Breeding for increased nitrogen use efficiency: a review for wheat (*Triticum aestivum* L.)

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Abstract

Nitrogen fertiliser is the most used nutrient source in modern agriculture and represents significant environmental and production costs. In the meantime, the demand for grain is increasing and production per area *((crop yield?))* has to increase. In this context, breeding for an efficient use of nitrogen became a major breeding objective. In wheat, nitrogen is required to maintain a photosynthetically active canopy ensuring grain yield and to produce storage protein in the grain hence end-use quality. In different situations of nitrogen management, the genetic, metabolic and physiological factors influencing nitrogen uptake and utilisation are reviewed. Their implications for breeding are discussed.

Key words: breeding - Nitrogen Use Efficiency - *Triticum aestivum*

CONTENT

DEFINITION OF NUE AND RATIONALE FOR ITS IMPROVEMENT

The concept of nitrogen use efficiency (NUE) has been widely used to characterize plant behaviour regarding different levels of nitrogen (N) availability. It is important to distinguish the concept of NUE and the NUE as a phenotypic trait.

Indeed, several definition and evaluation methods have been suggested, some of which are actually named “nitrogen use efficiency” (reviews in Good et al. 2004, Fageria et al. 2008). Moll et al. (1982) defined the most widespread NUE trait definition, at least among breeders, computed as the grain weight divided by the total N available to plant, and separated it into two components:

$$NUE = NUpE \times NUtE$$

with NUpE the N uptake efficiency calculated as the N in plant at harvest divided by the N available in soil, and NUtE the utilisation efficiency calculated as the grain dry mass divided by the total amount of N in plant at harvest. When different genotypes are compared, the computation of these components faces two main issues: (i) the complex estimation of N available to crop, and (ii) the estimation of the total amount of N in the plant.

N available to crop results from residual N before sowing, aerial N deposition, mineralization, and the actual availability of applied N. Thus, estimation of N available to crop is complex and an often used proxy has been the total amount of applied mineral N fertiliser summed to an estimation of residual N in soil. On 15 barley genotypes, Bingham et al. (2012) compared different methods to estimate available N. The first one was independent from genotype and used only residual soil N after winter and applied N fertiliser. The two others were dependent on the genotype and required a control without N fertilisation (N^0). Available N for the fertilized treatment (N^T) was then estimated either (i) by adding the total plant N at harvest for N^0 to the applied N fertiliser or (ii) by adding to the former the amount of soil N at harvest. Bingham et al. (2012) showed that genotype rankings were very similar between the three methods and that the simplest method can be used to start with.

However, these can lead to NUE overestimation in low N situations and NUE underestimation in high N situations (Cormier et al. 2013), making comparisons and/or joint analyses of different studies difficult. Within a large collection of genotypes, Cormier et al. (2013) suggested estimating available N from the distribution of the total plant N at harvest. They proposed to use N absorbed by the top 5% genotypes as an estimation of N that was available to the whole series.

To estimate the total amount of N in the plant, usually only the aerial parts are sampled. Not taking into account N in the roots artificially increases NutE and decreases NupE. However, measuring the quantity of root N (in the first 30 cm of soil layer) of a set of cultivars grown at two N levels, Allard et al. (2013) showed that only a small fraction of total N is partitioned to the roots (about 4 % or 10 kg ha^{-1} at harvest, following remobilisation of root N to grains). Here again the genotype rankings were very similar with or without root N.

Looking at the successes and debates that agitated other scientific communities may help to improve the approaches on wheat NUE. Ecologists developed another decomposition of NUE. Originally called "nitrogen utility", Hirose (1971) defined it as the flux ratio of dry mass productivity for a unit of N taken up from the soil. Berendse and Aerts (1987) suggested dividing it into two components to make it biologically meaningful in a context of perennial species in a steady-state system (*i.e.* annual biomass production = annual biomass loss; annual N uptake = annual N loss). Thus, NUE was defined as the product of the nitrogen productivity rate (NP; dry mass growth per unit of plant N) and the mean time residence of N (MRT). Later, Hirose (2011) revisited this definition and specified how it should be calculated to make it also suitable for non-steady state systems such as annual crops.

Compared to Moll et al. (1982), this definition has the interest to deliver a dynamic vision of NUE directly related to photosynthetic activity along the plant cycle. Nevertheless, it only focuses on N utilisation, as plant efficiency to extract N from the soil is not taken into account. However, in annual crops, this is an important parameter to consider as substantial amounts of N fertiliser are applied, implying environmental and economic issues.

In a similar way, in the water use efficiency (WUE) community, it has been explicitly decided not to account for water available to plant. The focus has been on viewing yield as the final objective through Passioura's (1977) seminal equation:

$$GY = WU \times WUE \times HI$$

with WU the water use *((used?))* (mm transpired), WUE the water use efficiency (kg above-ground dry matter / mm transpired) and HI the harvest index (kg grain / kg above-ground dry matter).

Following NUE formalisation by Moll et al. (1982), NUtE would then be equivalent to *((become?))* WUE × HI. NUpE would be an equivalent to WU divided by the quantity of water available to plant. The approach could be taken further by simply targeting nitrogen use (NU) as kg N absorbed by the plant instead of NUpE; in much the same way that WU is seen as (arguably) the most important target in improving water response (Blum 2009). This would also avoid dividing an already rather imprecise variable (NU) by an even more imprecise one (available N). Yet, environmental and economic issues are different in NUE where minimizing the loss of fertiliser applied (*e.g.* by leaching) and maximizing N uptake for increasing grain protein concentration lead to focus also on NUpE. Moreover, not to account for N available to crop implies to use genotypes dependent methods (*e.g.* repeated controls) to compare variety behaviour between different stress intensities or to characterize genotype × stress interactions, leading to confounding effects.

Criticisms of the initial WUE equation have heavily contributed to identify and prioritize approaches and traits. The first has been to recognize that the three terms of the equation are clearly not independent (Blum 2009, Tardieu 2013). Typically, as WU increases, WUE decreases because WU scales to biomass (Blum 2009), as does N absorption (Sadras and Lemaire 2014, Lemaire et al. 2007). Consequently, an excessively narrow focus on WUE may be counterproductive (Blum 2009). Although, the underlying physiological reasons for this are very different between nitrogen and water, framing the nitrogen community in much the same way as the water community could help in placing the focus on NU and on systematically accounting for total biomass when evaluating NU, as advocated by Sadras and Lemaire (2014).

As in water and ecologist communities, research on NUE can also be disconnected from the NUE definition of Moll et al. (1982) and focus on a dynamic approach. Indeed, NUpE and NUtE are calculated at the end of the crop cycle. However, total N in plant varies during the cropping season and

has a critical interaction with HI: once grains are growing, they become a N sink, and growers, breeders, and the wheat industry have to manage the contradictory objectives of high yields and high protein contents (Feil 1997, Jeuffroy et al. 2002, Oury and Godin 2007). First of all, pre-anthesis and post-anthesis phases should be clearly separated. Regarding the post-anthesis phase, the grain protein deviation (GPD; deviation from the yield-protein regression) criterion suggested by Monaghan et al. (2001) and Oury and Godin (2007) allows to specifically breed for high protein content without the associated yield penalty. The GPD analysis of Bogard et al. (2010) showed that this metric was tightly related to the deviation between pre-anthesis N uptake and post-anthesis N uptake, meaning the obvious: crops that are both high yielding and high in protein content absorb large quantities of nitrogen. In other words, the analysis of Bogard et al. (2010) places NU as a key factor without focusing on NUpE. Looking now to the pre-anthesis phase has the advantage of not having to deal with the yield-protein trade-off. Studying N impacts on yield, grain number per area can become the criterion to target instead of yield. Indeed, it allows to get rid of kernel weight elaboration, which occurs post-anthesis. And as suggested by Meynard (1987), at least in western European situations, N will essentially have an impact on grain number per area, and kernel weight will often add noise due to other stresses. This would also mean that HI would essentially be replaced by a fertility index implying complex phenotyping although it may allow a better characterization of N response regarding *((according to?))* the phenologic stage.

NUE has been the subject of a wealth of literature and underpinning projects for its improvement. There seems to be consensus on the need to increase progresses on NUE in breeding. To the best of our knowledge, NUE has not been the target of dedicated breeding improvement. Rather, it has been improved through indirect selection for yield, in environments targeted by breeding programs. Sadras and Richards (2014) have suggested that indirect selection for yield serves as a benchmark for any alternative approach. Several studies have evaluated a posteriori breeding improvement of NUE (Ortiz-Monasterio et al. 1997a, Guarda et al. 2004, Muurinen et al. 2006, Cormier et al. 2013). In the case of France, Cormier et al. (2013) quantified NUE improvement at 0.13 kg DM/kg N/year. Supposing an average French yield of 7 t/ha and assuming a reference NUE value between 37.8 kg DM/kg N (Cormier et al. 2013) and 33.3 kg DM/kg N (average value for wheat used in French balance

sheet N recommendation methods; Meynard 1987), this equates to a saving of around 6-8 kg N/ha after 10 years of genetic improvement. From an economic standpoint, the variations in (fertiliser N / grain price) ratios essentially determine the quantity of N applied. The impacts of this volatility on on-farm NUE and required N savings can be translated into two examples. First, 10 years of breeding (*i.e.* a saving of 6-7 kg N/ha) can compensate for a variation of N : grain price ratio from 5 to 6, *i.e.* 16% of the total observed volatility over the past 10 years (Cohan 2009). Second, over the same 10-year period, this price ratio has varied from 3 to 9 (Sylvester-Bradley and Kindred 2009) leading to a necessity to increase NUE from 23.8 to 28.6 kg DM/kg N requiring almost 40 years of breeding progress.

Overall, this leads us to conclude that breeding needs to tackle NUE more efficiently than it has been doing at the current rate.

