Faculty of Resource Science and Technology

Transformation of *Alcohol Dehydrogenase1* in Tomato

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Declaration

I declare that the thesis entitled " Transformation of *Alcohol Dehydrogenase1* in Tomato" hereby submitted for the STF 3015 Final Year Project 2 at the University Malaysia Sarawak (UNIMAS) is my own work and have not been previously submitted by me at another University for any degree. I cede copyright of the thesis in favor of the University Malaysia Sarawak (UNIMAS). Formulations and ideas taken from other sources are cited as such. This work has not been published.

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List of Abbreviations

%	percentage
µg	microgram
µL	microlitre
bp	basepair
CAM	Chloramphenicol
DNA	Deoxyribosenucleic Acid
g	gram
kb	kilobasepair
L	liter
LA	Luria Agar
LB	Luria Broth
M	molar
mg	milligram
mL	mililiter
mM	milimolar
PCR	Polymerase Chain Reaction
Rif	Rifampicin

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ABSTRACT

Alcohol dehydrogenase (Adh) is a versatile enzyme that contributes to biological pathways in plants such as germination, fermentation, stress tolerance in stresses like osmotic, oxygen and wound. It catalyzes the interconversion of alcohols, aldehyde and ketone. Previous works have detected the presence of Adh in sago palm, and subsequently isolated and characterised the gene. The objective of this study is to study the expression of *Adh1* in tomato. *Adh1* gene is studied with transformation into tomato plants using *Agrobacterium*-mediated transformation with *Agrobacterium tumefaciens*. Seeds of tomatoes were transformed with *A.tumefaciens* strain LBA4404 carrying the pGSA1131 binary vector. Transformants were confirmed using polymerase chain reaction (PCR). Confirmation with PCR is done with the use of specific primers for *Adh1* gene. DNA fragments of 1.1 kb were successfully amplified with the use of *Nco1* and *BamH1* primers from total DNA of transgenic plants. Reconfirmation tests with similar primers and bar primers were run to reconfirm the insert. There were total 16 transformants being analysed with 13 successfully transformed.

Keywords: *Adh 1*, *Agrobacterium tumefaciens*, seed transformation, tomato

ABSTRAK

Alcohol dehydrogenase (Adh) adalah sejenis enzim yang terlibat dalam aktiviti biological dalam tumbuhan seperti germinasi, fermentasi, toleransi tekanan seperti tekanan osmotik, oksigen dan perlukaan. Ia memangkinkan proses interconversi alkohol, aldehid and keton. Penyelidikan lepas telah mengesan kewujudan Adh dalam sagu, dan telah pun memencilkan dan mengkategorikan gen itu. Objecktif utama untuk kajian ni adalah untuk menkaji pengeksperesan Adh1 di dalam tomato. Gen Adh1 dikaji melalui transformasi ke dalam tumbuhan tomato melalui transformasi Agrobacterium-pengantaraan dengan menggunakan Agrobacterium tumefaciens. Biji benih tomato ditransformasikan dengan A. tumefaciens strain LBA4404 yang mengandungi vektor pGSA1131. Transformasi ini dianalisi dengan menggunakan reaksi rantai polimerase (PCR). Analisasi melalui PCR telah dijalankan dengan menggunakan pencetus yang spesifik untuk gen Adh1. DNA bersaiz 1.1 kb telah berjaya diamplifikasikan dengan menggunakan pencetus dengan penanda Nco1 and BamH1 daripada DNA tumbuhan transgenik. Pengesahan menggunakan pencetus penanda gen Bar telah dijalankan untuk mengesahkan kemasukan gen. Terdapat 16 transgenik tumbuhan dianalisasi dan 13 dijumpai berjaya ditransformkan.

Kata kunci: *Adh 1, Agrobacterium tumefaciens, transformasi biji benih, tomato*

1.0 Introduction

The discovery and development of recombinant DNA technology has provided a great tool to analyze gene function of whole organisms which able to improve agriculture productivity. The main constraints to increase crop production include low yields, disease and pest problems, seasonality offruiting, long juvenility period, the perishable nature offruits, and adaptation of crops to marginal lands (Daud, 1992).

