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The Influence of Photoperiod on the Regulation of Root and Callus Initiation of Perle Noir (*V. vinifera* L.): Expression of MADS-Box Gene

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Abstract

To study the influence of photoperiod on roots differentiation in the Tunisian grapevine (*Vitis vinifera* L.) cultivar Perle noir, roots and callus initiation were analyzed under three different conditions of day length: long day (LD), short day (SD) and darkness (D). The photoperiod influenced the number of callus and roots per cuttings; it has a significant effect on the roots and callus initiation. Expression profile analysis of six MADS-box genes (*VTM*8, *VSEP*2, *VAG*12, *VAG*17-1, *VAG*17-2 and *VSOC*1.3) during root and callus development is in agreement with the above-mentioned observation. The expression of the MADS-box genes during root and callus development fluctuated in a tissue-dependent manner. These data suggest that all genes are expressed in roots under three photoperiods. Total darkness gives the number of the most important root per cutting compared to the other two conditions. This photoperiodic condition gave the most important expression of the studied genes *VAG*12, *VAG*17-2, *VAG*17-1, *VTM*8 and *VSEP*2 transcripts were not found in callus grown in the dark or in LD conditions, respectively. *VSOC*1.3 transcripts were not found in callus grown in the dark or in SD conditions, respectively. Transcript abundance of *VTM*8 and *VSOC*1 was highest in LD.

Keywords

Grapevine, Root, Photoperiod, MADS-Box Genes, Callus, *Vitis vinifera* L.

1. Introduction

Certain shoot and root cells can be activated to produce a new one, each with a new meristem at its tip. The

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number and location of this organ are not predetermined. Therefore, each plant can integrate information from its environment into the decision about root and shout formation. Plants are dependent on the resources that are available in their immediate vicinity. Nutriment availability and distribution are in constant flux in the environment. Plants must be able to sense these changes and respond appropriately. The presence of active stem cell population and the ability to generate new ones allow the plant to adapt its morphology to its unique and changeable environment [1]. The formation of the lateral roots in the root system provides a good model for studying how plant development is coordinated with environment. For *Arabidopsis*, lateral roots originate from mature non-dividing pericycle cells arrested in the G2 phase of cell cycle [2]. The primordium then enlarges through the parent tissues to develop a meristem at the tip, and begins to grow as a mature lateral root [3]. The first visible event in lateral root is a series of anticlinal divisions in the root pericycle [4] [5]. The passage from pericycle cell to lateral root is unknown. Some studies suggest that specific pericycle cell exists. This suggests that environmental conditions influence lateral root formation [6]. Auxins play a central role and may interact with endogenous factors stimuli such as light [7]. Author [8] chooses two classes of *Arabidopsis thaliana* mutants altered in adventitious root formation. The super root mutant spontaneously makes adventitious roots and the argaunote 1 (Ago 1) mutants, which unlike super root is barely able to form adventitious roots. The defect in adventitious root observed in Ago 1 is correlated with light hypersensitivity and the deregulation of auxin homeostasis specifically in the apical part of the seedlings. The authors conclude that adventitious roots are regulated by GH3 genes and therefore perturbing auxin homeostasis in a light-dependent manner. Light may intervene to control adventitious root of *Arabidopsis*. Plants must be able to sense fluxing in grapevine that were notably absent through the 20th century [9]. Most of the information on adventitious formation in *Vitis spesis*, their hybrids, and rootstocks has been proprietary [10]. Grape Canes (*Vitis vinifera* L) does not contain preformed adventitious root primordia [11]. Favre (1973) [12] was the first to study the histological detail of adventitious root formation in *Vitis* canes, and identify the initial step as the appearance of "swelling" or hypertropic nuclei within clusters of cells of the interfascicular cambium. The second stage was identified as the initiation of periclinal divisions among the cambium cells followed by the third stage identified as the organization of morphogenetic fields. The formation of adventitious root is correlated with the convergence of multiple environmental and endogenous factors like internal compounds type PGRs auxin, cytokinin, spermine, spermidine; carbohydrates sugar as well as genetic background [13] [14]. Plants use light as a source of information to optimally adapt growth and development to the ambient environment. The most prominent process regulated by photoperiod is the flowering induction, but photoperiod has also been reported to control a wide variety of other developmental processes, including stem elongation, bud dormancy, axillary branching, leaf growth, and the formation of storage organs [15]. MADS-box genes encode transcription factors, the expression analysis of these genes reveals a close link with the grapevine growth and development process. In grapevine, Expression of MADS-box genes has been detected in reproductive and vegetative organs [16]. Expression of *VAGL*12 was detected in roots and fruits and during flower development. The expression level of *VAGL*12 and *VAGL*17 [17] [18] suggested their role in vegetative development, which had later been evidenced for some of them in root development [19] [20]. Expression of VAGL17-1 and VAGL17-2 was detected in roots and during reproductive development in grapevine and *Arabidopsis* [17] [18]. Here, we report our studies detailing the influence of the photoperiod on the regulation of root initiation of Perle noir cultivar (*V. vinifera* L.). We were further interested in understanding of the genetic and molecular control of root initiation in the grapevine.

