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Differential expression of *3β-HSD* and *mlncRNAs* in response to abiotic stresses in *Digitalis nervosa*

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Abstract: *Digitalis nervosa* is an important medicinal plant species belonging to the family of Scrophulariaceae that has the potential to be used for heart failure. 3β-hydroxysteroid dehydrogenase (*3β-HSD*) is a key gene in the biosynthesis of cardenolides for making digitalis effective compounds, hence identification of this gene is important for genetic engineering purposes towards increasing the yield of cardiac glycosides. In addition, mRNA-like non-coding RNAs (mlncRNAs), a class of long non coding RNAs, play key roles in various biological processes and may affect cardenolides pathway in digitalis plants. In the present work, full sequence of *3β-HSD* was isolated from *Digitalis nervosa*. Gene expression patterns of *3β-HSD* along with three mlncRNAs including *mlncRNA23*, *mlncRNA28* and *mlncRNA30* were studied and the results indicated that they are differentially expressed in different tissues including roots, stems and leaves, with the highest expression levels in leaves. Moreover, the transcript levels of these genes affected by the cold and drought stresses. The results obtained from the present study is important in order to understand the potential role of mlncRNAs in digitalis plants, especially in response to abiotic stresses.

Key words: Digitalis*; 3β-HSD*; Cardiac glycosides; *mlncRNA*; Gene expression.

Introduction

The genus Digitalis (generally known as the "foxglove") is a member of the family Plantaginaceae and comprises 23 species (1). Some species of Digitalis such as *Digitalis lanata* Ehrh. and *Digitalis purpurea* L. are economically important due to their pharmaceutical applications. The only species of the genus *Digitalis* in Iran is *Digitalis nervosa* and it is growing in the north and west of the country (2). This species is a biennial plant, its flowers due to the regular veins is similar to nerve fibers and hence is called nervosa (3). Digitalis plants are the main source of important pharmaceutical cardiac glycosides compounds such as digoxin and digitoxin (4). In general, cardiac glycosides (cardenolides) of Digitalis species are used in different types of heart failure, epilepsy and arthritis (5, 6). *Digitalis purpurea* has high utilization to treat congestive heart failure, particularly arrhythmia (6, 7). The main active compounds of *D. purpurea* are cardiac glycosides (cardenolides) (6, 8). *D. nervosa* has medicinal properties similar to *D. purpurea* (9), which makes it interesting for further studies. Additionally, the essential oils of *D. nervosa* are rich in *trans*-pinocamphone, which is recognized as a neurotoxic compound that can block γ-aminobutyric acid (GABA)-gated chloride channel (9). Besides, the essential oils of *D. nervosa* have also plenty of caryophyllene oxide (5.9%), which is well known as a preservative in foods, drugs, and cosmetics (9).

The biosynthetic pathway of cardiac glycosides includes three pathways: terpenoid backbone, steroids, and cardenolides (10). It has been reported that considering cholesterol as the starting point for cardenolides biosynthesis about 20 enzymes are involved in the formation of cardenolides in Digitalis (11). From this point cholesterol can be metabolized to pregnenolone (pregn-5-en-3β-ol-20-one; Figure 1), which is the first committed step towards glycosides synthesis (12). Once pregeneolone is formed, it can be converted to progesterone by 3β-hydroxy steroid dehydrogenase (3β-HSD) (13, 14). Conversion of progesterone to pregnanolone is then catalyzed by the enzyme progesterone 5β-reductase (P5βR) (15, 16). As cardiac glycosides structures are composed of several hydroxyl groups, it is assumed to be the effect of direct hydroxylation which are catalyzed by different monoxygenases. The important enzymes involving in cardenolide biosynthesis pathway are 3β-hydroxy steroid dehydrogenase (3β-HSD), keto steroid isomerase (KSI), progesterone 5β-reductase (P5βR) and malonyl-coenzyme A:21-hydroxypregnane21-O-malonyltransferase (21MAT) (11, 17). 3β-HSD is one of the key enzymes in the biosynthetic pathway of cardenolide which contribute in the early step of cardenolide biosynthesis, has bi-functional activity, comprising oxidation of pregnennolone to isoprogestron and reduction of 5β-pregnane-3-20-dione to 5β-pregnane-3β -ol-20-on (13, 18).

