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Association Mapping and Marker Development of Genes for Starch Lysophospholipid Synthesis in Rice

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Abstract: Phospholipids are a major kind of lipids in rice grains and have fundamental nutritional and functional benefits to the plant. Their lyso forms (lysophospholipids, LPLs) often form inclusion complexes with amylose or independently influence the physicochemical and functional properties of rice starch. However, the genetic basis for LPL synthesis in rice endosperm is largely unknown. Here, we performed a preliminary association test of 13 LPL compositions among 20 rice accessions, and identified 22 putative main-effect quantitative trait loci responsible for all LPLs except for LPC14:0 and LPE14:0. Five derived cleaved amplified polymorphic sequences and one insertion/deletion marker for three LPL-synthesis-related candidate genes were developed. Association analysis revealed two markers significantly associated with starch LPL traits. These results provide an insight into the genetic basis of phospholipid biosynthesis in rice and may contribute to the rice quality breeding programs using functional markers.

Key words: rice; starch lysophospholipid; phospholipid biosynthesis; grain quality; QTL; molecular marker; association mapping

Rice (*Oryza sativa* L.) has long been cultivated and consumed worldwide as a vital nutritional source. It satisfies over 21% of the daily calorie needs of the world's population (Zhan et al, 2014). According to the reported world rice statistics from the International Rice Research Institute (http://www.irri.org), the global paddy rice and milled rice productions were 700 and 565 million tons in 2015, respectively. Besides the sustained challenge of rice yield, with the increasing of living standards, rice quality improvement has also been a new mission for meeting the consumer's demands (Ren et al, 2015).

To date, functional lipids in rice grains have greatly attracted considerable attentions owing to their nutritional and healthy benefits. As a major class of complex lipids in cereal grains, phospholipids (PLs) serve as one of the necessary bioactive components of cell membranes by forming lipid bilayers. Increasingly emerging evidences have shown that PLs play a pivotal biological role in clathrin-mediated endocytosis, phagocytosis and macropinocytosis (Bohdanowicz and Grinstein, 2013). According to different involved hydrophilic heads, such as choline, ethanolamine, inositol and serine, the PLs in organisms are mainly comprised of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidylserine and the corresponding lyso forms of different PLs (Choi et al, 2005; Liu et al, 2013). In contrast to other classes of lipids, PLs in rice grains only account for a relatively minor proportion, however, they make a great contribution to the physicochemical and nutritional properties of grains via combining with

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starch to form complexes in endosperm (Maniñgat and Juliano, 1980; Putseys et al, 2010; Tong et al, 2015). For example, Tong et al (2015) found that lysophospholipids (LPLs) have significant correlations with pasting properties, the important traits of eating quality of rice, such as cool paste viscosity, breakdown and consistency. Although a number of experiments investigating the components, fractions, contents, structural and distribution features of rice PLs have been reported (Choi et al, 2005; Yoshida et al, 2011), the broadly diverse results are possibly owing to the different genotypes and environmental effects (Choi et al, 2005; Liu et al, 2014; Tong et al, 2014).

The results obtained when PLs begin to accumulate in plants are controversial. For example, Shewry et al (1973) found that PL synthesis occurred as early as seed germination imbibition, whereas others indicated it took place during the stage of rice seed ripening and was unchanged during storage (Nakamura et al, 1958; Perry and Harwood, 1993). However, the importance to uncover the biosynthesis of PL compositions in plants cannot be overstressed. Hence, a lot of efforts were made to illuminate the underlying genetic network. Several PL-synthesis-related enzymes and biosynthetic genes have been reported. For instance, *acetyl-CoA carboxylase* (*Acc1*) influences the length distribution of acyl-chain by regulating the relative proportion of C16 versus C18 fatty acids during lipid synthesis (Hofbauer et al, 2014). *INO1*, a structure gene encoding inositol-3-phosphate synthase of PL biosynthesis, has been found in yeast (Gaspar et al, 2011). Three major genes *cdsA*, *pgsA* and *pgpP* participate in the PL biosynthetic steps of cytidine triphosphate to cytidine diphosphate-diacylglycerol, cytidine diphosphate-diacylglycerol to phosphatedylclycerolphosphate, and phosphatidylclycerolphosphate to phosphatidyldlycerol, respectively (Martin et al, 1999; Kuhn et al, 2015). Two Mg^{2+}-dependent phosphatidic acid phosphohydrolases (PAH1 and PAH2) have been characterized in *Arabidopsis*, and they catalyze the first committed step of choline synthesis and define a special PC biosynthetic pathway at endoplasmic reticulum (Eastmond et al, 2010; Farquharson, 2010). Fatty acid desaturase 2 is negatively correlated with oleic acid composition while positively correlated with linoleic acid composition in maize (Li et al, 2013). The seed gene *phospholipase D* (*PLD*) controlling the degradation of PL membranes of oil bodies has also been mapped in rice (Suzuki, 2011a, b).

