First identification of auxin glycosyltransferase MdUGT74BP1 from apple

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Abstract

The glycosylation modification of auxin is considered to be one of the important mechanisms for regulating the dynamic balance of plant active hormones in different tissue cells. It is closely related to maintaining the dynamic balance of auxin content in plants, plant growth and development, and environmental response. In this study, we successfully cloned a glycosyltransferase gene *MdUGT74BP1* from an apple using molecular cloning technology. After constructing its prokaryotic expression vector, the active enzyme protein was purified and an *in vitro* enzymatic reaction revealed that *MdUGT74BP1* may be a glycosylated growth element and its analogs. The overexpression of *MdUGT74BP1* into *Arabidopsis thaliana* revealed that, compared to the wild-type, the *MdUGT74BP1*-overexpressing line showed an auxin-deficient phenotype. After exogenously spraying auxin IBA, the free auxin and auxin sugar esters of each plant were extracted and tested by HPLC. Compared with mutant plants, the auxin sugar ester content in the overexpressed lines increased significantly, while the free auxin content decreased significantly. These results further illustrate that *MdUGT74BP1* functions as glycosylated auxin in plants. We used qRT-PCR technology to detect the auxin pathway-related genes in each strain, revealing that the expression of the related genes was consistent with the phenotype. In conclusion, this study was the first to successfully identify glycosyltransferase *MdUGT74BP1* from apples, providing a theoretical and practical basis for the development and utilization of apple germplasm resources.

Introduction

Plant hormones regulate cell growth and development separately or cooperatively in terms of cell division and growth, growth and differentiation of tissues and organs, flowering and fruiting, maturity and senescence, dormancy and germination, and *in vitro* tissue culture (Bechtold et al. 2018). As the flexibility and diversity of hormonal mechanisms depend on their concentration and dynamic balance, to ensure the normal growth and development of plants and improve their environmental adaptation, the endogenous hormone levels of plants should be strictly controlled. Auxin was the first identified phytohormone, defined as an organic substance promoting the elongation and growth of plant cells at low concentration (Weijers et al. 2016). Auxin has an indole ring or benzene ring as its basic structure. Auxin forms an important class of plant endogenous hormones. At the cellular level, auxins can stimulate cell division and branch cell elongation, inhibit root cell growth, promote xylem and phloem cell differentiation, participate in cell wall formation and nucleic acid metabolism, ultimately regulating plant growth and morphogenesis (Salehin et al. 2015). The concentration and distribution of phytoactive auxins indole-3-acetic acid and indole-3-butyric acid are strictly regulated through synthesis, inactivation, and transportation pathways (Powers et al. 2020; Weijers et al. 2018).

There are two types of auxins: the first and most common form is free auxin, which stimulates plant growth and metabolism; it is has the strongest physiological effects (Peng et al. 2019). Among them, auxin IAA is the most widely studied. The biosynthesis of IAA mostly occurs at the site of cell division and growth, concentrated in the original leaf set, young leaves, and developing seeds. Mature leaves and root tips also produce auxin, but in very small amounts (Rinaldi et al. 2012). IAA biosynthesis occurs through
two pathways: one is tryptophan-dependent and the other is tryptophan-independent. According to the different intermediate products of IAA synthesis, the tryptophan-dependent biosynthesis pathway is usually divided into four branches: the indole acetaldehyde oxime pathway, the indole pyruvate pathway, the tryptamine pathway, and the indole acetamide pathway (Salehin et al. 2019). The other is bound auxin, which has very low to no biological activity but acts as hormonal storage in plant organs such as seeds. In most plant tissues, auxin binds to a variety of sugars, sugar alcohols, amino acids, and proteins (Jásik et al. 2016; Liu et al. 2017b).

There are two main pathways for auxin degradation: enzymatic degradation and photooxidation. Enzymatic degradation of auxin can be divided into decarboxylated degradation and non-decarboxylated degradation (Yesbolatova et al. 2019). Among these, decarboxylation degradation is an indole acetic acid oxidase discovered by Tang and Bonner in 1947, which oxidizes IAA and is widely distributed in higher plants (Tang et al. 1947). IAA oxidase is a peroxidase enzyme with an oxidase function. Its oxidation products include CO$_2$ and products such as 3-methylene oxindole (Katz et al. 2015). The degradation products that do not decarboxylate still retain the two carbon atoms from the IAA side chain, such as oxindole-3-acetic acid and dioxindole-3-acetic acid. In vitro indole acetic acid can be oxidized by riboflavin under strong light conditions, resulting in the formation of oxidation products like indole aldehyde and methylene oxindole (Shani et al. 2017). When IAA is applied to plants in field conditions, the above two degradation processes occur simultaneously. Synthetic auxins, such as NAA and 2,4-D, are not degraded by IAA oxidase, hence they have greater stability and can be retained in plants for longer. Therefore, in field conditions, synthetic auxin regulators are used instead of IAA (Niemeyer et al. 2020).