Plants undergo different environmental stresses during their growth and development such as anaerobic stress, drought, low temperature, dehydration, phytohormone and abscisic acid. In order to survive, plants adapt and respond to the environmental changes by altering their gene expression, cell structure and biochemistry. Plants such as tomato (*Lycopersicon esculentum*) is an important food globally. It is an important source of minerals and vitamins and also primary dietary source of lycopene, an antioxidant associated with several forms of human cancers, in particular prostate carcinoma (Levy *et al*., 1995; Carperle *et al*., 1996; Clinton *et al*., 1996).

Alcohol dehydrogenase (Adh) is one of the enzymes contribute to the stress-resistant of plant. Adh is part of the oxidoreductase family, catalyzes the interconversion of alcohols, aldehyde and ketone with the use of NAD^+ or $NADP^+$ as electron acceptor. This enzymatic reaction is reversible and a variety of primary or secondary alcohols, and hemiacetals (Wothington, 2012). Adh present in most organisms, ranging from bacteria to animals and plants, with yeast being the most active form of the enzyme (Wothington, 2012).

Sago palm is one of the local commodity in Sarawak. It is an important source of starch. However, it takes relatively long period of time to reach maturity (7 to 12 years) (Sim and Ahmed, 1978). This restricted the sago palm improvement under conventional breeding method. *Alcohol dehydrogenase* (*Adh*) gene derived from sago palm is used in this study. *Adh* expression in sago palm tissues had been studied and full length *msAdh1* cDNA was isolated by Roslan *et al.* (2008, 2010). However, the function of this *Adh1* was not fully characterised.

1.1.Problem Statement

- The functionality of *Adh* was not fully studied and characterised.

1.2.Objectives of Research

Objectives of this research are to:

- Transform tomato with sago palm *msAdh1* cDNA gene.
- Study the expression of sago palm *msAdh1* cDNA in tomato.

2.0 Literature Review

2.1 Tomato

Tomato is a globally important crop as an edible food. Tomatoes are usually used fresh or as processed products. Standard of fresh tomatoes do not meet high requirement of consumers, and breeders are now focusing on concentrating on improving sugar and acid levels and vine-ripened fruit (Speirs *et al*., 1998). Development of flavour and aroma volatiles in ripening tomato fruit has been studied extensively (Buttery *et al*., 1987, 1988, 1989; McGlasson *et al*.,1987; Baldwin *et al*., 1991). Among 400 volatile compounds found in ripening tomato (Baldwin *et al*., 1991), important components of flavour and aroma that have been identified.

Tomato contains alcohol dehydrogenase 2 (Adh2) enzyme (alcohol: NAD^+ oxidoreductase; EC 1.1.1.1). The enzyme is shown to accumulate in the fruit during ripening (Bicsak *et al*., 1982; Longhurst *et al*., 1994; Chen and Chase, 1993). Tomato alcohol dehydrogenase 1 (Adh1) enzyme is found on pollen, seeds and young seedlings (Tanksley, 1979) and is not associated with functions in fruit ripening (Speirs *et al*., 1998). Accumulation of Adh2 enzyme in late ripening process and the large increase in flavour volatiles in fruit as well as Adh's role in interconversion of volatile aldehydes and alcohols suggest that Adh play important role in flavour development (Longhurst *et al*., 1994).

Tomato has been frequently used in transformation studies (Abu-El-Heba *et al.*, 2008; Biradar, 2008). An efficient regeneration and transformation system was studied in both direct transformation via biolistic gun and indirect transformation via *Agrobacterium* mediated transformation (Abu-El-Heba, *et al*. 2008). A test on promoter trapping activity of pNU435 vector was conducted in tomato

transformation to observe the transformation and expression of gene inserted (Biradar, 2008). Tomato is a plant with fast growth rate which can be regenerate easily.