2. Material and Methods

2.1. Plant Material

Grapevine *V. vinifera* L. cv. Perle noir grafted onto the rootstocks of Richter 99 resulting from a mass selection in Tunisia was collected in January in the vineyards of GOVPF (Groupement Obligatoire Des Viticulteurs et Producteurs De Fruits).The vineyards are located in the region of Belli in the Governorate of Nabeul (latitude 10˚25', longitude 36˚37', 20 meters above sea level). Fertilization and further manipulations in the vineyard were performed according to commercial standards. Cuttings were collected at the proximal part of the cane and limited to three nodes, successively named N0 (the proximal), N1 and N2. The length of the cuttings was standardized to 21 cm, the thickness was approximately 10 mm, and only the eighth bud (when counting from the base) was retained. A thousand cuttings were directly put after the collect in 1.5-L beakers containing tap water and transferred to a culture chamber with a constant temperature of 25˚C.

2.2. Growth Conditions

The canes were kept in three different photoperiods with an irradiance of 10,000 $\mu E \cdot m^{-2} \cdot s^{-1}$: 8 h light/16 h dark (Short Day), 16 h light/8 h dark (Long Day) and continuous darkness (Darkness). One hundred cuttings were used in each day length. Buds reaching the stage of budburst (T0) were recorded every day. The plant material was divided into two parts: the first was reserved for physiology studies and the second for molecular studies. Roots and callus were counted and collected 40 days after budburst. Statistical analyses were carried out using ANOVA and the LSD test to determine the effect of photoperiod on the number of roots and the number of calli per cuttings. All statistical analyses were performed using 100 samples.

2.3. Analysis of Transcript Level

RNA Extraction

Total RNA was isolated from approximately 100 g of roots and callus frozen tissue powder using the SpectrumTM Plant Total RNA Kit (Sigma-Aldrich Switzerland located in Buchs, St Gallen, Switzerland) according to the manufacturer's instructions. The quality and quantity of the total RNA prepared was determined by spectrophotometer analysis. The RNA was diluted in distilled water and measured the absorbance at 260, 280 and 320 nm. One µg of RNA was treated with RNase-free DNase (Fermentas) according to the manufacturer's instructions, one unit of the enzyme completely degrades 1 µg of DNA in 10 min at 37˚C, and the remaining RNA was reverse transcribed using an oligo dT primer and the Invitrogen Reverse Transcriptase. Total RNA (1 mg) was reverse transcribed in a reaction mixture of 20 ml containing PCR buffer (Invitrogen), 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 20 units of RNase inhibitor, 50 units of reverse transcriptase (Invitrogen), 2.5 mM oligo(dT)18 and diethyl pyrocarbonate-treated water. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed as follows. Diluted cDNA (2 µL) were used for qRT-PCR reactions to detect *VTM*8, *VSEP*2, *VAG*12, *VAG*17-1, *VAG*17-2 and *VSOC*1.3. Each experiment included three technical replicates for each three biological replicates for each photoperiod. Transcript levels were determined by qRT-PCR using the 7300 Real-Time PCR System (Bio-Rad) and SYBR Green dye (Bio-Rad). Reactions were performed in a final volume of 15 µl containing 7.5 µl of 23 Power SYBR Green PCR Master Mix, 333 nM of forward and reverse specific primers and a 1:10 dilution of cDNA. After enzyme activation at 95˚C for 10 min, amplification was carried out in a two-step PCR procedure with 40 cycles of 15 s at 95˚C for denaturation and 1 min at 60˚C for annealing/extension Transcript levels were calculated using the standard curve method and normalized against the grapevine *εF*-1*α* gene for expression analyses (**Table 1**).

