Chapter 4
Recent Advances in *Rhizobium*–Legume Interactions: A Proteomic Approach

Javed Musarrat, Almas Zaidi, and Mohammad Saghir Khan

Abstract Nitrogen-fixing symbioses between legumes and rhizobia over the years have played a major role in sustainable agricultural ecosystems. Owing to specific interactions with rhizobia, the leguminous plants form specialized nitrogen-fixing organ called as nodule, wherein rhizobia dwell and bring out the conversion of atmospheric nitrogen (N) to its usable form. This symbiosis in turn may abate the demand for external application of nitrogenous fertilizers while growing legumes under natural soil environment. Contemporary genomic research has provided a better understanding of the *Rhizobium*–legume interaction at molecular level. Several genomic approaches have been employed to define and demonstrate the involvement of rhizobial genomes in the symbiotic events. The genomes of two rhizobial species namely *Mesorhizobium loti*, the symbiont of several *Lotus* species, and *Sinorhizobium meliloti*, the symbiont of alfalfa, have now been completely sequenced, which have revealed interesting information about the genome evolution and structure, plant–microbes communication, and physiological diversity among the microsymbionts of legumes. While for legumes, numerous expressed sequence tags representing tens of thousands of different genes involved in root nodule formation and nitrogen fixation from three major legume species, *Glycine max*, *Medicago truncatula,* and *Lotus japonicus* have been deposited in the public domain. Currently, biological research is directed to understand gene expression and function involved in rhizobia–legume interaction. In this context, proteomics with continually evolving set of novel techniques to study all facets of protein structure and function is being considered as a promising and effective tool in

J. Musarrat (✉)
College of Science, King Saud University, Riyadh 11451, Saudi Arabia
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, India
e-mail: musarratj1@yahoo.com

A. Zaidi and M.S. Khan
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, India

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the postgenomic era to explore further the intricacies of symbiotic process. It is likely that the proteomics approach may reveal the newer possibilities for better understanding the complex interactions of rhizobia and legumes, and also the mechanisms as to how rhizobia survive under stressed environment. The major breakthroughs from the contemporary proteome-level investigations into legume–rhizobia interactions are discussed.

4.1 Introduction

The capability of leguminous plants to form a symbiotic association with plant growth promoting rhizobacteria (PGPR) belonging to the order rhizobiales in the family rhizobiaceae with the potential of transforming atmospheric nitrogen into usable form (Newton 2000) has exerted a profound impact on legume productivity (Zaidi et al. 2003; Zaidi and Khan 2007; Franche et al. 2009). Legumes are grown globally on approximately 250 million ha, which in symbiotic association with heterogeneously distributed soil bacteria belonging to the genera *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Phylorhizobium*, *Azorhizobium,* and *Sinorhizobium*, provides about 90 million metric tons of N per year (Kinzig and Socolow 1994). Members of these genera are collectively called rhizobia (Vance 2001), which includes a taxonomically and physiologically diverse group of the α and β subclasses of the proteobacteria. Legumes adequately nodulated by rhizobial species are reported to fix up to 300 kg of N per ha annually, which is equivalent to 625 kg of urea fertilizer. Indeed, the rhizobia empower legumes to generate protein-rich foods that are extremely important both for humans and animals and thus play a major ecological and economic role. Rhizobial strains, which serve as suitable inoculants, are able to (1) colonize the soil and tolerate environmental stresses (2) compete with indigenous rhizobia or other naturalist microflora (3) form effective nodules (4) fix atmospheric nitrogen, and (5) have no deleterious effects on nontarget hosts (Brockwell et al. 1995; Howieson 1999). Strains of rhizobia, however, differ widely in their survivability as well as nodulating and nitrogen-fixing efficiency in different agro-ecosystems leading to a variation in legume productivity.

The interaction between rhizobia and its specific legume host, generally referred to as symbiosis, has been well documented (Long 1989; Pawlowski et al. 1996; Fauvart and Michiels 2008; Chang et al. 2009; Catherine et al. 2009; Herder and Parniske 2009). Also, many bacterial and some plant genes affecting nodulation have been characterized (Javier et al. 2007; Laranjo et al. 2008; Wei et al. 2009; Lu et al. 2009). The formation of nitrogen-fixing nodules (Oldroyd and Downie 2008) on legumes requires coordinated expression of several bacterial and plant genes. Initial stages of nodule formation require expression of specific nodulation (nod) genes by rhizobia leading to the synthesis of a group of signal molecules that induce nodule morphogenesis. Inside nodule, the nitrogen-fixing form of rhizobia, referred to as bacteroids, are surrounded by a host-derived membrane called the

peribacteroid membrane (PBM), which controls molecular exchanges between the bacteroid and the legumes (Panter et al. 2000). The elicitor or *Rhizobium* nod factor responsible for nodule initiation is a lipochitin oligosaccharide (LCO) and plays a pivotal role in the induction of symbiotic developmental responses in legumes, leading to the formation of nodules onto the root systems of growing legumes (Spaink 1996; Rolfe et al. 2003).

