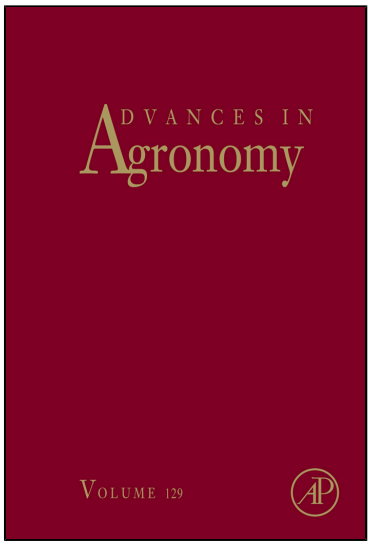

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CHAPTER ONE

Advances in Host Plant and Rhizobium Genomics to Enhance Symbiotic Nitrogen Fixation in Grain Legumes

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Abstract

Legumes form symbiotic relationship with root-nodule, rhizobia. The nitrogen (N_2) fixed by legumes is a renewable source and of great importance to agriculture. Symbiotic nitrogen fixation (SNF) is constrained by multiple stresses and alleviating them would improve SNF contribution to agroecosystems. Genetic differences in adaptation tolerance to various stresses are known in both host plant and rhizobium. The discovery and use of promiscuous germplasm in soybean led to the release of high-yielding cultivars in Africa. High N_2-fixing soybean cultivars are commercially grown in Australia and some countries in Africa and South America and those of pea in Russia. SNF is a complex trait, governed by multigenes with varying effects. Few major quantitative trait loci (QTL) and candidate genes underlying QTL are reported in grain and model legumes. Nodulating genes in model legumes are cloned and orthologs determined in grain legumes. Single nucleotide polymorphism (SNP) markers from nodulation genes are

available in common bean and soybean. Genomes of chickpea, pigeonpea, and soybean; and genomes of several rhizobium species are decoded. Expression studies revealed few genes associated with SNF in model and grain legumes. Advances in host plant and rhizobium genomics are helping identify DNA markers to aid breeding of legume cultivars with high symbiotic efficiency. A paradigm shift is needed by breeding programs to simultaneously improve host plant and rhizobium to harness the strength of positive symbiotic interactions in cultivar development. Computation models based on metabolic reconstruction pathways are providing greater insights to explore genotype–phenotype relationships in SNF. Models to simulate the response of N_2 fixation to a range of environmental variables and crop growth are assisting researchers to quantify SNF for efficient and sustainable agricultural production systems. Such knowledge helps identifying bottlenecks in specific legume–rhizobia systems that could be overcome by legume breeding to enhance SNF. This review discusses the recent developments to improve SNF and productivity of grain legumes.

1. INTRODUCTION

Globally, the average harvested area under pulses (beans, broad beans, chickpea, cowpea, lentils, lupins, peas, and pigeonpea) was 70 million ha from 2008 to 2012, with a total annual production and mean productivity of 62 million tons and 1.07 t ha^{-1}, respectively (http://www.faostat.fao.org/, assessed on December 10, 2013). The average production in comparison to the previous five years (2003–2007) increased by 11%, which was largely due to increase in acreage across growing areas. Asia's share of global pulses production was 28.7 million tons, Africa and American continent each shared 13 million tons, while Europe 5 million tons and Oceania 2 million tons.

Some grain legumes are widely produced, while others were restricted to specific continents. For example, Asia (10.4 million tons), the American continent (7.2 million tons), and Africa (4.2 million tons) were the largest producer of dry beans, while cowpea is predominantly produced in Africa (5.4 million tons). Pea production is dominated by the American continent (3.7 million tons) and Europe (3.5 million tons), while chickpea (9.1 million tons) and pigeonpea (3.5 million tons) are mostly confined to Asia. Both Asia and American continent contributed equally to lentil production (1.8 million tons). Such differences in pulses production were also noted at the subcontinent level as well. For example, dry beans were predominantly produced in South and Central America where they are originally from as well as in South and Southern/Eastern Asia, East Asia, and Southern or Eastern Africa. Cowpea is largely produced in West Africa; chickpea and pigeonpea in

South Asia; lentil in South Asia and North America; and peas in Canada, the northern United States and Eastern Europe (http://www.faostat.fao.org/, assessed on December 10, 2013).

Meanwhile, different than the previously mentioned food legumes, soybean and groundnut are the major leguminous oil crops. The global acreage of soybean for the period from 2008 to 2012 averaged 101.7 million ha, with a total annual harvest of 247 million tons and average productivity of 2.43 t ha^{-1}. The groundnut global acreage during the same period was 24.6 million ha on average with 39.6 million tons produced annually and average grain yield of 1.61 t ha^{-1}. The average global production in comparison to previous five years (2003–2007) increased by 17% in soybean and 8.8% in groundnut, which was largely to increased acreage (6.8% in groundnut and 12% in soybean) and partly due to productivity gains (groundnut, 2% and soybean, 4.6%).

Soybean has been predominantly produced in the American continent and Asia, with about 86% and 12% global production (247 million tons), respectively. Groundnut was the major legume crop in Asia and Africa, which together contribute 91% of its global production (64% and 27%, respectively). Within the Americas, South America contributed 49.4% of the global soybean production, followed by North America (36.0%). Finally, East Asia (6.2%) and South Asia (4.7%) produce smaller amounts of soybean. Groundnut production in Asia was mostly from East Asia (39.4%) and to a lesser extent from South Asia (17.3%) and Southeast Asia (6.9%), while the production in Africa was dominated by West Africa (15.8%), and to a lesser extent by Central and Eastern Africa (8%). North and South America contributed 5.3% and 2.6% of the global groundnut production, respectively.

Grain legumes, rich in protein or oil, carbohydrate, fiber, minerals, and vitamins, are characterized by low glycemic index (GI). Foods with low GI are generally associated with several long-term health benefits (Guillon and Champ, 2002; Duranti, 2006; Panthee et al., 2006; http://www.extension.usu.edu). The proteins in the seeds are low in sulfur-containing amino acids, cysteine, methionine, and tryptophan, but they are very rich source of another essential amino acid, lysine, which is low in the cereals (Duranti, 2006).

Legume starch and fiber have useful functional properties and can readily be used in food products. Resistant starch in legume seeds results in large amounts of butyrate upon fermentation by colonic bacteria. This short-chain fatty acid is rapidly absorbed in the colon to provide additional energy to animal models (de Fillippo et al., 2010), and they prevent

the establishment of potentially pathogenic intestinal microbes (Hermes et al., 2009). Dietary fibers play an important role in shaping microbial diversity in human gastrointestinal tract. The evidence to date suggests that grain legume kernel-derived fiber stimulates the growth of colonic bifidobacteria and contributes to colon health (Guillon and Champ, 2002; Smith at al., 2006; Fernando et al., 2010; He et al., 2011). The dietary fibers have also been found to have beneficial effects in reducing the risk of heart disease, diabetes, obesity, and some form of cancers (Marlett et al., 2002; Duranti, 2006; Jenkins et al., 2006). The isoflavones in legumes play a role in plant defense (Padmavati and Reddy, 1999), root nodulation (Subramanian et al., 2007), and also in human health (Jung et al., 2000).

Legume grains contain nonbeneficial antinutrients (such as protease inhibitors, tannin and phytic acid). However, the health benefits of tannins and phytates can be substantial and the effects of protease inhibitors are largely minimized during their processing and cooking. Even some protease inhibitors such as the major family of Bowman–Birk-type inhibitors found in many legume seeds has potential anti-inflammatory and cancer preventive properties within the gastrointestinal tract (Kennedy, 1998; Clemente and Domoney, 2006; Clemente et al., 2011, 2012).

The health benefits of legume consumption provide a strong base for the development of legume crops and their products as pro-nutritional, health-promoting foods (Clemente et al., 2012). One word of caution is that some grain legumes are reported to cause detrimental effects on human health due to toxins. For example, faba bean seeds contain glycosides, vicine and convicine, which cause a favism disease in genetically susceptible humans (Crépon et al., 2010). The grasspea contains neurotoxic amino acid, beta oxalyl-L-alpha, β-diaminopropionic acid (β-ODAP), which could lead to the crippling disease (neurolathyrism) in humans (Getahun et al., 1999; Geda et al., 2005).

Overall, however, legumes provide a range of nutritional and agroecosystems services to the societies, e.g., as important sources of protein-rich food and feed, oil, fiber, minerals and vitamins, improve soil fertility by contributing nitrogen through atmospheric N_2 fixation in symbiosis with rhizobia; improve soil structure and increase soil organic carbon status; reduce the incidence of pest and diseases in cropping systems; and increase the overall productivity and economic benefits of the production systems they are part of (Dwivedi et al., 2005; Peoples et al., 2009; Köpke and Nemecek, 2010; Chianu et al., 2011; Lupwayi et al., 2011).

Legumes also contribute to mitigating the climate change effects by reducing fossil-fuel use, ammonia fertilizer production or by providing feedstock for the emerging bio-based economies where fossil-fuel sources of energy and industrial raw materials are replaced in part by sustainable and renewable biomass resources; thus, legumes are an important component of sustainable production systems for human prosperity (Lupwayi et al., 2011; Jensen et al., 2012). Grain legumes are, without doubt, an important component of sustainable production systems for human prosperity and have a critical role in crop and cropping system diversification. For example, some legumes release unavailable phosphorous in the soil for recovery by the legumes themselves or other plants (Sinclair and Vadez, 2012). Legume crop residues have better nutritional quality than cereals straw for use as fodder for farm livestock (López et al., 2005; Blümmel et al., 2012).

A critical aspect of legume plants is that they form symbiotic relationship with root-nodule bacteria, rhizobia. The rhizobia are gram-negative bacteria from a limited set of clades, largely belong to *Alphaproteobacteria* (here onward referred as alpha-rhizobia) (Gyaneshwar et al., 2011) and grouped into distinct genera (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (renamed as *Ensifer*)), species, and symbiovars (Rogel et al., 2011). Other alpha-rhizobia-nodulating genera reported are *Methylobacterium*, *Devosia*, *Ochrobactrum*, *Aminobacter*, *Microvirga*, *Shinella* and *Phyllobacterium*. In addition, rhizobia such as *Burkholderia*, *Cupriavidus*, and *Pseudomonas* (formerly *Ralstonia*) of *Betaproteobacteria* (here onward referred as beta-rhizobia) subclass and even selected ones from *Gammaproteobacteria* are also able to form symbiotic association with legumes (Chen et al., 2001; Moulin et al., 2001; Sprent, 2007; Balachandar et al., 2007; Ormeño-Orrillo et al., 2013). *Burkholderia phymatum* as well as other species from the genus *Burkholderia* are highly promiscuous, effectively in nodulating several important legumes, including common bean (Gyaneshwar et al., 2011). In contrast, some rhizobium species have narrow host ranges compared to others with broad host ranges.

Symbiovar represents a group of bacterial strains distinguishable from other strains of the same species on the basis of physiological or biochemical characters, which can be shared by different species due to lateral gene transfer. Symbiovars have been reported in *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* genera; for example, ciceri in chickpea rhizobium species (*Mesorhizobium amorphae*, *Mesorhizobium tianshanense*, *Mesorhizobium ciceri*, *Mesorhizobium mediterraneum* and *Sinorhizobium meliloti*);

gallicum (*Rhizobium gallicum* and *Rhizobium giardinii*), giardinii (*R. giardinii*), mediterranense (*Sinorhizobium fredii* and *S. meliloti*), mimosae (*Rhizobium etli*), and phaseoli (*R. gallicum*, *R. giardinii*, *Rhizobium leguminosarum*, *R. etli*, and *Rhizobium phaseoli*) in common bean rhizobium species; or viciae in common vetch rhizobium species (*R. leguminosarum* and *Rhizobium pisi*) and faba bean rhizobium species (*Rhizobium fabae*) (Rogel et al., 2011). Symbiovars have not been reported in *Betaproteobacteria*.

Central Brazil and South Africa are the principal centers of diversity of β-proteobacteria (Gyaneshwar et al., 2011), and they may be regions where nitrogen fixation first arose among the legumes. Nitrogen (N) fixation is the process by which certain plants, including legumes, take nitrogen gas from the atmosphere; incorporate the molecules into their tissue, and subsequently into the ground, thus improving their own growth as well as soil health and overall productivity of the farming systems.

Symbiotic nitrogen fixation (SNF) is therefore a natural process of significant importance in world agriculture. The N_2 fixed by the legume crops represents a renewable source of nitrogen for agricultural soils. Globally, legumes in symbiosis with soil rhizobia are reported to fix 20–22 million tons (or 20–22 Tg) of nitrogen each year in agricultural production systems (Herridge et al., 2008). Large differences were, however, noted in the proportion of atmospheric N_2 fixed by the grain legume crops, e.g. 75% of the total nitrogen in plant was derived from SNF by faba bean; 62–94% by soybean, groundnut, pea, and lentil; 54–58% by cowpea, chickpea, and pigeonpea; and 39% by common bean.

Regional differences in the amount of shoot N were observed for these legume crops. For example, soybean was reported to fix 193 kg N ha^{-1} in Africa and 300 kg N ha^{-1} in South America; common bean 75 kg N ha^{-1} in North America; groundnut 100–116 kg N ha^{-1} in South and Southeast Asia; pea 130 kg N ha^{-1} in Europe; cowpea 63–84 kg N ha^{-1} in South Asia and Africa; chickpea and faba bean 70 and 82 kg N ha^{-1} respectively in Oceania; lentil 122 kg N ha^{-1} in West Asia; and pigeonpea 58 kg N ha^{-1} in South Asia.

Several factors contribute to the differences in N_2 fixation efficiency (Zahran, 1999; Hungria et al., 2006a; Mohammadi et al., 2012). Most importantly, factors that directly influence legumes growth (such as water, nutrient availability, pathogens and pests, crop husbandry practices and natural resource management that either limit the presence of effective rhizobia in the soil or enhance competition for soil mineral N) are critical to the amount of atmospheric N_2 fixed by the legume–rhizobium symbiosis

(Peoples et al., 2009; Weisany et al., 2013). An increase in soil concentration of nitrate can inhibit N_2 fixation quite severely.

The results described above argue for the use of rhizobia inoculation in large-scale agronomic systems, and this has been attempted at various times around the world. Among the greatest success stories was the inoculation of soybean with *Bradyrhizobium* in Brazil. The breeding of soybean cultivars in environments that made grain yield highly dependent on SNF, and the continuous selection of *Bradyrhizobium* strains appropriate for the newly released cultivars largely contributed to the higher grain yield and benefited the Brazilian economy. Estimates are that more than US$ 10 billion are saved per crop season in Brazil by SNF with soybean (Alves et al., 2003; Hungria et al., 2006b; Hungria and Mendes, 2015). Cowpea is the predominant crop in large part of the semiarid regions of Brazil. Cowpea inoculated with strain BR3267 showed grain productivity similar to the plants receiving 50 kg N ha^{-1} in the dryland areas in Brazil (Martins et al., 2003).

Many soils in Africa are severely depleted of nitrogen, and most often, smallholder farmers often cannot access or afford to apply chemical fertilizer. A pan-African project (N_2 fix for Africa), aimed at improving soil nitrogen fertility, has recently benefited to more than 250,000 smallholder farmers across eight countries with better legume genotypes and rhizobium inoculants. These, in addition to phosphorus fertilizer and improved crop management practices, often doubled the legume grain yields, thereby leading to an increase of US$ 335 $year^{-1}$ net household income on average for the farmers involved with the project (http://www.iita.org/2013-press-releases/-/asset_publisher/CxA7/content/putting-biological-nitrogen-fixation-to-work-for-smallholder-farmers?redirect=%2Fhome).

Research on host plant–rhizobium system in the past was heavily dependent on improving the rhizobium bacteria. However, the influence of the bacterial strain in influencing N_2 fixation is likely to be relatively small compared to that through regulation by the plant under stress conditions; as is the case with most of the grain legumes grown under marginal lands in developing countries (Sinclair and Vadez, 2012).

Plant breeding research in the 1980s and 1990s focused at combining high symbiotic nitrogen efficiency into improved genetic backgrounds in common bean and soybean, with some germplasm and breeding lines with high N_2 fixation being released. The International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria) succeeded in identifying promiscuous soybean germplasm and in further developing high-yielding promiscuous cultivars that were released elsewhere in Africa. There is now a broad and

greater acceptance on the need to simultaneously improve both host plant and rhizobium to harness the strength of the positive symbiotic interactions in cultivar development. This becomes more important in the view of advances made in genomics of rhizobium and several model SNF legumes. For example, some grain legumes have sufficient genomic data so that researchers can use the sequence variation in these crops to identify genetic markers associated with increased SNF and transfer these valuable alleles into improved genetic backgrounds. A large number of specific genes influencing the legume–rhizobia interactions have been cloned or analyzed with forward and reverse genetics. Likewise, the sequence variations among rhizobium genomes may provide insights to the genetic basis of promiscuity in rhizobium that may help in identifying other promiscuous rhizobium strains.

DNA markers can be used as tags in the genome while developing high-yielding cultivars with inherent capability to meet their nitrogen requirements through symbioses, which will reduce the need for inorganic fertilizers, and thus protect the environment (Fan et al., 2010; Sebilo et al., 2013). This review provides an overview of the genomic-led advances and other interventions that harness host plant–rhizobium symbiosis towards increasing grain legumes productivity.

2. HOST PLANT AND ENVIRONMENTAL STRESS FACTORS IMPACTING SNF

2.1 Host–Rhizobium Physiological and Biochemical Factors

Ontogenetic interactions between photosynthesis and SNF in legumes are of critical importance. This importance of photosynthesis for SNF in legumes has been inferred from various physiological studies that altered the availability of photosynthetic products and resulted in corresponding change in SNF (Wilson et al., 1933; Hardy and Havelka, 1975). For example, it has been shown that the photosynthesis rates at different stages of development in bean and pea are related to SNF in the root nodules, while the net carbon exchange rate of each leaf in these two pulses varied directly with carboxylation efficiency and inversely with the CO_2 compensation point. The net carbon exchange of the lowest leaves, which supplies fixed carbon to root nodules decreased in parallel with H_2 evolution from root nodules (Bethlenfalvay and Phillips, 1977). Furthermore, it is known that the photosynthates are imported into nodules, and are used as

carbon skeletons in ammonia assimilation (Larrainzar et al., 2009). When photosynthates are not metabolized due to partial or complete blockage of SNF, the accumulation of starch likely occurs (Ben Salah et al., 2009). An appropriate amount of SNF in bacteroids, however, can be achieved by maintaining the levels of photoassimilates, which are mainly sucrose at threshold levels (Ben Salah et al., 2009, 2011).

In a recent study on the role of nitrogen and carbon metabolism on SNF in cowpea, Rodrigues et al. (2013) reported that plants co-inoculated with *Bradyrhizobium* species and/or two plant growth-promoting bacteria (PGPB: *Paenibacillus durus* and *Paenibacillus graminis*) induced higher nitrogen content in nodules, total nitrogen accumulation, and shoot dry weight compared in the triple inoculation with other combinations when evaluated at the beginning of senescence. This increased nitrogen performance was positively correlated with the nodule sucrose content, but not with the content of total soluble carbohydrates, reduced sugars, and starch. Furthermore, their research showed that higher SNF under triple inoculation treatment was not significantly associated with sucrose synthase activity, but was weakly associated with soluble acid invertase activity in nodules at the beginning of senescence. Glutamate synthase, glutamine synthetase, and glutamate dehydrogenase were stimulated by double (*Bradyrhizobium* species plus *P. durus* or *Bradyrhizobium* plus *P. graminis*) and triple inoculation compared with only *Rhizobium* inoculation. These authors concluded that the inoculation with *Bradyrhizobium* species and PGPB is favorable for stimulating SNF activity in cowpea. However, legumes are not C-limited under symbiotic conditions (Neves and Hungria, 1987; Kaschuk et al., 2009), and indeed, that SNF can stimulate photosynthesis and vice versa (Kaschuk et al., 2012).

2.2 Mineral Nutrition of the Host Plant, High Nitrates in Soils, and Starter Nitrogen

Mineral nutrition of the host plant can affect SNF via host plant growth and development as well as through the process of nodule development and function as this process rests on the symbiosis between the rhizobium and the legume. The essential mineral nutrients required for legume SNF are those required for a normal establishment and functioning of the symbiosis. They are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), boron (B), molybdenum (Mo), chlorine (Cl), nickel (Ni), and cobalt (Co). Each essential nutrient performs

specific physiological and biochemical roles, and is required in optimum concentrations in the medium for the establishment and function of symbiosis between the legume host and the rhizobium. The role of mineral nutrients on SNF has been reviewed elsewhere (O'Hara et al., 1988; Zahran, 1999; O'Hara, 2001; Weisany et al., 2013), and we provide a synthesis below.

Among the major nutrients, phosphorus is essential for both nodulation and N_2 fixation. Indeed, nodules are strong sinks for phosphorus; as a consequence, symbiotic nitrogen-fixing plants require more phosphorus than those supplied with mineral fertilizers. The mode of nitrogen nutrition of legumes affects their phosphorus requirement (Cassman et al., 1981a,b). For achieving the potential of SNF by legumes, an adequate supply of phosphorus is a prerequisite because some legumes do not get established in conditions of insufficient soil P (Sahrawat et al., 2001). Mycorrhizal infection of roots of legumes stimulates both nodulation and nitrogen fixation under low phosphorus soil conditions (Redecker et al., 1997).

A relationship between SNF, P concentration, and soil pH exists that is important to researchers and agronomists alike. Soil pH in the neutral range optimizes the availability of all nutrients. In acidic pH soils, the availability of nutrients such as Ca, Mg, and P becomes limiting; on the other hand in soils with pH in the alkaline range the toxicity of sodium is the likely stressful that affects nodulation and nitrogen fixation. Thus soil pH is an important soil characteristic that indicates the availability of plant nutrients. Moreover, soil pH also directly influences nodulation and SNF through its effect on the numbers of naturally occurring rhizobium in noncultivated soils (Brockwell et al., 1991).

A review of the published literature on the effects of starter N application on SNF by legumes indicates mixed results relative to the basal application of small amount of mineral N. However, it is widely accepted that in high fertility soils, especially those rich in organic matter, the application of starter N is not necessary; and at times can reduce nodulation and SNF in crops such as soybean (Mendes et al., 2003; Hungria et al., 2006b) and bean (Vargas et al., 2000). In soybean, the application of N at later stages of plant growth also do not promote yield (Hungria et al., 2006b). On the other hand, in soils of low to very low in fertility and organic matter, the application of starter N at rate of 20–30 kg ha^{-1} has generally been reported to be beneficial to the growth and yield of several legumes (Erman et al., 2009; Sulfab et al., 2011; Sogut et al., 2013). Clearly, there is no single recommendation on starter N application because the beneficial effect of the basal N to

legumes depends on the fertility status of the soil relative to N concentration in the soil and the N needs of the plant.

