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Nitrogen remobilization and its importance in nitrogen use efficiency (NUE) of crops

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ABSTRACT

Nitrogen (N) remobilization during grain filling from pre-anthesis N uptake and stored in different tissues of crop N use efficiency (NUE). N is remobilized from to sink (young leaves or grains) with the help of nitrate/amino acid transporters. Nearly 80% of grain N in cereals is derived from N remobilized from vegetative tissues. Remobilization of N within the plant takes place from older leaves to young leaves, leaves to grains, senescing organs to grains, from storage parts to grains. Enzymes involved in N remobilization include glutamine synthetase (GS), glutamate dehydrogenase (GDH), asparagine synthetase (AS) and proteases. Among them, cytosolic GS plays a key role during N remobilization in cereals. There are various senescence-associated genes (SAG) involved in N remobilization from older degrading leaves to younger leaves and grains. Autophagy (ATG) is an important mechanism involved in the degradation of stored N in the form of various proteins to amino acids, which are transported to long-distance in the form of glutamine and asparagine via phloem tissue. There is a complex network of genes, mechanisms, and factors associated with N remobilization, which needs to be considered for improving NUE of crops.

Key words: Autophagy (ATG), Glutamine synthetase (GS), Nitrate transporters, N remobilization efficiency (NRE), NUE, Senescence associated genes (SAG)

Nitrogen (N) is an essential mineral element required for plant growth and development and it is considered to be the important factor limiting crop productivity, next to water. Crop production is highly dependent on the exogenous supply of N fertilizer in almost all agricultural lands (Kraiser *et al.* 2011). It is estimated that ~ 60% of N inputs are in excess for major food crops, and only 30%-50% of applied N is taken up by crops, and the unused N is lost to the environment (West *et al.* 2014). Efficient use of N fertilizer application and development of crops with high N utilization ability concerning increasing crop productivity and maintaining environmental sustainability is therefore, important (Zeigler and Mohanty 2010).

In general, NUE is defined as the yield of plant biomass per unit N available for uptake, ie the ratio between the total biomass of output (e.g. grain yield) and N input (e.g. N supplied in fertilizers). N utilization by the plants can be subdivided into two processes; N uptake: the ability of the plant to remove N from the soil as nitrate (NO_3^-) and ammonium ions (NH_4^+), and N utilization: efficiency of the N use to produce grain yield. NUE can be represented by two main components: N Uptake Efficiency (NUpE) and N Utilization Efficiency (NUtE). NUtE itself subdivided into two components, N Assimilation Efficiency (NAE) and N Remobilization Efficiency (NRE) (Avice *et al.* 2014). To obtain high NUE, N originating from uptake has to be utilized properly and remobilized from the source organs to sink organs, hence N remobilization (NR) is also a major determinant of NUtE (Gaju *et al.* 2011) and an efficient assimilation needs to be correlated to an enhanced NR to improve the seed N filling, which is essential to improve seed yield, in a context of N input reduction. NR is an important component of NUE in crops during vegetative as well as the reproductive stage which should be taken into consideration for better NUE in crops. Therefore, breeding crops with improved NUE represents an effective approach in increasing crop productivity with less N Fertilizer input. Conventional breeding and molecular genetics have been employed to improve NUE in crops. Currently, many candidate genes have been identified for improving NUE in crop plants, and these candidate genes exist in pathways relating to uptake, assimilation, remobilization, and storage of N (Thomsen *et al.* 2014). NUE is a network of various traits to dissect (Xu *et al.* 2012). Presently molecular breeding and genetic engineering approaches are required to improve NUE.

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Despite the significant contribution of NUE to crop productivity and the environment, understanding of the detailed mechanisms to improve NUE is relatively limited due to inherent complexity (Basra and Goyal 2002). Therefore, the purpose of this review is to present an overall examination of genetic, physiological and agronomical aspects of NUE and related traits, specifically by improving the N remobilization (NRE) or N recycling capacity of crops.

Physiology of N metabolism

N uptake: N metabolism can be divided into uptake, assimilation, and mobilization. Plant roots can absorb various forms of N, including nitrate, ammonium, and organic molecules, which are mainly amino acids. Absorption of N takes place in the form of nitrate under aerobic conditions, and as ammonium during anaerobic conditions. Following the first identification of a nitrate transporter, CHL1/AtNRT1.1 in *Arabidopsis thaliana* (Tsay *et al.* 1993), several nitrate transporters have been identified in *Arabidopsis* as well as in crop plants like rice and wheat, which are shown to be involved in nitrate uptake from soil (Kiba *et al.* 2012). In other studies, AtAMT1;1, AtAMT1;3, and AtAMT1;5 were identified in *Arabidopsis thaliana* as transporters involved in ammonium uptake from the external environment (Gazzarrini *et al.* 1999), while OsAMT1;1 and OsAMT3;1 were functionally characterized as ammonium uptake transporters from rice (Bao *et al.* 2015). In rice (*Oryza sativa* L.) OsAMT2 gene family is involved in ammonium uptake (Suenaga *et al.* 2003). Generally, plants adapted to acidic soils (calcifuge species) or adapted to low soil redox potential (e.g. wetlands) prefers ammonium ions. The calcifuge plant species belonging to genus Erica, including *Pinus massoniana* L., *Dicranopteris dichotoma* T., prefers ammonium rather than nitrate. Grassland species, tall fescue and reed canary (*Phalaris arundinacea*), and crops containing high carbohydrates such as sweet potato (*Ipomoea batatas*), potato (*S. tuberosum* L.), and tea trees (*Camellia sinensis*) also prefer ammonium nutrition (Li *et al.* 2013).