To understand the plant–microbe interactions, several model organisms have been chosen, which provide either genomic or expressed sequence tag (EST), used to identify gene transcripts, and are instrumental in gene discovery and gene sequence determination, a prerequisite for large-scale protein identification (Cordwell et al. 1995; Kaneko et al. 2000; Galibert et al. 2001; Kaneko et al. 2002; MacLean et al. 2007). Genome size is influenced by environmental factors, and the soil-dwelling species such as rhizobia tend to have larger genomes (Bentley and Parkhill 2004). For example, *Bradyrhizobium japonicum* has the largest chromosome size (approximately 9.2 Mb), and the variation in chromosome sizes among the rhizobial species may in part be due to the presence of extra-chromosomal (plasmid) DNA. Thus, the complexity and heterogeneity of microbial populations within soil requires a large inventory of genes in order to maximize survival of free-living cells (Bentley and Parkhill 2004; Young et al. 2006), while the ability to establish a symbiotic relationship with a host plant imposes an additional genetic requirement upon rhizobia. The genomes of *Rhizobium leguminosarum* bv viciae (Young et al. 2006), *Rhizobium etli* (González et al. 2006), and two photosynthetic *Bradyrhizobium* strains (Giraud et al. 2007) have recently been completed, which has increased the number of available complete rhizobial genome sequences to seven, including sequences obtained for *B. japonicum* USDA 110 (Kaneko et al. 2002), *Mesorhizobium loti* (Kaneko et al. 2000), and *Sinorhizobium meliloti* (Barnett et al. 2001; Capela et al. 2001; Finan et al. 2001; Galibert et al. 2001). Following the success of *Arabidopsis*, the genome sequencing of two legume species, *Lotus japonicus* (Japanese trefoil) and *Medicago truncatula* (barrel medic) was launched (Mathesius et al. 2001; Young et al. 2005) and a substantial amount of information about their gene structures as well as physical and genetic maps has been made public. For instance, 176 Mb (89 Mb finished, 9 Mb at phase 2, and 78 Mb at phase 1) and 189 Mb (122 Mb finished, 37 Mb at phase 2, and 30 Mb at phase 1) nonredundant sequences of the *L. japonicus* and the *M. truncatula* genomes, respectively, has been released. These correspond to approximately 40% of the entire genomes of both *L. japonicus* and *M. truncatula* with estimation of more than 60% coverage of the euchromatic regions, and cover 69% and 58% of public ESTs of *L. japonicus* and *M. truncatula*, respectively (Sato et al. 2007). Now, since the genome sequence of both the symbiotic partners, that is, rhizobia and some legumes have been completed, how can we understand the biological mechanisms of interaction between the two symbionts? To explain such interaction further, Djordevic and his group from Australia described the use of proteomics to study flavonoid induced proteins in *R. leguminosarum* (Guerreiro et al. 1997). Later on, they identified differentially displayed proteins expressed during the symbiotic interaction between *S. meliloti* 1021 and the legume *Melilotus alba,* and characterized

Table 4.1 Proteomic studies in the field of legume–*Rhizobium* interactions

Rhizobia	Proteins characterized by 2 DE	Identified proteins	References
Rhizobium sp. NGR234/ *R. fredii*/*Sinorhizobium meliloti*	16	0	Krause and Broughton (1992)
R. leguminosarum bv. *trifolii* strain ANU843	1700	12	Guerreiro et al. (1997)
Bradyrhizobium japonicum	12	1	Winzer et al. (1999)
B. japonicum	17	17	Panter et al. (2000)
S. meliloti	600	100	Natera et al. (2000)
R. leguminosarum	16	10	Morris and Djordjevic (2001)
R. leguminosarum	4	12	Guerreiro et al. (1997)
R. leguminosarum	22	5	Guerreiro et al. (1998)
S. meliloti	52	23	Guerreiro et al. (1999)
S. meliloti	189	52	Chen et al. (2000a)
S. meliloti	60	11	Chen et al. (2000b)
S. meliloti	51	7	Bestel-Corre et al. (2002)
S. meliloti	41	11	Bestel-Corre et al. (2002)
Rhizobium etli CE3	5	0	Encarnación et al. (2003)
Sinorhizobium medicae	50	5	Reeve et al. (2004)
S. meliloti strain 1021	67	67	Djordjevic et al. (2003)
R. etli strain EBRI 26	49	0	Shamseldin et al (2006)
S. meliloti strain 2011	10	1	Shamseldin et al (2006)
Rhizobium sp. VMA301	16	0	Mandal et al (2009)
B. japonicum	100	68	Hempel et al. (2009)

Modified from Bestel-Corre et al. (2004)

novel proteins produced during symbiosis by comparing proteome of free-living bacteria and bacteroids of *S. meliloti* (Siria et al. 2000). Since then, proteomic studies have focused on mutualistic symbioses of legumes with nitrogen-fixing rhizobia (Table 4.1) in order to identify proteins that are specifically induced by microbes (Vij 2003; Mathesius 2009). These studies have led to new insights into the detection of microbial signal molecules by plants, the balancing of defence responses, nutrient exchange, and the alteration of plant development by microbes.

