Accelerated senescence and nitrogen remobilization in flag leaves enhance nitrogen use efficiency in soft red winter wheat

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ABSTRACT

Wheat (Triticum aestivum L.) production requires a large amount of nitrogen (N) supply because growers aim to achieve high grain yield and appropriate grain protein content simultaneously. A comprehensive understanding of the mechanisms that underpin efficient N usage at limited N in wheat can facilitate the development of new N-saving varieties in this major crop. Here, we performed comparative analysis of flag leaf responses to N availability in soft red winter wheat with contrasting N use efficiency (NUE); VA08MAS-369 (high NUE) and VA07W-415 (low NUE). This study demonstrated that accelerated senescence along with enhanced breakdown of protein and starch in flag leaves was correlated with higher grain yield, NUE for grain yield, and NUE for grain protein under N limitation. The more dramatic reductions in flag leaf N compounds and carbohydrate reserves in VA08MAS-369 were linked with significantly elevated expression of genes and enzymes associated with these metabolic pathways in this high NUE genotype. Consistent with the gene expression data, nitrate reductase, glutamine synthetase, and NAD-dependent glutamate dehydrogenase activities were highly induced under limited N in VA08MAS-369. It was previously reported that accelerated senescence contributes to increased grain protein content in wheat under regular N supply. This study provides molecular and physiological evidence that vigorous senescence and N remobilization also benefit grain yield under N deprivation.

Introduction

Wheat (Triticum aestivum L.) is the most widely grown crop, cultivated on 220 million ha worldwide (FAO Stat, 2016). Wheat requires a high level of nitrogen (N) application relative to most other crops because growers aim to achieve both high yield and appropriate grain protein content. Due to its large cultivation area and N requirement, 18% of the total N fertilizers produced globally are applied to wheat, the largest amount of N supply in crop production (Ladha et al., 2016).

Among all nutrients applied to crops, N is generally the most expensive for growers (Han et al., 2015). In addition, the production cost of N-based fertilizers has constantly been rising. However, wheat absorbs only 20 to 40% of supplied N in the same season (Raun & Johnson, 1999; Sylvester-Bradley & Kindred, 2009). Non-absorbed N can be lost to waterbodies and atmosphere, leading to water pollution and greenhouse gas emission (Good & Beatty, 2011; Hirel et al., 2007). Thus, improvement of N use efficiency (NUE) in wheat not only reduces grower costs, but also addresses the environmental issues caused by N runoff and denitrification in wheat-growing regions.

Plant NUE depends on multiple steps including N uptake, assimilation, and remobilization. First, plants absorb inorganic N such as nitrate and ammonium, most of which is subsequently assimilated into organic N. Organic N and unassimilated inorganic N are stored in mature tissues, whereas they are utilized for growth in expanding tissues. In wheat and other grasses, mature leaves serve as sinks to store N before anthesis. However, upon anthesis, leaves become N sources to support grain filling. In wheat, up to 95% of the grain N is derived from the remobilization of N stored in the aboveground tissues before anthesis (Palta & Fillery, 1995; Van Sanford & MacKown, 1987). Although grain N accumulation largely relies on pre-anthesis N uptake and post-anthesis N remobilization, assimilation of newly absorbed N and export of reduced N products still occur in senescing flag leaves after anthesis (Martin, 1982; MacKown & Van Sanford, 1986).

Flag leaves are the major N source that supplies a large amount of assimilates for grains at the post-anthesis stage.
Flag leaves may serve as a model tissue to represent the whole-plant N status because the amount of N and activity of glutamine synthetase, the key enzyme for N assimilation, in flag leaves are strongly correlated with those at the whole-plant level in wheat (Kichey et al., 2006). For these reasons, post-anthesis changes in the content of metabolites, activities of enzymes, and expression of genes related to N assimilation and remobilization have been characterized in wheat flag leaves (Gregersen & Holm, 2007; Kichey et al., 2005; MacKown & Van Sanford, 1986; Peeters & Van Laere, 1994). However, the role of flag leaf metabolism and gene expression in the regulation of NUE remains unclear in wheat due to the lack of information obtained from comparative analyses using high and low NUE genotypes.