At present, since the enzymes participating in starch PL biosynthesis in rice have just been scarcely identified, the specific biosynthetic pathway of starch PLs in rice is still unclear. A possible and modified biosynthetic network of PLs in rice is outlined based on the studies on animals and yeasts (Fig. 1) (Kinney, 1993; Liu et al, 2013). Cytidinediphosphate-diacyglycerol and 1,2-diacylglycerol are both originated from phosphatidic acid after the acylation by cytidine-diphosphate-diacylglycerol synthase and phosphatidate-phosphohydrolase. Their main synthetic pathways have been reported (Fig. 1) (Liu et al, 2013). Especially, PE maybe produced from the reactions of three enzymes, phosphatidylserine decarboxylase, aminoalcoholphosphotransferase (AAPT) and ethanolaminephosphotransferase, while PC is probably produced by displacement through phospholipid N-methyltransferase, AAPT and cholinephosphotransferase. Interestingly, AAPT is a common enzyme mediating the biosyntheses of PC and PE. LPLs, such as lysophosphatidic acid, are possibly produced from the hydrolysis of diacylphospholipids by phospholipase A_2 (Fig. 1).

Quantitative trait loci (QTLs) responsible for lipid synthesis in rice have been reported (Liu et al, 2009; Qin et al, 2010; Shen et al, 2012; Ying et al, 2012). Ying et al (2012) identified 29 QTLs associated with fatty acid composition and oil concentration, and some of them are strongly associated with the rice ortholog genes *acyl-CoA:diacylglycerol acyltransferase* (*DGAT*) and *acyl-ACP thioesterase* (*FatB*). Shen et al (2012) identified three fat-content-related QTLs that are stably expressed in different environments and populations. Similarly, 7 and 14 QTLs for rice lipids were identified via different doubled haploid populations under diverse environments, respectively (Liu et al, 2009; Qin et al, 2010). These QTLs may contribute to improving rice nutritional quality via marker-assisted breeding. However, QTL analysis of rice starch lipids has not been reported.

The objectives of this study were to identify the QTLs or genetic loci for rice starch LPL content and composition across different environments, identify LPL-synthesis-related candidate genes and develop molecular markers for these candidate genes, and confirm whether these molecular markers are associated with LPL traits.

MATERIALS AND METHODS

Rice materials

Two sets of rice accessions were used. Set 1 planted at

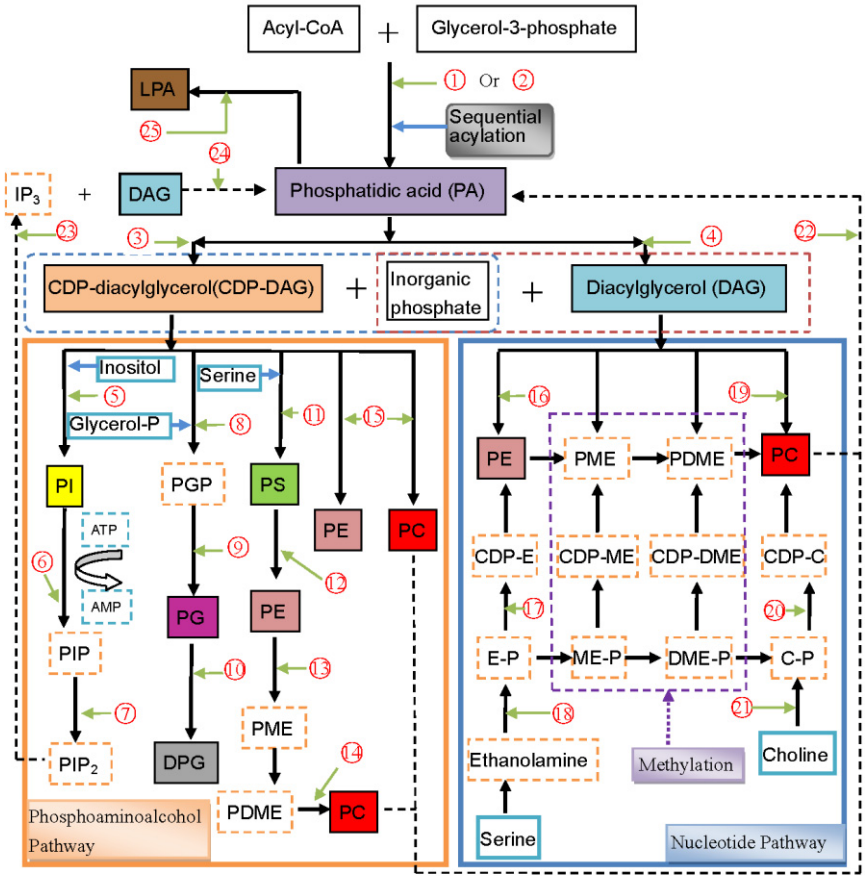

Fig. 1. Conceivable biosynthetic pathways of phospholipids in cereals.

Drawn based on Kinney (1993), D'Arrigo and Servi (2010) and Liu et al (2013).

