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RESEARCH ARTICLE

Quantitative Trait Loci Associated with Foliar Trigonelline Accumulation in *Glycine Max* L

Youngkoo Cho,[1,2,4] Victor N. Njiti,[3,4] Xinbo Chen,[1,4,5] Kanokporn Triwatayakorn,[3,4]
My Abdelmajid Kassem,[3,4] Khalid Meksem,[3,4] David A. Lightfoot,[3,4] and Andrew J. Wood[1,4*]

[1]*Department of Plant Biology, Southern Illinois University-Carbondale, Carbondale, IL 62901, USA*
[2]*Department of Biology, Eastern New Mexico University, Portales, NM 88130, USA*
[3]*Department of Plant, Soil, and General Agriculture, Southern Illinois University-Carbondale, Carbondale, IL 62901, USA*
[4]*Center for Excellence in Soybean Research, Teaching and Outreach,*
Southern Illinois University-Carbondale, Carbondale, IL 62901, USA
[5]*Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA*

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The objective of this study was to utilize a *Glycine max* RIL population to (1) evaluate foliar trigonelline (TRG) content in field-grown soybean, (2) determine the heritability of TRG accumulation, and (3) identify DNA markers linked to quantitative trait loci (QTLs) conditioning variation in TRG accumulation. Frequency distributions of 70 recombinant inbred lines showed statistically no significant departure from normality ($P > .05$) for TRG accumulation measured at pod development stage (R4). Six different molecular linkage groups (LGs) (B2, C2, D2, G, J, and K) were identified to be linked to QTLs for foliar TRG accumulation. Two unique microsatellite markers (SSR) on two different linkage groups identified QTL significantly associated with foliar TRG accumulation: a region on LG J (Satt285) ($P = .0019$, $R^2 = 15.9\%$) and a second region on LG C2 (Satt079) ($P = .0029$, $R^2 = 13.4\%$).

INTRODUCTION

Plant structural and functional genomics have been extremely powerful tools for gene discovery and analysis [1]. However, in the postgenomics era, understanding small molecule biochemical networks (ie, metabolomics) will play a central role in deciphering plant ontogeny and physiology [2]. Several initiatives are underway to characterize and engineer the metabolic flux through a number of key biochemical pathways [3]. The nicotinamide biochemical network is fundamental to cellular physiology and encompasses essential molecules such as NAD^+, $NADP^+$, nicotinamide, and nicotinic acid [4, 5].

Soybean (*Glycine max (L) Merrill)* contains a wide range of bioactive phytochemicals including alkaloids such as nicotinic acid betaine (ie, trigonelline, TRG) [6]. Although, being long considered as a storage form of nicotinic acid, TRG can re-enter the nicotinamide metabolic pathway by demethylation, and the ability of exogenous TRG to affect the plant cell cycle [7] and mediate leaf movement [8] has been well documented. In soybean, TRG accumulates within leaves in response to NaCl-stress [5, 9] and water deficit-stress [10] and is postulated to function as a compatible solute and/or osmoprotectant [11]. Foliar TRG concentration is developmentally controlled, accumulating in preflowering plants and declining as plants progressed to pod development and seed filling [10]. Pfeiffer et al [12] have also used TRG as a biochemical marker for interspecific weed competition. In coffee, roasting-induced breakdown products of TRG are important volatile flavor components [13].

Because of the economic importance of soybean, considerable effort has been devoted to the development of genetic linkage maps using RFLP [14], RAPD [15], AFLP [16], and microsatellite markers (SSR) [17]. These genetic tools have been used to identify quantitative trait loci (QTLs), which condition the variation of a number of important agronomic traits in soybean including aluminum tolerance [18], seed protein content [19], insect resistance [20], and resistance to *Fusarium solani* sudden death syndrome (SDS) and soybean cyst nematode (SCN) [21]. Using a interspecific cross in coffee, Ky et al [22] have identified a single QTL located on linkage group (LG) G ($\log_{10}$ of the odds ratio (LOD) score = 3.56) correlated with variations in TRG content of coffee beans.

Essex and Forrest are two soybean cultivars which contrast with each other in terms of disease resistance [15], water-deficit tolerance [9], isoflavone content [23], yield potentials [24], and foliar trigonelline content [4, 9, 10]. To date, 107 polymorphic SSR markers have been identified within 18 linkage groups with a distance of 2823 centimorgans (cM, Haldane units), and mapped in 100 recombinant inbred lines (RILs) developed from a cross of Essex by Forrest [23, 25]. The distances and orders of these markers defined in these parents were similar to the values determined by other research groups who have reported

the soybean genome to be 3000 cM encompassing 20 linkage groups [17, 26, 27].