Auxin receptors are specialized hormone receptors substances that bind to specific hormones and trigger physiological and biochemical reactions; (Salehin et al. 2019). There are two main theories concerning the exact location of auxin receptors in cells: one is believed to be on the plasma membrane, the other in the cytoplasm or nucleus (Zhang et al. 2019). The former promotes cell wall relaxation, while the latter promotes the synthesis of nucleic acid nuclear proteins.

When auxin and receptors bind together, one of the important functions of signal transduction is to activate some transcription factors. These transcription factors can promote the expression of specific genes after they enter the nucleus. Among them, the auxin response factor (ARF) plays a functional role in auxin signaling (Neogy et al. 2019; Chandler et al. 2016). These transcription factors are divided into two broad categories: one is an early gene or a primary response gene, which causes the activation of existing transcription gene (Bi et al. 2019); the other is an advanced gene or a secondary response gene, which is responsible for a long-term hormonal response, regulated by some early genes (Powers et al. 2019). It is currently believed that the auxin signaling process is related to the degradation of AUX/IAA protein and the activation of the ARF protein (Sathyan et al. 2019).

The glycosylation of plant small molecules is a common physiological phenomenon and one of the central mechanisms by which plant cells maintain their metabolic balance (Liu et al. 2017a). Glycosyltransferase is an enzyme specifically responsible for catalyzing this glycosylation reaction: it
transfers active sugar groups from nucleoside sugars, usually from uracil nucleoside diphosphate-glucose, to a series of plant small molecule compound receptors including hormones (e.g., auxin and cytokinin), secondary metabolites (e.g., flavonoids), heterogeneous compounds (such as herbicides and insecticides), pathogen infestations and toxic substances inside and outside the plant (Li et al. 2019). Glycosylation changes the biological activity, water solubility, stability, transport characteristics of cells and whole organisms, subcellular localization, remove the toxicity of endogenous and exogenous substances, and reduce the mutual recognition and binding characteristics of receptors in plant small molecule compounds (Li et al. 2018). Glycosyltransferase has multiple functions and biological activities, its substrates are wide, and glycosylation can also produce a cascade effect. Therefore, the glycosylation of glycosyltransferase on small molecules affects many plant growth and developmental aspects (Chen et al. 2020).

Many mechanisms can regulate the dynamic balance of plant endogenous hormones, such as hormone biosynthesis, degradation, transport, or formation of conjugates with other molecules, among which auxin glycosylation is considered to be an important regulator of endogenous auxin horizontal approach (Li et al. 2017). The glycosylation of phytohormones refers to the formation of conjugates between hormones and sugar molecules and is thought to be an important way to regulate the level of endogenous active hormones in plants (Vavra et al. 2017). At present, it is generally believed that this process is a form of inactivation of phytohormones and that plant growth and development are regulated through the synergistic effect of glycosylation products.

Jackson and colleagues first identified auxin glycosyltransferase UGT84B1 in Arabidopsis thaliana, of which the recombinant protein can glycosylate auxin to form the corresponding sugar ester in vitro. They further showed that the catalytic activity for IAA and IBA reached 159 and 112 mkat/kg, respectively, and revealed that UGT84B2, UGT75B1, and UGT75B2 glycosyltransferases only have weak activity against IAA. Jackson et al. continued to research UGT84B1 transgenic plants and extracted overexpressed plant proteins. In response to the IAA reaction, it was found that the amount of 1-O-indole acetyl sugar ester produced by the products of the enzymatic reaction was significantly higher than that of the wild-type. Correspondingly, when IAA was added exogenously, the content of 1-O-indole acetyl sugar ester in overexpressed plants was significantly higher than that of the wild-type. This experiment proves that UGT84B1 has the same enzymatic activity against plant endogenous auxins as shown in vitro. In terms of plant phenotype, the overexpressed Arabidopsis produced a series of auxin-deficient phenotypes compared to the wild-type. This result further suggest that the glycosylation of auxin plays an important role in plant growth and development (Jackson et al. 2001).

Subsequently, Tognetti et al. identified the IBA glycosyltransferase UGT74E2 from Arabidopsis and demonstrated that Arabidopsis UGT74E2 regulates plant morphogenesis and the response to drought and salt stress through the glycosylation of IBA. A biochemical analysis found that UGT74E2 has a strong substrate preference for IBA, therefore, it does not hinder the glycosylation of IAA. By detecting changes in the content of endogenous hormones in plants, UGT74E2 maintains catalytic specificity for IBA in plants. Similarly, both IBA-glс (a glocalization product) and free-state IBA increased and the level of
IAA also decreased. Changes in the concentration of these endogenous auxins and conjugates alter the homeostatic state (or the hormone balance), resulting in a series of overexpressed phenotypes, such as plants shorter than the wild-type, with more branches, and different rosette leaf morphology. Furthermore, such concentration changes significantly improved the tolerance of overexpressed plants to drought and salt stress, indicating that glycosylation may regulate the plants’ hormonal response, as well as their growth and development (Tognetti et al. 2010).