mRNA-like non-coding RNAs (mlncRNAs), a class of long noncoding RNAs (lncRNAs), have been identified as a new group of non-coding RNAs (ncRNAs) which show characteristics of mRNA and play important roles in the fundamental processes of cell growth,

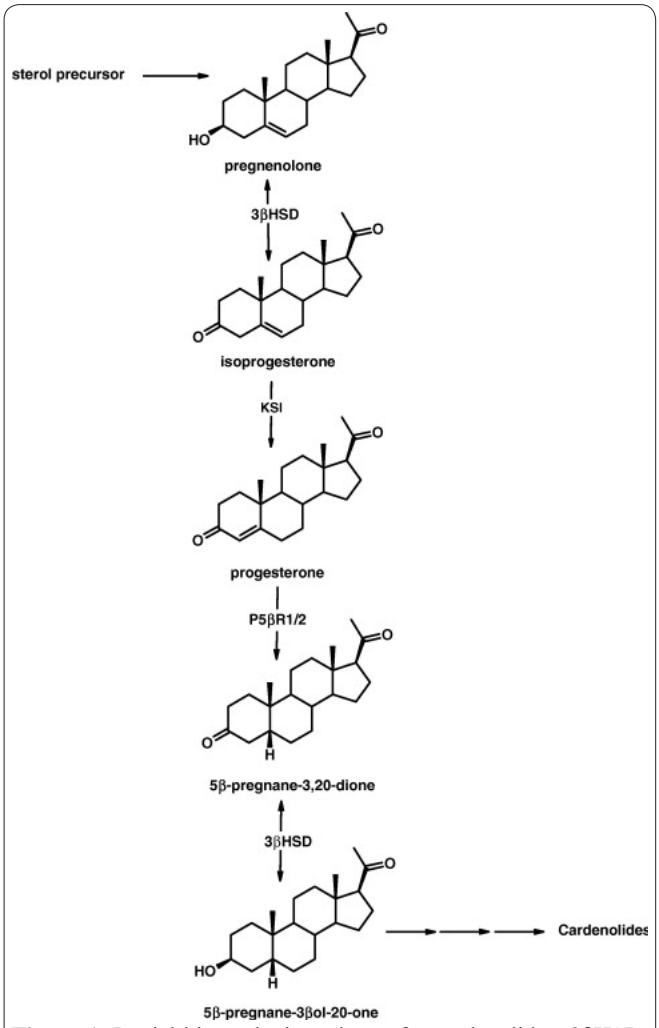

Figure 1. Partial biosynthetic pathway for cardenolides. 3βHSD, 3β-hydroxysteroid dehydrogenase; KSI, Δ^5-3-ketosteroid isomerase; P5βR1/2, progesterone 5β-reductase 1 or 2 (50).

development and response to the stresses in plants (19). Moreover, some mlncRNAs have significant roles in epigenetic pathways (20). This RNAs play important roles in the regulation of gene expression in eukaryotes (21). However, little is known about detailed expression pattern of mlncRNAs in Digitalis. To date, approximately 140 unigenes involving in cardiac glycoside biosynthesis and mlncRNAs associated with secondary metabolism and stress responses have been identified in foxglove (10). Nevertheless, the correlation of the unigenes and mlncRNAs is not known yet.

In the present work, based on the key role of 3β-HSD in the biosynthesis pathway of cardenolide in foxglove plants, isolation and expression of *3β-HSD* in *D. nervosa*, were investigated. Furthermore, we organized the experiments to outline the profiling of three characterized mlncRNAs in *D. nervosa*, as important regulators of gene expression. Information regarding the genes involved in the biosynthesis of cardenolides will be very useful to establish alternate strategies for their increased production.

