Chapter 2
Hairy Root Composite Plant Systems in Root-Microbe Interaction Research

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Abstract Plant-associated microbes are key determinants of plant health and productivity. Research on model plant species has helped discover fundamental plant mechanisms that determine the outcomes of these microbial interactions. However, the species-specific nature of several key plant-microbe interactions necessitates research in non-model plant species. A major bottleneck for research on non-model species is the lack of efficient transformation methods. *Agrobacterium rhizogenes*-mediated hairy root composite plant system is a transformative tool that has enabled a multitude of transgenic approaches to be efficiently used in non-model species. This chapter provides a snapshot of research using key examples to highlight how the tool had helped advance the frontier of root-microbe interaction research focusing on arbuscular mycorrhizal symbiosis, nodulation, pathogen responses, and microbiome research. Limitations of and recent developments in hairy root composite plant systems are also discussed.

Keywords *Agrobacterium rhizogenes* • Arbuscular mycorrhizal symbiosis • Nodulation • Nematode • Microbiome • Symbiosis • Hormone

Introduction

The key influence of plant-associated microbes on plant growth, health, and yield has drawn increasing interest toward plant-microbe interaction research (Busby et al. 2017). A largely underexplored subtopic is root-associated microbes and their interactions with plants. In the early 1900s, classical microbiology studies pioneered by Lorenz Hiltner determined that the highest microbial density and

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diversity in soils occur very close to plant roots (Hinsinger and Marschner 2006). For example, root surface scrapings had multiple-fold more colony-forming units compared to soil samples 0.5 cm away from the roots (Clark 1940). Carbon-rich energy sources provided by the plant are the key drivers of such microbial enrichment. Indeed, plants release on average 10–15% (Jones et al. 2009; Dennis et al. 2010) of their photosynthetic assimilates into the rhizosphere. Such deposition does not appear to be a random release of carbon by the plant but an active recruitment of specific microbes for colonization and/or rhizosphere presence. Increasing evidence indicates that the plant species influences the composition of root microbial communities (Mougel et al. 2006; Weisskopf et al. 2006; Micallef et al. 2009). Indeed, an intricate coevolution of plants and rhizosphere microbial communities was suggested by the observation that resident plants or their root exudates are capable of maintaining the biomass and diversity of soil fungal communities to a much greater extent than nonresident/introduced plants (Broeckling et al. 2008). When *Arabidopsis* plants or root exudates were present, fungal communities in soils with a history of growing *Arabidopsis thaliana* showed increased biomass and diversity. This increase was not observed when a "nonresident" plant species (*Medicago truncatula*) or its root exudates were present in the same soil. Additional experiments over three generations indicated that resident plants or their root exudates are capable of maintaining the biomass and diversity of soil fungal communities to a much greater extent than nonresident plants (Broeckling et al. 2008). Similarly, invasive weeds might manipulate native rhizosphere microbial communities to their advantage, perhaps an evolutionary advantage enabling survival and dominance in new environments (e.g., Inderjit et al. 2006). However, pathogenic microorganisms appear to have evolved to utilize these plant "recruitment" signals to identify and colonize their hosts. For example, soybean roots release isoflavonoids, a group of specialized metabolites, to potentiate nitrogen-fixing rhizobia bacteria for colonization and phytoalexins to protect against pathogens (reviewed by Paiva 2000). Zoospores of the root rot pathogen *Phytophthora sojae* are chemotactic to isoflavonoids (Morris and Ward 1992) suggesting that the pathogen might use these molecules to find their host.

This intricate coevolution between the plant and associated microbial communities including pathogenic and symbiotic organisms warrants specific research on the plant species of interest. In other words, plant-microbe interaction studies using model organisms such as *A. thaliana* can address a number of fundamental questions intrinsic to the plant, but not the unique research needs for the majority of plant-microbe interactions that are species-specific. Key examples include symbiotic nodulation and AM fungal interactions as well as a number of species-specific plant-pathogen interactions. In addition, the composition of rhizodeposits varies substantially among different plant species including unique and species-specific rhizodeposit compounds (e.g., isoflavonoids that are legume-specific) requiring research on specific plant species to understand their influence on root-microbe interactions.

Need for an Efficient Transformation System in Non-model Plant Species

A number of plant species suffer from the lack of certain advantages that most model plant species have. The most crucial of these advantages are (a) the availability of genetic and genomic datasets and (b) the ability to efficiently generate transgenic lines for various functional experiments. Key examples of the approaches enabled by transgenic technology include the loss or gain of function assays, genetic complementation, evaluation of marker gene expression, cellular localization of proteins and tagging of organelles and compartments, and in vivo biomolecular interaction studies. Advances in high-throughput sequencing technologies have enabled the acquisition of transcriptomic, if not genomic, sequence information for a number of non-model plant species. The number of plant species with genome/chromosome assemblies in NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/browse/) has grown from just a single species (*A. thaliana*) to 102 entries in 2017. On the other hand, the number of transformable plant species has been largely limited to specific plant families. Among the different methods used to transfer foreign genes into the plant, the utilization of a disarmed strain of *Agrobacterium tumefaciens* is the most efficient and predictable. The protocols for *Agrobacterium*-mediated transformation of most plant species involve tedious procedures such as tissue culture and regeneration with a few exceptions such as *Arabidopsis* (Clough and Bent 1998), flax (Bastaki and Cullis 2014), *Setaria viridis* (Saha and Blumwald 2016), and Brassicales such as canola (Lu and Kang 2008), for which a simple floral dip method has been developed. Genetic transformation protocols for a number of other plant species utilize a plant tissue culture phase which requires specialized infrastructure and trained personnel (Anami et al. 2013). They also suffer from poor efficiency and thus do not allow high-throughput plant production required for most functional genomic research programs. While transient approaches such as leaf infiltration and protoplast transformation have been used for gene expression assays, they suffer from the lack of cellular context and are not suitable for biological processes lasting several days such as many root-microbe interaction studies.

Hairy Root Composite Plants as a Complementary Solution to Stable Plant Transformation

The lack of an effective transformation method is a major bottleneck for research in a number of plant species. However, this is being addressed in part by the use of *A. rhizogenes,* a close relative of *A. tumefaciens* and a naturally occurring pathogen of plants (Riker et al. 1930). Both organisms are capable of transferring T-DNAs into the plant. While *A. tumefaciens* infection results in largely unstructured galls, *A. rhizogenes* infection results in neoplastic, transformed "hairy" roots that closely resemble wild-type plant roots in cellular organization. *A. rhizogenes* possesses

root-inducing (Ri) plasmid-containing root locus (*rol*) genes *rolA*, *rolB*, and *rolC*. T-DNA derived from this plasmid is integrated into the plant genomic DNA (Chilton et al. 1982), and infected plant cells form a mass of undifferentiated cells which subsequently give rise to a "hairy root." Hairy roots have been generated from a broad range of diverse dicotyledonous plant families and even some gymnosperms.

