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Identification and Bioinformatic Analysis of the ASAT Gene Family in *Oryza sativa*

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Abstract Rice bran oil is rich in bioactive sterol ferulates that are important not only for seed development, drought stress and lipid metabolism, but also for grain nutrition quality and human healthy. However, its complete compositions and associated metabolic pathways are poorly understood. *Arabidopsis thaliana* Acyl-CoA Sterol Acyltransferase1 (AtASAT1) is the only one in the superfamily of Membrane-Bound O-Acyltransferases that has ASAT activity, which prefers saturated fatty acyl-CoAs as acyl donors and cycloartenol as the acyl acceptor. Overexpression of *AtASAT1* in *Arabidopsis* results in a strong accumulation of cycloartenol fatty acyl esters accompanied by an increase of the whole sterol esters contents in seeds. Here we present our bioinformatic based analysis data aiming to identify more plant ASAT family members and to in silico study of them in rice. We identified 105 ASAT Family members from public genome database of 10 plant species including *Arabidopsis* and tomato, and the phylogenetic tree of ASAT family constructed revealed evolutionary relationships of ASAT family members between oil-bearing crop and non-oil-bearing species, and between monocotyledons and dicotyledons. We further investigated into physical and chemical properties of 14 OsASAT proteins, and chromosome distributions, structures and promoter sequences, and expression patterns of corresponding *OsASAT* genes, providing a solid theoretical basis for further study of the biological functions of OsASAT proteins.

Keywords Sterol esterification; ASAT; Protein structure; Molecular evolution; Expression pattern

Rice (*Oryza sativa*) is the main grain consumed by about 3.5 billion people all over the world. And rice bran is a by-product of rice milling process, which is rich in bioactive chemicals, such as polyphenols, peptides and lipids (such as phytosterol ferulate), and have the ability to remove free radicals and prevent diseases (Saji et al., 2019). At present, the most studied rice bran oil is sterol ferulate, which is a mixture of sterol and ferulic acid esterification. It is generally believed that rice bran oil mainly exists in the bran, embryo and endosperm of mature rice grains (Hernandez et al., 2016). During rice grain maturation, sterol ferulate is involved in maintaining the integrity and fluidity of plant cell lipid membranes, membrane lipid homeostasis, and regulating membrane permeability, fluidity, signal transduction and membrane binding enzyme activity in cell division (Kumar et al., 2015; Valitova et al., 2016). More and more studies have shown that sterol ferulate have positive health effects, such as lowering cholesterol, antioxidant (Faulds, 2010; Bumrungpert et al., 2019; Lai et al., 2019). Although rice bran oil has a variety of confirmed and expected biological activities, the biosynthesis pathway of sterol ferulate in plants is not clear (Zhu, 2015). From its molecular structure, the biosynthesis of sterol ferulate in plants may begin with the synthesis of sterol and ferulic acid, and then the esterification of ferulic acid and sterol. It has been reported that acyltransferase is involved in the biosynthesis of sterol ferulate (Kumar et al., 2015).

Sterol acyltransferases are a family of enzymes that catalyze the transfer of fatty acid groups to the hydroxyl group at C-3 position of the sterol backbone. Sterol acyltransferases are categorized into acyl-CoA: sterol acyltransferases (ASAT) and phospholipids: sterol acyltransferases (PSAT) depending on whether the fatty acid donor substrate is a long-chain acyl-CoA or a phospholipid. It is reported that phytosterol acyltransferase activity is mainly related to the membrane components of different species (Banas et al., 2005; Chen et al., 2007; Bouvier-Navé et al., 2010) and has broad-spectrum characteristics of participating in phytosterol metabolism. For

example, sterol acyltransferase activity increases during seed development in rape and tobacco (Harker et al., 2003). And Arabidopsis AtASAT1 and tomato SLASAT1 not only participate in sterol ester synthesis, but also affect the development of plant organs and tissues. But so far, ASAT enzymes in other plants have not been cloned and identified.

AtASAT1 is the only member with *ASAT* gene activity in Arabidopsis MBOAT gene family. The protease AtASAT1 encoded by *AtASAT1* prefers saturated fatty acyl-CoAs as acyl donors and cycloartenol as the acyl acceptor. Overexpression of *AtASAT1* in Arabidopsis results in a strong accumulation of cycloartenol fatty acyl esters accompanied by an increase of the whole sterol esters contents in seeds (Chen et al., 2007; Bouvier-Navé et al., 2010). In addition, the content of sterol esters contents in leaves and seeds of *atasat1* mutant decreased significantly, and it was also proved that *AtASAT1* was closely related to the balance of free sterols during leaf senescence in *Arabidopsis thaliana* (Bouvier-Navé et al., 2010). Tomato genome contains as many as 8 genes that encode ASAT proteins, among which *Solyc11g012260* gene, or *SLASAT1*, encodes the largest protein in the family (444 amino acid residues), which is also the gene with the highest homology with *AtASAT1* (49%). *SLASAT1* was used to supplement Arabidopsis *asat1-1* mutant. And it was found that the enzyme had obvious sterol substrate preference and could significantly increase the synthesis of sterol cycloartenol and 24-methylene cycloartenol (Lara et al., 2018). And tomato SLASAT1 also has dual roles in tomato plant and fruit development and stress response and has obvious substrate preference for phytosterol precursor cycloartenol and 24-methylene cycloartenol (Lara et al., 2018). It is worth noting that AtASAT1 is located in endoplasmic reticulum (ER) and SLASAT1 is located in plasma membrane (PM). These studies show that phytosterol acyltransferases are indeed involved in the esterification of alcohols and acids, but the species and biological functions of sterol acyltransferases in rice have not been elucidated. The molecular evolution characteristics, expression patterns and biological functions of sterol acyltransferases in different species have not been systematically compared.

