**Stem Lodging Resistance-1 controls stem strength by positively regulating the biosynthesis of cell wall components in *Capsicum annuum* L.**

Qing Li1,2,5, Canfang Fu1,2, Bozhi Yang1,2, Huiyang Yu1,2, Huan He1,2, Qing Xu1,2, Wu Miaoj, Rongyun Liu3, Wenchao Chen4, Zhuqing Zhang4, Xuexiao Zou1,2,*, Bowen Hu1,2,*, Lijun Ou1,2,*

1 Engineering Research Center of Education, Ministry for Germplasm Innovation and Breeding New Varieties of Horticultural Crops, Key Laboratory for Vegetable Biology of Hunan Province, College of Horticulture, Hunan Agricultural University, Changsha 410125, China; liqing01@caas.cn (L.Q.); fucanfang-24@stu.hunau.edu.cn (F.F.C.); yangbozhi@hunau.edu.cn (Y.B.Z.); yuhuiyang1234@126.com (Y.H.Y.); hh-17872310735@stu.hunau.edu.cn (H.H.); xuqing979693@stu.hunau.edu.cn (X.Q.).

2 Yuelushan Lab, Changsha 410128, China; liqing01@caas.cn (L.Q.); fucanfang-24@stu.hunau.edu.cn (F.F.C.); yangbozhi@hunau.edu.cn (Y.B.Z.); yuhuiyang1234@126.com (Y.H.Y.); hh-17872310735@stu.hunau.edu.cn (H.H.); xuqing979693@stu.hunau.edu.cn (X.Q.).

3 Hunan Xiangyan Seed Industry Co., Ltd, Changsha, 410100, China; miaowu55555@163.com (M.W.); lry@lpht.com.cn (L.R.Y.).

4 Vegetable Research Institute, Hunan Academy of Agricultural Science, Changsha, 410125, China; chenwenchao@hunaas.cn (C.W.C.); zhangzhuqing@hunaas.cn (Z.Z.Q.).

5 Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Key Laboratory of Synthetic Biology, Ministry of Agriculture and Rural Affairs, Agricultural Genomics Instituteate Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, 518120, China; liqing01@caas.cn (L.Q.).

Corresponding author.

E-mail: ou9572@hunau.edu.cn (O.L.J.); hubowen@hunau.edu.cn (H.B.W.); zouxuxexiao@hunau.edu.cn (Z.X.X.).

Tel: 86-17872349212 (O.L.J.); 86-15974232817 (H.B.W.); 86-13807481788 (Z.X.X.).

© The Author(s) 2024. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution License https://creativecommons.org/licenses/by/4.0/, which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Abstract

Lodging presents a significant challenge in cultivating high-yield crops with extensive above-ground biomass, yet the molecular mechanisms underlying this phenomenon in the Solanaceae family remain largely unexplored. In this study, we identified a gene, CaSLR1 (Capsicum annuum Stem Lodging Resistance 1), which encodes a MYELOBLASTOSIS (MYB) family transcription factor, from a lodging-affected Capsicum annuum EMS mutant. The suppression of CaSLR1 expression in pepper led to notable stem lodging, reduced thickness of the secondary cell wall, and decreased stem strength. A similar phenotype was observed in tomato with the knockdown of SlMYB61, the orthologous gene to CaSLR1. Further investigations demonstrated that CaNAC6, a gene involved in secondary cell wall (SCW) formation, is co-expressed with CaSLR1 and acts as a positive regulator of its expression, as confirmed through yeast one-hybrid, dual-luciferase reporter assays, and electrophoretic mobility shift assays. These findings elucidate the CaNAC6-CaSLR1 module that contributes to lodging resistance, emphasizing the critical role of CaSLR1 in the lodging resistance regulatory network.

Keywords: Pepper; Stem Lodging Resistance1; cell wall components; stem strength.

Introduction

Stems are essential for plant growth and development, as they not only facilitate the transport of water and nutrients but also provide critical structural support. Stem lodging can drastically affect crop production, significantly reducing yield and quality while increasing harvesting costs (Li et al., 2022). Consequently, research into stem lodging resistance (SLR) is vital for advancing sustainable agricultural practices. Many studies have shown that lodging resistance is closely related to the thickness of the SCW (Fan et al., 2017; Liu et al., 2018; Li et al., 2022). Cellulose, hemicellulose and lignin are the main components of the cell wall, enhancing the mechanical strength of plants (Zhang et al., 2021; Coen & Cosgrove, 2023). In the brittle stem mutant of rice, there was a notable decrease in the stem’s mechanical strength and its cellulose content (Fan et al., 2018). Lignin is considered an important factor in lodging resistance across different varieties (Li et al., 2021). Enhancing the expression of cell wall structural genes like 4-coumarate: CoA ligase 3 (4CL3) and phenylalanine ammonia-lyase (PAL) within the lignin biosynthesis pathways bolstered cell wall thickening, thus reinforcing mechanical support in rice (Liu et al., 2018). Therefore, stem cell wall components play critical roles in enhancing mechanical strength and maintaining stability in stems.

Previous studies have shown that secondary wall NACs (SWNs) transcription factors and MYB transcription factors (e.g. MYB46, MYB83) mediated transcription networks played key regulatory roles in plant SCW formation (Ardiyana & Rejab, 2015; Zhong et al., 2011). Studies on cotton and Arabidopsis thaliana have showed that concurrent disruption of secondary wall-associated NAC domain protein 1 (SND1) and NAC secondary wall thickening promoter 1 (NST1) results in stem droop, indicating functional redundancy among NAC transcription factors in stem development (Fang et al., 2020; Zhong et al., 2015; Zhong & Ye, 2015). Notably, inhibiting AtMYB46 alone can lead to a stem droop phenotype (Zhong et al., 2007), suggesting that this MYB TFs play a
crucial role in regulating stalk development. SWNs bind to specific promoter sequences, known as secondary NAC binding elements (SNBEs), in MYB46 and MYB83, thereby regulating SCW biosynthesis (Negi et al., 2017; Zhong et al., 2021). Nonetheless, our understanding of the pivotal role MYB TFs play in regulating plant cell wall development remains limited.

Pepper (Capsicum annuum L.) is an important vegetable crop due to its distinctive spicy components (Liu et al., 2017; Zou et al., 2020). However, during production, pepper often faces challenges such as flower and fruit drop after stem lodging, which severely impairs pepper’s yield and quality (Li et al., 2022). Despite its importance, research on stem lodging resistance in peppers is notably limited. In this study, we identified a distinct stem lodging mutant slr1 (stem lodging resistance 1) through ethylmethane sulfonate (EMS) mutagenesis. Genetic analysis revealed that the slr1 phenotype is likely governed by a recessive gene located on chromosome 8, encoding MYB61. Knockdown of CaSLR1 resulted in stem bending and lodging, a phenotype was also observed in CRISPR/Cas9-mediated knockout of SIMYB61, a homolog of CaSLR1 in tomato. Additionally, knockdown CaSLR1 led to the downregulation of numerous genes associated with cell wall formation, consequently causing thinner secondary cell walls and reduced stem strength. Furthermore, we identified a SCW formation-related gene, CaNAC6, co-expressed with CaSLR1, with CaNAC6 positively regulating the expression of CaSLR1. This suggests that CaSLR1 may serve as a central hub in the secondary cell wall (SCW) regulatory network, enhancing stem lodging resistance in pepper. Our study provides theoretical support for understanding the molecular regulatory mechanisms in stem development. It offers the target genes for molecular designing breeding programs aimed at enhancing lodging resistance in peppers and tomatoes.

