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The phosphate-responsive protein MdEXO enhances postharvest firmness in apple by modulating brassinosteroid biosynthesis and cell wall metabolism

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Abstract

Fruit softening is a critical determinant of shelf life and quality of apples (*Malus domestica*). While the degradation of cell wall components is a well-established driver of this process, the specific involvement of brassinosteroids (BRs) in postharvest apple softening and how their upstream regulators influence cell wall integrity perception remain unclear. In this study, transcriptomic analysis identified *EXORDIUM* (*MdEXO*) as a gene whose expression declined during storage. Real-time quantitative PCR (RT-qPCR) assays and in situ hybridization confirmed this downregulation and revealed a positive correlation between *MdEXO* expression level and fruit firmness. Further characterization showed that the *MdEXO* protein was phosphate-responsive and localized to the cell wall. Functional studies demonstrated that overexpression of *MdEXO* significantly enhanced fruit firmness, increased protopectin and cellulose content, and reduced soluble pectin levels. These changes were accompanied by the downregulation of key cell wall degradation-related genes, including *polygalacturonase 1* (*MdPG1*), *pectate lyase 5* (*MdPL5*), and β -*galactosidase 9* (*Md β -Gal9*). Additionally, *MdEXO* overexpression increased endogenous BR levels by upregulating the BR biosynthetic gene *DWARF 4* (*MdDWF4*). On the contrary, treatment with brassinazole (BRZ), the BR biosynthesis inhibitor attenuated the firmness-promoting effect of *MdEXO* overexpression. Furthermore, VIGS-mediated transient silencing of *MdEXO* in apple fruits produced the opposite phenotypic effects compared to the overexpression lines. These results demonstrate that *MdEXO* delays apple fruit softening by modulating BR biosynthesis, offering new insights into the hormonal regulation of postharvest fruit quality.

Keywords *Malus domestica*, Fruit softening, *MdEXO*, Brassinosteroids, Cell wall metabolism

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Introduction

Fruit softening, a ubiquitous physiological process following ripening, directly influences the postharvest shelf life, quality, and commercial value of fleshy fruits (Paniagua et al. 2014). As a globally significant economic crop, apple (*Malus domestica*) undergoes complex physiological and biochemical changes during postharvest storage. Excessive softening not only reduces fruit quality but also increases susceptibility to pathogens (Gapper et al. 2013; Liu et al. 2020; Bose et al. 2021; Wei et al. 2023).

The textural quality of fruit is determined by the structural integrity of the primary cell wall and middle lamella, which are composed primarily of polysaccharides such as cellulose, hemicellulose, and pectin (Wolf-Dieter 1998). Softening during ripening and storage is driven largely by the disassembly of this polysaccharide network, a process facilitated by the action of various hydrolytic enzymes (Paniagua et al. 2014). These enzymes, including pectin methylesterase (PME), polygalacturonase (PG), pectin lyase (PL), and β -galactosidase (β -Gal), degrade key pectin and hemicellulose components (Tucker et al. 2017; Wang et al. 2018a).

The critical role of specific cell wall-modifying enzymes in softening has been demonstrated through functional studies across different species. In tomato, down-regulation or mutation of the *PL* gene enhances fruit firmness and extends shelf life by inhibiting cell wall disruption and cell separation (Uluşik et al. 2016; Yang et al. 2017; Wang et al. 2019). Similarly, PL activity has been shown to promote softening in strawberry (Santiago-Doménech et al. 2008; Zhang et al. 2022). In apple, the pectin-degrading enzyme PG is directly involved in postharvest texture changes: *MdPG1* expression is specifically induced during storage, and while its overexpression induces cell separation, silencing of *MdPG1* reduces pectin degradation and delays softening (Atkinson et al. 2002, 2012). These examples highlight the essential and conserved role of targeted cell wall disassembly in regulating fruit texture.

In addition to direct enzymatic degradation, fruit softening is tightly regulated by a complex network involving plant hormones and other signaling molecules. In apple, the transcription factors MdeIL2 and MdCBF2 directly upregulate *MdPG1* expression, integrating ethylene and low-temperature signaling pathways to promote softening (Tacken et al. 2010). Recent research has further elucidated this ethylene response, revealing that the MdWRKY31-MdNAC7 transcriptional cascade directly activates the cell wall-modifying enzyme gene *MdXTH2*, orchestrating a key branch of the ethylene-mediated softening program (Wang et al. 2024). Similarly, abscisic acid (ABA) accelerates softening in strawberry (*Fragaria × ananassa*) by inducing the expression of cell wall-modifying genes such as *FaRGL1*, *FaXyl1*, and *FaXTH1* (Bustamante et al. 2009; Molina-Hidalgo et al. 2013; Nardi et al. 2014). In addition to hormonal regulation, metabolic pathways also play a critical role. For example, the accumulation of metabolites like D-galacturonic acid and D-glucuronic acid inhibits apple fruit softening by interfering with the function of class 1 non-symbiotic hemoglobin (MdHb1) (Zhao et al. 2025), highlighting a non-hormonal layer of regulation. Brassinosteroids (BRs) have

been recognized as positive regulators of ripening in both climacteric and non-climacteric fruits. In tomato, BRs are synthesized during ripening, and BR signaling is integral to this process (Li et al. 2016; Hu et al. 2020). Although BRs are known to delay softening in apples (Ji et al. 2021; Liu et al. 2022), the upstream regulators and the mechanisms linking them to the perception of cell wall integrity remain unclear.

The *EXORDIUM* (*EXO*) gene family, implicated in BR-mediated growth responses (Coll-Garcia et al. 2004), represents a potential molecular link between BR signaling and cell wall dynamics. This study identified *MdEXO* as a phosphate-responsive, cell wall-localized gene whose expression declines during apple storage. Functional analysis demonstrated that overexpression of *MdEXO* enhances firmness by remodeling cell wall components and suppressing cell wall degradation-related genes. Notably, *MdEXO* was found to regulate fruit firmness through a novel hormonal mechanism: it upregulates BR biosynthesis via *DWARF 4* (*MdDWF4*), and its firmness-promoting effect is abolished by a BR biosynthesis inhibitor. These findings position *MdEXO* as a novel regulator that delays apple softening by modulating BR levels.

Materials and methods

Plant materials and treatments

Mature ‘Luli’ apple fruits were harvested at 140 days after blooming (DAB) for transient infiltration assays. Uniformly mature, mechanically undamaged, and disease-free fruit were transported to the laboratory on the day of harvest and stored in a cold room at 4–10°C until use. Orin apple callus was maintained on MS medium. Transient fruit infiltration and stable callus transformation were performed following a previously described protocol (Xiang et al. 2024).

For brassinazole (BRZ) treatment, fruit samples or callus tissue that had been transiently transformed were immersed in a 10 μ M BRZ solution (Ji et al. 2021). All experiments included at least three independent biological replicates, conducted at the National Research Center for Apple Engineering and Technology, Tai’an, Shandong, China.

Subcellular localization of MdEXO

The *MdEXO* coding region was PCR-amplified and fused in-frame with green fluorescent protein (GFP). The resulting constructs were placed under either the cauliflower mosaic virus (CaMV) 35S promoter or the endogenous *MdEXO* promoter. Along with GFP-only control plasmids, the assembled constructs were electroporated into *Agrobacterium tumefaciens* GV3101. Bacterial cultures were grown to an OD₆₀₀ of 0.6–0.8, harvested, and

resuspended in induction buffer containing acetosyringone. The suspensions were kept at room temperature for approximately 2 h to activate virulence genes before infiltration into *Nicotiana benthamiana* leaves.

Leaf tissues were harvested 48 h post-infiltration, briefly incubated in propidium iodide (PI) to stain cell walls, and mounted in liquid medium for imaging. Confocal microscopy was performed on a Zeiss LSM 880 (Airyscan, German) using sequential scanning to avoid spectral crosstalk. GFP and PI signals were captured using standard emission windows, and Z-stack images were obtained for subcellular localization assessment (Xie et al. 2025).

RNA extraction, reverse transcription quantitative PCR (RT-qPCR), and RNA in situ hybridization

Total RNA was extracted from transiently infiltrated apple fruit tissues and apple callus using the OmniPlant RNA Kit, which includes an on-column DNase digestion step to eliminate genomic DNA. RNA integrity was verified by agarose gel electrophoresis and A260/280 ratio measurement. First-strand cDNA was synthesized from 1 µg of RNA using the PrimeScript kit under standard conditions.

RT-qPCR was performed in 20 µL reactions containing SYBR Green master mix and gene-specific primers. Amplification specificity was confirmed by melt-curve analysis. Relative transcript abundance was normalized to 18S rRNA (Defilippi et al. 2005). Each assay included three to four independent biological replicates, each quantified in triplicate. Primers are listed in Supplementary Table S1.

For RNA in situ hybridization, digoxigenin-labeled probes were synthesized *via* T7 polymerase-mediated in vitro transcription. Plant tissues were fixed in formalin-acetic acid-alcohol (FAA), dehydrated through an ethanol series, embedded in paraffin, and sectioned to 8–10 µm thickness. Sections were dewaxed, rehydrated, incubated with proteinase K, and hybridized overnight at 50–55°C. After stringent washes, signals were detected using alkaline phosphatase-conjugated anti-digoxigenin (anti-DIG) antibodies and chromogenic substrates (Wang et al. 2014).