The ability of hairy roots to grow in the absence of exogenously supplied plant hormones (unlike organ culture system) has been exploited to generate hairy root cultures, where these roots grow and branch profusely under axenic in vitro conditions. Hairy root cultures have been utilized for stable transgenic plant generation, secondary metabolite production, and root-microbe interaction studies (Georgiev et al. 2012). However, the need for in vitro maintenance and propagation conditions does not make hairy root cultures as suitable systems for a number of research questions especially root-microbe interactions involving intricate and complex signaling that occurs throughout the entire plant. For example, systemic signaling plays a key role in plant responses to nitrate (Zhang et al. 1999), autoregulation of nodulation (Delves et al. 1986), and phosphorus uptake regulation (Doerner 2008). The absence of stem tissues in hairy root cultures would make it virtually impossible to perform these studies.

Hairy root composite plants where hairy roots are induced from and left attached to shoot cuttings can address some of the concerns surrounding hairy root cultures. Both in vitro and "ex vitro" methods have been developed for the generation of composite plants consisting of a wild-type shoot with transgenic roots (Hansen et al. 1989; Collier et al. 2005). These methods are transformative especially for root-microbe interaction studies due to the reduced time required to generate transgenic plant tissues and the ability to be maintained independent of tissue culture. Research on two key plant-microbe interaction processes, nodulation and endomycorrhizal symbiosis, in legumes benefitted significantly from the adoption of composite hairy root composite plant systems for research (Boisson-Dernier et al. 2001).

An added advantage of the hairy root composite plant system is the adaptability of transformation vectors for use in stable transgenic plant generation. Therefore, candidate gene constructs can be efficiently screened by generating composite plants and can subsequently be moved directly into *A. tumefaciens* for a stable transgenic plant generation. In fact, methods for direct regeneration of stable transgenic plants from transformed hairy roots have also been developed (e.g., *M. truncatula;* Crane et al. 2006). This chapter presents a summary of plant-microbe interaction research enabled by the use of hairy root composite plant systems, highlighting key applications and discussing future potential. The goal here is not to present a comprehensive overview of discoveries made using hairy root composite plants but rather provide a snapshot using key examples of studies on arbuscular mycorrhizal (AM) symbiosis, legume nodulation, pathogenic interactions, and microbiome discovery.

Hairy Root Composite Plant Systems in Root-Microbe Interaction Research

Arbuscular Mycorrhizal Symbiosis

AM symbiosis is the most widespread association between plants and fungi (reviewed by Parniske 2008). Approximately 65% of all land plant species are capable of establishing a mutualistic interaction with the exclusively subterranean fungi of the phylum *Glomeromycota*. AM interactions appear to have evolved ~450 million years ago and might have played a key role in land colonization by plants. AM fungi are obligate biotrophs that depend entirely on carbon provided by a host plant for fungal metabolism and reproduction. The obligate symbiotic nature of these fungi necessitates cocultivation of the fungus with the plant both for maintenance and evaluation of plant-AM fungal interaction for research. The key plant rhizosphere signal that potentiates AM fungal colonization is the plant hormone strigolactone. AM fungi respond by producing chitin oligomers which are perceived by specific plant receptors. A signal transduction pathway including the receptor-like kinase SYMRK/DMI2, nuclear envelope-localized cation channels, the nuclear membrane calcium pump MCA8, and components of the nucleopore complex initiates calcium oscillations in the nucleus. These oscillations are decoded by a nuclear localized calcium and calmodulin dependent kinase CCamK/DMI3 together with its interacting partner IPD3/CYCLOPS. Subsequent activation of several transcription factors including NSP1, NSP2, and RAM1 activates gene expression and prepares the plant cells for colonization through the formation of the pre-penetration apparatus. Upon reaching primed cortical cells, AM hyphae form highly branched structures named arbuscules that facilitate the exchange of nutrients (reviewed by Gobbato 2015).

Root organ culture methods were instrumental in “clean” inoculum production for AM fungal research. While it was possible to get AM fungi to colonize excised and cultured roots, the maintenance of the coculture was not optimal as the roots detached from the plant require specific nutrient conditions and microenviornments for continued growth and branching. The ability of hairy roots to grow and branch in the absence of exogenous hormones was exploited by Mugnier and Mosses (1987) for cocultivation of AM fungi with plant roots. This hairy root culture system has been successfully used to advance research on plant-AM fungal interactions including studies on nutrient exchange, gene discovery, and functional analysis. However, it suffers from the fact that these roots are detached from the plant and may not truly reflect native physiological conditions. Composite plants on the other hand more closely reflect native conditions and have been successfully used for gene regulation assays (promoter element discovery), protein localization, and loss of function assays in the plant host or AM fungi.

Gene Regulation and Intracellular Markers

One of the most transformational uses of hairy root composite plants in AM symbiosis research is the development of transgenic root systems expressing marker genes. This enabled the identification of plant responses at the earliest stages of mycorrhizal colonization. *MtENOD11/12* genes are expressed in arbuscule-containing cortical cells of plants colonized by *Glomus* (Journet et al. 2001). Therefore, hairy root explants expressing the *pMtENOD11-gusA* fusion were generated and used to easily identify sites of AM fungal hyphal penetration in epidermal and cortical cells (Chabaud et al. 2002). Another landmark study was evaluated in vivo cellular dynamics within *M. truncatula* root epidermal cells using green fluorescent protein labeling of the microtubular cytoskeleton, actin filaments, and ER (Genre et al. 2005). Real-time imaging coupled with GFP tagging of cytoskeletal/ER components has revealed a complex multistep host response that precedes fungal entry. The plant cell synthesizes a transcellular apoplastic compartment that separates the penetrating hypha from the host cytoplasm. This novel structure comprising microtubules, microfilaments, and ER is assembled within a column of cytoplasm created during the progressive migration of the nucleus across the epidermal cell and defines the future path taken by the infection hyphae. An added resource developed later was a set of fluorescent protein fusions that label the nucleus, endoplasmic reticulum, Golgi apparatus, *trans*-Golgi network, plasma membrane, apoplast, late endosome/multivesicular bodies, transitory late endosome/tonoplast, tonoplast, plastids, mitochondria, peroxisomes, autophagosomes, plasmodesmata, actin, microtubules, periarbuscular membrane, and periarbuscular apoplastic space (Ivanov and Harrison 2014). These markers were expressed from the constitutive *AtUBQ10* promoter or the AM symbiosis-specific *MtBCP1* promoter to enable tracking of these cellular organelles/structures during AM fungal symbiosis or other processes. These resources have been validated in *M. truncatula* using hairy root composite plant system and should be easily adaptable for use in other species. The use of these markers to investigate AM symbiosis revealed that root cells undergo major cellular alterations in the nuclei, cytoskeleton, tonoplast, and plastids to accommodate their fungal endosymbiont. Other key examples of gene regulation discoveries made using hairy root composite plants include detailed promoter analysis studies of the *VfLb29* leghemoglobin gene promoter using a transcription fusion in transgenic *Vicia faba* and *M. truncatula* roots (Vieweg et al. 2004; Genre et al. 2005) and localization of phosphate transporter genes to periarbuscular membranes in soybean (Tamura et al. 2012) and *M. truncatula* (Pumplin and Harrison 2009).

