Impact of Next-Generation Sequencing Technology in Plant–Microbe Interaction Study

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Abstract Next-generation sequencing (NGS) technologies have revolutionized the biological research during the last few years. Nowadays due to this high-throughput technique, it is quite easy to produce huge amount of sequencing data at low cost. In the past years, plant–microbe interaction study was not an easy task. This review will give a broad idea about the importance of NGS in plant–microbe interaction study specifically for those microorganisms which play a great role in the interaction. Due to difference in sequencing systems, it is quite tough to overcome the problem regarding different types of errors. We are emphasizing on the importance of NGS data in plant–microbe interaction including the analysis of different microbial communities (using amplicon sequencing, Cross linking and sequencing of hybrids etc.). Screened research articles which are based on plant–microbe interaction study were used here to conclude the novel methods of plant–microbe interaction.

Keywords Next generation sequencing • Plant microbiota • Amplicon sequencing • Metagenomics • Transcriptomics

1 Introduction

Plants survive in adverse environmental conditions due to the association of various microorganisms which are present below ground in the rhizosphere and above the ground in the phyllosphere. Lorenz Hiltner a German Scientist defined the term rhizosphere which means roots surrounded by soil. He discovered many important microbes which play a major role in plant growth and health. From the time of Hiltner to the present day, various researches have been done to learn about the mechanism of plant–microbe interactions (Hartmann et al. 2008; Bulgarelli et al. 2013). These beneficial microorganisms are present as endophytes residing within the plant or as epiphytes residing on plant surface or near the roots. The importance

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of rhizosphere is more than that of phyllosphere because it is not only directly involved in plant nutrition and health but also involved in the good maintenance of microorganisms. These microorganisms are involved in carbon sequestration, ecosystem functioning, and nutrient cycling in terrestrial ecosystems (Berg 2009; Newton et al. 2010).

The microbial communities associated with plants are recognized specifically based on the microenvironment posed by crops and their cultivars. Thus, plants can be categorized based on the distinct microenvironments such as endorhiza, phyllosphere, spermosphere, carposphere, etc. These microenvironments are maintained with the help of various environmental factors for specific natural life. An interesting fact which apprehensions about the microbial populations on plant system is that their survival mechanism like how they are surviving nicely although they are coming from different sources (Berg et al. 2005; Vorholt 2012). Scientists revealed this fact with the help of the dormant pathogenic bacteria which usually get colonized although they act as a good platform for seed microbiome. In contrast plants are always interacting with diverse microbes present in wind or water, and some have capacity to colonize the phyllosphere (Fürnkranz et al. 2012; Bragina et al. 2012).

Insights into the rhizosphere microorganism plant interactions could be obtained due to the advancement in molecular biology techniques as well as in bioinformatics (Hartmann et al. 2009). Using stable isotope probing (SIP) approach, Haichar et al. (2008) revealed the structures of plant–host habitat and various bacterial communities. Other good examples of work that utilized molecular biology and bioinformatics are of Lundberg and Bulgarelli individually in *Arabidopsis thaliana*, in which they identified only specific bacterial communities present in the roots of *Arabidopsis thaliana* the model plant. Two bacterial species are present in the roots of *Arabidopsis*: *Proteobacteria* which are responsible for the regulation of growth-promoting factors and *Actinobacteria* which are responsible for the production of antimicrobial compounds (Bulgarelli et al. 2012; Lundberg et al. 2012). The abovementioned workers proposed that *Arabidopsis* itself is involved in the recruitment of a group of microbes which benefit its basic functions during specific environmental conditions.

Due to the commensal lifestyle of some microbes, neither they do any damage to the plant nor do they involve in plant growth promotion. The mechanism behind plant–microbe interaction is still not completely known. So, various questions arise for plant-related microbiota such as "Who are they?," "How they are surviving their life in particular environment?," "How do they interact with plant system?," "How they are beneficial for each other?," and "How they are affecting the plant growth and development directly or indirectly?" Solving all above questions will help to understand the whole mechanism of plant–microbe interaction and also help to identify those microorganisms which can be used in the near future to increase crop yield. In agriculture, plant microbiome interactions act as a fuel to increase the yield naturally (Berg 2009). Some good examples are stress protection products, biofertilizers, biocontrol, etc. Nowadays, there are vast growing markets for these bioproducts, but they are suffering from some specific problems like unpredictable

possessions under field, short shelf life, and risk calculations. Advancement in biotechnology has played a major role for development of advanced bioproducts using "omics" approach. In this area NGS has a great influence on the (a) discovery of new possessions for biocontrol along with plant growth-promoting factors, (b) optimization of different processes, (c) stabilization of outcome under field trial, and (d) risk calculation studies.

To answer the abovementioned questions, it is necessary to mine and annotate the genes involved in the plant–microbe associations from the genomes of both partners. Whole genome study of entire microbial communities, in other words metagenomics studies, will provide insights into the composition of such communities. Information on the physiological aspects of these microbial communities vis-à-vis their association with plants can be obtained from metagenomics studies (Niedringhaus et al. 2011). Reverse genetics approaches can be used to study the metabolic activities and gene regulatory mechanisms of the microbial cells that are in association with plants. In biological research next-generation sequencing (NGS) technologies have great impact because they provide a new platform to answer all those questions which possibly could not be solved before because of financial and technical restrictions. NGS technologies have provided the opportunity for finding answers to crucial questions in plant–microbe interactions with great speed (Schadt et al. 2010).

In this present review, we have discussed the importance of NGS in plant–microbe interaction studies. We have presented an overview of the specific requirement as well as function of different types of sequencing systems including their sources of errors and biases and other important matters. Specific focus is on the advantages of NGS techniques in studying microorganisms associated with plants. This review gives a brief outlook about what the scientist community will probably study in the near future.

