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EPIGENETIC REGULATION IN DEVELOPMENT OF *PRUNUS PERSICA* DURING REGENERATION USING COTYLEDON EXPLANT

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Abstract

Epigenetic factors recruit gene expression machinery toward organogenesis development during the differentiation process. Epigenetic factors' interaction relationships with Plant growth regulators (PGRs) signaling are focused in this study. Expression profiles of some dedifferentiation and differentiation-distinctive genes were quantified throughout the regeneration process of *Prunus persica* from cotyledons explant. Auxin; indole-butyric acid (IBA) and indole-acetic acid (IAA), and Cytokinin; thidiazuron (TDZ) and 6-benzyladenine (BA) with different combinations were used to test their ability to induce callogenesis and shoot differentiation. TDZ in combination with IAA revealed highest shoot regeneration percentage (70%) followed by combination of BA with IAA and scoring 66%. Our findings demonstrated that methyltransferases-responsive genes are accumulating at extremely low levels during callus formation while they show highest level of expression during shoot formation on both TDZ and IAA, and BA and IAA combinations, and very low expression level on TDZ and IBA (differentiation failure medium). In a similar manner, *DICER-LIKE1* (*DCL*1) displayed high expression levels during the differentiation process on TDZ and IAA and BA and IAA and Extremely Low expression levels during callus formation on all PGRs combinations as well as cotyledons explant. It was observed that the balance between the functions of the two protein groups Polycomb group (PcG), and its counteracting Trithorax group (TrxG) is manipulating plant development process. *SHOOT MERISTEMLESS* (*STM*), a member of Class 1*KNOX* gene family, revealed significant high expression in both dedifferentiated and differentiated calli.

Key words
Prunus persica, Epigenetic, Plant Growth regulators, DNA methyltransferase, Histone methylation, Polycomb group, Trithorax group, miRNA, Expression pattern.

Introduction

Peach (*Prunus persica* L.) is one of the most important fruit in the world (Ravi *et al.*, 2018). Tissue culture technique is described as a significant stressful agent for plant (Rathore *et al*., 2014) and may enhance epigenetic managements in *Prunus persica*. The impact of epigenetic activity in gene expression regulation is increasing intensively through over last years using modern molecular biology protocols (Bemer, and Grossniklaus 2012; de la Paz Sanchez *et al*., 2015). Adventitious shoot regeneration of peach was previously developed using leaf explants (Hassanein and Dorion, 2005; Zhou *et al*., 2010), cotyledons of mature seed explant (Pooler and Scorza, 1995; Padilla *et al*., 2006), immature cotyledon explants (Mante *et al*., 1989 and Pérez-Clemente *et al*., 2004), and nodal segment explants (Pérez-Jiménez *et al*., 2012). Different basal media were used for shoot regeneration in peach, i.e.; Murashige and Skoog medium (Mante *et al*., 1989; Pooler and Scorza, 1995) and woody plant medium (WPM) (Sabbadini *et al*., 2019 and Zong *et al*., 2019). Also, Gentile *et al*., (2002) used a combination of Quoirin and Lepoivre, 1977 medium and Murashige and Skoog medium, 1962. Adventitious shoot regeneration of peach has been previously obtained from the proximal region of cotyledons on TDZ with IBA (Mante *et al*., 1989; Pooler and Scorza, 1995). In addition, shoot regeneration was established from peach leaves (Gentile *et al*., 2002 and Sabbadini *et al*., 2019) and nodal segment explants (Pérez-Jiménez *et al*., 2012) on BA and α-naphthaleneacetic acid (NAA). Recently, Zong *et al*., 2019 developed an efficient shoot regeneration system from leaf explants on BAP and IBA.

Epigenetic activities were previously described during and after exposure to *in vitro* plant regeneration systems (De-la-Peña *et al*., 2012; Yang *et al*., 2013). Epigenetic factors are controlling plant developmental processes by involving in

transcriptome activation or repression (Wójcikowska *et al*., 2020). In this article, some of these significant genes were selected to evaluate their activity and function through both callus formation and differentiation process. *CHROMOMETHYLASE* 3 (*CMT3*), and *DOMAINS REARRANGED METHYLTRANSFERASE* 2 (*DRM2*) are two genes representing methyltransferase (Kankel *et al*., 2003). CMT3 targets methylation of 5′-CNG-3′ while DRM2 methylates CHH (Finnegan and Kovac 2000; Cao and Jacobsen, 2002). In certain cases, DNA methylation changes are inherited by regenerates progeny (Koukalova *et al*., 2005).

Polycomb group proteins (PcGs) is playing the fundamental role in the regulation of many vital processes development like; a transition from gametophyte to sporophyte stage, a transition from vegetative to reproductive stage, and embryogenesis and organogenesis as well (Turck *et al*., 2007; Kim *et al*., 2012). PcG is forming Polycomb Repressive Complexes (PRCs) to drive chromatin modification and gene silencing. *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) is a sole copy gene (Köhler *et al*., 2003) that belongs to the PRC2 family. Heterochromatin Protein 1 (HP1) is the major manager of chromatin function and structure (Canzio *et al*., 2014). *LIKE HETEROCHROMATIN PROTEIN* 1 (*LHP*1) protein interacts with ATRX that regulates the deposition of H3.3 histone in plants (Jackson *et al*., 2002; Mimida *et al*., 2007; Hennig and Derkacheva, 2009). As an antagonistic effect of PcG, PICKLE (PKL) is encoding by *CYTOKININ-HYPERSENSITIVE 2* (*CKH2*) gene. *PKL* is involving in plant hormones; auxin, gibberellin, and ABA signaling pathway in plant and consequently is affecting the developmental process in the plant (Eshed *et al*., 1999; Ori *et al*., 2000; Perruc *et al*., 2007).