E, Ethanolamine; ME, Methylethanolamine; DME, Dimethylethanolamine; IP_3, inositol 1,4,5-triphosphate; LPA, Lysophosphatidic acid; C, Choline; P, Phosphate; CDP, Cytidinediphosphate; PE, Phosphatidylethanolamine; PME, Phosphatidylmethylethanolamine; PDME, Phosphatidyldimethylethanolamine; PC, Phosphatidylcholine; PS, Phosphatidylserine; PI, Phosphatidylinositol; PIP, Phosphatidylinositol phosphate; PIP_2, Phosphatidylinositolbisphosphate; PGP, Phosphoglycerol phosphate; PG, Phosphatidylglycerol; DPG, Diphosphatidylglycerol; ①, Glycerol-3-phosphate acyltransferase; ②, 1-monoacylglycerol-3-phosphate acyltransferase; ③, CDP-diacylglycerol synthase (CTP: phosphatidatecytidylyltransferase); ④, Phosphatidatephosphohydrolase; ⑤, PI synthase (CDP-diacylglycerol: myo-inositol phosphatidyltransferase); ⑥, PI 4-kinase (ATP: phosphatidylinositol-4-phosphotransferase); ⑦, PIP kinase (ATP: phosphatidylinositol 4-phosphate 5-phosphotransferase); ⑧, PGP synthase (CDP-diacylglycerolphosphatidyltransferase); ⑨, PGP phosphatase (phosphatidylglycerol-phosphate phosphohydrolase); ⑩, CDP diacylglycerolphosphatidyltranserase; ⑪, PS synthase (CDP-diacylglycerol: L-serine O-phosphatidyltransferase); ⑫, PS decarboxylase; ⑬, PE N-methyltransferase; ⑭, Phospholipid N-methyltransferase; ⑮, Aminoalcoholphosphotransferase; ⑯, Ethanolaminephosphotransferase (CDP-ethanolamine: 1,2-diacylglycerol cytidylyltransferase); ⑰, Ethanolamine phosphate cytidylyltransferase (CTP: ethanolamine phosphate cytidylyltransferase); ⑱, Ethanolamine kinase (ATP: ethanolamine phosphotransferase); ⑲, Cholinephosphotransferase (CDP-choline: 1,2-diacylglycerol cytidylyltransferase); ⑳, Choline phosphate cytidylyltransferase (CTP: choline phosphate cytidylyltransferase); ㉑, Choline kinase (ATP: choline phosphotransferase); ㉒, Phospholipase D (PLD) (Phosphatidylcholinephosphatidohydrolase); ㉓, Phospholipase C (PLC); ㉔, Diacylglycerol (DAG) kinase; ㉕, Phospholipase A_2 (PLA_2).

Lingshui, Hainan Province, China, in 2010 and 2011 included 20 non-waxy rice accessions (G01–G20), of which 8 belong to japonica subspecies, 8 indica subspecies and 4 the aus group (Xu et al, 2014). Set 2 planted at Hangzhou, Zhejiang Province, China, in 2012 had 13 local accessions (R01–R13), 2 waxy accessions and 11 non-waxy accessions, including 3 japonica subspecies and 8 indica subspecies (Liu et al, 2014).

Rice starch LPLs

The endosperm LPL contents of the above two sets of rice have been previously reported (Liu et al, 2014; Tong et al, 2014, 2015).

Genotypes and association mapping

For the 20 non-waxy rice accessions (G01–G20), the public genotype data were available on the website of Gramene (http://www.gramene.org/). A total of 32 655 common single nucleotide polymorphism (SNP) sites were downloaded (Xu et al, 2014). Association mapping was performed using the genome association and prediction integrated tool (Lipka et al, 2012).

Analyses of population structure (Q) and optimum number of populations were conducted using the STRUCTURE software (Xu et al, 2014). When comparing the values of Bayesian information criterion (BIC), the optimal model based on kinship (K), population structure and principal components (P) for individual LPL trait was selected (Xu et al, 2014). The BIC results with the best model were presented at the significance level of $P < 0.001$.

DNA extraction

Genomic DNA was extracted from the fresh leaf tissues of plants grown in the greenhouse using a modified cetyltrimethyl ammonium bromide procedure according to Doyle (1991). DNA concentrations were estimated using a NanoDrop 2000 spectrophotometer (Thermo, San Jose, CA).

Sequencing, development of derived cleaved amplified polymorphic sequences (dCAPS) and insertion/deletion (InDel) markers, and genotyping

According to the physical positions of QTLs, putative genes responsible for LPL biosynthesis were identified. For these candidate genes, part of their sequences in the 20 rice accessions of Set 1 were amplified by appropriate PCR primer sets (Supplemental Table 1), which were synthesized by Shanghai Sangon Company (Shanghai, China).