2 Next-Generation Sequencing (NGS) Platforms

Currently, there are different types of platforms available for NGS. Instruments used for NGS can be classified as second- and third-generation sequencing technologies (Liu et al. 2012). It is quite difficult to categorize these instruments (Pareek et al. 2011); nonetheless we summarized in Table 1 all available methods for second- and third-generation sequencing. Roche 454, Illumina, and Life Technologies instruments come under second-generation sequencing technology. The second-generation sequencing technology is based on SOLiD (the Sequencing by Oligonucleotide Ligation and Detection) and sometimes Ion Torrent sequencers (Schadt et al. 2010). By Pacific Biosciences, the PacBio RS is the only single system which is commercially available for third-generation sequencing.

Table 1 Different NGS platforms

Company name	Sequencing principle	Sequencing platforms	Method for library construction	Modifications of nucleotides	Major sequencing error	Signal identification
Illumina	Flexible sequencing by synthesis	Illumina	Bridge PCR amplification	End blocked fluorescent nucleotides	Substitutions	Optical measurement of fluorescent emission
Life Technologies (2005–2008)	Sequencing by ligation	SOLiD 4	PCR on microbeads	Two base encoded fluorescent oligonucleotides	Substitutions	Optical measurement of fluorescent emission
Life Technologies (2008–2010)	Semiconductor-based sequencing by synthesis	Ion PGM	PCR on microbeads	None	Indels	Transistor-based detection
Pacific Biosciences	Single-molecule, real-time DNA sequencing by synthesis	PacBio RS	Not applied	Phosphor (P)-linked fluorescent nucleotides	Indels	Real-time optical detection of fluorescent dye
Roche	Pyrosequencing	454 FLX titanium	PCR on microbead	None	Indels	Optical detection of light

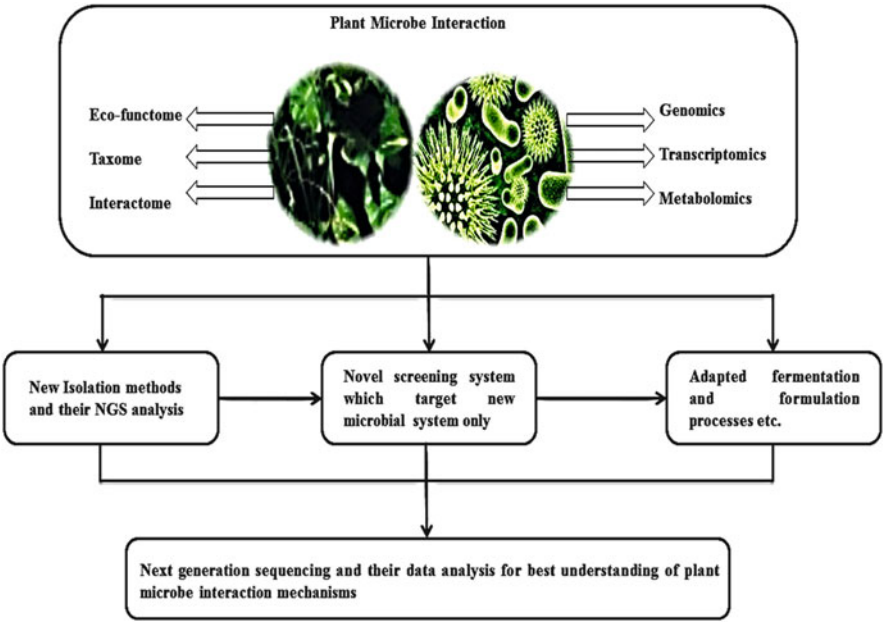

Fig. 1 Different approaches of studying plant-microbe interaction using NGS

3 Plant Microbiota and NGS

Plants' natural habitats are consisting high diversity of microorganisms. Because of good correlation between above ground and below ground, a large number of microbial diversities can be expected (Heijden et al. 1998; Thompson and Milos 2011). In agricultural systems, sometimes under intense environmental conditions, some microbes grow well and also benefited the plants. Conversely, natural ecosystems are especially well managed by mosses, plant growth-promoting bacteria, etc. Therefore, using NGS study of plants with respect to its eco-function, taxome, and interactome including the study of genomics, transcriptomics and metabolomics of microorganisms will ultimately reveal the hidden mechanism behind its interaction and survival (Fig. 1).

3.1 Shotgun Sequencing and Metagenomics Study

Till today, only few studies in metagenomics related to plant-associated microorganisms were completed using shotgun sequencing (Table 3). Currently, Roche 454 sequencing technology is mostly used for these types of studies. Recently, Mendes et al. (2014) found that the epiphytic rhizosphere microbiome is present in soybean, and they also characterized their taxonomic as well as functional

composition. Also, beneficial functions which help in plant growth and nutrition were identified by Sessitsch et al. (2012) where they used Sanger sequencing technology for metagenomics study of plant-associated microorganisms (Sessitsch et al. 2012). Unno and their co-workers found that metagenomes present in rhizosphere increased the growth of plant due to the presence of phytic acid. Some of the unique genes were identified that encode enzymes for phytic acid utilization, for example, citrate synthase (Unno and Shinano 2013). In the same year, another metagenomics study was done by Chhabra et al. (2013), where they constructed a fosmid library in *E. coli*. The major finding of their study was they screened an assay which has mineral phosphate solubilization capacity (Chhabra et al. 2013).