Materials and methods

Plant materials and growth conditions
The elite inbred line ‘6421’, which serves as a foundational breeding stock extensively utilized across China, was treated with 1% EMS (medial lethal dosage) to construct a mutant library (Yang et al., 2016). We sown ten thousand mutants and two hundred WT seeds, and then individual plant was conducted self-cross. Next, we harvested the self-cross seeds from individual plant to construct the M1 generation, and selected M2 generation with stem lodging from M1 self-cross seeds. Using the same planting screening method as the M2 generation, we obtained a genetically stable stem lodging mutant slr1 M3 generation (Arisha et al., 2015). WT and slr1 plants were planted in a greenhouse with 65% relative humidity. Various tissues were gathered from the growing plants for gene expression analysis, including roots, stems, leaves, blooms, fruits, and anthers.

Scanning electron microscopy (SEM) and determination of stem components
Fresh tissues were collected, taking care to minimize mechanical damage, such as bruising, pulling, and squeezing. Base on a previously described method (Liu et al., 2023), the tissue blocks were placed in electron microscopy fixative for 2 h and then preserved at 4 °C for transportation. The tissues were observed with an SEM, and images were taken.
The 45-day-old stems from WT, slr1, pTRV2, and pTRV2-CaSLR1 lines were collected. Cellulose, hemicellulose, and lignin contents were determined using kits (Cellulose content kit product code: G0715W, hemicellulose content kit product code: G0716W, lignin content kit product code: G0708W48, Suzhou Grace Biotechnology Co. Ltd., Suzhou, China).

**Candidate gene mapping**

We identified the chromosome region of candidate target genes using MutMap method (Takagi et al., 2015). Leaf samples were taken from 30 of the WT and slr1 plants of the F2 segregating population. We extracted the genomic DNA (DNA extraction kit, Product code: DP360, Tiangen Biotech Co. Ltd., Beijing, China) of thirty upright and thirty lodging plants from F2 population, respectively (Zong et al., 2018). Next, we constructed two mixed DNA pools and performed the whole-genome resequencing on the HiSeq™ PE150 platform (Illumina, Inc., San Diego, CA, USA). Reads data were filtered by Fastp (Chen et al., 2018), and clean reads were mapped to the reference of ‘Zhangshugang’ (http://ted.bti.cornell.edu/ftp/pepper/genome/Zhangshugang/) genome (Liu et al., 2023) using bwa mem v0.7.12 (Li & Durbin, 2009a). Duplicated reads derived from PCR were marked with samblaster. The generated BAM file was utilized to identify single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) using GATK (McKenna et al., 2010). Structural variations were identified by manta (Chen et al., 2016). SNPs were filtered using bcftools (Danecek et al., 2017) with the parameters QD < 2.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0. The SNP-index value was calculated to locate the traits, with the wild-type (WT) parent selected as the reference parent to compute the SNP-index of the two offspring.

To fine-map the candidate genes, we detected SNP co-segregation via the Kompetitive Allele-Specific PCR (KASP) genotyping technique. Based on a previously described method (Wang et al., 2023), we designed allele-specific primers based on the 200 bp sequence upstream and downstream of the SNPs, and shown in the [Supplementary Table S1](#). A total of 483 F2 individuals were used for KASP genotyping. The SNP genotyping detection system PARMS (penta-primer amplification refractory mutation system) (Gentides Biotech Co., Ltd) was used according to the manufacturer's protocol. The primers were synthesized by the Gentides Biotech Co. Ltd (Wuhan, China). A 10 μl reaction system was prepared, including 5uL 2× PARMS (containing fluorescent universal primers FAM, HEX), 0.7uL specific amplification primers, 1uL (50ng) DNA template and 3.3 uL double distilled H2O. Signals were detected using the LightCycle® 96 Real-Time PCR system (Roche, Basel, Switzerland) (Wang et al., 2023).

**Phylogenetic tree construction and subcellular localization of CaSLR1**

MAFFT v7.475 was used (Rozewicki et al., 2019) for multiple CaSLR1 protein sequence alignment. Then, the default parameters and maximum likelihood of the IQ-TREE v2.1.2 software was used to construct the phylogenetic tree (Nguyen et al., 2015) with the bootstrap value set as 1000. Finally, Itol ([https://itol.embl.de/](https://itol.embl.de/)) was used to visualize the results.
Using One Step Cloning Kit (Product code: C112-02, Vazyme Biotech Co. Ltd., Nanjing, China), as cited by Liu et al. (2022), we insert the coding sequence (CDS) of CaSLR1 into the pYBA1132 expression vector to construct the pYBA1132-CaSLR1-GFP vector. The leaves of the 3-week-old Arabidopsis ‘Columbia’ plants were used for protoplast isolation. The pYBA1132-CaSLR1-GFP and the empty PYBA1132-GFP vectors were proliferated and extracted. They were then used to co-transform Arabidopsis protoplasts with a nuclear marker (Ghd-RFP) and cultured under low light conditions for 8-10 h. Fluorescence was observed and captured via an LSM800 laser scanning confocal microscope (Zeiss, Germany).

RT-qPCR analysis

Total mRNA was extracted using the TRIzol reagent (Product code:ET121-01, TransGen Biotech), and 2 μg of total RNA was reversed into cDNA via a reverse transcription kit (Product code: AG11701, Accurate Biology [Hunan] Co. Ltd., Changsha, China). RT-qPCR was implemented using SYBR Green (Product code: AG11701) (Zhang et al., 2023) on a LightCycle® 96 Real-Time PCR system (Roche, Basel, Switzerland). The actin gene expression, as an internal control, in pepper was normalized.

Virus-induced gene silencing (VIGS)

To decrease the expression of target genes in the WT, we conducted virus-induced gene silencing following a previously established method with slight modifications (Zhang et al., 2022). We identified specific regions of CaSLR1 via online tool SGN VIGS (https://vigs.solgenomics.net) (Song et al., 2023; Sun et al., 2020). Unique primers were used to amplify the cDNA of pepper stems, yielding a product of approximately 300 base pairs. Next, we used the One Step Cloning Kit (Product code: C112-02) (Liu et al., 2022) to clone the fragment into the pTRV2 vector, which has been double-digested with EcoRI (Product code: FD0274) and BamHI (Product code: FD0054) (Thermo Fisher Scientific (China) Co., Ltd.). The vectors were then transformed into Agrobacterium strain GV3101. The whole cotyledons of ten-day-old seedlings were infected by this Agrobacterium strain GV3101. After six weeks, we collected the first and second internode stems above the cotyledon for scanning electron microscope analysis, and determined the contents of cellulose, lignin, and hemicellulose in same samples. The stem strength of the first internode was measured eight weeks post-infection.