TRAITS INFLUENCING N-UPTAKE EFFICIENCY

Root size and morphology

Nitrate is readily leached down the soil profile. Consequently, the primary root traits to improve for enhanced N capture include rooting depth and rooting density, especially for post-anthesis N uptake (Foulkes et al. 2009). A deeper relative distribution of roots could comprise *((right verb?))* part of an ideotype to maximize N capture and further improvements in root architecture could focus on root proliferation at depth in wheat (Carvalho and Foulkes 2011). Indeed, root length density (root length per unit volume of soil) is often below a critical threshold of 1 cm/cm (Barraclough et al. 1989, Gregory and Brown 1989) for potential nitrate capture at lower depths in the rooting profile (Ford et al. 2006, Reynolds et al. 2007).

Genetic variation in root system size has been widely reported in wheat (*e.g.* O'Toole and Bland 1987, Hoad et al. 2001, Ehdaie and Waines 2003, Ford et al. 2006), but root distribution varies strongly with soil characteristics, nutrient availability and mechanical impedance. In wheat, the use of synthetic wheat derivatives, incorporating genes from the diploid wild species *Triticum tauschii* (D genome)

with roots distributed relatively deeper (Reynolds et al. 2007) may help in the development of cultivars with deeper rooting systems. In addition, the wheat-rye translocation in 'Kavkaz' for the short arm of chromosome 1 (1RS) has been observed to have increased root biomass at depth (Ehdaie et al. 2003). And tall landraces from China and Iran have larger root biomass than semi-dwarf cultivars descended from CIMMYT breeding material (Ehdaie et al. 1991, Ehdaie and Waines 1993, 1997, Ehdaie 1995). It may also be possible to increase root length density at depth without extra carbon input by modifying specific root length (root length per root biomass; Carvalho et al. 2014). Although it is well established that plants respond to N deficiency by increasing the ratio of root biomass on total plant biomass (root dry weight ratio; RDWR) due to the functional equilibrium between the growth of the root and shoot (Barraclough et al. 1989, Dreccer et al. 2000, Robinson et al. 2001), there are to date no reports of genetic variation in the dynamic responses of RDWR to N supply.

Direct selection for root system architecture traits (length, biomass, density, lateral root dispersion) has been associated with improved water and/or nutrient uptake in wheat (Hurd 1964), upland rice (Price et al. 2002) and maize (Lynch 2007). Indirect selection for lower canopy temperatures might also be taken as an indication of a greater root uptake capacity, but higher stomatal conductance would produce a similar signal (Reynolds et al. 2009). Root hairs provide another potential mechanism to maximize N capture and two genes for root hair elongation, RTH1 and RTH3 *((italics?))*, have been identified in maize (Hochholdinger and Tuberosa 2009). Root architecture and root functions are likely to be multigenic and hence much more difficult to select for (Hall and Richards 2013). Therefore, breeding for root characteristics has seldom been implemented to date, principally because of the difficulties of scoring root phenotypes directly and the absence of suitable proxy measurements. Nevertheless, marker-assisted selection may be especially useful to pyramid multiple traits, such as root angle, root length, root weight and root to shoot ratio, which are associated with main effect quantitative trait locus (QTL) in wheat (Hamada et al. 2012, Sharma et al. 2011, Bai et al. 2013), even if a better understanding of the biology of these traits and the potential synergies and trade-offs between traits is required (Lynch et al. 2007). For example, the expression of length and density of

root hairs may be synergistic (Ma et al. 2001) and there may be antagonistic interactions between biomass allocation to different root classes due to competition for assimilates (Walk et al. 2006).

Root N transporter systems

In most countries, the commercial mineral forms of N commonly applied to crops are anhydrous ammonia, urea, ammonium sulphate and ammonium nitrate (Robertson and Vitousek 2009, Andrews et al. 2013). In addition, farmyard manure is also able to supply a considerable amount of N fertilisation (Hooda et al. 2000, Körschens et al. 2013). Mineral N fertilisers are particularly soluble for easy assimilation by crops. Both urea and ammonia are converted to nitrate (NO_3^-) at different rates depending on the nature of the soil and of the climatic conditions (Jarvis et al. 2011). Thus, NO_3^- is the main source of N for most crop species, whether inorganic or organic N is provided to the plant (Nasholm et al. 2009, Gioseffi et al. 2012).

Ammonium (NH_4^+) is the ultimate form of inorganic N available to the plant. Most of the NH_4^+ incorporated by the plant into organic molecules originates from NO_3^- reduction, although metabolic pathways such as photorespiration, phenylpropanoid metabolism, utilisation of N transport compounds and amino acid catabolism can generate NH_4^+ (Lea and Miflin 2011). In cultivated soil, NH_4^+ concentration is generally ten times lower than NO_3^- concentration (Nieder et al. 2011), but substantial amounts of ammonium (NH_4^+) can remain despite active nitrification by soil microorganisms. Both NO_3^- and NH_4^+ enter the root apoplast by diffusion or mass flow (Crawford and Glass 1998) *((NO_3^- mostly by mass flow but NH_4^+ mostly by diffusion: sentence needs rewording))*. Then, there are taken up via an active transport system by means of proteins termed high and low affinity transporters and located in the root cell plasma membrane (Loqué and von Wirén 2004, Glass 2009, Dechorgnat et al. 2011).

In higher plants, there are basically three different NO_3^- transport systems that operate depending on the NO_3^- concentration in the surrounding root environment. The first one is an inducible high affinity transport system (iHATS) that is induced in the presence of low concentration of NO_3^- in the range of 1 to 200 µM depending on the plant species (Pace and McClure 1986, Sidiqui et al. 1990). In wheat, it

was reported that the iHATS has a Michaelis constant (K_m) value of approximately 27 µM and requires 10 hours for full induction by NO_3^- (Goyal and Huffaker 1986). The second one is a constitutively expressed high affinity transport system (cHATS) that is present even in the absence of NO_3^-. Both systems exhibit a typical Michaelis-Menten saturation profile when the external NO_3^- concentration reaches a certain threshold. The third one is represented by a non-saturable low-affinity transport system (LATS) that dominates when NO_3^- in the external medium exceeds 250 µM, operating in the 0.5-1 mM concentration range (Sidiqui et al. 1990, von Wirén et al. 1997). Recent studies on NO_3^- channels of transporters showed that NO_3^- transport systems can also play versatile roles in sensing NO_3^- in plant development, pathogen defence and stress response (Wang et al. 2012). Although NH_4^+ ions can be passively taken up by plant roots, different root NH_4^+ transporters systems (Ludewig et al. 2007) allow the direct uptake of NH_4^+ ions and operate across a wide range of NH_4^+ concentrations (Loqué and von Wirén N. 2004). However, it is likely that in agricultural soils, NH_4^+ uptake operates mainly through the low affinity transport system (LATS), which is part of the NH_4^+ permeases in the Ammonium Transporter / Methylammonium Permeases / Rhesus (AMT / MEP / Rh) family (von Wirén and Merrick 2004). The K_m values for NH_4^+ influx in different species ranges between 1 and 200 µM (Bradley and Morris 1991, Wang et al. 1993), fitting with the average NH_4^+ soil concentration which rarely rises beyond 50 µM (Marshner 1995). In wheat, it was reported that the iHATS has a K_m value of approximately 50 µM and requires six hours for full induction by NH_4^+ (Goyal and Huffaker 1986).

NO_3^- transporters in higher plants are represented by two main gene families, namely the NRT1 PTR (Nitrate Transporter, Peptide Transporter) Family (NPF), which now regroups the previous NRT1 / PTR genes, and the NRT2 family also called the Major Facilitator Superfamily (MFS; Léran et al. 2014). An excellent review describing the different members of the NO_3^- and NH_4^+ transporters and the regulatory mechanisms affecting root N uptake systems, especially on the model species Arabidopsis, has recently been published by Nacry et al. (2013). This review emphasizes that expression and activity of most N uptake systems are regulated both by the concentration of their substrate and by a systemic feedback control of metabolites representative of the whole plant N status. In cereals in general and wheat in particular, there is far less information on root NO_3^- and NH_4^+

transport systems and their regulations. This is mainly because most of the pioneer work was conducted using the model plant Arabidopsis, due to the ease of obtaining mutants and transgenic plants altered in the expression of the different NO_3^- and NH_4^+ transporters (Miller and Smith 1996, von Wirén and Merrick 2004, Miller et al. 2007, Garnett et al. 2009, Xu et al. 2012). Nevertheless, gene structure and phylogeny of high or low affinity transport systems have been studied in a number of grasses including rice, maize, sorghum, *Brachypodium* and wheat (Plett et al. 2010, Yin et al. 2007, Girin et al. 2014). Moreover, a comprehensive overview of the complex phylogeny and gene expression patterns of 16 members of the NPF family in wheat has been recently published (Buchner and Hawkesford 2014). This study highlighted the complex pattern of expression of the nitrate transporters, mainly due to the presence of multiple co-orthologous genes that are differentially expressed according to the plant tissue, NO_3^- availability and to leaf senescence during the N assimilation and N remobilisation processes. In the wheat NO_3^- HATS system, earlier studies have also demonstrated that five genes are induced by abscisic acid when NO_3^- is not present. In contrast to the inhibitory effect of glutamine generally observed in other species, glutamine was able to induce the expression of NRT2 *((italic?))* genes in the absence of NO_3^- (Cai et al. 2006).

In addition, it also has to be considered that under agronomic conditions, both efficiency and the regulation of NO_3^- uptake systems may be enhanced by the presence of mycorrhizal associations (Hawkins et al. 2001), humic substances (Cacco et al. 2000), allelopathic compounds such as coumarin (Abenavoli et al. 2001) and plant growth-promoting bacteria (Mantelin and Touraine 2004), or inhibited when the CO_2 concentration is rising in the atmosphere (Bloom et al. 2014). Therefore, when studying the genetic basic of inorganic N uptake, environmental interactions must be taken into account together with the capacity of the plant to capture and transport NO_3^- or NH_4^+. This implies that, in combination with modelling approaches (Bertheloot et al. 2011), further research is required to obtain an understanding of the regulation of the NO_3^- and NH_4^+ HATS and LATS throughout the entire plant developmental process (Kong et al. 2013). It will also be necessary to evaluate the contribution of direct NH_4^+ uptake to the wheat N economy, as the available information on the NH_4^+ transport systems both at the molecular and physiological levels remains fragmentary in wheat (Causin and Barneix 1993, Søgaard et al. 2009) and other cereals such as maize (Gu et al. 2013) and rice (Gaur

et al. 2012). However, for wheat that preferentially uses NO_3^- instead of NH_4^+ as the main N source, an increase in NH_4^+ uptake may not be beneficial to the plant when the ion is applied to the soil (Angus et al. 2014).

