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Optimization of DNA Extraction and PCR Amplification Protocols Using the Model Plant *Arabidopsis thaliana*

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Abstract. *Arabidopsis thaliana,* a small spring annual plant is used as a model in plant genetics and biotechnology due to some advantages such as its relatively rapid life cycle (8 weeks from germination to production of seeds) and small genome size. Because of these advantages, a series of loss of function and gain of function mutant plants were generated using ecotypes of this plant in order to research the involvement of proteins and phytohormones in different physiological processes. The most commune approach to test the obtained mutants for their homozygosis in relation to the T- DNA (transfer DNA) insert is by the use of the Polymerase Chain Reaction (PCR) method. There were always problems regarding the isolation and amplification of DNA mentioned in the specific literature; this was the main reason why this research was focused on comparing two extraction protocols and two PCR master mixes. The results obtained were satisfying leading us to the conclusion that both extraction methods and amplification master mixes can be implemented further into the research and maybe be also transferred to species phylogenetically related with the model plant.

Keywords: *Arabidopsis thaliana*, T-DNA, glycine-rich RNA-binding protein (GRP), DNA, PCR

INTRODUCTION

In 1907, F. Laibach determined the number of chromosomes of a small spring annual plant called *Arabidopsis thaliana* and in 1943 he exposed the possibility of using it as a model plant for genetic research (Laibach, 1943). E. Reinholz, Laibach's student, in her thesis published in 1947 describes the first collection of mutant plants (induced mutation) (Reinholz, 1945) and in the 60s many researchers like Rédei, JH van der Veen in Holland, J. Veleminsky in Czechoslovakia and G. Röbbelen in Germany analyzed the properties and possible uses of this plant for laboratory experiments (Redei, 1992). Since then, *Arabidopsis thaliana* is used as a model in plant genetics and biotechnology due to its advantages like the small genome, relatively rapid life cycle and limited space needed for growth (Meinke *et al*., 1998).

Another advantage is the fact that since the 60s a series of loss of function and gain of function mutants using the T-DNA (transfer DNA) inset technique were created in order to study the involvement of several genes and proteins in different physiological processes (Azpiroz-Lenehan and Feldmann, 1997). Unlike the transposable elements and the point mutations, which often undergo excision after integration into the genome, the integration of T-DNA is stable over several generations (Krysan *et al*., 2001).

Arabidopsis thaliana has been used so far to study the genes involved in the response to environmental factors (genes responsible for tolerance to low temperatures were identified) (Kim et al., 2007a, b, Kim *et al*., 2008) and in the resistance to pathogens (Chisholm *et al.*, 2006, Luna *et al*., 2011). It was also analyzed how phytohormones like ethylene, jasmonic acid or abscisic acid are involved in several plant metabolic processes (Cao *et al.*, 2006, Schmidt *et al.*, 2010).

The easiest way to test the resulted mutants for their homozygosis in relation to the T- DNA insert is through the Polymerase Chain Reaction (PCR) method. There are still some

inconveniences of this technique due to difficulties regarding the DNA extraction and amplification.

Flavonoids, phenols and polysaccharides attach to nucleic acids over the DNA extraction process and can interact in the produced reactions. Polyphenols liberated from vacuole through the cell lysis process are oxidized with the help of cellular oxidases and go through permanent interferences with nucleic acids inducing a colour reaction that gives the DNA a brownish pigment (Varma *et al*., 2007), hence giving the DNA a resistance to restriction enzymes and polymerases (Katterman and Shattuck, 1983). There is several commercial extraction kits present nowadays on the market of molecular science but these are not necessarily suitable for all plant species and also they are known to be rather expensive (Li et al., 2008). A series of extraction protocols were developed for the extraction of nucleic acids from plants but due to the fact that these can contain large amounts of several distinctive substances according to species, it is highly improbable that only one nucleic acid extraction process could be suitable for all plant species present on the earth surface (Loomis, 1974).

The main purpose of this study was to compare some protocols for DNA extraction and amplification in order to establish the most appropriate method to verify the homozigosity of the *Arabidopsis thaliana* T-DNA insertion lines.

MATERIALS AND METHODS

Plant materials

Leaves from two *Arabidopsis thaliana* T-DNA insertion mutants labelled *grp7-1* (knock-out mutant for the glycine-rich RNA-binding protein 7) and *grp2* (knock-out mutant for the glycine-rich RNA-binding protein 2) were collected from plants grown in green house under normal (22℃, 16 hours light/8 hours dark, 70% humidity) cultivation conditions. The obtained leaves were either processed after their collection or they were conserved in liquid nitrogen or stored at -80℃ until further processing.