Soft red winter wheat is a class of wheat with higher yield and lower grain protein content than hard wheat (Thomason et al., 2009). Soft red winter wheat has good milling and baking properties for cookies, cakes, and crackers, whereas hard wheat is suitable for bread, rolls, and all-purpose flour. Their water requirement for growth is also different; soft red winter wheat is mainly grown in higher rainfall areas, while hard wheat is usually cultivated in semi-arid regions. Thus, these wheat classes have been bred toward different target traits independently using distinct selection criteria. Most wheat NUE studies have exclusively analyzed hard wheat. NUE research in soft red winter wheat is limited, but it was reported that the effect of N supply on vegetative N remobilization during grain filling and spike N accumulation at harvest is distinct in soft red winter wheat and hard wheat (Henson & Wain, 1983; Morris & Paulsen, 1985; Van Sanford & MacKown, 1987). These results emphasize the necessity of further investigation to determine the regulatory mechanisms underlying N utilization in this understudied wheat class.

In this study, we characterized flag leaf responses to N supply at the post-anthesis stage using two soft red winter wheat accessions with contrasting NUE. Time-course observation of major N and carbohydrate (C) compounds along with expression analysis of gene associated with N transport, assimilation, and remobilization in flag leaves revealed key physiological and molecular components contributing to NUE. Comparison of these data with agronomic NUE-related traits provided possible mechanisms of NUE regulation in soft red winter wheat.

### Materials and methods

#### Plant materials and growth conditions

This study used two soft red winter wheat genotypes, VA08MAS-369 (McCormick/GA881130ES) and VA07W-415 (PI72014415). These accessions were selected based on the result of our previous field study (Pavluluri et al., 2015). Sterilized seeds were grown in plastic pots (5.7 cm (L) x 3.8 cm (W) x 5.4 cm (H); 1 plant/pot) containing Metro Mix 360 potting soil (Sun Gro Horticulture, Vancouver, Canada) in a greenhouse (natural light, 22 °C day/13 °C night) until the 3-leaf stage. Subsequently, plants were exposed to vernalization treatment at 8 °C under 8 h light (80 μmol m⁻² s⁻¹)/16 h dark cycles for 8 weeks. Vernalized seedlings were transplanted into pots (2.4 L; 1 plant/pot) containing a mixture of 50% (v/v) Metro Mix 360 and 50% (v/v) sand and grown in the greenhouse (natural light, 18 °C day/7 °C night). Initially, all plants were treated with equal fertilizer application; ammonium nitrate (5 mg N/plant) and a half-strength of Hoagland's solution without N (50 ml/plant) twice a week. After three equal N applications, plants were treated with either normal (5 mg N/plant) or low (1 mg N/plant) N. N was supplied with a half-strength Hoagland’s solution lacking N twice a week. The total amount of N applied per plant for the entire period of the experiment was 100 mg for normal N and 31 mg for low N. When different N application started at the maximum tiller stage, the light period and temperature in the greenhouse were changed to 16 h light (22 °C)/8 h dark (13 °C) to stimulate flower development. Flag leaves were collected at midday at four different time points: 0, 5, 10, and 15 day after anthesis (DAA). Immediately after sample collection, all leaf tissues were frozen in liquid nitrogen and stored at −80 °C until use. Plants that were not used for flag leaf collection were grown until maturity for yield component analysis.

#### Analysis of NUE-related traits

Grains and straw harvested were dried in an oven at 65 °C for 3 d, and their dry weight was measured. Grain yield was calculated as grain weight (g) per plant. The concentrations of N in grains and straw were measured via dry combustion using a Vario MAX cube CNS (Elementar, Frankfurt, Germany) following the protocol of Kim et al. (2011). Grain protein content was calculated by multiplying grain N content by 6.15, a conversion value for soft red winter wheat. NUE for grain yield (NUEY) and grain protein (NUEP) were computed as follows.

NUEY = Grain yield (g)/N supplied (g); NUEP = Grain protein content (g)/N supplied (g) (Moll et al., 1982; Van Sanford & MacKown, 1987).
Quantification of carbohydrate and nitrogen compounds

The concentrations of chlorophyll, starch, total soluble sugars, nitrate, and ammonium were measured as described in Tamang et al. (2017). Flag leaf protein was quantified as described in Alpuerto et al. (2016). For total amino acid analysis, 75 mg of frozen tissues were homogenized in 450 µL of 0.83 M perchloric acid on ice. After centrifugation at 21,000 g at 4 °C for 20 min, the supernatant (300 µL) was neutralized with 75 µL of 1 M bicine (pH 8.3) and 70 µL of 4 M KOH. The precipitated salt was removed by centrifugation. The neutralized supernatant (80 µL) was mixed with 50 µL of 0.2 mM sodium cyanide resolved in 8 M sodium acetate and 50 µL of 168 mM ninhydrin resolved in 100% (v/v) 2-methoxyethanol. Following incubation at 100 °C for 15 min, 1 mL of 50% isopropanol was added immediately to the mixture. After cooling, absorbance at 570 nm was measured using a spectrophotometer. Glycine was used as the standard. Ninhydrin reacts with both amino acids and ammonium. Therefore, the ammonium contribution in each sample was calculated using the standard curve for ammonium and the ammonium content data, and then subtracted from the ninhydrin value.