Another field of investigation is the use of urea as a synthetic fertiliser in conventional agriculture (Andrews et al. 2013, Karamos et al. 2014). Indeed, to date, urea is mainly used as a source of N fertiliser (through soil mineralization after application) and the contribution of plant urea uptake and metabolism in a physiological and agricultural context has not been thoroughly investigated. Nevertheless, it is well known that plants possess leaf and root transporters to absorb urea as an intact molecule, and can hydrolyse and use it very efficiently (Witte 2011). Two distinct transport processes for urea have been identified in rice exhibiting a linear or a Michaelis-Menten kinetics (Wang et al. 2012). Moreover, encouragingly, when a rice urea transporter was overexpressed in Arabidopsis a positive effect was observed both on urea uptake at low concentration and on plant growth (Wang et al. 2012). In wheat, compared to other inorganic N sources, urea uptake was very low. Moreover, its kinetics of uptake was difficult to measure (Criddle et al. 1988). However, in some cases when applied at an optimum timing after anthesis, an increase in grain protein content or yield has been observed (Gooding and Davies 1992, Rawluk et al. 2000). More recently, in spring wheat, it has been shown that seed yield and N uptake were generally greater with polymer coated urea than urea alone (Malhi and Lemke 2013). Even if the efficiency of foliar application of urea in wheat and other cereals remains questionable, it is attractive in terms of environmental benefit. Thus, more research is required both at physiological and molecular levels.

Interaction with micro-organisms

Plant roots, including those of wheat, release a variety of organic substrates (*e.g.* organic acids, and sugars), exudates and other rhizodeposits (Nguyen 2003). This creates a particular fraction of soil in contact with roots named rhizosphere and favourable to the development of microorganisms. Plant rhizosphere is largely colonized by soil microorganisms, at levels of typically 10^8 to 10^9 bacteria per gram of rhizosphere soil and 1 to 1.5 m of fungal filaments per cm^2 of root surface (Moënne-Loccoz et

al. 2014). This microbial community contains a broad range of taxa differing from bulk soil community due to the selective effects of roots (Buée et al. 2009). Some of them, including pathogens as well as non-pathogenic microorganisms, may enter roots and reside within intercellular space or even within plant cells (Behl et al. 2012, Moënne-Loccoz et al. 2014). This also occurs in wheat (Germida and Siciliano 2001).

The composition and physiological activities of root-associated microbial communities is influenced by many factors, such as soil characteristics, farming practices, climatic conditions, and wheat genotypes (Mazzola et al. 2004). Indeed, rhizodeposition can differ between wheat cultivars (Wu et al. 2001) leading to differences in various aspects of the rhizosphere microbial ecology (Germida and Siciliano 2001). Therefore, it would be of prime interest to develop breeding strategies tailored both to suppress root pathogens and promote root colonization by plant-beneficial microbial partners (Lammerts van Bueren et al. 2011), especially those with the potential to enhance (i) N availability in the rhizosphere, (ii) root system and architecture, (iii) systemic plant metabolism and (iv) microbial phytoprotection (Fig. 1). This is all the more relevant since breeding is typically carried out under optimal conditions. Thus, phenotypic traits involved in interaction between plant and growth-promoting rhizobacteria may have been neglected (den Herder et al. 2010).

Soil microorganisms in the rhizosphere are major players in the availability of N for plant roots (Richardson et al. 2009). On one hand, N availability for roots may be reduced by microbial competition as various soil bacteria and fungi use ammonium and nitrate as N sources (Nelson and Mele 2006) and/or transform nitrate to gaseous N by denitrification (Herold et al. 2012). Nevertheless, plants can limit denitrification by releasing inhibitory secondary metabolites (Bardon et al. 2014), but so far this property is not documented in cultivated cereals. However, attempts are currently made to introduce into wheat a chromosome of *Leymus racemosus*, a wild relative of wheat, containing the ability for biological nitrification inhibition (Subbarao et al. 2007, Ortiz et al. 2008). On the other hand, N availability is enhanced by microbial mineralisation of organic N yielding ammonium in the rhizosphere. This entails proliferation of bacterial and fungal decomposers, as well as protozoan predators (Bonkowski 2004) and mycorrhizal fungi (Atul-Nayyar et al. 2009). In wheat, this priming effect reaches higher levels at the flowering stage (Cheng et al. 2003) and root colonization by

mycorrhizal fungi as well as positive mycorrhizal effects on plant nutrition and yield is genotype-dependent (reviewed in Behl et al. 2012). N availability for roots is also improved by N fixation. Thus, the community of N fixers (functional group) plays a key role for plant N nutrition (Hsu and Buckley 2009). Unlike in legumes, in wheat and other cereals, conversion of N_2 into NH_3 does not entail root-nodulating rhizobia but it can be perfomed by other non-nodulating N-fixing bacteria and part of the N fixed may be acquired by the plant (Behl et al. 2012). N-fixing bacteria occur naturally in soils including in the wheat rhizosphere (Nelson and Mele 2006, Venieraki et al. 2011). And inoculation with N fixers may enhance wheat yield (Kapulnik et al. 1987, Hungria et al. 2010, Behl et al. 2012, Neiverth et al. 2014). Their diversity and activity fluctuate with both plant species (Perin et al. 2006, Reardon et al. 2014) and cultivar (Coelho et al. 2009) including in wheat (Christiansen-Weniger et al. 1992, Manske et al. 2000, Venieraki et al. 2011). For example, the N-fixing bacterium *Klebsiella pneumonia* strain 342 can relieve N deficiency and enhance plant N levels (Iniguez et al. 2004) depending on cultivar (Manske et al. 2000).

Enhanced acquisition of water and mineral nutrients can be expected if the root system colonizes soil more extensively. Under in vitro conditions, wheat inoculation with rhizosphere bacteria may enhance root number and/or length, as well as root hair elongation (Dobbelaere et al. 1999, Combes-Meynet et al. 2011). These inoculation effects on root system architecture and biomass have been also evidenced in soil-grown wheat (Baldani and Baldani 2005, Veresoglou and Menexes 2010). Indeed, many bacteria and fungi modify root system architecture by manipulating plant hormonal balance, in particular by producing phytohormones such as auxins (Ortíz-Castro et al. 2009), cytokinins (Cassán et al. 2009, Moubayidin et al. 2009), or gibberellins. Gibberellins are produced by several rhizosphere bacteria and fungi (Bottini et al. 2004), including wheat strains (Upadhyay et al. 2009), thereby promoting primary root elongation and lateral root extension. For example, the wheat bacterium *Azospirillum brasilense* Sp245 synthesizes abscisic acid, which modifies lateral root development, and inoculation resulted in higher abscisic acid concentration in *Arabidopsis* (Cohen et al. 2008). Other root-branching signals especially 2,4-diacetylphloroglucinol (Brazelton et al. 2008) and nitric oxide (Creus et al. 2005) may also be implicated, including in wheat (Pothier et al. 2008, Couillerot et al. 2011). Their effects appear to take place via an auxin signal transduction pathway (Brazelton et al.

2008, Molina-Favero et al. 2008). Microbial interference with ethylene metabolism in roots may also be responsible for modifying wheat root system architecture (Upadhyay et al. 2009) by a direct microbial production of ethylene (Graham and Linderman 1980), or a reduction of ethylene concentration in plant roots by the deamination of ethylene precursor 1-aminocyclopropane carboxylic acid (Prigent-Combaret et al. 2008), thereby diminishing ethylene-mediated root growth repression (Glick 2005).

Microorganisms can induce systemic changes in plant physiology. For instance, a wide range of Arabidopsis genes displayed different expression levels upon inoculation with the plant-beneficial bacterium *Pseudomonas putida* (Srivastava et al. 2012). Microbial inoculation may also modify plant proteomic profiles (Mathesius 2009) and metabolomics profiles, both for primary metabolites (including rice shoot contents in amino acids; Curzi et al. 2008) and secondary metabolites in maize (Walker et al. 2012) and wheat (Fester et al. 1999). There are also indications that some rhizosphere bacteria may directly affect N metabolism in plants. Oil seed rape (*Brassica napus* L.) roots inoculated with *Achromobacter* strain U80417 displayed enhanced net influx rates of NO_3^- (Bertrand et al. 2000). Added to that, genes coding for two nitrate transporters (NRT2.5 and NRT2.6) were expressed at higher levels in Arabidopsis upon inoculation with *Phyllobacterium brassicacearum* STM196 (Mantelin et al. 2006). Tomato exposure to the bacterial metabolite 2,4-diacetylphloroglucinol increased the net root efflux of amino acids (Phillips et al. 2004). And in wheat, nitrate reductase activity of *Azospirillum brasilense* Sp245 inside roots is thought to contribute to N assimilation (Baldani and Baldani 2005). However, information is scarce and relevance for wheat remains to be further investigated.

A range of root-associated microorganisms promote plant health, by inhibiting root pathogens and/or triggering systemic induction of plant defence mechanisms (Couillerot et al. 2011, Almario et al. 2013). For instance, wheat inoculation with the bacterium *Pseudomonas fluorescens* Q8r1-96 resulted in cultivar-dependent, defence-related transcript accumulation in roots (Maketon et al. 2012). Thus, microbial phytoprotection effects are also important to consider and investigate.

