Identification of Soat1 as a Quantitative Trait Locus Gene on Mouse Chromosome 1 Contributing to Hyperlipidemia

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Abstract

We previously identified two closely linked quantitative trait loci (QTL) on distal chromosome 1 contributing to major variations in plasma cholesterol and triglyceride levels in an intercross derived from C57BL/6 (B6) and C3H/HeJ (C3H) apolipoprotein E-deficient (apoE⁻/⁻) mice. Soat1, encoding sterol o-acyltransferase 1, is a functional candidate gene located underneath the proximal linkage peak. We sequenced the coding region of Soat1 and identified four single nucleotide polymorphisms (SNPs) between B6 and C3H mice. Two of the SNPs resulted in amino-acid substitutions (Ile147Val and His205Tyr). Functional assay revealed an increased enzyme activity of Soat1 in peritoneal macrophages of C3H mice relative to those of B6 mice despite comparable protein expression levels. Allelic variants of Soat1 were associated with variations in plasma cholesterol and triglyceride levels in an intercross between B6.apoE⁻/⁻ and C3H.apoE⁻/⁻ mice. Inheritance of the C3H allele resulted in significantly higher plasma lipid levels than inheritance of the B6 allele. Soat1 variants were also significantly linked to major variations in plasma esterified cholesterol levels but not with free cholesterol levels. Trangenic expression of C3H Soat1 in B6.apoE⁻/⁻ mice resulted in elevations of plasma cholesterol and triglyceride levels. These results indicate that Soat1 is a QTL gene contributing to hyperlipidemia.

Introduction

Hyperlipidemia, comprising elevated levels of plasma cholesterol, triglyceride, or both, is a major risk factor for atherosclerotic cardiovascular disease [1]. Although a small subset of hyperlipidemia cases are caused by rare mutations that result in Mendelian traits segregating in families, the common forms of hyperlipidemia involve multiple genes and exhibit significant gene-environment interactions [2]. Recent genome-wide association studies (GWAS) have been remarkably successful in identifying novel genetic loci contributing to lipid metabolism [3], although it is challenging to establish causality between a genetic variant and trait in humans due to small gene effect, complex genetic structure, and environmental influences.

One effective approach to the identification of complex trait genes is the use of mouse models. Apolipoprotein E-deficient (apoE⁻/⁻) mice develop spontaneous hyperlipidemia and atherosclerosis even when fed a low-fat diet [4],[5]. Using intercrosses derived from apoE⁻/⁻ mouse strains, we and others have identified distal chromosome 1 as a major region contributing to hyperlipidemia [6],[7],[8]. QTL analysis of an intercross derived from C57BL/6 (B6) and C3H/HeJ (C3H) apoE⁻/⁻ mice has suggested that two closely linked loci in the distal chromosome 1 region account for major variations in plasma HDL, non-HDL cholesterol, and triglyceride levels [7]. The distal locus corresponds to Hdlq5, a HDL QTL identified in advanced intercross lines derived from B6 and NZB/B mice [9]. Subsequent studies have identified Apoa2 as the causative gene of Hdlq5 [10]. The proximal locus overlaps with Cq1 (158.6 Mb), a locus identified in a B6×KK-Ay intercross for plasma cholesterol concentrations [11]. Soat1 is a functional candidate gene close to the linkage peak of the proximal QTL. It encodes an enzyme in the endoplasmic reticulum that catalyzes the formation of cholesteryl esters from cholesterol and fatty acyl coenzyme A [12]. In mammals, two Soat genes have been identified: Soat1 is ubiquitously expressed and is responsible for cholesteryl ester formation in the brain, adrenal glands, macrophages, kidneys, and the liver, and Soat2 is expressed in the liver and intestines. Soat1 deficiency results in a significant reduction in non-HDL levels of apoE⁻/⁻ and LDLR⁻/⁻ mice fed a chow or Western diet [13]. Human genetic studies indicate that Soat1 variants are associated with elevations in plasma concentrations of HDL cholesterol and apoA-I among subjects with hyperlipidemia [14]. In the present study, we tested whether Soat1 was a QTL gene...
contributing to naturally occurring variation in plasma lipid levels, especially under the circumstances of hyperlipidemia, in mice.