2.2 Alcohol dehydrogenase 1

Alcohol dehydrogenase (ADH, EC 1.1.1.1) is an enzyme that account for the conversion of pyruvate into ethanol in fermentation pathway in various organisms, include bacteria, animals and plants. *Adh* superfamily constitutes of large group of enzymes which subdivided into three distinct categories: medium chain dehydrogenase/ reductases (MDRs), short-chain dehydrogenase/ reductases and iron-activated *Adh*s (Reid & Fewson, 1994; Kallberg *et al*., 2002). It is encoded by multigene family in flowering plants. It is involved in different biological activities like germination and abiotic stresses in plants (Manicol & Jacobsen, 2001; Fukao *et al*., 2003; Benz *et al*., 2007). *Adh* gene has been manipulated in different levels to study the effect of stress response and synthesis of secondary metabolites, the protein activities (Christine *et al*., 1990) and effect of balance of some flavor aldehydes and alcohols (Speirs *et al.*, 1998).

Expression of *Adh* vary according to different type of plants. *Adh* was detected in leaf and roots. It is highly expressed in young shoots of sago palm (Roslan *et al*., 2008), in leaves of sunflower that treated with high phosphorous (Padmanabhan & Sahi, 2011), in roots of flood-intolerant plants like *Arabidopsis* and pea under anaerobic condition (Chung & Ferl, 1999; Kato-Noguchi, 2000). The higher expression level in different tissue and developmental stage may be due to exposure to stresses and cells dividing (Bailey-Serres & Chang, 2005). *Adh* was previously detected in sago palm, a flood tolerant plant (Roslan *et al*., 2008), and the gene was isolated and characterised (Wee & Roslan, 2012).

2.3 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transfer system is a biological-based transformation system, a biomimetic process. In 1970s, the prospect of using *Agrobacteium tumefaciens* for rational gene transfer of exogenous DNA into crops was revolutionary. Genetic transformation of plants was viewed as a prospect in science (Darbani *et al*., 2008). Despite the development of other non-biological transformation methods (Sanford, 1988; Potrykus, 1991; Arencibia *et al*., 1992, 1995), *Agrobacterium*-mediated transformation is still popular and among the most effective especially to dicotyledonous plants. This method has also been used for monocotyledonous plants which was thought to be impossible like rice (Hiei *et al*., 1994), banana (May *et al*., 1995), corn (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997), sugarcane (Enriquez-Obregon *et al*., 1997,1998), forage grasses like Italian ryegrass (*Lolium* multiflorum) and tall fescue (*Festuca arundinacea*) (Bettany *et al*., 2003).

Agrobacterium is a natural transformation candidate that naturally transfer DNA into nucleus of plant cells and stably incorporate the DNA into the plant genome. *Agrobacterium tumefaciens* is a causative agent of crown gall disease of dicotyledonous plants which form a tumourous phenotype. During infection by *A. tumefaciens*, DNA, segment of T-DNA (transferred DNA) which carried on Ti-plasmid (tumour-inducing) is transferred to plant cell. T-DNA is delimited by 25-bp direct repeats that fank the T-DNA, only *cis* element within the borders will be transferred (Zupan & Zambryski, 1995).

3.0 Materials and Methods

3.1 Plant Material

Dried mature seeds of MT1 strain tomatoes were obtained from Malaysian Agricultural Research and Development Institute (MARDI). Seeds were surface sterilised with 1.05% (v/v) sodium hypochlorite (20%). They were then further sterilised with 70% ethanol, rinsed with sterile distilled water five times (Ismail *et al.*, 2005).

3.2 Bacterial Strain and Vector

Agrobacterium tumefaciens strain LBA4404 and *Escherichia coli* XL1-Blue harbouring binary vector pGSA1131/*msAdh1* were used. (Personal communication; Ms. Mastura Sani, post-graduate student from Genetic Engineering Laboratory, Universiti Malaysia Sarawak, Malaysia). The vector has T-DNA region containing *msAdh1* cDNA. *E.coli* XL1-Blue was cultured in LB with 30 mg/ml CAM in 37°C, 140 rpm shaking incubator for overnight.

3.3 Plasmid Extraction

The plasmid pGSA1131/*msAdh1* in *E.coli* XL1-Blue was extracted with the use of EURx Gene MATRIX Plasmid Miniprep DNA Purification Kit according to the protocol provided by the manufacturer. The plasmid extracted was stored in -20°C for further use.