MATERIALS AND METHODS

Plant material and field experiment

A cross of Essex [28] by Forrest [29] was made to generate RILs as described by Hnetkovsky et al [30] and Chang et al [31]. The RILs have been advanced to the $F_{5:14}$ generation from never less than 300 plants per RIL per generation. Seventy $F_{5:14}$ RILs were used for analysis of QTL associated with foliar TRG accumulation. Seventy RILs were planted on July 10, 2000 at the Southern Illinois University Agronomy Research Center (Carbondale, Ill, USA) in stoy soil, fine-silty, mixed, mesic, Aquic, Hapludalfs. Randomized complete block design was used with three replicates and two-row plots. Rows were planted 0.75 m apart and 3.0 m long. Plots were planted with approximately 17 seeds m^{-1}. For determination of TRG concentration, leaf samples were taken at pod development (R4) stage [32] from each plot.

DNA isolation

One hundred RILs ($F_{5:14}$) were planted in the same pots in the greenhouse (temperature range was 21–30°C, 16-h photoperiod). Approximately, 3 g of leaves were harvested from 2-week old soybean seedlings and were immediately frozen in liquid nitrogen. The frozen leaves were ground very fine with liquid nitrogen and genomic DNA was extracted as described by Paterson et al [33]. DNA concentration was determined fluorometrically and diluted to 15 ng/μL for further use as template in PCR reactions.

Microsatellite amplification

Microsatellite markers from all 20 linkage groups were selected at 25 cM intervals from the soybean genetic map [17]. The primer pairs were purchased from Research Genetics, Inc, (Huntsville, Ala, USA). The microsatellite primers were labeled by phosphorylating the 5′ end with 5 μL (γ-32P) ATP (3000 Ci/mmol) for 30 minutes at 37°C with 10 units of T4 polynucleotide kinase (Pharmacia, Piscataway, NJ, USA). The PCR amplifications were performed with genomic DNA from populations ($F_{5:14}$) of recombinant inbred lines in 96-well microtitre plates using a Perkin Elmer GeneAmp 9600 as described by Akkaya et al [34]. The PCR products were separated by electrophoresis on a 5% (wt/vol) polyacrylamide denaturing gel [35]. Two negative controls (with no template DNA), along with the two parents DNA as positive controls, were run in all the amplifications.

Extraction, isolation, and analysis of trigonelline

Approximately 0.5 g of fresh leaf tissue was extracted in MeOH at 4°C in the dark and TRG was isolated by ion exchange chromatography as described by Cho et al [9]. TRG was measured spectrophotometrically at 264 nm (UV-VIS spectrophotometer Lambda 12, Perkin-Elmer, Norwalk Conn, USA) and quantified using authentic TRG standards (Sigma, St Louis, Mo, USA).

Mapping quantitative loci for TRG

RILs were classified as either Essex or Forrest type (heterozygotes were excluded) for polymorphic DNA markers and compared with TRG concentration by a 1-way analysis of variance (ANOVA) (SAS Institute Inc 1992). Mapmaker/EXP 3.0b (Whitehead Institute, Cambridge, MA 02142) [36] was used to estimate map distance as cM between linked markers, and to construct a linkage map (heterozygous lines were excluded). The LOD for grouping markers was set at 2.0 and its maximum distance was 30 cM. To identify intervals associated with QTL regulating foliar TRG accumulation, the marker map and TRG accumulation data were simultaneously analyzed with Mapmaker/QTL 1.1 [37] with the F_2-backcross model for trait segregation [30, 31, 38]. Position of the QTL was inferred from the LOD peaks at individual loci detected by maximum likelihood test at positions every 2 cM between adjacent linked markers. The SSR used in this study have been previously identified in different soybean populations [39].

Statistical and genetic analysis

ANOVA of foliar TRG accumulation was conducted using the general linear models (GLM) procedure [40]. Variance components were estimated for TRG based on either fresh weight (FW) or dry weight (DW) basis. Narrow sense heritability (h^2) was estimated by variance components on line mean basis [41]: $h^2 = \sigma_A^2/(\sigma_E^2/2 + \sigma_A^2)$, where σ_A^2 is additive genetic variance, σ_E^2 is the error variance.

