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Regulatory mechanism of GA_3 on tuber growth by DELLA-dependent pathway in yam (*Dioscorea opposita*)

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Abstract

Key message **Endogenous and exogenous GA_3 responses to *DoEXP* and *DoXTH* depend on the *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox3*, *DoGA2ox4*, *DoGID1a,* and *DoDELLA1* to regulate yam tuber growth.**

Abstract Yam tuber undergoes significant alteration in morphogenesis and functions during growth, and gibberellins (GA) are considered potentially important regulators of tuber growth. However, it is little known about the regulation of GA metabolism and GA signaling components genes in tuber growth of yam. In this study, the cloning and expressions of GA_3 level, GA metabolism and signaling genes, and cell wall genes in tuber growth in response to GA_3 and GA biosynthesis inhibitor paclobutrazol (PP_{333}) treatments were studied. The contents of GA_3 accumulated at the tuber growth, with the highest levels in the early expansion stage. *DoGA20ox1*, *DoGA3ox1*, and four *DoGA2ox* genes were significantly abundant in the early expansion stage of tuber and gradually declined along with tuber growth. Three *DoGID1* and three *DoDELLA* genes were showed different expression patterns in the early expansion stage of tuber and gradually declined along with tuber growth. Five *DoEXP* and three *DoXTH* genes expression levels were higher in the early expansion stage than in other stages. Exogenous GA_3 increased endogenous GA_3 levels, whereas the expression levels of *DoGA20ox1*, *DoGA3ox1*, *DoGID1a*, and *DoDELLA1* were down-regulated in the early expansion stage of tuber by GA_3 treatment, *DoGA2ox3* and *DoGA2ox4* were up-regulated. PP_{333} application exhibited opposite consequences. Thus, a mechanism of GA_3 regulating yam tuber growth by DELLA-dependent pathway is established.

Keywords Gibberellin · Tuber growth · GA biosynthesis and signaling · Gene expression · Yam

Introduction

Yams are monocotyledonous plants belonging to the family *Dioscoreaceae*, and the tuber is its harvested organ. Yam tuber is an important storage source of starch, sugars, proteins, amino acids, vitamins, and small amounts of essential minerals. It is widely cultivated in tropical and subtropical regions. This biological process of yam tuberization could be divided into three phase: initiation, expansion, and maturation stage, and the expansion stage could be further divided into three periods: early expansion, middle expansion, and late expansion stage (Gong et al. 2016; Matsumoto et al. 2005). Tuber expansion was initiated, which was accompanied rapidly by a massive deposition of starch and storage proteins (Aksenova et al. 2012). It is very important to study the mechanism of tuberization for improving the yield and quality of yam.

Various endogenous and environmental factors influenced yam tuberization (Yoshida et al. 2007). It has been shown

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that high sucrose level, low nitrogen levels, short days, and high temperature promoted yam tuber tuberization (Feng et al. 2007; Chen et al. 2010; Agele et al. 2010; Yoshida et al. 2007). Plant hormones play an important role in tuber tuberization of yam; for example, gibberellins (GA), indole acetic acid (IAA), and abscisic acid (ABA) play a key role at the beginning of the tuber expansion stage, and trans-zeatin (tZ) and jasmonic acid (JA) are also involved in tuber growth (Chen et al. 2007; Gong et al. 2016). GA is involved in growth and development processes including seed germination, stem elongation, flower induction and development, and fruit set. Exogenous GA increased tuber weight, caused a significant tuber yield in yam (Gong et al. 2015, 2016; Yoshida et al. 2008), while inhibited tuber expansion in potato (Cheng et al. 2018; Hartmann et al. 2011). It shows that there is a different role of GA in regulating tuber growth between potato and yam, but the mechanism of GA-regulating tuber growth in yam is unknown.

The biosynthesis and signal transduction pathways of GA have been determined in arabidopsis and rice (Murase et al. 2008; Ueguchi-Tanaka et al. 2007; Sun 2011; Hedden and Phillips 2000). The biosynthesis of bioactive GAs has involved the action of two enzymes: GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). The final level of bioactive GAs is also regulated by inactivation, which is catalyzed by the GA 2-oxidase (GA2ox) enzyme (Salazar-Cerezo et al. 2018; Hedden and Sponsel 2015; Hedden and Thomas 2012). It showed that these genes took part in the control of tuber growth by affecting endogenous GA levels. The overexpression in potato showed that *StGA20ox1* participated in the biosynthesis of bioactive GAs, resulting in the high growth plants, which required a short photoperiod for tuber growth (Carrera et al. 2000). *StGA3ox2* delayed tuber induction and decreased average tuber weight (Bou-Torrent et al. 2011; Roumeliotis et al. 2013), while *StGA2ox1* degraded GA and promoted tuber tuberization (Kloosterman et al. 2007). The GIBBERELLIN-INSENSITIVE DWARF1 (GID1) and DELLA protein are the key components of the signal transduction pathways of GA (Murase et al. 2008; Ueguchi-Tanaka et al. 2007). DELLA protein is the key inhibitor of GA signaling pathway (Fleet and Sun 2005). GA signaling pathway is mainly realized by removing the inhibition of DELLA protein. After binding with active GA and sensing gibberellin signal, GA receptor GID1 transmits the signal to DELLA protein and induces a series of downstream reactions (Sun 2011; Li et al. 2018b; Van de Velde et al. 2017). It is necessary to study the effects of bioactive GA synthesis and signaling-related genes on tuberization in yam because of the differences of GA on tuber growth in potato and yam. Gibberellin 2-β-dioxygenase gene (Gibberellin regulatory protein gene) regulated GA_3 production in yam, and in turn, affected tuber growth (Ao, 2020). GA20ox2, GID1C2, and DELLA2 were responsive to GA_3 in yam tuber growth (Xing et al. 2020), and the expression of *DoGID1A* increased during yam bulbil sprouting (Long et al. 2019). This implies that GA metabolism and signaling components genes may regulate yam tuber growth. Until now, the mechanism of GA_3 regulation of these genes expression in yam is unknown.

Expansins (EXP) and xyloglucan endotransglycosylase/hydrolase (XTH) play important roles in crop growth and development requiring cell wall extension, modification, and cell enlargement (Xu et al. 2014; Yang et al. 2020; Kushwah et al. 2020; Ratke et al. 2018). Several reports have shown that EXP and XTH genes participated in tuber growth. Overexpression of *IbEXP1* suppressed the proliferation of cambium and metaxylem in the formation of sweetpotato storage roots (Noh et al. 2013). *StEXP* was expressed highly in potato tuber (Li et al. 2017). Furthermore, EXP and XTH genes acted as positive regulators in Chinese yam tuber growth (Zhou et al. 2020).

In our previous study, endogenous GA_3 played important roles in the early expansion stage (Gong et al. 2016), and exogenous GA_3 increased tuber weight and yield in *Dioscorea opposita* var. Guihuai 16 (Gong et al. 2015). Despite GA_3 may be closely related to yam tuber growth, little is known about GA metabolism and signaling components genes that mediated the regulation of tuber growth. In this study, we analyzed all potential GA-related, EXP, and XTH genes in the GH16 tuber transcriptomics (The data in the NCBI SRA database: PRJNA533985) and found that *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox1*, *DoGA2ox2*, *DoGA2ox3*, *DoGA2ox4*, *DoGID1a*, *DoGID1b*, *DoGID1c*, *DoDELLA1*, *DoDELLA2*, *DoDELLA3*, *DoEXP1*, *DoEXP2*, *DoEXP3*, *DoEXP4*, *DoEXP5*, *DoXTH1*, *DoXTH2*, and *DoXTH3* had the complete open reading frame (ORF), and shown significant differential expression during tuber expansion stage. Our work aimed to investigate the GA_3 level, potential GA-related genes, and EXP and XTH genes expression of tuber growth in response to GA_3 and PP_{333} treatments. The results will provide useful information for the mechanism of GA-regulating tuber growth.

