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ESTABLISHMENT AND IN-HOUSE VALIDATION OF STEM-LOOP RT PCR METHOD FOR MICRORNA398 EXPRESSION ANALYSIS

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MicroRNAs (miRNAs) belong to the class of small non-coding RNAs which have important roles throughout development as well as in plant response to diverse environmental stresses. Some of plant miRNAs are essential for regulation and maintenance of nutritive homeostasis when nutrients are in excess or shortage comparing to optimal concentration for certain plant species. Better understanding of miRNAs functions implies development of efficient technology for profiling their gene expression. We set out to establish validate the methodology for miRNA gene expression analysis in cucumber grown under suboptimal mineral nutrient regimes, including iron deficiency. Reverse transcription by "stem-loop" primers in combination with Real time PCR method is one of potential approaches for quantification of miRNA gene expression. In this paper we presented a method for "stem loop" primer design specific for miR398, as well as reaction optimization and determination of Real time PCR efficiency. Proving the accuracy of this method was imperative as "stem loop" RT which consider separate transcription of target and endogenous control. The method was verified by comparison

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of the obtained data with results of miR398 expression achieved using a commercial kit based on simultaneous conversion of all RNAs in cDNAs.

Key words: miRNA gene expression analysis, Real time PCR, iron deficiency

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding molecules identified in plants, animals and viruses. miRNAs are commonly 21-22nt long and they negatively regulate expression of protein-coding genes. In animals, miRNA sare usually incompletely complementary to 3'untranslated regions (UTRs) ofthe messenger RNA molecules (mRNAs), and they exert their function throughout a variety of mechanisms, including translational repression, mRNA cleavage or deadenylation (ROGERS and CHEN, 2013). Peculiarity of plant miRNAs is a near-perfect pairing with target mRNA and induction of gene repression through degradation of their target transcripts (BRODERSEN *et al*., 2008; HE and HANNON, 2004). Interestingly, plant miRNAsdo not strictly bind untranslated regions of target transcripts; more commonly they are able to bind their targets within coding regions (LELANDAIS-BRIÈRE *et al*., 2010). Also, 3'ends of plant miRNAsare methylated by RNA methyltransferase protein (HEN1). This unique feature makes plant miRNAs more stable and resistant to enzymes which target hydroxyl groups of the terminal nucleotide (YU *et al*., 2005). Plant miRNA target transcriptsare often key regulators of plant development and stress responses (RUBIO-SOMOZA and WEIGEL, 2011; SUNKAR *et al*., 2012). Nutrition deficiency is one of prevailing stresses that affect crop plats. For instance, iron deficiency is a considered as one of the major limiting factors for crop production causing considerable yield losses of economically important crops all over the world. Iron is essential microelement for all living organisms including plants, with numerous important physiological functions such as respiration and photosynthesis (RÖMHELD and NIKOLIC, 2007). Thus investigation of the contribution of miRNA in plant response to iron deficiency is significant and intriguing.

Given that miRNAs are very short molecules with variable GC content (5-95 %) and have extremely high homology within the same family, it was challenging to establish the efficient methods for individual miRNAs gene expression profiling (VÁLÓCZI *et al*., 2004; CHEN *et al*., 2005). Analysis of miRNA gene expression by Northern blot has lot of limits. However, Real-time PCR would be more appropriate approach for detection and precise miRNA quantification, particularly in the case when amount of material is low. Reverse transcription is the procedure that precedes the quantification in real time PCR. Since miRNAs molecules are very short and do not contain a polyA tail, conventional methods for cDNA synthesis by random hexamers or oligodT primers are inadequate. In order to solve this problem, the stem loop primers that can hybridize and then reversely transcribe specific miRNA are commonly used (CHEN *et al*., 2005). However; approaches based on specific stem loop RT primers have significant limitations as endogenous controls cannot be simultaneously reversely transcribed.

In this paper we present the establishment and in-house validation of a method for miRNA gene expression analysis based on stem loop primers. miR398 was chosen to set up this procedure as this micro RNA is highly stress-responsive and proposed to regulate plant responses to a variety of biotic and abiotic stresses (JAGADEESWARAN *et al*., 2009). Additional aim of this work was to investigate the profile of miR398 expressionin response to iron defficiency in cucumber. In this work we investigated the effect ofdifferent suboptimal iron concentrations on levels of miRNA398. Also, we validated actin and U6 as house-keeping genes for Fe deficiency experiments.