Table 1. List of primers used for RT PCR *εF*-1*α* was chosen as a reference gene.

Gene	Primers	Annealing Temperature (˚C)
*VTM*8	5'GTCTAAACATGGGTGGGACTT3' 5'CATCCAAGGTGGGAGGGATA3'	55
*VSEP*2	5'CTTCAATAACCAACGGGAAGA3' 5'TCTCAAACTAATGGCACACAGT3'	55
*VAG*12	5'GTTTGTTGTGTGCTCCAAGC3' 5'CGAAATGAATGTATGGGCAAG3'	55
*VAG*17.1	5'TAATTTAGTAGGTGATGCTGCTGCT3 5'AGATGGAGTATATGTGGTTGGTAGA3'	55
*VAG*17.2	5'CTTCAATAACCAACGGGAAGA3' 5'TCTCAAACTAATGGCACACAGT3'	55
*SOC*1.3	5'GTTCATAGGACCACCAGAAAGA3' 5'GCTAGTGCTGCTGTTAGACT3'	55
εF-1α	5'-GAACGTTGCTGTGAAGGATCTC-3' 5'-CGCCTGTCAACCTTGGTCAGTA-3'	55

3. Results

Our experiment shows that some cuttings give calls, others give both callus and roots and further give only callus (**Figure 1**).

3.1. Effect of Photoperiod on the Number of Roots

The numbers of roots in the grapevine cv. Perle noir fluctuated depending upon the photoperiod. The two statistical analysis including and excluding the cuttings without roots showed that there were significant differences between cuttings grown under D and LD conditions and between D and SD conditions respectively (**Table 2**) but there were no significant differences between cuttings grown under SD and LD conditions. Darkness conditions induce an increase of root number per cutting.

3.2. Effect of Photoperiod on the Number of Callus

We investigated the effect of photoperiod on the numbers of callus in each of the cuttings in this cultivar. Long day conditions induced an increase in callus number; it was significantly higher in cuttings grown in LD conditions than in cuttings grown in SD and D conditions respectively (**Table 2**). We showed that the highest number of roots per cuttings for Perle noir cultivar was obtained in the total darkness. The SD and the LD conditions has a median number per equivalent cuttings root. The percentage of rooting is also most important in total darkness conditions (**Table 2**).

3.3. Analysis of MADS-BOX Genes

Quantitative RT-PCR was used to determine the expression level of MADS-box genes in roots upon changes in photoperiod.

3.3.1. Analysis of *VTM*8, *VSEP*2, *VAG*12, *VAG*17-1, *VAG*17-2, and *VSOC*1.3 Transcripts in Roots

MADS-box genes expression level in roots is according with the results of Riquelme *et al*. 2009 [16] which studied vine chromosome genes and their implications in different organs. The transcriptional expression levels of these genes fluctuated differently in each tissue depending on the photoperiod. The expression of all genes was higher in D than in SD or LD conditions (**Figure 2**). The highest expression was found in the total darkness. It was 10.5 times higher for *VAG*17-2, 10.5 times for *VSEP*2, 9 times for *VAG*17-1, 6.9 times for *VAG*12, 10 times

Figure 1. Roots and callus obtained in our experience.

Table 2. Number of root and callus per cutting under three photoperiod conditions.

