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) & indole-acetic acid (IAA) and Cytokinin; thidiazuron (TDZ) & 6-benzyladenine (BA) with different combinations were used to test their ability to induce callus formation and shoot differentiation. TDZ in combination with the IAA revealed the highest shoot regeneration percentage as it was 70% followed by the combination of the BA & IAA with a shoot regeneration frequency of 66%. Our findings demonstrated that methyl transferases-responsive genes are accumulating at extremely low levels during callus formation while they expressed at a higher level during shoot formation on both TDZ & IAA and BA & IAA combinations and very low level on TDZ & IBA (differentiation failure medium). In a similar manner, DICER-LIKE1 (DCL1) displayed higher expression levels during the differentiation process on TDZ & IAA and BA & IAA and extremely low expression levels during callus formation on all PGRs combinations as well as cotyledons explant. We showed that the balance between the functions of the two protein groups Polycomb group (PcG) and its counteracting Trithorax group (TrxG) is manipulating the plant development process. SHOOT MERISTEMLESS (STM), a member of Class 1KNOX gene family, revealed significant high expression in both dedifferentiated and differentiated calli.

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

Introduction

The peach (Prunus persica L.) is the major commercial stone fruit of the world (Scorza and Sherman, 1996). Tissue culture technique is described as a significant stressful agent for the 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 year’s 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 the nodal segment explants (Pérez-Jiménez et al., 2012). Different basal media were used for adventitious shoot regeneration of peach, i.e, MS salts (Murashige and Skoog 1962; Mante et al., 1989; Pooler and Scorza 1995) and woody plant
medium (WPM) (Sabbadini et al., 2019; Zong et al., 2019). Gentile et al., (2002) used a combination of LP Salts (Quoirin and Lepoivre, 1977) and MS salts. 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 from peach leaves (Gentile et al., 2002 and Sabbadini et al., 2019) and nodal segment explants (Pérez-Jiménez et al., 2012) on BA and ±-naphthaleneacetic acid (NAA) (Zong et al., 2019) was developed as 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 & 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 (LHP1) 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 homebox (KNOX) transcription factors function during Prunus persica regeneration. As it 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 and is very vital for the replication process in the eukaryotic cells. PCNA is involving in DNA replication & DNA repair, chromatin remodeling & epigenetics (Moldovan et al., 2007).

This Study is aiming to explore molecular and epigenetic changes associated with callus and shoot enhancing protocols during tissue culture steps 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.

Materials and Methods

Seeds sterilization, preparation and Explants preparation

Peach seeds cv. Nemaguard was used in this investigation. Seeds shell were cracked by hand, removed from the shell and pre-sterilized by immersion in 75% Clorox solution supplemented with two to three drops of Tween 20 for 25 min. Followed by rinsing three times for 5 min in sterile distilled water Fig. 1. Then, sterilized seeds were incubated in sterile distilled water for 60 h under shaking condition at 17p C for breaking dormancy. For easy removal of testa by replacing the water every 12 h. Turgid seeds were sterilized again as described previously (San and Yildirim 2009). Cotyledon explants were used to carry out regeneration steps.

Shoot regeneration and plant let development

For selecting the best callus formation medium, cotyledon explants cultured on Petri dishes containing six different media based on either MS (Murashige and Skoog, 1962) or woody plant medium (WPM) with 3 % (w/v) sucrose and 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 to 5.8. Hormone-free medium was used as control table 1. Each treatment has 5 plates contain 6 explants/plate, with a total number of 30 explants. Cultures were incubated at 25°C in the dark as described by (Pooler and Scorza, 1995) for one month.

Produced callus was then sub-cultured to WPM fresh media and incubated for one more month at the light condition for shoot initiation.

To elongate the produced shoots, they were transferred to WPM supplemented with 1.5 mg/l GA3 and incubated for one month at the light.

For selecting the proper medium forming roots, elongated shoots were 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 the medium containing IBA.

Rooted shoots were then acclimatized on pots containing Petmos: perlite equal volume under greenhouse condition.

The expression patterns of epigenetic-regulatory genes

Some of the epigenetic-regulatory factors that have key roles in recruit the switch action between regeneration phases were selected to be investigated in this study; methyltransferase genes; CMT3 & DRM2, polycomb group genes; FIE2 & LHP1 and its counteracting Trithorax group genes; PKL1, PCNA class1 KNOX and Dicer Like DCL1 gene. WPM media with three PGRs combinations were used to induce callus formation from peach cotyledon explants; dedifferentiation media1 (DDM1 contains; 1.6 mg/l TDZ & 0.5mg/l IBA), dedifferentiation media2 (DDM2 contains; 1.6 mg/l TDZ & 0.5 mg/l IAA) and dedifferentiation media3 (DDM3 contains; 1.5 mg/l BA & 0.5 mg/l IAA). While the WPM media with the same three PGRs combinations were used to induce shoot differentiation from the induced callus; differentiation media1 (DM1 contains; 1.6 mg/l TDZ & 0.5 mg/l IBA), differentiation media2 (DM2 contains; 1.5 mg/l TDZ & 0.5 mg/l IAA) and differentiation media3 (DM3 contains; 1.5 mg/l BA & 0.5 mg/l IAA).