For gel-based analysis, PCR was performed in 50 μL system with 50 ng genomic DNA, 25 μL of 2× Master Mix (including 2× PCR buffer, 4 mmol/L $MgCl_2$, 0.4 mmol/L dNTPs, 50 U/mL high-fidelity *Taq* DNA polymerase) and 5 μL of each 10 μmol/L primer. PCR was run under the following conditions: pre-denature at 94 °C for 5 min; followed by 34 cycles of denature at 94 °C for 45 s, anneal at 55 °C for 1 min and extension at 72 °C for 45 s; and final extension at 72 °C for 8 min. Later, 2 μL PCR products were detected by 2% denaturing agarose gels, and the remaining PCR products were sent to Shanghai Sangon Company (Shanghai, China) for sequencing.

For detection of SNPs in the polymorphic sites of the sequenced candidate genes, the derived cleaved amplified polymorphic sequence markers were developed based on the web-based free software dCAPS Finder 2.0 (Konieczny and Ausubel, 1993; Neff et al, 1998). The InDel marker was developed using software Primer Premier 5.0. These markers were used to genotype both the two sets of rice materials. Amplified PCR products (3 μL) were digested with 1 U restriction endonuclease. Then, 8% denaturing polyacrylamide gel was used to separate the digested products, and gel images were acquired using the VersaDoc Imaging System Model 3000 (Bio-Rad Laboratories, USA).

Statistical analysis

Analysis of variance with the general linear model procedure was used to detect the associations between different endosperm LPL traits and marker alleles, which was conducted using the software TASSEL (version 2.1). The *P*-value of significance was set at $P < 0.05$.

RESULTS

Identification of QTLs for starch LPLs

A preliminary association test was conducted based on the optimal model, and 22 main-effect QTLs were identified for all the individual LPLs excluding LPC14:0 and LPE14:0 which distributed on all chromosomes except for chromosomes 3 and 7 (Table 1 and Fig. 2). The Manhattan plots for individual LPL contents showing significant peaks represented the identified main-effect QTLs on different chromosomes (Fig. 3). Three QTLs responsible for LPC16:0 were identified. *qC160-6-1* was detected on chromosome 6 in 2011, while *qC160-6-2* and *qC160-8* were identified on chromosomes 6 and 8 in 2012, respectively. For LPC18:1, two significant QTLs were discovered on chromosomes 1 and 2, respectively, in these two years. Two loci for LPC18:2, *qC182-9* and *qC182-11*, were identified. The QTL *qC183-1* for LPC18:3 was consistently detected on chromosome 1 in both 2011 and 2012. Four QTLs for total LPC were identified, including *qC-5*, *qC-9* and *qC-12* in 2011 and *qC-10* in 2012. Only one QTL for LPE16:0, *qE160-1*, was detected on chromosome 1 in 2011. Similarly, *qE181-2* for LPE18:1 and *qE183-1* for LPE18:3 were consistently discovered on chromosomes 2 and 1, respectively, in two years. Three QTLs for LPE18:2 were detected only in 2011. Only one QTL for total LPE, *qE-10*, was discovered on chromosome 10 in 2011. A total of three QTLs were detected for total LPL. Among them, *qL-10* was detected in two years.

Candidate gene identification and sequencing, marker development, and genotyping

Based on the physical positions of the associated QTLs,

Table 1. Genome-wide significantly associated QTLs for rice lysophospholipids (LPLs).

Trait	Year	Model	QTL	Chr	Position [a] (bp)	*P*-value	Major allele	Minor allele	Minor allele frequency	Allelic effect (R^2)
LPC16:0	2011	K	*qC160-6-1*	6	26 685 967	4.72×10^{-3}	C	T	0.30	0.8011
	2012	K	*qC160-6-2*	6	3 235 560	4.41×10^{-3}	G	A	0.25	0.5573
			qC160-8	8	8 481 084	3.64×10^{-3}	A	G	0.40	0.5872
LPC18:1	2012	Q+K	*qC181-2*	2	14 522 544	3.15×10^{-3}	G	A	0.20	0.8346
			qC181-1	1	27 729 601	4.20×10^{-3}	C	T	0.30	0.8107
LPC18:2	2011	K	*qC182-9*	9	15 125 638	4.37×10^{-3}	C	T	0.20	0.5585
	2012	K	*qC182-11*	11	17 847 796	4.07×10^{-3}	A	T	0.30	0.5698
LPC18:3	2011	K	*qC183-1*	1	23 517 183	3.27×10^{-3}	A	C	0.25	0.6047
	2012	K	*qC183-1*	1	23 517 183	4.89×10^{-3}	A	C	0.25	0.5411
TLPC	2011	ANOVA	*qC-5*	5	23 465 186	4.20×10^{-3}	G	A	0.25	0.5647
			qC-9	9	15 125 638	2.62×10^{-3}	C	T	0.20	0.6409
			qC-12	12	21 175 392	3.73×10^{-3}	T	A	0.25	0.5835
	2012	K	*qC-10*	10	5 339 258	4.36×10^{-3}	G	A	0.25	0.5589
LPE16:0	2011	ANOVA	*qE160-1*	1	6 971 525	3.33×10^{-3}	G	A	0.25	0.6016
LPE18:1	2011	Q+K	*qE181-2*	2	14 522 544	3.26×10^{-3}	G	A	0.20	0.7802
	2012	Q+K	*qE181-2*	2	14 522 544	2.17×10^{-3}	G	A	0.20	0.8364
LPE18:2	2011	K	*qE182-1*	1	23 585 360	3.23×10^{-3}	C	T	0.25	0.6064
			qE182-4	4	31 271 474	4.23×10^{-3}	T	C	0.20	0.5637
			qE182-11	11	17 943 118	3.82×10^{-3}	T	A	0.35	0.5798
LPE18:3	2011	K	*qE183-1*	1	23 517 183	4.27×10^{-3}	A	C	0.25	0.5623
	2012	K	*qE183-1*	1	23 517 183	4.33×10^{-3}	A	C	0.25	0.5600
TLPE	2011	ANOVA	*qE-10*	10	5 339 258	4.69×10^{-3}	G	A	0.25	0.5477
TLPL	2011	ANOVA	*qL-9*	9	15 125 638	2.92×10^{-3}	C	T	0.20	0.6229
			qL-10	10	5 339 258	4.67×10^{-3}	G	A	0.25	0.5482
			qL-12	12	21 175 392	4.93×10^{-3}	T	A	0.25	0.5399
	2012	K	*qL-10*	10	5 339 258	4.19×10^{-3}	G	A	0.25	0.5649

