Interactions of Meristem-Associated Endophytic Bacteria

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Abstract

Generally, all endophytes should be considered as a community that interacts with other symbiotic organisms, such as mycorrhiza. Even though an endophyte may colonize the plant systematically, communities colonizing the plant shoots normally differ to a degree from the root-associated endophytes. Meristem-associated shoot endophytic bacteria are often found as contaminants in plant tissue cultures started from shoot tips (buds) or embryos. Whereas root endophytic bacteria are reasonably well studied with respect to location and interactions with the host, not much is known about endophytes associated with shoot meristems. Endophytic bacteria have been localized in the meristematic tissues of buds and flowers by in situ hybridization and transmission electron microscopy. Meristem-associated endophytes may share some growth-promoting traits with the root endophytes, but likely additional mechanisms of actions exist. For example, such endophytes can produce adenine derivatives that induce growth of the host tissue. These endophytes may also affect the plant development by various ways. Some of them can co-synthesize secondary metabolites together with the plant host. Many more mechanisms remain to be determined by methods such as genomics and metabolomics, which are valuable tools for characterizing the interactions between the plant and endophytic bacteria.

1 Introduction

The studies on endophytic bacteria are often done on the plant root tissues (Rosenblueth and Martinez-Romero 2006). However, the root-associated communities typically differ from the shoot-associated ones on their diversity and function (Moore et al. 2006; Mano et al. 2006, 2007;

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Izumi et al. 2008; Yrjälä et al. 2010; Compant et al. 2011). The study by Yrjälä et al. (2010) on hybrid aspen seedlings showed that the most frequently cultured leaf endophyte was *Methylobacterium fujisawaense*, whereas the roots mainly contained bacterial species of *Burkholderia fungorum, Pseudomonas koreensis*, and *Rahnella aquatilis*. Izumi et al. (2008) compared the endophytic populations of pine, birch, and rowan in the below- and aboveground tissues using cultivation-dependent and cultivation-independent analyses. They found a clear difference between the bacterial communities and also showed that a higher number of strains are found in the roots than in the stem and leaf tissues, whereas there was no difference between stem and leaf communities. Cultivation-dependent analyses of grape vine (Compant et al. 2011) and rice (Mano et al. 2006, 2007) have given similar results. In this chapter, the shoot tissues, especially the meristematic tissues in shoot tips (buds), flowers, seeds, and seedlings, are discussed with respect to endophytic bacteria and their interactions with the plant host, possibly affecting plant growth and development. A number of growth-promoting traits are shared between epiphytes and endophytes, as some of the species do occupy both niches. However, most endophytes inhabit only the specific niche of the plant interior (Izumi et al. 2008; Yrjälä et al. 2010), and more than likely, they have specific traits and roles within the plant tissue. In this chapter, we discuss the role of bacterial endophytes in the plant shoot tissues in the light of the most recent discoveries.

2 Plant Shoot-Associated Endophytes

The endophytic bacteria of shoot tissues are often isolated from plant tissue cultures, which are started from the meristems of the shoot tips, or seed embryos. For example, endophytic bacteria have been detected in the tissue cultures of papaya (Thomas et al. 2007), banana (Thomas et al. 2008), hazelnut (Reed et al. 1998), sour cherry (Kamoun et al. 1998), various species of poplar, larch, black locust and Norway spruce (Van Aken et al. 2004; Ulrich et al. 2008), and Scots pine (Laukkanen et al. 2000; Pirttilä et al. 2000). The range of bacterial species isolated from plant tissue cultures is wide, *Paenibacillus, Bacillus, Pseudomonas*, and *Methylobacterium* probably being the most commonly reported genera (Pirttilä et al. 2000; Ulrich et al. 2008).