Histochemical staining for superoxide and hydrogen peroxide

For histochemical detection of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), transiently infiltrated apple fruit tissues and apple calli were sampled and subjected to vacuum-assisted infiltration. $O_2^{\bullet-}$ was detected using 0.5 mg/mL nitroblue tetrazolium (NBT) in 10 mM potassium phosphate buffer (pH 7.8), while H_2O_2 was stained

with 1 mg/mL 3,3'-diaminobenzidine (DAB) in 10 mM Tris-HCl (pH 5.0). After infiltration, the samples were incubated in the dark at 28°C for 8 h. The staining solution was then removed, and the tissues were decolorized by boiling in a bleaching solution consisting of ethanol:lactic acid:glycerol (3:1:1, v/v/v) for approximately 5 min until complete chlorophyll removal. After cooling, samples were preserved in absolute ethanol. Stained tissues were examined under a stereomicroscope (DM2500 LED, Leica, German), and images were captured under consistent lighting conditions for documentation and comparative analysis (Zhang et al. 2020).

Determination of $O_2^{\bullet-}$ and H_2O_2 contents

$O_2^{\bullet-}$ content was quantified using the hydroxylamine oxidation method (Lin et al. 2020). Extracts in phosphate buffer were sequentially incubated with hydroxylamine hydrochloride, sulfanilic acid, and α -naphthylamine. After complete color development, absorbance at 530 nm was recorded and compared to standard curves.

H_2O_2 concentration was measured using a commercial kit. Samples were extracted with Reagent I, and colorimetric reactions were initiated by stepwise addition of Reagents II–IV. Final absorbance was measured at 415 nm.

Enzyme activity assays

Superoxide dismutase (SOD) activity was assessed using NBT photoreduction assay (Zhu et al. 2022). Plant tissues were homogenized in ice-cold phosphate buffer, and the crude extract was cleared by centrifugation. Freshly prepared reaction mixtures were maintained under low-light conditions due to riboflavin sensitivity. Enzyme activity was inferred from the ability of extracts to inhibit NBT reduction, which was monitored spectrophotometrically.

Ascorbate peroxidase (APX) activity was determined by monitoring the rate of ascorbate oxidation triggered by H_2O_2 (Hu et al. 2012). Extracts were prepared in an antioxidant-containing buffer to stabilize APX. Absorbance at 290 nm was recorded immediately after H_2O_2 addition, and the linear rate of decrease was used to calculate enzyme activity.

Catalase (CAT) activity was measured by tracking the decomposition of H_2O_2 at 240 nm (Hu et al. 2012). Extracts were pre-cleared to reduce turbidity. The reaction was initiated by adding H_2O_2 , and the decrease in absorbance was monitored continuously to determine initial reaction rates.

The activities of PG, PL, and β -Gal were determined using enzyme-linked immunosorbent assays (ELISA). Briefly, 0.1 g of fresh fruit tissue or callus was homogenized in extraction buffer. Following the instructions

provided in the ELISA kit, absorbance readings were taken at 540 nm (for PG), 235 nm (for PL), and 400 nm (for β -Gal) using a microplate reader (GENios Plus, Tecan Group Ltd., Switzerland).

Determination of malondialdehyde (MDA) and proline contents

MDA was quantified using the thiobarbituric acid (TBA) assay (Ma et al. 2019). Extracts were mixed with TBA solution and heated to facilitate adduct formation. After clarification, absorbance at 450, 532, and 600 nm was measured to correct for background interference and calculate MDA levels.

Proline content was determined using the classical ninhydrin method (Ma et al. 2019). Homogenates in sulfosalicylic acid were heated to release free proline, which then reacted with acidified ninhydrin and was extracted with toluene. The organic phase was quantified at 520 nm.

Determination of phytohormone contents

Levels of major phytohormones (ABA; auxin, IAA; gibberellins, GA; exogenous cytokinin, CTK; BR) were assessed using ELISA kits. Samples were extracted with the organic solvents or buffers recommended by the manufacturer, purified by centrifugation, and incubated in antibody-coated wells. Absorbance readings were converted to concentrations based on standard curves.

Determination of cellulose, protopectin, and soluble pectin

Cellulose content was determined using a micro-scale kit. Approximately 0.3 g of material was homogenized in Extraction Buffer 1 and incubated at 90°C to ensure the complete removal of soluble components. The pellet was washed repeatedly to minimize contamination and then extracted in Extraction Buffer 2 for an extended period to release bound cellulose fractions. After drying and weighing, the residue was solubilized in concentrated sulfuric acid and kept on ice to prevent thermal degradation. After centrifugation, the clarified extract was combined with a colorimetric reagent and incubated at high temperature for chromophore formation, followed by measurement at 620 nm.

Protopectin and soluble pectin concentrations were measured spectrophotometrically from 0.05 g of tissue according to the manufacturer's protocol.

Determination of sugar and acid contents

Titrate acidity was measured by neutralization titration using standardized NaOH (Wang et al. 2022). For HPLC profiling, 0.1 g of tissue was extracted in aqueous solution, filtered through 0.22- μ m membranes, and injected into a chromatographic system equipped with an appropriate column for organic acids and sugars.

Detection was performed using UV or refractive index settings, depending on the analyte. Total soluble sugars were quantified using the phenol–sulfuric acid method, with absorbance measured at 630 nm (Ranjbar et al. 2020; Wang et al. 2025; Yu et al. 2025).

RNA-Seq and data analysis

'Red Delicious' fruit flesh samples collected at three storage stages were frozen in liquid nitrogen, ground into powder, and subjected to RNA extraction. Library construction and sequencing were performed by 'MetWare' Biotechnology (Wuhan, China) using Illumina platforms (NovaSeq6000, USA). Quality-filtered reads were aligned to the reference genome, and gene-level quantification was carried out using featureCounts (Version 2.0.8). FPKM values were computed from normalized read counts.

Differential expression analysis was conducted using DESeq2 (Version 1.50.2), with statistical significance determined by Benjamini–Hochberg-adjusted *P* values. Genes with adjusted *P* < 0.05 and $\log_2|\text{Fold Change}| \geq 1$ were considered differentially expressed. Principal component analysis (PCA) and correlation analyses were performed to assess sample consistency.

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean $\pm$ standard deviation (SD) of three biological replicates. Statistical analyses were performed using ANOVA, followed by Tukey's Honestly Significant Difference (HSD) test.

Results

Identification and characterization of *MdEXO*

To identify genes involved in postharvest softening, transcriptomic analysis was performed on 'Red Delicious' apples after 0, 15, and 45 days of storage. PCA revealed clear separation between the three time points with high reproducibility (Fig. 1a). Comparative analysis identified numerous differentially expressed genes (DEGs) (Fig. 1b), with 132 genes common across all comparisons (Fig. 1c). Among these, *MdEXO* (MD08G1186100) was bioinformatically predicted to localize to the cell wall, with its expression progressively declining during storage (Fig. 1d). qPCR analysis confirmed this down-regulation and demonstrated a strong positive correlation between *MdEXO* transcript levels and fruit firmness (Fig. 1e, f). *MdEXO* consists of a single exon encoding a 312-amino-acid protein with a conserved PHOSPHATE-INDUCED (Phi) domain, showing the highest similarity to a homolog in *Pyrus communis* (Fig. S1a–c). In situ hybridization visually confirmed the temporal decline of *MdEXO* mRNA during storage (Figs. 1g, S2), and