Materials and methods

Field experiment and sampling

GH16 (Guihuai 16) was planted at the Farm of Guangxi University in 2018–2019. Its healthy tubers' germination and planting patterns were consistent as the previous description (Gong et al. 2016). According to the life cycle of GH16 tuber, GA_3 and PP_{333} were treated as previously describing, 200 mg/L exogenous GA_3 and PP_{333} was sprayed on the leaves of GH16 on June 20, which was the beginning of tuber expansion of GH16 (Gong et al. 2016), and water was

used as the control (CK). Each experiment consisted of three plots: each plot included 60 tuber plants and arranged in a randomized complete block design. Tubers were collected at early expansion stage (0, 1, 3, 5, 7, 15, 30 days), middle expansion stage (60 days), late expansion stage (90 days), and maturation stage (120 days) after spraying. For each experiment, each plot represented a biological replicate. Five plants were selected randomly from every repetition each time. The distal ends (5 mm long) of five fresh tubers from a plot were washed with distilled water, cut down into pieces, and mixed as a biological repetition. All samples were immediately frozen in liquid nitrogen and stored at − 80 °C. The experiments were conducted from 2018 to 2019.

Quantitation of endogenous GA_3

Extraction and quantitation of endogenous hormones were carried as previously describing (Gong et al. 2016). The extracted and purified samples were subjected to UHPLC–QqQ-MS/MS before it was separated on the Agilent C18 column. The mobile phase A, consisting of 0.1% formic acid in the water, the mobile phase B, consisting of 100% methanol, were used for chromatographic separation, and the gradient changes were 65% A and 35% B for initial conditions which were maintained for 2 min, changing linearly to 0% A, 100% B for 2 min, and finally maintained at 65% A, 35% B for 3 min. The conditions of mass spectrometry were used in ESI mode.

Gene cloning and bioinformatics analysis

Genes of potential GA related, EXP, and XTH in GH16 tuber were searched from differentially expression by DEGseq and were functionally characterized by NR database in previous transcriptome database (The data in the NCBI SRA database: PRJNA533985) (Zhou et al. 2020). All gene sequences were identified by BLASTx and predicted complete ORF were submitted to NCBI (Table S1). The sequences of the primers are shown in Table S2. Total RNA was extracted from different tuber growth stages, using the MiniBEST Plant RNA Extraction Kit (TaKaRa), and cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa). Multiple alignments of the deduced amino acid sequence were performed with the sequences from different species using Clustal W and GeneDoc software, and a phylogenetic tree was constructed using the neighbor-joining method and 1000 bootstraps in MEGA 6.0, accession numbers of protein for organisms are shown in Table S3. The basic physical, chemical properties, and conserved domains of the proteins were predicted by the Expert Protein Analysis System(http://expasy.org/proteomics) and SMART software(http://smart.embl-heidelberg).

Quantitative real-time PCR analyses

Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent Kit (TaKaRa), and specific primers were designed to generate a 100–200 bp PCR product by Primer Premier 5.0 (Table S4). Real-time RT-qPCR was performed on a CFX96 Real-Time PCR Detection System (BIO-RAD), using iTaq™ universal SYBR® Green Supermix (BIO-RAD), according to the manufacturer's protocol. All reactions were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to estimate relative expression level. *DoActin* was used as internal controls which were designed to generate a 150 bp PCR product from corresponding cDNAs by Primer Premier 5.0. All gene expressions had three biological replicates. Heat map was constructed using HemI program.

Accession numbers

Sequence data from this article can be found in the NCBI databases using following accession numbers: *DoGA20ox1*(MW377784), *DoGA3ox1*(MW377785), *DoGA2ox1*(MW377786), *DoGA2ox2*(MW377787), *DoGA2ox3*(MW377788), *DoGA2ox4*(MW377789), *DoGID1a*(MW377790), *DoGID1b*(MW377791), *DoGID1c*(MW377792), *DoDELLA1*(MW377793), *DoDELLA2*(MW377794), *DoDELLA3*(MW377795), *DoEXP1*(MW377796), *DoEXP2*(MW377797), *DoEXP3*(MW377798), *DoEXP4*(MW377799), *DoEXP5*(MW377800), *DoXTH1*(MW377801), *DoXTH2*(MW377802), *DoXTH3*(MW377803).

Yeast two-hybrid assays

Y2H assays were performed with the Matchmaker Gold Yeast two-hybrid system (Clontech). *DoGID1a, DoGID1b*, and *DoGID1c* full-length ORFs were inserted into the *Nde*I-*EcoRI* site of the pGBKT7 bait vector (GAL4 binding-domain), respectively. *DoDELLA1, DoDELLA2, and DoDELLA3* were fused into the *Nde*I-*XhoI* site of the pGADT7 prey vector (GAL4 activation-domain), respectively. The sequences of the primers are shown in Table S5. Bait and prey vectors (200 ng) were transformed into Y2H Gold yeast strains, using Yeastmaker yeast transformation system 2. A positive colony was picked from QDO/X/A. One microliter of the positive yeast was spread in a 96-well plate containing medium with or without 100 μMGA_3. The growth of yeast colonies was observed 3 days after incubation at 30 °C. All assays were repeated three times.

Statistical analyses

The data based on three independent biological replicates were analyzed using SPSS19.0 software. Data were presented in tables and bar graphs as the mean ± standard error (SE). Statistical significance for gene expression among treatments was determined by Student's *t* test, one-way Analysis of Variance (ANOVA), and significant value set at $\alpha = 0.05$.

Results

Endogenous GA_3 quantities response to GA_3 and PP_{333} application

In this study, whether gibberellin is involved in tuber growth, GA_3 levels were detected in the early expansion stage (0, 1, 3, 5, 7, 15, and 30 days), middle expansion stage (60 days), late expansion stage (90 days), and maturation stage (120 days) after spraying with GA_3 or PP_{333}.

In the control, the content of endogenous GA_3 in tuber increased first, and then decreased, it had the highest values on 3 days and was higher in the early expansion stage than in other stages. There were less changes among middle, late, and mature stages (Fig. 1). Compared to the control, exogenous GA_3 could significantly increase the contents of endogenous GA_3 from 1 to 30 days and showed one obvious peak level of 224.08 ng/g on 3 days, then it decreased significantly from 60 to 120 days. There was an opposite trend after exogenous PP_{333} application, it remarkably decreased the content of endogenous GA_3 from 1 to 15 days, then had no change on other days. The endogenous GA_3 levels were higher in tuber expansion stage, especially the early expansion stage, suggesting that the GA_3 application had a significantly positive effect on increasing endogenous GA_3 content.

Isolation and characterization of GA_3 metabolism and signal component genes

To explain the accumulation profile of GA_3 during tuber growth, one GA20ox gene (*DoGA20ox1*), one GA3ox gene (*DoGA3ox1*), four GA2ox genes (*DoGA2ox1, DoGA2ox2, DoGA2ox3*, *DoGA2ox4*), three GA receptor genes (*DoGID1a, DoGID1b,* and *DoGID1c*), and three DELLA genes (*DoDELLA1, DoDELLA2, DoDELLA3*) were obtained with complete open reading frame (ORF), which found in the GH16 tuber transcriptomics, and these genes have shown significant differential expression during tuber expansion stage (Table S1).

DoGA20ox1 contains an ORF of 1167 bp encoding a protein of 388 amino acids. Conserved domains analysis indicated that DoGA20ox1 belonged to 2-oxoglutarate-dependent deoxygenates family, including DIOX-N domain and 2OG-FeII-Oxy domain (Fig. S1A). Phylogenetic tree analysis indicated that DoGA20ox1 clustered into monocot group, and showed a close relationship with *Oryza sativa*, *Zea mays, and Triticum aestivum* (Fig. 2A).

DoGA3ox1 contains an ORF of 1053 bp encoding a protein of 350 amino acids. Conserved domains analysis indicated that DoGA3ox1 belonged to 2-oxoglutarate-dependent deoxygenates family, including DIOX-N domain and 2OG-FeII-Oxy domain (Fig. S1B). Phylogenetic tree analysis indicated that DoGA3ox1 clustered into the dicotyledon