**Results**

**Soat1 sequence variation**

As part of an effort to find causal genes for the proximal lipid QTL on distal chromosome 1, all genes within the confidence interval (154.9–172.8 Mb) were perused for sequence differences in coding or promoter regions between B6 and C3H mice by querying public accessible databases (http://www.ncbi.nlm.nih.gov/SNP/ MouseSNP.cgi, http://cgdjax.org/tools/diversityarray.shtml, and www.ensembl.org). 16 likely candidate genes whose sequence variations may lead to changes in either the structure or quantity of a gene product were identified, which included Qsox1, Cep350, Tdrd5, Nphs2, Soat1, Tor1aip1, sec16b, Lztr2, Tyr, AB48100, LOC100040571, 1700015E13Rik, Nos1ap, Olfml2b, and Aif6. Among them, Soat1 is a gene located close to the linkage peak and is also involved in lipid metabolism. We sequenced the coding region of Soat1 by using cDNA as template and found four SNPs between B6 and C3H mice (Figure 1) [the accession number of the C3H/HeJ Soat1 gene sequence in the NCBI GenBank is bankit838062 DQ903181]. Two SNPs, A/G at position 439 and C/T at 613, led to amino-acid substitutions with isoleucine (Ile) to valine (Val) at amino acid residue 147 (Ile147Val) and histidine (His) to tyrosine (Tyr) at residue 205 (His205Tyr), respectively. The other two SNPs, A/C at 421 and C/T at 613, were synonymous base changes.

Enhanced enzyme activity

Soat1 activity was determined in vitro using cell homogenates prepared from peritoneal macrophages of B6.apoE2/2 and C3H.apoE2/2 mice. The enzyme activity was optimally solubilized with the zwitterionic detergent CHAPS at concentrations of 1–3% (Figure 3A). At all the concentrations used, the Soat1specificity activity was nearly twice as high in C3H as in B6 (P<0.05). The expression of Soat1 in peritoneal macrophages was examined by western blot analysis (Figure 3B). Densitometry of Soat1 bands was comparable between the two strains [442±60 vs. 421±113 (optical density); P = 0.87].

Association with variation in plasma lipid levels

We then examined whether Soat1 variants were associated with variations in plasma HDL, non-HDL cholesterol, and triglyceride levels in female F2 mice derived from B6.apoE2/2 and C3H.apoE2/2 mice. As shown in Table 1, inheritance of two copies of the C3H allele (CC genotype) resulted in significantly higher triglyceride, HDL, and non-HDL cholesterol levels than inheritance of two copies of the B6 allele (BB genotype) at the Soat1 locus (P<0.05 for each trait).

Linkage to plasma esterified cholesterol

Because Soat1 is an enzyme that catalyzes free cholesterol to cholesterol esters, we determined whether the Soat1 locus was linked to variations in plasma esterified and free cholesterol levels in the BXH cross. QTL analysis of F2 mice revealed that loci on chromosome 1 were responsible for major variations in plasma esterified cholesterol and free cholesterol levels (Figure 4). The interval mapping graph for chromosome 1 showed that the proximal peak of linkage curves for esterified cholesterol overlapped precisely with the Soat1 locus (Figure 5), which had a LOD score of 4.2 and explained 8% of the variance. In contrast, free cholesterol was controlled by two significant QTLs on chromosome 1, near markers D1Mit45 (94.9 Mbp) and D1Mit270 (172.7 Mbp), respectively, and a suggestive locus near marker D9Mit297 (33.8 Mbp) on chromosome 9. The QTL near marker D1Mit45 had a significant LOD
score of 4.6 and accounted for 9\% of the variance, and the QTL near marker D1Mit270 had a LOD score of 3.4 and explained 7\% of the variance. The suggestive QTL near marker D9Mit297 (33.9 Mbp) for free cholesterol had a LOD score of 3.3 and accounted for 6\% of the variance, and this QTL overlaps with Cq4 and Cq2 mapped in B6×KK-A F2 and KK×KK F2 crosses [11].

Analysis of transgenic mice

To directly evaluate the role of Soat1 in hyperlipidemia, we constructed transgenic mice that expressed C3H Soat1 and crossed the mice with B6.apoE\(^{-/-}\) mice for more than six generations. The expression of Soat1 protein in transgenic mice was analyzed by western blotting, and it was found in the liver, kidney, spleen, and the lung but not in the aorta, heart, or skeletal muscle (Figure 6). Real-time PCR analysis revealed that Soat1 mRNA expression levels in the liver were 2-fold as high in transgenic mice as in non-transgenic littermates (optical density: 37.2±2.3 mg/dl; P = 0.0048) (Figure 7). Plasma triglyceride levels were also higher in transgenic mice (78.1±5.1 vs. 67.0±5.5 mg/dl), although the difference was not statistically significant (P = 0.17).

