Journal of Integrative Agriculture 2026, 25(3): 977–988

Available online at ScienceDirect

Journal of Integrative Agriculture



RESEARCH ARTICLE

Genome-wide identification of the peach *LOB/LBD* genes and the positive role of the PpNAP4–PpLOB1 module in peach fruit softening

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Highlights

- PpLOB1 positively regulates peach softening.
- PpNAP4 activates *PpLOB1* expression.

Abstract

Softening of fleshy fruits during ripening and postharvest is a programmed physiological process that substantially impacts fruit quality and shelf life. However, the molecular mechanism underlying peach softening remains largely unknown. Lateral organ boundary (LOB) domain (LBD) proteins are pivotal regulators of plant growth and development. To date, certain LOB/LBD transcription factors are seemingly implicated in fruit softening. In this study, we identified 42 *LOB/LBD* genes in the peach genome. Expression analysis showed a significant upregulation of *PpLOB1* transcripts toward peach fruit ripening. PpLOB1 was classified into Class II subgroup, and showed high sequence similarity to several softening-related LOB/LBD transcription factors. Transient transformation assays showed that *PpLOB1* positively modulates peach softening. Further experiments demonstrated that PpLOB1 directly targets and activates the promoters of *pectate lyase 1* (*PpPL1*) and *PpPL15*, thereby contributing to the regulation of fruit softening. Additionally, PpNAP4 up-regulates *PpLOB1* expression by binding to its promoter. Meanwhile, our findings revealed that PpNAP4 and PpNAP6 cooperatively modulate the expression of *PpLOB1*. Taken together, our findings revealed a novel regulatory module involving PpNAP4 and PpLOB1 that modulates peach fruit softening.

Keywords: LOB/LBD, PpLOB1, PpNAP4, peach, softening

1. Introduction

Lateral organ boundary (LOB) domain (LBD) proteins are a group of plant-specific transcription factors (TFs) characterized by three conserved domains: a zinc finger-like motif CX2CX6CX3C (C-block) domain, a Gly-Ala-Ser (GAS) block domain, and a leucine zipper-like module LX6LX3LX6L domain (Majer and Hochholdinger 2011). Based on the number of domains present, LOB/LBD TFs are classified into two classes: Class I and Class II (Kong *et al.* 2017; Liang *et al.* 2022). Class I proteins possess all three domains and comprise most LOB/LBD TFs. In contrast, Class II members lack the leucine zipper-like coiled-coil domain. Genes of Class I are further grouped into four sub-groups: I A, I B, I C, and I E; and those in Class II are divided into sub-groups II A and II B. To date, LOB/LBD proteins have been reported to play important roles in various plant growth and developmental processes, such as organ development, anthocyanin synthesis, nitrogen metabolism, and plant responses to abiotic or biotic stress factors (Xu *et al.* 2016; Zhang *et al.* 2020).

Received 8 November 2024; Received in revised form 29 December 2024; Accepted 26 January 2025; Available online 20 February 2025
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doi: 10.1016/j.jia.2025.02.043

In all, 43 *LOB/LBD* genes have been identified in *Arabidopsis*, over 20 members of which have been functionally characterized (Xu *et al.* 2016; Ohashi-Ito *et al.* 2018; Cho *et al.* 2019). Comprehensive analysis of their phylogenetic relationship and functional characterization revealed that LOB/LBD TFs within the same clade often exhibit similar biological functions (Zhang *et al.* 2020). For instance, members of Class I A are primarily involved in the regulation of aboveground organ-related developmental phenomena, such as leaf polarity, pollen development, and floral organ formation; whereas Class I B proteins regulate developmental processes of belowground organs, such as lateral root initiation. Further, Class II LOBs/LBDs are mainly implicated in metabolic pathways during plant development, including anthocyanin biosynthesis and nitrogen response. Recently, several LOB/LBD TFs from horticultural plants have been functionally characterized. For example, tomato SlLBD40 is shown to be a negative regulator of drought stress, and knocking out *SlLBD40* significantly enhances drought tolerance in tomato lines (Liu *et al.* 2020). In turn, in citrus, CsLOB1 contributes to canker disease susceptibility by directly targeting and activating *Cs2g20600*, a gene of RING finger TF that is in response to pathogenic bacteria infection (Duan *et al.* 2018). And in apples, MdLBD16a functions in both root primordium development and callus tissue formation (Wang *et al.* 2023).

Besides that, LOB/LBD TFs have been implicated in the regulation of fruit growth and ripening, with the developmental stages having a significant impact on final quality and marketability. In tomatoes, SlLBD40 positively modulates fruit size by orchestrating mesocarp cell expansion, leading to a significant enhancement of fruit growth (Liu *et al.* 2024). During the ripening stage, SlLOB1 primarily regulates tomato softening by directly targeting multiple genes encoding cell wall-degradation enzymes such as *expansion 1* (*EXP1*), *endoglucanase 2* (*CEL2*), *alpha-xylosidase* (*XY*), *trichome birefringence-like* (*TBL*), and *pectate lyase* (*PL*) (Shi *et al.* 2021). In bananas, three ethylene-inducible genes, *MaLBD1*, *MaLBD2*, and *MaLBD3*, show increased expression levels during ripening, and transcriptional activation to the softening-related gene *MaEXP1/2* (Ba *et al.* 2014).

Peach is an importantly economic crop in temperate regions, and a typical climacteric fruit that undergoes rapid softening during ripening, leading to substantial losses during storage and transport (Carrari and Fernie 2006; Klee and Giovannoni 2011). Hence, unraveling the regulatory mechanisms of fruit ripening and softening is vital for peach production. To date, studies have underscored the pivotal roles of numerous TFs, including NACs, MYBs, HD-ZIPs, MADSs, and ERFs, in peach ripening and softening (Zhou *et al.* 2015; Gu *et al.* 2019; Dai *et al.* 2023; Xu *et al.* 2023). As for LOB/LBD TFs, Wu *et al.* (2024) recently reported that peach PpLBD16 activates the cell wall modification-related genes, *expansin-B2* (*PpEXPB2*) and *subtilisin-like protease 1.7* (*PpSBT1.7*), thus regulating auxin-mediated lateral root initiation process. However, the function of LOBs/LBDs in fruit ripening and softening in peaches remains unclear.

The NAC (NAM/ATAF1/2/CUC2) protein family is one of the largest TF families in higher plants, and many NACs have been shown to play significant roles in fruit ripening and softening (Olsen *et al.* 2005; Forlani *et al.* 2021). For example, in tomato, SlNOR-like1, SlNAM1, and SlNAM2/3 directly target the promoters of ripening-related genes, thus contributing to fruit maturation (Gao *et al.* 2018, 2021; Lin *et al.* 2022). In turn, in apples, NAC18.1 serves as a robust indicator of fruit softening (Migicovsky *et al.* 2021); while in strawberries, the NAC TF FaRIF is a key regulator that controls multiple ripening processes, including abscisic acid (ABA) synthesis and signaling, volatile compound production, and cell wall modification and degradation (Martin-Pizarro *et al.* 2021). Furthermore, some NAC proteins function as upstream regulators of other TFs, thereby indirectly regulating fruit ripening and softening. In particular, papaya CpNAC3 mediates ethylene signaling by specifically targeting the promoters of *CpERF9* and *CpEIL5* during fruit ripening (Fu *et al.* 2021). In apple, MdNAC52 is involved in anthocyanin biosynthesis via the activation of *MdMYB9* and *MdMYB11* expression (Sun *et al.* 2019). Meanwhile, some NAC TFs physically interact with other TFs to synergistically regulate fruit ripening. A case in point, tomato SlNAC4 interacts with RIN and NOR, thus contributing to carotenoid synthesis during fruit ripening (Zhu *et al.* 2014). Similarly, the papaya TF CpNAC2 forms a protein heterodimer with CpEIN3a, to jointly modulate carotenoid accumulation during fruit ripening (Fu *et al.* 2017). Overall, NAC proteins employ diverse regulatory mechanisms to participate in fruit ripening and softening processes, showcasing their substantial modulatory capacity.

