiency, in contrast, did not seem to induce the development of suberin lamellae (Fig. 3l, p) and suberin visualizations in zone B appeared to be comparable to that of the control. Qualitative and relative suberin composition was consistent with previously published data (Kreszies et al. 2019; Ranathunge et al. 2017) and also in between growth conditions, which is why only sums of aliphatics and aromatics are shown (Fig. 6a, b). Suberin amounts of both fractions of control conditions are in agreement with the control in the ABA treatment. Comparing aliphatic amounts of the nitrogen deficiency treatment to the control, significant increases could be observed in zone A and zone B. Zone C showed a slight trend of increased suberization, which was not confirmed statistically. Similar trends were observed for aromatics, however, none of these differences were significant. In strong contrast to this, phosphorus deficiency treatment resulted in significantly decreased aliphatic suberin amounts in zone A and B and the aromatic fraction, again, insignificantly followed this trend. Same as with nitrogen starvation, no change of aliphatic suberin could be observed in zone C. All mentioned observations are supported by the histochemical investigation (Fig. 3).

None of the deficiency stimuli of this study exerted osmotic stress on roots, which means that observable effects are solely due to the deficiency conditions employed, and no secondary effects in this regard need to be considered. Quantitative changes in suberin amounts can be attributed exclusively to differences in endodermal development, since no exodermis formation could be observed. Root lengths and Casparian band and suberin lamellae deposition in the control conditions fit very well to that reported previously for barley cultivar Scarlett (Kreszies et al. 2019, 2020b) cultivated under the very same growth conditions (same plant age and climate chamber).

However, the newly introduced nutrient deficiency stimuli employed in this study provoked significantly different reactions regarding root morphology and suberin deposition. Upon nitrogen starvation, roots were significantly longer (Fig. 5), which is in agreement with nitrate as well as ammonium deficiency treatments previously investigated with rice, maize, and barley (Armand et al. 2019; Melino et al. 2021; Plett et al. 2016; Ranathunge et al. 2015). Low nitrogen conditions also significantly enhanced aliphatic suberin amounts in zone A and zone B (Fig. 6a), fitting very well to the histochemical observations indicating earlier and stronger suberization (Fig. 3g, k, o). Such enhanced suberization of the root tip has only been reported via microscopy in salt-stressed barley (Knipfer et al. 2020), which is a combination of osmotic and ionic stress. In contrast, under severe osmotic stress zone A remained entirely unsuberized, while in zone B and C the suberin lamellae were enhanced (Kreszies et al. 2019). Under nitrogen depletion, there was not only a shift of onset of suberization to even before 12.5% distance. Additionally, the endodermis was fully suberized near the root tip (Fig. 3o). This increased suberin accumulation resulting from NO_3^- limitation is in line with other studies on barley (Armand et al. 2019; Melino et al. 2021) and also indicated on the gene expression level for maize (Plett et al. 2016), but conflicting with opposite findings on NH_4^+ in rice (Ranathunge et al. 2015) and NO_3^- in castor bean (Schreiber et al. 2005a). These reported differences could be attributed to the species investigated; especially dicotyledonous plants might react differently as monocots. In the case of rice, low dosages but not an entire deficiency of ammonium have been investigated (Ranathunge et al. 2015), whereas most other studies mentioned focused on nitrate reduction, and ammonium was rarely even supplemented in the hydroponic nutrient solution. As was hypothesized for rice and castor bean, a reduced amount of suberin lamellae could help to maintain a high uptake of the lacking essential nutrient, but this may not be valid in the case observed here. As the endodermis serves as a bidirectional barrier (Enstone et al. 2003), one might imagine that increased suberization in turn also aids in retaining nitrogen, which can still be taken up via high-affinity nitrate transporters into the root (Melino et al. 2021). Endodermal suberization appears to be highly dependent on plant species and precise environmental conditions. The fact that no changes in suberin contents took place in zone C despite strong reactions in zone A and B may indicate that a certain threshold value of suberin for its physiological function has been reached. In the mature root part of zone C the endodermis needs to be already completely suberized because water and solute transport in this root region is mainly longitudinal via the xylem to the shoot (Ranathunge et al. 2017). This would be supported by the finding that suberin amounts of zone B approach, but never surpass, those of the most developed zone C. Still, the increased suberin amounts due to changes in nitrogen status might lead to physiologically important decreased water and solute transport properties (Armand et al. 2019; Ranathunge et al. 2015).

Phosphorus deficiency, which similarly to nitrogen starvation resulted in increased root lengths (Fig. 5), contrarily affected root suberization. Especially in zone A and B aliphatic suberin amounts were significantly reduced (Fig. 6a), which was also reflected in fluorol yellow staining of suberin lamellae (Fig. 3h, l, p). Identical to nitrogen limitation, zone C was not significantly affected, and the aromatic suberin

fraction exhibited the same yet insignificant trend of the aliphatic domain. These findings are conflicting with microscopy-based reports (Armand et al. 2019) where decreased root lengths, increased root:shoot surface areas, and an increased suberization of the endodermis, most remarkably at 25 and 50% distance upon phosphorus deficiency treatment with barley were found. This resulted in significantly decreased osmotic hydraulic conductivity (Armand et al. 2019). In contrast, Andersen et al. (2018) histochemically identified low phosphorus to significantly reduce endodermal suberization in *Arabidopsis*, and Schraut et al. (2005) found phosphorus starvation to not statistically affect, if not even slightly increase, water flow (J_v) in maize roots. However, quantitative chemical suberin analysis, as provided here for the first time in the context of phosphorous deficiency, should be more specific than solely microscopic investigations (Kreszies et al. 2020b).

4.5 Heavy Metal Accumulation

Most studies to date investigating the effects of heavy metal accumulation in crop plants have been based on histochemical investigations (partly reviewed in Kreszies et al. 2020b), and very little is known about quantitative effects and root transport properties (Table 3). The studies have in common, that exposure of roots to heavy metals such as cadmium (Cd) always reduced the root length and enhanced the development of suberin lamellae (Líška et al. 2016; Lukačová et al. 2013; Redjala et al. 2011; Vaculík et al. 2009, 2012). For example, Líška et al. (2016) were able to show that gel-grown maize plants exhibited unilateral suberization of the endo- and exodermis after unilateral treatment with 50 μM cadmium, which points towards a highly elaborate sensing and reaction mechanism. By using 1 μM radiolabeled $CdCl_2$, Redjala et al. (2011) reported that growth in hydroponics, which by microscopy was found to induce lesser suberin deposition as aeroponic cultivation, resulted in increased uptake of cadmium if compared to aeroponics. This indicates increased membrane permeability towards heavy metal ions induced just by the method of cultivation (Redjala et al. 2011). The only study known to deliver quantitative information is that of Zeier (1998), who found 100 μM of $CdCl_2$ to significantly increase the suberin content in the endodermis and the hypo-/exodermis of maize, clearly confirming the notion of previously mentioned histochemical analyses.

4.6 Silicon Fertilization

Silicon (Si) supplementation has been reported to differentially influence the deposition of suberin lamellae in maize and rice but was typically only based on microscopical observations. Based on these, some studies reported enhanced suberization (Fleck et al. 2011, 2015; Lukačová et al. 2013), whereas others found no effect or even reduced suberin lamellae development (Vaculík et al. 2009, 2012). Very interestingly, only a few chemical analyses were carried out for silicon addition to maize

and rice roots. In these cases, no effects on aliphatic suberin could be observed, but the aromatic fraction was even significantly decreased sometimes (Fleck et al. 2015; Hinrichs et al. 2017). In barley, slightly increased root lengths upon silicon treatment, but no significant effects on suberin amounts by analytical methods were observed. The addition of silicon in osmotic stress conditions (−0.8 MPa induced by PEG8000) (Table 2) did not affect root lengths or the degree of suberization if compared to a PEG8000 treatment without silicon (Kreszies et al. 2020b). Contradictory observations of histochemistry obtained by many studies might be attributed to possible formations of silica aggregates that could either be able to interfere with the binding of fluorol yellow stain or lead to quenching of the fluorol yellow signals. This emphasizes the importance of combining qualitative microscopy with quantitative chemical analyses (Kreszies et al. 2020b). Transport properties after silicon application have been investigated in sorghum roots with and without osmotic and salt stress (Liu et al. 2014, 2015). In both studies it was found, that silicon treatment alone did not influence root hydraulic conductivity. However, the supplementation of silicon was able to significantly alleviate reductions in root hydraulic conductivity that are normally induced by osmotic and salt stress. These effects were attributed to enhanced aquaporin gene expression induced by silicon under stress conditions (Liu et al. 2014, 2015).

4.7 Hypoxia

The effects of oxygen deprivation are best described with rice plants (Table 4). Oxygen deficiency led to increased suberin amounts in the exodermis, both of the aliphatic and aromatic fractions, and in parallel radial oxygen was decreased starting from 20 mm behind the root tip (Kotula et al. 2009). Ranathunge et al. (2011a) confirmed these findings of effects on root morphology and suberin content of the exodermis, but also investigated changes in the endodermis as well as water and nutrient transport properties. They were able to show, that in addition to the exodermis also the endodermis is reinforced by suberin deposition under stagnant conditions. This did not correlate with decreased hydraulic conductivity (Lp_r(HY) as well as Lp_r(OS)). However, solute permeability of NaCl was significantly reduced, which was attributed to resulting specific pore sizes in the suberin lamellae, which would be capable of filtering Na^+ ions, but not water molecules (Ranathunge et al. 2011a). The fact that oxygen leakage through the cortex (Kotula et al. 2009) but not water transport was reduced by increased suberization was argued to be caused by the differential pathways employed by dissolved oxygen and water: oxygen travels in a diffusional manner, whereas water moves in hydrostatic bulk flow (Ranathunge et al. 2011a). Also in rice, the first mutant- and microdissection-based genetic evidence for a barrier formation in the hypo-/exodermis has been reported (Shiono et al. 2014a, b). By using permeability tests, *reduced culm number1* (*rcn1*) mutants of rice, which were defective in an ABC transporter gene, were shown to be incapable of forming an efficient exodermal barrier under stagnant conditions. Interestingly, the endodermal development was similar to the wildtype and it represented a barrier to apoplastic

tracers. Suberin biosynthesis genes were found to be most upregulated near the root tip, but no effect on lignin-associated genes could be observed. Quantitative suberin evaluation indicated that the absence of an exodermal barrier was occurring in parallel with a significant reduction of the aliphatic suberin fraction. However, this decrease of aliphatics was accompanied by an increase of the aromatic suberin amount, which in turn was not able to compensate for the lost barrier properties (Shiono et al. 2014a, b).

Another study incorporating chemical analyses based on a further monocotyledonous plant was carried out with two accessions of *Hordeum marinum*, a close relative of *H. vulgare* growing close to sea water (Kotula et al. 2017). Their findings were similar to that observed in rice plants and showed reduced root lengths and increased amounts of aliphatic suberin upon hypoxia. Microscopy indicated the development of an exodermis. This newly formed barrier resulted in significantly reduced radial oxygen losses in the accession that was shown to have the most pronounced enhancement of the exodermis upon oxygen deficiency (Kotula et al. 2017). Colmer et al. (2019) tried to identify molecules that could be involved in the perception of low oxygen conditions. They focused on small organic acids that are produced by anaerobic microorganisms upon hypoxia, being acetic, propionic, butyric, and hexanoic acid. Indeed, most of the acids in a specific concentration were able to significantly decrease root lengths and radial oxygen loss. Histochemical analysis, however, was not able to identify considerable changes in suberization in endodermis as well as hypo-/exodermis. Gene expression studies indicated an upregulation of suberin genes after treatment with propionic and butyric acid, which could potentially be responsible for providing the increased barrier properties to oxygen diffusion (Colmer et al. 2019). In a recent review focusing on the effect of low soil oxygen on root morphology and anatomy of maize, wheat, and rice it was summarized that an enhanced suberin formation under waterlogged conditions is always observed (Pedersen et al. 2020).

5 Conclusion

Responses in development and suberization of barley seminal roots (cv. Scarlett) towards different environmental stress factors are highly variable (Fig. 7). In response to osmotic stress and ABA treatment, the aliphatic suberin fraction exhibited significant increases, whereas the aromatics did show no or only weak increases (this study, Kreszies et al. 2019, 2020b). Especially root zone B (25–50%) showed the most intens responses towards environmental stimuli (Tables 1, 2, 3, and 4). Some stimuli specifically also triggered reactions in the most apical root segment (zone A; i.e. ABA and nitrogen deficiency, this study), which indicates the high plasticity of roots adapting their endodermal development to the variable environmental stress factors (Fig. 7). The weakest responses were observed in the basal root parts (50–100% or zone C), where suberization was already very high under control conditions. It is also obvious that barley cv. Scarlett seems to be incapable of forming an exodermis

Table 4 Summary of studies reporting about effects of hypoxia on root morphology, suberization reactions, and hydraulic properties of monocotyledonous crop species

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
O_2	*Oryza sativa*	Hydroponics (solution specified in Ranathunge et al. 2003)	30d (6d G + 7d C + 17d T)	0.0-0.2 mg/l O_2 by addition of 0.1% (w/v) agar to nutrient solution and flushing with nitrogen gas	Microscopy: investigation at apical 10-60 mm GC: zonation into apical 5-15/15-25/25-35/35-45/45-55/55-65 mm, separation of OPR ROL measurement	↓ RL	↑ Aliphatics and aromatics, most remarkably in 5-35 mm in EX	↓ ROL after 20 mm	-	Kotula et al. 2009
O_2	*Oryza sativa*	Hydroponics (solution specified in Miyamoto et al. 2001 and Ranathunge et al. 2003)	30-40d (6d G + 7d C + 17-27d T)	0.0-0.2 mg/l O_2 by addition of 0.1% (w/v) agar to nutrient solution and flushing with nitrogen gas	Microscopy: investigation at apical 5/10/20/30/40/50/70 mm GC: zonation into 60-90/80-250 mm, separation of CC/OPR RPP and pressure chamber	↓ RL = SA (more and thicker roots)	↑ Aliphatics and aromatics, most remarkably in 60-90 mm in EN and EX	= for Lp_r(HY) = Lp_r(OS) ↓ P_{sr} of NaCl	-	Ranathunge et al. 2011a
O_2	*Oryza sativa* 2 cultivars and 2 *rcn1* mutant lines	Hydroponics (solution specified in Colmer 2003)	23d (?d G + 9d C + 14d T)	0.1% (w/v) agar addition to nutrient solution	Microscopy: investigation at apical 20 mm and 5-20 mm below the root-shoot junction GC: no zonation, separation of CC and OPR Permeability tests Laser microdissection: separation of CC/CP/OPR of apical 15-25 mm zone qPCR	↓ RL	Comparing WT in stagnant to aerated: ↓ Aliphatics and aromatics in EN ↑ Aliphatics and aromatics in EX Comparing *rcn1* mutants to WT in stagnant: ↑ Aliphatics and aromatics in EN ↓ Aliphatics ↑ Aromatics in EX	Comparing WT in stagnant to aerated: ↓ Apoplastic tracer penetration over EX Comparing rcn1 mutants to WT in stagnant: ↑ Apoplastic tracer penetration over EX	↑ most remarkably in CC and OPR of 15-25 mm	Shiono et al. 2014b
O_2	*Oryza sativa*	Hydroponics (solution specified in Colmer 2003)	23d (2d G + 21d C + 9h T)	<1.0 mg/l O_2 by addition of 0.1% (w/v) agar to nutrient solution and flushing with nitrogen gas	Laser microdissection: separation of CC/CP/OPR of basal 12.5-22.5mm below the root-shoot junction Microarray analysis and qPCR	-	-	-	↑ most remarkably in OPR (Lignin-associated genes were not found to be upregulated)	Shiono et al. 2014a

(continued)

Table 4 (continued)

Abiotic stimulus	Species	Cultivation technique	Plant age[a]	Details	Methods[b]	Root morphology[c]	Suberization reaction[d]	Transport properties[e]	Suberin gene expression[f]	Reference
O_2	*Hordeum marinum* 2 accessions	Hydroponics (solution specified in this paper)	28-36d (7d G + 7d C + 14-22d T)	0.0-0.3 mg/l O_2 by addition of 0.1% (w/v) agar to nutrient solution	Microscopy: investigation at apical 15-25/35-45/55-65/75-85 mm GC (to RL): zonation into apical 5-45/45-85 mm, no separation ROL measurement	↓ RL (no data shown, but described)	↑ Aliphatics, in both zones with exception of 45-85 mm in one accession = (trend to ↑) Aromatics, in both zones with exception of 45-85 mm in one accession in EN and EX combined (microscopy indicates changes to come from EX rather than EN)	↓ ROL (in the accession forming the stronger ROL barrier in EX)	-	Kotula et al. 2017
Organic acids (produced by anaerobic microorganisms due to low O_2)	*Oryza sativa*	Hydroponics (solution specified in this paper)	28d (3d G + 21d C + 4d T)	Addition of 0.01/0.025/0.05 mM of four organic acids (acetic, propionic, butyric, hexanoic acid) to non-aerated solution	Microscopy: investigation at apical 50 mm qPCR ROL measurement	↓ RL induced by 0.025 or 0.05 mM of propionic, butyric, or hexanoic acid	=, at apical 50 mm in any of the treatments in EN and HY/EX	↓ ROL induced by all four organic acids behind apical 10 mm	↑ induced by 0.025 and 0.05 mM of propionic and butyric acid, respectively (Lignin-associated genes were upregulated by 0.05 mM propionic, butyric and hexanoic acid)	Colmer et al. 2019

[a] G, germination; C, cultivation under control conditions; T, treatment

[b] Only the most relevant methods for this summary are given. Microscopy, fluorescence or confocal; LR, lateral root; GC, gas chromatography; DW, dry weight; RL, root length; ROL, radial oxygen loss; zonation, axial division of roots into zones; no zonation, analysis performed with whole roots; separation, isolation of specific tissue layers; CC, central cylinder; OPR = outer part of the root; ECW, endodermal cell walls; RHCW, rhizo-/hypodermal cell walls; CP, cortical parenchyma; qPCR, quantitative PCR; RPP, root pressure probe; CPP, cell pressure probe

[c,d,e,f] =, no observable trend; ↓ and ↑, observed trends (histochemistry) or significant changes (gas chromatography)

[c] SA, surface area

[d] EN, endodermis; HY, hypodermis; EX, exodermis; ROL, radial oxygen loss; WT, wildtype

[e] Lp_r, root hydraulic conductivity; Lp_r(HY), hydrostatic hydraulic conductivity (apoplastic, symplastic, transcellular combined); Lp_r(OS), osmotic hydraulic conductivity (symplastic, transcellular combined); P_{sr}, solute permeability; σ_{sr}, reflection coefficient; J_v, water flow

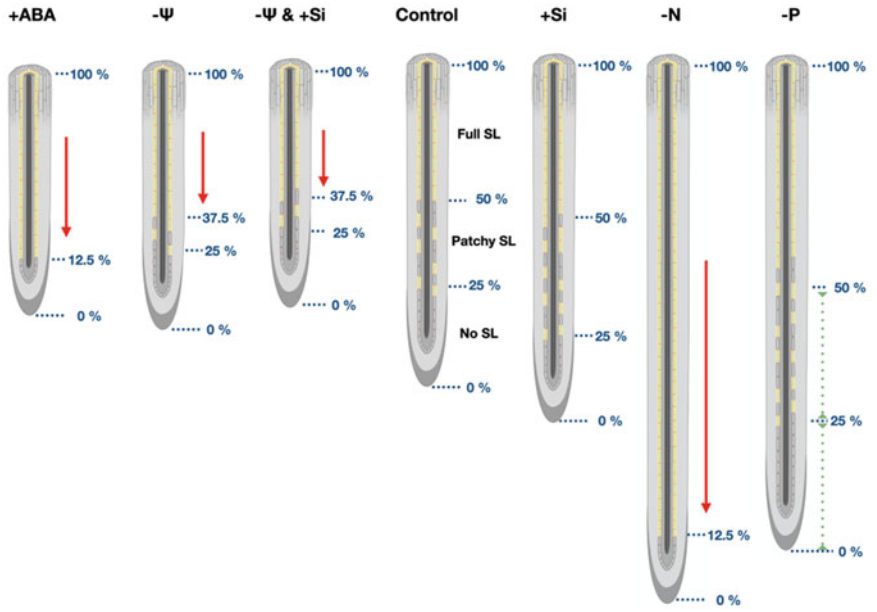

Fig. 7 Schematic representation of various abiotic environmental stimuli on seminal root development of barley cv. Scarlett. Under control conditions, barley seminal roots show in the youngest root zone from 0–25% relative root length only Casparian bands and no suberin lamellae. At 25–50% follows patchy suberin lamellae including passage cells. From approximately 50% relative length to the base of the seminal root the whole endodermis is completely suberized. In response to ABA, osmotic stress, and osmotic stress with silicon supplementation barley seminal root lengths are decreased and the fully suberized zone is shifted more towards the tip region (red arrow) because passage cells get suberized. Thus, under osmotic stress with and without silicon supplementation the patchy suberin lamellae root segment gets smaller, while it is completely missing after the addition of ABA. Furthermore, ABA treatment resulted in an earlier onset of suberization at around 12.5% relative distance. In response to supplementation with silicon or under nitrogen or phosphate deficiency barley seminal roots are significantly longer compared to the control. However, there was no effect on the suberization pattern by additional silicon supplementation. Under nitrogen deficiency, suberization is enhanced (red arrow) along the whole root similar to the ABA treatment. In contrast under phosphate deficiency, suberin amounts were reduced compared to the control (green dotted lines) in the younger half of the root. Data of this study was combined with those of Kreszies et al. (2019, 2020a, b). Only main roots and no lateral roots are shown for simplification. Red dots indicate Casparian bands, yellow lines indicate suberin lamellae (SL). +ABA, abscisic acid addition; −Ψ, osmotic stress by PEG8000; −Ψ & +Si, osmotic stress with silicon supplementation; +Si, silicon supplementation; −N, nitrogen deficiency; −P, phosphorus deficiency

in response to any of these stress conditions (Fig. 7). This, however, may only be concluded for cv. Scarlett, as other genotypes (e.g. wild barley) or barley species (Kotula et al. 2017; Kreszies et al. 2020a; Reissinger et al. 2003) have been reported to form an exodermis as a reaction to biotic as well as abiotic stimuli. We conclude, that the degree of suberin accumulation is essentially independent of absolute root

length, while it strongly and differentially responds to external environmental stimuli (Fig. 7).

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Hitting Hard Times: Effect of Abiotic Stress on Root Physiology

Shraboni Ghosh and Srayan Ghosh

Abstract Plants are exposed to a plethora of challenging situations throughout their lifecycle. As plants are sessile in nature, they have developed sophisticated signaling pathways to cope with the changing environment. However, most of the research till now is focused on aerial parts of the plant. Root although is the hidden part of a plant but performs many indispensable functions for the plant's survival. Furthermore, many abiotic stresses are first perceived by roots of a plant. Therefore, understanding how roots behave during stressful environment can be very useful for raising stress-tolerant crops along with increasing crop productivity. This chapter focuses on how roots sense different external stimuli and respond towards it. We have discussed molecular, physiological, anatomical changes in roots in response to various abiotic environmental cues.

1 Introduction

Being sessile in nature, plants encounter an array of adverse conditions throughout their lifecycle. Any environmental fluctuation may lead to variations in growth and development of a plant. The fluctuations can be in terms of temperature, excess or shortage of water, nutrient, soil salinization etc. These physical or chemical factors are termed as abiotic stress. Such environmental factors are considered as the main reason behind agronomic losses which is approximately half of the total produce all over the world (Bray et al. 2000).

A plant may respond differently under various abiotic stress conditions. Furthermore, individual abiotic stress may involve distinctive acclimation response and combined stresses may have responses that are also unique in nature (Mittler 2006). Both upper and lower parts of the plant are affected by various external stimuli. The aerial system i.e., shoot and underground system i.e., roots respond differentially towards various environmental factors. Shoot responses to environmental stimuli are easier to study as compared to root responses. Therefore, majority of the studied

S. Ghosh (✉) · S. Ghosh
National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India

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responses are specific to aerial part of the plant only. Being the supporting system of a plant, roots provide all raw materials from the soil required for plant sustenance. The main function of root is to provide water and mineral nutrients to the whole plant. Apart from that, roots are also involved in forming symbiotic associations with microbes, supporting the whole plant in the substratum and functioning as storage reservoirs. Therefore, proper development of root system is very crucial for overall functioning of the plant. Growth and development of root depends upon multiple factors like nutrient status of soil, temperature, moisture, salinity, and other soil properties (Ryan et al. 2016). Several abiotic stress conditions are first perceived by roots. After sensing the stress, roots tend to change their direction of growth in order to cope with changing environments. During the course of evolution, roots have evolved strategically, to overcome such stress conditions. Abiotic stress conditions may also promote transcriptional changes in roots of a plant. Root adaptations under stressful situations support the whole plant during adverse conditions. Certainly, understanding root physiology is a challenging task. But due to development of novel techniques, root biology has emerged as an upcoming field of plant science. This chapter highlights the phenotypical, anatomical, gene expression changes in roots under abiotic stress conditions. Additionally, tools for studying root responses are also discussed.

2 Perception of Abiotic Stress by Roots

Whenever a plant is exposed to any unfavorable condition, the stress receptors are activated to sense the change of environment. Identification of such sensors is very challenging due to lack any specific ligands, but they play a critical role in developing stress tolerance (Lamers et al. 2020). Till now, only a few such receptors or sensors have been identified. The identified sensors are mainly integral membrane proteins or membrane-anchored receptors.

Different external stimuli can create alterations in membrane fluidity. To overcome these changes plasma membrane adopts component adjustments to carry out their native function (Zheng et al. 2011). The roots have capability to sense changes in osmotic potential that help them to sense insufficiency of water and salt concentration in the soil. OSCA1 (reduced hyperosmolality-induced calcium increase 1), a calcium-permeable channel (Fig. 1) is recognized as a putative osmosensor. *osca1* mutant shows reduction in root length and leaf area indicating its role in sensing osmotic stress (Yuan et al. 2014a). HK1 (Histidine Kinase1) was identified an osmosensor in Arabidopsis (Nagaraj et al. 2013), however, HK1 doesn't play any role in transcriptional regulation under water deficit conditions (Sussmilch et al. 2017). In case of salt stress, Feronia receptor kinase was recognized as an external sensor in Arabidopsis (Feng et al. 2018). MOCA1, a glucuronosyltransferase was found to be functioning in response to salt-mediated signal transduction (Jiang et al. 2019). Fluctuations in surrounding temperature disturb the membrane fluidity. Plants have developed

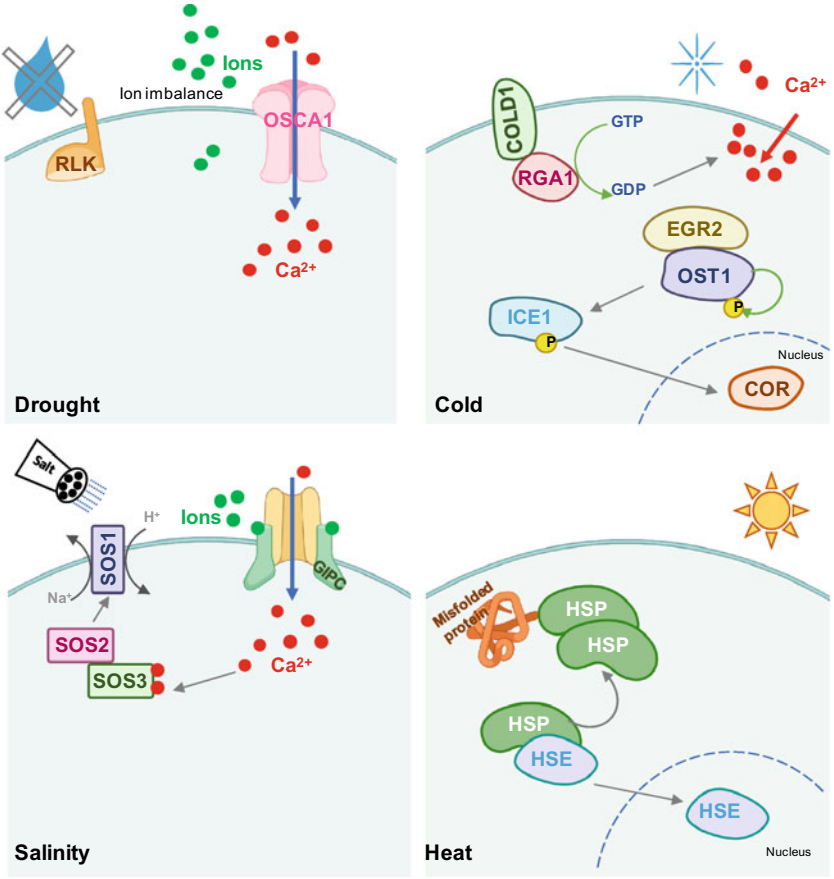

Fig. 1 Model showing stress sensing mechanism in plant roots. Water deficit conditions are sensed by several transmembrane proteins. OSCA1 is stimulated by increased membrane tension when subjected to osmotic stress, leading to influx of Ca2+ to persuade downstream signaling cascade. Drought results in separation of cell membrane from cell wall, which is recognized by RLKs restricted in the plasma membrane. In response to low temperature, COLD1 interacts with RGA1, which leads to enhanced GTPase activity; further calcium influx channel is stimulated. OST1 undergoes autophosphorylation, then it phosphorylates ICE1, resulting in initiation of COR gene expression. Elevated salt levels are sensed by binding of monovalent cations to GIPC sphingolipids. After binding of Na+ ions, calcium influx channel is triggered, which leads to activation of SOS pathway. Heat stress is sensed by detection of denatured proteins by HSPs, which later free HSE to stimulate expression of heat stress related genes.

distinct sensors to detect changes in membrane fluidity. For instance, COLD1 facilitates calcium flux during cold stress conditions in rice (Fig. 1), indicating its potential role in cold stress signaling (Ma et al. 2015). High temperature has similar effects on both root and shoots but roots can behave independently of other organs (Bellstaedt et al. 2019). Change in temperature is also sensed by phyB (a red/far-red

light receptor). Therefore, phytochrome B is considered as a thermosensor, but it gets activated in presence of light and mediates heat-induced signal transduction cascade (Legris et al. 2016). Elevated temperatures augment the rate of conversion of activated phyB (Pfr) into inactive phyB (Pr), leading to amplified stability of PIF4 (phytochrome interacting factor 4) and consequently suppression of expression of light induced genes (Ma et al. 2016). Plants also have certain channel proteins like CNGCs (cyclic nucleotide-gated channels) and GLR (glutamate receptor-like channels) that are involved in generation of calcium signaling during unfavorable conditions (Swarbreck et al. 2013).