2.1 Shoot Tissues as a Niche for Endophytic Bacteria

Compared to roots, plant shoot tissues are exposed to UV radiation, rapidly fluctuating temperatures and alternations in relative humidity. Shoot tissues contain more methanol, as methanol is mostly produced by the shoot tissues, contributing to methanol emissions to the atmosphere (Nemecek-Marshall et al. 1995). When exogenously applied to shoots, methanol induces plant growth, whereas root application results in toxic effects for the plant (Ramírez et al. 2006). Another significant difference between the shoot and root tissues as a niche for endophytes is photosynthesis, which exclusively occurs in the shoots. The few studies performed suggest that photosynthetic products are not consumed by endophytic bacteria, neither is photosynthetic efficiency affected by them. For example, the poplar endophyte *Enterobacter* sp. 638 has no effect on photosynthesis, stomatal conductance, photosynthetic water use efficiency or the maximum and operating efficiency of photosystem II (Rogers et al. 2012). Another example is the endophyte *Methylobacterium extorquens* DSM13060, isolated from shoot tips of Scots pine, which cannot utilize glucose or fructose as the energy source (Pirttilä et al. 2000). It is not well understood how the endophytes of shoot tissues enter the plant. Likely, some strains enter from the leaf surface through the epiderm or stomatal cells. In this case, their origin would be the water or air (wind). A number of shoot endophytes can be vertically transmitted, that is, through the seeds, although this has not exclusively been proved. Endophytes have been isolated from the seeds and even pollen (Cankar et al. 2005;

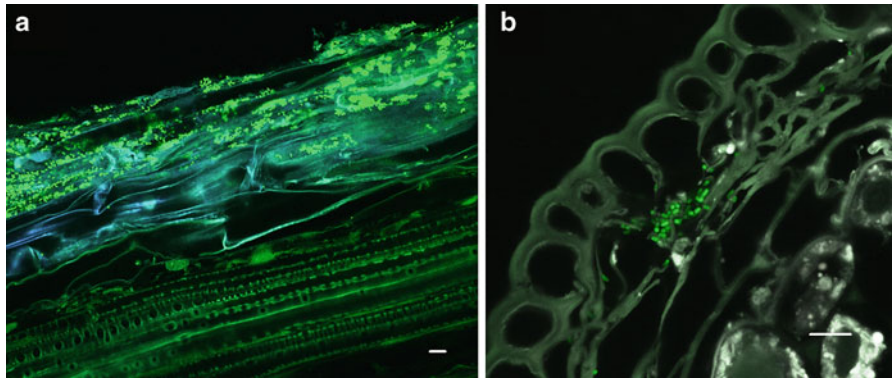

Fig. 5.1 Colonization of Scots pine seedling by GFP-tagged *Methylobacterium extorquens* DSM13060. (**a**) A longitudinal section of the pine root epiderm and cortex highly colonized by the bacteria 12 days after inoculation. (**b**) A cross section of the shoot, showing bacteria inside the cortex tissue 7 months after inoculation (scale bar = 10 μm)

Madmony et al. 2005; Pirttilä 2011), and a seed-inoculated endophyte has been shown to colonize the seedling tissues in *Eucalyptus* (Ferreira et al. 2008). A third likely source is the soil. When endophytes colonize plant shoots through the roots, they need to find a way to transfer further to the shoot tissues, and xylem has been proposed in several studies as the means of transportation after the first discovery of bacteria inhabiting xylem vessels (Bell et al. 1995). Our recent studies on colonization of Scots pine seedlings by the GFP-tagged *M. extorquens* DSM13060 indicate that all three routes can occur (Fig. 5.1; Koskimäki et al. unpublished).

2.2 Detection and Localization of Endophytic Bacteria in Shoot Meristematic Tissues

The traditional methods developed for the detection of endophytes relied on techniques dependent on plating of the bacteria. For example, surface-sterilized plant tissue was plated and the colonies growing on the medium after a specific incubation time were studied further. The endophytic bacteria associated with meristematic tissues were often isolated from plant tissue cultures, which had been started from surface-sterilized plant material. As a result, only cultivable strains were typically studied further, and the methods were also selective for species that preferred the growth conditions used. However, most endophytes are likely not cultivable (Koskimäki et al. 2010; Tejesvi et al. 2010) and a higher number of endophytes have been found by culture-independent methods than by culture-dependent ones (Yang et al. 2001; Podolich et al. 2007; Tejesvi et al. 2010; Yashiro et al. 2011). Therefore, culture-independent techniques, such as in situ hybridization (Pirttilä et al. 2000), and PCR-based methods, for example, denaturing gradient gel electrophoresis (DGGE) (Yang et al. 2001; Izumi et al. 2008), restriction fragment length polymorphism (RFLP) (Ardanov et al. 2012), and direct sequencing (Koskimäki et al. 2010), have been developed and applied for the study of single endophytic bacterial strains or whole communities. However, the methods based on amplification of bacterial 16S rDNA are often hampered by the similarity between bacterial, plant mitochondrial, and chloroplast sequences and need careful designing of primers specific for the bacteria (Sessitsch et al. 2002; Ardanov et al. 2012). Endophytes can be localized in the plant tissue by various microscopic methods. Transmission electron microscopy (TEM) enables very high magnification of the plant tissue and study of the location of bacteria in the cellular compartments, although distinguishing the bacterial cells in the sample requires specific expertise. Another weakness of the method is that TEM gives no information on the species of the

