Ashok Shrawat · Adel Zayed
David A. Lightfoot *Editors*

Engineering Nitrogen Utilization in Crop Plants

Springer

Engineering Nitrogen Utilization in Crop Plants

Ashok Shrawat · Adel Zayed
David A. Lightfoot
Editors

Engineering Nitrogen Utilization in Crop Plants

Springer

Editors
Ashok Shrawat
Biotechnology
Monsanto Company
Chesterfield, MO
USA

Adel Zayed
Biotechnology
Monsanto Company
Chesterfield, MO
USA

David A. Lightfoot
Department of Plant, Soil and Agricultural Systems
Southern Illinois University
Carbondale, IL
USA

ISBN 978-3-319-92957-6 ISBN 978-3-319-92958-3 (eBook)
https://doi.org/10.1007/978-3-319-92958-3

Library of Congress Control Number: 2018942929

Printed on acid-free paper

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This book is dedicated to the speakers of our "Engineering Nitrogen Use Efficiency Workshop," which has been held every year since 2003 at the Plant and Animal Genomics Conference, San Diego, USA. This tome is planned to be the first of many volumes on this topic because NUE Workshop speakers excel every year.

Please contact us with presentations for future Workshops.

Thanks to all.
Ashok Shrawat, David A. Lightfoot,
and Adel Zayed

Preface

This book celebrates 14 years of the "Nitrogen Use Efficiency Engineering" workshop at the Plant and Animal Genome Conference every January in San Diego, California. Each chapter was volunteered by a presenter.

Nitrogen is important. It an essential component in cellular physiology being scarce in bio-available forms. In contrast, oxygen, carbon, and hydrogen are much more bio-available. Nitrogen is present in numerous essential compounds including nucleoside phosphates and amino acids that form the building blocks of nucleic acids and proteins, respectively. In plants, nitrogen is used in large amounts in photosynthetic pigments, defense chemicals, and structural compounds. However, inorganic N is difficult to assimilate. Dinitrogen in the atmosphere is highly inert. Reduction to ammonium requires the energy of a lightning bolt, petrochemicals, or 12 ATP dephosphorylations per molecule within a nodule or other anaerobic environment. Global warming may increase the frequency of lightning storms and hence raise NO concentrations. Warmer, more stressed crops will require more nitrogen fertilizers to be applied and heavy rains will increase losses due to runoff.

Warmer days will cause more of the applied ammonium fertilizers to escape from the cell as ammonia gas. Photorespiration, increased by heat stress, releases tenfold more ammonium than is assimilated from the environment, and plants only re-assimilate $\sim$98% of this. Consequently, a haze of ammonia gas is found floating above a photosynthetic canopy. That ammonia may be lost on the wind or returned to the plant or soil by rains or dew falls. Any improvements to these nitrogen cycles can have a massive positive impact on the efficiency of agriculture, reduce its carbon footprint, and over geological timescales reverse some of the anthropogenic contributors to global warming.

The assimilation of ammonium has a second major problem associated with it. Ammonia is assimilated releasing one acidic proton per molecule. There is enough flux to reduce the pH of even well-buffered soils to concentrations that inhibit plant growth, both directly, and by the release of toxic concentrations of micronutrients (Al and Mn in particular). Reduction within a nodule or other anaerobic environment compounds this problem by releasing two protons per ammonium produced. Soil acidification is a worldwide problem on a massive scale. Nitrates and nitrites

provide a solution to the acidification problem, as their reduction to ammonium absorbs 3–4 protons. So a pH-balanced fertilizer should theoretically be a four to one mixture of ammonium and nitrates. Nitrates and nitrites are the ions produced by those lightning bolts that provide about 10% of the world's reduced nitrogen each year. However, they are not without costs and problems. Nitrite is highly toxic to photosynthesis and respiration and so must be immediately reduced to ammonium. Plants produce massive amounts of nitrite reductase for this purpose. Nitrate is benign, easy to store and transport and consequently is the major form of inorganic N found in plants. However, plants still produce tenfold more nitrate reductase than is absolutely needed for assimilation, growth, and yield. Why? This remains unclear.

The major problem with nitrates and nitrates in the environment is that they are water soluble and so are rapidly leached from soils. So much is lost from agricultural soils, industrial activity, and human waste treatments that the world's rivers, lakes, and oceans are significantly fertilized. Algae are the microorganisms that benefit the most from this fertilizer. Unfortunately, they run low on other nutrients (P,K) and so produce toxins to kill other organisms to obtain the limiting nutrients through their decomposition. In addition, they absorb much of the waters' oxygen at night killing even toxin resistant aerobes. Finally they bloom, blocking the light needed for photosynthesis by submerged organisms. Millions of acres of oceans are affected.

The major problem with nitrates in the human diet is that they are metabolized to a potent carcinogen (nitrosamine) in the acid of the human stomach problems. High nitrate and so nitrosamine amounts in human diets are associated with many different cancers as well as fertility problems. However, nitrates are naturally excreted in human and animal saliva for the purpose of producing some nitrosamines in the gut. This is because the combination of acid and nitrosamine effectively kills many human and animal pathogens. *Helicobacter pylori* is one example. This microbe causes stomach ulcers that left untreated often become cancerous. *H. pylori* is endemic and became more abundant as lifestyles became more stressful. Consequently, several epidemiological studies found diets high in nitrate to be healthy in the 1990s and beyond, whereas before that they were significantly unhealthy. Clearly, then the healthiest option is a low nitrate diet and low stress lifestyle. *H. phylori* and like pathogens and the lesions they cause are better treated with drugs than nitrosamines.

Microbes in the soil take up the bulk of all applied fertilizers before the plant can. Ammonium can be assimilated or oxidized to nitrite, nitrate, nitrous oxide, or dinitrogen by microbial activities. Plants have to absorb N from microbes by force, using highly efficient enzymes, or by trade through symbiosis. During symbiosis, the microbes are provided with sugars in return for ammonium. The microbes may be free-living in the rhizosphere or housed in specialized structures such as nodules. Symbiotic microbes produce a variety of chemical signals to elicit the delivery of sugars from the plants, and these systems are ripe for manipulation by biotechnology approaches.

Because soil particles do not naturally have many N-containing minerals, and because N can be readily lost from the rooting environment, N is the nutrient element that most often limits plant growth and agricultural yields. As noted above, nitrogen is found in the environment in many forms and comprises about 80% of the Earth's atmosphere as triple-bonded nitrogen gas (N_2). However, this large fraction of N is not directly accessible by plants and must be bonded to one or more of three other essential nutrient elements including oxygen and/or hydrogen through N-fixation processes, and carbon through N-assimilation processes. Plants are able to absorb a little NH_3 from the atmosphere through stomata in leaves, but this is dependent upon air concentrations. The ions NO_3^- and NH_4^+ are the primary forms for uptake in by plants. The most abundant form that is available to the plant roots is NO_3^-, and the most abundant form in leaves is NH_4^+. The process of nitrification by soil bacteria readily converts fertilizer NH_4^+ to NO_3^-. Relative nitrogen uptake is also dependent on soil conditions. Ammonium uptake is favored by a neutral pH and NO_3^- uptake is favored by low pH. Nitrate also does not bind to the negatively charged soil particles; therefore, it is more freely available to plant roots, especially through mass flow of soil water than is NH_4^+, which binds to negatively charged soil particles and so moves primarily by diffusion. The assimilation of NH_4^+ by roots causes the rhizosphere to become acidic, while NO_3^- causes the rhizosphere to become more basic.

In conclusion, the assimilation of inorganic nitrogen is a key process in the productivity of all crop plants, and there are many steps at which metabolic improvements can be made. In future, the ability to provide active nodules to non-legumes may provide a new impetus for agriculture, biotechnology, and crop science. Making crop N in foods and feeds will be critical advances. Reducing N loss to air and water will be critical. Therefore, we editors and workshop organizers thank the contributors for their work, often a lifetime avocation that began as a vocation!

Chesterfield, USA Ashok Shrawat
Chesterfield, USA Adel Zayed
Carbondale, USA David A. Lightfoot

Contents

Chapter 1
Developing Crop Varieties with Improved Nutrient-Use Efficiency

David A. Lightfoot

Environmental Issues

Nitrogen is an essential component in cellular physiology. Only oxygen, carbon, and hydrogen are more abundant (Marchner 1995; Andrews et al. 2004). Nitrogen is present in numerous essential compounds, including pigments, nucleoside phosphates, and amino acids, that underlie photosynthesis, nucleic acids, and proteins. In plants, nitrogen is used in the largest amounts in photosynthesis, pigments, defense chemicals, and structural compounds. However, inorganic N is difficult to assimilate. Dinitrogen in the atmosphere is highly inert. Reduction to ammonium requires the energy of a lightning bolt, petrochemicals, or 12 ATP dephosphorylations per molecule within a nodule or other anaerobic environment (Kaiser et al. 1998; Reid et al. 2011). Global warming may increase the frequency of lightning storms and hence raise NO concentrations. Warmer, more stressed crops will require more nitrogen fertilizers be applied and heavy rains will increase losses due to runoff.

Warmer days will cause more of the applied ammonium fertilizers to escape from the cell as ammonia gas (Lightfoot et al. 1999; 2001; 2007; 2008; 2009; 2010). Photorespiration, increased by heat stress, releases tenfold more ammonium than is assimilated from the environment, and plants only re-assimilate a portion of this. Consequently, a haze of ammonia gas is found floating above a photosynthetic canopy. That ammonia may be lost on the wind or returned to the plant or soil by rains or dew falls. Any improvements to these nitrogen cycles (Carvalho et al. 2011; Tercé-Laforgue et al. 2004a, b) can have a massive positive impact on the efficiency of agriculture, reduce its carbon footprint, and over geological timescales, reverse some of the anthropogenic contributors to global warming.

D. A. Lightfoot (✉)
Department of Plant, Soil and Agricultural Systems: Genomics and Biotechnology, Center for Excellence, The Illinois Soybean Center: Southern Illinois University, Carbondale, IL 62901, USA
e-mail: ga4082@siu.edu

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_1

The assimilation of ammonium has a second major problem associated with it Jahns et al. 1999; 2000; Koivunen et al. 2004a, b). Ammonia is assimilated releasing one acidic proton per molecule (Marchner 1995). There is enough flux to reduce the pH of even well-buffered soils to concentrations that inhibit plant growth, both directly and by the release of toxic concentrations of micronutrients (Al and Mn in particular). Reduction within a nodule or other anaerobic environment compounds this problem by releasing two protons per ammonium produced (Indrasumunar et al. 2011). Soil acidification is a worldwide problem on a massive scale.

Nitrates and nitrites provide a solution to the acidification problem, as their reduction to ammonium absorbs 3–4 protons (Marchner 1995). So a pH-balanced fertilizer should theoretically be a four-to-one mixture of ammonium and nitrates. Nitrates and nitrites are the ions produced by those lightning bolts that provide about 10% of the worlds reduced nitrogen each year. However, they are not without costs and problems. Nitrite is highly toxic to photosynthesis and respiration, and so must be immediately reduced to ammonium. Plants produce massive amounts of nitrite reductase for this purpose. Nitrate is benign, easy to store and transport, and consequently is the major form of inorganic N found in plants. However, plants still produce tenfold more nitrate reductase than is absolutely needed for assimilation, growth, and yield (Wang et al. 2000; Kleinhofs et al. 1980). Why this is, remains unclear.

The major problem with nitrates and nitrates in the environment is that they are water soluble and so are rapidly leached from soils (Moll et al. 1982; Lee and Nielsen 1987; David et al. 1997). So much is lost from agricultural soils, industrial activity and human waste treatments that the world's rivers, lakes, and oceans are significantly fertilized (Cherfas 1990; Burkholder et al. 1992). Algae are the micro-organisms that benefit the most from this fertilizer. Unfortunately, they run low on other nutrients (P, K) and so produce toxins to kill other organisms to obtain the limiting nutrients through their decomposition. In addition, they absorb much of the waters oxygen at night killing even toxin-resistant aerobes. Finally they bloom, blocking the light needed for photosynthesis by submerged organisms. Billions of acres of oceans are affected worldwide.

The major problem with nitrates in the human diet and saliva cycle is that they are metabolized to a potent carcinogen (nitrosamine) in the acid of the human stomach (Moller et al. 1990; Mirvish 1985; Duncan et al. 1998; Tannenbaum et al. 1978). Antioxidants in popcorn and tea can help reduce them. High nitrate and so nitrosamine amounts in human diets are associated with many different cancers as well as fertility problems. However, nitrates have uses; they are naturally excreted in human and animal saliva for the expressed purpose of producing some nitrosamines in the gut. This is because the combination of acid and nitrosamine effectively kills many human and animal pathogens. *Helicobacter pylori* is one example. This microbe causes stomach ulcers that left untreated often become cancerous. *H. pylori* is endemic and became more abundant as lifestyles became more stressful. Consequently, several epidemiological studies found diets high in nitrate to be healthy in the 1990s, and beyond, whereas before that, they were significantly unhealthy. Clearly, then the healthiest option is a low-nitrate diet and

low stress lifestyle. *H. pylori* and like pathogens and the lesions they cause are better treated with drugs than nitrosamines.

Microbes in the soil take up the bulk of all applied fertilizers before the plant can (Trenkel 1997; Cabello et al. 2004; Garcia-Teijeiro et al. 2009). Ammonium can be assimilated, directly by GS, or oxidized to nitrite, nitrate, nitrous oxide, or dinitrogen by microbial activities. Plants have to absorb N from microbes by force or trade, using highly efficient enzymes in force, and by trade through symbiosis [reviewed by (Indrasumunar et al. 2012)]. During symbiosis (endo- or ecto-), the microbes are provided with sugars in return for ammonium (Zhao et al. 2005). The microbes may be free-living in the rhizosphere (ecto-) or housed in specialized structures such as nodules (endo-). Symbiotic microbes produce a variety of chemical signals to elicit the delivery of sugars from the plants, and these systems are ripe for manipulation by biotechnology approaches. The humate industry appears to be manipulating the ecto-systems (Pracharoenwattana et al. 2010; Ohno et al. 2010; Lehmann and Kleber 2015; Taha and Osman 2017). The oleaginous carbon seems to be assimilated by microbes in return for nitrogen released to the plant

Plant Assimilations

Because soil particles do not naturally have many N containing minerals, and because N can be readily lost from the rooting environment, N is the nutrient element that most often limits plant growth and agricultural yields (Specht et al. 1999; Duvick 2005; Krouk et al. 2010). As noted above, nitrogen is found in the environment in many forms and comprises about 80% of the earth's atmosphere as triple bonded nitrogen gas (N_2). However, this large fraction of N is not directly accessible by plants and must be bonded to one or more of three other essential nutrient elements including oxygen and/or hydrogen through N-fixation processes, and carbon through N-assimilation processes (Marchner 1995). Plants are able to absorb a little NH_3 from the atmosphere through stomata in leaves, but this is dependent upon air concentrations. The ions NO_3^- and NH_4^+ are the primary forms for uptake in by plants. The most abundant form that is available to the plant roots is NO_3^- and the most abundant form in leaves is NH_4^+. The process of nitrification by soil bacteria readily converts fertilizer NH_4^+ to NO_3^- (Trenkel 1997). Relative nitrogen uptake is also dependent on soil conditions. Ammonium uptake is favored by a neutral pH and NO_3^- uptake is favored by low pH. Nitrate also does not bind to the negatively charged soil particles; therefore, it is more freely available to plant roots, especially through mass flow of soil water than is NH_4^+, which binds to negatively charged soil particles and so moves primarily by diffusion. As noted above, the assimilation of NH_4^+ by roots causes the rhizosphere to become acidic, while NO_3^- causes the rhizosphere to become more basic.

Nitrogen uptake and assimilation summates a series of vital processes controlling plants' growth and development (Lam et al. 2003). Nitrate, nitrite, and ammonium uptakes (and re-uptakes following losses) occur against massive

concentration gradients that require lots of energy to generate and maintain. In agriculture, plants are spaced sufficiently that they have an excess of captured light energy relative to the N and C supplies. Transgenic plants over-expressing low affinity nitrate uptake transporter Nrt1 increased the constitutive but not the induced nitrate uptake. Equally, plants transgenic with Nrt2.1 the high affinity nitrate transporter increased nitrate influx under low N conditions (Fraisier et al. 2000).

Transgenic plants expressing an ammonium transporter increased NUE (Gupta et al. 2008, 2011). Glutamate receptors in transgenic plants provided better growth. Equally, the uptake of short peptides had positive effects. All these transport-associated phenotypes would be desirable in agricultural production systems directed toward greater efficiency and lower environmental impacts, and a stack of the three transgenes would be of interest.

Nitrate acquired in the roots can be reduced in the shoot or the root, or even stored in vacuoles in the root or shoot for later assimilation. However, nitrate must be reduced to a useable form. This occurs via a two-step process catalyzed by the enzymes nitrate reductase (NR) and nitrite reductase to form NH_4^+. Both enzymes are produced in massive excess compared to the flux needed through the pathway, and mutants that reduce their amounts by 90% do not have phenotypes (Kleinhofs et al. 1980). Equally, some transgenic plants over-expressing NR increased nitrate reduction but were not altered in phenotype (Crete et al. 1997; Curtis et al. 1999; Djannane et al. 2002; Lea et al. 2004). However, two studies of NR over-expressing transgenic plants did record altered phenotypes including increased biomass, reduced drought stress (Ferrario-Méry et al. 1998, 2002), and improved NUE and yield during N limitation (Loussaert et al. 2008). These phenotypes would be desirable in agricultural crops. The coupling of NR to photosynthesis should be possible by transformation of plants with a ferredoxin-dependent NR from cyanobacteria.

The ability to fix dinitrogen is restricted to the bacterial world, but is widespread among microbes (Ferguson and Indrasumunar 2011). Many different *nif* gene families exist, suggesting selection for variation has been favorable for species. The activity of *nif* requires an anaerobic environment, so transferring the enzymes to plants will be difficult. To date, transgenics in this field are bacterial, as in hydrogenase enhanced microbes, or if plant, they are designed to improve the chances of nodule occupancy by improved bacterial strains. Strains that are most likely to set up nodule occupancy are rarely the most efficient nitrogen fixers. Plants also often fail to maintain effective nodules through flowering and pod set. Soybean and common bean for example have senescent nodules by flowering (Sinclair et al. 2007). Some species do have indeterminate nodules and it would be a valid goal of biotechnology to transfer this trait to major legume crops.

The N acquired as NH_4^+ does not require reduction upon uptake into the root, thus providing some energy savings to the plant over that of the NO_3^- form (Marchner 1995). However, it does require assimilation to avoid loss, and at high concentrations (>10 mM) toxicity to the plant occurs (Meyer et al. 1997; 2006; Godon et al. 1996). This can occur if a double application is made by inaccurate

GIS systems. Various studies have shown that under conditions of excessive NH_4^+ uptake, most plant species will transport this N source to the shoot, which is more sensitive to ammonium ions.

One important process to build key macromolecules in any living organism is the acquisition and utilization of inorganic forms of nitrogen during metabolism (Lea and Miflin 2011). Plants use amino acids as well as their precursors and catabolic products for important metabolic activities. Various other roles of amino acids include nitrogen storage and transport and the production of a very large number of secondary compounds including structural lignin compounds, light-absorbing pigments, phenolics and plant hormones. Plants convert the available inorganic nitrogen into organic compounds through the process of ammonium assimilation which occurs in plants by two main pathways. The first and primary pathway involves a reaction with glutamate to form glutamine which is catalyzed by glutamine synthetase (GS, EC 6.3.1.2) and requires an energy source of adenosine triphosphate (ATP). There are several isoenzymes of GS based on their location in the plant (Ortega et al. 2006). In the cytosol, GS1 is composed of 3–4 different subunits. There is only one isoform in the root plastids or shoot chloroplasts (GS2) with one subunit. Expressed in germinating seeds or in the vascular bundles of roots and shoots, the cytosolic form (GS1) produces glutamine for intracellular nitrogen transport. GS2 located in root plastids produces amide nitrogen for local consumption, while GS2 in the shoot chloroplasts re-assimilates photorespiratory ammonium (Lam et al. 2003). GS1 is encoded by a set of 3–6 paralogs in different crop species, so hetero-hexamers can form. However, the Kms hardly differ. Amino acid identity between GS1 isoforms is very high and is even similar to GS2. GS2 has a short peptide extension at the C-terminus that might be involved in regulation by phosphorylation. Alleles of the GS1- and GS2-encoding genes do exist that differ in their regulation. Alleles of GS appear to underlie QTL determining NUE and yield (Cañas et al. 2009, 2010). Transgenic analyses have been made of GS2 but not GS1. Among 12 studies in 9 plant species, the phenotypes reported included enhanced accumulation of N, growth under N starvation, herbicide (PPT) tolerance, leaf-soluble protein, ammonia, amino acids, and chlorophyll. Some genes and constructs decreased growth; salt, cold and drought tolerance; seed yield and amino acid content (Cai et al. 2009). Therefore, the use of GS transgenics in agriculture will be useful and desirable but only with careful attention to regulation and expression (Hemon et al. 1990; Coque et al. 2008 Seebaueret al. 2004; 2010).

The glutamine molecules produced by GS are used by a whole series of transaminases to produce the 20 protein amino acids and some nonprotein amino acids. Cardinal among the transaminases is the reaction catalyzed by glutamate synthase (GOGAT, EC 1.4.1.14 and 1.4.7.1) to form glutamate (Forde and Lea 2007). There are two common isoenzymes of GOGAT including a ferredoxin-dependent GOGAT (Fdx-GOGAT) and an NADH-dependent GOGAT (NADH–GOGAT). While both forms are plastidic, the Fdx-GOGAT enzyme is predominately found in photosynthetic organs and the NADH–GOGAT enzyme is found more in non-photosynthetic tissues such as in roots and the vascular bundles of developing

leaves (Lea and Miflin 2011). An NADPH-dependent GOGAT can be found in certain organs and in many bacteria. Plants transgenic with the NADH-dependent plant GOGAT have been reported. Phenotypes included enhanced grain filling, grain weight, total C and N content, and dry weight. Phenotypes were very similar to the benefits reported from alanine dehydrogenase and asparagine synthase (Good et al. 2007; Shrawat et al. 2008), suggesting the transaminases are acting on a common pathway.

The second pathway for ammonium assimilation also results in the formation of glutamate through a reversible reaction catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.2), with a lower energy requirement than GS/GOGAT. There are also at least two forms of GDH that occur in plants that include an NADH-dependent form found in mitochondria, and an NADPH-dependent form localized in the chloroplasts of photosynthetic organs. In addition, there are enzymes capable of aminating reactions that resemble GDH. GDHs present in plants serves as a link between carbon and nitrogen metabolism due to the ability to assimilate ammonium into glutamate or deaminate glutamate into 2-oxoglutarate and ammonium. However, due to the reversibility of this reaction, the assimilatory role of GDH is severely inhibited at low concentrations of ammonium. Additionally, GDH enzymes have a low affinity for ammonium compared with GS which further limits their assimilatory effectiveness. It has been suggested that the NAD-requiring form of GDH may be involved in carbon rather than nitrogen metabolism (Coruzzi and Bush 2001) with glutamate catabolism providing carbon skeletons for both the TCA cycle and the energy production during carbon or energy deficit. Alternate functions for GDH have also been proposed in which it has been assigned the role of re-assimilating excess ammonium, due to the limited ability of the GS/GOGAT cycle, during specific developmental stages (Limami et al. 1999; Loulakakis et al. 2002), such as during germination, seed set, and leaf senescence (Coruzzi and Zhou 2001. In contrast to plant GDHs, those found in microbes are very active in the assimilation of ammonium. Plants did not have the opportunity to incorporate this type of NADPH-dependent GDH because the bacterial lines that gave rise to chloroplasts do not contain *gdh*A genes. The few cyanobacteria with GDH activity have acquired genes by transgenesis or cellular fusions. Transgenic plants in six crop species have been produced that express *gdhA* genes from 3 microbes (Ameziane et al. 2000; Mungur et al. 2005; 2006; Abiko et al. 2010). Phenotypes in plants include increased biomass, water deficit tolerance, nutritional value, herbicide resistance, N assimilation, NUE, WUE, amino acid, and sugar content. GDH genes used in this way are being evaluated for commercialization (Nolte et al. 2004; 2009; 2016). One problem faced by this and the alanine dehydrogenase transgenics is a dependence on soil type for some of the beneficial effects. GDH seems to provide a growth advantage on silty–loam clay soils common in the southern Midwest of the USA. In contrast, the alanine dehydrogenase transgenics seem to work best on sandy soils. Combining the technologies or altering their regulation might provide stable beneficial effects in many soil types and locations.

A variety of other enzymes exist that are capable of aminating reactions. Each will be a candidate for over-expression in transgenic plants (Nelson et al. 2007; Castiglioni et al. 2008; Centtury et al. 2008; Goldman et al. 2009; Vidal et al. 2010). Phenylalanine ammonia lyase has been used in many transgenic plants. Equally, the enzymes of cyanide assimilations (cysteine metabolism) might be more active than previously thought and could be manipulated. Alteration of the enzymes of heme and chlorophyll biosynthesis might be tried again. The *E. coli hemA* gene was functional *but hemB* became insoluble in plant chloroplasts (unpublished).

In conclusion, the assimilation of inorganic nitrogen is a key process in the productivity of crop plants, and there are many steps at which metabolic improvements can be made (Pathak et al. 2008; Pennisi 2008). In future, the ability to provide active nodules or at least nitrogenases to non-legumes will provide an impetus for biotechnology (Harrigan et al. 2010; Valentine et al. 2011; Rubio et al. 2012; Wang et al. 2012). Nutritional value will be another breakthrough. Simple yield at the grain elevator worked well for 50 years, but now yield of milk and meat from feed has become a major new initiative, reducing waste as feces in the process. In addition, combining new breeding methods, new assays, genome editing, existing transgenes, and new promoters for their regulation will provide for new avenues in crop NUE improvement.

References

Abiko T, Wakayama M, Kawakami A, Obara M, Kisaka H, Miwa T, Aoki N, Ohsugi R (2010) Changes in nitrogen assimilation, metabolism, and growth in transgenic rice plants expressing a fungal NADP(H)-dependent glutamate dehydrogenase (*gdhA*). Planta 232(2):299–311

Ameziane R, Bernhard K, Lightfoot DA (2000) Expression of the bacterial *gdhA* encoding glutamate dehydrogenase in tobacco affects plant growth and development. Plant Soil 221:47–57

Andrews M, Lea PJ, Raven JA, Lindsey K (2004) Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Ann Appl Biol 145:25–35

Burkholder JM, Noga EJ, Hobbs CH, Glasgow HB Jr (1992) New 'phantom' dinoflagellate is the causative agent of major estuarine fish kills. Nature 358:407–410

Cabello P, Roldán MD, Moreno-Vivián C (2004) Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150:3527–3546

Cai H, Zhou Y, Xiao J, Li X, Zhang Q, Lian X (2009) Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. Plant Cell Rep 28(3): 527–537

Cañas RA, Quilleré I, Christ A, Hirel B (2009) Nitrogen metabolism in the developing ear of maize (Zea mays): analysis of two lines contrasting in their mode of nitrogen management. New Phytol 184(2):340–352

Cañas RA, Quilleré I, Lea PJ, Hirel B (2010) Analysis of amino acid metabolism in the ear of maize mutants deficient in two cytosolic glutamine synthetase isoenzymes highlights the importance of asparagine for nitrogen translocation within sink organs. Plant Biotechnol J 8:966–978

Carvalho Jde F, Madgwick PJ, Powers SJ, Keys AJ, Lea PJ, Parry MA (2011) An engineered pathway for glyoxylate metabolism in tobacco plants aimed to avoid the release of ammonia in photorespiration. BMC Biotechnol 11:111–121

Castiglioni P et al (2008) Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol 147:446–455

Century K, Reuber TL, Ratcliffe OJ (2008) Regulating the regulators: the future prospects for transcription-factor-based agricultural biotechnology products. Plant Physiol 147(1): 20–29

Cherfas J (1990) The fringe of the ocean—under siege from land. Science 248:163–165

Coruzzi G, Bush DR (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiol 125:61–64

Coque M, Martin A, Veyrieras JB, Hirel B, Gallais A (2008) Genetic variation for N-remobilization and post-silking N-uptake in a set of maize recombinant inbred lines. 3. QTL detection and coincidences. Theor Appl Genet 117(5): 729–747

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants, emerging 'matrix effects'. Curr Opp Plant Biol 4:247–253

Crete P, Caboche M, Meyer C (1997) Nitrite reductase expression is regulated at the post - transcriptional level by the nitrogen source in Nicotiana plumbaginifolia and Arabidopsis thaliana. Plant J 11:625–634

Curtis IS, Power JB, de Llat AAM (1999) Expression of chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. Plant Cell Rep 18:889–896

David MB, Gentry LE, Kovacic DA, Smith KM (1997) Nitrogen balance in and export from an agricultural watershed. J Env Qual 26:1038–1048

Djannane S, Chauvin JE, Meyer C (2002) Glasshouse behavior of eight transgenic potato clones with a modified nitratereductase expression under two fertilization regimes. J Exp Bot 53:1037–1045

Duncan D, Dykhuisen R, Frazer A, MacKenzie H, Golden M, Benjamin N, Leifert C (1998) Human health effects of nitrate. Gut 42:334–340

Duvick DN (2005) The contribution of breeding to yield advances in maize (*Zea mays* L.). Adv Agron 86:83–145

Ferrario-Méry S, Valadier MH, Foyer C (1998) Over expression of nitrate reductase in tobacco delays drought—induced decreases in nitrate reductase activity and mRNA. Plant Physiol 117:293–302

Ferrario-Méry S, Valadier MH, Valadier MH, Godefroy N, Miallier D, Hirel B, Foyer CH, Suzuki A (2002) Diurnal changes in ammonia assimilation in transformed tobacco plants expressing ferredoxin—dependent glutamate synthase mRNA in the antisense orientation. Plant Sci 163:59–67

Ferguson BJ, Indrasumunar A (2011) Soybean nodulation and nitrogen fixation, Chapter 4. In: Hendricks BP (ed) Agricultural research updates, vol 1, ©2011 Nova Science Publishers, Inc. ISBN: 978-1-61324-292-6

Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation, and signaling. J Exp Bot 58(9):2339–2358

Fraisier V, Gojon A, Tillard P, Daniel-Vedele F (2000) Constitutive expression of a putative high affi nity nitrate transporter in Nicotiana plumbaginifolia: evidence for post transcriptional regulation by a reduced nitrogen source. Plant J 23:489–496

Garcia-Teijeiro R, Lightfoot DA, Hernandez JD (2009) Effect of a modified urea fertilizer on soil microbial populations around maize (Zea mays L.) roots. Commun Soil Sci Plant Anal 40:2152–2168

Godon C, Krapp A, Leydecker MT, Daniel-Vedele F, Caboche M (1996) Methylammonium-resistant mutants of *Nicotiana plumbaginifolia* are affected in nitrate transport. Mol Gen Genet 250(3):357–366

Goldman BS, Darveaux B, Cleveland J, Abad MS, Sayed M (2009) Genes and uses for plant improvement. United States Patent Application 2009/0070897

Good AG, Johnson SJ, DePauw MD, Carroll RT, Savidov N, Vidamir J, Lu Z, Taylor G, Stroeher V (2007) Engineering nitrogen use efficiency with alanine aminotransferase. Can J Bot 85:252–262

Gupta, Liu J, Dhugga KS, Simmons CR (2008) Manipulation of ammonium transporters (AMTs) to improve nitrogen use efficiency in higher plants. US Patent Application #12/045,098

Gupta, Liu J, Dhugga KS, Simmons CR (2011) Manipulation of ammonium transporters (AMTs) to improve nitrogen use efficiency in higher plants. US Patent Application 13/043,109

Harrigan GG, Ridley WP, Miller KD, Sorbet R, Riordan SG, Nemeth MA, Reeves W, Pester TA (2010) The forage and grain of MON 87460, a drought-tolerant corn hybrid, are compositionally equivalent to that of conventional corn. J Agric Food Chem 57:9754–9763

Hemon P, Robbins MP, Cullimore JV (1990) Targeting of glutamine synthetase to the mitochondria of transgenic tobacco. Plant Mol Biol 15:895–904

Indrasumunar A, Searle I, Lin M-H, Kereszt A, Men A, Carroll BJ, Gresshoff PM (2011) Nodulation factor receptor kinase 1a controls nodule organ number in soybean (*Glycine max* L. Merr). The Plant J. 59:61–68

Indrasumunar A, Kereszt A, Gresshoff PM (2012) Soybean nodulation factor receptor proteins, encoding nucleic acids and uses therefor. US Patent Application 2011/0231952

Jahns T, Kaltwasser H (2000) Mechanism of microbial degradation of slow release fertilizers. J Polym Environ 8:11–16

Jahns T, Schepp R, Siersdorfer C, Kaltwasser H (1999) Biodegradation of slow-release fertilizers (methyleneureas) in soil. J Environ Polym Degrad 7:75–82

Kaiser BN, Finnegan PM, Tyerman SD, Whitehead LF, Bergersen FJ, Day DA, Udvardi MK (1998) Characterization of an ammonium transport protein from the peribacteroid membrane of soybean nodules. Science 281:1202–1206

Kleinhofs A, Kuo T, Warner RL (1980) Characterization of nitrate reductase-deficient barley mutants. Mol Gen Genet 177:421–425

Koivunen M, Horwarth WR (2004a) Effect of management history and temperature on the mineralization of methylene urea in soil. Nutr Cycl Agro Ecosyst 68(1):25–35

Koivunen M, Morisseau C, Horwarth WR, Hammock BD (2004b) Isolation of a strain of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) utilizing methylene urea (ureaformaldehyde) as nitrogen source. Can J Microbiol 50(3):167–174

Krouk G, Crawford NM, Coruzzi GM, Tsay YF (2010) Nitrate signaling: adaptation to fluctuating environments. Curr Opin Plant Biol 13:266–273

Lam HM, Wong P, Chan HK, Yam KM, Chow CM, Corruzi GM (2003) Overexpression of the ASN1 gene enhances nitrogen status in seeds of Arabidopsis. Plant Physiol 132:926–935

Lea US, ten Hoopen F, Provan F, Kaiser WM, Meyer C, Lillo C (2004) Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. Plant J 35: 566–573

Lea PJ, Miflin BJ (2011) Nitrogen assimilation and its relevance to crop improvement, chapter 1. In: Foyer C, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era. Annual plant reviews 42. Wiley Blackwell, New Delhi, India

Lehmann J, Kleber M (2015) The contentious nature of soil organic matter. Nature 528. https://doi.org/10.1038/nature16069

Lee LK, Nielsen EG (1987) The extent and costs of groundwater contamination by agriculture. J Soil Water Conserv 42:243–248

Lightfoot DA, Long LM, Vidal ME (1999) Plants containing the gdhA gene and methods of use thereof. US Patent #5,998,700

Lightfoot DA, Long LM, Vidal ME (2001) Plants containing the *gdhA* gene and methods of use thereof. US Patent #6,329,573

Lightfoot DA, Bernhardt K, Mungur R, Nolte S, Ameziane R, Colter A, Jones K, Iqbal MJ, Varsa EC, Young BG (2007) Improved drought tolerance of transgenic *Zea mays* plants that express the glutamate dehydrogenase gene (*gdhA*) of *E. coli*. Euphytica 156:106–115

Lightfoot DA (2008) Blue revolution brings risks and rewards. Science 321:771–772

Lightfoot DA (2009) Genes for use in improving nitrate use efficiency in crops, chapter 7. In Wood AJ , Jenks MA (eds) Genes for plant abiotic stress. Wiley Blackwell, New York, pp 167–182

Lightfoot DA and Fakhoury A (2010). Methods of using plants containing the *gdhA* gene: Aflatoxin reduction and fungal rot resistance. Patent Pending 2012/708,174

Limami A, Phillipson B, Ameziane R, Pernollet N, Jiang Q, Roy R, Deleens E, Chaumont-Bonnet M, Gresshoff PM, Hirel B (1999) Does root glutamine synthetase control plant biomass production in lotus japonicus L.? Planta 209(4):495–502

Loulakakis KA, Primikirios NI, Nikolantonakis MA, Roubelakis-Angelakis KA (2002) Immunocharacterization of Vitis vinifera L. ferredoxin-dependent glutamate synthase, and its spatial and temporal changes during leaf development. Planta 215:630–638

Loussaert DF, O'Neill D, Simmons CR, Wang H (2008) Nitrate reductases from red algae, compositions and methods of use thereof. United States Patent Application 20080313775

Marchner H (1995) Mineral nutrition of higher plants. Academic Press, London, UK

Meyer R (1997) A genetic analysis of response to ammonium and methylammonium in Arabidopsis thaliana. MS thesis, SIUC, Carbondale IL, pp 118

Meyer R, Yuan Z, Afzal J, Iqbal J, Zhu M, Garvey G, Lightfoot David A (2006) Identification of *Gsr1*: a locus inferred to regulate gene expression in response to exogenous glutamine. Euphytica 151:291–302

Mirvish SS (1985) Gastric cancer and salivary nitrate and nitrite. Nature 315:461–462

Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency to nitrogen utilization. Agron J 74:562–564

Moller H, Landt J, Pederson E, Jensen P, Autrup H, Jensen OM (1990) Endogenous nitrosation in relation to nitrate exposure from drinking water and diet in a Danish rural population. Cancer Res 49:3117–3121

Mungur R, Glass ADM, Goodenow DB, Lightfoot DA (2005) Metabolic fingerprinting in transgenic Nicotiana tabacum altered by the Escherichia coli glutamate dehydrogenase gene. J Biomed Biotech 2:198–214

Mungur R, Wood AJ, Lightfoot DA (2006) Water potential during water deficit in transgenic plants: Nicotiana tabacum altered by the Escherichia coli glutamate dehydrogenase gene. Plant Growth Regul 50:231–238

Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG et al (2007) Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci USA 104:16450–16455

Nolte SA, Young BG, Mungur R, Lightfoot DA (2004) The glutamate dehydrogenase gene gdhA increased the resistance of tobacco to glufosinate. Weed Res 44:335–339

Nolte SA (2009) Metabolic analysis of resistance to glufosinate in gdhA transgenic tobacco. PhD dissertation, PLB, SIUC, pp 244

Nolte SA, Young BG, Tolley L, Lightfoot DA (2016) Metabolic fingerprint of the resistance to glufosinate in tobacco transgenic with the glutamate dehydrogenase gene gdhA. Crop Sci 57:1–15

Ohno T, He ZQ, Sleighter RL, Honeycutt CW, Hatcher PG (2010) Ultrahigh resolution mass spectrometry and indicator species analysis to identify marker components of soil and plant biomass-derived organic matter fractions. Environ Sci Technol 44:8594–8600

Ortega JL, Moguel-Esponda S, Potenza C, Conklin CF, Quintana A, Sengupta-Gopalan C (2006) The 3'untranslated region of a soybean cytosolic glutamine synthetase (GS) affects transcript stability and protein accumulation in transgenic alfalfa. Plant J. 45:832–846

Pathak RR, Ahmad A, Lochab S, Raghuram N (2008) Molecular physiology of plant nitrogen use efficiency and biotechnological options for its enhancement. Curr Sci 94:1394–1403

Pennisi E (2008) The blue revolution, drop by drop, gene by gene. Science 320:171–173

Pracharoenwattana I, Zhou W, Keech O, Francisco PB, Udomchalothorn T, Tschoep H, Stitt M, Gibon Y, Smith SM (2010) Arabidopsis has a cytosolic fumarase required for the massive allocation of photosynthate into fumaric acid and for rapid plant growth on high nitrogen. Plant J. 62(5):785–795

Reid DE, Ferguson BJ, Hayashi S, Lin Y-H, Gresshoff PM (2011) Molecular mechanisms controlling legume autoregulation of nodulation. Annal Bot 108:789–795

Rubio-Wilhelmi Mdel M, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM (2012) Ammonium formation and assimilation in P(SARK)::IPT tobacco transgenic plants under low N. J Plant Physiol 169(2):157–162

Seebauer J, Moose SP, Fabbri B, Crossland L, Below FE (2004) Amino acid metabolism in young maize earshoots: implications for assimilate movement and nitrogen signaling. Plant Physiol 136:4326–4334

Seebauer JR, Singletary GW, Krumpelman PM, Ruffo ML, Below FE (2010) Relationship of source and sink in determining kernel composition of maize. J Exp Bot 61:511–519

Shrawat Ashok K, Carroll Rebecka T, DePauw Mary, Taylor Gregory J, Good Allen G (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. Plant Biotechnol J 6:722–732

Sinclair TR, Purcell LC, King CA, Sneller CH, Chen P, Vadez V (2007) Drought tolerance and yield increase of soybean resulting from improved symbiotic N fixation. Field Crops Res. 101:68–71

Specht JE, Hume DJ, Kumudini SV (1999) Soybean yield potential–a genetic and physiological perspective. Crop Sci 39:1560–1571

Taha SS, Osman AS (2017) Influence of potassium humate on biochemical and agronomic attributes of bean plants grown on saline soil. J Hortic Sci Biotechnol https://doi.org/10.1080/14620316.2017.1416960

Tannenbaum SR, Fett D, Young VR, Land PD, Bruce WR (1978) Nitrite and nitrate are formed by endogenous synthesis in the human intestine. Science 200:1487–1491

Tercé-Laforgue T, Dubois F, Ferrario-Méry S, Pou de Crecenzo M-A, Sangwan R, Hirel B (2004a) Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion cells when ammonia is provided either externally or released during photorespiration. Plant Physiol 136:4308–4317

Tercé-Laforgue T, Mäck G, Hirel B (2004b) New insights towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (*Nicotiana tabacum*) plants grown under different nitrogen regimes. Physiol Plant 120:220–228

Trenkel ME (1997) Improving fertilizer use efficiency. Controlled-release and stabilized fertilizers in agriculture. International Fertilizer Industry Association, Paris

Valentine V, Benedito VA, Kang Y (2011) Legume nitrogen fixation and soil abiotic stress, chapter 9. In: Foyer C, Zhang H (eds) Nitrogen metabolism in plants in the post-genomic era. Annual plant reviews 42. Wiley Blackwell, New Delhi, India

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A. 107:4477–4482

von Wiren N, Gazzarrini S, Gojon A, Frommer WB (2000) The molecular physiology of ammonium uptake and retrieval. Curr Opin Plant Biol 3(3):254–261

Wang R, Guegler K, LaBrie ST, Crawford NM (2000) Genomic analysis of a nutrient response in arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. Plant Cell 12(8):1491–1510

Wang X, Peng F, Li M, Yang L, Li GJ (2012) Expression of a heterologous SnRK1 in tomato increases carbon assimilation, nitrogen uptake and modifies fruit development. Plant Physiol 169(12):1173–1182

Zhao Y, Li W, Zhou Z, Wang L, Pan Y, Zhao L (2005) Dynamics of microbial community structure and cellulolytic activity in agricultural soil amended with two biofertilizers. Eur J Soil Biol 41:21–29

Part I
Molecular and Physiological Aspects of Nutrient Use Efficiency

Chapter 2
Improving Nitrogen Use Efficient in Crop Plants Using Biotechnology Approaches

Perrin H. Beatty and Allen G. Good

Introduction

Plants require a source of fixed, or biologically reactive nitrogen (N) to produce molecules such as nucleotide bases and amino acids, in order to make macromolecules like DNA and proteins that are then required for the genome, cellular structures and overall growth. Low or insufficient available N limits the plant growth and yield (both biomass and grain) of crop plants. Plants obtain fixed-N from the soil as ammonia, nitrate, urea, amino acids and peptides. Some plants, such as legumes and poplar trees, form a symbiosis with diazotrophic bacteria where they exchange ammonia from the diazotrophs for carbon (C) molecules and a protected living niche from the plant. Diazotrophic bacteria express a nitrogenase enzyme complex that allows them to reduce atmospheric N_2 to ammonia (NH_3) in a process called biological nitrogen fixation (BNF; Beatty et al. 2015). This enzyme complex has only been found expressed functionally in bacterial species. For centuries, organic N fertilizers (livestock and green manure) have been used to increase crop production. Since the commercialization of the Haber–Bosch process to synthesize ammonia from atmospheric N_2, synthetic N fertilizers have also been used to increase yields (Smil 2004). Synthetic nitrogen fertilizer use has increased by a factor of nine over the last 50 years, and with the predicted global population of 9 billion by 2050, plus the need to reduce hunger and malnutrition, nitrogen fertilizer use will need to increase in order to increase crop yields (Lassaletta et al. 2014). In 2012, the Food and Agriculture Organization of the United Nations (FAO) suggested that food production will need to increase by 60% between 2005/07 and 2050 to meet the

P. H. Beatty (✉)
Dept. Oncology, University of Alberta, Edmonton, AB T6G 2R7, Canada
e-mail: pbeatty@ualberta.ca

A. G. Good
Department of Biological Sciences, University of Alberta,
Edmonton, AB T6G 2E9, Canada

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_2

needs of 9 billion people. Reducing our global yield gaps to produce more food is essential; however, the estimate increase in N fertilizer application needed to do this is 45–73%. However, ramping up our agricultural outputs needs to be done as sustainable as possible, while also lowering N fertilizer application rates (from either organic or synthetic sources), so as to reduce or even eliminate environmental damage from N pollution.

Synthetic N fertilizers are costly to produce and expensive to buy and transport; therefore, they are mainly used by farmers in developed countries or countries with synthetic N fertilizer subsidies (Good and Beatty 2011a). Farmers in developing countries tend to not have the resources to buy or transport synthetic N fertilizer. In addition, these small-holder farmers are frequently growing crops on nutrient poor soils, leading them to produce crop yields far below the optimal yield which means they have little to no produce to take to market. This has resulted in a cycle of poverty because low yields do not allow an investment in fertilizers or improved crop management. The advent of Norman Borlaugh's green revolution of the 1960s, where crop plants were bred for high yields, allowed many farmers in developing countries in North and South America to break out of this poverty cycle by growing high yielding varieties (Borlaug 1972; Good and Beatty 2011a). However, these high yielding crop varieties were bred to use high levels of N fertilizer and so promoted non-sustainable agricultural practices such as excess N fertilizer application.

Crop plants tend to have low nitrogen use efficiency (NUE), taking up approximately 40–50% of the applied N. The rest of the applied N fertilizer that is unavailable to the plant enters the environment as N pollution (Good and Beatty 2011a; Galloway et al. 2014; Ng et al. 2016; Beatty and Wong 2017). Global crop NUE has decreased from the early 1960s when it was 68%, to the current value of 47%. This indicates that on a global scale, over half of the N fertilizer inputs to agriculture are lost to the environment. Therefore, unless our cropping systems can improve their N use efficiency in a substantial way, increasing N fertilizer inputs will only provide a low gain in crop yield with further N pollution to the environment (Lassaletta et al. 2014).

Excess N fertilizer can pollute the environment in many different ways, depending on the form of fixed-N in the fertilizer. Nitrate-N fertilizer is water soluble and so easily leaches into waterways, leading to drinking water contamination and proliferation of algal blooms that in turn cause dissolved oxygen levels to drop and subsequent loss of marine life and diversity. A classic example of water pollution in the USA is the 1.57 million metric tons of nitrogen (mainly as nitrate) that is spewed into the Gulf of Mexico from the Mississippi River (US Geological Survey; https://ks.water.usgs.gov/pubs/fact-sheets/fs.135-00.html), resulting in the formation of a hypoxic dead zone that can be seen from space. Nitrate left in the soil (not taken up by the plants) is chemically reduced by denitrifying soil bacteria to form nitrite (NO_2^-), nitric oxide (NO gas), nitrous oxide (N_2O gas) and ultimately, biologically inert N_2 (gas). The gaseous reduced N compounds are released into the atmosphere causing environmental damage. Nitric oxide is a precursor chemical that leads to tropospheric ozone pollution, and nitrous oxide is a greenhouse gas (GHG) with 296 times the global warming potential (GWP) of CO_2 plus it is an

ozone-depleting chemical (Galloway et al. 2004; Davidson et al. 2015). Seventeen per cent of global GHG emissions are from agriculture and a third of the agricultural GHG emissions are from N fertilizers (Strange et al. 2008). Ammonia N fertilizer is volatized to gaseous ammonium (NH_4^+), especially in wet soils and eventually leads to acid rain and dust formation. Acid rain causes soil nutrient and mineral depletion and loss of microbial diversity and is a health hazard for humans and other animals (Erisman et al. 2008). As an additional example of the environmental costs of agriculture N, researchers in the UK recently conducted a life cycle assessment of the pollution sources from the production of a loaf of bread (Goucher et al. 2017). Following the production of the bread from growing the wheat to packaging and transporting the loaf showed that the ammonium nitrate fertilizer supplied to the wheat was directly linked to 48% of the GWP, 39% eutrophication potential and 42% of the human toxicity potential.

We cannot ignore the past, present and future anthropomorphic changes to the Earth's climate and how this will affect agriculture, food production, food security, freshwater availability, available arable land, biodiversity, human health and the environment. It has become obvious that we need to focus on improving the agricultural industry to make it more sustainable and environmentally friendly, even with the realization that we need to increase yields. Please refer to the publications listed here for further information on anthropomorphic changes and sustainable agriculture (Leip et al. 2014; Zhang et al. 2015; Haines et al. 2017).

Measuring NUE

Agronomists have shown that although crop yields may be increasing, the NUE of many crop varieties is declining, in part due to high to excessive use of N fertilizers (Lassaletta et al. 2014). If NUE is calculated based on applied N, rather than soil available N, then as the applied N levels increase the crop NUE declines, even with an increase in yield. This is largely what explains the overall decrease in global NUE, although now that trend has been reversed in a number of key developed countries (Lassaletta et al. 2014). An analysis of the 50 year trend (1961–2009) within 124 countries in crop yield and N fertilizer inputs including; organic plus synthetic fertilizer, biological nitrogen fixation and atmospheric deposits, showed that some countries have improved both their crop NUE and yield whereas other countries have not (Lassaletta et al. 2014). Interestingly, Lassaletta et al. (2014) also saw that countries with a higher proportion of their N inputs from BNF than from synthetic N fertilizer also had better NUE. Other researchers have pointed out that this alternative approach to N fertilizer, of using BNF as an N input, could also improve NUE. The growth of BNF-symbiotic plants, such as legumes, as an N-source is under-utilized globally, given that most countries only dedicate a few per cent of arable land to legume crops (Crews and Peoples 2004) (Table 2.1).

NUE has been defined in many different ways (Table 2.2; Good et al. 2004); however, it is basically a ratio of the harvested product (as grain or biomass) to the

Table 2.1 N-related genes can be grouped into two classes made of a total of six gene families. Adapted from McAllister et al. 2012 and Han et al. 2015

Gene families	Target genes used in biotechnology	Gene name	References
Class 1 Growth and development			
Signalling	G-protein γ subunit	*DEP1*	Sun et al. (2014)
	Mitogen-activated kinase	*SMG1*	Duan et al. (2014)
	SNF1-related kinase	*SnRK*	Wang et al. (2012)
	Early nodulin-like protein	*ENOD*	Bi et al. (2009)
Regulation; Transcription factors and small RNA	DNA binding one zinc finger	*DoF1*	Li et al. (2013)
	bHLH transcription factor	*SAT1*	Chiasson et al. (2014)
	Nuclear factor Y	*NFY*	Chen et al. (2015)
	NAM, ATAF1,2 and CUC2	*NAC1, 2*	Yang et al. (2015)
	NAC-a6	*NAC005*	Christiansen et al. (2016)
	F-box protein	*APO*	Terao et al. (2010)
	Arabidopsis nitrate regulated 1	*ANR1*	Zhang and Forde (1998)
	ATL31 UBI-ligase	*Atl31*	Sato et al. (2011)
	PII regulatory protein	*GLB1*	Hsieh et al. (1998)
Senescence	Cytokinin oxidase/dehydrogenase	*CKX*	Ashikari et al. (2005)
	Stay-green protein	*SGR*	Park et al. (2007)
Class 2 N metabolism pathways			
Transporters	Nitrate transporter	*NRT*	Tsay et al. (1993)
	Ammonium transporter	*AMT, SAT1*	Yuan et al. (2007)
	Lysine histidine transporter	*LHT*	Hirner et al. (2006)
	Hexose transporter	*STP13*	Schofield et al. (2009)
	Amino acid permease	*AAP1*	Rolletschek et al. (2005)

(continued)

Table 2.1 (continued)

Gene families	Target genes used in biotechnology	Gene name	References
Amino acid biosynthesis	Alanine aminotransferase	*AlaAT*	Shrawat et al. (2008)
	Asparagine synthetase	*ASN*	Lam et al. (2003)
	Aspartate aminotransferase	*aspAT*	Ivanov et al. (2012)
	Asparaginase	*ASNase*	Zhou et al. (2009)
	Glutamate dehydrogenase	*GDH*	Abiko et al. (2010)
	Glutamine synthetase	*GS*	Brauer et al. (2011)
	Glutamate synthase	*GOGAT*	Tamura et al. (2011)
N-assimilation	Nitrate reductase	*NR*	Lea et al. (2006)
	Nitrite reductase	*NiR*	Takahashi et al. (2001)
C:N metabolism and storage	Rubisco small subunit	*Rubisco*	Masle et al. (1993)
	Ferredoxin NADP(H) reductase	*FNR*	Hanke et al. (2008)
	Isopentenyl transferase	*IPT*	Rubio-Wilhelmi et al. (2011)
	Cell wall invertase	*CIN*	Wang et al. (2008)

Table 2.2 Definitions, formula and inherent statistical considerations for NUE calculations

#	Term	Numerator	Denominator	Formula	Measurement	Statistical considerations
1	Nitrogen use efficiency (grain)	Yield	Ns	NUE = Gw/Ns	Grain yield	Ratio
2	Utilization efficiency (grain)	Yield	Nt	UtE = Gw/Nt	Grain yield	Ratio
3	Nitrogen use efficiency (biomass)	Yield (Biomass)	Nt	NUE = Sw/Nt	Biomass, feed, food	Ratio
4	Nitrogen use efficiency (grain)	Yield	Np	NUE = Gw/Np	Grain yield	Ratio
5	Agronomic Efficiency	Difference in yield	N supplied	AE = (GwF − GwC)/ N fert	Yield response	Numerator is difference, calculation a ratio
6	Physiological efficiency	Difference in yield	N supplied	PE = (GwF − GwC)/ Nfert − Ncon	Yield response	Numerator is difference, calculation a ratio
7	Efficiency of fertilizer uptake	N in plant	N supplied	UpE = Nt/Ns	Uptake of N by plant	Ratio, unaffected by total yield

Gw Grain weight
Ns Total N supplied to plant*
Nfert N Fertilizer applies (kg/ha)
Soil N SN
Total N − SN + Nfert
*In reality, Ns could be N applied, or total N available (N applied + soil N)

N available or N applied. NUE calculations are explained in more detail in Table 2.2. NUE can be partitioned into two processes within the plant; nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE; Good and Beatty 2011b; McAllister et al. 2012). Researchers have studied the genetics of both of these processes on overall NUE (Han et al. 2015). NUpE describes the plants' ability to take up biologically active N from the rhizosphere, into the plant. NUtE describes the plants' ability to utilize the N it has acquired and remobilize N over the course of seed development. The remobilization or re-harvesting of N from N-containing macromolecules in leaves (source tissue) and translocation of the N to seeds (sink tissue) is a very important part of the NUtE of a plant and so is often measured as the N remobilization efficiency of a plant. It is often the case that NUpE is more important to the plants overall NUE under low N conditions while under high N conditions variation for the two components of NUE are more evenly distributed (Beatty et al. 2010). Many researchers have pointed out that increasing the efficiency of either N uptake or N utilization (or both) could lead to an increase in crop NUE (Good and Beatty 2011b; Han et al. 2015).

Measuring NUE is challenging because of the number of different ways in which the data can be presented and analysed, and some of the inherent statistical issues that these measurements raise. While there are a number of different definitions of NUE, Table 2.2 provides a list of the main equations that have been used in determining a crop's NUE (Good et al. 2004). There are two inherent problems in these measurements. First, all of the measurements are ratios, which statisticians loath (Curran-Everett 2013). The rationale for the use of ratios often seems to be the desire to control for the influence of N fertilizer (the variable in the denominator) on yield. However, there are a number of limitations to the indiscriminate use of ratios. Allison et al. (1995) demonstrate that given linearity, a zero intercept between the numerator and denominator is necessary for a ratio to remove the confounding effects of the denominator; and seemingly minor departures from a zero intercept can have major consequences on the ratio's ability to control for the denominator. Given that plants will almost always yield something under no applied N, it is difficult to believe this would be the case. There are also a number of other key statistical assumptions that yield and NUE data violate. These include the fact that the ratio of two normally distributed variables cannot be normally distributed and that the use of ratios cannot easily take nonlinear effects between the numerator and denominator into account. All of these may violate the assumptions of subsequent parametric statistical analyses. The difficulty with ratios can be recognized by the fact that two genotypes may differ significantly in yield, but if the denominator N varies at the same rate, the two genotypes will have the same NUE but one has higher yield, something that any producer would clearly want, but that this calculation obscures. Additionally, the numerator, in Eqs. 5 and 6 (Agronomic and Physiological efficiency), is the difference between two variables (GwF and GwC), which may or may not be correlated to each other. Intuitively, we can see that if the first crop genotype had a higher GwF and GwC than genotype 2, but the difference between the two was the same, then they would appear to both have the same NUE.

The second difficulty with these measurements is that they rarely take into account the variability in available N (the denominator). Almost all calculations of N use efficiency will use a numerator that can be measured with some accuracy and where the variance components of yield can be carefully determined (site, year, genotype). The same cannot be said for the denominator, the measurement of N supply or available N. Soil N levels are challenging to measure in a reliable way and depending on the environment, researchers will use different depths of sampling, or measure different forms of N (Nitrate N, total N, ammonia N) as the measure of available N. Second, the variation in N levels will often differ significantly over very short distances in a field (Sylvester-Bradley and Kindred 2009 Kindred et al. 2014). Almost all researchers we have consulted sample multiple soil plots for a single field site, but these are then averaged to generate a single measure of N for the entire field site. Under these conditions, it is impossible to provide any level of statistical confidence in assessing whether one genotype in a trial was more nitrogen use efficient than another. Therefore, researchers need to be careful in defining NUE in their study and in recognizing the statistical difficulty of being able to say that one genotype differs significantly from another in field conditions.

Genetic Attempts to Modify/Improve N Metabolism

The literature is full of research articles aimed at improving NUE, by modifying and/or improving genes involved in root structure, N transport, primary N metabolism, N use developmental and regulation genes, N remobilization genes and others (Table 2.1; please refer to Reddy and Ulaganathan (2015) for a thorough review). These gene targets were selected for bioengineering experiments based on identification from either; genes of known N metabolism function, quantitative trait loci (QTL) genetic surveys of NUE plants, differentially regulated genes in transcriptomics studies of N-efficient plants or of the N-limitation responsive genes (microarray refs). The QTL and transcriptomic studies often identify the same genes as important for NUE, such as ammonium transport (*AMT*), nitrate transport (*NRT*), glutamine synthetase (*GS*) and glutamate synthase (*GOGAT*; Li et al. 2017; Xu et al. 2017). These genes do encode proteins that are integral for N metabolism. Over-expression of these genes tends to result in (depending on the gene) increased tissue N levels, increased amino acid levels and in some cases, an increase in biomass or seed number. These results are encouraging for an NUE trait; however, some unknown factor is still missing and the plants are not necessarily NUE. Also, the vast majority of these studies were done in a greenhouse and the NUE of the bioengineered plants were not measured under field trial conditions. NUE is a complex trait that appears to be influenced by a large array of seemingly diverse genes. As with most science research, it appears unlikely that there will be a "magic bullet" gene for NUE.

Although the focus of this Chapter is on biotechnological approaches to improving NUE, there are plant-bred cultivars that show high NUE compared to

older varieties. For example, there are modern maize, rice, wheat and barley varieties that can grow better and produce more yield under limited N conditions than their older counterparts (Le Gouis et al. 2000; Anbessa et al. 2010; Beatty et al. 2010; Chen et al. 2013; de Carvalho et al. 2016). Genetic screens conducted on some of these NUE varieties to find NUE-trait-associated genes have found that in some cases, primary N metabolism appear to be important for the trait (Quraishi et al. 2011). For example, in a recent breeding study, *TaGS1* was highly expressed post-anthesis and *TaGS2* was highly expressed pre-anthesis in an N-efficient winter wheat cultivar compared to an N-inefficient winter wheat cultivar (Zhang et al. 2017). Four metabolic regulation points were pinpointed as being involved with GS, across different tissue types, in the winter wheat. However, in a study of NUE genes in barley, it was found that a few NUE-associated genes co-segregated with field evaluated QTLs for the NUE trait, but many do not (Han et al. 2016). These results highlight again the inherent complexity of NUE. However, to date, the molecular mechanisms leading to higher NUE in these hybrids and cultivars are still not understood (Hawkesford 2011).

While every researcher has their own favourite gene or gene system, the genes that we would argue are of the most interest are ones where there has been significant genetic variation between two genotypes, for some component of NUE and where the trait and gene have been mapped through genetic analysis. Additional valuable data can be generated on a target gene using phenotype analysis from biotechnology approaches such as over-expression and knockout/down studies showing the effects of either a gain or loss of function, respectively. These two approaches could also be fine-tuned if paired with certain promoters to regulate gene expression in certain tissues or in a specific developmental stage. This will be discussed further in this Chapter. What is less compelling are studies that demonstrate differences between two genotypes and then correlate that phenotype with physiological or expression differences of the gene of interest.

N-related genes can be divided into two broad classes of genes; N metabolism pathways and growth and development. These two main classes can be further divided into a total of six gene families. The N metabolism pathways class includes genes associated with transport, amino acid biosynthesis, assimilation and a catch-all group of other genes (Han et al. 2015). The growth and development class includes genes associated with signalling and regulation with transcription factors and small RNA molecules (Fischer et al. 2013; Han et al. 2015).

N Metabolism Pathway Genes

Using the genes involved with N transport, assimilation and primary N metabolism as bioengineering gene targets for improved NUE in crop plants seemed the most promising for the obvious reason that they are integral for N uptake and N-assimilation (reviewed in Good and Beatty 2011b; McAllister et al. 2012; Fischer et al. 2013; Thomsen et al. 2014). However, numerous over-expression studies with

a variety of these gene targets have shown little progress in improving NUE of crop plants, particularly in the field (Brauer et al. 2011).

Additionally, there are a number of genes that are involved in C metabolism and given the tight link between C and N metabolism, there was the hope that modification of these genes might enhance N uptake (McAllister et al. 2012). However, the manipulation of these genes and the effect on NUE has been discussed in recent reviews (McAllister et al. 2012; Reddy and Ulaganathan 2015) and will not be considered further.

There is an amino acid biosynthesis gene, *AlaAT*, that when over-expressed in canola and rice shows an NUE phenotype when grown in the greenhouse and the field (Good et al. 2007; Shrawat et al. 2008). This gene codes for alanine aminotransferase (AlaAT, EC.2.6.1.2), an enzyme that catalyses the reversible production of alanine and 2-oxoglutarate from pyruvate and glutamate and so is involved in N metabolism downstream of GS and GOGAT. Interestingly, transcriptome analysis of alanine aminotransferase over-expressing (AlaAT-ox) rice lines compared to wildtype (WT), under low, medium or high N supply, did not identify any of the known N transport and N-assimilation genes as differentially regulated, instead the highly differentiated genes were regulatory, transcription factor, TCA cycle, secondary metabolism and unknown function genes (Beatty et al. 2009, 2013). Due to the altered expression of the TCA and secondary metabolite-associated genes, research into understanding the AlaAT-ox NUE phenotype was focused on measuring N-containing metabolites and the N-flux balance in the transgenic plants (described in Beatty et al. 2016). Exactly how over-expression of AlaAT alters plant N use to allow for increases in N uptake and/or N assimilation or N remobilization and ultimately NUE, is not well understood yet. Recently, four *AlaAT* genes were found in the genome of Poplar seedlings. All four genes were induced by exogenous N but one of the genes, *PnAlaAT3*, was mainly expressed in roots and regulated by glutamine and its related metabolites (Xu et al. 2017). Bioengineering crop plants with *AlaAT* is discussed further below.

Growth and Development Genes

The growth and development genes include signalling factors, transcription factors and senescence-associated genes. Senescence is one of the key regulated processes in plant development that remobilizes nutrients like nitrogen from source tissues (e.g. vegetative organs like leaves) to sink tissues (e.g. seeds), leading to the death of the source tissues and eventually the whole plant (Park et al. 2007). For some cereals, such as rice, wheat and barley, up to 90% of the N in the source tissues is remobilized to the grain which highlights the importance senescence is to yield (Diaz-Mendoza et al. 2016). In perennial grasses like switch grass, senescence occurs every year and does not cause the death of the plant. N is remobilized from the source tissues to the seeds, as with the cereals, but N is also remobilized to the roots for storage. Natural variation in N remobilization efficiency is seen amongst

switch grass accessions with a range of 20–61% remobilization from shoots recorded in one set of experiments (Yang et al. 2015).

Various transcription factors from families such as APETALA-2-like, MYB, Dof, NAC, bHLH and others have been used as bioengineering targets for NUE (Yanagisawa et al. 2004; Yaish et al. 2010; El-Kereamy et al. 2012; Chiasson et al. 2014; Yang et al. 2015). The zinc finger protein Dof1, belonging to the "DNA binding with one finger" transcription factor family have been studied in a number of plants species (Yanagisawa et al. 2004). These Dof proteins have been associated with regulation of genes involved in; vascular development, light signalling, carbohydrate metabolism, phloem sugar transport, photosynthetic carbon assimilation, flowering time, dormancy, response to phytohormones, storage protein synthesis and phytochrome signalling (Noguero et al. 2013). The Dof1 gene from maize (*ZmDof1*) has been shown to upregulate the expression of phosphoenolpyruvate carboxylase (*pepC*), the initial carbon fixing enzyme of C4 plants and a key component of the TCA cycle, in rice (Kurai et al. 2011). The expression of *ZmDof1* in Arabidopsis and potato has been shown to modulate the C/N network, promoting nitrogen uptake and increasing plant growth under low nitrogen conditions (Yanagisawa et al. 2004). However, in contrast with the positive agronomic phenotypes shown in rice, potato and Arabidopsis, an attempt to translate these agronomic outcomes to Populus was not successful (Lin et al. 2013).

In a QTL rice study, heterotrimeric G proteins (*dep1* gene) were found to be involved in a signalling role that coordinated nutrient regulation and plant development. The presence of the N insensitive *dep1-1* allele conferred enhanced N uptake and N-assimilation and ultimately high N harvest index and yielding rice plants (Sun et al. 2014).

In addition, there have been a number of other transcription factors that have been identified as biotechnology targets including *PvNAC1* and *PvNAC2, from Panicum virgatum* L. (switch grass) which are members of the NAM, ATAF and CUC (NAC)-family transcription factors. Expression of *PvNAC1* in an *Arabidopsis* stay-green mutant suppressed its senescence defect. Expression of *PvNAC1* in WT *Arabidopsis* triggered early leaf senescence and remobilization. Over-expression of *pvNAC2* in switch grass resulted in increased aboveground biomass associated with increased transcript levels of key nitrogen metabolism genes in leaves and nitrate and ammonium transporter genes in roots. The results indicate that NAC TFs play conserved roles in leaf senescence (Yang et al. 2015).

Promoter Regulation of NUE Genes

We have argued in several manuscripts that it is critical to regulate any gene of interest with the appropriate promoter and/or regulatory signals (Good et al. 2007; Beatty et al. 2009,2013). However, there are now other examples where gene regulation is critical for the desired phenotype. As discussed in the previous section, expression of *ZmDof1* in Arabidopsis and potato increased nitrogen uptake and plant growth under low nitrogen conditions (Yanagisawa et al. 2004). However,

when the *UBI4* promoter drove strong constitutive gene expression of *ZmDof1* in wheat, there were a number of detrimental effects (Pena et al. 2017). In contrast, the maize *rbcS* promoter that was used to drive a light regulated gene expression specifically to the mesophyll cells in the leaf blades and sheaths in C3 crops, resulted in positive agronomic effects in wheat (Matsuoka et al. 1994). There are numerous studies that report detrimental effects in plants bioengineered to over-express a transgene of interest in specific tissues, illustrating that proper pairing of promoter and target gene is essential for positive biotechnological outcomes (Cheon et al. 2004). The AlaAT over-expression in both canola and rice was driven by root-specific promoters. This positive promoter and target gene pairing has been proposed as part of the reason that these bioengineered plants exhibit an NUE phenotype (Good and Beatty 2011b). This is discussed further below. Recently, Chen et al. (2017) bioengineered rice with a rice nitrate transporter promoter and gene construct, *pOsNAR2.1:OsNAR2.1*, in a cisgene experiment and determined that the exogenous transgenic lines showed high nitrate transporter expression when driven by its native promoter. They grew the over-expression lines and WT plants under low, medium and high N supply and saw that the biomass and total N of the transgenic lines increased significantly compared to WT under all three N conditions. They also grew their transgenic lines in the field and measured grain yield, agricultural nitrogen use efficiency (ANUE), and dry matter transfer and found that each of these parameters increased by 21–22% compared to WT. This study is a good example of using an appropriate promoter to drive expression of the gene target. In other words, improving NUE using biotechnology is not only about finding the "best" target gene, it is also about finding the best promoter to pair with the target gene in order to drive expression of the gene at the right time, right place and right strength.

Alanine Aminotransferase: A Case Study on the Road to Commercialization

The unpredictability of transgenic approaches has been discussed, as the manipulation of genes such as *NR*, *NiR*, *GS* and *GOGAT* had been hypothesized to affect NUE. However, modifications of the expression of these genes have not produced consistent NUE phenotypes (McAllister et al. 2012). Meanwhile, the observation that crop plants over-expressing AlaAT have enhanced NUE (Good et al. 2007; Shrawat et al. 2008) has been considered surprising, since AlaAT was previously not thought of as a key component of N metabolism.

As discussed previously, the funding that supported the initial discovery work was not intended to produce nitrogen efficient canola (*Brassica napus*). The initial proposal was based on the hypothesis that increasing the amount of an osmotic compound (alanine) would enhance drought tolerance. Serendipity played a significant role in the discovery of the initial NUE phenotype in 1995, since additional nutrients were inadvertently left out of the watering solution, such that the plants

were significantly N stressed (Good and Beatty 2011a, b; Good et al. 2007). AlaAT's role in post-hypoxic recovery, C_4 photosynthesis, and its evolutionary conservation and role(s) in other organisms has been well documented (McAllister and Good 2015). Over-expression of a barley *AlaAT* in *Brassica napus* (canola) under the control of a tissue-specific promoter called *btg26* resulted in increased yield and biomass under N limiting conditions compared to control plants (Good et al. 2007; Good and Beatty 2011a, b). Subsequent AlaAT expression studies utilizing constitutive promoters indicated that tissue-specific expression is required to produce this NUE phenotype in canola, and that this phenotype is observed under N limiting conditions only (Good et al. 2007).

In cereal crops, AlaAT over-expression has been tested in rice, utilizing a rice *btg26* homologue, *OsAnt1* (Shrawat et al. 2008), and in barley, and wheat. Rice plants over-expressing AlaAT and grown in N limiting conditions showed increased biomass (denser, bushier plants with increased tiller number) and yield, as well as increases in total N and key metabolites (Shrawat et al. 2008).

Field Trials

The first proper field trials of the AlaAT gene occurred in the winter of 2003/2004 in Southern California and were reported in Good et al. (2007). Since then, multiple field trials have been conducted in Canada and the USA by both Arcadia Biosciences and Monsanto (Table 2.3). Despite the fact that AlaAT and the technology associated with its over-expression was the first example of a transgenic NUE crop considered for commercialization, to date no commercial variety of *Brassica napus* or any other crop has been released where the claim has been made that it is more nitrogen efficient. Field trials of canola over-expressing AlaAT have revealed that transgenic plants are able to maintain yields even with 40% less N application relative to the amount used in conventional production (Good et al. 2007). However, despite the number of field trials that have been approved which list NUE as the experimental trait, we are unaware of any data from these field trials. Table 2.3 provides a list of the field trials that were approved by key regulatory agencies and include those countries where we know that versions of this gene have been tested over a number of years. Specifically this includes Australia, Canada and the USA. We are personally aware that the initial Arcadia trials involved the rice *OsAnt1:AlaAT* construct we reported on (Shrawat et al. 2008) in canola. However, we cannot determine the nature of either the specific gene or the regulatory components used for the other trials conducted by different companies since almost all of the trial-associated documentation is listed Confidential Business Information (CBI, Table 2.3). As with canola, to date there is no evidence of varietal registration of this trait in any crop plant. While a number of different genes appear to have been evaluated in the field, other than *AlaAT*, we are unaware of a case where different genes have been over-expressed and promoted an NUE phenotype that was measured in field trials.

Table 2.3 Field trials of crops that listed nutrient use efficiency as the crop trait, with the organization that conducted the research, the crop species, the years of the field trials and the trial locations listed

Organization	Crop species	Years	Locations
USA[1]			
Monsanto	Soybean (*Glycine max*)	2004–2006	HI
Arcadia Biosciences	Rapeseed (*Brassica napus*)	2005–2014	CA, ID, ND,
Pioneer Hi-Bred International Inc.	Corn (*Zea mays*)	2006–2017	AR, CA, DE, GA, HI, IA, IL, IN, KS, MD, MN, MO, NE, PR, SD, TN, TX, WI
Arcadia Biosciences	Rice (*Oryza sativa*)	2006–2013	CA
Pioneer Hi-Bred International Inc.	Soybean (*Glycine max*)	2008–2015	AR, CA, DE, HI, IA, IL, IN, KS, KY, LA, MD, MN, MO, MS, ND, NE, OH, PR, SD, TN, WI
Forster & Assoc. Consulting	Sugarcane (*Saccharum officinarum*)	2009–2015	ID, MN, ND
Ceres Inc	Switch grass	2009–2012	AZ, GA, TN, TX
Monsanto	Rapeseed (*Brassica napus*)	2009	ND, MN, MT
Monsanto	Corn (*Zea mays*)	2010–2015	AR, CA, GA, HI, IA, IL, IN, KS, MD, MN, MO, NE, PR, SD, TN, TX, WI
Arcadia Biosciences	Wheat (*Triticum aestivum*)	2010–2016	ID, ND
Arcadia Biosciences	Barley (*Hordeum vulgare*)	2010	ID, ND, CA
Biogemma USA	Corn (*Zea mays*)	2011–2012	CA, IA, ID, IL, NE, PR
Limagrain Cereal Seeds	Wheat (*Triticum aestivum*)	2011	ND
University of Nebraska/Lincoln	Wheat (*Triticum aestivum*)	2012–2015	NB
Southern Illinois University	Corn (Zea mays)	2012–2014	IL
Ses Vanderhave NV	Sugar beet (*Beta vulgaris*)	2013	MN, ND
University of Illinois	Corn (Zea mays)	2013–2016	IL
Michigan State University	Potato (*Solanum tuberosum*)	2013–2016	MI
Ceres Inc	Sorghum	2013	TX
J. R. Simplot Company	Potato (*Solanum tuberosum*)	2014–2016	ID, OR

(continued)

Table 2.3 (continued)

Organization	Crop species	Years	Locations
Arcadia Biosciences	Cotton	2016	CA, PR
Biogemma USA	Wheat (*Triticum aestivum*)	2016	ID, ND, WA
Canada[2]			
Monsanto Canada Inc.	Canola/Rapeseed (*Brassica napus*)	2007–2012	Alberta, Manitoba, Sask.
Monsanto Canada Inc.	Wheat (*Triticum aestivum*)	2012	Alberta, Manitoba, Sask.
AgQuest	Wheat (*Triticum aestivum*)	2012, 2015	Manitoba
Australia[3]			
CSIRO	Barley (*Hordeum vulgare*)	2010, 2012, 2013	NA
CSIRO	Wheat (*Triticum aestivum*)	2010, 2012, 2013	NA
Sugar Research Australia Ltd	Sugarcane (*Saccharum officinarum*)	2009	NA

[1] http://www.isb.vt.edu/search-release-data.aspx
[2] data.aspx http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/ir-2
[3] http://www.inspection.gc.ca/plants/plants-with-novel-traits/approved-under-review/eng/

Beyond NUE, Using Biotechnology to Engineer N-Fixing Crop Plants

The theory that the NUE of crop plants could be vastly improved by engineering plants to fix their own N from atmospheric N_2, thereby synchronizing plant N demand with plant N supply, has been brought up in the literature for a century (Burrill and Hanson 1917; Merrick and Dixon 1984). Certainly in recent years, this idea has resurfaced as a viable research goal, due to a number of reasons including technological advances, complete genomic sequences and understanding of how nitrogen fixation works at both genetic and enzymatic levels (Curatti and Rubio 2014; Li et al. 2016). Currently, there are research projects underway in the UK, USA, China, Spain and Australia, aimed at introducing biological nitrogen fixation (BNF) to plants (Beatty et al. 2015). Three main approaches have been suggested to do this, one way is to convince non-BNF-symbiotic plants such as *Setaria* and other cereals to nodulate and share C and N with rhizobial diazotrophic (N-fixing) bacteria, like legumes (Rogers and Oldroyd 2014; Oldroyd and Dixon 2014). Another method is to synthesize a novel diazotrophic bacteria: plant association that would benefit both partners (Mus et al. 2016). The plant would be bioengineered to produce and excrete a specific C molecule and the diazotrophic bacteria would be bioengineered to recognize and metabolize that C molecule and in return give the

plant fixed-N. The third method involves transforming the plant with the genes required to express functional nitrogenase complex proteins and cofactors, therefore allowing the plant to directly fix N_2 into ammonia (Beatty and Good 2011; Lopez-Torrejon et al. 2016; Ivleva et al. 2016; Allen et al. 2017; Buren et al. 2017).

What was once a dream is now actually doable in the not-too-distant future (Vicente and Dean 2017). It is conceivable that N fixing crops could be the ultimate method to improve NUE. This would benefit small-holder farmers with little to no access to N fertilizers, intensive scale farmers by greatly lowering their N fertilizer costs, and it also vastly reduces N pollution and the environmental damage associated with nitrate leaching into aquatic ecosystems and ammonia volatization into the atmosphere (Beatty et al. 2015).

Conclusions

To date, it has been difficult to improve nutrient use efficiency using either classical breeding methods or transgenic approaches. We believe that this is likely due to the fact that nutrient metabolism in plants is a complex process and cannot be easily manipulated, without there being pleiotropic negative effects. However, advances in nutrient use efficiency are also plagued by the challenges in properly measuring soil nutrient levels and the challenges of eventually having to develop a plant that performs better in the field. Incremental improvements in NUE have occurred over the last few decades but have resulted from increases in yield, not any measureable improvement in a plants ability to take up N efficiently. We would suggest that similar incremental improvements in NUE may occur through introducing specific transgenes; however, these are likely to be relatively small and will be faced with the challenge of overcoming the regulatory costs associated with plants that have been genetically modified via biotechnology methods (McDougall 2011; Rothstein et al. 2014). In the more short term, it is clear that we need to focus on improved agronomic management approaches, as these have been successful in reducing N inputs while maintaining yield.

References

Abiko T, Wakayama M, Kawakami A, Obara M, Kisaka H, Miwa T, Aoki N, Ohsugi R (2010) Changes in nitrogen assimilation, metabolism, and growth in transgenic rice plants expressing a fungal NADP(H)-dependent glutamate dehydrogenase (gdhA). Planta 232:299–311

Allen RS, Tilbrook K, Warden AC, Campbell PC, Rolland V, Singh SP, Wood CC (2017) Expression of 16 nitrogenase proteins within the plant mitochondrial matrix. Front Plant Sci 8

Allison; Do you have a Reference for 1995? The other one I can get on Sunday but it is on the work computer. Allen

Allison DB, Paultre F, Goran MI, Poehlman ET, Heymsfield SB (1995) Statistical considerations regarding the use of ratios to adjust data. Int J Obes Relat Metab Disord: J Int Assoc Study Obes 19:644–652

Anbessa Y, Juskiw P, Good A, Nyachiro J, Helm J (2010) Selection efficiency across environments in improvement of barley yield for moderately low nitrogen environments. Crop Sci 50:451

Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, Matsuoka M (2005) Cytokinin oxidase regulates rice grain production. Science 309:741–745

Beatty PH, Anbessa Y, Juskiw P, Carroll RT, Wang J, Good AG (2010) Nitrogen use efficiencies of spring barley grown under varying nitrogen conditions in the field and growth chamber. Ann Bot 105:1171–1182

Beatty PH, Carroll RT, Shrawat AK, Guevara D, Good AG (2013) Physiological analysis of nitrogen-efficient rice overexpressing alanine aminotransferase under different N regimes. Botany 91:866–883

Beatty PH, Fischer JJ, Muench DG, Good AG (2015) Environmental and economic impacts of biological nitrogen-fixing (BNF) cereal crops. In: Biological nitrogen fixation. Wiley, pp 1103–1116

Beatty PH, Good AG (2011) Future prospects for cereals that fix nitrogen. Plant Sci (New York, NY) 333:416–417

Beatty PH, Klein MS, Fischer JJ, Lewis IA, Muench DG, Good AG (2016) Understanding plant nitrogen metabolism through metabolomics and computational approaches. Plants (Basel) 5

Beatty PH, Shrawat AK, Carroll RT, Zhu T, Good AG (2009) Transcriptome analysis of nitrogen-efficient rice over-expressing alanine aminotransferase. Plant Biotechnol J 7:562–576

Beatty PH, Wong JL (2017) Nitrogen use efficiency. In: Encyclopedia of applied plant sciences. Academic Press

Bi Y-M, Kant S, Clark J, Gidda S, Ming F, Xu J, Rochon A, Shelp BJ, Hao L, Zhao R, Mullen RT, Zhu T, Rothstein SJ (2009) Increased nitrogen-use efficiency in transgenic rice plants over-expressing a nitrogen-responsive early nodulin gene identified from rice expression profiling. Plant Cell Environ 32:1749–1760

Borlaug N (1972) The green revolution, peace, and humanity. In: Haberman FW (ed) Nobel lectures, peace 1951–1970. Amsterdam

Brauer EK, Rochon A, Bi YM, Bozzo GG, Rothstein SJ, Shelp BJ (2011) Reappraisal of nitrogen use efficiency in rice overexpressing glutamine synthetase1. Physiol Plant 141:361–372

Buren S, Young EM, Sweeny EA, Lopez-Torrejon G, Veldhuizen M, Voigt CA, Rubio LM (2017) Formation of nitrogenase NifDK tetramers in the mitochondria of Saccharomyces cerevisiae. ACS Synth Biol

Burrill, Hanson (1917) Is symbiosis possible between legume bacteria and non legume plants?. University of Illinios, Urbana, Ilinios

Chen J, Fan X, Qian K, Zhang Y, Song M, Liu Y, Xu G, Fan X (2017) pOsNAR2.1:OsNAR2.1 expression enhances nitrogen uptake efficiency and grain yield in transgenic rice plants. Plant Biotechnol J. https://doi.org/10.1111/pbi.12714

Chen M, Zhao Y, Zhuo C, Lu S, Guo Z (2015) Overexpression of a NF-YC transcription factor from bermudagrass confers tolerance to drought and salinity in transgenic rice. Plant Biotechnol J 13:482–491

Chen X, Chen F, Chen Y, Gao Q, Yang X, Yuan L, Zhang F, Mi G (2013) Modern maize hybrids in Northeast China exhibit increased yield potential and resource use efficiency despite adverse climate change. Glob Chang Biol 19:923–936

Chiasson DM, Loughlin PC, Mazurkiewicz D, Mohammadidehcheshmeh M, Fedorova EE, Okamoto M, McLean E, Glass ADM, Smith SE, Bisseling T, Tyerman SD, Day DA, Kaiser BN (2014) Soybean SAT1 (Symbiotic Ammonium Transporter 1) encodes a bHLH transcription factor involved in nodule growth and $NH4^+$ transport. Proc Nat Acad Sci 111:4814–4819

Christiansen MW, Matthewman C, Podzimska-Sroka D, O'Shea C, Lindemose S, Mollegaard NE, Holme IB, Hebelstrup K, Skriver K, Gregersen PL (2016) Barley plants over-expressing the NAC transcription factor gene HvNAC005 show stunting and delay in development combined with early senescence. J Exp Bot 67:5259–5273

Cheon BY, Kim HJ, Oh KH, Bahn SC, Ahn JH, Choi JW et al (2004) Overexpression of human erythropoietin (EPO) affects plant morphologies: retarded vegetative growth in tobacco and male sterility in tobacco and Arabidopsis. Transgenic Res 13:541–549. https://doi.org/10.1007/s11248-004-2737-3

Crews TE, Peoples MB (2004) Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agric Ecosyst Environ 102:279–297

Curatti L, Rubio LM (2014) Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Sci 225:130–137

Curran-Everett D (2013) Explorations in statistics: the analysis of ratios and normalized data. Adv Physiol Educ 37:213–219

Davidson EA, Suddick EC, Rice CW, Prokopy LS (2015) More food, low pollution (mo fo lo Po): a grand challenge for the 21st century. J Environ Qual 44:305–311

de Carvalho EV, Afférri FS, Peluzio JM, Rotili EA, Dotto MA, de Faria LA (2016) Genetics parameters and association of NUE methods in maize under different nitrogen levels/Parâmetros genéticos e associação de metodologias de EUN no milho sob diferentes doses de nitrogênio 3

Diaz-Mendoza M, Velasco-Arroyo B, Santamaria ME, González-Melendi P, Martinez M, Diaz I (2016) Plant senescence and proteolysis: two processes with one destiny. Genet Mol Biol 39:329–338

Duan P, Rao Y, Zeng D, Yang Y, Xu R, Zhang B, Dong G, Qian Q, Li Y (2014) SMALL GRAIN 1, which encodes a mitogen-activated protein kinase kinase 4, influences grain size in rice. The Plant J 77:547–557

El-Kereamy A, Bi YM, Ranathunge K, Beatty PH, Good AG, Rothstein SJ (2012) The rice R2R3-MYB transcription factor OsMYB55 is involved in the tolerance to high temperature and modulates amino acid metabolism. PLoS ONE 7:e52030

Erisman JW, Sutton MA, Galloway J, Klimont Z, Winiwarter W (2008) How a century of ammonia synthesis changed the world. Nature Geosci 1:636–639

Fischer JJ, Beatty PH, Good AG, Muench DG (2013) Manipulation of microRNA expression to improve nitrogen use efficiency. Plant Sci 210:70–81

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vöosmarty CJ (2004) Nitrogen cycles: past, present, and future. Biogeochemistry 70:153–226

Galloway JN, Winiwarter W, Leip A, Leach AM, Bleeker A, Erisman JW (2014) Nitrogen footprints: past, present and future. Environ Res Lett 9:115003

Good AG, Beatty PH (2011a) Fertilizing nature: a tragedy of excess in the commons. PLoS Biol 9:e1001124

Good AG, Beatty PH (2011b) Biotechnological approaches to improving nitrogen use efficiency in plants: alanine aminotransferase as a case study. In: The molecular and physiological basis of nutrient use efficiency in crops, pp 165–191

Good AG, Johnson SJ, De Pauw M, Carroll RT, Savidov N, Vidmar J, Lu Z, Taylor G, Stroeher V (2007) Engineering nitrogen use efficiency with alanine aminotransferase. Can J Bot 85:252–262

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9:597–605

Goucher L, Bruce R, Cameron DD, Lenny Koh SC, Horton P (2017) The environmental impact of fertilizer embodied in a wheat-to-bread supply chain. Nat Plants 3:17012

Haines A, Harris F, Kasuga F, Machalaba C (2017) Future earth—linking research on health and environmental sustainability. BMJ 357

Han M, Okamoto M, Beatty PH, Rothstein SJ, Good AG (2015) The genetics of nitrogen use efficiency in crop plants. Ann Rev Genet 49:269–289

Han M, Wong J, Su T, Beatty PH, Good AG (2016) Identification of nitrogen use efficiency genes in barley: searching for qtls controlling complex physiological traits. Front Plant Sci 7:1587

Hanke GT, Endo T, Satoh F, Hase T (2008) Altered photosynthetic electron channelling into cyclic electron flow and nitrite assimilation in a mutant of ferredoxin:NADP(H) reductase. Plant, Cell Environ 31:1017–1028

Hawkesford MJ (2011) An overview of nutrient use efficiency and strategies for crop improvement. In: The molecular and physiological basis of nutrient use efficiency in crops. Wiley-Blackwell, pp 3–19

Hirner A, Ladwig F, Stransky H, Okumoto S, Keinath M, Harms A, Frommer WB, Koch W (2006) Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant Cell 18:1931–1946

Hsieh M-H, Lam H-M, van de Loo FJ, Coruzzi G (1998) A PII-like protein in Arabidopsis: putative role in nitrogen sensing. Proc Nat Acad Sci 95:13965–13970

Ivanov A, Kameka A, Pajak A, Bruneau L, Beyaert R, Hernández-Sebastià C, Marsolais F (2012) Arabidopsis mutants lacking asparaginases develop normally but exhibit enhanced root inhibition by exogenous asparagine. Amino Acids 42:2307–2318

Ivleva NB, Groat J, Staub JM, Stephens M (2016) Expression of active subunit of nitrogenase via integration into plant organelle genome. PLoS ONE 11:e0160951

Kindred DR, Milne AE, Webster R, Marchant BP, Sylvester-Bradley R (2014) Exploring the spatial variation in the fertilizer-nitrogen requirement of wheat within fields. J Agric Sci. https://doi.org/10.1017/s0021859613000919

Kurai T, Wakayama M, Abiko T, Yanagisawa S, Aoki N, Ohsugi R (2011) Introduction of the ZmDof1 gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions. Plant Biotechnol J 9:826–837

Lam H-M, Wong P, Chan H-K, Yam K-M, Chen L, Chow C-M, Coruzzi GM (2003) Overexpression of the ASN1 gene enhances nitrogen status in seeds of Arabidopsis. Plant Physiol 132:926–935

Lassaletta L, Billen G, Grizzetti B, Anglade J, Garnier J (2014) 50 year trends in nitrogen use efficiency of world cropping systems: the relationship between yield and nitrogen input to cropland. Environ Res Lett 9:105011

Lea US, Leydecker M-T, Quilleré I, Meyer C, Lillo C (2006) Posttranslational regulation of nitrate reductase strongly affects the levels of free amino acids and nitrate, whereas transcriptional regulation has only minor influence. Plant Physiol 140:1085–1094

Le Gouis J, Béghin D, Heumez E, Pluchard P (2000) Genetic differences for nitrogen uptake and nitrogen utilisation efficiencies in winter wheat. Eur J Agron 12:163–173

Leip A, Leach A, Musinguzi P, Tumwesigye T, Olupot G, Stephen Tenywa J, Mudiope J, Hutton O, Cordovil CMDS, Bekunda M, Galloway J (2014) Nitrogen-neutrality: a step towards sustainability. Environ Res Lett 9:115001

Li H, Hu B, Chu C (2017) Nitrogen use efficiency in crops: lessons from Arabidopsis and rice. J Exp Bot

Li S, Zhao B, Yuan D, Duan M, Qian Q, Tang L, Wang B, Liu X, Zhang J, Wang J, Sun J, Liu Z, Feng Y-Q, Yuan L, Li C (2013) Rice zinc finger protein DST enhances grain production through controlling Gn1a/OsCKX2 expression. Proc Nat Acad Sci 110:3167–3172

Li XX, Liu Q, Liu XM, Shi HW, Chen SF (2016) Using synthetic biology to increase nitrogenase activity. Microb Cell Fact 15:43

Lin W, Hagen E, Fulcher A, Hren MT, Cheng Z-M (2013) Overexpressing the *ZmDof1* gene in Populus does not improve growth and nitrogen assimilation under low-nitrogen conditions. Plant Cell Tissue Organ Cult (PCTOC) 113:51–61

Lopez-Torrejon G, Jimenez-Vicente E, Buesa JM, Hernandez JA, Verma HK, Rubio LM (2016) Expression of a functional oxygen-labile nitrogenase component in the mitochondrial matrix of aerobically grown yeast. Nat Commun 7:11426

Masle J, Hudson GS, Badger MR (1993) Effects of ambient CO2 concentration on growth and nitrogen use in tobacco (Nicotiana tabacum) Plants transformed with an antisense gene to the small subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase. Plant Physiol 103:1075–1088

Matsuoka M, Kyozuka J, Shimamoto K, Kano-Murakami Y (1994) The promoters of two carboxylases in a C4 plant (maize) direct cell-specific, light-regulated expression in a C3 plant (rice). Plant J 6:311–319

McAllister CH, Beatty PH, Good AG (2012) Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10:1011–1025

McAllister CH, Good AG (2015) Alanine aminotransferase variants conferring diverse NUE phenotypes in Arabidopsis thaliana. PLoS ONE 10:e0121830

McDougall P (2011) The cost and time involved in the discovery, development and authorisation of a new plant biotechnology derived trait. Crop Life Int

Merrick M, Dixon R (1984) Why don't plants fix nitrogen? In: Trends in biotechnology, pp 162–166

Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA, Kouri ED, Paramasivan P, Ryu M-H, Oldroyd GED, Poole PS, Udvardi MK, Voigt CA, Ané J-M, Peters JW (2016) Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl Environ Microbiol 82:3698–3710

Ng JM-S, Han M, Beatty PH, Good A (2016) "Genes, Meet Gases": The role of plant nutrition and genomics in addressing greenhouse gas emissions. In: Edwards D, Batley J (eds) Plant genomics and climate change. Springer, New York, NY, pp 149–172

Noguero M, Atif RM, Ochatt S, Thompson RD (2013) The role of the DNA-binding one zinc finger (DOF) transcription factor family in plants. Plant Sci 209:32–45

Oldroyd GED, Dixon R (2014) Biotechnological solutions to the nitrogen problem. In: Current opinion in biotechnology, pp 19–24

Park S-Y, Yu J-W, Park J-S, Li J, Yoo S-C, Lee N-Y, Lee S-K, Jeong S-W, Seo HS, Koh H-J, Jeon J-S, Park Y-I, Paek N-C (2007) The senescence-induced staygreen protein regulates chlorophyll degradation. Plant Cell 19:1649–1664

Pena PA, Quach T, Sato S, Ge Z, Nersesian N, Changa T, Dweikat I, Soundararajan M, Clemente TE (2017) Expression of the maize Dof1 transcription factor in wheat and sorghum. Front Plant Sci 8:434

Quraishi UM, Abrouk M, Murat F, Pont C, Foucrier S, Desmaizieres G, Confolent C, Riviere N, Charmet G, Paux E, Murigneux A, Guerreiro L, Lafarge S, Le Gouis J, Feuillet C, Salse J (2011) Cross-genome map based dissection of a nitrogen use efficiency ortho-metaQTL in bread wheat unravels concerted cereal genome evolution. Plant J 65:745–756

Reddy MM, Ulaganathan K (2015) Nitrogen nutrition, its regulation and biotechnological approaches to improve crop productivity. Am J Plant Sci 06:2745–2798

Rogers C, Oldroyd GE (2014) Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. J Exp Bot 65:1939–1946

Rolletschek H, Hosein F, Miranda M, Heim U, Gotz KP, Schlereth A, Borisjuk L, Saalbach I, Wobus U, Weber H (2005) Ectopic expression of an amino acid transporter (VfAAP1) in seeds of Vicia narbonensis and pea increases storage proteins. Plant Physiol 137:1236–1249

Rothstein SJ, Bi YM, Coneva V, Han M, Good A (2014) The challenges of commercializing second-generation transgenic crop traits necessitate the development of international public sector research infrastructure. J Exp Bot 65:5673–5682

Rubio-Wilhelmi MdM, Sanchez-Rodriguez E, Rosales MA, Blasco B, Rios JJ, Romero L, Blumwald E, Ruiz JM (2011) Cytokinin-dependent improvement in transgenic PSARK:IPT tobacco under nitrogen deficiency. J Agric Food Chem 59:10491–10495

Sato T, Maekawa S, Yasuda S, Domeki Y, Sueyoshi K, Fujiwara M, Fukao Y, Goto DB, Yamaguchi J (2011) Identification of 14-3-3 proteins as a target of ATL31 ubiquitin ligase, a regulator of the C/N response in Arabidopsis. Plant J 68:137–146

Schofield RA, Bi YM, Kant S, Rothstein SJ (2009) Over-expression of STP13, a hexose transporter, improves plant growth and nitrogen use in Arabidopsis thaliana seedlings. Plant, Cell Environ 32:271–285

Shrawat AK, Carroll RT, DePauw M, Taylor GJ, Good AG (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. Plant Biotechnol J 6:722–732

Smil V (2004) Enriching the earth: Fritz Haber, Carl Bosch, and the transformation of world food production, 1st edn. MIT, Cambridge, MA

Strange A, Park J, Bennett R, Phipps R (2008) The use of life-cycle assessment to evaluate the environmental impacts of growing genetically modified, nitrogen use-efficient canola. Plant Biotechnol J 6:337–345

Sun H, Qian Q, Wu K, Luo J, Wang S, Zhang C, Ma Y, Liu Q, Huang X, Yuan Q, Han R, Zhao M, Dong G, Guo L, Zhu X, Gou Z, Wang W, Wu Y, Lin H, Fu X (2014) Heterotrimeric G proteins regulate nitrogen-use efficiency in rice. Nat Genet 46:652–656

Sylvester-Bradley R, Kindred DR (2009) Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency. J Exp Bot 60:1939–1951

Takahashi M, Sasaki Y, Ida S, Morikawa H (2001) Nitrite reductase gene enrichment improves assimilation of NO2 in Arabidopsis. Plant Physiol 126:731–741

Tamura W, Kojima S, Toyokawa A, Watanabe H, Tabuchi-Kobayashi M, Hayakawa T, Yamaya T (2011) Disruption of a novel NADH-glutamate synthase2 gene caused marked reduction in spikelet number of rice. Front Plant Sci 2

Terao T, Nagata K, Morino K, Hirose T (2010) A gene controlling the number of primary rachis branches also controls the vascular bundle formation and hence is responsible to increase the harvest index and grain yield in rice. Theor Appl Genet 120:875–893

Thomsen HC, Eriksson D, Møller IS, Schjoerring JK (2014) Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? Trends Plant Sci 19:656–663

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM (1993) The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell 72:705–713

Vicente EJ, Dean DR (2017) Keeping the nitrogen-fixation dream alive. Proc Nat Acad Sci 114:3009–3011

Wang E, Wang J, Zhu X, Hao W, Wang L, Li Q, Zhang L, He W, Lu B, Lin H, Ma H, Zhang G, He Z (2008) Control of rice grain-filling and yield by a gene with a potential signature of domestication. Nat Genet 40:1370–1374

Wang X, Peng F, Li M, Yang L, Li G (2012) Expression of a heterologous SnRK1 in tomato increases carbon assimilation, nitrogen uptake and modifies fruit development. J Plant Physiol 169:1173–1182

Xu Z, Ma J, Qu C, Hu Y, Hao B, Sun Y, Liu Z, Yang H, Yang C, Wang H, Li Y, Liu G (2017) Identification and expression analyses of the alanine aminotransferase (AlaAT) gene family in poplar seedlings. Sci Rep 7:45933

Yaish MW, El-Kereamy A, Zhu T, Beatty PH, Good AG, Bi YM, Rothstein SJ (2010) The APETALA-2-like transcription factor OsAP2-39 controls key interactions between abscisic acid and gibberellin in rice. PLoS Genet 6:e1001098

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. Proc Nat Acad Sci United States Am 101:7833–7838

Yang J, Worley E, Torres-Jerez I, Miller R, Wang M, Fu C, Wang Z-Y, Tang Y, Udvardi M (2015) PvNAC1 and PvNAC2 Are associated with leaf senescence and nitrogen use efficiency in switchgrass. BioEnerg Res 8:868–880

Yuan L, Loqué D, Ye F, Frommer WB, von Wirén N (2007) Nitrogen-Dependent Posttranscriptional Regulation of the Ammonium Transporter AtAMT1;1. Plant Physiol 143:732–744

Zhang H, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279:407–409

Zhang X, Davidson EA, Mauzerall DL, Searchinger TD, Dumas P, Shen Y (2015) Managing nitrogen for sustainable development. Nature 528:51–59

Zhang Z, Xiong S, Wei Y, Meng X, Wang X, Ma X (2017) The role of glutamine synthetase isozymes in enhancing nitrogen use efficiency of N-efficient winter wheat. Sci Rep 7:1000

Zhou Y, Cai H, Xiao J, Li X, Zhang Q, Lian X (2009) Over-expression of aspartate aminotransferase genes in rice resulted in altered nitrogen metabolism and increased amino acid content in seeds. Theor Appl Genet 118:1381–1390

Chapter 3
Transcription Factor-Based Genetic Engineering to Increase Nitrogen Use Efficiency

Yoshiaki Ueda and Shuichi Yanagisawa

Abbreviations

GOGAT	Glutamine oxoglutarate aminotransferase
GS	Glutamine synthetase
NiR	Nitrite reductase
NR	Nitrate reductase
NRT	Nitrate transporter
NUE	Nitrogen use efficiency
NUpE	Nitrogen uptake efficiency
NUtE	Nitrogen utilization efficiency
PEPC	Phosphoenolpyruvate carboxylase
PHT	Phosphate transporter
RNAi	RNA interference
TF	Transcription factor

Introduction

Nitrogen abundance is one of the most important edaphic factors affecting plant growth. Indeed, the huge increases in crop yields over the last century are primarily due to the large input of nitrogen fertilizers (Tilman et al. 2002). However, less than half the amount of applied nitrogen fertilizer is taken up and utilized by crops, and the remainder is destined to be lost through leaching and volatilization (Tilman et al. 2002; Good et al. 2004), eventually leading to environmental pollution and human health hazards. To lessen the burden to the environment and risks to human health, it is urgently necessary to develop crops that require less fertilizer input or

Y. Ueda · S. Yanagisawa (✉)
Laboratory of Plant Functional Biotechnology, Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo, Tokyo 113-0032, Japan
e-mail: asyanagi@mail.ecc.u-tokyo.ac.jp

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_3

use applied nitrogen more efficiently. Since transcription factors (TFs) coordinately regulate the expression of a number of genes related to a particular event, TF-based genetic engineering represents a promising approach for generating crops suitable for cropping systems requiring less fertilizer input. In this chapter, we summarize the general concept underlying the genetic engineering of nitrogen use with TFs and discuss TFs that can potentially be used to genetically modify nitrogen use efficiency (NUE) in crops.