Jin et al. identified a new auxin glycosyltransferase, UGT74D1 in Arabidopsis thaliana. Through in vitro biochemical analyzes, it was found that UGT74D1 could glycosylate IAA, indole-3-propionic acid, naphthyl acetic acid, and other auxins, in addition to glycosylation-modified IBA. When this gene was overexpressed in Arabidopsis thaliana, the phenotype of transgenic plants changed significantly: compared with that of transgenic plants of overexpressed UGT84B1 and UGT74E2, which were similar and different, respectively. There are three glycosyltransferase genes responsible for auxin regulation in plants, hence it is thought that in addition to their preference for catalyzed substrates, these auxin glycosylation-modified genes have different functions. (Jin et al. 2014).

This study was the first to successfully identify glycosyltransferase MdUGT74BP1 from apples, providing a theoretical and practical basis for the development and utilization of apple germplasm resources.

Materials and methods

Carriers, strains and plant materials

The cloning vector pBluescript II SK (+) (abbreviated as pBSK), prokaryotic expression vector pGEX-2T, plant expression vector pBI121, E.coli strain E.coli DH5α, XL1-Blue, Agrobacterium tumefaciens GV3101 are all kept in our laboratory.

The plant material used in this study is mainly Arabidopsis thaliana, of which wild-type Arabidopsis thaliana is the ecological type Col-O of Columbia.

Plant RNA extraction

1. Quickly transfer the ultra-low temperature frozen plant material to a liquid nitrogen pre-cooled mortar, add liquid nitrogen quickly Grind into a uniform powder; (2) Quickly transfer the material to a pre-cooled centrifuge tube, add 1ml of Trizol solution for every 50–100 mg of tissue material, vortex and mix well, place at room temperature for 5min, centrifuge at 12000 rpm, 4°C for 5 min. Carefully draw the supernatant and transfer into a new centrifuge tube; (3) Add 0.2 mL of chloroform to the above homogenate lysate, close the cap of the centrifuge tube, and shake vigorously by hand for 15 s. After mixing well, let stand at room temperature for 5 min, centrifuge at 12000 rpm, 4°C for 15 min; (4) Draw the supernatant and transfer to another new centrifuge tube, add equal volume of isopropanol to the supernatant and invert to mix thoroughly Keep at room temperature for 10 min; (5) Centrifuge
at 12000 rpm for 10min at 4°C, carefully discard the supernatant; (6) Slowly add 1 mL of 75% ethanol along the wall of the centrifuge tube to wash the precipitate, centrifuge at 12000 rpm at 4°C for 5 min, and carefully remove the supernatant. Leave to dry at room temperature, add appropriate amount of DEPC-treated double-distilled water to dissolve RNA; (7) After the RNA is completely dissolved, determine the OD value and concentration of the RNA sample by ultraviolet spectrophotometer, and detect the quality of RNA by agarose gel electrophoresis. After the reverse transcription is completed, the remaining RNA is stored at -80°C.

**Vector construction**

The RNA obtained above was reverse transcribed through a reverse transcription kit to obtain cDNA, and primers (Table S1) were designed from both ends of MdUGT74BP1 to amplify the target gene. After connecting to the intermediate vector pBSK, it is sent for sequencing and compared with the correct sequence published on the Internet. After 100% correct, the intermediate vector plasmid containing the target gene is extracted. After digestion, the target gene is connected to the prokaryotic expression vector pGEX-2T and the plant expression vector pBI121 was verified by bacterial solution PCR and plasmid digestion, and the verification was correct, which means that the vector was successfully constructed.

**Protein purification**

The verified correct E.coli XL1-Blue strain with the recombinant plasmid pGEX-UGT was inserted into 5 mL LB medium with 50 µg/mL Amp resistance, and cultured at 37°C overnight to activate the strain; (2) Take 750 µL of the above-mentioned cultured bacterial solution overnight and transfer it to 75 mL 2×YT medium (containing 50 µg/mL Amp) to expand the culture at 20°C; (3) After OD₆₀₀ = 1.0, add IPTG to the final concentration of 1 mM, Continue culturing at 20°C for 24 h; (4) Centrifuge at 4500 rpm for 10 min to collect the cells; (5) Suspend the cells obtained above in 2 mL Spheroblast buffer, immediately add 14 mL 1/2 concentration Spheroblast buffer; (6) Place on ice, Shake frequently during 30 min; (7) Centrifuge at 4500 rpm for 10 min, collect cells, add 5 mL of 1×PBS (containing 0.2 mM PMSF), process on ice for 30 min, and shake gently several times during the period; (8) Centrifuge at 10,000 rpm for 20 min, put on Transfer the supernatant to a new 10ml centrifuge tube, add 100 µL of 50% Glutathione Sephrose 4B agarose beads, combine at room temperature for 30 min, pay attention to mixing and shake the centrifuge tube during the period; (9) centrifuge at 500 g for 5 min, use a pipette Carefully aspirate the supernatant, and then transfer the Glutathione Sephrose 4B agarose bead homogenate to a 1.5 mL centrifuge tube; (10) Add 500 µL 1×PBS to wash the beads, mix upside down; (11) Centrifuge at 500 g for 5 min, discard the supernatant; (12) Repeat the first two steps and wash the beads a total of 3 times; (13) Add 50 µL Elution buffer, mix gently and resuspend the matrix. Shake on the DNA mixer at room temperature for 10 min to elute the fusion protein from the matrix; (14) Centrifuge at 500 g for 5 min, and gently pipette the supernatant into a new Eppendorf tube to obtain the purified protein solution; (15) Repeat the two steps of the previous step to obtain three times of eluate and combine them, and then measure the concentration of purified protein.
In vitro enzymatic reaction