endophytic organism. By TEM, endophytic bacteria have been detected in ultrathin sections of buds of linden (*Tilia cordata* L.) and needles of blue spruce (Doronina et al. 2004; Pirttilä et al. 2008).

In situ hybridization can be used for localization of endophytic bacteria by species, genus, class, or phylum. Pirttilä et al. (2000, 2003) developed oligonucleotide probes to detect endophytes in pine tissues. Using probes specific for eubacteria, *Methylobacterium* spp., a *Pseudomonas fluorescens* subgroup, and *Mycobacterium* spp., the corresponding endophytes were identified in the cells of scale primordia, the meristems, and around the resin ducts of Scots pine buds (Pirttilä et al. 2000, 2003, 2005) and in the cells of growing callus culture (Pirttilä et al. 2002). The advantage of using the in situ hybridization method is that besides localizing the microbes, it reflects the changes in the metabolic activity of the microbes when the probes are hybridized to transcripts such as ribosomal RNA (DeLong et al. 1989). Therefore, the location and metabolic activity of endophytes in the Scots pine shoot tips were dependent on the growth season when studied by in situ hybridization throughout the year. Endophytes were not detected at all during pine dormancy and rarely found in the elongating shoot tips during growth season. The highest endophytic metabolic rates were detected in tissues of spring and autumn, prior to growth or differentiation of the bud (Pirttilä et al. 2005). In addition to buds, endophytic bacteria are detected in reproductive organs. Madmony et al. (2005) isolated *Enterobacter cloacae* from pollen and fertilized ovules of different *Pinus* sp., and Pirttilä (2011) detected endophytes in inflorescences and seed embryos of *Pinus sylvestris*. Bacteria in the genera *Pseudomonas* and *Rahnella* were found in seeds of Norway spruce (Cankar et al. 2005). Furthermore, several endophytic bacterial species in the taxa *Gammaproteobacteria* (relatives of *Pseudomonas* sp.) and *Firmicutes* (relatives of *Bacillus pumilus* and *B. cereus* group members) have been isolated from flowers, fruits, and seeds of grapevine (Compant et al. 2011). In another recent study, species of *Kocuria*, *Acinetobacter*, *Enterobacter*, and *Staphylococcus* were isolated from seeds, endocarp, and mesocarp of different *Carica papaya* variety fruits (Krishnan et al. 2012). Johnston-Monje and Raizada (2011) studied recently the endophytic microbes in the seeds of various *Zea* sp. and by culture-independent methods identified *Clostridium* and *Paenibacillus* spp., and by culturing, bacteria in the genera *Enterobacter*, *Methylobacterium*, *Pantoea*, and *Pseudomonas*. Molecular methods provide additional tools for studying bacterial colonization and localization. These methods have commonly been used for studying microbes in the rhizosphere. Genetic tagging of endophytic bacteria with genes encoding for fluorescent reporter proteins allows detailed monitoring of the colonization process inside the plant tissues by using laser scanning confocal microscopy (LSCM) (Poonguzhali et al. 2008; Prieto et al. 2011). Broad host-range plasmid vectors and transposon systems with stable site-directed insertions to bacterial chromosome provide several alternatives suitable for transformation of most bacterial species (Koch et al. 2001; Ramos et al. 2011). Advances in the development of novel reporter protein derivates, which are brighter and more photostable than the conventional ones, have supplied new means to overcome the extensive autofluorescence of plant tissues, which often hinders the colonization studies by LSCM (Shaner et al. 2007; Lagendijk et al. 2010). Combination of LSCM with advanced genetic tagging methods presents a valid, noninvasive alternative for complex endophyte-host interaction studies to be performed with live or fixed plant tissues. In our recent interaction study, a dual labeling strategy was used to monitor simultaneously the endophytic colonization and gene expression of *Methylobacterium extorquens* DSM13060 in Scots pine (*Pinus sylvestris* L.) seedlings. *M. extorquens* DSM13060 was tagged chromosomally with green fluorescent protein (eGFP) under constantly active promoter by using Tn5 transposon. To assess the bacterial gene activity during the endophytic lifestyle, another reporter protein "mCherry" regulated by a selected promoter region was subsequently transformed to the same bacterial strain. Activation of the mCherry reporter verified that the selected

promoter and the gene regulated by it were functioning in the endophytic conditions. At the same time, the dual reporter experiment provided detailed information about methylobacterial colonization and localization in the pine tissues (Fig. 5.1, Koskimäki et al. unpublished).