4.2 Rhizobia–Legume Interactions: An Overview

Symbiosis in general, is an interaction between two species, where the association results in a mutually beneficial relationship. All legume crops in general are capable of establishing nitrogen-fixing root nodule symbiosis with rhizobia. Nitrogen-fixation efficiency within a single legume species, however, vary more than tenfold, providing a tremendous opportunity for engineering legumes for

nitrogen fixation. Similarly, only a very few rhizobial species can induce root nodules and infect them, but fix nitrogen only poorly or not at all. Such rhizobial species predominates in the rhizosphere and pose a serious threat to legume improvement in conventional soils (Kiers et al. 2007; López-García et al. 2009). In this context, the data collected so far for signal exchange, infection, and early nodulation, however, suggests that even though 99.99% of the rhizobial population can interact with roots, but cannot necessarily produce an effective infection (Pérez-Giménez et al. 2009). The symbiosis between legumes and rhizobia is hence, a result of complicated interconnection that leads to the formation of nodules on root systems or stems of legumes (Prell and Poole 2006). Competition for nodulation is, however, a major problem, where bacterial adhesins (required for root colonization), can play a significant role in symbiosis (Elías et al. 2009). Generally, the overall symbiotic process includes (1) plant infection (2) nodulation and nodule maturation (3) senescence (4) release of rhizobia and (5) persistence of rhizobial populations in soil. Of these, the early interaction period comprises less than 10% of the whole symbiotic cycle, while the steps from nodule senescence to rhizobial persistence in soil occupy more than 60% of it (Pérez-Giménez et al. 2009).

The process of nodule formation involves a cascade of events that starts with movement of rhizobia toward the chemical signals released by legume hosts (Redmond et al. 1986), which ultimately results in the physical contact between the two symbiotic partners. During this preinfection stage, rhizobia, however, essentially be able to colonize and attach firmly to the root surfaces, out compete the neighboring organisms, respond to nodulation *(nod)* gene-inducing flavonoid compounds and possibly other plant factors (present in seed and root exudates) and release lipo-oligosaccharide signals (Nod-factors) (Cullimore et al. 2001) that are important for induction of nodulation. Studies have shown that flavonoid compounds cause the initiation of legume–rhizobial interactions by attracting rhizobia to host roots leading this to curl and affect many of the symbiotic events like (1) stimulated growth, (2) modified composition of bacterial cell wall components, (3) induced expression of *nod* genes leading to production of Nod factors, induce expression of the TTSS, and (4) induced expression of plant cell wall degrading enzymes, required for successful root infections by rhizobia (Cooper 2004). A number of specific plant proteins, referred to as nodulins, are also expressed during infection, nodule maturation, and maintenance to support the nitrogen-fixation process (Sánchez et al. 1991). The Nod factors in association with polysaccharides (Skorupska et al. 2006) and effector proteins (Dai et al. 2008; Kambara et al. 2009; Elías et al. 2009) allow rhizobia to attach to root hairs and to penetrate within the root through a tubular structure called the infection thread (Cermola et al. 2000), where the cell wall gets disrupted and rhizobial cells come into direct contact with the host-cell plasma membrane (Brewin 2004). The plant cell membrane then outgrows and bacteria are taken up into the plant cell lumen by endocytosis. Once the rhizobia are endocytosed within a host-membrane-bound compartment called symbiosome (Roth et al. 1988), a horizontally acquired organelle, the bacteria differentiate into a new endosymbiotic form, the nitrogen-fixing bacteroids.

Rhizobia, in general, produce both indeterminate (e.g., *M. truncatula* or *Pisum sativum*) and determinate (e.g., *L. japonicus* or *Glycine max*) types of nodules. Indeterminate nodules are characterized by different zones (1) the distal meristem, where bacteria are internalized, (2) an inter zone with amyloplast accumulation and differentiation of bacteroids, (3) a fixation zone that includes plant cells: in this zone, the bacteria differentiate to become nitrogen-fixing bacteroids, leghemoglobin of the plant cytoplasm protect nitrogenase from oxygen toxicity yet it facilitate bacteroid respiration (Ott et al. 2005), and (4) a senescence zone in the basal region that contains degrading bacteroids and collapsing plant cells (Pawlowski and Bisseling 1996). In comparison, determinate nodules are typically round shaped and are derived from the cessation of meristem activity after nodule initiation and growth of the nodule mainly by cell expansion. Such bacteroids cause the enzymatic reduction of atmospheric nitrogen to ammonia and make this N accessible to host plants and allow plants to grow even in the absence of an external nitrogen source (Jones et al. 2007). In return, the bacteria are supplied with carbohydrates mainly succinate and malate (Prell and Poole 2006) in a protected environment. The host plants, however, regulate the number of nodules formed, the maturation of nodules, and the nitrogen fixation of the nodules. The amino acid cycling has also been reported in the *Rhizobium*–legume symbiosis (Lodwig et al. 2003). However, bacterial differentiation and nitrogen fixation depends on the microaerobic environment and other support factors provided by the plants. In addition, the polysaccharide composition of the rhizobial cell wall (EPS, LPS, and cyclic glucans) are also reported to be important for successful infection, invasion, and nodule development, bacterial release from infection threads, bacteroid development, suppression of plant defence response and protection against plant antimicrobial compounds (Gibson et al. 2008; Jones et al. 2008; Robledo et al. 2008). And hence, there is a strong suggestion that production of a variety of symbiotically active polysaccharides may allow rhizobial strains to adapt to changing environmental conditions and interact efficiently with legumes. Recently, tyrosinase (EC 1.14.18.1), a monophenol oxidase responsible for the synthesis of the black pigment known as melanin, and a plasmid-encoded product in many rhizobial species including *R. etli* has been found to be involved during early symbiosis where it provides resistance against reactive oxygen species (ROS) and phenolic compounds generated as part of the plant protective responses (Silvia et al. 2007).