3 Activation of Signaling Cascade

After stress perception, one of the earliest responses is stimulation of secondary messengers like calcium ions or reactive oxygen species (ROS) inside the cell. This transient increase is regulated by ligand-sensitive calcium channels. These messengers along with phytohormones can initiate cascade of signaling events leading to expression of stress responsive genes (Kollist et al. 2019). Along with calcium, ROS such as hydrogen peroxide, superoxide and hydroxyl ions are also accumulated in response to abiotic stress (Hasegawa et al. 2000). ROS may act as an intermediate signal for activation of downstream signaling (Price et al. 1994). Additionally, plants have well developed Mitogen activated Kinase family consisting of several MAP kinase kinase kinases (MAPKKK), MAP kinase kinases (MAPKK), and MAP kinases (MAPK). These modules like MAPK3, 4, and 6 are upregulated in response to various abiotic stresses (de Zelicourt et al. 2016). External cues like high salt and water deficit conditions stimulate SnRK2 family of protein kinases. In Arabidopsis, majority of SnRK2s are induced upon osmotic stress (Boudsocq et al. 2004). Beside these, protein kinases like TOR (TARGET OFRAPAMYCIN) and SnRK1 (Snf1-RELATED PROTEINKINASE1) are also involved in nutrient and energy signaling (Robaglia et al. 2012).

When plants are under salinity stress, a calcium dependent pathway known as SOS (Salt Overly Sensitive) gets activated (Zhu 2002). In this pathway, root specific SOS3 senses the elevation of calcium generated by excess sodium in the environment (Fig. 1). SOS3 then activates SOS2, a serine/threonine protein kinase. Further, SOS2 phosphorylates SOS1, a Na+/H+ antiporter that extrudes sodium ions back to the soil (Zhu 2002). SOS1 is found specifically in root epidermal cells and xylem parenchyma cells. SOS1 is also controlled by other proteins like CIPK8 (Calcineurin-B like protein 10 (CBL10)-interacting protein kinase 8) (Yin et al. 2020). During high salt condition, AtANNEXIN4 works as a calcium permeable transporter to activate SOS pathway (Ma et al. 2019). The role of HKT1 in salt stress is very well established. After perception of salinity stress, HKT1 gets upregulated in roots. It regulates sodium ions level in xylem by transporting it to the neighboring xylem parenchyma cells. SOS1 mediate transport of sodium ions from roots to leaves while

HKT appears to control it. However, it remains to be established whether SOS1 and HKT are expressed together or not.

In response to cold stress, many transcription factors are upregulated including CBFs (C-repeat Binding Factors). The CBFs further activate other downstream genes like COR (cold responsive) genes. The CBF genes are controlled by expression of a bHLH transcription factor, ICE1 (Inducer of CBF Expression) (Chinnusamy et al. 2007). In case of heat stress, ROS and NO act as secondary messengers. They activate HSFs (Heat Shock transcription Factors) to induce heat responses in plants (Katano et al. 2018). High temperature also stimulates expression of HSPs (Heat Shock Proteins) like HSP70 and HSP90, which function as chaperones to maintain protein homeostasis (Scharf et al. 2012).

In case of drought stress, protein phosphorylation plays an important role for stress adaptation. Several protein kinases are also involved in drought triggered signaling pathways. For instance, activation SRK2C which specifically expressed in root tips is initiated upon osmotic stress (Umezawa et al. 2004). Other ABA-activated SnRK2 may also activate other transcription factor responsible for stress tolerance.

4 Hormone Signaling in Roots During Abiotic Stress

To survive under stressful conditions, plants modulate their growth and development process. This modulation is achieved by coordinated action of phytohormones (Table 1). Their action may be near or distant to the organ of stress perception (Davies 2010). For example, roots synthesize phytohormones like cytokinin, gibberellins and ABA, these hormones are further transported to different tissues, where they perform their function. Among various phytohormones, ABA performs a vital role in maintaining the overall health of a plant. ABA can provide stress tolerance against several abiotic stresses like cold, drought, salinity (Rikin and Richmond 1976; Hsu and Kao 2003). Similarly, heavy metals like cadmium, nickel, zinc and aluminum also elevates ABA level in plants (Fediuc et al. 2005).

Table 1 Effect of abiotic stress on hormone signaling

Stress	Hormonal response in roots	References
Cold stress	Decrease in ABA	Verma et al. (2019)
Drought stress	Increase in ABA, SA, JA, Strigolactones	de Ollas et al. (2013, 2015), Ha et al. (2014), Muñoz-Espinoza et al. (2015)
Flooding stress	Decrease in ABA and JA; increase in ethylene	Arbona and Gómez-Cadenas (2008), Pedersen et al. (2021)
Heat stress	Decrease in ABA and JA	Vives-Peris et al. (2017)
Heavy metal stress	Decrease in ABA and JA; Increase in SA and auxin	López-Climent et al. (2011), Bankaji et al. (2014)
Salt stress	Increase in ABA	Ha et al. (2014)

Water deficit conditions are initially sensed by root. ABA sends signal to shoot to lower different physiological responses like transpiration, leaf expansion etc. (Wilkinson et al. 2012). ABA along with auxins control root growth under drought conditions. Even though auxins are the key players controlling root growth but cytokinin and ABA can also regulate root system architecture (Blilou et al. 2005; Munns and Sharp 1993). In response to dehydration stress, a slight rise in JA level was observed prior to ABA accumulation in Arabidopsis roots (de Ollas et al. 2015). Moreover, ABA also participates in other interactions like elevation of DELLA proteins (Rowe et al. 2016), which are known gibberellin repressors; inhibition of auxin biosynthesis (Yuan et al. 2014b). Gibberellin signaling is generally suppressed during adverse conditions, wherein majority of its function is mediated by DELLA proteins (Magome et al. 2008). DELLA proteins are often associated with salinity stress. In Arabidopsis, quadruple -*della* mutant showed impaired reduction in primary root growth (Achard et al. 2006). Also, GA-deficient biosynthetic mutant *ga1-3* demonstrated improved stress tolerance under high salt condition (Achard et al. 2006).

5 Root Morphology and Anatomy

Apart from morphological changes, abiotic stresses may also influence anatomical features of a root. Investigation of root anatomy might also be useful in generating stress-tolerant crops. The internal anatomy generally comprises of epidermis, cortex, endodermis, pericycle, xylem and phloem. Cellular pattering or development of these structures is altered during unfavorable conditions. Usually, deep root system with large xylem diameter is ideal for increasing water uptake capacity in dry areas (Comas et al. 2013). Nevertheless, small and fine roots with higher root length allow plants to augment their water uptake efficiency under water deficit condition (Comas et al. 2013; Thangthong et al. 2016). Drought stress induces ABA signaling in endodermis initials, leading to microRNA-mediated protoxylem formation (Bloch et al. 2019). In the stele region, expression of secondary wall associated genes get upregulated in response to different environmental cues (Iyer-Pascuzzi et al. 2011). Under stressful situations, tissue development gets severely retarded. For example, drought stress may affect cell differentiation in root elongation zone (Yamaguchi and Sharp 2010). Other than this, the roots show shrinkage in cell size, rise in vascular tissues and cell wall thickenings.

According to Bheemanahalli et al. (2019), greater stele to root diameter ratio improves hydraulic conductance and narrow xylem diameter facilitates water movement under dehydration stress. In case of water-logged conditions, formation of aerenchyma in root is one of the major adaptive responses. The aerenchyma is formed by cell lysis and deflation in the root cortex.

Increase in surrounding temperature may result in decrease in cell size and elevation in number of xylem vessels in roots (Bañon et al. 2004). When a plant is under heavy metal stress, root is the first part that encounters the metal ions. Heavy metal

like Chromium is known to accumulate higher in roots than shoots (Han et al. 2004). These elements enter the root via epidermis, and modify root anatomy. Sometimes, cellular patterning can be affected by the heavy metals. The anatomical alterations can be in terms of change in root diameter, size of root cells etc. (Stohs et al. 2000).

6 Changes in Root System Architecture in Response to Abiotic Stress

During ambient conditions, a plant grows steadily investing on both vegetative and reproductive growth. But when conditions change, they try to curb growth of each organ to acclimatize in unfavorable environment. Recently, morphological adaptations in root under abiotic stress have gained much attention from researchers all over the world. In general, hormonal signaling controls root development, but it is often influenced by different environmental factors (Petricka et al. 2012; Jung and McCouch 2013). External stimuli majorly affect RSA of a plant (Table 2). This may lead to changes in number or position of roots, rate of root elongation, root growth angles etc. Such changes result from synchronized action of both genetic and environmental factors. Among various abiotic stresses, drought and salt stress are primarily perceived by roots. Many crop species fail to grow above the ground in absence or shortage of water in soil. Therefore, plants with better root system at greater depths of the soil profile are more tolerant to drought. To overcome drought, plant roots display negative gravitropism to direct root growth towards water. Unlike shoot, root grows continuously in search of water to support the whole plant (Spollen and Sharp 1991). More importantly, growth of primary root remains unaffected during water deficit condition while lateral root formation gets severely affected. Notably, only development of lateral roots is inhibited, initiation of lateral root remains unaffected (Deak and Malamy 2005). Several transcription factors are involved in drought specific root adaptations like members of NAC domain family (Ooka et al. 2013). Unlike drought stress, high salt condition has an opposing action on the primary root where growth of primary root is strictly retarded than lateral roots (Julkowska et al. 2014). During salt stress, roots play a pivotal role in compartmentalization of sodium and chloride ions to maintain plant survival (Julkowska et al. 2017). In addition to cellular changes, roots show induction of several genes responsible for maintaining the root system architecture.

Just like any other abiotic stresses, nutrient deficiency in soil has a negative influence on RSA of a plant. Various soil nutrients like phosphorous, potassium, nitrogen etc. have specific effects on RSA. For e.g., low phosphate conditions are known to diminish primary root growth and root angle (Williamson et al. 2001). On the other hand, it fastens lateral root growth in terms of initiation and elongation (Williamson et al. 2001). The primary root growth under low phosphate condition can be restored by lowering the iron level, indicating their crosstalk signaling (Ward et al. 2008). Low nitrate condition does not have any effect on primary root but it induces lateral

Table 2 Genes controlling root system architecture in plants under abiotic stress

Stress	Gene	Plant source	Response	References
Drought stress	*WOX11*	Rice	Controls number of crown roots, lateral roots and root hair	Cheng et al. (2016)
	LRD2	Arabidopsis	Regulates formation of lateral roots	Deak and Malamy (2005)
	bHLH17, WRKY28	Arabidopsis	Induces longer roots	Babitha et al. (2013)
	DRO1	Rice	Increases root angle	Uga et al. (2013)
	ZFP34	Wheat	Increase root to shoot ratio	Chang et al. (2016)
	NAC5, NAC9, NAC10	Rice	Increase in root length and diameter	Jeong et al. (2013), Redillas et al. (2012), Jeong et al. (2010)
	EXPB23	Wheat	Enhances root growth	Li et al. (2015)
	MYB84	Soyabean	Increases primary root length	Wang et al. (2017)
	SNAC1	Cotton	Rises number of roots	Liu et al. (2014)
Heat stress	*PIMT1* and *PIMT2*	Arabidopsis	Increases root elongation rate	Ghosh et al. 2020a
	FBA1	Wheat	Increases root length	Li et al. (2018a, b)
	GolS2	Chickpea	Increases root growth	Salvi et al. (2018)
Salt stress	*RCc3*	Rice	Improves root system architecture	Li et al. (2018a, b)
	HKT1	Arabidopsis	Enhances lateral root development	Julkowska et al. (2017)
	SSR1	Poplar	Enhances lateral root emergence rate	Fang et al. (2017)
	MYBS1	Medicago	Increases primary root length	Dong et al. (2017)
	RPK1	Rice	Boosts root morphogenesis	Zou et al. (2014)
	RSS3	Rice	Maintains root growth	Toda et al. (2013)

(continued)

Table 2 (continued)

Stress	Gene	Plant source	Response	References
	SNAC1	Cotton	Increases in number of roots	Liu et al. (2014)
	SIM	Wheat	Augments root length	Yu et al. (2017)
	CYP79B2	Arabidopsis	Maintains lateral root growth	Julkowska et al. (2017)
	WRKY17	Maize	Improves root growth	Cai et al. (2017)
	SnRK2.4, SnRK2.10	Arabidopsis	Maintains lateral root	McLoughlin et al. (2012)
Heavy metal stress	*WRKY28*	Rice	Reduces total root length and lateral root number	Wang et al. (2018)
	EXPB2	Soyabean	Augments primary and lateral roots	Guo et al. (2011)
Nutrient deficiency	*TAR2*	Arabidopsis	Increases lateral root number	Ma et al. (2014)
	PSTOL1	Rice	Maintain root growth during phosphorus deficiency	Gamuyao et al. (2012)

root formation (Zhang and Forde 1998). Certain nitrate transporters like NRT2.1 and NRT1.1 promote lateral root initiation and elongation during nitrate supply (Little et al. 2005; Remans et al. 2006). Under low nitrate condition, other nitrate transporters like NRT1.8 is induced to procure nitrate from xylem sap (Li et al. 2010).

Generation of reactive oxygen species (ROS) is a common phenomenon witnessed during abiotic stress. ROS accumulation is controlled by activity of antioxidative enzymes on the other hand antioxidative enzymes are protected by protein repairing enzymes (Ghosh et al. 2020b; Kamble and Majee 2020). External environmental factors can affect RSA by regulating ROS assembly (Tyburski et al. 2009). ROS homeostasis in root tips can also adjust root growth (Yang et al. 2014). Recently, ROS equilibrium has come up as a chief player of RSA regulation by acting on primary root growth or lateral root emergence (Dunand et al. 2007; Tsukagoshi et al. 2010; Manzano et al. 2014). Variation in superoxide and peroxide levels in root tips can also influence root growth (Dunand et al. 2007).

7 Tools for the Study of Root Responses to Abiotic Stresses

Root is a vital organ of a plant and it supports the whole plant body. Being the hidden part of the plant, it often gets overlooked by the scientists for stress adaptation. Moreover, it is very difficult to study the physiological attributes under stress conditions. Any kind of abiotic stress may affect plant roots at physiological or molecular level. To explore such responses, many tools or techniques have been generated. One of the easiest ways to study root specific response is half-root stress technique. In half-root stress techniques, roots of a plant are kept under two separate treatments. This technique is based on a unique irrigation approach. It not only meets all requirements of a plant but also reduces water loss to increase crop productivity. Abiotic stress conditions like drought (Half-root drought stress or HRD), salinity (Half-root salinity stress or HRS) or nutrient deficiency (Half-root nutrient stress or HRN) are frequently studied through this approach (Iqbal et al. 2020). HRD stress may serve as a water conserving strategy that may alleviate the effect of global warming and sparsity in rains. In half-root stress approach, the stressed part of the plant senses alteration of environment, it then generates specific chemical signals, and these chemical signals are further recognized by the other half of the plant which acclimatizes in the changing environment. Therefore, half-root stress is a brilliant way to study adaptive responses under numerous external stimuli.

Recently, root phenotyping has gained much attention from different research groups all over the world. Root phenotyping can be done *in vitro* or in soil. To cope with extreme environmental conditions, plants often modulate their growth pattern. Changes in root architecture are one of the clever strategies adopted by plants. Therefore, it is very important to study the behavioral pattern of roots during stress. Under controlled conditions, root system architecture can be easily examined using plate-based experiments or hydroponics (Qiao et al. 2019). The phenotypical differences in RSA can be scanned or recorded as photographs. These photographs are further analyzed with the help of softwares like WinRHIZO, EZ-Rhizo, Root system analyzer, Root Nav, SmartRoot, Optimas analysis software, GiA Roots, Root reader, Root trace or IJ-Rhizo macro etc. (Armengaud et al. 2009; Clark et al. 2011; Galkovsky et al. 2012; Pierret et al. 2013, Schnepf et al. 2015). These tools facilitate measurement of various root related parameters like root length, root diameter, root angle etc. The main challenge arises in soil-based phenotyping or phenotyping at field-level. For soil-grown plants, techniques like Magnetic Resonance Imaging (MRI) and X-ray computed tomography (X-ray CT) are very popular. Both are non-destructive in nature and enables characterization of root growth patterns in soil however, these techniques are futile for large scale field studies (Morris et al. 2017). Shovelomics is a tool which allows study of roots at field level. In this technique, soil is completely removed; roots are washed carefully and analyzed for various root traits (Grift et al. 2011; Perkons et al. 2014; Bucksch et al. 2014). Another computational tool, Generator of Root Anatomy in R (GRANAR) provides digital versions of root anatomical network of monocot plants (Heymans et al. 2020). In addition to root phenotyping, other approaches like genomics, transcriptomics, metabolomics,

lipidomics are utilized to study root responses in plants. The -omic based techniques allow advancement of root phenotyping data at molecular level.

8 Conclusions

Plants face numerous adverse conditions throughout their life cycle. To overcome such situations, they acquire complex mechanisms involving perception of stress and activation of stress-signaling cascades. This triggers expression of stress responsive genes and several hormones. Whenever roots sense a change in the environment, they alter their direction of root growth. Therefore, RSA is a fundamental attribute to analyze in the field of agriculture. Exploring RSA of a plant in response to abiotic stress will allow us to develop approaches to generate crops with increased yield along with stress tolerance.

Being the hidden part of the plants, roots have been overlooked by the researchers. In the past few years, non-destructive phenotyping techniques have provided a novel platform for root-related studies. Development of such strategies will underpin efforts to generate plants with upgraded root systems. Moreover, these approaches will address the problem of global food security, where improved root phenotypes will be selected for future crops. Further investigation in this field would reveal important insights of roots during abiotic stress tolerance.

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An Approach in Updating Plant Metabolomics in Roots to Tolerate Anaerobic Submergence Stress

M. K. Adak, Arijit Ghosh, Indraneel Saha, and Debabrata Dolui

Abstract An overabundance of environmental extremities classically called abiotic stresses has been the integral part of plant's growth and development. Since then plants are also well adapted with its full genetic potential in two responses: susceptibility and resistance. These are coordinated with the expression of genes in up/down regulation according to genotypic plasticity at varying degrees as well as durations. Among the stressors most of those are perceived through root system of plants directly from soil like drought, salinity, metals and metalloids, pH variability, chemicals toxicity, hypoxia/anoxia etc. With the expression potential of gene(s) and its induction roots are also able to epigenetic regulation in tolerance of the stress factors where without interference of DNA sequence are also most important. Epigenetic regulation is also inheritable in nature but rather than any alteration of DNA sequence it involves the nuclear protein (histone) amendment as well as chemical modifications like methylation. In roots tissues certain conserved DNA sequences in chimeric manner in a precise and stringent regulation process tunes the responses to stresses that differs from rest of the flanking sequences. With the most modern–state-of art including high throughput sequencing at different platforms epigenetic regulation in roots genomics has reached a significant milestones to characterize stress. Thus, breeding with roots genomics now has set an alternative approach where world environmental climatic changes are ameliorated or minimize in crops to a significant extent. This chapter would encase various aspects of roots epigenetic responses to abiotic stresses in overall aspects of technology and its usefulness in crop sustenance.

1 Introduction

The phenotypes or morphological appearance of plants is the combinational results of a number of dynamic interaction of different molecules like nucleic acid, proteins, carbohydrates, organic acids, fat residues, phenolics and many others metabolites.

M. K. Adak (✉) · A. Ghosh · I. Saha · D. Dolui
Plant Physiology and Plant Molecular Biology Unit, Department of Botany, University of Kalyani, Kalyani, West Bengal 741235, India

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These are all well in coordination plants' developmental status as well as environmental inputs like light, temperature, salts, water, air, humidity etc. This demands a precise phenotypic explanation, particularly, for genome analysis excluding transcriptome, proteomes and metabolomes also. However, this approach not necessarily means effectivity in understanding as well as characterizing the total plant biology. So, an integrated approach is still in demand where metabolic studies with sequences of many reactions may be the linking for genes expression and phenotypic countenance. Thus, the question of metabolomes comes relevant to context of whole system biology of plants. This also brings special is of especial significance to satisfy the objective how plant system is responding to environmental variations. As already understood that gene expression ensures the potential of plants responses to adjust the stress, the accumulation and types of metabolites represent the keys to those adverse conditions. With understanding of different and other omics in deciphering the system analysis how metabolomics would be useful in assessment of stress tolerance in equally important. The metabolic profiling under set of environmental variations would be better in correlation for molecular and physiological activities of plants even under controlled or ambient condition of habitat. Even those metabolic profiling could be used as markers for selection pressure for better rootstocks in breeding programme.

2 Metabolomics Approaches for Deciphering the Stress Tolerance Under Submergence

Like other strategies covering genomics, proteomics, transcriptomics with high through put sequencing of genes, transcripts and peptides, metabolomics also approaches plants stress tolerance through study of metabolomes. In plant system, may be in a single cell, tissues, organs, however, in a specific growth or oncogenic state, metabolomics forwards the collection of biochemical reactions in a specific or collective paths. These could be more informative with plant's responses to a stressful environment with different level of accumulation of biometabolites. In *Sensustricto* it is within the 1200 kD of low molecular weight chemical residues (reaction precursors, intermediates and products in a reaction cycle), either primary or even secondary also are come under consideration of metabolomics. Almost the cases those are included the processed gene products either in structural of enzymatic proteins for a biochemical cycle(s) and thereby directly or indirectly present the functionality or viability of the tissues. With other predominant or supplementary functions these compounds are integral or indispensable in sense of plant structure or biomass, cell wall residues and cytoplasmic constituents, signaling residues, antioxidants and other moieties in plant's resistance to stress.

Irrespective of taxonomical hierchy in plant kingdom it covers around 200,000–1000,000 chemical residues as metabolites under varying concentration are documented those are quite flexible in their chemical diversity and property. Still, the

proper purification and identification through presumed annotation may be the full proof because of complexities and amalgamation with major and minor residues. By metabolic profiling may be the option in an wider identification of compounds with their predicted conformation and following tally with reaction kinetics. Therefore, the metabolomics would be more lenient to follow the abiotic stress paraphernalia with multidisciplinary approaches where a number of essentials must be the mandate like proper designing of the experiment, calibration of a vigorous protocols for data harvesting, finery in chemical analysis, efficient work station for data analysis, data combination with other omics and finally the validation of plants biological explanation. In more development metabolic profiling and metabolic finger printing a huge or vast array of data on metabolites could be procured without any earlier supposition. So, with global metabolome expression of any plants under any stress bio informatics tools may be the better option to corroborate the hypothesis/predicted data with metabolites accumulated in real. This may give the satisfactory results with higher level of accuracy as well as meticulousness. With modern state of art an initial analytical separation following detection and identification are the most predominant domain to start the metabolomics.

3 Submergence Stress: A Significant Scope for Metabolomics Study

Flood occurrence in the form of water logging or submergence poses a significant vulnerability for survival, growth and productivity. According to FAO it covers at least 10% of the total cultivable land is flooding prone with recurrent loss of grain yield in rice (Manik et al. 2019). Submergence due to flooding is the resultant for induced hypoxia/anoxia for a prolong duration for root system. The inadequate O_2 concentration in capillary water within the rhizosphere creates the well-known anoxic stress with significant loss of energy yielding metabolism like ATP synthesis (Ruf et al. 2019). The major changes in metabolomes are consisted, however, not limited with cytosolic acidification (lower pH values) as a function of impeded H^+/ATP ase activity. Even with the transient occurrence of submergence due to flash flood apart from growth and productivity, plants are characterized by expression of core-hypoxic responsive genes (Kuroha et al. 2019). The later is mostly focused with rearrangement of root specific metabolomes or metabolites distribution. A set(s) of gene and its expression is mostly targeted to anoxia/hypoxia responses which may come under categories of: alcoholic/lactate fermentation, interconversion of sucrose starch residues, stress induced metabolites for osmotic adjustment like compatible solutes, the hypoxia induced growth suppressing metabolites like ethylene/ABA/GA occurrence and their interplay, generation and lysis of oxygen/nitrogen moieties with oxidative redox (Fischer et al. 2020). Likewise, of those selected genes, alanine amino transferase is set as a reliable bio-marker with its hyper accumulation of alanine in rice roots under prolong submergence (Lothier et al. 2020). On the other hand, the

allocation of reduced carbons into sucrose exerts a flux in partitioning of starch and other polysaccharides in roots. The land races with quiescence (an adaptation to reserve the energy in expense of growth suppression under complete submergence) followers are more used with starch phosphorylase expression and activities. Rice genotypes with Sub1 possessing traits are more practiced with transformation of complex polysaccharides from simpler reducing/non reducing soluble sugars into roots, culm and leaf sheath (Morel et al. 2019). The oxygen deficiency in roots, particularly, under complete submergence makes it different for the bio-metabolome pattern in roots than shoots which has fairly revelation to the aerobic condition either directly from environment or photosynthetic oxygen. In comparison to other stress, submergence must be linked to a composite of stress where almost the environmental extremities are the concerned except high irradiance (Chevrier et al. 2020). Still, in fully grown or adult plants the tissues/organ specific metabolic differentiation is incomplete under submergence stress as its depth and duration of water stagnation may significantly vary. Accumulation of reserved carbohydrates as found in rice culm and leaf sheath is more common under stress along with few stress metabolites like γ-amino butyric acid (GABA), alanine, polyols (Ghatak et al. 2018). A gradual change in plant's low molecular weight organic acids like malic acid from TCA cycle pool may characterize submergence sensitivity. This is operated in homeostasis with amino acid depletion in roots to demark the hypoxia/anoxia under submergence stress. In sugar metabolomics under waterlogged rice a linear decline in few amino acids may suggests inhibition of protein synthesis in contiguous with sugar export from source (leaf sheath, cum) to sink (roots and other submerged tissues).It is more discriminating for the nitrogen metabolomes in submerged roots where nitrate assimilatory reactions remain more sensitive (Srivastava et al. 2019).The oxygen deprivation in roots would circumvent the impaired energy yielding metabolism where ATP dependent nitrate reduction is down regulated. Alternatively, roots may be more lenient to NAD(P)H dependent reduction with lesser sensitivity to hypoxia (León et al. 2020). Still, leaf sheath and culm are not much affected in nitrate reduction as compared to roots since exposed to air and more accessible to photosynthetic oxygen in mesophyll tissues. In waterlogged cotton, soybean other metabolites like ureides, glutamines are inhibited to translocate into leaves from roots (Lothier et al. 2020). The translocation efficiency under water logging is fully or partially dependent on metabolite exchange through root cell sap (in xylem/phloem conduits). This is illustrated with *Ricinus* where phloem sap does not vary with sugar concentration in roots but sap flow rate as well as flow area within the phloem sieve tube significantly found reduced (Shen et al. 2020). This is well consistent with other cereals like *Zea* where labelled carbon (^{14} C-sucrose) fed in leaves had not metabolized into TCA cycle intermediates in roots under anoxia. Contrarily, derivatives of glucose, however, non-metabolites moiety like ^{14}C-deoxyglucose had the minimum rate in translocation to roots under submergence induced anoxia (Maranas 2017). Despite of these there found few enigmas while someone takes the study of submergence metabolomics in roots that how it connects with areal shoots, whether phloem loading and unloading is independent through flux of metabolites. Under submerged

roots there is a tend to increase the phloem sugar loads that may exert an feedback inhibition on sugar export and downstream metabolism in shoots.

4 Areas Under Coverage of Root Metabolomics Under Submergence Stress

Under submergence plants have to experience a number of other abiotic stressors those may change directly or indirectly on morphological, anatomical, physiological, biochemical and cellular activities. Semi aquatic species like rice, roots survival under submergence are complemented by two sets of phenomena: escape and quiescence strategies. Both of those are targeted to assure to cope up the oxygen deficits either in form of hypoxia or anoxia. In the escape strategy the gas exchange and its rate from plants areal shoot to environment is increased with distinguishable features like those of epinasty, elongation of leaf sheath and internodes etc. Contrarily, energy expense is lesser in escape strategies with restriction of vegetative growth under water regimes. (The high energy depending phenomena like protein synthesis, DNA replication, cell wall synthesis are, secondary growth process are well regulated under quiescence with an observable alteration of cellular respiration from aerobic to anerobic (da Veiga Moreira et al. 2015). In sustenance to those the submergence tolerant cultivars are induced to sustain oxidative phosphorylation and allied metabolisms with formation of special tissues like aerenchyma in roots. Additionally, a number of metabolic activities like gas filled film in air spaces in leaf mesophyll is other support to coordinate the gas diffusion under anerobic/hypoxic condition under submergence (Liang et al. 2020). Along with high through put metabolomics a huge number of data are processed from roots responding to stress, particularly, in cereal crops. Both Gel based/free systems in roots proteomics could support the types of metabolites produced in different tissues in roots through either labeling or non labeling to know flow of reaction under stressed condition (Huihui et al. 2020). Since water logging or flooding stress may couple other facets of abiotic stresses the commonness and similarities of metabolites flux would more flexible to identify the nature of compounds in roots even at sub cellular levels also. Identification of complex proteins and metabolites are often challenged by presence of high number and amount of proteases, oxidative enzymes, and phenolics in tissues (Hashim et al. 2020). Still, metabolomic techniques like TCA precipitation, GITC extraction, SDS lysis, phenol phase separation and others chromatograms are the most uses for study of metabolomes in roots for any plants. Metabolomics coupled with proteomics had also great advantages in detection and analysis of target protein in roots even under post submergence period dried soil (Yan et al. 2020a, b). This releases the data on hyper/hypo-regulation of different target enzymes and their contribution in metabolic fluxes in common responses for saturated-dehydrated soil. More specifically, the organ specific secondary metabolites like a plethora of phenolics glycosides would be well in concern for submergence induced moisture deficit as well as oxidative revelation

Table 1 Submergence induced metabolic fluxes and their changes in roots for different cereal crops

Crop species	Stresses	Nature of metabolites fluxes	References
Rice	Submergence	Ethylene, Glycolic acid, Glyoxalic acid	Wu et al. (2017)
Wheat	Hypoxia/anoxia	Polyamines, proline, Glycine betaine, Glutein	Yan et al. (2020a, b)
Barley	Low light, alkaline pH	Tri acetic acid, Citric acid, lactic acid, ethanol, glydine	Park et al. (2009)
Sorgham	Oxidative stress, ROS accumulation	TCA, electron transportproteins, Cyto chrome c, amino acids, nucleotides	Khan et al. 2015
Maize	Salinity, alkalinity, ROS	Bicarbonate metabolism, stress protein, Fermented metabolites, Fe-S proteins, Ferulic acid, Siderophore	Ashraf et al. (2018)
Oat	Soilmoisture deficits, ionic imbalances	Secondary metabolites, Cell cycle proteins, H^+/ATP ase, DNA-nucleotides, chaperones	Akey and Morrison (1983)
Bajra	High irradiances, low temperature	Stress metabolites, jasmonic acid,phloem sapresidues, polysaccharides, inulin	Damame et al. (2017)

for tolerance species like cereal crops (Lobo et al. 2020). Even metabolomics coupled with analytical techniques for proteomics study has elucidated specific metabolites which have sparingly regulation in enzymatic cascade and also for post translational modifications for specific environmental extremities like low irradiance, anerobic exposure, ionic/metallic variation, pH sensitivity etc under submergence (Khan et al. 2020) (Table 1).

