Fruit Research

ARTICLE

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https://doi.org/10.48130/FruRes-2023-0007

Fruit Research **2023**, 3:7

Effects of TDZ and IBA on DNA methylation during callus induction in pear (*Pyrus ussuriensis* Maxim)

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Abstract

One of the essential epigenetic processes in higher eukaryotes, DNA methylation is essential for maintaining genome integrity and influencing gene expression. There is limited research on how plant growth regulators (PGR) affect DNA methylation during the pear callus formation process. In this study, using methylation-sensitive amplification polymorphism (MSAP), DNA methylation in pear callus caused by various quantities of thidiazuron (TDZ) and indole-3-butyric acid (IBA) was compared. The concentrations of TDZ and IBA could affect the callus induction rate and methylation level. It was discovered that induction rate and methylation were negatively correlated. The rate of pear callus induction was highest (54%) while the medium suppled with 3.0 mg·L^{-1} TDZ and 4.0 mg·L^{-1} IBA, although the matching DNA methylation level was lowest (27.96%). Additionally, there were significant difference in the level of genes' expression between different treatments and both methylation and demethylation are regulated by these genes. As for genes related to auxin and cytokinin, gene expression analysis revealed that their levels of expression after different concentrations of TDZ combined with IBA present in differences significantly. Our findings shed light on DNA methylation mechanisms of plant tissue culture (PTC) dedifferentiation.

Citation: Zhou F, Zhou S, Cui S, Liu J, Yang Y, et al. 2023. Effects of TDZ and IBA on DNA methylation during callus induction in pear (*Pyrus ussuriensis* Maxim). *Fruit Research* 3:7 https://doi.org/10.48130/FruRes-2023-0007

INTRODUCTION

Fruit trees are important agricultural resources because of their diversity and adaptability. Due to the growing market for fruits, improving the breeding of high-quality fruit tree varieties and breeding efficiency has become a major focus of research. Traditional fruit tree breeding techniques have some difficulties because of their long juvenile period, high heterozygosity, and incompatibility. PTC is an essential asexual propagation method that can increase propagation coefficients, shortening seedling periods, and ensure the stable inheritance of desirable traits.

The process of PTC consists of continuous dedifferentiation (callus formation) and redifferentiation (plant regeneration) stages[1,2]. Studies have demonstrated that growth regulators in plant are crucial *in vitro* culture. The callus from *Dendrocalamus sinicus* hypocotyls was induced on Murashige-Skoog (MS) medium containing 2.10 mg·L^{-1} 6-benzylaminopurine (6-BA) and 3.10 mg·L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and the induction rate was 88.87%[3]. *Cnidium officinale* root explants showed a maximum callus induction rate of 75% on MS medium containing 2.3 mol·L^{-1} 2,4-D and 2.2 mol·L^{-1} benzyladenine (BA)[4]. In *Salvia tebesana* shoot apical meristem explants, the callus induction rate could reach 100% when using 1-naphthylacetic acid (NAA) (0.5 and 1.0 mg·L^{-1}) or in combination with 6-benzylaminopurine (BAP) (0.5 mg·L^{-1}) and 2,4-D (0.5, 1.0, and 1.5 mg·L^{-1}) in conjunction with BAP (0.5 mg·L^{-1})[5]. PGR are therefore essential for the development of callus.

And epigenetic variations often occur in PTC[6]. A crucial epigenetic modification mode is DNA methylation[7–9], it is also affected during callus induction[10,11]. The relative expression level of DNA methyltransferase gene *DNA METHYLTRANSFERASE 1* (*MET1*), *DNA METHYLTRANSFERASE CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLASE 2* (*DRM2*) and *REPRESSOR OF SILENCING 1* (*ROS1*) has also changed during the callus formation[11–13]. The most popular PGR used to produce callus in varied plants are auxin and cytokinin[14–16]. The induction effect was affected by the concentration and combination of PGR, plant species and explant[17]. Previous research discovered that under the induction of 2,4-D, the level of DNA methylation in wheat mature embryos decreased and then increased[10]. The medium containing 1.0 mg·L^{-1} 6-BA and 0.1 mg·L^{-1} 2,4-D, whereas DNA methylation level and the relative expression level of DNA methyltransferase-related genes (*MET1*, *CMT3*) were lowest had the highest rate of callus induction (91.0%)[11]. Nevertheless, there are few reports on how growth regulators affect DNA methylation during the formation of pear callus.