MATERIALS AND METHODS

Plant materials and growth conditions

Cucumber (*Cucumissativus L. cv*. Chinese long) seeds were soaked in 1 mM $CaSO_4$ overnight and germinated in quartz sand moistened with saturated $CaSO_4$. The 4-day-old seedlings were then transferred to a complete nutrient solution (4 plants per 2.5-L plastic pot) containing (in mM): 0.7 K_2SO_4, 0.1 KCl, 2.0 $Ca(NO_3)_2$, 0.5 $MgSO_4$, 0.1 KH_2PO_4, and (in µM): 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$, 10 H_3BO_3. Iron was supplied as Fe(III)-EDTA at different concentrations: 0.5 µM (low Fe), 5 µM (moderate Fe) and 50 µM (adequate Fe).

Plants were grown for two weeks under controlled environmental conditions with a photoperiod of 16 h : 8 h and temperature regime of 24°C : 20°C (light : dark), photon flux density of 250 µmol m^{-2} s^{-1} at plant height, and relative humidity of about 70%. The pH of nutrient solutions was adjusted to 6.0 and checked daily. The nutrient solutions were renewed completely every 2 days and continuously aerated. Leaves and roots were collected and immediately frozen in liquid nitrogen. All samples were stored at -70°C prior to use.

Chlorophyll determination

Chlorophyll content in the fully expanded leaves was approximated non-destructively using a portable Chlorophyll Meter SPAD-502 device (Minolta Camera Co., Osaka, Japan).

Determination of Fe in plant tissues

The extracellular Fe contamination was removed from the intact roots during reductive extraction with bipyridyl and sodium dithionite according to the method of BIENFAIT *et al*., 1985, followed by washing with deionised water. Plant organs were then separated and oven dried at 70°C for 48 h. Pulverized dry plant material (0.2 g) was digested in conc. HNO_3 and H_2O_2 mixture (3:2 v/v) and Fe concentration was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; SpectroGenesis EOP II, Spectro Analytical Instruments GmbH, Kleve, Germany).

RNA extraction

Total RNA was extracted using the method described by TRINDADE *et al*., 2010, with slight modifications. Plant tissues (about 500mg) were frozen in liquid nitrogen and ground into a fine powder using mortar and pestle. 3.5ml of extraction buffer containing 8.0M guanidine hydrochloride, 20mM 2-(N morpholino)ethanesulfonic acid (MES), 20mM ethylenediaminetetraacetic acid (EDTA), 2% β-mercaptoethanol was added. After vigorous vortexing equal volume of phenol pH 4.3 was added and samples were centrifuged at 5000rpm for 20min. The supernatants were transferred into fresh tubes and equal volume of SEVAG - phenol: chlorophorm:isoamylalcohol, in ratio 25:24:1 was added and centrifuged at 5000rpm for 20min. SEVAG purification was repeated. Upper phase containing nucleotides was removed to a fresh tube containing 2 volumes of absolute ethanol and 1/20 volumes of 4M sodium acetate (pH5.2) in order to precipitate total RNA. Samples were kept at -20°C overnight and centrifuged on 5000rpm/30min. Pellet was washed by 70% ethanol and after air drying RNAs were resuspended in 300µl of DEPC water. Prior to further procedures, purity and quantity of obtained RNAs was determined using NanoVue. In order to remove DNA contamination from total RNA extracts,

samples were treated with Ambion DNA *free* kit for DNase treatment according to the manufacturer's protocol.

Primer design

The miRNA398 sequence was obtained from the miRBase sequence database. Stem-loop primer for miRNA398 reverse transcription (RTmiR398: 5'-gtcgtatccagtgcagggtcc gag gtattcgcactg gat acgaccagg gg-3') was designed according to VARKONYI-GASIC *et al*., 2007. Additional forward primer (For398: 5'-gcggcggtgtgttct cag gtc g-3') specific for the miRNA398 sequence was designed so as to be the same as miRNA398 sequence but without the last six nucleotides at the 3' end of the miRNA. Universal primer corresponding to universal backbone of stem-loop primer was used as reverse primer in further amplification. Its sequence was obtained from VARKONYI-GASIC *et al*., 2007 (Universal primer: 5'-gtgcagggtccgaggt-3'). Additionally, two pairs of primers for house-keeping gene amplification were designed. One pair was specific for actin (CuAct1: 5'-gctggcatatgttgctct tg-3' and CuAct2: 5'-cgatggtgatgacttgtc ca-3') and another one for U6 spliceosomal RNA (U6f: 5'-gtg cag ggtccgagg t-3' and U6r:5'-gccccgatgcactgcctcttc-3).'