In order to screen and purify the substrate of the recombinant protein, first select several types of major hormones as the substrate for the reaction, and then expand the screening range of the auxin substrate for MdUGT74BP1 to carry out the reaction. The reaction system is as follows: 0.5 M Tris-HCl (pH = 8.0) 20 μL; 50 mM MgSO4 10 μL; 200 mM KCl 10 μL; 0.1 mol/L UDP-glucose 5 μL; 10% β-mercaptoethanol 2 μL; 100 mmol/L substrate 2 μL; purified UGT protein 2–4 μg; ddH2O up to 200 μL. The reaction was carried out in a constant temperature water bath at 30°C for 3 hours. The reaction was terminated by adding 20 μL of 240 mg/mL trichloroacetic acid (TCA). The liquid nitrogen was quickly frozen and stored at -20°C for HPLC analysis.

Kinetic constant analysis: First, analyze the linear relationship between the time of enzyme protein and substrate reaction, according to the above reaction system. After the reaction system is prepared, react in a 30 degree water bath for 10 min, 20 min, 30 min, 40 min, 60 min, 90 min, 120 min and 180 min respectively. After the reaction is completed, add 20 µL of 240 mg/mL trichloroacetic acid (TCA) reaction termination liquid. Keep in medium for subsequent HPLC analysis. Next, the kinetic constant of the reaction between the enzyme protein and the substrate is measured. Set different concentration gradients within the substrate concentration range of 0–1 mM 0.025 mM, 0.05 mM, 0.075 mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.4 mM, 0.5 mM, 1.0 mM, respectively react with the quantitative enzyme protein, The reaction is carried out at the optimum temperature and PH value for 10 minutes. After the reaction is completed, 20 µL of 240 mg/mL trichloroacetic acid (TCA) reaction termination solution is added. After liquid nitrogen is quickly frozen and stored at -20 degrees, use For subsequent HPLC analysis. Finally, the enzyme activity was analyzed based on HPLC peak.

HPLC analysis conditions of auxin and its analogs

HPLC analysis conditions: the instrument was Shimadzu LC-20AT (Shimadzu, Japan). The instrument is equipped with a diode array detector SPD-M20A, an autosampler SIL-20A, a system controller CBM-20A, a degasser DGU-20A3 and a workstation LC solution (Ver 1.21). The chromatography column was an Ultimate XB-C18 (150 mm×4.6 mm, 5 µm) reverse phase column. The detection wavelength of each substance peak is between 190–430 nm. The auxin substrate mobile phases are methanol (mobile phase A) and water (mobile phase B). Both mobile phases contain 0.01 phosphoric acid (H₃PO₄). The binary high-pressure concentration gradient method was used for elution, with a flow rate of 1 ml/min and an elution time of 35 min. The detection conditions of the following substrates are: indoleacetic acid, detection wavelength is 210 nm, 10%-48% mobile phase A; indolecarboxylic acid, indolepropionic acid, indolebutyric acid and naphthaleneacetic acid, detection wavelength is 280 nm, 10%-70% mobile phase A; 2,4-D and chlorhexidine, the detection wavelength is 287 nm, 10%-100% mobile phase A.

Real-time PCR
Real-time PCR system is as follows: SYBR Premix Ex Taq (2x) 10.0 µL; Primer 1 (10 µM) 0.4 µL; Primer 2 (10 µM) 0.4 µL; dH2O 7.2 µL; template cDNA 2.0 µL. After the Real-time PCR system is prepared according to the above system, it is amplified according to the following Real-time PCR amplification program: Stage1 pre-denaturation; Reps 1 95℃ for 30 s; Stage 2 55℃ for 1 min; Reps 40, 95℃ for 5 s, 60℃ for 30 s; Melt Curve Stage 95℃ for 15 s, 60℃ for 1 min, 95℃ for 15 s.