DNA extraction

To isolate the DNA from the tissue of interest two types of protocols chosen from the literature were used. Because the *Arabidopsis thaliana* vegetal tissue does not contain a large number of inhibitors that are known to cause impediments during this step, there was no need to use chloroform or phenol in the DNA extraction protocol.

The first protocol used was obtained from the Department of Molecular Cell Physiology, University Bielefeld, Germany. It was labeled as "simple DNA prep" protocol and followed the steps outlined below:

1. Grind the vegetal material in a 2 ml tube frozen in N_2 with the help of a piston
2. Add 500 µl Wisconsin-Buffer and mix the composition with the piston
3. Spin at full speed 5 min
4. Remove 350 µl supernatant to a new 1,5 ml tube
5. Add 350 µl isopropanol and mix through inversion
6. Spin at full speed for 10 min and remove the supernatant
7. Leave the Pellet to dry for 15 min
8. Resuspend the DNA Pellet in 160 µl TE–Buffer
9. Shake for 30 min the tubes in a tube-shaker at 500 rpm and room temperature.

The Wisconsin-Buffer contains the following substances: 0.2 M Tris HCl, pH 9; 0.4 M LiCl; 25 mM EDTA; 1% (w/v) SDS

The second protocol was provided by the Department of Defense Genetics, KVL University, Denmark. This method was labeled as "protocol for micro tubes" and contains the following steps:

1. Grind leaf in a 1.5 ml tube with a piston for 15 s in liquid nitrogen.

Add 400 ml extraction buffer (200 mM Tris pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% (w/v) SDS)

2. Vortex 5 min.
3. Wait 5 min.
4. Spin at full speed for 3 min (15.000 rpm)
5. Remove 300 µl of supernatant to new tube
6. Add 300 µl isopropanol and mix, wait 2 min at RT
7. Spin at full speed for 3 min
8. Rinse with 500 µl 70% (v/v) EtOH and air – dry (in the incubator at 37°C approximately 10 min) and resuspend the pellet in 25 µl TE

Quantification of DNA

To quantify the extracted DNA the NanoDrop device was used. This device performs measurements of absorption at a wavelength of λ = 260 nm, λ = 280 nm and λ = 230 nm, being able to display not only absorption extensions, but also the DNA concentration in the test sample.

PCR analysis

The composition of the PCR reaction used two types of master mix both ready to use, one was provided by Rovalab (*2XRed PCR Master Mix*) and the other was provided by Bioline (*MyTaq Red DNA Polymerase*). The amplification was carried out in a 25 µl reaction mixture containing 5 µl 5X Red Buffer, 1 µl Forward Primer, 1 µl Reverse Primer, 0.15 µl Taq Polymerase (for the Bioline master mix) and 12.5 µl 2X Red Buffer, 1 µl Forward Primer, 1 µl Reverse Primer (for the Rovalab master mix). In both cases the sample of DNA used was 2 µl and H_2O was added up to 25 µl. Preparation of the reaction mixture was carried out in a laminar flow hood under sterile conditions in order to prevent any contamination of the samples. The primer combination used was: RP (AGA TGA ACG TAC CGA TTT GGG A), LP (CGT GAT ATG TCC CCA ACC ACT ACG A) (for the mutant *grp-1*), RP (TCC CCA GAG AGC TAG CTT TTC), LP (CCT TCA GAA CAG TCT CTC GCC) (for the mutant *grp2*) and LbB1 (ATT TTG CCG ATT TCG GGA C) the primer used for the identification of the T-DNA insert. Amplification was performed using a thermocycler, PalmCycler (Corbett Research) programmed as follows: 1 minute at 95 °C – initialization step, 35 cycles at the following temperature profile: 15 seconds at 95 °C – denaturation, 15 seconds at 48 °C – primer annealing, 10 seconds at 72 °C – elongation, 10 minutes at 72 °C – final elongation, for an indefinite time at 4 °C – final hold.

Statistical analysis

For the evaluation of the quantitative and qualitative characters of the isolated DNA the Student's t test was used. This test allows the comparison of the significant difference between two sets of data which follow a normal distribution. The samples analyzed were considered to be independent from each other one being used as control. The statistic interpretation was generated using GraphPad Prism 6.01 trial version a commercial scientific 2D graphing and statistics software published by GraphPad Software, Inc., California.

RESULTS AND DISCUSSIONS

This research was focused on comparing two types of DNA extraction protocols in order to establish the most suitable one for the isolation of nucleic acids from *Arabidopsis thaliana* leaf tissue. This step is very important in the following molecular genetic studies. A good quality and quantity of isolated DNA is crucial for molecular techniques such as polymerase chain reaction (PCR) and other genomic analysis.