Gene Function Discovery

Hairy root composite plant systems also enabled gene function discovery through loss or gain of function assays. An example of a comprehensive use of hairy root composite plants for gene function discovery in AM symbiosis is the use of RNAi, transcriptional fusions, and translational fusions to evaluate the role of *Vapyrin*, an

AM fungi-responsive gene in *M. truncatula* (Pumplin et al. 2010). Evaluation of AM fungal colonization in Vapyrin RNAi roots demonstrated that it is essential for arbuscule formation and efficient for epidermal penetration by AM fungi; promoter-GUS fusions showed that *Vapyrin* is induced transiently in the epidermis coincident with hyphal penetration and then in the cortex during arbuscule formation. Translational fusions demonstrated that the Vapyrin protein is cytoplasmic and that it accumulates in small puncta that move through the cytoplasm in cells containing AM fungal hyphae. An example of the use of gene-specific silencing in hairy roots to evaluate the function of closely related genes came from soybean (Indrasumunar et al. 2015). A leucine-rich repeat (LRR) receptor kinase (SymRK; also termed NORK) is required by legumes to establish a root endosymbiosis with Rhizobium bacteria as well as mycorrhizal fungi. Soybean has duplicated SymRK homeologues, but no mutants for these genes are available in soybean. Specific *GmSymRKβ* gene silencing resulted in a larger reduction of nodulation and mycorrhizal infection compared to that of *GmSymRKα*, suggesting it has the major activity of the duplicated gene pair. Other key examples include the discovery of the role in AM symbiosis for an ubiquitin-like protein that interacts with the symbiotic CCaMK (Kang et al. 2011), a carotenoid cleavage dioxygenase (Floss and Walter 2009), and a phosphate transporter (Maeda et al. 2006) in *Lotus japonicus*.

While the above studies are examples of reverse genetics to evaluate signaling components, such an approach has been used for metabolic engineering to determine the roles of specific enzymes/metabolites in AM fungal symbiosis. Colonization by AM fungi induces the accumulation of certain apocarotenoids in *M. truncatula*. Two isoforms of 1-deoxy-D-xylulose 5-phosphate synthase (DXS1 and DXS2) are crucial for this metabolic pathway, but only one of the isoforms (DXS2) is associated with AM fungal symbiosis. Specific silencing of MtDXS2 revealed that downstream isoprenoid products of this gene are crucial to sustain mycorrhizal functionality at later stages of the symbiosis (Floss et al. 2008).

Hormone Responses

The ability of hairy roots to grow independent of exogenous hormones is primarily due to their capacity to synthesize auxin and cytokinin (Cardarelli et al. 1987). While the levels of auxin in hairy roots are comparable to that in wild-type roots, they were reported to be more sensitive to auxin (Shen et al. 1988). This typically raises a concern about the suitability of hairy root composite plants to study hormone biology. However, a number of studies have successfully demonstrated that with appropriate controls, they can be used for hormone biology studies. Indeed, the role of auxin in the symbiotic interaction between *M. truncatula* and the AM fungus *Glomus intraradices* (recently named as *Rhizophagus irregularis*) was evaluated recently through the use of the synthetic auxin response marker DR5:GUS and alteration of auxin perception (Etemadi et al. 2014). DR5:GUS was preferentially expressed in root cells containing arbuscules suggesting that auxin activity might be crucial for cellular colonization of AM fungi and/or arbuscule formation. In

agreement, downregulation of auxin receptor genes through overexpression of microRNA393 (miR393) led to underdeveloped arbuscules in three different plant species.

During the establishment of AM symbiosis, an endogenous increase in jasmonic acid (JA) occurs. Enhanced expression of two full-length cDNAs coding for the JA-biosynthetic enzyme allene oxide cyclase from *M. truncatula* was observed during mycorrhization with *G. intraradices*. Antisense-mediated suppression of MtAOC expression in hairy roots resulted in lower JA levels and a remarkable delay in colonization with *G. intraradices* (Isayenkov et al. 2005). These roots had decreased number of arbuscules, but their structure was not altered indicating a crucial role for JA in the establishment of AM symbiosis.

Another interesting example is the manipulation of reactive oxygen species (ROS) generated by respiratory burst oxidative homologs (*Rboh*s) in common bean. Downregulation of *RbohB* in *Phaseolus vulgaris* was shown to suppress ROS production and abolish *Rhizobium* infection thread (IT) progression but also to enhance AM fungal colonization. On the other hand, overexpression of *RbohB* significantly enhanced ROS production, enhanced nitrogen fixation, and delayed nodule senescence but impaired AM fungal colonization (Arthikala et al. 2014, 2015).

Host-Induced Gene Silencing

The most exciting application of hairy root composite plant systems in AM fungal research is the use of host-induced gene silencing (HIGS), which causes RNA interference in the fungus using a transgenic host plant (Helber et al. 2011). The lack of suitable methods to transform AM fungi makes HIGS an excellent approach for loss of function studies in the fungus. HIGS involves the transformation of the host plant with a silencing construct targeted to a fungal gene, and this method was used to successfully knock down the expression of the target gene in the AM fungus, *Rhizophagus irregularis* (formerly *Glomus intraradices*). Eleven AM fungal genes predicted to encode secreted proteins were inducible both by treatment with the plant hormone and strigolactone and during symbiosis. An RNAi construct designed to specifically target one of these genes (SIS1) was expressed in hairy roots of *M. truncatula*, resulting in significant suppression of SIS1 expression in the intraradical mycelium indicating successful HIGS. Silencing of SIS1 led to reduced colonization and formation of stunted arbuscules suggesting a crucial role for this AM fungal gene in symbiosis (Tsuzuki et al. 2016).