TRAITS INFLUENCING N-UTILISATION EFFICIENCY

Nitrate assimilation

After being taken up by the roots, nitrate NO_3^- is then reduced to nitrite NO_2^- in the cytosol through the reaction catalysed by the enzyme nitrate reductase (NR; EC 1.7.1.1) using NADH / NAD(P)H / NADPH as electron donors. The NR enzyme represents the first step in the pathway of NO_3^- assimilation. They are positively regulated by NO_3^- and light at the transcriptional level; and are down-regulated at the post-transcriptional level by reversible phosphorylation during the dark period (Kaiser et al. 2011). In hexaploid wheat, two genes encoding NADH-NR have been identified (Boisson et al. 2005). NO_3^- reduction is followed by the reduction of NO_2^- to NH_4^+ catalysed by the enzyme nitrite reductase located in the plastids (NiR; EC 1.7.7.1; Sétif et al. 2009). NiR forms a complex with ferredoxin that provides electrons for the reduction of NO_3^- to NH_4^+ (Sakakibara et al. 2012). NH_4^+ is then incorporated into the amino acid glutamate through the action of two enzymes. The first reaction catalysed by the glutamine synthetase (GS; EC 6.3.1.2; Lea and Miflin 2011) is considered as the major route facilitating the incorporation of inorganic N into organic molecules in conjunction with the second enzyme glutamate synthase (GOGAT; EC 1.4.7.1; Suzuki and Knaff 2005), which recycles glutamate and incorporates C skeletons in the form of 2-oxoglutarate into the cycle. Then, the amino acids glutamine and glutamate are used as amino group donors to all the other N-containing molecules, notably other amino acids used for storage, transport and protein synthesis and to nucleotides used as basic molecules for RNA and DNA synthesis (Lea and Miflin 2011; Fig. 2).

In higher plants, including wheat, it exists several isoenzymic forms of GS and GOGAT which are located in different cellular compartments and differentially expressed in organs or cell types according to the developmental stage. Indeed, the GS enzyme exists as a cytosolic form (GS1) present in a variety of organ and tissues such as roots, leaves, phloem cells and a plastidic form (GS2) localised in chloroplasts and in plastids of roots and etiolated tissues. The relative proportions of GS1 and GS2 vary within the organs of the same plant and between plant species, each GS isoform playing a specific role in a given metabolic process, such as photorespiratory ammonia assimilation, nitrate reduction, N translocation and recycling (Lea and Milfin 2010). In wheat and other C3 cereals, both at

the transcriptional and at enzyme activity levels, GS2 predominates throughout the entire plant developmental cycle, although its activity can decrease by half after the flowering period. One GS1 isoenzyme is constitutively expressed in the phloem while others are generally induced in the cytosol of senescing leaves (Kichey et al. 2005, Christiansen and Gregersen 2014, Yamaya and Kusano 2014). Detailed analyses of gene expression and cellular localisation of the different wheat GS isoenzymes were performed in developing and senescing leaves as well as in a number of reproductive tissues (Kichey et al. 2005, Bernard et al. 2008). These studies highlighted that the complex GS isoenzyme pattern of expression was not only due to the hexaploid nature of the wheat genome, but also to the morphological complexity of leaves. In order to clarify the function of the different GS isoenzymes, a phylogenetic approach was taken, due to the lack of mutants or transgenic plants. This allowed the clustering of the different genes encoding GS into different classes of biological functions, which were not necessarily conserved between C3 and C4 cereals (Thomsen et al. 2014). In the same way, the enzyme GOGAT also exists in two forms that have specific roles during primary N assimilation or N recycling. A ferredoxin-dependent isoenzyme (Fd-GOGAT) is mainly involved, in conjunction with GS2, in the reassimilation of photorespiratory ammonia. A pyridine nucleotide-dependent isoenzyme (NADH-GOGAT; EC 1.4.1.14) is involved in the synthesis of glutamate in photosynthetic and non-photosynthetic organs or tissues, to sustain plant growth and development (Lea and Miflin 2011).

Glutamate can also be generated by the incorporation of ammonia into 2-oxoglutarate by the glutamate dehydrogenase (GDH; EC 1.4.1.2; Lea and Miflin 2011). However, a number of experiments using ^{15}N-labelling techniques and mutants deficient in GS and GOGAT have demonstrated that over 95 % of the ammonia available to the plant is assimilated via the GS / GOGAT pathway (Lea and Miflin 2011). Later on, it was clearly shown that GDH operates in the direction of glutamate deamination to provide organic acids, notably when the root and leaf cells are carbon-limited (Labboun et al. 2009, Fontaine et al. 2012). Recently, the hypothesis that GDH could play an important role in controlling not only glutamate homeostasis (Forde and Lea 2007, Labboun et al. 2009), but also the level of downstream and upstream carbon and N metabolites through the changes of its hetero-hexameric structure, has been put forward (Tercé-Laforgue et al. 2013). This function, which may also have a signalling role at the interface of C and N metabolisms, may be of importance when there is a shortage

of C under stress conditions or during several phases of plant growth and development. Moreover, transgenic studies performed on a number of model and crop species (Tercé-Laforgue et al. 2013) as well as quantitative genetic approaches performed on maize (Dubois et al. 2003) and wheat (Fontaine et al. 2009) strongly suggest that the reaction catalysed by NAD(H)-GDH is involved in the control of plant growth and productivity. Thus, further research is required to validate the function of GDH in crops such as wheat.

Over the last two decades, our knowledge of the various pathways involved in the synthesis of amino acids, particularly those derived from glutamate and glutamine, has been increased through the use of mutant and transgenic plants in which amino acid biosynthesis was altered. Amino acid biosynthesis is also of major importance for cereal growth and productivity (Howarth et al. 2008). Nevertheless, we will not cover it in this review as there are excellent reviews extensively describing the current knowledge of this complex pathway and its regulation (*e.g.* Lea and Azevedo 2007).

Leaf and canopy photosynthesis per unit N

Up to 75% of N in wheat leaves is located in mesophyll cells and is involved in photosynthetic processes, mainly as the chloroplastidic enzyme Rubisco (Evans 1983). Thus, responses in N-limited crops often include reductions in total leaf area, leaf expansion and duration, leaf N and chlorophyll content, leaf stomatal conductance, and photosynthesis per unit of leaf area (Sylvester-Bradley et al. 1990, Monneveux et al. 2005). These responses reduce radiation interception and radiation-use efficiency (above-ground biomass per unit radiation interception; RUE) and hence biomass (Foulkes et al. 2009b) and yield. Canopy and leaf processes affecting photosynthesis per unit of N uptake include: (i) radiation interception per unit of N uptake, (ii) optimizing vertical N distribution in relation to light in the canopy and (iii) leaf photosynthesis per unit of leaf N.

For a radiation interception of 95 %, assuming a light extinction coefficient (K) value of 0.5, a green area index (green canopy area per unit of ground area; GAI) of 6 is required. Indeed,

$$K = - \ln (I / Io) / L$$

where Io is the incident radiation and I is the amount of radiation not intercepted by a canopy having a GAI = L.

At anthesis, modern wheat cultivars produce canopies with GAI values around 6, hence achieve full interception at this stage (*e.g.* Moreau et al. 2012, Gaju et al. 2014). The only realistic way to increase fractional interception in the pre-anthesis phase is to increase fractional interception at the start of the stem-elongation phase. However, in wheat, it is already around 60-70 % (Shearman et al. 2005, Moreau et al. 2012). Thus, only marginal improvement seems possible. Physiological avenues for increasing fractional interception specifically under low N supply may be possible through an increased specific leaf N area (leaf area per unit leaf N; SLN) and/or a higher light extinction coefficient. Genetic variation in SLN has been associated with embryo size (Lopez-Castaneda et al. 1996) and earlier canopy closure (Rebetzke and Richards 1999). The light extinction coefficient is mainly influenced by leaf angle. For modern wheat cultivars, light extinction is approximately 0.55 for photosynthetically active radiation (Thorne et al. 1988, Abbate et al. 1998, Moreau et al. 2012). These values are associated with semi-erect to erect leaf angles which help to reduce light saturation in the upper canopy leaves, boosting RUE. A higher value of K seems unlikely to be desirable due to the trade-off with RUE. Although desirable, more prostrate leaves during early vegetative growth and more upright leaves during later vegetative growth may be difficult to achieve in practice. In summary, although genetic gains in radiation interception per unit of N uptake may be possible during stem elongation, these gains seem likely to be small.

N distribution in canopies in relation to light attenuation also affects photosynthesis per unit of N uptake. Considering that the leaf N gradient is “optimal” in accordance with the “optimization theory” (Field 1983, Hirose and Werger 1987, Anten et al. 1995, Moreau et al. 2012), theoretical studies indicated that leaf N maximizes canopy photosynthesis when it parallels the light gradient, *i.e.* when the light (K_L) and N (K_N) extinction coefficients are equal. In wheat, observed N gradients are generally less steep than predicted with the “optimization theory”, however do demonstrate that SLN follows an exponential gradient with vertical depth in the canopy (Critchley 2001, Pask 2009, Moreau et al. 2012). Possible reasons for this discrepancy have been discussed in detail by Kull (2002). There

is relatively little information on genetic diversity in the vertical distribution of N in relation to light in the canopy. Nevertheless, Berteloot et al. (2008) demonstrated with two French winter wheat cultivars (Apache and Isengrain) that the vertical distribution of N at anthesis was close to the optimum, as defined in the "optimization theory", and only differed significantly at the end of grain filling. Similarly, genetic differences were not found for five spring wheat genotypes grown in the Netherlands (Bindraban 1999). Moreau et al. (2012) analysed the vertical distribution of leaf N and light at anthesis for 16 wheat cultivars experimented in field trials in France and the UK in two seasons under two N levels. The N extinction coefficient with respect to light ($K_N : K_L$) varied with N supply and cultivar. A scaling relationship was observed between ($K_N : K_L$) and the size of the canopy for all the cultivars in the different environmental conditions. Interestingly, the scaling coefficient of the ($K_N : K_L$ - green area) index relationship differed among cultivars, suggesting that cultivars could be more or less adapted to low N environments.