Total RNA isolation and 1st cDNA synthesis

Tissue samples collected 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 Nano Drop 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 20¼l reaction volume using Revert Aid 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>
<thead>
<tr>
<th>Media No.</th>
<th>Basal Medium</th>
<th>TDZ mg/l</th>
<th>BA g/l</th>
<th>IBA mg/l</th>
<th>IAA mg/l</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>MS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>MS</td>
<td>1.6</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>MS</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>MS</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>WPM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>WPM</td>
<td>1.6</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>WPM</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>WPM</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>0.5</td>
</tr>
</tbody>
</table>
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 $2^{-\Delta\Delta CT}$ 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).

Results

Shoot regeneration and plantlet development

In this investigation, the regeneration system of *Prunus persica*, Nemaguard peach was established using cotyledon as explant. Two basal mediums; MS and WPM supplemented with different PGRs (IAA, IBA, TDZ and BA) with different concentrations to select the best regeneration medium for callus formation and shoot differentiation. Hormone-free MS and WPM were used as a control. Results showed that explants on all different MS media did not undergo callus formation while callus was successfully formed on WPM with different combinations of PGRs. Among the three WPM media, DDM1 medium was the best for callus induction (33.3%) followed by DDM2 (23.33%) and DDM3 (10%) Fig. 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 & 0.5 mg/l IBA combination failed to perform shoot differentiation. While the calli on DM2 and DM3 containing IAA 0.5 mg/l & 1.6 mg/l TDZ or 1.5 mg/l BA succeeded to develop into shoots with a percentage of 71 and 66%, respectively.

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 root 0.5 g/l bio-stimulant commercial product DISPER ROOT™ with a concentration of was added to IBA-medium. Results demonstrated that DISPER ROOT enhances the root formation as it gave a 40% root formation.

Thereafter, rooted shoots were acclimatized under greenhouse condition. Regeneration steps are illustrated in Fig. 3.

Gene expression analysis

![Fig. 2: Percentage of callus developed and shoots formation on different WPM media composition.](image)

Table 2: List of the primers used in qPCR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>NCBI reference</th>
<th>Sequences</th>
<th>Amplicon length (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pp_PCNA-F</td>
<td>Proliferating Cell Nuclear Antigen</td>
<td>XM_007205663.2</td>
<td>CCTTCACAAAGGAGCCACC</td>
<td>118</td>
<td>86.5</td>
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<tr>
<td>Pp_PCNA-R</td>
<td></td>
<td></td>
<td>GGAGCAAGTGAACCTGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp_FIE2-F</td>
<td>Fertilization Independent Endosperm 2</td>
<td>XM_007211395.2</td>
<td>CTACAAATCCAGCCCCCTGTT</td>
<td>130</td>
<td>84.5</td>
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<tr>
<td>Pp_FIE2-R</td>
<td></td>
<td></td>
<td>GCCAAATAGTCCCCATCCCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp_PKL-R</td>
<td>Pickle</td>
<td>XM_020567165.1</td>
<td>TTAGACCCTGACCCAGAAGAG</td>
<td>129</td>
<td>81.3</td>
</tr>
<tr>
<td>Pp_PKL-R</td>
<td></td>
<td></td>
<td>TCCCCACACATTCCAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp_DRM2-F</td>
<td>Domains Rearranged Methyltransferase 2</td>
<td>XM_007200985.2</td>
<td>TTGAACTGCGTCTAACCTC</td>
<td>165</td>
<td>80.5</td>
</tr>
<tr>
<td>Pp_DRM2-R</td>
<td></td>
<td></td>
<td>TGGGGCAACCTATTTCCTC</td>
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<tr>
<td>Pp_CMT3-F</td>
<td>Chromomethylase 3</td>
<td>XM_020565570.1</td>
<td>TCTGACCTTCCCCCTGTTGTA</td>
<td>220</td>
<td>79.3</td>
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<td>Pp_CMT3-R</td>
<td></td>
<td></td>
<td>CACCCCTTCTTGGGGGATT</td>
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<td></td>
</tr>
<tr>
<td>Pp_LHP1-F</td>
<td>Like Heterochromatin Protein 1</td>
<td>XM_007219400.2</td>
<td>GAAGTCTGGTCTGCTGGAGG</td>
<td>126</td>
<td>81.0</td>
</tr>
<tr>
<td>Pp_LHP1-R</td>
<td></td>
<td></td>
<td>TTTTGCCCTGGATT</td>
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<td></td>
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<tr>
<td>Pp_STM-F</td>
<td>ShootMeristemless</td>
<td>GQ281774.1</td>
<td>CCTACTGAGATGGCTGCCCT</td>
<td>120</td>
<td>82.0</td>
</tr>
<tr>
<td>Pp_STM-R</td>
<td></td>
<td></td>
<td>CTGAGGAAAGAATGACGTGTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp_DCL1-F</td>
<td>Dicer-Like 1</td>
<td>XM_020569133.1</td>
<td>TGCTTCCCTGATGTTGGA</td>
<td>177</td>
<td>83.4</td>
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<tr>
<td>Pp_DCL1-R</td>
<td></td>
<td></td>
<td>CCCTGTTCTACCTTGTTG</td>
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</tbody>
</table>
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. *CMT3, DRM2, PCNA, FIE2, LHP1, PKL, KNOX* are integrated with chromatin modifier while *DCL1* is a post transcription modifier.

