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Assessment of tissue distribution and subcellular localization of miR-302 and miR-21 by means of in situ hybridization technique

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Abstract

MicroRNAs (miRNAs) are a group of short non-coding RNAs implicated in numerous fundamental cellular processes, and their disregulations have been linked to several pathologic conditions, mainly cancers. Determining tissue distribution of miRNAs is a prerequisite for understanding their exact functions during development, tissue homeostasis and abnormality. In situ hybridization is a powerful technique to delineate the sub-cellular localization and tissue distribution patterns of mRNAs as well as miRNAs. Due to the important role of miRNAs in tumorigenesis, we optimized an ISH technique for detection of two well-known miRNAs (miR-302 and miR-21) in formalin-fixed paraffin-embedded (FFPE) tumor samples along with a pluripotent embryonal carcinoma cell line, NTERA-2 (NT2). After fixation of cells on slides/sectioning of FFPE blocks, proteinase K digestion, probe concentration, antibody development and light sensitive color reaction were optimized for both the FFPE samples and cell line. Signals for U6 snRNA, as an internal control, were detected in the nuclei of the cells. MiR-21 and miR-302 expression was detected in the cytoplasm of FFPE samples of seminoma carcinoma and in NT2 cell line, respectively. In this study, we optimized ISH for miRNA detection in FFPE samples and NT2 cell line.

Keywords**:** *in situ* hybridization, microRNA, miR-302, miR-21

Introduction

MicroRNAs (miRNAs) are short (18-22 nt) non-coding RNAs that post-transcriptionally regulate gene expression by direct degradation of their mRNA targets or inhibition of their translation. MiRNAs have prominent roles in regulation of cell proliferation, differentiation and apoptosis. They have been also implicated in different pathological conditions, especially in tumorigenesis. Contribution of miRNAs to carcinogenesis was first introduced by Calin et al. (Calin et al., 2002). Since then an enormous body of investigations has focused on their mechanism of action in cancer initiation and progression, as well as recognizing their role as potential cancer biomarkers and therapeutic targets (Schetter et al., 2008; Spizzo et al., 2009; Iorio et al., 2005). In this aspect, several techniques have been utilized or innovated to predict and experimentally validate new miRNAs, quantify their expression and localize their distributions within tumor tissues.

In situ hybridization (ISH) is carried out by a labeled oligonucleotide (called a probe) which is designed to complementary hybridize to a specific sequence (DNA, RNA, miRNA) inside the cells, and therefore precisely localizes its presence within the cell or tissue section (Carter et al., 2005). DNA probes are usually used for diagnostic purposes and chromosomal abnormalities (Zitzelsberger et al., 1994), while RNA probes are used for gene expression analysis (Jorgensen et al., 2010). Due to their critical role in tissue homeostasis, development and diseases, miRNA localization and expression timing is of vital importance in understanding their exact role in development and initiation and progression of diseases. ISH technique allows miRNA expression detection at the cellular level, and therefore it demonstrates the cellular source of a given miRNA. However, it does not provide a precise quantitative measurement of miRNA expression level.

The main challenges in ISH technology for miRNA detection are: 1) the unstable nature of RNA which demands RNase-free procedures during the whole experiment, 2) the small sizes of the miRNAs, and 3) the very similar sequences of some miRNAs (Jorgensen et al., 2010; Nuovo, 2010a). To improve the specificity and affinity of

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probe binding, different technologies have been employed. Among them locked-nucleic acid (LNA) substituted oligonucleotides sound promising. LNA is a nucleotide with a 2'-O, 4'-C-methylene-β-D-ribofuranosyl nucleotide bond that locked in an RNA mimicking sugar. With an increase in the melting temperature (Tm), oligonucleotides containing LNAs show higher hybridization affinity to the target RNA and an increase in the signal to background ratio. Moreover, they have higher binding specificity which makes them suitable for specific miRNA hybridization, since the miRNAs have very short length and some of them might differ in only 1-2 nucleotides (Vester and Wengel, 2004; Silahtaroglu et al., 2004; Silahtaroglu et al., 2007; Nuovo, 2010b).

Here, we are reporting an optimized ISH procedure for miRNA detection in FFPE samples of seminoma carcinoma and a human embryonal carcinoma cell line, NT2.

Materials and Methods

Bacterial strains, plasmids and media

Before starting the procedure, all the glasswares were incubated at 180ºC for 8 h to remove any potential RNase contamination. All the solutions were prepared with DEPC-treated water, and if it was possible, autoclaved after preparation. It is very important to maintain RNase-free conditions during the whole procedure. Once the procedure starts, it cannot be halted or the slides allowed to be air dried. The only steps that can be prolonged are the washing steps with phosphate buffered saline (PBS). In general, the whole protocol of ISH could be divided in 2 days.

Deparaffinization

FFPE blocks of seminoma carcinoma were collected from department of pathology, Shariati hospital (Tehran-Iran). Blocks were sectioned into 4-6 µm thickness, and were fished out from a water bath filled with DEPC (Cinnagen, Iran) treated water. For being firmly attached on slides, sections were incubated at 37°C and for overnight, instead of heating them on 60°C hot plate. On first day, the sections were deparaffinized using xylene for 10 times immersion and then 2 times of incubation each for 5 minutes, then slides were rehydrated with serial dilution of ethanol (100, 96 and 70%) each for 10 times immersion and then incubated for 5 minutes within each dilution. Finally slides were incubated with PBS (Invitrogen, USA) for 5 minutes.

Prehybridization treatment

Proteinase K (Fermentas, Lithuania) was diluted to 15 µg/mL concentration in proteinase K buffer (Tris-Cl 100 mM, EDTA 50 mM, NaCl 500 mM; PH=8) and slides were incubated with this solution for 20 minutes at 37°C in ThermoBrite hybridizer (Fisher Scientific, USA). After protein digestion, slides were washed with fresh PBS (prepared with DEPC treated water) for 5 minutes and then the tissues were fixed in increasing serial dilutions of ethanol (70, 96 and 100 %) each for 10 immerse and 1 minute of incubation. Slides were then air-dried under laminar hood.

Hybridization

Hybridization buffer (50% Formamide, 5X SSC (20x SSC preparation will be discussed further on), 0.1% Tween 20, 9.2 mM citric acid, 50 µg/mL heparin (Sigma, Germany; Cat # H3393), 500 µg/mL yeast RNA (Sigma, Germany; Cat # R6750)) was prepared freshly. 5' digoxigenin (DIG)-labeled miRCURY LNA microRNA detection probes (Exiqon, Denmark) were diluted to 50 nM in hybridization buffer and slides were incubated in 15 µl of the hybridization solution, covered with coverslips and sealed with fixogum (Germany). Slides were then incubated in ThermoBrite hybridizer for 3 minutes at 90°C to denature the dsRNAs, followed by 1 hour at hybridization temperature (Tm of the probe -21°C). Melting temperature for each LNA probe depends on the GC content of the oligonucleotides sequence and it is provided by the company in the probes' data sheet. Table 1 shows the probe sequences and hybridization temperatures for each miRNA and U6 snRNA.

Table 1. Probe sequences and hybridization temperatures for each gene.

Probe name	Sequence	Hybridization temperature
hsa-miR-302	5'- AAGCATGGAAGCACTTA-3'	47 ºC
hsa-miR-21	5'-TCAACATCAGTCTGATAAGCTA-3'	51 ºC
hsa/mmu/rno-U6 snRNA	5'-CACGAATTTGCGTGTCATCCTT-3'	55 ºC

Probes are labeled at 5' end and they have an extra nucleotide as 5'-overhang which facilitates the hapten (DIG) recognition by the antibody.

Stringency wash

Descending serial dilutions of standard sodium citrate buffer (SSC) (20x solution: NaCl 300 mM, Sodium Citrate 30 mM; PH=7) were prepared (5x, 1x, 0.2x) and stringency wash was performed for 5 minutes in each solution at hybridization temperature (47ºC for miR-302, 51ºC for miR-21 and 55ºC for U6) for each probe, followed by 5 minutes incubation in 0.2x SSC and 5 minutes in PBS, both at room temperature. Tissue sections are not allowed to dry out during this and subsequent stages.

Blocking and antibody treatment

Using PAP pen (Sigma, USA), a hydrophobic barrier was delineated around the tissue sections, then the slides were incubated with 2% sheep serum (Sigma, USA) in BSA/PBS-T (2 mg/mL) for 15 minutes at room temperature in humidifying chamber to block nonspecific binding of the first antibody. Then alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche, Germany) was diluted 1:800 in blocking buffer and covered the slides for overnight at 4 □C, in humidifying chamber.

Second day; Alkaline Phosphatase reaction

Slides were washed with PBS (3 times each for 5 minutes at room temperature) and incubated with Alkaline Phosphatase buffer (Tris-Cl 100 mM, $MgCl_2$ 50 mM, NaCl 100 mM, 0.1% Tween 20; PH= 9.5) for 3 times, each for 5 minutes at room temperature. Light-sensitive color reaction was performed with 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate (NBT/BCIP) ready-to-use tablets (Roche, Germany) for 3 hours at 30ºC in a humidified chamber. BCIP is a substrate for alkaline Phosphatase, and dephosphorylated BCIP itself can be oxidized with NBT. Both reduced NBT and oxidized BCIP generate a blue insoluble precipitant. Then slides were dehydrated in ascending serial dilutions of ethanol (70, 96 and 100%), before being counterstained with nuclear fast red (for miRNAs) or eosin (for U6 snRNA) for visualization of signals with light microscopy (Figure 1).

In situ Hybridization for NT2 cell culture

NT2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Media) (Gibco, USA), supplemented with 10% FBS (Sigma, USA). At the confluency of 60-70%, cells were trypsinized and the pellet was resuspended in 1 mL of DMEM. After checking cells' morphology with microscope, various numbers of cells were attached on coated slides with Cytospin centrifuge (Hettich, Germany). Different speeds and spin times were tested, before reaching the optimized conditions. Slides were then fixed with freshly made 4% paraformaldehyde (1 g paraformaldehyde, 25 mL PBS; PH=7.4) for 10 minutes followed by 3 times washing with PBS, each for 2 minutes. Slides were again washed with PBS-Triton X100 (0.5%) for 10 minutes and then hybridization and other steps of the procedure were performed as described for FFPE tissue sections.

Results

Optimizing ISH conditions with U6 snRNA

Different Proteinase K treatments (10, 20 and 30 minutes) and different probe concentrations (30 nM and 60 nM) were employed to optimize ISH conditions for FFPE samples. Incubation with 15 µg/ml Proteinase K for 30 minutes and 60 nM probe concentration in hybridization step were found to be the optimized conditions for signal detection of U6. Hybridization temperature for U6 probe was 55ºC. Blue signals of U6 snRNA were detected in the nuclei of the cells. Similar conditions were found to be applicable for NT2 cell line (Figure 2). In each experiment, a negative control with no probe treatment was used to confirm the specificity of hybridization. These slides were treated only with hybridization buffer, and as expected generated no visible signal.

MiR-21 cytoplasmic signals were detected in FFPE samples

We used the same obtained optimum conditions of ISH to detect miR-21 in the cytoplasm of seminoma carcinoma. The hybridization temperature was 51ºC and stringency washes were also performed at the same temperature. The signal intensity of miR-21 was compared with the intensity of U6 snRNA signals (Figure 3). No signal was observed in negative controls which further proves the specificity of miR-21 signals.

MiR-302 is localized within the cytoplasm of NT2 cells

We selected NT2 cell line due to the reports on high expression of miR-302 cluster in these cells (Hohjoh and Fukushima, 2007; Lee et al., 2008). We initially used hematoxilin and eosine (H&E) staining to optimize NT2 cell attachment and fixation situations. We then used 9 x 10^3, 18 x 10^3 and 36 x 10^3 cells and 800, 1000 and 1400 rpm and

6 minutes of spin for cell attachment to the slides. Moreover, we checked methanol and paraformaldehyde fixation for the cells. Finally, 8 x 10^3 cells and 800 rpm for 6 minutes of spin were selected as the suitable situations for attachment of the cells. The hybridization temperature for miR-302 probe was 47ºC. Our ISH results showed a cytoplasmic localization of miR-302 in this cell line (Figure 4). In our negative controls which had no probe treatment, there was no blue signal that was observed for miR-302 (data not shown).

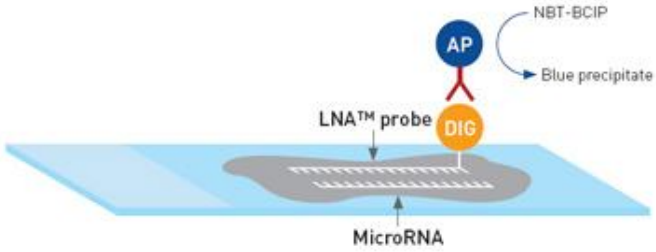

Figure 1. A schematic diagram of in situ hybridization procedure with LNA probe.

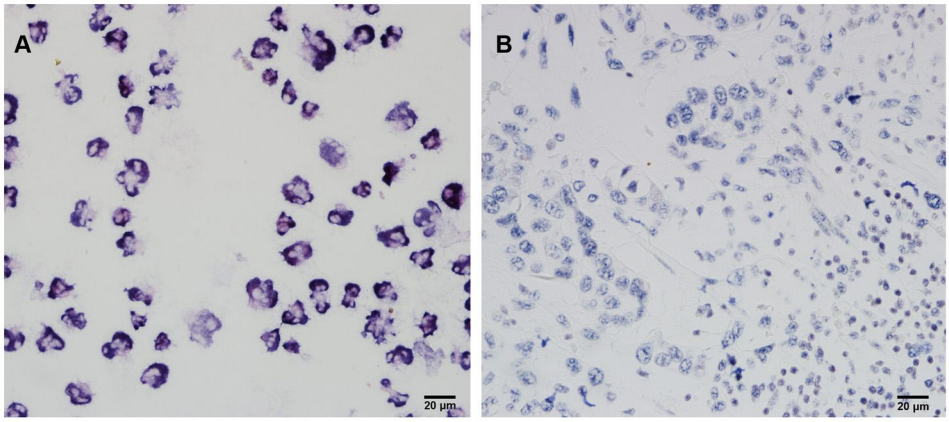

Figure 2. Nuclear signals of U6 snRNA; A) in NT2 cell line, B) in seminoma carcinoma FFPE tissue.

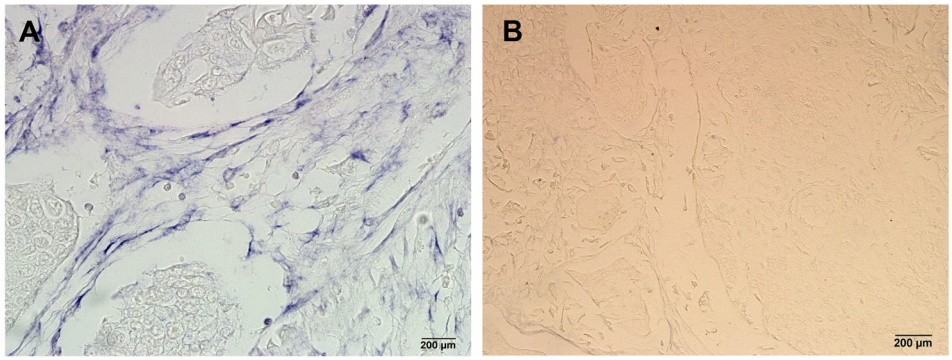

Figure 3. A) MiR-21 cytoplasmic signals in FFPE tissue of seminoma carcinoma, B) Negative control without probe treatment.

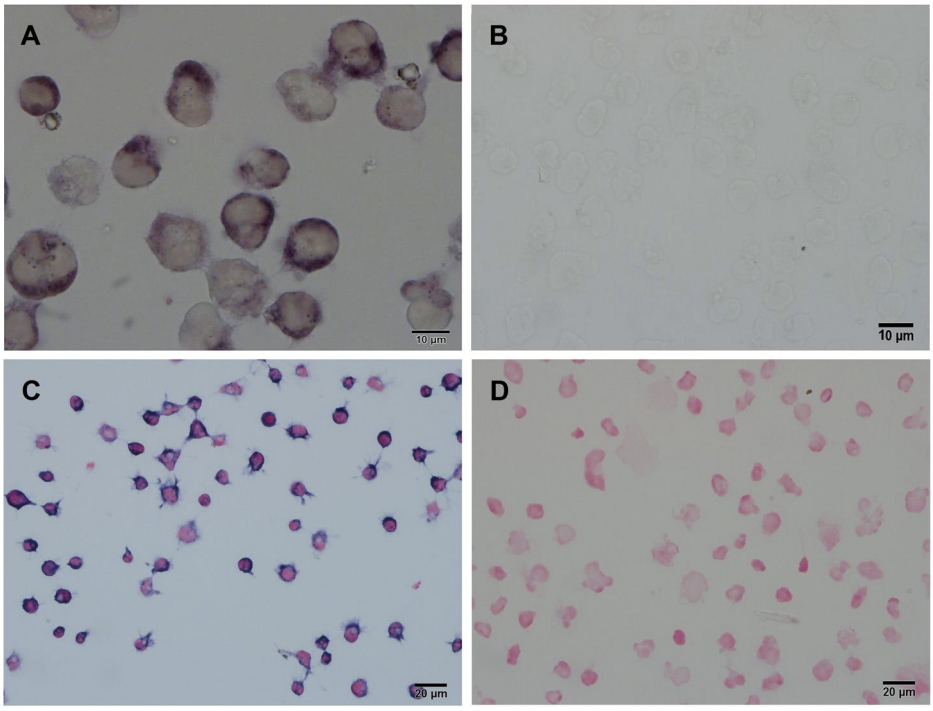

Figure 4. Cytoplasmic signals of miR-302, A) without counterstain, B) Negative control, C) slides were counterstained with nuclear fast red, blue signals show miR-302 localization, D) Negative control which is counterstained with nuclear fast red. There is no blue cytoplasmic signal in negative controls without probe treatment.