In this study, we identified 42 *LOB/LBD* genes in the peach genome. Among these genes, *PpLOB1* exhibited an increased expression toward fruit ripening and softening. Additionally, transient overexpression of *PpLOB1* in peaches resulted in increased expression of softening-related genes, thereby accelerating fruit ripening. Next, we found that PpLOB1 directly bound to and activated the promoters of *PpPL1* and *PpPL15*, which are two key genes involved in cell wall degradation. Furthermore, PpNAP4, a core regulatory TF in peach ripening, was found to transcriptionally activate the expression of *PpLOB1*, acting as its upstream regulator. Transient gene overexpression and silencing assays in peaches confirmed that PpNAP4 positively regulated *PpLOB1* expression. Recent studies have verified the interaction between PpNAP4 and PpNAP6 (Geng *et al.* 2022), and subsequent dual-luciferase reporter (DLR) assays showed that co-expression of *PpNAP4* and *PpNAP6* resulted in a significantly higher activation of the *PpLOB1* promoter. Overall, our findings revealed a novel regulatory module fulfilling roles in peach fruit softening that involves the PpNAP4 and PpLOB1 TFs, thus providing new insights into the mechanism of fruit ripening and softening.

2. Materials and methods

2.1. Plant materials

Peach (*Prunus persica* cv. Jinlinghuanglu, melting flesh) trees cultivated at the Horticulture Experimental Station in Yangling, Shaanxi, China, were used for the experiments reported herein. Fruit samples were collected at six

developmental stages: namely 25, 40, 55, 70, 85, and 100 days after full bloom (DAFB). Fifteen fruits were harvested on each sampling timepoint, and then randomly divided into three sub-groups that served as three biological replicates. Fruit firmness was assessed using a GY-4 fruit penetrometer equipped with an 8-mm diameter probe (Top Instrument Co., Hangzhou, China), with which a fraction of the epicarp on both sides of the peach suture was peeled to enable probe attachment. The pulp from each subgroup was sliced, quick-frozen in liquid nitrogen, mixed, and stored in an ultra-low temperature freezer for subsequent analysis.

2.2. Identification of *LOB/LBD* genes in the peach genome

The 43 identified LOB/LBD proteins of *Arabidopsis* were retrieved from The *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org/). These amino acid sequences were used as baits to search for LOB/LBD proteins in the peach genome (Genome Database for Rosaceae, http://www.rosaceae.org/) with the Basic Local Alignment Search Tool (BLAST). To validate the identified proteins, the Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd/) and the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) were used to filter the LOB/LBD TFs. The corresponding isoelectric points (PI) and molecular weights were calculated using the ExPASy ProtParam tool (https://web.expasy.org/protparam/). Additionally, the web server package Cell-PLoc 2.0, (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) was used to predict the subcellular localization of the proteins. Finally, the online software Search Tool for Retrieval of Interacting Genes/Proteins (STRING) was utilized to predict the interacting proteins.

In addition, the amino acid sequences of LOBs/LBDs from *Arabidopsis*, peaches, and other species were used to construct a phylogenetic tree, with which the related sequences were listed in Appendix A. Multiple alignments were performed using ClustalW with the default parameters, and the phylogenetic tree was constructed using the neighbor-joining method in MEGA 7 software.

2.3. RNA extraction, gene expression analysis, and molecular cloning assay

Total RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, following the protocol outlined by Chang *et al.* (1993). Double-stranded cDNA synthesis was performed using the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR ver.2 (Accurate Biotechnology, Changsha, China). Subsequently, real-time quantitative PCR (RT-qPCR) was performed using the SYBR Green Primers Pro *Taq* HS qPCR Kit (Accurate Biotechnology, Changsha, China) according to manufacturer instructions, with the Bio-Rad iQ5 real-time PCR system utilized in this assay. Gene transcriptional levels were determined using the $2^{-\Delta\Delta Ct}$ method, and the primers were detailed in Appendix B. The means of the reference genes, *PpTua5* (*Prupe.6G004100*) and *PpCYP2* (*Prupe.8G233900*), were used as the internal controls.

Synthetic double-strand cDNA obtained from mature peaches (100 DAFB) was used as the template for fragment cloning of specific genes, with the corresponding primers listed in Appendix C. Specific sequence cloning was performed using the PrimeSTAR Max DNA Polymerase (Takara Bio, Beijing, China). DNA fragments were purified using the Universal DNA Purification Kit (TianGen, Beijing, China). Additionally, the Ready-to-Use Seamless Cloning Kit (Sangon Biotech, Shanghai, China) was used for seamless cloning of specific fragments into plasmids.

2.4. Subcellular localization and transcriptional activation assays of PpLOB1

The coding region of *PpLOB1* without the stop codon was cloned into the pCMBIA2300 plasmid, resulting in the recombinant vector named 35S: PpLOB1-GFP. The constructed vector, along with the pCMBIA2300 empty vector (35S: GFP), were separately transformed into *Agrobacterium tumefaciens* GV3101 (AngYuBio, Shanghai, China) and used for tobacco (*Nicotiana benthamiana*) leaf transformation. Specifically, the *Agrobacteria* strains were cultured to OD_{600}=0.8 in an incubator at 28°C. And then, the bacteria were collected by centrifugation at 5,000 r min^{-1} for 10 min. Following that, bacteria was resuspended in a MES buffer (containing 10 mmol L^{-1} $MgCl_2·6H_2O$, 10 mmol L^{-1} MES, and 150 mmol L^{-1} acetosyringone, pH=5.6), and incubated for 2 h without shaking at room temperature before using for the transformation assay. Three leaves from different tobacco plants were infected with *A. tumefaciens* using a 1 mL syringe. Subsequently, the tobacco plants were cultured at 25°C for 48 h, after which the injected leaves were detected for the fluorescence signal using a laser scanning confocal microscope (Leica SP8 CARS, Germany).

The full-length coding sequence (CDS) of *PpLOB1* was cloned into pGBKT7 (BD) plasmids, to generate recombinant vectors BD-PpLOB1. This BD-PpLOB1 plasmid was introduced into the AH109 yeast strain, and then cultured on SD/–Trp and SD/–Trp/–His/–Ade/+X-α-Gal mediums. The growth status of the strain was used to assess its transcriptional activation. Concomitantly, the same procedures were conducted for BD empty vectors (BD EV) and BD-p53+AD-T cells, which served as negative and positive control, respectively.

2.5. Transient overexpression and gene silencing assays in peach fruit

The CDS of *PpLOB1* was cloned into the pGreen II 62-SK vector (designated SK-PpLOB1) for transient overexpression in peaches. Recombinant SK-PpLOB1 and pGreen II 62-SK empty vectors (SK EV) were transformed into *A. tumefaciens* GV3101 (AngYuBio, Shanghai, China), separately. Each strain was cultured in Luria-Bertani (LB) medium, harvested by centrifugation at 5,000 r min^{-1} for 5 min, and resuspended in MES buffer (OD_{600}=0.8). Prior to infection, the suspensions were incubated for approximately 2 h at room temperature without shaking. Peach fruits at 95 DAFB were injected using syringes, following the method outlined in our previous study (Dai *et al.* 2023). Five days later, all the fruits were collected and transported to the laboratory. Fruit firmness of the

infection site was measured using a GY-4 fruit penetrometer (Top Instrument Co., Hangzhou, China) as described by Li *et al.* (2016).