Nodulation

Root nodules are specialized nitrogen-fixing structures in roots of leguminous plants. They result from a well-coordinated symbiotic association between plants and rhizobia bacteria. Nodules are classified into two major types based on

meristem persistency: indeterminate and determinate (reviewed by Hirsch 1992). Indeterminate nodules are oblong and possess a persistent nodule meristem analogous to lateral roots. Examples of plants that form indeterminate nodules include temperate legumes, viz., pea, *M. truncatula*, and clover. In contrast, determinate nodules are spherical and lack a nodule meristem. Examples of plants producing determinate nodules include tropical legumes, viz., soybean, common bean, and *L. japonicus*. Despite these differences, most of the signaling elements identified so far are conserved between the two types of nodules.

The interaction between the symbiotic partners starts with the exchange of chemical signals. Legumes release specific flavonoids (a group of small phenolic compounds) as signal molecules into the soil. Perception of these compounds by compatible rhizobia bacteria leads to the production of specific lipochitooligosaccharide (LCO) bacterial signals (reviewed by Cooper 2007). LCOs from compatible rhizobia are in turn perceived by the host legumes through a receptor complex, comprised of a leucine-rich repeat receptor (e.g., *MtDMI2/LjSymRK*) and LysM receptor kinases (e.g., *LjNFR1, MtLYK3, LjNFR5, MtNFP*). A signal transduction pathway that includes an E3 ubiquitin ligase (e.g., MtPUB1), membrane microdomain-associated proteins (e.g., *MtFLOT2, MtSYMREM1*), cation channels (e.g., *LjCASTOR* and *LjPOLLUX/MtDMI1*), and nucleoporins (e.g., *LjNUP85* and *LjNUP133)* transduces the signal to the nucleus in the form of organized calcium spikes. Decoding of these signals by a CaCMK (MtDMI3/LjCCaMK) and a nuclear-localized coil-coil protein (MtIPD3/LjCYCLOPS) leads to activation of relevant transcription factors and gene expression. Within hours of LCO perception, the root hairs are deformed, and transcription of nodulation-specific genes begins in the root cells. Transcriptional regulation of these genes is mediated by transcription factors that belong to NIN, GRAS, NF-YA, and ERF families. Cells within the pericycle and cortical layers of the root initiate processes for cell division by ~24 h after LCO perception. By 48 h, the root hairs curl tightly to entrap the bacteria, and "infection threads" formed through invagination of the infected root hairs subsequently transport the bacteria to the dividing cortical cells. Bacteria colonize these nodule primordia cells and differentiate into membrane-enclosed bacteroids, and a mature nitrogen-fixing nodule forms in 2–3-week period (reviewed by Oldroyd 2013; Kang et al. 2016).

Gene Regulation and Cellular Localization of Gene Products

Rhizobial inoculation of hairy root cultures does not result in nodule formation. Nevertheless, hairy root composite plants can nodulate successfully and efficiently as wild-type plants (Kang et al. 2016). They have been widely used for several key discoveries in nodulation research. One of the earliest and most frequent uses for hairy root composite plants in nodulation research is to evaluate gene regulation and determine sites of expression of nodule-specific and/or nodulation-associated genes. A number of researchers generated series of 5′ deletions of promoters and evaluated transcriptional GUS fusions in hairy root composite plant with the goal of

discovering *cis* elements that confer nodule-specific gene expression. Examples include the evaluation of *P. vulgaris* glutamine synthetase (Shen et al. 1992); evaluation of rice *ENOD40* in soybean (Kouchi et al. 1999); discovery of cross-species regulation of a nodule-specific cysteine protease (Vincent et al. 2000); transcriptional regulation of *V. faba ENOD12* (Frühling et al. 2000), a soybean *PEPCase* (Nakagawa et al. 2003), and *L. japonicus ENOD40* promoters (Gronlund et al. 2005); and conservation of autoregulation gene expression between soybean and Lotus (Nontachaiyapoom et al. 2007) and the *ENOD8* esterase in *M. truncatula* (Coque et al. 2008). The use of translational fusions for cellular localization of proteins involved in nodule development has only been minimally utilized compared to AM fungal symbiosis. The most likely reason is the relatively difficult microscopy procedures required for proper visualization of fusion protein constructs in nodule cells. Nevertheless, hairy root composite plants have been used to discover that a membrane microdomain protein, GmFWL1, localizes to the tip of the soybean root hair cells in response to rhizobial inoculation (Qiao et al. 2017) and that the Rho-like GTPase, LjROP6, localizes to the plasma membrane and cytoplasm during Lotus nodule development (Ke et al. 2012).

Gene Function Discovery

One of the unique uses of hairy root composite plant systems in nodulation research is to evaluate species-specific roles of lectins in determining host specificity. Legume lectin stimulates infection of roots by rhizobia. Interestingly, introduction of the pea lectin gene into white clover hairy roots enables heterologous infection and nodulation by the pea symbiont *R. leguminosarum* biovar *viciae* (Diaz et al. 1989; van Eijsden et al. 1995). Pea lectin-transformed red clover hairy roots form nodule primordium-like structures after inoculation with pea-, alfalfa-, and Lotus-specific rhizobia, which normally do not nodulate red clover. Even exogenous application of lipochitin oligosaccharides derived from a broad range of rhizobia was active resulting in induction of cortical cell divisions and cell expansion. These indicated a broadened response to oligochitin signals in the transformed roots.

While forward genetic studies have contributed significantly to our knowledge on nodulation signaling and development, duplications in legume genomes (Young and Bharti 2012) have posed issues with the use of such an approach. Reverse genetic approaches where candidate genes were specifically selected based on their expression pattern and/or putative annotation have also equally contributed to the discovery of a number of genes involved in nodulation signaling and development. These studies relied heavily on hairy root composite plant technology. Indeed, even for characterization of genes discovered via forward genetics, hairy root composite plant technology was utilized for complementation, cellular localization, and/or expression assays.