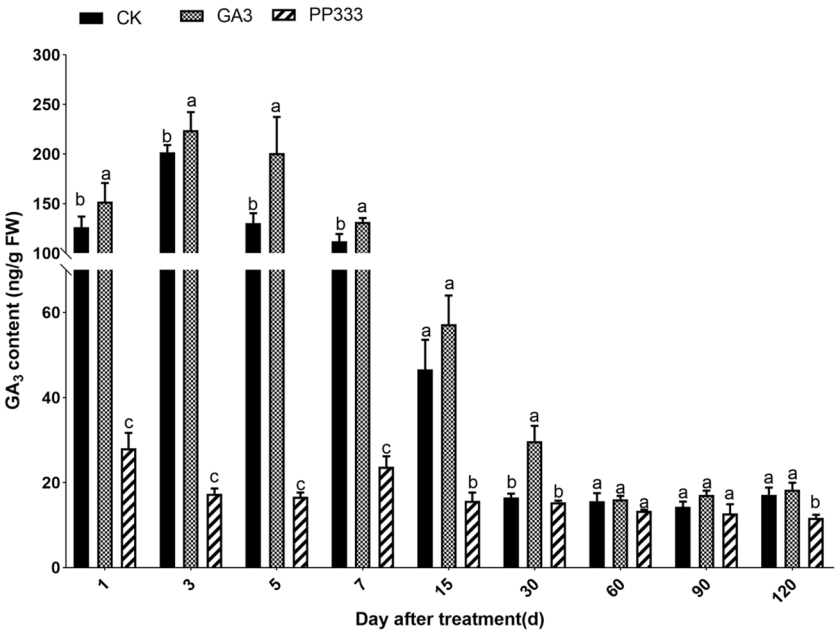

Fig. 1 Comparison of endogenous GA_3 contents after GA_3 and PP_{333} treatment. The data represent the mean ± standard error (SE) of three biological with three replicates each. Letters indicate significant at 5% levels, among different treatment. The analyzed tuber stage: early expansion stage (0–30 days), middle expansion stage (60 days), late expansion stage (90 days), and maturation stage (120 days). The same as below

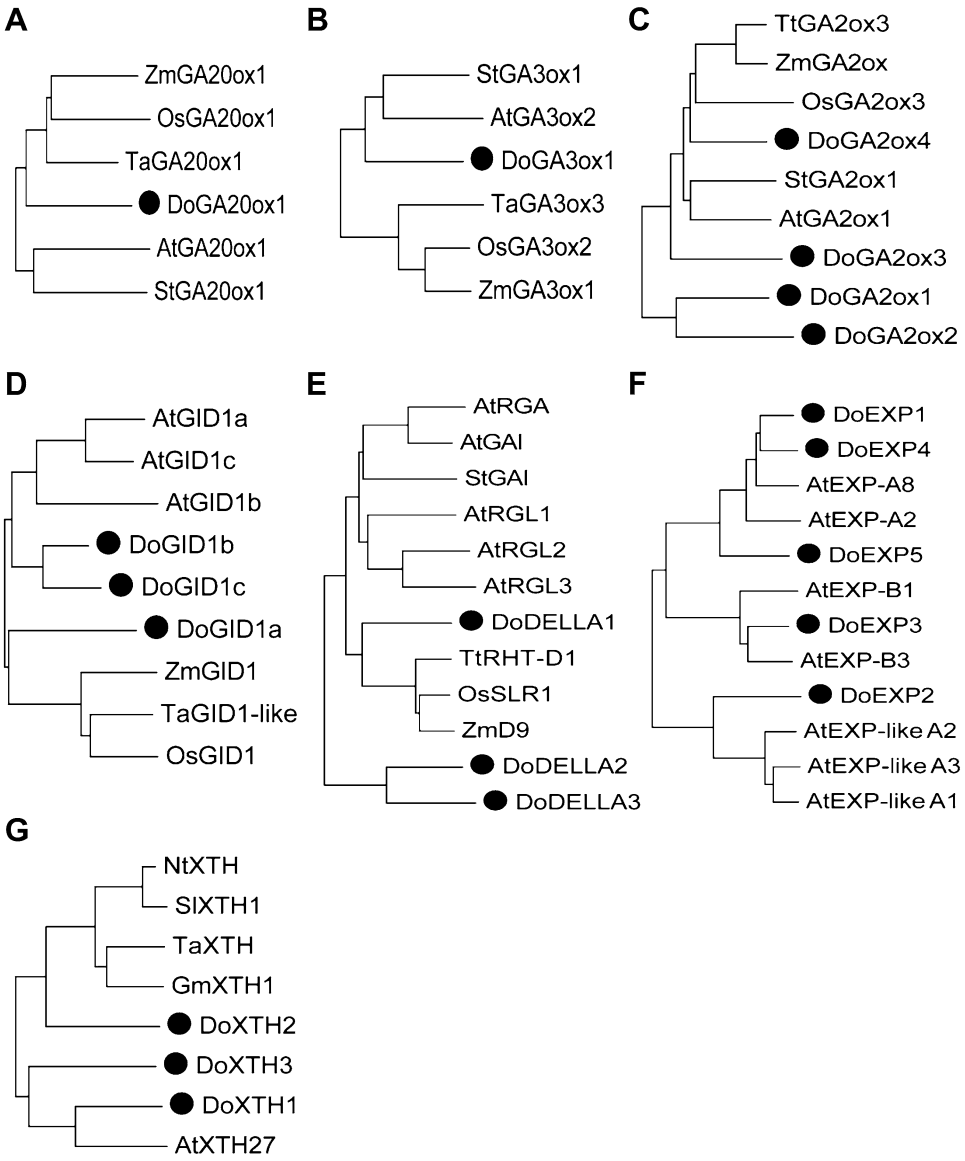

Fig. 2 Phylogenetic trees of the amino acid sequence alignment of GA metabolism and signal components genes of yam tuber. **A** *DoGA20ox1*. **B** *DoGA3ox1*. **C** *DoGA2ox* genes (*DoGA2ox1*, *DoGA2ox2*, *DoGA2ox3,* and *DoGA2ox4*). **D** GA receptor genes (*DoGID1a*, *DoGID1b*, and *DoGID1c*). **E** *DoDELLA* genes (*DoDELLA1*, *DoDELLA2*, *DoDELLA3*). **F** *DoEXP* genes (*DoEXP1*, *DoEXP2*, *DoEXP3*, *DoEXP4*, and *DoEXP5*). **G** *DoXTH* genes (*DoXTH1*, *DoXTH2*, and *DoXTH3*). Ortholog species information showed in Table S3

group and showed a close relationship with *Arabidopsis* and *Solanum tuberosum* (Fig. 2B).

DoGA2ox1, *DoGA2ox2*, *DoGA2ox3*, and *DoGA2ox4* were 525, 756, 852, and 1050 bp in ORF-encoding proteins of 174, 251, 283, and 349 amino acids, respectively. Conserved domains analysis indicated that DoGA2oxs belonged to 2-oxoglutarate-dependent dioxygenases family, including 2OG-FeII-Oxy domain (Fig. S1C). Phylogenetic tree analysis indicated that DoGA2ox1 and DoGA2ox2 clustered into same group, and DoGA2ox4 had highly homologous to the GA2ox in *Zea mays, Triticum aestivum* (Fig. 2C).

DoGID1a, *DoGID1b,* and *DoGID1c* were 1149, 381, and 1065 bp in ORF-encoding proteins of 382, 126, and 354 amino acids, respectively. Conserved domains analysis indicated that DoGID1s had the conserved HSL motifs HGG and GXSXG, with the amino acids related to HSL activity are S, D, and V (Fig. S1D). Phylogenetic tree analysis indicated that DoGID1a had highly homologous to the GID1 in *Zea mays*, *Triticum aestivum*, and *Oryza sativa* (Fig. 2D). Interestingly, DoGID1b and DoGID1c clustered into dicotyledonous group.

DoDELLA1, *DoDELLA2*, *DoDELLA3* were 1908, 1749, and 1407 bp in ORF-encoding proteins of 635, 582, and 468 amino acids, respectively. DoDELLAs contain highly conserved domains including DELLA domain and GRAS domain (Fig. S1E). Phylogenetic tree analysis indicated that the DoDELLA1 proteins had close relatives with *Zea mays*, *Oryza sativa*, and *Triticum aestivum* (Fig. 2E). Interestingly, DoDELLA2 and DoDELLA3 protein clustered into same group.

Isolation and characterization of cell wall genes

DoEXP1, *DoEXP2*, *DoEXP3*, *DoEXP4*, and *DoEXP5* were 770, 810, 555, 762, and 777 bp in ORF-encoding proteins of 256, 269, 184, 253, and 259 amino acid, respectively. Conserved domains analysis indicated that DoEXPs had the cellulose-binding-like domain (CBD), GGACG motif, and FRRV motif (Fig. S1F). Phylogenetic tree analysis indicated that DoEXPs clustered into the three groups. DoEXP1, DoEXP4, and DoEXP5 clustered into α-expansin protein, DoEXP3 clustered into β-expansin protein, and DoEXP2 clustered into α-expansin-like protein (Fig. 2F).

