Nitrogen use efficiency is regulated by interacting proteins relevant to development in wheat

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Summary
Wheat (Triticum aestivum) has low nitrogen use efficiency (NUE). The genetic mechanisms controlling NUE are unknown. Positional cloning of a major quantitative trait locus for N-related agronomic traits showed that the vernalization gene TaVRN-A1 was tightly linked with TaNUE1, the gene shown to influence NUE in wheat. Because of an Ala^{180}/Val^{180} substitution, TaVRN-A1a and TaVRN-A1b proteins interact differentially with TaANR1, a protein encoded by a wheat orthologue of Arabidopsis nitrate regulated 1 (ANR1). The transcripts of both TaVRN-A1 and TaANR1 were down-regulated by nitrogen. TaANR1 was functionally characterized in TaANR1::RNAi transgenic wheat, and in a natural mutant with a 23-bp deletion including 10-bp at the 5′ end of intron 5 and 13-bp of exon 6 in gDNA sequence in its gDNA sequence, which produced transcript that lacked the full 84-bp exon 6. Both TaANR1 and TaHOX1 bound to the Ala^{180}/Val^{180} position of TaVRN-A1. Genetically incorporating favourable alleles from TaVRN-A1, TaANR1 and TaHOX1 increased grain yield from 9.84% to 11.58% in the field. Molecular markers for allelic variation of the genes that regulate nitrogen can be used in breeding programmes aimed at improving NUE and yield in novel wheat cultivars.

Introduction
Nitrogen (N) is the most important nutrient for plant development and growth, and soil is often supplemented with N fertilizer to ensure successful seed production and high grain yield for non-N-fixing food crops such as wheat (Triticum aestivum L.), rice (Oryza sativa L.) and maize (Zea mays L.) (Santi et al., 2013). A sevenfold increase in the use of N fertilizer was found to be associated with a twofold increase in food production over the past four decades (Hirel et al., 2007; Shrawat et al., 2008). Given the projected increase in the world’s human population to over 9 billion by 2050, a further threefold increase in N input is expected to be needed to meet the world’s demand for major crop products (Cormier et al., 2013; Schroeder et al., 2013).

Although N fertilizer has the most direct and efficient approach for increasing crop production, the synthetic N fertilizers supplied to soils have immediate and adverse effects on the environment and climate. Only 30%–35% of added N fertilizers are taken up and used by wheat plants in the year of application, and the remaining 65%–70% (assuming fertilizer–soil equilibrium) is lost, predominantly as nitrous oxide, through gaseous plant emission, soil denitrification, surface run-off, volatilization and leaching, which contributes to atmospheric greenhouse gases and environmental pollution (Gaju et al., 2011; Raun and Johnson, 1999). Developing varieties of wheat that require less N input yet maintain the same or higher grain yields is an economically and environmentally sustainable goal in international agriculture.

The response of plants to added N involves genes in several pathways, including uptake, assimilation and translocation, as well as recycling and remobilization of N within the plants, and these responses differ according to genotype, environment, N level and plant age (Krapp, 2015). Wheat is traditionally divided into two types: winter wheat that requires the plant to be exposed to low temperatures during a winter season to accelerate its transition from vegetative to reproductive development (vernalization), and spring wheat, which has no requirement for vernalization (Pugsley, 1971). Compared to spring wheat, winter wheat requires significantly more N to achieve maximum grain yield, because it has a longer growing season with greater potential for leaching, volatilization and run-off losses (Goos and Johnston, 1999). Dual-purpose winter wheat cultivars planted in the Southern Great Plains (USA) require more N, because N is removed in grazed forage (MacKown and Carver, 2007). Thus, it is of interest to determine which genes regulate nitrogen use efficiency (NUE) when limited N...
fertilizers are supplied to winter wheat. Multiple genes involved in the response to N that influence root/shoot growth, N metabolism and N content have been identified in Arabidopsis thaliana. The N-related genes are divided into two classes: those involved in growth, such as Arabidopsis nitrate regulated 1 (ANR1) (Zhang and Forde, 1998) and superroot (Boerjan et al., 1995), and those involved in nitrogen metabolism pathways, such as the SAT1 transporter for initial ammonia uptake (Kaiser et al., 1998), CHL2 for nitrate reductase (LaBrie et al., 1992) and genes encoding three enzymes involved in ammonium assimilation (i.e. glutamine synthetase (GS) (Peterman and Goodman, 1991), glutamate synthase (GLU1) and glutamate dehydrogenase (GDH1) (Lam et al., 1995)). Other genes involved in the reduction of nitrate to nitrite, and the subsequent reduction of nitrite to ammonium, include nitrate transporter (NRT) (Tsay et al., 1993) and nitrite reductase (NiR) (Leydecker et al., 2000). In rice, heterotrimeric G proteins were found to regulate NUE (Sun et al., 2014). In wheat, TaNaC2-SA, a transcription factor encoding a NAC (NAM, ATAF and CUC), was found to bind to the promoter region of the genes encoding nitrate transporter and glutamine synthetase, resulting in an enhanced ability of roots to acquire nitrogen and increased grain yield (He et al., 2015). TaNFYA-B1, a subunit of nuclear factor Y, stimulated root development and up-regulated the expression of the genes encoding nitrate transporters in roots, resulting in a significant increase in nitrogen uptake and grain yield (Qu et al., 2015).

In Arabidopsis, quantitative trait loci (QTLs) mapping is an efficient method to determine which genomic regions contribute to NUE in different N environments (Loudet et al., 2003; Rauh et al., 2002). Hundreds of wheat QTLs or genomic regions are associated with N-related agronomic traits, but each accounts for only a small part (<25%) of the total phenotypic variation (An et al., 2006; Cormier et al., 2014; Habash et al., 2007; Mickelbart et al., 2015; Quarrie et al., 2005; Xu et al., 2014), which has limited molecular cloning of the mapped QTLs. A glutamate synthase (GoGAT) gene on wheat chromosome 3B contributed to NUE in wheat based on physical mapping, sequence analysis and functional validation of an NUE MetaQTL (Quraishi et al., 2011). In the previous study, the TaVRN-A1 locus on chromosome 5A was mapped to colocalate with a QTL for NUE in wheat, but it was unknown how this locus contributed to NUE in wheat. In this study, we evaluated a population of recombinant inbred lines (RILs) of winter wheat under contrasting N fertilization regimes and identified a major QTL for N-related agronomic traits. We cloned the gene associated with this QTL and further identified interactors of the protein encoded by the cloned gene.

**Results**

**Genetic identification of a major QTL for NUE**

Two populations from the same 96 RILs and the parental lines ‘Jagger’ and ‘2174’ were initially grown in a greenhouse on Kirkland silt loam soil in which N was severely deficient but other macronutrients were sufficient (Table S1), under long-day conditions and constant temperature, but without vernalization (Figure S1a). Under such conditions, the winter wheat plants remained in the vegetative stage and showed similar phenotypes until 11 weeks after planting (Figure S1b). When plants were starved of N, two different levels of N, 14 mg N kg⁻¹ soil (25 N, equivalent to 25 kg N ha⁻¹) and 56 mg N kg⁻¹ soil (100 N, equivalent to 100 kg N ha⁻¹), were used to fertilize the plants. Parental lines started to show differences in their morphological traits 3 weeks after N fertilization (Figure S1c).