5 Compartmentalization of Metabolic Flux in Roots Under Submergence

The quite natural adaptation under submerged roots is radial oxygen movement from shoot to roots through functioning of aerenchyma (Pedersen et al. 2021). The metabolic flux that characterizes plants roots are the development of lysigenous cavities through programmed cell death that subsequent follows in lysis of the cortical cells. In roots of terrestrial species like *Zea*, *Triticum* etc. such an adaptation may not be availed by the plants specially under aerobic condition (Pegg et al. 2020). Still, those upland species may be induced with such lysigenous cavities under oxygen

deficit waterlogged condition. In typical semi aquatic cereal like *Oryza* such adaptation is readily also available under aerobic condition that may over expressed when plants are transferred to complete submergence of hypoxia/anoxia (Nakamura and Noguchi 2020). The metabolite compartmentalization is strictly sensed by plants for the special compounds like ethylene under submergence. Even with illustration with aquatic species ethylene biosynthesis and its involvement in special tissues may characterize the submergence tolerance (Chakraborty et al. 2021). In chemical reaction ethylene accumulation in rhizosphere and its diffusion through aerenchyma sets a special ecological niche for submergence sensitive plants that may differ a set of metabolites in cellular compartmentalization. Ethylene is produced from methionine residues in a more complex cycle compatible to polyamine biosynthesis in a competitive manner (Sauter et al. 2013). One intermediates like1-amino-cyclopropane-1-carboxylicacid (ACC) is subsequently metabolized by ACC synthase (ACS) and ACC oxidase (ACO) to ethylene is the limiting factor for sensitivity to submergence for the species. The expression levels and polymorphisms of ACS1 and ACO5 increases to contribute,ethylene accumulation in the roots (Lee and Yoon 2018). The formation of aerenchyma is more compounded with the generation of reactive oxygen species (ROS) through a respiratory burst oxidase homolog H (RBOHH) which is in parallel overexpressed with ethylene (Fig. 1).

Another set of metabolite compartmentalization is limited in cortical tissues of rice roots where auxin signaling is distinctly perceived (Jun et al. 2011). A set of auxin response factors (ARFs) is regulated at specific auxin response element (AREs) in auxin induced genes where indole acetic acid binding proteins are most favored (Kim et al. 2020). In rice there recorded at least 25 ARF genes and 31 IAA genes which are highly variable in expression variable perception of hypoxia/anoxia (Wu and Yang 2020). IAA proteins are characterized with an most conserved sequence motif auxin-dependent proteolysis (Yan et al. 2020a, b). The correlation between ethylene formation and aerenchyma formation is well evident from auxin dependent mutation in roots gain of function (dominant-negative) iaa13. This mutant is characterized by a single amino acid substitution at the upstream (AUX/IAA domain II) of IAA13 protein (Yamauchi et al. 2019). Through the functional analysis of the iaa13 mutant it comes in understanding for its involvement of inducible aerenchyma in rice roots. This is more established with the exercise of auxin inhibitor(s) where aerenchyma formation had been restricted under oxygen deficit or hypoxic condition of waterlogging (Yamauchi et al. 2020). This is also coordinated with ethylene biosynthetic gene activities to support more with the facts of auxin involved aerenchyma formation. In rice roots there proposed a mechanism where IAA-ethylene coordinated aerenchyma formation in relation to submergence tolerance. This also establishes that auxin is not only involved in constitutive aerenchyma formation but also tends to form same tissues in rice roots. This is also evident from *Arabidopsis* where ACC application would be a key factor for inhibition of lateral roots in accompany with increased auxin concentration in apical portion of roots. This is equally contradicted with ETHYLENE INSENSITIVE2 (EIN2) mutant for ethylene signaling gene under submergence of *Arabidopsis* roots (Negi et al. 2008). This is further noticed that auxin inhibitor simultaneously down regulates the expression of ACS1 and ACO

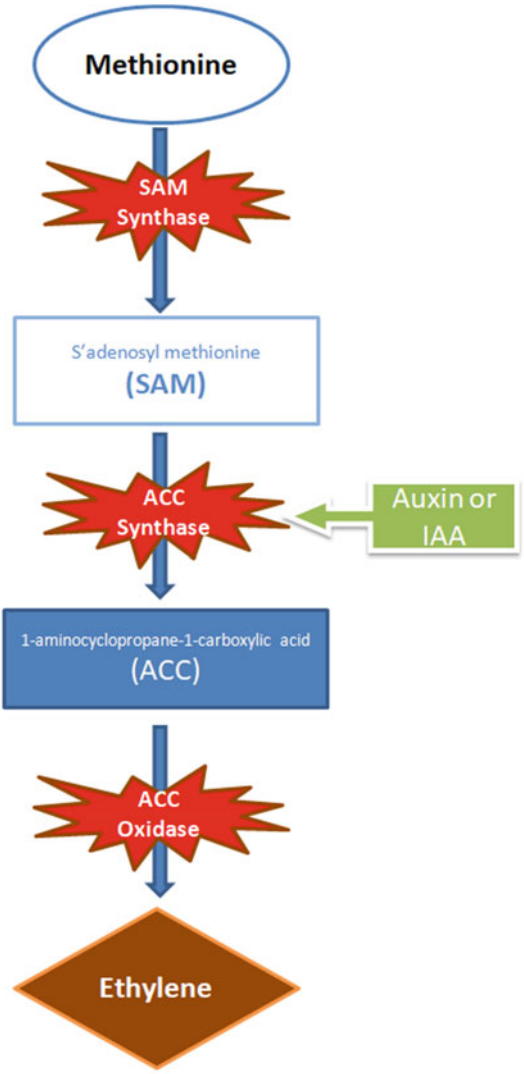

Fig. 1 Possible mechanism of auxin regulated ethylene biosynthesis

gene activities under stagnant water logging condition. This is experimentally proved in maize roots also where application of auxin also indices the lateral roots formation with ethylene hyper expression, however, irrespective of anerobic condition (Yu et al. 2015). So, cellular compartmentalization of ethylene and auxin in lateral root formation would be another key to metabolite compartmentalization in submerged roots sensitivity, particularly, under anoxia (Fig. 2).

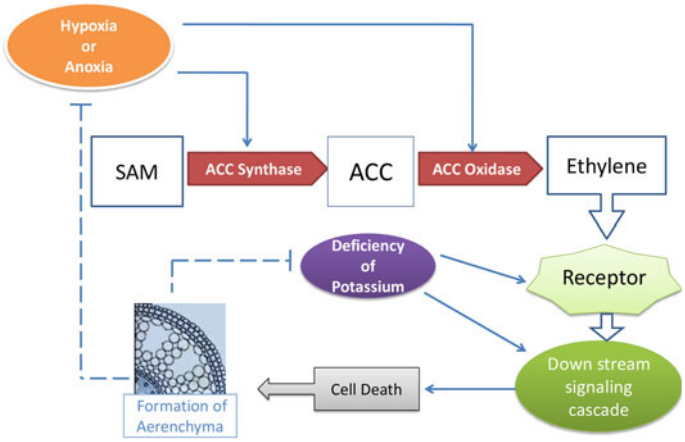

Fig. 2 Possible pathway of ethylene biosynthesis and its down stream regulation in rice root under hypoxia or anoxia

6 Metabolic Fluxes of Reaction Oxygen Species in Roots Under Submergence

Flooding and heavy downpour could cause the stagnation of water in the form of waterlogging/submergence leading to soil compaction and/or erosion, inundation, reduced oxygen tension or hypoxia/anoxia and finally plants damages. Stress hormones predominantly ethylene, ABA are the factors for induction of genes under inundation as well as perceive signaling form submerged soil to plant roots insides (Voesenek and Bailey-Serres 2015). In roots meristem and cortical cells the accumulation of ethylene can stimulate the endo plasmic reticulum (ER) sited transmembrane protein named ethylene insensitive transmembrane protein 2 (EIN-2) which in downstream also induced a set of transcription factors Ethylene insensitive 3 (EIN3). The later one the most important for the ethylene responses element (ERS) to activate those genes induced by the ethylene (Yu et al. 2017). Under anoxia ethylene can control a number of other growth regulators like GA, ABA etc. for cellular modification of quiescence or escape strategies under in survival strategies of plants. Herein the role of cellular redox would be another module in reaction with root cells for elongating and adventitious roots through cell wall lysis. This is as comparable to the programmed cell death as commonly available in rice, maize like cereal roots tissues (Basu et al. 2020). About the source, types and function of ROS to modulate the cellular redox, it is the hydrogen peroxide (H_2O_2) that function as a secondary messenger to ensure peroxidation reactions in membrane lipid lysis of endodermal layers of roots.

In a fine orchestration H_2O_2 can induce the ROS paths with a number of variants like super oxide anion (O_2^-), hydroxyl radical (OH^-), hydroxonium ions ($OH^{\cdot}$), singlet oxygen ($^{1/2}O_2$)etc. In fact, rice roots are well adapted to anerobic condition

either by anerobic respiration or alcoholic fermentation where these ROS are realized as byproducts (Sun et al. 2020). A well-known enzymatic cascade on different cellular organelle or non-cellular space that starts with respiratory burst oxidase homologue (RBOH) commonly NADP(H) oxidase in involved in ROS generation (Hong et al. 2020). In plants there recorded at least ten such genes in a multigene family to accommodate load of ROS generation as in *Arabidopsis*. The kinetics of different ROS are quite variable according to their chemical stability through the tissues when developed with anerobic stress.

On downstream development of O_2^- in roots are well sensitized with super oxide dismutase (SOD) into H_2O_2 (Saha et al. 2020). The later one being soluble ROS but more thana free radical can stimulate the cellular responses in two ways: anti-oxidation by peroxidase and elicitation of some other enzymatic reactions. Likewise, H_2O_2 in turn can stimulate few other ethylene response factors (ERFs), alcohol dehydrogenase (ADH) like anerobic proteins in roots under submergence. In a wider metabolic sense plant RBOHs are well characterized with other developmental processes where Ca^{+2} is most crucial. RBOHs could also moderate the Ca^{+2} efflux inside the cells which also bears relevance with adventitious root development (Demidchik et al. 2018). This is thoroughly studied in *Arabidopsis* where a homologue (*At* RBOHc) has been cloned with gentle NaCl treatment for development of lateral roots. Intestinally, those roots are good sensitive to other stresses like pathogenic or elicitations by symbiotic association (Sakuraba et al. 2015). With other variants in *Arabidopsis* like *At* RBOHd, *At* RBOHf is well coordinated in expression with transcript level under minimum salt differences in the growth media. More so, At RBOHd is reported with ABA signaling network for regulation of stomatal guard cells in a systematic response with other elicitations. As for e.g. signaling transduction for wound and biotic invasion, irradiances, heat and cold shock, abundances of salt and metals are the regular entities to exercise the ROS involvement and its consequent metabolism under roots in regulation of stress sensitivity (Luo et al. 2021). Undoubtedly, At RBOHd and its homologues are involved in stress perception to anoxia/hypoxia in roots but not much established in any direct relationship with ethylene metabolism. In earlier reports ROS like H_2O_2 was found to be reduced in *ein 2–5* as well as *rbohD-ko* mutant when subjected to hypoxia stress. The major hypoxia induced genes like Aldh in rice was down regulated in expression in such rbohD-ko mutant (Kim et al. 2019). So, there are ample scope to further study for the interactive session of ethylene and H_2O_2in roots not only for submergence tolerance but also other responses like seedling root growth, pigmentation as well as anoxia gene functioning.

7 Metabolomics in Roots for Re-Oxygenation Phenomena on Post Submergence

While flooding plants are partially or fully inundated but plants are exposed to high oxygen tension as but water level subsides. This creates another environment of high oxygen concentration coupled with strong irradiance (Striker 2012). The achlorophyllous tissues in leaves and culm under hypoxia are more sensitive to oxidative stress that sets the secondary impact on submergence sensitivity. The roots are more aerated along with loss of membrane permeability for K^+ and other osmolytes. The water deficit in root tissues may turn over to hyper turgidity and metabolically can not complete with ATP generation through non/poor functioning of oxidative energy metabolism (Rachmawati et al. 2019). At metabolic level for re oxygenation is evident with lipoxygenase activities in substantial accumulation of malondialdehyde content. A fall in compatible solute biosynthesis in roots leads to reduced permanent wilting percentage and finally ensures dehydration (Ayala et al. 2014). In reference to rice the major submergence tolerance regulator SubA, an ethylene response factor (ERFs) imparts the tolerance to oxidative and dehydration factors making submergence a multiple or composite stress. A number of ERFs are cloned from both rice and *Arabidopsis* where post submergence induced re-oxygenation are encountered with metabolic residues like ABA (ref). In fact, in rice roots re-oxygenation induces several motifs in ABA response element (ABRE) to bind with APETALA like factors (Saha et al. 2021). In a synchronized regulation of GAREs by respective factors, mostly bZip classes of proteins roots are maximized in oxidative stress tolerance by adopting quiescence strategies. For the later roots could regulate sugar utilization in aerobic respiratory flux by activation of genes like

Although the genetic mechanism of submergence survival for rice varieties containing the SUB1A gene has been elucidated, the downstream metabolic effects have not yet been evaluated. In this study, the metabolomes of Oryza sativa ssp. japonica cv. M202 and cv. M202(Sub1) were profiled using 1H NMR spectroscopy to compare the metabolic effect of submergence stress and recovery on rice in the presence or absence of SUB1A. Significant changes were observed in the NMR resonances of compounds in pathways important for carbohydrate metabolism. The presence of SUB1A in M202(Sub1) was correlated with suppression of carbohydrate metabolism in shoot tissue, consistent with the role of SUB1A in limiting starch catabolism to fuel elongation growth. The absence of SUB1A in M202 was correlated with greater consumption of sucrose stores and accumulation of amino acids that are synthesized from glycolysis intermediates and pyruvate. Under submergence conditions, alanine, a product of pyruvate metabolism, showed the largest difference between the two varieties, but elevated levels of glutamine, glutamate, leucine, isoleucine, threonine, and valine were also higher in M202 compared with the M202(Sub1) variety. The identification and characterization of alanylglycine (AlaGly) in rice is also reported. After 3 days of submergence stress, AlaGly levels decreased significantly in both genotypes but did not recover within 1 day of desubmergence with the other metabolites evaluated. The influence of SUB1A on dynamic

changes in the metabolome during complete submergence provides new insights into the functional roles of a single gene in invoking a quiescence strategy that helps stabilize crop production in submergence-prone fields.

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Role of Heavy-Metal Resistant Bacteria Isolated from Rhizosphere in Bioremediation and Plant Development

Debjani Mandal and Abhishek Basu

Abstract Heavy metal toxicity of soil and groundwater is a global menace. Bacteria isolated from the rhizosphere often exhibit the dual activity of plant growth promotion and bioremediation (and/or assisted phytoremediation) of heavy metals like arsenic, mercury, copper, cobalt, etc., contaminating the soil. Plant Growth Promoting Rhizobacteria (PGPR) evolve survival mechanisms by expression of different sets of genes or operon under heavy metal and xenobiotic stress. Different molecular pathways are activated within the PGPR for biodegradation, biotransformation, bioaccumulation, bioadsorption and/or biovolatilization of the pollutants. PGPR possess mechanisms to solubilze phosphate and potassium and fix nitrogen, hence, can be used as biofertilizer. They also produce phytohormones, volatile organic compounds and hydrolytic enzymes responsible for promotion of plant growth. Therefore, PGPR have commercial applications in enhancement of agricultural production and reclamation of heavy metal contaminated soils.

1 Introduction

Soil is a rich source of nutrients. It is a hub of various microflora and microfauna, site of diverse metabolic activities and centre of multiple interactions between different forms of life. Metal ions are absolutely essential for various biochemical reactions. Metalloenzymes use metal ions as cofactors. Metabolic pathways like electron transport chain, photosynthesis, transport and storage of different metabolites etc., require metal ions. Metals also play vital role, directly or indirectly, in initiation, activation, regulation and inhibition of various microbiological pathways and interactions between soil, plants and microbes. Heavy metals and metalloids are natural

D. Mandal · A. Basu (✉)
Department of Molecular Biology and Biotechnology, Sripat Singh College affiliated to University of Kalyani, Jiaganj, Murshidabad, West Bengal, India
e-mail: abhishek@mbbtsripatsinghcollege.in

D. Mandal
e-mail: debjani@mbbtsripatsinghcollege.in

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Table 1 Symptoms of heavy metal toxicity in plants

Plants	Heavy metal/metals	Symptoms
Barley	Cadmium, mercury	Symptoms similar to water deficiency
Wheat	Arsenic	Reduced seed germination and seedling growth
Helainthus annuus L	Arsenic	Reduced plumule and radical length
Elodea densa	Manganese, copper, cadmium, zinc, nickel	Reduced chlorophyll content, reduced photochemical efficiency of PS(II)
Thalassia hemprichii	Copper, zinc, lead, cadmium	Reduced chlorophyll and carotenoid content, reduced quantum yield
Phaseolus vulgaris	Zinc	Inhibited RUBISCO activity
Erythrina variegate	Cadmium	Reduced RUBISCO activity, reduced CO_2 fixation
Brassica juncea	Arsenic	Altered auxin level

constituents of various compounds that occur in Earth crust in far less amount than their toxic concentration to various life forms. Anthropogenic activities like mining, smelting, application of heavy metals containing compounds like pesticides, herbicides, fungicides, and use of heavy metals in glass, paper, wood and electronics industry, have led to increase in concentration of these heavy metals in soil (Hao et al. 2020; Järup 2003). Heavy metal toxicity in soil has affected the survival and growth of various life forms on Earth including human. Heavy metals like arsenic, mercury, chromium, nickel, cadmium are well known for their carcinogenic and mutagenic effects. These heavy metals have been classified as group 1 carcinogen by International Agency for Research on Cancer. Heavy metal toxicity leads to decline in the species richness and diversity in soil. Plants show symptoms of heavy metal toxicity like reduced growth, chlorosis of leaves, nutrient and water imbalance, root injury, alteration in seed germination, low biomass accumulation, senescence and ultimately death (Singh et al. 2016). Table 1 depicts the symptoms of heavy metal toxicity in plants. The symptoms of heavy metal toxicity in microbes are reduced enzyme activity and cell division, disruption of cell membrane, denaturation of proteins and nucleic acids, etc. (Igiri et al. 2018). We will now discuss the response of different life forms against metal induced stress.

2 Response of Different Organisms to Metal Intoxication and Metal Starvation

Metals cannot be synthesized or degraded. Its concentration can be regulated to an optimum level according to the cellular demands. Metals are indispensable for various

physiological processes involving metalloenzymes. In absence of specific metal, the activity of metalloenzymes is reduced, inhibited or become non-specific. Different metals are used as cofactors for different metalloenzymes. The need of metal in a cell should be carefully regulated by regulating two important parameters-the total concentration of metal in the cell and the labile pool of metal available and accessible for incorporation into various cellular enzymes, proteins and nucleic acids (Hao et al. 2020). Excessive metal outside the cell can alter the morphology, composition, size and growth patterns of microbial community. Metal intoxication within the cell can lead to mis-metalation of various metalloenzymes with non-specific metals leading to physiological imbalance and ultimately stunted growth and reproduction. In case of both metal starvation and intoxication, the first step is to sense the presence of metal inside the cell. Metalloregulatory proteins are specialized proteins regulated by metals. Binding of metals to these regulatory proteins causes allosteric transition in their conformation and alters their DNA binding affinity, modulating transcription of the genes responsible for metal homeostasis. This set of genes includes metallochaperones, metal importers, metal efflux proteins, transporters, etc. (Hao et al. 2020). Regulatory proteins sense and regulate sufficiency, limit and excess of metal, and modulate expression of sets of genes involved in metal homeostasis. Instead of directly sensing the metal, metalloregulators can indirectly sense the direct product of metal homeostasis. These metal regulators are very specific for the metals they bind to. They show high affinity for the specific metal responsible for the allosteric transition in them. The specific sequences of RNA can also act as switch for metal sensing. These riboswitches can sense metal either by directly binding to the metal or indirectly binding to the corresponding metabolic product. They adapt to a specific conformation on metal binding and sequester or present terminator or anti-terminator elements leading to regulation of definite sets of genes (Hao et al. 2020). Metal starvation is generally responded by upregulating the various specific and non specific metal import pathways and alternate pathways, which are either metal independent or do not require the limiting metal. In addition to this, the limiting metal is released from the store during metal starvation. Along with these, the efflux processes that lead to export of metal out of the cell and limiting metal dependent pathways are downregulated. On the contrary, metal intoxication is responded by downregulation of the import system and upregulation of the efflux system, metal sequestration by release of various extracellular polymers and siderophores, metal binding by abundant metabolites within the cell, storage of metal in different compartments of the cell, enzymatic detoxification (oxidation, reduction, methylation and demethylation) etc. (Hao et al. 2020). Excessive heavy metal in the local environment sometimes induces tolerance and resistance in the bacteria. Bacteria employ various strategies to respond to heavy metal toxicity. Bacteria encode for myriad of proteins, chaperones, enzymes and transporters responsible for heavy metal resistance in them. In the next section mechanism of heavy metal resistance in bacteria has been discussed.

3 Molecular Mechanism of Heavy Metal Resistance in Bacteria

Urbanization and modern agricultural activities have increased the use of heavy metal containing compounds in our daily life. Heavy metals are used in medical treatment due to their antimicrobial property, in agricultural field as manure, pesticides, fungicides, weedicides, and in animal husbandry as feed supplement to promote growth and prevent diseases in animal. This has led to the entry of heavy metals from various sources into the food chain and increase toxicity due to its biomagnification in the food chain. The surplus use of heavy metals has destroyed the environmental and human health (Hao et al. 2020; Nascimento and Chartone-Souza 2003). Heavy metals target cellular processes and induce oxidative stress, protein dysfunctioning, DNA damage and also alter membrane integrity. Microbes are also sensitive to environmental changes. Presence of heavy metals in excess induces a selection pressure on the microbe of the local environment. This changes the composition of the microbial community and allows the evolution of heavy-metal resistant microbes. Bacteria employ various survival strategies to cope with the heavy metal toxicity. One bacterium might become resistant to multiple metals if exposed for a prolonged period of time (Hao et al. 2020; Nascimento and Chartone-Souza 2003). We will now discuss some of the heavy metal resistance mechanisms in bacteria.

3.1 Mechanism of Mercury Resistance in Bacteria

Mercury is used as antibacterial and antifungal agents in agricultural fields. It is also used as catalyst in various industrial processes like amalgam formation during gold extraction. Other anthropogenic activities like burning of coal and petroleum products also add mercury in the environment. Inorganic mercury exists in two forms (Hg^0 and Hg^{2+}). The major form of mercury is Hg^0 that occur naturally in Earth's atmosphere. Mercury vapour (Hg^0) undergoes oxidation in presence of ozone and water to form the mercuric ion Hg^{2+}. Mercuric ion enters the water system and undergoes bacterial conversion into methylmercury. Methylmercury is the most common form of organic mercury. Release of industrial effluents into water body also adds mercury into them, which is again converted to methylmercury and is taken up by fish and other aquatic organisms. Consumption of methylmercury contaminated sea foods cause methylmercury poisoning in human (Foster 1987; Misra 1992; Nascimento and Chartone-Souza 2003). Mercury compounds have strong affinity for sulphur containing biomolecules like enzymes and proteins, which makes it very toxic to the biosystem. *Mer* genes confer mercury resistance in bacteria. These genes are mostly plasmid encoded but can also be present on transposons and bacterial chromosome. *Mer* genes are induced and regulated at transcriptional level and are involved in reduction and detoxification of inorganic and organic mercury. Organic

mercury like methylmercury is detoxified by organomercurial lyase. Mercury resistant bacteria have *mer* operon. *Mer* operon is a cluster of genes responsible for mercury resistance. In most of the bacteria the order of genes in *mer* operon is *merR, merT*, *merP*, *merA*, and *merB*. Some microbes might have *merC* and *merD* genes along with *merT* and *merP* genes. *merR* encodes a metal regulatory protein that senses the presence of mercury and activate the *mer* operon transcription, in presence of inducing concentration of Hg^{2+} ions by binding to the promoter operator region of *mer* operon. In absence of inducing concentration of Hg^{2+} ion, MerR represses the transcription of *mer* genes. MerP and MerT are periplasmic and cytoplasmic proteins, respectively, which transport mercury bound to their cysteine residues. *merA* gene encode for a flavoprotein that is involved in NADPH dependent reduction of Hg^{2+} to Hg^0. This mercury reductase enzyme is activated by substrate inhibitory concentration of mercuric ion and organomercurials. Such type of mercury detoxification is carried out by *Pseudomonas*, *E. coli, Staphylococcus aureus*, etc. (Foster 1987; Misra 1992; Nascimento and Chartone-Souza 2003). Hg^{2+} can easily diffuse across the outer membrane of the bacteria. In the cell, Hg^{2+} binds to the cysteine residues of MerP which then transport it to MerT on the cytoplasmic membrane. MerT transfers the mercuric ion to mercury reductase (MerA). Mercury reductase convert Hg^{2+} to Hg^0. *Mer* operon is present in many gram positive and gram negative bacteria like in transposon Tn21, Tn501, Tn5053 of plasmid NR1, PVS1 and pMR from *Shigella flexneri, Pseudomonas aeruginosa* and *Xanthomonas* sp., respectively. Plasmids pDU1358, pPB and pI258 of *Serratia marcescens*, *Pseudomonas stutzeri* and *Staphylococcus aureus*, respectively, also have *mer* operon. Mercury resistant bacteria are classified into two classes- narrow spectrum and broad spectrum mercury resistant bacteria. Narrow spectrum mercury resistant bacteria can only detoxify inorganic mercury compounds by the mercury reductase enzyme. Broad spectrum mercury resistant bacteria are resistant to both organic and inorganic mercury compounds. In addition to the mercury reductase, these bacteria have enzyme organomercurial lyase encoded by *merB* gene (Foster 1987; Misra 1992; Nascimento and Chartone-Souza 2003). Organomercurial lyase cleaves the C-Hg bond (organometallic linkage) in organomercurials to yield Hg^{2+}. Mercury reductase then converts Hg^{2+} into volatile metallic mercury Hg^0 in a NADPH dependent reduction process. Some bacteria might have a second regulator that downregulate the *mer* operon by binding to the promoter-operator region of the operon very weakly. This regulator is encoded by *merD* gene. Therefore, *mer* operon encodes all the genes required for mercury detoxification. Bacteria also employs other strategies for mercury resistance which include decrease in cell permeability and reduced uptake of Hg^{2+} ion, sequestration of mercury in different compartments of cell, decomposition and inactivation of mercury with H_2S, etc. (Fig. 1, Table 2) (Foster 1987; Misra 1992; Nascimento and Chartone-Souza 2003).

