Cloning and characterization of an *Arabidopsis thaliana* arsenic reductase gene (*ACR2*)

Master thesis in Molecular Biology (30 hp)

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Abstract

Arsenic is a toxic metalloid existing everywhere in the nature. It is toxic to most organisms and considered as human carcinogen. Arsenic contamination leads to severe health problems with diseases like damage of skin, lung, bladder, liver and kidney as well as central nervous system. It is very likely that too much chemicals such as cadmium and arsenic in the consumed foods can also lead to increased birth defects like spinal bifida. In some regions of South-East Asia, like Bangladesh, Burma, Thailand and India, arsenic contamination of human population via either food chain or drinking water is now considered as a national threat for mankind. As arsenic can be found everywhere in nature it may come in contact with food chain very easily through either water or cultivated crops. In South-East Asia the major cultivated crop is rice and it is the staple food for people in many countries like Bangladesh, Burma and Thailand. Cultivation of rice plants requires water either from rainfall or irrigation. Irrigated water in some regions of South-East Asia is highly contaminated with arsenic and by this way arsenic is accumulated in the rice corn which consumed not only by human but also by animals, birds and fishes. In order to avoid arsenic contamination in human food it is essential to find out a way to inhibit arsenic uptake in cultivated plants. Alternatively, we can also find out a way to metabolize arsenic “in plant”. In my experiment I have used Arabidopsis thaliana as a model plant to isolate an arsenic reductase (ACR2) gene. This gene has been reported to be involved in metabolism, transport and sequestration of arsenic in plants. My thesis works include studies of the ACR2 gene based on characterization of the corresponding SALK mutants. All plants were exposed to arsenics under in vitro conditions. It was observed that the SALK mutants were more sensitive to arsenics in comparison with the wild type control plants. ACR2 gene was cloned from the genomic DNA of A. thaliana by using Phire Plant Direct PCR kits using database sequences as primers. The amplified product was first ligated to the vector pKOH152 and then transferred to E. coli DH5α competent cells. Recombinant bacterial colonies were screened by colony PCR to confirm the insertion of ACR2. The band (1.3 kb) obtained in gel image indicates that the ACR2 gene was cloned successfully. For further confirmation of these results the cloned gene should be sequenced.
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Abbreviations

AAMS  Atomic Absorption Mass Spectrophotometer
ACR2  Arsenic reductase 2
As    Arsenic
As$^{{\text{III}}}$ Arsenite
As$^{{\text{V}}}$ Arsenate
CFU   Colony forming unit
DMAA  Dimethyl arsenic acid
DNA   Deoxyribonucleic acid
E. coli Escherichia coli
LB    Luria-Bertani
MMAA  Monomethyl arsenic acid
MRP1  Mitochondrial ribosomal protein 1
MRP2  Mitochondrial ribosomal protein 2
MS    Murashige and Skoog
ND    Nano drop
NEB   New England Biolab
PCR   Polymerase chain reaction
PCS1  Phytochelatin Synthase 1
Pht1  Phosphate transporter 1
TE    Tris EDTA
1. Introduction

Arsenic (As) is a toxic metalloid particle, available ubiquitously in earth’s crust. In addition, As is also present in the surface, ground water and atmosphere (Moore et al., 1977). Natural processes such as weathering of rocks and volcanic emissions as well as human activities like mining, combustion of fossil fuels, smelting of ores or the application of arsenical herbicides, pesticides and wood preservatives are the sole sources leading to As contamination in the environment (Smedley and Kinniburgh, 2002). Arsenic exists in either organic or inorganic form but is usually not encountered in its elemental state. Generally the inorganic fraction contains oxygenated As anions or more complex As salts. The As salts usually contain sulphur and iron. Among these As salts, the arsenopyrite (FeAsS) is the most abundant (Brewstar MD, 1994). The most common inorganic forms of arsenic in the environment are Arsenate (AsV) and arsenite (AsIII). On the other hand, the environmental organic arsenicals may derive from herbicides, pesticides and preservatives.

