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DEVELOPMENT OF ROOT TIPS SYNCHRONIZED SYSTEM FOR THE MODEL LEGUME *MEDICAGO TRUNCATULA* UPON REPLICATION STRESS

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Abstract

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Up to now the synchronized cell cultures were developed for alfalfa (*Medicago sativa)* and they served as a valuable tool for the identification and understanding of key regulators of the cell cycle. These cell culture systems were characterized with some disadvantages like continuous use of mitogenic compounds and conditions that differ from the stable physiological state in the plant meristem cells. The present procedure, described in this study is an easy and fast system for root tips synchronization of *Medicago truncatula*, upon hydroxyurea treatment. The system was originally developed for *Arabidopsis thaliana* and adapted for the model legume. The present study opens up possibility to investigate gene of interest that are involved in *Medicago truncatula* cell cycle progression.

Keywords: Medicago truncatula, cell cycle, root tips synchronization system, cyc like F-box protein

Abbreviations: 2,4-Dichlorophenoxyacetic acid; BAP 6-Benzylaminopurine; NAA a-Naphthaleneacetic

Introduction

The overall control of the cell cycle is broadly similar between plants and other eukaryotic organisms. Cell division cycle or cell cycle includes two active phases: the S phase when DNA synthesis occurs and the M phase (or mitosis) during which the compacted chromatin into chromosomes is equally distributed to the daughter cells. Regulatory gap phases G1 and G2 precede the S and M phases, respectively. The G1/S and G2/M–phase transitions are considered to be the major control points in the cell cycle at which decisions are taken with respect to division, differentiation, programmed cell death, or adoption of quiescent state. Both transitions are controlled by serine/threonine kinases that became functional by binding to a regulatory protein known as cyclins, and are therefore termed as cyclin-dependent kinases (CDKs), being the key regulators of the cell cycle

Usually the expression pattern of CDKs is constitutive throughout the cell cycle. Meanwhile the periodic expression of cyclins provides the characteristic cell cycle phase-specific timing of CDKs activities (Inze and De Veylder, 2006).

Functional cell cycle research needs the availability of synchronized cell cultures, which are valuable tool for understanding role of genes acting during cell cycle phases. In the available systems for synchronized cell cultures, cells are cultivated in the continuous pressure of mitogenic compounds, which often leads to genomic instability. Recently established *Arabidopsis thaliana* system based on root tips (Cools et al., 2010) synchronized by hydroxyurea opens up the possibility to investigate cell cycle genes or gene of interest in the stable endogenous conditions in organized meristems cells. The successful use of the above mentioned synchronization system for *Arabidopsis thaliana* root tips induced by hydroxyurea (HU) was confirmed in high-throughput experiments describe by (Cools et al., 2011).

In plants F-box proteins have been poorly investigated and functionally characterized. For example in the model plant *A. thaliana*, less than 5% of them were studied. These proteins are involved in the regulation of multiple develop-

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mental processes, such as leaf senescence and branching (Woo et al, 2010; Stirnberg et al., 2007) flower development (Imazumi et al., 2005; Chae et al., 2008) phytohormone signaling (Guo et al., 2003; Dill et al., 2004; Binder et al., 2007) circadian rhythms (Han et al., 2004) and defense responses (Kim et al., 2002). These proteins contain a F- box motif. The F- box motif is responsible for binding to SKP1 (S-phase-kinase-associated protein 1) in the formation of SCF [SKP1 (S-phase-kinase-associated protein 1), Cullin-1, F-box protein] ubiquitin ligase complex. The SCF complex is the largest family of E3 ubiquitin-protein ligases.

