Saad Sulieman · Lam-Son Phan Tran
Editors

Legume Nitrogen Fixation in Soils with Low Phosphorus Availability

Adaptation and Regulatory Implication

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Preface

The essential role of legumes in agriculture is well recognized. The capacity of legumes to fix atmospheric dinitrogen (N_2) in partnership with rhizobia provides an input-saving and resource-conserving alternative, thereby reducing the need for chemical fertilizers while enhancing overall crop productivity. As leguminous crops are inextricably linked to the surrounding environment, their mere inclusion in various cropping systems does not always ensure the attainment of the estimated optimal levels of symbiotic N_2 fixation in the field. Yield instability represents one important drawback in the development of grain legumes. Thus, special concerns have to be paid to the factors limiting legume growth in order to obtain a more stable level of legume production. Obviously, a major constraint to legume production is low phosphate (Pi) availability in soil. Low Pi availability is especially problematic for legumes, since legume nodules responsible for N_2 fixation have a high Pi requirement. This has become a hot issue because of how the mechanisms controlling N_2 fixation in legumes are affected by Pi deficiency; this is not fully understood. Understanding how nodule N_2 fixation responds to low Pi availability is crucial for improving legume production and maintaining agricultural sustainability in the context of global Pi crisis. In this thoughtful and provocative new book volume, we provide a concise and up-to-date presentation of the state-of-the-art knowledge of how current and projected future Pi scarcity will affect legume growth and their symbiotic N_2-fixing capabilities.

Due to the scarcity of Pi and the significant reduction in global Pi reserves, there is an urgent need to develop cultivars that are more efficient in acquiring Pi from soil and/or in using Pi more efficiently. Genetic improvement of Pi-use efficiency in legumes would be more economic and practical than relying on chemical-Pi fertilizers alone. Legume species or genotypes with higher Pi-use efficiency would be extremely useful. In recent years, much progress has been made in understanding how Pi starvation affects nodulated legume performance. Thus, we consider that it is timely to examine the physiological and molecular responses of nodules to Pi deficiency to identify common principles. In particular, we are interested in knowing whether we are now in a position to evaluate our acquired knowledge that might be applied by plant breeders, particularly through some of the new methods of

genetic engineering, to develop such unique and more adaptive cultivars with higher symbiotic efficiency. A wide range of senior undergraduate and graduate students, as well as researchers, including molecular biologists, physiologists, ecologists, and plant breeders in many disciplines related to crop productivity, will find the perspectives and analyses offered by this volume an exciting contribution to the development of our understanding in the area of plant stress physiology.

The *Legume Nitrogen Fixation in Soils with Low Phosphorus Availability: Adaptation and Regulatory Implication* volume contains thirteen chapters that collectively present the most comprehensive and authoritative review for the impacts of Pi scarcity on legumes and their symbiotic performance. The first nine chapters provide a foundation for the four subsequent chapters devoted to individual species. The attractive candidate species, namely, *Medicago truncatula* (Chap. 10), common bean (Chap. 11), soybean (Chap. 12), and white lupine (Chap. 13) represent the main focus of research on Pi deficiency in legumes. At the outset, we selected three principal objectives:

First, the contributors should be recognized internationally for their previous achievements in legume research.
Second, wherever possible, groups of authors with mutually complementary expertise should be invited to collaborate in writing individual chapters.
Third, the volume should reflect worldwide progress in legume's acclimation to Pi scarcity and, in particular, the remarkable achievements in recent years.

The authors, we believe, are to be congratulated on the in-depth coverage and quality of their contributions, given their busy schedules and various commitments. Numerous colorful figures and tables have been provided to facilitate the comprehension of the presented materials.

We gratefully acknowledge all the authors who joined this book project by contributing their valuable work and for their cooperation in bringing out this volume in time. We would like to express our appreciation to Springer US for taking up the publication of this volume. Special thanks and gratitude are extended to Dr. Kenneth Teng (Editor, Plant Breeding and Biotechnology, Springer New York), Mr. Jeffrey Taub (Senior Production Editor, Springer New York), and all other staff members of Springer involved in the production of this endeavor. We hope that readers will enjoy reading this volume, whose detailed and up-to-date information and knowledge may pave the way to promising areas for future research.

Khartoum North, Shambat, Sudan — Saad Sulieman
Yokohama, Japan — Lam-Son Phan Tran

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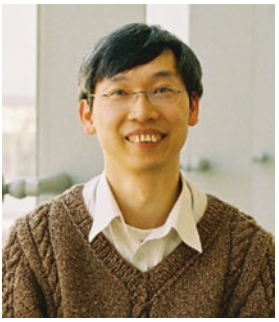
About the Editors

Dr. Saad Sulieman graduated from the University of Khartoum in 1998 with distinction in agronomy and was awarded many prizes for being the best graduate. He joined the Department of Agronomy, Faculty of Agriculture, University of Khartoum, as a teaching assistant in 1999. Subsequently, he obtained an M.Sc. in crop sciences at the University of Khartoum (2002) and then a Ph.D. in agricultural chemistry (2009) at Georg-August University of Göttingen, Germany, with a scholarship from the German Academic Exchange Service (DAAD). During his Ph.D. pursuit, he focused on investigating the physiological characterization of symbioses contrasting tolerance in nitrogen fixation to major soil limitations. After completing his Ph.D., he continued to work as a research assistant at the same institute for a year and a half. He also worked as a postdoctoral fellow at RIKEN Center for Sustainable Resource Science (CSRS), Yokohama, Japan, for one year (2013) with a postdoctoral fellowship from the Japan Society for the Promotion of Science (JSPS). Recently, he has been awarded a Georg Forster Research Fellowship for experienced researchers from the Alexander von Humboldt (AvH) Foundation. Throughout his career, he has taught many students at the B.Sc. and M.Sc. levels. Sulieman was promoted to lecturer in 2005, assistant professor in 2009, and associate professor in 2013. Between 2014 and 2016, he was the head of the Department of Agronomy, University of Khartoum. Throughout the years of his research career, he has attended many domestic and international meetings, conferences, seminars, and workshops. He has authored or coauthored more than 35 publications. The main focus of his research is the field of molecular plant physiology of abiotic stress.



Dr. Lam-Son Phan Tran is head of the Signaling Pathway Research Unit at RIKEN Center for Sustainable Resource Science, Japan. He obtained his M.Sc. in biotechnology in 1994 and Ph.D. in biological sciences in 1997, from Szent Istvan University, Hungary. After doing his postdoctoral research at the National Food Research Institute (1999–2000) and the Nara Institute of Science and Technology of Japan (2001), in October 2001, he joined the Japan International Research Center for Agricultural Sciences to work on the functional analyses of transcription factors and osmosensors in *Arabidopsis* plants under environmental stresses. In August 2007, he moved to the University of Missouri–Columbia, USA, as a senior research scientist to coordinate a research team working to discover soybean genes to be used for genetic engineering of drought-tolerant soybean plants. His current research interests are elucidation of the roles of phytohormones and their interactions in abiotic stress responses, as well as translational genomics of legume crops with the aim to enhance crop productivity under adverse environmental conditions. He has published over 110 peer-reviewed papers with more than 80 research and 30 review articles and contributed 8 book chapters to various book editions published by Springer and Wiley-Blackwell and to the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. He has also edited 7 book volumes for Springer, including this one.

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Chapter 1
The Role of Legume-*Rhizobium* Symbiosis in Sustainable Agriculture

Takuji Ohyama

Abstract The world's population is increasing rapidly, and world food production needs to be commensurate with the demands of human consumption. To increase cultivated acreage would be very difficult, so we need to promote crop production, or the efficient use of existing croplands. The use of chemical nitrogen (N) fertilizers in the twentieth century promoted crop production by 4–10 times, and has supported food production over the past 100 years. However, the cost of chemical N fertilizers is high for farmers in developing countries, and their production requires a lot of fossil fuel. In addition, the inappropriate or excess application of chemical N fertilizers causes environmental problems, such as contamination of ground water by nitrates, and air pollution and global warming due to nitrous oxide. On the other hand, most leguminous crops, such as soybeans, beans, chickpeas, and groundnuts, and legume forage crops such as alfalfa and clover can fix atmospheric dinitrogen (N_2) by symbiosis with soil microorganisms (collectively termed rhizobia). The supply of N by symbiotic N_2 fixation via legume-rhizobium symbiosis is the most important source of N in agro-ecosystems. This renewable and environmentally sustainable N source also ensures soil restorative agents for maintaining soil fertility and sustainable crop production. Legume crops provide an important source of protein, oil and carbohydrate for human diets and livestock feeds. The production of legumes depends on symbiotic N_2 fixation, and this process is affected by various environmental conditions, as well as the supply of water and mineral nutrients, especially the availability phosphorous—the main theme of this book.

Keywords Chemical N fertilizer • Global population increase • Legume-*Rhizobium* symbiosis • N_2 fixation • Root nodules • Sustainable agriculture

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1.1 Current Situation of Global Population and Food Security

1.1.1 Estimate of Global Population Change and Food Production

The world's population was only about 1 billion in 1800, doubled in 100 years to 2 billion in 1900, then tripled in the next 100 years to 6 billion in 2000. The total human population of the world reached 7.3 billion in 2015, and is still increasing. In 2015, about 60% of the global population (4363 million) lived in Asia, 16% (1186 million) in Africa, 10% (738 million) in Europe, 9% (634 million) in Latin America and the Caribbean, 5% (358 million) in Northern America, and 0.5% (39 million) in Oceania (Table 1.1) (United Nations Department of Economic and Social Affairs/Population Division 2015a). China (1376 million) and India (1311 million) are the two largest countries in the world in terms of population, followed by the USA (322 million), Indonesia (258 million), Brazil (208 million), Pakistan (189 million), Nigeria (182 million), Bangladesh (161 million), the Russian Federation (143 million) and Mexico (127 million) in 2015 (Table 1.2).

The United Nations estimates that the world population will reach 8.5 billion in 2030, 9.7 billion in 2050, and 11.2 billion in 2100 (Table 1.1) (United Nations 2015b). The population in Asia after 2030 will be relatively stable at around 5 billion; however, the population in Africa is estimated to increase markedly from 2050 to 2100 (4.4 billion), and to become comparable to the Asian population by 2100 (4.9 billion) (Table 1.1). The population in Europe will decrease slightly from 2050 (707 million) to 2100 (646 million). By 2050, the population in India is estimated to reach 1.71 billion, overtaking that in China (1.35 billion), followed by Nigeria (399 million), the USA (389 million), Indonesia (321 million), Pakistan (310 million), Brazil (238 million), Bangladesh (202 million), the Democratic Republic of the Congo (195 million), and Ethiopia (188 million) in 2050 (Table 1.2). From 2015 to 2050, the total increase will be about 2.38 billion, with 394 million in India, 216

Table 1.1 Population of the world and major areas, 2015, 2030, 2050, 2100

		Population	(million)	
Major area	2015	2030	2050	2100
World	7349	8501	9725	11,213
Africa	1186	1679	2478	4387
Asia	4363	4923	5267	4889
Europe	738	734	707	646
Latin America and the Caribbean	634	721	784	721
Northern America	358	396	433	500
Oceania	39	47	57	71

Data from United Nations Department of Economic and Social Affairs/Populaion Division (2015a)

Table 1.2 Ten countries with the largest populations, 2015 and 2050

Rank	Country	2015 Population (million)	Country	2050 Population (million)
1	China	1376	India	1705
2	India	1311	China	1348
3	USA	322	Nigeria	399
4	Indonesia	258	USA	389
5	Brazil	208	Indonesia	321
6	Pakistan	189	Pakistan	310
7	Nigeria	182	Brazil	238
8	Bangladesh	161	Bangladesh	202
9	Russian Federation	143	Democratic Republic of the Congo	195
10	Mexico	127	Ethiopia	188

Data from United Nations (2015b)

million in Nigeria, 121 million in Pakistan, 118 million in the Democratic Republic of the Congo, 89 million in Ethiopia, 84 million in Tanzania, 67 million in the USA, 65 million in Indonesia, and 63 million in Uganda.

The main point to note in these population changes in the world as estimated by the United Nations is that the most rapid increase will occur in the poorest area (Africa) and countries (African and Asian countries). Stable food supply by self-production in these areas will be very difficult due to the severe weather conditions and poor soils in these countries.

From the Technical Summary of Status of the World's Soil Resources (FAO, ITPS 2015), between 1961 and 2000, global population grew by 98%, but food production rose by 146% and food production per person increased by 24%. Crop yields have more than doubled and, quite remarkably, the area of arable land in use increased by only 8%. Arable land per person reduced substantially from 0.45 ha to 0.25 ha during this period. To achieve any increase in cultivated acreage would be very difficult, thus we need to promote crop production, or the efficient use of croplands.

1.1.2 Human Nutrition

All human beings need to eat a certain amount of food, from either plant or animal products, to support life. Most higher plants are "photoautotrophs", i.e., organisms capable of synthesizing their own food materials themselves from simple inorganic substances such as carbon dioxide, water, and minerals, using light energy. However, all animals, including humans, are "heterotrophs", i.e., organisms that are dependent for nutrition on organic substances derived from other organisms. Humans are omnivorous and eat many kinds of plant and animal products, such as crops,

vegetables, fruits, meat, milk, fish, eggs, etc., unless these are dangerous due to natural toxins or harmful microbial contamination, or just taste bad. There are a wide variety of foods all over the world depending on the region, culture, religion, history and natural habitat of traditional crops and animal products. Asian people traditionally depend on rice, soybean, vegetables and fish, and European people generally depend on wheat, meat and dairy products. However, the fundamental requirements of human nutrition are common to all.

There are three macronutrients (carbohydrates, proteins, lipids) and two micronutrients (minerals and vitamins). Sometimes water is included in the list of human nutrients (Vorster 2009), although water does not contain any organic substances or energy. Carbohydrates, such as starch and sugars, and lipid or oil provide energy and carbon materials for maintaining metabolism and growth in humans. On the other hand, protein in food is digested to amino acids and used for protein synthesis in the body.

Human nutrition is complex and the quantity and quality of essential nutrients in foods are important for the maintenance of life (Voster 2009). Optimal, balanced nutrition is a major determinant of health, and can promote health and well-being, as well as prevent ill-health and treat disease (Vorster 2009). However, hundreds of millions of food- and nutrition-insecure people still exist globally, leading to the coexistence of both under-nutrition and inappropriate nutritional behavior such as overconsumption in the world today.

The amount of food necessary for humans differs depending on their age, sex, body weight and the area in which they live (Ministry of Health, Labor and Welfare, Japan 2016). It was recommended that Japanese men and women aged 40 years old need food containing about 2300 kcal and 1750 kcal per day, respectively. On the other hand, world average food consumption in 2005/2007 was about 2772 kcal per person per day; this is expected to increase to 3070 kcal in 2050, and to 3200 kcal in 2080 (Table 1.3; Alexandratos and Bruinsma 2012). It is calculated that 7.3 billion

Table 1.3 Projections of food requirement in 2050 and 2080

Year	2005/2007	2050	2080
Energy (kcal person^{-1} day^{-1})	2772	3070	3200
Cereals, food (kg person^{-1} year^{-1})	158	160	161
Cereals, all uses (kg person^{-1} year^{-1})	314	330	339
Meat, food (kg person^{-1} year^{-1})	38.7	49.4	55.4
Oilcrops,[a] food (kg person^{-1} year^{-1})	12.1	16.2	16.9
Oilcrops,[a] all uses (kg person^{-1} year^{-1})	21.9	30.5	33.8
Cereals, production (million tons/year)	2068	3009	3182
Meat, production (million tons/year)	258	455	524
Paddy rice yield (tons/ha)	3.32	4.30	4.83
Arable land area (million ha)	1592	1661	1630

(Modified from Alexandratos and Bruinsma. World Agriculture Towards 2030/2050; The 2012 Revision) (FAO 2012)

[a]Oil equivalent

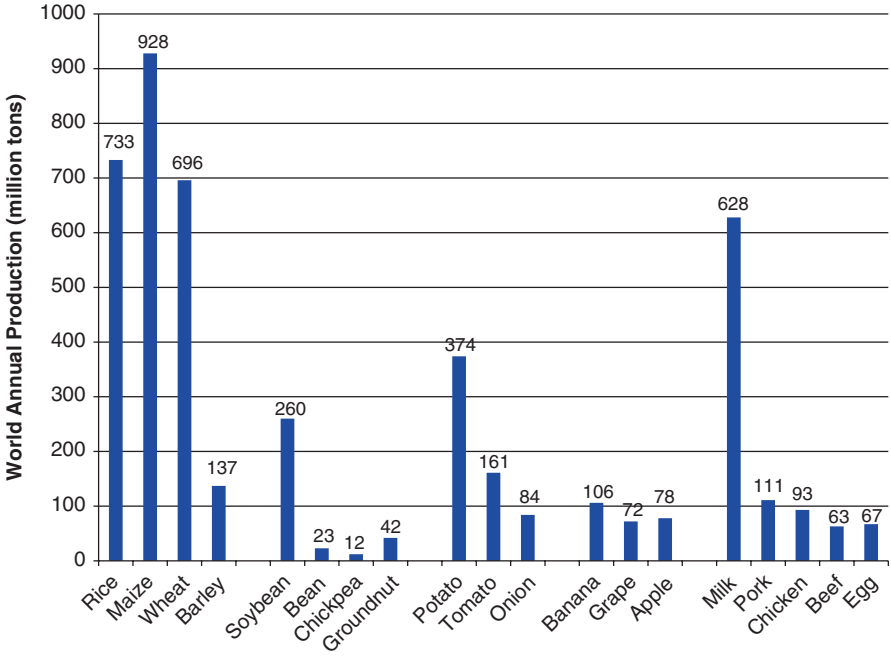

Fig. 1.1 World annual production of major foods (Average 2011–2013) (Data from MAFF, Japan based on FAOSTAT (2015.3))

people in 2015 and 9.7 billion people in 2050 will need 7.4×10^{12} and 10.9×10^{12} M cal per year, respectively.

As shown in Fig. 1.1, maize (928 million tons/year averaged over 2011–2013), rice (733), wheat (696), and barley (137) are major cereals, which provide mainly carbohydrates and starch, as does potato (374). Soybean (260) provides protein and oil, and beans (23), chickpeas (12) and groundnuts (42) are the major legume crops. Vegetables, such as tomato and onion, and fruits, such as banana, grape, and apple, are major foods. Regarding animal-derived foodstuffs, milk (628) production is the highest, followed by pork (111), chicken (93), egg (67) and beef (63). Although the production and consumption of dairy foods and meats are increasing rapidly, the world total production of cereals still far exceeds that of animal products. This is partly because relatively poor people in Asia and Africa depend mostly on plant products rather than animal products, and also because the requirement of cereals for animal feed is also increasing.

Proteins in our body are in a state of homeostasis, which means proteins are broken down constantly, and almost the same amount of protein is newly synthesized. Therefore, although the total protein content in our body is relatively constant, we need to take in a certain level of protein daily. It was estimated that every Japanese person needs 0.65 g protein (kg body weight)$^{-1}$ day^{-1}, which is equivalent to 39 g protein for a 60 kg person each day, i.e., 14.2 kg per year. The world's population

Table 1.4 Comparison of nutrient contents in 100 g edible part of major crops, vegetables, fruits, dairy foods and meets

Food	Energy kcal	Water g	Protein g	Lipid g	Carbohydrate g	Minerals g
Rice	350	15.5	6.8	2.7	73.8	1.2
Maize	350	14.5	8.6	5.0	70.6	1.3
Wheat	337	12.5	10.6	3.1	72.2	1.6
Barley	341	14.0	10.9	2.1	72.1	0.9
Soybean	417	12.5	35.3	19.0	28.2	5.0
Bean	336	15.5	23.9	2.0	55.0	3.6
Chickpea	374	10.4	20.0	5.2	61.5	2.9
Groundnut	562	6.0	25.4	47.5	18.8	2.3
Potato	76	79.8	1.6	0.1	17.6	0.9
Tomato	19	94.0	0.7	0.1	4.7	0.5
Onion	37	89.7	1.0	0.1	8.8	0.4
Banana	86	75.4	1.1	0.2	22.5	0.8
Grape	59	83.5	0.4	0.1	15.7	0.3
Apple	54	84.9	0.2	0.1	14.6	0.2
Milk	67	87.4	3.3	3.8	4.8	0.7
Pork (loos)	291	58.0	18.3	22.6	0.2	0.9
Chicken (thigh)	253	62.9	17.3	19.1	0	0.7
Beef (chuck roll)	318	56.4	16.2	26.4	0.2	0.8
Egg	151	76.1	12.3	10.3	0.3	1.0

(Modified from All Guide, Standard Tables of food composition in Japan (2009))

thus requires about 100 million tons of protein annually, either from meat, fish, milk, egg, beans or cereals.

Table 1.4 compares the compositions of major foods per 100 g edible parts (All Guide, Standard Tables of Food Composition in Japan 2009). In rice grains, carbohydrate, mainly starch, is the major component, accounting for 74% of grain weight. The carbohydrate contents in grains are similar in maize, wheat, and barley. Unlike cereal grains, starch is usually not present in mature soybean seeds. The composition of soybean seeds produced in Japan is as follows (per 100 g): energy 417 kcal, water 12.5 g, protein 35.3 g, lipids 19.0 g, carbohydrate 28.2 g, and minerals 5 g. The composition, especially the higher protein content, is quite different from cereal grains. Other legume crops, such as bean, chickpea, and groundnut, also contain relatively high protein content (20–25 g) compared with cereals, although less than in soybean. The lipid content in groundnut (47.5 g) and soybean (19 g) is much higher than in cereals grains (2–5 g).

Soybean seed is one of the most important protein sources for both humans and livestock. The amino acid composition of soybean seeds is relatively well balanced from amino acid scores, although soybean seeds contain a relatively low amount of the sulfur-containing amino acids, methionine and cysteine (Fig. 1.2) (Ohyama et al. 2013a). On the other hand, rice protein contains higher levels of methionine

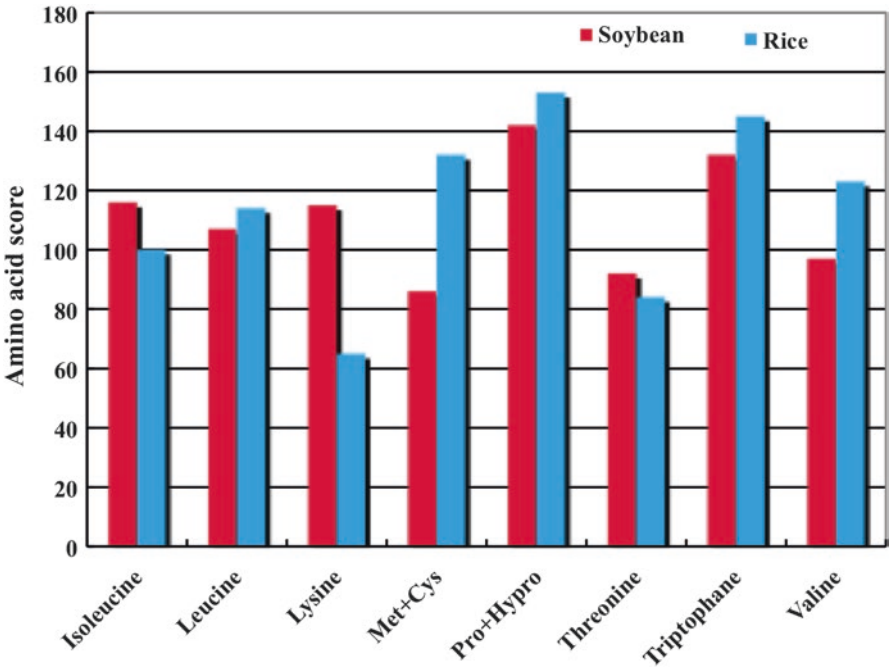

Fig. 1.2 Comparison of amino acid scores of essential amino acids in soybean seeds and rice grain (Figure from Ohyama et al. (2013a))

and cysteine, in spite of a low level of lysine. Therefore, by eating soybean and rice together, the balance of amino acid composition is compensated, and becomes very good.

1.1.3 Food Security

"Food security" is defined as "access for all, at all times, to a sustainable affordable supply of nutritionally adequate and safe food for normal physical and mental development and healthy, productive life" (Vorster 2009). Conversely, "food insecurity" is the state in which people live with hunger and fear starvation. To ensure food security, it is important to supply safe foods continuously. Being free from contamination of harmful microorganisms, toxins and other hazardous substances that cause disease is also important for food safety (Vorster 2009).

FAO estimates indicate that 12.5% of the world population (868 million people) is undernourished in terms of food energy intake, 26% of the world's children are stunted, 2 billion people suffer from micronutrient deficiencies, and 1.4 billion people are overweight or obese (FAO 2013).

Many complex and interacting factors contribute to malnutrition on different levels (Vorster 2009):

1. "Individual level" related to food and nutrient intake, health status, growth, and physical activity.
2. "Household level" related to family size and composition, gender equity, food distribution in household, income, availability of food, and access to food.
3. "National level" related to health, education, sanitation, agriculture, war, political instability, urbanization, population growth, natural disasters, and decreased resources.
4. "International level" related to social, economic and political structures, trade agreements, population size, population growth distribution, and environmental degradation.

World demand for crops is increasing due not only to global population growth, but also to increased biofuel production, and changing dietary preferences, with a move towards meat and animal products rather than plant foods (Cassidy et al. 2013). According to a 2011 analysis, 75% of all agricultural land, including crop and pasture land, is dedicated to animal production. Currently, 36% of the calories produced by the world's crops are being used for animal feed, and only 12% of those feed calories ultimately contribute to the human diet (Cassidy et al. 2013). The calorie conversion efficiency of milk, eggs, chicken meat, pork, and beef are 40%, 22%, 12% 10% and 3%, respectively, and the protein conversion efficiency of milk, eggs, chicken meat, pork, and beef are 43%, 35%, 40%, 10% and 5%, respectively (Cassidy et al. 2013).

A sufficient supply of sustainable food is essential in each country. Food balance sheets in 2003 showed that the self-sufficiency rates of grain [percentage of grain production per total amount of (production + import − export)] were highest in Australia (333%) and Argentina (249%), while Thailand (162%), Myanmar (131%), Vietnam (127%), Laos (123%), and Cambodia (122%) were relatively high in Asia. China (100%), India (98%), Indonesia (89%), and the Philippines (82%) are well balanced. However, Japan (27%) and Korea (28%) were in a critical situation (Ohyama et al. 2013a). Meat consumption increases rapidly in developing countries. However, meat production requires a large amount of crops. The production of 1 kg chicken, pork and beef requires about 2, 5 and 8 kg crops, respectively. Therefore, it is important that humans depend directly on protein from plant sources rather than from animal sources in order to provide sufficient food for the increasing population.

FAO and ITPS (Intergovernmental Technical Panel on Soils) reported the Status of the World's Soil Resources in 2015—the International Year of Soils (FAO and ITPS 2015). This is of particular relevance to the Sustainable Development Goals that the international community has pledged to achieve. They reported that 33% of land today is moderately-to-highly degraded due to erosion, salinization, compaction, acidification, and chemical pollution of soils (FAO and ITPS 2015). Sustainable soil management is a key foundational concept of this report, and is defined as follows: "Soil management is sustainable if the supporting, provisioning, regulating,

and cultural services provided by soil are maintained or enhanced without signify impairing either the soil functions that enable those services or biodiversity". In the report, the following actions have the highest priority: (1) Sustainable soil management can increase the supply of healthy food for the most food insecure people among us; (2) the global stores of soil organic matter should be stabilized or increased, and (3) humanity is close to the global limits for total chemical nitrogen fertilizer and regional limits for phosphorous use. Therefore, we should act to stabilize or reduce global nitrogen (N) and phosphorus (P) fertilizer use, while simultaneously increase fertilizer use in regions of nutrient deficiency.

1.2 N Chemical Fertilizers

1.2.1 N as a Major Essential Element of Plants

Most higher plants are photoautotrophs, and can synthesize all the compounds they need from carbon dioxide, water, light energy and inorganic nutrients absorbed from the roots. Aside from nutrient requirements, plants also need essential elements, which are classified as macro-elements (N, K, Ca, Mg, P and S), and microelements (Cl, Fe, Mn, B, Zn, Cu, Mo and Ni).

N is a major essential element for all organisms, being a main constituent of proteins, nucleic acids, and other indispensable organic compounds. Despite the high concentration of N_2 (about 78% by volume) in air, its concentrations in soil, rocks and sea water are relatively low, and the availability of N is often a limiting factor for crop production and natural plant growth. Major forms of inorganic N in soil are ammonium and nitrate, which plants absorb from their roots. Some plants, e.g., legumes, can fix atmospheric N_2 symbiotically in association with N_2-fixing soil bacteria, rhizobia.

1.2.2 Global N Cycling

N is cycled globally on land, in the atmosphere and in the ocean (Matsushima et al. 2010). Although the concentration of N_2 in air is high, all "eukaryotes", including plants, animals, and fungi, cannot use it by themselves. Only a wide variety of "prokaryotes", which have enzymes of the "nitrogenase complex" can fix N_2 to ammonia, and thus use N for the synthesis of organic compounds. There are two types of "biological N_2 fixation" (BNF) in agricultural systems, crops, pastures and fodder, as well as natural ecosystems (Herridge et al. 2008): free-living BNF, and symbiotic or plant-associated BNF.

As shown in Fig. 1.3, the total amount of annual global BNF was estimated at about 122 T gN (= million tons of N), of which 50–70 T gN is estimated to be present in agricultural systems (Herridge et al. 2008). About 100 T gN is synthesized

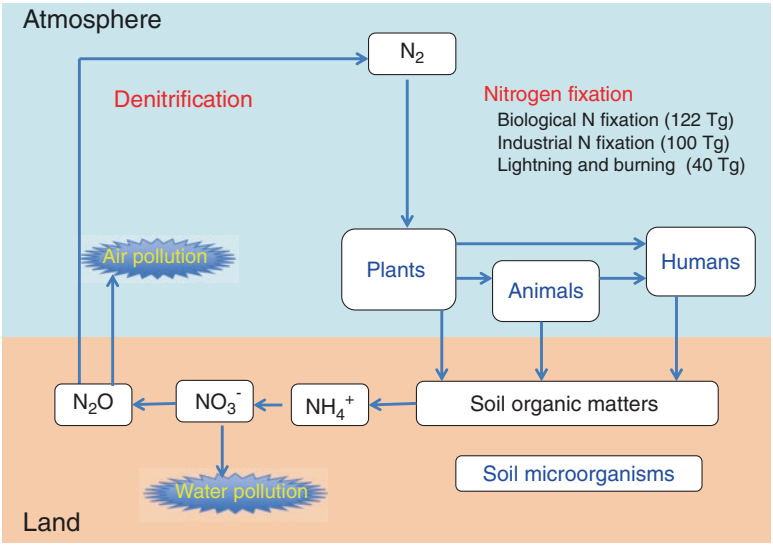

Fig. 1.3 Global nitrogen cycle

artificially by the Harbor-Bosch process, of which 80 T gN is used for N fertilizers. Due to lightning and burning of fuel, atmospheric N_2 is oxidized to NO_x; the total amount of N contributed from these processes is estimated to be 40 T gN. Plants can use the fixed N in the form of either ammonia or nitrate, and animals depend on the organic matter produced by plants. Humans eat foods originating from plant or animal products. The waste and dead bodies of organisms are broken down mainly by soil microorganisms, and the residual soil organic matter—humus—is deposited in the soil. Soil organic matter is eventually decomposed to minerals by soil microorganisms, and ammonia or ammonium (NH_4^+) is released through the mineralization process; NH_4^+ is readily oxidized to nitrate (NO_3^-) by nitrifying bacteria in nitrification processes in upland fields. And some part of NO_3^- is reduced to nitrous oxide (N_2O) by denitrifying bacteria, and finally reduced to N_2. The N cycle is related to environmental issues, e.g., NO_3^- elution to underground water, rivers and pond water, which may cause water pollution, and emission of N_2O—a potent global warming gas (Matsushima et al. 2010)—causes air pollution. Heavy use of N chemical fertilizers may aggravate these serious environmental problems.

1.2.3 Chemical N Fertilizers

Since human beings started agriculture about 10,000 years ago, maintenance of soil fertility has been of great concern to farmers and landowners, because sustainable crop production depends on soil fertility. Until the early 1800s, farmers used mainly

livestock excreta or plant residues as fertilizers, or used crop rotation agriculture to recover or maintain soil fertility. In 1876, Liebig elucidated the "mineral element theory" in which he proposed that plants take up minerals for their food, and this theory became widely accepted (Marschner 1995). Liebig's achievements led to a rapid increase in the use of mineral fertilizers, such as Chili saltpeter and superphosphate, in the nineteenth century. Superphosphate was the first industrial fertilizer, and was produced by Lawes and Gilbert in 1843. Around 1910, the Haber-Bosch method for producing ammonia from N_2 and H_2 was established, and chemical N fertilizers could be produced inexhaustibly.

The 1960s saw a dramatic increase in agricultural production—the so-called "the Green Revolution". This success was due mainly to the adaptation of modern crop cultivars that had superior lodging resistance by semi-dwarf trait, and these responded to applied N fertilizers more intensively than did traditional cultivars (Matsushima et al. 2010). As a result of the Green Revolution, the yield of major cereal crops became several times higher than that of previous traditional farming to meet the food demands of an increasing world population.

However, people began to notice that there are both advantages and disadvantages to the use of chemical fertilizers compared with organic fertilizers or compost.

The advantages of chemical fertilizer are as follows (Gowariker et al. 2009):

1. Individual nutrients, such as N, P, or K, can be supplied separately by chemical fertilizers depending on crop requirements and nutrient availability in the soil. Organic fertilizers contain a wide range of nutrients.
2. The effect of chemical fertilizer is generally rapid after its application, because they are usually water-soluble chemical compounds. Organic fertilizers release nutrients by decomposition of organic compounds by soil microorganisms, thus nutrient availability is usually slow.
3. The concentrations of nutrients in chemical fertilizers are very high, ~100 times higher than in organic fertilizers. Thus, small amounts of chemical fertilizers can provide sufficient nutrients. Also, chemical fertilizers are easy to apply, either manually or by using agricultural machines. The cost of labor and transportation are lower than with organic compost.
4. Chemical fertilizers are relatively clean and odorless, and they do not contain toxic compounds such as heavy metals, pesticides, harmful pests, insects or weed seeds, which some organic composts may contain.
5. Chemical fertilizers are chemically stable, and can be stored for a long time if kept in plastic bags.

Disadvantages of chemical fertilizers are as follows:

1. Production of chemical fertilizers, especially N fertilizer, requires a large amount of energy consumption derived from fossil fuels. This causes the increase in CO_2 concentration in the air, resulting in global warming.
2. Some of chemical fertilizers originate from mineral resources, such as phosphate rock and potash ore, and the estimated amounts of affordable reserves are limited, especially for phosphate rock.

Table 1.5 The consumption of nitrogen fertilizer (T gN) in the world and Asian countries

		Consumption	(T gN)	
Major area	1961	1971	2002	2012[a]
World	11.6	33.5	86.5	119.7
Americas	3.58	8.97	17.5	23.6
Europe	5.49	15.43	13.1	14.1
Oceania	0.04	0.14	1.3	1.4
Africa	0.35	0.95	2.6	3.3
Asia	2.12	8.04	52.0	77.3
China	0.54	3.37	29.1	45.1
India	0.25	1.80	10.5	16.8
Japan	0.63	0.68	0.53	0.43

(Modified from Matsushima et al. Global nitrogen cycling and its availability from soil, nitrogen assimilation in plants 2010). 2012[a] (FAOSTAT 2016)

3. Dependence on chemical fertilizers may cause a decrease in the input of organic matter to agricultural fields. This may lead to a decrease in soil organic matter, soil fertility, and thus to a deterioration in the physical, chemical and biological properties of the soil.
4. Excess or inappropriate use of chemical fertilizers cause environmental problems, such as NO_3^- pollution in ground water, eutrophication of rivers, lakes and oceans, and air pollution by nitrous oxides such as N_2O, which also cause global warming.
5. Chemical fertilizers are expensive, especially for small farmers in poor developing countries.

As shown in Table 1.5, the total consumption of chemical N fertilizers increased rapidly from 11.6 T gN in 1961 to 120 T gN in 2012. In Europe and Japan, consumption became stable or decreased after 1971 due to efforts to decrease excess and inefficient use of chemical N fertilizers. However, most other areas increased their use of N fertilizers. The amount of consumption varies widely, and is much lower in Africa and Oceania compared with Asia, the Americas, and Europe. To buy N fertilizer is economically difficult for small farmers in poor areas experiencing population explosions, e.g. as in areas of Africa and Asia. The high price of N fertilizer depends on production costs, due mainly to fossil fuel required to generate the high temperature and high-pressure conditions needed to react N_2 and H_2, as well as producing H_2 gas from fossil oils. The cost also reflects packaging, storage, transportation, and profit margins for the producing and trading companies.

The price of N fertilizers remained relatively stable from 1975 to 2000, but increased dramatically (~3-fold) from about $ 200 per ton in 2000 to about $ 600 per ton in 2013 (Fig. 1.4) (Schnitkey 2014). Poor family farmers cannot afford to buy N fertilizers at such a high price.

To avoid the excess use of N fertilizer, one of the best ways to save on cost and avoid environmental problems without decreasing crop yield is to increase “N use efficiency”. Takahashi et al. reported that the recovery rate of the basal dressing of

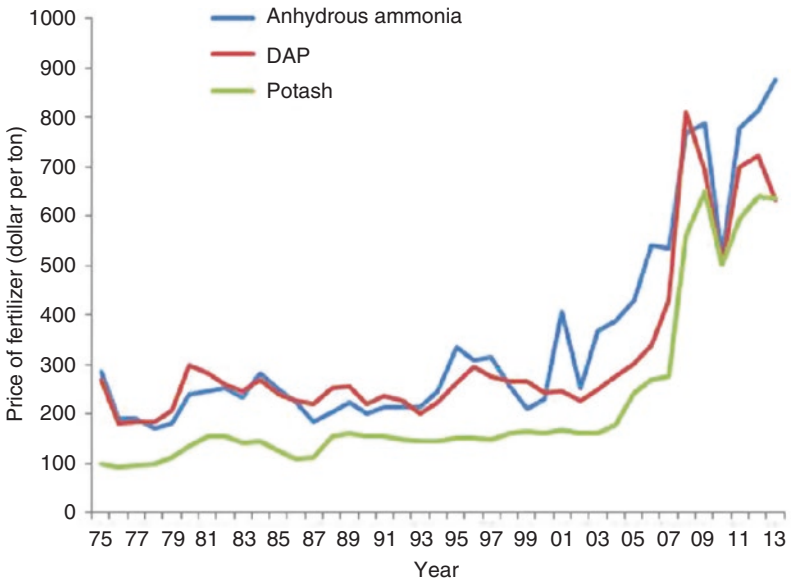

Fig. 1.4 Fertilizer prices, spring of year, 1970–2013 (Figure from Schnitkey (2014), Farmdoc daily Homepage)

chemical N fertilizer was only 10% in soybean cultivation using ^{15}N labeled ammonium sulfate, but that the basal deep placement of coated urea—a controlled release N fertilizer—was 62% (Takahashi et al. 1992). The recovery rate is widely affected by timing, placement, amount of application, and the form of N fertilizers. An optimum fertilizer treatment should be devised for each crop for each specific area and soil conditions. An alternative way of saving on chemical fertilizer is to use BNF. Symbiotic N_2 fixation by legume plants and rhizobia is the most potent BNF system available.

1.3 Legume Nodule Symbiosis

1.3.1 Legume Nodule Symbiosis: The Most Important N Source in Agroecosystems

As shown in Fig. 1.3, the total amount of BNF per a year is estimated at about 122 T gN, and is equal to, or greater than, the annual production of industrial N fixation (100 T gN). Therefore, BNF is still the most important N source in agroecosystems. The most important N_2-fixing agents in agricultural systems are the symbiotic associations between crop and forage/fodder legumes and rhizobia (Herridge et al. 2008). Herridge et al. calculated annual inputs of fixed N to be 18.5 T gN for oilseed

legumes and 2.95 T gN for pulses. They calculated soybean to fix 16.4 T gN annually, representing 77% of the N fixed by crop legumes. The estimates of annual N_2 fixation inputs except legumes are 12–25 T gN by pasture and fodder legumes, 5 T gN by rice, 0.5 T gN for sugarcane, <4 T gN by non-legume crops, and <14 T gN by extensive savannas (Herridge et al. 2008).

The advantages and disadvantages of symbiotic N_2 fixation for agriculture are as follows (Zahran 1999):

Advantages (Peoples et al. 2009; Giller and Cadisch 1995):

1. Unlike expensive chemical fertilizers, symbiotic N_2 fixation is low cost, indeed usually free, for farmers. When efficient indigenous rhizobia already dominate in the soil, farmers do not need to use any agricultural materials to establish nodule formation and N_2 fixation. When legume crops are planted on the virgin land, the inoculation of compatible rhizobia may be necessary to establish the legume-rhizobia symbiosis. The rhizobia inoculant is available commercially in many countries as biofertilizers (FNCA biofertilizer manual 2006) at relatively low cost compared with chemical N fertilizers.
2. Nitrogen use efficiency is very high, at nearly 100% for plant growth. Most of the N fixed by root nodules is assimilated and transported to host plant shoots through xylem vessels to support vegetative and reproductive growth, and the N_2 fixation rate is controlled to meet plant N demands. A small part of the fixed N may be lost by nodule degradation, root exudation or plant senescence as plant residues, but this N increases soil fertility by remaining in the soil.
3. Because of the high N use efficiency of symbiotic N_2 fixation, nitrification of fixed N is very low and NO_3^- leaching and denitrification loss are negligible in agricultural land (Hungria and Mendes 2015). Therefore, symbiotic N_2 fixation is an ecologically friendly measure.

Disadvantages:

1. It is difficult to control the rhizobium strains, especially the occupancy of principal indigenous rhizobia that has already been established (Deaker et al. 2015). When a selected strain is inoculated onto seeds, it will produce some nodules around the basal part of the primary roots, but most nodules formed in the lateral roots are infected by indigenous strains already present in the soil. Furthermore, the newly inoculated strain is easily lost after a few years (Ohyama et al. 2013b).
2. Symbiotic N_2 fixation is a physiologically high cost system that depends on the high consumption of photoassimilates compared with N absorption from roots. Therefore, N_2 fixation alone will result in poor growth and low yield. Symbiotic N_2 fixation will also suffer severe damage under stress conditions, such as drought or water logging, compared with N absorption from roots.
3. Application of a large amount of chemical N fertilizer causes decreased nodule mass and N_2 fixation activity. So, unlike in cereal crops, no dramatic increase in legume yield can generally be expected upon the application of chemical N fertilizers.

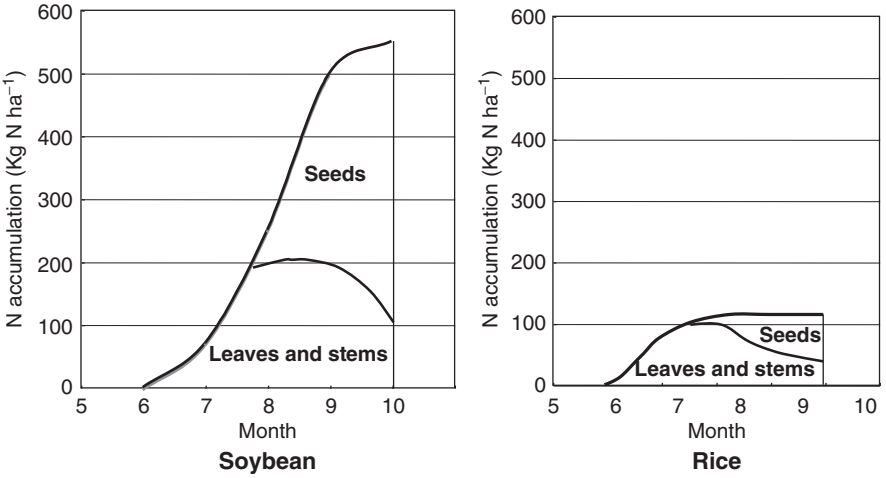

Fig. 1.5 Comparison of N accumulation in soybean and rice plant during vegetative and reproductive stages (Figure from Ohyama et al. (2013b))

1.3.2 N Metabolism and Seed Yield of Legume Crops

Legume crops such as soybean plants assimilate a large amount of N during both vegetative and reproductive stages of growth, and the total amount of N assimilated in a plant is highly correlated with soybean seed yield. One t of soybean seed requires about 70–90 kg N, which is about four times more than in the case of rice (Fig. 1.5) (Ohyama et al. 2013b).

Soybean plants assimilate N from three sources: (1) N derived from symbiotic N_2 fixation by root nodules (Ndfa), (2) N absorbed from soil mineralized N (Ndfs), and (3) N derived from applied fertilizer (Ndff) (Fig. 1.6) (Ohyama et al. 2009). For maximum seed yield of soybean, it is necessary to use both N_2 fixation and absorbed N from roots (Harper 1974, 1987). When only N_2 fixation is available to the plant ,vigorous vegetative growth does not occur, which results in reduced seed yield. On the other hand, a heavy supply of N often severely depresses nodule development and N_2 fixation activity and induces nodule senescence, which also results in reduced seed yield. In addition, a heavy supply of N from fertilizer or from the soil causes luxuriant shoot growth, which results in lodging and poor pod formation. Therefore, in Japan, for soybean cultivation, no, or only a small amount of, N fertilizer is applied as a "starter N" to promote initial growth.

The protein concentration in soybean seeds is about 4 times higher than in cereal seeds such as rice grains (Table 1.4). Soybean plants assimilate about 20% of total N until the initial flowering stage, and 80% of N during the reproductive stage (Fig. 1.5). On the other hand, rice assimilates about 80% of N until flowering. Therefore, the continuous assimilation of N after the initial flowering stage is essential for good growth and high seed yield in soybean cultivation (Ohyama et al. 2013b).

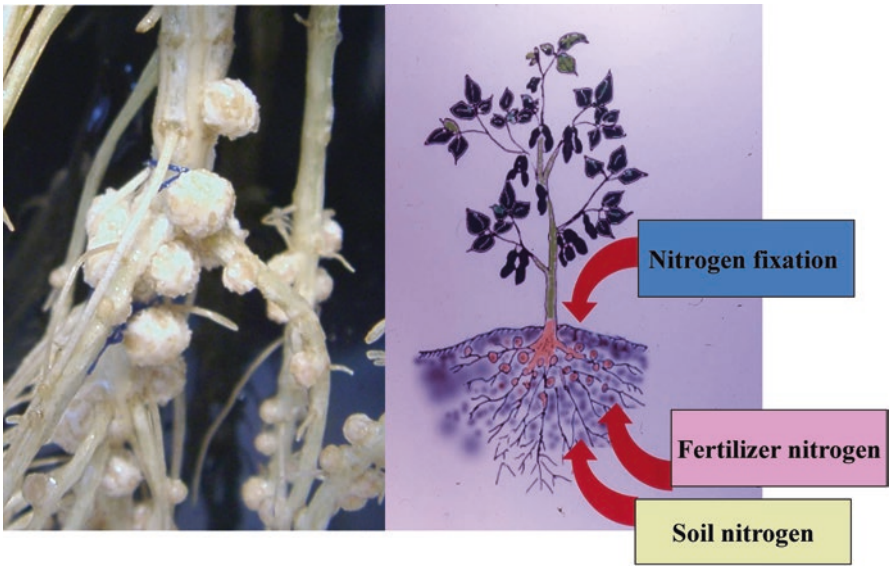

Fig. 1.6 Nodulated soybean roots cultivated with hydroponics, and three sources of N for soybean plants (Figure from Ohyama et al. (2009))

To obtain high seed yield of leguminous crops such as soybean, good nodulation and high (and long-lasting) N_2 fixation activity are essential. Nodule formation and nodule growth are influenced by various soil (water content, pH, mineral nutrition) and climatic (solar radiation, temperature, rainfall, etc.) conditions. In addition, legumes can absorb inorganic N, such as nitrate (NO_3^-) and ammonium (NH_4^+) from soil or fertilizers. Usually, high yield of soybean is obtained in fields with high soil fertility. By supplying a constant but low concentration of N either from soil or organic manure, good soybean growth will occur without depressing nodulation and N_2 fixation activity. However, it is well known that a high concentration of mineral N depresses nodule formation and N_2 fixation activity. Especially, NO_3^-, the most abundant inorganic N in upland soils, rapidly and reversibly inhibits nodulation and N_2 fixation of soybean, when nodulated roots are in direct contact with soil solution containing NO_3^- (Fujikake et al. 2003; Ohyama et al. 2011).

1.3.3 Symbiotic N_2 Fixation Related to Other Minerals

Seventeen elements are essential for the growth and reproduction of plants (Kovacevic et al. 2011). Whereas carbon (C) and oxygen (O) are derived from air, and hydrogen (H) from water, other elements are derived mainly from soils. The primary macronutrients are N, P and potassium (K), and secondary macronutrients are sulfur (S), calcium (Ca) and magnesium (Mg). Micronutrients are iron (Fe),

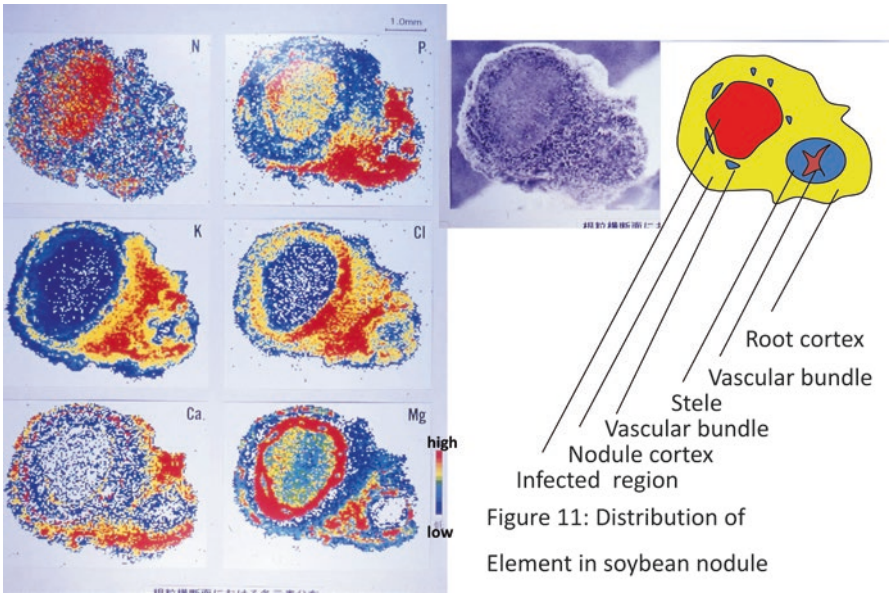

Fig. 1.7 Distribution of various minerals in nodulated roots of soybean (Figure from Ohyama et al. (2009))

manganese (Mn), boron (B), zinc (Zn), copper (Cu), molybdenum (Mo), nickel (Ni) and chlorine (Cl). Nutrient removal of one ton of soybean grain and corresponding biomass are ~100 kg N, 23–27 kg P_2O_5, 50–60 kg K_2O, 13–15 kg CaO, and 13–16 kg MgO (Kovacevic et al. 2011).

Soybean nodules are highly organized complex organs, as shown by the distribution of minerals examined by EPMA (Electron Probe X-ray Microanalysis) (Mizukoshi et al. 1995). Figure 1.7 shows the distribution of minerals in nodulated roots. The concentrations of N and P are higher in the central symbiotic region compared with the surrounding nodule cortex. Conversely, K, Cl and Ca are low in the central region of the symbiotic zone compared with the nodule cortex. Mg accumulates specifically in the inner and outer cortex inside sclerenchyma cells but not outside.

A sufficient supply of P is known to be important for symbiotic N_2 fixation in legume nodules. Concerning P application, Jabbar and Saud (2012) reported that the number and dry weight of soybean nodules are increased significantly by higher P application, up to 120 K gP/ha, under salt stress conditions. Tsvetkova and Georgiev (2003) reported that P deficiency treatment (0.1 mM phosphate) and an excess supply of phosphate (3 mM) in hydroponic soybean culture decreased the whole plant fresh and dry mass, and nodule weight, number and N_2 fixation activity compared with the optimum concentration of 1 mM.

1.3.4 Factors Affecting Soybean Yield

Soybean plants are very susceptible to environmental factors, such as climate and soil conditions. N_2 fixation by root nodules is very important for soybean growth and seed yield; however, it is difficult to obtain optimum conditions for nodulation and N_2 fixation, because nodulation and N_2 fixation are more susceptible to biotic and abiotic environmental factors than root growth and N absorption. Nodule formation and N_2 fixation are sensitive to external factors such as climate, soil properties, pests, etc., and internal factors such as competition among neighbor plants or competition among organs, pods, leaves, roots and nodules within a plant. Therefore, many stress conditions, such as drought stress, decrease in oxygen supply due to water logging, high or low pH, nutrient imbalance etc., may depress nodule formation and N_2 fixation activity. In addition, low population density of compatible bradyrhizobia, or the dominance of inefficient strains of indigenous bradyrhizobia in the field may decrease N_2 fixation activity. Inoculation of efficient strains of bradyrhizobia may promote soybean growth and seed yield.

Global warming may influence legume crop cultivation, including symbiosis with rhizobia (Sulieman and Tran 2015). From the Fifth Assessment Report of the United Nations Intergovernmental Panel on Climate Change (IPCC), warming of the atmosphere and ocean system is unequivocal, and there is a clear human influence on the climate (IPCC Fifth Assessment Report 2014). It was predicted that the global surface temperature increase by the end of twenty-first century is likely to exceed 2–4 °C relative to the late twentieth century. The key risks of global warming are identified with high confidence, span sectors and regions; such as (1) risk of death, injury, ill-health, or disrupted livelihoods in costal zones and small islands due to storm surges, coastal flooding, and sea level rise; (2) risk of severe ill-health for urban population due to inland flooding; (3) systemic risks due to extreme weather events leading to breakdown of infrastructure and critical services; (4) risk of mortality and morbidity during periods of extreme heat; (5) risk of food insecurity and the breakdown of food systems linked to warming, drought, flooding, and precipitation variability and extremes, particularly for poorer populations in urban and rural settings; (6) risk of loss of rural livelihoods and income due to insufficient access to drinking and irrigation water and reduced agricultural productivity, particularly for farmers and pastoralists with minimal capital in semi-rid regions; (7) risk of marine and coastal ecosystems and biodiversity; and (8) risk of loss of terrestrial and inland water ecosystems and biodiversity (IPCC Fifth Assessment Report 2014).

Concerning food security and food production systems, the IPCC report predicted that all aspects of food security are potentially affected by climate change, including food access, utilization, and price stability. For the major crops (wheat, rice and maize) in tropical and temperate regions, climate change without adaptation is projected to negatively impact production for local temperature increases of 2 °C or more above late twentieth century levels, although individual locations may benefit.

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Chapter 2
P Deficiency: A Major Limiting Factor for Rhizobial Symbiosis

Alvaro Sanz-Saez, Fermín Morales, Cesar Arrese-Igor, and Iker Aranjuelo

Abstract Together with nitrogen (N), phosphorus (P) has been described as the main plant macronutrient limiting growth. Although P is abundant in many soils, its availability for plants is low. For this reason, P is provided to plants largely through the application of P-enriched fertilizers. However, since rock phosphate reserves (the main source of P) are predicted to be depleted in the near future, it is crucial to understand the processes linked with a better P use efficiency. P is a target structural constituent of energetic compounds (ATP, ADP), nucleic acids, phosphate sugars, etc., that are essential for cell metabolism and plant development. Current knowledge highlights that low P availability negatively affects above- and below-ground organ growth, as a consequence, in part, of poor photosynthetic performance. While essential for all plants, the P requirement of N_2-fixing plants has been described as larger than that of non N_2-fixing plants, mainly as a consequence of the large P demand for biological N_2 fixation (BNF) processes. Moreover, three main factors have been suggested to affect BNF under low P conditions: carbon supply, N-feedback and O_2 diffusion have been identified as the main factors conditioning N_2 fixation under low P availability conditions. In this chapter, we summarize current knowledge regarding P content in plant performance, with special emphasis on N_2-fixing plants and the symbiotic relationship.

Keywords Biological N_2 fixation • Growth • Legumes • Nodule • P deficiency • Rhizobium • Symbiosis

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2.1 Introduction

The world's population is expected to increase from 7.5 billion in 2016 to 9.3 billion in 2050, and to 11.2 billion by 2100 (UN 2015). Along with the increase in population, dietary changes towards a preference for meat products due to the emergence of a middle class in developing regions such as Asia and South America will increase the demand for seed crops. To meet this demand, present crop production should be doubled by the end of the century. In the past, during the 1960s, similar challenges were faced, and the population doubled at the same pace as crop production due to unforeseen advances in plant germplasm, the unprecedented use of fertilizers such us the NPK, and the use of new irrigation techniques (Dyson 1999). The use of NPK fertilizers is paramount for crop production. Although nitrogen (N) is among the most abundant elements, it is critical for the growth of most plants due to its unavailability for absorption by plants (Vance et al. 2000). Plants assimilate N from two main sources: through absorption of the N available in soil, and through the N_2 fixation that occurs in the nodules of leguminous plants thanks to symbiosis with bacteria from the *Rhizobiaceae* family. The discovery of industrial N_2 fixation during the 1930s makes the use of N almost ubiquitous in agriculture, although this technique is energetically and environmentally costly (Vance 2001). Potassium (K) is a limited macronutrient on our planet, but, with current reserves, the K supply is thought to be enough to last for centuries (USGS 2014). Phosphorus (P) is the second most limiting element for plant growth after N (Vance et al. 2000), and, unlike N, global supplies of P are not infinite. The amount of phosphate (Pi) reserves readily available for its use in agriculture has been a matter of discussion in the last 10 years (Vaccari 2009; Cooper et al. 2011; Edixhoven et al. 2014; Scholz and Wellmer 2016), with some authors claiming that Pi reserves will last until the end of the century, and others that these reserves will last for at least 350 years. Therefore, the study of reserves available for future use in agriculture and other commodities, and understand the keys to P availability in soil and the effects of its deficiency in crop plants is paramount when facing the challenge of feeding an increasing population in an ever-changing world.

2.1.1 P Availability for Plants

P is an abundant element in natural and agricultural soils, and is found in three main "pools". (1) The soluble P pool is a very small fraction of the total P available in the soil. This fraction is very important because it is the only fraction with mobility in the soil, and is the pool from which plants absorb P in the form of orthophosphate. (2) The active P pool, which is found in the solid phase, contains inorganic Pi that is absorbed by soil particles, Pi that reacts with cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Al^{3+}, and organic P, which is mineralized by the action of soil microorganisms (Vance 2001). The active pool is the fraction that replenishes the soluble pool when plants take up the available Pi from it. The P fertility of a soil will depend on how

easily Pi is released into the soluble form. At the same time, Pi derived from a fertilizer can be immobilized through adsorption reactions with several metals and pass to the third-fixed pool. This fraction is very insoluble, and Pi is sequestered from the active and soluble pools, making Pi less available for plant acquisition.

Therefore, although P is an abundant mineral in soils, its availability is limited for plant absorption depending on how it moves through the different pools in the soil. A factor that affects Pi availability and mobility in soils is the pH. Reactions that reduce Pi availability can occur at any pH, but they are more accentuated in acidic ($pH \leq 5.5$) or in alkaline ($pH \geq 7.3$) soils (Schachtman et al. 1998). Approximately 30% of total land area is acidic soils; of these lands, it is estimated that 50% of the total potential arable land is acidic (soils with a $pH < 5.5$) (von Uexküll and Mutert 1995). In these conditions, the Pi added with fertilizers reacts with oxidized Al and Fe, forming very stable and insoluble aggregates that pass directly to the fixed pool, making this mineral unavailable for plant absorption (Zheng 2010). At the same time, acidic soils allow the dilution of heavy metals such as Al, which, at high concentrations, are toxic for plants (Horst 1995; Horst et al. 2010). On the other hand, in alkaline soils, Pi reacts mainly with calcium cations (Ca^{2+}) in different reactions, forming less soluble products such as dibasic calcium phosphate dihydrate, octocalcium phosphate, and hydroxyapatite (Busman et al. 2002). The precipitation of Pi from fertilizer in alkaline and acidic soils is one of the main causes of the low efficiency of Pi fertilizers. Due to problems of Pi availability in agricultural soils, crop yield is limited on around 40% of world's arable land (Vance 2001). To produce 7 tons ha^{-1} of a field crop in intensive agriculture, it is necessary to add between 90 and 120 kg P ha^{-1} (Bumb and Baanante 1996). However, only 20% of the applied Pi is removed by the crop in the 1st year (Mousavishalmani et al. 2002). This inefficient plant Pi uptake in comparison with the high amounts of Pi added in the fertilizer might end up in contamination of subterranean aquifers, rivers, lakes and seas; contributing to water eutrophication, and the uncontrolled growth of cyanobacteria and other microorganisms that consume all the oxygen dissolved in the water, causing the death of a large part of the ecosystem, as happened in the Mississippi delta in 2008 (Vaccari 2009).

Research on how to improve the P use efficiency of crops, and how to increase availability of the fixed Pi pool should be undertaken. Considering the objective of how to improve crop P use efficiency, several authors have highlighted two main strategies: (1) increase Pi acquisition through the roots, and (2) improve P conservation (Marschner and Dell 1994; Peoples et al. 1995; Harrison 1997; Gilroy and Jones 2000; Vance et al. 2000; Vance 2001). For better Pi acquisition, several strategies that result in expanded root surface have been applied, such as increased root growth in low P situations and increased root hair development (Gilroy and Jones 2000). Another strategy is to choose species and genotypes with organic acid synthesis and exudation that allow better Pi solubility in acidic soils. In this respect, white lupin (*Lupinus albus*) roots release organic acids such as malate and citrate that are able to solubilize unavailable inorganic Pi (Vance 2001); at the same time, this species also releases acid phosphatase into the rhizosphere, which helps solubilize organically immobilized P (Vance 2001). Although this species does not have a high relevance

in the crop market, its use in intercropping has been shown in several countries, such as in India (Ae et al. 1990). Taking a more molecular approach, the enhancement of Pi transporter and aquaporins in the roots has had proven efficacy (Ragothama 1999). Likewise, research into genes implicated in the symbiosis between plant and mycorrhizal and rhizobium organisms, and the overexpression of such genes, can increase P- and N-use efficiency (Marschner and Dell 1994; Peoples et al. 1995; Harrison 1997; Vance et al. 2000; Vance 2001). Mycorrhizal symbiosis increases root area by at least two-fold thanks to the fungal hyphae; at the same time, fungal secretions solubilize easily the unavailable Pi in the soil, increasing P use efficiency for the plant (Marschner and Dell 1994; Harrison 1997).

2.1.2 Phosphate Rock as a Limited Source of P Fertilizers

As stated above, the application of P fertilizers is important to maintain and increase crop production. More than 60% of the P applied in fertilizers comes from phosphate rock (PR), with the remaining 40% coming from recycled P derived from organic residues such us manure, crop scraps and human excreta (Cordell 2010; Liu et al. 2008; Smit et al. 2009). PR is a finite, non-renewable, resource; of the total PR mined, 82% is used for fertilizer production, 5% for livestock feed, and a small percentage in food additives and detergents (Schröder et al. 2010). PR is usually found in sedimentary rock deposits of marine origin, such as former continental shelves—an example of this would be part of the Sahara Desert (Edixhoven et al. 2014). The entire PR is mined and extracted in combination with other materials, which reduces its purity. Around 95% of P in PR is present as calcium-phosphate. According to the United States Geological Survey (USGS), deposits of PR in combination with impurities are commonly known as "PR reserves". However, to receive this consideration, the PR found in those deposits has to be extractable economically with current methods of extraction at the time of classification (USGS 2011). Depending on the source of the PR reserves, the purity of Pi that can be extracted can vary between 5% and 50% of the total mined material. If the PR found in a deposit is not economically extractable at the time of classification due to high concentration of impurities and/or difficulties in the mining process, the USGS classify this deposit as "PR reserve base". Each year, the USGS produces a mineral commodity report that includes annual production, estimation of PR reserves, and estimation of PR reserve base of the top 15 countries with more PR reserves or production. Reserve estimates are not stable, and can change for various reasons (Cooper et al. 2011). For example, they can increase with the discovery of a new deposit; they can be upgraded from reserve base to reserve if new techniques for extraction improve, or if the PR market price increases, encouraging mining companies to open new mines in veins that otherwise would remain unexploded. Conversely, PR reserves can be downgraded to PR reserve base if the market price drops. Although the nature of the estimation of PR reserve is volatile, in the last

10 years the predictions made by the USGS have varied considerably (Cooper et al. 2011; Edixhoven et al. 2014: Scholz and Wellmer 2016). In 2009, the USGS estimated that the PR reserves were around 16,000 million tons (Mt), meanwhile in 2011, the USGS accounted for PR reserves of 65,000 Mt, a 4-fold increase in the prediction (Jasinsky 2011). These differences in the estimation of the PR reserves between different years make it very difficult to predict how long the PR reserves are going to last. The differences in the estimations are due mainly to recalculation of the base reserve estimates of reserves for Morocco, which, according to the USGS, increased from 5500 Mt in 2009, to 50,000 Mt in 2011 (USGS 2011; Edixhoven et al. 2014; Scholz and Wellmer 2016). Some studies based on the USGS estimates before 2009, predicted that PR reserves could be depleted within 60–100 years (De Haes et al. 2009; Smit et al. 2009; Vaccari 2009; Cordell 2010). However, other recent studies based on more optimistic estimations of PR reserves such as USGS (2011), also called PR peak theory, estimate that PR reserves will be available for at least the next 200–300 years (Edixhoven et al. 2014; Scholz and Wellmer 2016). Independently of the estimations of PR used to predict PR reserves, due to the specific conditions needed for PR formation, the occurrence of PR is limited geographically to very specific regions, and therefore a very few countries hold the majority of PR reserves and production. In total, 17 countries account for almost 95% of the total reserves and production (Cooper et al. 2011). Of these, Morocco accounts for 77% of global reserves with 50,000 Mt, followed by China, Algeria, Syria, Jordan, and the United States (Jasinski 2011), with the United States and China being the biggest producers and consumers of PR (Cooper et al. 2011). As the PR demand is linked strongly to crop, food and biofuel production, the Food and Agriculture Organization of the United Nations (Alexandratos and Bruinsma 2012) estimates that PR demand will grow at a pace of 2% until 2050. At that time, several authors predict that PR demand will stabilize, and even drop slightly due to the scarcity of PR reserves in the main PR producers, such as the United States, with a 15% of world's total production, and China, with 37% (Cordell 2010; Cooper et al. 2011). According to Cooper et al. (2011), if PR production continues increasing at its current pace of 2%, China and United States would deplete their most accessible PR reserves within 55 years. If that happens, Morocco—the country with the biggest PR reserves in the world (77%) but with a moderate share of PR production (15%)—would have to improve its extraction and production industries to fill the gap left by the United States and China (Cooper et al. 2011). In this scenario, it is predicted that the demand for PR would decrease, and that there would be increased use of organic P sources such as manure, due to the higher price of the raw material as well as political uncertainties caused by one country controlling almost 90% of all PR sources (Cooper et al. 2011). Therefore, finding new technologies to make the P immobilized in the soil available to crops, and/or to find new methods to recycle the P used in agriculture or found in organic matter, will be essential to face the future scarcity of P, and the threat of resource monopoly from P producer and reserve holder countries.

2.2 General Importance of P to Plants

Legumes include important agricultural crops, as their high protein content is of primary importance for human food and animal feed. Forage legumes provide a good source of protein, with multiple positive effects on both human and animal nutrition. In addition, the ability of most legumes to establish symbiotic relationships with soil bacteria allows them to obtain their N requirements from N_2 fixation in nodules, and therefore avoids the heavy usage of chemical N fertilizers (Peoples et al. 1995; Irigoyen et al. 2014). The symbiotic relationship between rhizobial bacteria and legumes provides access to atmospheric N_2. Biological N_2 fixation (BNF) provides to the legumes and the surrounding plants an additional N source that is of great value in impoverished soils. As observed by Peoples et al. (1995), this symbiotic relationship is the main source of N_2 fixation in terrestrial ecosystems (provides 50% of BNF) and reduces the need to fertilize soils with chemical compounds, which leads to additional economic and environmental benefits. P is one of the essential macronutrients, being a structural component of many metabolites and required for numerous plant biochemical processes. It functions prominently in DNA (genetic information carrier) and RNA (responsible for genetic information translation), and is abundant in bio-membrane phospholipids, playing an important role in the interaction with surrounding ions (Marschner 1995). Most phosphate esters are intermediates in metabolic pathways of biosynthesis and degradation. Moreover, molecules with energy-rich phosphates are required for starch (ATP), sucrose (UTP) and cellulose (GTP) biosynthesis (Marschner 1995).

2.2.1 P Availability and Plant Growth

Despite relatively high soil total P content, it is limiting and often not easily available to plants due to low P solubility, P fixation to soil particles, and slow replenishment of the extracted P from the soil by the labile pool (Holford 1997; Ribot et al. 2008). Optimal plant P requirement is in the range 0.3–0.5% dry weight (Marschner 1995). P limitation manifests first by heavily affecting growth, and later affects photosynthesis (Halsted and Lynch 1996), typically reducing growth rates (Bottrill et al. 1970; Plesnicar et al. 1994). Moreover, P deficiency has also been described to induce a reduction in the number of leaves (Lynch et al. 1991), premature senescence of the leaves formed (Marschner 1995), and a reduction in leaf surface area or expansion (Terry and Ulrich 1973; Fredeen et al. 1989). The latter is related to the extension of epidermal cells, due to their low P content (Treeby et al. 1987), and a decrease in root hydraulic conductivity (Radin 1990). In contrast to shoot growth, root growth is much less impaired under P deficiency (Marschner 1995; Vysotskaya et al. 2016) and can even be enhanced (Anuradha and Narayanan 1991), coincident with higher root P retention and net P translocation from the shoot (Smith et al. 1990) and correlated with an increase in partitioning of carbohydrates (particularly

sucrose) towards the roots (Khamis et al. 1990). Formation of "proteoid roots" is another type of response of certain plant species to P deficiency (Marschner 1995). Also, the ability of other species to associate with mycorrhizal fungi allows them to take up P from the nutrient-poor soils that generally induce P deficiency.