As is considered as a human carcinogen and has been defined as a group 1 carcinogen. It is toxic for most organisms and is placed at the highest health hazard category by the international agency for research on cancer (National Research Council, 2001; Naidu et al., 2006). Medicinal properties of arsenic have been demoralized by humans for a considerable time. For example, the German pharmacologist Paul Ehrlich introduced the arsenic compound arsphenamine for the treatment of syphilis in 1909 (Van den Enden E, 1999). Arsenic compounds have also been used for the treatment of diseases such as amoebic dysentery, trypanosomiasis, sleeping fever and promyelocytic leukemia (National Academy of Sciences, 1977). Human needs a small amount of As but high dose of As can create serious health problems even death. The US Environmental protection agency reported that among five most toxic substances arsenic and cadmium are found at contaminated superfund sites (Johnson and Derosa, 1995). High levels of As in drinking water and soil have been reported around the world but the condition is worst in India and Bangladesh where around 400 million people are living at a high risk of arsenic poisoning (Clarke, 2003). The consumption of drinking water contaminated with high amounts of As was reported to cause human health problems in Argentina, China, India (West Bengal), Burma, Indonesia, Malaysia, Taiwan, United States and Bangladesh (Chakraboti et al., 2003). Most of the people of these countries use shallow ground water as drinking water and use this water for irrigating the crops. In Bangladesh, the concentration of As in ground water has been found to exceed the safety level (0.05mg AsL−1 of water is the Bangladeshi standard) in 59 out of 64 districts and approximately 80 million people are exposed to arsenic poisoning. The natural contamination of shallow hand tube wells in Bangladesh with arsenic has caused widespread human exposure to this toxic element through drinking water (Karim, 2000; Paul et al., 2000).
Due to irrigation with arsenic contaminated water; fruit plants, vegetables, grains and almost all crops can be contaminated with arsenic (Tripathi et al., 2007). Most of the human and animal foods are proceeded from cultivated plants. The consumption of these As contaminated grains, leaves or straw by cattle leads to the accumulation of As in their meat and milk. Humans and animals consuming arsenic contaminated meat, milk, crops or foods cause arsenic toxicity in their cells leading to lethal diseases. Long time arsenic exposure causes diseases such as neurological, vascular and genotoxic alterations (Easterling, Styblo et al., 2002). Rice is the staple food in Bangladesh. Due to the consumption of As contaminated rice or drinking water, approximately 30 million people are affected by As toxicity. In 1993, the As contamination was reported in ground water (Khan et al., 1997) and in 1998, the British geological Survey researched on the arsenic contaminated ground water in Bangladesh. In this research, 41 types of arsenic contaminations were detected in ground water. The laboratory tests conducted as a part of this study showed that approximately 35% of the ground water contains arsenic in a concentration of 0.05 mg/L (Smith et al., 2000). The World Health Organization estimated that chronic exposure to arsenic may reach epidemic proportions estimating that 10% people in the most contaminated areas may die due to arsenic poisoning related diseases (Smith et al., 2000). Since the financial costs connected with repairing the environmental damage by using physical remediation methods like reburial and excavation are high, these technologies are unacceptable for cleaning up the large areas of the planet that need arsenic remediation.

It has been reported that the concentration of soil arsenics in As contaminated areas ranges from 57 to 83 mg/kg dried soil whereas the concentration of As in soil from non-contaminated area ranges from 4 to 8 mg/kg dry soil (Abedin, Feldman et al., 2002). The maximum dietary amount of arsenic is 1.0 mg/kg of dried food. For example, rice produced in the south-west part of Bangladesh contains 1.8 mg of arsenic/kg dried rice (Guo, Zhu et al., 2007). In some mammals, including human, the arsenites (As\textsuperscript{III}) are methylated and converted to some relatively less harmful substances like monomethyl arsenic acid (MMAA) and dimethyl arsenic acid (DMAA). In human and other animals, these methylated arsenic acids can be excreted through urine (Hughes, Devesa et al., 2008). Different resistant mechanisms have been progressed by plants in opposition to arsenic, for example some plants show resistance by decreasing its uptake potentiality (Bleeker et al., 2006). Arsenic accumulation in tissues is found in Pteris vittata where gametophyte tissues are resistant to arsenate (As\textsuperscript{V}) (Gumaelius et al., 2004). However, there has been no research performed yet to define the mechanisms by which As\textsuperscript{V} and As\textsuperscript{III} can be removed from the plants.