Quantitative RT-PCR analysis

RNA extraction and cDNA synthesis were performed following the protocol described by Fukao and Bailey-Serres (2008). Quantitative RT-PCR was performed using the procedure described by Alpuerto et al. (2016). The 2-ΔΔCt method (Schmittgen & Livak, 2008) was used to calculate the relative expression of each gene. The list of primers and annealing temperatures used for this study is available in Table S1. The primers were designed to cover expression of the genes from all three hexaploid wheat genomes (Buchner et al., 2015; He et al., 2015).

Enzyme assays

The specific activity of nitrate reductase was assayed following the modified method of Ferrario-Méry et al. (1998). Leaf tissue (50 mg) was homogenized in 1 mL of a cold extraction buffer containing 50 mM MOPS-KOH (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium fluoride, 2 mM β-mercaptoethanol, 1 mM sodium molybdate, 10 mM flavin adenine dinucleotide, 1 mM nicotinamide adenine dinucleotide, 1% (w/v) polyvinylpyrrolidone (PVP), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were allowed to stand for 30 min on ice and then centrifuged at 12,000 g for 15 min at 4 °C. The supernatant (50 µL) was mixed with 450 µL of the reaction buffer containing 50 mM MOPS-KOH (pH 7.5), 1 mM sodium fluoride, 0.17 mM NADH, 10 mM potassium nitrate, and either 10 mM magnesium chloride or 5 mM EDTA. Following 8 or 16 min incubation at 25 °C, the reaction was terminated by the addition of 250 µL of 1% (w/v) sulfanilamide in 3 M HCl and 250 µL of 0.02% (w/v) n-naphthylethylenediamine dihydrochloride. After 10 min incubation at 25 °C, the absorbance at 540 nm was measured with a spectrophotometer. Sodium nitrite (0–500 µM) was used as the standard. The activation state of nitrate reductase was calculated by the enzyme activity quantified in the presence of magnesium chloride divided by the activity in the presence of EDTA.

Glutamine synthetase activity was measured using the modified protocol of Gibon et al. (2004). Crude protein was extracted from 50 mg of tissue in a cold extraction buffer (0.5 mL) containing 50 mM HEPS-KOH (pH 7.5), 10 mM magnesium chloride, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethoxy ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM benzamidine, 1 mM 6-aminocaproic acid, and 1 mM PMSF. The extract was placed on ice for 30 min and then centrifuged at 12,000 g for 15 min at 4 °C. The supernatant (50 µL) was mixed with a reaction buffer (950 µL) consisting of 50 mM HEPS-KOH (pH 7.5), 10 mM magnesium chloride, 10% (v/v) glycerol, 2 mM EDTA, 0.2 mM sodium metavanadate, 40 µM p1,p5-di (adenosine-5’)pentaphosphate, 1 mM phosphoenol pyruvate, 5 mM ATP, 0.6 mM NADH, 1 U/mL pyruvate kinase, 0.7 U/mL lactate dehydrogenase, 2 mM ammonium chloride, and 45 mM glutamate. For blank reactions, ammonium chloride and glutamate were omitted from the reaction buffer. The coupled NADH oxidation were monitored in the reaction mixtures at 340 nm for 30 min at 25 °C. The rate of NADH oxidation was calculated from the extinction coefficient of NADH, 6.22 mM⁻¹ cm⁻¹.

NAD-dependent glutamate dehydrogenase activity was assayed using the method of Turano et al. (1996) with the following modifications. Leaf tissue (50 mg) was homogenized in a cold extraction buffer that consists of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5% (v/v) glycerol, 0.05% (v/v), Triton X-100, 0.5% (w/v) PVP, and 1 mM PMSF. The extracts were placed on ice for 30 min and then centrifuged at 12,000 g for 15 min at 4 °C. The reaction mixture contained 50 µL of each crude extract, 100 mM Tris-HCl (pH 9.3), 1 mM calcium chloride, 0.25 mM NAD, and 35 mM glutamate in a final volume of 1 mL. For blank reactions, glutamate was omitted. The NADH oxidation was measured at 340 mM for 3 min at 25 °C using a spectrophotometer.
Results

Effect of N input on yield, yield components, and NUE-related traits

We grew two representative soft red winter wheat genotypes with contrasting NUE, VA08MAS-369 and VA07W-415, under normal and low N conditions, and then analyzed their grain yield, yield components, and NUE-related traits (Figure 1). At regular N input, the two genotypes displayed similar grain yield (grain weight per plant). Low N application reduced grain yield in both genotypes, but VA08MAS-369 maintained higher grain productivity than VA07W-415. Yield component analysis revealed that higher grain yield in VA08MAS-