As mentioned above, together with N, P is major limiting mineral governing plant development (Vance et al. 2000). Plant growth inhibition caused by low P availability might be more marked in N_2-fixing legumes, because symbiotic plants require more P than non-symbiotic plants. This differing dependence is due to the fact that symbiotic N_2 fixation is a high-P-demand process. Nodules have been described to concentrate up to 20% of total plant P (Gunawardena et al. 1992). P concentration in nodules is three-fold higher and is less affected by P deficiency than in other organs (Vadez et al. 1996). Although P is necessary for both growth and N_2 fixation, plants involved in BNF are particularly sensitive to P scarcity because P deficiency has greater impact on the N_2 fixation process than on shoot growth. Thus, P deficiency prevents nodulation or stops nodule growth when plants have formed nodules in, among others (see Divito and Sadras 2014 for a meta-analysis), white clover, lupins, bean, soybean and *Virgilia divaricata* (Almeida et al. 2000; Kouas et al. 2005; Miao et al. 2007; Kleinert et al. 2014; Vardien et al. 2014). As a consequence, nitrogenase (N_{ase}) activity decreases (Schulze et al. 2011; Divito and Sadras 2014; Kleinert et al. 2014), and the proportion of whole plant N derived from the symbiotic process decreases (Almeida et al. 2000). However, there is genetic variability within legume species to be exploited, and some mungbean (cultivar T-77; Gunawardena et al. 1992) and cowpea (Ankomah et al. 1996) cultivars are capable of good growth and high N_2 fixation, satisfying their total N requirements, in low P soils.

2.2.2 *Plant Photosynthesis Is Affected by P Deficiency*

P deficiency may, or may not, impact photosynthesis (Bottrill et al. 1970; Brooks 1986; Lauer et al. 1989; Rao and Terry 1989b, 1990). In *Eucalyptus globulus*, for instance, reductions in photosynthesis can be as large as 55% (Turnbull et al. 2007). P may affect photosynthesis via diffusional and biochemical processes. Some studies have argued that P affects photosynthesis through anatomical effects on stomata (Yang et al. 2016) or stomatal function (Fig. 2.1). In fact, P deficiency decreases stomatal conductance (Brooks 1986; Rao and Terry 1989a; Xu et al. 2007; Yang et al. 2016)—a process in which ATP is involved (Agbariah and Roth-Bejerano 1990). When P deficiency is sensed, the transcription levels of photosynthetic proteins decrease, resulting in reduced photosynthetic rates due to biochemical limitations (Rao and Terry 1989b, 1990; Qiu and Israel 1992; Warren 2011). In the chloroplast stroma, severe inhibition of starch biosynthesis occurs below ca. 5 mM Pi (Marschner 1995). Therefore, a low P supply limits photosynthesis through both stomatal and non-stomatal biochemical processes (Fig. 2.1).

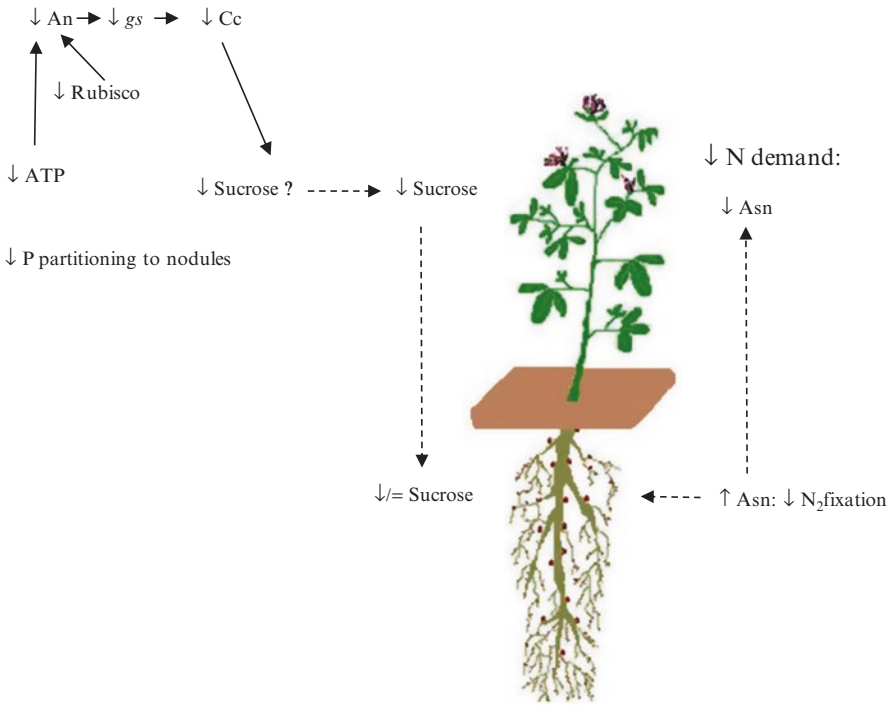

Fig. 2.1 Model representing the most visible changes in carbon and nitrogen primary metabolism of leaves and nodules of N_2 fixing plants exposed to P deficiency. This figure is a tentative summary representing the main findings described in the literature. *Arrows up* (↑) and *down* (↓) represent compounds/reactions whose content/activity is up- or down-regulated. *An* net photosynthesis, *Asn* asparagine, *Cc* chloroplast CO_2 concentration, *gs* stomatal conductance (Based on Aranjuelo et al. (2014))

The simplest explanation for a positive P relationship with photosynthesis is that Pi is one of the primary substrates of photosynthesis (Walker and Sivak 1986). Hence, there are dramatic and rapid effects on the photosynthetic machinery of sudden changes in internal Pi concentrations (Rao and Terry 1995). For instance, P_i limitation has a greater effect on C reduction than on light reactions (Lauer et al. 1989), lowering ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content, specific activity and efficiency (Brooks 1986; Lauer et al. 1989). It is known that the presence of Pi accelerates Rubisco activation (Sawada et al. 1992). Both accumulation of leaf starch (and/or sucrose) owing to reduced sink demand (Pieters et al. 2001), or insufficient Pi for operation of triose phosphate transport (Herold 1980), can impair photosynthesis. Furthermore, photosynthetic metabolism is particularly sensitive to Pi insofar as ribulose-1,5-bisphosphate (RuBP) pool size (Brooks 1986; Fredeen et al. 1989) and regeneration efficiency (Brooks 1986; Plesnicar et al. 1994) can be reduced in P-limited plants. Evidence comes from experiments carried out with spinach (Brooks 1986), soybean (Fredeen et al. 1989) and sunflower (Plesnicar et al. 1994). Nevertheless, the amount of P, and the fraction

involved in the primary processes of photosynthesis are variable and often small (Bieleski 1973), which makes it less clear why correlations of P with photosynthesis are so strong. In line with this, levels of Pi that reduces photosynthesis in sucrose-accumulating species might barely influence photosynthesis in starch-accumulating species (Walker and Sivak 1985). This is because starch biosynthesis liberates Pi from reduced C, thereby making Pi available for different metabolic reactions.

In many cases, positive relationships between P supply and photosynthesis are best explained by leaf P content, rather than by active (cytoplasmic located) P pool (Turnbull et al. 2007). In a plant with an adequate P supply, 85–95% of total Pi is located in vacuoles, from which release is usually slow (Marschner 1995). Under optimal P nutrition, Pi concentration in the cytosol is ca. 6 mM (Marschner 1995). Better relationships with leaf P content can be explained, at least in some cases, because P affects photosynthesis indirectly through effects on N allocation to the photosynthetic machinery. In *Pinus pinaster*, for example, there is a strong positive correlation between P supply, P storage as orthophosphate, and N allocated to Rubisco (Warren and Adams 2002). This may come about because, at longer time scales, P deficiency causes accumulation of carbohydrates that downregulate expression of genes coding for the photosynthetic machinery (Krapp and Stitt 1995).

2.3 Nodule Performance Under Low P Conditions

It has been postulated that legumes, since they are capable of fixing atmospheric N_2, will have an advantage in plant growth over non-N_2-fixing plants (Rogers et al. 2006). Within the symbiotic relationship established between plants and rhizobia, the plant provides photoassimilates to nodules while the bacteria return ammonium to the plant for the further synthesis of amino acids (Fig. 2.2). High metabolic activity of nodules demands a large supply of energy and C skeletons (in the form of sucrose) to the nodules (Aranjuelo et al. 2014). In order to fuel N_{ase} activity, photosynthetically fixed C is supplied to symbiosomes mainly in the form of dicarboxylic acids (such as malate), to produce energy and reducing power in the tricarboxylic acid (TCA) cycle. Organic C supply to nodules is also employed as C skeletons to synthesize N-transport compounds. In exchange for C supply, fixed N is transferred from the bacteroids to the plant cytosol either as ammonia and/or alanine and aspartic acid, where they are assimilated into ureides or amides. Typically, in tropical legumes (such as soybean and cowpea), ureides are the major form of N transported. In contrast, in temperate legumes (such as *Medicago* and *Pisum*), amides are reported as the major organic N compounds transported. The exchange of C and N between plant and nodules is well known, and it is clear that amino acids play a pivotal role in the interaction between both metabolisms (Lea and Forde 1994). However, the role of amino acids in nodule metabolism has led to controversy (Lodwig and Poole 2003), and the lack of knowledge about the metabolic fluxes of amino acids between plants and nodules is remarkable. Due to the dependency of C and N metabolism between bacteroids and plant, it is necessary to identify the

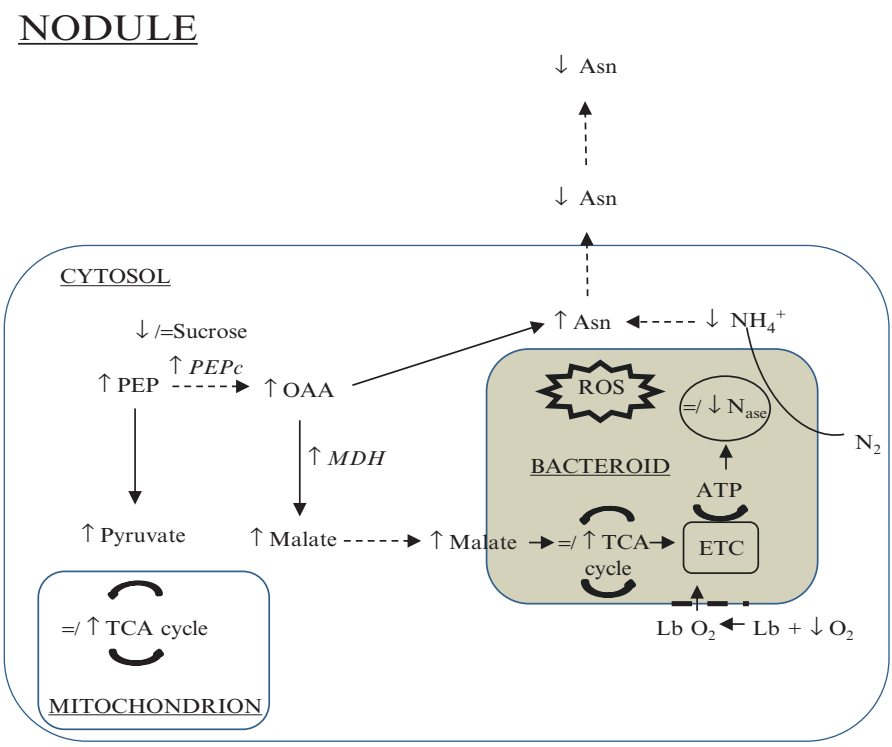

Fig. 2.2 Model representing the most visible changes in carbon and nitrogen primary metabolism of N_2 fixing nodules exposed to P deficiency. This figure is a tentative summary representing the main findings described in the literature. *Arrows up* (↑) and *down* (↓) represent compounds/reactions whose content/activity is up- or down-regulated. *Asn* asparagine, *ETC* electron transport chain, *Lb* leghemoglobin, *MDH* malate dehydrogenase, *Nase* nitrogenase, *OAA* oxaloacetate, *PEP* phosphoenolpyruvate, *PEPc* phosphoenolpyruvate carboxylase, *ROS* reactive oxygen species, *TCA* tricarboxylic acid pathway (Based on Sulieman et al. (2013) and Aranjuelo et al. (2014))

amino acid exchange in order to understand the importance of amino acids in this symbiotic relationship (Lodwig and Poole 2003).

The large P demand in nodules is linked with the large nodule energy requirements (Schulze et al. 1999). As described by Plaxton (2004), nodule and energy generating metabolism depends strongly upon the availability of P. P starvation has proved to negatively affect nodule P and adenylate levels (Le Roux et al. 2006; Vardien et al. 2016). According to published reports, the reductions in Pi adenylate pools, which accompany P, have important implications for nodule functioning. Reduced levels of ADP and P, and increased ADP/ATP ratios, have been reported to result in restricted electron flow in the cytochrome pathway (Vardien et al. 2016). In order to minimize the effects of P deficiency on BNF during low P supply, the nodules require very flexible mechanisms to maintain functional P homeostasis (Vardien et al. 2016). As observed by Vardien et al. (2016), in low P conditions, nodules

develop very flexible P recycling and internal P conservation mechanisms, rather than enhanced mechanisms aimed at acquiring external P. Within this context, according to Nasr Esfahani et al. (2016), when exposed to low Pi conditions, chickpea plants tend to reallocate Pi from both leaves and roots to their nodules (Fig. 2.1). A sharp reduction (78%), observed particularly in the Pi level in roots, would help prevent the complete depletion of nodular P level (Nasr Esfahani et al. 2016). Moreover, under P deficiency conditions, plants have been characterized to be able to remobilize P from internal resources, such as nucleic acids and phospholipids (Hernández et al. 2009). In this regard, the induction of genes involved in membrane-phospholipid degradation has been reported in different plant species (Hernández et al. 2009).

Previous studies conducted in N_2-fixing plants exposed to P deficiency and other stressful growth conditions (Almeida et al. 2000; Aranjuelo et al. 2014; Sulieman et al. 2013, 2014; Nasr Esfahani et al. 2016) remark that the main processes limiting nodule functioning (summarized in Fig. 2.2) are: (1) carbohydrate availability, (2) accumulation of nitrogenous compounds, (3) oxygen (O_2) permeability, and (4) accumulation of reactive oxygen species (ROS):

2.3.1 Carbohydrate Availability

Jakobsen (1985) observed that the impaired nodule performance under P deficient conditions is associated with the above-mentioned inhibition of photosynthetic apparatus, and the consequent depletion in nodule carbohydrate availability. As previously described, nodule N_2 fixation requires C provided by the host, mostly in the form of malate, for bacteroid respiration. The fact that low P availability negatively affects photosynthetic apparatus implies that less C is delivered to nodules, with consequent impact on nodule performance (Jakobsen 1985). The high level of metabolic activity in nodules requires a large amount of C supplied by the host plant to the nodule. It has been estimated that, during the photoperiod, up to 45% of photoassimilates may be exported towards the nodules (Gordon et al. 1987). So, within this context, a decline in nodule performance under low P conditions could be associated with the inhibition of the photosynthetic machinery. However, according to Almeida et al. (2000), while leaf photosynthesis can be negatively affected by P, poor growth or poor performance of the nodules cannot be associated with limitations in C availability. According to the same study, starch accumulated in the leaves, and the concentration of water soluble carbohydrates in the nodules of *Medicago truncatula* was highest under P deficiency. In a recent study with N_2-fixing *M. truncatula* plants exposed to sub-optimal P conditions, the authors observed that nodule sucrose content was relatively stable, and not markedly affected by low P supply (Sulieman et al. 2014). Such results suggest that, under P-limiting conditions, there appeared to be no shortage of carbohydrate provision from the host plant to the nodules. Moreover, in agreement with this conclusion, it

was observed that previous studies (Le Roux et al. 2008; Sulieman et al. 2013), also detected large increases in organic acids, such as malate, in nodules subjected to P deprivation. Malate is believed to be one of the major substrates entering the N_2-fixing bacteroids (Schulze et al. 2002). This dicarboxylic acid is the major energy source for bacteroids and plant mitochondria in different species, and is used for $NH4^+$ assimilation as the C skeleton in the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. It has been shown (Aranjuelo et al. 2014) that an accumulation of organic acids, in nodules under environmental constraints, reflects an impairment of BNF prior to a decline in the glycolytic pathway or sucrose supply from the shoots. On the other hand, a decrease in organic acids is due either to an impaired sucrose synthase activity or decreased glycolytic flux in nodules leading to a diminished symbiosome performance (González et al. 2001; Gálvez et al. 2005). Within this context, increases in nodule organic acid content detected in N_2-fixing plants grown with low P have been associated with the stimulation of nodule respiration derived from the large energy requirements (Schulze and Drevon 2005; Le Roux et al. 2008). Moreover, the large nodule carbohydrate demand is sustained by the increase in nodule dark CO_2 fixation (Le Roux et al. 2008; Cabeza et al. 2014). In nodules subjected to low P, an increase in alternative glycolytic pathway (phosphoenolpyruvate, PEP, metabolism via phosphoenolpyruvate carboxylase, PEPc) has been associated with an increase in malate levels (Le Roux et al. 2006, 2008; Cabeza et al. 2014). In a previous study where transcript and metabolite analyses were characterized in nodules of N_2-fixing bean plants exposed to P stress, it was revealed that the glycolysis/C-fixation pathway is induced in P-stressed nodules (Hernández et al. 2009).

For C metabolism, a major point of divergence in glycolysis is at the PEP branchpoint. Three enzymes at this branchpoint implicated in primary PEP metabolism are pyruvate kinase (PK), PEPc and phospho*enol*pyruvate phosphatase (PEP phosphatase) (Le Roux et al. 2008). Previous transcriptomic studies conducted in P-deficient plants showed that the over-expression of PEPc and malate dehydrogenase (MDH) in P-deficient plants was linked to an increase in nodule malate (and other organic acid) content (Hernández et al. 2009; Cabeza et al. 2014). Apart from the generation of pyruvate via conventional glycolysis, pyruvate can alternatively be synthesized from malate via the combined action of cytosolic PEPc, MDH and mitochondrial malic enzyme (ME) (Le Roux et al. 2008). From this perspective, apart from supplying anaplerotic C (to replenish TCA-cycle intermediates), the increase in PEPc would be a response to increased demands for pyruvate and/or Pi recycling (Juszczuk and Rychter 2002). These findings support the fact that stimulation of the PEPc pathway in P-deficient nodules ensures nodule respiration.

Although PEPc has a target role in alternative routes of C metabolism under P stress, the enzyme has also a target role in N_2 fixation. In amide-exporting legumes, this route via PEPc is vital to provide C skeletons for the assimilation of NH_4^+ (Le Roux et al. 2006). Furthermore, PEPc provides a portion of the C for both malate and aspartate synthesis. The double implication of PEPc on C and N metabolism implies

that there is a competition of organic acids for the TCA cycle and for amino acid synthesis (Le Roux et al. 2008; Hernández et al. 2009). Moreover, it was further reported that excessive malate accumulation in P-deficient nodules inhibits N_2 fixation and subsequent N assimilation.

2.3.2 Accumulation of Nitrogenous Compounds

A deleterious impact of low P availability in nodule performance has also been associated with processes of plant N adjustment. According to Almeida et al. (2000), the slower growth of plants exposed to low P implies that plant N requirements are also diminished. Consequently, these authors observed that depleted N_2 fixation is a consequence of an adjustment between plant N sink/source. Accordingly, the host plant would decrease the N_2 fixation rate in order to adjust to real N requirements. In accordance with what has been described in nodules subjected to other stresses such as drought and salinity (Aranjuelo et al. 2014), the decrease in N_{ase} activity under low P availability conditions has been associated with the accumulation of nitrogenous compounds in nodules (Almeida et al. 2000; Sulieman et al. 2013, 2014; Nasr Esfahani et al. 2016). The accumulation of these compounds can originate from decreases in carbohydrate fluxes to the nodules and the consequent decrease in the transport of nitrogenous compounds to the plant (Sulieman et al. 2013, 2014). According to Sulieman et al. (2013), the reduction in host plant growth would result in a reduction in N demand for the newly fixed N_2—a component known to be highly expensive in terms of C energy. As a result, a shoot-derived N-signal would probably be sent out to the nodule through the phloem to induce a down-regulation of N_{ase} activity (Sulieman et al. 2013, 2014). The fact that analyses of total amino acids in the phloem sap of *M. truncatula* P-stressed plants strongly increased (as compared with a control treatment) would support the hypothesis that amino acid build up is involved in the poorer nodule performance (Sulieman et al. 2013). The authors also observe that asparagine, or a precursor that interacts with nodule machinery and down-regulates the N_{ase} activity, might be the shoot signal (Sulieman et al. 2013). In addition to asparagine, other compounds, such as glutamate, glutamine, ureides, γ-aminobutyric acid (GABA), proline, polyamines, or a combination of these compounds, have been proposed as signaling candidates involved in nodule N-feedback signaling (Sulieman et al. 2014 and references therein). However, according to what observed by Nasr Esfahani et al. (2016) increases in nodule amino acid content could be also associated to other processes. When exposed to low P conditions, activities of the main enzymes associated with N metabolism increased, with the consequent accumulation of identified amino acids. Alternatively, the increase in the availability of amino acids could also have been attributed to protein degradation in P-deficient nodules (Nasr Esfahani et al. 2016).

2.3.3 O_2 Permeability

O_2 is required by the nodule for respiration processes; however, O_2 regulation is critical for BNF since most N_{ase} are sensitive to its presence (Aranjuelo et al. 2014). Nodule permeability to O_2 via the regulation of the O_2 diffusion barrier has been suggested as a key factor conditioning N_{ase} performance (Hunt and Layzell 1993). Previous studies have shown that water stress causes a diminishment in the permeability to O_2 diffusion, which leads to a reduction in nodule respiration, and, therefore, a lower production of energy via ATP synthase (Serraj and Sinclair 1996). Nodule cortex permeability to O_2, and consequently nodule O_2 content, has also been described to be affected by P availability (Jebara et al. 2005). Previous studies conducted with soybean, common bean and alfalfa exposed to low P reported an increase in cortex permeability (Ribet and Drevon 1995; Jebara et al. 2005; Schulze and Drevon 2005). According to those studies, such an increase might be a response to enhanced O_2-limitation due to wasteful O_2 alternative respiration. However, Jebara et al. (2005) showed that a decline in nodule permeability to O_2 of P deficiency in common bean plants, and that high P supply increases nodule conductance to O_2 diffusion. Another, not mutually exclusive, hypothesis is that the increase in nodule permeability might be the result of a higher shoot N demand per nodule mass under P deficiency (Ribet and Drevon 1995).

2.3.4 Oxidative Stress

Oxidative stress has been identified as another mechanism responsible for N_2 fixation inhibition under stressful growth conditions (Aranjuelo et al. 2014). Some environmental conditions (such as drought or salinity) have been described (Aranjuelo et al. 2014) to cause an O_2 content imbalance (necessary to ensure successful nodule performance) responsible for nodule senescence. The imbalance in O_2 control is associated with the formation of ROS, which could produce cellular damage. ROS production and removal is a complex process that requires a tight biochemical control involving enzymatic and non-enzymatic detoxification mechanisms that have been developed by plants (Aranjuelo et al. 2014). Despite its proven relevance in N_2 fixation, information on the impact of P deficiency in nodule oxidative status is scarce. A recent study (Nasr Esfahani et al. 2016) conducted in chickpea plants revealed that, in P-deficient conditions, the plants develop different strategies to overcome oxidative stress. As revealed by the authors, the increase in nodule pyruvate and GABA availability of P-deficient plants could be associated with their ROS scavenging properties and adjustment of cellular redox status. Moreover, Nasr Esfahani et al. (2016) also showed that increased activity of the main enzymes involved with the NH_4^+ assimilation (such as NADH-dependent glutamate dehydrogenase, NADH-GDH) was induced in nodules in response to low Pi stress. Such an increase, together with the above-mentioned increase in glutamate content, would help prevent potential ROS development derived from toxic accumulation of NH_4^+.

2.4 Conclusions

This chapter has reviewed the importance of P availability to plants, taking the perspective of a future P fertilizer shortage. As shown, P deficiency has strong impact in plants, functioning at both aboveground and belowground levels. P deficiency has been shown to affect negatively N_2-fixation and photosynthesis (as Pi is a direct subtract of the photosynthesis) and, consequently, plant growth. It should also be noted that effects derived from P-deficiency are more severe in non-fixative plants than in N_2-fixing plants. Although the effect of P-deficiency has been studied in the past, such studies were focused mainly on growth characterizations, especially of aboveground organs. However, in the current chapter, it is remarked that, as highlighted by studies carried out in recent decades, plant growth under P-deficiency conditions will be also conditioned by the inhibitory effect of P-deficiency in nodule performance. While, classically, carbohydrate availability, accumulation of nitrogenous compounds, together with O_2 diffusion and oxidative stress, have been identified as target process that mediate nodule functioning, as noted in this chapter, still little is known about the impact of P-deficiency in such processes. Overall, these advances emphasize the need to increase our knowledge on the P availability effect in N_2 fixation.

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Chapter 3
The Influence of Phosphate Deficiency on Legume Symbiotic N_2 Fixation

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Abstract Legumes are well recognized for their nutritional and health benefits as well as for their impact in the sustainability of agricultural systems. Phosphate (Pi) deficiency is a major nutritional factor limiting legume production, particularly in acidic and calcareous soils. Pi deficiency limits N_2 fixation, since it has been described to have a strong impact on the growth and survival of both rhizobia and host plant. Legumes have evolved complex mechanisms to cope with Pi limitation. The maintenance of symbiotic N_2 fixation seems to be a key aspect to assure legume productivity in low Pi environments. During the last decades, physiological components and molecular players underlying Pi-deficiency adaptation responses have been elucidated. Molecular, biochemical, physiological, and morphological responses are triggered to stimulate soil Pi uptake or to optimize its intracellular use efficiency and allocation over all plant organs. Research conducted on model species

D. Martins and A. Macovei have equal contributions to this manuscript.

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such as *Medicago truncatula* or important pulses such as common bean, soybean, and white lupin have provided valuable clues about the different mechanisms ensuring Pi homeostasis in nodules under Pi-limited conditions. In this chapter, we provide a general overview on the recent achievements on the impact of Pi deficiency on symbiotic N_2 fixation in a broad range of legume species. A critical discussion of the main results and open questions is provided, highlighting the different approaches used to address the current needs of agriculture under the climate change context.

Keywords Legumes • Pi deficiency • Phosphorus • Nodulation • Nitrogen assimilation • Symbiotic N_2 fixation

3.1 Introduction

Legumes are well recognized for their nutritional and health benefits as well as for their impact in the sustainability of agricultural systems (Araújo et al. 2015). Legume cultivation can play a central role as they represent the second major crop of agricultural importance worldwide, covering about 14% of the total cultivated land (Aranjuelo et al. 2014). When considering the nutritional impact of leguminous plants, namely, grain legumes which are also known as pulses, the international Food and Agriculture Organization (FAO) from the United Nations recognized the importance of these products by declaring 2016 the International Year of Pulses (FAO 2016). The standard *Codex Alimentarius* (2007) defines pulses as the dry seeds of leguminous plants, distinguished from the leguminous oil seeds by their low fat content, and includes species like beans (*Phaseolus* spp.), lentils (*Lens* spp.), peas (*Pisum sativum*), chickpea (*Cicer arietinum*), faba beans (*Vicia faba*), and cowpeas (*Vigna* spp.).

Legumes are of particular interest in agriculture because of their high nutritional value and innate ability to form symbiotic relations with dinitrogen (N_2)-fixing bacteria that often results in the formation of N_2-fixing nodules. This symbiotic relationship with soil bacteria from the genus *Rhizobium* that use the solar energy captured by the plant to break the triple bonds in inert atmospheric N_2 and form reactive nitrogen (N) species such as ammonium (NH_4^+) enables a more efficient plant growth, development, and seed production (Goh et al. 2013). Hence, this attribute provides legumes with a considerable advantage over the nonleguminous crops, especially when grown on soils with low N content (Brockwell et al. 1995). Both the World Health Organization (WHO) and FAO recommended increasing legume production because of their nutritional value and ability to fix N_2 in the soil (FAO 2013). This unique association comes from evidence suggesting that methods assigned to improve ecosystem sustainability can be closely related with goals of improving nutrition. A case study in Malawi revealed that the use of legumes to restore soil fertility also triggered an enhanced consumption of their products (Kerr et al. 2013). From a nutritional point of view, pulses had been even designated as “the poor man’s meat” mainly because of their high source of proteins and affordable costs, while containing also a vast

array of micronutrients in large quantities. Legume seeds are rich in most B vitamins as well as in iron, calcium, and zinc. Some crops like groundnut (*Arachis hypogaea* L.) and soybean (*Glycine max* L. Merr.) also contain higher amounts of fat, thus contributing to increased energy rates (Messina 1999). Additionally, the leaves of common bean and cowpea have similar nutrient concentrations with the seeds and even have higher amounts of micronutrients, as it also contains provitamin A and vitamin C (Messina 1999).

Besides, the decrease in the addition of N fertilizers has an overall positive impact on both agricultural costs and ecological integrity, as leaching of nitrate (NO_3^-) in the water supply can result in severe groundwater contamination (Crews and Peoples 2004). Moreover, a recent study conducted on a long-term N addition to the soil found that this treatment negatively impacted on the legume–rhizobium mutualism causing up to 30% reduction in plant biomass (Weese et al. 2015). From an agronomical point of view, when legumes are included in the intercropping systems, defined as the simultaneous cultivation of two or more crop species in the same field, it enhances the yield by improving resource-use efficiency (Li et al. 2003; Zhang et al. 2015; Xue et al. 2016).

When considering the essential nutrients needed for the development of crops with high yield potential, N and phosphorus (P) are the most limiting factors. N is an essential element required for successful plant growth as it is used by plants to synthesize amino acids, the building blocks of proteins, and other vital compounds, such as chlorophyll, nucleic acids, and enzymes. Among the signs of N deficiency in plants, stunt growth and chlorosis are the most encountered (Marschner and Marschner 2012). Although reactive N species such as NH_4^+, NO_3^-, and nitrite (NO_2^-) account for less than 5% of the total N in soil, they represent the main forms absorbed by most plants (Liu et al. 2014b). Hence, inorganic and organic fertilizers are applied to maintain the nutritional condition of different cropping systems, but this intensive use of chemicals although it has a positive impact on crop yield it also negatively impacts on the environment. In a report released by the US Department of Agriculture (USDA), it was stated that besides the increased agricultural productivity, the N addition also causes ozone-induced injury to crops and forests, eutrophication of aquatic ecosystems, biodiversity losses, and global climate changes (Ribaudo et al. 2011). Thus, by using legumes in rotation and intercropping systems, the use of N fertilizers can be substantially reduced, and the soil natural balance of N can be sustainably restored.

P is an essential macronutrient for plant and crop yield (Rodríguez and Fraga 1999). Phosphate (Pi) is the main P form that plant roots can absorb but its concentration is often limited for plant uptake (Chiou and Lin 2011). After uptake, it either remains as Pi or is assimilated by forming an ester with a hydroxyl group of a carbon (C) chain (e.g., sugar phosphates) or attaches to another Pi by forming an energy-rich pyrophosphate bond (e.g., adenosine triphosphate, ATP) (Marschner and Marschner 2012). Numerous metabolites include Pi in their molecular composition, such as sugar phosphates, nucleotides and nucleic acids, phospholipids, phosphoproteins, and energy-rich compounds like ATP (Czarnecki et al. 2013). Moreover, Pi play a major role in energy transformation and regulation of various

enzymatic activities involved in photosynthesis, respiration, energy generation, and nucleic acid biosynthesis (Sulieman and Tran 2015). Based on these assumptions, it becomes clear that the low availability of Pi in soil imposes serious limitations for legume growth and development.

The present chapter is designed to provide a general overview and critical discussions on the recent achievements regarding the study of Pi-deficiency responses in a broad range of legume species, with an emphasis on the symbiotic N_2 fixation (SNF) while highlighting different approaches used to address the current needs of agriculture and industry under the climate change context.

3.2 P is an Essential Nutrient for Legumes

A huge challenge derives from the fact that Pi has a low availability in the soil as it forms insoluble complexes with several minerals, while it is also poorly recovered from fertilizer addition because it is absorbed mainly by the soil and is not available for plants lacking specific adaptations (Balemi and Negisho 2012). With more than 40% of the world soils being deficient in Pi (Vance et al. 2003), the chemical fertilizer application alone is not a cost-effective way for increasing crop production in many Pi-limiting soils (Tilman et al. 2002). In turn, the addition of Pi fertilizers also has a high impact on the soil pH as well as on several morphological plant traits such as length and surface area of the roots, root architecture, root clusters (Shane and Lambers 2005), and even the root association with soil bacteria and fungi (Smith et al. 2000). Hence, plant adaptation strategies to overcome Pi deficiency include improved uptake efficiency, acquired through modification of the root morphology, exudation of chemical compounds into the rhizosphere, and association of roots with mycorrhiza (Vance et al. 2003), as well as improved utilization efficiency to produce higher dry matter per unit of Pi absorbed (Richardson et al. 2011). Research performed during field studies revealed that some legumes, such as white lupin (*Lupinus albus* L.), are able to better fix Pi when compared with other crops, mainly because of cluster-root (CR) formation (Abdolzadeh et al. 2010; Thuynsma et al. 2014). However, Pi deficiency negatively impacts on the leguminous plants' ability to fix N_2 by limiting the growth and function of the nodules. This aspect is further addressed in Sect. 3.4.2. This was thought to be a consequence of lower photosynthesis rate that limits the supply of C to the nodules (Sa and Israel 1991). Another study showed that low Pi inhibits nodulation and N_2 fixation more than it affects plant growth and that this is due to the presence of an N-feedback mechanism induced by low Pi (Almeida et al. 2000). In addition to the low C supply and N-feedback regulation, other studies have documented that also the oxygen (O_2) supply and oxidative stress are factors which can negatively affect nodulation and symbiotic efficiency (Arthikala et al. 2014; van Noorden et al. 2016). Nevertheless, mounting evidences are showing that Pi homeostasis in N_2-fixing plant results from a coordinated interplay between the host plant and symbiotic microorganisms (Sulieman and Tran 2015).

3.2.1 *The Agricultural Scenario of Legumes and Pi Deficiency Under Climatic Changes*

Nowadays, climate change represents one of the most stringent problems that the world is facing, with several ramifications over the global environmental impact, agriculture and food security, as well as overall humanity life quality. The assessment reports on the climate change provided by the Intergovernmental Panel on Climatic Change (IPCC) underline the vulnerability of both human and natural systems and advocate that agriculture holds great potential to aid in mitigating these adverse effects while further supporting food security goals (IPCC 2007). With the predicted increase in the atmospheric carbon dioxide (CO_2) levels, rise in temperature, higher frequency of weather instability, water scarcity, soil salinisation, etc., the agricultural sector must be prepared to tackle all these multifactorial issues and, at the same time, boost food productivity for the increasing population (FAO 2015). In a global effort to reduce the effects of climatic change, the Paris Agreement was set in motion, and 186 countries had published their action plan for ways to reduce greenhouse emissions (COP21 2015). Among these milestones, the implementation of sustainable, climate smart agriculture is essential to adapt to climate change. This emphasizes issues like the enhancement of agricultural productivity to increase food security while decreasing greenhouse gas emissions and increasing C sinks, as well as raising the adaptive capacity at multiple levels (Campbell et al. 2014).

From an agronomical point of view, grain legume production, although often used to promote environmental services, falls behind when compared with major grain cereals. A recent study estimated the variability and yield of grain non-legume in the EU and USA across a timeframe of more than 50 years (Cernay et al. 2015). The results of this study revealed that in EU the yield of legume crops vs. non-legume crops is highly variable, whereas in the USA, these differences are considerably lower, and EU imported more than 60% of its legume supply from the USA during this timeframe. The authors also suggest improving the cropping and intercropping system in EU in order to increase the local supply of legume grains. In fact, several studies pointed out the intercropping systems represent promising management practices that have an overall benefic impact on both crop yield and soil environment. In this sense, cereal/legume intercropping is an effective way to bring the legume production up to speed with the cereal crops while improving Pi uptake and N_2 fixation in soils with low Pi and N content. Field studies on intercropped faba bean and maize reported a 20% increase in production without fertilizer addition and up to 38% enhancement when Pi was added to the soil (Li et al. 2003). Another recent study showed that wheat grown together with faba bean also presented a significant enhancement of Pi uptake in Pi-deficient soils; however, no increase in biomass was observed, and this might have been due to the slow growth rates of the two species (Li et al. 2015a). These two studies underline the importance of choosing the best partner crops for intercropping systems, encouraging further studies of this broad agricultural field. A big-scale experimental study was conducted in 16 different sites covering a broad range of climate environments, from Atlantic to continental and

temperate to arctic, to assess the grass–legume mixture efficiency (Suter et al. 2015). This multifactorial study used five different grass species intercropped with white clover (*Trifolium repens*) and assessed plant productivity and N yield average together with the climatic influence. As concerned the total N yield, a positive effect of grass–legume mixture was observed that was however strongly affected by temperature and the proportion of legume present over the 3 years during which the study was conducted (Suter et al. 2015). But more importantly, this study, along with other studies summarized in the present review, underlines the importance of legumes in contributing to the development of recourse-efficient and environmentally-friendly agricultural practices to mitigate the challenges arouse by the global climatic changes.

3.2.2 Pi Deficiency and Legume Production Under Abiotic Stresses: Consequences and Adaptive Strategies

As legumes are an essential part of sustainable farming systems, an increasing body of research is continuously being carried out to better understand and mostly improve its uses in agriculture and food industry. The SNF which takes place in the root nodules represents a highly renewable and environmentally sustainable source of N. However, this symbiotic association between plant roots and bacteria is quite sensitive to environmental changes. Abiotic stresses, such as drought, extreme temperatures, salinity, and heavy metals, have a negative impact on legume production (Aranjuelo et al. 2014; Araújo et al. 2015). As abiotic stresses also influence soil conditions, both the plant and their symbiotic partners are affected, but the rhizobia display a higher degree of tolerance to stress manifested through more efficient maintenance of homeostasis (Priefer et al. 2001). In turn, this can have a positive impact also on the plant host (Xu et al. 2012). However, the symbiotic process is highly affected by abiotic stresses, and this was hypothesized to be related to a concerted accumulation of sucrose and reduction of malate during the bacteroid's respiration process (Ramos et al. 1999). As a consequence, also the N status of the plant is affected. This has been proposed to work via an N-feedback inhibition of nitrogenase activity that evolved either from a direct N-feedback inhibition in nodules or by an indirect N-feedback process due to N signaling from the aerial parts of the plant (Serraj et al. 2001). Aside from abiotic stress, nutrient availability also represents a major constrain for legume yields, with limited Pi availability receiving the most attention (Tesfaye et al. 2007; Sulieman and Tran 2015).

As stated previously, P is implicated in many biological processes driving seed germination, root development, and fruit ripening. Pi is usually translocated into the actively growing meristems. However, during plant maturation, Pi is transported into the fruits, where high-energy requirements are needed for fruit ripening and seed formation (Goh et al. 2013). Hence, Pi deficiencies during this period can affect both seed development and normal crop maturity. Pi deficiency is quite difficult to diagnose from a morphological point of view, with crops usually displaying no particular symptoms except for a general stunting during early growth.

Some evidences could include abnormal leaf coloration due to the accumulation of anthocyanins observed in some species (Sarker and Karmoker 2011) or root morphological changes (Wang et al. 2015). Conversely, biochemical diagnosis is more adequate as several studies reported enhanced carbohydrate concentrations (Hammond and White 2011), decreased soluble and insoluble protein content, as well as increased levels of proline and phenolic compounds in the root (Hernández et al. 2007; Sarker and Karmoker 2011). In white lupin, it was shown that sugars and Pi-stress signaling are closely interrelated (Uhde-Stone et al. 2003; Liu et al. 2005), while in common bean, it was evidenced that sugar is allocated to the roots in higher amount during Pi deprivation (Rychter and Randall 1994). The activity of sucrose synthase, a key enzyme of C metabolism, on legume nodules was affected by mild water deficit (Gonzalez et al. 1998), while reduced amounts of organic acids (oxalic, malic, and malonic acids) were also observed in common bean Pi-stressed roots when compared to Pi-sufficient roots (Hernández et al. 2007). From a physiological point of view, Pi deficiency has a direct negative effect on photosynthesis through a decrease in the photosynthetic ability per unit of leaf area also associated with a morphological reduction of the leaf area (Chaudhary et al. 2008). Altogether, these evidences support the assumption that a clear connection between C, N, and P metabolism exists.

The most studied abiotic stress factor interacting with the available soil Pi pools is water deficit or drought. Several studies performed under field conditions reported evidence of interactions between these two particular conditions. For instance, studies conducted in the Mediterranean region reported that lentil plants performed better under Pi fertilization during dry years (Matar et al. 1992). Another study showed that clover plants grown in dry soil and high Pi soil maintained better leaf water potential when compared with plants grown under low Pi, with only the low Pi plants actually showing clear symptoms of water stress (Singh et al. 1997). The effects of Pi deficiency and water deprivation are mostly encountered at the root level. In soybean, low Pi availability and water deprivation resulted in a gradual decrease of plant's vegetative development as well as nodule number, while the root length density was increased with more than 20% as compared with control plants in order to avail a greater area for nutrition (Gutiérrez-Boem and Thomas 1999). In addition to root growth, the formation of mycorrhizae as well as other rhizospheric microbial communities can substantially help alleviate the symptoms of both Pi deficiency and water stress (Gupta et al. 2015). This is because generally the soil arbuscular mycorrhizae fungi have a benefic influence on plant growth mainly due to their ability to uptake nutrients, especially Pi (Lambers et al. 2008). As water deficit is known to reduce the Pi availability, mycorrhizal association was shown to aid plants survival at multiple levels, from nutritional (Pi an N uptake) to physiological (stomatal regulation by osmotic adjustments) and cellular (enhanced activity of antioxidant enzymes) effects (Ruiz-Lozano 2003). In a study on berseem clover (*Trifolium alexandrinum*) grown in the semiarid conditions of the Mediterranean region, it was shown that the formation of mycorrhizae leads to enhanced biomass and increased Pi and N uptake, thus playing an important role in plant growth and development (Saia et al. 2014).

Many genes and gene networks are responsible for governing the adaptation of plants to both Pi starvation and water scarcity. Aside from the obvious genes implicated in the response to stress, also genes involved in the regulation of root system architecture as well as genes coding for Pi transporters were studied in legumes. In soybean, the *GmEXPB2* gene coding a β-expansin gene is mainly expressed in roots and greatly induced by Pi starvation (Guo et al. 2011). By using overexpression and silencing approaches, the authors demonstrated that the *GmEXPB2* gene is involved in root hair elongation, having a high impact on Pi uptake during low Pi availability and water deficit. Another example is the *PvTIFY* transcription factor characterized in common bean as being involved in the regulation of genes involved in Pi deficiency and jasmonate pathway (Aparicio-Fabre et al. 2013). As the jasmonate hormone regulates a myriad of processes, it is essential for plant growth and development as well as defense responses and abiotic stresses such as drought (Browse 2009). In common bean, a global comparative gene expression analysis reported that several *PvTIFY* genes as well as genes involved in the jasmonate biosynthetic pathway were responsive to Pi deficiency, suggesting that *PvTIFY* is either directly or indirectly (via the jasmonate pathway) involved in Pi adaptation mechanisms (Aparicio-Fabre et al. 2013).

To gain better insight on how to efficiently use leguminous plants to tackle the climatic change scenario in agriculture, an overview on the knowledge of the legume SNF process, along with the physiological and molecular aspects related to Pi deficiency, is required. Hence, the current information regarding these aspects is presented in the next subchapters.

3.3 Legume SNF: Back to Basics

Legume–rhizobia symbiosis provides synergistic advantages to both partners, allowing the host plant to benefit from the N_2-fixing ability of bacteria, which in turn are supplied with C compounds as energy sources for growth. Such a process, which has a tremendous importance in the agronomic context, still deserves to be investigated, particularly at the molecular level due to the extreme complexity of the regulatory pathways underlying plant–bacteria interactions, nodule development and metabolism, and adaptive strategies activated in response to adverse environments.

3.3.1 Free-Living Rhizobial Biology: Diversity, Growth, and Stress Tolerance

Life on planet Earth is dependent on essential elements among which N that is incorporated into proteins and nucleic acids. Although several living organisms are able to assimilate N in the form of NH_4^+, NO_2^-, NO_3^-, or urea ($CO(NH_2)_2$), only a restricted number of microorganisms can perform the so-called process of biological N_2 fixation (BNF), namely, the conversion of atmospheric N_2 molecule

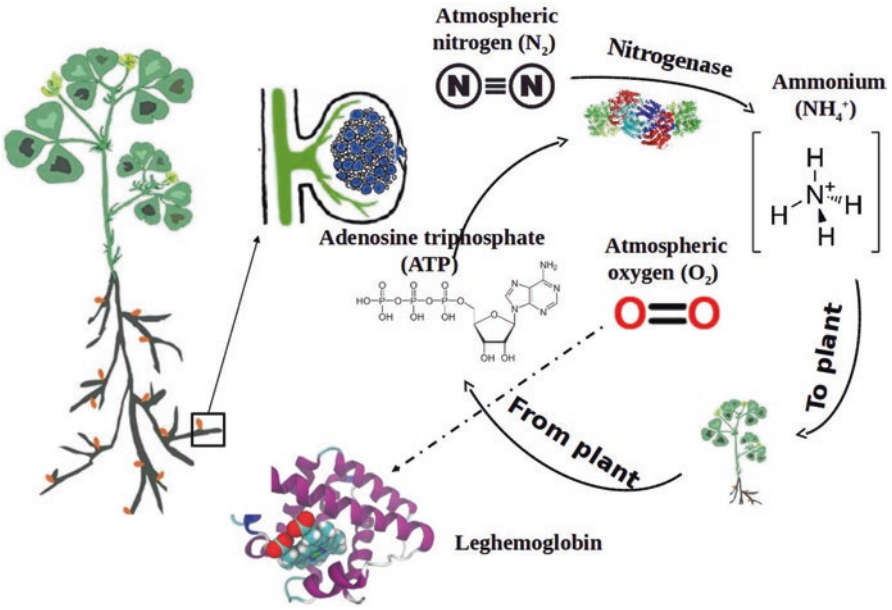

Fig. 3.1 Schematic representation of symbiotic N_2 fixation by nodulating legumes. N_2 is fixed by the bacteria in the nodules through the activity of nitrogenases. This requires high amounts of energy (ATP) which it reserves from the plant. Nitrogenases function in anaerobic conditions, so the leghemoglobin, produced within the legume–bacteria cooperation, binds the O_2 creating an anaerobic environment

(containing a highly stable covalent triple bond) to NH_4^+. A schematic overview of this process in the legume nodules is presented in Fig. 3.1. This conversion is catalyzed by nitrogenases, a family of complex metalloenzymes (see Hu and Ribbe 2015). Recent studies, including measurements in tropical forests, estimated the global BNF rate in terms of 58 Tg N per year (Vitousek et al. 2013; Sullivan et al. 2014). The gaseous N_2 molecule, which accounts for approximately 78% of the Earth's atmosphere, dissolves into water at the ocean surface allowing biological fixation in the marine ecosystems (Zehr and Ward 2002). Due to the relevance N_2-fixing organisms for life, it is essential to assess their presence, diversity, and distribution on our planet. This will allow a more accurate quantitative and qualitative estimation of the process on a global scale (Reed et al. 2011). Novel N_2-fixing organisms showing different degree of fixation rates have been recently discovered in various habitats, like the tropical forests (Batterman et al. 2013).

Free-living N_2-fixing bacteria (diazotrophic) include *Azotobacter* spp. (obligately aerobic, heterotrophic Gram-negative bacteria in the class of γ-*Proteobacteria*) widely distributed in natural and agricultural soils of temperate regions (Sahoo et al. 2014) and members of the genus *Clostridium* (endospore-forming obligate anaerobes) (Amon et al. 2010). *Cyanobacteria* represent another example of free-living N_2-fixing bacteria located in terrestrial and marine environments (Bullerjahn and

Post 2014; Singh et al. 2016). Among soil actinomycetes, the genus *Frankia* is well known for its N_2-fixing ability. *Frankia* free-living strains are found in different ecological niches in soil as components of microbial consortia (Chaia et al. 2010). The well-known N_2-fixing rhizobia are distributed within two different classes of *Proteobacteria*, namely, *α-Proteobacteria* and *β-Proteobacteria*. The α-proteobacterial genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Bradyrhizobium* contain bacteria present under free-living and symbiotic conditions, the latter allowing symbiotic association with a compatible host plant. Differences between the two physiological conditions, particularly at the level of cell surface structure, have been highlighted based on comparative proteome analyses carried out in *M. loti* (Tatsukami et al. 2013). The complexity of rhizobia ecology and evolution is reflected by their different lifestyle since symbiotic rhizobia, including highly mutualistic N_2 fixers as well as non-fixing parasites, coexist with nonsymbiotic strains lacking the ability to infect plants (Denison and Kiers 2004). Several studies have focused on the role played by rhizobia as natural endophytes of relevant cereal crops, able to promote plant growth and enhanced grain yield, independent on root nodulation and BNF (Yanni et al. 1997; Ji et al. 2010; Chen and Zhu 2013). For instance, in rice, it was demonstrated that rhizobia can colonize the plant roots without inducing nodulation. In this case, the bacteria reside mainly in the intercellular spaces, in contrast with the root nodule symbiosis where the bacteria have an intracellular colonization. Moreover, it was shown that common symbiosis genes (*DMI3*, *CASTOR*, *CYCLOPS*) are not required for the endophytic colonization of rice roots by rhizobial strains (Chen and Zhu 2013).

α-Proteobacteria include *Methylobacterium*, *Blastobacter denitrificans*, and *Devosia neptuniae*. *Methylobacterium* is a facultative methylotrophic bacterium associated with the plant phyllosphere (Minami et al. 2016), while the aquatic bacterium *B. denitrificans* is able to establish a symbiotic relationship with the flood-tolerant legume *Aeschynomene indica* (van Berkum et al. 1995). *D. neptuniae*, another non-rhizobia, Gram-negative, strictly aerobic short, and motile rod-shaped microorganism, is able to establish N_2-fixing root nodule symbiosis with *Neptunia natans*, a unique aquatic legume from tropical/subtropical regions (Rivas et al. 2003). β-*Proteobacteria* include β-rhizobia such as *Burkholderia*, *Cupriavidus* (formerly *Ralstonia*) *taiwanensis*, and *Herbaspirillum lusitanum* (Gyaneshwar et al. 2011). Both *Burkholderia* and *Cupriavidus* have been found associated with species of the genus *Mimosa*, mainly native to the New World, while *H. lusitanum* has been characterized as an endophyte able to colonize agronomically relevant cereals (James et al. 1997).

The ability of rhizobia to withstand environmental stresses is directly correlated with their agronomical relevance as N_2-fixing and growth-promoting bacteria since they can provide crop plants with enhanced tolerance to abiotic stresses like drought and salinity. It has been reported that *M. truncatula* plants nodulated with *Sinorhizobium medicae* or *S. meliloti*, having different degree of N_2 fixation efficiency, showed a significant delay in drought-induced leaf senescence compared to non-nodulated plants (Staudinger et al. 2016). Free-living rhizobia growth is impaired under osmotic stress induced by excessive salts in soil, with negative consequences on nodulation and symbiotic capacity. The use of salt-tolerant rhizobia strains, able

to perform N_2 fixation in high-salinity soils, represents a promising strategy and sustainable alternatives to chemical fertilization (Rejili et al. 2012). In a recent study, *Rhizobium leguminosarum* bv. *viceae* strains were isolated from the salt-tolerant *P. sativum* cultivar "Resal" at sites with contrasting climatic conditions across Portugal (Cardoso et al. 2015). By screening its tolerance to salinity, several changes in the bacterial protein profiles were determined. While rhizobia from southeast Portugal were able to withstand water shortage in soil, the bacteria from northwest Portugal were more susceptible to water stress (Cardoso et al. 2015). These findings highlight the need for extensive screening of stress-tolerant rhizobial populations in order to enhance the strain collections useful for agronomical applications.

Increased desiccation tolerance is another desired trait that would support survival of rhizobia on legume seeds during drying and storage. The response to desiccation stress in prokaryotes involves complex physiological responses and activation of protection mechanisms against cellular damage, e.g., the production of exopolysaccharides, induction of stress-responsive proteins, repair of membrane/DNA damage, and accumulation of intracellular sugars (Vriezen et al. 2007; Cytryn et al. 2007; Vanderlinde et al. 2010). Osmoprotectants are accumulated within rhizobia cells in response to osmotic and desiccation stress, either by de novo synthesis or by active uptake systems. The presence of the nonreducing disaccharide trehalose, involved in the protection of macromolecules against denaturation during desiccation stress, significantly improved desiccation tolerance in *Bradyrhizobium japonicum* (Streeter 2003) and *R. leguminosarum* bv. *trifolii* (McIntyre et al. 2007). More recently, an investigation performed on peat-cultured *R. leguminosarum* bv. *trifolii* TA1 and *B. japonicum* CB1809 strains revealed multifactorial features associated with increased desiccation tolerance which requires intracellular trehalose accumulation together with enhanced expression of proteins involved in cell envelope protection, DNA damage repair, and oxidative stress response (Casteriano et al. 2013). Stress-specific molecular responses have been analyzed by Mhamdi et al. (2015) in strains belonging to *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium* genera challenged with sodium chloride (NaCl) and polyethylene glycol (PEG). A negative correlation between cell viability and lipid peroxidation was observed, while increased levels of C19:0 cyclo-fatty acid were detected in *Sinorhizobium*- and *Mesorhizobium*-tolerant strains, as a protective mechanism for preserving membrane integrity. Superoxide dismutase (SOD) and catalase (CAT) activity were enhanced in response to both stress agents to provide protection against free radical species (Mhamdi et al. 2015).

3.3.2 Nodulation

Root nodule symbiosis observed in legumes is a fascinating example of successful symbiosis between plants and bacteria, characterized by a perfect balance between the host and the microsymbiont. Legumes facing N shortage secrete secondary metabolites, such as flavonoids, as signaling molecules that attract compatible symbiotic rhizobia by triggering the expression of the bacteria nodulation genes

(Abdel-Lateif et al. 2012). The latter encode enzymes required for the synthesis of the nodulation factor (NodF), a lipo-chitooligosaccharide which induces the plant molecular responses leading to root nodule symbiosis (Oldroyd et al. 2011). NodFs bind plant receptors which belong to the receptor-like kinases (RLKs) class, key components of the plant innate immunosystem, thus posing the question about the possible common features between the rhizobial–plant and pathogen–plant interactions and the possibility to exploit the plant immune response to modulate rhizobial infection and host range (Toth and Stacey 2015).

In many legumes, rhizobia enter the root epidermis using infection threads (ITs) or tubular plant-derived structures surrounded by a membrane which is contiguous with the plant cell plasmalemma and a layer of cell wall material. Rhizobia are trapped within the IT, and they remain isolated from the host cell cytoplasm. Fournier et al. (2015) reported on the use of live tissue imaging to monitor the early steps of the rhizobia–plant interaction, focusing on the transition from the entrapment of bacteria within the root hair cell to the formation of the IT. The authors suggest a new model in which the so-called infection chamber first gives rise to a globular apoplastic compartment that contains the bacteria resembling the structure of the future IT. Subsequently, the infection chamber is remodeled with a transition from radial morphology to the tubular structure typical of ITs (Fournier et al. 2015). ITs deliver rhizobia into the actively dividing cortical cells that will give rise to the nodule primordium, subsequently converted into the nodule, a new root organ. Within the nodule, bacteria differentiate into bacteroids, the SNF sites. Ammonia (NH_3) is supplied to the host plant which, in turn, provides rhizobia with carbohydrates. Terminal bacteroid differentiation is accomplished through cell enlargement (up to ten times compared to the size of the free-living bacteria), genome endoreduplication, and membrane permeabilization (Mergaert et al. 2006), and the entire process is ruled by plant antimicrobial peptides, named nodule-specific cysteine-rich (NCR) peptides, showing similarities to defensin-type factors (Haag et al. 2013; Alunni and Gourion 2016).

Legumes produce indeterminate or determinate nodules, differing in morphology and developmental program. Legumes from the inverted repeat-lacking clade (IRLC) (e.g., *P. sativum* L., *V. faba* L., and *M. sativa* L.), which develop indeterminate nodules, are able to secrete antimicrobial peptides that trigger endoreduplication of the bacterial genome and transition to a quiescent state. The rhizobial BacA (bacteroid development factor) protein is required for the uptake of plant-derived NCR peptides which rule bacteroid differentiation (Marlow et al. 2009). Bacteroids are surrounded by peribacteroid solution and peribacteroid membranes derived from the plasma membrane of the host plant cell. The peribacteroid solution contains high sugar levels, particularly inositols (Teijma et al. 2003), and molecules able to induce differentiation of rhizobia into bacteroids (Ohkama-Ohtsu et al. 2015).

Due to the high metabolic costs of nodulation, nodule number is tightly regulated by mechanisms that still need to be fully understood. As described later on in this chapter, Pi plays a major role on this process. The role of signaling peptides in the local and systemic control of nodule and lateral root formation, particularly at the early stage of nodule development, has been reviewed by Djordjevic et al.

(2015). Reactive oxygen species (ROS) accumulated through the activity of respiratory burst oxidative homologues (RBOHs), namely, NADPH oxidases, play key roles in several plant signal transduction pathways, among which are those that regulate the symbiosis between legumes and N_2-fixing bacteria (Arthikala et al. 2014). It has been previously demonstrated that ROS levels are transiently enhanced in tips of actively growing *Phaseolus vulgaris* root hair cells following exposure to NodFs (Cardenas et al. 2008), while ROS production is reduced in *M. truncatula* roots following the first hour of treatment with NodFs. Such a decrease is associated with the downregulation of the *MtRBOH2* and *MtRBOH3* genes (Lohar et al. 2007), whereas the use of inhibitors of NADPH oxidases in *M. truncatula* impairs both ROS production and the early rhizobial interaction in root hairs (Peleg-Grossman et al. 2007).

The complex molecular events underlying rhizobial infection and nodule organogenesis are under phytohormone control. Ethylene is a negative regulator of the legume–rhizobia symbiosis, acting at different stages during nodule formation by suppressing the signaling pathways triggered by NodFs (Guinel 2015). Larrainzar et al. (2015) investigated these issues using "omics" approaches to monitor the transcriptional changes occurring in roots of *M. truncatula* inoculated with *Sinorhizobium medicae*. The use of *M. truncatula* mutants showing reduced sensitivity to NodFs as well as the ethylene insensitive/Nod factor-hypersensitive mutant *sickle* revealed thousands of candidate genes modulated by NodFs and ethylene, allowing the prediction of key nodes controlling perception/transduction of signals brought to plants by NodFs (Larrainzar et al. 2015). The role of auxin signaling in rhizobial infection is not completely clarified, although it is possible that auxin regulates induction of cell division associated with infection (Breakspear et al. 2014; Schaller et al. 2015). Cytokinins are required for nodule development with cytokinin signaling responses occurring in both nodule primordia and developed nodules as recently showed by Reid et al. (2016) who found that the *Lotus japonicus Ckx3* (cytokinin oxidase/dehydrogenase) gene was induced by NodF during the early phase of nodule initiation. Jasmonate is also an emerging player in the control of symbiotic nodulation. A jasmonate ZIM-domain (JAZ) protein interacting with leghemoglobin in *Astragalus sinicus* was identified by Li et al. (2015b) who demonstrated its requirement for maintenance of proper nodule number, bacteroid development, and nitrogenase activity and highlighted a novel role for jasmonate during legume–rhizobia symbiosis.

Autoregulation of nodulation (AON) is a systemic signaling pathway which limits the number of nodules formed by the host legume plant when symbiosis with rhizobia takes place (Mortier et al. 2012). According to the most recent working hypothesis, developed in the model legume *M. truncatula*, root signaling peptides are translocated to the shoot where they bind to the shoot receptor complex SUNN (supernumerary nodules, belonging to the class of leucine-rich repeat receptor-like kinases or LRR-RLK), inducing a signal transduction pathway which impairs nodule formation. This mechanism requires the interaction of SUNN receptor with CRN (CORYNE) and CLV2 (CLAVATA) proteins which are essential players in the control of root meristem activity (Crook et al. 2016).

3.3.3 Nodule Function: Nitrogenase Activity

Atmospheric N_2 is made available to the biosphere through BNF catalyzed by nitrogenase, a metalloprotein that consists of two components, the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase) (Seefeldt et al. 2009). Electrons are delivered from Fe protein to MoFe protein in a reaction which requires the hydrolysis of at least two ATP molecules for each transferred electron. The Fe protein is extremely sensitive to O_2, and all diazotrophs maintain an anaerobic environment around nitrogenase.

Several methods have been developed to quantify the rates of N_2 fixation in terrestrial ecosystems, among which are ^{15}N isotope dilution (McAuliffe et al. 1958), acetylene reduction assay (ARA) (Hardy et al. 1968), N accretion (Knowles 1980), ^{15}N natural abundance (Shearer and Kohl 1986), and N difference (Weaver and Danso 1994). Stable isotope methods are generally considered the most accurate for measuring of SNF (Danso 1995). The ^{15}N isotope-based tracer techniques, which have significantly contributed to expand the knowledge of the dynamics occurring within the soil/plant system, rely on the Ndff equation: $N = N_l + N_s + N_o$ (where N is the total amount of N in the plant, N_l is the N recovered from the labeled pool, N_s is the N recovered from the unlabeled soil pool, and N_o is the N found in the plant at the beginning of the experiment) (Barraclough 1995). The discovery that the nitrogenase enzyme responsible for N_2 fixation also reduces acetylene (C_2H_2) to ethylene (C_2H_4) (Dilworth 1966) provided a useful assay for the quantification of the N_2 fixation process. ARA is still widely used because it provides a highly sensitive and inexpensive way to quantify relative nitrogenase enzyme activity in N_2-fixing tissues. Both acetylene and C_2H_4 are easy to measure by flame ionization gas chromatography. Thus, ARA can provide a simple, inexpensive measure of nitrogenase activity. There are two significant complications that can limit the use of the ARA for quantifying N_2 fixation: (1) the assay measures total electron flow through nitrogenase but only a proportion of electron flow is actually directed toward N_2 reduction (2) and this proportion can change depending on genetic and environmental factors. It has been reported that acetylene itself causes partial suppression of nitrogenase activity by limiting O_2 diffusion (Minchin et al. 1986). Recommendations and refinements to the original protocol have been presented by Vessey (1994) who underlined the importance of several key parameters, e. g., tissue preparation, time, and gas sample storage.

Hydrogen (H_2) evolution under normal atmospheric N_2 and O_2 levels (80:20 vol/vol) can be used to measure the apparent nitrogenase activity (ANA), namely, the situation where electrons are used to reduce both H^+ and N_2 (Hunt et al. 1987). H_2 evolving from the nodules provides an indirect, nondestructive measure of the N_2 fixation activity of nitrogenase. When N_2 in the air around nodules is replaced by argon (Ar), the electron flow through nitrogenase is directed onto H^+. The resulting H_2 evolution can be measured as a simple and nondestructive way to estimate total nitrogenase activity (TNA). The relative efficiency of nitrogenase in terms of electron allocation can be calculated as 1-ANA/TNA (electron allocation coefficient, EAC) (Fischinger and Schulze 2010). A mathematical model can be used to estimate

the rates of O_2, CO_2, N_2, and H_2 exchange from legume nodules under steady-state conditions of N_2 fixation. Based on this model, the rates of gas exchange, relative growth rate (RGR), TNA, EAC, uptake hydrogenase activity (HUP), and chemical features of the resulting N-containing molecules have been calculated with results that were in agreement with those obtained through experimental activity (Layzell et al. 1988). When considering the effects of nodule features on the rates of gas exchange, apparent respiratory cost (CO_2/NH_3), and sucrose cost (sucrose consumed/NH_3), ureide-producing nodules were estimated to consume 8% less sucrose per N fixed when compared to amide (asparagine)-producing nodules. However, ureide-producing nodules would show an apparent respiratory cost of 5% higher than that in amide-producing nodules. In both ureide-producing and amide-producing nodules, the apparent respiratory cost of N_2 fixation (CO_2/NH_3) was mainly dependent on EAC, followed by TNA, nodule RGR, and nodule size. EAC is modulated by the competitive inhibition of H_2, while the degree of inhibition is affected by the nodule's permeability to gas diffusion. Moloney et al. (1994) tested this hypothesis by measuring EAC in soybean nodules exposed to different partial pressures of H_2 and N_2, with or without changes in TNA or nodule permeability to gas diffusion. Results were compared with predictions from a mathematical model that combined equations for gas diffusion and competitive inhibition of N_2 fixation (Moloney and Layzell 1993). Both empirical and theoretical data revealed the same trend, namely, that decreases in EAC values were associated with increases in external pH_2, decreases in external pN_2, and decreases in nodule permeability to O_2 diffusion. The ability of the model to predict EAC provided strong support for the hypothesis that H_2-mediated inhibition of N_2 fixation plays a major role in the in vivo control of EAC. The model also hypothesized that the presence of a variable barrier to gas diffusion affects the H_2 and N_2 concentration in infected cells and consequently the degree of H_2 inhibition. Continuous measurement of H_2 evolution has been reported only for periods shorter than 48 h since H_2 analyzers are highly sensitive to humidity, and the analyzers react to O_2 pressure in the gas flow and temperature in their surroundings (Gordon et al. 2002). A new method for the non-invasive measure of nodule activity during prolonged time periods has been described by Cabeza et al. (2014, 2015) who provided novel insights into the regulation of N_2 fixation, such as the occurrence of daily rhythms in nodule activity.

The combination of chromatographic techniques with the use of isotopes as tracers has become an efficient tool for the study of metabolic fluxes in plants (Freund and Hegeman 2016). Nuclear magnetic resonance (NMR) in combination with isotope labeling is used in plant metabolomics to decipher metabolic fluxes, as a rapid, selective, highly reproducible, and site-specific tool. Isotope ratio mass spectrometry (IRMS) is also considered as one of the most suitable techniques for measuring isotopic ratios and isotopic enrichments due to high precision, sensitivity, and accuracy (Freund and Hegeman 2016). An IRMS-based method, allowing the measurement of $\delta^{13}C$ and $\delta^{15}N$ values of amino acids within the plant and symbiotic bacteria, has been described by Molero et al. (2011). The method revealed the pattern of C and N exchange between leaves and nodules, highlighting that γ-aminobutanoic acid (GABA), and glycine may represent an important form of C transport from leaves to the nodules.

3.3.4 Nodule Energy Status

Legume nodules are sites of intensive C and energy turnover, particularly in P-rich plant organs (Schulze et al. 2006). A correlation of SNF rates with the adenylate ratios, namely, ATP–ADP or adenylate energy charge AEC = ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]), has been highlighted (Wei et al. 2004). Early studies by de Lima et al. (1994) showed that exposure of soybean nodules to 10% O_2, stem girdling, or NO_3^- fertilization resulted in decreased AEC values in nodules. Wei et al. (2004) used nonaqueous centrifugal density gradient fractionation of the central infected zone tissue of soybean nodules to recover adenylate pools from subcellular compartments. When nodules were switched from air to Ar/O_2, AEC in the plant cytosol significantly increased, whereas AEC of the mitochondrial compartment in this central zone tissue remained stable. AEC values in the bacteroid compartment did not change in the short term but a decline was then observed after 60 min. Wei et al. (2004) also provided a simulation model in which ATP hydrolysis was linked to glutamine synthetase and asparagine synthetase activity required for the assimilation of fixed N, as well as to the translocation of C_4 acids across the symbiosome membrane. The large distances between sites of ATP production and hydrolysis were predicted to generate gradients in ATP, ADP, and AMP within the cytosol of the infected cell (Wei et al. 2004).

3.4 Physiological and Molecular Aspects of Pi Deficiency in Legumes

The Pi starvation response is a multifaceted adaptation that aims to improve Pi acquisition, and the internal recycling of Pi is composed of metabolic, physiological, and morphological components (Salazar-Henao et al. 2016). Pi acquisition by plants from the external environment is influenced by N metabolism. Early studies reported that pretreatments with NH_4^+ and NO_3^- can enhance Pi uptake in *Zea mays* roots (Smith and Jackson 1987). The uptake of NH_4^+ is associated with proton (H^+) release and decrease in the rhizosphere pH, which in turn facilitates Pi solubility and uptake (Zhao et al. 2009), and this might be possibly due to enhanced activity of the plasma membrane H^+-ATPase (Zeng et al. 2012). On the contrary, when NO_3^- is provided, Pi uptake is affected (Watanabe et al. 1998). More recently, Zhu et al. (2016) investigated these issues in two rice (*Oryza sativa* L.) cultivars, "Nipponbare" and "Kasalath," which differ in the ability of reutilizing Pi from their cell wall. This study demonstrated that NH_4^+ positively modulates the pectin content and pectin methylesterase activity in root cell walls under Pi deficiency, resulting in Pi remobilization from the cell wall and increased availability of soluble Pi in roots and shoots. Moreover, increased nitric oxide (NO) levels were detected in rice roots supplied with NH_4^+ as unique N source (Zhu et al. 2016).