Of late, substantial progress has been made in the identification of genes involved in plant–microbe symbioses and decoding their functions (Franken and Requena 2001). However, despite substantial progress in biological sciences, the mechanisms regulating legume root nodule development are still inadequately explained, and very few regulatory genes have been cloned and characterized. For instance, ethylene response factor (EFD) required for the formation of functional nitrogen-fixing nodules and upregulated during nodulation in *M. truncatula* has recently been characterized (Vernie et al. 2008). With the completion of the genome sequence of certain rhizobial species, proteomics techniques may now further be employed to understand the expression of the gene products in *Rhizobium*–legume interactions in natural conditions.

4.3 What is Proteomics?

The word "proteome" is a blend of "protein" and "genome" and was coined by Marc Wilkins in 1994 in the symposium: "2D Electrophoresis: from protein maps to genomes" in Siena, Italy (Wilkins 2009), which represents the complete set of proteins that are determined by the genome. Analogous to genomics, proteomics is the study of proteins and describes mainly the structure and functions of the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism, or system at a given time (Wilkins et al. 1996; Anderson and Anderson 1998; Kav et al. 2007). Proteome analysis has been used to compare the simultaneous accumulation of hundreds of plant proteins in response to a variety of bacterial signaling molecules. The term "proteomics" was first coined by Klose (1975) to make an analogy with genomics, the study of the genes, and after genomics, proteomics is often considered the next step in the study of biological systems because knowledge of where and when proteins are expressed is essential for understanding biological events. Proteomics can broadly be classified into "classical proteomics" for protein identification and "functional proteomics" for the detailed characterization of protein structure and function as well as protein–protein interactions (Yarmush and Jayaraman 2002). Proteomics in general involves the use of two-dimensional electrophoresis (2DE), which allows the separation of denatured protein polypeptides according to their isoelectric points and molecular weights combined with high-throughput mass spectrometry (MS) identification methods. Moreover, the peptide mass fingerprinting or de novo sequencing and bioinformatics tools (Fig. 4.1) are used to identify and characterize the proteins, their activities and interactions (Jungblut and Wittmann-Liebold 1995; Pasquali et al. 1996; MacBeath 2002; LaBaer and Ramachandran 2005). However, proteomics is much more complicated than genomics mostly because while an organism's genome is more or less constant, the proteome differs from cell to cell and from time to time. Interestingly, the study of proteins introduces another level of complexity at the level of the posttranslational modification (PTM) and the biological relevance of such modifications. These changes in PTM during the growth and development of organisms (including plants) or in response to stress (including disease) cannot be deduced from studies investigating genome sequences and/or transcript abundance but can only be deciphered through proteomics (Dubey and Grover 2001; Gygi et al. 1999; Park 2004; Thurston et al. 2005). Even though proteome analysis possesses high resolution power and sensitivity, there may be limitations in the analysis of total cellular protein. The inability to detect some proteins may indicate that they (1) are present in relatively low amounts (2) are not soluble (3) comigrate with more abundant proteins or (4) are of very low molecular masses and are not resolved in the second dimension. Despite these problems, proteome analysis may provide a sensitive new tool to examine plant–microbe interactions (Guerreiro et al. 1997) under natural conditions.

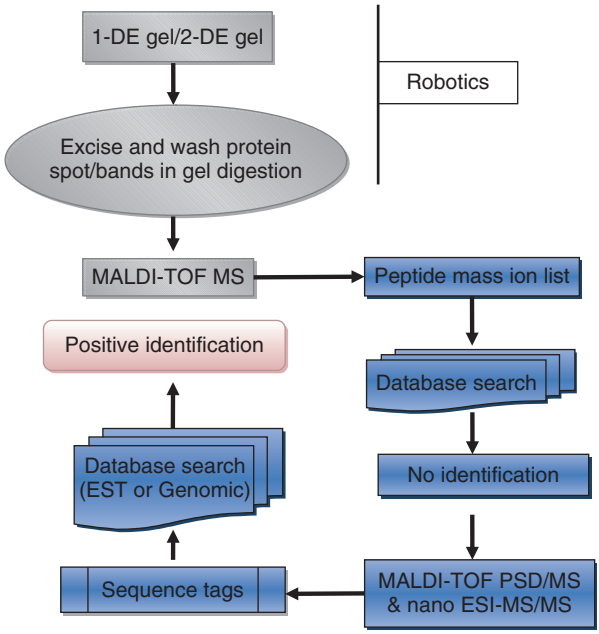

Fig. 4.1 A typical flow chart for the analysis of proteomes by MS. Proteins are separated by 1-DE or 2-DE visualized and selected for identification. The protein spots are excised and used to determine the mono-isotopic peptide ion masses by MALDI-time-of-flight (TOF) MS