Discussion

ISH is a robust technique for visualization of nucleic acids at cellular and tissue levels. We were able to detect miR-21 sub-cellular localization in seminoma carcinoma tissues. MiR-21 is a well-known oncomir and its overexpression has been reported along with cancer cell proliferation, apoptosis, tumor invasion, and metastasis (Reis et al., 2012; Folini et al., 2010; Yan et al., 2011). The optimized procedure can be used for other FFPE samples with minor changes due to tissue specific characters.

Our study on NT2 cells showed cytoplasmic distribution of miR-302. Different studies have reported this miRNA to be localized both at nucleus and cytoplasm depending on its role and its targets, which points out to its complex level of function Liao et al., 2010; Jeffries et al., 2011). In this study, we detected both miRNAs in the cytoplasm of the studied cells or tissues and for each specimen different procedure is needed. During fixation and paraffinization of tumor tissues, some cross-links form between proteins and RNAs inside the cells. Due to their small size, miRNAs are totally covered with cellular proteins. So, we need Proteinase K digestion in order to strip the miRNA, but this step is not needed for miRNA detection in cell lines. Following the protein digestion, we used different procedures for miRNA fixation in different samples. The present protocol is a robust and practical procedure for miRNA detection which will provide more accurate data at the tissue level.

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Deletion of *RAD52* in *Saccharomyces cerevisiae* severely decreases frequencies of *Agrobacterium* genetic transformation mediated by either an integrative or a replicating binary vector

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Abstract

Agrobacterium tumefaciens is capable to transfer genes across kingdoms. It can genetically transform not only plant cells, but also many other bacterial, algal, fungal, animal and human cells. This depends on the interactions among a variety of both *Agrobacterium* and host genes. Inside the host cell, *RAD52* which is involved in DNA repair is a key gene determining integration of T-DNA by homologous recombination. Here, using *Saccharomyces cerevisiae* haploid strains BY4741 and BY4742, a *rad52* diploid deletion strain was constructed in yeast BY4743 background. This model organism was employed to show that *RAD52* deletion severely decreases frequencies of *Agrobacterium* genetic transformation mediated by either an integrative T-DNA or a circular non-integrative T-DNA. Indeed, the frequencies of such *Agrobacterium*-mediated transformation (AMT) decreased by *ca*. 25-fold, compared to wild type BY4743. Hence, host *RAD52* deletion might affect AMT by a mechanism which differs from its only involvement in DNA repair in yeast.

***Keywords*:** RAD52, AMT, Agrobacterium, Saccharomyces

Introduction

Agrobacterium tumefaciens is naturally a soilborne phytopathogen which genetically transforms plants and causes crown gall disease in numerous plants (De Cleene and De Ley, 1976). In laboratory, it is capable to transform cells from many non-plant organisms including yeast, *Saccharomyces cerevisiae* (Soltani et al., 2008). This ability of *Agrobacterium* is based on a large tumor inducing (Ti) plasmid, which contains a set of virulence (*vir*) genes that can mobilize a segment of the Ti-plasmid, i.e. the T-DNA. This T-DNA, in a single stranded form (T-strand), is transported to the host cell where it can integrate into the host genome. Meanwhile, *Agrobacterium* also transfers a number of its virulence proteins to the host cell through its type IV secretion system (Vergunst et al., 2000, Schrammeijer et al., 2003). Delivered virulence proteins protect the T-strand from host nucleases, target it to the nucleus and possibly cooperate with host proteins to integrate it into the host genome (Tzfira et al., 2004).

Over the last decade, the yeast *S. cerevisiae* has become an excellent model host to study the host factors involved in *Agrobacterium*-mediated transformation (AMT). One of the key groups of identified host genes which affect AMT of yeast is *RAD52* epistasis group (Soltani, 2009). AMT of *S. cerevisiae* can result in random insertion of the T-DNA into the yeast genome by non-homologous end joining (Bundock and Hooykaas, 1996). However, when DNA sequences homologous to those of *S. cerevisiae* genome are present, the DNA fragment will mostly integrate into the genome by homologous recombination. When T-DNA contains a *S. cerevisiae* replicator such as an autonomously replicating sequence (ARS) or the replicator of the 2µ plasmid, the T-DNA will be maintained in the yeast cell as a replicative plasmid (Bundock et al., 1995; Piers et al., 1996), after circularization of the T-DNA. In *S. cerevisiae*, the integration of T-DNA by homologous recombination is very efficient. By using the yeast *S. cerevisiae* as a model, it was found that the proteins mediating T-DNA integration are the proteins involved in double strand break (DSB) repair of the genomic DNA (van Attikum et al., 2001). Rad52 is essential for T-DNA integration at double strand breaks by homologous recombination (van Attikum and Hooykaas, 2003). However, Yku70 is essential for T-DNA integration at double strand breaks by non-homologous end joining (van Attikum, 2003). Thus, in *S. cerevisiae* the Rad52 and Yku70 proteins play critical roles in determining whether

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the T-DNA is integrated via homologous recombination or via nonhomologous end joining (van Attikum, 2003). Hence, it is speculated that *RAD52* deletion in host cell might affect T-DNA integration by influencing DNA repair system. However, the effect of *RAD52* deletion on AMT is not investigated by replicating plasmids.

Here, the effect of host *RAD52* deletion on AMT frequencies is assayed by either a homologous integrative or a replicating non-integrative binary vector. To this end, yeast *rad52* diploid deletion mutants were constructed in *S. cerevisiae* BY4743 background. Then, AMT frequencies were assayed in *rad52* deletion mutants of *S. cerevisiae*, using both a homologous integrative and a replicating binary vectors.

Materials and Methods

Bacterial strains, plasmids and media

Two derivatives of *A. tumefaciens* strain LBA1100, containing either pRAL7100 (integrative T-DNA) or pRAL7101 (replicating T-DNA) binary vectors (Bundock et al., 1995), were used. The T-DNA of pRAL7100 contains the *URA3* gene flanked by the yeast *PDA1* sequences that allows integration into the yeast *PDA1* locus via homologous recombination. The T-DNA of pRAL7101 contains the *URA3* gene and the yeast 2µ origin of replication. Following AMT the T-DNA part of this plasmid will be maintained in yeast nucleus as an extrachromosomal replicating plasmid after circularization. *Agrobacterium* was grown and maintained as described by Hooykaas et al. (2006). *Escherichia coli* XL1-Blue was used for plasmid amplification.

Yeast strains and media

Saccharomyces cerevisiae haploid strains BY4741 (*MAT*a *his3Δ leu2Δ met15Δ ura3Δ*) and BY4742 (*MATα his3Δ leu2Δ lys2Δ ura3Δ*), obtained from Invitrogen (Groningen, the Netherlands), were used as the wild type strains to construct yeast diploid strain BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ/leu2Δ lys2Δ/LYS2 MET15/met15Δ ura3Δ/ura3Δ*), and to construct *rad52* diploid deletion strain in BY4743 background. Yeast BY strains were grown in liquid YPD medium at 30°C and *rad52* deletion mutant strain was grown in liquid YPD medium containing G418 (150 µg/ml) at 30ºC.

Construction of rad52 diploid deletion strain

To construct *rad52* diploid deletion strains in yeast BY4743 background, primers P3 and P4 (table 1) were designed to amplify *KANMX* cassette (1.6 kb), flanked by *loxP*, from pUG6 plasmid (Güldener et al., 1996) and 45 bp sequences upstream and downstream of *RAD52* gene by PCR. The amplified fragment was gel purified, and was transferred to yeast haploid strains BY4741 and BY4742 by Lithium Acetate transformation method (Gietz et al., 1995) and the deletion strains were selected on YPD medium containing G418 (150 µg/ml).The integration at *RAD52* locus was confirmed by colony PCR using four primers: P5 and P6 (635 bp), and P7 and P8 (560 bp) (table 1) to check upstream and downstream of integrated fragment. Then, the *rad52* haploid deletion strains were mated (Stansfield and Stark, 2007) to obtain a homozygous diploid deletion strain in BY4743 background, and were selected on MY medium (Zonneveld, 1986) supplemented with histidine (20 µg/ml), leucine (30 µg/ml)) and uracil (20 µg/ml). Construction of diploid deletion strains were confirmed by a duplex colony PCR using the universal primers of MAT locus (Table 1) (Huxley et al., 1990).

Chemical and Agrobacterium-mediated transformation of yeast strains

The lithium acetate transformation protocol as described by Gietz et al. (1995) was used to transfer the amplified fragments to yeast BY4741 and BY4742 strains. The original *Agrobacterium*-mediated transformation (AMT) protocol (Bundock et al., 1995) was used for transformation of yeast mutants. Yeast wild type strain BY4743 was used as a control.

Results

Construction of homozygous rad52 diploid deletion mutants

To construct *rad*52 diploid deletion in yeast BY4743 diploid background, the *KANMX* cassette from pUG6 was amplified using primers with 45 bp homologies to upstream and downstream regions of yeast *RAD*52 gene, i.e. P3 and P4. The amplified fragment was evaluated by gel electrophoresis, and subsequently was transferred to yeast haploid strains of BY4741 and BY4742. Colony PCR by primer combinations of P5 and P6, and P7 and P8 amplified the expected fragments, 635 bp and 560 bp respectively, in several yeast *rad*52 deletion mutants of BY4741 and BY4742 strains (figure 1). Upon this, *rad*52 haploid deletion strains of BY4741 and BY4742 background were mated to create BY4743 diploid strains. Then, yeast diploid strains were selected on MY medium supplemented

with histidine, leucine and uracil. Colony duplex PCR by *MAT* locus primers i.e. P9, P10 and P11, amplified the expected fragments of 492 bp and 369 bp in novel diploid deletion mutants, confirming their homozygosis (figure 2). This led to the construction of *rad*52 homozygous diploid deletion mutants in yeast BY4743 background.

Table 1. Primers designed and used in this study.

Oligo	Sequence (5'-3')
Universal primers for pUG6-KanMx cassette	
P1	CCagctgaagcttcgtacgc
P2	GCATAGGCCACTAGTGGATCTG
45 bp sequences from *RAD52*	
Upstream	TTGCCAAGAACTGCTGAAGGTTCTGGTGGCTTTGGTGTGTTGTTG
Downstream	AACGCTTCCTGGCCGAAACAAATAAAAAATTTGCATCATTATTTA
Primers to amplify *KANMX*-45bp *RAD52*	
P3	TTGCCAAGAACTGCTGAAGGTTCTGGTGGCTTTGGTGTGTTGTTG CCagctgaagcttcgtacgc
P4	TAAATAATGATGCAAATTTTTTATTTGTTTCGGCCAGGAAGCGTT GCATAGGCCACTAGTGGATCTG
Primers to check for *rad52* deletions	
P5 (*RAD52*)	AATGCAAACAAGGAGGTTGC
P6 (*KANMX*)	TCAGAAACAACTCTGGCG
P7 (*RAD52*)	CGACACATGGAGGAAAGAAAA
P8 (*KANMX*)	CTTCATTACAGAAACGGCT
Universal primers of *MAT* locus	
P9	AGTCACATCAAGATCTTTATGG
P10 (*MAT*α)	GCACGGAATATGGGACTACTTCG
P11 (*MAT*a)	ACTCCACTTCAAGTAAGAGTTTG

AMT of yeast rad52 homozygous diploid deletion mutants

Using *S. cerevisiae rad*52 homozygous diploid deletion mutant as the host, AMT was performed by either pRAL7100 (containing a homologous integrative T-DNA) or pRAL7101 (containing a replicating circular T-DNA) binary vectors. Data from two experiments (with two repeats in each one for *rad*52 deletion) are represented in Table 2 and Table 3. Analyses of AMT frequencies and relative frequencies in *rad*52 diploid deletion strains in comparison with wild type strain BY4743, as control, are represented in Table 4. As seen, relative frequencies of AMT in *rad*52 deletants are decreased to *ca*. 4% of the wild type strain, both with integrative and replicating binary vectors.

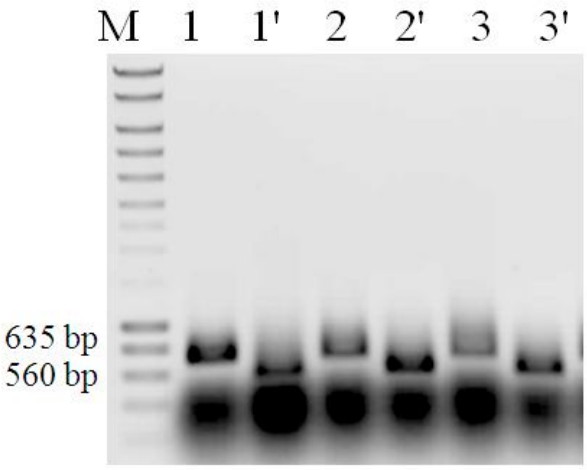

Figure 1. *RAD*52 substitution by *KANMX* cassette in deletion mutants (No.1-5) of yeast haploid strains of BY4742 (No. 1 and 2) and of BY4741 (No. 3,). Bands in lanes 1, 2, 3 obtained from primer combinations P5 and P6 (635 bp), and in lanes 1', 2', 3' obtained from primers combinations P7 and P8 (560 bp). M: DNA molecular ladder of 10 kb.

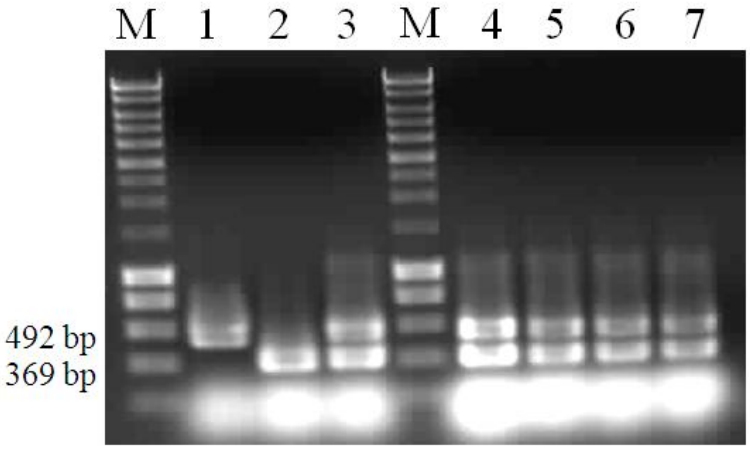

Figure 2. Presence of both *MAT*a and *MAT*α loci in yeast *rad*52 diploid deletion mutants constructed by mating haploid strains of BY4742 and BY4741. Lane 1: *MAT*a in haploid strain BY4741 (492 bp); lane 2: *MAT*α in haploid strain BY4742 (369 bp); lane 3: *MAT*a and *MAT*α in diploid strain BY4743; lanes 4, 5, 6, 7: *MAT*a and *MAT*α in *rad*52 diploid deletion strains in yeast BY4743 background. M: DNA molecular ladder of 10 kb.

Table 2. AMT of yeast wild type strain BY4743 and its *rad*52 deletion diploid strain by using a homologous integrative T-DNA.

pRAL7100 (integrative T-DNA)	Experiment 1			Experiment 2		
	Number of transformants	Output × 10^{-5}	Frequency	Number of transformants	Output × 10^{-5}	Frequency
Wt BY4743	143	385	0.371	180	228	0.789
rad*52*Δ	Repeat1: 6	102	0.059	4	162	0.025
	Repeat2: 1	97	0.010	2	203	0.009

Frequencies are depicted as the number of uracil prototrophic colonies divided by the output number of yeast cells. Transformations were performed using the standard transformation protocol (Bundock et al., 1995).

Table 3. AMT of yeast wild type strain BY4743 and its *rad*52 deletion diploid strain by using a replicating non-integrative T-DNA.

pRAL7101 (replicating T-DNA)	Experiment 1			Experiment 2		
	Number of transformants	Output × 10^{-5}	Frequency	Number of transformants	Output × 10^{-5}	Frequency
Wt BY4743	215	471	0.456	154	339	0.454
rad*52*Δ	Repeat1: 3	155	0.019	2	125	0.016
	Repeat2: 2	164	0.012	3	147	0.020

Frequencies are depicted as the number of uracil prototrophic colonies divided by the output number of yeast cells. Transformations were performed using the standard transformation protocol (Bundock et al., 1995).

Table 4. Analyses of frequencies and relative frequencies of AMT in yeast *rad*52 diploid deletion strain, performed either by an integrative or a replicating T-DNA.

	pRAL7100 (integrative T-DNA)			pRAL7101 (replicating T-DNA)		
	Average of AMT frequencies	Relative frequency	Relative frequency (%)	Average of AMT frequencies	Relative frequency	Relative frequency (%)
Wt BY4743	0.580	1.001	100	0.455376	1.001	100
	Repeat1: 0.042	0.072	7	0.017677	0.039	4
rad*52*Δ	Repeat2: 0.010	0.017	1	0.016302	0.036	4

Discussion

Agrobacterium-mediated transformation is a method of high interest for genetic transformation of both plants and fungi. In addition to agrobacterial proteins involved in AMT, it is evident from previous experiments that host proteins highly influence the fate of introduced transgene, T-DNA, thereby affecting the AMT frequencies (Citovsky et al., 2006). A group of such proteins, identified in model eukaryote *S. cerevisiae*, are *RAD*52 epistasis group of proteins (Soltani, 2009), which are initially involved in regulation of DNA double-strand breaks (DSBs) repair by homologous recombination (HR). Regulation of DSBs in the yeast *S. cerevisiae* involves the recruitment of Rad52, a central recombination protein, to sites of DNA breaks. The Rad52 protein plays a role in strand exchange and the annealing of single strand DNA, especially upon entry into S phase (Barlow and Rothstein, 2010). Thus, proper functioning of Rad52 affects double strand break repair by initiating and/or directing of many aspects of HR. It is shown that *rad*52 deletion affects the T-DNA integration by HR (van Attikum and Hooykaas, 2003). This has led to the speculation that *RAD*52 might affect AMT by influencing DNA repair by HR.

