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RESEARCH

QTL Analysis of Shoot Ureide and Nitrogen Concentrations in Soybean [*Glycine max* (L.) Merr.]

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ABSTRACT

Nitrogen (N) fixation in soybean [*Glycine max* (L.) Merr.] is more sensitive to drought than photosynthesis, and high concentrations of shoot ureide and N are associated with sensitivity of N_2 fixation to drought. Genotypic differences in ureide and N concentration were evaluated using a mapping population of 97 recombinant inbred lines derived from a cross between 'KS4895' and 'Jackson'. For three irrigated environments, broad-sense heritability for ureide and N concentration was 0.73 and 0.59, respectively. Under irrigated conditions, five quantitative trait loci (QTLs) for ureide concentration were identified using composite interval mapping (CIM). Multiple interval mapping (MIM) identified two QTLs with locations similar to those identified with CIM. Four QTLs for N concentration were detected using CIM, and one QTL was identified with MIM with a similar position as that identified with CIM. A QTL on Gm13 for shoot ureide and N appeared to be pleiotropic. In the drought environment, two QTLs were identified using CIM for both shoot ureide and N; a QTL for ureide concentration on Gm19 mapped to the same position as a ureide QTL under irrigated conditions, but the additive effect was opposite in sign. A search for metabolic genes in QTL regions predicted for the pleiotropic effect of N and ureide (Gm13, carbonic anhydrase) and for ureide (Gm19, inosine–uridine nucleoside hydrolase). These QTLs may be useful in selecting lines drought tolerant for N_2 fixation.

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Abbreviations: CIM, composite interval mapping; EST, expressed sequence tag; LG, linkage group; LOD, logarithm of odds; LRT, likelihood ratio test; LS, least square; MIM, multiple interval mapping; PCR, polymerase chain reaction; QTL, quantitative trait locus; RIL, recombinant inbred line; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

Nitrogen is nutritionally important to plants and animals. It is a basic element of energy-transfer molecules (such as adenosine triphosphate [ATP]), nucleic acids (such as DNA), chlorophyll, and a crucial component of amino acids, and hence proteins. In soybean [*Glycine max* (L.) Merr.], the symbiosis with *Bradyrhizobium japonicum* allows the crop to grow independently from inorganic N fertilizer. Under drought conditions, however, N_2 fixation is more sensitive than leaf gas exchange (Durand et al., 1987; Kuo and Boersma, 1971; Sinclair, 1986) and photosynthesis (Djekoun and Planchon, 1991; Serraj and Sinclair, 1997). Consequently, reliance on N_2 fixation in soybean makes the plant vulnerable to N deficiency under limited soil-moisture conditions.

The initial product of N_2 fixation is NH_3, and the NH_3 is assimilated into the ureides, allantoin and allantoate, in soybean

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(Sheoran et al., 1982). Ureides are exported from the nodule through the xylem (Schubert, 1986; Walsh, 1995). Under drought conditions, ureide concentration increases in shoot (deSilva et al., 1996; Serraj and Sinclair, 1996) and nodule (King and Purcell, 2005) tissues despite a decrease in ureide production from N_2 fixation.

Interestingly, there are genotypic differences in the degree to which ureides accumulate during drought. Genotypes in which N_2 fixation is drought sensitive accumulate large amounts of ureides, whereas genotypes in which N_2 fixation is drought tolerant accumulate substantially less ureides (King and Purcell, 2001, 2005; Purcell et al., 1998, 2000; Serraj and Sinclair, 1997).

The accumulation of high tissue ureides during drought, especially in genotypes in which N_2 fixation is drought sensitive, led to the hypothesis that ureides might serve as a signal molecule and elicit a decrease in N_2 fixation during drought (deSilva et al., 1996; Purcell et al., 1998, 2000; Serraj and Sinclair, 1996). Further experimentation indicated that ureides do not function directly as a signal molecule to decrease N_2 fixation (Ladrera et al., 2007; King and Purcell, 2005) but that the general N concentration of a genotype is associated with the ability to continue N_2 fixation during drought (King and Purcell, 2006). Across a wide range of the major soybean ancestors, those genotypes with low shoot N concentration under well-watered conditions had prolonged N_2 fixation under drought compared with genotypes with high shoot N concentration (King and Purcell, 2006). Therefore, it appears that drought tolerance for N_2 fixation could be associated with low shoot ureide or N concentration under well-watered conditions.

Because of the importance of N_2 fixation for N nutrition in soybean and the sensitivity of N_2 fixation to drought, improving the drought tolerance of N_2 fixation is a key step in improving soybean crop performance during drought (Sinclair et al., 2010). Unfortunately, little information is available on the population genetics of N_2 fixation, ureide, or N nutrition in soybean.

In the current research, we developed a recombinant inbred population from a cross between 'KS4895' and 'Jackson'. Previous research has characterized N_2 fixation in Jackson as being drought tolerant and having relatively low concentrations of shoot ureides and N (King and Purcell, 2005, 2006; Purcell et al., 2000). In contrast, N_2 fixation of KS4895 is drought sensitive and has relatively high concentrations of shoot ureides and N. Our objectives were to determine heritability of shoot ureide and N concentration and to identify quantitative trait loci (QTLs) associated with these traits under irrigated and drought conditions.

MATERIAL AND METHODS

Population Development

KS4895 (PI 595081, Maturity Group IV; Schapaugh and Dille, 1998) was used as the female parent in a cross with Jackson (PI 548657, Maturity Group VII; Johnson, 1958). Seeds from F_1 plants were bulk-threshed. Generations were advanced from the F_2 to the F_3 or F_5 generation by the single-seed descent method. For the last generation, all the seeds from individual F_3 or F_5 plants were threshed and used to establish 17 F_3– and 80 F_5–derived rows to generate recombinant inbred lines (RILs). The RILs were selected to have a fairly narrow range of maturities during generation advancement. Combining F_3– and F_5–derived RILs for genetic analysis increased mapping resolution (Charlson et al., 2009). Furthermore, the homozygosity of the subpopulations was similar at 91% (F_3 derived) and 94% (F_5 derived).

Phenotyping of Ureide and Nitrogen Concentration

Preliminary experiments (King and Purcell, unpublished results, 2010) indicated that shoot ureide and N concentrations increased during vegetative development but were relatively stable between the R2 and the beginning of the R5 developmental stages (Fehr and Caviness, 1977). The R4 or R5 period of development was associated with the greatest differences among genotypes in shoot ureide and N concentrations (King and Purcell, unpublished results, 2010). Therefore, sampling at these stages was chosen for phenotyping in the irrigated field trials.

Shoot biomass samples from three to five plants were collected from the R4 to early R5 stage under irrigated conditions, whereas shoot biomass samples were collected at R2 under drought conditions. Biomass samples were dried, weighed, and ground to pass through a 6-mm sieve. Subsamples were finely ground to pass through a 1-mm sieve.

