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Identifying nitrogen-use efficient soft red winter wheat lines in high and low nitrogen environments

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ABSTRACT

Nitrogen use efficiency (NUE) is of great interest to wheat (*Triticum aestivum* L.) breeders because it addresses the daunting prospect of feeding the burgeoning population under the constraints of limited land resources and a warming climate. In this study, we evaluated a 56 entry panel of SRW breeding lines and cultivars from the eastern US wheat region in 2014 for NUE and related traits. The 56-entry block was grown at Lexington and Princeton, KY at two N rates (0 and 112 kg ha^{-1}) in a complete factorial design. We measured normalized difference vegetative index (NDVI), biomass, harvest index, N harvest index, N uptake efficiency, N utilization efficiency, post-anthesis N uptake, N remobilization efficiency and overall NUE. Breeders usually apply high rates of N fertilizer to their plots in order to maximize genetic yield potential. Our study indicates that without screening breeding lines in low N environments concurrently, it will not be possible to identify high NUE genotypes. Post-anthesis N uptake, was highly correlated with yield ($r = 0.79$) under high N, but heritability of this trait was close to zero. Heritability of NUE, on the other hand was moderately high ($h^2 = 0.65$). Five breeding lines ranked within the top 10 for NUE in both low and high N environments. NDVI was found to be both heritable and highly correlated with yield across N environments ($R^2 = 0.78$). Genome wide association studies of NUE and related traits revealed QTL associated with NUE (chromosome 2B), uptake efficiency (chromosome1B) and utilization efficiency (chromosomes 1A and 3A). In accord with other studies, these QTL are of small effect and will likely only be useful in genomic selection as opposed to marker-assisted selection.

1. Introduction

Feeding ten billion people in a sustainable manner has become the most daunting challenge facing agriculture. Winter wheat (*Triticuum aestivum* L.) is an important component of the national and global food supply; as population increases, worldwide demand for wheat will continue to grow. As wheat breeders acknowledge the need for increased yields on a fixed land area, one strategy frequently mentioned is breeding for nitrogen use efficiency (NUE). Beyond increasing the food supply, this approach would enhance sustainability: excess nitrogen (N) fertilizer has been shown to have adverse environmental impacts such as N_2O emissions and eutrophication of freshwater and marine ecosystems (Sieling and Kage, 2008). Without an increase in N use efficiency, however, reduced N fertilizer use could decrease crop yields and quality if the plant experiences N deficiency (Cassman et al., 2003). The idea of breeding for NUE is not new, (see e.g. Moll et al. (1982)); yet it still has not gained traction as a strategy that can be easily implemented in a breeding program. NUE has been described in several different ways (Cormier et al., 2016) but a widely accepted definition is grain yield produced per unit soil N supply (soil N and fertilizer N; Moll et al., 1982). NUE defined in this way is the product of N uptake efficiency (NUpE) and N utilization efficiency (NUtE) (Moll et al., 1982; Nyikako et al., 2014).

Many studies have shown genetic variation in NUtE and NUpE (Van Sanford and Mackown, 1986; Ortiz-Monasterio et al., 1997; Foulkes et al., 1998; Muurinen et al., 2006; Barraclough et al., 2010; Gaju et al., 2011, 2014). These studies typically report genotype × N supply interaction that affects NUE, and, by definition, NUpE and NUtE (Ortiz-Monasterio et al., 1997; Foulkes et al., 1998; Muurinen et al., 2006; Barraclough et al., 2010; Gaju et al., 2011, 2014). To ensure maximum expression of genetic potential, most wheat breeding trials are conducted in high N environments. Therefore

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breeders select genotypes that perform well under what might be considered optimal conditions. The selected lines may not perform well where N is limiting. As a result, selection in low and high N environments is likely needed to identify genotypes with the potential to perform well under optimal as well as N-limiting conditions. One issue that has hindered implementation of selection for NUE is the high cost in time and resources of measuring certain NUE traits. Yet, it is critical to determine which are the "must have" traits to accurately measure NUE under low and high N conditions (Brancourt-Hulmel et al., 2005). Total N uptake at anthesis can give insight into growth of yield-generating leaves, floret fertility, amount of stem reserves, and creation of a deep root system, yet it is very costly to measure. Total N at maturity, also arduous to measure, provides information on the remobilization efficiency of N from the biomass to the grain (Cox et al., 1986; Swain et al., 2014). It has been suggested that wheat genotypes with superior N uptake, storage, and translocation capabilities will allow for further gains in NUE, along with stay-green genotypes whose slower senescence allows for a longer grain filling period through continued N uptake and translocation (Bogard et al., 2011; Swain et al., 2014).

To mitigate the cost and difficulty of measuring such traits it has been suggested that rapid and efficient selection of high NUE genotypes may be possible through canopy spectral reflectance (CSR; Li et al., 2014). CSR devices measure the amount of light reflected/absorbed by the plant's canopy surface that is affected by genotypic variation and environmental stress. A CSR index, such as the normalized difference vegetative index (NDVI), has been shown to have high correlations with wheat grain yield, biomass and N concentration (Ma et al., 1996; Raun et al., 2001; Crain et al., 2012; Prasad et al., 2007a,b). The CSR estimates of biomass and N content can be used to estimate NUpE and NUtE. The relationship of CSR with biomass is of great interest, since biomass is related to NUE and yield (Crain et al., 2012).

Though NUE has been extensively studied, traits or selection criteria that can be used in a breeding program are lacking. Therefore the objectives this study were to: identify high NUE wheat genotypes grown under low and high N environments at multiple locations, estimate heritability of NUE and related traits, and conduct genome-wide analyses of NUE to determine whether there were useful genetic markers associated with traits that determine NUE in SRW wheat.