A striking and particular fact of P nutrition in legumes is that N_2 fixation actually requires higher amounts of Pi for optimal functioning when compared with non-leguminous plants (Sulieman et al. 2013a; Tesfaye et al. 2007; Chiou and Lin 2011; Sulieman and Tran 2015), suggesting that legumes are more prone to suffer from Pi deficiency. This has been linked to the nodule development and energetic transformation, especially for the synthesis of mitochondria and symbiosome membranes (Mus et al. 2016). Several reports from the literature have unveiled the effects of Pi deficiency in several species from models like *M. truncatula* (Cabeza et al. 2014) to crops such as common bean (Araújo et al. 2008; Olivera et al. 2004) and white lupin (Thuynsma et al. 2014) or the legume pastures white clover (Almeida et al. 2000; Høgh-Jensen et al. 2002), alfalfa (Schulze and Drevon 2005), and stylo (*Stylosanthes* spp.) (Liu et al. 2016). All these legumes show different strategies to enhance Pi acquisition or improve its remobilization under Pi limitation (Fig. 3.2).

3.4.1 Impact of Pi Deficiency in Root and Shoot Traits

Alterations in morphological, anatomical, and architectural root traits are among the best described responses to Pi deprivation in an attempt to maximize Pi uptake from the soil (Negi et al. 2016; Mori et al. 2016; Yuan et al. 2016). The mechanisms by which

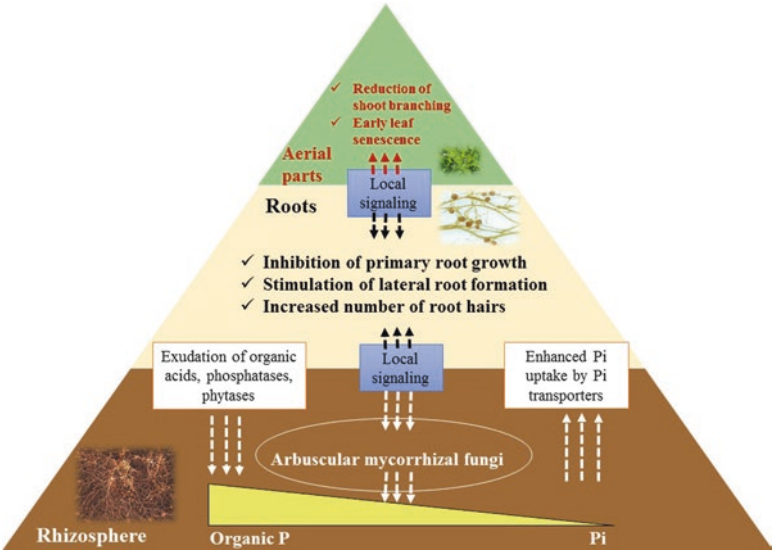

Fig. 3.2 Schematic representation of morphological, physiological, and biochemical responses to phosphate (Pi) deficiency in plants. Aside from the morphological changes in root architecture, Pi uptake is also facilitated by the expression of high-affinity Pi transporters. In addition to this, the exudation of organic acids, phosphatases, and phytases also mobilizes additional Pi resources. At the aerial parts level, high Pi deficiency can be associated with reduced shoot branching and early leaf senescence

roots are able to perceive Pi starvation remain not totally understood. In this context, root caps in the root–soil interface are expected to have an important role in sensing nutrient deficiency and respond to it (Svistoonoff et al. 2007). Coupled to the typical growth arrest of primary root in response to Pi deprivation, another well characterized response in legumes is root branching. Root branching contributes to increase topsoil foraging, maximizing Pi acquisition in the sense that the roots are searching for Pi-enriched patches. Common bean genotypes with increased basal root whorl number showed improved performance under low Pi availability in soils (Miguel et al. 2013).

Cluster root (CR) formation is a desirable trait to improve Pi acquisition when global Pi resources became scarce, representing a common adaptive strategy of legumes. Coupled to this, the colonization of mycorrhizae leads to higher Pi use efficiency as well as increased total N content in plants (Schulze et al. 2006; Sprent and James 2007). In a study conducted on *L. albus* and *L. pilosus*, Wang et al. (2015) showed that the CR percentage was strongly and negatively correlated with plant Pi status in species-specific manner. In *L. albus*, low Pi status at the shoot level is reflected in the amount of sucrose translocated to the roots (Wang et al. 2015). The main results of this study indicated that sucrose appears to have an important role in CR formation simultaneously acting both as a C source and a long-distance signal reporting the shoot Pi status to the root system. Interestingly, *L. angustifolius* does not form CR under Pi starvation, but it developed a strategy for achieving critical Pi uptake in low Pi environments by altering shoot growth and root architecture and secreting carboxylates from roots (Chen et al. 2013). On the other side, Pi supplementation seems to inhibit lupin's ability to form CRs, and also this response is dependent on the studied species (Abdolzadeh et al. 2010). Such studies bring evidence of genotype-dependent performances under low Pi availability suggesting that a determinant genetic control of this trait exists.

The response of two legume tree species, namely, *Virgilia divaricata* and *V. oroboides*, was investigated during low Pi conditions (Magadlela et al. 2014). While the growth of *V. divaricata* was not affected by Pi deficiency, the same was not observed in *V. oroboides*, which showed lower performance under such conditions. The authors were able to link the poor performance of *V. oroboides* to a lower Pi uptake, as well as a differential allocation of N and C nutrients, which were directed more to the aerial parts in the detriment of the roots and nodules, resulting in a decline of N nutrition, growth respiration, and overall photosynthetic costs. On the other hand, *V. divaricata* increased the Pi allocation to nodules and benefited N nutrition, while maintaining its photosynthetic costs. This is an example of different adaptation strategies to Pi starvation through the alteration of biomass allocation (Magadlela et al. 2014). Consequently, the selection of genotypes with better performance in low Pi soils could lead to sustainable crop production under Pi-limited environments.

The coordination of shoot and root responses to Pi deprivation involves trafficking of systemic signals, such as sugar, microRNAs (miRNAs), and hormones, through the vasculature (Lin et al. 2014). Nevertheless, the morphological responses of roots are thought to be chiefly controlled by the local concentration of Pi (Ticconi et al. 2004). However, it may be assumed that local and systemic signals are integrated and that long-distance signals might also influence some, if not all, root morphological responses (Salazar-Henao et al. 2016).

3.4.2 *Impact of Pi Deficiency in SNF*

In low Pi soils, legumes depending on N_2 fixation respond positively to Pi fertilization and show increased N content in shoots and roots (Richardson and Simpson 2011). Pi-limited grain legumes can provide normal SNF rates for as long as 3 weeks, taking advantage only of seed P reserves (Schulze et al. 2006). Due to the high Pi requirement of N_2-fixing nodules, shortage of Pi has a particularly large impact on legumes relying on SNF. Pi deficiency seems to affect nodulation and N_2 fixation to a greater extent than the aboveground plant growth (Almeida et al. 2000; Olivera et al. 2004). Low Pi availability resulted in a decline of N_2 fixation, while a sudden removal of Pi from the medium totally blocked the nodule growth and changed the relative growth rate of both shoots and roots in white clover (Høgh-Jensen et al. 2002, Almeida et al. 2000). On the other side, higher Pi applications significantly inhibited nodule function also in soybean and *M. truncatula* (Qin et al. 2012; Sulieman et al. 2013a), suggesting that a tight regulation of the Pi pools is needed to ensure efficient legume growth and development. Hence, the abovementioned studies provided strong evidence that elevated Pi concentrations in nodules are essential for N_2 fixation during Pi deficiency. Almeida et al. (2000) proposed that in Pi-deficient plants, the impairment of SNF could be a result of several factors among which (1) the impairment of the host plant growth, (2) the growth and functioning of the nodule, and (3) the interaction among these factors.

Pi allocation to nodules plays a key role to assure essential N nutrition (Magadlela et al. 2014). Other species that increase the allocation of Pi in the nodule upon Pi starvation include *M. truncatula*, *M. sativa*, and *L. luteus* (Sulieman et al. 2013b; Kleinert et al. 2014). Increased Pi uptake is mediated by the high affinity of Pi transporters in the nodules (Qin et al. 2012). Research has shown that, under normal condition, around 20% of plant total P is allocated to the nodule (Kouas et al. 2005), while considerably higher levels of Pi are required under low Pi conditions (Thuynsma et al. 2014). An upgrading of N_2 fixation ability in nodules can compensate for the reduction in the number of nodules observed under low Pi conditions (Almeida et al. 2000; Qin et al. 2012; Schulze et al. 2006). Alfalfa nodules grown under the Pi-deficient conditions are smaller but have had a higher O_2 uptake per N_2 reduced, coinciding with increased nodule permeability and conductance (Schulze and Drevon 2005). Nodule permeability to O_2 through the regulation of O_2 diffusion represents a key factor for the appropriate functioning of the nitrogenase enzyme (Schulze 2004). Thus, increased O_2 uptake appears to be a mechanism to adjust nodule metabolism to Pi deficiency in indeterminate N_2-fixing nodules such as in alfalfa, as previously shown for determinate (common bean and soybean) nodule forms.

The effects of long-term Pi deficiency and subsequent recovery on bacteroid mass/number per unit nodule mass and the energy status of soybean nodules were investigated by Sa and Israel (1991). The continuous Pi deficiency significantly decreased the whole-plant dry mass, Pi and N content, and specific nitrogenase activity, as compared to the Pi-sufficient control. The whole nodules of Pi-deficient controls contained 70–75% lower ATP concentrations than nodules of Pi-sufficient

controls. The energy charge and ATP concentrations in the bacteroid fraction of nodules were not significantly affected by Pi treatment. However, ATP and total adenylate concentrations and AEC values in the plant cell fraction of nodules were significantly decreased to 91%, 62%, and 50%, respectively, by the Pi deficiency. The specific activity of nodules (N_{fixed} per unit nodule biomass), AEC, and ATP concentration in the plant cell fraction increased to the levels of non-stressed controls after alleviation of external Pi limitation. The bacteroid mass per unit nodule mass and bacteroid N concentration did not increase to the level of non-stressed controls until 7 days after alleviation of external Pi limitation.

3.4.3 Pi Deficiency Induces the Excretion of Organic Acids and Pi-Releasing Enzymes

Legume genotypes with contrasting utilization of P for SNF are interesting systems to study the molecular mechanisms underlying N_2 fixation impairment under Pi deficiency. Many studies have focused on the role that acidic phosphatases play in intra- and/or extracellular Pi scavenging and recycling during Pi starvation (for review, see Plaxton and Tran 2011). Plants grown under limited Pi supply can increase the activity of phosphatases in roots to hydrolyze organic P compounds in the soil, thus improving plant Pi acquisition (Araújo et al. 2008; Plaxton and Tran 2011). However, little information is available about the role of these enzymes for internal plant metabolism at limited Pi conditions.

Phytate is one of the major organic forms of P in the soil but this P form is unavailable to plants unless mineralization takes place. Phytases are capable of hydrolyzing phytate to a series of lower phosphate esters of myoinositol and phosphate contributing for increasing the rhizospheric Pi contents for root uptake. It is argued that phytase activity in nodules would contribute to the adaptation of the rhizobia–legume symbiosis to low Pi environments as seen in *P. vulgaris* nodules (Araújo et al. 2008). In this context, Lazali et al. (2013) investigated the expression profiles of phytase genes in two recombinant inbred lines of *P. vulgaris* characterized by contrasting N_2 fixation ability under Pi deficiency, inoculated with *R. tropici* CIAT 899 strain and grown under low or high Pi supply. They detected accumulation of phytase transcripts in the nodule cortex and infected zone of both lines, but phytase gene expression was significantly enhanced in the *P. vulgaris* line with high N_2 fixation ability in the absence of Pi. This finding was well correlated with an increase in phytase (33%) and phosphatase (49%) activities and enhanced SNF efficiency (34%). The authors underlined a possible role of phytase activity within nodules in helping adaptation of the rhizobia–legume symbiosis to low Pi environments.

Plant acid phosphatases (APases) catalyze the hydrolysis of Pi from a group of phosphomonoesters and anhydrides with optimal activity pH below 7.0 (Duff et al. 1994). Increased APase activities and exudation is considered an efficient strategy for plants to mobilize and utilize organic P. This strategy was recently associated with the performance of stylo, a dominant pasture legume widely grown in tropical

and subtropical areas where acid soils cause Pi deficiency (Chandra 2009). The utilization of extracellular deoxyribonucleotide triphosphate (dNTP) and the underlying physiological and molecular mechanisms were examined for two stylo genotypes with contrasting Pi efficiency (Liu et al. 2016). The results showed that the Pi-efficient genotype (TPRC2001-1) was superior to the Pi-inefficient genotype (Fine-stem) when using dNTP as the sole Pi source. Moreover, Pi starvation can increase root-associated APase activities in stylo, which might be caused by enhanced expression levels of the purple acid phosphatases (PAP) in roots of both stylo genotypes. Furthermore, the higher expression levels of *SgPAP7* and *SgPAP10* in TPRC2001-1 roots contribute to its higher root-associated APase activities and thus facilitate greater utilization of extracellular dNTP by TPRC2001-1 than by Fine-stem. Other studies have also highlighted that some members of PAP gene family have a potential role in plants' response to symbiosis with rhizobia or arbuscular mycorrhizal fungi under Pi-limited conditions (Li et al. 2012).

3.4.4 Novel Clues on the Impact of Pi Deficiency in Legumes

Recently, Sulieman and Tran (2015) provided a comprehensive review of the complex mechanisms used by legumes to control Pi homeostasis in nodules, when Pi levels decrease. One of these strategies allows maintaining higher Pi concentration in nodules compared to other organs. Indeed, up to 20% of total plant P is found in the nodules, and under limiting Pi conditions, the Pi levels in nodules are even enhanced. A significant example is provided by Thuynsma et al. (2014) who showed that *L. albus* responds to Pi shortage by increasing CR production, a highly expensive metabolic process, to improve Pi supply to nodules. Expansion of CRs results in larger root surface area and exudation of organic acids which facilitate Pi absorption (Thuynsma et al. 2014). Increasing Pi acquisition also involves uptake mediated by high-affinity Pi transporters (Jia et al. 2011; Qin et al. 2012; Liu et al. 2014a), while root colonization by mycorrhizae results in the efficient translocation of high Pi amounts into the host plant (Wang et al. 2011). In addition, induction of APase is triggered by Pi starvation as a universal strategy in higher plants (Araújo et al. 2008), while addition of N to soil with low Pi content increases as well the activity of extracellular phosphatases (Tredeser and Vitousek 2001).

In most cases, the improved response toward Pi starvation results from a concerted action of multiple strategies, as seen in white lupin. The efficient C use and N assimilation process together with enhanced nodulation in CR zones and elevated organic acid production in nodules makes this species highly adaptable to Pi deficiency (Schulze et al. 2006). A complex cross talk of regulatory processes and molecular player, acting both locally or at the whole-plant level, assures Pi homeostasis in nodules under Pi starvation. Many genes responsible for the regulation of Pi homeostasis have been identified in *Arabidopsis* (Lin et al. 2009), while in legume species, such studies are relatively fewer, though a number of genes associated with this process were also identified in several legumes (Table 3.1). On the

Table 3.1 Genes involved in the regulation of phosphate (Pi) homeostasis in legumes

Gene	Accession No.	Description	References
PvPHR1	TC2883	Pi response 1; MYB transcription factor which activates a subset of Pi starvation-induced genes	Hernández et al. (2007)
CaPHT1;4	LOC101515444	High-affinity Pi transporter 1;4, involved in Pi acquisition and mobilization	Nasr Esfahani et al. (2016)
PvSIZ1	TC2445	SUMO E3 ligase; facilitates the sumoylation of PHR1 and regulates the expression of several Pi starvation-responsive genes	Valdés-López and Hernández (2008)
CaPHO1	LOC101494472	Phosphatase 1; involved in Pi loading into the xylem	Nasr Esfahani et al. (2016)
PvPHO2	TC1095	Ubiquitin E2 conjugase; regulates Pi uptake, allocation, and remobilization	Valdés-López and Hernández (2008)
LjPHO3	Lj6g3v2006830	Sucrose/H^+ symporter which regulates Pi starvation responses	Qin et al. (2016)
PvSPX1 *PvSPX2* *PvSPX3*	EF191350 GU189405 GU189406	SPX domain-containing proteins which regulate the expression of several Pi starvation-responsive genes involved in Pi uptake, allocation, and remobilization	Yao et al. (2014)
LjPT4 *MtP4*	chr1.CM2121.10.r2.a AY116211	Pi transporter 4; accumulates in specific domains of the periarbuscular membrane and give plants access to the Pi absorbed from the extraradical mycelium, via the H^+ energy gradient produced by H^+-ATPases	Volpe et al. (2016)
PvTIFY	TC1670	Transcription factor involved in the regulation of Pi deficiency	Aparicio-Fabre et al. (2013)

Accession numbers are retrieved from DFCI/common bean gene index, NCBI, and Phytozome
Pv Phaseolus vulgaris, *Ca Cicer arietinum*, *Lj Lotus japonicus*, *Mt Medicago truncatula*

other hand, the development of post-genome methodologies, such as global analysis of coding and noncoding transcriptomes, proteomes, and metabolomes integrated in solid bioinformatics platforms, has noticeably improved our knowledge and holistic understanding of various plant functions, including the response to environmental stresses (Mochida and Shinozaki 2011). In this subsection, we describe some studies in which the use of omics considerably increased our knowledge on the response of legume N_2 fixation under Pi deficiency.

BNF and Pi concentration in different organs of *M. truncatula* during a whole-plant Pi depletion experiment was investigated by Cabeza et al. (2014). N_2 fixation activity per plant diverged from that of fully nourished plants beginning at day 5 of the Pi depletion process, since fewer nodules were formed, while the activity of the existing nodules was maintained for as long as 2 weeks into Pi depletion. RNA-seq analysis revealed several mechanisms underlying nodule adaptation to Pi deprivation. Among the 1140 differentially expressed genes, several genes for Pi remobilization

from organic structures and nodule malate formation were up-regulated, while genes involved in fermentation were downregulated. During Pi depletion, nodule metabolism is shifted for acclimating nodules to low Pi availability before the tissue itself is depleted. Among those, reduced activity of fermentation pathways, increased CO_2 fixation, and upregulation of phosphatases contribute to mobilize Pi from organic structures. This enhanced turnover of the limited Pi quantities available allowed plants to uphold the high N_2 fixation rates of existing nodules well into the Pi depletion process.

The adaptation of *Mesorhizobium*–chickpea to Pi limitation was deeply investigated by Nasr Esfahani et al. (2016). Chickpea (*C. arietinum* L. cultivar ILC482) was inoculated with two symbionts (*Mc*CP-31 and *Mm*SWRI9) of the genus *Mesorhizobium*. ILC482 is a high-yielding elite Kabuli variety with relatively high adaptability to water scarcity (Rozrokh et al. 2012). *Mc*CP-31-inoculated plants showed bigger nodule dry mass and enhanced SNF than the *Mm*SWRI9 ones. Metabolic profiling revealed that differential responses in C and N metabolism-related metabolites were observed between *Mm*SWRI9- and *Mc*CP-31-inoculated plants in response to Pi deficiency. Pi deficiency significantly increased the levels of amino acid as asparagine, homoserine, isoleucine, 3-cyano-L-alanine, methionine, lysine, tyrosine, and phenylalanine in the *Mm*SWRI9-induced nodules. On the other side, the *Mc*CP-31-induced nodules showed significantly increased levels of glutamine, GABA, L-alanine, 3-cyano-L-alanine, aspartate, glutamate, threonine, lysine, and 5-oxoproline. The total level of identified sugars was increased by 68.8% in *Mc*CP-31-induced nodules, whereas the level of total detected sugars in *Mm*SWRI9-induced nodules remained unchanged under Pi starvation. When analyzing the differences in terms of organic acids, the levels of malate, glycolate, malonate, and isocitrate were decreased in the *Mm*SWRI9-nodulated roots, whereas the levels of α-ketoglutarate, malate, succinate, citramalate, galactonate, threonate, itaconate, and threonic acid-1,4-lactone displayed significant accumulation in the *Mc*CP-31-nodulated roots under Pi-deficient conditions. These metabolomic data results unshaded the complex cross talk among numerous signaling pathways in the regulation of *Mesorhizobium*–chickpea adaptation to Pi limitation. Nevertheless, it cannot be disregarded that other master players, such as miRNAs, might also contribute to regulation of SNF capacity in chickpea under low Pi availability (Nasr Esfahani et al. 2016).

MiRNA-mediated regulation of gene expression plays essential roles in almost all biological processes in plants including the modulation of legume response to Pi starvation (Jones-Rhoades et al. 2006; Zeng et al. 2010; Branscheid et al. 2010; Peláez et al. 2012; Ramírez et al. 2013). MiRNAs, as a class of small noncoding (20–24 nucleotides) RNAs, act at the posttranscriptional level leading to gene silencing through mRNA cleavage and translation repression based on sequence complementation (Bartel 2004). An miRNA macroarray was used to detect miRNAs in the leaves, roots, and nodules of control and Pi-deprived common bean plants (Valdés-López et al. 2010). In this study, several miRNAs that have never been reported as Pi stress responsive in other plant species (e.g., miR157, miR160, miR165/miR166, miR169, miR393, pvu-miR2118) were differentially regulated under Pi deficiency in one or more common bean organs. MiR172, which targets the

transcription factor APETALA2-related (AP2), was the only miRNA detected exclusively in common bean nodules, and its expression was slightly increased under Pi deprivation. MiR172 and AP2 have been previously associated with Pi starvation in roots and nodules of common bean (Hernández et al. 2009), as well as *M. truncatula* (Lelandais-Briere et al. 2009). Although miR172-mediated improvement of SNF in common bean *R. etli* was functionally validated (Nova-Franco et al. 2015), its role in Pi-mediated responses needs to be further elucidated.

The identification of genotypes with improved performances under Pi-limited environments opens new possibilities to understand which are the molecular players underlying such response. In an attempt to identify proteins responsive to Pi deficiency in the Pi-efficient soybean cultivar BX10, Sha et al. (2016) conducted a proteomic study, in which the protein accumulation profiles of shoots and roots were studied under Pi-deficient and Pi-sufficient conditions. Among the 88 proteins identified, 61 were responsive to Pi deficiency, most of them being described for the first in response to Pi starvation. Interestingly, several proteins associated with energy metabolism (e.g., vacuolar ATPase subunit B, ATP synthase CF1 α subunit) and cell cycle and division (e.g., patellin, actin-depolymerizing factor 1) were accumulated under Pi deficiency, indicating possible mechanisms activated to ensure of Pi homeostasis.

3.5 Conclusions

Legumes are well recognized for their nutritional and health benefits as well as for their impact in the sustainability of agricultural systems, due to their ability to form symbiosis with N_2-fixing microorganisms. Nevertheless, legume N_2 fixation has a high-energy cost, and Pi deficiency strongly hampers legume production, especially in low Pi soils typical of most of the tropical regions. The identification of legume genotypes with contrasting utilization of Pi for SNF, resulting from running breeding programs, has provided excellent models to study the molecular mechanisms underlying N_2 fixation impairment under Pi deficiency.

Legumes have evolved a complexity of mechanisms to cope with Pi limitation, which relies on strategies that aim to enhance Pi acquisition or improve its remobilization under Pi limitation. Roots have been the privileged studied organ, not only because they are the first organ to sense Pi shortage but also because of their role in SNF. The maintenance of SNF seems to be a key aspect to assure legume productivity in low Pi environments. To ensure proper SNF under Pi shortage, several strategies to maintain higher Pi concentrations in nodules were described. They include morphological and architectural changes in root and shoot traits, alteration in overall C and N whole-plant metabolism, and exudation of organic acids or Pi-releasing enzymes coupled to an enhanced Pi uptake system, mediated by specific Pi transporters. Many genes, enzymes, and miRNAs are involved in enhanced SNF under Pi-limited environments through the use of simple molecular biology and functional biology approaches. Nevertheless, the recent emergence of "holistic" omics-based studies will certainly make significant advances beyond the current state of the art.

Although considerable research efforts have been carried out to understand the molecular and regulatory mechanisms responsible for SNF under Pi limitation, the knowledge on this topic is still scarce. This also includes studies looking for alternative and optimized legume–rhizobium associations or the development of improved strains. In addition to this, in natural ecosystems, the interaction with other environmental factors that affect soil properties, such as water deficit or soil acidity, has to be considered. Previous studies have shown that all these factors impact on O_2, C, and N availability, crucial for N_2 fixation homeostasis. More studies exploring the combination of abiotic stresses, focused on enhanced SNF, should be integral part of legume improvement breeding programs. They are expected to address efficiently the current and future demands of modern agriculture and food production presently exacerbated by the variability in global climate change.

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Chapter 4
Metabolism and Transport of Carbon in Legume Nodules Under Phosphorus Deficiency

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Abstract Phosphorus (P) is an essential element for plant growth but is largely unavailable for root uptake due to the formation of insoluble complexes. Therefore, P deficiency is a wide-spread agricultural dilemma. However, in addition to P, nitrogen (N) and carbon (C) metabolisms are intricately linked to plant physiological events and are major determinants in the plant and nodule responses to P deficiency. These responses can be measured in terms of growth, photosynthesis, and respiration. It has been shown that during P stress, plant growth also affects the C requirement of biological N_2 fixation (BNF), and this has been proposed as a means of BNF regulation. Furthermore, the sink effect of nodules, at various levels of developmental and functional stages, has been observed via alterations in photosynthesis and respiration. The photosynthetic and respiratory C costs of BNF and nodule growth make considerable contributions to the overall C budget of the symbiosis. However, the use of overall C budget may mask the separate allocation of C to above and belowground organs during P deficiency. Moreover, the division of respiratory energy toward new tissue growth and nutrient acquisition may not be evident in the overall C budget. In this chapter, we review the recent contributions made in the arena of C metabolism of nodules during P stress and will aim to gain a better understanding of the underlying physiological and transcriptional events which give rise to changed in the C budget and allocation.

Keywords Alternative pathways • P stress • Respiratory costs • Carbon budget

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4.1 Introduction

The ability of legumes to form symbiotic relationships with both rhizobia (for N acquisition) and mycorrhizal soil fungi (for P acquisition) has allowed these plants to grow in nutrient poor soils worldwide (Kaschuk et al. 2009). Biological dinitrogen fixation (BNF) is carried out exclusively by the bacterial nitrogenase enzyme, which breaks the stable triple bond present in atmospheric dinitrogen (N_2) to finally produce ammonia (NH_3) (Hungria et al. 2005; Kaschuk et al. 2009). Ammonium (NH_4^+) is exported to the plant in the form of amides (glutamine or asparagine) or ureides (allantoin or allantoic acid) to finally be incorporated into other N-containing molecules (Coruzzi 2003; White et al. 2007). Rhizobial BNF can supply sufficient N for legume growth and maintenance of N-containing photosynthetic components even during nutrient limiting conditions (Warren and Adams 2006).

4.2 Photosynthesis and CO_2 Fixation

Rhizobial symbiosis is well known to affect whole plant photosynthesis by improving plant nutrition and increasing rates of photosynthesis per leaf area unit (Kaschuk et al. 2009). Assimilates produced during photosynthesis is partitioned to the various sink organs via phloem transport, with a portion of these assimilates dedicated for rhizobial growth, maintenance, and metabolic function (Yin and Van Laar 2005; Kaschuk et al. 2012). The assimilate pool must further also supply carbon (C) to roots, shoots, and reproductive organs, if present (Yin and Van Laar 2005). The reliance of the plant on BNF will lead to increased C costs associated with bacterial nitrogenase activity, biomass of nodules, protein turnover, and final N assimilation into various amino acids and intermediates (Kaschuk et al. 2009). These extra costs will lead to increased phloem loading and photosynthetic rates. This rise is used to compensate for nodule C costs at the source, in a nutrient-independent manner, by increasing photosynthetic rates. Symbiosis can thus act as a C sink; by stimulating photosynthesis rates, more C is fixed per unit nutrient N, which results in higher photosynthetic nutrient use efficiency (Kaschuk et al. 2012).

The increased N mass fraction in leaves may also stimulate photosynthesis rates in a nutrient-dependent manner (Kaschuk et al. 2009). N is essential for the synthesis and maintenance of key photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll. The increase in leaf N supply should thus stimulate both Rubisco activity and electron transport (ET) rates. The rate of photosynthesis may however not increase above saturating leaf N conditions, with any further increases leading to decreased photosynthesis rates (Yin and Van Laar 2005). The improved nutritional state that the symbiosis provides is critical to stimulate photosynthetic rates and increased plant growth. There is however a C cost involved in maintaining the activity of this symbiosis (Kaschuk et al. 2012). It has been shown for various legume species, including both ureide (soybean, cowpea) and amide exporters (broad bean, lupins), that rhizobial symbiosis can consume between 4% and 16% of recently fixed

photosynthetic C (Mortimer et al. 2009; Larimer et al. 2010; Thuynsma et al. 2014). This C is almost exclusively used during BNF, with a theoretical consumption of between 3.3 and 6.6 g C.g N^{-1} (both plant and bacterial species dependent). This is more energetically expensive when compared to N uptake, which generally does not exceed 2.5 g C.g N^{-1} (Larimer et al. 2010; Udvardi and Poole 2013). The efficiency of nutrient supply through the symbiosis is thus crucial, and suboptimal efficiency will lead to symbiotic plants being outperformed by nonsymbiotic plants.

Photosynthesis is limited by various environmental factors and intrinsic biochemical processes. The availability of water, nutrients (N and P), cofactors, and leaf temperature all affect photosynthesis rates. Furthermore the activity of Rubisco, regeneration of ribulose-1,5-bisphosphate (RuBP) (Li et al. 2009; Kaschuk et al. 2012;), ET rates, ATP and NADPH generation, and triose-phosphate utilization (TPU) limit photosynthesis biochemically (Lambers et al. 2006; Makoi et al. 2010). These biochemical factors are further affected by specific environmental factors, Rubisco activity being limited by atmospheric [CO_2], the availability of light affecting ET rates, and TPU being limited by C sink strength (Araya et al. 2006; Kaschuk et al. 2012). Generation of energy supply (via ATP and NADPH) and RuBP regeneration is limited by the availability of P. Indeed, P availability further also affects the sucrose-starch ratio in the chloroplast, with starch accumulation in leaves usually being an indicator of P deficiency (de Groot et al. 2001, 2003). Rhizobial symbiosis could thus act to remove the limitation of both Rubisco activity and ET rates through increased mass N fraction in leaves. The C costs incurred from this symbiosis could additionally alleviate the TPU limitation on photosynthesis (Kaschuk et al. 2009). P deficiency has also been shown to affect photosynthesis rates by limiting carboxylation capacity, while N deficiency limits the rate of light harvesting and ET rate (de Groot et al. 2003). During short-term P deficiency, photosynthesis is not limited by ATP availability or activation of Rubisco. Increased sink strength rather stimulates TPU export, recycling Pi and activating photosynthetic regulatory enzymes (Rychter and Rao 2005). During long-term P deficiency, photosynthesis rates decrease due to limited activation of the Calvin cycle, either due to lack of ATP and/or C skeleton intermediates/substrates. This leads to low carbohydrate production in the leaves and a subsequent reduction in photoassimilate loading into the phloem (de Groot et al. 2001).

Low-P availability can also inhibit photosynthesis rates via feedback regulation (Hermans et al. 2006; Hernandez et al. 2007; Hammond and White 2008). The rate of end-product synthesis in leaves, including sugar, starch, and amino acids, largely determines the rate at which Pi is cycled back to photosynthesis (Paul and Pellny 2003). Photophosphorylation and chloroplast ATPase activity are also sensitive to low cellular Pi concentrations, and their activity may become inhibited as a result (Wissuwa et al. 2005). This can be offset by increasing the demand for sucrose by shading/increased sink strength, which increases recycling of Pi back to the chloroplast (Wissuwa et al. 2005). The increased expression of sucrose phosphate synthase, cytosolic fructose-1-bisphosphatase, and UDP glucose pyrophosphorylase during low-Pi conditions further help to maintain the flow of C to sucrose (Hammond and White 2008). Hurry et al. (2000) demonstrated altered RuBP regeneration and increased expression of enzymes in the Calvin cycle, including Rubisco, during

low-temperature conditions, which inhibit photosynthesis, in a manner similar to low cellular Pi availability. These adaptations promote the synthesis and partitioning of C to sucrose and increase Pi liberation and recycling. Low availability of cellular Pi could thus mediate long-term adaptive control of photosynthesis via short-term feedback regulation (Paul and Pellny 2003).

4.3 Respiration

The principal substrates of plant respiration are sucrose and starch. These substrates are oxidized via glycolysis, the TCA cycle and the mitochondrial electron transport chain (ETC), with a large proportion of C allocated to biosynthesis of intermediates (Plaxton and Podestá 2006). During P deficiency, these essential energy and biosynthetic processes must still continue. Plants exhibit great metabolic plasticity in response to P deficiency by utilizing alternative glycolytic and mitochondrial enzymes, reducing metabolic rates, and utilizing alternative C sources, thus recycling and conserving valuable cellular Pi resources (Vance et al. 2003; Vance 2008).

With the onset of severe Pi deficiency, pyrophosphate (PPi) can be used as an alternative energy donor and bypass the ATP-dependent breakdown of sucrose (Plaxton and Podestá 2006). The constant production of PPi via anabolic synthesis of macromolecules leads to constant PPi pools, functioning to protect the limited ATP pool during P deficiency (Plaxton and Podestá 2006). An additional glycolytic bypass reaction involves non-phosphorylating NADP-dependent glyceraldehyde-3P dehydrogenase (NADP-G3PDH), which circumvents the use of both ATP and NADH during glycolysis (Vance 2008; Balemi and Negisho 2012). The final metabolic bypass involves the combined action of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and NAD-malic enzyme (NAD-ME) (Plaxton 2004; Le Roux et al. 2006). During P deficiency, the activity of pyruvate kinase (PKc) is severely limited due to the large (up to 80%) decline in intracellular ATP and ADP (Plaxton 2004; Le Roux et al. 2006). The action of PEPC, MDH, and ME bypasses PKc, producing malate and releasing Pi. The malate is transported into the mitochondria and used for acetyl-CoA production, maintaining the flux of C through glycolysis and the TCA cycle during P deficiency (Vance et al. 2003).

Phosphate supply is also critical to the maintenance of mitochondrial respiration and the ATP-ADP ratio (Vance et al. 2003). The decrease in ADP levels inhibits respiration by adenylate control (Vanlerberghe 2013). Two alternative pathways of respiration have been identified that bypass Pi-consuming steps in the mitochondrial ETC: (1) the rotenone-insensitive NAD(P)H dehydrogenase (ND2) and (2) alternative/cyanide-resistant oxidase (AOX) ETC (Vance 2008). The electrons from the TCA cycle are instead accepted by a rotenone-insensitive NAD(P)H dehydrogenase, which reduces NAD(P)H to $NAD(P)^+$, reducing the final ATP yield due to decreased H^+ pumping into the intermembrane space (Rasmusson et al. 2008). Three different subfamilies have beein identified in plant NDA, NDB, and NDC, located either on the inside or outside of the inner mitochondrial membrane

(van Dongen et al. 2011). Changes in expression of ND2 are closely followed by changes in AOX expression. The function of this co-regulation could be to cope with the large metabolic changes during stress conditions (Rasmusson et al. 2009).

The AOX enzyme catalyzes the oxidation of ubiquinol and the reduction of oxygen to water (Vance et al. 2003; Plaxton and Podestá 2006). Expression in tobacco cells during P deficiency leads to increased expression of reactive oxygen species (ROS), reduced rates of respiration, and C accumulation (Vanlerberghe 2013). Furthermore, AOX function is critical in dampening ROS generation during high respiration rates, when P is resupplied in P-deficient conditions (Vanlerberghe 2013). AOX has also been implicated in oxygen homeostasis; when oxygen levels are high, AOX can remove excess oxygen and thus also minimize ROS production in the mitochondria (van Dongen et al. 2011). This bypass reaction is another mechanism by which plant cells can modulate their metabolism in response to P deficiency, granting a great deal of metabolic flexibility during P-limiting conditions (Vanlerberghe 2013).

Although these non-phosphorylating pathways reduce the requirement for Pi during glycolysis, they do not contribute to the production of the proton motive force (PMF), required for ATP synthesis (van Dongen et al. 2011). This deficiency is overcome by uncoupling proteins (UCP) embedded in the inner mitochondrial membrane, which increases the transfer of protons into the mitochondria (van Dongen et al. 2011). UCPs have also been shown to be involved in amino acid and lipid synthesis.

This could be due to increasing oxidation of reducing equivalents leading to increased catabolism through both glycolysis and the TCA cycle. In this way C can be used to synthesize new organic molecules independent of the cell's energy demand (van Dongen et al. 2011).

Organic acid exudation from plant roots is well documented in many plant species under normal growth conditions. (Vance 2008). Organic root exudates are functionally diverse and affect rhizosphere composition, biochemistry, and soil microbial populations. These changes can directly affect plant growth, plant-microbe signaling, and nutrient acquisition from the soil (Shane et al. 2004, 2006; Vance 2008; George et al. 2011). Increased organic acid exudation will also lead to loss of C. It is estimated that up to 65% of daily fixed C is allocated to organic acid exudation (Schulze et al. 2006). This C is supplied by both, photosynthate originating from leaves and dark CO_2 fixation in the root tissue. The rate of CO_2 fixation in roots is comparable to rates in nodules, with photosynthesis supplying 65% and dark fixation providing 35% of anapleurotic C (Johnson et al. 1994). Increased CO_2 fixation is also accompanied by increased enzyme activity and mRNA expression of PEPC, MDH, and citrate synthase (Hammond and White 2008).

4.4 C and N Budget

C metabolism is regulated to efficiently utilize both Pi and N during limited availability (Paul and Pellny 2003). The production of photosynthetic machinery is a large N investment and provides the necessary C skeletons required for amino acid synthesis.

The C supplied by photosynthesis can also regulate the expression of enzymes involved in photosynthesis (Paul and Pellny 2003). When C accumulates in the leaves, storage and utilization enzymes are activated, while photosynthesis is repressed. Increased demand for C can have the opposite effect and will stimulate photosynthesis rates (Bihimidine et al. 2013). The metabolism of C thus acts as a self-regulatory mechanism to ensure efficient investment of both N and Pi during limited availability.

4.4.1 C and N Interaction Between Leaves and Root Systems

In leaves, C and N metabolisms converge at the supply of 3-PGA from photosynthesis and the Calvin cycle. It serves as an important precursor molecule to the amino acid precursors PEP, pyruvate, oxaloacetic acid (OAA), and 2-oxoglutarate (α-ketoglutaric acid). Primary regulation of glycolysis occurs at the consumption of PEP by both PK and PEPC, while secondary regulation occurs at the conversion of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate via PPK and PPi-dependent PFK. This points to bottom-up regulation of glycolysis, with PK and PEPC regulation integrating N assimilation and glycolysis (Plaxton and Podesta 2006; Balemi and Negisho 2012). The actions of PEPC and PK play an important role in PEP partitioning for the generation of OAA, needed for aspartate synthesis and 2-oxoglutarate, a substrate for the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle. The coordination of C and N metabolism must thus be tightly regulated and occurs through long distance sensing of NO_3^-/NH_4^+ (Zheng 2009).

While N is taken up by the roots and transported to the leaf, C assimilation and metabolism mainly occur in the leaf. Thus, photosynthesis and the root system must coordinate and regulate C and N metabolism (Zheng 2009). This is most evident when photosynthesis rates recover in plants deficient in N resupplied with N. Similarly when C supply is increased, N uptake and assimilation is increased. The assimilation of N is not only regulated by C but also by the relative abundance of cellular N pools (Eveland and Jackson 2011; Zhu et al. 2011). Plant glutamate receptors have been implicated in sensing changes in glutamate and the C/N ratio. The C-induced expression of glutamine synthase and C-induced repression of asparagine synthetase are both relieved by the addition of organic N (Kusano et al. 2011). Both inorganic and organic N may thus serve as signals to report on N status, leading to control and regulation of N uptake and assimilation, as well as regulation of C metabolism and assimilation. Similarly high concentrations of rhizosphere N can lead to decreased nodule viability and nitrogenase activity (Schulze 2004).

This feedback regulation involves N compounds transported in the phloem, to the nodules, which regulate N_2 fixation (Sulieman et al. 2010). The plants own N sink strength thus regulates N_2 fixation rates by modulating nitrogenase activity through sensing of the C-N ratio via phloem translocatable N-containing compounds. This mechanism of N signaling has been observed in nodulated *Trifolium repens*, *Pisum sativum*, *Vicia faba*, *Phaseolus vulgaris*, and *Medicago truncatula* (Almeida et al. 2000; Schulze 2003; Fischinger et al. 2006; Sulieman et al. 2010).

Sulieman et al. (2010) identified asparagine as the shoot derived N signal in *M. truncatula*, while Fischinger et al. (2006) suggested glutamate as the signal molecule in *P. vulgaris*. Other candidate signaling compounds include glutamine, ureides, γ-aminobutyric acid, proline, and polyamines (Sulieman et al. 2014). Sulieman et al. (2014) showed that the whole-plant N-feedback mechanism in *M. truncatula*, grown under P deficiency or with combined N (NH_4NO_3), is closely linked to nodule C metabolism. This supports the hypothesis that nodule amino acid content regulates nitrogenase activity, possibly by downregulating C supply to the nodules. The reduced C supply, in the form of reduced organic acid supply to the nodules, could thus be another form of coordinated metabolic regulation to assure the demand for C and supply of N are tightly coordinated (Sulieman et al. 2014).

4.4.2 Photosynthate Assimilation into Amino Acids

The N_2-fixing enzymes are only present only in the symbiotic bacteria and not in the plant itself (Valentine et al. 2011). Through the action of the nitrogenase enzyme, NH_3 is produced in the nodule and quickly diffuses out of the alkaline protoplasm of the bacteroid into the acidic peribacteroid space, becoming protonated and forming NH_4^+ (Vessey et al. 2005). Through the action of a specific ion channel for monovalent cations, NH_4^+ is transported through the peribacteroid membrane to the cytoplasm of the infected plant cell (Lea and Miflin 2010). In the plant cytosol, NH_4^+ is incorporated into glutamate and glutamine by the action of GS/GOGAT (Vessey et al. 2005). Importantly, aspartate and asparagine are also synthesized from glutamate and glutamine; together these four amino acids function to translocate organic N from sources to sinks (White et al. 2007).

The second enzyme in the N assimilation pathway is GOGAT and functions with GS in a metabolic cycle, where an amide group from glutamine is transferred to 2-oxoglutarate to produce two glutamate molecules. GOGAT has been shown to be essential not only in N assimilation but also in the transport of organic N sources and nodule formation (Mulley et al. 2011). *P. sativum* plants grown with mutated rhizobial GOGAT showed delayed nodule formation and a large downregulation of organic N transport out of the bacteroid (Mulley et al. 2011).

The final step in NH_4^+ assimilation is the incorporation of N into other amino acids via transamination reactions. Aspartate aminotransferase (Asp-AT) functions as an P-dependent aminotransferase in the production of aspartate (White et al. 2007). Aspartate is produced when Asp-AT catalyzes the reversible transfer of an amino group from glutamate to OAA, generating aspartate and 2-oxoglutarate (White et al. 2007). In C3 plants, Asp-AT also plays an important role in the malate-aspartate shuttle that allows the transfer of reducing agents from the mitochondria and chloroplasts to the cytosol (White et al. 2007).

Asparagine synthetase and asparaginase are two other enzymes involved in N metabolism (Lea and Miflin 2010). Although neither of these enzymes is involved in primary N assimilation, their activity is important under physiological stress.

Under many physiological stresses, plants divert glutamine to asparagine instead of glutamate (Lea and Miflin 2010). Asparagine synthetase catalyzes the ATP-dependent transfer of an amide group from glutamate to aspartate to produce one molecule of glutamate and asparagine. The inert nature and higher N per C content of asparagine is ideal for long-term storage and transport of N when C supply is low. Asparaginase is activated when stress conditions have subsided and carry out hydrolysis of aspartate to aspartic acid, releasing NH_4^+. This NH_4^+ is then cycled back to the GS/GOGAT system (Lea and Miflin 2010).

4.5 Source/Sink C Partitioning

Plants have limited internal nutrient resources and are faced with limited availability of external mineral and nutrient resources. The photoassimilates produced in the leaves must be loaded into the phloem and distributed to the various sink organs (Lynch and Ho 2005). The priority of the sink determines the supply of available photosynthate (Minchin and Lacointe 2005). Changes in the mineral nutritional status of plants will lead to changes in C resource allocation (Hermans et al. 2006). Nutrient deficiency can change C partitioning by regulating phloem transport and loading of C to sink organs or by depressing sink demand for C (Marschner et al. 2011).

Sucrose is the main form of C transported in the phloem, and its source to sink transport is essential for plant growth, with up to 80% of fixed C being exported from mature leaves (Hammond and White 2008; Lemoine et al. 2013). The availability of sucrose for export is however determined by various factors of plant metabolism including (1) the rate of C fixation, (2) the starch-sucrose ratio, (3) vacuolar sucrose pools, and (4) TPU export from the chloroplast. When these factors are affected, the amount of available sucrose changes, and this affects the source/sink relationship (Lemoine et al. 2013). Source leaves maintain sucrose production and phloem export during P deficiency, allowing root growth, even though overall photosynthetic rates and shoot growth are reduced (Marschner et al. 2011). Accumulation of sugars in the phloem signals the onset of P deficiency, even before photosynthesis is affected (Hammond and White 2008).

Legumes generally respond to P deficiency by increasing C allocation to the roots, thereby increasing the root-shoot ratio and area for soil exploration and P acquisition (Vance et al. 2003; Hermans et al. 2006). Plants exposed to P deficiency allocate up to 40% of daily assimilated C to root respiration (Lynch 2015). This increased metabolism, due to the increased root-shoot ratio, may be an important factor in reduced plant productivity during P deficiency. Changes in root architecture and type can help to conserve C. Adventitious roots, root hairs, and cluster roots can be induced during P deficiency. The greater specific root length of these rootlets increase the area and volume of soil explored for P acquisition. Furthermore, these rootlets require less C to produce when compared to normal roots, and the invested C can easily be recovered upon senescence (Vance 2008; Lambers et al. 2008; Niu et al. 2012; Thuynsma et al. 2014). Root exudation of organic acids

during P deficiency is another C requiring process. Plants can lose up to 10% of net fixed photosynthate via root exudation (Minchin and Lacointe 2005).

Nodules are also known to be strong sinks for both C and P, not readily releasing nutrients back to the plant (Richardson et al. 2009; Høgh-Jensen and Schjoerring 2010). Up to 15% of daily assimilated C can be consumed by the microsymbiont (Minchin and Lacointe 2005). The rate of BNF largely regulates the C required by the microsymbiont due to the large amount of energy expended on this process. The establishment, growth, and maintenance of nodule biomass also require C input (Minchin and Witty 2005; Kaschuk et al. 2009). Since N is essential to the plants' energy-producing machinery, both C and P resources are diverted to the nodule during short-term P deficiency to maintain BNF rates. With persisting nutrient deficiency however, nodule biomass and size decrease due to limited availability of C and P for nodule maintenance and growth as well as a decreased demand for N from the host plant (Schulze et al. 2006; Thuynsma et al. 2014).

4.6 Sucrose as a Systemic Signal of Phosphate Stress

According to Marschner et al. (1996), nutrient deficiency affects photoassimilate partitioning to sinks directly through phloem loading and transport or indirectly by impacting sink demand. Sugar concentrations in plants and phloem sap increase due to reduced plant growth or mineral deficiency, and it remains unclear as to whether this higher phloem sugar concentration is a stress response and/or a stress signal (Peuke 2010). Photosynthesis is negatively affected by P deficiency due a lack of Pi availability in chloroplast, but translocation of sucrose into the phloem is maintained and in some cases found to be increased during the first 6 days of phosphate starvation (Hermans et al. 2006; Lemoine et al. 2013). During P deficiency, the root surface area is increased by root hair initiation and elongation (Hammond and White 2008), and this change in root architecture induces increased sucrose allocation to the roots. In roots of white lupin (*Lupinus albus*), *LaPT1*, a phosphate transporter-encoding gene, and *LaSAP1,* a secreted acid phosphatase, are rapidly induced during phosphate starvation. Interruption of the shoot-to-root transport of sucrose by phloem girdling in phosphate-starved plants did not induce expression of *LaPT1* or *LaSAP1*, indicating that sucrose transported from the shoot to the root is necessary for phosphate starvation signaling (Liu et al. 2005). Application of exogenous sucrose to the roots repressed the induction of *LaPT1* and *LaSAP1* expression by Pi deficiency. Using common bean (*P. vulgaris* L.) in analogous experiments produced similar findings (Liu et al. 2010). Overall, these studies suggest that the phloem translocation of sucrose/photosynthates may play a crucial role in systemic signaling of Pi deficiency (Zhang et al. 2014).

As mentioned previously, it remains unclear whether the phloem sugar concentration is a stress response and/or a stress signal (Peuke 2010). The proposed role of sucrose as a systemic signal of Pi deficiency is based on the findings that phloem translocation of sucrose increases during early stages of Pi deficiency, sucrose

responsive genes are induced by Pi deficiency, exogenous application of sugar induces many *PSR* genes, removal of sucrose represses *PSI* gene expression, and lastly that sucrose is essential in most of the Pi starvation responses (Zhang et al. 2014). By imposing dark treatment to inhibit photosynthesis, applying sucrose exogenously, girdling of the phloem, as well as imposing gene defects, we may be obscuring or blocking the translocation of essential phloem transported signals other than sucrose.

4.7 C Metabolism in Relation to Nodule Structure

Plants of the Papilionoideae subfamilies, species of Trifolieae, and Fabeae tribes form indeterminate nodules, while plants belonging to the Phaseoleae and Loteae tribes develop desmodioid (determinate) nodules (Sprent and James 2008). The striking difference in metabolism between these two-nodule structure is that amides (glutamine and asparagine) is the major N forms exported from indeterminate nodules, mainly in Mediterranean legumes, whereas ureides (allantoin and allantoic acid) are the major N forms exported from determinate nodules, mainly tropical legumes (Streeter 1992). Legume nodules are a major sink in the root system, consuming 12–17 g of carbohydrates per gram of N fixed, which covers the costs of the nitrogenase reaction and NH_3 assimilation (Vance and Heichel 1991; Streeter 1992). Therefore, nodule respiration is mostly due to bacteroid metabolism, with approximately 15–30% of photosynthates transferred to the root system and an additional 30% of that photosynthate being used as C skeleton of N compounds for translocation to the shoots (Vance and Heichel 1991; Streeter 1992). The determinate, ureide-synthesizing nodules require less C than the indeterminate, amide-synthesizing nodules (Todd et al. 2006). Experimental determination of C and N budgets of ureide- and amide-forming legumes carried out by Atkins (1991) indicated that those based on ureides are generally more economical of C, with a C input of 1.4 g C g^{-1} N fixed in cowpea (*Vigna unguiculata* L. Walp.) compared to a minimum of 3.9 g C g^{-1} N fixed in lupin (*Lupinus* sp.), which forms indeterminate nodules. Furthermore, López et al. (2008) reported that C metabolism in *Lotus japonicus*, which develops determinate nodules, was less sensitive to salinity stress than *M. truncatula*, which develops indeterminate nodules. Enzymatic activities responsible for the C supply to the bacteroides to fuel N_2 fixation, such as sucrose synthase, alkaline invertase, MDH, and PEPC, were less affected by salinity stress compared to *M. truncatula*.

Therefore, during P stress is likely that nodules are able to alter the N export products to favor more C economical ureide export product. The benefit of ureide export during P stress is that a less C-consuming and more N-dense form of N is being exported to shoots, compared to amino acids or NH_4 (Atkins 1991; Todd et al. 2006). The preference of more ureides exported during P stress concurs with a study by Le Roux et al. (2009), where legumes accumulated more ureides relative to amino acids during P stress. The potentially lower C costs of ureide export may affect the plant's C budget, resulting in a positive impact on legume plant growth.

4.8 Respiratory C Costs of N_2 Fixation

A common response to P deficiency by legumes is increased relative biomass allocation to belowground organs. The resulting increase in root-shoot ratio presumably enhances P acquisition by promoting N_2 fixation (Nielsen et al. 2001; Magadlela et al. 2014; Thuynsma et al. 2014). Increased biomass allocation or diverting of carbohydrates to various plant parts is a major adaptation for plant survival and success during environmental stresses, including P nutrient stress (Nielsen et al. 2001; Mortimer et al. 2008; Magadlela et al. 2014;Thuynsma et al. 2014; Vardien et al. 2014). Ingestad and Ågren (1991) concluded that the main process controlling biomass allocation occur in the shoot, where the dominant factors are the ability of the plant to retain photosynthetic C for growth and the consequences of plant nutritional status on photosynthetic rates. The C resources produced during photosynthesis are either utilized in respiration, used for construction of plant tissues, or exported to the rhizosphere as organic acids and make up most of the dry weight of its organs (Nielsen et al. 2001). The plant's developmental stages and environmental conditions affect the magnitude of these carbohydrate allocations. Therefore, the success of plants under stress conditions may be determined by their ability to control carbohydrate utilization for metabolic energy (Nielsen et al. 2001; Magadlela et al. 2014; Thuynsma et al. 2014). It has long been suggested that in legumes, BNF requires an overall higher C budget in nutrient poor soils, specifically with regard to P.

The effects of P deficiency may be direct, as P is needed by nodules for their growth and metabolism or indirect. The high requirement of P may be linked to its role in C and energy metabolism of the nodule; therefore, as P deficiency may affect C supply to the nodules, the symbiotic bacteria will have greater respiratory demand on the host during BNF (Sa and Israel 1991; Valentine et al. 2011). This coincides with findings of Le Roux et al. (2009), Magadlela et al. (2014), and Vardien et al. (2014) who showed that nodule construction cost and growth respiration of different legume plants increased with P deficiency. Thus, P deficiency leads to a reduction of both nodulation and SNF (Magadlela et al. 2014; Vardien et al. 2014).

Nielsen et al. (1998) reported that root respiration of bean plants grown under low-P conditions, represented as a fraction of the whole-plant C budget, was approximately twice that of plants grown under moderate P stress. C expend in root respiration can amount to 20–30% of net photosynthesis under favorable conditions (Lambers et al. 1996) and found to increase under unfavorable conditions, such as P deficiency (Nielsen et al. 1998). Previous studies have reported that legume during environmental stresses, including P deficiency, reduce BNF in favor of soil mineral N assimilation, as it is less costly to assimilate mineral N than foster BNF (Magadlela et al. 2016). Interestingly, the success of two legume plants within the same genus growing in similar stress conditions can differ in response to stress by their ability to control carbohydrate allocation (Magadlela et al. 2014). This was found in two tree species,*Virgilia divaricata* and *V. oroboides* from a Mediterranean-type ecosystem of the Cape fynbos (Magadlela et al. 2014).

V. divaricata appeared to be more adapted to low-P conditions compared to *V. oroboides*; this was due to *V. divaricata*'s ability to maintain its photosynthetic costs and increase its root nodule C allocation under low-P conditions (Magadlela et al. 2014). In this regard,*V. divaricata* increased its allocation of resources to new root nodule growth during P stress, thus resulting in a greater efficiency to acquire atmospheric N_2 than did *V. oroboides* (Magadlela et al. 2014). However, enhanced nodule functions can also impose a high C cost on host reserves during P stress, where an increase of tissue construction costs. These findings agree with previous studies (Lynch and Beebe 1995; Mortimer et al. 2008; Nielsen et al. 1998; Magadlela et al. 2014).Vardien et al. (2014) also found an increase in the proportion of C allocation to root nodules of *V. divaricata* under low-P supply, along with increased P recycling and Fe concentrations in the nodules, thus increasing BNF efficiency. When some legumes are dependent on BNF and are able to develop cluster roots during nutritional stresses, there may be a trade-off in resource allocation between root nodules and cluster roots (Lynch and Ho 2005; Thuynsma et al. 2014).

Both root nodules and cluster roots require resources, including P, N, and C for maintenance, growth, and functionality; due to resource deficiency, competition may arise between sink organs for valuable resources (Lynch and Ho 2005; Schulze et al. 2006; Thuynsma et al. 2014). Thuynsma et al. (2014) showed evidence of increased cluster root formation during P deficiency and a decline during sufficient P in *L. albus*. Moreover, nodule biomass increased during sufficient P supply and decreased during P deficiency. The significant difference in specialized belowground organ allocation to cluster roots and root nodules suggests that these adaptations may be related to tolerance to P deficiency, as cluster root biomass decreased during sufficient P supply (Thuynsma et al. 2014). Furthermore, in *L. albus* cluster roots during P deficiency, there was an increase in the expression of phosphate transporters *LaPt1* and *LaPt2* (Lui et al. 2001). This functional plasticity means that valuable resources such as C and P are redirected to cluster roots and root nodules during P stress. On the other hand, the increased C costs of cluster roots during P deficiency appears to improve P nutrition of nodules to maintain BNF function during P stress (Lynch and Ho 2005; Schulze et al. 2006; Thuynsma et al. 2014).

4.9 Transcriptomics as a Tool to Identify Key Genes Involved in Nutritional Stresses and Metabolic Cross Talks in Legumes

Ultimately, the whole physiology of an organism is guided by the expression of genes that respond to developmental and environmental cues. The sequencing and annotation of entire genomes is facilitating the generation of inventories of genes involved in cell signaling as well as metabolic processes, not only of structural

genes (e.g., those coding for enzymes) but also for master regulatory genes, such as transcription factors, that regulate transcription of entire genetic networks involved in specific processes (Benedito et al. 2008).

Although the genomes and transcriptional profiles of model and major crop species are well known, orphan species (e.g., smaller crops, noncommercial native plants) are lagging behind. By the same token, while transcriptional studies have been extensively carried on many plant species using simple variables, the interaction effects of important agronomical environmental conditions are essentially lacking. And while model species already enjoy an extensive gamut of transcriptional profiles, experiments considering multiple nutrient deficiencies are very limited. For example, the effects on gene transcription or metabolism in different organs and tissues of combined low P and low N, or soil acidity, are still largely undetermined at the comprehensive, genomics level. Considering that gene transcription is a major control point of gene expression, it is fundamental to characterize transcriptional modulation of genes involved with metabolic adaptation under low nutrient availability, including various stress interactions. Experiments of this nature are challenging due to the number of treatments needed and the refined data analysis required to identify key genes and processes at play. Notwithstanding, this information is much needed to understand real stress situations plants face in the field.

It is also important to recognize that in many metabolic processes, especially for structural genes coding for key enzymes, major regulation points are downstream transcription, such as activity modulation through allosterism, protein modifications, or degradation, as well as substrate compartmentation. Therefore, in vitro analyses of enzyme activity must be interpreted with caution. Furthermore, while integrating enzymology with transcriptomics, it is important to identify members of gene families that are responsible for enzyme activity in specific tissues or conditions. For example, let's consider two cases of key enzymes involved in P × C metabolism in a model legume. We identified eight GS genes in the genome of *M. truncatula* (Fig. 4.1). Most GS expression derives from five homologs, with constitutive expression and little variation among organs, nodule developmental stages, tissues of the mature nodule, or NO_3^- application to nodulating roots. Therefore, variation of GS activity must be regulated posttranscriptionally, and a genetic approach to understand its role in metabolism must take into account expression of multiple genes. In the same way, we identified six PEPC homologs in *M. truncatula* (Fig. 4.2). All of them are found actively transcribed throughout organs, tissues, and conditions. However, how much each of these gene products is contributing to the general PEPC activity, whether they present similar properties, and the impact of each to BNF under optimal or stress conditions are unanswered questions. Therefore, comprehensive transcriptional analyses considering all metabolic and regulatory genes involved with nutritional deficiencies are still needed in legume species, especially for cases of concomitant deficiency situations and other stresses, such as C metabolism of nodules under P stress.

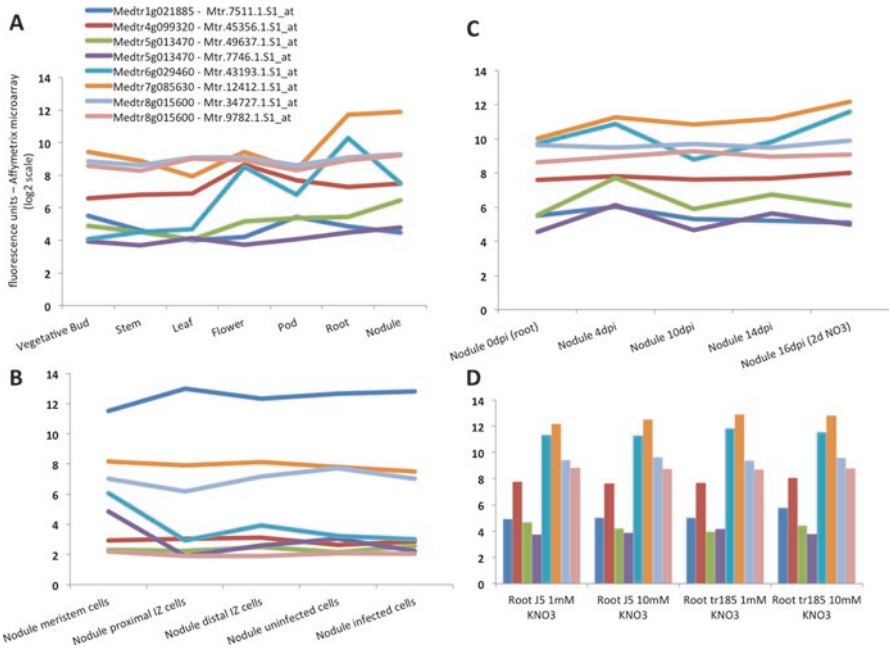

Fig. 4.1 Expression of genes coding for glutamine synthetase (GS) in the model legume, *Medicago truncatula*. (**a**) GS gene expression in various organs of the mature, nodulating plant growing under optimal conditions (Benedito et al. 2008). (**b**) GS gene expression in specific cell types of the mature nodule (Limpens et al. 2013). (**c**) GS gene expression during nodule development. Samples are as following: non-inoculated roots (0 dpi); nodule bumps (4 dpi, early nodule development); young, functional nodules (10 dpi); fully mature nodule (14 dpi); 14-day-old nodules were treated with 2 mM KNO_3 for 2 days (16dpi (2d NO_3)) (Benedito et al. 2008). (**d**) GS gene expression of two genotypes in non-inoculated whole-root systems in low and high nitrate conditions (Bourion et al. 2014). Data is based on the Affymetrix microarray platform (The *Medicago* Gene Atlas version 3: Benedito et al. 2008; He et al. 2009; http://mtgea.noble.org/v3)

4.10 Conclusion

Rhizobial symbiosis improves plant nutrition and increases rates of photosynthesis. These assimilates are partitioned to various sinks, and the assimilate requirements of rhizobial growth, maintenance, and metabolic function can be considerable. It is clear that legumes have an exquisite capacity to alter C metabolism in leaves and nodulated root systems during P stress. These alterations involve several primary pathways for sugar metabolism, organic acid synthesis, and amino acid assimilation and recycling. Owing to the requirement of the ubiquitous organic C skeletons, these alterations in the pathways are often integrated. It was recently found that the whole plant N-feedback mechanism in *M. truncatula* is closely linked to nodule C metabolism, lending further support to the hypothesis that nodule amino acid content regulates nitrogenase activity, possibly by downregulating C supply to the nodules. It is the integration of these alterations in pathways, where perhaps much of the future work will reveal the extent of metabolic plasticity within nodules, where the control may reside in various systems of functional regulation.

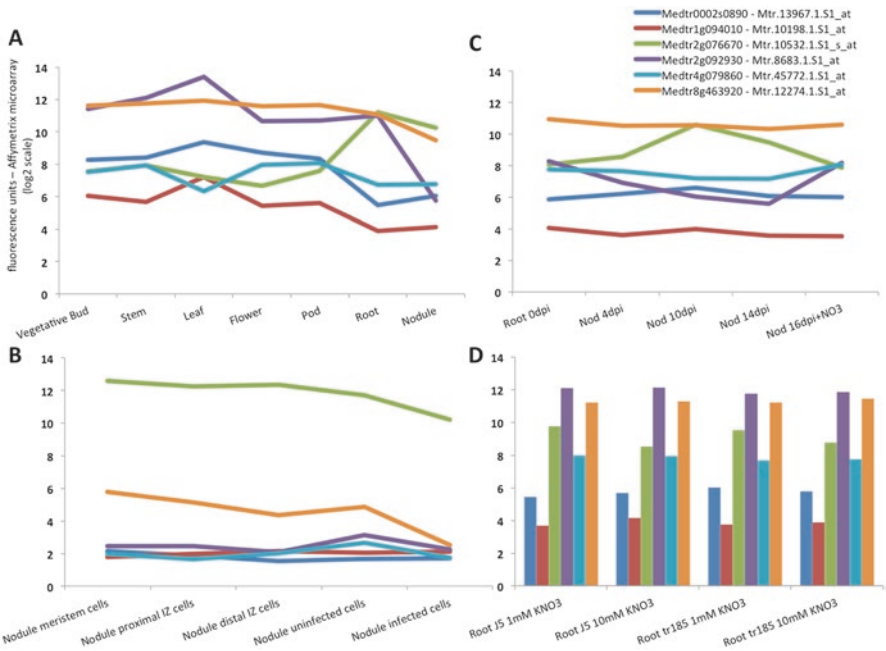

Fig. 4.2 Expression of genes coding for PEP carboxylase (PEPC) in the model legume, *Medicago truncatula*. (**a**–**d**) Samples are the same as in Fig. 4.1

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Chapter 5
Oxygen and the Regulation of N_2 Fixation in Legume Nodules Under P Scarcity

Aleysia Kleinert, Marcellous le Roux, Yun Kang, and Alex J. Valentine

Abstract Oxygen (O_2) is vital for nodule metabolism owing to its role in mitochondrial respiration for ATP generation, a vital component in N_2 fixation. However, the concentration of O_2 must be carefully regulated, because O_2 can also reduce inhibit nitrogenase. Phosphorus (P) deficiency can increase the nodule's permeability to O_2 and thereby exert a deleterious effect on N_2 fixation. Although the mechanism by which the P deficiency increases the O_2 permeability is not known, it has been attributed to a reduction in the O_2 diffusion barrier within the nodule. In order to maintain N_2 fixation, the nodules have several adaptations at the structural and metabolic levels to prevent and ameliorate these negative impacts. These adaptations will be evaluated in terms of structural and metabolic responses to O_2 diffusion. The structural responses are based on the physical barrier of cortical cells and their intercellular spaces, while the metabolic responses include respiratory alteration and binding of O_2 by leghemoglobin. We provide a critical evaluation of the current knowledge regarding the P effect on O_2 permeability and propose new theories on potential mechanisms of P deficiency on the O_2 diffusion barrier.

Keywords Oxygen • P stress • N_2 fixation • Nodule • Structural adaptations • Metabolism

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5.1 Introduction

The reduction of atmospheric dinitrogen (N_2) by mature bacteroids is an extremely energy-intensive process fueled by photosynthetically derived carbon from the plant. Nodules require energy for the fixation of atmospheric N_2 (Kleinert et al. 2014), synthesis of exported organic solutes of nitrogen (N) (Magadlela et al. 2016), as well as for growth and maintenance (Kleinert et al. 2014). Large amounts of oxygen (O_2) are required to generate this energy, and nodules therefore have a considerably higher rate of O_2 consumption than other plant tissues (Mortimer et al. 2009). O_2 inhibits the functioning of nitrogenase and the regulation of bacteroid metabolism in the O_2-limited environment of the nodule presents the bacteroid with problems. Therefore, energy, reductant, and carbon pools must be carefully balanced to ensure optimum rates of N_2 fixation (Lodwig and Poole 2003).

The O_2 concentration in the infection zone of nodules is maintained at approximately 18 nmol (Layzell and Hunt 1990). The mechanism of O_2 control in legumes has not yet been fully elucidated, but regulation appears to consist of three processes (Udvardi and Poole 2013):

1. An O_2 diffusion barrier exists in the outer cell layers of nodules which limits the diffusion of O_2 into the infected zone.
2. Bacteroids and plant mitochondria have high respiration rates for O_2 consumption.
3. Leghemoglobin has a high binding affinity for O_2 in the cytoplasm and delivers it to the infected cells for consumption by bacteroids and mitochondria.

The effect of O_2 on nodule physiology can be further exacerbated during phosphorus (P) deficiency. Many metabolites occur as orthophosphate (Pi) monoesters, while the phosphoanhydride bonds of compounds such as ATP function to transfer energy from energy-yielding process of photo-oxidative and substrate-level phosphorylation to the energy-dependent cellular processes of biosynthesis, ion pumping, and mechanical work (Plaxton and Tran 2011). The responses of nodules to O_2 and P stress may also depend on the morphological differences between nodules of different legumes.