CRISPR/Cas9-mediated CaSLR1 homolog encoding gene SIMY61 knockout in tomato

The homologous sequence of SIMY61 (Solyce01g102340) was obtained by aligning CaSLR1 to the tomato genome (Solanum lycopersicum). Based on the CDS of SIMY61, we designed CRISPR target sites with the assistance of the CRISPR-P 2.0 website (Hao et al., 2017). Two pairs of PCR primers for two CRISPR targets "TTGAACTTCATGCAGTTCTT" and "ATAACGAGATAAAGAATCTG" were designed (Supplementary Table S1). Next, the PGTR plasmid served as a template for PCR, and the product was cloned into the CRISPR expression vector pKSE401 by Golden Gate cloning (Chen et al., 2017). The vector was used to transform Agrobacterium tumefaciens EHA105 cells, constructing pKSE401-expressing strain Cri-SIMY61 for inoculation. Tomato (Micro Tom) cotyledons were transformed using a previously established method (Ouyang et al., 2005).
RNA-seq analysis

Total mRNA was extracted from the stems of the WT, slr1, pTRV2, and pTRV2-CaSLR1 lines using TRIzol reagent (Product code: ET121-01, TransGen Biotech Co., Ltd., Beijing, China) in three biological replicates from each group. Upon assessing the sample quality, we constructed the library and subsequently sequenced on the Novaseq platform (Modi et al., 2021). For RNA-seq processing, Fastp v0.20.0 was employed to filter the raw data, removing low-quality reads and adaptor sequences to obtain high-quality clean data. (Saremi et al., 2022). The clean reads were mapped to the pepper genome (Zunla_1, NCBI genome code: 4072) using HISAT2 v2.1.0 (Qin et al., 2014). Next, StringTie v2.2.3b (Pertea et al., 2016; Shumate et al., 2022) was utilized to compare the transcript assembly and quantify gene expression in the dataset. DESeq2 was used to quantitatively determine the differentially expressed genes (DEGs) with the criteria $|\log2FC| \geq 1$ and FDR $\leq 0.05$. (Love et al., 2014; Thawng & Smith, 2022). Based on the GO and KEGG databases, The DEGs were annotated via BLASTALL v2.2.26 (https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/) with an e-value set to 1e-5.

Weighted gene co-expression network analysis (WGCNA)

Based on gene expression levels, we constructed a co-expression network using the WGCNA package in R. Firstly, we normalized expression data to FPKM + 1 value and then transformed it to log2. The correlation between module genes was subsequently analyzed using WGCNA (Wang et al., 2023). The WGCNA hierarchical network was utilized to construct a co-expressed gene network module. BLASTALL v2.2.26 (https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/), with an e-value set to 1e-5, was used to annotate the module genes via GO and KEGG databases and Perl scripts.

Yeast One-hybrid assay (Y1H)

To explore the interactions of CaNAC6 with CaMYB61, Y1H assays were conducted. The ORFs of CaNAC6 were cloned into the pGADT7 backbone as prey (Xie et al., 2020). A tandem of three SNBEs of the CaMYB61 promoter was cloned into the pABAI backbone as bait. Both vectors were transformed into Y1H Gold yeast strain cells using Clontech's small-scale transformation method. Glass beads were used to coat SD/-Leu /AbA until no bacterial liquid flow was observed. Samples were then cultured on plates at 30°C in an incubator for 5-7 days.

Dual luciferase reporter analysis (LUC)

To validate the activation of CaMYB61 by CaNAC6, the coding sequence (CDS) of CaNAC6 was cloned into the pGreenII 62-SK backbone to construct an effector vector. The promoter sequence of CaMYB61 was then inserted into the pGreenII 0800-LUC (luciferase) backbone to drive the expression of LUC gene, creating a reporter vector. These vectors were transformed into GV3101 cells, which were subsequently injected into tobacco leaves and kept in the dark. After 2-3 days, we used a 5200 Imaging System (Tanon Science & Technology, Shanghai, China) to capture the fluorescence signal on leaves. Subsequently, we used a Dual-Luciferase® Reporter Assay System (Product code: E1910, Promega, Madison, WI, USA) to measure the fluorescence values of LUC and REN (Renilla luciferase). Each sample underwent testing with a minimum of six biological replicates.
Electrophoretic mobility shift analysis (EMSA)

The CDS of CaNAC6 was inserted into the PGX-6P-1 GST backbone and then transformed into Escherichia coli BL21 (DE3). Expression induction resulted in the production of the NAC6-GST fusion protein. Following the manufacturer's instructions, the LightShift chemiluminescence EMSA kit (Product code: Bes5003, Biotechnology Co., Ltd., Guangzhou, China) was utilized to detect the interaction between CaNAC6 and the SNBE of the CaSLR1 promoter. The synthesized SNBE element was annealed with a biotin-labeled double-stranded DNA probe (Novagen Biotechnology Co., LTD, Beijing, China). The mutant biotin-labeled and unlabeled probes were incubated with the NAC6-GST fusion protein to assess its binding capability. Following the binding reaction, the complex underwent electrophoresis on a 6% acrylamide gel and was subsequently transferred to a nylon membrane. After rinsing, the nylon was positioned in a membrane box, and the X-ray film was exposed for 5 minutes. Gel shift was facilitated using Anti-streptavidin-HRP (horseradish peroxidase) conjugated antibody (Product code: HRP-66001, Reda Henghui Technology Development Co. Ltd., Beijing, China).

Statistical analysis

The experimental data were analyzed using Microsoft Excel 2013. A histogram was generated using GraphPad Prism v7.04. Statistical significance was evaluated using Student's t-test. All qRT-PCR (quantitative Real-time PCR) analyses were carried out with at least three replicates.

Results

Phenotypic characteristics of slr1

To explore the phenotypic characteristics of stem lodging development in slr1, we compared the growth parameters of WT and slr1 mutants. At 45 days, all slr1 mutant plants exhibited evident stem lodging and twisting compared to the WT, whereas this lodging phenomenon was not observable at 25 days of seedling growth (Fig. 1a, Fig. 1b and Supplementary Fig. S1a). Notably, the slr1 mutant exhibited abnormal reproductive development with delayed first flower and fruit set, accompanied by a significant reduction in the number of fruits and germination rate. (Supplementary Fig. S1b-S1d). Furthermore, we compared the growth differences between 45-day-old WT and slr1 mutants. Compared to WT, the aboveground, root, total fresh weights, and first internode length of slr1 mutants were significantly reduced by 33.38, 40.82, 37.11, and 50%, respectively (Supplementary Fig. S1e-S1h).

However, no significant difference was observed in the leaf length-width ratio (Supplementary Fig. S1i). Since cellulose, hemicellulose, and lignin are critical components in lodging resistance (Coen & Cosgrove, 2023; Zhang et al., 2021), the contents of cell components in the stems of WT and slr1 were measured. Compared to WT, the cellulose, hemicellulose, lignin, and crude fiber contents of slr1 mutants significantly decreased by 49.75, 36.81, 24.36, and 21.89%, respectively (Fig. 1c-f).