T-DNA containing a *S. cerevisiae* replicator, like ARS or 2µ replicators, doesn't integrate into the host genome. Instead, after circularization, it will be maintained in the yeast cell as a replicating plasmid (Bundock et al., 1995; Piers et al., 1996). Here, both integrative and replicating binary vectors were assayed for AMT of yeast *rad*52 deletion strains. As it is shown in Table 4, the AMT frequencies mediated by HR-integrative binary vector pRAL7100 were decreased by *ca.* 25 fold, compared to the wild type BY4743. This is in agreement with previous results reported by van Attikum and Hooykaas (2003). However, it is further found that AMT frequencies obtained by the non-integrative binary vector pRAL7101, which will be remained as a circular replicating plasmid in the yeast cell, were also decreased by *ca.* 25 fold, compared to the wild type BY4743. This raises the question about the real effect of host *rad*52 deletion on the T-DNA fate inside the cell. To address this, *RAD*52 deletion may affect the replication of introduced T-DNA, in addition to its affection on host cell integrity. In support of the first hypothesis it is shown that the Rad52 is positively regulated upon entry into S phase, but repressed during the intra-S phase checkpoint (Barlow and Rothstein, 2010). Although HR is inhibited at stalled replication forks, for cell survival in the event of fork collapse, HR is necessary during S phase (Lisby et al., 2001). This implicates the function of *RAD*52 for the replication fork restart and thus proper DNA replication. Such functions might support the proper replication of introduced replicating T-DNA as well. Hence, in the absence of *RAD*52, replication of T-DNA may be suppressed and subsequently the AMT frequencies are reduced. On the other hand, *rad*52 deletion may have complicated effects on the cell integrity which are not fully explored yet, affecting the introduction of T-DNA into the cell and/or the expression of its genes.

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Computational Analyses for Identification Novel MicroRNAs from Cattle and Sheep

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Abstract

MicroRNAs (miRNA) are a class of noncoding and regulatory RNA molecules about 22 nucleotides in length. MicroRNAs regulate gene expression by an RNA interfering pathway through cleavage or inhibition of the translation of target mRNA. Many miRNAs have been reported for their important roles in developmental processes in various animals, but there is limited information about cattle and sheep miRNAs. The comparative genomics approach due to their conserved nature is a good source for the miRNAs discovery. Cattle and sheep are ideal model organisms for biological and comparative genomics studies. In our study, a computational method based on expressed sequence tag (EST) analysis was used for detection of cattle and sheep miRNAs. In cattle, 25 miRNA candidates found by homology searching frequently clustered at certain chromosomes and 28 miRNAs in sheep had been detected. Our results show that the cattle and sheep miRNA database can be providing useful information for investigating biological functions of miRNAs in cattle and sheep. Furthermore, the bioinformatics approach is a good manner for studying these functions.

***Keywords*:** microRNAs, comparative genomics, homology searching, sheep, cattle

Introduction

MicroRNAs (miRNAs) are non-coding RNAs, which are approximately 22 nucleotides (nt), and can regulate the expression of target genes by binding to complementary sites. It is more prevalent for miRNAs to down-regulate the expression of target genes by binding to the complementary sites in transcripts and cause transcript degradation or translational repression (Bartel, 2004; Pillai et al., 2007). However, recent studies found that miRNAs could increase protein translation by binding to the complementary promoter sequences (Place et al., 2008; Vasudevan et al. 2007).

The miRNAs in different organisms are involved in many gene regulation processes like; growth and development, transformed genes inactivation, cell signaling pathways, external stresses, cancer related proteins and defense against the invading viruses (Kidner and Martienssen, 2005).

The first miRNA was discovered in larval mutants of the nematode *Caenorhabditis elegans* and was identified as a down-regulator of gene expression. Larval worms with mutations in the lin-4 gene showed defects in the timing of cell division, and the miRNA encoded by the lin-4 gene silenced the expression of lin-14 mRNA (Lee et al., 1993). Hundreds of miRNAs have been identified by small RNA cloning and computational analysis in plants and animals (Bentwich et al., 2005). The view of miRNA biogenesis and maturation holds that compartmentalized stepwise processing of miRNAs takes place first in the nucleus and then in the cytoplasm. The primary transcripts of miRNAs (pri-miRNAs) are processed in the nucleus by the RNase III enzyme, Drosha, partnered by DGCR8 (or Pasha in *C. elegans*), to stem-loop intermediates of about 70 nt known as pre-miRNAs. The pre-miRNAs are then transported by Exportin-5 to the cytoplasm for cleavage by Dicer (another RNase III enzyme) and maturation to their active forms, which are taken in by an RNA interfering silencing complex. The miRNAs drive their target mRNAs to be cut or translationally inhibited, depending on the complementarity of the miRNA sequences to their targets (Plasterk, 2006).

There are basically two kinds of approaches to identify miRNAs. One is to sequence size-

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fractionated cDNA libraries. Many known miRNAs have been identified by this method (Fu et al., 2005). Mentioned method allows the identification of both conserved and unconserved miRNAs, but a limitation of which is that some miRNAs are expressed at low levels, and expressed at very specific stages or in rare cell types. In contrast, computational strategies, which may especially miss those that are not phylogenetically conserved, provide an efficient way to predict miRNAs and their targets by surveying genomic sequences or other databases like expressed sequence tags (ESTs). Which are based on the secondary structure characteristics, phylogenetic conservation of both sequence and structure, and thermodynamic stability of hairpins. Computational approaches have been successfully applied in vertebrates (Huang et al., 2008), insect (Singh and Nagaraju, 2008), and plant (Jin et al., 2008).

Cattle and sheep are two of the most important agricultural livestock for meat production, but there is limited information about their miRNAs (miRBase 18.0, November 2011) (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008). The aim of this study was identification more miRNAs in cattle and sheep in order to increase our knowledge and understanding of the gene regulatory networks in these livestock.

Materials and Methods

Computational identification of cattle miRNAs

In order to identify potential cattle miRNAs, we downloaded all of the known mammalian (human, mouse, pig, sheep, and dog) miRNAs registered in the database of miRBase 18.0 (http://www.mirbase.org/) (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008) and regarded them as starter reference sequences. Cattle miRNAs registered in the database were used to test the prediction of miRNA candidates. Cattle genomic survey sequences, mRNA, cDNA, RefSeq protein database, and EST sequences were obtained from NIH Genbank nucleotide databases (http://www.ncbi.nlm.nih.gov/). After removal of repeat miRNAs obtained from the database of miRBase, the rest of the unique miRNAs were blasted against the cattle genome (BTAU4.0). Sequences encoding protein were ignored by blasting the RefSeq protein database, and the secondary structures of the rest of the sequences flanked with 50–60 nt of genomic sequence were predicted by the software Mfold (version 3.2) (Zuker, 2003), at 37° C and 1 M NaCl. The sequences were considered to be miRNA candidates if they met the criteria as previously described by Zhang et al. (2006), with some modifications according to the features of most of the known miRNA hairpin precursors: (1) a sequence can fold into an appropriate stem-loop hairpin; (2) there is a mature miRNA sequence site in one arm of the hairpin structure; (3) there are predicted stem-loops without large loops or bulges in the miRNA* sequences; (4) predicted mature miRNAs have no more than four nucleotide substitutions compared with known miRNAs; and (5) predicted secondary structures have a minimum free energy (dG) higher than -18.8° C and paired bases within the stem-loop hairpin ≥16.

Computational identification of sheep miRNAs

Animal miRNAs and their precursor sequences were obtained from miRBase (Release 18:0 November 2011 at http://www.mirbase.org/) (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008). The Sheep ESTs (total 338483 sequences) were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/). A five-step prediction method was used to identify Sheep miRNAs. First, alignment of known animal miRNAs was conducted by BioEdit software package (Hall, 1999) to remove redundant sequences. Second, we used remaining miRNAs as query sequences for BLAST searched against the Sheep ESTs with BLASTN, the parameter settings were as follows: E-value cut-off was 10, the number of descriptions and alignments were 1000. Third, the ESTs with no more than two mismatched nucleotides were chosen to blast with the known pre-miRNA sequences. Fourth, the ESTs which have >90% similarity with the corresponding known precursor sequences were selected to remove the repeated sequences and the protein-coding sequences by BLASTN and BLASTX program. The last step was to apply Mfold (version 3.2) (Zuker, 2003), to further identify the pre-miRNAs. Four criteria were used: (1) the A+U content of the precursor sequences should range from 30 to 70%; (2) the mature miRNA should locate on one arm of the hairpin structure, and the corresponding positions of the mature miRNAs in their pre-miRNAs were nearly identical, which was calculated by δ –len (A,B) [13]. We used δ -len cut-off 10 as the default parameter; (3) the minimum free energy (MFE) of the secondary structure for each potential pre-miRNA was less than -20 kcal/mol; (4) the hairpin must include at least 16 bp within the first 22 nt of the miRNA, and should not contain large internal loops or bulges, particularly not large asymmetric bulges, as described in

reference (Clop et al., 2006). The Sheep miRNA, oar-mir-134, was selected for conservation and phylogenetic analyses. The analyses of oar-mir-134 with human (*H. sapiens*), mouse (*M. musculus*), rat (*R. norvegicus*) and cattle (*B. Taurus*) orthologues were done by the publically available weblogo: a sequence logo generator (Crooks et al., 2004) and ClustalW (Larkin et al., 2007) to generate cladogram tree using neighbor joining clustering method respectively.

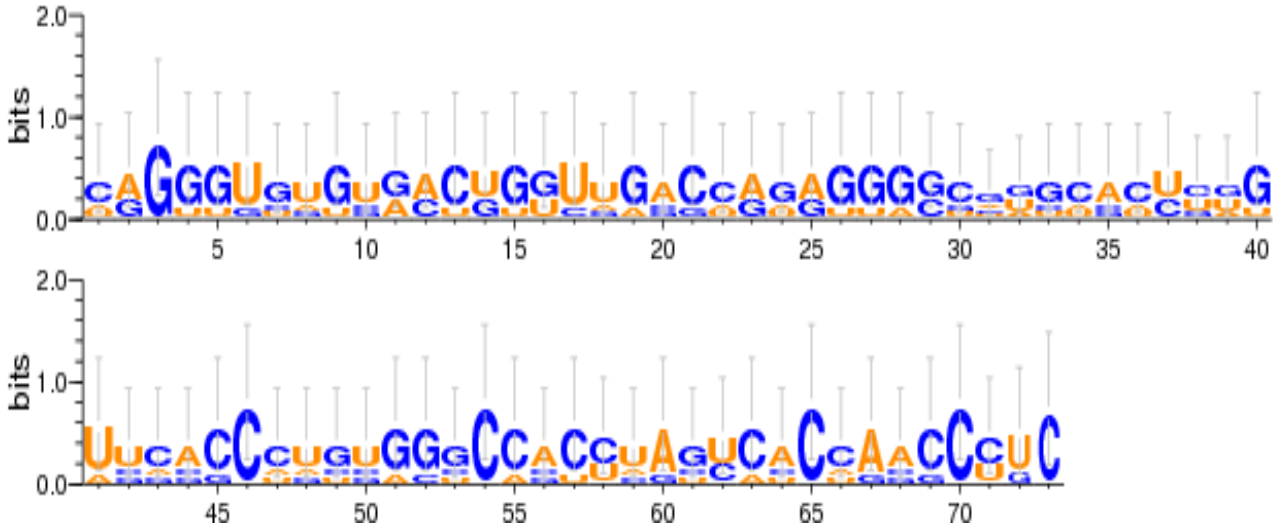

Figure 1. The miRNAs conservation studies. Alignment of sheep (*O. aries*) pre-miRNAs with human (*H. sapiens*), mouse (*M. musculus*), rat (*R. norvegicus*) and cattle (*B. Taurus*) was generated using Weblogo.

Results

Identification of 25 cattle miRNA candidates by computational prediction in order to identify potential cattle miRNAs by homology searching was carried out. We downloaded all of the miRNAs for humans, mice, pigs, dogs, and sheep registered in the miRBase (http://www.mirbase.org/). After removal of repeated miRNAs, 251 miRNAs were considered as starter references to blast the cattle genome. Of the 11121 sequences targeted within the 0–4 mismatch, only 1052 had a match of at least 16 nt, with >80% homology to known miRNAs, and were within two mismatches of a known miRNA of less than 19 nt. Of those sequences, 94 were fragmented sequences of encoding protein or of known noncoding RNA. The secondary structure of the remaining sequences was predicted by the program Mfold 3.2, and only 81 hairpins met the criteria for miRNAs. After blasting against the cattle genome EST database, we finally identified 25 cattle miRNA candidates. The cattle miRNA candidates were designated with the matched known miRNAs. The result showed that many of the candidates were likely expressed from two or three miRNA genes located on different chromosomes, such as bta-let-7a, bta-let-7f, bta-mir-1 and bta-mir-124a.

The homology search through comparative genetics is a rational approach to find interesting findings. The homology based search and applying in silico approach resulted 28 miRNAs in sheep. These 28 miRNAs are predicted from the analyses of 338483 sheep ESTs. First, we used a computational approach to exploit Sheep miRNAs in sheep EST database. Following a set of strict filtering criteria, we finally identified 28 Sheep miRNAs.

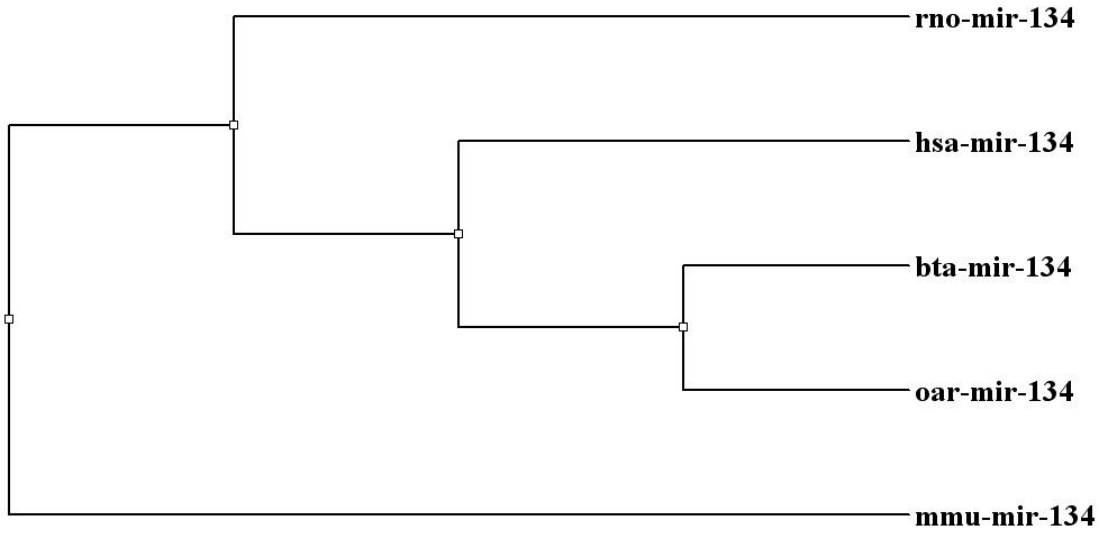

Figure 2. The sheep miRNAs phylogenetic analysis. The Phylogenetic analysis of the sheep pre-miRNAs with human (*H. sapiens*), mouse (*M. musculus*), rat (*R. norvegicus*) and cattle (*B. Taurus*) was done with the help of Clustalw and cladogram tree was generated using neighbor joining clustering method. The Phylogenetic tree showed that sheep is more closed to *B. Taurus* (bta).

Discussion

MicroRNAs represent a large class of gene regulatory molecules that control fundamental cellular processes in animals, including control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation, and organ development (Bartel, 2004; Ambros, 2004). miRNAs and their targets seem to form complex regulatory networks (Lewis et al., 2005). Identification of miRNAs would benefit biologists by improving our understanding of the miRNA regulatory networks. Small RNA cloning and genetic manipulations were tackled to discover novel miRNAs, and computational algorithms were also successfully applied to identify hundreds of miRNAs (Grad et al., 2003; Lagos-Quintana et al., 2002; Lai et al., 2003; Ruby et al., 2006). Computational prediction would overcome the difficulty of biochemically discovering low-abundance miRNAs.

In this study, we identified 25 miRNAs in cattle by homology searching. Coutinho et al., (2007) reported 129 mature cattle miRNAs identified by homology searching and small RNA cloning from immune-related tissues. They started the search, however, only from human miRNA stem-loop sequences or combined the predicted human miRNAs. Consequently, their prediction ignored other miRNAs not found in humans but in other mammals, such as mice, pigs, sheep, and dogs. Furthermore, their prediction was based on the draft genome of cattle, at almost the same time; Gu et al. (2007) discovered 59 distinct miRNAs from cattle adipose and mammary gland tissues by small RNA cloning. Combining the two reports, about 130 miRNAs have been discovered from adipose, mammary gland, and immune-related tissues in cattle. To identify more miRNAs in cattle, Long et al. (2009) searched experimental miRNAs based on the cattle genome. They also cloned miRNAs from four tissues of brain, liver, lung, and heart, given that miRNAs were probably expressed with tissue specificity. Our study results covered all miRNAs identified in previous studies (Coutinho et al., 2007; Gu et al., 2007; Long and Chen, 2009). This indicated that cattle miRNA candidates were predicted at very high accuracy.

We finally identified 28 Sheep miRNAs. Among which, 13 identified miRNAs sequences were identical or highly similar (1 or 2 nt mismatches) with those of known miRNAs in related species, which agreed with that most miRNAs are conserved among mammalian species. The minimum free energy (MFE) of all precursor sequences was lower than -20 kcal/mol. However, the MFE of its homologs in other species also has a higher value, for example, -16.7 kcal/mol in *Homo sapiens* and -15.2 kcal/mol in *Pan troglodytes* (Ambros et al., 2003; Lagos-Quintana et al., 2001).