Separate reverse transcription of target miRNAs and endogenous controls –Method1 (M1)

In this method reverse transcription was done using miRNA specific stem loop primer or random hexamers. Reverse transcriptions were performed in total volume of 20µl starting with 1 µg of total RNA and 1 µM stem-loop RT primers specific for miR398. Primer denaturation was performed for 5 min at 65^{o}C. Further, reactions were incubated on ice for 2 minutes. Mastermix was prepared by adding 4µl 5X Reaction buffer (Thermo Scientific), 2 µl 10mM dNTP (Thermo Scientific), 0.5 µl RiboLock RNase Inhibitor (Thermo Scientific) and 1 µl RevertAid Reverse Transcriptase (Thermo Scientific) per each reaction. After incubation for 10 min at 25^{o}C, reverse transcription was done at 43^{o}C for 1 hour. Final transcriptase deactivation was performed at 70^{o}C for 10min.

Alongside, exactly the same amount of RNAs used for reverse transcription with specific RT miR398 were reversely transcribed under the same conditions as previous but using Random hexamers (Thermo Scientific) as RT primers.

Simultaneous reverse transcription of all RNA species– Method2 (M2)

For simultaneous reverse transcription miScript II RT kit (Qiagen) was used **according** to the **manufacturer's** protocol. In a reverse transcription reaction miRNAs and other noncoding RNAs (ncRNAs) are polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT priming. Naturally polyadenilated mRNAs are also converted into cDNA by reverse transcriptase using oligo-dT priming.

Real-time PCR

Prior to SYBR Green assay, all cDNAs were diluted 1:5 with nuclease-free water. In the case of M2, aliquots of the same cDNA sample were used for Real-time PCR with primers designed for target micro RNA398 as well as for actin and U6 as endogenous controls. In the case of M1, cDNA samples obtained using stem-loop RT primer were used as template for PCR with microRNA398 specific and universal primer. cDNA samples obtained using random primer were used as template for actin or U6 primers. Reactions were performed in a 25 µl volume containing

300nM of each primer and 1X SYBER Green PCR master mix (Thermo Scientific). Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystem) with the following regimen of thermal cycling: 2min at 50°C, 10min at 95°C and 40 cycles of (95°C for 15sec, 60°C for 1min). Each PCR reaction was done in triplicate and no-template controls were included. Amplification of PCR products was detected in real time and results were analyzed with 7500 System Software (Applied Biosystem) and presented as 2^{-ddCt}, i.e. $2^{-(dCta-dCtd)}$, where (**a**) is related to adequate Fe, and (d)is related to Fe deficiency.

Statistical analysis

In order to test statistical significance of possible difference between results of miR398 expression obtained by MethodM1 and MethodM2 we used nonparametric Mann-Whitney U test. We set our null hypothesis as follow: there is no significant difference between results obtained applying Method1 and Method2. The level of significance was set at 0.05.

RESULTS AND DISCUSSION

PCR efficiency analysis

For in- house "stem loop" PCR optimization, miR398 was chosen due to its significantly alterable expression under abiotic stresses. It is involved in direct regulation of plant responses to various forms of abiotic stresses such as oxidative stress, water deficit, salt stress, ultraviolet stress, copper and phosphate deficiency.[15]

Specific miR398 stem loop RT primer (**RTmiR398**) was designed in such way to contain a universal backbone which forms a stem-loop structure, caused by complementarity between the nucleotides within the primer sequence. The specificity of RT miR398 primer is placed at the 3' end, representing a six-nucleotide extension which is a reverse complement to the 3' end of miRNA398.