Figure 1. Effect of nitrogen application on grain yield, yield components, and NUE-related traits in two wheat genotypes, VA08MAS-369 (369) and VA07W-415 (415). Nitrogen Use Efficiency for Yield (NUEY) = grain yield/nitrogen supplied. Nitrogen Use Efficiency for Protein (NUEP) = grain protein content/nitrogen supplied. Data represent means ± SE (n = 8 plants x 3 replicates). Bars not sharing the same letter are statistically significant (P < 0.05).
369 under low N supply resulted from greater grain number per plant and spike number per plant in this genotype. Reduced N application suppressed straw biomass accumulation in VA07W-415, but not in VA08MAS-369. Harvest index declined in response to low N in both genotypes, but a significant genotypic difference was not detected.

Grain N concentration clearly decreased under limited N in both genotypes although no genotypic difference was observed under N deprivation (Figure 1). In contrast with grain N, straw N concentration was unaltered by distinct N supply in the two genotypes. We also calculated the amount of N accumulated in grains and straw per plant. Limited N input largely decreased grain N accumulation per plant in both genotypes at a similar rate. On the other hand, low N reduced straw N accumulation per plant only in VA07W-415, primarily due to reduced straw biomass under N limitation. Finally, we compared the total amount of N accumulated in the whole plant (i.e., grain + straw). This value significantly declined under restricted N in both genotypes, but more N was detected in VA08MAS-369.

In cereal crops, a widely accepted definition of plant NUE for yield (NUEY) is the grain yield per unit of supplied N (Moll et al., 1982). This concept is also extended to NUE for grain protein (NUEP), which is defined as total grain protein divided by the amount of applied N (Van Sanford & MacKown, 1986). In this study, NUEY and NUEP were similar in both genotypes under normal N conditions (Figure 1). At low N, however, VA08MAS-369 exhibited higher NUEY and NUEP.

**Impact of N input on grain N and C compounds**

To evaluate the influence of N supply on the accumulation of major N and C compounds in grains, we quantified the concentrations of protein, total amino acids, starch, and total soluble sugars. Limited N input significantly reduced the accumulation of protein in grains of VA08MAS-369 and VA07W-415, but these values were not significantly distinct between the two genotypes at low N (Figure 2a). The level of total amino acids declined at low N in VA07W-415, whereas this level was not altered in VA08MAS-369 (Figure 2b). Reduced N application conversely increased the amount of starch in VA08MAS-369, but not in VA07W-415 (Figure 2c). The content of total soluble sugars was elevated at low N input in both genotypes, with higher accumulation in VA07W-415 (Figure 2d).

![Figure 2](image_url) Effect of nitrogen application on grain carbohydrate and nitrogen compounds. The concentrations of protein (a), total amino acids (b), starch (c) and total soluble sugars (d) were compared in grains of the two wheat genotypes with contrasting nitrogen use efficiency under normal and low N conditions. Data represent means ± SE (n = 3). Bars not sharing the same letter are statistically significant (P < 0.05).
Effect of N supply on the post-anthesis accumulation of major N and C compounds in flag leaves

Upon anthesis, leaves undergo senescence in which N and C compounds stored in leaves are remobilized into grains (Hirel et al., 2007; Kant et al., 2011). Flag leaves serve as the major N source that provides a large amount of assimilates for grains at the post-anthesis stage (Simpson et al., 1983). To monitor the post-anthesis accumulation of major N and C compounds in flag leaves under regular and low N conditions, we quantified the concentrations of protein, total amino acids, ammonium, nitrate, starch, and total soluble sugars along with chlorophyll, an indicator of leaf senescence. At normal N input, chlorophyll content declined in both genotypes after anthesis although the amount of chlorophyll was greater in VA80MAS-369 than VA07W-415 at most time points (Figure 3a). In contrast, the chlorophyll level declined more rapidly in VA08MAS-369 under limited N, indicating that leaf senescence was accelerated in this high NUE genotype. Similar to chlorophyll degradation, the post-anthesis decline of protein and total amino acids occurred more quickly in VA08MAS-369 than VA07W-415 under limited N supply (Figure 3b, 3c). The concentration of ammonium was decreased or unaltered within 5 DAA in all genotypes and treatments, but was elevated at 10 and 15 DAA (Figure 3d). Low N application suppressed the accumulation of ammonium in both genotypes over all time points, with higher concentrations in VA07W-415 at 5, 10, and 15 DAA. The amount of nitrate increased after anthesis in all genotypes and treatments (Figure 3e). Under N deprivation, no significant difference was detected between the two genotypes.