Nitrate reduction: After nitrate is absorbed by roots, it is sequentially reduced by nitrate reductase (NR) to nitrite and then by nitrite reductase (NiR) to ammonium (Xu *et al.* 2012). Assimilation of ammonium to glutamine takes place mainly in leaves via the GS-GOGAT cycle (Thomsen *et al.* 2014), with a minor contribution by roots. Root assimilated amino acids are transported to shoots via the xylem. Nitrate also transported directly to leaf for assimilation and stored in the form of various amino acids and proteins. During grain filling, N stored in leaves in the form of amino acids are exported to their sites of usage; i.e. to the developing grins via phloem loading (Tegeder and Masclaux-Daubresse 2018). It has long been predicted that the reduction of nitrate is the major control point in nitrate assimilation, because of the complex regulation of NR enzyme at both the transcriptional and post-translational level, and deactivation of NR by phosphorylation and binding of a 14-3-3 protein (Bachmann *et al.* 1996; Huber *et al.* 1996).

Ammonia assimilation: Primary sources of ammonium include direct uptake by roots from the soil, reduction of nitrate and atmospheric N, whereas secondary sources consist of amino acid catabolism following protein degradation, photorespiratory N cycling and ammonium produced in mitochondria by phenol metabolism (phenylalanine ammonia lyase) (Keys *et al.* 1978). Glutamine synthetase (GS) is a key enzyme responsible for ammonium assimilation, because it catalyzes the adenosine triphosphate (ATP) dependent fixation of ammonium to the δ-carboxyl group of glutamate to form glutamine. The enzyme glutamate synthase (GOGAT) is involved in the conversion of glutamine and 2-oxoglutarate to two molecules of glutamate, thus providing glutamate for ammonium assimilation. The net outcome of the GS-GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids through the action of aminotransferases or transaminases (Forde and Lea 2007). Specific amino acids can subsequently become precursors for all N containing organic molecules, such as proteins, and nucleic acids. The product of GS; glutamine, is itself the main form of organic N for transport in the phloem of rice and the xylem of poplar (Sauter and van Cleve 1992). GS is located in both cytoplasm (GS1) and plastid (GS2) of plant cells, the former being encoded by up to five genes and the latter by one (Hirel *et al.* 2011). GOGAT is present in distinct ferredoxin (Fd) and NADH-dependent forms, both of which are located in the plastid. While the role of the chloroplast (GS2) pathway in the leaves of C_3 plants in the reassimilation of ammonia liberated during the conversion of glycine to serine which occurs inside mitochondria (Blackwell *et al.* 1990) in the photorespiratory N and carbon cycle.

N transport within plants: The major percentage of assimilated N is used for protein synthesis, while smaller amount is present in other macromolecules like nucleic acids, and a large number of different primary and secondary metabolites. Amino acids, like glutamine, asparagines play an important role in metabolism and involve in long-distance transport of reduced N in the phloem. In legumes, ureides (allantoin and allantoic acid) comprise up to 90% of the total N source (Herridge *et al.* 1978) and are the main N compounds involved in long-distance transport (Tegeder and Masclaux-Daubresse 2018). The ureides; allantoin and allantoic acids are generated during purine catabolic pathway as intermediate compounds, which occurs in roots and are transported to the shoot. The ureides are degraded in shoots to produce ammonium ion for reassimilation with the help of enzyme allantoinase (ALN). Ureide permease (UPS), which is localized in the plasma membrane, is known to be a ureide transporter, and involved in the root to shoot transport of ureides (Pelissier and Tegeder 2007).

N storage within plants: Storage of N in leaves is mainly in the forms of nitrate, amino acid, and proteins (Nordin and Nasholm 1997; Tegeder and Masclaux-Daubresse 2018). Most of the N stored in leaves is stored as protein. Nitrate mostly stored in leaf vacuoles, accounting for 58-

99% of total leaf nitrate (Granstedt and Huffaker 1982). N absorption by plants is always faster than is required for their current growth, resulting in its accumulation in the tissue and the formation of N reserves. The stored N can act as a buffer pool for asynchrony between N supply and demand for growth, and N storage is a predominant strategy for the efficient utilization when suffering from deficiency (Rossato *et al.* 2001). Most of the plant N is stored in the leaf blades, followed by stems and then leaf sheaths.

Stems have long been identified as a major N pool, and in most studies leaf sheaths are included. These storage pools have an important function in high input situations where they can improve N uptake efficiency when pre-anthesis supplies are abundant. The requirement for N is higher after anthesis. Stored N is a buffer pool for maintaining leaf expansion and synthesizing photosynthetic proteins in early leaf growth (Lehmeier *et al.* 2013). Relatively high quantities of N may accumulate in short or long term storage pools before and after their transport. The type and amount of N compounds deposited in various source organs, tissues or sub-cellular compartments (e.g. vacuole and plastids) will have considerable effects on N transport and partitioning to sinks. Also, the formation of N storage pools seems to be an effective strategy for controlling cytosolic and apoplastic N concentrations, and thereby influencing N acquisition, transport, and assimilation by feed-forward or feedback regulatory processes. Under N limitation, nitrate could be discharged into the cytoplasm in mature leaves. Increased N supply significantly increases the concentration of free amino acids in leaves. Proline, glutamine, and arginine can be used as N storage sources, and their use varies by species (Nordin and Nasholm 1997).