Our study highlights Knotted1-like homeobox (KNOX) transcription factors function during *Prunus persica* regeneration. KNOX is the main factor in maintaining shoot apical meristem in the plant (Smith *et al*., 1992). Apical meristem produces the entire aerial part of the vascular plants KNOX influences the plant development and growth controlling hormone homeostasis in the meristem (Vollbrecht *et al*., 2000) as well. One of the most important gene expression regulators is DICER-LIKE1 (DCL1) (Chen, 2010). DCL1 is RNase III-like its function is the process of hairpin-like structure precursor transcripts to mature miRNAs, miRNA is a small 21–22 nucleotides regulatory RNAs (Millar and Waterhouse, 2005; Voinnet, 2009.).

Proliferating cell nuclear antigen (PCNA) is a homotrimer protein that is performing as a processivity element for the DNA polymerase **δ** and is very vital for the replication process in the eukaryotic cells. PCNA is involving in DNA replication and DNA repair, chromatin remodeling and epigenetics (Moldovan *et al*., 2007).

This Study is aiming to explore molecular and epigenetic changes associated with callus induction and shoot differentiation of *P. persica*. Therefore, this study is the first published data that evaluates the activities of DNA methyltransferase and other key regulators genes affecting the developmental process in *P. persica*.

Material and Method

Seeds sterilization and explants preparation

Peach seeds *cv*. Nemaguard was used in this investigation. Seeds sterilization and explants preparation was done as described by San and Yildirim (2009), where seeds shell were cracked by hand, removed from the shell, subsequently, pre-sterilized in 75% Clorox supplemented with two to three drops of Tween 20 for 25 min., then rinsed three times in sterile distilled water. For easy testa removal, sterilized seeds were then incubated for 60 h under shaking condition in sterile distilled water which was replaced every 12 h. Turgid seeds were sterilized once more as previously described. Cotyledon explants were used to carry out regeneration steps (Figure 1).

Fig. 1: Peach seeds preparation: (A) Shelled seed, (B) seeds without testa and (C) Cotyledon explant.

Shoot regeneration and plantlet development

For selecting best callus induction medium, cotyledon explants were cultured onto Petri dishes containing six different media based on either Murashige and Skoog, 1962 or woody plant medium (WPM) with 3 % (w/v) sucrose supplemented with different combinations of TDZ, BA, IAA and IBA according to Perez-Clemente *et al*. (2004). Media were solidified using 2.5g/l phytagel, pH was adjusted at 5.8. Hormone-free medium was used as control (Table 1). Cultures were then incubated in dark for one month in the growth chamber at 25°C ±2 as described by Pooler and Scorza, 1995. For shoot differentiation, produced calli were then sub-cultured onto fresh media based on WPM (No. 5-8, table 1) and incubated for one more month in growth chamber under cool white fluorescent light (16 h light/8 h dark cycle). For shoot elongation, shoots were consequently transferred to WPM supplemented with 1.5 mg/l GA3 and then incubated for one more month as previously mentioned light condition. For selecting the proper medium forming roots, elongated shoots were then transferred on WPM supplemented with 2.5% sucrose, 1.5 mg/l NAA or 2.5 mg/l IBA. For enhancing root formation, root bio-stimulant

commercial product DISPER ROOT™ at a concentration of 0.5 g/l was added to IBA containing medium. Rooted shoots were then acclimatized on pots containing peat moss + perlite (1:1 by volume) in greenhouse.

The expression patterns of epigenetic-regulatory genes

Epigenetic-regulatory factors that have key roles in recruiting the switch action between regeneration phases were selected to be investigated i.e.; methyltransferase genes; *CMT*3&*DRM*2, polycomb group genes; *FIE*2&*LHP*1 and its counteracting Trithorax group genes; *PKL*1, *PCNA* class1 *KNOX*, and Dicer Like *DCL1* gene. WPM media with three PGRs combinations were used for callus induction: dedifferentiation medium 1 (DDM1) containing 1.6 mg/l TDZ&0.5 mg/l IBA: dedifferentiation medium 2 (DDM2) containing 1.6 mg/l TDZ&0.5 mg/l IAA and dedifferentiation medium 3 (DDM3) containing 1.5 mg/l BA&0.5 mg/l IAA. While, the same WPM media with the same three PGRs combinations were used to differentiate shoots from the induced calli: differentiation media1 (DM1) containing 1.6 mg/l TDZ&0.5 mg/l IBA; differentiation media 2 (DM2) containing 1.6 mg/l TDZ & 0.5 mg/l IAA and differentiation media3 (DM3) containing 1.5 mg/l BA&0.5 mg/l IAA.