In order to fully reveal the biological function, expression pattern and protein structure of ASAT family in rice, compare the evolution characteristics of ASAT protein in monocotyledons and dicotyledons, and predict the role of rice ASAT family members in rice sterol ester metabolism and their effects on rice growth, development and stress response, bioinformatics analysis was used in this study. We identified 105 ASAT Family members from public genome database of 10 plant species including Arabidopsis and tomato, and the phylogenetic tree of ASAT family constructed revealed evolutionary relationships of ASAT family members between oil-bearing crop and non-oil-bearing species, and between monocotyledons and dicotyledons. We further investigated into physical and chemical properties of 14 OsASAT proteins, and chromosome distributions, structures and promoter sequences, and expression patterns of corresponding *OsASAT* genes, providing a solid theoretical basis for further study of the biological functions of OsASAT proteins in the metabolic pathway of rice bran oil.

1 Results and Analysis

1.1 Identification of ASAT family

AtASAT1 protein sequence was used as query, and the blastp program was used to search the protein data of three food crops rice (*Oryza sativa*, Japonica Group, asSEsmbly Build 4.0), maize (*Zea mays*, asSEsmbly B73 RefGen_v4), and sorghum (*Sorghum bicolor*, asSEsmbly Sorghum_bicolor_NCBIv3), four oil crops rape (*Brassica napus*, asSEsmbly Bra_napus_v2.0), soybean (*Glycine max*, asSEsmbly Glycine_max_v2.1), peanut (*Arachis hypogaea*, asSEsmbly arahy.Tifrunner.gnm1.KYV3), and sesame (*Sesamum indicum*, asSEsmbly S_indicum_v1.0) and two cash crops tomato (*Solanum lycopersicum*, asSEsmbly SL3.0), and tobacco (*Nicotiana tabacum*, asSEsmbly Ntab-TN90), and 128 candidate family members were obtained. After using conserved domain analysis and tblastn to remove redundant sequences, 105 candidate ASAT families were obtained. Among them, 6 rice OsASAT identified were homologous with the reported Arabidopsis AtASAT1, and the 8 tomato SLASAT identified were also consistent with the reported (Lara et al., 2018), which confirmed the reliability of the results. We named 105 ASAT homologous proteins as "species+ASAT+number" (Table 1) according to their position on the chromosome. And 14 candidate OsASAT family members in rice were selected as key analysis objects.

1.2 Evolutionary relationship of ASAT protein in different species

In order to explore the phylogenetic relationship of ASAT family proteins in plants, 105 ASAT proteins from 9 different plant species were identified, and the maximum likelihood method was used to construct the phylogenetic tree of ASAT (Figure 1). It is obvious that the genetic distance between Arabidopsis AtASAT1 and peanut, rape and rice is close, which means this protein may be more similar to peanut, rape and rice in origin and evolution. We also noticed that the OsASAT proteins of rice were distributed in all evolutionary branches, and the genetic distances of OsASAT2, OsASAT2, OsASAT3, OsASAT4, OsASAT5, OsASAT6 and OsASAT9 were close to Arabidopsis AtASAT1. And the genetic distances of OsASAT1, OsASAT7, and OsASAT13 were close to tomato SLASAT1. These results suggest that different OsASAT proteins have different functions, some of which are similar to Arabidopsis homologous proteins, others are similar to tomato homologous proteins. On the other hand, the ASAT proteins of rice, maize and sorghum in food crops showed clustering phenomenon, which indicated that the homology between proteins was good, and the time of evolution divergence was closer. And it is worth noting that in typical oil crops, such as peanut, soybean and rape have relatively close ASAT family proteins, while sesame ASAT proteins are scattered. These results suggest that the ASAT protein family may play a role in the evolution or differentiation of oil and food crops, and also suggest that ASAT protein may have different enzyme activities or functions in different crops. We pay more attention to OsASAT, so the following analysis mainly focuses on OsASAT.

1.3 Whole genome analysis of OsASAT encoding gene in Rice

14 OsASAT protein coding genes were identified in rice database by homologous search method, which were unevenly distributed on chromosomes 1, 2, 4 and 7 (Table 2; Figure 2). We noticed that the *OsASAT* on chromosomes 2 and 4 formed two gene clusters respectively (Figure 2). There is a great difference in the size of *OsASAT* gene. The longest cDNA is 4 599 bp, while the shortest cDNA is only 435 bp (Table 2). In addition, the structure of *OsASAT* gene varies greatly among individuals. Among them, 7 have no introns, while the remaining genes have different numbers of introns (1~9) (Figure 3). The results of selection site analysis (Table 2) showed that the non synonymous sites of the *OsASAT* gene family were more than the synonymous sites, indicating that they were relatively conservative in the process of evolution, that is, the rice *OsASAT* gene family tended to purify selection.

