Identification and Functional Characterization of the *GALACTINOL SYNTHASE (MoGolS1)* Gene in *Melissa officinalis* Plants

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Galactinol and raffinose accumulation in plants is associated with stressful environmental conditions such as cold, heat, or dehydration by the action of *galactinol synthase (GolS)* in the raffinose family of oligosaccharides biosynthetic pathway from UDP-galactose. Moreover, several reports mentioned that *GolS* transcription is up regulated by various environmental stresses like cold, heat, dehydration. Therefore, to determine whether *MoGolS1* was induced with the abiotic stress we analyzed the expression pattern of the gene under various abiotic stresses like heat, cold, abscisic acid, sucrose and salt concentration in the lemon balm plants grown in standard MS medium. The *MoGolS1* gene was 981-bp in length encoding 326 amino acids in its sequence and shared 77 and 76% sequence similarity with *Arabidopsis thaliana galactinol synthase4 (AtGolS4)* and *AtGolS1* genes respectively. The *MoGolS1* gene was strongly expressed by the abiotic stress induced by sucrose, ABA or heat shock. It was also expressed in responses to cold, manitol and NaCl. *MoGolS1* gene induction with various stresses may be possible for its crucial function in abiotic stress tolerance in plants, providing a good engineering target for genetic engineering.

Key words: abiotic stress, abscisic acid, galactinol synthase, *Melissa officinalis*, sucrose

Introduction

Soluble sugars including raffinose family oligosaccharides (RFOs) have been implicated in mitigating the effects of environmental stresses on plants [Downie *et al.*, 2003]. Their accumulation in plants is associated with stressful environmental conditions such as cold, heat, or dehydration [Santarius, 1973; Santarius and Milde, 1977; Hinesley *et al.*, 1992; Ashworth *et al.*, 1993; Wiemken and Ineichen, 1993; Bachmann *et al.*, 1994; Taji *et al.*, 2002] when they presumably assist sucrose in ameliorating plant stress [Zhao *et al.*, 2004]. It is also proposed to play significant roles in protecting cellular integrity during desiccation or dehydration and provides substrates for energy generation during seed germination [Downie *et al.*, 2003].

Galactinol is reported to be involved in tolerance to drought, high salinity and cold stresses which is produced from UDP-Gal and *myo* inositol by the action of Galactinol synthase (*GolS*). Because the only known function of galactinol is the formation

of RFOs [Saravitz *et al.*, 1987; Liu *et al.*, 1995], which is putatively the committed enzyme step in the RFO biosynthetic pathway [Pridham and Hassid, 1965; Saravitz *et al.*, 1987; Smith *et al.*, 1991; Peterbauer and Richter, 2001] from UDP-galactose. Thus *GolS* catalyses the first step in the biosynthesis of galactinol and RFO from UDP-galactose and plays regulatory roles in carbon partitioning between sucrose and RFOs [Saravitz *et al.*, 1987] under the abiotic stress conditions, and that galactinol and raffinose function as osmoprotectants in drought-stress tolerance of plants.

The *GolS* gene is thought to play potential roles in catalyzing metabolic steps, and provides an important experimental tool for manipulating the level of RFOs as osmoprotectants. These gene products are reported to function in the accumulation of osmoprotectants; detoxification, protection of cells; protein turnover; stress-signalling pathways; and transcriptional regulations [Bohnert *et al.*, 1995; Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997].

GolS activity in kidney bean seeds is increased on exposure of plants to cold, and the expression of *GolS* genes is induced by cold stress in *Arabidopsis* and *Ajuga reptans* plants [Liu *et al.*, 1995; Sprenger and Keller, 2000]. During cold acclimation, RFOs accumulate in leaves of *A. reptans* [Bachmann *et al.*, 1994]. *AtGolS1* and *AtGolS2* is reported to be induced by drought and high-salinity stresses, while *AtGolS3* is induced by cold.