One of the earliest studies used antisense suppression of *nodulin-35*, encoding a nodule-specific uricase from *Vigna aconitifolia* (moth bean) to determine that a

reduction in ureide biosynthesis limits the availability of symbiotically fixed nitrogen to the plant (Lee et al. 1993). This crucial experiment led to the conclusion that ureide-producing legumes developmentally control nitrogen assimilation. Similarly, antisense suppression of two small GTP-binding proteins whose cellular function is vesicular transport revealed their role in the biogenesis of the peribaceroid membrane (Cheon et al. 1993). Antisense nodules were smaller in size and showed lower nitrogenase activity. Coupled with other observations, they appear to play specific roles in the expansion of infected cells and bacteroid release. The results from this study revealed the crucial role of peribacteroid membrane in nodulation and nitrogen fixation. More recent examples where RNAi was successfully used in hairy root composite plants to determine gene function during nodule development include the discovery of roles of a phosphate transporter (Maeda et al. 2006) and a novel RING finger protein (Shimomura et al. 2006) in *L. japonicus*, an apyrase (Govindarajulu et al. 2009), a membrane microdomain protein FWL1 (Libault et al. 2010), and a lipoxygenase (Hayashi et al. 2008) in soybean. Hairy root composite plant technique has enabled discoveries in non-model species with limited genetic resources. The role of a nodule-specific cysteine protease gene in nodule senescence was discovered in the green manure legume *Astragalus sinicus* via RNAi in hairy roots (Li et al. 2008). Knockdown of a translationally controlled tumor protein (TCTP) from the woody leguminous tree *Robinia pseudoacacia* which was upregulated in the infected roots resulted in the impaired development of both nodule and root hair. Subsequent analyses revealed potential involvement of this protein in symbiotic cell differentiation and in preventing premature aging of the young nodules (Chou et al. 2016). The roles of small CLE peptides in systemic inhibition of nodulation via the nodulation autoregulation pathway were determined through overexpression of these peptides in wild-type and autoregulation mutants (Okamoto et al. 2009; Mortier et al. 2010; Lim et al. 2011).

Conserved biochemical function of the key nodulation signaling transcription factor NSP1 was identified through complementation experiments in hairy roots (Heckmann et al. 2006). Close similarities in rhizobial response phenotypes enabled the cloning of NSP1 in *L. japonicus* based on the preexisting knowledge in *M. truncatula*. In hairy root transformations, LjNSP1 and MtNSP1 complemented both *Mtnsp1-1* and *Ljnsp1-1* mutants, indicating an evolutionarily conserved biochemical function. The nodule autoregulation receptor kinase (GmNARK of soybean and HAR1 of *L. japonicus*) is essential for the systemic autoregulation. The expression patterns of a 1.7-kb *GmNARKpr::GUS* in soybean hairy roots and a 2.0-kb *LjHAR1pr::GUS* construct in stable transgenic *L. japonicus* plants were strikingly similar and localized to living cells within vascular bundles, especially phloem cells in leaves, stems, roots, and nodules. Interestingly, the same expression pattern was detected in transgenic *L. japonicus* plants carrying the *GmNARKpr::GUS* construct (Nontachaiyapoom et al. 2007). The ability of promoters from orthologous genes from soybean and *L. japonicus* to interchangeably drive phloem-specific expression suggested high evolutionary conservation of gene regulation and function.

Metabolic Engineering and Hormone Biology

The earliest signals during the establishment of symbiosis between the plant and rhizobia bacteria are the release of flavonoids by the plant roots. These compounds had also been hypothesized to play a role in regulating auxin transport during nodule development, but genetic evidence was not available. In order to evaluate the roles of flavonoids in nodule development, RNA interference in hairy root composite plants was employed to silence key enzymes involved in flavonoid biosynthesis in *M. truncatula* and soybean. Flavonoid-deficient *Medicago* roots showed increased auxin transport and were unable to initiate nodules providing the first genetic evidence for the indispensable role of flavonoids in nodulation (Wasson et al. 2006). Subsequently, key enzymes associated with specific branches of flavonoid biosynthesis were silenced to reveal that flavones and flavonols (or related compounds) have distinct, critical roles during nodulation (Zhang et al. 2009). Flavones are essential as Nod gene inducers to stimulate Nod factor production once the bacteria enter the roots, while flavonols (or related compounds) are essential, most likely, as auxin transport regulators in *M. truncatula*. Indeed, PIN auxin transporters are expressed in *Medicago* nodules, and silencing their expression results in reduced nodule formation (Huo et al. 2006). RNAi silencing of isoflavone biosynthesis in soybean also led to increased auxin transport and reduced nodulation. However, a genistein-hypersensitive *B. japonicum* mutant that can synthesize the Nod signal in the presence of very low levels of isoflavone *nod* gene inducers was able to successfully nodulate these roots (Subramanian et al. 2006). Thus, flavonoid-mediated modulation of local auxin transport at the site of rhizobial infection is crucial during indeterminate nodulation of Medicago, but not during determinate nodulation of soybean (Subramanian et al. 2007). Hairy root composite plants were crucial tools in these studies that discovered distinct key roles for flavonoids during nodulation. Recently, RNAi in hairy roots and chemical supplementation were used to demonstrate the crucial role of flavonoids in actinorhizal nodulation of *Casuarina glauca* (Abdel-Lateif et al. 2013) underscoring the use of the technique in non-model species.

Hairy root composite plants were used to evaluate auxin activity and the role of auxin sensitivity during nodule development and discover key regulatory mechanisms by which hormone balance is achieved during nodule development. Auxin-responsive marker gene expression has been observed during both determinate and indeterminate nodule initiation (Mathesius et al. 1998; Boot et al. 1999; Pacios-Bras et al. 2003; Takanashi et al. 2011), suggesting that auxin might play a key role in nodule initiation. It was subsequently shown using stable and/or hairy root transgenic plants that auxin activity is very low during nodule formation and is suppressed in the nodule infection zone during post-initiation stages of determinate nodule development (Suzaki et al. 2012; Turner et al. 2013). Enhanced sensitivity to auxin in the nodule primordium was associated with reduced sensitivity to cytokinin and resulted in reduced nodule formation (Turner et al. 2013). In agreement, exogenous auxin inhibited nodule formation in *M. truncatula* (van Noorden et al. 2006),

and conversely resistance to auxin resulted in enhanced nodule development in this species (Kuppusamy et al. 2009). A central player in regulating auxin sensitivity in soybean nodules was discovered to be microRNA160 that negatively regulates a set of repressor auxin response factor transcription factors. Hairy root composite plant system was utilized to localize miR160 activity through a fluorescence sensor and to evaluate the role of miR160 in dictating hormone sensitivities and nodule development through loss and gain of function assays and hormone rescue experiments. Results from these experiments indicated that the miR160-ARF10 signaling module dictates developmental stage-specific sensitivities to auxin and cytokinin and directs proper nodule development (Nizampatnam et al. 2015). Similar experiments demonstrated a role for miR167-ARF8 module in dictating auxin sensitivity during nodule development as well (Wang et al. 2015). These discoveries would not have been possible or would have been significantly delayed if not for the availability of hairy root composite plant technologies. This effective transformation tool was employed for a wide range of transgenic manipulations including loss or gain of gene and/or microRNA expression to alter auxin signaling, localization of microRNA activity, quantitative evaluation of marker gene expression, and co-expression of marker gene and gene modification constructs.