4.3.1 How Proteomics Can Be Useful in Rhizobium*–Legume Symbiosis Studies?*

Currently, the proteome analysis of plant–microbe interactions is not well established, though the majority of researches have been directed toward understanding the proteome of microbial partner, probably due to ease of culturing as such organisms are single celled and the availability of complete genome sequence (Djordjevic et al. 2003). However, the research is required to discover new proteins involved in symbiotic relationship and hence to understand the proteomic basis of legume–*Rhizobium* interactions, the PTMs, identification of specific isoforms of proteins involved in certain metabolic pathways, and the construction of biochemical pathways in which the newly identified proteins can act. Proteomics of the rhizobia–legume symbiosis was started a decade ago (Krause and Broughton 1992), and there was renewed interest in the technique several years after, with studies focusing either on nodule proteins (Winzer et al. 1999; Panter et al. 2000; Natera et al. 2000; Morris and Djordjevic 2001) or on the isolated rhizobia (Guerreiro et al. 1997, 1998, 1999; Chen et al. 2000a, b). The nodule-forming rhizobia are currently underrepresented in the available databases compared to numerous agronomically

important bacteria. Most of the genes identified so far have been found associated with infection, polysaccharide production, or nitrogen fixation (van Rhijn and Vanderleyden 1995; Gray and Rolfe 1990; Fischer 1994). With increasing interest in the complex regulatory changes that occur during *Rhizobium*–legume interactions under different environments, together with the ever-increasing availability of mutants, it is desirable to characterize the *Rhizobium* gene expression via proteome analysis (Kav et al. 2007). After the completion of the genome sequence of *S. meliloti* and the determination of a 410-kb DNA region of *B. japonicum* chromosome, harboring potential symbiosis-specific genes, the focus is shifted to employ proteomics to identify the proteins induced during symbiosis (Giel et al. 2007) or to analyze protein–protein interaction in the nitrogen-fixing bacterium (Shimoda et al. 2008). During symbiotic studies, proteomic analysis is likely to provide a broad spectrum of the proteins produced by both partners, that is, rhizobia and legumes. In this context, the recent discovery of several plant receptor kinases responsible for the early detection and signal transduction of Nod factor perception (Endre et al. 2002; Stracke et al. 2002) and autoregulation of nodule numbers (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2002), suggests that many early plant–microbe signaling events are regulated by phosphorylation events and key receptor kinases. Among legumes, proteomic analysis has mainly focused on *M. truncatula*, for which a proteome reference map has been established (Mathesius et al. 2001). Nevertheless, the proteomics approaches have been used to study protein patterns in several rhizobial species including *R. leguminosarum*, *R. etli,* and *S. meliloti* (Guerreiro et al. 1997, 1999; Encarnación et al. 2003). The first two-dimensional protein map of *R. leguminosarum* bv. *trifolii* strain ANU843, grown in defined medium in the presence and absence of flavonoid, resolved over 1,700 constitutive proteins representing about 30% of the estimated genomic output. While the global expression pattern of proteins was largely unaltered by the treatment, yet four inducible proteins were detected, which together with other 20 constitutively expressed proteins were sequenced to develop internal standards for the construction of a two-dimensional *Rhizobium* protein database. Of the identified proteins, NodE was present throughout the growth of the cells but decreased quantitatively during stationary phase cells, whereas NodB was not detected in the later stages of growth. Two of the induced proteins did not match with any known nodulation gene product, with one of these being present in mid-late log and stationary phase cells and possessing four consecutive His residues at the N terminus. Also, the reference maps for the *S. meliloti* during the early and late exponential growth phases, as well as protein patterns for bacteroids, were established before the genome sequence of *S. meliloti* became available (Guerreiro et al. 1999). The *S. meliloti* genome sequence paved the way for more sophisticated profiling of protein patterns. Djordjevic et al. (2003) used a combination of two-dimensional (2D) gel electrophoresis and peptide mass fingerprinting to investigate the protein patterns of nodule bacteria and of cultured bacteria in response to various stress conditions. About 1,180 protein products derived from 810 genes (13.1% of the predicted genes), demonstrated that the proteomic analysis is a powerful approach for global analysis of protein profiles. A large number of studies that include proteomic and

transcriptomic approaches have been initiated with the wide spread acceptance of these methods in the investigation of rhizobia–legume symbioses.

An obvious difference in the proteome of both symbiotic bacteria and cultured bacteria has been observed and putative nodule-specific and nodule suppressed proteins were identified. The data were further analyzed using metabolic pathway prediction programs and used to assess the biochemical and genetic changes. While considering the central carbon and nitrogen metabolism, the data revealed a greater similarity between the proteomic and biochemical findings and suggested that a highly specialized nutrient exchange occur between the nodule bacteria and the host plants. Proteins embossed in nodule bacteria are associated with vitamin synthesis and stress-related processes, like heat shock, chaperoning, detoxification of ROS, regulation of stress, and osmo-regulation. These findings clearly demonstrate the level of the shift in metabolism that occurs when *S. meliloti* invade legumes and form an effective symbiosis (Djordjevic 2004).