The identified sheep's miRNA secondary structures showed that there are at least 16 nt engaged in Watson–Crick or G/U base pairings between the mature miRNA and the opposite arms (miRNAs*) in the stem region except few, where the reference miRNAs have also less base pairings and the hairpin precursors do not contain large

internal loops or bulges. Similar findings were reported by many groups (Barozai et al., 2008; Barozai et al., 2012; Glazov et al., 2009; Hossain et al., 2009; Barozai et al., 2011; Barozai et al., 2011).

The sheep pre-miRNA (oar-mir-134) conservation and phylogenetic analyses with human (*H. sapiens*), mouse (*M. musculus*), rat (*R. norvegicus*) and cattle (*B. Taurus*) revealed that approximately miRNA (miR 134) sequences showed conserved nature in these animals (Fig. 1). The Phylogenetic analysis of the same miRNA (mir-134) sequences suggested that the sheep is more closed to *B. Taurus* (Fig. 2). The results are in agreement with the previous reported works (Lai et al., 2003; Sheng et al., 2010; Huang et al., 2010).

In conclusion, this study identified the 25 and 28 miRNAs in cattle and sheep, respectively. The miRNAs are the good functional genomic resources to understand the gene regulatory mechanism in cattle and sheep. These cattle and sheep's miRNAs will be useful in the near future for the improvement of meat production from this most important livestock animal. The identification of miRNA targets and expression profiles at a genome wide level, perhaps help us to understand why miRNAs are clustered at a genomic locus, too.

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Selection and optimization of single cell oil production from *Rodotorula* 110 using environmental waste as substrate

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Abstract

Micro-organisms such as bacteria, yeasts, molds and algae that accumulate lipid more than 20% of their biomass are called oleaginous. Microbial lipid has high similarity to the oil obtained from plants and animals. Microbial lipids are renewable sources that can be used for different purposes such as biodiesel production. Production of oil from yeasts has more advantages than that from plants. Accordingly, isolation of oleaginous yeasts with high ability of lipid production is very valuable. In this study we isolated 176 yeasts from 34 soil samples, from which 68 could produce lipid. The strains were screened by an enrichment technique in glycerol and then Sudan black B staining. After lipid extraction by Bligh and Dyer method, the best strain, *Rodotorula* 110**,** was selected. This strain proved to comprise lipid, dry biomass and lipid productivity at levels of 8.9 g/l, 15.29 g/l and 58.2% in optimized conditions, respectively. Then lipid production by the selected strain was evaluated on corn stalk and wheat straw hydrolysate as sole carbon sources. Lipid content on these media was 38.9% and 43.4%, respectively. The extracted lipid was analyzed by thin layer chromatography and FTIR spectroscopy.

Keywords**:** Oleaginous yeast**,** lipid extraction**,** Microbial lipid, FTIR spectroscopy

Introduction

Oleaginous micro-organisms such as yeasts, fungi and micro algae can accumulate high amounts of reserved lipids under appropriate cultivation condition, so their potential as lipid producing sources has attracted high attentions. Unicellular yeasts have high growth rate and can accumulate lipid in separate lipid bodies (Li et al., 2008; Drucken, 2008; Mullner and Daum, 2004; Melickova et al., 2004). They can also use low cost fermentation media such as waste material of agricultural and industrial products (Amaretti et al., 2010). Storage lipids are in the form of tri acyl glycerol (TAG) so different types of fatty acids are the main target for improving the biotechnological products. Phospholipids, sphingolipids, glycolipids, sterols and carotenoids as well as other lipid compounds are used for production of bioactive molecules used in cosmetics, nutritional and pharmaceutical products (Schorken and Kempers, 2009). Microbial oil has potential to substitute the plant oil in market. Micro-organisms that produce lipid more than 20% of their biomass are called oleaginous (Wynn and Ratledge, 2005; Amaretti et al., 2010).

The great part of microbial lipid is TAG which contains long chain fatty acids and is comparable with conventional plant oil (Kosa and Ragauskas, 2010; Pan et al., 2009). Some yeasts have evolved sophisticated metabolic pathways that allow them to grow on lipid substrates. Some of oleaginous yeasts can metabolize pentoses. This shows the ability of TAG production from lignocelluloses substrates and other low cost materials (Sabirova et al., 2010; Li et al., 2007, Zheng et al., 2012). Yeasts spp. that are known as oleaginous include *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* (Pan et al., 2009).

Rhodotorula is a pigmented yeast that can be identified easily by distractive orange/red colonies when grow on Sabouraud's Dextrose Agar. This color is the result of pigments that yeasts produce to block certain wavelengths of light and protect them. The color of colonies is very different from cream to orange, red, pink or yellow. This yeast has high potential of lipid production and some of its strains can accumulate lipid on xylose and lignocelluloses

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substrate as sole carbon sources. This ability is important for using these strains in industrial processes (Postgate, 1994; Dai et al., 2007).

Lipid accumulation occurs when one of the nutrients (usually nitrogen) is exhausted. At the same time, there are excess amount of carbon such as glucose in the medium. The cell response to exhaustion of nutrients is that they don't grow and multiply, but they continue to take up glucose from the medium. The surplus sugar is used for lipid biosynthesis. Under nitrogen limited condition the first requirement for the cells is to cease production of energy (i.e. , ATP) that is no longer needed for the synthesis of macromolecules, such as proteins and nucleic acids; because the cells do not grow and divide any more. During nitrogen limitation, oleaginous and non oleaginous yeasts continue to assimilate carbon but only oleaginous organisms metabolize it and increase the ATP/AMP ratio. These cells become larger when lipid particles grow (Meng et al., 2009; Fei et al., 2008; Raschke and Knorr, 2009; Fakas et al., 2008; Wynn and Ratlege, 2005).

The purpose of this study was isolation of oleaginous yeast with high potential of lipid production and evaluating the efficiency of lipid extraction by Bligh and Dyer method from oleaginous yeasts. Optimization of medium condition and its effect on increasing lipid production was evaluated. Analysis of produced lipid was done by FTIR spectrometry. Also the potential of lipid production by this strain on agricultural residues was evaluated.

Materials and Methods

Isolation and selection of oleaginous yeast

34 soil samples were collected to isolate yeasts. The soils were from peanut, walnuts, sunflower and almond gardens. A mass of 1 g of soil was added in to 50 ml enrichment medium which includes (g/L): glycerol 100, $(NH_4)_2SO_4$ 1, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5 and yeast extract 0.2 in a 250 ml erlenmeyer flask, then incubated at 30ºC for 96 h with shaking at 180 rpm (Pan et al., 2009).

Then 0.5 ml of this pre-cultured yeasts was added to solid medium which includes glucose (20 g/L), $(NH_4)_2SO_4$ (2 g/L), KH2PO4 (0.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $CaCL_2.2H_2O$ (0.1 g/L) and 2% agar. The plates were incubated at 28ºC for 48 h. After incubation, the yeast colonies were isolated for screening process (Zheng et al., 2012).

Screening for oleaginous yeasts by qualitative methods

Lipid production of isolates was evaluated by Sudan black B staining. The potential oleaginous yeast colonies were maintained on YPD slant which contains (g/L): glucose 20, yeast extract 20 and peptone 20 at 4ºC.

Determination of lipid production in isolated yeast

After qualitative analysis by Sudan Black B staining, oleaginous yeasts were cultured in nitrogen limited medium for 5 days. This medium includes glucose (40 g/L), $(NH_4)_2SO_4$ (2 g/L), KH_2PO_4 (7 g/L), NaH_2PO_4 (2 g/L), $MgSO_4.7H_2O$ (1.5 g/L) and yeast extract (1 g/L). 50 ml of this medium in 250 erlenmeyer flask was used on a shaker at 180 rpm and 28ºC. Before culturing in nitrogen limited medium, the yeast colonies were activated in inoculation medium, containing (g/L): glucose (15), $(NH_4)_2SO_4$ (5), KH_2PO_4 (1), $MgSO_4.7H_2O$ (0.5) and yeast extract (0.5) grown at 28ºC at 180 rpm for 48h (Pan et al., 2009; Kraisintu et al., 2010).

Lipid extraction was done, based on Bligh and Dyer method with few modifications (Pan et al., 2009). 50 ml of the sample was centrifuged at 5000 rpm for 10 min. After that the yeasts were washed with 50 ml of distilled water. Then 10 ml of HCl (4M) was added and incubated at 60 ºC for 3h. Then acid hydrolyzed mass was stirred with 20 ml chloroform/methanol mixture (1:1) at room temperature for 3 h. At the end centrifugation was done at 5000 rpm for 3-5 min at room temperature to separate the aqueous upper phase and organic lower phases. Then the lower phase containing lipid was recovered with pasteur pipette and evaporated in the vacuum. After that the dry lipid was weighed.

The ability of using xylose and subsequently lipid production with xylose as sole carbon source was investigated. Then the best strain was cultured on the nitrogen-limited medium which includes (g/L): xylose (40), $(NH_4)_2SO_4$ (1), KH_2PO_4 (7), $MgSO_4.7H_2O$ (1.5) and yeast extract (1) grown at 28ºC and 180 rpm in shaker incubator for 72 h (Pan et al., 2009).

Determination of single cell oil productivity (lipid content)

Lipid content in each trial condition was determined by the following equation (Kraisintu et al., 2010):

SCO productivity (Lipid content) = SCO Weight (g/L) / Cell dry weight (g/L) × 100

Analysis of single cell oil production by FTIR spectroscopy

Lipid production in oleaginous yeast was verified by sudan black staining at first, further confirmation of certain oil compounds was detected by FTIR (Fourier Transform Infrared) spectroscopy using JASCO FT/IR-6300, Japan device. The range of spectrum, analyzed by device, was set from 400 cm^{-1} to 4000 cm^{-1}. Triolein (bought from sigma Aldrich) was used as control sample for comparing with the produced single cell oil.

Effects of nutrient composition, pH, rpm, incubation time and temperature on lipid production

Effects of nutrient composition such as glucose and ammonium sulfate concentration and physical parameters such as pH, rpm, temperature and time of incubation on lipid production were evaluated. Glucose concentration was varied at 35, 55, 75, 95, 115g/L. Effect of combined organic nitrogen sources (yeast extract and peptone at 1 g/L) and inorganic compounds (ammonium sulfate and ammonium chloride at 1g/L) on lipid production were investigated. Also lipid production was evaluated under different ammonium sulfate concentration such as 0.5, 1, and 1.5 g/L. Other factors were variable; pH rates of 5, 5.5, 6, 6.5; temperatures of 25ºC and 35ºC; rpm of 150 and 200; and times of incubation of 24 h, 48 h, 72 h and 96 h (Kraisintu et al., 2010).

Lipid production using wheat bran and corn stalk as sole carbon sources

Before using wheat bran and corn stalk, they must be prepared by acid hydrolysis. For this purpose the materials were ground, then hydrolyzed by using sulfuric acid (5%). This digestion was performed at a solid:liquid ratio of 1:8, upon completion of the process they were autoclaved at 110°C for 25 min. Then the suspension was centrifuged to remove unhydrolyzed residues (Dai et al., 2007). 10 ml of this suspension was brought to 45 ml with sterile water. After adjusting the pH at 6, the other components (the same as nitrogen-limited medium) were added. 5 ml of the inoculation was added and incubated at the optimum condition.

Results

Isolation of oleaginous yeasts

174 yeasts were isolated, from which 68 were oleaginous according to our first qualitative analyses by Sudan black B staining. The yeast lipid bodies were obvious as black droplets inside the oleaginous yeast cells under optical microscope. Some of the strains had multi-lipid bodies. These strains were selected for lipid extraction after cultivation in nitrogen limited medium. The result of lipid extraction, dry biomass, lipid productivity, and the ability of xylose assimilation were shown in table 1.

As the results show the strain *Rodotorula* 110 (Yr_2) produced the highest amount of lipid. Also this strain could produce lipid in a medium with xylose as a sole carbon source. Yr_2 produced 6.17 g/L lipid per 17.82 g/L dry biomass and its lipid productivity was 34.62%. So this strain was selected for further investigation.

Effect of nitrogen source on lipid production in yeast

In the nitrogen limited condition the excess amount of carbon in the medium is used to produce lipid bodies by oleaginous yeasts. The effects of organic nitrogen such as yeast extract and peptone and inorganic nitrogen such as ammonium sulfate and ammonium chloride were determined in the nitrogen limited medium with 40 g/L glucose. Results showed that lipid production by this strain reached to its highest level at the presence of yeast extract and ammonium sulfate, although there was no significant difference for production examine between the evaluated nitrogen sources (table 2).

Effect of glucose and xylose as carbon sources on lipid production in yeast

Production of lipid in the medium with glucose as a sole carbon source was more efficient. Therefore glucose was used as carbon source for evaluating the yeast capacity for lipid production under different conditions. Lipid production on xylose with *Rhodotorula* 110, was 5.15 g/L in this study. Xylose is one of the main sugars in the hydrolysis of Lignocellulosic materials and environmental wastes, so oleaginous yeasts with capacity of using it are valuable from economical point of view.

Effect of glucose and ammonium sulfate concentration on lipid production

Table 2 shows that when glucose concentration increased from 35 g/L to 55 g/L and 75 g/L, the lipid production was also raised; but increasing of glucose concentration to 95 g/L and 115 g/L had reverse effect on lipid production because optimum concentration of glucose depends on the yeast strain and each strain has a different capacity for tolerating osmotic effect of sugar in the medium.

About ammonium salt the second concentration (1g/L) was the best one for lipid production. It has

been shown that nitrogen is necessary for growing but the limited condition is also important for lipid production.

Effects of physical parameters

The best temperature for lipid production by this strain was 25ºC and had significant effect on lipid production. Time of incubation had significant effect on lipid production too, but when agitation rates increased the lipid production decreased slightly. Lipid production after 24 h, 48 h and 72 h was 4.6 g/L, 6.03 g/L 8.85 g/L, respectively ; but after 96 h this production decreased to 7.95 g/l. The best pH for lipid production was 6.5 and lipid production at this pH with other optimized factors was 8.9 g/L.

Lipid production using agricultural residues

The results of lipid production in these media are shown in table 3. The results obtained in our study were excellent and showed high potential of lipid production by this yeast strain (Yr2).

FTIR spectroscopy analysis of lipid products

Microbial lipid graphs obtained from the FTIR analysis are shown in figure 1. Comparison of two graphs shows the highest similarity between extracted oil from oleaginous yeast with the standard oil of triolein. Significant peaks were created between 1670 to 1820 cm^{-1}, confirming presence of carbonyl groups. There are peaks between 2850 to 2929 cm^{-1} that show presence of methyl groups. All of the mentioned peaks confirm that the produced oil can be converted to biodiesel potentially (Elumalai et al., 2011; Lin-Vien,1991). Biodiesel compounds are analyzed based on the European standard of EN 14078 (European Standard EN 14078). FTIR has also been used for analyzing and confirmation of biodiesel based on the methy and ethyl ester of long chain fatty acids in products from *Chloralla vulgaris* and *Senedesmis* sp. (Elumalai et al., 2011).

Table 1. Results of lipid extraction, dry biomass, lipid productivity and Xylose assimilation for oleaginous colonies

strain	Lipid yield (g/L)	Dry biomass(g/L)	Lipid productivity (%)	Xylose assimilation
Y6	4.45	14.21	31.35	w
Yc1	2.13	8.61	24.78	++
Yb1	2.87	11.19	25.67	+
Yb2	2.19	9.18	23.88	++
Yd1	1.97	8.90	22.13	-
Yg1	2.06	9.17	22.53	++
Yq2	2.36	9.78	24.18	++
Ya2	2.35	10.06	23.35	-
Yr2	6.17	17.82	34.62	+
YK	2.16	8.08	26.78	+
YL2	1.94	8.16	23.81	-
Yq3	2.29	10.18	22.45	++
Yq1	2.36	10.02	23.57	+
Yt4	6.04	18.25	33.09	-
Yv2	3.86	13.87	27.88	+
Yh1	1.71	7.53	22.68	+
Yv1	1.92	8.37	22.92	+
Yu2	2.03	8.00	25.38	+
Yv3	3.04	10.72	28.37	+
yL3	3.67	14.22	25.85	++
Am1	3.95	16.91	23.39	-
An	2.91	11.80	24.33	+
Yu1	3.18	12.02	26.50	++
Ab1	4.97	15.47	32.15	+
Ak1	3.99	16.09	24.82	+
Yy1	1.95	8.83	22.17	++
Aa1	5.35	17.98	29.77	++
Ab2	5.09	15.15	33.62	-
Ad1	3.33	12.74	26.18	-
Ag1	4.88	19.93	24.48	+
Ai1	3.90	16.18	24.15	+
Aj	3.14	13.12	23.92	+
Ao1	4.13	15.08	27.38	w
Ao2	4.07	16.35	24.89	++
Ao3	3.30	14.04	23.55	++
Ap1	4.15	15.88	26.13	++

Ap2	4.07	16.15	25.25	++
Ap3	3.88	16.32	23.81	+
Aq1	4.44	17.65	25.16	+
Aw3	5.79	17.45	33.18	+
As1	4.09	16.44	24.87	+
Aa4	5.13	18.98	27.05	+
Ad2	5.03	15.95	31.54	w
Ai2	4.76	19.16	24.88	++
Ak2	3.67	14.56	25.22	++
AL2	3.89	15.46	25.17	+
Am2	4.15	16.07	25.85	++
Aq2	3.87	15.54	24.95	++
Aq3	3.45	14.03	24.63	++
Ar2	4.24	16.13	26.31	++
At1	3.94	16.80	23.45	++
Ay1	4.98	16.89	29.50	w
Az1	3.76	15.92	23.67	++
Az2	3.23	13.38	24.13	++
Bc2	3.77	15.86	23.78	+
Bd1	3.56	16.09	22.15	+
Bm	4.87	17.21	28.31	w
Bn	4.17	19.67	24.27	++
Be1	4.18	17.53	23.84	+
Bd2	3.25	9.79	33.20	++
Bc4	3.00	10.60	28.29	++
Bc1	3.57	12.83	27.82	-
Bb1	2.78	12.28	22.64	+
Ba1	4.41	17.28	25.52	w
Av2	4.12	16.54	24.90	+
Av1	3.92	16.21	24.18	++
Y29	3.92	16.03	24.45	+
At2	2.83	12.45	22.73	+

w: weak assimilation, +: positive (strains with ability of xylose assimilation), ++: high assimilation of xylose, - : negative (strains that could not grow on xylose as sole carbon source).