It has long been established that nitrates in the soil inhibit root infection, nodule development, and nitrogenase activity. Likewise, adequate nodulation is necessary for maximizing SNF by a legume (Atkins et al., 1984; Imsande, 1986; Sanginga et al., 1996). Moreover, poor and scanty nodulation is generally not able to satisfy the N needs of the plants, and therefore they rely on soil N to grow and produce (Zahran, 1999).

2.3 Drought, Salinity, and Heat Stress

Agricultural operations during crop production especially tillage, soil, nutrient and water management practices, and the use of crop protection practices greatly influence the population and efficacy of rhizobia in diverse production systems (Zahran, 1999; Hungria and Vargas, 2000; Giller, 2001). However, it has been observed that rhizobia can survive and exist in drier areas, but their population densities are at their lowest ebb under dry soil conditions. Therefore, drought seriously affects SNF, in addition to of course the effect of drought on the growth and development of the host legume. At times, it is hard to separate the effect of drought from that of heat stress as these two generally occur simultaneously especially in semiarid and arid tropical regions (Wery et al., 1994; Sinclair and Serraj, 1995; Zahran, 1999; Giller, 2001; Serraj and Adu-Gyamfi, 2004). Suitable strains of rhizobia that can survive and perform under moisture shortage and heat stress conditions in symbiosis with legumes are of critical importance, and research attention have been devoted to this aspect with some success (Busse and Bottomley, 1989; Hunt et al., 1981; Osa-Afina and Alexander, 1982; Rai and Prasad, 1983; Hungria et al., 1993; Giller, 2001).

To make the symbiosis effective under water and heat stresses, legumes tolerant to these stresses need to be combined with effective *Rhizobium* strains appropriate for each legume species and each type of growing environment. There are legume landraces and cultivars tolerant of high N_2 fixation under drought or high temperature (Keck et al., 1984; Rai and Prasad, 1983; Venkateswarlu et al., 1983, 1989; Devi et al., 2010).

Apart from high-alkaline low P soils with drought and heat stresses, salinity is one of the major constraints to growing of legumes in the semiarid and arid regions of the world. Salts have direct detrimental effects on the crop and have deleterious effect on the microbial populations including rhizobium (Tate, 1995; Serraj and Adu-Gyamfi, 2004). For salt-affected

environments, salinity tolerance of both the host legume and the rhizobium are a prerequisite.

Genetic variability in response to salts has been reported in legumes. However, the expression of tolerance to salts is a complex phenomenon, and is conditioned by several edaphic and environmental factors including soil characteristics, climatic conditions, and the stage of the crop growth. For example, faba bean, common bean, and soybean have been reported to be relatively more salt tolerant than pea as judged by nitrogen fixation (nitrogenase activity) during the growing season (Abdel-Wahab and Zahran, 1981). It has also been reported that the legume–rhizobium symbiosis and nodulation are more sensitive to salts than the actual rhizobium in some legume systems (Velagaleti and Marsh, 1989; El-Shinawi et al., 1989; Subbarao et al., 1990).

The inoculation of legumes with salt-tolerant strains of rhizobia will most likely improve SNF in salt-affected environments; however, to potentially exploit this advantage, the tolerance of the host legume is more important and essential to form a successful symbiosis in salt-affected environment than the bacterial strain alone (Craig et al., 1991). Salt-tolerant rhizobium strains have been reported which make these rhizobia highly valuable inocula to improve the productivity of the leguminous plants cultivated under saline environments (Ogutcu et al., 2010; Sharma et al., 2013).

Heat stress under high temperature is common not only in semiarid and arid regions, but also in the tropics and is a major impediment to SNF by legumes. As in the case of drought, the heat stress also adversely affects rhizobium effectiveness and efficacy, host legume growth and development, and symbiosis (Michiels et al., 1994; Hungria and Kaschuk, 2014). High root temperatures strongly affect rhizobium populations and SNF in legumes including soybean, guar, groundnut, cowpea, and beans. The critical temperatures for SNF vary widely (from 30 to 42 °C) with the legume and rhizobium strain (optimum temperature range is 28–31 °C). However, legumes (Hungria and Franco, 1993) and rhizobium strains (Graham, 1992) have the capacity to adapt to high temperature stress, leading to effective symbiosis and SNF.

One mechanism of heat tolerance is the synthesis of heat-shock proteins, which has been reported in both heat-tolerant and heat-sensitive bean-nodulating rhizobium strains at different temperature (Graham, 1992; Michiels et al., 1994). Research has also indicated that temperature stress consistently promoted the production of a protein with a relative mobility of 65 kDa in four strains of tree legume rhizobium. The 65 kDa detected under heat stress

was heavily overproduced and is specific to heat stress, but not overproduced under salt stress (Zahran et al., 1994). There is the need to better understand the role of synthesis and production of heat-shock protein so that it can be employed for improving the symbiosis between legumes and rhizobia.

Clearly, SNF by legumes in the tropical regions is constrained by multiple stresses as discussed above. Under these stress situations, the SNF contribution to the production systems remains sub-optimal at best. Therefore, there is a need to intensify research to make the symbiosis between host legumes and rhizobia optimum by alleviating stresses related to nutrient deficiency, water deficit, heat and salts, among others, which will lead to improving SNF in agroecosystems.

3. GENOMICS-LED INTERVENTION TO SELECT FOR PROMISCUOUS GERMPLASM

3.1 Selection Environment for Evaluating Germplasm and Breeding Populations for SNF

Several environmental, edaphic, plant, and mineral nutrient-related factors affect SNF and yield of grain legumes (see Sections 2.2 and 2.3). To evaluate and select legume cultivars for potential SNF, the selection environment (controlled conditions in greenhouse or in the field) has to be well defined so that reliable and repeatable results can be obtained and compared across sites and among researchers.

The basic methodology for SNF experiment would depend on the objective of the study. For example, if the aim is to select promiscuous germplasm for farmer's field conditions, especially relative to soil fertility status, the selection can be made without any nutrient amendments in both controlled and field conditions. However, if the purpose is to select for a cultivars' overall SNF potential, then the growing conditions in greenhouse or in the field need to be optimized, especially relative to nutrients and soil moisture. In controlled condition, greenhouse selection of cultivars, the environmental factors such as temperature, photoperiod, and humidity in addition to nutrients and soil moisture need to be optimized. In such controlled condition selection, the role of certain specific factors such as the lack or excess of individual nutrients, the effect of salt stress or water deficit among others can be further studied. The inputs from such studies can become a part of the dynamic standard methodology for the selection of legume cultivars for SNF potential in variable environments. In summary, it is important to follow a standard methodology under both controlled

conditions in the greenhouse and selection in the field for high SNF potential and yield of grain legumes evaluated.

The initial evaluation of a large number of germplasm lines or cultivars is best done under controlled conditions in a greenhouse under balanced plant nutrition, which implies providing optimum soil moisture and supplying all nutrients that are deficient minus N in whatever supply format that takes. The temperature and other climatic factors for the most part should be maintained close to conditions that crop is likely to experience during the growing season in the field. However, it should be emphasized that the final and most realistic evaluation of a cultivar has to be done under well-defined practical agricultural conditions in the field.

For evaluation of the potential of germplasm for SNF and yield, the conditions relative to soil, nutrient and water management should be standard and defined. It is known that nutrients such as P greatly influence SNF and yield of legumes, in addition to the factors of environment, rhizobium, and plant along with interactions among these (see Sections 2.2 and 2.3). If the objective is to evaluate the potential for SNF and yield of legumes, then all plant nutrients except N should be maintained in the range of sufficiency. The soil moisture regime also has to be defined similarly (optimum or sub-optimum). Following evaluation of cultivars under controlled greenhouse conditions and in the field to establish the potential for SNF and yield, the promising cultivars will be advanced to farmers' fields for similar trait evaluations. However, it is important to characterize and record the environmental, rhizobium, soil, and crop management practices used during on-farm evaluations.

To summarize, the selection environment needs to be defined during the evaluation of promiscuous grain legume germplasm for SNF under controlled condition in the greenhouse, in the field under research station management conditions, and finally under on-farm conditions in farmers' fields. In addition, the methodology has to be dynamic in nature and would vary with the objective of the selection. This systematic strategy will provide rich data sets to facilitate identification of grain legume cultivars with superior SNF and yield capacity under controlled, semicontrolled, and on-farm conditions (Van Kessel and Hartley, 2000; Rengel, 2002).

3.2 From Conventional to High-Throughput Assays to Phenotype N_2-Fixing Traits

Research on SNF suggests that several plant traits are associated with nitrogen fixation in grain legume crops, including nodule number and nodule

weight, root and shoot weight, total biomass, and percent and total atmospheric N_2 fixed (see Section 3.3.3). An accurate estimation of the total atmospheric N_2 fixed and phenotyping of traits associated with nitrogen fixation is a prerequisite to detect genetic variation associated with nitrogen fixation in crop germplasm. The most commonly used methods in the past include (1) difference method, (2) xylem-fluid method, (3) acetylene reduction activity (ARA), and (4) ^{15}N method (Herridge and Danso, 1995). The "difference method" is based on comparing the differences in the quantity of N absorption between plant with and without SNF capability (a cereal crop or non-nodulating legume isoline is used as non-nitrogen-fixing controls). Meanwhile, the "xylem-fluid method" is based on the fact that the SNF process results in ureide accumulation in this tissue while a non-SNF process produces nitrate (NO_3). The acetylene reduction activity method is based on the function of the nitrogenase enzyme, which converts acetylene (C_2H_2) to ethylene (C_2H_4), while the "^{15}N method" uses isotopically labeled fertilizer or naturally abundant ^{15}N to calculate rates of N_2 fixation.

All these methods have strengths and weaknesses. For example, in the difference method, the estimates are either overestimated or underestimated, while xylem-fluid method is difficult to perform in field conditions. The acetylene reduction activity method is fast, inexpensive, and easy to perform but nitrogenase activity declines rapidly in the presence of acetylene, with a concurrent reduction in respiration, thus it results in underestimating the atmospheric N_2 fixed; however, this method may be used as an initial screen to identifying qualitative differences in nitrogenase activity in the germplasm. The ^{15}N method is suitable and viable but ^{15}N and the instrumentation used for its detection is expensive and the process is often time-consuming with only a few specialized laboratories setup to perform the assay (Hardy et al., 1968: Minchin et al., 1983, 1986).

Digital image analysis allows rapid and nondestructive phenotyping of various parameters after segmentation of an image and extraction of quantitative features from the segmented objects of interest (Hatem and Tan, 2003). This technique is now increasingly being used in agricultural and food science research. Likewise, the differences in leaf chlorophyll content have been used to differentiate promiscuous and nonpromiscuous soybean germplasm (Gwata et al., 2004). Using a Minolta SPAD spectrometer in parallel with a leaf digital image analysis procedure based on a commercially available still camera, Vollmann et al. (2011) phenotyped a large number of soybean breeding lines including near-isogenic families, grown in the field across three seasons, for leaf chlorophyll content, nodulation,

agronomic, and seed traits, which revealed that nodulating and non-nodulating soybean lines significantly differ in chlorophyll content from the V5 soybean developmental growth stage (five fully developed leaves) onwards. The chlorophyll content of the soybean breeding lines was significantly correlated ($r = -0.937$) with the green color value (RGB color model) of leaf image analysis at the R3 soybean developmental growth stage (beginning of pod growth). Furthermore, both chlorophyll content (SPAD values) and leaf image analysis parameters (color values from RGB and HSB color models) were correlated with 100-seed weight, seed protein, and oil contents.

The results described above indicated that leaf parameters related to photosynthesis and nitrogen fixation could be utilized to determine the nitrogen status of a soybean crop and subsequently in forecasting seed-quality parameters of the harvestable product. Additional uses of digital imagery are needed for other legume crops to validate the utilization in different types of germplasm and soil conditions.

Nodulation status in the field for the most part has been determined by destructively harvesting roots from soil by coring, trenching, or uprooting of plants (Grubinger et al., 1982), requiring significant effort and time to excavate and separate roots from the soil. This procedure of determining nodulation is destructive, labor intensive and time-consuming. To address the concerns of manual and destructive sampling, Gray et al. (2013) developed a minirhizotron imaging system as a novel in situ method for assessing the number, size, and distribution of nodules in field-grown soybean exposed to elevated atmospheric CO_2 and reduced precipitation. They detected 134–229% greater nodule numbers in soybean grown under reduced precipitation and elevated CO_2 due to greater nodule density per unit root length, which demonstrated the potential of their imaging system to reveal changes in nodule production and distribution in response to environmental change. A further test may be needed in order to know whether this technique can be applied in plant breeding programs to identify promiscuous legume germplasm.

3.3 Genetic Variation and Traits Associated with SNF

3.3.1 Variability for SNF in Germplasm

Variation in plant genetic resources provides the basis and the raw material that plays a fundamental role in crop improvement programs. The CGIAR Consortium, USDA and other national agricultural research organizations hold large collections of germplasm, both cultivated and are wild or weedy

relatives of legume crops. A survey of literature during the periods from 1978 to 2013 revealed that limited germplasm sets have been screened for SNF and only in selected legumes, with most of these research targeting common bean, cowpea, and soybean (Table 1).

Published reports have identified a number of germplasm accessions that fix more atmospheric N_2 compared to others under similar conditions. For example, Pulver et al. (1985) evaluated 400 germplasm accessions and identified 10 highly promiscuous soybean accessions (Malayan from Nigeria; Obo from Central African Republic; Hernon 237 from Tanzania; Indo# 180, 216, 226, and Orba from Indonesia; TGm 119 and TGm 120 from East Africa; and M 351, a breeding line originated from a Malayan and Clemson Non Shattering cross), that showed effective symbiosis with soil rhizobia (25 indigenous rhizobia strain) across five diverse environments in Nigeria, ranging from the high rainfall, acid-soil zone (4°N) to the semiarid Northern Guinea Savannah (11°N). The soils in these locations were low in N and without the history of soybean cultivation. These highly promiscuous genotypes were later on included by the International Institute of Tropical Agriculture (IITA) to develop highly promiscuous and productive soybean cultivars (http://cdn.intechweb.org/pdfs/14933.pdf).

Large differences in N_2 fixation (42–93 mg N_2 per plant) among soybean cultivars have also been noted in cultivars from North America and Brazil. J 200, Bossier, Ivaí, and BR 29 accumulated 30% more N_2 than the mean of the cultivars evaluated (63.7 mg N_2 plant^{-1}) (Hungria and Bohrer, 2000).

A pioneering experiment involving about 20 common bean germplasm accessions evaluated across six countries (Austria, Brazil, Chile, Colombia, Mexico, and Peru) identified country-specific high N_2-fixing lines with those from Austria showed sixfold differences in N_2 fixation (Hardarson et al., 1993). Meanwhile those from the IAEA program in Austria selected for low and high SNF showed sixfold differences in N_2 fixation. Selected phosphorus (P) deficiency-tolerant common bean germplasm showed higher SNF (127.7 mg N_2 plant^{-1}) compared to 47.8 mg N_2 per plant observed in the P deficiency sensitive lines under limited P supply (70 m mol P per plant) (Vadez et al., 1999). Climbing beans were found to fix more N_2 than bush type beans (Rennie and Kemp, 1983).

Some cowpea genotypes such as Fahari, Pan311, Glenda, TVu11424, Mamlaka, Botswana White, Ngonjii, Encore, IT90K-76, IT84S-2246, IT93K-2045-29, CH14, and Vuli-1 fixed 102–182 kg N_2 ha^{-1} compared with ITH98-46, which derived only 49.6 kg N_2 ha^{-1}; and most importantly, these high N_2-fixing genotypes produced greater biomass and grain yield

Table 1 Summary of results on the screening of germplasm and cultivars for symbiotic nitrogen fixation (SNF) in chickpea, common bean, cowpea, groundnut, mungbean, and soybean

Number of germplasm screened	Summary of the range variation for SNF	References
Chickpea		
6	Variation in percent N_2 fixed (76.6–86.7%), aboveground biomass (154–283 mg per plant), and belowground biomass (271–386 mg per plant); with cultivar Sierra, Troy, and Almaz had high proportion of N fixed (85–87%)	Abi-Ghanem et al., 2012
40	Proportion of plant N_2 fixed among the subset of genetically diverse USDA chickpea core collection accessions ranged from 47% to 78%, while these accessions showed fourfold differences in total N fixed (TNF) (0.02–0.084 g per plant); ILC 235 from Iraq produced the greatest TNF, 0.084 g per plant	Biabani et al., 2011
Common bean		
4	Pubela, the highest N_2 fixer; targeted traits for improving SNF and yield include moderate number of nodules, leaf ureide content, total biomass at flowering, and nodule effectiveness	Kabahuma, 2013
30	European germplasm showed large genotypic variability for SNF, with accessions PHA 0013, PHA 0014, PHA 0034, and PHA 0053 as potential candidate for high SNF	Rodiño et al., 2005
220	Phosphorous deficiency-tolerant lines (G 19348, BAT271, ICA Pijao, G15839, G3456, G17722, G11088, G11087, G11087, G19839, G21130) at 50 days after sowing had fixed 127.7 mg N_2 per plant compared to 47.8 mg per plant in sensitive lines under limiting P supply (70 m mol P per plant)	Vadez et al., 1999

(*Continued*)

Table 1 Summary of results on the screening of germplasm and cultivars for symbiotic nitrogen fixation (SNF) in chickpea, common bean, cowpea, groundnut, mungbean, and soybean—cont'd

Number of germplasm screened	Summary of the range variation for SNF	References
9-30	High N_2-fixing cultivars included Riz 44 and Bat 322 in Austria; Honduras 35 and Carioca in Brazil; Red Mexican INIA and Don Timoteo in Chile; A 268 in Colombia; ICTA San Martin, ICTA Panamos and ICTA Quenack-Ché in Guatemala; Azufrado, Negro Colima and Negro Poblano in Mexico; Cabalero, Caraota, Blanco, Bayo Norma, Canario G-62-2-6 and Bayo G-7.5-9 in Peru; with cultivars from Austria showing 6 fold differences in N_2 fixation	Hardarson et al., 1993
5	High N_2-fixing lines (WBR 22-3, 22-8, 22-34, 22-50, and 22-55) selected from an advanced back cross-population involving ICA Pijao × Puebla 152	Bliss et al., 1989
26	N_2 fixed varied between 40 k ha^{-1} and 125 kg ha^{-1} depending on the cultivar, with climbing beans fixing higher N than bush beans	Rennie and Kemp, 1983
18	Five to sixfold differences as measured by acetylene reduction between cultivars, 23.4−120.1 $N_2(C_2H_2)$ fixed; relative N_2 fixation increased as the average seasonal nodule weight increased	Westermann and Kollar, 1978
Cowpea		
7	Costela de Vaca had significantly higher atmospheric N_2 fixed (45 kg ha^{-1}) than other landraces (22−30 kg N_2 ha^{-1}); however, it had lowest grain yield (381 kg ha^{-1}) and harvest index (0.14) compared with others (grain yield, 889−1147 kg ha^{-1}; harvest index, 0.37−0.45)	de Freitas et al., 2012
9	N_2 fixed in intercropping system (with maize) ranged from 11.4 to 51.7 kg ha^{-1}, with IT99k-377-1 being the highest N_2 fixer	Egbe and Egbo, 2011
32	Cultivar, Fahari obtained 80.9% of its N from symbiotic fixation and fixed ~182 kg N_2 ha^{-1}, followed by Pan311, Glenda, TVu 11,424, Mamlaka,	Belane et al., 2011

	Botswana White, Ngonji, Encore, IT90K-76, IT84S-2246, IT93K-2045-29, CH14, and Vuli-1 (contributing 102–160 kg N ha^{-1}), while ITH98-46 derived only 48.3% of its shoot N from symbiotic fixation, and contributed ~49.6 kg N_2 ha^{-1}; genotypes that fixed more N also produced more biomass and grain yield	
9	Large variability in N_2 fixation among cultivars in Ghana (49–155 kg N ha^{-1}) and South Africa (51–155 kg N ha^{-1}); cultivars responded differently to fixed N_2, the highest N_2-fixing Omandaw cultivar (155 kg N ha^{-1}) in Ghana had 74 kg N_2 fixed in South Africa, while Fahari which fixed highest N_2 (155 kg ha^{-1}) in South Africa had only 84 kg N_2 ha^{-1} fixed in Ghana	Pule-Meulenberg et al., 2010
30	N_2 fixation ranged from 14.1 kg N ha^{-1} by cv. TVu1509 to 157 kg N ha^{-1} by IT84S-2246 in 2005 and from 16.7 kg N ha^{-1} by cv. ITH98-46 to 171.2 kg N ha^{-1} by TVu11424ed	Belane and Dakora, 2009
7	N_2 fixation among 7 genotypes evaluated for two years on two acid soils low in available P ranged from 29 to 51 kg ha^{-1}, which was significantly increased with P application; IT89KD-391 and IT90K-59 efficient in N_2 fixation and P uptake	Jemo et al., 2006
7	Host–rhizobium specific response detected for acetylene reduction activity acetylene reduction activity as measure of N_2 fixation: Highest acetylene reduction activity (18.72 μmol C_2H_4 h^{-1} per plant) in Diongoma with rhizobium strain ISRA 312, while with NGR 234, it was Mougne which had highest acetylene reduction activity (14.91 μmol C_2H_4 h^{-1} per plant); Mouride had the lowest acetylene reduction activity with both rhizobium strains (1.60 and 1.85 μmol C_2H_4 h^{-1} per plant)	Fall et al., 2003

(*Continued*)

Table 1 Summary of results on the screening of germplasm and cultivars for symbiotic nitrogen fixation (SNF) in chickpea, common bean, cowpea, groundnut, mungbean, and soybean—cont'd

Number of germplasm screened	Summary of the range variation for SNF	References
16	Percentage of N_2 derived from the atmosphere (%Ndfa) and amount of % Ndfa (mg per plant), respectively, ranged from 33.3% to 74.5% and from 220 to 960 mg per plant, with cultivar Ndoute being the highest *Ndfa*, 960 mg N and 38 g N per plant in shoot and root, respectively	Ndiyae et al., 2000
Faba bean		
6	Percent atmospheric nitrogen fixed by six Ethiopian cultivars ranged from 59% to 84%, with total N_2 ranging from 218 to 362 kg N ha^{-1}	Nebiyu et al., 2014
16	Percent N_2 fixed varied from 40% to 83% across years and locations	Duc et al., 1988
Groundnut		
6	Total N varied from 48 to 162 mg per plant, while total fixed N from 41 to 132 mg per plant, with KKU 72-1 being the highest N_2 fixer	Pimratch et al., 2004
6	Nitrogenase activity (μmoles C_2H_4 per plant h^{-1}) ranged from 27 to 89, while total N_2 fixed from 262 to 557 mg per plant; NcAc 2821 being the highest in nitrogenase activity and total nitrogen fixed	Nigam et al., 1985
30 F_2 derived families	NC 6 superior in nodule number and weight, and nitrogenase activity, with more variation for these traits in F_5/F_6 families	Arrendell et al., 1985

Lupin		
80	Significant differences among cultivars for nodulation, root and shoot dry weight; PI# 250094, 457921, 237719, 457924, and 250572 were the source lines for high N_2 fixation traits	Robinson et al., 2000
Mungbean		
7	Percent atmospheric N derived among parents varied from 27% to 53% and fixed N_2 varied from 0.023 to 0.104 g per plant; the % N derived from atmosphere and fixed N_2 among 21 F_1 hybrids varied from 38% to 61% and from 0.010 to 0.168 g per plant, respectively	del Rosario et al., 1997
Pea		
7 and RILs (Cameor × Ballet)	Tenfold variation in nodule (nodule number, nodule weight, total nodule projected area) and twofold variation in root (number of lateral roots, total root length and root biomass) traits at four-leaf stage in germplasm; tenfold differences and transgressive segregants for nodule number, nodule biomass, and total projected nodule area per plant at the beginning of flowering,	Bourion et al., 2010

(*Continued*)

Table 1 Summary of results on the screening of germplasm and cultivars for symbiotic nitrogen fixation (SNF) in chickpea, common bean, cowpea, groundnut, mungbean, and soybean—cont'd

Number of germplasm screened	Summary of the range variation for SNF	References
Soybean		
31	Twice the variation in nodule number (23–57 nodules per plant) and nodule mass (24–57 mg per plant) among cultivars; nodulation unrelated to the maturity groups	Salvucci et al., 2012
25	Several fold differences in nodule number (2–21) and nodule weight (0.05–0.3 g per plant); TGx 1921-2F the only genotype with potential to use in breeding for high N_2 fixation	Ojo et al., 2007
152	Large differences in number of nodules (35–72 per plant), nodule weight (145–289 mg nodule per plant), and N_2 fixed (42–93 mg N per plant); J-200, Bossier, Ivaí and BR-29 accumulated 30% more N_2 than averaged 63.7 mg N per plant; large differences in nodule efficiency, from 246 mg N_2 g^{-1} nodule in EMG 304 to 460 mg N g^{-1} nodule in RS6	Hungria and Bohrer, 2000
17	Large variation in number of nodules (40–103 per plant) and Nodules weight (201–465 mg per plant)	Sinclair et al., 1991
400	Highly promiscuous germplasm, Malayan, Obo, Hernon 237, Indo# 180, 216, 226, Orba, TGm 119, TGm 120, and M-351, having effective symbiosis with soil rhizobia at all five sites tested in Africa	Pulver et al., 1985

(Belane et al., 2011). Of these, Fahari, Glenda, and Apagbaala were the most promiscuous cowpea germplasm or cultivars in trapping diverse strains across the evaluations made in Ghana, Botswana, and South Africa. For example, Fahari trapped nine strains, Glenda seven strains, and Apagbaala six strains. However, it must be added that the high N_2-fixing germplasm are not always more productive. For example, de Freitas et al. (2012) found that costela de vaca accumulated significantly higher atmospheric N_2 (45 kg ha^{-1}) than did other landraces (22–30 kg N_2 ha^{-1}). However, this cultivar gave lowest grain yield (381 kg ha^{-1}) and had lowest harvest index (0.14) compared to other cultivars (grain yield, 889–1147 kg ha^{-1}; harvest index, 0.37–0.45).