LPC14:0, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LPC16:0, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LPC18:1, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LPC18:2, 1-linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LPC18:3, 1-linolenoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LPE14:0, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine; LPE16:0, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine; LPE18:1, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine; LPE18:2, 1-linoleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine; LPE18:3, 1-linolenoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine; TLPC, Total lysophosphatidylcholine; TLPE, Total lysophosphatidylethanolamine; TLPL, Total lysophospholipid; Q, Population structure; K, Kinship; ANOVA, Analysis of variance; Chr, Chromosome.

[a], Position in base pairs for the leading SNP of rice sequence.

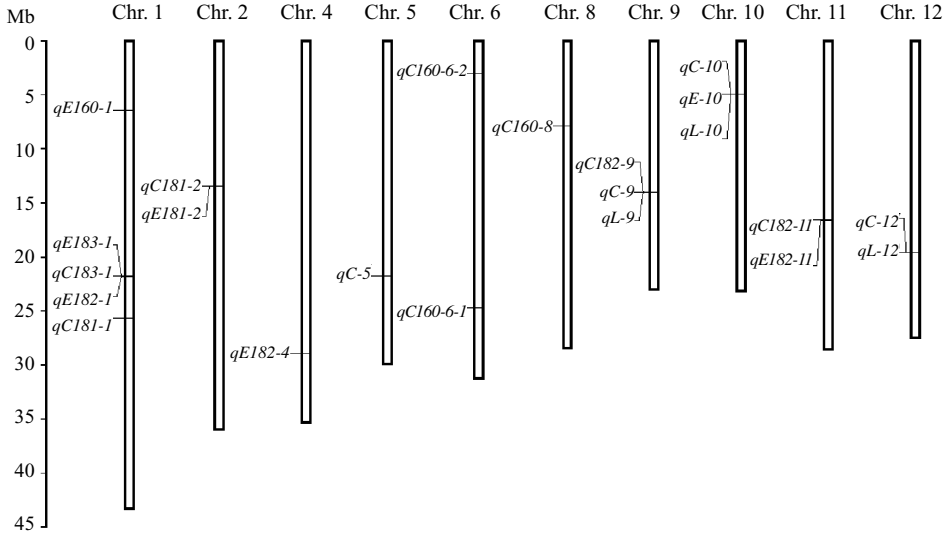

Fig. 2. Location of QTLs for rice starch lysophospholipids (LPLs) on chromosomes (Chr).

C160, C181, C182, C183, E160, E181, E182, E183, C, E and L in the QTLs represent LPC16:0, LPC18:1, LPC18:2, LPC18:3, LPE16:0, LPE18:1, LPE18:2, LPE18:3, total lysophosphatidylcholine, total lysophosphatidylethanolamine and total lysophospholipid, respectively.

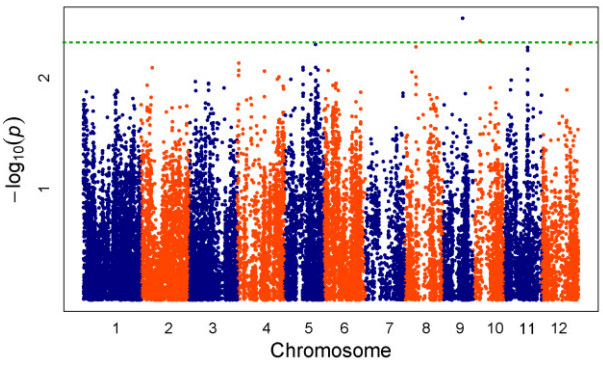

Fig. 3. Manhattan plots of association test for total lysophospholipid.