Table 2. Results of Yr2 cultivated in nitrogen limited medium at different conditions.

condition	**Lipid production (g/l)**	**Biomass (g/L)**	**lipid productivity(%)**
Nitrogen source			
Yeast extract and $(NH_4)_2SO_4$	6.29	17.57	35.78
Yeast extract and NH_4Cl	6.15	17.9	34.35
Peptone and $(NH_4)_2SO_4$	6.11	17.68	34.37
Peptone and NH_4Cl	6.18	17.88	34.55
Carbone source			
Glucose	6.3	17.45	36.1
Xylose	5.15	17.16	30
Glucose concentration(g/l)			
35	4.35	12.42	35
55	6.52	16.21	40.2
75	7.13	12.7	56.1
95	5.84	10.97	53.2
115	5.71	11.42	50
$(NH_4)_2SO_4$ (g/l)			
0.5	6.9	12.36	55.8
1	7.2	12.78	56.3
1.5	6.7	12.4	54
Temperature			
25ºC	7.23	12.86	56.2
35ºC	6.35	11.73	54.1
rpm			
150	7.34	13.04	56.28
200	6.83	12.64	54
Time of incubation			
24h	4.6	9.58	48
48h	6.03	11.59	52
72h	8.85	15.39	57.5
96h	7.95	14.14	56.2

pH			
5	8.83	15.49	57
5.5	8.35	14.54	57.4
6	8.58	14.84	57.8
6.5	8.9	15.29	58.2

Table 3. Lipid production on agricultural residues as sole carbon sources.

condition	**Lipid production (g/l)**	**Biomass (g/L)**	**lipid productivity (%)**
wheat bran	6.50	14.97	43.4
Corn stalk	5.84	15.01	38.9

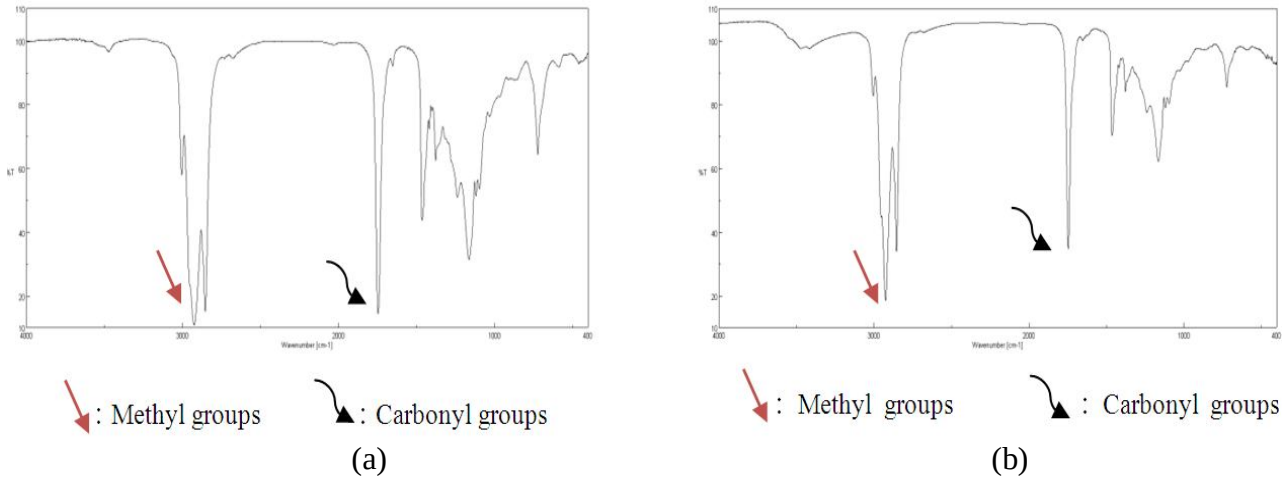

Figure 1. FTIR analytical graphs performed on the standard oil triolein (a) and the oil product from *Rhodotorula* 110 (b).

Discussion

SCO production from lignocellulosic materials, containing xylose, was carried out by some investigators (Zhao, 2005; Papanikolaou, 2008; Meester et al., 1996). Yeast strains that can use xylose in lignocellulosic hydrolysate have potential of industrial application (Dai et al., 2007). Evans and Ratledge (1984) evaluated lipid production in *Candida curvata* and its lipid content was 50% on xylose. Li et al. (2005) reported that lipid production in *Rhodosporidium toroloides* AS2.1389 was 10.6 g/L when using 100 g xylose. Pan et al (2009) isolated oleaginous yeasts with assimilating capacity of xylose and the best yeast strain could produce 5.8 g/L lipid while using 40 g/L xylose. Lignocellulosic materials are a good substrate for microbial oil production because of being low cost and also being abundance (Zheng et al., 2012; Khot et al., 2012). Lipid production on xylose with *Rhodotorula* 110, was 5.15 g/L in this study and with optimization of cultivation condition higher lipid production can be obtained.

About the effect of carbon source on lipid production, Ratledge (2002) reported that glucose was the most important factor on lipid production in oleaginous yeasts. Syed et al (2006) reported that increasing of glucose concentration to very high degrees had inverse effect on lipid production because of increasing osmotic potential of the medium; the same effect of glucose concentration on lipid production was obtained in our study.

About using environmental residues as sole carbon sources for lipid production in yeasts, Dai et al. (2007) used corn stalk and rice straw as sole carbon sources using *Rhodotorula glutinis*. The results for lipid content were 11.78% and 5.74% on corn stalk and rice straw, respectively. The yeast strain in our study had high potential of lipid production on lignocellulosic materials as sole carbon sources. The lipid productivity of this strain was 43.4% and 38.9% on wheat bran and corn stalk, respectively.

The results showed that this strain (*Rhodotorula* 110) is a high lipid producing yeast with potential of industrial applications. Its lipid yield reached to 8.9 g/L with lipid content of 58.2% after 72 h at 25ºC when cultivated in nitrogen limited medium. This medium included: 75 g/L glucose, 1 g/L ammonium sulfate and 1 g/L yeast extract with pH adjusted on 6.5 in shaking flask at 150 rpm. It could assimilate xylose and produce lipid on

lignocellulosic hydrolysate as sole carbon source. This ability shows the potential of this native strain for industrial lipid production.

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Designing of a *35S::DREB1A* molecular construct to produce environmental stress tolerant plants

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Abstract

Environmental stresses affect plant growth and cause losses worth hundreds of million dollars to agricultural industry each year. Many genes are induced in response to environmental stresses. The *DREB1A* gene is a stress-inducible transcription factor which its ectopic over-expression improves plant tolerance to environmental stresses. To produce environmental stress tolerant plants carrying the *DREB1A* gene, the full length cDNA of the *DREB1A* gene was amplified from *Arabidopsis thaliana* Col-0 plants by gene specific primers and cloned into pGEMT-Easy vector, and transformed into *E.coli*. Presence of the *DREB1A* gene was confirmed by restriction analysis as well as DNA sequencing. A 668-bp *Xba*I/*Bam*HI digested fragment of *DREB1A* gene from the *pGEMT::DREB1A* construct was sub-cloned into the pBI121 binary vector. The recombinant plasmids were transferred into *Agrobacterium tumefaciens* cells (strain LBA4404) and screened on LB medium supplied with kanamycin/rifampicin (50 mg/l). Positive bacterial colonies were selected based on colony-PCR analysis and saved for further application in plant materials.

***Keywords*:** abiotic stress, *DREB1A* gene, transgenic plants, *Agrobacterium tumefaciens*

Introduction

The world population is increasing with an alarming rate, while food products are decreasing due to the adverse effects of various abiotic stresses. For the most nations of the world it is a major area of concern to cope with increasing food requirements by minimizing the effects of harsh environmental conditions. Drought, high salinity, heat and freezing temperatures are main stresses which reduce growth and production of crop plants (Mahajan and Tuteja, 2005). Heat and low temperatures induce mechanical damages on the cell, while drought and salinity disturb the water balance and ion homeostasis (Serrano and Navarro, 2001). Plant tolerance to these stresses, is well known to be a multigenic trait resulted from expressions of many genes under stress conditions. Identification of genes related to the Ca^{2+} signaling pathway and environmental stress response showed interaction and correlation between different stress response pathways (Farooq et al., 2009). Although the roles of Ca^{2+} signaling pathway and Abscisic Acid (ABA) are not avoidable in stress response, transcription factors (TF) play central role in plant responses to biotic and abiotic stresses (Chaves and Oliveira, 2004).

The CBF/DREB TFs family, bind to the CRT/DRE elements, have been shown to be induced by drought, salinity, heat and low temperature stresses (Yamaguchi-Shinozaki and Shinozaki, 1994). Most of the stress responsive genes contain the DRE or DRE-related core motifs in their promoter regions. Several reports indicate that over-expression of the DREB TFs leads to an increase in the expression of stress responsive genes which in turn result in plant tolerance to the environmental stresses (Jaglo et al., 1998; Kasuga et al., 1999; Hsieh et al., 2002).

The *DREB1A* gene is a member of the CBF/DREB TFs family, expressed in most plant tissues and developmental stages. The expression of *DREB1A* gene, induced by low temperature, strongly up-regulates more than 50 downstream stress responsive genes (Ito et al., 2006; Hong et al., 2009). This would result in adaptation of plants to the stress conditions and exercise specific tolerance mechanisms. Over-expression of the *DREB1A* gene in crop plants can also result in high tolerance to the abiotic stresses, thereby increasing the efficiency of plant production (Zhao et al., 2007; Hong et al., 2009; Li et al., 2011). Interestingly, the *DREB1A* over-expressing wheat plants consistently had a higher total number of heads and better head development (Pellegrineschi et al., 2004).

We chose *DREB1A* gene to design a molecular construct useful for production of environmental stress tolerant plants. The *DREB1A* gene has many advantages such as central role in stress response signaling pathway, comprehensive effect on the environmental stress tolerance, short length and low

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number of exons, expression in all plant tissues and developmental steps, conservation in most of plant families, involvement in regulation of many downstream stress responsive genes and overcoming of silencing problem in host plants. Here, we report isolation of the *DREB1A* gene from *Arabidopsis thaliana* leaves followed by its cloning into the pGEMT-Easy vector and pBI121 binary vector, and subsequent transformation of *E.coli* and Agrobacterium cells.

Materials and Methods

Materials and growth conditions

The *Arabidopsis thaliana* wild type (WT) plants accession Columbia (Col-0) was used in this study. Seeds were surface sterilized by soaking in 70% (v/v) ethanol for 30 sec followed by soaking in 20% mercuric chloride for 10 min, and five times rinses in sterile distilled water. Sterilized seeds were placed on Murashige and Skoog (MS) medium solidified with 0.8% (w/v) agar. Seeds were stratified in darkness at 4°C for 4 days before transferring to growth chamber. After this period, they were grown for 4 weeks at 22-24°C under a 16/8-h light/dark photoperiod.

The *E.coli* (strain DH5α) cells were cultured on solid or liquid LB medium and incubated at 37°C for 16 h. The *Agrobacterium tumefaciens* (strain LBA4404) cells were also cultured on LB medium supplemented with 50 mg/l rifampicin and incubated at 28°C for 48 h. The bacterial cells containing pGEMT-Easy vector were selected on LB medium supplemented with 100 mg/l ampicillin and the pBI121 plasmid containing cells were screened on LB medium supplied with 50 mg/l kanamycin.

DNA extraction and PCR analysis

Leaves were frozen in liquid nitrogen and DNA was extracted according to Dellaporta et al. (1983). The following PCR primers were used to amplify the *DREB1A* full length cDNA: 5′-AGCTCTAGAATGAACTCATTTTCTGCTTTTT CTG- 3′ (forward) and 5′-AGCGGATCCTTAATAACTCCATAACGATAC GTCG- 3′ (reverse). The restriction sites for *Xba*I and *Bam*HI were also included, respectively. The amplification reaction contained 1xPCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, and 0.2 µl TaqDNA polymerase (1 u/µl). The final concentration of each primer was 0.4 µM. The amplification reaction protocol included an initial denaturation at 95°C for 3 min, followed by 35 amplification cycles of 95°C for 30 sec, 64°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 5 min. The PCR amplification products were separated on 1% (w/v) agarose gels using TBE buffer.

Fragment cloning into the pGEMT-Easy vector

The *pGEMT-Easy* vector has single 3′-T overhangs at the insertion site which greatly improve the efficiency of ligation of a PCR product into the plasmids (Promega, USA). Hence, after purification of the PCR products by purification kit (BioNEER, Korea) they were ligated into the pGEMT-Easy vector. For this, 2 µl of the purified PCR product was mixed with 1 µl of 10x ligation buffers, 1 µl of T_4DNA ligase and 1 µl of pGEMT-Easy vector in final volume of 10 µl. Reaction tubes were incubated overnight at 4°C. *E.coli* competent cells were prepared by ice-cold 50 mM $CaCl_2$ solution (Sambrook and Russel, 2006). 5 µl of the overnight ligation mixture was added to 100 µl fresh competent cells and left on ice for 30 min. Transformation was performed by the heat shock method by 45 sec incubation at 42°C, followed by a 2 min cooling period on ice. An aliquot (900 µl) of SOC solution was added and cells were incubated at 37°C for 3 h. The cells were transferred onto LB-Ampicillin-IPTG-XGal (LAIX) medium for blue/white colonies screening and incubated overnight at 37°C. The transferred plasmids were purified by plasmid isolation kit (BioNEER, Korea) according to the supplied protocol.

Restriction enzyme and sequence analysis

Restriction analysis was performed at 37°C for 3 h. The 20 µl restriction reaction contained 2 µl of 10x *Bam*HI or Tango buffer, 0.5 to 2 µl of *Xba*I with or without *Bam*HI (10 u/µl), 1 to 2 µl (0.05 ng) of plasmids which were extracted from blue/white colonies. At the end of the reaction, the restriction enzymes were inactivated at 80°C for 20 min. The digested products were separated on 1% (w/v) agarose gels. *Xba*I/*Bam*HI digested fragment was cleaned using a DNA purification kit (BioNEER, Korea), and then used for ligation into the pBI121 binary vector.

For confirmation of the cloned fragment, recombinant plasmids were sequenced with forward and reverse primers of *DREB1A* gene. The identity of the sequenced fragment was checked by BLAST (Basic Local Alignment Search Tool) searches available in TAIR (http//www.arabidopsis.org/Blast/).

Gene over-expression construct

To make over-expression construct, pBI121 binary vector was used. The *DREB1A* gene was

recovered as *Xba*I/*Bam*HI fragment from the pGEMT-Easy vector and subcloned into the same sites of pBI121. The 10 µl ligation reaction contained 1 µl of 10X ligation Buffer, 1 µl of T_4DNA ligase, 2 µl of *Xba*I/*Bam*HI digested pBI121 vector, and 2, 4 or 6 µl of gene digested fragment. Reaction tubes were incubated at 4°C for 16 h. By this strategy the *DREB1A* gene was placed under the control of CaMV 35S promoter and nopaline synthase (nos) terminator. The *pBI121::DREB1A* was introduced into *Agrobacterium tumefaciens* LBA4404 cells by freeze-thaw (5 min in liquid nitrogen and then 5 min at 37°C) transformation procedure (Sambrook and Russel, 2001). One ml fresh liquid LB was added and the cells were incubated at 28°C for 2 h. The cells were transferred onto the LB medium, supplied with kanamycin/rifampicin (50 mg/l) and incubated at 28°C for 48 h. Recombinant bacteria were confirmed by PCR analysis and saved for further application in plant materials.

Results

Here, we designed the *pBI121::DREB1A* molecular construct *in-silico*, by using the clone manager software. The sequences of *DREB1A* gene, pGEMT-Easy vector and pBI121 binary vector were scanned to select suitable no-cutter restriction enzymes and *Xba*I and *Bam*HI were selected. These sites were added to the 5′ ends of the primers. Presence of these two different sites has several advantages in next steps of cloning.

DNA extraction was performed from 2 to 3 weeks old *Arabidopsis thaliana* plants and the *DREB1A* fragment was amplified accordingly. At the end of the PCR reaction, a 700 bp DNA fragment was produced with the same size as with the *DREB1A* gene. The PCR products were cloned into the pGEMT-Easy vector after purification successfully. This experiment was further confirmed either by blue/white selection method or restriction digestion of the purified plasmids (figure 1). The plasmids were cut with both *Xba*I and *Bam*HI and a band of around 700 bp, corresponding to the *DREB1A* gene, was obtained (figure 1, lane 3). Finally, sequencing analysis and Blast program confirmed that the *DREB1A* gene was successfully amplified and inserted into the vector without any mutation.

We chose pBI121 binary vector for next step of cloning. *XbaI/BamHI* digested fragment, corresponding to the *DREB1A* gene, was purified from the gel and directly ligated into the cut pBI121 plasmid using *XbaI/BamHI* restriction sites. The pBI121 vector is a low copy number plasmid and it is very difficult to isolate enough amounts of pBI121 plasmid for restriction analysis. Therefore, we used colony-PCR to screen the growing colonies on the selective medium. Production of a band for DREB1A fragment would indicate that the *pBI121::DREB1A* construct had been made successfully.