From various studies, metagenomics data of microbial communities are now available especially from *Arabidopsis thaliana*, clover, rice, tomato, soybean, and tamarisk (Ottesen et al. 2013). These analyses confirm the consistent nature of metaproteome of bacteria belonging to phyllospheres of various plant species (Knief et al. 2012; Vorholt 2012). Additionally, these metagenomic datasets revealed the presence of microbial community at phylum level. On the other hand, the comparative analysis of data (metagenomic and metaproteomic) between rhizosphere and phyllosphere in rice confirmed the presence of very complex microbiota and a very clear vision about metagenomic and proteomic composition (Knief et al. 2012). The analysis of phyllosphere metagenomic datasets in incorporation with other metagenomic datasets used to monitor some genes which are involved in energy generation from light, i.e., photosynthesis (Atamna-Ismaeel et al. 2012; Vorholt 2012).

Some especial kind of metagenomic project was also completed to obtain a whole sequence of plant pathogens which can't be cultured. *Candidatus Liberibacter asiaticus* which is the main causative agent of citrus huanglongbing is transmitted through phloem feeding insects. Metagenomics study is performed using 454 sequencing technology (Delmotte et al. 2009; Duan et al. 2009). Sequencing resulted as ~38 contigs which were further confirmed by PCR reactions. Complete genome data analysis exposed that there was huge reduction in its genome with respect to highly divergent member of the family *Rhizobiaceae* because of their intracellular lifestyle (Fig. 2).

3.2 Study Through Amplicon Sequencing

Nowadays, most popular method to study plant–microbe interaction is amplicon sequencing (Fig. 2). This method is mostly used to distinguish between the rhizosphere and phyllosphere communities. Roche 454 sequencing is mostly used for this purpose, but some researchers also used the Ion PGM platform or the Illumina MiSeq. The amplicon size in phyllosphere studies varied from ~1000 to 10,000 reads per sample (Yergeau et al. 2014a, b), but longer reads can also be obtained with the help of 454 FLX+ instrument (Jiang et al. 2013; Perazzolli et al. 2014). From the last few years, NGS amplicon sequencing was exclusively used for

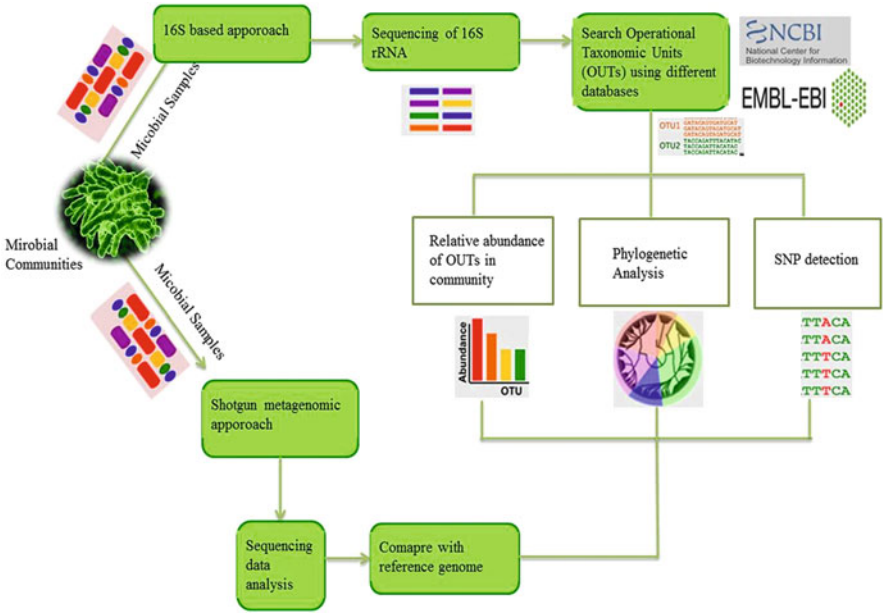

Fig. 2 Basic pipeline to study metagenomics using shotgun sequencing and amplicon sequencing for microbial communities

bacterial or fungal communities study (Kavamura et al. 2013), where the study of phyllosphere communities of bacteria based on the 16S rRNA gene and fungal communities was based on the ITS region (Bokulich et al. 2014). *chiA* is the only marker gene found by amplicon sequencing in plant microbiota (Cretoiu et al. 2012). The key objectives for this particular study were to find the diversity of this gene in different habitats.

Amplicon sequencing method is used to understand the reason of plant colonization in phyllosphere due to particular type of plant microbiota (Maignien et al. 2014). Also, the amplicon sequencing solved the numerous questions regarding plant microbiota in rhizosphere like biogeographical distribution of various microorganisms (Gottel et al. 2011; Peiffer et al. 2013), factors affecting host–plant interaction (Navarrete et al. 2013), factors affecting plant growth as well as nutrition (Lundberg et al. 2012), different soil types (Zhang et al. 2013), etc. Some scientists focused on the exploration of endomycorrhiza and ectomycorrhizal (Badri et al. 2013a, b). From various studies now it has been clear that plant plays a major role in microbiota selection (Rastogi et al. 2012; Reisberg et al. 2013); also its various mechanisms that affect the whole controlling process have been studied (Badri et al. 2009; Bodenhausen et al. 2013).

However, the importance of colonization of various plant compartments (Barriuso et al. 2010; Redford et al. 2010; Rosenzweig et al. 2012), role of specific treatments during plant cultivation like irrigation (Lumini et al. 2010; Dumbrell et al. 2011; Dohrmann et al. 2013), various aspects of bioremediation, major

impacts of herbicides, and effects of genetically modified plants in agriculture were also studied (Yu et al. 2012; Williams et al. 2013; Ottesen et al. 2013; Bell et al. 2014). All the above selected publications are indicating the importance of amplicon sequencing in the field of plant–microbial interaction study. Surely, further study will explore the other important factors related to plant–microbial interaction.