For the virus-induced gene silencing (VIGS) assay, specific fragments of *PpLOB1* were cloned into pTRV2 vectors to generate TRV2-PpLOB1 plasmids. The recombinant plasmids pTRV1 (TRV1) and pTRV2 empty vectors (TRV2 EV) were transformed into *A. tumefaciens* GV3101, respectively. Each strain was incubated, harvested, and resuspended in the MES buffer (OD_{600}=0.8). Before infection, suspensions of TRV1 and TRV2-PpLOB1 were mixed at a ratio of 1:1 (v/v), and the same procedure was performed for TRV1 and TRV2 EV.

Similarly, transient overexpression and VIGS assays of *PpNAP4* were also performed as described above.

2.6. Extraction and measurement of pectin lyase (PL) enzyme

The method of Marín-Rodríguez *et al.* (2003) was used to extract PL enzymes from peach pulp, and determination of PL activity was performed with the procedures outlined by Collmer *et al.* (1988). One unit (1 U) of PL activity was defined as the production of 1 nmol unsaturated digalacturonan per min, per kg of peach pulp.

2.7. Dual luciferase reporter (DLR) and luciferase imaging assays

Approximately 1.5–2 kb promoter sequences of *PpPL1* (*Prupe.1G060900*), *PpPL15* (*Prupe.5G161300*), and *PpLOB1*, were individually amplified and cloned into the pGreen-0800-LUC plasmids, to serve as reporters designated as pro-*PpPL1-LUC*, pro-*PpPL15-LUC*, and pro-*PpLOB1-LUC*, respectively. The SK EV, SK-PpLOB1, SK-PpNAP4, and SK-PpNAP6 plasmids were used as effectors. The DLR assays were conducted following previously published methods (Dai *et al.* 2023), in which the infection procedures in tobacco leaves were consistent with those described in Section 2.5. Various combinations of effectors and reporters were infiltrated into fully expanded tobacco leaves at a ratio of 6:1 (effectors: reporters, v/v). Renilla luciferase (REN) and firefly luciferase (LUC) activities were measured using a Dual-Luciferase Assay Kit (TransGen, Beijing, China). Luciferase imaging assays were performed using an Optical *in vivo* Imaging System, Lumazone PyloN2048B (Princeton Applied Research, Tennessee, USA).

2.8. Electrophoretic mobility-shift assay (EMSA)

The CDSs of *PpLOB1* and *PpNAP4*, without the stop codon, were individually cloned into pET-28a plasmids, and the recombinant vector was transformed into *E. coli* BL21 (DE3) (AngYuBio, Shanghai, China) for prokaryotic expression. Protein induction followed the established protocols in previous studies (Cao *et al.* 2021, 2023). Induction of protein expression was achieved by applying 1 mmol L^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) when the bacterial culture reached a density of 0.6–0.8 at 600 nm (OD_{600}). The induction culture proceeded for 16 h, then the bacterial cells were collected by centrifugation (4°C, 5,000 r min^{-1}, 5 min) and resuspended in 1× PBS buffer. Ultrasonic fragmentation was performed fully on ice using an ultrasonic soundproof box (Xinyi-650N, Ningbo, China). Ni-NTA Complete His-Tag Purification Resin (Roche, Basel, Switzerland) was used for protein purification following manufacturer instructions. Purified PpLOB1-His, PpNAP4-His, and His-tag proteins were used for subsequent EMSAs.

EMSAs were conducted using the Chemiluminescent EMSA-Shift Kit (Beyotime, Shanghai, China) following the manufacturer instructions. Double-stranded probes labeled with biotin were used as biotin probes, while unlabeled oligonucleotides served as competitors for binding TFs. All probes used for EMSAs were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Electrophoretic separation of the reaction mixture was performed on 6% native polyacrylamide gel (PAGE), followed by transferring onto a positively charged nylon membrane (PALL). Membrane imaging was performed using a ChemiDoc MP03030611 System (Bio-Rad, Hercules, CA, USA).

2.9. Statistical analysis

Student's *t*-test and Duncan's multiple range tests were conducted using IBM SPSS Statistics 23. Figures were generated using Origin 2018 and Microsoft Office PowerPoint (PPT), and the heat map was constructed using the Toolbox for Biologist (TBtools).

3. Results

3.1. Identification of LOB/LBDs in the peach genome

Using the identified LOB/LBD protein sequences of *Arabidopsis* as baits, we identified 42 LOB/LBD members in the peach genome. These candidates were further validated by CDD and SMART to confirm the presence of a conserved LOB/LBD domain (DUF260; Pfam number: PF03195). As shown in Table 1, we detailed the chromosomal locations, length of the amino acid (aa) sequences, predicted isoelectric points (pIs), molecular weights (MWs), and the predicted subcellular localization. These *LOB/LBD* genes were located across all eight peach chromosomes, with ten members located on the second chromosome. The deduced protein lengths ranged from 99 (Prupe.1G033400) to 366 (Prupe.4G055400) amino acids, with molecular weights ranging from 10.81 kDa (Prupe.1G033400) to 41.18 kDa (Prupe.3G049100). In turn, pIs varied between 9.38 (Prupe.6G223100) and 4.88 (Prupe.2G238900), indicating the potential functional diversity in various cellular microenvironments. Additionally, all LOB/LBD member proteins were predicted to be localized in the nucleus.

3.2. *PpLOB1* exhibits high expression level during peach ripening

A remarkable decrease in firmness was observed concomitant with peach ripening (Fig. 1-A). Further, subsequent analysis of the expression patterns of *LOB/LBD* genes in peach fruits at six developmental stages revealed that *Prupe.4G105700* showed a continuously increasing expression pattern throughout fruit development, peaking at ripening (100 DAFB) (Fig. 1-B; Appendix D). In contrast, the expression of all

Table 1 Detailed information of 42 LOB/LBD genes in peach and their encoded proteins[1)]