The uptake and aggregation of As in plants depend on different factors such as (a) type and age of the plant and (b) type and the concentration of arsenics in the soil. Plants generally uptake arsenic in the anionic forms of As\textsuperscript{III} and As\textsuperscript{V}; both of these anionic forms have distinct cytotoxic effects (Quaghebeur and Rengal, 2003). As\textsuperscript{III} reacts with the sulfhydryl groups of proteins and enzymes and slows down cellular activity resulting in death (Ullrich- Eberius et al., 1989). On the other hand, As\textsuperscript{V} is an analog of phosphate, an important plant
nutrient. It contests with phosphate for getting absorbed into the roots and cytoplasm where it might hamper phosphate metabolism making unstable ADP-As\textsuperscript{V}, and leads to the disruption of energy flows in cells (Meharg, 1994). This plant characteristic is useful in phytoremediation strategies but this system has some limitations. Most of the plants appear to have high levels of endogenous As\textsuperscript{V} reductase activity that reduces up to 95\% of total As\textsuperscript{V} to As\textsuperscript{III} (Pickering et al., 2000; Dhankher et al., 2002). In most plants, As\textsuperscript{III} is sequestered in roots. This prevents the translocation of As into leaves, stems and other organs including reproductive organs. The enzymatic reduction of As\textsuperscript{V} to As\textsuperscript{III} in roots through this mechanism can be adopted to engineer plants which will efficiently translocate arsenic to above ground tissues (Pickering et al., 2000; Dhankher et al., 2002).

Arsenic is readily taken up by plant roots, in most cases as As\textsuperscript{V}, the dominant form of As in aerobic environments (Meharg and Hartley-Whitaker, 2002). Some specific transporters have been identified which are considered to mediate a large part of the observed As\textsuperscript{V} influx. These include the \textit{A. thaliana} phosphate transporter 1 (Pht1) gene; and 1:4 high and medium affinity phosphate uptake systems (Shin et al., 2004). The metabolism and transport of As in the model plant \textit{A. thaliana} has been depicted in Figure 1. \textit{A. thaliana} is one of the most commonly used models for studying molecular and cellular biology of flowering plants.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{arsenic_metabolism_diagram.png}
\caption{The uptake, transport and metabolism of arsenic in \textit{Arabidopsis thaliana} plant through different parts of the \textit{A. thaliana} plant (Adjusted from Lund et al., 2009).}
\end{figure}

\textit{A. thaliana} has a small genome that already has been sequenced (Koncz, Chua et al., 1992). This plant has a rapid life cycle which is about six weeks from seed germination to ripening of seed. In the molecular level, four genes have been recognized to determine the uptake, transport and regulation of As in \textit{A. thaliana}. These are Phytochelatin Synthase 1 (\textit{PCS1}), arsenate reductase 2 (\textit{ACR2}), mitochondrial ribosomal protein 1 (\textit{MRP1}) and mitochondrial ribosomal protein 2 (\textit{MRP2}). \textit{PCS1} interacts with As and forms a complex in
the vacuole and can be easily pumped out from the plant. \textit{PCS1} is comparatively larger than \textit{ACR2}. The size of \textit{ACR2} gene is 1.358 kb long.

**Aim**

The objective of the present master thesis was to determine the total uptake of As in wild-type and mutant plants of \textit{A. thaliana} (SALK) and to clone the As reductase gene \textit{ACR2} from wild-type \textit{A. thaliana} plants into \textit{E. coli} cells. The uptake of As in wild and mutant type plants could be determined by atomic absorption spectrophotometry. It would define the role of \textit{ACR2} gene in causing As metabolism in \textit{A. thaliana} plants. A polymerase chain reaction (PCR) based cloning was adopted for cloning the \textit{ACR2} gene into \textit{E. coli} cells. This would enable the generation of large copies of \textit{ACR2} gene for cloning it into a suitable vector.

2. Materials and Methods

2.1. \textit{In vitro} growth of wild type and mutants of \textit{A. thaliana}

\textit{A. thaliana} seeds were initially vernalized for one week at 4°C temperature. The seeds were then germinated in a full strength (43.6 g/L) Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose and pH 5.8 under controlled conditions like 25°C temperature, 70% humidity and 16 hours light period. After 10 and 17 days of seed sowing of \textit{A. thaliana}, the rate of germination and growth of plants was analyzed morphologically. For As treatment, seeds were germinated on MS media containing 0 µM, 25 µM arsenite along with 25 µM arsenate and 50 µM arsenite along with 50 µM arsenate. Each treatment was replicated eight times.