The analysis of comparison between the two extraction protocols tested showed significant differences between the quantities of DNA extracted from *grp7-1* plants. The quantity extracted with the "simple DNA prep" protocol ranged within the values of 461 ng/µl and 887 ng/µl whereas by using the protocol "for micro tubes" the quantities were a lot smaller varying between 3.5 ng/µl and 99 ng/µl (Fig. 1).

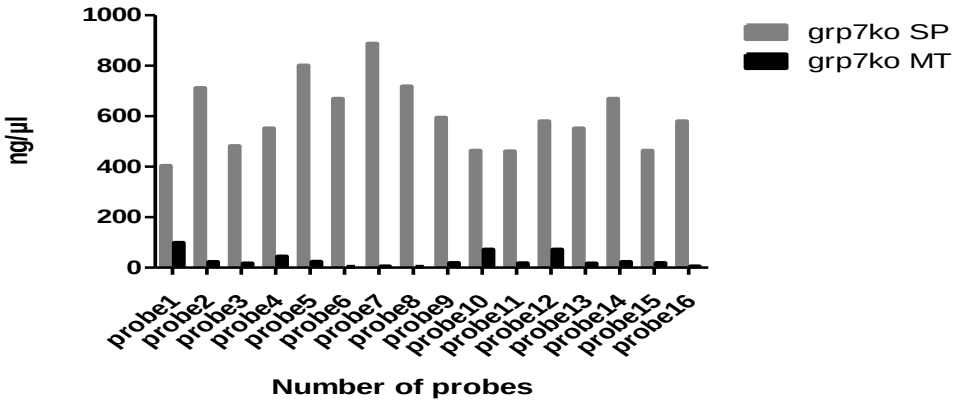

Fig.1. Quantity of isolated DNA from *grp7-1* leaf tissue. Sixteen independent mutant plants were tested for their homozigosity in regard to the T-DNA insert. The values present on the figure represent the mean of three successive measurements of the extracted DNA. The significance is shown for P<0.05.

Significant differences were observed also in regard to the DNA extracted from *grp2* plants. The values obtained using the "simple DNA prep" protocol ranged between 373 ng/µl and 703 ng/µl in contrast to the ones obtained using the protocol "for micro tubes" which varied from 10 ng/µl to 103 ng/µl (Fig. 2).

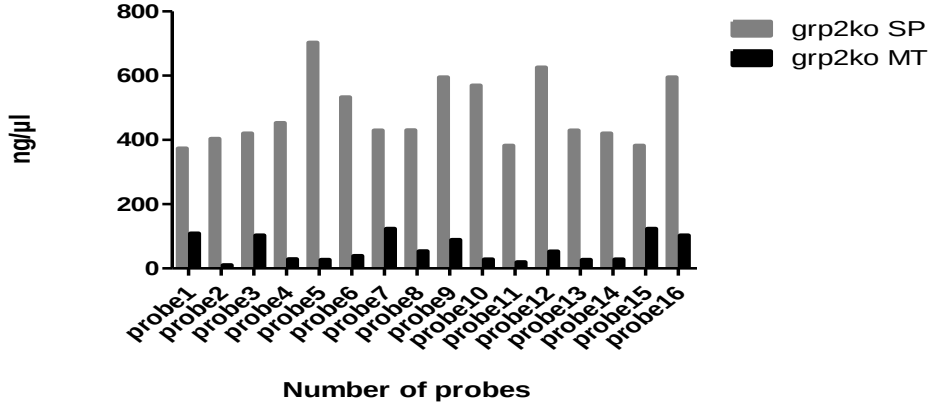

Fig.2. Quantity of isolated DNA from *grp2* leaf tissue. Sixteen independent mutant plants were tested for their homozigosity in regard to the T-DNA insert. The values present on the figure represent the mean of three successive measurements of the extracted DNA. The significance is shown for P<0.05.

Similar high quantities of isolated DNA were obtained by Abu-Romman in 2010 from leaf tissue harvested from *Salvia officinalis*. The protocols used by this researcher were still different and more complex than the ones tested during this study. The reason consists of the fact that sage is known to have high amounts of polyphenols which interact with the nucleic acids (Abu-Romman, 2010). Lower amounts of extracted DNA similar to those recovered in this study using the protocol "for micro tubes" were obtained also by other researches focused on *Melanoxylon brauna* a *Fabaceae* (Borges et al., 2012) and on isolation of DNA from young flower petals of some medicinal plant species (Ibrahim, 2011). In both cases the protocols used were different using CTAB (cetyltrimethylammonium bromide) instead of SDS (sodium dodecyl sulphate) as a detergent.