Auxin/cytokinin is essential for callus formation, and the transcription level of PGR related genes changes with the formation of callus[18,19]. Some previous studies have found that *AHK 2* and *AHK 4* encode cytokinin receptors[20–23]; *ARR 5* and *APRR 5* can be induced by cytokinin[24]; *CKX 7* catalyzed cytokinin oxidation[25]. The production of indole-3-acetic acid (IAA) -amino acid conjugates is catalyzed by auxin responsive genes *GH 3.1* and *GH 3.6*, it provides a defense mechanism against excess auxin in plants[26]. Auxin transport genes *Pin-like 3*, *Pin-likes 5*, and *Pin-likes 6* participate in auxin homeostasis by regulating auxin metabolism[27]. Therefore, in this study we selected these related genes for relative expression verification.

The MSAP and qRT-PCR technologies were applied in this study to examine changes of DNA methylation and gene expressions. Also the gene expression of auxin and cytokinin in pear (*Pyrus ussuriensis* Maxim) callus induced by TDZ and IBA was investigated. At the same time, the effects of TDZ and IBA on DNA methylation and auxin and cytokinin related gene expression in callus induction of pear leaf were discussed. Our findings shed new light on the plant dedifferentiation DNA methylation mechanism.

MATERIALS AND METHODS

Plant material and callus induction

The test tube pear (*Pyrus ussuriensis* Maxim) buds were cultured using the MS medium as the basic medium and 1.0 mg·L^{-1} of 6-benzylaminopurine (6-BA), 0.2 mg·L^{-1} of 3-indolebutyric acid (IBA), 3% (w/v) sucrose, and 0.6% (w/v) agar[28]. After bringing the pH to 5.8, it was autoclaved for 20 min at 121 °C. The culture temperature was 25 °C, and 16 h was set as the photoperiod. To induce callus, the leaves of pear *in vitro* cultured for one month were applied as explants. From the test-tube plantlets, only fully developed leaves (those closest to the stem) were selected. Two leaves from each of the 115 plants from each treatment were selected, for a total of 30 leaves from each treatment that were measured. As a technical repeat, the 30 leaves were separated into three groups, each with 10 leaves. The callus induction medium was NN69 medium with different concentration combinations of TDZ (1.0, 3.0, and 5.0 mg·L^{-1}) and IBA (2.0, 4.0, and 6.0 mg·L^{-1}), 30 g·L^{-1} of sucrose, and 7 g·L^{-1} of agar. Leaves of pear similar in size and from the same growth period were used. The two ends of the leaves were cut and five wounds were made in the middle of the leaves perpendicular to the main pulse. The explants were then laid flat in callus induction medium and cultured in darkness for 2 weeks. Following 2 weeks of induction, callus was collected for further analysis.

DNA extraction

Using a Plant Genomic DNA Kit (Vazyme, Nanjing, China), the genomic DNA of the pear (*Pyrus ussuriensis* Maxim) was isolated. 1% agarose gels were used to assess DNA quality. −20 °C was used to keep DNA samples.

Reactions of digestion and ligation

DNA samples were digested using *Eco*R I + *Hpa* II and *Eco*R I + *Msp* I. *Hpa* II and *Msp* I recognize and digest the identical site (5′-CCGG-3′), but their sensitivities differ to DNA methylation. *Hpa* II can recognize and digest non-methylation sites and single-chain methylation sites but cannot digest double-chain methylation sites; that is, it is unable to enzymatically digest sites that contain mCCGG, CmCGG, and mCmCGG. *Msp* I can recognize and digest non-methylation sites and double-stranded medial cytosine methylation sites and cannot enzymatically digest single-stranded lateral cytosine methylation sites; that is, it cannot enzymatically digest UmCCGG and mCmCGG sites. As a result, several polymorphic PCR amplification bands can represent various DNA methylation states in MSAP analysis.

Pre-amplification and selective amplification

The fragment length polymorphism program was used to guide two amplification processes[29]. In Supplemental Table S1, the primer combinations needed for amplification are displayed. Supplementary Table S2 displays the primer combinations' base sequences. Pre-amplified PCR reactions had a total volume of 20 μL and contained 2 μL of ligation products and 0.5 μL of *E* and *H* preamplification primers (e.g., Supplemental Table S2), Taq polymerase 1 U (Shenggong), 4 μmol·L^{-1} of dNTPs (Shenggong), and 2 μL of 10× PCR buffer (Shenggong). 26 cycles of 30 s each at 94 °C, 1 min at 56 °C, and 1 min at 72 °C were used to amplify the DNA fragments. The pre-amplified product was then diluted with ddH_2O for selective amplification. 0.5 μL of E and H selective amplification primers were added into the diluted product (e.g., Supplemental Table S2), Taq polymerase 1 U, 4 μmol·L^{-1} of dNTPs, and 2 μL of 10× PCR buffer supplemented with ddH_2O to 20 μL. The PCR conditions used were 13 cycles at 94 °C for 30 s, 65 °C for 30 s (reduced by 0.7 °C per cycle), 72 °C for 1 min, and 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min.