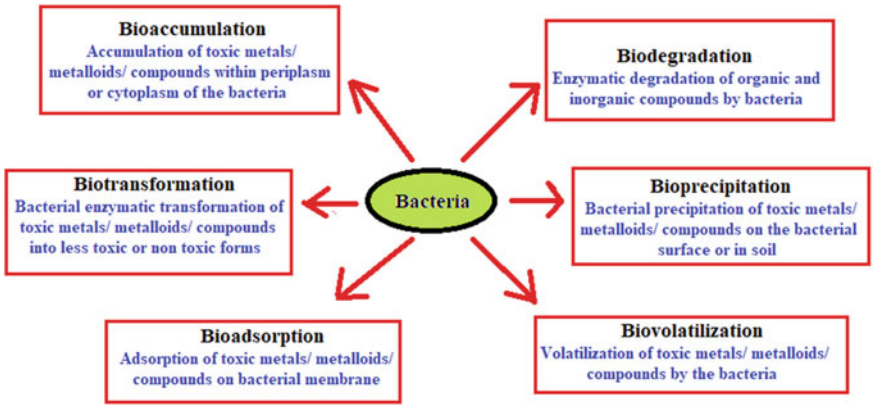

Fig. 1 Different mechanisms of bioremediation employed by heavy metal resistant bacteria

Table 2 Mechanism of heavy metal resistance employed by different bacteria

Bacteria	Mechanism of heavy metal resistance
Pseudomonas putida	Intracellular sequesteration of copper, zinc and cadmium
Rhizobium leguminosarum	Intracellular sequesteration of cadmium
Pseudomonas syringae	Sequesteration of copper in the periplasm
Geobacter spp	Reduction and precipitation of iron
Desulfuromonas spp	Reduction of toxic sulphur compounds
Geobacter sulfurreducens	Conversion of Cr^{6+} to Cr^{3+}
Geobacter metallireducens	Conversion of Cr^{6+} to Cr^{3+}
Klebsiella planticola	Precipitation of cadmium as insoluble sulfides
Vibrio harveyi	Precipitation of soluble lead as complex lead phosphate salt
Bacillus spp	Biomethylation of Hg^{2+} to gaseous methylmercury
Clostridium spp	Biomethylation of Hg^{2+} to gaseous methylmercury
Geobacter metallireducens	Reduction of Mn^{4+} to Mn^{2+} and U^{6+} to U^{4+}

3.2 Mechanism of Arsenic Resistance in Bacteria

Arsenite and arsenate, the two inorganic forms of arsenic are toxic to all life forms. Arsenite binds to thiol containing metabolites like reduced glutathione, lipoic acid, etc., and inhibit metabolically significant enzymes. Arsenate being the structural analogue of phosphate interferes with phosphate containing biochemical reactions. Arsenic toxicity shows symptoms of arsenicosis, keratosis, melanosis and cancer of liver, lungs, kidney, etc., in the long run. Arsenic is widely used in manufacturing of pesticides, fungicides, weedicide, herbicide, paints, paper and glass. Plants uptake arsenic from agricultural field and accumulate it in roots (mostly), leaves, stem and grains. Some hyperaccumulating plants accumulate arsenic at high concentration in the vacuoles. They are tolerant to high concentration of arsenic in soil. Various microorganisms also show tolerance and resistance to this heavy metal (Mandal et al. 2017). Arsenite oxyanion resemble the structure of glycerol and thus, is taken up by Glpf aquaglyceroporin, whose natural substrate is glycerol. The arsenic resistance genes in microbes are plasmid or chromosomally encoded. For example, pI258 plasmid of *Staphylococcus aureus* encodes genes for arsenite, arsenate and antimony resistance. Separate sets of genes are involved in resistance of these three chemical species (Silver et al. 1981). R733 plasmid of *E. coli* includes *ars* genes for arsenate reductase, arsenate efflux proteins and regulators (Rosen et al. 1988). Arsenic resistance genes are also present in transposon Tn2502 of pYV plasmid of *Yersinia enterocolitica* (Ye et al. 2007). Chromosomal homolog of arsenic resistance genes work in association with plasmid encoded genes for arsenic resistance. Chromosomal *ars* operon of *Pseudomonas aeruginosa* Dk2 encodes for organoarsenical efflux permease (encoded by *arsJ* gene) that transport *1-arseno-3-phosphoglycerate* (a highly unstable organoarsenical) out of the cell (Chen et al. 2016). Members of the group Eubacteria and Archeae have genes for arsenite oxidation. In *Alcaligenes faecalis* NCIB8687 the region encoding arsenite oxidase enzyme is of 71 kb. The two genes *asoA* and *asoB* along with twenty other genes are involved in arsenite resistance in this bacterium. *asoA* and *asoB* encode for the large molybdopterin containing and the small Rieske subunit of arsenite oxidase. The enzyme arsenite oxidase oxidizes arsenite into arsenate, the less toxic form of arsenic. The other putative genes encode for arsenite ATPase membrane transporter and efflux system. The arsenite oxidase operon *aoxABCD* has been identified in *Centibacterium arsenoxidans,* which also encode for arsenite detoxification and efflux system (Silver and Phung 2005). Arsenate operon in general might contain three, four or five genes, classifying the operon into three types (*arsRBC, arsRABC and arsRDABC*). *ArsR* gene encodes for a repressor protein that represses the transcription of *ars* operon. Binding of arsenic to this trans acting metalloregulatory protein, dissociate it from DNA and initiate the transcription of *ars* genes. ArsD is a metallic chaperone and inducer independent repressor that binds weakly to the promoter operator sequence. The primary role of ArsD is to bind and transfer arsenite to ArsA ATPase. ArsA is stimulated by both arsenite and antimony and interacts with membrane embedded efflux pump ArsB. These two hydrophobic proteins together transport arsenic outside the cell. ArsA also

associates with other membrane proteins. ArsB with twelve transmembrane domains can either use ATPase activity of ArsA or cell membrane potential to extrude arsenic species out of the cell. *ArsC* encode for an arsenate reductase that convert arsenate into arsenite prior to its extrusion. ArsC uses glutathione, redoxin or thioredoxin as electron source for the reduction process (Fekih et al. 2018). *Saccharomyces cerevisiae* have two independent transport systems for arsenic. Acr3p is a plasma membrane transporter involved in arsenite extrusion conferring arsenic resistance to *S. cerevisiae.* Ycf1p is another transporter protein of ABC transporter superfamily involved in storage of arsenite into vacuole in an ATP dependent manner. Chromosome XVI of *Saccharomyces cerevisiae* encodes for Acr1, Acr2 and Acr3, all three of which responds to arsenic stress. Acr1 might be sensitive to both arsenite and arsenate. Acr2 is an arsenate reductase and Acr3 is a plasma membrane embedded arsenic efflux transporter (Ghosh et al. 1999). Bacteria also have genes for detoxification of organic arsenicals. For example *Campylobacter jejuni,* a food borne pathogen is resistant to organic arsenicals like roxarsone (*4-hydroxy-3-nitrobenzene arsonic acid*) which is used as feed additive in poultry farming. *Campylobacter jejuni* consists of *ars* operon with four genes-*arsP, arsR, arsC* and *acr3*. The regulator ArsR, arsenate reductase ArsC, efflux transporter Acr3 and organoarsenical transporter ArsP together confers resistance to arsenic in the pathogen. ArsP have eight transmembrane helix and transport trivalent organoarsenicals mainly (Shen et al. 2014; Chen et al. 2015a). Some bacteria might also contain proteins like ArsH, ArsM, ArsK, ArsI, etc. For example ArsH of *Pseudomonas putida* is an organoarsenical oxidase that converts trivalent methylated and aromatic arsenicals into pentavalent species (Chen et al. 2015b). *ArsM* gene is established to encode for As (III) S-adenosylmethionine methyltransferase which methylate arsenite into volatile trimethyl arsine (Qin et al. 2006). *ArsI* encode Fe^{2+} dependent dioxygenase involved in demethylation of methylarsonic acid (Yoshinaga and Rosen 2014). ArsK is an arsenic efflux transporter that confers resistance to all type of arsenic compounds except pentavalent arsenate. It reduces accumulation of roxarsone, methylarsenite, arsenite, etc. It is induced by arsenite, antimonite, roxarsone and methylarsenite (Shi et al. 2018). Therefore, microbes have developed strategies to resist, tolerate, detoxify and export inorganic and organic arsenic compounds (Fig. 1, Table 2).

3.3 Mechanism of Copper Resistance in Bacteria

Copper is used as bactericide in agriculture field. It is an essential micronutrient for plant growth and development. Excess copper in soil induces stress in plants and microbes. Microbes encode genes that provide resistance to copper. Copper resistance genes are mostly encoded by plasmid. For example pPT23D plasmid of *Pseudomonas syringae pv.* tomato contain copper operon with four genes which are induced by copper only. The operon *copABCD* is under the regulation of copper inducible promoter followed by a constitutive promoter with two regulatory genes *copR* and *copS*. Both copper inducible promoter and the two regulatory genes are

essential for proper expression of copper resistance genes. CopA and CopC are two periplasmic copper binding proteins that limit the copper concentration in periplasm. *CopB* encode for an outer membrane protein that sequester copper outside the outer membrane. CopD is an inner membrane protein involved in copper transport. These proteins function together to reduce the copper concentration within the cytoplasm (Mellano and Cooksey 1988; Lim and Cooksey 1993). Plasmid pRJ1004 of *E. coli* has *pcoABCDRSE* operon that encode for proteins dealing with periplasmic copper toxicity (Rouch et al. 1985). *E. coli* also have two chromosomally encoded copper systems—*cue* system and *cus* system. *Cue* is the main system involved in copper transport. It has three important genes-*cueR, cueO* and *copA*. CueR is a copper responsive regulator and CueO is periplasmic multi-copper oxidase involved in oxidation of Cu^{+} to Cu^{2+}. CopA is an ATPase that transports copper out of the cytoplasm (Bondarczuk and Piotrowska-Seget 2013). *E. coli*, *Enterococcus hirae* and *Mycobacterium tuberculosis* also have two other regulatory proteins CopY and CsoR that negatively regulate the transcription of copper resistance genes under copper limiting condition. After entering into the cell Cu^{2+} can be reduced to Cu^{+}, which is more toxic in nature. Cu^{+} is effluxed out of the cell using the *cus* like *RND* system or it undergoes further oxidation to Cu^{2+} by CopA or PcoA or CueO like periplasmic copper oxidase and ATPase. CopA is a periplasmic copper binding ATPase with eight transmembrane segments. It is mainly involved in transport of copper from cytoplasm by oxidizing Cu^{+} to Cu^{2+} (Rademacher and Masepohl 2012; Bonderczuk and Piotrowska-Seget 2013; Martínez-Bussenius et al. 2017). Chromosomally encoded copper resistance genes are also present in *Acidithiobacillus feroxidans* ATCC23270 and ATCC53993. *Acidithiobacillus feroxidans* ATCC23270 have more than ten genes in its genome that are involved in copper homeostasis. Three genes *copA1, copA2, copB* encode ATPase, which transport copper. Three genes *cusA*, *cusB* and *cusC* encode for inner membrane antiporters that use proton motive force to efflux copper out of the cytoplasm. Two genes *cusF* and *copC* encode for periplasmic metallochaperones. Rus and AcoP are periplasmic copper binding protein (Martínez-Bussenius et al. 2017). Therefore, copper resistance mechanism in bacteria involves cytosolic and periplasmic metallochaperones, outer and inner membrane copper binding proteins, antiporters, transporters, oxidase and regulators (Fig. 1, Table 2).

3.4 Mechanism of Cobalt Resistance in Bacteria

Cobalt is a naturally occurring element in the Earth's crust. Airplane exhaust, burning of coal, volcanic eruption, etc., adds more cobalt to the environment. Cobalt is an important cofactor of various enzymes present in microbes, plants, animals and human. It is a component of vitamin B_{12}. Cobalt toxicity affects iron-sulphur proteins like succinate dehydrogenase, sulphide reductase, nitrate reductase, aconitase B, etc. It misbalances the iron homeostasis and induces sulphur assimilation. It generates oxidative stress in various life forms. Cobalt competes for iron at different sites and

replaces iron from the active sites of various enzymes. Cobalt also target proteins with cysteine or thiol groups, due to its affinity for sulphur atoms. Altogether, cobalt interferes with homeostasis of other metals (Nies 1992; Barras and Fontecave 2011). Cobalt has similar coordination property like iron and nickel. Therefore, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Mg^{2+} are usually transported into cell by divalent cation uptake system with broad specificity. Co^{2+} is imported by importers like FeoB (import Fe^{2+} mainly), CorA (Mg^{2+} importer), ZnpT (Zn^{2+} transporter) and by NikABCDE nickel uptake system. As cobalt is imported non-specifically by various divalent cation importers, mere downregulation of these importers won't be a solution to cobalt toxicity. Therefore, bacteria evolved plasmid encoded metal resistance genes to deal with various metal intoxications. These genes encode for efflux system inducible under stress condition to reduce the accumulation of metal inside the cytosol (Nies 1992; Barras and Fontecave 2011). For example, *Alcaligenes eutrophus* encodes for *czc* system with *czcA, czcB, czcC, czcD* and *czcR* genes. Zn^{2+} is the main cation exported by this system. CzcA is a cation proton antiporter involved in the efflux of Co^{2+}, Cd^{2+} and Zn^{2+}. CzcB is the ancillary protein with cation binding subunit. CzcC is the modifier, which changes the substrate specificity of the system when required. CzcD and CzcR are the regulators of the *czc* system (Nies 1992). *E. coli* chromosome have *rcnRAB* gene cluster, which control cobalt and nickel efflux. RcnR is the regulator controlling the homeostasis of copper by regulating CsoR (copper repressor) and sulphur by regulating CstR (presulphide sensing transcriptional repressor). RcnA is a cobalt efflux pump which work in conjugation with RcnB (periplasmic protein). Binding of cobalt to transacting metalloregulatory protein RcnR leads to its dissociation from DNA and initiate the transcription of *rcnA* and *rcnB* genes. RcnRAB system is also present in *Salmonella enterica* and many other bacteria. Cobalt stress also induces the expression of *nfuA gene*, and *iscRSUA* and *suFABCDSE* operons, all of which are involved in iron-sulphur biogenesis under normal and stress condition (Barras and Fontecave 2011). Therefore, efflux pumps, antiporters, metalloregulatory proteins, periplasmic proteins, etc., are activated in response to cobalt toxicity (Fig. 1, Table 2).

4 Application of Heavy Metal Hypertolerant Bacteria in Bioremediation of Heavy Metal Toxicity

Heavy metal pollution is a serious and complex environmental issue. Various physical and chemical methods are applied to remove heavy metals from the contaminated site. Common methods like ion-exchange, precipitation, membrane filtration, chemical extraction, adsorption, etc., are employed to remove heavy metals from groundwater, drinking water and wastewater. But these methods have some disadvantages like high cost of implementation, constant maintenance, high learning curve, etc. Also, they are unsuitable for removal of very low concentration of heavy metals, often add other toxic chemicals to the environment and are affected by various physico-chemical

factors like pH, temperature, organic matter content, nature of sample, etc. Bioremediation is the biological removal, reduction, detoxification and degradation of toxic compounds into less toxic or non toxic forms. Many microbes have adapted to heavy metal stress by evolving heavy-metal resistant genes which encode detoxification systems and resistant mechanisms in them, as discussed in the previous section (Fig. 1). These heavy metal resistance mechanisms can be exploited for bioremediation, which is an effective, cheap, eco-friendly and low input technology. Microbes can be used for bioadsorption, biotransformation, bioprecipitation, bioaccumulation and biovolatilization of toxic heavy metals and xenobiotics. These methods involve surface adsorption of heavy metals on the active groups of bacterial membrane, enzymatic transformation of toxic heavy metallic compounds into less toxic forms, precipitation of heavy metals on bacterial surface, accumulation of toxic metal within the bacterial cell and volatilization of toxic metals from the local environment, respectively (Tarekegn et al. 2020). Many heavy-metal resistant bacteria have been used for removal of heavy metals from the contaminated sites. For example *Acinetobacter* sp. and *Arthrobacter* sp. reduce concentration of chromium by 78% if applied in consortium (De et al. 2008). *Micrococcus luteus* could reduce lead concentration significantly (Puyen et al. 2012). *Bacillus megaterium* and *Bacillus subtilis* also showed reduction in lead concentration from 2.13 to 0.03 mg/L and 0.04 mg/L, respectively, in tannary effluents after 20 days. In the same study, *Bacillus subtilis* also reduced the concentration of cadmium from 0.4–0.03 mg/L (Table 2 and 3) (Abioye et al. 2018). Therefore, heavy-metal resistant microbes could be exploited for bioremediation of heavy metal toxicity. This biological technique can be easily applied on a large

Table 3 Bioremediation efficiency exhibited by different heavy metal hypertolerant bacteria

Bacteria	Reduction in heavy metal concentration	Bioremediation efficiency
Pseudomonas aeruginosa	Cadmium-100–17.4 mg/L	75% after 72 h
Brevibacterium iodinium	Lead-100–2 mg/L	Greater than 87% after 96 h
Alcaligens faecalis	Cadmium-100–19.2 mg/L	70% after 72 h
Immobilized *Bacillus subtilis*	Chromium-570–2 mg/L	99.6%
Pseudomonas aeruginosa	Lead-100–1.8 mg/L	98% after 96 h
Pseudomonas aeruginosa and Bacillus subtilis (in consortium)	Chromium-570–2 mg/L	99.6%
Bacillus megaterium	Lead-2.13–0.03 mg/L	98.6% after 20 days
Bacillus subtilis	Lead-2.13–0.04 mg/L	98% after 20 days
Bacillus subtilis	Cadmium-0.4–0.03 mg/L	92.5% after 20 days
Bacillus megaterium	Cadmium-0.4– 0.06 mg/L	85% after 20 days
Immobilized *Pseudomonas aeruginosa*	Chromium-570.4–4 mg/L	99.3%
Alcaligens faecalis	Copper-100–19.2 mg/L	70%
Pseudomonas aeruginosa	Copper-100–17.4 mg/L	75%

scale in a cost effective manner, even for very low metal concentration with minimal monitoring and exhibit some advantages over physical and chemical techniques.

5 Phytoremediation of Heavy Metal Pollution

Plants require nonmetals and compounds like ammonia nitrogen, phosphate, borate, sulfate, etc., for their growth and development. Metals like copper, zinc, magnesium, iron, calcium, potassium, manganese, molybdenum, etc., also play a vital role in various physiological processes of plants. These nutrients must be in aqueous phase to be absorbed by plant roots. The uptake and transport mainly involved the apoplasm (root cells) of the plant. However, the symplastic pathway (from cell to cell, crossing the root cell membrane) is also involved in transport of root minerals to the upstream of the plant. Nonessential minerals and contaminants like arsenic, selenium, chromium, mercury and other micronutrients like copper, zinc, cadmium, lead, cobalt, etc., also easily enter the plant roots, when present in easily soluble form. The minerals and contaminants can become an environmental concern when present in excess. These contaminants are also taken up through passive channels or transport proteins in addition to the water uptake system (Tsao 2003). Heavy metals are cytotoxic, mutagenic and carcinogenic in nature (Rajkumar et al. 2009). Heavy metals hamper the normal physiological processes of plants. Plants employ various mechanisms to deal with these toxic metals. Plants release various biochemical root exudates that facilitate the precipitation, sequesteration or complexation of metals at the rhizosphere. Plant roots can irreversibly bind the contaminant and prevent its entry. They can also uptake the contaminant through roots and sequester it into vacuole, the storehouse of the cell. This will prevent the transport of the contaminant into other plant parts. Plants also might release some enzymes in order to reduce the toxicity of inorganic substance or change its speciation making it available for incorporation into organometallic compounds (Tsao 2003). In spite of these strategies, plants may fail to reduce the toxicity of heavy metals, when present in excess amount. Some plants can accumulate heavy metal contaminants at a concentration much greater than the toxic concentration of the metal. These plants, also known as hyperacuumulators can store a specific metal up to 1% of their dry weight (that is 10,000 mg per kg) depending on the type of inorganic element. Members of genus *Brassica*, *Pinus*, *Salicornia*, *Thlaspi*, *Atriplex*, *Helianthus*, *Kochia*, and *Pelargonium* are hyperaccumulators of various metals. Hyperaccumulators often produce root exudates that react and transform toxic compounds into less toxic or nontoxic forms (phytotransformation). These are then taken up by plant roots and stored in vacuoles (phytosequesteration). The root exudates can also sequester, immobilize and precipitate contaminants in soil or on the root surface. Toxic metals can also be sequestered within root tissues (Tsao 2003). Sulfate, hydroxides, oxides, carbonates and carboxylates are often released from roots to precipitate or form metal complexes in soil. Root exudates can also change the soil pH, which leads to precipitation of various ionic species. Root exudates can convert the oxidation state of the metals and can

alter their solubility, bioavailability and bioadsorption by roots. Secondary metabolites like terpenoids, flavonoids, alkaloids, etc., are also released by roots for defense against pathogens. Plants also secrete chemicals against other plants for their defense. These biochemicals alter the microenvironment of the soil. Mucigel secreted by plant protect the plant root, increases the root penetration in soil and promote plant growth (Tsao 2003). Plants also produce proteins and enzymes that can degrade complex compounds into simpler ones. For instance, nitroreductase enzyme when released by plant, can break nitroaromatics like *trinitrotoluene*, *hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine*, etc. Similarly, dehalogenases, phenoloxidases, nitrilases and phosphatases released as root exudates, can degrade halogen components of contaminants, phenolic compounds, pesticides, herbicides and insecticides, respectively. Degradation of organic and inorganic contaminants by plant root exudates is called phytodegradation (Tsao 2003). Phytovolatilization is the process of volatilizing organic compounds and certain metalloids by plants. Contaminants present in water soluble form are taken up by plant roots. Water and the solutes form a continuous path in the plant from soil to root to leaves. Water along with some metalloids and volatile organic compounds are transpired out through the leaves. In addition to phytosequestration, phytotransformation, phytodegradation and phytovolatilization, plants can also use phytoextraction and phytostabilization as strategies to combat heavy metal stress. Plants can extract and subsequently remove the contaminants into terrestrial plant tissues (phytoextraction). Root exudates can also stabilize the contaminants in the soil preventing its uptake by plants (phytostabilization). Phytoextraction, phytostabilization, phytovolatilization, phytotransformation, phytodegradation and phytosequestration, are the mechanism of phytoremediation, where plants are used to remediate and revitalize metal/metalloid contaminated soil (Tsao 2003) (Fig. 2, Table 4). Phytoremediation is an eco-friendly and cost effective method with intangible benefits to soil ecosystem. Phytoremediation can not only remove the toxic metals and metalloids but also improve soil quality by enhancing sequestration of soil carbon,

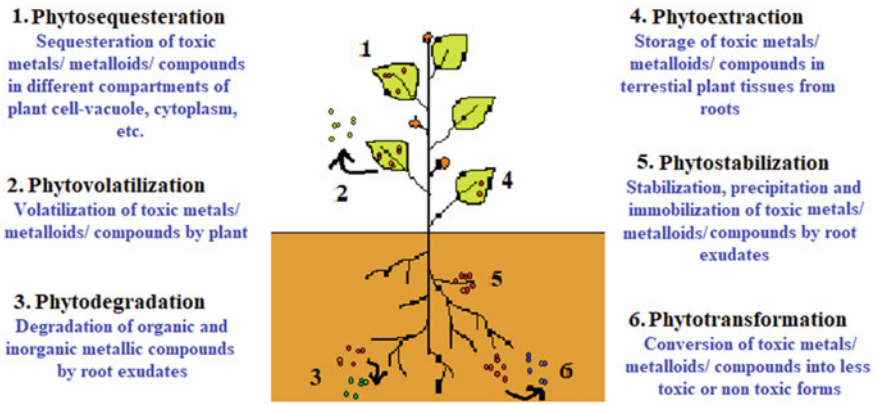

Fig. 2 Different strategies of phytoremediation of heavy metal toxicity

Table 4 Proteins involved in heavy metal uptake, accumulation and translocation in different compartments of hyperaccumulating plants

Hyperaccumulating plants	Increase in heavy metal uptake/accumulation/tolerance/resistance	Proteins involved
Thlaspi caerulescens	Zinc	Over-expression of ZIP (Zinc regulated transporter, iron regulated transporter protein): ZTN1, ZTN2 for zinc uptake
Arabidopsis halleri	Zinc	Over-expression of ZIP6, ZIP9 for uptake of zinc
Pteris vittata	Arsenic	Over-expression of phosphate/arsenate transporter in roots for uptake of arsenate
Astragalus bisulcatus	Selenium	Over-expression of sulphate transporters for uptake of selenium
Arabidopsis thaliana	Cadmium	Involvement of AtMRP1 and AtMRP2 transporters in transport of Phytochelatin-cadmium complex in vacuole
Arabidopsis halleri	Zinc, cadmium, cobalt, nickel	Over-expression of MTPs (Metal Tolerance Proteins): MTP1, MTP8, MTP11 for transport of heavy metals from cytosol to vacuole
Arabidopsis thaliana	Manganese	Over-expression of MTP8 and MTP11 for vacuolar transport of manganese
Stanleya pinnata	Selenium	Over-expression of sulphate transporters for uptake of selenium
Arabidopsis thaliana	Iron, manganese	Over-expression of NRAMP1 (Naturally resistant associated macrophage protein) for iron and manganese uptake
Thlaspi caerulescens	Zinc, cadmium, cobalt, nickel	Over-expression of MTPs (Metal Tolerance Proteins): MTP1, MTP8, MTP11 for transport of heavy metals from cytosol to vacuole

production of biomass and biofuel and maintenance of biodiversity (Teng et al. 2015). Effective phytoremediation require well developed root system in contact with soil. Rhizosphere is the soil region of 1–3 mm surrounding the individual roots. Rhizosphere is the site of high biological activities. It is the habitat of large population of microbes, some of which enhance the growth of plant. The rhizosphere surface should be large enough in order to have greater surface area for phytoremediation. Some plants like *Poa* sp. have shallow but dense fibrous root system that extends only few inches within the soil. Clovers (*Trifolium* sp.) and grasses (like *Lolium* sp.) have roots that can reach 1–4 feet below the soil surface. Larger surface area of rhizosphere is more advantages in phytoremediation than longer roots. Phytoremediation is a type of bioremediation with higher public acceptance, eco-friendly nature, low cost and low learning curve (Tsao 2003). Phytoremediation involves application of heavy metal hyperaccumulating plants at the contaminated site. But hyperaccumulators are mostly small and slow growing as heavy metals could affect their growth and metabolic rates. Also, the nature of phytoremediation employed by a plant depends upon the type of contaminant, ability of the contaminant to pass through plant root membrane, properties of contaminant, process of decontamination, type of plant species, surrounding microflora and fauna, soil type, etc. Phytoremediation technique predominantly includes plants but also involves interaction between plants, soil, contaminants, microflora and fauna. Therefore, significant change at any stage can affect the efficiency of plants to remove, detoxify, sequester immobilize, volatilize and extract toxic metal and metalloid from soil (Tsao 2003; Rajkumar et al. 2009). Rhizosphere often has heavy-metal resistant bacteria that can tolerate or resist heavy metal toxicity. These bacteria can assist and speed up the phytoremediation process of plants and can promote the growth of plant as discussed in the following sections.

Phytoremediation was used for integrated waste management in the town of Arcata, situated along the northern coast of California. The wastewater including the sewage of this town was treated in two stages. After the conventional sedimentation, filtering and chlorine treatment lots of dangerous pollutants, including toxic metals were still present in the wastewater. In the second stage, the wastewater was passed through six connected marshland containing suitable plant, algae, fungi and bacteria for phytoremediation, rhizoremediation and bioremediation resulting in neutralization and absorption of the pollutants present in the wastewater. These marshlands possess rich biodiversity of flora and fauna and constitute a wild life sanctuary. This is a real time example of phytoremediation in action.

6 Role of Rhizospheric Heavy-Metal Resistant Bacteria in Enhancement of Plant Growth

The A Horizon of the soil usually has 10^5–10^8 microbial cells per gram of dry soil. There is a decrease in the population of microbes with increasing depth of

the soil. The B and C horizon have 10^3–10^6 microbial cells per gram of dry soil, whereas groundwater usually has 10^5 cells per cubic milliliter of water. Rhizosphere is the zone surrounding the plant roots and is under its direct influence. The rhizosphere soil shows 10–100 fold higher population of microbes than the bulk soil (Tsao 2003). Rhizosphere is a highly competitive ecosystem where all species fight to colonize the best root zones. It is the habitat of variety of bacteria that have the ability to degrade different type of contaminants. Some rhizospheric bacteria are resistant to heavy metals, organic pollutants and amide herbicides. Heavy-metal resistant bacteria isolated from rhizosphere can sequester heavy metals and decompose organic and inorganic compounds (bioremediation). They can decrease metal phytotoxicity and accumulation in plant, thereby promoting plant growth (Khatoon et al. 2020). They can fix atmospheric nitrogen, mineralize and solubilize insoluble potassium and phosphate for plant (biofertilization), suppress phytopathogens, induce plant resistance mechanism (bioprotection) and promote production of phytohormones in plants (biostimulation). Plants release sugars, amino acids, flavonoids, proteins and organic acids. These molecules serve as messenger for rhizospheric bacteria and promote their activity (Khatoon et al. 2020). For example *Azospirillum brasilense* can promote growth of plant by biostimulation, biofertilization and bioremediation. It can produce *indole-3-acetic acid*, fix atmospheric nitrogen and alter heavy metal uptake in rice and other cereals. *Bacillus subtilis* show biostimulation, bioprotection, bioremediation and biofertilization in maize, chickpea, tomato etc. It produces *indole-3-acetic acid*, cytokinin, catalase and lipopeptides, and degrades xenobiotics for plants. *Azospirillum* sp. supplies sufficient amount of nitrogen in crop field which improves the yield and productivity of the land. *Acetobacter diazotrophicus* also plays the role of biofertilizer by fixing atmospheric nitrogen (Khatoon et al. 2020). *Rhizobiales* is an order of gram negative bacteria having agronomic importance. Some species of this order undergo symbiotic relationship with leguminous plant and provide the advantage of nodulation, atmospheric nitrogen fixation and plant growth in absence of external sources of nitrogen. *Rhizobia* can remove multiple types of organic pollutants like hydrocarbons, chlorinated compounds, phenolic compounds, pesticides, etc., from the environment (Teng et al. 2015). Members of genus *Rhizobium* degrade toxic compounds and therefore, make the order *Rhizobia*, important tool for heavy metal bioremediation. Members of *Rhizobia* confer heavy metal resistance by various mechanisms which include volatilization, adsorption, accumulation and sequestration of heavy metals. *Rhizobium* species with metal resistance genes can encode for efflux, detoxification and sequestration system in order to respond to the heavy metal toxicity. These genes are upregulated in presence of toxic metal and metalloids and confer heavy metal resistance to the bacteria (Teng et al. 2015). For example, *Mesorhizobium amorphae* CCNWGS0123 encode for CusA and CusB protein which participate in efflux of copper out of the cell. Similarly, arsenic resistance genes responsible for detoxification and resistance to arsenic, is present in *S. meliloti,* a member of *Rhizobiales*. Another rhizospheric bacteria *Pseudomonas putida* KT 2440 with *arsM* gene is involved in arsenic methylation and volatilization leading to arsenic removal. *Rhizobia* promote phytoextraction, phytotransformation, phytostabilization and phytovolatilization by the adjacent plants.

Rhizobia secrete enzyme like ACC (*1-aminocyclopropane-1-carboxylate*) deaminase, siderophores or organic acids to sequester and trap toxic pollutants in the soil, thereby reduce the symptoms of heavy metal stress in plants. It can also alter the redox state of metals and increase its complexation and bioavailability. Toxic metals can be adsorbed on the bacterial surface or can accumulate within the bacterial cell (Teng et al. 2015). Bacteria can volatilize or transform the toxic compounds into simpler ones by cytosolic or periplasmic or membrane embedded metal binding proteins. They can also fix atmospheric nitrogen and solublize phosphorus, thereby making them available to plants. They can induce plants to synthesize phytohormones. *Rhizobium* sp. RP5 secretes siderophores and increases the bioavailability of nickel and zinc to plants. *Bradyrhizobium* sp. relieves the stress of cadmium, zinc and nickel in *Vigna radiata*. *Cupriavidus taiwanesis* can overcome the low availability of metals and remove metal and metalloids for its symbiont *Mimosa pudica*. Therefore, Rhizobia and other rhizospheric bacteria aid in phytoremediation of metal by the symbiont plants and increase the plant biomass and soil fertility, as well as decrease the concentration of the metal in the local environment (Teng et al. 2015). The phenomenon of assisted phytoremediation (where microorganisms facilitate phytoremediation by plants) is a low input biotechnology technique that does not require addition of bacterial inoculants repeatedly at the contaminated site. But the effectiveness of this technique can be influenced by other competitive native bacteria present at the contaminated site. These bacteria can reduce the survival and bioremediation ability of *Rhizobium* and other rhizospheric bacteria. In addition, changing environmental condition like limitation of nutrients, change in pH, etc., can also affect the efficiency of these bacteria (Teng et al. 2015). Bacteria residing in the rhizosphere and promoting plant growth are also termed as plant growth promoting rhizobacteria (PGPR). These bacteria often exhibit tolerance or resistance to heavy metals when isolated from contaminated soil. They can convert, absorb, precipitate, accumulate or efflux heavy metals. These bacteria are of particular importance for their bioremediation potential and plant growth promotion. Serpentine soils with high pH contain high concentration of heavy metals like nickel, cadmium, cobalt and low concentration of calcium and other macronutrients. Presence of nickel at very high concentration leads to significant toxicity in plants growing in serpentine soil. Hyperaccumulating plants thriving in serpentine soil are mostly nickel hyperaccumulators. Serpentine soils are model for studying evolution of metal resistance in plants and plant growth promoting microorganisms. Hyperaccumulating plants like *Thlaspi goesingense, Thlaspi caerulescens, Alyssum bertoloni and Alyssum murale*, *Sebertia acuminate*, etc., are usually observed in serpentine soil. Serpentine soil is the habitat of various heavy-metal resistant bacteria that are hypertolerant to nickel and zinc toxicity (Rajkumar et al. 2009). Many heavy-metal resistant bacteria have been isolated from rhizosphere of *A. murale* and other hyperaccumulating plants. Most of the isolated strains are resistant to copper, cobalt, nickel, zinc, cadmium, chromium, arsenic, mercury and lead. Examples of heavy-metal resistant bacteria isolated from rhizosphere of plants are *Arthrobacter rhombi, Clavibacter xyli, Microbacterium arabinogalactolyticum, Rhizobium mongolense, Variovorax paradoxus,* etc. Such heavy-metal resistant rhizospheric bacteria also promote growth of plants at

different metal contaminated sites (Rajkumar et al. 2009). For example, inoculation of siderophore producing *Pseudomonas* sp. and *Bacillus megaterium* increased plant growth and enhanced nickel hyperaccumulation in *Brassica juncea,* without any visible symptoms of nickel toxicity. Other plant growth promoting bacteria like *Pseudomonas* sp. and *Pseudomonas jessenii* isolated from rhizosphere of serpentine soil protected *Ricinus communis* against heavy metal toxicity. These bacteria were applied at the site of nickel, copper and zinc contamination. They promote plant growth by producing and utilizing IAA as sole nitrogen source, and solubilizing phosphate and making it available for plants (Rajkumar et al. 2009). Interaction between PGPR like *Pseudomonas* sp. with *Rhizobium* indicated towards a synergistic process with potential nodule formation and better nitrogen fixation. Horizontal transfer of genes important for nodule formation and nitrogen fixation might have taken place between *Rhizobia* and *Pseudomonas* and *Burkholderia* which allowed them to form nodules in roots of *Robina pseudoacasia.* Therefore, combined application of rhizobacterial species is advantageous over application of a single species of nitrogen fixing bacteria at the contaminated site (Khatoon et al. 2020). Application of heavy-metal resistant plant growth promoting bacteria can reduce the cost of agricultural production by reducing the need for chemical fertilizers and increasing the bioavailability of nutrients. Once added as inoculant they increase their population within a short period of time due to their short doubling time. They often trigger weak defense response in plant than fungal elicitors and might facilitate sustainable and balanced relationship between bioremediation partners. These bacteria might also change the constitution and amount of root exudates and increase the availability of nutrients to the plants (Teng et al. 2015). Leguminous plants and nitrogen fixing bacteria often associate together in a symbiotic relationship, which gives various advantages to plants and bacteria. Plants provide nutrients like carbohydrates, inorganic minerals, etc., to the rhizospheric bacteria. Rhizospheric bacteria form a protective sheet around the plant and prevent contact between toxic contaminants and plant. The symbiotic relationship between plants and microbes is highly effective in removing environmental contaminants and promoting ecological sustainability (Fig. 3). However, the effectiveness of this method depends on the type of plant species, nature, diversity and richness of microbial community, toxicity, bioavailability of contaminants, physical and chemical properties of soil, organic matter content, pH, texture, etc. Plant growth promoting rhizospheric bacteria must be able to enhance the growth, development and yield of the plant. It must have broad spectrum of action and should be able to suppress the pathogenic infection in plants. It should have low doubling time, high rhizosphere competence and compatibility with other *Rhizobium* species (Teng et al. 2015; Khatoon et al. 2020). The next section describes few mechanisms employed by heavy metal resistant plant growth promoting bacteria for enhancement of plant growth.

