Review

Genetic approaches to enhancing nitrogen-use efficiency (NUE) in cereals: challenges and future directions

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Abstract. Over 100 million tonnes of nitrogen (N) fertiliser are applied globally each year to maintain high yields in agricultural crops. The rising price of N fertilisers has made them a major cost for farmers. Inefficient use of N fertiliser leads to substantial environmental problems through contamination of air and water resources and can be a significant economic cost. Consequently, there is considerable need to improve the way N fertiliser is used in farming systems. The efficiency with which crops use applied N fertiliser – the nitrogen-use efficiency (NUE) – is currently quite low for cereals. This is the case in both high yielding environments and lower yielding environments characteristic of cereal growing regions of Australia. Multiple studies have attempted to identify the genetic basis of NUE, but the utility of the results is limited because of the complex nature of the trait and the magnitude of genotype by environment interaction. Transgenic approaches have been applied to improve plant NUE but with limited success, due, in part, to a combination of the complexity of the trait but also due to lack of accurate phenotyping methods. This review documents these two approaches and suggests future directions in improving cereal NUE with a focus on the Australian cereal industry.

Introduction

Nitrogen (N) is generally the main nutritional driver of yield and quality in cereals because it is the mineral nutrient required by plants in the largest amount (Marschner 1995). Over 1 Mt of N fertiliser is applied in Australian agriculture each year with N use a major input cost for Australian cropping; now being at the same level as fuel (ABARES 2013). Nitrogen fertiliser costs are volatile because of the dependence on costs of the fossil fuel used in their production and this is unlikely to change in the near future. Unfortunately the recovery of the applied N is low, with only 33% of the applied N ending up in the grain (Raun and Johnson 1999). A major component of this is due to the poor uptake efficiency of cereals with only 40% of the applied N being taken up by the fertilised crop (Peoples *et al.* 1995; Sylvester-Bradley and Kindred 2009). Use of N fertiliser impacts the environment in several ways. First, greenhouse gas emissions from fertiliser production are significant, as emission of nitrous oxides, which are 300 times more potent as greenhouse gases than carbon dioxide, from unused N in soils account for 23% of the total greenhouse gas emissions from Australian agriculture (Commonwealth of Australia 2015). Second, leaching and run-off of N fertiliser leads to N pollution of groundwater, rivers and oceans. This occurs in high and low rainfall regions because early season waterlogging in dry regions can leach N out of the root zone. Third, the best known negative effects of N pollution are

associated with algal blooms and fish kills, and recent literature suggests that the dramatic increase in reactive nitrogen inputs to the biosphere are of a greater scale than the increase in human derived carbon dioxide inputs and may have similar or larger scale effects on the biosphere (Rockström *et al.* 2009). In response to the known environmental impacts of excess N fertilisation, European countries are introducing systems so that farmers now must account for all applied N fertiliser (Sutton *et al.* 2011).

Nitrogen-use efficiency: defining the goal

The focus of this review is on rain-fed cropping in Australia – a predominantly Mediterranean climate with low productivity, i.e. the average wheat yield <1.5 t ha^{-1} compared with 8.5 t ha^{-1} in well watered production areas such as the UK and northern Europe (FAOSTAT 2014). Although low yielding, this is a very different situation from that in regions such as sub-Saharan Africa where N fertiliser application is low as are yields, although this situation offers some very different challenges (Bänziger *et al.* 1997). This review focusses on the potential to increase nitrogen-use efficiency (NUE) in cereals in water-limited, but relatively high N environments.

NUE defined

NUE is a measure of how applied nitrogen fertiliser is utilised by the plant. The definitions of NUE have recently been reviewed,

and some of those listed by Good *et al.* (2004) are shown in Table 1. The simplest definitions reflect the amount of biomass or grain production per unit of N:

$$NUE = Sw/N, \qquad (1)$$

where Sw is shoot weight and N is N content of shoots (DW), and

$$NUE = Gw/Ns, \qquad (2)$$

where Gw is grain weight and Ns is N supply (g plant^{-1})

Further equations describe the two main components of NUE, the efficiency with which N is taken up from the soil (uptake efficiency) and the efficiency with which N is converted to grain (utilisation efficiency).

$$UPE = Nt/Ns, \qquad (3)$$

where Nt is total N in plant and Ns is N supply (g plant^{-1})

$$UTE = Gw/Nt, \qquad (4)$$

where Gw is grain weight and Nt is total nitrogen in the plant. Perhaps the most practically useful definition of NUE in this context is the physiological efficiency,

$$PE = (Gw_F - Gw_C)/(N_F \text{ uptake} - N_C \text{ uptake}), \qquad (5)$$

where Gw_F is grain weight (fertilised), Gw_C is grain weight (unfertilised), N_F uptake is plant N (fertilised) and N_C is plant N (unfertilised).

Eqn 5 takes into account the crop performance in comparison to an unfertilised control, or between two levels of N supply.

How can NUE be improved? Management versus plant improvement

Agronomy

The low NUE of cereal crops can in part be enhanced through improved N fertiliser management techniques (Keeney 1982; Cassman *et al.* 2002). Much of this improvement can be achieved through better matching of supply to plant demand, split applications being an important example. Better weather predictions will help match N supply to seasonal demand, i.e.

if rainfall that would allow a good finish to the season is predicted then more N can be applied in advance, enabling maximum yield and quality (Hayman *et al.* 2007).

Improving plant potential

Although improved fertiliser practice is an important component of improving NUE, there is great scope for improving plant potential for higher NUE. Historically, low N fertiliser costs have resulted in plant breeding efforts focussed on high yields with sufficient to excessive N levels. It has been suggested that this strategy has resulted in new cultivars with poor NUE when compared with older cultivars (Simmonds 1979; Kamprath *et al.* 1982). This theory has lost traction because of results showing that under low N, more N responsive modern varieties still perform better than historical varieties (Ortiz-Monasterio *et al.* 1997; Tollenaar and Wu 1999; Ding *et al.* 2005; Echarte *et al.* 2008). Although modern cultivars may yield higher than older ones under low N, there is considerable scope to increase yield on reduced N, maintaining yield potential but decreasing the N required doing so.

N nutrition of plants

Forms of nitrogen

The main form of nitrogen fertiliser applied to Australian cereal crops is urea, followed by various ammonium phosphates and ammonium sulfate (ABS 2014). Independent of the type of fertiliser applied, the major sources of N in cropping soils are nitrate (NO_3^-) and ammonium (NH_4^+), with NH_4^+ being ~10% of the NO_3^- concentration (Wolt 1994; Miller *et al.* 2007). Due to its predominance in the soil solution, NO_3^- is the predominant N form taken up by crop plants. The uptake of nutrients by plants is dependent on roots accessing them from the soil. For nutrients with high mobility in the soil, such as NO_3^-, and even for the less mobile NH_4^+, under most conditions root morphology is thought to be of considerably less importance than for nutrients with low soil mobility such as phosphorus (Burns 1980; Robinson and Rorison 1983).

