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SUMMARY

Although the biochemical and genetic basis of lipid metabolism is clear in *Arabidopsis*, there is limited information concerning the relevant genes in soybean. To address this issue, here we constructed three-dimension genetic networks using six seed oil-related traits, fifty-two lipid-metabolism-related metabolites and 54,294 SNPs in at most 286 soybean accessions. As a result, 284 and 279 candidate genes were found by phenotypic and metabolic genome-wide association studies and multi-omics analyses, respectively, to be significantly associated with seed oil-related traits and metabolites; six seed oil-related traits were found by MCP and SCAD analyses to be significantly related to thirty-one metabolites. Among the above candidate genes, 36 genes were found to be associated with oil synthesis (27), amino acid synthesis (4) and TCA cycle (5), and four genes *GmFATB1a*, *GmPDAT*, *GmPLDα1* and *GmDAGAT1* are known oil-synthesis-related genes. Using the above information, 133 three-dimension genetic networks were constructed, in which 24 are known, e.g., pyruvate-*GmPDAT*-*GmFATA2*-oil content. Using these networks, *GmPDAT*, *GmAGT* and *GmACP4* reveal the genetic relationships between pyruvate and the three major nutrients, and *GmPDAT*, *GmZF351* and *GmPgs1* reveal the genetic relationships between amino acids and seed oil content. In addition, *GmCds1*, along with average temperature in July and rainfall, influence seed oil content across years. This study provides a new approach for three-dimension network construction and new information for soybean seed oil improvement and gene function identification.

Keywords: seed oil related traits, lipid related metabolites, mGWAS, three-dimension genetic networks, soybean

Significance Statement

One hundred and thirty-three three-dimension genetic networks among seed oil-related traits, lipid-metabolism-related metabolites and genes in soybean were constructed for the first time using phenotypic and metabolic genome-wide association studies and multi-omics analyses. These networks were tried to explain the genetic relationships among seed oil-related traits, oil-synthesis-related carbon metabolites, and oil-synthesis-related amino acids.

INTRODUCTION

Scientists have focused on the genetic basis of seed oil-related traits in soybean for a long time, with the purpose of improving seed oil content and quality in this crop (Fang *et al.,* 2017). However, the significant negative correlation between seed oil and protein contents (Chaudhary *et al.,* 2015; Patil *et al.,* 2017) has resulted in very slow progress in improving soybean quality by means of conventional breeding (Charron *et al.,* 2005). Recently, metabolites, which act as a bridge between trait phenotype and its genes, have been shown to usually determine crop nutritional traits like seed oil content and its composition via a wide range of intermediate compounds such as fatty acids, phospholipids and carbohydrates (Wen *et al.,* 2015; Chen *et al.,* 2016). Although many genes have been found to be associated with seed oil-related traits and lipid synthesis, these studies have usually involved phenotypic genome-wide association studies (GWAS) and linkage analysis (Hwang *et al.,* 2014; Meng *et al.,* 2016; Fang *et al.,* 2017; Van & McHale, 2017; Leamy *et al.,* 2019; Zuo *et al.,* 2019; Zhang T *et al.,* 2019). Therefore, modern crop breeding necessitates the construction of three-dimension genetic networks among seed oil-related traits, genes and oil biosynthesis metabolites.

To date many genes have been reported to be involved in seed oil biosynthesis in *Arabidopsis*. For example, *GPAT* (Li *et al.,* 2007), *PDHC* (Shen *et al.,* 2006), *ACCase* (Roesler *et al.,* 1994), *KASI* (Xiong *et al.,* 2017), *FATB* and *FATA2* (Bonaventure *et al.,* 2003; Moreno *et al.,* 2012) were found to be involved in the synthesis of short chain fatty acids; *DGAT* and *PDAT* (Jako *et al.,* 2001; Zhang *et al.,* 2009; Pan *et al.,* 2013; Fan *et al.,* 2013) were found to be involved in triacylglycerol (TAG) biosynthesis; *LACS* (Lü *et al.,* 2010; Katavic *et al.,* 2014) was found to be involved in the synthesis of very long-chain fatty acid; *PLP2/PLA2A* (La *et al.,* 2009; Yang *et al.,* 2012), *Pgs1* or *PGP1* (Tanoue *et al.,* 2014), *Cds1* (Zhou *et al.,* 2013), *LPEAT2* (Jasieniecka-Gazarkiewicz *et al.,* 2017), and *TIM/PDTPI* (López *et al.,* 2016) were found to be involved in lipid synthesis; *OLE1* (oleosin) was found to be involved in the storage of lipid droplets (Siloto *et al.,* 2006; Shimada *et al.,* 2010). Although a hundred genes relating to lipid synthesis have been reported to participate in the process of carbohydrate metabolism (Zhang *et al.,* 2018), few genes have been reported to be related to the TCA cycle and amino acid synthesis

(Wen *et al.*, 2015; Zhang *et al.*, 2018). In *Arabidopsis*, *SDH1* (Huang *et al.*, 2013), *ACO1* (Park *et al.*, 2018), *MDH* (Selinski *et al.*, 2019), *FUM1* (Zubimendi *et al.*, 2018), *IDH-V* (Lemaitre *et al.*, 2006) and *2OGDH* (Araújo *et al.*, 2014) were reported to participate in the reaction of TCA cycle; *AGT* (Zhang *et al.*, 2002), *P5C1* (Giberti *et al.*, 2004), *MTO* (Goto *et al.*, 2002), *HMT2* (Ranocha *et al.*, 2000) and *AtBCAT* (Diebold *et al.*, 2002) were reported to participate in the amino acid metabolism.

In soybean, some transcription factors and genes encoding other functional proteins have been reported to be responsible for seed oil biosynthesis. The transcription factors *GmDof4*, *GmDof11* (Wang *et al.*, 2007), *GmbZIP123* (Song *et al.*, 2013), *GmLEC1a/GmLEC1b* (Zhang *et al.*, 2017), *GmWRI1a* (Chen *et al.*, 2017), *GmMYB73* (Liu *et al.*, 2014), *GmDREBL* (Zhang *et al.*, 2016), *GmNFYA* (Lu *et al.*, 2016), *GmLEC2* (Manan *et al.*, 2017) and *GmZF351* (Li *et al.*, 2017) were found to participate in the regulation of lipid accumulation. The functional genes *GmDGAT1* or *GmDAGAT1* (Lardizabal *et al.*, 2008; Chen *et al.*, 2016), and *GmOLE1* (desaturase) (Zhang D *et al.*, 2019) were reported to play a key role in plant diacylglycerol/triacylglycerol (DAG/TAG) biosynthesis, and *GmPLD* (phospholipase D) and *GmLPAT* (lysophosphatidyl acyltransferase) (Zhao *et al.*, 2012; Zhao, 2013) were found to regulate lipid synthesis. However, rare oil synthesis genes have been reported to be related to TCA cycle or amino acid synthesis in soybean.

As we all know, metabolites have a significant influence on signal transmission, material synthesis and decomposition and other differentiation processes in each cell (Chen *et al.*, 2014, 2016; Wen *et al.*, 2015). Using metabolome-based genome-wide association studies (mGWAS) and metabolome profiling analysis, recently, some genes have been identified to be associated with primary or secondary metabolites, which are responsible to complex traits (Chen *et al.*, 2016; Wu *et al.*, 2018). For example, *OMT1* encoding 5-hydroxyferulic acid O-methyltransferase in *Arabidopsis* was found to regulate 5-hydroxyferulic acid glucoside (Wu *et al.*, 2018), which influences the synthesis of lignins and sinapoyl esters (Tohge *et al.*, 2007); *Os07g32060* encoding flavone 5-*O*-glucosyltransferase in rice was found to regulate 5-*O*-glucoside, which influences the synthesis of flavonoids (Chen *et al.*, 2014); *Os12g27220* and *Os12g27254*

encoding spermidine hydroxycinnamoyl transferases in rice was found to regulate N-hydroxycinnamoyl spermidines, which influences phenolamides biosynthesis (Dong *et al.*, 2015); *Os02g57760* encoding nicotinic acid N-methyltransferase in rice was found to regulate trigonelline, which influences grain width (Chen *et al.*, 2016). At present the studies on soybean mGWAS are relatively limited.

As described above, the genetic relationships are derived mainly from either seed oil-related traits and genes, or metabolites and genes. In modern breeding strategies, it is very necessary to construct three-dimension genetic networks among seed oil-related traits, metabolites and genes. To address this issue, six seed oil-related traits, fifty-two lipid-related metabolites and 54,294 SNP markers in at most 286 soybean accessions were used to conduct single- and multi-locus GWAS (Zhou *et al.*, 2015; Zhou *et al.*, 2015; Wang *et al.*, 2016; Tamba *et al.*, 2017; Zhang *et al.*, 2017; Wen *et al.*, 2018; Ren *et al.*, 2018) for seed oil-related traits and metabolites, and genetic relationships between seed oil-related traits and metabolites were also established by the minimax concave penalty (MCP) (Zhang *et al.*, 2006) and smoothly clipped absolute deviation (SCAD) (Fan & Li, 2001) analyses. Candidate genes for seed oil-related traits and metabolites were predicted by bioinformatics, comparative genomics, and transcriptomics. Using the above results, 133 three-dimension genetic networks were constructed in this study. Using these networks, some new genetic relationships were uncovered, e.g., pyruvate and the three major nutrients, and amino acids and seed oil content. In addition, we also discuss the reasons of different seed oil contents across different years. Thus, this study provides a new approach for constructing three-dimensional genetic networks, which reveal some new genetic relationships among seed oil content, some metabolites (three major nutrients, malic acid, and amino acids) and genes. These relationships are useful for soybean quality improvement and gene function identification.

RESULTS

Distributions for six seed oil-related traits and fifty-two metabolites in soybean

Seed oil-related traits in this study are seed oil content and its five oil constituents, including stearic acid, palmitic acid, oleic acid, linoleic acid and linolenic acid. These traits were measured

from 286 soybean accessions between 2014 and 2016. The averages plus standard deviations across the three years for the above six traits were 17.92 ± 2.16, 3.54 ± 0.46, 11.65 ± 1.21, 24.79 ± 4.53, 52.29 ± 3.63 and 7.73 ± 1.58 (%), respectively, and their average coefficients of variation (CV) across the three years were 12.03, 10.33, 12.92, 18.24, 6.95 and 20.40 (%), respectively (Table S1). Clearly, these traits have large variation and are typical quantitative traits. Although the trends for five seed oil constituents in the three years are almost the same (Figure 1a-e), seed oil content in 2016 (16.67 ± 1.92, %) was significantly lower than those in 2014 (19.06 ± 2.18, %) and 2015 (18.03 ± 2.37, %) (P-value < 0.001).