three QTLs were found to be closed to the loci *Os06g0204400* (5 276 639–5 286 101 bp on chromosome 6) (*qC160-6-2*), *Os06g0649900* (26 575 128–26 579 581 bp on chromosome 6) (*qC160-6-1*) and *Os11g0546600* (20 174 656–20 176 341 on chromosome 11) (*qC182-11* and *qE182-11*), respectively, which encode AAPT, PLD and phospholipase A_2 (PLA_2), respectively. Therefore, these three candidate genes involved in PL metabolism were re-sequenced to investigate the associations between functional nucleotide polymorphisms and variations in individual LPLs. Specific primers (data not shown) were designed according to the sequences downloaded from National Center of Biotechnology Information to amplify the partial sequences of these three candidate genes (< 800 bp). After sequence alignment of these three genes in the 20 rice accessions of Set 1, several InDels and SNPs were discovered (Fig. 4). Among these polymorphisms, a 7-bp insertion in *AAPT* and five SNPs in *AAPT*, *PLD* and *PLA_2* were selected for developing molecular markers. Six functional molecular markers (Table 2) were developed and used to genotype 33 rice accessions from Set 1 and Set 2. Among the 20 accessions of Set 1, genotyping results (Table 3 and

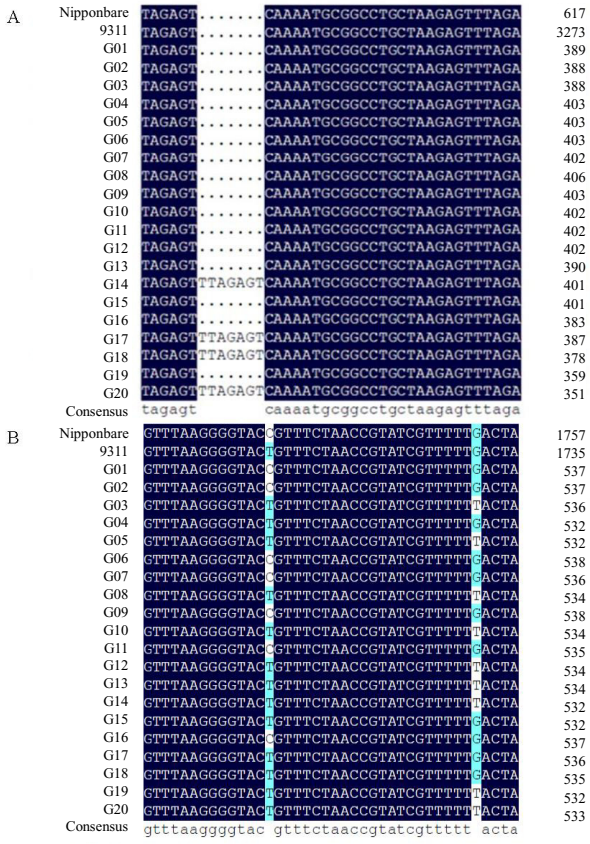

Fig. 4. Part sequence alignments of *AAPT* and *PLA_2* genes.

A, InDel found in *AAPT* gene among G01–G20; B, Single nucleotide polymorphism found in *PLA_2* gene among G01–G20.

AAPT, Aminoalcoholphosphotransferase; PLA_2, Phospholipase A_2.

Fig. 5) showed that T/A and A/G variants in *AAPT*, C/A and G/C variants in *PLD*, and G/T variant in *PLA_2* were consistent with the sequencing results.

Association test between starch LPL traits and molecular markers

Taking account into the trace LPL content in waxy

Table 2. Primers for enzyme digestion of several single nucleotide polymorphisms.

Primer	Type	Sequence (5′–3′)	PCR product (bp)	Restriction enzyme	Enzyme digestion product (bp)
AAPT1-F	InDel	CCAGCCTTGTTTCAATACCTG	134/127	–	–
AAPT1-R		AAATGTAGGAAGTTTTTACTTGC			
AAPT2-F	dCAPS	AAGAGCAAGTAAAAACTTCCTAAATT	222	*Apo*I	22, 200
AAPT2-R		GATACAAATGCCCAAATACCA			
AAPT3-F	dCAPS	CAAAGATCAATGCTGGGTAATTTC	184	*Sph*I	22, 162
AAPT3-R		AGGTAAATCAGTTCACCTGTGCA			
PLD1-F	dCAPS	CATCCTGCACTAAAAACAGTTGAAT	214	*Hinf*I	22, 192
PLD1-R		TGCACAACACCAGAGCCCCACC			
PLD2-F	dCAPS	CCTCCCAAAGTTTAGGCGGAAAAGGCC	187	*Hpa*II	27, 160
PLD2-R		TCAAAGCTCACAATAGCAGAATA			
$PLA_2$1-F	dCAPS	TTCCTATTGTTTCTTCCTCCCTCTT	230	*Hinf*I	20, 210
$PLA_2$1-R		GAAAAAACAAAATTAAAAAAGAGT			

dCAPS, Development of derived cleaved amplified polymorphic sequences.
Underlined letter means the mismatch base.