2. Materials and methods

2.1. 2014 Experiment

The experimental material used in this study was derived from the TCAP (http://www.triticeaecap.org/) elite eastern mapping panel, comprising 280 elite soft winter wheat breeding lines and cultivars from seven winter wheat breeding programs (Ohio, Missouri, Virginia, Kentucky, Maryland, Illinois, and New York). These lines are adapted to the wheat producing region of the U.S. that stretches from the northeast to the southern corn belt and are primarily F_4-derived from different crosses selected for a wide range of genetic backgrounds. In 2014 a subset of the larger mapping panel was grown at two locations: Spindletop Farm and the West Kentucky Research and Educational Center at Princeton (PRN), KY (37°6′7.37″ N, 87°52′13.62″W) where the soil series is a Crider silt loam [fine-silty, mixed, active, mesic, Typic Paleudalfs]. The experimental material consisted of 56 winter wheat genotypes, comprising one block of the TCAP eastern elite wheat panel. This block of entries from the original TCAP panel was chosen because it contained breeding lines from the University of Kentucky (UK), University of Illinois and Purdue breeding programs that represented a sample of the diversity contained within the UK wheat breeding program. The experimental material was planted on 14 Oct. 2013 at Princeton and 24 Oct. 2013 at Lexington in a randomized complete block design under fertilizer regimes of 0 kg ha^{-1} and 112 kg ha^{-1} actual N. Each genotype/N level treatment was replicated twice at each location. The experimental unit was a single 6-row yield plot 3.3 m in length, 1.2 m wide. In the 112 kg ha^{-1} treatment, N was applied in a 34 kg ha^{-1} and 78 kg ha^{-1} split, 13 March and 9 April 2014 at Princeton and 21 March and 17 April 2014 at Lexington.

2.2. Field sampling and data collection

At each location, 20 pre-N application soil samples were taken per treatment per rep at a depth of 30.5 cm using a soil probe, mixed by hand and air dried. Monthly soil samples were taken using the same sampling protocol following N application in the spring.

Prior to N application, vegetative material was harvested from 10 genotypes that were known to differ in agronomic traits such as heading date and height. Nitrogen status of each genotype within each N environment at both locations was measured at Feekes 10 (Zadoks 45; boot stage) using the hand held CSR device, Crop Circle®. The device was held approximately 56 cm above the plot and walked along the length of the plot at a steady pace in order to generate NDVI values for each plot. Heading date (50% of spikes emerged from flag leaf sheath) and anthesis date (50% of spikes with anthers extruded) were measured in each plot. Plot length and height were measured at the soft dough stage. At both locations a 30.5 cm length of row was harvested from each plot at anthesis (Feekes 10.51; Zadoks 60) and at harvest maturity for whole plant N analysis. Harvest maturity was determined when grain was hard and could not be split by a thumbnail (Feekes 11.4; Zadoks 92). At anthesis, all biomass was ground in bulk for N analysis. At harvest maturity, biomass was separated into grain and non-grain (stems, leaves and chaff) for N analysis. Plots were harvested with a small plot combine at Princeton and Lexington on 24 June and 2 July 2014, respectively. Harvested grain from each plot at both locations was collected in harvest bags to measure yield, moisture content, and test weight.

2.3. Data processing and analysis

Soil samples collected within each treatment and location were extracted for ammonia and nitrate using the KCl method (Crutchfield and Grove, 2011). Prior to N application, soil N was estimated to be 42.1 kg ha^{-1} at PRN and 40.6 kg ha^{-1} at LEX in the low N environment. The total N supply after N application (112 kg ha^{-1}) in the high N environment was 154.6 kg ha^{-1} at PRN and 152.6 kg ha^{-1} at LEX.

Biomass sampled prior to N application was air dried in the greenhouse, ground to a fine powder using a UDY cyclone grinder, and analyzed by the FlashEA 1112 combustion analyzer to measure N concentration. The vegetative samples collected from each plot at anthesis and maturity were treated similarly, but protein concentration was determined with a Near-Infrared Reflectance (NIR; Perten instrument DA7200) instrument. Whole grain sub-samples from each plot and location were run through the NIR instrument to measure grain protein. Grain protein concentration was divided by 5.7 to convert to grain N concentration. Total plant N uptake was determined by summing: grain N content (yield * % N) plus mature biomass N content (biomass * % N). Post-anthesis N uptake (PANU) was calculated by subtracting above-ground N content at anthesis from above-ground total plant N at maturity. Nitrogen remobilization efficiency (NRE) was defined as (biomass N at anthesis – biomass N at maturity/biomass N at anthesis (Barbottin et al., 2005; Gaju et al., 2011)). Nitrogen-use efficiency (NUE) and NUE components (nitrogen uptake efficiency (NUpE) and nitrogen uti-

Table 1
Least squares means of agronomic and N use traits[a] from a 2014 study of 56 SRW wheat lines grown at Lexington (LEX) and Princeton (PRN), KY under 112 (HN) and 0 (LN) kg ha^{-1} N. Level of significance for environment (E), genotype (G) and genotype × environment interaction (G*E) from the ANOVA is shown for each trait.

Environment	Height (cm)	Grain Yield (kg ha^{-1})	Grain N (%)	NDVI	NUE (kg ha^{-1} grain/(kg ha^{-1} soil N)	NUtE (kg ha^{-1} grain/(kg ha^{-1} plant N)	NUpE(kg ha^{-1} plant N/(kg ha^{-1} soil N)	NRE
PRN Low N	68.1	2371.5	1.74	0.43	42.1	52.7	0.80	79.8
PRN High N	85.8	4559.3	1.71	0.70	29.6	57.7	0.51	88.2
LEX Low N	68.6	2673.7	1.67	0.46	63.4	48.9	1.30	65.3
LEX High N	76.6	4740.9	1,72	0.66	31.1	53.9	0.58	80.9
Environment	<0.0001	<0.0001	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.01
Genotype	<0.0001	<0.0001	<0.01	<0.01	<0.0001	<0.0001	<0.05	<0.01
G × E	NS	NS	NS	NS	NS	<0.05	NS	NS

[a] NDVI, normalized vegetative difference index; NUE, nitrogen use efficiency; NUtE, nitrogen utilization efficiency; NUpE, nitrogen uptake efficiency; NHI, nitrogen remobilization efficiency.