Materials and Methods

Plant material

The seeds of Foxglove plant (*Digitalis Nervosa* Staud & Hochst) obtained from Iranian Biological Resource Center, Tehran, Iran. The seeds were washed and grown in a controlled-environment greenhouse with 16 h light/8 h darkness photoperiod and 25/20ºC (day/night). To assess expression across different tissue types, (root, stem and leaf) samples were collected.

Cold and drought stress treatments

For cold stress treatment, plants were transferred to a cold chamber at 4ºC, but control plants were placed on a growth chamber at 25ºC under the same light conditions. Moreover, drought-stress treatment was performed on plants in the greenhouse by reducing the irrigation to half for two weeks before sampling but the irrigation regime was normal for control plants in vegetative phase. Control plants for drought-stress experiment were placed on a growth chamber at 25ºC under the same light conditions and normal irrigation regime. For tissue sampling leaves of control and treated plants (3 biological replicates) at 1, 5, 10 and 24 hours after each stress were collected and immediately frozen in liquid nitrogen. The samples were kept at -80 ºC until further use.

RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples with Trizol reagent (Cinagen, Tehran, Iran). RNA quality was assessed by running on agarose gel electrophoresis. Besides, RNA quantity was measured using a NanoDrop Spectrophotometer (Nanodrop ND-1000). The DNase was used for the removal of genomic DNA contamination. cDNA was synthesized from 500ng of RNA using M-MuLV reverse transcriptase (Vivantis).

Isolation of Δ5-3β-hydroxysteroid dehydrogenase (3β-HSD) and sequence analysis

To isolate a gene encoding 3β-HSD, a primer pairs (called 3β-HSD1) was designed (Table 1). First the sequences of *3β-HSD* from seven Digitalis species obtained from the NCBI (Supplementary Figure 1). A multiple sequence alignment for all *3β-HSD*s was generated using ClustalW online software (22) Based on the sequence alignments, a primer pair (Table 1) was designed to amplify a fragment approximately 800 bp length. Followed by the confirmation of *3β-HSD* sequences, a new primer pairs were designed (called 3β-HSD2) for

Table 1. Nucleotide Sequence of primers used in this study.

Transcript	Forward primer (5'→3')	Reverse primer (5'→3')
3β-HSD1	ATGTCGTCAAAGCCAAGGT	CTAACGCACGACGGTGAAG
Ubiquitin	CTCTTCATTTGGTGTTGAGGCTTC	TGCCTTCACATTATCGATGGTGTC
3β-HSD2	GCTACGCGGTGGAGAAATAC	ATCTGACTCGGTGTCATTCC
mlncRNA23	ACCCGGTCGTAACCCGATCA	CCAAACGAGCTGGGTCAGATCTC
mlncRNA28	CAGCCTTAGGACTTCAGTGACCC	CCAGTGACCTTACCTTCTCGAGC
mlncRNA30	TGGGCCATGTAGCACCACAC	GTTGAAACGACGACGACGACTGTGG

the study of *3β-HSD* gene expression (Table 1).

RT-PCR analysis

Primer sequences of *ubiquitin*, mlncRNA23 (JO460543), mlncRNA28 (JO460815), and mlncRNA30 (JO462174) were used from the report of Wu et al., 2012. *Ubiquitin* was used as reference gene to analyze the relative gene expression (Table 1). All PCR amplifications were conducted in 20 µl reaction using Taq DNA polymerase (Vivantis) (0.5 unit) and specific forward and reverse primers (10 pmols) for selected genes (Table 1). The PCR thermal condition was as follows: initial denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 s, annealing at 50-55ºC (depending on primers) for 30 s, 72ºC for 30-70s (depending on amplicon length), and a final extension at 72ºC for 7 min. PCR products were visualized by separating on 1.5% agarose gel and subsequently staining with ethidium bromide. DNA bands intensity were converted to quantitative data by GelQuant.NET and relative gene expression was calculated based on the intensities of the target genes (*3β-HSD* or *mlncRNAs*) bands normalized relative to that of *ubiquitin* bands.