**Obtaining transgenic lines**

Transformation of Arabidopsis thaliana by floral infestation: (1) Short-day cultivation (12 h light/12 h dark) Arabidopsis thaliana, selected and taken the vigorous plants for the next experiment; (2) Generally, after the tillering is about 10 cm high, remove the two or three freshly opened small flowers at the lower end of the inflorescence. The remaining unopened inflorescence is the best inflorescence. Drop the bacteria onto the top of the inflorescence and allow it to spread evenly throughout the inflorescence; (3) The Agrobacterium to be transformed is inoculated in the liquid medium containing the corresponding antibiotics, and the bacteria are activated by shaking at 200 rpm and 200 rpm. The solution was inoculated into 50 mL of LB containing corresponding antibiotics at a ratio of 1:100 or higher, and cultivated to about OD₆₀₀ = 0.8; (4) After centrifuging 50 mL of bacterial solution, resuspend in 10 mL of 5% sucrose solution, add 2 µL of Silwet L-77 (According to the dosage of 0.2 ml/L Silwet L-77), it is used for transformation of Arabidopsis after mixing; (5) It is better to infect in the evening or evening, and the inflorescence of Arabidopsis after infection Wrap the cling film and place it in the dark overnight. Remove the cling film and put it under light for normal growth the next day. After a week, you can infect the newly grown inflorescence again to improve the conversion efficiency; (6) When the carob is ripe to 70%-80%, stop watering, wait for all the seeds to be harvested after maturity, and select resistant transformed seedlings.

Screening of transgenic plants: (1) Spread the sterilized seeds evenly on the MS solid medium plate (containing 40 mg/L Kan antibiotic), blow dry on an ultra-clean workbench, and seal the dish; (2) Seal the dish Put it in a refrigerator at 4℃, place it in the dark for 3 days for vernalization, and then grow it in the cultivation room; (3) Transgenic plants with successful transformation have green cotyledons and roots grow normally, which is normal green seedlings (called T1 generation transformation) Plants), instead of transformed seedlings are albino seedlings; (4) When the green seedlings of T1 generation grow to 4–6 true leaves, they are transferred to nutrient soil for growth, after maturity, each plant collects seeds and then conducts the above Kan Antibiotic screening, screening out transgenic lines (T2 generation) in accordance with the ratio of green seedlings: white seedlings = 3:1, and transferring the green seedlings to nutrient soil for growth. After harvesting the seeds of each plant, Kan antibiotic screening and screening All green plants (T3 generation) were produced, and the seeds of T3 generation plants were collected to be homozygous transgenic lines for subsequent experiments.

**Root length experiment**
After the seeds of Arabidopsis thaliana strains were surface-disinfected, the seeds were spotted on a 1/2 MS medium plate, darkened and vernalized for 3 days at 4°C, and transferred to an artificial climate room for vertical cultivation for about 2–3 days. The root length of the seedlings is about 0.5-1.0 cm, and the seedlings with relatively uniform growth conditions are transplanted onto 1/2 MS medium containing different concentrations of plant hormones, and the root tips of each seedling are marked with a marker pen to artificially cultivate. After 7 days, take a picture, use NIH-Image software to measure the root length of each seedling.

**Auxin sugar ester extraction**

After disinfecting the surface of the seeds of different strains, they were spotted on MS plates. After 3 days of vernalization at 4°C, take out and cultivate in an artificial climate room for 14 days. Take carefully 1 g of complete plant material for each group of samples. After pre-cultivating in MS liquid medium for 12 h, the control group continues to cultivate in MS liquid medium for 24 h. The seedlings of the treatment group were transferred to MS liquid medium containing 100 µM IBA for 24 h. After the treatment is completed, quickly remove it, absorb excess water on absorbent paper, quick freeze in liquid nitrogen, and then extract the auxin sugar esters according to the following method. (1) Quickly freeze 1 g of the processed material in liquid nitrogen, grind the material into a powder in liquid nitrogen, and transfer it to a 10 mL centrifuge tube; (2) Add 10 mL 80% methanol to extract, then add internal reference (Picloram); (3) After homogenizing slowly, let it stand at room temperature for 1 h, repeatedly inverting and mixing during this period; (4) Centrifuge at 5000 g for 5min; (5) Filter the supernatant after centrifugation through filter paper, and transfer the filtrate to a new centrifuge tube; (6) After vacuum evaporation in a rotary evaporator, dissolve the powder on the inner wall of the flask with 1 mL of pure methanol, dissolve it and transfer it to a 1.5 mL centrifuge tube, centrifuge at 16000 g for 20 min at high speed; (7) filter the supernatant to a new one In the centrifuge tube, the analysis sample was detected using the HPLC conditions of the previous biochemical analysis IBA.

**Data statistics and analysis**

All data in this study were statistically analyzed using Excel 2017 and SPSS 20.0 software, and the data presentation methods were expressed as mean ± standard deviation. The two-sample mean t test was used for the experimental group and the control group. One-way ANOVA was used for comparison between the groups. When the data difference between the two groups was significant, \( P<0.05 \), and when the data difference between the two groups was extremely significant, \( P<0.01 \), with statistical significance.