Table 2
Correlations among agronomic and N traits[a] in 56 SRW wheat lines grown in low N (0 kg ha^{-1}) and high N (112 kg ha^{-1}) environments at Lexington and Princeton, KY, 2014. Correlations in low N environment above diagonal; high N environment below diagonal. Correlations were estimated among trait least squares means (df = 54).

		NHI	TB-MAT	Grain Yield	Grain N (%)	BN-ANTH	TPN-MAT	PANU	NUE	NUtE	NUpE	NRE
		Low N Environment										
NHI			0.68**	0.24	0.13	0.07	0.75**	0.08	0.25	0.94**	0.75**	0.10
TB-MAT		0.52**		0.77**	−0.57**	0.27*	0.75**	0.23	0.76**	0.31*	0.31*	−0.09
Grain Yield		0.17	0.84**		−0.61**	0.28*	0.82**	0.24	0.99**	0.63**	0.80**	0.11
Grain N (%)		0.42**	−0.33*	−0.36**		−0.04	−0.24	−0.15	−0.59**	−0.72**	−0.59**	0.08
BN-ANTH		−0.10	−0.29*	−0.37**	0.31*		0.29*	−0.76**	0.28*	0.12	0.29*	0.49**
TPN-MAT	High N Env.	0.72**	0.67**	0.25	0.76**	−0.11		0.29*	0.82**	0.10	0.98**	0.01
PANU		0.30*	0.66**	0.79**	−0.01	−0.65**	0.80**		0.22	−0.01	0.26*	0.56**
NUE		0.17	0.84**	0.99**	−0.35**	−0.37**	0.75**	0.79**		0.61**	0.81**	0.10
NUtE		0.89**	0.43**	0.54**	−0.84**	−0.42**	−0.14	0.16	0.54**		0.10	0.21
NUpE		0.72**	0.67**	0.75**	0.26*	−0.11	0.99**	0.80**	0.75**	−0.14		0.003
NRE		−0.02	−0.60**	−0.72**	0.05	0.73**	−0.69**	−0.97**	−0.73**	−0.20	−0.69**	

[a] NHI, N harvest index; TB-MAT, total biomass(grain plus biomass) at maturity; BN-ANTH, biomass N at anthesis; TPN-MAT, total plant N at maturity; PANU, post anthesis N uptake; NUE, N use efficiency; NUtE, N utilization efficiency; NUpE, N uptake efficiency; NRE, N remobilization efficiency.
* P < 0.05.
** P < 0.01.

lization efficiency (NUtE)) were calculated as follows: NUpE = Total plant N/soil N (Pre-N soil N plus fertilizer N), NUtE = yield/total plant N, NUE = (NUpE) * (NUtE) (Moll et al., 1982). NUE defined in this manner is simply grain yield divided by a constant.

2.4. Statistical analysis

Analysis of variance (ANOVA) was performed with the General Linear Models procedure (Proc GLM; SAS, 2011) to determine genotype and treatment effects across locations and N environments. The model used was:

$$Y_{ijkl} = \mu + LOC_i + R(LOC)_{ij} + ENV_l + G_k + ENV * G_{lk} + LOC * G_{ik} + LOC * ENV_{il} + LOC * ENV * G_{ilk} + E_{ijkl}$$

Where: Y_{ijkl} = the observation in the kth genotype in the jth rep in the ith location and lth N environment, μ = the overall mean, LOC_i = the effect of the ith location, $R(LOC)_{ij}$ = the effect of jth rep within the ith location, ENV_l = the effect of the lth N environment, G_k = the effect of the kth genotype, ENV * G_{lk} = the effect of the interaction of the lth N environment with the kth genotype, LOC*G_{ik} = the effect of the interaction of the ith location with the kth genotype, LOC*ENV_{il} = the interaction effect of the ith location and lth N environment, ENV*LOC*G_{lik} = the effect of the interaction of the lth N environment and ith location with the kth genotype, E_{ijkl} = the residual error. LSMEANS were calculated for locations, treatments and genotypes.

Broad sense heritability estimates and confidence intervals from the combined analysis of data from four environments (2 locations × 2 N levels) were generated after Knapp et al. (1985). PROC CORR (SAS, 2011) was used to analyze the relationship among traits on an entry mean basis.

2.5. Association analysis

The TCAP elite mapping panel was genotyped using the Illumina Infinium™ SNP genotyping assay with 90 K SNP markers (Cavanagh et al., 2013). Genotyping was conducted at the USDA-ARS Biosciences Research Laboratory, Fargo, ND, U.S. The markers from this assay were mapped in 8 bi-parental mapping populations and produced chromosome positions for about 44.7% of the markers (Wang et al., 2014). A subset of the original 28000 plus markers provided by the lab of Dr. Clay Sneller at Ohio State University was generated by removing those markers with minor allele frequency <10% and with missing data >5% and then a SNP tagging method was used to define 3919 independent markers. Further details can be found in Huang et al. (2016). Genome wide association studies (GWAS) were carried out with the Genomic Association and Prediction Integrated Tool (GAPIT) which uses the compressed mixed linear model approach (Lipka et al., 2012).