In addition, SEM was performed to compare the difference in stem structure in WT and slr1 mutant. The results revealed that the hollow vessels within the xylem of the slr1 mutant was obvious presence, whereas the WT exhibited more xylem fiber cells (XFCs) (Fig. 1g). Conversely, in the WT, XFCs with thickened SCWs were
observed at the periphery of secondary xylem, while parenchyma-like cells (PCs) were prevalent in the corresponding tissues of *slr1* (Fig. 1h). These findings suggest that the abnormal development of xylem led to stem lodging in the *slr1* mutant.

**Fig.1 Phenotype characteristics of wild-type (WT) and *slr1* mutant plants.** (a) Plants at the beginning of flowering, scale = 6 cm. (b) Stem from WT and *slr1* mutants, scale = 1 cm. (c) Cellulose content (%). (d) Hemicellulose content (%). (e) Lignin content (%). (f) Coarse fiber content (%). The results were expressed as the mean ± SE (n = 3). **P < 0.01 and ***P < 0.001, as determined by Student's *t*-test. (g) Secondary xylem from the WT and *slr1* plants. Scale = 30 μm. XFC: xylem fiber cells, Ve: vessel. (h) The periphery of the secondary xylem in the WT and *slr1* plants. Scale = 30 μm. XFC: xylem fiber cell, PC: parenchyma cell, Red five-pointed star showed thickened secondary XFC walls.
Identification of the target gene in slr1 mutant

To map the target genes associated with stem lodging, we created an F2 segregating population with slr1 as the female parent and WT as the male parent for crossbreeding. We obtained a total of 483 F2 individuals, comprising 402 wild-type phenotypes and 81 mutant phenotypes. The Chi-square test revealed that the F2 population significantly deviated from the Mendelian 3:1 segregation ratio. (Supplementary Table S2). To preliminarily map genes related to stem lodging, we conducted Mutmap analysis using the parents and two mixed DNA pools from the F2 population (Supplementary Table S3). Based on the identified homozygous SNP loci, the frequency of allele gene SNPs was assessed by analyzing DNA sequencing variations, and ΔSNP index values were obtained. A distinct peak on chromosome 8 was observed (126,314,622-165,820,095; approximately 39.50 Mb), indicating significant differences in the ΔSNP index (Fig. 2a). Further, a series of KASP (Competitive Allele-Specific PCR) markers were developed using SNPs information from the candidate region for genotyping (Supplementary Table S1). Next, based on phenotypic data of individual plants from the F2 population, we performed genotyping to identify candidate region. The result indicated that the candidate region was located in SNP153164345 and SNP154539577 markers, with a physical distance of about 1.37 Mb. (Fig. 2b; Supplementary Table S4).

Additionally, analysis of chromosomal structural variations between slr1 and WT revealed a deletion spanning approximately 105 Kb (Chr08:153762245-153867356) within the candidate region of 1.37 Mb (Fig. 2c), covering five genes: CaZ8g15200, CaZ8g15210, CaZ8g15220, CaZ8g15230, and CaZ8g15240 (Fig. 2d). Based on the annotation information, CaZ8g15200 was identified as ARATH TF PIF3 (CaPIF3), primarily involved in photomorphogenesis (Dong et al., 2020); CaZ8g15210 as fibrous sheath CABYR-binding protein (CaFSCB); CaZ8g15220 as carbohydrate esterase (CaCE); CaZ8g15230 as a hypothetical protein FXO38_26921 (CaFXO); and CaZ8g15240 as a MYB61 TF (CaMYB61). Using cDNA from the WT and slr1 stems as templates, the amplification of coding sequences from five genes detected the specific band only in the WT, but not in slr1 (Fig. 2e). RT-qPCR analysis failed to detect any of the five genes in slr1 (Fig. 2f). Therefore, PCR and RT-qPCR revealed a large-fragment deletion in slr1 mutant.
Fig. 2- Mapping of the genes related to stem lodging using Mutmap. (a) Mutmap indicated a preliminary map region. The candidate chromosomal region of about 39.50 Mb was marked in a yellow arrow. (b) Candidate regions were narrowed down by genotyping. NP, WT (chromosomal cyan filling). MP, slr1 (chromosomal orange filling). The normal phenotype plants marked in cyan represent individual plants with chromosomal exchanges in the F2 population, including WT-24 and WT-73. The plants with a lodging phenotype were highlighted in orange. Figure illustrated individual plants with chromosomal exchanges in the F2 population, such as M-467, M-139, and M-242. The candidate region was identified as the chromosomal domain between the two red SNP markers. (c) Analysis of the chromosomal structural variation within the range of 153.16-154.53 Mb. Vertical axis: Depth coverage rate between WT and slr1; Abscissa axis: chromosome position. (d) Approximately 105 Kb was deleted in
the genome of slr1 mutant, including CaZ8g15200, CaZ8g15210, CaZ8g15220, CaZ8g15230, and CaZ8g15240. 
(e) The agarose gel electrophoresis was conducted using PCR products of five genes from both WT and slr1 plants. 
The genes and their respective PCR product sizes are as follows: CaZ8g15200 (CaPIF3, 2181 bp); CaZ8g15210 
(CaFSCB, 2385 bp); CaZ8g15220 (CaCE, 810 bp); CaZ8g15230 (CaFOX, 1098 bp); and CaZ8g15240 (CaSLR1, 
1263 bp). (f) The expression levels of the five genes in the stems of the WT and slr1 plants. The results are 
presented as mean ± SE (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, and n.s, not significant, as determined by the 
Student's t-test.

*CaSLR1 mediates stem lodging by affecting SCW thickness and stem strength in peppers and tomatoes*

MYB61 has been identified as a regulator influencing cellulose and SCW formation (Huang et al., 2015). 
Consequently, CaZ8g15240 was recognized as a crucial candidate gene and designated CaSLR1. Utilizing VIGS 
technology to silence CaSLR1 in WT plants resulted in noticeable twisting and lodging characteristics in their stems 
(Fig. 3a). RT-qPCR analysis confirmed a significant down-regulation of CaSLR1 expression in the stems, 
validating the successful silencing of CaSLR1 (Fig. 3b). These findings elucidated the role of CaSLR1 in mediating 
stem lodging development in pepper.

Given that SCW component biosynthesis affects plant mechanical support (Coen & Cosgrove, 2023; Zhang et 
al., 2021), SEM analysis of the stems from CaSLR1-silenced and control (pTRV2) plants (Fig. 3c) revealed a 
denser mechanical structure in the xylem of control plants, whereas the mechanical tissue of the xylem in CaSLR1- 
silenced plants exhibited cell wall collapse (Fig. 3d). Particularly, the cell walls of xylem fiber cells in CaSLR1- 
silenced plants appeared thinner compared to controls (Fig. 3e), indicating that silencing CaSLR1 leads to 
differences in xylem development. Moreover, the contents of the SCW components were significantly reduced 
(Fig. 3f). Stem strength analysis demonstrated that control plants exhibited a puncture strength of 1222.97 mg, 
whereas CaSLR1-silenced plants could withstand only 501.47 mg (Fig. 3g), indicating a reduction in stem strength 
due to the thinning of SCW.