For groundnut, NC 6, NCAc 2821, and KKU-72-1 have been identified as high N_2-fixing germplasm (Nigam et al., 1985; Arrendell et al., 1985; Pimratch et al., 2004). NC6 and KKU-72-1 are Virginia bunch types, released for cultivation in the United States and Thailand, respectively, while NCAc 2821 is a Virginia runner type germplasm line. Biabani et al. (2011) reported fourfold differences in the total N_2 fixed (0.02–0.84 g $plant^{-1}$) in a subset of genetically diverse USDA chickpea core collection accessions, with accession ILC 235 from Iraq being the greatest N_2 fixer.

The formation of representative subsets in the form of core (Frankel, 1984) and mini-core (Upadhyaya and Ortiz, 2001) collections has been suggested as the gateway to discover new sources of variation for enhanced utilization of agronomically beneficial germplasm in crop breeding. In several grain legume crops, the core or mini-core collections were formed based on passport, characterization, and evaluation data; e.g. for chickpea, common bean, cowpea, faba bean, lentil, mungbean, pea, pigeonpea, and soybean (Dwivedi et al., 2005; Upadhyaya et al., 2006; Mahalakshmi et al., 2007; Liu and Hou, 2010; Kwon et al., 2012; Khazaei et al., 2013; Qiu et al., 2013). More importantly, molecularly profiled and genotypically diverse reference sets derived from composite collections are also available in chickpea, common bean, cowpea, groundnut, and pigeonpea (http://gcpcrgrinfo.net). These reduced subsets represent most of the diversity that is present in the entire collection of a given species and therefore they are ideal resources to identify high N_2-fixing germplasm.

3.3.2 Genotype, Environment, and Strain Interactions

Knowledge of the genotype × environment and genotype × rhizobium strain interactions is a prerequisite to identifying germplasm and rhizobium strains for effective symbiosis in legumes. Research in cowpea suggests high genotype × location (environment) and genotype × rhizobium strain

interactions. For example, the highest N_2-fixing Omondaw cultivar (155 kg N_2 ha^{-1}) in Ghana fixed only 74 kg N_2 ha^{-1} in South Africa, while Fahari, the highest N_2-fixing line (155 kg N_2 ha^{-1}) in South Africa fixed only 84 kg N_2 ha^{-1} in Ghana. The low N_2-fixing line (ITH98-46), however, had maintained its low N_2-fixing ability (49–51 kg N_2 ha^{-1}) at sites in both the countries (Pule-Meulenberg et al., 2010). Likewise, cowpea cultivar Diongoma with rhizobium strain ISRA 312 in Senegal showed highest acetylene reduction activity (18.72 μmol C_2H_4 h^{-1} per plant), a measure of N_2 fixation; while with rhizobium strain NGR 234, it was Mougne that gave highest acetylene reduction activity (14.91 μmol C_2H_4 h^{-1} $plant^{-1}$). Mouride, another cowpea cultivar, had however the lowest acetylene reduction activity with both rhizobium strains (1.60–1.85 μmol C_2H_4 h^{-1} per plant) (Fall et al., 2003). Such differences in responses to rhizobium strains should be factored while selecting for efficient host–rhizobium symbiosis for increasing productivity of grain legume crops.

3.3.3 Relationships of SNF with Agronomic Traits

An understanding of the nature of associations between nitrogen-fixing traits (nodule number, nodule weight, root and shoot weight, percentage of atmospheric N fixed, shoot nitrogen, and nitrogenase activity) among each other and also their correlations with agronomic traits (seed yield and 100-seed weight) should prove useful in the selection of productive and high N_2-fixing progenies in plant breeding. Available reports suggest highly significant and positive correlation between nodule number and nodule weight in chickpea, groundnut, mungbean and soybean; between nodule weight and N_2 fixation in common bean, cowpea, groundnut, mungbean, and soybean; nodule weight with root or shoot weight in chickpea, common bean, groundnut, mungbean, and soybean; shoot weight with nodule number, nodule weight, and N_2 fixation in groundnut, common bean, mungbean, and soybean; root weight with nodule number, nodule weight, and N_2 fixation in common bean and soybean; seed yield with nodule number, nodule weight and N_2 fixation in common bean, groundnut, and mungbean; and 100-seed weight with N_2 fixation in common bean and groundnut (Table 2).

High positive correlation coefficients mean that simultaneous improvement for more than one trait can be practiced by plant breeding program; i.e., high N_2 fixation and productivity traits can be selected simultaneously to develop high N_2-fixing and productive cultivars. A high and positive correlation of leaf color score with total N_2 fixed, nodule weight, and shoot and

Table 2 Summary of results on the relationships among nodule number, nodule weight, % N_2 in shoot, total N_2 fixed, nitrogenase activity, root weight, shoot weight, harvest index, seed yield, and 100-seed weight in chickpea, common bean, cowpea, groundnut, mungbean, and soybean

Number and type of materials	Trait combination	Correlation coefficient	References
Chickpea			
39 USDA core collection accessions	Nodule number and nodule weight	0.676*	Biabani et al., 2011
	% N_2 fixation with root weight and shoot weight	0.157*−0.208*	
	Nodule weight with root weight and shoot weight	0.247*−0.294*	
	Total plant weight (root, shoot, nodule) and nodule weight	0.357*	
	Root weight and shoot weight	0.786*	
Common bean			
50 Iranian germplasm	Grain yield, 100-seed weight, and harvest index with nodule number, N% in shoot, and total N_2 fixed	0.208*−0.584*	Golparvar, 2012
	Nodule number with total N% in shoot and total N_2 fixed	0.466*−0.517*	
	Total N% in shoot and total N_2 fixed	0.671*	
47 Andean, Meso-American gene pool	Root and nodule weight linearly correlated with mg N fixed per plant	0.71*−0.74*	Vadez et al., 1999

(*Continued*)

Table 2 Summary of results on the relationships among nodule number, nodule weight, % N_2 in shoot, total N_2 fixed, nitrogenase activity, root weight, shoot weight, harvest index, seed yield, and 100-seed weight in chickpea, common bean, cowpea, groundnut, mungbean, and soybean—cont'd

Number and type of materials	Trait combination	Correlation coefficient	References
	Shoot weight and mg N fixed per plant	0.46*	
	Acetylene reduction activity (μmol C_2H_4 per plant) and mg N fixed per plant	0.38*−0.54*	
8 cultivars	Nodule weight with mg N_2 fixed per plant	0.84*	Westermann and Kolar, 1978
	Plant weight and seed yield with mg N_2 fixed per plant	0.55*−0.74*	
Cowpea			
32 genotypes and two years	Biomass with plant N fixed; shoot weight with shoot N fixed	0.91*	Belane et al., 2011
	Biomass with nodule specific activity	0.31*	
7 genotypes, two soil types, and 2 years	Nodule weight with N_2 fixed	0.34*−0.46*	Jemo et al., 2006
	Phosphorus uptake and N_2 fixed	0.34*−0.44*	
	N_2 fixed and grain yield	0.42*	
	Nodule weight and grain yield	0.37*	
Groundnut			
7 genotypes and two years	Total N_2 fixed with nodule weight, shoot weight, and 100-seed weight	0.63*−0.97*	Pimratch et al., 2004
	Leaf color score with total N_2 fixed, nodule weight, shoot weight, and biomass (root and shoot) weight	0.63*−0.97*	
30 $F_{2:6}/F_{2:7}$ lines, 2 years and 3 samplings	N_2 fixed with nodule number, nodule weight, nitrogenase activity, specific nitrogenase activity, and shoot weight	0.55*−0.87*	Arrendell et al., 1985

30 F_1's, and six parents	Nitrogenase activity with nodule number, nodule weight, total nitrogen, shoot and root weight	0.54*−0.97*	Nigam et al., 1985
Mungbean			
21 F_1's and 7 parents	Nodule weight and nodule number	0.74*	del Rosario et al., 1997
	N_2 fixed with nodule number and nodule weight	0.67*−0.69*	
	Shoot weight with nodule number, nodule weight, and N_2 fixed	0.71*−0.95*	
	Seed yield with nodule number, nodule weight and N_2 fixed	0.60*−0.70*	
Soybean			
25	Nodule number and nodule weight	0.856*	Ojo et al., 2007
152 North American cultivars, three maturity groups	Nodule number and nodule weight	0.579*	Hungria and Bohrer, 2000
	Nodule weight with root weight, shoot weight, % shoot N, total N in shoot, and total N per plant	0.622*−0.697*	
	Shoot weight with total shoot N, and total N per plant	0.911*−0.915*	
17 genotypes and 3 years evaluation	Nodule number and nodule weight	0.835*−0.906*	Sinclair et al., 1991
	Root weight with nodule number and nodule weight	0.314*−0.670*	
	Shoot weight with nodule weight, nodule number and nodule weight	0.396*−0.840*	

* Weight, refers to dry weight of the sample.

biomass weight revealed that leaf color score could be used as an indicator of high N_2-fixing trait in groundnut (Pimratch et al., 2004). Leaf color has also been found associated to be with high N_2-fixing ability in soybean (Gwata et al., 2004). Low P in the soil-plant system adversely impacts N_2 fixation (Vance, 2001). The significant relationships between N_2 fixed and total P uptake in cowpea (Jemo et al., 2006) suggests that genetic differences for N_2 fixation under P deficiency are due to differences in P-uptake efficiency (Pereira and Bliss, 1987, 1989). Similar results in common beans are empirical. More studies are needed involving diverse germplasm and breeding populations to elucidate such relationships prior to exploiting them as indices in breeding for high N_2 fixation in agriculturally important grain legume crops.

In summary, the presence of variation for nitrogen fixation in the germplasm collection and the existence of moderate to high association between N_2-fixing and agronomic traits mean that nitrogen fixation per se may be improved by introgressing positive alleles from germplasm into locally adapted grain legume cultivars. Three-way interaction involving host (plant genotype), rhizobium (strain variability and effectiveness), and environment (location effect) may however complicate this breeding task, which should be factored in while selecting for high N_2 fixation. The identification of promiscuous germplasm and their use in crosses and breeding may help develop promiscuous grain legume cultivars, as demonstrated in soybean (http://cdn.intechweb.org/pdfs/14933.pdf).

3.4 Abiotic Stress and N_2 Fixation

SNF is highly sensitive to drought, which causes decreased N accumulation, and yield of legume crops. The major factors contributing to decline in nitrogen fixation under drought stress include oxygen limitation, carbon shortage, and regulation by nitrogen metabolism (Serraj et al., 1999). The evidence to date suggests that drought exerts a local inhibition of nitrogen fixation in pea and soybean (Marino et al., 2007; Ladrera et al., 2007; Gil-Quintana et al., 2013a), and in the model legume, *Medicago truncatula* (Gil-Quintana et al., 2013b). Further, proteomic analysis using partial drought treatment or split-root system experiment (Marino et al., 2007), which allow half of the root system to be irrigated at field capacity while the other half remained deprived of water, indicates that plant carbon metabolism, protein synthesis, amino acid metabolism, and cell growth are among the processes most altered in soybean nodules under drought stress (Gil-Quintana et al., 2013a).

In spite of diverse physiological reactions to drought stress and the level of N_2 fixation inhibition caused by water deficit, the legume species have shown significant genetic variation in their ability to fix N_2 under drought. For example, genotypes with N_2 fixation tolerance to water deficit include Jackson, R01–416, R01-581F, Volstate and PI# 222547, 374163, 423886, 429328, 471938, 507039, 227557, 507414, and 578315B in soybean (Serraj and Sinclair, 1996, 1998; Sinclair et al., 2000; Chen et al., 2007; Jyotsna Devi and Sinclair, 2013); SER 16, SXB 412, NCB 226, and Calima in common bean (Jyotsna Devi et al., 2013); and KK 60-3, Tifton 8, and ICGV# 86015, 98353, and 98348 in groundnut (Pimratch et al., 2008a,b; Jyotsna Devi et al., 2010; Pimratch et al., 2013).

A pertinent question given the results described above is, what would be the best approach to identify N_2-fixing-tolerant germplasm that is also adapted to drought? Assessing germplasm directly for N_2 fixation may not be a logically feasible and cost-effective approach. It is suggested that reduced subsets of germplasm (see Section 3.3.1) could be first evaluated under water-stressed conditions to identify germplasm with enhanced adaptation to drought, which may subsequently be screened for N_2 fixation tolerance under drought. Such an approach has been successful in identifying N_2-fixing tolerant germplasm in groundnut and common bean (Pimratch et al., 2008a,b; Jyotsna Devi et al., 2010; Jyotsna Devi et al., 2013; Pimratch et al., 2013).

In soybean, the ureide levels in petioles harvested from well-watered plants is negatively correlated with the relative amount of N_2 fixed under dry conditions (Serraj and Sinclair., 1997). Sinclair et al. (2000) used a three-stage screening process to identify N_2-fixing tolerant soybean germplasm under drought. The first stage of screening involved the measurement of petiole ureide levels in a large number of germplasm to select 10% of the accessions with low petiole ureide levels. The selected accessions were then subjected to field evaluation to a sustained water-deficit period of approximately three weeks to select accessions ($\sim$10%) with high N based on an ARA enabling a large number of plant introductions to be discarded at first-stage screen itself, followed by concentrating on select germplasm in later stage evaluations. Using this approach, Sinclair et al. (2000) reported eight high-N_2-fixing soybean germplasm lines under drought.

Soil salinization is also a major constraint to SNF for legume growth (Munns, 2002). Salinity affects photosynthesis, nitrogen, and carbon metabolism (Soussi et al., 1999; Balibrea et al., 2003). Genotypic differences in

salt tolerance and for nitrogen fixation have been reported in common bean. Common bean germplasm BAT477 and Flamingo showed tolerance to SNF under moderate salinity, which depended largely on the ability of these genotypes to maintain adequate leaf area and an abundant and efficient nodular system (Saadallah et al., 2001; Tajini et al., 2012).

The evidence to date in common bean, groundnut, and soybean suggests that selection for enhanced drought adaptation or salinity tolerance has had an influence on the N_2 fixation ability under these stresses, which clearly demonstrate that genetic variability exists for all these traits. Therefore drought-adaptation and salinity tolerance are good candidate traits for breeding programs intent on developing high-N_2-fixing grain legume cultivars. However, these abiotic stresses (drought, salinity and heat) also influence the survival and effectiveness of the symbiotic rhizobium strains in the host–rhizobium partnership for increased N_2 fixation (see Section 2.3). Obviously, there is an urgent need to develop an integrated strategy that combines genetic tolerance to abiotic stress with efficient rhizobium resources in breeding of grain legume cultivars that have high N_2-fixing capacity under drought and or salt-stressed conditions.

3.5 Identifying Promiscuous Germplasm for Use in Breeding

Germplasm that nodulate effectively with diverse indigenous rhizobia strains are considered promiscuous, and the characteristic is termed promiscuity (Kueneman et al., 1984). Such germplasm form symbiotic association with available indigenous rhizobium strains in the soil and fix atmospheric nitrogen effectively with them, while nonpromiscuous germplasm would require predetermined rhizobium strain to fix atmospheric nitrogen. Cowpea-type rhizobia in Africa are indigenous and abundant. With the discovery of 10 promiscuous germplasm in soybean from tropical Africa and Southeast Asian countries (Pulver et al., 1982, 1985), IITA soybean breeders introduced the promiscuity trait into improved genetic background with high grain yield potential. Such cultivars would not need genotype-specific rhizobium strains or the need for large-scale inoculum production, delivery, and application at farm level.

To date, a number of dual purpose (grain and fodder) promiscuous soybean breeding lines with varying crop duration (early, medium, and late maturing types), designated as TGx (Tropical *Glycine* cross) that nodulate effectively with indigenous *Bradyrhizobium* species, have been developed and found promising (for nitrogen fixation and grain yield) when tested in several countries in Africa. For example, as of now, 21 IITA-bred TGx

lines have been released as cultivars, mostly in Nigeria, but some in Benin, Democratic Republic of Congo, Ethiopia, Ghana, Togo, and Uganda.

Substantial loss to soybean productions occurs if the cultivars are not resistant to pod shattering, rust and phosphorus deficiency. This last trait of phosphorous is especially important for atmospheric nitrogen fixation. IITA breeders have successfully combined promiscuity, phosphorus deficiency, and resistance to pod shattering with high yield potential into an improved genetic background (Giller and Dashiell, 2006; Tefera et al., 2009a, 2010; http://cdn.intechopen.com/pdfs/14933/InTech_Breeding_for_promiscous_soybean_at_iita__pdf). For example, some of the early maturing promiscuous soybean advanced lines, when evaluated during the 1980–1996 crop seasons at two locations in the Guinea Savanna region of Nigeria had on average 53% increase in grain yield (from 1117 to 1710 kg ha^{-1}), with an average annual genetic gain of 24.2 and 22.8 kg ha^{-1} (Tefera et al., 2009b). For medium and late maturing promiscuous soybean lines, the reported genetic gain in grain yield was 23.6 and 22.2 kg ha^{-1} $year^{-1}$, respectively (Tefera et al., 2010).

Other research has suggested the existence of enormous diversity in rhizobium species that nodulate on common bean and cowpea. The predominant rhizobium species reported from the center of origin of common bean is *R. etli*. Subsequently, several rhizobium species such as *R. leguminosarum* bv. *phaseoli*, *R. gallicum* bv. *phaseoli*, and *R. giardinii* bv. *phaseoli* or *Rhizobium tropici*/*Rhizobium leucaenae*/*Rhizobium freirei* adapted to acid soils and high temperatures have been found nodulating on common bean. The existence of a large number of rhizobium species capable of nodulating on common bean supports the idea that common bean is a promiscuous host (Martínez-Romero, 2003).

Cowpea, meanwhile, also develops symbiotic relationships with a variety of nodulating bacteria. For example, Guimarães et al. (2012) found 62 of 119 bacterial strains isolated from agricultural soils in the western Amazon of Brazil using cowpea as a trap plant, thus exhibited differences in symbiotic efficiency with 68% of strains promoting a significant increase in shoot dry matter of cowpea compared with the control (no inoculation and low levels of mineral nitrogen), which support the relevance of promiscuity in cowpea. Such promiscuity in trapping cowpea indigenous rhizobia was also reported from the symbiotic functioning of *Bradyrhizobium* species from Africa (Pule-Meulenberg et al., 2010). Notably, Pule-Meulenberg et al. (2010) found that cowpea genotypes such as Fahari, Glenda, and Apagabaala were most promiscuous across Botswana, Ghana, and South

Africa. Clearly, more research is needed to discover promiscuity and its use in breeding to develop highly promiscuous and productive grain legume cultivars.

3.6 QTL Associated with SNF Traits

Genetic research in chickpea, common bean, cowpea, groundnut, mungbean, and soybean using diallel or biparental mating populations involving six generations (parents, F_1, F_2, BC_1, and BC_2) revealed that traits associated with SNF are controlled by both additive and non-additive genes, with some evidence of epistatic interactions. The magnitude of these genetic effects varies depending on mating design and parental materials involved in the generation of appropriate genetic populations. For example, predominant non-additive genetic variation accounts for nodule number, nodule weight, nitrogenase activity, and shoot weight in cowpea (Miller et al., 1986), while non-additive genetic variation explains nodule number and shoot weight, and additive genetic variation for nodule weight in common bean (Franco et al., 2001). Likewise, predominant non-additive genetic variation was important for nodule number in chickpea (Bhapkar and Deshmukh, 1982), while predominant additive genetic variance was significant for nodule number, nodule weight, shoot weight, total nitrogen, percentage of N derived from atmosphere and nitrogen fixed per plant in mungbean (del Rosario et al., 1997). In groundnut, predominance of both additive and non-additive genetic effects and evidence of epistatic interactions are reported. For example, there are reports of predominant non-additive genetic variation occurs for nodule number, nodule weight, shoot weight, total nitrogen, and nitrogenase activity (Isleib et al., 1980; Nigam et al., 1985); predominant additive genetic variation for nodule weight, shoot weight, and leaf score (Phudenpa et al., 2005); and significant epistatic interactions for nodule number, nodule weight, shoot weight, and nitrogenase activity (Phillips et al., 1989).