Other key examples where hairy root composite plants were used to discover/demonstrate the roles of hormones during nodulation include RNAi silencing of the cytokinin receptor homolog cytokinin response 1 in *M. truncatula* to demonstrate a key role for the hormone in nodulation (Gonzalez-Rizzo et al. 2006), the expression of a dominant negative abscisic acid (ABA) signaling component to identify a negative role for ABA in *Medicago* nodulation (Ding et al. 2008), the expression of a salicylate hydroxylase to reduce endogenous salicylic acid levels to discover a negative role for this hormone in rhizobial infection and nodulation in *Medicago* and *Lotus* (Stacey et al. 2006), and the expression of cytokinin oxidase to reduced cytokinin levels and demonstrate opposite roles for the hormone in lateral root formation and nematode and rhizobial symbiosis in *Lotus* (Lohar et al. 2004). While not exhaustive, the above list provides a snapshot of the range of different approaches enabled by the use of hairy root composite plant technology to discover the roles of hormones during nodule development. It is worth noting that in the majority of these studies, most hormone-associated phenotypes have been reproducible in hairy roots albeit their slightly altered sensitivity to auxin and cytokinin. For example, the expression of the synthetic auxin marker DR5 in soybean hairy roots (Turner et al. 2013) closely resembles that in root tips and nodules of stable transgenic *L. japonicus* (Suzaki et al. 2012). Reduction in cytokinin levels by the expression of a cytokinin oxidase in *Lotus* hairy roots resulted in increased lateral root numbers as observed in stable transgenic *Arabidopsis* (Lohar et al. 2004). Therefore, despite slightly altered sensitivity to plant hormones, hairy roots appear to be useful tools in studying hormone biology.

Resources for Nodulation Studies in Non-model Plant Species

A number of protocols for hairy root composite plant generation as well as inoculation with AM fungi or rhizobia have been developed for different non-model species. The system developed for peanuts (*Arachis*) is crucial as it enables experiments to evaluate the nonclassical features associated with its nodulation (Sinharoy et al. 2009). Similarly, hairy root generation methods were developed for *Discaria trinervis*, an actinorhizal plant (belonging to the Rosales order). *Frankia* is able to efficiently nodulate *D. trinervis* transgenic roots and nitrogen fixation rates, and feedback inhibition of nodule formation by nitrogen was similar in control and composite plants (Imanishi et al. 2011). In fact, the *MtENOD11*, a marker for early infection-related symbiotic events in model legumes, was expressed in infection zones in root cortex and in the parenchyma of the developing nodule in *D. trinervis* transgenic roots. The unique intercellular infection described in this species can be studied in detail because of hairy root composite plant technology. However, it should be noted that in *Elaeagnus angustifolia* (Russian olive), hairy roots produced unusual pseudoactinorhizal structures that appeared similar to those produced by Frankia (Berg et al. 1992). Therefore, careful experimental design, e.g., uninoculated controls, and thorough evaluation might be needed when adopting hairy root composite plant systems for new plant species. Pea is recalcitrant to transformation and grows poorly on plates, and these qualities have hampered molecular research despite the availability of a number of mutants. A transformation technique using hairy roots and methods for rhizobial inoculation to study early cellular events giving rise to nodule primordia was developed for pea to overcome these challenges (Clemow et al. 2011). Other resources developed for model organisms but can be easily adapted for use in non-model organisms include (a) the plant transformation vector, pHairyRed, that enables high-throughput, nondestructive selection of hairy roots carrying the construct of interest (Lin et al. 2011) and (b) an RNAi system for analyses of gene function by reverse genetics (Sinharoy et al. 2015). A vector system, based on copper-controllable gene expression that provides control over place as well as time of expression of an introduced gene, is an excellent tool that enables functional studies in nodules without pleiotropic effects on root or plant growth (Mett et al. 1996). This system allowed nodule-specific conditional (copper-induced) expression of antisense constructs of aspartate aminotransferase-P2 in transgenic *L. corniculatus* plants. When expression was induced by the addition of copper ions to the plant nutrient solution, aspartate aminotransferase-P2 activity declined dramatically, and a decrease of up to 90% was observed in nodule asparagine concentration. One can envision modification of such a system for use with other inducible expression constructs.

Plant-Pathogen Interactions

Plants are under constant threat from pathogenic microorganisms that attack them, causing detrimental effects on plant health. In agriculture, this results in significant yield losses. Plants deploy pattern recognition receptors to detect microbe- or pathogen-associated molecular signatures. Successful pathogens can evade detection by secreting effector proteins that mask these molecular signatures or inhibit relevant plant signaling. Plants also possess other receptors that act largely inside the cell to detect these effectors. Successful recognition of the pathogen results in rapid and massive transcriptional reprogramming involving a number of plant transcription factors and context-specific co-regulators that are crucial for host immunity (Birkenbihl et al. 2017; Tang et al. 2017).

The majority of plant-pathogen interaction studies have been performed on non-transgenic plants' probability due to the availability of genetic variation, i.e., resistant vs. susceptible genotypes. Basic research on the discovery and understanding of mechanisms of plant resistance to pathogens has heavily utilized transgenic technology on model organisms with the assumption that they might be well conserved across species. However, hairy root composite technology has been used to study a number of species-specific pathogenic interactions on non-model species. Notable are mechanisms of plant resistance against nematodes and the roles of species-specific phytoalexin molecules in plant defense.

Plant-Nematode Interaction

A number of plant parasitic nematodes are obligate pathogens that typically require a plant host to complete their lifecycle. The ability of hairy roots to grow under axenic conditions attracted the interest of researchers as the roots can be used as a vehicle to propagate such nematodes. For example, reproduction of *Meloidogyne javanica* was compared on hairy root cultures from different plant species (Verdejo et al. 1988). While some plant species yielded more females and eggs than others, those roots that grew at moderate rates and produced many secondary roots supported the highest reproduction. The reared nematodes completed their life cycles on new transformed root cultures or greenhouse tomato plants suggesting that no alterations in pathogen biology occurred due to growth on transgenic hairy roots. Similarly, while certain nematodes have broad host specificity enabling research on model plant species, some require the use of native hosts. A gene that confers resistance to root-knot nematode was cloned from the myrobalan plum (*Prunus cerasifera*). This gene confers complete-spectrum, heat-stable, and high-level resistance to the nematode. Hairy root composite plant system was used to determine that this gene can confer the same level of resistance in a complementation assay (Claverie et al. 2011). Other examples include the overexpression of salicylic acid methyltransferase in susceptible backgrounds to confer resistance against soybean cyst nematode (Lin et al. 2013), expression of a nematode gene encoding the secreted

fatty acid- and retinol-binding protein in tomato hairy roots to identify its role in parasitism (Iberkleid et al. 2015), and overexpression of a terpene synthase gene to enhance resistance against soybean cyst nematode (SCN) (Lin et al. 2017). Even in cases where map-based cloning was used to identify candidate genes, hairy root systems were instrumental in confirming function. An excellent example is the discovery of copy number variation of three different genes in the *rhg1-b* locus of soybean, each of which contributes to resistance (Cook et al. 2012). Overexpression of the individual genes in the susceptible cultivar roots was ineffective, but overexpression of the genes together conferred enhanced SCN resistance. The experiments required the evaluation of a number of different plant transformation constructs which required a quick and efficient transformation system such as hairy roots.