Polyacrylamide gel electrophoresis and data analysis

The products of PCR were buffered and denatured for 10 min at 94 °C. Vertical electrophoresis analysis was performed with 6 % denaturing polyacrylamide gel. After silver staining, only the clear and repeatable bands were further converted to binary character matrices, where '+' denotes the presence of bands and '−' denotes no bands. The four types of identified bands are Type I (+, +), Type II (+, −), Type III (−, +), and Type IV (−, −) (e.g., Supplemental Table S3). Type I, no methylation in both *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I digested; Type II, methylation appeared only in *Eco*R I/*Hpa* II degested; Type III, methylation appeared in *Eco*R I/*Msp* I digested; Type IV, methylation appeared in *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I digested. The calculation formula of DNA methylation rate is as follows: Total amplified products = I + II + III; Total methylated products = II + III + IV; Per-methylated ratio (%) = [(III +IV)/(I + II + III + IV)] × 100; Hemi-methylated ratio (%) = [II/(I + II + III + IV)] × 100.

To identify variations in methylation during callus induction, non-methylated and methylated fragments were categorized in accordance with MSAP fragments. Site alterations in methylation status came in four different types: type A, maintaining methylation; type B and C, demethylation; type D, remethylation, in which type B, C, and D are polymorphic methylation bands. Type A is to maintain methylation type, including A1, A2, A3; Type B including B1, B2, B3, B4, B5, B6, B7, B8, is demethylated; Type C includes C1, C2, C3, C4 and C5, is also demethylated; D type includes D1, D2, D3, D4, D5 and D6, which are remethylation type (Supplemental Fig. S1). Type B, type C and type D are polymorphic methylation bands. The percentages of methylation patterns were defined as A(%) = 100 × A / (A + B + C + D); B(%) = 100 × B / (A + B + C + D); C(%) = 100 × C / (A + B + C + D); D(%)= 100 × D / (A + B + C + D).

Quantitative Real-time PCR Analysis

Using an EASY Spin Plant RNA Kit (TianGen, Beijing, China), total RNA from callus was obtained, and A NanoDrop 2000C equipment (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the purity of the RNA. M-MLV reverse transcriptase was used to synthesize cDNA from 1 g of total RNA (Vazyme, Nanjing, China). For qRT-PCR amplification, the following conditions were used: 5 minutes at 95 °C, 45 cycles at 60 °C, 15 seconds at 95 °C, and 30 seconds at 72 °C. The FastStart Essential DNA Green Master Kit was used in the standard mode with the Roche 480 real-time PCR system (Basil,

Switzerland). The total reaction system was 20 μL including 2 μL cDNA in 10-fold dilution. The *Pyrus* actin gene was used as a reference to normalize the qRT-PCR data, and the method via $2^{-\Delta\Delta Ct}$ was used to calculate the relative gene expression level[30]. Supplemental Table S4 lists the primers used.

RESULTS

Analysis of DNA Methylation level in pear callus induced by TDZ and IBA

Twenty pairs of primers used amplified a total of 2,534 clearly recognizable bands, including 838 methylation sites, accounting for 33.07% of all amplified bands. When TDZ was 3.0 mg·L^{-1} and IBA was 4.0 mg·L^{-1}, the induction rate was 54% (Fig. 1 and Table 1), and the methylation rate of callus was 27.96% (Table 2). The callus induction rate was 24.00% and 11.33%, respectively, when TDZ was 3.0 mg·L^{-1} and the IBA concentration was either 2.0 or 6.0 mg·L^{-1} (Fig. 1 and Table 1), and the methylation rate was 29.91% and 26.37%, respectively (Table 2). Callus induction rates were as low as 14.67% and 4.67%, respectively when IBA concentration was 4.0 mg·L^{-1} and TDZ concentration was either 1.0 mg·L^{-1} or 5.0 mg·L^{-1} (Fig. 1 and Table 1), and the callus methylation rate was 32.92% and 31.15%, respectively (Table 2). As the IBA concentration increase, the methylation level decreased. As the TDZ concentration increased, the methylation level first fell and subsequently rose. After the medium was supplemented with 3.0 mg·L^{-1} TDZ and 4.0 mg·L^{-1} IBA, the callus' methylation rate was 27.96%.