General Concept of Genetic Engineering of NUE Using TFs

Many processes associated with the use of nitrogen sources in the soil by plants include sensing, uptake, assimilation, and translocation, as well as remobilization within individual plants. For instance, during de novo nitrogen assimilation, plants acquire nitrogen from the rhizosphere predominantly in inorganic forms, such as nitrate and ammonium, through nitrate transporters (NRTs) or ammonium transporters (AMTs) on the root surface (Miller and Cramer 2004; Nacry et al. 2013). Ammonium that was taken up directly by the plant or produced via the reduction of nitrate is assimilated into carbon skeletons (2-oxoglutarate) provided via photosynthesis and the carbon metabolic pathway (Yanagisawa 2014). Nitrate reduction needs a supply of reducing power from NAD(P)H and the reduced form of ferredoxin, and ammonium assimilation requires NAD(P)H and ATP. Accordingly, de novo nitrogen assimilation involves numerous enzymes and transporters associated with several metabolic pathways. Furthermore, the activities of some transporters and enzymes are not mediated by a single polypeptide. For instance, OsNRT2.1 must be associated with an accessory protein OsNAR2.1 to fully exhibit its nitrate uptake activity (Yan et al. 2011). The finding that the transporters involved in nitrate and ammonium uptake are post-translationally regulated (Lanquar et al. 2009; Wang et al. 2012; Xu et al. 2012) points to another difficulty. Due to these facts, genetically modifying the expression levels of only one gene involved in nitrogen uptake or metabolism may not significantly improve nitrogen uptake and utilization (Fig. 3.1). In fact, simply overexpressing *NRT* or *AMT* genes does not always increase the uptake of these inorganic nitrogen sources (Yuan et al. 2007; Katayama et al. 2009). Similarly, increasing the expression levels of genes encoding nitrate reductase (NR) or nitrite reductase (NiR) alone did not always lead to an obvious increase in growth or yields, although some differences in metabolite and amino acid levels were observed (Good et al. 2004). Inconsistent results have been also reported with plants overexpressing glutamine synthetase (GS) or glutamine oxoglutarate aminotransferase (glutamate synthase; GOGAT) genes, which sometimes failed to increase the activity levels of these enzymes or led to growth retardation (reviewed in Good et al. 2004). Thus, alternative approaches that do not rely on the constitutive upregulation/downregulation of a single gene are more promising for altering nitrogen uptake and utilization in plants.

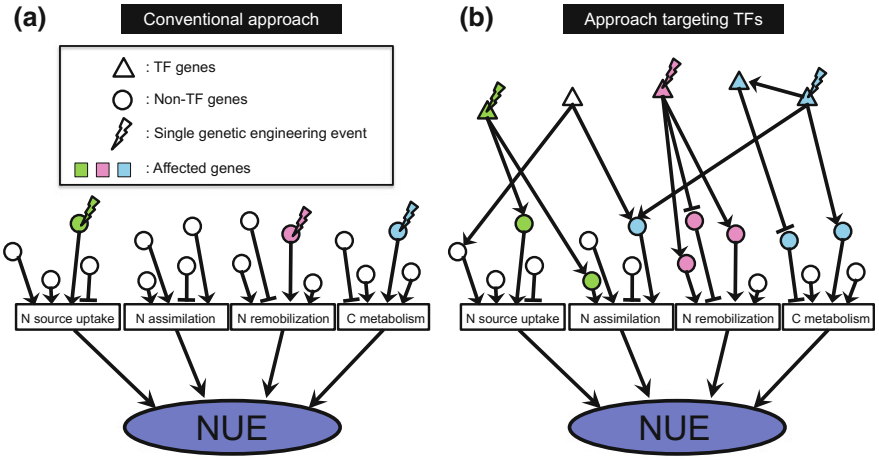


Fig. 3.1 Conceptual scheme explaining the effectiveness of TF-based genetic engineering to achieve higher NUE. A number of genes regulating different aspects of nitrogen uptake and nitrogen utilization (circles) coordinately contribute to NUE. Engineering via conventional approaches targeting a single gene (**a**) may lead to failure to improve NUE owing to the complexity and the large number of genes involved in NUE. On the other hand, the approach targeting TFs (triangles; **b**) can simultaneously influence a number of downstream genes involved in different aspects of NUE, likely resulting in successfully increasing NUE. An arrow indicates that the gene positively regulates the downstream component, while a T sign indicated that the gene has an inhibitory effect on the downstream component

In general, TFs coordinately regulate the expression of a set of target genes by binding to specific DNA sequences known as *cis*-regulatory elements, which differ for each TF. Higher plants harbor numerous TFs (Riechmann et al. 2000; Jin et al. 2014), which utilize complex transcriptional regulatory mechanisms to control various developmental processes and environmental responses (Riechmann and Meyerowitz 1998; Rubio et al. 2001; Dubos et al. 2010; Moreau et al. 2016). Hence, modifying the expression of TF genes represents an alternative approach to altering the expression of a single gene when coordinately modifying the expression of a number of genes is required to affect the intended trait. For instance, Arabidopsis PHR1, a GARP-type TF, binds to the nucleotide sequence GNATATNC in the regulatory regions for a number of genes that participate in phosphorus uptake and usage, such as genes encoding phosphate transporters (PHTs) and acid phosphatases, and then activates these genes to induce the phosphate starvation response (Rubio et al. 2001; Wu et al. 2013). In rice, overexpressing the rice homolog of *PHR1* led to enhanced phosphate uptake and better grain yields, suggesting that *PHR1* or *PHR1* homologs could be utilized in diverse crop plants (Guo et al. 2015). Another good example of genetic modification to produce agriculturally improved traits using TFs is the enhancement of drought, cold, and salinity stress tolerance by overexpressing *DREB*, encoding a transcriptional activator of the AP2/ERF TF family. Overexpressing Arabidopsis *DREB1* or

the rice homolog *OsDREB1* conferred higher tolerance against these stresses in rice through the activation of drought and cold stress tolerance-related genes whose expression is under the control of the DREB-binding sequences (Ito et al. 2006; Yamaguchi-Shinozaki and Shinozaki 2006). These successful examples suggest that TF-based genetic engineering is indeed an effective approach for engineering traits that require simultaneous modification of the expression levels of a set of related genes.

An Attempt to Increase NUE Using the Dof1 TF

The very first study involving TF-based genetic engineering to increase nitrogen utilization in plants was performed using the maize TF Dof1 (Yanagisawa et al. 2004). Dof TFs constitute a family unique to plants, including the green alga *Chlamydomonas reinhardtii*, suggesting that they originated prior to the divergence of terrestrial plants (Shigyo et al. 2006). Maize Dof1, the first Dof TF characterized, was originally identified as a TF that binds to the cauliflower mosaic virus (CaMV) *35S* promoter and the C4-type phosphoenolpyruvate carboxylase (PEPC) gene promoter in maize (Yanagisawa and Izui 1993; Yanagisawa and Sheen 1998). It was later shown that Dof1 also induced the expression of other carbon metabolism-related genes, such as genes encoding cytosolic orthophosphate dikinase and non-C4-type PEPC (Yanagisawa 2000), suggesting that it regulates a wide array of genes involved in carbon metabolism. In a subsequent study, the expression of *Dof1* in Arabidopsis under the control of the *35SC4PPDK* promoter (a derivative of the CaMV *35S* promoter) led to higher expression of genes involved in carbon assimilation, such as genes for non-C4-type PEPC and pyruvate kinase (Yanagisawa et al. 2004). These altered gene expression levels corresponded to higher PEPC and PK activity in the transgenic lines, as well as higher levels of amino acids, especially glutamine, and increased total nitrogen and carbon contents. In addition, the transgenic Arabidopsis plants produced higher biomass than wild-type plants under nitrogen-limiting conditions, thereby exhibiting more effective use of the limited nitrogen source in the growth medium (Yanagisawa et al. 2004).

The effectiveness of genetic engineering using *Dof1* was demonstrated in monocot species as well. Transgenic rice plants expressing maize *Dof1* exhibited higher expression of *PEPC* genes and higher PEPC activity in leaves than the wild type (Kurai et al. 2011). Furthermore, the transgenic plants exhibited upregulated expression of genes encoding enzymes involved in the TCA cycle, as well as enhanced nitrogen contents in various tissues, under both nitrogen-sufficient and nitrogen-deficient conditions (Kurai et al. 2011). In a recent study, *Dof1* was introduced into wheat under the control of the *rbc*S1 (rubisco small subunit 1 gene) promoter, resulting in higher PEPC activity and NUE under limited nitrogen conditions than in the wild type (Peña et al. 2017). Thus, Dof1 is a promising target TF for improving NUE by molecular breeding in both monocot and dicot species in the future.

TFs that Could Be Utilized for Genetic Engineering to Improve NUE

NUE is determined by two factors, nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE). The former is defined as the total amount of nitrogen acquired by plants divided by the total available nitrogen sources in the soil, while the latter is defined as the fraction of acquired nitrogen that is converted to grain or biomass (Xu et al. 2012). Improvement of NUpE is achieved by modifying either root architecture or nitrogen uptake activity per root surface area, whereas NUtE is greatly affected by the nitrogen assimilation activity, since plants cannot directly utilize acquired nitrate or ammonium without converting it to an organic form. Furthermore, NUtE is also affected by resource remobilization within a single plant, because a large portion of grain nitrogen (>70%) originates from the nitrogen pool that accumulates in the plant before anthesis, as shown in wheat using $^{15}NO_3^-$ labeling (Kichey et al. 2007). In this section, we therefore summarize our knowledge on TFs involved in modulations of root architecture in different nitrogen status, nitrogen uptake activity, assimilation activity, or remobilization of nitrogen (Table 3.1).

TFs Involved in Modifying Root Architecture

One of the earliest TFs found to regulate root architecture upon nitrogen supply is the Arabidopsis MADS-box TF, ANR1 (Zhang and Forde 1998). Arabidopsis lines with decreased *ANR1* transcript show abolished lateral root elongation upon nitrate supply (Zhang and Forde 1998), suggesting lower nitrate uptake in these lines. Another TF gene involved in modifying root architecture in different nitrogen status is Arabidopsis *NAC4* (Vidal et al. 2013) whose expression is regulated by a nitrate-inducible gene, *AFB3*, encoding an F-box protein that functions as an auxin receptor (Vidal et al. 2010). Knockout mutants of *NAC4* did not show marked increases in lateral root development upon nitrate supply, whereas wild-type plants did (Vidal et al. 2013). Thus, these TFs are potentially useful to improve the root architecture in response to nitrate supply and efficiently take up applied fertilizers in the field. In addition to the stimulation of lateral root growth upon nitrate supply, the preferential allocation of resources to roots growing in nitrate-rich patches of soil represents another important mechanism to adapt to a spatially non-homogeneous distribution of nitrate in the soil. A member of the TCP TF family in Arabidopsis, TCP20, underlies such a mechanism (Guan et al. 2014). The *tcp20* mutant did not show any obvious phenotypes when nitrate was uniformly supplied to the growth medium. However, it was nearly insensitive to local nitrate supply. This mutant showed similar lateral root growth in patches of medium containing high nitrate levels or no nitrate, whereas wild-type plants preferentially grew lateral roots in patches with high levels of nitrate. Because TCP20 binds to the promoter regions of *NIA1* (one of two NR genes in Arabidopsis) and *NRT1.1* (dual-affinity NRT gene)

Table 3.1 List of TFs that may be utilized for genetic engineering to increase NUE

Main target trait	Name	TF family	Origin	Target plant species	Promoter	Positive impact	Negative impact	Reference
Root morphology	ANR1*	MADS box	Arabidopsis	Arabidopsis	CaMV35S (cosuppression) (RNAi)		Defect in nitrate-induced lateral root proliferation	Zhang and Forde (1998)
	NAC4*	NAC	Arabidopsis	Arabidopsis	(Knockout)		Defect in nitrate-induced lateral root proliferation	Vidal et al. (2013)
	TCP20*	TCP	Arabidopsis	Arabidopsis	(Knockout)		Lower expression of *NRT1.1* and *NIA1*. Lack of preferential lateral root growth in high nitrate patches	Guan et al. (2014)
	PtaRAP2.11	AP2/ ERF	Poplar	Poplar	CaMV35S	Higher lateral root density and higher root and shoot biomass under low N supply		Dash et al. (2015)
	PtaNAC1	NAC	Poplar	Poplar	ET304 (*Populus* root-specific promoter)	Higher lateral root density and higher root biomass under low N supply		Wei et al. (2013)
Nitrogen source uptake	TaNFYA-B1	NF-Y	Wheat	Wheat	Maize ubiquitin	Higher nitrate uptake rate. Higher spike number	Lower number of grains per spike	Qu et al. (2015)

(continued)

Table 3.1 (continued)

	OsDOF18*	Dof	Rice	Rice	(Knockout)		Lower expression levels of ammonium transporter genes. Lower rate of ammonium uptake	Wu et al. (2017)
Nitrogen utilization	ZmDof1	Dof	Maize	Arabidopsis	Maize ubiquitin	Higher PEPC and PK activity. Higher N and C content. Higher biomass production under limited N supply		Yanagisawa et al. (2004)
	ZmDof1	Dof	Maize	Rice	Maize ubiquitin	Higher PEPC activity. Higher asparagine content. Higher biomass production under limited N supply		Kurai et al. (2011)
	ZmDof1	Dof	Maize	Wheat	Maize rbcS1 (Rubisco small subunit 1)	Higher PEPC activity. Higher biomass production under limited N supply		Peña et al. (2017)
Nitrogen utilization and uptake	NLP7	RWP-RK	Arabidopsis	Arabidopsis	CaMV35S	Higher biomass production. Higher nitrate uptake rate. Higher NR, GS, and PEPC activity and glutamine content		Yu et al. (2016a)
	HY5, HYH	bZIP	Arabidopsis	Arabidopsis	(Knockout)	Higher expression of *NRT1.1*	Lower expression of *NIA2* and lower NR activity in the light	Jonassen et al. (2008, 2009)

(continued)

Table 3.1 (continued)

	NIGT1/ HRS1, HHO1	GARP			(Knockout)	Higher expression of *NRT1.1*. Longer primary roots when grown with nitrate in the absence of phosphate		Medici et al. (2015)
	OsNIGT1*	GARP	Rice	Rice	Maize ubiquitin		Lower chlorophyll content when grown with nitrate as the sole N source	Sawaki et al. (2013)
	LBD37/38/ 39	ASL/ LBD	Arabidopsis	Arabidopsis	(Knockout)	Higher expression of *NIA1* and *NIA2*. Higher shoot glutamine content	Higher accumulation of anthocyanin	Rubin et al. (2009)
	TaNAC2-5A	NAC	Wheat	Wheat	Maize ubiquitin	Higher biomass production and grain yield. Higher N harvest index. Higher expression of *NRT*s and *GS2* genes		He et al. (2015)
	RDD1	Dof	Rice	Rice	Rice actin	Higher expression of *GS1;1*. Higher nitrate and ammonium uptake under limited N supply. Higher harvest index and grain yield	Smaller grain weight	Iwamoto and Tagiri (2016)
Internal nitrogen remobilization	OsNAP	NAC	Rice	Rice	(RNAi)	Higher grain weight and grain yield. Higher seed-setting rate. Delayed senescence	Lower protein and mineral concentrations in grains	Liang et al. (2014)

(continued)

Table 3.1 (continued)

	NAM-B1	NAC	Wild emmer wheat	Wheat	(RNAi)	Increase in nitrogen remobilization from leaves to grains. Higher grain protein and mineral content		Uauy et al. (2006)
Unknown	TaNF-YB4	NF-Y	Wheat	Wheat	Maize ubiquitin	Higher biomass production, spike number, and grain yield	Lower grain weight	Yadav et al. (2015)

The reported phenotypes are in comparison with the corresponding wild-type plants. "RNAi" indicates that the expression of the target gene was reduced by RNA interference. "Knockout" indicates that the gene was disrupted by the insertion of transfer-DNA. "Cosuppression" indicates that the transcript was absent despite introduction of the sense cDNA, probably due to cosuppression. Asterisk (*) indicates that only unfavorable NUE traits were observed in the study using knockout or overexpression lines, and thus there is only indirect evidence that the TF is useful for improving NUE. This list includes studies in which any alteration in phenotype was mentioned

and promoted their expression (Guan et al. 2014), TCP20 is probably a TF involved in modification of both root architecture and regulation of nitrate uptake and assimilation. Since these TFs were identified by negatively altered root architecture in their knockout mutants upon nitrate supply, it is necessary to examine whether these TF could be used to genetically engineer improved NUE.

Plants modify their root architecture during nitrogen deficiency in addition to upon nitrate supply. Among 11 genes that were identified as potential hubs for nitrogen starvation responses in *Populus* species by gene expression analysis, two TFs (PtaRAP2.11 and PtaNAC1) positively regulate primary root length and lateral root length and density in response to low nitrate conditions (Wei et al. 2013; Dash et al. 2015). In addition to the improved root growth, the *PtaRAP2.11* overexpression line also exhibited improved shoot growth under low nitrogen conditions in a soil experiment, suggesting their usefulness in genetic engineering of NUE.

In addition to TFs involved in modulations of root architecture in response to nitrogen status, a number of TFs are involved in root architecture either constitutively or in response to other signals [reviewed in Tian et al. (2014) and Yu et al. (2016b)]. These TFs may also be useful to improve nitrogen uptake in upland crop fields.

TFs Involved in the Modulation of Nitrogen Uptake Activity

Nitrogen uptake activity per root surface area, which is highly influenced by the activity of transporters expressed on the root surface, also affects nitrogen uptake by the whole plant. Genetically expanding nitrogen uptake activity is an alternative approach for the genetic improvement of NUpE. A few TFs involved in nitrogen uptake activity have already been identified. The wheat TF of the large NF-Y TF family (TaNFYA-B1) positively regulates the expression of some NRT genes, possibly via the binding of TaNFYA-B1 to the CCAAT boxes in the promoters of these transporter genes. Transgenic wheat plants constitutively overexpressing *TaNFYA-B1* had a larger root system compared to the wild type. Consequently, the transgenic plants had higher nitrate uptake rate and nitrogen content per plant, resulting in an increased number of spikes and grain yields under both control and nitrogen-deficient conditions in the field (Qu et al. 2015). Similarly, another wheat NF-Y TF, TaNF-YB4, also showed a positive effect on biomass production and grain yield, when it was constitutively overexpressed in wheat (Yadav et al. 2015). Although the genes regulated by TaNF-YB4 have not yet been identified, the authors proposed that this effect is likely due to higher nutrient uptake activity in the transgenic lines (Yadav et al. 2015). Unlike upland crop species, where nitrate is the predominant nitrogen source, ammonium is the predominant nitrogen source for lowland crop species such as rice (Miller and Cramer 2004). Thus, improving ammonium uptake is particularly important for the genetic improvement of lowland crop species. A recent study suggested that OsDOF18 is involved in regulating the expression of ammonium transporter genes and ammonium uptake, as determined using rice mutant lines lacking *OsDOF18* transcript (Wu et al. 2017).

TFs Involved in Regulation of Nitrogen Assimilation

Several TFs that positively or negatively regulate nitrogen assimilation in plants have been identified to date.

TaNAC2-5A, a nitrate-inducible NAC TF in wheat, induces the expression of plastidic GS gene (*GS2*) and NRT genes, which function in the uptake and translocation of nitrate, by directly binding to their promoter regions. Transgenic lines overexpressing *TaNAC2-5A* showed higher nitrate uptake rate, higher biomass production, higher grain yield, and higher harvest index [=(grain N accumulation)/(total N accumulation in aerial parts)], under both high and low nitrogen supply conditions, than observed in the wild type (He et al. 2015). Interestingly, although TaNAC2-5A belongs to a cereal-specific clade of the NAC TF family, the effectiveness of TaNAC2-5A was also demonstrated in Arabidopsis. Transgenic Arabidopsis lines expressing *TaNAC2-5A* had higher expression levels of NRT genes (*NRT2.1*, *NRT1.2,* and *NRT1.5*) and higher nitrate uptake rates than the wild type (He et al. 2015). TaNAC2-5A also directly or indirectly affected root architecture in Arabidopsis. Therefore, TaNAC2-5A may affect both nitrate uptake and utilization in both monocot and dicot species and then exert positive effects on biomass production.

Rice Dof daily fluctuations 1 (RDD1), a member of the Dof TF family, was originally identified as a TF involved in the circadian clock, with a marked diurnal oscillatory expression pattern in leaf blades (Iwamoto et al. 2009). A subsequent study revealed that *RDD1* transcripts are targeted by a microRNA, miR166, whose expression oscillates diurnally (Iwamoto and Tagiri 2016). By expressing *mRDD1* (*RDD1* carrying nucleotide substitutions in the miR166-recognition site and thus insensitive to miR166-mediated RNA degradation) in rice, RDD1 was shown to regulate genes involved in the uptake and utilization of various nutrients, such as cytosolic GS gene *GS1;1*, sucrose transporter gene *OsSUT2*, and sodium transporter gene *HKT1;1*, especially under low nutrient conditions (Iwamoto and Tagiri 2016). Compared to wild type, plants expressing *mRDD1* had higher nitrate and ammonium uptake activity in a hydroponic experiment. Furthermore, the *mRDD1*-expressors had higher harvest index [=(grain weight)/(shoot dry weight)] and higher total grain yields than wild type, possibly due to enhanced translocation of nutrients from leaves to grains. Therefore, RDD1 is another TF that regulates the uptake and utilization of nitrogen, as well as nutrient translocation.

Two basic leucine zipper family TFs, HY5 (LONG HYPOCOTYL5) and its closest homolog HYH (HY5 HOMOLOG), were found to induce NR activity in Arabidopsis under far-red and red light (Jonassen et al. 2008). It was later revealed that this effect is due to the specific induction of *NIA2* (one of two Arabidopsis NR genes) in the light (Jonassen et al. 2009). In agreement with this finding, the *NIA2* promoter, but not the *NIA1* promoter, harbors a Z-box and a GATA motif, which are putative binding sites of HY5 and HYH. In the same study, HY5 and HYH were shown to negatively affect the expression of *NRT1.1* in leaves and roots via an unknown mechanism (Jonassen et al. 2009).

Arabidopsis NIN-like protein (NLP) 6 and 7 are TFs that mediate a large portion of genome-wide gene expression reprogramming during primary nitrate responses (Konishi and Yanagisawa 2013; Marchive et al. 2013; Konishi and Yanagisawa 2014). NLP6 and NLP7 directly upregulate many early nitrate-responsive genes, such as *NRT2.1*, *NIA1*, *NIR,* a nitrite transporter gene (*AtNITR2*), and *GS2*, all of which are crucial for nitrate uptake or nitrogen assimilation (Konishi and Yanagisawa 2013; Marchive et al. 2013; Maeda et al. 2014). Consistently, transgenic Arabidopsis plants overexpressing *NLP7* exhibited higher expression of these target genes than wild type, resulting in higher nitrogen contents and biomass production, along with higher photosynthesis rates and PEPC activity (Yu et al. 2016a). These studies demonstrate that NLPs are indispensable TFs with widespread effects on the expression levels of genes determining nitrate uptake and nitrogen utilization in plants. Since the TFs NLP6 and NLP7 are post-transcriptionally regulated (Konishi and Yanagisawa 2013; Marchive et al. 2013; Liu et al. 2017), an approach other than simple overexpression of *NLP6* and *NLP7* might further improve NUE in plants.

LBD (LATERAL ORGAN BOUNDARY DOMAIN) proteins are a subfamily of the ASL/LBD TF family. The genes for these three proteins, *LBD37, LBD38*, and *LBD39*, are strongly induced upon nitrate supply, although ammonium or glutamine supply also induced their expression to a minor extent. These transcriptional repressors negatively regulate a number of genes involved in nitrate uptake and assimilation, such as *NRT1.1*, *NRT2.1*, *NIA1*, *NIA2*, and *GLN1.4* (cytosolic GS gene) (Rubin et al. 2009). Notably, these *LBD* genes also negatively affect the expression of glucose-6-phosphate dehydrogenase 2 gene involved in providing the carbon skeleton required for nitrogen assimilation. Consistent with these modifications in gene expression, mutant lines lacking either of these genes had higher glutamine contents than wild type (Rubin et al. 2009). Thus, knocking out these *LBD* homologs appears to have positive effects on nitrate uptake and its assimilation, although increased levels of anthocyanin in these mutant lines also suggested negative consequences of knockout of these *LBD* genes on growth due to their pleiotropic effects.

The GARP-type TF, NIGT1/HRS1, is a transcriptional repressor that is induced by nitrate supply (Sawaki et al. 2013; Medici et al. 2015). An Arabidopsis mutant line with mutations in *HRS1* and its closely related homolog, *HHO1*, showed better primary root growth in the absence of phosphate than wild-type plants, especially in the presence of nitrate. Moreover, higher expression of *NRT1.1* was observed in the double knockout line, suggesting that NIGT1/HRS1 might negatively affect nitrate uptake (Medici et al. 2015). The involvement of NIGT1 in nitrate uptake or utilization was also demonstrated in rice. *OsNIGT1*-overexpressing plants had lower chlorophyll contents than wild type when grown with nitrate as the sole nitrogen source, but not with ammonium, indicating that nitrate utilization was impaired in the transgenic lines (Sawaki et al. 2013).

TFs Involved in Nitrogen Remobilization

TFs involved in nitrogen remobilization and leaf senescence include NAM-B1, a NAC family TF originally identified in wild emmer wheat, and OsNAP, another NAC family TF in rice.

NAM-B1 is a TF that enhances resource remobilization to grains and hence affects the contents of proteins in grains, as well as minerals such as iron and zinc (Uauy et al. 2006). While wild emmer wheat carries the functional allele of *NAM-B1*, its function was lost during domestication due to the insertion of a single nucleotide, resulting in a frame-shift mutation. Knocking down *NAM* homologs in hexaploid wheat by RNA interference (RNAi) led to a stay-green phenotype but reduced grain protein and mineral contents due to decreased nutrient translocation from leaves (Uauy et al. 2006). Although grain yields were not directly tested in this study, the RNAi lines may increase the grain yields since the stay-green phenotype induces a longer grain-filling period (Gaju et al. 2011; Derkx et al. 2012; Xu et al. 2012).

OsNAP is also involved in nutrient remobilization from senescing leaves to grains in rice (Liang et al. 2014). Overexpression of *OsNAP* promoted leaf senescence, whereas reducing its expression by RNAi led to delayed senescence and an extension of the grain-filling period compared with wild type. The RNAi lines retained higher photosynthetic activity after anthesis, resulting in higher seed-setting rates, grain weight, and grain yield. However, the grains of the RNAi lines had reduced concentrations of protein and mineral nutrients such as nitrogen, phosphorus, iron, and zinc, likely due to reduced remobilization of elements to the grains, since the RNAi lines retained higher nutrient contents in their flag leaves.

These examples suggest that leaf senescence, nutrient translocation, and grain yields are complex traits, which are mutually influenced. Therefore, although TFs involved in nitrogen remobilization and leaf senescence could be used to genetically engineer improved NUE, a newly devised plan would be needed for the practical application of engineering of this type of TFs due to a trade-off between grain yields and protein and mineral contents in grains.

Precautions and the Challenges of TF-Based Engineering

Genetic engineering using TFs is a promising approach for improving nitrogen use, and a number of TFs with potential to improve NUE have been found to date. However, TF-based engineering of NUE may unexpectedly lead to unwanted traits from the viewpoint of agriculture. For instance, in the *OsNAP* RNAi lines, there appeared to be a trade-off between grain yields and grain protein/mineral contents. Thus, increasing grain yields by genetic engineering might lead to changes in the nutritional value of grains in these cases. Another example of unwanted traits produced by TF-based engineering is early flowering of rice plants expressing

mRDD1 (Iwamoto and Tagiri 2016). Although they have higher grain yields, their early flowering phenotype may affect field management and cropping systems if these strategies are to be adopted for current agricultural systems. Thus, attention should be paid to the possible side effects caused by the pleiotropic effects of TFs.

Another thing to consider is the effect of the environment. In several studies, the positive effects of the introduced TF genes were evident only under certain conditions, such as low nitrogen supply, as was the case for *PtaRAP2.1*, *PtaNAC1*, *TaNAC2-5A*, and *Dof1*. Similarly, experimental results obtained under artificial conditions might not always apply to different systems. For instance, transgenic tobacco plants expressing the Arabidopsis ammonium transporter gene *AMT1;1* showed increases in the rate of short-term ammonium uptake compared to wild-type plants when grown in nutrient solution, but the same plants failed to show enhanced nitrogen uptake and better growth when grown in soils supplied with ammonium (Yuan et al. 2007). Thus, the growth conditions and soil types in areas in which the genetically modified plants are to be grown should be kept in mind.

The last point to consider is the possible negative effects of constitutively expressing the target TFs. In some experiments aimed at increasing NUE, growth retardation was observed in transgenic plants, as reviewed by Good et al. (2004) and Xu et al. (2012). One of the ways to circumvent this problem might be the use of an inducible promoter. Since expressing *DREB1A* under the control of a strong constitutive promoter led to both increased drought and cold tolerance but severe growth retardation (Kasuga et al. 1999, 2004), the stress-inducible *rd29A* promoter was used to express *DREB1A*. This approach was successfully used to produce plants with increased cold or drought stress tolerance while minimizing the negative effect on plant growth in tobacco and Arabidopsis (Kasuga et al. 1999, 2004). Expressing introduced transgenes in a tissue-specific manner is another effective way to mitigate the possible negative effects of genetic engineering. Wei et al. (2013) used a root-specific promoter to drive *PtaNAC1* expression, which successfully increased root biomass without diminishing shoot biomass. Similarly, Peña et al. (2017) took advantage of the leaf-specific *rbc*S1 promoter to express maize *Dof1* in wheat, since *Dof1* expression driven by this promoter successfully increased biomass production, whereas the expression of this gene driven by a strong constitutive promoter led to drastic growth retardation (Peña et al. 2017). These examples suggest that the occasional growth retardation observed in genetic engineering experiments targeting high NUE might be mitigated by the use of an inducible or tissue-specific promoter.

Conclusions and Future Perspectives

During the long history of crop breeding, nitrogen uptake and utilization have dramatically improved. Indeed, modern wheat cultivars have higher NUE than old cultivars when grown under the same nitrogen conditions (Cormier et al. 2016). A number of genes involved in nitrogen uptake and assimilation were shown to be

under selective pressure during the modern breeding process in a specific rice group (Xie et al. 2015), and nitrogen use in crops has indeed improved over the past several decades through conventional breeding approaches. However, to further improve nitrogen use in crops and to enable the use of low-input cropping systems, more breakthroughs are needed. The nucleotide sequences of TF genes are generally more conserved among diverse ecotypes than those of other genes in Arabidopsis (Gan et al. 2011), suggesting that marker-assisted selection targeting TFs relying on naturally occurring sequence variation might be of limited use in crop species. In this situation, genetic engineering targeting TFs is a promising approach for improving nitrogen use in the future.

Our knowledge of the involvement of TFs in nitrogen use is steadily increasing. However, many of the studies performed to date have focused on the model plant Arabidopsis. More investigations are needed to determine whether similar mechanisms are conserved in other plant species, most importantly monocot species, which account for the majority of worldwide crop production. A recent cross-species comparison between Arabidopsis and rice led to the identification of conserved nitrogen-regulatory networks in rice (Obertello et al. 2015). This study, using information about protein–protein interactions, *cis*-binding sites, and orthologs between the species, showed that nitrogen treatment affects similar sets of genes in these species and also identified 23 core nitrogen-responsive TFs in rice. The identified TFs include orthologs of previously characterized Arabidopsis genes such as *LBD* and *HHO*. Thus, the mechanisms underlying nitrogen responses are similar between these species to a large extent, and the signaling mechanisms and TFs determining nitrogen responses/utilization in Arabidopsis are likely applicable to other plant species, at least (to some extent) in rice. Similar approaches would allow basic knowledge about Arabidopsis to be applied to other crop species in the future and would help define the TFs to be targeted to improve nitrogen use in these species. Recent technological advances, such as systematic evolution of ligands by exponential enrichment and protein-binding microarrays for high-throughput identification of TF binding sites (Franco-Zorrilla et al. 2014), should accelerate basic research about TFs in crops and pave the way for increasing the number of TFs to be utilized in genetic engineering targeting the improvement of nitrogen use in crops.

Acknowledgements This work was supported in part by JST CREST (no. JPMJCR15O5) and JSPS KAKENHI Scientific Research (nos. 18H03940, 26221102 and 18J01554). We apologize to the authors of the many publications that we were unable to cite due to space constraints.

References

Cormier F, Foulkes J, Hirel B, Gouache D, Moënne-Loccoz Y, Le GJ (2016) Breeding for increased nitrogen-use efficiency: a review for wheat (*T. aestivum* L.). Plant Breed 135:255–278. https://doi.org/10.1111/pbr.12371

Dash M, Yordanov YS, Georgieva T, Kumari S, Wei H, Busov V (2015) A systems biology approach identifies new regulators of poplar root development under low nitrogen. Plant J 84:335–346. https://doi.org/10.1111/tpj.13002

Derkx AP, Orford S, Griffiths S, Foulkes MJ, Hawkesford MJ (2012) Identification of differentially senescing mutants of wheat and impacts on yield, biomass and nitrogen partitioning. J Integr Plant Biol 54:555–566. https://doi.org/10.1111/j.1744-7909.2012.01144.x

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010) MYB transcription factors in *Arabidopsis*. Trends Plant Sci 15:573–581. https://doi.org/10.1016/j.tplants.2010.06.005

Franco-Zorrilla JM, López-Vidriero I, Carrasco JL, Godoy M, Vera P, Solano R (2014) DNA-binding specificities of plant transcription factors and their potential to define target genes. Proc Natl Acad Sci USA 111:2367–2372. https://doi.org/10.1073/pnas.1316278111

Gaju O, Allard V, Martre P, Snape JW, Heumez E, LeGouis J, Moreau D, Bogard M, Griffiths S, Orford S et al (2011) Identification of traits to improve the nitrogen-use efficiency of wheat genotypes. F Crop Res 123:139–152. https://doi.org/10.1016/j.fcr.2011.05.10

Gan X, Stegle O, Behr J, Steffen JG, Drewe P, Hildebrand KL, Lyngsoe R, Schultheiss SJ, Osborne EJ, Sreedharan VT et al (2011) Multiple reference genomes and transcriptomes for *Arabidopsis thaliana*. Nature 477:419–423. https://doi.org/10.1038/nature10414

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9:597–605. https://doi.org/10.1016/j.tplants.2004.10.008

Guan P, Wang R, Nacry P, Breton G, Kay SA, Pruneda-Paz JL, Davani A, Crawford NM (2014) Nitrate foraging by *Arabidopsis* roots is mediated by the transcription factor TCP20 through the systemic signaling pathway. Proc Natl Acad Sci USA 111:15267–15272. https://doi.org/10.1073/pnas.1411375111

Guo M, Ruan W, Li C, Huang F, Zeng M, Liu Y, Yu Y, Ding X, Wu Y, Wu Z et al (2015) Integrative comparison of the role of the PHOSPHATE RESPONSE1 subfamily in phosphate signaling and homeostasis in rice. Plant Physiol 168:1762–1776. https://doi.org/10.1104/pp.15.00736

He X, Qu B, Li W, Zhao X, Teng W, Ma W, Ren Y, Li B, Li Z, Tong Y (2015) The nitrate inducible NAC transcription factor TaNAC2-5A controls nitrate response and increases wheat yield. Plant Physiol 169:1991–2015. https://doi.org/10.1104/pp.15.00568

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol 47:141–153. https://doi.org/10.1093/pcp/pci230

Iwamoto M, Higo K, Takano M (2009) Circadian clock- and phytochrome-regulated Dof-like gene, *Rdd1*, is associated with grain size in rice. Plant Cell Environ 32:592–603. https://doi.org/10.1111/j.1365-3040.2009.01954.x

Iwamoto M, Tagiri A (2016) MicroRNA-targeted transcription factor gene *RDD1* promotes nutrient ion uptake and accumulation in rice. Plant J 2016(85):466–477. https://doi.org/10.1111/tpj.13117

Jin J, Zhang H, Kong L, Gao G, Luo J (2014) PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. Nucleic Acids Res 42:1182–1187. https://doi.org/10.1093/nar/gkt1016

Jonassen EM, Lea US, Lillo C (2008) *HY5* and *HYH* are positive regulators of nitrate reductase in seedlings and rosette stage plants. Planta 227:559–564. https://doi.org/10.1007/s00425-007-0638-4

Jonassen EM, Sévin DC, Lillo C (2009) The bZIP transcription factors HY5 and HYH are positive regulators of the main nitrate reductase gene in *Arabidopsis* leaves, *NIA2*, but negative regulators of the nitrate uptake gene *NRT1.1*. J Plant Physiol 166:2071–2076. https://doi.org/10.1016/j.jplph.2009.05.010

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 17:287–291. https://doi.org/10.1038/7036

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A combination of the *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiol 45:346–350. https://doi.org/10.1093/pcp/pch037

Katayama H, Mori M, Kawamura Y, Tanaka T, Mori M, Hasegawa H (2009) Production and characterization of transgenic rice plants carrying a high-affinity nitrate transporter gene (*OsNRT2.1*). Breed Sci 59:237–243. https://doi.org/10.1270/jsbbs.59.237

Kichey T, Hirel B, Heumez E, Dubois F, Le Gouis J (2007) In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. F Crop Res 102:22–32. https://doi.org/10.1016/j.fcr.2007.01.002

Konishi M, Yanagisawa S (2013) Arabidopsis NIN-like transcription factors have a central role in nitrate signalling. Nat Commun 4:1617. https://doi.org/10.1038/ncomms2621

Konishi M, Yanagisawa S (2014) Emergence of a new step towards understanding the molecular mechanisms underlying nitrate-regulated gene expression. J Exp Bot 65:5589–5600. https://doi.org/10.1093/jxb/eru267

Kurai T, Wakayama M, Abiko T, Yanagisawa S, Aoki N, Ohsugi R (2011) Introduction of the ZmDof1 gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions. Plant Biotechnol J 9:826–837. https://doi.org/10.1111/j.1467-7652.2011.00592.x

Lanquar V, Loque D, Hormann F, Yuan L, Bohner A, Engelsberger WR, Lalonde S, Schulze WX, von Wirén N, Frommer WB (2009) Feedback inhibition of ammonium uptake by a phospho-dependent allosteric mechanism in *Arabidopsis*. Plant Cell 21:3610–3622. https://doi.org/10.1105/tpc.109.068593

Liang C, Wang Y, Zhu Y, Tang J, Hu B, Liu L, Ou S, Wu H, Sun X, Chu J, Chu C (2014) OsNAP connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice. Proc Natl Acad Sci USA 111:10013–10018. https://doi.org/10.1073/pnas.1321568111

Liu K-H, Niu Y, Konishi M, Wu Y, Du H, Chung HS, Li L, Boudsocq M, McCormack M, Maekawa S et al (2017) Discovery of Nitrate-CPK-NLP signalling in central nutrient-growth networks. Nature 545:311–316. https://doi.org/10.1038/nature22077

Maeda S, Konishi M, Yanagisawa S, Omata T (2014) Nitrite transport activity of a novel HPP family protein conserved in cyanobacteria and chloroplasts. Plant Cell Physiol 55:1311–1324. https://doi.org/10.1093/pcp/pcu075

Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A (2013) Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nat Commun 4:1713. https://doi.org/10.1038/ncomms2650

Medici A, Marshall-Colon A, Ronzier E, Szponarski W, Wang R, Gojon A, Crawford NM, Ruffel S, Coruzzi GM, Krouk G (2015) AtNIGT1/HRS1 integrates nitrate and phosphate signals at the *Arabidopsis* root tip. Nat Commun 6:6274. https://doi.org/10.1038/ncomms7274

Miller AJ, Cramer MD (2004) Root nitrogen acquisition and assimilation. Plant Soil 274:1–36. https://doi.org/10.1007/s11104-004-0965-1

Moreau F, Thévenon E, Blanvillain R, Lopez-Vidriero I, Franco-Zorrilla JM, Dumas R, Parcy F, Morel P, Trehin C, Carles CC (2016) The Myb-domain protein ULTRAPETALA1 INTERACTING FACTOR 1 controls floral meristem activities in *Arabidopsis*. Development 6:1108–1119. https://doi.org/10.1242/dev.127365

Nacry P, Bouguyon E, Gojon A (2013) Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil 370:1–29. https://doi.org/10.1007/s11104-013-1645-9

Obertello M, Shrivastava S, Katari MS, Coruzzi GM (2015) Cross-species network analysis uncovers conserved nitrogen-regulated network modules in rice. Plant Physiol 168:1830–1843. https://doi.org/10.1104/pp.114.255877

Peña PA, Quach T, Sato S, Ge Z, Nersesian N, Changa T, Dweikat I, Soundararajan M, Clemente T (2017) Expression of the maize *Dof1* transcription factor in wheat and sorghum. Front Plant Sci 8:434. https://doi.org/10.3389/fpls.2017.00434

Qu B, He X, Wang J, Zhao Y, Teng W, Shao A, Zhao X, Ma W, Wang J, Li B et al (2015) A wheat CCAAT box-binding transcription factor increases the grain yield of wheat with less fertilizer input. Plant Physiol 167:411–423. https://doi.org/10.1104/pp.114.246959

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR et al (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. Science 290:2105–2110. https://doi.org/10.1126/science.290.5499.2105

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem 379:633–646. https://doi.org/10.1515/bchm.1998.379.6.633

Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR (2009) Members of the *LBD* family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. Plant Cell 21:3567–3584. https://doi.org/10.1105/tpc.109.067041

Rubio V, Linhares F, Solano R, Mart'in AC, Iglesias J, Leyva A, Paz-Ares J (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev 15:2122–2133. https://doi.org/10.1101/gad.204401

Sawaki N, Tsujimoto R, Shigyo M, Konishi M, Toki S, Fujiwara T, Yanagisawa S (2013) A nitrate-inducible GARP family gene encodes an auto-repressible transcriptional repressor in rice. Plant Cell Physiol 54:506–517. https://doi.org/10.1093/pcp/pct007

Shigyo M, Tabei N, Yoneyama T, Yanagisawa S (2006) Evolutionary processes during the formation of the plant-specific Dof transcription factor family. Plant Cell Physiol 48:179–185. https://doi.org/10.1093/pcp/pcl044

Tian H, De Smet I, Ding Z (2014) Shaping a root system: regulating lateral versus primary root growth. Trends Plant Sci 19:426–431. https://doi.org/10.1016/j.tplants.2014.01.007

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671–677. https://doi.org/10.1038/nature01014

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314:1298–1301. https://doi.org/10.1126/science.1133649

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010) Nitrate-responsive miR393/*AFB3* regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 107:4477–4482. https://doi.org/10.1073/pnas.0909571107

Vidal EA, Moyano TC, Riveras E, Contreras-López O, Gutiérrez RA (2013) Systems approaches map regulatory networks downstream of the auxin receptor AFB3 in the nitrate response of *Arabidopsis thaliana* roots. Proc Natl Acad Sci USA 110:12840–12845. https://doi.org/10.1073/pnas.1310937110

Wang YY, Hsu PK, Tsay YF (2012) Uptake, allocation and signaling of nitrate. Trends Plant Sci 17:458–467. https://doi.org/10.1016/j.tplants.2012.04.006

Wei H, Yordanov YS, Georgieva T, Li X, Busov V (2013) Nitrogen deprivation promotes *Populus* root growth through global transcriptome reprogramming and activation of hierarchical genetic networks. New Phytol 200:483–497. https://doi.org/10.1111/nph.12375

Wu P, Shou H, Xu G, Lian X (2013) Improvement of phosphorus efficiency in rice on the basis of understanding phosphate signaling and homeostasis. Curr Opin Plant Biol 16:205–212. https://doi.org/10.1016/j.pbi.2013.03.002

Wu Y, Yang W, Wei J, Yoon H, An G (2017) Transcription factor *OsDOF18* controls ammonium uptake by inducing ammonium transporters in rice roots. Mol Cells 40:178–185. https://doi.org/10.14348/molcells.2017.2261

Xie W, Wang G, Yuan M, Yao W, Lyu K, Zhao H, Yang M, Li P, Zhang X, Yuan J et al (2015) Breeding signatures of rice improvement revealed by a genomic variation map from a large germplasm collection. Proc Natl Acad Sci USA 112:E5411–E5419. https://doi.org/10.1073/pnas.1515919112

Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182. https://doi.org/10.1146/annurev-arplant-042811-105532

Yadav D, Shavrukov Y, Bazanova N, Chirkova L, Borisjuk N, Kovalchuk N, Ismagul A, Parent B, Langridge P, Hrmova M, Lopato S (2015) Constitutive overexpression of the *TaNF-YB4* gene in transgenic wheat significantly improves grain yield. J Exp Bot 66:6635–6650. https://doi.org/10.1093/jxb/erv370

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803. https://doi.org/10.1146/annurev.arplant.57.032905.105444

Yan M, Fan X, Feng H, Miller AJ, Shen Q, Xu G (2011) Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges. Plant Cell Environ 34:1360–1372. https://doi.org/10.1111/j.1365-3040.2011.02335.x

Yanagisawa S (2000) Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. Plant J 21:281–288. https://doi.org/10.1046/j.1365-313x.2000.00685.x

Yanagisawa S (2014) Transcription factors involved in controlling the expression of nitrate reductase genes in higher plants. Plant Sci 229:167–171. https://doi.org/10.1016/j.plantsci.2014.09.006

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. Proc Natl Acad Sci USA 101:7833–7838. https://doi.org/10.1073/pnas.0402267101

Yanagisawa S, Izui K (1993) Molecular cloning of two DNA-binding proteins of maize that are structurally different but interact with the same sequence motif. J Biol Chem 268:16028–16036

Yanagisawa S, Sheen J (1998) Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. Plant Cell 10:75–89. https://doi.org/10.1105/tpc.10.1.75

Yu L-H, Wu J, Tang H, Yuan Y, Wang S-M, Wang Y-P, Zhu Q-S, Li S-G, Xiang C-B (2016a) Overexpression of *Arabidopsis* NLP7 improves plant growth under both nitrogen-limiting and -sufficient conditions by enhancing nitrogen and carbon assimilation. Sci Rep 6:27795. https://doi.org/10.1038/srep27795

Yu P, Gutjahr C, Li C, Hochholdinger F (2016b) Genetic control of lateral root formation in cereals. Trends Plant Sci 21:951–961. https://doi.org/10.1016/j.tplants.2016.07.011

Yuan L, Loqué D, Ye F, Frommer WB, von Wirén N (2007) Nitrogen-dependent posttranscriptional regulation of the ammonium transporter AtAMT1;1. Plant Physiol 143:732–744. https://doi.org/10.1104/pp.106.093237

Zhang H, Forde BG (1998) An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. Science 279:407–409. https://doi.org/10.1126/science.279.5349.407

Chapter 4
Modeling Plant Metabolism: Advancements and Future Capabilities

Margaret N. Simons-Senftle, Debolina Sarkar and Costas D. Maranas

Introduction

High throughput tools such as transcriptomics and proteomics have emerged as important tools to systemically probe the processes controlling plant productivity and nitrogen use efficiency (NUE). However, we still lack a quantitative framework to integrate measurements at different levels. Gene and protein expression under N-limiting and N replete conditions have been used to identify key reactions and regulatory proteins involved in N metabolism. For instance, the overexpression of cytosolic glutamine synthase (GS1 and GS2) in rice grown in low nitrogen (N) led to a low yield and growth phenotype. However, these plants exhibited increased levels of core carbon (C) and N-containing metabolites in roots and shoots resulting in an unbalanced C:N metabolic ratio (Bao et al. 2014). As genome-scale metabolic (GSM) models are able to predict the flux through metabolic reactions within the plant, they can be relied upon to better understand the metabolism associated with various environmental and genetic factors.

Genome-Scale Metabolic Models and Flux Balance Analysis

Genome-scale metabolic (GSM) models are comprised of all known metabolic reactions that occur within an organism, tissue or compartment. The model is composed of three essential aspects: the reaction network, the biomass reaction, and gene–protein reaction (GPR) relationships. First, the model includes all internal reactions and metabolite transporters, including the uptake and export of metabo-

M. N. Simons-Senftle · D. Sarkar · C. D. Maranas (✉)
Department of Chemical Engineering, The Pennsylvania State University, University Park, Pennsylvania, USA
e-mail: costas@engr.psu.edu

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_4

lites and their associated metabolic costs. A coefficient matrix, known as the stoichiometric matrix, represents the stoichiometric coefficient of each metabolite within each metabolic reaction. Next, a proxy biomass formation reaction is appended that represents the accumulation of all biomass components. Finally, gene–protein reaction (GPR) relationships are developed to link the genes that are associated with a metabolic reaction. GPR relationships are developed using Boolean Logic gates where an "and" relationship represents a gene that is a subunit of a protein complex and an "or" relationship indicates an isozyme. Flux balance analysis (FBA) is used to predict the metabolic conversion from reactants to products (i.e., the flux in mmol gDW^{-1} h^{-1}) for each metabolic reaction (Orth et al. 2010). FBA assumes that the system has reached a pseudo-steady state by setting the production of each metabolite equal to its consumption (i.e., the change in concentration over time for each metabolite is equal to zero or $dx_i/dt = 0$). This steady-state assumption is represented by:

$$\sum_{j \in \text{Reactions}} S_{ij} v_j = \frac{dx_i}{dt} = 0, \forall i \in \text{Metabolites}$$

The stoichiometry of metabolite i in reaction j is represented by S_{-ij}, where S_{ij} is negative for reactants and positive for products. The flux or total metabolic conversion (represented in mmol gDW^{-1} h^{-1}) is represented by the variable v_j and the accumulation of metabolite i over time t is assumed to be zero. The feasible solution space is further constrained by including bounds for reactions that are known to be irreversible under physiological conditions. FBA seeks to maximize or minimize a user-defined objective function, which typically is the maximization of the flux through the biomass reaction. The biomass reaction assigns stoichiometric coefficients that represent the experimentally observed proportion of biomass components (in mmol gDW^{-1}) and represents biomass formation (in h^{-1}). It is critical to ensure that the molecular weight of the biomass component is normalized to 1 g $mmol^{-1}$ (Chan et al. 2017), especially when more than one biomass reaction is included in FBA, which may occur during multi-tissue growth of the plant. Applying FBA to GSM models is analogous to determining the max flow through a network of pipes toward a single drain of biomass (see Fig. 4.1a). The input pipes are analogous to the nutrients in the metabolic model, the output pipes represent the biomass reaction and other known secreted metabolites, and the complex system of pipes is analogous to the reactions connecting various metabolites. FBA identifies the flow through this network to maximize the flow out of one pipe.

While FBA identifies a unique maximum value for the objective function but usually not a unique reaction flux associated with the maximum value. In fact, any set of reaction fluxes determined by FBA is just one solution to the problem out of many alternatives. Flux variability analysis (FVA) is often used to determine the feasible range of each reaction's flux at the maximum objective function (Mahadevan and Schilling 2003). Conclusions drawn about a particular reaction must hold for the entire range of flux solutions determined by FVA. Alternatively,

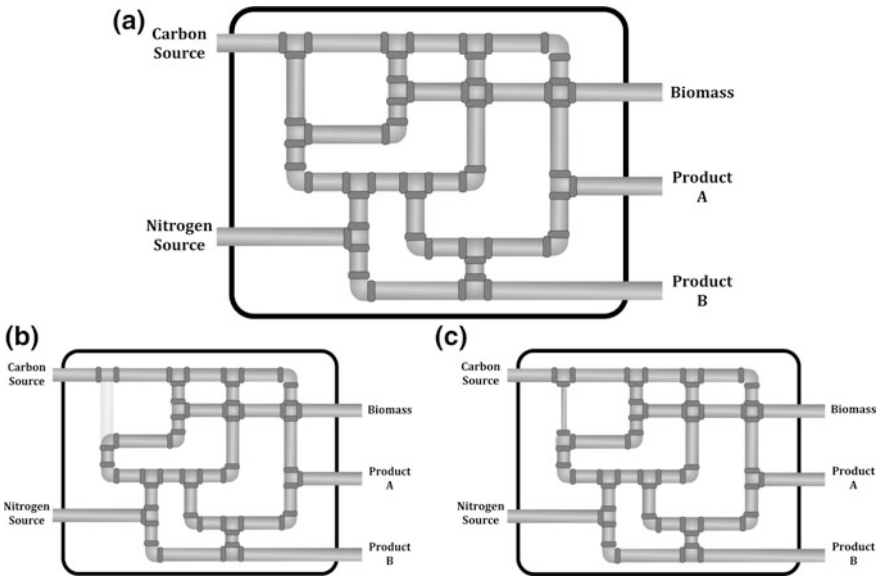


Fig. 4.1 Flux balance analysis (FBA) of a genome-scale metabolic (GSM) model is analogous to a set of pipes. **a** FBA solves for the flux through a reaction (i.e., the rate of conversion between reactants and products) in order to maximize an objective function, typically biomass formation. This is analogous to a set of pipes with a set of input flows (representative of the carbon and nitrogen sources) and output flows (representative of biomass and various products). FBA solves for the flux through each reaction (or pipe) by assuming the cell is operating at a pseudo-steady state. **b** To incorporate different types of experimental data, a class of methods termed the "switch" approach has been developed. This approach utilizes lowly expressed genes, proteins, or enzymes to turn off or remove a reaction (or pipe) for their associated metabolic reaction(s). **c** The "valve" approach utilizes data from multiple conditions to constrain the flux through a reaction by limiting the upper bound (i.e., decreasing the diameter of the pipe). This can cause a decrease in the reaction's flux when biomass is maximized, but is not required to alter the flux if the predicted flux before the inclusion of the constraint is low

parsimonious flux analysis (pFBA) (Lewis et al. 2010) is used to determine the minimum flux through all reactions required to maintain the maximum biomass formation identified through FBA. The pFBA formulation attempts to minimize the total enzyme load required to produce the optimal solution determined by FBA using the total sum of fluxes as a proxy. Many times constraining both the maximum biomass formation and the minimum sum of all fluxes does not result in a unique profile of fluxes and once again the variability must be assesses. This can be completed using a modification of FVA where both the maximum biomass level and the minimum total flux are set at their identified levels. Generally, the more constraints placed on the model (i.e., setting the maximum biomass formation and minimum sum of all fluxes) the smaller the variability for each reaction flux.

Platforms Available for Published Metabolic Models

GSM models are distributed using an SBML, MATLAB, or Excel format that can be readily uploaded into most published packages. For a thorough review of the software application packages, see (Lakshmanan et al. 2014; Dandekar et al. 2014). In addition to the packages reviewed, two new packages have emerged: COBRApy (Ebrahim et al. 2013), a python package that supports basic COBRA methods, and the Web-based US Department of Energy Systems Biology Knowledgebase (KBase) (Arkin et al. 2016). The COBRApy software package supports the next generation of metabolic modeling and uses Parallel Python (https://www.parallelpython.com) to split simulations across multiple CPUs enabling faster FVA simulations, which can be time intensive due to the large nature of plant metabolic models (Ebrahim et al. 2013). COBRApy allows users to develop their own constraints and objective functions allowing for more detailed modeling (Ebrahim et al. 2013). The KBase online interface allows users to create workflows that can be shared among researchers allowing for other users to quickly reproduce the simulations (Arkin et al. 2016). With KBase, users are able to perform standard FBA, pFBA, as well as gene and reaction knockouts (Arkin et al. 2016).

Plant GSM Models

The number of organisms with GSM models has dramatically increased since the first model of *Haemophilus influenzae* Rd published in 1999 (Edwards and Palsson 1999). Many microbial models were developed with increasing complexity and the first photoautotrophic model was developed in 2005 focused on central metabolism and photosynthetic reactions of *Synechocystis* sp. PCC 6803 (Shastri and Morgan 2005). The first plant genome-scale model was developed for barley seed (Grafahrend-Belau et al. 2009) in 2009 followed by a model of Arabidopsis (Poolman et al. 2009) published in the same year. In general, plant genome-scale models began as networks that contained all known metabolic reactions that occurred within the plant in any tissue or growth stage (Table 4.1). These non-compartmentalized reactions were developed for Arabidopsis (Poolman et al. 2009; de Oliveira Dal'Molin et al. 2010a, b) and maize (Saha et al. 2011). Plant genome-scale metabolic models were then created for specific tissues including the embryo in rapeseed (Hay and Schwender 2011a, b), seed in barley (Grafahrend-Belau et al. 2009), leaf in tomato (Yuan et al. 2016), leaf in rice (Poolman et al. 2013; Lakshmanan et al. 2013), and the leaf (Simons et al. 2014a, b; Seaver et al. 2015), embryo (Seaver et al. 2015), and seed (Seaver et al. 2015) in maize. Models of C4 metabolism have apportioned the metabolic flux between the bundle sheath and mesophyll cell types (Simons et al. 2014a, b; de Oliveira Dal'Molin et al. 2010a, b). More recently, plant metabolic models have included

inter-tissue transporters through the vascular tissue to model the whole-plant metabolic interactions for Arabidopsis (de Oliveira Dal'Molin et al. 2015) and barley (Grafahrend-Belau et al. 2013).

Table 4.1 A list of available genome-scale models for plants

Species	No. of associated genes	No. of reactions	Specificity	References
Arabidopsis thaliana	Not reported	1,406	Generic	Poolman et al. (2009)
Arabidopsis thaliana	1,419	1,567	Generic	de Oliveira Dal'Molin et al. (2010a, b)
Arabidopsis thaliana	Not reported	9,728	Whole-plant: root, stem, and leaf	de Oliveira Dal'Molin et al. (2015)
Brassica napus	Not reported	572	Embryo	Hay and Schwender (2011a, b)
Hordeum vulgare	Not reported	257	Seed (Endosperm)	Grafahrend-Belau et al. (2009)
Hordeum vulgare	Not reported	955	Whole plant: leaf, stem, seed, root, and phloem	Grafahrend-Belau et al. (2013)
Oryza sativa	Not reported	1,736	Leaf	Poolman et al. (2013)
Oryza sativa	629	326	Leaf central metabolism	Lakshmanan et al. (2013)
Oryza sativa japonica	148	1721	Leaf	Chatterjee and Kundu (2015)
Saccharum officinarum	3,881	1,588	Leaf: mesophyll and bundle sheath cells	de Oliveira Dal'Molin et al. (2010a, b)
Solanum lycopersicum L.	3410	2,143	Leaf	Yuan et al. (2016)
Sorghum bicolor	3,557	1,588	Leaf: mesophyll and bundle sheath cells	de Oliveira Dal'Molin et al. (2010a, b)
Zea mays	1,563	1,985	Generic	Saha et al. (2011)
Zea mays	5,824	8,525	Leaf: mesophyll and bundle sheath cells	Simons et al. (2014a, b)
Zea mays	2,322	2,635	Leaf	Seaver et al. (2015)
Zea mays	2,304	2,636	Embryo	Seaver et al. (2015)
Zea mays	2,280	2,636	Endosperm	Seaver et al. (2015)
Zea mays	11,623	1,588	Leaf: mesophyll and bundle sheath cells	de Oliveira Dal'Molin et al. (2010a, b)

Applications of Plant GSM Models

GSM models have been extensively used in a variety of applications in single-celled organism. However, due to the complex nature of plant metabolism, these applications are still being expanded to plant models. For a review of applications of microbial GSM models, see (Oberhardt et al. 2009). Plant genome-scale models have been used to examine the metabolism within a plant, predict the effect of genetic and environmental perturbations, and examine the metabolite transport between tissues and organs.

Elucidating Metabolic Fluxes and Identifying Knowledge Gaps

Metabolic models can be used to determine and predict knowledge gaps in an organism's secondary metabolism (Fritz et al. 2006). GSM models can systematically identify the set of metabolites that are known to be produced within the plant, but have no viable path given the set of metabolic reactions using an algorithm termed GapFind (Kumar et al. 2007). GSM models can be directly utilized to identify metabolites with a known biosynthetic pathway that have not yet been identified for the plant of interest. Optimization techniques (Kumar et al. 2007; Maranas and Zomorrodi 2016; Thiele et al. 2014) have been developed to identify the minimum number of reactions that must be added from either closely related organisms or the full database of known metabolic transformations to provide a feasible path to the metabolite of interest. Plants are able to produce an expansive pool of diverse chemicals, of which only a small fraction of the secondary metabolites have known synthesis pathways (Tatsis and O'Connor 2016). Computational frameworks have emerged to predict novel metabolic pathways to metabolites with unknown synthesis by utilizing known reaction rules (Li et al. 2004; Hatzimanikatis et al. 2005; Jeffryes et al. 2015). While this framework does not directly utilize GSM models, the gaps in metabolism can be quickly and systematically identified and GSM models can be used to ensure that the predicted pathways including cofactor production are viable given the plant's metabolism.

A multi-tissue model of maize was used to determine the reactions that are coupled to nitrogen uptake and the changes in metabolism due to the nitrogen source to aid in the understanding of nitrogen use efficiency (de Oliveira Dal'Molin et al. 2015). A coupling analysis (Price et al. 2004) was completed to determine reaction sets that contain highly or perfectly correlated reactions. Nitrate/nitrite reductase, glutamate translocation, and sucrose translocation are perfectly correlated (de Oliveira Dal'Molin et al. 2015), implying that a genetic perturbation that changes the flux through any one of the associated reactions will equivalently affect the other remaining reactions. The authors also examined the metabolic costs associated with nitrate vs. ammonium uptake and identified that even with the large network flexibility

in plants, nitrate uptake requires 17% more C fixation than ammonium to maintain the same growth (de Oliveira Dal'Molin et al. 2015). The higher required C fixation is linked to an increase in starch accumulation during the day, which is required to support growth during the night (de Oliveira Dal'Molin et al. 2015).

Predicting the Effects of Environmental and Genetic Perturbations

Given the significant resources required to develop transgenic crops (Rothstein et al. 2014), it is important to be able to predict the effect of a genetic change on metabolism (Beatty et al. 2016a, b). Microbial GSM models have been used to identify the lethality of each metabolic gene, including predicting synthetic lethal sets (Suthers et al. 2009; Pratapa et al. 2015). While this type of analysis can be utilized in a predictive manner, discrepancies in gene lethality compared to experimental results can help point the presence of isozymes or indicate the function of an unknown pathway (Chowdhury et al. 2015). A number of *ad hoc* reaction constraints have been included in GSM models to represent the complicated relationships between the reaction flux and the gene expression, protein concentration, or enzyme level (see Blazier and Papin 2012; Hyduke et al. 2013; Saha et al. 2014 for in-depth reviews of these algorithms). The simulation strategies fall into two main approaches known as the "switch" and "valve" approach. The "switch" approach is used to turn off or remove reactions based on a significantly low level of expression or activity, while the "valve" approach constrains the flux (to a non-zero flux) based on the change in expression, concentration, or level in one condition compared to a wild-type condition. Returning to our analogy with a pipe network, the "switch" approach would correspond to blocking all flow through one pipe (Fig. 4.1b), while the "valve" approach would be representative of replacing a pipe with a narrower pipe (Fig. 4.1c). The flux through the metabolic reaction (or pipe) is not forced to operate at the defined level; however, the constraints added in the "valve" approach decrease the maximum allowed flux through the reaction (or pipe). Both approaches take into account the underlying GPR relationships when determining the effect of the expression level on reactions.

A large-scale model of developing *Brassica napus* embryos (Hay and Schwender 2011a, b) was used to determine the percent of carbon uptake that was stored in biomass for a variety of carbon and nitrogen substrates. The model was used to test the carbon conversion efficiency for glucose, sucrose, and fructose each paired with ammonia, nitrate, alanine, asparagine, glutamine, and glutamate. The findings revealed that sucrose was always the most efficient carbon source followed by fructose, then glucose regardless of the nitrogen substrate (Hay and Schwender 2011b). Ammonia was the most efficient nitrogen source followed by alanine, glutamine, asparagine, glutamate and then nitrate, regardless of the carbon substrate (Hay and Schwender 2011b). When sucrose and ammonium were used as the sole carbon and nitrogen sources, a maximum theoretical carbon conversion efficiency

of approximately 70% was observed (Hay and Schwender 2011b). Compared to nitrate, ammonium utilization decreases the energy consumption required to synthesize nitrogen-containing organic compounds (Williams et al. 1987). The large-scale model of a developing *B. napus* seed was used to predict the variability in the composition of the organism's nutrient uptake given the predicted minimum total nutrient uptake required for the experimentally observed biomass formation (Hay and Schwender 2011a). When organic nitrogen sources (i.e., glutamine, glutamate, alanine, and asparagine) are available, glutamine is the only nitrogen source utilized because it has the highest carbon conversion efficiency (Hay and Schwender 2011a). Similarly, ammonium is the only nitrogen source taken up when inorganic nitrogen sources are supplied. The authors identified that PEP carboxylation differed between inorganic and organic nitrogen sources (Hay and Schwender 2011a). Flux in the direction of carboxylation is essential only when inorganic nitrogen is available (Hay and Schwender 2011a), suggesting an important link between nitrogen assimilation and carbon metabolism.

A GSM model of the maize leaf was developed to examine the metabolic changes between two glutamine synthetase mutants (i.e., the *gln1-3* and *gln1-4* mutants) and a low N supply condition compared to a wild-type N replete condition (Simons et al. 2014a, b). To simulate each condition, the cell-type specific reactions corresponding to the glutamine synthetase mutants were turned off (i.e., the glutamine synthetase reaction in the mesophyll cell was blocked for the *gln1-3* mutant and the glutamine synthetase reaction in the bundle sheath cell was blocked for the *gln1-4* mutant), the low N condition was supplied 1000-fold less nitrogen compared to the N replete condition, and the reactions corresponding to statistically lowly expressed genes and proteins were blocked using the "switch" approach (Simons et al. 2014a, b). The metabolism is significantly perturbed in the *gln1-3* and *gln1-4* mutants, with 49 and 45% of the metabolic reactions containing flux ranges that must change compared to the wild-type nitrogen replete condition, respectively (i.e., the flux range associated with the glutamine synthetase mutant does not overlap with the solution flux range associated with the wild-type condition) (Simons et al. 2014a, b). In contrast, only 7% of reactions in the low nitrogen supply condition have non-overlapping flux ranges compared to the nitrogen replete condition (Simons et al. 2014a, b). This indicates that there is a large metabolic work-around required in the glutamine synthetase mutants (Simons et al. 2014a, b).

Describing Metabolism Within Tissues or Organs

Plant cells contain a highly compartmentalized and complex metabolic network. By separating metabolic processes into various compartments, cells, and organs, the plant is able to obtain higher concentrations of a metabolite in the vicinity of related enzymes and create distinct organs that are specialized for a specific function. This compartmentalization is the backbone of C4 photosynthesis, which increases CO_2 concentration near RuBisCO by dividing carbon fixation between two cell types

within the leaf (i.e., the Mesophyll and Bundle Sheath cell) (Leegood 2002). The large degree of compartmentalization and specialization observed in plants requires a large number of metabolites to be transported throughout the plant. Within Arabidopsis, 882 recognized membrane transport proteins were identified (Ren and Paulsen 2005). Still, many transported metabolites and their associated proteins have yet to be identified (Linka and Weber 2010; Linka and Theodoulou 2013).

Plant metabolic models provide a promising avenue to model and predict the transport between a cell's internal compartments, between two cells, and between multiple organs. Draft plant GSM models can be created by including reactions based on location-specific molecular data sources and known metabolic transporters (Simons et al. 2014a, b). However, many draft models are not able to perform known metabolic function and additional ad hoc transporters and reactions must be included to restore network connectivity. Generally, the minimum number of reactions and transporters are added to restore network connectivity (Kumar et al. 2007; Jerby et al. 2010). In addition to the known transporters, 35 inter-organelle transporters were required to produce the biomass components within a maize metabolic model (Saha et al. 2011).

The transport of many metabolites requires energy as they are driven by electrochemical gradients that are maintained by ATP expenditure (Ramos et al. 1976; Sze 1984). Cheung et al. (2013) demonstrated that including accurate transport costs increases the accuracy of fluxes predicted by GSMs in central metabolism by comparing GSM model-predicted fluxes to fluxes predicted using ^{13}C metabolic flux analysis (MFA). Due to the challenge of identifying the transport mechanisms and energy demands associated with translocation, de Oliveira Dal'Molin et al. created a penalty weight to capture the coupling of transport to ATP hydrolysis (de Oliveira Dal'Molin et al. 2015). By varying this penalty weight, the authors were able to examine the effect of active transport on tissue translocation (de Oliveira Dal'Molin et al. 2015). With free tissue translocation, the GSM model predicts that nitrate taken up by the root is transported to the leaves where it is assimilated into glutamate (de Oliveira Dal'Molin et al. 2015). Under high-energy costs associated with tissue translocations, nitrate is assimilated in the root to avoid the transport costs (de Oliveira Dal'Molin et al. 2015).

Incorporation of GSM Models with Nonlinear Models

Detailed mechanistic models can be combined with GSM models providing a more accurate representation of metabolism by adding constraints, defining model inputs, or setting outputs based on complex models. Detailed models describe only a few reactions or physiological responses in high detail. However, these high-level nonlinear models can be combined with a GSM model to predict the whole-plant or whole-cell metabolic response to the environment or perturbation. This type of combination with a GSM model has been completed for maize to examine the metabolism along 15 segments of the developing leaf (Bogart and Myers 2016).

The rate between carbon fixation and oxygenase activity by Rubisco depends nonlinearly on the oxygen and carbon dioxide concentrations. This nonlinear relationship was incorporated into a maize GSM model by setting the flux through the associated reactions based on the nonlinear relationship (Bogart and Myers 2016). Using the developed model, the authors report the spatial transport of nitrogen through the leaf (Bogart and Myers 2016).

While this type of nonlinear model in combination with a GSM model has not yet focused on nitrogen metabolism and improving NUE, there is promise in combining the multiple types of models together. This combination can allow for a more thorough understanding of nitrogen metabolism by targeting responses that are outside of the realm of GSM models (see section "Incorporating Various Modeling Frameworks to Improve Nitrogen Use Efficiency").

Potential of Plant GSMs for Improving NUE in Plants

Plant GSM models have expanded in size and complexity in the last decade to new applications of examining the metabolite transport between tissues and incorporating new constraints to more accurately model metabolism. Plant GSM models provide a promising avenue to improve NUE by predicting the effect of genetic and environmental perturbations on metabolism. It has been predicted that the most effective NUE engineering strategies will target the flows of carbon and nitrogen through the metabolic network and will not simply focus on concentrations of individual metabolites (Beatty et al. 2016a, b). In addition, NIN-LIKE PROTEIN 7 (NLP7) was identified to significantly improve plant growth by coordinately enhancing both nitrogen assimilation and carbon assimilation, indicating the importance of the balance between carbon and nitrogen (Yu et al. 2016). Plant GSM models simultaneously model carbon and nitrogen metabolism providing a simulation technique for predicting the impacts of environmental and genetic perturbations on carbon and nitrogen metabolism.

Utilizing Optimization Techniques to Aid Metabolic Engineering

An arsenal of algorithms has been developed for microbial GSM models to successfully guide metabolic engineering (Fig. 4.2a). Algorithms such as OptKnock (Burgard et al. 2003), OptGene (Patil et al. 2005), OptReg (Pharkya and Maranas 2006), OptStrain (Pharkya et al. 2004), OptForce (Ranganathan et al. 2010), and EMiLio (Yang et al. 2011) have been used to predict the changes in reactions or their associated genes that lead to the increase of a product of interest. These optimization-based algorithms predict the gene knockouts, knockins, and/or knockdowns that maintain organism growth and increase the production of a

target metabolite. Among many other success stories, optimization algorithms using GSM models have aided in increasing sesquiterpene production in *Saccharomyces cerevisiae* (Asadollahi et al. 2009), ethanol yield in *S. cerevisiae* (Bro et al. 2006), and fatty acid production in *E. coli* (Tee et al. 2014). Because GSM models do not account for control mechanisms and their response to genetic perturbations, a "design-build-test-learn" cycle (Nielsen and Keasling 2016) has been proposed to improve genetic engineering and the predictions made by GSM models.

Plant metabolic networks have a high connectivity and plants have multiple isozymes even within the same cell (Sweetlove and Fernie 2013). To divert flux through reactions that will improve the plant's NUE, it is necessary to consider the whole plant's metabolism and engineer all alternative routes through the network (Sweetlove et al. 2017). Optimization techniques, such as OptGene (Patil et al. 2005), can be used to determine the minimum number of gene deletion strategies to increase NUE closer to the theoretical maximum.

Incorporating Various Modeling Frameworks to Improve Nitrogen Use Efficiency

By using detailed plant and environmental models, constraints can be elucidated that sharpen the predictions of GSM models (Fig. 4.2b). Crop models that simulate the effect of perturbations on specific fluxes, flux ratios, nutrient uptake, tissue growth rate ratios, or overall yield can serve as input or to impose constraints on the GSM model. Nonlinear and crop models can be used to expand the scope of GSM models to include irradiance (Rasse and Tocquin 2006), temperature (White et al. 2005), crop rotations (Kollas et al. 2015; Osman et al. 2015), soil properties (Liang et al. 2016), rainfall (Mishra et al. 2008; Hansen 2005), and climate data (Hansen 2005; Kang et al. 2009), which have previously been difficult to incorporate due to their indirect effect on metabolism.

Models, such as the carbon dynamic model (Rasse and Tocquin 2006) and the Water Heat Carbon Nitrogen Simulator (WHCNS) (Liang et al. 2016), can provide the necessary inputs or constraints on plant GSM models to expand their scope. A carbon dynamic model of *Arabidopsis* could utilize input data in the form of irradiance, CO_2 levels, temperature, and photoperiod to obtain information regarding the root to shoot allocation and sugar–starch partitioning (Rasse and Tocquin 2006), which can serve as constraints for a multi-tissue GSM model. By combining soil–crop models and the ability to represent various environmental conditions, Liang et al. developed a soil WHCNS model (Liang et al. 2016). The WHCNS model incorporates climate data, crop rotations, water availability, and fertilizer management to predict the soil water content, soil nitrate and ammonia concentration, crop nitrogen uptake, ammonia volatilization, root growth, leaf area index, crop yield, and the ratio of root, stem, and leaf dry matter (Liang et al. 2016). The WHCNS model was able to explain 84% of the variation in leaf area index and

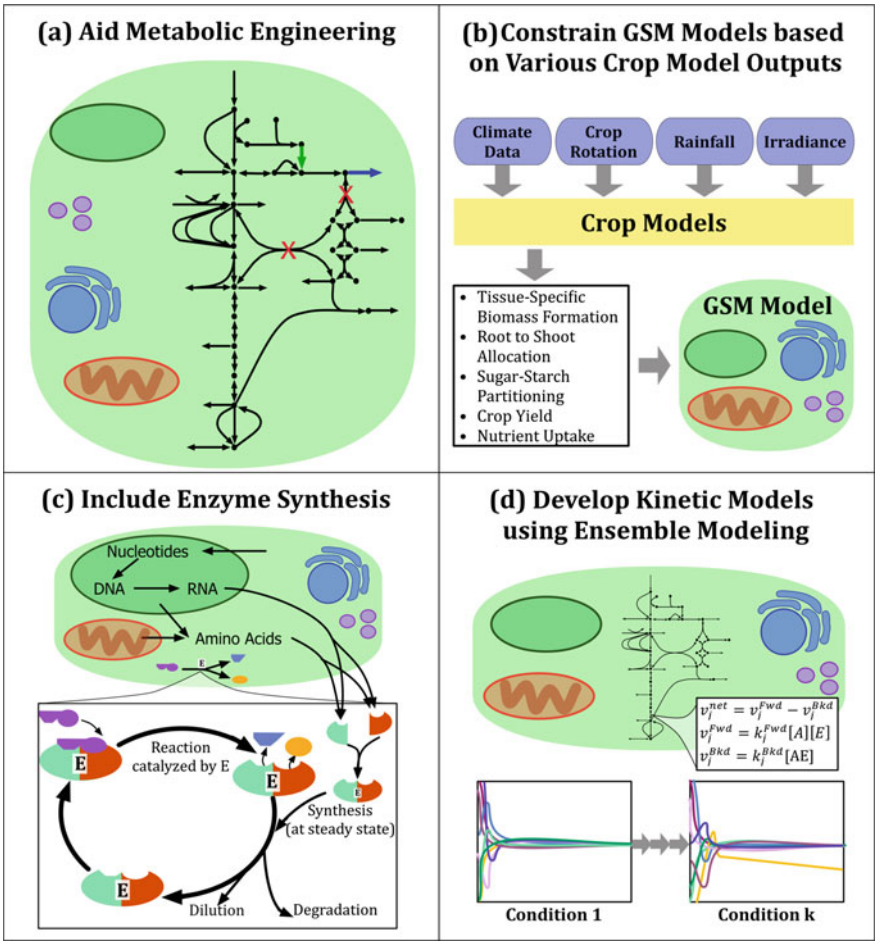


Fig. 4.2 Future applications of genome-scale metabolic (GSM) models. **a** GSM models can be used to improve nitrogen use efficiency (NUE) by suggesting genetic manipulations, such as reaction knockouts (represented by red x's) and overexpression (represented by the green arrow) in order to maximize the flux through a target reaction (represented by the blue arrow). **b** Crop models can serve as a link between data with non-intuitive relationships to metabolic effects and GSM models. Predictions made by the crop models can be incorporated as constraints in the GSM model. **c** Enzyme synthesis costs can be directly included in the model by accounting for the synthesis, degradation, and dilution rates of each enzyme. This allows for a more thorough model that can more closely be linked to transcriptomic and proteomic data (adapted from Lerman et al. 2012). **d** Kinetic plant models can be developed using ensemble modeling. Various sets of kinetic parameters that result in the expected solution are developed. Then the system is perturbed based on known effects and sets of parameters that do not recapitulate the known perturbations are removed (adapted from Khodayari and Maranas 2016)

dry matter (Liang et al. 2016). Crop outputs such as nitrogen uptake, crop yield, and the ratio of root, stem, and leaf dry matter can be directly incorporated into the GSM model predicting the changes in internal metabolism as a result of climate changes, crop rotation, water availability, and fertilizer management.

Combined models are required to elucidate the changes in metabolism as a consequence of known NUE factors that are not directly related to inputs of GSM models (Benincasa et al. 2011). The combinations of various types of models can be used to expand the scope of GSM models to environmental factors such as plant density, temperature, and light intensity. NUE depends directly on the nitrate and ammonium uptake, as well as the biological nitrogen fixation and amino acid uptake available from the soil (Beatty et al. 2016a, b). These soil properties are directly related to the crop rotation schedule (Carpenter-Boggs et al. 2000). NUE genes have also been related to carbon to nitrogen storage, nitrogen remobilization and senescence (McAllister et al. 2012).

Including Enzyme Synthesis Costs in GSM Models

Metabolism and Expression (ME) models (Fig. 4.2c) simulate the activity of transcription and translation providing a closer link to transcriptomic and proteomic data compared to standard GSM models. ME models, a major advancement in bacterial GSM modeling, expand metabolic reactions to include representative reactions for the production and degradation of the cell's macromolecular machinery. The metabolic conversion through a reaction then depends on the production of the associated enzyme. These models have been developed on a genome scale for *Thermotoga maritima* (Lerman et al. 2012) and *Escherichia coli* (O'Brien et al. 2013). A simplified model of *Synechocystis* sp. PCC 6803 was developed to model enzyme synthesis including light reactions, linear and cyclic electron transport, CO_2 uptake, carbon fixation, glycogen synthesis, TCA cycle, respiration, uptake of inorganic ions, and the synthesis of precursors of biomass formation (Rügen et al. 2015). The authors have developed an approach termed conditional FBA, which adds the following constraints: The total flux through a reaction is limited by the amount of the associated enzyme, the total enzyme production is bounded by the amount of ribosome, and light harvesting is constrained by the formation of pigments (Rügen et al. 2015). By incorporating these constraints, the authors were able to model the temporal organization and conditional dependences that occur within a diurnal organism matching with reasonable agreement several known properties of phototrophic metabolism.

Using the simplified *Synechocystis* model as a template, plant GSM models can be expanded to incorporate enzyme synthesis. Elucidating the metabolic changes throughout the day–night cycle is important to improving NUE as nitrogen uptake, nitrate reductase, and cytosolic glutamine synthetase change diurnally (Matt et al. 2001). The nitrate uptake rate depends on light intensity, which impacts the carbon uptake and growth (Delhon et al. 1996). Developing a ME model of plant

metabolism will be a beneficial tool, for more accurately incorporating the plethora of transcriptomic (Hayes et al. 2010; Usadel et al. 2009; Ko et al. 2016) and proteomic (Riter et al. 2011) data, even for cases that do not focus on the diurnal metabolic changes. Many transcriptomic and proteomic studies have focused on the changes in gene expression and quantity of protein expression under N-limiting (Amiour et al. 2012; Wang et al. 2003), water deficit (Opitz et al. 2014; Li et al. 2017; Shao et al. 2015), and long photoperiod (Wu et al. 2016) conditions. A more thorough understanding of metabolism can lead to improving NUE (Simons et al. 2014a, b), increasing drought tolerance, or enhancing photosynthetic efficiency by developing a model that can more accurately incorporate the observed changes captured in transcriptomic and proteomic data.

Kinetic Models of Plants

Flux balance analysis of GSM models assumes that for each metabolite there is no change in concentration over time. Kinetic models, however, are able to simulate the dynamic change in concentration over time by including enzyme parameters (Smallbone et al. 2010; Jamshidi and Palsson 2008). Kinetic models can also directly incorporate substrate concentrations, enzyme levels, and substrate-level regulatory barriers (Jamshidi and Palsson 2008; Khodayari and Maranas 2016). To model the organism's dynamic metabolism, reaction rate laws and their associated parameters must be included for each reaction that is catalyzed by an enzyme in the model. This includes a large number of parameters that are often unknown. An ensemble modeling approach (Tan et al. 2011) (Fig. 4.2d) samples a large number of parameters to create an ensemble of models. Parameter sets within this large number of models are removed based on their inability to replicate known effects of genetic or environmental perturbations (Tan et al. 2011). This ensemble modeling approach was recently applied to create a genome-scale kinetic model of *E. coli* that could capture a wide variety of perturbations (Khodayari and Maranas 2016). GSM models have been able to successfully predict the effect of genetic manipulations (Cardenas and Da Silva 2014; Xu et al. 2011; Lin et al. 2013). However, many designed mutants fail (Khodayari et al. 2014), making the ensemble approach to kinetic modeling essential to accurately predicting the effect of genetic manipulations. While genome-scale models of plants are much larger and more complex than *E. coli* models, this type of ensemble modeling approach can be expanded to include plant primary metabolism. As the required computational time is further optimized, a genome-scale kinetic model of the whole plant can be developed providing a powerful tool for predicting the effects of genetic and environmental perturbations and predicting genetic interventions.