To uncover the functional conservation of CaSLR1, we used the CRISPR/Cas9 system to knock out SIMYB61 
(Solyce01g102340), a homolog of CaSLR1, in tomatoes. Sequencing of the PCR product from the SIMYB61 
knockout (Cri-SIMYB61) lines revealed a seven-base edit at the target site (Fig. 3h). The Cri-SIMYB61 lines were 
self-crossed to obtain a stable genetic T1 generation, referred to as Cri-SIMYB61-T1. Compared to the wild-type 
(NC-T1), Cri-SIMYB61-T1 plants exhibited stem lodging (Fig. 3i). Additionally, the Cri-SIMYB61-T1 plants had 
fewer root branches (Fig. 3j) and shorter inflorescence branches (Fig. 3k) compared to NC-T1.

SEM analysis of cross-sectioned stems from NC-T1 and Cri-SIMYB61-T1 plants revealed significant 
differences. The pith (Pi) area in Cri-SIMYB61-T1 was enlarged compared to NC-T1, with stems from the latter 
containing abundant and well-developed granular plastids, while those from Cri-SIMYB61-T1 exhibited abnormal 
development (Fig. 3m). Moreover, the secondary cell walls (SCWs) of xylem fiber cells (XFCs) in NC-T1 were 
thicker, whereas those in Cri-SIMYB61-T1 were thinner (Fig. 3n), indicating that the edited SIMYB61 affected stem 
structure, resulting in thinner SCWs in XFCs in tomatoes. These results suggest that SIMYB61 knockout in
tomatoes leads to alterations in plant structure, notably in stem strength and stability, indicating a conserved function of SLR1 in both peppers and tomatoes.

Fig. 3 CaSLR1 mediates stem lodging by affecting SCW thickness and stem strength in peppers and tomatoes. (a) Virus-induced gene silencing of CaSLR1 in pepper, scale = 2.5 cm. (b) RT-qPCR analysis the relative expression levels of CaSLR1 in the CaSLR1-silenced line (pTRV2-CaSLR1) and control (pTRV2, NC) lines. (c) The stems of CaSLR1-knockdown and control lines; scale = 1 cm. (d) The xylem tissues from the stems of
the CaSLR1-knockdown and control lines, respectively; scale = 100 μm. (e) The secondary xylem from the stems of CaSLR1-knockdown and control lines, respectively; scale = 30 μm. (f) The contents of cellulose, hemicellulose, and lignin from the stems of the CaSLR1-knockdown and control lines, respectively. (g) The stem strength of CaSLR1-knockdown and control lines. The results were expressed as the mean ± SE (n = 3). **P < 0.01 and ***P < 0.001, as determined by Student's t-test. (h) SIMBY61, encoding a homolog of CaSLR1, was edited at the third exon in tomato (Micro-Tom). PAM: protospacer-associated motif. 7 bases (TAAAGAA) were edited. Target bases: ATAACGAGATAAAGTAATTCTG. PAM: TGG. (i) The phenotype of T1 generation from the knockout line (Crisi-SIMBY61) and no knockout line (negative control, NC). Scale = 5 cm. (j) The root from T1 generation. Scale = 5 cm. (k) The flower branches and inflorescences from T1 generation. Scale = 2 cm. (l) Scanning electron microscopy analysis of T1 generation stem. Scale = 500μm. Xy: xylem. PC: parenchymal cell. Pi: pith. (m) The parenchymal cell closed to xylem of T1 generation stem. Scale = 50μm. PC: parenchymal cell. The arrow points to the granular body. (n) The xylem fiber cell closed to secondary xylem from T1 generation stem. Scale = 50 μm. SCW: secondary cell wall. XFC: xylem fiber cell. Ve: vessel. The arrow points to SCW.

Subcellular localization and expression pattern of CaSLR1

A preliminary exploration of the function of CaSLR1, according to annotation information, revealed that CaSLR1 belongs to a typical MYB family with 1796 bp in length, consisting of three exons and two introns, and with a 1263 bp long CDS, encoding 421 amino acids (Supplementary Fig. S2a). CaSLR1 (protein ID: XP_016538869) and SlMYB61 (protein ID: XP_004230371) were identified to be the most closely related based on the comparative analysis of a phylogenetic tree. Interestingly, the homologous proteins of CaSLR1 were identified from other species such as Mus musculus, S. cerevisiae S288C, C. elegans, Oryza sativa, Nicotiana tabacum, Arabidopsis thaliana (Supplementary Fig. S2b), indicating the evolutionary conservatism of SLR1. CaPIF3, CaFSCB, CaCE, and CaFXO are species-specific, lacking homologous genes in both animals and microorganisms (Supplementary Fig. S3). Further, the sequence alignment analysis showed that two SANT/MYB domains in SLR1 were highly consistent between Capsicum annum (CaSLR1) and Solanum lycopersicum (SlMYB61), but they were slightly different among Capsicum annum (CaSLR1), Oryza sativa (OsMYB61), and Arabidopsis thaliana (AtMYB61) (Fig. 4a), hinting slightly different in evolution.

To further elucidate the function of CaSLR1, subcellular localization was examined by transiently transforming Arabidopsis protoplasts with the 35S:CaSLR1-eGFP expression vector. Confocal microscopy revealed green fluorescence distributed in the cell membrane and nucleus. Co-localization analysis with the nuclear marker Gpd-RFP confirmed the presence of CaSLR1 in the nucleus (Fig. 4b), suggesting its role in gene regulation.

To investigate the organ-specific expression patterns of CaSLR1 in peppers, qRT-PCR analysis was conducted. The results revealed that the expression of CaSLR1 was highest in the stems, with levels 8.01, 138.80, 11.97, 4.06, and 98.27-fold higher compared to the roots, leaves, flowers, fruits, and anthers, respectively, reaching significance (Fig. 4c). These results imply that CaSLR1 participates in regulating stem development.
Fig. 4 Subcellular localization and expression pattern of CaSLR1. (a) Multiple sequences alignment of MYB61. MYB61 includes two SANT/MYB domains: 9-63, marked green, and 66-114, marked orange. The CaSLR1 from the cultivated pepper species Capsicum annuum L.; SMYB61 from Solanum lycopersicum L.; OsMYB61 from Oryza sativa L.; and AtMYB61 from Arabidopsis thaliana L. (b) Subcellular localization of CaSLR1. Red fluorescent protein (Ghd-RFP) was used as the nuclear label. Scale = 10 μm. (c) Relative expression levels of CaSLR1 in the different tissues, roots, stems, leaves, blooms, fruits, and anthers of pepper plants. The results were expressed as the mean ± SE (n = 3). **P < 0.01 and ***P < 0.001, as determined by Student's t-test.

RNA-seq analyses between WT and slr1

To delve into the molecular mechanisms underlying stem lodging in slr1 mutants, RNA-seq was conducted using stem samples from 25- and 45-day-old WT and slr1 mutants. After removing adaptor sequences, over 86% of clean reads were mapped to the reference genome “Zunla_1”, with a GC content of about 43% and a Q30 quality score exceeding 87% (Supplementary Table S5). Differential expression analysis revealed 545 differentially expressed genes (DEGs) at 25 days and 1,546 DEGs at 45 days in slr1 mutants compared to WT. Notably, 251 DEGs were common between the two time points (Supplementary Fig. S4a). The enrichment analysis of GO and KEGG uncovered that the DEGs were significantly enriched in pathways related to cell wall development, including plant-type SCW biogenesis and phenylpropanoid biosynthesis (Supplementary Fig. S4b-c,
Supplementary Table S6). Protein-protein interaction (PPI) analysis showed that a regulatory network centered around CaSLR1 was formed during stem development (Supplementary Fig. S4d).