5.2 Nodule Structure and Metabolism

The structures of nodules differ generally from legumes of tropical and temperate origin, although these morphological differences do not always follow this simple division. In tropical and subtropical legumes (e.g., soybean, common bean, and cowpea), the roots generally form determinate nodules (Fig. 5.1) with a closed meristem that at nodule maturity does not divide any further (Smith and Atkins 2002). Nodules from tropical legumes are spherically shaped and its infected cells lack vacuoles (Schubert 1986). Furthermore determinate nodules are known to harbor several bacteroids within a symbiosome, which results from fusion of separate

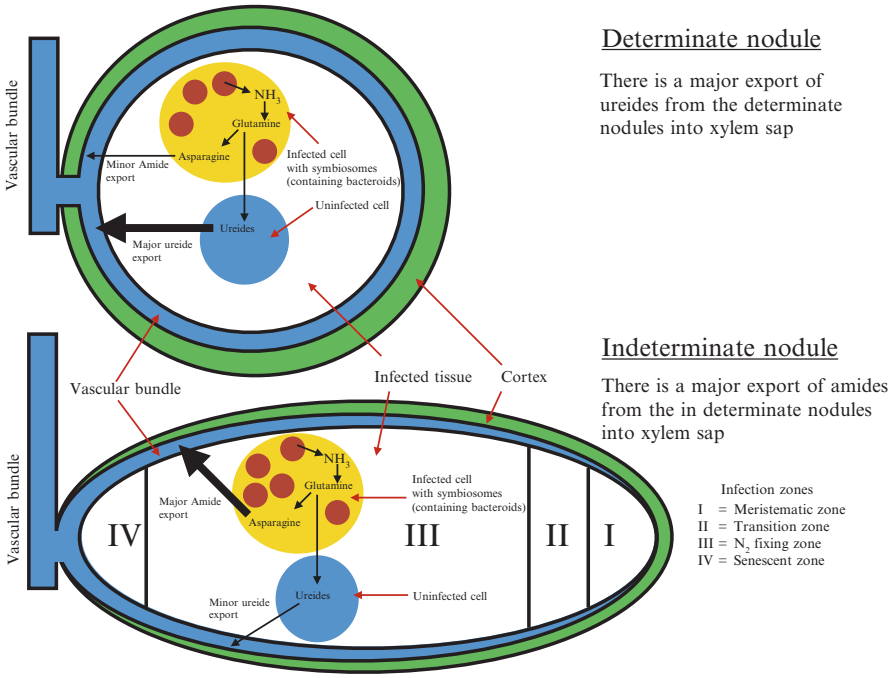

Fig. 5.1 The anatomical and metabolic differences between determinate and indeterminate legume nodules. Determinate nodules are generally from tropical origins and export fixed N_2 mostly as ureides. Indeterminate nodules generally originate from temperate regions and export fixed N_2 largely as amides (asparagine and glutamine)

symbiosomes and/or bacteroids dividing continually within the existing symbiosome (Prell and Poole 2006). In contrast, legumes from more temperate environments have nodules which are typically indeterminate (Fig. 5.1). These nodules are characterized by an open meristem, which allows for continual divisions right through the plant life cycle. This gives rise to a cylindrically shaped nodule in which the infected cells are vacuolated (Schubert 1986). Although various nodules differ in some structural details, the general organization of tissue regions is similar, with the infection zone normally confined to the central region of the nodule. The two types of nodules present an intriguing difference in metabolism.

In general, most temperate legumes (e.g., lupins, pea, clovers) usually transport their fixed N as amides, notably asparagine and glutamine (Fig. 5.1) (Streeter 1991). In comparison, the tropical legumes (e.g., soybeans, cowpea, common bean) export purine derivatives, most notably the ureides such as allantoin and allantoic acid (Fig. 5.1). The existence of these two metabolically distinct routes in legumes from different origins and nodule morphology is perhaps unsurpassed by other nutritional variations within plant systems (Streeter 1991; Le Roux et al. 2009). In terms of respiratory energy costs, the requirement of ATP and reductant per assimilated N does not differ significantly for ureide and amide

exporting legumes (Smith and Atkins 2002). Nonetheless, the organic N translocated as ureides has a lower C/N ratio than when N is exported as amino acids in amide exporting legumes (Smith and Atkins 2002). For these reasons, the ureide biosynthesis and export is commonly regarded as the more economical pathway in terms of C expenditure. Furthermore, photorespiration is generally promoted in tropical climates; so therefore the export of organic N as ureides may additionally serve to be more economical with C, compared to amino acid export. During P deficiency the export of more ureides, relative to amino acids, may be an adaptive advantage, especially when extremely low P supply can lead to a reduction in C supply. Although P stress will cause a decrease in the total N assimilation in the nodules and nodulated, the ratio of ureides being synthesized and exported, relative to amino acids, may change (Oliviera et al. 2004; Le Roux et al. 2009; Magadlela et al. 2016). The advantage of this adaptation during P stress is to export the form of organic N that is associated with a lower C economy. Compared to amino acids, the export of ureides represents a more N-dense form of organic N being exported to shoots (Atkins 1991; Todd et al. 2006). In spite of the anatomical and metabolic differences between these two groups of legumes, both amide and ureide exporting nodules are sensitive to O_2 supply. Moreover, P deficiency increases their respective O_2 permeabilities, so that biological N_2 fixation (BNF) is reduced (Ribet and Drevon 1995; Drevon and Hartwig 1997; Schulze and Drevon 2005; Le Roux et al. 2009).

5.3 Nodule O_2 Diffusion During P Deficiency

Irrespective of whether legumes have amide or ureide exporting nodules, the supply of P appears to be crucial to the control of O_2 permeability into the nodules. In this regard, these different types of legumes such as soybean (Ribet and Drevon 1995), common bean (Vadez et al. 1996), and alfalfa (Schulze and Drevon 2005) have all shown an increased O_2 diffusion into nodules during P stress. The effect of increases in O_2 flush inside the nodule resulting in the destruction the nitrogenase enzyme (Schulze and Drevon 2005; Avenhaus et al. 2016). This can occur in a relatively short time, from minutes to hours, with negative consequences to the capacity of nodules for fix N_2 (Schulze and Drevon 2005; Avenhaus et al. 2016). Besides P availability, nodule O_2 conductance is also affected by other abiotic stresses.

However, unlike drought or chilling stress, P deficiency has been repeatedly reported to increase nodule conductance to O_2 in many investigations, e.g., soybean (Ribet and Drevon 1995), common bean (Drevon et al. 2015; Bargaz et al. 2011), and alfalfa (Schulze and Drevon 2005). With one exception in common bean (Jebara et al. 2005), nodule O_2 conductance changes of five genotypes were compared under P deficiency conditions, and two genotypes showed decreased nodule conductance while the other three had no changes. Interestingly, in a similar study in common bean from the same research group (Bargaz et al. 2011), the nodule conductance of all five different genotypes were found to increase

significantly under P-deficient conditions. The reasons for this discrepancy are unknown; investigation of changes in nodule O_2 conductance under P limitation in genotypes of other legume species may help answer this question. The nodule conductance is calculated by dividing the permeability of the whole nodule population of one plant by the total nodule surface area (Schulze and Drevon 2005). Therefore, nodule O_2 conductance is affected by the nodule shape variance (few large nodules or many small nodules) when the nodule permeability is the same, which could partially explain the genotypic variance in nodule conductance in common bean under P limitation (Jebara et al. 2005). Given the negative impacts of an increase in O_2 permeability to nodule function, it stands to reason that legumes have several adaptions in place to reduce the effects of O_2 permeability. These adaptive mechanisms can be structural or functional and appear to operate at the levels of course and fine control.

The course control is thought to be a physical barrier to gas diffusion, which is located in the inner cortex (Fig. 5.2) of nodules (Hunt and Layzell 1993; Minchin 1997; Galvez et al. 2000), while the fine control of O_2 levels appears to reside in the oxygenated leghemoglobin gradients (Fig. 5.2) within the nodules (Thumfort et al. 1999). Broadly, these two levels of control can be separated into structural and metabolic adaptations, which will be evaluated in the following sections.

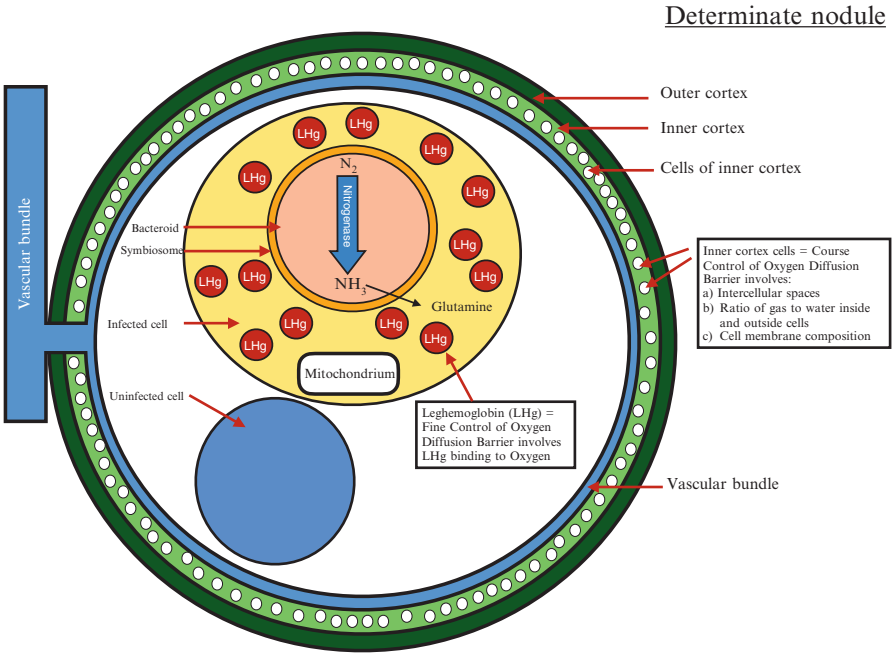

Fig. 5.2 The proposed O_2 diffusion barrier in legume nodules, using a determinate nodule as a model. The control of the O_2 diffusion barrier in the nodule is largely mediated by leghemoglobin-O_2 binding (fine control) and the physical barrier of inner cortical cells (course control)

5.4 Nodule Structural Adaptations to the Effect of P Stress on O_2 Permeability

N_2-fixing legumes have long been known to be sensitive to P deficiency, which probably is attributed to the key role of P in the cell energy metabolism and the high energy requirements for N_2 reduction by nitrogenase (Schulze and Drevon 2005). In nodulated plants, the nodule is usually the organ that has the highest P concentration (Bargaz et al. 2011; Schulze et al. 2006; Schulze and Drevon 2005). In multiple studies in various legume species, P limitation was generally shown to greatly reduce the number, size, biomass, as well as total surface area of nodules (Bargaz et al. 2011, 2013; Lazali et al. 2013; Sulieman et al. 2013; Schulze and Drevon 2005; Høgh-Jensen et al. 2002; Ribet and Drevon 1995). At the whole-plant level, however, the biomass ratio of nodule vs. shoot, root, or the entire plant could either increase, decrease, or remain relatively stable because the shoot and root growth, especially shoot growth, are similarly reduced by P limitation (Jebara et al. 2005; Kouas et al. 2005; Schulze and Drevon 2005). Differences in the nodule growth response to P deficiency appear to be related to plant species, genotypes, and the duration and the severity of the stress. Further structural control of the O_2 diffusion barrier is more apparent at the anatomical scale, involving the cortical cells of nodules (Fig. 5.2).

Drevon et al. (1998) suggested that osmoregulatory changes in nodule cortical cells may be responsible for the increased O_2 diffusion. The physiological purpose of this increased O_2 diffusion during P stress is unclear, but may be connected to ensuring sufficient adenylate levels for high N_2 fixation rates, in spite of the O_2 inhibition of nitrogenase (Schulze and Drevon 2005). Other legume species such as *Lupinus albus* control the O_2 diffusion in nodules during P stress by blocking the free spaces between cortical cells (Fig. 5.2) (De Lorenza et al. 1993; Iannetta et al. 1993; Schulze et al. 2006). In earlier studies, nodule O_2 conductance changes have been suggested to be regulated by variations of the intercellular spaces in the nodule inner cortex under conditions such as drought, chilling, salinity, or changes in rhizosphere O_2 concentrations (reviewed in Valentine et al. 2011; van Heerden et al. 2008; Serraj et al. 1995). The air space changes could result from occlusion of intercellular spaces and/or swelling or shrinking of the cells (Fig. 5.2). Speculations are that similar changes probably happen in the nodule under P-deficient stress. However, direct microscopic evidence is still lacking. The alterations in nodule water conductance under P deficiency have not been directly studied.

In one rare report, aquaporin transcripts were shown to increase by in situ hybridization in common bean (Drevon et al. 2015) under P stress. To our knowledge, independent studies on the changes in aquaporin function at transcript and protein levels under P limitation have not been performed. According to RNA-seq transcriptome profiling results of all the nodule aquaporins under P-deficient stress in *Medicago truncatula* (Cabeza et al. 2014), the majority of the nodule aquaporins are indeed up regulated, especially the very highly expressed aquaporins. Direct measurements on nodule water conductance changes under P limitation are required considering the possible indirect correlation between gene expression and protein

abundance/activity. The involvement of aquaporins poses an interesting possibility for the role of water relations as a possible mechanism of the O_2 barrier. This role of water relations is also supported by ion movements from the cortex during times of increased O_2 diffusion (Fig. 5.2).

The movement of K^+ ions from the cortex to the central zone of nodules, such as *Glycine max*, was shown to increase the nodule permeability to O_2 (Wei and Layzell 2006). This is consistent with a mechanism of K^+ acting in a water relations capacity within the cortex (Vessey et al. 1988; King and Layzell 1991; de Lima et al. 1994). In this regard, the removal of K^+ from the nodule cortex can facilitate the loss of water from these cortical cells to the xylem stream. The consequence is a change in the proportion of gas (high permeability) to water (low permeability) in the cortex and thereby an increase in O_2 permeability (Fig. 5.2). In this regard, the effect of P deficiency may be linked to a decline of K^+ influx into the cells via a ATP/ADP-dependent K-pump/channel or K^+ efflux via an outward K-pump/channel.

A further potential physical barrier to O_2 diffusion may reside in the structure of the cell membranes of infected cells or the symbiosomes (Fig. 5.2). Recent work has shown that P-deficient nodules can reduce the phospholipid component of their cell membranes (Vardien et al. 2016) and possibly replace them with sulfolipids and galactolipids. It may therefore also be possible that the removal of membrane phospholipids during P deficiency can contribute to the increase in O_2 permeability. This is an intriguing possibility, which should be further explored.

5.5 Metabolic Adaptations to the Effect of P Stress on O_2 Permeability

The responses of nodule N_2 fixation to O_2 may also be greatly accentuated under P stress, where the consequent metabolic changes within the nodule will attenuate these responses. Le Roux et al. (2006) reported constant Pi levels as well as ADP/ATP ratios in nodules after 14 days of P stress, while in the host roots there was a decline in Pi concentrations and adenylate levels. Several other authors also reported stable nodular Pi levels indicating that nodules may function optimally at low Pi concentrations (Al Niemi et al. 1997, 1998; Kleinert et al. 2014) and that the bacteroid fraction of nodules are able to realize their own P requirements by scavenging from host cells and not readily releasing P reserves back to host roots (Al Niemi et al. 1997, 1998; Colebatch et al. 2004). BNF measured in *L. luteus* declined during P deficiency, but the decline was mostly likely due to an indirect N-feedback effect of accumulated amino acids caused by a decline in plant growth (Kleinert et al. 2014). The decline in BNF also corresponded with a reduction in root-nodule CO_2 release rates and nodule O_2 uptake rates. A declining BNF, usually an energy-intensive process, would cause a decrease in the sink strength of nodules, and the lower respiration rates under P stress is confirmation of this. Similar reductions in the respiration rates of *Phaseolus vulgaris* during decreased nodular BNF were reported by Mortimer et al. (2008, 2009).

5.5.1 Alternative Glycolytic and Mitochondrial Electron Transport Chain Bypasses

The increase in O_2 uptake during P stress has been linked to the increase in the permeability of the O_2 diffusion barrier (Schulze and Drevon 2005; Bargaz et al. 2011; Drevon et al. 2015). Under P stress, the limitation of P*i* will reduce the ability of adenylate synthesis in nodules (Le Roux et al. 2006). However, since most of the research in area indicated that there is an increase in the O_2 uptake during P stress, this would imply that an alternative route to mitochondrial ATP phosphorylation might be in operation. This is supported by the engagement of the alternative oxidase during P stress, where a non-phosphorylating route is involved in O_2 consumption (Rychter et al. 1992).

The reduction in intracellular levels of ATP, ADP, and related nucleoside Ps which follows a drastic decline in cytoplasmic P_i levels, as experienced during prolonged P deficiency, would inhibit carbon flux through the ATP-dependent glycolytic steps (Plaxton and Podesta 2006). However, P_i-deficient plants need to generate energy as well as carbon skeletons to maintain their core metabolic processes. To this end, a cluster of at least six adenylate-independent glycolytic "bypass" enzymes have been identified in P-deficient plants (Plaxton and Podesta 2006) in addition to the inorganic pyrophosphate (PPi)-dependent H^+-pump (H^+-PPiase) of the tonoplast membrane (Plaxton and Tran 2011). These PSI bypasses facilitate glycolytic flux and vacuolar pH maintenance during periods of intense Pi stress when there is a decline in intracellular levels of adenylate and Pi levels (Plaxton and Tran 2011). Phosphoenolpyruvate carboxylase (PEPc) functions as the bypass enzyme together with malate dehydrogenase (MDH) and malic enzyme (ME) for the reaction catalyzed by ADP-limited cytosolic pyruvate kinase (PKc). The activity of this metabolic bypass during P_i stress when the ADP supply may be limiting for optimal PKc functioning would ensure continued pyruvate supply to the tricarboxylic acid cycle while at the same time releasing P_i back into the metabolic pool (Duff et al. 1989; Plaxton 2004). Several authors have reported an increase in PEPc activity for P_i -stressed samples compared to P_i-sufficient controls in *Brassica nigra* (Duff et al. 1989), *Brassica napus* (Nagano et al. 1994), and *Catharanthus roseus* suspension cells (Moraes and Plaxton 2000; Plaxton and Podesta 2006). Juszczuk and Rychter (2002) proposed that the increase in pyruvate synthesized via the alternative PEPc-MDH route could serve as a mechanism for oxidizing of reducing equivalents which accumulate during P stress. Schulze et al. (2006) found that P stress induced nodular enzyme activities of PEPc and MDH in *L. albus* plants. The two enzymes are central to carbon cycling and the energy substrates for N_2 fixation. Le Roux et al. (2006) reported no changes in nodular pyruvate levels synthesized from PEPc-derived malate during P deficiency, which implied that malate may have been used as a source for bacterial respiration inside the nodules due to the low nodular O_2 concentrations that would favor malate rather than pyruvate as the end product of glycolysis (Vance and Heichel 1991).

The decline in intracellular Pi and ADP levels during P deficiency will also impact on respiratory electron flow through the cytochrome pathway at sites of coupled ATP synthesis (Plaxton and Tran 2011). As with the glycolytic bypasses, the presence of nonenergy conserving pathways of mitochondrial electron transport provides a mechanism for respiratory flux to be maintained under limiting ADP and/or Pi conditions. Plants utilize the upregulation and/or increased engagement of nonenergy conserving (rotenone and/or cyanide-insensitive) pathways of the mitochondrial electron transport chain during P stress (Rychter and Mikulska 1990; Plaxton and Podesta 2006). This would allow the continued functioning of the mitochondrial citric acid cycle and electron transport chain with limited ATP production which would contribute to the survival of P-deficient plants (Plaxton and Tran 2011). The arrested growth and metabolism of P-deficient transgenic tobacco unable to synthesize a functional alternative oxidase (AOX) add weight to this idea (Sieger et al. 2005; Plaxton and Tran 2011). The lack of alternative oxidase during P stress appears to correlate with an increase in levels of proteins usually associated with oxidative stress. The continuation of respiration via the alternative oxidase which plays a role in maintaining the cellular redox and carbon balance also provides an essential adaptation whereby plant cells can control their response to Pi deficiency (Sieger et al. 2005).

5.5.2 Oxidative Stress Responses

The role of O_2 in oxidative stress is that the increase in O_2 permeability during P stress can lead O_2 being converted to reactive O_2 species (ROS). The study by Bargaz et al. (2013) is among the first to report on oxidative stress in nodules of N_2-fixing legumes during P deficiency. The limitation of P can result in imbalances in the antioxidant defense systems of plants, which lead to changes in mitochondrial membrane components and inhibition of electron transport through the cytochrome respiratory pathway (Juszczuk et al. 2001). This in turn can bring about an accumulation of ROS which can lead to oxidative stress (Bargaz et al. 2013). Plants employ antioxidative enzymes and nonenzymatic substances to act as free radical scavengers to detoxify ROS and protect cells from oxidative damage. Bargaz et al. (2013) found increased electrolyte leakage, malondialdehyde, and H_2O_2 in nodules of P-stressed plants, which all point to disturbances in cell membrane stability under P stress. Peroxidase activity was also found to be higher in P-stressed nodules than the P-sufficient treatment. This concurs with the findings of a 30% increase in peroxidase activity in *P. vulgaris* roots by Juszczuk et al. (2001). P-deficient nodules of *P. vulgaris* plants also exhibited an increase in phenol content under P deficiency (Bargaz et al. 2013). There have been reports that plants accumulating phenolic compounds are able to neutralize lipid radicals and therefore have important antioxidant properties for the protection of membranes (Bargaz et al. 2013).

5.5.3 *Leghemoglobin and P Nutrition*

The alteration of nodule metabolism during P-induced variations in O_2 supply represents a level of fine control which can be flexible over short or long periods of stress. In the area of control, the activity of leghemoglobin is vital because it binds to O_2. Leghemoglobin is localized within the cytosol of infected cells of the nodules and facilitates O_2 supply of the mitochondria of the bacteroids (Sherer et al. 2008). Moreover, it maintains a low-free O_2 concentration within these nodules, in order to protect the O_2-sensitive nitrogenase from irreversible inhibition by O_2 (Sherer et al. 2008, Schulze and Drevon 2005, Avenhaus et al. 2016). The short-term attenuation of the O_2 diffusion barrier by leghemoglobin is evident in a recent study of increased O_2 permeability into nodules (Fig. 5.2). It was found that the decline in N_2 was quickly followed by an upregulation in genes for nitrogenase formation. At the same time, a tightening of the O_2 diffusion barrier, presumably by leghemoglobin, reduced internal O_2 concentration and thereby protected the nitrogenase against further inhibition by O_2 (Avenhaus et al. 2016). During P deficiency, the role of leghemoglobin may become more important as the O_2 diffusion barrier is reduced during low P supply.

The interaction between P supply and leghemoglobin levels needs further investigation, since very little research has been focused on this. In one study, Miao et al. (2007) found that although P deficiency reduced N_2 fixation, it had no effect on leghemoglobin concentration in nodules of *G. max*. Interestingly, leghemoglobin may affect the levels of P compounds such as ATP. In a study on S deficiency, Sherer et al. (2008) found that reduced levels of leghemoglobin were also associated with a decline in ATP concentrations. This was attributed to the diminished leghemoglobin supply of O_2 to mitochondria, where the production of ATP was directly coupled to O_2.

5.6 Conclusions

The negative impact of excessive O_2 supply on nodule metabolism can be intensified during P deficiency. Although the precise mechanism of the O_2 inhibition has to date remained unclear, the exacerbated effects during P stress are even more elusive. Nonetheless, the adaptations to overcome this O_2 inhibition are known to be regulated at the structural and metabolic levels within nodules. Currently some of these mechanisms have been elucidated separately, but it is very likely that functional adaptation operates in a more integrated system. These integrated mechanisms may be the key to understanding the adaptations to P-induced O_2 stress. Clearly they need to be further explored with the modern tools of functional genomics, but in combination with classical approaches such as anatomy, physiology, and biochemistry.

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Chapter 6
Transport and Metabolism of Nitrogen in Legume Nodules Under Phosphorus Deficiency

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Abstract Leguminous plants, like all other plants, rely on a number of elemental nutrients for their growth, development, and metabolic processes. Nitrogen (N) and phosphorus (P) are some of the minerals that regulate the proper survival of a plant species. Legumes are one of the most important sources for the fixation of atmospheric dinitrogen (N_2). They harbor N_2-fixing bacteria (mainly rhizobacteria) in their roots that aid the process. These plant growth-promoting rhizobacteria in turn provide nutrients to the legumes for their proper growth. However, under nutritional stress conditions, the absorption and metabolism processes are affected. P deficiency is observed in a number of agricultural and environmental conditions. This deficiency adversely affects the metabolic processes of the legumes. Chief among this is the N transport and metabolism which governs the production, transport, and metabolism of many primary and secondary metabolites. The N metabolism, hence, has to be carefully regulated under P-stress conditions. The host plant as well as the nodules associated with their roots is involved in regulating the N metabolism and transport processes. Chief among these compensatory mechanisms are the alteration in catabolic and anabolic cycles, increasing the carbohydrate storage capacity and alteration of the N_2 fixation capability (Sulieman et al. 2008).

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This chapter provides insights into the changes observed in the N transport and metabolism in leguminous plants as a result of P deficiency and the mechanisms developed to respond to the P-stress conditions.

Keywords Nitrogen metabolism • Nodular modifications • Phosphorus deficiency • Legumes • Symbiosis • Plant adaptations

6.1 Introduction

Legumes, like other plants, are highly dependent upon nitrogen (N) for normal development and growth. They form a symbiotic relationship with the dinitrogen (N_2)-fixing bacteria (Adams et al. 2016). This metabolic process helps in improving the soil quality as well as the plant metabolism. The N_2-fixing bacteria help in the conversion of atmospheric N_2 to biologically consumable form. They account for more than half of the world's naturally fixed N (Canfield et al. 2010). These rhizobacteria commonly referred to as plant growth-promoting rhizobacteria (PGPR) belong to a number of families including *Rhizobiaceae*, *Phyllobacteriaceae*, *Hyphomicrobiaceae*, and *Bradyrhizobiaceae* (Bhattacharyya and Jha 2012). These bacteria are associated with the nodules of the legumes and help in the nitrogenase enzyme-mediated conversion of atmospheric N_2 into ammonia (NH_3) (Zhang et al. 2016). The symbiotic relationship between the leguminous plants and the soil bacteria helps in improving the soil quality and, thereby, improving the plant growth. The metabolic, physiological, and morphological characteristics of the plants are dependent and regulated by the availability of various organic and inorganic nutrients from the soil. Therefore, any biotic or abiotic stress factor can lead to an alteration in the overall status of the plant. Phosphorus (P) deficiency is one such abiotic stress factor that causes major modifications throughout the plant body (Babar et al. 2014). P is considered as an essential element in the nodulation process, hence, contributing to the N_2 fixation as well as the plant growth and survival processes (Hayashi et al. 2013). Figure 6.1 provides an insight into the alterations observed in N metabolism and other related processes in response to P scarcity. Both the catabolic and anabolic pathways, including photosynthesis, glycolysis, energy generation, carbohydrate and nucleic acid metabolism, are affected in response to P deficiency (Magadlela et al. 2015). Consequently, the leguminous plants adapt a set of compensatory mechanisms to modify their N consumption, transport, assimilation, and metabolism (Sulieman and Tran 2015). In this review, initially the N metabolic and transport processes within the legume body are discussed. Thereafter, the adverse effects of P deficiency on the legume and, in particular, nodule structure and function are presented. Toward the end, the compensatory metabolic and physiological mechanisms developed by the legumes to address the P deficiency are discussed.

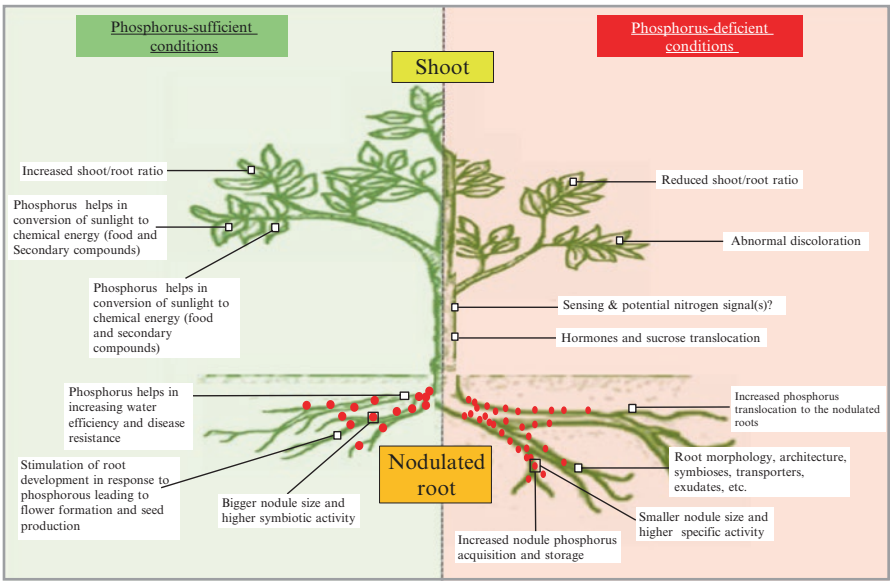

Fig. 6.1 The physiological and morphological adaptations of leguminous plants under phosphorus stress. All the processes of the shoots and roots are affected. The *left panel* shows the involvement of phosphorus in normal physiological mechanisms in legumes including photosynthesis, normal growth, and development. The *right panel* shows the adaptations of the legumes in response to phosphorus deficiency. A number of changes are observed including altered growth patterns, nodule formation, and nitrogen metabolism activities

6.2 Fate of N in Leguminous Plants

N is one of the most essential elements for the plant growth and survival. It contributes to the structural build-up of the plants and also forms an essential component of many biochemical pathways. Acquisition of N for carrying out the normal metabolic functions of the plants is of prime importance. In legumes, like other plants, the N levels are maintained by the absorption through the roots. Additionally, they develop symbiotic relationship with rhizobacteria that further facilitate the N absorption by the formation of nodules (Imadi et al. 2016, Fig. 1). The process known as symbiotic N_2 fixation is responsible for introducing millions of tons of N in plants annually. Rhizobacteria convert the atmospheric N_2 to NH_3 in special structures known as nodules, which is then exported to shoots in organic N forms. The understanding of the mechanisms involved in the biotransformation of N from elemental to plant-usable form is necessary as they play a pivotal role in legume growth. A number of conventional techniques have been employed earlier, but the advent of OMICS techniques has helped in deciphering the genetic, biochemical, molecular, and metabolic aspects of the rhizobia-legume relationship and its contribution to in planta fate of N (Bukhari et al. 2015).

6.2.1 Development of Nodules

Rhizobacteria activate the formation of nodules on the surfaces of legume roots. The bacteria release a variety of signaling molecules, primarily *Nod factors*, that interact with the protein kinases on the surfaces of the plant roots and through a Ca^{2+}-mediated process activate the nodule development genes (Okazaki et al. 2015). This signaling process leads to the formation of infections threads (cell wall-derived tubular structures that help in the binding of the rhizobacteria) and, later on, to the nodular development. This process helps in the formation of the symbiosome that comprises of bacteria surrounded by the plasma membrane of the legume cells (Clarke et al. 2014). The development process continues until a large number of bacteria are present within the plant's infected cell. Once a significant bacterial population is achieved, the N_2-fixing genes (*nif* and *fix*) help in the conversion of the bacteria to the fully functional forms known as bacteroids (Poza-Carrión et al. 2014). These bacterial forms undergo repeated endoreduplication or chromosome replication leading to the formation of larger progenitors of bacterial cells that are surrounded by highly efficient, uninfected vascular tissue (Suzaki et al. 2014). Such nodular structures are comprised of a growing zone (actively dividing bacteria), invasion zone (tissue penetrating bacteria), transition zone (growing, noninvasive stage), N_2-fixation zone (functionally viable rhizobacteria), and senescence zone (dead, degrading bacteria) based on the growth stage of the bacteria (Zgadzaj et al. 2015; Long 2015). Leghemoglobins, among many other molecules, are the proteins that play an essential role during the nodule formation process (Becana et al. 2015). The development of the nodules, therefore, facilitates the N absorption and assimilation process, thereafter.

6.2.2 Absorption of N by Legumes

Soil N is quite heterogeneous in terms of distribution as well as its chemical and physical forms. Plants rely on various systems to replenish their N needs. Chief among these is the N cycle that helps in the acquisition of the element through a series of steps. Most of the soil N is present in the form of complex organic compounds. Bacteria convert these heterogeneous compounds to ammonium (NH_4^+) through mineralization (Curtin et al. 2014). NH_4^+ is then converted to nitrate (NO_3^-) by the nitrification process (Stein and Klotz 2016). These are then converted to N gases (N_2/nitrous oxide/nitrogen dioxide/nitric oxide) through the denitrification process. The soil microbes can also utilize the inorganic N and immobilize it (Babar et al. 2016). The availability of N to the legume is, hence, dependent on the complex balance between mineralization, nitrification, and denitrification. A number of factors affect this sensitive balance including the water levels of the soil, moisture content of the atmosphere, aeration of the soil, diurnal cycle, and temperature and pH of the soil (Fowler et al. 2013). Moreover, the relative levels of various elements, especially carbon (C) and P, and their metabolic pathways also affect the degree to which the plants can absorb a particular element. The essential role of P in regulating various legume activities is summarized in Fig. 6.2.

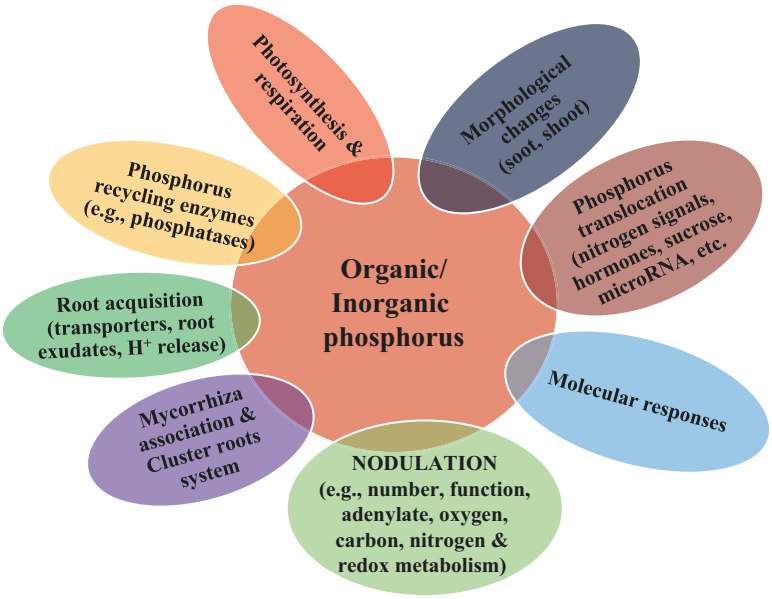

Fig. 6.2 The role of phosphorus in various biological processes within the legumes. Phosphorus affects multiple morphological, physiological, and metabolic processes of the legumes including the absorption, assimilation, and metabolism of nitrogen

N, whether in NO_3^- or NH_4^+ form, is transported through a number of specialized transporters. NO_3^- is transported across the plasma membrane by active transport. However, the net amount present within the cell is dependent on the balance between the active influx and the passive efflux of NO_3^-. NO_3^- movement is coupled with the movement of a pair of protons (H^+) out of the cell and is dependent on the ATP supply (Xu et al. 2012). The mechanism of transport at the levels of various organelles is different, for instance, an antiport mechanism has been suggested for the transport of NO_3^- across the tonoplast of the vacuoles (Babar et al. 2014). Moreover, the presence of a high-affinity transport system (HATS) and low-affinity transport system (LATS) has also been suggested for NO_3^- transport at various concentrations (Krapp et al. 2014). A gene family, represented by NO_3^- transporters (*NRT*), has been identified that is efficiently involved in the NO_3^- transport in various plants (Bai et al. 2013). In *Arabidopsis*, 52 different members of this transporter family have been identified (Lezhneva et al. 2014; Morcuende et al. 2007). Studies have reported the relative and transient expression of these NO_3^- transporters. Certain members, for instance, *NRT1*, are not very specific for NO_3^- and are also involved in the transport of certain amino acids and peptides (Léran et al. 2014). The efflux mechanisms for NO_3^- are more specific, are protein mediated, and operate through passive transport. The activation of the NO_3^- transport is, hence, controlled by the overall demand of the whole plant.

Similar to the NO_3^- transporters, legumes possess a number of genes that encode for the NH_4^+ transporters (*AMT*) (Takanashi and Yazaki 2014). The *AMT*s are constitutively expressed in the roots and considered responsible for the

absorption and transport of NH_4^+. Regulated by the diurnal cycles, these transporters are considered to play specific role in NH_4^+ absorption in all cellular types alike and not only by the root tissues (Von Wittgenstein et al. 2014). Yuan and colleagues reported the expression of the plant *AMT1* gene through the induction by rhizobacteria (Yuan et al. 2013). Various underlying mechanisms of action of these AMT transporters have been reported including potassium (K^+)-coupled transport, H^+-coupled transport, and channel-based (aquaporins) and/or ATP-mediated active transport (Li et al. 2014). Aquaporins have also been indicated in the efflux of NH_4^+ across various membranes.

Moreover, there are a number of gene families that are involved in the transport of amino acids, urea, N bases, and other N-containing heterocyclic compounds. A group of transporter proteins known as the oligopeptide transporters, for instance, are involved in the transport of these N compounds within the plant body (Léran et al. 2014). Most of these transport systems are nonselective and are involved in the movement of these compounds without any preference for any particular type of chemical entity. However, certain families of these proteins preferentially transport certain amino acids like lysine, histidine, and proline (Tegeder 2014). Such transporters are expressed in certain parts of the plant body and are actively involved in performing their functions in those particular plant parts. For example, the histidine and proline transporters are expressed at a greater level in the root of the plant. In addition to being selective for specific parts of the plant body, the expression of these transporter proteins is regulated by the environmental and nutritional conditions of the plant (Heath et al. 2012). Some transporters are likely to be expressed in environmentally harsh conditions like in the absence of certain mineral or organic compounds.

6.2.3 Assimilation of N in Legumes

After the acquisition of nitrogenous compounds, they have to be converted in specific forms that ensure the complete assimilation of these substances. The assimilation of NH_4^+, the end product of NO_3^- reduction (carried out by NO_3^- reductase and nitrite reductase), forms the most essential part of the process (Zhang et al. 2016). In nodulating legumes, the plants establish a symbiotic relationship with the rhizobacteria to enhance the absorption of NH_4^+ by the nodules. Furthermore, the secondary NH_4^+ assimilation, comprising of the photorespiration, phenylpropanoid biosynthesis, and/or amino acid metabolism, also depends on the essential steps of acquisition of NH_4^+ in required amounts (Balaur et al. 2013; Turgut-Kara and Cakir 2015). At the cellular level, the uptake of NH_4^+ by the roots leads to the generation of an electrochemical and pH gradient. The depolarization of plasma membrane and the accompanying liberation of H^+ lead to the acidification of the rhizosphere. The NH_4^+ is then incorporated into glutamate by the mediation of glutamine synthase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) (Betti et al. 2012; Pérez-Delgado et al. 2014). This is the prime source of incorporation of NH_4^+ into amino acids. Thereafter,

the NH_4^+/NO_3^- transport across the plasmalemma of root cells occurs through a H^+/NO_3^- symport system. NO_3^- reductase catalyzes the generation of nitrogen dioxide which is then converted to NH_4^+ in the plastids (Tortosa et al. 2015).

Legumes attain the atmospheric N_2 by establishing symbiotic relationship with rhizobacteria. The nodules, as mentioned earlier, are means developed by the plants which aid the N_2 fixation process. Regarding the assimilation of nitrogenous compounds, the energy and C skeletons are delivered as sucrose via the phloem (Atkins 2013, Fig. 3). The low concentration of sucrose and absence of fructose in the nodule apoplast help in the symplastic unloading from the phloem (Benitez-Alfonso 2014). This is associated with invertase activity and hexose transport. These sugars are degraded by the glycolytic pathway and then imported into the bacteroids and subsequently involved in the nitrogenase-catalyzed N_2 fixation process (Provorov and Vorobyov 2014). NH_4^+, the end product of N_2 fixation, is then assimilated by the GS/GOGAT cycle (Fig. 6.3). Amides and ureides are exported to the shoot through the xylem vessels (Flemetakis and Wang 2013; Todd 2016). A concentration gradient between the vessels, the symbiosome, and the cytosol helps in the release of NH_4^+ into the cytosol and the resultant nitrogenase activity.

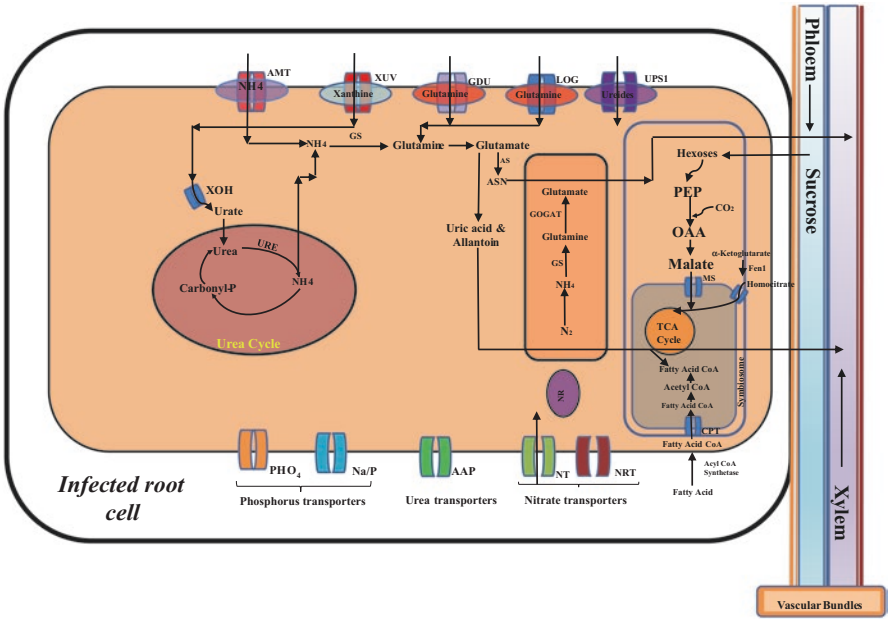

Fig. 6.3 A simplified presentation of carbon and nitrogen metabolic processes within the legume nodules. Various ions enter the system and drive the N_2 fixation metabolism. Once it has been processed, it is transported to the plant from where it is shifted to the shoots through the phloem tissue. The plants provide various ions to the bacteroid (H^+ proton, *Pi* inorganic phosphate, *ATP/ADP* adenosine tri/diphosphate, *Sst1* sulfate transporter 1, *MgtE* magnesium transporter, *Mol* molybdenum transporter, N_2 diatomic nitrogen, NH_4^+ ammonium ion (organic form), *GS* glutamate synthase, *GOGAT* glutamine oxoglutarate transferase, *Gln* glutamine, *Fen1* flap endonuclease) (Adapted from Udvardi and Poole 2013)

6.2.4 Vascular Transport of N in Legumes

The synthesis of nitrogenous compounds including amino acids occur mainly in the roots and the mature leaves that are then involved in the transport of N to other sinks like fruits, root tips, fruits, or seeds. In nodulated legumes, for instance, the ureides are the major form for the long-distance transport of N. The allantoin and allantoic acid are produced and then transported to the shoot where NH_3 is released and reassimilated into amino acids (Redden et al. 2013). Among the vascular tissues, xylem is involved in the transport of amides and ureides, while phloem is involved in the transport of organic N from leaves to other sink tissues. The membrane transport proteins are involved in the selective partitioning of amides and ureides among different organelles and cells (Tegeder 2014). Once the N enters the plant body through the root hairs and epidermal cells, the cell-to-cell transport occurs via plasmodesmata. The Casparian strip blocks the apoplasmic flow once the required amounts have entered the plant body (Okamoto et al. 2016). This is followed by the xylem-loading mechanism which is involved in the N export in the apoplasm, pericycle, or xylem parenchymal cells. Moreover, the N loading into the sieve element-companion cell complexes (SE-CC) of the phloem also occurs through the apoplasmic pathway (Liesche and Schulz 2013). Specialized transporter proteins, like NRT1.5 or SIAR1, are present in the plasma membrane of the pericycle cells and help in exporting the amino acids (including glutamine) for xylem loading (Jauregui et al. 2016; Müller et al. 2015). Similarly, BAT1 or bidirectional amino acid transporter releases organic N into the xylem apoplast for transport from the root to the shoot cells (Jewell et al. 2013). Moreover, it is also involved in the phloem unloading. Other examples of amino acid transporters include glutamine dumper (GDU), loss of GDU (LOG), and amino acid transporter (AAP) (Kan et al. 2015; Pratelli et al. 2012; Sze and Pilot 2014).

The conversion of N_2 to NH_3 in bacteroids is followed by the release of NH_4^+ and amino acids within the nodule cells. This leads to the synthesis of glutamine and asparagine and/or purine degradation, depending upon the legume species. The consequent production of ureide (allantoin) and then its transport through the vascular tissue follow. Much similar to the amino acid transporters, the ureide transporters help in specifically transporting it to the respective tissue. *GmUPS1* and *PvUPS* are generally involved in the ureide transport in the soybean, pea, and common bean (Carter and Tegeder 2016; Girke et al. 2014; Pélissier et al. 2004). Similarly, in ureide-transporting legumes, the N retrieved from the stem xylem acts as a temporary pool for storage. The recent discovery of *AtAAP2*, *PsAAP1*, and *PvAAP1* in Arabidopsis, pea, and common bean helped in revealing important ATP-dependent import systems in the respective plant species (Sze and Pilot 2014). Similarly, amino acid transporters like CAT6, CAT9, and ProT1 also facilitate the phloem loading of many legumes (Yang et al. 2010; Roychoudhury et al. 2015). Conversely, an aromatic neutral amino acid transporter, ANT1, helps in removing the organic N from the phloem (Sze and Pilot 2014). AMT is similarly involved in the transport of NH_3 in many leguminous plants (Guo et al. 2013).

The amount of N within the legume body helps in the control and maintenance of nodule development and N_2 fixation. UPS1 transporters not only facilitate the movement of ureides but also help in the nodulation mechanisms (Carter and Tegeder 2016). Since it is involved in the ureide delivery to the shoot, the repression of these transporters leads to the altered nodule development and N_2 fixation processes. On the other hand, the overexpression of *UPS1* and other amino acid transporters in the nodule cortex and the endodermis can potentially lead to an increased ureide and amide export (Baral et al. 2016). This is generally, in turn, related to an enhanced pooling of N in the shoot tissues and exaggerated shoot-root signaling processes (Foo et al. 2015). Hence, these transport systems are not only involved in transport of various forms of N between different tissues of the legumes but are also helpful in regulating the physiological and morphological characteristics of the plant.

6.2.5 N Utilization and Biotransformation in Nodules

The recent advances in the field of genomics have led to the identification of various molecular players involved in the N metabolism within the legume body. Genome-wide expression studies have helped in deciphering the role of various enzyme systems that aid the metabolism of N compounds. During the nodule development, many metabolic pathways are upregulated including glycolysis, purine and heme synthesis, carbon dioxide (CO_2) fixation, amino acid biosynthesis, and redox metabolism (Udvardi and Poole 2013; Verma et al. 2013; Dunn 2015; Pandey et al. 2015). Similarly, during the nodule development, a number of sensors and signaling mechanisms are developed that serve to identify and respond to the hypoxia, osmotic stress, and P limitation. Bacteroids act as a major P sink in the infected plants, and they generally pair up with phospholipids to form galactolipids that help in P conservation (Okazaki et al. 2013). Figure 6.3 represents the N utilization and biotransformation within the nodules of the leguminous plants.

A number of recent studies have helped in the identification of various metabolic pathways involved in the N biotransformation. These pathways act in conjunction with the carbohydrate and lipid bioprocesses leading, ultimately, to the nodule development and their physiological survival. However, the studies of the whole metabolome of the nodules have been unable to provide the exact information regarding the subcellular localization of these metabolic players. Over the past years, a number of studies have concentrated on studying the N metabolism and utilization within the bacteroids. In nodulating legumes, the C metabolism is mainly dominated by the organic forms (aromatic amino acids, C1 and C2 compounds) (Flemetakis and Wang 2013). The regulation of the metabolic processes is controlled by the legumes and surrounding conditions. The gene expression is intelligently controlled by the legumes, and many of these are species specific as well as age specific. The dicarboxylate, phenylalanine, and malonate catabolism are highly expressed in early root nodule bacteria, while as the bacteroids mature, dicarboxylate catabolism is the mainstay of this system (Ahsan and Salomon 2016).The dicarboxylate and the

malonate enzyme systems remain essential elements for N_2 fixation (Karunakaran et al. 2013). Malic enzyme and the combined activity of phosphoenolpyruvate (PEP) carboxykinase convert malate to pyruvate which is needed for acetyl-CoA synthesis and subsequently condensed with oxaloacetate (from malate dehydrogenase) in order to feed the TCA cycle (Udvardi and Poole 2013; Schenck et al. 2015). In many rhizobia, including *Rhizobium leguminosarum, Sinorhizobium meliloti*, and *Bradyrhizobium* species, the TCA cycle is the prerequisite for the N_2 fixation process (Udvardi and Poole 2013). However, in certain legume species, soybean, for instance, very different pathways are adopted for pyruvate metabolism (Allen and Young 2013). Many of the rhizobacteria receive dicarboxylates as the main C source for nitrogenase activity. Hence, they are dependent on sugars for the general biosynthesis and glycogen as a mean for storage of chemical energy (Kaur et al. 2016). The peribacteroid membranes possess a very selective permeability toward sugars, and, hence, gluconeogenesis appears to be an important process for bacteroids (Day et al. 1990). Moreover, redox balance and storage of C polymers by the bacteroids as poly-β-hydroxybutyrate (PHB) or even as lipid droplets are carried out by the bacteroids (Jiao et al. 2015). PHB is synthesized from two molecules of acetyl-CoA and mainly stored by the determinate nodules of legumes like common bean and soybean. Hence, the dependence of N_2 fixation and metabolism within the legume body is highly dependent upon the C metabolism (Sulieman et al. 2010). Rhizobacteria utilize the PHB, glycogen, lipid, or other products for regulating the C flux to facilitate the N-metabolic processes. The end products of the C metabolism are extremely useful in the N metabolism of the N_2-fixing bacteria (Sulieman et al. 2014). Within the nodule, the N combines with hydrogen to initially form NH_3, followed by NH_4^+. These ions help in the synthesis of various amino acids such as glutamine, glutamate, and aspartate which then facilitate the preparation of arginine, asparagine, nonprotein amino acids, and purine (Galili et al. 2016). Purines are subsequently converted to allantoin. All these components are then transferred to the xylem tissue for transport to other parts of the legumes. Within the root cells, the NO_3^- and NH_4^+ are converted to amides and ureides. These end products, along with NO_3^-, are transferred via the xylem tissue to the leaves and other aerial parts to produce amino acids, which are distributed through the phloem tissue throughout the legume body. GS and GOGAT enzyme cycle mediate the N metabolism as indicated above. Additionally, a number of ion transporters are also involved in these metabolic pathways like molybdenum transporter, sulfate transporter, and others (Gao et al. 2016; Zuber et al. 2013).

6.3 Plant Sensors and Cellular Communication

In legumes, plant sensors for detecting changes in the levels of elements, radicals, and compounds serve as an excellent means to facilitate the feedback mechanisms. In certain legumes, for instance, alfalfa and pea, there is a formation of indeterminate rooting nodules that arise from the inner root system and middle cortical cells and grow through the meristem (Xiao et al. 2014; Mortier et al. 2014). This occurs,

as discussed earlier, in response to the infection of the symbiotic bacteria to the inner most root system facilitating, thereby, the formation of infection threads. A number of genetic and cellular events are involved in this process. Initially, the plant roots generate a signal that activates the nodulation genes in rhizobia which in turn stimulates the induction of nodule meristem and the formation of an infection thread. Acyl homoserine lactones (AHLs) are believed to carry the instructions from the rhizobia to the plants (Zarkani et al. 2013). Similarly, during the N_2 fixation process, a series of nodulation genes are expressed that coordinate the process of nodule formation. These include the *nodA, nodB, nodC, nodE, nodF, nodL, nodM*, and *nodN* as observed in pea plants (Long 2015; Okazaki et al. 2015). In the following subsections, the cellular signaling of N and P within the legumes is discussed.

6.3.1 N Sensing and Signaling

Legumes play an important role as these plants serve as an excellent source to assimilate N in the environment. Contributing to the overall plant productivity, N strongly regulates the root architecture (Niu et al. 2012). For this purpose, NO_3^- and NH_4^+ transceptors provide the sensory components (Pellizzaro et al. 2015). The signals for N availability are mediated through an array of phytohormone pathways. Small molecules like microRNAs and peptides mediate the phytohormone signals resulting in the production of highly dynamic root responses (Lacombe and Achard 2016). These signals, hence, serve as an excellent means to facilitate the N-use efficiency within the legume plant. In these plants, the local as well as systemic controls mediate the N-associated root architecture regulation. High-N patches within the soil stimulate the lateral root elongation, while in case of the systematic pathways, root architecture is dictated by the plant's overall N status (Ruzicka et al. 2012). These pathways also adjust the nodule number. The earliest formed nodules stimulate the autoregulation process, which suppresses further nodule formation in the younger root regions. At the molecular level, the first induced nodules produce the root-derived signal (Q) that travel through the xylem to the shoot where they are sensed by the leucine-rich repeat receptor-like kinase (LRR-RLK) (Prince et al. 2014). Many analogues of this kinase system have been identified in nodulating legumes. Similarly, the Q sensing is followed by the production of shoot-derived inhibitor (SDI) that suppresses the subsequent nodulation of the root system (Huang et al. 2014). These signals, hence, regulate the systemic control through the rhizobia based N_2 fixation and regulation of nodulation in terms of number. Conversely, high NO_3^- levels within the local tissue also inhibit nodule formation.

The availability and concentration of N can change the overall root system architecture (RSA) of the plant. In many plants, these changes include the growth of primary roots, lateral roots, and their elongation (Fagard et al. 2014). Many transporters, including NRT1.1 (dual-affinity NO_3^- transporter) and NRT2.1 (high-affinity NO_3^- transporter), have been found to have NO_3^- signaling abilities, thereby controlling the growth of primary and lateral roots (Léran et al. 2013; Li et al. 2016).

These proteins can modulate the lateral growth rate in response to a high C/N ratio and low NO_3^- supply, respectively. Similarly, NRT1.1 regulates the root system architecture by altering the N transport and signaling mechanisms. A high localized NO_3^- concentration also stimulates lateral root elongation through NRT1.1-mediated pathways. The downstream signaling of the NRT1.1 involves the calcineurin B-like proteins (CBLs) and CBL kinases that act as Ca^{2+} sensors (Poovaiah et al. 2013; Miller et al. 2013). These proteins act as bridges between NO_3^- and Ca^{2+} signaling in the presence of compounds that decrease the availability of Ca^{2+} including Ca^{2+} chelators and other blocking agents (Choi et al. 2016).

Plant-derived microRNAs also affect the N-signaling pathways (Fischer et al. 2013). As demonstrated by transcriptomic analyses, the NO_3^- levels within Arabidopsis are affected by the microRNAs (Khraiwesh et al. 2012). Similarly, certain phytohormones including auxins, abscisic acid (ABA), and cytokinins also regulate the growth and development of plants. Auxins cause the inhibition of lateral growth of roots as well as an induction of transporter proteins (like NRT1.1) in high NO_3^- concentrations (Fernández-Marcos et al. 2013; van Noorden and Mathesius 2013). They also regulate the activation and expression of genes that are involved in auxin signaling pathways, auxin response factors (ARF), and auxins to indole acetic acid (IAA) ratio. ABA also regulates the root architecture in response to endogenous NO_3^- supply (Miyakawa et al. 2013). Similarly, cytokinin-dependent pathways also control the protein synthesis and development. *Isopentenyl transferase 3* (*IPT3*) encodes crucial enzymes that are expressed in response to varying concentration of NO_3^- (Hao et al. 2016). Cytokinins are also involved in carrying the sensory signals of N from the roots to the aerial parts of the plants (Hammond et al. 2004). Hence, a variety of signals facilitate the N signaling and related effector functions within the nodulating legumes.

6.3.2 P Sensing and Signaling

In order to react to P deficiency, the nodulating legumes must be able to identify the specific signals. The P signals mediate the metabolic processes including in the N metabolism. A number of recent studies conducted on Arabidopsis and numerous legumes have been able to identify the involvement of a number of transcription factors (TFs) including *MYB* (*PHRI*), *WRKY* (*WRKY75*), and *bHLH* (*OsPTFI*) in the signaling pathway of P (Pant et al. 2015; Devaiah et al. 2007; Yi et al. 2005). However, the exact genes that are involved in the process are not well characterized. It has also been studied that certain microRNAs interact with ubiquitin-conjugating enzyme (UBC) and play an important part in the response to P stress in Arabidopsis (Fujii et al. 2005). Microarray-based genomic studies are underway to identify the signaling and regulating genes involved in the process. In one published report on common bean roots, tens of genes have been found to contribute to the signal transduction and regulation (Hammond et al. 2004). Representatives of *MYB*, *UBC*, and *Bhlh* genes, families were also found to be up- or downregulated in expression. A number of

members of *MYB* superfamily have also been established as sensors of P deficiency. Among these, the *TC2883* had the greatest resemblance with a *MYB* gene implicated in response to P deficiency (Hernández et al. 2007). The ortholog in case of Arabidopsis is *PHRl*. Similarly, three genes encoding the *WRKY75*, *PHO2*, and *SIZ1* have been indicated to take part in the acquisition, homeostasis, and signal transduction rising from the P stress (Hernández et al. 2007). Similarly, *Capsicum annuum* putative U-box protein 1 (*CaPUB1*) is overexpressed in a number of nodulating legumes under a wide range of abiotic stress conditions (Hur et al. 2012). A number of research groups are currently focusing on the identification of sensors and signaling pathways involved in the P-stress conditions (Kucukoglu and Nilsson 2015).

At the metabolic level, P deficiency leads to an altered carbohydrate and amino acid metabolism that serves as indicative hallmarks of this abiotic stress. Many recent studies have indicated that both P signaling and carbohydrate metabolism are closely connected. In lupin, Arabidopsis and common bean, more carbohydrates are partitioned toward the root fractions when plants exposed to P scarcity (Balemi and Negisho 2012). The amount of useable carbohydrates is controlled by the genes induced by P deficiency. Similarly, the lack of sugars leads to an inhibition in the expression of stress-induced genes. It has been observed that the amount of sugars in P-stress roots is significantly higher in comparison to the adequately P-nourished root systems. The PRL1-associated protein, encoded by the *CV543658*, is expressed greatly in P-stressed bean roots and acts along with the SNF1 protein kinase system to oppress metabolism of glucose (Bhalerao et al. 1999). It also alters the synthesis of starch and blocks root elongation. Similarly, organic acids are also depleted in P-stressed roots primarily due to the altered activity of isocitrate dehydrogenase (ICD) in the TCA cycle. The molecular mechanisms involved in this process remain to be elucidated (Wang et al. 2014).

In conjunction with P stress and carbohydrate metabolic alteration, the N in terms of amino acid and protein metabolism are also affected. Many transcripts involved in P deficiency are similar to those expressed in other abiotic stress conditions including salinity, anoxia, and wounding. These stress factors lead to an enhanced peroxidation of lipids and an increased activity of the peroxidase enzymes (Hernández et al. 2007). Some published reports have revealed an increased expression of genes encoding these enzymes in the nodular structures of P-stressed plants. Moreover, it has been found that the same set of genes is upregulated in response to P deficiency that are overexpressed in N and K deficiency. In addition to the changes observed in the roots and the rest of the legume body, P deficiency reduces nodule number and biomass. Moreover, there is an inhibition in N_2 fixation capability due to the apparent reduction in the efficiency to transfer electrons to the functioning nitrogenase (Zhu et al. 2016; Vardien et al. 2016). However, they do preserve a certain level of metabolic activity which is attained mainly by affecting the photosynthetic process and the supply of nonstructural carbohydrates to the nodules. Importantly, the nitrogenase activity is particularly regulated under such conditions (Sulieman et al. 2013).

Additionally, the inorganic phosphate transporter system is overexpressed in the stress conditions (Vanek and Lehmann 2015; Nussaume et al. 2011). An enhanced expression of other types of transporters like the putative porin channel and acetyl glucosamine transporter is observed under such conditions (de María et al. 2007;

Zhukov et al. 2013). Moreover, an interplay between the balance of various plant hormones like auxin, cytokinin, and ethylene leads to the re-patterning of the root and shoot architecture. A decrease in the rate of photosynthesis has also been observed which can be related to an increased amount of carbohydrates within the P-stressed leguminous plants (Hernández and Munné-Bosch 2015). It can, hence, be concluded that P-stressful mechanisms are closely related to the N-metabolic pathways. The exact genes involved in these processes are not known. However, owing to the developments in the field of genomics, the interdependent nature of N, C, and P metabolisms has been deciphered.

6.4 Morphological and Physiological Adaptations in Legumes Under P Deficiency

Legumes have significant contributions to the topological environment and agricultural systems. They, like other plant species, are dependent upon the provision of suitable amount of nutrients. Any alteration or deficiency in the nutrient content leads to adversely affecting legume growth and productivity. Being an essential element for the normal growth and development, P should be available in appropriate quantities to the cultivated legumes. Leguminous plants have developed a number of strategies to cope with low-P availability. The adopted mechanisms could help in maintaining the survival of the legume-microbe symbiosis (Provorov and Vorobyov 2013). A number of morphological, physiological, and biochemical strategies are noticed that help in promoting the allocation and utilization of greater amount of P in the nodulating-root system.

6.4.1 N_2 Fixation

P deficiency is a widespread constraint for plant growth over the majority of the earth's land surface (Alexova and Millar 2013). Under such unfavorable conditions, most N_2-fixing legumes have the ability to maintain higher levels of metabolism in their root nodules. However, the exact mechanisms by which the metabolic processes are maintained are still poorly understood. A decreased level of P causes plant growth reductions, thereby decreasing the potential-N demand and N_2-fixation capability, and ultimately leading to a downregulation of nitrogenase activity (Nasr Esfahani et al. 2016). This response varies with the severity and duration of the P stress. The legume brings about two set of changes in response to any abiotic stress factor; short term and long term. In response to P deficiency, the nutrient uptake and plant growth are closely connected. This leads to significant morphological modifications in the plant development including a decreased specific leaf area (SLA), altered phenotypic plasticity and reduced shoot mass (Lambers et al. 2013). All these changes affect the photosynthetic machinery of P-deficient plant. Apart from

these changes at the shoot level, the roots respond to P scarcity by bringing about a major reduction in the nodule mass and nitrogenase activity per unit root mass. However, it is important to note that the per unit nodule mass itself is not markedly affected. Hence, the regulatory mechanisms might include an increased P partitioning to root tissue, increasing the C acquisition by reducing the lead area and increasing the N acquisition by maintaining nodule mass (Sulieman and Tran 2014). In long-term steady-state P deprivation, a diminished N_2 fixation capability of the legumes has been observed. Under such conditions, the N_2 fixation rate is decreased owing to an N-feedback mechanism (indicated by an increased asparagine concentration in the phloem) (Tawaraya et al. 2014).

6.4.2 Enzymatic Regulation

P deficiency leads to a significant alteration in the N-metabolic pathways. The activity of a number of enzymes is altered under P limitations. The substrates of these enzymes vary with some acting directly on the metabolic pathways involved in the legume physiological maintenance while others interacting with members of the symbiotic relationship of legumes and rhizobacteria. The exudation of organic acids and acid phosphatases through the roots and nodules enhance the solubility of the P-bound forms leading ultimately to an increase in the uptake of unavailable P resources (Lazali et al. 2013, 2015). As discussed earlier, the host plant modifies the nitrogenase symbiotic activity in response to P deficiency, thereby controlling the overall N_2 fixation efficiency. Similarly, the two enzymes, PEP carboxylase and malate dehydrogenase, that play a crucial role in the nodule-C catabolism as well as cluster roots are upregulated under P stress (Shane et al. 2013; Liang et al. 2013). The end products of the TCA cycle, mainly ATP, are involved in the metabolism of other elements and compounds, for instance N.

6.4.3 Metabolic Pathway Regulation

The metabolic processes are highly regulated within the root nodules under P deficiency. Soybean has been studied in depth to understand its functioning according to the nodule energy status. The examination of the soybean has helped in establishing that the diminished nitrogenase activity is associated with the hindered ATP-dependent reactions occurring in the plant cell fraction of nodules (Collino et al. 2015). A number of studies have also related the increase in N_2 fixation under low-P availability, owing to the altered nitrogenase activity. Three hypotheses have been put forward to explain the possible mechanisms involved in the regulation of N_2 fixation in legume nodules (Magadlela et al. 2014, Fig. 2). The first hypothesis is based upon the C supply regulation which states that the nitrogenase activity is regulated by the nodule assimilation and supply of various C substances (Sulieman et al. 2014).

Secondly, there is a possibility that the oxygen (O_2) supply might be regulating the overall status of the nitrogenase activity (Bargaz et al. 2013). Lastly, the N-feedback mechanism in the nodules controls the N_2 fixation capacity of the legumes. In the last few decades, evidence for the involvement of oxidative stress as part of the nodule response to P stress has also been described (Nasr Esfahani et al. 2016). Many researchers have correlated the differential C/N ratio as the ultimate drivers of the nitrogenase activity in legume nodules under P scarcity (Cauwanberghe et al. 2015; Goh et al. 2016). The N-feedback inhibition has been established as the master mechanism responsible for the downregulation of nitrogenase activity under many abiotic stress conditions including P deficiency. Similarly, the decrease in the anabolic processes also leads to a shortage in the provision of carbohydrates to the functioning nodules. A deficiency of P causes a decrease in the activity of enzyme and the rate of photosynthesis in the leaves, thus leading to an altered C provision. However, the concentration of starch in the leaves is elevated in a steady manner with the sink-limited photosynthesis (Pessarakli et al. 2015). In the nodules of P-stressed plants, a remarkable decrease in water-soluble carbohydrates has been observed in comparison to the roots. This might suggest that the nodules are comparatively weaker sink for such compounds and are not dependent on the C metabolism (Hermans et al. 2006). The sufficient availability of C source and the normal running of the TCA cycle, during the initial phases of P deprivation, ensure the provision of energy for the bioconversion of N_2 to NH_4^+ within the functioning bacteroids. However, over a longer period of time, the C as well as N substances are particularly exhausted, and the host plant as well as the nodules enters a highly stressed state leading, ultimately, to a decreased metabolic activity.

6.5 N-Feedback Regulation Under P Deficiency

The N metabolism serves as a major regulator of nodule growth and development. As discussed above, the host plant develops a number of mechanisms to ensure that the overall metabolic state is maintained under low P conditions. Similarly, the N_2 fixation is altered which set a series of signals to ensure that the overall homeostasis is maintained. The N metabolism and the associated N-feedback mechanisms under P-depleted conditions, hence, form the basis for regulating the whole-plant metabolism.

Under P suboptimal conditions, the symbiotic nodules are generally formed in the same manner as under normal, P-sufficient conditions (Sulieman and Tran 2014). The nodules continue to develop even after the plant does not have sufficient P level. However, in the long term, the development of nodules is highly dependent upon the continued provision of the macronutrients P in particular. In case the plant has fewer nodules, the size of the nodules is larger. However, in case there are a greater number of nodules, the size is decreased, accordingly (Vardien et al. 2014, Fig. 1). The mechanism of the size regulation is not very well understood. There are two possible hypotheses, generally, considered relevant to explain this feedback mechanism. Firstly, there is a possibility that the carbohydrate deprivation of the

nodules leads to the generation of a positive feedback mechanism in which a greater number of nodules are produced (Tajini and Drevon 2014). Contrarily, the alternate hypothesis is that there is a nitrite-based inhibition of the nitrogenase activity leading to a positive stimulus for the growth of a greater number of nodules in the roots of the leguminous plants (Singh and Singh 2016).

The feedback systems generally employed by leguminous plants are organized by cycling of nutrients within the plant. For instance, the uptake of many ions, including P, is highly regulated by the ion level in the phloem tissue. In general, the nodulating legumes have an abundant amount of N even under P-deprivation conditions. A group of transporters, enzymes, and ions are thought to contribute effectively to the whole-plant N homeostasis. It has been proposed that a potential N-signal within the phloem sap is sent back to regulate the nitrogenase activity and nodular growth. Higher concentrations of reduced N (amino acids and amides) in the phloem tissue are negatively correlated with the nodular growth and activity (Lin et al. 2014). Pending on the nodule-N export product, there are different players involved in this regulation. In the case of amide-exporting nodules (e.g., pea and lupin), amino acids such as glutamate, serine, and proline are mainly involved in the regulation process. Conversely, for ureide exporters, the amount of ureides and some amides (glutamine and asparagine) are the major modulators of nodule growth and activity. Hence, the nodule symbiotic activity will be regulated according to the potential-N demand. Within the symbiotic tissue, the phloem-N signal might move into the surrounding cortex, possibly changing the gaseous resistance level in the nodule cortex, thereby affecting the provision of O_2 to the infected zone. Accordingly, the translocation and metabolism of sucrose and/or the N assimilatory products out of nodules might be greatly affected. In accordance, the ^{15}N rhizodeposition studies have provided evidences that support an altered movement and assimilation of N in plants when exposed to P deficiency (He and Dijkistra 2015). Among the N-feedback potential regulators, asparagine has been characterized as a prime candidate that strongly modulates nitrogenase activity under P deficiency (Sulieman et al. 2010). It has been reported that phloem-feeding asparagine is translocated to the nodulating root and subsequently downregulated nitrogenase activity (Sulieman et al. 2013). Hence, this amide has been considered as one of the major signaling mediators that involve in the communication between the host plant and the nodules. Moreover, it has been also considered an essential compound involved in the transport and storage of N in many leguminous plants. Mechanistically, the involvement of this amide in restricting the C supply during the symbiotic communication and/or altering the O_2 diffusion barrier has been proposed (Sulieman et al. 2014).

6.6 Conclusion

It is very crucial for leguminous plants to have optimum-N concentrations for their growth and development. P is one of the most important nutrients for plant growth and metabolism. Not surprisingly, P deficiency causes negative impacts on plant N

and C concentrations and subsequently the overall growth and development. Although P is abundant in the soil, its availability is commonly reduced due to many reasons related to the physical and chemical characteristics of the soil. The inhibition of the P-dependent biochemical processes and C turnover leads to a suboptimal P availability. This is extremely detrimental to the legumes and causes a number of negative impacts on the nodular level such as the retardation of the nodule formation and downregulation of nitrogenase activity. To cope with P deficiency, nodulated plants develop a number of morphological, physiological, and biochemical strategies in respect with N transport and metabolism. For instance, a higher concentration of N and amino acids were detected in the shoot, phloem sap, and nodules of P-stressed plants. Accordingly, a whole-plant N-feedback mechanism was suggested to regulate nodulation under low-P conditions. Nonetheless, the satiety of the shoot-N signal remains to be elucidated. Fortunately, the rapid development of omics techniques has helped in deciphering many important molecular and cellular mechanisms relating to N metabolism in plants experiencing P deficiency. Understanding these mechanisms would help to open up a new horizon for the production of better P-adapted legumes that ensure proper growth and development. The plant biotechnologists and breeders are currently focusing on defining elite traits that could pave the road for designing the future P-adaptive cultivars.