The discovery of PCR-based DNA markers led to the construction of genetic linkage maps of varying intensity that has revolutionized the use of genomic-led approaches in applied crop breeding. To date, most of the grain legume crops have abundant PCR-based markers, more specifically microsatellites and SNPs, and fine mapping of the genomic regions associated with agronomically beneficial traits has just begun. Genetic research in the preceding paragraph clearly indicate that SNF is a complex trait and is possibly governed by various genes with varying effects, and dissecting its genetic basis may provide crop breeders more opportunities to harness

marker (QTL)-trait association in crop improvement (Dwivedi et al., 2007; Collard and Mackill, 2008). Common bean, pea, and soybean among the grain legume crops have been investigated to identify QTL associated with nitrogen fixation traits (Table 1.3).

For example, in common bean, Ramaekers et al. (2013) reported two major QTL each for percent N_2 fixed and total plant N_2 fixed, which contributed 17–21% phenotypic variations. More importantly, they detected two candidate genes underlying these QTL: an auxin-responsive transcription factor and AP2/ERF-domain-containing transcription factor. The former is associated with differences in growth and possibly yield and N accumulation between climbing and bush beans, while the latter with total amount of symbiotic nitrogen fixed. Further, the extensive conservation of gene order between chickpea and a model plant *M. truncatula* (Seres et al., 2007) have allowed researchers identify a candidate gene, *CaNSP2*, involved in nodulation pathway in chickpea, which had shown 85–86% sequence similarity to that reported for *NSP2* genes in pea and *M. truncatula* (Ali et al., 2014). In pea, 7 root QTL and 11 nodule QTL were detected in region of LG I close to *Af* gene (LG I-*Af*), with several QTL for root or nodule traits and seed N accumulation QTL mapped in similar regions that highlight the possibility of breeding new pea cultivars with increased root system size, sustained nodule number, and improved N nutrition (Bourion et al., 2010). Likewise, major QTL for shoot dry weight, nodule number, nodule dry weight, and ARA, each contributing 12–18% variation were noted in soybean (Tanya et al., 2005; Santos et al., 2013). Furthermore, association mapping and genotype by sequencing revealed few SNP markers on chromosome 5 and 14 closely associated with nodule number and nodule weight in soybean (http://www.proteinresearch.net/html_images/wsrc2013/18-february-session-1/352_agrama-f.pdf).

Recent findings, therefore, clearly indicate that molecular markers closely associated with desirable traits may be used to increase the efficiency and effectiveness of conventional breeding by indirect selection of desirable segregants in breeding populations. However, it should be noted that cross-validation of marker–trait association in independent samples and in different genetic backgrounds and environments is necessary to obtain unbiased estimates of QTL effects and the proportion of genetic variance explained by the detected marker-QTL association before using in applied crop breeding (Dwivedi et al., 2007).

Model legumes *M. truncatula* and *Lotus japonicus* are considered ideal for the study of host–microbe interactions, including SNF, because of their

Table 3 Quantitative trait loci associated with symbiotic nitrogen fixation traits in model legumes and grain legume crops

Cross and population	Rhizobia strain	Summary of the QTL associated with N_2-fixing traits	Reference
Model legume			
Lotus japonicus			
RILs (Miyakojima MG 20 × Gifu B-129)	*Mesorhizobium loti* MAFF303099	Thirty-four QTL associated with acetylene reduction activity plant^{-1}, acetylene reduction activity nodule weight^{-1}, acetylene reduction activity nodule number^{-1}, nodule number plant^{-1}, nodule weight plant^{-1}, stem length, stem length without inoculation, shoot dry weight without inoculation, root length without inoculation, and root dry weight without inoculation; acetylene reduction activity plant^{-1}, acetylene reduction activity nodule number^{-1}, nodule weight and stem length showed strong correlations and QTL co-localization suggesting these traits controlled by the same locus; QTL for acetylene reduction activity plant^{-1}, acetylene reduction activity	Tominaga et al., 2012

		nodule number^{-1}, nodule weight and stem length, co-localized around marker TM0832 on chromosome 4, were also colocalized with previously reported QTL for seed mass	
Medicago truncatula			
177 RILs (F803005.5 × DZA045.5)	*Sinorhizobium meliloti* strains (*Naut* and *Sals*)	Symbiotic signaling genes, *NFP* and *DM*13, co-localized with two QTL affecting average fruit weight and leaf number, suggesting that natural variation in nodulation genes may influence plant fitness; several QTL affecting multiple traits indicative of pleiotropy or tight linkage; unlike previous reports (Laguerre et al., 2007; Rangin et al., 2008; Heath, 2010; Heath et al., 2010), no evidence for G × G interactions in legume–rhizobium symbiosis detected, which could be either due to small effect loci that were undetected or more genotype–genotype combinations need to be tested	Gorton et al., 2012

(*Continued*)

Table 3 Quantitative trait loci associated with symbiotic nitrogen fixation traits in model legumes and grain legume crops—cont'd

Cross and population	Rhizobia strain	Summary of the QTL associated with N_2-fixing traits	Reference
Grain legume crops			
Common bean			
83 RILs (G2333 × G19839)	*Rhizobium tropici* CIAT899	Glasshouse studies − two QTL for percent N_2 fixed on linkage groups (LGs) b01 and b10 contributed 20−21% phenotypic variation, while another two QTL on LGs b04 and b10 contributed 17−18% phenotypic variation for total plant N_2 fixed; field evaluation—one QTL for percent N fixed on LG b04 and another for the total N fixed on LG01, both explaining 19−21% phenotypic variation DNA sequence comparison of markers closely linked to QTL detected two candidate genes underlying the QTL—auxin-responsive transcription factor and AP2/ERF-domain-containing transcription factor,	Ramaekers et al., 2013

		with former explaining differences in growth and possibly yield and N accumulation between climbing and bush beans, while the latter total amount of SNF in the field	
51 RILs (BAT-93 × Jalo EEP558)	*R. tropici* UMR-1899	Fifteen QTL for nodule number (NN), in absence of nitrogen, mapped on LGs 2, 3, 5, 6, 7, 10, and 11 and 5 while QTL associated with NN, in the presence of nitrogen, mapped on LGs 3, 5, 7, 10, 11; QTL detected in the absence of nitrogen contributed 34% variation, while those in the presence of nitrogen 28% variation for NN	Souza et al., 2000
RILs (BAT-93 × Jalo EEP558)		Four QTL for nodule number, together contributed 50% phenotypic variation	Nodari et al., 1993
Pea			
180 $F_{6:8}$ RILs and 153 $F_{6:9}$ RILs (Cameor × Ballet)	*Rhizobium leguminosarum* bv. *viciae*	32 QTL for root traits on six LGs, 8 for number of lateral roots, 21 for root length, and 3 for root dry matter; 26 QTL for nodule traits on 5 LGs, 9 for nodule number, 8 for nodule area, 4 for	Bourion et al., 2010

(*Continued*)

Table 3 Quantitative trait loci associated with symbiotic nitrogen fixation traits in model legumes and grain legume crops—cont'd

Cross and population	Rhizobia strain	Summary of the QTL associated with N_2-fixing traits	Reference
		nodule dry matter, and 3 for the relative part of the nodule dry matter; 7 of the 32 root QTL and 11 of the 26 nodule QTL detected in region of LGI close to the *Af* gene (LGI-*Af*), explaining 9–49% phenotypic variation; several QTL for root or nodule traits and seed N accumulation mapped in similar locations, highlighting the possibility of breeding new pea cultivars with increased root system size, sustained nodule number and improved N nutrition	
Soybean			
295 lines, two countries, two years	Indigenous rhizobium strains	Association mapping using genotype by sequencing approach-detected SNP markers associated with nodule number on chromosome 5, and nodule weight on chromosome 5 and 14	http://www.proteinresearch.net/html_images/wsrc2013/18-february-session-1/352_agrama-f.pdf
157 RILs (Bossier × Embrapa 20)		Two QTL for shoot dry weight on LGs E and L, three QTL for	Santos et al., 2013

	Bradyrhizobium japonicum SEMIA 5079 and *B. elkanii* SEMIA 587	nodule number on LGs B1, E, and I, and one QTL for the ratio of nodule dry weight/nodule number on LG1, explaining 15.4%, 13.8% and 6.5% phenotypic variation, respectively	
160 $F_{2:3}$'s (Embrapa 20 × BRS 133)	*B. japonicum* SEMIA 587 and *B. elkanii* SEMIA 566	Two genomic regions associated with nodule number (NN) and nodule weight (NW), contributing 7% and 10% phenotypic variation, respectively; epistatic interactions detected among nonlinked QTL for NN and NW	Nicolás et al., 2006
157 $F_{2:7}$ RILs (Bossier × Embrapa 20)	–	Twelve significant associations for shoot dry weight, nodule number and nodule weight in four LGs (B1, C2, D1b, and H), with all QTL having minor effects	dos Santos et al., 2006
136 RILs (SJ 2 × Suwon 157)	*B. japonicum* DASA 01026	5, 3, 4, and 2 QTL associated with nodule number (NN) per plant, nodule dry weight, plant dry weight (PDW), and acetylene reduction activity (ARA),	Tanya et al., 2005

(*Continued*)

Table 3 Quantitative trait loci associated with symbiotic nitrogen fixation traits in model legumes and grain legume crops—cont'd

Cross and population	Rhizobia strain	Summary of the QTL associated with N_2-fixing traits	Reference
		respectively, with most of these contributing small phenotypic variance, except for QTL on LG O contributing 17% variation to NN (Sat_038), 18% to NDW (Sat_274), 14% to PDW (Sat_274), and 12% to ARA (Sat_274), which may provide opportunity to select segregants with high N_2 fixation in segregating populations	
RIL (Pureunkong × Jinpumkong 2)		Ten mapped SNP located in nearby SSRs associated with seed protein, while QTL for nodule number and nodule fresh weight were closely linked to two mapped SNPs each; TC159475 in LG J positioned nearby both two QTL and Satt529, SSR marker associated with nodules per plant	Van et al., 2005

relatively small plant size and their well-sequenced genomes (Sato et al., 2008; Young et al., 2011). Recently among the grain legume crops, chickpea, pigeonpea and soybean genomes have also been sequenced (Schmutz et al., 2010; Varshney et al., 2012, 2013). Sequence comparison by Schmutz et al. (2010) with previously known nodulation genes from the model legumes identified 52 genes in soybean (28 nodulin and 24 regulatory genes), which probably represent true orthologous sets with *Medicago* or *Lotus* nodulation genes. Thirty-two of these genes had at least one highly conserved homologue gene, which were probably gene pairs arising from whole genome duplication of the *Glycine*, some 13 million years ago. Further analysis indicated that seven nodulin genes produced transcript variants; while none of the soybean regulatory nodulation genes produced transcript variants. The resequencing of diverse accessions and comparison of sequence variations with reference genomes may provide opportunities to mine allelic variations associated with agronomically beneficial traits, including SNF.

3.7 Cloning and Gene Expression Associated with SNF

3.7.1 Plant Genes and SNF

3.7.1.1 Model Legumes

M. truncatula and *L. japonicus* have been most extensively studied for host–microbe interactions and SNF. The genomes of both the model plants have been sequenced and annotated fully (Sato et al., 2008; Young et al., 2011); and a large number of genetic (mostly mutants defective in SNF) and genomic (markers and throughput assays, high density genetic maps) tools are available. They have been used to clone nodulation (NOD) and nitrogen-fixing (NIF) genes whose functions have been determined. Until 2010, a total of 26 genes in the model legumes have been cloned. These genes are involved in recognition of rhizobial nodulation signals, early symbiotic signaling cascades, infection and nodulation processes, and regulation of nitrogen fixation (Kouchi et al., 2010).

Orthologs of many of these nodulation genes are also found in grain legumes. Examples of orthologs in crops found by similarity to model legume genes include *PsSYM37* (orthologous to *LjNFR1* and *MtLYK3*), *PsSYM8* (*LjPOLLUX*, *MtDMI1*), *PsSYM9* (*LjCCaMK*, *MtDMI3*), *PsSYM35* (*LjNIN*, *MtNIN*), *PsSYM7* (*LjNSP2*, *MtNSP2*), *PsSYM10* (*LjNFRS*, *MtNFP*), *PsSYM19* (*LjSYMRK*, *MtDMI2*), and *PsSYM29* (*LjHAR1*, *MtSUNN*) in pea and *GmNFR5* (*LjNFR5*, *MtNFP*), *MsNORK* (*LjSYMRK*, *MtDMI1*), *GmNARK* (*LjHAR1*, *MtSUNN*), and *GmN56*

(*LjFEN1*) in soybean. In common bean, Galeano et al. (2012) developed a large set of intron-based SNP or indel markers based on cloned nodulation genes from model legumes (55 markers based on 33 Nod factor perception, signal transduction, calcium signal interpretation, and other NOD or NIF genes). These were based on summary of such genes in Stacey et al. (2006) and Kouchi et al. (2010). The second source of nodulation markers was a set of 162 soybean putative regulatory genes expressed during nodulation and in response to KNO_3 and KCl treatments that were reported by Libault et al. (2009), while the third source was a set of 179 nodule specific expressed sequence tags from common bean found in PhvGI Library (http://compbio.dfci.harvard.edu/cgi-bin/tgi/libtc.pl?db=phvest). These research advances are providing important clues to understanding both the molecular mechanisms underlying plant–microbe endosymbiotic associations and the evolutionary aspects of N_2-fixing symbiosis between legumes and rhizobium.

The formation of N_2-fixing nodules in legumes is tightly controlled by a long-distance signaling system in which nodulating roots signal to shoot tissues to suppress further nodulation (Ferguson et al., 2010). Schnabel et al. (2011) reported a mutant defective in this regulatory behavior and identified loss-of-function alleles of a gene designated as *ROOT DETERMINED NODULATION1* (*RDN1*). They showed that *RDN1* is an essential gene for normal nodule number regulation in *M. truncatula*. The *RDN1* promoter drives expression of the gene in cells of the vascular cylinder, suggesting that it could be involved in initiating, responding to, or transporting vascular signals. *RDN1* is a member of a small, uncharacterized, highly conserved gene family (*RDN* family) unique to green plants, including algae, and encodes a 357-amino acid protein of unknown function. A *sym1/TE7* gene in *M. truncatula,* an ortholog of *L. japonicus CYCLOPS*, which strongly impairs the symbiosome formation, encodes the recently identified interacting protein of DMI3 (IPD3) (Ovchinnikova et al., 2011).

The establishment of symbiosis involves specific developmental events occurring both in the root epidermis (site of bacterial entry) and at a distance in the underlying root cortical cells (site of cell divisions leading to nodule organogenesis). This activity depends on a molecular dialogue between the plant and the bacteria, which involves the production of lipochitooligosaccharide molecules (or Nod factors) by rhizobia. How these events are coordinated remains poorly understood. Using the lysine motif (LysM) domain-receptor like kinase gene *NFP* and the calcium- and calmodulin-dependent protein kinase gene *DMI3*, Rival et al. (2012) showed that

epidermal *DMI3* expression is sufficient for infection thread (IT) formation in root hairs. Epidermal expression of *NFP*, on the other hand, is sufficient to induce cortical cell divisions leading to nodule primordial formation, whereas *DMI3* is required in both cell layers for these processes. They therefore concluded that a signal, produced in the epidermis under the control of *NFP* and *DMI3*, is responsible for activating *DMI3* in the cortex to trigger nodule organogenesis in *M. truncatula*.

The symbiotic mutant *sen1* from *L. japonicus* forms nodules that are infected by rhizobia but that do not fix nitrogen. Hakoyama et al. (2011) identified the causal gene *SEN1* associated to this phenotype. *SEN1* encodes an integral membrane protein homologous to soybean's nodulin 21. They detected the expression of *SEN1* exclusively in nodule-infected cells, which increased during nodule development. Furthermore, they found that both symbiosome and bacteroid differentiation are impaired in the *sen1* nodules even at a very early stage of nodule development. This finding reveals that SEN1 protein is essential for nitrogen fixation activity and symbiosome or bacteroid differentiation in legume nodules.

Nodule development involves the distinct processes of nodule organogenesis, bacterial infection, and the onset of nitrogen fixation. Using *L. japonicus* mutants, uncoupled symbiotic stages and deep sequencing for the detection of candidate genes during expression studies, De Luis et al. (2012) identified miRNAs (microRNAs that were coded as miR genes for the study) involved in SNF. They showed that induction of an miR171 isoform, which targets the key nodulation transcription factor, Nodulation Signaling Pathway2, correlates with bacterial infection in nodules, while miR397 is systematically induced in the presence of active, N_2-fixing nodules but not in that of non-infected or inactive nodule organs. Likewise, miR397 is involved in nitrogen fixation-related copper homeostasis and belongs to the laccase copper protein family.

All plants have pectate lyase and polygalacturonase genes that are involved in cell wall degradation (Muñoz et al., 1998; Martin-Rodriguez et al., 2002; Høgslund et al., 2009). The infection of legumes by N_2-fixing rhizobia occurs via plant-made ITs. To allow rhizobial infection, the plant cell wall must be locally degraded for the formation of ITs. Xie et al. (2012) reported an *L. japonicus nodulation pectate lyase gene* (*LjNPL*), which is induced in roots and root hairs by Nod factors via activation of the nodulation signaling pathway and the NIN transcription factor. The mutant form *Ljnpl* produced uninfected nodules and most infection were arrested as infection foci in root hairs or roots while the few partially infected nodules

that did form contained large abnormal infections. This research demonstrated that legume-determined degradation of plant cell walls allow root infection to occur during the initiation of the symbiotic interaction between rhizobia and legumes.

The phytohormones cytokinin and auxin play essential roles in diverse aspects of cell proliferation and differentiation in plants. Auxin accumulates during the nodule development in *L. japonicus*. NODULE INCEPTION, a key transcription factor in nodule development, positively regulates auxin accumulation. Its accumulation is inhibited, however, by autoregulation of nodulation (AON) (Suzaki et al., 2012). The genetic mechanism regulating nodule organogenesis is relatively poorly characterized. Suzaki et al. (2013) noted that a mutation *tricot* (*tco*), which is a gain-of-function mutation of the cytokinin receptor, suppresses the activity of *spontaneous nodule formation 2* (*snf2*) in *L. japonicus*. Analysis of *tco* mutant showed that the gene *TCO* positively regulates rhizobial infection and nodule organogenesis, and is also involved in the maintenance of the shoot apical meristem (SAM). The *TCO* gene encodes a putative glutamate carboxypeptidase that had great similarity with the Arabidopsis ALTERED MERISTEM PROGRAM1 protein, which is involved in cell proliferation in the SAM. Thus, *TCO* is not only a novel gene for regulation of nodule organogenesis but also provide significant additional evidence for a common genetic regulatory mechanism in nodulation and SAM formation.

Nodulation is regulated principally by AON, dependent on shoot and root factors and is maintained by the nodulation autoregulation receptor kinase (NARK) gene in soybean. Reid et al. (2012) developed a bioassay to detect root-derived signaling molecules in the xylem sap of soybean plants that might function as AON. They identified an inoculation- and NARK-dependent candidate gene *GnUFD1a* that responded in both the bioassay and intact, inoculated plants. *GnUFD1a* is a component of the ubiquitin-dependent protein degradation pathway and provides new insight into the molecular responses occurring during AON, which may be used as a molecular marker to assist in identifying the factors contributing to the systemic regulation of nodulation in soybean.

As par of AON, host plants tightly control the number of nodules formed on their roots via a root-to-shoot-to-root negative feedback signaling loop. *CLR-RS* genes, which are expressed in the root, and the receptor kinase *HARI*, which functions in the shoot, mediate this autoregulation in *L. japonicus*. Okamoto et al. (2013) showed that an arabinosylated CLE-RS2 glycopeptide suppresses nodulation, and directly binds to the

HAR1 receptor kinase. Furthermore, they showed that CLE-RS2 glycopeptides are the long-sought mobile signals responsible for the initial step of AON.

NODULE INCEPTION (*NIN*) is a nodulation-specific gene that encodes a putative transcription factor and acts downstream of the common signaling pathways genes, *SYM*. Soyano et al. (2013) identified *LjNF-YA1* and *LjNF-YB1* as transcriptional targets of *NIN* in *L. japonicus*. The suppression of *LjNF-YA1* inhibited root-nodule organogenesis and loss of function of *NIN*, while the overexpression of *NIN* induced root-nodule primordium-like structures that originated from cortical cell in the absence of bacterial symbionts. Thus, *NIN* is a crucial factor for initiating nodulation-specific symbiotic processes. Moreover, ectopic expression of either *NIN* or the *NF-Y* subunit genes caused abnormal cell division during lateral root development, indicating that the *Lotus NF-Y* subunits can function to stimulate cell division. Hence, transcriptional regulation by *NIN*, including activation of the *NF-Y* subunit genes, induces cortical cell division, which is an initial step in root-nodule organogenesis.

3.7.1.2 Grain Legumes

Soybean and common bean are the most extensively studied grain legumes for the genes associated with SNF. To date, eight genetic loci, designated as *rj1*, *Rj2*, *Rj3*, *Rj4*, *rj5*, *rj6*, *rj7*, and *Rjfg1*, which were found naturally or by induced mutations, are known in soybean and are related to nodulation traits induced upon inoculation with compatible rhizobium strains (Hayashi et al., 2012a and references therein). Orthologs of some of these genes have also been reported in *L. japonicus*, *M. truncatula*, and *pea*. For example, *rj1* orthologs in *L. japonicus* (*LjNFR1*), *M. truncatula* (*MtLYK3*), and *pea* (*PsSYM37*); *rj5* and *rj6* orthologs in *L. japonicus* (*LjNFR5*), *M. truncatula* (*MtNFP*), and *pea* (*PsSYM10*); or *rj7* orthologs in *L. japonicus* (*LjNHAR1*), *M. truncatula* (*MtSUNN*), and *pea* (*PsSYM29*). In common bean, pilot amplification of 313 intron-based markers representing nodulation genes or genes expressed during nodulation were screened for single-strand conformation polymorphisms and any that were positive for intergene pool polymorphism were sequenced and converted to SNP markers that were assayed with the Sequenom technique (Galeano et al., 2012). These markers were named Bean SNP markers for nodulation (abbreviated BSn). A total of 178 SNPs were found in 65 sequenced regions of independent genes involved in nodulation. Allele-specific primers were designed in the flanking regions of these SNPs and were mapped. Confirmation of these genes and markers is

pending the full sequencing and public release of the common bean genome.

3.7.2 Plant Genes Expression and SNF

3.7.2.1 Model Legumes

Many genes are known to be associated with root-nodule development and activity in model legume *M. truncatula*. However, information on the precise stages of activation of these genes and their corresponding transcriptional regulators is lacking. By combining gene expression analyses using 70-mer oligonucleotide 16.4 K microarrays for both wild-type symbionts and nodulation defective symbiotic mutants, Moreau et al. (2011) identified more than 3400 differentially regulated genes and associated regulators, which they classified into four distinct stages of transcription reprogramming throughout nodulation. A small subset of gene expression regulators in this study were exclusively or predominantly expressed in nodules, whereas most other regulators were activated in response to abiotic or biotic stresses.