Colony-PCR products were separated on the gel (figure 2A). There is a band corresponding to DREB1A fragment on lane 3. This colony was used again in another colony-PCR test with positive and negative controls (figure 2B), to ensure that PCR is free from any possible contamination. The sub-cultured colonies were shown to be all transfermants of the *pBI121::DREB1A* construct (figure 2C). We used two different restriction enzymes to clone the DREB1A fragment into the pBI121 vector. Recombinant Agrobacterium cells containing *pBI121::DREB1A* construct are used for production of environmental stress tolerant plants.

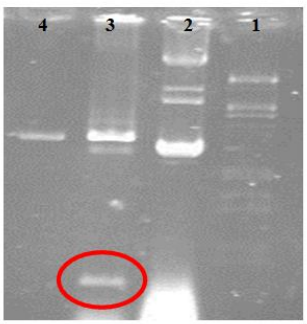

Figure 1. Restriction analysis of blue/white colonies isolated plasmids: lane 1 *Eco*RI/*Hind*III lambda DNA marker, lane 2 non-digested plasmid from white colonies, lane 3 *Xba*I/*Bam*HI digested plasmid isolated from white colonies which contain *DREB1A* fragment (circle), lane 4 none digested plasmid from blue colonies.

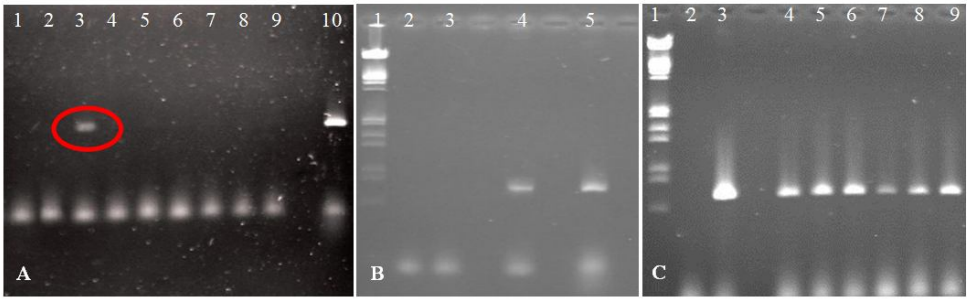

Figure 2. Agarose gel electrophoresis representing PCR products for confirmation of success rate of *DREB1A* gene cloning. A, lane 1 to 9 show colony-PCR products separation, Lane 3 has a band corresponding to DREB1A fragment, lane 10 show positive control with genomic DNA of *Arabidopsis thaliana*. B, lane 1 lambda DNA marker, lane 2 negative control with ddH_2O, lane 3 negative control with empty pBI121 vector, lane 4 colony-PCR containing *pBI121::DREB1A*, lane 5 positive control with genomic DNA of *A.thaliana.* C, lane 1 marker, lane 2 negative control with ddH_2O, lane 3 positive control with genomic DNA of *A. thaliana,* lane 4 to 9 colony-PCR from sub-cultured recombinant colonies.

Discussion

Environmental stresses have adverse effects on plant growth and seed production. We can overcome this problem with production of transgenic plant, over-expressing stress tolerance genes. It is very important to select a gene such as *DREB1A* which can confer high tolerance to various abiotic stresses. In this study we provided *pBI121::DREB1A* construct which could be applied for Agrobacterium transformation and consequently crop plant improvement. Transgenic plants would be more tolerant to the abiotic stresses such as drought, salinity, low and high temperatures (Vadez et al. 2007; Maruyama et al., 2009; Hong et al. 2009). It is also possible that over-expression of the *DREB1A* gene confer direct or indirect properties to plants to cope against heavy metal toxicity and harmful rays or mechanical injuries (Kohan and Bagherieh, 2011). These plants will have more stability and show more growth and productivity under stress conditions compared to the wild type plants.

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Plant regeneration via somatic embryogenesis and organogenesis in *Verbascum speciosum* Schard.

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Abstract

Plant regeneration was achieved in *Verbascum speciosum* Schard. via organogenesis and somatic embryogenesis by culture of mature embryo explants. Two types of calli, embryogenic and non-embryogenic, were induced from mature embryo explants on Murashige and Skoog (MS) medium supplemented with different concentrations of benzyl adenine (BA) and α-naphthalene acetic acid (NAA). In order to further proliferate the somatic embryoids, the yellow and friable embryogenic calli were transferred on MS medium containing 0.5 mg^{-1} charchol and 0.1 or 1 mg^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) or into MS medium containing 60 g^{-1} sucrose, 50 mgl^{-1} casein hydrolysate (CH), 0.5 mg^{-1} kinetin (Kin), 5 mg^{-1} 2,4-D and 0.5 mg^{-1} charchol. Among the 3 tested media, MS medium containing 0.1 mg^{-1} 2,4-D and 0.5 mg^{-1} charchol was more effective for proliferation of embryonic calli. Somatic embryos were transferred to hormone free MS medium for maturation and shoot regeneration. In addition, shoots and roots regenerated from non-embryogenic calli in hormone free MS medium or containing NAA and BA. Shoot buds were obtained from non-embryogenic calli and they were transferred to MS medium supplemented with 1 mg^{-1} BA or Kin for further growth and multiplication. Regenerated plants then were potted and maintained in the greenhouse.

***Keywords*:** embryogenic calli, shoot and root regeneration, *Verbascum speciosum*

Introduction

The genus *Verbascum* L. belongs to the tribe Verbasceae (Valdes, 1987) of the family Scrophulariaceae and Turkey, Iran and Pakistan are the main centers of its diversity (Zohary, 1973; Huber-Morath, 1978; Valdes, 1987). It is the largest genus within the large family of Scrophulariaceae includes 360 species throughout the world (Judd et al., 2002) and 42 species in Iran (Sharifnia, 2007). Among the species distributed in Iran, 15 species are endemic (Sharifnia, 2007). As hybridization is a very frequent phenomenon within *Verbascum* (Huber-Morath, 1978; Karaveliogullari et al., 2004), a high frequency of morphological variations has been an intricate in delimiting the species. The *Verbascum* species are adapted to the various habitats and different regions of Iran including rocky mountains, open forests, road sides and the margins of the rivers. The genus *Verbascum* is known for the presence of variety of compounds and many investigations led to isolation of saponins (Hartleb and Seifert, 1994), alkaloids (Dranarov and Hais, 1997), flavonoids (Afifi et al., 1993) and phenylethanoids (Akdemir et al., 2004).

Plant tissue culture is one of the most important conservation techniques because it offers great potential for rapid cloning from a minimum amount of plant material. In addition, it plays a key role in the production of plant material required for different purposes such as breeding, genetic and biotechnological research, and the acquisition of industrial raw material (Endress, 1994; Sahai et al., 2010). Although there are a few reports on the organogenesis and plantlet formation from leaf, petiole and root explants in *Verbascum thapsus* L. (Caruso, 1971; Turker et al., 2001), to date there is no report on tissue culture of *V. speciosum* Schard. Here we report a protocol for organogenesis and somatic embryogenesis from mature embryo culture of *V. speciosum.* This protocol can enable us to produce enough plant material of the species required for different purposes such as breeding, genetic and biotechnological researches.

Materials and Methods

Callus induction

Plants of *Verbascum speciosum* were collected at fruiting phase from Hamedan Province, west of Iran in July to August 2011. Voucher specimens

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were deposited in the herbarium of Bu-Ali Sina University (BASU), Hamedan, Iran. Different explants including leaves, petioles and capsules were washed with tap water, surface disinfested in 70% (v/v) EtOH and 5% sodium hypochlorite for 15 min, and then rinsed three times in sterile distilled water. Then mature embryos were dissected from the capsules. Different explants were cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with 0, 2.5 and 5 mgl^{-1} BA; and 0, 1.5 and 3 mgl^{-1} NAA along with 3% (w/v) sucrose and 1% (w/v) agar. The pH of all media was adjusted to 5.7-5.8 before sterilization by autoclaving at 121°C for 15 min. All cultures were kept at 5°C for 48 h and then incubated at 25 ± 2°C with 3000 lux light intensity from fluorescence cool tube light in culture room for 16 h. Reports of explants producing calli were recorded after 4 weeks and the calli were subcultured on fresh MS medium after 30 days of culture. All experiments were carried out in 3 replicates and 8 explants were used for each replication.

Embryogenic callus production

After 3 subcultures some embryonic regions produced on the calli in the media containing different concentrations of NAA and BA. Production of embryogenic regions was increased by further subcultures.

Embryogenic callus proliferation

In order to further multiplication the somatic embryoids, pieces of yellow and friable embryonic calli, produced in some concentrations of BA and NAA on the 30th day of culture, were transferred into 3 new media including 1) MS medium containing 0.1 mgl^{-1} 2,4-D along with 3% (w/v) sucrose and 0.5 mgl^{-1} charchol, 2) 1 mgl^{-1} 2,4-D along with 3% (w/v) sucrose and 0.5 mgl^{-1} charchol, and 3) MS medium containing 60 gl^{-1} sucrose, 50 mgl^{-1} CH, 0.5 mgl^{-1} Kin, 5 mgl^{-1} 2,4-D and 0.5 mgl^{-1} charchol. The data were recorded after 6 weeks of culture. Then, the embryonic calli with somatic embryos in earlier stages were transferred into hormone free MS medium for development of somatic embryos and shoot bud initiation.

Shoot and root multiplication

Shoot buds induced from non-embryogenic calli on MS medium containing different concentrations of BA and NAA, were transferred to MS medium supplemented with 1 mgl^{-1} BA or Kin for further growth and multiplication and data were recorded after 5 weeks. For root proliferation and plant regeneration, shoots along with thin roots were transferred to rooting medium comprises 3 mgl^{-1} NAA.

Plant acclimatization

For acclimatization, healthy plantlets with well-developed roots and shoots were chosen. They were removed from the culture media, washed in water to remove agar, and then transferred into the pots containing sterilized soil and perlite (1:1). The transplanted plants were covered with clear plastic bags to maintain humidity, placed in an acclimatization chamber, and watered with quarter strength MS mineral salt solution with 4 day intervals. After 2 weeks, the plastic bags were opened and the uncovered plants were maintained under natural day light conditions at 19-23°C in the laboratory. The survival frequency of the *in vitro* propagated plants was evaluated at the end of the 5th week of the acclimatization process.

Statistical analysis

Eight embryo explants or calli were used for each experiment in triplicates. The statistically significance of difference between means was estimated at the 5 percent level ($P < 0.05$) by the Completely Randomized Design.

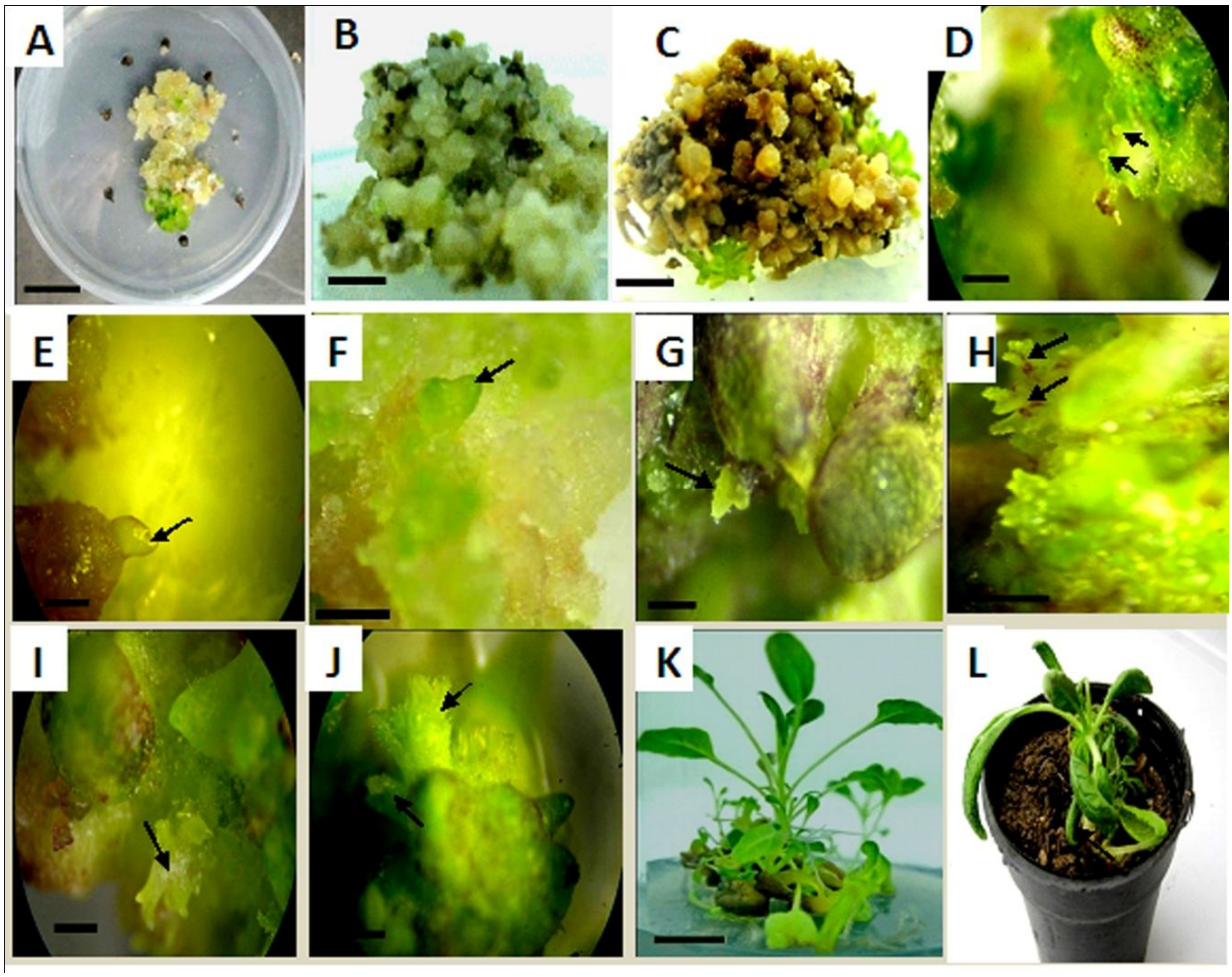

Figure 1. Somatic embryogenesis and plantlet regeneration from mature embryo explants in *Verbascum speciosum*. (A) Embryogenic callus induction in MS medium containing 2.5 mgl^{-1} BA without NAA after 6 weeks. (B) Embryogenic callus proliferation in MS medium containing 0.5 mgl^{-1} Kin, 5 mgl^{-1}, 2,4-D and 50 mgl^{-1} CH. (C) Embryogenic callus proliferation in MS medium containing 0.1 mgl^{-1} 2,4-D (Scale bar = 5 mm). (D) Embryogenic callus with globular somatic embryos in MS medium containing 0.1 mgl^{-1} 2,4-D (arrows). (E, F) Heart-shaped somatic embryos in hormone free MS medium (arrows). (G, H) Somatic embryos at torpedo stage in growth regulator free MS medium (arrows). (I, J) Somatic embryos at cotyledonary stage in hormone free MS medium (arrows). (K) Plant regeneration in hormone free MS medium (Scale bar = 10 mm). (L). Potted plant.

Results

Callus induction

Results showed that among 9 combinations of BA and NAA and different explants which were used, callus induction was successful only from mature embryo explants in 4 treatments. The highest frequency of non-embryogenic callus initiation was obtained in MS medium containing 1.5 mgl^{-1} NAA or 2.5 mgl^{-1} BA (table 1). However, the growth of calli formed at 1.5 mgl^{-1} NAA without BA was no longer promoted, but the produced adventitious roots vigorously. Calli which were induced on MS medium containing 2.5 mgl^{-1} BA, were heterogeneous and had morphogenetic potential. In addition, production of calli decreased by increasing BA. In the presence of only one growth regulator, NAA was more effective in root and BA in shoot regeneration. In MS medium without hormones both of shoots and roots were formed directly from the explants (table 2).

Embryogenic callus production

Two types of calli, embryogenic and non-embryogenic, were induced from mature embryo explants, which were distinguished well by their surface, color and texture. Non-embrogenic calli were soft, yellowish white and friable. Then, the calli turned green, hard, and compact with morphogenetic potential, while their peripheral portions remained yellowish white and friable as non-embryogenic calli after 10 weeks of culture. Embryogenic calli induction was observed only in MS medium supplemented with 2.5 mgl^{-1} BA without NAA (table 1). Somatic embryos were

observed on yellowish green embryonic calli after subculturing of the calli in the same fresh medium.

Embryogenic callus proliferation

The growth of yellow and embryonic friable calli was promoted by transferring them into new MS medium containing 0.1 or 1 mgl^{-1} 2,4-D and 0.5 mgl^{-1} charchol. Embryogenesis was increased significantly by 0.1 more than that by 1 mgl^{-1} 2,4-D (Table 3). The compact yellowish white calli spontaneously formed embryo-like structures (embryoids). However, no further differentiation was achieved in the embryos (figure 1).

In addition, transferring the embrogenic calli in new MS medium containing 60 gl^{-1} sucrose, 0.5 mgl^{-1} Kin, 5 mgl^{-1} 2,4-D, 50 mgl^{-1} CH and 0.5 mgl^{-1} charchol resulted in promotion of their growth, when they were transferred from MS medium supplemented with 2.5 mgl^{-1} BA without or 1.5 mgl^{-1} NAA (Table 3). The somatic embryos passed through each of the typical developmental stages in a growth regulator free MS medium after 8 weeks of culture, followed by conversion to plantlets (figure 1).