References

Bao A, Zhao Z, Ding G, Shi L, Xu F, Cai H (2014) Accumulated expression level of cytosolic glutamine synthetase 1 gene (OsGS1;1 or OsGS1;2) alter plant development and the carbon-nitrogen metabolic status in rice. PLoS ONE 9(4):e95581. https://doi.org/10.1371/journal.pone.0095581

Orth J, Thiele I, Palsson B (2010) What is flux balance analysis? Nature Biotechnol 28:245–248

Chan SHJ, Cai J, Wang L, Simons-Senftle MN, Maranas CD (2017) Standardizing biomass reactions and ensuring complete mass balance in genome-scale metabolic models. Bioinformatics btx453

Mahadevan R, Schilling CH (2003) The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. Metab Eng 5(4):264–276

Lewis NE, Hixson KK, Conrad TM, Lerman JA, Charusanti P, Polpitiya AD, Adkins JN, Schramm G, Purvine SO, Lopez-Ferrer D, Weitz KK, Eils R, Konig R, Smith RD, Palsson BO (2010) Omic data from evolved E. coli are consistent with computed optimal growth from genome-scale models. Mol Syst Biol 6:390. https://doi.org/10.1038/msb.2010.47

Lakshmanan M, Koh G, Chung BK, Lee DY (2014) Software applications for flux balance analysis. Brief Bioinform 15(1):108–122. https://doi.org/10.1093/bib/bbs069

Dandekar T, Fieselmann A, Majeed S, Ahmed Z (2014) Software applications toward quantitative metabolic flux analysis and modeling. Brief Bioinform 15(1):91–107. https://doi.org/10.1093/bib/bbs065

Ebrahim A, Lerman JA, Palsson BO, Hyduke DR (2013) COBRApy: constraints-based reconstruction and analysis for python. BMC Syst Biol 7, Artn 74. https://doi.org/10.1186/1752-0509-7-74

Arkin AP, Stevens RL, Cottingham RW, Maslov S, Henry CS, Dehal P, Ware D, Perez F, Harris NL, Canon S, Sneddon MW, Henderson ML, Riehl WJ, Gunter D, Murphy-Olson D, Chan S, Kamimura RT, Brettin TS, Meyer F, Chivian D, Weston DJ, Glass EM, Davison BH, Kumari S, Allen BH, Baumohl J, Best AA, Bowen B, Brenner SE, Bun CC, Chandonia J-M, Chia J-M, Colasanti R, Conrad N, Davis JJ, DeJongh M, Devoid S, Dietrich E, Drake MM, Dubchak I, Edirisinghe JN, Fang G, Faria JP, Frybarger PM, Gerlach W, Gerstein M, Gurtowski J, Haun HL, He F, Jain R, Joachimiak MP, Keegan KP, Kondo S, Kumar V, Land ML, Mills M, Novichkov P, Oh T, Olsen GJ, Olson B, Parrello B, Pasternak S, Pearson E, Poon SS, Price G, Ramakrishnan S, Ranjan P, Ronald PC, Schatz MC, Seaver SMD, Shukla M, Sutormin RA, Syed MH, Thomason J, Tintle NL, Wang D, Xia F, Yoo H, Yoo S (2016) The DOE systems biology knowledgebase (KBase). bioRxiv. https://doi.org/10.1101/096354

Edwards JS, Palsson BO (1999) Systems properties of the Haemophilus influenzae Rd metabolic genotype. J Biol Chem 274(25):17410–17416

Shastri AA, Morgan JA (2005) Flux balance analysis of photoautotrophic metabolism. Biotechnol Prog 21(6):1617–1626. https://doi.org/10.1021/bp050246d

Grafahrend-Belau E, Schreiber F, Koschutzki D, Junker BH (2009) Flux balance analysis of barley seeds: a computational approach to study systemic properties of central metabolism. Plant Physiol 149(1):585–598. https://doi.org/10.1104/pp.108.129635

Poolman MG, Miguet L, Sweetlove LJ, Fell DA (2009) A genome-scale metabolic model of Arabidopsis and some of its properties. Plant Physiol 151(3):1570–1581. https://doi.org/10.1104/pp.109.141267

de Oliveira Dal'Molin CG, Quek LE, Palfreyman RW, Brumbley SM, Nielsen LK (2010a) AraGEM, a genome-scale reconstruction of the primary metabolic network in Arabidopsis. Plant Physiol 152(2):579–589. https://doi.org/10.1104/pp.109.148817

Saha R, Suthers PF, Maranas CD (2011) Zea mays iRS1563: a comprehensive genome-scale metabolic reconstruction of maize metabolism. Plos One 6(7). https://doi.org/10.1371/journal.pone.0021784

Hay J, Schwender J (2011a) Metabolic network reconstruction and flux variability analysis of storage synthesis in developing oilseed rape (Brassica napus L.) embryos. Plant J 67(3):526–541. https://doi.org/10.1111/j.1365-313X.2011.04613.x

Yuan H, Cheung CY, Poolman MG, Hilbers PA, van Riel NA (2016) A genome-scale metabolic network reconstruction of tomato (Solanum lycopersicum L.) and its application to photorespiratory metabolism. Plant J 85(2):289–304. https://doi.org/10.1111/tpj.13075

Poolman MG, Kundu S, Shaw R, Fell DA (2013) Responses to light intensity in a genome-scale model of rice metabolism. Plant Physiol 162(2):1060–1072. https://doi.org/10.1104/pp.113.216762

Lakshmanan M, Zhang Z, Mohanty B, Kwon JY, Choi HY, Nam HJ, Kim DI, Lee DY (2013) Elucidating rice cell metabolism under flooding and drought stresses using flux-based modeling and analysis. Plant Physiol 162(4):2140–2150. https://doi.org/10.1104/pp.113.220178

Simons M, Saha R, Amiour N, Kumar A, Guillard L, Clément G, Miquel M, Li Z, Mouille G, Lea PJ, Hirel B, Maranas CD (2014a) Assessing the metabolic impact of nitrogen availability using a compartmentalized maize leaf genome-scale model. Plant Physiol 166(3):1659–1674. https://doi.org/10.1104/pp.114.245787

Seaver SM, Bradbury LM, Frelin O, Zarecki R, Ruppin E, Hanson AD, Henry CS (2015) Improved evidence-based genome-scale metabolic models for maize leaf, embryo, and endosperm. Front Plant Sci 6:142. https://doi.org/10.3389/fpls.2015.00142

de Oliveira Dal'Molin CG, Quek LE, Palfreyman RW, Brumbley SM, Nielsen LK (2010b) C4GEM, a genome-scale metabolic model to study C4 plant metabolism. Plant Physiol 154(4):1871–1885. https://doi.org/10.1104/pp.110.166488

de Oliveira Dal'Molin CG, Quek LE, Saa PA, Nielsen LK (2015) A multi-tissue genome-scale metabolic modeling framework for the analysis of whole plant systems. Front Plant Sci 6:4. https://doi.org/10.3389/fpls.2015.00004

Grafahrend-Belau E, Junker A, Eschenroder A, Muller J, Schreiber F, Junker BH (2013) Multiscale metabolic modeling: dynamic flux balance analysis on a whole-plant scale. Plant Physiol 163(2):637–647. https://doi.org/10.1104/pp.113.224006

Oberhardt MA, Palsson BO, Papin JA (2009) Applications of genome-scale metabolic reconstructions. Mol Syst Biol 5:320. https://doi.org/10.1038/msb.2009.77

Fritz C, Palacios-Rojas N, Feil R, Stitt M (2006) Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. Plant J 46(4):533–548. https://doi.org/10.1111/j.1365-313X.2006.02715.x

Kumar VS, Dasika MS, Maranas CD (2007) Optimization based automated curation of metabolic reconstructions. BMC Bioinf 8:212. https://doi.org/10.1186/1471-2105-8-212

Maranas CD, Zomorrodi AR (2016) Resolving network gaps and growth prediction inconsistencies in metabolic networks. In: Optimization methods in metabolic networks. Wiley, Inc, pp 119–135. https://doi.org/10.1002/9781119188902.ch6

Thiele I, Vlassis N, Fleming RM (2014) fastGapFill: efficient gap filling in metabolic networks. Bioinformatics 30(17):2529–2531. https://doi.org/10.1093/bioinformatics/btu321

Tatsis EC, O'Connor SE (2016) New developments in engineering plant metabolic pathways. Curr Opin Biotechnol 42:126–132. https://doi.org/10.1016/j.copbio.2016.04.012

Li CH, Henry CS, Jankowski MD, Ionita JA, Hatzimanikatis V, Broadbelt LJ (2004) Computational discovery of biochemical routes to specialty chemicals. Chem Eng Sci 59(22–23):5051–5060. https://doi.org/10.1016/j.ces.2004.09.021

Hatzimanikatis V, Li C, Ionita JA, Henry CS, Jankowski MD, Broadbelt LJ (2005) Exploring the diversity of complex metabolic networks. Bioinformatics 21(8):1603–1609. https://doi.org/10.1093/bioinformatics/bti213

Jeffryes JG, Colastani RL, Elbadawi-Sidhu M, Kind T, Niehaus TD, Broadbelt LJ, Hanson AD, Fiehn O, Tyo KE, Henry CS (2015) MINEs: open access databases of computationally predicted enzyme promiscuity products for untargeted metabolomics. J Cheminform 7:44. https://doi.org/10.1186/s13321-015-0087-1

Price ND, Schellenberger J, Palsson BO (2004) Uniform sampling of steady-state flux spaces: means to design experiments and to interpret enzymopathies. Biophys J 87(4):2172–2186. https://doi.org/10.1529/biophysj.104.043000

Rothstein SJ, Bi YM, Coneva V, Han M, Good A (2014) The challenges of commercializing second-generation transgenic crop traits necessitate the development of international public sector research infrastructure. J Exp Bot 65(19):5673–5682. https://doi.org/10.1093/jxb/eru236

Beatty PH, Klein MS, Fischer JJ, Lewis IA, Muench DG, Good AG (2016) Understanding plant nitrogen metabolism through metabolomics and computational approaches. Plants (Basel) 5(4). https://doi.org/10.3390/plants5040039

Suthers PF, Zomorrodi A, Maranas CD (2009) Genome-scale gene/reaction essentiality and synthetic lethality analysis. Mol Syst Biol 5:301. https://doi.org/10.1038/msb.2009.56

Pratapa A, Balachandran S, Raman K (2015) Fast-SL: an efficient algorithm to identify synthetic lethal sets in metabolic networks. Bioinformatics 31(20):3299–3305. https://doi.org/10.1093/bioinformatics/btv352

Chowdhury R, Chowdhury A, Maranas CD (2015) Using gene essentiality and synthetic lethality information to correct yeast and CHO cell genome-scale models. Metabolites 5(4):536–570. https://doi.org/10.3390/metabo5040536

Blazier AS, Papin JA (2012) Integration of expression data in genome-scale metabolic network reconstructions. Front Physiol 3:299. https://doi.org/10.3389/fphys.2012.00299

Hyduke DR, Lewis NE, Palsson BO (2013) Analysis of omics data with genome-scale models of metabolism. Mol BioSyst 9(2):167–174. https://doi.org/10.1039/c2mb25453k

Saha R, Chowdhury A, Maranas CD (2014) Recent advances in the reconstruction of metabolic models and integration of omics data. Curr Opin Biotechnol 29:39–45. https://doi.org/10.1016/j.copbio.2014.02.011

Hay J, Schwender J (2011b) Computational analysis of storage synthesis in developing Brassica napus L. (oilseed rape) embryos: flux variability analysis in relation to (1)(3)C metabolic flux analysis. Plant J 67(3):513–525. https://doi.org/10.1111/j.1365-313X.2011.04611.x

Williams K, Percival F, Merino J, Mooney HA (1987) Estimation of tissue construction cost from heat of combustion and organic nitrogen-content. Plant, Cell Environ 10(9):725–734

Leegood RC (2002) C4 photosynthesis: principles of CO2 concentration and prospects for its introduction into C3 plants. J Exp Bot 53(369):581–590. https://doi.org/10.1093/jexbot/53.369.581

Ren QH, Paulsen IT (2005) Comparative analyses of fundamental differences in membrane transport capabilities in prokaryotes and eukaryotes. PLoS Comput Biol 1 (3):190–201, Artn e27. https://doi.org/10.1371/journal.pcbi.0010027

Linka N, Weber AP (2010) Intracellular metabolite transporters in plants. Mol Plant 3(1):21–53. https://doi.org/10.1093/mp/ssp108

Linka N, Theodoulou FL (2013) Metabolite transporters of the plant peroxisomal membrane: known and unknown. In: del Río LA (ed) Peroxisomes and their key role in cellular signaling and metabolism. Springer Netherlands, Dordrecht, pp 169–194. https://doi.org/10.1007/978-94-007-6889-5_10

Jerby L, Shlomi T, Ruppin E (2010) Computational reconstruction of tissue-specific metabolic models: application to human liver metabolism. Mol Syst Biol 6:401. https://doi.org/10.1038/msb.2010.56

Ramos S, Schuldiner S, Kaback HR (1976) The electrochemical gradient of protons and its relationship to active transport in Escherichia coli membrane vesicles. Proc Natl Acad Sci U. S. A 73(6):1892–1896

Sze H (1984) H^+ -translocating ATPases of the plasma membrane and tonoplast of plant cells. Physiol Plant 61(4):683–691. https://doi.org/10.1111/j.1399-3054.1984.tb05191.x

Cheung CY, Williams TC, Poolman MG, Fell DA, Ratcliffe RG, Sweetlove LJ (2013) A method for accounting for maintenance costs in flux balance analysis improves the prediction of plant cell metabolic phenotypes under stress conditions. Plant J 75(6):1050–1061. https://doi.org/10.1111/tpj.12252

Bogart E, Myers CR (2016) Multiscale metabolic modeling of C4 plants: connecting nonlinear genome-scale models to leaf-scale metabolism in developing maize leaves. PLoS One 11(3): e0151722. https://doi.org/10.1371/journal.pone.0151722

Beatty PH, Klein MS, Fischer JJ, Lewis IA, Muench DG, Good AG (2016b) Understanding plant nitrogen metabolism through metabolomics and computational approaches. Plants 5(4):39. https://doi.org/10.3390/plants5040039

Yu LH, Wu J, Tang H, Yuan Y, Wang SM, Wang YP, Zhu QS, Li SG, Xiang CB (2016) Overexpression of Arabidopsis NLP7 improves plant growth under both nitrogen-limiting and -sufficient conditions by enhancing nitrogen and carbon assimilation. Sci Rep 6, Artn 27795. https://doi.org/10.1038/srep27795

Burgard A, Pharkya P, Maranas C (2003) OptKnock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. Biotechnol Bioeng 84:647–657

Patil KR, Rocha I, Forster J, Nielsen J (2005) Evolutionary programming as a platform for in silico metabolic engineering. BMC Bioinform 6, Artn 308. https://doi.org/10.1186/1471-2105-6-308

Pharkya P, Maranas C (2006) An optimization framework for identifying reaction activation/inhibition or elimination candidates for overproduction in microbial systems. Metab Eng 8:1–13

Pharkya P, Burgard AP, Maranas CD (2004) OptStrain: a computational framework for redesign of microbial production systems. Genome Res 14(11):2367–2376. https://doi.org/10.1101/gr.2872004

Ranganathan S, Suthers P, Maranas C (2010) OptForce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. PLoS Comput Biol 6:e1000744

Yang L, Cluett WR, Mahadevan R (2011) EMILiO: a fast algorithm for genome-scale strain design. Metab Eng 13(3):272–281. https://doi.org/10.1016/j.ymben.2011.03.002

Asadollahi MA, Maury J, Patil KR, Schalk M, Clark A, Nielsen J (2009) Enhancing sesquiterpene production in Saccharomyces cerevisiae through in silico driven metabolic engineering. Metab Eng 11(6):328–334. https://doi.org/10.1016/j.ymben.2009.07.001

Bro C, Regenberg B, Forster J, Nielsen J (2006) In silico aided metabolic engineering of Saccharomyces cerevisiae for improved bioethanol production. Metab Eng 8(2):102–111. https://doi.org/10.1016/j.ymben.2005.09.007

Tee TW, Chowdhury A, Maranas CD, Shanks JV (2014) Systems metabolic engineering design: fatty acid production as an emerging case study. Biotechnol Bioeng 111(5):849–857. https://doi.org/10.1002/bit.25205

Nielsen J, Keasling JD (2016) Engineering cellular metabolism. Cell 164(6):1185–1197. https://doi.org/10.1016/j.cell.2016.02.004

Sweetlove LJ, Fernie AR (2013) The spatial organization of metabolism within the plant cell. Annu Rev Plant Biol 64(64):723–746. https://doi.org/10.1146/annurev-arplant-050312-120233

Sweetlove LJ, Nielsen J, Fernie AR (2017) Engineering central metabolism—a grand challenge for plant biologists. Plant J 90(4):749–763. https://doi.org/10.1111/tpj.13484

Rasse DP, Tocquin P (2006) Leaf carbohydrate controls over Arabidopsis growth and response to elevated CO2: an experimentally based model. The New Phytol 172(3):500–513. https://doi.org/10.1111/j.1469-8137.2006.01848.x

White JW, Hoogenboom G, Hunt LA (2005) A structured procedure for assessing how crop models respond to temperature. Agron J 97(2):426–439

Kollas C, Kersebaum KC, Nendel C, Manevski K, Muller C, Palosuo T, Armas-Herrera CM, Beaudoin N, Bindi M, Charfeddine M, Conradt T, Constantin J, Eitzinger J, Ewert F, Ferrise R, Gaiser T, de Cortazar-Atauri IG, Giglio L, Hlavinka P, Hoffmann H, Hoffmann MP, Launay M, Manderscheid R, Mary B, Mirschel W, Moriondo M, Olesen JE, Ouml;zturk I, Pacholski JE, Ripoche-Wachter D, Roggero PP, Roncossek S, Rotter RP, Ruget F, Sharif B, Trnka M, Sharif B, Sharif B, Ventrella D, Waha K, Wegehenkel M, Weigel HJ, Wu LH (2015) Crop rotation modelling—a European model intercomparison. Eur J Agron 70:98–111. https://doi.org/10.1016/j.eja.2015.06.007

Osman J, Inglada J, Dejoux JF (2015) Assessment of a Markov logic model of crop rotations for early crop mapping. Comput Electron Agr 113:234–243. https://doi.org/10.1016/j.compag.2015.02.015

Liang H, Hu K, Batchelor WD, Qi Z, Li B (2016) An integrated soil-crop system model for water and nitrogen management in North China. Sci Rep 6:25755. https://doi.org/10.1038/srep25755

Mishra A, Hansen JW, Dingkuhn M, Baron C, Traore SB, Ndiaye O, Ward MN (2008) Sorghum yield prediction from seasonal rainfall forecasts in Burkina Faso. Agr Forest Meteorol 148 (11):1798–1814. https://doi.org/10.1016/j.agrformet.2008.06.007

Hansen JW (2005) Integrating seasonal climate prediction and agricultural models for insights into agricultural practice. Philos Trans R Soc B 360(1463):2037–2047. https://doi.org/10.1098/rstb.2005.1747

Kang YH, Khan S, Ma XY (2009) Climate change impacts on crop yield, crop water productivity and food security—a review. Prog Nat Sci-Mater 19(12):1665–1674. https://doi.org/10.1016/j.pnsc.2009.08.001

Benincasa P, Guiducci M, Tei F (2011) The nitrogen use efficiency: meaning and sources of variation-case studies on three vegetable crops in central Italy. Horttechnology 21(3):266–273

Carpenter-Boggs L, Pikul JL, Vigil MF, Riedell WE (2000) Soil nitrogen mineralization influenced by crop rotation and nitrogen fertilization. Soil Sci Soc Am J 64(6):2038–2045

McAllister CH, Beatty PH, Good AG (2012) Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10(9):1011–1025. https://doi.org/10.1111/j.1467-7652.2012.00700.x

Lerman JA, Hyduke DR, Latif H, Portnoy VA, Lewis NE, Orth JD, Schrimpe-Rutledge AC, Smith RD, Adkins JN, Zengler K, Palsson BO (2012) In silico method for modelling metabolism and gene product expression at genome scale. Nat Commun 3:929. https://doi.org/10.1038/ncomms1928

O'Brien EJ, Lerman JA, Chang RL, Hyduke DR, Palsson BO (2013) Genome-scale models of metabolism and gene expression extend and refine growth phenotype prediction. Mol Syst Biol 9:693. https://doi.org/10.1038/msb.2013.52

Rügen M, Bockmayr A, Steuer R (2015) Elucidating temporal resource allocation and diurnal dynamics in phototrophic metabolism using conditional FBA. Sci Rep 5, Artn 15247. https://doi.org/10.1038/srep15247

Matt P, Geiger M, Walch-Liu P, Engels C, Krapp A, Stitt M (2001) The immediate cause of the diurnal changes of nitrogen metabolism in leaves of nitrate-replete tobacco: a major imbalance between the rate of nitrate reduction and the rates of nitrate uptake and ammonium metabolism during the first part of the light period. Plant, Cell Environ 24(2):177–190. https://doi.org/10.1111/j.1365-3040.2001.00676.x

Delhon P, Gojon A, Tillard P, Passama L (1996) Diurnal regulation of $NO3^{-}$ uptake in soybean plants IV. Dependence on current photosynthesis and sugar availability to the roots. J Exp Bot 47(7):893–900. https://doi.org/10.1093/jxb/47.7.893

Hayes KR, Beatty M, Meng X, Simmons CR, Habben JE, Danilevskaya ON (2010) Maize global transcriptomics reveals pervasive leaf diurnal rhythms but rhythms in developing ears are largely limited to the core oscillator. PLoS ONE 5(9):e12887. https://doi.org/10.1371/journal.pone.0012887

Usadel B, Poree F, Nagel A, Lohse M, Czedik-Eysenberg A, Stitt M (2009) A guide to using MapMan to visualize and compare Omics data in plants: a case study in the crop species, Maize. Plant, Cell Environ 32(9):1211–1229. https://doi.org/10.1111/j.1365-3040.2009.01978.x

Ko DK, Rohozinski D, Song Q, Taylor SH, Juenger TE, Harmon FG, Chen ZJ (2016) Temporal shift of circadian-mediated gene expression and carbon fixation contributes to biomass heterosis in maize hybrids. PLoS Genet 12(7):e1006197. https://doi.org/10.1371/journal.pgen.1006197

Riter LS, Jensen PK, Ballam JM, Urbanczyk-Wochniak E, Clough T, Vitek O, Sutton J, Athanas M, Lopez MF, MacIsaac S (2011) Evaluation of label-free quantitative proteomics in a

plant matrix: a case study of the night-to-day transition in corn leaf. Anal Methods 3(12):2733–2739. https://doi.org/10.1039/C1AY05473B

Amiour N, Imbaud S, Clement G, Agier N, Zivy M, Valot B, Balliau T, Armengaud P, Quillere I, Canas R, Tercet-Laforgue T, Hirel B (2012) The use of metabolomics integrated with transcriptomic and proteomic studies for identifying key steps involved in the control of nitrogen metabolism in crops such as maize. J Exp Bot 63(14):5017–5033. https://doi.org/10.1093/Jxb/Ers186

Wang R, Okamoto M, Xing X, Crawford NM (2003) Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol 132(2):556–567. https://doi.org/10.1104/pp.103.021253

Opitz N, Paschold A, Marcon C, Malik WA, Lanz C, Piepho HP, Hochholdinger F (2014) Transcriptomic complexity in young maize primary roots in response to low water potentials. BMC Genom 15:741. https://doi.org/10.1186/1471-2164-15-741

Li PC, Cao W, Fang HM, Xu SH, Yin SY, Zhang YY, Lin DZ, Wang JN, Chen YF, Xu CW, Yang ZF (2017) Transcriptomic profiling of the maize (Zea mays L.) leaf response to abiotic stresses at the seedling stage. Front Plant Sci 8, Artn 290. https://doi.org/10.3389/fpls.2017.00290

Shao R, Xin L, Mao J, Li L, Kang G, Yang Q (2015) Physiological, ultrastructural and proteomic responses in the leaf of maize seedlings to polyethylene glycol-stimulated severe water deficiency. Int J Mol Sci 16(9):21606–21625. https://doi.org/10.3390/ijms160921606

Wu L, Tian L, Wang S, Zhang J, Liu P, Tian Z, Zhang H, Liu H, Chen Y (2016) Comparative proteomic analysis of the response of maize (Zea mays L.) leaves to long photoperiod condition. Front Plant Sci 7:752. https://doi.org/10.3389/fpls.2016.00752

Simons M, Saha R, Guillard L, Clément G, Armengaud P, Cañas R, Maranas CD, Lea PJ, Hirel B (2014b) Nitrogen-use efficiency in maize (Zea mays L.): from 'omics' studies to metabolic modelling. J Exp Bot 65(19):5657–5671. https://doi.org/10.1093/jxb/eru227

Smallbone K, Simeonidis E, Swainston N, Mendes P (2010) Towards a genome-scale kinetic model of cellular metabolism. BMC Syst Biol 4:6. https://doi.org/10.1186/1752-0509-4-6

Jamshidi N, Palsson BO (2008) Formulating genome-scale kinetic models in the post-genome era. Mol Syst Biol 4:171. https://doi.org/10.1038/msb.2008.8

Khodayari A, Maranas CD (2016) A genome-scale Escherichia coli kinetic metabolic model k-ecoli457 satisfying flux data for multiple mutant strains. Nat Commun 7:13806. https://doi.org/10.1038/ncomms13806

Tan Y, Rivera JG, Contador CA, Asenjo JA, Liao JC (2011) Reducing the allowable kinetic space by constructing ensemble of dynamic models with the same steady-state flux. Metab Eng 13(1):60–75. https://doi.org/10.1016/j.ymben.2010.11.001

Cardenas J, Da Silva NA (2014) Metabolic engineering of Saccharomyces cerevisiae for the production of triacetic acid lactone. Metab Eng 25:194–203. https://doi.org/10.1016/j.ymben.2014.07.008

Xu P, Ranganathan S, Fowler ZL, Maranas CD, Koffas MAG (2011) Genome-scale metabolic network modeling results in minimal interventions that cooperatively force carbon flux towards malonyl-CoA. Metab Eng 13(5):578–587. https://doi.org/10.1016/j.ymben.2011.06.008

Lin F, Chen Y, Levine R, Lee K, Yuan Y, Lin XN (2013) Improving fatty acid availability for bio-hydrocarbon production in Escherichia coli by metabolic engineering. PLoS ONE 8(10):e78595. https://doi.org/10.1371/journal.pone.0078595

Khodayari A, Chowdhury A, Maranas CD (2014) Succinate overproduction: a case study of computational strain design using a comprehensive escherichia coli kinetic model. Front Bioeng Biotechnol 2:76. https://doi.org/10.3389/fbioe.2014.00076

Chatterjee A, Kundu S (2015) Revisiting the chlorophyll biosynthesis pathway using genome scale metabolic model of *Oryza sativa* japonica. Sci Rep 5:14975

Chapter 5
Molecular Targets for Improvement of Crop Nitrogen Use Efficiency: Current and Emerging Options

Vikas Kumar Mandal, Narendra Sharma and Nandula Raghuram

Introduction

Food security is closely linked to nutrient availability for cropping, whereas its sustainability is directly linked to nutrient use efficiency. This is particularly true for nitrogen, which is quantitatively the most important component of all fertilizers. By 2050, there will be 70% increase in the global food demand, as the world population will increase to over 9.7 billion (Yang et al. 2012; York et al. 2016; Chen and Liao 2017). Unfortunately, the average N-use efficiency (NUE) in crops is about 30%, and the unutilized reactive N species that accumulate in the environment cause water and air pollution affecting health, biodiversity, and climate change (Sutton et al. 2013; Zhang et al. 2015). While short-term improvement of NUE at the farm level can be done using better agronomic practices, slow release fertilizers etc., the inherent ability of the crop to take up the available N and use it efficiently for maximal yield and minimal loss has to be tackled biologically.

A major biological challenge is that our idea of yield itself may vary between grain, fruit, seed, flower, leaf, and tuber depending on the crop. Another biological challenge is that out of the several dozens of definitions of NUE, very few are biologically relevant, such as uptake and utilization efficiency (Pathak et al. 2011; Yu et al. 2016). It is also not uncommon for yield-centric researchers to project N responsiveness as NUE. For example, a cultivar that keeps responding to increasing doses of N-fertilizer with slightly higher yield is misinterpreted as N-use efficient, even if its yield differential may keep falling with increasing N, making it actually less efficient. Such approaches also often push biologists to search for NUE within the narrow genetic pool of high-yielding varieties, rather than trying to find the true extent of genetic diversity that exists for NUE.

V. K. Mandal · N. Sharma · N. Raghuram (✉)
School of Biotechnology, Guru Gobind Singh Indraprastha University,
Dwarka 110078, New Delhi, India
e-mail: raghuram98@hotmail.com; raghuram@ipu.ac.in

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_5

The identification of the biological avenues for crop improvement toward NUE is hampered by the incomplete characterization of its phenotype and genotype (Pathak et al. 2011; Sinha et al. 2018). This is extremely important to identify contrasting varieties or to rank all the available germplasm in the increasing or decreasing order of NUE, so as to benefit from the fast-growing genomic data for association mapping. Some of the phenotypic traits associated with NUE so far include root length/number/branching/density, (Morita et al. 1988; Yang et al. 2012; Steffens and Rasmussen 2016), dense and erect panicle in rice (Sun et al. 2014), onset of post-anthesis senescence, and plant height in wheat (Gaju et al. 2011). This chapter is primarily focused on the recent advances in the molecular approaches to improve NUE in plants through the identification of the genes involved in N response and NUE and their manipulation by various means.

Molecular Aspects of N Response for NUE

N is present in soil in the form of nitrate (NO_3^-) or ammonium (NH_4^+) in aerobic or flooded (anaerobic/acidic) conditions, respectively. A small portion of N can also be absorbed in the form of amino acids or as urea directly by plants with the help of specific transporters. They are mainly absorbed through the roots and translocated throughout the plant through xylem. N-compounds are also recycled and remobilized from internal stores or senescing tissues through the phloem to the sites of demand, such as for grain filling in cereals. The genes involved in all these processes of N uptake, assimilation, and remobilization are important for N-use efficiency, which makes it a complex, quantitative trait. On an organism-wide scale, N response encompasses many more genes/processes that may contribute to NUE, including C metabolism, redox metabolism, and root/shoot development (Fig. 5.1). The molecular biology of N response has been elaborated in several reviews (Pathak et al. 2008; Krapp et al. 2014; Li et al. 2017; Sinha et al. 2018). Therefore, the following sections deal with various molecular targets that have been explored toward improvement of N-use efficiency.

Genes/QTLs Identified for NUE

Marker-assisted genetic mapping has helped identify many genes/QTLs for plant height, panicle weight, and panicle number such as *GS1*, *DEP1*, *NADH-GOGAT* to improve NUE. At the same time, many other genes involved in N transportation, assimilation, signaling, and regulation have been successfully used to improve NUE in rice and other plants (Fig. 5.1). Over-expression of *OsNRT2.3b* improved nitrate-uptake capacity, C metabolism, grain yield and thereby NUE by 40%. In addition, it also enhanced the uptake capacity of P and Fe by maintaining pH homeostasis (Fan et al. 2016). Therefore, modulation of the expression of N transporters has a beneficial impact on the overall plant NUE. However, it has also

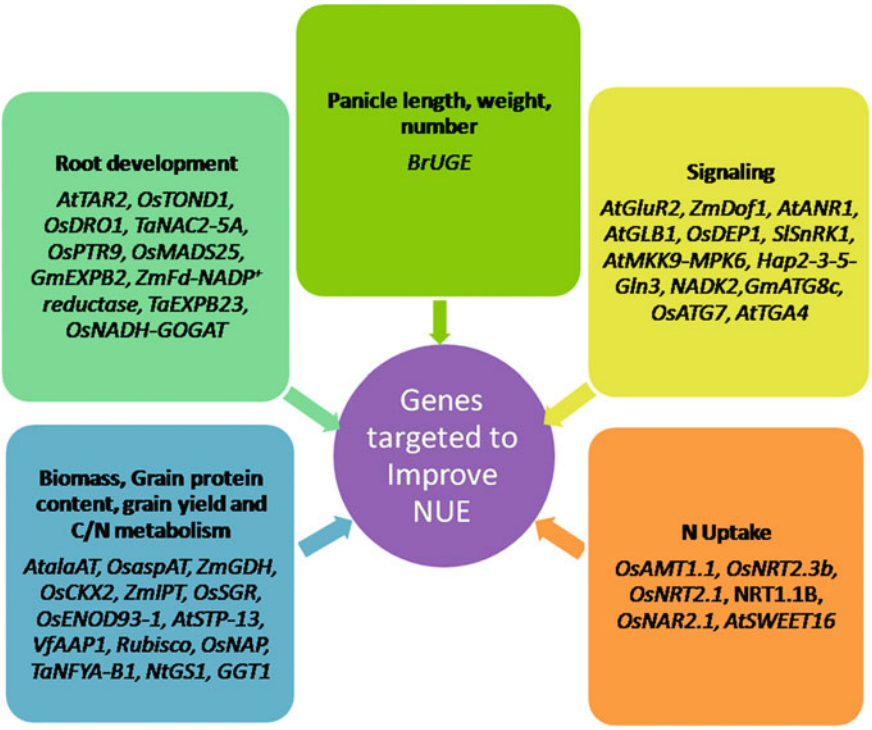


Fig. 5.1 N-responsive molecular targets of various physiological processes used to improve NUE.*AtalaAT*: *A. thaliana* Alanine aminotransferase; *OsaspAT*: *O. sativa* Aspartate aminotransferase; *ZmGDH*: *Z. maize* NADP-dependent glutamate dehydrogenase; *OsCKX2*: Cytokinin oxidase; *ZmIPT*: Cytokinin biosynthesis, *OsSGR:* Stay green, *OsENOD93-1*: Mitochondrial membrane protein; *AtSTP-13:* Hexose transporter, *VfAAP1: V. faba* Amino acid permease, Rubisco; OsNAP: NAC transcription factor, *TaNFYA-B1: T. aestivum* CCAAT-binding transcription factors, *NtGS1: N. tobacum* cytosolic glutamine synthetase, *AtGGT1:* glutamate: glyoxylate aminotransferase 1; *BrUGE*: *B. rapa* UDP-glucose 4-epimerase; *OsNADH-GOGAT*: NADH- dependent glutamate synthase; AtTAR2, *OsTOND1*: Tolerance Of Nitrogen Deficiency 1, *OsDRO1*: DEEPER ROOTING 1, *TaNAC2-5A*, *OsPTR9*: Peptide transporter/nitrate transporter, *OsMADS25*, *GmEXPB2: G. max* beta-expansin, *ZmFd-NADP*$^+$ reductase: Ferredoxin NADP$^+$ reductase, *TaEXPB23; AtSWEET1:* Sugars Will Eventually Be Exported Transporters; *AtGluR2*: Glutamate receptor*; AtGLB1:* PII regulatory protein*; OsDEP1:* (Dense and erect panicle 1) G protein γ subunit*; SlSnRK: S. lycopersicum* sucrose non-fermenting-1-related protein kinase 1; *AtMKK9-MPK6*: Mitogen-activated protein kinase; *Hap2-3-5-Gln3*: Hap2-3-5 binding domain and Gln3 activation domain; *AtNADK2*: NAD kinase (Wang et al. 2012; Klemens et al. 2013; Alvarez et al. 2014; Rothstein et al. 2014; Dellero et al. 2015; Wada et al. 2015; Abdula et al. 2016; Li et al. 2016; Chen and Liao 2017; Wan et al. 2017)

been reported that an increased N input may delay flowering time and consequent yield losses especially in high-latitude regions where late-season temperatures hamper grain filling (Li et al. 2017). Transgenic approaches using many other genes involved in N metabolism have also been implicated to improve NUE such as glutamate dehydrogenase (*GDH*), aspartate aminotransferase (*AspAT*), and

asparagine synthetase (*AS*). NLP proteins have been recently reported to increase crop yield by improving plant biomass under both N-rich and poor soil conditions (Xu et al. 2016).

Molecular Manipulation of Root System Architecture for NUE

In the past two decades, scientific community has established a strong basis to target root system architecture as an approach to improve NUE (Forde 2014; Fan et al. 2017; Li et al. 2017). Root system is comprised of embryonic (primary and seminal roots in Arabidopsis and cereals, respectively) and post-embryonic roots (lateral roots in Arabidopsis; lateral, brace, and crown roots in cereals). Studies carried out in maize helped us to understand the advantages of "steep, cheap, and deep" root morphology to absorb water and nutrients from soil (Lynch 2013). Long and thick primary roots help plants to acquire N from the deeper horizon, while fewer and longer lateral roots with steep root growth angles not only decreases the metabolic cost but also help in exploring greater volume of soil.

Numerous signaling mechanisms are involved in the adjustment of root development to heterogeneous N environments. Studies on the molecular control of N-responsive root development have been mainly carried out in Arabidopsis, though various homologs of the genes involved have also been found in rice and other plants (Forde 2014; Shahzad and Amtmann 2017). A summary of N-responsive regulators of root system architecture is provided in Table 5.1. Arabidopsis shows root adjustment towards different levels and forms of N in the surrounding rhizosphere with the help of various signaling molecules. This includes regulation of lateral root initiation in the xylem pole pericycle cells by *CEP5* (C-terminally encoded peptide) in an auxin and N-dependent manner (Roberts et al. 2016); inhibition of lateral root emergence during systemic low N signal by *CLE* (CLAVATA3/ESR-related) gene family which binds to *CLAVATA1* (CLV1 leucine-rich repeat receptor-like kinases (Araya et al. 2014, 2016; Okamoto et al. 2015); inhibition of primary root growth by *AFB3* (auxin receptors which are a part of the SCF^{TIR1}/AFB E3 ubiquitin ligase complex) in the presence of nitrate and promotion of lateral root growth by *AFB3/NAC4/OBP4*-signaling module (Vidal et al. 2010, 2013). Recently, several miRNAs (miR167, miR393, miR160 and miR171) and N-responsive transcription factors have been reported to regulate root system morphology (Table 5.1) under various N conditions in Arabidopsis and rice (Vidal et al. 2010; Yan et al. 2014; Bellegarde et al. 2017; Chien et al. 2017; Gifford et al. 2017; Sun et al. 2017; Undurraga et al. 2017). Generally lateral roots are much more sensitive to the fluctuating nutritional conditions and their response depends on the degree of stress in the surrounding region. Low N deficiency tends to promote lateral root initiation but moderate to severe N deficiency hampers further root emergence and elongation. Root morphology is also determined by the ratio of NO_3^-:NH_4^+. High NO_3^-:NH_4^+ ratio showed positive effect on the lateral root length, whereas low ratio has a contrary impact (Qin et al. 2017).

Table 5.1 N-responsive genes involved in the regulation of root system architecture

Gene/Protein name	Organism	Function	References
Tryptophan aminotransferase-related protein 2 (*TAR2*)	*Arabidopsis thaliana*	Maintenance of the root stem cell	Ma et al. (2014)
MADS-box transcription factor, Arabidopsis Nitrate Regulated1 (*ANR1*)	*Arabidopsis thaliana*	Root plasticity in response to NO_3^-. Promotes NRT1.1 dependent lateral root growth	Zhang and Forde (1998), Remans et al. (2006), Gan et al. (2012)
C-terminally encoded peptides (*CEPs*)	*Arabidopsis thaliana*	Act locally to inhibit lateral root initiation	Ohyama et al. (2008), Roberts et al. (2016)
(*CEPs*)	*Arabidopsis thaliana*	Acts as long-distance signaling molecule	Tabata et al. (2014)
CLAVATA3/Endosperm surrounding region-related peptides (*CLE*)	*Arabidopsis thaliana*	Overexpressed CLE1 to 7 inhibit lateral root development	Araya et al. (2014), Araya et al. (2016), Okamoto et al. (2015)
MiRNA167/Auxin Response Factor (*ARF8*)	*Arabidopsis thaliana*	Balancing between initiation and emergence of lateral roots	Gifford et al. (2017)
NAM, ATAF, and CUC transcription factor	*Arabidopsis thaliana*	Regulates primary and lateral roots development	Vidal et al. (2013)
MiRNA393/AFB3	*Arabidopsis thaliana*	Regulate development of Primary and lateral roots	Vidal et al. (2010)
miR444a/ANR1	*Oryza sativa*	Reduces nitrate induced lateral root formation	Yan et al. (2014)
EL5, a plant-specific ATL Family E3 Ubiquitin ligase	*Oryza sativa*	Maintains the viability of root apical meristem	Mochizuki et al. (2014), Nishizawa et al. (2015)
Arabidopsis plasma membrane H^+-ATPase isoform 2(*AHA2*)	*Arabidopsis thaliana*	Promotes primary and lateral root development	Mlodzinska et al. (2015)
OsMADS25	*Oryza sativa*	Promotes lateral and primary root development	Yu et al. (2015)
NAM, ATAF, and CUC transcription factor (*TaNAC2-5A*)	*Triticum aestivum*	Promotes root growth	He et al. (2015)
NUCLEAR FACTOR Y (*TaNFYA-B1*)	*Triticum aestivum*	Stimulates root development	Qu et al. (2015)
MADS-Box Transcription Factor (*GmNMHC5*)	*Glycine max*	Promoted lateral root not primary root.	Liu et al. (2015)
Nitrate assimilation-related component 1 (*OsNAR2.1*)	*Oryza sativa*	Lateral root formation	Huang et al. (2015)
MEKK1 kinase	*Arabidopsis thaliana*	Inhibit primary root growth and increased lateral root	Forde et al. (2013)
PHOSPHATE 1 (*PHO1*) and Root System Architecture 1 (*RSA1*)	*Arabidopsis thaliana*	Control root allometry	Rosas et al. (2013)

(continued)

Table 5.1 (continued)

Gene/Protein name	Organism	Function	References
MADs-box gene *AGL21*	*Arabidopsis thaliana*	Positively regulated lateral root development	Yu et al. (2014)
ABA–insensitive 2 (*ABI2*), calcineurin-like protein (CBL)-interacting protein kinase (*CIPK23*)	*Arabidopsis thaliana*	Inhibits lateral root development	Ho et al. (2009)
β-GLUCOSIDASE1 (*BG1*)	*Arabidopsis thaliana*	Lateral root development	Ondzighi-Assoume et al. (2016)

N Transporters as Targets to Improve NUE

Understanding the molecular mechanism of N uptake and its regulation is of great significance toward the improvement of NUE. Nitrogen is taken up from soil mainly in the form of nitrate (NO_3^-), ammonium (NH_4^+), amino acids or peptides, and urea with the help of substrate-specific transporters. Most of these transporters mediate active transport depending on the proton gradient across plasma membrane except few which act as channels and mediate passive transport of solutes. These transporters are classified into low affinity transport systems (LATS) and high affinity transport systems (HATS), as well as in terms of constitutive or inducible. LATS function at a relatively higher concentration of N (>0.5 mM) and have larger Km values (5 mM). On the other hand, HATS mediate transport at low N concentration (0.2–0.5 mM) and have smaller Km values (of about 50 μM). Analysis of tissue-specific expression under varying concentration of N is very important for an efficient N uptake and therefore determining crop yield (Li et al. 2017).

Five main families of nitrate transporters are present in plants: nitrate transporter 1/peptide transporter/nitrate peptide transporter family (*NRT1/PTR/NPF*), *NRT2*/nitrate nitrite porter (*NRT2/NNP*), chloride channels (*CLCs*), slow anion channel-associated 1 homolog 3 (*SLAC1/SLAH*), and aluminum-activated malate transporters (*ALMT*) (Li et al. 2017).

Many ammonium transporters (*AMTs*) have also been targeted to improve NUE by analyzing the phenotypic changes of specific overexpressing or mutant lines. AMTs belong to the AMT/MEP/Rhesus transporter family, which are highly conserved in bacteria, fungi, and plants with more than 700 homologs in bacteria and plants. In Arabidopsis, there are 6 *AMTs* and rice genome has 12 *AMTs* which have been classified into two subfamilies: *OsAMT1* and *OsAMT2* (Li et al. 2017; Xuan et al. 2017). The activities of these transporters are also controlled by phosphorylation, thereby preventing the accumulation of NH_4^+ to toxic levels within the plant system.

Urea uptake and metabolism within the plant and its evaluation as a target for NUE has not received requisite attention, despite the fact that most of Asian agriculture depends on urea fertilization. Urea transport occurs in plants through

five different types of urea transporters, out of which DUR3 type have high affinity and others have low affinity. DUR3 is a 1 urea/1H^+ symporter, whereas the low affinity urea transporters (tonoplast intrinsic protein, *TIP*) act as channels and are pH independent (Reddy and Ulaganathan 2015). Gene expression of DUR3-type transporters is controlled by ammonia, nitrate, and urea.

Nitrate and ammonium transporters are also sensitive to the pH changes of the rhizosphere, the apoplast, or the cytoplasm, as exemplified by modulation in the activity of *AtNPF6.3/AtCHL1/AtNRT1.1* and *OsNRT2.3b*. Similarly, water also affects N uptake and under the condition of drought stress, plants activate specific signaling pathways to overcome reduction in the N uptake. N starvation-induced basic leucine zipper (bZIP) transcription factor gene, *AtTGA4*, cytokinin synthesis gene isopentenyltransferase (*IPT*), and nodule inception-like 7 protein (*NLP7*) along with *NITRATE REGULATORY GENE2* (*NRG2*) are reported to regulate the process of N uptake under these conditions.

Other regulators of the N transporters include transcription factors (TF) such as MADS-box TF *ANR1*, LOB Domain-Containing proteins (*LBD37/38/39*), Nin like proteins (*NLP6*, *NLP7*), Hypersensitivity to Low Pi-Elicited Primary Root Shortening 1 (*HRS1*), TGACG Sequence-specific Binding Protein 1 (*TGA1*/4), Squamosa Promoter Binding Protein-Like 9 (*SPL9*), Auxin Signaling F-Box 3 (*AFB3*), Nitrate Regulatory Gene (*NRG2*), Teosinte Branched 1/Cycloidea/Proliferating Cell Factor 20 (*TCP20*), GATA transcription factor, High Nitrogen Insensitive 9 (*HNI9*), shoot-derived peptide signals such as bZIPTF, HY5, root-derived peptide signals such as CEP and CLE, and miRNAs such as miR393 and miR169a (Marchive et al. 2013; Chien et al. 2017; Xuan et al. 2017).

Apart from these transporters, roots also release exudates in the form of ions, organic compounds, and enzymes to improve nutrient acquisition efficiency (Chen and Liao 2017). Symbiotic association with arbuscular mycorrhizal fungi (AMF) also enables plants such as rice, maize, wheat, and soybean to acquire diffusible nutrients and fixed carbon beyond the rhizosphere and at the same time also reduces the inefficient use of applied N to the soil. It improves the N availability in the rhizosphere through varying the composition of rhizobial microbial community. Recently, Verzeaux et al. (2017) reported improved NUE in wheat by AMF-assisted increased N uptake and accumulation.

Components of N Sensing and Signaling as Targets to Improve NUE

Transcriptomic studies carried out in Arabidopsis, rice, maize, and several other plant species have provided ample support to the fact that N in the form of either nitrate, ammonium, nitric oxide or nitrogen metabolites (L-Glutamate) plays pivotal role in controlling many biological processes in plants, such as root development, crop yield, seed dormancy, flowering time, and leaf development (Wang et al. 2004; Forde et al. 2013; Sun et al. 2016; O'Brien et al. 2016; Noguero and

Lacombe 2016). During this, N mainly acts as a signaling molecule to regulate the expression of genes involved in nutrient transport, metabolism, glycolysis, gluconeogenesis, hormonal activities, etc., in both roots and shoots (Chakraborty and Raghuram 2011). Genes involved in these processes include transcription factors (MADS-Box Transcription Factor), phosphoenolpyruvate carboxylase, Gln synthetase, Asn synthetase, tryptophan amino transferase, ribosomal proteins, initiation factors and many more (Calatrava et al. 2017; Okumoto and Versaw 2017; Liu et al. 2017; Undurraga et al. 2017). Therefore, the knowledge of sensing and signaling components will further enhance our ability to develop improved crop variety (Table 5.2). For example, *NRT1.1* and *NRT2.1* sense changes in N concentration occurring in the external medium and initiate Ca^{2+}-mediated signaling cascade involving phospholipase C (*PLC*). *CHL1/NPF6.3/NRT1.1* acts as a dual affinity nitrate transceptor and therefore have the ability to sense both high and low concentrations of N. This property is dependent on the phosphorylation status which is under the tight control of CBL-interacting protein kinase23 (*CIPK23*) (Ho et al. 2009; Bouguyon et al. 2015; Riveras et al. 2015; Undurraga et al. 2017).

Plastid localized PII proteins in plants interact with N-acetyl-L-glutamate kinase (*NAGK*) and acetyl-CoA carboxylase to promote arginine synthesis and fatty acid synthesis, respectively. Glutamine binds to the C-terminal extension of PII proteins to enhance its ability to form complex with *NAGK* (Gent and Forde 2017).

Through the work carried out in yeast, target of rapamycin (TOR) was identified. In budding yeast, it is found to participate in signaling pathway including nutrient and hormonal signaling and then passing the information to downstream effectors. Plant genomes also have homologs of mammalian or yeast *TORC1* complex. Activity of TOR and sucrose non-fermenting 1 (Snf1) kinase (*SnRK1* in plants) complement with one another to maintain C/N homeostasis under different environmental conditions by regulating several biologically important processes such as photosynthesis, tricarboxylic-acid cycle, and N assimilation by mainly controlling protein synthesis (Dobrenel et al. 2016; Sesma et al. 2017). Similarly, general amino acid control non-derepressible 2 (*GCN2*) kinases also plays a very important role in controlling protein synthesis by causing phosphorylation of eIF2α initiation factor under N starvation. In Arabidopsis, there are 20 ionotropic glutamate-like receptors (*iGLR*) and 24 in rice which have important functional role in stomatal closure, root branching, and maintenance of primary root meristem (Weiland et al. 2014; Gent and Forde 2017).

Nitrogen requirements of crops are fulfilled by the legumes by the process of nodulation by symbiotic relationship with N-fixing bacteria. The availability of genetic mutants has enabled to carry out transcriptomic studies to find out the factors controlling nodulation. Generally, nodulation is promoted under low N supply and excess of N supply has a negative impact on the number of nodules formed. A number of mobile signaling molecules such as CLE peptides, TOO MUCH LOVE (*TML*), receptor-like kinases, *CORYNE* and *CLAVATA2*, *CEPs*, COMPACT ROOT ARCHITECTURE2 (*CRA2)*, nodule inception protein

Table 5.2 Potential targets to improve N sensing and signaling toward NUE

Gene/Protein Name	Organism	Function
PII protein	*Arabidopsis thaliana*	Nitrogen sensing
PII protein	*Arabidopsis thaliana*	Maintains plant C-N balance
PII protein	*Arabidopsis thaliana*	Arginine biosynthesis
TOR signaling pathway	*Arabidopsis thaliana*	Positive regulator of protein synthesis and a negative regulator of protein turnover
GCN2 protein kinase pathway	*Arabidopsis thaliana*	Phosphorylates translation initiation factor in response to uncharged tRNAs
Glutamate receptors	*Arabidopsis thaliana*	Act as amino acid gated Ca^{2+} channels
NRT2.1	*Arabidopsis thaliana*	long-distance transport of N
NRT1.1/AtNPF6.3	*Arabidopsis thaliana*	Transceptor for N
CLE (CLAVATA3/ESR-related) peptides and CLAVATA1 (CLV1) kinase	*Arabidopsis thaliana*	Expansion of roots in N-dependent manner
ELONGATED HYPOCOTYL5 (HY5) and a bZIP TF	*Arabidopsis thaliana*	Mobile signal mediates nitrate uptake
NF1 kinase	Wheat	Involved in signaling
2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KDa (*TAP46*)	*Arabidopsis thaliana*	Downstream effector of TOR protein

(*NIN*), NIN-like proteins (*NLP*), and miR172-EARLY NODULIN40 (*ENOD40*) module regulate nodulation (Murray et al. 2016).

In plants, nitric oxide plays a very important role in regulating many biological processes including seed germination, root development, senescence, plant immunity, and abiotic stress by controlling the expression of many regulatory components (Calatrava et al. 2017). Ammonium is also found to induce the expression of genes involved in N metabolism (*PEPC*, Gln synthetase, and Asn synthetase) and transport (Amino Acid Permease, *AAP1*) (Wang et al. 2004). It also affects ammonium uptake, assimilation, hormonal balance, and root system architecture by altering cytosolic pH and post-translational modification of proteins involved in these processes (Liu and Wirén 2017). In Arabidopsis, ammonia is sensed by an ammonium transporter (AtAMT1;1) whose activity is modulated by the calcineurin-B-like-interacting protein kinases (CIPK) proteins by phosphorylation (Xuan et al. 2017).

Molecular Targets Among the Genes of N Assimilation and Remobilization

Nitrate taken up inside the root cells is first reduced by nitrate reductase (*NIA*) to nitrite and then to ammonium by nitrite reductase (*NiR*). Two *NIA* genes exist in Arabidopsis and three in rice. Subsequently, nitrite moves into the plastid and is then metabolized into ammonium by the glutamine synthetase/glutamate synthase (*GS/GOGAT*) cycle. Ammonium is further incorporated into amino acids. This process of amino acid formation depends on the availability of photosynthates. GS is a very important enzyme for N assimilation and remobilization and there are two isoforms of the enzyme: *GS1* that carries out primary ammonium assimilation in roots or re-assimilation of ammonium in leaves and *GS2* that carries out assimilation of ammonium in chloroplast. Three-to-five members of GS have been found in different plant species; for example, there are three in rice. Depending on the electron donor specificity, there are two types of GOGAT, viz. ferredoxin-dependent (*Fd-GOGAT*) and NADH-dependent (*NADH-GOGAT*). *GLU1* and *GLU2* are two *Fd-GOGATs* and *GLT* is the only *NADH-GOGAT* gene present in the genome of Arabidopsis. Similarly, rice genome encodes one *Fd-GOGAT* and two NADH-*GOGAT*.

Single gene transgenics overexpressing the genes of primary N assimilation (NR, NiR and plastidic GS, GOGAT) did not radically improve NUE (Pathak et al. 2008, 2011; Krapp et al. 2014; Sinha et al. 2018). This was expected in a quantative, multigenic trait like NUE, which involves the coordinated expression of several genes including, but not limited to N-assimilation. This made regulatory targets more attractive than metabolic targets, but the inability to find specific nitrate response elements common to all N-responsive genes has delayed progress in this direction (Das et al. 2007; Pathak et al. 2009). Circadian clock master regulator, CIRCADIAN CLOCK-ASSOCIATED 1 (*CCA1*) also controls the expression of genes involved in N assimilation and thereby establishes a link between N metabolism and circadian clock (Gutiérrez et al. 2008). Kinases and phosphatases are also involved in the regulation of expression of genes coding for N assimilatory enzymes such as *NR*, *NiR*, *GS2,* and *Fd-GOGAT* (Undurraga et al. 2017). Another level of control of metabolism is carried out by transcription factors (*Dof*, *NLP7*, *GATA*), N metabolites (glutamine and glutamate), and miRNAs (Chien et al. 2017; Zuluaga et al. 2017). miR5640 targets phosphoenolpyruvate carboxylase (*PEPC*) which plays a very important role in maintaining C/N balance. The expression of *PEPC* and several other enzymes of tricarboxylic-acid cycle are also under the control of *Dof1* (*DNA BINDING WITH ONE FINGER*) TF (He et al. 2015). Castaings et al. (2009) reported the role NLP7 protein in N assimilation and sensing. All *NLP* proteins can bind nitrate-responsive cis-element *NRE* and mediate nitrate-dependent gene expression (Marchive et al. 2013; Xu et al. 2016; Yu et al. 2016) and improve C/N balance under both N-sufficient and N-deficient conditions. On the one hand, proper N assimilation is required for chloroplast development, synthesis of chlorophyll, and proteins such as Rubisco ((ribulose-1,5-bisphosphate

carboxylase/oxygenase and *PEPC*), whereas on the other hand, C assimilation provides energy source for N metabolism in the form of reducing equivalents (Ferredoxin and NADH) and C skeleton for synthesis of amino acids.

Remobilization of nitrate from source (leaves) to sink (developing parts) is also a significant determinant of NUE as it recycles organic N to the seeds during the grain-filling stage and therefore determines the crop yield. Leaf senescence is the underlying phenomenon of nutrient remobilization which facilitates the recycling of photosynthates to the developing seeds. Autophagy promotes senescence of aging plant parts. Several senescence-associated genes (ATG and metacaspases) are expressed at different stages of plant senescence (Havé et al. 2016). This process involves the participation of tissue-specific transporters which replenishes the N requirement during reproductive stage of plant development. Several reports suggest the regulators of this process, such as nitrogen limitation adaptation (*NLA*), which control the expression of *AtNRT1.7* by protein ubiquitination pathway. However, *NLA* is itself under the control of miRNA827 (Liu et al. 2016). Analysis of rice *GOGAT* mutant leads to the identification of another protein, viz. ferredoxin-dependent glutamate synthase (OsFd-GOGAT), to play a role in this process (Zeng et al. 2016). Fd-GOGAT plays a role in ammonium recycling by photorespiration.

Various Approaches to Identify More QTLs Associated with NUE

Modern technologies have improved our ability to study the regulation at the level of gene expression. These techniques include TARGET (Transient Transformation System for Genome-Wide Transcription Factor Target Discovery) and ChIP-Seq (Bargmann et al. 2013; Marchive et al. 2013) . A major challenge in crop improvement for nitrogen use efficiency (NUE) is that neither the phenotypic traits nor the genes/alleles determining NUE are clearly defined. Therefore, in this scenario it is very necessary to work with chemist and use analogs of different N sources and then carry out the phenotypic screening for NUE-related genes/loci. For example, by using chlorate, the toxic analog of nitrate, *OsNRT1.1B,* was identified as a critical QTL contributing to NUE divergence between rice subspecies (Hu et al. 2015). Similar strategy had earlier lead to the identification of several regulators of N assimilation in fungi and algae such as NIT2, NIT4, AREA, NIRA, and NIT2 (Castaings et al. 2009). Application of the new high-throughput measuring techniques such as genome-wide association studies (GWAS) also enables us to identify genes/QTLs regulating NUE. For example, Gifford et al. (2013) grew 96 Arabidopsis accessions under two N regimes and studied root phenotypic traits and identified *JASMONATE RESPONSIVE 1* (*JR1*) as one of the candidate genes. Similarly, *CALCIUM SENSOR RECEPTOR*, *PhzC, ROOT SYSTEM ARCHITECTURE 1*, and *PHOSPHATE 1* were discovered by high-throughput automated root image analysis

(Gifford et al. 2013; Rosas et al. 2013; Slovak et al. 2014). This technique helps us to study natural variations among different genotypes of a plant species and understand the complex regulatory mechanism behind NUE. Another such technique is semiautomated confocal microscopy with the help of which *KURZ UND KLEIN* (F-box family gene), was identified to play a significant role in root development (Li et al. 2017). Systems biology has also enabled us to identify novel interacting partners and further provides the missing knowledge about the components of signal transduction pathway of N sensing, signaling and metabolism (Gutiérrez et al. 2008; Vidal et al. 2013).

Conclusions

The last decade has witnessed tremendous progress in finding several molecular targets towards the improvement of N-use efficiency of plants. Several genes belonging to various processes have been identified including root development, N uptake, assimilation, and remobilization. In addition, genes involved in N sensing, signaling, and the regulation of the above processes have also emerged, including epigenetic regulation involving miRNA. While phenotype development has not kept pace with these developments, functional genomics and reverse genetics are opening newer opportunities for identification and validation of newer molecular targets. These developments strengthen the hope that improved crop varieties for NUE will become increasingly available for sustainable agriculture in the near future.

Acknowledgements This work was supported in part by research grant to NR and fellowship to NS from the Department of Biotechnology, Govt. of India under the Indo-UK Virtual Nitrogen Centre on Nitrogen Efficiency of Whole cropping Systems (NEWS) BT/IN/UK-VNC/44/NR/2015-16. VM is a recipient of DBT fellowship (BCIL/HRD/DBT-JRF/FLSP).

References

Abdula SE, Lee HJ, Kim J, Niño MC, Jung Y-J, Cho Y-C abd Cho Y-G (2016) *BrUGE1*transgenic rice showed improved growth performance with enhanced drought tolerance. Breed Sci 66(2), 226–233

Alvarez JM, Riveras E, Vidal EA, Gras DE, Contreras-López O, Tamayo KP, Aceituno F, Gómez I, Ruffel S, Lejay L, Jordana X (2014) Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of Arabidopsis thaliana roots. The Plant J 80(1):1–3

Araya T, Miyamoto M, Wibowo J, Suzuki A, Kojima S, Tsuchiya YN, Sawa S, Fukuda H, von Wirén N, Takahashi H (2014) CLE-CLAVATA1 peptide-receptor signaling module regulates the expansion of plant root systems in a nitrogen-dependent manner. P Natl A Sci 111 (5):2029–2034

Araya T, von Wirén N, Takahashi H (2016) CLE peptide signaling and nitrogen interactions in plant root development. Plant Mol Biol 91(6):607–615

Bargmann BOR, Marshall-Colon A, Efroni I, Ruffel S, Birnbaum KD, Coruzzi GM, Krouk G (2013) TARGET: A Transient Transformation System for Genome-Wide Transcription Factor Target Discovery. Mol Pla 6(3):978–980

Bellegarde F, Gojon A, Martin A (2017) Signals and players in the transcriptional regulation of root responses by local and systemic N signaling in Arabidopsis thaliana. J Exp Bot 68 (10):2553–2565

Bouguyon E, Brun F, Meynard D, Kubeš M, Pervent M, Leran S, Lacombe B, Krouk G, Guiderdoni E, Zažímalová E, Hoyerová K (2015) Multiple mechanisms of nitrate sensing by Arabidopsis nitrate transceptor NRT1.1. Nat Plants 1(3):15015

Castaings L, Camargo A, Pocholle D, Gaudon V, Texier Y, Boutet-Mercey S, Taconnat L, Renou J, Daniel-Vedele F, Fernandez E, Meyer C, Krapp, A (2009) The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. The Plant J 57(3):426–435

Calatrava V, Chamizo-Ampudia A, Sanz-Luque E, Ocaña-Calahorro F, Llamas A, Fernandez E, Galvan A (2017) How Chlamydomonas handles nitrate and the nitric oxide cycle. J Exp Bot 68 (10):2593–2602

Chakraborty N. and Raghuram N. (2011) Nitrate sensing and signaling in genomewide plant N response. In Nitrogen Use Efficiency in Plants, V. Jain, P. Anandakumar (eds) New India Publishing Agency, New Delhi. pp. 45–62

Chen L, Liao H (2017) Engineering crop nutrient efficiency for sustainable agriculture. J Integr Plant Biol 59(10):710–735

Chien P-S, Chiang C-B, Wang Z, Chiou T-J (2017) MicroRNA-mediated signaling and regulation of nutrient transport and utilization. Curr Opinion in Plant Biol 39:73–79

Das SK, Pathak RR, Choudhury D, Raghuram N (2007) Genomewide computational analysis of nitrate response elements in rice and Arabidopsis. Mol Genet Genomics 278(5):519–525

Dellero Y, Lamothe-Sibold M, Jossier M, Hodges M (2015) Arabidopsis thaliana *ggt1* photorespiratory mutants maintain leaf carbon: nitrogen balance by reducing RuBisCO content and plant growth. The Plant J 83(6):1005–1018

Dobrenel T, Caldana C, Hanson J, Robaglia C, Vincentz M, Veit B, Meyer C (2016) TOR signaling and nutrient sensing. Ann Rev Plant Biol 67(1):261–285

Fan X, Tang Z, Tan Y, Zhang Y, Luo B, Yang M, Lian X, Shen Q, Miller AJ, Xu G (2016) Overexpression of a pH-sensitive nitrate transporter in rice increases crop yields. P Natl A Sci 113(26):7118–7123

Fan X, Naz M, Fan X, Xuan W, Miller AJ, Xu G (2017) Plant nitrate transporters: from gene function to application. J Exp Bot 68(10):2463–2475

Forde BG (2014). Nitrogen signalling pathways shaping root system architecture: an update. Curr Opin Plant Biol 21:30–36

Forde BG, Cutler SR, Zaman N, Krysan PJ (2013) Glutamate signalling via a MEKK1 kinase-dependent pathway induces changes in Arabidopsis root architecture. The Plant J 75 (1):1–10

Gaju O, Allard V, Martre P, Snape JW, Heumez E, LeGouis J, Moreau D, Bogard M, Griffiths S, Orford S, Hubbart S (2011) Identification of traits to improve the nitrogen-use efficiency of wheat genotypes. Field Crops Res 123(2):139–152

Gan Y, Bernreiter A, Filleur S, Abram B, Forde BG (2012) Overexpressing the ANR1 MADS-box gene in transgenic plants provides new insights into its role in the nitrate regulation of root development. Plant Cell Physiol 53(6):1003–1016

Gent L, Forde BG (2017) How do plants sense their nitrogen status? J Exp Bot 68(10):2531–2539

Gifford ML, Banta JA, Katari MS, Hulsmans J, Chen L, Ristova D, Tranchina D, Purugganan MD, Coruzzi GM, Birnbaum KD (2013) Plasticity regulators modulate specific root traits in discrete nitrogen environments. PLoS Genet 9(9):e1003760

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2017) Cell-specific nitrogen responses mediate developmental plasticity. P Natl A Sci 105(2):803–808

Gutiérrez RA, Stokes TL, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero DC, McClung CR, Coruzzi GM (2008) Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. P Natl A Sci 105(12):4939–4944

He X, Qu B, Li W, Zhao X, Teng W, Ma W, Ren Y, Li B, Li Z,Tong Y (2015) The nitrate-inducible NAC transcription factor TaNAC2-5A controls nitrate response and increases wheat yield. Plant Physiol 169(3):1991–2005

Havé M, Marmagne A, Chardon F, Masclaux-Daubresse C (2016) Nitrogen remobilisation during leaf senescence: lessons from Arabidopsis to crops. J Exp Bot 68(10):2513–2529

Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. Cell 138 (6):1184–1194

Hu B, Wang W, Ou S, Tang J, Li H, Che R, Zhang Z, Chai X, Wang H, Wang Y, Liang C, Liu L, Piao Z, Deng Q, Deng K, Xu C, Liang Y, Zhang L, Li L, Chu C (2015) Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. Nature Genetics 47(7):834–838

Huang S, Chen S, Liang Z, Zhang C, Yan M, Chen J, Xu G, Fan X, Zhang Y (2015) Knockdown of the partner protein OsNAR2.1 for high-affinity nitrate transport represses lateral root formation in a nitrate-dependent manner. Sci Rep 5:18192

Klemens PAW, Patzke K, Deitmer J, Spinner L, Le Hir R, Bellini C, Bedu M, Chardon F, Krapp A, Neuhaus HE (2013) Overexpression of the vacuolar sugar carrier AtSWEET16 modifies germination, growth, and stress tolerance in Arabidopsis. Plant Physiol 163(3):1338–1352

Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince A-S, Chaillou S, Ferrario-Méry S, Meyer C, Daniel-Vedele F (2014) Nitrate transport and signaling in Arabidopsis. J Exp Bot 65(3):789–798

Li H, Hu B, Chu C (2017) Nitrogen use efficiency in crops: lessons from Arabidopsis and rice. J Exp Bot 68(10):2477–2488

Li X, Zeng R, Liao H (2016) Improving crop nutrient efficiency through root architecture modifications. J Integr Plant Biol 58(3):193–202

Liu KH, Niu Y, Konishi M, Wu Y, Du H, Chung HS, Li L, Boudsocq M, McCormack M, Maekawa S, Ishida T (2017) Discovery of nitrate–CPK–NLP signalling in central nutrient–growth networks. Nature 545(7654):311–316

Liu W, Han X, Zhan G, Zhao Z, Feng Y, Wu C (2015) A novel sucrose-regulatory MADS-box transcription factor GmNMHC5 promotes root development and nodulation in soybean (*Glycine max* [L.] Merr.). Int J Mol Sci 16(9):20657–20673

Liu W, Sun Q, Wang K, Du Q, Li W-X (2016) Nitrogen limitation adaptation (NLA) is involved in source-to-sink remobilization of nitrate by mediating the degradation of NRT1.7 in Arabidopsis. New Phytol 214(2):734–744

Liu Y, von Wirén N (2017) Ammonium as a signal for physiological and morphological responses in plants. J Exp Bot 68(10):2581–2592

Lynch JP (2013) Steep, cheap and deep: an ideotype to optimize water and N acquisition by maize root systems. Ann Bot 112(2):347–357

Ma W, Li J, Qu B, He X, Zhao X, Li B, Fu X, Tong Y (2014) Auxin biosynthetic gene TAR2 is involved in low nitrogen-mediated reprogramming of root architecture in Arabidopsis. The Plant J 78(1):70–79

Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A (2013) Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nat Commun 4:1713

Młodzińska E, Kłobus G, Christensen MD, Fuglsang AT (2015) The plasma membrane $H^{(+)}$-ATPase AHA2 contributes to the root architecture in response to different nitrogen supply. Physiol Plant 154(2):270–282

Mochizuki S, Jikumaru Y, Nakamura H, Koiwai H, Sasaki K, Kamiya Y, Kamiya Y, Ichikawa H, Minami E, Nishizawa Y (2014) Ubiquitin ligase EL5 maintains the viability of root meristems by influencing cytokinin-mediated nitrogen effects in rice. J Exp Bot 65(9):2307–2318

Morita S, Suga T, Yamazaki K (1988) The relationship between root length density and yield in rice plants. Jpn J Crop Sci 57(3):438–443

Murray JD, Liu C-W, Chen Y, Miller AJ (2016) Nitrogen sensing in legumes. J Exp Bot 68 (8):1919–1926

Nishizawa Y, Mochizuki S, Koiwai H, Kondo K, Kishimoto K, Katoh E, Minami E (2015) Rice ubiquitin ligase EL5 prevents root meristematic cell death under high nitrogen conditions and interacts with a cytosolic GAPDH. Plant Signal Behav 10(3):e990801

Noguero M, Lacombe B (2016) Transporters involved in root nitrate uptake and sensing by Arabidopsis. Front Plant Sci (7):1391

O'Brien JA, Vega A, Bouguyon E, Krouk G, Gojon A, Coruzzi G, Gutiérrez RA (2016) Nitrate transport, sensing, and responses in plants. Mol Plant 9(6):837–856

Ohyama K, Ogawa M, Matsubayashi Y (2008) Identification of a biologically active, small, secreted peptide in Arabidopsis by in silico gene screening, followed by LC-MS-based structure analysis. The Plant J 55(1):152–160

Okamoto S, Suzuki T, Kawaguchi M, Higashiyama T, Matsubayashi Y (2015) A comprehensive strategy for identifying long-distance mobile peptides in xylem sap. The Plant J 84(3):611–620

Okumoto S, Versaw W (2017) Genetically encoded sensors for monitoring the transport and concentration of nitrogen-containing and phosphorus-containing molecules in plants. Curr Opin Plant Biol 39:129–135

Ondzighi-Assoume CA, Chakraborty S, Harris JM (2016) Environmental nitrate stimulates abscisic acid accumulation in Arabidopsis root tips by releasing it from inactive stores. Plant Cell 28(3):729–745

Pathak RR, Ahmad A, Lochab S, Raghuram N (2008) Molecular physiology of plant N-use efficiency and biotechnological options for its enhancement. Curr Sci 94(11):1394–1403

Pathak RR, Das SK, Choudhury D, Raghuram N (2009) Genomewide bioinformatic analysis negates any specific role for Dof, GATA and Ag/cTCA motifs in nitrate responsive gene expression in Arabidopsis. Physiol Mol Biol Pla 15(2):145–150

Pathak RR, Lochab S, Raghuram N (2011) Improving nitrogen use efficiency. In Compr Biotechnol, vol 4, 2nd edn. Elsevier, Oxford, pp 209–218

Qin S, Sun X, Hu C, Tan Q, Zhao X, Xin J, Wen X (2017) Effect of NO_3^-:NH_4^+ ratios on growth, root morphology and leaf metabolism of oilseed rape (*Brassica napus* L.) seedlings. Acta Physiol Plant 39(9):198

Qu B, He X, Wang J, Zhao Y, Teng W, Shao A, Zhao X, Ma W, Wang J, Li B, Li Z (2015) A wheat CCAAT box-binding transcription factor increases the grain yield of wheat with less fertilizer input. Plant Physiol 167(2):411–423

Reddy MM, Ulaganathan K (2015) Nitrogen nutrition, its regulation and biotechnological approaches to improve crop productivity. Am J Plant Sci 6(18):2745–2798

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci USA 103(50):19206–19211

Riveras E, Alvarez JM, Vidal EA, Oses C, Vega A, Gutiérrez RA (2015) The calcium ion is a second messenger in the nitrate signaling pathway of Arabidopsis 1. Plant Physiol 177 (1):00961

Roberts I, Smith S, Stes E, De Rybel B, Staes A, Van De Cotte B, Njo MF, Dedeyne L, Demol H, Lavenus J, Audenaert D (2016) CEP5 and XIP1/CEPR1 regulate lateral root initiation in Arabidopsis. J Exp Bot 67(16):4889–4899

Rosas U, Cibrian-Jaramillo A, Ristova D, Banta JA, Gifford ML, Fan AH, Zhou RW, Kim GJ, Krouk G, Birnbaum KD, Purugganan MD (2013) Integration of responses within and across Arabidopsis natural accessions uncovers loci controlling root systems architecture. P Natl A Sci 110(37):15133–15138

Rothstein SJ, Bi Y-M, Coneva V, Han M, Good A (2014) The challenges of commercializing second-generation transgenic crop traits necessitate the development of international public sector research infrastructure. J Exp Bot 65(19):5673–5682

Sesma A, Castresana C, Castellano MM (2017) Regulation of translation by TOR, eIF4E and eIF2α in plants: current knowledge, challenges and future perspectives. Front Plant Sci 8:644

Shahzad Z, Amtmann A (2017) Food for thought: how nutrients regulate root system architecture. Curr Opin Plant Biol 39:80–87

Sinha VB, Jangam AP, Raghuram N (2018) Biological determinants of crop N use efficiency and biotechnological avenues for improvement. In: Masso C, Bleeker A, Raghuram N, Bekunda M, Sutton M (eds) Proceedings of the N2013. Springer

Slovak R, Göschl C, Su X, Shimotani K, Shiina T, Busch W (2014) A scalable open-source pipeline for large-scale root phenotyping of Arabidopsis. Plant Cell 26(6):2390–2403

Steffens B, Rasmussen A (2016) The physiology of adventitious roots. Plant Physiol 170(2):603–617

Sun C-H, Yu J-Q, Hu D-G (2017) Nitrate: a crucial signal during lateral roots development. Front Plant Sci 8:485

Sun H, Qian Q, Wu K, Luo J, Wang S, Zhang C, Ma Y, Liu Q, Huang X, Yuan Q, Han R, Zhao M, Dong G, Guo L, Zhu X, Gou Z, Wang W, Wu Y, Lin H, Fu X (2014) Heterotrimeric G proteins regulate nitrogen-use efficiency in rice. Nat Genet 46(6):652–656

Sun J, Ye M, Peng S, Li Y (2016) Nitrogen can improve the rapid response of photosynthesis to changing irradiance in rice (Oryza sativa L.) plants. Sci Rep 6(1):31305

Sutton MA, Bleeker A, Howard CM, Bekunda M, Grizzetti B, de Vries W, van Grinsven HJM, Abrol YP, Adhya TK, Billen G,. Davidson EA, Datta A, Diaz R, Erisman JW, Liu XJ, Oenema O, Palm C, Raghuram N, Reis S, Scholz RW, Sims T, Westhoek H, Zhang FS, with contributions from Ayyappan S, Bouwman AF, Bustamante M, Fowler D, Galloway JN, Gavito ME, Garnier J, Greenwood S, Hellums DT, Holland M, Hoysall C, Jaramillo VJ, Klimont Z, Ometto JP, Pathak H, Plocq Fichelet V, Powlson D, Ramakrishna K, Roy A, Sanders K, Sharma C, Singh B, Singh U, Yan XY, Zhang Y (2013) Our nutrient world: the challenge to produce more food and energy with less pollution. Global Overview of Nutrient Management, Centre for Ecology and Hydrology, Edinburgh on behalf of the Global Partnership on Nutrient Management and the International Nitrogen Initiative

Tabata R, Sumida K, Yoshii T, Ohyama K, Shinohara H, Matsubayashi Y (2014) Perception of root-derived peptides by shoot LRR-RKs mediates systemic N-demand signaling. Science (New York, N.Y.) 346(6207):343–346

Undurraga SF, Ibarra-Henríquez C, Fredes I, Álvarez JM, Gutiérrez RA (2017) Nitrate signaling and early responses in Arabidopsis roots. J Exp Bot 68(10):2541–2551

Vidal EA, Moyano TC, Riveras E, Contreras-López O, Gutiérrez RA (2013) Systems approaches map regulatory networks downstream of the auxin receptor AFB3 in the nitrate response of Arabidopsis thaliana roots. P Natl A Sci 110(31):12840–12845

Vidal EA, Viviana A, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis Thaliana. P Natl A Sci 107(9):4477–4482

Verzeaux J, Hirel B, Dubois F, Lea PJ, Tétu T (2017) Agricultural practices to improve nitrogen use efficiency through the use of arbuscular mycorrhizae: basic and agronomic aspects. Plant Sci 264:48–56

Wada S, Hayashida Y, Izumi M, Kurusu T, Hanamata S, Kanno K, Kojima S, Yamaya T, Kuchitsu K, Makino A, Ishida H (2015) Autophagy supports biomass production and nitrogen use efficiency at the vegetative stage in rice. Plant Physiol 168(1):60–73

Wan TE, Xue HE, TONG YP (2017) Transgenic approaches for improving use efficiency of nitrogen, phosphorus and potassium in crops. J Integr Agri 16 (12):60345–60347

Wang R, Tischner R, Gutiérrez RA, Hoffman M, Xing X, Chen M, Coruzzi G, Crawford NM (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of Arabidopsis. Plant Physiol 136(1):2512–2522

Wang X, Peng F, Li M, Yang L, Li G (2012) Expression of a heterologous SnRK1 in tomato increases carbon assimilation, nitrogen uptake and modifies fruit development. J Plant Physiol 169(12):1173–1182

Weiland M, Mancuso S, Baluska F (2014) Signalling via glutamate and GLRs in Arabidopsis thaliana. Funct Plant Biol 43(1):1–25

Xuan W, Beeckman T, Xu G (2017) Plant nitrogen nutrition: sensing and signaling. Curr Opin Plant Biol 39:57–65

Xu N, Wang R, Zhao L, Zhang C, Li Z, Lei Z, Liu F, Guan P, Chu Z, Crawford NM,Wang Y (2016) The Arabidopsis NRG2 protein mediates nitrate signaling and interacts with and regulates key nitrate regulators. The Plant Cell 28(2):485–504

York LM, Silberbush M, Lynch JP (2016) Spatiotemporal variation of nitrate uptake kinetics within the maize (*Zea mays L.*) root system is associated with greater nitrate uptake and interactions with architectural phenes. J Exp Bot 67(12):3763–3775

Yang JC, Zhang H, Zhang JH (2012) Root morphology and physiology in relation to the yield formation of rice. J Integr Agri 11(6):920–926

Yan Y, Wang H, Hamera S, Chen X, Fang R (2014) MiR444a has multiple functions in the rice nitrate-signaling pathway. The Plant J 78(1):44–55

Yu C, Liu Y, Zhang A, Su S, Yan A, Huang L, Ali I, Liu Y, Forde BG, Gan Y (2015) MADS-box transcription factor OsMADS25 regulates root development through affection of nitrate accumulation in rice. PLoS One 10(8):e0135196.

Yu LH, Miao ZQ, Qi GF, Wu J, Cai XT, Mao JL, Xiang CB (2014) MADS-box transcription factor AGL21 regulates lateral root development and responds to multiple external and physiological signals. Mol Plant 7:1653–1669

Yu LH, Wu J, Tang H, Yuan Y, Wang S-M, Wang Y-P, Zhu QS, Li SG, Xiang C-B (2016) Overexpression of Arabidopsis NLP7 improves plant growth under both nitrogen-limiting and -sufficient conditions by enhancing nitrogen and carbon assimilation. Scientific reports 6:27795

Zeng D-D, Qin R, Li M, Alamin M, Jin X-L, Liu Y, Shi C-H (2016) The ferredoxin-dependent glutamate synthase (OsFd-GOGAT) participates in leaf senescence and the nitrogen remobilization in rice. Mol Genet Genomics 292(2):385–395

Zhang H, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science (New York, N.Y.) 279(5349):407–409

Zhang X, Davidson EA, Mauzerall DL, Searchinger TD, Dumas P, Shen Y (2015) Managing nitrogen for sustainable development. Nature 528(7580):51

Zuluaga DL, De Paola D, Janni M, Curci PL, Sonnante G (2017) Durum wheat miRNAs in response to nitrogen starvation at the grain filling stage. PLoS One 12(8):e0183253

Chapter 6
From Arabidopsis to Crops: The Arabidopsis *QQS* Orphan Gene Modulates Nitrogen Allocation Across Species

Seth O'Conner, Andrea Neudorf, Wenguang Zheng, Mingsheng Qi, Xuefeng Zhao, Chuanlong Du, Dan Nettleton and Ling Li

Introduction

For decades, nitrogen fertilizers have been massively used to increase crop yields, but they negatively impact the environment in agriculture. New solutions are needed to improve the nitrogen use efficiency (NUE) of crops to increase yields and decrease the negative impacts on the environment (Han et al. 2015; Good et al. 2004; Lightfoot 2013). NUE is defined as the efficiency of uptake and utilization of the biologically reactive nitrogen from the growth environment. Different approaches are proposed, such as development of crops with improved NUE (Han et al. 2015; Hirel et al. 2007; Masclaux-Daubresse et al. 2010; McAllister et al. 2012; Beatty et al. 2009; Shrawat et al. 2008), the analysis of factors that interact with NUE (Han et al. 2015), plant metabolic engineering (Lau et al. 2014), and a metabolomics/computational approach for understanding NUE for an enhanced crop management and increased yields (Beatty et al. 2016). Here, we introduce

S. O'Conner · L. Li (✉)
Department of Biological Sciences, Mississippi State University, Starkville, MS 39762, USA
e-mail: liling@biology.msstate.edu

S. O'Conner · A. Neudorf · W. Zheng · L. Li
Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA

M. Qi
Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA 50011, USA

X. Zhao
Laurence H. Baker Center for Bioinformatics and Biological Statistics, Iowa State University, Ames, IA 50011, USA

C. Du · D. Nettleton
Department of Statistics, Iowa State University, Ames, IA 50011, USA

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_6

basic research on an Arabidopsis orphan gene qua quine starch (*QQS*) and its involvement in regulation of nitrogen allocation. *QQS* and its related network could be applied in agriculture for improved crops with increased protein content.

Regulation of Carbon and Nitrogen Allocation

The plant metabolic network regulates the allocation of carbon and nitrogen into different components such as protein, oil, and carbohydrate and determines plant composition (Eastmond 2006; Eastmond et al. 1997; Schiltz et al. 2004; Sulpice et al. 2014; Li et al. 2009; Melis 2013; Johnson and Alric 2013; Weselake et al. 2009; Ishihara et al. 2015). The regulatory mechanisms that interconnect the various fluxes across the metabolic network are being identified using a variety of approaches (Fernandez and Strand 2008; Liscombe and Facchini 2008; Mentzen and Wurtele 2008; Reiter 2008; Santos-Mendoza et al. 2008; Sweetlove et al. 2008; Usadel et al. 2008; Stitt 2013; Stitt et al. 2010; Thum et al. 2008). For example, one breakthrough was the finding that hexoses and other metabolites provide a mechanism to control carbohydrate allocation in part via modulation of transcriptional and post-transcriptional mechanisms (Koch 1996; Jang and Sheen 1994; Che et al. 2003; Baena-Gonzalez et al. 2007; Vidal and Gutierrez 2008). The Arabidopsis-specific orphan gene qua quine starch (*QQS*), one of the ~5% of expressed protein-coding genes in *Arabidopsis thaliana* that are unique to that single species (Arendsee et al. 2014), has been implicated in regulation of starch and protein metabolism (Li et al. 2009; Li and Wurtele 2015).

Carbon and nitrogen use is regulated at multiple levels (Xu et al. 2012). Global system analysis is leading to genes and processes that play a role in conversion of carbon and nitrogen to protein, lipid, and starch (Fukushima et al. 2014; Stitt 2013; Stitt et al. 2010; Thum et al. 2008). For example, the metabolites, such as trehalose 6-phosphate, sugar, and the amino acid precursor, shikimate, have been implicated in the process (Sulpice et al. 2014; Lastdrager et al. 2014). Gene networks mediated by the interaction of light and carbon signaling pathways in Arabidopsis have been defined by a combined approach with genetics, genomics, and systems (Stitt 2013; Thum et al. 2008; Yadav et al. 2014). Starch requires little cellular energy to synthesize and forms easily degradable, compact non-toxic storage units. As such, starch biosynthesis and degradation serves a central role in plant metabolism as the repository for reduced carbon produced in leaves during the day, as the supply of chemical energy and anabolic source molecules originating from sucrose during the night and as a potential storage of easily accessible energy at times of stress. The process is not fully understood, although most of the metabolic enzymes have been identified. Starch synthetic enzymes often are in families, with different members of each of these families often having unique biochemical functions (e.g., Kaplan and Guy 2004; Lao et al. 1999; Scheidig et al. 2002; Edner et al. 2007; Fulton et al. 2008; Laby et al. 2001; Chia et al. 2004; Critchley et al. 2001; Sparla et al. 2006; Lu and Sharkey 2004; Lu et al. 2006; Steichen et al. 2008; Zeeman et al. 2004). Starch

degradation and hexose/triose export involves a large number of enzymes, including families of DBEs, α-amylases, β-amylases, disproportionating enzymes, phosphorylase, glucan water dikinases, and glucose and maltose transporters (Doyle et al. 2007; Delatte et al. 2005, 2006; Lloyd et al. 2005; Smith et al. 2003; Wattebled et al. 2005, 2008; Yu et al. 2005; Zeeman et al. 2004; Streb et al. 2008; Kammerer et al. 1998; Niewiadomski et al. 2005; Walters et al. 2004; Weber et al. 2000, 2004; Niittyla et al. 2004; Schneider et al. 2002).

Owing to the central function of starch, starch production and degradation are highly likely to respond to environmental, circadian rhythm, metabolic, and/or hormonal signals (Li et al. 2007, 2009; Lu et al. 2005; Usadel et al. 2008; Weise et al. 2006). Such regulation is evident from the observation that changing the day length affects the rate of starch degradation in Arabidopsis leaves (Lu et al. 2005). Potential agents affecting starch metabolism include metabolites, the biosynthetic and catabolic enzymes themselves, and a wide variety of regulatory proteins, including previously unknown ones such as the *QQS* gene (Li et al. 2009). Starch regulation involves complex interactions among regulatory mechanisms that interact with global carbon and nitrogen allocation.

Identification of the QQS *Orphan Gene and the Role of* QQS *in Carbon and Nitrogen Allocation*

The starch synthase III, *ss3* knockout (KO), mutant of Arabidopsis is high in starch, but has a normal morphological phenotype (Zhang et al. 2005, 2008). We identified *QQS* as a gene whose expression is significantly altered in *Atss3* mutants relative to wild-type (WT) plants by transcriptome analysis of a microarray experiment (Li et al. 2009).

QQS encodes a 59-amino acid protein with no primary sequence similarity to genes of any other sequenced species, not even *A. lyrata* (Arendsee et al. 2014) or *A. halleri*. Like 15–35% of all eukaryotic genes (Gollery et al. 2006, 2007; Luhua et al. 2008), QQS has no known functional domains. As such, *QQS* is considered an orphan gene—unique to the species *Arabidopsis thaliana* (Gollery et al. 2006). *QQS* is one of the approximately 1,300 protein-encoding orphan genes of *A. thaliana*. Although all organisms, from bacteria to humans, have orphan genes, little is understood about their biological role. Lack of homology between sequence of QQS and any other protein provides no clue about its function. Clarifying the function of such genes should help to explain the overall species-specific regulatory and signaling networks.

Down-regulation of *QQS* results in increased starch and decreased protein, in otherwise normal appearing plants (Li et al. 2009; Li and Wurtele 2015). In contrast, *QQS* overexpression (OE) decreases starch accumulation and increases protein accumulation (Li and Wurtele 2015). Orphans are often disregarded, yet expression of the *QQS* orphan impacts both protein and starch levels and provides a

previously unidentified function in primary metabolism. Surprisingly but similarly, multiple soybean lines with different protein levels that expressed *QQS* (*QQS-E*) had decreased leaf starch, increased leaf protein, and increased seed protein (Li and Wurtele 2012, 2015; Li et al. 2015). Leaf starch and seed starch were decreased; leaf protein and seed protein were increased more than 10–20% in *QQS-E* maize and rice (Li et al. 2015). No aspect of growth or development was visually affected in all *QQS* mutants mentioned above. Thus, this species-specific gene can affect the composition of agronomic species thought to have diverged from Arabidopsis 100 million years ago (Hedges and Kumar 2009). No yield difference in *QQS-E* soybean and rice mutants has been identified (Li and Wurtele 2015; Li et al. 2015). Thus, a species-specific orphan gene can function across species to have effect on the primary metabolic function of carbon and nitrogen partitioning.

An experiment with elongated dark period followed by a diurnal cycle with normal light and dark period indicated that starch degradation was not affected in Arabidopsis *QQS* down-regulation mutants, and the increased starch content came from the increased starch biosynthesis (Li et al. 2009). The effects of *QQS* on starch and protein content are similar in leaves and seeds. We have found no significant differences between photosynthetic rates of *QQS-E* soybean and maize plants relative to their segregating WT siblings (Li et al. 2015). This indicates that changed carbon and nitrogen content in the ectopically expressed *QQS* mutants is not likely a result from an increase in the photosynthetic rate (Li et al. 2015).

Since our initial characterization of *QQS* in 2009 (Li et al. 2009), >30 papers have described the change of *QQS* expression level in response to environmental, genetic, and/or epigenetic perturbations, e.g., Seo et al. 2011; Silveira et al. 2013; and Ding et al. 2014. In Arabidopsis, *QQS* expression correlates positively with protein and negatively with starch under a variety of environmental conditions (Arendsee et al. 2014; Li and Wurtele 2012; Li et al. 2009; Li and Wurtele 2015). We proposed the hypothesis that *QQS* may function to adapt tolerance to stresses and may be a mediator of cross talk between primary metabolism and environmental perturbations.

The mechanism of *QQS* functions in another species has been a mystery. As tested by transgenesis indicated above, *QQS* can function across species barriers. The *QQS* transgene increases protein content in seeds of soybean, rice, and maize, independent of the genetic background of each host (Li and Wurtele 2015; Li et al. 2015). Our studies have provided the mechanism that possibly explains why the *QQS* orphan gene functions across species (Li et al. 2015). Yeast two-hybrid screening using QQS as bait identified Arabidopsis nuclear factor Y subunit C4 (AtNF-YC4, At5g63470) as a potential QQS interactor. Further studies (glutathione-S-transferase (GST) pull-down assays, bimolecular fluorescence complementation assays (BiFC), and co-immunoprecipitation (Co-IP) from Arabidopsis transgenic plants overexpressing MYC-tagged QQS (QQS-TAP)) have confirmed that QQS protein binds to AtNF-YC4 and to soybean, maize, and rice NF-YC4 homologs (Li et al. 2015). QQS did not interact with AtNF-YB7 in pull-down assays; the region of amino acids 73 to 162 of AtNF-YC4 (the AtNF-YC4 histone-fold-like domain) was indicated to be important for AtNF-YC4

binding to QQS (Li et al. 2015). Particularly, QQS-NF-YC4 protein complex appeared in the cytosol and the nucleus when QQS and AtNF-YC4 were co-expressed in tobacco leaf in vivo. One possibility is that predominantly cytosolic QQS (Li et al. 2009) and NF-YC bind in the cytosol and move into the nucleus, similar to the model for NF-YB (Kahle et al. 2005) moving into the nucleus as NF-YB-YC protein complex.

AtNF-YC4-OE in Arabidopsis looked similar to WT controls with decreased leaf starch accumulation and increased leaf protein content (Li et al. 2015), but there was lack of increased starch phenotype in the knockout mutant *Atnf-yc4*. AtNF-YC4 similarly functions as QQS in regulating carbon and nitrogen partitioning, and the redundant NF-YCs may have overlapped function, which are consistent with a model in which QQS interacts with AtNF-YC4 to change the allocation of nitrogen and carbon (Li et al. 2015).

NF-YC is a conserved transcription factor (Liang et al. 2014; Nardini et al. 2013), forming a heterotrimer complex with NF-YA and NF-YB proteins, and can alter transcription of some as yet not clearly defined genes (Nardini et al. 2013). They have been reported to regulate development, photosynthesis, flowering time, and tolerance to drought stress (Petroni et al. 2012; Laloum et al. 2013; Kumimoto et al. 2010; Liang et al. 2014). For example, the Arabidopsis gene AtLEC1, also designated as AtNF-YB9, promotes shoot meristem development (West et al. 1994), whereas CONSTANS is an AtNF-YA that modifies flowering time (Wenkel et al. 2006), and AtNF-YA9 plays a role in gametophyte viability (Levesque-Lemay et al. 2003). Our research is the first report providing evidence that NF-YC4 has a function in regulating primary metabolism (Li et al. 2015).

In short, mechanistic understanding of the transgenic effects of QQS was revealed by our discovery that QQS physically partners with the NF-YC4 protein to manifest its biological function. NF-YC4, a component of the NF-Y complex, a transcriptional regulator which is highly conserved across eukaryotes, possibly explains how QQS may be able to function across many species. Thus, the QQS orphan would be able to approach a biological network of a host via interactions with the conserved NF-Y complex protein.

Soybean, rice, and maize are major agronomic food crops. These findings are of major significance in impacting the world's nutritional supply of dietary protein. These data also indicate a possible avenue toward increasing protein composition in crop species, as protein deficiency is one of the greatest health problems worldwide, and plants provide the major protein sources to at-risk populations. Protein deficiency especially affects children, for whom crops such as rice and potato (protein-poor crops) are often their major dietary constituents, leading to a protein-poor diet. Furthermore, the consumption of proteins derived from plants has far less environmental impact than consumption of animal-derived protein sources. Thus, the ability to optimize protein productivity by use of higher protein plant-based foods would have far-ranging impacts to world health and sustainability.

QQS and Orphan Genes

To our knowledge, the *QQS* gene of Arabidopsis is the only plant orphan gene that has been significantly investigated. A number of orphan genes act as toxins or attractants (Arendsee et al. 2014). Three orphan genes appear to have a role in protection against oxidative stress via an as yet unknown mechanism (Luhua et al. 2008; Gollery et al. 2006, 2007). Several others have been experimentally shown to enable an organism to survive under abiotic stress; however, the mechanism by which these genes function is still to be discovered (Luhua et al. 2008; Gollery et al. 2006, 2007). At least one orphan gene from yeast appears to be essential (Khalturin et al. 2009). The vast majority of orphan genes have not been studied at all.

Taken together, previous data indicate that QQS may increase protein accumulation and decrease carbohydrate accumulation via its interaction with NF-YC4 protein, that this effect is irrespective of protein content level, and that the effect extends to the crop species diverged from Arabidopsis more than 100 million years ago (Hedges and Kumar 2009). The demonstration that an orphan gene from one species can interact with a metabolic network of another species via a conserved protein suggests new approaches to elicit phenotype changes including modulation of complex traits in crop species. In this study, we further provide evidence that overexpression of QQS interactor NF-YC4 can alter plant composition in crop species in soybean and maize, with similar growth and development as the control plants. Transcriptome analyses of the *QQS* mutant materials identified potential candidates for further study of nitrogen allocation.

Materials and Methods

Plant Selection and Growth

The *QQS*-expressing (*QQS-E*) soybean lines in the Williams 82 background were generated previously, and the plant composition and expression level of *QQS* have been quantified (Li and Wurtele 2015). The full-length coding sequences of *GmNF-YC4-1* (Glyma06g17780) and *ZmNF-YC4* (GrmZm2g089812) were each cloned into binary vector pB2GW7, as previously described (Li and Wurtele 2015; Li et al. 2015). The insert was expressed under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The 35S::GmNF-YC4-1 or 35S::ZmNF-YC4 fusion construct was introduced into *Agrobacterium tumefaciens* strain EHA101 and transformed into soybean (*Glycine max*) cultivar Williams 82 (Li and Wurtele 2015) or maize B104 at the Iowa State University (ISU) Plant Transformation Facility (PTF, http://www.agron.iastate.edu/ptf/index.aspx). The transformed soybean seeds and plants were delivered from the PTF at the T1 generation. The T1 generation was grown and self-fertilized in the growth chamber in Metro mix MM900 soil in pots with one plant/pot. The *GmNF-YC4-1-OE* T2 generation was planted in a field at Curtiss Farm in Ames, IA, for seed weight and seed

composition evaluation (T3 generation). The maize plants were delivered at the T0 generation and backcrossed to B104 in the greenhouse. The seeds from BC1 generation were planted in the field in South Woodruff Farm in Ames and backcrossed to B104 to generate BC2 kernels.

Arabidopsis plants were grown in a growth chamber at 22 °C under long-day conditions as described (Li et al. 2009). The seeds were planted on petri dishes, those harboring a "Bar" gene were selected with glufosinate as previously described (Jones et al. 2016), and plants were transferred to Sunshine Mix LC1 soil in pots at 12 d after planting. Soybean transformants, expressing *AtQQS* (*AtQQS-E*) or overexpressing *GmNF-YC4-1* (*GmNF-YC4-1-OE*), and maize plants overexpressing *ZmNF-YC4* were identified by PCR analysis for presence of the *QQS* or *NF-YC4* via vector-specific primers as described before (Li and Wurtele 2015). The vector-specific primers are pB2GW7-F: 5′-ACATTACAATTTACTATTCTAG TCGA-3′ and pB2GW7-R: GCGGACTCTAGCATGGCCG-3′; the control-gene primers are 18S-rRNA-F: 5′-GGGCATTCGTATTTCATAGTCAGAG-3′ and 18S-rRNA-R: 5′-CGGTTCTTGATTAATGAAAACATCCT-3′.

RNA-Seq

Total RNA was extracted from pooled Arabidopsis leaf samples of *QQS-OE* and Col-0 at the end of light period under long-day conditions in a growth chamber, or from soybean leaf samples of *QQS-E* and Williams 82 and from soybean seed samples of *QQS-E*, Williams 82, and low-protein PI 070456; the RNA was purified and sent to BGI Americas for sequencing as previously described (Li et al. 2015).

Two biological replicates were used for Arabidopsis *QQS-OE* mutant leaf samples, three were used for the Arabidopsis Col-0 controls, four for soybean *QQS-E* leaf samples, two for Williams 82 leaf controls, ten for soybean *QQS-E* seed samples and controls, and four for low-protein PI 070456 seed samples and controls. As previously described (Li et al. 2015), the cleaned reads were aligned using TopHat (Trapnell et al. 2009) and the mapped reads were counted. Similarly to the analyses as described before (Li et al. 2015), genes were tested using the method described by Lund et al. (2012) and QuasiSeq in the R package. Normalization was accomplished (Bullard et al. 2010). The *P* values and *q* values (Storey 2002) were calculated as previously described (Nettleton et al. 2006).

RNA Isolation and Real-Time PCR

The first trifoliate leaves of 23-d-old soybeans (∼100 mg fresh weight) were used for RNA isolation using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Two μg of RNA and SuperScript® III

First Strand kit (Invitrogen, Carlsbad, CA, USA) were used for cDNA synthesis. Quantitative real-time PCR (qRT-PCR) was performed using the cDNA and gene-specific primers of *GmNF-YC4-1* (GmNF-YC4-F: 5′-CCTCCCAGG CATGGCAGTCC-3′ and GmNF-YC4-R: 5′-CCATCAAGGCTCCGCTGG-3′). Each cDNA was amplified by quantitative PCR using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and iCycler real-time PCR system (Bio-Rad). *GmACTIN* (Glyma.15g050200, primers: GmActin-F: 5′-GAGC TATGAATTGCCTGATGG-3′ and GmActin-R: 5′-CGTTTCATGAATTCC AGTAGC-3′) was used as the reference gene to normalize the expression value in each sample, and the relative expression values were determined compared to that in the independent Williams 82 control samples, using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Liu et al. 2014).

Composition Analysis

Maize leaves were harvested individually (leaves from individual plant were stored in one envelope) and frozen in liquid nitrogen and kept in −80 °C freezer, at 63 days after planting, in the late afternoon. The entire third leaf from the top was harvested. Each plant was screened for genotype by PCR of genomic DNA of the leaf, determined as *ZmNF-YC4-OE* or sibling wild type using vector-specific primers as described above. Leaves from three plants of the same genotype (*ZmNF-YC4-OE* or sibling wild type) were crushed and pooled together as one sample. A small portion of each sample was used for the Lowry test; the larger portion was weighed to obtain the fresh weight, baked at 71 °C, and used to obtain the dry weight. The fresh and dry weights were used to calculate the moisture content. Nitrogen content per dry weight were determined by Kjeldahl method using the Kjeltec system 1002 with the dry samples, and protein content was calculated by multiplying 6.25. Protein content per dry weight was also determined by Lowry test kit following the manufacture's protocol (Fisher Scientific) using the frozen sample (the moisture content was used to convert fresh weight to dry weight).

Composition of soybean mature seeds (protein, oil, and fiber) and of maize mature seeds (protein, starch and oil) was analyzed with near-infrared spectroscopy at the Iowa State University Grain Quality Laboratory (http://www.extension.iastate.edu/Grain/Lab/) as described before (Li et al. 2015).

Statistical and Bioinformatics Analyses

For each experiment, plants were collected and analyzed in a randomized complete block design or completely randomized design. All plant composition tests were conducted with a minimum of three biological replicates. For all composition analyses, plant samples were assigned randomized numbers and provided to the

analysis facilities for determination in a randomized order with no designator of genotype.

Data are presented as mean ± SEM. Two sets of independent samples were compared using Student's t-test (two-tailed) with assumption of equal variances ($n \geq 3$). $P < 0.05$ was considered significant (*); $P < 0.01$ was considered very significant (**).

Estimates of gene size and gene uniqueness and gene annotations are based on the gene models described in Arabidopsis using the TAIR10 genome release (ftp://www.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/). The consensus sequences of soybean genome were used to identify the Arabidopsis orthologs. The annotation is available in https://soybase.org/genomeannotation/.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Genome Information Resource under the following accession numbers: *AtQQS* (At3g30720) and *AtNF-YC4* (At5g63470).