N remobilization (NR)

Plants mobilize nutrients and metabolites from older to younger leaves with the progression of growth, as well as under stress conditions such as N deficiency during vegetative as well as reproductive stage. Leaves perform the function of both source and sink. During pre-anthesis N uptake, leaves are the major sink organs for N storage and metabolism, but after anthesis older leaves are converted as source of N, as proteins are degraded to amino acids and are transported via phloem to developing grains. Most of the proteins found in seeds are derived from the amino acids recycled during senescence of leaves. In case of rice and wheat, 80% of grain N content is derived from leaves (Tabuchi *et al.* 2007). In *japonica* rice, approximately 80% of the total N in the panicle arises from remobilization through the phloem from senescing organs.

Approximately 80% of total leaf N is located in the chloroplasts mainly in the form of proteins and this is an important N pool for remobilization. Among chloroplast proteins, Rubisco (50% of total cellular proteins in C_3 and; 20% in C_4 plants) seems to serve as the major protein subjected to proteolysis and responsible for most N remobilized during leaf senescence for grain filling. N in leaves is recycled following protein hydrolysis and exported in the form of amino acids to grains, 60% to 95% of grain N comes from the remobilization of N stored in roots and shoots before anthesis. A less important fraction of seed N comes from post-flowering N uptake and N translocation to the grain. N deficiency conditions post flowering lead to significant reduction in size and N contents of grains (Dupont and Altenbach 2003).

In field grown wheat, remobilization efficiency of the N taken up before anthesis was shown to be variable depending on the genotypes examined (Cox *et al.* 1985). Remobilization of the N stored before anthesis and N uptake after anthesis is generally estimated by calculating the difference between the amount of total N present at anthesis and the amount of total N present at harvest in the different parts of the plant. The major forms of N in the phloem sap are glutamine and asparagines. In developing sink organs, the remobilized glutamine is reutilized for many biosynthetic reactions, via the GS-GOGAT pathway (Lea and Miflin 2003), which is mostly responsible for the metabolism of glutamine in rice. The low-affinity nitrate transporter NPF2.1 has been shown to mediate the mobilization of nitrate from older to younger leaves where it acts in the phloem loading of nitrate from older leaves to allow transport to younger leaves.

Two low-affinity nitrate transporters NPF1.1 and NPF1.2 are expressed in companion cells of the major vein and the expressions is higher in larger expanded leaves and, are involved in phloem loading of nitrate from mature leaves for redistribution in younger leaves (Kant *et al.* 2018). Proteins are degraded before they are remobilized to developing organs, which occurs mainly by three pathways: the chloroplast degradation pathway; the vacuolar and autophagic pathway; and the ubiquitin 26S proteasome pathway.There are multiple distinct routes or pathways involved in the degradation of the proteins, particularly carbonylated proteins. Proteosomal degradation or autophagy mechanisms are common pathways involved. It is found that for the degradation of carbonylated proteins, 26S proteasome pathway is involved rather than the autophagy (Jain *et al.* 2008).

Autophagy (self-eating), a conserved pathway in all eukaryotes, which involves membrane bound vesicles called autophagosomes which enclose cytosolic components and protein complexes, transferring them from the chloroplasts to the vacuole, by forming autophagic bodies and plays an important role in the remobilization of nutrient, such N under suboptimal nutrient conditions (Li *et al.* 2019). These are hydrolyzed by vacuole localized exo and endopeptidases which release amino acids for subsequent remobilization. Several ATG (Autophagy related) genes have been identified in plants. ATG8 and ATG12 are key genes in the autophagic system, with ATG8 participating in tagging proteins for degradation (Geng *et al.* 2008). Several autophagy genes have been reported to be induced during senescence, as well as under N limiting conditions (Guiboileau *et al.* 2012). Protein breakdown is also performed by proteases (Moureaux *et al.* 1979), and senescence-associated vacuoles (Otegui *et al.* 2005). In rice

(*Oryza sativa* L.), OsATG8a shows increased expression levels under N starvation conditions. Over expression of OsATG8a significantly enhanced the level of autophagy and the number of effective tillers in the transgenic rice. In addition, the transgenic lines accumulated more N in grains and the yield is significantly increased under normal N conditions (Li *et al.* 2019). The use of ^{15}N stable isotope labeling is a good alternative that generally used to study N remobilization. This can be achieved by applying ^{15}N nitrate to plants grown in hydroponics (Cliquet *et al.* 1990) or by infiltrating ^{15}N urea to the leaves of wheat plants grown the field.

Although NR seems to be a major lever for improving the NUE, the cellular mechanisms associated with NR from senescing leaves (proteolysis and the N export) remain largely an enigma. Studies on *Arabidopsis* reported the involvement of cysteine and serine proteases, and also the role of the proteasome in the degradation of proteins (the main form of N storage in leaves) into amino acids or peptides during senescence (Smakowska *et al.* 2014). The Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) can represent up to 65% of the soluble proteins in C_3 plants and 20-30% of total leaf N. Consequently, Rubisco is the major source of N available for remobilization and its budget is very relevant for the plant. Asparagine synthetase (AS) is an important enzyme for N remobilization during senescence because it is involved in the conversion of amino acids into transportable forms after protein degradation in senescing leaves (Fischer *et al.* 2007).