A total of 52 lipid-related metabolites in the pathways of the tricarboxylic acid (TCA) cycle, amino acid metabolism, oil synthesis and soybean isoflavone synthesis were measured from 214 soybean accessions in 2015. These metabolites are classified into organic acids, soybean isoflavone, phosphatidyl ethanolamines (PE), phosphatidyl cholines (PC), phosphatidyl inositols (PI) and amino acids. Organic acids measured in this study included pyruvic acid, succinic acid, fumaric acid, malic acid, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid; their phenotypic values varied from 175.87 to 50980.18, 1.35 to 515.01, 1.25 to 440.91, 18.61 to 5280.87, 0.9 to 342.63, 0.5 to 105.69, 0.15 to 112.67, 21.71 to 774.08 and 8.5 to 102.43 (μg/g), respectively; their CVs were 181.85, 123.82, 113.08, 82.37, 79.92, 75.57, 126.59, 90.47 and 45.02 (%), respectively. Soybean isoflavone measured in this study included daidzein, daidzin, genistein, genistin and glycitin; their phenotypic values varied from 0.23 to 163.78, 0.50 to 314.13, 0.22 to 87.65, 7.78 to 1611.42 and 0.002 to 238.69 (μg/g), respectively; their CVs were 107.06, 110.34, 104.93, 74.56 and 109.61 (%), respectively. The phenotypic values for PE (6), PI (6), and PC (6) with eighteen molecular species (for detail information, see Measurement in Experimental Procedures) varied from 3.02 to 2160.52, 0.00 to 30568.93, and 0.00 to 2830.26 (μg/g), respectively; their CVs were 91.88, 124.53, and 96.34 (%), respectively. A total of twenty amino acids were measured, their phenotypic values varied from 0.04 to 1864.51 (μg/g), and their CVs were from 41.89 to 236.48 (%). Detailed information for all the 52 metabolites is shown in Table S2. Clearly, these metabolites have large variations.

Genome-wide association studies for seed oil-related traits in soybean

Detection of main-effect quantitative trait nucleotides (QTNs) for oil-related traits With 286 soybean accessions, six seed oil-related traits measured from 2014 to 2016, along with 54,294 SNPs, were used to conduct phenotypic GWAS using GEMMA, mrMLM, FASTmrEMMA, ISIS EM-BLASSO, pLARmEB and pKWmEB. As a result, 334 significant QTNs were identified (Figure S1 and Table S3). Among these QTNs, they were distributed mainly on chromosomes 5, 6, 7, 8, 9, 13, 17, 18 and 19 (≥ 16 QTNs for each chromosome) and had 5.51% average proportion of total phenotypic variation explained by each QTN, and there were 56, 46, 50, 68, 75 and 39 QTNs, respectively, for palmitic, stearic, oleic, linoleic, linolenic acids and seed oil content. Thirty-five QTNs were detected in at least two environments, while 309 QTNs were identified in only one environment. A total of 77 significant QTNs for the above six oil-related traits were detected in at least two environments or two GWAS methods (Table S4). Among these common QTNs, there were 11, 17, 12, 18, 7, and 12 QTNs, respectively, for linolenic, linoleic, stearic, oleic, palmitic acids and seed oil content. Based on previous studies at https://www.soybase.org/GWAS/, there are many QTNs on chromosome 5 and almost no QTNs on chromosome 13. In this study, five significant QTNs were positioned within 38.0-41.0 Mb at the distal end of chromosome 5 and eight QTNs were positioned on chromosome 13.

Detection of QTN-by-environment interactions for oil-related traits The above datasets in GWAS were also used to detect QTN-by-environment interactions (QEs) using quantitative trait interaction (G × E) module in the PLINK software (Purcell *et al.,* 2007) (http://zzz.bwh.harvard.edu/plink/anal.shtml#qtgxe). As a result, 5, 1 and 3 significant QEs were found to be associated with linolenic acid, palmitic acid and stearic acid, respectively (Table S5). For example, the locus Chr18-4720420 was significantly associated with linolenic acid (P=6.53e-04).

Detection of QTN-by-QTN interactions for oil-related traits The above datasets in GWAS were again used to detect QTN-by-QTN interactions (QQs) using the online software PEPIS (http://bioinfo.noble.org/PolyGenic_QTL/) (Zhang *et al.*, 2016). As a result, 2, 2, 3, 1, 1 and 1 significant QQs were found to be associated with linoleic acid, seed oil content, palmitic

193 acid, oleic acid, stearic acid and linolenic acid, respectively (Table 6S). For example, the
194 epistasis between locus Chr13-20532852 bp and locus Chr13-20704034 bp was found to be
195 significantly responsible for linolenic acid (LRT=24.37).
196
197 ***Candidate genes for seed oil-related traits*** In order to determine candidate genes for
198 seed oil-related traits, we adopted the following analyses. First, we found all the genes between
199 the 100 kb upstream and downstream regions for each of the 334 significantly QTNs. Using
200 soybean metabolic pathway database, KEGG annotation (https://soycyc.soybase.org/) and soybean
201 genome annotation database and Gene Ontology terms (https://soybase.org/genomeannotation/),
202 then, all the above genes were used to mine the candidate genes or their *Arabidopsis* homologous
203 genes that were annotated in fatty acid biosynthesis, phospholipid biosynthesis, phospholipid
204 binding, phosphorylation and dephosphorylation, triacylglycerol biosynthesis, oxidoreductase
205 activity, electron carrier activity and TCA cycle pathways. As a result, 284 genes were found to
206 be associated with the above metabolic pathways.
207
208 Among the above 284 genes, twenty-two were found to be related to lipid metabolism pathways,
209 including 14 lipid biosynthesis related genes, 4 amino acid biosynthesis related genes and 4 TCA
210 cycle related genes. In oil biosynthesis related genes, *GmPDAT*, *GmDAGAT1*, *GmFATB1a*,
211 *GmKASI*, *GmPgs1*, *GmACC*, *GmFATA2*, *GmCds1*, *GmWRI1b*, *GmNFYA*, *GmDof11*,
212 *GmCYP78A10, Glyma.18g038400* and *GmBS1* were found to be associated, respectively, with
213 linolenic acid (LOD=4.15~4.20) and pyruvate (P-value=1.44e-05) (Liu, 2020), linolenic acid
214 (P-value=8.28e-09~1.58e-06) (Chen *et al.,* 2016), stearic acid (LOD=2.61~5.13) (Murad *et al.,*
215 2014), palmitic acid (LOD=3.09) (Xiong *et al.,* 2017), linoleic acid (LOD=4.86) (Tanoue *et al.,*
216 2014), oil content (LOD=3.11~5.31) (Roesler *et al.,* 2011), oil content (LOD=3.21) (Moreno *et*
217 *al.,* 2012), linolenic acid (P-value=1.56e-09) (Zhou *et al.,* 2013), palmitic acid (LOD= 3.59)
218 (Chen *et al.,* 2017), oleic acid (P-value=3.82e-06) (Lu *et al.,* 2016), linolenic acid (LOD=3.95)
219 (Wang *et al.,* 2007), linolenic acid (LOD=2.88) (Wang *et al.,* 2015), palmitic acid
220 (LOD=3.37~3.76) and palmitic acid (LOD=5.25) (Ge *et al.,* 2016). Among these genes,
221 *GmWRI1b*, *GmNFYA* and *GmDof11* have no annotations of biochemical metabolic processes;
222 *GmPDAT*, *GmDAGAT1*, *GmFATB1a*, *GmPgs1* and *GmFATA2* were differentially expressed

between wild and domesticated soybeans (Figure 2b and Table 1). In amino acid biosynthesis related genes, *GmAGT*, *GmBCAT*, *GmHMT2* and *GmP5C1* were found to be associated, respectively, with palmitic acid (LOD=3.39) (Zhang *et al.*, 2002), palmitic acid (LOD=4.70) (Diebold *et al.*, 2002), oleic acid (P=2.49e-09) (Ranocha *et al.*, 2000) and linoleic acid (LOD=3.84) (Giberti *et al.*, 2004). In TCA cycle related genes, *GmACO1* (*Glyma.01g162800*), *GmFUM1* (*Glyma.02g015700*), *GmSDH1* (*Glyma.01g175600*) and *GmMDH1* (*Glyma.13g104800*) were found to be associated, respectively, with oleic acid (P=4.34e-06) (Park *et al.*, 2018), linolenic acid (P=1.25e-06) (Zubimendi *et al.*, 2018), linoleic acid (LOD=3.29~3.68) (Huang *et al.*, 2013), and linolenic acid, P=2.24e-07) (Selinski *et al.*, 2019) (Figure 2a and Table 2).

Genome-wide association studies for acyl-lipid related metabolites in soybean

Genome-wide association studies for acyl-lipid related metabolites In 214 soybean accessions, fifty-two acyl-lipid related metabolites measured in 2015, along with 54,294 SNPs, were used to conduct metabolic GWAS using GEMMA, mrMLM, FASTmrEMMA, ISIS EM-BLASSO, pLARmEB and pKWmEB. As a result, 1,001 mQTNs were detected to be associated with the 52 acyl-lipid metabolites (Figure S2 and Table S7). Among these QTNs, they were distributed mainly on chromosomes 5, 7, 8, 13 to 18 and 20 (≥ 50 mQTNs for each chromosome) and had 6.63% average proportion of total phenotypic variation explained by each mQTN, and 230, 115, 66, 111, 96 and 383 SNPs were identified to be significantly associated, respectively, with 9 organic acids, 5 soybean isoflavones, 6 PEs, 6 PIs, 6 PCs and 20 amino acids in soybean (Figure S2). Forty-eight mQTNs were detected in at least two approaches (Table S8). In addition, there were some large-effect mQTNs, e.g., mQTNs Chr4-3969004, Chr5-2665256, Chr8-17117978 and Chr18-62242431 were found by ISIS EM-BLASSO to be associated, respectively, with glutamic acid (r^2=21.15%), PI (34:3) (r^2=9.31%), malate (r^2=4.97%) and isoleucine (r^2=6.75%), and mQTN Chr20-45754357 was found by mrMLM to be associated with pyruvate (r^2=6.18%).

Candidate genes associated with metabolites The methodologies of determining the candidate genes for acyl-lipid related metabolites were the same as those for the above seed

oil-related traits. First, we found all the genes between the 100 kb upstream and downstream regions for each of all the significantly mQTNs. Using soybean metabolic pathway database, KEGG annotation (https://soycyc.soybase.org/) and soybean genome annotation database and Gene Ontology terms (https://soybase.org/genomeannotation/), then, all the above genes were used to mine the candidate genes or their *Arabidopsis* homologous genes that were annotated in fatty acid biosynthesis, fatty acid activation, phospholipid biosynthesis, flavonoid biosynthesis, amino acid transporters, brassinosteroid biosynthesis, glycolysis, triacylglycerol biosynthesis, cellulose biosynthesis, jasmonic acid biosynthesis, and TCA cycle pathways. As a result, 279 genes were found to be associated with the above metabolic pathways.

Among the above 279 genes, twenty were found to be related to lipid metabolism pathways, including 17 oil biosynthesis related genes, one amino acid biosynthesis related gene, two TCA cycle related genes, and one lipid-related gene in previous studies. Among these lipid metabolisms related genes, six were the same as those for seed oil-related traits, including *GmPDAT*, *GmCds1*, *GmACO1*, *GmAGT*, *GmBS1*, and *GmPgs1*.