N transport into the root

Nitrate uptake by roots is facilitated by specific transporters belonging to high affinity and low affinity transport systems

Table 1.   Definitions of nitrogen use efficiency (NUE)
Adapted from Good *et al.* (2004)

No.	Term	Formula	Abbreviations	Comments	Ref
1	Nitrogen use efficiency	NUE = Sw/N	Sw, shoot weight; N, N content of shoots (DW)	Reflects increased biomass per unit applied N	Steenbjerg and Jakobsen (1963)
2	Nitrogen use efficiency (grain)	NUE = Gw/Ns	Gw grain weight; Ns, N supply (g per plant)	Reflects increased yield per unit applied N	Moll *et al.* (1982)
3	Uptake efficiency	UPE = Nt/Ns	Nt, total N in plant; Ns, N supply (g per plant)	Measures efficiency of uptake of N into plant	Moll *et al.* (1982)
4	Utilisation efficiency	UTE = Gw/Nt	Gw, grain weight; Nt, total nitrogen in plant	Fraction of N converted to grain	Moll *et al.* (1982)
5	Physiological efficiency	PE = (Gw$_F$ – Gw$_C$)/ (N$_F$ uptake – N$_C$ uptake)	Gw$_F$, grain weight (fertilised); Gw$_C$, grain weight (unfertilised); N$_F$ uptake, plant N (fertilised); N$_C$, plant N (unfertilised)	Measure efficiency of capture of plant N and conversion to grain yield	Craswell and Godwin (1984)

(HATS and LATS respectively), these transporters belonging to the NRT2 and NRT1/NPF families (Crawford and Glass 1998; Forde 2000; Tsay *et al.* 2007; Plett *et al.* 2010*b*; Léran *et al.* 2014). Although NH_4^+ is present in much lower concentrations than NO_3^- it is still an important N source and most plants grow best with a combination of the two N forms (Forde and Clarkson 1999). Ammonium uptake is via transporters belonging to the AMT families (Gazzarrini *et al.* 1999; Loque and von Wiren 2004; Ludewig *et al.* 2007).

Uptake of both NO_3^- and NH_4^+ changes in response to demand, in which plant growth rate has the largest influence (Clement *et al.* 1978; Lemaire and Salette 1984; Clarkson *et al.* 1986). Both NH_4^+ and NO_3^- uptake are subject to tight regulation, both being downregulated by high plant N status (Morgan and Jackson 1988; Henriksen *et al.* 1992; Jackson and Volk 1992; Aslam *et al.* 1993; Garnett *et al.* 2013). The regulation of N uptake seems to be in response to levels of downstream assimilates such as amino acids (Cooper and Clarkson 1989; Miller *et al.* 2008). Although the transporters responsible for NO_3^- and NH_4^+ uptake and some of the basics of the regulation of their transport are known for *Arabidopsis*, relatively little is known about how NO_3^- and NH_4^+ transport is regulated by crops in the field in response to supply and demand (Malagoli *et al.* 2005; Garnett *et al.* 2009; Garnett *et al.* 2013).

N storage and assimilation

Once taken up into the plant, NO_3^- can be either stored or assimilated. Nitrate not assimilated in the roots is loaded into the xylem via an electrochemically downhill process, either via non-specific anion channels or via specific transporters (Lin *et al.* 2008; Feng *et al.* 2011; Yan *et al.* 2011; Chen *et al.* 2012). Once in the shoots, NO_3^- can be assimilated, stored or transported back to the roots via the phloem. The majority of NO_3^- is stored in vacuoles (Miller and Smith 1996). Ammonium is not usually stored within the plant and is assimilated once within the root (Raven and Smith 1976). Nitrate assimilation occurs first via nitrate reductase (NR) to NO_2^- and then via nitrite reductase (NiR) to NH_4^+. Ammonium is then assimilated into organic N via glutamine synthetase and glutamate synthase (GS/GOGAT) (Oaks 1994; Lillo 2008; Thomsen *et al.* 2014). The assimilation of NO_3^- can occur in the roots or shoots and whether root or shoot predominates varies greatly between and within species and on N status and environmental conditions (Smirnoff and Stewart 1985).

Very little N is stored in the plant relative to carbon, the latter of which can be stored in large amounts as water soluble carbohydrates in the stem (van Herwaarden *et al.* 1998*a*). The majority of N within the plant is in proteins, with proteins such as Rubisco making up between 12 and 35% of the N in leaves of C_3 plants (Imai *et al.* 2008). Under N deficiency it is possible for plants to remobilise protein N from older tissue to growing leaves, and to cope with less protein N in younger tissue, but in both cases this leads to reduction in functions such as photosynthetic capacity (Hörtensteiner and Feller 2002).

N transport within the plant

Nitrogen is readily mobile within the plant and there is a constant cycling of N throughout the plant via the xylem and phloem (Cooper and Clarkson 1989). Nitrate is readily transported in both xylem and phloem but very little NH_4^+ is transported through the plant because of its toxicity (Schjoerring *et al.* 2002). The other main forms of N transported in the xylem and phloem are amino acids and amides (Pate 1973).

Remobilisation

The harvest index (HI), i.e., grain yield as a percentage of total biomass at harvest, is a good measure of the efficiency of biomass conversion into harvestable yield and this HI has continuously increased over the last 50 years (Sinclair 1998; Fischer 2011). Nitrogen harvest index (NHI), grain N as a percentage of total plant nitrogen at harvest, is the nitrogen equivalent of the HI. Ideally, as much of the total plant N as possible is catabolised from dying vegetative tissue and transported to the grain; however, there is considerable genetic variation in this trait and even greater variation caused by environmental factors (Barbottin *et al.* 2005).

Between 60 and 95% of grain N in wheat is taken up by the plant before anthesis (Hirel *et al.* 2007). This process results in the majority of N in the grain (51–92%) being derived from remobilisation of N from the vegetative parts of the plant (Van Sanford and Mackown 1987*a*; Barbottin *et al.* 2005; Kichey *et al.* 2007). The extent of remobilisation is dependent on N availability, environmental conditions and genotype and is greatly influenced by the extent of post anthesis N uptake (Moll *et al.* 1982; Borrell *et al.* 2001; Martre *et al.* 2003; Kichey *et al.* 2007).

Approaches to improve NUE

Increasing uptake efficiency

As discussed above there is considerable scope for improvement in the N uptake efficiency (NUpE) (Peoples *et al.* 1995; Sylvester-Bradley and Kindred 2009) and there are several ways this could be achieved.

Increasing uptake capacity

Increased NO_3^- uptake capacity may be achieved through better NO_3^- transporters, more effective regulation of the transport system or better storage and assimilation. Increasing the uptake capacity of roots is not simple because of the tight regulation of N uptake, N taken up surplus to requirements increasing plant N status, which, in turn, leads to feedback regulation and reduction in uptake capacity (Miller *et al.* 2007). There is genetic variability in root N uptake capacity (Dhugga and Waines 1989; Weiland 1989; Ortiz-Monasterio *et al.* 1997; Le Gouis *et al.* 2000) and it may be possible for this to be exploited by breeding programs or the information gained from understanding these differences can be used to direct the targeted manipulation of transport processes. However, Moose and Below (2009) point out that in maize, although yield per unit of N has increased over time in new hybrids through increased utilisation efficiency, uptake per plant has stayed the same, implying that N uptake improvement by conventional breeding approaches has reached its limit. If this limitation is real, then it is justification for non-conventional approaches such as those described later.

Changing root morphology

Due to the mobility of N in the soil, nutrient uptake modelling indicates that root morphology is of less relevance for N uptake than for nutrients such as phosphorus (Burns 1980; Robinson and Rorison 1983). However, given that NO_3^- uptake is dependent on water movement, root morphology would have a greater impact on NO_3^- uptake in drying soils (Garnett and Rebetzke 2013). Apart from drying soils, there are other circumstances where root morphology is of importance to N fertiliser uptake. In deep sands, NO_3^- is readily leached down the soil profile beyond recovery by crops. Plants with rapidly growing deep roots could prevent some of these losses and allow recovery of nutrient and water that would otherwise be inaccessible (Gastal and Lemaire 2002; Liao *et al.* 2004, 2006); however, this comes at a cost of greater carbon allocation to roots.