Glutamine was found at high levels in phloem sap and consequently, Glutamine synthetase (GS) is supposed to be largely involved in N remobilization processes during senescence in winter wheat (Kong *et al.* 2015) and maize (Schluter *et al.* 2013), especially the cytosolic form of GS (GS1) in the case of oilseed rape (Kong *et al.* 2015). Glutamate dehydrogenase (GDH) catalyzes the synthesis of glutamate as well as the deamination reaction of glutamate which provides ammonium for the GS activity in senescing leaves. Also, in case of high levels of ammonium, GDH can incorporate ammonium in α-ketoglutarate to produce glutamate. A significant quantity of glutamate has been found in phloem sap of oilseed rape (Tilsner *et al.* 2005) and an increase of both GDH activities in sliced leaves of oilseed rape was associated with a decrease in soluble proteins and ammonium, suggesting an important role for GDH in foliar N remobilization.

It is well established that amino acids delivered from leaves to roots through phloem transport can be recycled to the shoot in the transpiration stream. However, as the transpiration rate of developing seeds or grains is typically low, xylem to phloem transfer in the peduncle has been demonstrated to be important for the delivery of reduced N and other solutes to seeds (Feller and Fischer 1994). The efficient export of amino acids and the involvement of GS1 and GDH in N remobilization need to be confirmed in leaves as they senesce, because they are proposed to have an efficient N remobilization.

Table 1 Various parameters related to N movement within the plant parts (Cox *et al.* 1986)

N Remobilization (NR) (mg plant^{-1}) = N content at anthesis -N content at maturity

N Remobilization Efficiency (NRE) (%) = (N remobilization/N content at anthesis) × 100

N lost or gained (kg ha^{-1}) = N content at maturity - N content at anthesis

N at anthesis lost or gained (%) = (N lost or gained/N content at anthesis) × 100

N Harvest Index (NHI) = Grain N/total N content of aboveground parts at maturity

NUE (kg kg^{-1}) = Grain yield/N fertilizer application.

Mechanism of N remobilization

During the vegetative stage, young leaves and roots

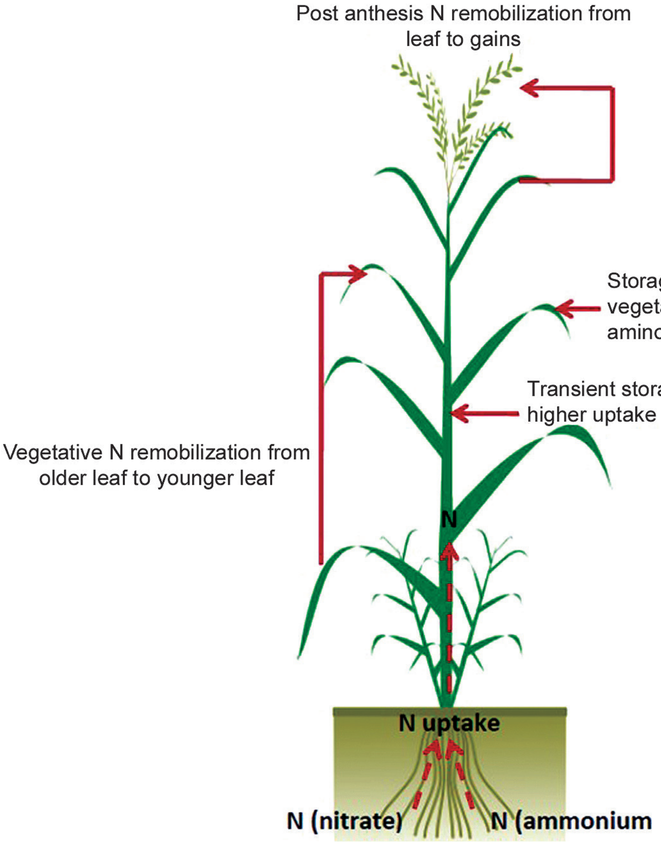

Fig 1 Overview of vegetative and post-anthesis N remobilization in crop plants (cereals).

act as sinks for nitrate uptake, assimilation, and storage of amino acids, and later on, during the reproductive stage, leaf proteins are degraded and amino acids, as well as other nitrogenous compounds, are released, then exported to reproductive organs as illustrated in Fig 1. This process occurs in various ways.

*N remobilization during the vegetative stage:*N remobilization also takes place during vegetative plant development, particularly from older to younger leaves along the axis of the plants *via* the sequential senescence process. Sometimes senescing leaves can fall with a high level of residual N (up to 3.5% of dry matter) leading to a significant return of N to the soil (Fageria *et al.* 2005), and decreases N remobilization to grains. During reproductive stages, the N amount in source leaves is largely remobilized to grains (86% of N present at the beginning of flowering in field conditions (Rossato *et al.* 2001), leading to a low residual N in dead leaves (less than 2% of dry matter), and resulting in the reduction of the risk of N pollution. In mesophyll cells of fully developed leaves, over 50% of the total N is present in the photosynthetic apparatus in chloroplasts. During vegetative stage older leaves function as source and younger leaves as sink and N is remobilized, subsequently post anthesis remobilization of N takes place from these leaves to grains.

N remobilization during reproductive senescence: Senescence is a highly regulated, genetically controlled process that results in the death of plant parts or the whole organism (Nooden *et al.* 1997; Munne Bosch 2008), leading to series of metabolic changes, remobilization and recycling of nutrients, and plants resume growth during grain filling, maturation or nutrient stress (Lim *et al.* 2007; Munne Bosch 2008). N remobilization during leaf senescence has been investigated in many plant species, like *Arabidopsis* (Diaz *et al.* 2008), tobacco (Masclaux-Daubresse *et al.* 2010), sunflower (Cabello *et al.* 2006), and maize (Martin *et al.* 2005). N remobilization from senescing leaves and its reuse by other parts of plants results in higher NUE.