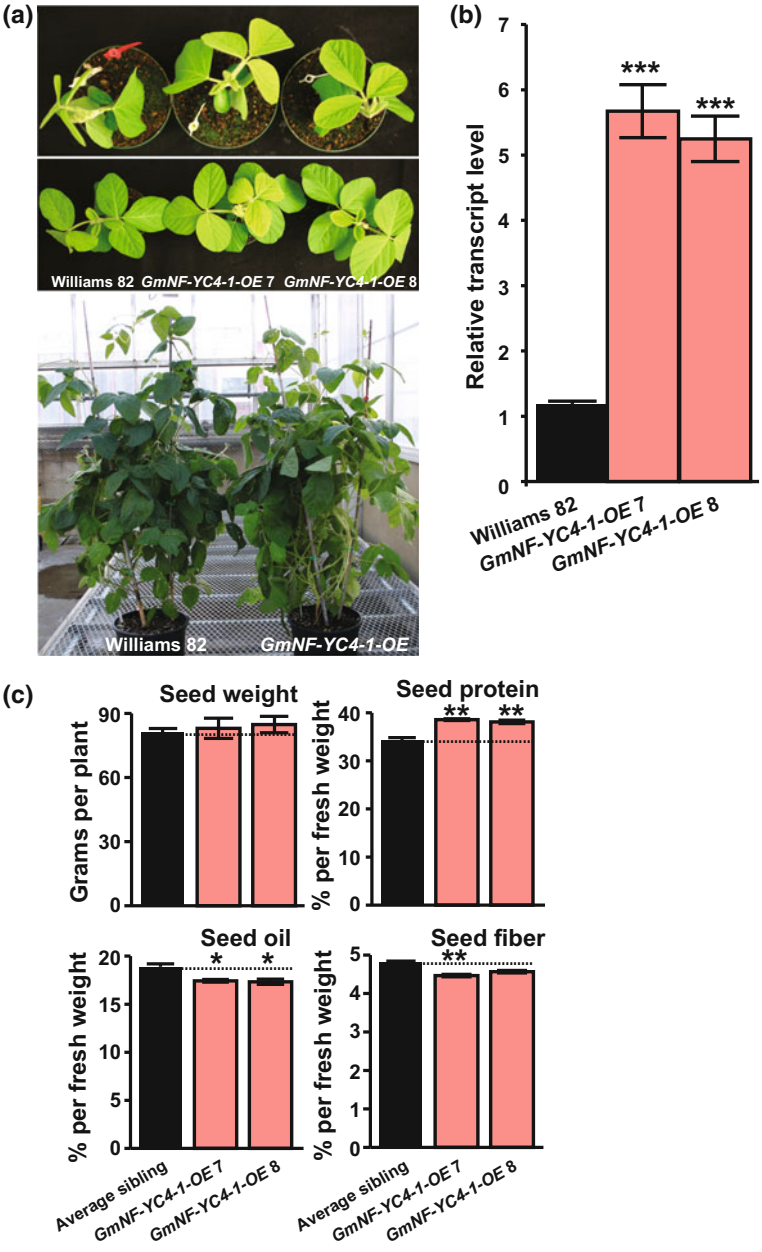
Results and Discussion

Overexpression of QQS Interactor NF-YC4 *in Transgenic Soybeans Regulates Nitrogen Allocation*

Soybean does not have a homolog of *AtQQS*, but it does have a highly conserved homolog that encodes NF-YC4 (Glyma06g17780) which interacts with QQS (Li et al. 2015). Plants that overexpressed *GmNF-YC4-1* (*GmNF-YC4-1-OE*) looked indistinguishable from Williams 82 control plants (Fig. 6.1a). The *GmNF-YC4* mRNA expression in two independent *GmNF-YC4-1-OE* lines was estimated to be 4.47-fold higher than that in the Williams 82 control plants ($P < 0.001$ for both) (Fig. 6.1b). The seed protein content increased by 8–11% in the *GmNF-YC4-1-OE* lines ($P < 0.001$ for both), while oil decreased by 2–6% ($P = 0.002$ and < 0.001) and fiber decreased by 3–6% ($P < 0.001$ for both), and no significant difference in yield per plant was observed (Fig. 6.1c). These data are consistent with our previous studies and support the idea that AtQQS can also interact with the GmNF-YC4-1 to promote an increase in the soybean protein content (Arendsee et al. 2014; Li and Wurtele 2015; Li et al. 2015).

Overexpression of *NF-YC4* in Transgenic Maize Regulates Nitrogen Allocation in the Leaves and Seeds

Similar to soybean, maize does not have a homolog of *AtQQS*, but it does have a highly conserved homolog that encodes NF-YC4 (GrmZm2g089812) which


(a)
Williams 82 GmNF-YC4-1-OE 7 GmNF-YC4-1-OE 8
Williams 82 GmNF-YC4-1-OE
(b)
Relative transcript level
***
***
Williams 82
GmNF-YC4-1-OE 7
GmNF-YC4-1-OE 8
(c)
Seed weight
Grams per plant
Seed protein
% per fresh weight
**
**
Seed oil
% per fresh weight
*
*
Seed fiber
% per fresh weight
**
Average sibling
GmNF-YC4-1-OE 7
GmNF-YC4-1-OE 8

◂**Fig. 6.1** Phenotype and composition of soybean transgenic lines overexpressing *GmNF-YC4-1*. **a** Visual phenotype and developmental patterns of *GmNF-YC4-1-OE* mutant lines were similar to the Williams 82 control plants (picture taken in the greenhouse). **b** Transcript level of *GmNF-YC4-1* in the *GmNF-YC4-1-OE* soybeans is significantly higher than that in the control line. *GmNF-YC4* mRNA transcripts were quantified by real-time PCR in transgenic and non-transgenic plants, compared to that in the independent Williams 82 control plants. **c** Seed weight per plant and composition of protein, oil, and fiber (seeds from T2 generation grown in the field). Composition was analyzed by near-infrared spectroscopy (NIRS), based on a 13% moisture content. All data in bar charts show mean ± SEM, $n = 3$ biological replicates, from two independent transformation events. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

interacts with QQS (Li et al. 2015). Maize B104 was transformable and derived from maize B73. The B104 genome is very similar to that of B73 which has been sequenced. Plants that overexpressed *ZmNF-YC4* (*ZmNF-YC4-OE*) looked similar to their segregated sibling control plants. The leaf protein content per dry weight was increased about 20% in *ZmNF-YC4-OE* when compared to WT, both by Lowry test (Fig. 6.2a) and by Kjeldahl method (Fig. 6.2b) ($P < 0.05$). The seed protein content, when compared to control, increased by 15-23% in the *ZmNF-YC4-OE* lines ($P < 0.001$), while starch decreased by 3% ($P < 0.01$), and no significant difference in oil was observed (Fig. 6.2c). These data are also consistent with our previous studies and support that AtQQS may interact with the ZmNF-YC4 to promote an increase in the maize protein content.

Identifying the Potential Soybean Transcripts Associated with the High-Protein Trait

We have a set of Arabidopsis and soybean plants with different combinations of protein content and *QQS* expression level (Table 6.1). As discovered in our previous study, *QQS* overexpression is associated with increased protein content in Arabidopsis leaf, soybean leaf, and soybean seeds. This set of materials, together with corresponding controls and a low-seed-protein soybean variety PI 070456 (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1115548), could be used to identify the potential soybean transcripts associated with the high-protein trait by RNA-sequencing (RNA-Seq).

There were 1445 transcripts differentially expressed in Arabidopsis *QQS-OE* leaf ($P < 0.01$) and 2249 transcripts differentially expressed in soybean *QQS-E* leaf ($q < 0.01$). The transcripts that were significantly altered in both Arabidopsis *QQS-OE* leaf and soybean *QQS-E* leaf are candidate genes potentially involved in regulation of protein accumulation.

Among them, fifteen transcripts may be positively associated with high protein in leaf (Table 6.2). They were expressed in WT Arabidopsis and soybean leaf, but had higher expression in Arabidopsis *QQS-OE* leaf and soybean *QQS-E* leaf. For example, Glyma02g03680 and its ortholog in Arabidopsis, AT1G24020 (Fig. 6.3a), are on this list. AT1G24020 is annotated as "MLP (member of the

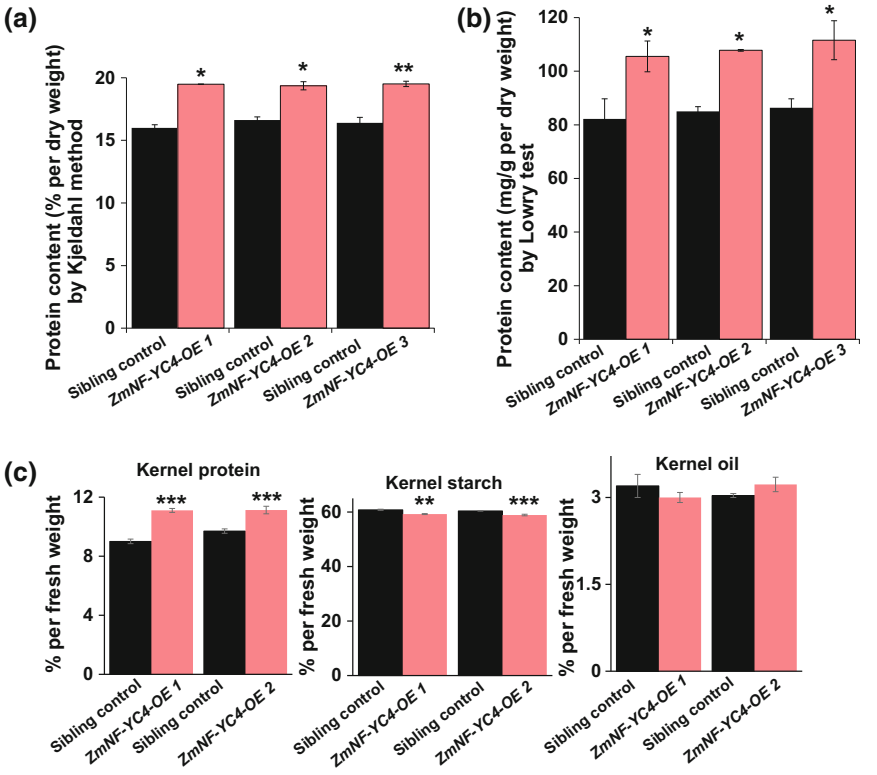


Fig. 6.2 Composition of maize transgenic lines overexpressing *ZmNF-YC4* planted in the field. **a** Protein content in leaf in *ZmNF-YC4-OE* mutant lines was higher by Kjeldahl method. **b** Protein content in leaf in *ZmNF-YC4-OE* mutant lines was higher by Lowry test. **c** Seed composition of protein, starch, and oil (BC2 generation). Composition was analyzed by near-infrared spectroscopy (NIRS), based on a 15% moisture content. All data in bar charts show mean ± SEM, $n = 3$ biological replicates, from multiple independent transformation events. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

Table 6.1 Materials with different *QQS* transcript level and protein accumulation

Genotype	Species	Tissue	***QQS transcript level***	Protein content
QQS-OE	*Arabidopsis thaliana*	Leaf	Increased *QQS*	High
Col-0	*Arabidopsis thaliana*	Leaf	Control	Control
QQS-E	*Glycine max*	Leaf	Ectopic *QQS*	High
Williams 82	*Glycine max*	Leaf	No	Control
QQS-E	*Glycine max*	Seed	Ectopic *QQS*	High
Williams 82	*Glycine max*	Seed	No	Control
PI 070456	*Glycine max*	Seed	No	Low

Table 6.2 Transcripts that may be positively associated with high-protein content in leaf and were differentially expressed in both Arabidopsis *QQS-OE* leaf and soybean *QQS-E* leaf

Soybean locus ID	Matched Arabidopsis locus ID	Transcript count (Mean ± SEM)			
		Arabidopsis leaf (Col-0)	Arabidopsis leaf (*QQS-OE*)	Soybean leaf (Williams 82)	Soybean leaf (*QQS-E*)
[a]Glyma02g03680	AT1G24020	307.3 ± 51.7	474.0 ± 16.0	292.5 ± 89.5	1204.3 ± 178.8
Glyma12g29100	AT1G60950	5278.3 ± 923.2	8130.0 ± 1082.0	868.5 ± 414.5	2398.3 ± 378.8
Glyma02g09510	AT1G67950	76.7 ± 12.4	109.0 ± 2.0	115.0 ± 11.0	239.5 ± 31.0
Glyma02g40290	AT2G30490	668.3 ± 25.6	1002.0 ± 27.0	716.5 ± 114.5	1705.0 ± 140.5
Glyma10g15730	AT2G36780	1.3 ± 0.3	9.5 ± 6.5	17.5 ± 12.5	121.5 ± 14.3
Glyma05g26450	AT2G39080	135.7 ± 21.7	194.0 ± 12.0	475.0 ± 278.0	620.0 ± 56.5
Glyma16g06930	AT3G01710	21.7 ± 6.0	42.0 ± 4.0	8.0 ± 6.0	23.8 ± 2.5
Glyma13g23630	**AT3G47650**	832.7 ± 21.8	1562.5 ± 3.5	547.0 ± 280.0	693.8 ± 59.1
Glyma17g12370	**AT3G47650**	832.7 ± 21.8	1562.5 ± 3.5	539.0 ± 281.0	610.5 ± 49.5
Glyma04g19020	**AT3G47650**	832.7 ± 21.8	1562.5 ± 3.5	403.5 ± 129.5	510.3 ± 46.4
Glyma06g08230	AT4G20325	44.7 ± 4.1	72.5 ± 0.5	36.5 ± 6.5	73.5 ± 4.6
Glyma14g38090	AT4G35160	9.7 ± 0.3	45.0 ± 15.0	151.5 ± 95.5	352.5 ± 64.3
Glyma10g28090	AT5G03560	29.7 ± 0.9	44.5 ± 2.5	268.0 ± 161.0	348.5 ± 36.6
Glyma04g31810	AT5G60910	30.0 ± 5.5	50.5 ± 3.5	4.0 ± 4.0	17.5 ± 1.6
Glyma08g17290	AT5G64670	141.0 ± 18.1	206.5 ± 9.5	123.5 ± 24.5	185.0 ± 13.7

[a]The accumulation of this transcript is visualized in Fig. 6.3a

*Bold font locus IDs indicate multiple soybean genes matched to the same Arabidopsis gene by sequence similarity

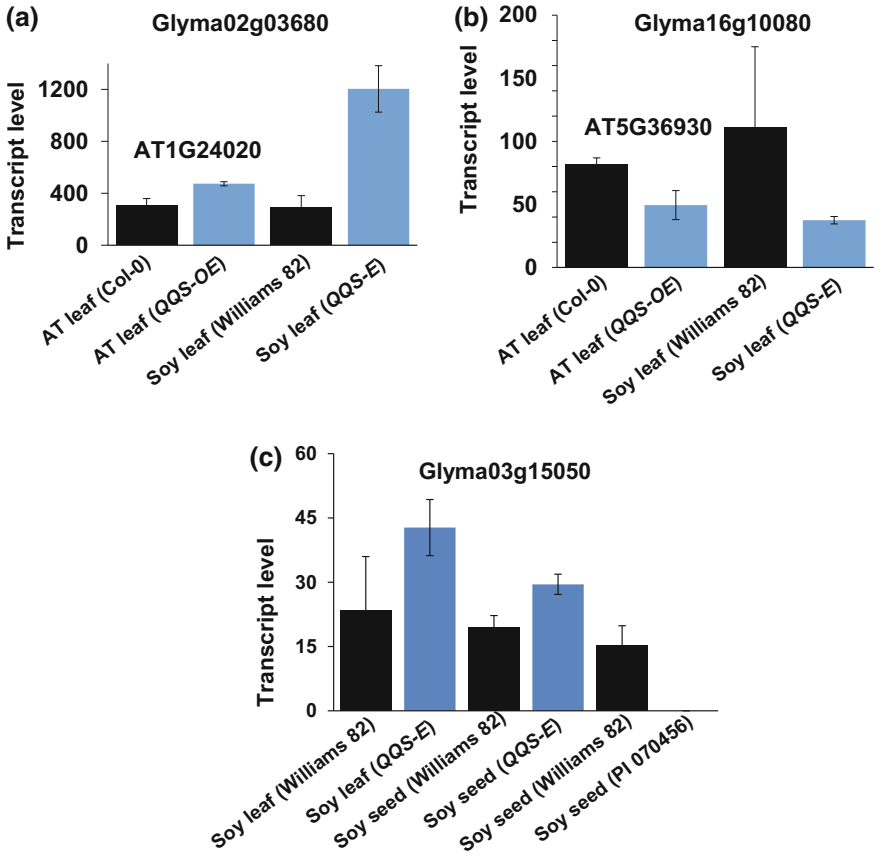


Fig. 6.3 Transcript accumulation in Arabidopsis and soybean leaf and soybean seed. **a** One transcript that may be positively associated with high protein in leaf. **b** One transcript that may be negatively associated with high protein in leaf. **c** One transcript that may be positively associated with high protein in leaf and seed. All data in bar charts show mean ± SEM, $n = 3$ biological replicates

Latex Protein family)-like protein 423", located in "chloroplast" and involved in "defense response" (Litholdo et al. 2016). Specifically, three soybean locus IDs and their Arabidopsis ortholog "AT3G47650" are on this list. AT3G47650 encodes a "DnaJ/Hsp40 cysteine-rich domain superfamily protein" and is located in "chloroplast". AT3G47650 was significantly down-regulated in cabbage leaf curl virus (CaLCuV)-infected rosette leaves at 12 days after inoculation (Ascencio-Ibáñez et al. 2008).

Among them, sixty-one transcripts may be negatively associated with high protein in leaf (Table 6.3). They were expressed in WT Arabidopsis and soybean leaf, but had a lower expression in Arabidopsis *QQS-OE* and soybean *QQS-E* leaf. As indicated by locus IDs in bold font, there were twelve groups of multiple soybean genes that genes

Table 6.3 Transcripts that may be negatively associated with high-protein content in leaf and were differentially expressed in both Arabidopsis *QQS-OE* leaf and soybean *QQS-E* leaf

Soybean locus ID	Matched Arabidopsis locus ID	Transcript count (Mean ± SEM)			
		Arabidopsis leaf (WT)	Arabidopsis leaf (*QQS-OE*)	Soybean leaf (WT)	Soybean leaf (*QQS-E*)
Glyma13g24520	**AT1G28380**	127.0 ± 17.0	76.5 ± 11.5	51.0 ± 50.0	6.5 ± 1.3
Glyma07g32010	**AT1G28380**	127.0 ± 17.0	76.5 ± 11.5	48.0 ± 41.0	8.5 ± 3.2
Glyma15g02000	AT1G52190	240.3 ± 102.8	102.5 ± 4.5	31.5 ± 27.5	3.3 ± 0.5
Glyma12g33230	**AT1G53050**	107.7 ± 11.0	69.5 ± 4.5	133.0 ± 82.0	66.8 ± 6.9
Glyma13g37230	**AT1G53050**	107.7 ± 11.0	69.5 ± 4.5	175.5 ± 107.5	105.0 ± 7.6
Glyma06g44730	**AT1G53050**	107.7 ± 11.0	69.5 ± 4.5	397.5 ± 216.5	251.0 ± 11.2
Glyma04g03150	**AT1G75410**	125.3 ± 11.1	82.5 ± 0.5	132.5 ± 56.5	48.3 ± 7.0
Glyma06g03200	**AT1G75410**	125.3 ± 11.1	82.5 ± 0.5	174.5 ± 104.5	56.0 ± 8.1
Glyma14g08120	AT2G16250	35.3 ± 3.9	18.0 ± 3.0	360.5 ± 282.5	191.3 ± 15.1
Glyma12g32520	**AT2G19130**	55.3 ± 5.9	29.0 ± 6.0	102.0 ± 73.0	65.3 ± 3.3
Glyma03g00530	**AT2G19130**	55.3 ± 5.9	29.0 ± 6.0	72.5 ± 68.5	8.5 ± 1.2
Glyma06g45590	**AT2G19130**	55.3 ± 5.9	29.0 ± 6.0	119.0 ± 84.0	54.0 ± 5.0
Glyma07g19120	AT2G38310	161.7 ± 14.1	97.5 ± 0.5	203.5 ± 173.5	58.3 ± 8.0
Glyma18g46750	**AT2G45910**	65.3 ± 7.3	36.5 ± 9.5	590.0 ± 298.0	352.5 ± 33.1
Glyma03g01110	**AT2G45910**	65.3 ± 7.3	36.5 ± 9.5	73.0 ± 72.0	2.3 ± 0.9
Glyma20g30030	**AT2G45910**	65.3 ± 7.3	36.5 ± 9.5	56.0 ± 17.0	26.8 ± 1.4
Glyma07g07650	**AT2G45910**	65.3 ± 7.3	36.5 ± 9.5	290.5 ± 245.5	37.8 ± 8.8
Glyma10g02370	AT2G47800	271.0 ± 36.1	139.5 ± 11.5	1176.5 ± 754.5	386.8 ± 52.1
Glyma20g34670	AT3G01680	149.0 ± 35.2	75.0 ± 5.0	6274.0 ± 5614.0	218.8 ± 36.8
Glyma16g28460	AT3G05650	78.7 ± 14.2	42.0 ± 4.0	60.5 ± 55.5	0.8 ± 0.5
Glyma02g28530	AT3G12020	139.0 ± 26.9	79.5 ± 9.5	266.5 ± 168.5	187.0 ± 13.8

(continued)

Table 6.3 (continued)

Soybean locus ID	Matched Arabidopsis locus ID	Transcript count (Mean ± SEM)			
		Arabidopsis leaf (WT)	Arabidopsis leaf (*QQS-OE*)	Soybean leaf (WT)	Soybean leaf (*QQS-E*)
Glyma13g34090	AT3G14840	370.7 ± 53.4	226.0 ± 10.0	78.5 ± 75.5	10.8 ± 1.4
Glyma07g37100	AT3G16910	477.3 ± 12.4	326.5 ± 16.5	612.5 ± 156.5	335.3 ± 33.9
Glyma09g40870	AT3G28040	353.7 ± 70.5	221.0 ± 5.0	513.5 ± 500.5	104.8 ± 12.2
Glyma11g09230	AT3G51480	31.0 ± 7.2	11.5 ± 3.5	187.5 ± 129.5	112.3 ± 14.6
Glyma07g37930	AT3G62900	95.3 ± 10.2	55.0 ± 2.0	104.0 ± 55.0	42.3 ± 7.1
Glyma10g27860	AT4G01720	35.0 ± 6.7	15.5 ± 3.5	66.0 ± 64.0	7.0 ± 1.2
Glyma09g04310	AT4G16150	151.3 ± 5.8	103.5 ± 3.5	313.5 ± 175.5	181.5 ± 16.8
Glyma11g00510	**AT4G23180**	156.3 ± 15.3	85.0 ± 14.0	12.0 ± 11.0	0.3 ± 0.3
Glyma20g27690	**AT4G23180**	156.3 ± 15.3	85.0 ± 14.0	33.5 ± 33.5	4.3 ± 1.1
Glyma18g45180	**AT4G23180**	156.3 ± 15.3	85.0 ± 14.0	89.5 ± 88.5	1.8 ± 0.6
Glyma20g27510	**AT4G23180**	156.3 ± 15.3	85.0 ± 14.0	10.5 ± 10.5	0.8 ± 0.5
Glyma20g27670	**AT4G23180**	156.3 ± 15.3	85.0 ± 14.0	11.5 ± 8.5	1.5 ± 0.6
Glyma10g32710	AT4G28600	94.0 ± 6.1	56.5 ± 9.5	137.5 ± 121.5	65.3 ± 5.1
Glyma04g05250	**AT4G32940**	260.7 ± 59.1	151.5 ± 0.5	453.0 ± 274.0	285.5 ± 24.1
Glyma14g10620	**AT4G32940**	260.7 ± 59.1	151.5 ± 0.5	1140.5 ± 734.5	424.8 ± 29.6
Glyma18g44930	AT5G01950	78.7 ± 13.9	35.5 ± 7.5	1214.5 ± 1196.5	58.8 ± 12.8
Glyma02g26890	**AT5G06600**	417.7 ± 41.2	279.5 ± 3.5	156.0 ± 107.0	79.3 ± 6.9
Glyma03g36450	**AT5G06600**	417.7 ± 41.2	279.5 ± 3.5	133.5 ± 123.5	25.0 ± 3.3
Glyma07g38510	AT5G19010	198.7 ± 27.3	123.0 ± 3.0	465.0 ± 359.0	210.3 ± 17.2
[a]**Glyma16g10080**	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	111.5 ± 63.5	37.5 ± 2.9
Glyma16g33910	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	290.5 ± 248.5	94.5 ± 15.6
Glyma19g07650	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	264.0 ± 245.0	25.5 ± 6.0

(continued)

Table 6.3 (continued)

Soybean locus ID	Matched Arabidopsis locus ID	Transcript count (Mean ± SEM)			
		Arabidopsis leaf (WT)	Arabidopsis leaf (*QQS-OE)*	Soybean leaf (WT)	Soybean leaf (*QQS-E)*
Glyma02g02790	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	115.0 ± 112.0	16.5 ± 4.1
Glyma16g10270	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	109.0 ± 99.0	41.5 ± 5.2
Glyma16g33930	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	10.5 ± 8.5	1.8 ± 0.3
Glyma08g41270	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	73.0 ± 61.0	13.3 ± 3.6
Glyma16g34000	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	12.5 ± 10.5	3.5 ± 0.3
Glyma16g23790	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	464.5 ± 353.5	254.0 ± 2.6
Glyma16g25110	**AT5G36930**	81.7 ± 5.2	49.5 ± 11.5	318.5 ± 292.5	58.3 ± 13.5
Glyma17g16150	AT5G47040	236.7 ± 32.2	135.5 ± 7.5	472.5 ± 292.5	283.5 ± 38.6
Glyma08g43780	AT5G48150	81.0 ± 2.5	37.5 ± 2.5	397.5 ± 202.5	178.5 ± 10.7
Glyma07g40100	**AT5G49760**	249.3 ± 13.2	158.0 ± 9.0	207.5 ± 205.5	45.0 ± 8.2
Glyma09g02210	**AT5G49760**	249.3 ± 13.2	158.0 ± 9.0	2217.0 ± 2166.0	276.5 ± 57.0
Glyma05g37680	**AT5G52430**	64.7 ± 9.9	38.5 ± 2.5	260.0 ± 170.0	109.8 ± 18.8
Glyma01g42970	**AT5G52430**	64.7 ± 9.9	38.5 ± 2.5	41.5 ± 37.5	5.3 ± 0.9
Glyma17g07520	AT5G57710	383.7 ± 7.4	245.5 ± 13.5	712.0 ± 438.0	441.3 ± 26.2
Glyma12g29630	AT5G60570	92.0 ± 4.7	60.0 ± 8.0	611.0 ± 353.0	300.5 ± 20.6
Glyma05g07380	AT5G60910	30.0 ± 5.5	50.5 ± 3.5	86.5 ± 33.5	260.3 ± 23.9
Glyma14g08800	**AT5G66850**	134.0 ± 22.3	66.0 ± 3.0	111.0 ± 81.0	44.3 ± 4.9
Glyma17g36380	**AT5G66850**	134.0 ± 22.3	66.0 ± 3.0	31.0 ± 29.0	2.0 ± 0.9

[a]The accumulation of this transcript is visualized in Fig. 6.3b

*Bold font locus IDs indicate twelve groups of multiple soybean genes that genes within each group matched to the same Arabidopsis gene by sequence similarity

within each group matched to the same Arabidopsis gene by sequence similarity. Specifically, Arabidopsis gene, AT5G36930 (Fig. 6.3b), is the Arabidopsis ortholog of ten soybean genes. It is annotated as "disease resistance protein (TIR-NBS-LRR class) family", located in "cytoplasm", and involved in "defense response". This provides another case to indicate that genes in disease resistance may be related to regulation of primary metabolism in nitrogen allocation (Qi et al. 2018).

There were 2249 transcripts differentially expressed in soybean *QQS-E* leaf ($q < 0.01$), 2314 transcripts differentially expressed in soybean *QQS-E* seed ($q < 0.001$), and 108 transcripts differentially expressed in low-protein PI 070456 seed ($P < 0.001$). Among them, there were 173 transcripts in common that were differentially expressed in soybean *QQS-E* leaf and seed. Interestingly, one gene, Glyma03g15050, was in common differentially expressed in soybean *QQS-E* leaf and seed, and low-protein PI 070456 (Fig. 6.3c). Its ortholog in Arabidopsis is AT3G54060, annotated as "myosin-M heavy protein", located in "nucleus". In Arabidopsis, it is expressed in reproductive organs in flower, plant sperm cell, and seed (https://www.arabidopsis.org/servlets/TairObject?id=36606&type=locus). Its transcript accumulation was higher in *QQS-E* soybean leaf and seed, and very low in low-protein PI 070456 seed. It may be associated with high-protein trait in soybean leaf and seed.

In conclusion, this research broadens our understanding of the Arabidopsis orphan gene *QQS* and its interactor *NF-YC4* as a modulator of plant composition and indicates that *NF-YC4* impacts carbon and nitrogen allocation to starch, lipid, and protein, increasing the protein content of soybean and maize. Seeds of transgenic crops expressing the *QQS* gene and *NF-YC4* yield increased protein without yield penalty. Multiple genes were identified as potential candidate genes in nitrogen allocation in *QQS* mutants. QQS and its related network open a new strategy to understand nitrogen allocation and create high-protein crops.

Acknowledgements We thank Diane Luth, Bronwyn Frame, and Kan Wang at ISU PTF for soybean and maize transformation; Kent Berns for field management; Charles Hurburgh and Glen Rippke at ISU Grain Quality Laboratory for NIRS analysis of soybean and maize seed composition; and Eurofins for Kjeldahl analysis. The transcriptome sequencing was conducted in conjunction with BGI. We thank BGI for contributing its expertise in genomic sequencing and bioinformatics analysis to provide processed sequencing data. This material is based in part upon work supported by: National Science Foundation (L.L. as Co-PI on MCB-0951170), United Soybean Board (2287 to L.L.), Iowa Soybean Association (to L.L.), Amfora Inc (to L.L.), ISU Research Foundation (to L.L.), and Center for Metabolic Biology at ISU.

References

Arendsee ZW, Li L, Wurtele ES (2014) Coming of age: orphan genes in plants. Trends Plant Sci 19:698–708

Ascencio-Ibáñez JT, Sozzani R, Lee T-J, Chu T-M, Wolfinger RD, Cella R, Hanley-Bowdoin L (2008) Global analysis of arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol 148:436–454

Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature 448(7156):938–942

Beatty PH, Klein MS, Fischer JJ, Lewis IA, Muench DG, Good AG (2016) Understanding plant nitrogen metabolism through metabolomics and computational approaches. Plants (Basel) 5:39

Beatty PH, Shrawat AK, Carroll RT, Zhu T, Good AG (2009) Transcriptome analysis of nitrogen-efficient rice over-expressing alanine aminotransferase. Plant Biotechnol J 7:562–576

Bullard JH, Purdom E, Hansen KD, Dudoit S (2010) Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinformatics 11:94

Che P, Weaver LM, Wurtele ES, Nikolau BJ (2003) The role of biotin in regulating 3-methylcrotonyl-coenzyme a carboxylase expression in Arabidopsis. Plant Physiol 131:1479–1486

Chia T, Thorneycroft D, Chapple A, Messerli G, Chen J, Zeeman SC, Smith SM, Smith AM (2004) A cytosolic glucosyltransferase is required for conversion of starch to sucrose in Arabidopsis leaves at night. Plant J 37:853–863

Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM (2001) A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in Arabidopsis. Plant J 26:89–100

Delatte T, Trevisan M, Parker ML, Zeeman SC (2005) Arabidopsis mutants Atisa1 and Atisa2 have identical phenotypes and lack the same multimeric isoamylase, which influences the branch point distribution of amylopectin during starch synthesis. Plant J 41:815–830

Delatte T, Umhang M, Trevisan M, Eicke S, Thorneycroft D, Smith SM, Zeeman SC (2006) Evidence for distinct mechanisms of starch granule breakdown in plants. J Biol Chem 281:12050–12059

Ding ZJ, Yan JY, Xu XY, Yu DQ, Li GX, Zhang SQ, Zheng SJ (2014) Transcription factor WRKY46 regulates osmotic stress responses and stomatal movement independently in Arabidopsis. Plant J 79:13–27

Doyle EA, Lane AM, Sides JM, Mudgett MB, Monroe JD (2007) An alpha-amylase (At4g25000) in Arabidopsis leaves is secreted and induced by biotic and abiotic stress. Plant Cell Environ 30:388–398

Eastmond PJ (2006) SUGAR-DEPENDENT1 encodes a patatin domain triacylglycerol lipase that initiates storage oil breakdown in germinating Arabidopsis seeds. Plant Cell 18:665–675

Eastmond PJ, Dennis DT, Rawsthorne S (1997) Evidence that a malate/inorganic phosphate exchange translocator imports carbon across the leucoplast envelope for fatty acid synthesis in developing castor seed endosperm. Plant Physiol 114:851–856

Edner C, Li J, Albrecht T, Mahlow S, Hejazi M, Hussain H, Kaplan F, Guy C, Smith SM, Steup M, Ritte G (2007) Glucan, water dikinase activity stimulates breakdown of starch granules by plastidial beta-amylases. Plant Physiol 145:17–28

Fernandez AP, Strand A (2008) Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. Curr Opin Plant Biol 11:509–513

Fukushima A, Kusano M, Mejia RF, Iwasa M, Kobayashi M, Hayashi N, Watanabe-Takahashi A, Narisawa T, Tohge T, Hur M, Wurtele ES, Nikolau BJ, Saito K (2014) Metabolomic characterization of knockout mutants in Arabidopsis: development of a metabolite profiling database for knockout mutants in Arabidopsis. Plant Physiol 165:948–961

Fulton DC, Stettler M, Mettler T, Vaughan CK, Li J, Francisco P, Gil M, Reinhold H, Eicke S, Messerli G, Dorken G, Halliday K, Smith AM, Smith SM, Zeeman SC (2008) {beta}-Amylase4, a noncatalytic protein required for starch breakdown, acts upstream of three active {beta}-Amylases in Arabidopsis chloroplasts. Plant Cell 20:1040–1058

Gollery M, Harper J, Cushman J, Mittler T, Girke T, Zhu JK, Bailey-Serres J, Mittler R (2006) What makes species unique? The contribution of proteins with obscure features. Genome Biol 7:R57

Gollery M, Harper J, Cushman J, Mittler T, Mittler R (2007) POFs: what we don't know can hurt us. Trends Plant Sci 12:492–496

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9:597–605

Han M, Okamoto M, Beatty PH, Rothstein SJ, Good AG (2015) The genetics of nitrogen use efficiency in crop plants. Annu Rev Genet 49:269–289

Hedges SB, Kumar S (2009) The timetree of life. Oxford University Press, New York, USA

Hirel B, Le Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot 58:2369–2387

Ishihara H, Obata T, Sulpice R, Fernie AR, Stitt M (2015) Quantifying protein synthesis and degradation in arabidopsis by dynamic 13CO2 labeling and analysis of enrichment in individual amino acids in their free pools and in protein. Plant Physiol 168:74–93

Jang JC, Sheen J (1994) Sugar sensing in higher plants. Plant Cell 6:1665–1679

Johnson X, Alric J (2013) Central carbon metabolism and electron transport in Chlamydomonas reinhardtii: metabolic constraints for carbon partitioning between oil and starch. Eukaryot Cell 12:776–793

Jones DC, Zheng W, Huang S, Du C, Zhao X, Yennamalli RM, Sen TZ, Nettleton D, Wurtele ES, Li L (2016) A clade-specific Arabidopsis gene connects primary metabolism and senescence. Front Plant Sci 7:983

Kahle J, Baake M, Doenecke D, Albig W (2005) Subunits of the heterotrimeric transcription factor NF-Y are imported into the nucleus by distinct pathways involving importin beta and importin 13. Mol Cell Biol 25:5339–5354

Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber A, Flugge UI (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: the glucose 6-phosphate/phosphate antiporter. Plant Cell 10:105–117

Kaplan F, Guy CL (2004) beta-Amylase induction and the protective role of maltose during temperature shock. Plant Physiol 135:1674–1684

Khalturin K, Hemmrich G, Fraune S, Augustin R, Bosch TC (2009) More than just orphans: are taxonomically-restricted genes important in evolution? Trends Genet 25:404–413

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol 47:509–540

Kumimoto RW, Zhang Y, Siefers N, Holt BF 3rd (2010) NF-YC3, NF-YC4 and NF-YC9 are required for CONSTANS-mediated, photoperiod-dependent flowering in Arabidopsis thaliana. Plant J 63:379–391

Laby RJ, Kim D, Gibson SI (2001) The ram1 mutant of Arabidopsis exhibits severely decreased beta-amylase activity. Plant Physiol 127:1798–1807

Laloum T, De Mita S, Gamas P, Baudin M, Niebel A (2013) CCAAT-box binding transcription factors in plants: y so many? Trends Plant Sci 18:157–166

Lao NT, Schoneveld O, Mould RM, Hibberd JM, Gray JC, Kavanagh TA (1999) An Arabidopsis gene encoding a chloroplast-targeted beta-amylase. Plant J 20:519–527

Lastdrager J, Hanson J, Smeekens S (2014) Sugar signals and the control of plant growth and development. J Exp Bot 65:799–807

Lau W, Fischbach MA, Osbourn A, Sattely ES (2014) Key applications of plant metabolic engineering. PLoS Biol 12:e1001879

Levesque-Lemay M, Albani D, Aldcorn D, Hammerlindl J, Keller W, Robert LS (2003) Expression of CCAAT-binding factor antisense transcripts in reproductive tissues affects plant fertility. Plant Cell Rep 21:804–808

Li L, Foster CM, Gan Q, Nettleton D, James MG, Myers AM, Wurtele ES (2009) Identification of the novel protein QQS as a component of the starch metabolic network in Arabidopsis leaves. Plant J 58:485–498

Li L, Ilarslan H, James MG, Myers AM, Wurtele ES (2007) Genome wide co-expression among the starch debranching enzyme genes AtISA1, AtISA2, and AtISA3 in Arabidopsis thaliana. J Exp Bot 58:3323–3342

Li L, Wurtele ES (2012) Materials and methods for modifying a biochemical component in a plant, U.S. Application 20120222167 A1, U.S. Patent 9157091, U.S. patent and trademark office

Li L, Wurtele ES (2015) The QQS orphan gene of Arabidopsis modulates carbon and nitrogen allocation in soybean. Plant Biotechnol J 13:177–187

Li L, Zheng W, Zhu Y, Ye H, Tang B, Arendsee ZW, Jones D, Li R, Ortiz D, Zhao X, Du C, Nettleton D, Scott MP, Salas-Fernandez MG, Yin Y, Wurtele ES (2015) QQS orphan gene regulates carbon and nitrogen partitioning across species via NF-YC interactions. Proc Natl Acad Sci USA 112:14734–14739

Liang M, Yin X, Lin Z, Zheng Q, Liu G, Zhao G (2014) Identification and characterization of NF-Y transcription factor families in Canola (Brassica napus L.). Planta 239:107–126

Lightfoot DA (2013) Nitrogen fixation and assimilation. In: Kole C (ed) Genomics and breeding for climate-resilient crops, vol 2. Target traits. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp 395–413

Liscombe DK, Facchini PJ (2008) Evolutionary and cellular webs in benzylisoquinoline alkaloid biosynthesis. Curr Opin Biotechnol 19:173–180

Litholdo CG Jr, Parker BL, Eamens AL, Larsen MR, Cordwell SJ, Waterhouse PM (2016) Proteomic identification of putative Microrna394 target genes in Arabidopsis thaliana identifies major latex protein family members critical for normal development. Mol Cell Proteomics 15:2033–2047

Liu JZ, Braun E, Qiu WL, Shi YF, Marcelino-Guimaraes FC, Navarre D, Hill JH, Whitham SA (2014) Positive and negative roles for soybean MPK6 in regulating defense responses. Mol Plant Microbe Interact 27:824–834

Lloyd JR, Kossmann J, Ritte G (2005) Leaf starch degradation comes out of the shadows. Trends Plant Sci 10:130–137

Lu Y, Gehan JP, Sharkey TD (2005) Daylength and circadian effects on starch degradation and maltose metabolism. Plant Physiol 138:2280–2291

Lu Y, Sharkey TD (2004) The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. Planta 218:466–473

Lu Y, Steichen JM, Yao J, Sharkey TD (2006) The role of cytosolic alpha-glucan phosphorylase in maltose metabolism and the comparison of amylomaltase in Arabidopsis and Escherichia coli. Plant Physiol 142:878–889

Luhua S, Ciftci-Yilmaz S, Harper J, Cushman J, Mittler R (2008) Enhanced tolerance to oxidative stress in transgenic Arabidopsis plants expressing proteins of unknown function. Plant Physiol 148:280–292

Lund SP, Nettleton D, McCarthy DJ, Smyth GK (2012) Detecting differential expression in RNA-sequence data using quasi-likelihood with shrunken dispersion estimates. Stat Appl Genet Mol Biol 11:1544–6115

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot 105:1141–1157

McAllister CH, Beatty PH, Good AG (2012) Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10:1011–1025

Melis A (2013) Carbon partitioning in photosynthesis. Curr Opin Chem Biol 17:453–456

Mentzen WI, Wurtele ES (2008) Regulon organization of Arabidopsis. BMC Plant Biol 8:99

Nardini M, Gnesutta N, Donati G, Gatta R, Forni C, Fossati A, Vonrhein C, Moras D, Romier C, Bolognesi M, Mantovani R (2013) Sequence-specific transcription factor NF-Y displays histone-like DNA binding and H2B-like ubiquitination. Cell 152:132–143

Nettleton D, Hwang JTG, Caldo R, Wise R (2006) Estimating the number of true null hypotheses from a histogram of p values. JABES 11:337–356

Niewiadomski P, Knappe S, Geimer S, Fischer K, Schulz B, Unte US, Rosso MG, Ache P, Flugge UI, Schneider A (2005) The Arabidopsis plastidic glucose 6-phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac development. Plant Cell 17:760–775

Niittyla T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC (2004) A previously unknown maltose transporter essential for starch degradation in leaves. Science 303:87–89

Petroni K, Kumimoto RW, Gnesutta N, Calvenzani V, Fornari M, Tonelli C, Holt BF 3rd, Mantovani R (2012) The promiscuous life of plant nuclear factor Y transcription factors. Plant Cell 24:4777–4792

Qi M, Zheng W, Zhao X, Hohenstein J, Kandel Y, O'Conner S, Wang Y, Du C, Nettleton D, Macintosh G, Tylka G, Wurtele E, Whitham S, Li L (2018) QQS orphan gene and its interactor NFYC4 reduce susceptibility to pathogens and pests. Plant Biotechnol J https://doi.org/10.1111/pbi.12961

Reiter WD (2008) Biochemical genetics of nucleotide sugar interconversion reactions. Curr Opin Plant Biol 11:236–243

Santos-Mendoza M, Dubreucq B, Baud S, Parcy F, Caboche M, Lepiniec L (2008) Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. Plant J 54:608–620

Scheidig A, Frohlich A, Schulze S, Lloyd JR, Kossmann J (2002) Downregulation of a chloroplast-targeted beta-amylase leads to a starch-excess phenotype in leaves. Plant J 30:581–591

Schiltz S, Gallardo K, Huart M, Negroni L, Sommerer N, Burstin J (2004) Proteome reference maps of vegetative tissues in pea. An investigation of nitrogen mobilization from leaves during seed filling. Plant Physiol 135:2241–2260

Schneider A, Hausler RE, Kolukisaoglu U, Kunze R, van der Graaff E, Schwacke R, Catoni E, Desimone M, Flugge UI (2002) An Arabidopsis thaliana knock-out mutant of the chloroplast triose phosphate/phosphate translocator is severely compromised only when starch synthesis, but not starch mobilisation is abolished. Plant J 32:685–699

Seo PJ, Kim MJ, Ryu JY, Jeong EY, Park CM (2011) Two splice variants of the IDD14 transcription factor competitively form nonfunctional heterodimers which may regulate starch metabolism. Nat Commun 2:303

Shrawat AK, Carroll RT, DePauw M, Taylor GJ, Good AG (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of alanine aminotransferase. Plant Biotechnol J 6:722–732

Silveira AB, Trontin C, Cortijo S, Barau J, Del Bem LE, Loudet O, Colot V, Vincentz M (2013) Extensive natural epigenetic variation at a de novo originated gene. PLoS Genet 9:e1003437

Smith AM, Zeeman SC, Thorneycroft D, Smith SM (2003) Starch mobilization in leaves. J Exp Bot 54:577–583

Sparla F, Costa A, Lo Schiavo F, Pupillo P, Trost P (2006) Redox regulation of a novel plastid-targeted beta-amylase of Arabidopsis. Plant Physiol 141:840–850

Steichen JM, Petty RV, Sharkey TD (2008) Domain characterization of a 4-alpha-glucanotransferase essential for maltose metabolism in photosynthetic leaves. J Biol Chem 283:20797–20804

Stitt M (2013) Systems-integration of plant metabolism: means, motive and opportunity. Curr Opin Plant Biol 16:381–388

Stitt M, Lunn J, Usadel B (2010) Arabidopsis and primary photosynthetic metabolism—more than the icing on the cake. Plant J 61:1067–1091

Storey JD (2002) A direct approach to false discovery rates. J Royal Stat Soc Ser B (Statistical Methodology) 64:479–498

Streb S, Delatte T, Umhang M, Eicke S, Schorderet M, Reinhardt D, Zeeman SC (2008) Starch granule biosynthesis in Arabidopsis is abolished by removal of all debranching enzymes but restored by the subsequent removal of an endoamylase. Plant Cell 20:3448–3466

Sulpice R, Flis A, Ivakov AA, Apelt F, Krohn N, Encke B, Abel C, Feil R, Lunn JE, Stitt M (2014) Arabidopsis coordinates the diurnal regulation of carbon allocation and growth across a wide range of photoperiods. Mol Plant 7:137–155

Sweetlove LJ, Fell D, Fernie AR (2008) Getting to grips with the plant metabolic network. Biochem J 409:27–41

Thum KE, Shin MJ, Gutierrez RA, Mukherjee I, Katari MS, Nero D, Shasha D, Coruzzi GM (2008) An integrated genetic, genomic and systems approach defines gene networks regulated by the interaction of light and carbon signaling pathways in Arabidopsis. BMC Syst Biol 2:31

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25:1105–1111

Usadel B, Blasing OE, Gibon Y, Poree F, Hohne M, Gunter M, Trethewey R, Kamlage B, Poorter H, Stitt M (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in the non-freezing range. Plant Cell Environ 31:518–547

Vidal EA, Gutierrez RA (2008) A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. Curr Opin Plant Biol 11:521–529

Walters RG, Ibrahim DG, Horton P, Kruger NJ (2004) A mutant of Arabidopsis lacking the triose-phosphate/phosphate translocator reveals metabolic regulation of starch breakdown in the light. Plant Physiol 135:891–906

Wattebled F, Dong Y, Dumez S, Delvalle D, Planchot V, Berbezy P, Vyas D, Colonna P, Chatterjee M, Ball S, D'Hulst C (2005) Mutants of Arabidopsis lacking a chloroplastic isoamylase accumulate phytoglycogen and an abnormal form of amylopectin. Plant Physiol 138:184–195

Wattebled F, Planchot V, Dong Y, Szydlowski N, Pontoire B, Devin A, Ball S, D'Hulst C (2008) Further evidence for the mandatory nature of polysaccharide debranching for the aggregation of semicrystalline starch and for overlapping functions of debranching enzymes in Arabidopsis leaves. Plant Physiol 148:1309–1323

Weber A, Servaites JC, Geiger DR, Kofler H, Hille D, Groner F, Hebbeker U, Flugge UI (2000) Identification, purification, and molecular cloning of a putative plastidic glucose translocator. Plant Cell 12:787–802

Weber AP, Schneidereit J, Voll LM (2004) Using mutants to probe the in vivo function of plastid envelope membrane metabolite transporters. J Exp Bot 55:1231–1244

Weise SE, Schrader SM, Kleinbeck KR, Sharkey TD (2006) Carbon balance and circadian regulation of hydrolytic and phosphorolytic breakdown of transitory starch. Plant Physiol 141:879–886

Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, Coupland G (2006) CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. Plant Cell 18:2971–2984

Weselake RJ, Taylor DC, Rahman MH, Shah S, Laroche A, McVetty PB, Harwood JL (2009) Increasing the flow of carbon into seed oil. Biotechnol Adv 27:866–878

West M, Yee KM, Danao J, Zimmerman JL, Fischer RL, Goldberg RB, Harada JJ (1994) LEAFY COTYLEDON1 is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. Plant Cell 6:1731–1745

Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182

Yadav UP, Ivakov A, Feil R, Duan GY, Walther D, Giavalisco P, Piques M, Carillo P, Hubberten HM, Stitt M, Lunn JE (2014) The sucrose-trehalose 6-phosphate (Tre6P) nexus: specificity and mechanisms of sucrose signalling by Tre6P. J Exp Bot 65:1051–1068

Yu TS, Zeeman SC, Thorneycroft D, Fulton DC, Dunstan H, Lue WL, Hegemann B, Tung SY, Umemoto T, Chapple A, Tsai DL, Wang SM, Smith AM, Chen J, Smith SM (2005) alpha-Amylase is not required for breakdown of transitory starch in Arabidopsis leaves. J Biol Chem 280:9773–9779

Zeeman SC, Thorneycroft D, Schupp N, Chapple A, Weck M, Dunstan H, Haldimann P, Bechtold N, Smith AM, Smith SM (2004) Plastidial alpha-glucan phosphorylase is not required for starch degradation in Arabidopsis leaves but has a role in the tolerance of abiotic stress. Plant Physiol 135:849–858

Zhang X, Myers AM, James MG (2005) Mutations affecting starch synthase III in Arabidopsis alter leaf starch structure and increase the rate of starch synthesis. Plant Physiol 138:663–674

Zhang X, Szydlowski N, Delvalle D, D'Hulst C, James MG, Myers AM (2008) Overlapping functions of the starch synthases SSII and SSIII in amylopectin biosynthesis in Arabidopsis. BMC Plant Biol 8:96

Part II
Nutrients as a Key Driver of Nutrient Use Efficiency

Chapter 7
Tackling Nitrogen Use Efficiency in Cereal Crops Using High-Throughput Phenotyping

Nicholas John Sitlington Hansen, Darren Plett, Bettina Berger and Trevor Garnett

Introduction

Nitrogen (N) is one of the most important plant mineral nutrients, essential for numerous biochemical and physiological processes. Greater use of N fertilizer played an important role in increasing yields during the 'green revolution' (Evenson and Gollin 2003). In recent times, however, cereal yield increases have stagnated, especially in developing countries (Lin and Huybers 2012; Ray et al. 2012, 2013). There has been a call for a second 'green revolution' to address flattening yields in a sustainable fashion, and increasing the nitrogen use efficiency (NUE) of cereal crops can play an important role.

Nitrogen use efficiency is important because inefficient N use is deleterious to the environment, expensive, and reduces the yield potential of crops. Cereal production utilizes 60% of all agricultural nitrogen applications but unfortunately, cereals generally recover less than half of the supplied N, causing wastage and pollution (Peoples et al. 1995; Raun and Johnson 1999; Fageria and Baligar 2005; Sylvester-Bradley and Kindred 2009). Therefore, increasing the NUE of cereals would have a significant environmental and economic impact (Ladha et al. 2016).

N. J. S. Hansen · D. Plett · B. Berger · T. Garnett (✉)
School of Agriculture, Food and Wine, Waite Research Institute, University of Adelaide, Adelaide, SA 5064, Australia
e-mail: trevor.garnett@adelaide.edu.au

N. J. S. Hansen · B. Berger · T. Garnett
The Plant Accelerator, Australian Plant Phenomics Facility, Waite Research Institute, University of Adelaide, Adelaide, SA 5064, Australia

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_7

NUE

For cereal production, NUE was defined by Moll et al. (1982) as grain production per unit N available in the soil. Nitrogen use efficiency is the combination of plant uptake efficiency (NUpE), how effectively the plants capture N, and utilization efficiency (NUtE), how well the plants use the N that is taken up (Good et al. 2004; Sadras and Richards 2014).

NUpE

Nitrogen uptake efficiency can be defined as the amount of N taken up by the plant as a proportion of the N available (both residual and added N) (Good et al. 2004). Nitrogen uptake efficiency is influenced by mass flow of soil water to the root, root morphology, transporter activity on the root surface, timing of N application, and microbial competition (Garnett et al. 2009).

Nitrogen is available to plants primarily as nitrate (NO_3-) and ammonium (NH_4^+). In cropping soils, N is predominantly available as NO_3-, with NH_4^+ being generally 10% of the NO_3- concentration (Wolt 1994); however, plants have been shown to perform better with a combination of NO_3- and NH_4^+ available (Forde and Clarkson 1999). Nitrate is readily mobile in the soil and moves to the root surface via mass flow, after which it is taken up by high and low affinity transporters (HATS and LATS, respectively) which are part of the NRT2 and NRT1/NPF families (Plett et al. 2010; Léran et al. 2014). Ammonium is much less mobile in soil than NO_3- and is taken into the root by the AMT transporter family (Howitt and Udvardi 2000; Ludewig et al. 2007; Gu et al. 2013). Transporters on the root surface have been targets for transgenic or genetic upregulation in order to increase uptake capacity (Fan et al. 2016); however, thus far, results have been mixed. They may have been unsuccessful as tissue N concentration is tightly controlled and negative feedback mechanisms prevent increased uptake (Garnett et al. 2015).

Altering root morphology has had limited success in improving N uptake. As NO_3- moves readily to the root via mass flow, changing root architecture is more effective for immobile nutrients such as phosphorus, than for NO_3- (Burns 1980). Burns showed that, due to the plastic nature of the root system, plants can cope with only 15% of their roots being exposed to NO_3-, leaving little imperative to increase root biomass from an NUpE perspective. Increased rooting depth may be useful in deep sandy soils to intercept highly mobile NO_3- being leached through the profile, or in deep soils with stored water and N at depth, however, in less porous soils increased root growth may be an inefficient use of carbon (C) (Garnett and Rebetzke 2013).

NUtE

Nitrogen utilization efficiency is the proportion of aboveground biomass N which is converted to grain (Good et al. 2004). This grain N is derived from tissue N that has been assimilated pre-anthesis and N that is taken up post-anthesis (Hawkesford 2017). Prior to anthesis the biomass acts as an N-sink; however post-anthesis those resources are remobilized to the grain as well as N that is assimilated post-anthesis (wheat/barley) (Martre et al. 2003) and post-silking (maize) (Rajcan and Tollenaar 1999). Harvest index (HI) is the ratio between the harvestable and shoot biomass and represents how efficient the plant is at assimilation and translocating resources to the grain (Sinclair 1998). Harvest index has increased greatly over the last 50 years through the development of semidwarf varieties (Sinclair 1998; Fischer 2011). Nitrogen harvest index (NHI), the ratio of grain N to aboveground biomass N, is synonymous with NUtE. In wheat, improvements in NUtE have been mainly due to improvements in the HI (Fischer and Wall 1976; Ortiz-Monasterio et al. 1997; Foulkes et al. 1998). However, in modern varieties NHI is high and consistent irrespective of N fertilization (Barraclough et al. 2010). In wheat, the remobilization of N can be quite efficient, with little residual N remaining in the straw (Hawkesford 2017).

The manipulation of remobilization has been shown to be possible via changes to the 'stay-green' traits which either reduce the rate, or delay the onset, of senescence (Thomas and Smart 1993). For crops such as sorghum, stay-green traits can be advantageous when they are grown with access to stored soil moisture as they can benefit from a longer period of photosynthesis, assimilating greater amounts of N into tissue, providing a greater source for grain filling (Borrell et al. 2001). However, for wheat/barley, this is not always ideal, for example, in Mediterranean climates which experience a hot-dry finish to the season with limited stored soil water. In these conditions, a rapid remobilization is preferable to shorten the period between anthesis and maturity (Garnett and Rebetzke 2013).

Efforts to Improve NUE, But No Progress

Efforts to improve NUE have ranged from improving agronomic practices, identifying significant QTL affecting NUE component traits (uptake and utilization) and transgenic approaches; however, these efforts have so far not resulted in NUE improvements. Breeding has historically taken place under plentiful N conditions, and it was hypothesized that this produced germplasm with reduced NUE, especially under low N conditions (Kamprath et al. 1982). More recently, this has been disproven by studies showing that newer varieties are more N efficient under low N conditions than older varieties in both wheat (Ortiz-Monasterio et al. 1997) and maize (Ding et al. 2005; Echarte et al. 2008). These improvements may have been incidental when breeding for yield; however, these gains are minimal and must now be improved using a more targeted approach.

Nitrogen use efficiency can be improved agronomically via better management practices including matching N fertilization to plant requirement, management of surface runoff, improving acidic soils, avoiding waterlogging to reduce anaerobic denitrification, and canopy management (Keeney 1982; Van Herwaarden et al. 1998a; Fageria and Baligar 2005). Although agronomic improvements will continue to play a central role in improving NUE, without improving the plant NUE progress will always be limited.

Genetic mapping in order to identify the QTL associated with NUE is an important step in its improvement and gains have been made in wheat (An et al. 2006; Quraishi et al. 2011; Xu et al. 2014), maize (Agrama et al. 1999; Gallais and Hirel 2004), rice (Cho et al. 2007; Wei et al. 2012), and barley (Mickelson et al. 2003). In one example in wheat, 11 major chromosomal regions responsible for NUE were identified (Quraishi et al. 2011). The loci identified in wheat are colocated with *Ppd* (photoperiod sensitivity), *Vrn* (vernalization requirement), and *Rht* (reduced height), which are all developmental genes, possibly controlling the amount of time that the plants can take-up and utilize N (Quraishi et al. 2011). For an extensive investigation of the genes identified, there are a number of recent reviews (Quraishi et al. 2011; Cormier et al. 2014; Garnett et al. 2015; Han et al. 2015).

The genetic variability of NUE within cereals has been shown to be high; especially under low N conditions (Dhugga and Waines 1989; Ortiz-Monasterio et al. 1997; Le Gouis et al. 2000), however, conventional breeding of elite lines has not resulted in NUE-improved germplasm. This is possibly because there is a large number of QTL influencing NUE (Garnett et al. 2015). This in turn requires a large population of backcrossed individuals in order to observe segregation at loci of interest and repeated measurements to assure confidence in the QTL measured, as the environmental impact is often more significant than the genotypic difference observed (Han et al. 2015).

Transgenic attempts to improve NUE have targeted amino acid biosynthesis, translocation/remobilization, signaling and N regulation, and C/N storage proteins for reviews consult (McAllister et al. 2012; Garnett et al. 2015). Some of the most promising transgenic approaches have overexpressed the genes responsible for glutamine synthetase (GS) (Brauer et al. 2011), glutamate dehydrogenase (Abiko et al. 2010) the rice nitrate transporter (NRT2.3/2.5) (Fan et al. 2016), and alanine aminotransferase (AlaAT) (Good et al. 2007). However, despite the concerted effort, neither transgenic nor conventional breeding has resulted in the commercial release of cereals with dramatically improved NUE.

Why Has No Progress Been Made?

Nitrogen use efficiency is a complex trait determined by a group of processes which transport the N molecules into the root, assimilate and utilized that N to produce biomass, and finally remobilize N to the grain. A large number of QTL are believed to be responsible for NUE but there has been very little overlap between mapping

studies (Garnett et al. 2015). Large numbers of QTL require large mapping populations and their repeated study to verify results. Furthermore, studies investigating NUE genetic variability have often been undertaken in single years, which does not take into account the significant environmental effects that are obvious in multi-year experiments (Barraclough et al. 2010; Hawkesford 2017). A minimum of three years of data per variety is recommended to account for the genotype x environment interaction (GxE) previously noted (Hawkesford 2017), suggesting some QTL studies may be compromised in this way.

Compounding the difficulty in identifying NUE QTL has been the use of inappropriate phenotyping methods. Ideally, NUE performance should be measured as the difference in plant growth and yield between high and low N. However, some QTL mapping studies investigating NUE have only utilized a single N level of fertilization, potentially missing QTL which are present at one or the other (Cormier et al. 2016). As described above, it is suggested there should be multiple years of field trials to reduce the E component in GxE. Studies in controlled environments, although having more control over E, need to be rigorous and repeatable. This has not always been the case. Pot experiments in controlled environments are criticized as sometimes having little bearing on field performance (Passioura 2006a), and this may in part be due to poor experimental setup, e.g., small pots, inappropriate watering levels, or poor growth conditions (Poorter et al. 2016). Hydroponics experiments allow tighter control of N levels but are further removed from the field than pots and results derived from these need to be validated in soil. A large number of studies reporting progress with NUE in transgenic plants have never advanced beyond the very basic phenotyping carried out in the initial publication. If controlled environment experiments were designed to be as comparable as possible to the field, their relevance to the field may be enhanced and field relevant progress made. However, often the methods used are poorly described in publications, and as with many field studies, there is an incomplete description of the growth environment. This is a critical oversight when trying to understand such an environmentally affected trait.

Can Modern Phenomics Help?

Modern phenomics, the study of plant growth, performance, and composition, utilizes new technologies to better characterize plant responses to the environment and also better describes the growth environment itself (Furbank and Tester 2011). Phenomics can aid in the phenotyping of NUE performance via nondestructive measurements of biomass, growth rates, and transpiration rates to observe germplasm differences over the course of their life cycle, adding a temporal dimension to the phenotype and providing more opportunities to understand final yields. Phenomics can also provide a platform wherein noninvasive biological data can be collected on a large number of plants simultaneously, providing observations of plant behavior that have been unavailable via traditional phenotyping techniques

and destructive harvests, e.g., chlorophyll fluorescence for photosynthetic performance or hyperspectral imaging for measuring leaf constituents. Finally, modern technologies allow much better quantification of the environment in which plants grow. In combination, these advances may enable progress in dissecting NUE that until now has been lacking.

Phenomics in Controlled Environments

Nitrogen use efficiency is a difficult trait to phenotype because the interaction with the environment can obscure genetic gains. Therefore, one way to improve the characterization of the genetic component of NUE is to provide a controlled, quantifiable and replicable environment within which to 'fine dissect' the component traits of NUE (Furbank and Tester 2011). Controlled environments provide this to different degrees, ranging from growth rooms, and glasshouses, to field-based installations such as rainout shelters (Rebetzke et al. 2012). To maximize value and allow replication of experiments, the controlled environment conditions should be well characterized and published with the phenotypic data (Billiau et al. 2012; Krajewski et al. 2015).

Controlled environment NUE phenotyping is often reliant on artificial illumination, the quantity and quality of which can vary significantly and is not often accounted for (Cabrera-Bosquet et al. 2016). In controlled environments, light quality varies greatly depending on the light source (Hogewoning et al. 2010). Given that light quality, not just intensity, can have major impact on plant growth, it needs to be quantified (Ugarte et al. 2010; Max et al. 2012; Dueck et al. 2016). It is now viable and relatively cheap to measure light quality, not just the intensity, and this should be done routinely and reported.

In addition to light quality, if experiments are to reflect field performance, the daily light incidence (mol m^{-2} d^{-1}) and temperature settings in controlled environments should reflect those of the target environment as much as possible. Meta-analysis of controlled environment experiments has demonstrated that experimental conditions often fall significantly outside desired climactic ranges, causing differences in specific leaf area and tillering among others, compared to the field and may affect NUE performance (Poorter et al. 2016a). For example, daily light incidence settings would be crucial when trying to tease apart the role of *Ppd* on NUE in wheat (Quraishi et al. 2011).

In addition to illumination and climate variation, controlled environment experiments can provide some control over soil homogeneity. Achieving uniform N across a field trial is nearly impossible and requires careful soil reserve depletion in previous seasons, but even then there can be considerable variation (Shaw et al. 2016). Controlled environment experiments can ensure a consistent level of soil structure and N content in all pots within the experiment, resulting in more precise N fertilization than in the field. Automatic watering systems in greenhouse phenotyping platforms also offer greater control over water application than the field or

conventional pot experiments which, often suffer from excessive watering levels causing hypoxia, affecting root growth (Passioura 2006b). These conditions could affect NUE phenotypes dramatically and are avoided in modern phenomics systems via the use of gravimetric watering systems, which can maintain soil water contents at levels more closely mirroring field conditions (Passioura 2006b).

When effective environmental monitoring is undertaken in controlled environments, it can become obvious that there are spatial differences that need to be taken account of in order to reduce error. The statistical design of experiments is crucial to achieve this (Brien et al. 2013). In order to account for the spatial variance within a greenhouse, a statistical design and analysis approach (blocked design) was more accurate than continually alternating the position of the plants within the experiment (Brien et al. 2013).

HTP Platforms

High-throughput phenotyping (HTP) platforms are specifically designed to automate the collection of plant biometric data. Most controlled environment HTP platforms are comprised of individual pots on conveyor belts (Fig. 7.1) which deliver the plants to a series of imaging cabinets and watering stations (although some HTP platforms are now based on moving whole benches of plants). Basic imaging is usually undertaken via red-green-blue (RGB) cameras but systems can also include fluorescence, thermal infrared (IR), near infrared (NIR), and hyperspectral imaging. The accurate estimation of biomass from digital images has been demonstrated in various crops, including barley (Honsdorf et al. 2014), rice (Yang et al. 2014), wheat (Golzarian et al. 2011), and sorghum (Neilson et al. 2015). Accurate growth curves can be derived from these images as shown in Fig. 7.2.

Forward genetics screens using HTP have become a powerful tool to identify relevant QTL and phenotype germplasm. The efficacy of genetic analysis of HTP data to identify relevant QTL has been demonstrated in maize (Muraya et al. 2017), barley (Chen et al. 2014; Honsdorf et al. 2014), rice (Campbell et al. 2015), and wheat (Parent et al. 2015). High-throughput phenotyping has been used in a forward genetics approach to investigate the genetic basis of maize growth traits and 988 QTL have subsequently been identified (Zhang et al. 2017). These traits include morphological traits, leaf architecture, biomass, and color. The use of HTP was crucial in these studies because many of the phenotypes studied were dynamic metrics, such as growth or transpiration rates, which could only be obtained on large populations via nondestructive HTP. High-throughput phenotyping has also been used to assess the response of sorghum to N and water limitation, via their growth, composition, and shape in a dose–response experiment (Neilson et al. 2015). This study aimed to optimize the use of HTP for the identification of plant phenotypes that correlated with performance under water and N stress. In addition to water stress and N treatment, HTP platforms have also allowed the phenotyping of salinity tolerance in barley (Meng et al. 2017) and rice (Al-Tamimi et al. 2016).

Fig. 7.1 A controlled environment high-throughput phenotyping platform at The Plant Accelerator, University of Adelaide, South Australia (2014). In the foreground are 480 wheat plants during an experiment. In the background are the automated doors leading to the imaging hall

Al-Tamimi et al. (2016) demonstrated that the growth curve analysis available in the HTP platform allowed the comparison of transpiration (gravimetrically), transpiration use efficiency (TUE), and relative growth rates (RGR) 1–13 days after salt application in 553 accessions. These phenotypes were then associated with specific genomic loci via genome-wide association study and have become targets for further research. The quantification of these phenotypes would not have been practical prior to HTP and genes with relatively small effects can now be identified for potential use in genomic selection approaches (Campbell et al. 2017). The use of HTP in (GWAS) shows promise for the identification of candidate genes for NUE improvement (Brown et al. 2014).

The same approach used for these complex and dynamic traits could also be used to fine dissect the component traits of NUE: NUtE and NUpE. The resolution of the growth observations allows for a dissection of growth rates at specific times during experiments and in response to changes in N or water availability. For example, the comparison of RGRs under specific N levels can identify germplasm which is able to rapidly establish biomass. Early biomass is advantageous for N uptake as N fertilizer application commonly occurs at seeding and early utilization minimizes losses. Furthermore, a major advantage of being able to measure growth is the temporal aspect of the response to N in plants. Growth analysis allows the timing of

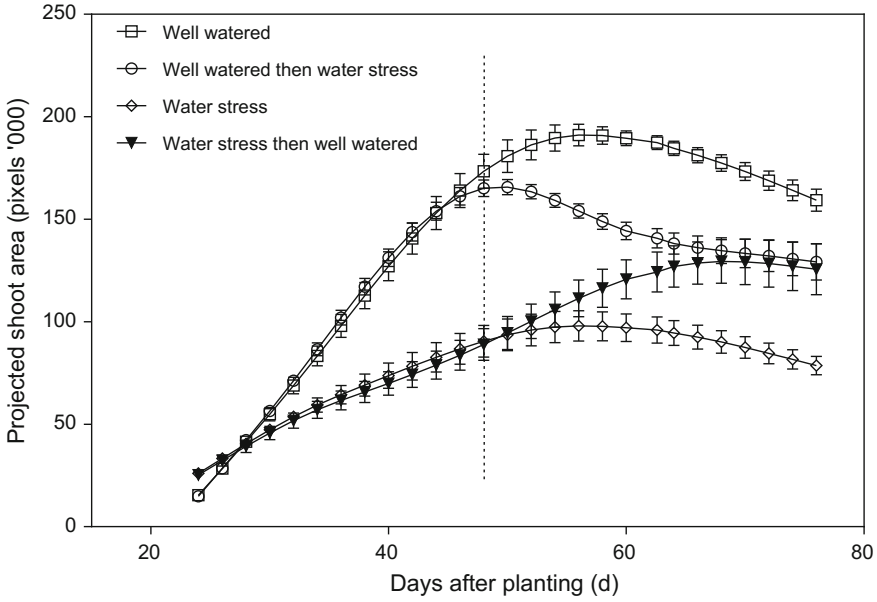


Fig. 7.2 Growth curves of a wheat cultivar under 4 water treatments: well-watered (open square), well-watered then water stress at day 48 (open circle), water stress (open diamond) and water stress until day 48 and then well-watered (closed triangle). These growth curves were derived from plant images captured on a high-throughput phenotyping platform and the amount of biomass is estimated from the number of pixels that the plants occupy of the image (projected shoot area). Change in water treatment is represented by the vertical dotted line. SEM shown of four replicates

N response to be determined, this being important in matching growth to fertilizer availability.

Nitrogen, specifically NO_3-, is freely mobile in moist soil but in drying soil its movement is restricted. The ability to control water availability in HTP systems allows the application of combined water and N stress. This can help identify which genotypes are able to respond to different N levels under drought conditions. Specifically, HTP platforms can identify germplasm which are N responsive under Mediterranean field conditions, the 'hot-dry finish' commonly experienced in wheat and barley production areas (van Herwaarden et al. 1998). Figure 7.2 (above) shows the growth curves of wheat and the influence of water treatment on their biomass.

Currently, measuring N uptake in cereals is dependent on destructive harvests or proxies such as chlorophyll content, which are limiting as they remove plant material from the experiment or in the case of chlorophyll, are inaccurate at high concentrations (Ecarnot et al. 2013). The interaction of electromagnetic radiation with molecules in the leaf makes spectral reflectance measurements a suitable method to assess leaf chemistry accurately and nondestructively (Kokaly 2001). Leaf or canopy spectrometry is versatile and has been demonstrated to estimate N in maize (Yendrek et al. 2016), wheat (Ecarnot et al. 2013), and rice (Sun et al. 2017).

Such noninvasive methods of phenotyping over time are ideal to tackle the dynamics of nitrogen partitioning throughout cereals (Garnett et al. 2013). Noninvasive phenotyping allows the observation of N uptake and partitioning as well as how these are affected by N availability and interactions with water. Comparing leaf-N contents between cultivars under changing N supply may provide insights into their respective N response capacities, i.e., germplasm that are able to maintain their leaf N content and growth under N scarcity. During remobilization, being able to measure leaf N directly would show the speed and efficiency of translocation, the different contribution of individual leaves and the interaction with water availability, and how this differs between germplasm.

Phenomics in the Field

Although controlled environment phenotyping systems provide extensive information on plant performance and allow the selection of material with putatively enhanced NUE, field performance is vital for translating research into commercial outcomes. As discussed, while field trials are essential, they are also problematic because of the inconsistent environmental conditions within one site, let alone between field environments. They are also challenging in terms of measuring growth parameters beyond yield at harvest. Advances in measurement technology and environmental monitoring mean that modern phenomics could have a major impact on phenotyping of NUE in the field.

Harvest yield is currently the standard measurement for NUE evaluation in breeding trials. Material being evaluated for NUE must have higher yields under the nitrogen treatments tested and, in the case of cereals such as wheat, maintain grain quality (Foulkes et al. 2009). Huge efforts globally have been expended on purely yield-based field evaluation of NUE with limited or no success in delivering higher NUE crops. Success may be improved with better environmental monitoring to better understand the E component of GxE. However, even if the environment is described to the best practice standards, if NUE performance is just based on yield, large amounts of potentially useful information is lost.

Modern field phenomics technologies facilitate the collection of this noninvasive range of plant characteristics such as leaf N, providing alternatives to destructive harvests. Total nitrogen uptake and remobilization can be ascertained from final biomass harvests and tissue N determination. However, as this is costly, time consuming and can compromise harvest yield measurement, they are not commonly carried out. Even if final biomass is measured, it provides no indication of the temporal nature of N uptake and remobilization. This can be important, for example, if early uptake of nitrogen is a major determinant of yield. Being better able to measure component traits that contribute to yield, and that may have greater heritability than yield per se (Rebetzke et al. 2016), has the potential to facilitate real improvement in NUE in the future.

As with controlled environments, field phenotyping will be most effective if combined with environmental monitoring. Climate data associated with field evaluations have often been lacking, relying on the nearest meteorological stations rather than weather stations onsite (Lovett et al. 2007). When phenotyping is undertaken in a site without adequate environmental observation, results may be attributed to genetic difference, when in fact they may be due to environmental conditions. Encouragingly, like in controlled environments, environmental monitoring in the field is becoming ubiquitous with decreasing cost and size of instruments. Ideally, each field site should have its own weather station that can also measure solar radiation. Nitrogen use efficiency and plant performance could then be normalized for weather conditions, solar irradiance, soil water, or tissue N content to provide better comparisons of phenotypes between research sites.

Soil greatly influences plant phenotypes; however, it is heterogeneous within and between field sites, resulting in environmental variation which needs to be accounted for (Lovett et al. 2007). An ideal field trial site would have a homogenous N and soil structure across the site. Achieving this would require resources beyond the scope of most research trials and so a compromise needs to be made between field preparation and variation. As field trial site uniformity cannot be achieved, effort should be concentrated in monitoring and evaluation. Regular soil testing should be undertaken during each experiment, and ideally the spatial variation characterized (Shaw et al. 2016). An idea of the soil disease load is important and is often available from local area mapping (Heap and McKay 2009). Field sites should also be mapped for salt, clay, and soil water via electromagnetic conductance EM38 measurements (Araus and Cairns 2014). Where possible, the heterogeneity of field sites should be quantified and the differences taken into account in experimental design.

The nondestructive phenotyping of modern phenomics allows the acquisition of much more information on plant performance compared to destructive harvests alone, allowing a much better understanding of the dynamics of traits. For this reason, numerous groups are working on improving field phenotyping capabilities with a variety of approaches being utilized to increase the precision, resolution, and throughput of phenotyping in situ by the conveyance of sensors over the crop canopy (Araus and Cairns 2014; Virlet et al. 2016).

Field HTP Technologies

The capacity of HTP in the field to characterize the performance of thousands of plants rapidly in situ is already available and the amount of data that can be collected can be challenging (White et al. 2012). The difference in the rate of data collection between HTP and conventional phenotyping is significant. A tractor boom-operated sensor bank containing multispectral cameras, ultrasonic sensors, and environmental monitoring instruments is able to collect height, canopy temperature, and reflectance ratios, which correlate well with yield, biomass, flowering

time, and N status at a throughput of 3,000 plots an hour. In contrast, the rate of manual phenotyping done by two people for the simple trait of 'plant height' is about 45 plots an hour (Tanger et al. 2017).

The sensors used in field phenotyping must be conveyed across the top of the plant canopy and many methods have been developed or utilized to do this. Systems range from ground-based gantry structures (Virlet et al. 2016), unmanned aerial vehicles (UAVs) (Sankaran et al. 2015), 'phenobuggies' (Crain et al. 2016; Rebetzke et al. 2016), or modified agricultural vehicles (Tanger et al. 2017), each having their own issues around sensor payload, resolution, cost, and speed. UAV drones and blimps fly above the canopy with sensor payloads generally weighing less than 5 kg, carrying RGB and multispectral cameras (Burger and Geladi 2006; Chapman et al. 2014). Field buggies or 'phenobuggies' range in complexity from a manually pushed trolley to larger motor and GPS-assisted vehicles (Fig. 7.3) (Deery et al. 2014; Crain et al. 2016) and are a convenient compromise between large payload and low-tech solutions. Agricultural vehicles such as tractors and quadbikes can be utilized with sensors attached to booms (Tanger et al. 2017). Ground-based methods can provide high spatial resolution observations due to the proximity of the sensor to the canopy, albeit at a lower throughput than UAVs. Unlike UAVs, ground-based platforms are not as restricted in their sensor payload and can carry heavier sensors such as short-waved infrared (SWIR) hyperspectral cameras (Eitel et al. 2014). Ground-based systems are disadvantaged under waterlogged conditions and may cause soil compaction after repeated

Fig. 7.3 A 'Phenomobile Lite' carrying sensors above a wheat trial, a noninvasive method to phenotype cereals in situ. HRPPC CSIRO Canberra, Australian Capital Territory

measurements. A permanent gantry structure avoids soil disturbance and can operate under wet conditions while maximizing the number of sensors conveyed, resulting in permanent high spatial resolution, where the detection of individual wheat ears in a plot is possible (Virlet et al. 2016). However, the disadvantages are cost, limited number of plots, and the fixed location requiring compromises between repeat experiments and necessary crop rotation (Andrade-Sanchez et al. 2013; Virlet et al. 2016).

For NUE phenotyping, RGB and multispectral cameras can be used to assess plant biomass, architecture, and chlorophyll-based indices such as normalized difference vegetation index (NDVI) (Holman et al. 2016). Spectral reflectance indices from multispectral cameras have demonstrated good correlation with wheat yield under irrigation (Babar et al. 2006) as well as many other physiological parameters (Peñuelas and Filella 1998). Although multispectral cameras cannot give a direct measure of plant N status, they can greatly expand the physiological parameters which can be collected nondestructively and that may correlate with NUE performance. Hyperspectral reflectance can also be utilized in the field to measure N directly in leaf tissue (Ecarnot et al. 2013). Light detection and ranging (LIDAR) (with and without a red laser) has also been used to simultaneously measure biomass and nitrogen distribution in the canopy (Eitel et al. 2014; Rebetzke et al. 2016).

Recent examples of high-throughput NUE phenotyping in the field have included the categorization of sorghum growth in response to N fertilization in order to assist with genomics-assisted breeding selection (Watanabe et al. 2017). When UAVs fitted with near-infrared green-blue (NIR-GB) cameras were used to predict canopy height, r^2 values of 0.678 at high-N and 0.842 at low N were found for correlations with actual canopy height. Alternatively in rice, ground-based HTP was utilized on a population of 1,516 recombinant inbred lines (RILs) to assess canopy height, temperature, and reflectance ratios which correlate well with biomass, leaf area index, flowering time, and nitrogen status (Tanger et al. 2017). These methods were able to identify the genomic regions associated with yield and yield-related traits in this large mapping population. High-throughput phenotyping facilitated this research and allowed it to be done significantly faster and at a lower cost than conventional phenotyping. More importantly, it allowed measurement of parameters that would have been impractical using manual measurement, and allowed them to be measured nondestructively on multiple occasions.

Conclusion

Little progress has been made in improving NUE of cereals (Garnett et al. 2015). This is despite the fact that the genomes of important cereal crops have been sequenced. Furthermore, genes, loci of interest, and regulatory networks influencing NUE have been identified but, as yet, no improved NUE cereals have been released commercially. Deepening genetic understanding may have provided false hope that improving cereal NUE could be easily achieved. The NUE research

carried out in the field and in controlled environments, although not yet leading to germplasm with improved NUE, has helped us better understand the complexity of the trait and, in particular, the major GxE interaction. Modern phenomics as detailed here gives us the opportunity to better characterize the environment, plant responses to the environment and, combined with continually increasing genetic information, offers the opportunity to make real progress in improving NUE.

Acknowledgements Funding was received from the Australian Research Council (LP130101055, IH130200027), and the National Collaborative Research Infrastructure Strategy (NCRIS).

References

Abiko T, Wakayama M, Kawakami A, Obara M, Kisaka H, Miwa T, Aoki N, Ohsugi R (2010) Changes in nitrogen assimilation, metabolism, and growth in transgenic rice plants expressing a fungal NADP (H)-dependent glutamate dehydrogenase (gdhA). Planta 232(2):299–311

Agrama HAS, Zakaria AG, Said FB, Tuinstra M (1999) Identification of quantitative trait loci for nitrogen use efficiency in maize. Mol Breeding 5(2):187–195

Al-Tamimi N, Brien C, Oakey H, Berger B, Saade S, Ho YS, Schmöckel SM, Tester M, Negrão S (2016) Salinity tolerance loci revealed in rice using high-throughput non-invasive phenotyping. Nature Commun 7:13342

An D, Su J, Liu Q, Zhu Y, Tong Y, Li J, Jing R, Li B, Li Z (2006) Mapping QTLs for nitrogen uptake in relation to the early growth of wheat (*Triticum aestivum* L.). Plant Soil 284(1–2): 73–84

Andrade-Sanchez P, Gore MA, Heun JT, Thorp KR, Carmo-Silva AE, French AN, Salvucci ME, White JW (2013) Development and evaluation of a field-based high-throughput phenotyping platform. Funct Plant Biol 41(1):68–79

Araus JL, Cairns JE (2014) Field high-throughput phenotyping: the new crop breeding frontier. Trends Plant Sci 19(1):52–61

Babar MA, van Ginkel M, Klatt AR, Prasad B, Reynolds MP (2006) The potential of using spectral reflectance indices to estimate yield in wheat grown under reduced irrigation. Euphytica 150(1):155–172

Barraclough PB, Howarth JR, Jones J, Lopez-Bellido R, Parmar S, Shepherd CE, Hawkesford MJ (2010) Nitrogen efficiency of wheat: genotypic and environmental variation and prospects for improvement. Eur J Agron 33(1):1–11

Billiau K, Sprenger H, Schudoma C, Walther D, Köhl KI (2012) Data management pipeline for plant phenotyping in a multisite project. Funct Plant Biol 39(11):948–957

Borrell A, Hammer G, Van Oosterom E (2001) Stay-green: a consequence of the balance between supply and demand for nitrogen during grain filling? Ann Appl Biol 138(1):91–95

Brauer EK, Rochon A, Bi YM, Bozzo GG, Rothstein SJ, Shelp BJ (2011) Reappraisal of nitrogen use efficiency in rice overexpressing glutamine synthetase. Physiol Plant 141(4):361–372

Brien CJ, Berger B, Rabie H, Tester M (2013) Accounting for variation in designing greenhouse experiments with special reference to greenhouses containing plants on conveyor systems. Plant Methods 9(1):5

Brown TB, Cheng R, Sirault XR, Rungrat T, Murray KD, Trtilek M, Furbank RT, Badger M, Pogson BJ, Borevitz JO (2014) TraitCapture: genomic and environment modelling of plant phenomic data. Curr Opin Plant Biol 18:73–79

Burger J, Geladi P (2006) Hyperspectral NIR imaging for calibration and prediction: a comparison between image and spectrometer data for studying organic and biological samples. Analyst 131(10):1152–1160

Burns IG (1980) Influence of the spatial distribution of nitrate and the uptake of N by plants: a review and a model for rooting depth. J Soil Sci 31:155–173

Cabrera-Bosquet L, Fournier C, Brichet N, Welcker C, Suard B, Tardieu F (2016) High-throughput estimation of incident light, light interception and radiation-use efficiency of thousands of plants in a phenotyping platform. New Phytol 212(1):269–281

Campbell MT, Du Q, Liu K, Brien CJ, Berger B, Zhang C, Walia H (2017) A comprehensive image-based phenomic analysis reveals the complex genetic architecture of shoot growth dynamics in rice (Oryza sativa). Plant Genome 10(2)

Campbell MT, Knecht AC, Berger B, Brien CJ, Wang D, Walia H (2015) Integrating image-based phenomics and association analysis to dissect the genetic architecture of temporal salinity responses in rice. Plant Physiol 168(4):1476–1489

Chapman S, Merz T, Chan A, Jackway P, Hrabar S, Dreccer M, Holland E, Zheng B, Ling T, Jimenez-Berni J (2014) Pheno-copter: a low-altitude, autonomous remote-sensing robotic helicopter for high-throughput field-based phenotyping. Agronomy 4

Chen D, Neumann K, Friedel S, Kilian B, Chen M, Altmann T, Klukas C (2014) Dissecting the phenotypic components of crop plant growth and drought responses based on high-throughput image analysis. Plant Cell 26(12):4636–4655

Cho Y-G, Kang H-J, Lee J-S, Lee Y-T, Lim S-J, Gauch H, Eun M-Y, McCouch SR (2007) Identification of quantitative trait loci in rice for yield, yield components, and agronomic traits across years and locations all rights reserved. no part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. permission for printing and for reprinting the material contained herein has been obtained by the publisher. Crop Sci 47(6):2403–2417

Cormier F, Foulkes J, Hirel B, Gouache D, Moenne-Loccoz Y, Le Gouis J (2016) Breeding for increased nitrogen-use efficiency: a review for wheat (T.aestivum L.). Plant Breed 135(3): 255–278

Cormier F, Le Gouis J, Dubreuil P, Lafarge S, Praud S (2014) A genome-wide identification of chromosomal regions determining nitrogen use efficiency components in wheat (*Triticum aestivum L.*). Theor Appl Genet 127(12):2679–2693

Crain JL, Wei Y, Barker J, Thompson SM, Alderman PD, Reynolds M, Zhang N, Poland J (2016) Development and deployment of a portable field phenotyping platform. Crop Sci 56(3):965–975

Deery D, Jimenez-Berni J, Jones H, Sirault X, Furbank R (2014) Proximal remote sensing buggies and potential applications for field-based phenotyping. Agronomy 4(3):349

Dhugga KS, Waines J (1989) Analysis of nitrogen accumulation and use in bread and durum wheat. Crop Sci 29(5):1232–1239

Ding L, Wang KJ, Jiang GM, Biswas DK, Xu H, Li LF, Li YH (2005) Effects of nitrogen deficiency on photosynthetic traits of maize hybrids released in different years. Ann Bot 96 (5):925–930

Dueck T, van Ieperen W, Taulavuori K (2016) Light perception, signalling and plant responses to spectral quality and photoperiod in natural and horticultural environments. Environ Exp Bot 121:1–3

Ecarnot M, Compan F, Roumet P (2013) Assessing leaf nitrogen content and leaf mass per unit area of wheat in the field throughout plant cycle with a portable spectrometer. Field Crops Res 140:44–50

Echarte L, Rothstein S, Tollenaar M (2008) The response of leaf photosynthesis and dry matter accumulation to nitrogen supply in an older and a newer maize hybrid all rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher. Crop Sci 48(2):656–665

Eitel JUH, Magney TS, Vierling LA, Brown TT, Huggins DR (2014) LiDAR based biomass and crop nitrogen estimates for rapid, non-destructive assessment of wheat nitrogen status. Field Crops Res 159:21–32

Evenson RE, Gollin D (2003) Assessing the impact of the green revolution, 1960 to 2000. Science 300(5620):758–762

Fageria NK, Baligar VC (2005) Enhancing nitrogen use efficiency in crop plants. In: Donald LS (ed) Advances in agronomy. Academic Press, pp. 97-185

Fan X, Tang Z, Tan Y, Zhang Y, Luo B, Yang M, Lian X, Shen Q, Miller AJ, Xu G (2016) Overexpression of a pH-sensitive nitrate transporter in rice increases crop yields. Proc Natl Acad Sci 113(26):7118–7123

Fischer R, Wall P (1976) Wheat breeding in Mexico and yield increases

Fischer RA (2011) Wheat physiology: a review of recent developments. Crop Pasture Sci 62 (2):95–114

Forde BG, Clarkson DT (1999) Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. Adv Bot Res 30:1–90

Foulkes M, Sylvester-Bradley R, Scott R (1998) Evidence for differences between winter wheat cultivars in acquisition of soil mineral nitrogen and uptake and utilization of applied fertilizer nitrogen. J Agric Sci 130(01):29–44

Foulkes MJ, Hawkesford MJ, Barraclough PB, Holdsworth MJ, Kerr S, Kightley S, Shewry PR (2009) Identifying traits to improve the nitrogen economy of wheat: Recent advances and future prospects. Field Crops Res 114(3):329–342

Furbank RT, Tester M (2011) Phenomics–technologies to relieve the phenotyping bottleneck. Trends Plant Sci 16(12):635–644

Gallais A, Hirel B (2004) An approach to the genetics of nitrogen use efficiency in maize. J Exp Bot 55(396):295–306

Garnett T, Conn V, Kaiser BN (2009) Root based approaches to improving nitrogen use efficiency in plants. Plant Cell Environ 32(9):1272–1283

Garnett T, Conn V, Plett D, Conn S, Zanghellini J, Mackenzie N, Enju A, Francis K, Holtham L, Roessner U, Boughton B, Bacic A, Shirley N, Rafalski A, Dhugga K, Tester M, Kaiser BN (2013) The response of the maize nitrate transport system to nitrogen demand and supply across the lifecycle. New Phytol 198(1):82–94

Garnett T, Plett D, Heuer S, Okamoto M (2015) Genetic approaches to enhancing nitrogen-use efficiency (NUE) in cereals: challenges and future directions. Funct Plant Biol 42(10):921–941

Garnett T, Rebetzke G (2013) Improving crop nitrogen use in dryland farming. Improving water and nutrient-use efficiency in food production systems. Wiley. pp 123–144

Golzarian M, Frick R, Rajendran K, Berger B, Roy S, Tester M, Lun D (2011) Accurate inference of shoot biomass from high-throughput images of cereal plants. Plant Methods 7

Good AG, Johnson SJ, De Pauw M, Carroll RT, Savidov N, Vidmar J, Lu Z, Taylor G, Stroeher V (2007) Engineering nitrogen use efficiency with alanine aminotransferase. Botany 85(3):252–262

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9(12):597–605

Gu R, Duan F, An X, Zhang F, von Wirén N, Yuan L (2013) Characterization of AMT-mediated high-affinity ammonium uptake in roots of maize (*Zea mays L.*). Plant Cell Physiol 54(9): 1515–1524

Han M, Okamoto M, Beatty PH, Rothstein SJ, Good AG (2015) The genetics of nitrogen use efficiency in crop plants. Annu Rev Genet 49:269–289

Hawkesford MJ (2017) Genetic variation in traits for nitrogen use efficiency in wheat. J Exp Bot 68(10):2627–2632

Heap JW, McKay AC (2009) Managing soil-borne crop diseases using precision agriculture in Australia. Crop Pasture Sci 60(9):824–833

Hogewoning SW, Trouwborst G, Maljaars H, Poorter H, van Ieperen W, Harbinson J (2010) Blue light dose–responses of leaf photosynthesis, morphology, and chemical composition of Cucumis sativus grown under different combinations of red and blue light. J Exp Bot 61(11):3107–3117

Holman F, Riche A, Michalski A, Castle M, Wooster M, Hawkesford M (2016) High throughput field phenotyping of wheat plant height and growth rate in field plot trials using UAV based remote sensing. Remote Sens 8(12):1031

Honsdorf N, March TJ, Berger B, Tester M, Pillen K (2014) High-throughput phenotyping to detect drought tolerance QTL in wild barley introgression lines. PLoS ONE 9(5):e97047

Howitt SM, Udvardi MK (2000) Structure, function and regulation of ammonium transporters in plants. *Biochimica et Biophysica Acta* (*BBA*). Biomembranes 1465(1):152–170

Kamprath EJ, Moll RH, Rodriguez N (1982) Effects of nitrogen fertilization and recurrent selection on performance of hybrid populations of corn. Agron J 74(6):955–958

Keeney DR (1982) Nitrogen management for maximum efficiency and minimum pollution. Nitrogen in agricultural soils. Madison, Wisconsin USA: American Society of Agronomy, **pp** 605–649

Kokaly RF (2001) Investigating a physical basis for spectroscopic estimates of leaf nitrogen concentration. Remote Sens Environ 75(2):153–161

Krajewski P, Chen DJ, Cwiek H, van Dijk ADJ, Fiorani F, Kersey P, Klukas C, Lange M, Markiewicz A, Nap JP, van Oeveren J, Pommier C, Scholz U, van Schriek M, Usadel B, Weise S (2015) Towards recommendations for metadata and data handling in plant phenotyping. J Exp Bot 66(18):5417–5427

Ladha JK, Tirol-Padre A, Reddy CK, Cassman KG, Verma S, Powlson DS, van Kessel C, de B. Richter D, Chakraborty D, Pathak H (2016) Global nitrogen budgets in cereals: a 50-year assessment for maize, rice, and wheat production systems. Sci Rep 6:19355

Le Gouis J, Béghin D, Heumez E, Pluchard P (2000) Genetic differences for nitrogen uptake and nitrogen utilisation efficiencies in winter wheat. Eur J Agron 12(3–4):163–173

Léran S, Varala K, Boyer J-C, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W, Geiger D, Gojon A, Gong J-M, Halkier BA, Harris JM, Hedrich R, Limami AM, Rentsch D, Seo M, Tsay Y-F, Zhang M, Coruzzi G, Lacombe B (2014) A unified nomenclature of nitrate transporter 1/peptide transporter family members in plants. Trends Plant Sci 19(1):5–9

Lin M, Huybers P (2012) Reckoning wheat yield trends. Environ Res Lett 7(2):024016

Lovett GM, Burns DA, Driscoll CT, Jenkins JC, Mitchell MJ, Rustad L, Shanley JB, Likens GE, Haeuber R (2007) Who needs environmental monitoring? Front Ecol Environ 5(5):253–260

Ludewig U, Neuhäuser B, Dynowski M (2007) Molecular mechanisms of ammonium transport and accumulation in plants. FEBS Lett 581(12):2301–2308

Martre P, Porter JR, Jamieson PD, Triboï E (2003) Modeling grain nitrogen accumulation and protein composition to understand the sink/source regulations of nitrogen remobilization for wheat. Plant Physiol 133(4):1959–1967

Max JFJ, Schurr U, Tantau H-J, Mutwiwa UN, Hofmann T, Ulbrich A (2012) Greenhouse cover technology. horticultural reviews. Wiley, pp 259–396

McAllister CH, Beatty PH, Good AG (2012) Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10(9):1011–1025

Meng R, Saade S, Kurtek S, Berger B, Brien C, Pillen K, Tester M, Sun Y (2017) Growth curve registration for evaluating salinity tolerance in barley. Plant Methods 13(1):18

Mickelson S, See D, Meyer FD, Garner JP, Foster CR, Blake TK, Fischer AM (2003) Mapping of QTL associated with nitrogen storage and remobilization in barley *(Hordeum vulgare* L.) leaves. J Exp Bot 54(383):801–812

Moll R, Kamprath E, Jackson W (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron J 74(3):562–564

Muraya MM, Chu J, Zhao Y, Junker A, Klukas C, Reif JC, Altmann T (2017) Genetic variation of growth dynamics in maize (Zea mays L.) revealed through automated non-invasive phenotyping. Plant J 89(2):366–380

Neilson EH, Edwards A, Blomstedt C, Berger B, Møller BL, Gleadow R (2015) Utilization of a high-throughput shoot imaging system to examine the dynamic phenotypic responses of a C4 cereal crop plant to nitrogen and water deficiency over time. J Exp Bot eru526

Ortiz-Monasterio R, Sayre K, Rajaram S, McMahon M (1997) Genetic progress in wheat yield and nitrogen use efficiency under four nitrogen rates. Crop Sci 37(3):898–904
Parent B, Shahinnia F, Maphosa L, Berger B, Rabie H, Chalmers K, Kovalchuk A, Langridge P, Fleury D (2015) Combining field performance with controlled environment plant imaging to identify the genetic control of growth and transpiration underlying yield response to water-deficit stress in wheat. J Exp Bot 66(18):5481–5492
Passioura JB (2006) The perils of pot experiments. Funct Plant Biol 33(12):1075–1079
Peñuelas J, Filella I (1998) Visible and near-infrared reflectance techniques for diagnosing plant physiological status. Trends Plant Sci 3(4):151–156
Peoples M, Freney J, Mosier A, Bacon P (1995) Minimizing gaseous losses of nitrogen. Nitrogen fertilization in the environment, pp. 565–602
Plett D, Toubia J, Garnett T, Tester M, Kaiser BN, Baumann U (2010) Dichotomy in the *NRT* Gene Families of Dicots and Grass Species. PLoS ONE 5(12):e15289
Poorter H, Fiorani F, Pieruschka R, Wojciechowski T, Putten WH, Kleyer M, Schurr U, Postma J (2016) Pampered inside, pestered outside? Differences and similarities between plants growing in controlled conditions and in the field. New Phytol 212(4):838–855
Quraishi UM, Abrouk M, Murat F, Pont C, Foucrier S, Desmaizieres G, Confolent C, Riviere N, Charmet G, Paux E (2011) Cross-genome map based dissection of a nitrogen use efficiency ortho-metaQTL in bread wheat unravels concerted cereal genome evolution. Plant J 65(5):745–756
Rajcan I, Tollenaar M (1999) Source: sink ratio and leaf senescence in maize: II. Nitrogen metabolism during grain filling. Field Crops Res 60(3):255–265
Raun WR, Johnson GV (1999) Improving nitrogen use efficiency for cereal production. Agron J 91(3):357–363
Ray DK, Mueller ND, West PC, Foley JA (2013) Yield trends are insufficient to double global crop production by 2050. PLoS ONE 8(6):e66428
Ray DK, Ramankutty N, Mueller ND, West PC, Foley JA (2012) Recent patterns of crop yield growth and stagnation. 3:1293
Rebetzke GJ, Chenu K, Biddulph B, Moeller C, Deery DM, Rattey AR, Bennett D, Barrett-Lennard EG, Mayer JE (2012) A multisite managed environment facility for targeted trait and germplasm phenotyping. Funct Plant Biol 40(1):1–13
Rebetzke GJ, Jimenez-Berni JA, Bovill WD, Deery DM, James RA (2016) High-throughput phenotyping technologies allow accurate selection of stay-green. J Exp Bot 67(17):4919–4924
Sadras VO, Richards RA (2014) Improvement of crop yield in dry environments: benchmarks, levels of organisation and the role of nitrogen. J Exp Bot 65(8):1981–1995
Sankaran S, Khot LR, Carter AH (2015) Field-based crop phenotyping: multispectral aerial imaging for evaluation of winter wheat emergence and spring stand. Comput Electron Agric 118:372–379
Shaw R, Lark RM, Williams AP, Chadwick DR, Jones DL (2016) Characterising the within-field scale spatial variation of nitrogen in a grassland soil to inform the efficient design of in-situ nitrogen sensor networks for precision agriculture. Agr Ecosyst Environ 230:294–306
Sinclair TR (1998) Historical changes in harvest index and crop nitrogen accumulation. Crop Sci 38(3):638–643
Sun J, Shi S, Gong W, Yang J, Du L, Song S, Chen B, Zhang Z (2017) Evaluation of hyperspectral LiDAR for monitoring rice leaf nitrogen by comparison with multispectral LiDAR and passive spectrometer. 7:40362
Sylvester-Bradley R, Kindred DR (2009) Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency. J Exp Bot 60(7):1939–1951
Tanger P, Klassen S, Mojica JP, Lovell JT, Moyers BT, Baraoidan M, Naredo MEB, McNally KL, Poland J, Bush DR, Leung H, Leach JE, McKay JK (2017) Field-based high throughput phenotyping rapidly identifies genomic regions controlling yield components in rice. 7:42839
Thomas H, Smart CM (1993) Crops that stay green1. Ann Appl Biol 123(1):193–219
Ugarte CC, Trupkin SA, Ghiglione H, Slafer G, Casal JJ (2010) Low red/far-red ratios delay spike and stem growth in wheat. J Exp Bot 61(11):3151–3162

Van Herwaarden A, Farquhar G, Angus J, Richards R, Howe G (1998a) 'Haying-off', the negative grain yield response of dryland wheat to nitrogen fertiliser. I. Biomass, grain yield, and water use. Aust J Agric Res 49(7):1067–1081

van Herwaarden AF, Angus JF, Richards RA, Farquhar GD (1998b) 'Haying-off', the negative grain yield response of dryland wheat to nitrogen fertiliser—II. Carbohydrate and protein dynamics. Aust J Agric Res 49(7):1083–1093

Virlet N, Sabermanesh K, Sadeghi-Tehran P, Hawkesford MJ (2016) Field scanalyzer: an automated robotic field phenotyping platform for detailed crop monitoring. Funct Plant Biol 44(1):143–153

Watanabe K, Guo W, Arai K, Takanashi H, Kajiya-Kanegae H, Kobayashi M, Yano K, Tokunaga T, Fujiwara T, Tsutsumi N, Iwata H (2017) High-throughput phenotyping of sorghum plant height using an unmanned aerial vehicle and its application to genomic prediction modeling. Front Plant Sci 8(421)

Wei D, Cui K, Ye G, Pan J, Xiang J, Huang J, Nie L (2012) QTL mapping for nitrogen-use efficiency and nitrogen-deficiency tolerance traits in rice. Plant Soil 359(1–2):281–295

White JW, Andrade-Sanchez P, Gore MA, Bronson KF, Coffelt TA, Conley MM, Feldmann KA, French AN, Heun JT, Hunsaker DJ (2012) Field-based phenomics for plant genetics research. Field Crops Res 133:101–112

Wolt JD (1994) Soil solution chemistry: applications to environmental science and agriculture. Wiley

Xu Y, Wang R, Tong Y, Zhao H, Xie Q, Liu D, Zhang A, Li B, Xu H, An D (2014) Mapping QTLs for yield and nitrogen-related traits in wheat: influence of nitrogen and phosphorus fertilization on QTL expression. Theor Appl Genet 127(1):59–72

Yang W, Guo Z, Huang C, Duan L, Chen G, Jiang N, Fang W, Feng H, Xie W, Lian X (2014) Combining high-throughput phenotyping and genome-wide association studies to reveal natural genetic variation in rice. Nat Commun 5

Yendrek C, Tomaz T, Montes CM, Cao Y, Morse AM, Brown PJ, McIntyre L, Leakey A, Ainsworth E (2016) High-throughput phenotyping of maize leaf physiology and biochemistry using hyperspectral reflectance. Plant Physiol 01447–02016

Zhang X, Huang C, Wu D, Qiao F, Li W, Duan L, Wang K, Xiao Y, Chen G, Liu Q, Xiong L, Yang W, Yan J (2017) High-throughput phenotyping and QTL mapping reveals the genetic architecture of maize plant growth. Plant Physiol 173(3):1554–1564

Chapter 8
Nitrogen Partitioning and Remobilization in *Arabidopsis* Under Sufficient and Depleted Conditions

Adel Zayed and Robert Crosby

Abbreviations

N	Nitrogen
NO_3	Nitrate
12S SSG	12S seed storage globulin protein
HYPO	Hypothetical protein
BBCH	BASF, Bayer, Ciba-Geigy, Hoechst (*BBCH*) scale
DAS	Days after sowing
DAT	Days after transplanting
RO	Reverse osmosis

Introduction

Nitrogen (N) is a key component of many important biological compounds in plants. When nitrogen is deficient in corn, plants exhibit premature senescence, delayed flowering, and yields are reduced to 60 bushels (bu) acre^{-1}, whereas optimally N-fertilized corn will easily yield more than 200 bu acre^{-1} (Sawyer 2015). N is taken up by roots then reduced and assimilated in leaves (Lemaitre et al. 2008), but its capacity to utilize these stores encounters several rate-limiting steps, ultimately leading to a plateau in yield despite increased N input. Long-term studies

A. Zayed (✉) · R. Crosby
Biotechnology, Monsanto Company, 800 North Lindbergh Blvd,
Mail Zone N2NB, St. Louis, MO 63167, USA
e-mail: adel.zayed@monsanto.com

R. Crosby
108 T.W. Alexander Drive, Research Triangle Park, Durham, NC 27709, USA

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_8

in Iowa showed that returns in corn yield response diminished substantially as the fertilizer N rate increased above 120 bu acre^{-1} (Sawyer 2015).