In oil biosynthesis related genes, *GmPDAT*, *GmLPEAT2* (*Glyma.03g019200*), *GmPDHC* (*Glyma.20g115500*), *GmLACS2* (*Glyma.11g122500*), *GmACP4* (*Glyma.20g230100*), *GmGPDH* (*Glyma.19g136100*), *GmPLDα1* (*Glyma.08g211700*), *GmPLP2* (*Glyma.05g049500*), *GmCds1* (*Glyma.18g055100*), *GmTIM* (*Glyma.13g146200*), *GmGPAT* (*Glyma.07g069700*), *GmPgs1* (*Glyma.18g302100*), *GmPLA2A* (*Glyma.14g081200*), *GmSAD* (*Glyma.14g121400*), *GmZF351* (*Glyma.06g290100*), *GmBS1* (*Glyma10g38970*), and *Glyma.08g323100* were found to be associated, respectively, with Pyruvate (P=1.44e-05) (Liu, 2020), PI (34:3) (P=7.12e-10) (Jasieniecka-Gazarkiewicz *et al.*, 2017), phenylalanine (LOD=4.05) (Zhang *et al.*, 2016), linolenic acid (P=2.63e-07) (Lü *et al.*, 2010; Katavic *et al.*, 2014), pyruvate (LOD=14.68) (Feng *et al.*, 2018), daidzin (LOD=4.71) (Shen *et al.*, 2006), malate (LOD=3.11) (Zhao *et al.*, 2012; Zhang G *et al.*, 2019), PI (34:3) (LOD=4.26) (La *et al.*, 2009), aspartic acid (LOD=5.65) (Zhou *et al.*, 2013), glycytin (LOD=3.41) (López *et al.*, 2016), serine (LOD=3.55) (Li *et al.*, 2007), isoleucine (LOD=6.75) (Tanoue *et al.*, 2014), PE (34:1) (LOD=3.92) (Yang e*t al* 2009), stearic acid (LOD=5.42) (Lindqvist *et al.*, 1996), phenylalanine (LOD = 3.96) (Li *et al.*, 2017), oleic

acid (LOD=3.26) (Ge *et al.*, 2016), and fumaric acid (LOD = 4.56). Note that gene *GmZF351* has no annotation of biochemical metabolic process, and eight genes (*GmPDAT*, *GmLPEAT2*, *GmSAD*, *GmLACS2*, *GmPLDα1*, *GmPLP2*, *GmTIM and GmZF351*) were differentially expressed between wild and cultivated soybeans (Figure 2b and Table 2). In genes related to amino acid biosynthesis, *GmAGT* (*Glyma.08g302600*) was found to be associated with palmitic acid (LOD=3.39) (Zhang *et al.*, 2002). In TCA cycle related genes, *GmIDH-V* (*Glyma.13g144900*) and *GmACO1* (*Glyma.01g162800*) were found to be associated, respectively, with γ-aminobutyric acid (LOD=2.78) (Lemaitre *et al.*, 2006) and glycytin (P=2.63e-07) (Park *et al.*, 2018) (Figure 3b and Table 3).

Genetic relationships between seed oil-related traits and lipid metabolism related metabolites in soybean

The MCP and SCAD algorithms were used to conduct multiple regression analysis of each seed oil-related trait on fifty-two acyl-lipid related metabolites, and the *t*-test was further used to determine the acyl-lipid related metabolites that were significantly associated with each oil-related trait. To reduce experimental error, the average of each seed oil-related trait in each accession across three years was used to conduct the above analysis. As a result, seed oil content, linoleic acid, linolenic acid, oleic acid and palmitic acid were found to be significantly associated, respectively, with 7, 5, 7, 2, 10 lipid metabolism related metabolites (Figure 3a and Table 3). Seed oil content had significant partial regression with genistein (0.526, P-value=0.002), PC (36:2) (0.679, P-value=1.09e-06), glutamic acid (0.243, P-value=0.038), daidzin (-0.842, P-value= 2.36e-06), PC (36:4) (-0.659, P-value=4.75e-06), PC (36:5) (-0.316, P-value=0.030) and aspartic acid (-0.172, P-value=0.034); linoleic acid had significant partial regression with fumarate (0.486, P-value=0.050), PC (36:5) (0.564, P-value=4.84e-05), daidzin (-0.911, P-value=0.003), PI (36:1) (-1.162, P-value=0.009) and stearic acid (-0.324, P-value=0.017); linolenic acid had significant partial regression with glycitin (0.664, P-value=0.008), PI (34:1) (1.367, P-value=4.19e-05), linolenic acid (metabolite) (-0.324, P-value=0.017), stearic acid (metabolite) (-0.633, P-value= 0.014), pyruvate (-0.026, P-value=0.050), fumarate (-0.662, P-value=0.017) and PI (34:2) (-1.420, P-value=0.045); oleic acid had significantly partial regression with daidzin (0.0732, P-value=3.11e-4) and isoleucine (-0.022, P-value=0.041);

palmitic acid had significant partial regression with daidzin (0.086, P-value=0.047), fumaric acid (0.220, P-value=1.09e-4), PC (36:2) (0.739, P-value=8.95e-4), PE (36:5) (0.383, P-value=1.24e-4), PI (34:1) (0.294, P-value=0.0387), tryptophan (0.142, P-value=0.004), aspartate (0.148, P-value=0.032), glutamic acid (-0.143, P-value=0.042), PC (34:2) (-1.020, P-value=0.002) and PI (36:2) (-0.162, P-value=0.005) (Table 1). No significant partial regression of stearic acid on acyl-lipid metabolites was identified.

Protein-by-protein interaction (PPI) analysis

The above 36 genes for seed oil-related traits and lipid related metabolites were used to identify the PPIs using the online software STRING (https://string-db.org/cgi/input.pl). As a result, the predicted values for 16 pairs of PPIs were larger than medium confidence value of 0.40 (Table S9), indicating the existence of significant PPIs. For example, Glyma13g16790.1 (GmPDAT) and Glyma18g36130.3 (GmFATA2) (0.69), GmCds1 (Glyma18g06190.1) and Glyma13g16790.1 (GmPDAT) (0.43), Glyma06g44440.1 (GmZF351) and Glyma13g16790.1 (GmPDAT) (0.43), Glyma08g22600.1 (GmPLDα1) and Glyma18g06190.1 (GmCds1) (0.69), Glyma05g03510.1 (GmPLP2) and Glyma13g16790.1 (GmPDAT) (0.57), Glyma13g16790.1 (GmPDAT) and Glyma08g08910.1 (GmKASI) (0.69), Glyma13g16560.1 (GmDAGAT1) and Glyma13g16790.1 (GmPDAT) (0.75), Glyma13g20790.1 (GmIDH-V) and Glyma02g01920.1 (GmFUM1) (0.92), and Glyma14g27990.1 (GmSAD) and Glyma20g25833.1 (GmFATB1a) (0.90). Clearly, the above two PPIs between GmDAGAT1 and GmPDAT (Liu, 2020) and between GmPDAT and GmFATA2 (Figure 4) were confirmed in vivo using luciferase complementation image assay. In addition, the interactions between GmIDH-V and GmFUM1, and between GmDAGAT1 and GmPDAT were reported, respectively, in Zhang et al. (2017) and Liu (2020), and the PPI between GmDAGAT1 and GmPDAT was further validated by the interaction between two loci Chr13-20532852 and Chr13-20704079 bp (Table S6).

Construction of three-dimension genetic networks from 6 soybean seed oil related traits, 23 lipid related metabolites, and 36 candidate genes in the pathways of fatty acids, amino acid synthesis and TCA cycle

First, primary metabolic networks in soybean were constructed. Making use of gene homogeneity, 28 genes having functional annotations in the above 36 candidate genes were incorporated into primary metabolic networks in *Arabidopsis thaliana* (Wen *et al.,* 2015; Zhang *et al.,* 2016; Li *et al.,* 2013). In the networks, there were 19 oil biosynthesis related genes, four amino acid biosynthesis related genes, five TCA cycle related genes, six seed oil related traits, and 43 metabolites (Figure 2a). Among the 19 oil biosynthesis related genes, 12 were differentially expressed between four cultivated and two wild soybeans (Figure 2b).

The above primary metabolic networks in soybean and all the above genetic information in this study were used to construct three-dimension genetic networks. In these networks, six oil-related traits, 23 lipid related metabolites, and the above 36 candidate genes were used to construct 133 genetic sub-networks, which belong to one of the three types listed below.

The first group included 33 sub-networks, in which each linked gene was identified commonly by phenotypic and metabolic GWAS. In isoleucine-*GmPgs1*-linolenic acid-*GmPDAT* sub-network, *GmPgs1* was identified to be associated commonly with isoleucine (metabolite) and linolenic acid (trait). In pyruvate-*GmPDAT*-linolenic acid-*GmCds1*, PE (34:1)-*GmPDAT*-linolenic acid-*GmDAGAT1* and PE (34:1)-*GmPDAT*-linolenic acid-*GmCds1* sub-networks, *GmPDAT* was identified to be associated commonly with linolenic acid (trait) and two metabolites [PE (34:1) and pyruvate]. In pyruvate-*GmAGT*-palmitic acid-*GmKASI* sub-network, *GmAGT* was identified to be associated with pyruvate (metabolite) and palmitic acid (trait). Among all the 33 sub-networks, five were known and the others were newly identified (Figure 3d and Table S10). To validate these results, five high-oil and five low-oil accessions were used to conduct hypothesis testing for each node (gene, metabolite or trait) in the above sub-networks. As a result, 5, 7, 14 and 7 sub-networks were found to have one, two, three, and four significant nodes, respectively, although the accessions used in traits and metabolite analyses had a little difference with those in gene expressional analysis (Table S11).

The second group included 84 sub-networks, which were derived from the significant association of oil-related traits with metabolites (Tables 1 and S10). In *GmPDAT*-pyruvate-linolenic acid-*GmDAGAT1* sub-network, pyruvate was significantly associated with linolenic acid

(P<0.050). In *GmLACS2*-linolenic acid (metabolite)-linolenic acid-*GmDof11* sub-network, linolenic acid (metabolite) was significantly associated with linolenic acid (P=0.045). In *GmTIM*-glycitin-linolenic acid-*GmPDAT*/*GmDAGAT1* sub-network, glycitin was significantly associated with linolenic acid (P= 0.008) (Table 1). Among all these sub-networks, 13 were known and the others were newly identified (Figure 3d and Table S10). Similarly, 15, 35, 31 and 3 sub-networks were found to have one, two, three, and four significant nodes, respectively (Table S11).

The third group included 16 sub-networks, which were derived from the interactions between the genes for oil-related traits and/or metabolites (Figure 3d and Table S10). In pyruvate-*GmPDAT*-*GmFATA2*-oil content and pyruvate-*GmPDAT*-*GmKASI*-palmitic acid sub-networks, the statistic scores for PPIs between *GmPDAT* and *GmFATA2* and between *GmPDAT* and *GmKASI* were 0.69 and 0.69, respectively. Moreover, luciferase complementation image assays (LCI) validated the protein interaction between GmPDAT and GmFATA2 (Figure 4). In phenylalanine-*GmZF351*-*GmPDAT*-linolenic acid sub-network, the statistic score for PPI between GmPDAT and GmZF351 was 0.43. In pyruvate-*GmPDAT*-*GmCds1*-linolenic acid sub-network, the statistic score for PPI between GmPDAT and GmCds1 was 0.43, while *GmPDAT* was significantly associated with linolenic acid and pyruvate. Among all these sub-networks, 6 were known and the others were newly identified. In the same way, 9, 1, and 6 sub-networks were found to have two, three, and four significant nodes, respectively (Table S11).