3 Interactions of Shoot Meristem-Associated Endophytes with Plant Host

Methanol present in the shoot tissues creates a good carbon source specifically for methylotrophs, which can utilize methanol and methane as the energy source (Fall 1996; Fall and Benson 1996). Because methanol is toxic for the plant, the removal by methylotrophs may already have significant benefits for the plant. Methanol applied to the plant surface increases plant shoot growth (Nonomura and Benson 1991; Ramírez et al. 2006), which suggests that methylotrophic bacteria transform methanol to products beneficial for the plant. For example, *Methylobacterium* spp. can participate in the biosynthesis of compounds commonly known as plant products (Zabetakis 1997; Koutsompogeras et al. 2007). Endophytic bacteria were recently detected in the receptacle vascular tissue and in the cells of achenes of raw strawberry. This study indicated that the biosynthesis of the strawberry flavor compounds DHMF and mesifuran is aided by the bacterial methanol dehydrogenase, as the bacterial methanol dehydrogenase and plant DMHF biosynthesis genes were localized by in situ hybridization in the same tissues or cells of the strawberry receptacle (Nasopoulou 2012). Independent of methylotrophy, many studies have reported the positive effect of shoot endophytic bacteria on tissue organogenesis and embryogenesis (Visser et al. 1994; Murthy et al. 1999; Pirttilä et al. 2004; Pohjanen et al. 2013). However, rarely specific, individual compounds are identified responsible for such effects. Phytohormones produced by endophytes are the most popular compounds suggested responsible for the morphological effects on plant host.

3.1 Endophytic Products

Production of plant growth hormones is typical for all plant-associated microbes. However, even though a microbe can produce plant growth hormones, it cannot be generalized to promote growth on all plant hosts, but the result depends on mutual interactions, as was discovered on *Solanum nigrum* endophytic bacteria (Long et al. 2008). Whereas gibberellin production can be considered a typical trait for root-associated bacteria, epiphytic and root endophytic bacteria most typically synthesize and secrete auxins (Brandl and Lindow 1996; Bastián et al. 1998; Costacurta et al. 1998; Doronina et al. 2002; Gamalero et al. 2003; Ivanova et al. 2001, 2008; Merzaeva and Shirokikh 2010). However, IAA has been identified as a product of a few endophyte species isolated from shoots. For example, the shoot endophytic *Pseudomonas stutzeri* strain producing IAA has been isolated from *Echinacea* tissue culture (Lata et al. 2006). The endophyte of poplar, *M. populi*, and the endophyte of pollen grains of *Pinus* spp., *Enterobacter cloacae*, are reported to produce IAA (Madmony et al. 2005; Taghavi et al. 2009). A number of pathogenic and beneficial plant-associated bacteria synthesize cytokinins (Akiyoshi et al. 1987; Timmusk et al. 1999; Garcia de Salamone et al. 2001). Methylotrophic epiphytic bacteria such as *Methylovorus mays* and *Methylobacterium mesophilicum* JCM 2829 also synthesize cytokinins (Ivanova et al. 2000, 2008). These results would indicate a significant role for plant growth hormones such as cytokinins in the plant growth promotion by plant-associated microbes. However, when cytokinin production and plant growth promotion were studied in the type strain *Methylobacterium extorquens* AM1, results indicated that cytokinin production might not be the factor contributing to plant growth (Koenig et al. 2002). *M. extorquens* was reported to produce tRNA-derived trans-zeatin, but when cytokinin-null (*miaA*) mutants incapable of cytokinin synthesis were generated, they stimulated germination of the heat-treated soybean seeds at the same level as the wild-type bacteria (Koenig et al. 2002).