Total RNA isolation and 1st cDNA synthesis

Tissue samples from each PGRs combination in both callus formation and differentiation phases were collected and immersed immediately on liquid nitrogen. Total RNA was extracted from all tissue samples using SV Total RNA Isolation System (Promega, USA) following the manufacturer's instructions, and the concentration of RNA was evaluated using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Followed by 1% agarose gel electrophoresis with ethidium bromide staining to assess the RNA purity and integrity. First-strand cDNA was achieved in 20µl reaction volume using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Quantitative Real-Time PCR (qRT-PCR) analysis

qRT-PCR reactions were carried out using a Stratagene MX3005P Real-Time System (Agilent, United States) with HERA SYBR® Green qPCR (willowfort, UK) as manufacturer's instructions. The reactions were performed as follows: 95°C for 2min, 40 cycles of 95°C for 7s, 60°C for 1min. Dissociation curves analysis were conducted at 95°C followed by 15s at 65°C and then heating to 95°C (0.5°C/s, with continuous fluorescence measurement) to assess the samples for primer dimers and non-specific targets. 18s was used as a reference control to normalize the expression of target genes (Kondo *et al.*, 2018). A list of selected oligonucleotide primers that were designed using Primer Quest (Integrated DNA Technologies) with the standard parameters is shown in (Table 2). Raw Ct-values data from the MX3005P detection system were exported to Microsoft Excel sheet and relative quantification gene expression levels were calculated using the 2^-ΔΔCT method (Schmittgen and Livak, 2008). The results of three independent biological and technical replicates were analyzed by Duncan's test using SPSS (20.0, IBM) and the results were presented as means ± SE. Furthermore, a Hierarchical Cluster Analysis (HCA) was created using the web server ClustVis (Metsalu and Vilo, 2015).

Media No.	Basal medium	TDZmg/l	BA g/l	IBA mg/l	IAA mg/l
1	MS	0	0	0	0
2	MS	1.6	0	0.5	0
3	MS	1.6	0	0	0.5
4	MS	0	1.5	0	0.5
5	WPM	0	0	0	0
6	WPM	1.6	0	0.5	0
7	WPM	1.6	0	0	0.5
8	WPM	0	1.5	0	0.5

Table 1: Different regeneration medium composition

Results

Shoot regeneration and plantlet development

In this investigation, the regeneration system of *Prunus persica*, Nemaguard was established using cotyledon explant. Two basal medium i.e.; MS and WPM supplemented with different PGRs (IAA, IBA, TDZ, and BA) with different concentrations were used to select best medium for callus formation and shoot regeneration. Hormone-free media based on MS and WPM were used as a control treatment. Results showed that explants on all different MS media did not undergo into callus, while callus was successfully formed on WPM with different combinations of PGRs. Among the three tested WPM media, DDM1 medium was the best for callus induction (33.3%) followed by DDM2 (23.33%) and DDM3 (10%) (Figure 2).

Subsequently, calli were sub-cultured on the same medium under the light condition for one more month for shoot differentiation. Callus sub-cultured on DM1 medium containing 1.6 mg/l TDZ and 0.5 mg/l IBA combination failed to perform shoot differentiation. While the calli on DM2 and DM3 containing IAA 0.5 mg/l and 1.6 mg/l TDZ or 1.5 mg/l BA succeeded to develop into shoots with a percentage of 71 and 66%, respectively.

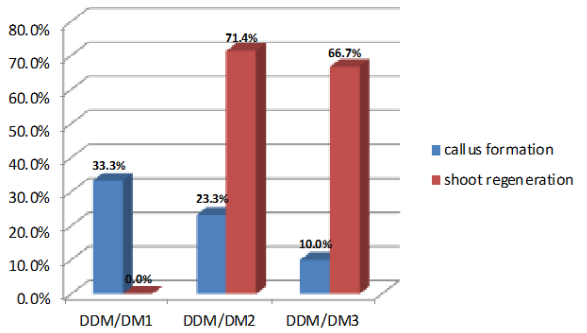

Fig. 2: Percentage of callus developed and shoots formation on different WPM media composition

Obtained shoots were elongated on the WPM medium supplemented with 1.5 mg/l GA3 for one month. Shoots for about 2-3 cm heights were then rooted on root formation medium. Shoots failed to produce any roots on medium containing 1.5 mg/l NAA, however, succeeded in forming short roots on 2.5 mg/l IBA medium with a percentage of 25%. To enhance root formation, 0.5 g/l bio-stimulant commercial product DISPER ROOT™ was added to IBA-medium. Results demonstrated that DISPER ROOT enhances the root formation as it gave 40% root formation.

Thereafter, rooted shoots were acclimatized under greenhouse condition. Regeneration steps were illustrated in figure (3).

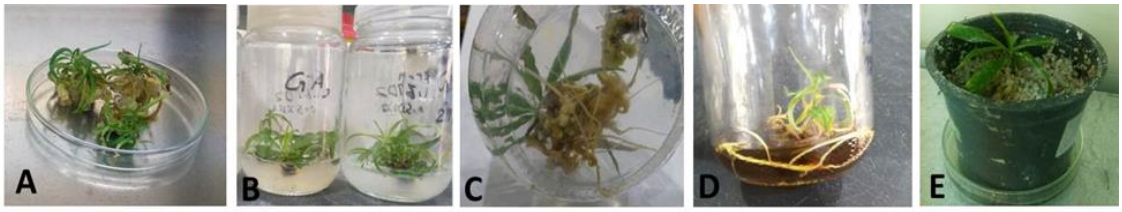

Fig. 3: Regeneration system of peach cv. Nemagurd: A). Adventitious shoot developed of obtained callus , B) Shoot elongation, C) Root formation on IBA-medium, D) root formation on IBA-medium supplemented with DisperRoot and E) Acclimatization under greenhouse conditions