Shoot and root multiplication

Shoot formation was induced from non-embryogenic calli. Transferring of the shoot buds into MS medium supplemented with 1 mgl^{-1} BA or Kin resulted in further growth and proliferation of them after 5 weeks of culture (figure 2). Results showed that Kin was found to be more effective than BA in shoot proliferation and the number of shoots per explant was higher in the presence of Kin when compared to BA, whereas the nodular structures started to differentiate into an organized structure of shoots. In addition BA had no effect on root induction. The highest frequency of shoot induction in the presence of 1 mgl^{-1} Kin was obtained, when the shoot buds were transferred from MS medium supplemented with 1.5 mgl^{-1} NAA and 2.5 mgl^{-1} BA. Root formation and plantlet regeneration were also observed in the same medium (table 4).

Plant acclimatization

Well-developed and rooted plantlets were transferred to sterile soil with perlite for acclimatization and covered with plastic bags to ensure high humidity around the plants. After 2 weeks, the plastic bags were removed and the plants were transferred to greenhouse. In the 5^{th} week of acclimatization, all of the plantlets survived and showed normal growth (figures 1 and 2).

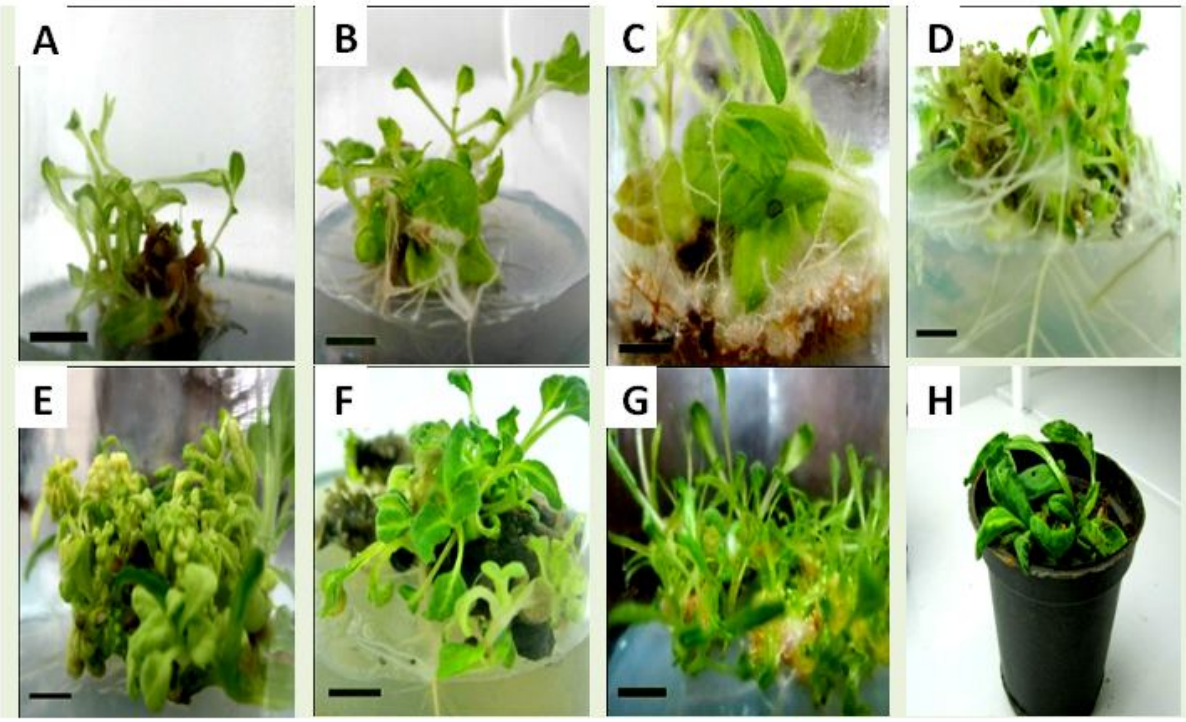

Figure 2. Shoot multiplication and plant regeneration in *Verbascum speciosium*. (A) Shoot regeneration on MS medium containing 1 mgl^{-1} each of BA and NAA after 6 weeks. (B, C) Root regeneration from the shoots on MS medium containing 3 mgl^{-1} NAA. (D, E) Shoot multiplication on MS medium containing 1 mgl^{-1} BA after 5 weeks. (F, G) Shoot multiplication on MS medium containing 1 mgl^{-1} Kin after 5 weeks (Scale bar = 10 mm). (H) Potted plant.

Table 1. Effect of various concentrations of BA and NAA on callus induction in *Verbascum speciosum* after 4 weeks.

Treatment (mgl^{-1})	Callus (%)	Embryogenic callus (%)
BA 0 + NAA 0	0^{c}	0^{b}
BA 0 + NAA 1.5	50^{a}	0^{b}
BA 0 + NAA 3	0^{c}	0^{b}
BA 2.5 + NAA 0	45.83^{ab}	16.67^{a}
BA 2.5 + NAA 1.5	41.66^{ab}	0^{b}
BA 2.5 + NAA 3	0^{c}	0^{b}
BA 5 + NAA 0	25^{b}	0^{b}
BA 5 + NAA 1.5	0^{c}	0^{b}
BA 5 + NAA 3	0^{c}	0^{b}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Table 2. Effect of various concentrations of BA and NAA on shoot and root regeneration in *Verbascum speciosum* after 6 weeks.

Treatment (mgl^{-1})	Shoot (%)	Root (%)
BA 0 + NAA 0	37.5^{a}	37.5^{a}
BA 0 + NAA 1.5	0^{c}	50^{a}
BA 0 + NAA 3	0^{c}	0^{c}
BA 2.5 + NAA 0	29.16^{a}	0^{c}
BA 2.5 + NAA 1.5	20.83^{ab}	37.5^{a}
BA 2.5 + NAA 3	0^{c}	0^{c}
BA 5 + NAA 0	12.5^{ab}	0^{c}
BA 5 + NAA 1.5	0^{c}	0^{c}
BA 5 + NAA 3	0^{c}	0^{c}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Table 3. Effect of 2,4-D, Kin and CH on embryogenic callus proliferation in *Verbascum speciosum* after 6 weeks.

Pretreatment (mgl^{-1})	1 mgl^{-1} 2,4-D	0.1 mgl^{-1} 2,4-D	0.5 mgl^{-1} Kin + 5 mgl^{-1} 2,4-D + 50 mgl^{-1} CH
BA 2.5 + NAA 0	65.33^{a}	66.44^{b}	66.7^{a}
BA 2.5 + NAA 1.5	66.44^{a}	88.89^{a}	77.7^{a}
BA 5 + NAA 0	0^{b}	100^{a}	0^{b}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Table 4. Effect of 1 mgl^{-1} BA or Kin on shoot multiplication in *Verbascum speciosum* after 5 weeks.

Pretreatment	BA (1 mgl^{-1})			Kin (1 mgl^{-1})		
(mgl^{-1})	Shoot (%)	Root (%)	Plant (%)	Shoot (%)	Root (%)	Plant (%)
BA 2.5 + NAA 0	34.44^{b}	0	0	100^{a}	0^{b}	0^{b}
BA 2.5 + NAA 1.5	66.44^{a}	0	0	100^{a}	88.67^{a}	100^{a}
BA 5 + NAA 0	33.22^{b}	0	0	100^{a}	0^{b}	0^{b}

Each number is the mean of 3 experiments with 8 replicates per experiment. Means with the same letters within columns are not significantly different at $p < 0.05$.

Discussion

Among different explants used in this experiment, calli were induced only on mature embryos. Two types of calli, embryogenic and non-embryogenic, were induced on MS medium supplemented with different concentrations of BA and NAA. Calli grown in the medium containing 2.5 mgl^{-1} BA were fast growing, with yellowish green and compact appearance. Those grown in 5 mgl^{-1} BA were however slow growing, with creamy-white and soft. These findings are inconsistent with the role of BA in callus induction in *V. thapsus* (Turker et al., 2001).

Somatic embryogenesis from various explants has been reported in many plant species (Williams and Maheswaran, 1986). It has been reported that growth and morphogenesis are controlled by the types and concentrations of plant growth regulators and the types of explants have also a considerable role in callus induction and somatic embryogenesis (Luo et al., 1999; Fatima et al., 2009). Results from present study showed that the choice of explants are crucial for inducing calli in *V. speciosum* and calli were not induced on young embryos, leaves and petioles. It is supporting the conclusion that the internal state of explant cells is of prime importance in the expression of somatic embryogenesis, with other conditions such as exogenous growth regulators simply enabling expression of this intrinsically determined pattern of development (Zimmerman, 1993; Williams and Maheswaran, 1986). Among the media tested, embryogenic calli were produced only on the medium containing 2.5 mgl^{-1} BA. It is a very rare phenomenon and there are several reports of somatic embryogenesis induced by BA along with 2,4-D or NAA in many plant species (Yasuda et al., 1985; Pasquale et al., 1994).

Result of transferring the embryogenic calli into 3 new media showed that MS medium containing 0.1 mgl^{-1} 2,4-D was more effective than the others in embryogenic calli multiplication. The present report showing the role of 2,4-D in embryogenic callus promotion in the genus *Verbascum*. The optimum concentrations of 2,4-D found for somatic embryo induction or promotion in *V. speciosum*, are not completely consistent with the works on other plant species. However, a high 2,4-D level (up to 2 mgl^{-1}) was reported necessary to enhance embryogenic callus production in many plant species (Nagarajan et al., 1986; Nolan et al., 1989; Luo et al., 1999).

Plants that are conventionally propagated adventitiously may be proliferated rapidly *in vitro* using not only the conventional organs as a source of explants, but also the other tissues not normally associated with vegetative reproduction (Meins, 1986). Shoots along with roots were regenerated directly from embryo explants in hormone free MS medium. Caruso (1971) observed that when stem segments of *V. thapsus* were grown on a simple nutrient medium without hormones, they gave a morphogenetic response and formed shoots. Torregrosa and Bouquet (1996), noted that regeneration could be obtained in the presence of BA alone, but auxin could positively modify the organogenic responses. Our results are inconsistent with those of Skoog and Miller (1957) in general and those of Turker et al., 2001 on *V. thapsus*; in which a low ratio of NAA to BA induces shoot formation, while the reverse condition induces root formation. In addition, MS medium supplemented with 1 mgl^{-1} Kin was more effective than BA, providing strong shoot proliferation (100%) from the explants with a higher number of shoots per explant. A similar result was achieved by stem segments in *V. thapsus* (Turker et al., 2001). The highest frequency of shoot proliferation was obtained, when the shoot buds were transferred from MS medium supplemented with 1.5 mgl^{-1} NAA and 2.5 mgl^{-1} BA. Plant regeneration was also observed in the same condition. *V. speciosum* represented a superior rhizogenesis potential and the calli formed at 1.5 mgl^{-1} NAA without BA or with 2.5 mgl^{-1} NAA, produced adventitious roots vigorously after further subcultures.

Results from present study showed that the choice of explants is crucial for inducing calli in *V. speciosum* and calli were induced only on mature embryos. Embryogenic and non-embryogenic were induced in different concentrations of BA and NAA. It has been reported that types and concentrations of plant hormones and the types of explants have also a considerable role in callus induction and somatic embryogenesis (Luo et al., 1999; Fatima et al., 2009). Embryogenic calli were produced only on the medium containing 2.5 mgl^{-1} BA. This rare phenomenon has been reported by some researchers (Yasuda et al., 1985; Pasquale et al., 1994).

Transferring the embryogenic calli into MS medium containing 0.1 mgl^{-1} 2,4-D was more effective than the other treatments in their multiplication. However, the optimum concentration of 2,4-D in *V. speciosum* is not completely consistent with other species.

Our results were consistent with those of Turker et al., 2001 on *V. thapsus*; in which a low ratio of NAA to BA induces shoots, while the reverse condition induces roots. In addition, MS medium

supplemented with 1 mgl^{-1} Kin was more effective than BA, providing strong shoot proliferation with a higher number of shoots per explant. The highest frequency of shoot proliferation and plant regeneration was obtained, when the shoot buds were transferred from MS medium supplemented with 1.5 mgl^{-1} NAA and 2.5 mgl^{-1} BA.

The *in vitro* culture protocol is established here can provide plant material for future physiological and biochemical studies on *V. speciosum*. These studies will include developing extraction and analytical procedures for the active ingredients of this medicinal plant species.

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In vitro propagation and conservation of *Diaphanoptera khorasanica* (Caryophyllaceae), a threatened endemic and potential ornamental species in northeast of Iran

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Abstract

Tissue culture methods provide tools to supplement traditional methods for collection, propagation and preservation of endangered plant species. In this study, *in vitro* propagation of *Diaphanoptera khorasanica* Rech.f., a rare and threatened plant species with limited distribution range and population was investigated. This species has a potential as an ornamental plant. Single node explants were provided from both adult and seedling sources. Several disinfection treatments were tried to permit selection of a suitable method. Different growth regulators were used for establishment, proliferation and rooting stages. Explants showed the highest establishment percentage after 5 min treatment with 1% sodium hypochlorite (NaOCl), cultured in MS medium containing 2.2 µM 6-benzylaminopurine (BAP) and 2.4 µM indole-3-butyric acid (IBA). The highest proliferation of explants from both adult and seedling source explants was obtained from media supplemented by BA treatment in contrast to TDZ. No significant differences were found between different concentrations of BAP and TDZ. Proliferated shoots in TDZ were longer and had more internode length and less vitrification, in comparison with those in BAP. *In vitro* rooting of proliferated shoots just induced in liquid half-strength MS medium and rooting was not observed in solid medium. The shoots that originated from adult plants gave rise to the highest rooting rate with 4.8µM α-naphthalene acetic acid (NAA) and 2.4 µM, but NAA rooted plantlets showed higher survival percentage in acclimatization step. This study was aimed towards developing an efficient protocol for *in vitro* propagation of *D. khorasanica* and conservation of this vulnerable species.

***Keywords*:** micropropagation, proliferation, nodal explant, preservation

Introduction

World biodiversity is declining at an unprecedented rate. During the period of 1996–2011, a total of 9098 plant species were added to the International Union for the Conservation of Nature (IUCN) red list of threatened species (IUCN, 2011a). During this period, there was also an increase of over 60% in the number of plants recorded as critically endangered. This is alarming and immediate conservation actions are required to protect these species. Although species conservation is achieved most effectively through the management of wild populations and natural habitats (*in situ* conservation), *ex situ* techniques can be used to complement *in situ* methods and, in some instances, may be the only option (Maunder et al., 1998; Ramsay et al., 2000). Biotechnological methods are now as essential components of plant genetic resource management (Benson et al., 2000), and they are becoming increasingly powerful for the conservation of rare and endangered plants.

In vitro propagation can offer considerable benefits for the rapid cultivation of endangered plant species, which have a limited reproductive capacity and exist in threatened habitats (Fay, 1994). In addition to micropropagation, plant tissue culture provides means for conservation of genetic resources. This is especially critical for rare and threatened endemic plant species as this method allows establishing cultures from a minimal amount of starting plant material for further multiplication (Benson et al. 2000). *In vitro* techniques have been found to be useful in the propagation of a large number of threatened plants (Amo-Marco and Lledo, 1996; Dhar and Joshi, 2005).

Diaphanoptera Rech.f. (Caryaphyllaceae: Silenoideae) is a regionally endemic genus which, comprises of six local or highly narrow endemic species in its distribution range, *D. khorasanica* Rech.f. and *D. stenocalycina* Rech.f. & Schiman-

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Czeika in northeast of Iran, *D. transhyrcana* (Preobr.) Rech.f. and Schiman-Czeika in west of Turkmenistan, *D. lindbergii* Hedge and Wendelbo, *D. afghanica* Podlech and *D. ekbergii* Hedge and Wendelbo in the north and northeast of Afghanistan (Rechinger and Schiman-Czeika, 1988). *Diaphanoptera* species are usually cushion-form herbal chamaephytes growing in different habitats, including plains with moderately saline soils and from low mountain steppes up to high mountain stony slopes.

D. khorasanica, the type species of the genus, has broad winged ribs on its calyx and thus is easily distinguished from the others (figure 1). This species, described in 1940, had been known until the publication of the Flora Iranica only from its *locus classicus* in serpentine hills of Robate Sefid between Mashhad and Torbate Heydarieh. According to Rechinger and Schiman-Czeika (1988) its re-collection through repeated travelling to the type locality and its surrounding area had been unsuccessful in 1948, 1975 and 1977. After the publication of Flora Iranica, more populations of *D. khorasanica* have been discovered in different areas of Khorassan-Kopedagh Mountains and mainly collected by and preserved in Herbarium of Ferdowsi University of Mashhad (FUMH). Because of the limited recorded localities, this species has been considered as vulnerable (Jalili and Jamzad, 1999) and faces a high risk of extinction in the wild in the medium-term future; therefore, instantly *in situ* and *ex situ* conservation is needed. Furthermore, since as low seed set makes seed propagation of this species difficult, *in vitro* multiplication might be used as an attractive alternative method for the reintroduction of this threatened species into the natural environment and reducing the risk of extinction (Chandra et al., 2006).

Taking these considerations into account the objectives of this study were to: (1) establish a simple and rapid micropropagation system for *D. khorasanica*, and (2) to assess the efficiency of the system in producing *in vitro* plantlets.

Materials and Methods

The distribution map of all the species of *Diaphanoptera* is mostly based on herbarium records in FUMH and geo-referencing of the distribution data in Flora Iranica (Rechinger and Schiman-Czeika, 1988) by DIVA-GIS 7.3 software. The species were evaluated for threat status and red-listed using IUCN categories and criteria (IUCN, 2011b).

Two kinds of plant resources were used in this experiment in order to investigate the possibility of *in vitro* propagation of *D. khorasanica* including both adult and seedling plants.