During the development of symbiosis, infection of legume root hairs by rhizobial species is considered the first of several complex events leading to nodule formation. To identify proteins involved in this process, proteomic studies have been conducted. For instance, Wan et al. (2005) have used proteomics to identify proteins in the soybean (*G. max*) root hairs after infection by *B. japonicum*. Root-hair protein preparations were obtained after 0, 3, 6, and 12 h exposure to *B. japonicum*. Mainly, the proteins previously identified as lipoxygenases, agglutinin, actin, peroxidase, and phenylalanine ammonia lyase, and some novel proteins such as phospholipase D and phosphoglucomutase have been reported. In a follow up study, a proteome-level analysis of the bacteroid of *B. japonicum* has been performed and proteins involved in nitrogen and carbon metabolism were identified along with numerous proteins related to protein synthesis, scaffolding, and degradation. Similar to the *M. alba–S. meliloti* interaction, the proteomes of *B. japonicum* inhabiting root nodules were significantly different from that of the culture-grown free-living bacterium (Sarma and Emerich 2006). Furthermore, while comparing the proteome-profiles of *B. japonicum* grown under in vitro condition and those obtained from bacteroid, proteins related to fatty acid and nucleic acid syntheses were considerably more abundant in cultured cells, whereas proteins related to nitrogen metabolism were present in higher amounts in bacteria living in nodules (Sarma and Emerich 2006) suggesting that the bacteroid state differs from the free-living state (Finan et al. 1991; Ampe et al. 2003; Barnett et al. 2004; Becker et al. 2004; Hoa et al. 2004) basically due to (1) changes in energy resources to support nitrogen fixation (2) changes in the protein degradation machinery and (3) enhanced expression of chaperonins to extend the life span of nascent proteins. Additionally, a small protein proteome of *M. truncatula* nodules establishes the presence of ribosomal proteins S6 and L24, a histone-like protein, and a peroxidase precursor (Zhang et al. 2006). In a similar study, the protein profiles of root hairs of *Vigna unguiculata* inoculated with *Rhizobium* sp. and a hair-deformation minus mutant of *Rhizobium* sp. were analyzed using 2-DE (Krause and Broughton 1992). Twelve symbiosis-specific proteins were observed and seemed to be associated with root-hair deformation and nodule development. These findings suggest that the

proteome analysis could serve as an attractive source for the study of root-hair infection by rhizobia and, in a more general sense, the functional genomics of a single, plant cell type. The results obtained also indicate that proteomic studies with legumes, lacking a complete genome sequence, are practical. Furthermore, the proteomic analysis of nodule cytosol proteins of soybean cv. Williams 82 led to conclude that of the 69 identified proteins, three were involved in glycolysis, which were further characterized to support their roles in the sucrose synthase pathway to provide malate for the bacteroids (Oehrle et al. 2008).The host-derived symbiosome membrane (SM) in intracellular symbioses represents both a structural and functional boundary between the two symbionts and hence, is strategically located to control the molecular interactions between the symbionts. Symbiosome membrane proteins from soybean nodules inoculated with wild-type and mutant *B. japonicum* were investigated using 2-DE, which revealed several quantitative differences between wild-type- and mutant-inoculated soybean nodules, including an observed significant downregulation of nodule-specific proteins in mutant-inoculated nodule (Winzer et al. 1999). While proteins of the PBM of soybean root nodules using combination of 2-DE and N-terminal sequencing was identified by Panter et al. (2000). This study identified homologues of hsp60 and hsp70 and presented evidence of the presence of a molecular machinery in the symbiosome for importing cytoplasmic proteins during co- or posttranslational modification. Proteins involved in the early stages of nodulation between the subterranean clover cultivar Woogenellup and two strains of *R. leguminosarum* bv. *trifolii* were identified using a comparative proteome approach (Morris and Djordjevic 2001). Proteins involved in nodule formation, early hormonal response upon infection and cell-wall strengthening and loosening were identified. Several symbiosis-related root proteins of *M. truncatula* were identified associated with the nitrogen-fixing bacterium *S. meliloti* using 2-DE, MALDI-TOF, and tandem MS (Bestel-Corre et al. 2002). In other studies, Natera et al. (2000) examined the proteome of *M. alba* root and nodules and the bacteroid of *S. meliloti*. In this study, *S. meliloti* proteins involved in carbon and nitrogen metabolism as well as protein synthesis were identified. In order to further characterize the changes that occur in *S. meliloti*, while it occupies the root nodule of *M. truncatula* and *M. alba*, a comparison of the proteomes of the nodule bacteria with that of *S. meliloti* cultured in the laboratory was performed using 2-DE and MS. This study identified nodule-specific proteins, nodule-suppressed proteins, stress-related proteins, transporters, and vitamin synthesis-related proteins, which suggested the presence of a highly specialized nutrient exchange system between the bacterium and the host (Djordjevic 2004). A proteome analysis of *M. alba* nodules revealed that over 250 proteins were differentially expressed in nodules compared to uninfected roots, and over 350 proteins changed in nodules compared to free-living rhizobia (Natera et al. 2000). Bestel-Corre et al. (2002) used a time-course analysis of root protein profiles to study *M. truncatula* inoculated with *S. meliloti*. A recent proteome study of early *M. truncatula* root responses to *S. meliloti* was carried out using 2D Difference in Gel Electrophoresis (DIGE). Within 24 h of inoculation of roots with rhizobia, 174 of approximately 3,700 proteins showed differential expression (van Noorden et al. 2007). Overall, 140