As the N-fertilized populations were grown under the same, continuous greenhouse conditions until they reached maturity, 10 N-related traits were phenotyped. A major QTL on the long arm of chromosome 5A was mapped associated with the N-related traits, but the colocalized QTLs largely differed in the log of the odds (LOD) for magnitudes of the total variation between the two contrasting N regimes (Figure 1a, b; Table S2). Furthermore, statistical analyses showed that interactions of the QTLs with N rate were significant or highly significant for five traits including heading date, leaf chlorophyll content, grains per spike, grain yield per plant and harvest index (Table S3). Therefore, these five traits should be regulated by the gene at the QTL. In the greenhouse study, winter wheat plants were not vernalized, to avoid the effect of vernalization; hence, these plants showed large variations in heading date. Those lines carrying the Jagger allele had an earlier average heading date and produced more grains, but lines carrying the 2174 allele showed delayed heading and produced fewer grains. The average grain yield of those lines carrying the Jagger allele was 0.84 g per plant, an increase of 140% compared with 0.35 g per plant for lines carrying the 2174 allele, when plants were grown in the same 100 N soil (Table S2). These results indicated that the N-regulated gene at the QTL affected development and thus grain yield. The QTL on wheat chromosome 5AL for the multiple N-related traits was temporarily designated QNue.osu-5A, and the gene causing the QTL was designated TaNUE1, the gene shown to have pleiotropic effects in hexaploid wheat.

To determine which gene is TaNUE1, fifteen recombinant events that occurred at QNue.osu-5A were used to fine map the QTL in the Kirkland soil and in the same greenhouse with long days and constant temperatures as used to find QNue.osu-5A (Table S4). These recombinant lines were generated from 6410 BC1F3 plants that were used to clone the gene responsible for vernalization requirement duration in winter wheat in our previous study (Li et al., 2013). PCR markers for the allelic variation of each gene were developed and mapped in the RILs (Figure S2). Seven of these recombinant lines were selected for testing in the commercial soil. Based on the segregation of heading date in these recombinant lines (Figure 1c; Table S4), the location of TaNUE1 was narrowed down to a region containing three candidate genes, TaCYB5, TaAGLG1, and TaVRN-A1 (Figure 1c).

The seven recombinant lines were also tested in a field with Teller loam soil with 7.5 ppm NO₃⁻ N, representing an N-stress condition (Table S1). As shown in Figure 2, plants carrying the Jagger TaNUE1 allele showed an increase of 18.1% in grain yield (Figure 2a), due to a significant increase in grains/spike (Figure 2b) and biomass (Figure 2c), compared with plants carrying the 2174 TaNUE1 allele in the low-N scenario. These field results further confirmed that TaNUE1 in the targeted region affected grain yield in the low-N scenario.

**Identification of candidate genes for TaNUE1**

Among the three candidate genes, only TaVRN-A1 showed allelic variation in the promoter and introns, but there was no significant difference in gene expression between the two TaVRN-A1 alleles before N was used and within 2 weeks after N was used (Figure S3). The result suggested that the effects of TaNUE1 on the mapped traits should rely on its protein sequence but not the transcript level.

Among the three candidate proteins for TaNUE1, there were no differences between the proteins encoded by the two alleles of TaCYB5; this gene was thus excluded as a candidate. At the protein sequence, TaAGLG1 had one amino acid substitution,
whereas TaVRN-A1 had two. Next, we tested whether the two MADS proteins, TaAGLG1 and TaVRN-A1, interacted with the wheat orthologue of ANR1 in Arabidopsis, which is also a MADS-box protein. ANR1 has a key role in regulating lateral root growth in response to changes in the external NO$_3^-$/CO$_2$ supply (Zhang and Forde, 1998). TaANR1 (GenBank Accession Number AM502900) had 70% similarity to the Arabidopsis ANR1 (GenBank Accession Number CAB09793). MADS-box proteins may form a protein complex, in which one MADS-box protein may positively or negatively regulate the expression of another MADS-box gene via a direct interaction (Jack, 2004). TaVRN-A1, TaAGLG1 and TaANR1a from Jagger were expressed in Escherichia coli. In pull-down assays, no interaction was observed between TaANR1a and TaAGLG1 (Figure 3a). However, the two TaVRN-A1 proteins encoded by the Jagger and 2174 alleles interacted differently with the TaANR1a protein (Figure 3b–e).

The alignment of TaVRN-A1 and TaANR1a proteins suggested that they might have interacting sites in the K box (Figure S4). The critical sites at which the Jagger TaVRN-A1a and 2174 TaVRN-A1b proteins interact with TaANR1a were identified by three
experiments. TaVRN-A1a(85–191) and TaVRN-A1b(85–191), which included both Leu$^{117}$/Phe$^{117}$ and Ala$^{180}$/Val$^{180}$ substitutions, interacted differentially with TaANR1a (Figure 3b). Similarly, TaVRN-A1a(131–191) and TaVRN-A1b(139–191), which included an Ala$^{180}$/Val$^{180}$ substitution but not a Leu$^{117}$/Phe$^{117}$ substitution, interacted differentially with TaANR1a (Figure 3c). However, TaVRN-A1a(85–179) and TaVRN-A1b(85–179), which included a Leu$^{117}$/Phe$^{117}$ substitution but not an Ala$^{180}$/Val$^{180}$ substitution, interacted with TaANR1a in a similar manner (Figure 3d). The intensity of interacted TaANR1a proteins was significantly stronger in the pull-down TaVRN-A1b protein than the pull-down TaVRN-A1a protein including the Ala$^{180}$/Val$^{180}$ substitution (Figure 3e). These comparative in vitro interaction studies indicated that the differential interaction of TaVRN-A1a and TaVRN-A1b with TaANR1a was caused not by the Leu$^{117}$/Phe$^{117}$ substitution but by the Ala$^{180}$/Val$^{180}$ substitution.

Next, TaVRN-A1 and TaANR1a were confirmed to interact with each other in vivo using bimolecular fluorescence complementation (BiFC). When TaANR1a was cloned into a pEG101-YFP vector and the construct transformed into tobacco leaves, enriched yellow fluorescent signals of TaANR1a-YFP were detected predominantly in the nucleus (Figure S5). The same pattern was observed for TaVRN-A1 (Li et al., 2013). When TaVRN-A1a was cloned into a pEG201-YN vector, and TaANR1a was cloned into a pEG202-YC vector, and these constructs were simultaneously expressed in the same cell, yellow fluorescence was again observed in the nucleus, confirming the in vivo interactions between these two proteins (Figure 4a–d). The differential interactions of TaVRN-A1a and TaVRN-A1b with TaANR1a provided initial evidence that TaVRN-A1 might be the better candidate for TaNUE1.