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Galactinol Synthase gene of *Coptis japonica* (*CjGolS*) is involved in berberine tolerance [Takanashi *et al.*, 2008]. Over expression of Arabidopsis *GolS1* and *GolS2* had increased levels of galactinol and raffinose in the leaves compared with wild-type plants under control growth conditions in the transgenic plants were correlated with increased tolerance to methyl viologen (MV) treatment and salinity or chilling stress probably by scavenging hydroxyl radicals as a novel function to protect plant cells from oxidative stress [Taji *et al.*, 2002; Nishizawa *et al.*, 2008]. Furthermore, *GolS1* in Arabidopsis is reported as a novel heat shock factor (HSF) target gene, which is responsible for heat stress-dependent synthesis of raffinose, [Panikulangara *et al.*, 2004] and may have influence in heat shock tolerance in the plants. Although the roles of *GolS* genes have been elucidated in many plants, nothing is known about it in the Lemon balm (*Melissa officinalis*), a very important medicinal plant species. As a result, we therefore decided to report here after identification and functional characterization in wild type lemon balm and transgenic Arabidopsis plants.

Materials and Methods

Plant growth conditions and treatments. *In vitro* regenerated containerized lemon balm (*M. officinalis* L.; *Lamiaceae*) plants grown in solid Murashige and Skoog (MS) medium 4 g/L Murashige and Skoog, 2% sucrose solidified with 7 g/L agar at pH 5.7 prior to autoclaving) were used for this study. Approximately 2 cm long explants, including shoot tip and one pair of leaves, were transferred to the culture medium and incubated in a controlled environment of 25.0°C in standard photoperiod (16 h light and 8 h dark) with a light intensity of 40 $\mu\text{mol}/\text{m}^2\text{s}$ provided by cool-white florescent lamps. For stress treatment, explants were grown in solid MS medium for 15 days and subjected to various treatment conditions. To examine the effects of sucrose concentration on gene expression, uprooted (15 days after planting) plants were exposed to liquid MS medium supplemented with 250 mM sucrose for 0, 6, 12 or 24 h. The effect of abscisic acid (ABA) was determined by placing the uprooted plants into liquid medium having 100 μM ABA for 0, 1, 3 or 6 h. The plants were cultured in medium with 300 mM NaCl or Mannitol for 0, 1, 3 or 6 h for assessing the effect of salts stress on the gene expression. For heat shock treatment, the plants grown in solid medium were placed at 37°C for 0, 1, 3, 6 or 12 h. Cold stress was provided by placing the plants in container at 4°C for 0 h, 12 h, 1 day, 2 day, 3 day or 6 day. Harvested roots, shoots, and leaves were frozen in liquid nitrogen and stored at -80°C until analyzed. T_1 generation seeds of transgenic Arabidopsis were surface sterilized and stratified at 4°C for 3 days and allowed to germinate on MS medium at 23 $\pm$ 1°C under a 16 h light/8 h dark

cycle.

All of the chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO) except for the ABA and fluridon, which were purchased from Duchefa Biochemie (Postbus, Haarlem, The Netherlands), random primer DNA labeling system from Invitrogen (San Diego, CA) and the ethanol, which were purchased from Merck (Darmstadt, Germany).

Lemon balm cDNA library construction (Commercial service). At the beginning of this study there was no information about genomic or expressed sequence tag (EST) for the lemon balm in the database (<http://www.ncbi.nih.gov/BLAST/>). Therefore we prepared the EST from the lemon balm cDNA library. Total RNA was purified with TRIzol from the lemon balm leaves grown with 250 mM sucrose in MS medium for 20 days. Poly(A)⁺ was purified with PolyA Tract mRNA isolation system (Promega, Madison, WI) and cDNA was synthesized with cDNA synthesis kit (Stratagene, La Jolla, CA). cDNAs were directionally cloned with *EcoRI* on the 5' and *XhoI* on the 3' end into the Uni-ZAP XR vector (Stratagene). The vector containing the DNA was then transformed into phage and Primary Lambda Phage Library was constructed by Gigapack III Gold Packaging Extract (Stratagene). EST was established by transforming into the host cells SOLR strain and nucleotide sequencing of the genes.