Plant growth hormone production is not common to all endophytes, especially those associated with meristematic tissues. Even in the strains producing plant growth hormones, the levels vary greatly (Ivanova et al. 2008). These results indicate that other possibly more prominent methods of growth promotion by endophytes exist. The endophytes isolated from Scots pine shoot tips, *Methylobacterium extorquens* DSM13060 and *Pseudomonas synxantha* DSM13080, produce compounds that extend the viability and affect the morphology of callus tissues in vitro (Pirttilä et al. 2004). The most common plant growth hormones were not identified responsible for these effects, but adenine and adenine ribosides were produced by *M. extorquens* DSM13060 (Pirttilä et al. 2004). Adenine induces plant growth in tissue culture, but the mode of action is unknown (George and Sherrington 1984). Adenine riboside is the metabolite of adenine (Baumann et al. 1994) and found abundant in the vascular cambial region of *Pinus sylvestris* (Moritz and Sundberg 1996). Therefore, adenine and adenine riboside are potential plant-growth-promoting products of shoot endophytes. A trait often associated with endophytic bacteria is production of the enzyme aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme transforms the ethylene precursor ACC to ammonia and 2-oxobutanoate, preventing ethylene signaling. Ethylene is a plant hormone acting in seed germination and various stresses, such as bacterial colonization. It has been suggested that ACC deaminase increases plant growth and development in stressful conditions by decreasing plant ethylene levels (Glick 2005). For example, the root endophyte *Burkholderia phytofirmans* PsJN carries a gene encoding ACC deaminase, and inactivation of this gene results in loss of the ability to promote root elongation in canola seedlings (Sun et al. 2009). Whereas the ACC deaminase-carrying endophytes are often isolated and studied in the rhizosphere or roots, a recent study performed on cut flowers indicates that bacteria were able to colonize the shoot where ACC deaminase prolonged flowering (Ali et al. 2012). However, an analysis of sequenced endophyte genomes suggests that ACC deaminase is less important than anticipated (Frank 2011). The *Methylobacterium extorquens* DSM13060 isolated from Scots pine buds carries the gene for ACC deaminase. When activation of this gene was studied by promoter fusion with a fluorescent protein, it was rarely active during endophyte colonization of pine seedlings (Koskimäki et al. unpublished). This might indicate a smaller role of ACC deaminase in the plant shoot-colonizing endophytes. Epiphytic methylotrophs can synthesize vitamin B_{12} (Nishio et al. 1977; Ivanova et al. 2006, 2008), which has been suggested a plant-growth-promoting product of endophytes, as well (Ivanova et al. 2008). Vitamin B_{12} comprises a group of compounds that have trivalent cobalt as the cofactor. Generally, vitamin B_{12} is the coenzyme for isomerization and transmethylation reactions in the biosynthesis of compounds containing methyl groups. Enzymes requiring vitamin B_{12} as the coenzyme are found in many flowering plants that cannot synthesize vitamin B_{12} (Holland and Polacco 1994). In mosses, epiphytic methylotrophs increase the biomass, amount, length, and the degree of branching of gametophytes (Koopman and Kutschera 2005), which are also obtained by exogenously applied vitamin B_{12} (Basile et al. 1985). However, our recent reporter gene studies on the shoot endophyte *M. extorquens* DSM13060 suggest a smaller role for bacterial vitamin B_{12} production in the plant-endophyte interaction, than previously suggested (Koskimäki et al. unpublished).

3.2 Interaction Web in the Full Plant Microbiome

The interactions between various plant-associated microbes are often studied in isolated in vitro conditions using single strains. These studies are usually concentrated on the roots because of the well-known benefits of root fungal and bacterial symbionts, mycorrhiza, and rhizobia, respectively. Mutualistic interactions can be found between mycorrhizal fungi and a group of bacteria, called mycorrhizal helper bacteria (MHB; Garbaye 1994). Furthermore, interactions between different plant-growth-promoting rhizobacteria (PGPR, Bashan and de-Bashan 2005) have been shown beneficial