Regulation of TaVRN-A1 in normal and transgenic wheat plants

A link between TaVRN-A1 and N regulation was discovered not only via its protein interaction with TaANR1, but also through its expression being regulated by N. In a separate experiment, four RILs carrying the Jagger TaVRN-A1a allele were tested in the same Kirkland soil and the same commercial soil. When N fertilizer was applied to plants grown for 11 weeks without fertilizer, transcription of the TaVRN-A1a allele was greatly down-regulated after 3 weeks (Figure 5a).

Next, the RNA interference (RNAi) approach was used to disrupt TaVRN-A1a expression in Jagger, and two individual transgenic plants (T20 and T40) were successfully generated. Transgenic T1 populations were tested in the same Kirkland soil used to identify QNue.osu-5A. Transcription of TaVRN-A1a was greatly reduced in the positive transgenic plants compared with nontransgenic plants (Figure 5b). Furthermore, the down-regulatory effects of TaVRN-A1a by RNAi were reflected in the heading date—for nontransgenic plants, this was 130 days, while it was 138 days for the positive transgenic plants (Figure 5c). In the positive plants, transcription of TaVRN-A1a was further down-regulated by N, compared with unfertilized positive plants (Figure 5b). After N fertilization, the heading date of positive transgenic plants was delayed to 143.4 days (Figure 5c). When T2 populations of T20 (Figure 5d) and T40 (Figure 5e) lines were tested in the commercial soil without vernalization, a typical positive plant carrying TaANR1::RNAi demonstrated delayed heading, fewer fertile tillers and decreased grain yield, compared with either the nontransgenic plants or the wild-type Jagger.

Down-regulation of TaVRN-A1 transcripts by N was also observed in six of seven winter wheat cultivars/lines that were naturally vernalized under field conditions. Vernalized plants were moved into a greenhouse where nutrients, temperature and light were controlled. Transcription of TaVRN-A1 significantly decreased 3 weeks after application of N (Figure 5g). Down-regulation of TaVRN-A1 (Figure 5f) and delayed heading and increased biomass by N (Figure 5h) were also observed in spring wheat cv. Bobwhite.

Function of TaANR1 in a natural mutant and transgenic wheat

Two pieces of evidence confirmed that the TaANR1 orthologue is also regulated by N in wheat. Firstly, a natural mutant of TaANR1 was found to have a genetic effect on wheat development and growth. When conserved primers for homoeologous TaANR1 genes were designed to test expression profiles, TaANR1 transcripts were found not visibly in leaves but predominantly in roots in both Jagger and 2174. The PCR product was 250 bp
in size in Jagger, but showed an additional band besides the 250-bp band in 2174 (Figure 6a). Sequence analysis indicated that the cDNA products with the single band from Jagger were a mixture of homoeologous TaANR1 transcripts from chromosome 2A (TGACv1_scaffold_093384_2AL) and chromosome 2D (TGACv1_scaffold_159992_2DL). In 2174, the cDNA products

Figure 3  In vitro interactions between MADS proteins. TaANR1 is a protein with MBP-tag, and TaVRN-A1 and TaAGLG1 are the proteins with HIS-tag. (a) The protein interaction of TaANR1a (60.1 kDa) and Jagger TaAGLG1a protein (23.79 kDa) or 2174 TaAGLGb (23.78 kDa). Diamonds represent an area where no interacting proteins are observed between the TaANR1a and TaAGLG1 proteins. (b) The protein interaction of TaANR1a with TaVRN-A1 (85–191) including both Leu117/Phe117 and Ala180/Val180 substitutions. The interacting proteins of TaANR1a with the 2174 TaVRN-A1b (15.8 kDa) indicated by a star are stronger in protein band intensity than the interacting proteins of TaANR1a with the Jagger TaVRN-A1a (15.7 kDa) indicated by a triangle. (c) The protein interaction of TaANR1a with TaVRN-A1 (139–191) including Ala180/Val180 substitution only. The interacting proteins of TaANR1a with the 2174 TaVRN-A1b (9.3 kDa) indicated by a star are stronger in protein band intensity than the interacting proteins of TaANR1a with the Jagger TaVRN-A1a (9.3 kDa) indicated by a triangle. (d) The protein interaction of TaANR1a with TaVRN-A1 (85–179) including Leu117/Phe117 substitution only. The interacting proteins of TaANR1a with the 2174 TaVRN-A1b (14.5 kDa) indicated by a star are similar in intensity than the interacting proteins of TaANR1a with the Jagger TaVRN-A1a (14.4 kDa) indicated by a triangle. At least three independent replicates were performed for each of these interactions. M indicates a protein marker. (e) Comparison of interacted TaANR1 proteins between TaVRN-A1a and TaVRN-A1b proteins.

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of the upper band were from TaANR1 on chromosome 2D, and the cDNA products of the lower band were from TaANR1b on chromosome 2A; however, the cDNA fragment of the TaANR1b gene was 166 bp in size because the 84-bp exon 6 was missing. To determine whether this missing exon 6 was caused by an exon-skipping event, or by a deletion event at the gDNA level, the gDNA products of this gene were cloned and sequenced. Results showed that the lack of exon 6 in its cDNA was caused by a 23-bp deletion event comprising 10-bp at the 5′ end of intron 5 and 13-bp of exon 6 in the gDNA sequence of 2174 (Figure 6b). This included the AG splice site at the 5′ end of intron 5, resulting in the loss of the full 84-bp exon 6 in its mRNA, and thus 28 amino acids. These missing amino acids were included in the interaction site of TaANR1 with TaVRN-A1 (Figure S4).

Based on the 23-bp indel, a polymorphic PCR marker for TaANR1 was developed (Figure 6c). When phenotypic data of the RIL population used to map QNue.osu-5A were analysed for genetic effects on TaANR1, there was a difference of 5.2 days in the Southern Great Plains (Table S5). Secondly, three individual transgenic Jagger plants with TaANR1::RNAi were successfully generated, and their T1 populations were tested in the commercial soil. TaANR1 transcript was down-regulated in positive plants compared with nontransgenic plants, and TaANR1 transcription was down-regulated by N in the positive transgenic plants and nontransgenic plants (Figure 6e). Based on this result, the heading date of transgenic plants was 2.5 days earlier — this confirms that TaANR1 has a minor and repressive effect on heading. A typical positive plant carrying TaANR1::RNAi showed an earlier developmental stage but fewer tillers during the juvenile phase compared with a nontransgenic plant (Figure 6f).

TaANR1 and TaHOX1 bound to the Ala180/Val180 position of TaVRN-A1

The Ala180 in the TaVRN-A1 protein was found to be critical for maintaining the ability to interact with TaHOX1, a homeobox protein involved in wheat heading date, and the mutated Val180 in TaVRN-A1 decreased the ability to bind with TaHOX1 (Li et al., 2013). In this study, the Ala180 in the TaVRN-A1a protein was less able to interact with TaANR1a than the mutated Val180 in TaVRN-A1b (Figure 3b–d). These results suggest that TaVRN-A1, TaANR1 and TaHOX1 might form a protein complex in wheat. To test whether TaANR1 interacts with TaHOX1, these two proteins were analysed for in vitro and in vivo interactions.