Over the past decade a model legume *M. truncatula,* has been proposed for molecular genetics and biochemical research. It is generally accepted that knowledge about certain shared characteristics of legumes, such as the pathways involved in symbiosis with rhizobia and synthesis of flavonoids, isoflavonoids, saponinis and glycosides, is considerably transferable from models to crops. Information from the model species may be useful for forage legumes, e.g. alfalfa and clovers, even for the study of agronomic traits (yield and growth habits), because *M. truncatula* are related to the forage legume species alfalfa. Model legume can also provide useful information for the improvement of other crops such as tomato, sunflower, cotton, corn and rice. The biochemistry of legumes is distinct from that of other plant groups and many unique molecules with biomedical application, such as isoflavones and plant sterols, are found among legume metabolites.

The objective of present study was the establishment of a root tips synchronized system for the important model legume species *Medicago truncatula*. The first goal was to find optimal synchronization settings and cell cycle phase marker genes. The efficiency of synchronization was confirmed by flow cytometric analysis and validate with transcriptional analysis of cell cycle marker genes. The second goal of the study was to link the expression of investigated gene encoding Cyclin like F-box protein from *Medicago truncatula* to the respective cell cycle phase in the background of wild type plants and overexpressed and knockdown mutants generated by *Agrobacterium*-mediated transformation of the model legume plant.

Materials and Methods

Plant material, growth conditions

Seeds from the highly regenerable genotype *Medicago truncatula* cv. 'Jemalong 2HA' (Nolan et al., 1989) were surface wounded by sand paper and sterilized with 6% (*v/v*) solution of sodium hypochlorite (commercial bleach) for 15 min, rinsed at least three times with sterile distilled water. The germinated seedlings were then propagated by cuttings. *In vitro* plant materials were grown in Magenta boxes (60 x 60 x 96 mm, Sigma) in a growth chamber at 24°C, with a 16-h photoperiod and light intensity of 30 μmol m^{-2} s^{-1}.

For synchronization experiments sterilized WT seeds were plated on square plates containing agar-solidified Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) and grown 24 h at 4ºC in dark condition. Seedlings were then grown for 5 days on MS basal medium. Root growth were measured on 24, 48, 72, 96, 120 h after transfer on five different concentrations of hydroxyurea (HU) /1, 2, 3, 4, 5 mM/ as well as on control MS medium. For validation of synchrony by qRT-PCR using cell cycle phase marker genes, root tips from WT seedlings were treated with 1mM HU and harvested at regular time intervals (0; 4; 6; 10; 14; 18; 22; 26 h) after HU treatment.

In vitro cuttings from wild type plants and confirmed transgenic overexpressed and knockdown lines from *Medicago truncatula* were grown on (MS) agar medium. Cuttings with equal size of root and shoot part were selected for treatment with 1mM HU at the two time points: 6 h and 18 h, and then the root tips (1 mm) were harvested.

Construction of expression vectors for genetic transformation

The recombinant plasmids were generated using the GATEWAY cloning system (Invitrogen Life Technologies, Inc., www.lifetechnologies.com). The Entry clones were constructed by inserting the ORF sequences of *F-box* gene (MT2G007220, Plaza 2.5, NP7270344 derived from Genebank accessions AC155889, TC178783, www.mwdicago.org) into the pDONR221 donor vector. Expression clones were generated by recombining the Entry clones of cyclin-like F-box into *pK7WG2* destination vector for overexpression, under the control of *CaMV 35S* promoter and *nptII* gene for plant selection (Karimi et al., 2007).

RNA interference (RNAi) method, which is a powerful reverse genetic tool to study gene function in *Medicago truncatula*, (Limpens et al., 2004), was used to silence the cyclin-like F-box expression in a sequence-specific manner. As RNAi mechanism is based on the formation of double stranded RNA (dsRNA), Xwin Razor software was used to *in silico* predict region of the gene with high silencing capacity, optimal for synthesis of dsRNAs. In order to create cyclin-like F-box RNAi construct, *pK7GWIWG2D(II)* hairpin RNA expression vector was used.