Data analysis

The experiments were arranged and analyzed based on completely randomized design with three replications. Analyses were conducted using SPSS (version 19) statistical software. Mean comparisons were carried out using Duncan's multiple range test at a probability level of 0.05 ($p < 0.05$).

Results

Isolation and sequence analysis of 3β-hydroxysteroid dehydrogenase

A 780 bp fragment of 3β-hydroxysteroid dehydrogenase was isolated from *D. nervosa* (Figure 2a) using designed primers (Table 1; Supplementary Figure 1). The isolated PCR product was successfully sequenced by Bioneer Inc., Seoul, Korea (Figure 2b). The obtained sequence was then blasted (BLASTx; search the protein database for homologs of the given sequence) to find homologous sequences in the NCBI Databases based on homologous similarity. Result revealed that the specific hit is NAD(P)-dependent dehydrogenase, short-chain alcohol dehydrogenase family with evalue of 6.42e-36 (Supplementary Table 1). The BLASTx sequence similarity score high-probable function protein. Moreover, the ubiquity of 3β-HSD is clear and it is highly conserved throughout the genus Digitalis (Supplementary Table 1). The isolated cDNA contains an open reading frame of 780 nucleotides encoding 259 amino acids and has 100 percent similarity with the 3β-HSD from *D. lanata* (Figure 3).

Expression profiles of 3β-HSD, mlncRNA23, mlncRNA28 and mlncRNA30 across different tissues

Based on the importance of 3β-HSD as a key gene in cardenolides biosynthesis and also regulatory role of mlncRNAs, the patterns of their expression across different organs and tissues are important. Therefore, transcript expression analysis of *3β-HSD* and three *mlncRNAs* in several tissues of Iranian foxglove (*D. nervosa*) was performed using semi-quantitative RT-PCR. The relative gene expression was normalized using *ubiquitin* as reference gene, which its stability has been shown in previous study (10). The results showed that under normal conditions, *3β-HSD* exhibited variable expression in different tissues with the highest expression levels in the leaf and the lowest level of transcripts (not detected) in the root tissue (Figure 4). Moreover, mlncRNAs transcripts were differentially expressed across tissues in *D. nervosa*. It was found that the expression levels of mlncRNAs were also high in leaves and low in roots. The differences in gene expression levels were statistically significant ($P < 0.05$). It seems almost all the transcripts are accumulated in the aerial parts (leaf and stem) of the plants probably because it is the active site of biosynthesis (Figure 4).

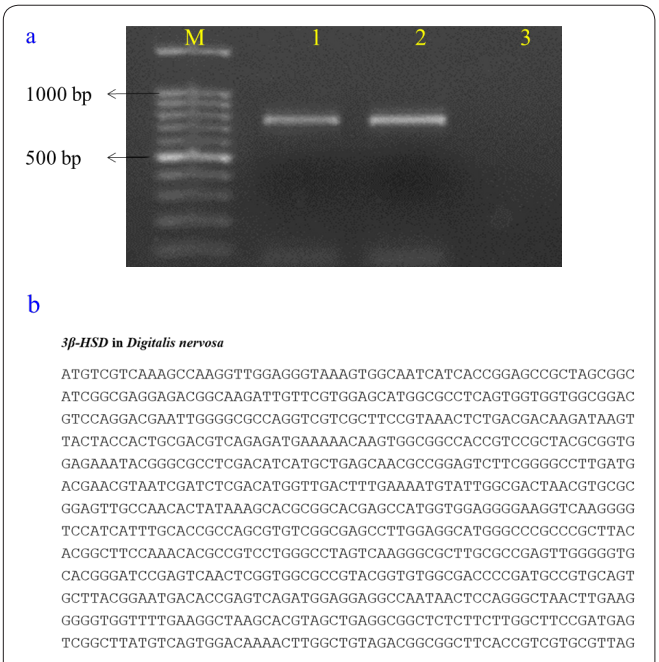

Figure 2. a) Agarose gel electrophoresis of PCR amplified products using species-specific PCR primer sets. Lane1 amplified fragment from leaf cDNA, lane2, amplified fragment from stem cDNA and lane3 negative control. Lane M, 1 kb DNA size marker (Vivantis). b) cDNA sequence (coding sequence) of the 780 PCR-amplified fragment from *Digitalis nervosa*.

