Transcriptomic analysis of *Medicago truncatula* calli with *MtWOX9-1* overexpression

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Somatic embryogenesis (SE) is the development of embryo-like structures from somatic plant tissues. This process rarely can be observed in nature, but for many plant species, in vitro protocols are developed, which allow to obtain somatic embryos formation directly from tissues of plant explant or from the embryogenic callus. SE is widely used for plant propagation and transformation; therefore, the search for SE stimulators and revealing of the mechanisms of their functioning are very important for biotechnology. Among the SE regulators, proteins of the WOX family play significant roles. WOX (WUSCHEL-RELATED HOMEOBOX) is a homeodomain-containing transcription factor family. Different WOX genes function in different plant organs and tissues, maintaining meristem activity and regulating cell proliferation and differentiation. Recently, we have shown that transcription factor *MtWOX9-1*, belonging to the WOX family, can stimulate SE in the *Medicago truncatula* callus culture. In this research, transcriptomic analysis of highly embryogenic calli with *MtWOX9-1* overexpression was performed in comparison to wildtype calli. It was shown that *MtWOX9-1* overexpression led to the activation of several groups of genes, including genes related to cell division, tissue differentiation, and seed development. Enriched GO pathways included several groups related to histone methyltransferase activity as well as DNA methylation and chromatin binding, suggesting major epigenetic changes that occur in call overexpressing *MtWOX9-1*. Using Medicago Truncatula Gene Expression Atlas, we also identified a group of genes coding for transcription factors that were both coexpressed with *MtWOX9-1* in different plant organs and differentially expressed in our samples. These genes are putative targets of *MtWOX9-1*, and they may act in the same pathway with this regulator during SE.

Key words: somatic embryogenesis; *Medicago truncatula*; plant regeneration; transcription factors; transcriptomic analysis.

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Транскриптомный анализ каллусов *Medicago truncatula* со сверхэкспрессией гена *MtWOX9-1*

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Соматический эмбриогенез (СЭ) – это развитие зародышеподобных структур из соматических тканей растений. Этот процесс редко можно наблюдать в природе, однако для многих видов растений разработаны протоколы культивирования в условиях in vitro, с помощью которых можно добиться формирования соматических эмбрионов напрямую из тканей растительного экспланта или из эмбриогенного каллуса. СЭ широко применяют в биотехнологии для размножения и трансформации растений, и в связи с этим поиск стимуляторов СЭ изучение механизмов их работы представляют собой актуальную задачу. Белки WOX играют важную роль в регуляции СЭ. WOX (WUSCHEL-RELATED HOMEOBOX) – семейство гомеодомен-содержащих регуляторов СЭ и изучение механизмов их работы представляют собой актуальную задачу. Белки WOX играют важную роль в регуляции СЭ. WOX (WUSCHEL-RELATED HOMEOBOX) – семейство гомеодомен-содержащих транскрипционных факторов. Различные гены WOX функционируют в разных органах и тканях растений, поддерживая активность меристем и регулируя пролиферацию и дифференцировку клеток. Ранее нами было обнаружено, что транскрипционный фактор *MtWOX9-1*, принадлежащий к семейству WOX, способен стимулировать соматический эмбриогенез в каллусной культуре у *Medicago truncatula*. В настоящем исследовании проведен сравнительный анализ транскриптома высокосоматических каллусов со сверхэкспрессией гена *MtWOX9-1* и транскриптома каллусов дикого типа. Показано, что сверхэкспрессия *MtWOX9-1* вызывает активацию нескольких групп генов, включая гены, связанные с делением клеток, дифференцировкой тканей, а также с развитием семян. Среди обогащенных наборов генов в терминах GO мы обнаружили несколько групп с активностью метилтрансфераз гистонов, метилированием ДНК и связыванием с хроматином, что предполагает существенные эпigenетические изменения, происходящие в каллусах со сверхэкспрессией *MtWOX9-1*. Используя базу данных Medicago Truncatula Gene Expression Atlas, мы идентифицировали также группу генов, кодирующих транскрипционные факторы, которые козэкспрессируются с *MtWOX9-1* в различ-
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Introduction

Somatic embryogenesis (SE) is a process of regeneration by which plants use somatic cells to grow embryo-like structures, which eventually can give rise to the new plant. This process isn’t observed often in nature, but when plant explants are cultivated in vitro, several factors can induce direct SE or SE from callus tissue. Such factors include specific hormones, the concentration of nitrogen compounds (Reinert et al., 1967), the stress impact (Nic-Can et al., 2016), etc. Most of existing methods of SE induction in vitro include treatment with hormones and mechanical injury, and it is supposed that SE acts as the mechanism of defense against stressful in vitro conditions.