Table 1. Effects of TDZ and IBA on the differentiation of pear leaf explants.

PGR Combination		No. of total explants	No. of callus	Induction rate (%)
2.0 mg·L^{-1} IBA	3.0 mg·L^{-1} TDZ	50	13	24.00 ± 3.06^{b}
		50	14	
		50	9	
4.0 mg·L^{-1} IBA		50	26	54.00 ± 2.00^{a}
		50	26	
		50	29	
6.0 mg·L^{-1} IBA		50	10	11.33 ± 4.67^{cd}
		50	5	
		50	2	
1.0 mg·L^{-1} TDZ	4.0 mg·L^{-1} IBA	50	7	14.67 ± 2.91^{c}
		50	5	
		50	10	
3.0 mg·L^{-1} TDZ		50	26	54.00 ± 2.00^{a}
		50	26	
		50	29	
5.0 mg·L^{-1} TDZ		50	2	4.67 ± 1.76^{d}
		50	4	
		50	1	

Samples were collected 2 weeks after induction in the dark. Only callus with sizes greater than or equal to 2 mm × 2 mm × 2 mm were counted. All analysis were repeated three times and the mean ± S.E. was calculated. Callus induction rate = number of callus in existence/total number of explants × 100%. Columns with different letters are significantly difference at $P < 0.05$ by Duncan's multiple range test.

Analysis of DNA methylation patterns in pear callus induced by TDZ and IBA

Methylation was mainly maintained in each treatment, both methylation and demethylation frequently happened simultaneously. Three percent of fragments were demethylated when the TDZ concentration was between 1.0 mg·L^{-1} and 3.0 mg·L^{-1}, and 2.36% of fragments were demethylated when TDZ was between 3.0 mg·L^{-1} and 5.0 mg·L^{-1} (Table 3). 5.88% of fragments were demethylated when the IBA concentration ranged from 2.0 mg·L^{-1} to 4.0 mg·L^{-1}, and 1.68% of fragments were demethylated when IBA concentration changed from 4.0 to 6.0 mg·L^{-1} (Table 3).

The NCBI pear (*Pyrus bretschneideri* Rehd) genome (www.ncbi.nlm.nih.gov/genome/12793) was chosen to contain three highly homologous DNA methylation maintenance-related genes (*PbMET1*, *PbCMT3*, *PbDRM2*) and demethylation-related genes (*PbROS1*), and the relative expression level of methylation and demethylation-related genes in this process were compared by qRT-PCR. This was done in accordance with the protein sequence of DNA methylation-related enzyme According to the findings, when IBA 4.0 mg·L^{-1}, *PbMET1*, *PbCMT3*, and *PbDRM2* reduced first and subsequently increased when TDZ concentration increased. In callus generated by 1.0 mg·L^{-1} TDZ, where the methylation level was 32.92%, the expression of genes associated to methylation was highest (Fig. 2a and Table 2). In callus generated by 3.0 mg·L^{-1} TDZ, the expression of methylation-related genes was the lowest and

Table 2. Comparison of DNA methylation level in pear callus induced by different TDZ and IBA concentrations.

Plant growth regulator	TDZ 3.0 mg·L^{-1}			IBA 4.0 mg·L^{-1}		
	IBA (mg·L^{-1})			TDZ (mg·L^{-1})		
MSAP band type	2.0	4.0	6.0	1.0	3.0	5.0
I	396	407	416	379	407	389
II	53	37	31	48	37	49
III	63	72	64	72	72	53
IV	53	49	49	66	49	74
Total amplified bands	512	516	516	499	516	491
Total methylated bands	169	158	144	186	158	176
Methylated ratio (%)	29.91	27.96	26.37	32.92	27.96	31.15
Permethylated ratio (%)	20.53	21.42	20.00	24.42	21.42	22.48
Hemi-methylated ratio (%)	9.38	6.55	6.37	8.50	6.55	8.67