Amino acid sequence comparison and phylogenetic analysis. The *MoGolS1* gene was identified from the lemon balm EST following nucleotide sequencing with BLASTN program (<http://www.ncbi.nlm.nih.gov>). Nucleotides of *MoGolS1* genes was translated into amino acids by using Expasy translation tool (www.expasy.ch/tools/dna.html). The nucleotide and amino acid sequences of *MoGolS1* and its related genes from other plant species were obtained from the nucleotide and protein databases of NCBI (<http://www.ncbi.nlm.nih.gov>). Amino acid sequences were then compared and phylogenetic trees were constructed using a multiple sequence alignment program, CLUSTAL W software (<http://workbench.sdsc.edu>).

RNA-gel blot analysis. Wild type lemon balm explants were grown on a standard MS medium for 15 days and exposed to various treatments for required period of time. Total RNA was purified using aurintricarboxylic acid (ATA) and lithium chloride [Lee *et al.*, 2002] or RNeasy Plant Mini kit (QIAGEN, Hilden, Germany). Northern analysis was performed following the procedure reported Kwon *et al.* [2007]. Fifteen microliter of total RNA was fractionated by electrophoresis on 1.2% (w/v) agarose gels (containing 1 $\times$ 3-(N-morpholino) propanesulfonic acid (MOPS) and 0.66 M formaldehyde) and transferred on to a Hybond nylon membrane (Amersham, Buckinghamshire, UK) with 20 $\times$ SSC by capillary action and UV cross linked. For Northern hybridization, DNA probes for *MoGolS1* was

amplified with gene specific primers (5' GCGCAGTAGTGAAC AACTTC 3' forward and 5' CTCGACACACCTGATGGCTA 3' reverse to amplify 383-bp fragment of *MoGolS1* from lemon balm cDNA. Probes were labeled with α -³²P dCTP using a random primer DNA Labeling System (Invitrogen). After pre-hybridization for 1h, the hybridization of α -³²P-random-primed probes onto RNA blots was carried out at 65°C overnight. The RNA blot was then washed twice in 1% SSC, 0.1% SDS and once in 0.1% SSC, 0.1% SDS at 42°C prior to autoradiography.

Molecular cloning of *MoGolS1* gene. The 5' and 3' Rapid Amplification of cDNA Ends (5'- and 3'-RACE) of candidate genes were performed (Commercial service) to identify open reading frame (ORF). Full length DNA fragments were then amplified from the lemon balm cDNA with gene specific primers (5' ATGGGGAGGAAGAAAAGGCA 3' forward and 5' AGCAGCAGAAGGGGCGAGGTA 3' reverse for *MoGolS1*) and inserted into the TOPO entry vector (Spectinomycin resistance) and transformed into *E. coli* chemically competent cells through Gateway Cloning System (Invitrogen) following manufacturer's recipe. After confirmation of successful transformation by colony polymerase chain reaction (PCR) (with M13 forward primers and gene specific reverse primers) and nucleotide sequencing the genes were cloned into the destination vector PMDC 32 for over expression and PMDC83 for 35S::gene::green fluorescent protein (GFP) localization using recombinant kits (Invitrogen) and transformed into *E. coli* (DH5 α) through electroporations. The transformation was confirmed again with colony PCR (with gene specific forward and reverse primers) and nucleotide sequencing. Finally the genes were inserted into the *Agrobacterium tumefaciens* and were transformed with Col-0 Arabidopsis plants by floral dipping. Transgenic T₁ Arabidopsis plants were screened with hygromycin resistance.