3.4 Competent Cell Preparation and Electroporation

Transformation of *A. tumefaciens* with pGSA1131/*msAdh1* extracted from *E.coli* XL1-Blue was performed via electroporation with the use of Gene Pulser Xcell™ Electroporation Systems. Preparation of *A. tumefaciens* competent cells was performed according to the protocol provided by the manufacturer with the use of 10% glycerol. The electroporation of the competent cells prepared was done according to the instruction manual provided by the manufacturer. The time constant recorded was 5.1 milliseconds with the voltage 2.37 kV. The recovered bacteria culture was plated on Luria Agar and left to grow in room temperature in dark for 2 days.

3.5 Colony Polymerase Chain Reaction (Colony PCR)

The colonies growed were picked and growed overnight in LB at room temperature. 100 µL of culture was inoculated for colony PCR. The culture was centrifuged at 13,500 rpm for 6 mins at room temperature. The pellet was suspended with sterile distile water. It was brought to incubator at 95°C for 5 mins and vortexed 5 times in between. It was then centrifuged at 13,500 rpm for 6 mins at room temperature. 3 µL of the DNA was subjected to colony PCR according to the PCR setup described in Table 1 and thermal cycle profile in Table 2.

3.6 Tomatoes Transformation and Regeneration

This protocol was adapted from Genetic Engineering Laboratory of Universiti Malaysia Sarawak, Malaysia (Personal communication; Ms. Nabella Holling, post-graduated student from Genetic Engineering Laboratory, Universiti Malaysia Sarawak, Malaysia).

3.6.1 *A. tumefaciens* Preparation

A. tumefaciens LBA4404 containing pGSA1131/*msAdh1* were grown overnight in Luria Broth at room temperature on a rotary shaker at 180 rpm for 2 days. The culture was sub-cultured to fresh LB until the OD_{600} reached 0.5 -0.6. It was then centrifuged at 3,500 rpm for 10 mins at room temperature. The pellet was resuspended in infiltration media.

3.6.2 Seed Preparation

About 100 seeds were washed with sterile distilled water then immersed in sterile distilled water and placed in 4°C overnight.

3.6.3 Infiltration

Seeds in water were placed in a sonicator for 10 mins. Seeds were dabbed to dry and added to infiltration broth prepared. It was then brought to shaker to incubate for 3 hours at 100 rpm in room temperature.

3.6.4 Post-Infiltration

Infiltration broth was drained from seeds. Seeds were treated with 500 mg/ml carbenicillin. Seeds then rinsed with sterile distilled water several times before being sow into ground. The soil was kept moist and dark. Seeds should germinate after two weeks.

3.7 Screening for Insertion

3.7.1 Plant DNA Extraction

Genomic DNA was extracted from young leaves of mature plants using GF-1 Plant Nucleic Acid Extraction Kit following the protocol provided by the manufacturer.

3.7.2 Polymerase Chain Reaction (PCR)

Molecular analysis on the plant DNA was performed using polymerase chain reaction (PCR). The PCR was conducted according to the PCR setup and the thermal cycle profile which shown in Table 1 and Table 2 respectively.

The primers used to amplify *msAdh1* cDNA fragment are *Nco1* (forward primer) with the sequence of 5'-GCA ATT CCA TGG CAA GCA GTG TTG GTC AA-3' and *BamH1* (reverse primer) with the sequence of 5'-ACC AAG GAT CCA TAG TGG TGG TGG TG-3'. A negative control was run to monitor the validity of the PCR. The expected size of product is 1.1 kb. The PCR products were then subjected to 1% agarose gel electrophoresis.

Table1: PCR Setup

Reagents	**Volume (µL)**
GoTaq PCR Master Mix	7.5
Forward Primer (*Nco1*)	1
Reserve Primer (*BamH1*)	1
Nuclease-free dH_2O	4.5
Genomic DNA	1
Final Volume	15

Table2: PCR Thermal Cycle Profile (*Nco1* and *BamH1* primers)

Program	**Temperature (°C)**	**Duration**	**Cycle**
Initial Denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	64	30 sec	35
Elongation	72	45 sec	35
Final Elongation	72	5 min	1

3.8 Reconfirmation for Insertion

Reconfirmation of insertion was done by polymerase chain reaction (PCR). There were 2 PCR performed with the use of different primers for screening. Both PCR products were subjected to 0.8% agarose gel electrophoresis.