To explore the co-expression patterns of CaSLR1, WGCNA was performed. The Principal Component Analysis model indicates a high consistency among the three repetitions (Supplementary Fig. S5a). WGCNA revealed that 30 branches were clustered, each represented by a unique color (marked with different colors) (Supplementary Fig. S5b-c). Notably, the antiquewhite4 module, which encompassed the candidate gene CaSLR1 (Zunla_1 reference genomic gene ID: Capana08g001690), was delineated (Fig. 5a, Supplementary Table S7) (Fig. 5a, Supplementary Table S7). GO enrichment unveiled significant enrichment of genes within the antiquewhite4 module in various cellular components, particularly those related to SCW formation, plant-type SCW biogenesis, xylan O-acetyltransferase activity, and xylan biosynthetic process (Fig. 5b, Supplementary Table S8). This highlights the potential role of CaSLR1 in orchestrating these crucial processes during stem development.

The expression patterns of the SCW biosynthesis-related genes within the antiquewhite4 module, such as cellulose synthase A catalytic subunit, β-1,4-xylosyltransferase IRX10L, UDP-glucuronate: xylan α-glucuronosyltransferase, and fasciclin-like arabinogalactan protein exhibited consistent down-regulation during stem development in slr1, particularly evident after stem lodging in slr1 plants (the 45-day-old plants; slr1_2) (Fig. 5c, Supplementary Table S9). This suggests a correlation between stem lodging and the biosynthesis of cell wall components in pepper. RT-qPCR confirmed the downregulation of cell wall formation-related genes from the antiquewhite4 module, including NAC transcription factor 7 (CaNAC7), glucuronoxylan 4-O-methyltransferase 1 (CaGXM1), pectinesterase 53 (CaPE53), and glycine-rich cell wall structural protein 1-like (CaGRP1L), in stems of 45-day-old slr1 mutants (slr1_2), affirming the reliability of the results (Fig. 5d).
Fig. 5 WGCNA analysis. (a) Genes cluster dendrogram analysis in which different gene modules are represented by branches and various colors. (b) GO enrichment analysis of the genes in the antiquewhite4 module. Key enriched pathways are shown in red font. (c) The expression heatmap of SCW biosynthesis-related candidate genes in the antiquewhite4 module. (d) RT-qPCR analysis of four SCW biosynthesis-related genes in the antiquewhite4 module, CaNAC7: NAC transcription factor 7; CaGXM1: glucuronoxylan 4-O-methyltransferase 1; CaPE53: pectinesterase 53; and CaGRP1L: glycine-rich cell wall structural protein 1-like. The results were expressed as the mean ± SE (n = 3). ***P < 0.001, as determined by the Student's t-test.

**CaNAC6 binds to the promoter of CaSLR1 to affect the SCW biosynthetic pathway**

NAC and MYB transcription factors (TFs) are recognized as the "master switches" controlling SCW formation (Zhong et al., 2011). Within the antiquewhite4 module, a CaNAC6 (Capana10g001138) TF was found to be co-expressed with CaSLR1 (Supplementary Fig. 4d, Supplementary Table S7). NAC29, a homologous protein of CaNAC6, has been implicated in regulating SCW deposition in rice (Zhang et al., 2018). NAC TFs contribute to SCW biosynthesis by binding to SNBE elements that existed in MYB promoters (Negi et al., 2017). We identified three SNBEs elements within 2000 bp promoter in CaSLR1 (Fig. 6a).

To confirm the binding between CaNAC6 and the CaSLR1 promoter, a Y1H assay was conducted. Yeast cells were co-transformed with pAbAi-proSLR1 along with either pGADT7-NAC6 or pGADT7 vectors (NC) and cultured on SD/-Leu medium supplemented with 500 ng/mL of AbA. The results showed that the co-transformed
pGADT7-NAC6 and pAbAi-proSLR1 were able to promote yeast cells' growth on the AbA selective medium, unlike the negative control cells (Fig. 6b).

Further, to confirm whether CaNAC6 could regulate CaSLR1 expression, we injected with 62SK-CaNAC6 and proSLR1-LUC into tobacco leaves via the LUC system. Compared to the negative control (62SK and proSLR1-LUC), the ratio of LUC to REN was 5.92-fold higher. However, substituting proSLR1-LUC with proSLR1-mut-LUC resulted in a decreased ratio to 1.79, indicating a significant reduction in gene transcriptional activation (Fig. 6c-e). To demonstrate that CaNAC6 was directly bind to the three SNBE elements of CaSLR1, an EMSA assay was conducted using purified CaNAC6 recombinant protein fused with a GST tag (Supplementary Fig. S6). The finding revealed that CaNAC6 was bound to the biotin-labeled SNBE1, 2, and 3 probes, with binding strength inversely proportional to the concentrations of competitor probes. However, mutation of the SNBEs core sequence abolished the binding, indicating the specificity of the interaction (Fig. 6f). Our results suggest that CaNAC6 positively regulated the expression of CaSLR1.

Fig. 6 CaNAC6 binds to the promoter of CaSLR1 and induces its transcription. (a) The position of the three secondary wall NAC binding elements (SNBEs) in the 2000 bp long promoter of CaSLR1. (b) The potential binding of CaNAC6 with CaSLR1 promoter in yeast. SD/-Leu, medium lacking leucine. SD/-Leu/500 AbA, medium lacking leucine and added with 500 ng/mL aureobasidin A (AbA). (c) Schematic of the vector used for the Dual-luciferase reporter system. LUC, firefly luciferase. REN, renilla luciferase. (d) LUC images of the tobacco leaves after transient infiltration. (e) The ratio of LUC to REN activity. The results were expressed as the mean ± SE (n = 6). ***P < 0.001, as determined by the Student's t-test. (f) The binding of CaNAC6 with the SNBE elements of CaSLR1 was determined using an EMSA assay.

CaSLR1 regulates genes expression associated with SCW formation pathways in pepper stems
To further explore the underlying molecular mechanism of CaSLR1-mediated stem development in pepper, RNA-seq was used to analyze RNA samples extracted from stems of 45-day-old CaSLR1-silenced and control plants (pTRV2). After filtering out adaptor sequences, more than 87% clean reads were obtained, which were then mapped to the pepper "Zunla_1" reference genome. The primary quality Q30 was above 92.9% (Supplementary Table S10). In total, we identified 2105 DEGs, including 1132 up-regulated and 973 down-regulated genes (Supplementary Fig. S7). The GO analysis revealed significant enrichment of DEGs in the cellular components associated with the plant-type cell wall and cellulose synthase complex. In terms of biological processes, enrichment was observed in cell wall organization, phenylpropanoid metabolism, and cellulose microfibril organization, indicating that CaSLR1-silencing affected the expression of genes related to cell wall components (Supplementary Table S11).