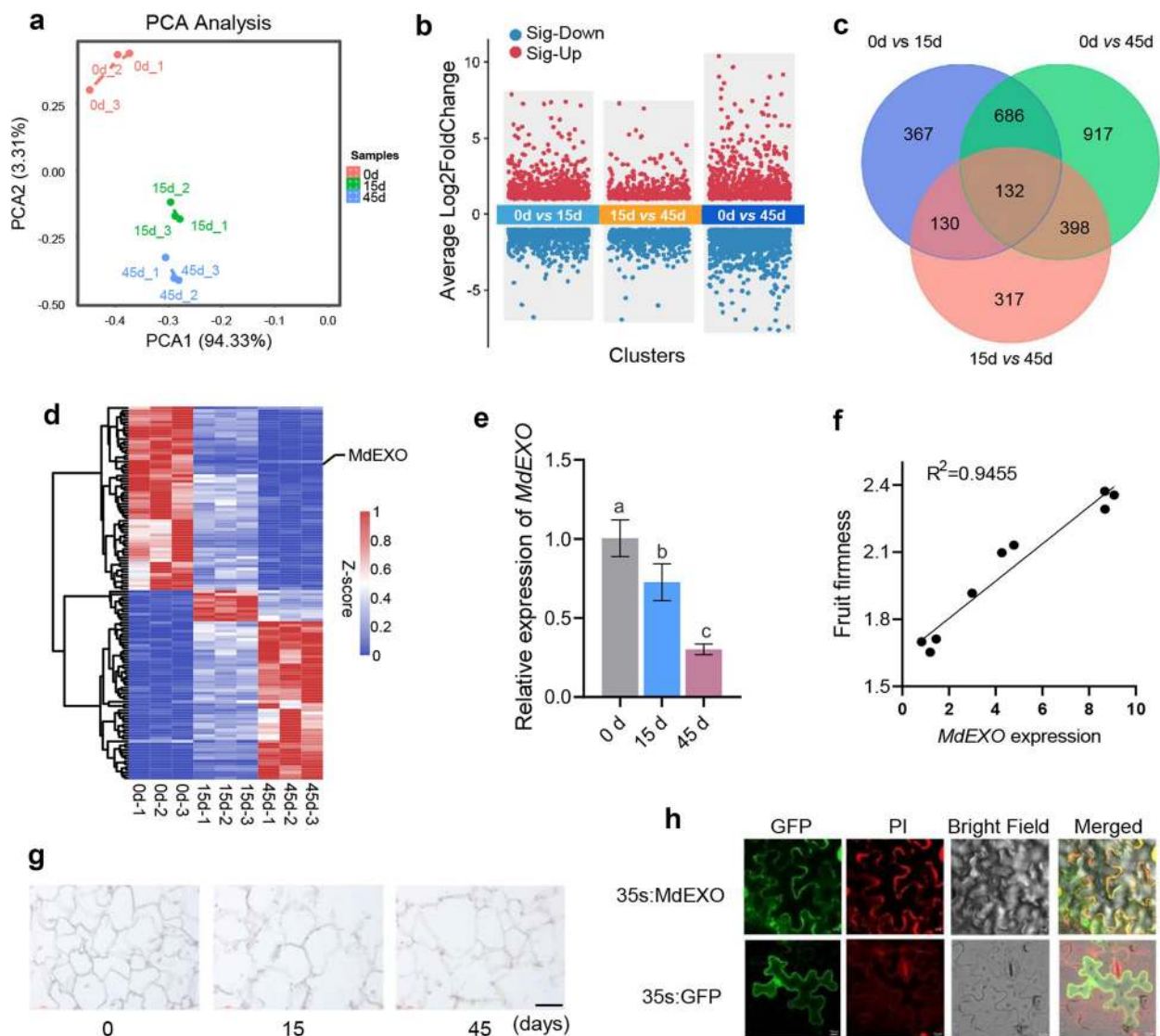


Fig. 1 Changes in the expression of genes and subcellular localization of *EXORDIUM* (*MdEXO*) in apple (*Malus domestica*) during postharvest storage. **a** Principal component analysis (PCA) of apple fruits at different postharvest storage stages. **b** Scatter plot of the average log₂-fold change (log₂FC) in different gene clusters. log₂FC ≥ 2. **c** Venn diagram of differentially expressed genes (DEGs) in apple fruits at different postharvest storage stages. **d** Heatmap of DEG expression profiles. **e** Relative expression level of *MdEXO* in fruits during different storage stages. **f** Analysis of the correlation between fruit firmness and *MdEXO* expression levels during different postharvest stages. **g** RNA in situ hybridization analysis of *MdEXO* expression in postharvest fruits. Scale bar = 100 μm. **h** Subcellular localization of *MdEXO* based on transient expression in tobacco leaves. Scale bar = 10 μm. GFP, green fluorescent protein; PI, propidium iodide. The data are presented as the mean ± SD of at least three biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

subcellular localization assays confirmed its cell wall localization (Fig. 1h).

***MdEXO* positively regulates fruit firmness by modulating cell wall composition**

To determine its function, *MdEXO* was transiently overexpressed or silenced in apple fruit (Fig. 2a). qPCR confirmed successful manipulation of expression (Fig. 2b). *MdEXO* overexpression significantly enhanced fruit

firmness, while silencing reduced firmness (Fig. 2c). Analysis of cell wall components showed that *MdEXO* overexpression increased protopectin and cellulose content while decreasing water-soluble pectin (WSP); silencing resulted in the opposite effects (Fig. 2d–f). These results were corroborated in stable *MdEXO*-overexpressing apple calli, which exhibited higher levels of protopectin and cellulose and lower WSP compared to wild-type (WT) controls (Fig. 2g–k). These results suggest that

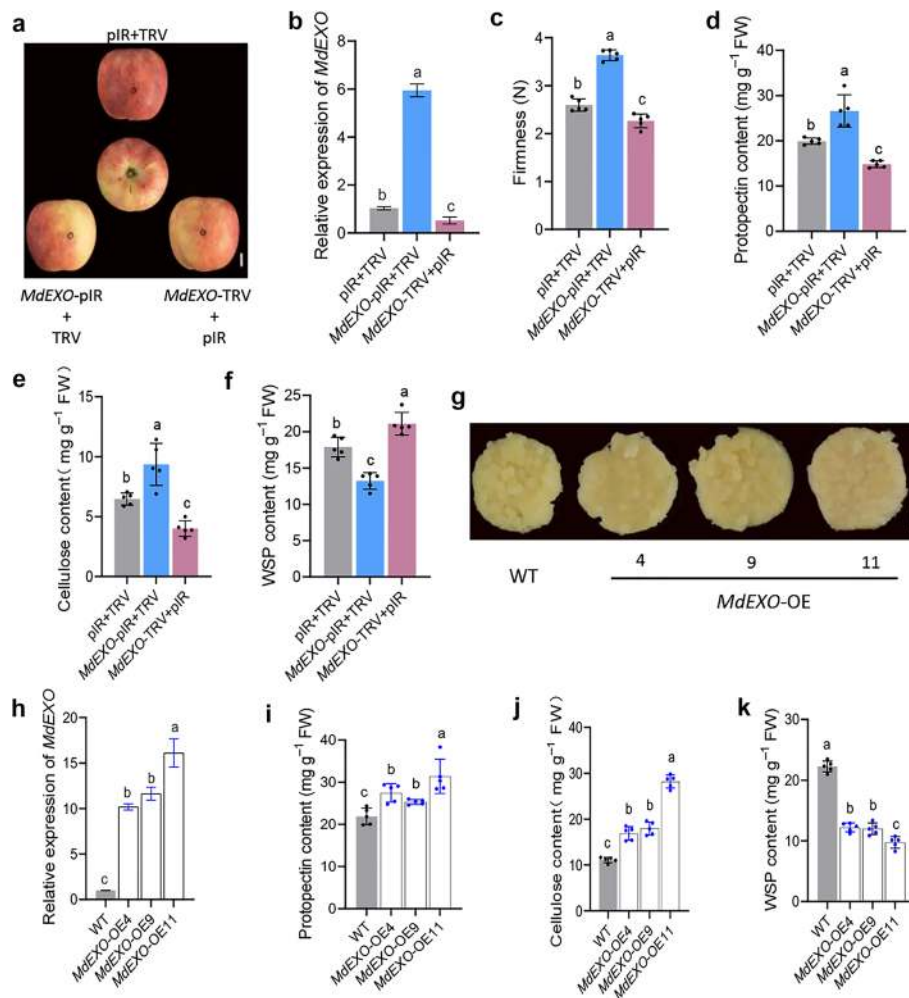


Fig. 2 *EXORDIUM* (*MdEXO*) enhances fruit firmness in apples (*Malus domestica*). **a** Phenotype of ‘Luli’ apple fruit injected with different combinations of overexpression and silencing vectors. Scale bar = 2 cm. pIR, protein interaction reporter; TRV, tobacco rattle virus. **b** RT-qPCR analysis showing the relative expression level of *MdEXO* around the injection sites of apple fruit. **c–f** Fruit firmness (**c**), protopectin content (**d**), cellulose content (**e**), and water-soluble pectin (WSP) content (**f**) around the injection sites in apple fruit. FW, fresh weight. **g** Phenotypes of apple calli overexpressing *MdEXO* (*MdEXO*-OE) and wild-type (WT). Scale bar = 1 cm. **h** RT-qPCR analysis showing the relative expression level of *MdEXO* in WT type and transgenic apple calli. **i–k** Protopectin content (**i**), cellulose content (**j**), and WSP content (**k**) in WT type and transgenic apple calli. The data are presented as the mean $\pm$ SD of at least three biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

MdEXO inhibits softening by preventing the degradation of major cell wall polymers.

MdEXO overexpression alters activities of enzyme and expressions of genes for cell wall degradation

The disassembly of cell wall polymers, driven by enzymes such as PG, PL, and β -Gal, plays a pivotal role in fruit softening. To investigate whether *MdEXO* overexpression exerts its firmness-preserving effect by altering cell wall degradation, we measured the activities of PG, PL, and β -Gal enzymes, as well as the expression of key genes—*MdPG1*, *MdPL5*, and *Md β -Gal9*—in apple fruit

following transient manipulation of *MdEXO* expression. As a result, *MdEXO* overexpression significantly inhibited the activities of PG, PL, and β -Gal, while silencing led to a pronounced increase in their activities (Fig. 3a–c). Similarly, qPCR analysis showed that *MdEXO* overexpression suppressed the transcript levels of these genes, while silencing led to their upregulation (Fig. 3d–f). This transcriptional regulatory role of *MdEXO* was further confirmed in stable transgenic systems: in apple calli overexpressing *MdEXO*, the activities of PG, PL, and β -Gal enzymes, as well as the expression of *MdPG1*, *MdPL5*, and *Md β -Gal9*, were significantly lower than in