2.2. Amplification of \textit{ACR2} gene

\textit{A. thaliana} seeds were germinated in the laboratory in soil. Primary green leaf tissues were used as the tissue source for \textit{ACR2} gene. The amplification of \textit{ACR2} gene was carried out directly from these fresh leaves following the standard protocol Phire Plant Direct PCR Kit (Finnzymes, 2009). According to this protocol a small leaf sample was placed in 20 µL of dilution buffer. The leaf was crushed with a 100 µL pipette tip by pressing it briefly against the tube wall. For the amplification of \textit{ACR2} gene, two types of primers were designed such as 1\textsuperscript{st} amplification primers (outer primers) and 2\textsuperscript{nd} amplification primers (inner primers). The 1\textsuperscript{st} amplification primers were designed using computer software Oligo 6 and the 2\textsuperscript{nd} amplification primers were designed using In-Fusion Advantage PCR Cloning Kit User Manual (Clontech, 2010). According to this protocol, the 15 bases of the 5’ end of the primer were homologous to 15 bases at one end of the vector pKOH152 and the 3’ end, the sequence was specific to the \textit{ACR2} gene.
The *ACR2* gene was amplified by two steps, first step was amplified by using 1\textsuperscript{st} amplification primers and after that the second step of amplification was done with the product of 1\textsuperscript{st} amplification primer by using 2\textsuperscript{nd} amplification primers. The 1\textsuperscript{st} amplification reaction mixture is shown in Table 1 and the 1\textsuperscript{st} amplification PCR cycles and reaction setups are shown in Table 2.

Table 1. Reagents, stock concentration, final concentration, volume and master mix used in the polymerase chain reaction with 1\textsuperscript{st} amplification primers

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock conc.</th>
<th>Final conc.</th>
<th>Volume (μL)*</th>
<th>Master Mix (μL)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>-</td>
<td>-</td>
<td>0.50</td>
<td>3</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>5 X</td>
<td>1X</td>
<td>10.0</td>
<td>60</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10 μM</td>
<td>0.5 μM</td>
<td>0.50</td>
<td>3</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>10 μM</td>
<td>0.5 μM</td>
<td>0.50</td>
<td>3</td>
</tr>
<tr>
<td>Phire Hot Start DNA polymerase</td>
<td>2 u/μl</td>
<td>0.5 U/20 μl</td>
<td>1.00</td>
<td>6</td>
</tr>
<tr>
<td>Deionised water</td>
<td>N/A</td>
<td>N/A</td>
<td>37.50</td>
<td>225</td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td></td>
<td>50.00</td>
<td>300</td>
</tr>
</tbody>
</table>

* = volume was taken for one reaction  
** = volume was taken for six reactions

Buffer contains MgCl\textsubscript{2} in Finzymes’ Phusion High-Fidelity PCR kit.

Table 2. PCR reaction set up and different cycle in the thermocycler machine for amplification of *ACR2* gene by 1\textsuperscript{st} amplification primers

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98°C</td>
<td>5 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>2</td>
<td>98°C</td>
<td>5 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>63.6°C</td>
<td>5 sec</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>40 sec</td>
<td>Extension</td>
</tr>
<tr>
<td>5</td>
<td>Go to step 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72 °C</td>
<td>1 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>7</td>
<td>4 °C</td>
<td>For ever</td>
<td>Holding</td>
</tr>
</tbody>
</table>

The 2\textsuperscript{nd} amplification reaction mixture is shown in Table 3 and the 2\textsuperscript{nd} amplification PCR cycles and reaction setup has been shown in Table 4.

Table 3. Reagents, stock concentration, final concentration, volume and master mix used in the polymerase chain reaction with 2\textsuperscript{nd} amplification primers

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock conc.</th>
<th>Final conc.</th>
<th>Volume (μL)*</th>
<th>Master Mix (μL)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>16.8 ng/μL</td>
<td>5 ng/20μL</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>1.00</td>
<td>4</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>5 X</td>
<td>1X</td>
<td>10.0</td>
<td>40</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10 μM</td>
<td>0.5 μM</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>10 μM</td>
<td>0.5 μM</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>Phire Hot Start DNA polymerase</td>
<td>2 u/μL</td>
<td>0.5 U/20 μl</td>
<td>1.00</td>
<td>4</td>
</tr>
<tr>
<td>Deionised water</td>
<td>-</td>
<td>-</td>
<td>36.75</td>
<td>147</td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td></td>
<td>50.00</td>
<td>200</td>
</tr>
</tbody>
</table>

* = volume was taken for one reaction  
** = volume was taken for four reactions

Table 4. PCR reaction set up and different cycle in the thermocycler machine for 2\textsuperscript{nd} amplification reactions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98°C</td>
<td>5 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>2</td>
<td>98°C</td>
<td>5 sec</td>
<td>Denaturation</td>
</tr>
</tbody>
</table>
2.3. Gel electrophoresis