A forward primer (**For398)** specific for the miRNA398 sequence was designed to be the same as miRNA398 sequence but without the last six nucleotides at the 3' end of the miRNA. In order to increase the primer length and melting temperature, a randomly selected nucleotide extension is added to the 5' end of the forward primer. Universal primer (**Up**) corresponding to universal backbone of stem-loop primer was used as reverse primer in amplification (VARKONYI-GASIC *et al.,* 2007). In addition, two pairs of primers were designed for Cucumber endogenous control amplification: one for actin (**CuAct1** and **CuAct2)** and another for small U6 RNA (**U6f** and **U6r**). cDNA sequences of actin and U6 rRNA were obtained from Cucurbit Genomics Database.

The estimation of the efficiency of PCR reaction for each of three pairs of primers was based on a standard curve generated from a two-fold serial dilution of cDNA pool (Fig 1). Mean quantification cycle (Ct) values of each two-fold dilution were plotted against the logarithm of the cDNA dilution factor. An estimation of PCR efficiency was derived from the equations:

$$E=(10^{-1/S} - 1) \times 100,$$

where S represents the slope of the linear regression (PFAFFL, 2004). Based on the slopes of these standard curves, the calculated PCR amplification efficiencies were: 107% (For398/Up) 94% (CuAct1/CuAct2) and 111% (U6f/U6r). Linear correlation coefficients (R2) were ranged from 0.9986 to 0.9992 (Fig 1).

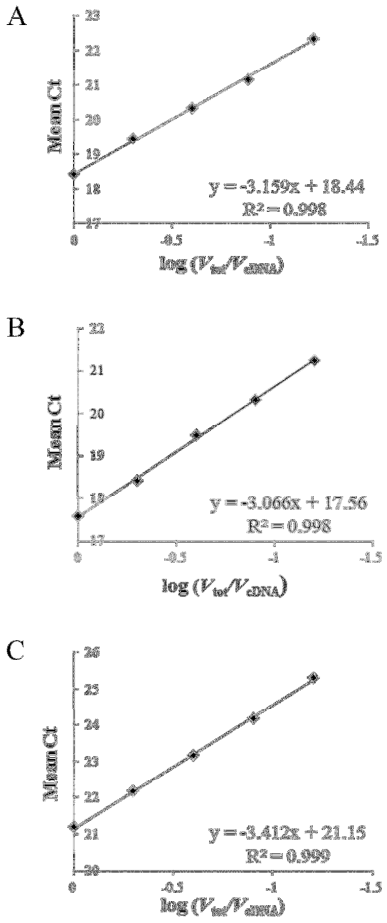

Figure 1.PCR efficiency.Standard curves of miR398**(A)**, U6**(B)**and actin **(C)**generated from a two-fold serial dilution of cDNA pool. Equation of regression curves and linear correlation coefficients (R2) are indicated in the bottom of each graph.

Melting curve analyses were performed following the RT-qPCR. The specificity of the amplicons was confirmed by the presence of a single peak and representative traces are shown (Fig 1). Electrophoretic separation of the amplicons showed a single fragment of the expected size in all cases, with no visible primer-dimer products (data not shown). To identify a more suitable house-keeping gene for gene expression analysis in Fe deficiency experiments we compared ddCt values calculated for each sample with both actin and U6 as internal controls. As expression ratios of these two internal controls were almost identical in all samples regardless experimental conditions we concluded both U6 and actin could be appropriate endogenous controls.

Cucumber miR398 expression at different levels of Fe supply

In this study, we also investigated the changes of miRNA398 gene expressionin cucumber plants at different levels of Fe supply, *viz.* low (severe Fe deficiency), moderate (mild Fe deficiency) and adequate (no Fe deficiency) supply of Fe. The concentrations of Fe and chlorophyll contents are shown in Table I. According to data obtained from measurements of leaf Fe concentrations, the plants were divided into three groups: 1. plants with adequate Fe concentration (adequate Fe), 2. moderately iron deficient plants (moderate Fe) and 3. Plants with low iron content (low Fe).

Table 1.Chlorophyll content and Fe concentrations in leaves of cucumber plants

	Chlorophyll, SPAD-unit	Leaf Fe concentration,µg g^{-1} DW
Adequate Fe	>30	>70
Moderate Fe	20-25	30-50
Low Fe	<10	>20

Furthermore, expression of miRNA398 was analyzed in these three groups. A dramatic decrease of miRNA398 expression was found in both leaves and roots of Fe deficient cucumber plants comparing to control plants with adequate Fe supply (Fig2). However, there was no significant difference in miR398 expression between moderate Fe and low Fe plants.