1.4 Analysis of physical and chemical properties of rice ASAT family proteins

In order to further clarify the protein structure and properties of ASAT family members in rice, we characterized the basic physical and chemical properties of 14 OsASAT family proteins in rice (Table 3). The results showed that the sequence of OsASAT protein ranged from 264 to 403 amino acids, and there were some differences among individuals. The molecular weight of family members is between 28.7 and 43.95 kD, which is similar to that of Arabidopsis AtASAT1, but there are also some differences among members. The positively charged residues were more than negatively charged residues, and the theoretical isoelectric points were more than 7, ranging from 9.48 to 10.92, indicating that OsASAT family proteins are rich in basic amino acids. The analysis of the above proteins by ProtScale showed that the aliphatic index of OsASAT proteins was more than 80, ranging from 100.58 to 113.02, indicating that the OsASAT family proteins had high fat solubility and thermal stability. However, the OsASAT family proteins showed strong hydrophobicity at the same time, and the hydrophobic index was more than 40. Because the proteins with strong hydrophobic waters are generally unstable, and the proteins with instability index more than 40 are also unstable proteins (Gasteiger et al., 2005), it can be concluded that 14 OsASAT family proteins are unstable proteins in theory.

Table 1 Name list of identified ASAT proteins from 9 tested plant species

Plant species	Protein ID of NCBI	Protein name	Plant species	Protein ID of NCBI	Protein name	Plant species	Protein ID of NCBI	Protein name
<i>Arachis hypogaea</i>	XP_025642946.1	AhASAT1	<i>Glycine max</i>	XP_003534825.1	GmASAT2	<i>Sorghum bicolor</i>	XP_002447562.1	SbASAT1
	XP_025687047.1	AhASAT2		XP_006586110.1	GmASAT3		XP_002448060.1	SbASAT2
	XP_025626860.1	AhASAT3		NP_001304471.2	GmASAT4		XP_002468615.1	SbASAT3
	XP_025688598.1	AhASAT4		XP_003530769.2	GmASAT5		XP_021318245.1	SbASAT4
	RYR79873.1	AhASAT5		XP_003530770.1	GmASAT6		XP_002438765.1	SbASAT5
	RYR79871.1	AhASAT6		NP_001240009.1	GmASAT7		XP_002444113.1	SbASAT6
	XP_025629805.1	AhASAT7		XP_014625754.1	GmASAT8		XP_002456060.1	SbASAT7
	XP_025613083.1	AhASAT8		XP_006603172.1	GmASAT9	<i>Sesamum indicum</i>	OQU87244.1	SbASAT8
	XP_025645988.1	AhASAT9		XP_014625980.1	GmASAT10		XP_011095962.1	SiASAT1
	XP_025694018.1	AhASAT10		XP_006602175.1	GmASAT11		XP_011097128.1	SiASAT2
	RYR00612.1	AhASAT11		ACU22791.1	GmASAT12		XP_011097818.1	SiASAT3
	XP_025668812.1	AhASAT12		KRG98638.1	GmASAT13		XP_020554122.1	SiASAT4
	QHN90994.1	AhASAT13		KRG98662.1	GmASAT14		XP_010312742.1	SIASAT1
	XP_025608479.1	AhASAT14		KRG98660.1	GmASAT15		XP_004241733.1	SIASAT2
	RYR28133.1	AhASAT15		XP_016453487.1	NtASAT1	<i>Solanum lycopersicum</i>	XP_025884084.1	SIASAT3
	QHO37416.1	AhASAT16		XP_016453488.1	NtASAT2		XP_004250264.2	SIASAT4
	QHO26646.1	AhASAT17		XP_016453485.1	NtASAT3		XP_025887953.1	SIASAT5
<i>Brassica napus</i>	XP_013663174.1	BnASAT1	<i>Nicotiana tabacum</i>	XP_016506397.1	NtASAT4		XP_025887954.1	SIASAT6
	XP_013698964.1	BnASAT2		XP_016499306.1	NtASAT5		XP_010314632.1	SIASAT7
	XP_013659822.1	BnASAT3		XP_016504134.1	NtASAT6		XP_004250553.3	SIASAT8
	XP_013698974.1	BnASAT4		XP_016463428.1	NtASAT7		PWZ05946.1	ZmASAT1
	XP_013654655.1	BnASAT5		XP_015624132.1	OsASAT1		NP_001353564.1	ZmASAT2
	XP_013711394.1	BnASAT6		XP_015634629.1	OsASAT2		ACG25815.1	ZmASAT3
	XP_013685657.1	BnASAT7		KAF2934556.1	OsASAT3		NP_001131179.2	ZmASAT4
	XP_013714610.1	BnASAT8	<i>Oryza sativa</i>	XP_015634567.1	OsASAT4	<i>Zea mays</i>	PWZ45391.1	ZmASAT5
	XP_013680812.1	BnASAT9		KAF2933153.1	OsASAT5		ACG41736.1	ZmASAT6
	XP_013652138.1	BnASAT10		XP_015635081.1	OsASAT6		ACG43872.1	ZmASAT7
	XP_013665788.1	BnASAT11		EAZ22913.1	OsASAT7		NP_001151683.2	ZmASAT8
	CDY17327.1	BnASAT12		KAF2923240.1	OsASAT8		ONM18562.1	ZmASAT9
	CDY12004.1	BnASAT13		XP_025882516.1	OsASAT9		ACG39975.1	ZmASAT10
	CDY36739.1	BnASAT14		KAF2944735.1	OsASAT10		NP_001340789.1	ZmASAT11
	CDY20770.1	BnASAT15		EAZ12916.1	OsASAT11		ACG37962.1	ZmASAT12
	CDY20768.1	BnASAT16		XP_015647081.1	OsASAT12		NP_001150127.2	ZmASAT13
	CDY20766.1	BnASAT17		XP_015625046.2	OsASAT13		ACG31412.1	ZmASAT14
<i>Glycine max</i>	XP_003534380.1	GmASAT1		EAZ22980.1	OsASAT14		PWZ32010.1	ZmASAT15