The resulting constructs were introduced into *Agrobacterium tumefaciens* strain C58C1, which was maintained on

Table 1
List of primers used for construction of vectors and qRT-PCR expression analyses

Mt-F gene overexpression	ATGCGTACAATCGAGCCTACA
Mt-R gene overexpression	TTCTATTCGAATTATCCCACGG
Mt-F gene inactivation	GTACAAAAAAGCAGGCGATGAGATTTGCCGGTTCAG
Mt-R gene inactivation	GTACAAGAAAGCTGGGTAACTGGAAATCAGTACGAAATGC
Mt-Cyc F-box-F	AGCAGTGCAAGCTGGATTTT
Mt-Cyc F-box-R	TCCCCCTCATTGACAGAAAC
Mt-CycA3;4-F	CTTCTATGGTGGCTGCATCA
Mt-CycA3;4-R	CTTGCAAAGAGCCACCTTTC
Mt-Cyclin- F	GGTGGAACAAGCTCAGAAGC
Mt-Cyclin- R	CAAAATCCTTGGGCACAACT
Mt-Cyclin1- F	GAGCATACACCCGGAAAGAA
Mt-Cyclin1- R	AGCGGCAGCTAGTTGAGAAG
Mt-CycA G2/M -F	TTTGTACGTGCAGCTCAAGG
Mt-CycA G2/M -R	TTTGGCCAGGAAAATTGAAG
Mt-CDK- F	ACCGGAAAACAATGATGCTC
Mt-CDK- R	GTTGCAGCTCAGAATCACCA
Mt-CycA2 G2/M -F	GTGGCTAAGCCTGCTCAATC
Mt-CycA2 G2/M -R	CACACTGGTTGGCACTCATC
Mt-histone H2A-F	CAGTTCCCTGTCGGACGTAT
Mt-histone H2A-R	TCAGATCCTTGCTTGCATTG
Mt-histone H4- F	GCACACTCATTGCTGAAGGA
Mt-histone H4-R	AGCAGCTGCATTGGCTATTT
Mt-Actin-F	TCAATGTGCCTGCCATGTATGT
Mt-Actin-R	ACTCACACCGTCACCAGAATCC
Mt-Ubiquitin-F	GCAGATAGACACGCTGGGA
Mt-Ubiquitin-R	AACTCTTGGGCAGGCAATAA

agar (1.5%) solidified YEB nutrient medium, supplemented with 100 mg/L rifampicin (Rif), 100 mg/L spectinomycin (Sp) and 50 mg/L gentamycine (Gm).

The sequences of primers used for construction of vectors are given in Table 1.

Composition of plant media for regeneration/transformation

Leaf and petiole explants collected from 35-day-old *in vitro* plants were used as explants for genetic transformation. Transgenic plants of *M. truncatula* were produced by application of combined protocol described by (d'Erfurth et al., 2003; Chabaud et al., 1996; Iantcheva et al., 2009). Leaf and petiole explants were wounded by scalpel blade and pre-cultivated on a solid callus induction medium Shab, (SH macro, micro nutrients and vitamins plus 5 mg/L auxin 2.4 D and 1 mg/L cytokinin BAP), (Schenk and Hildebrandt 1972) for a two-day period in darkness. Pretreated explants were inoculated with bacterial suspension (OD_{600} = 0.5) for 1 h on horizontal shaker at 100 rpm. The transformed plant material was co-cultivated for further 48 h, then transferred for a period of two weeks to selective SHab medium with Km (50 mg/L) and carbenicillin (Cb, 400 mg/L) for selection of the transformed tissue and removal of *Agrobacterium*.