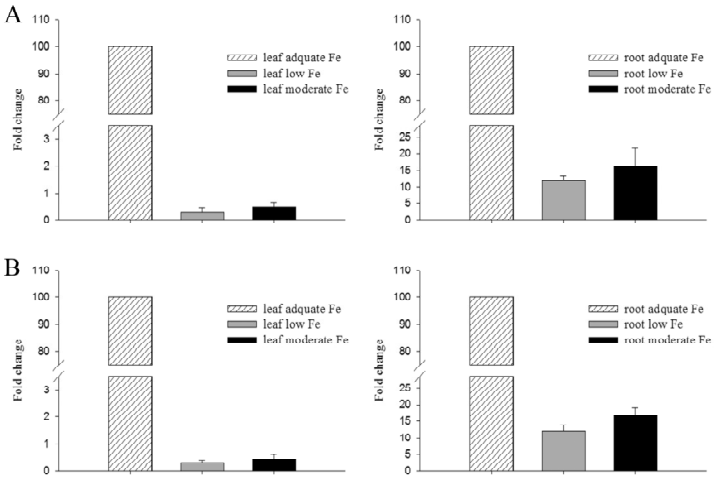

Figure 2.Expression analysis of miR398 under Fe deficiency stress in cucumber leaves androots. Expression analysis performed by Method 1 based on stem loop RT(**A**) and simultaneous conversion of all RNA species using miScript kit(**B**).

The expression data in cucumber leaves and roots showed the same pattern. Recently, it has been demonstrated that Fe deficiency resulted in considerable changes of the gene expression

(WATERS *et al*., 2012). Clearly, Fe deficiency leads to switching on mechanisms that increase Fe uptake and promote more efficient utilization of Fe within plant, which also includes repression of Fe-containing protein translation (e.g. FeSOD). For instance, Fe deficiency, causes up-regulation of Cu,Zn superoxide dismutase genes and down-regulation of Fe superoxide dismutase (FeSOD) genes (WATERS *et al*., 2012). SOD activity is necessary for plant defense against oxidative stress and thus FeSOD activity can be replaced by CuSOD activity. In order to increase CuSOD synthesys Fe deficient plants downregulate miRNA398 which directly repress CuSOD translation. Our experiment indicates that miR398, already known as key factor in regulation of transcription of Cu-containing proteins (BOUCHÉ, 2010), is down-regulated by different intensity of Fe deficiency stress (severe and mild Fe stress, respectively) in cucumber, probably allowing CuSOD to be expressed and to take over FeSOD function as adaptation to Fe limitation.

Verification of results obtained by "stem loop RT PCR technology"

The stem-loop RT-PCR method has been extensively used for detection and quantification of miRNAs (CHEN *et al*., 2005). The advantages of this method include high specificity and sensitivity, combined with ease of use and low cost. The high specificity and sensitivity are achieved through two steps, a miRNA-specific stem-loop reverse transcription, followed by amplification using a miRNA-specific forward primer. However, the endogenous controls need to be transcribed in a separate reaction and with different primer(s). Therefore, we verified the results using a method where the same cDNA template is used for simultaneous reverse transcription of miRNA and endogenous controls. Verification was carried out using a commercial kit based on polyadenilation of all RNA species and therefore suitable for reverse transcription using a common oligodT. The advantage of this approach is simultaneous reverse transcription of the miRNAs and endogenous control, but the specificity is lower and the sensitivity is reduced.

Exactly the same RNA samples of cucumber roots and leaves analyzed by Method M1, were subjected to miScript kit (Method M2). miRNAs and other noncoding RNAs (ncRNAs) were polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT primers. At the same time naturally polyadenilated mRNAs are also converted into cDNA by reverse transcriptase using oligo-dT priming. On first sight, our resultsof miRNA398 gene expression confirmed that separate transcription of target miRNA by stem loop primers and transcription of endogenous control by random hexamers(M1) (Fig 2A) is satisfactory and comparable to the results obtained using simultaneous conversion of all RNA species into cDNA (M2) (Fig.2B). The lack of significant difference between results obtained by M1 and M2 was confirmed using statistical non parametric Mann-Withney test (Table III). Results subjected to statistical analysis were miRNA398 fold change values calculated from delta delta Ct (ddCt) (Table 2).