Photosynthesis rate per unit of N affects NUtE. In C3 cereals such as wheat, the net light-saturated rate of leaf photosynthesis (Amax) typically increases to 20-30 μmol $CO_2/m^2/s$ at leaf N concentrations of 2 g N/m^2. Assuming an asymptotic relationship between Amax and leaf N concentration (Evans 1983, Sinclair and Horie 1989), there may be scope to decrease SLN whilst maintaining Amax. Indeed, since leaves of modern wheat genotypes typically accumulate more than 2.0 g N/m^2 under favourable conditions (Critchley 2001, Pask et al. 2012), NUtE could be increased by selecting for lower SLN to decrease the transient "storage" N components of leaves. A sensitivity analysis using the wheat Sirius crop model predicted that decreasing SLN in the range of 1-2 g/m^2 increased NUE by 10-15% when N was limiting (Semenov et al. 2007). However, under well fertilized conditions decreasing SLN below 2 g/m^2 may not be beneficial since the SLN required for maximal RUE in field-grown winter wheat in the UK and New Zealand was estimated to be 2.1 g/m^2 (Pask et al. 2012). Alternatively, increasing SLN above current values of 2-3 g/m^2 seems unlikely to be advantageous overall for NUtE as leaves may operate well below light saturation in the canopy (Reynolds et al. 2000), mesophyll cell size, leaf size and light interception may be reduced (Austin et al. 1982) and many chloroplasts may end up in a light-limited state due to intra-leaf shading in thick leaves. Genetic variability in SLN amounts to 1.4-2.6 g/m^2 for 144 durum wheat genotypes (Araus et al. 1997), 2.1-2.4 g/m^2 for 17 durum wheat

cultivars (Giunta et al. 2002) and 1.4-2.2 g/m^2 for 16 bread wheat cultivars (mean over a high and low N treatment; Moreau et al. 2012). SLN heritability in wheat is largely unknown. However, it is encouraging that the heritability for straw (leaf lamina, leaf sheath and stem) N at anthesis for winter wheat was > 0.60 under low N (Laperche et al. 2006) indicating that selection should be possible.

Rubisco catalyses a wasteful reaction with oxygen that leads to the release of previously fixed CO_2 and NH_3 and the consumption of energy during photorespiration. Consequently, at the metabolic level, there are several avenues to increase photosynthetic efficiency. These include: (i) relaxing the photo-protected state more rapidly, (ii) reducing photorespiration through ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) with decreased oxygenase activity, (iii) improving Rubisco activity, (iv) faster regeneration of ribulose-1,5-bisphosphate (RuBP) and (iv) introducing carbon-concentrating mechanisms associated with C4 photochemistry into C3 plants (see recent reviews by Reynolds et al. 2000, Parry et al. 2003, 2011, Long et al. 2006, Murchie et al. 2009, Zhu et al. 2010). These strategies all require modification of the photosynthetic components, which can only be achieved through genetic manipulation. Potential improvements in C3 cereals available from reduced photorespiration were estimated around 30 % and those from other mechanisms in the 15-22 % range (Long et al. 2006).

Alternatively, it may be possible to increase Amax by decreasing respiration in crops, although this has received less attention than photosynthesis partly due to difficulties in measurement. Respiration may consume 30% to 80% of the carbon fixed (Atkin et al. 2005) and is commonly divided into growth and maintenance components, each exerting differing effects. Respiration, increasing with temperature and depending on phenological stage (McCullough and Hunt 1993, Foulkes and Murchie 2011) may be positively but non-linearly related to photosynthesis. High respiration rates (especially at night) can increase reactive oxygen species, leading to cell damage and affecting pollen viability (Prasad et al. 1999). Recent work highlighting the importance of increased night time temperature with climate change on productivity in wheat (Tester and Langridge 2010, Lizana and Calderini 2013) and the high sensitivity of respiration to temperature in general, suggests that the environmental responses of crop respiration to temperature changes is an important area on which to focus.

Post-anthesis N remobilisation and senescence dynamics

In wheat, 35-42 % of the N in the above-ground crop at anthesis is in the leaf lamina, 14-20 % in the leaf sheath, 20-31 % in the true stem and 16-23 % in the ear, under optimal N supply (Pask et al. 2012, Barraclough et al. 2014, Gaju et al. 2014). Under low N conditions, the proportion of the N in the ear increases relative to that in the other plant components (Barraclough et al. 2014, Gaju et al. 2014). In field experiments in the UK and New Zealand, on winter wheat, the accumulation and remobilisation of structural, photosynthetic and reserve N was estimated in crop components under high N and low N conditions (Pask et al. 2012). At anthesis, reserve N accounted for 44 % of above-ground N in optimally fertilised crops, and was principally located in the true stem, but was observed in all crop components in non-limiting fertiliser N treatments. The efficiency of post-anthesis N remobilisation of true stem reserve N in the true stem was low (48 %) compared to the leaf sheath (61 %) and leaf lamina (76 %), and in well fertilised crops significant quantities of non-remobilized reserve N remained in true stem at harvest.

A high capacity to absorb N in the true stem before flowering could theoretically favour a high maximum rate of N uptake, hence higher NUpE (Foulkes et al. 2009). In addition, favouring a greater capacity to store N in non-photosynthetic organs (*i.e.* stem internodes) may enable the translocation of a larger amount of N to grains without reducing plant photosynthetic capacity (Bertheloot et al. 2008), although the respiratory cost of maintaining a large non photosynthetic pool of storage N is unclear. In wheat, genetic variation in stem N content at anthesis is reported (Triboï and Ollier 1991, Critchley 2001, Pask et al. 2009, Barraclough et al. 2014, Gaju et al. 2014), as well as in post-anthesis N remobilisation efficiency from the stem (Kichey et al. 2007, Pask et al. 2009, Gaju et al. 2014). In maize, studies reported an early remobilisation of N from the stem before the leaf lamina (Beauchamp et al. 1976, Friedrich and Schrader 1979). Thus, high stem N remobilisation efficiency would potentially favour high NUtE through delayed senescence of the leaf lamina.

'Stay-green' *((mixture of " and ' in the manuscript))* phenotype refers to the capacity of a genotype to retain green leaf area for longer than a standard genotype during grain-filling (Thomas and Smart 1993). Although under optimal conditions, wheat crops are in general little limited by the assimilate

supply during grain filling (Dreccer et al. 1997, Borrás et al. 2004, Calderini et al. 2006); under low to moderate N fertiliser levels there is evidence that yields can be limited by post-anthesis assimilate supply (Bogard et al. 2011, Gaju et al 2011). 'Stay-green' phenotypes and broader genetic variation in senescence have been reported in hexaploid wheat (Silva et al. 2000, Verma et al. 2004, Joshi et al. 2007, Christopher et al. 2008, Chen et al. 2010, 2011, Bogard et al. 2011, Gaju et al. 2011, Naruoka et al. 2012, Derkx et al. 2012). N dynamics are an important factor in the maintenance of green leaf area in sorghum, with 'stay-green' in sorghum hybrids linked to changes in the balance between N demand and supply during grain filling resulting in a slower rate of N translocation from the leaves to the grain (Borrell and Hammer 2000, van Oosterom et al. 2010a,b). The latter study showed that the onset and rate of leaf senescence were explained by a supply-demand framework for N dynamics, in which individual grain N demand was sink determined and was initially met through N translocation from the stem and rachis, and then if these N pools were insufficient, from leaf N translocation. A correlation between post-anthesis N remobilisation efficiency and the onset of the rapid phase of canopy senescence was reported under low N conditions amongst 16 wheat varieties grown at sites in the UK and France (Gaju et al. 2014). A transcription factor (*NAM-B1*) accelerates senescence and increases N remobilisation from leaves to grains in wheat (Uauy et al. 2006). Candidate regulatory genes which were members of the WRKY and NAC transcription factor families were related to senescence in controlled environment conditions (Derkx et al 2012). In a winter wheat doubled-haploid mapping population, QTLs affecting leaf senescence and grain yield and/or grain protein concentration were identified associated with QTLs for anthesis date, showing that the phenotypic correlations with leaf senescence were mainly explained by flowering time influencing post-anthesis N availability (Bogard et al. 2011).

These results suggested that a better understanding of the mechanisms determining post-anthesis N remobilisation and senescence associated with environmental characterization, particularly on their N availability during the post-anthesis period, would offer scope to raise grain yield and/or grain protein content in wheat cultivars.

Optimizing grain protein concentration and composition

Structural and metabolic proteins are present in the starchy endosperm cells of the grain, and the predominant protein fraction in this tissue is the gluten storage proteins, comprising a mixture of monomeric gliadins and polymeric glutenins. These groups of proteins are present in approximately equal amounts and together account for about 60-70 % of the total N in the endosperm tissue. The gluten proteins confer viscoelastic properties to dough crucial for processing wheat into baked food such as bread, pasta and noodles. A precise balance of gliadin and glutenin proteins is also required, as glutenins are predominantly responsible for dough elasticity (strength) required for bread-making and gliadins for dough viscosity and extensibility required for making biscuits and cakes. The qualitative composition of the grain protein is a genetic characteristic, caused in part by differences in protein synthetic capacity (Shewry and Halford 2002, Ravel et al. 2009), whilst the rate, duration and grain protein quantitative composition (*i.e.* the ratio between the different protein fractions; Martre et al. 2003) can be modified by environmental conditions.

An inverse relationship exists between the grain protein concentration and grain yield (Kibite and Evans 1984, Simmonds 1995, Oury et al. 2003, Oury et Godin 2007, Bogard et al. 2010), making the simultaneous genetic improvement of yield quantity and bread-making quality a difficult task. The physiological basis of this inverse relationship relates to competition between carbon and N for energy (Munier-Jolain and Salon 2005) and an N dilution effect by carbon based compounds (Acreche and Slafer 2009). The grain protein deviation (GPD) is the deviation from the regression between grain yield and grain protein concentration (GPC). GPD can be used to identify genotypes having higher GPC than expected from their GY (Monaghan et al. 2001) and wheat lines that have a positive GPD amongst groups of wheat lines (Oury et al. 2003, Bogard et al. 2010, 2011). Genetic variability in GPD has been related to post-anthesis N uptake (Kichey et al. 2007, Bogard et al. 2010, 2011) which is in part associated with anthesis date (Bogard et al. 2011). Since the majority of grain N originates from remobilisation (Pask et al. 2012, Gaju et al. 2014), rather than from post-anthesis uptake, mechanisms to enhance reserve N accumulation in the canopy and efficiency of N remobilisation should also be addressed in the genetic improvement of GPD (Hawkesford 2014). This may be the case using the already mentioned *NAM-B1* allele (Uauy et al. 2006) that increases N remobilisation

efficiency. An alternative to develop high quality and N efficient wheat lines is to modify grain protein composition to maintain dough strength and elasticity parameters with a lower GPC. In this sense, Guarda et al. (2004) observed that grain quality of cultivars introduced in Italy from 1900 to 1994 was increased although GPC was decrease.