Figure 1 Phylogenetic tree of ASAT proteins

Note: At: *Arachis hypogaea*; An: *Arachis hypogaea*; Bn: *Brassica napus*; Gm: *Glycine max*; Nt: *Nicotiana tabacum*; Os: *Oryza sativa*; Si: *Sesamum indicum*; Sl: *Solanum lycopersicum*; Sb: *Sorghum bicolor*; Zm: *Zea mays*

1.5 Localization, secondary structure and phosphorylation site analysis of rice ASAT family proteins

In order to analyze the biochemical properties and molecular functions of OsASAT family proteins, we analyzed the localization, secondary structure and phosphorylation sites of OsASAT family proteins (Table 4). The results of protein subcellular localization prediction showed that OsASAT protein localization is diverse, suggesting that it may have a variety of biological functions. The proportion of OsASAT family proteins located in chloroplast and plasma membrane is the largest, which is similar to that of Arabidopsis and tomato (Chen et al., 2007; Lara et al., 2018). The protein secondary structure showed that there were 2 similar α -helix and 7 similar β -sheet in the secondary structure of OsASAT family members, and all of them had the characteristics of α -helix (49.86%~57.8%) and random coil (26.69%~35.17%), which may be due to the conservative domain MBOAT_2 α -helix (37.41%) and random coil (42.69%) accounted for the highest proportion. The results of transmembrane domain analysis showed that there were 5 to 8 transmembrane helical structures in OsASAT protein, which was slightly lower than that in Arabidopsis AtASAT1, which indicated that OsASAT protein was probably located in membrane-related structures, such as plasma membrane, chloroplast membrane or mitochondrial membrane,

which was consistent with the prediction of subcellular localization of OsASAT protein mentioned above. The analysis of phosphorylation sites showed that there were few phosphorylation sites in OsASAT family proteins, ranging from 15 to 27, and most of them were concentrated in serine. And prediction results of signal peptides showed that no signal peptides were detected in 14 OsASAT family proteins, indicating that these proteins are likely to be non-secretory proteins, which is the same as Arabidopsis AtASAT1.

Figure 2 Distribution of *ASAT* genes on rice chromosomes

Note: The chromosome number is indicated above each bar. Boxes: The gene cluster. The scale on the left is in base pair (bp)

Table 2 Basic information of ASAT encoding gene family members in rice

Gene ID	Gene name	Chrome	Starting point	Ending point	Gene length (bp)	Synonymous sites	Non Synonymous sites
LOC_Os01g01010.1	<i>OsASAT1</i>	Chr1	4754	5797	1044	93.5	332.5
Os02g0100050	<i>OsASAT2</i>	Chr2	392	2696	2305	96.83	329.17
LOC_Os02g01020.1	<i>OsASAT3</i>	Chr2	9298	13896	4599	96.33	329.67
LOC_Os02g01030.1	<i>OsASAT4</i>	Chr2	14061	18598	1125	90	336
LOC_Os02g25700.1	<i>OsASAT5</i>	Chr2	15044756	15045814	1059	112.5	313.5
LOC_Os02g26840.1	<i>OsASAT6</i>	Chr2	15759511	15760551	1041	118	308
LOC_Os04g01006.1	<i>OsASAT7</i>	Chr4	2032	3679	855	93.83	332.17
LOC_Os04g01008.1	<i>OsASAT8</i>	Chr4	4951	8636	2463	101.5	324.5
LOC_Os04g20880.1	<i>OsASAT9</i>	Chr4	11720537	11721807	1035	114.83	311.17
LOC_Os04g40580.1	<i>OsASAT10</i>	Chr4	24098305	24099665	1050	121	305
LOC_Os04g40590.1	<i>OsASAT11</i>	Chr4	24101138	24102493	1038	123.5	302.5
LOC_Os07g01010.1	<i>OsASAT12</i>	Chr7	2362	3134	435	96	330
LOC_Os07g35570.1	<i>OsASAT13</i>	Chr7	21293128	21294251	1124	116.5	309.5
LOC_Os07g44030.1	<i>OsASAT14</i>	Chr7	26311992	26313267	1276	104.33	321.67

Note: Cyto: Cytoplasm; Chlo: Chloroplast; ER: Endoplasmic reticulum; Mito: Mitochondria; Nucl: Nucleus; Plas: Plasma membrane; Vacu: Vacuole

Figure 3 Analysis of gene structure of *OsASAT* genes

Note: The yellow and blue bars represent the CDS and the un-translated region (UTR), respectively, while the black line represents the intron. Numbers (0, 1, 2) stand for the intron phases