Accession number	Chromosome location	Length (aa)	pI	MW (kDa)	Predicted location
Prupe.1G033400	Pp01: 2316907–2317203 (–)	99	6.56	10.81	Nucleus
Prupe.1G311000	Pp01: 30221710–30222995 (–)	219	8.99	23.36	Nucleus
Prupe.1G411200	Pp01: 35911445–35912561 (+)	163	6.70	18.63	Nucleus
Prupe.1G424400	Pp01: 36583627–36585209 (+)	224	9.07	23.97	Nucleus
Prupe.2G014200	Pp02: 1258279–1259278 (–)	127	8.71	14.36	Nucleus
Prupe.2G146500	Pp02: 20283293–20286346 (–)	233	8.39	25.01	Nucleus
Prupe.2G146600	Pp02: 20297644–20301214 (+)	253	7.70	25.81	Nucleus
Prupe.2G191100	Pp02: 23050006–23050840 (–)	155	6.44	17.76	Nucleus
Prupe.2G218000	Pp02: 24768922–24770351 (–)	232	6.28	25.19	Nucleus
Prupe.2G238900	Pp02: 25939342–25940572 (+)	224	4.88	25.23	Nucleus
Prupe.2G253300	Pp02: 26673933–26674988 (–)	190	6.93	20.41	Nucleus
Prupe.2G256200	Pp02: 26822009–26822789 (+)	168	5.91	18.72	Nucleus
Prupe.2G261000	Pp02: 27037614–27039990 (–)	224	9.01	24.44	Nucleus
Prupe.2G290000	Pp02: 28417169–28417788 (+)	135	8.79	14.84	Nucleus
Prupe.3G018800	Pp03: 1331569–1332633 (+)	152	6.40	17.17	Nucleus
Prupe.3G049100	Pp03: 3462265–3463871 (+)	363	6.54	41.18	Nucleus
Prupe.3G071200	Pp03: 5116190–5117439 (+)	137	8.73	15.32	Nucleus
Prupe.3G126300	Pp03: 11621998–11623827 (+)	231	8.72	25.91	Nucleus
Prupe.3G236200	Pp03: 23105567–23107562 (–)	303	7.97	32.41	Nucleus
Prupe.3G273200	Pp03: 25292930–25294403 (+)	308	6.05	34.33	Nucleus
Prupe.3G290800	Pp03: 26084384–26086476 (–)	176	8.55	19.05	Nucleus
Prupe.4G055400	Pp04: 2664536–2666157 (+)	366	6.46	40.59	Nucleus
Prupe.4G086800	Pp04: 4269215–4271632 (–)	266	6.44	28.72	Nucleus
Prupe.4G105700	Pp04: 5462280–5464991 (–)	175	8.25	18.95	Nucleus
Prupe.4G271500	Pp04: 22166073–22167786 (–)	178	5.08	19.73	Nucleus
Prupe.4G138200	Pp04: 7693174–7696786 (–)	179	7.66	19.54	Nucleus
Prupe.5G167800	Pp05: 14475532–14478272 (+)	193	8.23	21.31	Nucleus
Prupe.5G184500	Pp05: 15326615–15328494 (–)	362	5.52	41.07	Nucleus
Prupe.6G043900	Pp06: 3193275–3194560 (+)	254	7.69	27.76	Nucleus
Prupe.6G050400	Pp06: 3550586–3551713 (–)	177	6.57	19.78	Nucleus
Prupe.6G093900	Pp06: 6478501–6480396 (+)	215	5.52	23.22	Nucleus
Prupe.6G223100	Pp06: 22810918–22812912 (+)	249	9.38	26.88	Nucleus
Prupe.6G365600	Pp06: 30684868–30688290 (–)	247	8.25	26.87	Nucleus
Prupe.7G011300	Pp07: 1504646–1506252 (+)	264	6.29	28.50	Nucleus
Prupe.7G137900	Pp07: 15439876–15443815 (+)	313	7.10	34.38	Nucleus
Prupe.7G145700	Pp07: 15900446–15901215 (+)	217	5.45	24.47	Nucleus
Prupe.7G208700	Pp07: 19146787–19148111 (+)	243	5.17	27.17	Nucleus
Prupe.7G209000	Pp07: 19170795–19174752 (+)	191	5.89	21.56	Nucleus
Prupe.8G142100	Pp08: 15940850–15942038 (–)	222	7.01	24.09	Nucleus
Prupe.8G227300	Pp08: 20376706–20377659 (+)	212	5.52	23.40	Nucleus
Prupe.8G227400	Pp08: 20384712–20386007 (+)	240	5.84	26.43	Nucleus
Prupe.8G227500	Pp08: 20391365–20392688 (–)	216	8.53	23.55	Nucleus

[1)] pI, isoelectric point; MW, molecular weight.

other *LOB/LBD* genes remained relatively low across fruit development. Therefore, *Prupe.4G105700* was designated as *PpLOB1* and selected for further investigation of potential involvement in peach ripening and softening. The expression levels of *PpLOB1* in other tissues were also analyzed, including flower buds (FB), blooming flowers (BF), young leaves (YL), mature leaves (ML), and young branches (YB) (Fig. 1-C). Compared to its high expression levels in fruits, *PpLOB1* was expressed relatively low in these tissues.

3.3. Phylogenetic analysis of the LOB/LBD proteins

Using amino acid sequences from *Arabidopsis*, peach, and other plant species, a phylogenetic analysis was performed to gain an insight into the evolutionary relationship between PpLOB1 and other LOB/LBD TFs. Proteins were classified into two distinct subfamilies; Class I comprised most of the LOB/LBD proteins, whereas Class II included a smaller subset (Fig. 2). Specifically, PpLOB1 was classified as a member of Class II, along with six other peach LOB/LBDs, five *Arabidopsis* homologs, tomato SlLBD47 and SlLOB1, and banana MaLBD4, indicating the potentially functional similarity among these proteins. Particularly, in tomatoes, SlLBD47 is specifically expressed in fruits (Gupta and Gupta 2021), and SlLOB1 plays a significant role in the softening process during fruit ripening (Shi *et al.* 2021). These findings suggested that Class II TFs might participate in the regulation of fruit development and ripening.

Due to its close phylogenetic affinity to PpLOB1, *Arabidopsis* AT1G31320 (LBD4) was selected as a reference for predicting interacting proteins (Fig. 2; Appendix E). The

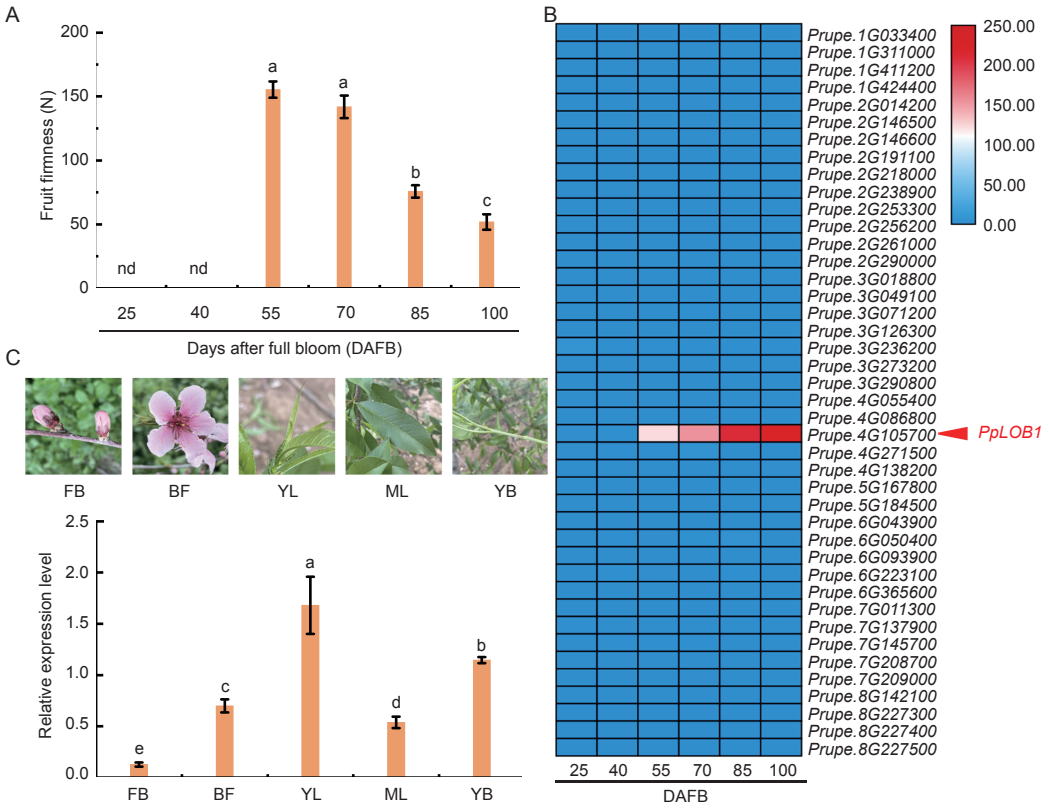


Fig. 1 Gene *PpLOB1* shows increased expression during fruit ripening and softening. A, peach firmness during development. 'nd' stands for not detected. B, transcription patterns of peach *LOB/LBD* genes during fruit development, and specific data used in the heatmap are shown in Appendix D. C, expression levels of *PpLOB1* in various tissues including flower buds (FB), blooming flowers (BF), young leaves (YL), mature leaves (ML), and young branches (YB). Bars indicate ±SD (*n*=3). Expression of *PpLOB1* at 25 DAFB is set to 1.