Agarose gel electrophoresis was used to separate the amplified ACR2 gene fragments based on the size of the fragments. The electricity and gel provide the accurate friction to separate the DNA fragments on the basis of their size. The smaller fragments run faster (Reece, 2004) than the larger ones. The gel was prepared with 0.8% of agarose in 1X TAE buffer. The loading dye used was 6X. Two types of DNA ladders (1 kb and 2 kb) were used. After gel electrophoresis, the ACR2 gene samples were chosen for digestion. The concentration was measured by Nanodrop® ND-1000 Spectrophotometer (Saveen Werner, USA) after amplification and purification.

2.4. Preparation of inner fragments by cloning enhancer

2µL of cloning enhancer was mixed with 5 µL of inner fragments, heated at 37°C for 40 minutes in heat block followed by heating at 80°C for 40 minutes. This mixture was stored at -20°C until further use. Preparation of linearized vector by restriction digestion was performed to achieve a successful In-Fusion reaction. The plasmid pKOH152 was digested by two restriction enzymes BamHI (High fidelity) and BbvCI.

2.5. Restriction digestion of plasmid pKOH152

Plasmid pKOH152 was provided by Dr. Kjell-Ove Holström, University of Skövde, Sweden. The concentration was measured by the Nanodrop® ND-1000 Spectrophotometer (Saveen Werner, USA). To achieve a successful In-Fusion reaction, the plasmid pKOH152 was digested by two restriction enzymes BamHI (High fidelity) and BbvCI. For cleavage analysis, the digested plasmid DNA fragments were run in the gel electrophoresis. The vector map of pKOH152 showed that it has BamHI (High fidelity) digestion sites at 2788 position and BbvCI restriction sites at 4878 position which are shown in Appendix I.

2.6. Fusion and Transformation

The gene ACR2 was fused to completely digested plasmid pKOH152 following the In-Fusion advantage PCR cloning kit protocol (Clontech, 2010). After incubation, the fusion mixture was diluted with 40 µL TE buffer to prepare 50 µL fusion mixtures. From this mixture, 2.5 µL was transformed to 50 µL competent cell line DH5α (New England Biolab) by heat shock method and the cells were grown in SOC medium following the High Efficiency Transformation Protocol (Clontech, 2010). Followed by this, 100 µL, 250 µL
and 500μL of transformed cells were spread on LB media plates containing 100 mg/L ampicillin and incubated at 37°C for overnight. Positive and negative control plates were set simultaneously. The positive control was performed with a circular vector of known concentration and the negative control was performed with known amount of linearized vector.

2.7. Observation of bacterial colony on plates

After overnight incubation, the colony forming units (CFUs) were observed on ampicillin-containing LB plates and compared to the controls. 24 bacterial colonies were randomly taken from three LB plates inoculated with 100 μL, 250 μL and 500μL of transformed cells. These bacterial colonies were cultured in LB broth containing-ampicillin to prepare fresh culture for isolation of recombinant plasmid. The plasmid isolation was performed by following Miniprep spin protocol (Qiagen, 2004) and concentration was measured by the Nanodrop® ND-1000 Spectrophotometer (Saveen Werner, USA).

2.8. Restriction digestion test

After isolation, the recombinant plasmids were digested with AscI for screening the right colony. Restriction digestion was done at 37°C for overnight. After incubation, the samples were treated for 20 minutes at 65°C to inactivate the restriction enzyme such that the restriction enzyme cannot play any role during fusion. The digested samples were run in gel electrophoresis to verify the right cloned colony.

2.9. Colony PCR

Five colonies chosen from the 24 above mentioned colonies were taken from the transformed LB agar plates for colony PCR. A master mixture was prepared and colony PCR was performed in PIKO Thermal Cycler (Finnzymes Instruments, EU) by following inner primer amplification protocol. For colony PCR, 2nd amplification primers were used i.e. the forward primer was 5´-GAGTAAAGAAGAACCATGGGGAGAAGCATATTTTC-3´ and reverse primer was 5´-ATTCGGCCGGCCTCAGCCTAGGGCGCAAT-3´. The colony PCR products were then analyzed on agarose gel electrophoresis to verify its exact band size (1.3 kb).