Table 3. Summary of alleles of three candidate genes.

Accession	AAPT1	AAPT2	AAPT3	PLD1	PLD2	PLA_21	Accession	AAPT1	AAPT2	AAPT3	PLD1	PLD2	PLA_21
G01	D	T	A	C	G	G	G18	I	A	G	C	C	G
G02	D	T	A	C	G	G	G19	D	T	A	C	G	T
G03	D	T	A	A	C	T	G20	I	A	G	A	C	T
G04	D	T	A	A	C	G	R01	D	T	A	C	C	G
G05	D	T	A	C	C	T	R02	D	T	A	C	C	G
G06	D	T	A	C	G	G	R03	D	T	A	C	G	T
G07	D	T	A	C	G	G	R04	D	T	A	C	C	G
G08	D	T	A	C	C	T	R05	D	T	A	C	C	T
G09	D	T	A	C	G	G	R06	D	T	A	C	C	G
G10	D	T	A	A	C	T	R07	D	T	A	C	G	G
G11	D	T	A	C	G	G	R08	I	A	G	C	C	G
G12	D	T	A	C	C	T	R09	D	T	A	C	C	T
G13	D	T	A	C	C	T	R10	I	A	G	C	C	T
G14	I	A	G	C	C	T	R11	D	T	A	C	C	T
G15	D	T	A	C	C	G	R12	D	T	A	C	C	T
G16	D	T	A	C	G	G	R13	I	A	G	C	C	T
G17	I	A	G	C	C	G							

AAPT, Aminoalcoholphosphotransferase; PLD, Phospholipase D; PLA_2, Phospholipase A_2; I, Insertion; D, Deletion.

rice, the association analysis between molecular markers and endosperm LPLs was performed based on 31 non-waxy accessions and all the 33 rice accessions. It was found that PLD1 marker was significantly associated with LPC16:0 in 31 non-waxy rice accessions but not in all the 33 rice accessions. Contrarily, PLA_21 marker was significantly associated with LPC18:1 in all the 33 rice accessions but not in 31 non-waxy rice accessions (Table 4). Some other markers were also significantly correlated with several individual LPL traits in 31 or all the 33 rice accessions, but these results were not in accordant with the target QTLs detected in 20 rice accessions (data not shown).

DISCUSSION

Although biosynthesis of the lipids, such as triacylglycerol and PLs, and their metabolisms in plants have received substantial attention (Bessoule and Moreau, 2004; Ambrosewicz-Walacik et al, 2015; Xu and Shanklin, 2016), the understanding of plant PL synthesis pathway and its regulation was limited to several genes responsible for *Arabidopsis* phospholipid biosynthesis, such as *Phosphoethanolamine N-methyltransferase* (*PEAMT*), *Choline kinase* (*CKI*), cytidine triphosphate-*phosphocholinecytidylytransferase* (*CCT*)

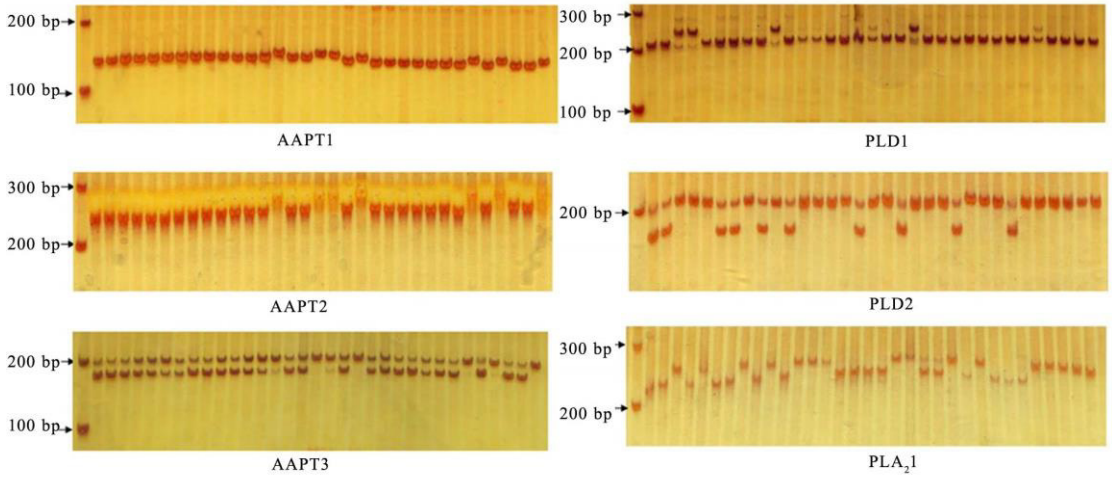

Fig. 5. Polymorphism of InDel and simple nucleotide polymorphisms in *AAPT*, *PLD* and *PLA_2* genes in rice accessions G01–G20 and R01–R13.
AAPT, Aminoalcoholphosphotransferase; PLD, Phospholipase D; PLA_2, Phospholipase A_2.
Lanes from left to right in each part are marker, G01–G20 and R01–R13, respectively.