DoXTH1, *DoXTH2*, and *DoXTH3* were 987, 882, and 879 bp in ORF-encoding proteins of 328, 293, and 292 amino acids, respectively. Conserved domain analysis indicated that DoXTHs had the conserved catalytic domain (DEIDFEFLG) and N-glycosylation site (Fig. S1G). Phylogenetic tree analysis indicated that DoXTH1 and DoXTH3 had highly homologous to the XTH in *Arabidopsis thaliana* (Fig. 2G). Interestingly, DoXTH2 was clustered into a different group with DoXTH1 and DoXTH3.

Expression profiles of genes related GA biosynthesis and signaling during tuber growth

The expressions of *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox1*, *DoGA2ox2*, *DoGA2ox3*, *DoGA2ox4*, *DoGID1a*, *DoGID1b*, *DoGID1c*, *DoDELLA1*, *DoDELLA2,* and *DoDELLA3* were assessed during tuber expansion stage. *DoGA20ox1* expression levels showed higher transcript from 1 to 15 days, and gradually declined along with tuber growth, reaching low levels on 120 days (Figs. 3A and 8). The expression level of *DoGA3ox1* increased first, then decreased with tuber growth, and had the highest levels on 30 days and 60 days (Figs. 3B and 8). All *DoGA2ox* genes were expressed in tuber growth, showed different accumulation patterns from 1 to 15 days, gradually declined from 30 to 120 days (Figs. 3C, D, E, F, and 8). The expression level of *DoGID1a* and *DoGID1c* increased from 1 to 60 days, decreased gradually at late stages of tuber growth (Figs. 4A, C and 8). The expression level of *DoGID1b* also decreased gradually with tuber growth (Figs. 4B and 8). The expression of *DoGID1c* was in general lower than that of *DoGID1a* and *DoGID1b* in tuber growth. The expression level of *DoDELLA1* increased gradually in 3 days and decreased gradually at late stages of tuber growth (Figs. 4E and 8). Conversely, *DoDELLA2* and *DoDELLA3* steadily increased or decreased along with tuber growth, respectively (Figs. 4E, F and 8).

To assess whether the increased GA_3 levels after application GA_3 resulted from transcriptional regulation, the expression levels of GA biosynthesis and signaling genes after GA_3 or PP_{333} treatment were measured during tuber growth. It showed that *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox3*, *DoGA2ox4*, *DoGID1a*, and *DoDELLA1* expression displayed in response to GA_3. At the early expansion stage (1, 3, 5, 7, 15, and 30 days), GA_3 treatment decreased *DoGA20ox1*, *DoGA3ox1*, *DoGID1a*, and *DoDELLA1* expression levels, while PP_{333} treatment enhanced their expression levels. In the meanwhile, GA_3 treatment increased *DoGA2ox3* and *DoGA2ox4* expression levels, while PP_{333} treatment decreased their expression levels, compared to the controls (Figs. 3, 4, and 8).

Properties of DoGID1-DoDELLA interaction in Y2H assays

To further characterize the biochemical properties of DoGID1s and DoDELLAs, Y2H assays were performed. Y2H assays showed that DoGID1a, DoGID1b, and DoGID1c interacted with all DoDELLAs, except DoGID1a and DoDELLA3 (Fig. 5). The binding results confirmed the essential GA-induced assembly of stable GA-DoGID1-DoDELLA complex in yeast (Fig. 5). DoDELLA1 and DoDELLA2 were effectively able to interact with DoGID1a, DoGID1b, and DoGID1c in the presence of GA_3 (growth in GA_3 medium and darker color), except for DoGID1b-DoDELLA3 and DoGID1c-DoDELLA3 (growth in GA_3 medium). Conversely, DoGID1a did not bind to DoDELLA3, even in the presence of GA_3. Nevertheless, this may be different in the case of DoGID1s and DoDELLAs, where sequence and conformational differences may confer some levels of specificity in DoGID1s and DoDELLAs. These data showed DoGID1s interacted with DoDELLA1 and DoDELLA2 in a GA-mediated manner in Y2H assay.

Expression profiles of cell wall genes during tuber growth

To understand the EXP and XTH genes response to tuber growth, the expression levels of five *DoEXP* and three *DoXTH* genes of the cell wall were detected in tuber growth. The expression levels of *DoEXP1*, *DoEXP2*, and *DoEXP5* genes were higher in the early expansion stage than in other stages except for *DoEXP3* and decreased gradually in late stages. *DoEXP4* was increased in the tuber growth stage (Figs. 6 and 8). The expression level of *DoXTH1*, *DoXTH2,* and *DoXTH3* increased on 30 days and decreased gradually in late stages (Figs. 7 and 8). Compared to the control, GA_3 treatment increased significantly the expression levels of five *DoEXP* and three *DoXTH* genes from 1 to 30 days, while that of were reduced by PP_{333} treatment from 1 to 30 days (Figs. 6, 7 and 8). Interestingly, GA_3 treatment increased significantly increased *DoEXP4* and *DoEXP5* expression during tuber growth. According to the expression level and pattern, *DoEXP4* and *DoEXP5* have more roles in tuber cell expansion than other genes.

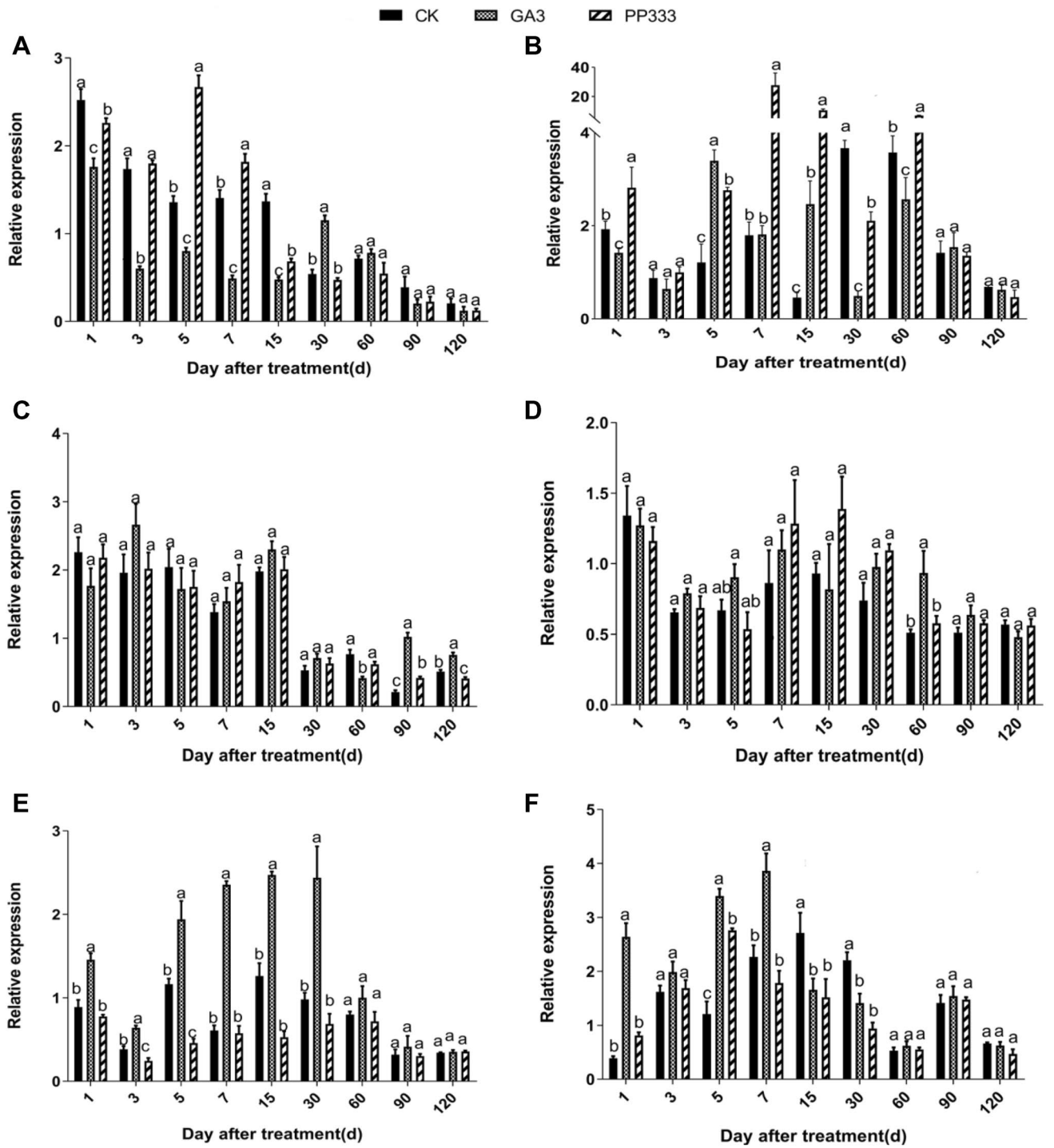

Fig. 3 Effects of GA_3 and PP_{333} on the expressions of *DoGA20ox1* (**A**), *DoGA3ox1* (**B**), *DoGA2ox*1 (**C**), *DoGA2ox2* (**D**), *DoGA2ox3* (**E**), and *DoGA2ox4* (**F**) of tubers in different developmental stages