Somatic embryo development occurs in general through the same stages as development of zygotic embryo, and therefore it is used as the model for studying embryogenesis. SE also has a lot of biotechnological applications in transformation of plants, artificial seeds production and micropropagation.

The way by which embryogenic cells are chosen among explant or callus cells is not fully investigated. In Medicago truncatula, one of SE model objects, somatic embryos are often derived from mesophyll cells near the damaged surface, which have to dedifferentiate, but some are derived from the stem-like vascular procambium cells (Wang et al., 2011; Rose, 2019). Auxin gradients are shown to play key role in determination of cells that will give rise to the embryo (Su et al., 2009).

Dedifferentiating cell, which will be capable to form somatic embryo later, undergoes a number of changes, including mitochondrial fusion and increase in peroxisomes (Tiew et al., 2015) and P-bodies (RNA processing bodies) numbers (Bhullar et al., 2017). The first one is used to provide a kind of quality control for mitochondrial populations in new generations (Rose, McCurdy, 2017). The second one is a kind of stress response, whereas the third one plays role in posttranscriptional gene regulation and cell reprogramming.

Genetic cascade that induces dedifferentiation of cell and development of somatic embryo is not fully uncovered. At the present time correlation with SE has been established for LEAFY COTYLEDON1 (LEC1), BABY BOOM (BBM), AGL15, SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) and other genes (Fehr, 2015). Among the regulators of SE, proteins of WOX family also play important roles. WOX (WUSCHEL-RELATED HOMEOBOX) is a homeodomain-containing transcription factors (TFs) family. Different WOX genes function in different plant organs and tissues, maintaining meristem activity and regulating cell proliferation and differentiation.

The most well-studied family members are WUS and WOX5 genes, which are expressed in the organizing and quiescent center cells of the shoot and root apical meristem (SAM and RAM), respectively, and regulate their development (Laux et al., 1996; Sarkar et al., 2007). The mechanism of WUS action in stem cell niche is related to stimulation of cytokinins activity, by which it represses the differentiation of SAM cells (Leibfried et al., 2005). WUS is also expressed in the floral meristem, where it stimulates AGAMOUS expression, providing termination of floral meristem activity (Lenhard et al., 2001).

WOX5 is functional analog of WUS gene in root. It is expressed in the quiescent center in the root apical meristem (Sarkar et al., 2007) and in different irregular meristems, such as nodule meristems (Ospiova et al., 2012) and meristem-like structures of agro bacterial and spontaneous tumors (Lebedeva et al., 2015; Vinogradova et al., 2015).

The participation in zygotic and somatic embryogenesis was demonstrated for many WOX family genes. For example, WUS is an important stimulator of SE in different species, such as Arabidopsis thaliana (Su et al., 2009), Capsicum chinense (Solis-Ramos et al., 2009) and Gossypium hirsutum (Bouchabké-Coussa et al., 2013; Xiao et al., 2018). WOX5 is also involved in SE, participating in RAM development in somatic embryos (Su et al., 2015). Expression of WOX1 and WOX3 homologs was observed during SE process in different objects, such as C. chinense (Valle-Gough et al., 2015), Vitis vinifera (Gambino et al., 2011) and Picea abies (Alvarez et al., 2015). The WOX11 and WOX12 genes are expressed in the early stages of callus and adventitious roots development in Arabidopsis, stimulating the cambium cells proliferation (Liu et al., 2014). The expression of WOX11 homolog during SE was shown in V. vinifera (Gambino et al., 2011).