3.8.1 First PCR Screening

The first reconfirmation test performed was via the same PCR setup and the thermal cycle profile used previously as represented in Table 1 and Table 2 respectively. The PCR was done on all sixteen samples and both positive and negative controls.

3.8.2 Second PCR Screening

The second reconfirmation test performed with PCR using *Bar* gene primers, *Bar-3-F* (forward primer) with the sequence of 5'-ATGAGCCCAGAACGACGCC-3' and *Bar-3-R* (reverse primer) with the sequence of 5'-ATCTCGGTGACGGGCAGG-3'. The expected PCR product was of the size 549 bp. The PCR setup was the same as described in Table 1 with replaced primers. The PCR thermal cycle profile was conducted according to Table 3.

Table3: PCR Thermal Cycle Profile (*Bar* gene primer)

Program	**Temperature (°C)**	**Duration**	**Cycle**
Initial Denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	59	30 sec	35
Elongation	72	45 sec	35
Final Elongation	72	5 min	1

4.0 Results

4.1 Confirmation of *msAdh1* cDNA insertion in pGSA1131

The plasmid extracted from *E.coli* XL1-Blue habouring pGSA1131 was subjected to 1% agarose gel electrophoresis (Figure 1). The *msAdh1* cDNA gene was further confirmed via PCR. The product obtained is of the desired size of 1.1 kb. (Figure 2).

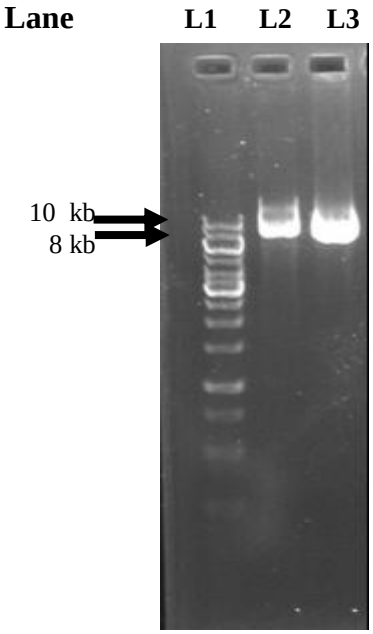

Figure 1: Plasmid pGSA1131 extraction. Lane 1 represent the 1 kb ladder marker; lane 2 and 3 represent the pGSA1131 extracted.

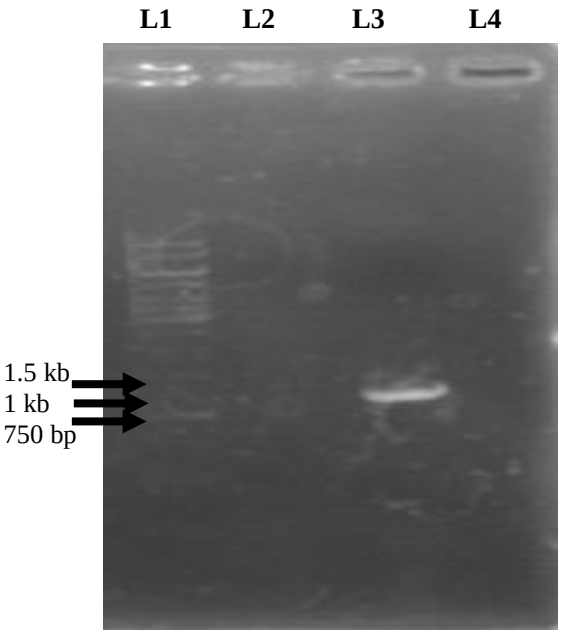

Figure 2: Agarose gel electrophoresis (1%) of PCR product. Lane 1 represent the 1 kb ladder marker , lane 2 represent negative control and lane 3 represent the PCR product.

4.2 *A. tumefaciens* Transformation

The *A. tumefaciens* transformation via electrophoration were left to grow in LA plates. The bacteria growed on both plates with different Rifampicin concentration. Three colonies were picked from plate from Picture 2 and subjected to Colony PCR. PCR products of desired size were observed from 2 colonies (Figure 4).