For wheat grown for feed, distilling *((distillation?))* and biofuel markets (high ratio of starch to protein required), a higher NUtE will be associated with a lower GPC. The minimum GPC reported is in the range 6.8-7.2 % (Martre et al. 2006, Kindred et al. 2008, Bogard et al. 2011), equivalent (assuming a conversion ratio of 5.7 between GPC and grain N %) to 1.2-1.3 % grain N %. It is not certain whether it is possible to decrease the N % below this it appears to be a minimum obligatory, approximately 1.5 % (Sinclair and Amir 1992), for the synthesis of essential amino acids and structural and metabolic proteins *((the start of the sentence needs to be reworded for clarity))*. After which, the synthesis of grain storage proteins typically increases the grain N concentration to 2.1-2.3 % (about 12-13 % protein, typical of milling wheat).

PHENOTYPING FOR NUE

Root phenotyping methods

The lack of high-throughput and large-scale phenotyping methods for root traits remains a bottleneck to gene discovery and selection for such traits in breeding programs (Fiorani and Schurr 2013). ~~Recent~~ *((1995 is not so recent))* Progress in root measurement methodology has enhanced our ability to visualise, quantify and conceptualise root system architecture traits and their relationship to plant productivity (Lynch 1995). However, laboratory screens have focused mainly on seedlings, with seedlings growing on germination paper or in growth pouches (*e.g.* Hund et al. 2009, Bai et al. 2013, Atkinson et al. 2015). Thus, although several screening tests have been designed to generate accurate and robust data from seedlings grown under artificial conditions, these phenotypes have only rarely been extrapolated to field conditions partly because of the pronounced plasticity of root growth and development processes. Laboratory-based methods can be limited in their ability to reproduce field-

like conditions (Passioura 2006, 2010, Poorter et al. 2012). For example, soil-environment × genotype interactions significantly affect the root length of wheat cultivars grown in sandy soil compared to agar plates (Wojciechowski et al. 2009). Encouragingly, seedling root traits based on paper-based germination screens were shown to be linked to mature plant traits such as height and yield in a Savannah × Rialto DH population (Atkinson et al. 2015) and plant height in a Avalon × Cadenza DH population (Bai et al. 2013). At an intermediate scale, the use of soil-filled root-observation chambers (rhizotrons or clear-pot) (e.g. Lobet and Draye 2011, Nagel et al. 2012, Richard et al. 2015) and non-destructive digital imaging techniques offers some promises (Manschadi et al. 2006, 2010), as X-ray computed tomography (Gregory et al. 2003, Lontoc-Roy et al. 2006, Hargreaves et al. 2009, Mooney et al. 2012, Mairhofer et al. 2013), magnetic resonance imaging (Metzner et al. 2015) and mini-rhizotrons (MacFall and Johnson 2012, Lontoc-Roy et al. 2006, Vamerali et al. 2012, Poorter et al. 2012).

Field phenotyping methods for roots in cereals were reviewed by Manske et al. (2002) and Polomski and Kuhn (2002), including the use of rhizotrons, mini-rhizotrons and assessments of root parameters from soil cores (root washing and root counts/image analysis). There are two relatively high-throughput field phenotyping techniques: the core break method (Köpke 1979) and shovelomics (Trachsel et al. 2011). In the core break method, a root auger is used to take soil-root cores from the field, the cores are then broken transversely and the roots on the exposed cross sections counted (Manske 2001). The number of roots visible is then used to estimated root length density and mass from established calibrations. A field study in Australia on a range of genotypes (cultivars, NILs and RILs) by Wasson et al. (2014) indicated that the core-break method can directly identify variation in deep root traits to speed up selection. Shovelomics involves the excavation and visual scoring of crown roots extracted from the field. Results in maize have been shown to be well correlated with total plant depth and root system total length (Trachsel et al. 2011). Finally, soil coring, root washing and scanning has been successful in describing root system architecture traits of adult plants in the field and in controlled environment conditions, and has been widely used as a standard technique to compare new methods against (Metzner et al. 2015). The measurement of the root system architecture traits from images is carried out using appropriate software. The most commonly used is the

commercial WinRHIZO (Regent instruments, Canada) and the public domain ImageJ (Schneider et al. 2012).

The development of methods that measure changes in the root DNA concentration in soil could eliminate the need for separation of roots from soil and permit large-scale phenotyping of root genotypes and responses to environmental stresses in the field (Huang et al. 2013).

Canopy phenotyping methods

A major limitation to improving yield and N stress tolerance in wheat is obtaining high-throughput accurate phenotypes on thousands of breeding lines. Promising technologies for high-throughput field phenotyping include spectral reflectance to estimate biomass, canopy size and N content. Spectral reflectance indices (SRI) are based on the capacity of canopies to absorb and reflect specific wavelengths of solar radiation according to their structural and physiological characteristics. Currently, the most widely applied SRI are based on the relative reflectance in the visible (400–700 nm) and in the near infrared (700–1,100 nm) due to the absorption of light by chlorophyll and associated pigments [e.g. the normalized difference vegetation index (NDVI) (Araus et al. 2001)]. Using ground-based spectroradiometers, SRI have been developed to estimate crop biomass (Babar et al. 2006), green canopy area (Aparicio et al. 2002), leaf chlorophyll (Babar et al. 2006), stay-green (Lopes and Reynolds 2012), grain yield (Gutierrez et al. 2012a, 2012b *((elsewhere, this has been written as 2012a,b))*), and grain protein content (Apan et al. 2006). The recent development of field-portable spectroradiometers measuring wavelengths up to 2,500 nm increases the capacity to phenotype wheat performance under N stress environments. In this sense, associations have been established between SRI measured during grain filling and grain yield and C isotope discrimination of the grain (Lobos et al. 2014). The challenge in the development of such techniques is to reach high-throughput both for data acquisition and processing as well as to derive metrics that are meaningful with regards to canopy structure and function.

Alongside spectral reflectance, promising remote-sensing technologies for field-based phenotyping include chlorophyll fluorescence imaging to measure photosynthesis (Murchie and Lawson 2013,

Romer et al. 2011) and infrared thermometry as a proxy for canopy photosynthesis (Olivares-Villegas et al. 2007, Saint Pierre et al. 2010). To date, the latter has been mainly applied under heat-stressed or water-stressed environments. Another remote-sensing technique that is now being adopted for field-based phenotyping in cereals to survey directly the 3D distribution of canopies is laser imaging detection and ranging (Lidar). This technology provides accurate estimates of crop height, cover, canopy structural properties (Lefsky et al. 2002, Omasa et al. 2007, Hosoi and Omasa, 2009), crop biomass and N content (Eitel et al. 2014). Furthermore, laser scanning coupled with fluorescence has potential to evaluate photosynthetic performance (Romer et al. 2011). Additional techniques relevant to NUE field-based phenotyping are stereo- and colour imaging to determine canopy structure and ear density (Berger et al. 2010) and near infrared spectroscopy to measure protein and N content using calibrations derived from N combustion analyses (White et al. 2012). A full review of the above phenomics technologies is beyond the scope of this article. Fortunately recent reviews of such phenomics methodologies are available (Furbank and Tester 2011, White et al. 2012, Araus and Cairns 2014).

Challenges that can limit the potential of ground-based sensor platforms (*e.g.* tractor-mounted sensors, phenomobiles) include the non-simultaneous measurement of different plots and vibrations resulting from uneven field surfaces. Some of these limitations can be addressed using high resolution and low-altitude aerial platforms such as small unmanned aerial vehicles. The availability of unmanned aerial vehicles has rapidly increased in recent years and several types, ranging from multicopters and helicopters to fixed wing, are now available (Lelong et al. 2008, Zhang and Kovacs 2012, Araus and Cairns 2014). These aerial platforms have an advantage over ground-based sensing platforms in generating surface maps in real time, and measuring plant parameters from several plots at a time. However, high quality camera systems often still exceed the payload *((payload the right word?))* of available drones. Automation of data processing and difficulties in extraction of meaningful parameters are other reasons which presently restrict fast methodological advances. Satellites platforms on the other hand are currently limited by frequency of measurements and spatial resolution.

BREEDING FOR NUE

Estimation of genetic progresses

Grain yield and the N demand to maximize yield evolved simultaneously (Guarda et al. 2004, Sylvester-Bradley and Kindred 2009), leading to an equal NUE of old and recent cultivars at their respective N optimum (Sylvester-Bradley and Kindred 2009). But when old and recent varieties are compared in the same N conditions, a significant genetic improvement of NUE was measured in various studies at different N levels (Table 1).

Ortiz-Monasterio et al. (1997) reported an NUE genetic progress of +0.4-1.1 %/year depending on the N levels in spring CIMMYT varieties cultivated between 1962 and 1985. In the same way, Cormier et al. (2013) estimated genetic progress at +0.30-0.37 %/year between 1985 and 2010 using 195 European elite winter varieties at optimal and sub-optimal N levels. Only Muurinen et al. (2006), studying 17 spring wheat cultivar released between 1901 and 2000, observed a poorly significant genetic improvement of NUE ($P = 0.055$).