Table 3 Basic physiological and chemical features of rice ASAT proteins

Protein name	Number of amino acids (aa)	Molecular weight (kD)	pI	Number of negatively charged residues (Asp+Glu)	Number of positively charged residues (Arg+Lys)	Total number of atoms	Instability index	Aliphatic index	Hydrophobic index
OsASAT1	348	38.76	10.2	16	32	5558	57.65	113.02	0.615
OsASAT2	346	36.77	9.84	18	30	5245	44.48	103.41	0.605
OsASAT3	347	36.76	9.98	17	31	5241	47.41	100.86	0.557
OsASAT4	264	28.70	10.92	9	29	4109	43.15	103.56	0.438
OsASAT5	352	38.52	9.97	16	29	5477	40.82	102.36	0.559
OsASAT6	381	40.61	9.48	22	32	5772	44.19	100.58	0.553
OsASAT7	344	37.30	9.69	21	34	5341	52.69	110.76	0.622
OsASAT8	348	37.22	10.76	19	33	5344	44.96	112.82	0.599
OsASAT9	403	43.95	9.66	27	43	6257	53.93	101.09	0.424
OsASAT10	349	37.35	10.76	19	33	5361	44.77	112.49	0.603
OsASAT11	345	37.06	10.01	19	30	5299	36.79	108.46	0.63
OsASAT12	336	36.35	9.93	21	35	5188	53.38	102.74	0.556
OsASAT13	354	37.98	9.93	22	36	5420	52.61	103.36	0.590
OsASAT14	356	39.68	10.14	17	33	5687	55.91	111.57	0.578

1.6 Sequence alignment and functional domain analysis of rice ASAT family proteins

In order to further clarify the structure and properties of rice ASAT family proteins, we carried out structural alignment and functional domain prediction of rice ASAT family proteins (Figure 4). The results showed that there were 12 conserved motifs with high similarity in the *OsASAT* protein sequence (Figure 4A), among which 7 motifs were completely consistent with Arabidopsis AtASAT1. It is worth noting that the distribution of conserved motif 1~9 of *OsASAT* family proteins is basically the same, showing the characteristics of high conservation. Subsequent online SMART detection of conserved motifs showed that motif 1 is a MBOAT superfamily domain, which usually ends with G (glycine) at the C-terminal of the protein (Figure 4B).

1.7 Analysis of cis-acting elements of rice *ASAT* gene

In terms of gene function, promoter is an important cis-acting element of gene expression, which determines the timing, location and quantitative expression of specific genes. In order to further study the evolution rate and characteristics of rice *OsASAT* gene family, we analyzed the cis-acting elements of *OsASAT* gene (Figure 5). The prediction results of cis-acting elements on the 2000 bp sequence up-stream of the transcriptional initiation site of *OsASAT* gene showed that there was a total of 1 756 cis-acting elements in the promoter of *OsASAT* gene family,

but the number of cis-acting elements varied greatly among gene individuals. Compared with other *OsASAT*, *OSTAT2* contained the least cis-acting elements. The largest proportion of cis-acting elements in all detected promoters were transcriptional core promoters such as CAAT-box (18.2%) and TATA-box (16.4%), followed by pressure response elements MYC (3%) and MYB (2.1%), and light response elements G-box (1.6%), plant hormone response elements ABRE (1.2%) and CGTCA-motif (1.1%), and so on. The above results showed that the expression of *OsASAT* gene is regulated by a variety of internal and external signals, which may be involved in response to stress and play an important role in normal plant growth and development and hormone balance *in vivo*.

Table 4 Protein structures, subcellular localization and phosphorylation sites a of rice ASAT

Protein name	Alpha-helix (%)	Beta turn (%)	Extended strand (%)	Random coil (%)	Number of predicted TMHs	Subcellular localization	Threonine	SEsrine	Tyrosine	Total sites
OsASAT1	53.16	4.31	14.08	28.45	6	Chlo, Plas	4	14	2	20
OsASAT2	57.8	3.18	9.54	29.48	7	Chlo, Mito	4	9	2	15
OsASAT3	51.87	3.75	11.82	32.56	5	Chlo, Mito	4	10	1	15
OsASAT4	54.55	4.17	8.33	32.95	5	Chlo, Vacu., Plas	2	18	0	20
OsASAT5	52.84	4.26	14.2	28.69	8	Chlo, Vacu, Plas	3	15	1	19
OsASAT6	51.18	2.62	11.02	35.17	7	Cyto, Vacu, Plas, Pero	5	11	3	19
OsASAT7	54.65	2.62	13.08	29.65	5	Chlo, Plas, Vacu, E.R.	4	15	2	21
OsASAT8	52.3	6.32	11.49	29.89	7	Chlo, Mito	5	12	1	18
OsASAT9	51.61	3.47	10.17	34.74	5	Plas, Chlo, Vacu, Nucl	6	18	3	27
OsASAT10	49.86	6.3	11.17	32.66	7	Chlo, Mito	9	16	2	27
OsASAT11	56.23	5.51	11.3	26.96	8	Chlo, Mito	4	9	1	14
OsASAT12	52.68	6.25	11.61	29.46	6	Chlo, Plas, Mito, E.R.	9	15	2	26
OsASAT13	53.39	4.8	9.89	31.92	7	Chlo, Plas	9	16	2	27
OsASAT14	51.97	3.65	14.33	30.06	7	Plas, Vacu, E.R.	5	14	2	21

Figure 4 Alignment of Arabidopsis AtASAT1 with rice ASATs and prediction of conserved motif patterns among them
Note: A: The distribution and sequence of motifs; B: Highly conserved motif patterns of ASAT family