proteins were identified by MALDI-TOF/TOF. These proteins included a large number of enzymes involved in energy, carbohydrate, amino acid, and flavonoid metabolism, which could reflect the metabolic adaptations in the roots as part of the developmental changes in preparation for nodule initiation. A more detailed study of the *S. meliloti* proteome under free-living and symbiotic conditions identified 810 gene products involved in at least 53 metabolic pathways (Djordjevic et al. 2003; Djordjevic 2004). Of these, several proteins appeared to be nodule specific, which included enzymes required for nitrogen fixation, heme synthesis as well as heat-shock and stress-related proteins, and proteins involved in detoxification processes. In addition, a number of transport proteins, in particular ABC transporters, showed specific changes between free-living (cultured) and nodule-inhabiting rhizobia, highlighting the specialized changes in nutrient transfer that develop in a functioning nodule (Djordjevic 2004). These investigations concluded that a substantial change occurred both within the bacterium grown under different circumstances and the temporal changes within the inoculated plant. In other study, the proteins of the PBM of soybean nodule bacteroids and their possible involvement in protein processing and the biogenesis and function of the PBM is reported (Panter et al. 2000).

4.3.2 Survival of Rhizobia in Stressed Environment: A Proteomic Approach

Microbial communities, in general, have evolved a wide range of strategies that accord them to confront with varying environmental challenges. The dynamism among microbes to overcome such adverse situations is critical for their survival and growth, which depends on rapid and efficient control of genetic expression and metabolic responses (Nystrom 1998). Of the heterogeneous microbial populations, a few can survive in strictly undesirable environments while others can be affected adversely under stressed conditions. When bacteria encounters adverse environments like heat, heavy metals, salt and nutrient limitations, and other factors, the level of expression increases substantially. For example, bacteria initiate a program of gene expression in response to osmotic stress by high salt concentrations, which are manifested as a set of proteins produced in increased amounts in response to the stress. Like other bacteria, soluble proteins from the salt-tolerant *R. etli* strain EBRI 26 separated by two-dimensional gel electrophoresis and visualized by Commassie staining demonstrated that the expression of at least six proteins of varying molecular weight were increased following 4% NaCl compared to *R. etli* grown in medium without salt. These proteins analyzed by MALDI-TOF after digestion with trypsin revealed a very good peptide mass fingerprint data, which could not be identified since the genome sequence of *R. etli* is not yet published. In another experiment, the soluble proteins from salt-induced or nonsalt-induced cultures from *R. etli* strain EBRI 26 when labeled separately with different fluorescent cyano-dyes

prior to two-dimensional gel electrophoresis affirmed that 49 proteins were differentially expressed after the addition of sodium chloride. Of these, 14 proteins were over expressed and 35 were downregulated. Similar experiments using *S. meliloti* strain 2011 identified four overexpressed and six downregulated proteins. Among the overexpressed protein was a carboxy-nospermidin decarboxylase, which plays an important role in the biosynthesis of spermidin (polyamine) while enzyme catalase was among the downregulated proteins. These proteins may play a role in salt tolerance (Shamseldin et al. 2006).

Another important unfavorable factor that adversely affects the growth of bacteria is the high acid environment. However, rhizobial species have also evolved mechanisms to cope with such deleterious environmental factor, where proteomics play a key role in identifying the proteins responsible for tolerance to high acidity. For example, the proteome analysis of *B. japonicum* USDA110 revealed 568 and 628 protein spots of cells grown at pH 4.7 and pH 6.8, respectively, (Puranamaneewiwat et al. 2006). Of these, only 84 protein spots with at least threefold differential expressions were further identified by MALDI-TOF MS. The annotated proteins were assigned to four different classes (1) proteins produced only at pH 4.7 (15 proteins such as D-alanine aminotransferase, 2-haloalkanoic acid dehalogenase, and periplasmic mannitol-binding protein) (2) proteins produced under both conditions but strongly induced at pH 4.7 (27 protein spots such as triosephosphate isomerase, UTP-glucose-1-phosphate uridylyl transferase, and glyceraldehyde 3-phosphate dehydrogenase) (3) proteins downregulated during growth at pH 4.7 (25 proteins such as GroEL, acyl-CoA dehydrogenase, and ATP synthase beta chain) and (4) proteins specific to growth at pH 6.8 (17 proteins such as ATP dependent protease ATP-binding subunit, N-utilization substance protein A, and 2-isopropylmalate synthase). The data of the differential protein expression can be a basis for mechanism elucidation of the acid response in *B. japonicum* USDA110. In a similar study, to elucidate the mechanisms of pH response in an acid-tolerant *Sinorhizobium medicae*, Reeve et al. (2004) have identified acid-activated gene transcription and now complement this approach by using a proteomic analysis to identify the changes that occur following exposure to acidity. Protein profiles of persistently or transiently acid-stressed *S. medicae* cells were compared to those grown in pH neutral, buffered media. A total of 50 pH-regulated proteins were identified; N-terminal sequences for 15 of these were obtained using the Edman degradation. Transient acid exposure downregulated GlnA and GlnK and upregulated a hypothetical protein while consistent acid exposure downregulated ClpP, an ABC transporter, a hypothetical protein, a lipoprotein, the Trp-like repressor WrbA1 and upregulated DegP, fructose bisphosphate aldolase, GroES, malate dehydrogenase, and two hypothetical proteins. These findings implicate proteolytic, chaperone, and transport processes as key components of pH response in *S. medicae*.