No significant interaction was detected between TaANR1a and TaHOX1 in vitro (Figure S6). However, TaANR1a interacted with TaHOX1 in vivo (Figure 4e–h). This indicated that TaANR1a and TaHOX1 bound to the Ala180/Val180 position of TaVRN-A1. The
Jagger TaHOX1a protein has a leucine residue at position 99 that is substituted with a proline residue in the 2174 TaHOX1b protein. Both TaHOX1a and TaHOX1b showed in vivo interactions with TaANR1a. These results suggest that the differential interactions between TaVRN-A1, TaANR1 and TaHOX1 proteins might modify N-related traits.

Figure 5 Regulation of TaVRN-A1 by N in normal and transgenic wheat plants. (a) Regulation of TaVRN-A1 transcript levels by N in Jagger grown in two different soils. (b) TaVRN-A1 transcript levels in TaVRN-A1::RNAi transgenic Jagger wheat grown in the Kirkland soil. (c) Heading date of transgenic Jagger wheat grown in the Kirkland soil. TaVRN-A1::RNAi(+) indicates positive transgenic plants, whereas TaVRN-A1::RNAi(−) indicates nontransgenic plants. (d) Comparison of typical transgenic positive plants carrying TaVRN-A1::RNAi(+), and nontransgenic plants of the T20 line at the heading stage. (e) Comparison of a typical transgenic plant carrying TaANR1::RNAi of the T40 line, and wild-type Jagger plants at the heading stage. (f) Effects of N on TaVRN-A1 transcript level in the spring wheat cultivar Bobwhite. (g) Effects of N on TaVRN-A1 transcript level in eight vernalized winter wheat cultivars/lines. The dark columns indicate that N was utilized, whereas the grey columns indicate without N. 1. Jagger; 2. OK12716R/W; 3. OK11D25056; 4. Bentley; 5. Duster; 6. Gallagher; 7. Ruby Lee; 8. IBA; (+N) indicates that N was applied to the plants. Cultivars 1–4 carry the TaVRN-A1a allele, whereas cultivars 5–8 carry the TaVRN-A1a allele. (h) Effects of N on heading date in the spring wheat cultivar Bobwhite. Gene transcript levels were calculated using the 2−ΔΔCT method, where CT is the threshold cycle. Primers for TaVRN-A1 and actin used as an endogenous control are provided in Table S7. The values represent mean expression levels (n = 7–16), and the bar indicates standard error. Asterisk indicates that the difference was significant between the two alleles (P < 0.05).
Genetic effects of TaVRN-A1, TaANR1 and TaHOX1 on grain yield in the field

To evaluate the combined effects of favourable alleles of TaVRN-A1, TaANR1 and TaHOX1 on grain yield, we tested the effects of natural mutations on grain yield in the 96 RIL population under field conditions for 2 years and in two locations. This approach circumvented regulatory roadblocks associated with cultivating genetically modified crops.

When tested in the field at high N levels in the 2007–2008 growing season, lines carrying the Jagger TaVRN-A1 allele achieved a higher grain yield by an average of 2.82% compared with plants carrying the 2174 TaVRN-A1 allele (Figure 7a). The Jagger TaVRN-A1 allele increased grain yield by 2.92% in the 2014–2015 growing season, when a population of the same RIL was grown in a field with sufficient N to attain high grain yield (Figure 7b). The TaANR1a in Jagger was observed to increase grain yield. Lines carrying the Jagger allele achieved higher grain yields by an average of 3.39% (2.8% in the 2007–2008 growing season, and 3.97% in the 2014–2015 growing season), compared with plants carrying the 2174 allele (Figure 7a, b). TaHOX1a had a repressive effect on grain yield. Lines carrying the Jagger TaHOX1a allele achieved a lower grain yield by a decrease of 4.46% from 4.69% on average in the 2007–2008 growing, and 4.22% in the 2014–2015 growing season compared with plants carrying the 2174 TaHOX1b allele (Figure 7a, b). Together, TaVRN-A1a and TaANR1a in Jagger and TaHOX1b in 2174 significantly increased grain yield by an average of 10.71% (9.84–11.58%) (Pr < 0.05), based on the average grain yield of the 96 RILs tested in the field for 2 years and in two locations.

Discussion

In this study, we used two contrasting N fertilization regimes to map QNue.osu-5A and identified TaVRN-A1 as TaNUE1 in winter wheat. In previous studies, TaVRN-A1 was cloned as a gene for variations in the qualitative vernalization requirement between

Figure 6 Function of TaANR1 in natural mutant and transgenic plants. (a) Comparison of TaANR1 expression patterns in root and leaf samples between Jagger and 2174. Primers ANR-MS-F2 (5'–GAATGGGTGCTCAAAGGACCTGCAGG–3') and ANR-MS-R2 (5'–GGAGTTCTTGAATTCTCGGTTAACCTGCA–3') were designed to amplify the 250-bp Jagger allele and the 166-bp 2174 allele. (b) A diagram of 23-bp indel in locations and sequences of TaANR1 between the Jagger and 2174 alleles. The splicing sites at 5' end (GT) and 3' end (AG) of intron 5 are highlighted in red. (c) A PCR marker for 23-bp indel between the Jagger TaANR1a allele and the 2174 TaANR1b allele. Primers ANR-MF1 (5'–ATCCACTAAAGCTCAGTTCGAC–3') and ANR-MR1 (5'– GGAGTTCTTGAATTCTCGGTTAACCTGCA–3') were designed to amplify the Jagger allele (286 bp) and the 2174 allele (263 bp). M: DNA marker. (d) Genetic effect of TaANR1 on heading date of RILs in the commercial soil and Kirkland soil. (e) Regulation of TaANR1a transcripts by N and RNAi transgenic wheat. Transcript levels were determined using values calculated by the 2^(-ΔΔCT) method, where CT is the threshold cycle. The values represent mean expression levels (n = 8), and the bars indicate standard errors. (f) Comparison of a typical transgenic plant carrying TaANR1a::RNAi and nontransgenic plant at the juvenile stage.

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spring wheat and winter wheat (Yan et al., 2003) and for the quantitative vernalization requirement between weak winter wheat and strong winter wheat using a positional cloning approach (Li et al., 2013). The TaVRN-A1 locus on chromosome 5AL was also mapped and directly linked to key wheat and barley traits such as frost tolerance, spike architecture and plant height, tiller and spikelet number, leaf length, grain yield, nitrogen uptake and NUE (An et al., 2006; Deng et al., 2015; Fowler and Gusta, 1977; Habash et al., 2007; Hayes et al., 1993; Kato et al., 2000; Quraishi et al., 2011; Roberts, 1990; Snape et al., 1985). In addition to the Vrn-A1 locus, the Vrn-D1 locus was associated with QTL for traits related to NUE components in wheat in the same population of the Chinese Spring 9 SQ1 population (Habash et al., 2007). However, the genes in the mapped regions that controlled NUE and the related agronomic traits were unknown.

