Bioinformatics and Expression Analysis of Acid Invertase LcSAI in Litchi (Litchi chinesis Sonn.) Fruit: Postprint

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Abstract

Acid invertase, as a key enzyme in sucrose metabolism, exhibits significantly higher expression and enzymatic activity in reducing sugar-accumulating lychees than in sucrose-accumulating lychees. To comprehensively elucidate the biological characteristics of acid invertase, this study employed bioinformatics approaches to systematically analyze the fundamental physicochemical properties, protein secondary structure, hydrophilicity/hydrophobicity, transmembrane domains, signal peptide, phosphorylation sites, conserved domains, and phylogeny of lychee fruit acid invertase LcSAI; and utilized qRT-PCR technology to examine LcSAI expression in various tissues of 'Feizixiao' and at different fruit developmental stages. The results revealed that lychee fruit acid invertase is a hydrophilic, unstable protein localized in the vacuole, lacking a signal peptide; its protein secondary structure is predominantly composed of random coils and extended strand motifs distributed throughout the entire protein; it possesses a transmembrane region at the N-terminus containing two conserved domains—the N-terminal Pfam DUF3357 domain and Glyco_{32} domain—belonging to the glycoside hydrolase family 32 superfamily; phylogenetic analysis demonstrated homology with the longan acid invertase gene; LcSAI expression levels across different tissues followed the hierarchy: male flower > root > young stem > seed > young leaf > female flower > pericarp > old leaf, with stage-specific expression patterns during fruit development. This study provides a data basis for further investigation into the regulatory mechanism of the LcSAI fruit acid invertase gene in the sucrose metabolic pathway.

Full Text

Bioinformatics and Expression Analysis of Acid Invertase LcSAI in Litchi (Litchi chinensis Sonn.) Fruit

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Abstract

Acid invertase serves as a key enzyme in sucrose metabolism, with significantly higher expression and enzyme activity observed in reducing sugar-accumulating litchi cultivars compared to sucrose-accumulating types. To comprehensively understand the biological characteristics of acid invertase, this study employed bioinformatics methods to systematically analyze the basic physicochemical properties, protein secondary structure, hydrophilicity/hydrophobicity, transmembrane domains, signal peptides, phosphorylation sites, conserved domains, and phylogenetic evolution of litchi fruit acid invertase LcSAI. Additionally, qRT-PCR was utilized to examine LcSAI expression patterns in different tissues and at various fruit developmental stages of 'Feizixiao' litchi. The results revealed that litchi fruit acid invertase is a hydrophilic, unstable protein localized to the vacuole, lacking a signal peptide. The protein's secondary structure consists primarily of irregular coils and extended strands distributed throughout the protein. LcSAI contains a transmembrane region at the N-terminus and two conserved domains: the Pfam DUF3357 domain and the Glyco_{32} domain, classifying it within the glycosyl hydrolase gene family 32 superfamily. Phylogenetic analysis indicated homology with longan acid invertase genes. Expression levels varied across tissues, following the pattern: male flower > root > young stem > seed > young leaf > female flower > peel > mature leaf, with stage-specific expression during fruit development. This study provides a data foundation for further investigation into the regulatory mechanisms of LcSAI in sucrose metabolism pathways.

Keywords: Litchi, acid invertase, bioinformatics, expression analysis

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Litchi (Litchi chinensis Sonn.) is a prestigious fruit in southern China, celebrated as the "King of Lingnan Fruits." Sugar content is intimately linked to fruit yield and quality, playing a critical role throughout fruit development and maturation. Litchi is a direct sugar-accumulating fruit, with sucrose, glucose, and fructose as the primary sugar components. Among these three sugars, fructose exhibits the highest sweetness—approximately three times that of glucose and 1.8 times that of sucrose—making their relative contents and ratios crucial determinants of fruit sweetness and flavor. Investigating sugar composition for-

mation characteristics is therefore essential for improving fruit intrinsic quality. Litchi pulp contains high sugar content that varies among cultivars; for instance, 'Nuomici' pulp sugar content accounts for 15.3% of fresh weight or 91.3% of dry weight, while 'Feizixiao' comprises 14.6% of fresh weight or 78.7% of dry weight.