Figure 5 Cis-acting element structures in promoter regions of rice *ASAT* family members

1.8 Cluster analysis of expression patterns of rice *ASAT* gene family

Analyzing the expression characteristics of *OsASAT* gene in biotic stress and abiotic stress, different developmental stages and tissue space can provide a basis for further analysis of the function of *OsASAT* protein. Firstly, we used the genevestigator (<https://genevestigator.com/>) to draw the spatial expression heat map of rice *OsASAT* in 36 different rice tissues (Figure 6). And it is obvious that *OsASAT* gene has obvious tissue expression specificity. Among them, *OsASAT10* is only expressed in a few vegetative tissues, while *OsASAT14* is expressed to varying degrees in more vegetative organs, especially in Internode tissues and young roots. However, the expression of these two genes in reproductive organs is so weak that it seems impossible to participate in the development of reproductive organs. *OsASAT4* and *OsASAT9* were specifically up-regulated in bud and flower (stamen, anther, pollen, pistil), which may play a major role in the development of rice reproductive organs. In addition, the highest expression levels of them were found in the seedling and coleoptile, suggesting that they may be involved in seed germination and seedling establishment. *OsASAT6* was highly expressed in roots and reached the highest level in internodes. It is worth noting that all *OsASAT* genes are expressed in caryopsis or rice panicles, which supports our speculation that *OsASAT* may be involved in steryl ferulate synthesis (Figure 6A).

The results of the line chart of *OsASAT* expression at 9 different developmental stages (Figure 6B) showed that the dynamic expression profiles of each *OsASAT* gene had their own characteristics. Among them, *OsASAT4* showed a sustained and stable high-level expression, and the expression level was significantly higher than that of other *OsASAT* genes. Except for *OsASAT1*, the expression of other *OsASAT* genes was up-regulated at the fruiting stage (flowering stage, milk stage and dough stage), suggesting that they may be involved in the process of seed development. Expression patterns of rice *ASAT* genes in 48 tissues at different developmental stages (Figure 6C) showed that no significant gene expression changes were found in the diurnal tissues except *OsASAT10* and *OsASAT14* in the leaf sheath, indicating that the expression patterns of each gene were stable during the diurnal variation. In addition, the spatiotemporal expression of most *OsASAT* genes has a broad spectrum, but several *OsASAT* genes have opposite expressions during specific organ development, such as *OsASAT5* and *OsASAT14*

(anther development), *OsASAT11* and *OsASAT14* (pistil development), *OsASAT5* and *OsASAT9* (embryo development), and *OsASAT10* and *OsASAT9* (endosperm development).

These results reflect the possible functional redundancy among these *OsASAT* genes from one aspect. We noticed that during embryo development, the expression of *OsASAT5* was high and stable, while the expression of *OsASAT3* and *OsASAT10* was steadily up-regulated, and the expression of *OsASAT4*, *OsASAT11* and *OsASAT14* was up-regulated at first and then down-regulated, suggesting that they may be involved in the regulation of embryo development. In the process of endosperm development, *OsASAT10* has ultra-high expression while *OsASAT9* shows ultra-low down-regulation, the expression of *OsASAT5* continues to up-regulate, and the expression of *OsASAT14* is down-regulated at first and then up-regulated, suggesting that they play different roles in different processes of endosperm development. And it must be pointed out that the genevitagator platform lacks the data of *OsASAT2*, *OsASAT7*, *OsASAT8*, and *OsASAT12*.

Then, we collected the mRNA expression data of ASAT protein coding genes in roots and seedlings under different hormone treatments from RiceXPro and carried out gene expression pattern cluster analysis (Figure 7). The results showed that each gene showed tissue expression specificity in hormone response. In root tissue (Figure 7A), gibberellin, auxin and brassinosteroid promoted most of *OsASAT* gene expression up-regulation, while jasmonic acid inhibited most gene expression. And gene expression showed significant differences with treatment of cytokinin and abscisic acid, and the expression of single gene changed significantly with hormone concentration, in which *OsASAT11* expression was very sensitive to abscisic acid. In the seedlings (Figure 7B), the gene expression changed greatly caused by each hormone treatment. Interestingly, cytokinin and gibberellin inhibited *OsASAT11* expression, while abscisic acid and jasmonic acid promoted *OsASAT11* expression. Jasmonic acid significantly inhibited the expression of *OsASAT5* and *OsASAT10*, while abscisic acid and gibberellin inhibited and promoted the expression of *OsASAT14*, respectively. Unlike other *OsASAT*, the *OsASAT1* expression is less affected by hormones. These results indicated that the response of members of *OsASAT* gene family to hormones is specific at both tissue and hormone levels, which is not exactly the same, reflecting the diversity of their biological functions. It should be noted that the RiceXPro platform lacks the data of *OsASAT2*, *OsASAT6*, *OsASAT7*, *OsASAT8*, *OsASAT12*, and *OsASAT13*.

Because sterol esters are reported to be involved in plant stress response, which is related to drought stress and membrane lipid metabolism (Kumar et al., 2018), we also obtained RNA-Seq data (GSEs74793) of Azuena rice ASAT protein family coding genes from AIPuFu (www.aipufu.com) under drought and heat for different times and analyzed their expression changes under abiotic stress such as drought and heat (Figure 8). As shown by heat map and hierarchical cluster analysis, *OsASAT* gene family was differentially expressed in different periods of drought and heat. Heat or drought promoted the high expression of *OsASAT3* and *OsASAT4*, while inhibited the expression of other *OsASAT* genes. These results showed that *OsASAT* gene may have functional division and coordinated control under abiotic stress, while *OsASAT3* and *OsASAT4* genes may play a role in abiotic stress of drought and heat in rice.