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Chapter 7
Examples of Belowground Mechanisms Enabling Legumes to Mitigate Phosphorus Deficiency

Mohamed Lazali and Adnane Bargaz

Abstract Legumes improve agricultural sustainability through symbiotic dinitrogen (N_2) fixation which constitutes a major input into agroecosystems and may provide an ecologically acceptable complement or substitute for mineral nitrogen fertilizers. However, low soil nutrient availability, notably phosphorus (P), is among the most nutrient limitations for legumes whose sensitivity to P deficiency has been attributed to low soil P availability and higher P requirements during the symbiotic N_2 fixation process. In response to P deficiency, plants use various adaptive strategies to improve soil P availability and their uptake efficiency, which involves modifications in nodulated-root architecture, rhizosphere acidification, and induction of genes involved in P use efficiency such as high-affinity P transporters and P-hydrolyzing phosphatases enzymes. This chapter reports numerous legume tolerance strategies to P deficiency that link morphological, physiological, and molecular responses. Stimulation of the root's extracellular potentialities to improve solubilization and acquisition of the rhizosphere soil P as well as optimization of intracellular use efficiency and allocation of P has been described. Coincident with most knowledge on legume performance under P deficiency, exploration of biotic factors with synergistic and complementary interactions for the benefit of both plants and soil microorganisms is increasingly adopted. A holistic understanding of the key mechanisms underlying legume tolerance to abiotic constraints will be valuable for strategies to improve symbiotic N_2 fixation and sustainable agriculture in a world of increasing population and declining renewable resources.

Keywords Legumes • Abiotic stress • Phosphorus • N_2 fixation • Nutrient use efficiency • Microorganisms

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7.1 Introduction

Symbiotic dinitrogen fixation (SNF) by legumes plays an important role in sustaining crop productivity and maintaining soil fertility, especially in marginal lands. The importance of legumes and SNF is anticipated to expand with the development of sustainable agriculture. However, SNF is particularly sensitive to some environmental constraints such as low phosphorus (P) availability (Bargaz et al. 2015; Drevon et al. 2015). Its deficiency, especially in legumes, affects nodule formation and functions giving the highly intricate symbiotic relationship between the rhizobial bacteria and the host plant legume (Alikhani et al. 2006). By contrast to nitrogen (N), there is no atmospheric source of P that could be made available to plants (Ezawa et al. 2002), and soils generally contain only a small amount of available P that is readily available for plant uptake. Therefore, P is applied as fertilizer to replenish the soil as to satisfy plant requirements (Richardson et al. 2009).

The availability of P to the legumes is limited by various properties of the soil itself and is largely determined by solubilization of P-containing compounds and P diffusion rates in the soil (Hinsinger 2001). Phosphate readily chelates to metal cations, clay particles, and organic soil material rendering it unavailable for plant uptake (Abel et al. 2002; Vance et al. 2003). Soil P is also influenced by pH, ionic strength, adsorption, and dissolution from these particles (Vance et al. 2003). Slow soil diffusion rates and fast root uptake transporters cause a rapid depletion of P in the rhizosphere, leading to irregular P distribution in the soil (Lambers et al. 2006). Organic and inorganic compounds readily interact and bind to P (Raghothama 1999).

Improving P nutrition of legumes under P-deficient conditions has generally involved two potential physiological strategies, depending on their ability to adjust their external and internal P requirements (Drevon et al. 2015). The first strategy involves plant–soil interactions such as modification of soil exploitation by roots, improved interactions with soil microorganisms such as mycorrhizal fungi, root endophytic microbes, and rhizosphere modifications that increase P availability (Hinsinger 2001; Richardson et al. 2011). The second strategy involves efficient partitioning and subsequent utilization of internal P through the use of plants that yield more per unit of P uptake (Vance et al. 2003; Lambers et al. 2010). Phosphorus efficiency can be achieved through P acquisition efficiency, defined as the ability of the plant or genotype to acquire P from low P soils or by internal P use efficiency processes that determine the ability of a genotype or species to convert P into growth or yield once it is acquired from the soil (Schröder et al. 2011; Simpson et al. 2011; Lazali et al. 2016a). This chapter aims to highlight the biochemical and molecular mechanisms involved in legumes' tolerance to abiotic constraints. Recent knowledge on legumes will be explored focusing on the belowground soil–root interface in order to understand the mechanisms involved in mitigating P deficiency.

7.2 Absorption and Mobilization of Inorganic P

7.2.1 High-Affinity P Transporters

Inorganic P concentration in plant tissues has been measured at 5–20 mM (Raghothama 1999; Nussaume et al. 2011), whereas the level of P available fraction in soils is typically less than 10 μM (Hinsinger 2001). This sharp concentration gradient between the plant and the soil illustrates a crucial role of Pi transporters (Fig. 7.1). An energy mediated co-transport process, driven by protons generated by a plasma membrane H^+-ATPase, has been proposed as the mechanism of inorganic P (Pi) uptake in plants (Schachtman et al. 1998; Raghothama 1999). In plants, two P uptake systems have been identified, a high-affinity system that is either increased or de-repressed under P deficiency and a low affinity system that is constitutively expressed (Rausch and Bucher 2002; Javot et al. 2007). Plants can possess multiple P transporters of each system. Among the Pi transporter families, the *Pht1* family has been most widely studied due to its key roles in Pi acquisition from the soil and Pi translocation within the plants. Genes encoding the high-affinity Pi transporters are

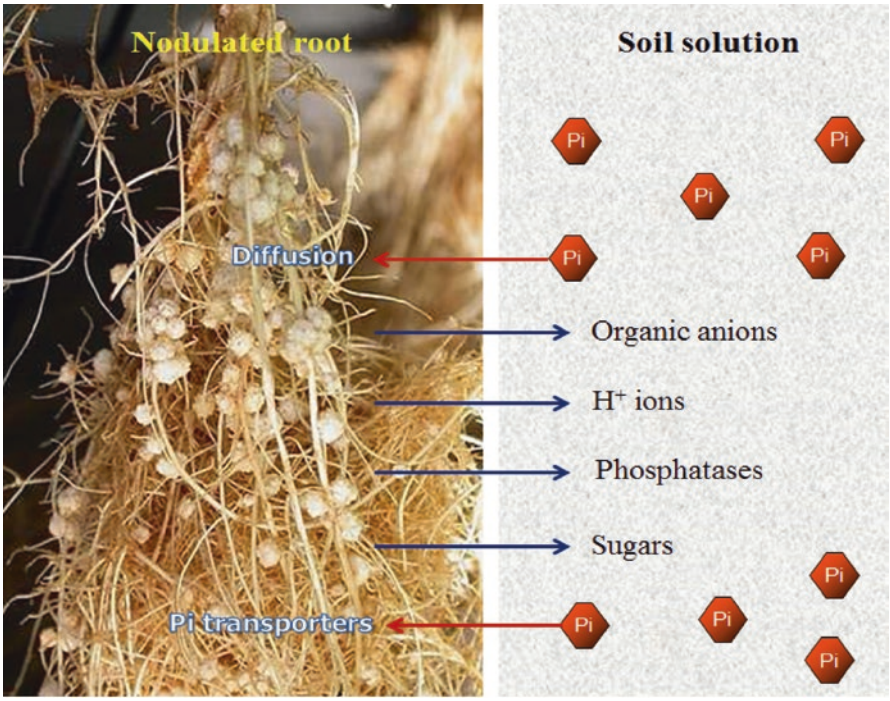

Fig. 7.1 Mechanisms of phosphorus acquisition by nodulated legume. The different mechanisms used by legumes to improve their absorption and mobilization of inorganic P include (i) higher exudation of organic acid and phosphatases, (ii) greater release of H^+ ions, and (iii) overexpression of high-affinity P transporters

preferentially expressed in Pi-starved roots (Leggewie et al. 1997; Liu et al. 1998). The expression of these genes in the root hairs and root epidermis indicates that these transporters are specifically targeted in those cell layers that are exposed to relatively high concentrations of Pi, thereby facilitating its uptake (Liu et al. 2008).

Studies by Muchhal and Raghothama (1999) show that Pi transporters are distributed along the entire length of the roots of Pi-starved plants. Moreover, a relatively uniform rate of Pi uptake was observed along the proteoid root axis. These data support the hypothesis that the entire root system retains the potential to transport Pi at an increased rate in response to Pi starvation (Muchhal and Raghothama 1999; Nussaume et al. 2011). The number of Pi transporter encoding genes that have been cloned from plants is rapidly increasing. In soybean, the high-affinity Pi transporter, *GmPT5*, regulates Pi entry from roots to the region of plant tissues in nodules and maintains Pi homeostasis in nodules, particularly under P-limiting conditions (Qin et al. 2012). The existence of multiple high-affinity Pi transporters is certainly a reflection of the complexity of the Pi transport process within plants. The study of the *Pht1* family of Pi transporters is particularly appropriate, due to their presence in the plant plasma membrane (Smith et al. 2000; Rausch and Bucher 2002; Smith and Barker 2002; Bucher 2007). Transcriptional activation of Pi transporters in response to Pi starvation seems to be a major regulatory mechanism for Pi uptake. Apparently, P deficiency rapidly induces the expression of genes that encode Pi transporters leading to increased transcription and protein synthesis, the assembly of the proteins in the plasma membrane of the outer cell layers of roots, and enhanced Pi uptake (Muchhal and Raghothama 1999). In *Arabidopsis*, the detection of P deficiency can occur within 12–24 h after removal of Pi from the medium (Muchhal and Raghothama 1999; Misson et al. 2004). A rapid increase in the number of Pi transporters occurs before the appearance of any visible P deficiency symptoms, suggesting that signals are initiated as a consequence of subtle changes in some cellular Pi pools. These findings are important steps toward resolving the complete signal transduction cascade starting from P limitation in the soil. However, the key questions such as how plants actually perceive soil P and whether it is the presence or the absence of soil P that acts as a signal need to be addressed to improve our current understanding of the signaling pathway.

7.2.2 pH Rhizospheric Variations

Legumes' roots can be responsible for considerable changes of rhizosphere pH (Hinsinger 1998; Jaillard et al. 2001) which arise mostly from the release of H^+ or OH^-/HCO_3^- to counterbalance a net excess of cations or anions entering the roots, respectively (Fig. 7.1). In that respect, N plays a prominent role because (i) it is the mineral nutrient that is taken up at the highest rate by most plant species (Marschner 1995; Alkama et al. 2009), and (ii) it occurs in the soil as various species that bear different charges: it can be taken up as a cation (NH_4^+) or as an anion (NO_3^-) or even as an uncharged species (N_2) in the case of N_2-fixing plants such as legumes living in

symbiotic association with N_2-fixing bacteria. It is thus expected that plants relying on nitrate will rather release OH^-/HCO_3^- and induce an alkalinization of the rhizosphere, while those relying on ammonium will release H^+ and strongly acidify their rhizosphere (Gahoonia and Nielsen 1992; Tang et al. 2009). Acidification of the rhizosphere is also expected to occur in the rhizosphere of N_2-fixing legumes which have a net positive excess of cations over anions entering their roots (Kouas et al. 2009; Alkama et al. 2012; Blossfeld et al. 2013). Legumes relying on N_2 fixation generally take up more cations than anions and thus extrude proportionally more H^+ than OH^- at the root–soil interface for compensation of electrical positive charges and regulation of cytosolic pH in their plant cells (Tang et al. 2004; Kouas et al. 2008).

The availability of P in the rhizosphere is influenced significantly by changes in pH and root exudates which can either directly or indirectly affect nutrient availability and/or microbial activity (Richardson and Simpson 2011). Acidification of the rhizosphere in response to P deficiency has been demonstrated for a number of species (Hinsinger et al. 2003; Devau et al. 2009) and can alter the solubility of sparingly soluble inorganic P compounds or affect the kinetics of Pi adsorption–desorption reactions in soil and the subsequent availability of Pi and various micronutrients in soil solution (Fig. 7.2). Numerous studies with phosphate rocks provided evidence that H^+ release by plant roots can considerably increase the dissolution of phosphate rocks and hence the bioavailability of P in the rhizosphere (Bolan et al. 1997; Hinsinger 1998; Yan et al. 2002). Several reports have shown that some species such as buckwheat, oilseed rape, and legumes were particularly efficient at using P from phosphate rocks, as related to their peculiar ability to release H^+ (Zoysa et al. 1998).

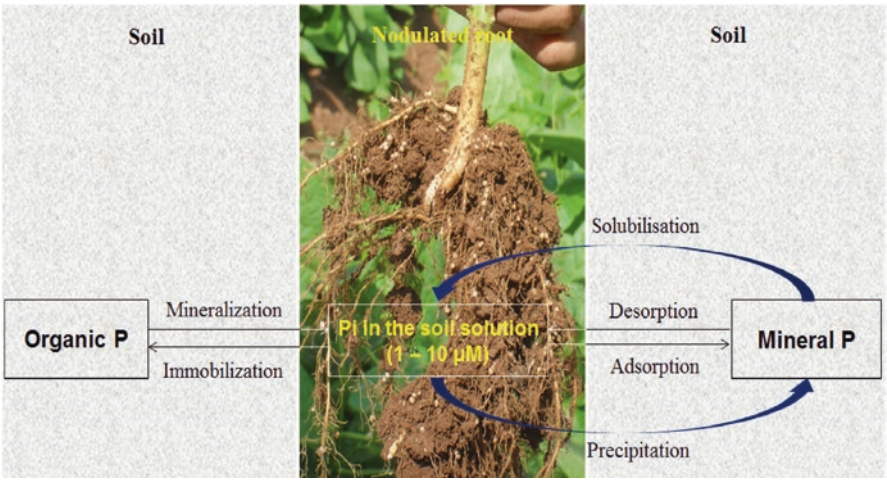

Fig. 7.2 Forms and availability of phosphorus in soil. The legume can only use inorganic phosphate (Pi) pool available in the soil solution. The Pi pool is maintained by the mineral P pool more or less readily available via the phenomena of desorption and/or solubilization. It can also be powered by the mineralization of the organic P

7.2.3 *Exudation of Organic Anions*

Under normal growth and development conditions, legumes' roots exude a wide variety of organic compounds including simple sugars, organic acids, amino acids, growth hormones, and polysaccharides (Vance et al. 2003). Exudation of organic compounds from roots can alter rhizosphere chemistry, soil microbial populations, competition, and plant growth (Marschner 1995). Exuded compounds are functionally diverse and can be involved in a wide array of processes ranging from signaling in plant–microbe interactions to allelopathy and nutrient acquisition (Harrison 1997). During nutritional stresses such as deficiency and/or toxicity situations, roots show enhanced synthesis and exudation of several organic anions (Ryan et al. 2001; Neumann and Römheld 2007). Convincing evidence now exists for exudation of malate and citrate as a principal mechanism in alleviating the edaphic stress of P deficiency and Al toxicity. The release of organic acids allows for the chelation of Al^{3+}, Fe^{3+}, and Ca^{2+}, and subsequent displacement of inorganic P from bound or precipitated forms (Hinsinger 2001; Ryan et al. 2001), and may also cause organic P to become more susceptible to hydrolysis by phosphatases (APases) (Braum and Helmke 1995). Organic anions are commonly released from roots in association with protons which results in an acidification of the rhizosphere (Kouas et al. 2009; Alkama et al. 2012). In addition to this change in rhizosphere pH, organic anions can also directly facilitate the mobilization of P through reduced sorption of P by alteration of the surface characteristics of soil particles, desorption of Pi from adsorption sites, and through chelation of cations that are commonly associated with Pi in soil (Jones and Darrah 1994; Jones 1998). The effectiveness of organic anions in mobilizing P from soil is highlighted by studies with white lupin which exudes significant amounts of citrate (and to some extent malate) from cluster roots that are formed in response to P deficiency (Neumann and Martinoia 2002; Vance et al. 2003; Lambers et al. 2013). Release of large quantities of citrate has been reported to be associated with strong rhizosphere acidification in cluster roots of white lupin (Neumann and Römheld 1999). In this particular case, Dinkelaker et al. (1989) showed that the efflux of citrate was far larger than the measured excess of cations over inorganic anions taken up by the roots of white lupin. In that case, Dinkelaker et al. (1989) suggested that in that case, H^+ released to accompany the efflux of citrate was a major component of the observed acidification of the rhizosphere around cluster roots. Increased organic anion efflux from roots in response to P deficiency also occurs in other species including chickpea and pigeon pea and to a lesser extent in lucerne (Veneklaas et al. 2003; Wouterlood et al. 2004; Pearse et al. 2006). Also, P-efficient genotypes of common bean were shown to have significantly higher levels of organic acid exudation than inefficient genotypes (Yan et al. 2004). A study by Tesfaye et al. (2001) has shown that enhanced organic acid exudation resulted in an improved tolerance to Al toxicity and enhanced P uptake of transformed *Medicago sativa* plants with nodule-enhanced forms of malate dehydrogenase (MDH) cDNA. This study has reported a 1.6-fold increase in root-tip MDH enzyme activity along with a significant increase in root exudation of several

organic acids (i.e., citrate, oxalate, malate, succinate, and acetate) as compared to non-transformed *M. sativa* (Tesfaye et al. 2001). Similarly, recent findings by Liang et al. (2013) have suggested that *Glycine max* adaptation to both P deficiency and Al toxicity is likely due to higher root malate exudation. In addition, these organic acids comprise a wide variety of compounds that vary with plant species, plant age, and environmental constraints (Neumann and Römheld 1999).

7.3 Mineralization of Organic P for Legume Symbiotic N_2 Fixation

7.3.1 Phosphatases: Activity and Gene Expression

Utilization of organic P by plants and microorganisms requires mineralization of substrates by APases enzymes that may be of either plant or microbial origin. Phosphatases are implicated in (i) providing P during seed germination from stored phytate (Brinch-Pedersen et al. 2002; Lazali et al. 2014), (ii) internal remobilization of P (del Pozo et al. 1999; Baldwin et al. 2001; Bargaz et al. 2015), (iii) and release of P from soil organic P esters by exudation of enzymes into the rhizosphere (Miller et al. 2001). Both intra- and extracellular APases are prominent in plants, and their activities have traditionally been used as markers for P deficiency (Duff et al. 1994; Baldwin et al. 2001; Lazali et al. 2016b). Intracellular forms are mostly localized in the vacuole and function as soluble proteins (Tang et al. 2013). Although extracellular APases occur in the root apoplast and are frequently released from cell suspension cultures, extracellular types are usually localized in the cell wall, outer surface of root epidermal cells, and the root apical meristem. Increased activity of APases occurs in response to P deficiency as part of P-starvation responses. In plants, this includes the release from roots of extracellular APases that are considered to be important for capture and recycling of organic P lost from roots or to allow greater access to soil organic P (Richardson et al. 2005; Bargaz et al. 2015). The release of APases into the rhizosphere is a typical and almost universal P-starvation response in higher plants (Turner et al. 2002), including N_2-fixing legumes such as soybean and white lupin whose APases activity increased steadily during both root and nodule development and reached a peak in mature stage suggesting this enzyme as a key element for functional nodules (Ozawa et al. 1995; Gilbert et al. 1999).

Furthermore, recent analyses of several intracellular APases have shown that the localization of tissue-specific cDNA of phosphoenolpyruvate phosphatase, trehalose 6-P phosphatase and fructose 1,6 bisphosphatase were mainly expressed in infected cells and nodule cortex of *Phaseolus vulgaris* (Bargaz et al. 2012, 2013; Lazali et al. 2016b). These studies provided the first evidence that phosphoenolpyruvate phosphatase and trehalose 6-P phosphatase were differentially expressed among nodule tissues of two P-efficiency recombinant inbred lines of *P. vulgaris* (RIL115 and RIL147) and suggested the abundance of their transcripts in infected

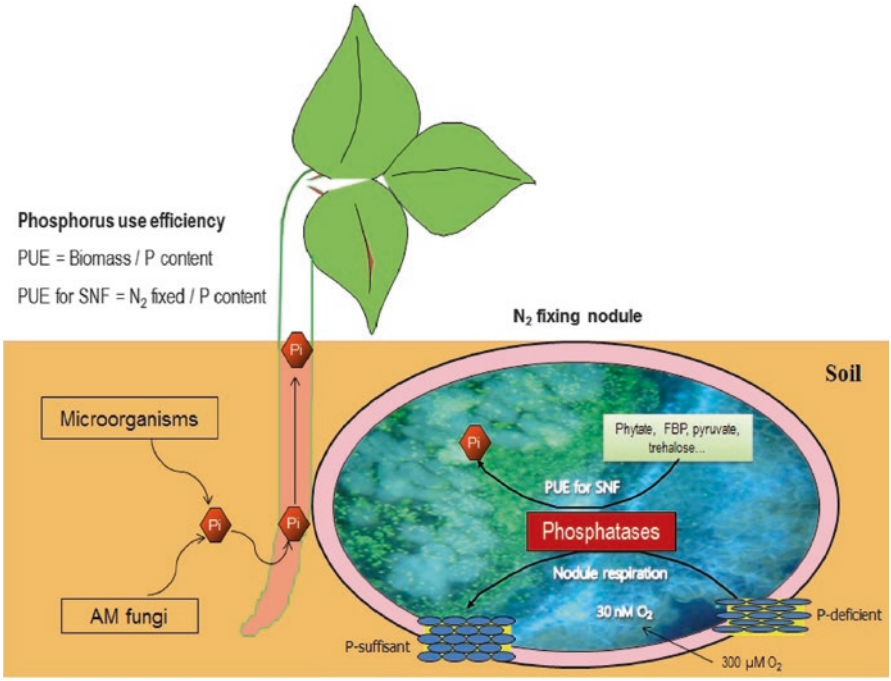

Fig. 7.3 Conceptual illustration of the functional roles played by phosphatases and plant–soil–microbe interactions for enhanced P efficiency in legumes. Phosphorus efficiency can be achieved through P acquisition efficiency, defined as the ability of the legume to acquire P from low P soils, or by P use efficiency, and the ability of a genotype to convert P into growth or yield once it is acquired from the soil. *FBP* fructose 1,6 bisphosphate, *PUE* P use efficiency, *SNF* symbiotic nitrogen fixation

cells at the vicinity of inner- and in outer-cortex cells to be involved in the adaptation to P deficiency (Fig. 7.3). This overexpression under P deficiency was coupled with increased enzyme activity, improved symbiotic efficiency, and increased N2 fixation and seems to play an important role in tolerance to low P availability (Araujo et al. 2008; Kouas et al. 2009; Drevon et al. 2015). The conceptual illustration in Fig. 7.3 that synthesizes mechanisms describing the involvement of APases in N_2 fixation during P deficiency suggests a role in controlling nodule O_2 permeability, a candidate mechanism that is also supposed to be tightly influenced by nodule P status (Bargaz et al. 2011; Alkama et al. 2012; Lazali and Drevon 2014). The postulate that APase in nodule cortex may somehow influence nodule permeability to O_2 diffusion is substantiated by the positive correlation between phytase activity and permeability to O_2 diffusion in nodules of *P. vulgaris* (Lazali and Drevon 2014). The role of APase in the nodule respiration might be substantiated by the accumulation of Pi in the inner cortex that would induce an increase in the size of these cells, which has been associated with an increased nodule respiration (Drevon et al. 1998; Bargaz et al. 2013).

7.3.2 *Phytases*

Among the wide range of APases, phytase is the only enzyme that has the specific capacity to degrade phytate, yielding a series of lower Pi esters of myo-inositol and Pi (Zhang et al. 2008; Lazali et al. 2013). On the basis of variations in structure and catalytic mechanisms, phytases can be divided into cysteine phytases (CPhy), purple acid phytases (PAPhy), histidine acid phytases (HAPhy), and b-propeller phytases (BPPhy) (Mullaney and Ullah 2003; Chu et al. 2004; Jorquera et al. 2008; Lei et al. 2013). Phytase enzymes are widely distributed in nature. The main sources are plants, microorganisms, and some animal tissues (Cookson 2002; Konietzny and Greiner 2002). Previous research has revealed that phytases from microbial origin are the most auspicious for their application in commercial biotechnological production of enzymes due to their catalytic properties and ease in enzyme production (Haefner et al. 2005; Lei et al. 2013). They mineralize organic P into inorganic P and increase P availability for plant uptake (Cookson 2002: Araujo et al. 2008; Lazali et al. 2013). A number of phytases differing in properties have been extensively screened and characterized from microorganisms, nodulated legumes and seeds (Table 7.1).

Several reports have evaluated the phytase activities of bacteria isolated from soil such as *Bacillus* sp. (Kim et al. 1998; Choi et al. 2001), *Pseudomonas* sp. (Richardson and Hadobas 1997), and *Enterobacter* sp. (Yoon et al. 1996). It has also demonstrated that plants have limited capability to access P directly from inositol Pi (Hayes et al. 1999; Richardson et al. 2001). This is attributed to poor availability of inositol Pi in soil solution, low extracellular APase (i.e., phytase) activity in roots, and low efficacy of enzyme–substrate interactions in soil (George et al. 2007; Richardson et al. 2009). Plant access to P from inositol Pi in soil is mediated primarily by microorganisms with extracellular phytase activity and their interactions within the rhizosphere (Richardson et al. 2001; Richardson and Simpson 2011). In addition, interactions between phytases and soil may further alter or reduce the efficacy of the enzyme–substrate

Table 7.1 Distribution of the different catalytic classes of phytases

Phytases	Source	Species	References
HAPhy	Bacteria	*Escherichia coli* appA	Golovan et al. (2000); Rodriguez et al. (1999)
	Fungi	*Aspergillus niger* phyA	Zhang et al. (2007)
		Candida krusei WZ-001	Quan et al. (2002)
	Plants	*Phaseolus vulgaris* (nodules)	Lazali et al. (2013)
		Arabidopsis thaliana	Mullaney and Ullah (1998)
BBPhy	Bacteria	*Bacillus amyloliquefaciens* DS11	Oh et al. (2001)
		Bacillus subtilis	Kerovuo et al. (1998)
		Sphingomonas sp. SKA58	Lim et al. (2007)
PAPhy	Fungi	*Aspergillus niger*	Ullah and Phillippy (1994)
	Plants	*Glycine max*	Hegeman and Grabau (2001)
		Phaseolus vulgaris (seeds)	Lazali et al. (2014)

HAPhy histidine acid phytase, *BBPhy* b-propeller phytase, and *PAPhy* purple acid phytase

interactions (George et al. 2007), and inositol Pi adsorbed to soil mineral or clay constituents appears to be protected against enzyme activity (Giaveno et al. 2010).

Seed P remobilization by phytase has been shown to positively influence the establishment and development of the rhizobial symbiosis (Valverde et al. 2002). Utilizing in situ reverse transcription polymerase chain reaction, Lazali et al. (2014) characterized the in situ localization of phytase transcript, which showed high expression in *P. vulgaris* seeds. This study demonstrated that the most intensive phytase gene expression was found in the embryo and cotyledons as compared to lower expression in radicles. This seedling phytase gene was reported to exhibit high homology (90%) with the previously characterized purple APase "*Gm*PAP02" in soybean (Lazali et al. 2014). Similarly, phytase cDNA in common bean nodules displayed 94% homology with *Gm*Phy07 (Lazali et al. 2014). These authors also reported that phytase enzyme activity seemed to be tissue specific and likely to vary from seeds to nodules as seed phytase activity was almost 10 times higher than in nodules.

7.4 Plant–Soil–Microbe Interactions for Enhanced P Efficiency in Legumes

As many soils worldwide have a moderate to high capacity to retain P, most of the fertilizer and manure applied to soil for P are rapidly bound by the soil minerals that are not subjected to rapid release. Thus, there is a need to develop plants that are more P efficient at low/deficient soil P, especially in the least developed and developing countries. Phosphorus-efficient plants are required to reduce inefficient use of various P inputs and to minimize potential P loss to the environment (Richardson et al. 2011; Lazali et al. 2016c).

Microorganisms are an important component of soil and directly or indirectly influence the soil's health through their beneficial or detrimental activities. Rhizospheric microorganisms mediate soil processes such as decomposition, nutrient mobilization and mineralization, storage release of nutrients and water, and N_2 fixation and denitrification (Richardson and Simpson 2011). Furthermore, the organisms possessing a Pi-solubilizing ability can also convert the insoluble phosphatic compounds into soluble forms (Kang et al. 2002; Pradhan and Sukla 2005) in soil and make them available to the crops. However, studies on Pi solubilization by nodule bacteria has been substantially less, despite it is known that P is the most limiting factor for N_2 fixation by rhizobia-legume symbiosis. There are only a few reports of Pi solubilization by rhizobia (Chabot et al. 1996) and the nonsymbiotic N_2 fixer, *Azotobacter* (Kumar et al. 2001).

Recognition that microorganisms are important for P mobilization in soil has led to research effort directed at improving plant P nutrition (Fig. 7.3). Soil microorganisms can also influence the availability of P to plant roots as they are involved in a number of biogeochemical processes that affect P transformation in soil (Richardson 2001). Most of the soils are low P available; thus, microorganisms and plants have developed similar and complementary mechanisms for hydrolysis, uptake and

mobilization, and uptake of P. Microbes are more competitive than plants for the uptake of P in the rhizosphere (Marschner et al. 2011). In addition, microorganisms can affect plant growth and nutrient uptake by release of growth-stimulating or growth-inhibiting substances that influence root physiology and root system architecture (Ryu et al. 2005; Govindasamy et al. 2009). For example, P efficient common bean genotypes produce shallower basal roots under low P availability compared with P-inefficient genotypes, where P acquisition significantly correlates with basal root shallowness (Liao et al. 2001). It has been estimated that variation in root growth angle among closely related genotypes is associated with up to 600% increase in P acquisition and 300% increase in yield in common bean (Liao et al. 2001). Furthermore, root parameters and associated microbes were positively interconnected in a legume-cereal intercrops with improved growth under P-deficient conditions (Bargaz et al. 2016; Latati et al. 2016).

There are also several reports on the improvement of nodulation and plant growth of legumes by Pi-solubilizing bacteria. For example, Rosas et al. (2006) reported on the positive effect of Pi-solubilizing bacteria *Bradyrhizobium japonicum* and *Pseudomonas putida* on the root and shoot growth of soybean. Similar results were observed by Kumar and Chandra (2008), who showed that Pi-solubilizing bacteria improved the symbiotic performance of introduced rhizobia in field-grown lentils. In another study, *Pseudomonas* spp. inoculated together with rhizobia significantly increased the number of pods per plant, number of seeds per pod per plant, and seed yield per hectare of soybean (Argaw 2012). Phosphate-solubilizing bacteria are known to help plants to acquire more P from soil, thus stimulating P uptake by plants and also improving nodulation and N_2 fixation (Elkoca et al. 2008). El-Azouni (2008) observed significant increases of dry matter, N and P uptake, and yield of soybean grown in Egyptian soil inoculated with the Pi-solubilizing fungi *Aspergillus niger* and *Penicillium italicum*.

Among soil microorganisms, arbuscular mycorrhizal fungi (AMF) have been found to be essential components of sustainable soil-plant systems (Smith and Read 2008). Arbuscular mycorrhizal fungi increased plant Pi uptake (Irshad et al. 2012; Casieri et al. 2013), micronutrients (Burkert and Robson 1994; Wahbi et al. 2016), and soil aggregation (Tisdall 1994) and act as antagonists against some plant pathogens (Duponnois et al. 2005). Moreover, it has been demonstrated that plants inoculated with AMF utilize more soluble Pi from rock phosphate than non-inoculated plants (El Faiz et al. 2015). Advantages of AMF symbiosis over the non-mycorrhizal state of the same plant genotype are inextricably associated with root architecture, in that plants with extensive, branched root systems, very fine roots, and long root hairs tend to show relatively low improvement in growth when they are mycorrhizal, even in low P soils (Schweiger et al. 1995). The proportion of root length that is colonized by AMF also tends to decrease with increasing soil P, and this is often taken to mean that the plant is suppressing the AMF symbiosis (Richardson et al. 2011). Arbuscular mycorrhizal fungi are believed to enhance the Pi nutrition of plants by scavenging the available P due to the large surface area of their hyphae and by their high-affinity Pi uptake mechanisms (Nussaume et al. 2011). Furthermore, rhizobia and Pi-solubilizing fungi, when used as seed inoculant, increased the grain

yield of chickpea under field conditions (Dudeja et al. 1981). Similarly, the effect of interactions between three Pi-solubilizing fungi, namely, *A. niger*, *A. fumigatus*, and *Penicillium pinophilum*, and N_2-fixing *Rhizobium leguminosarum* biovar *viciae* showed significantly greater positive effects on growth, nutrient uptake, and consequently, the yield of *Vicia faba* under field conditions (Mehana and Wahid 2002).

7.5 Conclusions and Future Prospects

The recent knowledge reported in this chapter highlights legumes' potential to cope with the most abundant abiotic stress worldwide, through key mechanisms including constitutive and stress-induced responses. Advanced knowledge at the physiological and molecular level has enabled breeding for candidate genes and genetic engineering for legume crops that are better adapted to stressful conditions. In spite of this, the extent of the stress tolerance in plants largely depends on factors that vary among genotypes and environmental conditions as well as the complexity and severity of the imposed stress. The last factor also includes the situation where multiple abiotic stress occur at the same time, which highlights the important challenges that research in plant breeding needs to address in order to develop legumes with tolerance to multiple constraints.

A greater understanding of P dynamics in the soil–rhizosphere–plant continuum is of utmost importance as it can provide a significant basis for optimizing P management to improve P use efficiency in crop production. It remains challenging to obtain greater insight on the mechanisms and regulation of P acquisition and internal utilization efficiency. The research thrust should be directed toward the improvement of P efficiency that often features more to P acquisition efficiency under limited P supply and to P use efficiency under high-P conditions. Therefore, the perfect breeding approach should take into account the improvement of both P acquisition and P utilization efficiency in the given species under different P supply conditions in various soil types. It is paramount establishing an integrated P management strategy that involves manipulation of soil, plant, and rhizosphere processes, development of P-efficient crops, and improving P recycling efficiency for current and a future agriculture more adapted to multiple environmental uncertainties.

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Chapter 8
Role of Plant Hormones and Small Signalling Molecules in Nodulation Under P Stress

Eloise Foo

Abstract Plant hormones and other mobile signalling elements play key roles in regulating nodulation and N_2 fixation in legumes. This includes many hormones associated with regulating general growth and development, such as cytokinin, auxin, gibberellins and strigolactones and plant hormones associated with response to stress, including ethylene. Mobile peptides and microRNAs have also shown to have significant roles in regulating nodule initiation, organogenesis and nutrient response. In this chapter we will discuss the roles of these small signalling molecules in nodulation, highlighting specific examples of their interactions with phosphorous (P) stress. P-induced small peptides and microRNAs have been identified in legumes, but the role of these signals in regulating nodulation response to P stress has not been directly investigated. Similarly, relatively few studies that have specifically examined the role of plant hormones in P response of nodulation and areas for future research are highlighted.

Keywords Autoregulation of nodulation • Auxin • Cytokinin • Ethylene • microRNA • Phosphate limitation • Strigolactones

8.1 Introduction

Nodulation requires sufficient macro- and micronutrients for nodule formation and function. One of the biggest constraints on nodulation is phosphorus (P) deficiency, as many soils have limited P availability (Tesfaye et al. 2007; Karmakar et al. 2015). Plants strictly regulate nodulation in response to P availability. As outlined in previous chapters, P level can be maintained in nodules under short-term P starvation (e.g. Al-Niemi et al. 1998; Qin et al. 2012), while long-term P limitation can result in reduced nodule formation and function (e.g. Sulieman et al. 2013). This response to long-term P limitation includes modulating the number of nodules formed (initiation and organogenesis), the size of the nodules (expansion) and their function (N_2 fixation) and the timing of nodule senescence.

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Generally, P availability to the roots acts both locally and systemically to regulate root processes. This has been revealed most elegantly in split-root studies, which show that the limitation of P to one side of a root system influences processes on the other side of the root system including root growth, root architecture, cluster root formation, gene expression, mycorrhizal colonisation and nodulation (Burleigh and Harrison 1999; Gentili and Huss-Danell 2003; Shane et al. 2003; Balzergue et al. 2010; Breuillin et al. 2010). This does not appear to be via altered P itself but is correlated with altered P levels in the shoot. This suggests that the response to P is mediated by shoot-derived signal(s) that may include roles for small signals such as microRNA (miRNA), sugar, peptides and mobile hormones (e.g. Scheible and Rojas-Triana 2015). Local effects of P limitation may act through P itself or be mediated by signalling molecules (for review see Zhang et al. 2014).

The role of plant hormones and other small, mobile signalling molecules (miRNA and peptides) in modulating nodule number or function in response to P has been relatively underexplored. P limitation is known to influence the synthesis and, in some cases, response to many of these growth-modifying substances. In addition, many hormones and small signalling molecules play a role in regulating nodulation. In this chapter, the literature examining the roles of a range of plant hormones, both growth promoting and stress related, in the P response of nodulation will be explored. Roles for other small signalling molecules (miRNA and peptides) and their associated response pathways will also be examined and Future directions in this area are identified.

8.2 Autoregulation of Nodulation and the P Response

The number of nodules formed on a plant is under strict control to ensure that plants balance nitrogen gained with carbon expended. A central regulator of this response is the autoregulation of nodulation (AON) pathway, which acts at a local and systemic level to monitor and control nodulation (Reid et al. 2011a; Soyano and Kawaguchi 2014). The AON pathway consists of a root-shoot-root signalling system that was originally identified in split-root studies, where prior exposure of one side of the root system to the rhizobia limited subsequent nodulation on the other side of the root system (e.g. Kosslak and Bohlool 1984; Caetano-Anollés and Gresshoff 1990).

The identification of legume mutants with a huge excess of nodules, termed "hyper/supernodulation", has helped define the genes and signals regulating this negative feedback system (Reid et al. 2011a; Soyano and Kawaguchi 2014). Grafting and split-root studies with these mutants have identified genes that control signals that move from root to shoot and from shoot to root and have led to the current model of the AON pathway (Fig. 8.1). In this model exposure to rhizobial bacteria and early nodulation events induce the expression of genes that encode mobile CLAVATA3/ESR (CLE)-related peptides (Okamoto et al. 2009, 2013; Mortier et al. 2010; Lin et al. 2010; Reid et al. 2011b). The generation of such

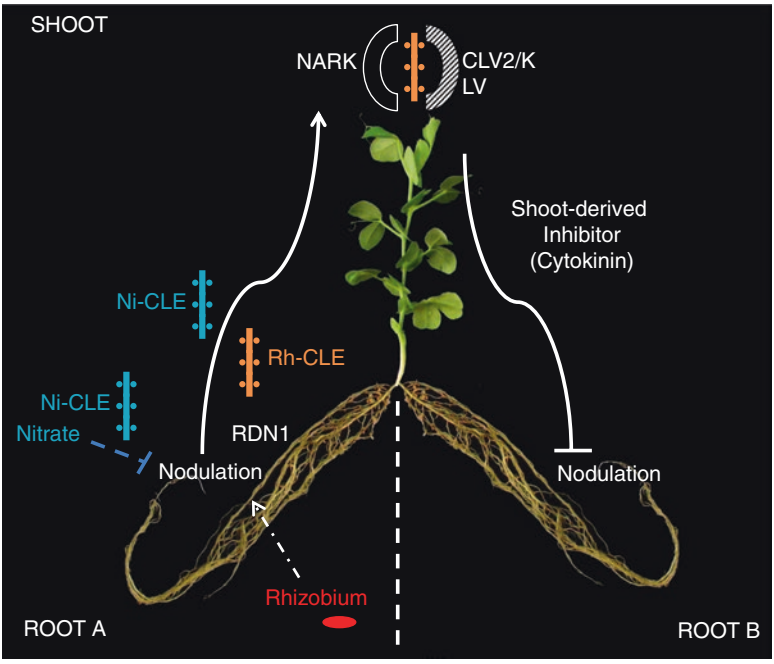

Fig. 8.1 Diagram of a plant with split-root system (root A and root B) indicating the major proteins and mobile signals that act in the autoregulation of nodulation (AON) pathway. Interaction with compatible rhizobium in root A induces upwardly moving Rh-CLE peptide, thought to be influenced by the action of RDN1. The Rh-CLE moves to the shoot and interacts with proteins (NARK, CLV2, KLV) that in turn generate a downward moving inhibitor of nodulation, recently suggested to be cytokinin. This signal suppresses nodulation in root A and root B. Nitrate induces Ni-CLE peptides that act locally and systemically to suppress nodulation

root-derived signals is thought to be influenced by the action of root-determined nodulation 1 (RDN1) (Novak 2010; Schnabel et al. 2011). These CLE peptides are thought to move to the shoot whereupon recognition by the NARK leucine-rich repeat-receptor kinase (LRR-RK) induces a downward moving signal that limits further colonisation (Delves et al. 1986; Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003; Schnabel et al. 2005). This downward moving signal was recently proposed to be shoot-derived cytokinin (Sasaki et al. 2014). Other shoot-acting receptors, KLAVIER (KLV) and CLAVATA2 (CLV2), have been proposed to complex with NARK in the shoot to control nodule numbers in the root (Miyazawa et al. 2010; Krussel et al. 2011). As noted above, a mutation in any part of this feedback pathway results in mutant plants with uncontrolled nodule number.

Elements of the AON pathway are known to play important roles in nitrate suppression of nodulation. Plant mutants with lesions in NARK, KLV and RDN1 have significant reductions in response to nitrate (e.g. Carroll et al. 1985; Jacobsen and Feenstra 1984; Oka-Kira et al. 2005). One CLE peptide is specifically induced by nitrate and acts locally and systemically to suppress nodulation

(Okamoto et al. 2009; Okamoto and Kawaguchi 2015; Jeudy et al. 2010). Given this important role for elements of AON in the N response, it is surprising that there is relatively little known about the role of AON in the P response. As the AON system primarily controls nodule initiation and spacing, rather than nodule function or senescence, it is likely that any role for the AON pathway relates to limiting nodule number under P limitation.

One study using the *nark* mutant of pea found that the suppression of nodule number observed in wild-type plants grown under P limitation was not seen in *nark* mutants, which displayed a supernodulation phenotype under P-deficient and P-sufficient conditions (Fig. 8.2; Foo et al. 2013b). A study in *Lotus japonicus* discovered that the expression of several genes that encode CLE peptides are elevated in response to P application to the roots (Funayama-Noguchi et al. 2011). This was recently confirmed by independent studies (Handa et al. 2015). Future studies are required to clarify the role of these P-induced CLE peptides, including their role in P control of nodule number and to identify the signalling elements required for CLE response.

It is important to note that although elements of the AON pathway also appear to play a role in limiting mycorrhizal colonisation (reviewed by Staehelin et al. 2011), there is no evidence that the AON pathway is required for P limitation of mycorrhizal colonisation (Wyss et al. 1990; Foo et al. 2013b). However, this does not preclude AON elements playing a role in P regulation of nodulation, since P suppresses mycorrhizal but promotes rhizobial colonisation, different regulatory mechanisms would be expected to control the P response of the two symbioses.

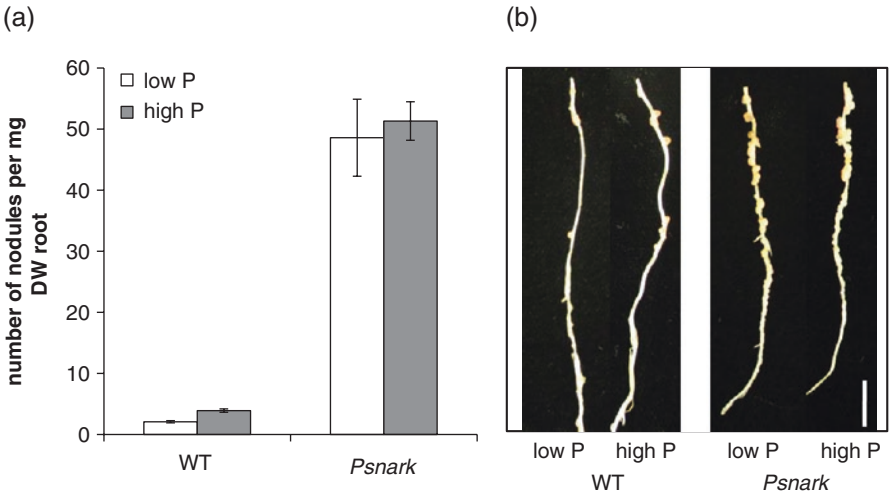

Fig. 8.2 Effect of phosphate fertilisation on the development of nodules in *Psnark* (autoregulation mutant) and wild-type pea (cv. Frisson). Plants were fertilised with 0.05 (low P) or 5 mM (high P) NaH_2PO_4. (**a**) Nodule number per mg root dry weight and (**b**) photo of nodules on secondary root of wild type and *Psnark* mutant plants (tertiary roots have been removed), scale bar = 1 cm (Adapted from Foo et al. 2013b)

8.3 miRNA

miRNAs are small non-coding RNAs that regulate the expression of other genes to influence plant development (Borges and Martienssen 2015). Recent studies have revealed important roles for miRNAs in the P response of root growth and mycorrhizal symbioses. In *Arabidopsis*, miR399 is a miRNA that is induced under P starvation that targets transcripts of the *PHO2* gene for degradation (Chiou et al. 2006; Pant et al. 2008). As the *PHO2* gene encodes an ubiquitin-conjugating enzyme that in turn negatively regulates the expression of a subset of genes that respond to P starvation including phosphate transporters, miR399 acts as a promoter of the P starvation response. Given the strong promotion of mycorrhizal symbioses by P limitation, Branscheid et al. (2010) explored the role of the miR399 family in mycorrhizal development and response to P in the mycorrhizal host *Medicago truncutula*. Branscheid et al. (2010) found that miR399 may act in mycorrhizal plants to suppress direct P uptake by keeping *PHO2* expression low, rather than acting as a signal to induce mycorrhizal colonisation under low P. However, the role of *miR399* in the P response of nodulation was not examined.

Orthologues of *miR399* have also been identified in soybean, and they have been shown to be elevated in response to P limitation (Xu et al. 2013). In addition to targeting *PHO2*, soybean miR399 orthologues also target *GmPT5* transcripts for degradation. *GmPT5* encodes a nodule-specific high-affinity phosphate transporter that is important in maintaining P homeostasis during P limitation by facilitating P transport into the nodule (Qin et al. 2012). Thus, in soybeans under severe/long-term P stress, *miR399* may downregulate P import into the nodule by targeting *GmPT5*. A connection between a miR399 orthologue and the regulation of the expression of genes in the P response pathways was also demonstrated in bean (Valdes-Lopez et al. 2008, 2010). However, the role of this response pathway in modulating nodule formation or function in response to P stress was not assessed. Further studies are required to provide direct evidence for the role of P response miRNAs, including but not limited to miR399, in modulating nodule formation and/or function in response to P stress and could employ the miRNA overexpressor lines in *M. truncutula* and soybean described above.

8.4 Strigolactones

Strigolactones are the most recently characterised group of plant hormones. Strigolactones are derived from carotenoids, and our current understanding of strigolactone biosynthesis is that, with some exception, plants produce both canonical strigolactones (e.g. strigol- and orobanchol-type) and noncanonical strigolactones (e.g. methyl carlactonoate) (Abe et al. 2014; Seto et al. 2014). Strigolactones act as an attractant for the mycorrhizal fungi, inducing spore germination and hyphal branching (Akiyama et al. 2005; Mori et al. 2016). In legumes strigolactones also exert a primarily positive role in nodule formation (Soto et al. 2010; Foo and Davies 2011; Liu et al. 2013; Lopez-Raez et al. 2017). Mutants or transgenic plants

deficient in strigolactones produce fewer nodules than wild-type plants, although the nodules that do develop are of a normal size and appear to be functional (Foo and Davies 2011; Liu et al. 2013), indicating strigolactones are likely to influence nodule initiation but not nodule organogenesis or function.

The biosynthesis of canonical strigolactones is strongly suppressed by high P, and this is mediated by systemic signals (e.g. Balzergue et al. 2010; Breuillin et al. 2010; Yoneyama et al. 2015). However, studies by Foo et al. (2013a) found that strigolactones are not required to mediate the suppression of nodule formation under long-term P limitation. Like wild-type plants, mutant pea plants totally deficient in strigolactones due to deletion of a key biosynthesis gene *CCD8* still suppressed nodule number under P limitation (Foo et al. 2013a). Indeed, P suppression of mycorrhizal symbioses was also maintained in these strigolactone-deficient lines, indicating that despite strong regulation of strigolactones by P, factors in addition to strigolactones mediate the regulation of symbioses in response to P availability.

8.5 Cytokinin

Cytokinin is known to play several key roles during nodulation (Ryu et al. 2012). In the roots cytokinin is necessary and sufficient to activate cortical cell divisions and nodule organogenesis. Mutants disrupted in the cytokinin receptors MtCRE1 and LjLHK1 show strongly reduced nodulation (Gonzalez-Rizzo et al. 2006; Murray et al. 2007), and gain-of-function mutants in CRE1 receptor result in spontaneous nodules in the absence of rhizobia (Tirichine et al. 2007). Cytokinins also play diverse roles in the AON system, with an application of synthetic cytokinin inducing an accumulation of nodulation-induced CLE peptides (Mortier et al. 2012), and recent work suggesting the AON system may suppress nodulation by inducing synthesis of shoot-derived cytokinin (Sasaki et al. 2014).

To date no studies have directly assessed the role of cytokinin in altered nodule formation or function under P stress. However, studies in non-legumes have indicated cytokinin is a negative regulator of the P stress response. For example, in *Arabidopsis* and rice cytokinin treatment represses the induction of many genes induced by P limitation (Martín et al. 2000; Franco-Zorrilla et al. 2002; Karthikeyan et al. 2002; Wang et al. 2006), and this was relieved in *Arabidopsis* mutants disrupted in the CRE1 or HK3 cytokinin receptors (Franco-Zorrilla et al. 2002, 2005). However, cytokinin appears to act locally to repress the induction of P starvation genes, as in split-root experiments neither exogenous cytokinin nor experiments with *cre1 hk3* double mutants disrupted systemic upregulation of the P-responsive genes (Franco-Zorrilla et al. 2005). As noted above, orthologous cytokinin receptor mutants are available in legumes, and it would be fascinating to use these mutants as a tool to examine the role of cytokinin signalling in the P regulation of nodulation. Given that the local cytokinin response appears to be a promoter of nodule formation, the derepression of the P limitation response by cytokinin could be one way that plants maintain nodule function under short-term P limitation.

8.6 Ethylene

Ethylene is a key regulator of nodulation in many legume species and in general plays a negative role in several key steps including infection thread formation, organogenesis, nodule positioning and regulation of total nodule number (Guinell 2015). The application of ethylene suppresses nodule formation in species that form indeterminate nodules, and disruption of ethylene signalling by chemical or genetic means in these species results in the development of significantly more nodules (Lee and LaRue 1992; Penmetsa et al. 2008; Foo et al. 2016). Interestingly, recent studies have indicated ethylene may also have some positive effects early in the rhizobial symbiosis (Larrainzar et al. 2015). Some rhizobial bacteria themselves express 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzymes that degrade ACC, the immediate precursor of ethylene, and on contact can reduce ethylene biosynthesis in plants. Studies with rhizobia with altered ACC deaminase activity indicate that it is this rhizobial driven change in ethylene biosynthesis that promotes nodule formation (e.g. Ma et al. 2003).

The production of ethylene is elevated by P deficiency in several legume species (Borch et al. 1999; Li et al. 2009). These studies also reported that P-limited plants were more sensitive to ethylene in root architecture assays. This is consistent with the well-characterised role of ethylene in modulating many aspects of root development under P deficiency in non-legumes (for review see Zhang et al. 2014). For example, ethylene contributes to the elevated root hair density and elongation observed in response to low P (Zhang et al. 2003; He et al. 2005), a particularly important phenotype for nodulation as it is the site of rhizobial infection in many legume species. It is interesting to note that ethylene's role in influencing root hair responses to P appears to be independent of the ethylene signalling pathway (Schmidt and Schikora 2001; Nagarajan and Smith 2012). In many cases, ethylene-signalling mutants have been valuable tools to examine the role of ethylene in the P response of root architecture and could be similarly employed in legumes to examine ethylene's role in P regulation of nodule formation. It would also be interesting to examine how P limitation may influence rhizobial ACC deaminase activity, given that, as outlined above, rhizobia themselves may modulate ethylene biosynthesis *in plant*a.

8.7 Auxin

Auxin is a central regulator of plant development, and important roles for auxin in nodule initiation and organogenesis have been described (for review see Mathesius et al. 2015). Auxin has been shown to accumulate at the site of nodule formation, and application studies indicate that auxin concentration/sensitivity may need to be maintained within a certain range and at particular stages/cell types for effective nodule formation. This includes modulating auxin transport through the action of auxin influx and efflux carries at a local level in the root and possibly influencing

auxin transport from the shoot to the root. The latter was revealed during studies with *Lotus japonicus har1* mutants (disrupted in the shoot-acting *NARK* orthologue of the AON pathway), which displayed increased auxin transport from shoot to root and unlike wild-type plants was unable to reduce auxin transport after inoculation with rhizobia (van Noorden et al. 2006). Rhizobia themselves can also produce auxin, although bacterially-derived auxin appears to influence nitrogen fixation rather than nodule formation (Spaepen et al. 2007; Pii et al. 2007). It is also possible that auxin produced by rhizobia influences root architecture, such as enhancing lateral root development and thus sites for nodule formation.

Auxin has been implicated in modifying root architecture in plants grown under P-limited conditions. However, there are considerable inconsistencies in the literature (for review see Zhang et al. 2014). For example, studies employing exogenous auxin and auxin transport inhibitors have concluded that either altered auxin transport or altered auxin signalling may explain the changes in root architecture observed during P limitation (e.g. López-Bucio et al. 2002; Nacry et al. 2005). Similarly, *Arabidopsis* mutants with altered auxin sensitivity or transport have been reported to exhibit no change (Williamson et al. 2001) or dramatic suppression (Nacry et al. 2005) of P starvation-induced changes in root development. This suggests that auxin-dependent and auxin-independent processes may regulate the P response of root architecture.

P limitation has also been reported to influence the exudation of plant flavonoids, including some flavonoids that are thought to act in mobilisation of P from the soil (e.g. Malusà et al. 2006). Some of these flavonoids produced by legumes attract rhizobial bacteria to legume roots. Thus, modified production of flavonoids under P limitation could alter nodule formation by influencing early signalling between the plant host and bacteria, although this has not been tested directly. It is interesting to note that in addition to their role as rhizosphere attractants, flavonoids have also been suggested to play a role *in planta* to enhance nodule formation by influencing auxin accumulation (Mathesius 2001).

The lack of well-characterised mutants that influence auxin synthesis, transport or response in legumes hampers investigation of the role of auxin in the P response of nodulation. However, studies could employ application approaches to modify auxin content, transport or response or use legumes transformed with auxin-response reporter lines (e.g. Mathesius et al. 1998; van Noorden et al. 2006) to examine the potential roles for auxin in altering nodule formation and/or function in response to P stress.

8.8 Other Hormones

The other hormones not covered above have also been implicated in regulating nodule formation and function, including the stress hormones abscisic acid, jasmonic acid and salicylic acid and the growth-promoting hormones gibberellins and brassinosteroids (for review see Ferguson and Mathesius 2014). However, there is

relatively little evidence that these hormone play important role(s) in the P responses of roots in non-legume systems (see Zhang et al. 2014).

One study has highlighted a possible link between gibberellin signalling though DELLA proteins and the P starvation response in *Arabidopsis* (Jiang et al. 2007). P limitation induced changes in root architecture, and this was blocked by exogenous gibberellin or DELLA-deficient mutants but enhanced in gain-of-function DELLA lines. However, this influence of gibberellin did not include upregulating P uptake. This has been extended, with the P starvation-induced MYB62 transcription factor shown to alter gibberellin biosynthesis in *Arabidopsis* (Devaiah et al. 2009). Given that this interaction appears to operate predominantly in leaves, it is not clear if altered gibberellin production in shoots would have implications for root processes, although gibberellin precursors have been shown to be mobile (e.g. Regnault et al. 2015). In the legume *M. truncatula* expression of the DELLA genes has been shown to be downregulated in response to P addition (Floss et al. 2013), and gibberellin signalling through DELLA has been shown to be an important suppressor of mycorrhizal formation (Foo et al. 2013c; Floss et al. 2013; Yu et al. 2014). Studies examining the formation and function of nodules under P stress in the range of gibberellin synthesis and/or response mutants available in legumes will be an important step in testing if gibberellin plays a role in these responses.

8.9 Conclusion

An examination of the role of hormones and other small signalling molecules in the P response of nodulation has identified significant gaps in our understanding (Table 8.1). Despite evidence for a role for many plant hormones in the response of root architecture and metabolism to P limitation and evidence that these same hormones influence nodulation, few studies have directly examined the role of plant hormones in altered nodule formation and/or function under P stress. Indeed, the synthesis and/or signalling of many plant hormones are known to be modified under P stress, and these changes might be expected to influence nodule formation and/or function. In some cases, well-characterised legume mutants with altered synthesis or perception of some of these plant hormones are available (cytokinin, ethylene and gibberellin), and future studies should employ these mutants to delineate the relationship between the P response of nodulation and hormone action. In the absence of hormone mutants, application studies, hormone quantification and application studies may be informative. Given that plant hormones often interact to control complex developmental processes, including nodulation, care should be taken to examine such relationships.

In addition to plant hormones, there is evidence that other small signalling molecules may also regulate the P response to nodulation. This includes emerging roles for CLE peptides, possibly perceived by elements of the AON pathway and miRNAs. Existing AON mutants and transgenic tools will also be crucial to establishing the precise signalling pathway that P may act through.

Table 8.1 Summary of the role of hormones and small signalling molecules in phosphorous (P) response of nodulation and suggested future studies

Hormone/signal	Role in P regulation of plant development?	Role in P regulation of nodulation?	Future studies
Autoregulation of nodulation pathway	Not tested	P-induced CLE peptides identified	Functional studies on P-induced CLE peptides in legumes (overexpression and knock out lines)
		NARK (CLE peptide receptor) required for P suppression of nodule number	Identify the signalling elements required for P-induced CLE response
miRNA (*miR399*)	Yes	*GmmiR399* orthologue – elevated by P limitation – degrades *GmPT5* (nodule-specific high-affinity P transporter) transcripts *PvmiR399* orthologue required for the regulation of P-responsive genes	Identify other nodule associated P-regulated miRNA Examine P response of nodulation in miRNA overexpression and knock out lines of legumes
Strigolactones	Yes	Not required for P regulation of nodule number	–
Cytokinin	Yes	Not tested	Examine P response of nodulation in legume cytokinin receptor mutants
Ethylene	Yes	Not tested	Examine P response of nodulation in legume ethylene signalling mutants Examine P limitation influence on rhizobial ACC deaminase activity
Auxin	Yes	Not tested	Monitor effect of chemicals that modify auxin content, transport or response on P response of nodulation Employ legumes transformed with auxin-response reporter lines to examine auxin role in response of nodulation to P stress

For details of references, please see text

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Chapter 9
Molecular Communication and Nutrient Transfer of Arbuscular Mycorrhizal Fungi, Symbiotic Nitrogen-Fixing Bacteria, and Host Plant in Tripartite Symbiosis

Chunling Chang, Fahad Nasir, Lina Ma, and Chunjie Tian

Abstract Plants colonized by Arbuscular mycorrhizal fungi (AMF) greatly enhance Phosphorus (P) and Nitrogen (N) acquisition, especially by extra radical mycelium. On the other hand, soil bacteria referred to as rhizobia establish a symbiotic relationship with legume plants by making novel root organ known as nodules, which fix atmospheric dinitrogen (N_2) and transfer it to the host plant. The symbiotic relationship of both AMF and rhizobia with the same host leguminous plants is termed a "tripartite symbiosis". This tripartite interaction allows legume plants to grow well in nutrient-deficient soils. Sophisticated and complex molecular communication exists between the AMF, rhizobia and host plant during tripartite symbiosis. In this chapter, we focus on some common features of the molecular dialogue shared during tripartite symbiosis. AMF and the nodulation process of rhizobia requires molecular recognition, regulation and specialized complex signaling molecules. For instance, plants secrets strigolactone (SL), which activates and up-regulates the *mycorrhizal factor* (*myc factor*) genes of AMF, which make an association with plant root hairs. SL exudates of plant roots also play a crucial role in rhizobial symbiosis, with SL-biosynthesis mutants of *Pisum sativum* and *Lotus japonicus* plants showing reduced nodule number. On the other hand, specific flavonoids molecules secreted

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by legume plants not only trigger the rhizobial *nodulation factor* (*nod factor*) genes responsible for nodule formation, but are also vital for hyphal growth of AMF. Moreover, the small polysaccharides, glycoproteins, and proteins (e.g., chitin-related compounds) responsible for stimulating transcription for enzymes involved in the synthesis of flavonoids are considered to be of fungal origin. Thus, establishment of tripartite symbiosis likely requires coordinated gene regulation synchronized by mutual exchange of diffusible signal molecules to induce the expression of genes involved in activation of a common symbiotic pathway and in colonization by microbial symbionts. Another common feature between AMF and rhizobia is that both benefit from carbohydrates provided by the host plant, which uses these symbionts as a source of energy. Finally, after the exchange of common signaling and the establishment of tripartite symbiotic interactions, the genes responsible for P and N metabolism and translocation are up-regulated, which increases the P and N supply to the host plant, especially in nutrient-scarce conditions, and ultimately increases agricultural productivity. However, to date, our knowledge of the synergistic or antagonism effects of the tripartite symbiosis on different beneficial microbes remains sparse, and requires further investigation in future studies.

Keywords Tripartite symbiosis • Phosphorus • Nitrogen • Signaling • Flavonoids • Strigolactone • Arbuscular mycorrhizal fungi • Rhizobia • Legume

9.1 Introduction

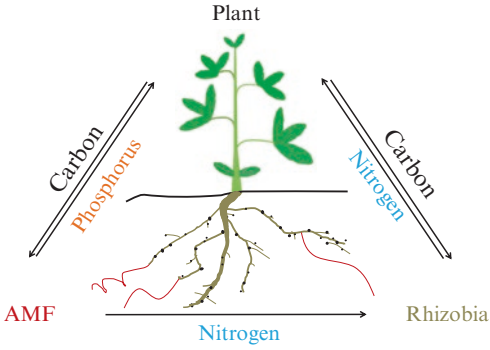
Root endosymbiosis is an association between host plant and soil microorganisms, involving intracellular accommodation of microbes within host cells, and having a widespread distribution in nature. Arbuscular mycorrhizal fungi (AMF) and rhizobia are the best examples of such symbiotic relationships between plants and microorganisms in the rhizosphere, and represent two major groups of plant-associated microbial mutualists (Sprent 2001).

The term "Mycorrhiza" is derived from two Greek words "myco" and "Rrhiza," and means symbiosis between a fungus and a plant root (Alizadeh 2011). AMF belong to the phylum Glomeromycota, and cannot synthesize their own lipid (food), and thus live as obligate symbionts in nature, in turn providing essential nutrients to their host. The symbiotic relationship between AMF and host plant is thought to have evolved more than 400 million years ago. AMF currently colonize more than 80% of terrestrial plant species by forming extensive hyphal branching networked systems (Trappe 1987). The hyphal network consists of two different types of mycelium system: the "intra-radical mycelium" (IRM) grows in, and creates many hyphal branches between, the parenchymatous cells of the host root, while the "extra-radical mycelium" (ERM) extends to the outside from root cells and increases the root surface area, which recruits and allocate more nutrients, especially P, and N to the host plant (Smith and Read 2008). The bidirectional flow or exchange of nutrients and other molecules between the two partners is termed the "reciprocal reward"

from which both partners benefit. AMF also allocate nutrients to specific plant species by determining the co-existence of plant species and altering plant diversity.

Nitrogen-fixing bacteria, formally known as "rhizobia," are prokaryotic microorganisms that invade the roots of legumes after detection of specific flavonoid chemical signals, and, as a result, induce the formation of specialized organs called nodules. After nodule formation, the rhizobia fix N_2 and provide it to the host plant allowing better growth in N scarce soils, while in return rhizobia receive carbon (C) from the host plant for energy and developmental purposes. Legume-associated rhizobia play a leading role in local as well as global N cycling, and also influence the productivity and species diversity in both agricultural and natural ecosystems (Sprent 2001; Van Der Heijden et al. 2008). Each rhizobial species can make symbiotic association only with a specific subset of host plant species, or, in other words, we can say that rhizobia species have a restricted choice of host plant selection. For example, *Sinorhizobium meliloti*, a soil bacterium, forms a symbiotic relationship with alfalfa (*Medicago sativa*) and medic (*Medicago truncatula*), but not with other leguminous plants species such as clover or soybean (Peters et al. 1986; Van Der Heijden et al. 2008). The same phenomenon is seen with other rhizobial species like *Rizobium trifolii*, which nodulates white clover (*Trifolium repens*) but is unable to colonize vetch (*Vicia*), and vice-versa.

In addition to rhizobial symbiosis, legumes also establish an interaction with beneficial AMF, which helps the host plant to be well nourished in nutrient-deficient soil by providing access to nutrients, in particular P. Plant growth of legumes is usually enhanced by symbiosis of AMF and rhizobial symbionts simultaneously (Barea et al. 1992). Thus, in this regard, with some exceptions, legumes are special plants species, which establish symbioses with both AMF and rhizobia simultaneously by favoring a three-way symbiotic interaction that provides benefits to all three partners, i.e., a "tripartite symbioses" (Barea et al. 1992). After establishment of tripartite symbiosis, legume plants take up essential nutrients such as P and N, and trade these elements with the host in exchange for sources of photosynthetically fixed C (Fig. 9.1).



Note: Brown dot refers to Rhizobia
Red curve refers to AMF

Fig. 9.1 Nutrient transfer model between arbuscular mycorrhizal fungi (AMF), rhizobia and host legume plant during tripartite symbiosis

9.2 Molecular Communication of AMF and Rhizobia During Tripartite Symbiosis

It has been shown that the establishment of both AMF and root nodules share some common features in early signaling. Here, we describe the molecular communication, common chemical signals, and nutrient complementation in rhizobia and AMF by installing the symbiotic relation with the same host plant, namely "tripartite symbiosis".

9.2.1 Signaling Molecules in the Rhizosphere During the Pre-symbiotic Interaction of AMF and Rhizobia

AMF and rhizobia establish a tripartite symbiosis as a result of signals exchanged during the pre-symbiotic phase, in which there is a mutual recognition of diffusible signals produced by the host plant and its microbial partners. Previous studies have shown that, during symbiosis, host plant roots secrete a phytohormone called strigolactone (SL), which strongly stimulates AMF spore germination and hyphal branching (Buee et al. 2000). A diffusible signal molecule in AMF is produced and activated in response to plant-secreted SL, which in turn triggers the common symbiotic pathway of the host plant, based on the genetic requirement necessary for their recognition (Fig. 9.2a) (Oldroyd 2013; Pfeffer et al. 1999; Smith and Read 1996). Myc factors are a mixture of sulfated and non-sulfated lipochitooligosaccharides (LCOs), which are structurally similar to the Nod factors (NFs) produced by rhizobia (Maillet et al. 2011). Recent investigations have also shown that AMF also produce LCOs; these Myc-LCOs stimulate mycorrhization in diverse plants species. At an early stage of both AMF and rhizobial symbiosis, the diffusible signals activate a signaling pathway called the common symbiotic pathway (CSP), which is required for successful establishment of AMF and rhizobia (Catoira et al. 2000). In contrast, host leguminous plant roots also secrete secondary metabolites known as "flavonoids", which are recognized by rhizobia. Rhizobia use an NF-dependent strategy for nodule formation during the symbiotic interaction. After perception of flavonoid signaling, *NF* gene expression occurs, which, at a later stage, results nodule organogenesis (Fig. 9.2b) (Denarie et al. 1996; Long 1996; Oldroyd 2013). NFs possess a chitooligosaccharide backbone generally made up of four to five *N*-acetylglucosamine (GlcNAc) residues with β1–4 linkages (Oldroyd 2013). In general, with a few exceptions, NF are vital for the symbiotic association between rhizobial species and host legumes, as rhizobia and legume mutants defective in the production and perception of NF, respectively, can no longer form the symbiotic association (Denarie et al. 1996; Downie and Walker 1999; Giraud et al. 2007; Oldroyd and Downie 2004). It has been shown experimentally that exogenous application of 0.1 μM GR24 (a synthetic SL analogue) in legume plants (*M. sativa* and *M. truncatula*) also induced nodulation, while a relatively higher concentration

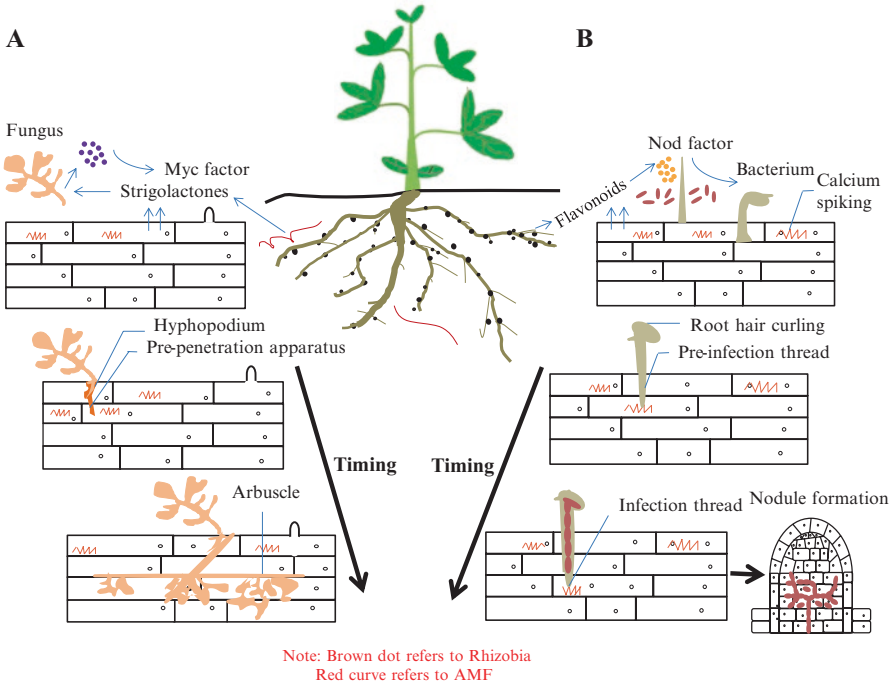


Fig. 9.2 Rhizobial and mycorrhizal colonization (Modified from Oldroyd 2013). (**a**) Host plant secrets a signaling molecule known as strigolactone (SL) in the rhizosphere, which promotes AMF symbiosis. AMF induce spore germination and hyphal branching after perception of SL signaling. In the next step, AMF produce mycorrhizal factors (Myc factors), which include lipochitooligosaccharide (LCOs) that trigger the symbiotic signaling pathway in the root region, which leads to calcium oscillations. AMF infect the host root cell via an infection peg from the hyphopodium, and allow hyphal growth inside the root epidermal cell. The route of hyphal invasion in the plant cell is predicted by a pre-penetration apparatus, and finally the fungus colonizes the host plant root cortex through intercellular hyphal growth. (**b**) The host plant releases flavonoids to rhizobia in the rhizosphere, which produce Nod factors, which are recognized by the host plant. Like Myc factors, Nod factor perception activates the symbiosis signaling pathway, leading to calcium oscillation, initially in epidermal cells, but later also in cortical cells, preceding their colonization. Rhizobia gain entry into the plant root by root hair cells that grow around the bacteria attached at the root surface, trapping the bacteria inside a root hair curl. Infection threads are invasive invaginations of the plant cell that are initiated at the site of root hair curls, and allow invasion of the rhizobia into the root tissue. The nucleus relocates to the site of infection, and an alignment of ER and cytoskeleton, known as the pre-infection thread, predicts the path of the infection thread, which ultimately leads to nodulation

ceased nodule organogenesis in *M. truncatula* (De Cuyper et al. 2015; Soto et al. 2010). It was also revealed that SL-biosynthetic mutant lines of *Pisum sativum* and *Lotus japonicus* produced few nodules, and could be rescued by exogenously applied GR24 (Foo and Davies 2011; Foo et al. 2013; Liu et al. 2013). Moreover, rhizobium infection and application of LCOs also induced the expression of SL-biosynthetic pathway genes (*MtD27*, *MtCCD8* and *MtCCD8*) in *M. truncatula* root hairs (Breakspear et al. 2014; van Zeijl et al. 2015).