3. Results

3.1. NUE in high and low nitrogen regimes

Least squares means (lsmeans) from a combined analysis of agronomic and N use traits measured in the 2014 experiment are presented in Table 1. The impact of the high N regime was pronounced, especially at Princeton. Anthesis date occurred 2.3 days later, on average, under high N at Princeton, while the difference between N treatments at Lexington was only 1 day (data not

Table 3
SRW wheat breeding lines evaluated for Nitrogen deficiency tolerance (NDT) in several traits[a] as part of a 56 entry panel grown at two N levels Lexington and Princeton KY, 2014.

Entry	NDT NUE	NDT NUtE	NDT NUpE	NDT NHI	NUE LOW N	NUE HIGH N
KY03C-2049-02	208.38	120.43	297.52	122.22	55.13	26.45
IL99-26442	203.30	90.25	222.82	92.46	58.09	28.57
KY03C-1221-06	196.68	93.43	212.95	91.67	51.54	26.20
IL07-20743	193.55	90.87	229.13	89.86	60.12	31.06
KY02C-3005-25	193.21	98.23	195.71	95.89	57.18	29.59
KY04C-1128-38-1-5	194.12	67.67	150.23	70.78	55.14	28.40
KY03C-1237-32	190.15	82.83	232.43	86.82	54.56	28.69
KY05C-1617-17-17-3	192.29	88.28	219.34	87.44	57.45	29.88
IL07-6861	187.65	107.77	174.06	100.83	56.54	30.13
KY03C-1192-37	189.14	96.83	196.62	99.56	56.68	29.97
KY02C-1121-75	**191.27**	**87.11**	**220.10**	**87.56**	**63.09**	**32.99**
IL07-21847	188.00	92.71	205.51	92.50	55.84	29.70
IL08-34020	182.58	96.93	191.93	94.93	48.45	26.53
KY03C-1195-10-1-5	187.31	116.96	283.69	117.06	54.48	29.09
KY03C-1237-15	182.78	82.18	224.33	83.33	52.88	28.93
KY02C-1058-03	181.78	90.30	203.88	89.22	51.24	28.19
KY04C-3006-33-14-3	182.19	85.94	218.95	88.64	52.94	29.06
IL01-11934	**179.25**	**77.22**	**123.94**	**73.84**	**60.39**	**33.69**
KY93C-1238-17-1	**181.78**	**94.18**	**193.82**	**94.90**	**62.02**	**34.12**
KY06C-1003-139-8-3	**179.07**	**89.63**	**201.33**	**84.92**	**58.64**	**32.75**
KY02C-1076-07	179.62	91.34	195.86	91.47	53.82	29.97
KY05C-1381-77-7-5	177.58	92.92	192.73	84.23	54.28	30.56
IL07-19334	176.65	88.10	205.30	91.63	51.68	29.25
KY03C-2047-06	176.22	89.23	199.33	90.50	53.80	30.53
KY04C-2006-41-1-1	178.77	90.78	196.77	89.78	58.34	32.64
FOSTER	173.94	97.17	179.06	92.57	52.93	30.43
KY03C-2047-02	172.32	94.57	180.75	91.62	52.88	30.69
03207A1-7-3-1	172.21	96.95	183.57	88.44	44.94	26.09
IL07-23420	175.32	92.46	189.07	90.97	56.12	32.01
KY03C-2314-08	**171.74**	**95.49**	**179.07**	**92.37**	**60.84**	**35.42**
Mean	**174.3**	**90.7**	**190.5**	**90.4**	**52.7**	**30.3**
Standard Error	**1.8**	**1.5**	**4.5**	**1.5**	**0.6**	**0.3**

[a] NUE, nitrogen use efficiency, NUtE, nitrogen utilization efficiency, NUpE, nitrogen uptake efficiency, NHI, nitrogen harvest index. Entries highlighted in bold had grain yield ranks in the top 10 in both N environments.

shown). Plant height at Princeton was increased by 25%; at Lexington there was an 11% increase in response to N. Grain yield at Princeton increased 92% in response to the high N treatment; at Lexington the increase was 77% (Table 1). Interestingly, N regime did not significantly affect grain N concentration (data not shown). Biomass N concentration at maturity, averaged across N treatments was 80% higher at Lexington than at Princeton, though the difference between low and high N regimes was about the same at either location: 30% (LEX) vs 28% (PRN). The difference in N uptake efficiency (NUpE) from low to high N varied widely between locations from a 36% decrease at Princeton to a 55% decrease at Lexington. The same pattern was seen with NUE where a difference among N regimes of 103% was recorded at Lexington, vs a 42% difference at Princeton (Table 1).

Significant differences ($P < 0.05$) were observed among genotypes for most of the agronomic and N use traits measured (Table 1). Genotype × environment (N level/site combination) interaction was significant ($P < 0.05$) only for NUtE among the agronomic and N use traits measured. Genotype × N level interaction was significant for several traits at Lexington only; thus the results of the analysis by N level/site combination are presented separately.

Correlations among agronomic and N use traits are shown in Table 2. It was of particular interest to see how correlations were affected by N regime. In contrast to the report of Gaju et al. (2014), we did not observe a strong effect of maturity on agronomic or N traits. Under low N, the only statistically significant correlation with anthesis date involved biomass N at anthesis indicating that when N was limiting, later flowering types accumulated more total N at anthesis (data not shown). Total biomass at maturity, under low N, was strongly correlated with yield and all N traits except PANU and NRE. Under high N, correlations between total biomass at maturity and all N traits were significant. The correlations between yield and other traits were similar under the two N regimes with these striking exceptions: (1) under low N, yield was not correlated with NRE, while under high N there was a correlation of −0.72; (2) yield was highly correlated with total plant N at maturity under low N, though only weakly correlated under high N (Table 2). Grain N content showed the expected negative correlation with yield under both N regimes, though it was of greater magnitude when N was limiting. The other difference between low and high N with respect to correlations with grain N content involved biomass N at anthesis which was significant only under high N. NRE showed a strong negative correlation with N uptake efficiency under high N, but a non-significant correlation under low N (Table 2).