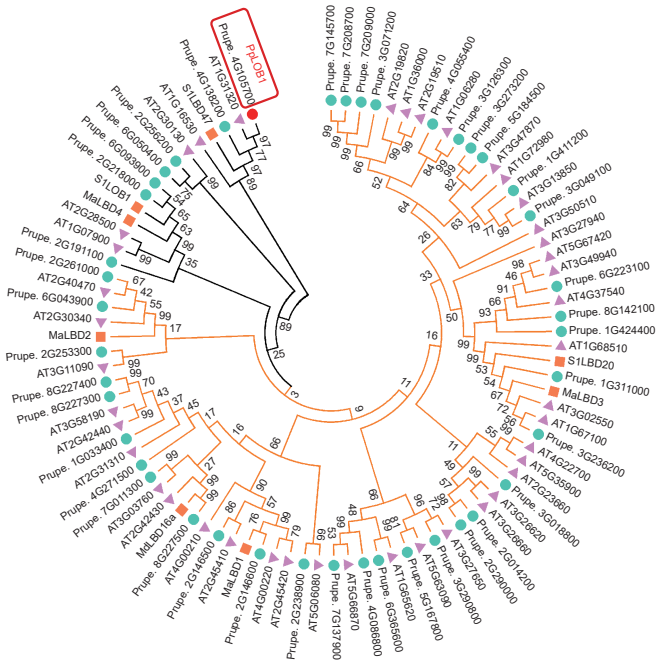


Fig. 2 Phylogenetic tree of LOB/LBD transcription factors (TFs) from peach (*Prunus persica*), *Arabidopsis* (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*) and banana (*Musa acuminate*). LOB/LBD proteins are classified into two groups: Class I (orange branches) and Class II (black branches).

predicted functional partners encompassed a range of TFs and phytohormone signaling molecules, including WOX4/14, Dof5.3, TDR, BRN1-2, CLE41/44, KNAT1, RAS1, and AHL11, which are implicated in a multitude of developmental processes (Appendix E). Consequently, we hypothesized that PpLOB1 might exert pleiotropic effects during peach development, analogous to its *Arabidopsis* counterpart.

3.4. Subcellular localization and transcriptional self-activation of PpLOB1

To further elucidate the characteristics of PpLOB1, intracellular localization and transactivation assays were performed. The full-length CDS of *PpLOB1* without the stop codon, fused with *GFP* under control by the *CaMV 35S* promoter, was transiently expressed in tobacco leaves. As shown in Fig. 3-A, the 35S:PpLOB1-GFP fusion protein showed a strong fluorescent signal predominantly in the cell nucleus, with weaker fluorescence in the cytosol or cytomembrane. Nuclear localization of PpLOB1 was consistent with the results observed for most LOB/LBD TFs, whereas its presence in other subcellular structures suggested its potential role in diverse cellular microstructures.

The transcriptional activation capacity of PpLOB1 was assessed in yeast strains harboring the recombinant BD-PpLOB1 vector. Results revealed that BD-PpLOB1, along with the positive control, showed robust growth and turned blue when cultured in SD/–Trp/–His/–Ade/+X-α-Gel medium,

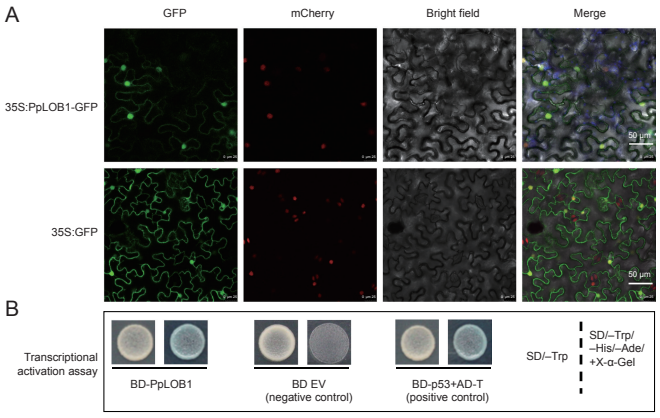


Fig. 3 Subcellular localization and transcriptional activation assays of PpLOB1. A, subcellular localization assay using tobacco leaves. B, transcriptional activity assay in the yeast AH109 strain.

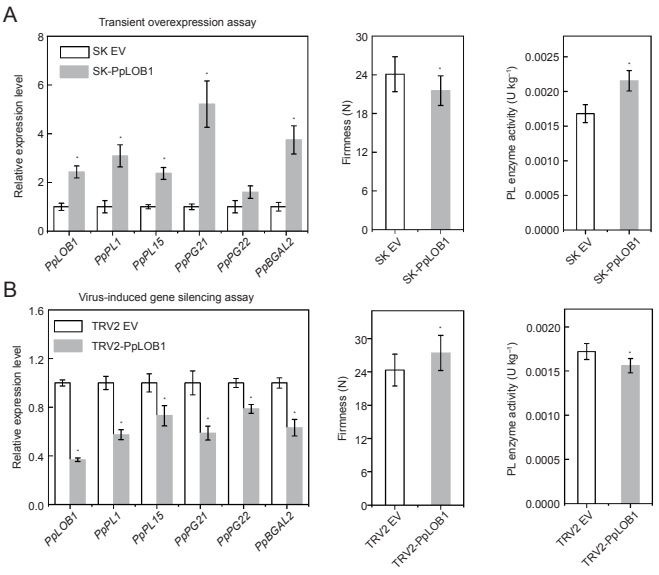


Fig. 4 Transient transformation assays of *PpLOB1* in peach fruit. A, transient overexpression of *PpLOB1* influences peach softening and the expression of related genes. B, virus-induced gene silencing assay (VIGS) in peach fruit. Asterisks indicate significant differences relative to the controls (P<0.05).

indicating the activation for reporter gene *LacZ* (Fig. 3-B). In contrast, the BD empty-vector (BD EV) control did not survive in the selection medium. These findings confirmed that PpLOB1 has trans-activation activity and may function as a transcriptional activator.

3.5. Transient transformation of *PpLOB1* affects the peach softening process

Transient overexpression and gene silencing assays were conducted in peach fruits to investigate the role of PpLOB1 in fruit softening. In the overexpression assay, the transcriptional level of *PpLOB1* in SK-PpLOB1 fruit was approximately 2.4-fold higher than control (SK EV) (Fig. 4-A). Concomitantly, four softening-associated genes, *PpPL1*, *PpPL15*, *PpPG21* (*Prupe.4G261900*), and *PpBGAL2* (*Prupe*.*3G050200*), were significantly induced in *PpLOB1* overexpressing fruit. Additionally, the firmness of SK-PpLOB1 fruit was notably lower than SK EV (Fig. 4-A), and PL activity was clearly enhanced by *PpLOB1*.

Meanwhile, in VIGS assay of *PpLOB1*, the expression of five softening-related genes was significantly suppressed, and firmness was slightly higher than control (Fig. 4-B). In turn, PL activity significantly decreased owing to *PpLOB1* silencing. Altogether, these observations suggested that PpLOB1 is involved in the pathways underlying the regulation of peach fruit softening.