DISCUSSION

One-dimension genetic networks among genes (Lin *et al.*, 2017) or metabolites (Sauvage *et al.*, 2014), and two-dimension genetic networks between traits and genes (Wang *et al.*, 2007) and between metabolites and genes (Wen *et al.*, 2015; Chen *et al.*, 2016) are frequently reported in previous studies. Recently, Shi et al. (2020) reported one two-dimension network between metabolites and traits in wheat. As we know, metabolites act as a bridge between traits and genes (Fiehn, 2002). Thus, it is very important and necessary to construct three-dimension genetic networks among traits, metabolites and genes. In these networks, 36 candidate genes were obtained from pGWAS and mGWAS, 23 metabolites were significantly associated with five

oil-related traits, and all the genetic information was used to construct 133 three-dimension genetic sub-networks. This study is novel in three aspects. To the best of our knowledge, first, this study reports the first 3D genetic networks in soybean. Among these sub-networks, 60 were found to be partly validated in previous molecular biology studies (Table 4), 21 were found to be involved in known KEGG metabolic pathways (https://www.kegg.jp/kegg/pathway.html) (Table S10), and 112 were newly identified in this study. Then, a series of GWAS approaches were used and all the significant QTNs across various environments or approaches were used to mine candidate genes in this study. This is because that the combination of several GWAS approaches has been recommended in a series of studies so as to improve the power in QTN detection (Chang et al. 2018; He et al. 2019; Li et al. 2019; Xu et al. 2019; Zhang et al. 2019a), and in practice some true genes for the traits of interest are found to be linked with the QTNs detected by only one GWAS method or in one environment (Zhang et al. 2019b). Finally, quite constructive, reasonable and interesting issues in these sub-networks have been discussed in this study. The results provide the theoretical basis for both functional identification of seed oil-related genes and quality improvement in soybean breeding.

Using the three-dimension genetic networks, we may mine some candidate genes to uncover some genetic relationships, for example, pyruvate and the three major nutrients, and amino acids and seed oil content. In this discussion we will focus on these relationships (Figure 5 and Table 4).

GmPDAT, GmAGT **and** ***GmACP4*** **reveal the genetic relationships between pyruvate and three major nutrients**

Nutrients mainly include amino acids, fatty acids and carbohydrates. In the amino acid metabolism, the absence of pyruvate affected the synthesis of amino acids (Orsi *et al.*, 2004; Feng *et al.*, 2018), and *AGT* participated in the metabolism of aspartic acid in *Arabidopsis thaliana* (Zhang *et al.*, 2013). In this study, *GmAGT* was found to be associated commonly with pyruvate (metabolite) and palmitic acid (trait) in the pyruvate-*GmAGT*-palmitic acid-*GmBS1/GmWRI1b* sub-network (Table 5), indicating the genetic relationship of *GmAGT* with

both pyruvate and palmitic acid.

Pyruvate and adenosine triphosphate (ATP) are the basic molecules in the synthesis of acetyl-CoA, while acetyl-CoA is the main precursor in fatty acid synthesis (Weiss *et al.,* 1974). Meanwhile, *ACP* acts as a carbon carrier for fatty acid synthesis, and *GmPDAT* and *GmDAGAT1* have been reported to be related to oil synthesis (Lardizabal *et al.,* 2008; Chen *et al.,* 2016; Liu, 2020). In this study, pyruvate was found to be significantly associated with linolenic acid (P=0.050) (Table 1) and both *GmPDAT* and *GmACP4* in the *GmACP4*-pyruvate-linolenic acid-*GmDAGAT1* sub-network (Table 5). We deduce that pyruvate may regulate the synthesis of fatty acids through the action of *GmACP4*, *GmPDAT* and *GmDAGAT1*.

In addition, pyruvate is an important product of glycolysis (Chen *et al.,* 2019). Based on the above information, therefore, *GmPDAT*, *GmAGT* and *GmACP4* may be key genes in the genetic relationships between pyruvate and three major nutrients.

GmPDAT, GmZF351 **and** ***GmPgs1*** **reveal the genetic relationship between amino acids and seed oil content**

Although seed oil content in soybean is negatively correlated to seed protein content, knowledge about the molecular mechanism of the negative correlation is limited (Chaudhary *et al.,* 2015; Patil *et al.,* 2017). Warrington et al. (2015) and Patil et al. (2017) revealed the significant correlation of crude protein with amino acid, especially for threonine. Note that threonine was the upstream mediator of isoleucine (Guo *et al.,* 2015). If isoleucine content changed, threonine content would be influenced, followed by the protein and oil contents. In this study, *GmZF351* was found to interact with *GmPDAT* in the detection of PPIs; *GmZF351* and *GmPDAT* were found to be associated with phenylalanine and linolenic acid (Table 4), respectively; *GmZF351* was reported to increase TAG content in soybean seed (Li *et al.,* 2017). In addition, *GmPgs1* was found to be significantly associated with isoleucine and linolenic acid in this study (Table 5), while *Pgs1* participated in the biosynthesis of phosphatidylglycerol (Tanoue *et al.,* 2014). Thus, *GmPDAT*, *GmZF351* and *GmPgs1* may be key genes in amino acid and oil synthesis, which may reveal the genetic relationship between amino acids and seed oil synthesis.

GmCds1, along with average temperature and rainfall, reveals interannual variation of seed oil content in soybean

Paired *t*-test showed that all the six oil-related traits in 286 soybean accessions have significantly higher in 2015 and 2016 than in 2014 (P-values<1e-04; Figure 1 and Table S12). Here we would discuss the reasons.

From the genetic perspective, several types of evidence were obtained. In this study, *GmPDAT* was found to be significantly associated with both pyruvate and linolenic acid; *GmCds1* was found to be significantly associated with linolenic acid; the interaction between the locus Chr18-4720420 and environment was found to be significantly associated with linolenic acid. Around Chr18-4720420, *GmCds1* is mined and annotated with phosphatidylglycerol biosynthesis in the soybean metabolic pathway database. Zhou et al. (2013) showed that *CDS* can influence the biosynthesis of phosphatidylglycerol in *Arabidopsis*. Meanwhile, *GmCds1* had significantly higher expression in cultivated soybeans than in wild soybeans (Figure 2b). More importantly, soybean seeds in the plants with overexpression and interference of *GmPDAT* showed significant changes in linolenic acid and linoleic acid as compared with the controls (Liu, 2020). As we know, CDS and PAP, along with PA as substrate, can form CDP-DAG and DAG, respectively (Nakamura, 2017). In extreme environments, thus, *GmCds1* may affect the synthesis of DAG, which may reduce the synthesis of TAG with the aid of *GmPDAT*, possibly resulting in the decrease in seed oil-related traits.

In addition, we conducted two analyses for environmental factors. First, we conducted correlation analysis between seed oil-related traits and average temperature from June to September in 2011, 2012, and 2014 to 2016. As a result, average temperatures in early and all the July were found to have significant correlation with linoleic acid (r=0.907, P-value=0.007; r=0.831, P-value=0.020), respectively (Table S13). Then, we calculated the rainfall from June to September. As a result, the rainfall in 2015 and 2016 was 1.57 and 1.42 times larger than that in 2014 (Table S14), while seed oil content decreased by 5.4% and 12.5% in 2015 and 2016, respectively, as compared with that in 2014.

Therefore, *GmCds1* and *GmPDAT*, along with average temperature in July and the rainfall, may influence the change of seed oil-related traits across years.

EXPERIMENTAL PROCEDURES

Association populations for phenotypic and metabolic GWAS

As described by Zhou et al. (2015), the 286 soybean accessions were randomly selected from 6 geographic regions in China using a stratified random sampling method, and included 14 wild, 153 landrace, and 119 bred accessions. All the accessions were planted in three-row plots in a completely randomized design at the Jiangpu Experimental Station of Nanjing Agricultural University (Nanjing, 31°14′N, 118°22′E) in 2014, 2015 and 2016. The plots were 1.5 m wide and 2 m long. Seeds for each accession in 2014 to 2016 were harvested from the middle row in three-row plots and used to measure seed oil content, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid at State Key Laboratory of Crop Genetics and Germplasm Enhancement of Nanjing Agricultural University. Among the 286 accessions in 2015, 214 were selected at 55 days after flowering (DAF) and used to measure acyl-lipid related metabolites at Beijing Pufeng Technology Co., Ltd. (Table S15). The mixture with at least three pods each from different plants for each accession was stored at -80°C before extraction and extracted for metabolite profiling.

Measurement for six oil-related traits in 286 soybean accessions

Approximate 10 g of seeds was collected from five plants per accession. Based on the method of Baydar and Akkurt (2001), five fatty acids (stearic, palmitic, oleic, linoleic and linolenic acids) (Fang *et al.*, 2017; Zhang G *et al*., 2019; Zuo *et al*., 2019) for each accession were measured by gas chromatography with a flame ionization detector and a Permabond FFAP stainless steel column (50 m × 0.2 mm × 0.33 μm, ThermoFisher Scientific, Waltham, MA) at Nanjing Agricultural University in 2014, 2015 and 2016. After drying at 70°C for 3 h, approximately 2 g of mature and well-rounded seeds were milled to a fine powder with an electric grinder. Solid fractions were filtered out using a 0.20-mm sieve weigh 0.03 g of soybean powders into a 2 mL

tube adding 0.5 mL of 2 mg/mL heptadecanoic acid (used as an internal standard) and 1 mL N-hexane shaking 30 secs, placed at room temperature for 5 h. 750 μL of the hexane layer was transferred to a new 2 mL tube adding 0.5 mL of 0.4M KOH-methanol shaking 2 min placed at room temperature for 2 h. The hexane layer was transferred to a new 2 mL tube centrifugation for 5 min at 6000 r/min, keep 500 μL of supernatant for further GC analysis. 1 μL of the prepared sample was injected into the Trace GC system (Thermo Fisher Scientific), which was equipped with a DB-23 column (Agilent Technologies, 60 m × 0.25 mm × 0.25 μm) at a split ratio of 1:20. The oven was programmed as follows: 150°C for 1 min, ramp to 200°C at 4°C/min, ramp to 220°C at 3°C/min, and finally ramp to 250°C at 25°C/min, holding 5 min with 1.1 mL/min helium as carrier gas (Lisec *et al.,* 2006; Marques *et al.,* 2006). Using methyl heptadecanoate (C17) as internal standard, oil content was calculated by the method introduced by Zhou et al. (2016).

Measurement for 52 acyl-lipid related Metabolites using LC–MS

A liquid chromatography–mass spectrometry system was used for the relative quantification of widely targeted metabolites in pods harvested 55 DAF. The beans were crushed using a mixer mill (MM 200, Retsch) by MIX-3000 (Hangzhou Miou Instrument), 100 mg dried powder was weighted and extracted overnight at 4°C with 1.0 ml pure methanol acetonitrile water (1:1). Centrifuge sample at 14,000 × g and 4°C for 15 min. 1 μL of the prepared sample was injected into the LC-20AD system (Shimadzu). Separation was performed in a C18 column (150 × 2.1 mm, 3.5 μm) using solvent A water (containing 0.01% heptafluorobutyric acid, 0.1% formic acid) and solvent B acetonitrile (containing 0.01% heptafluorobutyric acid, 0.1% formic acid) as mobile phases, column temperature, 50°C. The following MS conditions were used: gas temperature, 325°C; drying gas, 11 L/min; nebulizer, 40 psig; fragmentor, 120 V; and skimmer, 65 V. The instrument was set to acquire over the m/z range 40-1,200 with an acquisition rate of 1.2 spectra/s (Nygren *et al.,* 2011). Quantification of metabolites was carried out using standard curve method (Nygren *et al.,* 2011; Wen *et al.,* 2015; Thiele *et al.,* 2012).