Regarding the quality of the isolated DNA there were no significant differences observed in the comparison of the two protocols used. The values obtained ranged between 2.1 and 2.19 (A260/280) for the "simple DNA prep" protocol and 1.44 and 2.3 (A260/280) for the protocol "for micro tubes" in case of the *grp7-1* tested plants (Figure 4). Regarding the quality of the DNA extracted from the *grp2* plants the values varied between 2.09 and 2.18 (A260/280) for the "simple DNA prep" protocol and 1.44 and 2.18 (A260/280) for the protocol "for micro tubes" (Figure 5).

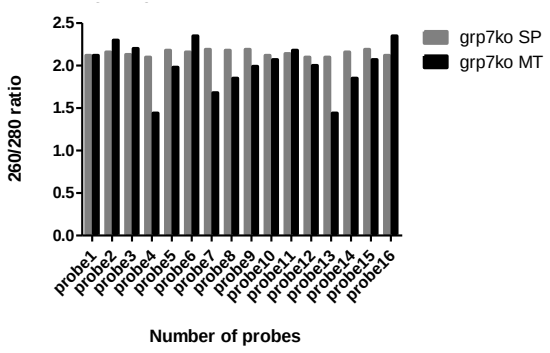

Fig.4. Quality of the isolated DNA from *grp7-1* leaf tissue. The values present on the figure represent the mean of three successive measurements of the extracted DNA. The significance is shown for P<0.05.

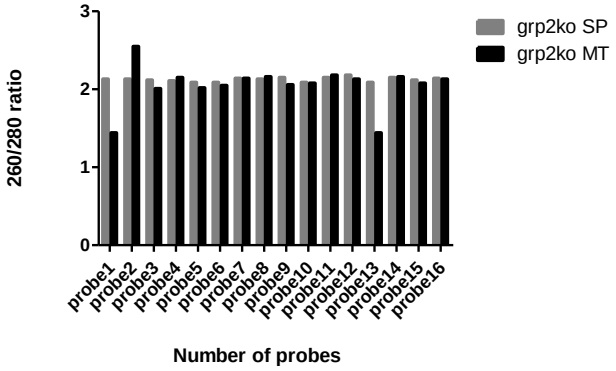

Fig.5. Quality of the isolated DNA from *grp2* leaf tissue. The values present on the figure represent the mean of three successive measurements of the extracted DNA. The significance is shown for P<0.05.

Similar results concerning the quality of the isolated DNA from plants were obtained also by researchers like Krizman et al, in 2006, in analysing different extraction protocols for different vegetal tissue originated from oregano (*Origanum vulgare*), hemp (*Cannabis sativa*), hop (*Humulus lupulus*) and coffee (*Coffea arabica*) (Krizman *et al*., 2006) or Wang who focused on analysing extraction protocols on *Reaumuria soongorica*, a desert shrub species (Wang *et al*., 2011).

Both master mixes used in the amplification of the isolated DNA generated good results in regard to the desired products. There were no significant differences in the quality of the generated products obtained after the amplification and the gel electrophoresis (Fig. 6 and Fig. 7).

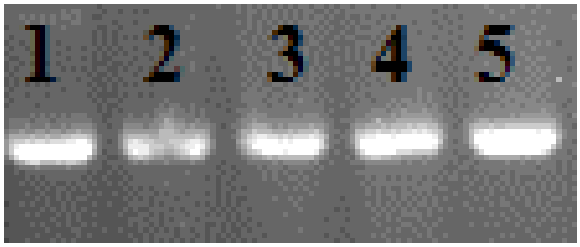

Fig.6. Amplification products. PCR products generated by the amplification using the Bioline produced master mix of the DNA isolated from grp2 leaf tissue. Five independent plants were analysed.

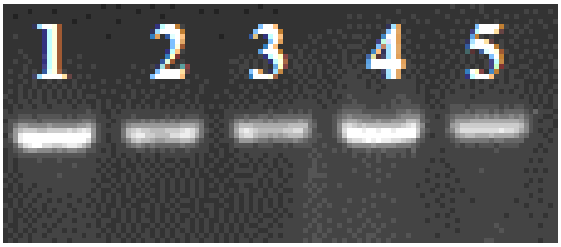

Fig.7. Amplification products. PCR products generated by the amplification using the Rovalab produced master mix of the DNA isolated from grp2 leaf tissue. Five independent plants were analysed.

CONCLUSION

The "simple DNA prep" protocol provided by the group from Germany generated better results regarding the quantity of the isolated DNA in contrast to that obtained from Denmark. In regard to the quality both protocols used provided good results. The samples were not contaminated with other cellular compounds. Also both master mixes tested generated good amplification products suggesting that they can be used with success during other experiments.

The results obtained during this study support the use of these two types of protocols and master mixes in the isolation and amplification of DNA from *Arabidopsis thaliana* plants and maybe also within family related species. Still, additional studies are needed to see if these protocols can be applied to the isolation of DNA from other species that are not related phylogenetically.

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