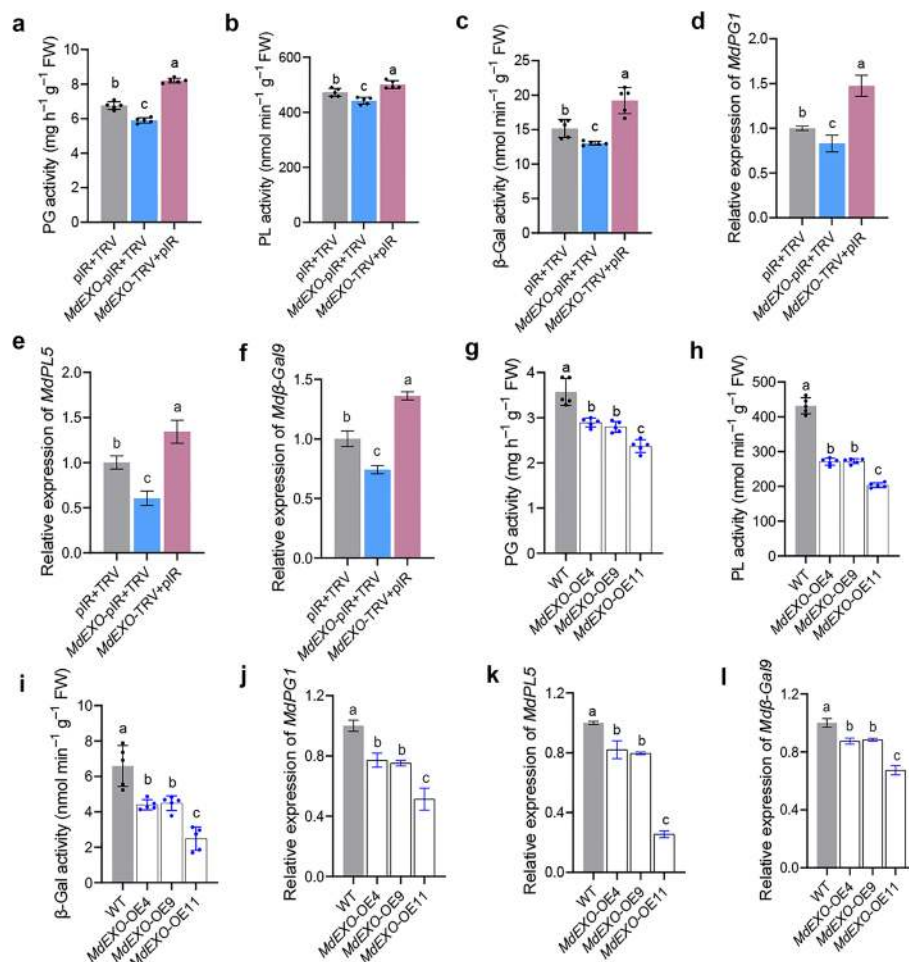


Fig. 3 Effects of *Malus domestica* EXORDIUM (*MdEXO*) overexpression on the expression levels of genes and activities of enzymes related to cell wall degradation. **a–c** Activities of cell wall degradation enzymes, including polygalacturonase (PG; **a**), pectate lyase (PL; **b**) and β-galactosidase (β-Gal; **c**), in the injection sites of apple fruit. FW, fresh weight; pIR, protein interaction reporter; TRV, tobacco rattle virus. **d–f** Relative expression levels of cell wall degradation genes, including *MdPG1* (**d**), *MdPL5* (**e**) and *Mdβ-Gal9* (**f**), at the injection sites of apple fruit. **g–i** Activities of PG (**g**), PL (**h**) and β-Gal (**i**) in apple calli. **j–l** Relative expression levels of *MdPG1* (**j**), *MdPL5* (**k**) and *Mdβ-Gal9* (**l**) in apple calli. Data are presented as the mean ± SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

the WT control (Fig. 3g–l). These results demonstrate that *MdEXO* inhibits the activity of cell wall-degrading enzymes and delays fruit softening, at least in part, through the transcriptional repression of key cell wall degradation genes.

Overexpression of *MdEXO* enhances antioxidant capacity and reduces reactive oxygen species (ROS) accumulation

Given the established connection between fruit softening and the disruption of antioxidant capacity and ROS homeostasis, the role of *MdEXO* in redox balance was investigated. Histochemical staining using NBT and DAB on transiently transformed apple fruits revealed that *MdEXO*-overexpressing fruits exhibited significantly fainter staining compared to the control (CK), while

MdEXO-silenced fruits showed more intense staining (Fig. 4a). Quantitative analysis confirmed that *MdEXO* overexpression significantly reduced the levels of O₂•⁻, H₂O₂, and MDA, while increasing proline accumulation. In contrast, *MdEXO* silencing resulted in the opposite effects (Fig. 4b–e). Additionally, the activities of key antioxidant enzymes—SOD, CAT, and APX—were significantly elevated in *MdEXO*-overexpressing fruits but markedly reduced upon *MdEXO* silencing (Fig. 4f–h).

These findings were corroborated in stable *MdEXO*-overexpressing apple calli. Compared to WT calli, transgenic lines displayed lighter NBT and DAB staining (Fig. 4i), along with reduced levels of O₂•⁻, H₂O₂, and MDA, and increased proline content (Fig. 4j–m). Consistently, the activities of SOD, APX, and CAT were significantly higher

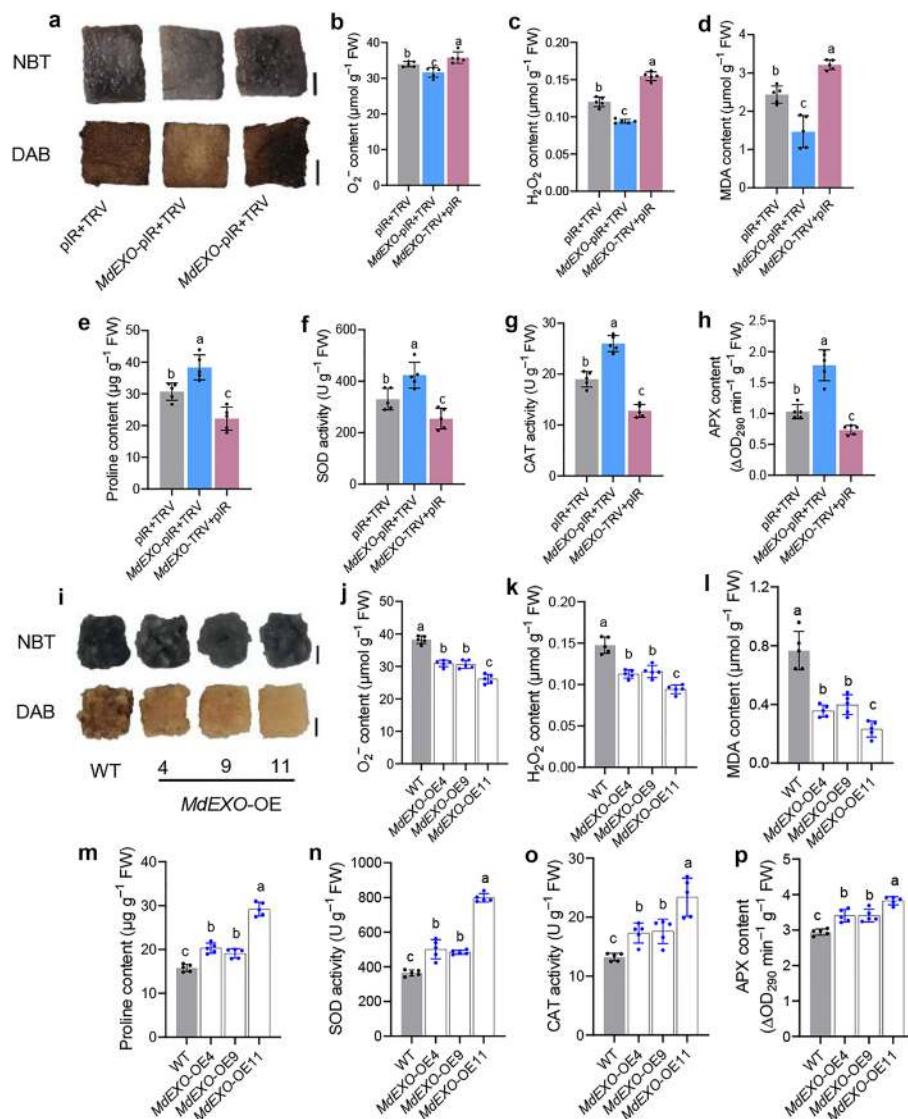


Fig. 4 Effect of *EXORDIUM* (*MdEXO*) overexpression on the antioxidant capacity of apples (*Malus domestica*). **a** nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) staining of the injection site of apple fruit; darker colors indicate higher levels of superoxide anion ($O_2^{\cdot-}$) and H_2O_2 . Scale bar = 1 cm. pIR, protein interaction reporter; TRV, tobacco rattle virus. **b–e** The contents of $O_2^{\cdot-}$ (**b**), H_2O_2 (**c**), malondialdehyde (MDA; **d**), and proline (**e**) around the injection site of apple fruit. FW, fresh weight. **f–h** Activities of superoxide dismutase (SOD; **f**), catalase (CAT; **g**), and ascorbate peroxidase (APX; **h**) around the injection site of apple fruit. **i** NBT and DAB staining of *MdEXO*-overexpressing (*MdEXO*-OE) and wild-type (WT) apple calli; darker colors indicate higher levels of $O_2^{\cdot-}$ and H_2O_2 . Scale bar = 1 cm. **j–m** The contents of $O_2^{\cdot-}$ (**j**), H_2O_2 (**k**), MDA (**l**), and proline (**m**) in apple calli. **n–p** SOD (**n**), CAT (**o**) and APX (**p**) activities in apple calli. The data are expressed as the mean $\pm$ SD of five biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

in the transgenic calli (Fig. 4n–p). These results demonstrate that *MdEXO* strengthens the antioxidant defense system and enhances ROS scavenging, thus mitigating fruit softening by regulating oxidative metabolism.