The genes WOX2, WOX8, and WOX9 play an important role in the zygotic embryogenesis, defining the differentiation of specific embryo domains: apical (WOX2), central (WOX9) and basal (WOX8) (Breuninger et al., 2008). In A. thaliana, WOX2 and WOX8 expression was detected in egg cell, though the Nicotiana tabacum homologs of these genes were shown to be de novo transcribed in zygote right after fertilization (Zhou et al., 2018). The WOX2 homolog in Larix decidua is expressed during early embryogenesis, both somatic and zygotic (Rupps et al., 2016). The WOX9 homologs are microspore embryogenesis markers in Brassica napus (Malik et al., 2007) and SE markers in V. vinifera (Gambino et al., 2011). Besides, expression of the WOX9 homolog MtWOX9-like was demonstrated during SE in M. truncatula (Kurdyukov et al., 2014).

In our previous studies, three new M. truncatula genes of the WOX family, that are expressed during SE, were found: MtWOX9-1, MtWOX11-like and STENOFOLIA (MtWOX1) (Tvorogova et al., 2015). It was further shown that overexpression of STF or MtWOX9-1 stimulates the emergence of somatic embryos (Tvorogova et al., 2016, 2019). In the present work, we concentrated on studying the functions of the MtWOX9-1 gene, analyzing how its overexpression affects the expression profile of embryogenic callus.

Materials and methods

Plant growth, cultivation and sample collection. M. truncatula line R-108 (Hoffmann et al., 1997) and transgenic line with MtWOX9-1 overexpression were used in the analysis.
Line with MtWOX9-1 overexpression, obtained through ar-gobacterial transformation of R-108 line plants with pMDC32 vector containing MtWOX9-1 coding sequence under the control of 35S promoter (Wolabu, 2015), was kindly provided by the laboratory of Dr Million Tadege.

M. truncatula seeds were sterilized in sulfuric acid for 10 minutes, rinsed 10 times with sterile water, and then put onto 1 % agar and left to germinate at 4 °C for 7 days. After germination, seedlings were transferred into the soil (Terra Vita, Russia) mixed with vermiculite (2:1). Plants were grown at 21 °C at 16 h photoperiod. Before in vitro cultivation, leaves of 30 day old plants were sterilized in 70 % ethanol for 1 minute, then in 50 ml solution of 0.5 % hypochlorite with two drops of Tween-20 for 10 minutes, and then rinsed 5–7 times with sterile water. In vitro cultivation and obtaining of embryogenic calli was performed as described previously (Tvorogova et al., 2016).

Two biological replicates for R-108 wildtype calli (wt from this point onward) and two biological replicates for MtWOX9-1 overexpressing calli (w9o from this point onward) were taken at 35th day of cultivation (5th day of cultivation of hormone-free medium). Plant material was divided into two parts, the first for transcriptomic analysis and the second for quantitative PCR (qPCR) analysis.

Transcriptome sequencing and bioinformatic processing. RNA extraction, library preparation and sequencing was performed by the Genoanalitica company (Moscow, Russia). Total RNA was extracted from calli with Trisol reagent and PureLink RNA Micro Kit (Invitrogen) according to manufacturer instructions. Quality was checked with BioAnalyser and RNA 6000 Nano Kit (Agilent). PolyA RNA was purified with Dynabeads® mRNA Purification Kit (Ambion). Illumina library was made from polyA RNA with NEBNext® Ultra II RNA Library Prep Kit for Illumina® (NEB) according to manual. Sequencing was performed on HiSeq1500 with 50 bp read length.

Trimming of adapter sequences was performed with Trimomatic (Bolger et al., 2014). Filtration of ribosomal RNA was performed with SortMeRNA (Kopylova et al., 2012) with rRNA sequences from M. truncatula Jemalong A17 genome assembly v5r1.6 used as reference database (Pecrix et al., 2018). Alignment on reference genome (assembly Medtr17_4.0) was performed with HISAT2 (Kim et al., 2015), and reads were counted with Stringtie (Pertea et al., 2015) with the usage of reference genome mentioned before and without de novo assembled transcripts. DESeq2 (Love et al., 2014), GSEABase (Morgan et al., 2019), and WGCNA (Langfelder, Horvath, 2008) R packages were used for differential expression analysis, GO gene enrichment analysis, and coexpression analysis, respectively.