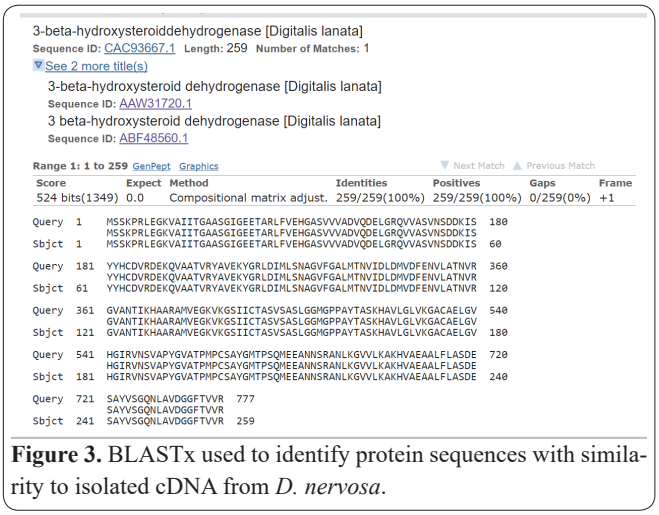

Figure 3. BLASTx used to identify protein sequences with similarity to isolated cDNA from *D. nervosa*.

3β-HSD, mlncRNA23, mlncRNA28 and mlncRNA30 transcript levels under drought condition

It is demonstrated that cardenolides play a role in

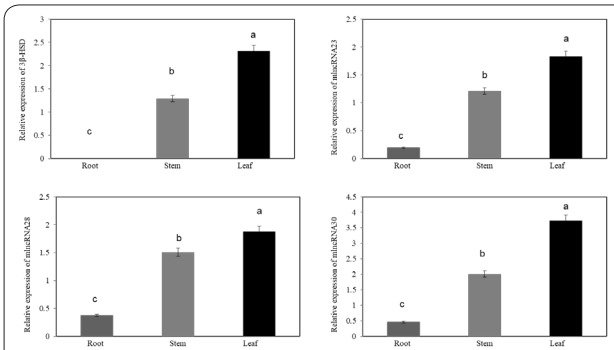

Figure 4. Tissue specific expression profiles of *3β-HSD* and three *mlncRNAs* in root, stem and leaf of *D. nervosa* under normal conditions. Different letters indicate statistically significant differences between tissues (mean ± SEM, n=3, Mean comparison by Duncan's test, p<0.05).

plant defense against abiotic and biotic stresses; therefore, the study of their key transcripts such as 3β-HSD and potential regulatory genes such as mlncRNA under different stress conditions is essential. In this regard expression profiles of *3β-HSD* and three mlncRNAs in the Iranian foxglove (*D. nervosa*) under normal and drought stress were investigated. The quantitative expression analysis revealed that 3β-HSD and the three mlncRNAs are differentially expressed during the time course of drought stress (Figure 5). The mlncRNAs and 3β-HSD genes under drought stress showed significant decreasing in time course of drought stress experiment (1 to 24 hours) in relation to control plants (Figure 5). All the mlncRNA genes had a high mRNA level during the early hours of drought stress while the transcript levels were decreasing when plants were exposed to longer time drought stress (Figure 5). The relative gene expression in control plants remained almost constant (Figure 5).