MdEXO modulates sugar metabolism to improve fruit quality

To assess the impact of *MdEXO* on fruit quality, key metabolites, including soluble sugars and organic acids, were

analyzed. Transient overexpression of *MdEXO* significantly increased the contents of total soluble sugars, sucrose, fructose, and glucose, whereas silencing *MdEXO* substantially reduced these components (Fig. 5a–d). Notably, neither manipulation of *MdEXO* expression significantly affected titratable acidity or malic acid content (Fig. 5e, f). Consistent with these results, stable *MdEXO*-overexpressing calli also accumulated higher levels of soluble sugars, sucrose, fructose, and glucose compared to WT controls, without

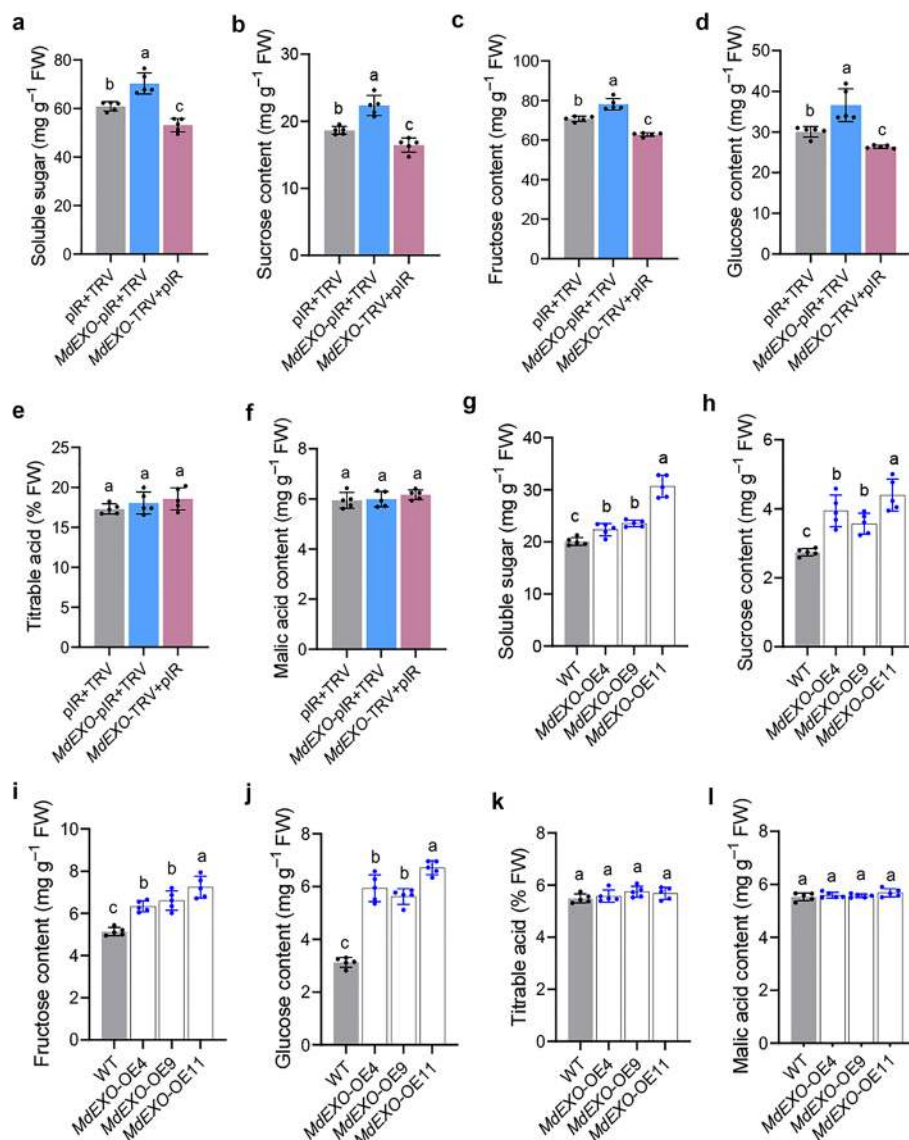


Fig. 5 Effects of *EXORDIUM* (*MdEXO*) silencing on the sugar and acid contents of apple (*Malus domestica*) fruits. **a–f** Contents of soluble sugars (**a**), sucrose (**b**), fructose (**c**), glucose (**d**), titrate acids (**e**), and malic acid (**f**) around the injection site in apple fruit. FW, fresh weight; plR, protein interaction reporter; TRV, tobacco rattle virus. **g–l** Contents of soluble sugars (**g**), sucrose (**h**), fructose (**i**), and glucose (**j**), titrate acids (**k**), and malic acid (**l**) in apple calli. WT, wild-type; *MdEXO*-OE, *MdEXO* overexpressing lines. The data are presented as the mean $\pm$ SD of five biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

significant changes in organic acid content (Fig. 5g–l). These results indicate that *MdEXO* specifically enhances sugar accumulation without affecting acid metabolism, ultimately contributing to improved fruit quality.

MdEXO positively regulates BR biosynthesis

As EXO proteins are involved in BR-mediated growth, the potential influence of *MdEXO* on BR homeostasis in apple was examined. Quantification of endogenous BR levels in transiently transformed fruits showed that

MdEXO overexpression significantly increased BR accumulation, while its silencing led to a notable decrease (Fig. 6a). Mechanistically, *MdEXO* overexpression significantly upregulated the expression of *MdDWF4*, a key enzyme in BR biosynthesis, whereas silencing had the opposite effect (Fig. 6b). In contrast, the transcript levels of the BR receptor gene *BRASSINOSTEROID INSENSITIVE 1* (*MdBR11*) and the BR catabolism-related gene *BR11 SUPPRESSOR 1-LIKE 1* (*MdBAS1*) remained unchanged (Fig. 6c, d). Stable *MdEXO*-overexpressing