After the appearance of callus along the edge of the wounded areas, the explants were transferred to CIM medium (MS macro, micro nutrients and vitamins plus 2 mg/L zeatin, 1 mg/L 2.4D) with fresh Km and Cb with the same concentration for another two weeks in order to finish process of callus initiation. After forming the clearly visible callus tissue, explants were transferred to the 09-03 medi-

um (MS macro, micro nutrients plus 0.9 mg/L BAP and 0.3 mg/L NAA, in order to form green embryo zones), containing only the antibiotic Cb at a concentration of 200 mg/L (to prevent eventual Agro infection) without the selective agent Km for 2 weeks. This step without selective pressure was applied for easier embryo formation and subsequent development of embryo structures on MS1 medium (0.05 mg/L BAP and 250 mg/L casein hydrolysate) for two passages of 20-25 days. With the appearance of embryo structures in late cotyledonary stage, the plant material was transferred to selective MS medium containing 50 mg/L Km. The putative transgenic rooted plantlets were cultivated on the same medium. After sampling for analyses, they were transferred to soil and grown in a greenhouse for seed production.

Root length measurements

Measurements of the root length were performed 5 days after seed germination and assessed every 24 h over 5 consecutive days. The length of at least 20 roots of wild type plants were measured for each data set. The images were analyzed using ImageJ 1.41 software.

Flow cytometry

Excised root tips (1 mm) of wild type seedlings treated with 1 mM HU for different time intervals (0; 6; 10; 14; 18; 22; 26; 30 h) were chopped with a razor blade in 200 µl CyStain UV Precise nuclei extraction buffer and DNA was stained by adding 800 µl staining buffer (Partec). Measurments were carried out with a flow cytometer CyFlow (Partec) and the samples analyzed with the CXP Analysis software (Partec). Six to eight root tips were analyzed for each of three technical repeats and for each of the above mentioned time points.

Selection of cell-cycle marker genes

Using the PLAZA 2.5 webtool, orthologs of *Arabidopsis* cell-cycle marker genes were identified for *Medicago truncatula*. Several genes corresponding to S, G2/M marker genes and to histone genes were selected. In total 8 genes were used for further expression analyses. Transcript levels were measured in two independent synchronization experiments using RT-qPCR. Expression levels in the 0h was arbitrarily set to one. *Selected genes:* MT1G018680-Cyclin-A3-4 ortholog of AT5G43080; MT5G088980 cyclin ortholog of AT4G37490; MT5G023790-cyclin-1 ortholog of AT2G17620; MT8G095930 cyclin A like ortholog of AT1G44110; MT1G075610 Medicago CDK ortholog of AT1G76540; MT2G102520 Cyclin A2 ortholog of AT1G80370; MT1G011850 histone H2A ortholog of AT1G52740; MT4G128150 histone H4 ortholog of AT2G28740.

qRT-PCR analysis

Gene expression studies were carried out using total RNA extracted from root tips (20 for each time point; 1 mm in size) of *M. truncatula* WT seedlings, T_1 overexpressed (OE) and T_1 knockdown (RNAi) lines. RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) and cDNA prepared via reverse transcription with the First Strand cDNA Synthesis Kit (Fermentas). Relative expression levels were analysed in triplicate in a 7300 Real-time PCR System (Applied Biosystems). Transcript levels were measured in two independent synchronization experiments. The expression levels of two endogenous reference genes, ACT and UBQ10, were used for data normalization. The sequences of primers used for qRT-PCR analysis are given in Table 1.

Statistical analysis

Measurement experiments were repeated three times and triplicate assays were performed for each experimental data set. Data were analysed using repeated measures ANOVA via SPSS 11.5 software. P values of < 0.05 were considered as statistically significant. Results are expressed as means ± standard deviation (SD).

Results and Discussion

Root growth length upon HU treatment

In most of the cases synchronization of the cell cultures were based on depletion of important growth nutrients or by using drugs – known as replication inhibitors such as aphidicolin or hydroxyurea. Hydroxyurea is a monoxydroxyl-substituted urea anti-metabolite that is used as an anti-neoplastic drug for treatment of myeloproliferative disorders. The application of HU is based on its anti-proliferative property that causes inhibition of the small subunits of the ribonucleotide reductase, a protein that is responsible for maintenance of the dNTP pool in proliferating cells (Saban and Bujak, 2009). The application of cytotoxic drug reduces the concentration of available nucleotides required for DNA replication and consequentially induces cell-cycle arrest (Wang and Liu, 2006).