Quantification of galactinol. The galactinol dihydrate standard and plant extracts treated with six different conditions were analyzed by ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometer (UPLC/Q-ToF MS, Bio-Rad, Berkeley, CA). UPLC was performed using a Waters ACQUITY UPLC™ system (Waters Corp., Milford, MA). The chromatography was performed on an Waters ACQUITY BEH HILIC column (100 mm × 2.1 mm, 1.7 μ m). The mobile phase consisted of (A) 0.1% formic acid in distilled water and (B) 0.1% formic acid in acetonitrile. The Ultra Performance Liquid Chromatography (UPLC) gradient condition was optimized as follows: linear gradient from 90 to 30% B (0–3.0 min), held at 30% B (3.0–3.7 min), and returned to the initial condition at 90% B (3.7–5.0 min). The flow rate was 300 μ L/min and the injection volume was 5 μ L. The column and autosampler was maintained at 35 and 15°C, respectively. The Q-ToF MS was performed using Q-TOF Micro mass detector (Waters,

Manchester, UK). Mass spectra of all samples were acquired using electrospray ionization in negative ion mode and ion preparation in single ion monitoring mode. The capillary voltage, sample cone voltage, extraction cone voltage, desolvation temperature, and source temperature were set at 2800, 28, 1.2 V, 300, 100°C, respectively. Leucine-enkephalin was used as a reference ion to measure accurate mass. Data acquisition and processing were conducted with MassLynx version 4.1 (Waters). QuanLynx software (ver. 4.0, Micromass, Manchester, England) was used for generation of a calibration curve and quantification of analytes. For quantitative analysis of galactinol, standard solutions at five concentration levels between 0.05 and 5 μ g/mL were used. Linearity of the multi-point calibration curves was determined by plotting the peak area ratios of the analyte using QuanLynx software. The galactinol levels in plant extracts treated with six different methods were quantified based on the calibration curve.

Results

Identification of lemon balm *Galactinol Synthase* gene.

During EST sequencing, we noticed interesting gene *GolS* in the lemon balm cDNA library which was very similar to other *GolS* genes from Arabidopsis and other plants. We decided to study on that gene too in the present research work. By using reverse transcription (RT)-PCR and 5'-3'- rapid amplification of cDNA ends (RACE), we obtained the full length *galactinol synthase* genes from lemon balm (*M. officinalis* L.), which we named *MoGolS1* genes. The *MoGolS1* was 981-bp in length containing open reading frame (ORF) encoding 326 amino acid in its sequence. The amino acid comparison shows that it shared 77 and 76% amino acid sequence with *Arabidopsis thaliana galactinol synthase* (*AtGolS*)4 and *AtGolS1* genes respectively. The *MoGolS1* also shares a considerable percentage (60–70%) of amino acid identity with *GolS* genes from other plants having distinct characteristics of hydrophobic pentapeptides (APSAA) at the conserved carboxy terminus of the sequences (Fig. 1A). A phylogenetic tree for important plant *GolS* genes including *MoGolS1* was constructed by using the full-length deduced amino acid sequencing (Fig. 1B).

***MoGolS1* gene transcription is up-regulated by sucrose concentration.** In our preliminary experiment we observed maximum transcription of *MoGolS1* gene in the plants exposed to 250 mM sucrose (data not shown). We therefore treated the plants in liquid medium with 250 mM sucrose for 0, 6, 12 or 24 h prior to purify total RNA for northern analysis. Sucrose concentration as well as treatment duration significantly enhanced the transcription levels of *MoGolS1* genes in the wild type lemon balm plants. Figure 2 (A and B) exhibits that *MoGolS1* gene was predominantly expressed in leaves