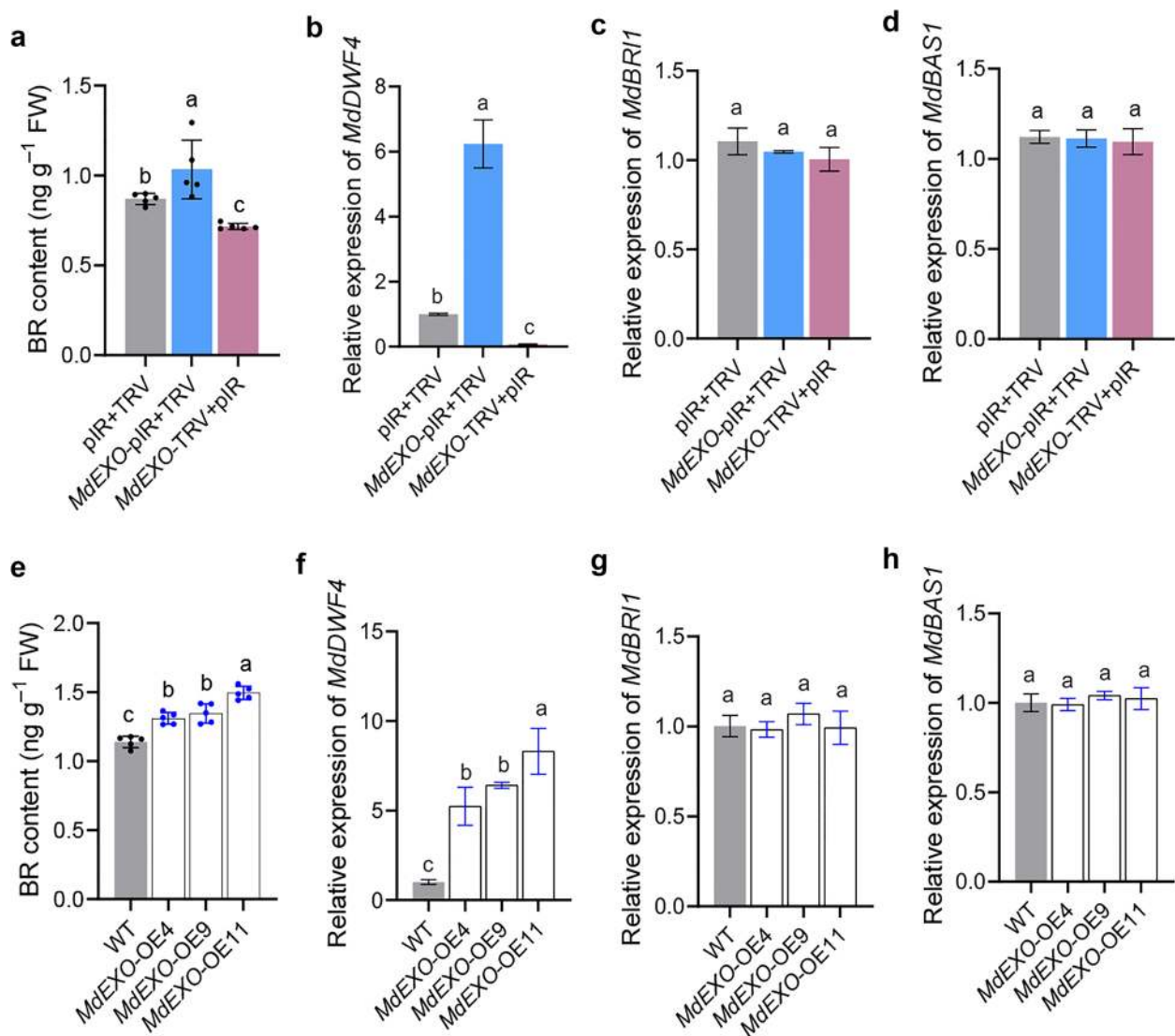


Fig. 6 Effect of *Malus domestica* EXORDIUM (*MdEXO*) silencing on brassinolide (BR) content. **a** BR content around the injection site in apple fruit. FW, fresh weight; pIR, protein interaction reporter; TRV, tobacco rattle virus. **b–d** Relative expression levels of *DWARF 4* (*MdDWF4*; **b**), *BRASSINOSTEROID INSENSITIVE 1* (*MdBRI1*; **c**), and *BRI1 SUPPRESSOR 1-LIKE 1* (*MdBAS1*; **d**) around the injection site in apple fruit. **e** BR content in apple calli. WT, wild-type; *MdEXO*-OE, *MdEXO* overexpressing lines. **f–h** Relative expression levels of *MdDWF4* (**f**), *MdBRI1* (**g**), and *MdBAS1* (**h**) in apple calli. The data are presented as the mean $\pm$ SD of at least three biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

calli exhibited similar results, with significantly higher BR levels (Fig. 6e) and increased *MdDWF4* expression (Fig. 6f), while *MdBRI1* and *MdBAS1* expression levels remained unaffected (Fig. 6g, h). These results indicate that *MdEXO* promotes BR biosynthesis primarily by transcriptionally activating *MdDWF4*.

The *MdEXO*-mediated inhibition of fruit softening depends on BR biosynthesis

To elucidate the functional relationship between *MdEXO* and BR metabolism in fruit softening, a pharmacological

approach was employed using BRZ (10 μ M), a specific BR biosynthesis inhibitor. Treatment of *MdEXO*-overexpressing fruits with BRZ significantly reduced endogenous BR levels (Fig. 7b) and downregulated *MdEXO* expression (Fig. 7c), thereby reversing the enhanced firmness to control levels (Fig. 7d). This effect was associated with reduced protopectin (Fig. 7e) and cellulose (Fig. 7f) contents, along with increased accumulation of WSP (Fig. 7g). Similar experiments in stable *MdEXO*-overexpressing apple calli confirmed these results: BRZ treatment suppressed both BR content (Fig. 7h) and

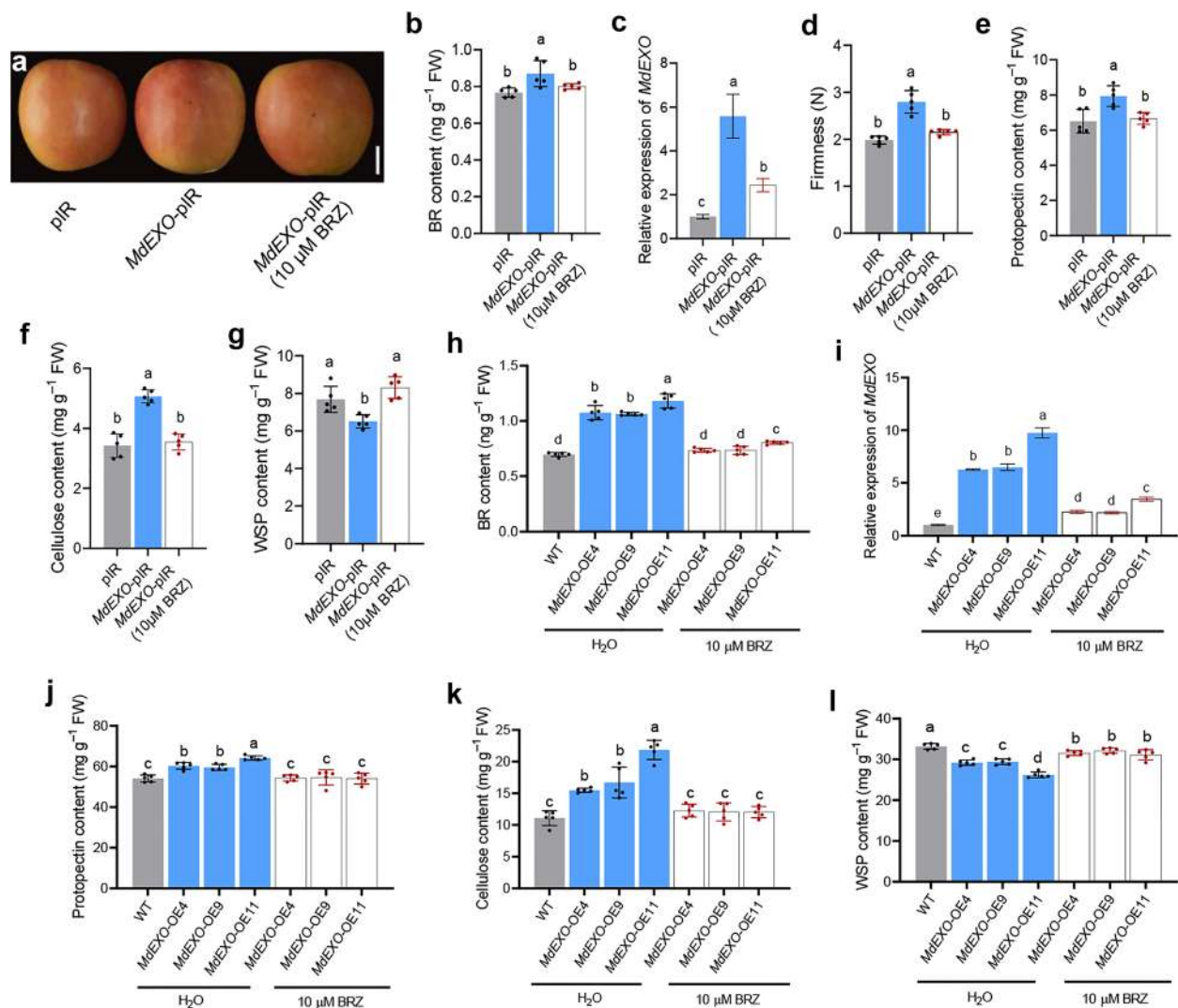


Fig. 7 Effects of brassinazole (BRZ) treatment on *Malus domestica* EXORDIUM (*MdEXO*)-overexpressing (*MdEXO*-OE) fruits and calluses. **a** Phenotype of 'Luli' apple fruit injected with the overexpression vector and treated with 10 μM BRZ. Scale bar = 2 cm. pIR, protein interaction reporter. **b** Brassinolide (BR) content in *MdEXO*-OE apple fruits treated with BRZ. FW, fresh weight. **c** Relative expression level of *MdEXO*. **d–g** Fruit firmness (**d**), protopectin content (**e**), cellulose content (**f**), and water-soluble pectin (WSP; **g**) around the injection site of BRZ-treated apple fruit. **h** BR content in BRZ-treated apple calli. WT, wild-type. **i** Relative expression level of *MdEXO* in apple calli. **j–l** Contents of protopectin (**j**), cellulose (**k**), and WSP (**l**) in BRZ-treated apple calli. The data are presented as the mean ± SD of at least three biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

MdEXO expression (Fig. 7i), leading to a decrease in protopectin (Fig. 7j) and cellulose (Fig. 7k), while WSP levels increased (Fig. 7l).

Additionally, the impact of BRZ on cell wall-degrading enzyme activity and the expression of associated genes was analyzed. In transiently injected, *MdEXO*-overexpressing apple fruit, BRZ treatment significantly restored the activities of PG, PL, and β-Gal enzymes (Fig. 8a–c), as well as the expression of *MdPG1*, *MdPL5*, and *Mdβ-Gal9* (Fig. 8d–f). This was further confirmed in *MdEXO*-overexpressing apple calli, where BRZ treatment restored the

activities of PG, PL, and β-Gal enzymes, along with the expression of *MdPG1*, *MdPL5*, and *Mdβ-Gal9* (Fig. 8g–i). These data indicate that BRs play a key role in maintaining fruit firmness and that *MdEXO*'s suppression of softening is dependent on endogenous BR biosynthesis.