NUE is an integrative trait, thus its improvement could be the result of modification on several components. An increase in N harvest index (NHI) was assessed at +0.15 %/year by Brancourt-Hulmel et al. (2003) and at +0.12 %/year by Cormier et al. (2013). This improvement is independent of the semi-dwarf allele introgressions (Gooding et al. 2012) and is associated with a decrease of N content in straw at maturity (Cormier et al. 2013). It may result from a better translocation (portion of N absorbed after anthesis and allocated to the grain) and/or a better N remobilisation. In summary, these results highlighted a breeding impact on N utilisation. An increase in N uptake was also observed (Ortiz-Monasterio et al. 1997, Guarda et al. 2004, Sylvester-Bradley and Kindred 2009). Nevertheless, this conclusion has to be balanced as Foulkes et al. (1998) who studied 27 cultivars released from 1969 to 1988 concluded that at zero N input, N offtake in grain decreased. Moreover, Cormier et al. (2013) could not conclude on this point due to a too low genetic variance for N uptake in a variety panel of 214 recent European elites.

To conclude, both N uptake and N utilisation may have been increased by breeding with a relative efficiency affected by the N levels (Ortiz-Monasterio et al. 1997, Le Gouis et al. 2000). We should

point out that this improvement is an indirect effect of breeding for grain yield at a constant N level as no specific targeted selection for NUE has been conducted.

Impact of G × N interactions on direct/indirect selection efficiency

In wheat, varieties are commonly selected and registered under high N conditions. Thus, genetic progresses in low N condition results from an indirect selection. Numerous studies detected significant G × N interactions for agronomic traits (*e.g.* Ortiz-Monasterio et al. 1997a,b *((idem))*, Le Gouis et al. 2000, Laperche et al. 2006a, Barracough et al. 2010, Cormier et al. 2013) meaning that genetic values of varieties differ between different N levels. Significance of G × N interactions directly affects the correlations of genetic values between different N levels, hence the best varieties at high N may not be the best at low N. In other words, when G × N interactions are significant, indirect selection efficiency (ISE) is reduced. Nevertheless, selecting at high N for low N can be efficient when heritabilities in high N are higher than in low N. Indeed, a balance between the ability to select (heritabilities), and the genetic correlation between the environment used to select and the one where varieties will be tested is required. This balance is easy to understand when we have a look at the ISE formula (Falconer and Mackay 1996):

$$ISE = r_{G12} \times h_2 / h_1$$

where varieties are tested in condition 1 but selected in condition 2, h_1 and h_2 are the respective heritability square roots in the two conditions and r_{G12} the genetic correlation between conditions, considering an equal selection intensity in both conditions.

In wheat, studies reported both genetic variance decrease and environmental variance increase at low N compare to HN. Thus, heritabilities are usually lower under low N conditions (Brancourt-Hulmel et al. 2005, Laperche et al. 2006a), and indirect selection at high N can be an effective strategy to breed for low N conditions. However, few studies directly quantified this indirect selection efficiency

(Brancourt-Hulmel et al. 2005, Przystalski et al. 2008, Annicchiarico et al. 2010, Cormier et al. 2013, Sarcevic et al. 2014). These studies have to be compared regarding N stresses and the number of genotypes used. Using 270 breeding lines tested during two years in the same environment (northern France), Brancourt-Hulmel et al. (2005) assessed an ISE of 0.65-0.99 for grain yield with an N stress which implied a mean yield reduction of 35 % and genetic correlations between 0.83 and 0.89. Cormier et al. (2013) tested 225 commercial varieties. Comparing high N and low N, the mean yield reduction was 20 % and trait heritabilities were stable. Thus, ISE was mainly dependent on genetic correlation. For grain yield it was estimated at 0.78. For the other agronomic traits investigated, ISE was between 0.25 and 0.99. The other studies used fewer genotypes. In Sarcevic et al. (2014), 19 varieties were tested and yield reduction was only 10 %, promoting high genetic correlations. Moreover, genetic correlations were allowed to exceed 1. As results, ISE for grain yield was high (1.04), as for grain N yield (1.34) and most grain quality rheological parameters (0.81-1.00). Using datasets from seven European countries comparing organic and non-organic cropping systems, Przystalski et al. (2008) found an ISE ranging from 0.86 to 1.02 for grain yield (calculated from the published results) under a N stress inducing a mean yield reduction of 27 %. However, this result seems overestimated regarding the unbalanced dataset and the method used. Annichiario et al. (2010) studied three datasets containing 7, 11, and 13 genotypes under two production systems (organic and conventional). Yield reduction ranged from 14 % to 28 % and ISE ranged from 0.89 to 1.20 for grain yield, but there were no consistent genotype × production system interactions, and/or heritabilities in organic system were lower than in conventional system mostly due to higher experimental error.

When dataset size is sufficient to properly estimate genetic correlation and N stress is substantial, ISE for grain yield is high but may not exceed one. Consequently, regarding breeder financial issues, indirect selection is efficient in moderate N stresses but it does not overpass direct selection in low N conditions. This was already observed in maize (*Zea mays*), for which selection under high N for performance under low N was predicted significantly less efficient than direct selection under low N when the relative yield reduction due to N stress exceeded 43 % (Bänziger et al. 1997). Concerning varieties recommendation, the approach is different as the goal is not to increase a trait mean value but to advise wheat growers, hence to predict the top ranking varieties, meaning that we should focus on

variety rankings between high N and low N conditions. Here again, to apply results from high N to low N experiments is not an easy task. Indeed, even with a high genetic correlation between high N and low N conditions, the probability to predict the top varieties in low N from high N ranking is low (0.55 for a genetic correlation of 0.8 in the simulation study of Przystalski et al. (2008)).

Molecular breeding

Molecular breeding can be defined as the use of molecular information to develop new genotypes. This molecular information can arise at different levels of the metabolic process: from genes through proteins to metabolites. In complex traits such as NUE, a lot of regulation pathways at different levels occur (*e.g.* transcription factor, post-transcriptional modification, allosteric regulation). These pathways depend on N levels (Howarth et al. 2008, Ruuska et al. 2008, Wan et al. 2013), organs (Ruuska et al. 2008), genotypes (McIntyre et al. 2011, Tenea et al. 2012), and stage (Ruuska et al. 2008, Wan et al. 2013). In the creation of genetically modified (GM) crop, this complexity makes promoter choice critical. Reviews of transgenic efforts to improve NUE in plant were published by Pathak et al. (2011) and McAllister et al. (2012). Using the example of research on alanine aminotransferase (AlaAT), a successful transgenic approach to increase NUE in oil seed rape (Good et al. 2007) and rice (Shrawat et al. 2008) actually *((currently?))* tested in wheat, they concluded that enzymes and proteins other than those involved in primary N uptake and assimilation may be good targets potentially due to less post-transcriptional controls.

Indeed, it has been believed for a long time that due to their strategic position along the N assimilatory pathway, NR, NiR, GS, and GOGAT enzymes were major checkpoints controlling plant NUE. However, the first results of modifications of these genes had not produced completely relevant NUE phenotypes. Nevertheless, there is some evidence that increasing NR activity improves NO_2^- assimilation in Arabidopsis (Takahashi et al. 2001). Moreover, it seems that wheat genotypes exhibiting a higher NR activity have a greater potential for N utilisation under non-limiting N supply with a well-coordinated system of N uptake and assimilation (Vouillot et al. 1996, Anjana et al. 2011). And recently, it was reported that overexpression of a tobacco NR gene in wheat increased the seed

protein content, without the need for increased N fertilisation (Zhao et al. 2013). Such an interesting finding could rekindle the possibility of using NR as a breeding target to improve wheat NUE, yield and grain quality. Far fewer studies have concerned the enzyme NiR in wheat.

In wheat, indirect evidence of the role of the GS enzyme in the control of NUE was also provided through correlation studies that suggested that the leaf enzyme activity could be used as a marker to monitor plant N status (Kichey et al. 2007). In addition, a number of quantitative trait loci (QTL) related to grain yield and grain protein content co-localizing with structural genes encoding either cytosolic GS1 (Habash et al. 2007, Fontaine et al. 2009, Gadaleta et al. 2014) or plastidic GS2 (Gadaleta et al. 2011, Bordes et al. 2013) were identified. However, functional validation of these candidate genes will be necessary to demonstrate their impact on wheat productivity (Swarbeck et al. 2011). A recent association analysis of one of the genes encoding cytosolic GS (TaGS1a) suggested that the enzyme had an important function in the control of a number of yield-related traits (Guo et al. 2013), as did its plastidic counterpart (Gadaleta et al. 2011).

Following the discovery that in rice mutants deficient in one of the two forms of NADH-GOGAT there was a considerable reduction in spikelet number (see Yamaya and Kusano 2014 for a review), studies on the wheat enzyme were also undertaken. Based on a quantitative genetic study in which colocalization between QTL for NUE and NADH-GOGAT was observed (Quraishi et al. 2011), it was proposed that in wheat and other cereals the gene could be used to improve grain filling either using genetic manipulation, or by selecting the best alleles (Salse et al. 2013). In durum wheat, it was also found that there is a strong correlation between NADH-GOGAT gene expression and grain protein content (Nigro et al 2013), thus indicating that unlike in a C4 plant such as maize (Martin et al. 2006), it is not cytosolic GS1 but NADH-GOGAT that is one of the major checkpoints controlling NUE in C3 cereals. Such a finding reinforces the current concept that NUE may be unique, depending not only on the species examined but also on the genetic variability within the species (Hirel et al. 2007, Simons et al. 2014).

Regarding marker assisted selection, to deal with N pathway complexity of regulation, we may think that the easiest screening would be based on protein or metabolite. Kusano et al. (2011) wrote a good review on metabolic approaches focusing on N metabolism. In wheat, Howarth et al. (2008) assessed

the impact of N supply on amino acid content during senescence. Moreover, various proteomic studies were performed at different growing stages and organs (Bahrman et al. 2004a, 2004b, 2005, Altenbach et al. 2011, Tétard-Jones et al. 2013). Nevertheless, these approaches are limited to the exploration of a narrow genetic diversity (Table 3). In fact, due to affordable cost (time and price), most molecular information available is at the genome level as genetic molecular markers. This information was used in association mapping studies on NUE related traits (Table 4) mostly using biparental design such as doubled haploids (DH) populations (An et al. 2006, Laperche et al. 2006, 2007, 2008, Habash et al. 2007, Fontaine et al. 2009, Li et al. 2010, Zheng et al. 2010, Bogard et al. 2011, 2013) or recombinant inbred line (RIL) populations (Garcia-Suarez et al. 2010, Li et al. 2010, Guo et al. 2012, Sun et al. 2013, Xu et al. 2013). Three studies covered a broader genetic diversity (Li et al. 2010, Bordes et al. 2013, Cormier et al. 2014) using large association panels. Discovering QTL co-localising with known N uptake or assimilation enzymes and new QTL, these studies provided new insights on NUE genetic determinism.