We also analyzed the accumulation of C compounds such as starch and total soluble sugars in flag leaves (Figure 3f, 3g). At normal N input, drastic changes in the starch level were not observed during 0–15 DAA. However, restricted N application significantly increased the concentrations of starch at anthesis (0 DAA), which rapidly declined by 15 DAA in the two genotypes with more rapid degradation in VA08MAS-369. Similar to starch, limited N supply increased the amount of total soluble sugars in both genotypes. Breakdown of total soluble sugars was observed at 15 DAA under N deprivation, but its concentration was similar in the two genotypes.

Influence of N input in the accumulation of mRNAs associated with leaf senescence, C and N metabolism in flag leaves

To determine whether physiological responses observed at distinct N application are linked with expression of genes associated with these processes, we monitored the transcript accumulation of representative genes involved in leaf senescence, C and N metabolism in flag leaves at the post-anthesis stage (Figure 4). For this analysis, we did not use RNA extracted from flag leaves at 15 DAA because high-quality RNA samples could not be obtained from these senesced leaves. **SENESCENCE-ASSOCIATED GENE 12 (SAG12)** is a senescence-inducible gene encoding a C1A cysteine protease. This gene has been used as a marker for leaf senescence in various plants including wheat (Cha et al., 2016; Guo & Gan, 2014; Sykorova et al., 2008). The level of SAG12 transcript was elevated after anthesis in all genotypes and N treatments, with stronger accumulation at limited N. Of the two genotypes, VA08MAS-369 contained more transcript of SAG12 under N deprivation, which corresponded with rapid leaf senescence and protein degradation in this genotype (Figure 3a, 3b).

Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is the most abundant protein in leaves and broken down to mobile N forms needed during grain filling (Kant et al., 2011). The abundance of mRNAs encoding Rubisco small (RbscSSU8) and large subunits (RbscLU) declined in flag leaves during 0–10 DAA. Limited N supply suppressed the accumulation of RbscSSU8 in both genotypes, with lower transcript levels in VA08MAS-369 over all time points. Unlike RbscSSU8, the amount of RbscLU was not largely affected by N deprivation in the two lines, but it appeared that this transcript was reduced more rapidly in VA08MAS-369 than VA07W-415 under low N.

We also analyzed mRNA accumulation of two representative α-amylase genes expressed in leaves, *Amy2* and *Amy4* (Buchner et al., 2015). The level of *Amy2* transcript was similar in VA08MAS-369 and VA07W-415 under N limitation. However, the abundance of *Amy4* was constantly higher in VA08MAS-369 over all time points, which was linked with more rapid starch degradation in flag leaves of this genotype at low N (Figure 3f).

We extended this analysis to genes associated with N transport, assimilation, and remobilization (Figure 5). Plants possess two classes of nitrate transporters: NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY (NPF) and NITRITE TRANSPORTER 2 (NRT2) (O’Brien et al., 2016). NPF transports a wide range of substrates including nitrate, peptides, amino acids, dicarboxylates, glucosinolates, indole-3-acetic acid (IAA), and abscisic acid (ABA) (Leran
The members of this family are categorized into eight subfamilies based on phylogenetic relationships, but substrate specificity is not conserved within each subfamily. Unlike NPF, most NRT2 transport only nitrate (Von Wittgenstein et al., 2014). Because most N transporter studies have been performed in roots, the role of these transporters in leaves is not well known. We selected two NPF7 genes and one NRT2 gene that are expressed in leaves and whose transcript accumulation was correlated with grain yield in wheat (He et al., 2015). The levels of NPF7.1 and NPF7.2 were more abundant in VA08MAS-369 than VA07W-415 at all time points under N deprivation.

**Figure 3.** Effect of nitrogen application on the accumulation of chlorophyll (a), protein (b), total amino acids (c), ammonium (d), and nitrate (e), starch (f), and total soluble sugars (g) in flag leaves at the post-anthesis stage in the two wheat genotypes with contrasting nitrogen use efficiency. Data represents means ± SE (n = 3). Bars not sharing the same letter are statistically significant at each time point (P < 0.05).
The accumulation of NRT2.1 mRNA was significantly higher at 10 DAA in VA08MAS-369 under N deficiency.