Fifty-two acyl-lipid related metabolites measured in this study included 9 organic acids (pyruvic,

succinic, fumaric, malic, palmitic (metabolite, m), stearic (m), oleic (m), linoleic and linolenic acids (m)), 5 soybean isoflavone (daidzein, daidzin, genistein, genistin and glycitin), 6 PEs [PE (34:1) (16:0/18:1), PE (34:2) (16:1/18:1), PE (36:2) (18:1/18:1), PE (36:3) (18:2/18:1), PE (36:4) (16:0/20:4) and PE (36:5) (16:1/20:4)], 6 PCs [PC (34:1) (16:0/18:1), PC (34:2) (16:0/18:2), PC (36:2) (18:0/18:2), PC (36:3) (18:1/18:2), PC (36:4) (18:1/18:3) and PC (36:5) (20:4/16:1)], 6 PIs [PI (34:1) (16:0/18:1), PI (34:2) (16:0/18:2), PI (34:3) (16:1/18:2), PI (36:2) (18:0/18:2), PI (36:3) (18:0/18:3) and PI (36:4) (16:0/20:4)], and 20 amino acids (alanine, arginine, γ-aminobutyric acid, phenylalanine, glycine, glutamic acid, glutamine, methionine, lysine, tyrosine, leucine, proline, tryptophan, serine, threonine, aspartic acid, asparagine, isoleucine, valine and histidine). The number of biological replicates for each accession was two.

GWAS for oil-related traits and acyl-lipid related metabolites

The preprocessing procedures for phenotypic and metabolic GWAS were as follows. Only SNPs with MAF ≥ 0.05 and missing rate < 0.1 in the mapping populations were used in the GWAS; the lines with more than 90% missing for trait phenotypes or metabolites were filtered out; the metabolites with more than 50% missing in 214 lines were excluded (Liaw *et al.*, 2002). The population structure was calculated using the Bayesian clustering program fastStructure (Raj *et al.*, 2014). Six oil-related traits in 286 accessions and 52 acyl-lipid related metabolites in 214 accessions, along with the above SNP information, were used to conduct phenotypic and metabolic GWAS using GEMMA (Zhou & Stephens, 2012), mrMLM (Wang *et al.*, 2016), ISIS EM-BLASSO (Tamba *et al.*, 2017), pLARmEB (Zhang *et al.*, 2017), FASTmrEMMA (Wen *et al.*, 2018) and pKWmEB (Ren *et al.*, 2018) methods. The K matrix was calculated in the above GEMMA and mrMLM programs. The threshold for significant QTN in phenotypic and metabolic GWAS was set at P-value ≤ 1/54,294=1.84e-05 for GEMMA and LOD ≥ 2.5 for the others (Xu *et al.*, 2018; Zhang *et al.*, 2019a). All the mQTNs were obtained from each biological replicate.

The interactions between QTNs and environment (QEs) were detected using quantitative trait interaction (G × E) module in PLINK 1.9 (http://zzz.bwh.harvard.edu/plink/anal.shtml#qtgxe) (Purcell *et al.*, 2007), and the critical P-value for significant QEs was set at 0.001.

The QTN-by-QTN interactions (QQs) were detected using the online software PEPIS (Zhang *et al.,* 2016) (http://bioinfo.noble.org//PolyGenic_QTL//Home.gy), and the critical P-value for significant QQs was set at LRT ≥ 13.815. The protein-protein interactions for candidate genes in phenotypic and metabolic GWAS were detected using the online tools STRING (https://string-db.org//) (Jensen *et al.,* 2009).

Genetic association analysis between oil-related traits and metabolites

MCP (Zhang *et al.,* 2006), SCAD (Fan & Li 2001) and *t*-test were used to construct the genetic relationships between six oil-related traits and 52 acyl-lipid related metabolites. To reduce experimental error, the average of each seed oil-related trait in each accession across 2014 to 2016 was used to conduct the above analysis. Statistical significance was calculated using *F*-test for the total regression of each oil-related trait on several metabolites and *t*-test for the regression of each oil-related trait on each metabolite. *, ** and *** indicated significant probability levels 0.05, 0.01 and 0.001, respectively.

Candidate gene identification

Candidate genes for each oil-related trait and metabolite were mined in two steps. First, all the genes between the 100 kb upstream and downstream regions for each of the significantly QTN or mQTNs were mined. Then, we downloaded the soybean metabolic pathway database, KEGG annotation (https://soycyc.soybase.org/) and soybean genome annotation database and Gene Ontology terms (https://soybase.org/genomeannotation/), and identified the genes or their *Arabidopsis* homologous genes, which were annotated with fatty acid biosynthesis, fatty acid activation, phosphatidylglycerol biosynthesis, flavonoid biosynthesis, amino acid transporters, brassinosteroid biosynthesis I, glycolysis, triacylglycerol biosynthesis, cellulose biosynthesis, jasmonic acid biosynthesis, and TCA cycle.

Differentially expressed gene based on RNA-sequenced data

Four cultivated soybeans (accession No. 101, 236, 257 and 276) with high seed oil content (20.9,

22.3, 17.2, and 17.8 (%), respectively) and two wild soybeans (accession No. 265 and 272) with low seed oil content (11.9 and 12.5 (%), respectively) were selected for RNA-seq analysis. Seeds were collected at five seed development stages (15, 25, 35, 45, and 55 DAF) for RNA extraction in 2014. Total RNA was extracted using *TRIzol* reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was analyzed in an Illumina Hiseq 2500 Sequencer. Sequence reads were aligned using SAM format (Li *et al.,* 2009). The raw reads were cleaned by removing reads with adapters and those of low quality. Clean reads were mapped to reference sequences using SOAPaligner/soap2 (http://soap.genomics.org.cn/ soapdenovo.html). Mismatches no more than two bases were allowed in the alignment. The gene expression level was calculated by using Reads Per kb per Million reads (RPKM method) (Mortazavi *et al.,* 2008).

Construction and visualization of three-dimension genetic networks among oil-related traits, metabolites and candidate genes

In the three-dimension genetic networks, oil-related traits, metabolites and candidate genes were the nodes of the networks, and the genetic relationships between oil-related traits and candidate genes, between metabolites and candidate genes, between oil-related traits and metabolites, and between candidate genes were the edges of the networks. The genetic relationships between oil-related traits and candidate genes were derived from phenotypic GWAS, ones between metabolites and candidate genes were derived from metabolic GWAS, ones between oil-related traits and metabolites were derived from the MCP, SCAD and t-test analyses, and ones between candidate genes were derived from the detection of both QQs and PPIs. Three-dimension genetic networks with the above nodes, edges and interactions were constructed by open-source software Cytoscape (Saito *et al.,* 2012).

Hypothesis tests for the differences of traits, metabolites and gene expressional levels in subnetworks between five high-oil and five low-oil soybean accessions

Five high-oil (accession nos. 95, 146, 159, 183, and 215; the average oil content: 18.85 ± 0.81 (SE) (%)) and five low-oil (accession nos. 214, 260, 261, 270, and 271; the average oil content:

13.83 ± 1.69 (%)) soybean accessions were selected to conduct hypothesis tests for the differences of traits and metabolites in the constructed subnetworks, while four high-oil (accession nos. 101, 236, 257, and 276) and two low-oil (accession nos. 265 and 272) soybean accessions were selected to conduct hypothesis tests for the expressional level differences of genes in the constructed subnetworks. Trait phenotype for each accession was the average across three years (2004 to 2006), metabolite in pods harvested 55 DAF was measured by LC-MS in 2015, and the expressional levels of genes at 15 DAF were measured by the RPKM values based on RNA-sequenced data. The *t* test was adopted in the hypothesis testing.

Cloning and generation of plant LUC vectors

Soybean (*Glycine max* Willimas 82) and *N. benthamiana* plants were grown at 16-hlight/8-h dark at 25°C for 30-60 d. Soybean total RNA was isolated using the trizol reagent (Invitrogen, Foster city, CA, USA), the first-strand cDNA was then synthesized using M-MLV reverse transcriptase (Promega). PCR-amplified DNA fragments were cloned into the N-LUC (LUC-luciferase) and C-LUC vector (Chen *et al.*, 2008, Zhang *et al.*, 2018). Full length CDS of *GmPDAT* and *GmFATA2* were cloned into the BamHI and SalI sites of JW-771-N, as well as KpnI and SalI sites of JW-772-C, to produce N-gene and C-gene recombination vectors for the luciferase complementation image assays (LCI) (Krenek *et al.*, 2015). Primers are listed in Table S16.

Detection of interactions in vivo

As described by Zhang et al. (2018), the recombinant plasmids like N-*GmPDAT* + C-*GmFATA2*, N-*GmPDAT*+C-LUC, N-LUC + C-*GmGmFATA2* or N-LUC+C-LUC were transfected into *Agrobacterium tumefaciens* (GV3101). After growing 48h under the condition of 16h-light and 8h-dark, leaf abaxial epidermis were daubed with 1mM luciferin (promega, E1602), the resulting luciferase signals were captured by Tanon-5200 image system (Tanon, Shanghai, China). These experiments were repeated three times to get similar results.

DATA AVAILABILITY STATEMENT

Supporting Information is available from the Wiley Online Library or from the author.

AUTHOR CONTRIBUTIONS

YMZ conceived of the project and its components. JYL, PL, YWZ, JFZ, GL, XH and YMZ performed field experiments, bioinformatics analysis and real data analysis. JYL and JFZ performed experimental LCI assays. YMZ, JYL and JMD wrote and revised the manuscript. All authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

ABC1	activity of bc1 complex homolog 1
ACC	acetyl coenzyme-A carboxylase
ACP4	acyl carrier protein (ACP)-4
ACO1	acyl-CoA oxidase 1
AGT	alanine glyoxylate aminotransferase
ATP	adenosine triphosphate
DAF	days after flowering
DG	diacylglycerol
DGAT/ DAGAT	acyl-CoA: diacylglycerol acytransferase

FATA	fatty acid thioesterase A
FATB	fatty acid thioesterase B
FUM1	fumonisin synthase gene 1
GPDH	glycerol phosphate dehydrogenase
GWAS	genome-wide association study
IDH-V	isocitrate dehydrogenase V
LACS	long-chain acyl-CoA synthetase
LTP	lipid transfer protein
MDH	malate dehydrogenase
mGWAS	metabolome-based genome-wide association studies
mrMLM	Multi-locus random-SNP-effect mixed linear model
OLE	oleosins
P5C1	pyrroline-carboxylic acid synthase 1
PDAT	phospholipid:diacylglycerol acyltransferase
PDHC	pyruvate dehydrogenase complex
PC	phosphatidylcholine
PE	phosphatidyl ethanolamine
PI	phosphatidylinositol
PPI	protein-protein interaction
PLDα1	phospholipase Dα1
Pgs1	phosphatidylglycerolphosphate synthase 1
QTN	quantitative trait nucleotides
RPKM	reads Per Kilobases per Millionreads
LCI	luciferase complementation image assay
SAD	sinapyl alcohol dehydrogenase
SDH1	succinate dehydrogenase1
SNP	single nucleotide polymorphism
TAG	triacylglycerol
TIM	translocases inner mitochondrial membrane

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Chromosomal distribution of oil-related trait QTNs for linoleic acid (blue), oleic acid (red), palmitic acid (green), stearic acid (pink), linolenic acid (navy blue) and seed oil content (black) on the soybean genome positions (*x* axis, cM).