Analysis of gene expression was accomplished on 4 different samples (roots_lowFe, roots_modFe, leave_lowFe and leave_modFe). In case of M1 we have considered results of miRNA398 expression from two biological experiments. Within each of them four repeats of reverse transcription followed by Real time PCR quantification was performed, resulting in eight repetitions. In the case of M2, two reverse transcriptions from two biological experiments were performed. Because two sets of samples M1 and M2 (Table 2) are small (n1=8 and n2=4), we used the Mann Whitney U testto determine whether these two groups of results are not different.

Table 2. Fold change of miR398 expression in roots and leaves of cucumber plantsgrown inmoderately iron (moderate Fe) or intense iron deficiency (low Fe). Fold changes (2^{-ddCt}) are calculated relatively to control plants grown under adequate Fe.

Variable	Root_LowFe		Root_ModFe		Leaf_LowFe		Leaf_ModFe	
Meth. / nr	M1	M2	M1	M2	M1	M2	M1	M2
1	11.23	13.41	17.38	14.65	0.27	0.22	0.45	0.22
2	12.62	10.97	15.12	16.41	0.21	0.42	0.39	0.68
3	11.04	10.42	14.21	19.97	0.12	0.29	0.47	0.47
4	13.23	12.98	15.81	16.19	0.27	0.24	0.28	0.34
5	10.98		17.98		0.31		0.38	
6	11.33		14.16		0.35		0.58	
7	10.32		18.63		0.66		0.81	
8	13.92		16.78		0.26		0.50	

*nr- number of repetitions, Meth. - methods

Table 3. Results of Mann-Withney non-parametric test.

Variable	z-value	p-value
Root_lowFe	0.17	0.86
Root_modFe	-0.34	0.73
Leaf_lowFe	0.00	1.00
Leaf_modFe	0.68	0.50

As all Z values shown in Table 3.are within range ±1.96, we concluded there was no significant difference between two methods (M1 and M2) that we applied for miRNA398 gene analysis. According to statistical analysis of data from our preliminary experiments concerning role of miRNA398 in nutrition stress, usage and synthesis of new stem loop primers for miRNA expression analysis in further experiments is adequate.

CONCLUSIONS

Stem loop qPCR is reliable and inexpensive method for quantification of miRNA gene expression, as we demonstrated for miRNA398 expression in iron deficient cucumber plants. U6

and actin are suitable genes for use as endogenous control since we have confirmed their stable expression in plants grown under different iron supply regimes. Strong reduction in miRNA398 expression in cucumber leaf and root at limited iron supply indicate that it plays an important role in response to iron deficiency stress.

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REFERENCES

BIENFAIT, H.F., W. VAN DER BRIEL, N.T. MESLAND-MUL (1985): Free Space Iron Pools in Roots. Plant Physiol., *78*: 596-600

BOUCHÉ, N. (2010): New insights into miR398 functions in Arabidopsis. Plant Signal Behav.**,** *5(6)*: 684–6

BRODERSEN, P., L. SAKVARELIDZE-ACHARD, M. BRUUN-RASMUSSEN, P. DUNOYER, Y.Y. YAMAMOTO, L. SIEBURTH, O. VOINNET (2008): Widespread translational inhibition by plant miRNAs and siRNAs. Science, *320*: 1185-90

CHEN, C., D.A. RIDZON, A.J. BROOMER, Z. ZHOU, D.H. LEE, J.T. NGUYEN, M. BARBISIN, N.L. XU, V.R. MAHUVAKAR, M.R. ANDERSEN, K.Q. LAO, K.J. LIVAK, K.J. GUEGLER (2005): Real-time quantification of microRNAs by stem–loop RT–PCR. Nucleic Acids Res., *33(20)*: e179

HE, L. and G.J. HANNON (2004): MicroRNAs: small RNAs with a big role in gene regulation. Nature , *5(7)*: 522-31

JAGADEESWARAN, G., A. SAINI, R. SUNKAR (2009): Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta, *229(4):* 1009-14