MdEXO regulates apple fruit softening via an integrated multi-pathway mechanism

PCA of the data from transiently transformed fruits revealed distinct clustering between *MdEXO*-overexpressing and -silenced fruits, which were clearly

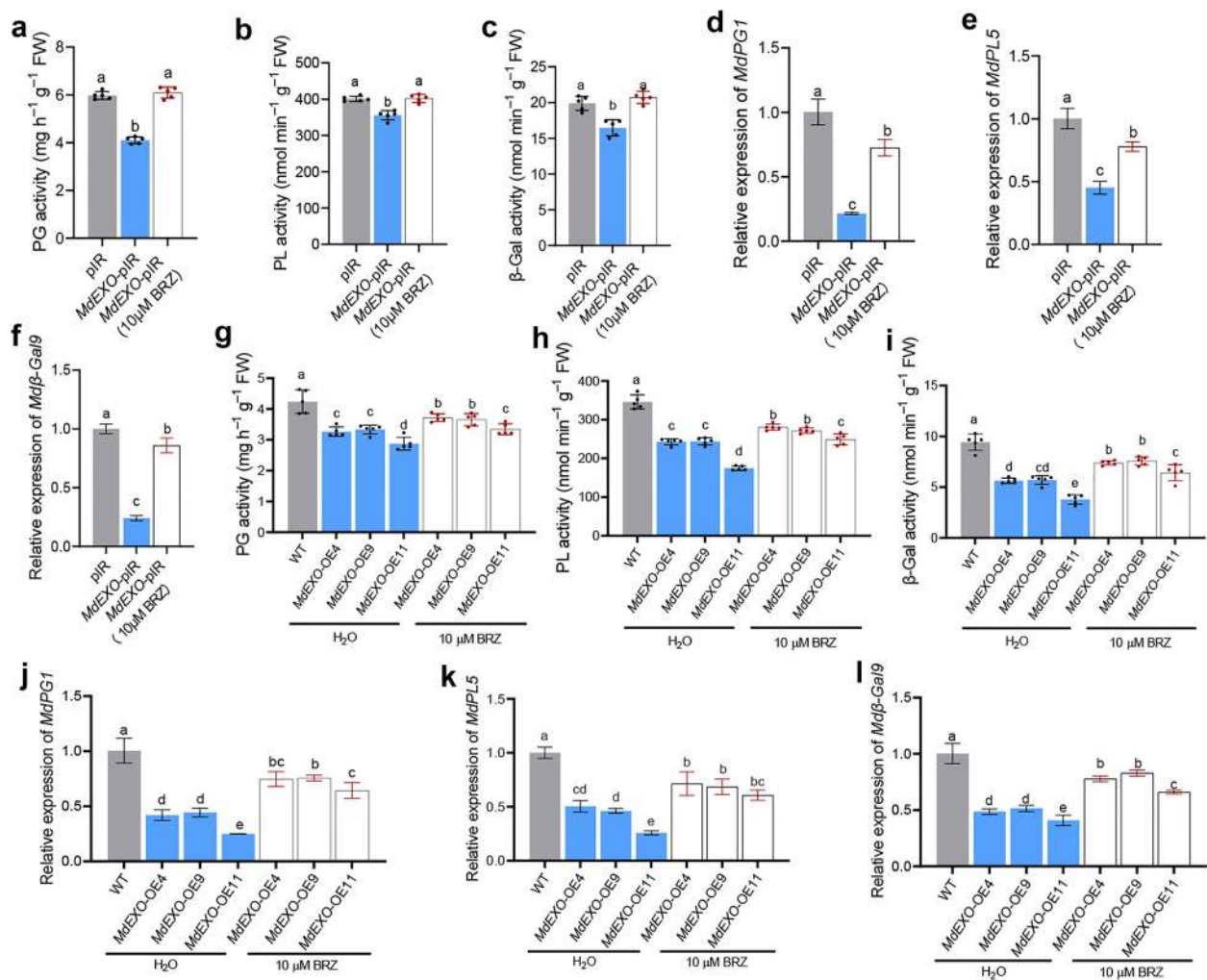


Fig. 8 Effects of brassinazole (BRZ) treatment on cell wall degradation enzyme activity and gene expression in *Malus domestica* EXORDIUM (*MdEXO*)-overexpressing (*MdEXO*-OE) fruits and calluses. **a–c** Activities of cell wall degradation enzymes, including polygalacturonase (PG; **a**), pectate lyase (PL; **b**), and β-galactosidase (β-Gal; **c**), at the injection sites of apple fruit. FW, fresh weight; pIR, protein interaction reporter. **d–f** Relative expression levels of cell wall degradation genes, including *MdPG1* (**d**), *MdPL5* (**e**), and *Mdβ-Gal9* (**f**) at the injection sites of apple fruit. **g–i** Activities of PG (**g**), PL (**h**), and β-Gal (**i**) in apple callus. WT, wild-type. **j–l** Relative expression levels of *MdPG1* (**j**), *MdPL5* (**k**), and *Mdβ-Gal9* (**l**) in apple callus. Data are presented as the mean ± SD from at least three biological replicates. Statistically significant differences ($P < 0.05$) are indicated by different lowercase letters

separated from the control, demonstrating that *MdEXO* significantly influences the physiological state of the fruit (Fig. 9a). *MdEXO* expression showed significant positive correlations with fruit firmness, protopectin, cellulose, antioxidant enzyme (SOD, CAT) activities, proline, sugar contents (soluble sugars, sucrose, glucose, fructose), BR levels, and *MdDWF4* expression. In contrast, *MdEXO* expression negatively correlated with ROS levels ($O_2^{\bullet-}$, H_2O_2), MDA content, and the expression of cell wall degradation-related genes (*MdPG1*, *MdPL5*, *Mdβ-Gal9*).

In summary, *MdEXO* regulates fruit softening through a multifaceted mechanism (Fig. 9b): (1) it upregulates *MdDWF4* to enhance BR biosynthesis, establishing a positive feedback loop that sustains cell wall integrity and

firmness; (2) it strengthens the antioxidant system, reducing oxidative damage and maintaining cellular structure; and (3) it independently promotes sugar accumulation, thus improving fruit quality. These findings uncover a novel regulatory role for *MdEXO* within BR signaling and expand the functional understanding of EXORDIUM-like proteins, offering valuable insights for strategies aimed at improving postharvest apple quality by regulating ripening and senescence.

Discussion

Fruit firmness is a key postharvest quality trait that primarily determines the commercial value of apples and is tightly regulated by cell wall metabolism and

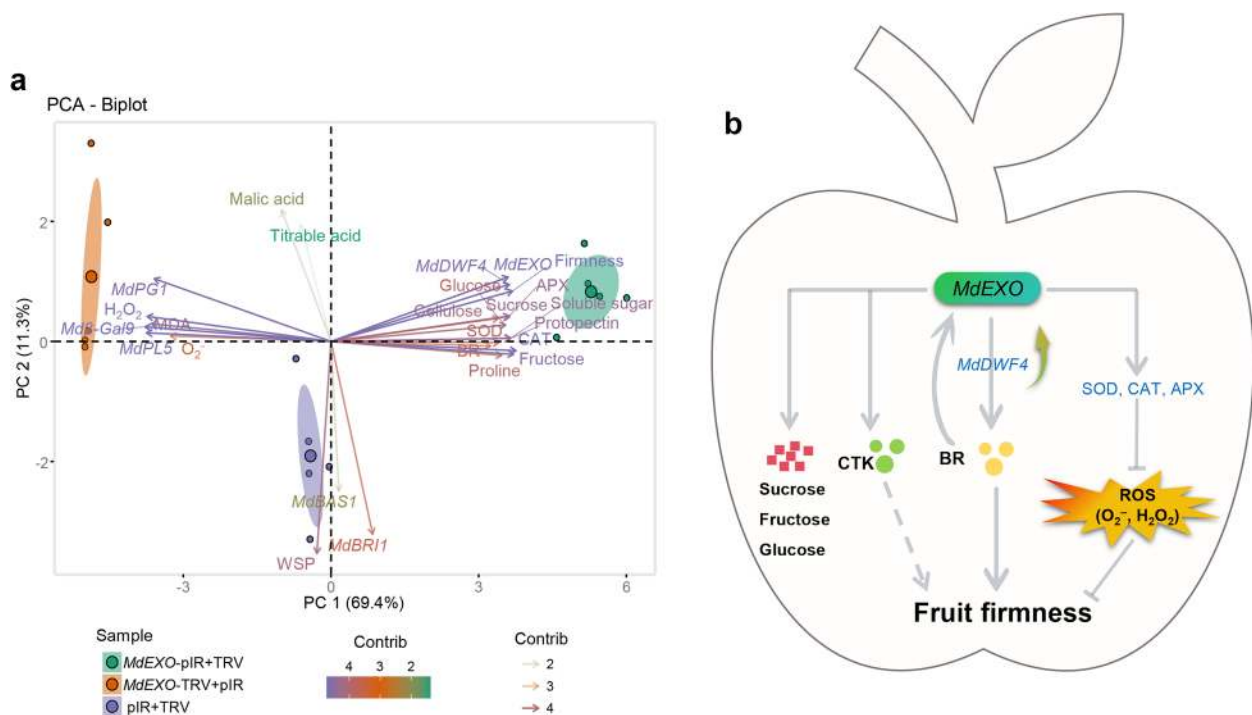


Fig. 9 Comprehensive analysis of the role of *EXORDIUM* (*MdEXO*) in regulating apple (*Malus domestica*) fruit firmness. **a** Principal component analysis (PCA) of various physiological and biochemical indicators in apple fruits subjected to transient injection (pIR+TRV, *MdEXO*-pIR+TRV, and *MdEXO*-TRV+pIR). The plot displays the distribution of samples, with PC1 and PC2 representing the contribution rates of variance. Circles in different colors represent different groups. pIR, protein interaction reporter; TRV, tobacco rattle virus. *MdPG1*, polygalacturonase 1; *MdPL5*, pectate lyase 5; *Mdβ-Gal9*, β-galactosidase 9; MDA, malondialdehyde; O₂⁻, superoxide anion; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; *MdDWF4*, DWARF 4; BR, Brassinolide; *MdBR11*, BRASSINOSTEROID INSENSITIVE 1; *MdBAS1*, BRI1 SUPPRESSOR 1-LIKE 1; WSP, water-soluble pectin. **b** A working model illustrating the role of *MdEXO* in regulating fruit firmness. The arrows and T-bars represent positive and negative regulation, respectively. CTK, exogenous cytokinin; ROS, reactive oxygen species

phytohormone homeostasis. Although BRs play a role in fruit softening, the regulatory network underlying their action, particularly the connection between BR signaling and cell wall integrity sensing in apples, remains poorly understood. This study identified *MdEXO*, a cell wall-localized gene from the EXORDIUM family, whose expression strongly correlates with fruit firmness during storage. Functional analyses demonstrated that *MdEXO* maintains fruit firmness by inhibiting the degradation of protopectin and cellulose. Moreover, *MdEXO* promotes endogenous BR biosynthesis, leading to the transcriptional repression of key cell wall-degrading enzyme genes, including *MdPG1*, *MdPL5*, and *Mdβ-Gal9*. These findings provide new insights into the mechanisms of fruit softening and highlight the critical role of cell wall-localized proteins in integrating structural signals with hormone-mediated regulatory pathways.