In order to select proper system settings different concentrations of HU influenced on root growth were tested. Five days old seedlings of *Medicago truncatula* cv. 2HA grown on MS basal medium were transferred to media containing five different concentrations of HU /1, 2, 3, 4, 5 mM HU/. Root growth was measured on 24, 48, 72, 96 h after transfer as well as on control MS medium. 96 h after transfer root growth was still detected on 1 and 2 mM HU and control. Higher concentrations than 2 mM, of applied drug, stopped growth of the roots after 48 to 72 h. Detailed measurements of root growth

are performed for 5 days treatment with 1 and 2 mM HU for (24, 48, 72, 96, 120 h) in order to select proper concentration for further analyses (Figure 1A). Results from statistical analysis of root growth dynamics for 24 h on the control MS medium showed significantly higher ($p < 0.01$) root growth rate, compared to the root growth on 1 and 2 mM HU. For the first 24 h there was not difference in root growth on 1 and 2 mM HU. Root growth for 48 h on MS medium was significantly higher compared to 1mM HU ($p < 0.01$) and 2 mM HU ($p < 0.001$). For 48 h we were able to observed also significant difference in root growth between 1 mM HU and 2 mM HU ($p < 0.01$). Measurements of the root growth for 72 h also confirmed significant difference on MS medium compared to 1 mM HU ($p < 0.001$) and to 2 mM HU ($p < 0.0001$) and between the two selected concentrations ($p < 0.001$). Later on, root growth was measured for 96 h and 120 h and significant difference was detected on MS medium compared to 1 and 2 mM HU ($p < 0.0001$) and between two concentrations for 96h ($p < 0.001$) and for 120 h ($p < 0.05$). On the base of the collected results concentration of 1mM HU was selected for establishment of root tips synchronization system in *Medicago truncatula*.

Flow cytometric analysis of HU treated root tips

For a detailed look on kinetics of cell cycle progression, flow cytometric analysis were performed on excised root tips of seedlings treated with 1 mM HU for different time intervals (0; 6; 10; 14; 18; 22; 26; 30 h). Under not-treated conditions time point 0h 37% of the cells had a 2C DNA content, 47% 4C and approximately 17% of the cells had a DNA content between 2C and 4C representing cells going DNA replication. During the first 6 h after transfer to HU the number of S cells increased up to 46%. As was mentioned above the application of HU reduces the concentration of available nucleotides that are required for DNA replication and consequentially induces accumulation of cells at the G1-to-S transition. Prolonged application of HU lead to increase of the number of 2C cells upon 6 h to 10 h treatment, which might be corresponds to a cell-cycle arrest in G1-to-S check point in the condition of depleted level of nucleotides. Further on 14 h treatment with HU (time point 14 h) the number of 4C cells started to increase indicating first cells completed S phase. The number of 4C cells continued to rise until the 18 h time point, after which it declined indicating the onset of M phase. The number of 4C cells started slowly to decrease at time points 22h, number of 2C cells increased pronouncedly and S phase nuclei slowly increased again until 30 h indicating the beginning of second round of S phase of the cell cycle progression. The performed flow cytometric analyses of *Medicago truncatula* root tips at the condition of treatment with 1mM replicative drug HU monitored a progression trough cell cycle and were characterized with a prolonged S phase started from time point 6h to 10h and second enter to S phase around 26 h. The second arrest in cell cycle progression, correspond to G2/M check point and was pointed from 14 h to 22 h of treatment with HU (Figure 1 B).

Expression analysis of HU treated root tips of Medicago truncatula

Cell cycle progression induced by 1mM HU was monitored via the expression profiles of cell cycle marker genes at the selected time point intervals (0; 4; 6; 10; 14; 18; 22; 26 h).