Ureides were extracted from 0.125 g of finely ground plant material in test tubes by adding 5 mL of 0.2 M NaOH. The test tubes were placed in a water bath at 100°C for 30 min, and then 1 mL was transferred to a 1.5-mL microfuge tube. Samples were centrifuged at 20,000 × *g* for 5 min, and 50 to 100 μL of the supernatant was analyzed for ureide concentration using the colorimetric procedure of Young and Conway (1942). Shoot N concentration was analyzed by the Dumas method (Kaya and Campbell, 1967) with a Leco FP-428 Determinator (Leco Corporation, St. Joseph, MO) at the Soil Testing and Plant Analysis Laboratory at the University of Arkansas.

Field Trials

A randomized complete block design was used for the field trials. The field trial in 2000 (three replications) was conducted under mild drought conditions at Keiser, AR (35°40′27″ N, 90°04′24″ W), using 75 of the F_5–derived RILs. The soil at Keiser was a Sharkey silty-clay (very-fine, smectitic, thermic Chromic Epiaquert) with approximately 1% organic matter. Row spacing at Keiser was 96.5 cm and plot length was 5.1 m.

In the irrigated environments a combination of F_3– and F_5–derived lines were used, but a different number of RILs was used each year due to the availability of sufficient seed quantities. Field trials in 2005 had three replications with 95 RILs, trials in 2007 had one replication and 91 RILs, and trials in 2011 had two replications and 86 RILs. The irrigated trials were conducted at Fayetteville, AR (36°5′4″ N, 94°10′29″ W). All entries were planted into single- or two-row plots (50.8-cm row spacing and 6.1-m row length). Populations were sown on 9 June 2005, 31 May 2007, and

1 June 2011. Irrigation was supplied with an overhead sprinkler system when soil moisture reached an estimated 35-mm deficit (Purcell et al., 2007). The soil type at Fayetteville was a Taloka silt loam (fine, mixed, thermic Mollic Albaqulf) with 1.1% organic matter and a pH of 6.6. Jackson and KS4895 were used as tolerant and sensitive cultivars for N_2 fixation (Purcell et al., 2000), respectively, in all three years (2005, 2007, 2011).

Statistical Analysis

The SAS 9.2 (2008) statistical software package (SAS Institute Inc., Cary, NC) was used for analysis of variance, heritability, least square mean (LS mean), normality, randomization, phenotypic correlation, and parent independent *t* test. Analysis of variance was performed by year on the phenotypic data collected in 2000, 2005, and 2011. Because data in 2007 were unreplicated, it was not evaluated using analysis of variance but was combined with other irrigated environments (2005, 2011) for analysis in a multiyear model. All classifications were treated as random variables. A mixed model was used for analysis of variance and heritability calculations, and normality tests. PROC GLM was used to estimate LSMEANS, and PROC GPLOT and PROC UNIVARIATE were used to test for normality in each year.

DNA Extraction

Young leaf tissue was collected from the 97 RILs and two parental lines of KS4895 and Jackson described above. Each leaf sample was put on dry ice in a cooler soon after the tissue was collected and moved to the laboratory. The tissue was freeze-dried in a lyophilizer (Model 18DX48SA, Botanique Preservation Equipment, Inc.), ground to a fine powder with a tissue pulverizer (Garcia Manufacturing, Visalia, CA), and then stored at −20°C. DNA was isolated using the modified quick extraction tool, Maxwell 16 automated machine (Promega, Madison, WI). Absorbance was determined at 260 and 280 nm with a spectrophotometer and used to estimate DNA concentration.

Marker Genotyping

Simple sequence repeat (SSR) amplicons were evaluated for polymorphisms on parental lines. Markers were amplified using the polymerase chain reaction (PCR), and polymorphic markers were identified using polyacrylamide gel electrophoresis or by separating PCR products by size using an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA).

The Illumina GoldenGate Assay, using the BeadStation 500G (Illumina, Inc., www.illumina.com [accessed 10 July 2013]), was performed as described by Fan et al. (2003), using the 1536-SNP USLP 1.0 array created by Hyten et al. (2010). The allele calls were made with Illumina GenomeStudio SNP analysis software (www.illumina.com) based on the fluorescence emission. Eight single nucleotide polymorphisms (SNPs) that were not included in the USLP 1.0 marker set were genotyped using a KASP reaction (K-Bioscience, Hoddesdon Herts, UK). Reaction results were read on a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) using Endpoint genotyping.

Linkage Test

A total of 664 informative markers (493 SNPs and 171 SSRs) were used for the genetic map. Markers that had an unexpectedly high number of double-crossover events were removed. The χ^2 goodness-of-fit test (df = 2) was conducted to detect deviation from the expected 1:1 Mendelian segregation. In the χ^2 test, the Bonferroni-adjusted significant $\langle$ criterion was set to 0.0001 (0.05/664). Map Manager QTX (Manly et al., 2001) was used to create raw data files for Mapmaker 3.0/Exp (Lander et al., 1987), which was initially used for linkage tests and grouping. Initially, a logarithm of odds (LOD) criterion from 6 to 15 was used to establish linkage groups (LGs) or sub-LGs. All unlinked sub-LGs were joined at a LOD value of 3. The Kosambi mapping function (Kosambi, 1944) was used for all linkage analysis. Since the BARC-SNP marker name was very long, we chose to reference the SNP number submitted to GenBank. We removed the first common letter (s) and the first common four digits (1079) to use MapMaker 3.0 and Map Manager QTX.

Genetic Map Construction

The R/qtl library (Broman et al., 2003) in R (2.9.2) was used for the final construction of the genetic map. The recombination fraction was estimated by Maximum Likelihood with the Expectation-Maximization algorithm (Lander et al., 1987). The default iteration maximum number was 10,000 and 0.000001 was used as the tolerance value. A genotyping error frequency of 1% was assumed in R/qtl for linkage distance estimation. The marker order was tested with the "Ripple" command with window size of 3. Two input files (*.cro and *.map) that were derived from R/qtl were used to construct the named-marker genetic map in WinQTLCartographer 2.5.010 (Wang et al., 2011).

QTL Analysis Based on a Single-QTL Model

The software package WinQTLCartographer 2.5.010 was used for the QTL analysis. The Maximum Likelihood approach (Weller, 1986) using the Expectation-Maximization algorithm (Meng and Rubin, 1993) was used for parameter estimation of composite interval mapping (CIM; Zeng, 1994) with stepwise selection, an α value of 0.05, and a window size of 1 cM. The parameters included a minimum distance of 5 cM between the putative map positions of adjacent putative QTLs. A minimum 1-LOD declination value was required between two adjacent putative peaks (if both peaks exceeded the LOD threshold value) to declare the two peaks as separate QTLs. A permutation test (1000 repeats) (Churchill and Doerge, 1994) was applied to empirically determine the critical likelihood ratio test (LRT) value for declaring the existence of a QTL. The LRT threshold values varied depending on the measured trait, water treatment, and year, thereby necessitating specification of LRT thresholds for each trait–water–year combination.