Figure S2. Chromosomal distribution of metabolic QTNs for amino acids (grey), daidzin group (green), organic acid (blue), fatty acid (orange), and PC, PE and PI (pink) on the soybean genome (*x* axis, cM).

m1: alanine; m2: arginine; m3: γ-aminobutyric acid; m4: phenylalanine; m5: glycine; m6: glutamic acid; m7: glutamine; m8: methionine; m9: lysine; m10: tyrosine; m11: leucine; m12: proline; m13: tryptophan; m14: serine; m15: threonine; m16: aspartic acid; m17: asparagine; m18: isoleucine; m19: valine; m20: histidine; m21: daidzin; m22: daidzein; m23: glycitin; m24: genistein; m25: genistin; m26: pyruvate; m27: succinic acid; m28: malic acid; m29: fumaric acid; m30: linoleic acid; m31: stearic acid; m32: linolenic acid; m33: oleic acid; m34: palmitic acid; m35: PC (34:1); m36: PC (34:2); m37: PC (36:2); m38: PC (36:3); m39: PC (36:4); m40: PC (36:5); m41: PE (34:1); m42: PE (34:2); m43: PE (36:2); m44: PE (36:3); m45: PE (36:4); m46: PE (36:5); m47: PI (34:1); m48: PI (34:2); m49: PI (34:3); m50: PI (36:2); m51: PI (36:3); m52: PI (36:4).

Table S1 | Phenotypic characteristics for seed oil related traits in 286 soybean accessions.

Table S2 | Phenotypic characteristics for metabolites (μg/g) in 214 soybean accessions.

Table S3 | Candidate genes in genome-wide association studies for seed oil-related traits.

Table S4 | 77 QTNs of seed oil related traits detected commonly in two years or by at least two methods.

Table S5 | Nine QTN-by-environment interactions for seed oil related traits in soybean.

693 **Table S6** | Ten QTN-by-QTN interactions for seed oil related traits in soybean.
694
695 **Table S7** | Candidate genes in genome-wide association studies for fifty-two metabolites.
696
697 **Table S8** | 48 metabolic QTNs detected by at least two GWAS approaches.
698
699 **Table S9** | 16 pairs of significant PPIs between 36 candidate genes derived from phenotypic and
700 metabolic GWAS
701
702 **Table S10** | 133 genetic sub-networks among oil related traits, metabolites and candidate genes.
703
704 **Table S11 |** The significances for the differences of traits (t), metabolites (m) and gene
705 expressional levels in 133 subnetworks between high-oil and low-oil soybean accessions
706
707 **Table S12** | Paired *t*-tests and their P-values for seed oil related traits between 2014 and the
708 others.
709
710 **Table S13** | Correlation analysis between seed oil-related traits and average temperature at the
711 seed developmental stages.
712
713 **Table S14** | Rainfall and annual average (mm) in 2014 to 2016
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715 **Table S15** | 214 accessions used to measure acyl-lipid related metabolites at 55 days after
716 flowering in 2015.
717
718 **Table S16** | Primers used in Luciferase complementation image assays.

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Table 1 | Twenty-two key candidate genes derived from genome-wide association studies for seed oil-related traits

Trait	Genome-wide association studies				Comparative genomics				P-value[§]	Reference
	Chr	Position	LOD score or P-value	Method, year[†]	Candidate genes		Arabidopsis homologs	Functional Annotation		
Oil content	18	42441603	1.47e-05	6, 2014	*Glyma18g36130*	*GmFATA2*	*AT4G13050*	Acyl-ACP thioesterase	0.050*	Moreno *et al.* 2012
	18	58420889	3.11~5.31	1, 2014; 3, 2014 & 2015	*Glyma18g50020*	*GmACC*	*AT5G15530.1*	fatty acid biosynthetic process	0.121	Turlapati *et al*. 2011
Linolenic acid	2	1549143	1.67e-08	6, 2015	*Glyma02g01920*	*GmFUM1*	*AT2G47510.1*	fumarase 1	0.083	Zubimendi *et al.* 2018
	5	247186	2.88	2, 2014	*Glyma05g00220*	*GmCYP78A10*	*AT1G74110*	control of seed size in soybean	0.086	Wang *et al.* 2015
	13	20274945	2.14e-6	6, 2014	*Glyma.13g104800*	*GmMDH1*	*AT2G22780.1*	peroxisomal NAD-malate dehydrogenase 1	0.070	Selinski *et al.* 2019
	13	20532852	8.28e-09~1.58e-06	6, 2014 & 2015	*Glyma13g16560*	*GmDAGAT1*	*AT2G19450.1*	diacylglycerol acyltransferase 1	0.013*	Chen *et al*. 2016
	13	20704034	3.17e-06	6, 2014	*Glyma13g16790*	*GmPDAT*	*AT2G19450.1*	diacylglycerol acyltransferase 1	0.016*	Liu *et al.* 2019
	13	40977541	3.95	2, 2016	*Glyma.13g40420*	*GmDof11*	*AT2G28510*	increase the content of total fatty acids and lipids	0.180	Wang *et al.* 2007
	18	4720420	1.56e-09	6, 2014	*Glyma.18g055100*	*GmCds1*	*AT2G45150.3*	phosphatidylglycerol biosynthesis I	0.170	Zhou *et al.* 2013
	18	62146771	4.86	4, 2015	Glyma18g54020	GmPgs1	AT2G39290.1	phosphatidylglycerolphosphate synthase 1	0.022*	Tanoue *et al.* 2014
Linoleic acid	1	51429468	3.29~3.68	4 & 5, 2016	*Glyma05g33940*	*GmSDH1*	*AT5G66760.1*	succinate dehydrogenase 1	0.055	Huang *et al.* 2013
	3	36244172	3.84	5, 2015	*Glyma03g28476*	*GmP5C1*	*AT5G14800*	1-pyrroline-5-carboxylate reductas	0.002*	Giberti *et al*. 2004
Oleic acid	1	49157127	7.08e-06	6, 2014	*Glyma01g36750*	*GmACO1*	*AT4G35830.1*	aconitase 1	0.031*	Park *et al.* 2018
	2	50913342	3.82e-06	6, 2014	*Glyma02g47380*	*GmNFYA*	*AT3G20910.1*	nuclear factor Y, subunit A	0.057	Lu *et al.* 2016
	3	39102918	1.45e-08	6, 2014	*Glyma03g31281*	*GmHMT2*	*AT3G63250.1*	homocysteine methyltransferase 2	0.176	Ranocha *et al.* 2000
Stearic acid	20	36599310	4.94~5.38	1 & 3, 2014; 2 & 4, 2015	*Glyma05g08060*	*GmFATB1a*	*AT1G08510.1*	fatty acyl-ACP thioesterases B	0.041*	Xue *et al*. 2013
Palmitic acid	4	4161316	4.70	1, 2014	*Glyma04g05190*	*GmBCAT*	*AT5G28680.1*	Serine/threonine protein kinase	0.322	Diebold *et al.* 2002
	8	6430244	3.71	1, 2016	*Glyma08g08910*	*GmKASI*	*AT5G46290.1*	beta-ketoacyl-acyl carrier protein synthase I	0.234	Xiong *et al*. 2017
	8	16829990	3.59	4, 2015	*Glyma08g24420*	*GmWRI1b*	*AT3G54320.1*	regulate the synthesis of fatty acids and triacylglycerols	0.098	Chen *et al*. 2017
	8	41399047	3.39	4, 2014	*Glyma.08g302600*	*GmAGT*	*AT2G13360.1*	glycine biosynthesis III		Zhang *et al.* 2002
	10	46681643	5.25	1, 2016	*Glyma10g38970*	*GmBS1*	*AT4G14720.1*	seed size related gene	0.106	Ge *et al.* 2016
	18	3091833	3.37~3.76	2 & 4, 2015	*Glyma.18g038400*	*Glyma.18g038400*	*AT3G55470.2*	phospholipid-binding protein		

[§]: The P-values were calculated using paired *t*-test from the average RPKM values at four stages between cultivated (high seed oil, n_1=4) and wild (low seed oil, n_2=2) soybeans, and their significances were marked by * (0.05 level); [†]: the methods ISIS EM-BLASSO, mrMLM, FASTmrEMMA, pLARmEB, pKWmEB and GEMMA were indicated by 1 ~ 6, respectively.

1074 **Table 2 | Twenty key candidate genes derived from genome-wide association studies for acyl-lipid related metabolites**

Trait	Genome-wide association studies				Comparative genomics				P-value[§]	Reference
	Chr	Position	LOD or P-value	Method[†]	Candidate genes		Arabidopsis homologs	Functional Annotation		
Pyruvate	8	41488353	4.21	5	*Glyma.08g302600*	*GmAGT*	*AT2G13360.1*	glycine biosynthesis III	NA	Zhang *et al.* 2002
	13	20743520	1.44e-05	6	*Glyma13g16790*	*GmPDAT*	*AT2G19450.1*	diacylglycerol acyltransferase 1	0.016*	Liu 2020
PE (36:3)	1	49466364	5.68	4	*Glyma01g36750*	*GmACO1*	*AT4G35830.1*	aconitase 1	0.031*	Park *et al.* 2018
Oleic acid	10	46505619	3.26	1	*Glyma10g38970*	*GmBS1*	*AT4G14720.1*	seed size related gene	0.106	Ge *et al.* 2016
PI (34:3)	3	1966012	7.12e-10	6	*Glyma03g02171*	*GmLPEAT2*	*AT2G45670.1*	predicted phosphate acyltransferase,	0.00*	Jasieniecka-Gazarkiewicz *et al.* 2017
	5	2665256	4.26	1	*Glyma05g03510*	*GmPLP2*	*AT1G12640.1*	phosphatidylcholine acyl editing	0.050*	La *et al.* 2009
Phenylalanine	20	34798928	4.05	2	*Glyma20g24830*	*GmPDHC*	*AT3G25860.1*	acetyl-CoA biosynthetic process from pyruvate	0.170	Zhang *et al.* 2016; Shen *et al*. 2006
Stearic acid	14	35956260	5.42	4	*Glyma14g27990*	*GmSAD*	*AT1G43800.1*	Plant stearoyl-acyl-carrier-protein desaturase family protein	0.032*	Du *et al*. 2016
Linolenic acid	11	9480133	2.63e-07	6	*Glyma11g13050*	*GmLACS2*	*AT1G49430.1*	long-chain acyl-CoA synthetase 2	0.043*	Lü *et al.* 2010; Katavic *et al.* 2014
Daidzein	15	7627221	4.33	1	*Glyma15g10520*	*GmACP4*	*AT4G25050.1*	acyl carrier protein 4	0.090	Feng *et al*. 2018
Daidzin	19	35006105	4.71	1	*Glyma19g31730*	*GmGPDH*	*AT3G26720.1*	Glycerol-3-phosphate dehydrogenase	0.231	Shen *et al.* 2006
Malate	8	17117978	3.11	1	*Glyma.08g211700*	*GmPLDα1*	*AT3G15730.1*	phospholipase D alpha 1	0.011*	Zhao *et al*. 2013
Glycytin	13	24389546	3.41	1	*Glyma13g20930*	*GmTIM*	*AT2G21170.1*	triose phosphate isomerase	0.031*	López *et al.* 2016
Aspartic acid	18	4792076	5.65	1	*Glyma.18g055100*	*GmCds1*	*AT2G45150.3*	cytidinediphosphate diacylglycerol synthase	0.170	Zhou *et al.* 2013
Serine	7	6389701	3.55	5	*Glyma07g07580*	*GmGPAT*	*AT4G00400.1*	triacylglycerol biosynthesis	0.381	Li *et al.* 2007
Isoleucine	18	62242431	3.30	1	*Glyma18g54020*	*GmPgs1*	*AT2G39290.1*	phosphatidylglycerolphosphate synthase 1	0.022*	Tanoue *et al.* 2014
Phenylalanine	6	47437352	3.96	1	*Glyma06g44440*	*GmZF351*	*AT1G03790.1*	Zinc-Finger Protein	0.011*	Li *et al*. 2017
PE (34:1)	14	6990732	3.92	5	*Glyma14g08920*	*GmPLA2A*	*AT2G26560.1*	phospholipase A 2A	0.045*	Yang et *al* 2009
γ-aminobutyric acid	13	24115317	2.78	4	*Glyma13g20790*	*GmIDH-V*	*AT5G03290.1*	isocitrate dehydrogenase V	0.097	Lemaitre *et al*. 2006
Fumaric acid	8	43127956	4.56	5	*Glyma.08g323100*	*Glyma.08g323100*	*AT5G55380.1*	long-chain-alcohol O-fatty-acyltransferase	0.316	

1075 [§]: The P-values were calculated using paired *t*-test from the average RPKM values at four stages between landrace (high seed oil, n_1=4) and wild (low seed oil, n_2=2) soybeans, and their significances were marked by * (0.05 level); [†]: the
1076 methods ISIS EM-BLASSO, mrMLM, FASTmrEMMA, pLARmEB, pKWmEB and GEMMA were indicated by 1 ~ 6, respectively.