Table 1: List of the primers used in qPCR

	Gene name	NCBI reference	Sequences	Amplicon length (bp)	Tm (°C)
Pp_PCNA-F	Proliferating Cell Nuclear Antigen	XM_007205663.2	CCTTCACAAAAGCGACCAC	118	86.5
Pp_PCNA-R			GGAGCCAAGTAGAACCTGATG		
Pp_FIE2-F	Fertilization Independent Endosperm 2	XM_007211395.2	CTACAATCCAGCCCTCCTGT	130	84.5
Pp_FIE2-R			GCCAAATAGTCCCATCCTCA		
Pp_PKL-F	Pickle	XM_020567165.1	TTAGACCCTGACCCAGAAGAG	129	81.3
Pp_PKL-R			TCCCACACCATTTCCAGAG		
Pp_DRM2-F	Domains Rearranged Methyltransferase 2	XM_007200985.2	TTGAACTGCGTGCTAACCTC	165	80.5
Pp_DRM2-R			TGGGGCAACCTTATTTCTTC		
Pp_CMT3-F	Chromomethylase 3	XM_020565570.1	TCTGACCTTCCTGCTGTTGA	220	79.3
Pp_CMT3-R			CACCCTTTCTCTTCGGGATT		
Pp_LHP1-F	Like Heterochromatin Protein 1	XM_007219400.2	GAAGTCTGGTTCTGTGAAGAGG	126	81.0
Pp_LHP1-R			TTTTCTGCCCCTGATTGATT		
Pp_STM-F	Shoot Meristemless	GQ281774.1	CCTACTGCGAGATGCTGACT	120	82.0
Pp_STM-R			CTGAGGAAGAATGAACTGTGAGA		
Pp_DCL1-F	Dicer-Like 1	XM_020569133.1	TGCTTCCTGATGTGATTGGA	177	83.4
Pp_DCL1-R			CCCTGGTCTCACTTGGTTGT		

Gene expression analysis

Dynamic patterns of epigenetic change and chromatin remodeling during the *in vitro* regeneration process of *Prunus persica* from cotyledon explant under PGRs signals were investigated in this study. Some important key-marker genes related to methylation and chromatin remodeling were selected for their stability and movability during the processes. *CMT*3, *DRM*2, *PCNA*, *FIE*2, *LHP*1, *PKL*, *KNOX* are integrated with chromatin modifier while *DCL*1 is a post transcription modifier.

***CMT*3&*DRM*2 expression patterns**

The two methyltransferases genes were quantified throughout the regeneration process. Very low expression levels were observed during callus formation on DDM1, DDM2, and DDM3 media with folds change of 0.3, 0.1, and 0.3 respectively for *CMT*3, and 0.7, 0.6, and 0.6 respectively for DRM2. Although *CMT*3 was significantly increased during shoot differentiation on DM2, and DM3 media by 6.5 and 1.1 folds respectively, it displayed a low expression level on DM1 by 0.3 fold. The Expression behavior of *DRM*2 was somehow similar to that of *CMT*3 as it showed significantly increased during shoot differentiated on DM2 and DM3 by 2 and 1.4 folds respectively and very low expression on DM1 by 0.4 fold. The lowest expression level for *CMT*3&*DRM*2 was detected on cotyledon explant showing 0.6 fold as shown in (Figure 4A&B).

***FIE*2&*LHP*1&*PKL*1 expression patterns**

*FIE*2 and *LHP*1 are two genes belonging to PcG proteins that are repressing gene expression by either trimethylation, or monoubiquitination respectively leading to chromatin condensation. While their counteracting TrxG proteins proposed to switch on gene expression**.** *FIE*2 expression levels were moderately high on dedifferentiated callus media; DDM1, DDM2, and DDM3 by 1.7, 1.4, and 1.5 folds respectively. On the other hand, the expression level of *LHP*1 exhibited enormous elevation during callus formation on DDM1 and DDM3 media compared to DDM2 medium by 10, 9, and 2.8 folds respectively. Whereas during the differentiation process *FIE*2 expression was significantly higher on differentiation DM3 medium compared to DM2, and DM1 media by 3.8, 2, and 1 folds respectively, and the cotyledon was 1.4-fold change. However, LHP1 expression level during the differentiation stage was moderately expressed on DM1 and DM3 and low expressed on DM2 by 1.6, 1.6, and 0.7 folds respectively as shown in (Figure 4C&D).

PKL belongs to TrxG was highly expressed during the callus stage on DDM1, and DDM3 media than on DDM2 media by 6, 5, and 2 folds respectively. In contrast, its expression level during the differentiation on DM2 medium was significantly higher than on DM1, and DM3 media by 5, 1, and 1.3 fold respectively and it expressed in a moderate level in cotyledon by 3 fold as shown in (Figure 4E).

***PCNA* expression pattern**

Similar to *CMT*3, *DRM*2, and *FIE*2, the expression patterns of *PCNA* were significantly high on, DM1, DM2, and DM3 media by 4, 2.5, and 6.6 folds respectively. On the contrary, its expression levels were decreased on DDM1, DDM2, and DDM3 media by 0.9, 0.25, and 0.8 folds respectively (Fig. 4F).

***STM* expression pattern**

The expression patterns of *STM*, the KNOX1-related gene, exhibited stable expression levels during callus formation and shoot differentiation stages. Its expression level was 0.7, 1.5, 0.8, 0.9, 0.6, and 1.2 folds on DDM1, DDM2, DDM3, DM1, DM2, and DM3 respectively. Whereas, its expression level exhibited a drastic decrease in cotyledon by 0.11-fold change (Figure 4G).

***DCL*1 expression pattern**

The expression level *DCL*1 exhibited high expression level during the differentiation stage on DM3 and DM2 by 2.4, and 1.4 folds respectively but its expression was markedly decreased under DM1 by 0.16 fold (Figure 4H).