To investigate the synergistic effects of DEGs in signal transduction and biochemical metabolism pathways, we performed KEGG enrichment analysis. The results revealed that DEGs were mainly enriched in 17 pathways, including hormone signal transduction, starch and sucrose metabolism, phenylpropanoid biosynthesis, etc. (Supplementary Table S12), indicating that CaSLR1 affected lignin biosynthesis. Research indicates that the protein encoded by the auxin pathway gene ARF binds to the promoter regions of essential genes associated with lignin biosynthesis, including caffeoyl-CoA O-methyltransferase (CCoAOMT2) or 4-coumarate: coenzyme A ligase (4CL3, 4CL7, and 4CL9), and activate their expression thereby affecting mechanical bending in bamboo (Wang et al., 2023).

Additionally, we explored the gene expression of cellulose, cell wall structural proteins, and hemicellulose biosynthesis pathways in CaSLR1 silenced plants. Heatmap analysis revealed a significant down-regulated in numerous genes associated with cell wall structural protein biosynthesis, such as COBRA-like (COBL) and cellulose synthase A catalytic subunit (CESA) (Fig. 7a). Similarly, genes related to hemicellulose biosynthesis, including IRX9H, IRX9, and xyloglucan endotransglucosylase protein 30 (XTH30), were also down-regulated (Fig. 7b). XTH30 is recognized for its crucial role in building and restructuring xyloglucan cross-links (Jan et al., 2004). Notably, the expression of genes essential for lignin biosynthesis, including caffeoyl-CoA O-methyltransferase-like (CCoAOMTL), laccase-4-like, laccase 2-like, laccase 11-like, and 4-coumarate-CoA ligase-like 1 (4CL1), and cinnamyl alcohol dehydrogenase 1 (CAD1), was also down-regulated (Fig. 7c), indicating that CaSLR1 may play a role in regulating genes expression associate with lignin biosynthesis. Furthermore, the down-regulated genes involved with cell wall formation was verified by qRT-PCR (Fig. 7d). The consistent between the qRT-PCR results and transcriptomic sequencing data validates the reliability of our findings. Therefore, we propose that CaSLR1 modulates the expression of secondary cell wall (SCW) biosynthesis genes, thereby influencing stem development in pepper.
Fig. 7 CaSLR1 regulates the expression of cellulose, hemicellulose, and lignin biosynthesis related genes in pepper stems. (a) The expression-related heatmap of genes involved in the cellulose biosynthesis pathway and cell wall structural proteins. (b) The expression-related heatmap of genes involved in the hemicellulose biosynthesis pathway. (c) The expression-related heatmap of genes involved in the lignin biosynthesis pathway. (d) qRT-PCR analysis verified the down-regulated genes related to SCW formation pathways using RNA-seq. The results were expressed as the mean ± SE (n = 3). **P < 0.01; ***P < 0.001, as determined by the Student’s t-test. (e) A hypothesis suggests that CaSLR1 regulated the stem lodging resistance. In WT, CaNAC6 binds to SNBE elements in the promoter of CaSLR1, thereby inducing its transcription. As a result, the stem accumulated cellulose,
hemicellulose, and lignin, which promotes the deposition of the SCW and increases stem strength, enabling the stem to remain upright. In contrast, in the mutant slr1, a deletion of CaSLR1 and its promoter abolished the binding of CaNAC6 to the SNBEs. The transcription of CaSLR1 was inhibited, and the accumulation of cellulose, hemicellulose, and lignin in stems was reduced. Accordingly, it resulted in the thinning of the SCW, weakening of stem strength, and finally, stem lodging. “×” marked that the effect was cancelled. *CESA: cellulose synthase; COBL4: COBRA-like 4; IRX9: irregular xylem 9; XTH30: xyloglucan endotransglycosylase protein 30; CCoAOMT1: caffeoyl-CoA O-methyltransferase 1-like; laccase 2L: laccase 2-like; 4CL1: 4-coumarate-Co-A ligase 1; CAD1: cinnamyl alcohol dehydrogenase 1; 4CCL7: 4-coumarate-Co-A ligase like 7; AAE2: Acyl-activating enzyme 2; ACSL4: Long chain acyl-CoA synthetase 4-like; IRX15LL: IRX15–LIKE–like.

Discussion

Stems serve as the primary load-bearing structure in plants, providing resistance against lodging. However, during stem development, cells within the vascular bundles, fibers, and vessels gradually lose their protoplasm, leaving only the cell walls to offer mechanical support (Ardiyana & Rejab, 2015). The mechanical strength and stability of the stem are largely influenced by cell wall components (Jamet & Dunand, 2020; Ahmad et al., 2020). Here, a typical pepper lodging mutant slr1, induced by EMS, exhibited constrictive xylem and defective secondary cell wall development, accompanied by a significant decrease in cellulose, hemicellulose, and lignin contents (Fig. 1c-1h). Via fine mapping and chromosome structure variation analysis, a deletion region of approximately 105 Kb on chromosome No. 8 was identified as the candidate region, including CaZ8g15200, CaZ8g15210, CaZ8g15220, CaZ8g15230, and CaZ8g15240 five genes (Fig. 2b-2c). Among five genes, CaZ8g15230 was not expressed in WT and slr1 mutants. Hence, we only focus on another four genes. CaZ8g15210 encodes carbohydrate esterase. Carbohydrate esterase family members have been suggested to aid in reducing cell wall rigidity by cleaving covalent linkages between lignin and glucuronoxylan (Seveso et al., 2024). CaZ8g15240 encodes the transcription factor MYB61. Previous research has demonstrated that *PbMYB308* binds with *PbMYB61* to negatively regulate the synthesis of stone cell lignin in pear fruits (Zhu et al., 2023). In *Arabidopsis thaliana*, the *AtMYB61* mutant exhibited reduced xylem formation and impaired xylem cell structure (Romano et al., 2012). Our results reveal that *CaSLR1* (CaZ8g15240) is homologous to *AtMYB61* (Supplementary Fig. 2b). Given that *CaSLR1* knockdown lines exhibit a lodging phenotype similar to slr1, it is considered a candidate causal gene. In the study, notably, the F2 population did not conform to the expected Mendelian 3:1 separation ratio, and only one peak was observed in the Mutmap analysis (Fig. 2a). Previous research indicated that germination and seedling establishment were compromised in *Arabidopsis myb61* mutants (Penfield et al., 2001). Our findings revealed that the *CaSLR1* mutation significantly decreased the number of fruits and the seed germination percentage. Specifically, the seed germination rate was 99% in WT but only approximately 52% in slr1 mutants (Supplementary Fig. S1a-S1c). We speculate that Caslr1 affects fruit and seed development, potentially explaining the abnormal F2 segregation ratio. Additionally, the slr1 mutant is caused by a substantial mutation. Contrary to the predominant point mutations induced by EMS, as by Xiong et al. (2023) reported, the genomic mutagenesis library confirmed that EMS can also
cause large fragment deletions (Lesa, 2006). For instance, 870 large homozygous deletions were detected in tetraploid wheat and 7,971 in hexaploid wheat (Krasileva et al., 2017), likely a consequence of using higher EMS dosage.