The EXORDIUM family encodes proteins containing a conserved PHI-1 domain and regulates cell division and organ growth in model plants (Farrar et al. 2003). This study extends the functional role of *MdEXO* to postharvest physiology and presents evidence for its positive

regulatory role in fruit firmness. Fruit softening primarily results from the depolymerization of the cell wall polysaccharide network (Tucker et al. 2017; Posé et al. 2019). Our data show that *MdEXO* inhibits the degradation of protopectin and cellulose, which correlates with the transcriptional repression of genes involved in cell wall depolymerization, consistent with known cell wall metabolic regulation. However, as a cell wall-localized protein, the mechanism by which *MdEXO* transduces extracellular structural signals to the nucleus to regulate downstream gene expression remains unclear and warrants further investigation. Given its cell wall localization, it is hypothesized that *MdEXO* may function to sense alterations in cell wall architecture or relay extracellular signals, triggering intracellular cascades that ultimately regulate the transcription of cell wall metabolic genes.

The cell wall localization of *MdEXO* positions it as a potential key mediator in the poorly understood signaling cascade that transmits extracellular information to the nucleus. Our findings that a wall-localized protein can regulate nuclear gene expression (e.g., *MdDWF4*) introduce a novel paradigm for “outside-in” signaling

in plants. This suggests that MdEXO may not function merely as a downstream effector but as an active participant in sensing cell wall dynamics or integrating environmental cues (potentially *via* its phosphate-responsive nature) to coordinate transcriptional reprogramming during postharvest physiology. Thus, MdEXO represents a significant conceptual advancement, providing a unique genetic entry point to unravel the fundamental mechanisms of extracellular matrix sensing that coordinate development and stress responses beyond fruit softening.

Given that EXO proteins are involved in BR signaling in *Arabidopsis* (Coll-Garcia et al. 2004; Schröder et al. 2009), it was hypothesized that MdEXO may regulate fruit softening through modulation of hormone pathways. Indeed, overexpression of *MdEXO* specifically increased endogenous BR levels and the expression of the BR biosynthetic gene *MdDWF4*, without affecting the core components of BR signal transduction. This places *MdEXO* upstream in the BR biosynthesis pathway. Notably, classical models describe *EXO* as a downstream responsive factor in BR signaling (Coll-Garcia et al. 2004), while our study uncovers a positive feedback regulation of BR biosynthesis by *MdEXO*, thus extending the current understanding of EXORDIUM protein functions. This feedback mechanism may be physiologically important for maintaining hormonal homeostasis during postharvest fruit development. BR functions in fruit softening vary across species—delaying softening in apple and peach (Ji et al. 2021; Liu et al. 2022; Meng et al. 2023) but promoting it in tomato and persimmon (Liu et al. 2021; He et al. 2018). In our study, treatment with the BR biosynthesis inhibitor BRZ reversed the phenotypic effects of *MdEXO* overexpression, providing direct evidence that *MdEXO* acts through the BR pathway and confirming the positive role of BR in maintaining apple fruit firmness. These opposing phenotypic effects suggest that, although the BR signaling pathway itself is conserved, its downstream target genes may be programmed for distinct physiological functions in fruits of different species. In fruits like apples, intended for long-term storage, BR signaling may be “redirected” to maintain cell wall integrity, while in rapidly ripening fruits like tomatoes, it promotes softening. The identification of *MdEXO* as an upstream regulator opens a new avenue for investigating the evolutionary origins of functional divergence in BR signaling across species.

Fruit softening is regulated by a complex interplay of endogenous hormones and their interactions. GA induce *Gibberellic Acid-Stimulated Arabidopsis* (*SIGASA1*) expression in tomato (*Solanum lycopersicum*) and suppress the transcription of cell wall remodeling genes, such as *PG2a* and *PL* (Su et al. 2023). IAA interacts with ethylene in a dose-dependent manner to modulate peach

softening (Ma et al. 2023). In ‘Orin’ apples, ABA accelerates softening by promoting ethylene biosynthesis through the upregulation of ethylene-regulating genes (Wang et al. 2018b). CTK treatments delay ripening and softening in banana by maintaining chlorophyll content (Huang and He 2023). In our study, *MdEXO* overexpression not only increased BR levels but also specifically elevated CTK content (Fig. S3), suggesting that *MdEXO*-mediated physiological effects may stem from the combined action of increased BR and enhanced CTK signaling. BR and CTK are known to work synergistically to regulate cell division, proliferation, and differentiation (Zhiponova et al. 2013; Oh et al. 2020). Since EXO is BR-inducible and involved in cell division regulation (Coll-Garcia et al. 2004), the concurrent accumulation of CTK and enhancement of BR signaling likely form a synergistic regulatory module.

During tomato fruit ripening, cell division halts, while nuclear endoreduplication becomes increasingly active, playing a critical role in coordinating energy allocation and metabolic processes. Nuclear endocycle activity is significantly correlated with the accumulation of sugars, organic acids, and other metabolites (Musseau et al. 2020). The elevated CTK levels induced by *MdEXO* may suppress endocycle progression, lower the global anabolic rate, and indirectly influence the biosynthesis of cell wall-modifying enzymes, providing a novel cellular perspective on its softening-delaying phenotype. However, whether apple fruit ripening is similarly regulated by the endocycle, and whether BR and CTK modulate nuclear cycle progression and cell wall gene expression *via* comparable transcriptional complexes, remains to be determined. Future research should focus on exploring the crosstalk between MdEXO and CTK-related pathways to deepen our understanding of its pivotal role in fruit maturation and senescence.

Conclusion

This study establishes *MdEXO* as a central regulator of postharvest fruit softening in apple. MdEXO forms a positive feedback loop—“*MdEXO*—BR—cell wall degradation repression”—suppressing the transcription of cell wall-degrading enzymes and delaying softening. This pathway represents a species-specific softening-inhibition mechanism in apples and highlights the variable roles of BR in fruit physiology across species. Additionally, *MdEXO* enhances antioxidant capacity and sugar accumulation, collectively improving storage quality. By positioning *MdEXO* as a key node linking cell wall integrity sensing with hormonal feedback regulation, this work not only advances our understanding of EXORDIUM protein functions but also provides valuable theoretical and genetic resources for improving apple postharvest quality.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-026-00101-1>.

Supplementary Material 1: Figure S1. Sequence analysis and phylogenetic tree of *Malus domestica* EXORDIUM (*MdEXO*) and its homologous genes. a Exons are represented by gray and orange boxes, with the orange boxes highlighting the conserved PHOSPHATE-INDUCED 1 (Phi-1) domain. Introns are denoted by lines. b Phylogenetic tree of *MdEXO* and its homologous proteins from various species, illustrating their evolutionary relationships. c Multiple sequence alignment of *MdEXO* and other EXO proteins, with the conserved Phi-1 domain indicated by a red box.

Supplementary Material 2: Figure S2. RNA in situ hybridization using a sense probe as the negative control. Scale bar = 100 μ m.

Supplementary Material 3: Figure S3. Phytohormone contents in transiently injected apple fruits and *Malus domestica* EXORDIUM (*MdEXO*)-overexpressing (*MdEXO*-OE) apple callus. a–d Hormone contents around the injection sites in apple fruit: auxin (IAA; a), abscisic acid (ABA; b), gibberellins (GA; c), and exogenous cytokinin (CTK; d). e–h Hormone contents in apple callus: IAA (e), ABA (f), GA (g), and CTK (h). WT, wild-type; pIR, protein interaction reporter; TRV, tobacco rattle virus.

Supplementary Material 4: Table S1. Primers used for gene cloning and RT-qPCR.

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Not applicable.

Authors' contributions

DGH conceived and designed the study. JCM and JCZ performed the experiments. WYW, YX, YWZ, CNM, FFP, FX and WJZ analyzed the data. DGH, JCM and JCZ wrote the paper. All authors discussed the results and commented on the manuscript.

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Data availability

All data generated or analyzed in the study are available within the paper and its supplementary files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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