In many plants during senescence, the content of organic N needed for remobilization is present in the form of proteins. Chloroplasts are first organelles that alter their structure and function because of the degradation of chlorophylls that direct the changes in leaf color, whereas mitochondria and nucleus remain undamaged until the final stage. The activity of the chloroplastic isoform of GS (GS2) enzyme, which is the primary N assimilating enzyme in rapidly photosynthesizing leaves, decreases during senescence, whereas the activity of the cytosolic isoform of GS (GS1), which synthesizes glutamine from ammonium provided by GDH, increases during senescence (Masclaux-Daubresse *et al.* 2010). As there is progress in senescence, vacuolar proteolysis releases free amino acids into the cellular pool.

During senescence, the enzymes GS1, GDH, and AS are induced because the N content available in protein and nucleic acid molecules is converted into transferrable amino acids, especially amides, glutamine, and asparagine. As the process of senescence continues, a chain of transamination reaction creates glutamate, which serves as a substrate for GDH enzymes. Then, deamination of glutamate occurs to form ammonia in the presence of GDH enzymes. This ammonia is further converted into glutamine in the presence of the enzyme GS. Glutamine is one of the important forms of N, transported during nutrient remobilization. It has been proved that there is a relationship between GS activity, N content of stem, and yield. GS activity has increased grain yield and stems N content in wheat and maize. Increased activity of GS1 in vascular bundles indicates its increased demand in healthy plants and helps in transporting the N from the senescing leaves. The presence of GS2 in photosynthetic tissues confirmed its role in the re-assimilation of photorespiratory ammonia (Kamachi *et al.* 1992).

After protein degradation during senescence, the amino acids released are loaded into the phloem. While all the amino acids are remobilized, glutamine, asparagine followed by glutamate, aspartate, serine, and alanine are predominant in phloem sieve tubes (Sanders *et al.* 2009). QTLs for N remobilization detected by ^{15}N tracer methods mainly coincide with QTLs for leaf senescence (Xu *et al.* 2012). N remobilization during leaf senescence is tightly regulated by chloroplastic and vacuolar protease activities as well as by the various long-distance transport pathways.

N remobilization from storage parts: Storage of nitrate provides an important supply for N remobilization to support vegetative growth. Nitrate can be stored inside the vacuole present in stem, leaf, and also in roots which are remobilized to economic parts during grain filling from vegetative parts or senescing parts. Stem, rather than the leaf, act as a storage organ for N. Land plants accumulate nitrate in the vacuole which is important for osmotic balance and as an N reserve. Leaf tissue or sap nitrate concentrations are used as indicators of N status in plants (Schepers *et al.* 1992), and measurements of leaf tissue nitrate primarily determine nitrate stored in the vacuole. Remobilization of vacuolar nitrate can be measured by removing all N supply from growing plants (van der Leij *et al.* 1998).

It is found that both *indica* and *japonica* types of rice can accumulate nitrate in the leaf when N is supplied with nitrate form (Fan *et al.* 2005), and it is evident that *japonica* rice has a greater capacity to store, and subsequently remobilize, vacuolar stored nitrate. During grain filling the ability of a plant to remobilize leaf stored N is an important factor for NUE in crops, and has been strongly implicated in quantitative trait locus (QTL) studies with cereals (Mickelson *et al.* 2003). It is confirmed that the stem is a crucial buffering organ for transient N storage in case of non-synchronism between the massive remobilization of N coming from source leaves and its further utilization by seeds.

N remobilization during seed germination: Growth of embryo is dependent on the remobilization of storage compounds in seeds, and during germination the storage compounds are broken down and remobilized to developing

Table 2 Details of genes expressed during senescence

Gene	Plant target/involved pathway	References
SAG2	*Arabidopsis thailana* Cysteine protease	Hensel (1993)
Sec1	*Zea mays* Cysteine protease	Smart *et al.* (1995)
LSC790	*Brassica napus* Cysteine protease	Buchanan-Wollaston and Ainsworth (1997)
SAG12	*Arabidopsis thailana* Cysteine protease	Lohman *et al.* (1994)
Sec2	*Zea mays* Cysteine protease	Smart *et al.* (1995)
UB17	*Solanum tuberosum* Polyubiquitin protein	Garbarino *et al.* (1995)
Atgsr2	*Arabidopsis thailana* Glutamine synthetase	Bernhard and Matile (1994)
Atgsr2	*Oryza sativa* Glutamine synthetase	Kamachi *et al.* (1992)
Atgsr2	*Raphanus sativus* Glutamine synthetase	Kawakami and Watanabe (1988)
Atgsr2	*Brassica napus* Glutamine synthetase	Buchanan-Wollaston and Ainsworth (1997)
DcACS1	*Carnation cultivar Polaris* Ethylene biosynthesis	Tanase and Onozaki (2015)
DcACO1	*Carnation cultivar Polaris* Ethylene biosynthesis	Tanase and Onozaki (2015)
DcCP1	*Dianthus caryophyllus* L. Cysteine protease	Tanase *et al.* (2013)
DcbGal	*Dianthus caryophyllus* L. β-Galactosidase	Tanase *et al.* (2013)
DsGST1	*Dianthus caryophyllus* L. Glutathione-S-transferase	Tanase *et al.* (2013)
.DcLip	*Dianthus caryophyllus* L. Lipase	Tanase *et al.* (2013)
PRODH	*Rosa hybrid* Proline metabolism	Kumar *et al.* (2009)
P5CDH	*Rosa hybrid* Proline metabolism	Kumar *et al.* (2009)
NECD	*Arabidopsis thailana* ABA biosynthesis	Buchanan-Wollaston *et al.* (2005)
AAO1	*Arabidopsis thailana* Aldehyde oxidases	Buchanan-Wollaston *et al.* (2005)
AAO3	*Arabidopsis thailana* Aldehyde oxidases	Buchanan-Wollaston *et al.* (2005)
RPK1	*Arabidopsis thailana* ABA-inducible receptor like kinase	Kuo and Ching (2004)
SEN4	*Arabidopsis thailana* Methyl jasmonate signaling pathway	He *et al.* (2005)
SEN5	*Arabidopsis thailana* Methyl jasmonate signaling pathway	He *et al.* (2005)
VPE	*Arabidopsis thailana* Methyl jasmonate signaling pathway	He *et al.* (2005)
LSC54	*Arabidopsis thailana* Metallothionein	Navabpour *et al.* (2003)
RBCS	*Arabidopsis thailana* Ribulose bisphosphate carboxylase	Morris *et al.* (2000)
LSC222	*Arabidopsis thailana* Chitinase	Hanfrey *et al.* (1996)
LSC460	*Arabidopsis thailana* Glutamine synthetase	Buchanan-Wollaston and Ainsworth (1997)