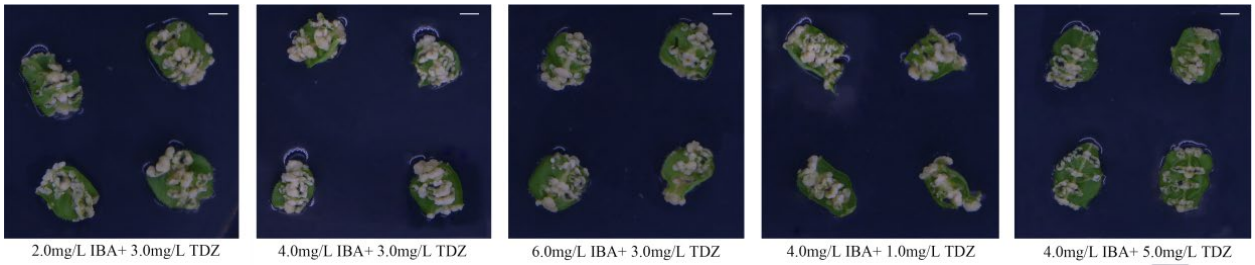

Fig. 1 Comparison of pear callus induced by different TDZ and IBA concentrations. Scale bars = 0.5 cm.

Table 3. MSAP pattern of pear callus induced by different TDZ and IBA concentrations.

Type	IBA (mg·L^{-1})						No.of band	Percentage	TDZ (mg·L^{-1})						No. of band	Percentage
	2.0		4.0		6.0				1.0		3.0		5.0			
	M	H	M	H	M	H			M	H	M	H	M	H		
A1	+	+	+	+	+	+	364	422 (88.66%)	+	+	+	+	+	+	351	418 (89.51%)
A2	+	−	+	−	+	−	45		+	−	+	−	+	−	47	
A3	−	+	−	+	−	+	13		−	+	−	+	−	+	20	
B1	−	−	+	+	+	+	6	28 (5.88%)	−	−	+	+	+	+	0	14 (3.00%)
B2	−	−	+	−	+	−	5		−	−	+	−	+	−	0	
B3	−	−	+	−	−	−	0		−	−	+	−	−	−	0	
B4	−	−	−	+	−	+	2		−	−	−	+	−	+	1	
B5	+	−	−	+	−	+	0		+	−	−	+	−	+	0	
B6	−	−	−	+	+	+	1		−	−	−	+	+	+	0	
B7	−	−	+	−	+	+	7		−	−	+	−	+	+	1	
B8	+	−	+	+	+	+	7		+	−	+	+	+	+	12	
C1	−	−	−	−	+	+	1	8 (1.68%)	−	−	−	−	+	+	2	11 (2.36%)
C2	−	−	−	−	−	+	3		−	−	−	−	−	+	5	
C3	+	−	+	−	+	+	1		+	−	+	−	+	+	1	
C4	+	−	+	−	−	+	0		+	−	+	−	−	+	1	
C5	−	−	−	−	+	−	3		−	−	−	−	+	−	2	
D1	+	+	+	+	−	+	5	18 (3.78%)	+	+	+	+	−	+	13	24 (5.14%)
D2	+	+	+	+	−	−	1		+	+	+	+	−	−	1	
D3	+	+	−	−	−	−	7		+	+	−	−	−	−	1	
D4	+	−	+	−	−	−	3		+	−	+	−	−	−	7	
D5	+	−	−	−	−	−	1		+	−	−	−	−	−	2	
D6	−	+	−	+	−	−	1		−	+	−	+	−	−	0	
Total							476								467	