3.3 Transcriptomic and Metatranscriptomic Studies Using NGS

NGS technologies have not restricted itself only for genomics sequencing but nonetheless also performed well in transcriptomic and metatranscriptomic studies in plants (Fig. 3). In present scenario, both Illumina and 454 technologies are the most extensively used technology for plant–microbe interaction study (Thakur et al. 2013). When the whole genomes of desired organisms are not sequenced, then NGS is used to explore the whole information regarding that particular organism (Tremblay et al. 2012). Additionally, some studied showed the parallel analysis of the host and the pathogen interaction using transcriptome data (Weßling et al. 2012). These types of analysis are mostly dependent on the plant–host interaction pattern (Zhuang et al. 2012).

Recently, some metatranscriptomic studies were done using these advanced technologies. Firstly, Chaparro and co-workers studied the metatranscriptome in *Arabidopsis* plant. They studied the role of different microbial communities in rhizosphere at different plant development stages. They also found that these microbial genes were also involved in the regulation of various metabolic pathways (Chaparro et al. 2014). Similar study has been performed by Yergeau and their colleagues where they compared different composition in the rhizosphere of willow with bulk soil (soil was contaminated with organic pollutants). In this study they confirmed that various genes involved in hydrocarbon degradation also genes involved in carbon and amino acid uptake upregulated in the rhizosphere (Fernandez et al. 2012; Yergeau et al. 2014a, b).

4 Major Challenges in Sequencing of Pathogen Genomes and Metagenomes

Whole genome sequencing started only after the successful completion of small segment DNA sequencing. These advanced technologies markedly increased the area of research in phytopathogen and also in study of intra-strain diversity of a pathogen species (Mardis 2008). The major challenge lies on the genome assemblies for eukaryotic filamentous phytopathogens because of their large genome size

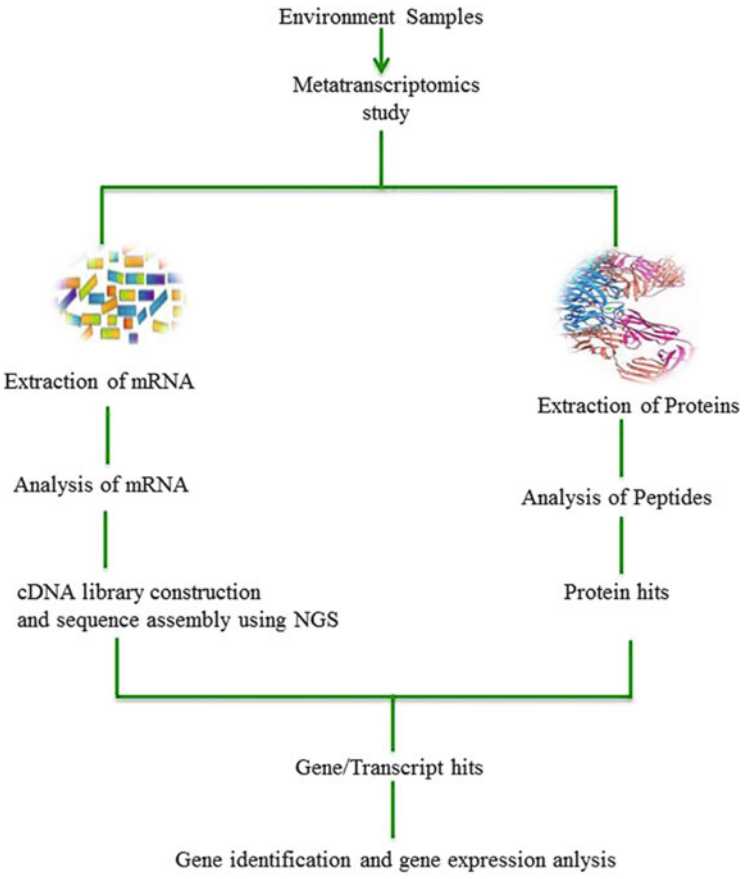

Fig. 3 Workflow for transcriptomic and metatranscriptomic study of plant-associated microbes for gene identification and expression analysis

approximately 18.7 Mb–180 Mb (Schirawski et al. 2010; Wicker et al. 2013). Their large genome size can be a reason for differences in ploidy levels also due to the presence of transposable elements (Schmidt and Panstruga 2011). Additionally, the presence of these elements causes troubles in the contigs assembly. They also cause difficulties to identify specific difference in karyotypes when they are compared to strains that differ in virulence. In spite of these challenges, scientists revealed some important mechanisms for different filamentous phytopathogens (Schmidt and Panstruga 2011). Currently, the major challenges in the study of plant microbiota are complex rhizosphere and diverse soil communities which prevent the completion of de novo assembly. Therefore, third-generation sequencing technologies such as PacBio can help to overcome these problems (Proctor 2011).

4.1 Modifications in Pattern-recognition Receptors and Their Functions

The innate immunity of plant is based on the microbe-associated molecular patterns (MAMPs) by pattern-recognition receptors (PRRs). Identification of novel PRRs is considered as less effective at the protein level because of very low abundance. A novel method called the 5C strategy was used to overcome this problem by Patrick Boyle. First of all the MAMP probe is interlinked with cognate PRR in the presence of UV rays and further both dissolved from the membrane. The chemical analysis and characterization demonstrates that this strategy is quite effective for cross-linking of the bacterial flagellin (MAMP flg22) to its PRR FLS2 and its purification (Albert et al. 2010). This methodology provided a new insight for detecting unknown interacting molecules of proteins of interest and also for the identification of novel PRRs. LRR-receptor kinases FLS2and EFR of *Arabidopsis thaliana* that detect the bacterial proteins flagellin and EF-Tu using a method based on chimeric variants (Albert and Felix 2010; Doehlemann and Hemetsberger 2013) is also one of the best example of pattern recognition receptor.