Table 4. Marker loci associated with starch lysophospholipids (LPLs) traits detected with analysis of variance (ANOVA) model in 31 non-waxy and all the 33 rice accessions.

Gene	Marker	Trait	Non-waxy accession (31)		All rice accession (33)		QTL
			*p*_Marker	R^2_Marker	*p*_Marker	R^2_Marker	
PLD	PLD1	LPC16:0	0.0149	0.1876	0.0984	0.0856	*qC160-6-1*
PLA_2	$PLA_2$1	LPC18:2	0.0902	0.0958	0.0277	0.1468	*qC182-11*, *qE182-11*

PLD, Phospholipase D; PLA_2, Phospholipase A_2.

and *AAPT* (Eastmond et al, 2010). Genetic analysis of rice PL is necessary not only for understanding PL biosynthesis in rice plants, but also for breeding rice varieties with optimized PL accumulation in grains. To our knowledge, only a few genes corresponding to rice starch LPL synthesis have been reported.

Association mapping is a mature and widely accepted technology to identify the genotype-phenotype relationships among diverse germplasms (Yang et al, 2014). For example, 74 QTLs significantly associated with maize kernel oil content and fatty acid composition have been identified by association mapping (Li et al, 2013). In this study, association mapping identified 22 main-effect QTLs for rice endosperm LPL concentration and composition on all chromosomes except chromosomes 3 and 7 (Fig. 2). This is the first study on the genetic basis of rice LPL content (Table 1 and Fig. 2). Especially, we discovered that six QTLs located on chromosomes 1, 2, 9, 10, 11 and 12, respectively, simultaneously controlled more than two LPL traits (Table 1 and Fig. 2). It maybe indicate that a tight physiological and biochemical link exists during the biosynthesis or regulation of these LPL components. It was worth noting that *qE-10*, *qC-10* and *qL-10* located on the close positions on chromosome 10 (Fig. 2), probably indicating a locus regulating starch LPL accumulation in rice. Additionally, *qE183-1*, *qC183-1*, *qE181-2* and *qL-10* were simultaneously detected in different environments (Table 1), which demonstrated that they were highly stable for LPE18:3, LPC18:3, LPE18:1 and total LPL across different environments. More importantly, the QTLs *qC160-6-2*, *qC160-6-1* and *qC182-11*/*qE182-11* were close to the loci *Os06g0204400*, *Os06g0649900* and *Os11g0546600*, respectively. Three genes were evidenced to encode AAPT, PLD and PLA2 which were vital enzymes mediating PL synthesis in plants (Fig. 1). Since the other novel QTLs with minor effect were only detected in one environment, they might be affected by environment or genotype × environment interaction. Additionally, it should be noted that no QTL was found for LPC14:0 and LPE14:0, which might due to the small population. Therefore, a large population is necessary for detecting the significant QTLs for LPC14:0 and LPE14:0.

Because of the small amount of SNPs used in the preliminary association test, we cannot confirm that the detected significant SNPs were right in the candidate genes. Thus, we re-sequenced the three candidate genes and discovered some SNPs and one InDel (Fig. 4). We further developed dCAPS to genotype some of these SNPs to test whether there were real associations between the SNPs of candidate genes and the LPL content in rice grains. If there were some relationships, these markers can be used for improving rice starch LPLs during molecular breeding. Analysis of variance showed that *PLD1* locus was significantly correlated with LPC16:0 and total LPC content ($P < 0.05$, Table 4). *$PLA_2$1* was significantly correlated with LPC18:1 and LPC18:2 contents ($P < 0.05$, Table 4). Thus, the gene markers of both *PLD1* and *$PLA_2$1* associated with *qC160-6-1* (LPC16:0) and *qC182-11* (LPC18:2) were confirmed (Tables 1 and 4). However, some gene markers were not associated with the target traits but with other traits. For example, the AAPT1, AAPT2 and AAPT3 markers were all significantly associated with both LPE18:2 and LPE18:3 contents, respectively (data not shown). Further studies using a large rice population and more SNPs are necessary for understanding the genetic architecture of rice starch LPLs.

In summary, a total of 22 QTLs responsible for individual LPL content were identified via a preliminary association test. Three candidate genes responsible for LPL biosynthesis were discovered, and their partial sequences were sequenced. Among the identified nucleotide variations, five dCAPS and one InDel markers for these three candidate genes were successfully developed. Two markers were confirmed to be associated with the target LPL traits. This study provides an insight into the genetic basis of LPL biosynthesis in rice and may contribute to the rice

quality breeding programs using the functional markers derived from the candidate genes.

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SUPPLEMENTAL DATA

The following material is available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Primers for re-sequencing three putative phospholipids synthesis related genes.

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