Heritability estimates and confidence intervals (90%) are presented in Table 4. These estimates are based on an analysis of the four environments (location × N regime) evaluated in 2014. Only PANU and biomass N (maturity) have confidence intervals that enclose zero, though several other traits approach that threshold. Anthesis date and height, as expected, were highly heritable.

3.2. Breeding choices and different nitrogen regimes

One of the measures of N use efficiency applied in this study was the ratio of yield under low N to yield under high N, termed nitrogen deficiency tolerance (NDT) by Wei et al. (2012) (Table 3). In a breeding program, when identifying lines in a preliminary test to continue testing, one might select the top 10 or 20% based on yield and other agronomic traits. In this study, if we selected the top 20% based on average grain yield over both N levels, the list of selected lines would include only 1 of the top 10 yielders in the high N environment. If the selection criterion had been yield under high N, the top 10 would have included lines with ranks 11, 18, 19, and 20 for yield NDT and we would have missed those lines with the high-

Table 4
Broad sense heritability (h^2) and 90% confidence interval (upper limit (UL); lower limit (LL)) for agronomic and N traits across N environments and locations in a 56 entry SRW wheat panel grown in low N (0 kg ha^{-1}) and high N (112 kg ha^{-1}) environments at Lexington and Princeton, KY in 2014 and a 280 entry wheat panel grown at high N at Lexington, KY in 2012.

Trait†	h^2	LL	UL
Anthesis date	0.80	0.72	0.86
Height	0.70	0.59	0.79
Yield	0.64	0.50	0.75
Harvest Index	0.29	0.02	0.49
Grain N Concentration	0.51	0.30	0.65
NDVI	0.51	0.28	0.64
Biomass N content -anthesis	0.25	0.12	0.43
Biomass N content- maturity	0.10	−0.38	0.31
Total plant N- maturity	0.32	0.04	0.52
PANU	0.28	−0.01	0.49
NHI	0.31	0.03	0.51
NUE	0.64	0.50	0.75
NUtE	0.58	0.41	0.71
NUpE	0.29	0.01	0.50
NRE	0.36	0.04	0.68

† PANU, post anthesis N uptake; NHI, nitrogen harvest index; NUE, nitrogen use efficiency; NUtE, nitrogen utilization efficiency; NUpE; nitrogen uptake efficiency; NRE, nitrogen remobilization efficiency.

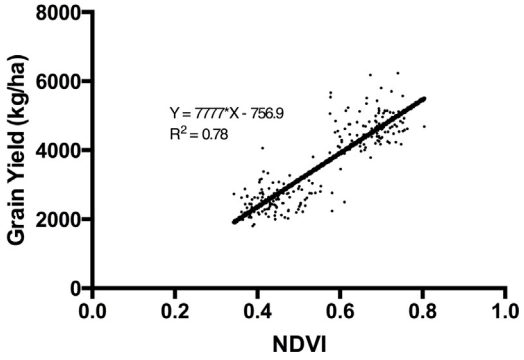

Fig. 1. Regression of grain yield on NDVI assessed on 56 soft red winter wheat breeding lines and cultivars grown at two N levels (0 and 112 kg ha^{-1}) Lexington and Princeton KY, 2014.

est NDT yields. Instead we focused on lines that ranked among the top 10 yielders in both environments; these lines are highlighted in bold in Table 3, which includes the top 30 lines ranked by NDT – yield. These lines in bold do not have the highest NDT values for overall NUE, NUtE, or NUpE, but it turns out they do have the highest NUE values of the lines shown under low N. It is clear that to select for NUE, breeding material must be evaluated under both low and high N conditions. In the present study we applied an approach commonly used by breeders, only "selecting" (by highlighting in bold) lines that yielded well in both N environments. If a line is to be widely grown it must be able to respond to high N; high NUE under low N conditions will not be enough to ensure its success. Furthermore, the only way for this to apply to the real world situation that wheat growers face is to incorporate an economic analysis into the overall performance analysis which was beyond the scope of this study. A complete picture would require a cost ascribed to very high N rates on the basis of N loss – reflecting an economic cost to growers as well as environmental costs.

The relationship between NDVI and grain yield is illustrated in Fig. 1. The two traits were very highly correlated, with an R^2 of 0.78, when entry means from low N and high N regimes were used for the analysis. Focusing on only one of the N levels does not yield the same result: under low N, the correlation was only 0.17; under high N, the correlation was significant, but the R^2 was only 0.27 (data not shown). In Fig. 2 grain yield was regressed on PANU in

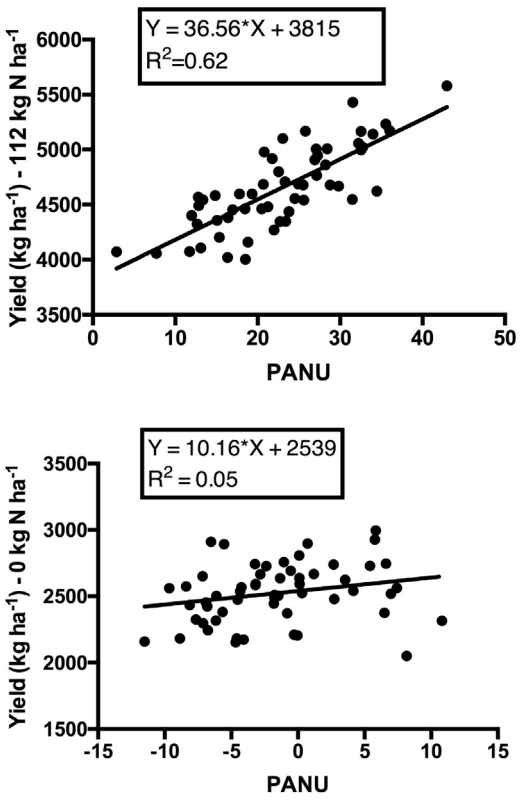

Fig. 2. Regression of grain yield on PANU (post anthesis N uptake) assessed on 56 soft red winter wheat breeding lines and cultivars grown at two N levels (0 and 112 kg ha^{-1}) Lexington and Princeton KY, 2014. Upper panel: high N environment; lower panel: low N environment.

each N environment. Under low N there was no significant linear relationship, in contrast with the strong relationship seen in the high N environment (R^2 = 0.62). Both of these figures illustrate the importance of evaluating of breeding material in both low *and* high N conditions.