	Darkness	Short Day	Long Day
Number root per cutting	8.20 ± 1.33	5.21 ± 0.91	5.13 ± 1.17
Number callus per cutting	5.20 ± 0.83	3.95 ± 0.95	6.05 ± 0.83
% rooting	71	52	56
% calling	32	28	38

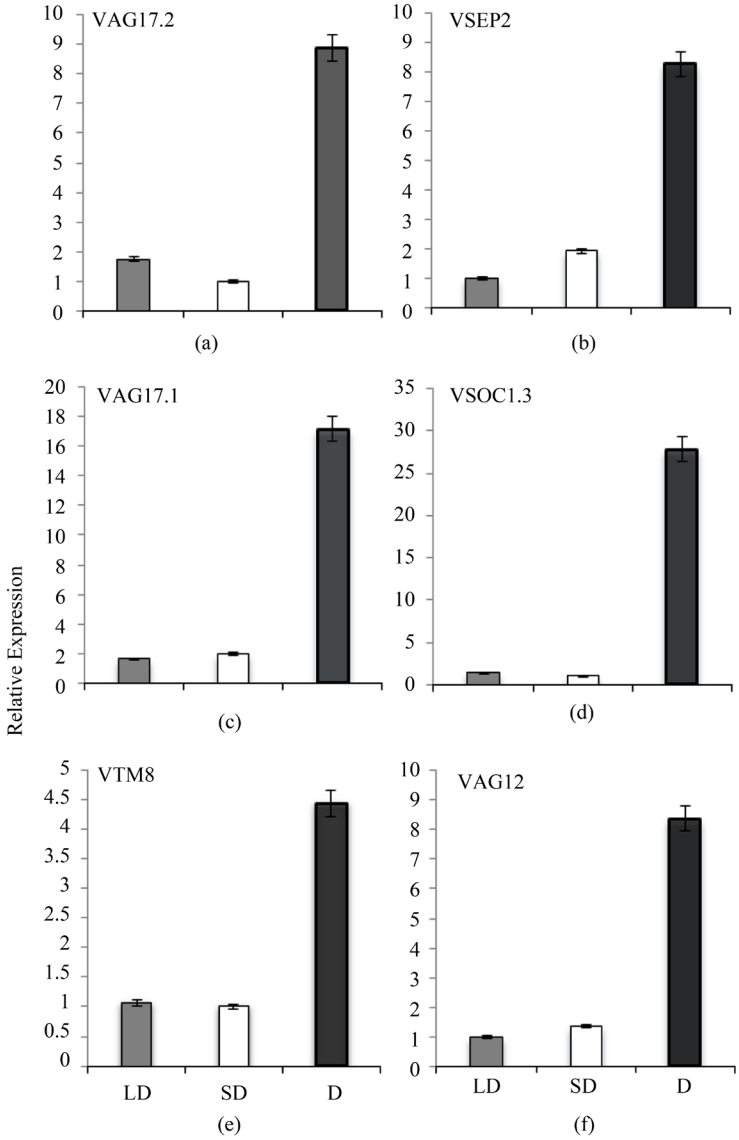

Figure 2. Expression of *VAG*17-2, *VSEP*2, *VAG*17-1, *VSOC*1.3, *VTM*8 and *VAG*12, and during root development under LD, SD and D conditions. The relative expression of the genes was estimated by quantitative RT PCR analysis using *εF*-1*α* as a reference gene. Error bars = SD of three PCR reaction. Each bar represents measurement from 3 technical replicates for each three biological replicates ± SEM.

for *VSOC*-3, 4.5 times for *VTM*8 than in the SD and LD conditions. The light limits the expression of these genes but not in the same manner. Transcripts of *VAG*17-2 were detected in roots in all three photoperiods. Compared to SD, the switch to LD resulted in an increase in the expression of this transcription factor ((**Figure 2(a)**). The transcript abundance of *VSOC*1.3 was highest in roots grown in dark (**Figure 2(d)**). Moreover, the expression levels of *VAG*12 mRNA observed was two times higher in LD grown cuttings than the SD grown cuttings (**Figure 2(f)**). Whereas, the transcript abundance of *VSEP*2 was two times higher in SD grown cuttings than in LD grown cuttings (**Figure 2(b)**).