Discussion

QTLs for plasma HDL on mouse distal chromosome 1 have been reported many times in numerous crosses [15]. QTLs for plasma triglyceride and non-HDL on distal chromosome 1 have also been reported in several crosses, including three BXH crosses [6], [7], [8], [16]. In the intercross between B6.apoE\(^{-/-}\) and C3H.apoE\(^{-/-}\) mice, we have observed that QTLs for HDL coincide with QTLs for triglyceride and non-HDL on distal chromosome 1 [7], suggesting that these plasma lipid phenotypes are controlled by the same genes. We have also observed two distinct peaks of the linkage curves for plasma triglyceride or non-HDL with the distal peak near marker D1Mit270 (172.7 Mbp) and the proximal peak near marker D1Mit425 (158.6 Mbp) [7], suggesting the existence of two QTLs for plasma lipids in the distal chromosome 1 region. Two other studies have also suggested the existence of a lipid QTL near 81.6 cM on mouse chromosome 1 [7], [11].

The distal QTL overlaps with Hdlq5 [9], and there is conclusive evidence that this QTL is responsible for the increased HDL phenotype in transgenic mice.

Table 1. Statistical association between allelic variation at the Soat1 locus and plasma lipid levels in F2 mice derived from B6.apoE\(^{-/-}\) and C3H.apoE\(^{-/-}\) mice.

| Trait          | BB (n = 61) | BC (n = 112) | CC (n = 45) | Variance (%) | P value |
|----------------|------------|-------------|-------------|--------------|---------|
| Triglyceride   | 154±17     | 175±44      | 185±52      | 5            | 2.1×10⁻³ |
| Non-HDL        | 701±187    | 803±201     | 911±262     | 10           | 1.0×10⁻⁵ |
| HDL            | 26±20      | 38±30       | 38±27       | 3            | 3.5×10⁻² |

Measurements are presented as means ± SD. The unit for these measurements is mg/dl. Variance (%) accounted for by the Soat1 locus is expressed as the percentage of the total phenotypic variance detected in the F2 cohort. BB, homozygous for C57BL/6 alleles; CC, homozygous for C3H alleles; BC, heterozygous for C57BL/6 and C3H alleles. The percentage of variance explained by Soat1 genotype and likelihood ratio P values are shown. doi:10.1371/journal.pone.0025344.g002
evidence supporting Apoa2 (92.6 cM) to be the causal gene for the QTL. Indeed, sequence analysis of the Apoa2 coding region in many mouse strains has revealed a number of nucleotide differences [10], [17]. Apoa2 variants are associated with variation in HDL cholesterol levels of mice [10],[18]. Transgenic overexpression of Apoa2 elevates plasma HDL, non-HDL cholesterol, and triglyceride levels [19], and Apoa2 deficiency reduces plasma HDL, non-HDL, and triglyceride levels in mice [20].

The present study strongly suggests that Soat1 is the causal gene for the proximal QTL. Multiple polymorphisms have been found in the coding region of this gene between the two parental strains that had been used in our previous studies to map the lipid QTL, and two of the polymorphisms lead to amino acid substitutions in the protein product. These polymorphisms led to changes in the function of Soat1 enzyme. As macrophages express only Soat1, we measured its activity in these cells. The present finding that C3H
had significantly higher Soat enzyme activity than B6 despite comparable protein expression in macrophages indicates that these SNPs have resulted in increases in Soat activity. This study has also provided several lines of other evidence supporting Soat1 expression in the liver of transgenic and non-transgenic littermates. Each lane represents non-transgenic mice, respectively. C, expression of transgenic and non-transgenic (control) littermates. The expression level of Soat1 was expressed as copy number relative to 10,000 copies of GAPDH mRNA. Results are means ± SE of 6 and 5 transgenic and non-transgenic mice, respectively. C, expression of Soat1 protein in the liver of transgenic and non-transgenic littermates. Each lane represents an individual mouse.