3.3. Genome-wide association study

Results from the GWAS are presented in Table 5. Only traits for which there was a SNP with a LOD score >3 are listed. The analysis of the 2014 study was done using BLUPs generated by a mixed model analysis of each N environment separately as well as BLUPs from a combined analysis over both environments. The false discovery rate (FDR) for anthesis date with the 56-entry panel was <0.10, which is a good indicator of the viability of this analysis on a small number of genotypes. Three possible QTL were noted on 2A, 6A and 4B, the first two in agreement with Cormier et al. (2014). Other agronomic traits with significant QTL in the 2014 study include biomass (anthesis), grain plus biomass (maturity), and grain yield. A number of N use traits had at least one significant QTL, ranging in magnitude of effect from −7.4 (NUE) to 6.9 (N concentration at maturity) % of the mean of the respective trait (Table 5).

4. Discussion

Nitrogen use efficiency and the traits related to it have been studied exhaustively over the years (e.g., Van Sanford and Mackown, 1986; Gaju et al., 2011, 2014) and yet there remains uncertainty over the genetic underpinning of these traits. This is not surprising, given the complexity involved in the response of the wheat plant to nitrogen, whether native or exogenous. It is very difficult to achieve accuracy and precision in measuring these traits;

environmental effects are pronounced, and experimental noise is often excessive in these studies. Nevertheless researchers persist with these studies in the belief that it is only by understanding the genetic basis of N response that we can fashion an appropriate breeding strategy for developing NUE cultivars.

The goal of our 2014 experimental design was to create, by manipulating fertilizer N, an environment where N was the primary factor that limited grain yield. It appears that this was achieved according to the data in Table 2. Yield in the low N environment was on average, 54% of that in the high N environment. The conventional wisdom has long been that high grain yield in winter wheat is predicated on biomass and N accumulation prior to anthesis. Sinclair and Jamieson (2006) note that high amounts of N accumulated by anthesis are a key factor in grain number (the yield component most closely associated with yield determination), and that in agreement with Fischer (1993) this can be explained in terms of maintenance of leaf tissue that continues to photosynthesize. Abbate et al. (1995) surmised that the relationships between grain number per unit ground area and N at anthesis may show that the effect of N fertilization on grain yield could be explained as a consequence of crop growth prior to anthesis. Ferrisea et al. (2010), in a study of durum wheat, found that N remobilization was not affected by N fertilization, but they did observe that yield and kernel number per unit area were correlated with crop dry matter and N at anthesis.

The breeding lines in Table 3 are the top 30 lines for NDT-NUE, and of those highlighted in bold none ranks lower than 10th in both the low and high N environments. At first glance they do not appear noteworthy for the traits listed, except for NUE in the individual environments where they are top ranking. A study of their performance with respect to the many traits we measured does not reveal anything exceptional, nor does it indicate a pattern (data not shown). While it would be gratifying to identify a physiological trait, preferably one easy to measure, that explained much of the NUE variation in the study, that has not happened.

A glance at the NUE literature reveals that in most studies many traits are measured both to gain understanding of the underlying mechanisms and in hopes of identifying a few traits that stand out in terms of explaining variation in NUE. Further elucidation of relationships among traits in the two N environments was attempted by using a stepwise regression approach with grain yield as the dependent variable. Under high N conditions, the equation for the best fit model ($R^2 = 0.99$) was grain yield = −4432.3 + 53.0*biomass N (anthesis) + 53.7*PANU + 82.9*NUtE. In the low N environment, the best fit model ($R^2 = 0.99$) was grain yield = −2529.0 + 50.1*biomass N (Maturity) + 50.2*NUtE. These equations reinforce the correlations in Table 2 and suggest a different set of explanatory traits for each N environment. Under high N, biomass dry weight and N content at anthesis were significant regressors, as were PANU and NUtE. Our results indicate that when N is abundant, both pre and post anthesis periods are important in explaining yield variation. This is illustrated when grain yield is regressed on PANU in each N environment (Fig. 2). Under high N there appears to be a strong relationship between yield and PANU. Under N – constrained conditions, however, this association unravels, likely because the plant ran out of N. It is not clear if the apparent high N relationship between yield and PANU is real or an artifact of the way in which PANU is calculated in which yield is used in the numerator. Further, PANU is one of the few traits in this study that was not found to be heritable (Table 4). As noted above, when multiple regression was used to model low N response, biomass N at maturity along with NUtE (grain yield/total plant N) accounted for most yield variation. If these results are confirmed, there are implications for future research. At low N, there would be no need to make costly anthesis measurements of either biomass dry weight or N concentration. Under elevated N levels,