Fig. 1. Multiple sequence alignment and phylogenetic tree of *GolS* amino acids from various higher plants. (A) Deduced amino-acid sequence comparison of the *MoGolS1* with *GolS* from other plant species (indicated in the left margin) by CLUSTAL W program. The identical residues are shaded in dark gray with black letters. Residues with at least 75% to 50% conservation are shaded in light gray. Amino acid residue numbers are indicated on the left in the alignment. *Triticum aestivum GolS1* (*TaGolS1*; Accession AB250356.1), *T. aestivum GolS2* (*TaGolS2*; AB250357.1), *Zea Mays GolS1* (*ZmGolS1*; NM_001112278.1), *Z. mays GolS2* (*ZmGolS2*; NP_001105749.1), *Z. mays GolS3* (*ZmGolS3*; EU962434.1), *Arabidopsis thaliana GolS1* (*AtGolS1*; AB062848.1), *A. thaliana GolS2* (*AtGolS2*; AB062849.1), *A. thaliana GolS3* (*AtGolS3*; AB062850.1), *A. thaliana GolS4* (*AtGolS4*; NM_104734.3), *A. thaliana GolS6* (*AtGolS6*; NP_567741.2), *A. thaliana GolS7* (*AtGolS7*; NM_104732.1), *Melissa officinalis GolS1* (*MoGolS1*). (B) A phylogenetic tree derived from the amino acid sequence comparison in (A) above.

irrespective to the treatment. However, the expression level was significantly higher in the sucrose treated leaves for 24 h followed by 12 h than the control leaves (Fig. 2B). Although there was a constitutive expression of the gene in the roots, no transcription was detected in the stem tissues whether the plants were treated with sucrose.

ABA enhances the *MoGolS1* transcription in lemon balm plants. As we know that high concentration of sucrose induces

oxidative stress to the plants mediated by ABA dependent or independent pathway, we wondered whether the ABA induced the transcription level of *MoGolS1* gene in the lemon balm plants. We treated the uprooted lemon balm plants in liquid MS medium supplemented with 100 μ M ABA for 0, 1, 3 or 6 h before extracting the total RNA for northern hybridization. Figure 3A shows that *MoGolS1* transcription level was significantly enhanced with the abiotic stress induced by ABA

Fig. 2. Northern blot analysis of *MoGolS1* genes in wild-type lemon balm leaves (L), stems (S), and roots (R). Lemon balm explants were grown on standard MS medium for 15 days and treated in liquid MS medium supplemented with 250 mM sucrose for 0, 6, 12 or 24 h. Total RNA was extracted with aurintricarboxylic acid (ATA) and lithium chloride and separated by electrophoresis on a formaldehyde agarose gel followed by transferring to a nylon membrane. Northern blots (15 μ g per lane) were hybridized with α - 32 P labeled cDNA probes of *MoGolS1* (A). Relative transcription level of the *MoGolS1* (B) genes was calculated by Multi Gauge software (version 3, Fujifilm corp, Tokyo, Japan) in leaves (white bar), stems (shaded bar) and roots (black bar) as ratio to the blank. Equal loading of RNA samples in each lane was verified by ethidium bromide staining of ribosomal RNA shown beneath the northern blots.

Fig. 3. Effects of ABA on *MoGolS1* gene expression in lemon balm plants. After 15 days of growth in solid MS medium, lemon balm plants were treated in liquid medium supplemented with 100 μ M ABA for 0, 1, 3 or 6 h. Total RNA was purified from leaf (L), root (R) and stem (S) tissues with aurintricarboxylic acid (ATA) and lithium chloride and northern hybridization was performed with α - 32 P labeled cDNA probes of *MoGolS1* (A). Equal loading of RNA samples (15 μ g in each lane) was verified by ethidium bromide staining of ribosomal RNA shown beneath the northern blots. The transcript level of *MoGolS1* (B) was determined in leaves (white bar), stem (shaded bar) and roots (black bar) as ratio of sample transcription to the blank.

over the incubation periods. The transcription started within very short period of incubation even within 1 h of treatment. Like sucrose, the *MoGolS1* expression level was considerably higher in leaf tissues compared to the stem and roots due to the ABA