3.3.2. Analysis of *VTM*8, *VSEP*2, *VAG*12, *VAG*17-1, *VAG*17-2, and *VSOC*1.3 Transcripts in Callus

The expression level was also analyzed in the callus under the three photoperiods. The gene expression level of

these genes fluctuates differently depending on the photoperiod (**Figure 3**). Transcripts of *VAG*17.1, *VTM*8 and *VSOC*1.3 were detected only in the callus grown in LD condition (**Figure 3(a)**, **Figure 3(d)**), it indicate that these genes are maybe specifically associated with callus development. The expression of *VAG*17.1 was important in Darkness and LD condition, it was two times higher in D (favorable condition of roots) grown cuttings than in LD (favorable condition of callus) grown cuttings and almost undetectable in SD grown cuttings (**Figure 3(b)**). The transcript levels of *VAG*17.2 mRNA were observed only in SD grown cuttings (**Figure 3(c)**). Transcript abundance of *VAG*12 and *VSEP*2 was highest in Darkness (optimum condition of the roots) than the SD and almost undetectable in LD (**Figure 3(e)** and **Figure 3(f)**).

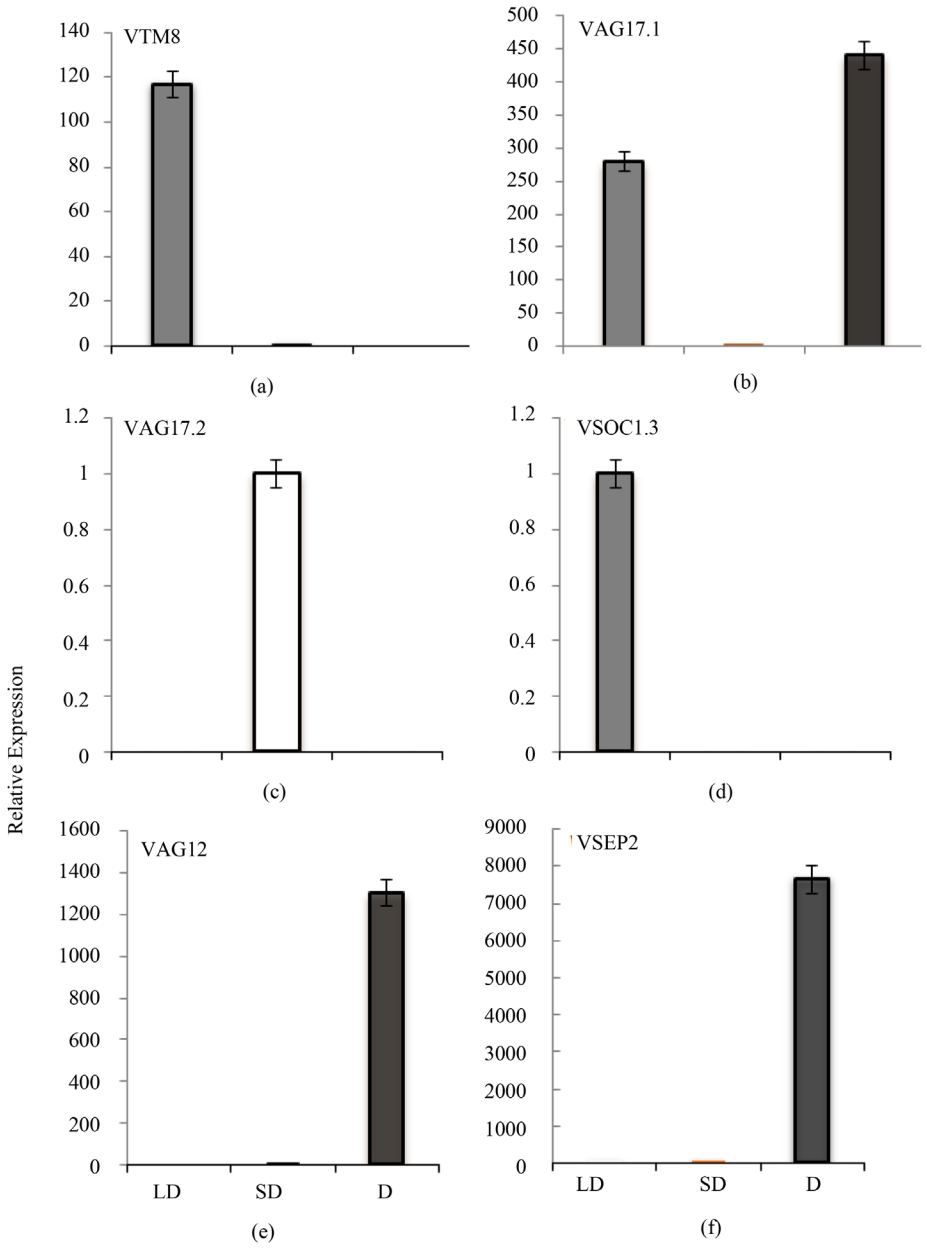

Figure 3. Expression of *VTM*8, *VAG*17-1, *AG*17-2, *VSOC*1.3, *VAG*12 and *VSEP*2, during call development under LD, SD and D conditions. The relative expression of the genes was estimated by quantitative RT PCR analysis using *εF*-1*α* as a reference gene. Error bars = SD of three PCR reaction. Each bar represents measurement from 3 technical replicates for each three biological replicates ± SEM.

Light is an important factor it increase or decrease the expression of the MADS-box genes. The expression for the calluses is 27 times greater for *VAG*17-1 that the roots, both *VAG*12 156 and 882 times higher for *VSEP*2 in total darkness that the roots. The intensity of the expression of *VTM*8 is 118 times for the callus that the roots and it is 138 times for *VAG*17-1 in long day. The intensity is almost identical for *VAG*17-2 call and *VAG*17-2 root short day. The order of magnitude is not the same; there is much in favor of callus for the roots. On the other hand expression presents a significant variability in total darkness dependent genes. The intensity of expression is as important in long day but never equaled optimal expression obtained in total darkness. In short day it is almost identical. We have seen that the genes studied are expressed roots. Their maximum values are obtained in total darkness. Three of these genes are also expressed in the callus, and also have maximum values at total darkness which is similar to the roots. There is a gene that has the maximum value in total darkness and the maximum value along the day condition for optimal expression calluses. Through these results we can hypothesize the common origin of roots and calluses. But the discrepancy