3.6. PpLOB1 regulates fruit softening by directly targeting and activating the promoters of *PpPL1* and *PpPL15*

Dual luciferase reporter (DLR) assays were performed to investigate the mechanism by which PpLOB1 modulates fruit softening. Previous studies demonstrated that *PpPL1* and *PpPL15* act as core regulators of peach softening, making them prime candidates for further exploration (Xu *et al.* 2022). Hence, the promoters of *PpPL1* and *PpPL15* were cloned into pGreen II 0800-LUC plasmids as reporters, while SK EV and SK-PpLOB1 were used as the effectors (Fig. 5-A). Upon co-transfection with SK-PpLOB1, the LUC/REN ratios of pro-*PpPL1*-*LUC* and pro-*PpPL15*-*LUC* were significantly enhanced compared to SK EV (Fig. 5-B). Additionally, fluorescence imaging revealed notably intense luminescence signals when *PpLOB1* and the respective promoters of *PpPL1* and *PpPL15* were co-expressed (Fig. 5-C).

Recently, the LOB/LBD recognition site (LOBRS) [C/T]TTCT[C/T][T/C] was identified using DNA affinity purification sequencing (DAP-seq) *in vitro* (Wu *et al.* 2024). As expected, LOBRS was detected in the promoters of *PpPL1* and *PpPL15* (Fig. 5-D). Further, EMSAs were conducted to validate the direct binding of PpLOB1 to promoters of *PpPL1* and *PpPL15*. The results demonstrated that the purified PpLOB1-His protein specifically bound to the biotin probes derived from the promoter sequences of *PpPL1* and *PpPL15*, as evidenced by distinctly shifted bands, whereas the His-tag alone did not. These findings demonstrated that PpLOB1 directly activates the promoters of *PpPL1* and *PpPL15*, thus regulating the softening process in peach fruit.

3.7. PpNAP4 acts as an upstream modulator of *PpLOB1*

Previous studies have shown that PpNAP4 functions as a positive regulator of peach ripening by orchestrating various processes including ethylene biosynthesis, fruit softening, sucrose accumulation, and aroma synthesis (Cao *et al.* 2021, 2023; Jin *et al.* 2022; Zhang *et al.* 2023). Notably, the expression trends of *PpNAP4* and *PpLOB1* were highly similar (Cao *et al.* 2021, 2023), and multiple NAC recognition sites (NACRSs) were identified in the promoter region of *PpLOB1* (Appendix F). Hence, we hypothesized that PpNAP4 serves as a direct upstream regulator of *PpLOB1*. Consistently, using DLR and fluorescence imaging assays, we verified that PpNAP4 activated the promoter of *PpLOB1*

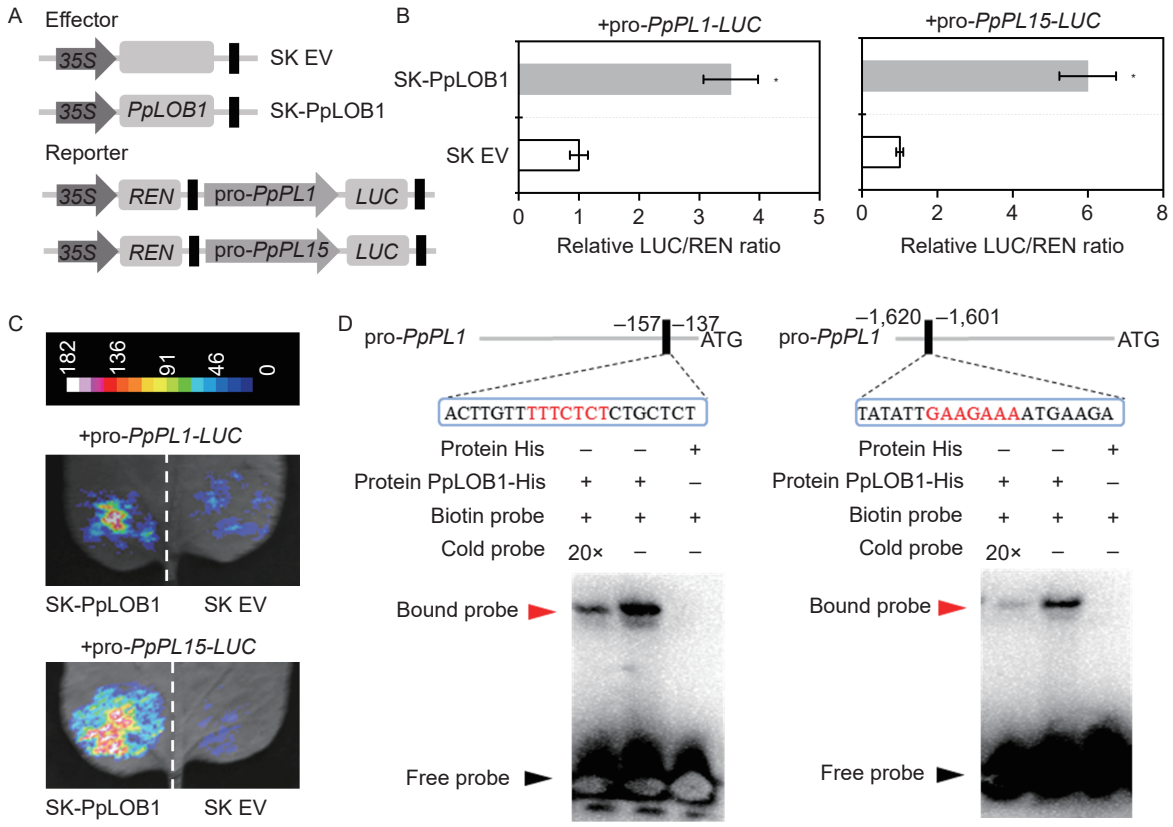


Fig. 5 PpLOB1 positively regulates peach fruit softening by transcriptionally activating the expression of *PpPL1* and *PpPL15*. A, schematic representation of plasmid construction. B, dual-luciferase reporter (DLR) assays conducted on tobacco leaves. Bars indicate SD (n=3), and asterisks represent significant differences relative to the controls (P<0.05). C, luciferase imaging assays conducted on tobacco leaves. D, electrophoretic mobility shift assays (EMSAs) of PpLOB1 and *PpPL1/15* promoter probes.

(Fig. 6-A–C). Additionally, the direct binding of PpNAP4 to the promoter of *PpLOB1* was also confirmed by EMSA (Fig. 6-D).

Transient transformation assays of *PpNAP4* in peaches were conducted to investigate its regulatory effects on *PpLOB1* expression. In the overexpression assay of *PpNAP4*, *PpLOB1* expression was clearly induced (Fig. 6-E). Conversely, *PpLOB1* transcription was significantly suppressed upon *PpNAP4*-silencing (Fig. 6-E). These results demonstrated that PpNAP4 is an upstream regulator of *PpLOB1*, that activates its expression by directly binding to its promoter.

3.8. PpNAP4 and PpNAP6 co-operatively regulate *PpLOB1* expression

Our previous studies demonstrated the involvement of PpNAP6 in ethylene biosynthesis during peach ripening (Dai *et al.* 2023). Recently, Geng *et al.* (2022) detected an interaction between PpNAP4 and PpNAP6; however, the biological significance of this interaction has not yet been elucidated. To investigate the potential cooperative regulation to *PpLOB1*, PpNAP4 and PpNAP6 were co-expressed in tobacco using DLR assays. The results indicated that their co-expression led to significantly higher activation of *PpLOB1* promoter than the individual TF, which was further confirmed by the results of the luciferase imaging assays (Fig. 7-A and B).