Fig. 4. Transcription levels of *MoGolS1* gene in lemon balm plants in response to mannitol. Whole plants were treated with 300 mM mannitol in liquid medium for 0, 1, 3, or 6 h. Total RNA was purified from the leaves (L), stems (S) and roots (R) and probed with *MoGolS1* (A) DNA fragments. Ethidium bromide-stained gel images of rRNA bands are shown beneath the northern blots. Relative transcript levels of *MoGolS1* (B) gene in leaves (white bar), stems (shaded bar) and roots (black bar) was shown as ratio of sample transcript to the blank.

treatment. Expression level of the gene in root tissue was almost constant irrespective to the treatments (Fig. 3B). However, transcription was gradually down-regulated in the stem tissues over the incubation period and it was distinct after 3 h of the treatment. This data implicated that the sucrose induced induction of *MoGolS1* gene in lemon balm plants might be ABA dependent.

***MoGolS1* gene expression in response to Mannitol, NaCl, cold or heat.** Since, *AtGolS1* and *AtGolS2* gene, two homolog of *MoGolS1* are reported to induce with dehydration and high salinity [Taji *et al.*, 2002], we examined whether the *MoGolS1* was expressed by dehydration stress induced by the application of mannitol and high salinity with NaCl. As expected, the transcription level of the *MoGolS1* gene was markedly enhanced in the leaves treated with mannitol for 1 h and NaCl after 3 h of incubation in the treatment medium (Fig. 4A and 5A). The maximum transcription of *MoGolS1* was detected just after 1 h of incubation in mannitol and 6 h in NaCl indicates that the transcription of *MoGolS1* in the mannitol was faster than the NaCl. Although irregular, weak expression of the gene was also detected in the stem tissues treated with both mannitol and NaCl without much difference. The transcription of *MoGolS1* in the root tissue was weak and constitutive (Fig. 5B).

To determine whether the cold stress enhanced the transcription level of *MoGolS1* gene in the lemon balm leaves, we treated the plants at 4°C prior to purify of total RNA. From the northern blot analysis, it was observed that the transcription level of *MoGolS1* gene increased in the leaves treated for 12 h or 1 day in cold. The transcription level of the gene after 2 days of cold treatment was

Fig. 5. Expression of *MoGolS1* gene in response to NaCl. Total RNA was purified from the leaves (L), stems (S) and roots (R) treated with 300 mM NaCl for 0, 1, 3 or 6 h in liquid medium. Northern blot analysis was carried out with RNA blot with α - 32 P labeled *MoGolS1* (A) DNA probes. Equal loading of RNA samples (15 μ g in each lane) was verified by ethidium bromide staining of ribosomal RNA shown beneath the northern blots. The transcript level of *MoGolS1* (B) was determined in leaves (white bar), stems (shaded bar) and roots (black bar) as ratio of sample transcript to the blank.

Fig. 6. Cold induced transcription of *MoGolS1* and *MoMYB3* genes in lemon balm leaves. Total RNA was purified from the leaves treated with cold (4°C) 0 h, 12 h, 1 day, 2 day, 3 day or 6 day. Northern hybridization analysis was carried out with RNA blot with α - 32 P labeled *MoGolS1* DNA probes. Equal loading of RNA samples (15 μ g in each lane) was verified by ethidium bromide staining of ribosomal RNA shown beneath the northern blot.

irregular in pattern (Fig. 6A). One of the homolog of the *MoGolS1*, the *AtGolS1* is known as a Nobel heat shock factor target gene, induced even in wild type Arabidopsis plants after heat stress [Panikulangara *et al.*, 2004]. We therefore investigated whether our *GolS1* gene (*MoGolS1*) was induced with heat shock treatment. Lemon balm plants (15 days after planting them in the MS medium) were treated with heat stress at 37°C for 0, 1, 3, 6 or 12 h before extracting total RNA from leaf, stem and root tissues. It was noticed that heat stress greatly enhanced the *MoGolS1* gene expression level in the lemon balm leaves after 3 h of incubation (Fig. 7A and B). Unlike sucrose or ABA treatment, considerable increasing of transcription level was also observed in the roots after 1h of incubation period. However, the expression level of *MoGolS1* gene in the stem tissue was almost similar irrespective to the treatment durations.