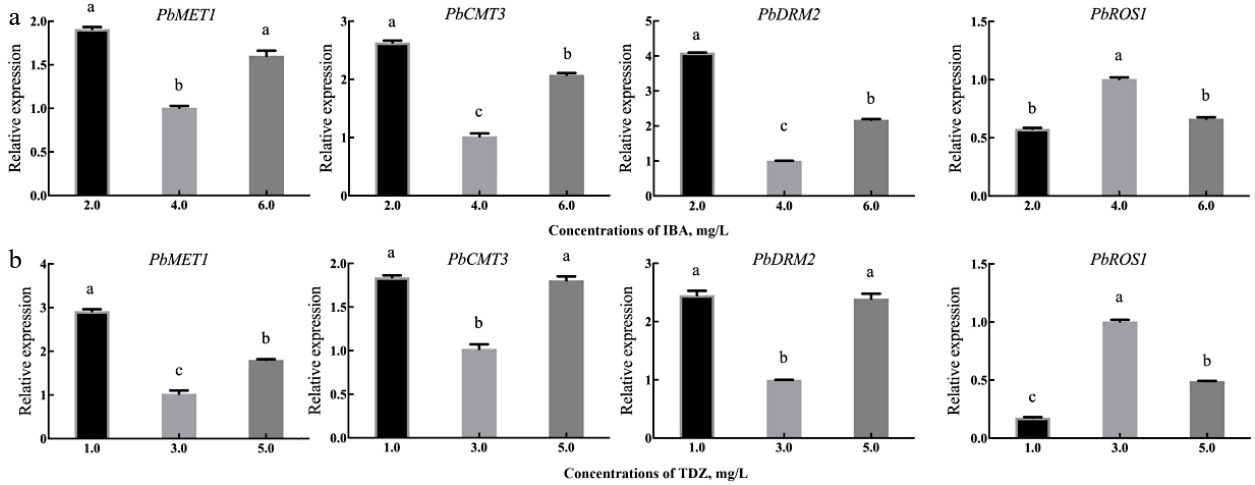

Fig. 2 Expression analysis of methylation related genes in pear callus induced by different TDZ and IBA concentrations.

the level of methylation was the lowest at 27.96% (Fig. 2a and Table 2). The relative expression of demethylation-related genes *PbROS1* showed an opposite trend to the changes of DNA methylation-related genes (Fig. 1a). The highest gene expression and lowest methylation levels were seen when the TDZ concentration was 3.0 mg·L^{-1} (27.96%, Table 2). When IBA concentration changed, the genes *PbMET1*, *PbCMT3*, *PbDRM2* and *PbROS1* showed similar changes (Fig. 2b). The callus generated by 2.0 mg·L^{-1} IBA had the highest methylation level and the highest expression of genes related to methylation, both of which were 29.91% (Table 2). In the callus generated by 4.0 mg·L^{-1} IBA, the amount of methylation was low (27.36%), and the expression of genes related to methylation was at its lowest (Table 2). When IBA was 4.0 mg·L^{-1}, *PbROS1* showed the highest gene expression and the lowest methylation level (27.96%, Fig. 2b and Table 2).

Analysis of expression of auxin and cytokinin-related genes in pear callus induced by TDZ and IBA

During callus formation, we studied the expression of genes associated to auxin and cytokinin during callus development. Significant alterations in the expression of associated genes were brought about by the treatment of leaves with various amounts of TDZ and IBA (Fig. 3). On the seventh day following treatment, *PbGH 3.1-1*, *PbPin-likes 3* and *PbCKX 7* expression was considerably lower in IBA 4.0 mg·L^{-1} and TDZ 3.0 mg·L^{-1}