3. Results

3.1. Effect of As on Growth of A. thaliana

Wild type (Colombia-1) and SALK mutants (N-59 and N-83) of A. thaliana were grown on MS media containing 0 μM, 25 μM AsIII along with 25 μM AsV and 50 μM AsIII along with 50 μM AsV. The growth of the plants was observed after 10 days (Figure 2) and 17 days (Figure 3).
Figure 2. Wild type (WT) Co-1, SALK mutant N-59 and N-83 were exposed with 0, 50 µM and 100 µM arsenite and arsenate after 10 days.

Figure 3. Wild type (WT) Co-1, SALK mutant N-59 and N-83 were exposed with 0, 50 µM and 100 µM arsenite and arsenate after 17 days.
Three weeks old A. thaliana plants were collected from the culture chamber, prepared dust and kept them in 4°C such that the As cannot be changed from As\textsuperscript{V} to As\textsuperscript{III}. Due to the unavailability of Atomic Absorption Mass Spectrophotometer (AAMS) machine at the Department of Molecular Biology at Skövde University, it was suggested that the measurement of total As would be conducted in Stockholm University. The samples were sent to the concerned laboratory at Stockholm University. The results obtained from AAMS for total As are awaited.

3.2. Amplification of \textit{ACR2} gene with outer and inner primers

The outer primers (1\textsuperscript{st} amplification primers) which were designed using the Oligo 6 software were used are as follows:

Sequence of forward primer: 5’-TCAGATTTCGGAAGAGAAGG-3’
Sequence of reverse primer: 5’-CGTTGCTTTTGGTTATGACTTT-3’
Product size 2.03kb

The other properties of primers are shown in Table 5:

<table>
<thead>
<tr>
<th>Primer</th>
<th>length</th>
<th>Tm (°C)</th>
<th>GC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>23 bp</td>
<td>64.6</td>
<td>43.3</td>
</tr>
<tr>
<td>Reverse</td>
<td>23 bp</td>
<td>63.1</td>
<td>34.7</td>
</tr>
</tbody>
</table>

Inner primers (2\textsuperscript{nd} amplification primers) for \textit{ACR2} gene are as follows:

Sequence of forward primer:
5’-GAGTAAAGAAGAACCATGGGGAGAAGCATATTTTC-3’

Sequence of reverse primer:
5’-ATTCGGCCGGCTCAGCCTCATTAGGCGCAAT-3’
Product size 1.3kb

The other properties of 2\textsuperscript{nd} amplification primers are shown in Table 6.

<table>
<thead>
<tr>
<th>Primer</th>
<th>length</th>
<th>Tm (°C) Total Seq.</th>
<th>Tm (°C) Gene Seq.</th>
<th>GC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>35 bp</td>
<td>72.7</td>
<td>64.1</td>
<td>40.0</td>
</tr>
<tr>
<td>Reverse</td>
<td>32 bp</td>
<td>85.0</td>
<td>65.8</td>
<td>59.3</td>
</tr>
</tbody>
</table>

At the 5’- end, the underlined part (15 bases) of the forward and reverse primers are homologous to one end of the vector pKOH152 and the 3’ end, the sequence was specific to the \textit{ACR2} gene. The melting temperature which has been shown in Table 6 is the melting temperature of total sequences i.e. the vector part and the gene part. The melting temperatures of gene sequences for forward and reverse primers are 64.1°C and 65.8°C respectively.

In order to get optimum results for amplification, High-Fidelity PCR kit was used. The amplification reactions were carried out with six different leaves and the PCR products were thereafter analyzed using gel electrophoresis (Figure 4).
Figure 4. PCR products amplified with 1\textsuperscript{st} amplification primers. Lane L1 and L2 is 2-log DNA ladder. Lane 1, 2, 3, 4, 5 and lane 6 show band for \textit{ACR2} at about 2.0 kb position which were selected for purification.

In lane 6, it can be observed that one thin band is near 0.9 Kb and also in lane 5 one thin band can be observed at 0.9 Kb. This might be due to the high amount of \textit{ACR2} gene in these lanes. The amplified \textit{ACR2} gene products were purified and the concentrations of the purified \textit{ACR2} gene products were measured. The concentration and purity obtained for \textit{ACR2} gene are shown in Table 7.