**Results and analysis**

*Apple glycosyltransferase gene* MdUGT74BP1 *determination*
The apple-based genome was previously sequenced by Velasco et al. (2010); The classification of apple glycosyltransferase genes was identified by Li et al. (2022). First, we used bioinformatics to compare the 44 amino-acid conserved sequences of glycosyltransferase (Table S1) with records in the NCBI protein blast. According to the annotation of the gene chip published on the internet, https://iris.angers.inra.fr/gddh13/, the gene MD15G1357700, which is thought to be a glycosyltransferase gene in apples, can glycosylated auxin compound, was screened (Table S2). The specific gene sequence has been shown (Table S3).

Using the NCBI Blast program (Fig. 1a), the amino-acid sequence of the apple gene MD15G1357700 was analyzed. The results showed that the amino-acid sequence showed the highest degree of similarity to UGT74E2 in Arabidopsis; Reference to the published apple evolutionary tree, thus MD15G1357700 was named MdUGT74BP1. Arabidopsis UGT74E2 is a glycosyltransferase for glycosylated auxin and its analogs; Arabidopsis UGT74E2 is a glycosyltransferase of glycosylated auxin and its analogues; therefore, we suspected that the function is similar to MdUGT74BP1. Using bioinformatics technology, we constructed the three-dimensional structure of the MdUGT74BP1 protein according to the gene and amino-acid sequence of MdUGT74BP1 (Fig. 1b).

**MdUGT74BP1 expression pattern analysis**

To investigate the spatiotemporal expression of the gene MdUGT74BP1, we obtained samples of a ten-year-old “New Star” apple tree in the fruit tree test garden of Shandong Agricultural University, including the new root system, annual stems, young and old leaves, flowers in full bloom, and peels 30 days after flowering; as well as young fruits, seeds, peels, mature fruits, and seeds 120 days after flowering. Then followed RNA extraction, reverse transcription into cDNA, and the insertion of design primers at both ends of the MdUGT74BP1 gene. Primer sequences has been shown (Table S4). The Real time PCR results show that the MdUGT74BP1 gene is mainly expressed in young peels and seeds (Fig. 2a).

Wild type apple seedlings was induced by auxin and growth analogs as well as 100 µmol/L of ICA, IAA, IPA, IBA, NAA, and 2,4-D, used to treat a 6-leaf one-core apple tree seedlings for 1, 3, 6, 12, and 24 h. After the treatment, RNA samples were extracted separately for each treatment, and after reverse transcription into cDNA, the expression of MdUGT74BP1 was detected. The experimental results show that MdUGT74BP1 is up-regulated by ICA, IAA, IPA, IBA, NAA, 2,4-D and that the up-regulation is most significant after 12 h (Fig. 2b). This result indicates that the MdUGT74BP1 gene is up-regulated by auxin and its analogs, which may be involved in auxin glycosylation.

**Identification of apple glycosyltransferase MdUGT74BP1 in vitro biochemical substrate and in vitro enzyme activity analysis**

After constructing the prokaryotic expression vector for the MdUGT74BP1 gene, the active purified prokaryotic expression protein was extracted *in vitro*. The *in vitro* enzymatic reaction is performed on the small molecule compounds of auxin and its analogs to find the corresponding substrate. The experimental results found that MdUGT74BP1 could glycosylate auxin and its analogs (Fig. 3). To
examine this process in more detail, we further analyzed its specific enzyme activity. The experimental results show that the specific enzyme activity of MdUGT74BP1 to ICA, IAA, IPA, IBA, NAA, and 2,4-D were 0.22, 1.45, 2.03, 2.53, 1.57, and 0.49, respectively (Table 1).

**Apple MdUGT74BP1 overexpression in Arabidopsis and phenotypic analysis**

To determine the function of MdUGT74BP1 in plants, we overexpressed the apple *MdUGT74BP1* gene into *Arabidopsis thaliana*. After three generations of screening, we obtained a homozygous-overexpressed line (Fig. 4a) which was similar to the wild-type control group. Comparatively, *OE3* was overexpressed by about 18 times and *OE9* was overexpressed by about 20 times. However, under normal conditions, the overexpressed lines showed auxin-deficient phenotype, short and multi-branched (Fig. 4b), indicating that MdUGT74BP1 may also be involved in the auxin metabolism pathway in plants.

Generally, auxin promotes root growth at low concentrations and inhibits it at high concentrations. In this study, we treated each *Arabidopsis* strain with exogenous auxin IBA and IAA, including wild-type and *MdUGT74BP1* overexpressed lines (Fig. 4c, 4d). Without treatment, there was no significant difference in root length; while for the treatment with IBA and IAA, the root length of the overexpressed lines was significantly longer than that of the wild-type. This may be because *MdUGT74BP1* overexpressed lines have a large amount of glycosyltransferase. Higher expression results in higher glycosylation, which may cause inactivation of some exogenous IBA and IAA, making the root elongation of the overexpressed body stronger than that of the wild-type, as a high concentration of auxin has an inhibitory effect.