LELANDAIS-BRIÈRE, C., C. SORIN, M. DECLERCK, A. BENSLIMANE, M. CRESPI, C. HARTMANN (2010): Small RNA diversity in plants and its impact in development. Curr. Genomics, *11(1)*: 14-23

PFAFFL, M.W. (2004): Quantification Strategies in real-time PCR. A-Z of quantitative PCR. Published by International University Line, La Jolla, CA, *3*: 87-112

ROGERS, K. and X. CHEN (2013): Biogenesis, Turnover, and Mode of Action of Plant MicroRNAs. Plant Cell, *25(7)*: 2383-99

RÖMHELD, V. and M. NIKOLIC (2007): Handbook of Plant Nutrition, CRC Press, Boca Raton: 329-350

RUBIO-SOMOZA, I. and D. WEIGEL (2011): MicroRNA networks and developmental plasticity in plants. Trends Plant Sci., *16(5)*: 258-64

SUNKAR, R., V. CHINNUSAMY, J. ZHU, J-K. ZHU (2007): Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends in Plant Sci., *12(7)*: 301-9

SUNKAR, R., Y.F. LI, G. JAGADEESWARAN (2012): Functions of microRNAs in plant stress responses. Trends Plant Sci., *17 (4)*: 196-203

TRINDADE, I., C. CAPITÃO, T. DALMAY, M.P. FEVEREIRO, D.M. DOS SANTOS (2010): miR398 and miR408 are up-regulated in response to water deficit in Medicago truncatula. Planta, *231*: 705-16

VÁLÓCZI, A., C. HORNYIK, N. VARGA, J. BURGYÁN, S. KAUPPINEN, Z. HAVELDA (2004): Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res., *32(22)*: e175

VARKONYI-GASIC, E., R. WU, M. WOOD, E.F. WALTON, R.P. HELLENS (2004): Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods, *3*: 12

WATERS, B.M., S.A. MCINTURF, R.J. STEIN (2012): Rosette iron deficiency transcript and microRNA profiling reveals links between copper and iron homeostasis in Arabidopsis thaliana. J. Exp. Bot., *63(16)*: 5903-18

YU, B., Z. YANG, J. LI, S. MINAKHINA, M. YANG, R.W. PADGETT, R. STEWARD, X. CHEN (2005): Methylation as a crucial step in plant microRNA biogenesis. Science, *307 (5711)*: 932-5

USPOSTAVLJANJE I VALIDACIJA „STEM-LOOP RT PCR" METODE ZA ANALIZU EKSPRESIJE MIKRORNK398 GENA

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Izvod

Mikro RNK (miRNK), klasa malih nekodirajućih RNK molekula, imaju značajnu ulogu tokom razvića, kao i u odgovoru na različite stresogene faktore spoljašnje sredine. Neke od biljnih mikro RNK imaju važnu ulogu u finoj regulaciji i održavanju homeostaze nutrijenata u uslovima kada je određeni element prisutan u višku ili manjku u odnosu na optimalnu koncentraciju za biljku. Za razumevanje bioloških funkcija određenih miRNK neophodno je razvijanje efikasne metodologije za praćenje njihove genske ekspresije. U okviru ovog rada pokazujemo uspostavljanje i validiranje metode za analizu miRNK genske ekspresije u krastavcu u uslovima deficijencije gvožđa. Reverzna transkripcija "stem-loop" prajmerima u kombinaciji sa „Real-time PCR" metodom je jedan od načina za kvantifikaciju ekspresije miRNK. Prikazan je postupak dizajniranja „stem-loop" prajmera specifičnih za miRNK398, optimizacija i određivanje efikasnosti odgovarajuće „Real-time PCR" reakcije. Ovaj pristup omogućio nam je uvid u promene nivoa ekspresije miRNK398 u model biljci gajenoj u različitim uslovima deficijencije gvožđa. Metod jeverifikovan poređenjem dobijenih podataka sa rezultatima ekspresije miRNK398 u istim uzorcima, primenom komercijalnog kita. Potvrđivanje tačnosti metode od velikog je značaja s obzirom na to da se zarazliku od „stem-loop RT" gde se vrši odvojena transkripcija target gena i endogene kontrole, komercijalnim kitom efikasno izvod i simultana konverzija svih RNK u cDNK..

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