Phytoalexins

Phytoalexins are low molecular mass secondary metabolites with antimicrobial activity produced by plants. Their production and/or release is typically induced in response to pathogen attack. A number of phytoalexins are of species-specific nature, and genetic studies to determine their function often necessitate the use of transgenic technology in the native species. Pisatin is an isoflavonoid phytoalexin synthesized by pea (*Pisum sativum* L.). In a pioneering study, hairy roots with reduced pisatin levels were generated by suppression of two key biosynthesis pathway genes, isoflavone reductase (IFR) which catalyzes an intermediate step in pisatin biosynthesis and (+)6a-hydroxymaackiain 3-*O*-methyltransferase which catalyzes the final step, and expression of a fungal gene encoding pisatin demethylating activity (Wu and VanEtten 2004). Hairy roots with reduced pisatin content were more susceptible, underscoring the hypothesis that phytoalexin production is a disease-resistance mechanism. Isoflavonoid derivatives have phytoalexin activity in soybean and a number of other legumes. Silencing of the key isoflavonoid biosynthesis enzyme, isoflavone synthase (IFS), or the 5′-deoxyisoflavonoid branch pathway enzyme, chalcone reductase (CHR), led to the breakdown of resistance against the root rot pathogen, *Phytophthora sojae*. Loss of resistance was accompanied by suppression of hypersensitive response (HR) cell death and suppression of cell death-associated activation of hydrogen peroxide and peroxidase. Results from these studies indicated that 5-deoxyisoflavonoids play a critical role in the establishment of cell death and race-specific resistance (Subramanian et al. 2005; Graham et al. 2007). In another study, soybean hairy root lines with suppressed expression of chalcone synthase 6 or isoflavone synthase 2 had significantly lower levels of isoflavones and their derivative coumestrol (Lozovaya et al. 2007). These roots failed to induce the production of soybean phytoalexin glyceollin, in response to the fungal pathogen *Fusarium solani* f. sp. *glycines* that causes soybean sudden death syndrome. In agreement, there was very high fungal growth on these roots indicating the importance of phytoalexin synthesis in root resistance to pathogens. Soybean hairy roots transformed with the resveratrol synthase and resveratrol oxymethyl transferase genes accumulated glycoside conjugates of the stilbenic compound

resveratrol and the related compound pterostilbene, which are normally not synthesized by soybean plants. Interestingly, the accumulation of these compounds increased their resistance to the soybean pathogen *Rhizoctonia solani* (Zernova et al. 2014). Another key example for the use of hairy root composite plants in phytoalexin research is the discovery of ATP-binding cassette (ABC) transporter located in the plasma membrane as the likely transporter of isoflavonoid phytoalexins (Banasiak et al. 2013).

A variety of novel approaches in plant-pathogen interaction research were possible because of the availability of hairy root composite plant systems. Examples include the use of *Medicago* hairy roots as a model to study the smut pathogen *Ustilago maydis* (Mazaheri-Naeini et al. 2015), discovery of the role in virulence of the *Phytophthora* effector PSR2 in soybean (Xiong et al. 2014), and evaluating pathogen-induced expression of the soybean GmaPPO12 promoter including the identification of potential regions crucial for induction (Chai et al. 2013). Another interesting approach is the expression of pathogen elicitors in plants to determine their role in pathogenesis. Such an approach has also been used in hairy root systems to induce the production and secretion of high-value plant metabolites. For example, the expression of oomycetal proteinaceous elicitor, β-cryptogein, in *Coleus blumei* hairy roots under the control of alcohol-inducible promoter caused significant decrease of soluble phenolics and rosmarinic acid in hairy root lines and increase of phenolics, rosmarinic acid, and caffeic acid in the culture medium (Vukovic et al. 2013). These data suggest that β-cryptogein might be a potential regulatory factor for phenolic secretion from the roots and can be utilized in commercial production systems for efficient extrusion into the culture medium.

Microbiome Research

Intricate coevolution does not appear to be limited to the plant host and specific microbes. Plant growth, development, and health are also influenced by interactions among members of the microbial communities and simultaneous interaction of the plant with multiple members of the community (Tkacz and Poole 2015). The influence of plant genotype and the environment on the composition and diversity of rhizosphere microbiota is subject of wide interest (Gottel et al. 2011; Bulgarelli et al. 2012; Peiffer et al. 2013; Philippot et al. 2013). In particular, how the microbiome composition is influenced by specific rhizodeposit compounds and/or rhizodeposition machinery has also been investigated (Walker et al. 2003; Bais et al. 2006). The availability of genetic mutants impaired in biosynthesis and transport of specific rhizodeposit compounds are crucial for the success of these studies (Badri et al. 2008, 2009). A number of species-specific compounds are likely to attract specific microbes that have the capacity to metabolize them as carbon source, or might serve as signal molecules to specific rhizosphere microbes (e.g., isoflavonoids in soybean). A major bottleneck in evaluating the roles of these compounds is the lack of a comprehensive collection of genetic mutants in all plant species. Hairy

root composite plants offer great potential for these studies as transgenic can be used to alter the activities of specific host genes and evaluate their roles in microbiome composition and activity.

Recently RNAi in hairy root composite plants was used to silence the biosynthesis of isoflavonoids in soybean with the goal of evaluating their role in shaping the rhizosphere microbiome (White et al. 2015, 2017). The results from these studies showed that hairy root transformation itself influenced the bacterial community structure. The most abundant phyla in proximal soils of untransformed roots were *Proteobacteria* (~79%) and *Bacteroidetes* (~8–11%) in agreement with a number of other plants. Interestingly, the abundance of *Proteobacteria* was much lower (~56–60%), whereas that of *Bacteroidetes* was higher (~16–22%) in proximal soils of hairy roots. This indicated that the hairy root transformation influenced rhizosphere bacterial communities even at the phylum level. Subsequent evaluation revealed that hairy root transformation impacted numerous bacterial families that were otherwise unaffected in proximal soils of untransformed soybean roots when compared to that of the bulk soil. However, the majority of the families (~74%) that were differentially abundant in the untransformed roots vs. bulk soil showed similar trends of differential abundance in hairy roots suggesting that these families can be successfully studied using hairy roots. Notable exceptions included *Sphingomonadaceae* which were enriched in untransformed roots but unaltered in hairy roots and *Acidobacteriaceae* whose abundance was reduced in untransformed roots but unaltered in hairy roots. Another study sought to obtain knowledge on microbiome of specific organic growing media that inhibit hairy root induction (Grunert et al. 2014a). The goal in this study was to utilize the knowledge to control hairy roots as it is a major disease in tomato. A comparison of the microbiomes of organic and inorganic growing media indicated potential competitive interactions of specific microbial families with *A. rhizogenes* (Grunert et al. 2014b) providing promising potential for disease suppression.