9.2.2 *Common Symbiotic Pathway (SYMRK) Crucial for Both AMF and Rhizobial Symbiosis*

Legumes use a common symbiotic pathway to establish rhizobia and mycorrhizal symbiosis, and both symbionts depend partly on overlapping genetic programmes (Duc et al. 1989; Wegel et al. 1998). Symbiotic receptor-like kinase (SymRK) was previously found to be required for root nodule development, which is likely active near the junction of fungal and rhizobia signaling cascades. *DMI1*, *DMI2, DMI3* and *NSP* genes were confirmed to be crucial for both nodulation and AMF formation in *M. truncatula*, and can also control the major NF signal-transduction pathway (Fig. 9.3a). In previous work, it was inferred that SymRK in *L. japonicus* and *DMI2* in *M. truncatula* were required for NF signaling and mycorrhizal establishment (Endre et al. 2002; Stracke et al. 2002). Kosuta et al. (2008) found that mycorrhizal fungi activate calcium oscillations in *M. truncatula* root hair cells, which requires *DMI1* and *DMI2* components of the common SymRK pathway, which are also necessary for NF-induced calcium (Ca^{2+}) spiking (Kosuta et al. 2008). CCaMK is also sufficient to induce nodule formation, which is also activated during mycorrhizal association, and leads to early mycorrhizal responses. Miwa et al. (2006) indicated that a *L. japonicas* SymRK pathway mutant was impaired in Ca^{2+} spiking, and was unable to form root nodules or to be infected by AMF (Miwa et al. 2006). So the genes *DMI1*, *DMI2*, *DMI3*, and *NSP* are responsible for nodule formation, and plants with mutations in these specific genes were unable to establish a symbiotic association with endomycorrhizal fungi. There are at least three steps to nodulation and endomycorrhization in *M. truncatula*, giving insight into evidence for a common signaling pathway in nodulation and mycorrhization (Fig. 9.3b). Also, the perception of NF requires NF receptors NFR5/NFP and NFR1/LYK3, in combination with a signaling pathway that is also involved in the establishment of mycorrhizal associations.

In summary, AMF and rhizobia share the SymRK pathway at both the molecular and genetic level after perception of microbial specific LCOs signals. In this regard, the common symRK pathway is crucial for the successful establishment of tripartite symbiosis with leguminous plants.

9.2.3 *Possible Role of MAPK Cascades During Tripartite Symbioses*

The interaction between SymRK and SIP2 is conserved in leguminous species (Chen et al. 2012). A SymRK-interacting protein (SIP2) represents a typical plant mitogen-activated protein kinase kinase (MAPKK), which was found to form protein complexes with SymRK. Mitogen-activated protein kinases (MAPKs) plays a vital role in stress responses in AMF signal transduction pathways (Liu et al. 2015; Widmann et al. 1999). On the other hand, a MAPK cascade is likely to be a

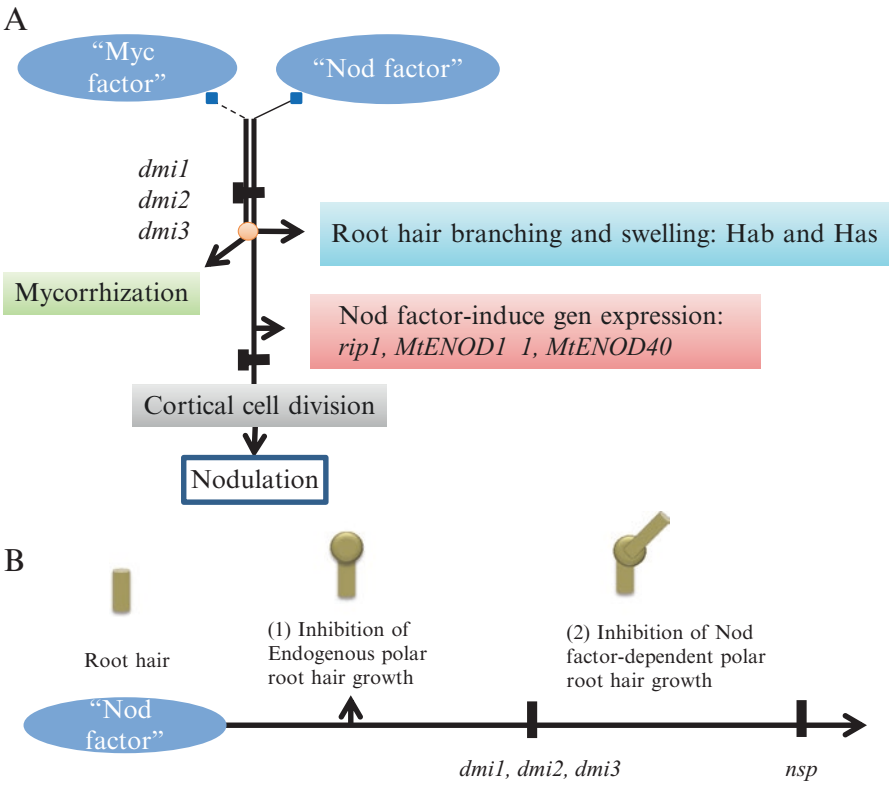


Fig. 9.3 Models for the intervention of *DMI1*, *DMI2*, *DMI3,* and *NSP1* in Nod Factor signal transduction (Modified from Catoira et al. 2000). (**a**) *MtENOD11*, *rip1,* and *MtENOD40* can be expressed in a Nod factor signal transduction pathway, as well as cortical cell division and nodulation. In the mutants of *dmi1*, *dmi2*, *dmi3*, and *nsp1*, *DMI1*, *DMI2*, and *DMI3* presumably intervene at one or more steps of the pathway downstream of Has and upstream of both Hab and *NSP*. The part of the signaling pathway controlled by *DMI1*, *DMI2*, and *DMI3* would be induced both by Nod factors and by potential mycorrhization signals ("Myc factors"), and would play a role in the preparation of the plant for infection by both rhizobia and endomycorrhizal fungi. *Hab* Root hair branching, *Has* root hair swelling. (**b**) Nod factors would (1) inhibit endogenous polar root hair growth, and (2) promote Nod-dependent growth. *DMI1*, *DMI2*, and *DMI3* are required for an element or elements of Nod factor signal transduction downstream of (1) and upstream of (2) or that Nod factors trigger (1) and (2) by different pathways. *Nsp* mutants are able to initiate polar growth in response to Nod factors, indicating that *NSP* is involved in a component of Nod factor signal transduction downstream of (2)

downstream target of the NF signaling pathway. So it is reasonable to speculate that AMF and rhizobia not only share the common SymRK pathway and perceive common plant-secreted chemical signals in tripartite interaction with host legumes, but that they also share some common components of MAPK signaling at the presymbiotic or symbiotic stage. However, our knowledge of the involvement of MAPK cascades during tripartite symbiosis is in its infancy, requiring further investigation at the molecular and genetic level.

9.3 P Metabolism in AMF and Rhizobia During Tripartite Symbiotic Association with the Host Plant

P is one of 17 essential elements required for plant growth, and is the least available in the soil (Bieleski 1973; Raghothama 1999). P is taken up by plants as inorganic phosphorus (Pi) via two major pathways: the first is via the root-soil interface, including root hair cells, which increase the available Pi; the second strategy is the mycorrhizal uptake pathway in extending extra-radical fungal hyphae (Bates and Lynch 1996; Jakobsen et al. 1992). P is absorbed by the ERM, with subsequent efflux into the interfacial apoplast, which is regulated by the concentration of intracellular metabolically active Pi with the hyphae. The fungal mycelia grow to up >100 times larger than root hairs, and can absorb more P in Pi-depleted zones. Neutral lipid is the main type of triacylglycerol (TAG) synthesized in the IRM and exported to the ERM, and is stored in AMF spores and vesicles (Bago et al. 1999).

It is well known that rhizobia play a key role in natural and agricultural ecosystems by fixing N_2 and promoting plant productivity (van der Heijden et al. 2016). Depending on symbiotic N_2 fixation, most legumes have a higher P requirement than the N fertilizer supplied; however, AMF enhance Pi uptake via their extensive mycorrhizal hyphal networks, and the subsequent P transfer to legumes likely facilitates legume growth and biological N_2-fixation in nutrient-poor grassland communities. Indeed, up to 20% of total P is allocated to nodules, as Pi deficiency also affects the growth of rhizobia (Ribet and Drevon 1995) and reduces the growth of nodules (Gunawardena et al. 1992). Rhizobia require more energy and P than legume roots, because the nodules are strong P sinks and N_2-fixation is an energy intensive process (Al-Niemi et al. 1998; Almeida et al. 2000; Sa and Israel 1991). Nasto et al. revealed that N_2-fixing legumes have higher AMF root colonization levels and greater root P activity than non-fixers (Nasto et al. 2014). As a result, P uptake enhances the extensive mycorrhizal hyphal network, and facilitates host plant growth by increasing symbiotic N_2 fixation in the host legume in nutrient-scarce environments.

Enhancement of the tripartite symbiosis due to the interaction between the two micro-symbionts is likely to continue at this stage, mainly through plant-mediated systemic mechanisms. Increased P supply by the fungus is then likely to be the main factor regulating the tripartite symbiosis.

9.4 N Metabolism in AMF and Rhizobia During Tripartite Symbiosis

The host plant transfers C to AMF, and AMF translocate P to the plant; however, they both compete for soil N. N is the nutrient whose unavailability most commonly limits plant growth in natural ecosystems. About 30% of total N uptake by roots

comes from the ERM of AMF. AMF absorb nitrate (NO_3^-) (Bago et al. 1996), ammonium (NH_4^+) (Johansen et al. 1996) and amino acids (Hodge et al. 2001) from the environment by ERM, and transfer N to the host plant to increase the availability of N forms. N uptake and incorporation into amino acids occurs via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, which has been found in ectomycorrhizal fungi, and the amino acids formed are then transported to the IRM (Chalot et al. 1994; Martin 1985). Arginine (Arg) is the major form of N synthesized and stored in the ERM, and is transported to the IRM where NH_4^+ is released for use by the plant cell (Tian et al. 2010). In the ERM of AMF, Arg may be associated with polyphosphate (PolyP), and can break down into inorganic N through accumulated N and transport with P.

Each year, about 40 million tons of N_2 is added to agricultural systems by N_2-fixing rhizobia symbiosis with legume plant roots (Udvardi and Poole 2013). Rhizobia nodulate specific leguminous host plants, resulting in the formation of root nodules, which fix atmospheric N_2 and enhance host plant growth. Approximately 6 g of photosynthetic C is used per gram fixed N (Vance and Heichel 1991). During the deformation stage, the plant perceives the rhizobial signal, as discussed above, and initiates a developmental program aimed at the formation of symbiotically N_2-fixing nodules (Denarie et al. 1996). After recognition of specific plant and bacterial signals, and activation of common symbiotic pathways, formation of infection threads and nodule organogenesis, N_2 fixation then starts in bacterial nodules followed by its transport to the plant. During the course of this process, rhizobia differentiate into a N_2-fixing form, typically known as bacteriod, in the nodules. NF also acts as symbiotic signaling molecules for initiating nodule development, which can be perceived or transduced by the host plant. Genetic analysis of rhizobia has led to the identification of *nod* genes, which are under the control of signals secreted by the plants into the rhizosphere. Through the symbiosis, rhizobia fix N_2 from the atmosphere for leguminous plants, and legumes can account for as much as 97% of the total plant N (Peoples and Craswell 1992).

Mycorrhizal colonization generally has a positive effect on nodulation. It is interesting that the two groups of microorganisms do not seem to compete for colonization sites (Ardakani et al. 2009); however, the nodule number and size of rhizobia increase in the presence of AMF (Tobar et al. 1996). Dual inoculation with AMF and rhizobia can increase plant growth and N_2 fixation to a greater extent than inoculation with either AMF or rhizobia alone (Antunes et al. 2006). Flavonoids are thought to be key signal compounds associated with the establishment of tripartite symbiosis, and specific flavonoids are necessary to stimulate nodulation and N_2 fixation (Day et al. 2000; Zhang and Smith 1995). When AMF Myc factors are present, the rhizobial NF has less chance to be cleaved by constitutive plant chitinases, which enhances nodulation (Antunes and Goss 2005). However, interaction among participants in the tripartite symbiosis has a significant impact on N_2 fixation (Goss and De Varennes 2002; Science and Antunes 2004).

9.5 C Metabolism During AMF and Rhizobial Tripartite Symbiosis with Host Legumes

C flow is a key process occurring during AMF and rhizobial symbiosis. AMF and rhizobia are believed to be completely dependent on their host plant for the C source. In the symbiotic state, significant C flow takes place from the plant to the fungus via internal fungal structures (Shachar-Hill et al. 1995). Plant root cells release sucrose (Suc), which is then hydrolyzed to hexose (Schaarschmidt et al. 2006), and finally taken up by the fungus (Schüßler et al. 2006). The exchange of C for P supply may be coupled (Bücking 2004; Bücking and Shachar-Hill 2005); once taken up by the fungus, C is incorporated into lipids in fungal mycelium (Bago et al. 2002; Pfeffer et al. 1999). To a certain degree, the lipid represents the major C source for supporting the extension of the ERM in the soil, and controls the C supply to regulate fungus lipid metabolism (Kiers et al. 2011). Increased carbohydrate availability stimulates C flux across the mycorrhizal interface and alters Pi uptake, allocation and transfer to the host plant during AMF symbiosis (Bücking and Shachar-Hill 2005), while during high plant P levels, C release to the fungus declines (Olsson et al. 2002). Moreover, C expenditure for N assimilation is high, and this high C cost has been suggested to be responsible for the decrease in ectomycorrhizal fungal diversity and growth in soil with high N availability.

On the other hand, the host provides bacteriods with C supply to increase N_2 fixation and successful symbiosis (Rogers et al. 2009). C transport and metabolism involves the delivery of Suc to the root nodule of the host legume, passing through the phloem, where it is cleavage by Suc synthase and enters into the glycolytic pathway, ultimately providing malate to the bacteriods. In return, bacteriods provide NH_4^+ to the host plant cell, which is finally assimilated into glutamine. Amides (glutamine and asparagine) and uriedes are transported to plant from root nodule via xylem as discussed in the section on P metabolism. Malate within the bacteriods is oxidized by Krebs' cycle and provides reductants for the nitrogenase complex and respiratory chain that fuels the nitrogenase complex with ATP. Many reports in the literature have confirmed that legumes have a competitive advantage over non-leguminous plants as the amount of CO_2 increases (Rogers et al. 2006; Soussana and Hartwig 1995; Zanetti et al. 1996). Additionally, various experiments have found that nodulated and non-nodulated genotypes within a legume species that fix N_2 are more responsive to elevated CO_2 than non-fixers (Ainsworth et al. 2002, 2004; Lüscher et al. 2000).

In particular, legumes form tripartite associations with AMF and rhizobia, making legumes a renewable source of N, and allowing them to grow in low P soils (Frey and SCHÜEPP 1992; Udvardi et al. 2005). In the same way, legumes can increase photosynthetic productivity upon AMF and rhizobia dual inoculation (Jia et al. 2004). It is clear that legumes provide the C for both AMF and rhizobia, as a kind of compensation strategy; the two symbionts supply nutrients, especially N and P, to the host plant, and accelerate host plant photosynthetic productivity, which finally leads to agricultural productivity in a sustained manner.

9.6 Concluding Remarks

Legumes are fascinating plants that make a tripartite interaction with AMF and rhizobia by sharing common chemical signaling molecules (flavonoids and SL) and symbiotic pathways, for instance SYMRK and MAPK cascade pathways. After perception of common chemical signals, and activation of common symbiotic pathways, the AMF and rhizobia establish a successful symbiotic relation with the host legume plant. These two symbionts play a key role in natural ecosystems as they enhance the availability of nutrients, as AMF supply P and other mineral elements to host plants, and rhizobia provide N for legumes. Moreover, dual inoculation of legumes with AMF and rhizobia not only enhances plant growth but also promotes rhizobial-N_2 fixation compared to single inoculation. In this regard, AMF indirectly help the plant with N uptake, by increased supply N to rhizobia for nodule development and N_2 fixation.

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Chapter 10
Adaptation to Phosphate Stress by N_2-Fixing Legumes: Lessons to Learn from the Model *Medicago truncatula*

Saad Sulieman and Lam-Son Phan Tran

Abstract The central importance of inorganic phosphate (Pi) in legume nutrition and agricultural sustainability has long been recognized. However, Pi concentration in soil solution is often low because it readily forms insoluble complexes with calcium in alkaline soils or with iron and aluminum oxides in acidic soils. Consequently, low Pi availability is frequently the most limiting nutrient for plant growth and development over the majority of the earth's land surface. Among the legumes, the model plant *Medicago truncatula* has emerged as a well-established system for characterization of Pi deficiency on leguminous plants at the physiological and molecular levels. The recent published reports have contributed to major advances in understanding the physiological, biochemical, and molecular mechanisms/pathways in *M. truncatula* root nodules to ameliorate low Pi stress. Metabolic and gene expression profiles of *M. truncatula* nodules induced by Pi stress reveal interesting features, including rearrangement of Pi, carbon, and nitrogen homeostases, enabling plants to cope with Pi scarcity. In this context, the separate impacts of Pi deficiency on such candidate regulatory pathways should be considered, but moreover their interacting and entwined networks cannot be excluded. Therefore, understanding the responsive and adaptive mechanisms conferring Pi tolerance to the *M. truncatula* symbiotic system is very important for the development of selection and breeding strategies.

Keywords *Medicago truncatula* • Adaptation • Nitrogenase activity • Nodule • Pi deficiency • *Sinorhizobium meliloti* • Symbiotic capacity

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10.1 Introduction

It is well established that legume symbiotic dinitrogen (N_2) fixation is dependent on the availability of inorganic phosphate (Pi), the only form of phosphorus that can be assimilated by plants (Rodiño et al. 2009; Qin et al. 2012; Nasr Esfahani et al. 2016). Unfortunately, legumes are continuously subjected to conditions in which availability and mobility of Pi are at lower extremities. Under such diminishing Pi supplies, legumes may lose the distinct advantage of a valuable unlimited source of nitrogen (N), especially for plants cultivated in Latin America, Asia, and Africa (Valdés-López and Hernández 2008; Zhang et al. 2014). Long-term performance and survival of legumes is, therefore, highly dependent on the metabolic reprogramming and genetic circuitry of plants to Pi scarcity (Cabeza et al. 2014a; Nasr Esfahani et al. 2016). To maintain their symbiotic performance under Pi limitations, legumes have developed a fascinating ability to evolve a diverse array of responsive and adaptive strategies (Li et al. 2011; Drevon et al. 2015; Sulieman and Tran 2015).

Sustainable cropping practices require plant researchers to identify and discover mechanisms that improve legume acclimation, as well as to exploit these Pi stress-responsive mechanisms to develop varieties with higher symbiotic efficiency. Accordingly, morphological, biochemical, physiological, and molecular adaptations of plants subjected to Pi stress have been the subject of many recent reviews (e.g., Plaxton and Tran 2011; Ha and Tran 2014; López-Arredondo et al. 2014; Sulieman and Tran 2015; Herrera-Estrella and López-Arredondo 2016). The knowledge on regulatory and acclamatory mechanisms involved in Pi acquisition and utilization by nodulating legumes will be useful in developing cultivars with effective N_2 fixation via conventional breeding and/or biotechnological approaches, hence, contributing greatly to the practice of economical and environmentally friendly crop agriculture (Sulieman et al. 2013a).

Among the legumes, *Medicago truncatula* (barrel medic) has received a special attention as one of the best platforms for Pi stress research (Jain et al. 2007; Tesfaye et al. 2007). This valuable species serves as one of the major evolutionary success stories to explore the genetic and molecular aspects of N_2-fixing symbiosis in leguminous plants (Larrainzar et al. 2014; Staudinger et al. 2016). Being closely relative to alfalfa (*M. sativa*), *M. truncatula* has been emerged as a model plant for studies of legume biology and has been the subject of structural and functional genomic investigations (Rose 2008; Sulieman and Schulze 2010a; Panara et al. 2012; Cheng et al. 2014). It has been particularly used in numerous studies aiming to reveal the negative impacts of Pi stress on plant growth and development. In this regard, many researchers have used the cultivar Jemalong (line A17) in association with *Sinorhizobium meliloti* (formerly called *Rhizobium meliloti*) to facilitate and characterize the legume metabolic adaptive mechanisms when subjected to Pi limitation (Sulieman et al. 2013a, b). Therefore, understanding the physiological and biochemical mechanisms conferring Pi stress tolerance to this symbiosis is very important for developing different selection and breeding strategies.

This chapter summarizes the knowledge currently being made toward understanding of Pi stress in the model *M. truncatula* that may contribute to the understanding of the acclimation of the nodulated legumes to Pi scarcity. Likewise, other chapters in

this volume exploring other attractive candidate species, namely, common bean (Sánchez-Correa and Valdés-López 2017, Chap. 11), soybean (Zogli et al. 2017, Chap. 12), and white lupine (Uhde-Stone 2017, Chap. 13), have also provided valuable genomic and genetic evidence in understanding plant responses and adaptations to Pi limitations. Collectively, these four plant systems represent the main focus of low Pi stress research in legumes (Valdés-López and Hernández 2008). Although these four systems show several common adaptive mechanisms, such as the root architecture modifications, exudation of Pi-mobilizing compounds, and higher Pi conservation in nodules, they also possess some specific responsive mechanisms, for instance, specific shoot-to-nodule signaling communication, formation of proteoid roots, and different transcriptome changes occurring in roots and nodules in response to Pi starvation.

10.2 Whole-Plant Regulation of *M. truncatula* Nodulation Under Pi Deficiency

An effective symbiosis between *M. truncatula* and *S. meliloti* is dependent on a balanced physiological interaction enabling the microsymbiont to fix atmospheric N_2 (Sulieman and Tran 2013). Under Pi-limited conditions, the symbiotic interaction between both partners is regulated by still poorly understood control mechanisms (Hernández et al. 2009; Nasr Esfahani et al. 2016). However, based on the published literature, there is more than one systemic regulatory circuit controlling nodule functioning in *M. truncatula* under Pi scarcity (Sulieman et al. 2014). It has been suggested that whole-plant responses of *M. truncatula* to low Pi concentrations involve a great deal of coordination of sensing and long-distance signaling communication (Tesfaye et al. 2007; Sulieman et al. 2010; Cabeza et al. 2014a). This regulation can be achieved through various regulatory switches, which leads to a new metabolic arrangement in *M. truncatula*, increasing diverse metabolic processes involved in energy supply and cell growth. One of the major challenges we are currently facing is the identification of the regulators that orchestrate these metabolic rearrangement and determination of the regulatory networks that control plant responses to Pi deficiency. Here, we outline the current advances in research on the complex network of *M. truncatula* responses to low Pi stress and discuss several possible strategies that could help to develop low Pi-tolerant symbiotic legumes.

10.2.1 Nodule Formation

The understanding of the physiological basis of nodule formation under Pi scarcity has greatly advanced since the exploration of *M. truncatula* symbiosis. Early nodule initiation was almost unaffected at lower levels of Pi in the external nutrient solution (Tang et al. 2001; Sulieman et al. 2008, 2010). Based on the performed trials, low Pi (~1 μM) supply was sufficient to establish optimal nodulation when a large number

of bacteria were present. Hence, it is hypothesized that Pi reserve in *M. truncatula* seeds sufficiently support rhizobial infection during the onset of nodule formation. However, more Pi supply is critically required to boost the formation of nodules at advanced stages of their development (Høgh-Jensen et al. 2002; Chaudhary et al. 2008; Drevon et al. 2015). At this premise, plants managed to maintain the activity of the existing nodules at a considerable level, while on a whole-plant basis N_2 fixation was restricted by a virtual suppression of the formation of new nodules during Pi deficiency (Cabeza et al. 2014a). The observed repression of nodule formation by prolonged Pi deficiency was supported by differential expression of various genes involved in nodule formation and symbiosome development (Cabeza et al. 2014a).

The role played by quorum sensing in regulating the survival and development of *M. truncatula-S. meliloti* symbiosis has been recently investigated under Pi stress (Pakdaman et al. 2014). To ensure the survival and successful symbiosis under Pi limitation, this pathway helps coordinate the functional processes of free-living and symbiotic *S. meliloti* according to the local density of the bacterial population (McIntosh et al. 2009). Hence, a cross talk between Pi availability and bacterial density in the growing substrate is established to regulate the quorum-sensing pathway in bacteria, thereby affecting the flavonoid biosynthesis in plant roots and the subsequent steps involved in the symbiotic communication between *M. truncatula* and *S. meliloti* (Mathesius et al. 2003; Pakdaman et al. 2014).

Several reports have suggested a putative function for strigolactones in legume nodule formation, especially under Pi deprivation (Soto et al. 2010; Foo et al. 2013; Liu et al. 2013). Strigolactones are a class of plant hormones whose biosynthesis is activated in response to Pi deficiency. In *M. truncatula*, it was recently showed that *MtD27* gene is involved in strigolactone biosynthetic pathway in plant roots upon Pi stress. This gene is strongly induced by lipochito-oligosaccharides (also known as Nod factors) excreted by rhizobia through a symbiotic signaling cascade (van Zeijl et al. 2015). Based on the published data, *MtD27* is co-expressed with a couple of genes (*MtCCD7* and *MtCCD8*) in nodule primordia, as well as nodule meristem and distal infection zone of mature nodule at subsequent stages of progress. Hence, a putative function for these strigolactone biosynthetic genes was suggested during several stages of *M. truncatula-S. meliloti* symbiosis following Pi starvation (van Zeijl et al. 2015). In this context, further information on strigolactones and the role played in nodulation under Pi stress is provided by Eloise Foo in this volume (Chap. 8).

10.2.2 Nodule Function

Pi deficiency adversely affects nitrogenase activity and nodule N_2-fixation capacity in leguminous plants (Chaudhary et al. 2008; Rotaru and Sinclair 2009; Valentine et al. 2011; Sulieman and Tran 2015). With the aid of H_2 evolution, an elaborated system that enables continuous (up to several weeks), high time resolution (measurements can be taken every second) and noninvasive in vivo measurements of nitrogenase activity has been recently developed (Cabeza et al. 2015) (Fig. 10.1a).

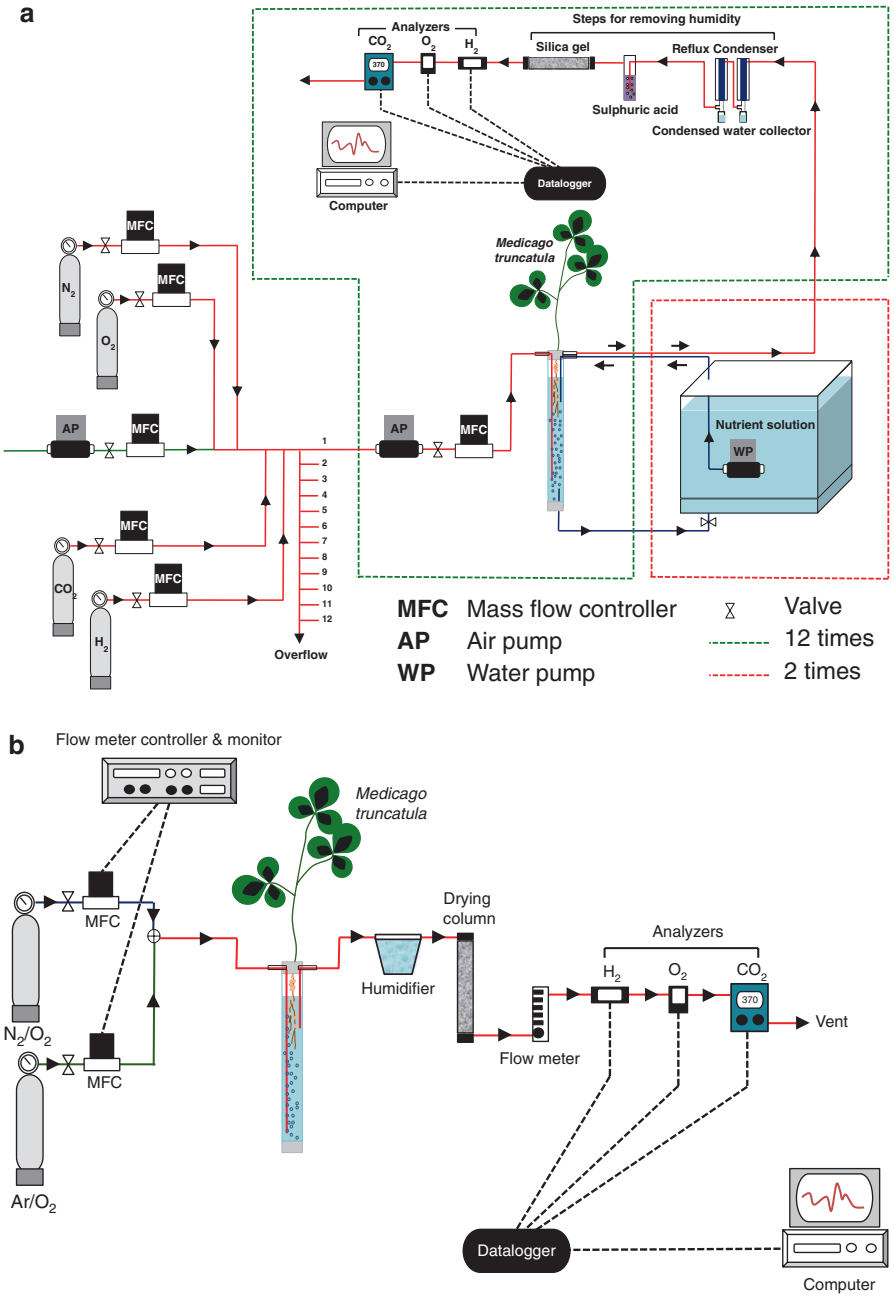

Fig. 10.1 (**a**) Experimental setup for long-term, continuous, and noninvasive measurement of nodule H_2 evolution (Cabeza et al. 2015). (**b**) Experimental setup for classical open-flow gas measurement system used for measuring the nitrogenase activity (H_2 production)

The new set up was made based on the old classical open-flow gas measurement system (Fig. 10.1b). Although the classical system provides point measurements of N_2 fixation, it has been widely used by many researchers interested in the physiological and biochemical aspects of nodule N_2 fixation (Gordon et al. 2002; Schulze and Drevon 2005; Schulze et al. 2006; Fischinger and Schulze 2010; Fischinger et al. 2010). Unlike the old set up, *M. truncatula* exhibited vigorous growth in the new system when being solely dependent on N_2 fixation (Cabeza et al. 2015).

Importantly, the characterization of *M. truncatula* plants exposed to Pi deficiency was precisely estimated for wild-type and genetically manipulated (i.e., mutants) plants. Rather than a steady decline upon exposed to Pi deficiency, a daily consistent rhythm in nodule activity has been discovered (Cabeza et al. 2014b, 2015). Over the day/night cycle, Pi starvation obviously reduced the nitrogenase activity after several hours of light exposure, followed by a recovery period in the afternoon. This recovery was further extended after a short decline during the dark period of the light/dark regime. Collectively, a significantly decreased activity per day was quantified in Pi-stressed *M. truncatula* plants. Apparently, the discovered different daily patterns of nodule activity under Pi stress necessitate, in some cases, us to re-evaluate those regular point measurements of nitrogenase activity (Cabeza et al. 2014b). Alternatively, it might indicate a circadian effect on nitrogenase activity under low Pi conditions, which is of high interest for further in-depth studies.

10.2.3 Proton Release and Acidification of the Rhizosphere

Various plants have developed numerous strategies to cope with Pi deficiency resulted from low Pi availability in soils (Hinsinger 2001; Hinsinger et al. 2003). One of the widespread plant responses in N_2-dependent *M. truncatula* grown under a continuously low Pi supply is the increased acidification of the rhizosphere (Tang et al. 2001; Schulze et al. 2011). In accordance, the higher root acidification capacity related to proton (H^+) extrusion has been demonstrated in the rhizosphere of common bean (Alkama et al. 2012). Due to the higher uptake of excessive cations over anions in nodulating plants under Pi deprivation, the net H^+ efflux has been enormously increased in the rhizosphere (Tang et al. 2001, 2009; Liu et al. 2016). Hence, Pi deficiency affects the cation-anion uptake equilibrium and consequently H^+ release at root-soil interface for compensation of electrical positive charges and regulation of cytosolic pH in the root cells. The increase in rhizosphere acidification suggests an involvement of nodulated-root-acidification capacity in the adaptation of *M. truncatula* to Pi deficiency. This trait can alter the rhizosphere chemistry and facilitate the Pi acquisition at the root-soil interface, especially in soils containing Ca-Pi such as neutral and calcareous soils or even acidic soils fertilized with Pi rocks (Zhang et al. 2014).

Based on the published reports, the responses to Pi stress in terms of nodulated-root H^+ efflux widely differ between *S. meliloti*-Jemalong A17 symbiotic combinations harboring different symbiotic efficiencies (Schulze et al. 2011). Although the amounts of specific H^+ efflux, i.e., per unit root biomass, were the highest at the lowest

level of Pi supply (5 µmol Pi plant^{-1} week^{-1}) for both *S. meliloti* strains 102F51 and 2011, the H^+ efflux from plants inoculated with *S. meliloti* 102F51 was greater than that of *S. meliloti* 2011-inoculated plants at the same Pi level (Schulze et al. 2011). These findings are in agreement with the study of Sulieman and Schulze (2010a) that showed that *S. meliloti* 2011 strain has a lower symbiotic efficiency compared with *S. meliloti* 102F51. Hence, we assume that the H^+ efflux ability of *M. truncatula* Pi-starved plants is closely associated with its nodule symbiotic efficiency.

10.2.4 Pi Recycling

In order to acclimate to low Pi availability, *M. truncatula* plants have evolved a strategy to stabilize cellular Pi homeostasis in nodules and keep high rates of nitrogenase activity as long as possible (reviewed in Sulieman and Tran 2015). Since nodules represent a preferential Pi sink among various plant organs, Pi concentration in Pi-starved nodules can reach up to threefold of that in other organs (Jebara et al. 2005; Schulze and Drevon 2005; Hernández et al. 2009; Sulieman et al. 2013a, b). During Pi starvation, plants are able to provide a sufficient assimilate supply for bacteroids in Pi-deprived nodules until the Pi level in leaves reaches a lower threshold concentration, which is necessary to preserve the organ functioning during Pi limitation (Cabeza et al. 2014a). While keeping searching for new Pi resources, Pi mobilization in nodules takes place that would contribute to a higher turnover of the scarce Pi, thereby ameliorating the negative effects of Pi deficiency under low Pi availability. In conformity, purple acid phosphatases and Pi transporters (e.g., PHO1) were consistently upregulated to mobilize scarce Pi from organic substrates in response to Pi deprivation (Cabeza et al. 2014a). For instance, intracellular acid phosphatases can scavenge and remobilize Pi from a broad spectrum of Pi monoesters, hence, considered among the major intricate arrays of biochemical and physiological adaptation to Pi deficiency (Li et al. 2012; Vardien et al. 2016). In this context, further information about phosphatases and their roles in maintaining nodule Pi homeostasis under Pi stress is described elsewhere in this volume.

10.2.5 Assimilate Supply and Carbon Turnover

The role of carbon (C) metabolism in regulation of N_2 fixation under Pi stress has been reviewed in Chap. 4 (Kleinert et al. 2017a). Downregulation of C turnover in diverse grain legume nodules has been widely observed under Pi-deprived conditions. The root nodules are a unique organ with intense C metabolic activity and hence are exceptionally Pi-rich plant tissues. In order to maintain the functioning system under conditions of limiting Pi, legumes tend to modulate their nodule C-respiratory pathways, and thus contributing to a higher internal P-use efficiency (Valentine et al. 2011; Nasr Esfahani et al. 2016). These metabolic rearrangements have been strongly

supported by various studies on the C expenditure for maintaining nitrogenase activity (nodules respired C per unit reduced N) in Pi-deficient treatments.

Although Pi starvation has a strong impact on leave CO_2 assimilation and carbohydrate turnover, photosynthate importation in the form of sucrose that primarily fuels the symbiotic process is relatively stable in *M. truncatula* nodules under Pi limitations (Cabeza et al. 2014a; Sulieman et al. 2013b) (Fig. 10.2). This finding implies that photoassimilate transport into the nodules of Pi-starved plants normally functions. The ability of plants to adapt and buffer assimilate supply to nodules was supported by the artificial sucrose feeding to the phloem of Pi-stressed plants, which showed insignificant effect (Cabeza et al. 2014a). The sufficient photoassimilate supply to *M. truncatula* under Pi stress was also supported by the supernodulating mutant (*SUNN*) nodules that are often in excessive number, with small size and low specific activity compared with the wild-type (Jemalong A17) nodules (Cabeza

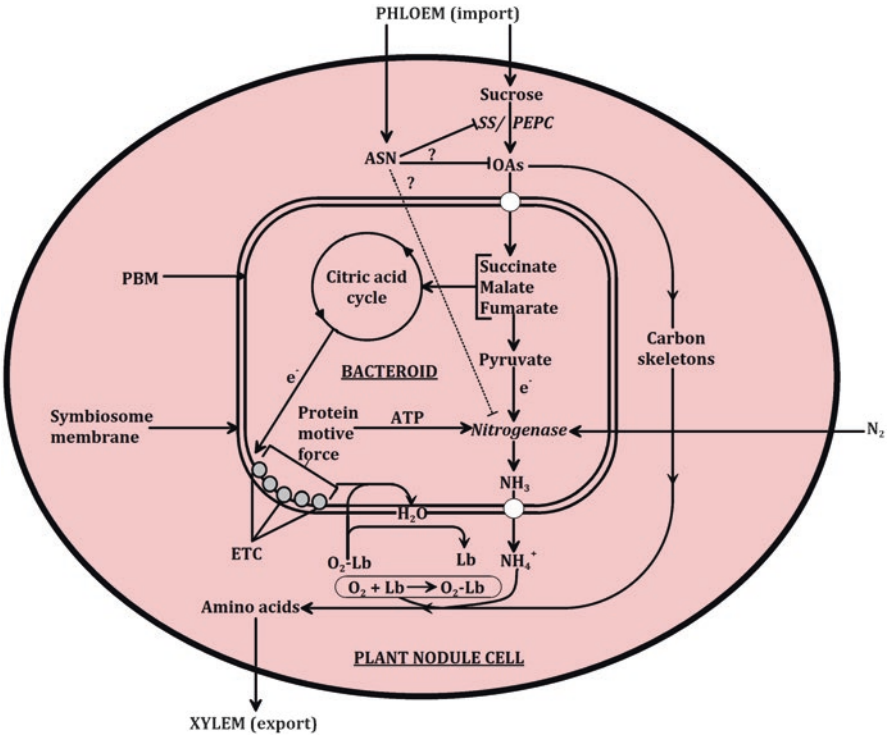

Fig. 10.2 Schematic representation showing the possible interaction of phloem-imported asparagine (ASN) with nodule carbon metabolism and the sequence of events leading to decreased N_2 fixation in *Medicago truncatula* nodules subjected to phosphate starvation. *Dotted bar* indicates the possible direct effect of the shoot signal (unproved yet). The amide ASN might be able to cross the peribacteroid membrane (PBM) and acts to downregulate the nitrogenase activity (Redrawn from Sulieman et al. 2014). *ETC* electron transport chain, *Lb* leghemoglobin, *OAs* organic acids, *PEPC* phosphoenolpyruvate carboxylase, *SS* sucrose synthase

et al. 2014b). The nodule transcriptome analysis in *M. truncatula* revealed a complex effect of Pi deficiency 5 day after the Pi depletion process (Cabeza et al. 2014a). For instance, genes involved in the maintenance of nodule C-fluxes were differentially expressed under Pi stress. Among them, two genes coding for sucrose synthases (SSs) were downregulated by Pi deficiency. Moreover, the transcriptome data suggested a possible role played by invertases in nodule sucrose metabolism. Both SSs and invertases catalyze the transformation of sucrose to dicarboxylic acids (Nasr Esfahani et al. 2014a, b).

It is generally accepted that legume plants fuel nodule N_2 fixation by dicarboxylic acids synthesized via glycolytic degradation of sucrose to malate (Sulieman and Tran 2013; Udvardi and Poole 2013) (Fig. 10.2). When growing under Pi-deficient conditions, expression of genes involved in nodule CO_2 fixation and malate formation were upregulated, while genes involved in fermentation (pyruvate decarboxylase, alcohol dehydrogenase, quinohemoprotein ethanol dehydrogenase, L-lactate dehydrogenase) were downregulated in *M. truncatula* (Cabeza et al. 2014a). The downregulation of these genes would strongly help redirect the C flow from the fermentation processes to upgrade malate formation. Moreover, the transcriptome data revealed that two genes encoding phosphoenolpyruvate carboxylases (PEPCs) were upregulated under Pi stress. Nodule CO_2 fixation through the activity of PEPCs is of vital importance for malate formation. In support of this finding, Sulieman et al. (2013b) reported that a pronounced increase in total malate level (~ 3 mg g^{-1} FW nodule) in *M. truncatula* nodules was coincident with the lowest level of Pi supply (1 μ M).

10.2.6 Oxygen Supply and Oxidative Stress

Symbiotic N_2 fixation is a high energy-consuming process that occurs under extremely low oxygen (O_2) concentrations. A sufficient O_2 supply to infected cells is essential for mitochondrial and bacteroid respiration, but excessive levels of O_2 can damage nitrogenase and increase production of activated O_2 species. Thus, an appropriate control of O_2 supply is essential for the efficient functioning of legume nodules. The effect of Pi starvation on O_2 diffusion into the nodule is a striking and intriguing feature of its adaptation to Pi stress. Generally, different models have been proposed to facilitate the O_2 diffusion into nodules of leguminous plants when Pi supply is limited (reviewed by Kleinert et al. 2017b, Chap. 5). Among the reported concepts, leghemoglobin (Lb) (Fig. 10.2) and O_2-diffusion barrier (ODB) are shown to be the major factors responsible for the maintenance of O_2 concentration around bacteroids (Sujkowska et al. 2011). For a better adaptation, Pi-stressed legumes form smaller nodules, which may also facilitate O_2 diffusion by the increased nodule surface area/nodule volume ratio (Schulze 2004; Schulze and Drevon 2005; Thuynsma et al. 2014). This finding was supported by increased respiration rates and unchanged N_2 fixation. The physiological role of increased O_2 uptake under Pi deficiency is still poorly understood, but it has been suggested that

it may contribute to maintaining a sufficient adenylate charge for high nitrogenase activity (Kouas et al. 2009; Lazali et al. 2014; Valentine et al. 2011). Thus, the nodule permeability that controls the nodule respiration and energetic metabolism appears to be a key factor in determinate (Ribet and Drevon 1995; Vadez et al. 1996) as well as indeterminate (Schulze and Drevon 2005) nodule functioning.

In accordance with an increase in nodule conductance to O_2 diffusion under Pi deficiency, higher abundance of several transcripts, including those encoding phosphoenolpyruvate phosphatase, trehalose-6P phosphatase, and ascorbate peroxidase, in the cortical and infected cells was observed in response to Pi deficiency (Sulieman and Tran 2015 and references therein). Some published reports have evidenced the significant contributions achieved by these enzymes in nodule permeability to O_2 diffusion and coping with oxidative stress in N_2-fixing plants (Bargaz et al. 2013). This is in principle agreement with the high levels of the enzymatic antioxidants in the nodule cortex. Unfortunately, little is known about the oxidative stress-related and O_2-associated functions-related mechanisms that affect nitrogenase activity within the nodules of *M. truncatula* exposed to Pi stress. Recently, an elegant study from Schulze's lab successfully addressed the acclimation processes of *M. truncatula* nodules to raising external O_2 concentrations (Avenhaus et al. 2016). Briefly, the sudden increase in O_2 concentration around nodules was coincided with a rapid destruction in nitrogenase enzyme. In order to maintain N_2 fixation, a quick neoformation of the enzyme was additionally documented. This adaptive response was explained by the increased formation of nodule-specific cysteine-rich (NCR) peptides accompanied with nicotianamine, which appears to be crucial to supply bacteroid with iron. However, little attention has been given to the relationship between nodule Pi status and nodule permeability in this study. Obviously, details concerning structural, biochemical, and molecular modifications of O_2-flux to bacteroids of Pi-starved *M. truncatula* nodules are still lacking. It is therefore an important area for future research.

10.2.7 N-Feedback Regulation

A growing body of evidence supported a loss of autonomy in regulation of the function of the microsymbiont under various types of stresses, including Pi deficiency. The overall control of nitrogenase activity of the bacteroids is widely regulated by the host plant as a consequence of N satiety (Fischinger et al. 2006; Soussana and Tallec 2010; Sulieman and Schulze 2010b; Sulieman 2011; Cabeza et al. 2014b). An overwhelming amount of circumstantial evidence pointed to the fact that a downregulatory impact on nodule functioning under Pi deficiency results from a phloem mobile shoot-born factor (Almeida et al. 2000; Kouas et al. 2009; Sulieman et al. 2013a, b, 2014). For a better maintenance of plant N homeostasis, specific potential signaling molecules spill over into the phloem and are sent back to the nodules to modulate their activity by systemic feedback mechanisms (Fig. 10.3). In conformity, it was shown that ^{15}N labeling (ammonium) of leaves resulted in a rapid subsequent labeling of nodules (Sulieman et al. 2010). The detailed microstructural

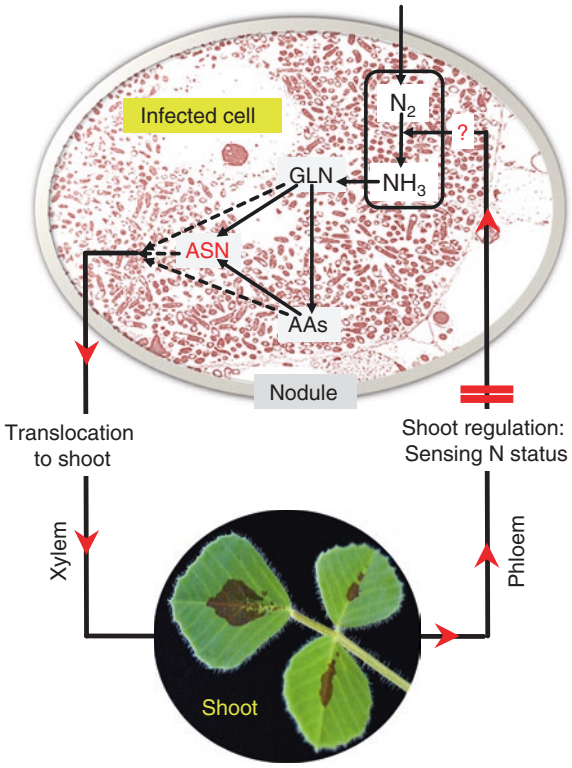

Fig. 10.3 Asparagine (ASN) production and the N-feedback effect on N_2 fixation in the model plant *Medicago truncatula* (Redrawn from Sulieman and Tran 2013). *AAs* amino acids, *GLN* glutamine, *N* nitrogen

and chemical investigations (Schubert 2007) as well as the transcriptional analyses (Ruffel et al. 2008) have provided further support for these homeostatic regulatory pathways. Although the identity of potential signals is not completely resolved yet, numerous physiochemical findings have indicated some metabolites, such as glutamine and γ-aminobutyric acid (GABA), that are potentially involved in sensing plant N status (Sulieman and Tran 2013; Sulieman 2015 and references therein).

An important approach in understanding the complex regulation of nodulated *M. truncatula* under Pi stress is the use of targeted analyses of defined metabolites. A few regulatory N-rich metabolites have been identified as prime mediators of adaptation of *M. truncatula* to low Pi stress. Of special interest was the significantly increased pool of asparagine (ASN) in nodules accompanied by a strong inhibitory tendency when ASN was artificially fed to the phloem of intact nodulated *M. truncatula* plants (Sulieman et al. 2010; Sulieman 2015). The level of this amide in plants was surprisingly found to be increased when nodulated legumes were exposed to Pi deficiency (Table 10.1). These data provided strong arguments in favor of a major role played by this amide in long-distance regulation of nodule activity.

Table 10.1 Phosphate deficiency stimulates asparagine accumulation in various organs of symbiotic legumes that transport fixed nitrogen as amides and ureides

Species	Variety	Microsymbiont	Origin	References
Chickpea (*Cicer arietinum* L.)	ILC482	*Mesorhizobium mediterraneum* SWRI9	Nodule	Nasr Esfahani et al. (2016)
Common bean (*Phaseolus vulgaris* L.)	Contender	*Rhizobium leguminosarum* biovar *phaseoli*	Nodule	Mortimer et al. (2012)
Lotus japonicus (Regel) K. Larsen.	Gifu	NA	Root	Keller (2003)
Barrel medic (*Medicago truncatula* Gaertn.)	Jemalong A17	*Sinorhizobium meliloti* 2011	Nodule	Sulieman et al. (2013a)
		Sinorhizobium meliloti 102F51	Nodule	Sulieman et al. (2014)
				Sulieman et al. (2008)
			Phloem sap	Sulieman et al. (2013b)
			Phloem sap, nodule	Sulieman et al. (2010)
White clover (*Trifolium repens* L.)	Milkanova	*Rhizobium leguminosarum* biovar *trifolii* WPBS5	Phloem sap	Høgh-Jensen et al. (2002)
		Rhizobium leguminosarum biovar *trifolii* RBL 5020	Root, nodule	Almeida et al. (2000)

NA not available

Nevertheless, the biological significance of ASN in the whole-plant-based downregulation of symbiotic N_2 fixation has been a matter of continual debate in recent years (Sulieman and Tran 2013) (Figs. 10.2 and 10.3). The challenge now is to understand how ASN and other regulatory and signaling components mediate the many responses that plants require to adapt to Pi stress. Because of the difficulty to unambiguously determine the ASN origin, it is very challenging to interpret specific aspects of the observed physiological changes in whole organs, such as nodules that consist of the two symbiotic partners, solely with biochemical investigations. Hence, determining changes in gene expression by transcriptomic approaches would be very helpful in discriminating between influences on nodule metabolism originating from the host plant or its microsymbiont partner.

As illustrated by the case of ASN, a close synchronization between the whole-plant N-feedback mechanism and the nodule C metabolism in regulating *M. truncatula* symbiotic activity was recently reported by our research group (Sulieman et al. 2013b, 2014) (Fig. 10.2). The accumulation of ASN in nodules of Pi-stressed plants caused a concurrent significant restriction of cellular organic acid (i.e., succinate) provision to the functioning bacteroids, which might subsequently lead to downregulation of nodule metabolism and nitrogenase (H_2 evolution) activity. Unlike the situation in several other leguminous species, succinate was the predominant dicarboxylic

acid quantified in the nodules of *M. truncatula* (Sulieman and Schulze 2010a; Sulieman et al. 2013a, b). However, the identity of the shoot-born factor that links the perception of the Pi alteration and the signal transduction pathway and leads to the C alterations, as yet, is purely speculative. Alternatively, other factors, such as amino acid cycling or O_2 limitation, may also be involved in the regulation of nodule activity, as discussed elsewhere (Schulze and Drevon 2005; Sulieman et al. 2010; Sulieman and Schulze 2010b). Indeed, the better understanding of the systemic signals and the mechanisms that transduce these signals into metabolic and morphologic responses might provide novel avenues to improve crop yield using lower-Pi inputs.

10.2.8 Mycorrhizal Association

Similar to rhizobial symbiosis, legumes form endosymbiotic association with mycorrhizal fungi, which provides the host plant with diverse mineral nutrients (predominantly Pi) in exchange for photosynthetically derived sugars (Horváth et al. 2011; Smith et al. 2011; Nouri et al. 2014). The establishment of mycorrhizal root colonization requires an on-going molecular dialogue between the fungus and host plant leading to cellular reprogramming of plant root for endosymbiosis (Tesfaye et al. 2007; López-Arredondo et al. 2014; Wang et al. 2014). The ability of *M. truncatula* plants to form tripartite associations with mycorrhizal fungi and *S. meliloti* gives them access to sources of Pi and N that would ordinarily not be available to the host plants (Javot et al. 2007; Bonneau et al. 2013; Wang et al. 2014). In this context, *M. truncatula* has been adopted as a valuable model system for investigating the arbuscular mycorrhizal symbiosis with the obligate biotroph *Glomus* spp. (Hohnjec et al. 2006; Rose 2008; Horváth et al. 2015).

Mycorrhizal association led to a systemic transcriptional induction of genes involved in photosynthesis, amino acid metabolism, and protein synthesis in the shoots of *M. truncatula* plants in response to Pi deficiency (Liu et al. 2007). Transcriptional analyses revealed a specific upregulation of a high number of genes involved in protein synthesis, mainly ribosomal protein encoding genes, and those encoding proteins involved in the amino acid metabolism. The induction of these genes in the shoots of mycorrhizal plants likely accounts for the often observed increased growth of mycorrhizal plants during Pi limitation (Sulieman and Tran 2015). In addition, mycorrhizal symbiosis can systemically enhance the CO_2 uptake rate of plants as a result of an enhanced abundance and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and a higher turnover rate of the photosystems I and II (Caravaca et al. 2003; Boldt et al. 2011; Adolfsson et al. 2015). Rubisco is known to be one of the key chloroplast enzymes (comprising 25–50% of leaf total cellular N) in the photosynthetic process (Goicoechea et al. 2014; Irigoyen et al. 2014; Sulieman et al. 2015).

Numerous studies have highlighted the fact that mycorrhizal and root-nodule symbioses share some common signaling pathways (Stacey et al. 2006; Tesfaye et al. 2007). For instance, studies of mutants defective in nodulation have shown mechanis-

tic similarities between nodulation and mycorrhizal infection (Horváth et al. 2011). Several *M. truncatula* mutants are defective in both rhizobial and mycorrhizal symbioses, and the impaired genes are known as common symbiosis (*SYM*) genes (Parniske 2004). An example of these genes is the *IPD3* (*interacting protein of DMI3*), required for intracellular accommodation of N_2-fixing bacteria and mycorrhizal fungi (Horváth et al. 2011). The structural similarity between mycorrhizal and rhizobial symbioses is also supported by the characterization of the *DMI* "does not make infection" genes *1*, *2*, and *3* , involved in both nodular (Nod factor) and mycorrhizal factor (Myc factor) signaling pathways (Ané et al. 2004; Stacey et al. 2006). Indeed, the demonstration of the functional signaling pathways between the mycorrhizal and rhizobial symbioses will be an excellent target for plant breeding with the scope to improve crop plant productivity via nodular and mycorrhizal symbioses. A complete review of mycorrhizal symbiosis is beyond the scope of this communication, and more recent information relating to this pivotal association is described elsewhere in this volume (see Chap. 9).

10.3 Role of Genotypes and Strains in *M. truncatula* Adaptations to Pi Deficiency

The symbiotic efficiency in *M. truncatula*-*S. meliloti* combinations under Pi deficiency is reflected on plant growth and functioning (Schulze et al. 2011; Sulieman et al. 2013b). The parameters used to estimate the symbiotic effectiveness include, for instance, shoot and root dry weight, nodule number and dry weight, and N_2-fixing capacity. Overall, *M. truncatula* symbiotic attitude is determined by each symbiont's input as well as the microbe × plant genotype interactions. Contrasting (tolerant vs. sensitive) associations were identified for Pi-tolerant behaviors, involving the same *M. truncatula* genotype with different *S. meliloti* strains and vice versa. Using the model Jemalong A17 genotype, we have identified contrasting symbioses related to the microsymbiont partner: tolerant associations with *S. meliloti* 2011 and sensitive ones involving *S. meliloti* 102F51 under low Pi supply (Sulieman et al. 2013a, b). Surprisingly, this situation was reversible when plants are grown under non-stressful growth conditions (Sulieman and Schulze 2010a). The results presented suggest that at least one of the factors contributing to the differences in the efficiency of combinations with different *S. meliloti* strains is most likely related to the nodule C catabolism, i.e., the capacity to produce organic acids. Therefore, it remains important to elucidate the Pi-tolerant mechanisms of these symbiotic combinations in order to improve the agronomic performance of various cultivated leguminous plants (Sulieman et al. 2013b). Identification of species that possess higher symbiotic efficiency under conditions of suboptimal Pi supply is a strategy to overcome this soil constraint (Richardson et al. 2011). This, in turn, is the cornerstone for development of any future novel N_2-fixing crops that have high N_2-fixation capacity under Pi limitation by genetic engineering. Nonetheless, to date, the

physiological mechanisms underlying the higher/lower symbiotic efficiency in the *M. truncatula-S. meliloti* associations are still poorly studied.

10.4 Future Perspectives and Concerns

Because of the projected crisis in the availability of high-grade rock Pi minerals, plant acclimation to Pi scarcity has been a topic of extensive research during the last past decades (Vance 2010; Ha and Tran 2014; Zhang et al. 2014; Herrera-Estrella and López-Arredondo 2016). The interactions between N_2 fixation and Pi availability involve various intricate mechanisms that have investigated in different plant species with the aid of numerous molecular, genetic, proteomic, and physiological approaches. Among leguminous plants, *M. truncatula* has emerged as a model system for studying the general biology of legumes and for exploring the genetic and molecular aspects of N_2-fixing symbiosis. Identifying the physiological bases of adaptation of *M. truncatula* to low Pi conditions may accelerate the selection for increased Pi use efficiency and high productivity in Pi-depleted soils during crop breeding (Tesfaye et al. 2007; Vance and Chiou 2011). Figure 10.4 summarizes the main morphological, physiological, and biochemical characteristics by which

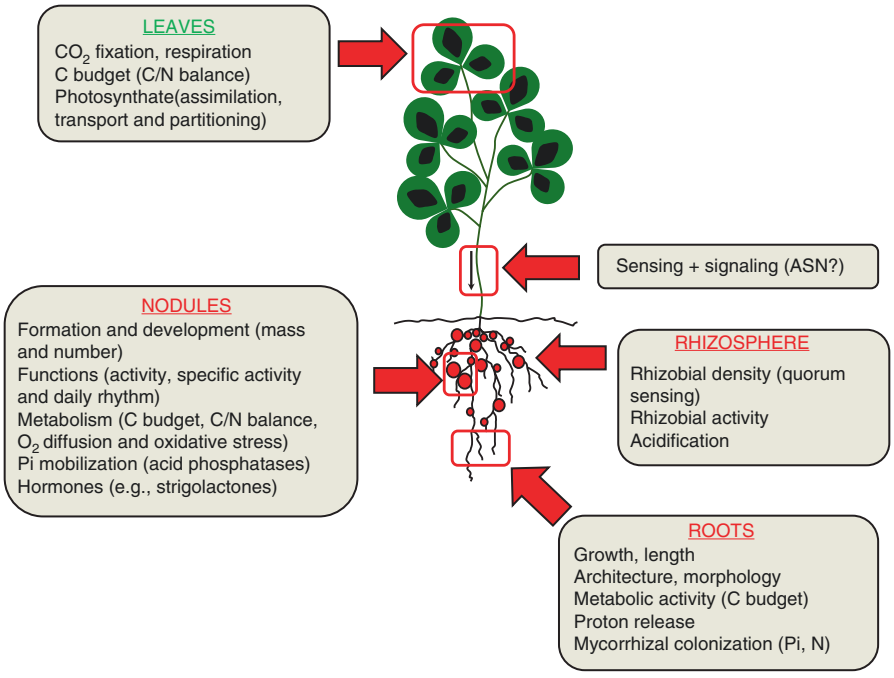

Fig. 10.4 Summary of the main morphological, physiological, and biochemical characteristics by which *Medicago truncatula* can be affected by Pi deficiency

M. truncatula can be affected by Pi scarcity. Nowadays, this possibility, with the integration of omics (including transcriptomics, proteomics, and metabolomics), plant biochemistry, and plant breeding disciplines, increasingly appears to be a more realistic goal. The development of legume varieties with effective N_2 fixation under Pi-limited conditions is a necessity to stabilize the legume productivity for sustainable farming practices compatible with increasing requirements for food and feed (Hernández et al. 2009; Qin et al. 2012; Sulieman and Tran 2013).

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Chapter 11
Physiological Mechanisms and Adaptation Strategies in Common Bean (*Phaseolus vulgaris* L.) Under P Deficiency

Maria del Socorro Sánchez-Correa and Oswaldo Valdés-López

Abstract Common bean is an excellent protein and mineral source for human consumption. Likewise, this legume, through symbiosis with rhizobia, incorporates atmospheric dinitrogen (N_2) into the food chain. Unfortunately, these properties are seriously affected when common bean plants grow on soils with low phosphate levels. Hence, in order to design strategies to develop elite cultivars able to produce high quality seeds and efficiently fix N_2 under phosphorus (P) deficiency, it is important to understand how common bean copes with this nutritional constraint. Over the two past decades, significant progress has been made in understanding the genetic and metabolic responses of common bean. In this chapter, we summarize and discuss recent advances in the understanding of the strategies that non-nodulating and nodulating common bean use to cope with P deficiency. We also discuss future directions and research priorities that may lead to a better understanding of the physiological and molecular adaptations that allow common bean to thrive under conditions of P deficiency.

Keywords Common bean • Nodules • Transcription factors • microRNAs • P deficiency

11.1 Introduction

Legumes are of considerable ecological importance because of their ability to establish symbiotic associations with bacteria (rhizobia) allowing them to fix the atmospheric dinitrogen (N_2) into forms (e.g., ammonia or amino acids) that can be assimilated by higher organisms (Venkateshwaran et al. 2013). Legumes are also agronomically important because a great variety of products can be obtained from them; for instance, forage for livestock, cooking oil, and even biofuels

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(Biswas et al. 2011; Jensen et al. 2012). Certainly, the most important characteristic of legumes is that many of them are an excellent source of protein and minerals for human consumption (Broughton et al. 2003; Castro-Guerrero et al. 2016).

Phaseolus vulgaris (common bean) is the principal source of non-animal protein for human consumption in the developing world (Castro-Guerrero et al. 2016). Besides the caloric intake, common bean grains also provide fiber, minerals, thiamine, folate, and a variety of flavonoids and secondary metabolites with medicinal properties (i.e., analgesic and neuroprotective) (Blair et al. 2013; Jha et al. 2015). Unfortunately, most arable soils in the developing world have a significant degree of phosphorus (P) deficiency, which negatively affects the nutritional quality of common bean seeds, and, consequently, the nutrition of many thousands of people from developing countries (Brevik 2013; Clair and Lynch 2010). Farmers from these countries have used P fertilizer as a principal strategy to address the low soil fertility and increase common bean productivity. Although this strategy has a beneficial effect on the nutritional quality of common bean, this practice has also led to soil contamination and water eutrophication (Savci 2012). Given these circumstances, in order to provide common bean seeds with high nutritional value, it is important to understand how common bean copes with P deficiency if we are to generate new strategies in the development of a sustainable agriculture. In this chapter, we discuss recent advances in our understanding of the strategies used by non-nodulating and nodulating common bean in order to cope with P deficiency. We also discuss the state of the art of the genetic mechanisms underlying common bean adaptations to P deficiency. Finally, we discuss future directions and research priorities that may lead to a better understanding of the physiological and molecular adaptations that allow common bean to thrive under conditions of P deficiency.

11.2 Morphological Responses to P Deficiency

Plants absorb P from the soil as inorganic phosphate (Pi). However, Pi interacts with different soil components (i.e., organic matter and metallic elements like iron, calcium and aluminum), which makes it inaccessible for plant uptake (Plaxton and Tran 2011). Under such conditions, plants seek potentially Pi-rich soil patches, which can be located in the topsoil layer. To do so, plants modify their root architecture (López-Arredondo et al. 2014). For instance, it has been reported that, under Pi-deficient conditions, *Arabidopsis thaliana* develops shorter primary roots, and increases the length and number of lateral roots and root hairs (López-Arredondo et al. 2014). Additionally, Pi-deficient *A. thaliana* plants also develop shorter leaves and shoots, which leads to an increase in the root-to-shoot dry weight ratio. Although these morphological responses have been observed in different plant species, there are plants that have some very particular responses to Pi deficiency. For instance, although a reduction in primary root growth has not been observed in common bean plants (Hernández et al. 2007; Ramaekers et al. 2010), a significant increase in the number of lateral and root hairs has been observed in different common bean

cultivars (Lia et al. 2001; Yan et al. 2004). Different studies indicate that activation of the shallower growth of basal roots is a morphological characteristic response of common bean to P deficiency (Lia et al. 2001; Ramaekers et al. 2010). Shallower growth of basal roots promotes adventitious rooting and lateral branching of the basal roots (Lia et al. 2001). Because shallower roots enhance topsoil foraging, where Pi is located, it has been proposed that this type of root plays an important role in the adaptation of common bean to Pi deficiency (Ramaekers et al. 2010). In fact, different quantitative trait locus (QTL) analyses have shown a positive correlation between the ability to increase the number and length of shallower roots, lateral roots, and root hairs with the efficiency in the Pi uptake (Yan et al. 2004). Additionally, it has been reported that the common bean Mesoamerican cultivar G19833, which is cataloged as resistant to Pi deficiency, develops more cortical aerenchyma in roots, which help remobilize Pi from the soil (Shen et al. 2002; Fan et al. 2003).

In nature, common bean interacts symbiotically with N_2-fixing rhizobia and forms a new organ called a nodule (Downie 2014). Inside the nodule, rhizobia fix the inert N_2 gas into assimilable-nitrogen (N) forms (i.e., ureides: alantoin and alantoic acid), making N available to common bean. Since this symbiosis is an energy-expensive process, host plants need to provide a high amount of Pi to achieve the amount of ATP that rhizobia need to fix N_2 (Vardien et al. 2016). Accordingly, under sub-optimal Pi conditions, common bean may reduce the number of nodules per plant by ~60–70%(Vadez et al. 1999). This adaptation helps common bean to maintain the symbiosis with rhizobia under Pi-deficiency conditions and obtain the N required for its metabolic needs. Interestingly, under Pi-deficient conditions nodules accumulate more Pi than the root fraction. This ensures that rhizobia have sufficient Pi levels to drive the N_2 fixation process (Hernández et al. 2007).

11.3 Pi Uptake Under Limiting Conditions

Along with root architecture modifications and a reduction in the number of nodules, plants have developed different metabolic adaptations that allow them to uptake Pi from the soil under Pi-limiting conditions. For instance, different plant species, including common bean, exude a variety of P-mobilizing compounds, such as organic acids (i.e., citrate, oxalate and malate), protons, and even phosphatases (Ramaekers et al. 2010; López-Arredondo et al. 2014). These root-exudates participate in the soil cationic-exchange that makes Pi available for plant uptake. Metabolomic analyses have determined the composition of root-exudates from Pi-deficient common bean plants. (Hernández et al. 2007; Tawaraya et al. 2014). Based on these studies, common bean root exudates contain different amino acids (e.g., asparagine, arginine and proline), N-compounds (e.g., putrescine, spermidine), sugars (e.g., fructose, mannose) and organic acids (e.g., citrate, fumarate). Since nodules are also exposed to Pi-deficiency, Hernández et al. (2009) performed a metabolomic analysis on nodules from Pi-deficient common bean plants. This latter study revealed that the concentration of different amino acids (e.g., β-alanine),

organic acids (e.g. citric acid), polyhydroxy acids (e.g., galactanic acid) and sugars (e.g., mannose) increased in response to Pi deficiency. Except for organic acids, it is not clear whether the other root-exudates and nodule metabolites identified in these studies have Pi mobilizing activity, or whether they play a different but relevant role in the adaptation of common bean to Pi deficiency.