Variations in sugar composition among litchi cultivars result from differential regulation by enzyme systems, whose activities correlate with gene expression patterns. Different litchi cultivars accumulate distinct primary sugars: 'Feizixiao' and 'Heiye' predominantly accumulate reducing sugars, whereas 'Wuheli' and 'Nuomici' primarily accumulate sucrose. These sugar profiles are regulated by sucrose metabolism-related enzyme activities (Wang et al., 2003; Yang et al., 2012). Research indicates that invertase is a key enzyme catalyzing sucrose cleavage into reducing sugars and fructose. 'Nuomici' litchi, which accumulates sucrose, shows almost undetectable acid invertase activity during fruit maturation, while 'Feizixiao', which accumulates reducing sugars, maintains high acid invertase activity, suggesting a close relationship between acid invertase activity and sugar accumulation patterns in litchi (Yang et al., 2013). Consequently, investigating the regulatory mechanisms of acid invertase in litchi fruit sugar accumulation holds significant importance.

Sturm et al. (1990) first cloned the acid invertase gene from carrot. With the rapid advancement of high-throughput sequencing technologies and bioinformatics, acid invertase genes have subsequently been isolated from numerous plants, including tomato (Sun et al., 2009), sugarcane (Niu et al., 2013), papaya (Yan et al., 2014), citrus (An et al., 2001), apple (An et al., 2003), goji berry (Wang et al., 2014), and dendrobium (Meng et al., 2017). However, systematic analysis of litchi acid invertase remains unreported. Based on our research group's transcriptome database of 'Feizixiao' fruit at different developmental stages (unpublished), we identified an acid invertase gene (Unigene0007246) containing a complete ORF. NCBI Blast alignment revealed 97.98% homology with the litchi acid invertase gene AFP23357.1 cloned from 'Nuomici' aril by Yang et al. (2013), with 13 single nucleotide differences, which we designated as LcSAI. To comprehensively understand the biological characteristics of acid invertase, this study employed bioinformatics and qRT-PCR techniques to systematically analyze the basic physicochemical properties, subcellular localization, protein secondary structure, hydrophilicity/hydrophobicity, signal peptides, transmembrane structures, phylogenetic evolution, and expression patterns of LcSAI in different tissues and fruit developmental stages, aiming to provide data support for future functional studies and applications of LcSAI.

1.1 Plant Materials

The experimental material consisted of 'Feizixiao' litchi harvested from the litchi orchard at the Institute of South Subtropical Crop Research, Chinese Academy of Tropical Agricultural Sciences. The litchi fruit acid invertase gene LcSAI (Unigene0007246) was retrieved from our research group's transcriptome database of 'Feizixiao' fruit at different developmental stages. The full-length

Unigene0007246 spans 2,226 bp, with a complete CDS of 1,929 bp encoding 643 amino acids.

1.2 Bioinformatics Analysis of Litchi Acid Invertase LcSAI

The basic physicochemical properties of LcSAI were predicted using the online ExPASY ProtParam tool (https://web.expasy.org/protparam/). Subcellular localization was analyzed using Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/Plant-multi/). Protein secondary structure prediction was performed using the SOPMA Secondary Structure Prediction Method (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=npsa_{sopma}.html). Hydrophilicity/hydrophobicity was analyzed using ExPASY ProtScale (https://web.expasy.org/protscale/). Signal peptide prediction was conducted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane structure analysis employed TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Phosphorylation sites were predicted using NetPhos (http://www.cbs.dtu.dk/services/NetPhos/). Conserved domains were analyzed using NCBI's Conserved Domain Architecture Retrieval Tool (https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps). Homology of the litchi acid invertase LcSAI sequence was compared via NCBI Blast. Multiple sequence alignment of candidate sequences was performed using ClustalX 1.83 software, followed by phylogenetic tree construction using MEGA 6.0 software with the Neighbor-Joining method to analyze evolutionary relationships with other species.