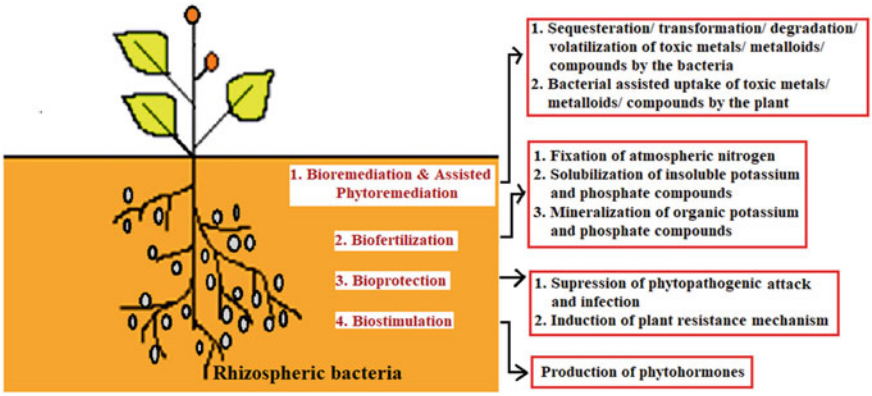

Fig. 3 Enhancement of plant growth and bioremediation of heavy metal pollutants by plant growth promoting Rhizobacteria (PGPR)

7 Mechanism of Action of Heavy-Metal Resistant Plant Growth Promoting Bacteria

7.1 Phosphate Solubilization

Phosphorus is one of the essential macronutrient essential for growth and development of plants. Phosphate participates in many metabolic pathways like photosynthesis, electron transport chain, respiration, root and seed growth and development, etc. Adsroption and chemical precipitation make phosphate less soluble or insoluble in soil. Though phosphate is present in high concentration in the soil, it is not available to the plants. Heavy metals in the soil also interfere with the uptake of phosphate by plants leading to reduced growth. Application of chemical fertilizers increases the agricultural production cost and changes the structure of soil ecosystem. Plant growth promoting bacteria that produce enzymes like phosphatase and phytase are involved in mineralization of complex organic phosphate compounds. Phytic acid which is the major component of organic phosphorus compound is broken down by the enzyme phytase. Phosphatase enzyme use organic phosphorus as a substrate and transform it into inorganic forms. Plant growth promoting bacteria also produce various organic acids that lower the pH of the soil and chelate mineral ions. These organic acids solublize the insoluble phosphate and make the phosphorus available for plant, without the application of chemical fertilizers (Rajkumar et al. 2009; Khatoon et al. 2020).

7.2 Potassium Solubilization

Potassium is also an essential macronutrients required for plant growth and development. It is essential for root hair development, growth of pollen tube, management of cellular osmotic balance, etc. Potassium also sometimes becomes unavailable to plants. Potassium solubilizing plant growth promoting bacteria produces organic acid like citrate, oxalate, acetate, etc. These acids cause extensive degradation and transformation of insoluble potassium containing compounds like clay silicates, mica, feldspar, granite, calcite, etc., into soluble forms, which is then taken up by the plants. For example, *Bacillus* sp. produces carboxylic acid that can solubilize potassium containing compounds (Khatoon et al. 2020).

7.3 Nitrogen Fixation

Nitrogen is an essential element for plant growth. However, plants cannot use the atmospheric nitrogen directly. There are examples of rhizobacteria which can fix atmospheric nitrogen in the soil as ammonia or release the nitrogen stored in decaying biomass as ammonia in the soil (ammonification). Further, the ammonia could be converted into nitrites and nitrates by bacterial action. Symbiotic rhizobacteria like *Rhizobium* sp. could fix nitrogen in the form, which could be utilized by the plant. Free living bacteria like *Azotobacter*, *Azospirillum*, etc. found in the rhizosphere could also fix nitrogen efficiently. *Rhizobium*, *Azotobacter* and some Cyanobacteria are used as biofertilizers to increase the nitrogen content of the soil.

7.4 Siderophore Production

Iron is the most important nutrient that participates in various physiological processes of the plant. Plants become deficient in iron supply under stress condition. Siderophores are organic molecules that show high affinity for Fe^{3+} ions. Siderophores can also form complexes with other bivalent heavy metal ions. Plant growth promoting bacteria produce siderophores of various types. For example, bacillibactins, pyoverdines, and cephalosporins are some of the common siderophores produced by these bacteria to chelate iron and make it available to plants. Siderophores decreases the free radical formation and protect phytohormones from oxidative damage. It increases the bioavailability and mobility of metals. It protects plants from pathogen by making iron unavailable to them. Therefore, siderophore producing plant growth promoting bacteria can reduce metal induced toxicity in plants. Such bacteria can also be used as biocontrol agent as they can reduce bacterial and fungal infection in plants (as antibacterial and antifungal agents) (Ahemad 2015; Khatoon et al. 2020).

7.5 *Production of Phytohormones*

Phytohormones are messenger molecules produced by plants participating in various physiological processes at very low concentration. Cell elongation, apical dominance, tissue differentiation, cell division, intracellular communications, etc., involves the action of phytohormones. Plants produce auxin in the form of *indole-3-acetic acid*. When the production of *indole-3-acetic acid* is low then it promotes primary root elongation. Higher production of *indole-3-acetic acid* inhibits primary root growth and promotes lateral and adventitious root formation. Phytohormones can alleviate biotic and abiotic stress condition. Plant growth promoting bacteria mainly produces auxin, which is involved in various physiological processes like cell elongation, division, differentiation, etc. *Indole-3-acetic acid* producing bacteria promote absorption of nutrients by proliferating plant roots. They also reduce metal adsorption and assist adaptation to heavy metal stress. They improve the antioxidant system and induce physiological changes promoting plant growth. For example, members of genus *Rhizobium*, *Pantoea*, *Agrobacterium*, *Bacillus, Pseudomonas*, etc., produce auxin and promote plant growth. Four tryptophan dependent pathways are involved in microbial *indole-3-acetic acid* biosynthesis. These four pathways engage *indole-3-acetamide*, *indole-3-pyruvic acid*, *indole-3-acetonitrile* and *indole-3-tryptamine* as intermediates. *Indole-3-pyruvic acid* pathway is the main pathway of *indole-3-acetic acid* production in plant growth promoting bacteria. The major precursor of *indole-3-acetic acid* is tryptophan and the production is catalyzed by aminotransferase and flavin containing monooxygenase (Rajkumar et al. 2009; Ahemad 2015; Khatoon et al. 2020).

7.6 *Production of* 1-Aminocyclopropane-1-Carboxylate *(ACC) Deaminase*

Ethylene is a gaseous plant hormone produced by all parts of plant in small amount. Ethylene production increases in plant during fruit development, ripening and under stress conditions like draught, salinity and metal induced stress. ACC is the immediate precursor of ethylene. Plant secretes ACC as one of its root exudates. ACC deaminase transforms ACC into ammonia and α-ketobutyrate. ACC deaminase is also synthesized by plant growth promoting bacteria. These bacteria utilize the ammonia after the degradation process and promote the secretion of more ACC from plant roots, which decreases the ACC concentration in plant and subsequently reduces the symptoms of adverse environmental stress in plants. ACC deaminase also improves the metal phytoremediation ability by facilitating longer root and greater root density in plants experiencing metal stress. These bacteria also increase metal mobility and bioavailability by producing varieties of organic acids, iron chelators and enzymes for plants. *Pseudomonas fluorescens* YsS6 is a free living ACC deaminase producing bacteria that alters the ethylene level and promotes nodulation in plants. *Pseudomonas* sp.

UW4 also show ACC deaminase activity and promote plant growth (Rajkumar et al. 2009; Ahemad 2015; Khatoon et al. 2020).

7.7 Production of Volatile Organic Compounds

Some plant growth promoting bacteria produce volatile organic compounds like *hydrogen cyanide*, *N, N-dimethyl hexa decyclamine*, *dimethyl disulfide*, etc., which can promote growth of host plant either by protecting it from harmful microbes or by increasing availability of minerals. For example *Arthrobacter agilis* UMCV2 produces *N, N-dimethyl hexa decyclamine* that protects the host plant from the attack of *Botrytis cinerea* and *P. cinnamomi*. *Dimethyl disulphide* act as elicitor of defense response in plants and show antagonistic action against *Botrytis cinerea.* Hydrogen cyanide also has an antagonistic role against various pathogens. It also increases the bioavailability of phosphate in rhizosphere. *Bacillus* spp. and *Pseudomonas* spp. are examples of volatile hydrogen cyanide producing bacteria (Khatoon et al. 2020).

7.8 Production of Hydrolytic Enzyme

Some plant growth promoting bacteria produce hydrolytic enzyme like cellulase, pectinase, etc. Cellulase when secreted from bacteria degrades cellulose (components of dead plant parts) into glucose which adds carbon to soil and promote plant growth (Khatoon et al. 2020).

7.9 Miscellaneous Actions of Plant Growth Promoting Bacteria

Brucella sp. K12 improved growth and yield of *Hibiscus esculentus* L. and reduced the Cr^{6+} concentration in soil and plant tissues. Other bacteria like *Microbacterium* sp. SUCR140 decreases Cr^{6+} toxicity in *Pisum sativum* and *Zea mays* and increased the overall growth of the plant. Vesicular–arbuscular mycorrhiza (VAM) is a symbiotic association between fungi and plant. This association also protects plant by restricting the uptake of toxic element like cadmium, nickel, lead, etc. VAM sequester toxic metals into their tissues, increases water uptake and provide resistance to plants against drought, salinity, etc. Fungi can extend their hyphae beyond the rhizosphere and collect nutrients from distant soil, increasing the nutrient uptake by plants.

8 Conclusion

Heavy-metal resistant plant growth promoting rhizobacteria is a potentially significant tool to combat the dual issue of metal contamination of soil and groundwater and low agricultural yield. The knowledge gathered about PGPR by different research groups all over the world could be translated into field application. Application of different PGPR as biofertilizers according to the need of the soil could supplement or substitute the use of harmful chemical fertilizers. Further, the bioremediation and the assisted phytoremediation potential exhibited by the rhizospheric bacteria could be exploited for construction of bio-filters and mitigation of heavy metal toxicity in soil, groundwater and wastewater.

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Conflicts of Interest On behalf of all authors, the corresponding author states that there are no conflicts of interest/competing interests.

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Understanding the Regulation of Root Development Towards Environmental Stresses for Crop Improvement

Ashis Sarkar and Swarnendu Roy

Abstract In the present time, agricultural production is under immense threat due to rapid changes in global climate. Environmental stresses are imparted by a number of factors which includes biotic factors like pathogenic microbes, fungus, herbivores etc. and abiotic factors like nutrients, light, temperature, salinity, water etc.; all of which have been known to profoundly obstruct plant growth and development. Plant root growth and functions are among the vital attributes that determine the performance of crop plants under stressed conditions. Root architecture has been known to be severely affected under the effect of stress which is reflected in the form of root organ deformation, decrease in lateral branching, root hairs, membrane integrity etc. This chapter therefore, details the elementary aspects of plant root development and the impacts of different environmental stresses on root development. At the same time, this chapter will also detail the insights of phytohormones and genetic regulation associated with root growth and development. Most importantly, the chapter will focus on the currently available strategies like plant-microbial consortium, transgenic development, and miRNA and CRISPR-Cas9 mediated genetic interventions that are specifically associated with the improvement of root architecture and developmental attributes that aids in conferring an enhanced level of stress tolerance in crop plants.

1 Introduction

Transition of plants from aquatic habitats to land is considered as one of the key events in the course of plant evolution. Plants are initially evolved in homogenous aquatic environment, as a result simple ancestral root like structures or sequential arrangement of few elongated cell after a wide cell fulfilled the requirements of water and nutrients absorption. Furthermore transition of aquatic plants from homogenous aquatic environment to sandy earth crust limited nutrient, water uptake

A. Sarkar · S. Roy (✉)
Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Raja Rammohunpur, Siliguri, West Bengal, India
e-mail: swarnendubotany@nbu.ac.in

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and other root functions (Sanchez-Calderon et al. 2013). Therefore, transition to land habitats compelled the plants to initiate momentous adjustment in structure of plant roots as well as other organs (Raven and Edwards 2001). Establishment of plants on land is supported by root, a specialised structure that supports the entire plant body and grows in the direction of gravity. Earliest plant communities inhabited the earth's sandy crust struggled for the establishment on land, for water and nutrient uptake (Raven and Edwards 2001). But over millions of years, sandy habitats have been eventually substituted by heterogeneous soil, which supported more advance vegetation with complex and sophisticated root system (Sanchez-Calderon et al. 2013). The complex root system in terrestrial plants is manifested by a wide range of root system architectures (RSA) ranging from unbranched to a composite branching system witnessed among the different species, which helps in attaining optimal functional performance for anchorage and mechanical support, and uptake of nutrient and water from heterogeneous soils (Sanchez-Calderon et al. 2013). Plant roots also play many additional functional roles like production and distribution of hormones, development of soil organic matter by adding organic carbon, nitrogen in coordination with soil microflora etc. (Fageria and Moreira 2011).

Root development has been most widely studied in *Arabidopsis* where it has been observed that the development process starts during early embryogenesis followed by several developmental events which gives rise to primary root and lateral branching (Scheres et al. 1994). Further progression of root system development is guided by the action of quiescent center (QC)—a set of mitotically less active stems cells residing in the root tip and surrounded by various other stem cell initials (Scheres et al. 1994). The root system architecture is deliberately designed during post-embryonic root developmental programme (PERDP), and is profoundly supervised by a number of genes (Lynch 1995; López-Bucio et al. 2003; Hodge et al. 2009). However, in response to adverse conditions the programme allows adjustments of phenotypic traits for acclimatization.

Various abiotic and biotic factors are known to significantly control root growth and development, such as few pathogenic viruses remarkably decreases root tuber and induces lateral root formation (Legg 2014), whereas some microbes and fungal pathogens invades in to the root tissues by using several mycotoxins and modifies root morphology by altering phytohormone gradient (Tattar 1989; Chen et al. 2017). Similarly, abiotic stresses also alters root traits through morphological, biochemical and molecular changes. For instance, deficiency of major nutrients induces lateral branching, supress primary root elongation; drought and salinity stress profoundly suppresses the lateral root formation (Xiong et al. 2006; Lynch 2011). Environmental stresses alters many aspects of plant root development, severely damages root architecture and reduces crop yields. In this context, improvement of crop plants via root system upgradation regarding stresses is essential to fulfil global food supply. Different approaches for the modification of root system architecture can evidently affect the water and nutrient uptake capability of plants under different stresses. Use of conventional plant–microbe interaction or modern molecular tools viz. transgenesis, miRNA and CRISPR-Cas9 have shown promising results to enhance root-specific traits and eventually agricultural production (Zhang 2015; Jaganathan et al. 2018;

Pan et al. 2020). Therefore, understanding of the genetic and phytohormones control of plant root development is also crucial for agricultural improvement via application of modern strategies.

In this connection, this chapter reviews the elementary aspects of root development and provides an insight to the genetic and phytohormones regulation of the developmental processes along with the impact of different abiotic and biotic stresses on root development. Additionally, this chapter will focus the currently available strategies and their utilization for the modulation of root-specific traits for improving crop performance and production under the effect of various biotic and abiotic stresses.

2 Root Development

In vascular plants, root is an essential part below the ground, which plays a key role in the anchorage and establishment of connection with soil for the uptake of water, nutrients and many essential minerals. The root system is majorly distinguished in to three types viz. taproots, fibrous roots and adventitious roots. In most of the dicotyledons taproot is found, which arises from the radicle of germinating embryo and comprises of a primary root along with branches. On the contrary, fibrous root system contains large number root nearly identical in size and is observed mainly in the monocots including cereals. However, adventitious roots are mainly developed in response to environmental stresses from the non-root tissues e.g. nodes, base of the stem, branches etc. (Steffens and Rasmussen 2016). Root development has been widely studied in the model plant *Arabidopsis* where the development is known to be initiated in the early embryogenesis stage and has been referred to as embryonic development (ED). The primary root and multiple lateral roots with further branching constitute the major proportion of the taproot system which is formed during the post-embryonic development (PED) stage. While in the cereals, the fibrous root systems mainly manifests the development of primary and seminal roots during ED, whereas in PED phase shoot-borne and lateral roots are developed. However, the basic phenomenon of root development in both the root types is more or less similar.

Broadly, there are two major phases in the development of roots—ED and PED. In the ED phase (Fig. 1), the developing embryo comprises of primary meristems, vital layers of tissues and body axis (Jürgens 2001; Willemsen and Scheres 2004; Capron et al. 2009; Sanchez-Calderon et al. 2013). This is followed by the initiation of mitotic division in the meristems that occurs at the time of germination and encompasses the PED phase. The development of the entire root system eventually takes place from the main body axis and the primary root meristems (Willemsen and Scheres 2004; Laux et al. 2004; Malamy 2005). The major events in the development of roots have been discussed in the following sections.

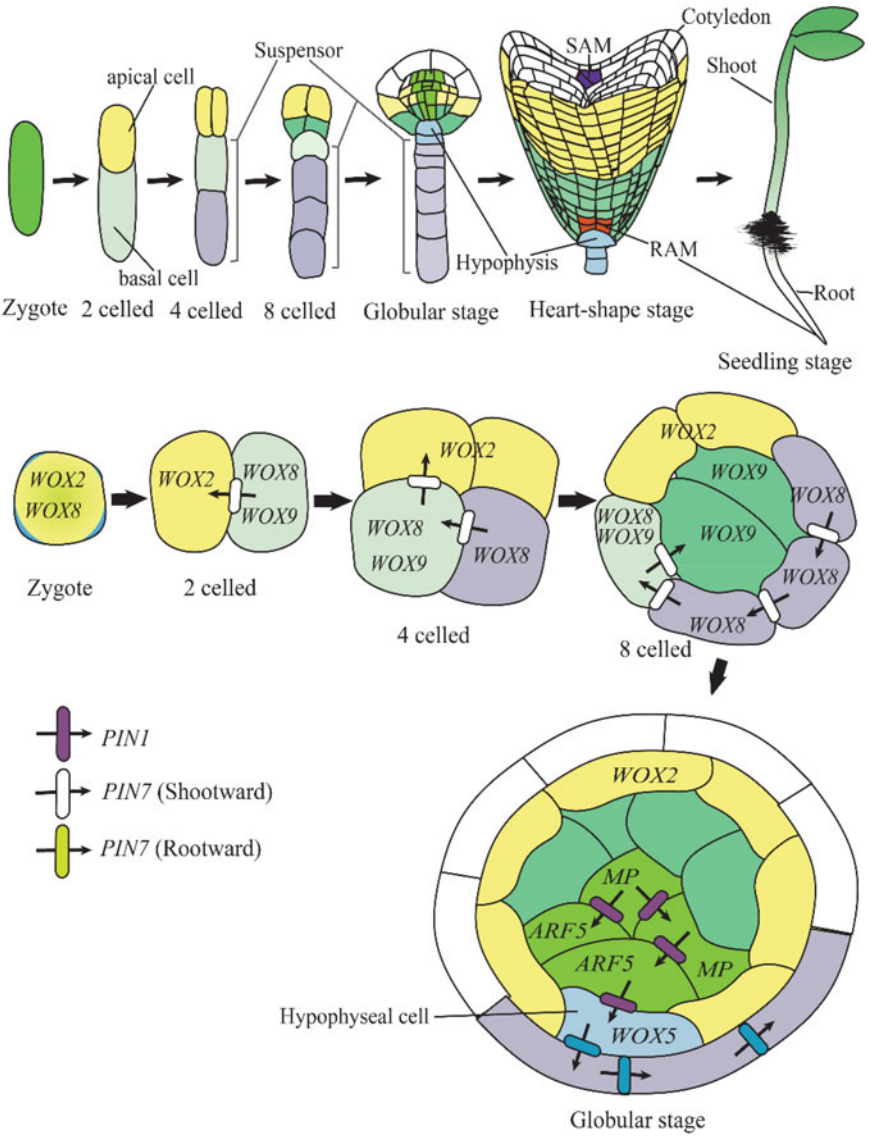


Fig. 1 Developmental stages during embryogenesis showing the involvement of major genes. The developmental stages of an embryo from a single cell to seedling stage have been shown at the top. The diagrammatic series at the bottom illustrates the top view of embryonic development stages (single cell to globular stage) to represent the specific role of the major genes (shown in different colours uniformly for both the diagrams). Differential expression of *WOX* genes have been shown in apical and basal lineage development. The expression of *WOX2*, *WOX8* and *WOX9* gene have been shown respectively by yellow, grey and deep-green coloured cells. Expression pattern of these genes establishes the plant body plain. Also, expression profiles of other genes - *MP* (green), *PIN1* (violet), shootward auxin transporters *PIN7* (white) and rootward auxin transporters *PIN7* (blue) have been shown. The expression of *PIN* proteins plays a fundamental role in auxin movement to establish auxin polarity and decisively determines the embryonic root development

2.1 Embryonic Development (ED) Phase

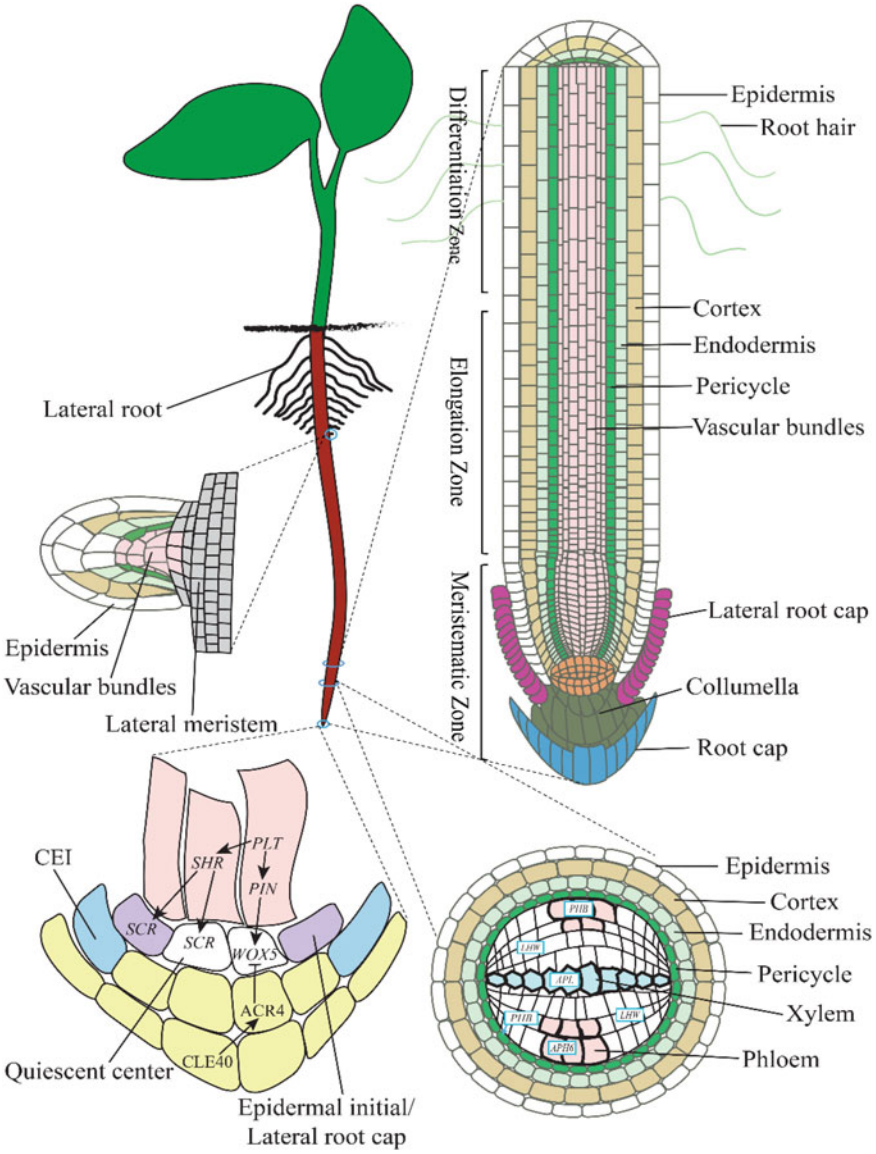
In angiosperms during early embryogenesis, polarized zygote generates two unequal daughter cells comprising of a smaller or comparatively sizable apical cell and a larger basal cell respectively (Mansfield and Briarty 1991). The two celled asymmetric embryo in *Arabidopsis* further gives rise to pro-embryo from the upper apical cell after successive vertical divisions and the basal cell develops the suspensor (Fig. 1), which links the embryo with the maternal tissue (Petricka et al. 2012). The top most cell of the suspensor develops into the progenitor cell or hypohysis, which gives rise to the root meristem. More precisely, the asymmetric division of the hypophysis induces two cells—the lens shaped upper cell which promotes the development of quiescent center (QC) and the basal cell which develops into the columella stem cells (Fig. 1**,** Petricka et al. 2012). In the meantime, the pro-embryo promotes the development of stem cells for the development of root epidermis, ground tissue and vascular bundles (Scheres et al. 1994). Further, a group of stem cells designated as root apical meristem (RAM) contributes to the growth of root apex from the embryonic root cells (Fig. 1). The activity of RAM is determined by the extent of cell division and differentiation as well as regulated by the influence of phytohormones (Kerk et al. 2000; Miyashima et al. 2013). The further development of root is initiated instantly following the germination of seeds in the PED phase which lasts all the way through the plant life.

2.2 Post-embryonic Development (PED) Phase

Post-embryonic development of the root in a way controls the fate of the plant growth and development. The developing root longitudinally divides into three zones viz. differentiation zone, elongation zone and meristematic zone (Fig. 2). The zone of maturation or differentiation zone (DZ) is composed of specialized cells, which plays the functional role of root such as absorption of water as well as nutrients, provides anchorage and mechanical strength (Motte et al. 2019). While the elongation zone (EZ) consists a group of specialised cells with adequate capability to increase the length many times than that of the breadth and the meristematic zone (MZ) is formed by a pool of stem cells, which can differentiate and elongate many times (Motte et al. 2019).

The maintenance of the MZ has been supported by a set of mitotically less active stem cells which are generated during embryogenesis and encircles the QC, known as RAM (Petricka et al. 2012). RAM plays a crucial role in root development by contributing newer cells. Among these stem cells, shootward and lateral cells of the QC give rise to endodermal, cortex and vascular cells. On the other hand, the rootward stem cells of the QC generates epidermis, lateral root cap (LRC), root cap and columella cells (Petricka et al. 2012). The QC plays a central role in maintaining the reservoir of undifferentiated stem cells and specifications of stem cell niche (Van

Den Berg et al. 1997). A continuous asymmetric division of the stem cells generates new group of stem cells, which further divides multiple times and shifts apart from the QC and eventually differentiate into different layers (Drisch and Stahl 2015). The order of post-embryonic development can be further described under the following sub-headings.