RESULTS

Performance of RILs

TRG concentration estimated on the basis of leaf fresh weight ranged from 60 μg g^{-1} FW to 150 μg g^{-1} FW, whereas the concentration based on leaf dry weight ranged from 250 μg g^{-1} DW to 650 μg g^{-1} DW (Figure 1). Statistically, the frequency distribution of RILs ($n = 70$) showed no significant departure from normality ($P > .05$) for TRG accumulation estimated on the basis of leaf dry weight (Figure 1a). The frequency distribution of the lines was slightly skewed toward Forrest for TRG concentration based on leaf fresh weight, but this skewness did not result in a significant departure from normal distribution ($P > .05$) (Figure 1b). All these inbred lines showed nondiscrete classes and continuous variation, and are also unimodel.

Although a large proportion of the recombinant inbred population was distributed within the TRG concentration range of female parent Essex, TRG concentrations among 70 RILs were much higher than any two parental values (44% based on leaf fresh weight, Figure 1a; 54% based on dry weight, Figure 1b). This result indicates transgressive segregation present in inbred populations derived from two different parents.

Variance components and heritability

Table 1 indicates that RILs ($n = 70$) significantly differed for TRG concentrations ($P < .01$), which were estimated on

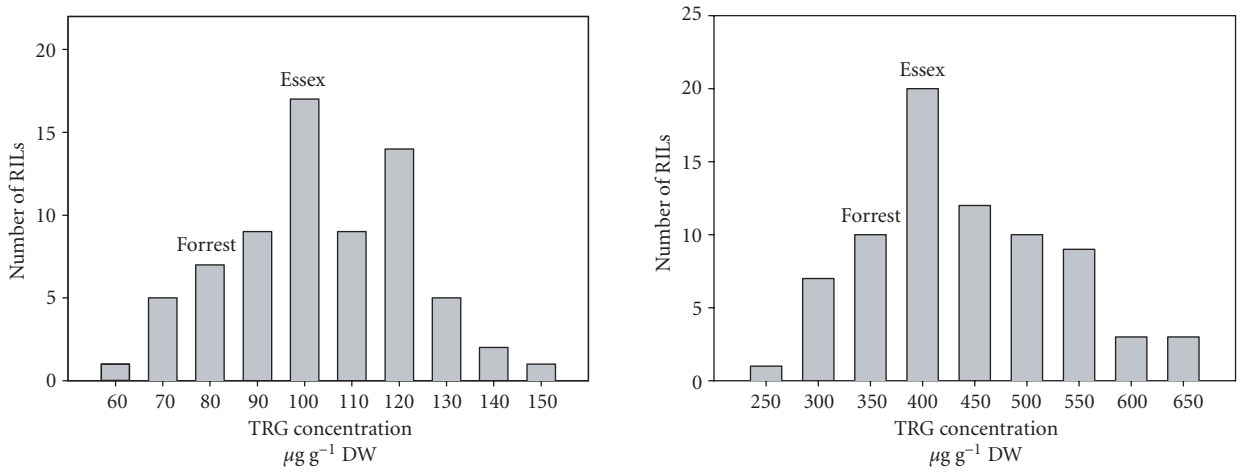

Figure 1. Trigonelline (TRG) concentration and normal distribution of RILs derived from a cross of Essex with Forrest. The mean trigonelline concentration for individual parents is presented. (a) Frequency distribution of TRG estimated on the basis of fresh weight of leaf sampled at pod setting stage; (b) on the basis of dry weight of leaf.

Table 1. Mean square, estimated ratio for additive and error variance, and narrow sense heritability on trionelline biosynthesis among 70 recombinant inbred lines derived from a cross between Essex and Forrest.

Source	Mean square	σ_A^2		σ_E^2		h^2
	Line[1]	Ratio (%)[2]		Ratio (%)		(%)
Fresh weight	724*	186	34.6	352	65.4	51.4
Dry weight	16.341+	5.068	45.0	8.167	55.0	62.1

*, + Significant at the 0.01 and 0.001 levels of probability, respectively.

1 degree of freedom = 69.

2 Ratios for σ_A^2 estimated by $(\sigma_A^2/\sigma_T^2)\times100$, where $\sigma_T^2 = \sigma_A^2 + \sigma_E^2$, and for σ_E^2 by $(\sigma_E^2/\sigma_T^2)\times100$, respectively; σ_A^2, σ_E^2, and σ_T^2 are the additive, error, and total component of variances, respectively.