Table 7. \textit{ACR2} gene concentrations of S-2, S-3, S-4, S-5, S-6 and their purity after NanoDrop

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (ng/µL)</th>
<th>(A_{260/280})</th>
<th>(A_{260/230})</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-2</td>
<td>11.4</td>
<td>2.43</td>
<td>0.87</td>
</tr>
<tr>
<td>S-3</td>
<td>12.1</td>
<td>2.10</td>
<td>0.98</td>
</tr>
<tr>
<td>S-4</td>
<td>12.8</td>
<td>1.68</td>
<td>0.62</td>
</tr>
<tr>
<td>S-5</td>
<td>14.8</td>
<td>1.89</td>
<td>0.86</td>
</tr>
<tr>
<td>S-6</td>
<td>16.8</td>
<td>1.99</td>
<td>0.86</td>
</tr>
</tbody>
</table>

The purified \textit{ACR2} gene products were amplified with 2\textsuperscript{nd} amplification primers using an annealing temperature of 85\degree C. The amplified \textit{ACR2} gene products were analyzed using the gel electrophoresis (Figure 5).
After amplification of ACR2 gene with 2nd amplification primers it was digested by AccI and SbfI restriction enzymes. The restriction digestions were carried out using sample 3 and 4 (sample B and A) with both endonucleases separately and mixture of these enzymes. Result has been shown in Figure 6.
3.3. Digestion of vector pKOH152

The plasmid pKOH152 was digested by restriction enzymes BamHI (High fidelity) and BbvCI and the digested gene products were run on gel electrophoresis (Figure 7). The vector map showed that it has BamHI site at 2788 position and BbvCI site at 4878 position as shown in Appendix I.

![Gel electrophoresis image](image-url)

Figure 7. Gel image shows two bands of the vector pKOH152 after its digestion with BamHI (High fidelity) and BbvCI restriction enzymes. Lane L is the 2 Kb DNA ladder and lane 1, 2, 3, 4, 5 shows the restriction digestion of pKOH152.

3.4. Cloning and Transformation

The transformed cells were diluted with TE buffer. From the diluted cell suspension, 100 µL, 250 µL and 500µL of transformed cell suspensions were spreaded on LB media plates containing 100 mg/L ampicillin. The inoculated plates were incubated at 37°C for overnight along with positive and negative control. After an overnight incubation, the numbers of CFUs were observed as shown in Table 8.

Table 8. Showing the number of CFUs on plates after transformation along with positive and negative control

<table>
<thead>
<tr>
<th>Types of plates</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>UC</td>
</tr>
<tr>
<td>Negative control</td>
<td>NG</td>
</tr>
<tr>
<td>100 µL transformed cells</td>
<td>7</td>
</tr>
<tr>
<td>250 µL transformed cells</td>
<td>15</td>
</tr>
<tr>
<td>500 µL transformed cells</td>
<td>32</td>
</tr>
</tbody>
</table>

UC=uncountable
NG=no growth
3.5. Screening

Screening was performed to verify the restriction digestion. For screening, 24 colonies were selected and were digested with AscI. Following restriction digestion, the digested samples were run on gel electrophoresis (Figure 8).

Figure 8. Gel electrophoresis picture of 24 colonies. 24 colonies were digested AscI restriction enzyme. Lane L1 and L2 indicate 1 Kb gel pilot DNA ladder. Out of 24 colonies five colonies (1, 12, 16, 17 and 18) shows band near 6 Kb.

3.6. Colony PCR

After doing colony PCR, the amplified products were visualized through agarose gel electrophoresis (Figure 9). Out of five colonies four colonies (12, 16, 17 and 18) demonstrated bands at the expected size. These bands were almost same with control (2nd amplification product) band.
4. Discussions

Arsenic is the 20th most plentiful component in the earth’s crust, and found all over the place in nature. Previous researches have shown that several genes in the model plant *A. thaliana* regulate arsenic uptake, metabolism and transport. Therefore, strategies are aimed in cloning of these genes from *A. thaliana* and to transfer these to other crops to avoid arsenic accumulation in the edible parts.

The treatment of wild type and mutants of *ACR2* gene *A. thaliana* plants with As showed that the mutant types were more affected by the toxic effects of As than their wild type counterparts. It clearly indicated that the functional *ACR2* gene has a prominent role in reducing As toxicity in wild type *A. thaliana* plants. A similar study involving silencing of *ACR2* gene in *A. thaliana* showed that the knock-down plant cell lines for the *ACR2* gene were more vulnerable to As\(^{V}\) than their wild type counterparts with functional *ACR2* gene (Parkash *et al.*, 2005). The SALK mutants have no functional arsenic reductase gene and therefore they are weaker than the wild types. In the present study, a more vigorous growth was observed for both wild type and mutant plants grown in the absence of As (As\(^{III}\) and As\(^{V}\)) than those being exposed to As (As\(^{III}\) and As\(^{V}\)). Moreover, the wild type and mutant plants which were exposed to 50µM As (As\(^{III}\) and As\(^{V}\)) exhibited more growth than those exposed to 100µM (As\(^{III}\) and As\(^{V}\)). It was also observed that plants exposed to As (As\(^{III}\) and As\(^{V}\)) for 17 days were about to die but in this case the mutant N-59 were more vigorous than mutant type N-83 (Figure 3). This reinforced the fact that arsenate is the most mobile form of arsenic in the majority of plant species. Since the *ACR2* genes of SALK mutants were knockdown, arsenate could have been trapped in roots. Therefore the plants might have accumulated more arsenate in the shoots and become dead.