'Stay green' is a trait whereby plants have delayed senescence allowing for a longer period of photosynthesis (Thomas and Smart 1993; Foulkes *et al.* 2007). In deep soils of the northern Australian wheat cropping zone this stay green characteristic has been related to deep rooted genotypes being able to extract water from deep in the soil profile (Christopher *et al.* 2008). For sorghum and maize this trait was thought to be related to higher specific leaf N, allowing greater N and carbon allocation to root growth (Borrell *et al.* 2001; Worku *et al.* 2007). Remobilisation of N in stay green genotypes can be reduced with reduced senescence, a lower N harvest index, but this is thought to be offset by increased N uptake from the soil during grain filling (Borrell and Hammer 2000). The usefulness of this trait may be limited to these deep soils were crops rely on stored water for growth.

Increasing utilisation efficiency

Modifying specific leaf N

Increasing the photosynthetic N utilisation efficiency (PsNUE) is another approach to improve NUE. By increasing the leaf area index and decreasing the specific leaf N, the radiation use efficiency could be increased (Gastal and Lemaire 2002). The key enzyme involved in carbon fixation is Rubisco and this enzyme makes up a large proportion of leaf N (Imai *et al.* 2008). Rubisco is also involved in photorespiratory losses which can be as high as 20% of total carbon fixation in C_3 plants and also liberates ammonia, which requires re-assimilation (Bauwe *et al.* 2010). Increasing the efficiency of Rubisco should improve PsNUE, and progress towards this goas has been made (Lin *et al.* 2014). In C_4 plants, such as maize, photorespiration is limited by structural and metabolic changes making them much more efficient in terms of carbon and N (Hibberd *et al.* 2008). Increasing PsNUE may have a downside since lower leaf N levels reduce the amount of N available for remobilisation.

Delayed senescence (stay green)

Stay green was introduced above in relation to uptake efficiency. Although the mechanisms are not fully understood, it appears that stay green is not necessarily linked to continued uptake of N and water by roots (Foulkes *et al.* 2009). Stay green can be linked to delayed senescence, which could be beneficial by enabling continued photosynthesis with age, but could be detrimental for NUE if it reduced remobilisation of N to the grain.

Increasing remobilisation efficiency

Nitrogen remobilised from vegetative parts to the grain accounts for between 60 and 92% of grain N (Van Sanford and Mackown 1987*a*; Palta *et al.* 1994; Barbottin *et al.* 2005; Kichey *et al.* 2007). The percentage of N remobilised to the grain is usually lower with high N supply (Moll *et al.* 1982) but is also dependent on the extent of post anthesis N uptake and environment (Borrell *et al.* 2001; Martre *et al.* 2003; Barbottin *et al.* 2005; Kichey *et al.* 2007). The efficiency of N remobilisation has been found to increase with water stress, so is likely linked to a reduced uptake of N from drying soils (Palta *et al.* 1994). The multitude of factors that affect remobilisation makes it a difficult trait to select for. Improved nitrogen content in wheat grains related to the Gpc-B1 locus was found to be due to early senescence regulated by genes from the NAC family of transcription factors (Uauy *et al.* 2006).

For wheat, grain protein is an important determinant of the price a grower gets for their crop. An inverse relationship has been observed between grain protein and yield, with higher yielding crops commonly showing decreased grain protein (Bogard *et al.* 2010). The nature of this relationship is not simple with the strength of the relationship varying considerably between studies and there being a significant genotype × environment interaction (Oury and Godin 2007). Selecting genotypes that deviate from this negative relationship, i.e., those genotypes that maintain protein with high yield, has been suggested as a tool for breeding programs aiming to address this issue (Monaghan *et al.* 2001).

Genetics of NUE

As with all complex traits, identification of important NUE-related genes may be accomplished using a genetic approach. However, as with other complex traits such as drought tolerance, the current understanding of the genetic elements providing NUE in cereal, oilseed and legume crops is quite limited. Though they are not the specific focus of this review, NUE related genetic studies in maize and rice are the most extensive of any crop and will be discussed here as they have potential to inform future work in cereals, oilseeds and legumes.

The mapping of quantitative trait loci (QTL) controlling NUE in crop plants will be useful for several reasons (Moose and Below 2009). First, the loci controlling NUE and its component traits can be discovered. Second, improved map-based cloning techniques makes QTL mapping a useful approach for discovering genes with an important impact on NUE and component traits. The discovery of molecular markers that are tightly linked to NUE loci enables selection for important NUE genes without extensive phenotyping of germplasm with multiple N treatments. Also, knowledge of NUE QTL will be important when incorporating NUE improvement transgenes into multiple genetic backgrounds to ensure maximum trait expression and stability.

Genotypic variation

A crucial first step in any genetic mapping and breeding approach is to identify the existing variation in available germplasm. As NUE is a complex trait, this involves measurement of a variety of individual component traits, the importance of each being variable between environments and crops.

Variation in NUE and related traits has been described in: wheat (Cox *et al*. 1985; Van Sanford and MacKown 1987*b*; Le Gouis and Pluchard 1996; Le Gouis *et al*. 2000); barley (Anbessa *et al*. 2009; Anbessa *et al*. 2010; Beatty *et al*. 2010); legumes (Harrison *et al*. 2004); oilseeds (Grami and Lacroix 1977; Yau and Thurling 1987; Svečnjak and Rengel 2006*a*, 2006*b*); maize (Bertin and Gallais 2000; Hirel *et al*. 2007); rice (Cassman *et al*. 1993; Tirol-Padre *et al*. 1996; Borrell *et al*. 1998; Ladha *et al*. 1998; Mae *et al*. 2006; Samonte *et al*. 2006); and the model plant species *Arabidopsis* (Chardon *et al*. 2010; Masclaux-Daubresse and Chardon 2011).

There are few studies that present data for relative grain yield between low and high N treatments. However, several of the studies above provide NUE calculations (e.g. grain yield per unit of available N) for a set of varieties at low and high N treatments separately. In a recent example, 10 field grown barley lines were evaluated for NUE (kg grain per kg available N) and the highest ranking lines had 20–40% higher NUE than the lowest ranking lines within individual N treatments and trial years (Beatty *et al*. 2010). Similar results have been found in several other studies mentioned above, indicating that there is useful variation within varieties currently grown by producers. However, these measurements can be difficult to interpret as NUE measurements and even rankings of the NUE of varieties often change substantially from year to year. This indicates that other environmental factors influence measurements of NUE in field trials and may mean future variety evaluations will need to occur in more carefully controlled conditions.

If there was no variability in elite germplasm it would be difficult to improve NUE via conventional breeding. It has been suggested that decades of breeding for yield without N limitation has resulted in N responsive plants that are poorly adapted to low N availability. However, studies in wheat and maize have shown that modern, N responsive germplasm out yield older less N responsive germplasm even under low N conditions (Hirel *et al*. 2007; Moose and Below 2009). As discussed above, there appears to be genetic diversity for NUE in elite germplasm; however, with such a complex trait and substantial environmental interactions, this makes the genetic components of NUE difficult to dissect.

Exotic germplasm may be useful in better understanding particular aspects of NUE and this knowledge could be utilised in designing breeding goals. The genetic variation available in exotic germplasm may also be useful in the genetic improvement of NUE and component traits. Exotic germplasm has not been extensively evaluated for NUE or component traits, partially because it is difficult to accurately measure and compare NUE in material with a broad range of growth habits and phenologies. This approach is being successfully used in maize breeding for a variety of traits and is being utilised in combination with association mapping studies (McMullen *et al*. 2009), which suggests there is promise for using exotic germplasm in NUE genetic studies and improvement strategies in small grained cereals.

QTL mapping studies

A variety of traits contributing to NUE have been evaluated in multiple populations and these studies are summarised in Table 2. In wheat and maize there are individual populations that have been most extensively focussed on. The wheat RIL population Arche × Recital has been characterised for a large variety of traits in both glasshouse and field studies (Laperche *et al*. 2006, 2007; Fontaine *et al*. 2009). The maize RIL population F2 × Io population has been used for multiple studies and the QTL for agronomic traits identified in older studies have been compared with newly identified QTL for biochemical traits to search for co-localisation of traits (Bertin and Gallais 2001; Hirel *et al*. 2001; Limami *et al*. 2002; Gallais and Hirel 2004; Coque *et al*. 2006; Coque and Gallais 2006). This appears to be a good approach to maximise the data extractable from each population, but it is likely that further study is needed in separate populations to identify further important NUE QTL and genes.