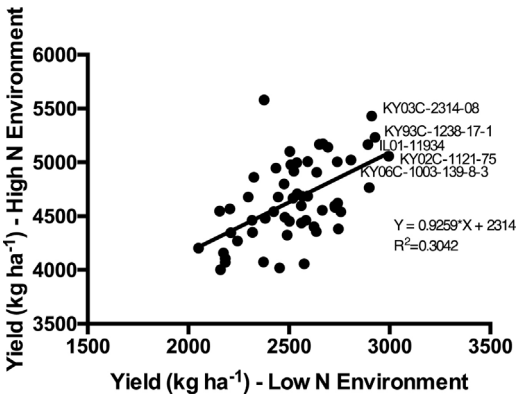

Fig. 3. Regression of grain yield under high N on grain yield under low N in 56 soft red winter wheat breeding lines and cultivars grown at two N levels (0 and 112 kg ha^{-1}) Lexington and Princeton KY, 2014. Entries named in the figure are those with yields ranked in the top 10 in both N environments.

however, biomass dry weight at anthesis, N content at anthesis and PANU all require anthesis measurements. These traits are laborious and expensive to measure, underscoring the need to find indirect estimators. As shown in Fig. 1 the use of NDVI as an indirect estimator when growing material in two N environments is encouraging. However the relatively high R^2 for the model reflects the two clusters of points at each end of the line that define the two N levels, more than genetic variation for NUE. The five lines highlighted in Table 3 have NDVI values above and below the mean, though the best NUE candidate, discussed below, has the highest NDVI values of the five in both environments (data not shown).

Fig. 3 shows the relationship between low and high N environments with respect to grain yield. In our study, the spread of the data points (entry lsmeans) about the regression line is more diffuse than was observed by Gaju et al. (2011); this difference is reflected in the fit of the regession lines: $R^2 = 0.84$ in Gaju et al. (2011) vs $R^2 = 0.30$ in our study (Fig. 3). In one sense, the lack of alignment between the two N environments is good news for the breeder because it suggest genotype × N interaction (G × N) that might be exploited in selecting for NUE. In our study, G × N was significant at Lexington but not at Princeton, thus muddying the water.

In the four panels that comprise Fig. 4, NUE (and thus grain yield) is regressed on its components, uptake and utilization efficiency in low and high N environments. One genotype, IL01-11934 is highlighted in the four panels because it tells an interesting story. Under low N, this line achieved high NUE but its NUpE lsmean is among the lowest observed; the same is true for its NUtE value. This means that the plant took up relatively little N but still managed to produce high grain yield, ranking 5th under low N. When N was not limiting, IL01-11934 still performed well (yield rank = 6th) but its N uptake was such that it ranked among the highest lines for that trait. This breeding line would seem to have the combination of attributes that would make it an ideal candidate in an NUE selection scheme. For reasons mentioned earlier, breeders must look for lines that are “opportunistic” as well as efficient, that are able to take up and utilize N when it is abundant.

Genome wide association studies can identify some potentially useful QTL that might ultimately be used in a molecular breeding program after validation. As was done with yield and NUE analysis, it was informative to carry out the GWAS for each N environment separately. With the caveat that the number of lines in this panel was quite small, the analysis was still useful because it identified SNPs for a number of traits that were not identified in the combined analysis (Table 6). Cormier et al. (2014), in an elegant GWAS of NUE, emphasized the importance of pre-grainfill N status, both N

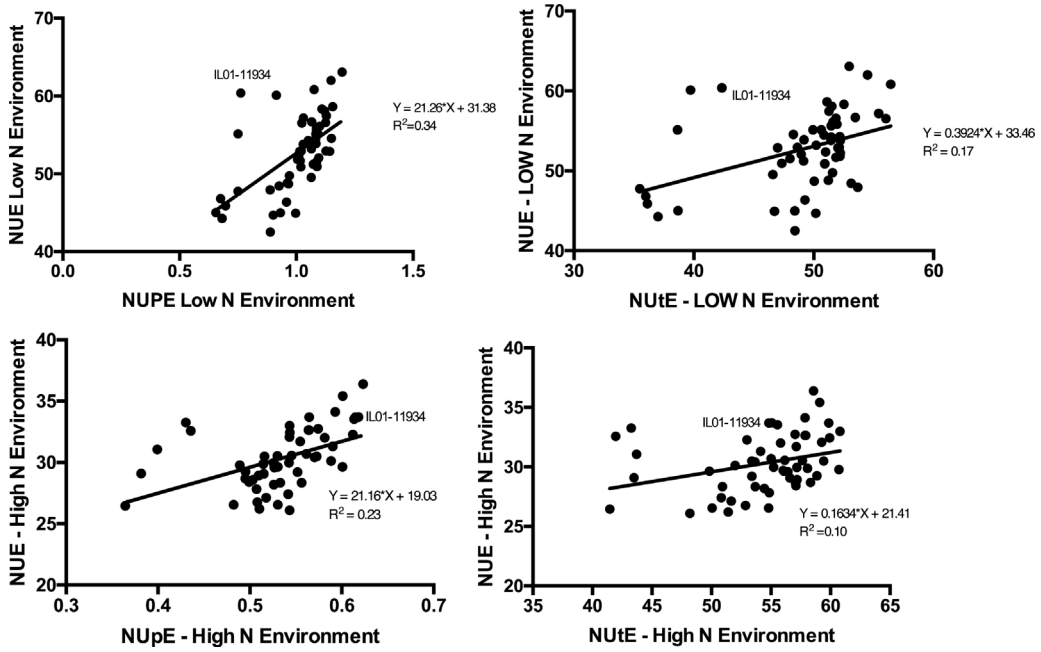

Fig. 4. Regression of NUE on NUpE in the low N environment upper left; regression of NUE on NUtE in the low N. Environment upper right; regression of NUE on NUpE in the high N environment lower left and regression of NUE On NUtE in the high N environment lower right.

Table 5
GWAS of 56 SRW wheat lines grown in low N (0 kg ha^{-1}) and high N (112 kg ha^{-1}) environments, Lexington and Princeton, KY 2014. Only SNPs with LOD score >3 are shown. Effect of SNPs is expressed in percent of the mean of each trait.