The plant plasma membrane-localized NADPH oxidases, known as respiratory burst oxidase homologues (RBOH), play crucial roles in plant growth and development. Marino et al. (2011) reported seven putative RBOH-encoding genes in *M. truncatula*. The expression analysis of these *MtRboh* genes in *M. truncatula* tissues revealed that one of the genes, *MtRbohA*, was significantly upregulated in *S. meliloti*-induced symbiotic nodules. Its expression was, however, restricted to the N_2-fixing zone of the nodule. Furthermore, using *S. meliloti bacA* and *nifH* mutants defective to form efficient nodules, they showed strong link between nitrogen fixation and *MtRbohA* upregulation. Phytohormone cytokinin regulates many aspects of plant development, including symbiotic nodule organogenesis. Using a combination of transcriptomic, biochemical, and molecular approaches, Ariel et al. (2012) unveiled new- and posttranscriptional networks acting in symbiotic nodule organogenesis downstream of the CRE1 signaling pathway, and identified two novel transcription factor, NSP2 and bHLH476, linked to *M. truncatula* nodulation, thereby suggesting their recruitment in legumes into specific symbiotic functions.

Limpens et al. (2013) obtained a comprehensive gene expression map of an indeterminate *Medicago* nodule and identified genes that show specific enriched expression in the different cells or tissues. They used Affymetrix Medicago GeneChips and laser-capture micro-dissection to isolate specific cells and tissues obtained from the nodule infection zone divided into a distal (where symbiosome formation and division occur) and proximal (where

symbiosomes were mainly differentiating) regions as well as infected cells from the fixation zone containing mature nitrogen-fixing symbiosomes of *M. truncatula*. Further validation of expression profiles of these genes, by comparison to published genes expression profiles and experimental verification, indicated that the data can be used as digital "in situ," which offers a genome-wide insight into genes specifically associated with subsequent stages of symbiosome and nodule cell development, and can serve to guide future functional studies.

3.7.2.2 Grain Legumes

The temporal and spatial regulation of genes and gene networks in grain legumes has been an area of recent study with gene expression tools. In soybean, Hayashi et al. (2012b) used an RNA-seq approach based on the Illumina GAllx platform and the specific root tissues (the Zone Of Nodulation, ZON) known to respond to *Bradyrhizobium japonicum* inoculation to identify new genes involved in nodulation. They used a twofold difference as minimum criterion for detection of differences in gene expressions and detected 2915 differentially expressed genes in this soybean tissue, of which 1677 were upregulated in response to nodulation, whereas 1238 were downregulated. Of these, 407 upregulated genes and 150 downregulated genes exhibited a greater than fivefold changes in expression. More importantly, the expression of many genes, including an *endo-1,4-β-glucanase*, *a cytochrome P450* and a *TIR-LRR-NBS receptor kinase*, was transient, peaking quickly during the initiation of nodule ontogeny. They also detected a set of differentially regulated genes acting in the gibberellic acid biosynthesis pathway, suggesting a novel role of gibberellic acids in nodulation.

The soybean genome contains 18 members of the 14-3-3 protein family, but little is known about their association with specific phenotypes. Radwan et al. (2012) found that *Glyma0529080 Soybean G-box factor 14-3-3c* (*SGF14c*) and *Glyma08g12220* (*SGF14l*) genes, which encode 14-3-3 proteins, have an essential role in soybean nodulation. They detected increased abundance of *SGF14c* mRNA in nodulated soybean roots at 10, 12, 16, and 20 days after inoculation with *B. japonicum*. Both transcriptomic and proteomic analyses showed that mRNA and protein levels were significantly reduced in the *SGF14c*/*SGF14l*-silenced roots, which exhibited reduced numbers of mature nodules. The host cytoplasm and membranes, except the symbiosome membrane, were severely degraded in the failed nodules, suggesting a critical role of one or both of these 14-3-3 proteins in early development stages of soybean nodules. Nguyen et al. (2012) noted 240

phosphoproteins that were significantly regulated (>1.5-fold abundance change) in soybean root hairs, reflecting a critical role of phosphorylation during the initiation of the *B. japonicum* infection process. Other recently identified genes associated with rhizobial infection, nodule primordium development, nodule organogenesis, and nodule number in soybean include *GS52*, *Control of nodule development* (*CND*), and *GmFWL1* (Govindarajulu et al., 2009; Libault et al., 2009, 2010; Tanaka et al., 2011).

Rhizobium strain, *B. japonicum* CPAC 15, is widely used in commercial inoculants in soybean production in Brazil. Using suppressive subtractive hybridization technique combined with Illumina sequencing and soybean roots, de Carvalho et al. (2013) analyzed global expression of genes in soybean roots of a Brazilian cultivar Conquista. They detected 3210 differentially expressed transcripts at 10 days after inoculation, which they grouped into seven classes of genes related to nitrogen fixation-related processes. During nodulation, they found that a higher percentage of genes were related to primary metabolism, cell wall modifications, and antioxidant defense system, and identified putative functions of some of these genes for the first time in *Bradyrhizobium*-soybean symbiosis. By proteomic analysis, they were able to identify two proteins; a putative glutathione-S-transferase (*Glyma12g28670.2*) and sucrose synthase (*Glyma15g20180.3*), which had 1.47-fold change vis-á-vis the non-inoculated conditions. Other proteins described for the same symbiotic association, using similar approach, include sucrose synthase (nodulin-100), β-tubulin, rubisco activase, glutathione-S-transferase, a putative heat-shock 70-kDa protein, pyridine nucleotide-disulphide oxidoreductase, and a putative transposase (Torres et al., 2013).

In common bean, a set of newly cloned genes has elucidated aspects of the nodulation process. For example, Quiceno-Rico et al. (2012) cloned and characterized two cDNAs (*PvuTRX1h* and *PvuASH1h*) from common beans that encoded polypeptide homologues of trithorax group proteins that play critical roles in the regulation of transcription, cell proliferation, differentiation, and development in eukaryotes. Quantitative RT-PCR analyses of transcript abundance in roots and nodules, at different developmental stages, demonstrated that *PvuTRX1h* is abundant at the early stages of nodule development, whereas *PvuASH1h* functions at the stages of highest N_2-fixing activity of the nodules. This finding suggests that these genes could participate in the formation of nodules in common bean.

In another example from common bean, Montiel et al. (2012) identified nine members of the *Rboh* gene family and found that the transcript of one

of *PvRbohB* accumulated abundantly in shoots, roots, and nodules. They detected *PvRbohB* promoter activity in meristematic regions of common bean roots, as well as during the IT progression and nodule development. Further research showed that RNAi-mediated *PvRbohB* downregulation in transgenic roots reduced reactive oxygen species production and lateral root density, and greatly impaired nodulation. This study suggested that NADPH oxidase is crucial for successful rhizobial colonization and probably maintains proper IT growth and shape, thereby confirming previous research linking *Rboh* to nodule nitrogen fixation in *M. truncatula* (Marino et al., 2011). More recently, *PvRbohB* was noticed to significantly upregulate in *Phaseolus vulgaris*-mycorhiza (*Rhizophagus irregularis*) symbiosis as being involved in downregulation of *PvRbohB* transcription by RNAi silencing induced early hyphal root colonization, which leads to significant increase in mycorrhizal colonization in *PvRbohB*-RNAi roots (Arthikala et al., 2013). This finding indicates that *PvRbohB* has a role both during the plant-rhizobial symbiosis and in symbiotic interactions of arbuscular mycorrhizal (AM) symbiosis.

Receptor for activated C kinase (RACK1) is a highly conserved, eukaryotic protein of the WD-40 repeat family, involved in plant signal-transduction pathways that were studied in common bean by Islas-Flores et al. (2011), who found that the *PvRACK*1 mRNA transcript increased during *P. vulgaris* nodule development at 12–15 days post-inoculation. This study suggested an important role for the *RACK*1 gene after nodule meristem initiation and rhizobium nodule infection. Downregulation of *PvRACK*1 transcription by RNAi silencing resulted in a reduced nodule number, impaired nodule cell expansion, and smaller nodule size. These results indicate that *PvRACK*1 has a pivotal role in the cell expansion and in symbiosome and bacteroid integrity during nodule development. Overexpression of the *PvRACK*1 transcript led to an increased susceptibility to heat stress, and this negatively influenced normal nodule development (Islas-Flores et al., 2012).

Trehalose (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside) is a nonreducing disaccharide involved in growth, development, and differentiation in plant cell (Paul et al., 2008). Barraza et al. (2013) showed that trehalose accumulation in common bean, triggered by *PvTRE*1 downregulation, led to a positive impact on the legume–rhizobium symbiotic interaction increasing trehalose content, bacteroid number, nodule biomass, and nitrogenase activity all resulting in improved SNF. Thus, genetic modification of trehalose degradation could be an alternative approach for improving SNF.

Nodule number on legume roots after rhizobial infection is controlled by the plant shoot through autoregulation and mutational inactivation of this mechanism, which leads to hypernodulation. A *Sym*28 locus, which encodes a protein similar to the *Arabidopsis* CLAVATA2 (CLV2) protein, is involved in autoregulation in pea and inactivation of the *PsClv*2 gene in four independent sym28 mutant alleles resulted in hypernodulation. This finding suggests that pea *Sym*28 is the *PsClv*2 gene (Krusell et al., 2011). The cosegregation of hypernodulation and fascination alleles in this study further confirm the earlier evidence that two traits are linked (Sagan and Duc, 1996).

4. GENOMICS-LED INTERVENTION TO SELECT FOR EFFECTIVE RHIZOBIUM STRAINS

4.1 Rhizobium Genetic Resources, Host Specificity, and Diversity

The "rhizobium" definition is based on the ability to elicit nodule formation in leguminous plants. This practical definition may cause confusion, as some non-nodulating bacteria named "rhizobium" are in fact not able to induce nodule formation. Rhizobia, identified so far, belong to two bacterial classes, *Alphaproteobacteria* and *Betaproteobacteria*; accordingly they are called alpha- and beta-bacteria, respectively. In alpha bacteria rhizobial strains are present in the genera *Sinorhizobium* (syn. *Ensifer*), *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, *Devosia*, *Ochrobactrum*, *Aminobacter*, *Microvirga*, *Shinella*, and *Phyllobacterium*. In beta-bacteria, rhizobia are present within strains of the genera *Burkholderia* and *Cupriavidus* (*Ralstonia*). Although the ability to infect legumes by rhizobia does not follow the taxonomy, some rhizobial strains are specific to certain plant species, e.g. *R. leguminosarum* bv. viciae, for species of genera *Pisum*, *Vicia*, *Lathyrus*, and *Lens*, while others such as *S. fredii* strain NGR234 are able to nodulate a range of leguminous plants. In particular *S. fredii* NGR234 is able to nodulate more than 120 genera of legumes and the nonlegume species *Parasponia andersonii* (Pueppke and Broughton, 1999).

While beta-rhizobia are mainly found in association with tropical legumes (Moulin et al., 2001; Chen et al., 2005; Amadou et al., 2008; Gyaneshwar et al., 2011), alpha-rhizobia are more widespread and nodulate tropical to temperate legumes, including pastures, trees, and grain legumes; and consequently they are the most studied rhizobia. *Sinorhizobium* (syn. *Ensifer*) is the most studied genus, accounting for 2005 records in PubMed

(http://www.ncbi.nlm.nih.gov/; accessed on December 5, 2013) and 6728 in ISI Web of Knowledge (http://apps.webofknowledge.com; accessed on December 5, 2013), followed by the genera *Rhizobium* and *Bradyrhizobium*.

Both alpha- and beta-rhizobia can live as free bacteria in soil or plants, but, when conditions are suitable, may form symbiotic associations with leguminous plants (van Rhijn and Vanderleyden, 1995). However, several strains of these rhizobial species do not possess the ability to induce nodule formation (van Rhijn and Vanderleyden, 1995). Probably due to this heterogeneous life style (both free-living and symbiont) rhizobia have large genomes, often composed by several replicative elements (a chromosome, plus additional elements as chromids, megaplasmids, or plasmids) (Harrison et al., 2010; Pini et al., 2011; Black et al., 2012; Galardini et al., 2013a).

Genetic diversity within single rhizobial taxa is usually very high. In fact, within the same species several biovars have been identified, each with different plant host specificity. For example, there are four biovars (bv. mediterranense, bv. lancerottense, bv. medicaginis, bv. meliloti) in *S. meliloti* (Villegas et al., 2006; Mnasri et al., 2007; Leònó-Barrios et al., 2009; Rogel et al., 2011) and three biovars (bv. trifolii, bv. phaseoli and bv. viciae) in *R. leguminosarum*. As in the case of rhizobia, biovars are defined by both genetic methods and mostly by symbiotic capabilities toward the host plant, the more appropriate term "symbiovar" has been recently proposed (Rogel et al., 2011). The symbiovar thus reflects an assembly of genes suitable for host specificity, providing the basis for the identification of genetic determinants of symbiotic specificity and exploitation of rhizobial genetic resources. For example, the symbiovar tropici has been recently described to encompass the symbiotic plasmid of the *R. tropici*/*R. leucaenae*/*R. freirei* (Ormeño-Orrillo et al., 2012).

A large genetic polymorphism exists in natural populations of rhizobia, especially in strains isolated as symbionts of root nodules in the species *S. meliloti* (Paffetti et al., 1996, 1998; Carelli et al., 2000). Analyses performed by several molecular techniques (RAPD, BOX-PCR, PCR-RFLP, AFLP, MLST, etc.) revealed that the diversity of strains isolated from individual plants or few plants populations is so high that each isolate is often characterized by a unique molecular fingerprint (Paffetti et al., 1996; Biondi et al., 2003b; Grange and Hungria, 2004; Alberton et al., 2006; Bailly et al., 2006; Talebi Bedaf et al., 2008). Consistently, several strains of *S. meliloti* have been shown to harbor a large number of multi-copies mobile genetic elements (such as insertions sequences, transposons, and mobile introns), generating a high and dynamic genetic diversity (Biondi et al.,

1999, 2003a, 2011). This diversity is particularly concentrated in the megaplasmid harboring symbiotic and nitrogen fixation genes (Giuntini et al., 2005; Galardini et al., 2011; Mengoni et al., 2013). The development of a cultivation-independent approach for the analysis of the genetic diversity of *S. meliloti* populations (Trabelsi et al., 2010b) allowed detection of even higher number of putative strains relative to those identified through cultivation techniques (Pini et al., 2012).

The geographic location and the host plant seem to directly influence the extent of genetic differentiation of rhizobia (Paffetti et al., 1996, 1998; Carelli et al., 2000). Several studies have been performed looking at the genetic diversity of rhizobial symbiont of the same crop species in different soils and locations (Kaschuk et al., 2006; Giongo et al., 2008; Adhikari et al., 2012; Rashid et al., 2012; Lopez–Lopez et al., 2013). Moreover, biogeographic patterns in rhizobial population diversity were also reported. For instance, in *S. meliloti* genetic differences among strains isolated from different regions of Iran were related to the geographical distance among sites (Talebi Bedaf et al., 2008). Similar patterns were also reported for soybean-nodulating rhizobia (Han et al., 2009; Zhang et al., 2011); and evidence was also found for natural selection in the symbiotic genes (Bailly et al., 2006) and homologous recombination (gene exchange) in the *nod* genes region (Bailly et al., 2007), which may promote and drive strain genetic differentiation within the same species.

In conclusion, rhizobium genetic diversity is very high, both at the phylogenetic scale, with different rhizobial recipes in alpha- and beta-rhizobia, and at the intra-species level, due to the effect of natural selection, drift, and activity of mobile genetic elements in the rhizobial genomes. Moreover, a large number of non-nodulating rhizobia are present, whose functions in plant growth promotion and interactions with the nodulating rhizobia are still unknown.

4.2 Host–Rhizobium Interaction and Competition with Indigenous Rhizobium Strains

Rhizobia inoculation with elite strains can remarkably increase yield of important grain legumes worldwide (Kaschuk et al., 2010b); and in this regard soybean in South America is probably the most emblematic example (Hungria et al., 2006a,b; Hungria and Mendes, 2015). However, there are often reports of lack of responses to inoculation with elite strains, and in most cases the failure has been attributed to the indigenous or naturalized rhizobial population in the soils (from now both will be called as established

population of rhizobia) (Graham, 1981; Thies et al., 1991a,b). In the absence of established rhizobial populations, responses to inoculation can be impressive. For example, soybean is exotic in South America (Hungria et al., 2006a; Hungria and Mendes, 2015) and in areas cropped for the first time in Brazil, grain yield increases with inoculation ranged from 600 to 1600 kg ha^{-1} in the 1980s (Hungria et al., 2006a) and at present, with improved productive cultivars, the increase may vary up to 3600 kg ha^{-1} (Zilli et al., 2010).

But what happens after exotic soybean inoculant strains are established in soils? For several legumes, it has been reported that population as low as 10–20 cells g^{-1} of soil may inhibit responses to inoculation, once the inoculant strains are not able to compete with established population (Weaver and Frederick, 1974; Singleton and Tavares, 1986; Thies et al., 1991a,b; Hardarson, 1993). However, continuous research efforts with soybean in South America has shown that even in soils with populations of 10^3 cells g^{-1} of soil or higher, annual re-inoculation results in yield increases averaging 8–14% (Hungria et al., 2005a, 2006a,b; Hungria and Mendes, 2015). Similar results have been obtained with common bean, a crop probably considered as the most erratic in responding to inoculation (Hardarson, 1993). Field trials with elite strains in Brazil in soils with high populations have also shown that inoculation and re-inoculation of common bean may result in on average 20% increase in farmer's fields growing the crop under low technology, and an increase ranging 5–25% under high input technology (Hungria et al., 2000, 2003; Mendes et al., 2007). These results provide encouragement for the selection of elite strains for each legume, the production of inoculants of high quality, a systematic control of quality of inoculants, and the large-scale use of inoculation with elite strains (Hungria et al., 2005b). Consequently, the paradigm of impossibility of introducing new strains in soils with established populations of rhizobia might not be the rule; and the information should be delivered to researchers and farmers for evaluating and confirming the feasibility of inoculation of legumes in soils with established populations of compatible rhizobia.

Nevertheless, despite several successful stories, the inoculation can fail and the limitations therein must be diagnosed and overcome. The performance of the symbiosis depends on the rhizobial attributes of competitiveness (capacity of the strain to compete against other strains), infectiveness (capacity of forming nodules in stressed environment), and effectiveness (capacity of fixing nitrogen). These attributes are traded-off in plant selectiveness/promiscuity, rhizobial capacity to survive in the soil and compete

with other rhizobial strains, and to infect the plant and to fix nitrogen. Therefore, in future the success of SNF will depend on improving host plant, rhizobia, and environment system of the crop.

Starting with the host plant, in the case of soybeans in South America, the low N fertility of most soils and the high price of N-fertilizers have led plant breeders to selecting cultivars under low N conditions and inoculation with elite strains, favoring nitrogen fixation (Hungria and Vargas, 2000; Alves et al., 2003; Hungria et al., 2005a, 2006a; Hungria and Mendes, 2015). In the case of common bean, it has been difficult to convince the plant breeders, and this explains erratic responses to inoculation, and the reports of low contribution of SNF (Graham, 1981; Hardarson, 1993). Therefore, plant breeders should consider nitrogen fixation in the breeding programs as mandatory and a prerequisite for the future success of symbiosis.

Considering the micro-symbiont in the following years, rhizobial selection needs to be performed to match their effectiveness with the increasingly higher demand of more productive cultivars, to surpass the competitiveness of soil rhizobial population; and to overcome the challenge of production in stressed environment, including high temperatures, drought, soil salinity, and acidity (Hungria and Vargas, 2000; Hungria et al., 2005b; Hungria and Mendes, 2015). Relative to the competitiveness, it is also important to consider that established populations might be continuously segregating into more diverse communities, and thereby changing in effectiveness (Barcellos et al., 2007; Torres et al., 2012); and this calls for continuous monitoring of soil population.

Environmental stresses constrain plants, rhizobia, and symbioses to perform optimally (Hungria and Vargas, 2000); and the impending global climatic changes too need attention relative to the change in stresses. For the host plant, selection for tolerance to environment stresses, e.g. drought (Cattivelli et al., 2008) is a need of the future, but the breeding effort is to be performed in the presence of rhizobia and low N levels to assess potential nitrogen fixation. For the rhizobia, it has been shown that it is possible to select them for higher tolerance to environmental stresses such as higher temperature (Hungria et al., 1993). Differences in the symbiotic performance under environmental stresses may also vary with the host and the bacterium (Roughley et al., 1981; Ramos et al., 2003; Shiro et al., 2012; Hungria and Kaschuk, 2014) and thus there is a need to follow a holistic approach.

In cases when the inoculation fails, or there are problems in the production and distribution of inoculants, plants should be capable of establishing

symbiosis with indigenous or naturalized rhizobia (Herridge and Rose, 2000; Mpepereki et al., 2000). That has been the strategy followed leading to development of promiscuous soybean cultivars in Africa (Kueneman et al., 1984; Tefera, 2011). However, this strategy does not ensure the right combination of the plant with the most efficient rhizobia, as it is often reported for common bean (Graham, 1981). In addition, nowadays, in Africa, with better facilities to produce and distribute inoculants, the interest of farmers in nonpromiscuous soybean cultivars has increased due to the higher yield potential of these cultivars (N2Africa, 2013).

In North and South America, Europe and Australia, soybean cropping has always been based on nonpromiscuous cultivars. However, despite using inoculants, nodulation by the local population may not always be most effective in fixing nitrogen, and this remains a serious limitation (Herridge and Rose, 2000; Hungria et al., 2006a). In this context, without doubt, dealing with the capacity of the plant to avoid indigenous or naturalized strains that are very competitive, but have low capacity to fix nitrogen is a research challenge at present time and likely to continue in the coming decades.

Probably the most studied case of the problems faced with established population of rhizobia, limiting the introduction of new strains and the capacity to fix nitrogen, is that of *B. japonicum* serogroup USDA 123 in the mid-western United States. There are reports of occupation of 60–80% of the soybean nodules by this serogroup in the United States (Kvien et al., 1981; Cregan et al., 1989; Weber et al., 1989); and there have been reports from soils in Canada (Semu and Hume, 1979) and Korea (Kang et al., 1991). In Brazil, serogroup 123 is found in practically all soils, due to the establishment of strain SEMIA 566 in the 1960s and of CPAC 15 in the 1990s, belonging to the same serogroup (Mendes et al., 2004; Hungria et al., 2006a). To surpass the competitiveness problem, starting with serogroup 123 and then including other strains, one approach taken by plant breeders has been to identify soybean genes that restricted nodulation, increasing the likelihood that following inoculation with more efficient strains may establish symbioses (Caldwell, 1966; Devine and Breithaupt, 1980). As long as the manipulation restricts infection selectively, allowing only efficient strains to form nodules, it would be possible to manipulate rhizobia infection without compromising yields.