Nitrate assimilation still occurs in senescing flag leaves of wheat after anthesis (MacKown & Van Sanford, 1986; Martin, 1982). Nitrate reductase (NR) is the first enzyme in the nitrate assimilation pathway. The current study showed that the transcript of NR1 was reduced upon anthesis at normal and low N in both genotypes, but was significantly more abundant in VA08MAS-369 under limited N at 0 and 10 DAA. Glutamine synthetase (GS) and glutamate dehydrogenase (GDH) are key enzymes involved in N remobilization from senescent leaves to grains (Bernard & Habash, 2009; Masclaux-Daubresse et al., 2010). Of four GS mRNAs analyzed, GS1 was senescence-inducible and its transcript accumulation was highest in VA08MAS-369 over all time points under N limitation. The levels of GSr and GS2 transcripts remained relatively stable during 0–10 DAA, but GSr and GS2 mRNAs were more highly accumulated at 10 DAA and 0 DAA, respectively, under low N supply. Low N induced mRNA accumulation of GDH2 in both genotypes; the expression level was higher in VA08MAS-369 over all time points under limited N.

NAC is a large transcription factor family that regulates various abiotic and biotic stress responses in plants (Nuruzzaman et al., 2013). In wheat, overexpression of NAC2 increased the expression of NRT2.1, NPF7.1, NPF7.2, and GS2 in shoots and roots, resulting in enhanced grain yield and grain N concentration under low N supply (He et al., 2015). In the present study, the level of NAC2 transcript was induced by low N in both genotypes, with significantly higher accumulation in VA08MAS-369 (Figure 5). This increase in NAC2 mRNA was correlated with higher transcript accumulation of NPF7.1, NPF7.2, NRT2.1, and GS2 in the high NUE accession under limited N, confirming the significance of this transcription factor in NUE regulation.

**Impact of N supply on activities of enzymes associated with N assimilation and remobilization in flag leaves**

Our gene expression analysis indicated that mRNA accumulation of the representative genes encoding NR, GS, and GDH were more abundant in the high NUE accession, VA08MAS-369, at low N. However, these enzymes may be regulated at the post-transcriptional, translational, and post-translational levels. Indeed, the activity of nitrate reductase is controlled by protein phosphorylation and 14-3-3 proteins (Kaiser et al., 1999). Therefore, we assayed the activities of these three enzymes in the samples used for gene expression analysis (Figure 6). NR is a phosphoprotein, but both phosphorylated and dephosphorylated forms are equally active. Phosphorylated NR physically interacts with inhibitory 14-3-3 proteins (nitrate
reductase inhibitory proteins, NIPs) in the presence of Mg\(^{2+}\), inactivating this enzyme (Kaiser et al., 2001). In the presence of EDTA, maximal NR activity can be measured because the chelate compound prevents the binding of phosphorylated NR to 14-3-3 proteins. The present work indicated that maximal NR activity decreased upon anthesis when N was sufficiently supplied (Figure 6a). At low N, NR activity remained low during the post-anthesis period (0–15 DAA), but was higher in VA08MAS-369 at 15 DAA. In the presence of Mg\(^{2+}\), where part of NR is inactivated, the changing trend in NR activity was almost similar to the maximal NR activity, with higher activity in VA08MAS-369 at 5 and 15 DAA under limited N conditions. We also determined the NR activation state, but it was not altered by N supply in the two genotypes at either regular or low N (Figure 6a).

GS and GDH play a key role in N remobilization in senescing leaves (Bernard & Habash, 2009; Masclaux-Daubresse et al., 2010). GS activity was significantly elevated in response to low N in VA08MAS-369 at most time

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**Figure 5.** Relative mRNA levels of genes associated with nitrogen transport, assimilation, and remobilization in flag leaves of the two wheat genotypes with contrasting nitrogen use efficiency at normal and low N input. Data represents means ± SE (n = 3). Bars not sharing the same letter are statistically significant (P < 0.05). NPF7.1 and NPF7.2, group 7 of NPF family nitrate transporters; NRT2.1, a NRT2 family transporter; GS1, GSe, GSr, and GS2, glutamine synthase 1, e, r, and 2; GDH2, glutamate dehydrogenase 2; NAC2; a NAC transcription factor directly regulating the expression of NPF7.1, NPF7.2, NRT2.1, and GS2.
points, whereas the induction of GS activity under limited N was not prominent in VA07W-415 (Figure 6b). Similarly, low N considerably increased GDH activity in VA08MAS-369 over all time points, but the enzyme activity was unaltered by N limitation in VA07W-415 (Figure 6c).