In addition to organic or amino acids, plants also release different soil cations, primarily H^+ and Na^+ (Plaxton and Tran 2011). This cation release is driven by plasma membrane ATPases that are activated under Pi deficiency conditions (Yu et al. 2016). Cation pumping is extremely important because it drives the organic acid exudation, and is also involved in the release of Pi from soil particles and its consequent absorption by the plant (Yu et al. 2016). Indeed, plants that are extremely adapted to Pi deficiency, like *Lupinus albus*, show a high H^+-ATPase activity that is correlated with highly efficient Pi uptake (Yan et al. 2002; Tomasi et al. 2009; Cheng et al. 2011). This cation pumping activity has been also registered in different plant species, and the efficiency of the ATPase pump determines, in part, their level of tolerance to P-deficiency conditions (López-Arredondo et al. 2014).

Along with exudation of cations and Pi-mobilizing compounds, plants also release purple acid phosphatases (PAP)—a class of extracellular acid phosphatase (APase)—to the rhizophere (Plaxton and Tran 2011). Different evidence indicates that the activation and release of PAP is triggered by Pi deficiency (Robinson et al. 2012; Ramaekers et al. 2010). Likewise, it has been reported that PAPs have high activity in P-deficient soils (Ramaekers et al. 2010). Thus, it has been suggested that the principal role of PAP is to hydrolyze orthophosphate from external phosphomonoesters and phosphodiesters derived from decomposition biomaterial to provide Pi for plant uptake. PAP has been characterized genetically and biochemically in different plant species, including common bean, and findings indicate that the release of APase to the rhizosphere is a universal plant response to Pi deficiency (Liang et al. 2010, 2012; Robinson et al. 2012).

Once Pi is released from soil sources, plants need to transport it into the root cells. To increase Pi transport efficiency under limiting conditions, plants activate and localize Pi high affinity transporters (Pht) in the root plasma membrane (Plaxton and Tran 2011; López-Arredondo et al. 2014; Bouain et al. 2016). Through this type of transporter, plants ensure that cells have enough Pi for critical metabolic processes, such as the biosynthesis of organic acids. Increasing evidence has demonstrated that this high affinity Pi transport system is a co-transport system driven by H^+-ATPase and Na^+-ATPase (Plaxton and Tran 2011). Interestingly, most studies on the biochemical regulation of this co-transport system have been performed in common bean and in *L. albus* (Miller et al. 2001; Cheng et al. 2011). Additionally, transcriptomic studies in common bean have led to the identification of genes encoding different Pht, many of which are conserved in the model plant *A. thaliana*. However, these transporters have not been genetically characterized in common bean.

The responses described above are oriented to provide Pi for metabolic activities of different plant organs. However, different studies have demonstrated that, under Pi deficiency, most of the absorbed Pi is relocated into the nodules (Vadez et al. 1999; Vardien et al. 2016). This Pi allocation pattern is required to maintain Pi

homeostasis in the nodule, and to generate the amount of ATP required for N_2 fixation (Sulieman and Tran 2015). A portion of the Pi accumulated in the nodule is relocated from other organs via the vascular system. However, there is evidence indicating that symbiotic tissues and the bacteroid can active specialized Pi-transport systems allowing them to obtain Pi from both internal and external sources (Al-Niemi et al. 1998). In fact, it has been reported that the high-affinity Pi transporter *GmPT5* is expressed in the junction area between roots and nodules as well as in nodule vascular bundles (Qin et al. 2012). Additionally, ^{33}P uptake assays have demonstrated that *GmPT5* participates in Pi transport from roots to nodules under Pi-limited conditions (Qin et al. 2012). However, homologs of this transporter have not been identified and characterized in common bean.

11.4 Common Bean, Like Other Plants, Obtains Pi from Internal Sources

P is an integral part of different biological molecules, including ATP, phospholipids, and nucleic acids, and is also required for signal transduction and posttranslational protein modifications. Owing to its biological relevance, it is important that plants maintain P homeostasis at any physiological moment, even under conditions of Pi deficiency (López-Arredondo et al. 2014). To maintain P homeostasis under limiting conditions, plants regulate the activity of different P-dependent pathways to avoid P wasting. For example, different studies have demonstrated that P-dependent metabolic pathways can stay partially active by using pyro-phosphate instead of Pi (Plaxton and Tran 2011). Likewise, it has been observed that Pi-deficient common bean roots decrease ATP synthesis and activate the alternative oxidase (AOX) pathway—a non-energy conserving terminal in plant mitochondria—to avoid oxidative damage triggered by an accumulation of electrons in the mitochondria (Vanlerberghe 2013). Through these modifications, plants can channel Pi into metabolic pathways that allow them to thrive under P-deficient conditions. Another strategy to obtain Pi from internal sources is by degrading biological molecules that contains Pi. For instance, plants can activate different phospholipases to obtain Pi from phospholipids (Plaxton and Tran 2011). Under these circumstances, membranes experience lipid turnover, where phospholipids are replaced by glyco and sulfolipids (Andersson et al. 2005). Additionally, there are reports demonstrating that under Pi-limiting conditions, plants activate RNAase type H (RNsH), which liberates Pi from RNA to facilitate its remobilization (Bariola et al. 1994; Valdés-López and Hernández 2008; Plaxton and Tran 2011). Although the expression of different phospholipases and RNsH has been observed in Pi-deficient common bean roots and nodules, there is no evidence to date regarding its potential role in Pi scavenging in this important legume.

Different studies have documented the metabolic adaptations allowing nodules to obtain Pi from internal sources under Pi deficiency. For instance, it has been reported that the activity of acid phosphatases (APs) and phytases increases under Pi-limiting conditions in common bean nodules (Kouas et al. 2009).

Likewise, Lazali et al. (2016) reported that *fructose-1,6-bisphosphatase* (FBPase) is expressed in the inner nodule cortex, and that its activity was increased under Pi deficiency in common bean nodules. Interestingly, the activity of APs, phytase and FBPase has a positive correlation with Pi content and Pi use efficiency, suggesting that these enzyme play some role in Pi remobilization under Pi-limiting conditions in common bean nodules (Kouas et al. 2009; Lazali et al. 2016).

11.5 Genetic Control of the Responses to P Deficiency

Both morphological and biochemical responses to Pi deficiency are tightly regulated at different levels, including the transcriptional and posttranscriptional level (Zhang et al. 2014). Indeed, a growing body of evidence in different plants, including common bean, has demonstrated the participation of different transcription factors (TFs), microRNAs, and a variety of genes in the adaptation to P deficiency (Hernández et al. 2007, 2009; Valdés-López et al. 2010; López-Arredondo et al. 2014; Zhang et al. 2014). Despite limitations to our knowledge of the genetics of common bean, significant progress in the understanding of the genetic mechanism underlying common bean responses to Pi deficiency has been made in the past decade (Hernández et al. 2007, 2009; Tian et al. 2007). For example, it has been demonstrated that genes that participate in Pi recycling (e.g., *PvRNsH*, *PvAP*), transport (Pi transporter *PvPht1,4*) and homeostasis (microRNA *PvmiR399* and the E2 Ubiquitin conjugase *PvUBC24*: *PvPHO2*) are regulated by the TF PvPHR1, whereas some genes involved in carbon (C) metabolism (e.g., *phosphoenolpyruvate carboxylase*), organic acid biosynthesis (e.g., *malate dehydrogenase*), phosphate transport (*PvPHO1*), and signaling (e.g., *Receptor-like protein kinase-related*) are regulated by the TF PvTIFY (Valdés-López et al. 2008; Aparicio-Fabre et al. 2013). Likewise, it has been demonstrated that microRNAs *PvmiR399* and *PvUB24* are critical components in the maintenance of Pi homeostasis during limiting conditions in common bean (Valdés-López et al. 2008). Transcriptional analyses on Pi-deficient common bean plants have also led to the identification of several Pi-deficiency responsive genes that might play some role in C/N metabolism, lipid biosynthesis, and nutrient transport—processes that are important for plant survival under this nutritional constraint (Hernández et al. 2007, 2009; Tian et al. 2007). In the same way, a microRNA expression profile analysis in common bean revealed that the microRNAs pvu-miR1511, pvu-miR393, pvu-156/157 and pvu-miR399 were upregulated, whereas pvu-miR319, pvu-miR398 and pvu-miR408 were downregulated under P deficiency conditions (Valdés-López et al. 2010). Both transcriptional and microRNA expression profile analyses have been extended to nodules from Pi-deficient common bean plants (Hernández et al. 2009; Valdés-López et al. 2010). These studies have revealed that members of the C2C2(Zn), AP2/EREBP, WRKY, MYB, NC, TIFY, bZIP, GRAS and C3H-type 1(Zn) TF family increased their expression in response to Pi deficiency (Hernández et al. 2009). Likewise, the

microRNA expression profile revealed that the microNAs PvmiR319, PvmiR398, PvmiR172 and PvmiR156/157 increased their accumulation in response to Pi-limiting conditions, whereas accumulation of pvmiR1511, pvmiR1524 and pvmiR1532 decreased (Valdés-López et al. 2010). Although these transcriptional analyses have led to the identification of several genes and microRNAs that might play a vital role in the adaptation of common bean to Pi deficiency, few of them have been functionally characterized in non-symbiotic conditions. For example, it has been demonstrated that transgenic roots of common bean overexpressing the PAP *PvPAP3* obtain more Pi through the degradation of ATP and dNTPs than non-transgenic roots (Liang et al. 2010, 2012). Additionally, Yao et al. (2014) demonstrated that *PvSPX1* controls the expression of at least ten P-deficiency responsive genes (e.g., the P transporter *PvPHT2*; and PAPs *PvPAP1-PvPAP5*). Furthermore, Yao et al. (2014) also demonstrated that *PvSPX1* is involved in P uptake and in the formation of root hairs under Pi-limiting conditions.

Genome-wide DNA methylome analyses on *A. thaliana* and *Oryza sativa* revealed that Pi-deficiency triggered modifications in DNA methylation levels (Secco et al. 2015; Yong-Villalobos et al. 2015). The differentially methylated regions (DMRs) identified in these studies were found to be localized in transposons and in intergenic regions. Interestingly, the majority of the DMRs were noted in the vicinity of Pi-deficiency responsive genes, and a few DMRs were located in the gene body of P deficiency-responsive genes (Secco et al. 2015; Yong-Villalobos et al. 2015). Among the Pi deficiency-responsive genes affected by DNA methylation status was *SPX2*, which encodes a repressor of the master regulator PHR1, and the microRNA *miR827* was also identified (Yong-Villalobos et al. 2015). This finding strongly suggests that plant responses to Pi deficiency might be also regulated at an epigenetic level. Despite that fact that the DNA methylome of Andean and Mesoamerican common bean has been recently published (Kim et al. 2015; Crampton et al. 2016), there is no evidence as yet on the potential epigenetic control of the responses of this legume to Pi deficiency under both symbiotic and non-symbiotic conditions.

11.6 Conclusions and Future Perspectives

Undoubtedly, common bean is the most important non-animal source of protein and minerals for human consumption in the developing world. Likewise, common bean is an important source of secondary metabolites with medicinal properties. Additionally, common bean also incorporates atmospheric N_2 into the food chain. As mentioned earlier in this chapter, these properties are affected significantly by Pi deficiency. Hence, in order to develop elite common bean cultivars that can efficiently fix N_2 and provide seeds with high nutritional value, even when grown under Pi limiting conditions, it is imperative to understand how common bean copes with this nutritional constraint (Fig. 11.1). Despite the considerable progress made in the past 10 years, several gaps remain to be filled if we are to have a clear idea about

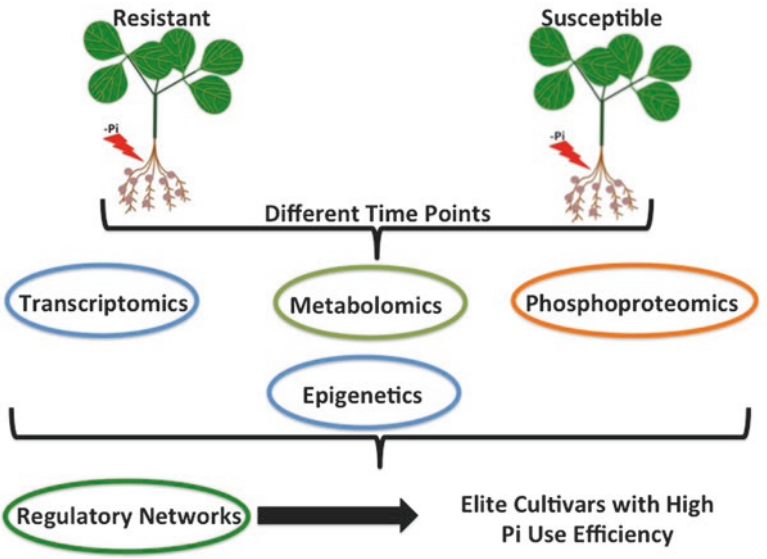

Fig. 11.1 Suggested pipeline to understand the mechanism underlying common bean adaptation to Pi deficiency. In this proposed pipeline, we suggest to use two cultivars, one resistant and other susceptible to Pi deficiency. We proposed to build a regulatory network based on Transcriptomics (for both coding- and non-coding RNAs), Epigenomics (DNA methylation and histone remodeling), Metabolomics, and phosphoproteomic data from both cultivars. The regulatory network(s) could be used to design breeding programs leading to generate elite cultivars with high Pi use efficiency

how common bean can thrive despite Pi deficiency. For instance, although many TFs have been identified, there is no information about what genes are targeted, or how they interact to coordinate and regulate common bean nodules responses to Pi deficiency. Likewise, although there is transcriptional information (for both coding and noncoding RNA) available, this is limited to particular plant organs (roots or nodules) and time points (10 and 21 days post treatment). Thus, it is necessary to generate transcriptomic information from different time points that capture the critical transcriptional responses of common bean to Pi deficiency. Similarly, more studies at metabolomic level are needed, which will give a better idea about metabolic adaptations to this nutritional condition. Furthermore, since protein phosphorylation is a critical step in signal transduction, it is imperative to perform phosphoproteomic analysis. The provision of transcriptomic and phosphoproteomic data will allow gene regulatory network analyses, which will help identify and trace the signaling pathways that control common bean responses to Pi deficiency under both non-symbiotic and symbiotic conditions. Likewise, it will be necessary to perform DNA methylation analysis and identify any histone modifications that occur under Pi deficiency conditions, which will allow us to understand some of the epigenetic control of common bean responses to Pi deficiency conditions. All these proposed analyses are easily achievable, because the genome from the two gene pools

(Mesoamerican and Andean) is available, and many genetic tools can be used in common bean. Thus, the availability of the common bean genome will contribute significantly to our understanding of how this important legume thrives and adapts to Pi deficiency conditions.

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Chapter 12
Physiological and Molecular Mechanisms and Adaptation Strategies in Soybean (*Glycine max*) Under Phosphate Deficiency

Prince Zogli, Lise Pingault, and Marc Libault

Abstract Phosphorus is a major plant macronutrient involved in many and different biological processes, such as energy transfer (ATP), photosynthesis, respiration, biosynthesis of nucleic acids and proteins, membrane biosynthesis (e.g., phospholipids), and signaling pathways. Legumes, including soybean, are highly dependent on the availability of scarcely available organic phosphorus in the rhizosphere, especially when considering the need for phosphorous during nodulation—a legume-specific mutualistic symbiotic interaction between plants and nitrogen-fixing soil bacteria. As a consequence, the limited assimilation of phosphorus greatly hinders the nodulation process, soybean growth and soybean yield. Thus, understanding how soybean responds to low-phosphorus situations is imperative for breeding towards low-phosphorus tolerance. Toward these aims, scientists are using powerful genetic and molecular technologies to identify soybean genes playing essential roles in plant resistance to low-phosphorus environments. Functional genomic studies on soybean, as well as on other legumes suitable for comparative genomic with soybean, have provided valuable information in recent years and hold bright promise for the future. In this chapter, taking advantage of the recent development of high-throughput sequencing technologies, the sequencing of the soybean genome, the development a various biotechnological and breeding platforms, and the molecular, cellular and physiological analyses of soybean response to phosphate (Pi) deprivation, we describe our current understanding of the adaptation of soybean plants to limited Pi availability.

Keywords Soybean • Functional genomic • Genome • Breeding • -Omic analyses phosphate deprivation • Bioinformatics

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12.1 Introduction: Soybean (*Glycine max*), a Major Crop Species

Soybean is ranked as one of the five major crops across the world in terms of area dedicated to its culture and production (Fig. 12.1; http://apps.fas.usda.gov/psdonline/circulars/production.pdf). Specifically, based on the most recent World Agricultural Production report (USDA Foreign Agricultural Service, http://apps.fas.usda.gov/psdonline/circulars/production.pdf), 319.73 million metric tons of soybean were produced in 2014–2015. In 2016–2017, soybean production is projected to increase to over 323 million metric tons. Among soybean producers, the USA produces around one-third of the total soybean production (i.e., 106.88 million metric tons in 2014–2015). Other major soybean producers include Brazil and Argentina.

Soybean is currently grown under different environmental conditions in tropical, semi-tropical and temperate regions where Pi availability is often limited due to soil erosion and acidity, the latter leading to the formation of minerals in combination with aluminum and iron causing Pi retention in soils. This retention is especially high in tropical and subtropical regions of the globe (http://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/use/maps/?cid=nrcs142p2_054014). The contrast existing between these environmental conditions clearly leads to specific morphological and physiological adaptations of soybean plants, which also affects their yield. To reflect these adaptations, soybean is categorized in various maturity groups (from 000 to IX) which, interestingly, have been correlated with the differential sizes of

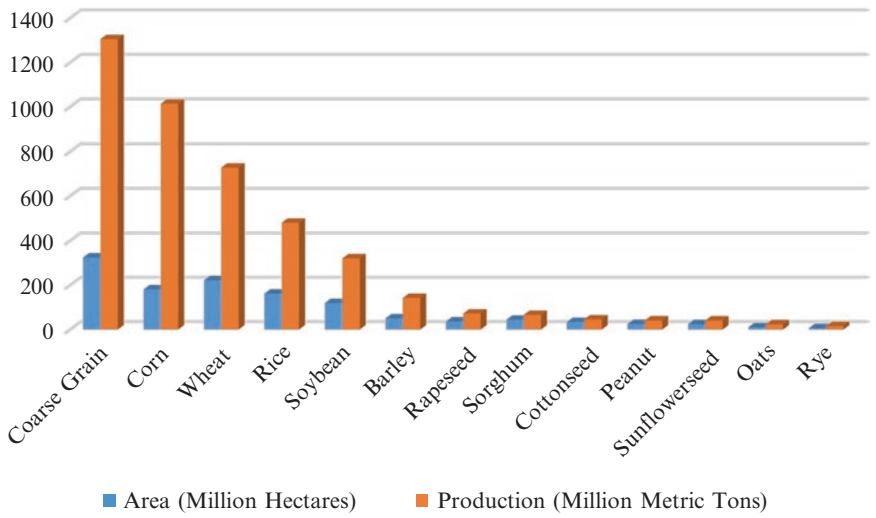

Fig. 12.1 Soybean is a major cultivated crop worldwide. Cultivated area (million of hectares; *blue*) and crop production (million metric tons; *orange*) in 2014 across the globe are reported for the 13 most important crops (Adapted from http://apps.fas.usda.gov/psdonline/circulars/production.pdf)

the soybean genome (Graham et al. 1994). Early maturing soybeans (groups 000–IV), which are grown in the northern regions of the USA (e.g., North Dakota), go through a quick development, while late maturing soybeans (groups V–IX), which are growing under warmer and humid conditions (e.g., Florida), develop more slowly.

The economic importance of soybean leads national agencies and researchers to seek understanding of soybean biology with the objectives of enhancing yield, nutritional quality and resistance to various biotic and abiotic stresses including Pi depravation (Abberton et al. 2016). These efforts include the establishment of breeding programs, and the development of genetic, genomic and molecular tools to understand soybean biology and to enhance soybean growth as well as productivity (http://soybeangenomics.missouri.edu/news/SoyGenStratPlan_2017-2021.pdf; http://unitedsoybean.org/about-usb/strategic-planning/). These programs include a specific focus on the performance of soybean growing under low-Pi conditions—a major limitation to soybean development and yield.

12.2 Soybean Genetic, Genomic and Molecular Resources

12.2.1 Soybean Genomic Resources

A major achievement in soybean biology was the publication in 2010 of the soybean genome sequence (Schmutz et al. 2010). This resource has now opened the possibility to link genetic and physical soybean maps Song et al. (2016) as well as to promote a deeper understanding of the molecular mechanisms controlling soybean resistance to various stresses, including low-phosphorus conditions.

More recently, several legume genome sequencing programs have also been completed [e.g. *Medicago truncatula* (Young et al. 2011), *Phaseolus vulgaris* (Schmutz et al. 2014) and the diploid ancestors of the cultivated peanut (Bertioli et al. 2016)]. These various genomic resources are available on the Phytozome platform (https://phytozome.jgi.doe.gov/pz/portal.html; Goodstein et al. 2012). The access to, and comparison of, these different genomes has opened avenues to better understand of legume evolution, and has enhanced comparative genomics between legume species. Such analysis requires the development of specific bioinformatics resources. For instance, Phytozome (Goodstein et al. 2012) proposes applications to highlight protein homology and gene ancestry. The Comparative Genomics platform (CoGe) (Lyons and Freeling 2008; Lyons et al. 2008) also provides resources to compare plant genomes and highlight gene orthology based on their micro- and macrosyntenic relationships. SoyKB (Joshi et al. 2014, 2012) also proposes access to comparative genomic tools but, in this case, focusing specifically on the characterization of homeologous soybean genes arising from the two whole duplications of the soybean genome (http://soykb.org/search/homologous.php).

Ultimately, comparative genomic studies lead to the transfer of biological information between species based on their close evolutionary relationships. For instance, comparison of the structure of legume genomes based on macrosynteny and microsynteny relationships helps to characterize the macroevolution between plant species (e.g., duplication, translocation, deletion, etc.), and to identify orthologous genes potentially sharing the same biological function [e.g., comparative transcriptomic and genomic analyses between *G. max* and *Arabidopsis thaliana* led to the characterization of the soybean genes potentially controlling floral transition (Libault et al. 2010a)].

Such analyses performed between legume and non-legume plants are highly dependent on the evolutionary relationships existing between plant species. For instance, due to the low level of synteny between their genomes, revealing gene orthology between monocotyledons and dicotyledons remains difficult. In contrast, the close evolutionary relationships existing between legumes facilitates comparative genomic analyses. Specifically, soybean belongs to the papilionoideae clade, where *M. truncatula*, *Lotus japonicus* and *P. vulgaris* (Cannon et al. 2010) are also listed. Papilionoideaes diverged from the Mimosoideae (*Chamaecrista fasciculata*) and the Caesalpinoideae (*Cercis* genus) about 60 million years ago (Mya). Subsequent to this divergence, the papilionoideae genome duplicated. After speciation between papilionoideaes, the soybean genome duplicated for a second time approximately 13 Mya (Gill et al. 2009; Schlueter et al. 2004, 2007), increasing the size and complexity of its genome. The most recent annotation of the soybean genome (i.e., Wm82.a2.v1) predicts a total of 56,044 protein-coding loci. Surprisingly, compared to the currently 50,894 annotated genes in *M. truncatula*, the most recent duplication of the soybean genome did not double the number of coding genes (Young and Bharti 2012), raising questions regarding the loss of paralogs following whole genome duplicaitons (WGDs) and their functional evolution. This close evolutionary relationship helped to characterize the pool of legume genes controlling nodulation (Qiao et al. 2016)—a plant-bacteria symbiotic biological process common to most legumes, and indeed almost exclusive to legumes (as a rare example, the non-legume *Parasponia* species are capable of developing root nodules).

12.2.2 Soybean Genetic Resources

To increase the performance of soybean plants under different unfavorable conditions, breeding programs to expand soybean diversity have been initiated (Tripathi and Khare 2016). For instance, the impact of limited availability of Pi on soybean development calls for the breeding of low-Pi-tolerant soybean cultivars. Conventional breeding relies on direct selection of breeds characterized by stable traits of interest over a wide range of growth environments.

Identifying molecular markers or quantitative trait loci (QTL) associated with low-Pi tolerance would facilitate the use of maker-assisted selection (MAS) for the development of low-Pi-tolerant varieties to mitigate the negative effect of low Pi on

yields. Efforts have been made so far to identify QTLs associated with important root traits in soybean, such as lateral root length and Pi efficiency (Table 12.1) (Zhang et al. 2009; Grant et al. 2010; Liang et al. 2014). Specifically, several QTLs associated with phosphorus content in soybean plants have been characterized. Li et al. (2005) characterized a total of 7 QTL mapped on two different linkage groups, Leaflet P1-1 and P1-2, which are associated with phosphorus content in leaves and roots (Li et al. 2005). In a different study, Liang et al. (2010) performed a large-scale analysis of the QTL associated with root architecture and phosphorus content in soybean plants (Liang et al. 2010). Among the 31 QTL identified in this latter study, 13 and 18 were associated with root architecture and plant phosphorus content, respectively. Interestingly, four of these QTLs are associated with both traits, supporting the central role of the root system in phosphorus uptake, and the concept that adaptation of root architecture is one of several plant responses to Pi deprivation. To enhance the access to these phosphorus-associated QTLs, Soybase (Grant et al. 2010) has implemented a search tool (http://soybase.org/search/objectlists.php).

However, in soybean, Pi efficiency-associated QTLs (Zhang et al. 2009, 2010; Li et al. 2005) have not been fully integrated into soybean breeding programs. The main reason for this is that markers such as restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR) or single nucleotide polymorphism (SNP) (Gore et al. 2002; Gutierrez-Gonzalez et al. 2011; Hyten et al. 2010b; Keim et al. 1990) that are used for QTL analysis, affect the efficiency and accuracy of QTL calling (Zhang et al. 2016). Hence, these analyses call for the need to link genetic and genomic information. Such information can be generated only using a multifaceted large data collection approach. The recent use of next generation sequencing technology to generate high-density genetic maps greatly enhances the resolution and the accuracy of QTL calling (Qi et al. 2014; Yu et al. 2011). A few of the next generation sequencing strategies developed so far for high density genetic map construction include: restriction site-associated sequencing (RADseq) (Miller et al. 2007), whole-genome resequencing (WGRS) (Xie et al. 2010, 2013), two-enzyme genotyping-by-sequencing (GBS) (Poland et al. 2012), genome-wide association studies (GWAS) (Zhang et al. 2015) and specific-locus amplified fragment sequencing (SLAFseq) (Sun et al. 2013).

To understand genome complexity and its related challenges, scientists have taken advantage of high-throughput sequencing technologies to construct RAD and reduced representation libraries (RRLs) (Davey and Blaxter 2010) for whole genome sequencing. For instance, RADseq consists of Illumina-based sequencing of short DNA tags that represent all the restriction enzyme sites available in a genome (Miller et al. 2007), enhancing SNP discovery and genotyping (Baird et al. 2008), and eliminating the need for generating the species-specific arrays previously required for microarray-based RAD marker analysis (Davey and Blaxter 2010). Because both RADseq and RRLseq are geared towards generating simplified libraries via restriction digest for sequencing, SNP discovery and genotype characterization are maximized (Hyten et al. 2010a; Wiedmann et al. 2008) while the challenges of sequencing complex genomes is minimized (Davey and Blaxter 2010;

Table 12.1 Predicted qualitative trait loci (QTL) associated with phosphorus use efficiency in Soybean

Number	QTL-associated gene	Gene annotation	Biological function	References
1	*Glyma.08G195200*	PROTEIN PHOSPHATASE PP2A REGULATORY SUBUNIT B	Protein phosphatase type 2A regulator activity	Guo et al. (2011)
2	*Glyma.08G195100*	GmACP1	Phosphatase activity	
3	*Glyma.08G194900*	Phosphoethanolamine	Phosphatase activity	
4	*Glyma.08G194100*	*Phospholipase D*	Protein binding activity	
5	*Glyma.08G194000*	CALCINEURIN B-LIKE PROTEIN 10	Calcium Ion binding activity	
6	*Glyma.02G266700*	ZINC FINGERDOMAIN CONTAINING PROTEIN	Hydrolase activity	Gore et al. (2002)
7	*Glyma.02G267500*	ZINC FINGER CCCH DOMAIN-CONTAINING PROTEIN 26-RELATED	Metal ion binding	
8	*Glyma.02G267900*	Tyrosine phosphatase family (Y_phosphatase3)	Tyrosine Phosphatase activity	
9	*Glyma.04G212900*	P-loop containing nucleotide triphosphate hydrolases	Hydrolase activity	
10	*Glyma.04G214000*	CELL WALL/VACUOLAR INHIBITOR OF FRUCTOSIDASE 2-RELATED	Pectin methylesterase inhibitor/enzyme inhibitor activity	
11	*Glyma.04G214400*	XYLAN ALPHA-GLUCURONOSYLTRANSFERASE 3-RELATED	Glycosyl transferase	
12	*Glyma.13G161900*	PURPLE ACID PHOSPHATASE 26	Acid phosphatase-related	
13	*Glyma.18G196200*	MRNA-CAPPING ENZYME	Phosphatase activity, specificity phosphatase, DNA binding	
14	*Glyma.18G198000*	Protein kinase domain	Protein kinase activity	
15	*Glyma.18G198200*	Threonine-specific protein kinase	Protein tyrosine kinase activity, ATP binding,	
16	*Glyma.18G198800*	Protein kinase domain	Protein phosphorylation,	
17	*Glyma.18G199000*	Protein kinase domain	Protein kinase activity	
18	*Glyma.18G199100*	Non-specific serine/threonine protein kinase	Protein kinase activity	
19	*Glyma.18G199200*	Protein kinase domain	Protein kinase activity	

Wiedmann et al. 2008). As a reflection of the power of RRLseq technology, this approach led to the prediction of 25,047 soybean SNPs, of which 80–92% were validated (Hyten et al. 2010a).

GBS, another multiplexed system, uses reduced-representation high-throughput sequencing libraries from restriction enzyme-fragmented DNA (Elshire et al. 2011). The system generates large SNP data useful for quantitative trait mapping (Elshire et al. 2011), as well as genome high-density molecular markers (Poland et al. 2012; Heffner et al. 2010). The data generated by GBS, besides SNP identification and QTL mapping/marker identification is also useful for GWAS (He et al. 2014; Fu et al. 2014). Though capable of generating substantial amounts of data, the occurrence of repetitive DNA in large or duplicated genomes such as the soybean genome, makes the unique alignment of sequence reads difficult, and makes SNP calling with GBS a big challenge (Treangen and Salzberg 2012). In complex genomes, there is also a tendency for acquiring sequence reads that are non-uniformly distributed (Beissinger et al. 2013).

The SLAFseq technique has been used recently to create high density maps and to identify QTLs in rice (Mao et al. 2015), cucumber (Xu et al. 2014) and soybean (Zhang et al. 2016; Qi et al. 2014; Li et al. 2014). The SLAFseq technique relies on deep sequencing and a double barcode system for efficient marker calling and accurate genotyping to generate high density maps without need for a reference genome. Applying SLAFseq to soybean recombinant inbred lines (RILs) derived from a cross between Nannong 94–156, a high-Pi efficiency line, and Bogao, a low-Pi efficiency line, (Zhang et al. 2016) constructed a high-density genetic map. The latter authors were able to identify QTLs that had already been reported (e.g. QTLs associated with *GmACP1* and *GmPT1*) as well as novel candidates. Using SLAFseq, Zhang et al. (2016) also demonstrate the capability to enhance QTL mapping compared to a previous study [e.g., q18-*1* QTL was mapped in a 0.7 cM interval compared to 3.9 cM as reported by Zhang et al. (2009) who applied a linkage mapping approach]. Both GBS and SLAFseq methodologies have also been widely used because of their simplicity and cost effectiveness (Jarquín et al. 2014; Sonah et al. 2013, 2015). To complement these technologies, an Illumina Infinium array (SoySNP50K iSelect BeadChip) for ~50,000 SNPs is currently available for large-scale genotyping (Song et al. 2013).

12.3 Soybean Adaptation to Pi Deprivation

Phosphorus is a macronutrient that is essential to plant growth and development (Penuelas et al. 2013). Phosphorus is involved in many metabolic processes, including energy transfer (ATP), photosynthesis, respiration, biosynthesis of nucleic acids and proteins, membrane biosynthesis (e.g., phospholipids) and signaling pathways (Vance et al. 2003; Johnston and Steen 2000; Khan et al. 2009). A variation in phosphate availability can induce changes such as an increase in asparagine concentration in both root and nodules (Rychter and Randall 1994), an increase by 50% in the

respiration rate at the root meristematic zone growing in Pi-rich environments (Wanke et al. 1998), and improved carbohydrate content in the roots of plants grown under Pi-deprived conditions (Almeida et al. 2000). As a consequence, limited availability of Pi is a major limitation to plant growth. Unfortunately, a low Pi content is a characteristic of much arable land (~70%), including land areas of soybean cultivation (Cakmak 2002; Hinsinger 2001; López-Arredondo et al. 2014). This is a consequence of soil erosion and the high fixation of inorganic phosphorus limiting its assimilation by plants (Sample et al. 1980). In order to maximize Pi uptake, plants, including soybean, adopt several physiological and molecular mechanisms to enhance the release of inorganic Pi. Gaining knowledge of these strategies, the mechanisms controlling them, and the gene regulatory networks involved, is essential to developing phosphorus-efficient soybean plants.

Those adaptive strategies rely on enhanced root growth; the proliferation of root hair cells, which is aimed at increasing the surface of exchange between the root system and the rhizosphere (Nielsen et al. 2001; Niu et al. 2013; Rouached et al. 2010); the production of plant root exudates [e.g., malate (Hinsinger 2001; Hoffland 1989; Miura et al. 2005; Sas et al. 2001)], or plant enzymes such as phosphatases, to enhance the release of organic Pi (Raghothama 1999; Vance 2003); the expression of high affinity Pi transporters to increase Pi uptake; and the promotion of arbuscular mycorrhizae symbiosis (López-Arredondo et al. 2014). At the molecular level, plant organs have developed specific responses to low Pi. For instance, transcriptome changes occurring in roots and nodules in response to Pi starvation are indicative of the redistribution of Pi between plant organs (Chen et al. 2011).

12.3.1 Enhanced Plant-Rhizosphere Interaction in Response to Pi Deprivation

The basal function of the root system is the uptake of water and nutrients, including Pi, from the soil. The root system architecture (RSA), which refers to the spatial distribution of all root parts in a particular growth environment, is a dynamic system affected by the external environment, including limited Pi availability. Specifically, in response to Pi deprivation, plants enhance their surface of interaction with the rhizosphere and their topsoil foraging capacity though an increase in primary root length, root branching, the number and length of lateral roots and enhancement of root hairs, as well as increased cluster-root formation (i.e., high density of short lateral rootlet enhancing the interaction of the plant with the rhizosphere) (Miura et al. 2005; Borch et al. 1999; Carswell et al. 1996; Dinkelaker et al. 1995; Jin et al. 2012; Kim et al. 2008; Lambers et al. 2011; Basu et al. 2007; Miguel 2011). Similarly, Zhao et al. (2004) highlighted the central role of the RSA on the Pi efficiency of soybean germplasms after applying a geographic information systems (GIS)-assisted approach (Zhao et al. 2004). This latter study suggested a possible co-evolutionary pattern among shoot type, root architecture and Pi efficiency and availability (Zhao et al. 2004). Among these characteristics, root hair

cells—epidermal root cells characterized by their lateral expansion—can contribute to almost 70% of the total surface area of the roots and can be responsible for up to 90% of the Pi acquired by plants (López-Arredondo et al. 2014). Therefore, optimal modifications of the RSA, including root hair cell morphometric and density, can be crucial for adaptation of soybean to low-Pi conditions.

The high plasticity of the RSA in response to low-Pi conditions has also been highlighted in other crop species such as maize (*Zea mays*), rice (*Oryza sativa*), and common bean (*P. vulgaris*). Hence, although the RSA differs between monocots and dicots, it can be argued that the main adaptive root traits are shared among all vascular plant species for the purpose of enhancing Pi acquisition (Niu et al. 2013). Any change in RSA is greatly influenced by hormone signaling and, as a consequence, hormone regulatory pathways play a critical role in regulating the uptake of phosphorus by soybean plants. For instance, functional genomic studies on both Arabidopsis and soybean plants demonstrated that the increase of lateral root formation in response to Pi deprivation was under the control of auxin through the ubiquitin-proteasome function of the auxin receptor, TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Perez-Torres et al. 2008). Molecular mechanisms controlling plant cell elongation are also important in the development of the root system and the uptake of Pi. For example, *GmEXPB2*, a gene encoding an expansin, is upregulated in response to phosphorus starvation, and regulates primary and lateral root elongation and Pi uptake efficiency (Guo et al. 2011, 2008).

12.3.2 Increased Pi Availability

To maximize the outcomes of these morphological changes, plants also release exudates, such as organic acids, into the rhizosphere to enhance the mobilization of Pi and its uptake (Brown et al. 2013). These acids are usually released by the plant in response to Pi deprivation and aluminum pollution (Ryan et al. 2001). Specifically, malate exudation under low-phosphorus conditions helps to solubilize phosphorus interacting with iron and aluminum oxides in acid soils, thereby enhancing phosphorus uptake by the plant (Hoffland 1989; Sas et al. 2001). Functional analyses in *A. thaliana* suggest that the aluminum-activated malate transporter, ALMT, is responsible for malate excretion (Hoekenga et al. 2006; Sasaki et al. 2004). Cloning and study of the soybean ALMT homolog, *Gm*ALMT1, confirmed the role of *ALMT* genes in malate exudation and tolerance to aluminum pollution (Liang et al. 2013). The transcriptomic regulation of *GmALMT1* by soil pH, phosphorus and aluminum also support the central role of *GmALMT1* in malate excretion (Liang et al. 2013). Taken together, these results show that *GmALMT1* plays a critical role in the adaptation of soybean to soil containing limited organic phosphorus. To maximize Pi availability, plants also secrete phosphatases. For instance, Gm*ACP1*, which encodes an acid phosphatase, is strongly up-regulated in phosphorus-efficient soybean accessions compared to low phosphorus-efficient accessions (Zhang et al. 2014). Overexpression of Gm*ACP1* in soybean roots led to an increase in phosphorus uptake (Zhang et al. 2014).

12.3.3 Enhanced Phosphorus Uptake

Another strategy adopted by plants to maximize Pi uptake is the use of high affinity Pi transporters (Gilroy and Jones 2000; Lynch and Brown 2001), which is advantageous because plants are constantly challenged by the task of taking up negatively charged Pi ions ($H_2PO_4^-$) across the negatively charged plasma membrane of the cell (Bucher 2007). Among the various plant Pi transporter families known to exist, the most studied Pi transporter proteins belong to the *PHT1* family (Bucher 2007; Qin et al. 2012a). This family encodes high affinity Pi transporters that are primarily up-regulated in response to Pi deprivation in the root hair cells and at the root apex (Javot et al. 2007a; Gordon-Weeks et al. 2003). Mining of the soybean genome sequence led to the identification of 14 PHT1 Pi transporter genes (PT1-14) (Qin et al. 2012a). With the exception of Gm*PT10*, which is induced in response to elevated phosphorus, the 13 remaining *PT* soybean genes are induced by Pi deprivation. In addition, each of the 14 Gm*PTs* is characterized by its tissue specificity (Qin et al. 2012a). For instance, *Gm*PT5 has been characterized as a regulator of Pi transport during the nodulation process (Qin et al. 2012b). Specifically, *Gm*PT5 controls entry of Pi from soybean roots to nodules. This role is essential during nodule development due to the large requirement for Pi during plant organogenesis, and in mature nodules where the fixation of atmospheric N_2 remains an energy-consuming biological process. Interestingly, supporting the balance existing between macroelements and the crosstalk among different nutrient-signaling pathways, Gm*PTs* are also up-regulated in response to nitrogen, potassium, and iron deficiency (Qin et al. 2012a). Through the complementation of a phosphorus-uptake defective yeast mutant, Qin et al. (2012a) also demonstrated that the soybean PT proteins, with the exception of GmPT6 and GmPT10, are high affinity transporters (Qin et al. 2012a; Tamura et al. 2012). Other studies have suggested *Gm*PT1 and *Gm*PT2 are low affinity transporters within the soybean plant (Wu et al. 2011). Further studies on these Pi transporters will greatly improve our understanding of soybean adaptation to soils depleted in phosphorus, paving the way for designing more precise breeding strategy(ies).

12.3.4 Mycorrhization: A Mutualistic Symbiosis to Enhance Phosphorylation

Mutualistic symbiosis also benefits plant phosphorus uptake. Specifically, about 80% of land plants enhance phosphorus uptake by promoting arbuscular mycorrhizal (AM) symbioses (Burleigh et al. 2002; Koide and Kabir 2000; Smith et al. 2000; Tibbett and Sanders 2002). AM symbiosis enables Pi to be scavenged from a large surface area, and delivery of Pi directly to the root cortical cells. To maximize the reallocation of Pi in plant cells, AM-inducible Pi transporters accumulate in

the periarbuscular membrane (Javot et al. 2007a, b). Specifically, among the 14 high affinity Pi transporters reported in soybean, only GmPT7, GmPT10 and GmPT11 are AM-inducible Pi transporters (Tamura et al. 2012). In addition to maximizing Pi uptake, AM symbiosis also results in increased phosphatase activity in soybean (Abdel et al. 2014).

12.4 Adaptation of Soybean Nodulation to Limited Pi Availability

12.4.1 Soybean Nodulation Is Highly Dependent on Pi Availability

Like in many plant growth systems, unavailability of Pi poses a major constraint to soybean plant growth (Kochian et al. 2004). This is especially true for soybean plants because of their engagement in the nodulation process. Nodulation is a legume-specific biological process resulting from the mutualistic symbiotic interaction between legumes and soil bacteria from the *genus* Rhizobia (Oldroyd 2013). This interaction is initiated by specific chemical recognition by the two partners. Upon recognition, rhizobia will infect the legume root—a process initiated in the root hair cell. The outcome of this symbiosis is the development of a new root organ named a "nodule," where the differentiated bacteria will fix and assimilate atmospheric nitrogen for the plant. In exchange, the plant will provide to the bacteria a sturdy source of carbon through the products of photosynthesis. This controlled and reciprocal exchange of various carbon and nitrogen compounds between the two partners is also highly dependent on Pi availability (Kochian et al. 2004; Robson and Broughton 1983; Vance 2001). Specifically, Pi deprivation is a serious limitation for legume development and nodulation because it affects nodule number and development as well as the biochemical activity of nitrogenase—the enzyme involved in the reduction of atmospheric nitrogen to ammonia (Vance et al. 2003; Tang et al. 2001; Olivera et al. 2004; Sa and Israel 1991; Le Roux et al. 2006; Ribet and Drevon 1995). As a reflection of the dependence of the nodulation process on Pi availability, the number of nodules developing on the surface of the soybean root system is regulated by Pi availability (Vadez et al. 1996). Also, in response to Pi deprivation, ATP, total adenylate concentration and energy charge decrease significantly in the nodule (Sa and Israel 1991). For instance, whole nodules in Pi-deficient controls contained 70–75% lower ATP concentrations than nodules of Pi-sufficient controls. At the biochemical level, fixation of atmospheric dinitrogen by nitrogenase requires a large amount of Pi through supplies of energy (i.e., ATP) and reducing agent (i.e., NADPH) according to the following chemical formula:

$$\mathbf{N}_2 + 8\mathbf{H}^+ + 8\mathbf{Fdred} + 16\mathbf{ATP} \rightarrow 2\mathbf{NH}_3 + 2\mathbf{H}_2 + 8\mathbf{Fd}^{\mathbf{ox}} + 16\mathbf{ADP} + 16\mathbf{Pi}$$

To enhance legume nodulation under limited Pi conditions, it is important to understand the molecular response of soybean nodules to Pi starvation. For instance, to identify genes potentially controlling plant responses to Pi deprivation, researchers have initiated a series of "-omic" studies. Proteomic studies identified 44 Pi-starvation responsive proteins from soybean nodules. Transcriptomic analysis through the use of quantitative RT-PCR technology correlated a change in transcript abundance with a modification in the amount of these Pi-regulated proteins (Chen et al. 2011). Taking advantage of comparative genomic strategies, knowledge gained from other legume plants can be transferred to soybean. For instance, due to its importance as a human dietary legume, and, because it is frequently cultivated in areas that lack sufficient Pi in the soil, the transcriptomic and metabolomic responses of common bean to Pi stress have been characterized (Broughton et al. 2003; Graham et al. 2006; Hernandez et al. 2007, 2009). These studies, as well as others conducted in non-legumes, have led to the identification of genes and miRNAs potentially regulating legume response to Pi stress (Hernandez et al. 2009; Valdes-Lopez et al. 2008, 2010). It will be interesting to see if soybean orthologs might share a similar response to Pi deprivation.

12.4.2 Physiological and Molecular Response of Soybean Nodulation Under Pi-Deprivation

Pi deprivation greatly hinders nodule development, nitrogen fixation and plant growth (Hernandez et al. 2009; Almeida et al. 2000; Chaudhary et al. 2008; Le Roux et al. 2009). To minimize the detrimental effect of low Pi on nodule development, developing nodules rely on the activation of specific Pi transporters (Al-Niemi et al. 1998). This is achieved through induction of expression of genes such as *GmPT5* that responsible for mobilizing and transporting Pi from other plant organs to the nodules (Qin et al. 2012b). A second strategy consists of increasing the pool of acid phosphatases (APase) to optimize Pi acquisition in developing nodules. Indeed, APase activity gradually increases in developing nodules to reach a peak of activity in mature nodules (Penheiter et al. 1997). Recent studies suggest that the induction of APase activity is highly dependent on Pi availability (i.e., APase activity is higher in low Pi conditions) (Araújo et al. 2008).

Expression of the *GmEXPB2* gene is upregulated in a P-dependent manner only in young nodules, and influences nodule formation and development (Li et al. 2015). The GmEXPB2 protein is known to be engaged in loosening the cell wall to facilitate greater rhizobial infection, as well increased cell division and elongation (Li et al. 2015). Since GmEXPB2 was shown to be involved in nodule vascular trace (NVT) and nodule vascular bundle development, it is thought that upregulation of GmEXPB2 promotes nodule organogenesis by facilitating nodule vascular bundle development, which allows for optimal import of substances such as sugars to the developing nodule (Li et al. 2015). Stabilizing nodule P homeostasis is another

adaptive strategy to enhance nitrogen fixation under P-deprived conditions (Qin et al. 2012b). The *GmSPX3* gene was recently identified as a regulator of P homeostasis in soybean (Yao et al. 2014).

12.4.3 Effect of AM of Soybean Nodulation Under Pi-Deprivation

Associating with both AM and Rhizobium in low Pi environments facilitates phosphorus and nitrogen uptake, thereby promoting plant growth and nodulation (Meng et al. 2015). Due to the cost of carbon allocation for AM symbiosis, AM association is dependent on nutrient, including Pi, availability, and the capacity of the plant to acquire nutrients (Smith et al. 2009). As a consequence, AM association is high in Pi-deprived conditions compared to Pi-sufficient conditions (Wang et al. 2011). A recent research report on GmNF-YA1a and GmNF-YA1b proteins suggested that these two proteins promote AM symbiosis due to minimized AM symbiosis in RNAi composite plants (Schaarschmidt et al. 2013). Though it is not clear how these GmNF-YA1s regulate establishment of AM symbiosis under low Pi conditions, the regulation of genes such as AM-inducible soybean phosphate transporters (Glyma.13g08720, Glyma. 14g28780,and Glyma.14g36650) was reported (Schaarschmidt et al. 2013). Thus, it is likely that GmNF-YA1a/b indirectly influences phosphate transport and nodulation through transcriptional regulation, since these phosphate transporters are known to regulate phosphate transport to nodules (Qin et al. 2012b; Tamura et al. 2012). Also, root exudates from Soybean grown in p-deficient soil have increased phosphatase activity in response to AM symbiosis, suggesting that AM-induced assimilation of phosphorus in low phosphorus soils may also involve the activity of acid and alkaline phosphatases (Abdel-Fattah et al. 2014).

12.5 -Omic Analyses of Soybean Response to Pi Deprivation

-Omic is a collection of large-scale biological data sets covering various molecular analyses. -Omic data sets covers various aspect of the biology of an organism including the collection of genes (genomics), epigenomic modifications (epigenome; i.e., methylation pattern of the genomic DNA, chemical modification of the histone tails, pool of non-coding RNAs) transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics). The integration of these various -omic data sets is currently leading to the development of systems biology approaches (Tripathi et al. 2015; Libault et al. 2010b; Jogaiah et al. 2013). Ultimately, the purpose of systems biology is to better understand the overall complexity in the response of an organism to environmental stresses and/or during developmental processes. Evolutionary biology also benefits from the comparison of various -omic datasets generated across different species to better understand the various molecular aspects of the evolution of living organisms (Libault et al. 2010a).

The release of the soybean genome sequence associated with the improvement of molecular and analytical technologies open avenues to further our global understanding of soybean biology. For instance, -omics analyses (e.g., epigenomic, transcriptomic, proteomic, metabolomics) (Shamimuzzaman and Vodkin 2012; Jones and Vodkin 2013; Severin et al. 2010) have been performed to study different aspects of soybean biology, including, among others, nutrient deprivation such as Pi deprivation (Chen et al. 2011), soybean nodulation (Brechenmacher et al. 2010, 2012; Nguyen et al. 2012; Libault et al. 2010c; Li et al. 2010), soybean response to pest infection (Mazarei et al. 2011; Matsye et al. 2011; Klink et al. 2007, 2009, 2010, 2011; Ithal et al. 2007a, b; Puthoff et al. 2007; Klink and Matthews 2009; Alkharouf et al. 2007; Wise et al. 2007), soybean seed development, and nutritional quality (Hajduch et al. 2006; Agrawal et al. 2008; Krishnan et al. 2009; Asakura et al. 2012).

12.5.1 Transcriptomic Analyses

Previous analyses using microarray technology revealed a total of 42 soybean genes that are expressed differentially between low-Pi-tolerant and -sensitive soybean accessions (Wang et al. 2016). Since then, high-throughput sequencing technologies have revolutionized our understanding of plant genomes, epigenomes, transcriptomes, structural genomics (i.e., nucleosome position and protein-DNA interactions such as the binding sites of transcription factors on the genomic DNA), and breeding strategies. For instance, the release of soybean transcriptome atlases (Libault et al. 2010a; Severin et al. 2010) has allowed precise mapping of the expression of soybean genes in various plant organs, including in root hair cells, in response to rhizobia inoculation (Libault et al. 2010c). High-throughput sequencing technologies have also been applied to the characterization of novel transcripts and non-coding RNAs (Fu et al. 2009).

Using high-throughput sequencing technology, 1612 Pi-deficiency-responsive genes, including *GmPHT1-3*, *GmPHT1-9*, *GmPHT1-14*, *GmSPX3*, *GmIPS1* and *GmPLDZ2*, were identified in soybean (Zeng et al. 2015). In soybean, the PHT1 gene family, which comprises 15 genes, encodes Pi transporters that are important for Pi uptake and transport (Fan et al. 2013; Song et al. 2014). More recently, 42 candidate genes were identified as induced by low-Pi stress by using low-Pi tolerant (Chundou, CD) and low-Pi sensitive (Yunhefengwodou, YH) soybean accessions (Wang et al. 2016).

12.5.2 Analytical Analyses

Plant transcriptional responses to environmental stresses do not always translate into proteomic changes (Libault et al. 2010d). It is thus imperative to perform proteomic studies as well as transcriptional studies in order to understand abiotic stress

tolerance mechanisms in soybean. Several liquid chromatography mass spectrometry (LC-MS)-based proteomic studies have been reported so far (Brechenmacher et al. 2009; Clarke et al. 2015; Mooney et al. 2004; Ohyanagi et al. 2012) including in response to Pi deficiency (Sha et al. 2016).

A more holistic approach is needed to fully characterize low-Pi response pathways in soybean. Just having a knowledge of genes, transcripts and proteins alone does not fully explain biological processes unless we also have knowledge of the metabolites involved. Thus, the use of metabolomics to identify and quantify the complete range of metabolites involved in low-Pi tolerance is imperative. Various platforms exist to reveal plant metabolomic content, e.g., gas chromatography mass spectrometry (GC-MS), fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), liquid chromatography mass spectrometry (LC-MS), capillary electrophoresis mass spectrometry (CE-MS), and nuclear magnetic resonance (NMR) (Putri et al. 2013a, b).

Phospholipids represent 30% of the total organic Pi in plants (Okazaki et al. 2015). Accordingly, plant growth under Pi-limited conditions shows a decreasing phospholipid membrane content that is complemented by an increase in non-Pi glycolipids such as digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol (SQDG). These glycolipids help to maintain cellular functions under low-Pi stress. In *A. thaliana* and rice plants growing under Pi-limited conditions, a new non-Pi glycolipid, identified as glucuronosyldiacylglycerol (GlcADG), also plays an important role in mitigating the stress induced in plants growing under low-Pi conditions (Okazaki et al. 2013).

12.6 Conclusions and Perspectives

There is no single optimal approach to enhancing soybean growth and yield under low-Pi conditions. Therefore, there is a need to apply a combination of approaches when developing breeding programs (Fig. 12.2). For instance, the combination of genomic selection with marker assisted selection is a feasible strategy to breed soybean lines characterized by their greater Pi acquisition. Genetic engineering can also be used to manipulate important genes in order to improve Pi acquisition and crop growth. However, this cannot be done without a deep knowledge of genes controlling low Pi-tolerance pathways, which will be revealed through the establishment of systems biology strategies. Initial work in this area has already shown that adopting genetic manipulation to enhance Pi tolerance in soybean is promising. As an example, Wang et al. (2009) overexpressed the *AtPAP15* gene in soybean and, as a consequence, improved Pi accumulation, plant dry weight, Pi content and yield (Wang et al. 2009). The recent development of new biotechnological tools also represents an opportunity to better understand soybean responses and adaptation to low Pi. In addition to the expanding capabilities of sequencing technologies, another promising tool for genomic manipulation is the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated protein (Cas) (CRISPR-Cas9) system. This system is viewed as a potent tool to precisely target genes of interest

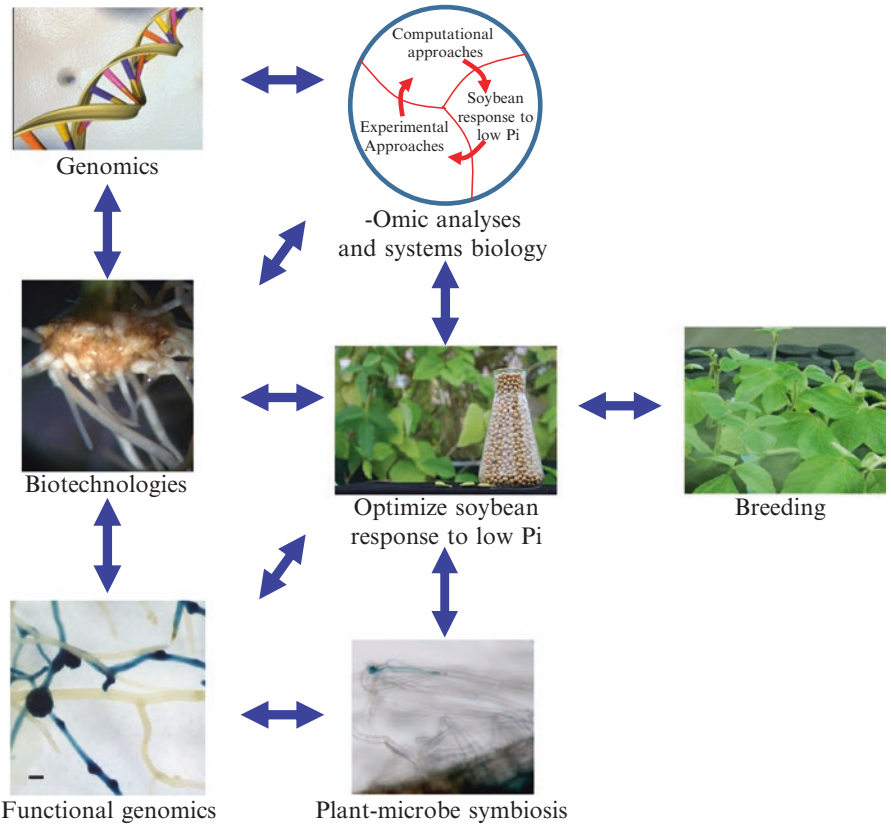

Fig. 12.2 The use and integration of various strategies will be essential to optimize soybean adaptation to limited Pi. These strategies include the use of molecular tools (e.g., genomics, functional genomics, biotechnologies and system biology), with breeding efforts and the optimization of plant–microbe symbiosis (i.e., both rhizobia and AM). (Sub-figures provided by Ms. Mehrnoush Nourbakhsh and Ms. Zhenzhen Qiao)

(Jinek et al. 2012). In prokaryotes, the CRISPR-Cas system is part of an immune mechanism used to remove foreign nucleic acid inserts (Koonin and Makarova 2009). The principle has been adapted and used successfully for genome editing in plants as well as in soybean (Jinek et al. 2012; Sun et al. 2015). Through -omics, there is also the prospect of identifying other key players in the Pi-acquisition for further studies using tools like the CRISPR-CAS. The CRISPR-CAS technology can also be used to create stable mutations for the functional study of genes involved in the Pi-acquisition and tolerance pathway(s).

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Chapter 13
White Lupin: A Model System for Understanding Plant Adaptation to Low Phosphorus Availability

Claudia Uhde-Stone

Abstract Phosphorus (P), in its ionized form (P_i), is one of the most limiting nutrients for plant growth and development. White lupin is a dinitrogen (N_2)-fixing legume that can increase P_i availability in soils. Under P_i deficiency, white lupin develops cluster roots, also known as proteoid roots. Cluster roots are densely packed lateral roots, resembling bottlebrushes. The resulting increase in root surface, together with coordinated biochemical responses, releases bound P_i and makes it available for plant uptake. The most noticeable biochemical responses that increase P_i availability include excretion of organic anions and phosphatases. As a consequence, white lupin can grow without addition of P_i fertilizer, and its ability to fix N_2 is less inhibited by P_i deficiency, compared to other legumes. However, formation of cluster roots requires additional carbon (C) and nitrogen (N). Thus, white lupin needs to carefully balance C use with the formation of cluster roots and nodules. High-throughput approaches, particularly RNA-seq, have revealed many of the genes involved in cluster root formation and function and are beginning to reveal networks that regulate P_i acquisition and use. A better understanding of white lupin's responses to low nutrients may help to overcome inhibition of N_2 fixation by low P_i availability in other legumes.

Keywords Cluster roots • *Lupinus albus* • N_2 fixation • Phosphate deficiency • Proteoid roots

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13.1 White Lupin: A Model for Plant Adaptations to Phosphate Deficiency

13.1.1 Introduction

Symbiotic dinitrogen (N_2) fixation is crucial for world food security, but can be severely limited by low phosphate ion (P_i) availability, which characterizes about 40–60% of the world's arable land (Vance et al. 2003; Batjes 1997; Tiessen 2008). About 90% of applied P_i fertilizer is obtained as mined phosphate rock (PR), a non-renewable resource (Baveye 2015; Vance and Chiou 2011). While global P_i reserves are being depleted, P_i levels in many agricultural soils are building up due to the use of P_i fertilizer. Much of the applied P_i is sorbed by soil particles and converted into organic forms unavailable to most plants (Lambers et al. 2006).

The development of more P_i-efficient crop plants could reduce the need for P_i fertilizer. To discover mechanisms that allow plants to grow better on nutrient-poor soils, scientists are turning to model plants that are well adapted to nutrient deficiency. White lupin is a N_2-fixing legume that can increase P_i availability in soils via specialized roots, known as cluster or proteoid roots. This chapter will explore how the morphological and biochemical responses in cluster roots increase soil P_i availability and the costs associated with these responses. Special emphasis is given to "omics" approaches that are beginning to unravel the molecular and physiological underpinnings of white lupin's adaptations to P_i deficiency. Such findings may be applied toward the development of crop plants that can thrive in nutrient-poor soils.

13.1.2 Lupin: An Ancient Crop for the New Age

With more than 300 lupin species found in both the Old and New Worlds, the genus *Lupinus* in the Fabaceae (legume) family has among the highest speciation rate known for any genus (Hughes and Eastwood 2006). Lupins occupy diverse habitats, from sea level to alpine tundra. Species include annuals and perennials, from miniature lupin (*L. bicolor*) to the 8 m high wooden trees of *L. jaimehintoniana* (Clements et al. 2005).

Cultivation of lupins began over 3000 years ago in Egypt and the general Mediterranean region and up to 6000 years ago in the central Andes region of South America (Clements et al. 2005; Uauy et al. 1995). The three lupin species of most agronomic importance are the "Old World" species white lupin (*L. albus*), narrowleaf (blue) lupin (*L. angustifolius*), and yellow lupin (*L. luteus*) (Brebaum and Boland 1995). Early Greek and Roman writers already mention the ability of lupin to improve soils as a green manure and rotation crop, reviewed by Clements et al. (2005). For example, Virgil (70–19 BC) mentions the use of lupins for crop rotation, and Cato the Elder (234–149 BC) recommends lupins for farming on various soils, as green manure, fodder, and grain.

Traditional white lupin contains high alkaloid levels and thus requires soaking. A *labrum lupiniarum*, a special vessel to remove the water-soluble alkaloids from lupin seeds, was common in ancient Roman households. Soaked seeds of white lupin, boiled and pickled, are still a popular snack in Mediterranean regions. In Germany during the 1930s, screening of millions of lupin seeds and leaves for low alkaloid levels initiated the development of "sweet" forms of lupin, including sweet white lupin (Sengbusch 1942). While areas of lupin cultivation declined in some countries, such as Germany, Poland, and the former USSR, lupin was discovered in other areas, particularly Australia, with its vast areas of coarse, nutrient-poor soils (Gladstones et al. 1998; Gladstones 1994; Clements et al. 2005).

Breeding efforts have continued in recent decades, primarily in Australia (Cowling et al. 1998; Yang et al. 2010; Lin et al. 2009), and lupins are entering a new phase of commercial use. While originally cultivated mainly as a green manure or forage, lupins are increasingly grown for their seeds, which can be used as an alternative to soybeans. Lupin grain is highly regarded as feed for livestock and as an ingredient in aqua-feeds (Lambers et al. 2013; Tadele 2015; Borquez et al. 2011). Lupin grain is gluten-free, and considered a prebiotic, a food that supports a healthy gut microbiome. Lupin seeds are high in protein (30–40%), dietary fiber (30%), and antioxidants but low in oil and starch. Accordingly, lupin grain shows great potential for increased use in human food, and breeding efforts are ongoing (Clements et al. 2005; Tadele 2015).

13.1.3 White Lupin Responds to P_i Deficiency by the Formation of Cluster Roots

Phosphorus (P) is an essential macronutrient for plants but is difficult to acquire. Plants take up P as negatively charged phosphate ions (P_i) in form of $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}, which in soils are often bound to cations such as Ca^{2+}, Mg^{2+}, and Al^{3+}, or converted into organic forms not amenable for plant uptake (Schachtman et al. 1998).

Plants have evolved a range of strategies that increase P_i uptake and availability (Marschner 1995). The most common of these strategies, employed by about 82% of higher plant species, is a symbiotic association with mycorrhizal fungi (Brundrett 2002). White lupin exhibits an exceptional tolerance for poor soils, despite a lack of such mycorrhizal symbiosis. Instead, white lupin develops cluster roots, also called proteoid roots, in response to P_i deficiency (Braum and Helmke 1995; Lambers et al. 2013; Gardner et al. 1982). Cluster roots enable white lupin to take up almost five times more P_i per unit root length than soybean, a mycorrhizal species that does not form cluster roots (Watt and Evans 2003).

Cluster roots are zones of dense tertiary lateral roots, resembling bottlebrushes, which increase the root surface for nutrient uptake (Keerthisinghe et al. 1998; Neumann et al. 1999). Of the species that form cluster roots, white lupin has been

most thoroughly studied. Physiological modifications in cluster roots that contribute to P_i acquisition include the excretion of large amounts of organic anions and protons that increase the availability of mineral-bound P_i, and other nutrients, such as iron (Fe), manganese (Mn), and zinc (Zn) (Marschner 1995; Dinkelaker et al. 1995; Gardner et al. 1982; Neumann and Martinoia 2002). Intercropping of white lupin was shown to increase P_i and trace elements in neighboring plants (Wiche et al. 2016; Cu et al. 2005) and shows potential for phytoremediation and mining (Wiche et al. 2015).

Another important feature of white lupin is its ability to fix N_2 in symbiosis with the soil bacterium *Bradyrhizobium* sp. In most legumes, symbiotic N_2 fixation is inhibited by low P_i concentrations, but N_2 fixation rates in white lupin appear less susceptible to inhibition by low P_i (Schulze et al. 2006). Taken together, the ability to fix N_2 and to increase availability of P_i and trace elements in poor soils makes white lupin an important crop for soil improvement and an illuminating model for the study of plant adaptations to nutrient deficiency.

13.2 Cluster Roots Increase P_i Availability in the Rhizosphere

13.2.1 Most Soil P_i Is Unavailable to Plants

P_i is an essential macronutrient for plant growth and development, required for molecules such as ATP, NADPH, nucleic acids, and phospholipids (Schachtman et al. 1998; Raghothama 1999). However, compared to other major nutrients, P_i is far less mobile and thus more difficult for plants to obtain. While total soil P_i may range from 500 to 2000 μM, typically only 0.1–10 μM are in the soil solution and available for uptake by plant roots (Hinsinger 2001). This is low compared to adequate P_i concentrations of most crops, which range from several to tens of μM P_i. The low availability of P_i is due to its high reactivity. The negatively charged P_i can adsorb onto positively charged minerals such as Fe and Al oxides. In addition, P_i anions tend to form insoluble complexes with cations of Al and Fe under acidic conditions, and Ca and Mg under neutral and alkaline conditions, rendering the complexed P_i unavailable to plants (Hinsinger 2001).

As a consequence, an estimated 5.7 billion hectares worldwide (about 67% of total farmland) contain too little available P_i for sustaining optimal crop production (Batjes 1997), making P_i a major limiting factor for plant growth. Any available P_i is rapidly taken up by roots, resulting in a rapid decline of available P_i in the rhizosphere (Lambers et al. 2008; Shen et al. 2011). Application of P_i fertilizer is not a sustainable solution. Large amounts of phosphate rock (PR) are mined and applied for crop production worldwide. Whether and when global P_i demand will surpass supply of PR is subject to intense debate. While some researchers have claimed that world reserves of PR are getting depleted at an alarming rate (Cordell et al. 2009; Rosemarin et al. 2011), others argue that such concerns in the past have always been

refuted by discoveries of new PR reserves (Ulrich and Frossard 2014; Scholz and Wellmer 2015). Much discussion has been triggered by a drastic increase of estimated global PR reserves from 16,000 Mt. PR in 2010 to 67,000 Mt. PR in 2014, mostly based on a 2010 report by the International Fertilizer Development Center (IFDC). Edixhoven et al. (2014) critically assessed the IFDC report and suggest that the report overestimates accessible PR reserves (Edixhoven et al. 2014). In response, Scholz and Wellmer (2015) point out that PR reserves are dynamic, i.e., new reserves can be expected to be found (Scholz and Wellmer 2015). What is certain, however, is the fact that PR is a finite resource essential for life, a fact that requires a collective effort to make better use of P_i than is currently the case.

In addition, heavy fertilizer use can lead to P_i runoff into nearby water systems and results in eutrophication and subsequent decline in biodiversity (Runge-Metzger 1995; Correll 1998; Cramer 2010). Moreover, inexpensive P_i fertilizer is not available in the tropics and subtropics, where the highly weathered soils are particularly prone to P_i deficiency (Batjes 1997).

Although agriculture depends on fertilizer to enhance yields, economic and ecological drawbacks have prompted the search for alternative approaches to maintaining the global food supply (Cramer 2010; Cordell et al. 2009; Gaxiola et al. 2011; Vance et al. 2003). To discover mechanisms that enable plants to grow better on poor soils, scientists are learning from plants that are especially well adapted to nutrient-poor soils, such as cluster root-forming plants.

13.2.2 Cluster Roots Arose Independently in Various Plant Families

Cluster roots are bottlebrush-like clusters of short lateral rootlets that are capable of making soil P_i more available, primarily due to the release of organic anions (Gardner and Boundy 1983; Dinkelaker et al. 1989). Cluster roots are an adaptation to poor nutrient availability, and many cluster root-bearing species, including white lupin, are pioneer species in disturbed environments (Skene 2000). Most are capable of N_2 fixation via *Rhizobia* and *Frankia* symbiosis (Skene 1998), but typically non-mycorrhizal, with some exceptions (Skene 2000; Neumann and Martinoia 2002; Lambers et al. 2006). Because mycorrhizal associations are believed to be an ancestral mode of P_i acquisition in higher plants, present-day non-mycorrhizal species must have lost their ability to form such association. Cluster root development is much faster than that of mycorrhizal associations, which may be an advantage particularly in dry climates (Neumann and Martinoia 2002). Indeed, cluster root formation in white lupin was shown to be stimulated in dry patches (Felderer et al. 2015), and many cluster root-bearing plants are well adapted to poor soils in seasonally dry regions.