Schematic diagram showing ***FIE*2&*LHP*1&*STM*** genes interaction during peach regeneration steps (Figure 5).

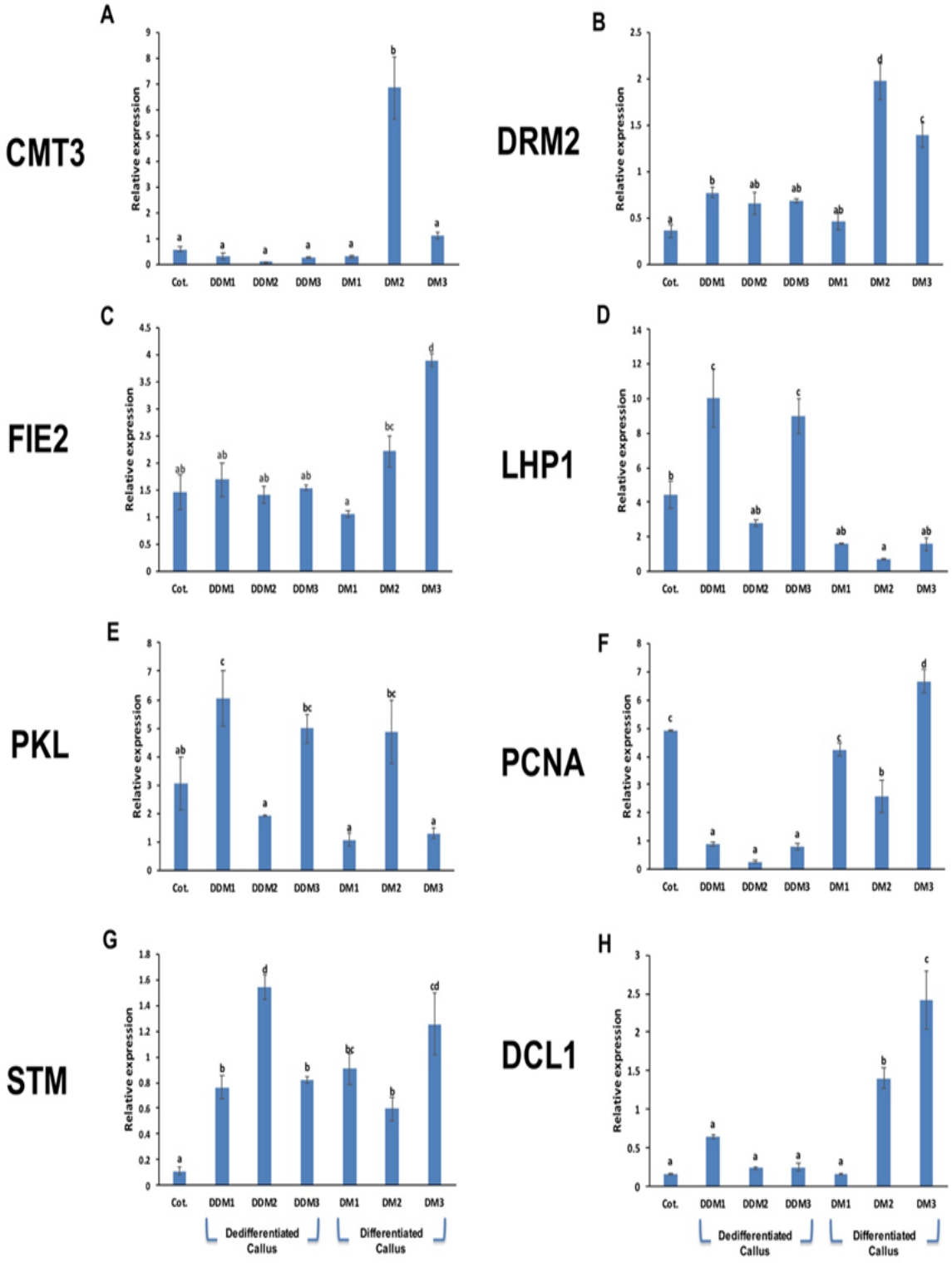

Fig. 4: Relative Expression analysis of chromatin modifier genes; (**a**)CMT3,(**b**)DRM2, (**c)** FIE2, (**d)** LHP1,(**e)** PKL,(**f)** PCNA, (**g)** KNOX, and (**h)** DCL1 in dedifferentiation and differentiation stages under three different combinations Duncan's multiple range tests and is represented at ($P \leq 0.05$).