The softness of stems relies on the elastic behavior of fibers, which is determined by both the overall load and the composition of the SCW. Overexpressing plants with p1300-SND1P-XND1 exhibited recumbent stems, while stem length remained largely unaffected (Zhang et al., 2020). Alterations in SCW formation and components within vascular cells have been shown to affect stem mechanical properties (Xiao & Anderson, 2016; Zhang et al., 2022). Our study reveal that the cellulose content of CaSLR1-knockdown lines is significantly reduced by 36.39% compared to pTRV2 controls (Fig. 3f). While both CaSLR1 and AtMYB61 influenced cellulose content, notable differences in stem erectness were observed between peppers and Arabidopsis. CaSLR1-knockdown lines displayed complete lodging with a prostrate posture (Fig.3a). However, the erectness of the inflorescence stems of the Atmyb61 mutant was not affected (Romano et al., 2012). In rice lines where OsMYB61 was overexpressed, cellulose content increased, while total lignin and xylose (one of the main components of hemicellulose) contents remained unchanged at the basal internodes (Huang et al., 2015). In our study, CaMYB61-knockdown lines exhibited reductions in hemicellulose and lignin contents. Additionally, the stem strength of CaMYB61-silenced lines decreased by approximately 59%. The differing effects of MYB61 on the pathways of hemicellulose and lignin biosynthesis in various species could be a significant factor contributing to the alterations observed in stem development.

Furthermore, MYB61, a crucial multifunctional protein, influences various metabolic processes in plant growth. OsMYB61 is directly regulated by Growth Regulator Factor 4 (GRF4), a known regulator of nitrogen using efficiency (NUE), which promotes biomass production in rice (Gao et al., 2020). In this study, biomass decreased in CaSLR1-silenced pepper lines and SLMYB61 knockout tomato lines (Supplementary Fig.S1f; Fig. 3i-3k). As a regulator of resource allocation, AtMYB61 expression was observed in the metabolic sink, particularly in the xylem and roots. Mutants of AtMYB61 exhibited reductions in lateral roots and xylem vessels (Romano et al., 2012). In slr1 mutant and CaSLR1-knockdown lines, the decreased contents of cellulose, hemicellulose, and lignin revealed that the CaSLR1 affected the formation of structural carbohydrates in plants. These findings support the conclusion that MYB61 influences resource allocation. Furthermore, despite the homology between CaSLR1 and AtMYB61, there are notable differences in their phenotypic effects. These distinctions may arise from variations in stem types between pepper and Arabidopsis inflorescences, as well as differences in amino acids sequences within conserved domains (Fig. 4a). Another contributing factor could be the variations in types of mutations, which lead to varying phenotypic outcomes. In this study, the deletion of the entire CaSLR1 gene exhibited a more pronounced effect (Fig. 1a), in contrast to the subtler phenotypes observed in AtMYB61-knockout lines in Arabidopsis (Romano et al., 2012). An intriguing avenue for future research is to investigate whether mutations in different regions of CaSLR1...
would impact the biosynthesis of cell wall components, thereby influencing the deposition of SCWs and potentially causing plant-specific variations.

A complex multilevel transcriptional network in plants acts on the biosynthetic genes of SCW components, thereby regulating the thickening process of SCWs (Hennet et al., 2020). Studies on Arabidopsis mutants have established a clear consensus that NAC and MYB TFs act as switches in regulating SCW biosynthesis (Hennet et al., 2020; Wang et al., 2020). MYB46 was one of the earliest discovered SCW master switch (Xiao et al., 2021). The down-regulation of MYB46 and MYB83 drastically repressed SCW thickening in both vessels and fibers, thereby causing collapsed vessels and reduced plant growth (Zhou et al., 2009). SND1 binds to the SNBE element in MYB46 promoter and activates its expression (Zhong et al., 2007). In this study, WGCNA indicated that CaSLR1 and CaNAC6 clustered in the same module (Fig. 5a). CaNAC6 exhibited homology to NAC29, which regulated SCW deposition in rice (Zhang et al., 2018). Overexpressing OsNAC29 lines exhibited thicker internodes and significantly increased cellulose content (Huang et al., 2015). Our study revealed that CaNAC6 specifically binds to SNBE elements in CaSLR1 promoter, thereby activating its expression (Fig. 6b-6d). Therefore, CaNAC6 and CaSLR1 are important regulators of SCW formation in pepper.

Secondary wall-associated NACs TFs activate the secondary MYB master switch, leading to the expression of downstream SCW formation-related genes (Wang et al., 2020). In our study, CaSLR1 regulated the expression of downstream genes involved in cellulose, hemicellulose, and lignin biosynthesis, including CESAs, COBL4, IRX9, XTH30, CCoAOMT1, laccase 2L, 4CL1, CAD1, 4CCL7, IRX15L (Fig. 7a-c). On the Golgi apparatus, CESAs assemble into cellulose synthase complexes, which are then transported to the plasma membrane for cellulose synthesis, ultimately depositing cellulose on one side of the cell wall (Polko et al., 2019). The spatial control of lignin chemistry depends on different combinations of laccases with nonredundant activities immobilized in cell wall layers (Blaschek et al., 2023). Given all this, CaSLR1 and CaNAC6 may be crucial master switch in the molecular network of regulating secondary cell wall formation in peppers. Based on our study, a hypothesis was proposed to elucidate the mechanism of stem lodging resistance in pepper. In the WT, CaNAC6 binds to the SNBEs in the promoter of CaSLR1, inducing the transcription of CaSLR1. Consequently, the expression of CaSLR1 promoted the accumulation of cellulose, hemicellulose, and lignin, resulting in thickened secondary cell walls (SCWs) and erect stems. Conversely, in the slr1 mutant, the complete deletion of CaSLR1 and its promoter prevents CaNAC6 from binding to the SNBE elements, reducing the accumulation of these structural components, leading to thinner SCW and stem lodging (Fig. 7e). These findings highlight the CaNAC6-CaSLR1 module that contributes to lodging resistance, underscoring the pivotal role of CaSLR1 within the regulatory network for lodging resistance.
Funding

This research was supported by the National Natural Science Foundation of China (32172584), the Natural Science Foundation of Hunan Province (2021JJ30339), the Hunan Provincial Innovation Foundation for Postgraduate (CX20200655), the National Natural Science Foundation of China (32002040).

Author contributions

QL performed experiments and analyzed data; CFF, HH and QX performed RNA-seq and RT-qPCR; BZY constructed F$_2$ population; HYY analyzed Mutmap sequencing data; WM, RYL, WCC and ZQZ performed field management; QL and BWH wrote the paper; ZXZ and LJO directed the project.

Data availability

BSA-seq and RNA-seq data generated in this study are available at the NCBI. BSA-seq data bioproject accession: PRJNA1115904; VIGS RNA-seq data bioproject accession: PRJNA1115904; WT and slr1 RNA-seq data bioproject accession: PRJNA1113712.

Conflict of interest

The authors declare that they have no competing interests.

Supplementary data

Supplementary data are available at Horticulture Research online.

Acknowledgements

We thank Dr. Xingyao Xiong and Dr. Feng Liu for their helpful discussions on this manuscript.

Reference


73. Zong Y, Song QN, Li C, Jin S, Zhang DB, Wang YP, Qiu JL, Gao CX. 2018. Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nature Biotechnology* 36, 950-+