either leaf fresh weight or dry weight at reproductive growth stage (R4). Ratios of additive (σ_A^2) and experimental error variances (σ_E^2), and heritability were estimated for TRG accumulation during reproductive growth stage. Proportions of additive (σ_A^2) to total variance ($\sigma_T^2 = \sigma_A^2 + \sigma_E^2$) were 34.6% for TRG concentration based on fresh leaf weight, and 45.0% based on dry leaf weight (Table 1). Proportions of error variance (σ_E^2) to the total were 65.4% for TRG concentration on a leaf fresh weight basis, and 55.0% based on leaf dry weight. Additive variances (σ_A^2) were relatively smaller than experimental error variances (σ_E^2) for both fresh and dry weight in RILs grown under conventional tillage fields. This indicates that TRG accumulation, as a polygenic trait, is dependent upon environment. Narrow sense heritabilities (h^2) for TRG concentration were 51.4% and 62.1% based on leaf fresh and dry weight, respectively (Table 1). Dry weight based narrow sense heritabilty (62.1%) was higher due to a large portion of additive variance (σ_A^2) against environmental error variance (σ_E^2).

Molecular markers associated with foliar TRG accumulation

Microsatellite markers relevant to QTL for foliar TRG accumulation were identified on the basis of 1-way ANOVA using 70 RILs (Table 2). Two independent chromosomal regions on two different molecular linkage groups were found to contain QTL for TRG accumulation (LG J and LG C2) (Table 2, Figure 2). A region on LG J identified by the microsatellite marker Satt285 was significantly associated with TRG accumulation based on leaf fresh weight ($P = .0019$, $R^2 = 15.9\%$). The interval containing the QTL spanned 14.4 cM between Satt285 and Satt249, had a peak LOD score of 2.0 and explained 12.9% of the total variation in TRG concentration. The region of Satt285 derived the beneficial

Table 2. DNA markers associated with QTLs for trigonelline biosynthesis in 70 RILs derived from the cross of Essex with Forrest.

Marker	LG	Trait[a]	R^2	*P* value	LOD[b]	QTL var.[c]	Mean ± SEM[d] ($\mu g\ g^{-1}$)	
							Essex	Forrest
Satt285	J	FW	15.9	0.0019	2.0	12.9	104 ± 3	88 ± 4
	J	DW	15.1	0.0018	1.9	12.1	444 ± 15	371 ± 17
Satt079	C2	FW	13.4	0.0029	2.1	15.2	90 ± 3	105 ± 3
	C2	DW	7.8	0.0198	1.5	11.0	385 ± 15	435 ± 14
Satt319	C2	FW	8.5	0.0175	—	—	92 ± 3	104 ± 3
Satt240	K	FW	8.6	0.0208	—	—	106 ± 3	95 ± 3
	K	DW	6.9	0.0348	—	—	445 ± 16	400 ± 13
Satt163	G	FW	7.8	0.0258	—	—	105 ± 3	94 ± 3
Satt275	G	DW	6.4	0.0368	—	—	437 ± 15	393 ± 14
CAA16	G	FW	7.7	0.0220	—	—	94 ± 3	105 ± 3
Satt574	D2	DW	7.2	0.030	—	—	431 ± 16	380 ± 13
Satt464	D2	FW	8.0	0.030	—	—	103 ± 4	92 ± 3
	D2	DW	7.5	0.030	—	—	436 ± 18	385 ± 13
Sat_083	B2	FW	7.8	0.0291	—	—	105 ± 3	94.5 ± 4
	B2	DW	6.1	0.0455	—	—	442 ± 15	399 ± 15

a Leaf FW and DW weight were used for the estimation of TRG concentration.
b LOD is an indicative of the probability based on the presence of a locus, not on its absence.
c Amount of variability in the trait explained by the marker loci based on Mapmaker/QTL 1.1.
d SEM is a standard error of the mean.

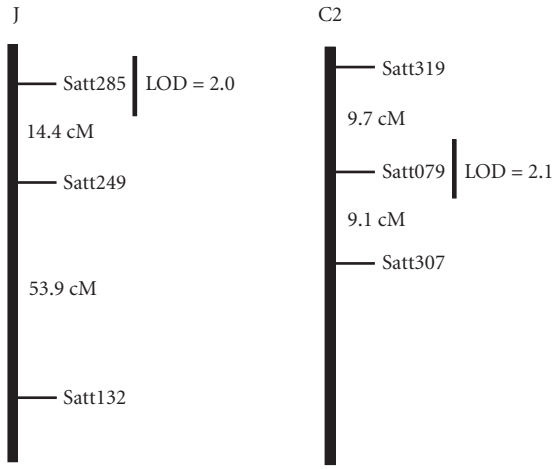

Figure 2. Location of microsatellite markers and three QTLs conditioning trigonelline biosynthesis in soybean grown under conventional field condition. The markers were assigned to the linkage groups C2, J, and L based on the soybean genetic linkage map [39]. END indicates the likely position of the telomere on designated linkage group. Names and distances of markers, and peak LOD score for the interval are given. The QTL LOD scores are from single locus analyses of additive gene effects using Mapmaker/QTL 1.1. Genetic distances are from the recombinant inbred line function of Mapmaker/EXP 3.0b.