1077 **Table 3 | The significant association of seed oil related traits with metabolites in soybean**

Seed oil related traits	Metabolite	Partial regression coefficient	*t*-test	*F*-test	Seed oil related traits	Metabolite	Partial regression coefficient	*t*-test	*F*-test
Linolenic acid	Glycitin	0.664	0.008**	4.61e-07***	Palmitic acid	Daidzin	0.086	0.047*	2.59e-15***
	Pyruvate	-0.026	0.050*			Fumaric acid	0.220	1.09e-4***	
	Fumaric acid	-0.662	0.017*			PC (34:2)	-1.020	0.002**	
	PI (34:1)	1.367	4.19e-05***			PC (36:2)	0.739	8.95e-4***	
	PI (34:2)	-1.420	0.045*			PE (36:5)	0.383	1.24e-4***	
	Linolenic acid (m)	0.444	0.045*			PI (34:1)	0.294	0.0387*	
	Stearic acid (m)	-0.633	0.014*			PI (36:2)	-0.162	0.005**	
Oil content	Daidzin	-0.842	2.36e-06***	3.62e-10***		Asparagine	0.148	0.032*	
	Genistein	0.526	0.002**			Glutamic acid	-0.143	0.042*	
	PC (36:2)	0.679	1.09e-06***			Tryptophan	-0.142	0.004 **	
	PC(36:4)	-0.659	4.75e-06***		Linoleic acid	Daidzin	-0.911	0.003**	3.11e-05***
	PC (36:5)	-0.316	0.030*			Fumarate	0.486	0.050*	
	Asparagine	-0.172	0.034*			PC (36:5)	0.564	4.84e-05***	
	Glutamic acid	0.243	0.038*			PI (36:1)	-1.162	0.009**	
Oleic acid	Daidzin	0.073	3.11e-4***	1.13e-4***		Stearic acid (m)	-0.324	0.017*	
	Isoleucine	-0.022	0.041*						

1078 *, ** and***: significances at the 0.05, 0.01and 0.001 levels, respectively.

Table 4 | Sixty genetic sub-networks that were partly validated by previous molecular biology studies

Sub-networks constructed in this study				Evidences from previous molecular biology studies	Sub-networks constructed in this study				Evidences from previous molecular biology studies
Group	**No.**	**Sub-network**	**Known§**		**Group**	**No.**	**Sub-network**	**Known§**	
I	3	Aspartic acid—*GmCds1*—Linolenic acid—*GmDAGAT1*	New	*GmCds1*—Linolenic acid (Zhou *et al*. 2013); Linolenic acid—*GmDAGAT1* (Chen *et al*. 2016)	II	34	*Glyma.08g323100*—Fumaric acid—Linolenic acid—*GmPDAT*	New	Linolenic acid—*GmPDAT* (Liu 2020)
I	4	Aspartic acid—*GmCds1*—Linolenic acid—*GmDof11*	New	*GmCds1*—Linolenic acid (Zhou *et al*. 2013); Linolenic acid—*GmDof11* (Wang *et al*. 2007)	II	35	*Glyma.08g323100*—Fumaric acid—Linolenic acid—*GmDAGAT1*	New	Linolenic acid—*GmDAGAT1* (Chen *et al*. 2016)
I	7	Aspartic acid—*GmCds1*—Linolenic acid—*GmPgs1*	New	*GmCds1*—Linolenic acid (Zhou *et al*. 2013); Linolenic acid—*GmPgs1* (Tanoue *et al*. 2014)	II	39	*Glyma.08g323100*—Fumaric acid—Linolenic acid—*GmDof11*	New	Linolenic acid—*GmDof11* (Wang *et al*. 2007)
I	11	Isoleucine—*GmPgs1*—Linolenic acid—*GmPDAT*	New	*GmPgs1*—Linolenic acid—Gm*Pgs1* (Tanoue *et al*. 2014); Linolenic acid—*GmPDAT* (Liu 2020)	II	41	*GmLACS2*—Linolenic acid (m)—Linolenic acid—*GmPDAT*	Known	Linolenic acid—*GmPDAT* (Liu 2020)
I	12	Isoleucine—*GmPgs1*—Linolenic acid—*GmDof11*	New	*GmPgs1*—Linolenic acid (Tanoue *et al*. 2014); Linolenic acid—*GmDof11* (Wang *et al*. 2007)	II	42	*GmLACS2*—Linolenic acid (m)—Linolenic acid—*GmDAGAT1*	Known	Linolenic acid—*GmDAGAT1* (Chen *et al*. 2016)
I	18	PE (36:3)—*GmACO1*—Oleic acid—*GmNFYA*	New	Oleic acid—*GmNFYA* (Lu *et al*. 2016)	II	46	*GmLACS2*—Linolenic acid (m)—Linolenic acid—*GmDof11*	New	Linolenic acid—*GmDof11* (Wang *et al*. 2007)
I	19	PE (34:1) —*GmPDAT*—Linolenic acid—*GmDAGAT1*	Known	*GmPDAT*—Linolenic acid (Liu 2020); Linolenic acid—*GmDAGAT1* (hen *et al*. 2016)	II	48	*GmSAD*—Stearic acid (m)—Linolenic acid—*GmPDAT*	Known	Linolenic acid—*GmPDAT* (Liu 2020)
I	20	PE (34:1) —*GmPDAT*—Linolenic acid—*GmPDAT*	Known	*GmPDAT*—Linolenic acid (Liu 2020); Linolenic acid—*GmPDAT* (Liu 2020)	II	49	*GmSAD*—Stearic acid (m)—Linolenic acid—*GmDAGAT1*	Known	Linolenic acid—*GmDAGAT1* (Chen *et al*. 2016)
I	22	Pyruvate—*GmAGT*—Palmitic acid—*GmBS1*	New	Palmitic acid—*GmBS1* (Ge *et al*. 2016)	II	53	*GmSAD*—Stearic acid (m)—Linolenic acid—*GmDof11*	New	Linolenic acid—*GmDof11* (Wang *et al*. 2007)
I	24	Pyruvate—*GmPDAT*—Linolenic acid—*GmCds1*	Known	*GmPDAT*—Linolenic acid (Liu 2020); Linolenic acid—*GmCds1* (Zhou *et al*. 2013)	II	56	*GmGPDH*—Daidzin—Oil content—*GmFATA2*	New	Oil content—*GmFATA2* (Moreno *et al*. 2012)
I	26	Pyruvate—*GmPDAT*—Linolenic acid—*GmDAGAT1*	Known	*GmPDAT*—Linolenic acid (Liu 2020); Linolenic acid—*GmDAGAT1* (Chen *et al*. 2016)	II	58	*GmCds1*—Asparagine—Oil content—*GmFATA2*	New	Oil content—*GmFATA2* (Moreno *et al*. 2012)
I	27	Pyruvate—*GmPDAT*—Linolenic acid—*GmDof11*	New	*GmPDAT*—Linolenic acid (Liu 2020); Linolenic acid—*GmDof11* (Wang *et al*. 2007)	II	61	*GmGPDH*—Daidzin—Palmitic acid—*GmBS1*	New	Palmitic acid—*GmBS1* (Ge *et al*. 2016)
I	30	Pyruvate—*GmPDAT*—Linolenic acid—*GmPgs1*	New	*GmPDAT*—Linolenic acid (Liu 2020); Linolenic acid—*GmPgs1* (Tanoue *et al*. 2014)	II	62	*GmGPDH*—Daidzin—Palmitic acid—*GmWRI1b*	New	Palmitic acid—*GmWRI1b* (Chen *et al*. 2017)
I	31	Pyruvate—*GmAGT*—Palmitic acid—*GmWRI1b*	New	*GmPDAT*—Linolenic acid (Liu 2020); Palmitic acid—*GmWRI1b* (Chen *et al*. 2017)	II	67	*Glyma.08g323100*—Fumaric acid—Palmitic acid—*GmBS1*	New	Palmitic acid—*GmBS1* (Ge *et al*. 2016)
II	1	*GmGPDH*—Daidzin—Linoleic acid—*GmPgs1*	New	Linoleic acid—*GmPgs1* (Tanoue *et al*. 2014)	II	68	*Glyma.08g323100*—Fumaric acid—Palmitic acid—*GmWRI1b*	New	Palmitic acid—*GmWRI1b* (Chen *et al*. 2017)