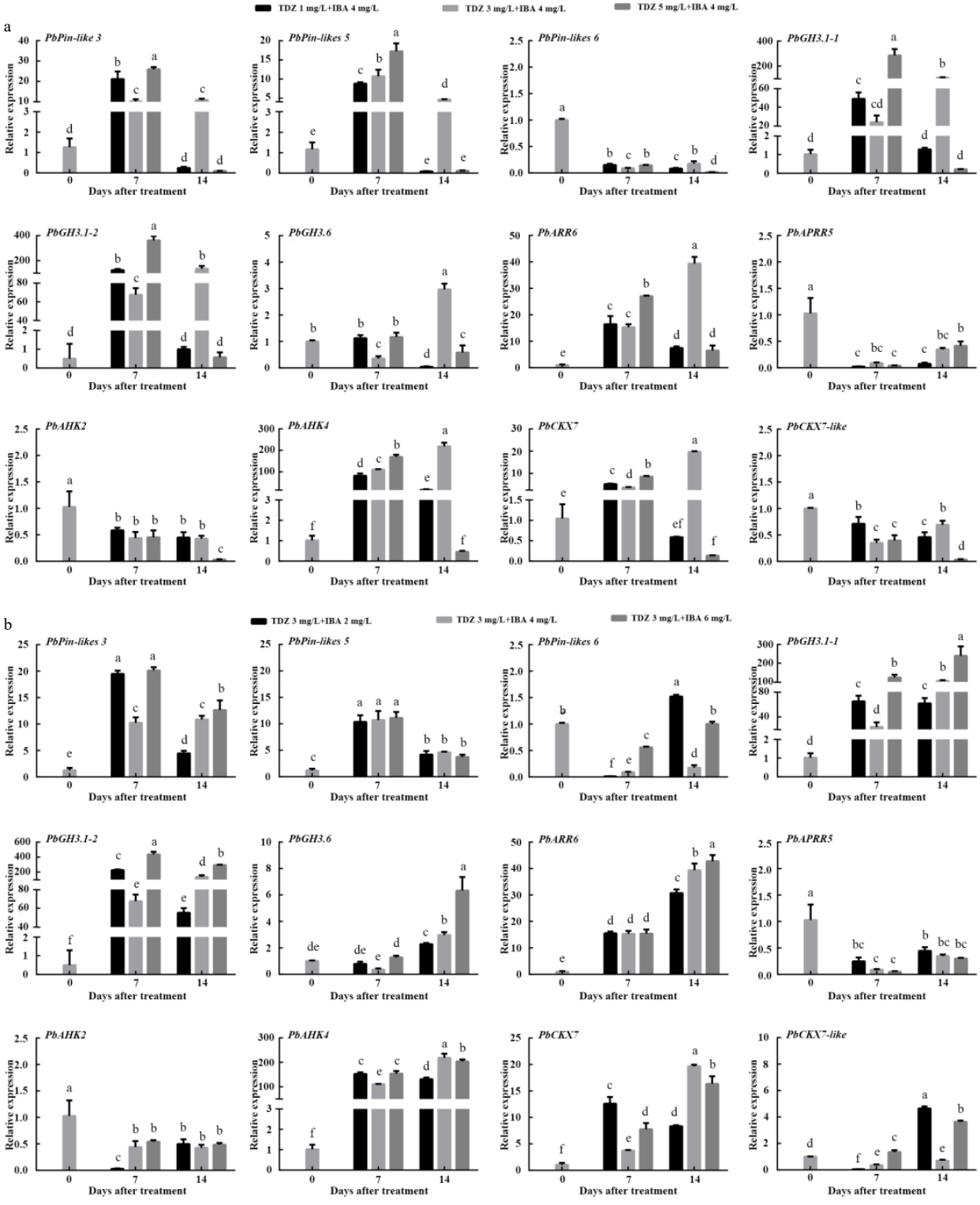

Fig. 3 Analysis of expression of auxin and cytokinin-related genes in pear callus induced by different TDZ and IBA concentrations.

treatment group, but *PbAHK 4* expression was significantly greater in the IBA 4.0 mg·L^{-1} and TDZ 1.0 mg·L^{-1} treatment group and decrease significantly in comparison to the other treatments (Fig. 3a). *PbGH 3.1-1* and *PbPin-likes 3* expression was lower significantly than that of IBA 6.0 mg·L^{-1} and TDZ 3.0 mg·L^{-1} treatments, but significantly up-regulated than that of other treatments seven days after treatment, whereas *PbCKX 7* and *PbAHK 4* expression was significantly lower than that of other treatments (Fig. 3b).

DISCUSSION

Studies into plant developmental processes[31], gene functions[32], micropropagation[33], and the creation of trans-

genic plants with properties[34] have all made substantial use of PTC. Much research has revealed that epigenetic alterations take place during the process of PTC[35–40]. DNA methylation, genomic imprinting, nucleolar dominance, maternal impact, gene silencing, transposon activation, and RNA editing have received the majority of epigenetics research attention[41]. One of the most significant epigenetic changes is DNA methylation. In plant genomes, 5-methylcytosine (5mC) is one of the most widely used epigenetic markers in plant genomes[8].

Previous studies have shown that PGR in plants affect callus induction during PTC[4,5,41]. In this study, we examined the dedifferentiation process in tissue culture because this process is important for regeneration, genetic transformation, and the establishment of a transient verification system. The differentiation direction of explants depends on cytokinin and auxin, which is advantageous to callus growth. In PTC, the most common growth regulators are two categories: auxins (NAA, IBA, 2,4-D and so on) and cytokinins (TDZ, 6-BA, BAP, and so on). The greatest callus induction rate was 86% in peanut (*Arachis hypogaea* L.) embryos grown on MS medium suppled with 5.5 mg·L^{-1} BAP and 1.5 mg·L^{-1} NAA[42]. When the kinetin (KT) was 4.65 μmol·L^{-1} and IBA was 19.60 μmol·L^{-1} to induce callus, the rate of induction was 97.75%[43]. In this work, we found that TDZ and IBA could affect callus induction. Callus development was greatest and the induction rate was highest (54%) with 3.0 mg·L^{-1} TDZ and 4.0 mg·L^{-1} IBA. Previous studies have demonstrated that PGR have a favorable impact on the development of callus in mangosteen stem explants[44]. The highest rate of callus induction was 75% when root explants generated callus on MS medium containing 2.3 μmol·L^{-1} of 2,4-D and 2.2 μmol·L^{-1} of BA[4]. On MS medium supplied with varied dosages of 2,4-D, NAA, and BAP, explants generated callus at a rate of up to 100%[5].