4.2 Modification of Fungi by Plant Viruses

Highly improved tool in plant biology is virus-induced gene silencing (VIGS) to know the function of genes through transient silencing. Peter and Donato confirmed the role of virus-induced gene silencing in filamentous fungi having essential mechanisms to replicate and infect in *Colletotrichum* species. Moreover, genetically modified viruses (GMV) can be used for the expression analysis of foreign genes like green fluorescent protein (GFP). GMV have been observed to be quite helpful in gene silencing experiments. As advanced techniques are very fast and effective so these techniques could be utilized for the manipulation of untransformable fungi such as biotrophic fungi (Lu et al. 2003; Mascia et al. 2014).

4.3 Gene Silencing and Epigenetics in Plants

In present scenario it has been found that heritable genetics or gene transfer from one species to another might be influenced by RNAi-mediated gene silencing. The model plant *Arabidopsis* infected with *Pseudomonas syringae* pv. tomato (PstDC3000) showed higher resistance to the pathogens. This mechanism confirmed that hypomethylated SA-dependent genes responsible for downregulation of genes; so indirectly involved to increase resistance in *Arabidopsis* (Luna et al. 2012). Parallel study has been performed in rice plants also. These results are

representing the possibility of heritable epigenetics in heritable variation within species (Stroud et al. 2013; Mascia et al. 2014).

5 Cultivation-Independent Methods for Plant Microbiota Study

In recent times, cultivation-independent methods in amalgamation through NGS are gaining new insight for analyzing arrangement and utilities of the plant-colonizing microbial communities. The sequencing data of especial genes from different microbial community containing taxonomically information regarding its DNA is known as marker gene analysis or in other words amplicon sequencing. It permits characterization of different microbial communities with respect to their relative abundances and identities. Massive sequence data are produced by marker gene sequencing. These data delivers noticeable facts related to taxonomy of different microbial communities in contrast with other profiling techniques (Nocker et al. 2007). Additionally, the metagenomics approach salvages the information contained within the whole genome of a particular microbial population through shotgun sequencing (Riesenfeld et al. 2004; Delmotte et al. 2009). Additionally, metagenomics study also allows the characterization, function analysis, and metabolic pathway involvement. Bioinformatics analysis helps to determine the possible prospective of microbial communities in corresponding metabolic pathways (Table 2). Not only metagenomics but also metatranscriptomics is playing a great role in the regulation of gene expression under different environmental conditions. Analysis mostly done by reverse transcription along with random shotgun sequencing of isolated RNA from the microbes. Further, these analyses complemented with metaproteomics or metaproteogenomics (Riesenfeld et al. 2004) which regulates the expression of various protein products under specific experimental conditions. For the completion of metaproteogenomics analysis, it needs a reference gene for protein identification (Knief et al. 2012).

6 Characterizing the Plant-Associated Microbiota

Even though numerous filamentous phytopathogen bacteria and only some of them acting like mutualists (Knief et al. 2011; Schenk et al. 2012; Knief 2014) have been studied in the research laboratory, the exact good cultivation surroundings for these plant-related bacteria and fungi are still mysterious, which inhibits their extensive study via isolate cultures (Hugenholtz 2002). The asymptomatic plants generally provide different surroundings for the survival of various plant-associated microorganisms. Different studies using NGS of these microbial habitats act as a major breakthrough in the discovery of novel taxa (Xu et al. 2012). Also they influenced

Table 2 Cultivation-independent methods for plant microbiota study

Techniques applied	Software used	Advantages	Disadvantages	Applications
Marker gene analysis	mothur, QIIME, and amplicon noise	Easy method for characterization of new and rare species	Biases during PCR amplifications	Discovery of novel species including their taxonomic profiling
Metagenomics	For assembly: IDBA-UD, Ray Meta, and MetaVelvet For profiling: MLTreeMap, AMPHORA, mOTU, and MetaPhlAn For function analysis: MG-RAST, IMG/M, and CAMERA	Unbiased profiling and it allows genomic studies of uncultured microbial species	Very lower coverage with respect to the marker gene sequencing Very low abundance Anticipated gene functions are not matched to expressed protein content	Discovery of novel species including their taxonomic profiling Also taxonomic binning and genome reconstruction as well as study of functional, metabolic potential, and evolutionary relationships
Metatranscriptomics	For de novo assembly: IDBA-MT For mapping: Bowtie2 and BWA-SW For function analysis: MG-RAST and CAMERA	Determination of novel transcripts and sensitive detection method	Presence of rRNA in samples	Study of active function and pathways analysis
Metaproteogenomics	Mascot (for protein identification); MG-RAST and CAMERA (for function)	Good estimation of functional activities with proteomics as compare to transcriptomics	Requires reference genes for protein identification	Study of active function and pathways analysis

Note: References for above discussed method Berg and Smalla (2009), Boisvert et al. (2012), Brady and Salzberg (2009), Caporaso et al. (2010), Glass et al. (2010), Kemler et al. (2013), Koopman and Carstens (2011), Leung et al. (2013), Markowitz et al. (2012), Namiki et al. (2012), Patil et al. (2011), Peng et al. (2012), Quince et al. (2011), Schloss et al. (2009), Segata et al. (2012), Shade et al. (2013), Stark et al. (2010), Sun et al. (2010), Sunagawa et al. (2013), Wu and Scott (2012)

some environmental factors for the discovery of novel effector proteins from microbial communities (Table 3).