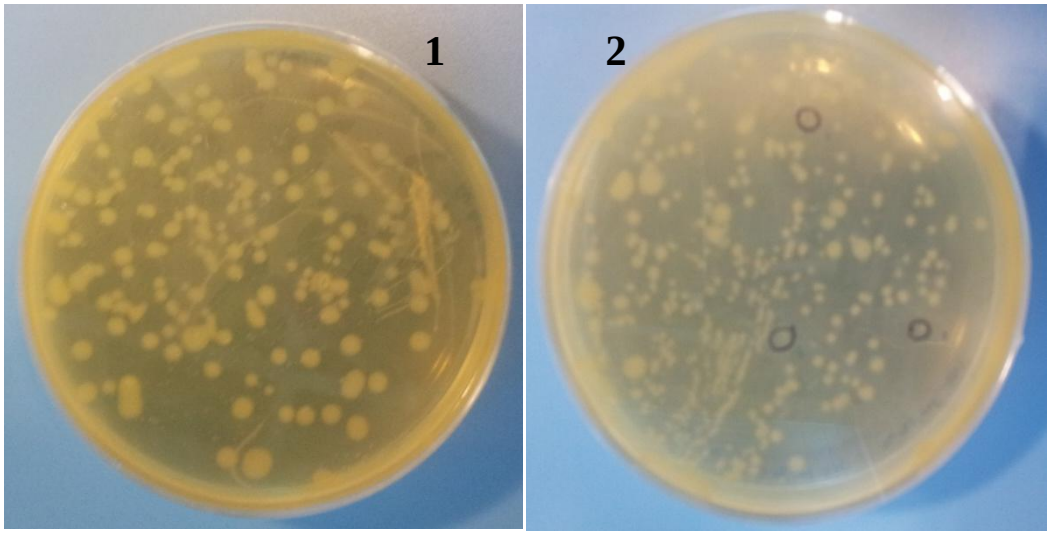

Figure 3: *A. tumefaciens* growth on LA upon transformation. Picture 1 represent the *A. tumefaciens* growth with 75 mg/ml Rifampicin and 30 mg/ml Chloramphenicol LA plate while Picture 2 represent the *A. tumefaciens* growth with 100 mg/ml Rifampicin and 30 mg/ml Chloramphenicol.

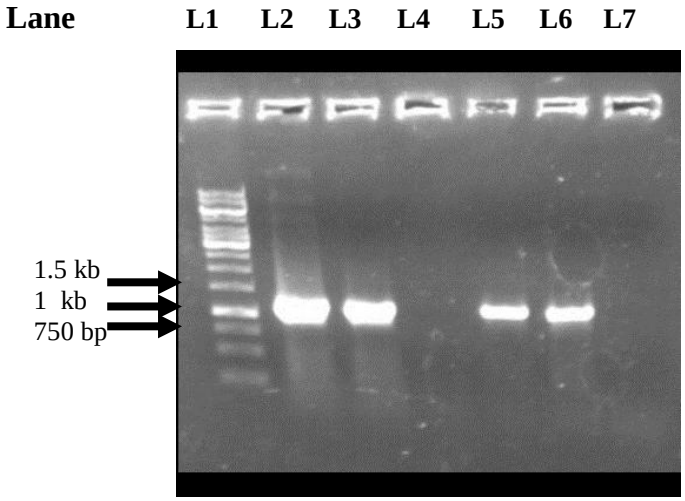

Figure 4: Agarose gel electrophoresis (1%) of colony PCR products. Lane 1 represent 1 kb ladder marker; Lane 2 and 3 represent positive controls; Lane 4 represent negative control; Lane 5 to7 represent colony 1 to 3 respectively.

4.3 Tomatoes Transformation

The growth of tomatoes was observed to be mature after 3 weeks after germination at day 4 upon plantation after transformation as shown in Figure 5. Sixteen mature plants were chosen to be analyzed. Genomic DNAs were extracted from the leaves as shown in Figure 6 and 7. Smearing was observed indicates the DNA extracted was of low purity. The bands of DNA were not bright indicate low DNA concentration.

Figure 5: Tomatoes growth after 3 weeks.