for the host plant (Madhaiyan et al. 2010). These microbes usually improve the growth and nutrition of the plant and, in the case of MHB, also the growth and sporulation of the fungal partner. Similarly, the mycorrhizal fungus can promote growth of the bacterial partner. For example, in *Pinus halepensis* roots, the ectomycorrhizal fungus *Suillus granulatus* improved the survival of *Pseudomonas fluorescens* in areas where the fungal colonization was the highest (Rincón et al. 2005). The interaction between microbes is often specific for the species or the strain. Studies combining epiphytic *Methylobacterium oryzae* strains with different rhizobacteria (Madhaiyan et al. 2010) or with arbuscular mycorrhiza (Kim et al. 2010) showed that the positive growth effect was dependent on the combination of microbes. Similarly, the root endophytic bacteria *Pseudomonas aeruginosa* and *Burkholderia cepacia* of oil palm were shown to act as mycorrhizal helper bacteria on two arbuscular mycorrhizal fungi, *Glomus clarum* and *Glomus intraradices*, but to exhibit antagonism on the pathogen *Ganoderma boninense* (Sundram et al. 2011). Although the microbial communities differ in the aerial parts from those of the roots (Izumi et al. 2008; Yrjälä et al. 2010) and there is a very low number of published examples of microorganisms interacting in the plant shoot tissues, a similar interaction between various members likely exists. For example, parallel to bacteria found in the hyphae of mycorrhizal fungi in the rhizosphere, Hoffman and Arnold (2010) revealed bacteria inhabiting the living hyphae of foliar endophytic fungi. Furthermore, Araújo et al. (2001) isolated several endophytic species from leaf tissues of citrus rootstocks and found that *Guignardia citricarpa*, one of the most abundant fungi among the isolates, stimulated growth of the endophytic *P. agglomerans* but had an inhibitory effect on growth of some endophytic *Bacillus* species.

Microbes can prevent or inhibit the growth of other strains by several ways. Direct growth inhibition can occur through secreted compounds, but antagonism includes also the competition for colonization sites, nutrients, and minerals (reviewed by Berg 2009). Endophytic *Bacillus subtilis* strain from the stem of the giant hogweed (*Heracleum sosnowskyi*, Manden) produces antifungal lipopeptide antibiotics and is able to protect tomato against the fungal pathogen causing tomato foot and root rot (Malfanova et al. 2011, 2012). *Bacillus mojavensis* isolated from kernels of maize is able to inhibit growth of the pathogenic fungus *Fusarium verticillioides* and reduce mycotoxin production (Bacon et al. 2001; Bacon and Hinton 1999), and a number of *B. mojavensis* strains were shown to produce a mixture of surfactins, which are toxic to several pathogens (Bacon and Hinton 2011). Another example comes from our study on shoot endophytic *Methylobacterium* sp. IMBG290, which induced resistance against the pathogen *Pectobacterium atrosepticum* in potato. The resistance was not due to produced toxins but dependent on the inoculum density of *Methylobacterium* sp., which was associated with changes in the structure of the existing, innate endophyte community. The changes correlated with resistance or susceptibility, suggesting that the whole endophytic community acted on the plant responses (Ardanov et al. 2012). Interaction between symbiotic microorganisms can also occur across various plant compartments (Novas et al. 2009; Liu et al. 2011), such as roots and shoot tips. These examples demonstrate that an endophyte strain isolated from the host plant should never be considered as an organism interacting with the plant host alone, but as a member of the full plant microbiome.

4 Conclusions

The plant shoot-colonizing bacterial endophytes are considerably less studied than bacteria living in the roots or in the rhizosphere. Due to easy access to culturable isolates in the root tissues, the great majority of studies worldwide are concentrated on root-colonizing endophytes (Rosenblueth and Martinez-Romero 2006). However, the shoot meristems can be considered one of the most important tissues of the plant, responsible for growth and development of new leaves and stems. The finding of bacterial endophytes in these tissues suggests that a balanced

interaction is essential for their proper function. How is the plant regulating the endophytes colonizing these tissues, and which role are the microbes playing in plant development? It is known that symbiotic microbes affect the development of animals (Troll et al. 2009). As endophytes have been occupying the plant interior for more than 400 million years (Krings et al. 2007), mutual evolution must have driven ways to subsist, adapt, and eventually refine the interaction to a balanced state. Development of genomic tools is effectively opening the doors to the secret world of bacterial endophytes and allowing further studies on their life inside the plant, as we have described in this chapter. Metabolomics is another tool that can provide a systemic view of the plant-microbe interaction at the level where genomics has no access (Scherling et al. 2009; Fester et al. 2011). Knowledge gained with these powerful methods will be helpful in defining the details of the plant-endophyte interaction in the plant shoot meristems.

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