Galactinol dihydrate level is decreased in the lemon balm leaves. Galactinol dihydrate standard was analyzed in the negative ion mode, and both galactinol and galactinol dihydrate

Fig. 7. *MoGolS1* gene expression in lemon balm plants under heat stressed condition. Northern blot analysis was carried out on RNA blot (15 μ g per lane) purified from wild-type lemon balm leaves (L), stems (S), and roots (R) treated with heat stress (HS) at 37°C for 0, 1, 3, 6 or 12 h with α - 32 P labeled cDNA probes of *MoGolS1* (A). Relative transcript levels of *MoGolS1* (B) in leaves (white bar), stems (shaded bar) and roots (black bar) shown was calculated as ratio of the sample transcript to the blank.

were detected at 341 and 377 Da as [MH]⁺ ions, respectively. Based on the mass spectrum, galactinol was chosen to be used for quantification since it showed higher ion intensity compared to galactinol dehydrate. LC/MS analysis as a single ion monitoring (SIM) at *m/z* 341 on a standard compound was performed to identify galactinol. Galactinol dihydrate standards were prepared at five different concentration levels, namely 5, 1, 0.5, 0.1, 0.05 μ g/mL. Calibration curve was generated with relative peak areas against concentration using QuanLynx software. The curves showed good linearity and the concentration coefficient was found to be in the range of 0.9996. To quantitate the changes in the level of galactinol depending on six different treatment methods, standard compound was identified in plant extracts using retention time and *m/z* value by LC/MS analysis. Detected peak areas of analytes with unknown concentrations were applied to the fitted calibration curve, and the galactinol concentrations of each sample were calculated. As shown in Fig. 8, the concentrations of galactinol were decreased in sucrose, ABA, NaCl, mannitol, heat or cold when compared to the control.

Discussion

Through EST sequencing, we identified *GolS* gene (*MoGolS1*) from lemon balm cDNA library. Plant *galactinol synthase* genes play multiple roles in the defense mechanisms to protect the plants from various stresses and adverse environmental conditions. We observed that the *MoGolS1* transcription was significantly up regulated in the leaves treated with 250 mM sucrose for 12 to 24 h compared to the control

leaves (Fig. 2A and B). Although there was no report about the transcriptional activation of *GolS1* gene with the higher concentration of sucrose, reports indicate that elevated level of sucrose induces osmotic stress to the plants [Javed and Ikram., 2008] which might be responsible for the sucrose induced transcription of the *MoGolS1* gene in the lemon balm leaves since Liu *et al.* [1995] mentioned the accumulation of GolS in response to the osmotic stress in plants. Moreover, Nagira *et al.* [2006] reported that elevated sucrose concentration (7%) increased the endogenous ABA level in the regenerated torenia plants. The osmotic stress induced by sucrose in the plants is proposed either ABA dependent or independent. To elucidate the factors affecting the sucrose induced elevated transcription of *GolS1* in the lemon balm leaves, we treated lemon balm plants with 100 μ M ABA for various period of time and performed northern hybridization. As expected, we noticed that the transcription level of the *GolS1* was strongly up regulated in the lemon balm leaves treated with ABA for 1 or 3 h (Figs. 3A and B) while Taji *et al.* [2002] reported a week induction of *AtGolS1* and *AtGolS2* by ABA but not *AtGolS3*.