(a) Rhizosphere

Generally roots evacuate approximately ~11–45% of photosynthetically fixed carbon, which consist of numerous carbon compounds; these carbon compounds are

Table 3 Revealing facts of plant-associated microbiota using NGS technology

Sequencing technology	Plant species and type of sequencing as well as sample	Major findings	References
Rhizosphere			
Pyrosequencing (Roche 454)	*A. thaliana* and marker genes, i.e., bacterial 16S rRNA sequencing and fungal ITS sequencing data	Role of ABC transporter mutant in different root exudate compositions from the wild type, with increased excretion of phenolic compounds and reduced sugar excretions, accompanied by higher abundances of OTUs related to beneficial rhizobacteria	Badri et al. (2009)
Pyrosequencing (Roche 454)	*A. thaliana* and bacterial 16S rRNA sequencing	Impact of soil bacteria on leaf metabolome	Badri et al. (2013a, b)
Pyrosequencing (Roche 454)	Oak and bacterial 16S rRNA sequencing data	Rhizosphere enrichment of proteo-bacteria relative to bulk soil	Uroz et al. (2010)
Pyrosequencing (Roche 454)	Sugar beet and PhyloChip (marker gene)	*Gammaproteobacteria* and *Betaproteobacteria* were enriched in soil so that it can suppress *Rhizoctonia solani* infection. On the other hand, *Pseudomonadaceae* strains protected the plant against infection. The protective mechanism of strain was lost in a mutant with a defective non-ribosomal peptide synthase gene	Mendes et al. (2011)
Pyrosequencing (Roche 454)	Maize and bacterial 16S rRNA sequencing data	Twenty-seven modern maize inbred strains were studied across five fields. The difference between field and bulk soil versus the rhizosphere accounted for most variation in diversity, and a weak genotype effect was observed within fields. *Burkholderiales*, *Oceanospirillales*, and *Sphingobacteriales* were found to be enriched relative to bulk soil, whereas *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia* were depleted	Peiffer et al. (2013)

(continued)

Table 3 (continued)

Sequencing technology	Plant species and type of sequencing as well as sample	Major findings	References
Shotgun sequencing (Roche 454)	Rice and shotgun metagenome and metaproteogenome	Three rice cultivars were studied in one field. *Alphaproteobacteria* (*Rhizobium* spp. and *Methylobacterium* spp.) and *Actinobacteria* (*Microbacterium*) dominated the phyllosphere. Methanol-based methylotrophy linked to *Methylobacterium* dominated the protein repertoire, as well as proteins linked to transport processes and stress response. In the rhizosphere, *Alphaproteobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria* were most abundant, and *Archaea* were present. There was also higher diversity. Proteins linked to methanogenesis and methanotrophy, as well as nitrogen fixation, were found	Knief et al. (2012)
454 GS Flx (Roche 454)	Wheat, oat, pea, and a sad1 oat mutant and metatranscriptome	Analyzed active rhizosphere microbiomes in soil, as well as rhizosphere for three plant species and a sad1 oat mutant that is deficient in producing antifungal avenacins. Interestingly, for the sad1 mutant, the non-fungal eukaryotic rhizosphere community was more strongly altered than the fungal community, suggesting that avenacins in vivo may have effects other than protecting from fungal pathogens	Turner et al. (2013)
Roche 454	*Glycine max* rhizosphere and bulk soil samples taken from mesocosm experiments with soil from soybean fields in Brazil	The rhizosphere community is selected from the bulk soil based on functions related to N, Fe, P, and K metabolism	Mendes et al. (2014)
Roche 454	Rhizosphere samples from greenhouse-grown *Lotus japonicus*; plants of the same age but two different developmental stages grown in presence of phytic acid	Differences in microbial community composition in the rhizosphere of the differently developed plants; identification of genes related to phytic acid utilization	Unno and Shinano (2013)

(continued)

Table 3 (continued)

Sequencing technology	Plant species and type of sequencing as well as sample	Major findings	References
Roche 454	Barley rhizosphere samples collected from an experimental field in Ireland with 15 years of barley monoculture under low-input mineral management regime	Identification of genes and operons involved in mineral phosphate solubilization in the rhizosphere	Chhabra et al. (2013)
		Phyllosphere	
Massively parallel sequencing (Roche 454)	*Quercus macrocarpa* and eukaryotic marker gene sequencing (ITS, 18S rRNA or 28S rRNA)	Different fungal communities were studied; they were found to be hyperdiverse and dominated by ascomycetes and *Alternaria*. *Epicoccum* and *Erysiphe* were the most abundant genera	Jumpponen and Jones (2009)
454 GS Flx (Roche 454)	Fifty-six tree species and bacterial 16S rRNA sequencing	Fifty-six tree species were studied in the same location except for *Pinus ponderosa*. *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, TM7, and *Firmicutes* were the most abundant	Redford et al. (2010)
Pyrosequencing (Roche 454)	*Tamarix* species and bacterial and archaeal 16S rRNA sequencing and eukaryotic marker gene sequencing (ITS, 18S rRNA or 28S rRNA)	Three *Tamarix* species (salt-secreting desert tree) were studied at four different locations. Both location and tree species determined microbial community structure	Finkel et al. (2011)
454 GS Flx (Roche 454)	Six tropical tree species and bacterial 16S rRNA sequencing	Six tree species had largely distinct microbial communities in the same location, with ~3–8% overlap of OTUs between species and ~10–18% overlap of OTUs within species. *Alphaproteobacteria*, *Gammaproteobacteria*, and *Acidobacteria* were abundant	Kim et al. (2012)
454 GS Flx (Roche 454)	Lettuce and bacterial 16S rRNA sequencing	Core genera of phyllosphere microbiota across 44 fields consisting of *Pseudomonas*, *Bacillus*, *Massilia*, *Arthrobacter*, and *Pantoea* species were studied	Rastogi et al. (2012)