4. Discussion

LOB/LBD TFs are regulatory elements essential for plant organ development. The first *LOB/LBD* gene, *AtLOB*, was identified in *Arabidopsis* (Bell *et al.* 2012). To date, many *LOB/LBD* genes have been identified in horticultural plants, including 56 members in tomato (*Solanum lycopersicum*) (Dong and Manghwar 2023), 58 genes in apple (*Malus domestica*) (Wang *et al.* 2023), 37 candidates in strawberry (*Fragaria vesca*) (Chen *et al.* 2018), 60 *LBDs* in white pear (*Pyrus bretschneideri*) (Song *et al.* 2020), 50 identified genes in grapevine (*Vitis vinifera*) (Grimplet *et al.* 2017), and 40 predicted genes in melon (*Cucumis melo*) (Tufekci 2023), among others. In peaches, LBD16 (Prupe.8G227500) reportedly participates in the modulation of lateral root initiation, and is the only LOB/LBD member that has been functionally characterized (Wu *et al.* 2024). In other species, certain LOB/LBD TFs are implicated in the regulation of fruit growth and development (Ba *et al.* 2014; Shi *et al.* 2021; Liu *et al.* 2024). Given the similar regulatory effects of homologous TFs (Zhang *et al.* 2020), we wondered whether certain LOB/LBD members in peach might play a role in fruit growth and ripening.

In this study, we found that *PpLOB1* mRNA levels were high during peach ripening, indicating a significant association with fruit maturation (Fig. 1). Subsequent evolutionary analysis classified PpLOB1 as a Class II LOB/

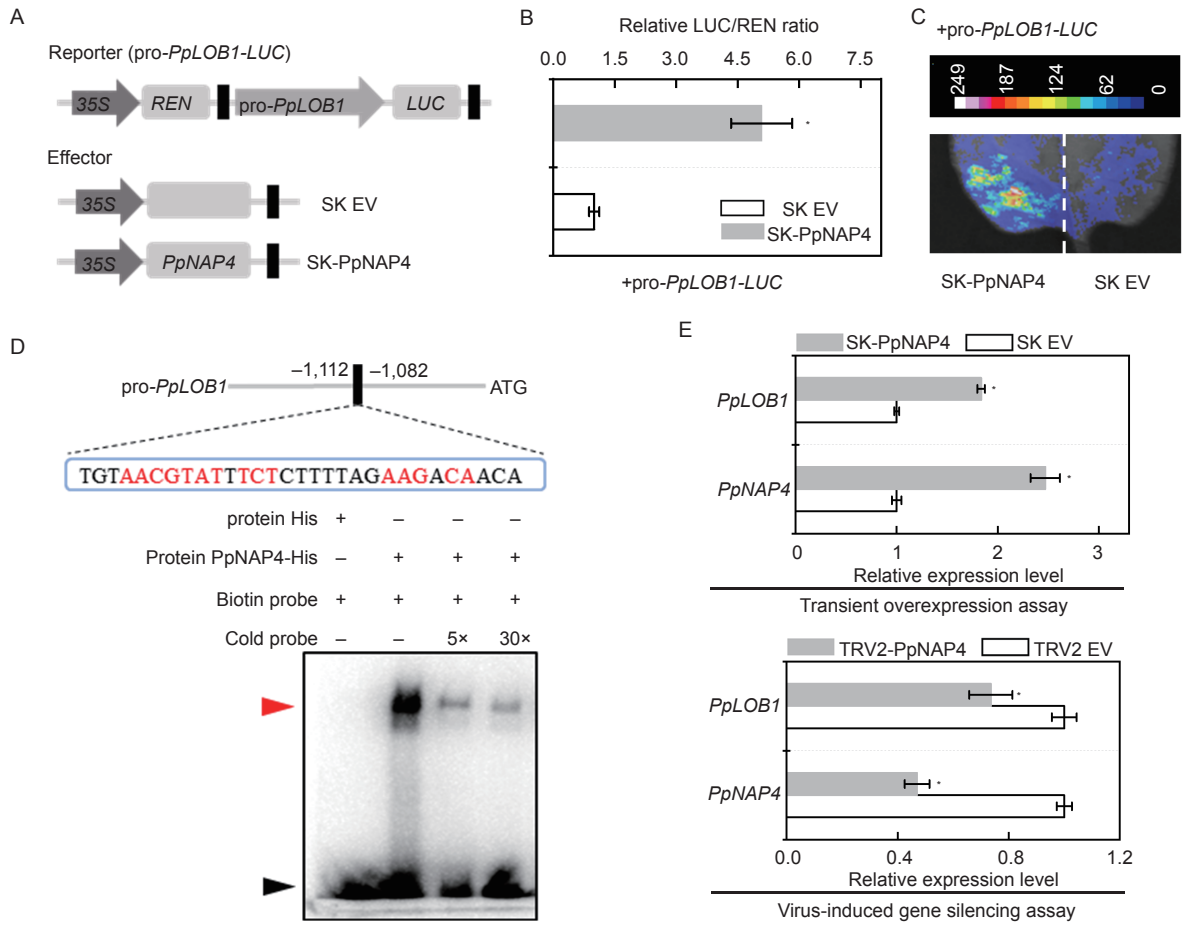


Fig. 6 PpNAP4 acts as the upstream regulator of *PpLOB1*. A, vector construction diagram. B, dual-luciferase reporter (DLR) assay using tobacco leaves. The LUC/REN ratio of the empty SK vector (SK EV) is set to 1. C, luciferase imaging assay of tobacco leaves. D, electrophoretic mobility shift assay (EMSA) of PpNAP4 binding to the *PpLOB1* promoter. E, transient transformation assays of *PpNAP4* in peaches. The transcription levels of the empty vector (EV) were set to 1. Bars indicate SD (n=3), and asterisks represent significant difference relative to the controls (P<0.05).

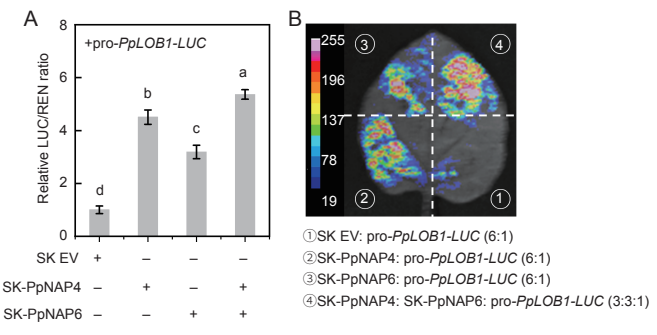


Fig. 7 PpNAP4 and PpNAP6 collaboratively modulate *PpLOB1* expression. A, dual-luciferase reporter (DLR) assay of indicated effectors and reporters in tobacco leaves. Symbols + and – indicate presence or absence, respectively. Vertical bars indicate the means±SD (n=3), and different letters indicate significant difference at 5% level between the combinations. B, luciferase imaging assay in tobacco leaves.

LBD TF, along with other functionally characterized members found in horticultural plants (Fig. 2). Particularly, SlLBD40 and SlLOB1 have been shown to play pivotal roles in tomato fruit development (Shi *et al.* 2021; Liu *et al.* 2024). These observations indicated the potential role of PpLOB1 in peach development. Additionally, the subcellular localization assay showed that PpLOB1 was mainly localized in the nucleus (Fig. 3-A), which is consistent with previous reports that most LOB/LBD TFs are localized in the nucleus (Ba *et al.* 2014; Ba *et al.* 2016; Duan *et al.* 2018; Liu *et al.* 2020; Song *et al.* 2020). In addition, we found weak but clear fluorescence signals of PpLOB1-GFP in the cytosol or cytomembrane, suggesting that PpLOB1 may have distinct roles in these subcellular components (Fig. 3-A), which warrants further investigation. Yeast strains containing the BD-PpLOB1 vector exhibited robust growth in SD/–Trp/–His/–Ade/+X-α-Gel medium (Fig. 3-B), indicating that PpLOB1 functions as a transcriptional activator.