embryo. Cytosolic GS (GS1) has been detected in different parts of the embryos of starch accumulating seeds, which is directly involved in N assimilation (Shewry and Halford 2002). Genetic studies in maize (Limami *et al.* 2002) showed the co-localization of a QTL for a high rate of germination with the Gln1-3 structural gene suggesting high levels of GS activity in the germinating seed could lead to faster germination.

Role of transporters in N remobilization

Several nitrate transporters have been characterized for their role in nitrate movement from the roots to the shoots, and the grains. The reallocation of nitrate from root to shoot occurs via the xylem vessels. A low-affinity nitrate transporter NPF7.3 located on the plasma membrane, is involved in the loading of nitrate into the xylem (Li *et al.* 2017). Two low-affinity nitrate transporters, NPF7.2 and NPF2.9, have potential negative role in root to shoot nitrate transport. OsNPF2.4, a low-affinity rice nitrate transporter, was reported for its functions in nitrate acquisition by root and long-distance nitrate transport from root to shoot in rice. OsNPF2.4 is located in the plasma membrane and is expressed mainly in the epidermis, xylem parenchyma, and phloem companion cells and was reported to promote root growth, and higher biomass and grain yield (Xia *et al.* 2014).

In the leaves, nitrate is either assimilated in the cytosol or is temporarily stored in vacuoles. A low-affinity nitrate transporter, NPF6.2 was characterized to have a role in regulating leaf nitrate homeostasis. NPF6.2 is located in the leaf petiole; *npf6.2* mutant plants had lower nitrate content in the leaf petiole (Kant *et al.* 2018). Plants mobilize nitrate, nutrients, and metabolites from old to young leaves with the progression of growth, as well as under stress conditions such as N deficiency. The low-affinity nitrate transporter NPF2.13 has been shown to mediate the mobilization of nitrate from older to younger leaves where it acts in the phloem loading of nitrate from older leaves to allow transport to younger leaves. NPF2.13 is localized in the phloem of minor veins in older leaves, with *npf 2.13* mutants retaining more nitrate in older leaves, possessing less nitrate in the phloem sap of older leaves and having reduced transport of radiolabeled nitrate to younger leaves.

Two high-affinity nitrate transporters NRT 2.4 and NRT2.5, which are involved in root nitrate uptake, also have potential roles in phloem nitrate transport in shoots, under N starvation conditions. NRT2.4 is expressed in the epidermis of lateral roots and the phloem parenchyma of shoots, and is thought to have a role in nitrate transport as mutant plants had lower nitrate levels when grown specifically under low N conditions. NRT2.5 is expressed in the epidermis and the cortex of the roots, as well as the minor veins of mature leaves, and is potentially involved in nitrate loading into the phloem. Nitrate can be remobilized to the seed, although it is not clear what proportion of nitrate assimilation this accounts for compared to assimilation in vegetative tissues or the whole plant. Low-affinity transporter NPF2.12 is expressed in vascular tissues of the silique and has a role in relocating nitrate to the seed, and it is found that mutant plants of *npf2.12* had lower nitrate levels in the seed as well as a higher seed abortion rate.

NPF5.5, contributes to nitrate transport to embryo (Leran *et al.* 2015). A high-affinity nitrate transporter NRT2.7 has a similar role, although expressed in mature seeds, with plants over-expressing NRT2.7 having higher nitrate content in the seed, while mutant had lower levels compared to wild type plants (Chopin *et al.* 2007). Amino acid transporters found to play central role in plant N metabolism and source-sink interactions (Tegeder 2018), as amino acids released by the degradation of proteins during reproductive growth, need to be transported to the developing grains via phloem tissues. Several amino acid transporters from amino acid permeases (AAP) family; AAP1, AAP2, AAP3, AAP6, AAP7, AAP8, and AAP16 have been reported to play important roles in amino acids transport (Kant *et al.* 2018).