Results from the present study indicated that it is TaVRN-A1 that is regulated by N and linked to the multiple agronomic traits. The conclusion was drawn based on three pieces of experimental evidence. Firstly, the segregated NUE and related agronomic traits were associated with three candidates at the TaVRN-A1 locus, and the TaVRN-A1 gene was the best candidate responsible for the traits. Secondly, the Ala180/Val180 substitution in the TaVRN-A1 protein caused differential interactions with TaANR1, establishing a connection between the vernalization gene and the N-regulated gene. The function of TaANR1 in wheat was validated by the down-regulation of TaANR1 transcripts by N, and the delayed effect of TaANR1 on heading date by RNA interference in transgenic plants and by analysis of a natural mutant. Lastly, RNA interference and N showed similar effects in the transgenic wheat in delaying heading date. However, precise effects of disrupted TaANR1 expression on NUE need to evaluate in a field experiment.

Three homoeologous genes, TaVRN-A1, TaVRN-B1 and TaVRN-D1, exist in common wheat. In this study, only allelic variation in TaVRN-A1 was found to link with the segregated NUE and related agronomic traits in the winter wheat population, and only TaVRN-A1 was tested for its interaction with TaANR1. The homoeologous TaVRN-B1 and TaVRN-D1 and their proteins should also have similar effects on the N-related agronomic traits, although the role of the genes in the N-related agronomic traits under different genetic backgrounds needs to be investigated. In cereal crops, NUE refers to the ratio of grain yield to N supplied by soil and fertilizer, which is dissected into two components: N uptake (NU) and N utilization efficiency (UTE) (An et al., 2006; Moll et al., 1982). In this study, we used grain yield to describe NUE for two alleles for each of the three genes tested at the same N level. The allelic effect of TaVRN-A1a on grain yield was increased to up to 140% when the population was grown in the Kirkland soil with limited N and in the greenhouse without vernalization. The enlarged effects observed in plants grown under low-N conditions facilitated positional cloning of TaNUE1. In addition, the cloned TaVRN-A1 allowed for identification of its interactors in the same pathway. The results from this study suggest that the differential interactions between TaVRN-A1,
TaANR1 and TaHOX1 proteins might modify N-related traits. It is a critical finding that TaANR1 and TaHOX1 competed to bind to the Ala^{180}/Val^{180} position of TaVRN1. The competition between TaANR1 and TaHOX1 for TaVRN-A1 is reflected in their effects on grain yield. As a competitor with TaANR1 for TaVRN-A1, TaHOX1a had a repressive effect on grain yield. These genes caused subtle modifications to grain yield in a common high-N scenario in the field, but genetically incorporating favourable alleles from the TaVRN-A1a and TaANR1a genes in Jagger, and the TaHOX1b gene in 2174, increased grain yield by an average of 10.71% (9.84% to 11.58%). Favourable alleles of these genes involved in regulating nitrogen pathways can be pyramided to improve NUE and grain yield in novel wheat cultivars.

Nitrogen has paradoxical effects on development. Lines started from seed and grown in the commercial soil showed accelerated plant development compared with those grown in the Kirkland soil. However, when N was supplied to the plants prior to stem elongation, the plant heading date was delayed. Plants accelerate the transition to the reproductive phase and flower faster with lower nutrients (Martinez-Zapater et al., 1994). This study indicated that when N was used to fertilize a plant prior to stem elongation, TaVRN-A1 transcripts were down-regulated, and the plant heading date was thus delayed. This explains the molecular mechanism underlying the observation that plants flower faster in N-deficient conditions. In winter wheat, TaVRN-A1 is induced by vernalization, light or plant age (Danyuk et al., 2003; Li et al., 2013; Murai et al., 2003; Tavdaviskis et al., 2003; Yan et al., 2003). Without vernalization, TaVRN-A1 is expressed in spring wheat because of the presence of multiple mutation forms, including indels of different forms in the promoter and the first intron (Dubicovsky et al., 2006; Fu et al., 2005; Pidal et al., 2009; Yan et al., 2003, 2004), a retrotransposon insertion in the 5' UTR (Chu et al., 2011), an miRNA binding site in a miniature inverted-repeat transposable element in the promoter (Yu et al., 2014) or the binding site of TaGRP2 in intron 1 (Kippes et al., 2015). This study demonstrated that N regulates TaVRN-A1 and flowering in both spring wheat and winter wheat cultivars.

**Materials and methods**

**Plant materials and experimental soils**

*Mapping QNue.osu-5A in RILs in low-N soil*

The population of RILs was generated from a cross between two locally adapted winter wheat cultivars, 'Jagger' and '2174' (Chen et al., 2009). A population of 96 RILs was used to initially map traits related to NUE. A Kirkland silt loam soil was collected from the Cimarron Valley Research Station, OK (USA); the soil was low in N, but had normal levels of other essential nutrients (Table S1). The soil was thoroughly mixed using a cement mixer before being distributed equally into pots. Three plants of a single RIL were grown in each pot (10 cm in diameter and 12 cm in height) with 1.8 kg soil in a greenhouse in Stillwater, OK. The greenhouse was maintained at a constant temperature of 25°C (day)/night with long days (16 h light/8 h darkness), and additional sodium lamps were supplied. The winter wheat population was not subjected to vernalization, to avoid interactive effects between low temperatures and the traits of interest.

Leaf greenness and total N of aboveground biomass were assessed from one of the replicates. Heading date was scored when a single plant had completely emerged from the boot. Leaf chlorophyll content was assayed using a SPAD 502 Chlorophyll Meter (Konica Minolta Sensing, Inc., Osaka, Japan). The dry weight of the shoot tissues in mature plants was weighed to determine biomass. Harvest index was the ratio of total grain weight to total aboveground biomass. Tiller number was counted from ten plants at the end of the stem elongation stage when tillers reached their maximum number. Spike number was counted at maturity, and the total number of grains from a plant was counted to calculate the number of grains per spike. Grains were dried at 65°C for 2 days; grain weight was the weight of the dried grain plus 13% standard moisture per thousand grains (thousand kernel weight). These yield components were phenotyped from three plants tested in the Kirkland soil in the greenhouse.

PCR markers for the allelic variation of each gene were developed and mapped in the RILs (Figure S2). Markers for three candidate genes were developed based on the sequences deposited in GenBank, including JQ915055 for Jagger and JQ915056 for 2174 of TaVRN-A1; JQ915057 for Jagger and JQ915058 for 2174 of TaAGLG1, JQ915059 for Jagger and JQ915060 for 2174 of TaCYB5.

*QTL validation and statistical analysis*

QNue.osu-5A was identified in the same population of recombinant inbred lines (RILs) grown under two contrasting N fertilization regimes in the same greenhouse. The genetic linkage group utilized to identify genes for stem elongation time (Chen et al., 2009) and vernalization requirement duration (Li et al., 2013) was used to discover QNue.osu-5A. WinQTLCart 2.5 (North Carolina State University, Raleigh) (Wang et al., 2007) was used to conduct analyses for the N-related traits using interval mapping (IM). A QTL was declared when the logarithm of the odds (LOD) score exceeded the threshold value of 2.5, and the significance level was at 0.05. LOD values and genetic partitioning of the total phenotypic variation are presented as generated from the QTL program, using standard procedures. One-way analysis of variance (ANOVA) was used to analyse the interactions of the QTLs with N rate for each of the 10 traits using the SAS procedure CORR (SAS Institute, Cary, NC). A QTL was statistically claimed to be regulated by N, when the genotype x nitrogen environment effect was significant.