qPCR expression analysis. Total RNA was extracted from calli with Purezol reagent (Bio-Rad, USA) according to manufacturer instructions. RNA was treated with DNase I (Thermo Scientific, USA) for DNA removal. cDNA synthesis was performed with RevertAid reverse transcriptase (Thermo Scientific, USA) with oligo-dT18 primer according to manufacturer instructions. cDNA samples were diluted with sterile water to the end volume of 100 μl. For qPCR, the reagent kit for qPCR with Eva Green (Syntol, Russia) was used. Quantitative estimation of analyzed gene expression was performed with 2-ΔΔCt method (Livak, Schmittgen, 2001). Actin gene (MTR_3g095530) and constitutive gene for histone-like protein H3L (MTR_4g097170) were used as reference genes, and their primer sequences (ActinF: TCAATGTCGCTCCCATG TATGT; ActinR: ACTCAACCGCTACCCA; H3LF: CTTT GCTTGGTGCTGTATGATGG; H3LR: ATTCAAAAG GGGCTGTCATA) were taken from literature (Ariel et al., 2010; Zhang et al., 2014). Primers for BHLH TF-like protein gene (Medtr1g107185, F: GAAACCAAAAACAACCA CTG; R: GACCTTCTGCTCCTACACAC), bZIP TF gene (Medtr7g104190, F: CGGATGGAGGTGACACAGAAC; R: CCTTGTTGATGGAAGTGATG) and MtWOX9-1 (Medtr2g015000, F: CCAGAACAGATACGACACAG AAC; R: TTAGGAAAACAGGGAAAAATAC) gene were selected using Primer3 Select online software (Untergasser et al., 2012).

Results

To analyze the mechanisms of MtWOX9-1 functioning during SE, 35 day-old wt and w9o calli of M. truncatula were obtained. At this stage, somatic embryos, visible as green spots on callus surface, started to appear on w9o calli (Suppl. Fig. 1, a), but not on wt calli which usually start form embryos later during cultivation. The RNA was extracted, reverse transcribed and sequenced from embryogenic wt and w9o calli (two biological replicates for each variant). According to qPCR expression, expression level of MtWOX9-1 was several thousand times higher in w9o samples than in wt samples (see Suppl. Fig. 1, b). 4 complementary DNA libraries were sequenced with an average depth of approximately 15 million of reads. After trimming and ribosomal RNA removing, about 12 millions of 35-bp reads were taken for analysis. After alignment with HISAT2, about 75 % of reads in each sample were uniquely mapped. Reads were counted by Stringtie, and correlation analysis performed for DESeq normalized counts demonstrated high correlation between biological replicates (see Suppl. Fig. 1, c).

After the analysis of differential expression with DESeq package and imposition of 0.01 adjusted p-value and 1.0 log2 fold change cutoffs, 3133 genes out of 51628 analyzed were found to be differentially expressed (Suppl. Table S1), with 1608 and 1525 up- and downregulated, respectively, in w9o calli in comparison to wt calli. qPCR expression analysis of two differentially expressed genes (DEGs) supported transcriptome analysis data (Suppl. Fig. 2).

To find new potential stimulators and repressors of SE, we assessed expression levels of genes coding TFs among DEGs (selection of DEGs with GO annotation number 0006355, “regulation of transcription, DNA-templated”). We also added there five DEGs from WOX family, which happened not to be included in this GO group, but, according to numerous data, should have TF function. We found 173 DEGs coding TFs of which 94 gene was upregulated, including several TFs from NF-Y family, B3 domain TFs, MADS-box TFs etc (Suppl. Table S2).
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As well as w9o calli appear to be more embryogenic than wt ones according to our data (Tvorogova et al., 2019), we supposed that MtWOX9-1 should specifically stimulate expression of genes related with embryo development and development of pods. To check this hypothesis, we selected the genes with specific expression in seedpod (expression level in seedpod is at least 4 times higher than average expression level measured in seedpod, leafblade, nodule, root, flower and bud) from the M. truncatula genome database (Krishnakumar et al., 2015). Among these genes, only 3.7 % (44 genes) were supposed that MtWOX9-1 should specifically stimulate expression of genes related with embryo development and development of pods. To check this hypothesis, we selected the genes with specific expression in seedpod (expression level in seedpod is at least 4 times higher than average expression level measured in seedpod, leafblade, nodule, root, flower and bud) from the M. truncatula genome database (Krishnakumar et al., 2015). Among these genes, only 3.7 % (44 genes) were differentially expressed in our experiment, but 75 % of this group of DEGs were upregulated (Fig. 1), which is consistent with our hypothesis.