3β-HSD, mlncRNA23, mlncRNA28 and mlncRNA30 transcript levels under cold condition

In response to cold stress condition, we also examined the expression profiles of of *3β-HSD* and three mlncRNAs in the Iranian foxglove (*D. nervosa*). The results indicated that all genes are differentially expressed in response to cold treatment (Figure 6). All the studied genes under cold condition showed significant decrease in the time course of cold exposure (1 to 24 hours) compared to the control condition (Figure 6). The lowest level of transcripts expression was obtained 24 hours after cold treatment. The relative gene expression in the control plants remained almost constant (Figure 6). Generally, the profiles of expression levels of the three *mlncRNAs* and *3β-HSD* were very similar.

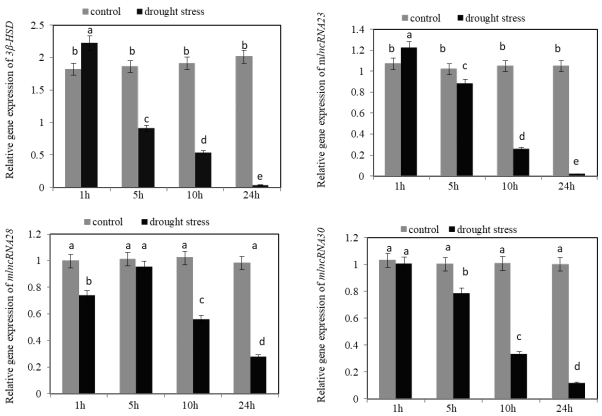

Figure 5. Profiling studies of gene expression in the leaf tissue under drought stress at a 24-hour time course. Different letters indicate statistically significant differences (mean ± SEM, n=3, Mean comparison by Duncan's test, p<0.05).

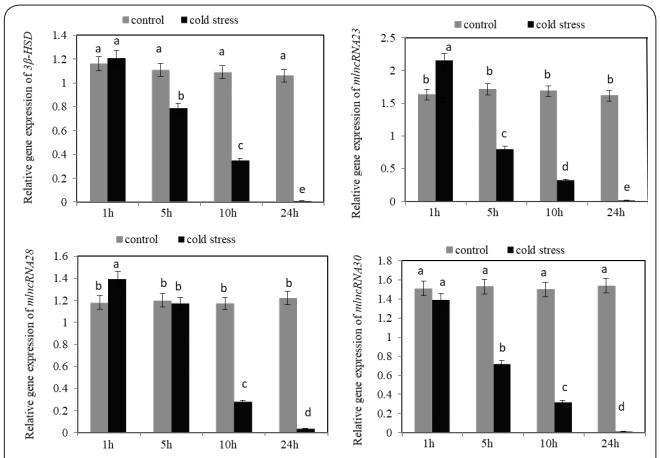

Figure 6. Profiling studies of gene expression in the leaf tissue under cold stress at a 24-hour time course. Different letters indicate statistically significant differences between time study expression in leaf tissue (mean ± SEM, n=3, Mean comparison by Duncan's test, p<0.05).

Discussion

Digitalis plants produce cardenolides which are important natural products for the treatment of cardiac insufficiency. The increase of cardenolides production is often minor and depends greatly on the physiological and developmental stage of the plant. Therefore, it is important to elucidate and study the factors that influencing cardenolides biosynthesis. Cardenolides production has a genetic basis and identification of enzymes and encoding genes involved in biosynthesis are of great importance. However, there is strong data for phenotypic plasticity, with the biotic and abiotic factors in environment probably affecting cardenolides production (23). 3β-Hydroxysteroid dehydrogenase (3β-HSD) and progesterone 5β-reductase (P5βR) are both supposed to be key enzymes in the biosynthesis of these natural products (11, 17). Therefore, the first aim of this study was the isolation of 3β-HSD cDNAs in *D. nervosa* which might be implicated in cardenolide biosynthesis. Based on comparative sequence analysis, an isolated 780 bp cDNA encodes a protein responsible for 3β-HSD activity and belongs to the 3β-HSD in other Digitalis species. A BLASTx search in the database revealed lower similarity of isolated sequence from *D. nervosa* with proteins from other species other than those corresponding to 3β-HSD in Digitalis (Supplementary Table 1). Gene expression analysis indicated that 3β-HSD transcript was present in leaf and stem tissues, although it was not detectable in roots. But, this gene under drought and cold stress conditions exhibited variation in its transcript levels and was responsive to drought and cold stress conditions. High expression of *3β-HSD* in the leaves can be related to biosynthesis of glycoside in this tissue. Herl and colleagues (2007) also isolated a full length of 3βHSD cDNA consisting of 780 nucleotides from *Digitalis lanata*. Genomic 3β-HSD sequences of