Additionally, adding large amounts of nitrogen fertilizer to fields is not the best solution for two reasons. First, environmental impacts of nitrogen runoff have become a major problem in modern agriculture. Plants are only able to utilize 30–50% of applied N to useful food products (Masclaux-Daubresse et al. 2010), and 8–9% is leached out of soil as nitrate (Cameron et al. 2013). This represents a major ecological problem, as leached nitrate pollutes surrounding waterways and oceans. Secondly, while worldwide N fertilization use has increased sevenfold in forty years (Hirel et al. 2007), and the cost of nitrogen to farmers is the most expensive input on well-watered fields (Masclaux-Daubresse et al. 2008). With increasing food demands, it is imperative for farmers to increase yields while reducing inputs. One way to achieve this is by finding genotypes that are more efficient at nitrogen metabolism.

Nitrogen metabolism can be divided into two phases: accumulation and remobilization (Hirel et al. 2007). The first phase, accumulation, occurs during the vegetative stage, where available nitrogen (usually supplied as nitrate) is absorbed by the roots. Nitrate is accumulated and stored in the leaves as nitrate for later use when uptake and accumulation become limiting. Genes with improved nitrogen accumulation are beneficial, as they can absorb and accumulate more N (Hirel et al. 2007), reducing input, cost, and nitrogen leaching to the environment. However, improving accumulation alone will not yield the most effective results. The next phase, remobilization, primarily occurs during the reproductive stage. At this stage in the life cycle, uptake and accumulation have become significantly slow, and stored nitrate from vegetative tissue starts to be remobilized to younger leaves and reproductive tissues (Hirel et al. 2007). During grain filling, 45–65% of grain N in corn and 60–95% of grain N in rice comes from remobilization (Hirel et al. 2007; Masclaux-Daubresse et al. 2008). Harnessing this genetic variability can aid in increasing yield (Hirel et al. 2007). Gene discovery for increased nitrogen utilization efficiency (NUE) should include both accumulation and remobilization to have the most impact on yield. During N depletion conditions, nitrate, a transportable N source, disappears fast from source tissues, indicating plants can mobilize N reserves to maintain N metabolism (Richard-Molard et al. 2008). Plants ability to withstand N deficiency is related not only to its ability to accumulate and store nitrate, but also transport nitrate to sink tissues (Richard-Molard et al. 2008) as uptake by source tissues is insufficient for high demands of sink tissues (Masclaux-Daubresse et al. 2008). Thus, it is necessary to study both accumulation and remobilization to have the best approach in finding genes with improved NUE.

There are two strategies for finding genotypes with improved nitrogen metabolism. The most common approach is to screen plants that can maintain standard yields with reduced nitrogen inputs. Genes identified by this approach can ultimately reduce nitrogen input and thereby reduce economic and environmental impacts; however, an increasing world food demand mandates an approach to increase yield with the same nitrogen input, requiring overcoming the plateau effect. This study was conducted to improve our understanding of the transition between

nitrogen accumulation, assimilation, and remobilization especially when nitrogen is limiting and to pinpoint the strategies by which plants can improve their remobilization efficiency.

We developed a hydroponics assay that can successfully evaluate genetic efficacy for both phases of nitrogen metabolism in *Arabidopsis thaliana*. Hydroponics is advantageous in allowing complete and precise control of nutrient media as well as characterization of intact root system which is difficult to evaluate in soil (Hirel et al. 2007). While there are few studies that investigate or characterize root morphology and its relationship to N supply, biomass, production, and yield (Hirel et al. 2007), it is necessary to evaluate the site of N uptake and observe modification of the root architecture as plants forage for nutrients (Lemaitre et al. 2008). In addition, to effectively evaluate remobilization, it is necessary to establish significant resources in the sink, which is directly impacted by photoperiod. Use of short-day (10 h) conditions in this study was effective in increasing nitrogen storage in source tissues more than long day, allowing differences in as low as 1 week of treatment initiation of nitrogen depletion to show exponential changes in seed yield.

Under these conditions, we demonstrated that plants became efficient under limited N: They fully assimilated all taken up inorganic N and distributed more N to the critical parts, seeds and roots, as a survival strategy. The method was also used to elucidate responses to limited N of two N-stress tolerant genes contrasting in their responses to limited nitrogen environment.

Results

Understanding Plant Response to Nitrogen Depletion Under Short- and Long-Day Conditions

Seed Yield Response to Nitrogen Depletion for Plants Grown Under Short- and Long-Day Conditions

Obtaining high seed yield is the ultimate goal in studying remobilization efficiency. As external N supply is reduced, vegetative tissue must remobilize N to the reproductive tissues to promote early flowering. If a gene improves remobilization efficiency, an increase in seed yield can result under N depletion, as an increased mobilization of N supply from vegetative tissue to the seeds will facilitate protein synthesis. Photoperiod can directly impact flowering and therefore seed production. In Arabidopsis, under short day (10 h) energy is mostly spent in increasing source tissue, while, under long day (16 h), energy is mostly spent on increasing sink tissue. For a remobilization screen, short day may be ideal, as increasing source capacity allows more resources for remobilization to sink tissues. However, with energy diverted to source under short day, the sink tissue could be too limiting to evaluate genetic differences in remobilization. It is also important to establish the

timing of the N depletion on the plant life cycle. Implementing the treatment too early or too late may not allow sufficient sensitivity to detect changes in resource redistributions.

To address the above concerns, plants were grown in this study under N sufficient conditions under short- or long-day conditions for 4 weeks after which one of four N depletion treatments was initiated, plus a control group grown in N sufficient conditions for the duration of the life cycle. Plants for both photoperiods were grown under N sufficient conditions until N depletion initiation, which was implemented on either 4, 5, 6, or 7 weeks, and continued until growth stage 9.7 (Lancashire et al. 1991). Due to rapid growth of reproductive tissue under long day, long-day plants were grown for 8 weeks after sowing, while short-day plants were grown for 10 weeks. The control population for both photoperiods was continually grown under N sufficient conditions.

In this study, our primary metric for evaluating remobilization efficiency was yield. Under both short- and long-day conditions, seed yield increased with increased duration of N sufficient conditions (Fig. 8.1). As expected, long day produced higher yield than short day. Short-day yield was more sensitive to N supply, with a significant reduction between 4 and 5 weeks duration of N depletion conditions. This shows short day to be more sensitive to N depletion conditions than long day, allowing better detection of efficacious genotypes. Because of the significant changes in yield between 4 and 5 weeks, N depletion initiation at 4 weeks under short-day conditions was selected as the threshold for tolerance due to potential sensitivity in detecting efficacious genotypes.

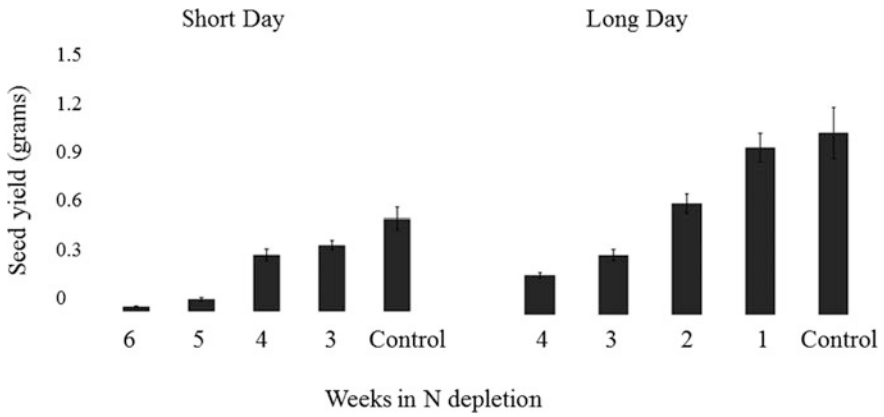


Fig. 8.1 Effect of seed yield under short and long day due to exposure of N depletion conditions. Short-day conditions were more sensitive in detecting differences in seed yield under N depletion. A significant shift was observed at 3 and 4 weeks under exposure to N depletion conditions under short day. Error bars = standard error

Dry Mass Distribution Response to Nitrogen Depletion for Plants Grown Under Short- and Long-Day Conditions

To understand the implication remobilization has on all plant tissues, dry weights were collected on roots, rosettes, inflorescence, and seeds. As expected, more biomass accumulated in the reproductive tissues under long day, whereas short day increased vegetative tissues (Fig. 8.2). Under long-day conditions, the distribution of biomass for each tissue type changed very little with prolonged exposure to N depletion conditions. As exposure to N depletion increased under long day, the total biomass was reduced, but the distribution each tissue contributed to total biomass remained the same, with inflorescence accounting for approximately 70% (Fig. 8.3). Plants exposed to short-day conditions not only changed the total biomass with exposure to N depletion conditions, but also changed the distribution of biomass. As plants were exposed to N depletion conditions for a longer interval, energy was diverted from increasing rosette and inflorescence to increasing yield and root tissue. Yield was increased to accelerate seed development before plant death, and roots were increased to forage for more nitrogen. Interestingly, the short-day control treatment had more biomass than the long-day control treatment, most likely due to increased exposure to N sufficient conditions due to longer life cycle. These data support the conclusion that short day is more sensitive than long day under N depletion conditions to detect potential genetic changes in remobilization efficiency.

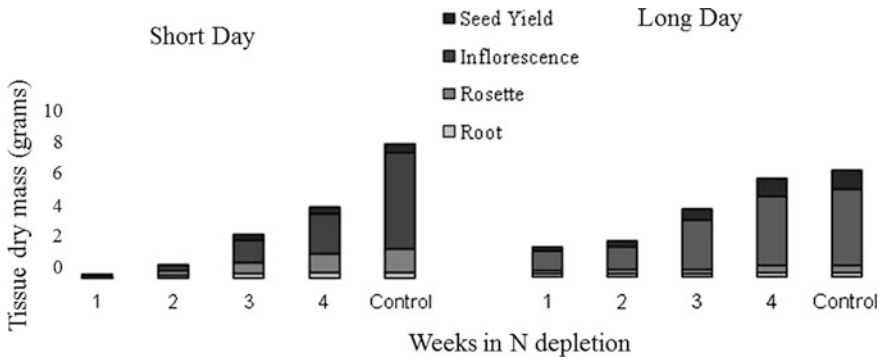


Fig. 8.2 Dry mass distribution (grams) under short and long day due to exposure of N depletion conditions. Biomass allocation was significantly impacted by N depletion, especially under short day. More biomass accumulated in source tissue under short-day conditions than long day at 3 and 4 weeks under N depletion and control treatment

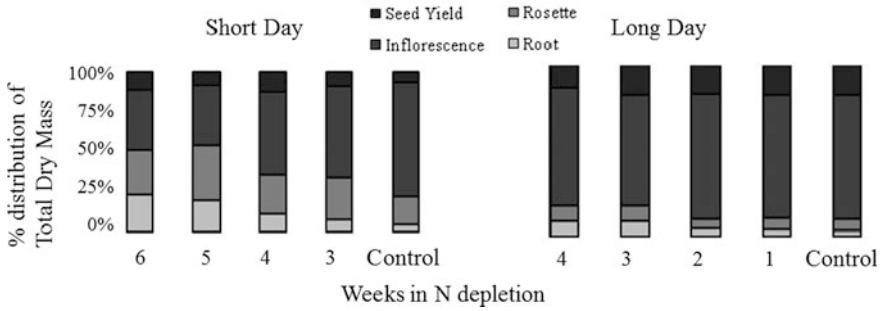


Fig. 8.3 % Dry mass of short- and long-day plants under exposure to N depletion conditions. Prolonged exposure to N depletion conditions significantly altered the biomass allocation under short-day conditions, but not under long-day conditions

Total N Response to Nitrogen Depletion for Plants Grown Under Short- and Long-Day Conditions

Total N (mg plant^{-1}) measures the N content accumulated and stored in the plant. Improved accumulation was observed by increases in total N under N sufficient conditions. Under N depletion conditions, excess stored N is remobilized to the reproductive tissues to promote an increase in seed yield, and one way to determine improved remobilization is observing increased depletion of stored N from vegetative tissue under N depletion conditions. Total N was collected for roots, rosettes, and seeds under short- and long-day conditions. Under short day, plants increased total N in source tissues, mostly as storage, in response to prolonged exposure to N sufficient conditions (Fig. 8.4). Under long day, plants had the largest amount of

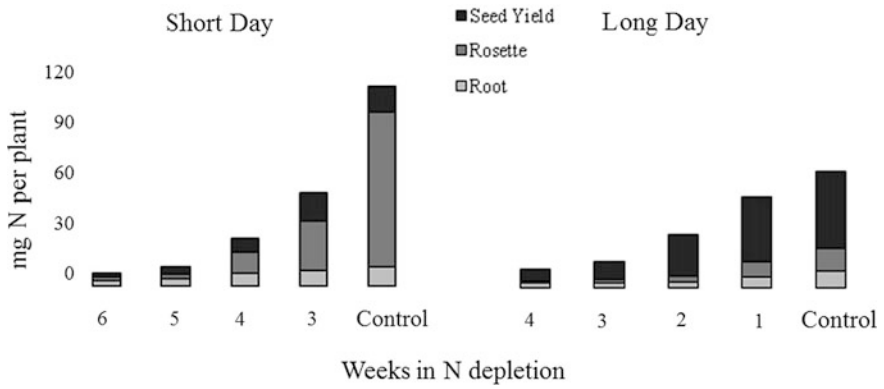


Fig. 8.4 Total N (mg plant^{-1}) of short- and long-day conditions under prolonged exposure to N depletion conditions. Total plant N was higher under short-day conditions than long day. As severity of N depletion conditions increased, N accumulation was reduced in rosette tissues and increased in roots and seeds, especially under short day

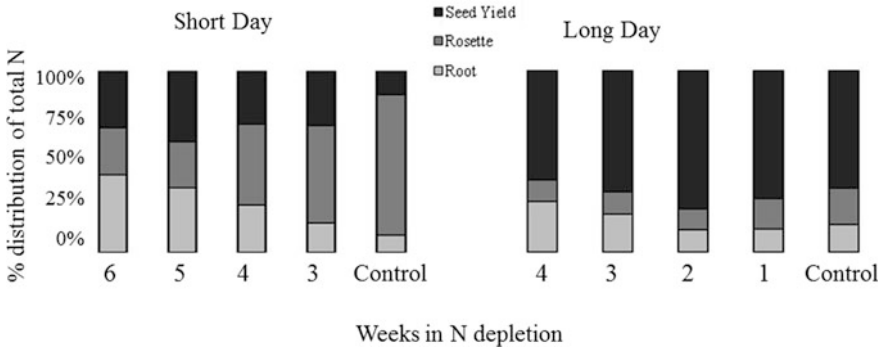


Fig. 8.5 % Total N distribution per plant of short and long day under prolonged exposure to N depletion conditions. As exposure to N depletion conditions increased, total N shifted from rosettes to roots and seeds, especially under short-day conditions

total N in seeds. Allocation of N under short day shows that total N was sacrificed in rosette tissue for seed and roots as N depletion conditions were prolonged, suggesting remobilization out of source and into sink tissues (Fig. 8.5). However, under long day, N depletion conditions had little impact on distribution of N, showing that short day is the preferred photoperiod for a remobilization screen.

Total NO_3 Response to Nitrogen Depletion for Plants Grown Under Short- and Long-Day Conditions

Total NO_3 content changes in different tissues in response to N treatment reflect how well plants accumulate, assimilate, and remobilize NO_3. To evaluate NO_3 accumulation and remobilization, we analyzed tissues for total NO_3 N. Under sufficient N supply, excess N is accumulated and stored as NO_3 in vegetative tissue for subsequent assimilation and remobilization to reproductive tissues. This occurs naturally during flowering, as accumulation slows and N must be assimilated and remobilized to the reproductive tissue for seed development. This can also occur prematurely to promote reproductive tissue growth prior to plant death when N supply is limited and accumulation slows. As a result, N is remobilized to the reproductive tissue to hasten seed development prior to plant senescence.

Under both short- and long-day conditions, NO_3 accumulated mainly in the rosettes, the main storage component in Arabidopsis plants (Fig. 8.6). As N supply was depleted, NO_3 reserves decreased. However, NO_3 levels under short-day conditions were exponentially decreased as N depletion became more severe, showing effective remobilization to seeds under limiting N. Under long day, NO_3 levels were also reduced, but not as severely as under short day. Under short day,

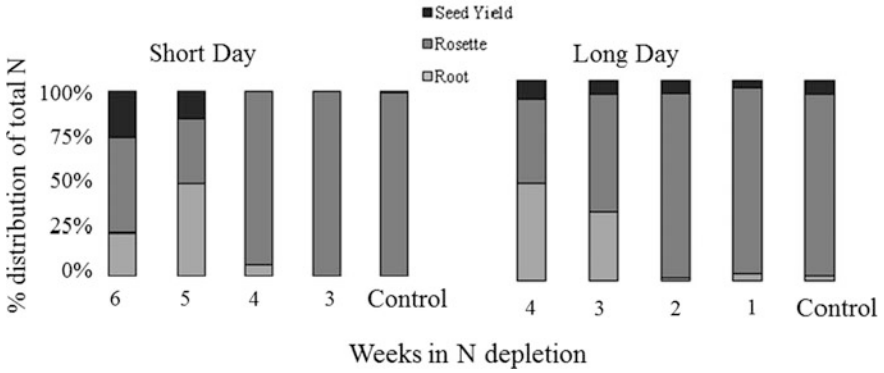


Fig. 8.6 % Distribution of total plant NO_3 of short and long day under N depletion conditions. A significant shift is observed in distribution of NO_3 at the two most severe N depletion treatments in both short- and long-day plants. At this treatment, nearly all stored NO_3 has been assimilated to promote seed set before plant death

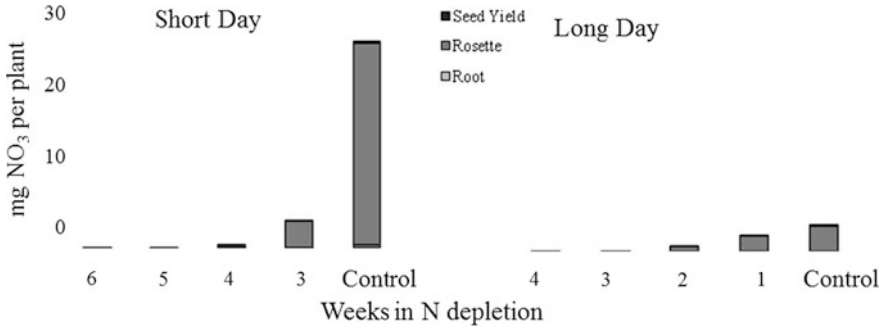


Fig. 8.7 Total plant NO_3 (mg plant^{-1}) of short- and long-day conditions under prolonged exposure to N depletion conditions. Nitrate accumulated mainly in the rosettes under control conditions and is depleted as exposure to N depletion conditions increases. Short-day plants accumulate and deplete NO_3 at a greater rate than plants grown under long-day conditions

the two most severe N depletion initiation treatments (5 and 6 weeks) completely eliminated all NO_3 reserves, showing these conditions to be too severe for evaluation of remobilization. Week 4 has trace amounts of NO_3, but enough to support high seed yield and total plant biomass (Fig. 8.7). As a result, these conditions were selected for a case study of two genes, 12S SSG and HYPO, found to have improved N uptake in a different nitrogen seedling assay as described below.

Evaluation of N Uptake Genes

Low Nitrogen Response Screen

A low nitrogen (0.2 mM) vertical plate screen was performed to identify the transgenic plants showing increased or decreased N accumulation and/or utilization when subjected to the low N environment. The down-regulation of the 12S seed storage globulin (12S SSG) gene (as antisense configuration) and the over-expression of a hypothetical protein (HYPO) gene (as sense configuration) were identified by this low N screen (Fig. 8.8). In response to low N stress, control seedlings exhibit three symptoms. First, root area increases as the plant forages for nutrients (Lemaitre et al. 2008). Secondly, as energy is diverted from the shoots to the roots, shoot fresh weight is reduced. Lastly, anthocyanin accumulates in the rosettes as a stress response, exhibiting red and purple coloring on leaves. The 12S SSG exhibited signs of low N tolerance to all three symptoms. This gene resulted in significantly higher fresh weights in both low N and sufficient nutrient media, greener rosette color, and a smaller root area. However, interestingly when the 12S SSG was subjected to a drought soil assay, significantly smaller seed yield was observed, suggesting sensitivity to water stress. Conversely, the HYPO transgenic exhibited only reduced anthocyanin accumulation as a low N-stress tolerance response, suggesting a different mode of action in N metabolism from the 12S SSG.

Total Nitrogen Changes in Response to Nitrogen Sufficient and Depletion Conditions

Under N sufficient conditions, the 12S SSG protein (line 2) accumulated significantly more total N than control in seed and inflorescence tissues as well as the roots, indicating increased N accumulation in roots and reproductive tissue (Figs. 8.9a–d). Under N depletion conditions, the 12S SSG had higher total N in seeds in both lines, indicating improved N remobilization to seeds under N

Fig. 8.8 Low N vertical plate screen identifies genes with altered N response. The 12S SSG exhibited three signs of low N tolerance: increased rosette fresh weight, decreased root fresh weight, and reduction in anthocyanin accumulation. The HYPO exhibited only the reduction in anthocyanin accumulation

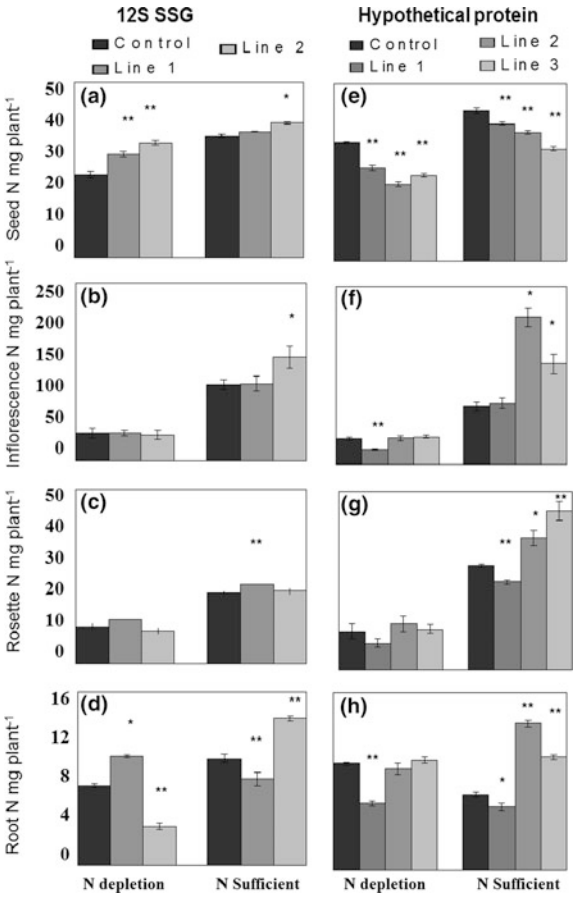


Fig. 8.9 Total nitrogen (mg plant^{-1}) allocation in transgenic and control tissues under N depletion and N sufficient conditions. Total N is higher than control in seeds for 12S SSG and lower than control for HYPO. 12S SSG had improved N accumulation under N sufficient conditions, and the stored N was utilized under N depletion conditions, preferentially to the seeds. The HYPO increased N storage under N sufficient in all tissues except seeds. The stored N was utilized under N depletion, with seed N content reduced even further. Total N for 12S SSG seed yield (**a**), inflorescence dry weight (**b**), rosette dry weight (**c**), root dry weight (**d**), HYPO seed yield (**e**), inflorescence dry weight (**f**), rosette dry weight (**g**), and root dry weight (**h**). * indicates significance <0.1; ** indicates significance <0.05. n = 3. Error bars = standard error

depletion conditions. All other tissues were neutral (except for a varied response in root tissue between the two transgenic lines), indicating increased N accumulation under N sufficient conditions was utilized evenly among these tissues when subjected to N depletion. Thus, the 12S SSG gene, which may be involved in increasing C and N to seeds (Hou et al. 2005), shows increased N accumulation in vegetative tissues and increased remobilization and assimilation of this excess N to the reproductive tissues under N depletion conditions.

The HYPO transgenic was also found to have increased N accumulation in inflorescence and root tissues, as well as rosettes in 2 of 3 transgenic lines. More total N was accumulated in the control line in vegetative and inflorescence tissues under N sufficient conditions, but when subjected to N depletion conditions, total N in the HYPO transgenic was comparable to control, indicating relatively enhanced N accumulation under N depletion conditions (Figs. 8.9e–h). However, unlike the 12S SSG, total N in seeds was found to be less than control at both conditions. This suggests that excess N was preferentially accumulated and utilized in vegetative tissue and inflorescence, whereas seed N was reduced. The increased N accumulation in vegetative and inflorescence tissues suggests this transgenic may increase N uptake, but the decrease in seed N suggests a lack of a role of this gene in N partitioning or long-distance transport.

Total Nitrate Nitrogen Changes in Response to N Treatment

For a gene to help improve remobilization efficiency, it should facilitate increased NO_3 storage under N sufficient conditions and efficiently remobilize these excess N stores under N depletion conditions. Under N sufficient conditions, both lines of the 12S SSG transgenic increased total NO_3 N storage over control at vegetative and inflorescence tissues, indicating the excess N was being stored as NO_3 in these tissues (Figs. 8.10a–d). The seeds had high N but comparable NO_3 levels as the control plants, indicating that the transgenic lines utilized excess N to assimilate proteins instead of storing it as NO_3. When subjected to N depletion conditions, all tissue types for transgenic line 2 of the 12S SSG gene were either comparable or lower than control, indicating the excess N stored as NO_3 under N sufficient conditions was effectively assimilated when the N deficiency was encountered. However, seed N was comparable to control at both conditions, indicating a normal sink–source relationship.

Similarly to the 12S SSG transgenic, the total NO_3 N content for 2 of the 3 HYPO transgenic lines under N sufficient conditions was higher than control plants, particularly in inflorescence (Figs. 8.10e–h). Additionally, under N depletion, NO_3 N content in vegetative and reproductive tissues of the HYPO transgenic lines was either comparable or less than the control, indicating efficient utilization of stored NO_3, also seen in the 12S SSG. However, seed NO_3 content of the HYPO transgenic was reduced in response to N deficiency, indicating a preference to retain NO_3 storage in vegetative tissue as opposed to seeds. Under N depletion conditions, the transgenic conserved NO_3 preferentially in vegetative tissue compared to the control, reducing NO_3 levels in seeds, illustrating an unusual source–sink relationship.

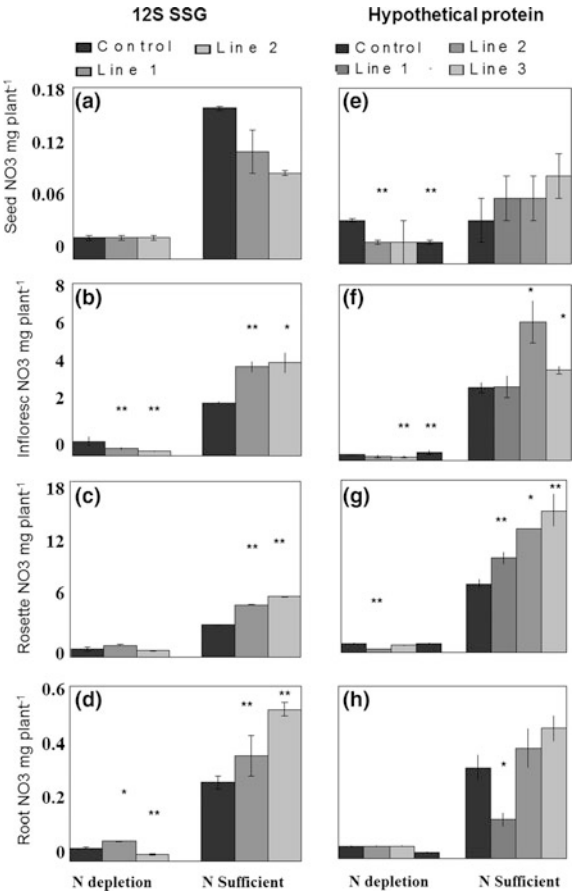


Fig. 8.10 Total nitrate (mg plant^{-1}) allocation in transgenic and control tissues under N depletion and N sufficient conditions. Total NO_3 increased in source tissues for both genes under N sufficient. Under N depletion, nitrate content in all three transgenic lines of the HYPO was reduced to half of the control line nitrate content. Total NO_3 N for 12S SSG seed yield (**a**), inflorescence dry weight (**b**), rosette dry weight (**c**), root dry weight (**d**), HYPO seed yield (**e**), inflorescence dry weight (**f**), rosette dry weight (**g**), and root dry weight (**h**). * indicates significance <0.1; ** indicates significance <0.05. n = 3. Error bars = standard error

Seed Yield Response to Nitrogen Depletion

In response to N depletion, wild-type seed yield is typically reduced compared to N sufficient, and this trend was observed in the control plants of the 12S SSG (12% reduction). Under N sufficient conditions, lines 1 and 2 of 12S SSG transgenics produced 8 and 14% more seed yield than control, respectively (Fig. 8.11). However, when subjected to N depletion conditions, both lines of the 12S SSG transgenic increased seed yield over the control, with line 2 increasing seed yield 45% over control. This suggests that the increased accumulation of N and storage as NO_3 in vegetative tissue of the 12S SSG transgenic under N sufficient conditions allows for increased reserves to be remobilized to the seeds under N depletion conditions, thereby increasing seed yield. The 12S SSG is believed to contribute to increasing C and N in seeds, allowing seeds to act as an N sink. When subjected to N depletion, N is more efficiently sent to seeds in the 12S SSG transgenic, resulting in even higher yield than control under N depletion compared to N sufficient.

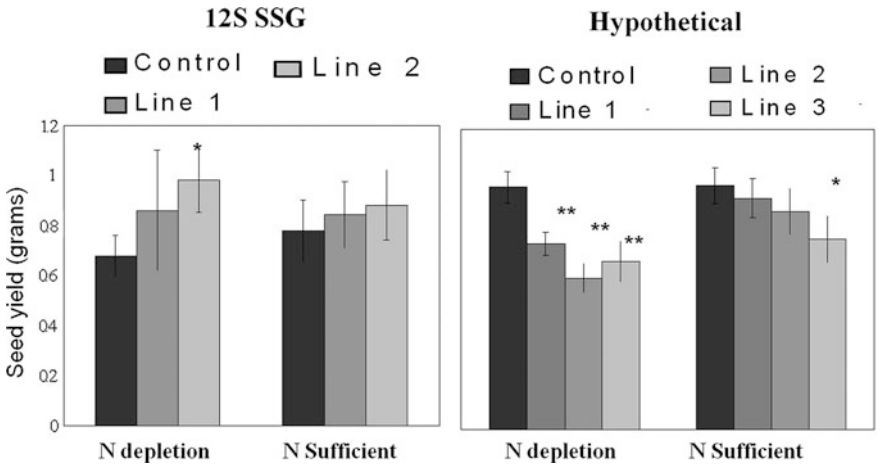


Fig. 8.11 Impact of N depletion and N sufficient conditions on seed yield. Under N depletion conditions, 12S SSG plants increased seed yield over N sufficient conditions, whereas the HYPO plants decreased seed yield. * indicates significance <0.1; ** indicates significance <0.05. n = 3. Error bars = standard error

Under N sufficient conditions, all three HYPO transgenic lines had reduced seed yield of 5–20% compared to control, despite an increase in total N and NO_3 accumulation in seeds. Under N depletion conditions, all three HYPO transgenic lines had even further reduction (23–37%) in seed yield compared to the control, indicating sensitivity of the HYPO transgenic lines to N depletion conditions. It suggests that N was not efficiently remobilized to seeds due to preferential retention of N and NO_3 in the source tissue, resulting in a significant decrease in seed yield of the HYPO transgenic lines under N-depleted conditions compared to the control.

Biomass Distribution Response to Nitrogen Depletion

To evaluate the effect that total N and total NO_3 N have on plant tissue biomass, we collected dry weights of plant tissues harvested from both N sufficient and N depletion conditions for both the 12S SSG gene and the HYPO transgenics. Typically, tissues with high N and NO_3 levels will also have increased biomass. Under N sufficient, only the inflorescence biomass of transgenic line 2 of the 12S SSG significantly increased over the control (Fig. 8.12c). However, under N depletion conditions, seed yield increased in line 2 (Fig. 8.12b), while root biomass increased in line 1 (Fig. 8.12e). As expected, rosettes of the control and the transgenic plants from both the 12S SSG and the HYPO genes exhibited early senescence and anthocyanin accumulation under N depletion conditions (Fig. 8.13). However, no significant differences in rosette biomass were observed between the control and the transgenic lines (Fig. 8.12d). This suggests that the

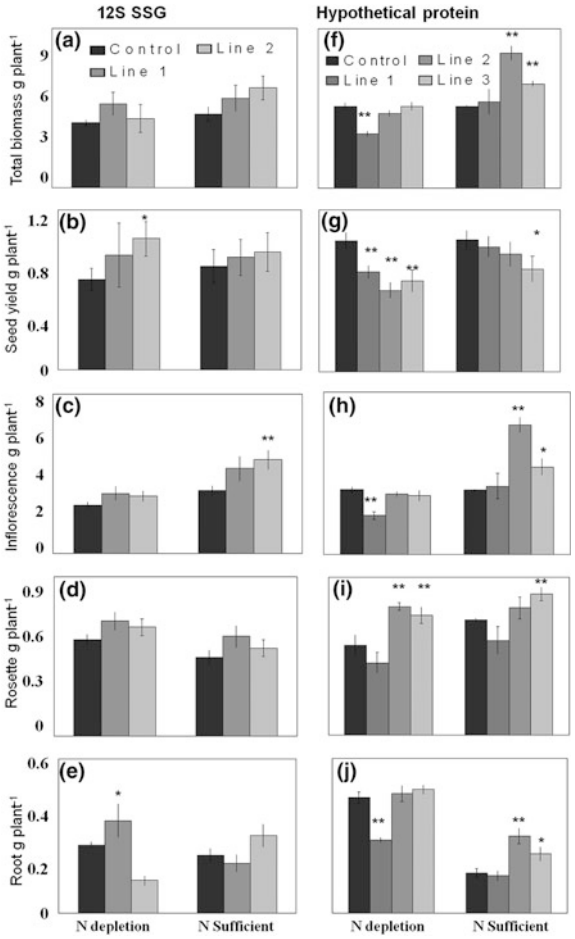


Fig. 8.12 Biomass allocation under N depletion and N sufficient conditions. 12S SSG total biomass (a), seed yield (b), inflorescence dry weight (c), rosette dry weight (d), root dry weight (e), and HYPO total biomass (f, seed yield (g), inflorescence dry weight (h), rosette dry weight (i), and root dry weight (j). Under N depletion conditions, the 12S SSG transgenic plants allocated more biomass to seed yield, whereas the HYPO transgenic plants allocated more biomass to rosette and less to inflorescence. * indicates significance <0.1; ** indicates significance <0.05. n = 3. Error bars = standard error

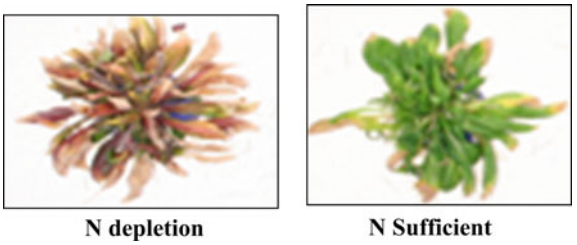


Fig. 8.13 Control plant rosettes under N sufficient at 10 weeks are green with little senescence. Under N depletion, control plants show increased anthocyanin accumulation and senescence

increased N and NO_3 content under both N sufficient and N depletion conditions in the 12S SSG transgenic lines did not translate to increased total biomass. However, seed yield increased in the 12S SSG transgenic lines under N depletion, most likely due to increased N remobilization from vegetative tissue to reproductive tissue.

The HYPO transgenic had an increase in total biomass under N sufficient conditions in 2 of 3 transgenic lines, primarily due to significant increases in inflorescence tissue (Fig. 8.12f). Interestingly, increased inflorescence did not translate to increased seed yield, and one transgenic line had decreased yield compared to control, while the remaining two transgenic lines were nonsignificant (Fig. 8.12g). Under N depletion conditions, all three transgenic lines of the HYPO transgenic had reduced seed yield compared to the control (Fig. 8.12g), while two of the three lines had increased rosette biomass (Fig. 8.12i). Since vegetative tissue had increased total N and NO_3 under N depletion and increased biomass, it suggests that the vegetative tissue growth was preferred over reproductive tissue growth under N depletion conditions in the HYPO transgenic lines. Thus, under N sufficient conditions, more biomass was accumulated in all tissues except seed, probably due to excess N storage in vegetative tissues. Under N depletion, excess N in vegetative tissue was utilized to promote vegetative growth, as opposed to being remobilized to the reproductive tissue (unlike 12S SSG), suggesting this gene to be inefficient at N remobilization compared to the control.

Discussion

Adaptation to limiting N conditions is an important survival strategy that plants use to successfully complete their life cycle under such sub-optimal environmental conditions. In developed countries, it is customary that farmers use large amounts of N fertilizers for most crops to help prevent fluctuating levels of N from impacting yield but, as a consequence, much of this N is wasted to the environment. By contrast, in developing countries N fertilizer is not readily available to many farmers. Therefore, in either case developing crops that have improved genetics for yielding well under limiting N conditions would be very advantageous (Kant et al. 2011). The goal of this study was to understand the response of plants to limiting N conditions in order to use this knowledge to improve NUE either by increasing yield under existing levels of N supply or by decreasing N application levels while maintaining current yield.

Considering the investment of plants in N acquisition, the remobilization of N during senescence is critical for efficient N usage and for plant survival. Senescence represents the final developmental act of the leaf, during which the leaf cell is dismantled in a coordinated manner to remobilize nutrients and to secure reproductive success (Thomas 2013). The onset of senescence is strictly regulated and occurs under optimal conditions in an age-dependent manner. However, upon exposure to environmental stress or nutrient deficiency, the plant can execute the senescence program as an adaptive response to promote survival and reproduction (Schippers et al. 2015). Arabidopsis exhibits two types of senescence: sequential and reproductive senescence. During sequential senescence, older leaves senesce and their nutrients are translocated to younger, growing parts of the plant. Reproductive senescence occurs at the whole-plant level and promotes seed

viability and quality (Noodén and Penney 2001). The current study showed that Arabidopsis plants exposed to increasing levels of N depletion had very limited growth, as judged by limited biomass accumulation, and underwent an apparent survival mode. As the severity of N depletion increased, plants not only fully assimilated all inorganic N stored in source tissues (Fig. 8.6), but also initiated their reproductive senescence earlier resulting in a more efficient redistribution of N and total biomass to roots and seeds (Figs. 8.3 and 8.5). Allocation of more N to the roots sustains root growth and supports the root system's sophisticated foraging strategy to find novel nutrient resources once those in the immediate vicinity become depleted (Guan et al. 2014; Higuchi et al. 2014). Upon exposure to N-depleting conditions, premature senescence of rosette leaves also promoted whole-plant survival through the allocation of a larger ratio of N and biomass distribution to the seeds (Figs. 8.3 and 8.5). The processes of senescence and remobilization provide the plant with phenotypic plasticity to help it adapt to adverse environmental conditions. Senescence and remobilization may not be the only strategy that plants use for survival under environmental stress conditions. For example, under salt stress the accumulation of Na^+ in older leaves might promote the survival of young tissues to ensure reproductive success. However, it remains to be demonstrated whether the remobilization of nutrients from salt-saturated leaves actually occurs (Schippers et al. 2015).

Development of genetic varieties with improved nitrogen use efficiency (NUE) is essential for sustainable agriculture. Understanding the mechanisms regulating

the processes of N accumulation, assimilation, and remobilization is crucial for the improvement of NUE in crop plants (Kant et al. 2011). The above results help us better understand the physiological basis of variation in NUE in plants and therefore may offer avenues to increase NUE in crop plants by genetically engineering plants with genes responsible for efficient N remobilization even when N is not limiting. To that extent, two of the genes (12S seed storage globulin precursor and a hypothetical protein) that were identified to have potential to improve plant tolerance to limited nitrogen were assessed for their N remobilization efficiency using the hydroponic method described here. Phenotypic and metabolic analysis of Arabidopsis plants transformed with these two genes showed that the gene encoding the 12S seed storage globulin precursor has improved accumulation, assimilation, and remobilization, leading to increased seed yield under N depletion conditions. Conversely, the hypothetical protein has improved accumulation in vegetative tissues, but reduced assimilation and remobilization, leading to reduced seed yield. These results demonstrate that screening for tolerance to limited N to discover genes and genotypes that can improve NUE in crop plants is not sufficient unless coupled with other methods that can further explore the potential of these genes and genotypes to fully utilized accumulated nitrogen in grain and seed filling through remobilization. This is especially critical as 40–90% of the nitrogen used in seed filling in various crops comes from remobilization.

Materials and Methods

Plant Transformation and Hydroponic Plant Growth

Plant transformation was performed by floral dipping method as described in Zhang et al. (2006). T_2 seeds were suspended in 0.1% agarose and stratified for three days at 4 °C for better germination. After stratification, seeds were sown on customized charcoal gray 25 mm × 9 mm foam "identi-plugs" (VWR, Batavia, Illinois) and saturated with deionized water. Plugs were placed in a 72 cell count plug flat (28 cm × 14 cm × 2.5 cm) and placed in a propagation dome with 4 L of germination nutrient solution (See "Nutrient Solution Preparation"). Light was prevented from contacting solution to prevent algal growth. Plants were grown in a walk-in growth room (TCR480, *Conviron*, Winnipeg, Manitoba, Canada) at standard conditions (22 °C day, 20 °C night, 65% relative humidity, 250 umol m^{-2} s^{-1} light intensity, and 16 h photoperiod) for three weeks. Nutrient solution volumes in the hydroponic trays were maintained at 4 L twice per day 0–5 days after sowing (DAS). To promote root elongation, solution volume was reduced to 3 L 5–13 DAS. At 11 DAS, seedling thinning was performed leaving one seedling per plug. Genes selected were identified as having altered nitrogen metabolism when grown on low nitrogen media (0.2 mM), consisting of Hoaglands recipe (Hoagland 1950) and agar media on petri plates (Cat # 4021, Nunc, Rochester, New York) for 21 DAS, in walk-in growth room at standard conditions. The two genes selected were 12S seed storage globulin in antisense configuration (At1g07750—"12S SSG") and a hypothetical protein in sense configuration (At3g49550—"HYPO"). HYPO had three transgenic events that were selected for experimentation, while 12S SSG had two transgenic events. A transgenic line containing an empty vector was used as the control.

Nitrogen Treatment

21 DAS plants at growth stage of 5.10 in BBCH scale, where inflorescence emergence is observed (Boyes et al. 2001), were transplanted to hydroponics trays containing vegetative nutrient solution (see "Nutrient Solution Preparation"). 20 seedlings from each transgenic line and 10 seedlings from the control line were transplanted. Each plug was placed in a small plug holder with dimensions 4.1 cm ID × 4.9 cm OD × 1.4 cm H (custom order, Caplugs, Buffalo, New York). During all stages of plant development, nutrient solution was monitored three times per week. Nutrient solution volume was adjusted with reverse osmosis (RO) water if water loss/uptake exceeded 10%. pH was maintained between 5.75 and 6.25. Electrical conductivity (EC) was monitored and controlled so not to exceed ±20%. Solution was replaced once per week. 35 DAS (14 days after transplanting [DAT], growth stage 6.0), T_0 timepoint tissue collection was performed on 40% of the population (8 plants per transgenic seed line). After T_0 tissue collection, 6 transgenic seedlings were subjected to nitrogen depletion conditions, while the

remaining 6 transgenic seedlings maintained nitrogen sufficient conditions. 70 DAS (49 DAT, 35 days after treatment, growth stage 9.7), a final harvest was performed for the remaining population from the nitrogen depletion and nitrogen sufficient treatments. For all plant harvests, dry biomass, total nitrogen, total carbon, and nitrate contents were collected on roots, rosettes, inflorescence, and seeds (when available).

Hydroponics System Description

Hydroponics system consists of one 100-L reservoir, which continuously pumped nutrient solution at a speed of 7 L per minute to each of three hydroponics trays. Dissolved oxygen concentration was enhanced using aquarium air-stones. Hydroponics tray dimensions were 121 cm × 61 cm × 53 cm. Nutrient solution in trays was kept at a depth of 15 mm and allowed to freely drain back to reservoir. Two hydroponics systems (2 reservoirs and 6 trays) were used. These systems were positioned across from one another to mitigate any environmental variability/location effect in the growth room. Each construct was tested in one tray in each hydroponics system, for a total of 2 trays per construct (Zayed et al. 2012).

Nutrient Solution Preparation for Hydroponics

Germination nutrient solution was prepared with the following recipe: 0.125x modified Coopers Nutrient Solution (Cooper 1975), containing macro elements 241uM KH_2PO_4, 721uM KNO_3, 260uM $MgSO_4$, 531uM $Ca(NO_3)_2$, as well as micro elements 3.5uM H_3BO_3, 0.2uM $CuSO_4$, 0.2uM $ZnSO_4$, 0.04uM $(NH_4)_6Mo_7O_{24}$, 29uM iron EDTA. 125uM MES was added as a pH buffer. Vegetative nutrient solution was prepared with the following recipe: 0.5x modified Noren Nutrient Solution (Norén et al. 2004), containing macro elements 4 mM NH4 NO_3, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 2.5 mM K_2SO_4, 1.5 mM $CaCl_2$, as well as micro elements 46uM H_3BO_3, 10uM $MnSO_4$, 0.8uM $ZnSO_4$, 0.3uM $CuSO_4$, 0.6uM MoO_3, and 20uM iron EDTA. 1 mM MES was added as a pH buffer. Solution for nitrogen depletion treatment was the same formulation, with exclusion of 4 mM NH_4NO_3.

Metabolic Analysis

Plant tissue samples were prepared for metabolic analysis by placing tissue in 50-mL Falcon tube and dried at 65 °C for 4 days to ensure complete desiccation. Dried samples were placed in a GenoGrinder 2000 (2000, Spec Sample Prep,

Metuchen, New Jersey) and ground to a fine powder with four, 7-mm zinc oxide beads at 1200 revolutions/min for 2 min. Total nitrogen and carbon were analyzed on ground tissue using a 2400 C/H/N analyzer (P/N, Perkin Elmer, City, State). Powder was placed in small tin capsules (P/N, Perkin Elmer, City, State) in 1.5–3.0 mg aliquots, then inserted in 2400 C/H/N analyzer for combustion. Total nitrate was analyzed on ground tissue using a commercially available nitrate reductase (YNaR 1U/96 well plate, Nitrate Elimination Co, Inc., Lake Linden, MI) and nitrate standard (LC17900-7, Lab Chem, City, State). Ground samples were suspended in 15 mM KOH and distributed to a 96 well plate (Cat# 3641, Costar, Costar, New York) in 10 ± 3 mg aliquots. 10 uL of suspended samples were filtered and transferred to clean 96 well plates. 90 uL of prepared nitrate reductase enzyme (YNaR 1U/96 well plate, Nitrate Elimination Co, Inc., Lake Linden, MI) and 50 uL each of color reagents, 1% sulfanilamide and 0.02% naphthylethylenediamine (S9251 and N9125, respectively, Sigma-Aldrich, St Louis, MO), were added to sample to produce color changes.

Reagents were prepared and added according to manufacturer's instructions (P/N, Nitrate Elimination Co, Inc, Lake Linden, MI). Commercially available NADH (N8129, Sigma-Aldrich, St Louis, MO) and color reagents were used to prepare reagents. Nitrate concentration was determined by a plate reader (Safire2-Basic, Tecan, Trading AG, Switzerland) at an absorbance reading of 540 nm.

References

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Görlach J (2001) Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. Plant Cell 13:1499–1510

Cameron KC, Di HJ, Moir JL (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162(2013):145–173

Cooper AJ (1975) Crop production in recirculating nutrient solution. Sci Hortic 3:251–258

Guan P, Wang R, Nacry P, Breton G, Kay SA, Pruneda-Paz JL, Davani A, Crawford NM (2014) Nitrate foraging by Arabidopsis roots is mediated by the transcription factor TCP20 through the systemic signaling pathway. Proc Natl Acad Sci USA 111:15267–15272

Higuchi K, Iwase J, Tsukiori Y, Nakura D, Kobayashi N, Ohashi H, Saito A, Miwa E (2014) Early senescence of the oldest leaves of Fe-deficient barley plants may contribute to phytosiderophore release from the roots. Physiol Plant 151:313–322

Hirel B, Le Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot 58(9):2369–2387

Hoagland DR (1950) The water-culture method for growing plants without soil. University of California, College of Agriculture, Agricultural Experiment Station, Berkeley, Calif, p 39:ill. (Circular; 347)

Hou X, Tong H, Selby J, Dewitt J, Peng X, He ZH (2005) Involvement of a cell wall-associated kinase, WALK4, in Arabidopsis mineral responses. Plant Physiol 139:1704–1716

Kant S, Bi YM, Rothstein SJ (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J Exp Bot 62(4):1499–1509

Lancashire PD, Bleiholder H, Boom TVD, Langelu¨ddeke P, Stauss R, Weber E, Witzenberger A (1991) A uniform decimal code for growth stages of crops and weeds. Ann Appl Biol 119: 561–601

Lemaitre T, Gaufichon L, Boutet-Mercey S, Christ A, Masclaux-Daubresse C (2008) Enzymatic and metabolic diagnostic of nitrogen deficiency in Arabidopsis thaliana Wassileskija accession. Plant Cell Physiol 49(7):1056–1065

Masclaux-Daubresse C, Reisdorf-Cren M, Orsel M (2008). Leaf nitrogen remobilisation for plant development and grain filling. Plant Biol (Stuttg) 10 Suppl 1:23–36

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot 105: 1141–1157

Noodén LD, Penney JP (2001) Correlative controls of senescence and plant death in *Arabidopsis thaliana* (Brassicaceae). J Exp Bot 52:2151–2159

Norén H, Svensson P, Andersson B (2004) A convenient and versatile hydroponic cultivation system for Arabidopsis thaliana. Physiol Plant 121:343–348

Richard-Molard C, Krapp A, Brun F, Ney B, Daniel-Vedele F, Chaillou S (2008) Plant response to nitrate starvation is determined by N storage capacity matched by nitrate uptake capacity in two Arabidopsis genotypes. J Exp Bot 59(4):779–791

Sawyer JE (2015) Nitrogen use in Iowa corn production. Iowa State University Extension and Outreach, Ames, Iowa

Schippers JHM, Schmidt R, Wagstaff C, and Jing HC (2015). Living to die and dying to live: the survival strategy behind leaf senescence. Plant Physiol 169(2): 914–930

Thomas H (2013) Senescence, ageing and death of the whole plant. New Phytol 197:696–711

Zayed A, Jones L, Tierney C, Miller M (2012) Hydroponics apparatus and methods of use. United States Patent Application # 20120277117. November 1, 2012

Zhang X, Henriques R, Lin SS, Niu QW, Chua NH (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc 1(2):641–646

Chapter 9
Field Testing for Improved Nitrogen Use Efficiency of Corn—From Whole-Plant Physiology to Agroecosystem Scales

Kevin R. Kosola

Introduction

Nitrogen is the mineral element most often limiting to yield of cereal crops; N fertilizer is also typically the largest variable cost for production of corn (Poffenbarger et al. 2017). Improving nitrogen use efficiency has the potential to improve agricultural sustainability by allowing growers to produce more grain with less nitrogen, while reducing losses of nitrogen to the environment. Best practices for nutrient management are based on the 4 R's (right form, right place, right timing, and right rate), which are focused around making sure nutrient availability matches with plant demand (Flis 2017). There is a potential fifth R for corn—the Right hybrids for the system—plants that can efficiently capture and convert N into grain and have nitrogen use-related traits that fit with the management system. Improvements in both nutrient management and plant nutrient use have and will continue to play a role in allowing farmers to continue to improve the sustainability of corn agronomic systems.

Corn production systems that provide more grain per unit fertilizer applied will have either (1) improved plant capture of the N applied, (2) improved utilization of N captured by the plant, or (3) a combination of both. There is an extensive literature focused on physiological factors influencing nitrogen use efficiency, with recent studies on modern hybrids (e.g., Chen and Vyn 2017; Ciampitti and Vyn 2011; Ning et al. 2017). There is also an extensive body of literature focused on agroecosystem level analysis of fertilizer use and recovery (e.g., Cassman et al. 2002). The two approaches are complementary, as the physiology of nitrogen acquisition and utilization depend in part upon soil nitrogen availability during development. This review provides a comparison and contrast of the different

K. R. Kosola (✉)
Monsanto Company, 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA
e-mail: krkoso@monsanto.com

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_9

definitions of corn nitrogen use efficiency in the literature, how they have been measured, and their use in characterizing corn nitrogen fertilizer use and management at scales ranging from the plant to the agroecosystem.

Corn cropping systems globally can vary widely in the typical quantity of fertilizer nitrogen applied, and how much is used by the crop (Morris et al. 2018; Vitousek et al. 2009). From both an economic perspective and an environmental perspective, the critical parameters to characterize crop fertilizer nitrogen use on the farm scale are recovery efficiency of fertilizer nitrogen (RE_N, the percent of fertilizer N captured by the crop) and agronomic efficiency of fertilizer nitrogen (AE_N, the incremental yield increase per unit N applied) (Cassman et al. 1996; Novoa and Loomis 1981). Along with information on the cost of fertilizer N, the price received for grain produced, and the yield response to N curve used to calculated AEN, it is possible to calculate an economically optimal rate of N for a system (Hong et al. 2007; Kyveryga et al. 2011; Morris et al. 2018; Raun and Schepers 2008). The economically optimal rate of nitrogen is rarely equivalent to the rate of nitrogen that gives maximal yield in a location. Because farmers cannot fully predict RE_N, AE_N, or the price received for grain for any given year and location, continuing to develop cropping systems that include N-efficient crops and flexible N management regimes that retain fertilizer N is an ongoing goal of agronomists, breeders, and farmers.

NUE Parameters in Crop Physiology

N Use to Produce Grain

Crop physiology studies of the components of corn nitrogen use include characterization of nitrogen acquisition (typically limited to aboveground biomass N) and utilization of captured nitrogen (Novoa and Loomis 1981). One simple analysis possible at physiological maturity is to measure total plant N and grain weight, allowing calculation of the conversion coefficient for use of plant N to produce grain. This metric has been termed "nitrogen utilization efficiency" (Huggins and Pan 1993) or "nitrogen internal efficiency" (Ciampitti et al. 2012) (Table 9.1). Mueller and Vyn (2016) cite Gastal and Lemaire (2002) as further breaking down N internal efficiency (NIE) into N conversion efficiency (NCE = total biomass production per unit N) and harvest index (grain per unit biomass). Where harvest index is relatively invariant in a system, which is typical for systems with adequate nutrition, N conversion efficiency (NCE) is equivalent to nitrogen internal efficiency (NIE).

Boomsma et al. (2009) defined nitrogen use efficiency as grain yield per unit N applied, or agronomic efficiency (Ladha et al. 2005, Table 9.1) in their experiments on the effects of density and N rate on corn traits. Ciampitti et al. (2012) use a definition of nitrogen use efficiency that is equivalent to agronomic efficiency and

Table 9.1 Traits measured to characterize nitrogen acquisition and use by corn

Trait	Definition	Citations
Nitrogen use efficiency	Yield increase over yield at zero N/N applied	Haegele and Below (2013)
Agronomic efficiency (AE_N)	Incremental yield increase per unit N applied	Cassman et al (1996), Novoa and Loomis (1981)
Nitrogen use efficiency (Lemaire)	Incremental yield increase per unit N applied = N uptake efficiency x N conversion efficiency x harvest index	Sadras and Lemaire (2014)
Nitrogen utilization efficiency (Huggins and Pan 1993) Nitrogen internal efficiency (Ciampitti et al. 2012)	Grain per unit N in plant	Ciampitti et al. (2012), Huggins and Pan (1993)
Nitrogen recovery efficiency	N taken up/Fertilizer N applied	Ciampitti et al. (2012)
Fertilizer use efficiency; N fertilizer recovery efficiency (RE_N)	Fertilizer N taken up/Fertilizer N applied	Cassman et al. (2002), Ciampitti et al. (2012)
N uptake efficiency	Total plant N at maturity/total available N	Huggins and Pan (1993), Moll et al. (1982)
N remobilization	(Stover N R6 – Total plant N R1)/(Total Plant N R1)	Gallais et al. (2007)
N supply	Fertilizer N applied + Available soil N	Huggins and Pan (1993)
N Available	R6 total plant N (zero N applied) + postharvest soil NO_3	Huggins and Pan (1993)
Post-silking N uptake	Total plant N R6 – Total plant N R1	Gallais et al. (2007)
Post-silking N uptake	Total plant N R6 – Total plant N R1	Gallais et al. (2007)
NLAI—N per unit LAI	Total shoot N/Leaf area index	Ciampitti et al. (2013a)
NNI—nitrogen nutrition index—vegetative stage only	$\%N_{measured}/\%N_{\underline{critical}}$	Sadras and Lemaire (2014)

The first column lists the name of the trait; the second column shows the definition of the trait, including calculations required; the third column provides a citation where the trait has been used as defined

further parse this into the product of NIE and "Nitrogen recovery efficiency", defined as R6 N content/total N applied (Table 9.1). In both cases, the agronomic efficiency was calculated using the grain yield relative to the zero-N plots.

Further refinement of sources of N used for grain filling requires additional measurements. Nitrogen uptake does not typically supply sufficient nitrogen to meet the full needs of grain protein formation (Ciampitti and Vyn 2013); nitrogen remobilization from pools of nitrogen acquired during vegetative growth is

common in grain crops of all types. The N budget method is common, due to its relevance and simplicity. Biomass N is measured at R1, before grain filling starts; the nitrogen remobilization ratio is determined by measuring total stover N at R6 and measuring proportion of R1 N lost from vegetative tissue during grain filling (Chen and Vyn 2017; Gallais et al. 2007; Table 9.1). Further partitioning sources of nitrogen has included budget-method analysis of N supply from additional tissues—stalk, leaf (sometimes divided among individual leaves or leaves above and below the ear), cob, and root (Ciampitti and Vyn 2011).

An alternative to N budget methods is the use of ^{15}N as a tracer to characterize internal plant N pools and fluxes. This was first described for corn by Crawford et al. (1982), who used ^{15}N labeling in a sand culture system to track N dynamics within the plant, finding that stalk and leaf N pools were the primary N sources for grain. Ma et al. (1998) used stalk infusion of field-grown corn to label vegetative plant N pools with a known quantity of ^{15}N tracer before grain filling and then measured N pools and ^{15}N enrichment at R6. Gallais et al. (2007) used a similar method; soil N was ^{15}N enriched at V6 to uniformly label vegetative N pools, and then ^{15}N enrichment was measured in stover and grain at R6 to calculate flux of stored vegetative N to grain. This has the advantage of requiring only one destructive sampling, at R6. Silva et al. (2017) have elaborated this method to provide ^{15}N soil labeling at multiple stages between V14 and R5, tracking the fate of N uptake during development.

Measuring N Uptake

Measuring crop capture of fertilizer N is also a key metric for nitrogen use efficiency. Differentiation of fertilizer N fraction is not essential for studies where the primary purpose is understanding variation in plant dynamics of N uptake and germplasm or transgenic variation in N acquisition. ERA studies (Chen and Vyn 2017) and characterization of heritability of NUE (Coque et al. 2008; Hirel et al. 2003) have identified post-silking N uptake as a key parameter, with more N-efficient inbreds (Coque et al. 2008) and more recent hybrids (Chen and Vyn 2017; Ciampitti and Vyn 2012, 2013, 2014) having increased post-silking N uptake. These experiments rely primarily on N budget methods based on destructive harvests, with post-silking N uptake determined as the difference between total N at R6 and total N at R1.

Systems Level Characterization—Tracking Fertilizer N Capture

Fertilizer use efficiency can be defined as crop capture of applied fertilizer; in on-farm data from North American corn production, RE_N was 37% (Cassman et al. 2002). Fertilizer use efficiency in the agroecosystem incorporates both individual

plant-level factors that affect fertilizer uptake (discussed above) with agroecosystem factors that affect nitrogen cycling and net crop availability of fertilizer nitrogen. The timing and form of nitrogen application and the potential effect of cover crops on nutrient retention during the off-season for annual crops interact with soil interactions with climate to determine crop availability of fertilizer nitrogen and available nitrogen inputs from legumes, mineralization of organic matter, and atmospheric N deposition (Blesh and Drinkwater 2013; Huggins and Pan 1993).

Characterization of efficiency of fertilizer N capture (RE_N) is essential for a full understanding of the economic and environmental components of N fertilizer use in agroecosystems. Quantitative analysis of RE_N has been carried out with ^{15}N-depleted fertilizer as a tracer (Broadbent 1980; Broadbent and Carlton 1980) and by the difference method (Cassman et al. 2002; Fixen et al. 2015; Huggins and Pan 1993). Both have inherent limitations in accuracy of values captured for indigenous N mineralization (Cassman et al. 2002). Indigenous plant-available soil N is measured by sampling plants from a zero-N applied plot and measuring biomass and N concentration, along with soil samples at harvest (Huggins and Pan 1993). N contained in the plants from the zero-N plots reflects plant-available pre-season residual N (which can be measured by pre-season soil sampling), N mineralization, and N deposition (from either irrigation or precipitation), with the caveat that any stimulation of N mineralization by fertilization is not included in the zero-N plot estimate (Huggins and Pan 1993). These soil samples should ideally be collected to span the soil profile contained in the plant root zone.

Logistical and cost concerns limit the ability to carry out destructive sampling at the scale necessary to characterize RE_N and/or detailed NUE components across regions or on a wide scale. If a zero-N check plot is available, the agronomic efficiency of N use, AE_N (grain produced per unit fertilizer N supplied), can be calculated without destructive sampling. Relative comparisons of AE_N are highly informative and useful for comparing across hybrids or other plant material, particularly where the intent is to provide information for region where the test cropping system is in common use and soil characteristics are similar across the region of interest (Bender et al. 2013a, b; Haegele and Below 2013; Huggins and Pan 1993).

Environmental Effects on NUE

N availability affects source–sink relations in the developing plant, with affects on leaf production, leaf area (Muchow 1988a, b; Muchow and Davis 1988; Muchow and Sinclair 1995; Sinclair and Muchow 1995), and radiation use efficiency (Muchow and Davis 1988), as well as strong effects on kernel number (Andrade et al. 2002; Uhart and Andrade 1995a, b). Nitrogen availability has strong effects on grain-filling duration due to effects on leaf senescence and leaf area duration (Ciampitti et al. 2013a; Roth et al. 2013; Thomas and Ougham 2014). Traits such as NIE and N remobilization that are a function of both grain and stover biomass and

N content are not a simple linear function of N availability. Stalk and leaf nitrogen are a primary source of remobilized N when post-silking N uptake cannot supply sufficient N to meet grain demands. Under increasing N supply, stover N continues to increase past the range of maximum yield; N remobilization can decrease to near zero, and NIE will also decrease as stalk N pools increase.

NIE and N remobilization have both been observed to decrease as soil N availability increases (Hirel et al. 2011; Masclaux et al. 2001; Pommel et al. 2006). The decrease of NIE under high N conditions is due to the saturable nature of grain yield response to available N and the capability of corn leaves and especially stalks to accumulate luxury amounts of N. When N fertilization exceeds crop N requirements, corn stalks will accumulate nitrate (Brouder et al. 2000; Isla and Blackmer 2007). The decrease in N remobilization is associated with increased post-silking N uptake and improved stay green, both factors changing source–sink carbon and N dynamics during the grain-filling period. The increased duration of photosynthesis is hypothesized to enable improved C supply to the root system, with consequent increased duration of N uptake and a decreased demand on stalk and leaf pools of N to supply grain N (Chen and Vyn 2017; Pan et al. 1986; Moll et al. 1982). Under very high N, it becomes difficult to detect differences in N remobilization; post-silking N uptake can meet most N demands during grain filling.

Increased N remobilization with decreased N supply represents a shift between post-silking N uptake and use of vegetative N pools to meet the N demand of grain filling. Under very low N, kernel number can be strongly reduced by N limitations, due to both decreased ovule number and increased kernel abortion (Uhart and Andrade 1995b). Under these extreme conditions, stalk N remobilization and NIE can decrease to near zero.

An example of a trait that changes nitrogen capture is the change in NUE due to the introduction of rootworm resistance. (Bender et al. 2013c; Haegele and Below 2013) have shown that yield response curves to nitrogen were substantially different between hybrids differing on only in the presence or absence of root expression of BT, leading to differences in corn rootworm pressure on the corn root system. Corn rootworm larvae eat newly emerged crown and brace roots, leading to a substantial decline in root biomass and functional root length for nutrient capture. Rootworm pressure had a strong effect on post-silking N uptake, and presumably also on RE_N. Rootworm protection effects on remobilization were not reported. This is consistent with the suggestion by Dobermann and Cassman (2002) that the lower RE_N average across farms compared to small-plot research results may be affected by pests, pathogens, and other external factors reducing yield potential.

Environmental Variation in N Availability

Agroecosystem N cycling characterization and crop physiology intersect in the study of environmental variation in crop response to fertilization, which is necessary to generalize results from experiment-station level experiments to results of

widely planted experiments, and from experiments to on-farm results. N availability in the field is a function of season-long-integrated N gains from fertilization, mineralization and N deposition from irrigation and atmospheric sources, and N losses from leaching, denitrification, microbial immobilization, and any weed competition (Huggins and Pan 1993). Because these are dynamic, time-integrated processes, single time-point soil samples provide only a snapshot of nitrogen pools in the soil, not an indication of fluxes through these pools. Soil biogeochemistry methods are available to monitor both pools and fluxes of N cycling (e.g., isotope pool dilution and buried bag incubations for N mineralization (Burger and Jackson 2003). Use of these methods in analysis of crop response to nutrient availability is a potential area of opportunity (Ruzicka et al. 2012))

In-season nitrogen application is referred to as split-rate N application, where crop nitrogen requirements are met by applying fertilizer across multiple times, potentially including preplanting and in-season application timing to more closely match the timing of crop N demand (Scharf et al. 2002). The bulk of the literature on split-rate N applications is focused on side-dress in the standard V6 to V10 window, due both to equipment factors and to the requirement for sufficient N before exponential growth phase (Scharf et al. 2002). Because weather has a strong effect on N cycling, field studies with in-season N applications have variable results (Xie et al. 2013). In-season applications as late as R1 have been found to result in increased yield (Nelson et al. 2011; Russelle et al. 1983). NUE improvements have been documented for corn with in-season N application (Ciampitti and Vyn 2011; Kovacs et al. 2015). Although under dry conditions, side-dress applications have been shown to decrease yield (Kovacs et al. 2015).

Characterization of the timing of crop N demand is not necessarily captured in standard methods to determine RE_N. To detect any changes among hybrids or transgenic plants in the timing of N demand, either repeated harvests (Bender et al. 2013b; Ciampitti et al. 2013a, b) or an indirect method or tracking N acquisition over time is needed. Modeling of critical N concentrations for vegetative growth (Gastal and Lemaire 2002; Lemaire et al. 2008; Sadras and Lemaire 2014; Ziadi et al. 2009) provides characterization of N and biomass dynamics during vegetative growth, but does not directly account for grain N requirements. The extensive body of work on remote sensing tools for adaptive N management provides a range of methods to derive a quantitative estimate of N dynamics (Franzen et al. 2016), at least during vegetative growth.

Spatial variation in N mineralization and other N cycling processes are always a factor influencing crop yield and will affect all of the methods available to characterize crop N use, e.g., (Wendroth et al. 2011; Zhu et al. 2009). Wendroth et al. (2011) have developed a unique design for field N fertilizer response studies. By applying variable rate N in a sine-wave pattern along the row, they could use Fourier transform analysis of yield to deconvolute spatial variation due inherent N availability from the spatial pattern of the N application.

There is a gap in our current ability to scale up detailed crop physiology measurements of nitrogen use efficiency components to whole field-level analyses. This gap would not be a concern if small-plot research data on RE_N matched up with

farm-scale data. Cassman et al. (2002) and Dobermann and Cassman (2002) indicated that RE_N estimates from experimental plots overestimate RE_N achieved by farmers for irrigated and rain-fed corn in the North-Central USA. They speculate that this is due to differences in the scale of farming operations and differences in N management. Differences in N management are expected to have an effect on RE_N; the "4 Rs" of N management recommendations (right fertilizer source at the right rate, with the right timing, in the right place) are founded on this understanding that economically optimal N fertilizer practices will lead to improved RE_N. Factors that decrease yield potential (weeds, pathogens, pests, heat stress, drought, etc.) are the most likely factors that lead to this difference.

Summary and Future Possibilities

Variation in nitrogen availability has consistently been observed to alter components of nitrogen use (Hirel et al. 2011; Masclaux et al. 2001; Pommel et al. 2006). High nitrogen availability within the range found on high-fertility sites with agronomically realistic fertilizer rates can reduce nitrogen remobilization near zero, reducing the ability to compare hybrid variation in this trait. Nitrogen internal efficiency (NIE) is reduced by high N, and post-silking N is increased by high N. Using a zero-N applied check to obtain an estimate of total plant N capture of intrinsically available soil N, when combined with measurements of soil N at planting and harvest as described by Huggins and Pan (1993), allows for comparison of results across experiments on a plant-available-N basis and provides the ability to correct for variation in soil N mineralization and other factors contributing to intrinsic N availability. Variation in timing of N availability could be quantified with this method using multiple harvests. As for any field experiment, spatial variation of intrinsic N availability between zero-N check plots and other experimental plots must be controlled for by proper blocking. Huggins and Pan (1993) point out that effects of fertilization on N mineralization are also absent in this estimate. When feasible, testing at a range of nitrogen rates mitigates the risk of high variation in N availability across locations or years and allows characterization of the response curve or reaction norm. The common practice of collection of metadata on other factors that can affect plant growth and yield is also valuable, particularly when experiments span a wide range of growing conditions. Infestations of corn rootworm (Bender et al. 2013c) or other root herbivores or pathogens can affect nutrient and water uptake (Stevens and Jones 2006), and stresses that affect kernel set will also affect nitrogen use efficiency components.

In this review, I have covered methods used in studies of nitrogen use efficiency from whole-plant to agroecosystem levels. While the focus of authors naturally depends upon their research objectives, there is an unfortunate proliferation of terms for the same components of nitrogen use efficiency. Use a common set of terminology based on prior literature, rather than generating new names for existing metrics. There are opportunities for future research and scholarship in linking

aspects of crop nitrogen use across different scales of organization. Molecular and physiological studies on traits influencing nitrogen acquisition and utilization should be linked with whole-plant and crop physiology field studies of N use and partitioning. These combined information sets will have great value in aiding the development of nutrient-efficient crops. Ideally, new plant varieties with the potential for improved crop nutrient use efficiency will be tested for their performance in agronomic ecosystems using the methods developed by agronomists, biogeochemists, and agroecologists.

References

Andrade FH, Echarte L, Rizzalli R, Della Maggiora A, Casanovas M (2002) Kernel number prediction in maize under nitrogen or water stress. Crop Sci 42:1173–1179

Bender RR, Haegele JW, Ruffo ML, Below FE (2013a) Modern corn hybrids' nutrient uptake patterns. Better Crops Plant Food 97:7–10

Bender RR, Haegele JW, Ruffo ML, Below FE (2013b) Nutrient uptake, partitioning, and remobilization in modern, transgenic insect-protected maize hybrids. Agron J 105:161–170

Bender RR, Haegele JW, Ruffo ML, Below FE (2013c) Transgenic corn rootworm protection enhances uptake and post-flowering mineral nutrient accumulation. Agron J 105:1626–1634

Blesh J, Drinkwater LE (2013) The impact of nitrogen source and crop rotation on nitrogen mass balances in the Mississippi River Basin. Ecol Appl 23:1017–1035

Boomsma CR, Santini JB, Tollenaar M, Vyn TJ (2009) Maize morphophysiological responses to intense crowding and low nitrogen availability: an analysis and review. Agron J 101:1426-1452

Broadbent FE (1980) Residual effects of labeled N in field trials. Agron J 72:325–329

Broadbent FE, Carlton AB (1980) Methodology for field trials with nitrogen-15 depleted nitrogen. J Environ Qual 9:236–242

Brouder SM, Mengel DB, Hofmann BS (2000) Diagnostic efficiency of the blacklayer stalk nitrate and grain nitrogen tests for corn. Agron J 92:1236–1247

Burger M, Jackson LE (2003) Microbial immobilization of ammonium and nitrate in relation to ammonification and nitrification rates in organic and conventional cropping systems. Soil Biol Biochem 35:29–36

Cassman KG, DeDatta SK, Amarante ST, Liboon SP, Samson MI, Dizon MA (1996) Long-term comparison of the agronomic efficiency and residual benefits of organic and inorganic nitrogen sources for tropical lowland rice. Exp Agric 32:427–444

Cassman KG, Dobermann A, Walters DT (2002) Agroecosystems, nitrogen-use efficiency, and nitrogen management. Ambio 31:132–140

Chen K, Vyn TJ (2017) Post-silking factor consequences for N efficiency changes over 38 years of commercial maize hybrids. Front Plant Sci 8

Ciampitti IA, Murrell ST, Camberato JJ, Tuinstra M, Xia Y, Friedemann P, Vyn TJ (2013a) Physiological dynamics of maize nitrogen uptake and partitioning in response to plant density and n stress factors: I. Veg Phase Crop Sci 53:2105–2119

Ciampitti IA, Murrell ST, Camberato JJ, Tuinstra M, Xia YB, Friedemann P, Vyn TJ (2013b) Physiological dynamics of maize nitrogen uptake and partitioning in response to plant density and nitrogen stress factors: II. Reprod Phase Crop Sci 53:2588–2602

Ciampitti IA, Vyn TJ (2011) A comprehensive study of plant density consequences on nitrogen uptake dynamics of maize plants from vegetative to reproductive stages. Field Crops Res 121:2–18

Ciampitti IA, Vyn TJ (2012) Physiological perspectives of changes over time in maize yield dependency on nitrogen uptake and associated nitrogen efficiencies: a review. Field Crops Res 133:48–67

Ciampitti IA, Vyn TJ (2013) Grain nitrogen source changes over time in maize: a review. Crop Sci 53:366–377

Ciampitti IA, Vyn TJ (2014) Nitrogen use efficiency for old versus modern corn hybrids. Better Crops Plant Food 98:19–21

Ciampitti IA, Zhang H, Friedemann P, Vyn TJ (2012) Potential physiological frameworks for mid-season field phenotyping of final plant nitrogen uptake, nitrogen use efficiency, and grain yield in maize. Crop Sci 52:2728–2742

Coque M, Martin A, Veyrieras JB, Hirel B, Gallais A (2008) Genetic variation for N-remobilization and postsilking N-uptake in a set of maize recombinant inbred lines. 3. QTL detection and coincidences. Theor Appl Genet 117:729–747

Crawford TW Jr, Rendig VV, Broadbent FE (1982) Sources, fluxes, and sinks of nitrogen during early reproductive growth of maize (Zea mays L.). Plant Physiol 70:1654–1660

Dobermann A, Cassman KG (2002) Plant nutrient management for enhanced productivity in intensive grain production systems of the United States and Asia. Plant Soil 247:153–175

Fixen P, Brentrup F, Bruulsema TW, Garcia F, Norton R, Zingore S (2015) Nutrient/fertilizer use efficiency: measurement, current situation and trends. In: Dreschel P, Dreschel P, Heffer P, Magen H, Mikkelsen R, Wichelns D (eds) Managing water and fertilizer for sustainable agricultural intensification. IWMI, IPNI, IPI, Paris, IFA, pp 8–38

Flis S (2017) The 4Rs in crop nitrogen research. Crops Soils 50:18–20

Franzen D, Kitchen N, Holland K, Schepers J, Raun W (2016) Algorithms for in-season nutrient management in cereals. Agron J 108:1775–1781

Gallais A, Coque M, Le Gouis J, Prioul JL, Hirel B, Quillere I (2007) Estimating the proportion of nitrogen remobilization and of postsilking nitrogen uptake allocated to maize kernels by nitrogen-15 labeling. Crop Sci 47:685–693

Gastal F, Lemaire G (2002) N uptake and distribution in crops: an agronomical and ecophysiological perspective. J Exp Bot 53:789–799

Haegele JW, Below FE (2013) Transgenic corn rootworm protection increases grain yield and nitrogen use of maize. Crop Sci 53:585–594

Hirel B, Harrison J, Limami A (2003) Improvement of nitrogen utilization. Improv Strategies Legum Biotechnol 10A:201–220

Hirel B, Tetu T, Lea PJ, Dubois F (2011) Improving nitrogen use efficiency in crops for sustainable agriculture. Sustainability 3:1452–1485

Hong N, Scharf PC, Davis JG, Kitchen NR, Sudduth KA (2007) Economically optimal nitrogen rate reduces soil residual nitrate. J Environ Qual 36:354–362

Huggins DR, Pan WL (1993) Nitrogen efficiency component analysis—an evaluation of cropping system differences in productivity. Agron J 85:898–905

Isla R, Blackmer AM (2007) A simplified test of cornstalk nitrate for better N management. Agron Sustain Dev 27:237–241

Kovacs P, Van Scoyoc GE, Doerge TA, Camberato JJ, Vyn TJ (2015) Anhydrous ammonia timing and rate effects on maize nitrogen use efficiencies. Agron J 107:1205–1214

Kyveryga PM, Blackmer TM, Caragea PC (2011) Categorical analysis of spatial variability in economic yield response of corn to nitrogen fertilization. Agron J 103:796–804

Ladha JK, Pathak H, Krupnik TJ, Six J, van Kessel C (2005) Efficiency of fertilizer nitrogen in cereal production: retrospects and prospects. In: Sparks DL (ed) Advances in agronomy, vol 87, pp 85–156

Lemaire G, van Oosterom E, Jeuffroy M-H, Gastal F, Massignam A (2008) Crop species present different qualitative types of response to N deficiency during their vegetative growth. Field Crops Res 105:253–265

Ma BL, Dwyer LM, Tollenaar M, Smith DL (1998) Stem infusion of nitrogen-15 to quantify nitrogen remobilization in maize. Commun Soil Sci Plant Anal 29:305–317

Masclaux C, Quillere I, Gallais A, Hirel B (2001) The challenge of remobilisation in plant nitrogen economy. a survey of physio-agronomic and molecular approaches. Ann Appl Biol 138:69–81

Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen-utilization. Agron J 74:562–564

Morris TF, Murrell TS, Beegle DB, Camberato JJ, Ferguson RB, Grove J, Ketterings Q, Kyveryga PM, Laboski CAM, McGrath JM, Meisinger JJ, Melkonian J, Moebius-Clune BN, Nafziger ED, Osmond D, Sawyer JE, Scharf PC, Smith W, Spargo JT, van Es HM, Yang H (2018) Strengths and limitations of nitrogen rate recommendations for corn and opportunities for improvement. Agron J 110:1–37

Muchow RC (1988a) Effect of nitrogen supply on the comparative productivity of maize and sorghum in a semi-arid tropical environment I. Leaf growth and leaf nitrogen. Field Crops Res 18:1–16

Muchow RC (1988b) Effect of nitrogen supply on the comparative productivity of maize and sorghum in a semi-arid tropical environment. III. Grain yield and nitrogen accumulation. Field Crops Res 18:31–43

Muchow RC, Davis R (1988) Effect of nitrogen supply on the comparative productivity of maize and sorghum in a semi-arid tropical environment II. Radiation interception and biomass accumulation. Field Crops Res 18:17–30

Muchow RC, Sinclair TR (1995) Effect of nitrogen supply on maize yield. II. Field model analysis. Agron J 87:642–648

Mueller SM, Vyn TJ (2016) Maize plant resilience to N stress and post-silking N capacity changes over time: a review. Front Plant Sci 7

Nelson KA, Scharf PC, Stevens WE, Burdick BA (2011) Rescue nitrogen applications for corn. Soil Sci Soc Am J 75:143–151

Ning P, Fritschi FB, Li C (2017) Temporal dynamics of post-silking nitrogen fluxes and their effects on grain yield in maize under low to high nitrogen inputs. Field Crops Res 204:249–259

Novoa R, Loomis RS (1981) Nitrogen and plant production. Plant Soil 58:177–204

Pan WL, Camberato JJ, Jackson WA, Moll RH (1986) Utilization of previously accumulated and concurrently absorbed nitrogen during reproductive growth in maize zea-mays influence of prolificacy and nitrogen source. Plant Physiol (Rockville) 82:247–253

Poffenbarger H, Artz G, Dahlke G, Edwards W, Hanna M, Russell J, Sellers H, Liebman M (2017) An economic analysis of integrated crop-livestock systems in Iowa, U.S.A. Agric Syst 157:51–69

Pommel B, Gallais A, Coque M, Quillere I, Hirel B, Prioul JL, Andrieu B, Floriot M (2006) Carbon and nitrogen allocation and grain filling in three maize hybrids differing in leaf senescence. Eur J Agron 24:203–211

Raun WR, Schepers JS (2008) Nitrogen management for improved use efficiency. In: Schepers JS, Raun WR (eds) Nitrogen in agricultural systems. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp 675–693

Roth JA, Ciampitti IA, Vyn TJ (2013) Physiological evaluations of recent drought-tolerant maize hybrids at varying stress levels. Agron J 105:1129–1141

Russelle MP, Hauck RD, Olson RA (1983) Nitrogen accumulation rates of irrigated maize. Agron J 75:593–598

Ruzicka DR, Hausmann NT, Barrios-Masias FH, Jackson LE, Schachtman DP (2012) Transcriptomic and metabolic responses of mycorrhizal roots to nitrogen patches under field conditions. Plant Soil 350:145–162

Sadras VO, Lemaire G (2014) Quantifying crop nitrogen status for comparisons of agronomic practices and genotypes. Field Crops Res 164:54–64

Scharf PC, Wiebold WJ, Lory JA (2002) Corn yield response to nitrogen fertilizer timing and deficiency level. Agron J 94:435–441

Silva ADO, Camberato JJ, Coram T, Filley T, Vyn TJ (2017) Applicability of a "multi-stage pulse labeling" N-15 approach to phenotype N dynamics in maize plant components during the growing season. Front Plant Sci 8

Sinclair TR, Muchow RC (1995) Effect of nitrogen supply on maize yield I. Modeling physiological responses. Agron J 87:632–641

Stevens GN, Jones RH (2006) Influence of root herbivory on plant communities in heterogeneous nutrient environments. New Phytol 171:127–136

Thomas H, Ougham H (2014) The stay-green trait. J Exp Bot 65:3889–3900

Uhart SA, Andrade FH (1995a) Nitrogen deficiency in maize.1. Effects on crop growth, development, dry-matter partitioning, and kernel set. Crop Sci 35:1376–1383

Uhart SA, Andrade FH (1995b) Nitrogen deficiency in maize.2. Carbon-nitrogen interaction effects on kernel number and grain-yield. Crop Sci 35:1384–1389

Vitousek PM, Naylor R, Crews T, David MB, Drinkwater LE, Holland E, Johnes PJ, Katzenberger J, Martinelli LA, Matson PA, Nziguheba G, Ojima D, Palm CA, Robertson GP, Sanchez PA, Townsend AR, Zhang FS (2009) Nutrient imbalances in agricultural development. Science 324:1519–1520

Wendroth O, Kersebaum KC, Schwab G, Murdock L (2011) Spatial relationships of soil properties, crop indices, and nitrogen application pattern with wheat growth and yield in a field. In: Ahuja LR, Ma L (eds) Methods of introducing system models into agricultural research. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp 229–259

Xie M, Tremblay N, Tremblay G, Bourgeois G, Bouroubi MY, Wei Z (2013) Weather effects on corn response to in-season nitrogen rates. Can J Plant Sci 93:407–417

Zhu Q, Schmidt JP, Lin HS, Sripada RP (2009) Hydropedological processes and their implications for nitrogen availability to corn. Geoderma 154:111–122

Ziadi N, Belanger G, Gastal F, Claessens A, Lemaire G, Tremblay N (2009) Leaf nitrogen concentration as an indicator of corn nitrogen status. Agron J 101:947–957

Chapter 10
Legume Nitrogen Utilization Under Drought Stress

V. Castañeda, E. Gil-Quintana, A. Echeverria and EM. González

Summary

Legumes account for around 27% of the world's primary crop production and can be classified based on their use and traits into grain and forage legumes. Legumes can establish symbiosis with N-fixing soil bacteria. As a result, a new organ is formed, the nodule, where the reduction of atmospheric N_2 into ammonia is carried out catalyzed by the bacterial exclusive enzyme nitrogenase. The process, highly energy demanding, is known as symbiotic nitrogen fixation and provides all the N needs of the plant, thus avoiding the use of N fertilizers in the context of sustainable agriculture. However, legume crops are often grown under non-fixing conditions since legume nodulation is suppressed by high levels of soil nitrogen occurring in chemically fertilized agro-environment. In addition, legumes are very sensitive to environmental stresses, being drought one of the significant constraints affecting crop production. Due to their agricultural and economic importance, scientists have carried out basic and applied research on legumes to better understand responses to abiotic stresses and to further comprehend plant–microbe interactions. An integrated view of nitrogen utilization under drought stress will be presented with particular focus on legume crops.

V. Castañeda · E. Gil-Quintana · A. Echeverria · EM.González (✉)
Department of Environmental Sciences, Public University of Navarra,
31006 Pamplona, Spain
e-mail: esther.gonzalez@unavarra.es

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_10

Drought Stress

Climate change is multifaceted and includes changing concentrations of greenhouse gases in the atmosphere (like CO_2), rising temperatures, changes in precipitation patterns, and increasing frequency of extreme weather events (Gray and Brady 2016). Thus, Earth´s climate is drastically changing leading to more intense and extended drought situations. Indeed, the area affected by drought has increased substantially since the middle twentieth century (Dai 2011), and the frequency of droughts is predicted to increase in regions that are already dry by the end of the twenty-first century. Drought observatories have estimated that around 40% of the land area is affected by drought and has an expectative in expansion due to the global climate change (Trenberth et al. 2013). It has been estimated that two-thirds of the potential yield of major crops are usually lost due to adverse growing environments (Bajaj et al. 1999; Daryanto et al. 2016). Abiotic stresses, above all water deficit, are the most important factors limiting crop productivity, with a growing importance due to the increase in climate alterations such as reduced rainfall (Lesk et al. 2016). Indeed, most climate change studies predict an increase in arid areas worldwide (Shu et al. 2007), aggravated by the rapidly increasing world population, which puts pressure on food and water demands (Somerville and Briscoe 2001). This problem arises not only from the limiting nature of water supplies, but also from the increased need for food production, which leads to an improper management of agricultural lands. For example, most crops are cultivated in lands and regions to which they are not optimally adapted, yielding up to 22% of their genetic potential due to improper climatic and soil conditions (Boyer 1982). Therefore, the understanding of plant drought stress tolerance has become an urgent matter, since it can allow us a better management and to minimize its harmful effects on crops.

Drought is defined as "the decrease in water inputs into an agro/ecosystem over time that is sufficient to result in soil water deficit (i.e., decrease in the available soil water)" (Gilbert and Medina 2016). Therefore, drought is a condition of climatic dryness severe enough to reduce soil moisture and water below the minimums necessary for sustaining plant, animal, and human life (Perez and Thompson 1996). This stress interferes with the optimal plant growth, physiology, and reproduction, ultimately causing a significant reduction in plant productivity (Farooq et al. 2009). Water deficit can be defined as any water content of a tissue or cell below the highest water content exhibited in the most hydrated state. Although the terms "drought stress" and "water-deficit stress" are usually employed indistinctively, water does not only become limiting for plant communities as a result of inadequate rainfall but also due to other environmental conditions such as excessive salinity in the soil solution or as a consequence of freezing temperatures. In this work, the term drought stress will be used referring to periods where water is withheld from the plant.