Figure 6 Spatio-temporal expression patterns of *OsASAT* genes

Note: A: Expression patterns of rice *ASAT* genes in 36 different tissues; 1: Germination; 2: Seedling; 3: Tillering stage; 4: Stem elongation stage; 5: Booting stage; 6: Heading stage; 7: flowering stage; 8: Milk stage; 9: Dough stage; B: Line charts of the kinetic expression patterns of rice *ASAT* genes along rice plant development; C: Expression patterns of rice *ASAT* genes in 12 tissues at different developmental stages

Figure 7 Cluster analysis of expression patterns of *ASAT* genes responding to hormone treatments in rice
Note: A: Underground root; B: Above-ground seedlings

Figure 8 Cluster analysis of expression patterns of *OsASAT* genes responding to abiotic stress

2 Discussion

Plant is famous for large and diverse chemical metabolites. Steryl ferulate, as an important secondary metabolite in plants, plays an important role in maintaining membrane lipid homeostasis, regulating membrane permeability, fluidity, signal transduction and membrane binding enzyme activity. For rice, steryl ferulate is an important component of rice bran oil. It is an important topic in rice bran oil metabolic biology to study the protease function and heredity and evolution of steryl ferulate metabolism pathway. Based on the chemical structure of steryl ferulate, it is inferred that OsASAT enzyme may be involved in the synthesis of steryl ferulate. So far, only ASAT enzymes from *Arabidopsis* and tomato have been cloned and identified, which are involved in sterol ester synthesis and sterol metabolism. The function of ASAT in other plants has not been reported. In addition, the synthesis and metabolic pathway of phytosteryl ferulate have not been revealed. The study on the evolutionary characteristics, expression patterns and biological functions of ASAT family molecules in different species is helpful not only to the cloning and functional identification of *ASAT* genes in other species, but also to the

understanding of rice bran oil biosynthesis pathway. In this study, we identified 96 ASAT family members from public genome database of 8 plant species including Arabidopsis and tomato, and focused on the bioinformatics analysis of 14 rice ASAT (OsASAT) proteins.

We constructed phylogenetic tree of 105 ASAT protein family members including Arabidopsis thaliana and tomato. The results showed that the origin and evolution of Arabidopsis AtASAT1 was more similar to peanut, rape and rice. The number of OsASAT protein is more than that of SIASAT, and both of them are scattered in the evolutionary tree. However, the genetic distance between tomato SIASAT1 and Arabidopsis AtASAT1 is farther, and the genetic distance between OsASAT2, OsASAT3, OsASAT4, OsASAT5, OsASAT6 and OsASAT9 in rice is closer to Arabidopsis AtASAT1. OsASAT1, OsASAT7 and OsASAT13, which are close to tomato SIASAT1. It is speculated that these proteins may have functions similar to sterol acyltransferases. On the other hand, there is good homology between OsASAT and ASAT proteins of maize and sorghum in food crops, and the time of evolution divergence is closer. The members of ASAT family identified in monocotyledons and dicotyledons showed good homology. Also, there are obvious clustering phenomena of ASAT proteins in typical oil crops and food crops, indicating that the ASAT protein family may play a certain role in the evolution or differentiation of oil crops and food crops. It should be noted that there is no one-to-one correspondence between the number and homology of plant ASAT protein family members, suggesting that ASAT is involved in the differentiation of different species and has experienced different evolutionary processes.

The OsASAT protein localization is diverse, and chloroplast and plasma membrane localization account for the largest proportion, which is similar to the results in Arabidopsis and tomato (Chen et al., 2007; Lara et al., 2018). At present, it has been confirmed that the biosynthesis of phytosterol and ferulic acid occurs in the cytoplasm (Suzuki and Muranaka, 2007; Li et al., 2008; Saulnier et al., 2012). But the enzyme system involved in the esterification of ferulic acid and sterol is not clear. And our analysis can provide a reference for the cellular localization of sterol ferulic acid esterification. We analyzed the synonymous and non synonymous sites of OsASAT coding genes, and concluded that OsASAT coding genes tended to purify selection. The appearance of ASAT gene cluster structure in different species suggests that the ASAT family coding genes may be replicated in the process of evolution, and then the new gene sequence and protein structure will be changed correspondingly through recombination and mutation, and finally show functional diversity. Gene clustering has a selective advantage in evolution, and it has been proved that gene clustering plays an important role in rice seed dormancy and resistance (Ye et al., 2020). The evolutionary characteristics of the clustering of ASAT coding genes in rice may be related to their specialized biological functions.

The molecular weight and secondary structure of OsASAT and AtASAT1 proteins are similar, indicating that the ASAT family members are conserved in evolution, which is consistent with the above results of synonymous and non synonymous sites analysis. The analysis of transmembrane helix structure showed that OsASAT protein was probably located in the membrane related structure. On the one hand, it complemented the results of subcellular localization. On the other hand, it might correspond to the characteristics of OsASAT protein, such as alkaline, high fat solubility and high instability index. It should be noted that 7 motifs of OsASAT protein sequence are identical to Arabidopsis AtASAT1, and motif 1 is a MBOAT superfamily domain. MBOAT family members include several key enzymes in lipid metabolism and protein acyltransferases involved in signal transduction, such as ASAT acyl-CoA: DGAT, LAT, and GOAT. These results provide a valuable molecular reference for the study of the function of OsASAT protein involved in lipid metabolism, especially in rice bran oil metabolism.