Using the PLAZA 2.5 webtool, orthologs of *Arabidopsis* cell-cycle marker genes were identified for *Medicago trun-*

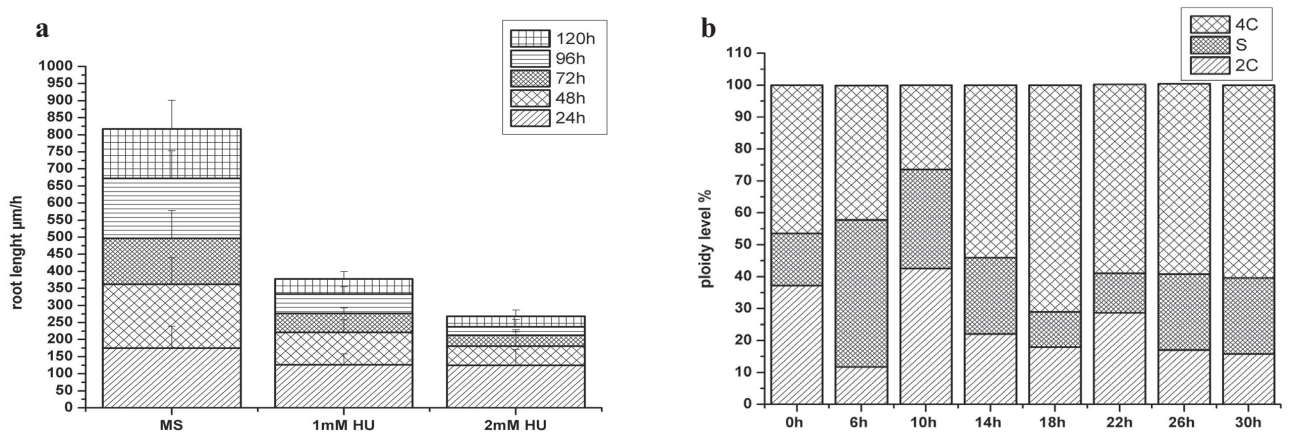

Fig. 1.

catula. In order to select correct marker gene for S phase and G2/M transition, six genes corresponding /see above/ were selected for further expression analyses.

The expression level of the S phase marker genes were plotted on Figure 1C. The transcript levels of CyclinA3-4 gene increased sharply to 4h after treatment, keeping almost constant level between 6 and 10 h. The expression reached a maximum at 10 h, followed by decrease in transcription. The second peak of expression (typical for the S phase cyclins) was formed at 18 h, followed by sharp decrease and raised again with the beginning of a second S phase – 26 h. Cyclin 1 possessed the same profile like CyclinA3-4, but its expression level was rather low than this of CyclinA3-4. The change of transcript level of Cyclin was very low, with a small peak at 6 h and started rising again at 26 h. The obtained results on expression profiles of selected marker genes pointed CyclinA3-4 (*Arabidopsis* ortholog AT5G43080 CyclinA3;1) as a proper marker gene for S phase. The monitored profiles of *M. truncatula* CyclinA3-4 and Cyclin1 possessed the timing and peaks of expression similar to those obtained for *Arabidopsis* S phase marker cyclin investigated by (Cools et al., 2010; Cools et al., 2011).

Data obtained for *Arabidopsis* root tips synchronization system also proved that HU treatment has an effect on transcription of histone genes. Transcript levels of two core histone genes (H2A and H4) also were examine during the progressed cell cycle. The level of histone H2A sharply increased from 0-6 h and peaked at 6 h and slowly decreased and started to rise again when cells started to progress through the second S phase -26 h, (Figure 1 D). The expression of the other core histone gene H4 followed the same profile but its peak appeared at time point 4 h, two hours earlier than H2A, which was in accordance with the data obtained for *Arabidopsis* (Cools et al., 2010).