Nevertheless, several difficulties persist to implement this knowledge in breeding, as NUE and its related traits appeared highly polygenic and genetic background specific. Thus, several small locus effects should be pyramided. Moreover, information quantity will raise with the recent development of several wheat SNP arrays (90K, Wang et al. 2014; 420K, 670K, and 820K). Genomic prediction methods may overpass these limitations and facilitate breeding but to now these methods are still at a development stage. Added to that, G × N and more generally of G × E remain a major trade-off in marker assisted selection leading to difficulties to develop new genotypes adapted to a broad range of environments and N levels.

Prospect on new strategy: heterosis

F1 hybrid wheat cultivars have been regularly registered in Central Europe, which represents more than half of the world's hybrid wheat production (Longin et al. 2012). Commercial hybrids may be produced with chemical hybridizing agents, which induce male sterility when applied at the right stage, but also based on photoperiodic sensitivity or on cytoplasmic male sterility. Limits to the use of

F1 hybrids are the cost of the seed, related to the difficulty to produce them on a regular basis coupled with the absence of high heterosis for yield.

However, hybrids may show particular characteristics for abiotic stress tolerance and NUE. Limited but consistent best-parent heterosis have been reported for grain yield under high yielding conditions, *e.g.* +4.3 % for 10 hybrids (Borghi et al. 1988), +7.3 % for 17 hybrids (Brears et al. 1988), +3.6 % for 430 hybrids (Morgan et al. 1989) in experiments conducted in field plots. On average in Europe on five studies, Longin et al. (2012) reported mid-parent heterosis around 10 %, ranging from 3.5% to 15%. It was also reported that the hybrids are more stable than pure lines (Mühleisen et al. 2014) indicating a higher tolerance to abiotic stresses.

Perezin et al. (1992) and Oury et al. (1994, 1995) reported either a higher grain protein content of the hybrids for the same yield or the same protein content despite a higher grain yield. These results tend to indicate a higher NUE and N uptake for hybrids compared to pure lines. Some studies also showed that best parent heterosis was higher at low N level than at high N level (Le Gouis and Pluchard 1996, Le Gouis et al. 2002). This was however not confirmed by Kindred and Gooding (2005) using four commercial hybrid, who observed a significant heterosis only at high N level. Le Gouis et al. (2002) observed a best-parent heterosis for total N at anthesis and harvest meaning a better N uptake, while Kindred and Gooding (2004) reported only little heterosis for total above-ground N but an increased N utilisation efficiency. N uptake mid-parent heterosis at flowering and maturity could be related to a more efficient root system. Indeed, heterosis was shown for different root characteristics such as root length, root dry matter, and root surface area (Kraljevic-Babalic et al. 1988, Wang et al. 2006, Li et al. 2013).

Conclusion

NUE is complex and determined by a wide diversity of physiological traits. Consequently, breeding for enhanced NUE can be achieved through selection on several components. However, compensations and regulations are numerous and dependent on the N regimes, genotypes and stage, leading to difficulties to create efficient NUE phenotypes. Nevertheless, ‘omics and association studies

provided interesting results allowing to prioritize route of improvement. Moreover, the development of high-throughput genotyping and phenotyping methods may accelerate research on a wide diversity.

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Author contributions

Definition of NUE and rationale for its improvement: FC and DG. Root size and morphology: JF. Root N transporter systems: BH. Interaction with micro-organisms: YML. Nitrate assimilation: BH. Leaf and canopy photosynthesis per unit N: JF. Post-anthesis N remobilisation and senescence dynamics: JF. Optimizing grain protein concentration and composition: JF. Phenotyping for NUE: JF. Breeding for NUE: FC and JLG. Coordination of contribution and manuscript editing: FC and JLG.

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((list format needs corrections hereafter to conform to journal style. Would be done more quickly using Endnote))

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TABLES

Table 1: Assessment of yearly percent genetic gain in nitrogen use efficiency (NUE) from direct comparison of old and modern cultivars

Period	Genotypes	N level (kg N/ha)	NUE (%/year)	Reference
1962-1985	8	0	1.2	Ortiz-Monasterio et al. 1997
		75	0.4	
		150	0.6	
		300	0.9	
1977-2007	24	0	0.35	Sylvester-Bradley and Kindred 2009
		200	0.58	
1985-2010	195	150	0.37	Cormier et al. 2013
		250	0.30	

Table 2: Efficiency of selection in high N environment for low N environment (Indirect Selection Efficiency - ISE) regarding yield reduction between high and low N trials

Genotypes	Yield reduction (%)	ISE	Reference
270	35	0.65-0.99	Brancourt-Hulmel et al. 2005
12-188	27	0.86-1.02	Przystalski et al. 2008
225	20	0.78	Cormier et al. 2013
19	10	1.04	Sarcevic et al. 2014

Table 3: List of 'omics studies related to nitrogen use efficiency in wheat

	Reference	Genotypes	N levels	Organs	Stage	Methods	data points
Proteomic	Bahrman et al. 2004a	2 (Arche, Récital)	0; 2; 8; and 20 mg N/ plant/day	Leaf	60 days	2D gel electrophoresis	524 spots
	Bahrman et al. 2004b						541 spots
	Bahrman et al. 2005		0.5 and 3.0 mM NO_3^-	root	2nd node		860 spots
	Altenbach et al. 2011	1 (Butte 86)	0 and 30 mg N/plant/DAP	grain	maturity		54N
	Tétard-Jones et al. 2013	1 (Malacca)	organic, conventional	flag leaf	ear emergence, anthesis, kernel milk stage		111N
Transcriptomic	Ruuska et al. 2008	1 (Janz)	1 mM KNO3 and 2 mM KNO3 + 3 mM Ca(NO3)2	lower leaves and stem, flag leaf, penult internode	anthesis, 9 DPA	cDNA microarray	36,000 sequences
	Howarth et al. 2008	1 (Hereward)	48 and 192 kg N/ha	leaf 2 and 3	senescence	GeneChip Affymetrix	55,052 transcripts
	McIntyre et al. 2011	8 (Seri × Babax population)	0; 44; 60 and 172 kg N/ha	stem	anthesis		
	Tenea et al. 2012	3 (Tommi, Centenaire,	organic, conventional	flag leaf	kernel milk stage		

		Cubus)				
	Wan et al. 2013	6 (Cordiale, Hereward, Istabraq, Malacca, Marksman and Xi 19)	100; 200 and 350 kg N/ha	caryopse	14, 21, 28 and 35 DPA	
Metabolomic	Howarth et al. 2008	1 (Hereward)	48 and 192 kg N/ha	leaf 2 and 3	senescence	Gas chromatography-mass spectrometry

Table 4: List of association mapping studies related to nitrogen use efficiency in wheat. HN: high nitrogen; LN: low nitrogen

Reference	Pop.	Genotypes	Origin	Marker	Map (cM)	Env	Year	Site	Treatment	Traits	QTL

An et al. 2006	DH	120	Hanxuan 10 × Lumai 14	395 (AFLP, SSR, EST)	3,904		2	1	2	LN=HN-150 kg N ha	5	34
Li et al. 2010	Panel +DH +RIL	260 +120 +142	Core collection Hanxuan 10 × Lumai 14 Xiaoyan 54 × Jing 411	3 TaGS2		1	1	1	2	LN HN	5	
Guo et al. 2012	RIL	131	Chuan 35050 × Shannong 483	719 (DArT, SSR, EST)	4,008	12	1	1	12	N, P, K	24	380
Sun et al. 2013						3	1	1	3	NO_3^-/NH_4^+ ratio	8	147
Xu et al. 2013	RIL	182	Xiaoyan 54 × Jing 411	555 (SRR, EST, *Glu* loci)		4	2	1	2	LN HN	14	126
Laperche et al. 2007	DH	222	Arche × Recital	190 (SSR, GLU-1A/1D, Rht-B1, SPA, Fd-gogat-D1, VRN-A1, B1)	2,164	14	2	4	2	LN=HN-100 kg N ha		233
Laperche et al. 2006a	DH	120			2,164	1	1	1			18	32
Laperche et al. 2008	DH	222			2,164	14	2	4	2	LN=HN-100 kg N ha	6	45
Zheng et al. 2010	DH	222		182 SSR	2,164	12	2	3	2	LN HN	4	131
Fontaine et al. 2009	DH	137-221		197 (SSR)	3,285	3	3	1	1		16	148
Habash et al. 2007	DH	91	CS × SQ1	449 (SSR + GS loci)	3,522	1	1	1	1		21	145
Garcia-Suarez et al. 2010	RIL	114	W7984 × Opata85			4	2	1	2	LN=0 ; HN=120 kg N ha	10	138
Bogard et al. 2011	DH	140	Toisondor × 3CF9107	475 (DArT, SSR, SNP)	2344	10	2	5	2	LN=(25-50)%HN	7	140
Bogard et al. 2013	3 DH	80 +80 +140	Toisondor × Quebon CF9107 × Quebon Toisondor × CF9107	741 (DArT, SSR, SNP)	2510	7	2	3	2	LN=25%HN	2	89

Bordes et al. 2013	Panel	196	Core collection	899 (DArT, SSR, SNP)		12	2	3	2	LN=HN-(35–120) kg N	8	54
Cormier et al. 2014	Panel	214	Commercial varieties	23,603 SNP	3,167	8	2	3	2	LN=HN-100 kg N	28	333

FIGURES

Figure 1: Summary of microbial effects.

Figure 2: Main N assimilation pathways in wheat.