Multiple-trait Analysis

Multiple-trait analysis was used to evaluate the significance of the interaction between a QTL and the field trial environments (H_o: QTL × Environment = 0, H_a: QTL × Environment ≠ 0). Multiple-trait mapping was based on the CIM method (Jiang and Zeng, 1995) in WinQTLCartographer 2.5.010. The multiple-trait test was applied with the three irrigated environments (2005, 2007, and 2011). Most of the procedures except for the permutation step were the same as those described for CIM. Because the same three traits in different years (2005, 2007, and 2011) were jointly

permutated, these traits were independently randomized to obtain the significant LOD value across the whole genome at an overall α value of 0.05. Therefore, four types of LOD threshold values were obtained (i.e., 2005, 2007, 2011, and joint-trait).

QTL Analysis Based on Multiple-QTL Model

QTL Network software, developed by Yang et al. (2007, 2008), was used to apply the multiple interval mapping (MIM) method (Kao et al., 1999). The QTL × QTL interaction and QTL × Environment interaction were also evaluated in this model. The candidate marker intervals were first identified via a marker pair selection method to prevent over-fitting of the model (Piepho and Gauch, 2001). Then, a one-dimension genome scan was executed to identify QTL controlling marker intervals. For the next step, all possible epistasis between marker intervals were identified. In a two-dimension genome scan, all possible combinations of two loci were tested to determine if they had a significant effect on ureide or N concentration, regardless of whether or not loci were in a QTL region (Yang et al., 2007, 2008). The statistical significance of all tests was executed with an *F* test. The Bayesian method via Gibbs sampling (Wang, 1994) was used for parameter estimation in MIM. Each sequential model was converted into a final model using the stepwise model selection procedure. Interacting loci pairs in the two-dimension genome scan were added to the model using a stepwise selection procedure. For each sequential model added by the stepwise procedure, a permutation test was conducted for new coefficient terms (Yang et al., 2007, 2008). A genome-wise threshold value of 0.05 was used as an overall α level for each sequential model using an *F* test. Therefore, the QTL Network software generated an *F*-statistic profile instead of a LOD profile.

Identification of Candidate Genes

SoyBase (www.soybase.org [accessed 10 July 2013]) and phytozome (www.phytozome.net [accessed 10 July 2013]) were used to search for candidate genes between flanking markers of QTLs. The Glyma 1.0 gene set in SoyBase provided predicted gene information. The homology-based gene prediction program, GenomeScan (Yeh et al., 2001), and expressed sequence tags (ESTs) identified in legumes using PASA (Program to Assemble Spliced Alignments, http://pasa.sourceforge.net [accessed 10 July 2013]; Haas et al., 2003) were jointly used to predict genes. All predicted genes between flanking markers were organized by gene ontology analysis (www.geneontology.org [accessed 10 July 2013]). SoyBase and KEGG (www.genome.jp/kegg/ [accessed 10 July 2013]) were used to investigate possible pathways.

RESULTS

Field Trial and Phenotype Data

Analysis of variance for shoot ureide and N concentrations was performed for data collected in 2005, 2007, and 2011 under irrigated field conditions. Although the data in 2007 had only one replication, it was included in the multiyear analysis. The main effects (RIL and year) and interaction effect (Year × RIL) were significant ($P < 0.0001$) for both traits, indicating that RIL means should not be averaged across years for subsequent QTL analysis. Instead, a LS mean was estimated for each RIL on a yearly basis. Analysis of variance conducted by year for 2000, 2005, and 2011 for ureide and N concentrations indicated a significant effect of RIL in all cases (Table 1).

The significance of the differences between the two parental means was tested by a two-tailed *t* test (Table 2). Under irrigated conditions in 2005 and 2011 (there were no parental data in 2007), ureide concentration was greater for KS4895 than for Jackson ($P = 0.001$ and $P = 0.045$, respectively), but N concentration between parents was not significantly different either year. The range of values of ureide and N concentrations among the RILs extended beyond that of the parents in both years, indicating the possibility of transgressive segregation. The population means for shoot ureide and N concentration in the KS4895 × Jackson population across the three years were 36.0 μmol g^{-1} and 2.71 g 100 g^{-1}, respectively. Average means for the two parents across the two years were 38.94 μmol g^{-1} and 2.84 g 100 g^{-1} for shoot ureide and N, respectively, indicating that midparent values were close to population means.

In the drought trial in 2000, both ureide ($P = 0.0005$) and N ($P = 0.03$) concentrations were greater for KS4895 than for Jackson (Table 2). The ureide concentration of KS4895 was greater than the maximum ureide concentration among the RILs while the minimum value among the RILs was lower than that of Jackson. For N concentration in 2000, the range among RILs extended beyond the parental values. The population means for both traits were near the midparent values.

The normality of each trait was tested with skewness, kurtosis, Q-Q plot, and residual plots (Table 2). The N concentration followed a normal distribution except in 2011 in which the distribution was slightly skewed to the right. The ureide concentration had a normal distribution in all environments. In general, both N and ureide concentrations followed a normal distribution.

Broad-sense heritability from progeny means was estimated for both traits across the three years (Table 1; Knapp et al., 1985). The estimated heritability for shoot ureide concentration was 0.73, and it ranged from 0.62 to 0.81 (95% confidence intervals). When using expected mean squares, broad-sense heritability was similar (0.74). The heritability of shoot N concentration was 0.59 with 95% confidence intervals ranging from 0.43 to 0.72. The heritability was estimated to be 0.61 using expected mean squares. Under mild drought conditions in 2000, broad-sense heritability for ureide and N concentrations were 0.60 and 0.65, respectively.

Shoot ureide and N concentrations of RILs averaged over replications in the irrigated trials each year had strong positive phenotypic correlations (Table 3), and when averaged over the irrigated environments, the phenotypic correlation was 0.72. The correlation in the

Table 1. Analysis of variance and broad-sense heritability (h^2) for ureide and nitrogen concentrations by years and over years (2005, 2007, 2011) for irrigated environments.

Water treatment	Year	Replications	RILs†	Ureide	h^2	Nitrogen	h^2
Drought	2000	3	75	*	0.60	**	0.65
Irrigated	2005	3	96	**	0.73	**	0.64
Irrigated‡	2007	1	90	–	–	–	–
Irrigated	2011	2	86	**	0.79	**	0.73
			Multiyear analysis for irrigated environments‡				
	Year			NS§		**	
	RIL			NS		**	
	Year × RIL		96	**	0.73	**	0.59

* Parental means were significant at $P = 0.05$.

** Parental means were significant at $P = 0.01$.

† Recombinant inbred lines.

‡ Analysis of variance was not performed for 2007 since it only had one replication, but it was included in the multiyear analysis.

§ NS, parental means were nonsignificant.

Table 2. Parental and population statistics for shoot ureide and nitrogen concentrations in the KS4895 × Jackson population.

