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Article

Integrating QTL mapping and transcriptomics to decipher the genetic architecture of sterol metabolism in *Brassica napus* L

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Abstract

Sterols are secondary metabolites commonly found in rapeseed that play crucial physiological roles in plants and also benefit human health. Consequently, unraveling the genetic basis of sterol synthesis in rapeseed is highly important. In this study, 21 individual sterols as well as total sterol (TS) content were detected in a double haploid (DH) population of *Brassica napus*, and a total of 24 quantitative trait loci (QTL) and 157 mQTL were identified that were associated with TS and different individual sterols. Time-series transcriptomic analysis showed that the differentially expressed genes (DEGs) involved in sterol and lipid biosynthesis pathways were enriched. Additionally, a regulatory network between sterol-related DEGs and transcription factors (TFs) was established using coexpression analysis. Some candidate genes were identified with the integration of transcriptomic analysis and QTL mapping, and the key candidate gene *BnSQS1.C03* was selected for further functional analysis. *BnSQS1.C03* demonstrated squalene synthase activity *in vitro* and increased the TS by 3.8% when overexpressed in *Arabidopsis*. The present results provide new insights into sterol regulatory pathways and a valuable genetic basis for breeding rapeseed varieties with high sterol content in the future.

Introduction

Brassica napus L. (AACC, $2n = 38$) is of great significance in the food and chemical industries [1], with ~70 million tons of rapeseed produced annually worldwide [2]. Previous studies have revealed that most agronomic characteristics of *B. napus* are quantitative traits, and their genetic mechanisms have frequently been studied using QTL analysis [3–5]. In addition to the genetic improvement of agronomic traits, the genetic enhancement of secondary metabolites, such as vitamins, glucosinolates, carotenoids, and phytosterols, has been increasingly garnering attention in recent years, particularly in *B. napus*, due to their health-promoting properties [6, 7].

Studies have shown that phytosterols can decrease low-density lipoprotein cholesterol and change sterol formation of human body [8, 9]. Phytosterols have also been shown to have anti-inflammatory [10, 11], cytoprotective [11], and cardiovascular protective effects [12]. Some special sterols have been confirmed to have significant health effects. For example, squalene is commonly used as an auxiliary medicine and as a natural cardiovascular protection drug [13, 14]. The antitumor activity of phytosterols have also been reported. For example, brassicasterol, a specialized sterol in rapeseed, has been shown to possess antiviral and anticancer functionalities [15]. Phytosterols are present in vegetable oils with varied concentrations and compositions and have been detected in rice bran oil, corn oil, and rapeseed oil [16, 17]. Therefore, increasing the phytosterol content might represent a new method to enhance the quality of edible oils [18, 19]. Phytosterols also have important physiological functions in plants; for example, they are important components of membrane structures [20, 21]. Sterols are precursors for brassinosteroid (BR) synthesis [22], and some sterols can also produce BR-independent effects [23, 24].

A correlation between sterol synthesis and lipid synthesis has been reported, revealing that the sterol upstream synthesis genes 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (*HMG1*) and acetyl-CoA carboxylase 1 (*ACC1*) are coregulated by adenosine 5′-monophosphate (AMP)-activated protein kinase (*AMPK*) [25]. The overexpression of fatty acid synthesis-related genes (including *ACC1*) can induce the expression of genes involved in several sterol biosynthetic pathways, resulting in increased levels of multiple sterols [26]. In addition, sterol is also involved in oil body formation, lipoglobulin synthesis, and lipid accumulation due to the presence of sterols in the membrane structure, which form lipid rafts and sterol ester vesicles on the membrane [27]. Genetic molecular analyses of sterols were performed in previous studies. For example, 45 QTL associated with sterol content in sunflower

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for the ortholog of *AtFK* on ChrA04). The orthologous of candidate genes and their annotation were obtained by BLASTn based on *A. thaliana* database (http://www.arabidopsis.org/).

Sterol content-related genes were collected from Gene Ontology database (http://geneontology.org/) by searching the keywords such as "sterol biosynthetic process", "sterol metabolic process", "sterol transport process", and "terpenoid biosynthesis". Candidate genes in the CIs of QTL were screened using the collected sterol-related genes to identify potentially important target genes within the intervals. Visualization of QTL intervals and candidate genes was conducted using Circos software [111].

Gene expression analysis of seeds during different developmental stages

Thirty lines with relatively higher TS and 30 lines with relatively lower TS in KN DH population were selected, respectively. Seeds from these sixty lines at 28 dpf, 35 dpf, and 42 dpf were collected and stored at −80°C for further transcriptomic analysis. Three replications were conducted for all the materials, resulting in a total of 18 samples that were sent for RNA sequencing in Qiantang Biotech Corporation (Suzhou, Jiangsu Province, China).

The original data were processed to remove the reads of low quality and joint contaminations to ensure the reliability of the results. HISAT was used to align clean reads to the reference genome (ZS11) [112]. Bowtie2 was utilized for aligning clean reads to the reference sequence, followed by RSEM for calculating the expression levels of genes and transcripts. According to the requirements, maSigPro [113] and DEseq2 [114] algorithms were applied to detect the time-series DEGs.