Searching for genes in soybean restricting nodulation started in the 1950s, and since then a number of nonlinked genes regulating soybean infection by *Bradyrhizobium* have been identified: (1) recessive alleles *rj*1that

confer restriction of nodulation with any *Bradyrhizobium* strain (Williams and Lynch, 1954; Caldwell, 1966); (2) gene *Rj*2, found in cultivars Hardee, CNSS, IAC-2, and Bonminori, that induces insufficient response to the serogroups of *Bradyrhizobium* spp. USDA 7, USDA 14, USDA 122, c1 and 6, resulting in the proliferation of cortex cells without formation of regular nodules (Caldwell, 1966; Vest et al., 1973; Devine et al., 1991; Hayashi et al., 2012); (3) *Rj*3, which restricts nodulation of the cultivars Hardee, CNS D-51, IAC-2, and Bonminori with *Bradyrhizobium elkanii* USDA 33 (Vest, 1970; Hayashi et al., 2012); (4) *Rj*4, found in cultivars Hill, Dunfield, Dare, Amsoy 71, Tracy, Akisengoku and Fukuyutaka, that induces inefficient nodulation with strains USDA 61, USDA 62, USDA 83, USDA 94, USDA 238, USDA 259, USDA 260, and USDA 340; it also restricts serogroup USDA 123 (Vest and Caldwell, 1972, Vest et al., 1973; Devine et al., 1990; Sadowsky and Cregan, 1992; Hayashi et al., 2012); (5) *rj*5 and *rj*6, that completely hampers nodulation, identified with chemical mutation corresponding to the same loci that *rj*1 (Harper and Nickell, 1995); (6) nodulating mutants *rj*7 or *nts*1, nitrate-tolerant symbiosis and *rj*8, also generated by chemical mutagenesis (Vuong et al., 1996). All these genes are not linked during segregation events, facilitating the construction of soybean genotypes that contain one or two combinations of these genes (Devine and O'Neill, 1989; Qian et al., 1996; Vuong and Harper, 2000; Hayashi et al., 2012).

Unfortunately, the strategy might not be applicable to all matches of soybean-*Bradyrhizobium* germplasm in Brazil. A screening on 152 commercial cultivars did not find any restriction to the nodulation of soybeans with the dominant *Bradyrhizobium* serogroups (Bohrer and Hungria, 1998; Hungria and Bohrer, 2000). There is only one report of nodulation restriction in Brazilian genebank, in which the cultivar IAC-2 did not form nodules when inoculated with strains CB 1809 and SEMIA 5039 (Peres and Vidor, 1980); however, the gene governing the restriction was not identified.

Soybean may also be nodulated by fast-growing rhizobia belonging to the genus *Sinorhizobium* (=*Ensifer*); and another approach that has been considered to overpass infection by established *Bradyrhizobium* is the inoculation with elite *S. fredii* strains (Cregan and Keyser, 1988; Buendía-Clavería et al., 1994). It was originally thought that *S. fredii* was specific for Asian soybean lines (Keyser et al., 1982; Devine, 1985), but later it was shown that 17% of 194 North American genotypes were effectively nodulated by strain USDA 257 (Balatti and Pueppke, 1992), and almost 70% of the Brazilian

cultivars were able to form effective nodules with *S. fredii* and *Sinorhizobium xinjiangensis* (Chueire and Hungria, 1997). Studies have identified that gene *Rfg*1 is related to the nodulation with *S. fredii* (Devine and Kuykendall, 1994). Soybean cultivar Peking carries the recessive allele of the gene *Rfg*1 (Devine and Kuykendall, 1994) that codifies for effective nodulation, while other cultivars, such as Kent (Devine, 1984) and McCall (Balatti and Pueppke, 1990) carry dominant alleles of the gene, resulting in ineffective and rudimentary nodule formation. However, competitiveness of *Sinorhizobium* against *Bradyrhizobium* is strongly influenced by pH, and the strain is apparently successful only under high pH (Hungria et al., 2001). As soils cropped with soybean are often acidic in pH, the interest in using *Sinorhizobium* to overpass the competitiveness with less effective *Bradyrhizobium* has not been further pursued.

It is noteworthy to mention that the isolation of most non-nodulating, hypernodulation and nodulation restriction genes by classic techniques has been confirmed by using the genomic techniques (Hayashi et al., 2012). Soybean genes related to the nodulation phenotypes were *rj*1 (*GmNFR*1α), *rj*5 (*GmNFR*5α), and *rj6* (*GmNFR*5β) for non-nodulation genes; *rj*7 (*NTS*1/*GmNARK*) for hypernodulating gene; *Rj*2 (*Rj*2), and *S. fredii* *Rfg*1 (*Rfg*1) for restriction nodulation phenotypes.

Supposing that a plant genotype does not totally exclude more dominant and less effective strains in the soil, partial restriction (sanction) or the stimulation of a chosen strain may be an option for plant breeding. In the United States, Weiser et al. (1990) identified 12 out of 382 soybean genotypes that could distinguish strains, which hardly form nodules with less efficient strains. As a suggestion, the mechanism of plant preference for more efficient strains could be related to lecthins playing a role in the adhesion of bacteria to the roots (Ishizuka et al., 1991, 1993). In Brazil, studies have also shown a degree of preference of cultivars for different strains (Hungria and Bohrer, 2000).

Kiers et al. (2007) reported strong evidence to show that earlier American soybean cultivars (Kabott, Pagoda, and Flambeau) were relatively more capable to sanction less effective rhizobia than the new cultivars (Maple Glen, AC Harmony, and AC Rodeo). Yet, it is not clear how plants perceive less efficient strains when they are saprophytically living in the soil before establishing symbiosis. Low specificity is a ubiquitous condition for all legumes all over the world (Perret et al., 2000), and the restriction to nodule formation seem to be exception rather than the rule (Bohrer and Hungria, 1998; Hungria and Bohrer, 2000). On the rhizobial side, diversity shifts due to environmental conditions (Andrade et al., 2002;

Kaschuk et al., 2006) and expands by their own dynamics (Barcellos et al., 2007; Torres et al., 2012). Furthermore, the expression of nodulation restricting genes is affected by environmental factors, such as temperature (Sadowsky et al., 1995). Therefore, the challenge is to orchestrate plant and bacterial genetics so that the best of each partner is used. Concomitantly to the rhizobial selection and the development of better inoculation strategies (e.g. Hungria et al., 2005a,b, 2006a; Hungria and Mendes, 2015), there is a need for developing plant breeding strategies that overcome the constraints related to rhizobial competition.

4.3 Host (Wild Relatives)–Rhizobium Symbiosis to Identifying Stress Tolerant Rhizobium Strains

Several environmental conditions can limit the growth and activity of N_2-fixing plants. The efficiency of N_2 fixation is related to both the physiological state of the host plant and to rhizobial partner (Zahran, 1999). Indeed, factors that impose limitations on the vigor of the host legume, as for instance salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, plant diseases, etc. can reduce the N_2-fixing potential of symbiosis. Consequently, several efforts have been carried out both on the improvement of plant traits to cope with unfavorable conditions (Dwivedi et al., 2005). The effects of salinity, drought, temperature, low pH, and heavy metals and high temperature have been most extensively investigated in identifying rhizobial strains in natural environments showing tolerance towards these stressors, which can be used as inocula on target plant germplasm (Rupela et al., 1991; Hungria et al., 1993, 2000; Zahran, 2001; Provorov and Tikhonovich, 2003; Roumiantseva, 2009; Elboutahiri et al., 2010; Tikhonovich and Provorov, 2011; Boukhatem et al., 2012).

Salt stress inhibits the initial steps of symbiosis (Zahran and Sprent, 1986; Coba de la Peña et al., 2003). The effects of salt stress on nodulation and nitrogen fixation of legumes have been examined in several studies (Zahran, 1999 and references therein). The reduction of N_2-fixing activity by salt stress seems to be related with a reduction in nodule respiration and in leghemoglobin production (Ferri et al., 2000). Several studies have been performed looking at salt-tolerant rhizobia, especially in subarid regions, where conditions may likely have contributed in selecting rhizobial strains with the ability to cope with osmotic stress (Mnasri et al., 2007; Trabelsi et al., 2010a). These studies showed the presence of rhizobia tolerant to high NaCl concentrations (up to 1 M), which can be used as inocula for crop production in saline soils. Phenotype Microarray™ experiments also

showed that such strains could be isolated from nonsaline soils with low salt concentration (as *S. meliloti* BL225C) tolerance to relatively high salt (600 mM NaCl) concentration (Biondi et al., 2009). These results are similar to those that showed recently on rhizobia nodulating *Acacia* sp (Boukhatem et al., 2012) and alfalfa (Elboutahiri et al., 2010), for which no correlation was found between salt, pH , and temperature tolerances of rhizobial strains and the corresponding edaphoclimatic characteristics of their regions of origin. More importantly, when rhizobial strains (*S. meliloti*) overexpressing part of the molecular mechanism devoted to osmotic response (as the *bet* system, involved in the production of the osmoprotectant glycine betaine, see Boscari et al., 2002) were constructed, they showed higher symbiotic efficiency under salt stress (Boscari et al., 2006).

Often linked to salt stress are both drought and temperature stresses (Räsänen and Lindström, 2003; Vriezen et al., 2007; Elboutahiri et al., 2010; Alexandre and Oliveira, 2013). In particular, as reviewed recently by Vriezen and co-workers (2007), desiccation is a critical step if proper inocula have to be prepared for spraying as biofertilizers as well as for long-term survival of the inoculated rhizobia under desiccating conditions in arid soils, but the molecular mechanisms of survival under these conditions may involve *bet* genes, as well as production of exopolysaccharides, which are still not fully understood (Vriezen et al., 2007). Several research groups have investigated the molecular basis of thermal tolerance in rhizobia (recently reviewed by Alexandre and Oliveira, 2013), and rhizobial strains that grow at 50 °C were isolated (Boukhatem et al., 2012) from nodules of *Acacia* sp. in Algeria.

Another important factor affecting symbiosis is low soil pH. In particular, low pH has been recognized as one of the factors limiting SNF for selecting rhizobial strains (as acidic pH), or by reducing phosphate availability (high pH). Low pH tolerant rhizobia have been isolated from soils (Elboutahiri et al., 2010) and for some strains a positive correlation was found between the salt tolerance and the adaptation to alkaline pH (Shamseldin and Werner, 2005). Intriguingly, when looking at single species, as *S. meliloti*, acid tolerance of strains was not correlated with the pH of the soil, suggesting the presence of micro-niches in the soil matrix or in the plant, which allow strains sensitive to low pH to thrive in acid soils. However, acid soils, as for instance those in the Mediterranean region have been chosen for the isolation of low pH-tolerant rhizobia (Loi et al., 2005). Indeed, many strains nodulating annuals plants in Mediterranean region (as several *Medicago* species) have been isolated and deeply characterized for their efficient symbiosis

under low soil pH (Reeve et al., 2006). For example, a putative transmembrane protein (LpiA) was shown to express under acid conditions; and this protein strongly enhanced the viability of cells exposed to lethal acid (pH 4.5) conditions. Moreover, for some of the most promising rhizobial strains in terms of low pH tolerance, genome sequences have been determined (Reeve et al., 2010a,b). Phenotype Microarray™ experiments were carried out (Biondi et al., 2009) and confirmed the sensitivity of strains of *S. meliloti* to low pH. There was a large variability in natural isolates for such trait, allowing such variability to both disclose the molecular determinants for acid tolerance in rhizobia and to devise appropriate inoculant in acid tolerant legume crops.

The third example of tolerance to environmental stress in rhizobia, which can be used for crop improvement, is trace metal tolerance. In last few years there has been an increasing interest in microsymbionts from wild legumes growing in soils rich in trace metals (e.g. nickel or copper). In particular the flora of serpentine soils has been studied in details. Serpentine soils are distributed all over the world and originate from an array of ultramafic rocks characterized by high levels of nickel, cobalt, and chromium, and low levels of N, P, K, Ca, and a high Mg/Ca ratio (Brooks, 1987). The flora of serpentine soil contains several endemics, including many legume species (Brady et al., 2005). The bacteria inhabiting serpentine soil and endophytes of serpentine plants have attracted the attention of many investigators (Mengoni et al., 2010 and references therein). Moreover, the biotechnological potential of metal tolerant bacteria for increasing plant growth under trace metal contamination has been investigated (Abou-Shanab et al., 2006; Rajkumar et al., 2009). In particular, several bradyrhizobial strains with tolerance up to 15 mM Ni (II) have been isolated from the endemic legume *Serianthes calycina* grown in New Caledonia serpentine soils (Chaintreuil et al., 2007). Legume species growing on mine deposits have been used as a source of metal tolerant rhizobia. Recently, a symbiont of *Anthyllis vulneraria*, a legume species, growing close to a zinc mine in the south of France has been isolated and identified as new species (*Mesorhizobium metallidurans*), highly tolerant of Zn (Vidal et al., 2009). The association between *A. vulneraria* and *M. metallidurans* has been demonstrated effective for the growth of the host plant in the soil contaminated by Zn, Pb, and Cd (Mahieu et al., 2011).

High temperatures can seriously limit SNF and this effect could become even more drastic in the future, as climate projections predict increases in annual average temperatures in many countries around the world. High

temperatures can affect N_2 fixation by reducing the viability of rhizobia in the soil (Hungria and Vargas, 2000); and in a study with *R. freirei* strain PRF 81, it has demonstrated that in response to heat stress several proteins were upregulated, with an emphasis on oxidative stress-responsive proteins (Gomes et al., 2012). High temperature also affect the exchange of molecular signals between the host plant and rhizobia (Hungria and Stacey, 1997), as well as other steps involved in nodulation and nodule functioning (Hungria and Franco, 1993; Hungria and Vargas, 2000; Hungria and Kaschuk, 2014). However, the feasibility of selecting rhizobial strains showing not only higher tolerance to high temperatures, but also higher capacity of establishing more tolerant symbiotic associations with the host plant has been demonstrated in common bean (Hungria et al., 1993, 2000) and could be applied to other legumes.

In conclusion, there are several rhizobial strains that have been isolated and characterized for tolerance to many environmental stresses; and in some cases also proved to be effective in improving legume growth under unfavorable conditions, as for instance as pioneer species for restoration ecology in marginal lands (Wang et al., 2005; Coba de la Peña and Pueyo, 2012), thanks to the nurse effect they provide toward other small shrubs and herbaceous species as in the case of the legume shrub *Retama sphaerocarpa* (Padilla and Pugnaire, 2006). However, often (as in the above mentioned cases of salt, pH, and thermal tolerances) there is no direct relationship between soil and climatic features of the region of origin of strains with their corresponding phenotypes, clouding our understanding of the evolutionary mechanisms and of the concept of selective pressure on soil bacteria.

4.4 Harnessing Sequence Diversity among the *Rhizobium* Genomes to Enhance Host–Rhizobium Symbiosis

Symbiosis is described as a close relationship between different biological species. Although biologists have been studying symbiotic relationships since the early nineteenth century, they have been little explored for the large degree of variability shown by symbiotic partners and only recently have stirred the attention of systems and computational biologists. Concerning rhizobium–legume symbiosis, most of the studies conducted so far in this system have been based on classical (molecular) genetics tools that have unveiled most of the molecular steps of the symbiotic process (Gibson et al., 2008). However, the large diversity of strains present in nature, often characterized by different symbiotic performances, in a continuous (quantitative) range of characters, has been poorly explored in molecular terms.

The variability in the plant-growth promotion phenotype observed in natural strains has raised the question whether the evolution of the host–rhizobium symbiosis follows a mutualistic or an antagonistic coevolution, especially in the presence of strains with reduced benefits for the host plant (Friesen, 2012). Such strains are either labeled as "defective" or "cheaters," depending on their fitness gain with respect to the host fitness gain. Early studies reported reduced ability of ineffective strains to compete in the host (Robinson, 1969) and later the presence of effective competitors has been demonstrated (Amarger, 1981; Triplett and Sadowsky, 1992). However, the presence of host sanctions posed on strains unable to efficiently fix nitrogen inside the nodules (Kiers et al., 2003), suggests that the host has an important role in driving the evolution of symbiotic traits. Such selection implies the emergence in rhizobial populations of genetic traits that positively affect plant fitness and a vast variability in such traits; in the symbiosis checkpoints are expected to be targeted, such as the early stages of host–bacteria signaling, root adhesion and invasion and of course nitrogen fixation. The many molecular mechanisms that have evolved in the legume symbioses, pose a serious challenge in tracking the variability of the genetic traits related to the symbiotic phenotype. This variability targets many of the key molecular players in symbiosis: the *nod* genes for instance have been found to be not necessary in the *Bradyrhizobium–Aeschynomene* symbiosis (Giraud et al., 2007). Even the *nif* gene cluster, encoding the nitrogenase complex has been found to exhibit a high variability in terms of the presence of various subunits, from 15 *nif* genes in *Bradyrhizobium* and *Azoarcus caulinodans* to just eight *nif* genes in *R. leguminosarum bv. viciae*, suggesting that other genes may be necessary for a correct assembly and functioning of the Nif complex. The regulation of the *nifA* gene also varies between rhizobia species, with the most notable examples being the absence of the FixJL two-component system in some rhizobia species (such as *R. leguminosarum bv. viciae* and *Cupriavidus taiwanensis*) (Masson-Boivin et al., 2009).

Such high variability at the molecular level has been highlighted through comparative genomics screenings. Inside alpha bacteria a conserved set of 264 genes (including core symbiotic genes) was found to be common to rhizobial species, irrespective of the overall alpha-rhizobia phylogeny (Young et al., 2006). A further study on a larger panel of alpha-bacteria, including free-living, symbiont and endophytic strains, showed the presence of 73 genes common to symbiotic α-rhizobia (Pini et al., 2011). However, a comparative study between alpha- and beta-rhizobia showed that inside the 214 known symbiotic genes there are no common genes that are exclusively

found in both alpha- and beta-rhizobial species. Only a limited number of genes were shown to be preferentially associated with rhizobia, including five *nif* genes (*nifBDEKN*) and three *nod* genes (*nodACD*) (Amadou et al., 2008), though there are rhizobial strains such as BTAi1 and ORS 278 lacking the *nodABC* operon (Giraud et al., 2007). Such variability in the gene repertoire associated with the symbiosis in rhizobia species is even more complicated when considering the natural genomic variability at the intraspecific level, which can account up to a significant fraction of the so-called "pangenome" (Medini et al., 2005). Interesting, genomic analysis of rhizobia strains has also highlighted that apparently no simple core symbiome exists, and that a systems biology approach to N_2-fixing symbiosis may be required to understand evolutionary relationships with the host plant (Black et al., 2012). Intriguing is also the sharing of symbiotic and virulence genes in pathogens and symbionts, also thereby revealing our poor knowledge about the evolution of SNF (Carvalho et al., 2010) and the possible common genetic program between rhizobial, actinorhizal and mycorrhizal symbioses (Tromas et al., 2012).

Despite this great variability in the symbiotic pathways, comparative genomics analyses are still able to correlate specific genetic traits to the variability in the symbiotic phenotype. Now several genome sequences of rhizobial taxa are present in public databases and for some species several genomes have been sequenced (Table 4), which paves the way for future comparative analyses. A broad spectrum of comparative analysis has been conducted to identify new genes related to symbiosis in the *S. meliloti* Rm1021 reference strain, using both rhizobial and nonrhizobial alphaproteobacterial species (Queiroux et al., 2012). The nonrhizobial genomes content has been subtracted from the *S. meliloti* Rm1021 genome by removing the common gene set; the resulting genes have been intersected with genomes of rhizobial species belonging to *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*, leading to a list of gene candidates that were subsequently confirmed by mutation experiments. This analysis highlighted a *sodM*-like gene whose mutation increases the strain competition in nodule occupancy, thus confirming the predictive power of such an approach.

Comparative analysis on a narrower phylogenetic spectrum should highlight genes that can better explain the intraspecific phenotypic variability. A notable example of this approach has been recently published by Sugawara et al. (2013), involving the analysis on the genome of several strains of *S. meliloti* (33) and *S. medicae* (13), but also based on a phenotypic characterization of symbiotic performances on 27 *M. truncatula* genotypes. Six plant

Table 4 Rhizobial strains with complete genome (www.ncbi.nlm.nih.gov assessed on 21st December, 2013)

Taxon	Number of genomes	Mean length (Mbp)	Mean GC content (%)	Mean number of genes
Azorhizobium caulinodans	1	5.37	67.3	4717
Bradyrhizobium BTAi1	1	8.49	62.8	7621
Bradyrhizobium CCGE	1	7.39	64.2	Nan
Bradyrhizobium ORS	3	7.46–7.86	64.1–65.7	6716–6716
Bradyrhizobium S23321	1	7.23	64.3	6892
Bradyrhizobium STM	1	8.43	63.8	Nan
Bradyrhizobium WSM1253	1	8.71	61.6	Nan
Bradyrhizobium WSM471	1	7.78	63.4	Nan
Bradyrhizobium YR681	1	7.83	64.7	Nan
Bradyrhizobium elkanii	2	8.68–9.48	62.0–64.4	Nan
Bradyrhizobium japonicum	4	8.87–9.21	61.5–64.1	8317–8826
Bradyrhizobium uid80709	1	7.31	65.8	Nan
Cupriavidus taiwanensis	1	6.48	65.0	5896

Mesorhizobium WSM4349	1	8.29	61.3	Nan
Mesorhizobium alhagi	1	6.97	62.4	Nan
Mesorhizobium amorphae	1	7.29	62.1	Nan
Mesorhizobium australicum	1	6.2	62.8	5792
Mesorhizobium ciceri	1	6.69	61.7	6264
Mesorhizobium loti	1	7.6	60.6	7272
Mesorhizobium metallidurans	1	6.23	62.0	Nan
Mesorhizobium opportunistum	1	6.88	62.9	6508
Methylobacterium nodulans	1	8.84	64.4	8308
Rhizobium 2MFCol3	1	6.55	60.1	Nan
Rhizobium 42MFCr	1	6.21	59.2	Nan
Rhizobium AP16	1	6.5	60.0	Nan
Rhizobium BR816	1	6.95	60.4	Nan
Rhizobium CCGE	1	6.92	59.8	Nan

(*Continued*)

Table 4 Rhizobial strains with complete genome (www.ncbi.nlm.nih.gov assessed on 21st December, 2013)—cont'd

Taxon	Number of genomes	Mean length (Mbp)	Mean GC content (%)	Mean number of genes
Rhizobium CF080	1	7.02	62.2	Nan
Rhizobium CF122	1	6.14	59.8	Nan
Rhizobium CF142	1	7.46	60.3	Nan
Rhizobium IRBG74	1	5.46	58.7	5478
Rhizobium JGI	2	1.23–2.07	59.1–59.4	Nan
Rhizobium NGR234	1	6.89	61.3	6362
Rhizobium PDO1	1	5.5	58.7	Nan
Rhizobium Pop5	1	6.5	61.1	Nan
Rhizobium etli	10	3.43–7.2	60.4–61.6	5963–6792
Rhizobium gallicum	1	7.22	59.3	Nan
Rhizobium giardinii	1	6.81	57.4	Nan
Rhizobium leguminosarum	18	5.24–8.0	58.6–61.0	6415–7143
Rhizobium lupini	1	5.27	58.3	Nan
Rhizobium mesoamericanum	1	6.45	57.8	Nan
Rhizobium phaseoli	1	6.62	60.8	Nan
Rhizobium tropici	1	6.69	58.9	6287
Sinorhizobium fredii	3	6.96–7.81	57.1–59.1	6743–7409
Sinorhizobium medicae	3	6.4–6.86	59.6–60.7	6213–6213
Sinorhizobium meliloti	19	6.69–8.94	59.9–62.4	6218–7092

Nan, annotation not available.

phenotypes were measured for each bacterial strain/plant genotype pairs, considering both nodule characteristics (number, color, and dry mass) and whole plant phenotypes (dry mass, height, and chlorophyll content), allowing the deception of two phenotypic clusters in which strains from both *S. meliloti* and *S. medicae* were present. The presence/absence patterns of specific genes involved in symbiosis was then compared with these two phenotypic clusters: genes encoding a type IV secretion system, the *hemN* gene (heme biosynthesis) and a relatively large cluster related to denitrification (*nirKV*, *norECBQD,* and *nosRZDFYLX*) were preferentially associated with the strains belonging to the cluster with higher symbiotic efficiency, even though they were not present in all the strains of the cluster, thereby suggesting the presence of other genes correlated with the symbiotic phenotype. Recently, two Community Sequencing Programs of the U.S. Department of Energy-Joint Genome Institute have focused on the sequencing of strains of *S. meliloti* with different symbiotic performances toward alfalfa, aiming to identify genes responsible for symbiotic differences (Galardini et al., 2011, 2013b). These analyses confirmed the importance of the denitrification cluster and of the *hemN* gene, which were found to be missing from the strain producing a lower plant growth, together with a copy of the *fixNOQP* operon for electron transport in low oxygen environments (Galardini et al., 2011). Very recently, a putative nickel transporter (*nreB*) of *S. meliloti* has been shown to be involved in symbiotic efficiency in the host legume *Medicago sativa*, possibly via modulation of urease activity (Pini et al., 2014), suggesting that several unsuspected genes present in rhizobial genome may be investigated and exploited for improving symbiotic performance.