◀**Fig. 2 Diagrammatic representation of the different portions of a plant root and the genes associated with the developmental process**. At the center, an illustration of seedling with primary and lateral roots have been shown. At the top right, L.S. of the primary root have been shown representing the three principle zones of a developing root viz. differentiation zone (DZ), elongation zone (EZ) and meristematic zone (MZ). To the left of the seedling, a lateral root initiation from the primary root have been shown representing the different cell initials in different colours. At the bottom right, T.S. of the root meristem zone showing the cellular organization have been presented. The expression of different genes such as *APL* (xylem), *PHB* (endodermis and phloem), *LHW* (pith) and *APH6* (phloem) have been shown that regulate the development of the different layers. In all the cases, different layers of the developing root are shown with different colours viz. epidermis (white), root cap (blue), cortex (antique gold), endodermis (sage), pericycle (green), vascular bundles (light pink), columella (emerald) and lateral root cap (purple). At the bottom left, an enlarged view of the root tip has been shown representing the meristematic cells like quiescent center (white), epidermis initial/lateral root cap cells (sky blue) and cortex/endodermis initial (CEI, light purple). Additionally, the genes regulating the development and maintenance of QC and RAM have been shown. Precisely, *WOX5* and *SCR* expression is associated with the maintenance of QC and other regulatory genes such as *PLT, PIN, SHR*, *ACR4* and *CLE40* functions in tandem to regulate the expression of *WOX5*

2.2.1 Development of Root Layers: Dermal Layers, Ground Tissues and Vascular Tissues

The layers of developed roots are broadly distinguished in to the outer protective layer of epidermis, several layers of ground tissues and the central layers of vascular tissues. The developing root of *Arabidopsis* contains a set of stem cells forming a rings below the QC, called epidermal initials (EI) (Dolan et al. 1994). The clonal studies have cited that these stem cells divides periclinally, the outward cells develops lateral root cap and the inner cells divides transversely to restore the EI further for the development of root epidermis (Dolan et al. 1993, 1994). Root hairs are generally introduced from the epidermal cell files attached to the underlying cortical cells, while the larger epidermal cells distantly situated from the cortical cells remain hairless (Dolan et al. 1994).

The successive asymmetric division of the RAM generates stem cell initials and daughter cells located upward of QC (Fig. 2), which further leads to the formation of root layer initials such as cortex-endodermal initials (CEI) which again asymmetrically divides multiple times to generate cortex-endodermal daughter cells (CED). The CED cells further gives rise to cell lineages for the development of ground tissues viz. cortex, endodermal layer etc. (Benfey and Scheres 2000). All the cells of the pericyclic layer can contribute to the development lateral roots, while in regular circumstances the pericycle cells adjacent to the internal xylem poles gives rise to the lateral roots (Malamy and Benfey 1997). On the other hand, vascular initials (VI) are formed by the stem cell initials (RAM) situated above the QC (Scheres et al. 2002). The vascular initials then divide repeatedly and generates the protoxylem and procambial cell lineages, and finally develops into water conducting xylem, sugar conducting phloem, conjunctive tissues and pith (Scheres et al. 2002).

2.2.2 Root Branching, Root Cap and Columella

In dicotyledons, the taproot system is composed of primary root and multiple lateral roots with their branches, however in monocotyledons adventitious roots gives rise to plentiful of lateral roots along with branches and consists of fibrous root system (Motte et al. 2019). In *Arabidopsis* pericycle cells neighbouring to the xylem poles in primary root functions as the initiation site of lateral roots. The xylem pole pericycle (XPP) cells subsequently divides (asymmetric division) and induces lateral root primordia, which eventually progresses into lateral root (Motte et al. 2019). During ED a group of stem cells situated opposite of shoot apex creates a group of progenitor cells, which later gives rise to root caps and columella in the PED phase (Dolan et al. 1993). Below the QC, root cap cells are constantly formed and also replaced repeatedly by the activity of RAM (Dolan et al. 1993). While, the lateral root cap is generated by periclinal division of the epidermis-lateral root cap stem cells and an anticlinal division of columella stem cells leads to the development of columella autonomously from root cap development (Dolan et al. 1993).

3 Phytohormonal Regulation and the Genetic Control of Root Development

Most of root developmental studies have revolved around the model plant *Arabidopsis* and few commercially significant cereal crops. Structural development of root is regulated by a specific set of genes particularly during the ED and PED phases (Petricka et al. 2012). Initiation of root development starts during the early embryogenesis phase and is strictly regulated by differential expression of several genes along with gradient of phytohormones. For instance, in *Arabidopsis*, during early embryogenesis, polarity of embryo (apical-basal) determines the fate of plant development. The apex positioned cell gives rise to the shoot meristem, whereas the basal cell forms embryonic root and root meristems in two cell embryo (Mansfield and Briarty 1991).

All the aspects of root development is strictly under the control of phytohormone gradient which in turn is dependent up on the genetic regulation. During embryogenesis, ordered distribution of auxin evidently decides the apical-basal axis and pattern of cell elongation and proliferation (Grieneisen et al. 2007). An elevated concentration of auxin determines lower mitotic activity in QC, intermediary auxin concentration determines the moderate mitotic activity in QC surrounding the stem cells, and subordinate auxin level in meristematic zone has been associated with cell differentiation, rapid cell proliferation and elongation (Grieneisen et al. 2007). Formation, organization and maintenance of RAM is also profoundly synchronized by the levels of auxin (Reed et al. 1998; Sabatini et al. 1999; Benjamins and Scheres 2008; Wang et al. 2014). Although auxin primarily determines the embryonic root development, other hormones also play vital role in the developmental aspects of root during both embryonic and post-embryonic phase. For instance, the cytokinins

govern several aspects of root development in synchronization with auxin (Garay-Arroyo et al. 2012). Cytokinins mainly affect cell differentiation of meristematic zone and inhibits expansion to contain RAM size (Dello Ioio et al. 2007). Cytokinin also affects the rate of cellular differentiation in vascular tissues at meristematic zone to elongation zone transition zone, while exogenous application does not affects meristematic cell proliferation and stem cell niche (SCN) activity (Dello Ioio et al. 2007). Other phytohormones e.g. gibberellins promotes root development by regulating cell proliferation and elongation (Fu and Harberd 2003), brassinosteroids regulates cell cycle progression and differentiation in root meristems (González-García et al. 2011), ethylene promotes cell hair differentiation and constrains cell elongation (Achard et al. 2003).

The development of embryonic roots is known to be regulated by *WUSCHEL HOMEOBOX (WOX) genes which plays a crucial role.* In *Arabidopsis,* dynamic expression of novel *WOX* genes *decides embryonic patterning, for instance,* the fate of apical and basal cells are regulated by *WOX2 and WOX8* transcription factors (Fig. 1) respectively, whereas *WOX5 expression* in hypophyseal cells lead to the establishment of the quiescent center (Haecker et al. 2004). *WOX* genes are also involved in modulating the auxin related pathways and thereby regulates the fate of root development (Haecker et al. 2004). The QC plays central role in allocation of meristem cells and stem cell niche and the maintenance of QC cell identity allied with the gene expression of *WOX5* transcription factor (Sarkar et al. 2007). *WOX5* expression exceedingly constrained in QC cells determined by a receptor-like kinase gene *ACR4*, which expressed in adjacent columella initials and columella cells (De Smet et al. 2008). Another crucial gene *SCARECROW* (*SCR)* plays an important role in the development of ground tissue (radial patterning) and also functions in the maintenance of QC (Di Laurenzio et al. 1996; Sabatini et al. 2003). Likewise, maintenance of QC and the auxin maxima in root meristem region has been known to be retained by another set of genes–*PLETHORA*s (*PLT*s), through the regulation of auxin efflux genes designated as *PIN FORMED* (*PIN*s) expression (Aida et al. 2004; Petersson et al. 2009). It has been well realized that the three major well studied pathways viz. *WOX5-ACR4-CLE40, SHR/SCR-RBR* and auxin-*PLT* pathways plays the major role in the maintenance of QC (Fig. 2; Matsuzaki et al. 2010; Cruz-Ramírez et al. 2012).

Embryonic root development is known to be regulated by numerous factors. Among these factors, the phytohormone auxin has been recognised as one of the major determinants of embryonic root development. Genetic abnormality in auxin related genes such as *PINFORMED* (*PIN1,3,4,7*), *AUXIN SIGNALLING F-BOX* (*ABF1,2,3*), *TRANSPORT INHIBITOR RESPONSE* (*TIR1*), *YUCCA* (*YUC1,4,10,11*), *TRYPTOPHAN AMINOTRANSFERASE RELATED* (*TAR1,2*), *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA1*) etc. leads to the deformity of root (Friml et al. 2003; Dharmasiri et al. 2005; Cheng et al. 2007; Stepanova et al. 2008). Another study reported, 23 *AUXIN RESPONSE FACTORS* (*ARFs*) induced by auxin which plays an important role in root development. Among them *ARF5*/*MONOPTEROS* (*MP*) plays a vital role in the specification of hypophysis and root formation (Hardtke and Berleth 1998). Recent studies have revealed

that along with *MP* genes, four *TARGET OF MP* (*TMO3,5,6,7*) genes are also co-expressed in pro-vascular cells, which controls the expression of *MP* gene and contributes in root development (Schlereth et al. 2010). Some reports have suggested that the usage of inhibitory chemicals against auxin transporters (*PIN*) affected the formation of hypophysis and roots during embryogenesis (Hadfi et al. 1998; Friml et al. 2003). During early embryogenesis (two cell stage) the lower cell supports the apical cell to transport auxin by *PIN7* (Friml et al. 2003). While, in the later stages of embryogenesis (globular stage) *PIN7* expressed in suspensor cells are generated from the basal cell which maintains the level of auxin in the pro-embryo for the further developmental process (Friml et al. 2003). This study also suggested *PIN1* and *PIN7* collectively regulates the differentiation of hypophysis and root development and also determines the establishment of pro-vascular tissues (Friml et al. 2003; Petricka et al. 2012).

Root functions are highly reliant up on the specification of cells, proper arrangement of the specific cells and their architecture. During PED phase of root development which mainly encompasses regulated cell division and formation of the cell initials generates the lineages of specific cells (Dolan et al. 1993). Current studies have revealed that the cellular specification of epidermal layer from single tissue layer and further root epidermis further specified in two types of cells, trichoblasts (hair-forming) and atrichoblasts (hairless) (Slovak et al. 2016). Molecular screening of hair and hairless cells revealed a number of genes involved in the determination of root epidermal cells. Among them *GLABRA2* (*GL2*), *ENHANCHER OF GLABRA3* (*EGL3*) *TRANSPARENT TESTA GLABRA* (*TTG1*), *WEREWOLF* (*WER*) etc. have been known to stimulate hairless root epidermal cells, in contrast *CAPRICE* (*CPC*) and mutation in hairless genes specifies hair cells in *Arabidopsis* (Galway et al. 1994; Rerie et al. 1994; Masucci et al. 1996; Lee and Schiefelbein 1999; Wada et al. 2002; Bernhardt et al. 2003). Further, cellular specification of ground tissues are also known to be controlled by numerous genes, however two genes have been mostly studied by researchers—GRAS family *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*). The studies also pointed out that the *SHR* and *SCR* both plays a significant role in the generation of ground tissue initial daughter cells from the cortex endodermis initial (CEI). Additionally another gene *SCHIZORHZA* (*SCZ*) regulates the formation of ground tissue stem cells and establishes the embryonic roots (Pernas et al. 2010). *SHR* genes evidently differentiates endodermis of ground tissues, whereas cortex and endodermis differentiation regulated by *SCR* genes activity (Benfey et al. 1993; Scheres et al. 1995).

In the root pro-cambium, gradient of cytokinin levels and a tissue specific *CYTOKININ OXIDASE2* enzyme determines the specification of the vascular cells, while another negative regulator of cytokinin signalling *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*) determines the morphogenesis of metaxylem and phloem vascular tissues in plant root (Mähönen et al. 2000, 2006). Cascade of genetic modulation is crucial for the developmental aspects of vascular bundles for instance, *SHR*/*SCR* are known to controlled by the expression of miRNA 165 and 166 in endodermis, which eventually reaches the stele and downregulates the expression of *PHABULOSA* (*PHB*) member of class III HD-Zip genes necessary

for establishment of patterning ground tissues, pericycle and xylem (Carlsbecker et al. 2010; Miyashima et al. 2011). Similarly, another gene *LONESOME HIGHWAY* (*LHW*) governs the bilateral symmetry and also maintains cell files of vascular bundles in root (Ohashi-Ito and Bergmann 2007). Differentiation of phloem cells is controlled by *ALTERED PHLOEM DEVELOPMENT* (*APL*) and also suppresses the xylem specification in phloem poles (Bonke et al. 2003). Root tips are protected by a layer of cells and are also constantly removed to make space for the growth of RAM and differentiation of root tissues. Root cap cells are originated from root cap initial cells, the fate of which is determined by the expression of *NAC* transcription factor family genes *FEZ* and *SOMBRERO*, which regulates the timing and orientation of root cap initials cell division (Willemsen et al. 2008).

4 Effect of Environmental Stresses on Root Development

The development of an organism is controlled by both genetic and environmental factors. Likewise, in plants a variety of environmental factors considerably influence the developmental aspects in roots (Fig. 3). These environmental factors can be broadly categorized in to abiotic and biotic factors.

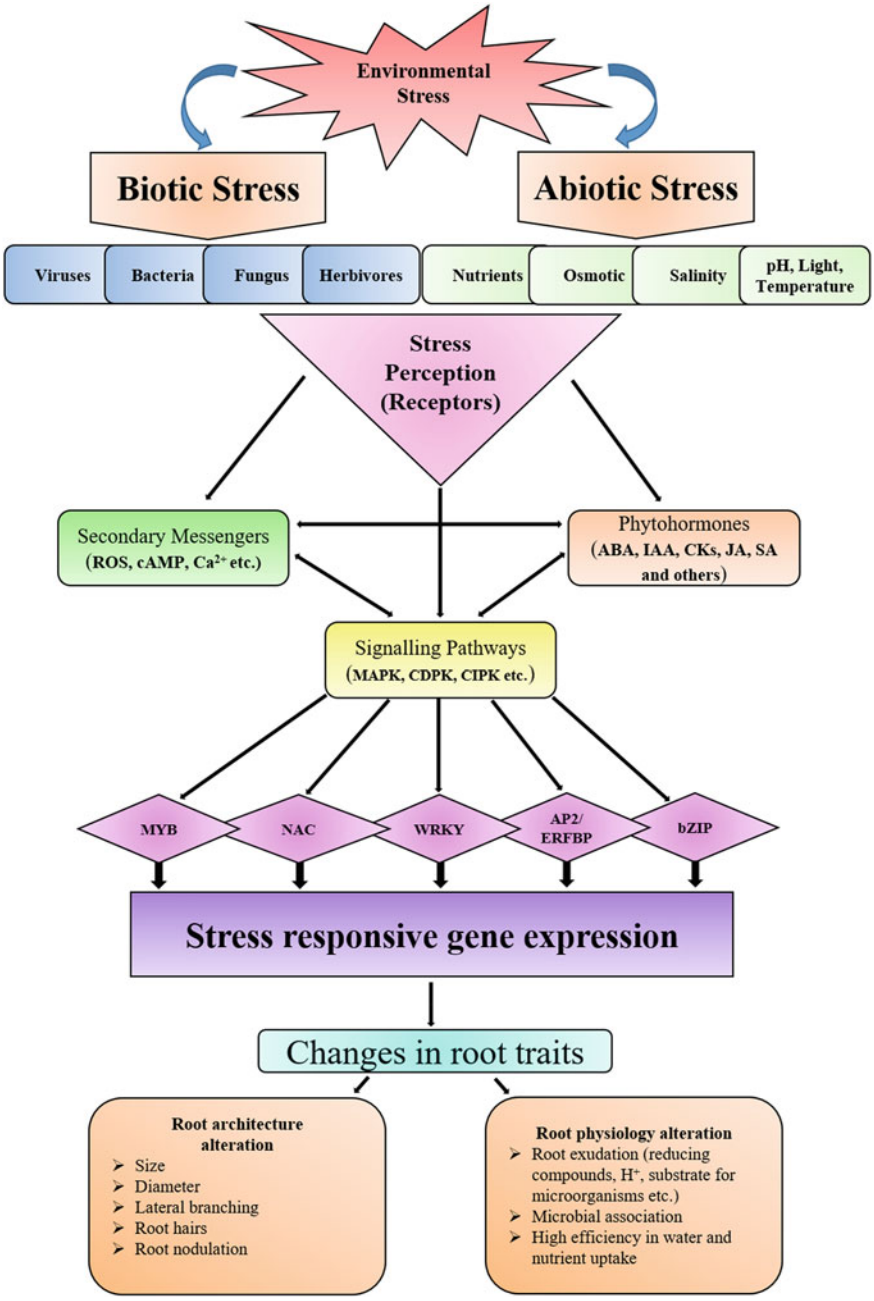
4.1 Abiotic Factors

Factors such as nutrients, water, light, temperature, organic and inorganic matter together regulates every aspect of plant development (Fig. 3). Inconsistency in the availability of these factors inflict stress on plants and hamper their growth. Some aspects of root development are also obstructed by the effect of abiotic stresses which has been discussed in the following sections.

4.1.1 Nutrients

Nutrients are one of the essential determinant of plant development and functions. In soil, nutrients are scattered heterogeneously, hence, it is very important for the plants to develop root system in such way that they can efficiently obtain the nutrients as per their requirement. Certain types of nutrients are likely to be rich in different layers of soil e.g. in the upper layer of soil phosphate is profusely found whereas nitrates are more abundant in deeper layers (Motte et al. 2019).

Nitrogen is a fundamental element and soil nutrient which is also an important constituent of proteins and nucleic acids. Plants are incapable of up taking nitrogen directly from the atmosphere or soil, hence the uptake of utilizable nitrogen (nitrates or ammonium) by roots takes place commonly (Krouk et al. 2010). The availability of nitrates or ammonium in soil is determined by the action of several microorganisms


Environmental Stress
Biotic Stress
Abiotic Stress
Viruses
Bacteria
Fungus
Herbivores
Nutrients
Osmotic
Salinity
pH, Light, Temperature
Stress Perception (Receptors)
Secondary Messengers (ROS, cAMP, Ca2+ etc.)
Phytohormones (ABA, IAA, CKs, JA, SA and others)
Signalling Pathways (MAPK, CDPK, CIPK etc.)
MYB
NAC
WRKY
AP2/ ERFBP
bZIP
Stress responsive gene expression
Changes in root traits
Root architecture alteration
Size
Diameter
Lateral branching
Root hairs
Root nodulation
Root physiology alteration
Root exudation (reducing compounds, H+, substrate for microorganisms etc.)
Microbial association
High efficiency in water and nutrient uptake

◀**Fig. 3** A schematic representation showing the perception and responses of plant roots towards environmental stresses. Plants senses environmental stresses broadly in the form of biotic and abiotic factors through wide-ranging receptors and generates ROS as primary response. After primary response, a network of secondary and phytohormonal signalling cascade induces specific transcription factors (e.g. *NAC*s, *MYB*s, *bZIP*s, *WRKY*s, *AP2/ERFBP* etc.). These stress responsive transcription factors expresses different transporters (like NHXs, nitrate transporters, phosphate transporters, aquaporin etc.), enhances root length and induces the formation of lateral roots to improve water and nutrient uptake. Some phytohormones in coordination with genes improves nodulation ability in plant roots and moreover helps the plant to adapt under different stresses

and application of nitrogen fertilizers. However, root development is highly impacted by the presence of ammonium which prevents root elongation and gravitropism. On the other hand, the presence of nitrates provokes root elongation (Motte et al. 2019). Among the dual functions of *NITRATE TRANSPORTER 1.1* (*NRT1.1*), auxin transport is activated under nitrate starvation, which lowers auxin accumulation in lateral roots leading to the suppression of lateral root primordia development (Krouk et al. 2010; Bouguyon et al. 2016). Lack of nitrogen in soil also induces signalling of *CLE-CLAVATA1* which results in the suppression of lateral root primordial outgrowth (Araya et al. 2014). Another study have suggested that during nitrogen starvation C-terminally encoded peptide downstream family (CEPDs) can induce multiple nitrate-rich genes (*NRT 1.1* and *NRT 2.1*) that regulates the development of roots and play an important role in the stimulation and elongation of lateral roots, and primary root development (Tabata et al. 2014; Vidal et al. 2015; Ohkubo et al. 2017). Recent reports have also demonstrated that the CEPD signalling pathway via *TCP 20* transcription factor controls the expression of *WOX5* in association with *SCR* and *PLT* (Guan et al. 2014; Shimotohno et al. 2018; Motte et al. 2019).While the effects ammonium on plant roots are uncertain, although some studies have claimed that ammonium can induce lateral roots and impedes the formation of primary root (Gruber et al. 2013; Ruiz Herrera et al. 2015).

Phosphorus is also a key element essential for the synthesis of nucleic acids, ATP, constitution of cell membrane, and growth and development of plants. Roots are extremely receptive to phosphate deficiency. Phosphate deficiency favours horizontal growth of the adventitious roots, and constrains primary root growth, and promotes axial branching to establish superficial rooting system which regulates the plants in the exploration of upper layers of soil (Lynch 2011; Péret et al. 2014). Studies reported that, phosphate starvation in *Arabidopsis* can result in the development of auxin sensitivity locally by magnifying auxin signalling and expression of *TIR1* genes which in turn leads to the alteration in lateral root primordia development (Nacry et al. 2005; Pérez Torres et al. 2009). Another report have established that a wide range of phosphate-starvation responsive genes regulated by *PHOSPHATE STARVATION RESPONSE1* (*PHR1*) transcription factor, also contributes to the development of lateral roots by modulating auxin-signalling (Bustos et al. 2010; Castrillo et al. 2017; Motte et al. 2019). Contrarily, over exposure of phosphate induces ethylene facilitated negative regulation of plant development and growth in *Arabidopsis* (Shukla et al. 2017). The study also highlighted the mechanism of plant

responses towards excessive phosphate in coordination with ethylene and metal ions that regulates the activity of apical root meristem and primary root length.

Sulphate is another major nutrient that play an imperative role in plant development. Like other nutrients sulphates are distributed in an uneven manner in soil. Sulphate-starvation induces considerably higher branching of root system through the initiation of lateral roots near the root tips as observed in case of *Arabidopsis* (López-Bucio et al. 2003). Root growth under sulphate deficiency was found to be regulated by nitrilase gene family, which plays an important role in auxin synthesis using indole-3-acetonitrile to adjust lateral branching and root growth (Rubio et al. 2001). Apart from the above discussed nutrients several other nutrients are also involved in the regulation of plant growth, but the mechanism of their role in root development has been less studied. A study have reported that deficiency of potassium initiates signalling cascades similar to other abiotic stresses which include phytohormone (mainly auxin, ethylene and jasmonic acid) signalling and upregulation of high affinity potassium transporters in roots (Ashley et al. 2006). Unavailability of macronutrients like iron and magnesium causes significant decline in the density of lateral roots, however insufficiency of manganese, zinc, calcium, and boron leads to change in lateral root density (Gruber et al. 2013; Kellermeier et al. 2013, 2014). However, excess manganese negatively regulates auxin biosynthesis and downregulates the expression of *PIN4* and *PIN7* (Zhao et al. 2017), whereas excess presence of iron adversely affects the formation of lateral roots by suppressing *PIN2* and upregulating *AUX1* to induce root elongation (Li et al. 2015).

4.1.2 Water

Water availability in the layers of soil varies greatly, hence plants are required to alter their root developmental strategies for extracting the required amount of water for growth and development. Water scarcity in soil is first sensed by plant roots which severely impacts all the developmental aspects. Under drought condition, growth of some crop plants are immensely disturbed which prevents shoot growth entirely, while roots tends to be relatively resilient under low water potentials (Spollen and Sharp 1991). Therefore, roots play an important function to provide resilience to the plants under scarcity of water. In response to drought, growth of lateral roots has been found to be highly affected, but development of primary roots was barely affected (Deak and Malamy 2005). Some reports have suggested that drought may cause suppression of the lateral root meristem and exceedingly reduce the formation of lateral roots, yet the generation of primordia remains unaffected (Deak and Malamy 2005; Malamy 2005). Suppression of lateral root development under the effect of drought extensively establishes an adaptive response for the enhanced level of persistence in plants during unfavourable conditions (Xiong et al. 2006). Under drought stress, *DEEPER ROOTING1 (DRO1)* gene expression results in the development of perpendicular roots and much deeper root system (Uga et al. 2013). In response to water stress, degeneration of amyloplasts in root cells predominantly take place in the columella region which assists hydrotropism in *Arabidopsis* and

radish (Takahashi et al. 2003). Phytohormones like abscisic acid (ABA), cytokinins and gibberellins produced in the roots which play an important role in the plant growth and development are also influenced by the availability of water. Although, auxins are the major determining factor for root growth, ABA and cytokinins are also reported to play key role in modulating the architecture of root system during osmotic stress (Munns and Sharp 1993; Billou et al. 2005). Phytohormones like, auxins and abscisic acid (ABA) play essential role in plant root development via complex signalling under drought stress (Sanchez-Calderon et al. 2013). The study also suggested that in response to drought stress, ABA mediated signalling facilitates polar transport of auxin that leads to primary root elongation, while lower accumulation of auxin in lateral roots suppresses lateral root primordia and inhibits lateral branching (Sanchez-Calderon et al. 2013). Mild drought stress on the other hand triggers upregulation of root morphology related enzymes (Xyloglucan endotransglucosylase) and down regulation of structural proteins (actin, tubulin) which leads towards polarized growth for the formation of root hairs (Sanchez-Calderon et al. 2013). Contrarily, under severe drought stress, overexpression of structural proteins and *MYB96* transcription factor induces lateral root growth to cope with drought (Seo and Park 2009; Sengupta and Reddy 2011).

4.1.3 Salinity

Salt stress is conceded as one of the major preventive factors in plant growth and development. NaCl and Na_2SO_4 are the primary salts which makes the soil saline, however some noticeable quantities of calcium and magnesium salts also contributes to soil salinity (Parihar et al. 2015). Salinity can modify nutrient disparity, reduce photosynthesis, induce oxidative damage, decreases water potential, disturbs cellular ion homeostasis, changes water status and eventually impacts plant growth and yield (Parihar et al. 2015). Plant roots are the first organ to perceive salt stress stimulus. NaCl induced stress has been found to alter the root morphology traits and plasticity in *Brassica napus* by promoting root hairs, increase in root surface area and induction of lateral roots (Arif et al. 2019). Transcriptomic analysis suggested in response to higher NaCl stress, rapid and substantial expression of aquaporin was witnessed in *Arabidopsis* roots, which was responsible for the positive changes in root morphology (Boursiac et al. 2005). Also, the regulation of ROS generation by NADPH oxidase as a response to salt stress and accumulation of excessive calcium in the cytosol of root hair cells leads to root elongation in *Arabidopsis* (Foreman et al. 2003).

4.1.4 Light, pH and Temperature

Many developmental aspects of plants are controlled by a group of photoreceptors which gets activated and functions at specific wavelengths of light. Root movement away from light or negative phototropism is regulated by blue light dependent receptors- PHOTs (Wan et al. 2012). In absences of light, very short and thinner roots

have been shown to be developed in *Arabidopsis* seedlings compared to light grown seedlings (Laxmi et al. 2008; Dyachok et al. 2011). Expression of blue light sensing CRY1 and CRY2 in *Arabidopsis* root was known to inhibit root growth by polar transport and modulation of auxin concentrations (Mo et al. 2015). Current studies have also revealed that activity of the photoreceptor PhyB and under heat stress effectively increases root growth (Hanzawa et al. 2013; Jung et al. 2016; Legris et al. 2016).

Soil pH ranging from 5.0–7.5 has slight impact on plant root development, while lower pH (<5) imparts a certain level of stress and thereby influences plant root development (Lynch et al. 2011). Acidic soils (pH < 5) tends to inhibit root growth through H^+ efflux in many plant species (Schubert et al. 1990). A fewer studies have reported the effects of higher pH on root development. A study suggested that at high pH (>7.5) which was linked to ammonium toxicity, root growth was significantly inhibited (Schenk and Wehrmann 1979). In alkaline soil, root growth of *Lupinus angustifolius* was also significantly inhibited by high pH > 6 due to the inhibition of cell elongation and increase in root diameter (Tang et al. 1992).

Among the abiotic factors temperature is also a major determinant of plant growth and development. Deviation of temperature in soil with time and depth, significantly impacts the development of roots. The optimum temperature for root growth differs from species to species and generally tends to be lower than that required for shoot growth (Lynch et al. 2011). Supraoptimal temperature can induce reduction of metaxylem diameter and limits the water conducting ability of wheat root (Huang et al. 1991). At lower temperature roots are more impacted than shoot which has been observed in the form of reduction of root elongation as well as root branching, decrease in enzyme activities, lignification of metaxylem vessels etc. (Covey-Crump et al. 2002; Gladish & Rost 1993; Huang et al. 1991; Pahlavanian & Silk 1988).

4.2 Biotic Factors

The plant root development is also influenced by many biotic factors e.g., viruses, microbes, fungus, pests etc. (Fig. 3). A number of studies have elaborated the effects of viral infection on plant development. The viral infection is wide spread in cultivated crops as well as wild varieties of plants. On entrance of viral particles plants are severely affected and limits the growth of both underground and aboveground plant organs (Andika et al. 2016). A common viral pathogen, *beet necrotic yellow vein virus* (BNYVV) infects plant roots and induces lateral root formation, increasing rootlets and eventually inhibits the formation of taproot in sugar beet (Tamada 2016). Other tuber crop disease causing viruses like *cassava mosaic gemini viruses* (CMGs), *sweet potato feathery mottle virus* (SPFMV) and *yam mosaic virus* (YMV) significantly reduces the tuber size and decreased the production of three major tuber crops in Africa (Legg 2014). Another study reported significant inhibition of root number and length even restrained of root primordia in a callus via alteration of phytohormone

level induced by *apple stem grooving virus* (ASGV) which primarily infects pear cultivars.

Apart from viruses, plant root development has been known to be considerably affected by other biotic factors. Plants are the host of microbiome, countless microbial population inside and outside of the roots, composed of pathogenic and beneficial bacteria, fungus, oomycetes and some archaea (Pascale et al. 2020). Pathogenic soil borne microbes infects numerous economically important plant species and reduces crop quality and yield. For instance, *Rhizobium rhizogenes* can infect a wide range of plant species by transforming t-DNA into plant genome and modulates plant development by reducing peduncles, wrinkle leaves, delayed flowering, hairy root induction etc. (Desmet et al. 2020). Some studies have reported that the soil borne pathogens like nematodes (*Gaeumannomyces graminis*) can produce cytokinin to interrupt root growth, while a deleterious microorganisms *Pseudomonas* sp. produces phytotoxins (e.g. cyanide) to interfere with the function of mycorrhiza and eventually prevents root development (Cahill et al. 1986; Bolton et al. 1989; Schippers et al. 1990; Nehl et al. 1997). Some other phytopathogens and root-associated insects directly inhibits plant growth or else act as vectors for numerous pathogenic fungus and microbes (Willsey et al. 2017).

Aside from microbial community, many fungi affects the root development by entering through wounds in the lower part of stem or by directly penetrating the healthy roots. As soon as infection of pathogenic fungus is established, development of plants or roots are severely reduced (Tattar 1989). A notorious root rot causing fungal pathogen *Phytopthora* has been known to infect extensive range of herbs, shrubs and trees. Infection with *Phytopthora* successively destroys small roots, and caused the formation of lesions in larger roots (Tattar 1989). Other fungi like clover root borers (*Hylastinus obscurus*) can introduce pathogenic fungus and infect healthy red clover root (Leath 1973). Some pathogenic nematodes can reduce root growth by exploiting the sugar reservoir in roots by damaging root tissues and eventually constrains root development via alteration in phytohormone level (Smith 2007). Herbivorous insects of both aboveground and belowground impacts in wide range of plant species through altering plant root morphological traits such as root biomass and branches, also hinders beneficial root-microbial interaction alongside adjustment of magnitude in phytohomonal and secondary metabolite of root (Johnson et al. 2016; Heinze 2020).