allele from Essex (Table 2). This region was also significantly (P = .0018, R^2 = 15.1) associated with dry weight TRG concentration and derived the beneficial allele from Essex (Table 2). A second region on LG C2 was identified by the microsatellite marker Satt079 was significantly (P = .0029, R^2 = 13.4%) associated with TRG concentration. The intervals were 9.7 cM between Satt079 and Satt319, and 9.1 cM between Satt079 and Satt307 (Figure 2). The interval had a peak LOD score of 2.1 and explained 15.2% of total variation in TRG concentration. The region of Satt079 derived the beneficial allele from Forrest (Table 2). This region was also significantly (P = .0198, R^2 = 7.8) associated with dry weight TRG concentration and derived the beneficial allele from Forrest (Table 2).

These two QTLs explained 28.1% of the total variation for foliar TRG accumulation, and the genomic regions derived the beneficial allele predominately from the male parent (ie, Forrest). Four regions identified by eight microsatellite markers (Satt319, 240, 163, 275, 574, 464, Sat_083, and CAA16; Table 2) on four linkage groups (B2, D2, G, and K) were marginally associated (P < .04) with TRG accumulation, however their LOD scores did not exceed the threshold value (Table 2). A majority (92%) of these minor regions derived the beneficial allele from the maternal parent (ie, Essex).

DISCUSSION

TRG accumulation could be polygenic and additive in the population based on the result of frequency distribution

with statistical normality ($P > .05$). If one or a few dominant genes control a character, a few genes should be conspicuous in their effects [42] and the frequency distribution of TRG accumulation should be skewed to one side and discontinuous. As shown in Figure 1, the variation of TRG accumulation among advanced homozygous inbred lines is continuous with no discrete classes segregating among the lines suggesting that the character is not controlled by a single major gene in conjunction with multiple minor genes. TRG accumulation might not be controlled by one major gene with large effects. Polygenic quantitative traits such as yield, vigor, and seed-quality usually exhibit nondiscrete classes and in general are sensitive to environmental influence [43].

Transgressive segregation is observed for TRG accumulation among the advanced population, which falls outside the phenotypic range of the parents [23]. It is possible within a segregating population that similar effects of additive gene action can be seen with overdominance and epistasis, but are due to heterozygosity of the population. The populations used for this study were homozygous inbred lines after a large number of generations of selfing. Therefore, the transgressive segregation in this population is more likely to be due to additive effects of polygenes. Additive gene effects on traits such as TRG accumulation are enhanced by each additional gene, either an allele at the same loci or gene at different loci. If nonadditive gene effects such as dominance (complete, partial, etc) are involved in a trait, the frequency distribution should not be normally distributed [44, 45].

Two chromosomal regions identified on linkage group J and C2 of the soybean gene map are significantly associated with foliar TRG accumulation. The chromosomal regions associated with four minor loci on various linkage groups explained that TRG accumulation could be controlled by different polygenes present on different linkage groups. Some of the QTLs detected with low stringency ($P < .1$) were consistently detected across different environments [46]. Expressivity of a large number of genes affecting TRG accumulation is relatively small. However, it should be additive for the trait, indicating that TRG accumulation is a polygenically inherited trait whose expression is often modified by growth environment.

This study indicates that TRG accumulation, a polygenic trait of intermediate heritability, is amenable to manipulation within a breeding program [37]. A drawback in conventional breeding programs is the time and expense required for the development of superior cultivars. The use of molecular markers is a powerful tool for improving breeding efficiency by ingressing only the desired trait (in this case TRG accumulation). Molecular markers including microsatellite DNA confer efficient allelic variance as well as codominace for genome mapping of *G max* Molecular markers can be used to define allelic loci of chromosomal segments underlying TRG biosynthesis under various environments [47]. The QTLs identified from this study will be invaluable for the alteration of TRG accumulation by marker assisted selection [30, 44]. Saturating the genomic regions surrounding the identified QTLs with macrosatellite markers will be critical to cloning the gene(s) that underlies these QTL.

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* Corresponding author.
E-mail: wood@plant.siu.edu
Fax: +1 618 453 3441; Tel: +1 618 453 5609