In addition, several studies have shown that PGR can cause changes in DNA methylation level during PTC[11,45–47]. Treatment of barley callus with 0.5 μmol·L^{-1} brassinolide (BR) led to a slight change in the DNA methylation level compared with the control group[47]. DNA methylation *in vitro* pitaya shoots was sensitive to low concentrations of gibberellin (GA), but the sensitivity of DNA methylation to high concentrations of GA was reduced[45]. Only a few research have looked at how TDZ and IBA affect DNA methylation during plant callus induction. In this process there have also been limited findings on methylation and demethylation. We found that different concentrations of TDZ and IBA during pear callus induction led to differences in DNA methylation. And methylation and demethylation related genes were also different significantly among treatments. When TDZ was 3.0 mg·L^{-1} and IBA was 4.0 mg·L^{-1}, the level of methylation was low (27.96%) and the callus induction rate was maximum (54%). Meanwhile, the methylation-related genes had the highest relative expression, while that of demethylation-related genes was lowest. On the medium supplied with 0.1 mg·L^{-1} 2,4-D and 1.0 mg·L^{-1} 6-BA, *Brassica napus* had the lowest level of methylation and the maximum rate of callus induction (91.0%), demonstrating that hormone function and callus differentiation were connected to methylation level[11]. The induction rate and the level of DNA methylation caused by 6-BA and 2, 4-D were compared, and it was discovered that there were substantial variations in both of these parameters[46]. These conclusions are supported by our experimental data.

The relative expression level of DNA methyltransferase and demethylase genes during callus development triggered by various doses of PGR has not yet been extensively studied. The relative expression level of genes involved with DNA methyltransferase (*MET1*, *CMT3*) in *Brassica napus* callus induced by varied doses of PGR were significantly different[11]. At the same time, many studies have shown that MET1 and DRM2 methyltransferases affect the formation of *Arabidopsis* callus[48]. According to a study by Chen et al., DNA methyltransferase genes (*CMT3*, *DRM2*) and DNA demethylase genes (*ROS1*) may control DNA methylation in the *Dimocarpus longan*[49]. We discovered that the relative levels of expression of DNA methyltransferase genes (*PbMET1*, *PbCMT3*, *PbDRM2*) and DNA demethylase genes (*PbROS1*) were significantly different in callus induced by different PGR. This was similar with the findings of previous research[11,48,49].

In order to comprehend how auxin and cytokinin-related genes are expressed during induction of pear callus. This study analyzed the expression of auxin response genes (*PbGH 3.1-1*, *PbGH 3.1-1*, *PbGH 3.6*), transport genes (*PbPin-likes 3*, *PbPin-likes 5*, *PbPin-likes 6*), cytokinin response genes (*PbARR 6*, *PbAPRR 5*, *PbAHK 2*, *PbAHK 4*) and cytokinin degradation genes (*PbCKX 7*, *PbCKX 7-like*) during callus formation. IBA and TDZ have an effect on auxin biosynthesis. When using various IBA concentrations, the auxin synthesis gene is considerably up-regulated as the concentration increases. It is worth noting that under the treatment of different dosages of TDZ, the auxin synthesis essential genes *PbGH 3.1-1*, *PbGH 3.1-2* and *PbGH 3.6* were significantly up-regulated on the 14 days following with TDZ 3.0 mg·L^{-1} and IBA 4.0 mg·L^{-1}. *PbPin-likes 6* was also considerably down-regulated in terms of transport, demonstrating that auxin is required to participate in the later stage of callus formation. The study of teaplant also confirmed that auxin played a vital role in the formation of callus[50]. The synthesis of cytokinins was more significantly affected by TDZ. Under the treatment of TDZ 3.0 mg·L^{-1} and IBA 4.0 mg·L^{-1}, *PbARR 5* and *PbAHK 4* were significantly up-regulated at 14 days after treatment, indicating that the synthesis and metabolism of cytokinins were mainly affected by TDZ concentration and played an vital role in callus formation[51].

In summary, different combinations of TDZ and IBA concentrations have an effect on the pear callus induction and DNA methylation. Methylation and demethylation occurred simultaneously during pear callus induction. TDZ and IBA affected callus formation through transcriptional regulation. Our study sheds new light on the DNA methylation process of plant callus induction.

ACKNOWLEDGMENTS

The authors acknowledge funding provided by the National Key Research and Development Program of China (2018YFD1000100) and the China Agricultural Research System (CARS-29-07).

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (https://www.maxapress.com/article/doi/10.48130/FruRes-2023-0007)

Dates

Received 6 October 2022; Accepted 20 February 2023; Published online 20 March 2023

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