When the studies have provided candidate genes located beneath QTL the candidate genes are often responsible for controlling photoperiod (Ppd-A1 and Ppd-B1), dwarfing (Rht-B1 and Rht-12) and vernalisation (Vrn-A1 and Vrn-D1). This was especially highlighted in a recent meta-QTL analysis which analysed the data from three previous mapping studies in wheat and showed these candidate genes under the 11 meta-QTL identified (Quraishi *et al*. 2011). These results are not surprising considering these traits control developmental time, thus affecting the time for the crop to uptake and assimilate N.

Another class of candidate genes discussed in several mapping studies are those encoding enzymes related to the assimilation and remobilisation of N. A large number of QTL identified across species have genes encoding glutamine synthetase (GS), glutamate synthase (GOGAT) or NO_3^- reductase (NR) located within the QTL interval. This suggests that these genes are important for NUE and may be suitable targets for further breeding and transgenic approaches to improving NUE. However, the QTL associated with these genes encoding enzymes are often extremely wide with small effects, thus, fine mapping studies and ultimately transgenic manipulation of the genes would be required to strengthen this hypothesis. Additionally, a recent study of the activity of several N assimilation enzymes in the maize B73 × Mo17 IBM population showed that only three of 81 identified QTL were *cis*-QTL, meaning the gene encoding the enzyme was located beneath the activity QTL (Zhang *et al*. 2010). This suggests that genes encoding regulatory proteins are more important for the actual activity of the N assimilation or remobilisation enzyme than the gene encoding the enzyme itself, thus regulatory proteins may be better targets for improving NUE through breeding and transgenic approaches.

The most common definition of NUE involves a relative measurement of growth traits or yield at low versus high N provision (physiological NUE). Surprisingly there are few studies that have mapped QTL for this type of relative measurement. Approximately half of the NUE QTL studies have been undertaken at one N level and many of the others have been undertaken with a combination of 'normal' N provision and 'no' N provision. It is difficult to compare between studies utilising 'no' N treatments as European soils without added fertiliser often contain N levels comparable to those found in fertilised Australian soils. Two studies from rice that mapped QTL for NUE compared between low and high N provision in rice (Lian *et al*. 2005; Feng *et al*. 2010) and more of this type of experiment would be useful to identify QTL for NUE. The

Table 2. Summary of studies that have identified QTL for traits associated with NUE

Abbreviations: DH, doubled haploid; DW, dry weight; RIL, recombinant inbred line; NR, nitrate reductase; GS, glutamine synthetase; GDH, glutamate dehydrogenase; IRIL, internated recombinant inbred line; GOGAT, glutamate synthase; GS, glutamate synthase; CSSL, chromosome segment substitution lines

Crop	Population	Environment	Population size	N treatment	Traits	QTL number	Variation explained (%)	Reference
Winter wheat	Hanxuan10 × Lumai14	Field and hydroponics	120 DH	Low, high	N uptake, DW, total N	34	4.3–21.9	An et al. (2006)
Wheat	Chinese Spring × SQ1	Glasshouse	95 DH	Normal	Leaf enzyme activities, agronomic traits, tissue N	164	2.7–30.5	Habash et al. (2007)
Wheat	Arche × Recital	Field	241 DH	Low, high	Yield, agronomic traits, N/protein content	79	3.3–33.0	Laperche et al. (2007)
Wheat	3 populations above	In silico	N/A	N/A	Meta QTL	11	N/A	Quraishi et al. (2011)
Winter wheat	Arche × Recital	Field	241 DH	Normal	Leaf enzyme activities, amino acids, N/protein content, agronomic traits	155	4.8–31.9	Fontaine et al. (2009)
Wheat	Arche × Recital	Glasshouse	120 DH	Low	Root architecture traits, DW	32	8.6–39.0	Laperche et al. (2006)
Wheat	Langdon × DIC6B	Field and glasshouse	N/A	N/A	Grain protein, zinc, iron content and senescence	1	NAC gene identified[A]	Uauy et al. (2006)
Wheat	Xiaoyan 54 × Jing 411	Field	182 RIL	Low, normal	Grain yield, yield components, tissue N, calculated efficiency parameters	117	1.6–35.2	Xu et al. (2014)
Wheat	Chuan 35050 × Shannong 483	Hydroponic	131 RIL	Low, middle, high	Root traits, DW, tissue N, calculated NUE	197	6.2–27.3	Wei et al. (2012)
Barley	Lewis × Karl	Field	146 RIL	Normal	Tissue N/protein contents, agronomic traits, yield	51	5.8–45.9	Mickelson et al. (2003)
Barley	Blenheim × Kym	Field	99 DH	Normal	Grain carbohydrate/N	16	N/A	Bezant et al. (1997)
Barley	H. vulgare ssp. sponteneum × Scarlett	Field	301 DH	Low, high	Agronomic and yield traits	82	0.1–56.1	Saal et al. (2011)
Barley	Lewis × Karl	Field	146 RIL	Normal	Peptidase activities	20	8.4–20.8	Yang et al. (2004)
Pea	Terese × K586	Field	139 RIL	Low	Agronomic and yield traits, tissue N content	117	8.0–90.0	Burstin et al. (2007)
Pea	Cameor × Ballet	Both	207 RIL	Low	Root/nodule traits, N accumulation/content traits	178	4.6–45.1	Bourion et al. (2010)
Medicago	Jemalong A17 × DZA315.16	Glasshouse	175 RIL	Low, high	Biomass, tissue N, calculated efficiency variables	34	6.0–52.0	Moreau et al. (2012)
Arabidopsis	Col-0 × Ler	Glasshouse	99 RIL	High	Root length, aerial mass, root mass on three N types	19	N/A	Rauh et al. (2002)
Arabidopsis	Bay-0 × Shah	Glasshouse	415 RIL	Low, high	Water, NO_3^-, chloride, PO_4^- content	42	2.0–21.0	Loudet et al. (2003a)
Arabidopsis	Bay-0 × Shah	Glasshouse	415 RIL	Low, high	Shoot dry matter, total N/ NO_3^-/amino acids	84	2.0–21.0	Loudet et al. (2003b)
Maize	B73 × G79	Field	214 RIL	Very low, high	Agronomic and yield traits	44	7.1–18.1	Agrama et al. (1999)
Maize	F2 × Io	Field	99 RIL	Low, high	Agronomic and yield traits	29	7.0–48.0[B]	Hirel et al. (2001)
Maize		Glasshouse	77 RIL	Low	Leaf NO_3^- content, NR and GS activities	13	28.0–52.0[B]	
Maize	F2 × Io	Field	99 RIL	Low, high	Tissue N content, yield components, N remobilisation	67	9.2–16.4	Gallais and Hirel (2004)
Maize		Glasshouse	77 RIL	Low	Leaf NO_3^- content, NR, GS and GDH activities	16	28.0–52.5[B]	