Like the bacterial partners, nitrogen-fixing legumes are also sensitive to stressed factors. For example, water limitation has been reported to reduce nitrogen fixation substantially (Guerin et al. 1990; Zahran 1999) possibly due to downregulation of key enzymes involved in symbiosis (Gordon et al. 1997; Arrese-Igor et al. 1999),

oxygen limitation (Diaz del Castillo et al. 1994), nitrogen feedback (Serraj et al. 2001; King and Purcell 2005) and a shortage in nodule carbon flux (Arrese-Igor et al. 1999). It is now well established that plants have evolved many adaptations to counteract dehydration by dehydration-responsive changes in expression of proteins, which may lead to cellular adaptation against water deficit conditions (Bray 2004; Blum 2005). For example, proteomics approach to identify dehydration-responsive extracellular matrix (ECM) proteins in several commercial varieties of a food legume like chickpea, is reported (Bhushan et al. 2007). The comparative proteomics analysis led to the identification of 134 differentially expressed proteins that include predicted and novel dehydration-responsive proteins. It has been demonstrated that over a hundred ECM proteins are presumably involved in a variety of cellular functions like cell wall modification, signal transduction, metabolism, and cell defence and rescue. Moreover, under water stressed condition, synthesis of trehalose (α-D-glucopyranosyl (1-1) α-D-glucopyranoside), an uncommon sugar in the plant kingdom (Mellor 1992) is increased either directly via bacterial osmo-regulatory mechanisms or indirectly via oxygen partial pressure and accumulates in the root nodule but can be exported from nodules . Under similar conditions, trehalose synthesis is triggered in cultured rhizobia (Hoelzle and Streeter 1990) and if the trehalose concentration is high enough, that is, if synthesis is high and hydrolysis low, then some trehalose may escape the confines of the nodule (Streeter 1980) and may be able to act as osmo-protectant in other parts of the plant.

Proteomics also help to evaluate the impact of sewage sludges polluted with heavy metals or polycyclic aromatic hydrocarbons, on legume–*Rhizobium* interaction. For example, although control sludge showed positive effects toward *M. truncatula* plants noninoculated or inoculated with *S. meliloti*, the polluted sludges exhibited clear negative effects on plant growth and root symbioses. A clear correlation was established between some symbiosis-related proteins and the levels of nodulation, revealing a potential use of this technology for environmental studies (Bestel-Corre et al. 2002). Sewage sludge-related proteins were also identified in nodulated *M. truncatula* roots (Bestel-Corre et al. 2002), and in cultured *S. meliloti* cells (Bestel-Corre et al. 2002), thus giving some supplementary information when these data were compared to physiological data. Likewise, *Rhizobium* sp. VMA301 was isolated from the root nodules of *Vigna mungo*, grown in arsenic contaminated field. The LC50 value of arsenite for VMA301 was found to be 1.8 mM. A total of 16 differentially expressed proteins were identified using RP-HPLC and MALDI TOF mass spectrometry from arsenite-induced whole cell lysate soluble proteins. Of these, nine proteins were upregulated and seven proteins were downregulated in comparison to the cells grown without arsenite. These differential protein expressions mitigated the toxic effect of arsenite and stimulate the detoxification process (Mandal et al. 2009). Such studies thus suggested that the proteins expressed by rhizobial species as revealed by proteomic studies under stressed environment may help rhizobia to establish an effective symbiosis even under derelict or stressed soils leading thereby to improve the performance of legumes under polluted soils.

4.4 Conclusion

The interaction between legumes and rhizobia though has been widely studied but looks still in an actively diversifying evolutionary phase. The genetic approaches adopted to understand the mechanistic basis of legume–*Rhizobium* interaction has provided significant insight into nodule development and function. However, the genes able to knock down the symbiotic events like nodulation, nitrogen fixation, and other symbiotic processes may not necessarily be the ones that were identified as essential for symbiosis through genetic screens. Moreover, numerous evidences suggest that genome architecture and even content are influenced greatly by the multiphasic lifestyle adopted by nodule bacteria. To further understand how legume–rhizobia partnership continues leading to successful nodulation and nitrogen fixation, proteomics in recent times has provided valuable insight into the symbiotic interaction of the rhizobia and their respective host legumes despite certain limitations associated with this technique. Proteomic analysis is hence, likely to extend our knowledge of the fascinating partnership that exists between legumes and rhizobia. Furthermore, the development of other functional genomic approaches, such as studies focusing upon the metabolomics of stem- and root nodule bacteria (Barsch et al. 2004; Colebatch et al. 2004) may help to extend our understanding of the interaction occurring between legumes and their corresponding rhizobial partners. However, the combination of proteome-based techniques along with information generated from genomic sequencing is likely to lead to a better understanding of various events occurring during *Rhizobium*–legume interactions, which may ultimately lead to model both legumes and rhizobia for enhancing legume productivity in both conventional and stressed soils across different geographical regions of the world.

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