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**Table 2.** Significant and suggestive QTL for plasma esterified and free cholesterol in F2 mice derived from B6.apoE−/− and C3H.apoE−/− mice.

| Chromosome marker (cM) | Trait            | LOD | SI (cM) | Variance (%) | Pemp | Model of inheritance |
|------------------------|------------------|-----|---------|--------------|------|----------------------|
| Soat1 (81.6)           | Esterified cholesterol | 4.2 | 75–89   | 8            | 0.002 | Additive             |
| D1mit206(95.8)         | Esterified cholesterol | 4.4 | 87–102  | 9            | 0.001 | Additive             |
| D1mit45(58.5)          | Free cholesterol  | 4.6 | 41–62   | 9            | 0.004 | Additive             |
| D1mit270(92.3)         | Free cholesterol  | 3.4 | 73–101  | 7            | 0.005 | Additive             |
| D9mit297(15)           | Free cholesterol  | 3.3 | 0–27    | 6            | 0.008 | Additive             |

*From Mouse Genome Informatics database at http://informatics.jax.org.
*Suggestive QTL and significant QTL were 2.4 and 3.4, respectively, for free cholesterol and 2.4 and 3.3, respectively, for esterified cholesterol as defined by 1000 permutation tests.
*Variance (%) indicates the percentage of the phenotypic variance at the peak marker.
*Support intervals (SI) were defined by a 1-unit decrease in LOD score on either side of the peak marker.
*Pemp, empirically determined P-value for the whole genome, was calculated using the permutation test function of MapManager QTb20.
*Model of inheritance was determined using the MapManager QT program. C3H was the high allele at all the markers, contributing to elevated free or esterified cholesterol levels.

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Figure 6. Characterization of Soat1 transgenic mice. A, expression of Soat1 protein in transgenic mice. PC, positive control: lane loaded with Soat1 protein; NC, negative control: lane loaded with buffer only. B, real-time PCR analysis of Soat1 mRNA expression in the liver of transgenic and non-transgenic (control) littermates. The expression level of Soat1 was expressed as copy number relative to 10,000 copies of GAPDH mRNA. Results are means ± SE of 6 and 5 transgenic and non-transgenic mice, respectively. C, expression of Soat1 protein in the liver of transgenic and non-transgenic littermates. Each lane represents an individual mouse.

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Soat1 and Hyperlipidemia

**Figure 6.** Characterization of Soat1 transgenic mice. A, expression of Soat1 protein in transgenic mice. PC, positive control: lane loaded with Soat1 protein; NC, negative control: lane loaded with buffer only. B, real-time PCR analysis of Soat1 mRNA expression in the liver of transgenic and non-transgenic (control) littermates. The expression level of Soat1 was expressed as copy number relative to 10,000 copies of GAPDH mRNA. Results are means ± SE of 6 and 5 transgenic and non-transgenic mice, respectively. C, expression of Soat1 protein in the liver of transgenic and non-transgenic littermates. Each lane represents an individual mouse.

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to be a QTL gene affecting plasma lipid levels. First, the parental strain C3H, which has higher Soat1 enzyme activity, exhibits higher plasma cholesterol and triglyceride levels than the B6 strain, which has lower enzyme activity [21]. Second, F2 mice with the C3H allele at the Soat1 locus had higher plasma cholesterol and triglyceride levels than those with the B6 allele. Third, the QTL for esterified cholesterol coincided with the QTLs for triglyceride, HDL, and non-HDL at the Soat1 locus, suggesting that a gene involved in cholesterol esterification affected plasma HDL and non-HDL cholesterol levels. Soat1 is such a gene that synthesizes cholesterol esters and potentially affects lipoprotein assembling. Finally, the direct evidence is that transgenic mice expressing C3H Soat1 had significantly elevated plasma levels of HDL and non-HDL cholesterol.