5 Strategies for Crop Improvement by Modulating Root-Specific Traits

Plants alter their growth and developmental patterns by regulating cell differentiation and proliferation under different environmental conditions (Sanchez-Calderon et al. 2013). Roots are the most essential organs of plant which controls or regulate the overall developmental aspects of the entire plant system. Therefore, manipulating

the developmental aspects of root has been on the prime focus of researchers all over the world for the improvement of functional traits and performance even under adverse conditions (Joshi et al. 2018). In this connection, several strategies have been evaluated to modulate the development of root specific traits starting from enriching the rhizosphere with microbes, genetic interventions for root modifications and so on. Some of the major strategies have therefore been detailed in the following sections and also in Table 1 which highlights the potential of root architectural changes in crop improvement under normal and stressed environments.

5.1 Root Modification by Microbial Consortia

In recent times plant–microbe association has been acknowledged as a simple and remarkable approach to improve crop production. The symbiotic association co-evolved in such a way that more than 90% of plant species are symbiotically associated with bacteria and fungi which contribute in beneficial attributes to the host plants (Bonfante and Genre 2010). Among symbiotically associated organisms, 80% belongs to arbuscular mychorrhizal fungi (AMF) (Parniske 2008). AMFs are obligatory biotrophs which can perceive stimulatory signals generated from root-exudates (like strigolactone) which helps in associating the microbial flora with the hosts (Parniske 2008; Bonfante and Genre 2010). In legumes most of the symbiotic association is presented by the microbes (rhizobia), which induces the formation of root nodules (Begum et al. 2001). After establishment of these symbiotic association, plant-symbionts exchanges nutrients continuously and modulates the overall growth and development. In this connection, AMFs are known for its capability to accumulate inorganic low water soluble phosphates and transportation inside the host roots in exchange of sugars (Parniske 2008). *Rhizobium* is known to fix atmospheric nitrogen biologically into inorganic forms (nitrate, ammonium) inside root nodules and then transfers it to the host roots (Begum et al. 2001). Some reports also suggested that AMFs can also transfers major nutrients like nitrogen in the form of inorganic (nitrate and ammonium) and organic (amino acids) molecules into plant through specialised transporters, whereas AMFs receive all the carbon and fatty acid requirements for their metabolism in return (Bonfante and Genre 2010; Maclean et al. 2017; Parniske 2008). Zhang et al. (2019) have also reported that exogenously inoculated AMF (*Funneliformis mosseae*) can considerably enhance the phytohormone (ABA, IAA, methyl jasmonate and brassinosteroids) levels, thereby improving the traits like formation of root-hairs and increase in root biomass and in this process confers an enhanced level of drought tolerance to the host plant. Also, the mixture of AMFs (*Glomus mosseae*) and PGPR (*Bacillus amyloliquefaciens*) have been shown to modify plant root architecture, enhances nutrient uptake, improves plant growth and eventually assists in the development of salinity resistance as observed in the studies with *Elaeagnus angustifolia* seedlings (Pan et al. 2020). Another important feature of symbiotic association is to induce systemic resistance (ISR) elicited by

Table 1 List of studies reporting the manipulation of root specific genes for enhancing stress tolerance in plants

Name of plants	Intervention with genetic modification/other resources	Specification of genes or other resources	Experimental details	References
Abiotic stress				
Rice	OsSWEET13	Sucrose transporter	OsSWEET13 knockout induces resistance against Xanthomonas oryzae	(Zhou et al. 2015a)
Soybean	GmF3H1 GmF3H2 GmFNSII-1	Isoflavone biosynthetic gene	Enhances isoflavonoid biosynthesis, induces root hair and provides resistances against soybean mosaic virus (SMV)	(Zhang et al. 2020)
	GmNMHC5	Nodule promoter	Promotes lateral root and nodule formation in transgenic soybean	(Liu et al. 2015)
	GmEXPB2	Nodule promoter	Induces root nodule and β-expansin expression	(Guo et al. 2011; Xie et al. 2015)
Rice	OsNPF2.4	NO3-Transporter	Enhances NO3- influx in roots thereby increasing nitrogen availability	(Xia et al. 2015)
Maize	ZmDof1	N absorption	Improves nitrogen assimilation by altering root morphology	(Kurai et al. 2011)
Wheat	TaNAC2-5A	Transcription Factor	Increases nitrate transporter and promotes root growth in low N	(He et al. 2015)
Rice	OsMYB2P-1	Transcription Factor	Enhances P-transporters and improves root efficiency in phosphate uptake	(Dai et al. 2012)
	OsPT2 OsPT6	Transporter	Enhances phosphate uptake in roots in transgenic rice	(Ai et al. 2009)
Maize	ZmPTF1	Transcription Factor	Improves root architecture and increases phosphate uptake in maize	(Li et al. 2011)

(continued)

Table 1 (continued)

Name of plants	Intervention with genetic modification/other resources	Specification of genes or other resources	Experimental details	References
Rice	OsARF12	Auxin response factor	Improves Fe uptake in transgenic rice	(Qi et al. 2012)
	OsHAK16p	Phosphate related transcription factor	Enhances WOX11 expression and improves root development	(Chen et al. 2015)
	OsNAC9 OsNAC10	Drought related transcription factors	Advances root water absorption capabilities and improves drought tolerance	(Jeong et al. 2010; Redillas et al. 2012)
	OsNAC5 OsEXPA8 DROI	Drought related transcription factors	Improves drought tolerance by positive modification of root traits	(Milad et al. 2019)
Groundnut	DREB1A	Drought related transcription factors	Increases root elongation and enhances water absorption under drought stress	(Gantait and Mondal 2018)
Rice	SNAC1	Stress responsive transcription factor	Improves root morphology and provides resistance against drought and salt stress	(Liu et al. 2014b)
Soybean	GmNAC11 GmNAC20	Stress responsive transcription factor	Induces root hair and improves root morphology in transgenic soybean	(Hao et al. 2011)
Rice	SOR1 DRO1	Stress related transcription factor	Advances root architecture and increases grain yield	(Kitomi et al. 2020)
Wheat	TaNHX2	Vacuolar Na + /H + antiporter (NHX)	Enhances vacuolar compartmentalization of Na + and K + ion and provide salinity resistance	(Mushke et al. 2019)

(continued)

Table 1 (continued)

Name of plants	Intervention with genetic modification/other resources	Specification of genes or other resources	Experimental details	References
	TaWRKY93	Stress responsive transcription factor	Improves root length and induces lateral roots under salinity, drought, low temperature and osmotic stress	(Qin et al. 2015)
Cotton	GhWRKY34	Stress responsive transcription factor	Enhances primary root length and increases lateral roots under salinity, drought, low temperature and osmotic stress	(Zhou et al. 2015b)
Rice	OsbZIP71	Stress responsive transcription factor	Improves root traits and provides drought, salinity and osmotic stress tolerance	(Liu et al. 2014a)
Soybean	GmbZIP1	Stress responsive transcription factor	Promotes root growth and improves abiotic stress tolerance	(Gao et al. 2011)
Rice	RCc3	Root-specific stress responsive gene	Improves the root phenotypes and yield parameters	(Li et al. 2018)
	OsMYB-R1	Transcription factor	Alters root morphology via inducing lateral root and improves drought and heavy metal tolerance	(Tiwari et al. 2020b)
Tomato	SIARF4	Auxin response factor	CRISPR-Cas9 mediated downregulation enhances root density and growth under osmotic and salt stress	(Bouzroud et al. 2019)
Maize	ARGOS8	Auxin related gene	sgRNA guided upregulation of ARGOS8 gene advances root traits and drought tolerance	(Shi et al. 2017)

(continued)

Table 1 (continued)

Name of plants	Intervention with genetic modification/other resources	Specification of genes or other resources	Experimental details	References
Tomato	SlLBD40	JA signaling pathway gene	Suppression of SlLBD40 alters root morphology and enhances drought resistance	(Liu et al. 2020)
Potato	miR397 miR398	Growth related miRNA	Enhances nitrate uptake under low nitrogen and improves crop yields	(Tiwari et al. 2020a)
Rice	osa-miR12477	Oxidative stress responsive miRNA	Improves root growth and increases salt stress resistance	(Parmar et al. 2020)
Arabidopsis	miR156	Growth related miRNA	Promotes root development and provides salt tolerance	(Cui et al. 2014)
Biotic stress				
Sorghum	Endophytic bacteria	Growth promoter	Application of multi-trait microbes improves root traits and provides drought tolerance	(Govindasamy et al. 2020)
Tomato	*Pseudomonas fluorescens* *Glomus mosseae*	Growth promoter	Combine application of microbe and AMF improves root architecture and improve phosphate uptake	(Gamalero et al. 2004)
Soybean	PGPR	Beneficial microbes	Application of two PGPR induces salt resistance via enhancing root morphology	(Egamberdieva et al. 2017)
Rice	OsSWEET13	Sucrose transporter	OsSWEET13 knockout induces resistance against Xanthomonas oryzae	(Zhou et al. 2015a)

(continued)

Table 1 (continued)

Name of plants	Intervention with genetic modification/other resources	Specification of genes or other resources	Experimental details	References
Soybean	GmF3H1 GmF3H2 GmFNSII-1	Isoflavone biosynthetic gene	Enhances isoflavonoid biosynthesis, induces root hair and provides resistances against soybean mosaic virus (SMV)	(Zhang et al. 2020)
	GmNMHC5	Nodule promoter	Promotes lateral root and nodule formation in transgenic soybean	(Liu et al. 2015)
	GmEXPB2	Nodule promoter	Induces root nodule and β-expansin expression	(Guo et al. 2011; Xie et al. 2015)

advantageous microbiomes in the roots of wide range of host plants conferring resistance to several pathogens (Pieterse et al. 2014). ISR mechanism has been primarily reported to be developed by the application of several plant growth-promoting bacterial (PGPR) genera like *Pseudomonas, Bacillus* and *Serratia,* which is dissimilar from phytopathogen induced 'systemic acquired resistance' or SAR (Kloepper et al. 2004; Pieterse et al. 2014). In recent times, ISR has been a well–studied mechanism in many plant growth-promoting fungus (PGPF) such as genus of AMFs like *Fusarium*, *Trichoderma*, *Serendipita* etc. (Shoresh et al. 2010; Jung et al. 2012).

A recent study on synergistic application of multi-trait endophytic bacteria (e.g. *Ochrobactrum* sp., *Enterobactercloacae*, *Enterobacter* sp. and *Microbacterium* sp.) have been known to restore osmotic balance through significant advancement in root traits (length, surface area, dry weight etc.) of drought stressed *Sorghum bicolor* plants thereby providing enhanced tolerance (Govindasamy et al. 2020). Similarly, the combined inoculation of microbe (*Pseudomonas fluorescens*) and AMF (*Glomus mosseae*) in tomato plants suggestively enhances the growth, alters the root architecture and improves phosphate acquisition under low phosphate condition (Gamalero et al. 2004). Enhanced rhizobial (*Bradyrhizobium japonicum)* colonization in soybean roots under nutrient stress also considerably improves root growth, symbiotic performance and thus helps to balance nutrient supply under stress (Egamberdieva et al. 2018). Two rhizosperic microbes *Mitsuaria* sp. And *Burkholderia* sp. from *Arabidopsis* have been shown to profoundly alter the physiological response, root system architecture and phytohormonal level to augment plant survival and drought resistance in *Arabidopsis* and maize (Huang et al. 2017). Under salinity stress, inoculation of two PGPR (*Bradyrhizobium japonicum* and *Pseudomonas putida*) considerably improved salt tolerance by improving the nodulation traits and root architectural traits like root length, surface area, volume etc. (Egamberdieva et al. 2017). The study also described that the combined application of two PGPR strains

significantly improved the uptake of nitrogen and phosphate, thereby improving plant growth and salt resistance in soybean at the same time (Egamberdieva et al. 2017).

5.2 *Root Architectural Engineering for Crop Improvement*

Most of current works in the field of crop improvement have focused on the modulation of root-specific genes which has been achieved by the production of transgenic plants aiding greater efficiency to confront abiotic stress. It has also been realized that the modification of root architecture holds the key for improved performance of crop plants and could even be crucial for supporting a second wave of green revolution (Hodge et al. 2009; Koevoets et al. 2016; Armanda et al. 2019). Therefore, researchers have recently focused on the strategies for improving crop productivity under low nutrient soils through the optimization of root morphology and architecture to boost the nutrient acquisition efficiency of plants (Hawkesford 2011). In transgenic crop plants like wheat, overexpression of transcription factor genes like *TaNAC2-5A* (*NAC* family transcription factor) increases root growth by enhancing NO_3 influx, while in soybean upregulation of *GmNMHC5* (MADS-box transcription factor) advances root growth by stimulating root nodulation and eventually improves crop yield even under nitrogen starvation (He et al. 2015; Liu et al. 2015). Modification of root architecture also benefits top soil foraging and better acquisition of nutrients (P, Mn, Cu, Ni etc.) with relatively low mobility and bioavailability in soil (Lynch 2011). For instance, upregulation of *OsMYB2P-1* and *ZmPTF1* transcription factors control root traits and alters root architecture in rice and maize respectively under low phosphate conditions (Li et al. 2011; Dai et al. 2012). Under phosphate starvation, in *Arabidopsis* and soybean the overexpression of *GmEXPB2* gene encodes expansin protein (situated in cell wall and plays key role in cell elongation) rapidly, which upsurges root length, alter root architecture and improves phosphate uptake (Guo et al. 2011; Xie et al. 2015). In a transgenic experiment enhancement in rice root elongation and accumulation of Fe has been reported to be controlled by the upregulation of an auxin response factor *OsARF12* (Qi et al. 2012). Deficiency of key nutrient like potassium can be alleviated by the overexpression of *OsHAK16p:WOX11* in rice which have shown promising results in terms of enhanced root growth with 72% increase in potassium uptake in addition to better grain production (Chen et al. 2015).

Root architectural and anatomical changes have also been known to confer better stress tolerance mechanism in crop plants. Under drought stress, root specific upregulation of *NAC* family genes (*OsNAC9* and *OsNAC10*) enhances root length, and provides drought resistance and also considerably improves grain yields in transgenic rice plants (Jeong et al. 2010; Redillas et al. 2012). Likewise in another study, upregulation of *OsNAC5, OsEXPA8,* and *DRO1* genes in transgenic rice modulates root phenotypes remarkably viz. enhancement in root length, thickness and number, and eventually advances root architecture for effective water absorption (Milad et al. 2019). In groundnut, overexpression of *DREB1A* gene induces the generation of elongated roots and thereby facilitates water absorption from deeper layer of soil

and improves pod yields and overall yield under drought stress (Gantait and Mondal 2018). Liu et al. (2014a) have also reported that the expression of rice *SNAC1* gene in transgenic cotton plants have shown promising results in terms of enhanced root growth and crop yield under drought and salt stress. Two other genes of soybean from *NAC* family *GmNAC11* and *GmNAC20* are known to regulate the expression of nuclear genes that modulates root functions through induction of root hairs during abiotic stress (Hao et al. 2011). The study also pointed out that the overexpression of *GmNAC20* induces the formation of lateral roots and improved stress tolerance against salinity and freezing, whereas *GmNAC11* only improved salt resistance (Hao et al. 2011). In a near-isogenic line of rice, *SOIL SURFACE ROOTING1* (*qSOR1*) and *DEEPER ROOTING1* (*DRO1)*—two homologous allele have been known to play a crucial role in controlling root growth angles under salt stress. The loss of function in *qSOR1* have been found to develop exceedingly modified root system architecture, which improved salt tolerance and increased crop yield (Kitomi et al. 2020). Apart from this, the vacuolar Na^+/H^+ antiporters (*NHX*) plays a crucial role in the alleviation of salt stress, for instance, transgenic sunflower with upregulated *TaNHX2* gene (wheat vacuolar antiporter gene) have shown superior growth performance as well as improved Na^+ and K^+ accumulation in leaves and roots on exposure to salinity (Mushke et al. 2019). A key transcription regulator family *WRKY* also confers stress responses. In a transgenic line of *Arabidopsis,* upregulation of *TaWRKY93* (class II *WRKY* transcription factor from wheat) and *GhWRKY34* (class II *WRKY* transcription factor from cotton) reforms root traits via enhancement in primary root length and lateral roots, which eventually helps the plants to resist salt, drought, low temperature and osmotic stress (Qin et al. 2015; Zhou et al. 2015a, b). In plants abscisic acid (ABA) signalling pathway and *bZIP* transcription factor synergistically contributes to resistance towards several abiotic stresses. In this connection, increased expression of *OsbZIP71* significantly improved salinity, drought and osmotic stress tolerance in transgenic rice via improvement in root and yield related attributes (Liu et al. 2014b). Similarly upregulation of *GmbZIP1* substantially improved salinity, drought and low temperature tolerance in multiple transgenic plants (Gao et al. 2011).

A unique gene *OsMYB-R1* from *MYB* transcription family, has also been known to improve both biotic and abiotic resistance in transgenic rice (Tiwari et al. 2020a, b). A network of calcium-dependent signalling pathway induced by overexpressed *OsMYB-R1*, was known to induce resistance against *Rhizoctonia solani* (causal organism of root and stem rot disease) in transgenic lines (Tiwari et al. 2020a, b). Also upregulation of *OsMYB-R1* increased lateral root formation, alters root morphology and improved cellular homeostasis by regulating crosstalk of auxin-salicylic acid signalling subjected to drought and heavy metal (Cr) stress (Tiwari et al. 2020a, b). A current study described the colonization of bacteria with multiple plant growth promoting traits improved drought stress by upregulating two drought responsive genes (*SbP5CS1*and *SbP5CS2*) and modulates root architecture in *Sorghum bicolor* (Govindasamy et al. 2020). In rice, overexpression of *RCc3* gene significantly increased auxin accumulation, local biosynthesis and polar transport in root, which in turn significantly modulated the root architectural phenotypes such as elevated

growth of primary root, adventitious roots and lateral roots that positively enhanced the yield related parameters (Li et al. 2018).

5.3 *Recent Techniques for the Modification of Root-Architecture*

Availability of advance genome editing tools along with sequenced genome of crop plants unlocks ocean of possibilities for the development of desirable traits. In recent times one of most versatile and effective techniques for crop improvement includes CRISPR/Cas9 system (Jaganathan et al. 2018). For instance, improvement the root specific traits in respect to higher density and growth has been achieved by CRISPR-Cas9 technique, which was used to repress the expression of *SIARF4* gene in a transgenic line of tomato increased root density and promoted root growth under osmotic and salt stress (Bouzroud et al. 2019). Five *SWEET* genes encodes sucrose transporters in rice has been found to be associated with the bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae*, knockout of these genes using CRISPR-Cas9 system considerably enhanced the resistance against the pathogen (Zhou et al. 2015a). Similarly, CRISPR-Cas9 mediated gene knockout of *PsPP2Ab1* in *Phytophthora sojae, a* pathogenic agent for root rot disease of soybean, promises effective disease management and improves crop through effectively minimizes the fungal vegetative growth and production of sporangia yield (Qiu et al. 2021). Also, targeted gene alteration in the soybean genes viz. *GmF3H1*, *GmF3H2* and *GmFNSII-1* significantly improved isoflavonoid contents, induced hairy root and commendably enhanced the resistance against soybean mosaic virus (SMV) by affecting the coat protein biosynthesis (Zhang et al. 2020). Another, CRISPR-Cas9 facilitated study have shown that sg-RNA targeted overexpression of *AUXIN REGULATED GENE INVOLVED IN ORGAN SIZE8* (*ARGOS8*) gene family which significantly improved the drought tolerance of plants via adjustment of root system in transgenic maize (Shi et al. 2017). Plant specific *SlLBD40* (gene of LBD family in tomato) gene associated with jasmonic acid signalling pathway plays an important role in development of lateral organ and root. In transgenic experimentations, CRISPR-Cas9 mediated suppression of *SlLBD40*, suggestively improved the level of drought tolerance in tomato (Liu et al. 2020).

Apart from the CRISPR-Cas9 technique, modern approach employing the miRNA based technique present new possibilities for the elevation plant stress tolerance and crop improvement. Micro-RNAs are found to be associated with a wide-ranging stress response mechanisms for instance, stimulation of stress responsive kinases, transcription factors, transporter genes, universal heat-shock proteins, F-box proteins, salinity related proteins, calmodulin binding proteins, different molecule transporter proteins, zinc-finger proteins etc. (Tiwari et al. 2020a, 2020b). This technique has also been employed for the modulation of root-specific traits for conferring enhanced tolerance to different stresses. In this connection, upregulation of miR397 and

miR398 in potato plants under nitrogen deprived condition manifested an enhanced rate of nitrogen absorption in roots due to the better functioning of nitrate transporters (Tiwari et al. 2020a, 2020b). A recent study on miRNA (*osa-miR12477*) targeted regulation of L-ascorbate oxidase (LAO) gene (involved in oxidative stress mitigation) and differential expression of *AP2/EREBP* transcription factor promoted root growth and significantly improved salt tolerance in sensitive rice varieties (Parmar et al. 2020). Similar miRNA based approach revealed that the expression of *GmNHXs* in soybean also manifested the improvement in root, stem and leaf tissue development under high salinity (Joshi et al. 2021). In *Arabidopsis* transgenic line overexpressing miR156 enhanced plant survivability and resistance against salt induced stress (Cui et al. 2014). The study also revealed that the upregulation of two downstream genes *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE9* (*SPL9*) and *DIHYDROFLAVONOL-4-REDUCTASE* (*DFR*) contributed towards anthocyanin metabolism, helped plants to adapt various environmental stress through miR156-*SPLs-DFR* signalling network (Cui et al. 2014). Direct participation of many miRNAs like miR156, miR167, miR393, miR396 and miR398 have also been well studied in the regulation of root development and overexpression of these miRNAs suggestively improved drought tolerance in plants (Sunkar et al. 2012).

6 Epilogue

Plant roots are one of the most vital organs that helps in anchorage and supply of essential elements and water which is elementary for survival and developmental processes. The roots are also under a direct control of the environmental factors and are often the first organs to perceive the stimulus of stress. Root performance therefore, determines the plant performance and survivability under various stressed condition. Also, the performance of roots determine the yield of crop plants. Henceforth, a well-developed root system is desirable for plant growth and development. In this connection, root system architecture, root organization, branching and development of lateral roots and hairs and so on determine the level of beneficial attributes conferred by the root system to the entire plant. All these aspects are also under the direct control of phytohormones and genetic regulation that are also known to be modulated by different abiotic and biotic stresses. Thus, understanding the regulatory genes and hormonal functions for root development holds the key for the improvement of root-specific agronomic traits. Under stress, several plant responses are triggered that aids in the modification of root system architecture that may help the plant to adapt the negative impacts of stresses. But roots are generally stress sensitive and therefore other strategies are required to be adopted to enhance their performance. In this regard, recent biotechnological advances have helped us in devising various strategies for the improvement of root-specific traits by targeting the expression of beneficial genes that can confer enhanced level of tolerance. Root architecture engineering employing the selective use of beneficial microorganisms

along with the intervention of genetic tools for the modulation of genetic and physiological regulation of root seems to be promising in this regard. However, further studies are required to be conducted in the future to achieve the most deserved success and also to translate the laboratory results to the agricultural field.

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In Vitro Biosynthesis of Natural Products in Plant Roots

Lebin Thomas, Zeeshan ur Rahman, Kuldeep Sharma, Devendra Nagar, Akanksha Vashishtha, Gaurav Kumar, and Siva P. K. Chetri

Abstract Various plants are composed of structurally and chemically diverse secondary metabolites. Especially plant root systems are remarkable accumulators of high-valued phytomedicines. But the production of different secondary metabolites in the root system can be limited. Also, enhanced and controlled synthesis of secondary metabolites in plants is challenging. Plant tissue culture holds great promise for ecofriendly, economical and industrial-scale production of phytochemicals and natural bioactive compounds. Use of elicitors for enhanced de novo synthesis of natural products from roots cultures can be used as effective alternative strategies for improved productivity of bioactive compounds. Elicitation involves signaling pathway, which transfers the extracellular stimulus to the inside plant cell, leading to molecular and physiological changes for their better survival, persistence and competitiveness, and activates the synthesis for different compounds. The present article sheds light on the use of different elicitors including abiotic (physical and chemical) and biotic (bacteria, fungi and hormones), which enhance the accumulation of secondary metabolites in the root system of medicinal plants.

L. Thomas
Department of Botany, Hansraj College, University of Delhi, Delhi, India

Z. ur Rahman
Department of Botany, Zakir Husain Delhi College, University of Delhi, Delhi, India

K. Sharma
Department of Botany, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

D. Nagar
Amity Institute of Environmental Sciences, Amity University, Noida, UP, India

A. Vashishtha
Department of Plant Protection, C. C. S. University, Meerut, UP, India

G. Kumar
Department of Botany, Hindu College, University of Delhi, Delhi, India

S. P. K. Chetri (✉)
Department of Botany, Dimoria College, Khetri, Assam Kamrup Metro, India

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1 Introduction

The natural products or secondary metabolites that have primarily an important role in the interaction of the plants with its environment are of immense therapeutic potential occur across all the taxonomic groups within the plant kingdom (Hussein and El-Anssary 2019). The natural products serve as attractants for pollinators and have diverse utilities of protection from environment, phytopathogens, insect pests and herbivores, etc. (Dixon 2001). Besides, secondary metabolites have industrial utilizations as agrochemicals, biopesticides, colors, flavors, food additives, fragrances, and pharmaceuticals. As an adaptation to the varying environments, plants can amend the production and preservation of diverse sets of bioactive secondary metabolites that include alkaloids, glycosides, flavonoids, terpenoids, volatile oils, tannins, resins, etc. are differentially distributed in different plant parts, i.e. roots, stems, leaves, flowers, seeds, etc. (Atanasov et al. 2015). Among the different plant parts, roots are rich in diversity of such therapeutic natural products and are being explored for the production and upregulation of the bioactive constituents. However, their production is much less than primary metabolites and dependent on the plant growth phase and presence of different elicitors (Oksman-Caldentey and Inzé 2004). Plant biotechnology, especially in vitro cultures were found to be effective for the production of different bioactive compounds such as anthraquinones, benzylisoquinoline alkaloids, diosgenin, ginseng, nicotine, etc. (Alamgir 2018).

Though research in this arena had gradually gained momentum in the last two decades, a proper understanding of the biosynthetic pathways of natural products in roots is limited. The lack of proper information in the field of in vitro studies on root natural product biosynthesis may be due to lack of convenient experimental systems (Loyola-Vargas and Miranda-Ham 1995). Here, we briefly review the current understanding on in vitro biosynthesis of natural products in plant roots and the various prospects and perspectives on their improved in vitro biosynthesis.

2 In vitro Production of Secondary Metabolites

The traditional method of secondary metabolite extraction from plant raw material or chemical synthesis are difficult and these have the disadvantages of seasonal plant growth, heterogeneous products, high risk of plant extinction, stereo-specificity, stringent conditions of biochemical reactions and high costs of production (Thakur et al. 2019). However, the biotechnological production of secondary metabolites with the use of in vitro plant cultures has benefits of homogeneous yield extracts and maintenance, which is independent of seasonal conditions (Pedreño and Almagro 2020).

Plant tissue culture methodology as a means of producing plant natural compounds has a long history and is an integral of plant biotechnology. Biotechnological production in plant cell culture is an attractive alternative and provides conditions useful for the production of plant natural compounds that are of high economic value (Misawa 1994; Verpoorte and Memelink 2002). A range of environmental and nutritional manipulations in the media composition are known to influence the biosynthetic pathways of secondary metabolites pertaining to the accumulation of the desired metabolite in the culture and various strategies have been developed to improve the production of secondary metabolites using plant cell cultures (Bourgaud et al. 2001; Matkowski 2008).

The hairy root cultures have also been developed as a favorable approach for plant secondary metabolite production, particularly when the synthesis or extraction of these bioactive compounds are unfeasible and involve any harm to the environment. The hairy roots are differentiated cultures of *Agrobacterium rhizogenes* mediated modified roots, which have the similar phytochemical pattern of the corresponding wild type organ. Hairy roots have higher stability and productivity that make them a valuable biotechnological tool for the production of plant secondary metabolites (Pistelli et al. 2010). There are certain methods of obtaining secondary metabolites from plant cell suspension and hairy root cultures particularly with several elicitation methods in small and large scale production. Increased and efficient product recoveries of metabolites that are released into the cultivation medium involve elicitation, inducing membrane permeability and in situ product release (Cai et al. 2012).

3 Elicitation

Usage of elicitors for induced up-regulation of metabolite content, known as elicitation has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites (Roberts and Shuler 1997). Elicitors are signals that activate the formation of plant natural products. Researchers have put their best efforts to utilize plant cell biosynthetic capabilities for obtaining enhanced useful products and for studying the plant metabolism (Misawa 1994; Verpoorte and Memelink 2002). According to their origin, elicitors can be divided on the basis of their source, i.e., abiotic and biotic. Abiotic elicitors are composed of non-biological sources such as salts or physical factors. Biotic elicitors originate from biological sources which may be exogenous or endogenous. Many plant natural product pathways were reported to be influenced by microbes such as fungi, bacteria, viruses, yeast, etc. and their use has gained prominence due to improved yield and quality of the natural products (Chandran et al. 2020) (Fig. 1).