Transient transformation assays and subsequent biochemical experiments revealed that PpLOB1 positively modulates fruit softening by directly targeting the promoters of *PpPL1* and *PpPL15* (Figs. 4 and 5). Notably, the identified PpLOB1 in this study is the first documented LOB/LBD TF involved in peach fruit ripening. Analogously, tomato SlLOB1 participates in the regulation of textural changes by upregulating several cell-wall catabolic genes (Shi *et al.* 2021). In bananas, three ethylene-inducible transcription

factors, MaLBD1/2/3, have been reported to be implicated in fruit softening by transcriptionally activating the expression of *MaEXP1/2* (Ba *et al.* 2014). Our results contribute significantly to the understanding of the mechanisms by which LOB/LBD TFs are involved in fruit softening.

Next, we aimed to identify upstream transcriptional modulators that regulate the expression of *PpLOB1* during fruit ripening. Previous studies have shown that several TFs act as upstream regulators of LOB/LBDs. In *Arabidopsis*, ARF7 and ARF19 bind to the auxin response factor recognition element (AuxRE) in the promoter of *LBD18*, thereby jointly modulating lateral root growth in response to auxins (Pandey *et al.* 2018). Similarly, SlMYC2 serves as an upstream regulator of *SlLBD40*, and SlLBD40 physically interacts with SlMYC2 to regulate cell expansion by promoting *SlEXPA5* transcription during tomato fruit development (Liu *et al.* 2024). In turn, Wuschel-related homeobox 11 (MdWOX11) activates *MdLBD29* expression in apples by binding to its promoter, thereby promoting adventitious root primordium formation (Mao *et al.* 2023). Likewise, rice *LBD16* has been demonstrated to be a direct downstream target of WOX11, controlling crown root development (Geng *et al.* 2024). The NAC TFs comprise one of the largest TF families in plants, and some *NAC*s have been identified as LOB/LBD target genes (Soyano *et al.* 2008; Han *et al.* 2021; Jia *et al.* 2022); however, whether certain NACs act as upstream transcriptional regulators of *LOB/LBD* genes has not been reported.

In peaches, multiple NAC TFs are involved in the regulation of fruit ripening and softening, including PpNAC.A59 (Guo *et al.* 2021), PpNAP4/PpNAC1 (Cao *et al.* 2021, 2023; Zhang *et al.* 2023), PpNAP6 (Dai *et al.* 2023), and BLOOD (BL) (Zhou *et al.* 2015). Particularly, PpNAP4 is a key player in peach fruit maturation and participates in multiple ripening-related processes, with the regulatory roles in peach softening have been well investigated (Cao *et al.* 2023; Zhang *et al.* 2023). Additionally, NACRS was detected in the *PpLOB1* promoter region (Appendix F), prompting an investigation into whether PpNAP4 acts as an upstream modulator of *PpLOB1*. Subsequent DLR experiments, luciferase imaging assays, and EMSAs confirmed the direct interaction and activation of PpNAP4 to the *PpLOB1* promoter (Fig. 6-A–C). Furthermore, transient transformation of *PpNAP4* significantly affected the expression of *PpLOB1* (Fig. 6-E). Previously, yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays demonstrated a physical interaction between PpNAP4 and PpNAP6 (Geng *et al.* 2022). Given the collaborative nature of NAC TFs, we hypothesized that PpNAP4 and PpNAP6 cooperatively regulated the expression of *PpLOB1*. Consistent with previously reported collaborative roles between NAC TFs (Fu *et al.* 2017, 2021), our experiments showed that the co-expression of PpNAP4 and PpNAP6 had a greater activation effect on the *PpLOB1* promoter than the individual TF (Fig. 7).

Altogether, our data allow us to propose a new mechanism by which PpNAP4 and PpLOB1 positively regulate peach softening (Fig. 8). According to this model, increased expression of *PpNAP4* at the onset of peach ripening leads to significant protein accumulation (Li *et al.* 2016; Cao *et al.* 2021). Then, PpNAP4 interacts with PpNAP6,

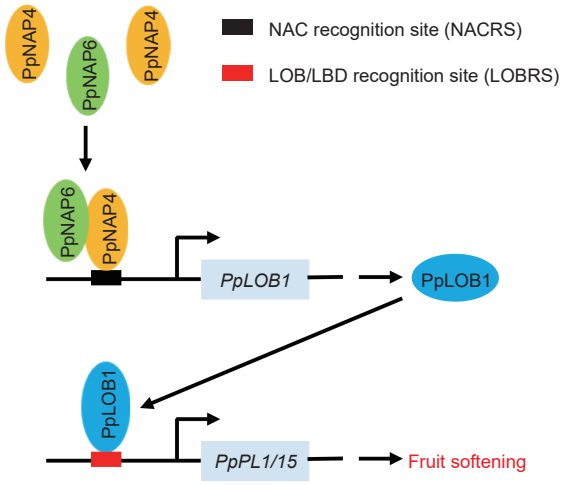


Fig. 8 The working model of PpNAP4–PpLOB1 regulating peach fruit softening. As peach ripening initiates, the accumulated PpNAP4 interacts with PpNAP6 to synergistically regulate *PpLOB1* expression. Activated PpLOB1 transcriptionally induces the expression of *PpPL1* and *PpPL15*, two core genes involved in cell-wall degradation. In conclusion, these transcription factors and structural genes jointly modulate peach fruit softening.

and induces *PpLOB1* expression by targeting its promoter. Subsequently, the accumulated PpLOB1 transcriptionally increases the expression of *PpPL1* and *PpPL15* through direct promoter binding. As a result, the induction of pectate lyase enzymes, which mediate cell-wall degradation and lead to fruit softening. Overall, our findings reveal a novel regulatory module involving PpNAP4, PpNAP6, and PpLOB1, that plays pivotal roles in orchestrating the peach fruit-softening process. Nonetheless, our study has certain limitations. A subcellular localization assay revealed that PpLOB1 is not exclusively localized in the nucleus, but is found in the cytosol or cytomembrane as well. However, the biological significance of such distribution remains unclear. Furthermore, the potential for the coordinated modulation of PpLOB1 and other regulatory factors is currently unclear. These aspects warrant further investigation to fully elucidate the role of PpLOB1.

5. Conclusion

A total of 42 LOB/LBD transcription factors were identified in peach through homologous alignment. Among them, PpLOB1 plays a key role in peach softening by directly targeting and activating the promoters of the *PpPL1* and *PpPL15* genes. Transient transformation assays indicated that *PpLOB1* positively modulates peach fruit softening. Additionally, we confirmed that PpNAP4 is a direct upstream regulator of *PpLOB1*. Another NAC protein, PpNAP6, is involved in *PpLOB1* transcription by interacting with PpNAP4. These results highlighted the modulatory effects of PpLOB1, PpNAP4, and PpNAP6 on peach fruit softening.

Acknowledgements

This work was supported by grants from the China Postdoctoral Science Foundation (2023M742867), the

High-level Innovation and Entrepreneurship Talents Program of QinChuangYuan, Shaanxi Province, China (QCYRCXM-2022-234) and the National Key R&D Program of China (2019YFD1000203).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used Kimi AI to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2025.02.043

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