Discussion

Yam is one of the most commercial tuber crops. Yam tuber growth and development are an attractive theoretical model for studying the development of underground organs. In recent years, GA has been used to explore the physiological factors affecting the growth of yam tubers. While endogenous GA has been detected in yam, GA_3 was higher in early expansion stage than other stages (Gong et al. 2016), and the application of GA_3 to yam produced new tubers and

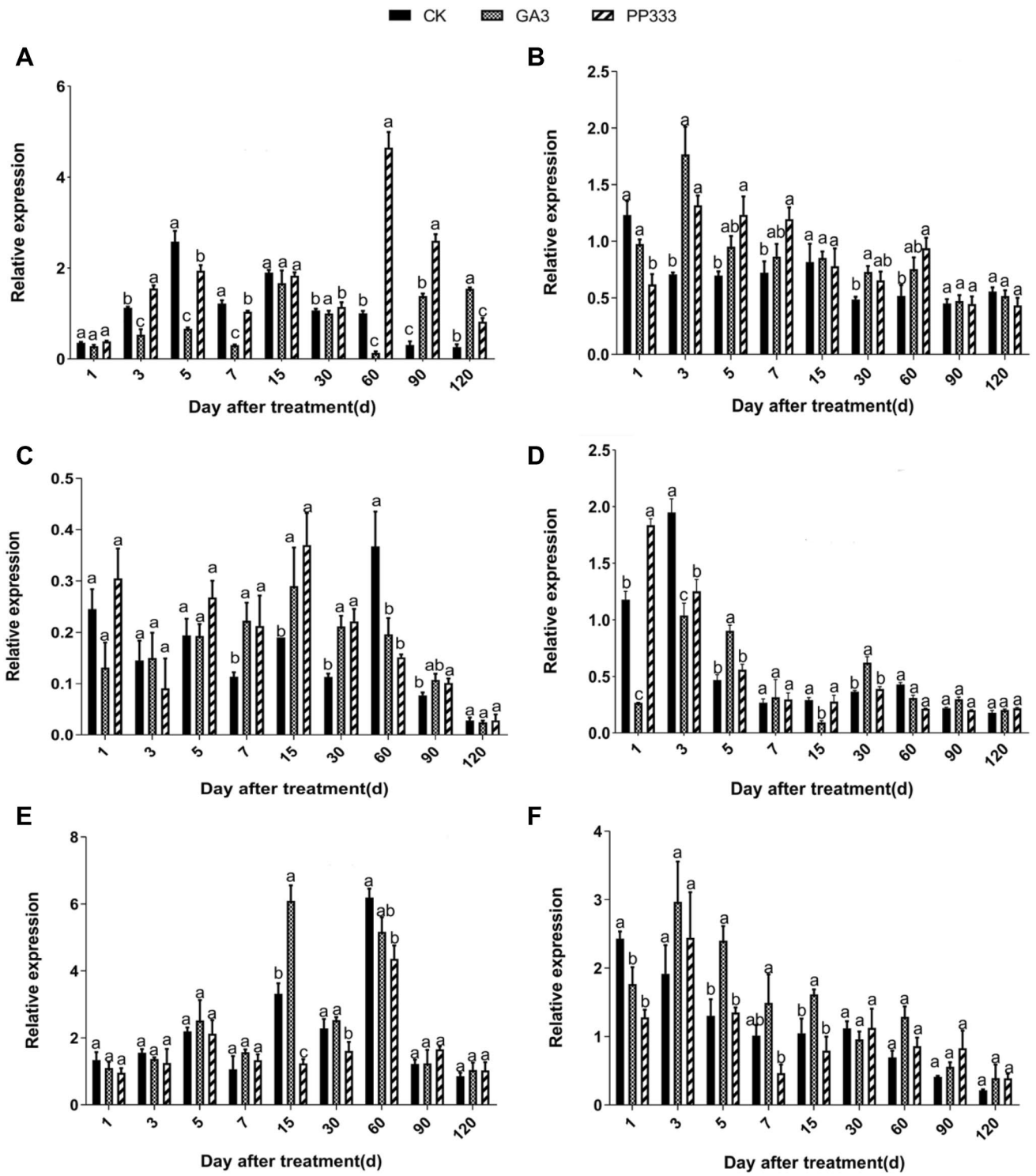

Fig. 4 Effects of GA_3 and PP_{333} on the expressions of *DoGID1a* (**A**), *DoGID1b* (**B**), *DoGID1c* (**C**), *DoDELLA1* (**D**), *DoDELLA2* (**E**), and *DoDELLA3* (**F**) of tubers in different developmental stages

promoted tuber expansion with an increase in tuber weight and yield (Yoshida et al. 2008; Gong et al. 2015). The concentration of bioactive GAs is determined by the balance between gene expression of biosynthesis and deactivation, in which GA20ox, GA3ox, and GA2ox genes encode key enzymes of biosynthesis and inactivation of GA, and GA binds its receptor, GID1, to form a complex pathway that mediates the degradation of DELLA proteins to regulate

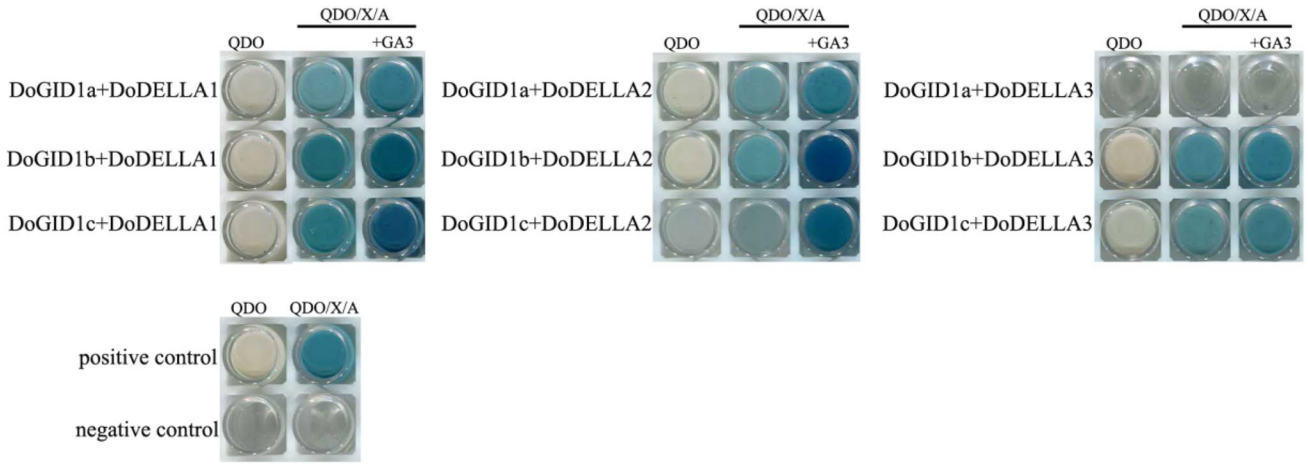

Fig. 5 Interaction between DoDELLAs and DoGID1s proceeding in a GA-dependent manner. The addition of 100 μM GA_3 to the medium-enhanced GID1-DELLA interactions

plant growth (Hedden and Sponsel 2015; Middleton et al. 2012; Hedden and Thomas 2012). However, the regulating mechanism of GA_3 on yam tuber growth by bioactive GAs remains elusive.