**CMT3 & DRM2 expression patterns**

The two methyl transferases 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 *CMT3* and 0.7, 0.6 and 0.6 respectively for *DRM2*. Although *CMT3* 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 *DRM2* was somehow similar to that of *CMT3* 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 *CMT3* & *DRM2* was detected on cotyledon explant showing 0.6 fold as shown in Fig. 4A & B.

**FIE2 & LHP1 & PKL1 expression patterns**

*FIE2* & *LHP1* 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. *FIE2* 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 *LHP1* 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 *FIE2* 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 & DM2 and low expressed on DM2 by 1.6, 1.6 and 0.7 folds respectively as shown in Fig. 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 DM2 media by 5, 1 and 1.3 fold respectively and it expressed in a moderate level in cotyledon by 3 fold as shown in Fig. 4E.

**PCNA expression pattern**

Similar to *CMT3, DRM2* and *FIE2*, the expression patterns of *PCNA* were significantly high on, MD1, MD2 and MD3 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 Fig. 4G.

**DCL1 expression pattern**

The expression level *DCL1* 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 Fig. 4H.

Schematic diagram showing *FIE2 & LHP1 & STM* genes interaction during peach regeneration steps Fig. 5.

**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 Fig. 6.

Discussion

In vitro tissue culture is a crucial tool in all plant science aspects especially modern biotechnology and in commercial and industrial production as well. In vitro
Epigenetic regulation in development of *prunus persica* during regeneration using cotyledon explant plants, tissue culture is enhancing the breeding of health crops by eliminating various viruses (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 & 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 (0.5mg/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 × 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 methyl transferases; CMT3 and DRM2 were determined to test their activity during the regeneration process. Both of CMT3 and DRM2 was suggested to control the expressions of some genes responsible for developmentals witch processes (Cao and Jacobsen, 2002 and Jullien, et al., 2012). Our data indicated that DRM2 and CMT3 were expressed on a low-level during callus stimulation on medium containing TDZ & IBA, TDZ & IAA and BA & 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 CMT3 expressed the higher level followed by DRM2 during differentiation and shoot regeneration on the differentiation medium with TDZ & IAA followed by the medium with BA & 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 & 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 & IAA and BA & 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 CMT3 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 CMT3 followed by DRM2 on TDZ & 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 DM3 and DRM2, DCL1, the processor of miRNA precursor, displayed significantly higher expression levels during shoot differentiation on TDZ & IAA and BA & IAA than on TDZ & 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 DCL1 elevating level under the differentiation stage is due to increase bulk-miRNAs transcription during shoot formation. Hence, our data demonstrated that DCL1 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). LHP1, the PRC1 related protein, is interacting with PRC2 in distinct pathways to perform a specific repression function. LHP1-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, FIE2 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 FIE2 and LHP1 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 FIE2 and the other chromatin remodeling genes; CMT3, DRM2, PKL and PCNA during dedifferentiated calli stage on all of PGRs combination media. This result was an indicator that LHP1 activity is enhanced during the callus development process. FIE2 but not LHP1 mRNAs exhibited extensively accumulating in differentiated tissue growing on BA & IAA followed by TDZ & IAA medium then TDZ & 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, LHP1 showed lower expression levels during the differentiated stage on all PGRs combination than FIE2. 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 Arabidops is 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 & IBA and BA & IAA than in TDZ & IAA. In contrast with differentiated tissue, PKL mRNA level was higher by treatment with TDZ & IAA than by treatment with TDZ & IBA and BA & 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 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 show differentiation than in cotyledons explant confirming our prospect. The highest expression level of STM has attained during callus formation on TDZ & IAA assuming 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 & IBA) confirms this fact as well. On the other hand, the high STM expression during the differentiation process on TDZ & IAA and BA & 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 stressful effects during the regeneration process may influence the epigenetic machinery under PGRs signaling is described. Moreover, the role of some essential genes controlling the plant-developmental process was presented. We are focusing on analyzing the accumulation patterns of those genes in both dedifferentiation and differentiation status using the q PCR approach. Expression accumulation of some essential endogenous chromatin modifier genes (DRM2, CMT3, FIE2, LHP1, PKL, DCL1 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 during the switch phase change from dedifferentiation status to differentiation status.

References


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