Based on flow cytometric analysis of HU treated root tips we observed an arrest at G2/M check point of cell cycle progression between 14 h to 22 h of treatment. The expression profiles of selected G2/M specific genes were plotted on Figure 1 E. The first of the selected G2/M marker genes CyclinA G2/M specific formed small peak of its expression at 4 h followed by decrease and again rise of induction reaching a maximum at 18 h treatment with HU, followed by slowly drop at time point 22 h and increased levels of its transcript were seen as cells progressed through the second S phase. The expression profile of the other two selected genes CyclinA2 G2/M specific and CDK, showed similar expression profiles, peaked at 14 h, followed by slowly decrease. The results obtained for expression profiles of G2/M selected marker genes for *M. truncatula* were in accordance with those observed for *Arabidopsis*.

On the base of the established root tips synchronized system for *Medicago truncatula*, the expression of investigated MtCyclin like F-box gene (MT2G007220, Plaza 2.5) was examined through the progression of cell cycle. Its expression was linked with prolonged S phase – started from time point 4h and maintained almost the same level until 10 h followed by slow decrease during G2/M phase and started to rise again with the beginning of second S phase (Figure 1 F).

Our further experiments were based on progeny of obtained transgenic lines. Quantitative differences of MtCyclin like F-box expression in T_1 lines with overexpression (OE) and T_1 lines with knockdown (RNAi) were determined by examining MtCyclin like F-box mRNA abundance levels using quantitative RT-PCR. Three individual plants were randomly selected and assayed for the expression level. The MtCyclin like F-box transcript level in the OE T_1 plants of *M. truncatula* was increased 0.5 times compared with the control ($p < 0.05$). In RNAi lines the expression level was significantly lower compared to WT control ($p < 0$ .001) Figure 1 G.

Later on experiments were based on available stable transgenic lines. The expression level of MtCyclin- like F-box was examined in synchronized root tips of transgenic overexpressed, knockdown lines and WT plants. The time point of 6 h and 18 h, corresponding to S phase and G2/M check point were selected to monitor the expression level of investigated MtCyclin like-F-box. Expression level of MtCyclin-like F-box at 6h time point showed maximum of induction in OE line, followed by WT and was lowest in the RNAi line. At the time point 18 h transcript level of OE line drop twice, slowly decreased in WT and remain the same in RNAi line (Figure 1H). The expression profile of the MtCyclin-like F-box in root tips harvested from cuttings from transgenic and WT plants display the same profile as those one established in WT seedlings synchronized root tips.

In addition, to the MtCyclin-like F-box the transcriptional response of the selected S phase cell cycle marker gene CyclinA3-4 in background of the OE and RNAi lines of synchronized *M. truncatula* root tips was investigated. Our experiments monitor that the expression level of S phase cell cycle marker CyclinA3-4 was comparable with this of MtCyclin like F-box in the background of overexpressed (OE), knockdown (RNAi) lines and WT plants (Figure 1 I). Most of the literature data confirm that two E3 ubiquitin-ligase complexes (SCF-related complexes and the Anaphase-Promoting Complex/Cyclosome (APC/C) complexes) were pointed to control the plant cell cycle and operate respectively at the G1-to-S and in the M-G1 transitions (Marrocco et al., 2010). Investigated MtCyclin-like F-box, is a part of the SCF complex. On the base of the obtained results, we proposed that its