seven digitalis species were also identified and all have identical size consisting of 780 nucleotides with one intron of 90-96 bp at the 5'end of the gene (13). All the related proteins share a high degree of sequence similarity (13). Analysis of TBLASTX revealed that the isolated 3βHSD cDNA from *D. nervosa* showed 100 percent identity of amino acids (or protein) with cDNA of *D. lanata* (Figure 3). The deduced 3βHSD protein from *D. lanata* shares significant similarities with putative alcohol dehydrogenase from other plant species (13). The expression of *3βHSD* from *D. purpurea* was also studied and its transcripts stayed almost constant over time and during plant development in normal condition (24). In *D. lanata* transcription rate of the 3βHSD gene was calculated using qPCR and leaves showed higher level in comparison with K3OHD cell line (14). These data confirm the relatively higher 3β-HSD enzyme activity in leaves (14). The high expression of *3β-HSD* and activity of its enzyme in leaves support that cardenolide biosynthesis takes place in leaves. Leaves of Digitalis plants are still the major source for the isolation of cardenolides that are applied for treatment of cardiac insufficiency in humans (4).

Non-protein-coding RNAs (npcRNAs), well-known as noncoding RNAs (ncRNAs), are transcripts that do not encode proteins but instead their functions remain on the RNA molecules (25, 26). In addition to the famous housekeeping ncRNAs including rRNAs, tRNAs, snoRNAs, and snRNAs, many new regulatory ncRNAs (riboregulators) have been identified and characterized using new genomic methods (27). The regulatory ncRNAs exist in both eukaryotes and prokaryotes and represent the abundant majority of all transcripts in the cells (28), which is revealed by the new genomic methods such as tiling arrays, whole transcriptome analysis, reverse transcription-RCR (RT-PCR) and computational prediction (29, 30, 31). Therefore, the growing number of these regulatory ncRNAs offerings that they could play a more important role than formerly assumed. Moreover, several transcriptional profiling studies have been done to investigate stress tolerance in plants and revealed remarkable differences between control and stress-treated plants in the relative expression levels of the genes encoding stress response regulators and their target proteins (32, 33). Although, only 1–2 % of entire transcribed genes, translated into proteins (34), and indeed, in addition to stress-inducible regulatory proteins and transcription factors, non-coding RNAs like microRNAs (miRNAs) are also recognized to regulate plant stress responses (35, 36). For example, role of miRNAs in regulation of drought-responsive genes have been reported (35, 37-39). Moreover, regulatory mRNA-like non-coding RNAs (mlncRNAs) affect cellular activity through specific sequences and/or RNA-folding structures (40). Therefore, according to the importance of ncRNAs, we organized this study to assess profiling of three newly identified mlncRNAs, during drought and cold stress conditions in *D. nervosa*. The transcript expression analysis revealed high expression level of the three mlncRNAs (mlncRNA23, mlncRNA28 and mlncRNA30) in leaves following by stem under drought and cold stress conditions. The three mlncRNAs which have been characterized in *D. purpurea* showed similarly high expression levels in leaves (10). Although the low conservation of mlncRNAs is a usual occurrence in living organisms (41), the three studied mlncRNAs in two species of Digitalis (*purpurea* and *nervosa*) displayed a high degree of homology at least in the selected fragments of the mlncRNAs. Consequently, these results could possibly support the existence of the particular mlncRNAs in both *Digitalis nervosa* and *Digitalis purpurea* and other digitalis species. Based on the analysis of data, differentially expressed mlncRNAs correlate highly with expression of *3β-HSD* (r= 0.98 with *mlncRNA23*, 0.98 with *mlncRNA28* and 0.995 with *mlncRNA30*). This indicates the occurrence of adjacent relationship between *3β-HSD* and the three mlncRNAs through an unknown mechanism. In rapeseeds (*Brassica napus*) cadmium-induced expression of lncRNAs seemed to be linked to their nearby protein-coding genes (42). In rainbow trout (*Oncorhynchus mykiss*) a positive correlation between the number of the differentially regulated lncRNAs and that of the protein-coding genes has been discovered (43). Our results propose that the expression of mlncRNAs is regulated at developmental stages like *3β-HSD*, and besides indicate the potential role of mlncRNAs in *D. nervosa* growth and development.