Species	Cross/population	Environment	Population	N treatment	Trait	No.	% variation	Reference
Maize	B73 × Mo17	Glasshouse	94 IRIL	Normal	10 leaf enzyme activities, shoot biomass	88	3.4–24.2	Zhang et al. (2010)
Maize	F2 × Io	Field	99 RIL	Low, high	Agronomic and yield component traits	62	9.0–23.3	Coque and Gallais (2006)
Maize	Ac7643S5 × Ac7729/TZSRWS5	Field	240 $F_{2:3}$	Low, high	Agronomic and yield components	72	0.4–21.3	Ribaut et al. (2007)
Maize	Z3 × 87–1	Glasshouse	94 RIL	Low, high	Root traits	23	11.0–43.7	Liu et al. (2008)
Maize	F2 × Io	Glasshouse	140 RIL	N/A	Seed germination and GS activity	9	7.3–18.2[B]	Limami et al. (2002)
Maize	F2 × Io	Field	53 RIL	Low, high	Tissue ^{15}N content	33	9.0–21.9	Coque et al. (2006)
Maize	Huang-C × Xu178	Field	213 $F_{2:3}$	Low, high	Stover nutrient/fibre/protein content	28	7.4–23.6	Xie et al. (2009)
Maize	Ye478 × Wu312	Field	218 RIL	Low, normal	Grain yield, leaf traits, flowering time, anthesis – silking interval	61	5.2–21.9	Cai et al. (2012)
Maize	F2 × Io	Field	100 RIL	Normal	Kernel and cob enzyme activities and amino acids	33	9.0–31.0	Cañas et al. (2012)
Maize	Ye478 × 9 inbred lines	Field	74 RIL	Low, normal	Yield and yield components	42	4.0–29.2	Liu et al. (2012)
Sugarcane	Q165 × IJ76–514	Glasshouse	168 F1	Low, high	DW, tissue N, GS activity, protein, internal NUE	281	3.0–19.0	Whan et al. (2010)
Rice	93–11 × Nipponbare	Hydroponic	119 RIL	Low, high	Plant height, root length, DW	44	5.5–33.2	Zhao et al. (2014)
Rice	Zhenshan 97 × Minggui 63	Field	127 RIL	Low, normal	Grain yield, biomass, tissue N, calculated efficiency	30	4.0–16.6	Wei et al. (2012)
Rice	Zhenshan 97 × HR95	Field	188 RIL	Low, medium, high	Grain yield and component traits	57	0.8–23.1	Tong et al. (2011)
Rice	Zhenshan97 × Minghui63	Glasshouse	239 RIL	Very low, normal	Root/shoot/plant weight; relative weight	52	1.2–17.5	Lian et al. (2005)
Rice	R9308 × Xieqingzao B	Glasshouse	238 RIL	Low, normal	Relative DW, root length, shoot height	7	9.1–14.5	Feng et al. (2010)
Rice	Nipponbare × Kasalath	Glasshouse	98 RIL	Normal	Leaf GS and GOGAT protein content	13	4.4–7.7	Obara et al. (2001)
Rice	Dasanbyeo × TR22183	Field	166 RIL	Low, normal	Tissue N content, yield component traits	78	6.9–32.1	Cho et al. (2007)
Rice	IR64 × Azucena	Glasshouse	82 DH	Low, normal, high	Agronomic and yield component traits	16	15.7–50.3	Senthilvel et al. (2008)
Rice	Koshihikari × Kasalath	Glasshouse	38 CSSL	Low, high	Seminal root length	9	N/A	Obara et al. (2010)
Rice	Taichung 65 × IRGC 104038	Glasshouse	161 RIL	Low, high	Seminal root length	8	7.5–19.4	Obara et al. (2011)

[A]QTL was identified in earlier studies in multiple environments and populations, the QTL was fine mapped and a single NAC gene was identified.
[B]Total variation explained by all QTL detected for a trait.

inherent difficulty in accurately measuring NUE traits in the glasshouse and field may be compounded when comparing relative trait values at low and high N rendering the relative values (and their associated QTL) prone to large errors. Most of the studies summarised in Table 2 describe large numbers of QTL for each measured trait and often these QTL do not appear in multiple studies.

Reproducibility of QTL mapping and the ability to fine map QTL and ultimately the cloning of the gene beneath the identified QTL depends on the quality of the phenotyping (in addition to the quality of the genetic resources). Very few genes have been identified as being responsible for NUE QTL. One such example is a NAC transcription factor gene identified from a population derived from wild emmer wheat and durum. The transcription factor is responsible for delayed senescence in wheat and also for the control of the grain protein, zinc and iron content (Uauy *et al.* 2006). Although the authors speculate the increased content is due to increased remobilisation of N, zinc and iron to the grain, it would require further analysis of N uptake and remobilisation to determine if this speculation is accurate. Regardless, cloning of genes underlying QTL will require improved phenotyping methods to improve repeatability of QTL identification as well as improved fine mapping. This will also allow development of perfect molecular markers for important NUE genes in breeding programs as well as the cloning of these genes for introduction into other germplasm through transgenic approaches.

Utilising genetic variation to improve NUE

Current QTL data are of limited use for improving NUE in varieties developed for Australian cropping. This is because it is a complex trait with a large number of QTL with little overlap between studies, and also because most studies have been carried out under conditions not relevant to the unique conditions of Mediterranean environments. It is essential to begin a dissection of the genetics of NUE relevant to the Australian cropping zone using appropriate mapping populations. Progress in this may be enhanced by conducting mapping studies in controlled environments in parallel with field-based studies.

In addition to the development of refined and high-throughput phenotyping of varieties and populations suitable for Australian cropping environments, efforts to improve NUE in crop plants will need to be underpinned by advances in platform technologies, especially biotechnology. Collins *et al.* (2008) identified several factors that will facilitate the identification and cloning of QTL. First, improvement of molecular platforms including single nucleotide polymorphism profiling, 'omics' profiling, tiling arrays and mapping approaches, such as association mapping (Rafalski 2010). Second, the development of new types of genetic materials and techniques, including multiparental approaches such as multiparent advance generation intercross (MAGIC) populations (Huang *et al.* 2012; Sannemann *et al.* 2015) and nested association mapping (NAM) populations (Yu *et al.* 2008); third, progress in bioinformatics will improve functional maps and comparative mapping; and finally, advances in sequencing technologies (e.g. genome and transcriptome), tools for functionally characterising genes, such as TILLING and RNA interference. Genome wide association studies (GWAS) may help with identifying genes

important to NUE by allowing greater analysis of diversity within a species, especially when combined with improved phenotyping methods (Heffner *et al.* 2009; Poland *et al.* 2012; Charmet *et al.* 2014; Cooper *et al.* 2014). The development and implementation of new and improved technologies will increase the rate of progress in genetic improvement of NUE of polypoloid crop species with large and complex genomes such as wheat.

Transgenics approaches to improving NUE

Compared with the conventional genetic approach, gene modification by transgenic approaches allows the introduction of a single gene without affecting the majority of the genome. This approach has been successfully applied to introduce valuable agronomic traits such as disease, pest or herbicide resistances into crops, and the GM crops carrying these traits have been cultivated in 22 countries, with large areas in the USA and Canada but also now in Argentina, Brazil, China and India (FAOSTAT 2014). Improving NUE by biotechnological approaches is also major priority for plant researchers. Although there is no NUE technology ready in the market, industrial parties have been investing substantially in NUE due to its huge potential. Good *et al.* (2004), Brauer *et al.* (2011) and McAllister *et al.* (2012) have presented comprehensive summaries of NUE and the molecular physiology approaches for improving it. Herein, we update the current biotechnological activities based on publically available information and further highlight some potential approaches which could be applied to improve NUE in crops in future.