Cluster roots occur in a wide range of distantly related plant families (Skene 1998), but are most common in the Proteaceae family, typically slow-growing sclerophyllous shrubs and trees common in soils of extremely low P_i availability in

the Mediterranean region, Western Australia, and South Africa (Dinkelaker et al. 1995). Cluster roots are also found in some members of other families adapted to low soil fertility, such as Betulaceae, Casuarinaceae, Cucurbitaceae, Cyperaceae, Elaeagnaceae, Fabaceae, Moraceae, Myricaceae, and Restionaceae (Neumann and Martinoia 2002). Not every genus of cluster root-forming families produces cluster roots, nor every species within a genus, indicating that cluster roots arose independently within different families (Skene 2000). Thus, despite their superficial similarity, cluster roots from different families will likely have underlying differences (Skene 2000).

Cluster root formation is primarily found in two of the five clades that comprise the genus *Lupinus* (Ainouche et al. 2004; Ainouche and Bayer 1999; Skene 2000). All members of these two clades can form cluster roots (Fig. 13.1), indicating that

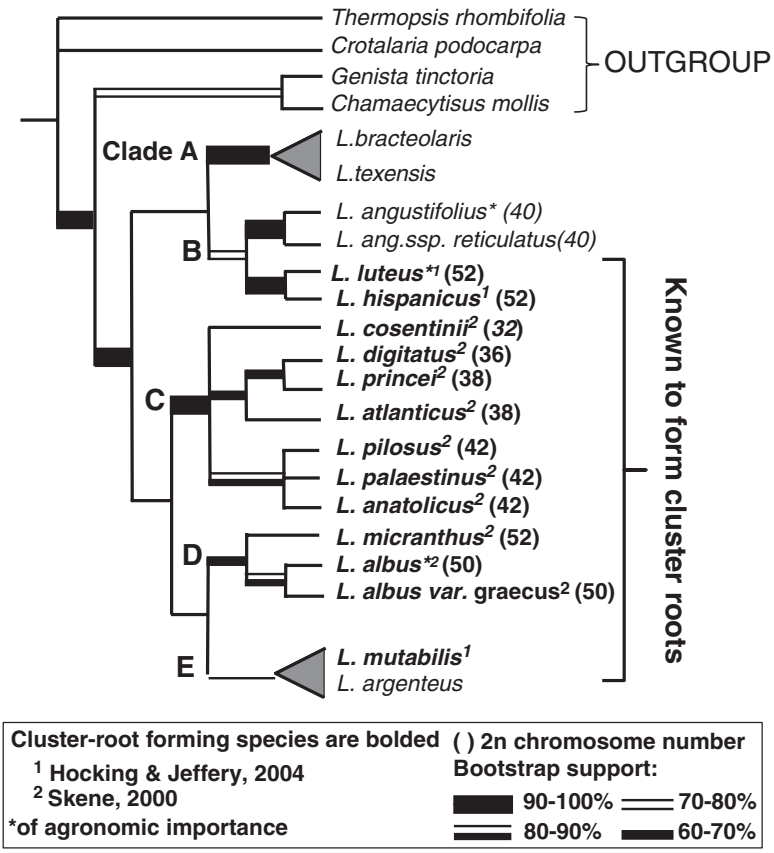

Fig. 13.1 Phylogenetic relationships among Old World lupines (Redrawn and modified from Ainouche et al. 2004). Species known to form cluster roots are bolded; 2n chromosome numbers are given in brackets. Species of most agronomic importance are indicated by *. Bootstrap confidence values (from 500 replicates) supporting the branches are given in the legends

cluster roots in white lupin arose in a common ancestor of these two sister clades (Skene 2000). However, lupins show a range of more or less clustered root structures (Lambers et al. 2013), and lupins from other clades, most notably *L. luteus*, have been shown to form cluster-like roots that release carboxylates (Fig. 13.1) (Hocking and Jeffery 2004). Yet others, such as *L. angustifolius*, do not produce cluster roots, but still release large amounts of carboxylates (Egle et al. 2003; Pearse et al. 2006b; Lambers et al. 2012).

An interesting aspect of cluster roots is the question if their formation requires unique, cluster root specific factors. The various degrees of cluster root formation in the genus *Lupinus*, and the independent rise of cluster roots in many unrelated genera, point toward formation of cluster roots by processes common to all roots, rather than the need for unique factors.

13.2.3 A Variety of Factors Influence Cluster Root Formation in White Lupin

Formation of cluster roots appears to be primarily induced by internal P_i deficiency, but external factors also play a role (Fig. 13.2). Split-root experiments revealed that cluster root formation is induced by low internal P_i of the shoot (Shen et al. 2005; Shane et al. 2003). However, the root genotype is also a determining factor, as

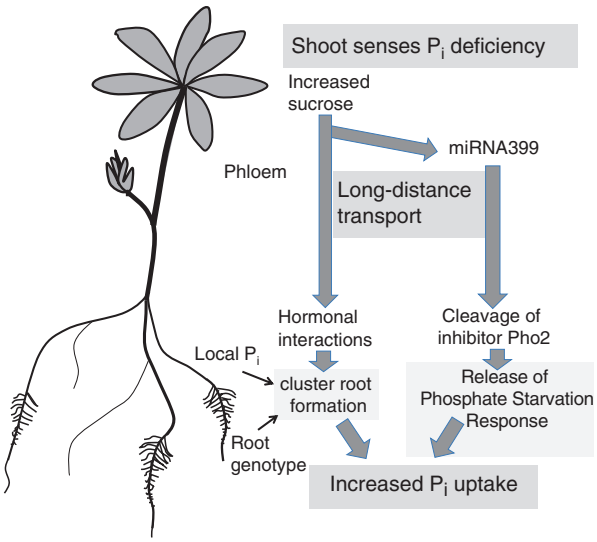

Fig. 13.2 Schematic overview of major factors known to influence cluster root formation and function in white lupin. In response to P_i deficiency, increased sucrose is translocated from shoot to root and, in interaction with plant hormones, triggers cluster root formation. Increased sucrose also induces miR399, which in turn activates the phosphate starvation response

shown in reciprocal grafting between white lupin and the noncluster root-forming *L. angustifolius*, where only plants with the white lupin root system formed cluster roots (Lambers et al. 2003; Marschner 1995).

In addition to internal P_i, external P_i plays a role in cluster root formation. Local P_i application actually induced cluster root formation and proton extrusion (Shu et al. 2007). On the other hand, localized root contact with high P_i reduced P_i deficiency-induced gene expression in white lupin cluster roots (Liu et al. 2005; Tesfaye et al. 2007). These opposing results indicate the involvement of local signals capable of detecting both high and low P_i availability.

Expression studies and external application of hormones and hormone antagonists indicate involvement of many hormones in cluster root formation, namely auxin, cytokinin, ethylene, gibberellin, brassinosteroids, and nitric oxide (Gilbert et al. 2000; Wang et al. 2010, 2015b; Meng et al. 2013; O'Rourke et al. 2013; Secco et al. 2014). External application of auxin was shown to induce cluster root formation, while cytokinin (Neumann et al. 2000; Gomez and Carpena 2014; Meng et al. 2013; Gilbert et al. 2000) and gibberellin (O'Rourke et al. 2013) had an inhibiting effect. Polar transport of auxin from shoot to root is known to induce lateral root formation (Jung and McCouch 2013) and thus was considered a potential shoot-borne signal for the induction of cluster roots (Gilbert et al. 2000; Neumann et al. 2000). However, shoot-to-root translocation of auxin was unaffected by P_i limitation, making auxin an unlikely candidate for long-distance signaling of P_i deficiency (Wang et al. 2015b). Similarly, removal of shoot apices, the main source of polar auxin transport, and application of auxin transport inhibitors at the shoot had no effects on cluster root formation of P_i-deficient white lupin (Meng et al. 2013).

Sucrose is another shoot-derived factor involved in the formation of cluster roots (Zhou et al. 2008; Wang et al. 2015c). Sucrose-induced cluster root formation was suppressed by application of an ethylene antagonist, indicating a possible interaction between sucrose and ethylene signaling (Wang et al. 2015b). A connection between sucrose and auxin signaling was shown in *Arabidopsis*, where increased sucrose accumulation is required for the Fe deficiency responses, with auxins acting downstream of sucrose in transmitting the Fe deficiency signal (Lin et al. 2016). Similarly, cluster root formation in white lupin may involve auxin as a downstream mediator of long-distance sucrose signaling (Fig. 13.2).

While sucrose accumulation appears to be an important long-distance signal for nutrient deficiency, it is not specific to P_i deficiency. Sucrose accumulation has been implicated in signaling of diverse processes, including Fe deficiency (Lin et al. 2016), which may result in considerable cross talk between nutrient deficiencies. Indeed, several genes identified as upregulated in cluster roots under P_i deficiency were also upregulated in cluster roots under Fe and N deficiency (Rath et al. 2010; Uhde-Stone et al. 2005). However, other P_i-responsive genes are specific to P_i deficiency (Rath et al. 2010), indicating that beside sucrose, additional signals may act to specify the nutrient deficiency present.

miRNAs have been shown to act as long-distance signals in nutrient deficiency responses, and are often induced specifically by a certain nutrient deficiency, while being suppressed by others (Liang et al. 2015). In *Arabidopsis*, miR399 was induced under P_i deficiency but downregulated under Fe and N deficiency (Paul et al. 2015).

Reciprocal grafting experiments revealed that miR399 serves as a long-distance signal of P_i deficiency from shoots to roots in *Arabidopsis* (Lin and Chiou 2008; Pant et al. 2008). Once reaching the roots, miR399 cleaves the inhibitor Pho2, thus activating P_i deficiency-responsive genes (Kuo and Chiou 2011; Pant et al. 2008). miR399 was found in the phloem sap of white lupin (Rodriguez-Medina et al. 2011), and expression of miR399 was upregulated under P_i deficiency (Zhu et al. 2010), indicating a potential role of miR399 as an additional long-distance signal of P_i deficiency in white lupin. Expression studies in common bean showed that miR399 induction in response to P_i deficiency requires photosynthetic carbon (C) assimilation, indicating that sucrose acts upstream of miR399 (Fig. 13.2) (Liu and Vance 2010).

Several hormones have been implicated in cluster root development (Wang et al. 2015b). Among these is nitric oxide, a diffusible bioactive molecule that functions in numerous plant processes, including lateral root formation (Correa-Aragunde et al. 2004, 2006). Wang et al. (2010) found accumulation of nitric oxide in P_i-deficient white lupin roots, particularly in cluster rootlet primordia. The use of specific inhibitors revealed that a nitric oxide-synthase-like enzyme and a xanthine oxidoreductase are required for the accumulation of nitric oxide in cluster roots (Wang et al. 2010). Application of an exogenous nitric oxide donor enhanced the formation of cluster roots in P_i-deficient, but not in P_i-sufficient white lupin. In contrast, Meng et al. (2012) found cluster root formation induced by an exogenous nitric oxide donor under $+P_i$. Interestingly, like cluster roots induced by external sucrose application, the cluster roots triggered by nitric oxide under $+P_i$ conditions did not secrete citrate.

In summary, results suggest that sucrose acts as a nutrient deficiency signal translocating from shoot to root, possibly interacting with ethylene signaling. Accumulation of sucrose in the root promotes other factors, such as auxin and nitric oxide, which contribute to the formation of cluster roots. Sucrose accumulation, combined with P_i deficiency, induces a parallel long-distance signal in form of miRNA399, which induces certain P_i starvation response genes. Other factors, such as additional miRNAs and hormones, are likely involved as well. While certainly simplified, this model can explain how external application of sucrose, auxin, and nitric oxide can trigger the formation of cluster roots under sufficient nutrient supply. However, these are typically not fully functional (Meng et al. 2012; Wang et al. 2015c), because without P_i deficiency, miR399 is not induced, and the P_i starvation response is not activated (Fig. 13.2).

13.2.4 Cluster Roots Display Morphological and Physiological Modifications

13.2.4.1 The Morphology of Cluster Roots Creates Nutritional Hotspots

In dicots, lateral roots initiate at random from the pericycle of primary roots (Vance et al. 2003). In contrast, cluster rootlets initiate at every protoxylem pole within a cluster zone (Skene 2000), resulting in a bottlebrush-like appearance. In comparison to typical roots, where root hair development occurs from a discrete number of

epidermal cells, cluster roots produce an overabundance of root hairs. The dense clustering of rootlets, together with abundant root hair growth, increases the root surface of cluster roots by more than 100-fold.

In contrast to typical lateral roots, cluster rootlets are of determined growth, meaning that they lose their apical growth meristem soon after initiation and cease to grow, reaching a final length of just a few millimeters. Determined root growth poses the problem of depletion of nutrients within the rhizosphere. Cluster rootlets are not optimized for soil exploration, but rather create short-term nutrient hotspots (Skene 2000), with a large surface area to take up mobilized nutrients, particularly P_i. Once most of these nutrients are "mined," typically after 2–3 days, cluster roots become senescent, recycling the nutrients invested in their growth.

Five main zones of cluster root development and function can be distinguished, preemergent, juvenile, immature, mature, and senescent (Fig. 13.3) (Neumann et al. 1999; Wang et al. 2014). Preemergent zones have increased numbers of lateral root meristems, but these have not yet broken through the surface. Just emerging rootlets form juvenile zones, while immature zones have fully expanded rootlets that however are not yet fully functional. Within about 2 days, immature cluster rootlets/mature and their physiological responses peak, before a few days later functional activity declines and rootlets become senescent. A cluster root may show all zones of development, and new cluster roots continue to form in the younger portions of the growing root system (Fig. 13.3).

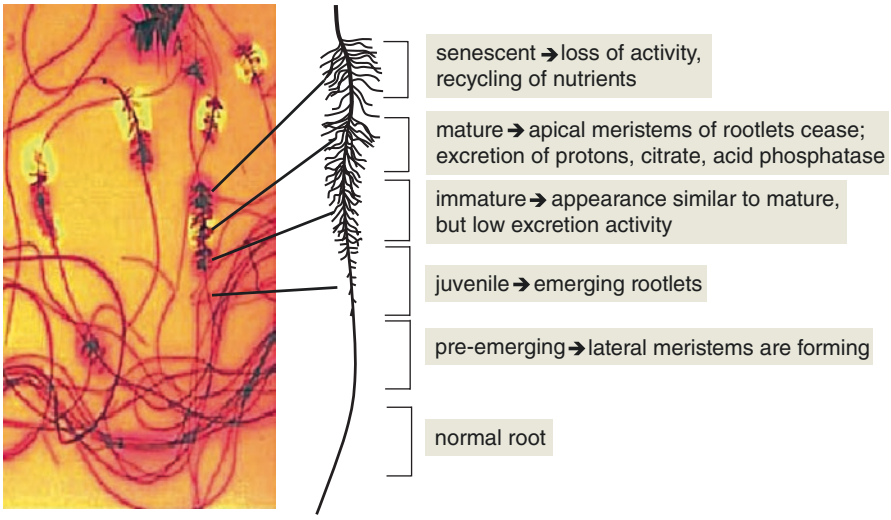

Fig. 13.3 Developmental stages of cluster roots in P_i-deficient white lupin (Modified from Massonneau et al. 2001 and Wang et al. 2014). The roots were placed between two plates of agar containing 0.04% (w/v) *bromocresol purple* as a pH indicator. The dye turns to *yellow* (pH < 5) when rootlets mature and acidify their surrounding and *purple* under alkaline conditions (pH > 7). The *yellow color* distinguishes active cluster root zones from immature and senescent zones. The different developmental stages of cluster roots are indicated in the schematic drawing

The determinate nature of cluster rootlets is not designed for competition, and other plants can benefit from the increased availability of mineral nutrients. Wheat intercropped with lupin was shown to acquire more P_i, Mn, and N than when grown alone, demonstrating white lupin's ability to facilitate mineral nutrition of other plants (Gardner and Boundy 1983; Cu et al. 2005).

13.2.4.2 Synchronized Biochemical Responses Mobilize P_i in Cluster Root Zones

Cluster roots of white lupin release copious amounts of citrate into the rhizosphere (Dinkelaker et al. 1989; Gerke et al. 1994). Citrate is capable of freeing inorganically bound P_i by two main mechanisms, ligand exchange and dissolution of P_i sorption sites (Gardner et al. 1982; Gerke et al. 1994). Citrate concentrations of 50–90 mmol per g soil have been reported, sufficient to make bound P_i available for plant uptake. Citrate exudation occurs in a spatial and temporal pattern. Using hydroponics, Neumann et al. (1999) found that citrate exudation occurred mainly in mature cluster root zones (Neumann and Römheld 1999). Dessureault-Rompré et al. (2007), using soil-filled rhizoboxes, reported one or more main bursts of citrate exudation from white lupin cluster roots in the afternoon (Dessureault-Rompré et al. 2007).

An interesting question is the mechanism by which citrate is exuded. Citrate occurs mainly as a trivalent anion in the cytoplasm. Sensitivity of citrate export to anion channel blockers (Neumann and Römheld 1999) and patch-clamp experiments on white lupin protoplasts (Zhang et al. 2004) support the hypothesis that anion channels are involved. Protons and other cations, such as potassium (K^+), sodium (Na^+), and magnesium (Mg^{2+}), appear to serve as counterions (Zhu et al. 2005b; Tomasi et al. 2009). Citrate exudation occurs primarily in mature cluster root zones and is associated with a simultaneous acidification of the rhizosphere to pH 4–5 (Fig. 13.3) (Dinkelaker et al. 1989). Tomasi et al. (2009) demonstrated a link between organic acid and proton exudations. Stimulation of the plasma membrane H^+-ATPase increased citrate exudation, while inhibition reduced it. Bursts of citrate exudation were associated with increased expression and activity of *LHA1* PM H^+-ATPase, further supporting a connection between citrate and proton extrusion (Tomasi et al. 2009).

Cluster roots also release large amounts of flavonoids, mainly genistein and hydroxygenistein derivates (Weisskopf et al. 2006b). Excretion of flavonoids is increased in $-P_i$ cluster roots, with highest excretion in juvenile and immature cluster root zones (Weisskopf et al. 2006b). Tomasi et al. (2008) showed that flavonoids can release P_i complexed with Fe, indicating a role of flavonoids in P_i mobilization (Tomasi et al. 2008). In addition, a role of flavonoids in repelling microbial citrate consumers in the rhizosphere has been suggested (Weisskopf et al. 2006a).

Mature cluster roots also excrete large amounts of acid phosphatase (APase, EC 3.1.3.2), which cleaves P_i from organic complexes (Gilbert et al. 1999; Wasaki et al. 2003; Tang et al. 2013). Tang et al. (2013) compared intra- and extracellular APase activity during cluster root development. Activity and gene expression of both

intracellular APase (LaSAP1) and extracellular APases (LaSAP2) increased under P_i deficiency. While intracellular APase activity remained relatively constant during cluster root development, activity of extracellular APase increased with cluster root maturation and was highest at the mature and senescent stage (Tang et al. 2013).

P_i concentrations within plant cells are typically 1000 times higher than those outside, and thus roots need to take up P_i against a steep concentration gradient. It is believed that high-affinity transporters mediate P_i uptake by cotransport, powered by a plasma membrane H^+-ATPase (Raghothama and Karthikeyan 2005). White lupin cluster roots show high expression of the high-affinity P_i transporter transcripts *LaPT1* and *LaPT2* (Liu et al. 2001). While *LaPT2* shows fairly similar expression independent of P_i status, *LaPT1* is highly induced by P_i deficiency in both normal and cluster roots.

Considering that diffusion of P_i in soil, rather than the maximum P_i inflow rate, is the limiting factor of P_i uptake from soil (Raghothama and Karthikeyan 2005), increased expression of high-affinity P_i transporters may only have a slight effect on P_i uptake (Lambers et al. 2006). But the highly synchronized morphological and biochemical changes in cluster roots act together, resulting in P_i mobilization and uptake within cluster root zones that is much greater than that of normal roots.

13.3 Balance of Carbon and P_i Budgets

13.3.1 Responses to P_i and N Deficiency Come with a Cost

Success under nutrient deficiency depends in part on a plant's ability to balance carbohydrate allocations and costs of competing responses to obtain resources. In response to low P_i and N availability, white lupin forms specialized root structures, cluster roots under P_i deficiency, and N_2-fixing nodules under N deficiency (Schulze et al. 2006; Thuynsma et al. 2014). In addition to the increased cost of root respiration and growth, large amounts of carboxylates are needed for both cluster root and nodule function. Cluster roots excrete vast amounts of organic acids into the rhizosphere, and nodules must supply the N_2-fixing bacteroids with sufficient carboxylates for the energy-demanding process of N_2 fixation. As more carbon (C) is diverted to the roots, less is available for the growth of photosynthetic tissues.

The effect of P_i supply on resource allocation (C, N, and P_i) in white lupin indicates a trade-off in resource allocation between cluster roots and nodules (Thuynsma et al. 2014). Under low P_i, a higher investment in cluster roots benefits the P_i nutrition of nodules, which require P_i to perform N_2 fixation. This investment may explain why white lupin's ability to fix N_2 appears less prone to inhibition by P_i deficiency, compared to other legumes (Schulze et al. 2006). When P_i is supplied in sufficient amounts, C partitioning shifts from cluster root toward nodule production (Thuynsma et al. 2014).

An understanding of costs, resource allocations, and trade-offs of nutrient stress responses is needed to develop crops better adapted to the various stresses of poor soils that become increasingly common on our planet.

13.3.2 Respiratory Costs Increase Under P_i Deficiency

C costs in response to P_i and N deficiency can be divided into costs for root growth, carboxylate needs, and respiration. Root respiration typically accounts for 10–30% of net photosynthesis (Lambers et al. 1996) but can increase under low P_i (Lynch and Beebe 1995) and low N availability (Van der Werf et al. 1992). While root respiratory costs increase, less C is allocated toward photosynthetic tissue; in addition, photosynthesis is impaired by severe P_i and N deficiency (Plaxton and Tran 2011; Hermans et al. 2006; Campbell and Sage 2006).

To support the increased respiration, more carbohydrates are needed. However, ATP and ADP levels decline dramatically under P_i starvation (Plaxton and Podestá 2006). Under severe P_i deficiency, plants switch to alternative pathways of glycolysis and mitochondrial electron transport to allow respiration to proceed (Plaxton and Tran 2011; Florez-Sarasa et al. 2014). Such alternative reactions bypass either the generation or the use of ATP. Florez-Sarasa et al. (2014) compared in vivo respiratory activities of the regular cytochrome oxidase pathway with the alternative oxidase pathway in cluster roots and normal roots of white lupin. The alternative oxidase pathway bypasses several proton-pumping steps and thus, generates less ATP while passing electrons down the electron transport chain. The study showed that once cluster rootlets cease to grow and ATP demand declines, activity of the cytochrome oxidase pathway decreases. In contrast, alternative oxidase activity remains high, to continue oxidation of NADH produced during citrate synthesis (Florez-Sarasa et al. 2014). While bypassing the generation of ATP is energetically wasteful, it can keep metabolism flowing under P_i deficiency.

13.3.3 Cluster Root and Nodule Function Require Additional Carboxylates

The amounts of citrate and malate released by cluster roots of white lupin in response to low P_i can range from 10% to more than 25% of plant dry weight (Johnson et al. 1994, 1996). The exudation of such large amounts of organic acids would be a waste of C under P_i-sufficient conditions. Thus under sufficient P_i, normal root growth is favored over cluster root formation (Thuynsma et al. 2014).

Organic acid synthesis in roots requires a combination of glycolysis, tricarboxylic acid cycle (TCA), and dark fixation of CO_2 by phosphoenolpyruvate carboxylase (PEPC) (Massonneau et al. 2001; Johnson et al. 1996). Dark fixation of CO_2 is a glycolytic bypass reaction that does not produce ATP and thus can continue under P_i deficiency when ADP and P_i are limited. Moreover, dark fixation can recover some of the CO_2 lost by respiration. PEPC catalyzes the carboxylation of PEP to form oxaloacetate, which is then reduced to malate. Malate can either be used directly, e.g., for excretion from cluster roots, or funneled toward the TCA (Plaxton and Tran 2011). Johnson et al. (1996) calculated that dark C fixation in roots of P_i-deficient white lupin via PEPC accounts for 25–34% of the C exuded as citrate and malate.

Nodules also use dark fixation to address their C needs, both for feeding N_2-fixing bacteroids and as skeletons for amino acid synthesis (Le Roux et al. 2009; Vance et al. 1983; Christeller et al. 1977). It has been estimated that dark fixation of CO_2 in root nodules provides 25–30% of the C required for N_2 fixation (Maxwell et al. 1984; Vance et al. 1983; Christeller et al. 1977). Increased dark CO_2 fixation in nodules correlates with increased transcript abundance and enzyme activities of nodule-specific PEPC and malate dehydrogenase in white lupin (Rosendahl et al. 1990; Schulze et al. 2002, 2006). Taken together, these results indicate that PEPC and malate dehydrogenase play a key role in dark fixation of C in both cluster roots and root nodules.

As organic acid synthesis increases under P_i deficiency, transcript abundance of enzymes involved in glycolysis and glycolytic bypass reactions are upregulated in $-P_i$ cluster roots (Fig. 13.4) (Uhde-Stone et al. 2003; Penaloza et al. 2002; O'Rourke et al. 2013). Some of the phosphorylation steps of glycolysis can be bypassed under P_i starvation (Plaxton and Tran 2011). Glycolytic bypass enzymes that use pyrophosphate (PP_i), rather than ATP, scavenge this intracellular P_i source that is otherwise unused (Plaxton 1996; Theodorou et al. 1992; Plaxton and Tran 2011). Several (PP_i)-dependent glycolytic bypass enzymes and an inorganic pyrophosphate H^+-pump (H^+-PP_iase) have been reported in plants (Plaxton and Podestá 2006). Many transcripts in formate and tetrahydrofolate (THF) metabolism are more abundant in $-P_i$ cluster roots, compared to $+P_i$ roots of white lupin (O'Rourke et al. 2013). Reconstruction of metabolic pathways based upon expression profiles suggests an additional pathway of CO_2 fixation in P_i-deficient cluster roots via the one-C metabolism (Fig. 13.4) (O'Rourke et al. 2013).

In summary, white lupin displays high metabolic plasticity to meet the increased C demands of both cluster roots and nodules. Metabolic bypass reactions, dark fixation of CO_2, and possibly one-C metabolism enable continuation of C metabolism when P_i is limited and funnel additional C toward organic acid synthesis.

13.3.4 White Lupin Produces Cluster Roots with Little Change in Biomass Allocation

The rate and plasticity of root growth are important factors for effective uptake of nutrients (Lynch 2011; Lynch and Ho 2005). White lupin responds to P_i and Fe deficiency by the formation of cluster roots (Gardner et al. 1982; Marschner 1995; Neumann and Martinoia 2002; Hagström et al. 2001), which can make up more than 50% of the total root mass (Keerthisinghe et al. 1998). While P_i deficiency in the shoot increases cluster root formation, P_i-rich as well as dry soil patches can induce local cluster root formation (Shu et al. 2007; Felderer et al. 2015), demonstrating a high plasticity of white lupin's root architecture. N availability also influences cluster root formation, depending on the type and amount of N. Cluster root formation has been shown to be stimulated by NH^+ application (Sas et al. 2002) but inhibited by high levels of N (Dinkelaker et al. 1995).

In response to low P_i, plants typically allocate a greater proportion of biomass to the root system (Lynch and Ho 2005; Lynch and Brown 2001). However, in a long-term experiment in soil, Shen et al. (2003) showed that formation of cluster roots

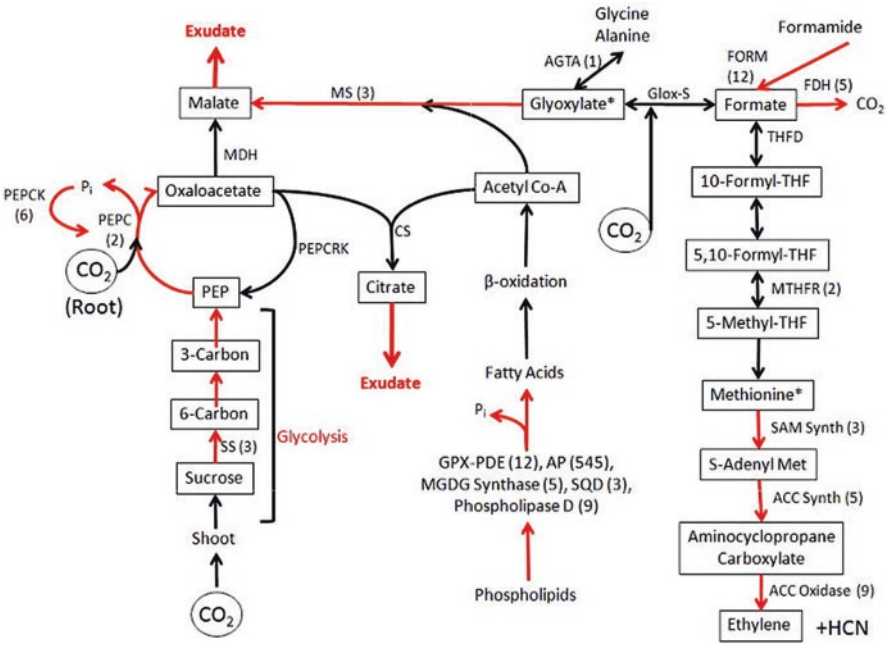

Fig. 13.4 Modifications in white lupin cluster root metabolism as evidenced by transcript expression (Figure adapted from O'Rourke et al. 2013) (Copyright American Society of Plant Biologists). Increased expression in $-P_i$ cluster roots determined by RNA-seq and confirmed by qPCR (indicated as fold change in parentheses). *Red arrows* represent genes shown to be upregulated under P_i deficiency. Increased sucrose metabolism via glycolysis and organic acid production provide C for malate and citrate exudation into the rhizosphere. Organic acids lost through exudation are replenished through anaplerotic pathways involving phosphoenolpyruvate carboxylase (PEPC) and a glyoxylate-like cycle malate synthase (MS). One-C metabolism is enhanced through a formate and tetrahydrofolate (THF) pathway. The THF pathway contributes to methionine and ethylene production. Increased expression of transcripts involved in phospholipid degradation release P_i for recycling and C for acetyl-CoA synthesis. Acetyl-CoA and glyoxylate provide C for malate synthesis through MS. Formate may be carboxylated to glyoxylate by a putative glyoxylate synthase (Glox-S). Additional abbreviations: *SS* sucrose synthase, *PEPCK* phosphoenolpyruvate carboxylase kinase, *PEPCRK* phosphoenolpyruvate carboxylase carboxykinase, *MDH* malate dehydrogenase, *CS* citrate synthase, *FORM* formamidase, *FDH* formate dehydrogenase, *AGTA* alanine glyoxylate transaminase, *THFD* tetrahydrofolate deformylase, *MTHFR* methylene-THF reductase, *SAMSynth* s-adenosylmethionine synthase, *ACCSyn* aminocyclopropane synthase, *ACC* oxidase, *PLA1* phospholipase A, *GPX-PDE* glycerophosphodiester-phosphodiesterase, *AP* acid phosphatase, *MGDG synthase* monogalactosyldiacylglycerol synthase, *SQD* sulfoquinovosyltransferase

under limiting P_i occurred before any reduction of shoot and root dry weight was observed, indicating that formation of cluster roots does not drastically increase biomass allocation to the roots (Shen et al. 2003). Funayama-Noguchi (2015) compared root growth, biomass allocation, respiratory properties, and root construction cost in P_i-deficient white lupin and *L. angustifolius*, a noncluster root-forming species (Funayama-Noguchi et al. 2015). Under P_i deficiency, white lupin produced cluster roots with little change in biomass allocation, while *L. angustifolius* significantly increased biomass allocation to roots. However, the construction cost of cluster roots

in white lupin was higher than that of *L. angustifolius* roots. In conclusion, both lupins use contrasting strategies to increase P_i availability under P_i deficiency: *L. angustifolius* produces large quantities of low-cost roots, while white lupin produces cluster roots at high costs, but with little change in biomass allocation (Funayama-Noguchi et al. 2015).

13.3.5 External C, Longer Days, and Brighter Light Can Increase Cluster Root Formation

Wasaki et al. (2005) characterized the effect of atmospheric CO_2 concentrations on cluster root formation and organic acid exudation in white lupin, using rhizoboxes (Wasaki et al. 2005). The study revealed that development of cluster roots was accelerated under elevated atmospheric CO_2, both with and without P_i fertilizer application. Increased development of cluster roots under elevated atmospheric CO_2 concentrations was also reported for white lupin grown in hydroponic culture (Watt and Evans 1999; Campbell and Sage 2002). While elevated atmospheric CO_2 concentrations increase cluster root formation, root zone CO_2 concentration in typical soils appears sufficient for optimal dark fixation of CO_2 in cluster roots (Cramer et al. 2005).

Cluster root formation appears to be regulated by a negative feedback loop: cluster roots enhance internal P_i status which suppress further cluster root formation (Pearse et al. 2006a). Wang et al. (2015a) tested the importance of growth rate, because an increased growth rate could dilute internal P_i status and thus prevent the negative feedback. The study showed a strong negative correlation between cluster root formation and internal P_i status, but only a marginal positive correlation with the growth rate (Wang et al. 2015a). These results are consistent with other studies showing that internal P_i concentrations are a major factor in cluster root formation (Shen et al. 2003).

To evaluate interactions between C and P_i supply, Cheng et al. (2014) assessed the effects of light intensity on sucrose accumulation and cluster root formation and function in white lupin. Intense light resulted in higher biomass production and a larger root/shoot ratio, particularly under sufficient P_i. Both low P_i and intense light increased cluster root formation and citrate exudation. Transcripts of PEPC (*LaPEPC3*) in cluster roots increased with higher light intensity, independent of P_i status. These findings support the hypothesis that C in excess of shoot growth capabilities is translocated to the roots as sucrose, serving both as a long-distance signal and as C source for cluster root formation and carboxylate exudation (Cheng et al. 2014).

13.3.6 Trade-Off in Costs Between Cluster Roots and Nodules

White lupin forms effective N_2-fixing nodules with *Bradyrhizobium* sp. (Schulze et al. 2006). Under P_i deficiency, P_i is preferentially allocated to nodules for maintenance of N_2 fixation, sometimes at the expense of plant growth (Hogh-Jensen et al. 2002).

In addition, N_2-fixing bacteroids consume large amounts of C, mainly in form of malate, both for feeding bacteroids to maintain N_2 fixation and for the production of amino acids for export and use (Schulze et al. 2002, 2006).

Thuynsma et al. (2014) found that white lupins grown after short-time high P_i supply preferentially allocated C and P_i resources to nodules, at the expense of cluster roots. This change in C and P_i allocations was accompanied in metabolic changes in the activities of enzymes related to organic acid synthesis. Because cluster roots increase P_i acquisition under low P_i, investment in cluster roots may benefit the P_i nutrition of nodules.

Schulze et al. (2006) evaluated whether white lupin can continue nodulation and N_2 fixation under P_i deficiency. Plants were grown without N, inoculated with *B. lupini*, and subjected to sufficient or no P_i. Nodulation and N_2 fixation appeared extremely tolerant of insufficient P_i for an extended time. At 21 days after inoculation, N_2 fixation rates did not differ between $+P_i$ and $-P_i$ supply. Nodulated white lupin shoots did not show any symptoms of nutrient stress when grown without any N and P_i for up to 27 days. Seeds provided some internal P_i, but plant P_i concentrations eventually turned low after 21 days without P_i supply, while N concentration and total N remained unaffected. White lupin grown with or without P_i had P_i concentration in nodules that were greater than in other organs. Eventually, between 21 and 37 days, shoots of plants grown without P_i developed symptoms of N and P_i deficiency, and both P_i concentration and N_2 fixation decreased (Schulze et al. 2006).

Thuynsma et al. (2014) reported reduced nodule biomass in response to low P_i supply, and Schulze et al. (2006) reported significantly less nodule biomass in plants grown 37 days without P_i, compared to $+P_i$ grown plants. However, Schulze et al. found that nodule biomass was unaffected after 3 weeks without P_i, but nodule size decreased, indicating more, but smaller nodules. Interestingly, these smaller nodules were primarily found in cluster root zones. Schulze et al. (2006) speculate that the increased number of nodules in $-P_i$ may be a result of increased auxin concentrations in cluster root zones. Accordingly, external application of the auxin analogue naphthalene acetic acid (NAA) stimulated both cluster root formation and nodule development in cluster root zones (Schulze et al. 2006).

Taken together, these studies indicate that cluster roots play a vital role in white lupin's ability to perform N_2 fixation under low P_i, but investment shifts from cluster roots toward nodules as soon as sufficient P_i becomes available.

13.4 "Omics" Approaches Toward a Better Understanding of P_i Adaptation in White Lupin

A major goal for crop production is the development of lines that perform well in P_i-deficient soils. Identification of genes that are differentially expressed in response to P_i deficiency will be useful for understanding P_i deficiency responses and aid in marker-assisted selection for crop improvement. High-throughput approaches, such as transcriptomics and metabolomics, have identified many of the genes and metabolites involved in cluster root formation and function and are beginning to reveal

networks that regulate P_i acquisition and use. However, evidence for cross talk between nutrient deficiency responses is accumulating, making it necessary to dissect P_i starvation responses from general nutrient stress responses (Rai et al. 2015; Jost et al. 2015).

Proteomics, genomics, ionomics, and epigenomics may hold key to important aspects of the P_i deficiency response but are currently understudied in white lupin. Genome editing has the potential to revolutionize functional analysis of genes in white lupin, particularly if stable transformation of white lupin plants can be established.

13.4.1 Transcriptomics Reveal the Molecular Basis of Cluster Root Formation and Function

Next-generation sequencing of cDNA, referred to as RNA-seq, is a powerful tool for analyzing gene expression on a global scale and is rapidly replacing EST and array-based approaches. O'Rourke et al. (2013) performed RNA-seq on roots and leaves of white lupin grown with and without P_i. This approach resulted in 277 million Illumina reads from 12 cDNA libraries: 3 biological replicates each of $+P_i$ and $-P_i$ leaves and $+P_i$ and $-P_i$ roots, the latter consisting primarily of cluster roots. Because the genome of white lupin has not yet been sequenced, the transcripts had to be assembled de novo and were used to build a reference transcriptome, the *L. albus* gene index (Table 13.1) (LAGI 1.0; http://comparative-legumes.org). The study identified over 50,000 active (RPKM $\geq$ 3) transcripts, representing about 7.8% of the white lupin genome. Of the 50,000 active transcripts, 2128 were differentially expressed ($\geq$ twofold change; p-value $\leq$0.05) in response to P_i deficiency, 1342 in leaves, 903 in roots, and 117 in both tissues. Table 13.2 lists selected genes that are highly induced in $-P_i$, compared to $+P_i$ roots.

Wang et al. (2014) used RNA-seq to investigate the changes in gene expression during white lupin cluster root development (Fig. 13.3) (Neumann et al. 1999; Massonneau et al. 2001). RNA-seq was performed on three pooled cDNA libraries of preemergent (PE), juvenile (JU), and mature (MA) cluster roots, respectively,

Table 13.1 Characteristics of white lupin reference transcriptomes LAGI01 (*L. albus* gene index 01) (O'Rourke et al. 2013) and LAGI02 (Secco et al. 2014)

	LAGI01	LAGI02
Number of contigs	125,821	65,097
Number of active[a] contigs	50,734	32,204
Average contig length	1,155 bp	1,625
Longest contig	15,514	26,640
% GC	39.6	39.4
Total base pairs	145,286,000	105,789,289

[a]*RPKM* (reads per kilobase per million) above a threshold of 3 (O'Rourke et al. 2013) or 5 (Secco et al. 2014)

Table 13.2 RNA-seq expression profiles of selected differentially expressed genes, confirmed by qRT-PCR

	$-P_i/+P_i$ ratio				
	Roots		Leaves		
Identifier	RNA-seq	qRT-PCR	RNA-seq	qRT-PCR	Annotation
LAGI01_35427	451	546	1.5	25	Purple acid phosphate
LAGI01_58965	37	13	1	18	Peroxidase
LAGI01_85917	27	30	1	−7	Ferric reductase
LAGI01_48402	23	12	2	1	Formamidase
LAGI01_72004	22	5	1	3	Ferric reductase 3
LAGI01_21605	19	33	2	2	MATE[a]
LAGI01_46294	17	14	1	5	Low-phosphate root (LPR1)
LAGI01_74540	16	4	1	1	Nodulin
LAGI01_77756	15	3	1	1	Malate synthase
LAGI01_30950	12	16	1	7	Cytochrome P450
LAGI01_51470	12	11	1	28	bHLH[a] transcription factor
LAGI01_66840	9	1	1	6	Unknown
LAGI01_48446	9	12	6	1	GPX-PDE[a]
LAGI01_42932	8	8	2	4	Phosphatase
LAGI01_3168	7	9	14	1	Phospholipase D
LAGI01_46560	6	22	4	7	SPX3[a] (SPX domain 3)

Adapted from O'Rourke et al. (2013) (Copyright American Society of Plant Biologists)
Negative values indicate higher expression in $+P_i$ than $-P_i$; expression ratios of 1 indicate no difference in expression between $+P_i$ and $-P_i$. Gene names that have been abbreviated are denoted by an [a] and are defined below
[a]*MATE* multi drug and toxin extrusion, *bHLH* basic helix-loop-helix, *GPX-PDE* glycerophosphodiesterase, *SPX* SYG1/Pho8/XPR1

resulting in 147 Mio Illumina reads. Mapping against the white lupin reference transcriptome (LAGI 1.0) yielded 103,147 unique sequences. Ratios of normalized reads were calculated to identify gene expression that changes during cluster root development (Wang et al. 2014).

Secco et al. (2014) performed RNA-seq analyses to compare gene expression in $-P_i$ roots (root tips, immature and mature cluster roots, respectively) with $+P_i$ roots (root tips and mature roots, respectively). RNA-seq libraries of three independent biological replicates for each treatment were paired-end sequenced. Paired-end sequencing produces reads from both ends of a cDNA, which can be mapped to a reference transcriptome. One hundred and thirty-three Mio high-quality reads were used for de novo assembly of the root transcriptome and merged with LAGI01 to generate an improved reference transcriptome, designated LAGI02 (Table 13.1), with 65,097 functionally annotated contigs (Secco et al. 2014). Of these, 747 genes were differentially expressed by more than eightfold in the developmental stages of cluster roots, giving molecular insights into the regulatory processes involved in cluster root formation.

13.4.1.1 Differential Expression Reflects Cluster Root Functions

Differential gene expression identified by RNA-seq confirmed known cluster root functions (O'Rourke et al. 2013), primarily in the mature stage of cluster root development (Secco et al. 2014; Wang et al. 2014). As expected, genes involved in P_i uptake (high-affinity P_i transporters), P_i scavenging (purple acid phosphatases, RNases, phospholipid degradation), rhizosphere acidification (ATPases), flavonoid metabolism (phenylpropanoid pathway), and organic acid synthesis were upregulated in $-P_i$ cluster roots. Interestingly, genes involved in C1 and acetyl-CoA metabolisms were also upregulated in P_i-deficient cluster roots, possibly contributing to organic acid synthesis (Fig. 13.4).

Transcript expression of MATE (multidrug and toxic compound extrusion) and ALMT (aluminum-activated malate transporter) genes were upregulated in mature cluster roots, coinciding with the exudation of citrate and flavonoids. MATE transporters have been implicated in the transport of citrate and flavonoids (Furukawa et al. 2007; Rogers et al. 2009; Durrett et al. 2007; Zhao and Dixon 2010), while malate efflux seems to be mediated by ALMTs (Sasaki et al. 2004). Interestingly, transcripts for nitrate, ammonium, and sulfate transporters were also upregulated in P_i-deficient cluster roots, indicating cross talk between nutrient deficiency responses.

13.4.1.2 RNA-Seq Unravels the Regulatory Network of Cluster Root Development

P_i deficiency affected the expression level of several transcription factors and hormone-related genes (Secco et al. 2014; O'Rourke et al. 2013; Wang et al. 2014). O'Rourke et al. (2013) identified 155 transcription factors differentially expressed in response to P_i deficiency. Secco et al. (2014) showed that expression of several transcription factors, such as *PLT*, *SCR*, *PHB*, *PHV*, and *AUX/IAA*, with known roles in the control of meristem activity and development were induced in cluster root tips. Genes involved in hormonal responses (*PIN*, *LAX*, *YUC*) and cell cycle control (*CYCA/B*, *CDK*) were also differentially expressed (Secco et al. 2014). Several genes coding for SPX proteins were differentially expressed in response to P_i deficiency (O'Rourke et al. 2013). Proteins containing the SPX domain are emerging as important players in P_i homeostasis (Zhou et al. 2015; Secco et al. 2012).

Transcripts involved in gibberellic acid homeostasis, reception, and signaling were differentially expressed in $-P_i$ cluster roots, indicating a formerly unknown role of gibberellic acid in cluster root development (O'Rourke et al. 2013). Transcripts for gibberellic acid 2-oxidase, involved in degradation of gibberellic acid, were upregulated in $-P_i$ cluster roots. These results are consistent with Gou et al. (2010), whose studies in poplar suggest that gibberellic acid mediates the repression of lateral root density by negatively regulating lateral root primordia (Gou et al. 2010).

Transcripts related to auxin and cytokinin homeostasis were also differentially expressed in $-P_i$ cluster roots, supporting the importance of auxin and cytokinin levels in cluster root formation. Separating the different developmental stages revealed that transcripts related to auxin, nitric oxide, and brassinosteroid were most strongly

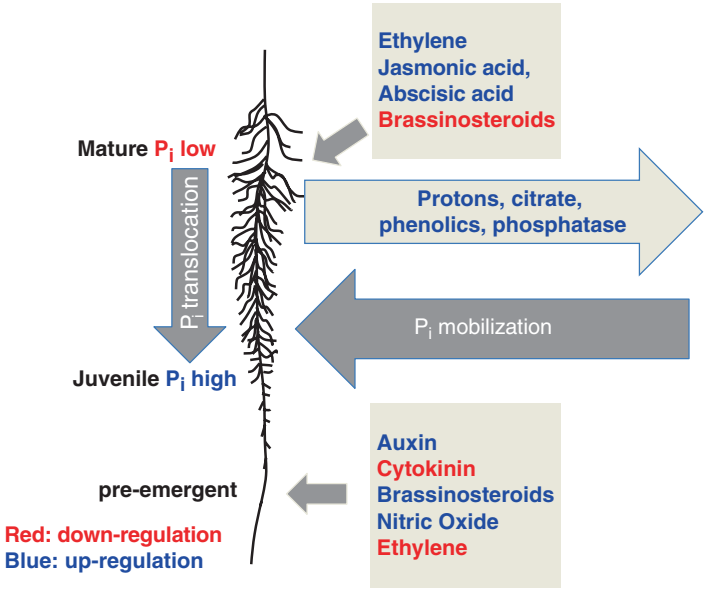

Fig. 13.5 Schematic representation of the role of selected phytohormones in the regulation of cluster root initiation and maturation (Modified from Wang et al. 2014). *Blue color* indicates upregulation, while *red color* indicates downregulation

expressed in preemerging cluster roots (Fig. 13.5), indicating their involvement in cluster root initiation (Wang et al. 2014). In contrast, transcripts related to abscisic acid, jasmonic acid, and ethylene were induced in mature clusters, indicating a role of these hormones in cluster root maturation (Fig. 13.5) (Wang et al. 2014). Ethylene has been shown to restrict primary root elongation and to promote lateral root formation (Nagarajan and Smith 2012). The upregulation of ethylene-related genes was accompanied by upregulation of an Fe deficiency regulated network, known to mediate ethylene-induced expression of Fe deficiency responses in other species (Wang et al. 2015b). O'Rourke et al. (2013) also reported induction of genes involved in ethylene production in $-P_i$ cluster roots, compared to $+P_i$ normal roots, confirming earlier findings that $-P_i$ lupin roots produce increased ethylene (Gilbert et al. 2000).

13.4.1.3 Expression Profiling Identifies microRNAs Associated with P_i Deficiency in White Lupin

microRNAs (miRNAs) have emerged as important players in regulating plant responses to P_i deficiency (Kuo and Chiou 2011). Mature miRNAs are short (about 21 nt) single-stranded noncoding RNAs, generated from a single-stranded RNA precursor with a hairpin secondary structure. miRNAs negatively regulate gene expression at the posttranscriptional level by base pairing to their target mRNAs, which in plants typically directs mRNA cleavage (Voinnet 2009).

Due to their small size, sequencing of mature miRNAs requires special library construction, and mature miRNAs are not included in the current RNA-seq approaches of white lupin. Secco et al. (2014) went around this problem by screening RNA-seq data for primary miRNAs transcripts (pri-miRNAs) (Secco et al. 2014). Most of the identified pri-miRNAs showed no differential expression in response to P_i deficiency, but miRNA156 showed an about two to threefold upregulation in P_i-deficient cluster roots.

O'Rourke et al. (2013) analyzed differentially expressed transcripts for complementary miRNA sequences (obtained from miRBase) to identify potentially miRNA targets, using the psRNATarget tool (http://plantgrn.noble.org/psRNATarget/). This approach identified 261 P_i-responsive transcripts potentially regulated by 127 miRNA families. Two of the 127 miRNAs identified in this analysis, miR399 and miR395, are well known to regulate P_i deficiency responses (Kuo and Chiou 2011).

Zhu et al. (2010) used microarray technology to identify miRNAs in white lupin that are differentially expressed in response to P_i deficiency. To this end, Zhu et al. used 771 publicly available mature plant miRNA sequences to design 40 nt probes which were then printed in array format. These microarrays were hybridized against white lupin RNA from $+P_i$ and $-P_i$ roots, stems, and leaves. The study revealed a set of about 30 miRNAs involved in white lupin's response to P_i deficiency and showed that miRNAs coordinately regulate a network of downstream genes. Interestingly, miR164 expression was reduced under P_i deficiency, while its target gene *NAC1* was upregulated. In *Arabidopsis*, NAC1 acts as a transcriptional activator transmitting auxin signals for lateral root development, suggesting that miR164 and NAC1 may play a role in auxin-mediated cluster root formation in white lupin (Zhu et al. 2010).

Some miRNAs are believed to be transported from shoot to root to signal the P_i status of the shoot to the root. Analysis of a cDNA library constructed from white lupin phloem exudate identified 12 small RNA clones (18–25 nt) with homology to miRNAs identified in other species, including miRNA 399 (Rodriguez-Medina et al. 2011). In *Arabidopsis*, miR399 was shown to be a long-distance signal from shoots to roots (Lin and Chiou 2008; Pant et al. 2008). P_i deprivation significantly increased miR399 levels in phloem exudate collected from fruits of white lupin, supporting a systemic signaling role of miR399 in the P_i starvation response in white lupin (Rodriguez-Medina et al. 2011).

13.4.2 Beyond Transcriptomics: White Lupin Proteomics, Metabolomics, and Ionomics Add to the Picture

13.4.2.1 Proteomics Are Only Beginning to Unravel the White Lupin Proteome

Transcriptomics have revealed thousands of white lupin genes differentially expressed in response to P_i deficiency. However, adaptation of plants to P_i deficiency likely involves posttranscriptional control of the proteome in form of post-translational modifications, allosteric regulation, and protein degradation (Alexova

and Millar 2013). While progress has been made in the proteomics of P_i starvation in several plant species, such as *Arabidopsis*, rice, and maize (Alexova and Millar 2013), the white lupin proteome in response to P_i deficiency has not been studied in greater detail. Tian et al. (2009) established a reference proteome of white lupin roots, and Rodriguez-Medina et al. (2011) identified proteins in phloem sap. Both studies used primarily 2D gel electrophoresis, followed by mass spectroscopy.

Tian et al. (2009) identified 170 proteins in developing white lupin roots. Proteins involved in primary and secondary metabolisms were abundant. Because membrane proteins are typically underrepresented in 2D gel analyses, Tian et al. (2009) separated microsomal membrane proteins by 1-D gel electrophoresis, and subjected these to LC-MS/MS (liquid chromatography–tandem mass spectrometry), putatively identifying a total of 74 proteins. ATPases, ABC transporters, aquaporins, and glycoproteins are potentially involved in cell signaling and recognition; ribosomal and heat shock proteins were abundant in the membrane fraction (Tian et al. 2009).

Rodriguez-Medina et al. (2011) analyzed white lupin phloem sap via 2D gel electrophoresis, followed by MS/MS (tandem mass spectrometry), and tentatively identified 86 proteins involved in metabolism (24%), protein modification/turnover (9%), redox regulation (8%), cell structural components (6%), stress and defense response (6%), and others. Particularly prominent proteins in the phloem sap were cyclophilin, ubiquitin, a glycine-rich RNA-binding protein, a group of proteins that comprise a glutathione/ascorbate-based mechanism to scavenge oxygen radicals, and enzymes involved in glycolysis, methionine, and ethylene synthesis (Rodriguez-Medina et al. 2011).

Taken together, these studies provide a useful reference for proteomic analysis in white lupin. However, more sensitive and high-throughput gel-free MS approaches, such as quantitative labeling with isobaric tags (iTRAQ), have the potential to substantially further our understanding of the white lupin proteome. Until recently, the lack of white lupin sequence data limited the ability to identify lupin proteins by MS (mass spectrometry), but the recently generated white lupin reference transcriptome (Secco et al. 2014; O'Rourke et al. 2013) provides a useful resource for the computational analysis of MS and MS/MS data in white lupin. Such proteomic analyses are needed to determine both differential expression and differential posttranslational modifications of proteins during white lupin's response to P_i deficiency.

13.4.2.2 Metabolomic Profiling Reveals Metabolic Changes During P_i Starvation

To better understand the metabolic consequences of P_i starvation, a number of studies have assessed metabolic changes in response to P_i deficiency, mainly focusing on root exudation (Dessureault-Rompré et al. 2007; Weisskopf et al. 2006b; Tomasi et al. 2009; Neumann and Römheld 1999), cluster root metabolites (Massonneau et al. 2001), and phloem sap (Rodriguez-Medina et al. 2011). More recently, Muller et al. (2015) used nontargeted metabolic profiling to gather insights into white lupin's physiological adaptations to P_i deficiency in shoots and roots (Fig. 13.6). In this study, white lupin plants were exposed to P_i starvation for 14 and 22 days,

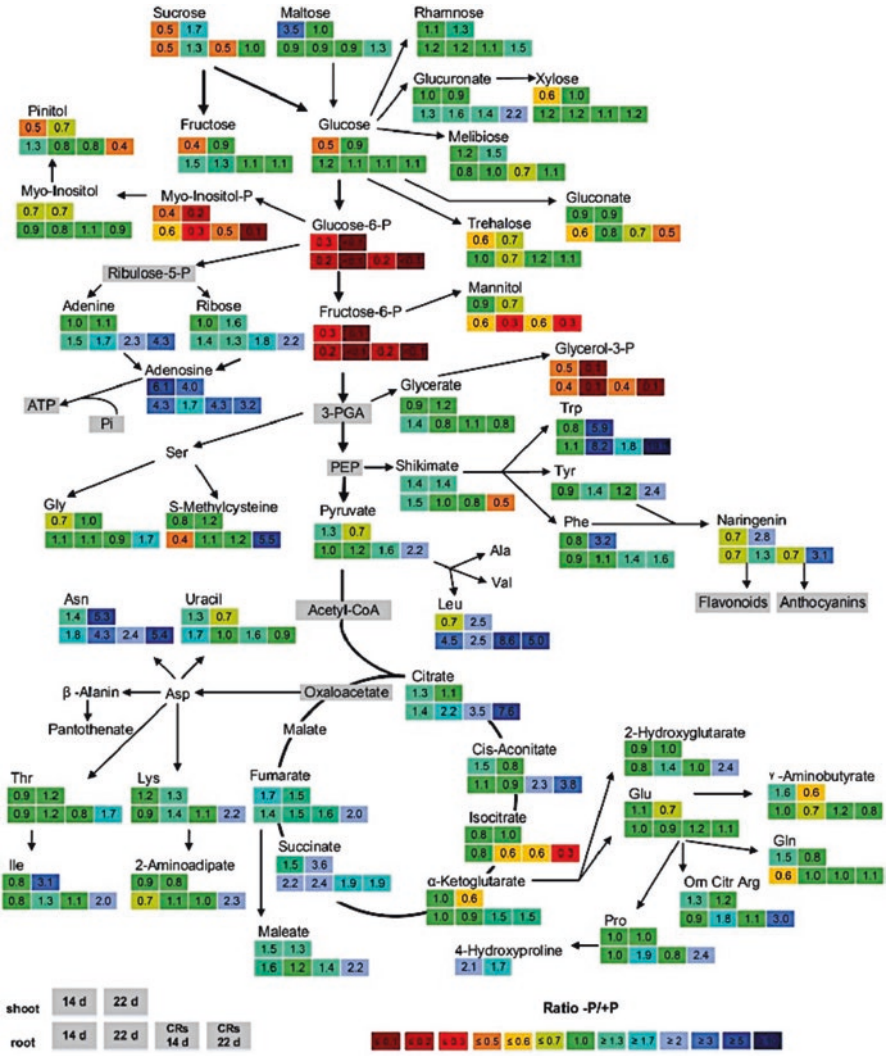

Fig. 13.6 Effect of P_i supply on the levels of metabolites from shoots and roots of 14- and 22-day-old white lupin plants (Adapted from Muller et al. 2015). Relative ratios ($-P_i/+P_i$), $n = 4$, are shown for metabolites with significant increases or decreases of at least 30% (1.3-fold/0.7-fold). Metabolites in *gray boxes* were not determined. The *upper two boxes* show the $-P_i/+P_i$ ratio of shoots after 14 days (*left box*) and 22 days (*right box*). The *lower four boxes* show the $-P_i/+P_i$ ratio of roots. From *left* to *right*: noncluster roots after 14 days, noncluster roots after 22 days, cluster roots after 14 days, cluster roots after 22 days

respectively. Metabolic changes in shoots, cluster roots, and noncluster roots during early and late P_i starvation responses were assessed by gas chromatography–mass spectrometry (GC-MS).

Muller et al. (2015) reported that organic acids and several shikimate pathway products showed increased levels in 22-day-old P_i-deficient roots and shoots, possibly

involved in carbohydrate partitioning toward the growing root system (Fig. 13.6). Ribose, adenine, and adenosine also accumulated in P_i-deficient lupin tissues, likely as a result of RNA and nucleotide degradation to scavenge internal P_i. Phosphorylated metabolites, such as glycerol-3-phosphate, were reduced in both shoots and roots, probably due to replacement of phospholipids with sulfo- and galactolipids (Plaxton and Tran 2011).

Half of the measured amino acids accumulated under P_i deficiency, possibly due to protein degradation and suppressed protein biosynthesis. An increase in certain amino acids, particularly asparagine, in $-P_i$ lupin roots has been previously reported (Johnson et al. 1996). Muller et al. found the rare aromatic amino acid tryptophan showed the strongest accumulation (Fig. 13.6). Such accumulation of tryptophan in roots has also been found in P_i-deficient *Arabidopsis* (Pant et al. 2015). Plants use tryptophan in the synthesis of auxin, which can induce cluster root formation (Wang et al. 2015b; Gilbert et al. 2000; Meng et al. 2013). The aromatic amino acids phenylalanine and tyrosine and the flavanone naringenin were also found to be increased in 22-day-old shoots and cluster roots of P_i-deficient white lupin (Fig. 13.6). These metabolites are part of the phenylpropanoid pathway by which flavonoids are synthesized. It is known that white lupin roots release flavonoids and isoflavonoids into the rhizosphere to mobilize soil P_i and to prevent microbial degradation of exuded organic acids (Tomasi et al. 2008; Weisskopf et al. 2006b). Taken together, metabolomic studies reveal the great impact of P_i deficiency on white lupin's metabolism, leading to a wide range of metabolic and physiological changes.

13.4.2.3 Ionomics Hold Potential to Reveal Networks of Mineral Nutrition

While metabolomics assess organic metabolites, ionomics quantify the mineral nutrient and trace element composition of organisms. Several high-throughput elemental profiling studies have analyzed how the plant ionome responds to environmental stimuli (Huang and Salt 2016). Baxter et al. (2008) discovered an ionomic regulatory network in *Arabidopsis* that is involved in both Fe and P_i homeostasis (Baxter et al. 2008). For example, ionomics revealed that a short root, a commonly used measure of the P_i deficiency response, may actually be caused by changes in Fe homeostasis (Ward et al. 2008; Ticconi et al. 2009). These findings highlight the importance of looking at the complete ionome, as opposed to only one element of interest (Ward et al. 2008; Baxter 2015). White lupin's release of citrate and proton excretion stabilizes heavy metals, such as As and Cd (Vázquez et al. 2006). White lupin is considered a promising tool not only for increasing soil nutrient availability but also phytoremediation (Cd, As, Pb, Th) and phytomining (La, Nd, Sc) (Wiche et al. 2015, 2016).

In recent years, ionomic studies have been performed in many plant species (Huang and Salt 2016), but currently, no high-throughput studies have been performed in white lupin. However, considering the advanced high-throughput, low-cost approaches available, such as ICP-MS (inductively coupled plasma mass spectrometry), ionomics hold potential to further our understanding of the ionomic network in white lupin and the genes involved in its regulation.

13.4.3 The White Lupin Genome Has Not Yet Been Sequenced

While next-generation sequencing has greatly advanced white lupin transcriptomics (Secco et al. 2014; Wang et al. 2014; O'Rourke et al. 2013), white lupin's genomics have received much less attention. At this point, genomes of ten legume species have been sequenced or have entered the process (O'Rourke et al. 2014), including *L. angustifolius* (Yang et al. 2013; Hane et al. 2016), but white lupin is not among them.

In absence of a white lupin genome sequence, genetic linkage maps provide important information for molecular breeding and comparative genomics. The first genetic linkage map of white lupin was published with about 300 genic (gene-based) and AFLP (amplified fragment length polymorphism) markers. Subsequently, high-resolution sequence-tagged site (STS) markers were developed and mapped (Croxford et al. 2008). Recently, diversity array technology markers, combined with AFLPs, were used to create a high-density genetic linkage map of white lupin, consisting of 38 linkage groups and spanning a length of 2169 cM.

Comparative genomic studies indicate conservation among legume genomes, giving hope that genomic information from model legumes, such as *Medicago truncatula*, can be applied to other legumes (Li et al. 2012; Zhu et al. 2005a; Cannon et al. 2009; Choi et al. 2004). However, Phan et al. (2007) found only 46% of markers showing synteny between *M. truncatula* and white lupin. This limited synteny is an indication that genomic information gathered on *M. truncatula* may not easily be transferred to white lupin (Phan et al. 2007).

Lupins are considered to be polyploid in origin, and white lupin has a large genome with a diploid chromosome number of 50 and a 2C DNA content of 1.16 pg (Naganowska et al. 2005). In fact, all cultivated *Lupinus* species have high chromosome numbers (Fig. 13.1) (Gustafsson and Gadd 1965; Ainouche et al. 2004). However, such ancient polyploidy may not greatly complicate functional analysis of white lupin genes, as mutations tend to segregate monofactorially, indicating a functionally diploid structure (Gustafsson and Gadd 1965).

13.4.3.1 Epigenomics May Enhance Our Understanding of White Lupin's P_i Starvation Response

In addition to genomic analysis, the importance of epigenomics in plant responses to abiotic stress is becoming apparent. Histone modifications and DNA methylation can be correlated with gene expression in response to abiotic stresses, such as water deficit, high salinity, and temperature shifts (Kim et al. 2015; Bobadilla and Berr 2016). For example, expression of a glycerophodiesterase (*GPXPD*) gene in tobacco in response to aluminum, salt, and cold stress is associated with demethylation in the coding region of the *GPXPD* gene (Choi and Sano 2007). It can be assumed that epigenetic changes are equally important in the response to P_i deficiency. White lupin epigenomics hold potential to further our understanding of white lupin's P_i starvation response.

13.4.3.2 Phenomics Could Bridge the Gap Between Genomics and Agricultural Traits

Variations for complex phenotypic traits are frequently controlled by many genetic loci, referred to as quantitative trait loci (QTLs), scattered throughout the genome. Phan et al. (2007) identified two QTLs for anthracnose resistance, and two QTLs for flowering time, as well as a Mendelian trait for alkaloid content in white lupin. Croxford et al. (2008) mapped QTLs for flowering time, alkaloid synthesis, and stem height.

QTLs for traits related to P_i deficiency tolerance have been identified in other species (Vance 2010; Reymond et al. 2006). P_i-responsive QTLs include the PUP1 QTL in rice; PSTOL1, a protein kinase designated as phosphate starvation tolerance 1 (Chin et al. 2011); a monogalactosyl diacylglycerol (MGDG) synthase; a glucose-6-phosphate transporter (G-6-PT) in *Brassica napus* (Shi et al. 2013); and low phosphate roots (LPR1 and LPR2), identified as multicopper oxidases in *Arabidopsis* (Ticconi et al. 2009; Reymond et al. 2006; Ward et al. 2008). Lupin genomics could facilitate the identification of P_i-responsive QTLs and give rise to genome-wide association studies (GWAS) in white lupin.

Phenomics hold potential to bridge genomics and phenotypical traits. A large number of white lupin accessions are stored and maintained in seed banks, displaying a relatively high level of genetic variations, as assessed by ISSR (inter simple sequence repeat) and AFLP (amplified fragment length polymorphism) markers (El-Sherif et al. 2016; Sbabou et al. 2010a). Such large germplasm collections can provide the basis for phenomic screens to decide on the best combinations for breeding programs. For example, in the early 1920, plant breeders realized the need of an easy detection for alkaloid content to screen large numbers of lupins. After adapting an appropriate test, Sengbusch screened millions of seeds and leaves for low alkaloid content, identifying several low alkaloid mutants of cultivated lupin species (Sengbusch 1942). Today, new technologies, such as noninvasive 3D imaging of root systems, promise high-throughput screening of germplasm collections for valuable traits (Furbank and Tester 2011; Gregory et al. 2009).

13.4.4 Reverse Genetic Tools Are Needed for Functional Analysis of White Lupin Genes

13.4.4.1 RNA Interference (RNA_i) Has Proven Feasible for the Knockdown of White Lupin Genes

Reverse genetic tools are needed to determine the function of genes identified by transcriptomics and other "omics" approaches in white lupin's P_i starvation response. RNA_i has been employed to silence white lupin genes of interest, such as LaMATE (*L. albus* multidrug and toxin extrusion) (Uhde-Stone et al. 2005), scarecrow transcription factor LaSCR1 (Sbabou et al. 2010b), and GPX-PDE

(glycerophosphodiester phosphodiesterases) (Cheng et al. 2011). LaMATE was found to be highly expressed in mature P_i-deficient cluster roots; thus, a potential role in citrate excretion was proposed. However, phenotypical analysis of LaMATE RNA_i mutants did not confirm a role of LaMATE in organic acid excretion (Uhde-Stone et al. 2005), suggesting other roles of LaMATE, such as loading of citrate into the xylem, or flavonoid transport. Suppression of the scarecrow transcription factor LaSCR1 in transformed roots of lupin resulted in decreased root numbers, indicating a potential role of LaSCR1 in maintaining root growth (Sbabou et al. 2010b). Knockdown of glycerophosphodiester phosphodiesterase (GPX-PDE) through RNA_i resulted in impaired root hair development and density, indicating a role of GPX-PDE in root hair development in P_i-starved white lupin (Cheng et al. 2011).

RNA_i in these studies was delivered by *Agrobacterium rhizogenes*-based root transformation. This transformation generates composite plants with transformed roots but wild-type shoots (Uhde-Stone et al. 2005). Currently, no protocol exists for stable transformation in lupin. Recently, Yamagishi et al. (2015) successfully employed peanut stunt virus (PSV) for transient transformation of whole lupin plants. In a proof of concept study, white lupin was inoculated with PSV harboring an RNA_i construct against the *LaPDS* gene (*L. albus* phytoene desaturase). The inoculated plants developed the expected phenotype of photo-bleaching. Moreover, PSV spread systemically in white lupin plants, and small interfering RNA of *LaPDS* was detected in leaves, roots, and cluster roots (Yamagishi et al. 2015).

13.4.4.2 Genome Editing Is a Promising Tool for the Functional Analysis of White Lupin Genes

While RNA_i "knocks down" gene expression, genome editing is a promising approach to completely "knock out" genes of interest. CRISPR/Cas, a genome editing tool that requires only a single RNA to target a specific DNA sequence, has been used to generate mutations in a growing number of plant species (Schiml and Puchta 2016; Liu et al. 2016; Puchta 2016; Tang et al. 2016; Barakate and Stephens 2016). A common strategy of genome editing is to induce targeted cuts in a DNA sequence of interest, which is then repaired by the cell via an inaccurate repair mechanism (nonhomologous end joining), typically introducing small indels at the target site. More controlled changes can be introduced by providing DNA for homology-directed repair (HDR). Variations of genome editing, such as multiplexing, controlled genomic deletions, and epigenetic modifications, are emerging rapidly (Puchta 2016; Schiml and Puchta 2016). CRISPR-Cas should be a useful addition in the molecular toolbox for functional analysis of genes involved in white lupin's response to P_i deficiency, especially if stable transformation of white lupin can be established.

13.5 Concluding Remarks

The legume white lupin serves as an illuminating model to investigate plant adaptations to P_i starvation. White lupin responds to P_i deficiency by formation of cluster roots. Highly synchronized morphological and biochemical changes within cluster root zones result in chemical mobilization of sparingly available P_i sources in soils.

High-throughput molecular techniques, particularly RNA-seq, rapidly increase our understanding of white lupin cluster root formation and function. Other areas of great promise, such as genomics, proteomics, ionomics, and epigenomics, are not yet fully utilized. In addition, more robust reverse genetic approaches need to be developed for the functional analysis of P_i deficiency-induced genes and the dissection of P_i-signaling pathways. Insights gathered from white lupin's response to P_i deficiency may help to identify new targets for plant breeding and engineering of crop plants that are better able to utilize poorly available soil P_i.

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