Endogenous GA_3 level in tuber growth is controlled by GA biosynthetic genes

GA_3 and GA_4 were detected in yam tuber (Gong et al. 2016). High endogenous GA_3 is responsible for the beginning of tuber dormancy and growth (Ao et al. 2020; Zhu and Hou 2011). Furthermore, GA20ox regulated GA_3 production in yam tuber (Ao et al. 2020). GA20ox2, GID1C2, and DELLA2 were responsive to GA_3 in yam tuber growth (Xing et al. 2020). It is suggested that GA_3 may be closely related to tuber growth, and GA-related genes are responses to GA_3 regulation in yam. In rice, GA_4 could be rapidly inactivated and degraded in GA_3-treated cells by GA-inactivating enzymes; however, GA_3 was not easily inactivated by inactivating enzymes and remained active after GA_3 treatment, which promoted GID1–SLR interaction (Ueguchi-Tanaka et al. 2007). Hence, it is necessary to investigate the GA_3 level and clarify the molecular mechanism in tuber growth. In this study, GA_3 had the highest level from 1 to 30 days in the early expansion stage, and then decreased in middle, late, and mature stages, which shows that the increment of GA_3 may induce cell expansion, and improve tuber growth. The result is similar to previous reports in yam (Gong et al. 2016) and other species. During carrot root growth, the highest GA levels were observed early enlarge stage (42 days), this level subsequently decreased (Wang et al. 2015). GA levels are critical for early tissue or organ development which is consistent with the role of GA during early plum fruit formation (El-Sharkawy et al. 2014).

However, little is known the correlation between GA levels and the gene expressions of GA metabolism components in yam tuber. In this study, the accumulation of GA_3 was consistent with the expression of *DoGA20ox1* during the early expansion stage of yam tuber, contrary to the expression of *DoGA2ox3* and *DoGA2ox4*. With the tuber growth, the content of GA_3 gradually decreased, and the expression of these genes also gradually decreased. Taken together, the decrease in endogenous GA_3 in yam tuber growth is explained by GA biosynthetic gene expressions. The down-regulation of *DoGA20ox1* transcript and the apparent accumulation of *DoGA2ox3* and *DoGA2ox4* reflect an important role for the genes related to GA_3 synthesis during tuber growth. This scenario is similar to the results observed in other species during plant development. GA accumulation in anthesis coincided with transient up-regulation of *VvGA20ox1*, *VvGA20ox3*, and *VvGA3ox2* in grapevine (Giacomelli et al. 2013). Hence, endogenous GA_3 accumulates in the early expansion stage by *DoGA20ox1*-active synthesis and *DoGA2ox3*- and *DoGA2ox4*-active inactivation. However, the *DoGA3ox1*, *DoGA2ox1*, and *DoGA2ox2* expression patterns were incompletely consistent with the GA_3 levels possibly because of the feedback mechanism, or other hormones may play vital roles in tuber growth (Gong et al. 2016), which indicating a complex mechanism in endogenous GA_3 accumulation.

DELLA participate in yam tuber growth

It is known that GID1 acts as GA receptors, whereas DELLA protein is a negative regulator of GA signaling (Murase et al. 2008; Ueguchi-Tanaka et al. 2007). Hence, the expression levels of *DoGID1* and *DoDELLA* genes were assessed during various tuber developmental stages. *DoGID1a* and

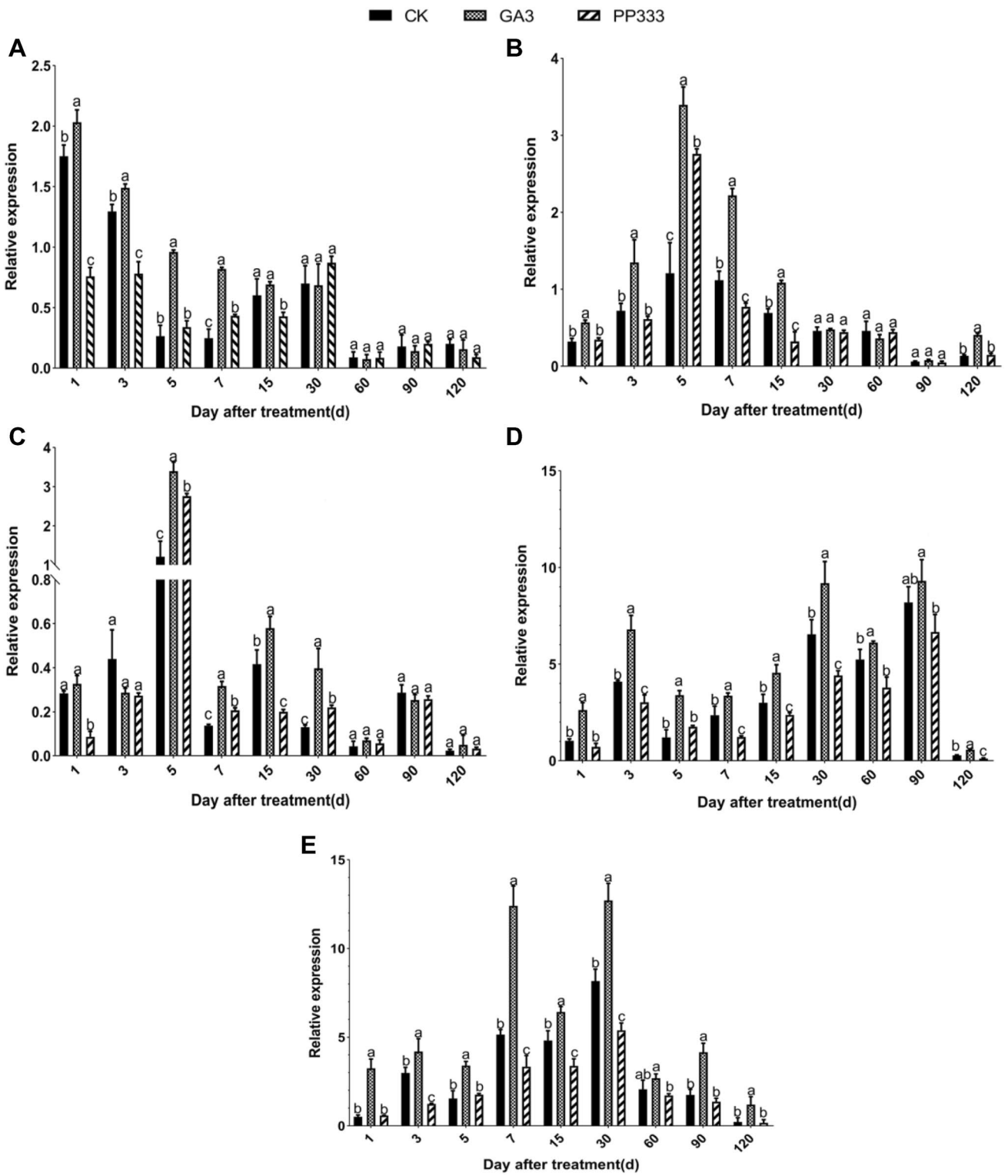

Fig. 6 Effects of GA_3 and PP_{333} on the expressions of *DoEXP1* (**A**), *DoEXP2* (**B**), *DoEXP3* (**C**), *DoEXP4* (**D**), and *DoEXP5* (**E**) of tubers in different developmental stages

DoDELLA1 genes were abundantly expressed in the early expansion stage. GA_3 was involved in tuber growth, particularly the early expansion stage. Similarly, GA is needed to organize the abundant cell expansion, division during tissue development. The abundance of the *DoGID1a* and *DoDELLA*1 genes during early tuber expansion stage