**Discussion**

The results presented here demonstrated that accelerated leaf senescence and nitrogen remobilization in flag leaves at the post-anthesis stage were associated with higher grain yield, NUEY, and NUEP under N deprivation in soft red winter wheat. In general, delayed onset and slow progression of senescence are linked to improved grain yield in wheat (Christopher et al., 2016; Gregersen et al., 2013). However, these observations were made when a sufficient amount of N was supplied to hard wheat accessions. The contribution of leaf senescence (slow vs. fast) to grain productivity may depend on N availability (high vs. low) and/or wheat classes (hard vs. soft wheat). Under N limitation, accelerated senescence can benefit efficient N remobilization. Indeed, the increased rate of senescence was closely associated with rapid protein and starch breakdown in flag leaves under N deficiency (Figure 3b, 3f). The progress in flag leaf senescence and starch degradation was consistent with the transcript accumulation of a senescence marker gene, SAG12 and α-amylase gene, Amy4 (Figure 4). In addition, the mRNA level of Rubisco small subunit gene was suppressed in VA08MAS-369 at all time points under N limitation. Reduced Rubisco biosynthesis in the high NUE genotype under low N availability may contribute to the efficient allocation of amino acids into grains because the limited amino acid resource is not used for
the production of the most abundant leaf protein. Altogether, post-anthesis senescence including chlorophyll, protein, and starch breakdown was more strikingly accelerated in VA08MAS-369 than VA07W-415 under N deprivation, contributing to efficient translocation of mobile N and C compounds into grains in the high NUE genotype.

Besides grain yield, grain protein content can also be influenced by the rate of senescence in wheat. For example, the introgression of Gpc-B1 into three spring wheat genotypes accelerated the rate of flag leaf senescence under normal N conditions, leading to increased grain protein content (Uauy et al., 2006a). Map-based cloning revealed that Gpc-B1 locus encodes a NAC transcription factor gene, TaNAM-B1 (Uauy et al., 2006b). Knockdown of four related TaNAM genes by RNAi in spring wheat reduced the rate of senescence at regular N supply, resulting in low grain protein. In the present study, accelerated senescence did not affect grain protein content, but rather increased grain yield under N deficiency (Figure 1, 2a, 3a).

The inconsistent effect of rapid senescence on grain yield and protein content may result from genotypic differences between soft and hard wheat and/or distinct N availability in these experiments. At low N, starch and soluble C were considerably accumulated in flag leaves of both genotypes at anthesis, relative to normal N application (Figure 3f, 3g). Increased accumulation of C reserves under N limitation is commonly observed in various tissues and species including wheat flag leaves (Ruuska et al., 2008; Scheible et al., 1997; Scofield et al., 2009; Wang & Tillberg, 1996). A possible explanation for this phenomenon is a distinct C requirement for N uptake and assimilation under low vs. high N supply. At high N, a large amount of C consumption is necessary to support energetically expensive N uptake and assimilation. In contrast, energy demand for these processes is lower under limited N, resulting in increased starch and soluble C accumulation. It has also been suggested that nitrate serves as a signaling molecule to suppress starch accumulation (Scheible et al., 1997). Distinct contributions of rapid senescence to grain yield vs. grain protein content at low and high N may be determined by the amount of C reserves available at anthesis. An increase in grain yield largely depends on the level of C transported from vegetative tissues because the major part of wheat grain is starch. At low N, a large quantity of C reserves is available at anthesis, leading to increased grain yield rather than grain protein content. At high N, the amount of C reserves is limited at anthesis, resulting in an elevation of grain protein content per grain when senescence is promoted. Further investigation at the molecular level is required to elucidate the regulation of N allocation to these competitive, yet commercially important traits under distinct N availability.

In senescing leaves, N remobilization is activated as N assimilation is inhibited (Smart, 1994; Xu et al., 2012). However, there is biochemical evidence that nitrate assimilation still occurs in flag leaves of wheat after anthesis (MacKown & Van Sanford, 1986; Martin, 1982). In the present study, the level of NR1 mRNA and NR activity was significantly higher in VA08MAS-369 than VA07W-415 after anthesis at low N (Figure 5, 6a), suggesting that the high NUE genotype maintained the capability of post-anthesis nitrate assimilation under N deficiency. Sustained nitrate assimilation at the post-anthesis stage may be crucial for wheat grain filling at low N. Indeed, at high and medium N, only 5–17% of the grain N came from the N assimilated after anthesis, whereas the proportion of the grain N derived from post-anthesis N assimilation was 37% at low N (Palta & Fillery, 1995). These data suggest that maintained capability of nitrate assimilation can benefit higher grain yield, NUEY, and NUEP in VA08MAS-369 under N deficiency.