We also performed gene enrichment analysis with GSEA-Base package using GO terms (Ashburner et al., 2000). Upregulated and downregulated genes were found to be enriched with genes from 17 and 21 GO “Molecular Function” groups, respectively (Fig. 2, Suppl. Fig. 3, Suppl. Table S3).

Interestingly, upregulated GO pathways included several groups related with histone methyltransferase activity as well as DNA and chromatin binding, suggesting major epigenetic changes occurring in w9o calli. We analysed A. thaliana homologs of upregulated histone methyltransferases and found that most of them are responsible for histone repressive marks associated with DNA methylation (Table).

49 and 40 “Biological Process” GO terms were overrepresented among upregulated and downregulated genes, respectively (Fig. 3, Suppl. Fig. 4, Suppl. Table S4). In accordance with the data above, genes from DNA methylation and gene silencing GO groups were found among upregulated genes.

To find the genes which may work together with Mt-WOX9-1, we performed coexpression analysis using Benedito et al. (2008) data from Medicago Truncatula Gene Expression Atlas and WGCNA R package (Langfelder, Horvath, 2008). We found 55 coexpression modules, containing from 35 to 4914 different genes and isoforms (Suppl. Table S5). The MtWOX9-1 module contained 2337 genes, out of which 225 genes were differentially expressed in our calli samples according to transcriptome analysis (Suppl. Table S6). We searched for TF genes among this group using selection of genes with GO annotation number 0006355, “regulation of transcription, DNA-templated”. We also added two DEGs from WOX family (including MtWOX9-1 itself), which happened not to be included in this GO group, but, according to numerous data, should have TF function.
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Proline dehydrogenase activity
Phosphoenolpyruvate carboxylase activity
Microtubule motor activity
Microtubule binding
Oxidoreductase activity, acting on the CH−NH2 group of donors
Structural constituent of cytoskeleton
Cytoskeletal protein binding
Histone-lysine N-methyltransferase activity
Lysine N-methyltransferase activity
Chromatin binding
Protein methyltransferase activity
Histone binding
Nutrient reservoir activity
Pectinesterase activity
Aspartyl esterase activity
Glucosyltransferase activity
Dioxygenase activity

Fig. 2. Overrepresented “Molecular function” GO pathways in upregulated genes.

| Gene                  | A. thaliana closest homolog | Histone mark                  | References                      |
|-----------------------|-----------------------------|-------------------------------|---------------------------------|
| Medtr1g048950         | SUVH5                       | H3K9me1/me2 (Repressive)      | Ebbs, Bender, 2006; Rajakumara et al., 2011 |
| Medtr5g016870         | CLF                         | H3K27me3 (Repressive)         | Schubert et al., 2006          |
| Medtr5g018850         | SUVR5                       | H3K9me2 (Repressive)          | Caro et al., 2012              |
| Medtr6g061200         | SUVH4/KRYPTONITE            | H3K9me1/me2 (Repressive)      | Johnson et al., 2004           |
| Medtr6g061270         | SUVH4/KRYPTONITE            | H3K9me1/me2 (Repressive)      |                                 |
| Medtr7g084090         | SUVH4/KRYPTONITE            | H3K9me1/me2 (Repressive)      |                                 |
| Medtr7g088370         | SUVH1                       | H3K4me3 (Activating)          | Li et al., 2016                |

The resulting TF gene list contained 18 genes, up- or down-regulated in w9o calli, including genes for three B3 domain proteins, several homeobox-containing factors, etc (Suppl. Table S7).