A previous study also showed that Soat1 deficiency reduces plasma total cholesterol levels of apoE−/− mice [13]. However, the previous knockout mice were generated using embryonic stem cells derived from 129/SvJ mice and then backcrossed onto the B6 background. Linkage would cause the retention of a significant segment of 129/SvJ chromosome harboring the targeted gene in recipient mice. Several genes adjacent to Soat1, including Apoa2, are polymorphic between B6 and 129/SvJ and could contribute to variations in plasma lipid levels. The present study of transgenic mice has excluded a possible interference from Apoa2 and thus provided more definite evidence on the role of Soat1 in modifying plasma lipid levels. In this study, we found that transgenic mice had an increased level of HDL cholesterol. In contrast, the effect of Soat1 on plasma HDL cholesterol was not observed in the knockout mice. An explanation for the discrepant results is that apoE−/− mice have an extremely low HDL cholesterol level, and thus it would be harder for a gene to exert an effect to further reduce than to elevate it. In humans, a missense variant (R526G) and a variant in the 5′ untranslated region (−77G>A) have been found in the Soat1 gene, and individuals with −77G>A variant have significantly higher plasma HDL concentrations than those without the variant among hyperlipidemic subjects [14]. Nevertheless, genome-wide association studies (GWAS) to date have failed to detect any association with lipid traits in humans. One probable explanation for this outcome is that the gene is large (64.89 kb) and includes many variants (794 SNPs in the NCBI dbSNP database). Only a small number of markers in Soat1 have been typed, and these markers may not have the strongest association.
In summary, we have provided reasonable evidence to support Soat1 to be a QTL gene, although further studies are needed to prove which SNP affects Soat1 function. Soat enzyme catalyzes the formation of cholesteryl esters from free cholesterol and fatty acyl coenzyme A. Because of its potential role in foam cell formation, this enzyme has been a target for developing therapeutic drugs for the past two decades. Theoretically, inhibition of the enzyme should block the esterification of cholesterol and prevent the transformation of macrophages into foam cells. However, some animal studies show that Soat inhibitors exert hypolipidemic effects and reduce atherosclerosis [22],[23],[24] while some studies show that inhibition of Soat activity promotes atherosclerosis [25]. In humans, administration of Soat inhibitors failed to reduce, rather increase, plaque volume [26],[27]. Studies with more specific inhibitors of Soat1 activity have also shown aggravation rather than alleviation of atherosclerosis in rabbits and mice [25],[28]. As Soat1 elevates plasma levels of both good (HDL) and bad (non-HDL) cholesterol, the present results may explain why Soat1 inhibitors are not clinically effective as expected and many have failed in clinical stages probably due to its effect on HDL cholesterol. Moreover, continued inhibition of Soat activity increases intracellular free cholesterol and limits the efflux of free cholesterol, which may induce cytotoxic effects within cells.

Materials and Methods

Ethics statement

All procedures were carried out in accordance with current National Institutes of Health guidelines and approved by the University of Virginia Animal Care and Use Committee (Assurance #A3243-01, Animal Protocol #3109).

Mice

B6.apoE/−/− mice were purchased from the Jackson Laboratory, and C3H.apoE/−/− mice were generated in our laboratory. The generation of F2 mice from B6.apoE/−/− and C3H.apoE/−/− mice was reported previously [7]. At 6 weeks of age, female F2 mice were started on a Western-type diet containing 21% butterfat, 34% sucrose, and 0.2% cholesterol (TD 80137, Harlan Laboratories) and maintained on the diet for 12 weeks. To generate transgenic mice, a clone containing the Soat1 gene in the pTARBAC2.1 vector was picked from the CHORI-34 Mouse C3H/HeJ BAC library constructed by the Pieter De Jong’s Laboratory at Children’s Hospital Oakland Research Institute. This clone contained the entire Soat1 gene, 142,565 bp upstream and 14,893 bp downstream from the 5’ and 3’ ends, respectively. The integrity of Soat1 was confirmed through partial sequencing and restriction enzyme digestion before the purified BAC DNA was microinjected into B6D2 F1 fertilized eggs at a concentration of 1 mg/ml. Transgenic founders were identified by PCR amplifications of both forward and reverse fragments of the BAC DNA with primers 5’-TCTTTCTCCGCACCCGACATAGAT-3’ and 5’-TTAGGAGCCACTGTGGTTAGCTGT-3’. Positive transgenic mice were backcrossed with B6.apoE/−/− mice for more than 6 generations and maintained in a heterozygous condition for the transgene.

Plasma lipid measurements

Plasma total cholesterol, HDL cholesterol, and triglyceride were measured as reported previously [29]. Non-HDL cholesterol was calculated by subtracting the HDL cholesterol levels from the total. Free cholesterol levels were determined using a kit from Wako (Richmond, VA). Briefly, 6 μl of plasma samples, lipid standards, and controls were loaded in a 96-well plate and then mixed with 150 μl of free cholesterol reagent. After a 5-min incubation at 37°C, the absorbance at 600 nm was read on a Molecular Devices (Menlo Park, CA) plate reader. Esterified cholesterol levels were calculated by subtracting free cholesterol levels from total cholesterol levels.