The structure of *OsASAT* genes varies greatly among individuals, but the genes with close evolutionary distance have similar structure and expression patterns, such as *OsASAT1* and *OsASAT4*. Consistent with the reports of other species (Chen et al., 2007; Lara et al., 2018), different *OsASAT* genes have different expression specificity in tissues and developmental stages, which proves that *OsASAT*, like *ASAT* genes in Arabidopsis and tomato, not only participates in the esterification of phytosterols and acids, maintains sterol homeostasis, but also affects the growth and development of plant nutrition and reproductive organs. On the other hand, the cis-acting element

found in the promoter region of the *OsASAT* gene family indicates that *OsASAT* plays an important role in both biotic and abiotic stress responses in rice, which is similar to the reported result that *SLASAT* has dual response to plant stress.

Based on all analysis data, especially the spatiotemporal expression data, and compared with Arabidopsis AtASAT1 and tomato ASAT, we speculated that OsASAT4, OsASAT5, OsASAT9, OsASAT10, OsASAT11 and OsASAT14 are most likely to be involved in the synthesis of rice bran oil. Study on the follow-up function of single enzyme evolution will provide a solid theoretical basis for further study of the analysis of rice bran oil metabolic pathway.

3 Materials and Methods

3.1 Data download and identification of Plant ASAT family members

AtASAT gene information was downloaded from the Arabidopsis information resource (TAIR, <http://www.arabidopsis.org/>), rice gene and protein sequence were downloaded from RiceData (<http://rice.plantbiology.msu.edu/>). The proteome sequences, genome sequences and gene annotation files of the other 8 species were derived from National center of biotechnology information (NCBI, <https://ftp.ncbi.nlm.nih.gov/genomes/>). Using Arabidopsis AtASAT1 protein sequence as query, ASAT homologous proteins (E-value= $1e^{-10}$) of other species were searched by Blast 2.5.0 software. After the primary ASAT family members were obtained, the redundant sequences were removed by tblastn. At the same time, the preliminary functional domains of the initial protein sequences were detected and analyzed by CDD website (<http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) and SMART website (<http://smart.embl-heidelberg.de/>), and finally the candidate members of ASAT family were screened. The ASAT homologous protein named as "the abbreviation of the first letter of the Latin name of the species+ASAT+number" according to its position on the chromosome.

3.2 Analysis of rice ASAT family proteins

The molecular weight and theoretical isoelectric point of the protein were predicted by ComputePI/MW on the online website ExPASy (https://web.expasy.org/compute_pi/). The first-order physical and chemical properties (such as the number of charged residues, total atoms, instability index, aliphatic index, and Hydrophobic index) were predicted by online software ProtParam (<https://web.expasy.org/protparam/>). The prediction of protein secondary structure was completed by SOPMA online tool (https://npsaprabhi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). Transmembrane structure was finished by TMHMM website (<http://www.cbs.dtu.dk/services/TMHMM/>). Protein signal peptides were detected by SignalP-5.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Phosphorylation sites were detected by NetPhos3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/>). Protein subcellular localization was completed by the integration of TargetP (<http://www.cbs.dtu.dk/services/TargetP/>), ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>) and WoLF PSORT prediction results. The amino acid sequence alignment of ASAT protein domain was completed by MEME (<http://meme-suite.org/>) online software Cluster W2.

3.3 Chromosome location, gene structure and promoter analysis of rice *ASAT* gene

The chromosome location of *OsASAT* gene was mapped by TBtools software. The structure of *OsASAT* gene was analyzed by online software GSDS (<http://gsds.cbi.pku.edu.cn/>). Gene synonymous sites were analyzed by software DnaSP (<http://www.ub.edu/dnasp/>). The analysis of cis-acting elements of promoter was completed by online software PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

3.4 Construction of phylogenetic tree of ASAT protein family

MEGA-X software (Kumar et al., 2018) was used to construct the phylogenetic tree of ASAT family proteins of 9 species. ClustalW2 was used to carry out multiple sequence alignment of protein sequence, with parameter of Gap Opening Penalty=10, Gap Extension Penalty=0.2, Delay Divergent Cutoff=30%. Then the maximum likelihood method was used to construct the phylogenetic tree, and with the parameter of Bootstrap=1000.

3.5 Gene expression and cluster analysis of rice ASAT family

We obtained the gene expression information of rice *ASAT* gene in hormone treatment, abiotic stress, different developmental stages and different tissues (including embryo and endosperm) through RiceXPro (<http://ricexpro.dna.affrc.go.jp/category-SEselect.php>), rice expression database AIPuFu (www.aipufu.com), and genevestigator platform (<https://genevestigator.com/>). And we derived expression map through platform.

Authors' contributions

SWL was the designer and executor of this study, completed the data analysis, drafted the manuscript, and participated in the experiment design and analysis. SJX conceived and directed of the study, guided experiment design, data analysis, and draft and revision of the manuscript. Both authors read and approved the final manuscript.

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