The mean value of each trait from plants carrying the same allele was used for ANOVA. The t-test was used to determine the significance level to compare mean differences between treatments. Correlation analysis examined the association of two phenotypes in the same treatment.

*Testing critical recombinant lines*

Fourteen recombinant BC1F2 and three recombinant BC1F3 lines (Figure 1c) were tested with the same Kirkland soil. Sixty plants of each recombinant line were tested under the same greenhouse conditions used to discover QNue.osu-5A. A commercial potting mix, Sunshine Redi-earth growing mix (Sungro Horticulture Canada Ltd.) was used in the experiments, which includes fine Canadian sphagnum peat moss, vermiculite and dolomitic lime-stone, and has a nutrient system delivering a supply of N for 6 weeks. Whenever appropriate, N from Miracle-Gro® Water Soluble All Purpose Plant Food or urea fertilizer was dissolved in water and poured into pots. The heading date was scored when a single plant head had completely emerged from the boot.

At the Cimarron Valley Research Station in the 2011–2012 growing season, seven critical recombinant lines were grown in a Teller soil with 7.5 ppm NO3−-N, representing a N-stress condition.
A forage crop was grown for two consecutive seasons, and no N was supplied to both lower and level the available N in the Teller soil. The experimental design was a randomized complete block with three replications. Yield components were phenotyped from 10 plants per line tested in the Teller soil in the field.

**Testing the RIL population in the field**

Yield experiments on 96 RILs of the Jagger × 2174 population were conducted for 2 years. The first experiment took place at the North Central Agronomy Research Station near Lahoma, OK, and the population was planted as a standard yield plot on October 30, 2007. The plot area (3 m × 1.26 m) was fertilized before planting according to soil-test recommendations for adequate N to attain a yield goal of approximately 3000–6700 kg/ha in this specific site (Edwards et al., 2012). The second experiment was conducted at the Agronomy Research Station in Stillwater, OK, and the population was planted as a head-row plot on 14 November 2014. At the experiment site, the top 30 cm of soil contained 40 ppm N, 16 ppm P$_2$O$_5$ and adequate K with a pH of 5.3. Diammonium phosphate (DAP, 8–5–14) fertilizer of 309 kg/ha and urea of 371 kg/ha were incorporated into the soil by cultivator tillage. The experimental design was a randomized complete block with two replications. Dry grains for each plot were weighed to determine grain yield.

**Regulation of TaVRN-A1 by N in different cultivars/lines**

Three cultivars/lines carrying the TaVRN-A1a allele (Bentley, OK12716W, and OK11D25056) and four cultivars carrying the TaVRN-A1b allele (Duster, Gallagher, IBA, and Ruby Lee) were first planted in the field for an annual variety trial at Stillwater on 14 October 2015. Plots were 3.0 m in length, with 30 cm between rows. At the experiment site, the top 30 cm of soil contained 47 ppm N, 39 ppm P$_2$O$_5$ and 66 ppm K$_2$O, with a pH of 5.3. Diammonium phosphate (DAP, 18-46-0) fertilizer of 56 kg/ha was also supplied in-furrow. On 24 February 2016, 130 days after planting, six plants of each cultivar, which were expected to have satisfied their vernalization requirement in the natural field condition, were transferred to pots with commercial soil in the greenhouse and N fertilizer at the 100 kg N ha$^{-1}$ level was supplied. Three weeks after fertilization, leaf samples from three plants were collected to test the regulation of gene expression by N. Unfertilized plants were used as controls.

**In vitro protein interactions**

The three cDNAs of TaVRN-A1a and TaVRN-A1b encoding proteins from 85–191 aa, 85–179 aa, and 139–191 aa; one cDNA of AGLG1a and AGLG1b encoding proteins from 1 to 180 aa; and one cDNA of TaHOX1a and TaHOX1b encoding proteins from 1 to 150 aa, were each cloned into a pSKB3 vector with a N-terminal 6x-HIS-tag (Li et al., 2013). The cDNAs encoding proteins from 111 to 240 aa (end) for TaANR1a, and from 111 to the end for TaANR1b, were cloned into a pMAL-c2X vector with an MBP-tag (New England BioLabs, Ipswich, MA, USA). The primers used for cloning are listed in Table S6. Constructs were expressed in *E. coli* (BL21 DE3). An Ni-NTA column (Qiagen, Germantown, MD, USA) was used to purify the expressed proteins from pSKB3 with the 6x-HIS-tag, and an amylase column (New England BioLabs) was used to dialyse and purify the protein fused with the MBP-tag.

The protein fused with the 6x-HIS-tag (500 μg/mL, 50 μL) was incubated in an Ni-NTA column (40 μL) for 1 h at 4°C in binding buffer with 10 mM imidazole, and the MBP-tag protein (500 μg/mL, 100 μL) was then added to the column. MBP-tag protein (500 μg/mL, 100 μL) alone was added to an Ni-NTA column as a negative control for nonspecific interactions between MBP-tag and the Ni-NTA column. Reactions were incubated overnight at 4°C. The interacting proteins in the resin were then washed with chilled binding buffer with 35 mM imidazole six or eight times until the MBP-tag protein disappeared in the negative control. The protein was eluted by boiling the samples in 40 μL of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 5 min. The eluted protein samples (5 μL) were analysed on a 12% SDS-PAGE gel stained with Coomassie Brilliant Blue. When the protein fused with the MBP-tag was incubated in the amylose column, the 6x-HIS-tag protein was added to the amylose column and then washed with column buffer until the 6x-HIS-tag protein disappeared in the negative control. Interacting proteins were analysed in pull-down assays.

**Subcellular localization and in vivo protein interactions**

The complete cDNA of TaANR1a and the cDNA fragment of TaHOX1a encoding its protein from 1 to 150 aa were cloned into pDONR207 with the BP Cloning Kit (Invitrogen) according to the manufacturer’s instructions. The LR Cloning Kit (Invitrogen, Waltham, MA, US) was then used to transfer TaANR1 to pEarleygate 101 (pEG101) for subcellular localization of TaANR1a. Primers used for constructs are provided in Table S6.