	2000		2005		2007		2011	
	Ureide	Nitrogen	Ureide	Nitrogen	Ureide	Nitrogen	Ureide	Nitrogen
	μmol g^{-1}	g N 100 g^{-1}	μmol g^{-1}	g N 100 g^{-1}	μmol g^{-1}	g N 100 g^{-1}	μmol g^{-1}	g N 100 g^{-1}
				Parental statistics				
KS4895	32.41***	3.02*	50.54**	2.98NS†	–	–	41.61*	2.74NS
Jackson	12.47	2.73	32.45	2.77	–	–	31.17	2.85
				Population statistics				
Mean	18.63	2.87	39.05	2.89	34.89	2.63	34.13	2.62
Minimum	9.82	2.62	26.33	2.49	12.55	1.97	17.99	2.03
Maximum	29.96	3.23	51.59	3.32	63.99	3.46	50.82	3.18
Skewness	0.41	0.37	0.00	0.09	0.32	0.15	0.11	0.22
Kurtosis	0.12	0.15	–0.37	–0.68	–0.44	0.34	–0.13	–0.35
Normality test‡	NS	NS	NS	NS	NS	NS	NS	***

* Parental means were significant at $P = 0.05$ as determined by a two-tailed t test.

** Parental means were significant at $P = 0.01$ as determined by a two-tailed t test.

*** Parental means were significant at $P = 0.001$ as determined by a two-tailed t test.

† NS, parental means were nonsignificant as determined by a two-tailed t test.

‡ Four types of tests (Shapiro–Wilk, Kolmogorov–Smirnov, Cramer–von Mises, and Anderson–Darling), skewness, kurtosis, Q-Q plot, and residual plot were considered to test normality. Normality tests were conducted on progeny means for each year.

drought environment was also positive with a correlation coefficient of 0.71. When shoot N was regressed on shoot ureide, the linear regression coefficient had a positive slope (Fig. 1). A shoot ureide increase of 1.0 μmol g^{-1} resulted in the shoot N increase of 0.02 g 100 g^{-1} (50:1 ratio) and the regression accounted for 51% of the variation between shoot ureide and N concentrations.

Genotype Data and Genetic Map Construction

A χ^2 test was used to evaluate segregation distortion, and 19 SNPs were eliminated that had significant segregation distortion. Markers assigned to each chromosome in the KS4895 × Jackson population had similar positions when compared with the estimated positions in SoyBase. Since the genetic map of the KS4895 × Jackson population was composed of SSR and SNP loci, the marker order was compared to that of the consensus map version 4.0. A LOD value of the original genetic map order (consensus map order) in a given chromosome was compared to that of the KS4895 × Jackson genetic map order. Most markers had the same marker order, but two- or three-marker sets in a

Table 3. Phenotypic correlations between shoot ureide and nitrogen concentrations in the KS4895 × Jackson population.

Year	Water treatment	Correlation coefficient†	95% confidence interval	
			Lower value	Upper value
2000	Drought	0.50***	0.31	0.66
2005	Irrigated	0.64***	0.50	0.74
2007	Irrigated	0.66***	0.53	0.77
2011	Irrigated	0.63***	0.48	0.74
2005–2011‡	Irrigated	0.72***	0.60	0.80

*** Parental means were significant at $P = 0.001$ as determined by a two-tailed t test.

†Pearson's product moment correlation coefficients were estimated assuming shoot ureide and nitrogen concentrations follow independent normal distributions.

‡Ureide and nitrogen concentrations were averaged over years for each recombinant inbred line to estimate phenotypic correlations.

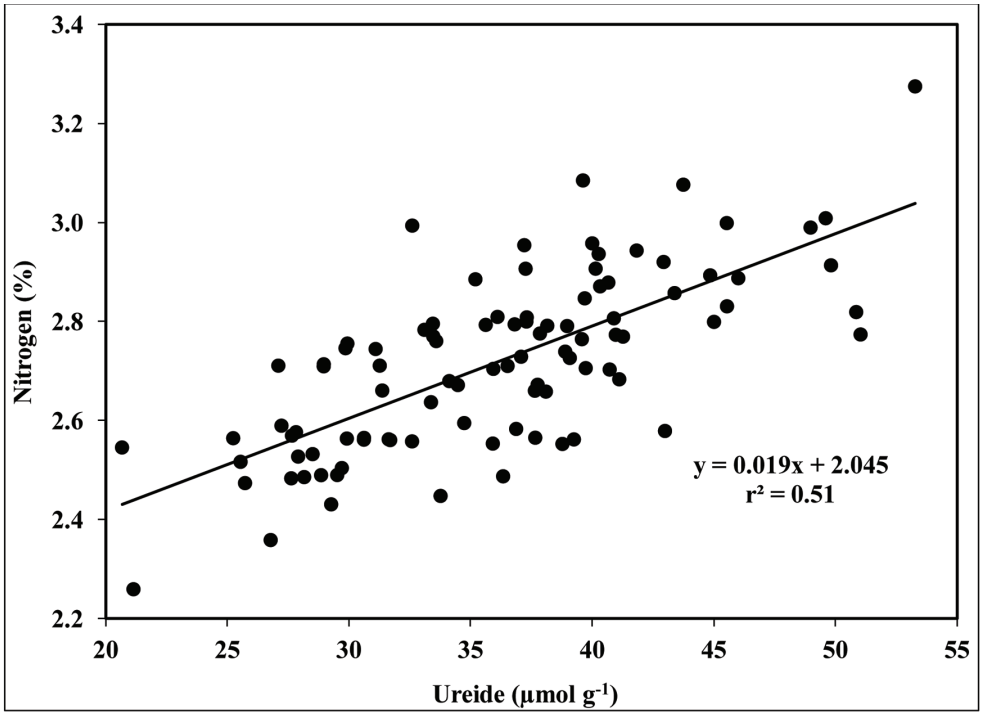

Figure 1. Relationship between shoot ureide and nitrogen concentration in a recombinant inbred population between KS4895 and Jackson, averaged over three irrigated environments.

given chromosome in the KS4895 × Jackson genetic map had minor differences in marker order. Minor map location differences are expected given the differences in population size between our population and those used to construct the consensus map. Additionally, others have reported minor differences in marker order between specific populations and the consensus map (Hyten et al., 2010).

A total of 664 markers (171 SSRs + 493 SNPs) was used to construct the genetic map using 97 RILs. At a LOD value of 3.0 or 3.5 all sub-LGs were joined and associated with their respective chromosomes. The total length of the genetic map was 2734.1 cM and was longer than that of the consensus map version 4.0 (2241.3 cM). In most cases, distances between adjacent markers were <20 cM, but marker-to-marker distances >30 cM did occur in some chromosomes.

QTL Analysis

Composite interval mapping and MIM were used as QTL models and all QTL results from CIM or MIM models under well-watered or drought conditions are shown in Table 4. Several QTLs for shoot ureide concentration were identified with the CIM model under irrigated field conditions in 2005, 2007, and 2011 with R^2 values ranging from 0.12 to 0.31 and additive effects of 2.33 to 5.99 μmol g^{-1}. Putative QTLs positioned at 117.5 cM (2007) and 124.3 cM (2011) on Gm19 were considered to be the same QTL based on the overlapping confidence interval (Table 4). The marker BARC-016145-02292 on Gm19 had the highest LOD value (10.6) and accounted for 31% of the variation of shoot ureide concentration. The marker BARC-017917-02456 on Gm13 had the highest additive effect (5.99 μmol g^{-1}). On Gm06 and Gm13, the KS4895 allele increased shoot ureide concentration.