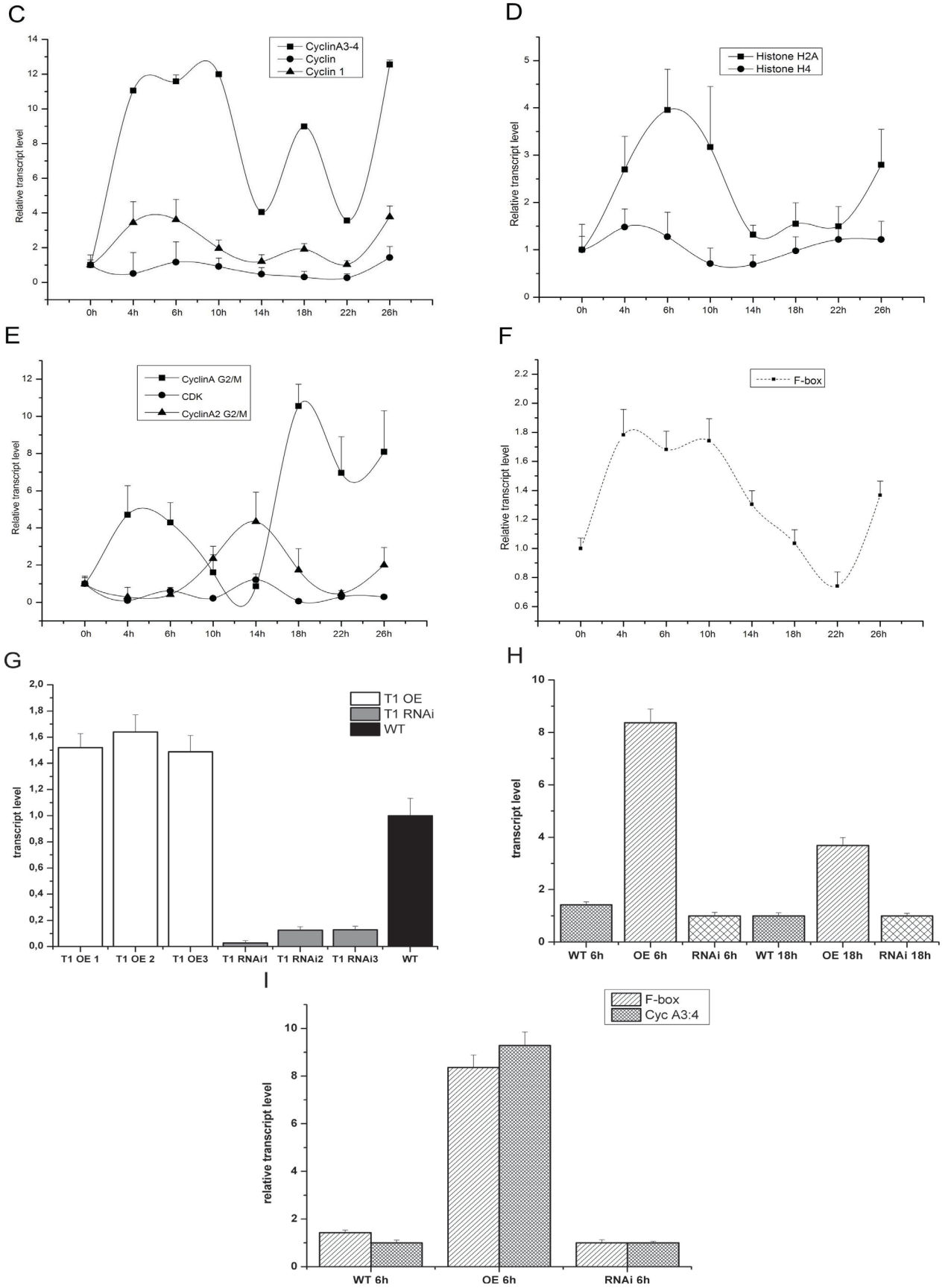

Fig. 1. C, D, E, F, G, H and I

function was to control the cell cycle, involved in degradation of targeted cell cycle proteins during S phase.

Conclusion

The established system for root tips synchronization is an effective method to study cell cycle genes and genes of interest involved in cell cycle in the model plant *Medicago truncatula* and mutants available. The system provides opportunities to test investigated genes in meristem cells in physiologically stable conditions.

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