Drought Is a Major Threat to Legumes Crops

Grain and forage legumes are grown on around 15% of the arable surface of the Earth, being the second most important crop after cereals attending to world first crop production (FAOSTAT; Graham and Vance 2003). The economic relevance of legume crops is related to both their importance as a protein source for animal feed and human nutrition and their use as raw material in the industry (Edgerton et al. 2008). Common bean, soybean, chickpea, pea, and faba bean are some of the most widely cultivated grain legumes, while cowpea, pigeon pea, lentils, and grass pea play an essential nutritional role in low-income regions of the world. Regarding forage legumes, plants in the Medicago, Trifolium, and Lotus genera are probably the most extended legumes for livestock production. Furthermore, the ability of legume plants to carry out nitrogen fixation in symbiosis with soil rhizobium bacteria provides an environmental-friendly source of reduced nitrogen in the biosphere, being an essential element in sustainable agriculture worldwide.

Despite the numerous advantages of the cultivation of legumes, one of the factors that limit their wider cultivation is the reduction of legume crop yields due to abiotic stress conditions, particularly drought. Three are the main factors contributing to this limitation of productivity: i) in intensive crop-based agricultural systems worldwide, legumes are commonly grown under rain-fed conditions. This is the case in the Mediterranean area (Jacobsen et al. 2012), USA, Brazil and Argentina, the three countries responsible for 87% world's soybean production (FAOSTAT, 2013), or Asia (Kumar and Abbo 2001); (ii) legumes are often grown in rotation after cereal harvest toward the end of the growing season when environmental conditions are more limiting for plant growth; and (iii) improvement in legume crop yields has not kept pace with those of cereals, for which higher yielding modern varieties have been developed (Jeuffroy and Ney 1997). The limitations described above, along with the predictions of an increasing world food demand (Postel 2000) and the rise in temperature at the global level, are driving forces for the investigation of legume responses to drought toward the ultimate development of new varieties with improved water use efficiency and drought tolerance.

Regulation of Nitrogen Fixation Under Drought

Legumes can establish symbiosis with N-fixing soil bacteria. As a result, a new organ is formed, the nodule, where the reduction of atmospheric N_2 into ammonia is carried out catalyzed by the bacterial exclusive enzyme nitrogenase. This process is known as symbiotic nitrogen fixation (SNF) and may provide all the N needs of the plant, avoiding the use of N fertilizers in the context of sustainable agriculture. The effects of drought on SNF occur at different steps of the symbiotic interaction: infection, nodule development, and nodule functioning. Under drought, both the

formation of new root hairs and the elongation of previously differentiated root hairs are limited and, as a consequence, the development of new plant–bacteria interactions and infection threads is greatly reduced (Worrall and Roughley 1976). Moreover, SNF is one of the physiological processes to first show stress responses in nodulated legumes, a decline that cannot be explained by the relatively slow decline in photosynthetic rates (Durand et al. 1987).

Although several hypotheses have been proposed to explain the decline in SNF during drought, the origin of the inhibitory signals, the molecular mechanisms involved, and the interaction among the factors responsible for the inhibition of SNF are not yet fully understood. It has been postulated that drought stress provokes an increase in nodular oxygen diffusion resistance and thus a decline in the oxygen level for bacteroid respiration (Durand et al. 1987). However, the increase of oxygen concentration in the rhizosphere of drought-stressed nodules does not fully restore NF rates, suggesting that other factors are also involved (Del Castillo et al. 1994; Del Castillo and Layzell 1995).

The availability of carbon in nodules as supply for bacteroid respiration and nitrogenase activity is the second regulatory mechanism suggested (Fig. 10.1). The main carbon source transported from the aerial part is sucrose, which is hydrolyzed in nodules by sucrose synthase (SuSy). The essential role of SuSy for NF has been shown for pea (Gordon et al. 1999) and the model legume *M. truncatula* (Baier et al. 2007). Indeed, Gordon et al. showed a correlation between SuSy activity decline and NF inhibition in stressed soybean nodules (Gordon et al. 1997). Moreover, SuSy has been shown to be the first enzyme to decline under drought stress in soybean (Gonzalez et al. 1995), pea (Gonzalez et al. 1998; Galvez et al. 2005) and common bean (Ramos et al. 1999), leading to the accumulation of sucrose and the depletion of organic acids, principally malate, in nodules. However, the SuSy-mediated NF inhibition seems not to take place in forage legumes such as *M. sativa* (Naya et al. 2007) and *M. truncatula* (Larrainzar et al. 2009). In these studies, significant declines in the SuSy activity were found only after the inhibition of NF and concomitant to malate accumulation, suggesting that carbon availability is not the limiting factor for the inhibition of NF in these plants. Moreover, in a recent metabolomic approach, the limitation of respiratory carbon substrates was demonstrated not to be the cause of NF inhibition in drought-stressed *M. truncatula* nodules (Larrainzar et al. 2009).

The third suggested factor implies an N-feedback mechanism involving the N-status of the plant. This theory has received much attention in ureide-exporter tropical legumes, mostly due to studies conducted in soybean. Legumes can be classified into amide- or ureide-exporters according to the compounds used for the transport of fixed N compounds. In general, amide-exporter legumes, such as *M. truncatula*, contain indeterminate-type nodules and are originated from temperate regions. These plants transport fixed nitrogen in the form of amides, mainly asparagine and glutamine. On the other hand, ureide-exporter legumes, such as soybean, are mostly tropical legumes with determinate-type nodules and transport mainly allantoin and allantoic acid. However, exceptions to this general pattern can be found. For instance, the temperate legume *Lotus* spp., with the determinate type

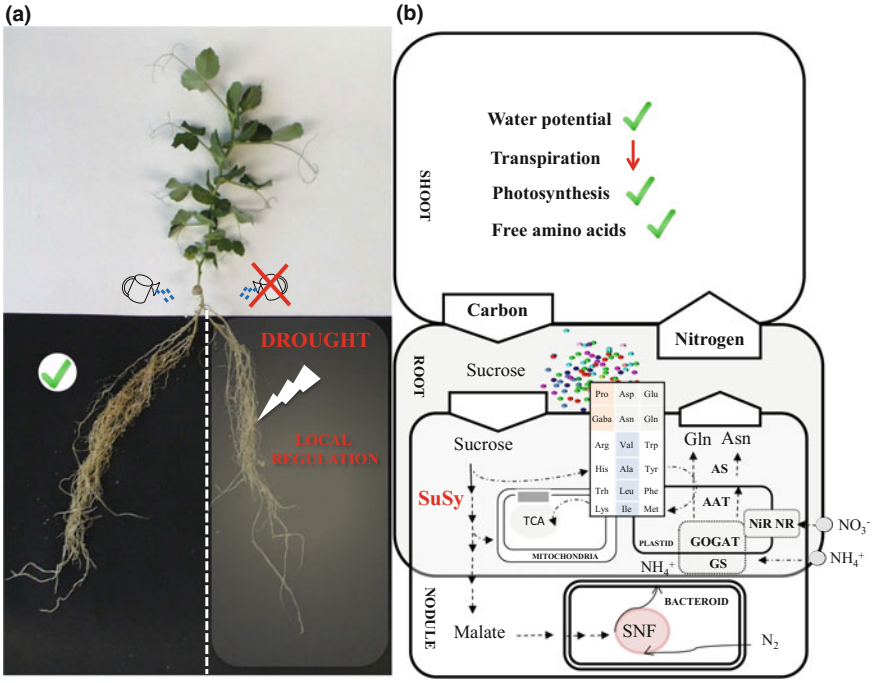


Fig. 10.1 **a** Split-root system set up as a tool to study the local or systemic nature of plant signaling processes. **b** Schematic representation of the main physiological and metabolic processes affected by drought at shoot, root, and nodule level. AAT, aspartate aminotransferase; AS, asparagine synthase; GS, glutamine synthetase; GOGAT, glutamate synthase; NR, nitrate reductase; NiR, nitrite reductase; SNF, symbiotic nitrogen fixation; SuSy, sucrose synthase; TCA, tricarboxylic acid cycle

of nodules, exports amides rather than ureides (Sprent 2001). Several N compounds have been suggested as inhibitory signal molecules; such are the cases of ureides (Serraj et al. 1999; Vadez and Sinclair 2000), glutamine (Neo and Layzell 1997), asparagine (Bacanamwo and Harper 1997; Vadez et al. 2000), and aspartate (King and Purcell 2005). The restriction on the export of N compounds, with their subsequent accumulation in the nodules in water-deficit conditions, has also been postulated (Pate et al. 1969; Walsh 1989a, b). Serraj et al. (2001a) refined the model by proposing two possible origins for the feedback inhibition: a direct feedback within the nodules and an indirect feedback due to N compound signals coming from the aerial part. A more recent study showed that ureides were accumulated in soybean nodules and not in leaves, suggesting a local regulation of NF (Ladrera et al. 2007). Recent works using Split-Root System-Based confirm the operation of local regulatory mechanisms controlling SNF in pea (Marino et al. 2007), *M. truncatula* (Gil-Quintana et al. 2013a), and soybean (Gil-Quintana et al. 2013b) under water-deficit conditions. The concomitant reduction in nitrogenase activity, malate content, and SuSy activity in the nodules of the unwatered split-root section

supports the existence of a local carbon-based regulation of SNF in pea (Marino et al. 2007). In addition, the general variations in amino acid and ureide content in leaves, roots, and nodules (Gil-Quintana et al. 2013a, b) challenged the widely accepted N-based systemic regulation hypothesis (King and Purcell 2005; Sulieman et al. 2010), reinforcing the direct feedback inhibition in the nodules hypothesis.

Drought Stress Effect on the Root System

Although legume crops may lend to a sustainable use of nitrogen fertilizers, the nitrogen-fixing process is mostly suppressed in nitrogen-fertilized agro-environments (Murray et al. 2017). Under these conditions, legume response to drought would be similar to that of other cultivated crops, even though attention should be paid to specific features of legume plants (Fig. 10.1). In herbaceous crops, most of the nitrate is reduced predominantly in the shoots via the reducing equivalents derived from photosynthesis (Scheurwater et al. 2002; Hachiya et al. 2016). Leaf nitrate reduction declines rapidly in response to drought in important crops such as maize (Foyer et al. 1998) or wheat (Fresneau et al. 2007) which correlate with the decline of the photosynthetic process. However, temperate legumes assimilate nitrate chiefly in the roots when growing under low N supply, while shoot nitrate assimilation becomes increasingly important as the nitrate concentration increases (Andrews 1986). Conversely, tropical legumes exhibit constant ratios of the shoot to root nitrate assimilation where this ratio is specific for each of the species (Andrews 1986). These features have not been tested for the current model plants for temperate and tropical legumes, *Medicago* and Lotus, respectively. Unlike nitrate, ammonium is chiefly assimilated in the roots by the coordinated activities of GS and GOGAT (Funayama et al. 2013; Guan et al. 2015; Trepp et al. 1999a, b). In the context of legume plants, ammonium nutrition would closely mimic the symbiotic N-fixing legumes since bacteroids assimilate very little of the fixed ammonia, which is mainly exported to the host plant (Brown and Dilworth 1975; Vance et al. 1994). In this context, legumes have been shown to be relatively tolerant to ammonium nutrition (Domínguez-Valdivia et al. 2008; Ariz et al. 2010).

Roots are the first organs that sense water deficit in soils and interact directly with edaphic water, and therefore drought responses of this organ are highly important. Several studies try to dissect the molecular response of roots of different legumes to drought stress (Micheletto et al. 2007; Zhang et al. 2014). In this context, the primary nitrogen assimilation pathway does not seem to be severely affected under drought stress conditions. This response seems coherent since drought affects cell growth and protein synthesis even at a very moderate level (Hsiao 1973) and hence nitrogen demand is expected to be reduced. With regard to carbon economy, Muller et al. (2011) highlighted a lack of correlation between carbon availability and sink organ growth under water-deficit stress.

Regarding nitrogen metabolism, drought provokes an overall accumulation of amino acids in roots of nodulated (Gil-Quintana et al. 2013a, b) and non-nodulated plants (Frechilla et al. 2000), thereby dismissing a possible nitrogen starvation in drought-stressed plants. In addition, changes in protein synthesis and degradation may strongly affect the pool of free amino acids. Taking as reference the amino acid composition of the *Arabidopsis* proteome, Hildebrandt et al. (2015) estimated that the pool size of the protein-bound amino acids varied less than tenfold. Therefore, inhibition of protein synthesis (Lyon et al. 2016) or enhancement of proteolytic activities (Kohli et al. 2012) could influence the overall accumulation of the free amino acid pools in drought-stressed tissues. Lyon et al. (2016) highlight the importance of protein turnover dynamics in drought recovery processes. On the other hand, pool sizes of the free amino acids, which are around 100- to 1000-fold smaller than the corresponding pools of protein-bound amino acids, are highly diverse (Gil-quintana et al. 2013a; Watanabe et al. 2013). This reflects the various functional roles of these compounds and their interaction with the synthesis of other relevant compounds such as nucleotides or hormones. Amino acid synthetic pathways mainly consume intermediates from glycolysis, the pentose phosphate pathway, and the citric acid cycle and the involved enzymes are mostly located in the plastid with some of them addressed to the cytosol (reviewed in Pratelli and Pilot 2014). In general, the primary products of nitrogen assimilation, Glu, Gln, Asp, and Asn, constitute the larger pools in plants (Coruzzi 2003) although they are not much induced during stress, and accordingly, primary nitrogen assimilation enzyme activities rarely increase in response to drought stress (Larrainzar et al. 2009). Conversely, other less abundant amino acids under control conditions such as Pro (Jacoby et al. 2011), branched chain amino acids (Joshi et al. 2010), Lys and Thr (Obata and Fernie 2012), and His and Trp (Larrainzar et al. 2009) respond individually to drought. Accordingly, the expression of different enzymes involved in the synthesis of some amino acids is eventually affected (Pratelli and Pilot 2014). Unlike the amino acid synthesis, catabolism is mainly addressed to the mitochondria or the cytosol (Hildebrandt et al. 2015). The involvement of mitochondria favors the nitrogen and carbon recycling during the senescence processes occurring under drought stress since mitochondria functionality remains longer than that of other organelles (Avila-Ospina et al. 2014). Araújo et al. (2011) pointed to protein degradation and amino acid catabolism as an alternative carbon source for respiratory processes in stressed plants. Research on amino acid metabolism needs to be expanded for a better understanding on intracellular compartmentalization (Mintz-Oron et al. 2012) dealing not only to photosynthetic tissue but also to those exhibiting a heterotrophic metabolism such as roots.

Furthermore, an active long-distance transport of the amino acids between root and shoot occurs involving both the phloem and the xylem vascular tissues (Jeschke and Hartung 2000). In legumes, amino acids are mainly transported via the xylem (Atkins et al. 1983) but concomitantly some amino acids may be transferred to the phloem to supply nitrogen directly to the sink (Zhang et al. 2010; Tegeder 2014). Recent studies have shown that transport of amino acids between shoot and roots determines nitrogen uptake and metabolism (Miller et al. 2008; Santiago and

Tegeder 2016). However, the role of long-distant transport of amino acids needs to be studied further to better understand the changes in the source–sink interactions occurring under drought. The amino acid exchange requires continuous inward and outward transport across membranes, and numerous genes encoding amino acid transporters have been described (Jack et al. 2000). The induction of proline transporters has been reported in *Arabidopsis* and rice exposed to drought and salt stress (Rentsch et al. 1996; Zhao et al. 2012). In addition, the expression level of different amino acid transporters showed a differential response to drought among shoots and roots in wheat, suggesting that they may play a role in the amino acid exchange among aerial and underground tissues (Wan et al. 2017).

Future Prospects

Legume crops can fix atmospheric nitrogen through their symbiotic association with N-fixing bacteria or by using chemical fertilizers. In this latter case, the legume root exhibits particular features such as a higher tolerance to ammonium and the ability to carry out nitrate reduction to a greater extent than other non-legume crops. Regarding nodulated plants, the nitrogen fixation process has been shown to be rapidly inhibited under moderate drought stress conditions. For those nitrogen-fertilized legumes, few studies have been carried out at root level, although it is widely known that nitrate reductase activity is severely affected in leaves. However, although the different nitrogen assimilation processes seem to be impaired in legumes, the general accumulation of nitrogen compounds occurring in the different tissues dismisses any possible nitrogen scarcity playing a pivotal role in the legume response to drought. Indeed, cell growth is one of the processes firstly affected by water-deficit stress at a moderate level and hence nitrogen demand is presumed to be lower under drought stress. Further knowledge on long-distant transport of nitrogen compounds and amino acid metabolism compartmentalization may contribute to improving legume nitrogen utilization under moderate water-stress conditions.

References

Andrews M (1986) Nitrate and reduced-N concentrations in the xylem sap of *Stellaria media*, *Xanthium strumarium* and six legume species. Plant, Cell Environ 9:605–608

Araujo WL, Tohge T, Ishizaki K, Leaver CJ, Fernie AR (2011) Protein degradation—an alternative respiratory substrate for stressed plants. Trends Plant Sci 16:489–498

Ariz I, Esteban R, García-Plazaola JI, Becerril JM, Aparicio-Tejo PM, Moran JF (2010) High irradiance induces photoprotective mechanisms and a positive effect on NH_4^+ stress in *Pisum sativum L.* J Plant Physiol 167:1038–1045

Atkins CA, Pate JS, Peoples MB, Joy KW (1983) Amino acid transport and metabolism in relation to the nitrogen economy of a legume leaf. Plant Physiol 71:841–848

Avila-Ospina L, Moison M, Yoshimoto K, Masclaux-Daubresse C (2014) Autophagy, plant senescence, and nutrient recycling. J Exp Bot 65:3799–3811

Bacanamwo M, Harper JE (1997) The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. Physiol Plant 100:371–377

Baier MC, Barsch A, Kuester H, Hohnjec N (2007) Antisense repression of the *Medicago truncatula* nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptome and metabolome. Plant Physiol 145:1600–1618

Bajaj S, Targolli J, Liu LF, David Ho TH, Wu R (1999) Transgenic approaches to increase dehydration-stress tolerance in plants. Mol Breed 5:493–503

Boyer JS (1982) Plant productivity and environment. Science 218:443–448

Brown CM, Dilworth MJ (1975) Ammonia assimilation by rhizobium cultures and bacteroids. J General Microbiol 86:39–48

Coruzzi GM (2003) Primary N-assimilation into amino acids in *Arabidopsis*. Arabidopsis Book 2: e0010

Dai A (2011) Drought under global warming: a review. Wiley Interdiscip Rev Clim Change 2:45–65

Daryanto S, Wang L, Jacinthe PA, Yu X, Luo L, Cui K (2016) Global synthesis of drought effects on maize and wheat production. PLoS ONE 11:e0156362

Del Castillo LD, Layzell DB (1995) Drought stress, permeability to O_2 diffusion, and the respiratory kinetics of soybean root-nodules. Plant Physiol 107:1187–1194

Del Castillo LD, Hunt S, Layzell DB (1994) The role of oxygen in the regulation of nitrogenase activity in drought-stressed soybean nodules. Plant Physiol 106:949–955

Domínguez-Valdivia MD, Aparicio-Tejo PM, Lamsfus C, Cruz C, Martins- Loução MA, Moran JF (2008) Nitrogen nutrition and antioxidant metabolism in ammonium-tolerant and -sensitive plants. Physiol Plant 132:359–369

Durand JL, Sheehy JE, Minchin FR (1987) Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water-deprivation. J Exp Bot 38:311–321

Edgerton SA, MacCracken MC, Jacobson MZ, Ayala A, Whitman CE, Trexler MC (2008) Prospects for future climate change and the reasons for early action. J Air Waste Manage Assoc 58:1386–1400

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: effects, mechanisms and management. Sustain Agric. Springer, Netherlands, Dordrecht, pp 153–188

Foyer CH, Valadier M-H, Migge A, Becker TW (1998) Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of Nitrogen and carbon metabolism in maize leaves. Plant Physiol 117:283–292

Frechilla S, González EM, Royuela M, Minchin FR, Aparicio-Tejo PM, Arrese-Igor C (2000) Source of nitrogen nutrition (nitrogen fixation or nitrate assimilation) is a major factor involved in pea response to moderate water stress. J Plant Physiol 157:609–617

Fresneau C, Ghashghaie J, Cornic G (2007) Drought effect on nitrate reductase and sucrose-phosphate synthase activities in wheat (*Triticum durum* L.): role of leaf internal CO_2. J Exp Bot 58:2983–2992

Funayama K, Kojima S, Tabuchi-Kobayashi M, Sawa Y, Nakayama Y, Hayakawa T, Yamaya T (2013) Cytosolic glutamine synthetase1;2 is responsible for the primary assimilation of ammonium in rice roots. Plant Cell Physiol 54:934–943

Galvez L, Gonzalez EM, Arrese-Igor C (2005) Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. J Exp Bot 56:2551–2561

Gilbert ME, Medina V (2016) Drought adaptation mechanisms should guide experimental design. Trends Plant Sci 21:639–647

Gil-Quintana E, Larrainzar E, Arrese-Igor C, González EM (2013a) Is N-feedback involved in the inhibition of nitrogen fixation in drought-stressed *Medicago truncatula*? J Exp Bot 64:281–292

Gil-Quintana E, Larrainzar E, Seminario A, Diaz-Leal JL, Alamillo JM, Pineda M, Arrese-Igor C, Wienkoop S, Gonzalez EM (2013b) Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean. J Exp Bot 64:2171–2182

Gonzalez EM, Aparicio-Tejo PM, Gordon AJ, Minchin FR, Royuela M, Arrese-Igor C (1998) Water-deficit effects on carbon and nitrogen metabolism of pea nodules. J Exp Bot 49:1705–1714

Gonzalez EM, Gordon AJ, James C, Arrese-Igor C (1995) The role of sucrose synthase in the response of soybean nodules to drought. J Exp Bot 46:1515–1523

Gordon AJ, Minchin FR, James CL, Komina O (1999) Sucrose synthase in legume nodules is essential for nitrogen fixation. Plant Physiol 120:867–877

Gordon AJ, Minchin FR, Skot L, James CL (1997) Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. Plant Physiol 114:937–946

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol 131:872–877

Gray SB, Brady SM (2016) Plant developmental responses to climate change. Develop Biol 419:64–77

Guan M, Møller IS, Schjoerring JK (2015) Two cytosolic glutamine synthetase isoforms play specific roles for seed germination and seed yield structure in *Arabidopsis*. J Exp Bot 66:203–212

Hachiya T, Ueda N, Kitagawa M, Hanke G, Suzuki A, Hase T, Sakakibara H (2016) *Arabidopsis* root-type ferredoxin:NADP(H) oxidoreductase 2 is involved in detoxification of nitrite in roots. Plant Cell Physiol 57:2440–2450

Hildebrandt TM, Nunes Nesi A, Araujo WL, Braun H-P (2015) Amino acid catabolism in plants. Mol Plant 8:1563–1579

Hsiao TC (1973) Plant responses to water stress. Ann Rev Plant Physiol Plant Mol Biol 24:519–570

Jack DL, Paulsen IT, Saier MH (2000) The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. Microbiol 146:1797–1814

Jacobsen SE, Jensen CR, Liu F (2012) Improving crop production in the arid mediterranean climate. Field Crops Res 128:34–47

Jacoby RP, Taylor NL, Millar AH (2011) The role of mitochondrial respiration in salinity tolerance. Trends Plant Sci 16:614–623

Jeschke WD, Hartung W (2000) Root–shoot interactions in mineral nutrition. Plant Soil 226:57–69

Jeuffroy MH, Ney B (1997) Crop physiology and productivity. Field Crop Res 53:3–16

Joshi V, Joung JG, Fei Z, Jander G (2010) Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress. Amino Acids 39:933–947

King CA, Purcell LC (2005) Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiol 137:1389–1396

Kohli A, Narciso JO, Miro B, Raorane M (2012) Root proteases: reinforced links between nitrogen uptake and mobilization and drought tolerance. Physiol Plant 145:165–179

Kumar J, Abbo S (2001) Genetics of flowering time in chickpea and its bearing on productivity in semiarid environments. Adv Agron 72:107–138

Ladrera R, Marino D, Larrainzar E, Gonzalez EM, Arrese-Igor C (2007) Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. Plant Physiol 145:539–546

Larrainzar E, Wienkoop S, Scherling C, Kempa S, Ladrera R, Arrese-Igor C, Weckwerth W, Gonzalez EM (2009) Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. Mol Plant-Microbe Interact 22:1565–1576

Lesk C, Rowhani P, Ramankutty N (2016) Influence of extreme weather disasters on global crop production. Nature 529:84–87

Lyon D, Castillejo MA, Mehmeti-Tershani V, Staudinger C, Kleemaier C, Wienkoop S (2016) Drought and recovery: independently regulated processes highlighting the importance of protein turnover dynamics and translational regulation in *Medicago truncatula*. Mol Cell Proteomics 15:6

Marino D, Frendo P, Ladrera R, Zabalza A, Puppo A, Arrese-Igor C, Gonzalez EM (2007) Nitrogen fixation control under drought stress. Localized or systemic? Plant Physiol 143: 1968–1974

Micheletto S, Rodriguez-Uribe L, Hernandez R, Richins RD, Curry J, O'Connell MA (2007) Comparative transcript profiling in roots of *Phaseolus acutifolius* and *P. vulgaris* under water deficit stress. Plant Sci 173:510–520

Miller AJ, Shen Q, Xu G (2008) Freeways in the plant: transporters for N, P and S and their regulation. Curr Opin Plant Biol 12:284–290

Mintz-Oron S, Meir S, Malitsky S, Ruppin E, Aharoni A, Shlomi T (2012) Reconstruction of *Arabidopsis* metabolic network models accounting for subcellular compartmentalization and tissue-specificity. Proc Nat l Acad Sci USA 109:339–344

Muller B, Pantin F, Genard M, Turc O, Freixes S, Piques M, Gibon Y (2011) Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. J Exp Bot 62:1715–1729

Murray JD, Cheng-Wu L, Chen Y, Miller AJ (2017) Nitrogen sensing in legumes. J Exp Bot 68:1919–1926

Naya L, Ladrera R, Ramos J, Gonzalez EM, Arrese-Igor C, Minchin FR, Becana M (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiol 144:1104–1114

Neo HH, Layzell DB (1997) Phloem glutamine and the regulation of O_2 diffusion in legume nodules. Plant Physiol 113:259–267

Obata T, Fernie AR (2012) The use of metabolomics to dissect plant responses to abiotic stresses. Cell Mol Life Sci 69:3225–3243

Pate JS, Gunning BES, Briarty LG (1969) Ultrastructure and functioning of transport system of leguminous root nodule. Planta 85:11–34

Perez E, Thompson P (1996) Natural hazards: causes and effects. Lesson 7-drought. Prehosp Disaster Med 11:71–77

Postel SL (2000) Water and world population growth. Am Water Works Assoc J 92:131–138

Pratelli R, Pilot G (2014) Regulation of amino acid metabolic enzymes and transporters in plants. J Exp Bot 65:5535–5556

Ramos MLG, Gordon AJ, Minchin FR, Sprent JI, Parsons R (1999) Effect of water stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris* L.). Ann Bot 83:57–63

Rentsch D, Hirner B, Schmelzer E, Frommer WB (1996) Salt stress-induced proline transporters and salt stress-repressed broad specificity amino acid permeases identified by suppression of a yeast amino acid permease-targeting mutant. Plant Cell 8:1437–1446

Santiago JP, Tegeder M (2016) Connecting source with sink: the role of *Arabidopsis* AAP8 in phloem loading of amino acids. Plant Physiol 171:508–521

Scheurwater I, Koren M, Lambers H, Atkin OK (2002) The contribution of roots and shoots to whole plant nitrate reduction in fast- and slow-growing grass species. J Exp Bot 53:1635–1642

Serraj R, Vadez V, Sinclair TR (2001) Feedback regulation of symbiotic N_2 fixation under drought stress. Agronomie 21:621–626

Serraj R, Vadez V, Denison RF, Sinclair TR (1999) Involvement of ureides in nitrogen fixation inhibition in soybean. Plant Physiol 119:289–296

Shu FH, Shang H, Glassgold AE, Lee T, Baker J, Bizzarro M, Wittig N, Connelly J, Haack H (2007) Model projections of an imminent transition to a more arid climate. Science 316:1181–1475

Somerville C, Briscoe J (2001) Genetic engineering and water. Science 292:2217

Sprent JI (2001) Nodulation in legumes. R Bot Gardens, Kew

Sulieman S, Fischinger SA, Gresshoff PM, Schulze J (2010) Asparagine as a major factor in the N-feedback regulation of N_2 fixation in *Medicago truncatula*. Physiol Plant 140:21–31

Tegeder M (2014) Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. J Exp Bot 65:1865–1878

Trenberth KE, Dai A, van der Schrier G, Jones PD, Barichivich J, Briffa KR, Sheffield J (2013) Global warming and changes in drought. Nat Clim Change 4:17–22

Trepp GB, Plank DW, Gantt JS, Vance CP (1999a) NADH-glutamate synthase in alfalfa root nodules.Immunocytochemical localization. Plant Physiol 119:829–837

Trepp GB, van de Mortel M, Yoshioka H, Miller SS, Samac DA, Gantt JS, Vance CP (1999b) NADH-glutamate synthase in alfalfa root nodules. Genetic regulation and cellular expression. Plant Physiol 119:817–828

Vadez V, Sinclair TR (2000) Ureide degradation pathways in intact soybean leaves. J Exp Bot 51:1459–1465

Vadez V, Sinclair T, Serraj R (2000) Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N_2 fixation in soybean. Physiol Plant 110:215–223

Vance CP, Gregerson RG, Robinson DL, Miller SS, Gantt JS (1994) Primary assimilation of nitrogen in alfalfa nodules—molecular features of the enzymes involved. Plant Sci 101:51–64

Walsh KB, Canny MJ, Layzell DB (1989a) Vascular transport and soybean nodule function.2. A role for phloem supply in product export. Plant, Cell Environ 12:713–723

Walsh KB, McCully ME, Canny MJ (1989b) Vascular transport and soybean nodule function—nodule xylem is a blind alley, not a throughway. Plant, Cell Environ 12:395–405

Wan Y, King R, Mitchell RAC, Hassani-Pak K, Hawkesford MJ (2017) Spatiotemporal expression patterns of wheat amino acid transporters reveal their putative roles in nitrogen transport and responses to abiotic stress. Sci Rep 7:5461

Watanabe M, Balazadeh S, Tohge T, Erban A, Giavalisco P, Kopka J, Mueller-Roeber B, Fernie AR, Hoefgen R (2013) Comprehensive dissection of spatiotemporal metabolic shifts in primary, secondary, and lipid metabolism during developmental senescence in *Arabidopsis*. Plant Physiol 162:1290–1310

Worrall VS, Roughley RJ (1976) Effect of moisture stress on infection of *Trifolium Subterraneum* L. by *RhizobiumTrifolii* Dang. J Exp Bot 27:1233–1241

Zhang JY, Cruz DE, Carvalho MH, Torres-Jerez I, Kang Y, Allen SN, Huhman D, Tang Y, Murray J, Sumner LW, Udvardi MK (2014) Global reprogramming of transcription and metabolism in *Medicago truncatula* during progressive drought and after rewatering. Plant, Cell Environ 37:2553–2576

Zhang L, Tan Q, Lee R, Trethewy A, Lee YH, Tegeder M (2010) Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in *Arabidopsis*. Plant Cell 22:3603–3620

Zhao H, Ma H, Yu L, Wang X, Zhao J (2012) Genome-wide survey and expression analysis of amino acid transporter gene family in rice (*Oryza sativa* L.). PLoS ONE 7:e49210

Chapter 11
Exploiting Genetic Variability of Root Morphology as a Lever to Improve Nitrogen Use Efficiency in Oilseed Rape

Julien Louvieaux, Hugues De Gernier and Christian Hermans

Innovation to Make a Step Change for Reducing the Environmental Impact of Nitrogen Fertilization

Asustained improvement in crop yield is required to meet the food demands of the rapidly growing world population. By 2050, a societal challenge will be to almost double food production from existing land areas in order to feed more than nine billion people (Lynch and Brown 2012; Ray et al. 2013; Pradhan et al. 2015), while facing yield-depressing consequences of climate change (Moore and Lobell 2015). Plant mineral nutrition drives all terrestrial food webs. In that context, the root organ is a pivotal yield determining factor because it is responsible for water and nutrient capture in the soil.

Nitrogen (N) is the essential macronutrient required in the greatest amounts by plants and it accounts for one to five percent of the dry matter (Marschner 2012). Plants need that element for life's building blocks, such as nucleic acids and proteins, and also for a variety of secondary metabolites (Xu et al. 2012). Plants cannot directly access to the main gaseous reservoir (N_2) in the atmosphere. From the soil, they mainly absorb inorganic N forms such as nitrate (NO_3^-) and ammonium

J. Louvieaux · H. De Gernier · C. Hermans (✉)
Laboratory of Plant Physiology and Molecular Genetics, Interfacultary School of Bioengineers, Université libre de Bruxelles, Campus Plaine CP 242, Bd du Triomphe, 1050 Brussels, Belgium
e-mail: chermans@ulb.ac.be

J. Louvieaux
Laboratory of Applied Plant Ecophysiology, Haute Ecole Provinciale de Hainaut Condorcet, Centre pour l'Agronomie et l'Agro-industrie de la Province de Hainaut, 11 rue Paul Pastur, 7800 Ath, Belgium

Present Address:
H. De Gernier
Center for Plant Systems Biology, VIB-UGent, Technologiepark 927, 9052 Ghent, Belgium

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_11

(NH_4^+), as well as N-containing organic compounds such as amino acids and peptides (Näsholm et al. 2009; Tegeder and Rentsch 2010). In agricultural ecosystems, N is continually depleted by such processes as exportation of N-containing crop residues from the plot, microbial denitrification and nitrate leaching (Fig. 11.1). Therefore, the soil N reserve must be replenished every so often in order to maintain high crop yield. Nitrogenous chemical fertilizers have been intensively dampened for decades but excessive nitrate concentrations are detrimental to people (methaemoglobinaemia) and environment (eutrophication, greenhouse gas emission) (Buckart and Stoner 2007; Wick et al. 2012; Richard et al. 2014). As much as two-thirds of the applied N may not be recovered in the harvested crop organs (Liu et al. 2010).

One way to reduce N-fertilizer input is to breed for crops with higher nitrogen use efficiency (NUE) (Kant et al. 2011; Han et al. 2015). The Green Revolution programs of the 1960s achieved to increase crop yield unprecedentedly but without considering NUE as a breeding criterion. The case of wheat is well documented. Barraclough et al. (2010) reported that UK breeders have selected high-yield genotypes but at high fertilizer inputs. At present, the improvement of NUE is central for promoting ecofriendly agriculture (Bouchet et al. 2016a; Li et al. 2017;

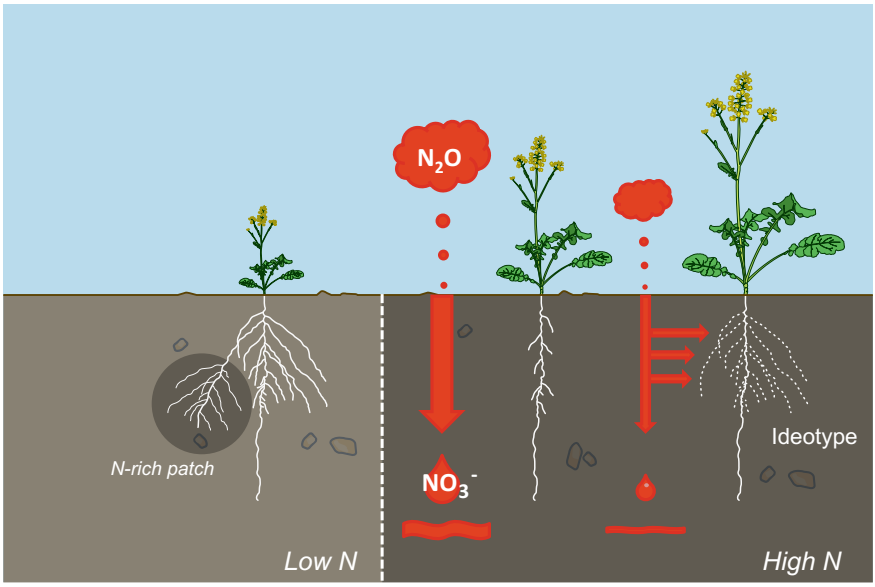


Fig. 11.1 Premises on ideal root architectural attributes to optimize nitrogen acquisition in time and space. Nitrate (NO_3^-) can leach through the soil and quickly be depleted in surface strata. A root system with rapid exploitation of deep soil would optimize the capture of that mobile resource. External nitrate has a dual effect on lateral root development: (i) a localized stimulation of N-starved roots elongation at the contact with rich nitrate source and (ii) a systemic inhibition of uniformly high nitrate concentrations on lateral root elongation. A plant ideotype that limits N-runoffs would rapidly develop a branched and deep root system

Stahl et al. 2017). Moll et al. (1982) gave the most basic and widely used definition for NUE as the harvested dry matter divided by unit of N available in the soil. NUE has two main components: the uptake efficiency (NupE), which describes the capacity to acquire N from the soil, and the utilization efficiency (NutE), the capacity to utilize the absorbed N to produce harvestable organs (Han et al. 2015). The latter one can be divided, in its turn, into the N assimilation (NAE) and the N remobilization (NRE) efficiencies. Both NupE and NutE components underlie distinct genetic mechanisms and differences exist between and within crop species.

Definitely, a second green revolution is anticipated for breeding crops that require fewer fertilizers and with optimized root morphology in order to enhance nutrient acquisition (Lynch 2007; Den Herder et al. 2010; McAllister et al. 2012). While current attempts to improve NUE are mostly focusing on NutE processes (McAllister et al. 2012; Havé et al. 2017), this chapter focuses on less conventional approaches that incorporate root morphology and N transport system to improve NupE (Garnett et al. 2009).

The Model Species *Arabidopsis thaliana* as a Resource Base for Understanding Nitrogen Acquisition in Plants

The root system architecture and the activity of N transporters are critically contributing to N capture in the soil (Gent and Forde 2017a, b). The manipulation of those two features can lead to NupE amelioration. Before tackling crop issues, we will first concisely review current knowledge in *Arabidopsis thaliana*, which is universally recognized as a model organism for fundamental plant biology research and also for translational research in crops (Lavagi et al. 2012; Forde 2014, Havé et al. 2017; Li et al. 2017; Nour-Eldin et al. 2017). Although Arabidopsis has no agronomic interest, it is close parent to *Brassica* crops (see previously). Therefore, it may be used as a resource base because of its comparatively smaller genome than those cultivated species, thereby facilitating genetic analysis.

The Nitrate Influence on the Root Morphology of Arabidopsis thaliana

Since plants are sessile organisms and cannot migrate toward more prosperous habitats, they have evolved mechanisms to adapt to fluctuations in water and nutrient availability. The root system architecture is a term that refers to the spatial configuration of the entire root organ in the soil (Lynch 1995). It exhibits a high degree of plasticity in response to nutrient availability (López-Bucio et al. 2003). In this case, nitrate is a major determinant of root morphology and biomass allocation between plant organs (Hermans et al. 2006; Forde 2014; Kiba and Krapp 2016).

Learning about mechanisms of root growth stimulation or repression by nitrate availability may help to draw strategies to optimize root system architecture and ultimately NUE.

The formation of lateral roots contributes to shape the root system architecture. Many of the advances in understanding the molecular mechanisms that regulate lateral root growth and development are driven from Arabidopsis studies. Lateral roots are formed from the pericycle (the outermost cell layer of the root vascular cylinder) through hormonal auxin-dependent cell cycle activation (Himanen et al. 2002; Laskowski and Ten Tusscher 2017). After asymmetric divisions, the founder cells will constitute a lateral root primordium that passes through the endodermal, cortical, and epidermal tissue layers of the parent root, before penetrating the soil (Lavenus et al. 2013; Vermeer and Geldner 2015; Birnbaum 2016). Malamy and Benfey (1997) described eight stages in the lateral root development from the initiation to the emergence (Fig. 11.2a). Diverse nitrate signaling pathways are depicted to act at multiple stages of lateral root development, depending on nitrate concentration and distribution in the growth medium (extensively reviewed in Forde 2014; Kiba and Krapp 2016; O'Brien et al. 2016; Gent and Forde 2017a, b; Sun et al. 2017; Undurraga et al. 2017).

In vitro culture systems are commonly used to observe the 2D root morphology of Arabidopsis in response to mineral nutrient supply (De Pessemier et al. 2013; Xiao et al. 2015). Here, we will describe the global and local nitrate effects on root morphology of seedlings grown in vertical agar plates (Fig. 11.2b–c) (Zhang et al. 2007). First, uniformly low nitrate levels stimulate lateral root development, which substantially increases the root surface area available for N acquisition (Fig. 11.2b). Nonetheless, lateral root growth can be restricted under severe N deficiency conditions for an extended period (Krouk et al. 2010; Gruber et al. 2013; Araya et al. 2014). Conversely homogeneous high nitrate levels inhibit lateral root elongation by preventing lateral root primordium activation at postemergence (Zhang et al. 1999). Second, when roots of N-deficient plants come into contact with nitrate, lateral root outgrowth is enhanced within the nitrate-rich patch (Fig. 11.2c) (Zhang et al. 1999). That response is known as the foraging capacity.

Lateral root developmental responses to nitrate supply have been associated, namely with the MADS-box transcription factor ARABIDOPSIS NITRATE REGULATED 1 (ANR1) (Zhang and Forde 1998; Gan et al. 2012), the microRNA 167a/b and AUXIN RESPONSE FACTOR 8 (ARF8) (Gifford et al. 2008) and with some nitrate transporters (see below). More and more players are being identified (Sun et al. 2017) but we cannot simply cover all of them in this section. Despite the evolutionary distance between Arabidopsis and crops, some of the above-mentioned genes may have a similar role in coordinating nitrate availability with the root development (Forde 2014).

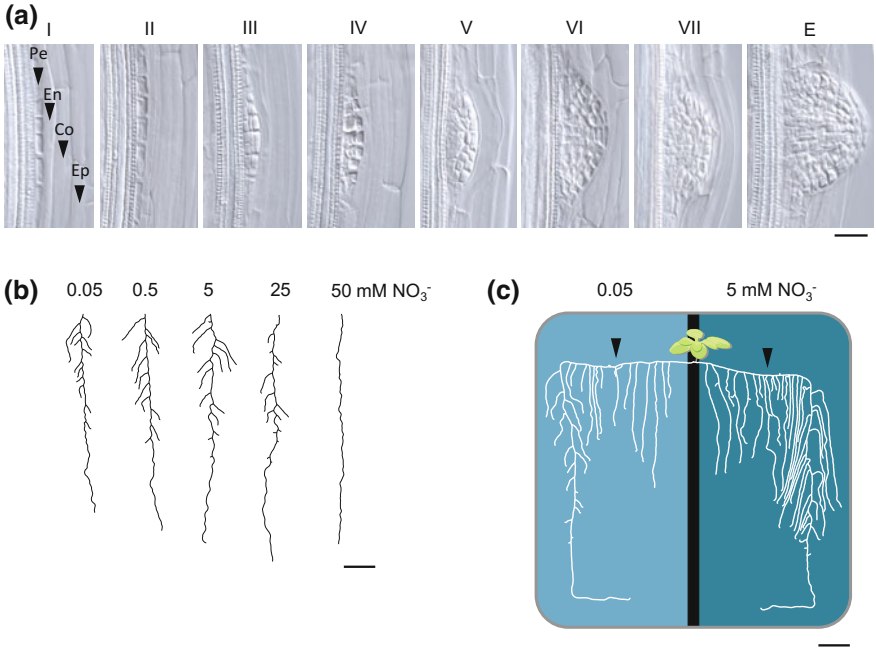


Fig. 11.2 Root morphological responses to nitrate supply of the model species *Arabidopsis thaliana* (in vitro culture). A. Temporal sequence of lateral root primordium development in *Arabidopsis thaliana* Columbia-0 (Col-0). Roman numbers (I to VII) represent seven stages of development and E, the emergence out of the parent root. Different tissue layers are indicated by arrowheads: pericycle (Pe), endodermis (Ed), cortex (Co), and epidermis (Ep). Scale bar: 20 µm. B. Root morphology in response to homogeneous nitrate supply. Col-0 seedlings grew for 14 days on vertical agar plates containing 0.05, 0.5, 5, 25, or 50 mM nitrate. Medium formulation is described in Hermans et al. (2010a). Growth conditions were constant temperature of 20 °C and light period of 16 h light (50 µmol photons m^{-2} s^{-1})/8 h darkness. Scale bar: 1 cm. B. Root morphology response to heterogeneous nitrate supply. Split-root experiment shows a distinct promotion of root growth in the right sector with high nitrate supply (5 mM) relative to the left deplete sector (0.05 mM). Col-0 grew for one week on homogeneous 5 mM nitrate medium and then primary root was pruned to two first-order lateral roots, at a distance of 1 cm below the hypocotyl. After one week, the two main lateral roots with identical size were placed on two agar sectors which were containing 0.05 or 5 mM nitrate and separated by a gap of 2 mm width. Arrowhead marks the position of the two root tips at the time of transfer. Illustration represents a root system 27 days after germination. Growth conditions were the same as in panel B. Scale bar: 1 cm

The Molecular Identification and Functional Characterization of Nitrate Transporters in Arabidopsis thaliana

The uptake of nitrate from the soil solution to the root is a process that occurs at the plasma membrane of root cells through the action of well-characterized transport systems (Fan et al. 2017). The low-affinity transport (LAT) system is active at high

(>0.5–1 mM) and the high-affinity transporter (HAT) system at low (<0.2 mM) nitrate concentrations, respectively. Both systems include inducible and constitutive carriers (Nacry et al. 2013). In Arabidopsis, nitrate transporters are encoded at least by four gene families: *NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY* (*NRT1/NPF*), *NITRATE TRANSPORTER 2* (*NRT2*), *CHLORIDE CHANNEL FAMILY* (*CLC*), and *SLOW ANION ASSOCIATED CHANNEL HOMOLOG* (*SLAC/SLAH*) (Léran et al. 2014; Noguero and Lacombe 2016). Since its isolation from an Arabidopsis mutant screen for resistance to chlorate (Tsay et al 1993), NRT1.1/NPF6.3 is the most extensively characterized of all nitrate carriers. That transporter has double-affinity for nitrate (Wang et al. 1998). Mechanistically, the phosphorylation of a threonine residue decouples the NRT1.1 homodimer configuration and switches the transporter from LAT (homodimer) to HAT (dimer decoupled) state (Liu and Tsay 2003; Parker and Newstead 2014; Sun et al. 2014). Besides facilitating nitrate uptake, NRT1.1 is also depicted as a transceptor (transporter/receptor) that emerges as a main hub for sensing environmental nitrate conditions and triggering different signaling pathways (Bouguyon et al. 2015). For example, nitrate sensed by NRT1.1 may elicit the production of second messengers (e.g., cytosolic calcium levels), which would consequently trigger cascades to change the expression levels of some nitrate assimilation pathway genes (Undurraga et al. 2017). Remarkably, both NRT1.1 and NRT2.1 modulate the root system architecture. The lateral root proliferation in nitrate-rich zones (as depicted in Fig. 11.2c) is demonstrated to rely in part on the dual auxin/nitrate transport activity of NRT1.1 (Remans et al. 2006a; Krouk et al. 2010; Mounier et al. 2014; Bouguyon et al. 2016). Similarly, NRT2.1 plays a key role in root morphological responses to N limitation (Little et al. 2005; Remans et al. 2006b).

Finally, enhancing nitrate acquisition by manipulating transporters has revealed to be a successful target for improving NUE (Fan et al. 2017). There are few examples in cultivated plants of higher nitrate transporter expression resulting into increased plant growth, yield, and NUE (Fu et al. 2015; Hu et al. 2015, Fan et al 2016; Chen et al. 2016, 2017).

Premises on Ideal Root Architectural Attributes to Optimize Nitrogen Acquisition in Oilseed Rape

One sensible target to improve NupE is enhancing soil exploration and N resource capture by plants (Lynch and Brown 2012). A common rationale shared by different authors (Lynch 2013, Li et al. 2016; Pierret et al. 2016) is that NupE can be improved by favouring a branched and deep root system that explores an important soil volume in order to prevent N leaching (Fig. 11.1). Field trials support that such root features underlie high NUE in crops (Yu et al. 2015). Also, some quantitative trait loci (QTLs) are involved concurrently in NUE and root morphology, suggesting that NUE can be improved through direct selection of root morphological traits (Li et al. 2015; Pestsova et al. 2016).

Our focus is on winter oilseed rape, which is the second most important oilseed crop in the world (Stahl et al. 2017). Oilseed rape (*Brassica napus* L., 2n = 4x = 38) has an allotetraploid genome resulting from the interspecific cross between turnip (*B. rapa*, A genome, 2n = 2x = 20) and cabbage (B. *oleracea*, C genome, 2n = 2x = 18). Besides *Brassica* species are closely related to the plant model *Arabidopsis thaliana* (2n = 2x = 10), which we have introduced (see previously). Winter oilseed rape is an increasingly important cash crop that diversifies cereal-dominated crop rotations and that can absorb N from the soil and to incorporate it in vegetative biomass during the autumn. Unfortunately, oilseed rape has a low ratio of seeds produced per N unit applied, around half that for cereals (Moll et al. 1982; Bouchet et al. 2014, 2016a) and the small recovery involves risks for N leaching. Increasing NUE is therefore essential to ensure the environmental and economic sustainability of that crop production (Bouchet et al. 2016a). There are indications that NUE is more strongly correlated with the NupE than with the NutE under limiting N fertilization in field conditions (Berry et al. 2010; Schulte auf'm Erley et al. 2011; Nyikako et al. 2014; Miersch et al. 2016). This suggests that NupE is a valuable target for the creation of N-efficient oilseed rape genotypes. Furthermore, there are indications that the seed yield is most closely correlated with root growth following stem extension at low N supply (Kamh et al. 2005). Another report indicates that an N-efficient cultivar is characterized by a high root production during the vegetative growth stage (Ulas et al. 2012). Therefore, all these elements advocate for root morphology optimization as a valuable strategy to improve NUE in oilseed rape.

An Example of High-Throughput Screening of Root Morphology in Laboratory Conditions and Validation upon Field Trial

Some recent studies started to explore the genetic diversity of root biomass production and root morphology in oilseed rape (Kamh et al. 2005; Schulte auf'm Erley et al. 2007; Rahman and McClean 2013; Fletcher et al. 2015; Bouchet et al. 2016a; Thomas et al. 2016a, b; Zhang et al. 2016) and very few ones focusing on the response to N supply (Leblanc et al. 2008; Lemaire et al. 2013). Here, we challenged a diversity set of winter oilseed rape genotypes in a controlled environment, for identifying contrasting root morphologies with different N treatments. Eventually, the crop performance was challenged in a field trial. Correlations between traits measured in laboratory and field conditions were established. That way, we tested our hypothesis that root biomass production and root morphological traits could be positive indicators of shoot biomass production and presumably of yield performance. The panel was composed of two inbred lines and 26 hybrid cultivars from different breeding companies (registration between 2009 and 2016). They were recommended by the French Technical Center for Oilseed Crops and

Industrial Hemp (Terres-Inovia, France) for the northern France growing conditions. Actually that selection reflects the current predominance of modern hybrid cultivars in crop-producing countries (Gehringer et al. 2007; Stahl et al. 2017). One of the main reasons is that hybrids are the most productive genotypes whatever the N nutrition conditions (Kessel et al. 2012).

The Phenotyping Seedlings in a Laboratory Environment

We used the pouch-and-wick system for observing root morphology (Adu et al. 2014, Thomas et al. 2016a, b). With that hydroponic setting, seedlings grew for seven days on vertical germination paper imbibed with a nutrient solution containing 0.2 mM (N−) or 5 mM (N+) nitrate, as the sole source of N (Fig. 11.3). Representative root organs are presented in Fig. 11.4. Biomass production and root morphological traits measured in hydroponics are defined in Table 11.1. On average for the panel, the shoot biomass (S, −16%) and the total biomass (R + S, −13%) were lower, and the root-to-shoot biomass ratio (R:S, +20%) was more important, whereas the root biomass (R) was not different at N− compared to N+ (Fig. 11.5a–

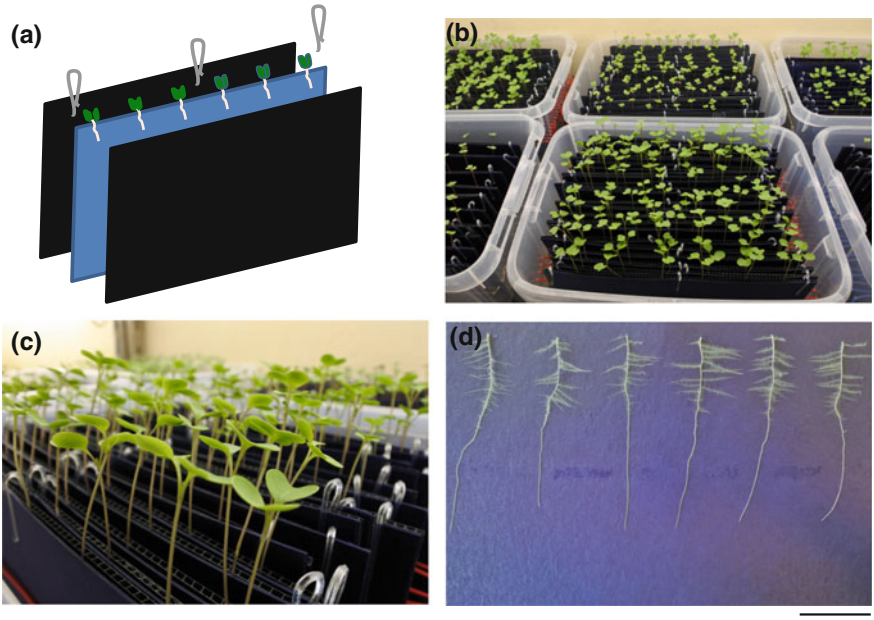


Fig. 11.3 Hydroponic pouch-and-wick system to observe root morphology of winter oilseed rape. **a** The system is made of two black rigid plates and a blue germination paper (20 × 30 cm) imbibed with a nutrient solution. **b** Containers (19 L capacity) can hold up to 25 systems. **c** Close-up of the systems. **d** The blue color of the germination paper allows to clearly discriminating the roots of seven-day-old seedlings. Scale bar: 5 cm

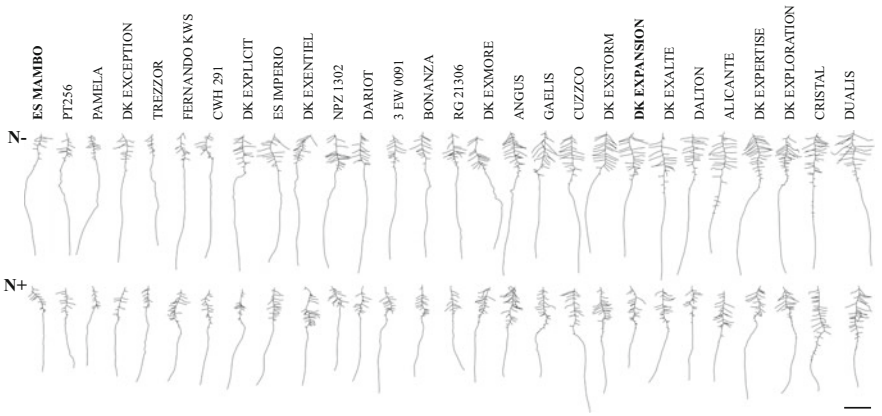


Fig. 11.4 Root morphologies of winter oilseed rape genotypes in response to nitrate supply. Pictures of representative root organs of 28 genotypes after seven-day growth in the hydroponic pouch-and-wick system with 0.2 mM (N−) or 5 mM (N+) nitrate supplies. Root organ scans were annotated with the RootNav image analysis software version 1.7.6 (Pound et al. 2013). Genotypes are ranked from the left to the right by increasing sum of lateral root lengths (ΣL_{LR}) value measured at N-. The formulation of the nutrient solution is derived from Hermans et al. (2010b). Growth conditions were constant temperature of 21 °C, photoperiod of 16 h light (150 µmol photons m^{-2} s^{-1})/8 h darkness and relative humidity of 70%. Scale bar: 2 cm

Table 11.1 Definitions of biomass production and root morphological traits measured in hydroponics

Biomass production traits	
R	Root biomass
S	Shoot biomass
R + S	Total biomass
R:S	Root to shoot biomass ratio
Root morphological traits	
L_{PR}	Length of primary root = L_{Z2} + L_{Z3} + L_{Z4}
L_{Z2}	Length of primary root zone 2, delimited between the first and last lateral root
L_{Z3}	Length of primary root zone 3, delimited between the hypocotyl junction and the first lateral root
L_{Z4}	Length of primary root zone 4, delimited between the last lateral root and the primary root tip
N_{LR}	Number of lateral roots >1 mm
ΣL_{LR}	Sum of lateral root lengths
ML_{LR}	Mean length of lateral roots = $\Sigma L_{LR}/N_{LR}$
TRL	Total root length = L_{PR} + ΣL_{LR}
$D_{LR} - Z_1$	Density of lateral roots in zone 1 = N_{LR}/L_{PR}
$D_{LR} - Z_2$	Density of lateral roots in zone 2 = $(N_{LR} - 1)/L_{Z2}$
SRL	Specific root length = $(L_{PR} + \Sigma L_{LR})/R$

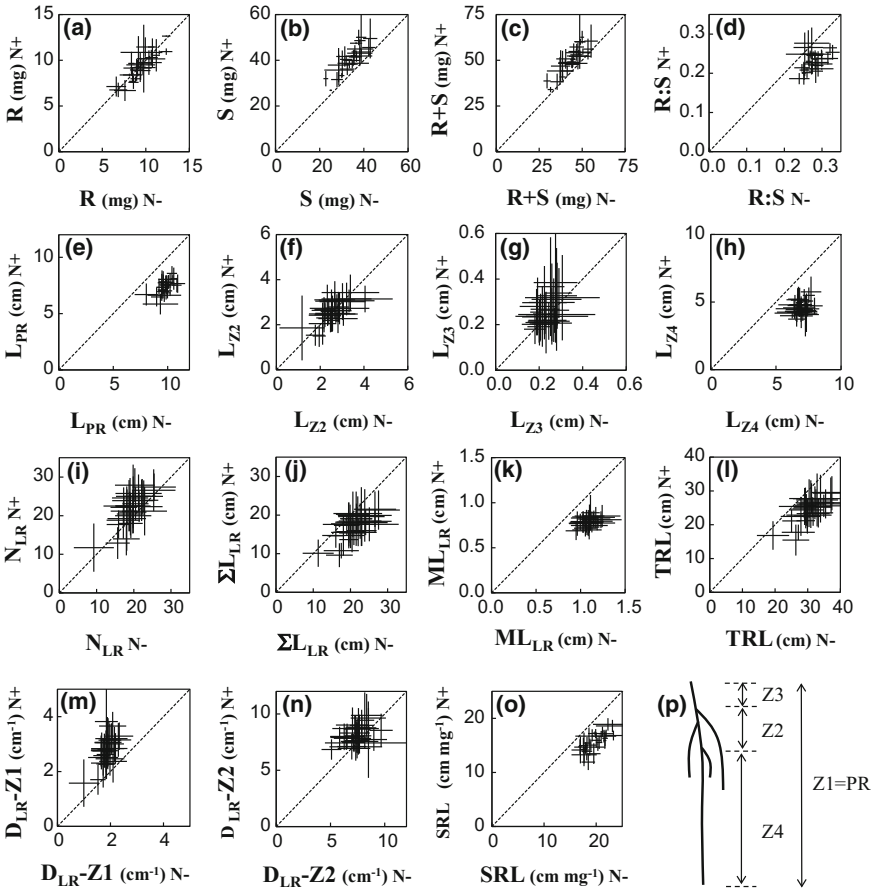


Fig. 11.5 Biomass production and root morphological traits of winter oilseed rape genotypes in response to nitrate supply (hydroponics). Twenty-eight genotypes grew for seven days in the pouch-and-wick system with 0.2 mM (N−) or 5 mM (N+) nitrate supplies. Growth conditions are detailed in the legend of Fig. 11.4. Traits measured at N− are plotted versus those measured at N+ . The different genotypes are represented by horizontal and vertical lines (standard deviations), which intersect at the mean values of the trait. The dashed line, with a slope of one, serves as a guide to compare the two nitrate conditions. **a** root biomass (R), **b** shoot biomass (S), **c** total biomass (R + S), **d** root to shoot biomass ratio (R:S), **e** length of the primary root (L_{PR}), **f** length of primary root zone 2 (L_{Z2}), **g** length of primary root zone 3 (L_{Z3}), **h** length of primary root zone 4 (L_{Z4}), **i** number of lateral roots (N_{LR}), **j** sum of lateral root length (ΣL_{LR}), **k** mean length of lateral root (ML_{LR}), **l** total root length (TRL), **m** density of lateral roots in zone 1 (D_{LR} − Z1), **n** density of lateral roots in zone 2 (D_{LR} − Z2), **o** specific root length (SRL), **p** definition of the primary root zones. $n = 2$ (6 pooled organs) for biomass traits, $n = 12$ for root morphological traits

d). Concerning the root morphological traits, the length of primary root (L_{PR}, +32%), the lengths of primary root zone 4 (L_{Z4}, +51%), the sum of lateral root lengths (ΣL_{LR}, +25%), the mean length of lateral roots (ML_{LR}, +37%), the total root length

(TRL, +27%), the density of lateral roots in zone 2 (D_{LR} − Z2, +4%), and the specific root length (SRL, +26%) were more important, the density of lateral roots in zone 1 (D_{LR} − Z1, −9%) was lower, while the lengths of primary root zone 2 and 3 (L_{Z2}, L_{Z3}) and the number of lateral roots (N_{LR}) did not change during N− compared to N+ conditions (Fig. 11.5e–o). All mentioned differences between N treatments were significantly ($P < 0.01$) different. We observed a large diversity of root morphologies between genotypes. For example, the differences between the two most extreme genotype values were in the range of 36 and 47% for $L_{PR,}$ and of 143 and 124% for ΣL_{LR}, respectively, at N− and N+ . The two inbred lines (ES MAMBO and PAMELA) had some of the poorest root morphological features among the panel (Fig. 11.4). This testifies the overall superiority of hybrids compared to inbred lines as reported by Thomas et al. (2016b).

The Assessement of On-Field Performance

After phenotyping root morphology at a young developmental stage, we challenged the performance of these 28 genotypes to the field conditions. Crop field trial was conducted at the Centre pour l'Agronomie et l'Agro-industrie de la Province du Hainaut (CARAH) in Belgium. The genotypes grew in microplots (13.5 m^2) with a randomized complete block design in four replicates. Culture conditions and fertilization level are described in Fig. 11.6 legend. Seed yield and seed quality traits, as well as optical indices, are listed in Table 11.2. The values of the seed yield and seed quality traits (Table 11.2) were falling in the range of the survey conducted by Stahl et al. (2017). Large variations of these traits were found among the diversity

Table 11.2 Definitions and values of traits measured in the field. Values are the means of 28 genotypes (each genotype assessed in four microplots) ± std

Seed yield and seed quality traits		
SY	Seed yield corrected to a standard water content of 9%	4,756 ± 442 kg ha^{-1}
TSW	1,000 seed weight with moisture adjusted to 9%	3.85 ± 0.25 g
NConc	Concentration of N in dry seeds	3.20 ± 0.09%
ProtConc	Concentration of protein in dry seeds	20.0 ± 0.6%
OilConc	Concentration of oil in dry seeds	47.0 ± 1.0%
SNU	Seed N uptake = NConc x SY	138 ± 12 kg ha^{-1}
ProteinY	Protein yield = ProtConc x SY	865 ± 73 kg ha^{-1}
OilY	Oil yield = OilConc x SY	2,037 ± 213 kg ha^{-1}
GLS	Glucosinolates concentration in dry seeds	13.1 ± 1.9 µmol g^{-1}
Optical indexes measured on the leaf canopy		
CHL	Chlorophyll index	51.2 ± 1.7
FLAV	Flavonol index	1.40 ± 0.06
NBI	Nitrogen balance index	36.8 ± 1.9

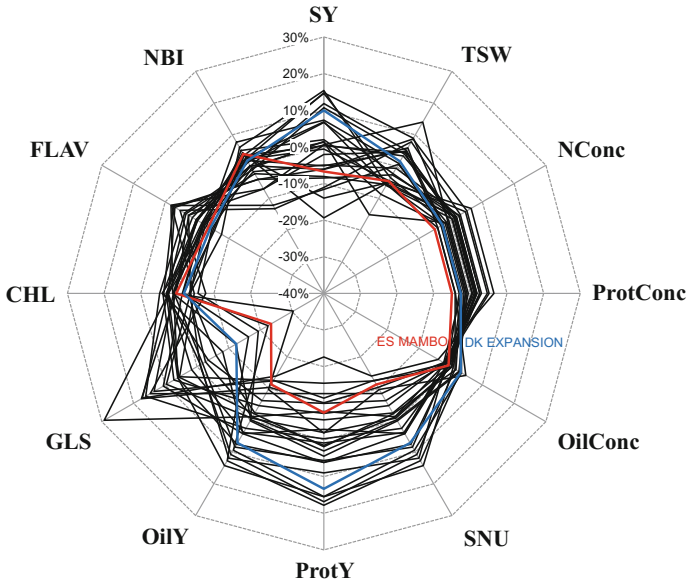


Fig. 11.6 Field traits of winter oilseed rape genotypes. The spider plot shows for 28 genotypes, the percentage variation of the normalized trait values relative to the average of the panel (0% indicates no difference compared to average). On each of the axes is plotted one trait: seed yield (SY), 1,000 seed weight (TSW), seed N concentration (NConc), seed protein concentration (ProtConc), seed oil concentration (OilConc), seed N uptake (SNU), seed protein yield (ProtY), seed oil yield (OilY), seed glucosinolates concentration (GLS), chlorophyll index (CHL), flavonol index (FLAV), and nitrogen balance index (NBI). SY was measured with the Delta plot combine harvester (Wintersteiger), NConc, ProtConc, OilConc and GLS with the XDS NIR analyzer (Foss), TSW with the Numigral seed counter (Chopin Technologies) and CHL, FLAV and NBI with the Dualex optical device (Scientific +) in leaves at flowering stage. ES MAMBO is indicated by the red curve and DK EXPANSION by the blue curve. Field experiments were conducted at CARAH in Belgium (50°36′41″ N, 3°45′20″ E) on a loamy soil using a complete randomized block design with four replicates. Date of sowing: September 8, 2015, seeding rate: 60 seeds m^{-2}, weed control: metazachlor, quinmerac, and clomazone at pre-emergence, N-fertilizer application: 90 kg N ha^{-1} at the beginning of the culture (September 29, 2015) and 82 kg N ha^{-1} at the beginning of the spring vegetation (March 21st 2016), date of harvest: July 30, 2016

set: 34% difference between the two most extreme genotypes for seed yield (YS), 29% for 1,000 seed weight (TSW), 10% for seed N concentration (NConc) and seed protein concentration (ProtConc), 4% for seed oil concentration (OilConc), 25% for seed N uptake (SNU), 40% for seed protein yield (ProtY), 25% for seed oil yield (OilY) and 59% for glucosinolate concentration (GLS) (Fig. 11.6). An optical sensor device was used to determine three indices at the leaf canopy level during the flowering stage: chlorophyll (CHL), flavonol (FLA), and nitrogen balance index (NBI), which is the ratio between the two first indices and an indicator of N sufficiency (Cerovic et al. 2015). Differences between the most extreme genotypes were 9% for CHL, 16% for FLA, and 59% for NBI (Fig. 11.6).

Correlations Between Phenotypic Traits

Pearson's product moment correlation coefficients (*cor*) were calculated between measured traits (Fig. 11.7). We will successively detail and discuss some significant ($P < 0.05$) inter-trait phenotypic correlations in hydroponics and field environments separately, then between these two data sets.

(i) During hydroponics, both R and S biomasses correlated strongly and positively with L_{PR}, N_{LR}, ΣL_{LR}, D_{LR} − Z1, and TLR (min. *cor* = 0.69 for R and 0.61 for S; max. *cor* = 0.84 for R and 0.75 for S), with some stronger correlations observed at N− (Fig. 11.7). We previously defined a root system ideotype for maximizing spatial N capture with profuse and profound root branching (Fig. 11.1). However, that strategy may express conflicting views because increased root production could negatively impact on aboveground biomass and final yield. Nonetheless, our concept proves here to be pertinent in laboratory setups, as genotypes with high total root length also produced high shoot biomass at two N

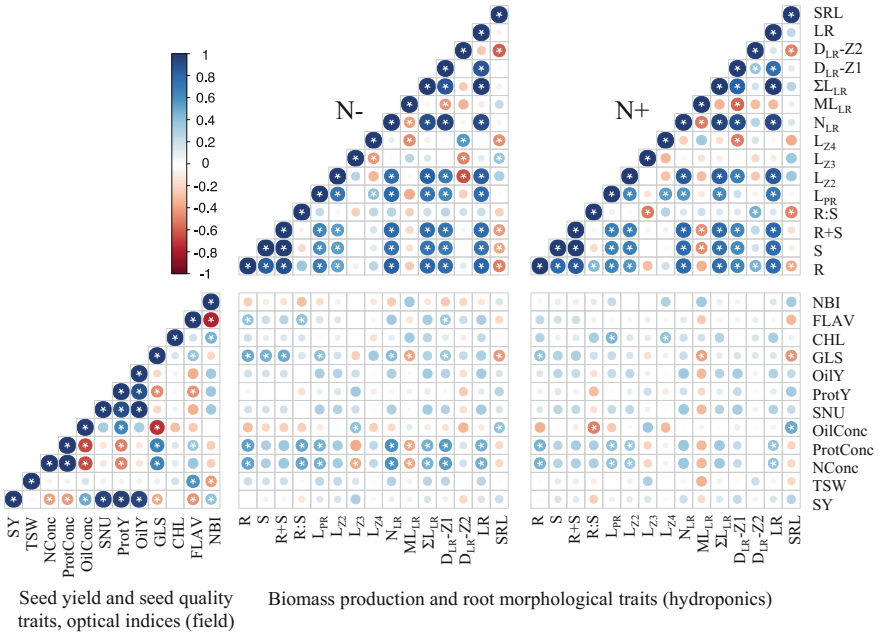


Fig. 11.7 Inter-trait phenotypic correlations in winter oilseed rape genotypes. Biomass production and root morphological traits were measured at 0.2 mM nitrate (N−) or 5 mM nitrate (N+) in hydroponics. Seed yield and seed quality traits as well as optical indices were measured in the field. The trait definitions are given in Tables 11.1 and 11.2. Circle area and color intensity indicate the strength of the correlation. Blue or red colors indicate positive or negative correlations. Star indicates a correlation coefficient significantly ($p < 0.05$) different from zero. Graphics are drawn with R package corrplot (version 0.77)

supplies (Fig. 11.7). This suggests that high root branching can be a positive NUE indicator, thereby supporting the long-term objective of our research.

(ii) During field trial, SY correlated negatively with NConc and ProtConc (*cor* = −0.42) but positively with OilConc (*cor* = 0.5), while OilConc correlated negatively with NConc and ProtConc (*cor* = −0.65 and −0.67 correspondingly) (Fig. 11.7). Since SNU, ProtY, and OilY are, respectively, the products of NConc, ProtConc, and OilConc with SY (Table 11.2), these traits implicitly show high positive correlations. It is well known that oil and protein concentrations in seeds are negatively correlated (Bouchet et al. 2016b; Stahl et al. 2017) and some identified QTLs affect these two traits in an opposite manner (Chao et al. 2017). A possible reason can be that oil and protein biosynthetic pathways happen in the endoplasmic reticulum during the same period of seed development, suggesting a competition with one another pathway for carbon resources utilization (Chao et al. 2017; Stahl et al. 2017). Glucosinolates (GLS) are toxic sulfur-containing secondary metabolites playing a role in plant defence against pests (Burow and Halkier 2017). Their consumption may be linked to a reduced number of cancer (e.g., colon) incidences (Wu et al. 2013) but too high GLS in food is goitrogen (Eisenbrand and Gelbke 2016). For that reason, research efforts have achieved to reduce GLS as low as 8–15 μmol g^{-1} in seeds of oilseed rape (Table 11.2) (Nour-Eldin et al. 2017). We found that GLS correlated positively with NConc and ProtConc (*cor* = 0.66 and 0.67 respectively) (Fig. 11.7), and this is probably because GLS is derived from amino acids. Furthermore, GLS negatively correlated with OilConc (*cor* = −0.74) (Fig. 11.7), comforting the results by Gu et al. (2017) who found a negative correlation between GLS and oil body size in seed cells. Finally, optical parameters measured at the flowering stage could somehow predict yield traits. FLA correlated negatively with SY (*cor* = −0.45) but positively with TSW (*cor* = 0.58), while NBI correlated positively with SY (*cor* = 0.39). This opens more considerations regarding to the "stay-green" strategy with delayed senescence to improve source to sink relationships (Bouchet et al. 2016a, Havé et al. 2017).

(iii) When comparing hydroponics and field data, R biomass and root morphological traits (L_{PR}, N_{LR}, ΣL_{LR}, D_{LR} − Z1, and TRL) correlated positively with seed NConc and ProtConc (*cor* ranging from 0.30 to 0.59) (Fig. 11.7). Topp et al. (2016) pointed out that there can be sometimes little correlation between root phenotypes collected in controlled and field environments. Nonetheless, Thomas et al. (2016b) already indicated that the primary root length measured with the pouch-and-wick culture system was a good predictor of emergence, early vigor, and seed yield in the field. Our preliminary results are encouraging us to conduct more field trials and to test the genotype-by-environment interactions in different pedoclimatic conditions and with different N fertilization levels, as emphasized by Bouchet et al. (2016b) and Stahl et al. (2017).

A Rhizotron Setting to Observe Root Growth Belowground

We previously depicted a high-throughput screen of 2D root morphology in controlled conditions. Nonetheless the field greatly differs from the laboratory environment. Hence, in the ground, the roots are exploring strata with different texture and with heterogeneous distribution of water and nutrients (Pierret et al. 2016). The microbial communities of the rhizosphere also impact on the root growth of their host plants (Verbon and Liberman 2016). Undeniably, observing crop rooting in situ remains an incredible challenge: A drawback is to extract an entire root system from the soil substrate without deterioration. We will illustrate how a rhizotron system can be used to examine root proliferation in deep soil horizons with a camera.

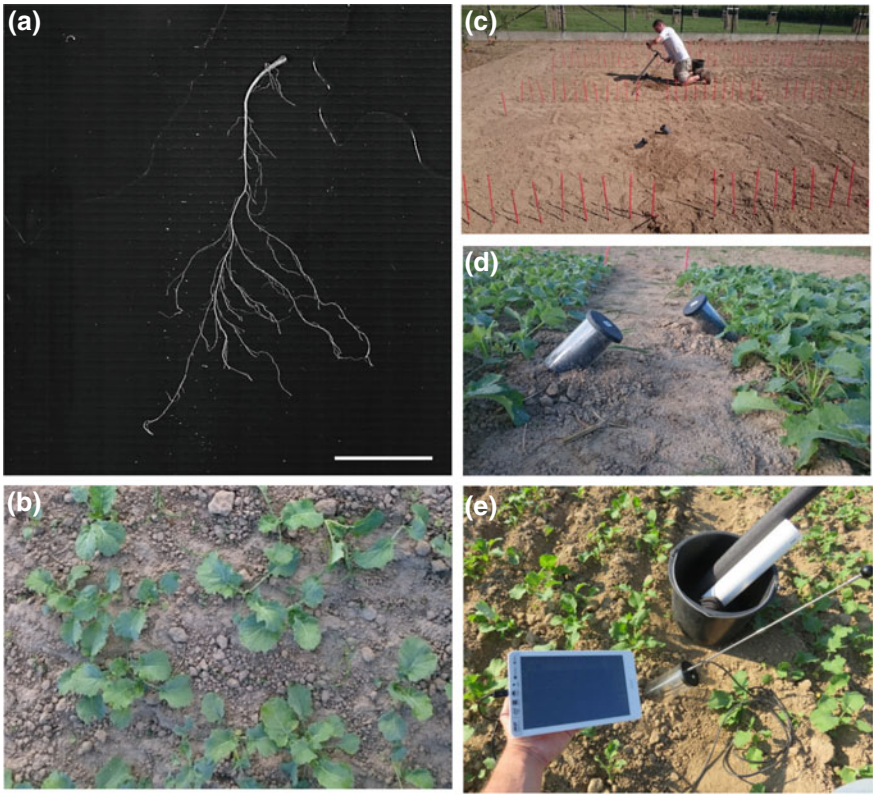


Fig. 11.8 Field observations of root morphology. **a**–**b** Soil coring of winter oilseed rape plantlets at stage BBCH 14 (four leaves unfolded). Root system after being washed (**a**). Scale bar = 5 cm. Aerial part prior to coring (**b**). **c**–**d** Tube rhizotron system for nondestructive observation of root development. Placement of clear acrylic tubes (6.35 cm inner diameter and 105 cm in length) at an angle of 60° between the field rows (**c**). Close-up of tubes sealed with plugs (**c**). The CI 600 in situ Root Imager (CID Bio-Science, USA) is inserted in the tube to capture digital images of roots in the soil

Common field observations of root organs consist of trench excavation or soil coring (Topp et al. 2016). Soil cores are obtained after washing roots from adherent soil substrate with caution to avoid damage (Fig. 11.8a, b). Because that process is tedious and destructive, it does not allow the simultaneous and over time observation of a large number of root organs. By overcoming that limitation, a rhizotron system gives the ability to nondestructively incorporate elements of time and depth to root density information over the seasons (Muñoz-Romero et al. 2010; Topp et al. 2016). In the next illustration, we have monitored root growth of two winter rapeseed genotypes (DK EXPANSION and ES MAMBO) with contrasting root morphologies, as identified in hydroponics (Fig. 11.4). Transparent tubes were installed down to 70 cm profile depth at an angle of 45° between the field rows (Fig. 11.8c–e). We observed the root intersections with an in situ camera and found that the root system rapidly developed within weeks after sowing until winter came (Fig. 11.9). During the spring, no marked root development around the tubes was visible.

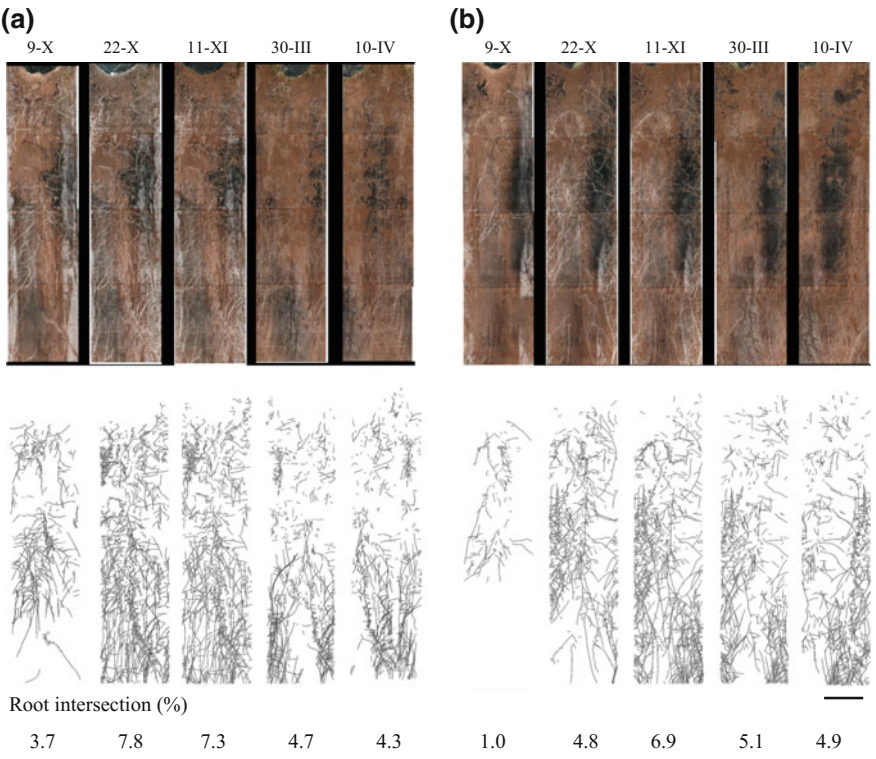


Fig. 11.9 Observation of root development belowground with a camera system. Original (top) and skeletonized (bottom) root intersection images of the two winter oilseed rape genotypes DK EXPANSION (**a**) and ES MAMBO (**b**) Images were taken at five different days during the culture (year 2015-206) with the CI-600 in situ Root Imager (CID Bio-Science, USA). Scale bar: 10 cm

These observations are in accordance with Barraclough (1989) who estimated after soil coring, that as much as two-thirds of the total crop root production were already achieved before winter at a depth of 0–180 cm. The author also reported that more than three quarters of the root total length were deployed in the top 40-cm horizon. Besides, our rhizotron system permitted to discriminate between the two genotypes. For instance, the hybrid cultivar DK EXPANSION had faster root proliferation than ES MAMBO during the first weeks after germination (Fig. 11.9), thereby confirming the hydroponic observations at a young developmental stage (Fig. 11.4).

Conclusion

This chapter illustrated how synergistic activities in laboratory and field environments can be used to gain insights on the root growth and development of winter oilseed rape. Such screen may speed up the delivery of genotypes with great morphological features. We provided an example of a root phenotyping procedure in controlled setups with a proof of concept evaluation. The rationale is that N-efficient genotypes are those with a dense root system to explore an important soil volume. In this study, the genotypes with high total root length values produced high aerial biomass. This indicates no trade-off between those traits during hydroponic culture. We are currently dissecting the genetic bases underlying nitrate-dependent root morphology changes using several genomic strategies that exploit the natural variation in large collections of genotypes. That information could be incorporated in crop improvement programs (marker-assisted selection with genes holding the promise to optimize root system architecture). Studies for enhancing NUE must be seen in a global strategy with multiple factors to be considered. Introgression of positive root traits and high N transport activity must be sought in genotypes with high N utilization efficiency.

Acknowledgements We thank László Kupcsik for setting up the pouch-and-wick system with Julien.Louvieaux

Sources of funding This work is supported by an incentive research grant from the Fonds de la Recherche Scientifique (F.R.S.-FNRS) and by Wiener Anspach Fondation at ULB. Christian. Hermans. is an F.R.S.-FNRS Research Associate

References

Adu M, Chatot A, Wiesel L, Bennett M, Broadley M, White P, Dupuy X (2014) A scanner system for high-resolution quantification in root growth dynamics of *Brassica rapa* genotypes. J Exp Bot 65:2039–2048

Araya T, von Wirén N, Takahashi H (2014) CLE peptides regulate lateral root development in response to nitrogen nutritional status of plants. Plant Signal Behav 9:e29302

Barraclough P (1989) Root growth, macro-nutrient uptake dynamics and soil fertility requirements of a high-yielding winter oilseed rape crop. Plant Soil 119:59–70

Barraclough P, Howart J, Jones J, Lopez-Bellido R, Parmar S, Shepherd C, Hawkesford M (2010) Nitrogen efficiency of wheat: genotypic and environmental variation and prospects for improvement. Eur J Agron 33:1–11

Berry PM, Spink J, Foulkes MJ, White PJ (2010) The physiological basis of genotypic differences in nitrogen use efficiency in oilseed rape (*Brassica napus* L.). Field Crops Res 119:365–373

Birnbaum KD (2016) How many ways are there to make a root? Curr Opin Plant Biol 34:61–67

Bouchet A-S, Nesi N, Bissuel C, Bregeon M, Lariepe A, Navier H, Ribière N, Orsel M, Grezes-Besset B, Renard M, Laperche A (2014) Genetic control of yield and yield components in winter oilseed rape (*Brassica napus* L.) grown under nitrogen limitation. Euphytica 199:183–205

Bouchet A-S, Laperche A, Bissuel-Belaygue C, Snowdon R, Nesi N, Stahl A (2016a) Nitrogen use efficiency in rapeseed. A review. Agron Sustain Dev 36:38

Bouchet A-S, Laperche A, Bissuel-Belaygue C, Baron C, Morice J, Rousseau-Gueutin M, Dheu JE, George P, Pinochet X, Foubert T, Maes O, Dugué D, Guinot F, Nesi N (2016b) Genetic basis of nitrogen use efficiency and yield stability across environments in winter rapeseed. BMC Genet 17:131

Bouguyon E, Brun F, Meynard D, Kubeš M, Pervent M, Leran S, Lacombe B, Krouk G, Guiderdoni E, Zažímalová E, Hoyerová K, Nacry P, Gojon A (2015) Multiple mechanisms of nitrate sensing by Arabidopsis nitrate transceptor NRT1.1. Nat Plants 1:15015

Bouguyon E, Perrine-Walker F, Pervent M, Rochette J, Cuesta C, Benkova E, Martinière A, Bach L, Krouk G, Gojon A, Nacry P (2016) Nitrate controls root development through posttranscriptional regulation of the NRT1.1/NPF6.3 transporter/sensor. Plant Physiol 172:1237–1248

Burkart MR, Stoner JD (2007) Nitrate in aquifers beneath agricultural systems. Water Sci Technol 56:59–69

Burow M, Halkier BA (2017) How does a plant orchestrate defense in time and space? Using glucosinolates in Arabidopsis as case study. Curr Opin Plant Biol 38:142–147

Cerovic ZG, Ghozlen NB, Milhade C, Obert M, Debuisson S, Le Moigne M (2015) Nondestructive diagnostic test for nitrogen nutrition of grapevine (*Vitis vinifera* L.) based on dualex leaf-clip measurements in the field. J Agri Food Chem 63:3669–3680

Chao H, Wang H, Wang X, Guo L, Gu J, Zhao W, Li B, Chen D, Raboanatahiry N, Li M (2017) Genetic dissection of seed oil and protein content and identification of networks associated with oil content in *Brassica napus*. Sci Rep 7:46295

Chen J, Zhang Y, Tan Y, Zhang M, Zhu L, Xu G, Fan X (2016) Agronomic nitrogen-use efficiency of rice can be increased by driving *OsNRT2.1* expression with the *OsNAR2.1* promoter. Plant Biotechnol J 14:1705–1715

Chen J, Fan X, Qian K, Zhang Y, Song M, Liu Y, Xu G, Fan X (2017) *pOsNAR2.1:OsNAR2.1* expression enhances nitrogen uptake efficiency and grain yield in transgenic rice plants. Plant Biotechnol J. https://doi.org/10.1111/pbi.12714

De Pessemier J, Chardon F, Juraniec M, Delaplace P, Hermans C (2013) Natural variation of the root morphological response to nitrate supply in *Arabidopsis thaliana*. Mech Dev 130:45–53

Den Herder G, Van Isterdael G, Beeckman T, De Smet I (2010) The roots of a new green revolution. Trends Plant Sci 15:600–607

Eisenbrand G, Gelbke HP (2016) Assessing the potential impact on the thyroid axis of environmentally relevant food constituents/contaminants in humans. Arch Toxic 90: 1841–1857

Fan X, Tang Z, Tan Y, Zhang Y, Luo B, Yang M, Lian X, Shen Q, Miller AJ, Xu G (2016) Overexpression of a pH-sensitive nitrate transporter in rice increases crop yields. Proc Natl Acad Sci USA 113:7118–7123

Fan X, Naz M, Fan X, Xuan W, Miller AJ, Xu G (2017) Plant nitrate transporters: from gene function to application. J Exp Bot 68:2463–2475

Fletcher RS, Mullen JL, Heiliger A, McKay JK (2015) QTL analysis of root morphology, flowering time, and yield reveals trade-offs in response to drought in *Brassica napus*. J Exp Bot 66:245–256

Forde BG (2014) Nitrogen signalling pathways shaping root system architecture: an update. Curr Opin Plant Biol 21:30–36

Fu Y, Yi H, Bao J, Gong J (2015) LeNRT2.3 functions in nitrate acquisition and long-distance transport in tomato. FEBS Lett 589:1072–1079

Gan Y, Bernreiter A, Filleur S, Abram B, Forde BG (2012) Overexpressing the *ANR1* MADS-box gene in transgenic plants provides new insights into its role in the nitrate regulation of root development. Plant Cell Physiol 53:1003–1016

Garnett T, Conn V, Kaiser B (2009) Root based approaches to improving nitrogen use efficiency in plants. Plant, Cell Environ 32:1272–1283

Gehringer A, Snowdon R, Spiller T, Basunanda P, Friedt W (2007) New oilseed rape (*Brassica napus*) hybrids with high levels of heterosis for seed yield under nutrient-poor conditions. Breed Sci 57:315–320

Gent L, Forde BG (2017a) How do plants sense their nitrogen status? J Exp Bot 68:2531–2539

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2008) Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci USA 105:803–808

Gruber BD, Giehl RF, Friedel S, von Wirén N (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. Plant Physiol 163:1611–1679

Gu J, Chao H, Wang H, Li Y, Li D, Xiang J, Gan J, Lu G, Zhang X, Long Y, Li M (2017) Identification of the relationship between oil body morphology and oil content by microstructure comparison combining with QTL analysis in *Brassica napus*. Front Plant Sci 7:1989

Han M, Okamoto M, Beatty PH, Rothstein SJ, Good AG (2015) The genetics of nitrogen use efficiency in crop plants. Ann Rev Genet 49:269–289

Havé M, Marmagne A, Chardon F, Masclaux-Daubresse C (2017) Nitrogen remobilization during leaf senescence: lessons from Arabidopsis to crops. J Exp Bot 68:2513–2529

Hermans C, Hammond JP, White PJ, Verbruggen N (2006) How do deficiencies of essential mineral elements alter biomass allocation? Trends Plant Sci 11:610–617

Hermans C, Porco S, Verbruggen N, Bush D (2010a) Chitinase-like protein CTL1 plays a role in the root system plasticity in response to multiple environmental signals. Plant Physiol 152:904–917

Hermans C, Vuylsteke M, Coppens F, Craciun A, Inzé D, Verbruggen N (2010b) The early transcriptomic changes induced by magnesium deficiency in *Arabidopsis thaliana* reveal the alteration of circadian clock genes expression in roots and the triggering of ABA-responsive genes. New Phytol 187:119–131

Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inzé D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14:2339–2351

Hu B, Wang W, Ou S, Tang J, Li H, Che R, Zhang Z, Chai X, Wang H, Wang Y, Liang C, Liu L, Piao Z, Deng Q, Deng K, Xu C, Liang Y, Zhang L, Li L, Chu C (2015) Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. Nat Genet 47:834–838

Kamh M, Wiesler F, Ulas A, Horst WJ (2005) Root growth and N-uptake activity of oilseed rape (*Brassica napus* L.) cultivars differing in nitrogen efficiency. J Plant Nutr Soil Sci 168:130–137

Kant S, Bi YM, Rothstein SJ (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J Exp Bot 62:1499–1509

Kessel B, Schierholt A, Becker H (2012) Nitrogen use efficiency in a genetically diverse set of winter oilseed rape (*Brassica napus* L.). Crop Sci 52:2546–2554

Kiba T, Krapp A (2016) Plant nitrogen acquisition under low availability: Regulation of uptake and root architecture. Plant Cell Physiol 57:707–714

Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K, Zazimalova E, Benkova E, Nacry P, Gojon A (2010) Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Dev Cell 18:927–937

Laskowski M, Ten Tusscher KH (2017). Periodic lateral root priming: what makes it tick? The Plant Cell 29:432–444

Lavagi I, Estelle M, Weckwerth W, Beynon J, Bastow RM (2012) From bench to bountiful harvests: a road map for the next decade of Arabidopsis research. Plant Cell 24:2240–2247

Lavenus J, Goh T, Roberts I, Guyomarc'h S, Lucas M, De Smet I, Fukaki H, Beeckman T, Bennett M, Laplaze L (2013) Lateral root development in Arabidopsis: fifty shades of auxin. Trends Plant Sci 18:450–458

Leblanc A, Renault H, Lecourt J, Etienne P, Deleu C, Le Deunff E (2008) Elongation changes of exploratory and root hair systems induced by aminocyclopropane carboxylic acid and aminoethoxyvinylglycine affect nitrate uptake and *BnNrt2.1* and *BnNrt1.1* transporter gene expression in oilseed rape. Plant Physiol 146:1928–1940

Lemaire L, Deleu C, Le Deunff E (2013) Modulation of ethylene biosynthesis by ACC and AIB reveals a structural and functional relationship between the $K^{15}NO_3$ uptake rate and root absorbing surfaces. J Exp Bot 64:2725–2737

Léran S, Varala K, Boyer JC, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W, Geiger D, Gojon A, Gong JM, Halkier BA, Harris JM, Hedrich R, Limami AM, Rentsch D, Seo M, Tsay YF, Zhang M, Coruzzi G, Lacombe B (2014) A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci 19:5–9

Li P, Chen F, Cai H, Liu J, Pan Q, Liu Z, Gu R, Mi G, Zhang F, Yuan L (2015) A genetic relationship between nitrogen use efficiency and seedling root traits in maize as revealed by QTL analysis. J Exp Bot 66:3175–3188

Li X, Zeng R, Liao H (2016) Improving crop nutrient efficiency through root architecture modifications. J Integr Plant Biol 58:193–202

Li H, Hu B, Chu C (2017) Nitrogen use efficiency in crops: lessons from Arabidopsis and rice. J Exp Bot 68:2477–2488

Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci USA 102:13693–13698

Liu KH, Tsay YF (2003) Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J 22:1005–1013

Liu J, You L, Amini M, Obersteiner M, Herrero M, Zehnder AJ, Yang H (2010) A high-resolution assessment on global nitrogen flows in cropland. Proc Natl Acad Sci USA 107:8035–8040

López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol 6:280–287

Gent L, Forde BG (2017b) How do plants sense their nitrogen status? J Exp Bot 68:2531–2539

Lynch J (1995) Root architecture and plant productivity. Plant Physiol 109:7–13

Lynch J (2007) Roots of the second green revolution. Aust J Bot 55:493–512

Lynch JP, Brown KM (2012) New roots for agriculture: exploiting the root phenome. Philos Trans R Soc Lond B Biol Sci 367:1598–1604

Lynch J (2013) Steep, cheap and deep: an ideotype to optimize water and N acquisition by maize root systems. Ann Bot 112:347–357

Malamy JE, Benfey PN (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development 124:33–44

Marschner H (2012) In: Marschner P (ed) Marschner's mineral nutrition of higher plants, 3rd edn. Academic Press, Elsevier

McAllister CH, Beatty PH, Good AG (2012) Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10:1011–1025

Miersch S, Gertz A, Breuer F, Schierholt A, Becker HC (2016) Influence of the semi-dwarf growth type on nitrogen use efficiency in winter oilseed rape. Crop Sci 56:2952–2961

Moll R, Kamprath E, Jackson W (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron J 74:562–564

Moore FC, Lobell DB (2015) The fingerprint of climate trends on European crop yields. Proc Natl Acad Sci USA 112:2670–2675

Mounier E, Pervent M, Ljung K, Gojon A, Nacry P (2014) Auxin-mediated nitrate signalling by NRT1.1 participates in the adaptive response of Arabidopsis root architecture to the spatial heterogeneity of nitrate availability. Plant, Cell Environ 37:162–174

Muñoz-Romero V, Benítez-Vega J, López-Bellido L, López-Bellido RJ (2010) Monitoring wheat root development in a rainfed vertisol: tillage effect. Eur J Agron 33:182–187

Nacry P, Bouguyon E, Gojon A (2013) Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating ressource. Plant Soil 370:1–29

Näsholm T, Kielland K, Ganeteg U (2009) Uptake of inorganic nitrogen by plants. New Phytol 182:31–48

Noguero M, Lacombe B (2016) Transporters involved in root nitrate uptake and sensing by *Arabidopsis*. Front Plant Sci 7:1391

Nour-Eldin HH, Madsen SR, Engelen S, Jørgensen ME, Olsen C, Andersen JS, Seynnaeve D, Verhoye T, Fulawka R, Denolf P, Halkier BA (2017) Reduction of antinutritional glucosinolates in Brassica oilseeds by mutation of genes encoding transporters. Nature Biotechnol 35:377–382

Nyikako J, Schierholt A, Kessel B, Becker HC (2014) Genetic variation in nitrogen uptake and utilization efficiency in a segregating DH population of winter oilseed rape. Euphytica 199:3–11

O'Brien JA, Vega A, Bouguyon E, Krouk G, Gojon A, Coruzzi G, Gutiérrez RA (2016) Nitrate transport, sensing, and responses in plants. Mol Plant 9:837–856

Parker JL, Newstead S (2014) Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1. Nature 507:68–72

Pestsova E, Lichtblau D, Christian Wever C, Thomas Presterl T, Therese Bolduan T, Milena Ouzunova M, Peter Westhoff P (2016) QTL mapping of seedling root traits associated with nitrogen and water use efficiency in maize. Euphytica 3:585–602

Pierret A, Maeght J-L, Clément C, Montoroi J-P, Hartmann C, Gonkhamdee S (2016) Understanding deep roots and their functions in ecosystems: an advocacy for more unconventional research. Ann Bot 118:573–592

Pound MP, French AP, Atkinson JA, Wells DM, Bennett MJ, Pridmore T (2013) RootNav: navigating images of complex root architectures. Plant Physiol 162:1802–1814

Pradhan P, Fischer G, van Velthuizen H, Reusser DE, Kropp JP (2015) Closing yield gaps: how sustainable can we be? PLoS ONE 10:e0129487

Rahman M, McClean P (2013) Genetic analysis on flowering time and root system in *Brassica napus* L. Crop Sci 53:141

Ray DK, Mueller ND, West PC, Foley JA (2013) Yield trends are insufficient to double global crop production by 2050. PLoS ONE 8:e66428

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A (2006a) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci USA 103:19206–19211

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A (2006b) A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. Plant Physiol 140:909–921

Richard AM, Diaz JH, Kaye AD (2014) Reexamining the risks of drinking-water nitrates on public health. The Ochsner J 14:392–398

Schulte auf'm Erley G, Wijaya K-A, Ulas A, Becker H, Wiesler F, Horst WJ (2007) Leaf senescence and N uptake parameters as selection traits for nitrogen efficiency of oilseed rape cultivars. Physiol Plant 130:519–531

Schulte auf'm Erley G, Behrens T, Ulas A, Wiesler F, Horst WJ (2011) Agronomic traits contributing to nitrogen efficiency of winter oilseed rape cultivars. Field Crops Res 124: 114–123

Stahl A, Pfeifer M, Frisch M, Wittkop B, Snowdon RJ (2017) Recent genetic gains in nitrogen use efficiency in oilseed rape. Front Plant Sci 8:963

Sun J, Bankston JR, Payandeh J, Hinds TR, Zagotta WN, Zheng N (2014) Crystal structure of the plant dual-affinity nitrate transporter NRT1.1. Nature 507:73–77

Sun C-H, Yu J-Q, Hu D-G (2017) Nitrate: a crucial signal during lateral roots development. Front Plant Sci 8:485

Tegeder M, Rentsch D (2010) Uptake and partitioning of amino acids and peptides. Mol Plant 3:997–1011

Thomas C, Graham N, Hayden R, Meacham M, Neugebauer K, Nightingale M, Dupuy L, Hammond J, White P, Broadley M (2016a) High-thoughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.). Ann Bot 118:655–665

Thomas CL, Alcock TD, Graham NS, Hayden R, Matterson S, Wilson L, Young SD, Dupuy LX, White PJ, Hammond JP, Danku JM, Salt DE, Sweeney A, Bancroft I, Broadley MR (2016b) Root morphology and seed and leaf ionomic traits in a *Brassica napus* L. diversity panel show wide phenotypic variation and are characteristic of crop habit. BMC Plant Biol 16:214

Topp CN, Bray AL, Ellis NA, Liu Z (2016) How can we harness quantitative genetic varation in crop root systems for agricultural improvement? J Integr Biol 58:213–225

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM (1993) The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell 72:705–713

Ulas A, Schulte auf'm Erley G, Kamh M, Wiesler F, Horst WJ (2012) Root-growth characteristics contributing to genotypic variation in nitrogen efficiency of oilseed rape. J Plant Nutr Soil Sci 175:489–498

Undurraga SF, Ibarra-Henríquez C, Fredes I, Álvarez JM, Gutiérrez RA (2017) Nitrate signaling and early responses in Arabidopsis roots. J Exp Bot 68:2541–2551

Verbon EH, Liberman LM (2016) Beneficial microbes affect endogenous mechanisms controlling root development. Trends Plant Sci 21:218–219

Vermeer JE, Geldner N (2015) Lateral root initiation in *Arabidopsis thaliana*: a force awakens. F1000Prime Rep 7:32

Wang R, Liu D, Crawford NM (1998) The Arabidopsis CHL1 protein plays a major role in high-affinity nitrate uptake. Proc Natl Acad Sci USA 95:15134–15139

Wick K, Heumesser C, Schmid E (2012) Groundwater nitrate contamination: Factors and indicators. J Environ Manag 111:178–186

Wu QJ, Yang Y, Vogtmann E, Wang J, Han LH, Li HL, Xiang YB (2013) Cruciferous vegetables intake and the risk of colorectal cancer: a meta-analysis of observational studies. Ann Oncol 24:1079–1087

Xiao Q, De Gernier H, Kupcsik L, De Pessemier J, Dittert K, Fladung K, Verbruggen N, Hermans C (2015) Natural genetic variation of *Arabidopsis thaliana* root morphological response to magnesium supply. Crop Pasture Sci 66:1249–1258

Xu GH, Fan XR, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Ann Rev Plant Biol 63:153–182

Yu P, Li X, White PJ, Li C (2015) A large and deep root system underlies high nitrogen-use efficiency in maize production. PLoS ONE 10:e012693

Zhang H, Forde BG (1998) An *Arabidopsis* MADS Box gene that controls nutrient-induced changes in root architecture. Science 279:407–409

Zhang H, Jennings A, Barlow PW, Forde BG (1999) Dual pathways for regulation of root branching by nitrate. Proc Natl Acad Sci USA 96:6529–6534

Zhang H, Rong H, Pilbeam D (2007) Signalling mechanisms underlying the morphological responses of the root system to nitrogen in *Arabidopsis thaliana*. J Exp Bot 58:2329–2338

Zhang Y, Thomas CL, Xiang J, Long Y, Wang X, Zou J, Luo Z, Ding G, Cai H, Graham NS, Hammond JP, King GJ, White PJ, Xu F, Broadley MR, Shi L, Meng J (2016) QTL meta-analysis of root traits in *Brassica napus* under contrasting phosphorus supply in two growth systems. Sci Rep 6:33113

Chapter 12
Genetic Improvement of Nitrogen Use Efficiency in Oilseed Rape

Andreas Stahl and Rod Snowdon

Oilseed Rape in the Global Context

Oilseed rape (*Brassica napus* L.), also known as rapeseed or canola, is mainly grown for its high-quality vegetable oil for human nutrition, for industrial use as a substitute for fossil oil, and for its high-value protein in the extraction meal. With a ten-year average production volume of around 58 M metric tons (2004–2013, http://faostat.fao.org/), oilseed rape is the third most important oil crop globally behind soybean and oil palm. During the past ten years, the average worldwide production area exceeded 30 M ha annually, particularly in Canada (6.5 M ha), China (7 M ha), the European Union (EU; 7.8 M ha), Australia (1.8 M ha), and the United States of America (0.5 M ha). Within the EU, France (1.43 M ha) and Germany (1.40 M ha) are the countries with the biggest production areas. In South Asia (in particular India), mustard (*Brassica juncea*) is the dominating from. The annual on-farm yield increases for the period from 1991 to 2010 in Canada, China, India, France, and Germany were estimated at 33, 37, 15, 21, 68 kg/ha, corresponding to 1.7, 2, 1.4, 0.6, and 1.7%, respectively (Fischer et al. 2014). More recent data from Canada report increases of 54 kg/ha and year (2.6%) between 2000 and 2013 (Morrison et al. 2016), reflecting a recent trend toward higher performing hybrid cultivars. Edible oilseed rape is grown as winter types in Europe, with a growing period of around 11 months, whereas early flowering spring-sown canola forms grown in Canada are harvested after only 4 months. Australian growth conditions suit intermediate types with European, Canadian, and Asian ancestry (Cowling 2007).