In addition, two QTLs for shoot ureide concentration were detected under mild drought conditions in 2000. These QTLs were located on Gm09 and Gm19. The QTL on Gm19 that was detected in 2000 under drought conditions accounted for 18% of the phenotypic variation and had a similar position as the QTL identified in 2007 and 2011. However, the QTL identified on Gm19 in 2000 (drought) and in 2007 and 2011 (irrigated) had opposite additive effects on shoot ureide concentration.

The two QTLs for shoot ureide concentration identified with the MIM model were located on Gm13 and Gm19 (Table 4). These QTLs had the same positions and nearest markers as QTLs identified in the CIM model. Narrow-sense heritability for these QTLs was 0.102 and 0.117, respectively. The additive effects for these QTLs on Gm13 and Gm19 were 3.11 and 2.34 μmol g^{-1}, respectively.

Multiple-trait analysis for shoot ureide concentration for irrigated conditions (2005, 2007, and 2011) was undertaken. In this analysis, ureide concentration for each year and combined over years (joint trait) was used to evaluate the stability of ureide QTLs across years (Jiang and Zeng, 1995). Jiang and Zeng (1995) found that the interval maximum likelihood-ratio values for the multiple-trait mapping roughly followed a χ^2 distribution with $2n + 1$ df (n = 3 as the number of jointly used traits). When the Bonferroni correction was applied in the current research, α was 0.0001 due to the use of 664 markers. Therefore, a

Table 4. Quantitative trait loci (QTLs)identified from composite interval mapping (CIM) and multiple interval mapping (MIM) analyses for shoot ureide and nitrogen concentrations in the KS4895 × Jackson population.

Gm no.	Nearest marker	Submitted SNP† no.	Year	Water treatment‡	R^2	QTL effect§	Favorable allele¶	QTL position	LOD#	$P > \chi^2$ or $P > F$	95% confidence interval or 95% LOD support interval††
								cM			cM
					CIM (shoot ureide)						
Gm09	BARC-060299-16598	ss107927867	2000	WD	0.16	1.58	KS4895	1.0	3.6	0.000019	0.0–3.1
Gm19	Satt561	–	2000	WD	0.18	–1.67	Jackson	122.5	4.2	0.000017	119.9–124.2
Gm06	BARC-054349-12493	ss107924020	2005	WW	0.17	–2.33	Jackson	2.0	3.7	0.000020	0.0–9.0
Gm13	BARC-017917-02456	ss107915800	2007	WW	0.20	–5.99	Jackson	9.5	5.1	0.000002	8.5–11.5
Gm19	BARC-044415-08701	ss107913933	2007	WW	0.11	4.11	KS4895	117.5	3.8	0.000020	110.6–121.1
Gm09	BARC-061897-17586	ss107928760	2011	WW	0.12	2.53	KS4895	65.8	5.0	0.000002	65.2–68.4
Gm13	BARC-013325-00484	ss107914640	2011	WW	0.12	–2.59	Jackson	79.0	4.8	0.000004	76.9–82.5
Gm19	BARC-016145-02292	ss107913069	2011	WW	0.31	4.52	KS4895	124.3	10.6	0.000000	119.3–124.6
					MIM (shoot ureide)						
Gm13	BARC-013325-00484	ss107914640	–	WW	–	–3.11	Jackson	79.0	–	0.000000	75.9–83.0
Gm19	BARC-016145-02292	ss107913069	–	WW	–	2.34	KS4895	124.2	–	0.000000	123.2–124.8
					CIM (shoot nitrogen)						
Gm13	BARC-014657-01608	ss107912991	2000	WD	0.24	–0.06	Jackson	49.6	6.3	0.000000	48.6–55.1
Gm17	BARC-057467-14765	ss107925962	2000	WD	0.12	0.05	KS4895	72.3	3.3	0.000020	65.4–73.3
Gm13	BARC-055499-13329	ss107924542	2007	WW	0.25	–0.16	Jackson	11.5	7.2	0.000000	9.4–13.9
Gm13	BARC-013325-00484	ss107914640	2007	WW	0.11	–0.10	Jackson	79.0	3.7	0.000020	77.0–82.3
Gm13	Satt490	–	2011	WW	0.11	–0.09	Jackson	40.2	4.0	0.000018	35.6–43.2
Gm13	BARC-013325-00484	ss107914640	2011	WW	0.14	–0.09	Jackson	80.0	4.3	0.000015	76.5–83.8
Gm16	BARC-019215-03395	ss107916233	2011	WW	0.20	0.12	KS4895	126.5	5.8	0.000000	120.2–129.5
					MIM (shoot nitrogen)						
Gm13	BARC-013325-00484	ss107914640	–	WW	–	–0.08	Jackson	79.0	–	0.000000	76.9–82.0

†Single nucleotide polymorphism.

‡Two types of water treatments were applied. WW and WD mean well-watered and water-deficit field conditions, respectively.

§QTL effects were defined as one-half the mean of Jackson alleles minus one-half the means of KS4895 alleles. The units for the effect on shoot ureide are μmol g^{-1} and the units for shoot N are g N 100 g^{-1}.

¶Favorable allele was defined as the alleles giving low shoot ureide or nitrogen concentration.

#Logarithm of odds.

††95% confidence intervals are shown for CIM and 95% LOD support intervals are shown for MIM.

χ^2 value of 29.87 was approximately equivalent to a LOD score of 6.5 (29.87 × 0.217). The threshold LOD value for the joint trait was 5.4, which was lower than 6.5 estimated according to Jiang and Zeng (1995). One QTL located on Gm19 (124.3 cM) had a significant QTL × Year interaction. The LOD values of 2011 and of the joint trait were greater than the threshold value of the joint trait. The estimated QTL × Year effect was 1.57 μmol g^{-1}. The multiple-trait analysis indicated that there was a significant difference in the ureide concentration QTL on Gm19 but that the other ureide concentration QTLs were stable across years. The fact that the QTL on Gm19 had a negative additive effect in 2000 (drought environment) and a positive effect in 2011 (Table 4) agrees with the QTL × Year interaction.

In the MIM model, there were no significant QTL × Year interactions for the shoot ureide concentration. However, there were significant interactions from two pairs of loci. Although these loci were not identified as QTLs, these interactions may be useful in a selection model for ureide concentration. As shown in Fig. 2, there were four loci (Gm02, Gm4, Gm5, Gm14) that had significant interactions under irrigated conditions. The interaction effect of two loci located on Gm4 and Gm5 was 2.29 μmol g^{-1} ($P < 0.00001$). Two markers, Satt565 and Sat_217, were used as the nearest markers. Two other loci located on Gm02 and Gm14 had a 1.86 μmol g^{-1} interaction effect ($P < 0.00017$). Two markers, BARC-062943-18169 (ss107929341) and BARC-015539-02002 (ss107915148), were used as the nearest markers.