Discussion
In this study, we analyzed transcriptome of embryogenic calli, affected by overexpression of SE stimulator MtWOX9-1. It is important to note that in this study we used a single line with MtWOX9-1 overexpression, therefore one cannot exclude the possibility that observed effects are related with specific location of transgenic insert in this line. However, according to our results (Tvorogova et al., 2019), the positive effect of MtWOX9-1 overexpression on embryogenic capacity was observed after several independent transformation events, therefore we can assume that the majority of the effects demonstrated in this study are not unique for specific transgenic line.

Analysis of gene groups activated in w9o calli allowed us to suggest that MtWOX9-1 overexpression and probably SE itself are associated with major epigenetic changes in embryogenic callus, such as DNA and histone methylation. Such changes are common for in vitro cultures (Kumar, Van Staden, 2017). Most of observed upregulated chromatin-related pathways were found to be repressive which is probably due to the start of differentiation of callus cells forming specific embryo tissues. Interestingly, such pathways as postembryonic development and seedling development were upregulated, suggesting important differences between zygotic embryogenesis and somatic embryogenesis, lacking dormancy stage.

As it was mentioned above, SE is the stress-induced process: temperature, wounding, starvation, heavy metal ions, and osmotic stress can lead to dedifferentiation of somatic cells and to other changes. In support of this, we also found some upregulated GO groups associated with stress response, including, for example, proline dehydrogenase and response to abscisic acid. Proline dehydrogenase enzyme is an important component of plants pathogen defense system which contributes to the hypersensitive response (HR) and disease resistance, and promotes the accumulation of reactive oxygen...
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### Fig. 3.
Overrepresented "Biological Process" GO pathways in upregulated genes.

| Pathway                                                                 | OddsRatio |
|------------------------------------------------------------------------|-----------|
| Proline catabolic process                                              | 10        |
| Cytokinesis by cell plate formation                                     | 20        |
| Mitotic cytokinesis                                                     | 30        |
| Cytokinetic process                                                    | 40        |
| DNA replication initiation                                             |           |
| Regulation of seedling development                                      |           |
| Microtubule-based movement                                             |           |
| DNA methylation                                                        |           |
| DNA modification                                                        |           |
| Galactose metabolic process                                            |           |
| Gene silencing by RNA                                                  |           |
| Base-excision repair                                                   |           |
| Ribonucleoside biosynthetic process                                    |           |
| Oxylipin biosynthetic process                                          |           |
| Glycosyl compound biosynthetic process                                 |           |
| Histone lysine methylation                                             |           |
| Response to abscisic acid                                              |           |
| Auxin-activated signaling pathway                                       |           |
| Negative regulation of gene expression                                 |           |
| Tissue development                                                      |           |
| Cell division                                                          |           |
| Mitotic cell cycle                                                      |           |
| Cellulose biosynthetic process                                         |           |
| Beta-glucan metabolic process                                          |           |
| Response to salt stress                                                |           |
| DNA packaging                                                          |           |
| Glucan biosynthetic process                                            |           |
| Microtubule-based process                                              |           |
| Cellular response to hormone stimulus                                  |           |
| DNA replication                                                        |           |
| Cellular carbohydrate biosynthetic process                             |           |
| Glutamine family amino acid metabolic process                          |           |
| Glucan metabolic process                                               |           |
| Protein methylation                                                    |           |
| Cellular polysaccharide metabolic process                              |           |
| Polysaccharide biosynthetic process                                    |           |
| Cell wall modification                                                 |           |
| Monocarboxylic acid biosynthetic process                               |           |
| Cytoskeleton organization                                              |           |
| Response to oxygen-containing compound                                 |           |
| Fatty acid metabolic process                                           |           |
| Post-embryonic development                                             |           |
| Negative regulation of metabolic process                               |           |
| Pectin catabolic process                                               |           |
| Response to organic substance                                          |           |
| Response to endogenous stimulus                                        |           |
| Galacturonan metabolic process                                         |           |
| Carbohydrate catabolic process                                         |           |
| Cell wall organization                                                 |           |
species (ROS) and oxidative cell death (Cecchini et al., 2011; Monteoliva et al., 2014).