In the main growing regions, oilseed rape has become one of the most important dicotyledonous crops, with an integral role as a break crop in cereal crop rotations

A. Stahl (✉) · R. Snowdon
Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
e-mail: andreas.stahl@agrar.uni-giessen.de

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_12

(Kirkegaard et al. 2016). The positive influence of its root system on soils, the phytosanitary effect, and residual nitrogen from plant residues have a strong positive influence on yields of subsequent cereals (Christen and Sieling 1993; Christen et al. 1992). European winter oilseed rape also provides soil coverage for almost the entire year, preventing soil erosion and reducing nutrient leaching.

As for most non-legume crops, nitrogen (N) is the nutrient that has to be supplied to oilseed rape in the highest quantities and the fertilization is imperative to achieve high-yield levels. Since oilseed rape has a relatively high acquisition of nitrogen during vegetative growth stages, but a comparatively low nitrogen recovery in harvested organs (seeds), its cultivation is often associated with an N-balance surplus that can potentially damage other ecosystems (Sieling and Kage 2008; Hirel et al. 2007). This unused nitrogen not only lowers the economic productivity of the crop, but can also exacerbate gaseous N-emissions, nitrate leaching, or run-off. Rockström et al. (2009) estimated that, on a global scale, pollution of ecosystems with excess nitrogen has already crossed an acceptable boundary. Furthermore, energy-dependent mineral fertilizer production by the Haber-Bosch process raises carbon dioxide emissions and lowers the greenhouse gas balance. Since oilseed rape is the primary feedstock for European biodiesel production, it must meet EU legislatory demands for reductions in greenhouse gas generation resulting from crop production, in order to enhance the sustainability standard of renewable energy. As an overall consequence, noteworthy increase of nitrogen use efficiency (NUE) in oilseed rape is essential to address these constraints and achieve more economical, sustainable vegetable oil and protein production in temperate agricultural zones.

Addressing NUE Throughout the Entire Production System

Since future sustainability standards must be regarded as an economic and ecological benchmark across the entire production system, NUE is always the result of Genotype × Nitrogen × Environment × Management interactions (G × N × E × M) within the overall cropping system (Dresbøll and Thorup-Kristensen 2014). These interdependences are best illustrated by examples. Firstly, NUE can be altered enormous by management practices, for example by precise adjustments in timing and dosage of N fertilization through sophisticated application methods. In this regard, state-of-the-art sensor technologies and a more accurate plant demand prediction are important issues (Pahlmann et al. 2017; Sieling and Kage 2010; Müller et al. 2008; Henke et al. 2007). Secondly, favorable or unfavorable alterations in one aspect of the N cycle normally also affect the subsequent plant growth conditions. For example, N that is unused by the previous crop might be at least partially usable by the subsequent crop (mostly wheat), depending on the environmental and management conditions. Furthermore, a large number of biotic and abiotic constraints, which are also influenced by N availability, can significantly limit the seed yield and thus the realized NUE. Obviously,

if plants are affected by diseases or drought they will not achieve comparable NUE levels to those of healthy or sufficiently rain-fed plants. In this context, however, maintaining crop health and reducing yield losses by management decisions or resistance breeding is rather an elimination of inefficiencies than a direct tool to engineer NUE. The same is true for the avoidance of seed yield loses by increased resistance to lodging or pod shatter (Raman et al. 2014). Although all these complex interactions certainly contribute to a more efficient use of N, in this chapter we focus on genetic improvement of NUE under optimal crop management practices, beyond solutions to biotic and non-nitrogen abiotic penalties. We outline which traits in particular must be addressed in order to improve NUE and how existing genetic potential in *B. napus* can be used to enhance NUE by breeding improved varieties of oilseed rape.

The Glass Half Full: NUE Is Already Constantly Improving

In a very simplistic interpretation, NUE can be defined at the end of the crop season as the seed yield per unit of plant-available N (Good et al. 2004; Moll et al. 1982). From this perspective, varieties attaining higher seed yields at a given nitrogen availability level can be claimed to be more N-efficient. It is extensively documented that—for different investigated time periods—modern varieties significantly outperform older varieties under the same environmental conditions, giving strong evidence that breeding drives improvement of NUE (Stahl et al. 2017; Lotze-Campen et al. 2015; Koeslin-Findeklee et al. 2014; Kessel et al. 2012; Gehringer et al. 2007). Interestingly Kessel et al. (2012) observed high correlations ($r = 0.96$) for seed yield between high and low nitrogen fertilization levels, demonstrating that the ranking of genotypes at reduced N levels is not tremendously different from high N levels. Hence, breeders have apparently indirectly selected more efficient varieties simply by targeting seed yield as the most important breeding aim. High-performing hybrid oilseed rape varieties tend to have a general yield advantage over inbreds (Wang et al. 2016; Gehringer et al. 2007), with heterosis levels between 4 and 63% (Becker 1987). In the context of NUE, heterosis is considered to be increased under limiting conditions. For European hybrid varieties, Wang et al. (2016) reported heterosis up to 20% under high N and up to 35% under limiting N conditions. However, the physiological and genetic basis for heterosis is not well understood in *B. napus*. As a consequence, strategies to further improve NUE should include investigation of driving forces of heterosis. Moreover, breeding progress is not solely expressed in higher yields but can also affect other plant traits. For example, modern cultivars including hybrids were found to have lower N concentration in senesced leaves than older varieties or synthetic *B. napus* accessions (Kessel et al. 2012).

NUE as the Final Result of Numerous Plant Growth Characteristics

However indirect selection for NUE has been successful in the past, achieving higher levels of NUE more rapidly in future breeding requires more directed selection. One strategy can be to evaluate diverse material under divergent N environments (Ceccarelli 1994), whereas another option is to test and select only under low-N environments in order to more directly select genotypes with a high NUE. An ideal genotype will capture a high percentage of available N, producing a higher seed yield per unit of acquired N and leaving minimal N in the plant residues at harvest (Moll et al. 1982). In this context it becomes obvious that NUE is not a single trait, but rather a conglomerate of traits that can be clustered in those that affect nitrogen-uptake efficiency (NupE) or nitrogen utilization efficiency (NutE) (Sattelmacher et al. 1994; Moll et al. 1982). In light of the fact that NUE is influenced by a great number of factors, favorable allele combinations for a trait influencing one factor might be masked by other traits and, thus, remain hidden and overseen in selection processes. In order to combine and pyramid complementary traits enhancing NUE, endpoint selection based on the yield is not sufficient to optimally exploit the genetic potential. Only a knowledge-based combination of numerous traits will lead to superior NUE. Therefore, we propose to dissect the complex breeding aim into sub-traits which must be assessed throughout the entire vegetation period (Stahl et al. 2016; Thurling 1991). To make this possible, a deeper understanding of individual target traits is required.

Acquisition of and Response to Nitrogen

For biomass development at the beginning of their lifecycle, oilseed rape plants have a high demand for uptake of nitrogen and other nutrients from the soil. Generally, members of the Brassicaceae family are considered to have a strong ability to acquire nitrogen and produce vegetative plant biomass (Cramer 1993). In particular, in case of European winter oilseed rape production, the 11-month growing season beginning shortly after the preceding crop allows uptake of N in autumn and consequent prevention of leaching. Numerous studies have pointed out that under low-N supply (residual soil N plus fertilizer) the relative contribution of NupE is more important than the NutE. On the other hand, in cases where N supply is high, the relevance of NutE rises while that of NupE is diminishing (Wang et al. 2016; Miersch et al. 2016a, b; Nyikako et al. 2014; Kessel et al. 2012; Schulte auf'm Erley et al. 2011; Berry et al. 2010). Nevertheless, several studies suggest that water and nitrogen acquisition can still be meaningful and an important contributor to NUE, in particular during flowering and post-anthesis, even under intensive growth conditions (White et al. 2013; Ulas et al. 2012; Schulte auf'm Erley et al. 2011; Berry et al. 2010; Kamh et al. 2005; Wiesler et al. 2001).

In this regard a stay-green habit is suggested to be advantageous, since the leaves still maintain a high level of assimilates for transporters in the roots to take up N. However, the direction of causality is not yet clarified (Kamh et al. 2005). Berry et al. (2010) calculated a yield increase of 16 kg/ha for each additional kilogram of N taken up after flowering.

For N acquisition, the root system (reviewed in Garnett et al. 2009) and early vigor might be potentially relevant to ensure crop establishment. Investigations of the root system during early plant developmental stages have been subjected to previous studies in *B. rapa* (Adu et al. 2014) and *B. napus*, either for early vigor and drought adaptation (Hatzig et al. 2014, 2015) or in conjunction with phosphorus acquisition (Shi et al. 2013). In order to select directly for specific root traits, or indirectly by use of genetic marker associations, it is necessary at least initially to phenotype large populations and determine relevant target traits or markers. For early developmental stages, Shi et al. (2013) used a doubled-haploid population (n = 190) to determine genomic regions associated with root traits. Indeed, they found a significant QTL on chromosome A03 associated with root architectural traits which also colocated with QTL identified in *B. napus*, *B rapa,* and Arabidopsis. However, for effective use of data generated at early developmental stages under controlled (artificial) environments, the transferability to field conditions and the relevance at later developmental stages is a critical point that is not necessarily given. The reasons for this poor transferability are manifold (reviewed in Poorter et al. 2016). Nevertheless, some studies have demonstrated relationships between root traits in artificial and field conditions (Thomas et al. 2016). For example, in a Canadian study with eight genotypes, a relationship was observed between root seedling traits and seed yield under field conditions (Koscielny and Gulden 2012). Most portions of the root system are thought to be fully developed prior to flowering (Le Deunff and Malagoli 2014; Rahman and McClean 2013; Barraclough 1989); however, few studies have been conducted on genetic diversity of the root system in *B. napus* beyond the early developmental stages, most likely because of the difficulties in accessing the root system. One of the first studies looking at the inheritance of the root system at more developed growth stages was conducted by Rahman and McClean (2013). In their mapping study, based on a segregating F2 population from a cross between spring-type and winter-type *B. napus*, the authors hypothesized a trigenic dominant control of root vigor per se. However, transferability of data from single biparental mapping populations to a general context is difficult, and crosses between winter-type and spring-type genotypes have an extremely high dependency on vernalization and flowering-time traits that can strongly mask other traits. Furthermore, to date there is only scarce knowledge available about the genetic variation of root traits in *B. napus* in relationship to N availability. Several studies agree that the total root length is much more relevant for nitrogen uptake than the root mass (Schulte auf'm Erley et al. 2007; Kamh et al. 2005). Moreover, a faster root penetration rate is suggested to reduce nitrogen leaching in deeper soil layers (Thorup-Kristensen 2006). By direct comparison between two extreme lines, (Kamh et al. 2005) showed that a higher root length in deeper soil layers positively influenced NupE. In addition, the

finding, that hybrids have longer roots than inbred lines (Koeslin-Findeklee et al. 2014) and can acquire up to 49% more N than their corresponding parents (Wang et al. 2016), suggests that the exploitation of heterosis could confer an N-uptake advantage. In order to access late root phenotypes in populations large enough to facilitate genetic mapping, Fletcher et al. (2014) used the "root pulling force"—the force needed to pull adult plants out of field soil—as a proxy for the root system size. The result was a strong correlation between flowering time and root pulling force; however, two major QTL for root system size were identified on chromosomes A08 and C07 that do not colocalize with flowering time and, thus, can be considered as candidate regions for further investigations of adult plant root architecture in *B. napus* (Fletcher et al. 2014).

A larger root system might be beneficial on the one hand in soil layers with a high occurrence of plant-available nutrients, but on the other hand it can also be associated with a metabolic cost for building and maintenance of the root system. In particular in cases where the root system size is not limiting for N acquisition, a large root system can counteract the benefits. Therefore, the ability of a genotype to react to divergent N levels deserves further attention. It is known that nitrate is not only a nutrient, but also an important signaling molecule that allows plants to adapt to different nitrogen levels (Zhang and Forde 1998, 2000). In recent studies of adult plant roots under controlled conditions (Hohmann et al. 2016), we revealed by a so-called shovel-omics approach tremendous genetic variation for root traits within the gene pool of *B. napus*. Arguably even more interesting is the observation of a strong genetic variation for the response to divergent N supply under field growth conditions (Bouchet et al. 2016).

In case of low nitrogen availability, plants frequently increase their root surface and alter their transporter activity (reviewed in Kiba and Krapp 2016), probably in order to capture more nitrogen, and diminish shoot growth. Hence, it is not surprising that the root-to-shoot ratio increases as a result of low N (Passioura 1983). Nitrogen uptake might be influenced by the root surface and thus by the number of lateral roots (Fig. 12.1). This in turn is controlled among others by the root-specific N-responsive gene *ANR1* (Gan et al. 2012), CLAVATA3/ESR (CLE) peptides, and the nitrated-inducible *CLAVATA1* (*CLV1*) leucine-rich repeat receptor-like kinase (Araya et al. 2014). There are examples that further control takes place by nitrogen on the transcriptional and post-transcriptional level (Coruzzi and Zhou 2001). Finally, in Arabidopsis the microRNAs such as miR167 and miR393 regulate the target genes *ARF8* and *AFB3,* respectively, which alter the root system architecture depending on the supplied N (Vidal et al. 2010; Gifford et al. 2008). Due to this genetic complexity, targeted genetic approaches to regulate NUE for breeding are difficult. In the foreseeable future, successful exploitation of genetic variation for root traits and their response to different environments will clearly require extensive, precise and standardized phenotyping pipelines, (Thorup-Kristensen and Kirkegaard 2016; Dorlodot et al. 2007) and appropriate statistical modeling approaches (Araya et al. 2016).

Fig. 12.1 Comparison of root morphology between elite winter oilseed rape genotypes with few lateral roots (left) and with intense lateral root network (right) grown under field conditions. (A. Stahl and R. Snowdon, unpublished data)

The Bottleneck in Rapeseed: Nitrogen Utilization

Flowering is characterized by a paroxysm of change from the vegetative to the generative developmental stage (Fig. 12.2). Associated with this is a change in source–sink relationships. Whereas, prior to flowering, the vegetative plant organs form the sink for nitrogen, post-flowering they now become the source for developing generative organs that now form the new N sink. Compared to other crops such as wheat and maize, the seed N of oilseed rape at harvest mainly originates from protein degradation in other plant segments (Masclaux-Daubresse et al. 2008; Malagoli et al. 2005; Rossato et al. 2001). Oilseed rape development is characterized by an early start of senescence processes and leaf abortion after flowering. Since not all of the nitrogen is remobilized, aborted leaves cause N loss from the plant-internal N pool to the upper soil layer of the field. Kessel (2000) measured that 10% of the total N uptake is lost by dropped leaves. The N losses in senesced leaves decline from flowering until maturity not only just because the previously aborted leaves are bigger, but also due to a higher N concentration in the leaves (Stahl 2016). Data from Wang et al. (2016) indicated that N remobilization into seeds depends rather on plant genetic effects than on level of N supply and that the responsible processes are not source limited. This is in agreement with the finding that phloem loading of amino acids is efficient and not below that found in other crops, suggesting a sink limitation (Tilsner et al. 2005). Taken together, one might

argue that—from an overall viewpoint—the desynchronization between source and sink activitiesin oilseed rape is a physiological bottleneck for increased NUE. Directly after flowering the source offers an overload of nitrogen that cannot be captured by the small, just developing sink. As a result, nitrogen is lost in high concentration via aborted leaves that are not able to fully remobilize their N reserves (Stahl 2016; Rossato et al. 2001). Gombert et al. (2010) support this hypothesis of sink-limited N remobilization, in particular at high N fertilization levels. At later stages, the sink is much more developed and is able to incorporate nitrogen more efficiently; however, meanwhile many leaves have already been lost and the only remaining source organs are the stems and roots (and later the silique walls) segments (Masclaux-Daubresse et al. 2008; Malagoli et al. 2005; Rossato et al. 2001). During this phase, the stem and taproot are considered to act as buffer organs (Rossato et al. 2001). Indeed, a direct comparison of extreme genotypes revealed that there is a genetic difference in the proportion of N that is remobilized from leaves and stems (Girondé et al. 2015a, b). In fact, however, the source–sink relationship is even more complex, because of an undetermined growth behavior and sequential entry into the generative phase. While the main raceme and early side branches have already finished flowering and switched to the generative phase, late side branches with their leaves are still developing and flower late. For this reason, the complete flowering period can take a few weeks.

This complex temporal chorography of flowering, senescence, and N remobilization makes it obvious that regulation of flowering time is essential for efficient allocation of plant-internal resource (Fig. 12.2). In a recent study on a diverse collection of European winter-type oilseed rape, early flowering was revealed to be beneficial for an increased NUE at low-N supply (Fig. 12.3). The notion that control of flowering time is a critical regulator for yield traits, and its potential target for manipulation by breeders, was already described by Schiessl et al. (2014) and Jung and Müller (2009). Further, genome-wide association studies in spring-type and winter-type canola oilseed rape (Schiessl et al. 2015; Xu et al. 2016), along with as biparental QTL mapping in spring-type *B. napus* (Luo et al. 2014; Quijada et al. 2006; Udall et al. 2006), identified important chromosomal regions with relevance for flowering-time regulation. Schiessl et al. (2015) found one region on chromosome A09, with common associations with plant height and seed yield, containing the AGL transcription factor and putative meristems regulator *Bn.FUL* (Melzer et al. 2008). In Arabidopsis, *FUL* plays a role in silique development via *NO TRANSMITTING TRACT* (Chung et al. 2013), suggesting a potential involvement in the new sink formation. Sequence variation in other important flowering time regulating genes, described by Schiessl et al. (2014), represents interesting diversity for analysis of N effects.

On the other hand, not only the regulation of flowering time is associated with yield and NUE performance of individual genotypes, but also the nitrogen (Fig. 12.4) and chlorophyll content, along with the timing and degree of senescence (Gregersen et al. 2013). It was confirmed that photosynthesis and chlorophyll contents—especially in siliques (Hua et al. 2014)—have a positive relationship to seed oil content (Wu et al. 2014). Recently, Qian et al. (2016b) discovered that a

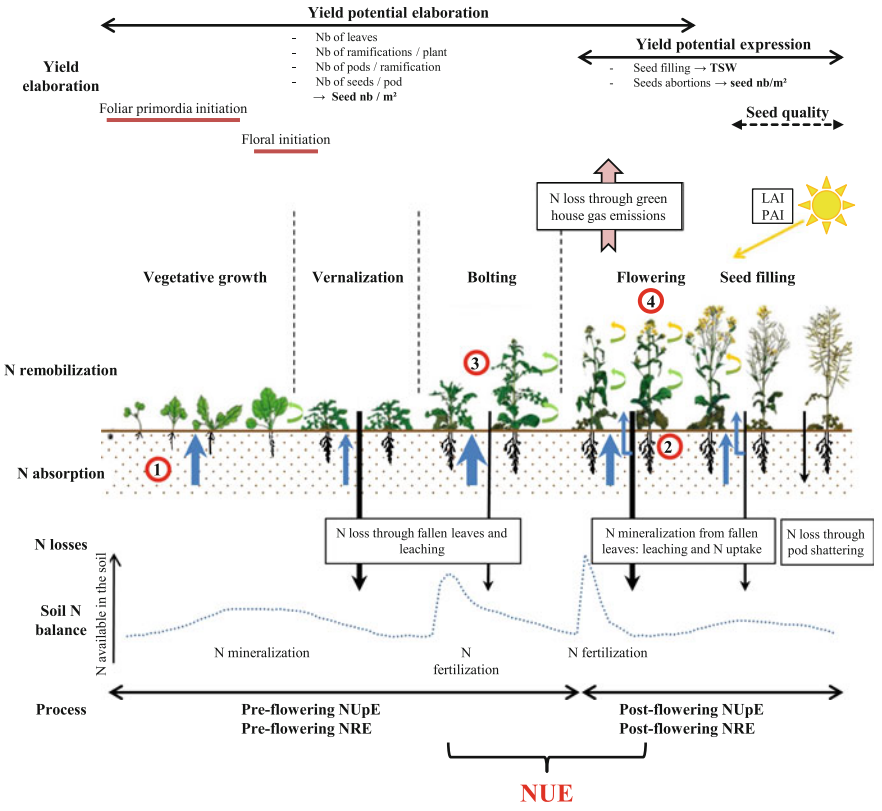


Fig. 12.2 Rapeseed N dynamics over the crop cycle. The figure depicts the interconnected relationships between plant growth, N dynamics, and yield elaboration over the crop cycle. N uptake is represented by blue arrows whose width indicates the relative amount of N absorbed at a given time point. N losses are shown with black arrows whose width indicates the relative amount of N lost at a given time point. The critical stages for the final establishment of nitrogen use efficiency (NUE) are noted as follows: pre-flowering nitrogen-uptake efficiency (NUpE) and sequential nitrogen remobilization efficiency (NRE) (1); post-flowering nitrogen-uptake efficiency (NUpE) (2); sequential and monocarpic NRE during the flowering and seed filling periods (3); and the interactions between NUE, leaf area index (LAI), and pod area index (PAI) (4). Thousand seed weight (TSW). (First published in Agronomy for Sustainable Development, Bouchet et al. 2016)

deletion of the stay-green gene *NON-YELLOWING 1* (*NYE1*) is associated with low chlorophyll and seed oil content in Asian *B. napus* forms. More interestingly, signatures of selection suggest an artificial selection of haplotypes carrying this deletion during breeding of oilseed rape, indicating a selective advantage in at least some environments. Related to this, a strong interdependency exists between senescence processes and the plant nitrogen status. On one side a shortage of nitrogen supply can induce senescence processes (Bi et al. 2007), whereby the onset of senescence affects the remobilization of plant-internal N. Delayed senescence, in turn, enables an extended period of sunlight usage for photosynthesis, which

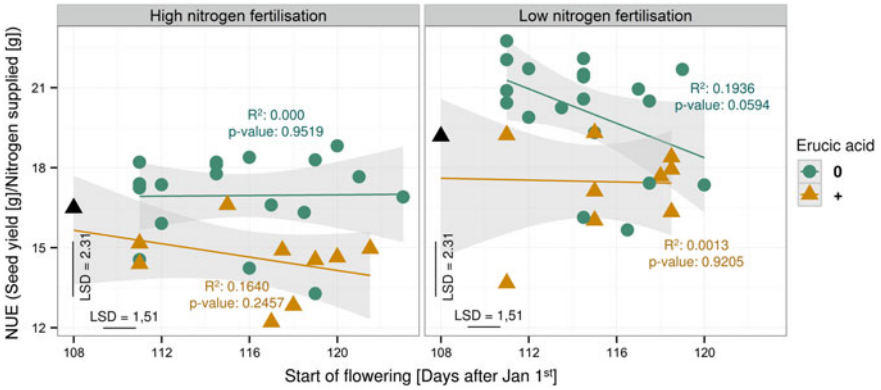


Fig. 12.3 Correlation nitrogen use efficiency (NUE) with flowering time at low nitrogen (right) and high nitrogen fertilization (left) in a highly diverse set of winter-type winter oilseed rape accessions separated into an old, non-adapted group with high erucic acid content (+) and rather modern group (0). Gray shaded areas depict 95% confidence interval. Cultivar Olimpiade is marked with black triangles (First published in Plant and Soil, Stahl et al. 2016)

potentially leads to higher yields (Kant et al. 2015; Gan and Amasino 1995), especially under abiotic stress (reviewed in Jameson and Song 2016). Furthermore, in light of nitrogen remobilization, a delayed continuous senescence can allow the source organs (at this stage mainly leaves) to continuously release stored leaf metabolites toward the sink. In particular under consideration of the disbalance between source and sink activation in oilseed rape, this delay might be advantageous as long as the genotypes do not stay green for too long and miss a final N remobilization from vegetative organs to the seeds. Alteration of source–sink relationships is strongly mediated by the hormone cytokinin, the presence of which delays leaf senescence, prevents degradation of photosynthetic proteins (Jameson and Song 2016; Guo and Gan 2014), and activates specific cell cycle genes (Schaller et al. 2014). Particularly under abiotic constraints such as nitrogen deficiency and drought stress, maintenance of cytokinin levels was shown to confer yield advantages in other crops (Peleg et al. 2011). Besides environmental influences, senescence is also under genetic control by so-called senescence-associated genes (SAGs). A number of SAGs have been identified not only in *Arabidopsis thaliana* (van der Graaff et al. 2006; Buchanan-Wollaston et al. 2005), but also in *B. napus* (Lee et al. 2015). In particular the gene *SAG12*, encoding a cysteine protease responsible for degradation of the photosynthetic apparatus, was found to be very responsive to senescence induction in *B. napus* (Koeslin-Findeklee et al. 2015). As a molecular marker for leaf senescence (Brunel-Muguet et al. 2013; Gombert et al. 2006), *SAG12* expression was revealed to be an even more sensitive indicator than SPAD measurements (Koeslin-Findeklee et al. 2014). Zhao et al. (2015) showed that moderate enhancement of cytokine levels may lead to increased branching, enhanced photosynthesis, better abiotic and biotic stress resistance, and ultimately higher yields. Cytokinins are not just an important regulator of senescence, but also

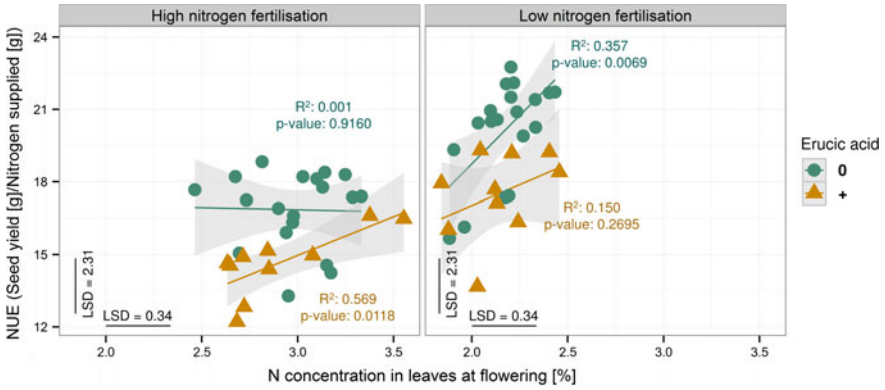


Fig. 12.4 Correlation nitrogen use efficiency (NUE) with N concentration in leaves at flowering at low nitrogen (right) and high nitrogen fertilization (left) in a highly diverse set of winter-type winter oilseed rape accessions separated into an old, non-adapted group with high erucic acid content (+) and rather modern group (0). Gray shaded areas depict 95% confidence interval (First published in Plant and Soil, Stahl et al. 2016)

play a regulatory role in shoot apical meristem, are relevant for inflorescence architecture (discussed in the next paragraph), enhance the cell division of the seeds (Jameson and Song 2016; Hwang et al. 2012; Brugiere et al. 2008; Emery et al. 2000; Singh et al. 1988), and are therefore considered to be key driver of seed yield (Han et al. 2014). It was observed for *B. napus* that source leaves, floral buds, and pods depend on cytokinin supplied from maternal sources immediately after anthesis. On the other hand, elongating pods and developing seeds produce their own cytokinin (Song et al. 2015). This finding underlines a differential regulation of yield components, suggesting that manipulation of different traits requires a dedicated spatial and temporal regulation of each target trait. The endogenous cytokinin level can be modified by altering genes encoding for the isopentyl transferase (IPT) which is the key biosynthetic enzyme and/or by the cytokinin oxidase/dehydrogenase which causes cytokine degradation (Zhao et al. 2015). Furthermore, it was demonstrated in rice that cytokinins could be directly altered in plant breeding programs. As an example, a loss-of-function mutant of the gene *Osckx2* led to increased cytokinin levels and 20% higher yield in rice (Ashikari et al. 2005).

NUE in Light of Seed Quality and Plant Architectural Traits

In principal there are two options to increase the sink for nitrogen and increase the amount of N that the plants extract from the field: (1) elevated seed protein concentration, and (2) increased seed yield per se. The seed protein concentration in oilseed rape generally varies between about 15 and 20%; however, in more diverse material a much broader spectrum can be observed. It is widely known that seed

protein has a negative correlation between seed yield and seed oil concentration, due to a competition for the same carbon resources (Zhao et al. 2006; Rathke et al. 2005). Oil yield is still the most important economic parameter for oilseed rape production (Funk and Mohr 2010), even though the valuable protein is attracting increasing rising attention. Hence, until now breeders have generally selected for genotypes high in oil content, with high per se yield and hence a high oil yield. For this reason, modern high-yielding varieties show higher oil concentration in seeds. In the past, this led to the paradoxical observation that higher seed N concentration appears to be negatively associated with an increased NUE in modern breeding pools (Koeslin-Findeklee et al. 2014; Stahl and Snowdon, unpublished data). As a consequence, increasing the seed protein concentration in order to raise NUE of rapeseed oil will only be a promising strategy if genotypes can be found that at least partially break the negative correlation between oil and protein content, or if the protein yield as an oil-extraction by-product receives the necessary economic attention. The suggestion to include protein in the ecological footprint calculation associated with NUE has some merit, but would justify breeding attempts toward enhanced seed protein content at the cost of prioritising oil content. A more reasonable scenario is selection for the sum of oil plus protein (Grami and Stefansson 1977), or at least including the protein content of the meal as a breeding goal in order to increase the recovered N in seeds. A monitoring conducted in Australia observed that both oil content in seeds and protein content in the meal increased by 0.09 and 0.05% per year respective between 1978 and 2012 (Potter et al. 2016), suggesting that the negative correlation between seed oil and protein can partially be broken.

Irrespective of seed quality traits, yield per se is by far the most important factor to increase NUE. Seed yield is strongly dependent on the principal plant architecture, since it has a major impact on optimized light capture for photosynthesis, along with the development of the primary yield components, number of siliques per plant, number of seeds per silique, and thousand seed weight (TSW), respectively. In rapeseed, the development of axillary meristems to develop side branches besides the main raceme is of fundamental relevance for yield formation. Although plant architecture and yield components show a high plasticity and are influenced by many environmental factors, for example the sowing density (Junior et al. 2012; Diepenbrock 2000), there is also a noteworthy genetic variance for yield-associated architectural traits that has not yet been directly targeted in plant breeding. A recent study of branching angle across a diverse population of 143 genotypes, including mainly spring but also 6 winter types, discovered genetic variation up to 50° (20°–70°) and a high heritability (Liu et al. 2016). In the same study, significant associations to branching angle were found on chromosomes A2, A3, A7, C3, C5, and C7. Another complementary study, also analyzing a DH population, demonstrated that plant architectural traits are in significant correlation with seed yields. On the other hand, QTL related to plant architecture were found to be more stable than yield-related QTL, since they are less strongly influenced by the environment. In general, the genes underlying the identified QTL could be grouped into the categories auxin/IAA, gibberellins, and transcription factors (Cai et al. 2016).

Another study investigated the heterosis for yield components in a winter-type *B. napus* DH mapping population between cv. Express and R53. Midparent heterosis for seed yield was found to be 30%, for number of siliques per area 19% and for number of seeds per siliques 11.2%. No heterosis was observed for thousand seed weight. A total of 33 QTL were identified that play a potential role in heterosis for yield and yield components. Furthermore, the authors found that a combination of dominance, overdominance and epistatic effects were involved in expression of heterosis (Radoev et al. 2008). Cai et al. (2014) conducted association mapping for six yield-related traits including architectural traits and yield components, finding 18 markers that were repeatedly detected over two years. To our knowledge, however, detailed genetic mapping studies of multiple plant architectural traits have not been performed under divergent nitrogen fertilization levels.

Collective alterations of plant architectural traits can be a strategy to enhance the harvest index (HI), the ratio of seed yield compared to the entire plant biomass. Genetic variation for HI was reported by Svečnjak and Rengel (2006). An extreme alteration in harvest index can be achieved by the use of dwarf genes to produce semidwarf hybrids, as those show reduced stem elongation and therewith a lower plant residual biomass. One prominent dwarfing gene is the *bzh* gene described and mapped on chromosome A06 by Foisset et al. (1996, 1995). In a recent study, Miersch et al. (2016a, b) used a doubled-haploid population of 108 genotypes to produce test hybrids, segregating into *bzh* semidwarf and normal-type hybrids, in order to assess the effects of the dwarf phenotype on NUE under high- and zero-N fertilization, respectively. The result was that semidwarf hybrids outperformed the normal-type hybrids for seed yield exclusively under N deficiency (Miersch et al. 2016a, b). Based on this result, it appears that the dwarf gene not only alters the plant length, but also shows pleiotropic effects on seed yield. Furthermore, branching on dwarf cultivars originates in lower stem sections, leading to a more even distribution of plant biomass across the vertical axis of the plant (Wang et al. 2004). In other studies using independent genetic backgrounds for semidwarf and normal-type rapeseed, no advantage of semidwarf hybrids for reduction of N leaching was observed (Sieling and Kage 2008; Koeslin-Findeklee et al. 2014). As an extension of the harvest index concept, the nitrogen harvest index can be used to express the proportion of N that is stored in seeds at harvest compared to the amount of N in the entire mature plant.

Use of Genetic and Genomic Resources in Breeding Programs

Oilseed rape (*B napus* L.) has a relatively young evolutionary age and is the allotetraploid hybrid of the diploid progenitor species *B. oleracea* (C genome donor) and *B. rapa* (A genome donor) (Chalhoub et al. 2014). Each of those species is in fact also the result of ancient polyploidization, showing triplication of an

ancestral genome (Parkin et al. 2014, 2005). In general, *B. napus* potentially has an extremely wide genetic and morphological variation (Bus et al. 2011). However, due to adaptation to local cropping systems and strict selection for double-low (00) seed quality (zero erucic acid content and low glucosinolate content), today's elite varieties represent a very small gene pool with only narrow genetic diversity (Qian et al. 2014; Hasan et al. 2006). On the other hand, genetic diversity is a prerequisite for plant breeders and avoidance of drift due to a low effective population size is essential for long-term breeding success. Hence, genetic replenishment of genetic diversity is essential for maintenance of breeding progress (Griggs et al. 2014; Cowling and Léon 2013; Cowling 2007). Therefore, breeders face the challenge to enrich their gene pools with genetic diverse material (Girke et al. 2012a, b), while at the same time attempting to avoid compromising the high standards they have already achieved for important adaptation and quality traits (Cowling et al. 2009). In *B. napus*, very large chromosome blocks with low genetic diversity and high linkage disequilibrium, containing essential QTL for major seed quality and flowering-time traits considerably hamper the re-enrichment of depleted gene pools (Qian et al. 2014). Interestingly, there is a genome bias in this phenomenon, with much larger conserved LD blocks in the C subgenome than in the A subgenome (Qian et al. 2014). Furthermore, conserved haplotype blocks can lead to inadvertent co-selection of linked traits. For example, Qian et al. (2016a) discovered a conserved haplotype block on chromosome A02 which contains an ortholog of the key glucosinolate biosynthesis genes *METHYLTHIOALKYMALATE SYNTHASE-LIKE 1* (*MAM1*), along with a number of chlorophyll-related genes. Strong linkage in repulsion can hinder trait combination, and hence breeders require a boost in recombination to exchange strongly conserved chromosome segments. Numerous studies have demonstrated that de novo allopolyploidization of *B. napus* from its ancestors is a promising strategy to induce homeologous chromosome recombination and overcome low recombination rates (Mason and Batley 2015; Mason and Snowdon 2016). In the context of increased NUE, Wang et al. (2014) demonstrated that production of resynthesized *B. napus* lines by interspecific hybridization has the potential to enlarge the genetic variation and allow the identification of more efficient genotypes. Also, Bouchet et al. (2016) and Stahl et al. (2016) found a very broad genetic variation for traits associated with nitrogen uptake and utilization efficiency across adapted and non-adapted *B. napus* genotypes. For hybrid breeding programs, the enrichment of genetic diversity and separation into heterotic pools is of fundamental importance (Zou et al. 2010).

Tools to Accelerate Breeding Progress

So far the evidence confirms that classical breeding approaches have obviously been successful to increase the seed yield tremendously and therewith NUEin oilseed rape (Stahl et al. 2017). However, future breeding programs can be

streamlined by implementing several emerging tools that could help further boost NUE breeding progress.

Candidate gene approaches are frequently suggested as an option to improve specific traits. Potential genes are known for some sub-traits influencing NUE, and research in *B. napus* can benefit from a close relationship to the model crucifer, *A. thaliana* (Bancroft et al. 2015; Cheung et al. 2009; Parkin et al. 2005), but targeting specific genes (e.g., through targetted mutations) is complicated by the genome complexity of the allopolyploid. The earlier genome triplication since the divergence forms *A. thaliana,* followed by the allopolyplodization and genome restructuring (Chalhoub et al. 2014), has greatly expanded gene copy number in *B. napus* (Lysak et al. 2005; Chalhoub et al. 2014). As a consequence, targeting single gene copies often does not result in a phenotypic change, since there are number of copies that might compensate each other. For example, Orsel et al. (2014) demonstrated that the sixteen *BnaGLN1* genes, coding for a cytosolic glutamine synthetase isoform, show divergent tissue-specific expression and environmentally dependent control. Moreover, one trait might be compensated by the superiority in other traits, so that modification of a single trait may not necessarily result in an improved phenotype (Boote et al. 2013).

In order to determine potential genomic regions which might be responsible for particular trait expression, high-resolution genotyping with high-density genome-wide markers is already state of the art and broadly used in genetic research and breeding (reviewed in Voss-Fels and Snowdon 2015; Snowdon et al. 2012). For *B. napus*, the Illumina Brassica Consortium Infinium array, released in 2012 and carrying functional assays for 52,157 markers (Clarke et al. 2016), is a very powerful tool for forward genetics, genomic prediction, or genomic selection (Ganal et al. 2012). Although this high number of markers is necessary for high-resolution genetic studies in diverse populations, in breeding populations with more conserved LD the extensive SNP information is often redundant. Filtering of SNP loci can lead to a much more effective chip, including only genome-specific SNPs that cluster clearly in both genomes (Clarke et al. 2016).

Although biparental QTL mapping can be useful to dissect agronomic traits in breeding populations (Wurschüm 2012), studies revealed that NUE is controlled by multiple small-scale effects, making the sole use of marker-assisted selection for NUE unrealistic (Bouchet et al. 2014). Nevertheless, the improvement of single NUE-influencing traits by marker-assisted selection, such as root morphology, plant architecture, or seed quality traits, could be potentially helpful. Indeed, several studies have identified chromosomal regions associated with yield-related traits that might be interesting for candidate gene searches (Basunanda et al. 2010; Radoev et al. 2008; Udall et al. 2006). However, there are extremely few examples for successful implementation of marker-assisted selection for QTL involved in quantitative traits (Bernardo 2016).

In contrast, high-density genome-wide markers are highly suited to genomic selection (GS) and predictive breeding strategies, and these offer unprecedented possibilities for improvement of complex traits such as NUE (Snowdon and Iniguez Luy Snowdon et al. 2012). Genome-wide association studies (GWAS) in *B. napus*

have elucidated traits from early developmental stages (Hatzig et al. 2015) until maturity and harvest (Körber et al. 2016). The latter study detected 112 SNP-trait associations for various traits using a species-wide diversity set comprising 405 *B. napus* winter and spring-type accessions.

The use of genome-wide marker data not only allows GWAS, but also can be used to estimate the breeding value of a genotype based on the calibration of a model by a training population which is extensively phenotyped in different environments. Genomic selection (GS) approaches attempt to predict the genotype-specific breeding value based on whole genome-wide marker profiles. Advantages of GS are the time and cost-effective (pre-)selection of breeding material (Jonas and de Koning 2013; Heffner et al. 2010, 2011; Crossa et al. 2010). Results from spring-type oilseed rape using a ridge-regression best linear unbiased prediction (BLUP) in combination with 500 cross-validations demonstrated that it is possible to predict seed yield and seed quality traits. Prediction accuracy ranged from 0.29 for seedling emergence, a trait with very low heritability, to 0.81 for the highly heritable trait oil content (Jan et al. 2016). Considerations of GxE effects (Cullis et al. 2010) into GS selection models can lead to noteworthy improvements in prediction accuracy. With a precise GS tool, breeders have the chance to preselect their material and, thus, at least partially replace expensive field trials or other extensive phenotypic screenings by genomic screenings. However, if the heritability for a trait is low, as for NUE, and the additive and nonadditive genetic variance is large, the training population must be large to achieve adequate prediction adequacy (Heffner et al. 2011). From this perspective, the optimized use and detailed integration into breeding program will still have to be evaluated. Nevertheless, traits such as NUE, for which phenotypic selection can be challenging, can potentially profit considerably from GS techniques. In future, the integration of crop growth models into GS models as it has been implemented in maize is an attempt to increase prediction accuracy by consideration of functional biological relationships (Technow et al. 2015). For rapeseed, genomic prediction of hybrid performance (Jan et al. 2016), along with modeling of nitrogen uptake (Malagoli et al. 2005) and plant-internal nitrogen partitioning (Malagoli and Le Deunff 2014), has each been conducted individually. However, approaches integrating both methods have yet to be developed and require further research.

Outlook

The acquisition of increasing quantities of large-scale “omics” data and their association with agronomic traits in suitable populations open the possibility to considerably improve our understanding of plant physiological processes of NUE and their genetic determinants (Raman et al. 2016). Ongoing developments in high-throughput data collection and high-performance computing facilitate in silico modeling of plants from genome to phenome, in order to resolve crop development in different environments (Zhu et al. 2016).

It stands to reason that progress in genomics can substantially help to adapt oilseed rape to future growth conditions (Nelson et al. 2016). As for most other important crops, next-generation sequencing and high-throughput genomics tools (Edwards et al. 2013) have provided access to the genomes of *B. napus* (Chalhoub et al. 2014) and its ancestors *B rapa* (Wang et al. 2011) and *B. oleracea* (Yu et al. 2013). As more and more *B. napus* genomes are resequenced (e.g., Schmutzer et al. 2015), we will get closer and closer to achieving a complete catalog of genes and variants in the species pangenome (Golicz et al. 2016a, b), and using this information to gain insight into gene presence–absence and/or copy-number variation associated with complex traits such as NUE. Ultimately, new breeding technologies based on genome-editing techniques (Cardi and Varshney 2016; Mao et al. 2013) will provide interesting new tools to discover, characterize, and engineer NUE in rapeseed.

References

Adu MO, Chatot A, Wiesel L, Bennett MJ, Broadley MR, White PJ, Dupuy LX (2014) A scanner system for high-resolution quantification of variation in root growth dynamics of *Brassica rapa* genotypes. J Exp Bot 65(8):2039–2048. https://doi.org/10.1093/jxb/eru048

Araya T, Kubo T, von Wiren N, Takahashi H (2016) Statistical modeling of nitrogen-dependent modulation of root system architecture in Arabidopsis thaliana. J Integr Plant Biol 58(3):254–265. https://doi.org/10.1111/jipb.12433

Araya T, Miyamoto M, Wibowo J, Suzuki A, Kojima S, Tsuchiya YN et al (2014) CLE-CLAVATA1 peptide-receptor signaling module regulates the expansion of plant root systems in a nitrogen-dependent manner. Proc Natl Acad Sci USA 111(5):2029–2034. https://doi.org/10.1073/pnas.1319953111

Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, et al (2005) Cytokinin oxidase regulates rice grain production. Science (New York, N.Y.) 309(5735), 741–745. https://doi.org/10.1126/science.1113373

Bancroft I, Fraser F, Morgan C, Trick M (2015) Collinearity analysis of Brassica A and C genomes based on an updated inferred unigene order. Data Br 3:51–55. https://doi.org/10.1016/j.dib.2015.01.004

Barraclough PB (1989) Root growth, macro-nutrient uptake dynamics and soil fertility requirements of a high-yielding winter oilseed rape crop. Plant Soil 119(1):59–70. https://doi.org/10.1007/BF02370269

Basunanda P, Radoev M, Ecke W, Friedt W, Becker HC, Snowdon RJ (2010) Comparative mapping of quantitative trait loci involved in heterosis for seedling and yield traits in oilseed rape (*Brassica napus* L.). Theor Appl Genet 120(2):271–281. https://doi.org/10.1007/s00122-009-1133-z

Becker HC (1987) Quantitative genetik und zuchtmethodik bei raps—versuch einer literaturübersicht. In: Bericht über die Arbeitstagung der Saatzuchtleiter in Gumpenstein Verlag der Bundesanstalt für alpenländische, Landwirtschaft, Gumpenstein, Austria, pp 67–82

Bernardo R (2016) Bandwagons I, too, have known. Theor Appl Genet 129(12):2323–2332. https://doi.org/10.1007/s00122-016-2772-5

Berry PM, Spink J, Foulkes MJ, White PJ (2010) The physiological basis of genotypic differences in nitrogen use efficiency in oilseed rape (*Brassica napus* L.). Field Crops Res 119(2–3):365–373. https://doi.org/10.1016/j.fcr.2010.08.004

Bi Y-M, Wang R-L, Zhu T, Rothstein SJ (2007) Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis. BMC Genomics 8:281. https://doi.org/10.1186/1471-2164-8-281

Boote KJ, Jones JW, White JW, Asseng S, Lizaso JI (2013) Putting mechanisms into crop production models. Plant, Cell Environ 36(9):1658–1672. https://doi.org/10.1111/pce.12119

Bouchet A-S, Laperche A, Bissuel-Belaygue C, Snowdon R, Nesi N, Stahl A (2016) Nitrogen use efficiency in rapeseed. a review. Agron Sustain Dev 36(2). https://doi.org/10.1007/s13593-016-0371-0

Bouchet A-S, Nesi N, Bissuel C, Bregeon M, Lariepe A, Navier H et al (2014) Genetic control of yield and yield components in winter oilseed rape (*Brassica napus* L.) grown under nitrogen limitation. Euphytica 199(1–2):183–205. https://doi.org/10.1007/s10681-014-1130-4

Brugiere N, Humbert S, Rizzo N, Bohn J, Habben JE (2008) A member of the maize isopentenyl transferase gene family, *Zea mays* isopentenyl transferase 2 (ZmIPT2), encodes a cytokinin biosynthetic enzyme expressed during kernel development. Cytokinin biosynthesis in maize. Plant Mol Biol 67(3):215–229. https://doi.org/10.1007/s11103-008-9312-x

Brunel-Muguet S, Beauclair P, Bataillé M-P, Avice J-C, Trouverie J, Etienne P, Ourry A (2013) Light restriction delays leaf senescence in winter oilseed rape (*Brassica napus* L.). J Plant Growth Regul 32(3):506–518. https://doi.org/10.1007/s00344-013-9317-9

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG et al (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. The Plant J Cell Mol Biol 42(4):567–585. https://doi.org/10.1111/j.1365-313X.2005.02399.x

Bus A, Körber N, Snowdon RJ, Stich B (2011) Patterns of molecular variation in a species-wide germplasm set of *Brassica napus*. Theor Appl Genet 123(8):1413–1423. https://doi.org/10.1007/s00122-011-1676-7

Cai D, Xiao Y, Yang W, Ye W, Wang B, Younas M et al (2014) Association mapping of six yield related traits in rapeseed (*Brassica napus* L.). Theor Appl Genet 127(1):85–96. https://doi.org/10.1007/s00122-013-2203-9

Cai G, Yang Q, Chen H, Yang Q, Zhang C, Fan C, Zhou Y (2016) Genetic dissection of plant architecture and yield-related traits in *Brassica napus*. Sci Rep 6:21625. https://doi.org/10.1038/srep21625

Cardi T, Varshney R (2016) Cisgenesis and genome editing. Combining concepts and efforts for a smarter use of genetic resources in crop breeding. Plant Breed 135(2):139–147. https://doi.org/10.1111/pbr.12345

Ceccarelli S (1994) Specific adaptation and breeding for marginal conditions. In: Rognli OA, Solberg E, Schjelderup I (Hg.): Breeding fodder crops for marginal conditions, Bd. 2. Springer, Netherlands, Dordrecht (Developments in plant breeding), p 101–127

Chalhoub B, Denoeud F, Liu S, Parkin IA, Tang H, Wang X et al (2014) Plant genetics. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science (New York, N.Y.) 345(6199):950–953. https://doi.org/10.1126/science.1253435

Cheung F, Trick M, Drou N, Lim YP, Park YK, Kwon SJ et al (2009) Comparative analysis between homoeologous genome segments of *Brassica napus* and its progenitor species reveals extensive sequence-level divergence. Plant Cell 21(7):1912–1928. https://doi.org/10.1105/tpc.108.060376

Christen O, Sieling K (1993) The effect of different preceding crops on the development, growth and yield of winter barley. J Agron Crop Sci 171(2):114–123. https://doi.org/10.1111/j.1439-037X.1993.tb00120.x

Christen O, Sieling K, Hanus H (1992) The effect of different preceding crops on the development, growth and yield of winter wheat. Eur J Agron 1992(1):21–28

Chung KS, Lee JH, Lee JS, Ahn JH (2013) Fruit indehiscence caused by enhanced expression of NO TRANSMITTING TRACT in *Arabidopsis thaliana*. Mol Cells 35(6):519–525. https://doi.org/10.1007/s10059-013-0030-0

Clarke WE, Higgins EE, Plieske J, Wieseke R, Sidebottom C, Khedikar Y et al (2016) A high-density SNP genotyping array for *Brassica napus* and its ancestral diploid species based on optimised selection of single-locus markers in the allotetraploid genome. Theor Appl Genet 129(10):1887–1899. https://doi.org/10.1007/s00122-016-2746-7

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants. Emerging 'matrix effects'. Curr Opin Plant Biol 4(3):247–253. https://doi.org/10.1016/s1369-5266(00)00168-0

Cowling WA, Buirchell BJ, Falk DE (2009) A model for incorporating novel alleles from the primary gene pool into elite crop breeding programs while reselecting major genes for domestication or adaptation. Crop Pasture Sci. 60(10):1009. https://doi.org/10.1071/cp08223

Cowling WA (2007) Genetic diversity in Australian canola and implications for crop breeding for changing future environments. Field Crops Res 104(1–3):103–111. https://doi.org/10.1016/j.fcr.2006.12.014

Cowling WA, Léon J (2013) Sustainable plant breeding. Plant Breed 132(1):1–9. https://doi.org/10.1111/pbr.12026

Cramer N (1993) Umweltverträglichkeit der N-Versorgung des Rapses. Raps 1993(11):4–7

Crossa J, de Los Campos G, Perez P, Gianola D, Burgueno J, Araus JL et al (2010) Prediction of genetic values of quantitative traits in plant breeding using pedigree and molecular markers. Genetics 186(2):713–724. https://doi.org/10.1534/genetics.110.118521

Cullis BR, Smith AB, Beeck CP, Cowling WA (2010) Analysis of yield and oil from a series of canola breeding trials. Part II. Exploring variety by environment interaction using factor analysis. Genome 53(11):1002–1016. https://doi.org/10.1139/G10-080

Diepenbrock W (2000) Yield analysis of winter oilseed rape (*Brassica napus* L.): a review. Field Crops Res 67(1):35–49. https://doi.org/10.1016/S0378-4290(00)00082-4

de Dorlodot S, Forster B, Pages L, Price A, Tuberosa R, Draye X (2007) Root system architecture: opportunities and constraints for genetic improvement of crops. Trends Plant Sci 12(10):474–481. https://doi.org/10.1016/j.tplants.2007.08.012

Dresbøll DB, Thorup-Kristensen K (2014) Will breeding for nitrogen use efficient crops lead to nitrogen use efficient cropping systems? A simulation study of G × E×M interactions. Euphytica 199(1–2):97–117. https://doi.org/10.1007/s10681-014-1199-9

Edwards D, Batley J, Snowdon RJ (2013) Accessing complex crop genomes with next-generation sequencing. Theor Appl Genet 126(1):1–11. https://doi.org/10.1007/s00122-012-1964-x

Emery Neil RJ, Ma Q, Atkins CA (2000) The forms and sources of cytokinins in developing white lupine seeds and fruits. Plant Physiol 123:1593–1604

Gan S, Amasino RM (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270(5244):1986–1988. https://doi.org/10.1126/science.270.5244.1986

Gan Y, Bernreiter A, Filleur S, Abram B, Forde BG (2012) Overexpressing the ANR1 MADS-box gene in transgenic plants provides new insights into its role in the nitrate regulation of root development. Plant Cell Physiol 53(6):1003–1016. https://doi.org/10.1093/pcp/pcs050

Ganal MW, Polley A, Graner E-M, Plieske J, Wieseke R, Luerssen H, Durstewitz G (2012) Large SNP arrays for genotyping in crop plants. J Biosci 37(5):821–828. https://doi.org/10.1007/s12038-012-9225-3

Garnett T, Conn V, Kaiser BN (2009) Root based approaches to improving nitrogen use efficiency in plants. Plant, Cell Environ 32(9):1272–1283. https://doi.org/10.1111/j.1365-3040.2009.02011.x

Gehringer A, Snowdon R, Spiller T, Basunanda P, Friedt W (2007) New oilseed rape (*Brassica napus*) Hybrids with high levels of heterosis for seed yield under nutrient-poor conditions. Breed Sci 57(4):315–320. https://doi.org/10.1270/jsbbs.57.315

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2008) Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci USA 105(2):803–808. https://doi.org/10.1073/pnas.0709559105

Girke A, Schierholt A, Becker HC (2012a) Extending the rapeseed gene pool with resynthesized *Brassica napus* II: Heterosis. Theor Appl Genet 124(6):1017–1026. https://doi.org/10.1007/s00122-011-1765-7

Girke A, Schierholt A, Becker HC (2012b) Extending the rapeseed genepool with resynthesized *Brassica napus* L. I: genetic diversity. Genet Resour Crop Evol 59(7):1441–1447. https://doi.org/10.1007/s10722-011-9772-8

Girondé A, Etienne P, Trouverie J, Bouchereau A, Le Cahérec F, Leport L et al (2015a) The contrasting N management of two oilseed rape genotypes reveals the mechanisms of proteolysis associated with leaf N remobilization and the respective contributions of leaves and stems to N storage and remobilization during seed filling. BMC Plant Biol 15:59. https://doi.org/10.1186/s12870-015-0437-1

Girondé A, Poret M, Etienne P, Trouverie J, Bouchereau A, Le Cahérec F et al (2015b) A profiling approach of the natural variability of foliar N remobilization at the rosette stage gives clues to understand the limiting processes involved in the low N use efficiency of winter oilseed rape. J Exp Bot 66(9):2461–2473. https://doi.org/10.1093/jxb/erv031

Golicz AA, Batley J, Edwards D (2016a) Towards plant pangenomics. Plant Biotechnol J 14 (4):1099–1105. https://doi.org/10.1111/pbi.12499

Golicz AA, Bayer PE, Barker GC, Edger PP, Kim H, Martinez PA et al (2016b) The pangenome of an agronomically important crop plant *Brassica oleracea*. Nature Commun 7:13390. https://doi.org/10.1038/ncomms13390

Gombert J, Etienne P, Ourry A, Le Dily F (2006) The expression patterns of SAG12/Cab genes reveal the spatial and temporal progression of leaf senescence in *Brassica napus* L. with sensitivity to the environment. J Exp Bot 57(9):1949–1956. https://doi.org/10.1093/jxb/erj142

Gombert J, Le Dily F, Lothier J, Etienne P, Rossato L, Allirand JM et al (2010) Effect of nitrogen fertilization on nitrogen dynamics in oilseed rape using 15 N-labeling field experiment. Z. Pflanzenernähr Bodenk 173(6):875–884. https://doi.org/10.1002/jpln.200800270

Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9(12):597–605. https://doi.org/10.1016/j.tplants.2004.10.008

Grami B, Stefansson BR (1977) Gene action for protein and oil content in summer rape. Can J Plant Sci 57(3):625–631. https://doi.org/10.4141/cjps77-092

Gregersen PL, Culetic A, Boschian L, Krupinska K (2013) Plant senescence and crop productivity. Plant Mol Biol 82(6):603–622. https://doi.org/10.1007/s11103-013-0013-8

Griggs D, Stafford SM, Rockström J, Öhman MC, Gaffney O, Glaser G et al (2014) An integrated framework for sustainable development goals. In: E&S vol 19, no 4. https://doi.org/10.5751/es-07082-190449

Guo Y, Gan S-S (2014) Translational researches on leaf senescence for enhancing plant productivity and quality. J Exp Bot 65(14):3901–3913. https://doi.org/10.1093/jxb/eru248

Fischer RA, Byerlee D, Edmeades GO (2014) Crop yields and global food security: will yield increase continue to feed the world? ACIAR Monograph no. 158. Australian Centre for International Agricultural Research, Canberra, xxii, 634 pp

Fletcher RS, Mullen JL, Heiliger A, McKay JK (2014) QTL analysis of root morphology, flowering time, and yield reveals trade-offs in response to drought in *Brassica napus*. J Exp Bot 66(1):245–256. https://doi.org/10.1093/jxb/eru423

Foisset N, Delourme R, Barret P, Hubert N, Landry BS, Renard M (1996) Molecular-mapping analysis in *Brassica napus* using isozyme, RAPD and RFLP markers on a doubled-haploid progeny. Theor Appl Genet 93(7):1017–1025. https://doi.org/10.1007/BF00230119

Foisset N, Delourme R, Barret P, Renard M (1995) Molecular tagging of the dwarf BREIZH (Bzh) gene in *Brassica napus*. Theor Appl Genet 91(5):756–761. https://doi.org/10.1007/BF00220955

Funk H, Mohr R (2010) Die Rapsabrechnung. UFOP-Praxisinformation, Union zur Förderung von Oelund Proteinpflanzen e.V. Berlin. http://www.ufop.de

Han Y, Yang H, Jiao Y (2014): Regulation of inflorescence architecture by cytokinins. Frontiers Plant Sci 5:669. https://doi.org/10.3389/fpls.2014.00669

Hasan M, Seyis F, Badani AG, Pons-Kühnemann J, Friedt W, Lühs W, Snowdon RJ (2006) Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. Genet Resour Crop Evol 53(4):793–802. https://doi.org/10.1007/s10722-004-5541-2

Hatzig S, Zaharia LI, Abrams S, Hohmann S, Legoahec L, Bouchereau A et al (2014) Early osmotic adjustment responses in drought-resistant and drought-sensitive oilseed rape. J Integr Plant Biol 56(8):797–809. https://doi.org/10.1111/jipb.12199

Hatzig SV, Frisch M, Breuer F, Nesi N, Ducournau S, Wagner M-H et al (2015) Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. Frontiers Plant Sci 6:221. https://doi.org/10.3389/fpls.2015.00221

Heffner EL, Jannink J-L, Sorrells ME (2011) Genomic selection accuracy using multifamily prediction models in a wheat breeding program. The Plant Genome 4(1):65–75

Heffner EL, Jannink J-L, Sorrells ME (2010) Plant breeding with genomic selection. Gain per unit time and cost. Crop Sci 50(5):1681. https://doi.org/10.2135/cropsci2009.11.0662

Henke J; Breustedt G, Sieling K, Kage H (2007) Impact of uncertainty on the optimum nitrogen fertilization rate and agronomic, ecological and economic factors in an oilseed rape based crop rotation. The J Agric Sci 145(05):455. https://doi.org/10.1017/s0021859607007204

Hirel B, Le Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot 58(9):2369–2387. https://doi.org/10.1093/jxb/erm097

Hohmann M, Stahl A, Rudloff J, Wittkop B, Snowdon RJ (2016) Not a load of rubbish: simulated field trials in large-scale containers. Plant, Cell Environ 39(9):2064–2073. https://doi.org/10.1111/pce.12737

Hua S, Chen Z-H, Zhang Y, Yu H, Lin B, Zhang D (2014) Chlorophyll and carbohydrate metabolism in developing silique and seed are prerequisite to seed oil content of *Brassica napus* L. Bot Stud 55(1):34. https://doi.org/10.1186/1999-3110-55-34

Hwang I, Sheen J, Muller B (2012) Cytokinin signaling networks. Ann Rev Plant Biol 63:353–380. https://doi.org/10.1146/annurev-arplant-042811-105503

Jameson PE, Song J (2016) Cytokinin: a key driver of seed yield. J Exp Bot 67(3):593–606. https://doi.org/10.1093/jxb/erv461

Jan HU, Abbadi A, Lucke S, Nichols RA, Snowdon RJ (2016) Genomic prediction of testcross performance in canola (*Brassica napus*). PLoS One 11(1):e0147769. https://doi.org/10.1371/journal.pone.0147769

Jonas E, de Koning J-D (2013) Does genomic selection have a future in plant breeding? Trends Biotechnol 31(9):497–504. https://doi.org/10.1016/j.tibtech.2013.06.003

Jung C, Müller AE (2009) Flowering time control and applications in plant breeding. Trends Plant Sci 14(10):563–573. https://doi.org/10.1016/j.tplants.2009.07.005

Junior J, Abrahão E, Mertz LM, Henning FA, Quilón IR, Maia MDS, Altisent JMD (2012) Changes in canola plant architecture and seed physiological quality in response to different sowing densities. Rev Bras de Sem 34(2012):14–20

Kamh M, Wiesler F, Ulas A, Horst WJ (2005) Root growth and N-uptake activity of oilseed rape (*Brassica napus* L.) cultivars differing in nitrogen efficiency. J Plant Nutr Soil Sci 168(1):130–137. https://doi.org/10.1002/jpln.200421453

Kant S, Burch D, Badenhorst P, Palanisamy R, Mason J, Spangenberg G (2015) Regulated expression of a cytokinin biosynthesis gene IPT delays leaf senescence and improves yield under rainfed and irrigated conditions in canola (*Brassica napus* L.). PloS One 10(1):e0116349. https://doi.org/10.1371/journal.pone.0116349

Kessel B (2000) Genetische Variation und Vererbung der Stickstoff—Effizienz bei Winterraps (*Brassica napus* L.) (In German). PhD dissertation, Universität Göttingen Göttingen, Germany

Kessel B, Schierholt A, Becker HC (2012) Nitrogen use efficiency in a genetically diverse set of winter oilseed rape (L.). Crop Sci 52(6):2546. https://doi.org/10.2135/cropsci2012.02.0134

Kiba T, Krapp A (2016) Plant Nitrogen acquisition under low availability: regulation of uptake and root architecture. Plant Cell Physiol 57(4):707–714. https://doi.org/10.1093/pcp/pcw052

Kirkegaard JA, Lilley JM, Morrison MJ (2016) Drivers of trends in Australian canola productivity and future prospects. Crop Pasture Sci 67(4):i. https://doi.org/10.1071/cpv67n4_fo

Koeslin-Findeklee F, Meyer A, Girke A, Beckmann K, Horst WJ (2014) The superior nitrogen efficiency of winter oilseed rape (*Brassica napus* L.) hybrids is not related to delayed nitrogen

starvation-induced leaf senescence. Plant Soil 384(1–2):347–362. https://doi.org/10.1007/s11104-014-2212-8

Koeslin-Findeklee F, Rizi VS, Becker MA, Parra-Londono S, Arif M, Balazadeh S et al (2015) Transcriptomic analysis of nitrogen starvation—and cultivar-specific leaf senescence in winter oilseed rape (*Brassica napus* L.). Plant Sci Int J Exp Plant Biol 233:174–185. https://doi.org/10.1016/j.plantsci.2014.11.018

Körber N, Bus A, Li J, Parkin IAP, Wittkop B, Snowdon RJ, Stich B (2016) Agronomic and seed quality traits dissected by genome-wide association mapping in *Brassica napus*. Front Plant Sci 7:386. https://doi.org/10.3389/fpls.2016.00386

Koscielny CB, Gulden RH (2012) Seedling root length in *Brassica napus* L. is indicative of seed yield. Can J Plant Sci 92(7):1229–1237. https://doi.org/10.4141/cjps2012-070

Le Deunff E, Malagoli P (2014) An updated model for nitrate uptake modelling in plants. I. Functional component: cross-combination of flow-force interpretation of nitrate uptake isotherms, and environmental and in planta regulation of nitrate influx. Ann Bot 113(6):991–1005. https://doi.org/10.1093/aob/mcu021

Lee B-R, Jin Y-L, Park S-H, Zaman R, Zhang Q, Avice J-C et al (2015) Genotypic variation in N uptake and assimilation estimated by 15 N tracing in water deficit-stressed *Brassica napus*. Environ Exp Bot 109:73–79. https://doi.org/10.1016/j.envexpbot.2014.08.004

Liu J, Wang W, Mei D, Wang H, Fu L, Liu D et al (2016) Characterizing variation of branch angle and genome-wide association mapping in rapeseed (*Brassica napus* L.). Front Plant Sci 7:21. https://doi.org/10.3389/fpls.2016.00021

Lotze-Campen H, von Witzke H, Noleppa S, Schwarz G (2015) Science for food, climate protection and welfare. An economic analysis of plant breeding research in Germany. Agric Syst 136:79–84. https://doi.org/10.1016/j.agsy.2015.02.005

Luo YX, Luo CY, Du DZ, Fu Z, Yao YM, Xu CC, Zhang HS (2014) Quantitative trait analysis of flowering time in spring rapeseed (*B. napus* L.). Euphytica 200(3):321–335. https://doi.org/10.1007/s10681-014-1140-2

Lysak MA, Koch MA, Pecinka A, Schubert I (2005) Chromosome triplication found across the tribe Brassiceae. Genome Res 15(4):516–525. https://doi.org/10.1101/gr.3531105

Malagoli P, Laine P, Rossato L, Ourry A (2005) Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (*Brassica napus*) from stem extension to harvest: I. Global N flows between vegetative and reproductive tissues in relation to leaf fall and their residual N. Ann Bot 95(5):853–861. https://doi.org/10.1093/aob/mci091

Malagoli P, Le Deunff E (2014) An updated model for nitrate uptake modelling in plants. II. Assessment of active root involvement in nitrate uptake based on integrated root system age: measured versus modelled outputs. Ann Bot 113(6):1007–1019. https://doi.org/10.1093/aob/mcu022

Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu J-K (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol Plant 6(6):2008–2011. https://doi.org/10.1093/mp/sst121

Masclaux-Daubresse C, Reisdorf-Cren M, Orsel M (2008) Leaf nitrogen remobilisation for plant development and grain filling. Plant Biol 10:23–36. https://doi.org/10.1111/j.1438-8677.2008.00097.x

Mason AS, Snowdon RJ (2016) Oilseed rape: learning about ancient and recent polyploid evolution from a recent crop species. Plant Biol (Stuttgart, Germany) 18(6):883–892. https://doi.org/10.1111/plb.12462

Mason AS, Batley J (2015) Creating new interspecific hybrid and polyploid crops. Trends Biotechnol 33(8):436–441. https://doi.org/10.1016/j.tibtech.2015.06.004

Melzer S, Lens F, Gennen J, Vanneste S, Rohde A, Beeckman T (2008) Flowering-time genes modulate meristem determinacy and growth form in *Arabidopsis thaliana*. Nat Genet 40(12):1489–1492. https://doi.org/10.1038/ng.253

Miersch S, Gertz A, Breuer F, Schierholt A, Becker HC (2016a) Influence of the semi-dwarf growth type on nitrogen use efficiency in winter oilseed rape. Crop Sci 56(6):2952–2961. https://doi.org/10.2135/cropsci2016.01.0044

Miersch S, Gertz A, Breuer F, Schierholt A, Becker HC (2016b) Influence of the semi-dwarf growth type on seed yield and agronomic parameters at low and high nitrogen fertilization in winter oilseed rape. Crop Sci 56(4):1573–1585. https://doi.org/10.2135/cropsci2015.09.0554

Moll RH, Kamprath EJ, Jackson WA (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization 1. Agron J 74(3):562. https://doi.org/10.2134/agronj1982.00021962007400030037x

Morrison MJ, Harker KN, Blackshaw RE, Holzapfel CJ, O'Donovan JT (2016) Canola yield improvement on the Canadian Prairies from 2000 to 2013. Crop Pasture Sci 67(4):245. https://doi.org/10.1071/cp15348

Müller K, Böttcher U, Meyer-Schatz F, Kage H (2008) Analysis of vegetation indices derived from hyperspectral reflection measurements for estimating crop canopy parameters of oilseed rape (*Brassica napus* L.). Biosyst Eng 101(2):172–182. https://doi.org/10.1016/j.biosystemseng.2008.07.004

Nelson MN, Lilley JM, Helliwell C, Taylor CM, Siddique KHM, Chen S et al (2016) Can genomics assist the phenological adaptation of canola to new and changing environments? Crop Pasture Sci 67(4):284. https://doi.org/10.1071/cp15320

Nyikako J, Schierholt A, Kessel B, Becker HC (2014) Genetic variation in nitrogen uptake and utilization efficiency in a segregating DH population of winter oilseed rape. Euphytica 199:3–11. https://doi.org/10.1007/s10681-014-1201-6

Orsel M, Moison M, Clouet V, Thomas J, Leprince F, Canoy A-S et al (2014) Sixteen cytosolic glutamine synthetase genes identified in the *Brassica napus* L. genome are differentially regulated depending on nitrogen regimes and leaf senescence. J Exp Bot 65(14):3927–3947. https://doi.org/10.1093/jxb/eru041

Pahlmann I, Böttcher U, Kage H (2017) Developing and testing an algorithm for site-specific N fertilization of winter oilseed rape. Comput Electron Agric 136:228–237. https://doi.org/10.1016/j.compag.2016.12.005

Parkin IAP, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC, Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with Arabidopsis thaliana. Genetics 171(2):765–781. https://doi.org/10.1534/genetics.105.042093

Parkin Isobel A P, Koh C, Tang H, Robinson SJ, Kagale S, Clarke WE et al (2014) Transcriptome and methylome profiling reveals relics of genome dominance in the mesopolyploid *Brassica oleracea*. Genome Biol 15(6):R77. https://doi.org/10.1186/gb-2014-15-6-r77

Passioura JB (1983) Roots and drought resistance. Agric Water Manag 7(1–3):265–280. https://doi.org/10.1016/0378-3774(83)90089-6

Peleg Z, Reguera M, Tumimbang E, Walia H, Blumwald E (2011) Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress. Plant Biotechnol J 9(7):747–758. https://doi.org/10.1111/j.1467-7652.2010.00584.x

Poorter H, Fiorani F, Pieruschka R, Wojciechowski T, van der Putten WH, Kleyer M et al (2016) Pampered inside, pestered outside? Differences and similarities between plants growing in controlled conditions and in the field. The New Phytol 212(4):838–855. https://doi.org/10.1111/nph.14243

Potter T, Burton W, Edwards J, Wratten N, Mailer R, Salisbury P, Pearce A (2016) Assessing progress in breeding to improve grain yield, quality and blackleg (Leptosphaeria maculans) resistance in selected Australian canola cultivars (1978? 2012). Crop Pasture Sci 67(4):308–316. https://doi.org/10.1071/CP15290

Qian L, Qian W, Snowdon RJ (2014) Sub-genomic selection patterns as a signature of breeding in the allopolyploid *Brassica napus* genome. BMC Genomics 15:1170. https://doi.org/10.1186/1471-2164-15-1170

Qian L, Qian W, Snowdon RJ (2016a) Haplotype hitchhiking promotes trait coselection in *Brassica napus*. Plant Biotechnol J 14(7):1578–1588. https://doi.org/10.1111/pbi.12521

Qian L, Voss-Fels K, Cui Y, Jan HU, Samans B, Obermeier C et al (2016b) Deletion of a stay-green gene associates with adaptive selection in *Brassica napus*. Mol Plant 9(12):1559–1569. https://doi.org/10.1016/j.molp.2016.10.017

Quijada PA, Udall JA, Lambert B, Osborn TC (2006) Quantitative trait analysis of seed yield and other complex traits in hybrid spring rapeseed (*Brassica napus* L.): 1. Identification of genomic regions from winter germplasm. Theor Appl Genet 113(3):549–561. https://doi.org/10.1007/s00122-006-0323-1

Radoev M, Becker HC, Ecke W (2008) Genetic analysis of heterosis for yield and yield components in rapeseed (*Brassica napus* L.) by quantitative trait locus mapping. Genetics 179 (3):1547–1558. https://doi.org/10.1534/genetics.108.089680

Rahman M, McClean P (2013) Genetic analysis on flowering time and root system in L. Crop Sci 53(1):141–147. https://doi.org/10.2135/cropsci2012.02.0095

Raman H, Raman R, Kilian A, Detering F, Carling J, Coombes N et al (2014) Genome-wide delineation of natural variation for pod shatter resistance in *Brassica napus*. PLoS ONE 9(7): e101673. https://doi.org/10.1371/journal.pone.0101673

Raman R, Diffey S, Carling J, Cowley RB, Kilian A, Luckett DJ, Raman H (2016) Quantitative genetic analysis of grain yield in an Australian *Brassica napus* doubled-haploid population. Crop Pasture Sci 67(4):298. https://doi.org/10.1071/cp15283

Rathke G-W, Christen O, Diepenbrock W (2005) Effects of nitrogen source and rate on productivity and quality of winter oilseed rape (*Brassica napus* L.) grown in different crop rotations. Field Crops Res 94(2–3):103–113. https://doi.org/10.1016/j.fcr.2004.11.010

Rockström J, Steffen W, Noone K, Persson A, Chapin III FS, Lambin EF et al (2009) A safe operating space for humanity. Nature 461(7263):472–475. https://doi.org/10.1038/461472a

Rossato L, Lainé P, Ourry A (2001) Nitrogen storage and remobilization in *Brassica napus* L. during the growth cycle: nitrogen fluxes within the plant and changes in soluble protein patterns. J Exp Bot 52(361):1655–1663. https://doi.org/10.1093/jexbot/52.361.1655

Sattelmacher B, Horst WJ, Becker HC (1994) Factors that contribute to genetic variation for nutrient efficiency of crop plants. Z Pflanzenernaehr Bodenk 157(3):215–224. https://doi.org/10.1002/jpln.19941570309

Schaller GE, Street IH, Kieber JJ (2014) Cytokinin and the cell cycle. Curr Opin Plant Biol 21:7–15. https://doi.org/10.1016/j.pbi.2014.05.015

Schiessl S, Iniguez-Luy F, Qian W, Snowdon RJ (2015) Diverse regulatory factors associate with flowering time and yield responses in winter-type *Brassica napus*. BMC Genomics 16:737. https://doi.org/10.1186/s12864-015-1950-1

Schiessl S, Samans B, Huttel B, Reinhard R, Snowdon RJ (2014) Capturing sequence variation among flowering-time regulatory gene homologs in the allopolyploid crop species *Brassica napus*. Frontiers Plant Sci 5:404. https://doi.org/10.3389/fpls.2014.00404

Schmutzer T, Samans B, Dyrszka E, Ulpinnis C, Weise S, Stengel D et al (2015) Species-wide genome sequence and nucleotide polymorphisms from the model allopolyploid plant *Brassica napus*. Sci Data 2:150072. https://doi.org/10.1038/sdata.2015.72

Schulte auf'm Erley G, Behrens T, Ulas A, Wiesler F, Horst W (2011) Agronomic traits contributing to nitrogen efficiency of winter oilseed rape cultivars. Field Crops Res 124 (1):114–123. https://doi.org/10.1016/j.fcr.2011.06.009

Schulte auf'm Erley EG, Wijaya K-A, Ulas A, Becker H, Wiesler F, Horst WJ (2007) Leaf senescence and N uptake parameters as selection traits for nitrogen efficiency of oilseed rape cultivars. Physiol Plant 130(4):519–531. https://doi.org/10.1111/j.1399-3054.2007.00921.x

Shi L, Shi T, Broadley MR, White PJ, Long Y, Meng J et al (2013) High-throughput root phenotyping screens identify genetic loci associated with root architectural traits in *Brassica napus* under contrasting phosphate availabilities. Ann Bot 112(2):381–389. https://doi.org/10.1093/aob/mcs245

Sieling K, Kage H (2008) The potential of semi-dwarf oilseed rape genotypes to reduce the risk of N leaching. The J Agric Sci 146(01). https://doi.org/10.1017/s0021859607007472

Sieling K, Kage H (2010) Efficient N management using winter oilseed rape. A review. Agron Sustain Dev 30(2):271–279. https://doi.org/10.1051/agro/2009036

Singh S, Letham DS, Jameson PE, Zhang R, Parker CW, Badenoch-Jones J, Noodén LD (1988) (1988): Cytokinin biochemistry in relation to leaf senescence. IV. Cytokinin metabolism in soybean explants. Plant Physiol 88:788–794

Snowdon RJ, Luy I, Federico L (2012) Potential to improve oilseed rape and canola breeding in the genomics era. Plant Breed 131(3):351–360. https://doi.org/10.1111/j.1439-0523.2012.01976.x

Song J, Jiang L, Jameson PE (2015) Expression patterns of *Brassica napus* genes implicate IPT, CKX, sucrose transporter, cell wall invertase, and amino acid permease gene family members in leaf, flower, silique, and seed development. J Exp Bot 66(16):5067–5082. https://doi.org/10.1093/jxb/erv133

Stahl A (2016) Detailed phenotypic assessment of genetic variation for nitrogen uptake and utilisation efficiency traits in a diverse panel of *Brassica napus* L. PhD dissertation, Universität Giessen, Giessen, Germany

Stahl A, Friedt W, Wittkop B, Snowdon RJ (2016) Complementary diversity for nitrogen uptake and utilisation efficiency reveals broad potential for increased sustainability of oilseed rape production. Plant Soil 400(1–2):245–262. https://doi.org/10.1007/s11104-015-2726-8

Stahl A, Pfeifer M, Frisch M, Wittkop B, Snowdon RJ (2017) Recent genetic gains in nitrogen use efficiency in oilseed rape. Front. Plant Sci. 8:963. https://doi.org/10.3389/fpls.2017.00963

Svečnjak Z, Rengel Z (2006) Nitrogen utilization efficiency in canola cultivars at grain harvest. Plant Soil 283(1–2):299–307. https://doi.org/10.1007/s11104-006-0020-5

Technow F, Messina CD, Totir LR, Cooper M (2015) Integrating crop growth models with whole genome prediction through approximate Bayesian computation. PLoS ONE 10(6):e0130855. https://doi.org/10.1371/journal.pone.0130855

Thomas CL, Graham NS, Hayden R, Meacham MC, Neugebauer K, Nightingale M et al (2016) High-throughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.). Ann Bot 655–665. https://doi.org/10.1093/aob/mcw046

Thorup-Kristensen K (2006) Effect of deep and shallow root systems on the dynamics of soil inorganic N during 3-year crop rotations. Plant Soil 288(1–2):233–248. https://doi.org/10.1007/s11104-006-9110-7

Thorup-Kristensen K, Kirkegaard J (2016) Root system-based limits to agricultural productivity and efficiency: the farming systems context. Ann Bot 573–592. https://doi.org/10.1093/aob/mcw122

Thurling N (1991) Application of the ideotype concept in breeding for higher yield in the oilseed brassicas. Field Crops Res 26(2):201–219. https://doi.org/10.1016/0378-4290(91)90036-U

Tilsner J, Kassner N, Struck C, Lohaus G (2005) Amino acid contents and transport in oilseed rape (*Brassica napus* L.) under different nitrogen conditions. Planta 221(3):328–338. https://doi.org/10.1007/s00425-004-1446-8

Udall JA, Quijada PA, Lambert B, Osborn TC (2006) Quantitative trait analysis of seed yield and other complex traits in hybrid spring rapeseed (*Brassica napus* L.): 2. Identification of alleles from unadapted germplasm. Theor Appl Genet 113(4):597–609. https://doi.org/10.1007/s00122-006-0324-0

Ulas A, Schulte auf'm Erley G, Kamh M, Wiesler F, Horst WJ (2012) Root-growth characteristics contributing to genotypic variation in nitrogen efficiency of oilseed rape. Z Pflanzenernähr Bodenk 175(3):489–498. https://doi.org/10.1002/jpln.201100301

van der Graaff E, Schwacke R, Schneider A, Desimone M, Flugge U-I, Kunze R (2006) Transcription analysis of arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol 141(2):776–792. https://doi.org/10.1104/pp.106.079293

Vidal EA, Tamayo KP, Gutierrez RA (2010) Gene networks for nitrogen sensing, signaling, and response in Arabidopsis thaliana. Wiley Interdiscip Rev Syst Biol Med 2(6):683–693. https://doi.org/10.1002/wsbm.87

Voss-Fels K, Snowdon RJ (2015) Understanding and utilizing crop genome diversity via high-resolution genotyping. Plant Biotechnol J 1086–1094. https://doi.org/10.1111/pbi.12456

Wang G, Ding G, Li L, Cai H, Ye X, Zou J, Xu F (2014) Identification and characterization of improved nitrogen efficiency in interspecific hybridized new-type *Brassica napus*. Ann Bot 114(3):549–559. https://doi.org/10.1093/aob/mcu135

Wang L, Mühling K-H, Schulte auf'm Erley G (2016) Nitrogen efficiency and leaf nitrogen remobilisation of oilseed rape lines and hybrids. Ann Appl Biol 169(1):125–133. https://doi.org/10.1111/aab.12286

Wang ML, Zhao Y, Chen F, Yin XC (2004) Inheritance and potentials of a mutated dwarfing gene ndf1 in *Brassica napus*. Plant Breed 123(5):449–453. https://doi.org/10.1111/j.1439-0523.2004.01014.x

Wang X, Wang H, Wang J, Sun R, Wu J, Liu S et al (2011) The genome of the mesopolyploid crop species *Brassica rapa*. Nat Genet 43(10):1035–1039. https://doi.org/10.1038/ng.919

White PJ, George TS, Dupuy LX, Karley AJ, Valentine TA, Wiesel L, Wishart J (2013): Root traits for infertile soils. Front Plant Sci 4:193. https://doi.org/10.3389/fpls.2013.00193

Wiesler F, Behrens T, Horst WJ (2001) The role of nitrogen-efficient cultivars in sustainable agriculture, In: Optimizing nitrogen management in food and energy production and environmental protection. In: Proceedings of the 2nd international nitrogen conference on science and policy. The Scientific World, vol 1

Wu X-L, Liu Z-H, Hu Z-H, Huang R-Z (2014) BnWRI1 coordinates fatty acid biosynthesis and photosynthesis pathways during oil accumulation in rapeseed. J Integr Plant Biol 56(6):582–593. https://doi.org/10.1111/jipb.12158

Wurschüm T (2012) Mapping QTL for agronomic traits in breeding populations. Theor Appl Genet 125(2):201–210. https://doi.org/10.1007/s00122-012-1887-6

Xu L, Hu K, Zhang Z, Guan C, Chen S, Hua W et al (2016) Genome-wide association study reveals the genetic architecture of flowering time in rapeseed (*Brassica napus* L.). DNA Res Int J Rapid Pub Rep Genes Genomes 23(1):43–52. https://doi.org/10.1093/dnares/dsv035

Yu J, Zhao M, Wang X, Tong C, Huang S, Tehrim S et al (2013) Bolbase: a comprehensive genomics database for Brassica oleracea. BMC Genomics 14:664. https://doi.org/10.1186/1471-2164-14-664

Zhang H, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279(5349):407–409. https://doi.org/10.1126/science.279.5349.407

Zhang H, Forde BG (2000) Regulation of Arabidopsis root development by nitrate availability. J Exp Bot 51(342):51–59. https://doi.org/10.1093/jexbot/51.342.51

Zhao J, Becker HC, Zhang D, Zhang Y, Ecke W (2006) Conditional QTL mapping of oil content in rapeseed with respect to protein content and traits related to plant development and grain yield. Theor Appl Genet 113(1):33–38. https://doi.org/10.1007/s00122-006-0267-5

Zhao J, Bai W, Zeng Q, Song S, Zhang M, Li X et al (2015) Moderately enhancing cytokinin level by down-regulation of GhCKX expression in cotton concurrently increases fiber and seed yield. Mol Breed New Strateg Plant Improv 35:60. https://doi.org/10.1007/s11032-015-0232-6

Zhu X-G, Lynch JP, LeBauer DS, Millar AJ, Stitt M, Long SP (2016) Plants in silico: why, why now and what?–an integrative platform for plant systems biology research. Plant, Cell Environ 39(5):1049–1057. https://doi.org/10.1111/pce.12673

Zou J, Zhu J, Huang S, Tian E, Xiao Y, Fu D et al (2010) Broadening the avenue of intersubgenomic heterosis in oilseed Brassica. Theor Appl Genet 120(2):283–290. https://doi.org/10.1007/s00122-009-1201-4

Chapter 13
The Importance of Organic Nitrogen Transport Processes for Plant Productivity and Nitrogen Use Efficiency

Mechthild Tegeder and Molly Perchlik

Abbreviations

AAP	Amino Acid Permease
AMT	Ammonium Transporter
CAT	Cationic Amino acid Transporter
DUR	Degradation of urea (urea transporter)
LHT	Lysine-Histidine-type Transporter
NRT	Nitrate Transporter
PTR	Peptide Transporter
NPF	NRT1/PTR Family
ProT	Proline Transporter
UmamiT	Usually Multiple Acids Move In and out Transporter
UPS	Ureide Permease

Summary

Plants need large amounts of nitrogen for growth, development, and reproduction. Generally, inorganic nitrogen is acquired from the soil or atmosphere and reduced in nodules, roots, or photosynthetically active source leaves to amino acids or ureides. These organic compounds present the main nitrogen forms transported from source to sink, and their regulated partitioning is critical for plant metabolism, growth, and efficient nitrogen use. Nitrogen uptake and long-distance transport of organic nitrogen from root to leaf to seed requires the function of plasma membrane transporters. Amino acid and ureide transporters are localized to critical positions along the path where they control nitrogen acquisition, export from nodules,

M. Tegeder (✉) · M. Perchlik
School of Biological Sciences, Washington State University, Pullman, WA 99164, USA
e-mail: tegeder@wsu.edu

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_13

xylem-to-phloem transfer, phloem loading, and seed import. These transporters present important targets for manipulation of nitrogen partitioning to improve seed yield and quality, and nitrogen use efficiency.

Introduction

Nitrogen (N) is an essential macronutrient that is needed in large amounts for plant growth and development. It is an important component of many biological molecules, including amino acids, which are the building blocks of proteins and enzymes. Amino acids are also used as N precursors or donors for the synthesis of nucleic acids, ureides, chlorophyll, and many other N metabolites, such as alkaloids, which are involved in plant defense (Lam et al. 1996; Epstein and Bloom 2005; Zrenner et al. 2006; Miret and Munné-Bosch 2014; Züst and Agrawal 2016). In addition, amino acids are the main long-distance N transport forms in most plant species.

Plants generally acquire inorganic N from the soil through uptake of nitrate and ammonium via transport proteins (Fig. 13.1; Loqué and von Wirén 2004; Krapp et al. 2014). Uptake of organic N compounds also occurs (e.g., amino acids and peptides), especially in ecosystems with low soil N mineralization and in cropping systems that use organic fertilizers such as manure or compost (Farley and Fitter 1999; Rentsch et al. 2007; Näsholm et al. 2009; Tegeder and Rentsch 2010). In addition, legumes can acquire atmospheric dinitrogen through a symbiotic relationship with bacteria that reside in root nodules. This fixed N is reduced to amino acids and ureides in nodules of temperate and tropical legumes, respectively (Bergersen 1971; Schubert 1986; Tegeder 2014). Inorganic N that is taken up by the root is assimilated into amino acids either in roots or in leaves, depending on the plant species, N availability, and the diurnal cycle (Fig. 13.1; Andrews et al. 1992; Lam et al. 1996; Stöhr and Mäck 2001; Ferrario-Méry et al. 2002; Miller et al. 2007; Xu et al. 2012; Krapp 2015). Amino acids that are taken up or synthesized in roots are either metabolized within root cells or transported to the shoot for use (Miflin and Lea 1977; Schobert and Komor 1990).

In plants that mainly reduce N in roots, the newly produced amino acids are translocated in the xylem transpiration stream to source leaves (Fig. 13.1; Miflin and Lea 1977; Schobert and Komor 1990). Along this path, amino acids can be transferred from the xylem to the phloem to directly supply developing sinks, such as young leaves, flowers, and seeds with N (Pate et al. 1975; van Bel 1984; Zhang et al. 2010). However, many plant species preferentially transport nitrate to source leaves where photosynthesis provides the reductants and carbon skeletons for amino acid synthesis (Andrews 1986; Lam et al. 1996; Lewis et al. 2000; Nunes-Nesi et al. 2010; Tegeder and Masclaux-Daubresse 2017).