Previous reports located TaVRN-A1 and TaHOX1 in the nucleus of the cell (Li et al., 2013). The TaANR1 in pDONR207 was fused to the C-terminal amino acid portion (175–239 aa) of YFP in the pEarleyGate202-YC vector (pEG202-YC) to test in vivo interactions with TaVRN-A1 (1–244 aa, end) or TaHOX1 (1–150 aa), which was fused to the N-terminal 174 amino acid portion (1–174 aa) of YFP in the pEarleyGate201-YN vector (pEG201-YN). Reciprocal empty vectors were also used as negative controls for interactions with TaANR1, TaVRN-A1 or TaHOX1 proteins. *Agrobacterium tumefaciens* strains (GV3101) carrying the BiFC constructs and p19 strain were used together to infiltrate *Nicotiana benthamiana* leaves 5 weeks after planting. Leaves were ready for BiFC imaging after 3 days of infiltration. Images were visualized under a BX-51 fluorescence microscope (Olympus). Images were taken with a bright filter (BF) to indicate the background of the leaves infiltrated with *A. tumefaciens* carrying constructs, or with an ultraviolet filter to indicate the position of the nucleus stained with 4’, 6-diamidino-2-phenylindole (DAPI), and with GFP and GFPA filters to indicate the presence of fluorescent proteins. Scale bars in all images are 50 μm.

**Transgenic wheat**

Two transgenic plants (T20 and T40) were generated using the *TaVRN-A1::RNAi* constructs, and three transgenic plants (T1, T13 and T14) were generated using the *TaANR1::RNAi* construct. The primers used to amplify the genes are provided in Table S6. Amplified genes were inserted at the enzyme digestion sites (Asc I/Vnr II, sense fragment; Spe I/As IS I, antisense fragment) of the RNAi vector pMCG161—this contains the BAR gene, which confers resistance to bialaphos. Constructs were transformed into...
the Jagger cultivar by particle bombardment using a published protocol (Sparks and Jones, 2014). Transgenic wheat plants were tested in the same Kirkland soil used to discover QNue-osu-5A. The T1 of three lines of TaANR1::RNAi were tested in the commercial soil. All transgenic lines were tested under the same greenhouse conditions used to discover QNue-osu-5A and without vernalization. Leaf samples were collected from TaVRN-A1::RNAi lines 77 days after planting and before N fertilizer at the 100 kg N ha\(^{-1}\) level was applied to positive and negative plants. Leaf samples with/without N were collected for RNA extraction 3 weeks after fertilization. Root samples of TaANR1::RNAi lines were collected 56 days after planting and before N fertilizer at the 100 kg N ha\(^{-1}\) level was applied. Root samples for RNA extraction were collected 1 and 2 weeks after fertilization. Four T1 lines of T20 and T40 were selected to generate a T2 population for phenotyping in the commercial soil.

**Quantitative RT-PCR**

Total RNA from shoots and roots was extracted using TRIzol\textsuperscript{®} reagent (Invitrogen) according to the manufacturer’s instructions. Root RNA was extracted using a modified method in the RNA precipitation step, whereby 0.25 mL of isopropanol was added, followed by 0.25 mL high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 mL TRIzol\textsuperscript{®} reagent. One microgram of RNA was treated with deoxyribonuclease I (Invitrogen), and complementary DNA (cDNA) was synthesized using a SuperScript\textsuperscript{™} II Reverse Transcriptase Kit (Invitrogen) with an oligo(dT)\(_{20}\) primer. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) and iQ\textsuperscript{™} SYBR\textsuperscript{®} Green Supermix (Bio-Rad, Hercules, CA, USA) with a three-step cycling program consisting of an initial denaturation step at 95°C for 3 min, followed by 39 cycles at 95°C for 15 s, 57°C for 30 s and 72°C for 30 s. Primers used in RT-qPCR to amplify TaVRN-A1, TaANR1 and actin are listed in Table S7. Gene transcript levels were described using the values calculated by the 2\(^{-ΔΔCt}\) method (Li et al., 2013), where CT is the threshold cycle. All samples were subjected to two technical replicates and at least six biological replicates.

**Acknowledgements**

We acknowledge funding from the Oklahoma Center for Advanced Science and Technology (OCAST). This study was also supported by USDA-NIFA T-CAP Grant No. 2011-68002-30029, the Oklahoma Wheat Research Foundation, and the Oklahoma Agricultural Experiment Station.

**Conflict of interest**

The authors declare no conflict of interest.

**References**

An, D., Su, J., Liu, Q., Zhu, Y., Tong, Y., Li, J., Li, J. et al. (2006) Mapping QTLs for nitrogen uptake in relation to the early growth of wheat (Tritium aestivum L.). Plant Soil, 284, 73–84.

Boerjan, W., Cervera, M.T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M. et al. (1995) Superroot, a recessive mutation in Arabidopsis, confers auxin overproduction. Plant Cell, 7, 1405–1419.

Chen, Y., Caner, B.F., Wang, S., Zhang, F. and Yan, L. (2009) Genetic loci associated with stem elongation and winter dormancy release in wheat. Theor. Appl. Genet. 118, 881–889.

Chu, C.G., Tan, C.T., Yu, G.T., Zhong, S., Xu, S.S. and Yan, L. (2011) A novel retrotransposon inserted in the dominant Vrn-B1 Allele confers spring growth habit in tetraploid wheat (Triticum turgidum L.). G3, 1, 637–645.

Cornier, F., Faure, S., Dubreuil, P., Heumez, E., Beaucâine, K., Lafarge, S., Praud, S. et al. (2013) A multi-environmental study of recent breeding progress on nitrogen use efficiency in wheat (Triticum aestivum L.). Theor. Appl. Genet. 126, 3035–3048.

Cornier, F., Le Gous, J., Dubreuil, P., Lafarge, S. and Praud, S. (2014) A genome-wide identification of chromosomal regions determining nitrogen use efficiency components in wheat (Triticum aestivum L.). Theor. Appl. Genet. 127, 2679–2693.

Danylyk, Jr., Kane, N.A., Breton, G., Limin, A.E., Fowler, D.B. and Sarhan, F. (2003) TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Physiol. 132, 1849–1860.

Deng, W., Caoao, M.C., Wang, P., Sato, K., Hayes, P.M., Finnegan, E.J. and Trevaskis, B. (2015) Direct links between the vernalization response and other key traits of cereal crops. Nat. Commun. 6, 5882–5889.

Dubcovsky, J., Lounkoian, A., Fu, D., Valarik, M., Sanchez, A. and Yan, L. (2006) Effect of photoperiod on the regulation of wheat vernalization genes VRN1 and VRN2. Plant Mol. Biol. 60, 469–480.

Edwards, J.T., Hunger, R.M., Smith, E.L., Horn, G.W., Chen, M.S., Yan, L., Bai, G. et al. (2012) ‘Duster’ Wheat: a durable, dual-purpose cultivar adapted to the southern Great Plains of the USA. J. Plant Regist. 6, 1–12.

Fowler, D.B. and Gusta, L.V. (1977) Influence of fall growth and development on cold tolerance of rye and wheat. Can. J. Plant Sci. 57, 751–755.

Fu, D., Szücs, P., Yan, L., Helguera, M., Skinner, J.S., von Zitzewitz, J., Hayes, P.M. et al. (2005) Large deletions within the first intron in VRN1 are associated with spring growth habit in barley and wheat. Mol. Genet. Genomics, 273, 54–65.

Gaju, O., Allard, V., Martre, P., Snape, J.W., Heumez, E., LeGouis, J., Moreau, D. et al. (2011) Identification of traits to improve the nitrogen-use efficiency of wheat genotypes. Field Crops Res. 123, 139–152.