For shoot N concentration under well-watered conditions, four QTLs were detected with the CIM model. These QTLs were located on Gm13 (3) and Gm16 (1) (Table 4). The QTLs positioned at 79 (2007) and 80 cM (2011) on Gm13 had overlapping confidence intervals and were considered the same QTL. The QTL effects ranged from 0.085 to 0.156 g 100 g^{-1}, and R^2 values ranged from 0.11 to 0.25. The marker BARC-055499-13329 showed the highest additive effect (0.16 g 100 g^{-1}) and accounted for 25% of the phenotypic variation. All QTL alleles from Jackson except for the one located on Gm16 contributed to a decrease of shoot N concentration.

Under mild drought conditions in 2000, QTLs on Gm13 (1) and Gm17 (1) were detected (Table 4). The QTL

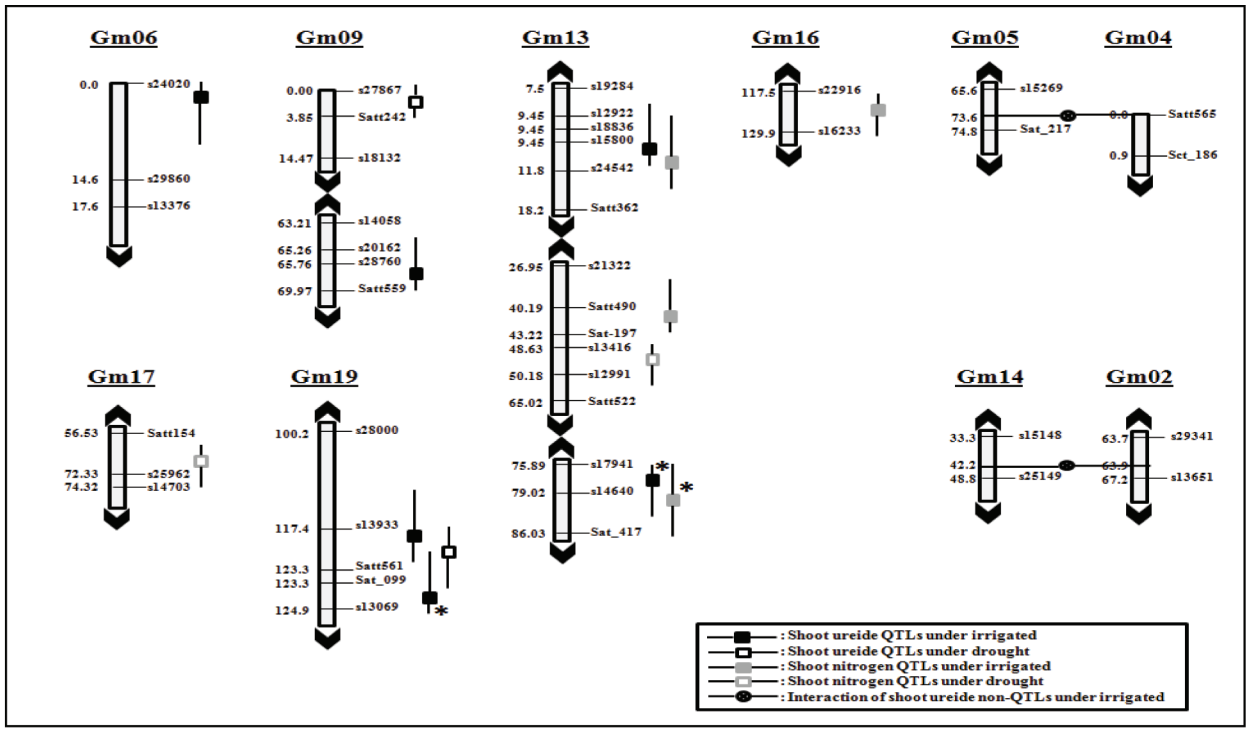

Figure 2. Location of quantitative trait loci (QTLs) for shoot ureide and nitrogen concentrations based on composite interval mapping in the KS4895 × Jackson population. The three QTLs with an asterisk were also identified with multiple interval mapping. The vertical lines on each side of QTLs represent 95% confidence intervals. The horizontal lines between Gm05 and Gm04 and between Gm14 and Gm02 represent chromosomal locations that were not QTLs but which interacted with ureide concentration under irrigated conditions.

allele from Jackson on Gm13 accounted for 24% of the phenotypic variation and decreased shoot N concentration, whereas the QTL allele from Jackson on Gm17 accounted for 12% of the variation and increased shoot N concentration.

In the MIM model for shoot N concentration, one QTL located on Gm13 (79 cM) was identified. This QTL had the same position and nearest marker as the QTLs identified in the CIM model for shoot N concentration and the QTL identified for shoot ureide concentration using CIM and MIM models. The narrow-sense of heritability of this QTL was 0.07 and the additive effect was 0.08 g 100 g^{-1}. In both models, there was no significant QTL × Year or other interactions, indicating that all N concentration QTLs were stable across years.

Figure 2 illustrates putative QTLs for both shoot ureide and N concentrations based on CIM and MIM. The solid black or gray square indicates QTLs detected under well-watered conditions. The open black or gray square denotes QTLs found when RILs were grown under drought conditions. On Gm13 there was a QTL for ureide concentration positioned at 9.5 cM and a QTL for N concentration positioned at 11.5 cM, which had overlapping support intervals. Likewise, the QTL positioned at 79 cM (shoot ureide) and 80 cM (shoot N) on Gm13 had overlapping support intervals. Considering the high phenotypic correlation between shoot ureide and N concentrations (Table 3 and Fig. 1), these QTLs appeared to have pleiotrophic effects for shoot ureide and N concentrations.

We investigated which traits were associated with QTL positions for shoot ureide and N concentrations using SoyBase (www.soybase.org/). Considering the LOD support intervals, Table 5 shows identified QTLs and nearby SSRs that have been reported for other traits. Quantitative trait loci for seed traits (weight, oil, protein, and steric acid), phenology, agronomic characteristics, and disease reactions have all been reported (Table 5) near where the QTLs for ureide concentration were identified in this study. Quantitative trait loci for similar traits have been reported near the QTLs for N concentration identified in our study including ones associated with reaction to cyst nematode, salt tolerance, drought index, and yield (Table 5).

Screening for Candidate Genes

A primary screening for candidate genes was executed. We evaluated QTL regions located on Gm13 (79 cM) and Gm19 (124.2 or 124.3 cM) (see Tables 4 and 6) to search for candidate genes between flanking markers. We chose these two QTL regions because the QTLs were well identified for CIM and MIM with a high R^2 and likelihood. For the QTL region on Gm13 (0.54 Mb between BARC-025915-05157 and BARC-013325-00484), we searched for candidate genes for both traits. Candidate genes for shoot ureide concentration also were investigated in the QTL region on Gm19 (1.94 Mb between Sat_099 and BARC-016145-02292). All predicted genes, between the flanking markers on Gm13 and Gm19, were organized by gene ontology terms (www.geneontology.