Several downregulated GO groups associated with stress response were also found, including phenylalanine ammonia-lyase activity (Wada et al., 2014) and chitin binding groups. Several other SE and cell division associated pathways were also found to be upregulated in w9o calli, including phosphoenolpyruvate carboxylase and pectin methyltransferase (PME) activity, cytokinetic process etc. Phosphoenolpyruvate carboxylase modulates cell division and elongation of cotton fibers, the ovule epidermal cells formed during flowering process (Li et al., 2010). Changes in the methylationstification status of pectins have been associated with cell wall remodeling that occurs during diverse plant developmental processes (Levesque-Tremblay et al., 2015). It was shown that PME expression level increased in heart-torpedo embryos and mature cotyledonary embryos (Pérez-Pérez et al., 2018). This tendency is kept during somatic embryogenesis process among both woody and herbaceous plants (Solis et al., 2016). Besides their roles in plant development, PMEs are also involved in stress response and pathogen defense (Ma et al., 2013).

WOX genes are master regulators of development, and their direct targets often encode for other TFs. The results of our coexpression analysis allowed to identify several TF genes which may be the direct targets of MtWOX9-1. Therefore, the next step of research will be to check the relations between MtWOX9-1 and these genes using molecular biology methods.

**Conclusion**

The observed differences between w9o and wt calli and control can be considered as the specific effect of MtWOX9-1 overexpression but, on the other hand, they may result from SE process itself, which tends to start earlier in w9o calli (Tvorogova et al., 2019). Thus, these data may be useful both for MtWOX9-1 target search and for the search for new SE-associated genes and SE stimulators.

**References**

Alvarez J.M., Sohlgberg J., Engström P., Zhu T., Englund M., Moschou P.N., van Arnold S. The WUSCHEL-RELATED HOMEO-BOX 3 gene PaWOX3 regulates lateral organ formation in Norway spruce. New Phytol. 2015;208(4):1078-1088. DOI 10.1111/nph. 13536.

Ariel F., Diet A., Verdenaud M., Gruber V., Frugier F., Cres-Alvarez J.M., Sohlberg J., Engström P., Zhu T., Englund M., Moschou P.N., van Arnold S. The WUSCHEL-RELATED HOMEO-BOX 3 gene PaWOX3 regulates lateral organ formation in Norway spruce. New Phytol. 2015;208(4):1078-1088. DOI 10.1111/nph. 13536.

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Bekker D.S., Sheahan M.B., Rose R.J. Transcriptional regulation of early embryo development in the model legume Medicago truncatula. Plant Cell Rep. 2014;33(2):349-362. DOI 10.1007/ s00299-013-1535-x.

Bolger A.M., Lohse M., Usadel B. Trimomatic: A Flexible Trimmer for Illumina Sequence Data. Bioinformatics. 2014;30(15):2114-2120. DOI 10.1093/bioinformatics/btu170.

Bouchabék-Coussa O., Obellianne M., Linderm D., Montes E., Maigard A., Vilaine F., Panettier C. Wusche1 overexpression promotes somatic embryogenesis and induces organogenesis in cotton (Gossypium hirsutum L.) tissues cultured in vitro. Plant Cell Rep. 2013;32(5):675-686. DOI 10.1007/s00299-013-1402-9.

Breuninger H., Rikirsch E., Herrmann M., Ueda M., Laux T. Differential expression of WOX genes mediates apical-basal axis formation in the Arabidopsis embryo. Dev. Cell. 2008;14(6):867-876. DOI 10.1016/j.devcel.2008.03.008.

Caro E., Stroud H., Greenberg M.V.C., Bernatavichute Y.V., Feng S., Groth M., Vashisti A.A., Wolschlegel J., Jacobsen S.E. The SET-domain protein SUVR5 mediates H3K9me2 deposition and silencing at stimulus response genes in a DNA methylation-independent manner. PLoS Genet. 2012;8(10):e1002995. DOI 10.1371/ journal.pgen.1002995.