In addition, some other families have been identified for their potential involvement in the transport of amino acids including a lysine histidine like transporter (LHT), oligopeptide transporters (OPT), cationic amino acid transporters (CAT), proline transporters (ProT), and aromatic and neutral amino acid transporters (ANT) (Tegeder & Masclaux□Daubresse., 2018). However, only a few of these transporters have been functionally characterized for their role in amino acid transport. LHT1, for example, is involved in amino acid uptake in the root epidermis and the supply to leaf mesophyll cells (Hirner *et al.* 2006), while OPT3 has a role in peptide and amino acid transport during early embryo development (Stacey *et al.* 2002).

Transcription factors associated with senescence and N remobilization

Single gene transgenics overexpressing the genes of primary N assimilation (NR, NiR and plastidic GS, GOGAT) did not radically improve NUE (Krapp *et al.* 2014). NUE is a multigenic trait, and involves the contributions of multiple genes to get a better response to the applied N. Kinases and phosphatases are also thought to play important roles in the regulation of expression of genes coding for N assimilatory enzymes such as NR, NiR, GS2, and Fd-GOGAT (Undurraga *et al.* 2017). In plants, major protein phosphatases include PP2Cs gene family that act as regulator during stress signaling (Moorhead *et al.* 2007; Singh *et al.* 2016). Protein phosphatase (PP2C9) is closely related with the improvement of the NUE in rice by enhancing N uptake and assimilation.Another level of control of metabolism is carried out by transcription factors: Dof, NLP7, GATA, N metabolites: glutamine and glutamate, and miRNAs (Curci *et al.* 2017).

miR5640 targets phosphoenolpyruvate carboxylase (PEPC) which plays a very important role in maintaining C/N balance. NLP7 proteins are involved in N assimilation and sensing (Castaings *et al.* 2009). All NLP proteins can bind nitrate responsive cis-element and mediate nitrate dependent gene expression and improve C/N balance under both N sufficient and N deficient conditions. All these transcription factors and genes mentioned are also responsible for N remobilization process during senescence and nutrient recycling. Autophagy promotes senescence and aging of plant parts. Several senescence-associated genes (ATG and metacaspases) are expressed at different stages of plant senescence (Have *et al.* 2016). This process involves the participation of tissue-specific transporters which replenishes the N requirement during the reproductive stage of plant development.

Several reports suggest the regulators of this process, such as N limitation adaptation (NLA), which control the expression of AtNRT1.7 by protein ubiquitination pathway. However, NLA is itself under the control of miRNA827 (Liu *et al.* 2016). Analysis of rice GOGAT mutant leads to the identification of another protein, viz. ferredoxin-dependent glutamate synthase (OsFd-GOGAT), to play a role in this process (Zeng *et al.* 2016). Fd-GOGAT plays a role in ammonium recycling by photorespiration. The key enzymes of N assimilation which are upregulated during senescence and N remobilization are GS and its isoforms; GS1.1, GS1.2, GS1.3, GS2, GDH, and AS. Among all these isoforms GS1.1 is found to play important roles during N remobilization. Predominant genes encoding the transcription factor HvNAC026, serine-type protease SCPL51, and the autophagy factors ATG7 and ATG18F were upregulated during senescence of flag leaves from plants with standard N supply. NAC transcription factor HvNAC026, the serine-type protease SCPL51, and the autophagy factors APG7 and ATG18F in crops like barley are major regulators and executors of N remobilization during barley leaf senescence (Hollmann *et al.* 2014).

OsLHT1 is an amino acid transporter for neutral and acidic amino acids, and disruption of OsLHT1 function inhibits the rice growth and yield which might be due disruption of amino acid transport from vegetative organs to developing grains (Wang *et al.* 2016). OsNPF7.2 plays a role in the intracellular allocation of nitrate in roots, and thus influences rice growth under high nitrate supply (Hu *et al.* 2016). Nuclear Factor-Y (NF-Y) family of transcription factors takes part in many aspects of growth and development in eukaryotes. They have been classified into three subunit classes, namely, NF-YA, NF-YB and NF-YC (Das *et al.* 2019). Transient expression of NF-YA genes in rice protoplasts promoted the transcripts of PR genes and NRT2 genes, while it reduced the transcripts of NRT1 genes under N limiting conditions in rice.

Genetics of N remobilization in crop plants

Because of the genetically determined links between yield, grain protein concentration, and leaf senescence onset, mapping of QTLs for leaf senescence has been attempted in various plant species. QTLs for leaf and rosette longevity have been mapped using the Arabidopsis Cape Verde Island Landsberg recombinant inbred line (RIL) population (Luquez *et al.* 2006) when cultivated under high or low N nutrition regimes. A link between N assimilation and leaf senescence was also revealed by mapping the QTLs for rosette yellowing using a Bay-0 × Shahdara RIL *Arabidopsis* population (Diaz *et al.* 2008). When plants were grown under low N nutrition, early yellowing senescence symptoms were differentially observed depending on genotype. Five QTLs for leaf yellowing senescence were mapped, and three major QTLs were detected on chromosomes 2 and 3, and colocalized with major QTLs for rosette dry matter, total amino acid concentration and total N concentration (Loudet *et al.* 2003).

QTLs for NUE have been mapped in several plant species, including maize, rice, and Arabidopsis (Loudet *et al.* 2003). No QTLs for N remobilization have been examined from rice and maize. However, QTLs related to N remobilization from senescing leaves for grain filling have been found in barley and durum wheat (Joppa *et al.* 1997; Mickelson *et al.* 2003) mapped a QTL on chromosome 6 for seed protein content in durum wheat. Many QTLs in the flag leaf at anthesis, mid grain development, and maturity, the difference in leaf N content between anthesis and maturity in barley have been mapped (Mickelson *et al.* 2003). The difference in leaf N content between anthesis and maturity is the best trait to estimate N remobilization efficiency (NRE) from the flag leaf, and three QTLs explaining variations in this trait were mapped on chromosomes 6 and 7.