Trait[a]	SNP	Chromosome	Position	P value	Effect (%)	R^2 (w/o SNP)	R^2 (w/SNP)	N env
Anthesis	BS00098857_51	6A	61.42	9.10E-06	1.9	0.12	0.56	Low
Date	BS00098857_51	6A	61.42	5.55E-05	12.3	0.14	0.48	Combined
	RAC875_c10850_316	4B	103.87	5.55E-05	5.8	0.14	0.48	Combined
	RFL_Contig3509_229	2A	430.56	5.55E-05	5.5	0.14	0.48	Combined
Biomass at Anthesis	Excalibur_c88792_93	1A	411.15	0.00091	5.1	0.016	0.27	Combined
N Concentration Anthesis	BS00093975_51	3A	75.27	0.00082	0.7	0.16	0.37	High
NDVI	IAAV5863	1B	364.19	0.00059	−1.7	0.06	0.32	High
N Concentration At Maturity	RAC875_c2253_1255	6A	89.33	0.00053	6.9	0.13	0.37	High
NUE	Ku_c63748_1264	2B	131.38	0.00078	−7.4	0.24	0.44	Combined
NUpE	TA001769-0538	1B	345.15	0.0007	−6.2	0.20	0.41	Combined
NUtE	Tdurum_contig46413_779	1A	89.29	0.00063	2.4	0.18	0.40	Combined
NUtE	Excalibur_c62042_175	3A	76.94	0.00065	2.4	0.13	0.37	High
PANU	Excalibur_c6714_246	5A	117.45	0.0009	0.71	0.0001	0.25	Low
Grain N Concentration	BS00099998_51	6B	160.39	7.32E-05	3.8	0.08	0.44	High
	Excalibur_c62042_175	3A	76.94	0.0001	−1.8	0.08	0.40	High
	Kukri_c33374_1048	2A	67.37	0.0006	1.2	0.08	0.33	High
Total Biomass At Maturity	RAC875_c43002_382	1A	439.42	0.0004	−0.7	0.23	0.46	High
Biomass N Maturity	Ex_c5594_2818	5B	214.95	0.00069	4.2	0.08	0.3303	Combined
Grain Yield	Ku_c63748_1264	2B	131.38	0.000642929	5.6	0.27	0.4664	Combined

[a] NDVI, normalized difference vegetative index; NUE, nitrogen use efficiency; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency.

concentration and biomass N at anthesis. The present study also identified SNPs associated with these traits (Table 6).

Ultimate value of potential QTL identified by GWAS depends on the magnitude of the QTL effect among other things. Interestingly, there was a significant SNP (4.2%) on chromosome 5B associated with increased biomass N at maturity, a trait that was significant in determining yield variation in the stepwise analysis of the low N environment (Table 6). There was a putative QTL (5.1%) associated with biomass at anthesis on chromosome 1A, a trait shown to be important in the stepwise regression analysis of yield in the high N environment. GWAS also revealed potential QTL associated with NUE and its components, uptake and utilization efficiency with effects ranging from −7.4 to 2.4% of the means of those traits (Table 6). In a recent review of N use efficiency Cormier et al. (2016) summarized studies in which putative N use QTL were reported. Though their own recent study (Cormier et al., 2014) had revealed 388 such QTL, the later review paper acknowledged the polygenic nature of the traits and the likelihood that most QTL are of small magnitude.

The state of the current conversation about NUE is reflected in two papers from these investigators: Cormier et al. (2013) and Cormier et al. (2014). In the former, a multi -location evaluation of European wheat cultivars, the authors observed apparent improvement in N utilization efficiency and no improvement in uptake efficiency, which was not heritable. Further, they note that NUE was increased most readily in low N environments. In the second paper, in which exhaustive GWAS of NUE was reported, the QTL of interest that emerged were associated with N concentration at anthesis, flowering date and total vegetative N at anthesis. Thus, one study indicates the importance of pre-anthesis events and the other highlights the role of remobilization of N during grain fill.

There is evidence in these recent papers of the importance of N taken up by anthesis, yet it is clear that utilization efficiency is critical to improving NUE. We see the same indications from the data reported herein.

Screening breeding lines in low and high N environments is critical to determining NUE. If environmental concerns and prohibitive input costs argue for lower N rates, we must be able to identify genotypes that can perform relatively well on lower N rates, as the four breeding lines in Table 5 did. We estimated heritability of a number of agronomic and N use traits in this study. In evaluating these traits and their potential as breeding targets, it is not only essential that they are heritable, but they must be positively associated with yield and relatively easy to measure. Of the traits measured, only NDVI meets these criteria. It is only moderately heritable, but can be measured very rapidly and in this study over two N levels it was highly correlated with yield (Table 3; Fig. 1). The strong linear relationship between the two traits reflects the differences in low and high N environments more than genetic variation, but it represents a starting point in identifying true NUE types. In research conducted in Oklahoma Prasad et al. (2007a,b) proposed using spectral reflectance as an indirect selection criterion. In spite of the vastly streamlined evaluation process – using NDVI instead of onerous anthesis and maturity measurements – breeders may be reluctant to plant another set of trials under low N simply because the tests have to be planted, screened and harvested. In the absence of a robust QTL that explains significant genetic variation in NUE, there may be no alternative.

One of the problems with NUE research is the tremendous expense of measuring traits like biomass at anthesis. Both spectral reflectance and GWAS offer the promise of increased efficiency and lower costs. Whether GWAS identifies individual QTL that are used in marker-assisted selection or whether genomic selection models and methods are devised for NUE, much QTL validation is required. In the interim, breeders will have to test their material in a low as well as a high N environment in order to identify the most efficient types. If canopy spectral reflectance can be shown to be very predictive of yield, breeders might be able to use it in place of yield testing in one of the environments.

Acknowledgments

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