Cecchini N.M., Monteoliva M.I., Alvarez M.E. Proline dehydroge-nase contributes to pathogen defense in Arabidopsis. Plant Physiol. 2011;155(4):1947-1959. DOI 10.1104/pp.111.167163.

Ebsb M.L., Bender J. Locus-specific control of DNA methylation by the Arabidopsis SUL3 histone methyltransferase. Plant Cell. 2006;18(5):1166-1176. DOI 10.1105/tpc.106.041400.

Fehér A. Somatic embryogenesis – Stress-induced remodeling of plant cell fate. Biochim. Biophys. Acta. 2015;1849(4):385-402. DOI 10.1016/j.bbamcr.2014.07.005.

Gambino G., Minuto M., Boccacci P., Perrone I., Vallania R., Gribau-do I. Characterization of expression dynamics of WOX homeo-domain transcription factors during somatic embryogenesis in Ficus vi-nifera. J. Exp. Bot. 2011;62(3):1089-1101. DOI 10.1093/jxb/erq349.

Hoffmann B., Trinh T.H., Leung J., Kondorosi A., Kondorosi É. A new Medicago truncatula line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection. Mol. Plant Microbe Interact. 1997;10(3):307-315.

Johnson L., Mollah S., Garcia B.A., Muratore T.L., Shabanowitz J., Hunt D.F., Jacobsen S.E. Mass spectrometry analysis of Arabidopsis histone H3 reveals distinct combinations of post-translational modifications. Nucleic Acids Res. 2004;32(22):6511-6518. DOI 10.1093/nar/gkh992.

Kim D., Langmead B., Salzberg S.L. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods. 2015;12(4):357-360. DOI 10.1038/nmeth.3317.

Kopylova E., Noël L., Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic Data. Bioinformatics. 2012;28(24):3211-3217. DOI 10.1093/bioinformatics/bts611.

Krishnakumar V., Kim M., Rosen B.D., Karamycheva S., Bidwell S.L., Tang H., Town C.D. MTGD: The Medicago truncatula genome database. Plant Cell Physiol. 2015;56(1):e1. DOI 10.1093/pcp/pcu179.

Kumar V., Van Staden J. New insights into plant somatic embryoge-nesis: an epigenetic view. Acta Physiol. Plant. 2017;39(9):194. DOI 10.1186/s11738-017-2487-5.

Kurdyukov S., Song Y., Sheahan M.B., Rose R.J. Transcriptional reg-ulation of early embryo development in the model legume Medicago truncatula. Plant Cell Rep. 2014;33(2):349-362. DOI 10.1007/ s00299-013-1535-x.

Langfelder P., Horvath S. WGCNA: an R package for weighted corre-lation network analysis. BMC Bioinformatics. 2008;9(1):55. DOI 10.1186/1471-2105-9-55.

Laux T., Mayer K.F., Berger J., Jürgens G. The WUSCHEL gene is a master regulator of development, and their cell fate. Biochim. Biophys. Acta. 2015;1849(4):385-402. DOI 10.1016/j.bbamcr.2014.07.005.

Lutova L.A. Initiation of spontaneous tumors in radish (Raphanus sativus): cellular, molecular and physiological events. J. Plant Physiol. 2015;173:97-104. DOI 10.1016/j.jplph.2014.07.030.
Leibfried A., To J.P.C., Busch S., Strehle S., Kehle A., Demar M., Kreplak J., Mayjonade B., Satgé C., Perez M., Cauet S., Marande W., Chantry-Darmon C., Lopez-Roques C., Bouchez O., Bérard A., De-Grève V.M. The relationship between stress and somatic embryogenesis. J. Exp. Bot. 2016;67(1):2711-2723. DOI 10.1093/jxb/erw275.

Su Y.H., Liu Y.B., Bai B., Zhang X.S. Establishment of embryonic shoot-root axis is involved in auxin and cytokinin response during Arabidopsis somatic embryogenesis. Front. Plant Sci. 2015;6:658. DOI 10.3389/fpls.2015.00658.

Tvorogova V.E., Fedorova V.A., Potenskoyava E.A., Kudriashev A.A., Kuznetsova, E.A. Potsenkoyava, Y.A. Fedorova, L.A. Lutova

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