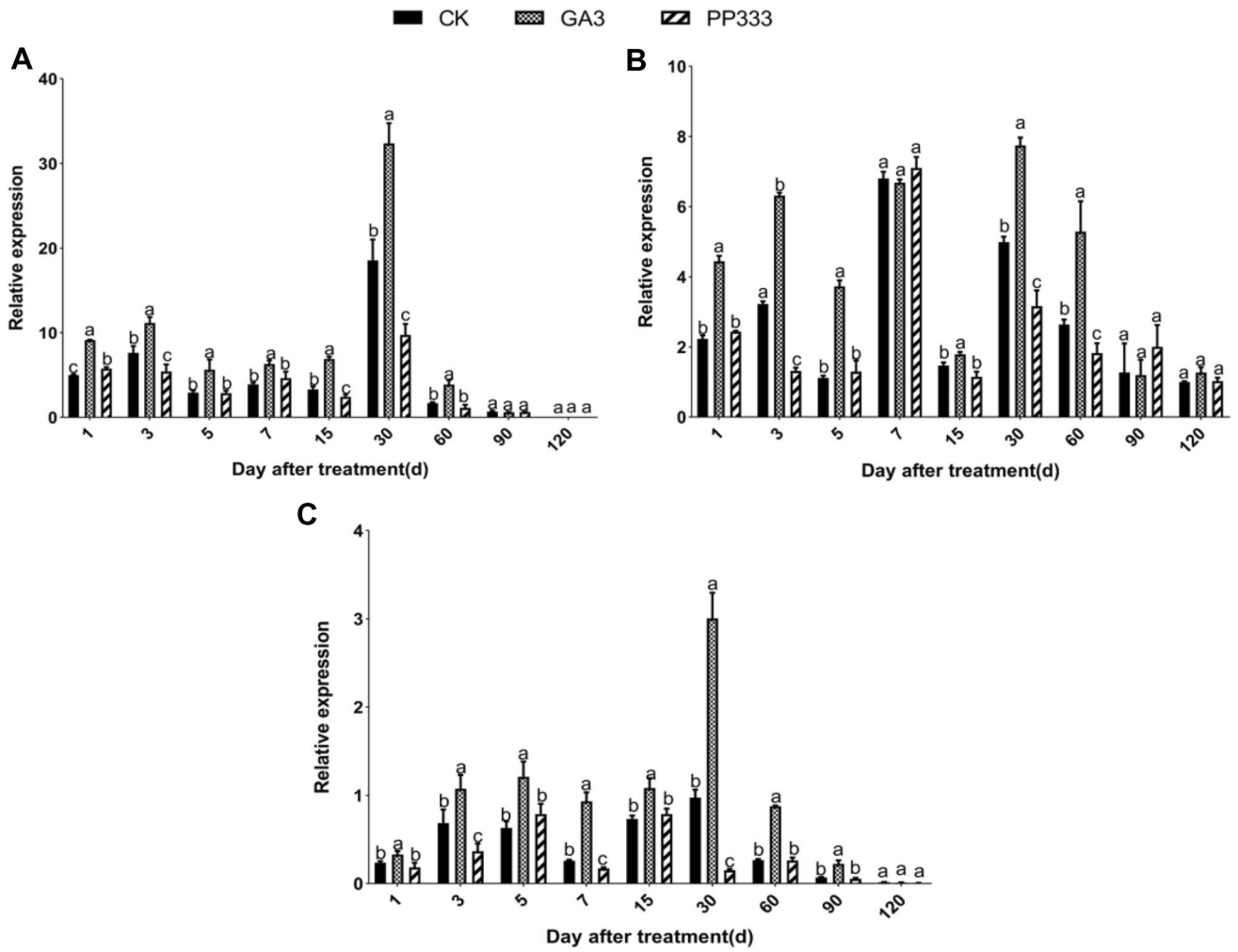

Fig.7 Effects of GA_3 and PP_{333} on the expressions of *DoXTH1*(**A**), *DoXTH2*(**B**), and *DoXTH3*(**C**) of tubers in different developmental stages

suggests a dominant task of *DoGID1a* and *DoDELLA1* genes in regulating GA response during this stage. Previous reports have indicated that GID1 and DELLA proteins play a key role in hypocotyl growth, stem and root elongation, bud dormancy, plant height, and fruit development in GA response (Griffiths et al. 2006; Lv et al. 2018; Li et al. 2018a). These results suggested that *DoGID1a* and *DoDELLA1* genes are active components of the GA signal network that regulate tuber growth.

Effect of exogenous GA_3 treatment on gene expression

In this study, GA_3 treatment increased endogenous GA_3 levels and PP_{333} treatment decreased endogenous GA_3 levels in tuber growth, which is consistent with the previous results. In pear, GA_{4+7} increased unpollinated ovaries GA_{4+7} levels to induce parthenocarpy (Liu et al. 2018). GA_3 application resulted in a significant increase in GA_3 levels along with a slight acceleration in fruit size and weight in plum fruit development (El-Sharkawy et al. 2014). It can be assumed that exogenous GA_3 applications increase relatively higher endogenous GA_3 levels in the tuber and improve yam tuber growth.

GA homeostasis in plants is maintained by feedback regulation. When GA levels were too high in plants, GA20ox and GA3ox were subject to negative feedback by decreasing their expression, GA2ox increased expression by positive feed-forward regulation (Fukazawa et al. 2017). In Cucumber, *CsGA20ox1*, *CsGA20ox2*, and *CsGA3ox1* were strongly repressed by GA_3 treatment, when *CsGA2ox1, CsGA2ox4*, and *CsGA2ox6* were simultaneously induced by GA_3 treatment (Sun et al. 2018). Exogenous GA application in tomato fruit led to down-regulation of *SIGA20ox*, *SIGA3ox* and up-regulation of *SIGA2ox* (Chen et al. 2016).

Taken together, we propose that the expression profiles of *DoGA20ox1*, *DoGA3ox1,* and *DoGA2ox* were regulated by GA availability in yam tuber, especially in the early

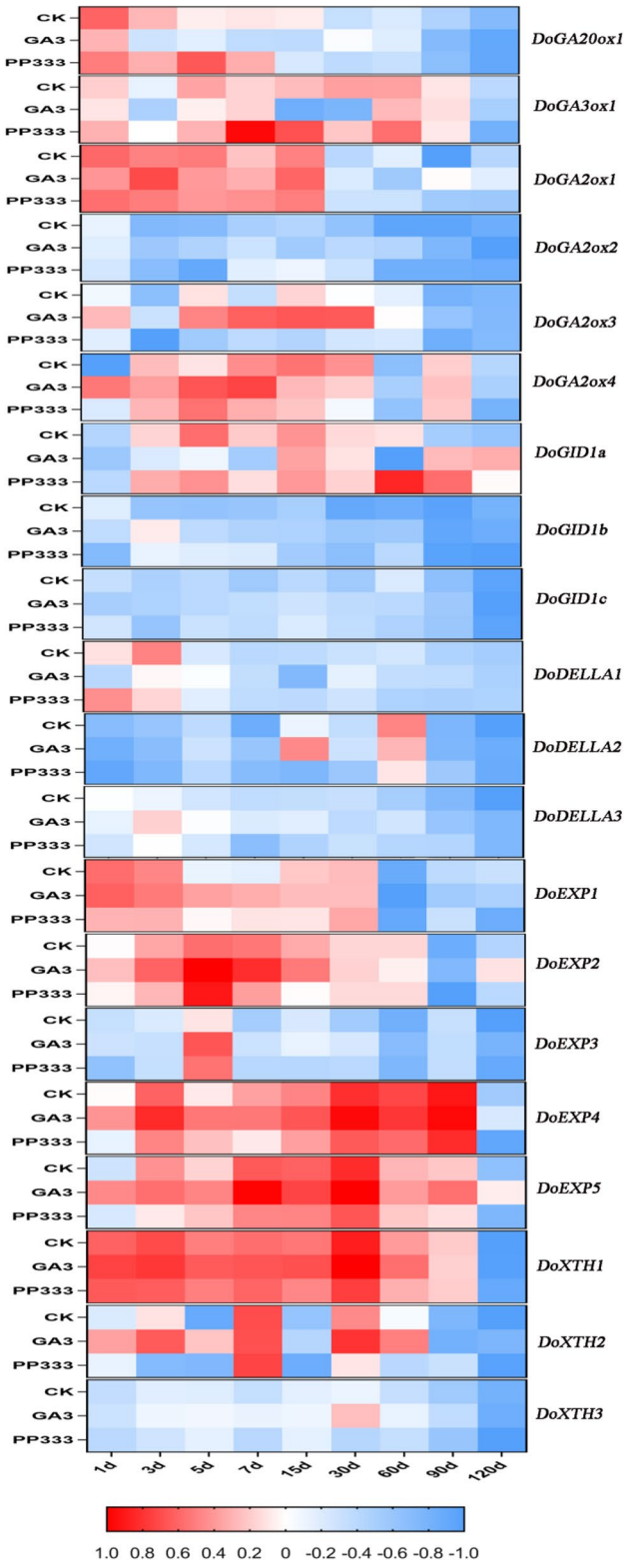

Fig. 8 Heat map of all gene expression in different developmental stages after GA_3 and PP_{333} treatment. The colors from red to blue indicate the up-regulation and down-regulation

expansion stage. The down-regulation of *DoGA20ox1* and *DoGA3ox1*, and up-regulation of *DoGA2ox3* and *DoGA2ox4* following by GA_3 application show a feedback mechanism in response to endogenous GA_3. On the other hand, PP_{333} application exhibited the adverse consequences. It suggests the existence of a negative feedback mechanism to regulate the expression of *DoGA20ox1* and *DoGA3ox1*, and positive feedback regulation of *DoGA2ox3* and *DoGA2ox4* in tuber growth. The feedback regulation may keep suitable concentration of active GA_3 after exogenous GA_3 application, to improve growth and prevent overgrowth simultaneously in tuber. In addition, no significant changes were observed for the expression of *DoGA2ox1* and *DoGA2ox2* in tuber growth of GA_3 and PP_{333} treatment, suggesting that individual members of the *DoGA2ox* gene family may play different physiological roles to response GA_3 treatment, or depend on tissue types and organs.