(continued)

Table 3 (continued)

Sequencing technology	Plant species and type of sequencing as well as sample	Major findings	References
Shotgun sequencing (Roche 454)	Clover, soybean, and *A. thaliana* and shotgun metagenome and metaproteogenome	Alphaproteobacterial genera (*Sphingomonas* and *Methylobacterium*) were studied. For *Methylobacterium* spp., multiple proteins related to methanol-based methylotrophy were detected; for *Sphingomonas* spp., many proteins related to carbohydrate uptake were found	Delmotte et al. (2009)
Illumina MiSeq	Samples from *Salmonella* enrichment cultures from outdoor-grown tomato (*Solanum lycopersicum*) and tomato leaves and fruits	Differences in metagenomic composition of replicate phyllosphere enrichment cultures; enrichment of *Paenibacillus* on *Salmonella*-selective media	Ottesen et al. (2013)
Roche 454	Leaf samples from field-grown soybean (*G. max*), Switzerland	High consistency in the microbial community composition and their proteomes on different host plants	Delmotte et al. (2009)
Roche 454	Psyllid infected with the endophyte "*Candidatus Liberibacter asiaticus*"	Complete genome sequence of the uncultured plant pathogen and insect symbiont "*Candidatus Liberibacter asiaticus*"	Duan et al. (2009)
		Endosphere	
Pyrosequencing (Roche 454)	Cottonwood trees and 16S rRNA sequencing and eukaryotic marker gene sequencing (ITS, 18S rRNA, or 28S rRNA)	The rhizosphere was enriched with *Acidobacteria* (~31%) and *Alphaproteobacteria* (~30%) relative to the endosphere. Most endophytes were *Gammaproteobacteria* (~54%) or *Alphaproteobacteria* (~23%). *Pezizomycotina* and *Agarimycotina* were abundant in both the rhizosphere and the endosphere	Gottel et al. (2011)
Pyrosequencing (Roche 454)	Pea and eukaryotic marker gene sequencing (ITS, 18S rRNA, or 28S rRNA)	Rhizosphere and endosphere fungal communities of diseased and healthy pea roots were studied across three fields in comparison to bulk soils. Health status and field both had significant effects on fungal community	Xu et al. (2012)

(continued)

Table 3 (continued)

Sequencing technology	Plant species and type of sequencing as well as sample	Major findings	References
		structure in roots, whereas only field was associated with significant changes of the rhizosphere and soil communities	

directly or indirectly taking part in primary and secondary metabolism (Badri et al. 2013a, b). These work as energy sources, antimicrobials for soil microorganisms (Berg 2009; Dennis et al. 2010), which leads to the fortification of bacteria in the rhizosphere (Uroz et al. 2010). Likewise, the rhizosphere microbial communities are shaped by plants with the help of growth-promoting factors. These beneficial microorganisms also have biocontrol activity which help to release microorganism-derived antimicrobials; in this manner they indirectly counteracting pathogens (Berendsen et al. 2012; Mendes et al. 2013). For example, disease-suppressive soils, which have the capacity to suppress plant diseases, act as a key microbial beneficial strain, and it is also relevant for antimicrobial gene cluster (Mendes et al. 2011; Hirsch and Mauchline 2012) (Table 3).

In plant rhizosphere the taxonomic profiling studies have endeavored various information regarding plant species, genotype, soil type, different growth stages, and various microbial community structures using NGS. Metagenomics study on rhizosphere and bulk soil microbial species revealed some noticeable consequence on the microbiota of rhizosphere for some plants (Uroz et al. 2010; Peiffer et al. 2013). But in several cases, bonding of microbial diversity of rhizosphere was very strongly related with specific soils (Berendsen et al. 2012). In most of the cases, host genetic makeup was considered as an inconsequential determinant for the rhizosphere microbiome (Lundberg et al. 2012). Therefore, soil bacterial biomes ultimately elucidate the alterations of fields that increase field-specific rhizosphere microorganisms for these plants. Besides all those things, pathogen-specific genes and their secretion were specifically found in root-associated microbiota; this indicates the significance of host innate immune system and plant–microbe interactions (Bulgarelli et al. 2012).

(b) Phyllosphere

We can say that phyllosphere is actually subject to harsh environmental conditions. These include high UV radiation, fluctuating temperatures, low water availability, as well as low nutrient availability. Due to cell wall metabolism, plants produce various organic compounds like amino acids, sugars, alcohol, some volatile carbon, etc. (Vorholt 2012). NGS technologies helped a lot to examine the importance of geographical proximity versus species identity of plant phyllosphere microbial communities (Delmotte et al. 2009). Several tree species select its own bacterial communities for excretion of different phytochemicals in the phyllosphere (Whipps et al. 2008). Some metaproteogenomic studies have been performed on few plants

like clover, soybean, and *Arabidopsis thaliana*. In terms of similarities, it has been seen that clover, soybean, and *Arabidopsis thaliana* microbial communities in phyllosphere were quite similar (Redford and Fierer 2009; Lopez-Velasco et al. 2011) as compared to the phyllosphere of rice which was more distinct (Rastogi et al. 2012; Schlaeppi et al. 2014) (Table 3).