Potential TFs of sterol-related DEGs were identified using the TF regulation network module of the BnIR (http://yanglab.hzau.edu.cn/BnIR) website. Cytoscape was employed to construct the network of TFs and their functional target genes. The degree value of each node, representing the number of associations with other nodes, was obtained from Cytoscape. Enrichment analysis and visualization of KEGG and GO were performed using clusterProfiler and ggplot2 in R software [115]. A regulatory map was constructed based on the pathway of *A. thaliana* in the KEGG pathway database (https://www.genome.jp/kegg/pathway.html).

cDNA cloning and sequence alignment

In the present study, the coding sequence (CDS) region of the candidate gene *BnSQS1.C03* was cloned from cDNA in two parents (KenC8 and N53–2) of mapping population. The CDS of *BnSQS1.C03* was cloned using the PMD18-T vector and *Escherichia coli* (*E. coli*) DH5α. Homologous sequences were aligned using the BLAST function of the online Brassica database (http://brassicadb.cn/#/BLAST/). Nucleotide sequence translation, alignment, and amino acid sequence alignment were performed using MEGA 7. Gene structure was analyzed and mapped using the gene structure display server (GSDS), an online software program (http://gsds.gao-lab.org/). Promoter cis-acting element analysis and amino acid sequence motif analysis were performed using the corresponding prediction tools from the MEME (https://meme-suite.org/meme/tools/meme) and PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The sequences of all primers used in the study are listed in Table S14.

Expression of soluble recombinant forms of *BnSQS1.C03* in *E. coli*

To express *BnSQS1.C03* in *E. coli*, the following steps were performed: The CDS of *BnSQS1.C03* was inserted into the pET28a vector, and the recombinant plasmid was then transformed into BL21(DE3) *E. coli* cells. The transformed cells were cultured in 2 × YT medium (containing 16 g/L yeast extract, 16 g/L tryptone, 5 g/L sodium chloride) with kanamycin. Induction was initiated with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C. After centrifugation and resuspension in buffer A (containing 0.27 g/L potassium dihydrogen phosphate, 1.42 g/L disodium hydrogen phosphate, 8 g/L sodium chloride, 0.2 g/L potassium chloride, and adjusted to pH 7.4 with concentrated hydrochloric acid). Cell lysis was achieved by sonication. The insoluble fraction was discarded, and the supernatant was purified using Ni-agarose resin, followed by elution with imidazole gradients. The purified proteins were analyzed by SDS-PAGE and concentrated using an ultrafiltration centrifuge.

Assay for SQS activity

The SQS activity assay was performed as described with minor modifications [65]. The reaction mixture, totally 100 μL, comprised of 25 μM FPP, 2 mM NADPH, 10 mM dithiothreitol, 5 mM $MgCl_2$, 15 μg squalene synthase (SQS), and 0.1 M PBS buffer (containing 0.027 g/L potassium dihydrogen phosphate, 0.142 g/L disodium hydrogen phosphate, 0.8 g/L sodium chloride, 0.02 g/L potassium chloride). The reaction was incubated for 30 minutes at 30°C and then terminated by boiling for 5 minutes. The sample was extracted with *n*-hexane, concentrated, and dissolved in acetonitrile. Detection was performed using an Agilent 1200 LC with a VWD detector, on a ZORBAX Eclipse C18 column, using 100% acetonitrile as the mobile phase at room temperature, with a detection wavelength of 210 nm.

Subcellular localization experiment

The CDS of *BnSQS1.C03* was cloned into the pCAMBIA-1303 plasmid, which contains the red fluorescent protein (mCherry) gene at the C-terminus, and the expression is driven by the *Cauliflower Mosaic* Virus (CaMV) 35S promoter. The subcellular localization vector was transformed into the *Agrobacterium tumefaciens* strain GV3101, and then it was infiltrated into the leaves of *Nicotiana benthamiana* following the previously reported procedure [116]. The fluorescent signal was detected using an FV1000 laser scanning confocal microscope (Olympus, Japan).

Vector construction and transformation into Arabidopsis

The wild-type *A. thaliana* that used in the experiment was Columbia-0 (Col-0). The T-DNA insertion mutant SALK_077057C of the homologous gene *AtSQS1* in *A. thaliana* was purchased from the Arashare website (http://www.arashare.cn). The *BnSQS1.C03* gene was integrated into the pCAMBIA-1303 vector through homologous recombination, with gene expression driven by the CaMV35S promoter. The recombinant vector was transformed into *A. tumefaciens* strain GV3101 by electroporation. The flower-dip method was used to transform the gene into Columbia-0 and SALK_077057C. Homozygous T2 and T3 generation transgenic lines were used for subsequent analysis.

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Author contributions

M.L., H.J., and L.Z. conceived and designed the project. Y.X. and G.L. performed the experiments and analyzed the data. Y.X. wrote the

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manuscript. M.L. and H.J. revised the manuscript. H.L., P.F., and S.Y. provided assistance during the experiment, and J.H. helped with the visualization of the data.

Data availability

The data that support the findings of this study are accessible in the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) with the accession number PRJNA1078081.

Conflict of interest statement

The authors declare that they have no conflict of interests.

Supplementary Data

Supplementary data is available at Horticulture Research online.

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