In order to study the expression level of the mlncRNAs under environmental stresses we next quantified the transcript expression level of the three mlncRNAs under cold and drought stress conditions. The results revealed that mlncRNAs were differentially expressed in response to these abiotic environmental conditions. Therefore, the mlncRNAs are more possibly stress-responsive (cold-responsive and dehydration-responsive). Additionally, a comparison of gene expression levels of *mlncRNAs* and *3β-HSD* under conditions of cold and drought specify the same transcriptional trend. Therefore, the similarity of mlncRNAs and 3β-HSD expression patterns in response to cold and drought stresses suggests that the cold and the dehydration signaling networks are probably overlapped as acknowledged in Wu et al., (2012). This exhibits the importance of mlncRNAs in stress responses and points the existence of a crosstalk among mlncRNAs in response to cold and drought stresses. To date, several lncRNAs have also been associated with responses to abiotic stress. It has been shown that in rice (*Oryza sativa*) several noncoding RNAs identified, whose expression were highly regulated by drought stress conditions (44). Studies in *Medicago truncatula* indicates that lncRNAs are likely involved in regulating plant's responses and adaptation to osmotic and salt stresses in complex regulatory networks with protein-coding genes (45). In *Populus tomentosa* known lncRNAs under nitrogen deficiency exhibited differentially expression and their complementary protein-coding genes involved in responses to Nitrogen deficiency were also identified (46). In rapeseeds (*Brassica napus*) some lncRNAs have been detected to be differentially expressed in cadmium-exposed plants (42). Gene expression has been assayed against different treatments (47-50). In *A. thaliana* the expression of 1832 identified lncRNAs were reported to be regulated by various abiotic stresses (51). In wheat (*Triticum aestivum*) 125 detected lncRNAs exhibited different expression level under drought and heat stress conditions (52). Several drought-responsive and tissue-

specific lncRNAs have also been discovered in maize (53). Additionally, differential expression of long noncoding RNAs in lines of rainbow trout (*Oncorhynchus mykiss*) in response to infection with *Flavobacterium psychrophilum* were reported (54).

In general, up to now many noncoding RNAs in several plant species have been identified and found to be stress-responsive. Transcript expression analysis of mlncRNAs provides essential information about tissue specificity and environmental regulatory, which is important for plant growth and development. Overall, three mlncRNAs and 3β-HSD showed closely the same expression levels in response to drought and cold stresses. The identification and expression pattern analysis of mlncRNAs from Digitalis plants represents a resource for investigating how the noncoding RNAs interact during cold and drought stresses and how they are involved in plant development regulation. Since plants exhibit several developmental plasticity to adapt their growth to alter environmental conditions, understanding how lncRNAs work, may elucidate new mechanisms involving in plants growth control. This may help to design new tools for biotechnological applications.

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Conflicts of interest
none

Author's contribution
Vahideh Salimi performed the experiments, Asad Maroufi conceived the work, designed experiments and wrote the paper, Mohammad1Majdi analyzed the data and contributed with writing paper.

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