is found mostly in non-expression of the three genes in complete darkness, the important expression of two genes in long day and another in short day. The physiological conditions of expressing roots is identical and reproducible for the 6 genes studied, the fact is with the cal. Added to this the very high values obtained only in the calluses. In the roots, the studied genes are expressed independently of photoperiod used. The intensity of the expression is always up to total darkness for callus, the intensity of the expression is generally much larger than in roots. Unlike the roots, the expression is absent *VTM*8 and *VSOC*1-3 in short day and total darkness. It is also absent for the gene *VAG*17.1 short day. For VAG17-2 gene expression is absent long day and total darkness. The expression is absent in long day and short day for *VAG*12 and *VSEP*2. If for roots, photoperiod conditions influence gene expression are reproducible, it is not the same for the calluses which actually poses the problem of similarity of these organs.

4. Discussion

The highest number of roots per cuttings for Perle noir cultivar was obtained in the total darkness. The SD and the LD conditions have a median number per equivalent cuttings root. The percentage of root is also most important in total darkness conditions than the LD and SD conditions. The MADS-box genes selected from the study of Riquelme *et al.* 2009 [16] were found in the roots of Perle noir cultivar, which supported the results of the authors mentioned above. Our results show that the intensity of the gene transcripts is variable with the photoperiod and they have a maximum value in the total darkness. The expression of the six genes is in the same direction as the physiological result namely that the number of root is most important in the total darkness.

The highest number of callus was found under LD conditions, the level expression of the studied genes in root fluctuated differently in callus. *VTM8* and *VSOC*1.3 gene have the highest expression in the LD and are probably associated with the physiological response and give the most important the number of callus. On a fundamental level, the study of the influence of the photoperiod on the number of roots and callus of the cuttings of Perle noir cultivar should be compared with the results obtained by Husen 2008 [21].

Husen 2008 [21] found the highest number of roots per bud under the total darkness conditions; on the other hand, the long day gives the highest number of callus per bud. Our results show that the expression level of 6 MADS-box gene studied in the roots goes in the same direction as the physiological results. For callus, the results would give the particular importance for genes *VTM*8 and *VSOC*1-3.

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