1.3 Expression Analysis of LcSAI in Different Tissues and Fruit Developmental Stages

Samples of different 'Feizixiao' tissues were collected, including young leaves from new shoots, mature leaves from fully developed shoots, stamens and pistils during flowering, young roots, and peel, pulp, and seeds from mature fruits. For fruit developmental stage expression analysis, pulp collection began on April 11, 2019 (1 week after anthesis) and continued weekly until fruit maturity on May 30, 2019 (8 weeks), totaling eight sampling time points. All samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA from different tissues was extracted using the Huayueyang Plant RNA Extraction Kit, and first-strand cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa). The qRT-PCR primers for LcSAI were LcSAI-qF: 5'-TCAGCAGGTGAGGAAGAAGG-3' and LcSAI-qR: 5'-TCAGGAGCCAATGTTGACCT-3', with LcActinFW: 5'-GTGGTTCTACTATGTTCCCTG-3' and LcActinRE: 5'-CTCGTCGTACTCATCCTTTG-3' serving as internal controls. Primers were synthesized by Guangzhou Aiji Biotechnology Co., Ltd. qRT-PCR was performed on a Roche 480 II instrument using SYBR Premix Ex TaqTM (TaKaRa) in 96-well plates with a 20 L reaction volume, generating 200 bp amplicons. The thermal cycling program consisted of initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Each sample was analyzed in

triplicate. Relative gene expression levels were calculated using the 2^-ΔΔCT method.

2.1 Basic Physicochemical Properties and Secondary Structure Analysis of LcSAI Protein

ExPASY analysis predicted the basic physicochemical properties of LcSAI. The protein comprises 643 amino acids with a molecular weight of 72,005.18 Da, a molecular formula of $C_{3270}H_{4944}N_{850}O_{962}S_{14}$, and a theoretical isoelectric point of 5.18. The estimated half-life is 30 hours, with an instability index of 39.30, classifying it as a stable protein. The aliphatic index is 83.69, and the grand average of hydropathicity is -0.262, indicating a hydrophilic nature. Leucine (Leu) is the most abundant amino acid at 9.2%, while cysteine (Cys) is the least abundant at 0.5%. Charged amino acid analysis revealed 71 negatively charged residues (Asp+Glu) and 47 positively charged residues (Arg+Lys) [Figure 1: see original paper]. Plant-mPLoc predicted vacuolar localization for LcSAI. The secondary structure comprises α-helices, β-sheets, irregular coils, and extended strands [Figure 2: see original paper], with irregular coils being most prevalent (359 aa, 55.83%), followed by extended strands (152 aa, 23.64%), α-helices (94 aa, 14.62%), and β-sheets being least common (38 aa, 5.91%).

Hydrophilicity/hydrophobicity analysis using ProtScale (Kyte & Doolittle algorithm, where >0 indicates hydrophobicity and <0 indicates hydrophilicity) showed that LcSAI possesses distinct hydrophobic and hydrophilic regions. Although the amino acid scores at positions 43 and 44 (3.156) were higher than the minimum value at position 395 (-2.689), the number of hydrophilic amino acids (392) exceeded hydrophobic residues (243), consistent with primary structure predictions and confirming its hydrophilic nature.

2.2 Signal Peptide Prediction and Transmembrane Structure Analysis of LcSAI Protein

Signal peptides are typically short peptide chains located at the N-terminus of amino acid sequences, generally 5-30 amino acids in length. SignalP 4.1 Server analysis using the euk network algorithm predicted that LcSAI lacks a signal peptide [Figure 3: see original paper], classifying it as a non-secretory protein synthesized in the cytoplasm without protein transport function.

Transmembrane regions are protein segments that span the cell membrane, typically forming α-helical structures of approximately 20-25 amino acid residues composed mainly of hydrophobic amino acids. TMHMM analysis revealed one transmembrane region in LcSAI at amino acid positions 43-63 [Figure 4: see original paper].

2.3 Phosphorylation Site and Conserved Domain Analysis of LcSAI Protein

NetPhos 3.1 Server analysis identified potential protein kinase phosphorylation sites, including 30 serine (Ser), 26 threonine (Thr), and 10 tyrosine (Tyr) residues [Figure 5: see original paper]. Conserved domain analysis using NCBI's Conserved Domain Architecture Retrieval Tool revealed two conserved domains: the N-terminal Pfam DUF3357 domain (positions 13-117) and the Glyco_{32} domain (positions 125-593), placing LcSAI within the glycosyl hydrolase gene family 32 superfamily [Figure 6: see original paper].