The genetic markers that can be related to the variability in the host-rhizobium symbiosis however, are not limited to gene presence/absence patterns, but can also be tracked down to the so-called panregulon, a term used to indicate the variability in the gene regulation inside a set of genomes; the presence or absence of a regulatory motif in the upstream region of a gene can also be taken into account when building a list of candidate genetic markers. A comparison of three *S. meliloti* strains for predicting the presence of known regulatory motifs belonging to eight transcriptional regulators involved in the symbiotic process analyzed key regulators of the symbiotic process, from early stages of host-bacteria recognition (NodD1, NolR), to bacteroid metabolism and nitrogen fixation (NifA, Fur, FixK, FixJ) and to competition (ChvI, NesR). The predicted regulons (i.e., the group of genes under the control of the same transcriptional regulator)

showed that there is variability in the symbiosis gene regulatory network, even though nearly half of this variability was due to the absence of the regulated gene itself rather than differences in the upstream regulatory motif. Several genes related to symbiosis and nitrogen metabolism were predicted to be differentially regulated in the three strains (Galardini et al., 2011).

In conclusion, the quest for a "super-rhizobium," that is, the search for genes that may increase symbiotic performance of strain, is going on. Several data are now available, through classical genetic experiments (screening of mutants, etc.) and whole genome sequences. However, no ultimate markers for the identification of the "best" strains can be defined, since the overall picture of gene interactions during the symbiotic processes is not fully understood, especially for those genes present in the dispensable genome fraction of rhizobial species. Consequently, more effort is needed toward the molecular characterization of gene functions and the modeling of genome–phenotype relationships.

4.5 Rhizobial Endophytes in Host and Nonhost on Plant Growth and Development

Some rhizobia can form symbiotic relationships with nonlegume species such as those of *Parasponia* genus (Cannabaceae) (for a review see Matiru and Dakora, 2004). The nodulation of *Parasponia* by rhizobia suggested that molecular mechanisms for plant-bacteria cross talk may be conserved and broader than expected. *P. andersonii* (Planch.) has been recently found to be nodulated by rhizobia belonging to four different genera (Op den Camp et al., 2012), with variable levels in nitrogen fixation efficiency, which suggested that such nontarget legumes could be reservoir for a balance between symbiotic and commensal (opportunistic) rhizobia. The rhizobial infection and nodule formation in nonlegume crops suggest potential extension of SNF to nonlegume crops such as cereals. However, apart from the specific case of *Parasponia*, rhizobia may behave as symbionts in host plants, but also as commensals in terms of rhizospheric or endophytic strains in nonhost plants such as rice and maize (Chi et al., 2005). In host and nonhost species, rhizobia also colonize the intercellular and intracellular spaces of epidermis, cortex, and vascular system (Figure 1). For instance, *S. meliloti* and other rhizobia species have been shown to enter rice roots and translocate to leaves (Chi et al., 2010), where they attain relatively high numbers (10^3–10^5 cell g^{-1} dry weight) comparable to those of commonly occurring endophytes (Mengoni et al., 2012; Pini et al., 2012). *A. caulinodans*, the rhizobial symbiont of plants from the genus *Sesbania*, may enter the root

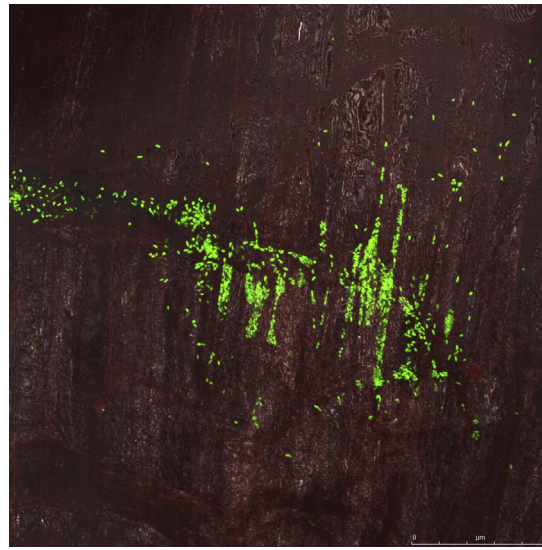

Figure 1 Confocal laser scanning microscopy (CLSM) of *Sinorhizobium meliloti* cells labeled with GFP (pHC60) colonizing vascular tissue and apoplastic space of *Medicago truncatula* plantlets. The used strain (Rm1021 ΔnodA) had a fix^{-}, nod^{-} phenotype as a consequence of the block in the synthesis of Nod Factor. Rm1021 ΔnodA strain was kindly provided by Dr D. Capela (CNRS, Toulouse, France). *We acknowledge Dr D. Nosi (University of Florence) for assistance in CLSM imaging.*

system of nonhost plant species, as several monocots and *Arabidopsis*, and invade apoplastic spaces between epidermal cells and the xylem (Cocking, 2003). Interestingly, the co-application of *A. caulinodans* and flavonoids such as naringenin and daidzein was shown to significantly enhance root colonization and xylem localization in *A. thaliana* (Stone et al., 2001). In wheat, the application of the flavonone naringenin increases rhizobial entry via cracks and promotes intercellular localization (Matiru and Dakora, 2004). Indeed, *nodD*1 gene product of *S. fredii* NGR234 responds to activation by phenolic compounds isolated from wheat extracts (le Strange et al., 1990); and rhizobia have been isolated as natural endophytes of several nonlegume crops such as rice, banana, carrot, and sweet potato (Rosenblueth and Martinez-Romero, 2006). Such nonsymbiotic interactions have been claimed as result of crop rotation also, which may have induced the rhizobia released from the legume crop root nodules to be close contact with the following cereal crop. For instance, *R. leguminsarum* bv. *trifolii* was isolated as a natural endophyte from roots of rice in the Nile delta (Yanni et al., 1997). Since rice has been grown in rotation with clover for about seven

centuries in the Nile delta, there would have been a selective pressure for rhizobial tight interaction with rice. A similar evolutionary scheme could have worked with maize and *P. vulgaris* grown in association for thousands of years in Mesoamerica (Gutiérrez-Zamora et al., 2001; Rosenblueth and Martínez-Romero, 2006) and with photosynthetic bradyrhizobia and African brown rice. Indeed, African brown rice generally grows in the same wetlands of the bradyrhizobia host plant *Aeschynomene* (Chaintreuil et al., 2000).

Rhizobial release of nodulation signals such as lipo-chitooligosaccharides is known to stimulate seed germination in a wide range of plant species by an unknown mechanism. Moreover, large increases in plant growth were observed in sorghum, soybean, and cowpea when supplied with lumichrome, a derivative of the vitamin riboflavin, which was identified as a signaling molecule, increasing root respiration rates by rhizobia (Volpin and Phillips, 1998; Phillips et al., 1999). This suggests that in planta release of lumichrome by rhizobial endophytes could be a factor in stimulating growth of cereals following rhizobial inoculation (Dakora et al., 2002; Ramírez-Puebla et al., 2012). In several species (e.g. cowpea, lupin, soybean, and maize), rhizobial inoculation showed similar effects on root respiration and stomatal conductance as did lumichrome application to roots, thereby suggesting a lumichrome-mediated action of rhizobia on plant physiology (Matiru and Dakora, 2005).

The ecological niches for rhizobia are wider than previously expected (soil and root nodules of legumes), including the endosphere (root, stem, and leaf tissues) of potentially all higher plants. Indeed, plant-association is a common trait within several bacterial classes; and both *Alphaproteobacteria* and *Betaproteobacteria* contain many plant-associated representatives and endophytic strains. Bacteria belonging to the genera *Azoarcus*, *Methylobacterium,* and *Enterobacter* have been shown to colonize plant tissue and also nitrogen fixation (Reinhold-Hurek and Hurek, 1998; Hurek and Reinhold-Hurek, 2003; Krause et al., 2006; Naveed et al., 2013). In a bioinformatics search for genes, which may confer both endophytic and symbiotic behavior, several genes encoding for membrane transporters were found associated with strains having endophytic behavior as well as a relatively high number of genes with unknown function in *Alphaproteobacteria* (Pini et al., 2011), which allow to speculate that several molecular determinants of endophytic interaction are still to be discovered and characterized.

Concerning the relationships between the fraction of nodulating rhizobia and that of rhizobia nonsymbiotically associated with plants

(endophytic or rhizospheric), a pivotal study was conducted several years ago (Segovia et al., 1991) on *R. leguminosarum* isolated from bean rhizosphere. This study demonstrated that the presence of a fraction of rhizospheric rhizobia, which once complemented with the plasmid harboring symbiotic genes, was able to form effective symbiosis with bean plants as well as control strains. More recently, using a quantitative PCR approach (Trabelsi et al., 2009), the presence of *S. meliloti* in the leaf tissue of *M. sativa* was noted (Pini et al., 2012). Since rhizobia were detected only by cultivation-independent methods, their genetic relationship with corresponding nodulating strains could not be determined. In particular, it is unclear if they come from root nodules or on the contrary if they constitute an independent population, not involved in symbiotic interactions with plant via root nodules. However, an *S. meliloti* strain, named H1, isolated from *M. sativa* leaves showed genomic features similar to the ex-nodulating strains (Galardini et al., 2013b), and retained indeed the ability to nodulate alfalfa (F. Pini, IRI-CNRS, France, personal communication). The capability to colonize all plant compartments suggests the occurrence of high genetic variability within rhizobial populations, and potential new ecological and functional roles for rhizobia are not investigated so far. Moreover, other studies involving *nodC* gene as rhizobial marker showed a higher diversity of *nodC* gene sequences amplified from DNA extracted from soil with respect to those from nodule isolates, suggesting the existence of other potential non-nodulating rhizobia in the chickpea and clover rhizosphere (Zézé et al., 2001). Those nonsymbiotic strains could likely be involved in other activities (e.g. lumichrome-mediated activity) of plant growth promotion, behaving as rhizospheric or endophytic strains.

5. CHALLENGES AND OPPORTUNITIES TO COMBINING HIGH SNF TRAITS INTO IMPROVED GENETIC BACKGROUND

5.1 Abiotic Stress Tolerance and Host–Rhizobium Symbiosis: a Breeding Challenge

Drought, extreme temperature, and salinity affect legume–rhizobium symbiosis by impairing the development of root hairs, and the site of entry of rhizobia into the host (Rupela and Kumar Rao, 1987; Räsänen and Lindström, 2003; Niste et al., 2013). Kantar et al. (2010) indicated that useful genetic variation exists to enhancing drought adaptation in both rhizobia and some legume hosts. A deep understanding of the regulation of the

SNF process will further contribute to select a drought-adapted, effective symbiosis, which will improve crop productivity in water-deficit agroecosystems. In this regard, proteomics provide the means for studying the root-nodule-symbiotic bacteria interactions (Muneer et al., 2012). Such studies also provide broad insights as to what proteins are produced by both the host plant and the rhizobium during the signal exchange and the signal-transduction pathways following photophosphorylation. For example, Fe-containing proteins are keys in SNF in the nodule, while the other proteins such as those related to SNF are affected by abiotic stress.

5.1.1 Plant–Rhizobium Interactions for Alleviating Abiotic stress(es)

Some microbes are known to affect rhizosphere soil physico-chemical properties. Research also shows that some microorganisms may influence the crop's adaptation to abiotic stresses such as drought, chilling injury, salinity, metal toxicity, and high temperature (Dimkpa et al., 2009; Cordeiro Brígido, 2012; da Silva Lobato et al., 2013; Grover et al., 2011). The extent and specificity of these existing plant–microbe interactions are, however, poorly understood (de Zelicourt et al., 2013). Further research is needed to understanding the association mechanisms, as to what factors are involved in the choice and selectivity of plant–microbial association and how these microbes provide tolerance to plants under abiotic stress. As noted by Coba de la Peña and Pueyo (2012), the ensuing information would assist in selecting and engineering rhizobia and legumes with enhanced adaptation to marginal and stressful environments.

5.1.2 Mycorrhizal Fungi Alleviate Abiotic Stress in Plants

An enhanced adaptation to drought through AM fungi association relates to their positive effects in facilitating water and nutrient uptake and their transport—especially P and other—insoluble mineral nutrients from the soil that lead to hydration of plant tissue (Rapparini and Peñuelas, 2014). The AM symbiosis enhances plant adaptation to drought through various combined physical, nutritional, physiological, and cellular effects (Ruiz Lozano, 2003). Although research advances are helping our understanding as to how AM confers enhanced crop adaptation in drought-prone environments, further information is needed to unravel the involvement of metabolites and their metabolic pathways. This knowledge should assist to elucidating the mechanisms in the drought avoidance and or in the AM symbiosis induced adaption.

5.1.3 Selecting for Nitrogen Fixation Drought Tolerance in Breeding Programs

Review of literature on this important aspect suggests that SNF in soybean is sensitive to even a modest soil water deficit (Sinclair, 1986; Sall and Sinclair, 1991; Serraj and Sinclair, 1997); and it has been indicated that a decline in N_2 fixation during soil drying and the associated yield reduction is indeed the result of inadequate N availability to the crop (Ray et al., 2006). Moreover, an obsolete and low-yielding soybean cultivar Jackson is reported to show no reduction in N_2 fixation during soil drying (Sall and Sinclair, 1991; Serraj and Sinclair, 1997). From the crosses involving Jackson (N_2 fixation tolerance to drought) and KS 4895 (a high-yielding line but sensitive to nitrogen fixation under drought stress), Sinclair et al. (2007) selected two advanced breeding lines that produced high grain yield than controls under moderate- to and low-yielding rainfed environments; and when evaluated for N_2 fixation under drying soil conditions in the greenhouse, these lines fixed more N than the sensitive parent, which suggests that using high N_2-fixing drought-tolerant germplasm, it is possible to select productive progenies with N_2-fixing tolerance under drought stress. These lines thus, offer great opportunities for increased yields under rainfed conditions as a result of reduced sensitivity to N_2 fixation under water-deficit condition. More recently, research on soybean has established that the genotypic differences for sensitivity to N_2 fixation under soil drying are strongly correlated with the shoot nitrogen concentration and shoot ureides under well-watered conditions, and with the shoot ureides concentration under drought conditions. It follows from this that shoot nitrogen concentrations under well-watered conditions could be used as a useful screening tool for evaluating soybean germplasm for drought tolerant N_2 fixation (King et al., 2014). Clearly, there is need for further research to test the use of shoot nitrogen as a criterion to identifying N_2 fixation drought-tolerant germplasm for use in breeding programs.

5.1.4 Overexpressing Trehalose-6-Phosphate Synthase Gene Improves Drought Tolerance and SNF

Trehalose is a nonreducing disaccharide (α-d-glucopyranosyl-1, 1-α-D-glucopyranoside) involved in stress tolerance in plants (López-Gómez and Lluch, 2012). *Rhizobium* and other related genera synthesize trehalose, which accumulate in bacteroids and in nodules (Müller et al., 2001). Suárez et al. (2008) found that common bean plants inoculated with *R. etli* overexpressing trehalose-6-phosphate synthase gene had more nodules, increased

nitrogenase activity and SNF, higher biomass and greater grain yield compared with plants inoculated with wild-type *R. etli*. Furthermore, the upregulation of genes involved was detected in stress tolerance and carbon and nitrogen metabolism. Thus, trehalose metabolism in rhizobia has shown to be important for signaling plant growth, yield, and adaptation to abiotic stress, and therefore its manipulation has a major impact on legume plants, including on SNF. More recently, Talbi et al. (2012) have shown that the inoculation of bean plants with an *R. etli* strain overexpressing *cbb3*$^{+}$ oxidase confers greater drought tolerance to SNF and increased plant dry weight.

5.2 Delayed Leaf Senescence in Relation to Photosynthesis, Symbiosis, and Productivity

Senescence is a developmental process that in monocarpic plants overlaps with the reproductive phase. Leaf senescence is associated with chlorophyll degradation and a progressive decline in photosynthetic capability (Matile et al., 1996). Germplasm lines with delayed leaf senescence (DLS), which is also known as the stay-green attribute have been found in many crops, including cowpea and soybean among the grain legumes (Abu-Shakra et al., 1978; Phillips et al., 1984; Gwathmey et al., 1992; Gwathmey and Hall, 1992; Gregersen et al., 2013). Such germplasm maintain chlorophyll and extend photosynthesis to fix carbon (C) in leaves or stems throughout a longer season than other cultivars that do not show the stay-green trait.

DLS in several crops is associated with a higher drought tolerance and a better performance under low nitrogen conditions (Gregersen et al., 2013). Cowpea and soybean germplasm with the DLS phenotype have shown variable agronomic performance. For example, Abu-Shakra et al. (1978) identified segregants in a soybean cross involving Lee68 × L63-1097, that yielded relatively well and maintained green leaves, while similar segregants in a cowpea cross (8517 × H8-9) survived (maintained green leaves longer) and performed well under heat and drought stress, but yielded poorly under nonstress conditions (Ismail et al., 2000). Furthermore, Abu-Shakra et al. (1978) found that DLS segregants in soybean had greater chlorophyll content, leaf protein, ribulosebisphosphate carboxylase activity and ribulosebisphosphate carboxylase protein in the leaves, and greater nitrogenase activity in the root nodules, while segregants with leaf senescence characteristics were inferior relative to these traits. More importantly, the DLS segregants flowered one week earlier and maintained their green leaves three weeks longer than those of the senescent types. Delay in leaf senescence in such cases did not however reduce total dry matter or pod dry weight

accumulation. These findings demonstrate that it is feasible to breed for DLS trait coupled with high nitrogenase activity and improved productivity in soybean.

Meanwhile, drought is recognized as the single, most prominent threat to agricultural production worldwide that accelerates leaf senescence, leading to a decrease in canopy size, loss in photosynthesis, and reduced yields. DLS has been found associated with enhanced adaptation to drought and increased biomass and grain yield under water stress (Ismail et al., 2000; Rivero et al., 2007; Gregersen et al., 2013). The DLS trait in cowpea and soybean offers an opportunity to dissect the genetic basis of stay-green trait in legumes. A diverse panel of cowpea germplasm and recombinant intred lines or RILs (IT93K-503-1 (showing adaptation to drought) × CB46 (drought susceptible)) was evaluated across four countries (Burkina Faso, Nigeria, Senegal, and the United States) under limited water conditions and SNP-genotyped using Illumina 1536 GoldenGate assay (Muchero et al., 2013). This research identified seven loci, five of which showed pleiotropic effects between DLS, biomass, and grain yield. Likewise, positive pleiotropy was noted in cowpea based on positively correlated mean phenotypic values ($r = 0.34$ to <0.87) and allele effects ($r = 0.07 < 0.86$) for DLS and grain yield across three African environments. Three of the five putative stay-green QTL (*Dro-1*, *Dro-3*, and *Dro-7*), which previously were reported to mediate DLS at the early vegetative stage in cowpea (Muchero et al., 2009), were identified in both RILs and diverse germplasm with resolutions of 3.2 cM or less for each of the loci, suggesting that these may be valuable targets for marker-assisted breeding in cowpea. Moreover, the colocation of delayed senescence with biomass and grain yield QTL suggests the possibility of using delayed senescence as criteria for postflowering adaptation to drought-prone environments in cowpea breeding. Incorporation of DLS into agronomically desirable genetic background may help to select for adaptation to drought, increased grain yield, and SNF in legumes.

Plant physiological processes that improve photosynthate acquisition, accumulation, and remobilization are important mechanisms for adaptation to drought stress. Knowledge of the genetic basis of these physiological processes may provide crop genetic enhancer opportunities to tailor plants with improved productivity. Using multi-environment data (RILs evaluated in eight environments differing in drought stress across Africa and South America) and mixed model, Asfaw et al. (2012) discovered nine QTL for 10 drought-stress tolerance mechanism traits, mapped on 6 of the 11 linkage groups in common bean, with significant QTL × environment interaction

for six of the nine QTL. QTL for SPAD chlorophyll meter reading and pod yield were the most consistent across environments. Further, candidate genes underlying major QTL for percent nitrogen fixed and total plant nitrogen fixed have been reported in common bean (see Section 3.6).

Photosynthates (C) are a limiting resource for plants. Legumes spend 4–16% of photosynthesis on each of the rhizobial and AM symbioses (Kaschuk et al., 2010a). Thus, growth and activity of both microsymbionts and the host plant would depend on C availability and competition between partners. Today, we know that C costs of the rhizobial and mycorrhizal symbioses for legumes are compensated by increases in the rates of leaf photosynthesis, independent of nutritional benefits (Kaschuk et al., 2009, 2010a). For example, Kaschuk et al. (2010a) reported on average 28% and 14% increase in photosynthetic rate due to rhizobial and mycorrhizal symbioses, respectively, and 51% due to dual symbioses. Likewise, the leaf P mass fraction increased by 13% due to rhizobial symbioses, while mycorrhizal symbioses increased leaf P by 6%, and dual symbioses increased leaf P by 41%. In contrast, neither of symbionts alone or together significantly increased the leaf N mass fraction, while the rate of photosynthesis increased substantially more than the C costs of rhizobial and mycorrhizal symbioses.