Current approaches

Glutamine synthetase (GS) is the most studied gene aiming to improve NUE both in monocot and dicot plants (Table 3). In some cases transgenic lines overexpressing GS displayed improvements in NUE, resulting in increased biomass and grain yield. However, dicot crops studies often failed to show any improved phenotypes. The different results may be due to the unfavourable match of recipient and donor species of the gene or the promoter choice (Good *et al.* 2004; Brauer *et al.* 2011). Negative results may also have been related to lack of knowledge of the complexity of GS1 and GS2 isoforms and the roles and distribution within the plant (Thomsen *et al.* 2014). Good *et al.* have developed a technology using alanine aminotransferase (AlaAT) (Good *et al.* 2007; Shrawat *et al.* 2008). When barley AlaAT was expressed under control of a stress inducible promoter *btg26* or *OsAnt1* in canola or rice, respectively, the transgenic lines showed significant improvement in NUE under relatively low nitrogen conditions. In their field studies the transgenic canola required 40% less N fertiliser than wild type plants to achieve the highest yield potential (Good *et al.* 2007). The same trend was also observed in rice glasshouse studies, although the reduction of N fertiliser (by 12%) was not as great as the case for canola (Shrawat *et al.* 2008). The AlaAT technology is currently the most advanced NUE technology both in monocot and dicot plants but the biological mechanism behind the technology is still poorly understood.

Glutamate dehydrogenase (GDH) is also an potential candidate gene for NUE. There are two publications describing transgenic approaches to constitutively express GDH in tobacco and rice aiming to improve NUE (Ameziane *et al.* 2000; Abiko

Table 3. Biotech approaches – List of genes used in transgenic approaches to improve nitrogen use efficiency gene list

Gene	Function	Source	Background	Promoter	Phenotype	Ref
N uptake						
NRT1.1 (NPF 6.3, CHL1)	Nitrate transporter	Arabidopsis	Arabidopsis	CaMV 35S	Nitrate uptake	Liu et al. (1999)
PTR6	Peptide transporter	Rice	Rice	Ubiquitin	Increased plant growth	Fan et al. (2014)
NRT2.1	Nitrate transporter	N. plumbaginifolia	N. tabaccum	CaMV 35S rolD	Nitrate uptake, content	Fraisier et al. (2000)
NRT3.1 (NAR2.1)	Nitrate transport component	Arabidopsis	Arabidopsis nrt3.1 mutants	CaMV 35S	Nitrate uptake	Okamoto et al. (2006)
AMT1.1	Ammonium transporter	Arabidopsis (Q57H)	Xenopus oocytes		Increased ammonium uptake	Loque et al. (2009)
		Rice	Rice	Ubiquitin	Increased ammonium uptake, reduced biomass	Kumar et al. (2006)
		Rice	Rice	Ubiquitin	Increased ammonium uptake and seed yield	Ranathunge et al. (2014)
AMT2.1	Ammonium transporter	Rice	Yeast		Ammonium uptake	Suenaga et al. (2003)
N assimilation/enzymes						
Nia1 (NR1)	Nitrate reductase	N. tabaccum	Lactus sative	CaMV 35S	Nitrate content, chlorate sensitivity, nitrate levels	Lillo et al. (2003)
		N. tabaccum (Ser521 mutation)	N. plumbaginifolia	CaMV 35S	NR activity, nitrate accumulation	Lillo et al. (2003)
Nia2 (NR2)	Nitrate reductase	N. tabaccum	Potato	CaMV 35S	Reduced nitrate levels	Djennane et al. (2002a); Djennane et al. (2002b)
NiR	Nitrite reductase	N. tabaccum	N. tabaccum, Arabidopsis	CaMV 35S	NiR activity	Crete et al. (1997)
GS1	Glutamine synthetase (cytosolic)	Spinach	Arabidopsis	CaMV 35S	NO_2^- assimilation	Takahashi et al. (2001)
		Soybean	Lotus	CaMV 35S	Decreased ammonium uptake	Vincent et al. (1997)
		Bean	Wheat	rbcS	Increased N uptake	Habash et al. (2001)
		N. tabaccum	N. tabaccum	CaMV 35S	Increased biomass	Fuentes et al. (2001)
		Pea	N. tabaccum	CaMV 35S	Increased biomass, protein	Oliveira et al. (2002)
		Pine	Poplar	CaMV 35S	Increased biomass	Man et al. (2005)
		Soybean	Pea	CaMV 35S, LBC3 (nodule), rolD(root)	Higher GS activity in some lines	Fei et al. (2003)
		Soybean	Pea	LBC3(nodule), rolD(root)	Increased biomass in some rolD lines	Fei et al. (2006)
		Maize	Maize	pCsVMV (OX)	Increased kernel number	Martin et al. (2006)
		Rice	Rice	CaMV 35S	Increased N, decreased seed yield	Cai et al. (2009)
		Rice	Rice	Ubiquitin	Enhanced NHI and UtE in growth camber	Brauer et al. (2011)
GS2	Glutamine synthetase (plastidic)	Rice	Rice	Soybean Rubisco	Photorespiration capacity up	Hoshida et al. (2000)
		N. tabaccum	N. tabaccum		Increased growth rate	Migge et al. (2000)
GOGAT	Glutamate synthase	N. tabaccum	N. tabaccum	CaMV 35S	Increased biomass	Chichkova et al. (2001)

Table 3. (continued)

Gene	Function	Source	Background	Promoter	Phenotype	Ref
AlaAT	Aminotransferase	Rice (Japonica)	Rice (Indica)	GOGAT (Japonica rice)	Increased grain weight	Yamaya et al. (2002)
		Barley	Canola	BnBtg26	Increased biomass and seed yield	Good et al. (2007)
			Rice	OsAnt1	Increased biomass and seed yield	Shrawat et al. (2008)
AspAT	Aspartate aminotransferase	Panicum miliaceum	N. tabaccum	CaMV 35S	Enzyme activity, PEPC activity	Sentoku et al. (2000)
GDHA	Glutamate dehydrogenase	E.coli	N. tabaccum	CaMV 35S	Increased plant biomass, DW, yield in field	Ameziane et al. (2000)
		Aspergillus	Rice	CaMV 35S	Increased DW, N, yield in field	Abiko et al. (2010)
ASN1	Asparagine synthetase	Arabidopsis	Arabidopsis	CaMV 35S	Enhanced N status in seeds	Lam et al. (2003)
GluR2	Glutamate receptor	Arabidopsis	Arabidopsis	CaMV 35S	Reduced growth rate	Kim et al. (2001)
NAGK	N-acetyl glutamate kinase	Brevibacillus laterosporus	Maize	Scubi4	Improved N usage	US Patent# 08692070
VP	Vacuolar H^+-pyrophosphatase	Wheat	Tobacco	CaMV 35S	Improved NUE, PUE	Li et al. (2014)
Transcription factor/regulatory						
ANR1	Transcription factor	Arabidopsis	Arabidopsis	CaMV 35S	Root length	Zhang and Forde (1998)
DOF1	Transcription factor	Zea mays	Arabidopsis	CaMV 35S	Nitrogen content 30% up, growth rate up under low N, reduced glucose level	Yanagisawa et al. (2004)
DOF1, GS1, GS2		Arabidopsis	Rice	Ubiquitin	Increased biomass, modulation of C and N metabolites	Baker et al. (1994); Kurai et al. (2011)
		Arabidopsis	Tobacco	PrbcS	Enhanced N metabolism	Wang et al. (2013)
GLB1	Regulatory protein	Arabidopsis	Arabidopsis	CaMV 35S	N and C sensing	Hsieh et al. (1998)
ENOD93–1	Early nodulin	Rice	Rice	Ubiquitin	Increased shoot biomass and seed yield	Bi et al. (2009)
At3 g21150	B-box domain protein 32	Arabidopsis	Arabidopsis	CaMV 35S	Altered C/N sensing, increased low N tolerance	US patent application no. 2008/0155706
AGL21 (At4 g37940)	Transcription factor	Arabidopsis	Arabidopsis	CaMV 35S	Tolerance to low N	US patent no. 7692067
YRP	Yield related protein				Increased yield	US patent application no. 2011/0010800
ATHB17	Homeobox-leucine zipper protein	Arabidopsis	Maize	Rice actin	Increased ear size	US patent no. 08895818 Rice et al. (2014)

et al. 2010). In both cases the transgenic plants showed increased biomass and seed yield in the field. Interestingly GDH was cloned from *Escherichia coli* or *Aspergillus*, but not from plants in either report. When *Nicotiana plumbaginifolia* GDH was expressed in tobacco, biomass was decreased (Terce-Laforgue *et al.* 2013).