Plants possess two classes of GS that are located in the cytosol or chloroplast. Cytosolic GS is the predominant form in senescing leaves and located in vascular bundles, suggesting its critical role in N remobilization from senescing leaves to grains (Kichey et al., 2005; Lam et al., 1996; Xu et al., 2012). In support of this notion, double knockout mutation of cytosolic GS genes in maize, gln1-3 and gln1-4, increased the amount of amino acids and total N left in leaves below ears after grain filling, resulting in reduced grain yield (Martin et al., 2006). Unlike cytosolic GS, chloroplastic GS is predominant in photosynthetically active leaves, primarily assimilating ammonium generated by nitrate reduction and photorespiration in chloroplasts (Lam et al., 1996). In wheat, three cytosolic GS isoforms (GS1, GSε, and GSγ) and one chloroplastic isoform (GS2) have been recognized (Bernard et al., 2008). The present work revealed that GS1 mRNA was induced in senescing flag leaves of the two genotypes regardless of N rates, with further increase in VA08MAS-369 under N limitation (Figure 5). In addition, the transcript abundance of another cytosolic GS, GSγ, was relatively stable over the course of this experiment, but the mRNA level was higher at 10 DAA in VA08MAS-369 at low N. In wheat, GS1 and GSγ are primarily expressed in senescing leaves under regular N application (Bernard et al., 2008). It is most likely that the significant induction of these two major GS isoform genes in response to N limitation primarily contributes to higher GS enzyme activity in post-anthesis flag leaves in VA08MAS-369 (Figure 6). Regarding chloroplastic GS2 that mainly works for N assimilation, its mRNA accumulation was more abundant in the high NUE genotype at anthesis (0 DAA) under N deprivation, but was relatively stable over the course of the experiment in both genotypes (Figure 5). This implies that the assimilation capability of ammonium to generate
glutamine through GS2 in the chloroplast is still active in senescing leaves even under N limitation, with higher activity in VA08MAS-369.

Although GDH catalyzes the reversible amination/deamination reaction between glutamate and 2-oxoglutarate, most physiological and genetic studies have demonstrated that deamination of glutamate is the major catalytic reaction (Bernard & Habash, 2009; Masclaux-Daubresse et al., 2010). The proposed role of deaminating NAD-dependent GDH is to supply a TCA intermediate, 2-oxoglutarate, to the TCA cycle, supporting continuous ATP production (Masclaux-Daubresse et al., 2008; Miflin & Habash, 2002). The resulting ammonium in this reaction will be assimilated into glutamine by cytosolic GS1, which will be transferred to grains. This mechanism may be more important at the later stage of leaf senescence because this process enables a large quantity of ATP synthesis via the TCA cycle and electron transport system even in C-starved leaves, supporting energetically expensive N transport and assimilation during grain filling. In the present study, NAD-dependent GDH was significantly induced under N deficiency in senescing leaves of VA08MAS-369, but not VA07W-415 (Figure 6c), consistent with the induction of GDH2 mRNA (Figure 5). In the same senescing leaves under low N, GS activity and GS1 mRNA accumulation were also higher in VA08MAS-369 (Figure 5, 6b). It is likely that increased GS and NAD-dependent GDH activities in VA08MAS-369 promote the remobilization of N compounds from source leaves to grains even under C-starved conditions at the latter senescence stage, leading to higher grain yield, NUE, and NUEP in this genotype. As discussed above, both post-anthesis assimilation of newly absorbed N and remobilization of stored N are critical for wheat grain filling at low N supply. These processes require the influx and efflux of mobile N forms in senescing leaves. The present work revealed that NPF7.1, NPF7.2, and NRT2.1, whose expression in leaves was correlated with grain yield at low N (He et al., 2015), was highly induced in VA08MAS-369 under N deprivation (Figure 5). The molecular function of these transporters in N influx and efflux is still unknown in wheat leaves. In rice, however, one of four NRT2 genes, OsNRT2.3b is involved in nitrate remobilization in leaves, and overexpression of this transporter gene increased grain yield and NUE (Fan et al., 2016). The results presented here suggest that these N transporters expressed in senescing leaves can aid in efficient N transport and remobilization in the high NUE genotype. Further research using transgenic approaches will determine the functional importance of these N transporters in N remobilization and NUE in wheat.

In conclusion, this study has demonstrated that accelerated leaf senescence and vegetative N remobilization benefit grain yield under N deprivation. Stronger expression of genes associated with N transport, assimilation, and remobilization reflected higher grain yield, NUE, and NUEP in VA08MAS-369 under limited N. A combination of elevated NAD-dependent GDH and GS activities may be a key mechanism underlying efficient N remobilization from senescing leaves to grains under low N availability. These processes enable continuous ATP production even under C starvation at the latter senescence stage, supporting energetically expensive N transport and assimilation during grain filling.

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