For grain protein in wheat, two QTLs were mapped on chromosomes 2 and 6. One of the QTL was cloned through positional cloning and fine mapping (Uauy *et al.* 2006). The locus encodes a NAC transcription factor, NAM-B1, which accelerates leaf senescence and increases nutrient remobilization from leaves to developing grains. The role of NAM-B1 was confirmed by reducing the RNA levels of multiple NAM homologs by RNA interference, this resulted in delayed senescence and reduced wheat grain protein, zinc and iron content. At the same time as the identification of the NAM-B1 gene at the QTL locus in durum wheat, functional analysis of an AtNAP homolog in Arabidopsis was reported (Guo and Gan 2006). In Arabidopsis, NAC family transcription factors is also involved in the control of leaf senescence progress. QTL co-localization strongly suggested that the major endo or amino-peptidases were not involved in leaf N remobilization or in the control of grain protein content. In contrast, QTL co-localization suggested that vacuolar carboxypeptidase isoenzymes would be involved in leaf N remobilization. The genetically determined links between yield, grain protein concentration, leaf senescence onset and severity have been confirmed by the co-location of QTLs in species such as maize, rice, and arabidopsis.

Strategies to improve N remobilization

Approaches to improve crop productivity and NUE have mainly concerned the genetic manipulation of inorganic N uptake, allocation, metabolism, remobilization and its regulation (Masclaux-Daubresse *et al.* 2010; Xu *et al.* 2012, Li *et al.* 2017). Inorganic N transporters and N assimilation genes have been overexpressed with some success by using constitutive promoters giving positive results (Xu *et al.* 2012, Li *et al.* 2017). In rice constitutively overexpressing the high-affinity nitrate transporters, OsNRT2.3 and OsNRT1.1 exhibited increased nitrate uptake, shoot biomass and grain yield (Fan *et al.* 2016). In rice, over-expression of OsNRT2.1 controlled by the root and leaf nitrate inducible OsNAR2.1 promoter resulted in increased above-ground plant biomass and grain yield (Chen *et al.* 2016). Similarly, targeted expression of the ZmNRT1.1A and ZmNRT.1B, and OsNPF6.5 transporters in maize and rice root cells, respectively, led to higher plant productivity (Hu *et al.* 2015; Allen *et al.* 2016).

NUE could also be increased in canola plants grown under low N supply that expressed a barley (*Hordeum vulgare*) alanine aminotransferase gene in their roots. All these transgenic approaches to enhance plant NUE is to N remobilization after anthesis form vegetative parts to grains. Using targeted expression of the yeast sulfur (S)-Methyl- Methionine Permease1 (MMP1) in the pea phloem of leaf minor veins, it was demonstrated that amino acid phloem loading is a major control point in source to sink allocation of N (Tan *et al.* 2010). Long-distance transport of S and N was increased in the transgenic plants, leading to improved biomass production, seed yield, and seed storage protein concentrations. When an amino acid transporter was overexpressed in the storage parenchyma of legume cotyledons, seed protein accumulation was successfully increased of the remobilization of stored N from vegetative organs to reproductive organs (Weigelt *et al.* 2008). The outcome was increased source to sink transport of amino acids, improved biomass and seed production, and increased seed protein concentrations.

The AAP1 pea plants were also used to determine that how changes in source to sink amino acid transport affect plant NUE (Perchlik and Tegeder 2017). AAP1 over expressers produced the same seed yield as the controls with only half the amount of N supplied. It will be important now to transfer these recent successes from the glasshouse to the field to resolve whether the engineered plants continue to outperform the control counterparts in their natural environment. In agronomy point of view split application of N during the vegetative stage is the most preferred strategy to get optimum N absorption, because N absorption declines after anthesis, and remobilization of N starts afterward. It is also important to find genotypes with N uptake and remobilization which can be used as donors for breeding programs to achieve enhance N economy and pollution-free crop production.

Conclusion and future perspectives

N is one of the yield-limiting factors in crop production. Hence, improving NUE is a major goal of crop improvement and increase yields of grains, reduce the input cost of crop production, and environmental pollution. Understanding of N uptake, its fate within the plant, and remobilization of N to grains is very important to improve NUE in crops. To improve NUE, N remobilization during vegetative and post-anthesis period plays an important role. By modifying the physiology of N metabolism and enhancing the N remobilization, NUE of crops can be maximized. This might be achieved, at least in part, by a better understanding of N metabolism and its regulation, and by identifying likely target genes for manipulation by either direct gene transfer or marker-assisted breeding. A more logical approach for improving NUE is to manipulate transcription regulators which can control a cascade of genes involved in a given process of nitrate uptake, transport, assimilation, remobilization, rather than modulating a single or handful of genes simultaneously. Remobilization of N takes place from non-senescing parts, senescing parts, and also from the N stored in various storage parts like stem, leaf, petiole, and root. The challenge faced by breeders to select plants with high N index (NHI) and efficient NRE, while maintaining yield and seed quality, is easier said than done. N remobilization is fundamental for plant N economy since it controls a large part of the N fluxes from source to sink organs. Fundamental knowledge of the mechanisms controlling N remobilization during plant development and in response to stress is increasing, and is required to improve N remobilization and N fertilizer economy. There are multiple regulatory controls at the gene and protein level to modify the activity of N remobilization and NUE, and it is important to take this complexity into account.

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