Recent Developments and Future Prospects

A number of root-microbe interactions result in distinct cell-type-specific responses. For example, localized responses occur in cells colonized by the microbe that are distinct from those in adjacent non-colonized cells. In root nodules, there are specific zones and cell types with distinct biological functions and gene expression patterns. Therefore, profiling gene expression and other molecular signatures at cell-type resolution has very high potential to better inform us about plant responses during their interaction with microbes. Cell-type-specific profiling is enabled by two recent methods, isolation of nuclei tagged in specific cell types (INTACT; Deal and Henikoff 2011) and translating ribosome affinity purification (TRAP; Mustroph et al. 2013; Reynoso et al. 2015). Both techniques require a well-characterized cell-type-specific promoter. With the availability of a number of promoters that respond specifically to specific microbes in colonized cells, the application of these methods

in root-microbe interactions is an exciting possibility using hairy root composite plants. In cells expressing the INTACT construct, nuclear envelope is tagged with a biotin ligase recognition sequence, and the co-expressed biotin ligase biotinylates them. This enables isolation of those tagged nuclei using streptavidin affinity purification. Therefore, if the construct were to be driven using a promoter that is specifically expressed in arbuscule-containing cells, nuclei from those cells can be isolated and molecular signatures evaluated. Similarly, cells expressing the TRAP construct have ribosomes assembled with a larger subunit protein that carries a FLAG peptide tag. These ribosomes (along with bound RNAs) can be affinity purified using an anti-FLAG antibody.

A suite of promoters that mark cell- or tissue-specific expression were developed for root development research in tomato (*Solanum lycopersicum*) using the hairy root composite plant system (Ron et al. 2014). These include promoters that drive expression in the stele, endodermis, QC and initials, phloem, maturing xylem, meristematic cortex cells, meristematic, elongating and mature cortex cells, lateral root cap and epidermal cells, and the tomato root meristem. Remarkably, the authors stated that the gene expression of reporters was indistinguishable in plants transformed by *A. tumefaciens* (when available) as compared with *A. rhizogenes*. This suggested that hairy roots are not only anatomically similar to wild-type roots but also have similar cell-type identity and developmental signaling pathways. Therefore, the use of hairy root composite plants to evaluate cell-type-specific responses to microbe interactions is very promising.

Another exciting development is the use of genome editing tools in hairy root composite plants. RNA-guided genome editing using the bacterial type II CRISPR/Cas9 system enables precise, site-specific deletions in the DNA (reviewed by Bortesi and Fischer 2015). A single-guide RNA that contains a guide sequence region of 19–22 bp that matches the target DNA sequence to be mutated guides the nuclease to the sequence-specific position on the DNA for cleavage. Sequence-specific guide RNAs can be designed against genes of interest to generate specific deletions and thus effectively generate null alleles. Hairy root composite plants have been used primarily to evaluate the efficacy of various guide RNAs and promoters to drive the components prior to investing time in generating stable transgenic plants (Jacobs et al. 2015; Michno et al. 2015; Jacobs and Martin 2016). However, currently the method suffers from relatively poor mutagenic efficiency. For example, when a sgRNA targeting SYMRK locus was expressed in *L. japonicus* hairy roots, only about 35% mutagenic efficiency was observed. The authors also evaluated two sgRNAs targeting three homologous leghemoglobin loci to obtain multigene knockouts. Only 20 out of 70 hairy root transgenic plants exhibited white nodules, with at least two leghemoglobin genes disrupted in each plant (Wang et al. 2016). Nevertheless, with improved efficiencies using more efficient nucleases (e.g., Murovec et al. 2017), and multiple guide RNAs, it might be possible to utilize CRISPR gene editing in hairy root composite plants for root-microbe interaction research.

Limitations and Considerations

Research summarized above provides a snapshot of the broad applications that have been enabled in root-microbe research because of hairy root composite plant systems. It should, however, be noted that the majority of monocotyledonous species are not or poorly amenable to *A. rhizogenes*-mediated hairy root transformation (Porter and Flores 1991). Therefore, the majority of these approaches are limited to dicotyledonous species. As discussed with some examples above, the method produces composite plants with transgenic roots and untransformed shoots. Therefore, shoots still have a "wild-type" phenotype with regard to the activity of the gene target when used for loss or gain of function assays. For example, if a particular compound is transported from the shoot to the root, silencing biosynthesis in the root alone may not be effective. The majority of procedures used for hairy root composite plant generation initiate multiple independent transgenic roots from the shoot explant. In many protocols, the gene cassette of interest is carried on a separate binary vector and not on the Ri plasmid. Therefore, only a portion of the hairy roots (typically 20–60%) carry the gene cassette of interest. This necessitates the use of a "visible" selectable markers (e.g., constitutively expressed fluorescent proteins) to enable separation of "non-transgenic" roots from roots useful for experimental procedures. Finally, each hairy root is an independent transgenic event, and therefore some level of variation is expected from root to root even in the same hairy root composite plant. Nevertheless, highly efficient procedures enable generation of a large number of hairy root composite plants in a small space within a relatively short period of time which can easily overcome a number of these limitations.

Conclusions

Hairy root composite plants have been and will continue to be a transformative tool in root-microbe interaction research. The tool has enabled the implementation of a variety of transgenic research approaches in multiple plant species that do not have effective transformation systems. Of particular note are legumes that are agriculturally significant due to their high protein grains and symbiotic nitrogen fixation capacity. The ability to monitor hormone and microbe-responsive marker genes, fluorescently tag cellular compartments and organelles, localize proteins of interest, and manipulate gene and microRNA activities and complementation assays for confirmation of gene function and determination of evolutionary conservation are key applications that have pushed the frontier of knowledge on legume-microbe interactions. Limitations of hairy root composite plant systems can be easily overcome with careful experiment design taking into consideration specific limitations such as the need for a visible selection marker, root-to-root variability, and an altered microbiome. Recent developments such as conservation of cell-type-specific markers and

the application of genome-editing tools are exciting opportunities that would enhance the utility of hairy root composite plants for discoveries in root-microbe interaction research.

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