Also, we found the expression pattern of *DoGID1a* and *DoDELLA1* in response to GA_3 or PP_{333} treatment in the early expansion stage. GA_3 application down-regulated *DoGID1a* and *DoDELLA1* expressions, while PP_{333} treatment up-regulated expressions in the early expansion stage. It is consistent with the previous results in grape and pear. The application of GA_3 increased endogenous GA_3 level, down-regulated *VvGID1* transcripts, and resulted in the degradation of VvDELLA protein in grape and pear (Acheampong et al. 2017, 2015; Liu et al. 2018). In addition, DoGID1s interacted with DoDELLA1 and DoDELLA2 in a GA-mediated manner in Y2H assay. The potential explanation for DoGID1a and DoDELLA1 in response to GA_3 is that exogenous GA_3 application increase endogenous active GA_3 concentration, and high-level GA_3 rapidly induced DoDELLA1 degradation, resulted in the *DoGID1a* expression decrease.

However, the expression patterns of *DoGA2ox1*, *DoGA2ox2* and *DoGID1b*, *DoGID1c*, *DoDELLA2*, and *DoDELLA3* were incompletely consistent with the GA_3 levels by GA_3 and PP_{333} application, and it possibly results from the feedback mechanism of GA_3 (Fukazawa et al. 2017) or their different roles in the control of active GA homeostasis. In the meanwhile, other hormones may also play vital roles in active GA biosynthesis and metabolism, indicating a complex mechanism of GA biosynthesis and catabolism (Okabe et al. 2019; Cong et al. 2019).

Previous studies in pear had demonstrated that exogenous GA_{4+7} barely changed the expression levels of encoded biosynthetic GA genes, since GA content was already high to support pear fruit development, while mainly influenced GA response genes, such as cell cycle and cell expansion genes (Liu et al. 2018). Some EXP and XTH genes associated with cell walls were found in the yam tuber expansion stage by transcript profiling of Guihuai 16 (Zhou et al. 2020). Plant organ growth depends on

cell differentiation and enlargement through GA regulation. Overall, all *DoEXP* and *DoXTH* genes expression levels were high in the early expansion stage, except *DoEXP4* was high in the late expansion stage. In the meanwhile, GA_3 had a higher level in the early expansion stage, which showed that the increment of GA_3 may regulate all *DoEXP* and *DoXTH* genes expression, induce cell expansion, and improve tuber growth. Exogenous GA_3 treatment in maize leaf could affect cell expansion and gives rise to a dramatic change in direction for cell expansion in growing cells (Nelissen et al. 2012). Cellular elongation required cell wall remodeling enzymes, including EXPs and XTHs, to rearrange cell wall matrix polymers for cell wall loosening (Hervieux et al. 2016; Cosgrove 2000). GA_3 could up-regulate EXPs and XTHs transcripts to promote cell elongation or cell wall modification in *Arabidopsis*, *Cucumber,* and *Persimmon* (Sanchez-Montesino et al. 2019; Sun et al. 2017; Han et al. 2016). GA_3 application up-regulated all *DoEXP* and *DoXTH* genes in the expansion stage of the tuber, while the PP_{333} treatment showed the opposite pattern. *DoEXP4*, *DoEXP5,* and *DoXTH1* genes performed higher responses to GA_3 in tuber growth. DELLA protein could regulate cell wall properties by repressing the *EXP8*, *EXP10*, and *XTH28* expression either directly or indirectly in *Arabidopsis* (Oh et al. 2009; Dello Ioio et al. 2008). These results suggest that the accumulation of GA_3 by the activities *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox3*, *DoGA2ox4*, and the degradation of DoDELLA1 proteins due to increase the formation of GA-DoGID1a-DoDELLA1, increase GA response genes expression, such as *DoEXP1*, *DoEXP2*, *DoEXP3*, *DoEXP4*, *DoEXP5*, *DoXTH1*, *DoXTH2*, and *DoXTH3*, and then enhance tuber growth by inducing cell expansion.

In brief, endogenous GA_3 biosynthesis is catalyzed by *DoGA20ox1* and *DoGA3ox1*, and its deactivation is catalyzed by *DoGA2ox3* and *DoGA2ox4* in yam tuber growth. High GA_3 promotes tuber growth by stimulating the degradation of the growth repressing DoDELLA1 proteins. In GA_3 treatment, the down-regulation of *DoGA20ox1* and *DoGA3ox1*, and up-regulation of *DoGA2ox3* and *DoGA2ox4* transcript accumulation are in response to a high endogenous GA_3 level as a feedback mechanism. A model is presented in Fig. 9. Exogenous GA_3 results in a significant increase in endogenous GA_3 levels along with a feedback mechanism by downregulating *DoGA20ox1* and *DoGA3ox1* expression levels and upregulating the expression of *DoGA2ox3* and *DoGA2ox4* to maintain GA homeostasis. A high GA_3 level can rapidly bind *DoGID1a* and allows the *DoDELLA1* to be targeted for degradation, leading to increase *DoEXP1*, *DoEXP2*, *DoEXP3*, *DoEXP4*, *DoEXP5*, *DoXTH1*, *DoXTH2,* and *DoXTH3* expression, cell division and cell expansion, and improve tuber growth. This work helps increase our understanding of the cross-talk between endogenous and exogenous GA_3 in yam tuber.

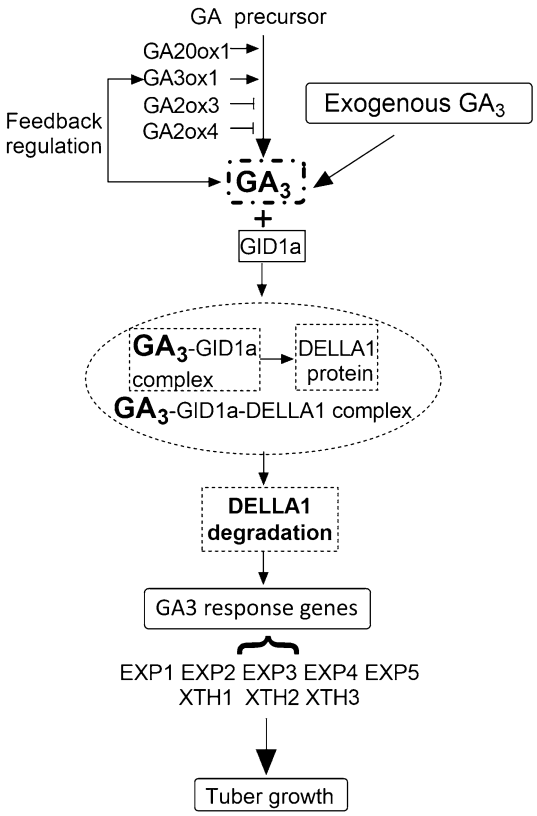

Fig. 9 Probable mechanism of endogenous GA_3 regulates tuber growth in yam. The accumulation of active GA_3 is controlled by the activities of *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox3,* and *DoGA2ox4*, and the degradation of DoDELLA1 proteins due to the formation of GA-DoGID1-DoDELLA. Active GA_3 improves GA response genes expression, such as *DoEXP1*, *DoEXP2*, *DoEXP3*, *DoEXP4*, *DoEXP5*, *DoXTH1*, *DoXTH2,* and *DoXTH3*, and enhance tuber growth

Conclusion

The profiles of GA_3 accumulation with tuber growth may be the result of the combined action of *DoGA20ox1*, *DoGA3ox1*, *DoGA2ox3*, and *DoGA2ox4* expression and DoDELLA1 protein degradation, and the formation of GA-DoDID1a-DoDELLA1, while promoting the expression of GA response genes *DoEXP1*, *DoEXP2*, *DoEXP3*, *DoEXP4*, *DoEXP5*, *DoXTH1*, *DoXTH2*, and *DoXTH3*.

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Author contributions Y.Y.Z. performed the experiments and wrote the manuscript. Y.T.L. and M.X.G performed the experiments. F.Q., J.Z., and D.X. provided technical assistance and research guidance. A.Q.W. and L.F.H. designed the research and revised the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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