Goos, R.J. and Johnston, B.E. (1999) Performance of two nitrification inhibitors over a winter with exceptionally heavy snowfall. Agron. J. 91, 1046–1049.

Habash, D.Z., Bernard, S., Schondelmaier, J., Weyen, J. and Quarrie, S.A. (2007) The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield. Theor. Appl. Genet. 114, 403–419.

Hays, P.M., Liu, B.H., Knapp, S.J., Chen, F., Jones, B., Blake, T., Franckowiak, J. et al. (1993) Quantitative trait locus effects and environmental interaction in a sample of North American barley germ plasm. Theor. Appl. Genet. 87, 392–401.

He, X., Qu, B., Li, W., Zhao, X., Teng, W., Ma, W., Ren, Y. et al. (2015) The nitrate-inducible NAC transcription factor TaNAC2-S5 controls nitrate response and increases wheat yield. Plant Physiol. 169, 1991–2005.

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

Jack, T. (2004) Molecular and genetic mechanisms of floral control. Plant Cell, 16, 51–57.

Kaiser, B.N., Finnegan, P.M., Tyerman, S.D., Whitehead, L.F., Bergersen, F.J., Day, D.A. and Udvardi, M.K. (1998) Characterization of an ammonium transport protein from the peribacteroid membrane of soybean nodules. Science, 281, 1202–1206.

Kato, K., Miura, H. and Sawada, S. (2000) Mapping QTLs controlling grain yield and its components on chromosome SA of wheat. Theor. Appl. Genet. 101, 1114–1121.

Kippes, N., Debernardi, J.M., Vasquez-Gross, H.A., Alpinar, B.A., Budak, H. and Kato, K. (2015) Identification of the VERNALIZATION 4 gene reveals the origin of spring growth habit in ancient wheats from South Asia. Proc. Natl Acad. Sci. USA, 112, 5401–5410.

Krapp, A. (2015) Plant nitrogen assimilation and its regulation: a complex and challenging process on nitrogen use efficiency in wheat (Triticum aestivum L.). Nat. Commun. 6, 1860.

Kraj, M., Van Breusegem, F., Guo, J. and Van Montagu, M. (2007) A novel retrotransposon in the dominant Vrn-1 Allele confers spring growth in tetraploid wheat (Triticum turgidum L.). G3, 1, 637–645.

Kumar, M., Tewari, M., Zhai, Y., Li, Y. and Yan, L. (2013) A multi-environmental study of recent breeding progress on nitrogen use efficiency in wheat (Triticum aestivum L.). Theor. Appl. Genet. 126, 3035–3048.

Kumar, M., Tewari, M., Zhai, Y., Li, Y. and Yan, L. (2013) A multi-environmental study of recent breeding progress on nitrogen use efficiency in wheat (Triticum aestivum L.). Theor. Appl. Genet. 126, 3035–3048.
mutants, chl2 and chl7, of Arabidopsis thaliana. Mol. Gen. Genet. 233, 169–176.

Lam, H.M., Coscigiano, K., Schulz, C., Melo-Oliveira, R., Tjaden, G., Oliveira, I., Ngi, N. et al. (1995) Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis. Plant Cell 7, 887–898.

Leydecker, M.T., Camus, I., Daniel-Vedele, F. and Truong, H.N. (2000) Screening for Arabidopsis mutants affected in the Nii gene expression using the Gus reporter gene. Plant Physiol. 120, 161–170.

Li, G., Yu, M., Fang, T., Cao, S., Carver, B.F. and Yan, L. (2013) Vernalization requirement duration in winter wheat is controlled by TaVRN-A1 at the protein level. Plant J. 76, 742–753.

Ludet, O., Chalillo, S., Mergout, P., Talbotec, J. and Daniel-Vedele, F. (2003) Quantitative trait loci analysis of nitrogen use efficiency in Arabidopsis. Plant Physiol. 131, 345–358.

Mackown, C.T. and Carver, B.F. (2007) Nitrogen use and biomass distribution in culms of winter wheat populations selected from grain-only and dual-purpose systems. Crop Sci. 47, 350–358.

Martinez-Zapater, J.M., Coupland, G., Dean, C. and Koornneef, M. (1994) The transition to flowering in Arabidopsis. In Arabidopsis (Meyerowitz, E.M. and Somerville, C.R., eds.), pp. 403–433. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mickelbart, M.V., Hasegawa, P.M. and Bailey-Serres, J. (2015) Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. Nat. Rev. Genet. 16, 237–251.

Moll, R.H., Kamprath, E.J. and Jackson, W.A. (1982) Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron. J. 74, 562–564.

Murai, K., Miyamae, M., Kato, H., Takumi, S. and Ogihara, Y. (2003) WAP1, a wheat APETALA1 homolog, plays a central role in the phase transition from vegetative to reproductive growth. Plant Cell Physiol. 44, 1255–1265.

Peterman, T.K. and Goodman, H.M. (1991) The glutamine synthetase gene of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell, 72, 705–713.

Wang, S., Basten, C.J. and Zeng, Z.B. (2007) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh. http://sta.tgen.ncsu.edu/qtcart/WQTLCart.htm.

Yu, X., Wang, R., Tong, Y., Zhao, H., Xie, Q., Liu, D., Zhang, A. et al. (2014) Mapping QTLS for yield and nitrogen-related traits in wheat: influence of nitrogen and phosphorus fertilization on QTLS expression. Theor. Appl. Genet. 127, 59–72.

Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T. and Dubcovsky, J. (2003) Positional cloning of the wheat vernalization gene VRN1. Proc. Natl Acad. Sci. USA, 100, 6263–6268.

Yan, L., Helguera, M., Kato, K., Fukuyama, S., Sherman, J. and Dubcovsky, J. (2004) Allelic variation at the VRN-1 promoter region in polyploid wheat. Theor. Appl. Genet. 109, 1677–1686.

Yu, M., Carver, B.F. and Yan, L. (2014) TamRII123 originated from a family of miniature inverted-repeat transposable elements (MITE) including one inserted in the Vrn-A1a promoter in wheat. Plant Sci. 215–216, 117–123.

Zhang, H. and Forde, B.G. (1998) MADS box gene that controls nutrient-induced changes in root architecture. Science, 279, 407–409.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Winter wheat plants in N-deficient soil before and after fertilization.

Figure S2 Markers for genes encompassing TaNUE1.

Figure S3 Comparison of TaVRN-A1 expression level in Jagger versus 2174.

Figure S4 Interacting site of TaVRN-A1 and TaANR1a proteins.

Figure S5 The subcellular location of ANR1a proteins.

Table S1 Characteristics of the soils used for NUE.

Table S2 LOD and variation of QNue.osu-5A on 10 traits related to N utilization.

Table S3 Regulatory effects of QNue.osu-5A by N.

Table S4 Wheat heading date of seven critical recombinant lines and parental lines tested in different soils.

Table S5 Wheat cultivars used for determining the frequency of TaANR1 alleles.

Table S6 Primers for protein–protein and protein–DNA interactions, and gene transformation.

Table S7 Primers for gene expression.