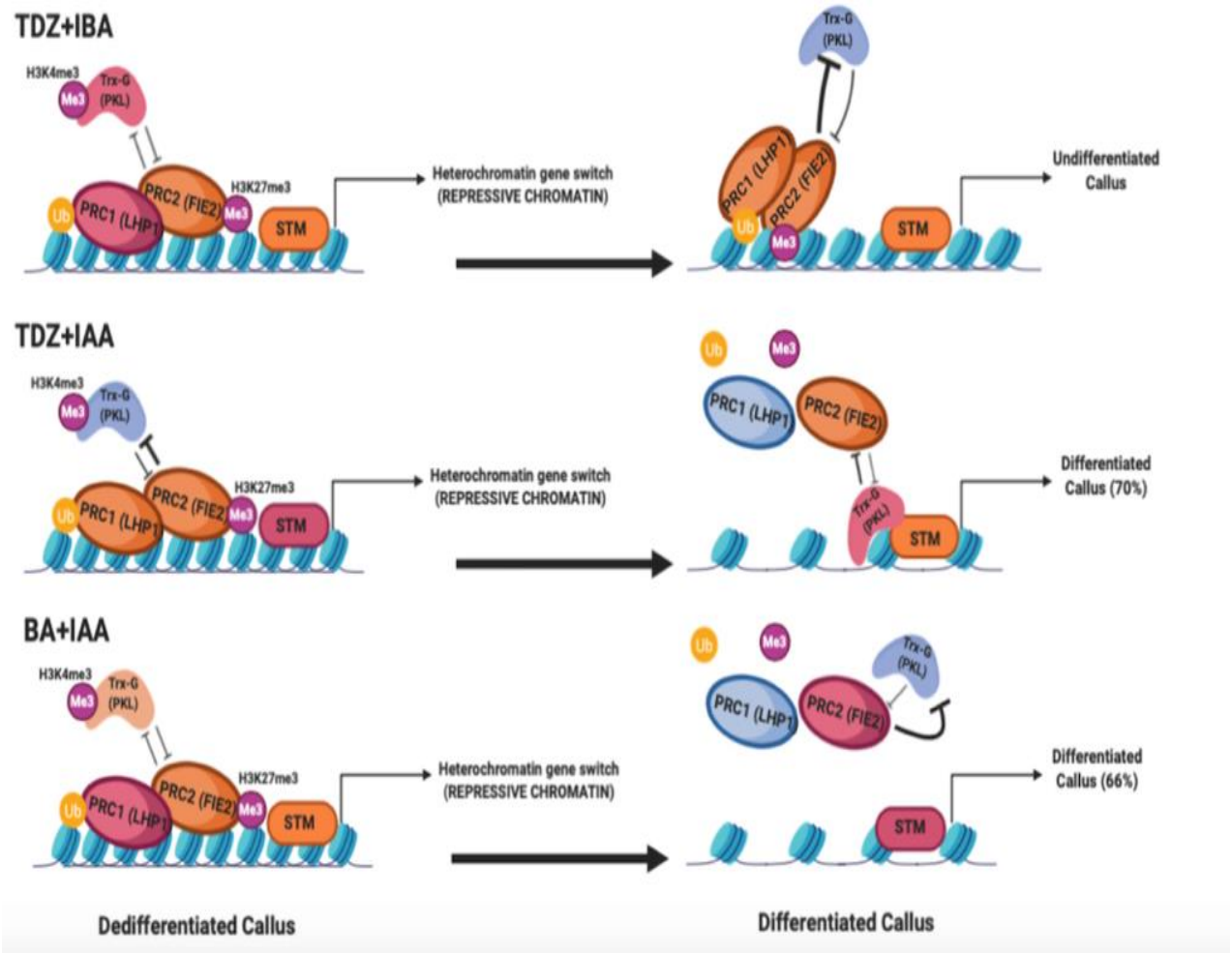

Fig. 5: Schematic diagram is showing the role of some chromatin modifiers genes in chromatin during the dedifferentiation and differentiation stages under three different combinations of callus induction media (TDZ+IBA, TDZ+IAA, and BA+IAA). The Red, orange, and blue colors represent high moderate and low moderate respectively.

Hierarchical Cluster Analysis (HCA)

The dynamics of epigenetic-integrated genes that were investigated in this study were subjected to hierarchical cluster analysis (HCA). Considering the status of the tissues under the different media conditions, the two main branches of the hierarchical clusters separated the Cotyledon, DDM1, DDM2, DDM3, and DM1 tissue status, on the one side, from DM2 and DM3, on the other side. Generally, the most changes in gene expression occurred on DM2 and DM3 differentiation media, and the expression patterns of DRM2, CMT3, and PKL on DM2 were higher than DM3. On the contrary, the expression patterns of DCL, FIE2, STM, and PCNA on DM3 were higher than DM2 and the expression patterns of LHP1 was down-regulated on DM2 and DM3 as well (Figure 6).

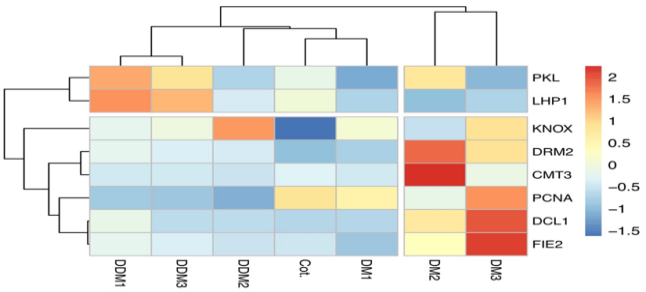

Fig. 6: Hierarchical clustering of the differentially expressed genes among the different treatments. The red color represents up-regulation and grey is for down-regulation.