DNA binding with one finger (DOF1) is one of the few examples of transcription factors used to improve N metabolism in plants. When DOF1 was overexpressed in *Arabidopsis*, the transgenic lines showed modification in N and C metabolism, and better growth under low N conditions (Yanagisawa *et al.* 2004). Similarly DOF1 overexpressing rice lines also displayed increased biomass and enhancement in N and C metabolism (Kurai *et al.* 2011). When a rice early nodulation gene OsENOD93–1 was overexpressed in rice, the transgenic lines showed improved NUE including biomass and seed yield increase under both N-limiting and non-limiting conditions (Bi *et al.* 2009). AGL21, a member of the MADS-box transcription factor family seems to be involved in lateral root formation, overexpressed lines produced more lateral roots than wild type *Arabidopsis* (Yu *et al.* 2014). This gene is also patented for the purpose of improvement of NUE.

Nitrogen transporters and other N assimilatory enzymes are well characterised as to their function. However, several attempts to improve NUE by manipulating these genes gave mixed results; especially no positive outcomes were observed with N transporter studies (Table 3). Most of the previous studies used ectopic promoters such as CMV35S to drive a gene of interest, and this might be the reason for the negative impact on NUE (Table 3). One approach to overcome the problem is to use tissue specific promoters and/or inducible promoters to target expression to when and where the gene is needed (Table 4). For example, salt tolerance was enhanced in *Arabidopsis* and rice by manipulating the expression of some key sodium transporters in the root in a cell specific manner (Moller *et al.* 2009; Plett *et al.* 2010*a*).

Longer term approaches

Because C_4 photosynthesis is 50% more efficient in plants than C_3, engineering C_4 photosynthetic system into C_3 plants became a plant scientists' big dream because plants can continue to photosynthesise when stomata are closed such as under water limitation. C_4 plants can fix CO_2 more efficiently with less Rubisco compared with C_3 plants. Therefore, in theory engineered C_3 crops with a C_4 system should also show traits of improved water and nitrogen use efficiency (Brutnell *et al.* 2010). Rice is probably the most studied crop in this regard (Hibberd *et al.* 2008; Taniguchi *et al.* 2008; Miyao *et al.* 2011). For example, four enzymes from C_4 pathway were introduced and successfully overproduced in the rice mesophyll cells, although no transgenic rice plants showed an improvement in photosynthesis compared with the control plants (Miyao *et al.* 2011). Also the International Rice Research Institute (IRRI) and associated partners have initiated a project to engineer C_4 rice (http://irri.org/c4rice, accessed 15 May 2015), demonstrating the potential of the technology.

Although it has been long known that N-fixation in non-legume plants by nodulated roots or associated with N-fixing nodule independent bacteria (Bond and Gardner 1957; Beatty and

Good 2011), agronomic research on this topic has been advanced more in the last decade (Bhattacharjee *et al.* 2008). When maize and wheat were inoculated with an N-fixing bacterium, *Klebsiella pneumonia* 342, the inoculated plants were rescued from N deficiency symptoms (Riggs *et al.* 2001; Iniguez *et al.* 2004), and the wheat plants also showed increased total N content (Iniguez *et al.* 2004). Another N-fixing bacterium isolated from sugarcane, *Gluconacetobacter diazotrophicus* was able to enhance growth of the inoculated sugarcane displaying increased biomass and N content which were equivalent with the plants fed with fertiliser at rate of 140 kg N ha^{-1} (Muthukumarasamy *et al.* 2006). Developing N-fixing crops with root nodule symbiosis has also been attempted from both N-fixing bacteria and non-legume host crop plants (Charpentier and Oldroyd 2010). There are several major issues to be solved before the technology will be broadly applied (e.g. host specificity of N-fixing bacteria, and insufficient carbon traffic between host plants and bacteria). However, this technology certainly has the potential to improve NUE in non-legume crops.

Cisgenesis is a term for gene modification of a new combination of promoter, gene of interest and terminator from the same species or sexually compatible species as used for conventional breeding. Cisgenic plants could contain a single or more cisgenes, but should not contain a transgene. The US Environment Protection Agency (USEPA) has been considering changing regulation of cisgenic plants to make them exempt from the regulatory process (Waltz 2011). The idea was also shared by the US Department of Agriculture (USDA) and the US Department of Health and Human Services (USDHHS). If the exemption of cisgenic plants becomes valid, the cost and process of commercialisation of genetically modified (GM) crops would be substantially reduced. Therefore, researchers should consider the cisgenic approach when they aim to release the GM products to the market.

Plant transformation is still a developing technology, with crops like wheat only recently being routinely transformed. In other crops such as barley, technique development has progressed such that elite germplasm is now being transformed, which have been recalcitrant to transformation previously. This enables evaluation of transgenes directly in relevant varieties adapted to Australian growing conditions, speeding up the time of delivery of improved germplasm to breeders. Technological advances in the transformation area are ongoing, and cleaner transformation methods, e.g. *Agrobacterium*-mediated transformation versus biolistics, marker free transgenics and targeted gene editing such as with CRISPR/Cas (Belhaj *et al.* 2013) are examples that could enhance probabilities of positive outcomes.

Interactions with other breeding goals

Drought

Due to unreliable rainfall, water is the dominant factor limiting yield for most Australian cereal growers and as such, is the factor that farmers must manage first. Although farmers cannot control rainfall, manipulating N is the major way growers can accommodate uncertain water availability. Farmers apply N at stem elongation dependent on actual and predicted water availability for that season to avoid 'haying off' (van

Table 4.   Examples of tissue-specific and inducible promoters in plants

Gene	Source	Expression patterns		Reference
		Inducible	Tissue-specificity	
Cor78	*Arabidopsis*	Cold, drought, salt, ABA, wounding		Ishitani *et al*. (1997)
Rci2A	*Arabidopsis*	Cold, dehydration		Capel *et al*. (1997)
Rd22	*Arabidopsis*	Drought, salt		Yamaguchi-Shinozaki and Shinozaki (1994)
Cor15A	*Arabidopsis*	Cold, dehydration, ABA		Baker *et al*. (1994)
GH3	Soybean	Auxin		Liu *et al*. (1994)
ARSK1	*Arabidopsis*	Salt	Root	Hwang and Goodman (1995)
PtxA	Pea	Salt	Root	GenBank no. X67427
SbHRGP3	Soybean		Root	Ahn *et al*. (1996)
KST1	Potato		Guard cell	Plesch *et al*. (2001)
KAT1	*Arabidopsis*		Guard cell	Plesch *et al*. (2000)
PRP1	Tobacco	Pathogen		Ward *et al*. (1993)
Hsp80		Heat		US patent no. 5187267
Alpha-amylase		Cold		PCT application no. WO96/12814
PinII		Wound		Euro patent no. 375091
RD29A	*Arabidopsis*	Salt		Yamaguchi-Shinozaki and Shinozaki (1993)
Viral RNA-polymerase			Plastid	PCT application no. WO95/16783, PCT application no. 97/06250
NRT1.1	*Arabidopsis*		Root primordial, root tips	Remans *et al*. (2006)
ANR1	*Arabidopsis*		Root primordial, root tips	Remans *et al*. (2006)

Herwaarden *et al*. 1998*a*, 1998*b*, 1998*c*). Some growers even apply multiple in season N applications to better match N demand with water supply. More accurate weather predictions would allow better management of N (Hayman *et al*. 2007), as would improve application practises; however, growers will continue to react to drought by varying N application. This has to be taken into account when targeting NUE, in this water limited environment, N availability will be limited through necessity. If this constraint is kept in mind, it seems unlikely any breeding for NUE will have a negative impact on water use efficiency (WUE) (Garnett and Rebetzke 2013).