The *in vitro* amplification performed in this study using 1\(^{st}\) and 2\(^{nd}\) amplification primers for *ACR2* gene showed that *ACR2* gene was successfully amplified, enabling the amplified segments to be used for cloning. The analysis of amplification products using electrophoresis indicated that sample 2 to 6 were amplified properly and demonstrated good quality bands. The size of *ACR2* bands in sample 2 to 6 were approximately 2.0 kb which were similar to the size of the DNA ladder used. In contrast to this, no band was observed for sample 1. The reason behind this might be that the reaction mixture was not mixed properly or sample was not loaded correctly in lane 1. Since PCR depends upon suitable reaction conditions like annealing temperature, DNA template, primers, reaction buffer, DNA polymerase concentration and magnesium ions (Mg\(^{2+}\)) (Martha *et al.*, 2001), a variation in any of these conditions might also prevented the optimum amplification for sample 1.

Since sample 6 showed more clear bands than other samples, it was chosen for conducting further PCRs with to get the amplified products. The gel image...
(Figure 6) showed that all samples were amplified properly and demonstrated good quality bands. The same samples were loaded in different lanes in different amount to verify the band brightness. Therefore, due to high amount of ACR2 gene in some lanes, some bands were more obvious than the rest. In comparison to the ladder (New England Biolab), ACR2 band was corresponding to approximately 1.3 kb which is the desired size.

Two samples (4 and 3) were selected for digestion from amplified inner fragments of ACR2 which were digested with AccI and Shfl (Figure 7). Lane 1 and 4 digested with AccI which cut at two positions at approximately 0.88 and 0.42 kb. Lane 2 and 5 digested with ShfI which cuts at approximately 1.4 kb, lane 3 and 6 digested with both AccI and ShfI.

The digestion results of vector pKOH152 (Figure 8) and vector map are shown in Appendix 1. The restriction position of the enzyme BamHI (High fidelity) in vector is at 4878 position and after digestion it cut at approximately 5.4 kb. The position of restriction enzyme BbvCI in vector at 2788 and after digestion it cut at approximately 1.9. After restriction digestion the vector pKOH152 size is 6.082 kb.

After linearization, the vector showing the appropriate bands (Figure 7) was fused with the ACR2 gene. The recombinant vector was then transformed to the DH5α competent cells. Out of 32 transformants obtained from 500µL of cell suspension of transformed cells, 24 colonies were selected for screening (results shown in Figure 9). It was observed that out of 24 colonies 5 colonies (1, 12, 16, 17 and 18) gave the expected bands. Therefore, it could be assumed that in these 5 colonies, the ACR2 gene has been inserted correctly. The results obtained in colony PCR indicated that out of 5 colonies, 4 colonies (12, 16, 17 and 18) demonstrated the bands of expected size which are approximately similar to those obtained in the 2nd PCR amplification. For further confirmation of these results sequencing of this gene is necessary and it will be compulsory part for continuation of this project in future.

5. Conclusion

Though the experimental data revealing As accumulation in wild type and mutated plants has been still lacking, the morphological studies indicate that the A. thaliana plants with mutated ACR2 gene were more sensitive to As compared with wild type control plants. The restriction analysis and the colony PCR conducted in this study verify that ACR2 gene was cloned into pKOH152.

6. Future perspectives

A comparative analysis of the results obtained from atomic absorption spectroscopic measurement of As from wild type and mutated A. thaliana plants should be further conducted. Moreover, sequencing analysis should be
performed for the cloned ACR2 gene to ensure that the target gene was cloned into the correct position within the plasmid vector. The cloned ACR2 gene can also be isolated from the host bacterial cells and used for generating genetically modified plant cells.

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8. References


8. Appendix I.

Map of pKOH 152 with different restriction sites.