The biotic elicitors are usually recognized by specific receptors bound to the cell membrane. A signal transduction system transfers the stimulus to the cell, leading to changes that ultimately as a defense response lead to the formation of phytoalexins (Baenas et al. 2014). The overall multifold ability of such elicitors is unique as well as multidimensional (Gorelick and Bernstein 2014). The genetic and physiological

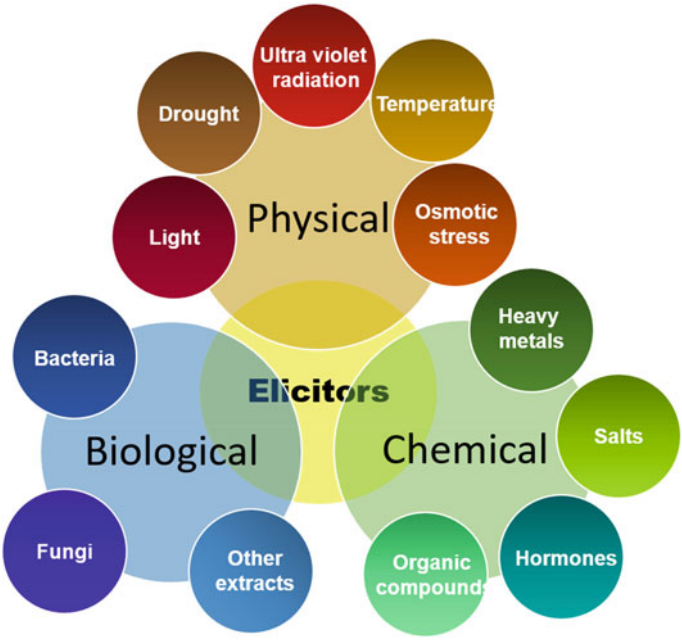

Fig. 1 The various physical, chemical and biological elicitors that affect the secondary metabolite production

state of the plant is the prime determining factors for the response of the plant to such stimulus (Ramirez-Estrada et al. 2016) (Fig. 2). Research work suggests that it can control a large number of biochemical control points, regulate the expression of key genes and transcription factors (Giri and Zaheer 2016). Therefore, elicitors have the ability to control a variety of cellular activities both at biochemical and molecular level (Zhao et al. 2005; Baenas et al. 2014). The specific elicitor recognition process

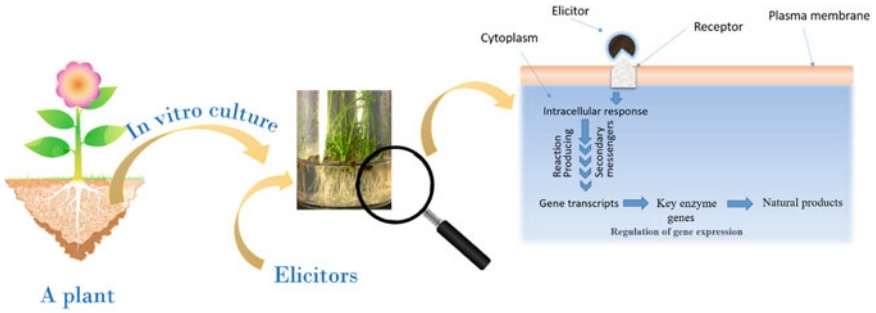

Fig. 2 The molecular induction of elicitors on in vitro cultured plant roots for secondary metabolite production

increases the plant's ability to respond to biotic and abiotic stresses with improved synthesis of signal compounds such as jasmonic acid, salicylic acid, ethylene, etc. which directly or indirectly affects the biosynthesis of natural products (Giri and Zaheer 2016). A wide range of elicitors had been used by researchers for the production of different bioactive compounds using root cultures of certain medicinal plants (Table 1).

Table 1 Use of different types elicitors for the production of secondary metabolites from the plant root system

Plants	Types of elicitors	Elicitor used	Secondary metabolites	References
Withania somnifera	Physical	pH 5.5	Withanolide A	Murthy and Praveen (2013)
Hypericum perforatum	Physical	Blue light	Naphthodianthrone derivatives, total phenolic compounds and hypericin	Najafabadi et al. (2019)
Hyoscyamus reticulatus L	Chemical	Iron oxide nanoparticles	Tropane alkaloids, hyoscyamine and scopolamine	Moharrami et al. (2017)
Trigonella foenum-graecum	Chemical	Zinc oxide nanoparticles	Trigonelline	Tariverdizadeh et al. (2021)
Adhatoda vasica	Chemical	Tryptophan and sorbitol	Vasicinone, vasicine, 2-acetyl benzyl amine and other pyrroloquinazoline alkaloids	Singh et al. (2017)
Fagopyrum esculentum	Chemical	Sucrose	Flavonoid content	Jeong et al. (2018)
Ipomoea aquatica	Chemical	NaCl	Anthocyanin	Kitayama et al. 2019
Perovskia abrotanoides	Hormonal	Methyl jasmonate	Cryptotanshinone and tanshinone IIA	Zaker et al. (2015)
Linum album	Hormonal	Indole acetic acid	Podophyllotoxin and 6-methoxypodophyllotoxin	Farkya and Bisaria (2008)
W. somnifera	Hormonal	Salicylic acid	Withanolide A, withanone and withaferin A	Sivanandhan et al. (2013)
Taverniera cuneifolia	Biological	*Rhizobium leguminosarum*	Glycyrrhizic acid	Awad et al. (2014)
Stevia rebaudiana	Biological	*Piriformospora indica* and *Azotobacter chroococcum*	Steviol glycoside	Kilam et al. (2015)

4 Physical Factors

There are various sources of abiotic origin such as physical factors which displays an effect on in vitro culture conditions by influencing the natural product biosynthesis in in vitro plant parts. Among the available physical factors which put an impact on in vitro biosynthesis of enhanced natural products include the variations in pH, temperature, light as well as a combination of these factors. Under in vitro conditions, these physical factors produce specific stress signals that trigger the up-regulation of the biosynthetic pathways of specific plant natural products. Such physical factors can be used as elicitors under in vitro conditions and there are various examples of research findings and updates on the broader use of several physical factors as elicitors for enhanced biosynthesis of natural products from plant roots.

4.1 pH

As a physical factor, the pH has an important role in maintaining the solidifying status of the culture media as well as optimizing the performance of the growth medium. The in vitro culture media can be modified from the standardized value to lower as well as higher values and this change in pH can act as elicitors for enhancement of natural products in the different plant parts. For most plants, an acidic pH of 5.8 is observed to be suitable for the production and accumulation of the metabolites. The root cultures of *Beta vulgaris* showed an increased release of betalain pigments after a brief exposure to an acidic medium. Furthermore, there was a retained capability in beet roots for re-growth and continued pigment accumulation (Mukundan et al. 1998). An acidic pH of 3.5 was found to increase the release of alkaloids in *Datura stramonium* and *Catharanthus roseus* and thiophenes in *Tagetes patula* root cultures, without affecting growth of subsequent subcultures (Saenz-Carbonell et al. 1993). The submerged adventitious root cultures of *Stevia rebaudiana* showed varied effect to differential pH conditions. The maximum fresh and dry biomass was obtained at a high media pH of 6.0, while a lower pH of 5.1 facilitated the production of steviol glycosides (steviosides, rebaudioside-A), but dulcoside, total phenolics and flavonoids were found to be increased at 5.8 pH in these roots (Ahmad et al. 2018). Similarly, adventitious root cultures of *Withania somnifera* showed optimal biomass at the initial medium pH of 5.8 and maximum withanolide A production at 5.5 pH (Murthy and Praveen 2013).

4.2 Temperature and Duration

Temperature has a very vital role for the optimum maintenance in vitro of growth conditions. However, variations from the normal temperature range of 25 °C either

lower or higher and the duration of temperature treatment can affect the metabolic regulations as it has significant effects on membrane permeability (Ramakrishna and Ravishankar 2011; Wang and Wu 2013). Although, scientific literature on the utility of temperature variations as an in vitro elicitor for improved accumulation of natural products in plant roots is limited, there are some reports of enhanced biosynthesis employing the aforesaid elicitor. Amongst the temperature ranges of 19.5, 24 and 32 °C employed as elicitor on the hairy root cultures of *Catharanthus roseus*, the temperature at 19.5 °C for 27 days displayed the optimum response with 2.56 mg/g dw of indole alkaloids accumulated in the hairy root cultures (Toivonen et al. 1992). The hairy roots (3-week-old) of *Beta vulgaris* revealed variations in pigment biosynthesis when treated with varying temperature of 40 °C, 45 °C and 50 °C for 30 min and 60 min, respectively. The exposure at the aforesaid temperatures for 60 min individually displayed the maximum (13.4, 40.2 and 47.5%, respectively) release of pigments (Thimmaraju et al. 2003). The hairy roots of *Pueraria candollei* var. candollei grown on liquid B5 medium when raised at temperatures of 25 and 32 °C resulted in a maximum response at 32 °C with a daidzein content of 31.0 mg/g dw synthesized in the hairy roots (Danphitsanuparn et al. 2012). A variation in temperature was also proved beneficial for in vitro production of astragalosides (2.657 mg/g dw) in *Astragalus membranaceus* hairy root cultures which was higher in comparison to that of 3-year-old field grown roots (Jiao et al. 2015).

4.3 Light

Light as a physical factor can act as an elicitor due to the broad range of its wavelength. The various wavelength ranges of light had been employed as elicitors for in vitro enhancement of secondary metabolites from plant parts as per available scientific literature. The hairy root cultures of *Oxalis tuberosa* modified in an *Agrobacterium rhizogenes*-mediated system had better growth and exuded constitutive levels of fluorescent compounds of harmine (7-methoxy-1-methyl-β-carboline) and harmaline (3,4-dihydroharmine) when irradiated with UV light (Bais et al. 2003). A metabolome analysis revealed an increase of 35 hydrophilic and 11 lipophilic metabolites in *Panax ginseng* adventitious roots irradiated with red (630 nm) and blue (465 nm) light-emitting diode (LED) light or fluorescent lamp (FL) light. Whereas, the LED light-irradiated roots had higher concentrations of sucrose, lower amino acids, alpha-tocopherol, beta-amyrin and phenolic acids (Park et al. 2013). For the adventitious root cultivation of *Hypericum perforatum*, biomass production was higher when grown in the dark and red light conditions, while a one-week blue light treatment was an effective stimulator for increasing the production of secondary metabolite of naphthodianthrone derivatives, including total phenolic compounds and hypericin (Najafabadi et al. 2019). The maximum accumulation of secondary metabolites, glycyrrhizic acid and liquiritin in roots of a medicinal plant *Glycyrrhiza uralensis* were obtained under an irradiance of 100 μmol m^{-2} s^{-1}, though these concentrations were negatively correlated with root biomass (Hou et al. 2010). However, light quality

can have varied effects on the regulation of different secondary metabolite production in a plant. Blue light treatment caused a down regulation of the genes involved in the tanshinone biosynthesis pathway in hairy root culture of *Salvia miltiorrhiza* though rosmarinic acid was slightly increased (Chen et al. 2018).

4.4 Combination

Under in vitro conditions, physical factors include a wide range of sources and each source has its own specific effect. However, for better utilization of the different physical factors as elicitors under in vitro conditions, the different combinations of physical factors in varied ranges has also been employed by the researchers. A combination of different physical factors is used to obtain an optimized root biomass and secondary metabolite production. The effect of different physical factors indicated that a lower temperature of 25 °C and an acidic pH of 5 are beneficial for maximal biomass and silymarin production in *Silybum marianum* hairy root cultures. It was suggested that low pH environment functions as an inducing signal for lipoxygenase activity, which subsequently caused a higher silymarin production (Rahimi and Hasanloo 2016). The hairy roots of *Panax ginseng* cultivated in large-scale bioreactors developed highest biomass in the presence of red light and ginsenoside in fluorescent light, though these were optimal under a 20 °C/13 °C day (12 h)/night (8 h) mode (Yu et al. 2005). A high concentration of chlorogenic acid, rutin, trans-2-decenal and total phenol along with antioxidant capacity of *Coriandrum sativum* were achieved with the combination of photosynthetic photon flux density (300 μmol m^{-2} s^{-1}) and root-zone temperature (30 °C) (Nguyen et al. 2019).

5 Chemical Factors

Other than the physical factors, there is a wide range of chemical factors such as metal salts, heavy metals, organic compounds, salts, etc. which acts as determining factors for in vitro biosynthesis of natural products in different plant parts. These chemical factors under in vitro conditions can induce chemical specific stress signals affecting the key enzymes of the biosynthetic pathways for positive up-regulation of some plant natural products.

5.1 Heavy Metals

Heavy metals are toxin to plant body (Rahman and Singh 2019). However, their low concentrations can act as elicitors for inducing production of secondary metabolites. Many heavy metal contaminated sites can be used for cultivation of medicinal plants for the enhanced production of various products. Use of heavy metal contaminated resources such as water and fertilizers can also be employed at non-contaminated sites. Heavy metal exposure to roots of plants may cause significant variations in quality and quantity of metabolites in different plant parts (Lajayer et al. 2017). Various metals such as Cu, Zn, Fe, Ni, Co, Pb, Mn, Ag, Cr, etc. have induced synthesis of different phytochemicals in different plants (Thakur et al. 2019). For instance, Thangavel et al. (2007) used zinc (Zn) and cadmium (Cd) for induction of phytochelatins from cell suspension cultures of red spruce (*Picea rubens* Sarg.; Thangavel et al. 2007). The hairy root cultures of medicinal plant *Hyoscyamus reticulatus* L. gave enhanced production of tropane alkaloids, hyoscyamine and scopolamine after the addition of iron oxide nanoparticles (FeNPs) (Moharrami et al. 2017). Copper oxide nanoparticles (CuO NPs) induced glucosinolate (GSL) and phenolic compounds in *Brassica rapa.* CuO NPs upregulated genes associated with oxidative stress in plants for increased production of bioactive compounds (Chung et al. 2019). In another study, amendment of CuO NPs to root cultures of chinese cabbage revealed elevated accumulation of nanomaterials, which altered gene expression level for phenolic compounds (flavonols, hydroxybenzoic and hydroxycinnamic acids) and glucosinolates (gluconasturtiin, glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin, glucoallysin, glucobrassicanapin, sinigrin, progoitrin, and gluconapin) (Chung et al. 2018). Cu stress to root suspension cultures of *Panax ginseng* promoted phenolics metabolism and lignin synthesis (Ali et al. 2006). The metabolic activity of *Hypericum perforatum* was found to be dependent on chromium (Cr) concentrations as well as number of days of metal exposure to seedlings, which induced the varying levels of bioactive compounds (protopseudohypericin, hypericin and pseudohypericin; Trilliiet et al. 2006). Zinc oxide nanoparticles too proved efficient elicitors for the production of trigonelline in hairy roots of fenugreek (Tariverdizadeh et al. 2021).

5.2 Organic Compounds

Organic compounds have an important role in culture media composition and for the maintenance and performance of in vitro culture conditions. Many organic compounds such as carbohydrates, amino acids, fatty acids can act as elicitors for the production of secondary metabolites. Compounds like tryptophan and sorbitol increased the production of bioactive compounds like vasicinone, vasicine, 2-acetyl benzyl amine and other pyrroloquinazoline alkaloids in cell cultures of *Adhatoda vasica* (Singh et al. 2017). Chitosan, a low-cost biopolymer stimulated the synthesis

of flavonoids in hairy root cultures of *Isatis tinctoria* L. (Jiao et al. 2018b). In another study, chitosan influenced formononetin and calycosin accumulation in hairy root cultures of a legume, *Astragalus membranaceus* (Gai et al. 2019). In a different study, compounds like chitosan, L-alanine and 1-naphthol enhanced the extracellular secretion of plumbagin in Plumbago indica root cultures (Jaisi and Panichayupakaranant 2016). The main role of chitosan as elicitor is in causing oxidative burst leading to expression of genes (associated with mitogen-activated protein kinase signaling (MAPK) cascades (Gai et al. 2019). Many folds increase in artemisinin accumulation in cell suspension cultures of *Artemisia annua* L. has also been reported (Salehi et al. 2019). Sucrose treatment influenced tyrosine and phenylalanine ammonia-lyase activities in buckwheat (*Fagopyrum esculentum* M.) sprouts, which promoted flavonoid content in plant (Jeong et al. 2018). The seedlings of licorice (*Glycyrrhiza uralensis*) experienced increased flavonoids yield and callus growth, when the appropriate concentration of sucrose, methyl jasmonate and phenylalanine were added in the medium (Guo et al. 2013).

5.3 Salts

Under in vitro conditions, different salts in varied concentrations can display their impact as stressors on the overall culture growth performance and accumulation of secondary metabolites. Salt stress causes osmotic and nutritional imbalances and leads to cellular dehydration in plants, under such induced stress conditions the biosynthesis of natural products is stimulated in different plant parts (Akula and Ravishankar 2011). Many salt sensitive plants have declined the production of bioactive compounds after salt exposure (Daneshmand et al. 2010). However, salt tolerant species have showed markedly improved production of some metabolites after addition of different salts. The treatment of 50 mM NaCl to the seedlings of wild type of water spinach (*Ipomoea aquatica*) potentially increased the production of anthocyanin and calcium without any deleterious effects on plant body (Kitayama et al. 2019).

6 Hormones

An effective method for enhancing secondary metabolites production in plants involve the application of hormones that function as biological elicitors. These are formed within pathogens or by the plant and can activate or inactivate many enzymes or ion channels utilizing different receptors (Patel and Krishnamurthy 2013). The plant hormones are chemical messengers that bind to specific target tissues for eliciting a physiological reaction and inducing genetic expression of various photosynthetic pathways that are applicable for an environment. The elicitors, salicylic acid (SA) and jasmonic acid (JA) are among the most common plant hormones that can

signal different gene expression (Thakur et al. 2019). The SA, that is known to signal systemic acquired resistance in plants and regulate resistance to many bacterial, fungal and viral pathogens can elicit the production of secondary metabolites in plants (Hayat et al. 2010; Thomas and Singh 2020). The phytohormone JA that regulates resistance to pathogens with an octadecanoid pathway, can further stimulate the biosynthesis of secondary metabolites in plants (Tamogami et al. 1997; Złotek et al. 2016). Jasmonates, including JA and methyl jasmonates (MeJA) are a family of cyclopentanone compounds which functions as an integral signal for the biosynthesis of the plant secondary metabolites of terpenoids, flavonoids, alkaloids and phenylpropanoids (Thakur et al. 2019). The treatment of SA and MeJA to the hairy roots of *Salvia miltiorrhiza* resulted in significant increase and accumulation of tanshinone, which are a group of active diterpenes used for treating cardiovascular disease (Hao et al. 2015). A low concentration of MeJA can stimulate the production of cryptotanshinone and tanshinone IIA in adventitious root cultures of *Perovskia abrotanoides* (Zaker et al. 2015). This accumulation was positively correlated to the expression of isopentenyl-diphosphate delta-isomerase, geranylgeranyl diphosphate synthase, copalyl diphosphate synthase and kaurene synthase genes (Hao et al. 2015). An application of SA to the hairy roots of *Withania somnifera* is known to enhance the production of biomass, and a variety of withanolides, viz. withanolide A, withanone and withaferin A (Sivanandhan et al. 2013). The MeJA could enhance synthesis of soluble biophenols of protocatechuic, gentisic, vanillic, caffeic, syringic, p-coumaric, ferulic, salicylic and cinnamic acids from *Panax ginseng* adventitious roots (Sivakumar and Paek 2005). In *Panax ginseng* adventitious roots, MeJA and SA altered phenolic synthesis enzymes to induce the accumulation of phenolic compounds. These hormones increased the total phenolics, flavonoids, ascorbic acid, non-protein thiols and cystein contents along with 1,1-diphenyl-2-picrylhydrazyl radical reducing activity. However, with MeJA incorporation, glucose 6-phosphate dehydrogenase, phenylalanine ammonia lyase and substrate specific peroxidase (caffeic acid peroxidase, quercetin peroxidase and ferulic acid peroxidase) activities were found to be higher, while SA treated roots had more proline and increased shikimate dehydrogenase, chlorogenic acid peroxidase and β-glucosidase activities (Ali et al. 2007).

The phytohormones, viz. abscisic acid (ABA), gibberellin (GA) and ethylene have shown effectiveness to induce production of phenolic acids in hairy roots of *Salvia miltiorrhiza*. These phytohormones increased phenylalanine ammonia-lyase and tyrosine aminotransferase activities that probably caused enhanced synthesis of different phenolic acids mainly caffeic acid, rosmarinic acid and salvianolic acid B (Liang et al. 2013). The GA can specifically accumulate in the endodermal cells of the root elongation zone with an active transport mechanism (Shani et al. 2013). The supplementation of a particular concentration of gibberellic acid (GA3) is a critical factor for optimizing secondary metabolite production. For light-grown *Echinacea purpurea*, a moderate GA-3 concentration of 0.025 μM facilitated development of thick, dense, purple-colored roots with high concentrations of different secondary metabolites (cichoric acid, caftaric acid, chlorogenic acid) production, increased phenylalanine ammonia lyase activity, cell viability and free radical scavenging

activity (Abbasi et al. 2012). Similarly, a treatment of indole-3-acetic (IAA) acid and gibberellic acid (GA) in optimal concentrations enhanced the growth parameters and accumulation of flavonoids and other phenolic compounds (4-hydroxybenzoic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin, quercetin) in *Fagopyrum esculentum*, with enhanced growth parameters of shoot length, root length and fresh weight (Park et al. 2017). An analysis of the effect of a broad range of phytohormones like GA, ABA and SA on growth and secondary metabolism of *Artemisia annua* hairy roots indicated that there are suitable conditions for stimulating total root lengths and artemisinin production (Weathers et al. 2005). For the hairy root line of *Linum album*, IAA induced thicker root tips and increased the concentration of podophyllotoxin and 6-methoxypodophyllotoxin (Farkya and Bisaria 2008). With an increased duration of application and concentrations of auxins, sorgoleone was enhanced in the hydrophobic root exudate of *Sorghum bicolor*, in which biosynthetic genes of *DES2*, *DES3*, *ARS1*, *ARS2* and *OMT3* were up-regulated (Uddin et al. 2011). Ethylene is a product of stress which is known for the inhibition of growth but application of ethylene on plant cells, might induce excretion of secondary metabolites from cells. For Ginseng (*Panax ginseng*), gaseous compositions of 10 ppm (57.9 g) and 20 ppm (55.17 g) ethylene caused an increase of dry weight in the adventitious root. This was correlated with enhanced uptake of phosphate, glucose and fructose (Jeong et al. 2006). With elevated auxin and ethylene, there is a rapid and unique signaling on the metabolome of *Arabidopsis* roots, particularly of phenylpropanoid, glucosinolate and fatty acid metabolism, though metabolites of one group exhibited similar modifications (Hildreth et al. 2020).

7 Beneficial Microorganisms

There are various biotic factors, especially include a wide range of microbes such as bacteria, fungi, etc. which can affect the in vitro culture growth conditions and stimulate the natural products biosynthesis in different plant parts, i.e. roots, stem and leaves. Similar to physical and chemical factors, under in vitro conditions, these biotic factors produce specific stress signals that can trigger the key enzyme genes of the biosynthetic pathways of particular plant metabolites and subsequently lead to their enhanced accumulation.

7.1 Bacteria

Different microbial forms are potential non-aggressive pathogens to plant roots and possess the ability to trigger a cascade of defensive response and production of secondary metabolites (Mañeroet al. 2012). Many bacteria and their exudates can be employed for elicitation of bioactive compounds from the plant roots. Use of *Agrobacterium rhizogenes* is very common in plant tissue culture to develop the

hairy root cultures (Wilczańska-Barska et al. 2012; Brijwal and Tamta 2015). The cultures of *Agrobacterium rhizogenes* ATC15834 has established a rapid and unlimited in vitro growth of root in *Pentalinon andrieuxii* (Apocynaceae) for the production of different phytochemicals including betulinic acid (Alejandro et al. 2020). Hairy root cultures of *Rauwolfia serpentine* using *A. rhizogenes* A4 were scaled up in a mechanically agitated bioreactor for the production of terpene indole alkaloids (Mehrotra et al. 2016). Many plant growth promoting rhizobacteria have played a key role in promoting root hairs and growth, and producing enhanced secondary metabolites in plants. The elicitation of glycyrrhizic acid (GA) was almost six times higher in root culture in *Taverniera cuneifolia* when grown in presence of *Rhizobium leguminosarum* (Awad et al. 2014). Another rhizobium, *Mesorhizobium amorphae* (GS3037) induced 1.7 folds saponin and up to 19 folds ginsenosides in a mutant adventitious root culture in *Panax ginseng* (Le et al. 2018). A Gram-negative bacterium, *Stenotrophomonas maltophilia* which acted as plant growth regulators of *Hypericum perforatum,* alsotriggered enhancement of hypericin and pseudohypericin in plant seedlings (Mañero et al. 2012). Two different bacteria, *Bacillus cereus* and *Staphylococcus aureus* were employed for the elicitation of hairy roots in *Datura metel* for the production of a tropane alkaloid, scopolamine which is used in pharmaceutical industry (Shakeran et al. 2017). An endophyte, *Bacillus altitudinis* also increased biomass and ginsenoside accumulation in adventitious roots of *P. ginseng* (Song et al. 2017). However, the combined effects of a chemical additive and endophyte showed negative effects on the growth of roots and the ginsenoside accumulation (Song et al. 2017). Exposure of *Pectobacterium carotovorum* lysate to hairy root cultures of *Scutellaria lateriflora* stimulated accumulation of wogonin, which is associated with phytoalexin activity (Wilczańska-Barska et al. 2012). In another way, some microorganisms can induce production of elicitor compounds like jasmonic acid in plants, which can act as transducer of elicitor signaling pathways for various activities in plants (Thakur et al. 2019). A different study used combined inoculation of a fungus, *Piriformospora indica* and bacterium, *Azotobacter chroococcum*, having properties of improving biomolecules, growth and secondary metabolite content in plants, enhanced antioxidant potential and steviol glycoside content in *Stevia rebaudiana* (Kilam et al. 2015). Different species of *Pseudomonas* have shown role in the production of various secondary metabolites like γ-terpinene, *trans*-sabinene hydrate, *cis*- sabinene hydrate and thymol from *Origanum* × *majoricum,* stevioside from *Stevia rebaudiana,* monoterpenes and phenolic compounds from *Tagetes minuta,* etc. (Banchio et al. 2010; Vafadar et al. 2014; del Rosario et al. 2013). The inoculation of *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors to root of commercial cultivars of blackberry plants modulated gene expression and increased secondary metabolites production. The main inducible metabolites were flavonoid, catechin, epicatechin, anthocyanin, quercetin, and kaempferol in the fruits of blackberry plants (Martin-Rivilla et al. 2021). A different study identified that two different metabolites produced by *Pseudomonas fluorescens* N21.4 triggered isoflavone content in soybean seedlings (Algar et al. 2012). An enhanced production of scopolamine by three different bacterial elicitors, *Pseudomonas aeruginosa* KCTC 1750, *Bacillus cereus* KCTC 1012 and *Staphylococcus aureus* KCTC 1916

was also induced in adventitious hairy root cultures of *Scopolia parviflora* (Jung et al. 2003).

7.2 Fungi

Various known fungal elicitors are most effective in enhancing the productivity of expedient secondary metabolites and of hairy root in plant cell culture. These elicitors can be the degradation products, metabolites, secreted substances or fermented liquid of fungi, while chemically constitute oligosaccharide (chitin, chitosan, cyclodextrins), proteins (some enzymes and substances of protein properties) and polyunsaturated fatty acids (Takeuchi et al. 2013; Zhai et al. 2017). The potential medicinal properties in roots of *Taverniera cuneifolia* mostly comes from an appreciable amount of glycyrrhizic acid. The utilization of live fungal cultures of *Mucor hiemalis* and *Aspergillus tenuis* as elicitors enhanced glycyrrhizic acid production to 4.90 ± 0.10 mg/g and 1.50 ± 0.07 mg/g, respectively in *T. cuneifolia* root cultures, which was higher than the frequently used elicitor of methyl jasmonate (Awad et al. 2014). The insecticide pyrethrins that naturally occur in the flowers of *Chrysanthemum cinerariaefolium*, were found to be elicited and enhanced in hairy roots induced from leaves. This enhanced pyrethrin yield occurred with the incorporation of the culture filtrate of an endophytic fungus, *Fusarium oxysporum*, resulting from an increased hairy root growth and higher biomass yield (Khan et al. 2017). The endophytic *Mucor fragilis* is known to be an effective fungal elicitor of different metabolites in *Salvia miltiorrhiza*, which is the most commonly used medicinal material. The mycelium extract of *M. fragilis* affected the regulation of the gene expression of primary and secondary metabolites, causing the accumulation of salvianolic acid, rosmarinic acid, stearic acid and oleic acid in *S. miltiorrhiza* hairy roots (Xu et al. 2021). Addition of *Aspergillus niger* derived elicitor resulted in a maximum accumulation of adventitious root metabolites including total flavonoids and glycyrrhetinic acid, other compounds including uralsaponin B, licorice saponin B2, liquiritin, and (3R)-vestitol in *Glycyrrhiza uralensis* (Li et al. 2016). Besides, there was an up-regulated expression of cinnamate 4-hydroxylase, β-amyrin synthase, squalene epoxidase and cytochrome P450 monooxygenase genes that are involved in the biosynthesis of bioactive compounds, along with an increased superoxide dismutase, catalase and peroxidase activities. Additionally, an immobilized *Aspergillus niger* was found to function as a superior elicitor in the *Isatis tinctoria* hairy root-fungus co-cultivation system for a high flavonoid production in optimized conditions (Jiao et al. 2018a). An endophytic *Penicillium citrinum* KACC43900 is known to have high plant growth promotion and gibberellin producing capacity when applied to roots of rice and *Atriplex gemelinii* seedlings (Khan et al. 2008). The culture filtrates of *Trichoderma atroviridae* and *Trichoderma harzianum* could elicit ginsenoside production in a cell suspension line of *Panax quinquefolius* (ginseng) that are absent in roots (Biswas et al. 2016). An in vitro chemotherapeutic paclitaxel synthesis from *Corylus avellana* cell suspension culture was found to be significantly enhanced with a combined

treatment of cell extract and culture filtrate derived from *Camarosporomyces flavigenus* growing in mid and late log phase (Salehi et al. 2020). A co-cultivation of growth-promoting endophytic *Piriformospora indica* that successfully colonize roots of *Centella asiatica* resulted in a prompt augmentation of root and shoot biomass. This was accompanied with a favored synthesis of the trisaccharide triterpene and asiaticosides in *Centella asiatica* (Satheesan et al. 2012).

8 Conclusion

In the present article, an overview of research information on the in vitro biosynthesis of natural products in plant roots employing both abiotic and biotic elicitors is briefly described. In case of abiotic elicitors, the impact of various physical and chemical factors, their combination, heavy metals, organic compounds and salts on in vitro biosynthesis of natural products in plant roots have been discussed as per previous scientific literature. The significance of biologically important plant–microbe interactions involving bacteria and fungi had revealed valuable scientific data on enhanced in vitro biosynthesis of natural products in plant roots. In the various research findings, the root exudates act as signal molecules during plant–microbe interactions.

The technological advancements in the detection and evaluation methods of highly sensitive bioactive compounds have led to the evaluation of a variety of such natural products for their roles in the rhizosphere interactions between plants, microbes and the soil and the biochemical mechanisms behind them. Under in vitro conditions, there is a significant scope for targeting enhanced accumulation of valuable medicinal natural products synthesized in some plant roots employing suitable elicitors singly or in combination and through modifications of the culture conditions. Further additions to the earlier research findings of plant interactions with more number of microbes and abiotic elictors and better perception of their regulatory interactions at molecular level will pave way for a higher level of understanding of the mechanisms of in vitro biosynthesis of natural products in plant roots.

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Soumya Mukherjee and František Baluška

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