**Apple MdUGT74BP1 alter the auxin metabolism pathway**

After the exogenous addition of auxin IBA to each strain, the auxin sugar ester was extracted and analyzed using HPLC (Fig. 5). Compared with the wild-type, the auxin sugar ester content in the overexpressed strains was significantly higher, while the concentration of free-state auxin was significantly lower. This result further shows that MdUGT74BP1 can glycosylate auxin, resulting in a large accumulation of auxin sugar esters and a lower concentration of free-state auxin, leading to a lack of auxin phenotype.

**Related gene expression**

Previous experiments have shown that UGT74E2 can glycosylate auxin in *Arabidopsis* plants. In particular, phenotypic experiments have shown that UGT74E2 expression changes affect the dynamic balance of auxin (Tognetti et al. 2010). To better understand the molecular mechanisms involved in the auxin anabolic process of MdUGT74BP1, RT-PCR was used to analyze the expression levels of some functional genes related to auxin anabolic metabolism in *Arabidopsis* wild-type and *MdUGT74BP1* overexpressed lines.
The YUC gene family in the auxin synthesis pathway catalyzes the direct conversion of indole pyruvate to IAA. Compared to the wild-type, our analysis found that the expression level of YUCCA1, YCAA2, YUCCA8, and YUCCA11 was effectively significantly up-regulated in overexpressed lines (Fig. 6a). Because of this, we hypothesized the overexpression of MdUGT74BP1 leads to a lower concentration of free-state auxin and promotes the up-regulation of auxin biosynthetic genes.

We also analyzed other auxin-responsive genes such as IAA1, IAA14, ARF12; and GH3-2 (Fig. 6b). Compared with the wild-type, our analysis found that the expression level of IAA1, IAA14, ARF12, and GH3-2 genes was down-regulated in overexpressed MdUGT74BP1 lines. This led us to confirmed our hypothesis that free-state auxin concentration decreases with MdUGT74BP1 overexpression and suggested that this promotes the down-regulated expression of auxin-responsive genes.

Discussion

Apple auxin glycosyltransferase MdUGT74BP1 enzyme activity

Glycosylation is an important ubiquitous physiological mechanism which maintains the homeostatic balance in plant cells (Liu et al. 2017a). Glycosyltransferase can transfer active sugar groups from nucleotide sugars, usually from uracil nucleoside diphosphate-glucose (UDP-glucose) to small molecule receptors, which can alter the biological activity of small molecules, such as solubility, stability, cell localization, and binding performance (Li et al. 2019).

So far, the auxin glycosyltransferase enzymes identified in the model plant Arabidopsis include UGT84B1 (Jackson et al. 2001), UGT74E2 (Tognetti et al. 2010), UGT74D1 (Jin et al. 2014), UGT84A2 (Zhang et al. 2017), and UGT75D1 (Zhang et al. 2016). There are few reports of similar enzymes in other species. In 2019, Liu and their colleagues identified the first glycosyltransferase gene in rice, OsIAGT1. They found that this enzyme was responsible for the glycosylation of auxin and its analogs. However, the authors note that there is no current research on the corresponding auxin glycosyltransferase gene in rice (Liu et al. 2019). While UGT84B1 efficiently glycosylates IAA in Arabidopsis, it also has a high catalytic influence on other auxins and their analogs (Jackson et al. 2001); while the most suitable substrate for UGT74E2 is IBA, which is suitable for other auxins. The catalytic activity of UGT84B1 is relatively low (Tognetti et al. 2010). The apple auxin glycosyltransferase gene MdUGT74BP1 identified here has the highest similarity to Arabidopsis glycosyltransferase UGT74E2, but its catalytic activity is higher than UGT74E2 with a similarly large catalytic range. These results indicate that during the plant evolutions, redundant mechanisms were formed and multiple glycosyltransferases could act against the same type of plant hormones. The question remains: why are there multiple auxin glycosyltransferases in plants? We believe that there may be a synergy between different auxin glycosyltransferase members. Indeed, their show preferences for different substrates, which enables the plant to fine-tune the dynamic balance of auxin. On the other hand, the spatial and temporal specific expression of these genes differs: although they have similar hormone modifications, because of the specific time and location of their role, their function
differs. Improves the plant's fine regulation of auxin, making the plant more sensitive to different environments during growth and development.

**Enzymatic properties of apple auxin glycosyltransferase MdUGT74BP1**

Our findings indicate that MdUGT74BP1 has different conversion efficiencies for different auxins and their analogs: IBA > IPA > NAA > IAA > 2,4-D > ICA. This result is consistent with current knowledge on auxin glycosyl. The conversion rate of transferase to different substrates is different. Further analysis of the structural formula for each substrate shows that the preference of MdUGT74BP1 during substrate selection may be related to the regioselectivity of the substrate structure, that is, the length of different auxin side chains determines whether auxin can be used by MdUGT74BP1. As a key factor for effective glycosylation, therefore, MdUGT74BP1 has the highest catalytic efficiency for substrate IBA and the lowest for ICA. It is unclear whether the catalytic efficiency of MdUGT74BP1 for different substrates in plants is consistent with its catalytic efficiency *in vitro*. Although the most suitable substrate for MdUGT74BP1 in *in vitro* biochemical reactions was found to be IBA, MdUGT74BP1 in plants may glycosylate naturally-occurring free auxins (such as IAA and IBA) at different sites due to their tissue specificity or related substrates.