Soat1 cDNA sequencing and genotyping

Total RNA isolated from the liver of B6 mice and C3H mice was reverse transcribed to cDNA with use of the Superscript RT-PCR kit (Invitrogen). The PCR primers used for amplification of...
Soat1 cDNA in both directions were as follows: 5′-AGGAAGCTT-GAATTCAAGTGG-3′/5′-CTTGGGTATGGTCCTGGA-3′; 5′-GGCAAGATTCACATCCACACG-3′/5′-AACAGCTA-CGACAAGTAGCTAAG-3′. After purification with a QiAquick PCR purification kit, PCR products were sequenced on an ABI Prism Cycle sequencer 310 (Applied Biosystems). The Soat1 polymorphism at T454C was used to screen the BXH intercross with the PCR restriction fragment length polymorphism (PCR-RFLP)-based method. PCR amplification on genomic DNA was performed using primers 5′-AGATTTGCTCTGAAGGGCC-GCAA-3′ and 5′-AGGCTAGCTGGACCCTTTCTGCAA-3′, and the 712 bp amplicon was then digested with CldI restriction enzyme (New England BioLabs, Hertfordshire, UK) according to the manufacturer’s instruction. The PCR product from the B6 allele but not that from the C3H allele was digested by CldI to 511 bp and 201 bp fragments. Thus, the BB allele should exhibit two bands, the CC allele one band, and the BC alleles three bands on an agarose gel.

Soat1 activity assay in macrophages

Peritoneal macrophages were prepared as we previously described [30]. Following a brief culture as a monolayer in RPMI medium containing 10% fetal bovine serum, macrophages were harvested by hypotonic shock and scraping, and the resultant cell homogenate was kept in a buffer (50 mM Tris, 1 mM EDTA at pH 7.8 with protease inhibitors) at concentrations of 2–4 mg/ml. To solubilize the enzyme, 1 M KCl and various concentrations of CHAPS were added. The enzyme activity of Soat1 was measured in duplicate in taurocholate/cholesterol/PC mixed micelles as described by Chang et al. [31].

Western blot analysis

The presence of Soat1 in various tissues of transgenic mice and in peritoneal macrophages of B6.aapoE−/− and C3H.aapoE−/− mice was determined by western blot analysis. Proteins were prepared as we previously described [32], separated by electrophoresis on 4–12% Tris-polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes. The membrane was probed with a rabbit polyclonal antibody against Soat1 (H-125, Santa Cruz), and signals were detected by the chemiluminescence method [Invitrogen]. The density of the bands was quantified with a densitometer (Molecular Devices, CA).

Real-time PCR analysis of Soat1 expression

Total RNA extracted from the liver of transgenic mice was treated with DNase I, and then reverse transcribed to cDNA as described above. cDNA was mixed with SYBR Green supermix reagent (Bio-Rad) and specific primers to assess expression of Soat1 and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) by real-time PCR. Primers used for Soat1 amplification were 5′-TGTGCATCAAGAAGTACCCAGGA-3′/5′-GGTGCCAGGA-AACCACCAAGTGA-3′ and for GAPDH were 5′-GGTGTGAACCGGA TTGGCCGTAAT-3′/5′-GGCCTTGACTGTGCGTGAATTTT-3′. Real-time PCR on each sample was run in triplicate on an iCycler iQ5 machine (Bio-Rad) under the condition of 50°C for 2 minutes, 95°C for 2 minutes, then 95°C 30 seconds, 60°C 30 seconds, and 72°C 30 seconds for 40 cycles as reported [33]. The expression level of Soat1 was expressed as mRNA copy number relative to 10,000 copies of GAPDH mRNA.

Statistical analysis

QTL analysis was performed as we previously described [7],[33] ANOVA was used for determining if the mean phenotype values of progeny with different genotypes at a specific marker were significantly different. The Student t test was used when only two means were compared. Differences were considered statistically significant at P<0.05.

Author Contributions

Conceived and designed the experiments: ZL ZY WS. Performed the experiments: ZL ZY TM QW ZS CCC WS. Analyzed the data: ZL ZY QW CCC WS. Contributed reagents/materials/analysis tools: WS. Wrote the paper: ZY WS. Obtained grants for support of this project: WS.

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