Table 5. Traits associated with quantitative trait locus (QTL) positions for shoot ureide and nitrogen in the KS4895 × Jackson population.

Trait	Gm no.	Year	Marker	Trait–Reference
Ureide	Gm19	2000	Sat_113	Seed weight (Stombaugh et al., 2004), seed oil (Qi et al., 2011), first flower date/plant height/lodging (Orf et al., 1999)
			Sat_099	Seed weight/reproductive period (Orf et al., 1999), reaction to *Fusarium solani* infection (Njiti and Lightfoot, 2006)
	Gm13	2007	Satt510	Seed oil (Specht et al., 2001), protein (Kabelka et al., 2004), reaction to *Sclerotinia sclerotiorum* infection (Arahana et al., 2001)
			Satt335	Seed protein (Hyten et al., 2004), plant height (Gai et al., 2007), pod maturity date (Li et al., 2008), lodging (Orf et al., 1999)
	Gm19	2007	Sat_113	Seed weight (Stombaugh et al., 2004), seed oil (Qi et al., 2011), first flower date/plant height/lodging (Orf et al., 1999)
	Gm13	2011	Sat_090	Protein content/plant height/steric acid content (Reinprecht et al., 2006)
	Gm19	2011	Sat_099	Seed weight/reproductive period (Orf et al., 1999), reaction to *Fusarium solani* infection (Njiti and Lightfoot, 2006)
Nitrogen	Gm13	2000	Sat_197	Stem strength (Chen et al., 2011)
			Satt144	Isoflavone content (Zeng et al., 2009), yield (Orf et al., 1999; Reyna and Sneller, 2001), genistein content (Zeng et al., 2009)
	Gm17	2000	Satt447	Alkaline salt tolerance (Tuyen et al., 2010)
			Satt669	Drought susceptibility index (Du et al., 2009)
	Gm13	2007	Satt510	Seed oil (Specht et al., 2001), protein (Kabelka et al., 2004), reaction to *Sclerotinia sclerotiorum* infection (Arahana et al., 2001)
			Satt335	Seed protein (Hyten et al., 2004), plant height (Gai et al., 2007), pod maturity date (Li et al., 2008), lodging (Orf et al., 1999)
			Sat_090	Protein content/plant height/steric acid content (Reinprecht et al., 2006)
	Gm13	2011	Satt490	Plant height (Kabelka et al., 2004), isoflavone content (Primomo et al., 2005)
			Sat_197	Stem strength (Chen et al., 2011)
			Sat_090	Protein content/plant height/steric acid content (Reinprecht et al., 2006)
	Gm16	2011	Sat_224	Reaction to cyst nematode (Guo et al., 2005)
			Satt431	Yield (Reinprecht et al., 2006), first flower date (Pooprompan et al., 2006), reaction to stem rot (Bachman et al., 2001)
				Reaction to cyst nematode (Glover et al., 2004)

Table 6. The classification of genes in quantitative trait locus (QTL) regions on Gm13 and Gm19 based on composite interval mapping, multiple interval mapping, and gene ontology analyses.

QTL position	Sequence length†	Flanking markers	Trait	Classification of genes	No. of predicted genes
cM	bp				
			Gm13		
79.0	40,148,892–40,685,899	BARC-025915-05157	Both	Transcription factor	7
		BARC-013325-00484		DNA or RNA related	1
				Metabolic process	7
				Proteolysis	2
				Others	16
				No function	12
			Gm19		
124.2/124.3	43,523,541–45,472,806	Sat_099	Ureide	Transcription factor	21
		BARC-016145-02292		DNA or RNA related	12
				Metabolic process	39
				Proteolysis	13
				Others	54
				No function	52

† Information of sequence length was acquired from Soybean Physical Map Resources at Soybase (http://www.soybase.org/pmd/index.php [accessed 12 July 2013]).

org; Table 6). The term *DNA or RNA related* refers to proteins involved in elongation, replication (helicase), recombination, mRNA splicing or processing, and release for translation. The term *Others* refers to kinase, transferase, ubiquitin ligase, and proteins involved in ribosome, membrane, exocytosis, ATP-binding, receptor, phosphorylation, auxin or cytokine-signal transduction, ion transport, cell wall modification, protein modification, pollen formation, immune response, Fe-S synthesis, polymerization, protein targeting, folding, and guanosine triphosphatase activity.

Because purine (for shoot ureide concentration) and nitrogen (for shoot N concentration) pathways are well

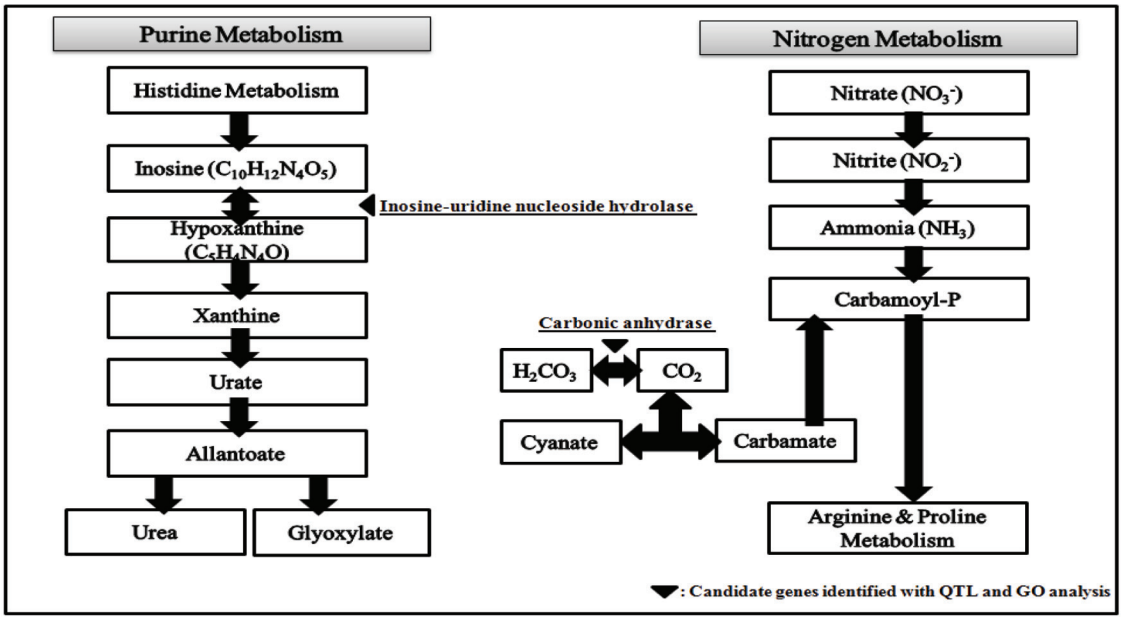

Figure 3. Metabolic pathways involved with candidate genes identified on Gm13 (carbonic anhydrase) and Gm19 (inosine–uridine nucleoside hydrolase) in quantitative trait locus (QTL) regions between flanking markers. GO, gene ontology.

known, we focused on predicted genes in a "Metabolic Process" class (Table 6). In Gm13, there were proteins associated with polysaccharide, lipid, hydrolase activity, and dehydratase, etc. One predicted gene, possibly associated with the shoot N concentration trait, was carbonic anhydrase (E.C. 4.2.1.1). Figure 3 shows how this gene is involved in nitrogen metabolism. The important roles of this enzyme are to maintain acid–base balance in other tissues and to influence the transport of CO_2. In SoyBase, locus and EST names of this gene were Glyma13 g39920 and Glyma13 g39920.1. The location of this gene was between 40,462,990 and 40,465,241 bp and its size was about 2.25 Kb (Table 6).