DISCUSSION

In vitro culture is a crucial tool in all plant science aspects especially modern biotechnology and in commercial and industrial production as well. Plant tissue culture is considered a main step in pathogen free plant production (Taskin *et al*., 2013; Rajasekharan and Sahijram, 2015). It can participate effectively in the rescue process of threatened plant species (Sarasan *et al*., 2006). Explants re-programming

during the regeneration process can drive physiological, morphological, and biochemical modifications. Otherwise, the somaclonal variation is a well-known term in tissue including transposons activation, DNA methylation, gene expression modifications, chromatin remodeling, and a wide spectrum of RNAs interference (Li *et al*., 2012; Ikeuchi *et al*. 2015, Han *et al*. 2018, Lee and Seo 2018, Kabita *et al*., 2019). PGRs supplemented to regeneration media along with both oxidative and physical stresses during this process can drive both genetic and epigenetic modifications. Several factors are affecting adventitious shoot regeneration production in peach-like; explant types; basal salts type; and PGRS combinations and concentration (Zong *et al*., 2019). In the present investigation, cotyledon derived from mature seeds as explants were used in the regeneration process of peach *cv*. Nemagurd. Cotyledon explants from mature seed were successfully used by Pooler & Scorza (1995); and Padilla *et al*. (2006) who found that cotyledons had the highest transformation rate in peach. Our results indicated that the WPM medium has a positive effect on callus induction of peach while MS medium gave negative results. Likewise, Zong *et al*., (2019), and Sabbadini *et al*., (2019) found that the WPM medium was the proper basal salt medium for developing shoot regeneration in peach. Otherwise, Mante *et al*. (1989); Pooler and Scorza, (1995), and Perez-Jime´nez *et al*. (2012) succeed to regenerate peach shoots on MS basal medium. It was observed that combinations of TDZ (1.6 mg/l) with IBA (0.5 mg/l) or with IAA (0.5mg/l) and combination of BA (1.5 mg/l) with IAA (0.5mg/l) were successful to induce callus with percentage of 33.3, 23.3, and 10, respectively. However, shoots were developed on both combination of TDZ with IAA, and BA with IAA with a percentage of 71 and 66 respectively. While the shoot failed to differentiate on medium with TDZ and IBA. In contrarily, the highest shoot regeneration of peach from cotyledon explants has been obtained on medium containing TDZ with and IBA by Mante *et al*. (1989); Pooler and Scorza (1995). In addition, Perez-Clemente *et al*. (2004) found that the presence of TDZ with IAA in the regeneration system of peach using immature cotyledon explants gave low regeneration percentage (8.5 %.). A method for adventitious shoot regeneration from leaves of micro-propagated peach was reported by Gentile *et al*., (2002) on a medium supplemented with BA and NAA, then transferred to an auxin-free medium. On the other hand, peach was regenerated from callus derived from the nodal segment explants (Pérez-Jiménez *et al*., 2012) on a medium containing BA and NAA. Zong *et al*., (2019) developed an efficient shoot regeneration system of Hansen 536 (*Prunus dulcis x Prunus persica*) from leaf explants on a medium containing BAP and IBA.

This study is focusing on a precise quantification of some genes' transcripts that are affecting callus formation and differentiation process throughout regeneration steps. Cotyledons explant of *Prunus persica* were exposed to different PGRs combinations to evaluate their ability to induce callus formation and shoot differentiation. Different combinations of cytokinin TDZ&BA and auxin IBA&IAA auxin were used and each gene expression was estimated on the three stages; cotyledon, dedifferentiation, and differentiation stage.

The expression pattern of the two methyltransferases; *CMT*3, and *DRM*2 were determined to test their activity during the regeneration process. Both of *CMT*3 and *DRM*2 was suggested to control the expressions of some genes responsible for developmental switch processes (Cao and Jacobsen, 2002 and Jullien, et al., 2012). Our data indicated that *DRM*2 and *CMT*3 were expressed on a low-level during callus stimulation on medium containing TDZ and IBA, TDZ and IAA, and BA and IAA combinations. In a similar manner, Huang *et al*. (2012) proved that methyltransferases-responsive genes are expressed in intensely low-level during the early stages of *Malus xiaojinensis* regeneration. While *CMT*3 expressed the higher level followed by *DRM*2 during differentiation and shoot regeneration on the differentiation medium with TDZ and IAA followed by the medium with BA and IAA. Our result presented that the TDZ in combination with the IAA was essential for the highest shoot regeneration frequency that reaches 70% followed by the combination of the BA and IAA with a shoot regeneration frequency of 66%. This result enables us to suggest that methyltransferase genes can be an excellent candidate to enhance epigenetic variations during the differentiation process under TDZ and IAA and BA and IAA estimation. This data is partially concordance with Taskin *et al*. (2015) who proposed that BdCMT3 plays a significant role during stress condition derived by callus formation and shoot regeneration. In a similar manner, Li *et al*. (2014) proved the incidence of the high expression level of *CMT*3 in *B. oleracea* during the tissue culture process. The expression levels of DNA methyltransferase were extensively affected by PGRs supplemented on tissue culture media (Huang *et al*., 2012). Thus we referred to the extremely low expression level of *CMT*3 followed by *DRM*2 on TDZ and IBA (differentiation failure medium) is due to the fact that the cultured cells tend to remove epigenetic markers when they preserve totipotency potential (Neelakandan and Wang, 2012).

In a similar manner like *DM*3 and *DRM*2, *DCL*1, the processor of miRNA precursor, displayed significantly higher expression levels during shoot differentiation on TDZ and IAA and BA and IAA than on TDZ and IBA (the differentiate failure media). And extremely low expression levels were recorded during callus formation on all PGRs combinations as well as cotyledons explant. In the beginning, it was supposed that miRNAs are contributing in homologous transcript cleavage *via* ARGONAUTE1; however, miRNAs were proved to act as a repressor of the transcription process and directed DNA methylation in the plant (Brodersen *et al*.,

2008; Chellappan *et al*., 2010). We supposed *DCL*1 elevating level under the differentiation stage is due to increase bulk-miRNAs transcription during shoot formation. Hence, our data demonstrated that *DCL*1 is essential for plant development regulation in agreement with (Poethig, 2009) and (Sunkar, 2010) who proposed that miRNA is participating in physiological regulation as well as stress tolerance in the plant.