In vitro culture of single nodes from adult plants

Young shoots (20 cm long) were collected from *D. khorasanica* plants in Kalat, Razavi-Khorasan Province in May 2009. The Collected shoots were washed with running tap water for 1 h and surface sterilized with 70% ethanol (for 30 seconds), and different concentrations plus time intervals of sodium hypochlorite (NaOCl) and mercury chloride ($HgCl_2$). After rinsing three times with distilled water, the terminal buds and leaves were removed. Single node explants, about 1.5 cm long, were cultured in Murashige and Skoog (MS) basal medium (Sigma-Aldrich, USA; pH adjusted to 5.8) with 2.2 µM 6-benzylaminopurine (BAP) and 1 µM IBA.

After four weeks of culture, the number of surviving explants, explants with developing axillary shoots and shoots per nodal explants were recorded. For the proliferation stage, developed axillary shoots were cultured in MS medium supplemented with BAP (2.2, 4.4, 8.8 µM) or TDZ (0.5, 1.1, 2.2 µM). The number of shoots per explant and shoot length were recorded after four weeks. Rooting of shoots was examined in two phases. In the first experiment, IBA and NAA (1.2, 2.4, and 4.8 µM) treatments were used in solidified ½ MS medium with 8 gr/l Agar. In the second one, shoots were either put in liquid $^1/_2$ MS medium with the same treatments of the first experiment or pulse treated for 15 seconds in 25 or 50 or 100 µM concentrations of IBA or NAA. Shoots were put on filter-paper bridge in liquid medium. Rooted shoots were gently washed with distilled water and transferred to plastic cups with drainage holes containing a 1:1 mixture of perlite and fine sand for acclimatization stage. For the initial seven days, plantlets were covered with a transparent cup and irrigated with a half-strength MS solution (sucrose free). After seven days, plantlets were exposed to the atmosphere and irrigated with both distilled water and a half-strength MS solution. Cultures were maintained at 24±1ºC and illuminated by white fluorescent tubes (40 µmol m-2 s-1) 16 h per day. Fifteen replicates were used for each treatment.

In vitro culture of single nodes from seedlings

Explants were obtained by germinating seeds collected from a natural population, namely, during June 2009. Seeds were surface sterilized with 70% ethanol (30 s), 1% NaOCl for 20 min followed by

three washes with sterile distilled water. Seeds were then germinated on the one-half strength hormone-free MS medium under a 16-h photoperiod provided by fluorescent light. Shoots formed after 5 weeks of culture. Uniform shoots were selected from *in vitro* seedlings and nodal stem segments with a pair of auxiliary buds excised and cultured in MS medium containing 2.2 µM BAP and 1 µM IBA. Proliferation and rooting treatments were done using similar treatment and methods used for adult source explants.

Data analysis

The data were analyzed by SAS (SAS Institute, Inc., Cary, NC) software. Mean values were separated according to Duncan's multiple range test at probability of 0.05 level. Percentage data were log transformed to achieve normal distribution.

Results

Biogeography and conservation

The distribution map of this species shows that they are mainly local endemics or distribute in a highly confined area, except for *D. khorasanica* which has a relatively wider distribution range in Khorassan-Kopedagh floristic province (figure 2). All species evaluated as threatened but in different categories. *D. khorasanica* is re-examined as a vulnerable species and the other species are red-listed as endangered or critically endangered plant species for the first time in the World based on IUCN criteria (table 1).

Adult source explants

Based on the results, 1% NaOCl for 5 min showed the best effect in disinfection of explants. Explants showed the highest establishment rate (less contamination and higher growth) after a 1% NaOCl disinfection treatment for 5 min in MS medium containing 2.2 µM BAP and 2.4 µM IBA. Bud break and growth of shoots were noticed in the nodal explants in the presence of both BAP and TDZ cytokinins in MS medium. The higher multiplication rates were obtained with BAP treatments with only significant differences between 2.2 µM BAP, and 1.1 µM TDZ based on shoot number and between 2.2 and 4.4 µM BAP and 1.1 and 4.4 µM TDZ based on the mean shoot length. Less vitrification percent was observed in different concentrations of TDZ, in comparison with BAP (table 2). Callus formation was seen in some explants in spite of IBA elimination in proliferation phase. Proliferated shoots showed very low root formation (less than 5%) in the solid ½ MS medium (figure 4). The highest rooting percentage was observed in liquid medium with the highest rate in 4.8 µM NAA and 2.4 µM IAA, and no evidence of root initiation or development was observed in pulse treated shoots (table 3). Root formation was observed later in NAA treated shoots, but shoots were stronger and more aggregate and subsequently less injured during transfer to acclimatization phase, so they showed higher acclimatization percentage (table 3) in comparison with rooted shoots with IAA and IBA treatments.

Seedling source explants

A high germination (80%) was observed after 2 or 3 weeks in ½ MS medium. Single nodes from one month old seedling showed good establishment in MS medium containing 2.2 µM BAP and 2.4 µM IBA. In proliferation phase, higher proliferation (based on shoot numbers) was observed in BAP treatments compared to TDZ ones, but only 8.8 µM BAP showed a significant difference with 1.1 and 4.4 µM TDZ. The percentage of vitrified shoots was higher (table 2) in medium containing BAP than the TDZ treatments (figure 3) and significant differences were found between BAP and TDZ treatments. The acclimation percentage of rooted shoots was lower in the seedling source explants, but still higher acclimation was recorded on the 4.8 µM NAA treated shoots (table 3).

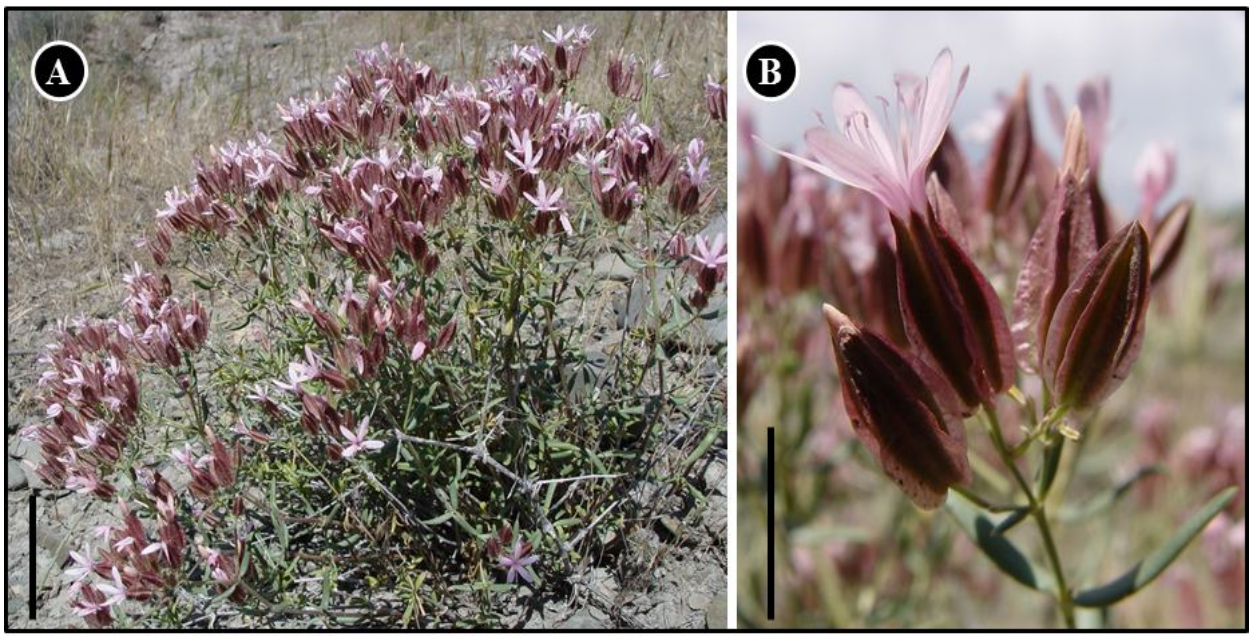

Figure 1. *Diaphanoptera khorasanica* in its natural habitat in Fereizi region, northern foothills of Binalood mountain range, Razavi Khorassan. A: lax cushion-form habit, scale bar = 5 cm; B: close-up view of the flowers, scale bar = 1 cm.

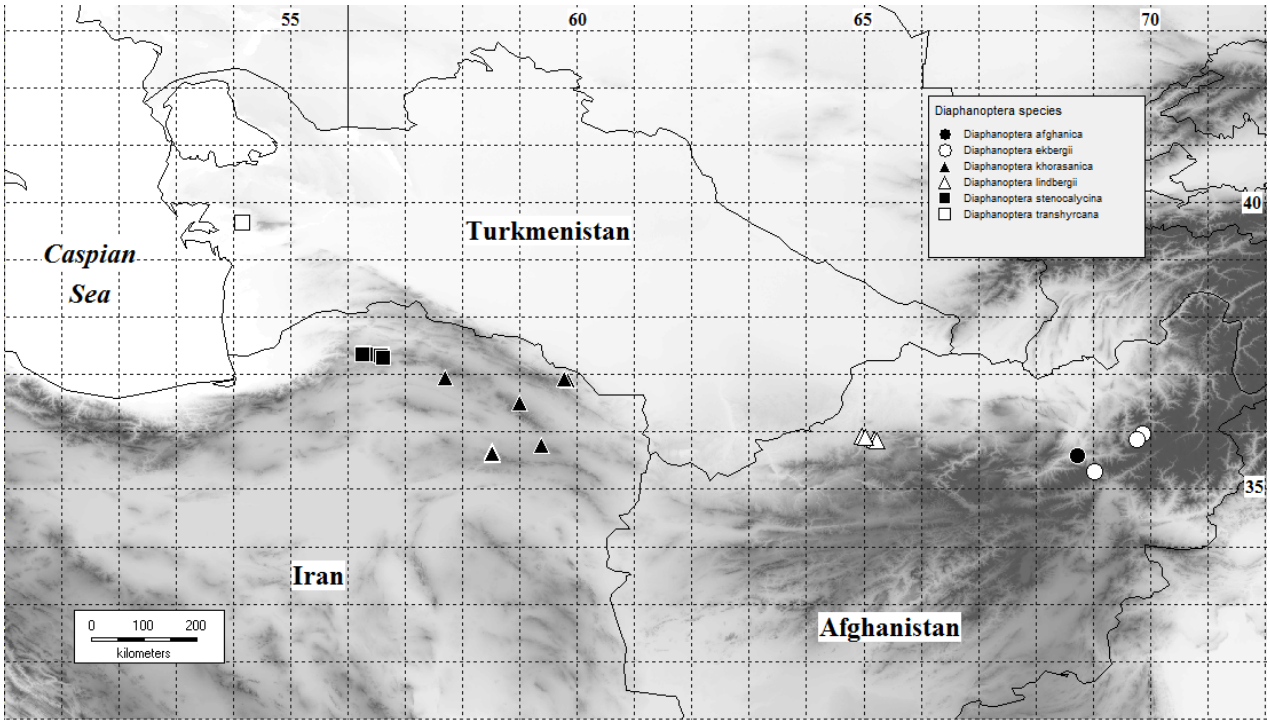

Figure 2. Distribution map of *Diaphanoptera* species in the World.

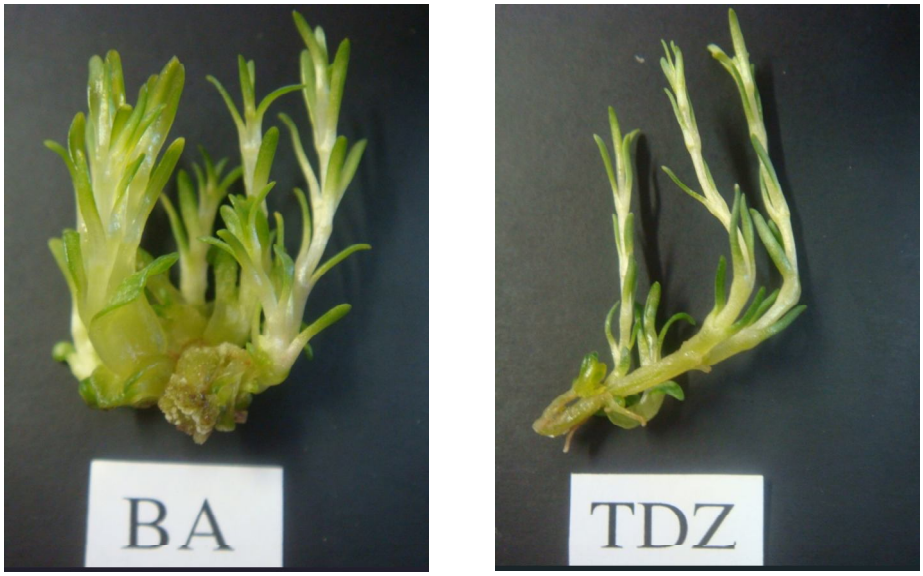

Figure 3. Comparison of proliferated shoots from adult plant in BAP and TDZ treatments.

Figure 4. Rooted shoot in liquid ½ MS medium and 4.8 µM NAA treatment.

Table 1. Threat status and biogeography of *Diaphanoptera* species. (Abbreviations: CR: Critically Endangered, EN: Endangered, VU: Vulnerable, IT: Irano-Turanian, KK: Khorassan-Kopetdagh, Afgh.: Afghanistan).

Species	Threat status (Criteria)	Habitat	Chorotype
D. khorasanica	VU (B1+B2ab (i, iv))	low to middle mountain steppes	IT ^{KK}
D. stenocalycina	CR (B2 bc (i, iii))	plains with saline soils	IT ^{KK}
D. transhyrcana	CR (B1+B2 a,b (iii))	low mountain steppes	IT $^{Aralo\text{-}Caspian}$
D. afghanica	CR (B1+B2 a,b (iii))	valley plains	IT $^{Afgh.}$
D. lindbergii	EN (B1+B2 a,b (iii))	low mountain steppes	IT $^{Afgh.}$
D. ekbergii	EN (B1+B2 a,b (iii))	high mountain stony steppes	IT $^{Afgh.}$

Table 2. Shoot proliferation results from adult and seedling sources of *Diaphanoptera khorasanica*.

Explant source								
	Adult				Seedling			
Cytokinin (µM)	Mean Shoot Number	Mean Shoot Length (cm)	Mean Number of Nodal Segment	Vitrification%	Shoot Number	Mean Shoot Length (cm)	Mean Number of Nodal Segment	Vitrification%
BAP								
2.2	5^{ab}	1.1^{bcd}	1.8^{ab}	67 b	2^{ab}	0.8^{b}	2.5^{a}	58 b
4.4	4.4^{abc}	1.6^{a}	2.1^{ab}	85 a	1.7^{abc}	0.9^{b}	3^{a}	76 a
8.8	4.6^{abc}	1.4^{ab}	1.8^{ab}	91 a	2.3^{a}	0.9^{b}	2.6^{a}	80 a
TDZ								
1.1	2.6^{c}	0.9^{d}	2.1^{ab}	0 e	1.5^{b}	1.9^{a}	3^{a}	0 d
2.2	3.4^{bc}	1.4^{abc}	2^{ab}	3.2 d	1.8^{ab}	1.4^{ab}	3.2^{a}	0 d
4.4	2.8^{bc}	1^{cd}	2.4^{a}	4.8 c	1.3^{b}	1.34^{ab}	2.6^{a}	4 c

Table 3. Rooting percentage from adult and seedling sources of *Diaphanoptera khorasanica*.

Auxin	Concenteration (µM)	Adult source		Seedling source	
		Rooting %	Acclimatization %	Rooting %	Acclimatization %
IAA	1.2	34 d	12 e	18 de	7 d
	2.4	56 a	18 c	25 ab	8 cd
	4.8	45 bc	18 c	22 bc	8 cd
IBA	1.2	23 g	10 f	15 f	7 d
	2.4	26 f	10 f	16 ef	8 cd
	4.8	42 c	15 d	20 cd	9 c
NAA	1.2	30 e	17 cd	17 ef	9 c
	2.4	48 b	26 b	22bc	12 b
	4.8	60 a	32 a	27 a	16 a

Discussion

D. khorasanica is a vulnerable species in spite of finding more populations in different mountain ranges of Khorassan-Kopetdagh after the Flora Iranica. None of populations of *D. khorasanica* are located in National Parks or Protected Areas of Khorassan; therefore, there is no *in situ* conservation for this species and more efforts are needed to preserve the remaining threatened populations.

The results showed that it was possible to propagate *D. khorasanica* using both adult and seedling sources. Shoots showed different percents of vitrification depending on cytokinin type and concentration that was in agreement with the other reports (Casas et al., 2010; Kharrazi et al., 2011). Vitrification is one of the most influential issues of the other species of Caryophyllaceae family such as *Dianthus caryophyllus* (Saher et al., 2005; Fernandez-Garcia et al., 2008; Casas et al., 2010) and *Colobanthus quitensis* (Zuniga et al., 2009). Up to 60% loss of plants has been reported in

micropropagation of different species because of vitrification (Pâques, 1991). The physiological basis of this changes is not clear although some have suggested the role of oxidative stress (Saher et al., 2004), changes in pectin methylation pattern (Fernandez-Garcia et al., 2008) or incomplete development of cuticular waxes (Olmos and Helin, 1998). The vascular connections at the root-to-shoot interface are important for the function of the vascular system and the viability and survival of the plants after transplanting. Smith et al. (1991) reported that the vascular connections between new *in vitro* roots and the micro cutting stems of maple, birch, and apple were often discontinuous since the *in vitro* roots were often subtended by callus or emerged from callus.

At the rooting phase, *D. khorasanica* shoots showed high sensitivity to the culture medium regarding to be solid or liquid and growth regulator; therefore, providing the optimized conditions for rooting is recommended. In spite of good rooting of shoots, this species showed low acclimatization percentage in *ex vitro* conditions (table 3). The possible reason might be the succulent nature of this species leaves that causes more intensification and vitrification of them during rooting phase in liquid mediumThe other possible reason for low acclimatization of rooted shoots might be callus formation at the end of these shoots. Consequently it is suggested that for obtaining normal plantlets with minimum vitrification rate, low concentrations of TDZ is more suitable. Complimentary experiments are under way to optimize the acclimatization step.

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