Thus, inoculation of legumes with rhizobia or mycorrhiza fungi, which results in a strong sink for the products of photosynthesis can improve the nutrient use efficiency and the proportion of seed yield in relation to the total plant biomass. The sink stimulation therefore, represents as adaptation mechanism that allows legumes to take advantage of nutrient supply from their microsymbionts without compromising on the total amount of photosynthesis available for plant growth (Kaschuk et al., 2010a). Furthermore, Kaschuk et al. (2010b) showed that increases in grain yield due to symbioses also resulted in increased seed protein but no change in lipid mass fractions, which confirm that legumes are not C-limited under symbiotic conditions.

The discovery of germplasm with delayed senescence and the fact the trait is associated with adaptation to drought and SNF, high N_2-fixing drought tolerant germplasm (see Section 3.4), legumes not being C-limited under symbiotic conditions, and genomic regions associated with adaptation to drought or candidate genes associated with nitrogen fixation in some legumes suggests that it is worthwhile approach to continue research in the search for such germplasm and integrate these into breeding programs to develop legume cultivars that are productive, high nitrogen fixer, resist drought stress, and nutritionally not inferior.

5.3 Selecting for High Nitrogen Fixation Ability into Improved Genetic Background

Early research during the 1980s and 1990s on plant breeding for enhanced SNF was mainly concentrated on common bean and soybean (Graham and Temple, 1984; Rosas and Bliss, 1986; Keyser and Li, 1992; Bliss and Hardarson, 1993; Herridge and Danso, 1995; Barron et al., 1999; Herridge and Rose, 2000; Mpepereki et al., 2000; Rengel, 2002). The emphasis during these decades was rather on exploring natural genetic variation for nitrogen fixation, discovering promiscuous host and rhizobium genetic resources, host–rhizobium specificity and interactions, the selection methods and environments, and plant physiological traits associated with high SNF.

Such efforts in plant breeding indeed resulted in the release of five high N_2-fixing cultivars of common bean in South America in 1990s (Bliss, 1993) and the finding of promiscuity in soybean, which was exploited by IITA breeders to develop several high-yielding promiscuous soybean advanced lines and cultivars, of which, few were released for cultivation in some countries in Africa (Mpepereki et al., 2000; see Sections 3.5 and 5.3). Likewise, an intensive effort by microbiologists and plant breeders led to release of several high N_2-fixing productive cultivars of soybean in Brazil and Argentina in South America (Hungria et al., 2006a). Search for effective soybean rhizobial strains and genotypes with high SNF capacity resulted in measurements that go up to 94% of total N plant's needs and rates higher than 300 kg N ha^{-1} (Hungria et al., 2005a, 2006a).

A large number of mutants with altered nodulation pattern (nod^{-}, no nodulation; $nod^{+/-}$, few nodules; fix^{-}, ineffective nodulation; nod^{++}, hypernodulation; nod^{++nts}, hypernodulation even in the presence of otherwise inhibitory nitrate levels) have been reported in several grain legume crops (Bhatia et al., 2001). Research showed that use of nodulation mutants have indeed contributed to the understanding of the genetic regulation of host–symbiotic interactions, and nodule development and nitrogen fixation (Sidorova et al., 2011 and references therein).

Among these mutants, hypernodulation mutants have shown poor grain yield. Despite the low yield of the mutants, hypernodulating mutants were used in legume breeding programs and intermediate hypernodulator segregants such as PS47 in soybean and K74a and K76a in pea were selected. PS47 fixed more N_2 and yielded as much as wild types, confirming previous reports that intermediate hypernodulators hold promise for use in breeding (http://www.regional.org.au/au/asa/1998/4/252song.htm). Similarly,

K74a and K76a outyielded Torsdag cultivar by 2- to 2.5-fold (Sidorova et al., 2011). The soybean cultivar "Nitrobean 60," which outyielded the check cultivar Bragg by 15% and contributed a high amount of fixed N_2 to the following cereal in crop rotation, was released in Australia (Bhatia et al., 2001).

For peas, an older set of mutants was used in breeding and a new cultivar "Triumph," originating from a cross between the commercial cultivar "Classic" and a donor of symbiotic effectiveness traits (K-8274) exhibited high grain yield and potential for legume–rhizobia–mycorrhizal symbioses. It was released in the central region of the Russia (Borisov et al., 2008). More recently, Sidorova et al. (2011) found dominant symbiotic pea mutants, which are characterized by their high grain yield and nitrogen fixation. They mapped the symbiotic genes on a *Pisum* chromosome. Using these symbiotic mutants with higher efficiency of nitrogen fixation in breeding program, Sidorova et al. (2011) isolated a series of high-yielding lines, characterized by their high root biomass with increased nitrogen content that were superior to the pea cultivars in both nodulation and nitrogenase activity. Farmers are growing these lines and minimizing the use of organic and mineral fertilizers in Russia.

The success in the development of promiscuous soybean germplasm and cultivars, which nodulate and fix atmospheric nitrogen with indigenous rhizobium strains, shows that such germplasm should be sought in other legumes to develop high N_2-fixing lines. Moreover, breeding for high SNF would be more effective if rhizobium strain selection is an integral part of the host breeding program. SNF is a complex physiological process, involving host, rhizobium, host–rhizobium interaction, and the growing environments. The use of DNA markers may therefore facilitate the identification of QTL associated with high SNF and their introgression into improved germplasm (Dwivedi et al., 2007; Collard and Mackill, 2008).

Candidate genes associated with high nitrogen fixation have been identified in the genomes of common bean (Galeano et al., 2012; Ramaekers et al., 2013), soybean (Schmutz et al., 2010), and model legume *M. truncatula* (Young et al., 2011; Stanton-Geddes et al., 2013). Furthermore, the genomes of model legumes, *Lotus japonicus* (Sato et al., 2008), *M. truncatula* (Young et al., 2011) and some of the grain legume crops, chickpea (Varshney et al., 2013), pigeonpea (Varshney et al., 2012), and soybean (Schmutz et al., 2010) have since been sequenced, with common bean soon to be published and available at the Phytozome Web site. Sequence variation of plant genes that determine the stability and effectiveness of symbiosis may be used for

developing DNA markers that will facilitate breeding of legume cultivars with high symbiotic efficiency (Zhukov et al., 2010).

The breeding populations should be exposed to selection for high symbiotic efficiency in soils that are low to deficient in N and P. Low soil N status is essential to select for the potential SNF, while P is the most critical nutrient for SNF. Moreover, nitrogen fixation is also sensitive to nitrate (NO_3) concentration in the growing medium, and therefore, the need to select for enhanced capacity to nodulate and fix nitrogen in the presence of nitrate in the soil. A visual assessment about the nodule number and nodule weight can be used as a simple selection criterion in early segregating generations while advanced lines should be evaluated for physiological traits associated with SNF (nodule number, nodule weight, nodule effectiveness, biomass, and shoot and root nitrogen). Percent atmospheric nitrogen and total nitrogen fixed should be assessed only on select breeding lines.

The increase in plant biomass production due to plant–microbe (rhizobia and mycorrhiza) symbiosis (Shtark et al., 2011) may be used as an indicator for an effective symbiosis in plants. It should however be supported by other parameters directly associated with nitrogen fixation because it may not hold true in some legumes and environments. Likewise, high-throughput assays and genes for assessing diversity and phylogenetic relationships among rhizobium strains may provide molecular tags to identify promiscuous rhizobia, which will obviate the need for inoculation, which is fret with problems for use especially in practical agriculture in the developing countries (Mukutiri et al., 2008).

5.4 SNF Projects to Harness Host–Rhizobium Symbiosis

The CGIAR Research Program "Leveraging legumes to combat poverty, hunger, malnutrition and environmental degradation" has included harnessing host-rhizobium symbiosis as one of the four priority key areas to capture unique legume ability to fix nitrogen, with a clear five-year outputs to identify germplasm with high SNF, the edaphic factors limiting SNF, and assess SNF potential of elite breeding lines. The activities to achieve the outputs in this global project are identifying environmental factors limiting SNF in chickpea, soybean and climbing beans; standardize screening protocols for SNF in target legumes; identify drought-prone site(s) with low available soil P to evaluate germplasm for SNF; access genotype × environment interactions for SNF; develop agronomic practices for high SNF in different agro-ecologies; access residual effect of SNF on the succeeding crop; identify genes/genomic regions associated with SNF

in chickpea; isolate and evaluate high nodulating and nitrogen-fixing indigenous rhizobia; access rhizobia diversity using 16S rDNA; develop protocols for mass production of efficient rhizobia strains; and strengthen capacity of stakeholders in SNF research and development (http://www.cgiar.org/our-research/cgiar-research-programs/cgiar-research-program-on-grain-legumes).

The National Science Foundation of the United States has awarded a grant for the project "Overcoming the domestication bottleneck for symbiotic nitrogen fixation in legumes" to research on nitrogen fixation in chickpea. The project is aimed at understanding how, and to what extent, crop domestication has impacted plants' ability to fix atmospheric nitrogen and identify genes that control nitrogen fixation capacity to develop more efficient nitrogen-fixing legume crops. The researchers plan to characterize the wild ancestors and the domesticated crop in search for genes whose function has been made less efficient during the course of domestication. Such genes could potentially be used either in classical plant breeding or molecular approaches to improve the capacity for nitrogen fixation in legume crops (http://news.ucdavis.edu/search/news_detail.lasso?id=9504).

The legume CRSP project of USA now known as the Legume Innovation Laboratory has an active component on enhancing SNF of leguminous crops grown on degraded soils in Eastern Africa. This research aims identifying production systems that enhance SNF, develop germplasm that benefits from symbiotic inoculation, and share the technological know-how with small-holder farmers to enhance bean production in sub-Saharan Africa. Researchers in this project has made considerable progress towards identifying common bean germplasm with superior SNF such as Puebla and BAT 477, which have been used as parents in crosses to generate populations for genetic studies and examine the potential for selecting advanced lines with high SNF. It is encouraging to note that this project has developed mapping populations, identified few genomic regions associated with SNF, and using conventional breeding and selection methods developed a few breeding lines with improved SNF (http://legumelab.msu.edu).

N2AFRICA is a large-scale, science-based "research-in-development" project focused on putting nitrogen fixation to work for smallholder farmers growing legume crop (common bean, cowpea, groundnut, and soybean) in Africa. N2AFRICA is funded by the Bill and Melinda Gates Foundation and the Howard G. Buffet Foundation and has many partners in the Democratic Republic of Congo, Ethiopia, Ghana, Kenya, Liberia, Malawi, Mozambique, Nigeria, Rwanda, Sierra Leone, Tanzania, Uganda, and

Zimbabwe. The stated goals of this project include to identify niches for targeting N_2-fixing legumes; test multipurpose legumes to provide food, animal feed, and improved soil fertility; promote the adoption of improved legume cultivars; support the development of inoculum production capacity through collaboration with private sector partners; develop and strengthen capacity for legume research and technology dissemination; and deliver improved cultivars of legumes and inoculant technologies to more than 225,000 smallholder farmers in eight countries of sub-Saharan Africa. (http://www.n2africa.org).

6. METABOLIC RECONSTRUCTION AND MODELING TO PREDICTING SNF

SNF is the biological process by which atmospheric nitrogen (N_2) acquired by bacteroids located in plant root nodules is converted into ammonium by nitrogenase. It involves a complex host–rhizobium symbiotic relationship orchestrated by the genetic and metabolic networks of both organisms (Dixon and Kahn, 2004). SNF results from the coordinated action of a variety of genes, protein, and metabolites, which in turn activate signal transduction cascades and transcriptional factors inside bacteroids. Central to all these processes are transporters of both the plant and the rhizobia, which transfer elements and compounds across various plant and bacterial membranes (Udvardi and Poole, 2013). The developmental processes prior to nitrogen fixation include exchange of a complex signals between the symbiotic partners triggers the invasion of the plant roots by rhizobial bacteria, the rhizobial bacteria then induce the plant roots to form nodule and penetrate into the plant cell through formation of infection thread and once inside the host cell, the rhizobial bacteria differentiate into nitrogen-fixing bacteroids, the site of nitrogen fixation (Jones et al., 2007). The universal features of symbioses known to date in legumes are the establishment of a microaerobic environment within nodules as a prerequisite for SNF, transport of reduced carbon (mainly in the form of dicarboxylic acids) from the plant to rhizobia, and transport of fixed nitrogen, ammonia, from the rhizobia to the plant (Lodwig et al., 2003; Prell and Poole, 2006; Prell et al., 2009).

6.1 Reconstructing Metabolic Network to SNF

Systemic understanding of nitrogen fixation requires the construction of a model to integrate genomic and high-throughput data in a hierarchical and coherent manner (Palsson, 2004). Using a constraint-based approach

and the flux balance analysis (FBA) (Price et al., 2004; Varma and Palsson, 1994), Resendis-Antonio et al. (2007) presented a genome-scale metabolic reconstruction (iOR363) model for *R. etli* CFN42—one of the rhizobia strain widely associated with nitrogen fixation in common bean, which includes 387 metabolic and transport reactions across 26 metabolic pathways. They analyzed the physiological capabilities of *R. etli* during stages of nitrogen fixation. Resendis-Antonio et al. (2007) found that FBA of reconstructed metabolic network for *R. etli* provided results in agreement with physiological observations. This reconstructed genome-scale metabolic network provides an important framework to compare model predictions with experimental measurements, and eventually generate hypotheses to improve nitrogen fixation. For example, the model clearly reproduces the utilization of oxidative phosphorylation, gluconeogenesis, and PHB biosynthesis pathways during nitrogen fixation, and suggests that a double gene deletion in PHB synthase and glycogen synthase could potentially increase SNF. Thus, the reconstruction and analysis provides template for studying symbiotic nitrogen-fixing bacteria to generate hypotheses, design experiments, and test predictable control principles for the metabolic network of *R. etli* (Resendis-Antonio et al., 2007).

A genome-based study of the metabolic activity in nitrogen fixation, involving *R. etli* bacteroids located at the root nodules of common bean, revealed 415 proteins and 689 upregulated genes that orchestrate nitrogen fixation. Furthermore, the constraint-based modeling simulated nitrogen fixation activity in such a way that 76.83% of the enzymes and 69.48% of the genes were experimentally proven and altered. A change of metabolic activity on these enzymes, as a result of gene deletion, induced different effects in nitrogen fixation, which is in agreement with observations made in *R. etli* and other *Rhizobiaceas*. Thus, a genome-scale study of metabolic activity in SNF may facilitate constructing computation model to integrate high-throughput data, describe and predict metabolic activity, and design experiments to explore the genotype-phenotype relationship in SNF (Resendis-Antonio et al., 2011).

S. meliloti 1021, of the genera *Medicago, Melilotus*, and *Trigonella*, is the most extensively studied strain for understanding the mechanisms of SNF and for legume–microbe interactions. Zhao et al. (2012) presented a manually curated model *i*HZ565 that provides an overview of the major metabolic properties of the SNF in *S. meliloti* 1021, which accounts for 565 genes, 503 internal reactions, and 522 metabolites. Moreover, the in silico predicted flux distribution was highly consistent with in vivo evidences,

which prove the robustness of the model. Furthermore, 112 of the 565 metabolic genes included in *i*HZ565 were found to be essential for efficient SNF in bacteroids under the in silico microaerobic and nutrient sharing condition. Thus, the model *i*HZ565 can be used as a knowledge-based framework for better understanding of the symbiotic relationships between rhizobia and legumes to uncover and better understand the mechanism(s) of nitrogen fixation in bacteroids and provide new strategies to improve SNF (Zhao et al., 2012). This knowledge of how transport and metabolism may be further integrated to achieve effective SNF and how varying during symbioses may help identify bottlenecks in specific legume–rhizobia systems that could be overcome by legume breeding aiming to enhance SNF (Udvardi and Poole, 2013).

6.2 Modeling to Predict Nitrogen Fixation

The improved prediction of SNF will help design efficient and sustainable agricultural production systems. There are several techniques available for direct measurement of SNF in the field. These are, however, time-consuming, expensive, and generate data relevant only to the time and place of measurement (see Section 3.2). Moreover, dinitrogen fixation by legume is sensitive to abiotic (drought, salinity, and heat) and biotic stresses, including soil mineral N (see Section 2.3). Models such as Sinclair, EPIC, Hurley Pasture, Schwinning, CROPGRO, SOILN, APSIM, Soussana, and STICS may be used for indirect measurement of N_2 fixation (Liu et al., 2011 and references therein). A detailed review on modeling clearly states that simulation by a dynamic model is preferable for quantifying SNF because of its capability to simulate the response of N_2 fixation to a wide range of environmental variables and crop growth status (Liu et al., 2011). The N_2 fixation rate in most simulation models is estimated from a predefined potential N_2 fixation rate, adjusted by the response functions of soil temperature, soil/plant water status, soil/plant N concentration, plant carbon (C) supply, and crop growth stage. Nonetheless, these models are imperfect, and need further improvement. The potential areas for improvement include consideration of photosynthetic C supply, refining soil mineral N concentration effects, characterization and incorporation of effects of excess soil water stress and water stress, and other factors used in simulation modeling (Liu et al., 2011).

Liu et al. (2013) proposed a new algorithm, which uses the aboveground biomass of legume crops to estimate the N_2 fixation rate that they incorporated into the SPACSYS model (Wu et al., 2007). When parameterized for

field pea and validated against published data from two northern European sites, Liu et al. (2013) found that model simulated the dynamic processes of N_2 fixation, N accumulation, and aboveground dry matter accumulation rate in pea. N_2 fixation was however, sensitive to low temperature and photosynthetic rate. Moreover, larger green leaf area and faster establishment in young pea plants coupled with a high photosynthetic rate would enhance N_2 fixation, suggesting that pea breeding aimed at cold tolerance and a high photosynthetic rate could increase N_2 fixation under a similar climate. Clearly, more emphasis should be placed to develop crop-specific models that accurately predict N_2 fixation for developing a sustainable production system.

7. PERSPECTIVES

Grain legumes contribute significantly to world food production, and provide a range of nutritional and agroecosystems services to the society. Legumes form symbiotic association with rhizobia to fix renewable source of N for agricultural soils. SNF is constrained by multiple stresses, and the contribution of SNF under stressed conditions is sub-optimal. There is a need to intensify research to make the symbiosis between host legumes and rhizobia optimum by alleviating stresses related to nutrient deficiency, water deficit, heat and salts, among others, which would improve SNF in agroecosystems. Genetic differences in adaptation to these stresses have been noted in both host plant and rhizobium species. More research efforts however should be directed to identifying and understanding the genetic and molecular basis of adaptation stress, both in host plant and rhizobium.

A systematic evaluation of germplasm is needed to select high N_2-fixing legume accessions for use in crop breeding. Reduced subsets, in the form of core or mini-core collections, representing diversity of the entire germplasm of a given species in the genebank, are suggested as a gateway to identify new sources of variability. Such subsets are available in most of the grain legumes (Dwivedi et al., 2005, 2007), and should be evaluated for SNF traits. The selection environment (with respect to nutrients) needs to be well defined to obtain reliable and reproducible results. Likewise, the germplasm and breeding populations should be ideally evaluated in soils low in N and P to detect genetic differences in N_2 fixation. Low soil N status is essential to select for the potential SNF, while P is the most critical element for SNF. SNF is a complex trait influenced by nodule number and nodule weight, root and shoots weight, total biomass, and percent and total

atmospheric N_2 fixed. Accurate phenotyping is the key to identifying high N_2-fixing germplasm and make rapid gain in breeding programs. Selected high-throughput assays have been applied to assess N_2 fixation in soybean. These assays need, however, to be evaluated further across legumes before proposing their use in breeding programs. Until that time, a visual assessment of leaf color score and nodulation may be used as simple selection criteria in early segregating generations. The advanced breeding lines, should on the other hand, be evaluated for detailed physiological traits associated with SNF, with only selected lines evaluated for percent atmospheric and total N fixed.

The last decade has seen the emergence of novel, intriguing aspects of rhizobial biology, potentially having profound impacts on SNF and crop production, even in nonlegumes. Following the discovery of beta-rhizobia, a revolution in the rhizobial concept emerged extending the ability to form N_2-fixing symbiosis with legumes outside *Alphaproteobacteria*. Indeed, different symbiotic combinations are possible, at least for alpha- and beta-rhizobia, which have evolved through horizontal transfer of few common genes followed by diversification and habitat specialization. Large variation in bacteria–plant interaction recipes are also present in the same rhizobial species, as highlighted by recent genome studies, which indicate the genomic region harboring symbiotic genes as the most variable in terms of type and number of genes, within rhizobial genomes. Such high genomic variation is then also reflected in many adaptive phenotypes of strains, such as those related to the tolerance to stresses, as for instance salinity, pH variation, high temperature, and the presence of pollutants in soil, and may be in the ability of elite strains to compete with indigenous rhizobia. Consequently, there is a plethora of different natural rhizobium strains, large enough to provide an optimal basis for the screening of genes and phenotypes to be used for producing other strains specific for different soils and host genotypes. Last but not least, there may be, as noted in various experiments, additional roles (not related to nodulation) of rhizobial symbionts for plant growth, in both legume and nonlegume species. These roles seem to be linked to the production of bioactive molecules, which stimulate plant growth (e.g. phytohormones, lumichrome, or the Nod factor), and also to the direct colonization of plant endosphere in cereal crops (such as rice), which in turn could have protective or antagonistic effects toward the colonization of the same environments by other commensal or pathogenic microorganisms. The future of rhizobial biology is then directed towards the screening and collection of strains with interesting phenotypes and to link, under a

systems biology view, such new or already known phenotypes with genomic information, providing genetic tools to screen and improve plant growth promoting performances of rhizobial strains.

The finding and utilization of promiscuous soybean germplasm led to the development and release of several high-yielding cultivars in some countries in Africa; while high N_2-fixing productive pea cultivars in Russia and a soybean cultivar in Australia originated from mutation, crossing, and selection. Furthermore, breeding in low N soils in South America has resulted in hundreds of soybean commercial cultivars with high SNF capacity. Soybean breeding elsewhere demonstrated that intermediate nodulators (not the hypernodulators) when used as parents led to high N_2-fixing productive segregants. Genes with varying effects seem to control N_2 fixation. To date, few major QTL and candidate genes, underlying QTL have been reported in grain and model legumes. Nodulating genes in model legumes have been cloned and their orthologs determined in grain legumes. SNP markers associated with nodulation genes are available in common bean and soybean.

Genomes of chickpea, pigeonpea, soybean, and of several rhizobium species have been decoded. Expression studies revealed few genes associated with SNF, in both model and grain legumes. The advances in host plant and rhizobium genomics are helping researchers to identify DNA markers that aid in breeding legume cultivars with high symbiotic efficiency. A paradigm shift is needed in breeding programs to simultaneously improve host plant and rhizobium to harness the strength of the positive symbiotic interactions in cultivar development.

Systematic understanding of nitrogen fixation requires the construction of a model to integrate genomic and high-throughput data in a hierarchical and coherent manner. Development of computation models based on metabolic reconstruction pathways may help identifying bottlenecks in specific legume–rhizobia systems, which could be overcome by legume breeding aiming to enhance SNF. Models to simulate the response of N_2 fixation under a wide range of environmental variables and crop growth are assisting researchers to quantify SNF for development of efficient and sustainable agricultural production systems.

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