**MdUGT74BP1 regulates auxin metabolic balance**

To study the function of auxin glycosyltransferase MdUGT74BP1 in plants, we overexpressed *MdUGT74BP1* into *Arabidopsis thaliana*. After three generations of screening, we obtained *MdUGT74BP1-*overexpressed homozygous lines, OE3 and OE9. Under normal culture conditions, the overexpressed lines exhibited a lack of auxin phenotype and were short and multi-branched. This result shows that MdUGT74BP1 in the plant can glycosylate auxin, causing a large accumulation of auxin sugar esters which significantly reduces the concentration of free-state auxin, resulting in the lack of auxin phenotype. To further determine the activity of MdUGT74BP1 glycosylated auxin and its structural analogs in plants, we first treated each *Arabidopsis* strain by exogenously adding auxin IBA and IAA to both the wild-type and *MdUGT74BP1-*overexpressed lines. Our results show that there was no difference in root length in the control plants, while treatment with IBA and IAA led to significant inhibition of the main root growth in each line, while the *MdUGT74BP1-*overexpressed strains showed high glycosyltransferase expression, which makes glycosylation more likely, resulting in the inactivation of some exogenous IBA and IAA and the up-regulation of main root elongation. This result indicates that apple MdUGT74BP1 is also involved in the auxin metabolism pathway.

The YUC gene family in the auxin synthesis pathway can catalyze the direct conversion of indole pyruvate to IAA. Our findings show that YUCC1, YCAA2, YUCCA8, and YUCCA11 were significantly up-regulated in overexpression lines, compared to the wild-type. We also analyzed the expression of auxin-responsive genes *IAA1, IAA14, ARF12* and *GH3-2*, revealing that these genes were significantly down-
regulated in overexpressed lines. Because of this, we speculate that *MdUGT74BP1* overexpression causes a decrease in free-state auxin concentration, promotes the up-regulation of auxin synthesis genes, and down-regulates auxin-responsive genes.

In summary, this study enables us to make important conclusions. The apple auxin glycosyltransferase gene (*MdUGT74BP1*) catalyzes naturally-occurring auxin and its structural analogs, thereby generating their corresponding sugars esters. This discovery lays the foundation for future research on how *MdUGT74BP1* regulates the dynamic balance of auxin through phytogenetics and provides glycosyltransferases and their specificity for the industrial production of specific sugar esters of auxins through an enzymatic synthesis biocatalytic method.

**Declarations**

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**Author contributions** PL and LJ conceived and designed the experiment. PL, WG, XT, AZ and RL conducted the experiments, performed data analysis and wrote the manuscript. PL, WG, XT, AZ, RL and LJ participated in material development, sample preparation and data analysis. PL and RL drafted proposals and corrected the manuscript. All authors read and approved the final manuscript.

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**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethics approval and consent to participate** All experimental studies on plants have complied with relevant institutional, national, and international guidelines and legislation.

**References**


Table 1

Table 1 is available in Supplementary Files section.

Figures
### Figure 1

Analysis of sequence information of MdUGT74BP1. **a** Alignment of amino acid sequence of MdUGT74BP1 with other Plant species. The C-terminal conserved glycosyltransferase PSPG domain was shown in red box. **b** Prediction of secondary structure of MdUGT74BP1 protein

<table>
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<tr>
<th>Sequence</th>
<th>Alignment</th>
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<td><strong>MdUGT74BP1</strong></td>
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Figure 2

MdUGT74BP1 expression pattern. a MdUGT74BP1 spatiotemporal expression pattern. b Expression of MdUGT74BP1 induced by auxin and auxin analogues. Note: *P<0.05, **P<0.01, n=3
Figure 3

HPLC analysis of reaction products from IAA and IBA catalyzed by MdUGT74BP1 and UGT74E2
Figure 4

Expression intensity of MdUGT74BP1 OE lines and their Phenotype. **a** Expression intensity of MdUGT74BP1 OE lines. **b** The phenotype of MdUGT74BP1 OE lines. **c** Root length of MdUGT74BP1 in medium supplemented with IAA and IBA. **d** Statistics of root length elongation of MdUGT74BP1 in medium supplemented with IAA and IBA. Note: *P<0.05, **P<0.01, n=3
Figure 5

HPLC detection of total flavonoids of various strains after IBA spraying
Figure 6

Related gene expression. **a** YUCCA related gene expression. **b** others related gene expression. Note: *P<0.05, **P<0.01, n=3

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MdUGT74BP1supplementtable1130.doc
- MdUGT74BP1tables1128.doc