(c) Endosphere

However, there are various endosphere microbial communities whose composition is still conserved and not known by the scientific community. They are trying hard to explore this area. The endosphere acted as an inhabitant for rhizosphere microbial community (Lundberg et al. 2012). In the *Arabidopsis thaliana*, three bacterial families from three different phyla were found that they enriched in the endosphere, even though their interaction patterns with different plants and the significance of these phyla for endosphere communities are still unknown. Model plant *Arabidopsis thaliana* and its close lineages share a taxonomically narrow root microbiota; due to common sharing of root microbiota, they comprise stable community of bacteria (*Actinomycetales*, *Burkholderiales*, and *Flavobacteriales*) (Schlaeppi et al. 2014). The bacterial communities present on root endophytes also initiate the inactivation of lignocellulose (Bulgarelli et al. 2012), which indicates their importance in controlling lignocellulolytic activity in endosphere niche. Other studies showed that bacteria like *Methylobacteriaceae* were present in roots of *Arabidopsis thaliana* (Lundberg et al. 2012). These bacteria act as a facultative methylotrophs so that they can metabolize methanol derived from plant in the phyllosphere microbial community and also to accomplish parallel role in the endosphere. Also, in some other plant species like pea root disease caused by fungal endosphere communities (Xu et al. 2012) (Table 3) showing the usefulness of plant microbiota for the plant survival.

7 Application of NGS Technologies in Future Studies Will Increase Understanding in Plant–Microbe Interactions

With the availability of up to third-generation sequencing platforms, a number of limitations have been already overcome. Particularly, libraries preparation and their sequencing become very easier and faster with respect to the costs per base and time. These technologies are providing in detail information related to physiological and chemical potential of microbial communities related to plants. A large number of limitations of NGS, for example, higher sequencing error or low-quality reads, can be reduced by using well-designed sequence data analysis methods. The third-generation sequencing will lead us in a new era where we can obtain even more and longer reads, improve day-by-day technologies, and remove all the errors in NGS analysis.

Some other limitation of NGS studies is a huge amount of sequences that represent unknown genes of known or unknown organisms. Sometimes for those

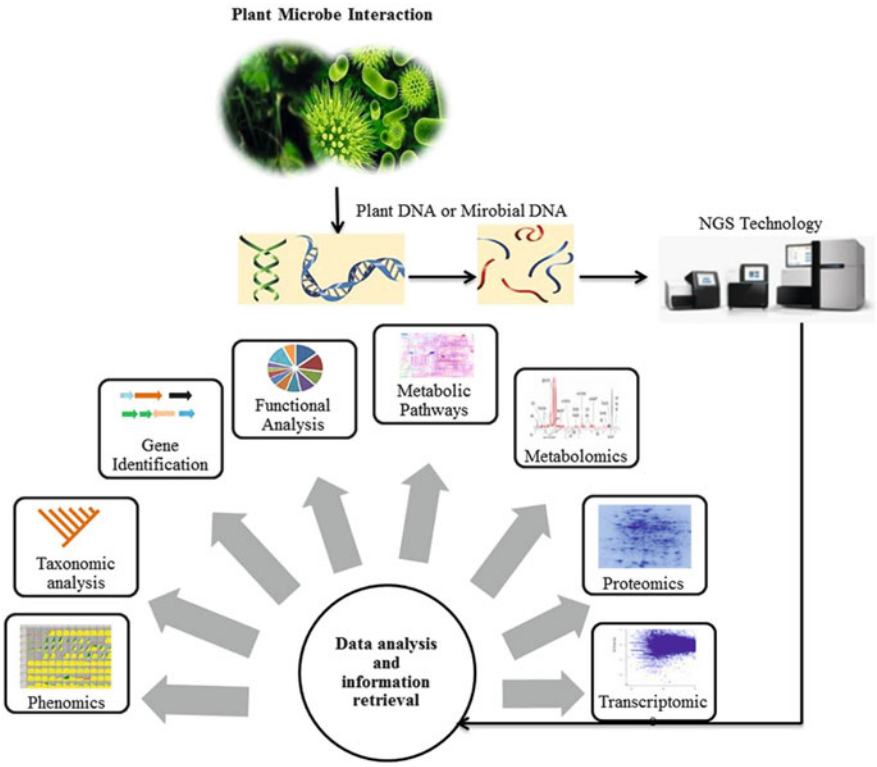

Fig. 4 Applications of NGS in various fields

sequences, no homolog is found in public databases that also creates a problem for further information retrieval. Linking those unknown genes and finding their function, their phylogeny, and their other properties are still very challenging tasks (Fig. 4). A large number of sequencing projects are going on for gathering the information for different types of microbial strains and their respective ecosystems to overcome these limitations (Brown et al. 2012). All these information play a vital role in the study of plant-associated microorganisms. This information may build a model system so that it can explain or predict microbial interactions in the phyllosphere, endosphere, and rhizosphere under various environmental conditions.

8 Conclusions

Novel methods based on NGS techniques will have a great impact on science field. They can help in the detection of new bio-resources and novel plant growth-promoting agents with their high-performance speed and good efficiency. Plant–

microbe interaction may play a vital role to create new perspectives for sustainable agriculture. Plants are the major source for the development of new microbes and various bioactive compounds because huge diversity is present within the plant microbiome. Using NGS techniques we can explore the novel microbes associated with plants which may help to plants for stress resistance during adverse environmental conditions, although a large number of successful information were already reported regarding diversity and specificity of the plant microbiome. Altogether, these researches open new insight for sustainable agriculture.

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