In leaves, the organic N compounds are either used for metabolism, stored as amino acids, ureides or proteins, or exported into the phloem to supply developing sink tissues with N (Ellis 1979; Millard 1988; Liu et al. 2005; Lee et al. 2014;

Table 13.1 Overview on genetic approaches used to increase nitrogen transporter expression in crop plants and their impact on seed yield, protein levels, and nitrogen use efficiency when plants were grown with varying nitrogen (N) supply

Species	Genetic approach	N supply	N regime	Seed yield	NUE	NUpE	NUtE	Protein	Additional phenotype	References
Rice	*PTR9/NPF8.20* OE	0 N	none	↑↑	↑↑			↕ seed	↑ biomass	Fang et al. (2013)
		0.25 mM $(NH_4)_2SO_4$	low	↑	↑			↑ seed	↑ biomass	
		5 mM $(NH_4)_2SO_4$	high	↑	↑			↑ seed	↑ biomass	
		2 mM $NaNO_3$	medium	↑	↑			↑ seed	↑ biomass	
		1 mM NH_4NO_3	medium	↑↑	↑↑			↕ seed	↑ biomass	
Rice	*NRT2.1* OE	180 kg urea/ha	low	↑	↑				↑ biomass	Chen et al. (2016)
									↑ biomass	
		300 kg urea/ha	normal	↑	↑				↑ shoot N	
Rice	*AMT1-3* OE	0, 0.02 g NH_4NO_3	low	↓	↓			↕ leaf	↓↓ biomass	Bao et al. (2015)
		0.2, 1 g NH_4NO_3	high	↓	↓			↑ leaf	↓ biomass	
Rice	*AMT1.1* OE	0.03 mM $(NH_4)_2SO_4$	low	↑					↑ biomass	Ranathunge et al. (2014)
		0.3 mM $(NH_4)_2SO_4$	optimum	↑					↕ biomass	
		3 mM $(NH_4)_2SO_4$	high	↕					↓ biomass	
Rice	*AMT1.1* OE	10 μM NH_4^+	low						↑ NH_4 uptake	Kumar et al. (2006)
									↓ biomass	
		2 mM NH_4^+	high						↑ NH_4 uptake	
									↕ biomass	

(continued)

Table 13.1 (continued)

Species	Genetic approach	N supply	N regime	Seed yield	NUE	NUpE	NUtE	Protein	Additional phenotype	References
Pea	*ScMMP1*	Peter's 20-20-20*	high	↑				↑ seed	↑ biomass	Tan et al. (2010)
Pea	*AAP1* OE	5 mM NH_4NO_3	low	↑	↑	↕	↑	↓ seed	↑ biomass	Zhang et al. (2015), Perchlik and Tegeder (2017)
		10 mM H_4NO_3	medium	↑	↑	↑	↑	↕ seed	↑ biomass	
		2x 10 mM NH_4NO_3	high	↑	↑	↑	↕	↑ seed	↑ biomass	
Soybean	*PvUPS1* OE	atmospheric N_2		↑					↑ N fixation	Carter and Tegeder (2016)

Arrows refer to an increase (↑), decrease (↓), or no change (↕). AAP, amino acid permease; AMT, ammonium transporter; N_2, atmospheric dinitrogen NH_4^+, ammonium; NO_3^-, nitrate; NPF; NRT1/PTR family; NRT, nitrate transporter; NUE, nitrogen use efficiency; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency; OE, overexpression; PTR, peptide transporter; *Pv*UPS1, *Phaseolus vulgaris* ureide transporter 1, *Sc*MMP1, *Saccharomyces cerevisiae* S-methyl-methionine transporter

*Peter's 20-20-20 contains 20% N consisting of 50% urea-N, 30% nitrate-N and 20% ammonium-N

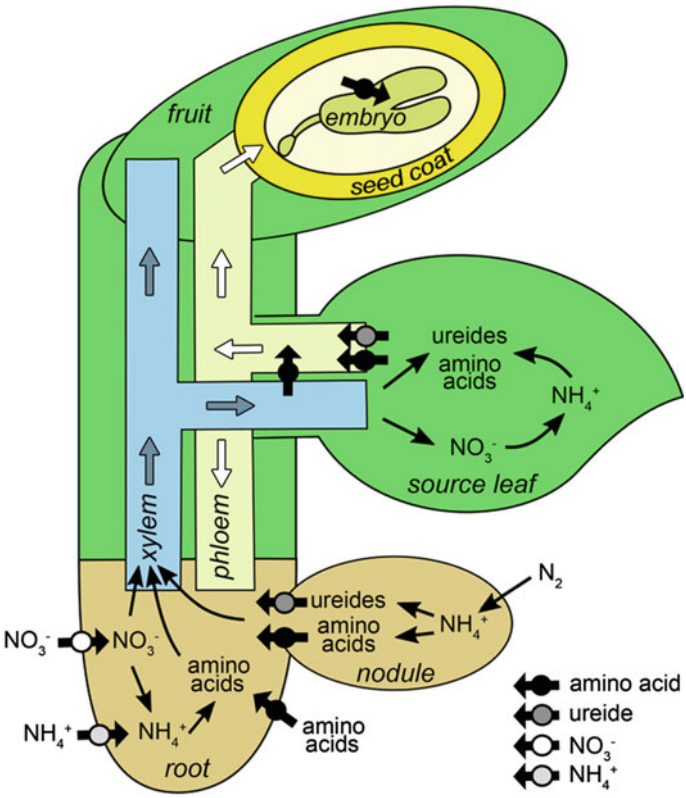


Fig. 13.1 Model of nitrogen (N) fixation, uptake, assimilation, and partitioning in plants. Legumes fix atmospheric N_2 through a symbiotic relationship with bacteria in root nodules. In tropical and temperate legumes, the fixed N is reduced to ureides or amino acids, which are transported from the nodules via the xylem to the shoot. Alternatively, plants take up amino acid and inorganic N (ammonium, NH_4^+; nitrate, NO_3^-), the later being from assimilated into amino acids in the roots or shoot. Root amino acids (and NO_3^-) are moved in the xylem to the shoot. Some of the xylem amino acids are transferred along the transport path to the phloem for direct N supply of sinks. However, the majority of amino acids are delivered with the transpiration stream to source leaves. Root-derived amino acids and ureides, and leaf-synthesized amino acids are loaded into the phloem for N supply of developing sinks such as fruits and seeds. Ureides are converted within the seed coat to amino acids. Amino acids are released from the seed coat into the seed apoplast followed by import into embryo (for details, see recent reviews Tegeder 2014; Tegeder and Masclaux-Daubresse 2017). Soil-to-root and root-to-shoot-to-seed N transport require a series of membrane transport steps. Some transporters (arrows with a circle) that are localized in key positions where they control N uptake and partitioning to sinks are indicated (see text and also Table 13.1)

Tegeder and Masclaux-Daubresse 2017). The N compounds enter the phloem primarily in leaf minor veins and are then moved toward sinks using an osmotic pressure gradient (Fig. 13.1; Pate 1980; Knoblauch et al. 2016). Once in the sink, N compounds are symplasmically unloaded from the phloem and move toward the

sink cells. Within the seed coat of seed sinks, organic N catabolism, transamination, and re-assimilation processes may occur (Atkins et al. 1975; Rainbird et al. 1984; Weber et al. 1995; Gallardo et al. 2007), followed by release of amino acids into the seed apoplast and import into the embryo for development and storage compound accumulation (Patrick 1997; Offler et al. 2003).

Efficient plant uptake, allocation, and use of N in source and sink are essential for plant biomass production and reproductive success (Fig. 13.2). In modern cropping systems, large amounts of industrially produced N fertilizers are supplied in order to guarantee N availability for maximum seed yields. However, many crop plants inefficiently acquire and use N, and an increase in N fertilization is often not proportional to increases in yield production (Ju et al. 2004; Delin and Stenberg 2014; Lassaletta et al. 2014; Mueller et al. 2014; Zhu et al. 2016). Depending on the crop species, soil conditions, and N supply, plants may take up less than half of the N fertilizer (Raun and Johnson 1999; Kumar and Goh 2002; Yang et al. 2015; Zhu et al. 2016). While a number of factors may impact N uptake and usage, including inorganic N import into the roots, N assimilation, and its regulation may (Kumar et al. 2006; Tsay et al. 2011; Ruffel et al. 2011; Nacry et al. 2013; Bao et al. 2015; Stahl et al. 2016), recent studies suggest that amino acid and/or ureide partitioning processes within the plant also play essential roles in plant productivity and N use efficiency (Fig. 13.2; Rolletschek et al. 2005; Schmidt et al. 2007; Weigelt et al. 2008; Tan et al. 2010; Zhang et al. 2010, 2015; Carter and Tegeder 2016; Santiago and Tegeder 2016).

Long-distance partitioning of amino acids and ureides requires plasma membrane-bound transport proteins that facilitate movement of the organic N from nodules or roots to leaves and finally to sinks (Fig. 13.1; Delrot et al. 2001; Rentsch et al. 2007; Tegeder 2014). This review discusses key transporters that control N root uptake, and root-to-shoot and leaf-to-seed partitioning of organic N, and examines their importance for plant growth, development, and seed production. Further, we evaluate the importance of amino acid transporters in source and sink for the efficiency of N uptake and use.

Importance of Nitrogen Root Uptake Systems for Plant Performance

Root N uptake from the soil is mediated by plasma membrane transporters, influenced by the availability of soil N, and controlled by plant N assimilation processes and the N demand of the plant (Fig. 13.1; Ruffel et al. 2011; Nacry et al. 2013; Stahl et al. 2016). A range of inorganic and organic N transporters with varying substrate specificities and affinities are present in roots (Wang et al. 1998; Sonoda et al. 2003; Yan et al. 2011; Haynes 2012; Fan et al. 2017). This diversity enables the root to regulate uptake in response to varying soil environments, including different N forms and concentrations.

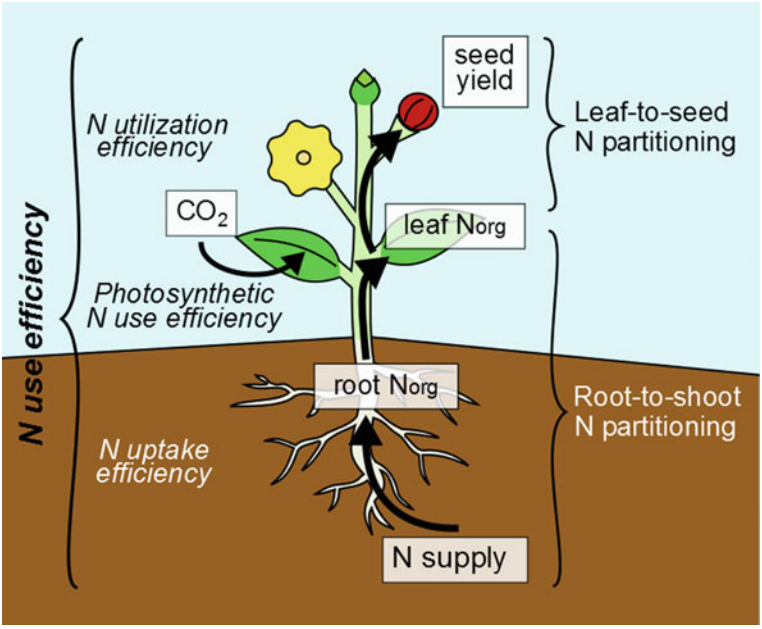


Fig. 13.2 Model on nitrogen (N) uptake, partitioning, and N use efficiency. Uptake of inorganic and organic N (N_{org}) and root-to-sink partitioning of N_{org} are indicated. N use efficiency (NUE) is defined as the amount of seed yield relative to N supply (Moll et al. 1982). NUE is comprised of two components: N uptake (NUpE) and utilization efficiency (NUtE). NUpE is the proportion of N in the shoot relative to the N supply, and it is influenced by the amount of N uptake and root-to-shoot N partitioning. NUtE describes the amount of shoot N used for seed production, which is influenced by leaf-to-seed N partitioning. Additionally, NUE is affected by photosynthetic N use efficiency, or the rate of CO_2 fixation per area leaf N (Commichau et al. 2006; Makino 2011)

Inorganic Nitrogen Uptake

Nitrate uptake is achieved by transporters of the NRT1 family (Nitrate Transporter1), also named NPF transporters (Nitrate Transporter1/Peptide Transporter Family), and the NRT2 family (Fan et al. 2017). NRT1 transporters are mostly low-affinity systems (Huang et al. 1999; Liu et al. 1999; Guo et al. 2002; Léran et al. 2014), while NRT2 transporters primarily function in high-affinity nitrate uptake (Wang et al. 1998; Okamoto et al. 2003; Li et al. 2007; Yan et al. 2011). Studies on natural variation and using manipulation of *NRT1* or *NRT2* expression have shown that NRT function affects N acquisition, plant productivity, and N use for seed production (Table 13.1). For example, overexpression of *ZmNRT1.1* and *ZmNRT1.3* in maize roots led to improved nitrate uptake, seed yield, and N use efficiency (Allen et al. 2016). Likewise, variation in *NRT1.1B/OsNPF6.5* expression in root and shoot tissue of the *Oryza sativa* (rice) subspecies *indica* and

japonica seems to correlate with efficient use of N for seed development (Hu et al. 2015). Further, *OsNRT2.1* overexpression in rice roots and leaves resulted in increased biomass, seed yield, and N use efficiency (Chen et al. 2016; 2017). Similar successes have also been reported when *NRT1* or *NRT2* transporters were constitutively overexpressed in rice (Table 13.1; Fang et al. 2013; Fan et al. 2016a, b; Feng et al. 2017).

Uptake of ammonium is regulated by saturable, high-affinity ammonium transporters (AMTs) and non-saturable, low-affinity systems (*i.e.*, aquaporins or cation channels) (Glass et al. 2002; Sonoda et al. 2003; Jahn et al. 2004; Loqué et al. 2005; Lea and Azevedo 2006; Guo et al. 2007; Bárzana et al. 2014). In *Arabidopsis thaliana*, six AMT genes are present, while 10 AMTs have been identified in rice. Up to date, genetic manipulation of ammonium transporters had relatively little success. For example, overexpression of *OsAMT1;1* and *OsAMT1;3* in rice under control of the CaMV-35S promoter resulted in increased ammonium uptake, but biomass and seed yield were either not changed or decreased (Table 13.1; Kumar et al. 2006; Bao et al. 2015). Nevertheless, the use of a ubiquitin promoter-*OsAMT1;1* construct led to more biomass and seed productivity (Table 13.1; Ranathunge et al. 2014). Overall, manipulation of ammonium transport processes to improve plant N uptake and use may be challenging, since alterations in cellular ammonium pools or excess ammonium can be toxic for the plant cell (Britto and Kronzucker 2002; Bittsánszky et al. 2015).

Uptake of Organic Nitrogen

High amounts of organic N may be found in cropping systems that rely on manure or compost for N nutrition (Khan 1971; Gregorich et al. 1994; Senwo and Tabatabai 1998). Although peptides, proteins, and other N compounds can be acquired by the plant, research on root uptake of organic N has mainly focused on amino acids (Rentsch et al. 2007; Komarova et al. 2008; Paungfoo-Lonhienne et al. 2008; Tegeder and Masclaux-Daubresse 2017). In Arabidopsis, five transporters have been shown to affect amino acid uptake by roots, and these include Amino Acid Permeases AAP1 and AAP5, Proline Transporter ProT2, and Lysine-Histidine-type Transporters LHT1 and LHT6 (Grallath et al. 2005; Hirner et al. 2006; Lee et al. 2007; Svennerstam et al. 2007, 2008, 2011; Lehmann et al. 2011; Perchlik et al. 2014; Ganeteg et al. 2017). However, all of these amino acid transporters were characterized using Arabidopsis mutants and it still remains to be examined if and how their increased expression in roots affects N acquisition and usage, and plant growth.

Soils often contain considerable amounts of urea (Kojima et al. 2006). Following microbial hydrolysis, a majority of the urea-N is accessible to the plant as ammonium, but direct urea uptake also occurs (Mérigout et al. 2008). As shown in Arabidopsis and rice, urea is actively taken up and transported within root tissues via the high-affinity urea transporter DUR3 (Kojima et al. 2007; Wang et al. 2012;

Bohner et al. 2015). Constitutive expression of rice *DUR3* in Arabidopsis *dur3-1* mutants resulted in increased urea uptake and shoot growth, suggesting manipulation of root urea import as a potential approach to improve plant performance (Wang et al. 2012).

Function of Nodule Ureide Transporters in Atmospheric Nitrogen Fixation and Plant Growth

Legumes can access the large atmospheric N pool through a symbiotic interaction with rhizobia that are housed in root nodules (Fig. 13.1). The final organic products available to the plant are amides in nodules of temperate legumes (e.g., pea, *Pisum sativum*) and the ureides allantoin and allantoic acid in case of tropical legumes such as soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) (Streeter 1979; Scharff et al. 2003; Tajima et al. 2004; Todd et al. 2006; Atkins and Smith 2007). While the molecular mechanisms for nodule amide transport processes remain to be identified, transport of ureides out of the nodules requires the function of UPS1 (Ureide Permease 1) proteins (Pélissier et al. 2004; Collier and Tegeder 2012; Carter and Tegeder 2016). When overexpressing the common bean *UPS1* transporter (Pélissier et al. 2004; Pélissier and Tegeder 2007) in soybean nodules, ureide transport from nodules to shoot and finally to seeds were increased resulting in a significantly improved seed yield (Table 13.1; Carter and Tegeder 2016). In addition, atmospheric N fixation was enhanced in the transgenic versus control nodules by around 100%. Together, these results support that organic N export out of the nodules is a key regulatory step in N fixation, shoot N supply, and seed development in legumes. The data further suggest significant improvements in plant N acquisition and use efficiency.

Role of Amino Acid Transporters in Root-to-Shoot Nitrogen Supply and Photosynthetic Use Efficiency

Amino acids that are taken up from the soil or synthesized in roots or nodules are translocated in the xylem mainly to photosynthetically active, transpiring leaves (Figs. 13.1 and 13.2; Miflin and Lea 1977; Schobert and Komor 1990). The rate of N transport from the root to leaves is influenced by the rate of N flux from the root cells to the xylem, transpiration rate and its associated hydrostatic pressure gradient between root and leaf, and import of N into the mesophyll cells (Engels et al. 1992; Gouia et al. 1994; Windt et al. 2006). Amino acid xylem loading requires the export of amino acids into the apoplast from either nodule or root endodermal cells, the pericycle or vascular parenchyma cells (Tegeder 2014). Root-localized Usually Multiple Acids Move In and out Transporters (UmamiTs) are predicted to function in this efflux step (Ladwig et al. 2012; Müller et al. 2015; Besnard et al. 2016).

Import of the organic N into the leaf cells is, at least in part, mediated by LHT1, since a mutation in the transporter results in decreased uptake of amino acids by mesophyll cells and their accumulation in the apoplast, overall negatively affecting growth (Hirner et al. 2006; Liu et al. 2010; Svennerstam et al. 2011). Future studies will need to address if an increase in amino acid import into the source leaf cells affects N flux rates from the roots to the shoot and, subsequently, root N uptake and assimilation.

Not all amino acids that are transported out of the root are directed to leaves. In particular during vegetative phase, up to 21% (van Bel 1984) of the organic N may be retrieved from the transpiration stream along the pathway for metabolism (Bailey and Leegood 2016), establishment of N storage pools (Streeter 1979; Millard 1988), or xylem-to-phloem transfer to directly supply growing sinks with N (Fig. 13.1; Dickson et al. 1985; Pate et al. 1975; van Bel 1984; 1990). Arabidopsis AAP6 is localized to the vascular parenchyma and is thought to be involved in amino acid removal from the xylem (Hunt et al. 2010), while AAP2 has been shown to function in amino acid loading into the transport phloem (Zhang et al. 2010). Mutants of *aap6* and *aap2* both demonstrated reduced phloem amino acid levels, and in *aap2* plants less N was transported to developing sinks, resulting in decreased seed protein levels (Hunt et al. 2010; Zhang et al. 2010). However, no negative effects were observed with respect to *aap2* seed yield or seed germination rates (Zhang et al. 2010). On the contrary, in *aap2* mutants, xylem allocation of amino acids to leaves was elevated, leading to increased carbon fixation (Zhang et al. 2010). Overall, leaf carbon metabolism and partitioning to *aap2* siliques and seeds were enhanced, which resulted in higher fatty acid levels per seed, seed number, and seed oil yields. This suggests that at least for oil (or starch) crop plants, optimizing N allocation to photosynthetically active source leaves presents a promising approach to increase seed carbon/oil/starch yields, and potentially photosynthetic N use efficiency (Makino and Osmond 1991; Escudero and Mediavilla 2003; Dordas and Sioulas 2008).

Influence of Phloem Loading of Amino Acids and Source-to-Sink Transport on Seed Development

Xylem-derived and leaf-synthesized amino acids are used for leaf metabolism, transiently stored in amino acid or protein pools, or loaded into the phloem for translocation to sinks (Fig. 13.1 and 13.2; Tegeder and Masclaux-Daubresse 2017). The amount of N that is allocated to sinks and used for seed development is affected by several physiological factors including N uptake, metabolism, and source-to-sink allocation (Habash et al. 2001; Tsay et al. 2011; Girondé et al. 2015). In particular during senescence, leaves are considered strong sources for amino acids, and effective N mobilization during leaf senescence and redistribution to sinks can significantly impact the efficiency of N utilization for seed development (Moll et al. 1982; Muurinen et al. 2007; Masclaux-Daubresse and Chardon 2011). The phloem is primarily comprised of sieve elements and companion cells (SEs/CCs),

which accommodate the long-distance transport of amino acids to sink (Kempers et al. 1998; Oparka and Turgeon 1999). Depending on the plant species and frequency of functional plasmodesmata, phloem loading occurs either symplasmically via plasmodesmata, or apoplastically involving cellular export and import processes (van Bel 1993; Rennie and Turgeon 2009). At least with respect to sucrose, many crop plants are considered to be apoplastic phloem loaders (Geiger et al. 1973; Winter et al. 1992; Aoki et al. 2004; Slewinski et al. 2009; Chen et al. 2012), and a similar phloem-loading mechanism is assumed for amino acids and other N-containing compounds (Servaites et al. 1979; Lohaus et al. 1995; Fischer et al. 1998). In apoplastic loading, amino acids are passively exported from parenchyma or bundle sheath cells into the cell wall space. In Arabidopsis, UmamiT18/SIAR1 is involved in this efflux step (Ladwig et al. 2012) and potentially BAT1 (Dündar and Bush 2009). The amino acids move within the apoplastic space to the SE-CC complex of the phloem where they are actively taken up (Dündar and Bush 2009; Ladwig et al. 2012; Santiago and Tegeder 2016). Based on localization studies in Arabidopsis, pea, and common bean, several members of the AAP transporter family have been identified as potential phloem loaders (Tegeder et al. 2007; Tan et al. 2008; Tegeder and Rentsch 2010; Tegeder and Ward 2012). However, up to date a function in amino acid import into the SEs/CCs has only been demonstrated for AAP8 (Santiago and Tegeder 2016). Analysis of Arabidopsis *aap8* mutants showed decreased amino acid import into the phloem resulting in reduced seed yield. The study suggests that amino acid transporter function in phloem loading regulates seed number and size, and is most probably important for efficient N utilization for seed development (Santiago and Tegeder 2016).

The importance of amino acid phloem loading for sink development has further been demonstrated by overexpressing a yeast sulfur (S)-methyl-methionine transporter (*i.e.*, *MMP1*) in the leaf phloem (and embryo) of pea plants (Tegeder et al. 2007; Tan et al. 2010). Long-distance transport of S-containing amino acids, including S-methyl-methionine, was increased in the transgenic plants, positively co-regulating amino acid metabolism and source-to-sink allocation, and seed N import (Tan et al. 2010). Together, this led to increased biomass production, seed yield, and seed protein levels. However, S import into the embryo was unchanged. S-methyl-methionine is converted in seed coats to methionine, and results suggest that the 'pulling force' for methionine uptake by the embryo was limited in the transgenic plants and that seed loading of methionine or other S compounds may present a bottleneck in increasing S-rich, high-quality seed storage proteins (Tan et al. 2010).

Amino Acid Transporter Function in Seed Sinks and Their Importance for Nitrogen Storage Pools

Developing fruits and seeds are major sinks for N during reproductive phase. Phloem unloading in seeds is generally assumed to occur via the symplasmic pathway through plasmodesmata (Patrick 1997). However, post-phloem transport of amino

acids into seeds involves both apoplastic and symplasmic transport routes dependent on the developmental stage and seed tissue (Peoples et al. 1985; Patrick 1997; Offler et al. 2003; Stadler et al. 2005; Müller et al. 2015). Symplasmic isolations occur between the outer and inner integuments of the seed coat (Schneitz et al. 1995; Stadler et al. 2005). Further, the maternal seed coat encircles the endosperm and the developing embryo, which are all symplasmically disconnected (Stadler et al. 2005). Overall, the lack of plasmodesmata necessitates a sequence of export and import steps to finally release the amino acids into the seed apoplast for uptake by the embryo. Arabidopsis transporters that are involved in amino acid movement toward the embryo for development and/or storage protein synthesis include UmamiTs, the Cationic Amino acid Transporter CAT6 and AAPs (Fig. 13.1; Hammes et al. 2006; Schmidt et al. 2007 Sanders et al. 2009; Ladwig et al. 2012, Müller et al. 2015).

The majority of N uptake by the embryo happens via the outer cotyledon epidermal cells exposed to the seed apoplast, although some of the N may move apoplastically and is taken up into the storage cells of the cotyledon parenchyma (Offler et al. 2003). Up to date, only AAP1 has been shown to function in import of amino acids into the embryo (Sanders et al. 2009). Studies with Arabidopsis *aap1* mutants resolved that decreased amino acid uptake by embryo epidermis cells (and potentially parenchyma cells) led to reduced seed protein levels. In addition, source leaf N metabolism and source-to-sink N allocation seemed negatively affected, ultimately resulting in decreased silique development (Sanders et al. 2009). These results suggest that transporter function in seed sinks controls seed N storage pool, and may negatively feedback regulate sink development. However, when overexpressing *AAP1* in the storage parenchyma cells of pea and *Vicia narbonensis* cotyledons, N uptake into the embryo was increased, but seed yields were not altered in the transgenic legumes (Rolletschek et al. 2005, Weigelt et al. 2008). Together with the function of phloem transporters (see above), this indicates that both amino acid loading into the phloem and import into the embryo may present bottlenecks for efficient use of N for seed development and establishment of seed N pools.

Effects of Concurrent Increases in Amino Acid Phloem and Seed Loading on Seed Yield and Plant Nitrogen Use Efficiency

Plants generally display a trade-off between seed number and seed protein accumulation (Martre et al. 2003; Seiffert et al. 2004; Gambín and Borrás 2010; Drechsler et al. 2015; Santiago and Tegeder 2016). The amount of amino acids loaded into the phloem and allocated to sinks could therefore impact fruit and seed number, and/or seed N level (Santiago and Tegeder 2016; Tan et al. 2010). In addition, seed import processes influence seed protein pools (Lemaître et al. 2008; Sanders et al. 2009; Tan et al. 2010, Drechsler et al. 2015; Santiago and Tegeder 2016; Rolletschek et al. 2005, Weigelt et al. 2008; see above). To increase both, the amount of N that is 'pushed' into the phloem and the amount that is 'pulled' into the

seed, recently an *AAP1* amino acid transporter was simultaneously overexpressed in the phloem and embryo of pea plants (Zhang et al. 2015). In these plants, phloem loading and seed import of amino acids were increased leading to higher seed numbers and significantly enhanced seed yields and seed storage protein levels when grown in very high N environments. In addition, N uptake and metabolism were upregulated, probably via feedback control.

The *AAP1* pea plants were further examined with respect to plant nitrogen use efficiency (NUE) and by evaluating the seed yield relative to high, moderate and low N applications (Table 13.1; Perchlik and Tegeder 2017). Regardless of the N supply, the *AAP1* plants performed better than controls and exhibited improved NUE. In addition, the transgenic plants achieved the same seed yield as controls with half the amount of N fertilizer. When analyzing the different components of NUE, specifically N utilization efficiency (NUtE) and N uptake efficiency (NUpE), some variations were observed (c.f. Fig. 13.2). Under high N, only NUpE efficiency was improved, while under low N, NUtE was enhanced. However, both NUpE and NUtE were significantly increased when N supply was moderate (Perchlik and Tegeder 2017). Overall, the data suggest that engineering amino acid partitioning from leaf to seed provides a promising approach for plant breeding not only to facilitate improved seed yield and quality, but also to support efficient plant N use.

Conclusions

Organic N allocation is an essential component for establishing seed yield and seed N pools. Nodule ureide transporters and amino acid transporters involved in N root-to-shoot movement, xylem-to-phloem transfer, phloem loading, and seed import are critical in regulating N partitioning to sinks. The function of these transporters also has significant impact on N soil uptake, N utilization in source and sink, and overall plant N use. Repression or targeted overexpression of key ureide and amino acid transport proteins can positively affect both N and carbon metabolism and partitioning in plants leading to increases in biomass production, seed development, and N use efficiency. However, current data mostly derive from studies with plants grown in controlled environments, and it will now be crucial test their performance under field conditions. Further, organic N transport processes from root to seed involve a series of export and import steps for a range of amino acids (and ureides), and many of the responsible transporters have not yet been characterized, although they may present promising targets to alter N allocation to specific organs or tissues and to optimize plant N use efficiency.

Acknowledgements M.T. acknowledges support from the US National Science Foundation (IOS-1457183) and the Agriculture and Food Research Initiative (AFRI) competitive award number 2017-67013-26158 from the USDA National Institute of Food and Agriculture.

References

Allen SM, Guo M, Loussaert DF, Rupe M, Wang H, Pioneer Hi-Bred International, Inc., EI Dupont De Nemours & Company (2016) Enhanced nitrate uptake and nitrate translocation by over-expressing maize functional low-affinity nitrate transporters in transgenic maize. U.S. patent application 14/770,863

Andrews M (1986) The partitioning of nitrate assimilation between root and shoot of higher plants. Plant, Cell Environ 9:511–519

Andrews M, Morton JD, Lieffering M, Bisset L (1992) The partitioning of nitrate assimilation between root and shoot of a range of temperate cereals and pasture grasses. Ann Bot 70: 271–276

Aoki N, Scofield GN, Wang X-D, Patrick JW, Offler CE, Furbank RT (2004) Expression and localisation analysis of the wheat sucrose transporter *TaSUT1* in vegetative tissues. Plant 219:176–184

Atkins CA, Smith PM (2007) Translocation in legumes: assimilates, nutrients, and signaling molecules. Plant Physiol 144:550–561

Atkins CA, Pate JS, Sharkey PJ (1975) Asparagine metabolism—key to the nitrogen nutrition of developing legume seeds. Plant Physiol 56:807–812

Bailey KJ, Leegood RC (2016) Nitrogen recycling from the xylem in rice leaves: dependence upon metabolism and associated changes in xylem hydraulics. J Exp Bot 67:2901–2911

Bao A, Liang Z, Zhao Z, Cai H (2015) Overexpressing of *OsAMT1-3*, a high affinity ammonium transporter gene, modifies rice growth and carbon-nitrogen metabolic status. Int J Mol Sci 16:9037–9063

Bárzana G, Aroca R, Bienert GP, Chaumont F, Ruiz-Lozano JM (2014) New insights into the regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize plants under drought stress and possible implications for plant performance. Mol Plant Microbe Interact 27:349–363

van Bel AJ (1984) Quantification of the xylem-to-phloem transfer of amino acids by use of inulin [^{14}C] carboxylic acid as xylem transport marker. Plant Sci Let 35:81–85

van Bel AJ (1990) Xylem-phloem exchange via the rays: the undervalued route of transport. J Exp Bot 41:631–644

van Bel AJ (1993) Strategies of phloem loading. Annu Rev Plant Phys Plant Mol Biol 44:253–281

Bergersen FJ (1971) Biochemistry of symbiotic nitrogen fixation in legumes. Ann Rev Plant Physiol 22:121–140

Besnard J, Pratelli R, Zhao C, Sonawala U, Collakova E, Pilot G, Okumoto S (2016) UMAMIT14 is an amino acid exporter involved in phloem unloading in Arabidopsis roots. J Exp Bot 67:6385–6397

Bittsánszky A, Pilinszky K, Gyulai G, Komives T (2015) Overcoming ammonium toxicity. Plant Sci 231:184–190

Bohner A, Kojima S, Hajirezaei M, Melzer M, Wirén N (2015) Urea retranslocation from senescing Arabidopsis leaves is promoted by DUR3-mediated urea retrieval from leaf apoplast. Plant J 81:377–387

Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in plants: a critical review. J Plant Physiol 159:567–584

Carter AM, Tegeder M (2016) Increasing nitrogen fixation and seed development in soybean requires complex adjustments of nodule nitrogen metabolism and partitioning processes. Curr Biol 26:2044–2051

Chen J, Fan X, Qian K, Zhang Y, Song M, Liu Y, Xu G, Fan X (2017) *pOsNAR2.1:OsNAR2.1* expression enhances nitrogen uptake efficiency and grain yield in transgenic rice plants. Plant Biotechnol J 15:1273–1283

Chen J, Zhang Y, Tan Y, Zhang M, Zhu L, Xu G, Fan X (2016) Agronomic nitrogen-use efficiency of rice can be increased by driving *OsNRT2.1* expression with the *OsNAR2.1* promoter. Plant Biotechnol J 14:1705–1715

Chen LQ, Qu XQ, Hou BH, Sosso D, Osorio S, Fernie AR, Frommer WB (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science 335:207–211

Collier R, Tegeder M (2012) Soybean ureide transporters play a critical role in nodule development, function and nitrogen export. Plant J 72:355–367

Commichau FM, Forchhammer K, Stülke J (2006) Regulatory links between carbon and nitrogen metabolism. Curr Opin Microbiol 9:167–172

Delin S, Stenberg M (2014) Effect of nitrogen fertilization on nitrate leaching in relation to grain yield response on loamy sand in Sweden. Eur J Agron 52:291–296

Delrot S, Rochat C, Tegeder M, Frommer WB (2001) Amino acid transport. In: Lea P, Gaudry JFM (eds) Plant nitrogen. INRA-Springer, Paris, France, pp 215–235

Dickson RE, Vogelmann TC, Larson PR (1985) Glutamine transfer from xylem to phloem and translocation to developing leaves of *Populus deltoides*. Plant Physiol 77:412–417

Dordas CA, Sioulas C (2008) Safflower yield, chlorophyll content, photosynthesis, and water use efficiency response to nitrogen fertilization under rainfed conditions. Indust Crops Prod 27:75–85

Drechsler N, Zheng Y, Bohner A, Nobmann B, von Wirén N, Kunze R, Rausch C (2015) Nitrate-dependent control of shoot K homeostasis by the nitrate transporter1/peptide transporter family member NPF7.3/NRT1.5 and the Stelar K^+ outward rectifier SKOR in Arabidopsis. Plant Physiol 169:2832–2847

Dündar E, Bush DR (2009) BAT1, a bidirectional amino acid transporter in Arabidopsis. Planta 229:1047–1056

Ellis RJ (1979) The most abundant protein in the world. Trends Biochem Sci 4:241–244

Engels C, Munkle L, Marschner H (1992) Effect of root zone temperature and shoot demand on uptake and xylem transport of macronutrients in maize (*Zea mays L.*). J Exp Bot 43:537–547

Epstein E, Bloom AJ (2005) Mineral nutrition of plants: principles and perspectives, 2nd edn. Sinauer Assoc. Inc., Sunderland, UK

Escudero A, Mediavilla S (2003) Decline in photosynthetic nitrogen use efficiency with leaf age and nitrogen resorption as determinants of leaf life span. J Ecol 91:880–889

Fan XR, Feng HM, Tan YW, Xu YL, Miao QS, Xu GH (2016a) A putative 6-transmembrane nitrate transporter OsNRT1.1b plays a key role in rice under low nitrogen. J Integr Plant Biol 58:590–599

Fan X, Tang Z, Tan Y, Zhang Y, Luo B, Yang M, Lian X, Shen Q, Miller AJ, Xu G (2016b) Overexpression of a pH-sensitive nitrate transporter in rice increases crop yields. Proc Natl Acad Sci USA 113:7118–7123

Fan X, Naz M, Fan X, Xuan W, Miller AJ, Xu G (2017) Plant nitrate transporters: from gene function to application. J Exp Bot 68:2463–2475

Fang Z, Xia K, Yang X, Grotemeyer MS, Meier S, Rentsch D, Xu X, Zhang M (2013) Altered expression of the *PTR/NRT1* homologue *OsPTR9* affects nitrogen utilization efficiency, growth and grain yield in rice. Plant Biotechnol J 11:446–458

Farley RA, Fitter AH (1999) Temporal and spatial variation in soil resources in a deciduous woodland. J Ecol 87:688–696

Feng H, Li B, Zhi Y, Chen J, Li R, Xia X, Xu G, Fan X (2017) Overexpression of the nitrate transporter, *OsNRT2.3b*, improves rice phosphorus uptake and translocation. Plant Cell Rep 36:1287–1296

Ferrario-Méry S, Valadier MH, Godefroy N, Miallier D, Hirel B, Foyer CH, Suzuki A (2002) Diurnal changes in ammonia assimilation in transformed tobacco plants expressing ferredoxin-dependent glutamate synthase mRNA in the antisense orientation. Plant Sci 163:59–67

Fischer WN, André B, Rentsch D, Krolkiewicz S, Tegeder M, Breitkreuz K, Frommer WB (1998) Amino acid transport in plants. Trends Plant Sci 3:188–195

Gallardo K, Firnhaber C, Zuber H, Héricher D, Belghazi M, Henry C, Küster H, Thompson R (2007) A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds: evidence for metabolic specialization of maternal and filial tissues. Mol Cell Proteomics 6:2165–2179

Gambín BL, Borrás L (2010) Resource distribution and the trade-off between seed number and seed weight: a comparison across crop species. Ann Appl Biol 156:91–102

Ganeteg U, Ahmad I, Jämtgård S, Aguetoni-Cambui C, Inselsbacher E, Svennerstam H, Schmidt S, Näsholm T (2017) Amino acid transporter mutants of Arabidopsis provides evidence that a non-mycorrhizal plant acquires organic nitrogen from agricultural soil. Plant, Cell Environ 40:413–423

Geiger D, Giaquinta R, Sovonick S, Fellows R (1973) Solute distribution in sugar beet leaves in relation to phloem loading and translocation. Plant Physiol 52:585–589

Girondé A, Etienne P, Trouverie J, Bouchereau A, Le Cahérec F, Leport L, Orsel M, Niogret MF, Nesi N, Carole D, Soulay F (2015) The contrasting N management of two oilseed rape genotypes reveals the mechanisms of proteolysis associated with leaf N remobilization and the respective contributions of leaves and stems to N storage and remobilization during seed filling. BMC Plant Biol 15:59

Glass AD, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, Okamoto M, Rawat S, Siddiqi MY, Unkles SE, Vidmar JJ (2002) The regulation of nitrate and ammonium transport systems in plants. J Exp Bot 53:855–864

Gouia H, Ghorbal MH, Touraine B (1994) Effects of NaCl on flows of N and mineral ions and on NO_3^- reduction rate within whole plants of salt-sensitive bean and salt-tolerant cotton. Plant Physiol 105:1409–1418

Grallath S, Weimar T, Meyer A, Gumy C, Suter-Grotemeyer M, Neuhaus JM, Rentsch D (2005) The AtProT family. Compatible solute transporters with similar substrate specificity but differential expression patterns. Plant Physiol 137:117–126

Gregorich EG, Monreal CM, Carter MR, Angers DA, Ellert B (1994) Towards a minimum data set to assess soil organic matter quality in agricultural soils. Can J Soil Sci 74:367–385

Guo F-Q, Wang R, Crawford NM (2002) The *Arabidopsis* dual-affinity nitrate transporter gene *AtNRT1.1* (*CHL1*) is regulated by auxin in both shoots and roots. J Exp Bot 53:835–844

Guo S, Kaldenhoff R, Uehlein N, Sattelmacher B, Brueck H (2007) Relationship between water and nitrogen uptake in nitrate-and ammonium-supplied Phaseolus vulgaris L. plants. J Plant Nutr Soil Sci 170:73–80

Habash DZ, Massiah AJ, Rong HL, Wallsgrove RM, Leigh RA (2001) The role of cytosolic glutamine synthetase in wheat. Ann Appl Biol 138:83–89

Hammes UZ, Nielsen E, Honaas LA, Taylor CG, Schachtman DP (2006) AtCAT6, a sink-tissue-localized transporter for essential amino acids in Arabidopsis. Plant J 48:414–426

Haynes RJ (2012) Uptake and assimilation of mineral nitrogen by plants. Mineral nitrogen in the plant-soil system. Elsevier Science, Orlando, Fl, USA, pp 303–378

Hirner A, Ladwig F, Stransky H, Okumoto S, Keinath M, Harms A, Frommer W, Koch W (2006) Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant Cell 18:1931–1946

Hu B, Wang W, Ou S, Tang J, Li H, Che R, Zhang Z, Chai X, Wang H, Wang Y, Liang C (2015) Variation in *NRT1.1B* contributes to nitrate-use divergence between rice subspecies. Nat Genet 47:834–878

Huang N-C, Liu K-H, Lo H-J, Tsay Y-F (1999) Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell 11:1381–1392

Hunt E, Gattolin S, Newbury HJ, Bale JS, Tseng HM, Barrett DA, Pritchard J (2010) A mutation in amino acid permease AAP6 reduces the amino acid content of the Arabidopsis sieve elements but leaves aphid herbivores unaffected. J Exp Bot 61:55–64

Jahn TP, Møller AL, Zeuthen T, Holm LM, Klærke DA, Mohsin B, Kühlbrandt W, Schjoerring JK (2004) Aquaporin homologues in plants and mammals transport ammonia. FEBS Lett 574: 31–36

Ju X, Liu X, Zhang F, Roelcke M (2004) Nitrogen fertilization, soil nitrate accumulation, and policy recommendations in several agricultural regions of China. Ambio 33:300–305

Khan S (1971) Nitrogen fractions in a gray wooded soil as influenced by long-term cropping systems and fertilizers. Can J Soil Sci 51:431–437

Kempers R, Ammerlaan A, van Bel AJ (1998) Symplasmic constriction and ultrastructural features of the sieve element/companion cell complex in the transport phloem of apoplasmically and symplasmically phloem-loading species. Plant Physiol 116:271–278

Knoblauch M, Knoblauch J, Mullendore DL, Savage JA, Babst BA, Beecher SD, Dodgen AC, Jensen KH, Holbrook NM (2016) Testing the Münch hypothesis of long distance phloem transport in plants. eLife 5:e15341

Kojima S, Bohner A, Gassert B, Yuan L, Wirén NV (2007) AtDUR3 represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots. Plant J 52:30–40

Kojima S, Bohner A, Von Wirén N (2006) Molecular mechanisms of urea transport in plants. J Membr Biol 212:83–91

Komarova NY, Thor K, Gubler A, Meier S, Dietrich D, Weichert A, Grotemeyer MS, Tegeder M, Rentsch D (2008) AtPTR1 and AtPTR5 transport dipeptides in planta. Plant Physiol 148:856–869

Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS, Chaillou S, Ferrario-Méry S, Meyer C, Daniel-Vedele F (2014) Nitrate transport and signalling in Arabidopsis. J Exp Bot 65:789–798

Krapp A (2015) Plant nitrogen assimilation and its regulation: a complex puzzle with missing pieces. Current Opin Plant Biol 25:115–122

Kumar A, Kaiser BN, Siddiqi MY, Glass AD (2006) Functional characterisation of *OsAMT1.1* overexpression lines of rice, *Oryza sativa*. Funct Plant Biol 33:339–346

Kumar K, Goh K (2002) Recovery of ^{15}N-labelled fertilizer applied to winter wheat and perennial ryegrass crops and residual ^{15}N recovery by succeeding wheat crops under different crop residue management practices. Nutr Cycl Agroecosys 62:123–130

Ladwig F, Stahl M, Ludewig U, Hirner AA, Hammes UZ, Stadler R, Harter K, Koch W (2012) Siliques are Red1 from Arabidopsis acts as a bidirectional amino acid transporter that is crucial for the amino acid homeostasis of siliques. Plant Physiol 158:1643–1655

Lam HM, Coschigano KT, Oliveira IC, Melo-Oliveira R, Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Ann Rev Plant Biol 47:569–593

Lassaletta L, Billen G, Grizzetti B, Garnier J, Leach AM, Galloway JN (2014) Food and feed trade as a driver in the global nitrogen cycle: 50–year trends. Biogeochemistry 118:225–241

Lea PJ, Azevedo RA (2006) Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. Ann Appl Biol 149:243–247

Lee BR, Lee DG, Avice JC, Kim TH (2014) Characterization of vegetative storage protein (VSP) and low molecular proteins induced by water deficit in stolon of white clover. Biochem Biophys Res Commun 443:229–233

Lee Y-H, Foster J, Chen J, Voll L, Weber A, Tegeder M (2007) AAP1 transports uncharged amino acids into roots of *Arabidopsis*. Plant J 50:305–316

Lehmann S, Gumy C, Blatter E, Boeffel S, Fricke W, Rentsch D (2011) *In planta* function of compatible solute transporters of the AtProT family. J Exp Bot 62:787–796

Lemaître T, Gaufichon L, Boutet-Mercey S, Christ A, Masclaux-Daubresse C (2008) Enzymatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana Wassileskija* accession. Plant Cell Physiol 49:1056–1065

Léran S, Varala K, Boyer JC, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W (2014) A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci 19:5–9

Lewis CE, Noctor G, Causton D, Foyer CH (2000) Regulation of assimilate partitioning in leaves. Funct Plant Biol 27:507–519

Li W, Wang Y, Okamoto M, Crawford NM, Siddiqi MY, Glass AD (2007) Dissection of the *AtNRT2.1:AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. Plant Phys 143:425–433

Liu KH, Huang CY, Tsay YF (1999) CHL1 is a dual-affinity nitrate transporter of Arabidopsis involved in multiple phases of nitrate uptake. Plant Cell 11:865–874

Liu Y, Ahn J-E, Datta S, Salzman RA, Moon J, Huyghues-Despointes B, Pittendrigh B, Murdock LL, Koiwa H, Zhu-Salzman K (2005) Arabidopsis vegetative storage protein is an insect acid phosphatase. Plant Physiol 139:1545–1556

Liu G, Ji Y, Bhuiyan NH, Pilot G, Selvaraj G, Zou J, Wei Y (2010) Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. Plant Cell 22:3845–3863

Lohaus G, Winter H, Riens B, Heldt HW (1995) Further studies of the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and in the sieve tubes. Plant Biol 108:270–275

Loqué D, Ludewig U, Yuan L, von Wirén N (2005) Tonoplast intrinsic proteins AtTIP2;1 and AtTIP2;3 facilitate NH_3 transport into the vacuole. Plant Physiol 137:671–680

Loqué D, von Wirén N (2004) Regulatory levels for the transport of ammonium in plant roots. J Exp Bot 55:1293–1305

Makino A, Osmond B (1991) Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiol 96:355–362

Makino A (2011) Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. Plant Physiol 155:125–129

Masclaux-Daubresse C, Chardon F (2011) Exploring nitrogen remobilization for seed filling using natural variation in *Arabidopsis thaliana*. J Exp Bot 62:2131–2142

Martre P, Porter JR, Jamieson PD, Triboï E (2003) Modeling grain nitrogen accumulation and protein composition to understand the sink/source regulations of nitrogen remobilization for wheat. Plant Physiol 133:1959–1967

Mérigout P, Lelandais M, Bitton F, Renou JP, Briand X, Meyer C, Daniel-Vedele F (2008) Physiological and transcriptomic aspects of urea uptake and assimilation in Arabidopsis plants. Plant Physiol 147:1225–1238

Miflin BJ, Lea PJ (1977) Amino acid metabolism. Annu Rev Plant Physio 28:299–329

Millard P (1988) The accumulation and storage of nitrogen by herbaceous plants. Plant, Cell Environ 11:1–8

Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM (2007) Nitrate transport and signalling. J Exp Bot 58:2297–2306

Miret JA, Munné-Bosch S (2014) Plant amino acids derived vitamins: biosynthesis and function. Amino Acids 46:809–824

Moll R, Kamprath E, Jackson W (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron J 74:562–564

Mueller ND, West PC, Gerber JS, MacDonald GK, Polasky S, Foley JA (2014) A tradeoff frontier for global nitrogen use and cereal production. Environ Res Lett 9:P054002

Müller B, Fastner A, Karmann J, Mansch V, Hoffmann T, Schwab W, Suter-Grotemeyer M, Rentsch D, Truernit E, Ladwig F, Bleckmann A (2015) Amino acid export in developing Arabidopsis seeds depends on UmamiT facilitators. Curr Biol 25:3126–3131

Muurinen S, Kleemola J, Peltonen-Sainio P (2007) Accumulation and translocation of nitrogen in spring cereal cultivars differing in nitrogen use efficiency. Agron J 99:441–449

Nacry P, Bouguyon E, Gojon A (2013) Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil 370:1–29

Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. New Phytol 182:31–48

Nunes-Nesi A, Fernie AR, Stitt M (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Mol Plant 3:973–996

Offler CE, McCurdy DW, Patrick JW, Talbot MJ (2003) Transfer cells: cells specialized for a special purpose. Annu Rev Plant Biol 54:431–454

Okamoto M, Vidmar JJ, Glass ADM (2003) Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant Cell Physiol 44:304–317

Oparka KJ, Turgeon R (1999) Sieve elements and companion cells—traffic control centers of the phloem. Plant Cell 11:739–750

Pate JS (1980) Transport and partitioning of nitrogenous solutes. Ann Rev Plant Physio 31:313–340

Pate JS, Sharkey PJ, Lewis OAM (1975) Xylem to phloem transfer of solutes in fruiting shoots of legumes, studied by a phloem bleeding technique. Planta 122:11–26

Patrick JW (1997) Phloem unloading: sieve element unloading and post-sieve element transport. Annu Rev Plant Biol 48:191–222

Paungfoo-Lonhienne C, Lonhienne TGA, Rentsch D, Robinson N, Christie M, Webb RI, Gamage HK, Carroll BJ, Schenk PM, Schmidt S (2008) Plants can use protein as a nitrogen source without assistance from other organisms. P Natl Acad Sci USA 105:4524–4529

Pélissier H, Frerich A, Desimone M, Schumacher K, Tegeder M (2004) PvUPS1, an allantoin transporter in nodulated roots of French bean. Plant Physiol 134:664–675

Pélissier H, Tegeder M (2007) PvUPS1 plays a role in source-sink transport of allantoin in French bean (*Phaseolus vulgaris*). Funct Plant Biol 18:282–291

Peoples MB, Pate JS, Atkins CA, Murray DR (1985) Economy of water, carbon, and nitrogen in the developing cowpea fruit. Plant Physiol 77:142–147

Perchlik M, Foster J, Tegeder M (2014) Different and overlapping functions of Arabidopsis LHT6 and AAP1 transporters in root amino acid uptake. J Exp Bot 65:5193–5204

Perchlik M, Tegeder M (2017) Improving plant nitrogen use efficiency through alteration of amino acid transport processes. Plant Physiol 175:235–247

Rainbird RM, Thorne JH, Hardy RW (1984) Role of amides, amino acids, and ureides in the nutrition of developing soybean seeds. Plant Physiol 74:329–334

Ranathunge K, El-kereamy A, Gidda S, Bi YM, Rothstein SJ (2014) *AMT1;1* transgenic rice plants with enhanced NH_4^+ permeability show superior growth and higher yield under optimal and suboptimal NH_4^+ conditions. J Exp Bot 65:965–979

Raun W, Johnson G (1999) Improving nitrogen use efficiency for cereal production. Agron J 91:357–363

Rentsch D, Schmidt S, Tegeder M (2007) Transporters for uptake and allocation of organic nitrogen compounds in plants. FEBS Lett 581:2281–2289

Rennie EA, Turgeon R (2009) A comprehensive picture of phloem loading strategies. Proc Natl Acad Sci USA 106:14162–14167

Rolletschek H, Hosein F, Miranda M, Heim U, Götz KP, Schlereth A, Borisjuk L, Saalbach I, Wobus U, Weber H (2005) Ectopic expression of an amino acid transporter (*VfAAP1*) in seeds of *Vicia narbonensis* and pea increases storage proteins. Plant Physiol 137:1236–1249

Ruffel S, Krouk G, Ristova D, Shasha D, Birnbaum KD, Coruzzi GM (2011) Nitrogen economics of root foraging: transitive closure of the nitrate–cytokinin relay and distinct systemic signaling for N supply vs. demand. Proc Natl Acad Sci USA 108:18524–18529

Sanders A, Collier R, Trethewy A, Gould G, Sieker R, Tegeder M (2009) AAP1 regulates import of amino acids into developing Arabidopsis embryos. Plant J 59:540–552

Santiago J, Tegeder M (2016) Connecting source with sink: the role of Arabidopsis AAP8 in phloem loading of amino acids. Plant Physiol 171:508–521

Scharff AM, Egsgaard H, Hansen PE, Rosendahl L (2003) Exploring symbiotic nitrogen fixation and assimilation in pea root nodules by in vivo ^{15}N nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry. Plant Physiol 131:367–378

Schobert C, Komor E (1990) Transfer of amino acids and nitrate from the roots into the xylem of *Ricinus communis* seedlings. Planta 181:85–90

Schmidt R, Stransky H, Koch W (2007) The amino acid permease AAP8 is important for early seed development in *Arabidopsis thaliana*. Planta 226:805–813

Schneitz K, Hülskamp M, Pruitt RE (1995) Wild-type ovule development in Arabidopsis thaliana: a light microscope study of cleared whole-mount tissue. Plant J 7:731–749

Schubert KR (1986) Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. Ann Rev Plant Physiol 37:539–574

Seiffert B, Zhou Z, Wallbraun M, Lohaus G, Möllers C (2004) Expression of a bacterial asparagine synthetase gene in oilseed rape (*Brassica napus*) and its effect on traits related to nitrogen efficiency. Physiol Plant 121:656–665

Senwo Z, Tabatabai M (1998) Amino acid composition of soil organic matter. Biol Fert Soils 26:235–242

Servaites JC, Schrader LE, Jung DM (1979) Energy-dependent loading of amino acids and sucrose into the phloem of soybean. Plant Physiol 64:546–550

Slewinski TL, Meeley R, Braun DM (2009) Sucrose transporter1 functions in phloem loading in maize leaves. J Exp Bot 60:881–892

Sonoda Y, Ikeda A, Saiki S, Wirén NV, Yamaya T, Yamaguchi J (2003) Distinct expression and function of three ammonium transporter genes (*OsAMT1;1–1;3*) in rice. Plant Cell Physiol 44:726–734

Stadler R, Lauterbach C, Sauer N (2005) Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in Arabidopsis seeds and embryos. Plant Physiol 139:701–712

Stahl A, Friedt W, Wittkop B, Snowdon RJ (2016) Complementary diversity for nitrogen uptake and utilisation efficiency reveals broad potential for increased sustainability of oilseed rape production. Plant Soil 400:245–262

Stöhr C, Mäck G (2001) Diurnal changes in nitrogen assimilation of tobacco roots. J Exp Bot 52:1283–1289

Streeter J (1979) Allantoin and allantoic acid in tissues and stem exudate from filed grown soybean plants. Plant Physiol 63:478–480

Svennerstam H, Ganeteg U, Bellini C, Näsholm T (2007) Comprehensive screening of Arabidopsis mutants suggests the Lysine Histidine Transporter 1 to be involved in plant uptake of amino acids. Plant Physiol 143:1853–1860

Svennerstam H, Ganeteg U, Näsholm T (2008) Root uptake of cationic amino acids by Arabidopsis depends on functional expression of amino acid permease 5. New Phytol 180:620–630

Svennerstam H, Jämtgård S, Ahmad I, Huss-Danell K, Näsholm T, Ganeteg U (2011) Transporters in Arabidopsis roots mediating uptake of amino acids at naturally occurring concentrations. New Phytol 191:459–467

Tajima S, Nomura M, Kouchi H (2004) Ureide biosynthesis in legume nodules. Front Biosci 9:1374–1381

Tan Q, Grennan AK, Pélissier HC, Rentsch D, Tegeder M (2008) Characterization and expression of French bean amino acid transporter PvAAP1. Plant Sci 174:348–356

Tan Q, Zhang L, Grant J, Cooper P, Tegeder M (2010) Increased phloem transport of *S*-methylmethionine positively affects sulfur and nitrogen metabolism and seed development in pea plants. Plant Physiol 154:1886–1896

Tegeder M (2014) Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. J Exp Bot 65:1865–1878

Tegeder M, Masclaux-Daubresse C (2017) Source and sink mechanisms of nitrogen transport and use. New Phytol. https://doi.org/10.1111/nph.14876

Tegeder M, Rentsch D (2010) Uptake and partitioning of amino acids and peptides. Mol Plant 3:997–1011

Tegeder M, Ward JM (2012) Molecular evolution of plant AAP and LHT amino acid transporters. Front Plant Sci 3:21

Tegeder M, Tan Q, Grennan AK, Patrick JW (2007) Amino acid transporter expression and localisation studies in pea (*Pisum sativum*). Funct Plant Biol 34:1019–1028

Todd CD, Tipton PA, Blevins DG, Piedras P, Pineda M, Polacco JC (2006) Update on ureide degradation in legumes. J Exp Bot 57:5–12

Tsay YF, Fan SC, Chen HY; Academia Sinica (2011) Method for changing nitrogen utilization efficiency in plants. U.S. Patent Application 12/832,234

Wang WH, Köhler B, Cao FQ, Liu GW, Gong YY, Sheng S, Song QC, Cheng XY, Garnett T, Okamoto M, Qin R (2012) Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis. New Phytol 193:432–444

Wang R, Liu D, Crawford NM (1998) The Arabidopsis CHL1 protein plays a major role in high-affinity nitrate uptake. Proc Natl Acad Sci USA 95:15134–15139

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. Plant Cell 7:1835–1846

Weigelt K, Küster H, Radchuk R, Müller M, Weichert H, Fait A, Fernie AR, Saalbach I, Weber H (2008) Increasing amino acid supply in pea embryos reveals specific interactions of N and C metabolism, and highlights the importance of mitochondrial metabolism. Plant J 55:909–926

Windt CW, Vergeldt FJ, de Jager PA, Van As H (2006) MRI of long-distance water transport: a comparison of the phloem and xylem flow characteristics and dynamics in poplar, castor bean, tomato and tobacco. Plant, Cell Environ 29:1715–1729

Winter H, Lohaus G, Heldt HW (1992) Phloem transport of amino acids in relation to their cytosolic levels in barley leaves. Plant Physiol 99:996–1004

Xu G, Fan X, Miller A (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182

Yan M, Fan X, Feng H, Miller AJ, Shen Q, Xu G (2011) Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges. Plant, Cell Environ 34:1360–1372

Yang L, Cao W, Thorup-Kristensen K, Bai J, Gao S, Chang D (2015) Effect of *Orychophragmus violaceus* incorporation on nitrogen uptake in succeeding maize. Plant Soil Environ 61:260–265

Zhang L, Tan Q, Lee R, Trethewy A, Lee Y, Tegeder M (2010) Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in Arabidopsis. Plant Cell 22:3603–3620

Zhang L, Garneau M, Majumdar R, Grant J, Tegeder M (2015) Improvement of pea biomass and seed productivity by simultaneous increase of phloem and embryo loading with amino acids. Plant J 81:134–146

Zhu S, Vivanco JM, Manter DK (2016) Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. Appl Soil Ecol 107:324–333

Zrenner R, Stitt M, Sonnewald U, Boldt R (2006) Pyrimidine and purine biosynthesis and degradation in plants. Ann Rev Plant Biol 57:805–836

Züst T, Agrawal AA (2016) Mechanisms and evolution of plant resistance to aphids. Nat Plants 2:15206

Chapter 14
New Screening Strategies for Dinitrogen Fixation in Soybean

Raphael Lemes Hamawaki, Curtis Wolf and Stella K. Kantartzi

Introduction

Soybean (*Glycine max* [L.] Merr.) is currently the most cultivated legume worldwide that is used for food and feed due to its high protein and oil content. The increasing demand for affordable and environmentally friendly agricultural products leads to the development of different strategies that will decrease the production cost, while mitigating environmental impacts. The world growing population that is expected to reach 9.7 billion people by 2050 (United Nations 2015) imposes a significant challenge to the modern agriculture. This new scenario demands not just advances in agricultural technologies like chemicals, fertilizers, and new cultivars but also requires a huge effort to minimize environmental impacts related to activities for the intensification of modern agriculture like soil erosion, loss of fertility, depletion of nutrient reserves, pollution of soil and water, and loss of genetic resources (Hamuda and Patko 2010). Particularly, in the field of plant nutrition one of the greatest challenges is to increase yield, while maintaining or reducing the input of plant nutrients.

Biological processes that allow plants to recover nutrients from the environment through symbiotic associations with fungi (mycorrhizae) and bacteria (rhizobia) are gaining more relevance in science. For instance, mycorrhizal networks were found to be associated with nutrient transferring from one plant to another in natural environments (van der Heijden 2016), and attempts have been made to transfer the

R. L. Hamawaki (✉) · C. Wolf · S. K. Kantartzi
Southern Illinois University, Carbondale, IL, USA
e-mail: hamawaki@siu.edu

S. K. Kantartzi
e-mail: stella.kantartzi@siu.edu

R. L. Hamawaki
Ministry of Education of Brazil, CAPES Foundation, Brasilia, Brazil

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3_14

capacity of dinitrogen fixation to non-legume crops (Mus et al. 2016). Since soybean is the most cultivated N_2-fixing crop, the impact of any improvement or reduction in N_2 fixation activity can be substantial when considered its impact in the worldwide soybean cultivation. Soybean is grown with almost no supply of nitrogenous fertilizers. Recent studies demonstrated that modern soybean cultivars have limited N_2 fixation capacity, and this fact causes the N requirements of high-yielding fields not to be met (Salvagiotti et al. 2008). Furthermore, compared with old cultivars, modern genotypes have a reduced biological nitrogen fixation (BNF) capacity (Van Kessel and Hartley 2000). Several studies have focused on the selection of enhanced rhizobia strains and on the improvement of soybean genotypes to increase the soybean N_2 fixation capacity.

The overall performance of BNF is determined by both the host plant and the symbiotic bacteria. Moreover, the efficiency of this relationship will be dictated by the capacity of these organisms to interact with each other in the presence of a diverse range of environmental factors (Keyser and Li 1992).

Therefore, in the context of the bacteria enhancement, the progress is evidenced by the new selected strains that have been released by both public and private institutions. These improved bacterial strains increase N_2 fixation in soybean fields through the use of liquid- and peat-based inoculants. On the host side, several attempts have been made to increase the N_2 fixation capacity of soybean genotypes through breeding techniques; however, little progress has been made, probably because only traits related to nodulation have been used to measure the BNF capacity. Therefore, further strategies need to be investigated in order to increase the BNF capacity of soybean genotypes. In this chapter, we aimed to present new screening strategies for improving the BNF capacity of soybean and discuss tentative approaches that might support the selection of soybean genotypes for enhanced dinitrogen fixation.

Soybean Symbiosis for N_2 Fixation

The symbiotic association between leguminous plants and rhizobia is traced back 65–149 million years in the Cretaceous period, and its evolution was mostly driven by the requirement of the host plant for an essential nutrient supply and of the microsymbiont for a safe and protected environment to survive and multiply (Devine and Kuykendall 1996). Throughout this period of evolution, a complex gene network was developed between the host and the bacteria. These genes activate and regulate all the mechanisms involved in the nodule formation and N_2 fixation, which begins with the host recognition and nodule initiation and ends with the nitrogenase synthesis and its activity throughout the symbiosis period. In the host, most genes are directly related to nodules and nodulation traits, whereas the genetic control of N_2 fixation is yet to be revealed.

Soybean is able to form nodules with fast-growing *Rhizobium* and slow-growing *Bradyrhizobium* genera. Despite this common ability, there are few genetic similarities between these two genera of rhizobia (Devine and Kuykendall 1996). On the host side, this characteristic demonstrates that soybean is widely adapted to

different genera and populations of rhizobia. However, whether or not this represents an advantage or imposes a limitation to soybean is being subject of investigation in the field of host genetic control of BNF.

Wide adaptation might also be seen as a disadvantageous characteristic if considered the infection of soybean roots by inefficient *Bradyrhizobium* strains, which ultimately causes a restriction in dinitrogen fixation. In this respect, some adaptive mechanisms that soybean acquired during its evolution are the capacity to restrict nodulation with inefficient strains. On the other hand, one important advantage that a broad adaptation to different rhizobia strains can offer is the ability that some genotypes have to nodulate promiscuously with wild rhizobia in the soil. This aspect is employed by breeders when newly developed cultivars are unable to form nodules without inoculation with certain strains of *Bradyrhizobium*. To reverse this process, soybean cultivars are backcrossed with soybean genotypes capable to nodulate promiscuously (Kueneman et al. 1984).

The Role of N_2 Fixation in Soybean Production

The great diversity and wide range of biological processes that N participates in plants make this nutrient one of the most absorbed and frequently the most limiting mineral element in plant nutrition. The total N consumed for the production of grain and oilseed crops represents 60% of all the three major nutrients (i.e., N, P, and K) supplied to agriculture (FAOSTAT 2013). Global farming is estimated to consume 100 Tg of N annually through the use of fertilizers, soil mineral N, and N_2 fixation (Herridge and Rose 2000); the annual contribution of the latter is likely to be in the range of 20–22 Tg (Herridge et al. 2008).

Soybean is a major oilseed crop worldwide accounting for 39% of the total land harvested and 30% of the total production of oil crops (FAOSTAT 2014). As a supplier of protein and oil, soybean is probably the most relevant N_2-fixing legume crop. Its cultivation is estimated to contribute annually 77% of the total N_2 fixed that represents 16.4 Tg of N_2 fixed globally (Herridge and Rose 2000).

The Need to Improve N_2 Fixation in Soybean

Despite the significant input of N and the raising environmental concerns regarding the use of fossil fuels for the production of N fertilizers, little attention has been paid in soybean breeding programs to the improvement of N_2 fixation. A previous study that analyzed 362 experiments in North America and Australia demonstrated an annual decline of 0.7% in the amount of N_2 fixed by soybean cultivars released from 1970 to 2000 (Van Kessel and Hartley 2000). In the last 30 years, the progress that has been achieved in soybean genetic improvement has been remarkable: 31.2 kg ha^{-1} yr^{-1} on average in the USA (Specht et al. 1999) and 28 kg ha^{-1}

worldwide (Wilcox 2004). Despite the progress in yield potential, many concerns have been raised regarding the capacity of current and future cultivars to meet their N demand solely by BNF and the available soil N (Salvagiotti et al. 2008).

Achievements in Breeding Soybean for Enhanced N_2 Fixation

The improvement of BNF in soybean can be approached from the host and the microsymbiont bacterial side. From the bacterial side, the selection of superior strains and enhancement of the symbiotic capacity of *Bradyrhizobium japonicum* through mutations have deployed highly efficient bacterial strains that have increased the total N_2 fixation of soybean plants (Keyser and Li 1992). From the soybean side, US soybean cultivars were combined with Asian genotypes, that nodulate promiscuously and fix N_2 with indigenous rhizobia strains, to develop progenies with high performance, nodulation, and N_2 fixation (Herridge and Rose 2000). Other breeding efforts to improve N_2 fixation capacity have been made, but with limited results. A soybean breeding program that aimed to increase symbiotic nitrate tolerance was initiated in Australia in 1980 (Herridge et al. 2008). The results showed that a high nitrate concentration in the soil can interfere with the initiation of nodulation at the beginning of plant development and the peak of N_2 fixation process.

Herridge and Rose (2000) selected 32 genotypes with different levels of symbiotic nitrate tolerance and crossed them with high-yielding cultivars. The distribution frequency of nitrogen fixation in the segregating populations did not deviate from the normal distribution, indicating that this trait might be quantitative and, thus, regulated by multiple genes. The study of quantitative traits is challenging, since they are heavily influenced by various environmental factors.

Approaches to Enhance N_2 Fixation Through Specific Genes

Aiming to accelerate the genetic improvement of soybean genotypes for N_2 fixation, several studies have been pursued on specific genes that regulate N_2 fixation and nodulation. Devine (1984) reported *rj*1, a gene that restricts the nodulation by some *Bradyrhizobium* strains, whereas Herridge and Rose (2000) identified *Rj*2, *Rj*3, and *Rj*4 and compared the level of nodulation restriction. However, the improvement of N_2 fixation through the control of rhizobia populations and their diversity is challenging.

Another approach to improve N_2 fixation in soybean is to increase the number of nodules using nitrate-tolerant symbiotic (*nts*) soybean. These genotypes, known as supernodulators, were developed using mutation breeding and are characterized by

a non-sensitive nodulation to soil nitrate levels (Carroll et al. 1985). Soybean has a mechanism, called autoregulation of nodulation (AON) that prevents excessive nodulation through a negative feedback system that prevents the plant from allocating energy to sustain nodules in soil types that can partially supply N to the plant (Caetano-Anolles and Gresshoff 1991). A mutation in the nodule autoregulation receptor kinase (*NARK*) modifies AON and results in a supernodulator phenotype (Searle et al. 2003). Although supernodulating mutants produce 10–20x more nodules than the wild type, they are 30–40% less productive and show a restricted root growth (Day et al. 1986; Gremaud and Harper 1989). For instance, the supernodulating cultivars Bragg, Williams, Elgin 87, and Enrei are on average 20–41% less productive than the parental lines (Wu and Harper 1991; Pracht et al. 1994; Herridge and Rose 2000). As a result, no supernodulating genotypes have been released as commercial cultivars.

Tentative Breeding Strategies

Evaluation of Traits Directly and Indirectly Related to N_2 Fixation

Soybean genotypes with a high N absorption and high N_2 fixation are more likely to be more productive; one reason is that they can meet the high N demand in periods that are critical for soybean development such as during the initial root development and pod-filling stages. The pattern of N accumulation derived from N_2 fixation in soybean does not follow that of total N accumulation by the plant. The N_2 fixation activity starts a few weeks after germination during the V2 and V3 stages, continues to increase during the plant development, and reaches a peak at the R3–R5 stage (Zapata et al. 1987). Afterward, the rate of N_2 fixation declines and this is due to the energy required for the pod-filling stage (Imsande 1989; Keyser and Li 1992). Therefore, at the early vegetative and late reproductive stages, the symbiosis can just provide a limited amount of N that is likely not to supply the total amount required by the plant (Keyser and Li 1992). The remaining N required is either absorbed from the soil or reallocated from the vegetative tissues. Thus, the plant relies on soil N which may limit plant productivity in cases of deficiency. As a result, an effective strategy for improving N_2 fixation through breeding would be to assess the genetic variability for high N_2 fixation in the early and late developmental stages.

In 2015, we conducted one cycle of greenhouse experiment to evaluate the N_2 fixation capacity of 25 genotypes with a diverse genetic background using the ^{15}N dilution technique (Table 14.1). The main objective of this approach was to identify traits that are reliable indicators of a superior N_2 fixation. Leaf and pod atom% ^{15}N excess represents the amount of the heavier stable isotope of N expressed as a percentage of the total N. Since the abundance of ^{15}N in the atmospheric N_2 is very

Table 14.1 Assessment of traits related to early and late N_2 fixation for 25 soybean genotypes/cultivars grown under greenhouse conditions

Genotype/ cultivar	Early N_2 fixation traits			Late N_2 fixation traits								
	SPAD readings	Leaf %N	Leaf atom% ^{15}N excess	Days to maturity	Shoot dry mass	Seed weight	Seed number	Mean seed weight	Pod %N	Nodule number	Nodule dry weight	Pod atom% ^{15}N excess
		%		number of days	mg/ plant	mg/ plant	number/ plant	mg/seed	%	number/ plant	mg/plant	
JTN-4307	31.5	2.38	0.4821	188	9,881	5,503	53.9	105	0.65	71.1	126	0.0145
JTN-5203	30.1	2.64	0.5093	179	5,350	2,552	35.3	75	1.15	21.4	36	0.0200
Osage	31.9	2.99	0.4614	193	12,463	4,903	52.1	95	1.02	68.2	179	0.0174
Ozark	30.6	2.29	0.4648	177	5,319	2,350	27.3	87	1.39	29.1	82	0.0176
R05-3239	33.8	2.42	0.4951	180	5,075	2,200	24.6	90	1.22	18.9	53	0.0233
Jake	28.5	2.21	0.5180	175	7,106	3,711	35.6	103	0.87	40.0	93	0.0170
Saluki 4910	30.2	2.27	0.5187	185	7,181	4,301	41.4	105	0.96	59.8	125	0.0191
Bragg	22.6	1.78	0.5248	210	12,113	2,662	31.6	81	0.73	101.0	189	0.0114
Davis	29.5	2.83	0.4597	195	10,494	3,027	30.3	98	0.84	48.6	88	0.0109
Enrei	38.3	3.02	0.4949	100	3,069	3,472	12.5	254	0.67	24.9	197	0.0512
Williams	27.6	2.17	0.4730	162	6,656	3,938	30.1	131	0.57	55.2	127	0.0295
PI 471938	27.9	2.09	0.5134	172	7,806	4,255	40.4	102	0.69	74.9	138	0.0186
Clark	25.9	1.93	0.5257	177	7,163	4,238	37.4	120	0.71	31.1	77	0.0177
Bossier	26.6	2.11	0.5033	219	13,644	2,645	29.9	90	0.77	106.1	239	0.0107
Centennial	31.3	2.01	0.4884	203	10,425	4,891	43.2	113	0.57	84.9	167	0.0116
Hardee	28.7	2.14	0.4873	224	12,213	2,815	31.8	84	0.79	72.0	136	0.0116
Jackson	29.4	2.12	0.5243	194	11,038	3,944	33.8	110	0.52	61.3	132	0.0136
S.J.2	25.5	2.01	0.5593	239	14,256	2,150	25.8	65	1.03	108.5	282	0.0090

(continued)

Table 14.1 (continued)

Genotype/ cultivar	Early N_2 fixation traits			Late N_2 fixation traits								
J-200	27.0	1.90	0.5105	235	16,094	1,211	15.2	62	1.37	90.4	141	0.0264
R01-416F	28.9	2.24	0.5024	169	7,063	3,875	36.2	102	1.06	43.9	124	0.0172
PI 96169B	32.2	1.78	0.5355	125	3,013	1,994	11.8	186	0.47	0.9	19	0.1005
PI 96171	38.5	2.93	0.4611	121	3,100	2,209	19.5	113	0.77	28.8	172	0.0543
SS2-2	33.5	2.87	0.4984	175	7,525	4,680	34.9	139	0.95	72.2	212	0.0210
Nitrasoy	27.6	1.65	0.5630	211	3,213	407	7.6	58	0.50	0.4	1	0.1023
D68-099	30.2	1.65	0.5653	215	2,988	435	9.3	56	0.67	0	0	0.0894
LSD ($p < 0.05$)	4.42	0.54	0.0455	20	2,448	1,387	13.1	53	0.35	27.8	70	0.0230
Minimum	12.7	0.87	0.0294	84	0	100	1.0	0	0.22	0	0	0.0048
Maximum	46.0	5.09	0.3143	249	25,800	9,100	82.0	1,014	3.99	254	842	0.1681
Mean	29.9	2.26	0.1392	188	8,170	3,248	30.7	108	0.84	52.4	125	0.0282
Median	30.0	2.09	0.1310	189	7,400	3,000	29.0	97	0.65	44	104	0.0142
SD	6.5	0.80	0.0591	41.7	4,950	2,019	17.5	69	0.55	47.5	117	0.0339
25%	25.3	1.65	0.1014	167	4,025	1,650	16.0	75	0.44	15.0	36	0.0095
75%	34.7	2.79	0.1619	221	11,800	4,700	43.0	123	1.05	76.0	180	0.0266

low (0.3663 atom% ^{15}N), the enrichment of a growing medium or soil with ^{15}N labeled fertilizer allows to estimate the amount of N that soybean plants uptake from the soil and also the remaining N derived from BNF. To calculate this difference, two non-nodulating soybean genotypes (i.e., Nitrasoy and D68-099) that rely exclusively on the N provided by the soil were used as controls. The N_2 fixation activity was assessed in the early and late growth stages to identify genotypes with a consistent N_2 fixation capacity throughout plant development.

The genotype effect was significant ($p < 0.001$) for all the evaluated traits. The leaf and pod atom% ^{15}N excess of the non-nodulating genotypes Nitrasoy and D68-099 as well as of PI 96169B were the highest among all the evaluated genotypes. The nodule number of nodulating genotypes ranged from 18.9 to 108.5 compared with 0–0.9 of the non-nodulating genotypes and PI 96169B. These results demonstrated a wide variation in nodulation capacity among the evaluated genotypes.

SPAD meter readings were used to estimate differences in BNF capacity based on chlorophyll content. N_2 fixation is related to the photosynthetic activity that provides carbohydrates to the microsymbiont and affects the Rubisco activity during photosynthesis (Vollmann et al. 2011). In our study, SPAD meter readings were tested as a trait related to early N_2 fixation. The results revealed a wide variation among the evaluated genotypes that ranged between 22.6 and 38.5 for the nodulating genotypes and between 27.6 and 30.2 for the non-nodulating genotypes.

Another important trait that can be a strong indicator of an enhanced N_2 fixation capacity is the plant biomass. The main reason for this association is the large N accumulation that is required to grow large plants. Thus, the selection for this trait implies the improvement of the ability of the plant to meet this higher N demand (Herridge and Rose 2000). Although larger soybean plants do not necessarily have higher yields, in breeding programs the use of a trait to estimate the plant‘s capacity to accumulate biomass may assist to point out the lines with enhanced capacity of N_2 fixation when selecting lines with similar yield potential.

The shoot dry mass of the nodulating genotypes was 3,013–16,094 mg compared with that of the non-nodulating genotypes 2,988–3,213 mg. These results showed that plants with high N_2 fixation are larger in size, and that they are greatly influenced by the allocation of N in the plant body.

Estimation of N_2 Fixation Capacity in Different Stages During the Plant Development

In the same experiment, we measured the early stage %Ndfa using leaf samples collected 5 weeks after germination. Additionally, pod samples were collected at the R7 developmental stage for the analysis of the late %Ndfa. The performance of each genotype regarding %Ndfa in the early and late evaluations are presented in Fig. 14.1.

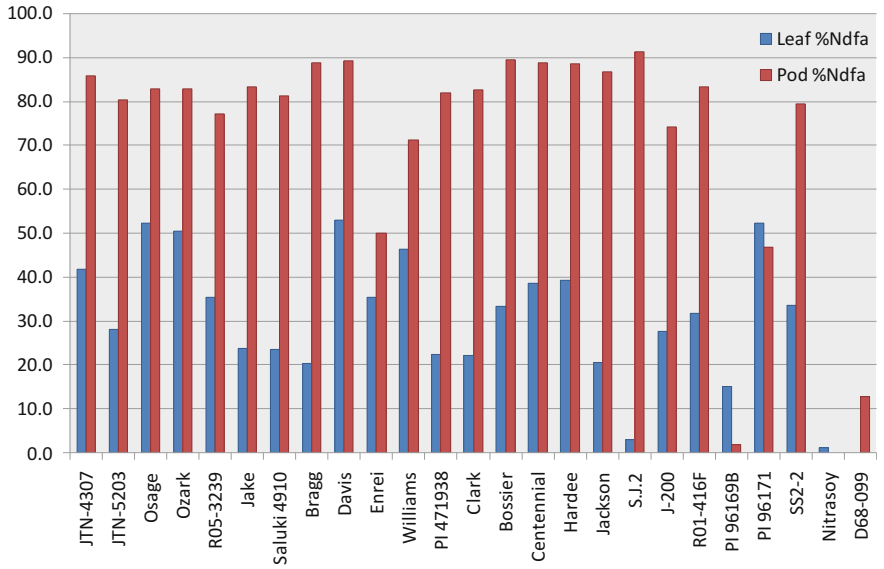


Fig. 14.1 Percentage of nitrogen derived from atmosphere (%Ndfa) for 25 soybean genotypes evaluated in early (leaf) and late (pod) growth stages under greenhouse conditions

Overall, the results indicated the high dependence of soybean plants on the N derived from N_2 fixation, except for the two non-nodulating lines Nitrasoy and D68-099. Additionally, our data revealed that the level of genetic variability for N_2 fixation capacity in the early growth stages was higher than that in late growth stages.

Differences among genotypes in the early and late measurements of N_2 fixation could be attributed to nodule aging. Nodules are formed on the main root at the beginning of plant development and last for 65 days on average; after this period, they become inactive (Bergersen 1958). In order to maintain a high rate of N_2 fixation after the R2 stage, the soybean plant forms a secondary set of nodules usually on the lateral and deep roots (Keyser and Li 1992; Imsande 1989; Zapata et al. 1987). The underlying genetic mechanism that controls the formation of nodules in different stages of plant development remains unknown, and thus, further studies are needed to more efficiently improve N_2 fixation capacity in soybean.

Evaluating the Association of Traits with N_2 Fixation

In soybean breeding programs, the N_2 fixation capacity is usually evaluated based on characters related to nodulation, because the direct measurement of total N_2 fixation and %Ndfa is costly. Previous genetic studies have identified quantitative trait loci (QTL) associated with the number, weight, and size of nodules (Hwang

et al. 2014; Santos et al. 2013). However, information on the effect of these traits on yield is limited, preventing their use in breeding programs.

A weak association between plant growth and nodulation has been reported for some N_2-fixing legume species in which non-nodulating and low-nodulating genotypes have similar yield to that of high nodulating genotypes (Rupela and Rao 2004). Abaidoo et al. (1999) evaluated the N_2 fixation capacity of soybean genotypes based on nodulation traits and concluded that the exclusive use of these traits to select genotypes for high N_2 fixation is inappropriate, and greenhouse conditions, and they recommended the use of additional parameters related to N_2 fixation capacity. The weak correlation between nodulation characters and total N_2 fixation could be attributed to factors that cause nodules to be inefficient to fix nitrogen, therefore limiting the amount of N to the plant.

One of the known causes for this low efficiency of dinitrogen fixation is the low competitiveness of strains supplied by inoculants in soils long cultivated with soybeans. In these areas, their capacity to colonize nodules is considerably low compared with that of native rhizobia. It has been reported that only 10% of soybean nodules are formed by strains provided by the inoculant. This means that 90% of the rhizobia strains present in nodules are of unknown origin and therefore can be less efficient for dinitrogen fixation. Ultimately, this causes an overall low efficiency of BNF and a limited yield (Kvien et al. 1981; Greder et al. 1986; Herridge and Rose 2000).

The capacity of *Bradyrhizobium* to fix atmospheric N_2 greatly varies under field conditions from high to extremely low. A previous study showed that the proportion of each strain that colonizes the soybean roots follows a normal curve (Bergersen 1970). Although soybean can compensate the inability of ineffective nodules to fix N_2 by increasing the photosynthates provided to the effective nodules, in situations in which ineffective nodules reduce the nodule mass of effective nodules, the total N_2 fixed will be decreased (Singleton and Stockinger 1983).

In summary, the assessment of nodulation traits to evaluate and select genotypes for N_2 fixation capacity is unreliable. Some indirect traits of N_2 fixation could serve as auxiliary tools to efficiently select genotypes for BNF. For instance, these traits could indirectly measure the capacity of the plant to maintain a high photosynthetic rate and supply carbohydrates to the pod and BNF, or the allocation of N_2 fixed in the seeds and plant tissues (Imsande 1989; Keyser and Li 1992).

In our greenhouse experiment we assessed traits that affect or are affected by N_2 fixation, which was estimated through the value of atom% ^{15}N excess (a parameter of the ^{15}N dilution technique). To identify the traits that interact with N_2 fixation, we tested the association of SPAD readings (i.e. measuring the greenness of leaves) with leaf %N at the early growth stages and with shoot dry mass, total seed weight, total seed number, days to maturity, pod %N, nodule number, and nodule dry weight at the late growth stages. The results yielded significant and negative correlations of early N_2 fixation with SPAD readings ($r = -0.45$; $p < 0.001$) and leaf %N ($r = -0.49$; $p < 0.001$). The late N_2 fixation (pod atom% 15 N excess) was significantly negatively correlated with shoot dry mass ($r = -0.51$; $p < 0.001$), seed number per plant ($r = -0.48$; $p < 0.001$), nodule number ($r = -0.39$; $p < 0.001$),

total seed weight ($r = -0.38$; $p < 0.001$), days to maturity ($r = -0.36$; $p < 0.001$), nodule dry weight ($r = -0.30$; $p < 0.001$), and pod %N ($r = -0.28$; $p < 0.001$). Additionally, a significant moderate correlation ($r = 0.45$; $p < 0.05$) was identified between early N_2 fixation and late N_2 fixation, indicating that N_2 fixation capacity is widely affected by or affects related traits, and that traits indirectly linked to N_2 fixation are possibly more strongly associated with N_2 fixation than those that are directly associated.

Since many of the tested correlations between the evaluated traits and the pod atom% ^{15}N excess were found to be significant, this confirms that the late N_2 fixation is affected or is being affected by these traits. Therefore, a multiple regression model was tested between all characters evaluated at the R7 stage and late N_2 fixation. Through this analysis, it was possible to identify those traits that are mostly associated with late N_2 fixation and determine whether fixation capacity can be accurately predicted at this stage. The multiple regression model revealed that the number of days to maturity, shoot dry mass, seed weight per plant, pod %N, and nodule dry weight significantly contributed to late N_2 fixation ($R^2 = 0.47$; $p < 0.001$). Although the model yielded a moderate value for R^2, it showed that multiple traits need to be evaluated in order to more accurately assess the N_2 fixation capacity of soybean genotypes.

Herridge and Rose (2000) highlighted the importance of screening multiple traits associated with N_2 fixation when selecting genotypes for enhanced BNF, since the related genes might naturally segregate. Additionally, Van Kessel and Hartley (2000) reported that to select genotypes for high N_2 fixation, it is critical to conduct field experiments in soils with low N content.

Selecting Genotypes with Enhanced N_2 Fixation Capacity

For breeding purposes and future molecular studies, it would be helpful to select genotypes with distinct characteristics that would allow identifying putative loci linked to BNF. To this end, we performed a cluster analysis using Ward's method based on early and late N_2 fixation measurements. As shown in Fig. 14.2, the 25 genotypes were classified into three different categories. Cluster 1 (red) included genotypes with an inconsistent behavior for N_2 fixation. For instance, JTN-4307 showed a relatively high late N_2 fixation, but low early N_2 fixation. Cluster 2 (green) included genotypes with a relatively high N_2 fixation, of which Davis showed the highest early and late N_2 fixation measurements. Cluster 3 (blue) included genotypes with a relatively low N_2 fixation, including the non-nodulating lines D68-099 and Nitrasoy.

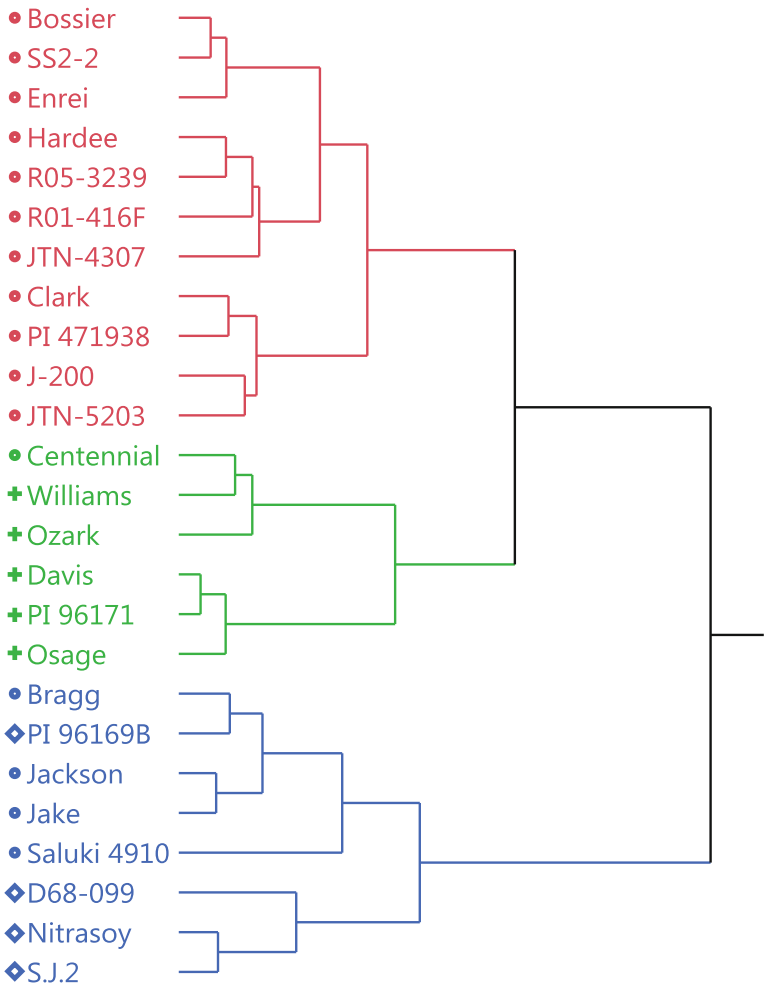


Fig. 14.2 Dendrogram of leaf and pod atom% ^{15}N excess for 25 soybean genotypes using Ward's method

Future Outlook

Previous studies have focused on improving the BNF capacity of soybean genotypes; however, the incorporation of the associated traits into soybean lines is yet to be achieved. Improving N_2 fixation in soybean is complex, since BNF is a multigenic character highly affected by the environment. Therefore, the use of strategies that are more appropriate for the improvement of quantitative traits may be more suitable to evaluate and select genotypes with enhanced BNF. We carried out a study to assess traits that are directly and indirectly related to BNF using soybean

genotypes with different genetic backgrounds, and found that these traits have a significant correlation with N_2 fixation activity compared with nodulation traits. Our results also suggested that at least two measurements of integrated N_2 fixation activity should be performed during plant development in order to more accurately select genotypes for BNF capacity. This approach may help to develop soybean lines with improved BNF that will reach their maximum yield potential without the additional use of nitrogenous fertilizers.

Acknowledgements This study was supported by the North Central Soybean Research Program and the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES-Process BEX 11900/13-0).

References

Abaidoo RC, Dashiell KE, Sanginga N, Keyser HH, Singleton PW (1999) Time-course of di-nitrogen fixation of promiscuous soybean cultivars measured by the isotope dilution method. Biology Fert Soils 30:187–192

Bergersen FS (1958) The bacterial component of soybean root nodule: changes in respiratory activity, cell dry weight, and nucleic acid content with increasing nodule age. J Gen Microbiol 19:312–323

Bergersen FJ (1970) Some Australian studies relating to long term effects of the inoculation of legume seed. Plant Soil 32:727–736

Caetano-Anolles G, Gresshoff PM (1991) Plant genetic control of nodulation. Ann Rev Microbiol 45:345–382

Carroll BJ, McNeil DL, Gresshoff PM (1985) Isolation and properties of soybean [*Glycine max* (L.) Merr.] mutants that nodulate in the presence of high nitrate concentrations. Proc Natl Acad Sci USA 82:4162–4166

Day DA, Lambers H, Bateman J, Carroll BJ, Gresshoff PM (1986) Growth comparisons of a supernodulating soybean (*Glycine max*) mutant and its wildtype parent. Physiol Plant 68:375–382

Devine TE (1984) Genetics and breeding of nitrogen fixation. In: Alexander M (ed) Biological nitrogen fixation. Plenum, New York, pp 127–154

Devine TE, Kuykendall LD (1996) Host genetic control of symbiosis in soybean (*Glycine max* L.). Plant Soil 86(1):173–187

FAOSTAT 2013. http://faostat3.fao.org/download/Q/QC/E. Accessed May 26 2016

FAOSTAT 2014. http://faostat3.fao.org/download/Q/QC/E. Accessed May 26 2016

Greder RR, Orf JH, Lambert JW (1986) Heritabilities and associations of nodule mass and recovery of *Bradyrhizobium japonicum* serogroup USDA 110 in soybean. Crop Sci 26:33–37

Gremaud MF, Harper JE (1989) Selection and initial characterization of partially nitrate tolerant nodulation mutants of soybean. Plant Physiol 89:169–173

Hwang S, Ray JD, Cregan PB, King CA, Davies MK, Purcell LC (2014) Genetics and mapping of quantitative traits for nodule number, weight, and size in soybean (*Glycine max* L. [Merr.]). Euphytica 195:419–434

Herridge D., Peoples M.B., Boddey R.M. (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18

Herridge D, Rose I (2000) Breeding for enhanced nitrogen fixation in crop legumes. Field Crops Res 65:229–248

Hamuda HEAF, Patko I (2010) Relationship between environmental impacts and modern agriculture. Obuda Univ e-Bull 1(1):87–98

Imsande J (1989) Rapid di-nitrogen fixation during soybean pod fill enhances net photosynthetic output and seed yield: A new perspective. Agron J 81:549–556

Keyser HH, Li F (1992) Potential for increasing biological nitrogen fixation in soybean. Plant Soil 141:119–135

Kueneman EA, Root WR, Dashielli KE, Hohengerg J (1984) Breeding soybeans for the tropics capable of nodulating effectively with indigenous *Rhizobium* spp. Plant Soil 82:387–396

Kvien CS, Ham GE, Lambert JW (1981) Recovery of introduced *Rhizobium japonicum* strains by soybean genotypes. Agron J 73:900–905

Mus F, Crook MB, Garcia K, Costas AG, Geddes BA, Kouri ED, Paramasivan P, Ryu M, Oldroyd GED, Poole PS, Udvardi MK, Voigt CA, Jean-Michel Ane, Peters JW (2016) Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl Environ Microbiol 82(3):3698–3710

Pracht JE, Nickell CD, Harper JE, Bullock DG (1994) Agronomic evaluation of non-nodulating and hypernodulating mutants of soybean. Crop Sci 34:738–740

Rupela OP, Rao DLN (2004) Breeding legumes for improved nitrogen (N2) fixation. In: Jain HK, Kharkwal MC (eds) Plant breeding: mendelian to molecular approaches. Narosa, New Delhi, pp 719–748

Salvagiotti F, Cassman KG, Specht JE, Walters DT, Weiss A, Dobermann A (2008) Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. Field Crops Res 108:1–13

Santos MA, Geraldi IO, Garcia AA, Bortolatto N, Schiavon A, Hungria M (2013) Mapping of QTLs associated with biological nitrogen fixation traits in soybean. Hereditas (Lund, Sweden) 150:17–25

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ, Gresshoff PM (2003) Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science 299:109–112

Singleton PW, Stockinger KR (1983) Compensation against ineffective nodulation in soybean. Crop Sci 23:69–72

Specht JE, Hume DJ, Kumudini SV (1999) Soybean yield potential-a genetic and physiological perspective. Crop Sci 39:1560–1570

United Nations, Department of Economic and Social Affairs, Population Division (2015) World population prospects: the 2015 revision, key findings and advance tables. Working paper no. ESA/P/WP.241

Van Kessel C, Hartley C (2000) Agricultural management of grain legumes: has it led to an increase in nitrogen fixation? Field Crops Res 65:165–181

Van der Heijden MGA (2016) Underground networking. Science 352(6253):290–291

Vollmann J, Sato T, Walter H, Schweiger P, Wagentristl H (2011) Soybean di-nitrogen fixation affecting photosynthesis and seed quality characters. Soil, Plant Food Interact, pp 496–502

Wilcox JR (2004) World distribution and trade of soybean. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production and uses. ASA, CSSA, ASSA, pp 1–13

Wu S, Harper JE (1991) Dinitrogen fixation potential and yield of hypernodulating soybean mutants: a field evaluation. Crop Sci 31:1233–1240

Zapata F, Danso SKA, Hardarson G, Fried M (1987) Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron J 79:172–176

Index

A. Shrawat et al. (eds.), *Engineering Nitrogen Utilization in Crop Plants*,
https://doi.org/10.1007/978-3-319-92958-3