In a "Metabolic Process" class on Gm19, there were proteins related with lipid, amino acid, carbohydrate, reactive oxygen species, sulfur, nucleic acid, phosphate, hydrolase activity, glycogen, glycolysis, and glucan. A likely predicted gene associated with the shoot ureide concentration trait is inosine–uridine nucleoside hydrolase (E.C. 3.2.2.1 or E.C. 3.2.2.8). Figure 3 shows how this gene is related with purine metabolism. The function of the enzyme encoded by this gene is to catalyze the hydrolysis of inosine to form hypoxanthine, which is a precursor of the ureides. In SoyBase, locus and EST names of this gene were Glyma19 g37830 and Glyma19 g37830.1. The location of this gene was between 44,924,907 and 44,927,967 bp and the size was about 3.06 Kb (Table 6). Both predicted genes were close to the nearest markers in QTL regions.

DISCUSSION

QTL Detection Power

Because of our relatively small population size (97 RILs), there is a possibility of overestimating QTL effects due to the Beavis effect (Xu, 2003). To increase our QTL detection power, we constructed a dense genetic map and reduced most of the larger gaps between markers and at the ends of chromosomes. We used codominant markers to optimize the linkage information (Allard, 1956). We used a RIL population rather than backcross population to have greater information content. We used CIM (as a single-QTL model) or MIM (as a multiple-QTL model with multiple-year data), and we used a 1000-permutation test to set up critical threshold values for each model.

The qtlDesign library in R (Broman et al., 2003; Sen et al., 2007) provided a convenient means to compute statistical power and detectable QTL effects based on the above listed parameters using a single-QTL model (CIM). A simulation estimated the minimum detectable QTL effect on shoot ureide concentration considering residual error variance (genetic variance + error variance) (39.91), desired power (0.8), 1000-permutation-based average threshold value in 2005, 2007, and 2011 (3.3), sample size (97), theta (0.2, recombination fraction), and the number of replications. The minimum detectable QTL effect was 3.04 μmol g^{-1}. The R^2 of a QTL exhibiting this minimum QTL effect was expected to be 18.81%.

In Table 4, real data indicated that R^2 values ranged from 11 to 31%. Two putative QTLs on Gm13 (5.99 μmol g^{-1}) and Gm19 (averaged 4.32 μmol g^{-1}), had effects >3.04 μmol g^{-1}. The other three QTLs did not have effects >3.04 μmol g^{-1} with CIM analysis. However, in MIM, one QTL on Gm13 (positioned at 79 cM) had a 3.11 μmol g^{-1} effect, although this QTL was detected with 2.34 μmol g^{-1} effect in CIM. It seemed that two QTLs on Gm06 and Gm09 had statistical power <0.8 considering the minimum QTL effect and R^2 of a QTL.

Although the assumptions in the simulation study were a bit different from our data, QTL results in the KS4895 × Jackson RIL population for the shoot ureide concentration were highly reliable. In the case of the shoot N concentration, we observed that R^2 and additive effects from the simulation and our data had similar ranges. It would be necessary to increase population size to more accurately detect QTLs or find smaller QTL effects.

Screening for Candidate Genes

We focused on the "Metabolic Process" class and found two candidate genes that had a metabolic association with ureide and/or N concentration. Proteins belonging to other classes (Table 6), such as transcription factors, might also be candidate genes to control shoot ureide and N concentrations. It is presumed that these proteins could interact with other molecules through downstream pathways and ultimately affect N_2 fixation in nodules. Moreover, these proteins could be regulated through upstream pathways by signal molecules.

Many studies have proposed candidate signal molecules such as nitrous oxide, reactive oxygen species, abscisic acid, and carbohydrate sources to have a decisive effect on N_2 fixation under drought stress. It has been reported that nitrous oxide decreased nitrogenase activity as an inhibitor (Trinchant and Rigaud, 1982). A recent study showed that nitrous oxide under flooding conditions attenuated the expression of the *nifH* and *nifD*, which are genes coding for parts of the nitrogenase protein in bacteroids (Sánchez et al., 2010). Reactive oxygen species were increased through oxidative stress under drought stress (Boo and Jung, 1999; Loggini et al., 1999; Moran et al., 1994; Wellburn et al., 1996), and their accumulation could damage chloroplast and mitochondria to decrease N_2 fixation and plant growth. Abscisic acid concentration was highly correlated with N_2 fixation rate, decline of sucrose synthase activity, and leghaemoglobin content in soybean and pea (*Pisum sativum* L.) (González et al., 1995, 2001). Tominaga et al. (2010) screened mutant lines of birdsfoot trefoil [*Lotus japonicus* (Regel) K. Larsen] with high concentrations of abscisic acid. The enhanced N_2 fixation 1 (*enf1*) mutant showed increased nodule number and N_2 fixation ability. The endogenous abscisic acid concentration of *enf1* was very low compared with that of the wild type.

It is not clear how a regulatory network affecting N_2 fixation functions or which signal molecules are involved in ureide and N concentrations. Under drought stress, a signal transduction pathway could be attenuated by multiple factors (Desikan et al., 2004; Neil et al., 2002). Unfortunately, we had limited information about the upstream or downstream pathways of each predicted gene in QTL regions on the Web-based sources. To eliminate ambiguous candidate genes, it also would be necessary to perform transcriptome analysis for both traits in nodule and bacteroid cells and to combine expression QTL with traditional QTL results. Fine mapping would be also helpful to identify candidate genes for these QTLs.

CONCLUSIONS

Previous studies (King and Purcell, 2005; Purcell et al., 1998; Serraj et al., 1999) noted that N_2 fixation was sensitive to drought in genotypes with high ureide or N concentrations under water-deficit or water-replete conditions. Two cultivars, KS4895 and Jackson, with highly significant and different ureide concentrations were selected as parents for a molecular mapping population. We detected five QTLs for ureide concentration on Gm06 (1), Gm09 (1), Gm13 (2), and Gm19 (1) and four QTLs for N concentration on Gm13 (3) and Gm16 (1) under irrigated conditions. Quantitative trait loci were also identified under drought conditions, but additional data from drought environments are needed. Further studies of these QTLs and of expression QTLs under drought conditions will be necessary to understand how genes involved in shoot ureide and N traits interact with other molecules in nodule and bacteroid cells to determine which lines can be selected for high N_2 fixation.

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