II	4	*GmGPDH*—Daidzin—Linoleic acid—*GmPDAT*	New	Linoleic acid—*GmPDAT* (Liu 2020)	II	73	*GmCds1*—Asparagine—Palmitic acid—*GmBS1*	New	Palmitic acid—*GmBS1* (Ge *et al.* 2016)
II	5	*Glyma.08g323100*—Fumarate—Linoleic acid—*GmPgs1*	New	Linoleic acid—*GmPgs1* (Tanoue *et al.* 2014)	II	74	*GmCds1*—Asparagine—Palmitic acid—*GmWRI1b*	New	Palmitic acid—*GmWRI1b* (Chen *et al.* 2017)
II	8	*Glyma.08g323100*—Fumarate—Linoleic acid—*GmPDAT*	New	Linoleic acid—*GmPDAT* (Liu 2020)	II	79	*GmGPDH*—Daidzin—Oleic acid—*GmNFYA*	New	Oleic acid—*GmNFYA* (Lu *et al.* 2016)
II	9	*GmSAD*—Stearic acid (m)—Linoleic acid—*GmPgs1*	Known	Linoleic acid—*GmPgs1* (Tanoue *et al.* 2014)	II	80	*GmACP4*—Pyruvate—Linolenic acid—*GmDAGAT1*	New	Linolenic acid—*GmDAGAT1* (Chen *et al.* 2016)
II	12	*GmSAD*—Stearic acid (m)—Linoleic acid—*GmPDAT*	Known	Linoleic acid—*GmPDAT* (Liu 2020)	II	82	*GmACP4*—Pyruvate-Linolenic acid—*GmPDAT*	New	Linolenic acid—*GmPDAT* (Liu 2020)
II	13	*GmTIM*—Glycitin—Linolenic acid—*GmPDAT*	New	Linolenic acid—*GmPDAT* (Liu 2020)	II	83	*GmACP4*—Pyruvate—Linoleic acid—*GmPgs1*	New	Linoleic acid—*GmPgs1* (Tanoue *et al.* 2014)
II	14	*GmTIM*—Glycitin—Linolenic acid—*GmDAGAT1*	New	Linolenic acid—*GmDAGAT1* (Chen *et al.* 2016)	II	81	*GmACP4*—Pyruvate—Linolenic acid—*GmLACS2*	New	Linolenic acid—*GmLACS2* (Katavic *et al.* 2014)
II	18	*GmTIM*—Glycitin—Linolenic acid—*GmDof11*	New	Linolenic acid—*GmDof11* (Wang *et al.* 2007)	III	1	Stearic acid (m)—*GmSAD*—*GmFATA2*—Oil content	Known	*GmFATA2*—Oil content (Moreno *et al.* 2012)
II	20	*GmPDAT*—Pyruvate—Linolenic acid—*GmPDAT*	Known	Linolenic acid—*GmPDAT* (Liu 2020)	III	2	Stearic acid (m)—*GmSAD*—*GmFATB1a*—Palmitic acid	Known	*GmFATB1a*—Palmitic acid (Chen *et al.* 2017)
II	21	*GmPDAT*—Pyruvate—Linolenic acid—*GmDAGAT1*	Known	Linolenic acid—*GmDAGAT1* (Chen *et al.* 2016)	III	8	Pyruvate—*GmPDAT*—*GmWRI1b*—Palmitic acid	New	*GmWRI1b*—Palmitic acid (Chen *et al.* 2017)
II	22	*GmPDAT*—Pyruvate—Linolenic acid—*GmCds1*	Known	Linolenic acid—*GmCds1* (Zhou *et al.* 2013)	III	9	Pyruvate—*GmPDAT*—*GmDAGAT1*—Linolenic acid	Known	*GmDAGAT1*—Linolenic acid (Chen *et al.* 2016)
II	25	*GmPDAT*—Pyruvate—Linolenic acid—*GmDof11*	New	Linolenic acid—*GmDof11* (Wang *et al.* 2007)	III	10	Phenylalanine—*GmZF351*—*GmPDAT*—Linolenic acid	New	*GmPDAT*—Linolenic acid (Liu 2020)
II	27	*GmAGT*—Pyruvate—Linolenic acid—*GmPDAT*	New	Linolenic acid—*GmPDAT* (Liu 2020)	III	12	Pyruvate—*GmPDAT*—*GmFATA2*—Oil content	Known	*GmFATA2*—Oil content (Moreno *et al.* 2012)
II	28	*GmAGT*—Pyruvate—Linolenic acid—*GmDAGAT1*	New	Linolenic acid—*GmDAGAT1* (Chen *et al.* 2016)	III	13	Pyruvate—*GmCds1*—*GmPDAT*—Linolenic acid	New	*GmPDAT*—Linolenic acid (Liu 2020)
II	32	*GmAGT*—Pyruvate—Linolenic acid—*GmDof11*	New	Linolenic acid—*GmDof11* (Wang *et al.* 2007)	III	15	PI (34:3) —*GmPLP2*—*GmPDAT*—Linolenic acid	Known	*GmPDAT*—Linolenic acid (Liu 2020)

1080 §: “known” sub-networks could be found in the KEGG PATHWAY website (https://www.kegg.jp/kegg/pathway.html), and “New” ones were constructed in this study.

Table 5 | The significances for the differences of traits (t), metabolites (m) and gene expressional levels in six subnetworks between high-oil and low-oil soybean accessions

Subnetwork	Node 1			Node 2			Node 3			Node 4			Reference
	High	**Low**	**P**-value	**High**	**Low**	**P**-value	**High**	**Low**	**P**-value	**High**	**Low**	**P**-value	
1	Pyruvate (m)			*GmAGT*ǂ			Palmitic acid (t)			*GmBS1*			Zhang *et al.* 2002; Ge *et al.* 2016
	1339.57±891.57§	437.61±62.53	0.043*	2.19±0.81	0.83±0.40	0.104	10.69±0.69	11.43±0.54	0.049*	19.54±1.71	10.71±1.72	0.018*	
2	Pyruvate (m)			*GmPDAT*			Linolenic acid (t)			*GmDAGAT1*			Liu *et al.* 2020; Chen *et al.* 2016
	1339.57±891.57	437.61±62.53	0.043*	5.68±0.63	1.52±0.54	0.005**	7.51±0.06	12.34±0.58	0.000**	11.54±2.09	1.16±0.47	0.007**	
3	Isoleucine (m)			*GmPgs1*			Linolenic acid (t)			*GmPDAT*			Tanoue *et al.* 2014; Liu 2020
	83.86±43.86	31.61±18.38	0.027*	7.5±1.51	3.33±0.08	0.035*	7.51±0.06	12.34±0.58	0.000**	5.68±0.63	1.52±0.54	0.005**	
4	Pyruvate (m)			*GmAGT*ǂ			Palmitic acid (t)			*GmWRI1b*			Zhang *et al.* 2002; Chen *et al.* 2017
	1339.57±891.57	437.61±62.53	0.043*	2.19±0.81	0.83±0.4	0.104	10.69±0.69	11.43±0.54	0.049*	16.67±2.76	9.23±1.15	0.036*	
5	Pyruvate (m)			*GmACP4*♯			Linolenic acid (t)			*GmDAGAT1*			Feng *et al.* 2018; Chen *et al.* 2016
	1339.57±891.57	437.61±62.53	0.043*	3.17±1.08	0.92±0.92	0.099	7.51±0.06	12.34±0.58	0.000**	11.54±2.09	1.16±0.47	0.007**	
6	Phenylalanine (m)			*GmZF351*			Linolenic acid (t)			*GmPDAT*			Li *et al.* 2017; Liu *et al.* 2020
	116.61±43.74	75.16±14.15	0.050*	64.71±16.19	14.64±7.29	0.025*	7.51±0.06	12.34±0.58	0.000**	5.68±0.63	1.52±0.54	0.005**	

* and **: significances at the 0.05 and 0.01 levels, respectively. §: average ± standard deviation. The trait phenotype for each accession was the average across three years (2014 to 2016). The *t* values for the traits (t) and metabolites (m) were calculated between five high-oil and five low-oil accessions, while the *t* values for gene expressional levels were calculated between four high-oil and two low-oil accessions. ǂ: *GmAGT* was found to have significant difference in expression (P=0.004) between four high-oil accessions and one low-oil accession (no. 265) at 15, 25 and 35 DAF, respectively; ♯: *GmACP4* was found to have significant difference in expression (P=0.033) between four high-oil accessions and one low-oil accession (no. 272) at 15, 25 and 35 DAF, respectively.

Figure Legends

Figure 1. Frequent distributions for seed oil content (f) and its constituents (a-e) in 286 soybean accessions. The results in 2014, 2015 and 2016 were indicated by green, yellow and navy-blue bars, respectively. Data are shown as the means ± standard deviation. *, ** and ***: the 0.05, 0.01 and 0.001 probability levels of significance, respectively, in the paired *t*-test (*n*=286).

Figure 2. The primary metabolic networks in soybean (a) and the expression profiling of 19 key seed oil-related genes identified in this study (b). These genes with red, pink and blue colors are in the pathways of oil biosynthesis, amino acid biosynthesis and TCA cycle, respectively. The metabolites and genes with grey color aren't identified in this study. *ABC1*, activity of bc1 complex homolog 1; *ACC*, acetyl coenzyme-A carboxylase; *ACO1*, acyl-CoA oxidase 1; *ACP4*, acyl carrier protein (ACP)-4; *AGD*, diaminopimelate aminotransferase; *BCAT*, branched-chain amino acid transaminase; *AGT*, alanine glyoxylate aminotransferase; *Agpat3*, acylglycerophosphate acyltransferase; *CDS1*, CDP-diacylglycerol synthase 1; *CM*, chorismate mutase; *DAGAT1*, diacylglycerol acyltransferase enzymes 1; *FATA*, fatty acid thioesterase A; *FATB*, fatty acid thioesterase B; *LACS*, long chain fatty acyl CoA synthetase; *FUM1*, fumonisin synthase gene 1; *GPAT*, glycerol-3-phosphate acyltransferase; *GPDH*, glycerol phosphate dehydrogenase; *HMT2*, homocysteine-S-methyltransferase 2; *IDH-V* isocitrate dehydrogenase V; *KASI*, β-Ketoacyl-ACP synthase I; *LPEAT2*, lyso-PE acyltransferase 2; *MDH*, malate dehydrogenase; *MTO*, mitochondrial tRNA modification gene; *P5C1*, pyrroline-carboxylic acid synthase 1; *PDAT1*, phospholipid diacylglycerol acyltransferase 1; *PDHC*, pyruvate dehydrogenase complex; *PDK1*, pyruvate dehydrogenase kinase 1; *Pgs1*, phosphatidylglycerolphosphate synthase; *PLA2A*, phospholipase A2; *PK*, pyruvate kinase *PLDα1*, phospholipase D gene 1; *PLP2*, proteolipid protein 2; *SAD*, sinapyl alcohol dehydrogenase; *SDH1*, succinate dehydrogenase1; *TIM*, translocases inner mitochondrial membrane. DAF: days after flowering. Domesticated soybeans include four high seed oil content accessions; wild soybeans include two low oil soybean accessions.

Figure 3. The significant associations of soybean seed oil-related traits with metabolites (a) and

three-dimension genetic networks among seed oil-related traits, metabolites and candidate genes (b and c). The red and green lines represent significantly positive and negative correlations between seed oil-related trait and metabolite, respectively. In three-dimension genetic networks, the nodes for oil-related traits and genes are indicated by red and yellow colors, respectively, and the other nodes are indicated by blue (PC, PE, and PI), green (amino acids), pink (isoflavone) and grey (organic acids) colors; the edges are indicated by the relationship among seed oil-related traits, metabolites and candidate genes; bold red and black lines represent known and newly identified sub-networks, respectively. I: the first group of sub-networks, in which the candidates are significantly associated commonly with oil-related traits and metabolites; II: the second group of sub-networks, in which oil-related traits are significantly related to metabolites; III: the third group of sub-networks, in which one interacted gene is related to oil-related traits, and another interacted one is related to metabolites.

Figure 4. Luciferase complementation image assay of the interaction of *GmPDAT* with *GmFATA2* in *Agrobacterium*-infiltrated *N. benthamiana* leaves under dark illumination. I and II represent bright and dark fields, and their treatments are the same. The image shows the interaction between *GmPDAT* and *GmFATA2* in *N. benthamiana* leaves, with the LUC images of *N. benthamiana* leaves co-infiltrated with the *Agrobacterium* strains containing N-*GmPDAT* and C-*GmFATA2* (experimental group, top left corner), N-LUC and C-*GmFATA2* (control, top right corner), N-*GmPDAT* and C-LUC (control, bottom left corner), and N-LUC and C-LUC (control, bottom right corner). LUC fluorescence was detected from 48 to 60 h after infiltration by confocal microscopy. The experiment was repeated three times with similar results.

Figure 5. The genetic relationships between pyruvate and three major nutrients, between amino acids and seed oil content, and between malate and seed oil content are dissected by *GmPDAT*, *GmAGT* and *GmACP4* (red), *GmPLDα* and *GmCds1* (pink), and *GmPDAT*, *GmZF351* and *GmPgs1* (blue), respectively, in the three-dimension genetic networks. The genes are in italic.