There are reports that other stresses also enhanced the *GolS* gene transcription. For examples, Taji *et al.* [2002] reported that *AtGolS1* and *AtGolS2* was induced by drought and high salinity but *AtGolS3* was induced by the cold stress, while Panikulangara *et al.* [2004] mentioned that Arabidopsis *GolS1* was heat inducible. To determine whether other types of abiotic stress also induced *MoGolS1* transcription in the lemon balm plants we carried out northern hybridization purifying RNA from lemon balm leaves, stems and roots treated with 300 mM mannitol or NaCl. The results show that transcription level of the *MoGolS1* gene was markedly enhanced in the leaves treated with mannitol after 1 h and NaCl after 3 h of incubation in the treatment medium (Figs. 4A and 5A). The maximum transcription of *MoGolS1* was detected just after 1h of treatment in mannitol and 6 h in NaCl indicates that the transcription of *MoGolS1* in the mannitol was faster than the NaCl. Although there was no report about the transcription of *GolS* gene by mannitol, strong induction of *AtGolS2* was reported by 250 mM NaCl for 2–8 h [Taji *et al.*, 2002]. As mentioned by Taji *et al.* [2002] and Panikulangara *et al.* [2004] that *GolS* gene also induced by the heat and cold stress, we treated the lemon balm plants with heat (37°C) or cold (4°C) before extracting total RNA. Our northern data shows that heat stress for 3 h greatly influenced the activation of *MoGolS1* transcript in the lemon balm leaves (Figs. 6A and B). Our result was supported by the findings of Panikulangara *et al.* [2004] who mentioned strong induction of *GolS1* gene both in wild type (WT) and heat shock factor3 (HSF3) TPs Arabidopsis plant subjected to heat stress (37°C) for 2h. The transcription level of *MoGolS1* gene also increased at moderate level in the leaves treated with cold for 12 h or 1 day. These data supported the

Fig. 8. Quantitation of galactinol dehydrate. For quantitative analysis of galactinol, standard solutions at five concentration levels between 0.05 and 5 μ g/mL were used. Linearity of the multi-point calibration curves was determined by plotting the peak area ratios of the analyte using QuanLynx software. The galactinol levels in plant extracts after stress treatment were quantified based on the calibration curve.

findings of Taji *et al.* [2002], who reported strong induction of *AtGolS3* transcription when Arabidopsis plants were treated with cold for 10–24 h at 4°C. Similar result was also reported by Downie *et al.* [2003] mentioning that tomato galactinol synthase (*LeGolS1*) gene induction in the leaves of seedlings was maximum when treated with cold (5°C) for 12 h. The transcription level of *MoGolS1* gene after 2 days of cold treatment was weak and irregular (Fig. 6A). Similar characteristics of gene expression was observed for *AtGolS1* which strongly induced by the drought, high salinity and weakly by ABA [Taji *et al.*, 2002] and heat shock [Panikulangara *et al.* 2004] was responsible for the various stress resistance in the Arabidopsis.

In contrast to the enhanced expression of *MoGolS1*, the concentration of galactinol dehydrate was lower after a stress treatment than control (Fig. 8). Their accumulation in plants is associated with stressful environmental conditions such as cold, heat, or dehydration [Santarius, 1973; Santarius and Milde, 1977; Hinesley *et al.*, 1992; Ashworth *et al.*, 1993; Wiemken and Ineichen, 1993; Bachmann *et al.*, 1994; Taji *et al.*, 2002]. We may think of hypotheses to explain these results. Although the *MoGolS1* gene is induced by abiotic stresses, the level of protein may not be fully functional in stress conditions. Or the *MoGolS1* gene is the only one gene which is responsible for playing a role in producing galactinol dihydrate in *M. officinalis*. Indeed, in our EST analysis we found only one *GolS* gene which was studied in current study.

In Conclusion, the *MoGolS1* gene was strongly expressed by the abiotic stress induced by sucrose, ABA, NaCl, cold or heat shock. However, its level is not enhanced much in response to mannitol. The *MoGolS1* gene induction with various stresses may be possible for its crucial function in abiotic stress tolerance in plants, providing a good engineering target for genetic engineering.

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