Plant development is controlled by the two counteracting groups; TrxG and PcG proteins. PcGs are transcription repressors involved in chromatin remodeling and histone methylation. PRC1 directs Histone H2A lysine monoubiquitination while RPC2 directs the spatial and temporal expression of many genes by mediating repression through the trimethylation of H3 histone at lysine 27 (H3K27me3) (Kim *et al*., 2012; Horst *et al*., 2016). The Performance of these functions *via* Polycomb Repressive Complexes (PRCs) is leading to chromatin condensation (Molitor and Shen, 2013 and Mozgova and Hennig, 2015). *LHP*1, the PRC1 related protein, is interacting with PRC2 in distinct pathways to perform a specific repression function. *LHP*1-PRC2 interaction is mediating the transcription suppression process during cell division (Liu *et al*., 2009; Rizzardi *et al*., 2011; Veluchamy *et al*., 2016; Wei *et al*., 2017). On the other hand, *FIE*2 core is a PRC2 related gene that has an ultimate function in developmental regulation through the plant life cycle and is highly conserved during the evolution of the plant (Butenko and Ohad, 2011; Derkacheva and Hennig, 2014). The accumulation patterns of *FIE*2 and *LHP*1 expression levels were identified by qPCR approach under PGRS-controlling callus and shoot formations. Interestingly, both *FIE2* and *LHP1* exhibited high expression but *LHP1* showed the enormously highest accumulation than *FIE*2 and the other chromatin remodeling genes; *CMT*3, *DRM*2, *PKL*, and *PCNA* during dedifferentiated calli stage on all of PGRs combination media. This result was an indicator that *LHP*1 activity is enhanced during the callus development process. *FIE*2 but not *LHP1* mRNAs exhibited extensively accumulating in differentiated tissue growing on BA and IAA followed by TDZ and IAA medium then TDZ and IBA (the differentiation failure medium). So, we suggested that BA has a positive feedback on elevating *FIE2* expression level during the differentiation process and shoot formation. This result was in agreement with the fact that FIE contributes in vital regulating both leaves and flower development in the vegetative phase (Kinoshita *et al*., 2001 and Chanvivattana *et al*., 2004). In contrast, *LHP*1 showed lower expression levels during the differentiated stage on all PGRs combination than *FIE*2. We conclude the continuous high expression levels of FIE2 during all phases of tissue culture protocol (dedifferentiated and differentiated calli) are due to the vital contribution of FIE2 in regulating various genes involved in the developmental process. Our conclusion is confirmed by Zhang *et al*. (2007) and Bouyer *et al*. (2011), they supposed that about from 20% to 35% of Arabidopsis genes are hypothetically regulated by PRCs complexes.

PKL is a member of TrxG group that catalyzes H3K4me3, the histone methylation mark. In an adverse function of PcG, TrxG is proposed to switch on gene expression and keep them active (Schuettengruber *et al*., 2011) PcG and TrxG complexes interact in either an antagonistic or cooperative mode depending on the developmental phase. PKL is involving in gene expression regulation, stress response controlling, and cell differentiation in the plant as well (Hollender and Liu, 2008; Kubo and Kakimoto, 2001). On the other hand, *PKL* mRNA level in callus was higher in TDZ and IBA and BA and IAA than in TDZ and IAA. In contrast with differentiated tissue, *PKL* mRNA level was higher by treatment with TDZ and IAA than by treatment with TDZ and IBA and BA and IAA. This result indicates that elevating and decreasing of *PKL* mRNA level is greatly affected by the incidence of auxin signal.

One of the most particular genes in the plant developmental process is *SHOOT MERISTEMLESS* (*STM*) that belongs to the class 1*KNOX* gene family. Since *STM* is required for the sustainable function of shoot apical meristem (SAM) and formation of *de novo* meristem (Scofield *et al*., 2014), it was expected to up-regulate throughout all steps of the regeneration process. In fact, *STM* function is accomplished by the induction of cytokinin synthesis to inhibit cellular differentiation and enhancing the cells to retain self-sustaining meristem status (San and Yildirim 2009). The author showed that *STM* expression was significantly higher during callus formation and shoot differentiation than in cotyledons explant confirming our prospect. The highest expression level of *STM* has attained during callus formation on TDZ and IAA assumingly that combination has a positive feedback on *STM* function to keep on the callus fate status. Moreover, the considerably high expression level during differentiation on the medium failed to undergo the differentiation process (TDZ and IBA) confirms this fact as well. On the other hand, the high *STM* expression during the differentiation process on TDZ and IAA and BA and IAA combinations is due to the maintenance of STM role during the organogenesis process in SAM development (Long *et al*., 1996 and Clark *et al*., 1996). This data enables us to propose that STM has a persistence role during the plant-developmental process.

Conclusion

In this study, an overlook of how stressing effects during the regeneration process may influence the epigenetic machinery under PGRs signaling was described. Moreover, the role of some essential genes controlling the plant-developmental process was presented**.** This research is focusing on analyzing the accumulation patterns of eight epigenetic genes in both dedifferentiation and differentiation

status using the qPCR approach. Expression accumulation of some essential endogenous chromatin modifier genes (*DRM*2, *CMT*3, *FIE*2, *LHP*1, *PKL*, *DCL*1, and *PCNA*), and the differentiation interacting gene (*STM*) were estimated during callus formation and shoot differentiation under PGRs signals. This expression analysis of the key regulators genes was an excellent indicator of gene activity changes during the switch phase from dedifferentiation status to differentiation status.

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