One WUE trait that may conflict with NUE is in breeding for high early vigour. Early vigour is one goal of programs aimed at improving WUE through reducing soil evapotranspiration and enabling greater access to soil water and nutrients (Rebetzke and Richards 1999). However, if this early vigour leads to greater biomass that then leaves the crop open to haying off, it would have a negative impact.

Quality

Improving NUE cannot be chosen at the expense of grain quality, more specifically in relation to N, grain protein. A simple way of improving NUE would be to drop grain protein targets, an option for maize aimed at the bioethanol production market. However, N efficient plants that yield well but do not have sufficient N to put into the grain are not ideal for cereals or oilseeds. As discussed earlier, there is already an inverse relationship between yield and grain protein the breaking of which is the target of breeding programs. Any germplasm with high NUE will always have to maintain standards of quality before release and this will have

to be taken into account. Improvement in NUE through both management and breeding will have growers better prepared for a wet finish to the season and will avoid quality drops that are common when this occurs.

Biofuels

Breeding for NUE should have little impact on biofuel production. Oilseed crops with improved oil composition for biofuel production will have minimal differences in N. Using cereal chaff for biofuel production would have little impact on NUE as it is desirable to remobilise as much N as possible out of the chaff and into the grain.

Future directions

Timeframes are based on the assumptions that efforts commence now, and germplasm delivery refers to delivery of a product to farmers (including breeding and pre-breeding activities).

Phenotyping (<5 years)

Better phenotyping is a high priority both for understanding the genetics of NUE and evaluating new material. Because of the major effect of environment on N availability and demand, this is a major factor confounding NUE trial results. Aside from rainfall, soil characteristics are another important influence on N availability and trial outcomes. Better characterisation of environmental data of field trials in general and within trial variation in environment will improve the quality of results. Root diseases further complicate trial results; however, this

problem is getting easier to manage with improved diagnostic methods.

However, advances in plant growth analysis and imaging capacity should enable development of suitable, high-throughput methods to evaluate germplasm in a highly accurate and repeatable manner both in the field and in controlled environments (Furbank and Tester 2011). Further, protocols to evaluate germplasm for NUE traits alone and their interactions with other environmental aspects (genotype × nitrogen × environment) will need to be developed. The N in this should also include management of N, i.e. if split N applications are routine then this needs to be incorporated into trial designs. Not only will this be necessary in the glasshouse, but in the field, using rainout shelters and irrigation to control for interactions between NUE and drought. The ideal measure of NUE of a variety will be relative yield at low and adequate N provision, but also include component traits (e.g. biomass at a defined point in the lifecycle). Controlled environments, although not equivalent to the field, do allow better dissection of environmental components to traits and also allow evaluation of transgenics where field measurements are made difficult due to regulatory constraints or where seed quantity is limited such as with newly developed populations.

Most studies have utilised phenotyping under low and high N provision separately. To identify QTL involved in physiological NUE, phenotypes need to be compared between the two treatments (e.g. the ratio of yield at low vs high N provision) to identify varieties that will yield well on a range of N provision. Another important factor is that trials must use N fertiliser practices that are consistent with local practices in aspects such as timing of fertiliser application.

Phenotyping root traits may be necessary to improve N uptake (Lynch 2007); however, above-ground traits are easier to measure and have been the target. There are examples of this approach in maize (Liu *et al*. 2008) and pea (Bourion *et al*. 2010), although further development in root imaging technologies will make this approach more feasible.

Exploring variation in existing varieties (<5 years)

Understanding of basic biology involved in N uptake and utilisation across the lifecycle and development of accurate phenotyping methods will allow evaluation or re-evaluation of NUE and component traits in existing varieties. Evaluations will be required in glasshouse and field trials over multiple years and in combination with interacting environmental factors.

Understanding the biology (5+ years)

As mentioned above, basic knowledge of the components of NUE and how they interact with other factors influencing plant growth, development and yield is lacking. This type of basic characterisation of the physiological and molecular biological factors involved in NUE throughout the lifecycle of the crop will be crucial for developing appropriate phenotyping methods and for evaluation of variation in existing and exotic germplasm as well as quantifying improvements made to NUE through traditional breeding and transgenic approaches. Advancing knowledge of the biology underlying NUE will

greatly facilitate interpretation of genomics information and inform choices for transgenic manipulation targets.

Utilising variation in existing populations and varieties (5+ years)

Existing mapping populations or those developed based on evaluation of NUE in existing varieties can be utilised for QTL and association mapping approaches. To date, association mapping has not been utilised in NUE trait mapping studies in cereals, but represents an important resource that should be utilised in future work with existing varieties and diverse germplasm projects. Ultimately the genes responsible for the NUE locus will be identified through fine mapping and cloning; however, beneficial NUE alleles may be moved into existing varieties using marker assisted conventional breeding before the actual cloning of the gene.

Utilising variation in exotic germplasm (5+ years)

Evaluation of NUE in exotic germplasm is a difficult task because of the wide variation in agronomic factors, including plant size, architecture, WUE, heading dates and yield. Component NUE traits identified in exotic germplasm can be backcrossed into existing varieties in order to develop germplasm for mapping the NUE loci and improving the NUE of the existing varieties. New technologies and techniques such as association mapping and sequencing platforms should benefit this area greatly and will speed the process of identifying and utilising beneficial exotic NUE alleles to improve existing varieties. Depending on the trait and the genetic complexity associated with the trait, transgenic approaches may be more suitable than conventional breeding approaches in this case.

Crops have been selected under high N, thus, potentially limiting NUE under reduced N application. To find variation it may be useful to assess more diverse germplasm. One effort has been made to introgress wild allelic variation into domesticated barley and this indicates the potential of this approach. This effort focussed on the introgression of NUE traits from *Hordeum spontaneum* into adapted barley (Saal *et al*. 2011). It may be difficult to introgress traits into commercial varieties if germplasm is too exotic.

Application of advanced genomics approaches to enhancing NUE (5+ years)

The genomics field is going through a stage of rapid development and the outcome has been that a whole new range of approaches are now not only possible but viable. Utilising these advances will make improvement of a complex trait like NUE much more likely than has previously been possible. New approaches moving away from bi-parental populations to advanced populations such as NAM and MAGIC populations are now being utilised in efforts to map NUE related traits. Next generation sequencing offers the opportunity for high throughput genotyping of germplasm at low cost and because of this, approaches such as genomic selection are now being utilised by breeding companies. Major advances in bioinformatics, aided by collaborations through international consortia such as the International Wheat Genome Sequencing Consortium, will enable progress in genetic improvement of wheat and barley to rival crops such as maize.

As summarised by Moose and Below (2009), the integration of quantitative trait mapping, transcriptomics, metabolomics and transgenics is the next step in breeding for NUE. The work of Zhang *et al.* is an example of such an integrative approach (Zhang *et al.* 2010, 2015).

Transgenic technologies (5+ years)

There is currently an existing transgenic technology – alanine aminotransferase (Good *et al.* 2007) – that is being evaluated in Australian cereals as part of a commercialisation process. At present there are no other published technologies that are being evaluated. It is most likely that the first transgenic NUE approaches introduced to Australian cereals and oilseeds will be through collaborative agreements with international biotechnology partners through access to their existing intellectual property.

Further transgenic approaches to improve NUE will be identified through hypothesis based identification of candidate genes, positionally cloning genes in QTL or screening mutagenised populations. Real progress will be made through improved transformation protocols, improved gene–promoter combinations, whole-pathway manipulation, stacking multiple genes and better phenotyping.

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