2.4 Phylogenetic Relationship Analysis of LcSAI

NCBI Blast analysis revealed that litchi LcSAI shares amino acid sequence homology with acid invertases from longan (AKJ70978, 95.18%), lacquer tree (AHB33921, 79.53%), citrus (XP_{024046763}, 77.81%), rubber tree (XP_{021687094}, 77.8%), cassava (AFH77956, 77.48%), peach (XP_{007218854}, 70.85%), cacao (XP_{017972919}, 75.43%), apple (AFU56882, 69.94%), pear (BAG30919, 70.35%), and grape (XP_{002265534}, 65.2%). Multiple sequence alignment of LcSAI with these species demonstrated high conservation, though the 5' N-terminal Pfam DUF3357 domain (13-117) showed lower conservation, and the Glyco_{32} domain (125-593) exhibited some variation among species. Phylogenetic tree construction using MEGA 6.0 [Figure 7: see original paper] indicated that LcSAI is most closely related to longan SAI from the same Sapindaceae family, representing orthologous genes. Apple, pear, and peach SAI proteins from Rosaceae clustered together, indicating close relationships, while cassava and rubber tree SAI from Euphorbiaceae also showed close phylogenetic affinity.

2.5 Expression Analysis of LcSAI in Different Tissues and Fruit Developmental Stages

qRT-PCR analysis revealed that LcSAI expression varied across 'Feizixiao' litchi tissues, with highest expression in male flowers, followed by roots, young stems, young leaves, and seeds, while female flowers, peels, and mature leaves showed relatively low expression [Figure 8: see original paper]. During fruit development, LcSAI expression in pulp peaked at week 2, declined sharply at week 3, gradually increased during weeks 4-6, and reached its minimum at week 8 [Figure 9: see original paper].

3 Conclusion and Discussion

Acid invertases are multi-gene encoded proteins that regulate vacuolar sink activity, osmotic pressure, sugar signaling, sucrose accumulation, sucrose concentration, stress responses, hormones, and cell elongation (Lu et al., 2017; Chikov et al., 2015; Zhang et al., 2014; Kulshrestha et al., 2013). Previous studies have

employed bioinformatics to systematically analyze acid invertase genes in goji berry (Lei et al., 2014; Wang et al., 2014), papaya (Yan et al., 2014), sugarcane (Niu et al., 2013), and dendrobium (Meng et al., 2017). In this study, we conducted comprehensive bioinformatics and expression analyses of the cloned litchi fruit acid invertase, predicting its basic physicochemical properties, subcellular localization, transmembrane structure, signal peptide, phosphorylation sites, and phylogenetic evolution. LcSAI is a hydrophilic protein containing one transmembrane structure and lacking a signal peptide, consistent with findings in sugarcane (Niu et al., 2014), goji berry (Lei et al., 2014), and papaya (Yan et al., 2014). The protein contains two conserved domains: the N-terminal Pfam DUF3357 domain (13-117) and the Glyco_{32} domain (125-593), classifying it within the glycosyl hydrolase gene family 32 superfamily. LcSAI shows high amino acid sequence similarity (85-95%) with longan and citrus sequences. Neighbor-Joining phylogenetic analysis revealed that litchi SAI is orthologous to longan acid invertase, while showing more distant relationships with apple, pear, peach, and grape.

Acid invertase genes exhibit constitutive expression across different developmental stages and tissues in plants, though expression levels vary among tissues. For example, goji berry LbSAI expression follows the pattern: flower > fruit stalk > fruit > stem > leaf > root (Wang et al., 2014). In dendrobium, SAI expression varies significantly across tissues and developmental stages, decreasing annually in leaves, increasing yearly in roots, and peaking in stems during the second year (Meng et al., 2017). Sugarcane SoSAI1 shows higher expression in immature and mature leaves than in old leaves from the elongation stage to early physiological maturity, while stem expression generally follows the pattern: young stem > mature stem > old stem (Niu et al., 2014). Our study demonstrates that LcSAI is expressed in all tissues but shows tissue-specific expression levels, with highest expression in male flowers, roots, seeds, young stems, and young leaves, and lowest expression in female flowers, mature leaves, and peel. LcSAI expression also exhibits stage-specific patterns during fruit development. These results align with sugarcane studies, where higher LcSAI expression in young tissues correlates positively with high acid invertase activity in rapidly growing young tissues and organs (Niu et al., 2014). This systematic bioinformatics and expression analysis of LcSAI in 'Feizixiao' based on transcriptome data establishes a foundation for future functional studies of litchi SAI genes and sugar accumulation mechanisms.

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Note: Figure translations are in progress. See original paper for figures.