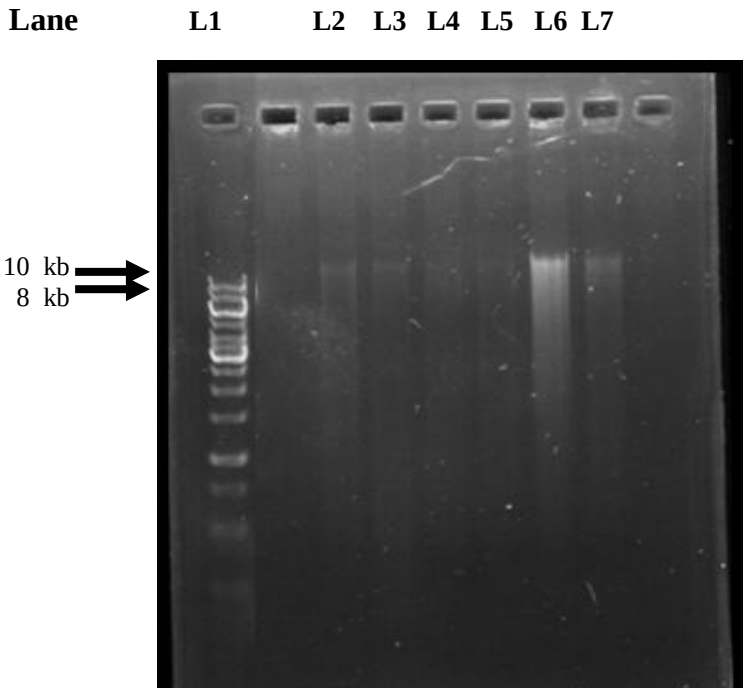

Figure 6: Agarose gel electrophoresis (1%) of plant DNA extracted. Lane 1 represents 1 kb ladder marker; Lane 2 to 7 represent plant DNA 1 to 6 respectively.

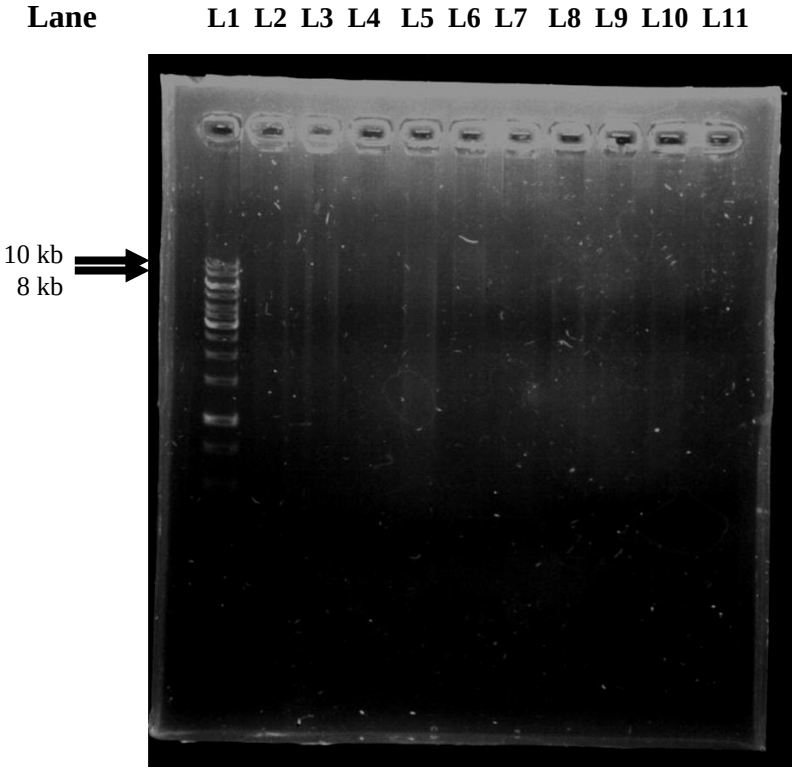

Figure 7: Agarose gel electrophoresis (1%) of plant DNA extracted. Lane 1 represents 1 kb ladder marker; Lane 2 to 11 represent plant DNA 7 to 16 respectively.